Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot tips

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Summary

The presented dissertation project analyzed factors affecting cryopreservation of potato shoot tips. The aim of this study was to find correlations between differences in preculture treatments of donor plants and corresponding changes of biochemical compounds and cryopreservation results. Furthermore ultrastructural investigations and thermal analyses were performed to get better insight into cellular conditions.

Increased shoot regeneration was found after alternating temperature preculture of donor *in vitro* plants prior to cryopreservation for 7 d at 22/8 °C day/night temperature with 8 h photoperiod (AT) compared to the original method using preculture with constant temperatures for 7 d at 22 °C with 16 h photoperiod (CT). The improvement in average shoot regeneration from 34.6 % to 58.7 % was observed in two cultivated potatoes *S. tuberosum* 'Désirée' and 'King Edward' and two wild species *S. acaule* and *S. demissum* using the DMSO droplet method.

Cold acclimation and increase in cold tolerances after AT could not be found for *in vitro* or greenhouse cultures grown in soil in any accession tested. Therefore, it can be concluded, that the improvement of cryopreservation results after AT is not directly related to cold acclimation and cold tolerances.

In comparative biochemical analyses total concentrations of soluble sugars (glucose, fructose, and sucrose) were higher for all accessions after AT, whereas starch and proline concentration were mostly decreased. The increase in soluble sugar contents could be the main reason for better cryopreservation results, because sugars act as cryoprotectant and stabilize membranes during cryopreservation.

In the proteomics part of this study a new protocol suitable for potato shoot tips was developed using trichloracetic acid/acetone precipitation, 13 cm 2D gels with pH gradient of 4-7, and ruthenium(II)-tris-(bathophenanthrolin-disulfonate) staining. Proteome analysis of shoot tips revealed only minor changes in protein expression in *S. tuberosum* 'Désirée' and *S. demissum* after AT. Identified proteins belong to the functional groups of metabolism, signal transduction, defence, transcription, energy and secondary metabolism. Most of the altered proteins were downregulated, assuming that AT does not induce major changes in the primary metabolic pathway of the plants. These results strongly suggest that AT does not cause stress, but rather physiological fortification in potato shoot tips and leads to reinforcement against the stress occurring during the steps of the cryopreservation protocol.

Ultrastructural analyses revealed major changes of cells after 2 h of DMSO treatment. Cells contained numerous small vesicles, while at the same time mitochondria and chloroplasts had increased in size and vacuoles had turned to irregular shape. Two days after rewarming, the meristematic dome area and part of the epidermis showed signs of extensive structural damage. Rupture of plasmalemma, plasmolysis and destruction of cell organelles as well as strong heterochromatization of nuclei were observed. Survival and regeneration of cells were found mainly in leaf primordial regions and seldomly in the meristematic dome. Shoot tips with AT showed minor damage compared to CT explants.

Thermal analysis using differential scanning calorimetry (DSC) revealed that shoot tips contained some crystallized water during cooling and melt-endotherm during rewarming after incubation in the DMSO solution. In *S. tuberosum* 'Désirée' additionally to ice formation very small glass transition at around -116 °C was found. AT had no significant effects on thermal behaviour of potato shoot tips. Success in the DMSO droplet cryopreservation method is evident, also with crystallization of water molecules during cooling and melting of ice during rewarming. Ice crystals might be located in the extracellular space or, when inside the cytoplasm they are so small, that survival and regeneration of tissue is not totally stopped.

Comparison of liquid media with agarose drops and solid media in the recovery phase showed improved regeneration on the latter with higher regeneration percentages, less callus formation and better plantlet structure.

Zusammenfassung

Die vorliegende Arbeit analysierte Faktoren, welche die Kryokonservierung von Kartoffelsprossspitzen beeinflussen. Das Ziel der Arbeit war es, Korrelationen zwischen verschiedenen Varianten der Vorkultur der Ausgangspflanzen und den Veränderungen in wichtigen biochemischen Komponenten zu finden. Weiterhin sollten Untersuchungen zur zellulären Ultrastruktur und die thermische Analyse detaillierte Einsichten in den Kryokonservierungsprozess bringen.

Die Sprossregeneration erhöhte sich nach einer Vorkultur der *In-vitro*-Ausgangspflanzen vor der Kryokonservierung von 7 Tagen mit Wechseltemperaturen bei 22/8 °C Tag/Nachttemperatur mit 8 h Lichtdauer im Vergleich zur bisher genutzten Methode, in welcher eine Vorkultur für 7 Tage mit konstanten Temperaturen bei 22 °C mit 16 h Lichtdauer angewendet wurde. Eine Erhöhung der durchschnittlichen Sprossregenerationsraten von 34,6 % auf 58,7 % wurde bei zwei Kulturkartoffeln, *S. tuberosum* 'Désirée' und 'King Edward', und bei zwei Wildarten *S. acaule* und *S. demissum*, unter Anwendung der DMSO-Tröpfchenmethode erreicht.

Bei keiner der getesteten Akzessionen wurde eine Kälteakklimatisierung und Erhöhung der Kältetoleranzen der *in vitro* Kulturen oder Gewächshauspflanzen in Erde nach der Vorkultur mit Wechseltemperaturen gefunden. Daraus wurde die Schlussfolgerung gezogen, dass die hier angewandte Wechseltemperatur in der Vorkultur keine Kälteakklimatisierung der Kartoffeln bewirkt. Somit können die verbesserten Kryokonservierungsergebnisse nicht auf eine Kälteakklimatisierung der Ausgangspflanzen zurückgeführt werden.

In einer vergleichenden biochemischen Analyse war die Gesamtkonzentration gelöster Zucker (Glukose, Fruktose und Saccharose) für alle getesteten Akzessionen nach der Vorkultur mit Wechseltemperaturen erhöht. Die Konzentrationen von Stärke und Prolin waren dagegen meist verringert. Die gefundene Erhöhung löslicher Zucker könnte der Hauptgrund für die verbesserten Ergebnisse der Kryokonservierung sein, da Zucker als Kryoprotektor fungieren und die Membranen während der Kryokonservierung stabilisieren.

Für die Proteinuntersuchungen konnte ein neues Protokoll zur Analyse von Kartoffelsprossspitzen erstellt werden. Dieses beinhaltet eine Trichloressigsäure/Aceton-Fällung, 13 cm 2D-Gele mit einem pH-Gradienten von 4-7 und Ruthenium(II)-tris-(bathophenanthrolin-disulfonat)-Färbung. Die Analyse zeigte nur geringe Veränderungen in der Proteinexpression der Sprossspitzen für *S. tuberosum* 'Désirée' und *S. demissum* nach der Vorkultur mit Wechseltemperaturen. Identifizierte Proteine gehören zu den funktionellen Gruppen des Metabolismus, der Signal-Transduktion, der Stressabwehr, der Transkription,

der Energie und des Sekundärmetabolismus. Die meisten der regulierten Proteine waren in der Expression vermindert, was bedeuten könnte, dass die Vorkultur mit Wechseltemperaturen keine Veränderungen im primären metabolischen Stoffwechselweg zur Folge hat.

Diese Ergebnisse lassen vermuten, dass die Vorkultur mit Wechseltemperaturen keinen Stress auslöst, sondern eher eine physiologische Stärkung der Kartoffelsprossspitzen darstellt und somit eine Kräftigung der Pflanzen gegen den Stress während der einzelnen Schritte des Kryokonservierungsprotokolls bewirkt.

Ultrastrukturelle Untersuchungen zeigten, dass größere Veränderungen in der Struktur der Zellen nach der zweistündigen Einwirkung der DMSO-Lösung stattfanden. Die Zellen beinhalteten viele kleine Vesikel, während gleichzeitig Mitochondrien und Chloroplasten vergrößert waren und Vakuolen eine unregelmäßige Struktur annahmen. Zwei Tage nach dem Erwärmen waren der meristematische Dom und ein Teil der Epidermis stark geschädigt. Die Zerstörung von Plasmamembranen und Zellorganellen wurde beobachtet. Die Zellkerne zeigten eine starke Heterochromatisierung. Eine weitere Schädigung der Zellen erfolgte durch Plasmolyse. Überlebende und sich regenerierende Zellen wurden hauptsächlich in den Blattprimordien gefunden und seltener im meristematischen Dom. Die Sprossspitzen zeigten nach der Vorkultur mit Wechseltemperaturen weniger zerstörtes Gewebe im Vergleich zu solchen, die unter konstant hoher Temperatur kultiviert worden waren.

Nach der Inkubation der Sprossspitzen in der DMSO-Lösung zeigte die thermische Untersuchung mittels Dynamischer Differenzkalorimetrie (DSC), dass sich in den Sprossspitzen eine geringe Menge an Eiskristallen während des Abkühlens bildete und diese beim Erwärmen schmolzen. Bei *S. tuberosum* ‚Désirée' konnte neben der Eisbildung auch ein schwacher Glasübergang bei ca. -116 °C detektiert werden. Die Vorkultur bei Wechseltemperaturen hatte keinen signifikanten Effekt auf das thermische Verhalten der Kartoffelsprossspitzen. Der Erfolg der DMSO-Tröpfchenmethode ist in der Lagerung einer großen Anzahl von Mustern bewiesen worden, obwohl eine Eisbildung während des Abkühlens und Schmelzen von Eiskristallen während des Erwärmens beobachtet worden ist. Die gebildeten Eiskristalle könnten sich entweder extrazellulär befinden oder aber intrazellulär und dann in einer so geringen Größe ausgeprägt sein, dass das Überleben und die Regeneration nach der Erwärmung nicht gefährdet sind.

Der Vergleich der Regeneration von Pflanzen auf flüssigem Medium mit Agarosetropfen und festem Medium zeigte eine verbesserte Regeneration auf dem letzteren, weniger Kallusbildung und bessere Pflanzenstruktur.

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Abbreviations

ABA	abscisic acid
AcN	acetonitrile
AT	alternating temperature preculture (7d 22/8 °C day/night temperatures)
ATP	adenosine triphosphate
B9	n-dimethylamino-succinamic acid
cCBB	colloidal Coomassie Brilliant Blue
CHAPS	(3-[(3-cholamidopropyl)-dimethyalmino] -propansulfate)
CT	constant temperature preculture (7d 22/22°C day/night temperatures)
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DSC	differential scanning calorimetry
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
EST	expressed sequence tag
GA_3	gibberellic acid
HCl	hydrogen chloride
HPLC	high pressure liquid chromatography
IEF	isoelectric focussing
IPG	immobilized pH gradient
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
LC	liquid chromatography
LN	liquid nitrogen
MALDI	matrix-assisted laser desorption/ionization
ME	mercaptoethanol
MS	basal medium after Murashige and Skoog (1962)
MSH	MS solution with 0.5 mg/l zeatin riboside, 0.5 mg/l indole acetic acid, 0.2 mg/l gibberellic acid, and 3 % sucrose
NCBInr	Nonredundant Database of the Nacional Center for Biotechnology Information
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PCA	principal component analysis

PAGE	polyacrylamide gel electrophoresis
PVS	plant vitrification solution
RFLP	restriction fragment length polymorphism
RuBP	ruthenium(II)-tris-(bathophenanthrolin-disulfonate)
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEM	transmission electron microscope
TFA	trifluoroacetic acid
Tg	glass transition
TIGR	The Institute for Genomic Research
TMDSC	temperature modulated DSC
TOF	time-of-flight
Tris	trishydroxymethylaminomethane

Chapter 1. Introduction

Cryopreservation is the storage of biological material at -196 °C in liquid nitrogen (LN) in such a way, that viability is maintained and regeneration is obtained after rewarming. In plant cryopreservation most of the protocols are carried out following an empirically optimized method. The physiological and morphological processes on cell and organ level in the different steps of the preculture, cryopreservation and post treatment up to the regeneration of the plant are so far not sufficiently analyzed and understood in detail. Without deeper understanding of such basic processes a further development of cryopreservation is difficult.
In this study potato is used as object for analyses because more than 1000 accessions of this plant have been cryopreserved effectively and are stored in the cryocollection of the Leibniz Institute of Plant Genetics and Crop Plant Research ([IPK]; IPK, 2007). This demonstrates the success of the applied DMSO droplet method. Furthermore, many other cryopreservation studies have been done with potato (Bajaj, 1977; Fabre and Dereuddre, 1990; Golmirzaie and Panta, 2000; Halmagyi et al., 2005; Kim et al., 2006b; Schäfer-Menuhr et al., 1994), which makes it a good object for comparative analysis. Potato is an important crop worldwide, whose breeding is traditionally based on clonal breeding. Therefore, the plants are highly heterozygous, and the seeds are not true to type. Thus, maintenance of cultivated potato accessions is not possible by seeds (Dodds et al., 1991). In genebanks, the plant material is maintained vegetatively. To preserve this crop for the long term, cryopreservation is the best option. Conclusions from experimental analyses within this study should give new results concerning the mode of action of the method, which can lead to improvement of the conservation method.

1.1. Potato as a worldwide important crop

Potato is one of the most frequently cultivated species on earth and ranks on place four in produced crops after maize, rice and wheat (FAOSTAT, 2007; Spooner and Bamberg, 1994). Annual production was 314 million tonnes in 2006 with China as the biggest producer (FAOSTAT, 2007). Among cereals and the root and tuber crops potato has the highest yield with 16.7 kg/ha. The cultivated potato is used for food, feed, medicine, processed foods (potato chips, alcoholic beverages) and industry products like plastic polymers (from potato starch). Thus, potato is clearly a crop of considerable world importance.

Potato belongs to the botanical family of *Solanaceae* and the genus *Solanum*, which consists of over 2000 species (Hawkes, 1978). Next to the cultivated potato *Solanum tuberosum,* seven other cultivated and 228 wild species exist, which belong to the section *Petota* containing the tuber-bearing potatoes (Hawkes, 1990). The origin of cultivated potatoes lies in the high Andes of South America and the costal strip of central to southern Chile (Hawkes, 1978). After the first potatoes were brought to Europe in the late 16th century, this crop plant was distributed and utilized all over the world mostly in temperate regions (Hawkes, 1990).
Today over 4000 varieties of *Solanum tuberosum* ssp. *tuberosum* exist (Hils and Pieterse, 2005). Because of the high number of varieties and related species, potato is known to have the richest genetic diversity of any cultivated plant (FAO, 2007; Messer, 2000). To prevent the loss of potato genetic resources, long term conservation of plant material is accomplished in genebanks and private collections worldwide (Tab. 1). For example in the IPK genebank (external branch Groß Lüsewitz, Germany), about 5900 accessions are conserved containing 2700 cultivated accessions and over 130 wild species (3200 accessions; IPK, 2007). Conservation of genetic resources in genebanks is important, because of their high value as breeding material, for research, industry and private needs.

1.2. Conservation of potato genetic resources

1.2.1. Field conservation

Cultivated potatoes are maintained vegetatively, because they produce highly heterozygous seeds (Dodds et al., 1991). For this reason collections are conserved clonally for preservation of individual genotypes. The simplest method of maintenance is cultivation in the field, where genetic integrity of the varieties can be verified directly (Schäfer-Menuhr, 1996). Potatoes are conserved as tubers, where the risk of loosing material through biotic and abiotic factors like diseases, pests, unfavorable weather conditions or other accidents, is very high. This method of conservation is widely applied for varieties which are needed frequently.

1.2.2. *In vitro* conservation

More stable conservation is given through *in vitro* cultures, where plants are cultivated under sterile conditions and constant environmental factors. To slow down the overall growth and lengthen the time of subculturing growth reducing conditions are used, such as application of ABA, mannitol, B9 and high sucrose concentration (Dodds et al., 1991; Thieme, 1992; Withers, 1985). In addition low temperatures and low light conditions lead to reduced growth

and, therefore, less work with the material. Advantages of *in vitro* storage are that preserved material is sterile, there is no risk of infections by insects or damage through inauspicious weather conditions and less work is needed than with field collections. Also cultures can be kept pathogen-free once viruses or other pathogens have been eliminated. Another advantage is that the varieties are available all year round (Schäfer-Menuhr, 1996). Disadvantages are that growth retardants alter plant morphology and can induce DNA methylation (Harding, 1994) and somaclonal variation (Kumar, 1994). Multiplication of plants is still necessary. Further mislabelling is another source of error and will not be as easily detected as in a field genebank (Schäfer-Menuhr, 1996). In the sterilization process, *in vitro* cultures are freed from superficially adhering contaminants. However, secondary infections through bacteria and fungi are possible. Endogenous bacteria, which grow within the cultures, are usually not a problem for *in vitr*o maintenance of potato. In the Groß Lüsewitz Cultivated Potato Collection Germany, situated in the Northern Branch Station of IPK, the potato *in vitro* collection is maintained mainly as micro tubers. These are induced through low temperature storage of plants at 9 °C and short day conditions (8 h photoperiod) and are stored afterwards at 4°C in the dark for up to two years (Thieme, 1992).

1.2.3. Cryopreservation

The best method to date for long term conservation of vegetatively propagated plants is cryopreservation, meaning storage of biological material in LN without losing viability. In cryopreservation, cell division, metabolic and biochemical processes are arrested and thus the plant material can be stored without deterioration or modification for a long period of time (Shibli et al., 2004; Zhu et al., 2006).

Advantages of cryopreservation are that material can be stored for theoretically indefinite time with low costs and little space. Work input is needed mainly at the beginning when samples are prepared and cooled. Once in storage, only refilling of LN is needed.

Other advantages are prevention of infections and genetic changes. Further, the degree of cleanliness is highest for cryopreserved explants after *in vitro* and field cultures. Cryopreservation is only useful for long term storage, because the material is normally not ready for immediate utilization and rewarming and growth of plant material takes some time.

Critical are the control and/or avoidance of intracellular ice nucleation. Ice nucleation affects the structural, osmotic and colligative integrity of cells causing physical ruptures and mechanical injury (Benson, 2008). Intracellular ice nucleation is prevented through freeze-induced dehydration, osmotic dehydration and evaporative desiccation. Extracellular ice

formation, as an example of freeze induced dehydration, withdraws free liquid water molecules through an osmotic gradient from the cytoplasma to intercellular spaces, where it crystallizes. Because of dehydration the cellular concentration of solutes rises and becomes too high to nucleate to ice crystals during cooling. This means that it solidifies without crystallization. This status is called the 'glassy state'. In this state the water molecules are amorphous and lack an organized structure but possess the mechanical and physical properties of a solid (Taylor et al., 2004). From the 'glassy state' water molecules can easily revert to liquid or devitrify to ice (Benson, 2008). Many cryopreservation protocols use chemical solutes like dimethyl sulfoxide (DMSO), sucrose and plant vitrification solutions (PVS, Sakai et al., 1990) to decrease the free water content in cells. DMSO and glycerol (both components of PVS2) are cell wall and membrane penetrable and increase cellular osmolality to avoid ice formation (Benson, 2008; Fig. 1). Sucrose being a disaccharide is a bigger molecule and can penetrate the cell wall, but not the plasma membrane (Tao and Li, 1986; Fig. 1). When cells are frozen, sucrose is concentrated in the cell wall space. Here it protects protoplasts from freeze-induced dehydration. It also can form a buffer layer between cell wall and the protoplast to protect the outer surface of the plasmalemma (Tao and Li, 1986).

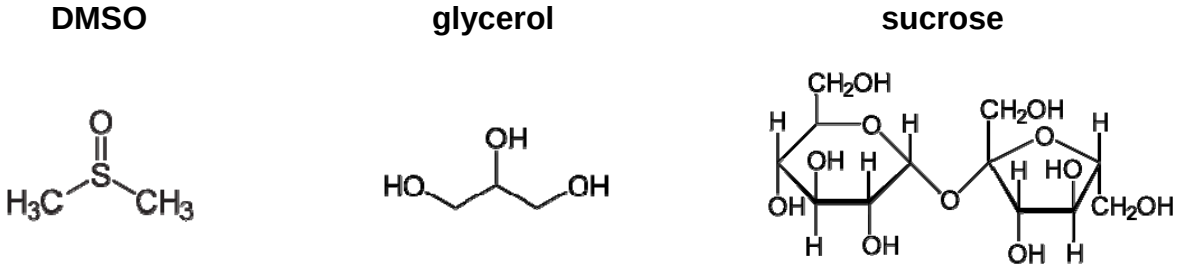


Figure 1. Chemical structures of DMSO, glycerol and sucrose (Falbe and Regitz, 1997, 1998).

To avoid ice formation during cryopreservation most protocols apply rapid cooling and rewarming techniques (e.g. quench cooling in LN ca. 1200 °C/min, Hirsh et al., 1985) to reach the direct transition of water structure from liquid to amorphous solid and *vice versa*.

Though endogenous bacteria usually are no problem for *in vitro* maintenance, they may be detrimental for rewarmed samples after cryopreservation. The samples are influenced through the stress, occurring during isolation, preculture, cooling and warming. After rewarming, bacteria can, therefore, dominate and kill the plant tissue.

Besides potato (Tab. 1), nowadays over 200 other plant species are cryopreserved all over the world (Engelmann, 1991; Harding, 2004; Reed, 2002; Sakai and Engelmann, 2007; Takagi, 2000). Material for plant cryopreservation are shoot tips, embryogenic cultures, excised

embryos, embryonic axes, buds, callus, hairy roots, pollen grains, protoplasts or cell suspension cultures (Engelmann, 1991; Panis and Lambardi, 2005; Reed, 2002). For cryopreservation of vegetatively propagated species, such as root and tuber crops, however, meristematic tissues are most common explants (Panis and Lambardi, 2005).

Table 1: European and American collections of potato (*Solanum sp.*) genetic resources. (VIR – N.I.Vavilov Research Institute of Plant Industry, Russia; IPK – Leibniz Institute of Plant Genetics and Crop Plant Research, Germany; CGN – Centre for Genetic Resources, The Netherlands; CRI – Crop Research Institute, Czech Republic; CPC – Commonwealth Potato Collection, Scotland/UK; CIP – International Potato Center, Peru; NRSP 6 – The US Potato Genebank, USA; PROINPA – The Potato Collection of the Proyecto de Investigacion de la Papa, Bolivia; INTA – The Potato Collection of INTA Balcarce, Argentina; CORPOICA – Corporacion Colombiana de Investigacion Agropecuaria, Columbia)

Institute	Collection total n	Cryopreservation collection n	Cryopreservation collection species	Literature
Europe				
VIR	9000	-	(no collection, but experiments started)	(Gavrilenko, 2008)
IPK	5900	1028	*S. tuberosum subsp. tuberosum*	(IPK, 2007)
CGN	2700	-	-	http://www.cgn.wur.nl/
CRI	2225	50	*S. tuberosum subsp. tuberosum*	(Faltus et al., 2008, Faltus, personnal communication 2008)
CPC	1300	-		http://www.scri.ac.uk/
America				
CIP	7544	446	*S. tub. subsp. andigena, S. phureja, S. stenotomum, S. goniocalyx, S. chaucha,* + hybrids	(Golmirzaie and Panta, 2000; Gonzalez-Arnao et al., 2008)
NRSP-6	nearly 5000	-	-	http://www.ars-grin.gov/
PROINPA	2288	-	(no collection, but experiments started)	www.proinpa.org
INTA	1842	-	(no collection, but experiments started)	(Mroginski and Rey, 2007, Clausen, personal communication 2008)
CORPOICA	900	-		http://www.redepapa.org/
The potato collection of Chile	687	-		http://www.uaustral.cl/

1.3. Cryopreservation of potato – literature review

1.3.1. Cryopreservation methods

In plant cryopreservation many different methods exist, which can be arranged in 3 major groups: two-step cooling, vitrification and encapsulation/dehydration (Benson et al., 2007; Keller et al., 2008b). For potato all of the above mentioned methods have been applied as well as combinations of them (Tab. 2; Gonzalez-Arnao et al., 2008; Keller et al., 2008a).

The first potato cryopreservation studies were done by Bajaj (1977; Tab. 2), who used a **two-step cooling procedure**. Herewith cell water content is reduced by freeze-induced dehydration. Firstly tuber sprouts and axillary buds are cryoprotected with different glycerol and/or sucrose solutions. Then they are cooled slowly in the vapor phase of LN and later directly in LN. Bajaj (1977) revealed between 7-18 % survival of tuber sprouts and axillary buds with this protocol. The method is rather complicated and time consuming (Schäfer-Menuhr et al., 1997) and needs special equipment (Kartha and Engelmann, 1994).

Simpler than the two-step cooling method is the **ultra-rapid freezing** method (Grout and Henshaw, 1978; Tab. 2). Shoot tips are isolated, precultured and cryoprotected with MS (Murashige and Skoog, 1962) medium containing sucrose and 10 % v/v DMSO. Afterwards each single shoot tip is directly plunged into LN on a tip of a hypodermic needle. Rewarming is done rapidly through plunging the needles directly into a vial of MS with benzyladenine at 35 °C. Then shoot tips are removed from solution and are put on regeneration medium. Grout and Henshaw (1978) could present one of the first successful cryopreservation protocols for potato shoot tips with survival rates of 20 % and shoot regeneration of 10 %. Important in this method is the rapid cooling, so that water is formed to amorphous ice and the rapid rewarming, so that devitrification does not occur on return to biological temperatures (Benson et al., 2006; Grout and Henshaw, 1978).

DMSO droplet method is an optimisation of the former method (Schäfer-Menuhr et al., 1994, Tab. 2). The term ‘droplet’ refers to droplets of cryoprotectant on an aluminium foil, into which the shoot tips are placed for cooling and rewarming. The original idea of using aluminium foils came from Kartha et al. (1982), who cryopreserved cassava shoot tips on foils in plastic Petri dishes using a two-step cooling method. Schäfer-Menuhr et al. (1994) used this idea for a fast cooling method of potato shoot tips. The foils make it easier to put a large number of shoot tips at once quickly into and out of LN, when compared with the hypodermic needle, where only one shoot tip at a time can be handled. Furthermore, aluminium is a very good heat conductor important for quick cooling and rewarming of

samples. For potato protocol, foils have the size of 20 x 7 x 0.03 mm (Schäfer-Menuhr, 1996), so that two foils fit into one cryovial. Concerning the state of water during cooling, no details of the ice nucleation characteristics of the droplet were presented in this study (Schäfer-Menuhr et al., 1994). But as observed in the study of Benson et al. (1992) using cassava, it is highly likely that vitrification occurs (Benson et al., 2006).

DMSO droplet method is applied in the IPK (Tab. 1) and up to date 1028 accessions are stored (IPK, 2007) with a mean regeneration percentage of 58 % (Kaczmarczyk et al., 2008). This collection includes the accessions formerly stored at German Collection of Microorganisms and Cell Cultures (DSMZ) and the Institute of Crop Science of the Federal Agricultural Research Centre (FAL, Braunschweig) (Schäfer-Menuhr et al., 1997), which have been integrated into the IPK collection (Keller and Dreiling, 2003).

In the **encapsulation/dehydration** method explants are encapsulated in sodium alginate beads prior to preculture followed by desiccation under a sterile air flow at room temperature or above silica gel (Fabre and Dereuddre, 1990; Grospietsch et al., 1999, Tab. 2). After desiccation material is slowly cooled (Fabre and Dereuddre, 1990) or put directly into LN. Rewarming follows slowly through exposure of beads in air at room temperature. For recovery, encapsulated samples are generally placed on culture medium without the need to extract the explants from their alginate coating (Gonzalez-Arnao and Engelmann, 2006). Limited growth recovery was obtained from slowly cooled shoot tips (Fabre and Dereuddre, 1990), whereas high survival could be obtained after rapid cooling (Grospietsch et al., 1999).

Vitrification is one of the main and most widely applied plant cryopreservation methods (Tab. 2). Sarkar and Naik (1998) applied vitrification protocol for potato shoot tips. Firstly explants are isolated and precultured on filter paper discs over half strength liquid MS supplemented with gibberellic acid (GA_3) and different combinations of sucrose and mannitol for 2 d. Then shoot tips are loaded firstly in 20 %, then in 60 % PVS2 solution (30 % w/v glycerol, 15 % w/v ethylene glycol, 15 % w/v DMSO in MS medium supplemented with 13.7 % w/v sucrose). Finally they are cryoprotected with 100 % PVS2 for 5 min in cryovials and afterwards plunged directly into LN. After quick rewarming in 35 °C water bath, PVS2 is removed and shoot tips are washed in dilution medium. Then shoot tips are cultivated on regeneration medium. Best results of this method gave survival of 54 % (Sarkar and Naik, 1998). In the International Potato Center (CIP) cryopreservation research began in 1995 using the vitrification method developed by Steponkus and collaborators (Golmirzaie and Panta, 1997; 1992). The vitrification solution in this method contained ethylene glycol : sorbitol : bovine serum albumin (50:15:6 wt %) and samples were cooled in

polypropylene straws. A number of 197 genotypes were cryopreserved with average survival of 46 % (Golmirzaie and Panta, 2000). In 2004, CIP in collaboration with Katholieke Universiteit Leuven, Belgium, adapted the droplet-vitrification method to potato (see below) originally designed for cryopreserving banana shoot tips (Gonzalez-Arnao et al., 2008; Panis et al., 2005).

Encapsulation/vitrification, which combines elements of vitrification and encapsulation/dehydration, alginate beads are precultured with sucrose solution, loaded with sucrose-glycerol-solution and dehydrated with PVS2 in cryotubes. Tubes are cooled directly in LN. Then cryotubes with beads are rewarmed rapidly in a water bath at 30 °C. PVS2 solution is removed. Beads are washed twice with 1.2 M sucrose solution and afterwards put on medium for regeneration (Hirai and Sakai, 1999, Tab. 2). In contrast to the encapsulation/dehydration method, beads are dehydrated by PVS2, which is much faster than desiccation in the laminar flow or over silica gel. Results of shoot formation after rewarming were also better for the encapsulation/vitrification (best with 70 %, Hirai and Sakai, 1999) compared to the encapsulation/dehydration (best with 59 %, Grospietsch et al., 1999).

Droplet vitrification, which combines elements of DMSO droplet method and vitrification, is a relatively new method for potato cryopreservation (Halmagyi et al., 2005; Panta et al., 2006, Tab. 2). Hereby shoot tips are treated as in the vitrification method, but before cooling, shoot tips are transferred to aluminium foils, which are put into cryovials and cooled in LN. After rewarming of vials in water bath, foils with explants are taken out and shaken in the unloading solution containing MS medium with 1.2 M sucrose, and afterwards shoot tips are put on regeneration medium. This method is quite successful; results are similar or better than in the vitrification method. The addition of foils makes the transfer of shoot tips into and out of LN easier, like in the droplet method.

In recent years the number of species cryopreserved using the combined droplet vitrification protocol with rapid cooling and rewarming is increasing (Gonzalez-Arnao et al., 2008; Halmagyi et al., 2005; Kim et al., 2006a; Kim et al., 2006b; Leunufna and Keller, 2005; Sakai and Engelmann, 2007; Sant et al., 2008; Senula et al., 2007; Yoon et al., 2006), whereas two-step cooling methods are used less often and mostly for callus and suspension cultures (Gnanapragasam and Vasil, 1992; Mikula et al., 2005). The reason for wider use of droplet vitrification lies in the fast and easy application, where no expensive cooling device as for two-step cooling is necessary.

Table 2. Cryopreservation methods applied for potato.

Method	Reference
two-step cooling	(Bajaj, 1977; Benson et al., 1989; Harding and Benson, 1994; Towill, 1981)
DMSO droplet method	(Barandalla et al., 2003; Schäfer-Menuhr and Schumacher, 1997; Schäfer-Menuhr et al., 1994; Schäfer-Menuhr et al., 1997)
encapsulation/dehydration	(Bouafia et al., 1996; Fabre and Dereuddre, 1990; Grospietsch et al., 1999)
encapsulation/vitrification	(Hirai and Sakai, 1999)
vitrification	(Golmirzaie and Panta, 2000; Kryszczuk et al., 2006; Sarkar and Naik, 1998; Zhao et al., 2005a)
droplet vitrification	(Halmagyi et al., 2005; Kim et al., 2006b; Panta et al., 2006)

1.3.2. Genetic stability

In theory all metabolic activities at temperatures of LN are reduced to zero, so that after rewarming from cryopreservation true-to-type plants are expected (Panis et al., 2001).

Potato is an example, where many studies on genetic integrity after cryopreservation have been performed. Morphological, phenotypic, cytological, biochemical and molecular comparisons were conducted revealing that plant material was genetically stable after cryopreservation.

Shoot tips of genotypically diverse (diploid, tetraploid and hexaploid) potato species exhibited normal developmental patterns (flowering, berry set and tuber formation) after regeneration from cryopreservation (Benson et al., 1996a). Cytological studies revealed that their ploidy status was maintained and chromosomal abnormalities were not observed (Benson et al., 1996a). Harding (1991) and Harding and Benson (2000) confirmed the stability of the ribosomal RNA genes (rDNA) as well as the nuclear-chloroplast DNA complex in the chloroplast genome in potato plants regenerated from cryopreserved shoot apices.

Between 96 and 252 regenerated plants from cryopreserved shoot tips of ‘Désirée’ and ‘Golden Wonder’ were compared to control material using microsatellite fragments. Microsatellite profiles in regenerated material were identical to those of the parental plants

and their progeny. This demonstrates the stable somatic inheritance of genomic regions containing the measured simple sequences (Harding and Benson, 2001).

In potato shoot tips using the DMSO droplet method, genetic stability was confirmed in a random sample of the collection using morphological parameters, flow cytometric measurements and restriction fragment length polymorphism (RFLP) analysis (Schäfer-Menuhr et al., 1997). The comparison of morphological parameters revealed only one abnormal and a few weak plants out of 98 varieties. The vitality was not stronger reduced than among control plants, which were not cryopreserved (Schäfer-Menuhr et al., 1997). No abnormalities were found for chromosome number and after RFLP analysis within 161 samples of regrown plants tested (Schäfer-Menuhr et al., 1996; Schäfer-Menuhr et al., 1997). Storage in LN for up to ten years was found to have no adverse effect on the regeneration rates (Keller et al., 2006; Mix-Wagner et al., 2002). Therefore, it seems to be safe to conclude that the cryopreservation technique does not induce somaclonal variation (Schäfer-Menuhr et al., 1997).

Dihaploid ($2n = 2x = 24$) potatoes and wild species *S. microdentum* and *S. pinnatisectum* produced more than one shoot after rewarming from cryopreservation (Ward et al., 1993). These shoots might have been originated from callus and could exhibit somaclonal variation. Ploidy levels of cryopreserved, regenerated plants were tested using flow cytometry. Only one tetraploid plant was found, which could be due to spontaneous doubling. Because the number of regenerated cryopreserved shoots, which showed abnormality was so low, it was concluded, that cryopreservation does not induce ploidy changes (Ward et al., 1993). The ability of plants derived from cryopreserved shoot tips to produce tubers was not affected by the cryopreservation process, unlike the formation of flowers, which was impaired compared to control, tuber-derived plants (Harding and Benson, 1994).

There is no evidence so far for direct function of DNA methylation in the change of genetic stability. Methylation changes in genomic DNA after cryopreservation were found in almond leaves (Channuntapipat et al., 2003), chrysanthemum shoots (Martín and González-Benito, 2006), and potato (Harding, 1997). DNA methylation can play a role in somaclonal variation, but with an unknown extent and unknown mechanistic basis of the process (Kaeppler et al., 2000). But Channuntapipat et al. (2003) and Harding (2004) suggest that these changes may not be induced by cryopreservation *per se* but are the results of the whole process of *in vitro* culture and regeneration.

1.3.3. Ultrastructural changes

Cryo-injury or cryopreservation damage of biological material can be caused by physical and biochemical events (Dumet and Benson, 2000). Mazur (1970; 1977) proposed a 'two-factor' hypothesis to account for cryo-injury based on the physical effects of ice crystal formation and the dynamic effects of freezing rate (Dumet and Benson, 2000). The first factor accounts for large intracellular ice crystals, which form during rapid cooling and cause mechanical damages. The second factor is the dehydration damage arising from extracellular ice crystal formation. At slow cooling rates, crystallization is induced extracellularly, so that damage arises through extreme osmotic dehydration, which occurs when intracellular, unfrozen water moves from the inside to the outside of the cell to compensate for the water vapor deficit as water freezes in the extracellular space (Steponkus et al., 1992).

Intracellular ice formation causes damages primarily on membranes (Li et al., 1979; Muldrew et al., 2004; Zhu et al., 2006). This damage can occur during freezing with ice crystallization or during thawing with recrystallization of ice. It has not yet been conclusively established whether intracellular ice formation is the cause of, or results from, damage to cellular components (Muldrew et al., 2004). Dowgert and Steponkus (1984) reported that in rye protoplasts, when cooled to the temperature of 50 % survival (LT_{50}), the plasma membrane was disintegrated through induced expansion. When protoplasts were isolated from cold-acclimated leaves, the predominant form of injury following cooling to -25 °C (LT_{50}) was loss of osmotic responsiveness. During freeze-induced dehydration, endocytotic vesicles formed in non-acclimated protoplasts whereas exocytotic extrusions formed on the surface of acclimated protoplasts. After thawing and osmotic expansion, the endocytotic vesicles remained in the cytoplasm and the protoplasts lysed before their original volume was regained. In contrast to this, the exocytotic extrusions were drawn back into the surface of acclimated protoplasts and they regained their original volume and surface area (Dowgert and Steponkus, 1984). Similar findings of membrane alterations and vesicle formation during freezing and rewarming were found by Steponkus (1984), who suggested that vesicles result from deletion of material of the plasma membrane during freeze-thaw cycles.

Ultrastructural studies on potato shoot tips were conducted by Grout and Henshaw (1980) and Golmirzaie et al. (2000). Histological and cytological differences to control explants could not be found directly after rewarming within the first 24 h. Afterwards both studies revealed that extensive damage was visible in shoot tip tissue after cryopreservation and rewarming. They reported cell wall rupture, rupture of epidermis, protoplast outflow, and anomalous nucleus shape of surviving and killed explants. Survival involved either regrowth of the shoot apex or

callus proliferation, although sometimes it was difficult to be certain whether the original apical meristem resumed growth or whether it was replaced by another meristem rapidly regenerated from the callus (Grout and Henshaw, 1980). Some studies indicate that the original meristem does not survive cryopreservation and that only small groups of cells are enough for regeneration of complete shoots (Fukai and Oe, 1990; Grout and Henshaw, 1980; Haskins and Kartha, 1980). Insufficient number of undamaged cells could lead to callus formation instead of shoot development (Grout and Henshaw, 1980).

The few studies describing histological and ultrastructural observations throughout the cryopreservation of potato (Golmirzaie et al., 2000; Grout and Henshaw, 1980) do neither include *S. tuberosum* cultivars nor the DMSO droplet method (Schäfer-Menuhr et al., 1994). Still, the ultrastructural changes occurring during this procedure can give important leads to understand and improve this method of crop preservation.

1.3.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) belongs to thermal analysis methods that can be used for measurement and determination of glass transitions and crystallization in cryopreservation (Bilavčík et al., 2007). It measures heat flow and water transitions in samples as a function of temperature and/or time (Benson et al., 2006; Höhne et al., 2003). These events are estimated from temperature differences between an empty reference and a filled sample pan, which are cooled and warmed at the same rate (Revilla and Martínez, 2002). The DSC instrument recalculates the differential heat flux and produces a heat differential graph. It analyses the physical state of water during cooling and heating. Transitions between liquid, amorphous glassy, and ice states can be detected by heat flow data through endo- and exothermic peaks. The point of change from the liquid to the glass states is the glass transition (Tg) temperature. Thermal profiles provide important information about the cryoprotective treatments required to obviate lethal ice formation and stabilize glasses (Benson et al., 2006).

Sherlock et al. (2005) used DSC to identify stable glass transitions during rewarming of beads containing *Ribes* meristems. Tg midpoint temperature ranged from -88 to -54 °C during rewarming. Benson et al. (1996b) applied DSC analysis to find phase change behaviour of water during cooling and rewarming of *Ribes* shoot tips using vitrification and encapsulation/dehydration methods. The study revealed that stable glasses are important, because during rewarming devitrification and ice growth can occur, which should be avoided. Benson et al. (1996b) demonstrated that cold acclimation and pre-growth in DMSO did not influence the nucleation and melt characteristics of *Ribes* shoot tips.

Volk and Walters (2006) found that exposure of mint and garlic shoot tips to PVS2 changed the behaviour of water within the shoot tips. Enthalpy of melting transitions decreased to about 40 J/g (compared to 333 J/g for pure H_2O), amount of unfrozen water increased to ~ 0.7 g H_2O/g dry mass (compared to ~ 0.4 g H_2O/g dry mass for unprotected shoot tips), and a glass transition at -115 °C was apparent. Santos and Stushnoff (2003) found that limited intracellular ice formation was not lethal in embryonic axes of *Citrus sinensis*, although survival was improved by further desiccation to completely eliminate ice formation. DSC analysis is a powerful tool to be applied for cryopreservation. Within this study thermal analysis was applied to potato shoot tips after preculture and cryoprotection using the DMSO droplet method. The results can give answer to the questions if and when glass transitions occur and how stable these glasses are. Further, crystallization can be detected and the amounts of frozen water can be determined. Interpretation of those results could lead to better understanding of fundamental processes of cryopreservation and support the improvement of the method.

1.3.5. Biochemical analysis and proteome research

1.3.5.1. Cold acclimation in plants

Plants can survive exposure to sub-zero temperatures and increase their freezing tolerance, when they are exposed to low, non-freezing temperatures. This process is known as cold acclimation (Smallwood and Bowles, 2002; Xin and Browse, 2000). It is a multigenic and quantitative trait with complex biochemical and physiological changes resulting in increased freezing tolerance (Hannah et al., 2005). Cold acclimation can be divided into two steps, the changes in metabolism, which are followed by induction of freezing tolerance. The changes in metabolism are considerable and affect growth and water balance, accumulation of compatible solutes (like proline, ß-alanine betaine, and glycine betaine), membrane and cell wall composition, antioxidant production, cold-regulated gene expression and protein levels (Hannah et al., 2005; Thomashow, 1999; Xin and Browse, 2000).

1.3.5.2. Cold preculture before cryopreservation

Cold precultures of donor plants before cryopreservation are known to improve results for woody (Chang and Reed, 2000; Niino and Sakai, 1992; Reed et al., 2003) and herbal species (Keller, 2005; Seibert and Wetherbee, 1977), which are able to cold-acclimate to low temperatures. Sometimes the effect of cold acclimation can be replaced by incubation in

sucrose solutions (Dumet et al., 2000). Also in other species like *Zoysia matrella*, *Z. japonica* and yam, which are not usually able to acclimate to cold temperatures, cryopreservation results could be improved after exposure to low temperatures (Chang et al., 2000; Leunufna and Keller, 2005).

1.3.5.3. Biochemical effects of cold preculture and pretreatments before cryopreservation

The aim of cold preculture or incubation in sucrose solutions (Grospietsch et al., 1999) is to reduce or avoid intracellular ice formation during cooling. During the precultures the metabolism of donor plants or explants changes. These changes can be well monitored through biochemical analyses.

Grospietsch et al. (1999) revealed an increase in soluble sugars and proline after sucrose preculture of potato shoot tips, which led to better cryopreservation results. Similar results, concerning increase in soluble sugars after preculture, were found in *Arabidopsis thaliana* suspension cells (Bachiri et al., 2000), in axillary buds of *Gentiana scabra* (Suzuki et al., 2006), and in sugar beet shoots (Vandenbussche et al., 1999). In potato, low temperature precultures were applied before cryopreservation using encapsulation/vitrification and droplet vitrification methods, but with weaker results when compared with the original protocols (Halmagyi et al., 2005; Hirai and Sakai, 2000; Kryszczuk et al., 2006). However, Kryszczuk et al. (2006) used an alternating temperature preculture of 7 d with 21/8 °C (day/night temperature) and could improve results in 4 potato accessions tested applying the DMSO droplet method. At IPK preliminary experiments showed that alternating temperature preculture of 7 d with 22/8 °C (day/night temperature; AT) led to improvement of the regeneration after cryopreservation using the DMSO droplet method (IPK, 2005). From field and greenhouse experiments it is known, that cultivated potatoes (*S. tuberosum*) belong to the group of frost sensitive potatoes and are unable to acclimate to low temperatures (Li, 1977), whereas other species like *S. acaule* and *S. demissum* belong to the frost resistant potatoes and are able to cold-acclimate (Li and Fennel, 1985). The determination of cold tolerances of *in vitro* plants using the above mentioned AT was expected to give insight if cold acclimation takes place. Determination of soluble sugars, starch and proteins should further show, which metabolites undergo changes under different preculture conditions.

Also osmolality can give information about the physiological state of *in vitro* plants. Osmolality describes the concentration of osmotically active particles in 1 l solution. It is a measure of the number of particles present in solution and is independent of the size or weight

of the particles. Osmolality or colligative properties of a solution can be measured using an osmometer, which, for example, determines the concentration of osmotically active particles that reduce its vapor pressure. The determined osmolality stands for the concentration of dissolved solutes in the cell. This concentration should be higher for those tissues with lower free water content, meaning that tissue with high osmolality reaches glass transition easier during cooling in cryopreservation.

1.3.5.4. Proteome studies in potato

Further biochemical characterization of explants can be accessed through the determination of the plant protein expression. Proteomics approaches are applied to analyze the complex functions of plants at different levels and are supported through the progress achieved in sensitive and rapid protein identification by mass spectrometry (Canovas et al., 2004).

In potato proteome studies have been done mainly on tuber tissue (Bauw et al., 2006; Delaplace et al., 2005; Lehesranta et al., 2006; Lehesranta et al., 2005) and in connection with phytopathogens such as *Erwinia carotovora* (Coulthurst et al., 2006), *Pectobacterium atrosepticum* (Mattinen et al., 2007) and the aphid *Macrosiphum euphorbiae* (Nguyen et al., 2007). Protein expression studies in combination with cryopreservation are a quite new field. Capentier et al. (2007) used banana as model to study protein expression associated with sucrose-mediated osmotic stress in the meristem. They developed a 2D protocol for small amounts of tissue and used polypeptide databases obtained by mass spectrometry for identification of proteins from closely related plant species. The results suggests that maintenance of an osmoprotective intracellular sucrose concentration, the enhanced expression of particular genes of glycolysis and the conservation of the cell wall integrity are essential to maintain homeostasis, and to acclimate and survive dehydration before cryopreservation (Carpentier et al., 2007). An optimised protocol using phenol was applied for the extraction of proteins from potato shoot tips. It was developed by the same research group, but its results were not further evaluated and no identification of proteins was presented (Carpentier et al., 2005). Therefore, a proteomics approach for potato shoot tips would be a new application area and of great value to study differences in preculture treatments and differences between species.

1.4. Aims of the dissertation project

The aim of this study is to find fundamental aspects in ultrastructure, biochemical compounds and thermal analysis which can explain the cryopreservation process and results. Potato is used as a model organism to analyze the various parameters involved in the DMSO droplet protocol for shoot tips of *in vitro* cultures (Fig. 2). The present work investigates the effect of preconditioning (AT), cryoprotection, cooling, rewarming and regeneration of cryopreserved apical shoot tips. Comparison between cold-tolerant wild species and cold-sensitive cultivated potatoes will be performed. Results should give answer to the question, whether cold acclimation can be correlated to cryopreservation results. The study aims at analyzing the factors affecting the recovery of cryopreserved shoot tips and to understand fundamental actions of cryopreservation. Therefore, the study also includes histological and ultrastructural analyses by transmission electron microscope (TEM) after different steps in the protocol. With these, the damaging influences of LN as well as repair and regeneration processes are intended to be studied. Furthermore, it was planned to perform biochemical analyses of soluble sugars, starch, amino acids, and proteins as well as thermal analyses by DSC. The synthesis of these results can help to find causes for genotype effects and to improve reactions of accessions with low regeneration. Moreover, it was intended to apply the results for methodical and/or technical improvement of cryopreservation for potato.

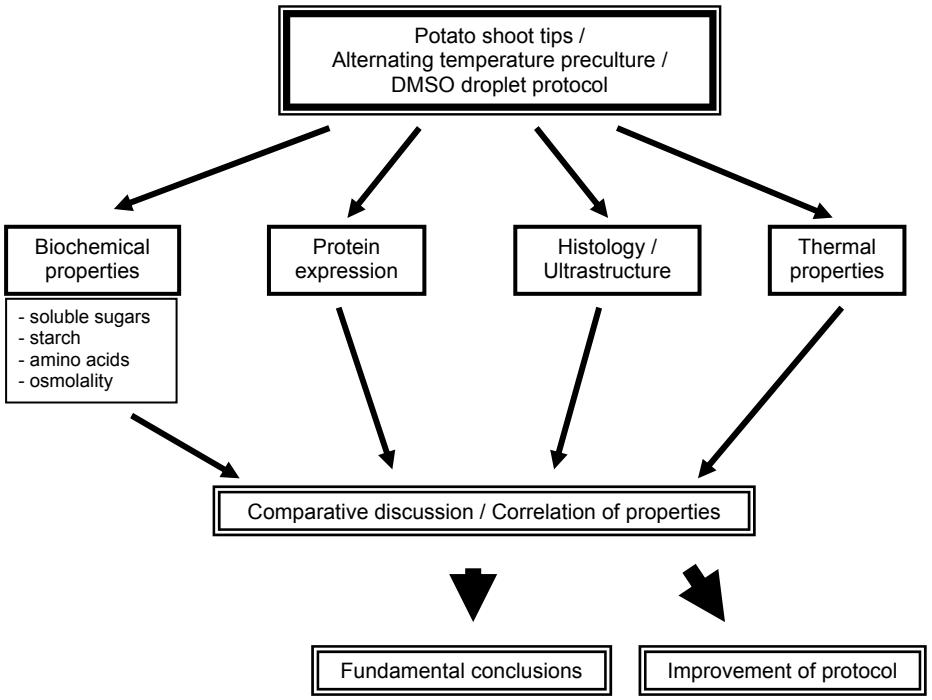


Figure 2. Overview of analyses of fundamental research in the field of cryopreservation of potato.

Chapter 2. Materials and Methods

2.1. Plant material

The varieties of *Solanum tuberosum* 'Désirée', 'Ackersegen', 'King Edward', as well as the material of *S. acaule*, and *S. demissum* were received from the Cultivated Potato Collection, Germany, situated in the Northern Branch Station of IPK in Groß Lüsewitz. 'Désirée', 'Ackersegen', and 'King Edward' were supplied as *in vitro* plants, *S. acaule* as tuber and *S. demissum* as seeds.

2.1.1. Explant sterilization

Tubers of *S. acaule* were put in the dark for sprouting for 8 weeks. Dark-grown sprouts were cut in nodal segments and rinsed in 70 % ethanol. Afterwards segments were placed into an Erlenmeyer flask and shaken in sodium hypochlorite solution with 3 % active chlorine for 10 min. Then segments were washed three times with sterile water under sterile conditions. Nodal segments were dried on filter paper and bleached ends were cut off. At last segments were put onto MS medium (Murashige and Skoog, 1962) with 3 % sucrose for the establishment of *in vitro* culture.

S. demissum seeds were also washed in sodium hypochlorite solution with 3 % active chlorine for 10 min and rinsed with sterile water afterwards. Seeds were placed on MS medium with 3 % sucrose for germination.

From each wild species one clone was chosen, multiplied and used for experiments.

2.1.2. Cultivation of *Solanum sp.*

Plants were grown *in vitro* on MS medium supplemented with 2 % sucrose and 10 g/l agar. They were maintained under a 16 h photoperiod at 22 °C (light intensity 70 µmol/[s m^2]) in a PERCIVAL incubator (Perry, Iowa, USA).

In the greenhouse, plants were grown in soil in 2.5 l pots under a 16 h photoperiod at 22 °C and relative humidity of 70 %.

2.2. Temperature programmes in preculture of donor plants

2.2.1. Constant temperature preculture (CT)

Plants were grown for 4 weeks under the conditions as described in 2.1.2.

2.2.2. Alternating temperature preculture (AT)

Plants were grown for 3 weeks under the conditions as described in 2.1.2., followed by one week at 22/8 °C (day/night temperature) under an 8 h photoperiod. Light intensity was 70 µmol/(s m^2) for *in vitro* cultures.

2.2.3. Preculture variants other than CT and AT

Plants were grown for 3 weeks under the conditions as described in 2.1.2., followed by different incubation times (3, 7, and 14 d) at 25/-1 °C (day/night temperature) under an 8 h photoperiod or constant temperature of 2 °C under an 16 h photoperiod (light intensity 70 µmol/[s m^2]).

2.3. Cryopreservation

2.3.1. Isolation, pretreatment and cooling

The protocol after Schäfer-Menuhr et al. (1994) was used with some modifications. Apical shoot tips (approximately 3 mm long and 0.5 mm thick, Fig. 19a) were isolated and placed in liquid MS solution with 0.5 mg/l zeatin riboside, 0.5 mg/l indole acetic acid (IAA), 0.2 mg/l GA_3, and 3 % sucrose (MSH) overnight. On the second day shoot tips were placed in cryoprotectant solution (MSH with 10 % DMSO) for 2 h. They were then transferred into 2.5 µl drops of cryoprotectant solution placed on a small piece of heat-sterilized aluminium foil and quickly cooled in LN. Foils were transferred to cryovials and stored in LN for 1 h.

2.3.2. Rewarming

Shoot tips were rewarmed by placing the aluminium foils in liquid MS medium at room temperature for 1 to 5 min. Afterwards liquid and solid regeneration media were compared. For regeneration on liquid medium, shoot tips were placed in drops of warmed agarose in small plastic Petri dishes. After solidification of the drops, liquid MSH solution was added (Schäfer-Menuhr et al., 1994). In contrast to the above mentioned method, shoot tips were placed directly after rewarming on solid medium (MSH with 10 g/l agar). Subsequently Petri dishes of both variants were cultivated for regeneration in a climate room with 25/20 °C (day/night temperature) and 16 h photoperiod (light intensity about 50 µmol//[s m^2]).

2.4. Determination of cold tolerances

The electrolyte leakage method is used to determine the lethal freezing temperatures (LT_{50}) of plants (Dexter et al., 1932). LT_{50} is the freezing temperature, at which the rewarmed tissue loses 50 % of its total ions (Palta and Li, 1978). In the experiment LT_{50} was calculated from conductivity measurements of ion efflux from plant material.

Leaves of greenhouse cultures or *in vitro* plants without roots were put in test tubes filled with 200 µl distilled water and cooled in a silicon-oil bath (HUBER CC 130) at a rate of 1.5 °C/h and temperature range of 0.0 to -13.0 °C. At -1.0 °C ice crystallization was induced and at certain temperature steps (0.0, -1.0, -2.5, -3.0, -4.0, -5.5, -8.0, -10.0, -13.0 °C) samples were rewarmed over ice. Control plants were kept on ice for the same time. When samples had thawed, 5 ml of distilled water was added and tubes were put on a shaker at 4 °C overnight. Electrolyte leakage was determined after freezing using a conductivity cell (Konduktometer HI 8820, HANNA Instruments). The tubes with samples were boiled in a water bath for 30 min to determine the total ion content in the leaves. Afterwards the ion concentration of the water surrounding the tissue was determined. Freeze damage is expressed as percentage electrolyte leakage before and after boiling of plant material in water. LT_{50} of the non-frozen control samples were subtracted from results for correction. For better comparison of results, LT_{50} was normalized to the maximal leakage of the first sample measured.

Each LT_{50} was determined as mean of three repetitions. For graphical analysis the software program PRISM 3.0 was used.

2.5. Determination of osmolality

In vitro plants without roots were mashed with a leaf press (Wescor® LP-27, Langenfeld) and then the plant sap was measured with a vapour pressure osmometer (Wescor VAPRO® 5520, Langenfeld). Three *in vitro* plants were used per each measurement, with ten repetitions per accession.

2.6. Determination of soluble sugars and starch

Soluble sugars and starch were quantified in shoot tips homogenized under LN, extracted with 80 % ethanol and measured in an enzyme-coupled assay according to Hajirezaei et al. (2006). For soluble sugar determination, the homogenized shoot tips were extracted in ethanol at 80 °C for 1 h. Samples were centrifuged at 14000 rpm at 4 °C for 5 min. Afterwards the supernatant was transferred to a new tube and dried in a SARANT Speed Vac® (SPD 101B)

for 2.5 h. Samples were resuspended with 250 µl distilled water. Samples of 10 µl were put together with the measuring buffer. It consisted of 290 µl of 100 mM imidazol (pH 6.9), 5 mM magnesium chloride, 90.4 mM NAD, 39.4 mM ATP and 2 U/ml $NADP^+$-linked glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*). To start the reactions, the respective enzymes were sequentially added to the following final concentrations: for glucose 1 U/ml hexokinase, for fructose 0.5 U/ml phosphoglucose isomerase, and for sucrose 11.5 U/ml invertase (ß-fructosidase). The change in absorbance was continuously followed at 340 nm using an Ultra Microplate Reader (EL 808, BIO-TEK Instuments).

Starch content was measured using the pellet of the extraction. The pellet was washed twice with 80 % ethanol. Than 200 µl 0.2 M potassium hydroxide was added to the pellet. The pellet was incubated at 4 °C overnight. Another volume of 200 µl of potassium hydroxide was added and the samples were incubated at 95 °C for 1 h. Then samples were neutralized with 70 µl 1 N acetic acid and centrifuged. The volume of 100 µl supernatant was added to 100 µl solution 1 (2 mg/ml amyloglucosidase in 50 mM sodium acetate, pH 5.2). Samples were incubated overnight at 37 °C, and afterwards glucose was measured in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase. Three experiments with seven to ten repetitions each, one repetition consisting of 20 to 50 shoot tips, were performed.

2.7. Determination of amino acids

Amino acids of shoot tips were measured using a reversed-phase HPLC system as described by Rolletschek et al. (2002). The same material was used as for soluble sugar and starch analyses.

2.8. Proteome analysis

Protein expression of shoot tips of *S. tuberosum* ‘Désirée’ and *S. demissum* were accomplished after CT and AT. Three biological replicates, each consisting of 3 technical replicates were analyzed per accession and treatment (total of 36 gels, Fig. 3).

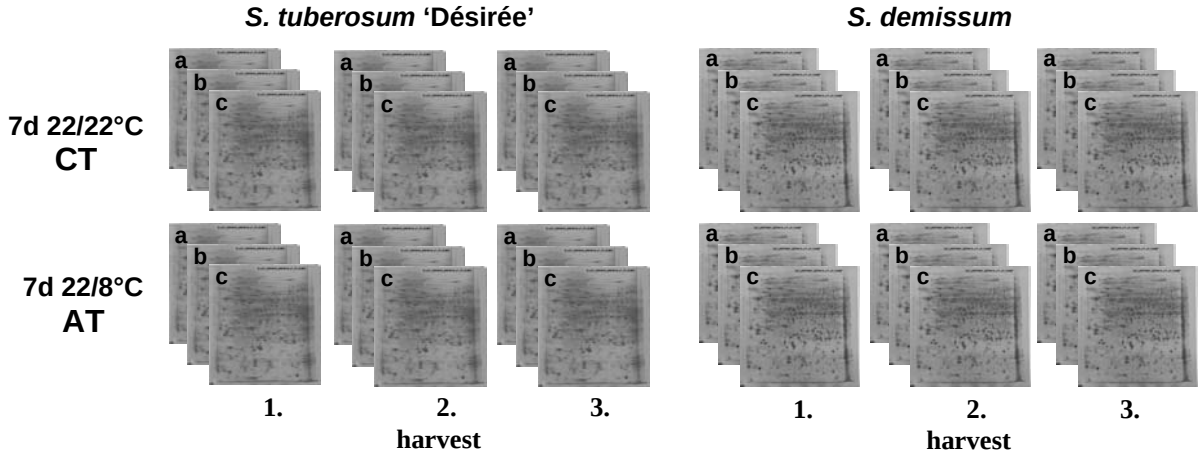


Figure 3. Overview of produced gels for potato proteome approach. 3 biological experiments (harvest 1-3) with each 3 replicates (a-c) were performed. Comparison of two potato species (*S. tuberosum* 'Désirée' and *S. demissum*) as well as comparison of two different precultures (CT, AT) were made.

2.8.1. Extraction

Two precipitation methods were compared. These were TCA/acetone precipitation (Amme et al., 2006) and phenol extraction/ammonium acetate precipitation (Carpentier et al., 2005).

Approximately 300 mg material per biological treatment (between 300 and 700 shoot tips) was ground under LN in a cooled mortar to homogeneous flour.

TCA/acetone precipitation: Aliquots of 100 mg flour were washed in 1 ml 10 % TCA/0.07 % 2-ME/acetone, cooled for 30 s in LN, and proteins were precipitated at -20 °C for 45 min. Samples were shaken after 5, 10, and 15 min. After centrifugation (18000 rpm, 4 °C, 5 min), supernatant was discarded. The pellet was resuspended in 1.5 ml acetone and 0.07 % 2-ME, shaken and put into ultrasonic bath for 5 min. Samples were cooled in LN for 30 s and incubated at -20 °C for 30 min. After centrifugation (18000 rpm, 4 °C, 5 min), supernatant was discarded. After repetition of acetone wash, pellets were dried in a vacuum centrifuge for 10 min. A pellet of 1 mg protein was dissolved in 50 µl RH-A buffer (8 M urea, 2 % CHAPS, 20 mM DTT, 0.5 % IPG) by incubating at 37 °C on a shaker for 1 h. Insoluble material was pelleted by centrifugation (18000 rpm, room temperature, 15 min; Amme et al., 2006).

Phenol extraction/ammonium acetate precipitation: Ground material was resuspended in 500 µl of ice-cold extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM potassium chloride, 1 % w/v DTT, 30 % w/v sucrose, complete protease inhibitor cocktail; Roche applied Science) and shaken for 30 s. Then 500 µl of ice-cold Tris buffered phenol (pH 8.0) was added, and the sample was shaken at 4 °C for 15 min. After centrifugation (6000 x g, 4 °C, 3 min) the phenol phase was collected, re-extracted with 500 µl of extraction buffer, and

shaken for 30 s. Subsequent to centrifugation (6000 x g, 4 °C, 3 min) the phenolic phase was collected and precipitated overnight with five times of the sample volume of 100 mM ammonium acetate in methanol at -20 °C. Pellet was air-dried, re-suspended in 100 µl lysis buffer (8 M urea, 2 % CHAPS, 20 mM DTT, 0.5 % IPG) and shaken at room temperature for 1 h (Carpentier et al., 2005).

2.8.2. Protein quantification

The protein concentration was determined using the 2D Quant Kit (Amersham/GE Healthcare) following the manufacturer's instructions.

2.8.3. Two-dimensional gel electrophoresis and protein staining

For ruthenium stained gels, 100 µg of protein and for cCBB stained gels 200 µg of protein were loaded by rehydration on IPG strips of 13 cm in length with a pH gradient of 4-7. The following parameters were used for separation on an IPGphor II unit (Amersham/GE Healthcare): 14 h rehydration, 1 h gradient to 250 V, 1 h gradient to 500 V, 1 h gradient to 4000 V and 5.35 h 4000 V with a total of about 25 kVh. After IEF, strips were equilibrated for 15 min in buffer A (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % v/v glycerin, 2 % w/v SDS, 20 mM DTT, 0.01 % bromphenol blue). Strips, designated for RuBP staining, were equilibrated additionally in buffer B (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerine, 2 % w/v SDS, 135 mM iodacetamide, 0.01 % bromphenol blue) for 15 min prior to SDS-PAGE.
Strips were than placed on top of an 11.25 % SDS polyacrylamide gel and covered with 0.5 % agarose. Separation in the second dimension was carried out using a Hoefer S600 apparatus (Amersham/GE Healthcare). Afterwards gels were washed for 5 min with water, and proteins were visualized with either RuPB staining using ruthenium(II)-tris-(bathophenanthrolin-disulfonate) sodium salt solution (Fluka) following the protocol of Lamanda et al. (2004) or with cCBB staining using GelCode®Blue Stain Reagent (Pierce Chemical Company) following the manufacturer's instructions.

2.8.4. Image acquisition and analysis

Image acquisition was performed using a UMAX Power Look III scanner (Umax Systems GmbH, Willich, Germany) with the MagicScan software (v4.5, Umax) for cCBB stained gels. The Fuji FLA-5100 (Fuji Film, Tokyo, Japan) with the image reader FLA-5000 v1.0 software was used for RuBP stained gels. Scanning parameters were: resolution 100 µm, 16 bit picture, excitation wavelength 473 nm, emission filter 580 nm. Progenesis SameSpots v2.0.27

software using default parameters was applied for comparative image analysis (Witzel et al., 2007).

2.8.5. Mass spectrometry

Identification of proteins was carried out using mass spectrometry. Excision of protein spots from the gel was performed manually. After washing with 400 µl 10 mM ammonium bicarbonate including 50 % AcN for 30 min, the gel pieces were dried. A volume of 7.5 µl trypsin solution (Sequencing Grade Modified Trypsin V511, Promega, Madison, USA, 10 ng/µl in 5 mM ammonium bicarbonate including 5 % AcN) was added to each sample for the digestion of proteins, followed by an incubation step at 37 °C for 5 h. The digestion was stopped by adding 1 µl 1 % TFA.

The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed as follows. A volume of 1 µl digest was mixed with 2 µl of the matrix solution (5 mg α-cyano-4-hydroxycinnamic-acid in 80 % v/v acetonitrile and 0.1 % w/v TFA) and 1 µl of this mixture was deposited onto the matrix-assisted laser desorption/ionization target (Bruker Daltonics, Bremen, Germany). The acquisition of Peptide Mass Fingerprint data was performed on a REFLEX III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Bruker Daltonics) operating in reflector mode. Spectra were calibrated using external calibration and subsequent internal mass correction under application of the flexAnalysis 2.4 software (Bruker Daltonics). Protein identification was performed with the Mascot search engine (Matrix Science, London, UK) (Perkins et al., 1999) searching the Nonredundant for *Viridiplantae* Database of the National Center for Biotechnology Information (NCBInr) and within a combined database of expressed sequence tags (EST) sequences from *Solanaceae* sequences from The Institute for Genomic Research (TIGR). Parameters for the search were the following: monoisotopic mass accuracy 200 ppm, missed cleavages 1, allowed variable modifications oxidation (methionine), propionamide (cysteine) and carbamidomethyl (cysteine).

Proteins not identified by matrix-assisted laser desorption/ionization mass spectrometry were subjected to Nano-Liquid-Chromatography-Electrospray-Ionization-Quadrupol-Time-Of-Flight (nanoLC-ESI-Q-TOF) tandem mass spectrometry. For the nanoLC-ESI-Q-TOF tandem mass spectrometry and the *de novo* sequencing experiments, 2 µl of the digest was subjected to nanoscale reversed phase liquid chromatography analysis on a nanoAcquity ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA). The mobile phase flow from the binary pump was used to preconcentrate and desalt the digest

samples on a 20 mm x 180 µm Symmetry 5 µm C18 precolumn (Waters Corporation) for 3 min at 4 µl/min with an aqueous 0.1 % formic acid solution. The peptides were subsequently eluted onto a 100 mm x 75 µm analytical 1.7 µm BEH C18 column (Waters Corporation) and separated at 0.6 µl/min with an increasing AcN gradient from 5 % to 40 % B in 30 min. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B of 0.1 % formic acid in AcN. The nanoscale LC effluent from the analytical column was directed to the NanoLockSpray source of a Q/TOF Premier hybrid orthogonal accelerated Time-of-Flight (oa-TOF) mass spectrometer (Waters Corporation, MS Technologies Centre, Manchester, UK). The mass spectrometer operated in a positive ion mode with a source temperature of 80 °C and a cone gas flow of 30 l/h. A voltage of approximately 2 kV was applied to the nano flow sample tip. The mass spectra were acquired with the TOF mass analyzer in V-mode of operation and spectra were integrated over 1 s intervals. Mass spectrometry and tandem mass spectrometry data were acquired in a continuum mode using MassLynx 4.1 software (Waters Corporation, Technologies Centre). The instrument was calibrated with a multi-point calibration using selected fragment ions of the collision induced dissociation of Glu-Fibrinopeptide B (SIGMA-ALDRICH Chemie GmbH, Taufkirchen, Germany). Automatic data directed analysis was employed for tandem mass spectrometry analysis on doubly and triply charged precursor ions. The product ion tandem mass spectrometry spectra were collected from *m/z* 50 to *m/z* 1600. Lock mass correction of the precursor and the product ions was conducted with 150 pmol/µl Glu-Fibrinopeptide B in 0.1 % formic acid in AcN/water (1:1, v/v) respectively, and introduced via the reference sprayer of the NanoLockSpray interface. ProteinLynx Global*SERVER* v2.3 software was used as a software platform for data processing, deconvolution and *de novo* sequence annotation of the spectra, and various database search types. The tandem mass spectrometry spectra searches were conducted with a protein *Viridiplantae* index of the SwissProt database and with the potato EST Gene index of the TIGR database. A 10 ppm peptide, 0.1 Da fragment tolerance, one missed cleavage, and variable oxidation (methionine) and propionamide (cysteine) were used as the search parameters.

2.9. Ultrastructural analysis

For ultrastructural studies, shoot tips were chemically fixed with 2 % glutaraldehyde and 2 % formaldehyde in cacodylate buffer (50 mM, pH 7.0) for 16 h. After three washes of 15 min each with the same buffer, the probes were postfixed with 1 % osmiumtetroxide for 1 h. The samples were washed with buffer and distilled water and subsequently dehydrated in a graded

ethanol series followed by embedding in agar low viscosity resin. Longitudinal sections of 3 µm were cut on a Reichert-Jung Ultracut S microtome (Leica, Vienna, Austria) and stained with methylene blue. Digital recordings were made on a Zeiss Axiovert microscope equipped with an Axiocam (Carl Zeiss, Jena, Germany). After ultra-thin sectioning (70 nm), samples were stained with 4 % uranyl acetate and examined in a Tecnai 20 TEM at 80 kV. Digital recordings were made with the MegaView III software (Soft Imaging System GmbH, Münster, Germany).

2.10. Differential scanning calorimetry

Thermal analysis of potato shoot tips and different solutions was undertaken using a differential scanning calorimeter (DSC) Q2000 with LN cooling system (TA Instruments - Waters, New Castle, Delaware, USA). The system was calibrated with indium (melting point 156.6 °C, enthalpy 28.71 J/g). Samples were weighed on a Sartorius microbalance ME235S (accuracy ± 10 µg; Sartorius, Göttingen, Germany) and placed in 40 µl aluminium pans, which were hermetically sealed. Water state assays were performed with different solutions, each containing 20 µl. These were MSH solution, cryoprotectant solution (10 % DMSO in MSH), 99.5 % w/v DMSO, and PVS2 (30 % w/v glycerol, 15 % w/v ethylene glycol, 15 % w/v DMSO, 13.7 % w/v sucrose in MS medium). Further, DSC measurements were made with freshly isolated and with cryoprotected (MSH with 10 % DMSO for 2 h) shoot tips of *S. tuberosum* 'Désirée' and *S. demissum* after CT or AT. 'Désirée' shoot tips were also measured after incubation in PVS2 for 20, 30, 40, and 60 min.

In most samples cooling and warming was measured in the DSC device. The following DSC programme was used for solutions, freshly isolated shoot tips and shoot tips incubated in cryoprotectant solution (10 % DMSO in MSH): samples were cooled at a rate of 10 °C/min to -160 °C, at -160 °C samples were held isothermally for 5 min before ramping up to 30 or 40 °C at a rate of 10 °C/min. DSC programme used for 'Désirée' shoot tips incubated in PVS2, was the same as above, but cooling was done only until -140 °C. For each single measurement 3 to 5 shoot tips were used for one run. The runs were repeated between 5 and 8 times, always with new material.

Some samples of *S. tuberosum* 'Désirée' were cooled in hermetically sealed pans outside the DSC directly in LN. Only warming was measured in DSC device. After transfer of pans in pre-cooled DSC at -180 °C the following programme was used: isothermally hold for 10 min at -180 °C and afterwards rewarming until 40 °C at a rate of 10 °C/min. For this analysis 3 repetitions were made.

Results were analyzed with the software programme Universal Analysis 2000 Version 4.3A (TA Instruments - Waters, New Castle, Delaware, USA). Onset temperatures of crystallization and melting, midpoint temperature of glass transition (Tg), and enthalpies were calculated. Onset temperatures are the points of intersection between the baseline and the slope of the peak's steepest portion (Sun, 1999). Enthalpies were determined from the area of the peaks above or below the interpolated baseline.

2.11. Chemicals

Chemicals were mainly purchased from Duchefa Biochemie B.V. (Haarlem, The Netherlands), Roche (Mannheim), Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt). Sucrose, sodium hypochlorite and ethanol were purchased from Carl Roth GmbH (Karlsruhe), agar by SERVA Electrophoresis GmbH (Heidelberg), agarose from Life Technologies (Grand Island, NY, USA) and LN from Praxair, Düsseldorf.

2.12. Enzymes

All biochemical enzymes were purchased from Roche (Mannheim).

2.13. Consumable supplies

Not included are consumable supplies, which belong to the standard equipment of a lab.

in vitro culture glasses (375 ml)	Thierfelder Fleischereibedarf, Giersleben
cryovials	Nunc GmbH & Co. KG, Wiesbaden
sterile plastic Petri dishes	Nunc GmbH & Co. KG, Wiesbaden
Dewar	KGW Isotherm, Karlsruhe

2.14. Statistical analysis of data

Statistical analysis for cryopreservation experiments was done using the chi square (χ^2) test. Differences between groups of data (sugar, starch, amino acid analysis) were calculated using the t-test, or if normality test failed, using the Mann-Whitney test. Statistical analysis was done using the software SigmaStat 3.1. (Systat Software, Inc.).

Chapter 3. Results

3.1. Cryopreservation

3.1.1. Optimization of exposure time and temperature conditions in preculture

Three cultivated potatoes (*S. tuberosum* 'Ackersegen', 'Désirée', and 'King Edward') and one wild species (*S. demissum*) were used as material to find optimal preculture conditions. Donor *in vitro* plants were exposed to 4 different temperature regimes: 22/22 °C, 2/2 °C, 22/8 °C, and 25/-1 °C (day/night temperatures), with 16 h photoperiod for constant temperatures and 8 h photoperiod for alternating temperatures. Furthermore, 3 different preculture durations (3, 7 and 14 d) were compared.

Best average results were received after preculture with 22/8 °C for 7 d in comparison with constant temperature conditions (22/22 °C 7 d, Fig. 4). Also plants precultured with 25/-1 °C showed high regeneration percentages, but with this temperature regime, sometimes plants died or showed yellow leaves before isolation of shoot tips so that these plants could not be used for the cryopreservation experiments. Concerning duration of preculture, regeneration of shoots after 7 d was, in average, better as or equal to that after 3 d, whereas regeneration percentages decreased after 14 d preculture in 'Ackersegen' and 'King Edward'. Because of these results, it was decided to use the alternating temperature preculture at 22/8 °C for 7 d (AT) in comparison with the constant temperature conditions at 22 °C for 7 d (CT) for further experiments. Two cultivated potatoes (*S. tuberosum* 'Désirée' and 'King Edward') and two wild potatoes (*S. acaule* and *S. demissum*) were chosen as material for further analyses.

3.1.2. Cryopreservation results after AT

In comparison to control conditions, an increase in shoot regeneration capacity was observed in *S. tuberosum* 'Désirée', 'King Edward', *S. acaul*e and *S. demissum in vitro* plants after rewarming from cryopreservation using AT (Tab. 3). Similar results were found in six other *S. tuberosum* varieties (data not shown). The average percentage of surviving shoot tips was 88.7 % after CT and 91.6 % after AT (including all green explants like shoots, leaves, and callus). However, only green shoots were counted for determination of the regeneration percentages. Mean shoot regeneration was 34.6 % after CT in experiments using agarose drops and liquid regeneration medium, which increased to 45.2 % in variants subjected to AT (Tab. 3).

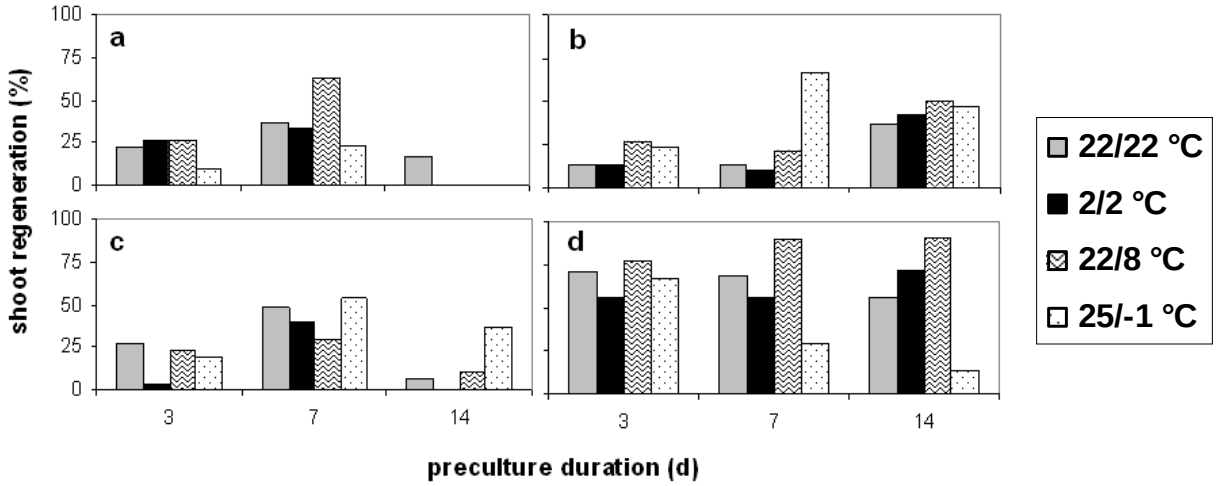


Figure 4. Shoot regeneration after cryopreservation using different preculture temperatures (see legend, day/night temperature) and different preculture durations. For each experiment 30 shoot tips were used. **a**) *S. tuberosum* 'Ackersegen', **b**) *S. tuberosum* 'Désirée', **c**) *S. tuberosum* 'King Edward', **d**) *S. demissum.*

Table 3. Shoot regeneration after 7 d preculture with CT and AT in control (- LN) and cryopreserved explants (+ LN). Comparison of survival (S, shoots, leaves and callus, in %) and shoot regeneration (R, only shoots, in %) using agarose drops in liquid and solid regeneration media. Results are means of three experiments with approximately 30 shoot tips tested per variant. Mean is calculated from total shoot tip number. Significances are calculated for the comparison within the same media and between precultures (no comparison between S and R). Values in bold with asterisk mark significant changes after AT (χ^2 test, *P < 0.05, **P < 0.01)

preculture		CT				AT			
regeneration media		liquid		solid		liquid		solid	
accession	LN	S	R	S	R	S	R	S	R
S. tuberosum 'Désirée'	-	100.0	97.8	100.0	100.0	100.0	98.9	100.0	100.0
	+	70.0	12.2	48.1	19.0	88.9	23.3	73.5	**44.6****
S. tuberosum 'King Edward'	-	100.0	93.3	95.8	95.8	100.0	88.0	100.0	98.6
	+	90.8	21.8	77.8	44.4	81.1	16.7	86.3	**75.0***
S. acaule	-	100.0	97.7	100.0	95.6	100.0	98.8	100.0	100.0
	+	94.3	36.8	80.2	25.3	97.7	**55.8***	83.5	33.0
S. demissum	-	100.0	91.2	100.0	97.7	98.9	97.7	100.0	98.8
	+	100.0	67.4	92.5	62.4	98.9	**85.6****	94.7	**82.1****
mean	-	100.0	95.0	99.1	97.2	99.7	95.7	100.0	99.4
mean	+	88.7	34.6	75.6	38.4	91.6	45.2	84.8	58.7

3.1.3. Comparison between liquid and solid regeneration medium

Improvement of the cryopreservation results was achieved after AT for both media used. Only *S. tuberosum* 'King Edward' showed a small, but not significant decrease in regeneration in agarose drops with liquid medium. After AT, the wild species *S. acaule* and *S. demissum* had significantly higher regeneration (Tab. 3) in agarose drops on liquid medium. The improvement in regeneration percentages was better on the solid medium, on which *S. tuberosum* 'Désirée', 'King Edward', and *S. demissum* showed significantly higher regrowth after AT (Tab. 3).

Average regeneration of plants for the 4 accessions tested was always higher using solid medium. Without AT shoot regeneration was 34.6 % in liquid medium and 38.4 % on solid medium, whereas with AT regeneration was increased to 45.2 % in liquid medium and to 58.7 % on solid medium (Tab. 3). Furthermore, the increase of regeneration after AT was better on solid regeneration medium, where *S. tuberosum* 'Désirée' improved regeneration by 25.6 %, *S. tuberosum* 'King Edward' by 30.6 % and *S. demissum* by 19.7 %. Only *S. acaule* showed smaller but no longer significant improvement of the regeneration compared to that in liquid medium. The improvement of results was especially found for cultivated potatoes, which had low regeneration when using the original protocol (Schäfer-Menuhr, et al. 1994).

3.2. Determination of cold tolerances

Experiments revealed that AT leads to better plant regeneration (Tab. 3). For this reason it was interesting to know, if there is a correlation between the better cryopreservation results with AT explants and the levels of cold tolerance of potato plants. For wild species such correlation can be assumed, because *S. acaule* and *S. demissum* are known to have the capacity to cold acclimate (Li, 1977; Mastenbroek, 1956). For all species it was not known yet, if they can acclimate to low temperatures under conditions of *in vitro* culture. Therefore, the lethal freezing temperature (LT_{50}) measurements of *in vitro* and soil cultured potato plants were performed to investigate, whether there are some links to the cryopreservation results.

3.2.1. Lethal freezing temperatures of *in vitro* cultures

All tested accessions did not express significant increase in freezing tolerance after AT. LT_{50} curves are shown as an example of *S. tuberosum* 'Désirée' after CT and AT (Fig. 5). Wild potatoes showed higher freezing tolerance when compared to cultivated potatoes, independent of preculture (Fig. 6).

3.2.2. Lethal freezing temperatures of greenhouse cultures

All accessions did not show significant increase in freezing tolerance after AT. Wild potatoes showed a higher freezing tolerance when compared to cultivated potatoes, independent of preculture (Fig. 7). Furthermore, freezing tolerances in wild potatoes grown in soil are higher when compared with *in vitro* cultures.

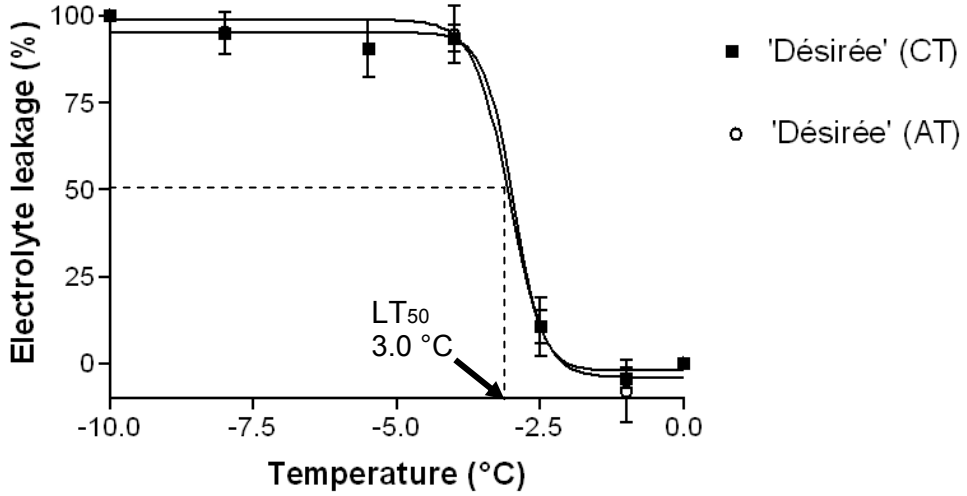


Figure 5. Example of electrolyte leakage of *in vitro* plants of *S. tuberosum* 'Désirée' after CT (squares) and AT (circles). Samples were measured after cooling to different temperatures. LT_{50} is marked by the dotted line with arrow. Means of 3 measurements per treatment ± standard error.

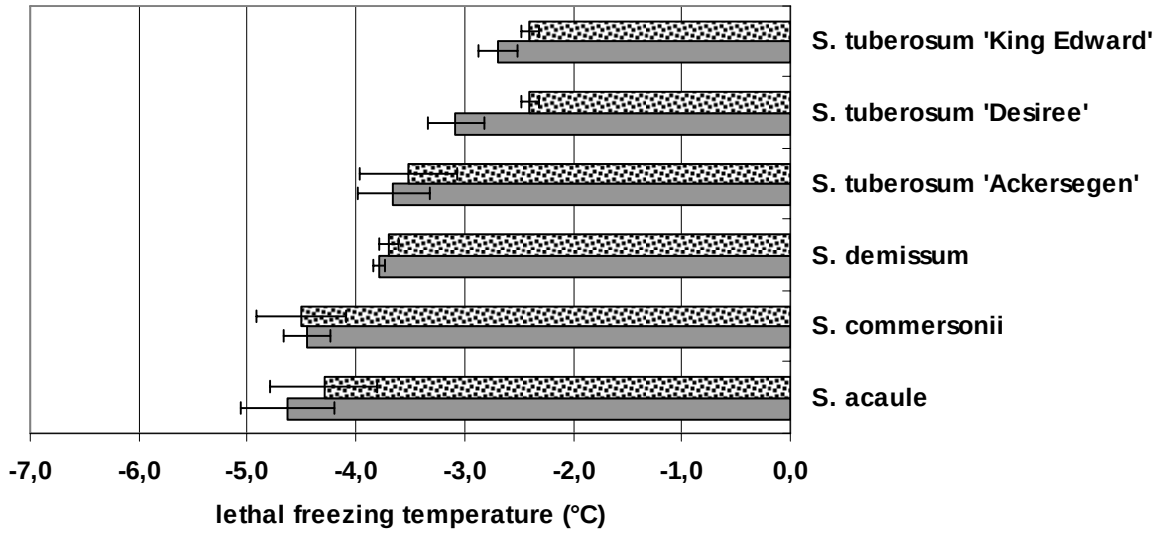


Figure 6. Lethal freezing temperatures of *in vitro* cultures (± standard error) after CT (filled bars) and AT (dotted bars). Means of three samples per accession.

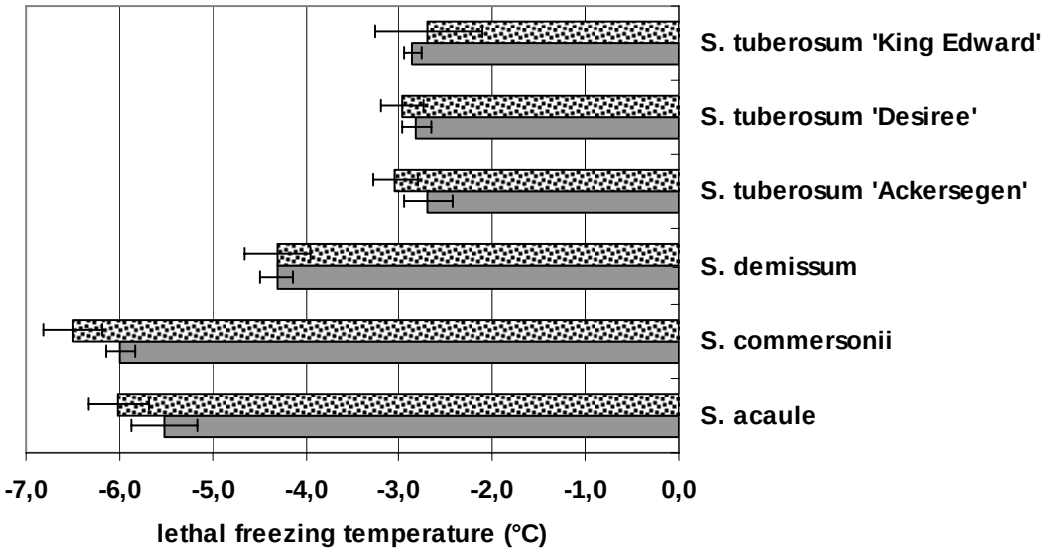


Figure 7. Lethal freezing temperatures of soil grown cultures (± standard error) after CT (filled bars) and AT (dotted bars). Means of two experiments for cultivated potatoes and means of three experiments for wild species, means of three samples per experiment.

3.3. Biochemical analysis

3.3.1. Osmolality after AT

The total osmolality was lowest in *S. demissum* with in average 150 mmol/kg, compared to the other samples which had average osmolalities of 215, 228, and 268 mmol/kg ('Désirée', 'King Edward', *S. acaule,* respectively*)*. Osmolality of *in vitro* plants decreased significantly in *S. tuberosum* 'King Edward' and nonsignificantly in 'Désirée' after AT. *S. demissum* and *S. acaule* in contrast, did not show significant changes after AT (Fig. 8).

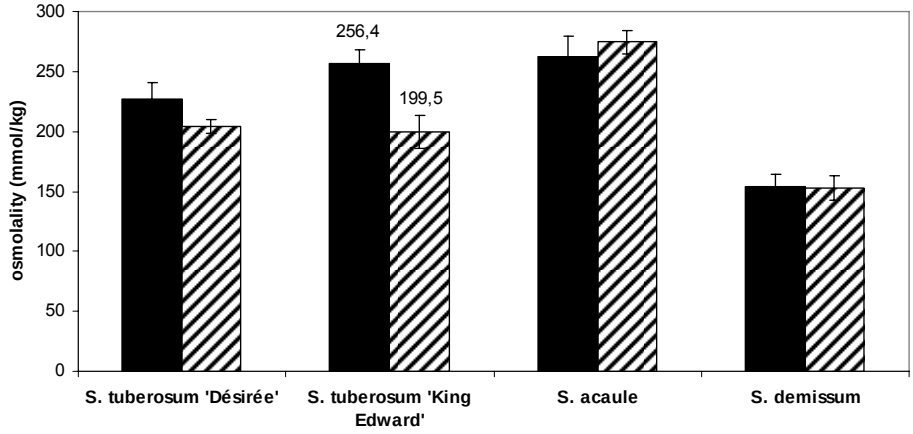


Figure 8. Osmolality of *in vitro* plants after CT (filled bars) and AT (hatched bars). Results are shown as means of 10 measurements, each consisting of 3 to 5 plants without roots. Bars represent means ± standard error. Bar pairs with indicated values show significant changes after AT in comparison to CT ($P < 0.05$*)*.

3.3.2. Determination of soluble sugars and starch

Total soluble sugar concentrations increased in three of four accessions after exposure to AT (Fig. 9). Total concentrations of soluble sugars were higher for the wild species *S. acaul*e and *S. demissum* compared to the cultivated accessions. In *S. tuberosum* 'Désirée' and *S. demissum* differences were statistically significant. The increase was mainly due to changes in glucose content. Fructose and sucrose showed only minor changes after AT (Fig. 10).

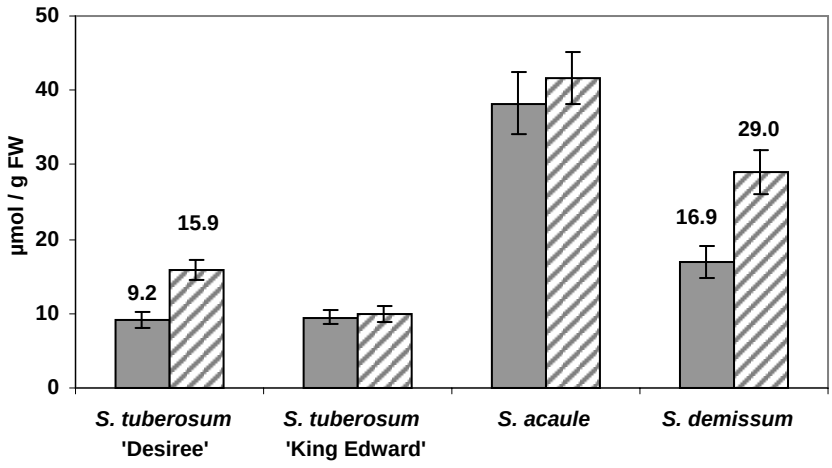


Figure 9. Concentrations of total soluble sugars after CT (filled bars) and AT (hatched bars). Results are shown as means of three experiments with 7 to 10 repetitions each, 20 to 50 shoot tips per repetition. Bars represent amount ± standard error. Bar pairs with indicated values show significant changes after AT in comparison to CT ($P < 0.01$ for *S. tuberosum* 'Désirée', $P < 0.05$ for *S. demissum)*.

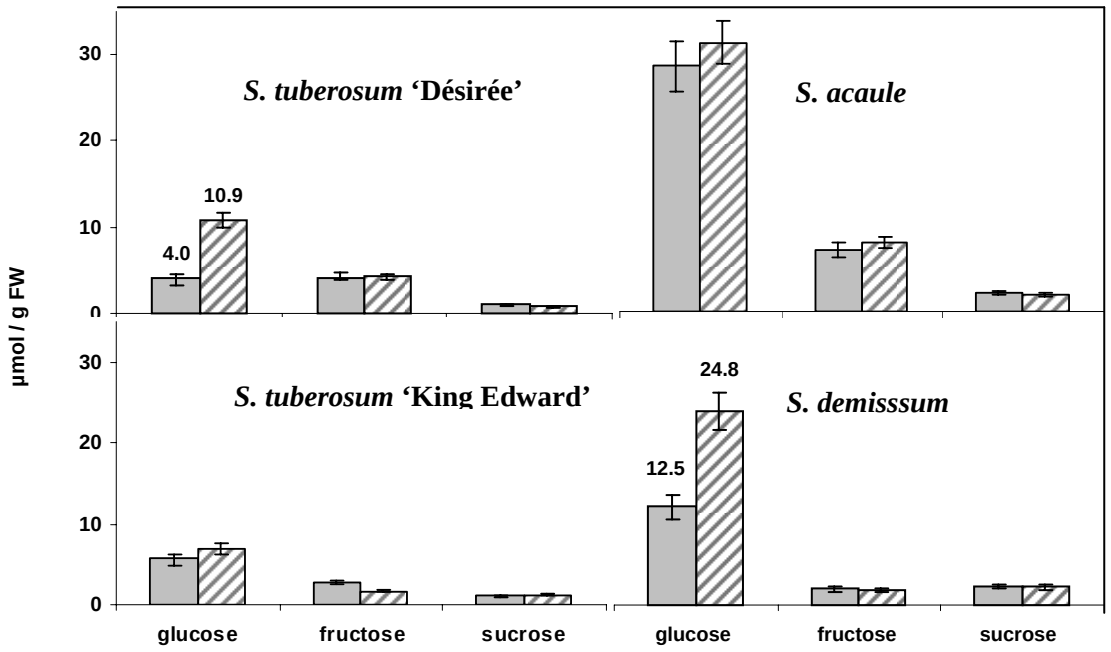


Figure 10. Concentrations of specific sugars after CT (filled bars) and AT (hatched bars). Results are shown as means of three experiments with 7 to 10 repetitions each, 20 to 50 shoot tips per repetition. Bars represent amount ± standard error. Bar pairs with indicated value show significant changes after AT in comparison to CT ($P < 0.001$)

Starch concentrations decreased significantly after AT in *S. tuberosum* 'Désirée', *S. demissum* but not significantly in *S. acaule*. In *S. tuberosum* 'King Edward' no significant change was found in starch concentration after AT (Fig. 11).

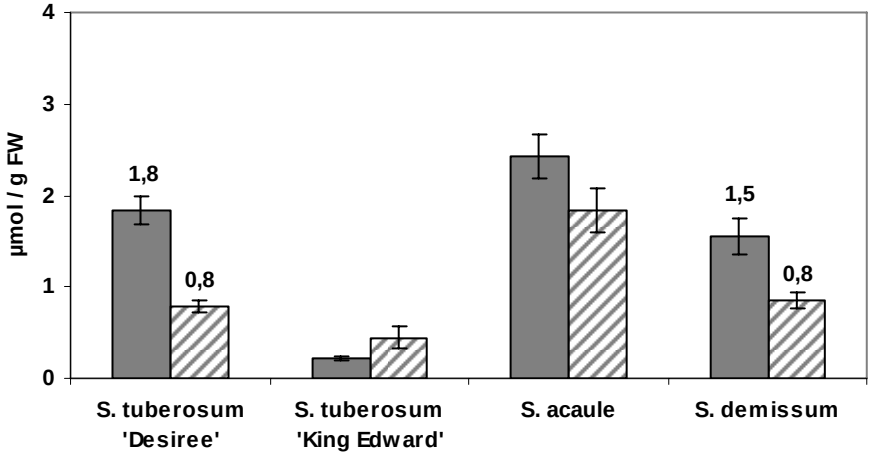


Figure 11. Starch concentration in shoot tips after CT (filled bars) and AT (hatched bars). Results are shown as means of three experiments with 7 to 10 repetitions each, 20 to 50 shoot tips per repetition. Bars represent amount ± standard error. Bar pairs with indicated value show significant changes after AT in comparison to CT ($P < 0.001$ for *S. tuberosum* 'Désirée', $P < 0.04$ for *S. demissum)*.

3.3.3. Determination of amino acids

Concentrations of seventeen essential amino acids were determined in shoot tips. Out of these, asparagine showed significant increase after AT in *S. tuberosum* 'Désirée', and *S. acaule,* whereas asparagine did not change significantly in *S. tuberosum* 'King Edward'. A slight decrease in asparagine concentration was detected in *S. demissum.* In all accessions asparagine was the amino acid with the highest concentration (Tab. 4). In contrast to asparagine most other measured amino acids decreased after the preculture with alternating temperatures, such as glutamine, glycine and proline (Tab. 4). Other amino acids had very low concentrations and did not change significantly (data not shown).

Table 4. Amino acid concentrations in µmol/g FW ± standard error in shoot tips after CT and AT. Results are shown as means of three experiments with 7 to 10 repetitions each, 20 to 50 shoot tips per repetition. Values in bold with asterisk mark significant changes after AT in comparison to CT ($P < 0.02$).

	CT				AT			
	S. tuberosum 'Désirée'	*S. tuberosum* 'King Edward'	*S. acaule*	*S. demissum*	*S. tuberosum* 'Désirée'	*S. tuberosum* 'King Edward'	*S. acaule*	*S. demissum*
Asparagine	1.28 ± 0.18	12.99 ± 1.75	3.31 ± 0.19	22.96 ± 2.57	**8.03* ± 0.53**	13.76 ± 1.66	**5.49* ± 0.55**	**21.84* ± 2.59**
Aspartate	0.39 ± 0.02	1.63 ± 0.18	0.58 ± 0.03	3.21 ± 0.30	**0.33* ± 0.02**	1.68 ± 0.11	**0.79* ± 0.07**	2.88 ± 0.16
Glutamate	0.69 ± 0.04	2.88 ± 0.27	0.90 ± 0.07	4.99 ± 0.29	**0.52* ± 0.04**	2.84 ± 0.17	0.99 ± 0.08	**3.92* ± 0.29**
Glutamine	0.80 ± 0.09	4.78 ± 0.52	4.22 ± 0.26	8.66 ± 0.56	**1.50* ± 0.11**	4.52 ± 0.26	**2.79* ± 0.27**	6.40 ± 0.28
Glycine	0.20 ± 0.02	0.35 ± 0.03	0.78 ± 0.06	0.69 ± 0.07	**0.13* ± 0.02**	0.42 ± 0.04	**0.33* ± 0.05**	0.40 ± 0.03
Proline	2.30 ± 0.23	4.91 ± 0.47	8.71 ± 0.88	8.78 ± 1.70	**0.27* ± 0.03**	5.17 ± 1.11	**2.62* ± 0.25**	6.84 ± 0.77
Serine	0.34 ± 0.02	2.53 ± 0.24	1.18 ± 0.07	4.69 ± 0.35	**0.24* ± 0.02**	3.00 ± 0.23	**0.68* ± 0.05**	**4.04* ± 0.29**

3.4. Proteome analysis

3.4.1. Establishment of an extraction protocol

A comparative protein analysis of *S. tuberosum* 'Desiree' and *S. demissum* shoot tips after CT and AT was conducted. The results should lead to the identification of proteins responsible for the improved cryopreservation results after the latter preculture.

The first step was to establish a new protocol suitable for potato shoot tips from *in vitro* plants, because it was not available yet. Two precipitation methods were compared. TCA/acetone precipitation (Amme et al., 2006) revealed slightly higher protein extraction efficiency with 2.7 µg protein per mg of fresh weight compared to phenol extraction/ammonium acetate precipitation with 2.1 µg/mg fresh weight (Carpentier et al., 2005). The TCA/acetone precipitation protocol was selected for the sample preparation for further analysis, because of slightly higher extraction efficiency and less streaking of protein spots on 2D gels (Fig. 12). Furthermore, it was more time-saving and less harmful to health as compared to the phenol extraction protocol.

3.4.2. Establishment of the staining protocol

RuBP staining revealed more protein spots (2300 spots for TCA and phenol extraction protocols each) than cCBB staining (1086 spots TCA protocol, 1150 spots phenol protocol, Fig. 12). Therefore, higher sensitivity was found with RuBP staining, meaning that for this procedure lower amounts of source material were needed. Only 100 µg protein per gel was required for RuBP in comparison to 200 µg protein for cCBB. RuBP staining was used for further analysis because of these results.

3.4.3. Selection of pH gradient

When the protein extracts were separated on 2D gels with pH gradient from 3-10, most of the spots were detected in the centre of the gel (Fig. 13a). Because of this, it can be estimated that most proteins were located in the pH gradient range between 4 and 7, and this was confirmed by 2D gel electrophoresis using a pH gradient from 4-7 (Fig. 13b). Here, the separation of protein spots was improved according to their isoelectric points and, therefore, it was used for further analysis.

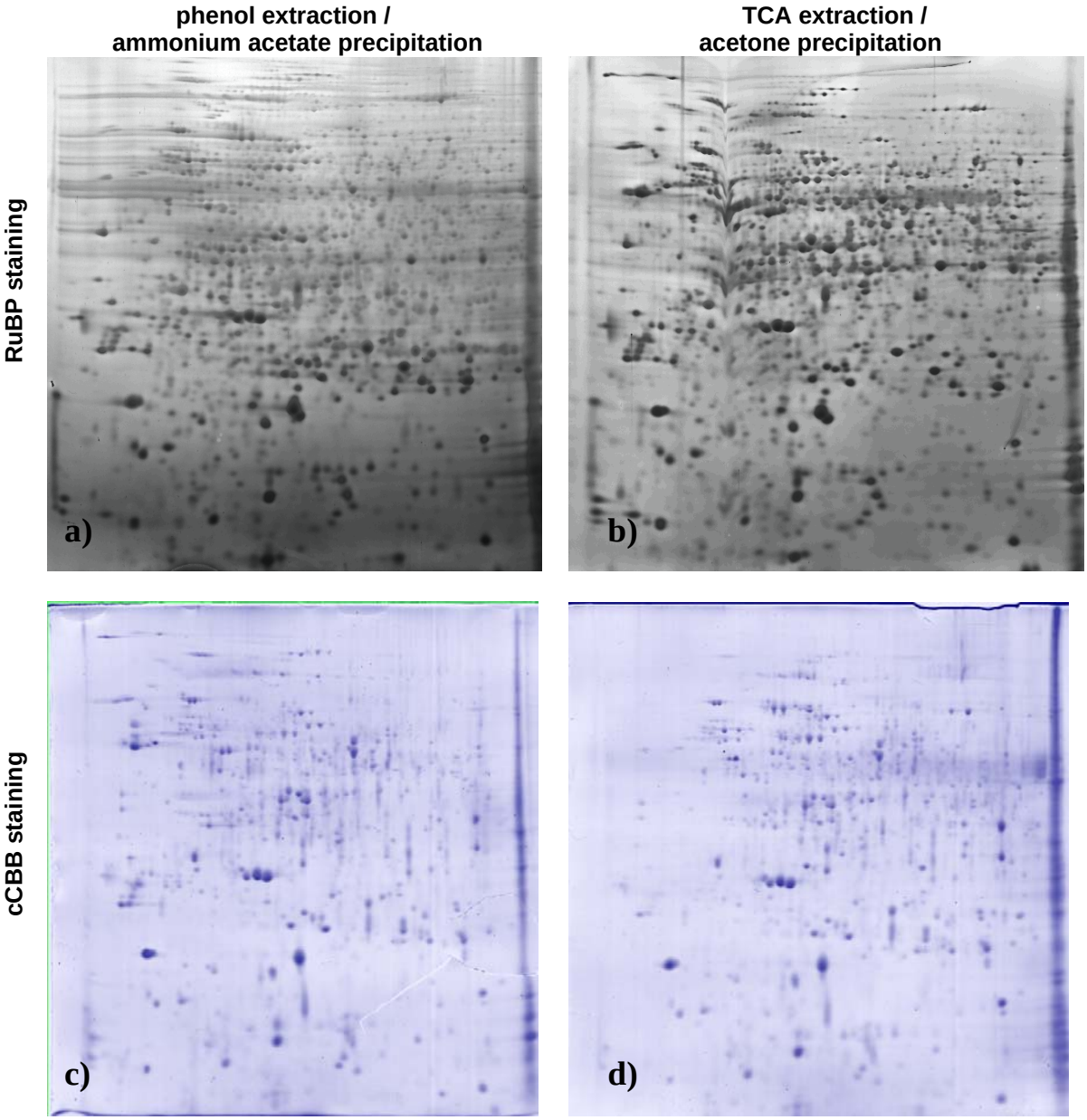


Figure 12a-d. Protocol optimization of proteome analysis for potato shoot tips (*S. tuberosum* 'Désirée'). Comparison of 2D gels with different extraction and staining methods. Two precipitation methods were compared. These were **a), c)** phenol extraction/ammonium acetate precipitation (Carpentier et al., 2005) and **b), d)** TCA/acetone precipitation (Amme et al., 2006). Furthermore, two staining procedures were compared. **a), b**) RuBP, 100 µg protein per gel, with **c), d)** cCBB, 200 µg protein per gel.

3.4.4. Comparison of protein spot patterns of two *Solanum* species

Spot pattern between biological repetitions, each consisting of 3 technical replicates, were compared for shoot tips after AT in comparison to CT. The results received varied from experiment to experiment. For example, in the first repetition one protein spot was upregulated, while the same spot was downregulated in the second and third repetitions. Therefore, a new analysis was done as follows.

A group of 9 gels per treatment, consisting of 3 biological replicates with each 3 technical replicates, was compared for shoot tips immediately after AT in comparison to CT. Spots were analyzed, which were expressed significantly different at least 1.5-fold between treatments. It was found that protein patterns were different between treatments and between accessions for both potato species analyzed.

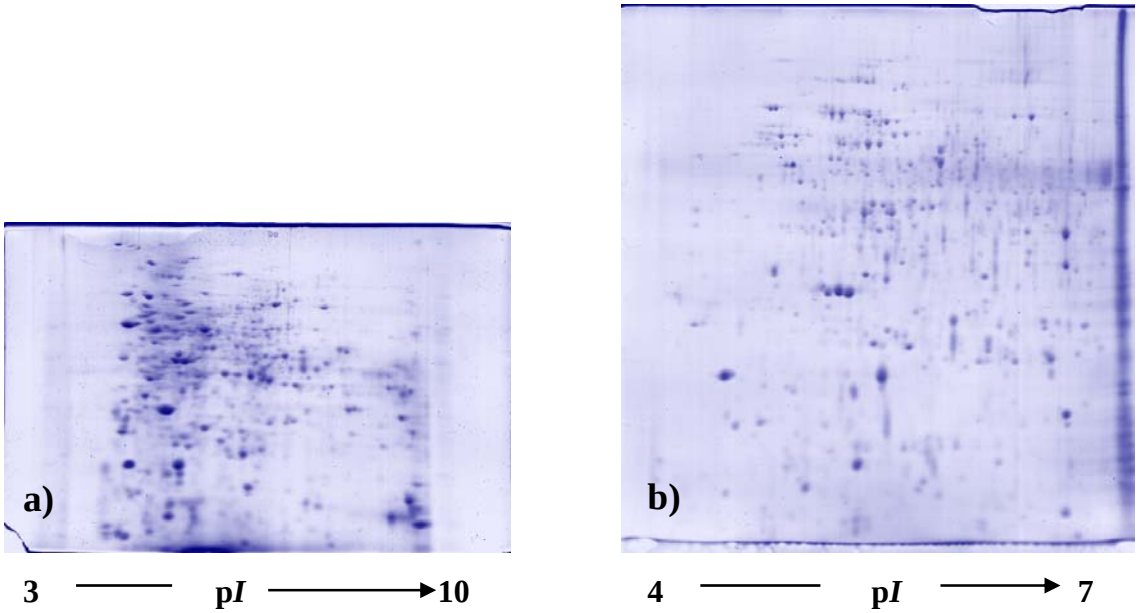


Figure 13a-b: Protocol optimization of proteome analysis for potato shoot tips (*S. tuberosum* 'Désirée'). Comparison of 2D gels with different pH ranges. IEF was carried out on IPG strips with pH gradients **a**) 3-10 (7 cm length) and **b)** 4-7 (13 cm length) and separation in second dimension on an 11.25 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (p*I* – isoelectric point).

On average, 2079 ± 139 spots per gel were visualized on 2D gels of *S. tuberosum* 'Désirée'. A total of 9 protein spots were found differently expressed after AT (Fig. 16, Tab. 5). Of these, 6 were found to be more abundant and 3 less abundant. For *S. demissum* in average 2369 ± 17 protein spots per gel were detected. A total of 64 protein spots were at least 1.5 times changed after AT, comprising 16 protein spots with higher abundance and 48 with lower abundance (Fig. 16, Tab. 5). In *S. demissum* more changed protein spots (2.7 %) between treatments were found in comparison to *S. tuberosum* 'Désirée' (0.4 %). Protein spots of *S. demissum* were mostly downregulated, whereas 'Désirée' had a higher percentage of upregulated protein spots (Tab. 5).

One changed spot was found in both of the two *Solanum* species (spot number 1 in 'Désirée', spot number 35 in *S. demissum*, Fig. 17). In both species this spot was upregulated after AT (Tab. 5). The reproducibility of experiments was higher in *S. demissum* with a lower standard

error of detected protein spots compared to the cultivated potato. This was confirmed by principal component analysis (PCA), in which the normalized spot volume of all gels was compared. The PCA of biological and technical repetitions of the 2D gels were grouped closer together in *S. demissum* than in 'Désirée' (compare Fig. 14 with Fig. 15). The proteins from shoot tips differently expressed after CT and AT were clearly separated in two groups in PCA for both potato species analyzed (Fig. 14, 15). The group of 'Désirée' gels with CT had the highest deviation.

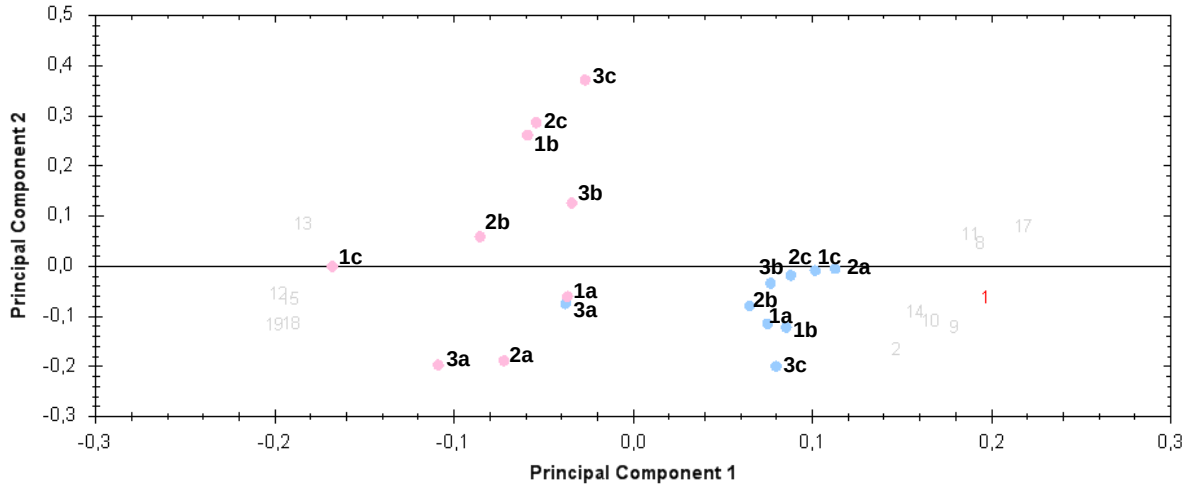


Figure 14: PCA based on differently expressed protein spots of *S. tuberosum* 'Désirée' technical replicates (see Fig. 3). Pink spots: gels with protein extract from CT explants, blue spots: gels with protein extract from AT explants.

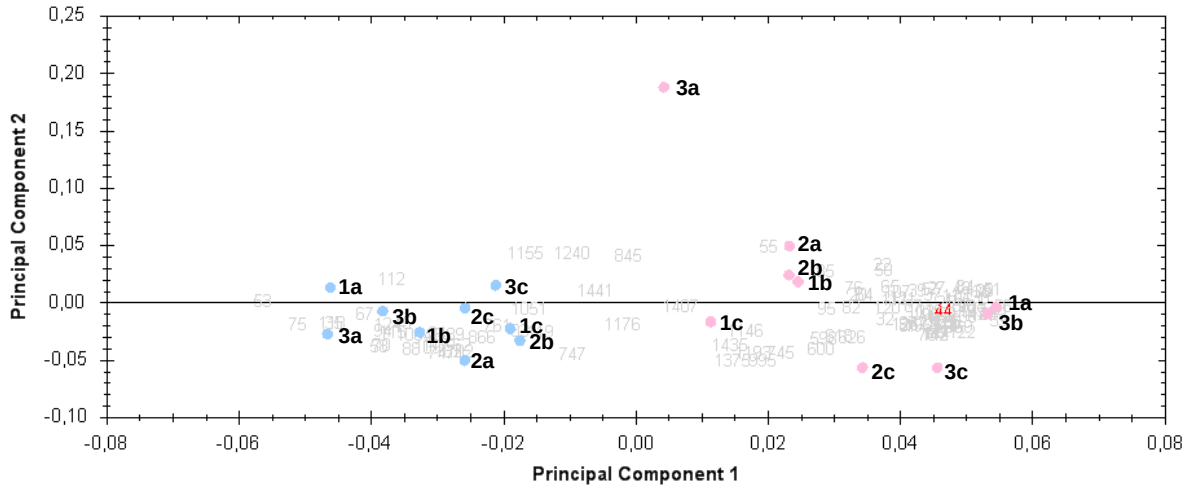


Figure 15: PCA based on differently expressed protein spots of *S. demissum* technical replicates (see Fig. 3). Pink spots: gels with protein extract from CT explants, blue spots: gels with protein extract from AT explants.

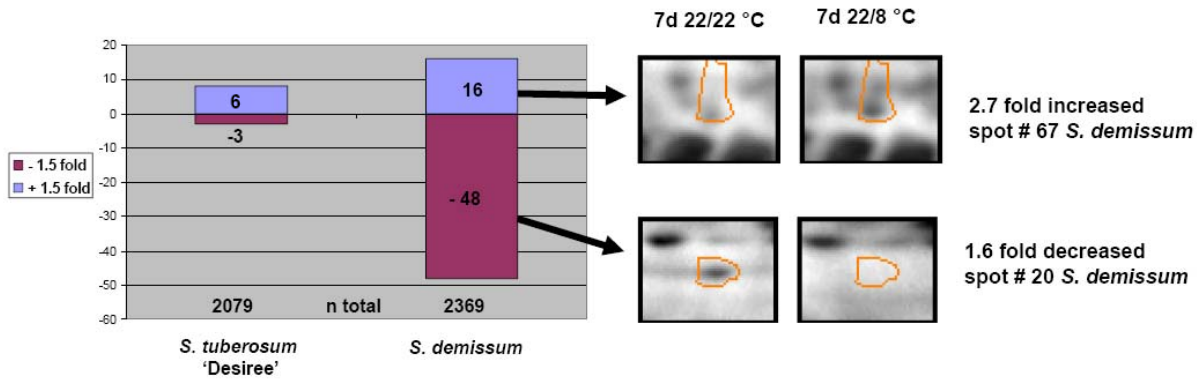


Figure 16. Numbers of spots expressed at least 1.5-fold differently between treatments (CT, AT). In *S. tuberosum* 'Désirée' out of 2079 spots per gel 9 spots were expressed differently in all gels. In *S. demissum* out of 2369 spots per gel 64 spots were at least 1.5-fold changed.

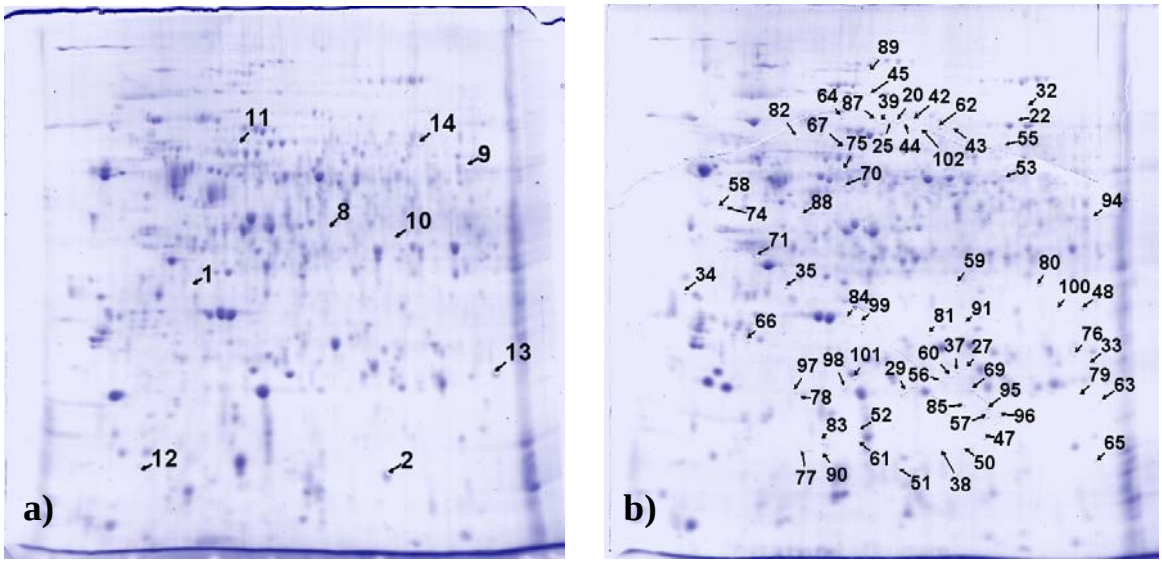


Figure 17a-b. Preparative 2D gels of *S. tuberosum* 'Désirée' (**a**) and *S. demissum* (**b**) protein extracts (200 µg protein per gel, cCBB staining, pH gradient 4-7). Protein spots, differently expressed in both treatments at least 1.5-fold, are marked with numbers.

3.4.5. Identification of changed proteins from potato shoot tips

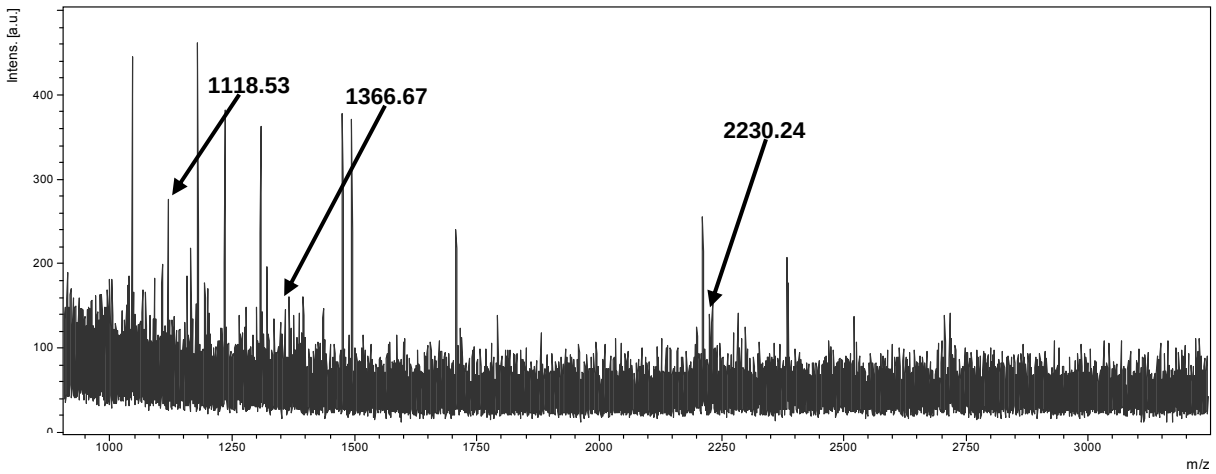
Protein spots that were collected from RuBP stained gels were too low in protein concentration for mass spectrometry-based identification. Therefore, preparative gels were made, loaded with 200 µg protein each, followed by cCBB staining for protein visualisation (Fig. 17). Most of the proteins were identified by MALDI-TOF mass spectrometry. After tryptic digestion of spots and mass spectrometry, the received spectra were used for protein identification, searching the NCBI *Viridiplantae* and TIGR *Solanaceae* database (Fig. 18).

If MALDI-TOF mass spectrometry was not successful for protein identification, *de novo* sequencing of peptides via LC-ESI-Q-TOF mass spectrometry was applied. Received amino acid sequences were compared with TIGR *Solanaceae* and Translated European Molecular Biology Laboratory (TrEMBL) databases for protein identification (Fig. 19). Not all proteins

could be identified by mass spectrometry (Tab. 5a, b). Reasons for this could consist of the low intensity of analyzed spots or the incompleteness of the EST potato database. Out of the differently expressed proteins 4 (44.4 %) could be identified for *S. tuberosum* 'Désirée' and 24 (37.5 %) for *S. demissum* using mass spectrometry (Tab. 5a, b). Successfully identified proteins were classified according to the functional catalogue by Bevan et al. (1998).

In *S. tuberosum* 'Désirée', identified proteins belong to the functional groups of metabolism (1), transcription (1), transporters (1), and others (1). In *S. demissum,* identified proteins belong to functional groups of metabolism (4), signal transduction (4), disease/defence (4), energy (2), transcription (2), secondary metabolism (1) and others (7; Tab. 5a, b).

a)



b)

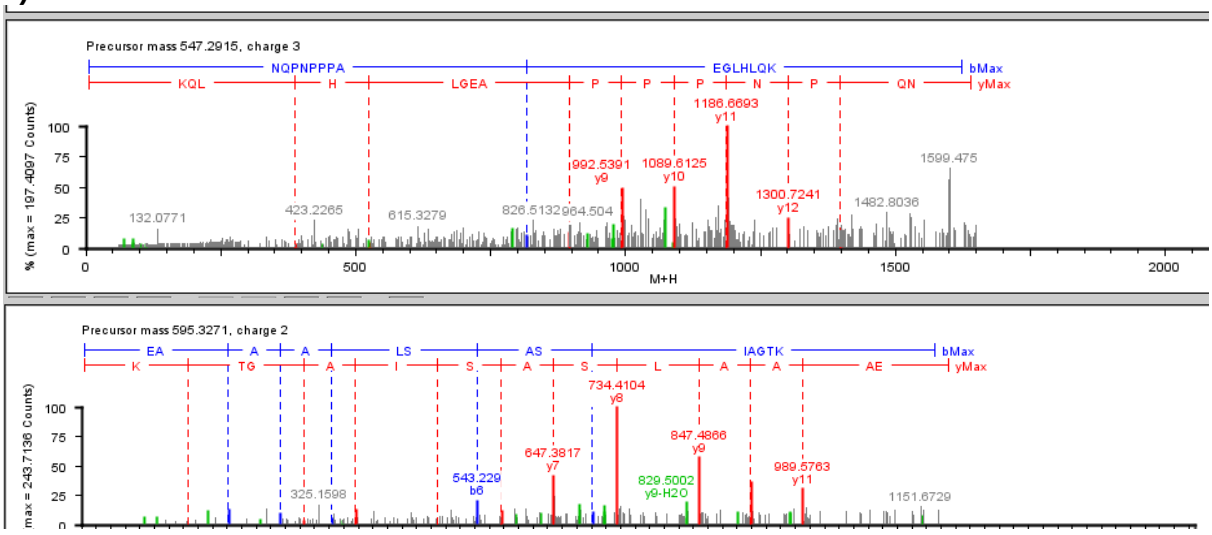
Start - End	Observed $[M + H]^+$	Mr (calc)	Matched Sequence
83 - 94	1366.6752	1365.7140	1 K.SATYDEIKQAIK.E
91 - 100	1118.5358	1117.5615	1 K.QAIKEESEGK.L
101 - 121	2230.2403	2229.0689	1 K.LKGILGYTEDDVVSTDEVGDS.-

c)

IVEGLMTTVHSITATQKTVDGPSSKDWRGGRAASFNIIPSSTAAKAVGKVLPSLNGKL
TGMSFRVPTVDVSVVDLTVRLQKSATYDEIKQAIKEESEGKLKGILGYTEDDVVSTD
EVGDS

Figure 18a-c. Example of a peptide fingerprint spectrum using MALDI-TOF mass spectrometry. Identification of spot 91 of *S. demissum* as glyceraldehyde 3-phosphate dehydrogenase (gi:125658297 from *Ficus albipila* / TC136143 from *Capsicum annuum*). **a)** MALDI-TOF spectrum of tryptic digestion from spot 91, **b)** table of peptides which were observed by MALDI-TOF and used for identification, **c)** protein sequence (in grey) and matching tryptic peptides (in black). Letters mark the amino acids. Protein identification was performed by searching for *Viridiplantae* in the NCBInr database.

a)



b) CLUSTAL 2.0.2. multiple sequence alignment

S. tuberosum 'Désirée'	N	Q	P	N	P	P	P	A	E	G	L	H	L	Q	K
A. thaliana	N	Q	P	D	P	A	P	S	E	N	L	H	I	Q	K
	*	*	*	:	*	.	*	:	*	.	*	*	:	*	*

S. tuberosum 'Désirée'	E	A	A	A	L	S	A	S	I	A	G	T	K
A. thaliana	Q	A	A	A	L	D	S	S	L	K	G	T	A
	:	*	*	*	*	.	:	*	:		*	*	

c)

MATGLGFFKLSFLLSLLSGGSDLLGPDAESGVAQIGKFPPSCNRIECPSYELVHSGNGY
EIRRYNNTVWVSTEPIPDISLVDATRTAFFQLFAYIQGKNEYHQKIEMTAPVISQVSPS
DGPFCESSFTVSFYVPKKNQPDPAPSENLHIQKWNSRYVAVRQFSGFVSDDSIGEQAA
ALDSSLKGTAWANAISKSKEDGVGSDSAYTVAQYNSPFEFSGRVNEIWLPFELDV

Figure 19a-c. Example of de *novo* sequencing of peptides via LC-ESI-Q-TOF mass spectrometry. Identification of spot 13 of *S. tuberosum* 'Désirée' as similar to SOUL like protein (TC 159433) of *Arabidopsis thaliana*. **a)** ESI-tandem mass spectrometry spectra of the m/z 547.2915 $[M+H]^+$ (upper panel) and m/z 595.3271 $[M+H]^+$ (lower panel) peptide ions derived from in-gel tryptic digestion of spot 13, **b)** Blast results of *de novo* sequenced peptides using CLUSTAL W software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) showed sequence similarity with a homologous protein from *A. thaliana*, **c)** Protein sequence (in grey) of *A. thaliana* and homologous tryptic peptides identified by LC-ESI-Q-TOF-mass spectrometry (in black). Letters mark the amino acids. Blast homology and similarity searches were conducted with a protein *Solanaceae* index of the TIGR database.

Table 5a. Identification of proteins in *S. tuberosum* 'Désirée'. Identification was done by database search against *Viridiplantae* protein index of the nonredundant NCBI database using peptide mass fingerprint data from MALDI-TOF mass spectrometry and nanoLC-ESI-Q-TOF tandem mass spectrometry analysis. The identification numbers are listed below. Theoretical values for molecular mass (Mr) and isoelectric point (pI) were calculated using ExPASy tools (http://www.expasy.ch). Expression ratio – level of protein expression in gels after AT in comparison to CT. P values were determined after ANOVA (Phoretix 2D Evolution software, Nonlinear Dynamics), *ni* not identified.

No.	Protein name	Species	Expression ratio	P	Identification number	p*I*	M_r (kDa)
***S. tuberosum* 'Désirée'**							
01 Metabolism							
12	GB|CAA58220.1|602594|STDN starch (bacterial glycogen) synthase	*Solanum tuberosum*	-1.5	0.0102	CN462276	6.9	66.6
04 Transcription							
14	similar to UP|Q9LFY7_ARATH, T7N9.4, partial (12%), Q93VV0_ARATH, protein name: AT3g09320/F3L24_19	*Arabidopsis thaliana*	-1.5	0.0344	Nt_TC13409	8.4	32.4
07 Transporters							
2	similar to TIGR_Ath1|At3g24530.1 68416.m03080 AAA-type ATPase family protein / ankyrin repeat family protein	*Arabidopsis thaliana*	+2.4	1.34E-07	Ca_TC4128		
others							
13	weakly similar to GB AAM64525 1 21592576 AY086962 SOUL like protein	*Arabidopsis thaliana*	-1.5	0.0054	St_TC159433	4.8	25.4
1	ni		+2.5	7.58E-10			
8	ni		+1.6	0.0042			
9	ni		+1.6	0.0010			
10	ni		+1.6	0.0156			
11	ni		+1.5	0.0282			

Table 5b. Identification of proteins in *S. demissum*. Description as in table 5a.

No.	Protein name	Species	Expression ratio	P	Identification number	p*I*	M_r (kDa)
S. demissum							
01 Metabolism							
20	AJ698900.1 GI:62953143, nitrogen starvation response protein	*Gibberella fujikuroi*	-2.7	0.0245	Lg_CN384657	4.7	26.6
43	AJ698900.1 GI:62953143, nitrogen starvation response protein	*Gibberella fujikuroi*	+1.8	0.0017	Lg_CN384657	4.7	26.6
81	weakly similar to UP|Q95TD6_DROME, SD07783p, partial (26%)	*Drosophila melanogaster*	-1.6	0.0095	Lg_BW685635	5.9	34.0
99	AJ698900.1 GI:62953143, nitrogen starvation response protein	*Gibberella fujikuroi*	-1.5	0.0030	Lg_CN384657	4.7	26.6
02 Energy							
79	UP|Q9ZSP7, Cytochrome b5 DIF-F, complete	*Petunia hybrida*	-1.6	0.0352	St_CK716997	5.9	17.1
91	homologue to UP|Q8VWN9_CAPAN, Glyceraldehyde-3-phosphate dehydrogenase precursor	*Capsicum annuum*	-1.5	0.0010	TC136143	8.9	44.4
04 Transcription							
59	similar to GB|AAB60730.1|2160167|F21M12 Identical to A. thaliana Myb-like protein	*Arabidopsis thaliana*	-1.7	0.0007	TC147862	6.4	95.8
65	similar to GB|AAB60730.1|2160167|F21M12 Identical to A. thaliana Myb-like protein	*Arabidopsis thaliana*	-1.6	0.0445	TC147862	6.4	95.8
10 Signal Transduction							
57	similar to UP|Q7XZU1_ARATH SAC domain protein 4, partial (4%)	*Arabidopsis thaliana*	-1.7	0.0004	Lg_BP887076	6.5	94.0
62	homologue to UP|Q40886_PETHY,Petunia Shaggy kinase 4, partial (33%)	*Petunia hybrida*	+1.6	0.0136	Lg_TC179204	8.7	46.4
69	similar to UP|O82469_MESCR, Protein phosphatase-2C, partial (85%)	*Mesembryanthemum crystallinum*	-1.6	0.0009	Lg_AW616779	4.7	39.6
97	similar to UP|Q9LT51, Xylulose kinase, partial (42%)	*Arabidopsis thaliana*	-1.5	0.0170	Ca_TC4802	5.4	61.9
11 Disease / defence							
58	weakly similar to UP|Q42393_CAPAN, Sn-1 protein, partial (46%)	*Capsicum annuum*	+1.7	0.0001	Lg_TC173131	5.6	16.9
66	weakly similar to UP|Q94G86_OLEEU, Beta-1,3-glucanase-like protein, partial (78%)	*Olea europaea*	-1.6	0.0095	St_TC142622	5.5	48.9
75	weakly similar to UP|Q42393_CAPAN, Sn-1 protein, partial (46%)	*Capsicum annuum*	+1.6	0.0002	Lg_TC173131	5.6	16.9

Table 5b. Continued

No.	Protein name	Species	Expression ratio	P	Identification number	p*I*	M_r (kDa)
76	UP|Q7XJI7_LYCES, Glycine-rich protein TomR2, complete	*Lycopersicon esculentum*	-1.6	0.0461	Lg_TC186298	5.7	24.0
20 Secondary Metabolism							
37	similar to UP|Q8GSM8 Squalene monooxygenase 1, partial (50%)	*Medicago truncatula*	-2.0	0.0003	Ca_TC5140	5.0	17.3
others							
29	similar to UP|Q944I0_ARATH, AT5g63440/MLE2_7, partial (92%)	*Arabidopsis thaliana*	-2.1	0.0071	St_TC142746	6.2	22.7
38	homologue to GB|AAL58922.1|18087583|AF462834 T4g35250/F23E12_190	*Arabidopsis thaliana*	-2.0	3.26E-05	TC134777	8.7	43.7
64	unknown protein		-1.6	0.0172	St BF153842		
71	unknown protein		+1.6	0.0037	St BF153842		
80	similar to GP|20259443 unknown protein	*Arabidopsis thaliana*	-1.6	0.0189	Lg_AI774640	5.6	71.3
90	unknown protein		-1.5	0.0257	Nt_DW003056		
101	unknown protein		-1.5	0.0113	Lg_AW617469		
34	ni		+2.0	0.0340			
35	ni		+2.0	1.51E-07			
45	ni		+1.8	0.0015			
53	ni		+1.7	6.07E-06			
67	ni		+1.6	0.0004			
70	ni		+1.6	0.0003			
74	ni		+1.6	0.0079			
88	ni		+1.6	0.0117			
89	ni		+1.5	0.0106			
94	ni		+1.5	0.0043			
102	ni		+1.5	0.0051			
100	ni		-1.5	0.0005			
98	ni		-1.5	0.0024			
96	ni		-1.5	0.0125			
95	ni		-1.5	0.0171			
87	ni		-1.6	0.0146			
85	ni		-1.6	0.0389			
84	ni		-1.6	0.0001			
83	ni		-1.6	0.0012			
82	ni		-1.6	0.0136			

Table 5b. Continued

No.	Protein name	species	Expression ratio	P	Identification number	p*I*	M_r (kDa)
78	ni		-1.6	0.0118			
77	ni		-1.6	0.0205			
63	ni		-1.6	0.0051			
61	ni		-1.7	0.0080			
60	ni		-1.7	0.0026			
56	ni		-1.7	1.12E-05			
55	ni		-1.7	0.0871			
52	ni		-1.7	0.0022			
51	ni		-1.7	2.92E-06			
50	ni		-1.8	0.0055			
48	ni		-1.8	0.0001			
47	ni		-1.8	0.0007			
44	ni		-1.8	0.0001			
42	ni		-1.9	0.0017			
39	ni		-1.9	0.0082			
33	ni		-2.0	0.0002			
32	ni		-2.0	0.0285			
27	ni		-2.2	0.0011			
25	ni		-2.2	0.0007			
22	ni		-2.4	0.0178			

3.5. Aspects of cell ultrastructure in shoot tips in regard to cryopreservation

3.5.1. Morphogenetic reactions of the explants after rewarming

During the first two days after rewarming all shoot tips remained greenish, making it impossible to separate living from dying ones. One day later, however, living shoot tips were still greenish and showed first signs of regrowth, whereas dying ones had lost their green colour now appearing whitish (Fig. 20b). Regeneration of leaves was usually visible six days after rewarming, and new plantlets could be obtained after four to eight weeks (Fig. 20c, d). The meristematic part of the shoot tips formed the center of regeneration. This means that only the meristematic area (meristematic dome and leaf primordia) remained green, whereas the bottom part of the shoot tip died. The green surviving tissue continued growing, often showing a globular shape. One or several new shoots developed from this globular structure (Fig. 20d). Also regeneration started with regrowth of young leaves followed by shoot formation from the base of these leaves. In some explants only growth of leaves or callusing was observed. The meristematic dome itself usually did not show regrowth after rewarming. The morphological features of shoot regeneration were the same in explants after CT and AT.

3.5.2. The cell ultrastructure in different steps of the protocol

Semi-thin sections of freshly isolated shoot tips showed heavy staining of the meristematic regions (Fig. 20e, f). In these living shoot tips nucleoli were darkly stained (arrows in Fig. 20f), whereas nuclei were rather weakly stained (Fig. 20f). Two days after cryopreservation and rewarming the meristem and epidermal layers of the leaf primordia were much weaker stained and showed dead cells with very dark nuclei (arrowheads in Fig. 20h). However, in the leaf primordia, cells were still alive and displayed mitotic activity (arrow in Fig 20h). These intact cells showed the same staining characteristics like cells in freshly isolated shoot tips (compare Fig. 20f with 20h).

The ultrastructural features of shoot tips of plants grown under CT were indistinguishable from those of plants subjected to AT (Fig. 21a-d). TEM studies of the meristematic regions showed cells with intact plasma membranes, undamaged cell walls and dense cytoplasm filled with ribosomes and many mitochondria, dictyosomes and chloroplasts (Fig. 21a-d). The large spherical nuclei displayed the typical features of plants with relatively small genome size, i.e., they were mainly euchromatic with small heterochromatic regions restricted to the nuclear periphery (Fig. 21a, c; Nagl and Fusenig, 1979). The high nucleo-cytoplasmic ratio in the

cells making up the dome area was typical for meristematic cells (Ishikawa, 1996; Towill, 1983).

Overnight incubation in MSH had no effect on the ultrastructural features, and cells looked similar to freshly isolated shoot tips (not shown). In contrast to this, subsequent incubation in cryoprotectant solution (MSH with 10 % DMSO) for 2 h led to the appearance of small vesicles in the cytoplasm (Fig. 22a) and swelling of mitochondria and chloroplasts (Fig. 22b - c). DMSO incubation also changed the shape of the large central vacuoles. In non-treated shoot tips, they were more or less spherical, whereas after DMSO treatment they became irregular in shape, often showing multiple cytoplasmic invaginations (compare Fig. 21a, c and 22d). Such changes after DMSO incubation were similar for both preculture treatments. Ultrastructural characteristics were unchanged directly after rewarming in comparison to that before cooling (not shown). Significant ultrastructural changes were found in cryopreserved explants 1 h after rewarming. Most cellular organelles had increased in size due to swelling. Some vacuoles and many small vesicles were observed especially close to the cell membranes (Fig. 23a-b). At this stage, the only difference between AT (Fig. 23a) and CT explants (Fig. 23b) was in the ultrastructure of the nuclei. In AT explants, nuclei had remained virtually unchanged with heterochromatic regions tightly appressed to the nuclear envelope (see Fig. 22a and 23a). In nuclei of CT explants, the heterochromatin was more clumped and often redistributed in non-peripheral regions (Fig. 23b).

Two days after the initiation of regeneration, control explants which had been cooled without cryoprotectant (Fig. 24 a-b) showed the typical characteristics of dead tissues. The cells of the meristematic dome were severely affected. The cytoplasm showed typical signs of extraction, meaning organelles were disintegrated and partly unhinged, indicating the rupture of the plasma membrane (Fig. 24b). Organelles could not be discerned (Fig. 24a-b) and nuclei were often heavily condensed (Fig. 24a), which is a clear sign for cell death.

Similar ultrastructural damages were also observed in the cryoprotected shoot tips (Fig. 24c). The latter, however, also contained groups of living subepidermal and epidermal cells in the leaf primordia (Fig. 24d - f). Though individual cellular organelles like chloroplasts often had an abnormal morphology (Fig. 24f), the cells as a whole were alive.

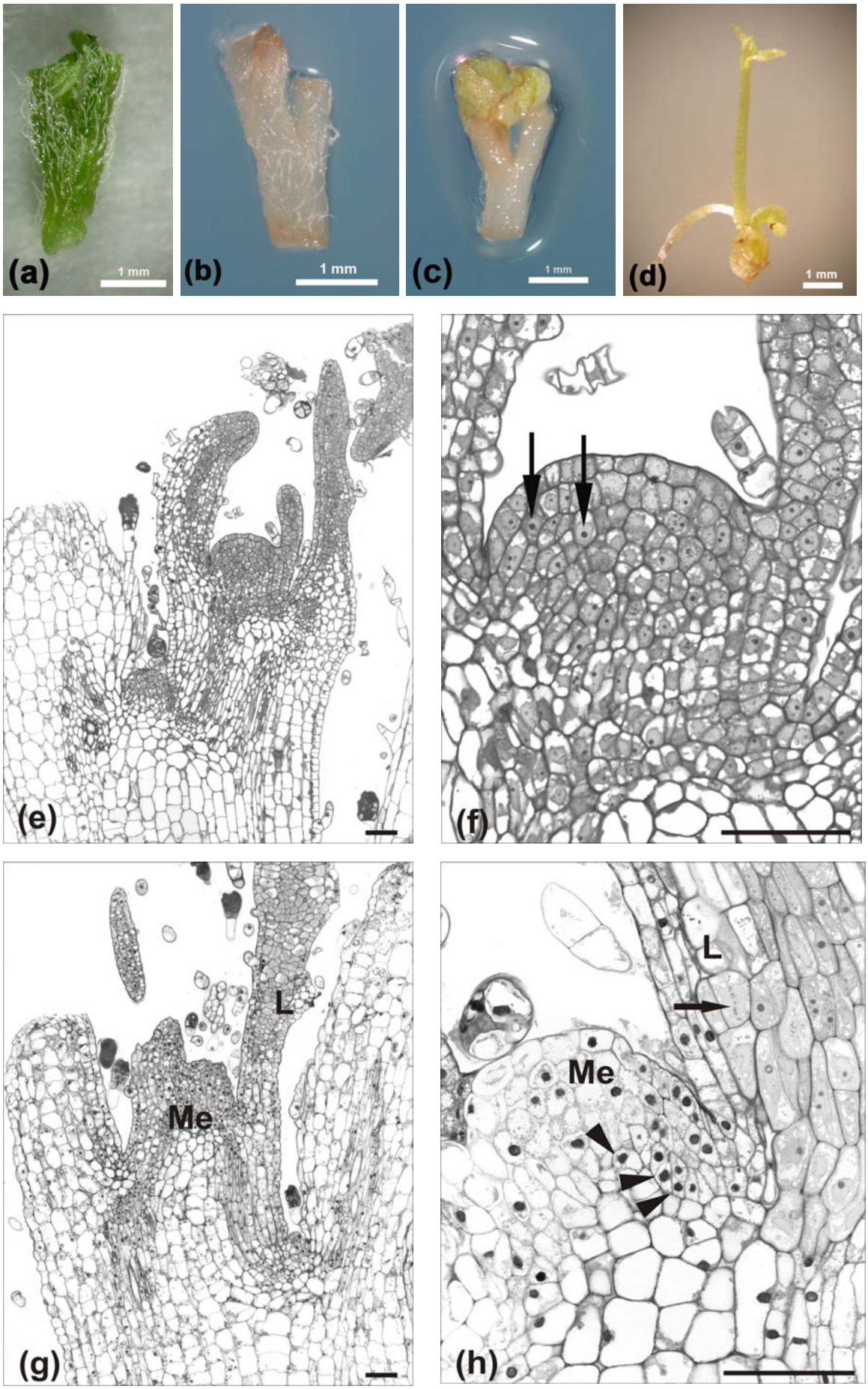


Figure 20a-h. Description next page.

Figure 20a-h. Morphology and histological structure of isolated shoot tips. **a)** freshly isolated untreated shoot tip, **b)** dead shoot tip 4 d after rewarming, **c)** vital shoot tip showing regrowth of leaves 6 d after rewarming, **d)** shoot regrowth 8 weeks after rewarming. **e), f)** methylene-blue stained semi-thin sections of isolated untreated shoot tips reveal heavy staining of meristematic regions (e); note the dark nucleoli (arrows) in the otherwise weakly stained nuclei, **g), h)** cryopreserved shoot tips 2 d after rewarming (after AT, overnight incubation and DMSO cryoprotection); note the much weaker staining of the meristematic regions. Detail studies show dead cells, characterized by small, very dark nuclei (arrowheads) in the meristematic dome and epidermal layer of the leaf primordium. Interestingly, parenchymal cells of the leaf primordia are still alive and display mitotic activity (arrow in h). *Bar* = 50 µm (e, f, g, h), *Me* meristem, *L* leaf primordium.

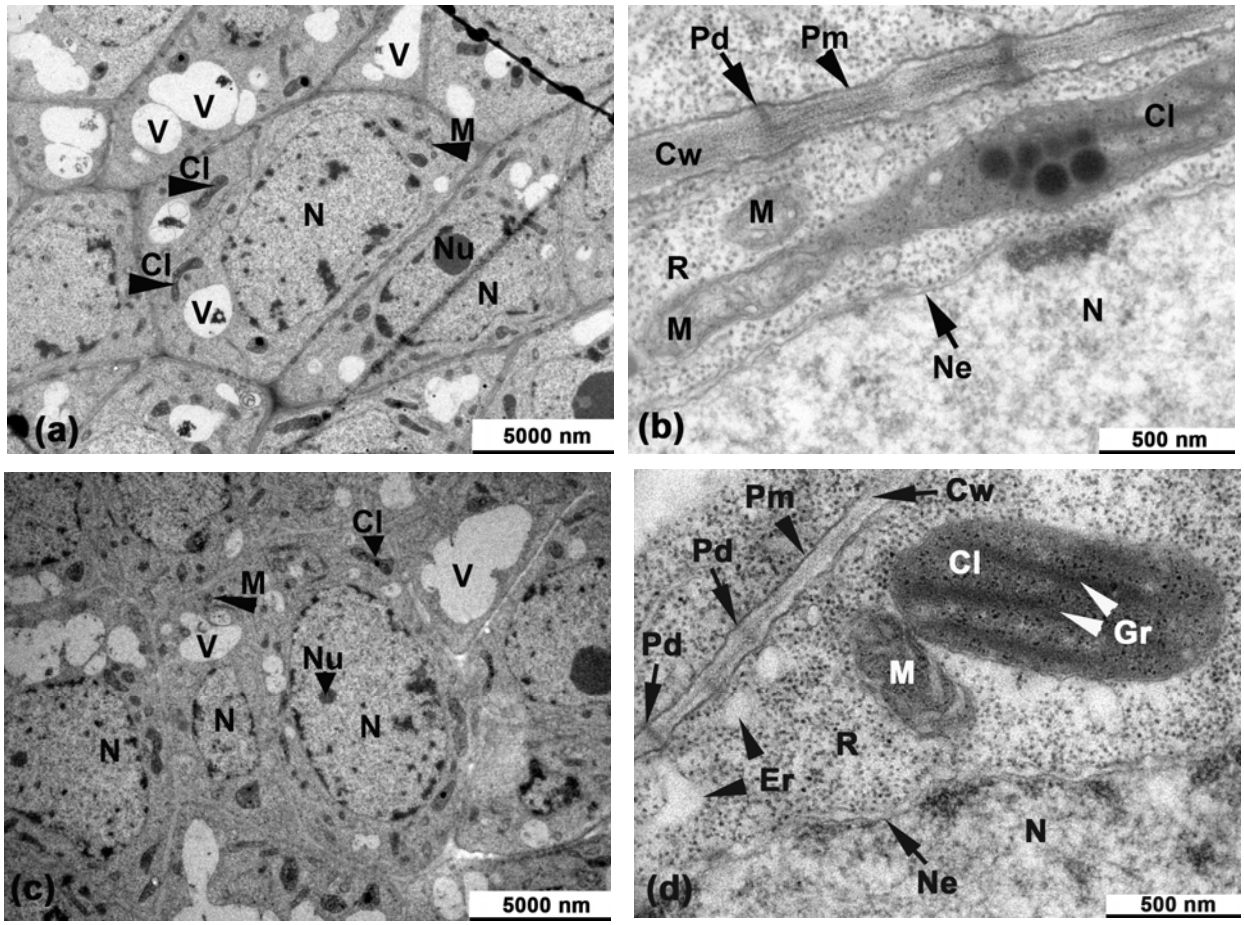


Figure 21a-d. Ultramicrographs of isolated shoot tip meristems. The ultrastructure of meristematic cells after CT **(a, b)** is indistinguishable from that of cells subjected to AT **(c, d)**. *Cl* chloroplast, *Cw* cell wall, *Er* endoplasmatic reticulum, *Gr* grana, *M* mitochondrion, *N* nucleus, *Ne* nuclear envelope, *Nu* nucleolus, *Pd* plasmodesma, *Pm* plasma membrane, *R* ribosomes, *V* vacuole.

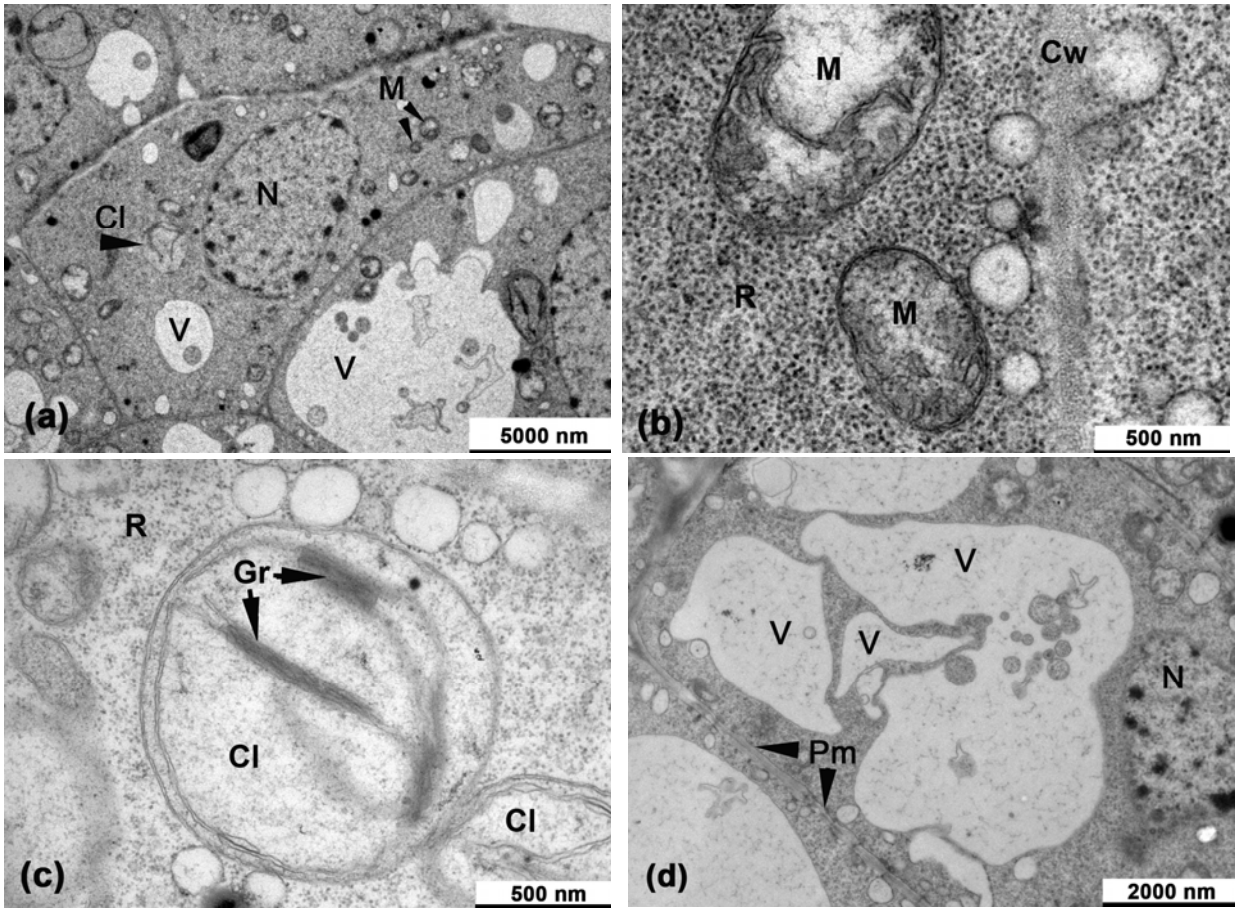


Figure 22a-d. Ultrastructure of isolated shoot tip meristems after AT and DMSO incubation for 2 h. **a)** cellular overview showing the presence of multiple small vacuolar structures, **b)** swollen mitochondria and small vesicle-like structures covered with ribosomes indicating that these represent dilations of the endoplasmatic reticulum, **c)** enlarged chloroplast and small vesicle-like structures, **d)** irregularly shaped large vacuoles with cytoplasmic invagination. *Cl* chloroplast, *Cw* cell wall, *Gr* grana, *M* mitochondrion, *N* nucleus, *Pm* plasma membrane, *R* ribosomes, *V* vacuole.

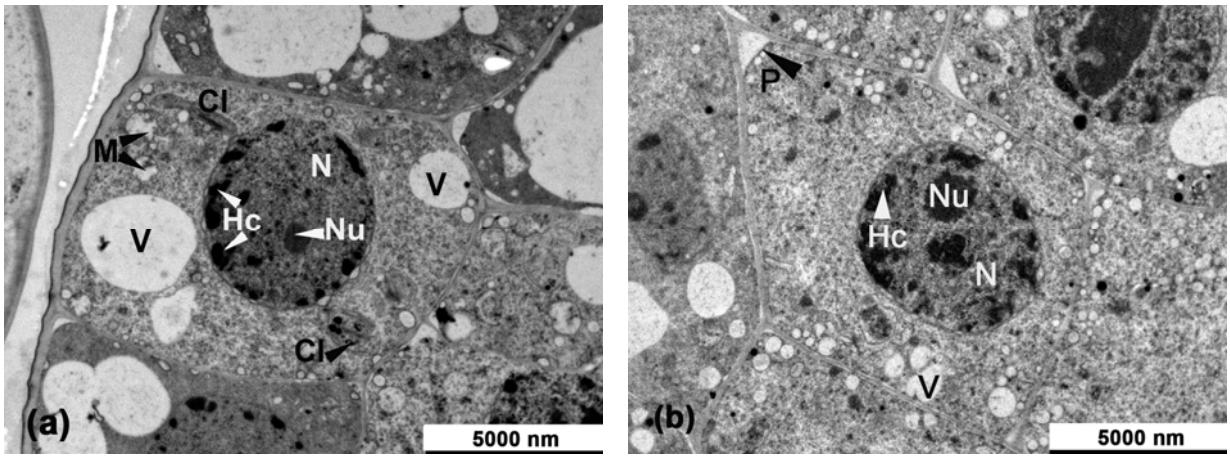


Figure 23a-b. Micrographs of cryo-stored shoot tip meristem cells 1 h after rewarming. a) meristematic cell of explant after AT containing nucleus with heterochromatin tightly appressed to the nuclear envelope, b) meristematic cell after CT showing nucleus with patches of heterochromatin distributed more freely throughout the nuclear interior. *Cl* chloroplast, *Hc* heterochromatin, *M* mitochondrion, *N* nucleus, *Nu* nucleolus, *P* plasmolysis, *V* vacuole.

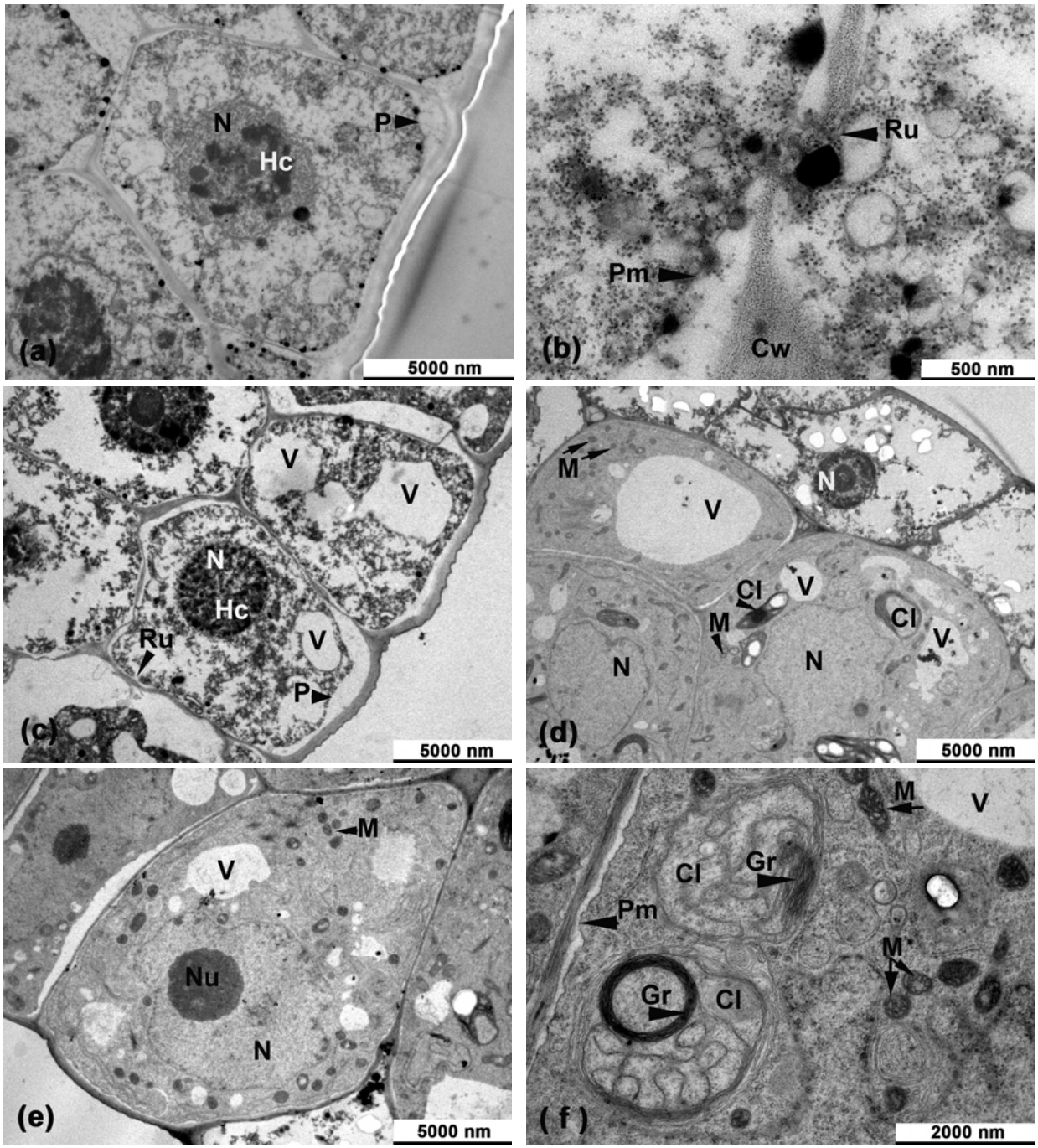


Figure 24a-f. Electron micrographs of meristematic cells 2 days after rewarming. **a)** CT explant after LN without cryoprotection (control), **b)** control showing rupture of plasma membrane, **c-f** AT explants with cryoprotection 2 d after rewarming, **c)** dead cells of meristematic dome area, **d)** dead epidermic cells and living parenchymal cells in leaf primordium, **e)** living epidermal cell in leaf primordium, **f)** aberrant appearance of chloroplasts in living epidermal cell. *Cl* chloroplast, *Cw* cell wall, *Gr* grana, *Hc* heterochromatin, *M* mitochondrion, *N* nucleus, *Nu* nucleolus, *P* plasmolysis, *Pm* plasma membrane, *Ru* rupture of plasma membrane, *V* vacuole.

3.6. Differential scanning calorimetry

3.6.1. Thermal analysis of solutions

MSH

MSH showed crystallization during cooling and glass transition and melting during rewarming. Tg was at -43.2 °C. The heat flow amount during melting was the highest in comparison to the other solutions with -333.2 J/g (Fig. 25a, Tab. 6).

10 % DMSO in MSH

The graph for the cryoprotectant solution (10 % DMSO in MSH) shows crystallization during cooling and two events during warming. Firstly Tg is detected at -121.6 °C and afterwards melting at -10.3 °C with heat flow of -226.0 J/g (Fig. 25b, Tab. 6).

99.5 % DMSO

Almost pure DMSO shows the same characteristics as MSH and cryoprotectant solution. Also crystallization during cooling and glass transition and melting during warming was found. Tg during warming was at -125.7 °C and melting point at 9.1 °C with heat flow of -170.9 J/g (Fig. 25c, Tab. 6).

PVS2

In contrast to the other solutions PVS2 did not show any crystallization or melting events. Two Tg's were detected. Tg1 at -115 °C was determined during warming and also visible during cooling. The specific heat capacity at Tg1 was the highest with 1.20 J/(g °C) in comparison to the other solutions measured (Fig. 27d, Tab. 6). Tg2 was detected at -46 °C and much smaller than Tg1 with specific heat capacity of 0.02 J/(g °C) (Fig. 25d, Tab. 6).

Table 6. Properties of different solutions in DSC analysis during rewarming (from -160 °C to 40 °C). Results are means of 5 experiments ± standard error.

Solution		Tg		Rewarming	
		Temperature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)
MSH		-43.20 ± 0.07	0.12 ± 0.00	-2.43 ± 0.02	-333.17 ± 0.83
10 % DMSO in MSH		-121.64 ± 0.04	0.24 ± 0.00	-10.29 ± 0.09	-226.05 ± 0.85
99.5 % DMSO		-125.67 ± 0.02	0.06 ± 0.00	9.12 ± 0.18	-170.94 ± 1.74
PVS2	Tg1	-114.52 ± 0.02	1.20 ± 0.01	_	_
PVS2	Tg2	-45.48 ± 2.51	0.02 ± 0.01		

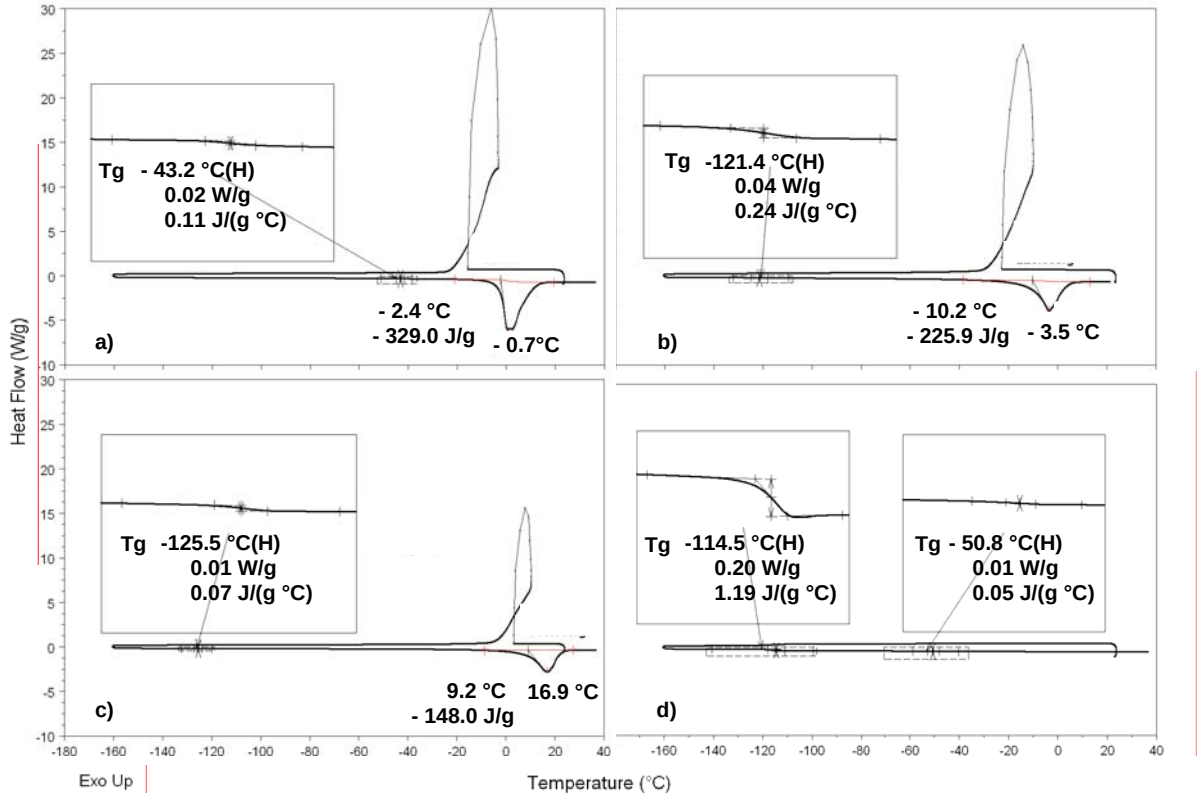


Figure 25a-d. Example of DSC thermograms of different solutions, **a)** MSH, **b)** 10 % DMSO in MSH, **c)** 99.5 % DMSO, and **d)** PVS2. Inlets showing Tg. Exothermic reactions (positive heat flow, upper part of curve) are shown during cooling. Calculation below curves showing negative heat flow (melting of ice, lower part of curve) during warming. Speed of cooling and warming 10 °C/min.

3.6.2. Comparison of thermal analyses of potato shoot tips after different precultures (DMSO droplet method)

3.6.2.1. Thermal analysis of freshly isolated shoot tips

Fresh, unprotected shoot tips showed clear crystallization during cooling and melting during warming. No glass transition was found (Fig. 26a, b, 27a, b; Tab. 7a, b). 'Désirée' had higher melt-endotherms in comparison to *S. demissum* independent of preculture (Fig. 28; Tab. 7a, b). Fresh shoot tips of 'Désirée' showed significant increase in heat flow during warming after AT of donor plants (324.5 ± 1.0 J/g) in comparison to CT explants (311.3 ± 1.1 J/g; Tab. 7a). Within *S. demissum,* the melt-endotherms during warming were independent of the preculture and did not show significant differences (Fig. 28, Tab.7b). The onset temperatures or melting points calculated during warming were -0.8 (after CT) and -0.9 °C (after AT) in *S. tuberosum* 'Désirée' and about 2 °C lower in *S. demissum* (-2.7 [after CT] and -3.0 °C [after AT]; Fig. 29; Tab. 7a, b). There were no significant differences with respect to onset temperatures and precultures within each species.

3.6.2.2. Thermal analysis of cryoprotected shoot tips

Shoot tips after CT and AT were analyzed. They were isolated, incubated overnight in MSH and cryoprotected in 10 % DMSO solution for 2 h (DMSO droplet method, Schäfer-Menuhr et al., 1994). Afterwards DSC measurements were performed. *S. tuberosum* 'Désirée' and *S. demissum* showed ice crystallization during cooling and melting during rewarming for all measurements. Cryoprotected shoot tips had lower heat flow during rewarming in comparison to freshly isolated shoot tips (Fig. 28, Tab. 7a, b). After cryoprotection, the onset temperature of melting was also significantly lower in comparison to freshly isolated shoot tips (Fig. 29, Tab. 7a, b). Heat flows and onset temperatures during melting were independent of preculture temperature and species (Fig. 26c, 26d, 27c, 27d, 28, and 29; Tab. 7a, b).

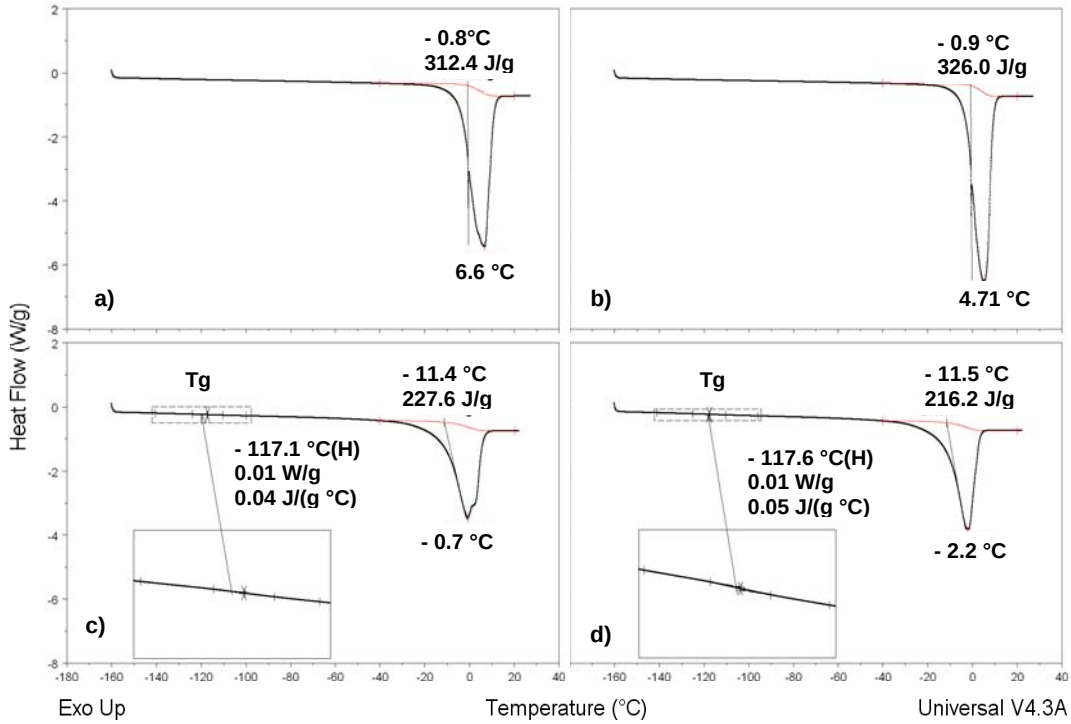


Figure 26a-d. Example of DSC warming thermograms of shoot tips of *S. tuberosum* 'Désirée'. Comparison of freshly isolated shoot tips with shoot tips isolated, incubated in MSH overnight and subsequently in10 % DMSO in MSH for 2 h as well as influence of CT and AT. **a)** fresh shoot tips CT, **b)** fresh shoot tips AT, **c)** cryoprotected shoot tips CT, **d)** cryoprotected shoot tips AT.

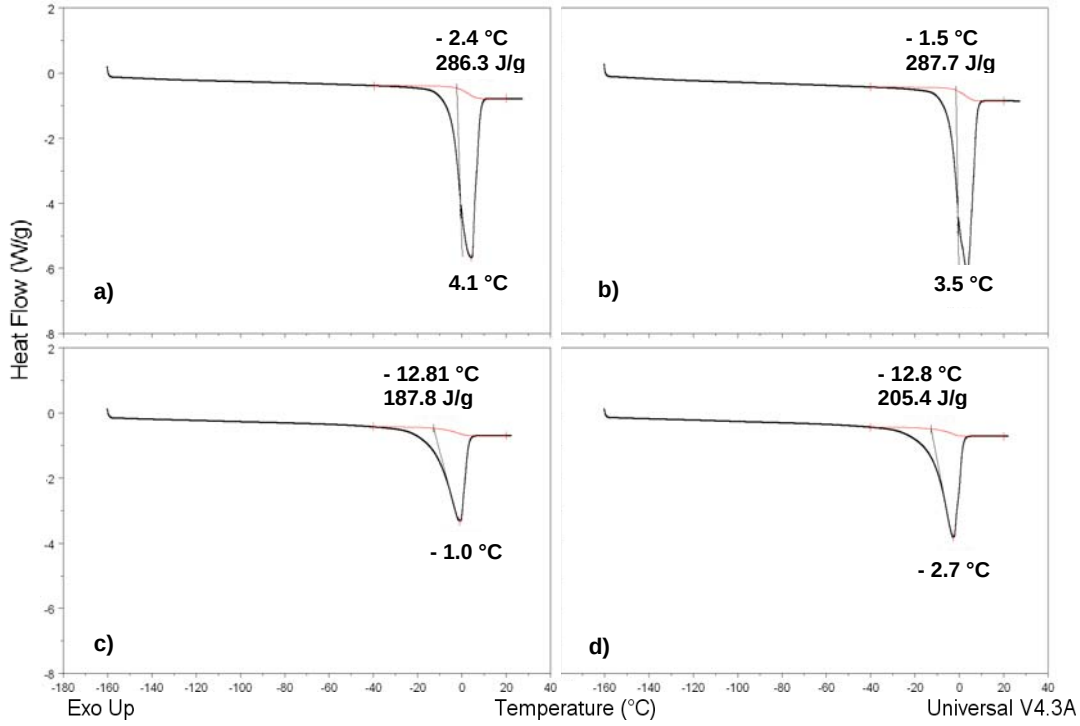


Figure 27a-d. Example of DSC warming thermograms of shoot tips of *S. demissum*. Comparison of freshly isolated shoot tips with shoot tips isolated, incubated in MSH overnight and subsequently in10 % DMSO in MSH for 2 h as well as influence of CT and AT. **a)** fresh shoot tips CT, **b)** fresh shoot tips AT, **c)** cryoprotected shoot tips CT, **d)** cryoprotected shoot tips AT.

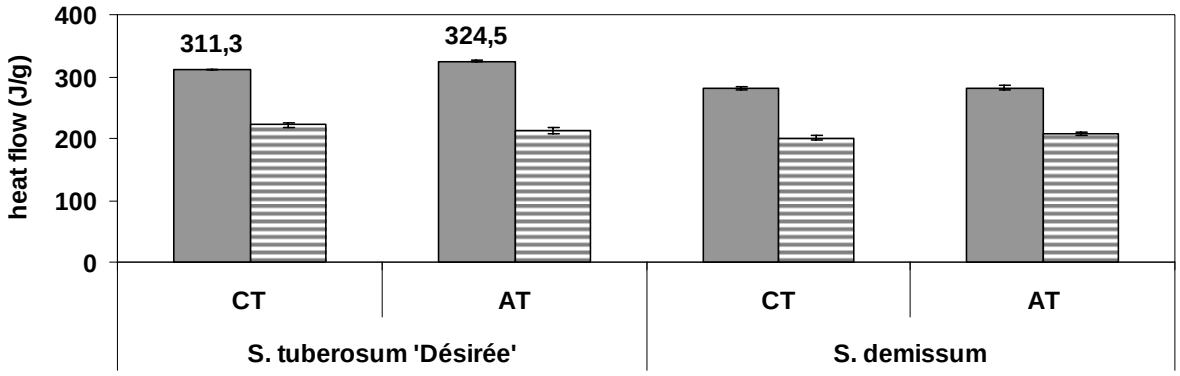


Figure 28. Heat flow enthalpies of the melt-endotherms (J/g ± standard error) during rewarming measured in DSC. Comparison of freshly isolated shoot tips (grey bars) with shoot tips isolated, incubated in MSH overnight and subsequently in 10 % DMSO in MSH for 2 h (striped bars). Results are shown for shoot tips after CT and AT of donor plants, means of 5 to 8 experiments, bar pairs with indicated value mark significant differences after AT in comparison to CT ($P < 0.02$).

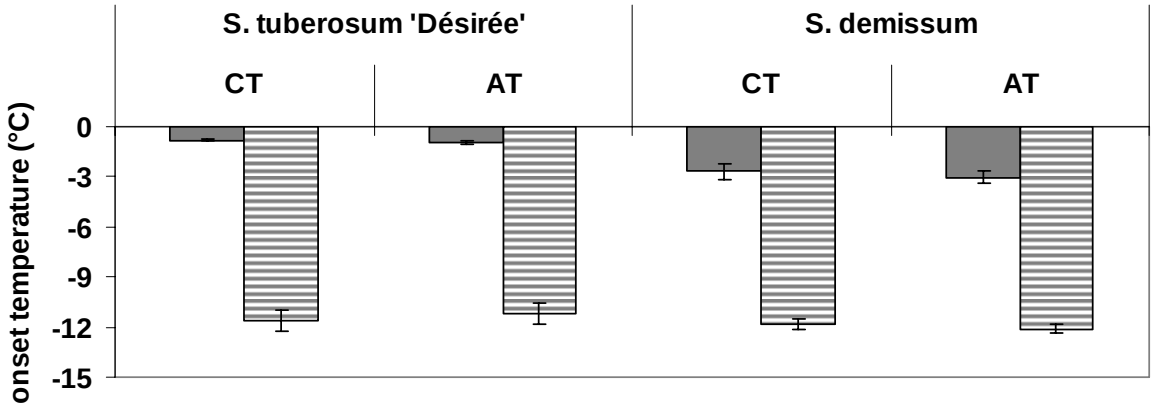


Figure 29. Onset temperatures ± standard error of the melt-endotherms during rewarming measured in DSC. Comparison of freshly isolated shoot tips (grey bars) with shoot tips isolated, incubated in MSH overnight and subsequently in 10 % DMSO in MSH for 2 h (striped bars). Results are shown for shoot tips after CT and AT of donor plants, means of 5 to 8 experiments.

Table 7a. Properties of Tg and melting (M) of *S. tuberosum* 'Désirée' measured by DSC of freshly isolated shoot tips (F) and shoot tips after cryoprotection (CP, 10 % DMSO in MSH for 2 h. Results are means of 5 experiments ± standard error.

	CT				AT			
	Tg		M		Tg		M	
	Temperature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)	Temperature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)
F	-	-	-0.79 ± 0.01	311.33 ± 1.10	-	-	-0.92 ± 0.10	324.48 ± 1.00
CP	-114.46 ± 1.16	0.03 ± 0.01	-11.58 ± 0.64	222.13 ± 3.32	-116.70 ± 0.59	0.05 ± 0.01	-11.22 ± 0.64	212.44 ± 4.16

Table 7b. Properties of Tg and melting (M) of *S. demissum* measured by DSC of freshly isolated shoot tips (F) and shoot tips after cryoprotection (CP, 10 % DMSO in MSH for 2 h). Results are means of 5 experiments ± standard error.

	CT				AT			
	Tg		M		Tg		M	
	Temperature (°C)	Specific heat capacity [J/(g °C)]	onset (°C)	Heat flow (J/g)	Temperature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)
F	-	-	- 2.67± 0.47	280.28 ± 2.38	-	-	-3.01 ± 0.41	282.16 ± 4.47
CP	-	-	-11.81 ± 0.32	200.59 ± 3.87	-	-	-12.11 ± 0.26	207.67 ± 2.45

In *S. tuberosum* 'Désirée', small Tg could be found during rewarming (Fig. 26c, d; Tab. 7a). This could not be observed for *S. demissum*. Heat capacity of 'Désirée' at Tg was 0.03 ± 0.01 J/(g °C) at -114.5 ± 1.2 °C for shoot tips with CT in comparison to 0.05 ± 0.01 J/(g °C) at -116.7 ± 0.6 °C for shoot tips after AT. No significant differences were found in Tg's between precultures.

3.6.2.3. Thermal analysis of potato shoot tips after direct cooling in LN

Shoot tips of *S. tuberosum* 'Désirée' were cryoprotected (DMSO droplet method), put into aluminium pans, sealed hermetically and cooled in LN outside the DSC device. The DSC measuring chamber was precooled to -180 °C and the pan with shoot tips was transferred quickly to the measuring chamber. Warming curves were monitored.

During warming two Tg's were detected. The first Tg was at -117 °C with specific heat capacity of 0.04 ± 0.01 J/(g °C). This was similar to Tg's detected with the cooling speed of 10 °C/min. A further Tg could be detected at -33 °C, which was not found in curves cooled with 10 °C/min. Specific heat capacity at the second Tg was higher with 0.35 ± 0.22 J/(g °C). The melting with heat flow of 189.89 ± 1.94 J/g was lower in comparison to slowly cooled explants (Tab. 8, Fig. 30).

Table 8. Properties of Tg's and melting of shoot tips of *S. tuberosum* 'Désirée' during rewarming after cryoprotection in DMSO solution for 2 h. Cooling outside DSC directly in LN, rewarming in DSC from -180 °C until 40 °C. Results are means of 3 experiments ± standard error.

Tg1		Tg2		Melting	
Temperature (°C)	Specific heat capacity [J/(g °C)]	Temperature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)
-116.97 ± 0.23	0.04 ± 0.01	- 33.22 ± 0.61	0.35 ± 0.22	-10.18 ± 1.00	189.89 ± 1.94

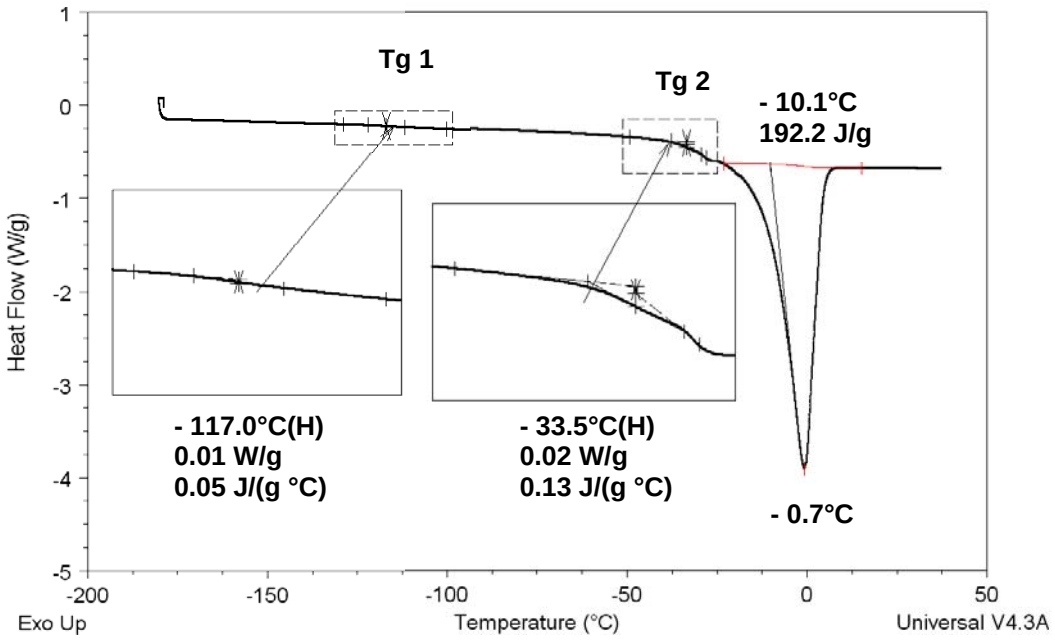


Figure 30. Example of DSC warming thermograms of shoot tips of *S. tuberosum* 'Désirée' cooled in LN. Inlets showing Tg's.

3.6.3. Comparison of different incubation times of potato shoot tips using PVS2 as cryoprotectant

DSC analysis was done after cryoprotection of shoot tips of *S. tuberosum* 'Désirée' in PVS2 for 20, 30, 40, and 60 min. For all curves Tg was detected between -103 and -116 °C. Specific heat capacity at Tg was highest with 0.5 J/(g °C) for samples incubated in PVS2 for 60 min. Some of the samples showed a second Tg between -41 and -75 °C (Tab. 9). Shoot tips incubated for 20 min were characterized by ice crystallization during cooling and the highest melting peak (111 ± 7 J/g) of all treatments during warming (Fig. 31, 32; Tab. 9). The longer the incubation time of shoot tips in PVS2, the lower was ice crystallization during cooling and the heat flow of melting during warming. The latter dropped from 111 J/g (20 min), over 85 J/g (30 min), 67 J/g (30 min) to 53 J/g after 40 min incubation. No melting was observed after an incubation time of 60 min (Fig. 31, 32; Tab. 9). Samples incubated for 30 min showed two different thermograms. Two out of 4 repetitions showed 2 Tg's (Tab. 9, 30 min A). The other 2 repetitions showed one Tg and recrystallization and melting (Tab. 9, 30 min B). Samples incubated for 40 min also showed one Tg and recrystallization and melting during warming.

Table 9. Properties of Tg, recrystallization (R) and melting (M) of *S. tuberosum* 'Désirée' after different incubation times in PVS2 during rewarming (-140 °C until 40 °C). After 30 min incubation time, two types of curves were detected (A, B). Results are means of 4 experiments ± standard error, * means of 2 experiments ± standard error, # one experiment (IT- incubation time).

	Tg1		Tg2		R		M	
PVS2 IT	Temper-ature (°C)	Specific heat capacity [J/(g °C)]	Temper-ature (°C)	Specific heat capacity [J/(g °C)]	Onset (°C)	Heat flow (J/g)	Onset (°C)	Heat flow (J/g)
20 min	-103.31 ± 0.72	0.32 ± 0.06	-72.43 ± 0.73	0.22 ± 0.02	-	-	-37.15 ± 1.92	110.87 ± 7.33
30 min A	-103.97* ± 0.40	0.36* ± 0.16	-74.76* ± 0.20	0.25* ± 0.11	-	-	-42.08* ± 0.83	85.29* ± 0.19
30 min B	-111.62* ± 6.32	0.17* ± 0.03	-	-	-97.24* ± 0.30	24.48* ± 7.91	-47.15* ± 0.14	67.19* ± 2.08
40 min	-115.72 ± 0.80	0.23 ± 0.09	-	-	-94.89 ± 1.31	36.13 ± 4.03	-46.37 ± 0.56	52.69 ± 1.48
60 min	-108.16#	0.48#	-40.73#	0.17#	-	-	-	-

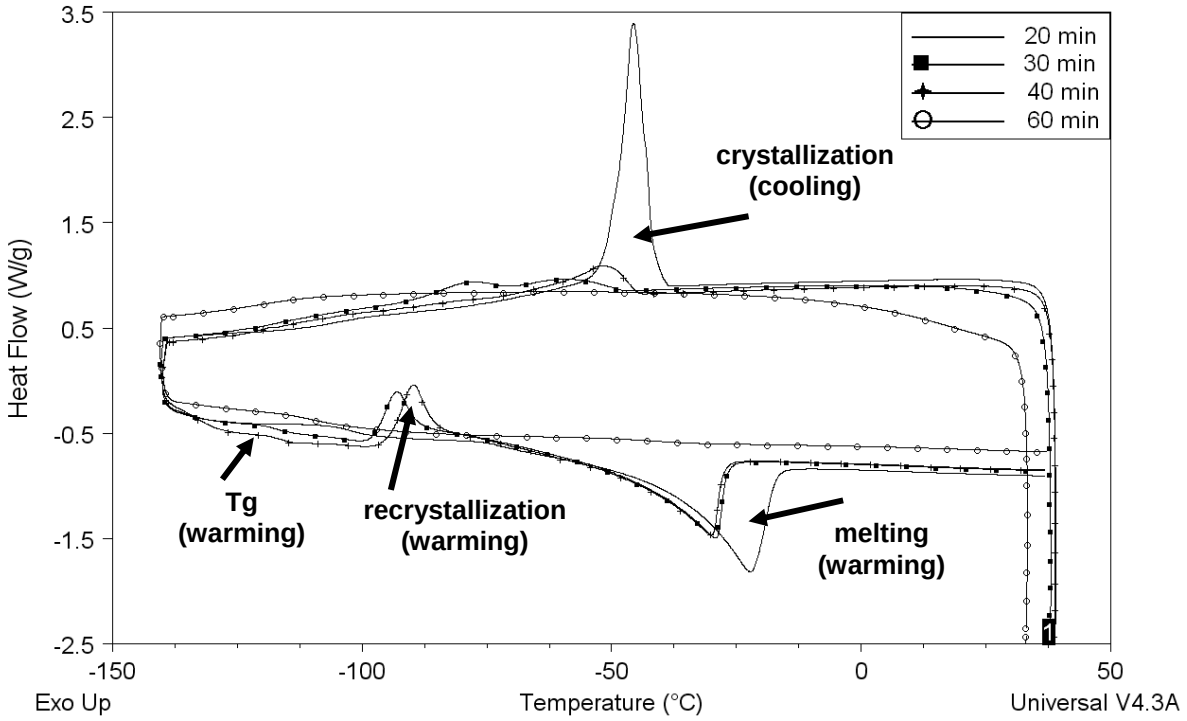


Figure 31. Example of DSC cooling and warming thermograms of shoot tips of *S. tuberosum* 'Désirée' after different incubation times in PVS2.

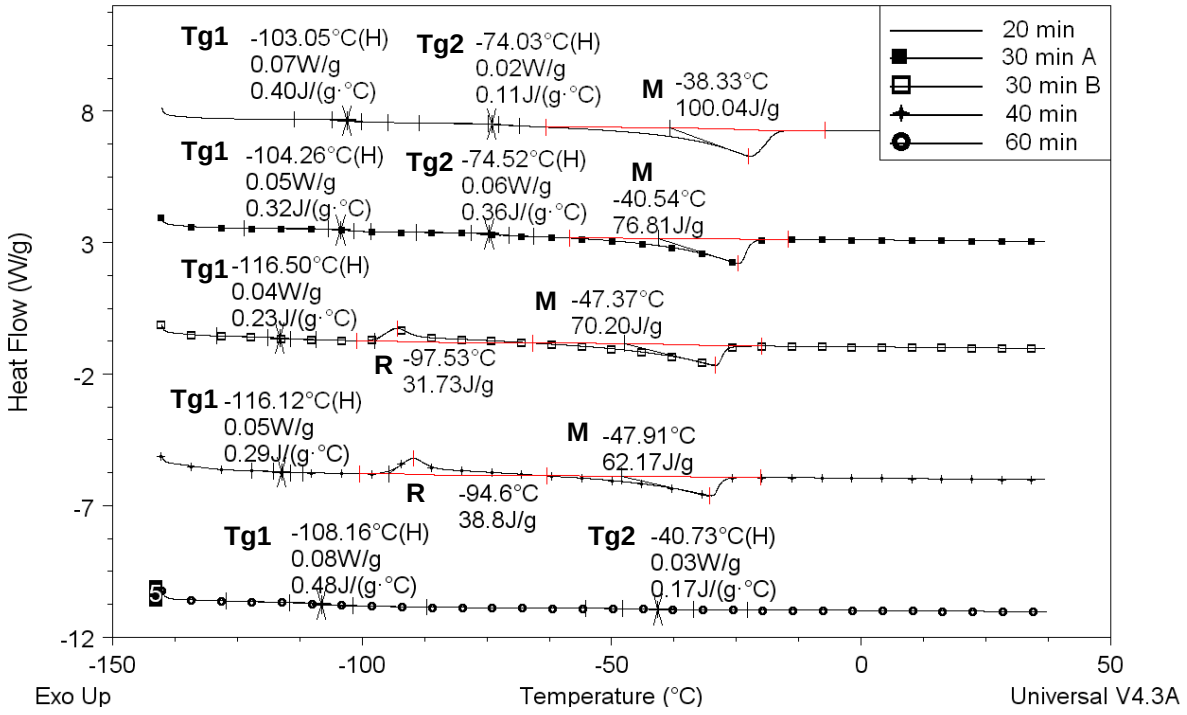


Figure 32. Example of DSC warming thermograms of shoot tips of *S. tuberosum* 'Désirée' after different incubation times in PVS2. Respective curves are shifted along the y-axis (R – recrystallization, M – melting).

Chapter 4. Discussion

4.1. Influence of alternating temperature preculture and solid regeneration medium on cryopreservation results

The specific AT of donor plants applied in the current study affects the cryopreservation results of potato positively using the DMSO droplet method. Similar results were found by Kryszczuk et al. (2006), who could demonstrate that cold preculture of 7 d with 21/8 °C (day/night temperatures) increased the regeneration rates of four tested cultivated potato accessions using the original protocol described by Schäfer-Menuhr et al. (1994). The AT always increased the regeneration percentage of all accessions, independent of the regeneration medium used. Only *S. tuberosum* 'King Edward' showed decrease in regeneration in liquid medium. Out of different tested preculture temperatures, the oscillation of 22 to 8 °C seems to be very important to improve cryopreservation results. In previous studies, other authors (Halmagyi et al., 2005; Hirai and Sakai, 1999) used preculture temperatures of constantly 4 °C. However, they failed to show any improvement of regeneration percentages after cryopreservation in comparison to original protocol conditions. Based on the presented results it was concluded that preculture temperatures lower than 8 °C might not be favourable for *in vitro* plants of potato. In agreement with the shown results, Zhao et al. (2005b) could also demonstrate that pretreatment of potato plants at 10 °C for 3 weeks yielded in improvement of cryopreservation results.

In the experiment using agarose drops with liquid regeneration medium, wild species showed better cryopreservation results in comparison with cultivated potatoes. Regeneration percentages of wild species were independent of preculture. An interrelationship between frost tolerance of the different species and their regeneration behaviour after cryopreservation was speculated. This was disproved in the next cryopreservation experiment using solid regeneration medium. In this case, both cultivated potatoes showed better regeneration than the wild species *S. acaule* after AT. From these experiments it can be concluded, that differences in cryopreservation results are not directly related to cold acclimation and cold tolerances. Results of cold tolerance tests are discussed in the section below.

Interestingly, the comparison of liquid and solid regeneration media showed that the solid medium was more suitable. In the latter case, the regeneration percentages after cryopreservation were higher for *S. tuberosum* 'Désirée' and 'King Edward', independent of

preculture. On solid medium less callus formation was found and the regenerated plants showed better structure of shoots, leaves and roots. The drier environment and contact with the medium on only one side of the explants could further reduce callus formation and enhance better shoot formation. Furthermore, the method using solid media was faster and easier in handling.

Based on the shown results it is concluded that the used system increases regeneration after cryopreservation for all accessions, but with highest improvements for such accessions with lower regeneration percentages. Solid regeneration medium showed better results concerning average regeneration percentage, plantlet formation and handling of method. Because of all these positive findings, the DMSO droplet protocol so far used in IPK is recently in change towards application of AT and solid medium for regeneration.

4.2. Biochemical changes after AT

Temperatures of 2 to 4 °C are usually used for experiments to induce cold acclimation in potato plants (Li et al., 1979, Swaaij et al., 1985; Stone et al., 1993). Li et al. (1979) defined the upper limit of acclimating temperature between 12 and 13 °C for *S. commersonii*. The AT chosen in this study, using oscillation between 22 and 8 °C, lies below this limit. Therefore, it was assumed, that cold acclimation takes place during preculture for wild species. From other cryopreservation studies it is known that cold acclimation improves regeneration (Chang and Reed, 2000; Niino and Sakai, 1992). The hypothesis was formulated, if cold acclimation could be confirmed through cold tolerance determination and biochemical characteristics, improved results after AT could be related to this aspect in this study.

Cultivated potatoes are known to be unable to cold-acclimate (Li et al., 1979). But cold precultures applied in tropical and subtropical species, which are also not able to cold-acclimate, have shown improved cryopreservation results (Leunufna and Keller, 2005, Chang et al., 2000). In cultivated potatoes cold acclimation tests have not been done with *in vitro* cultures so far. Therefore, in this study biochemical experiments were done on one side to find out, if cold acclimation takes place for tested accessions. On the other side, biochemical compounds should be characterized, which could explain better cryopreservation results after AT.

The process of cold acclimation is a multiple trait with complex physiological and biochemical changes (Hannah et al., 2005). A typical characteristic is the increased concentration of soluble sugars in cold-acclimated plants (Levitt, 1972). This could be found

in all analyzed potatoes, although increases were sometimes not significant. In comparison to CT, increase in soluble sugar content after AT was highest in *S. demissum* with 42 % and lowest in *S. tuberosum* 'King Edward' with 4 %. Double or higher increases, as reported by Li et al. (1979) in *S. acaule* and *S. tuberosum* after hardening at 2 °C for 15 d, could not be found.

Besides sugar accumulation also starch increases at low temperatures (Li and Fennel, 1985). This could not be confirmed in this study, because starch decreased in three accessions. Only *S. tuberosum* 'King Edward' showed no decrease in starch concentration after AT. Because of relatively small increases in sugars and mainly decrease in starch concentration, it was concluded, that the preculture used in this study is not cold enough to induce cold acclimation in potato plants. This was proven in the cold tolerance experiments. In these, none of the tested accessions showed cold acclimation or improved cold tolerance after AT independent of preculture and culture system (*in vitro* / greenhouse plants). From this result it became clear, that cold acclimation does not occur during AT, e.g. the above mentioned hypothesis could not be confirmed. The observed single changes can, however, be interpreted as part of the rather complex process of cold acclimation. They are, nevertheless, not sufficient to represent the whole acclimation process. The higher concentration of soluble sugars could be the reason for improvement of regeneration after cryopreservation. Soluble sugars have been shown to act as cryoprotectants in stabilizing membranes during cooling (Anchordoguy et al., 1987; Hincha, 1990) and lowering the cell water content osmotically (Hitmi et al., 1999) to prevent lethal intracellular ice formation.

Osmolality was the lowest in *S. demissum,* which had the best cryopreservation results independent of preculture and regeneration medium. This is in contrast to the hypothesis, that the tissue with highest osmolality would have best cryopreservation results. One explanation could be that the material used for osmolality measurements was different to that used for cryopreservation. In the first case whole *in vitro* plants without roots were measured, in the second one the shoot tip only. Therefore, it would be best to measure osmolality directly in shoot tips. This could not be done in this study, because of time limit and the high numbers of shoot tips needed for such a measurement. Another explanation would be that the pretreatment of shoot tips in MSH and cryoprotection in DMSO could have a greater influence on cryopreservation results than the osmolality measured in donor plants. Osmolality was lower in cultivated potatoes and slightly higher or the same in wild species after AT in comparison to CT. Better cryopreservation results could be explained by lower osmolality in the following way. During cryoprotection a faster import of DMSO solution in

shoot tips with low osmolality is possible, so that higher DMSO concentration within shoot tips could lead to better results. This would explain the low osmolality in *S. demissum,* which had the highest regeneration results. But this can only be part of the explanation, because, as seen in the cryopreservation results, also the type of regeneration medium was of big influence on regeneration results. More repetitions should be done to confirm this hypothesis. The concentration of DMSO in shoot tips after cryoprotection should be determined, as has been done by Kim et al. (2004).

The concentrations of essential amino acids were measured in the investigated accessions in order to find out whether there was a positive correlation between cryopreservation and endogenous amino acids. Highest concentrations were found in asparagine, glutamine, glycine and proline. Asparagine increased after AT in three analyzed accessions, but not in *S. demissum*, which showed minor decrease. The increase of the concentration of asparagine at low temperatures was already reported by Stewart and Larher (1980), who showed that asparagine positively correlated with freezing tolerance in apple. In the present study, glutamine concentration decreased in three analyzed accessions, but not in *S. tuberosum* 'Désirée'. Glutamine as well as asparagine belong to the class of nitrogen-containing amino acids (Lohaus et al., 1998; Urquhart and Joy, 1982). Usually they are produced in roots and in leaves from where they are transferred to the phloem to be transported to young leaves and vegetative apices. They do not go through the xylem, because of lack of transpiration within vegetative apices (Urquhart and Joy, 1982). It is not clear, why the glutamine concentration was lower after AT in the present study. One possible explanation might be that glutamine was immediately converted to other amino acids, nucleic acids and other nitrogen containing compounds. Most interesting was the change in concentration of proline. For three of the four tested accessions, decrease in proline concentration was measured in the shoot tips after AT. Usually proline is accumulated at low temperatures (Stewart and Larher, 1980). This amino acid is known to act as osmoregulator, protective agent for cytoplasmic enzymes and membranes and is a compound, which accumulates under stress (Swaaij et al., 1985).

In general it is known, that soluble nitrogen content increases in plants grown at low temperatures (Levitt, 1972). This could be confirmed in the current study for asparagine. Other amino acids mainly decreased in concentration after AT. Reasons for the decrease in amino acid concentrations could be that AT represented temperature conditions, which did change the amino acid metabolism only partly. Therefore, the typical effects of low temperature application were not detectable.

The applied alternating temperatures are similar to those in the natural environment of *Solanum* species and, thus, seem more favourable than the constantly warm temperatures, usually applied to *in vitro* cultures of potato. This may indicate that constantly warm temperatures could more likely induce stress rather than alternating temperatures.
To conclude, biochemical analyses revealed that no cold acclimation takes place after AT. Therefore, cold acclimation capacity and cold tolerance can not be directly related to cryopreservation results under the environmental conditions of this study. Improvement of cryopreservation results can be related to increase in soluble sugar concentration. Starch and amino acid concentrations mainly decreased after AT. To increase the soluble sugar concentration, which could perhaps further improve cryopreservation results, lower alternating temperatures like 22/2 °C or 15/2 °C (day/night temperatures) should be tested.

4.3. Changes in the proteome after AT

Characteristics after CT or AT were also determined on the proteome level. Proteome studies on potato shoot tips have only been done on a side study so far (Carpentier et al., 2005; CRYMCEPT, 2002 - 2005). Since shoot tips are the material for cryopreservation and the before-mentioned biochemical studies have been performed with shoot tips, also for this analysis shoot tips were investigated. The problem was to harvest enough shoot tips at a given time. For one biological experiment between 300 and 700 shoot tips had to be collected, because at least 100 µg of pure protein was needed for one gel. During the harvest, problems of standardization occurred. Although any effort was made to standardize plant growth, small differences between the plantlets were visible concerning height or strength of leaves and stems. A further problem was the excision procedure of shoot tips. It was difficult to excise always the same proportion and type of tissue. The size of the apical meristem itself also differs. Apical shoot tips of the cultivar ‘Désirée’ were bigger, more hairy and more difficult to excise than such of *S. demissum.* Another problem may be that the degree of injury, imposed by the excision procedures, differs from shoot tip to shoot tip. Similar difficulties were reported for proteome analysis of apical shoot tips of potato cultivars ‘Unicopa’ and ‘Ijsselster’ (CRYMCEPT Report, 2002 - 2005). After preparation of material, two different extraction protocols and two types of staining were compared to find a suitable protocol for potato tissue with a minimum of starting material. Until now the minimum of at least 1 to 2 g of material was needed for proteome analysis (Schlesier and Mock, 2006; Schlesier et al., 2004), but with the protocol established in this study, 150 mg of material is sufficient. The

protocol optimized in this study includes extraction with TCA/acetone precipitation and staining using RuBP. The 2D gels produced were very good in quality concerning resolution and numbers of detected spots, making the optimized protocol suitable for potato shoot tips.

In the first analysis of results, repetitions of 3 biological experiments were compared with each other. The results were rather different from experiment to experiment. For example, in the first repetition one protein spot was upregulated, while the same spot was downregulated in the second and third repetitions. The reproducibility was therefore not very high. Similar problems were also reported by the CRYMCEPT Report (2002 - 2005), were up- and-down regulation of matched spots was difficult to reproduce. Also Weckwerth et al. (2008) mentioned that achieving reproducibility of 2D gel analyses is laborious and dependent on sample origin and biological variability. Of course, a solution of this problem would be to perform more biological repetitions. But this was not possible, because of difficulties in collection of material and limit of time. Therefore, another way was gone for the evaluation of the analyses. All biological repetitions were put into one group, which was compared for both treatments (CT *vs.* AT). Results revealed 9 and 64 protein spots ('Désirée' and *S. demissum*), which were at least 1.5-fold changed in expression between the treatments. Of the differently expressed proteins found, the change in expression was not very high with a maximum intensity of 2.5-fold for spot 1 of 'Désirée' and a minimum intensity of – 2.7-fold for *S. demissum* spot number 20. Most of the spots had an expression of +/- 1.5 to 1.6-fold between preculture treatments, meaning, that no strong change of one or a group of certain proteins was detectable. Also the number of protein spots expressed differently after AT in comparison to CT was rather low with 0.4 % for 'Désirée' and 2.7 % for *S. demissum*.

During identification of proteins using mass spectrometry, it was found, that the protein concentration derived from the use of the RuBP-stained gels was not high enough. Therefore, new gels with higher protein concentration were made (200 µg protein, cCBB staining), which led to successful identification of a part of the analyzed spots. Most of the proteins were identified using MALDI-TOF, a few more using LC-ESI-Q-TOF-mass-spectrometry.

One of the identified proteins of *S. tuberosum* 'Désirée' was spot number 2, which was similar to an AAA-type ATPase family / ankyrin repeat family protein of *A. thaliana*. This protein belongs to the functional group of transporters (Bevan et al., 1998). AAA-type proteins (ATPases Associated with diverse cellular Activities) form a large protein family with manifold cellular functions (Frickey and Lupas, 2004). These proteins play a role in molecular chaperones, ATPase subunits of proteases, helicases or nucleic-acid-stimulated ATPases (Iyer et al., 2004). Ankyrin repeats are tandemly repeated modules of about 33

amino acids. They occur in a large number of functionally diverse proteins such as enzymes, toxins, and transcription factors mainly from eukaryotes (Bork, 1993). Bae et al. (2008) reported that *Arabidopsis* ankyrin repeat protein (AKR2A) plays an essential role with chaperone activity in the biogenesis of the chloroplast outer envelope membrane proteins. Works, which describe the function of this protein family in potato, are not published so far. But for tobacco, which belongs to the same botanical family as potato, ankyrin repeat-containing proteins are known to be required for salicylic acid signalling in the activation of plant defence after pathogen attack (Klessig, 2000). In this study in *S. tuberosum* 'Désirée' the described protein was 2.4 fold upregulated after AT. Because of the many functions of the AAA-type protein family / ankyrin repeat family proteins it is not possible to determine the specific function for the protein identified within this study.

Another identified protein in *S. tuberosum* 'Désirée' was starch synthase, which belongs to the functional group of metabolism (Bevan et al., 1998). Starch synthase catalyzes the reaction of ADP-Glucose to starch in plastids in 'source' organs likes leaves (Weiler, 2002) or it catalyzes starch production in amyloplasts in 'sink' organs like the potato tuber (Fernie et al., 2002). This enzyme was downregulated 1.5-fold after AT in shoot tip tissue in this study. Downregulation of starch synthase and, therefore, starch synthesis results in lower starch concentration. This fact correlates with the results of starch determination within this study. The starch concentration measured was also lower after AT for most of the accessions tested in comparison with CT explants. Although the change in expression for starch synthase was only small, decreasing tendencies in starch concentrations were confirmed through proteome analysis.

The nitrogen starvation response protein, identified in *S. demissum*, also belongs to the functional group of metabolism. Teichert et al. (2004) described this protein in the role of glutamine synthetase as an enzyme and as a possible regulator in the nitrogen regulation system. In this study, nitrogen starvation response protein was found in three analyzed spots; 20, 33, and 99. The expression was different between spots. Whereas for spot 20 and 99 the proteins were downregulated (-2.7 and -1.5-fold), for spot 33 the expression was upregulated (1.8-fold). The nitrogen metabolism seems, therefore, to be changed after AT. Resulting from these different expressions, it did not become clear, which function the nitrogen starvation response protein has within the processes investigated in this study.

For *S. demissum,* a protein (spot 66) similar to beta-1,3-glucanase like protein from *Olea europaea* was found to be downregulated 1.6-fold after AT. Beta-1,3-glucanase is a member of the 'pathogenesis-related' protein family and was detected in pollen tissue (Huecas et al.,

2001). This protein was assigned to the functional group of disease / defence proteins after Bevan et al. (1998). Sequence similarity of *S. demissum* to the described protein could mean that defence mechanisms within potato shoot tips were less after AT. But this function can only be speculated, because similarity was only weak and results should be proved in further experiments. Another protein, Sn-1, was identified for *S. demissum* also from the functional group of disease / defence proteins. Pozueta-Romero et al. (1995) isolated Sn-1 from red fruits of *Capsicum annuum*. Sn-1 was located at the subcellular level in the membrane of vacuoles. Sequence similarity for this protein showed homology to proteins involved in the plant's disease resistance response. In contrast to Beta-1,3-glucanase like protein, Sn-1 was upregulated in spot 58 (1.7-fold) and spot 75 (1.6-fold) after AT in this study.

In conclusion, no clear tendency in stress regulation and metabolic activity within identified protein spots could be found. Some proteins were up- and some were downregulated. The fact that one protein was found more than once with different regulations, make definite conclusions very difficult.

Although some proteins were identified, the majority of differently expressed proteins could not be specified. On one side, this was due to the low intensity of analyzed spots. On the other side, potato is one of the important model organisms, where DNA sequencing started, but so far the full genome sequence is not available (http://www.potatogenome.net). The database for potato EST is, therefore, not complete and in most cases homologies to other plant EST's were not similar enough for identification. Also algorithms for protein identification from mass spectra need to be improved in order to upgrade the plant proteomics approach (Weckwerth, 2008).

Proteome analysis after cold treatment at 6 and 10 °C for 7 d was done with *Arabidopsis thaliana* by Amme et al. (2006). They showed that identified proteins belonging to the cold-stress responses were mainly upregulated. However, in this study, for both accessions tested the majority of identified proteins belong to the groups of metabolism and signal transduction, which were mainly downregulated. This result was unexpected, because it was initially thought that the AT would induce metabolism and stress responses of the plant. This and the generally low change in protein expression as well as the low number of changed spots lead to the conclusion that the AT does not impose metabolic stress in the potato plants. This confirms the results obtained by soluble sugar, starch and amino acids determination.

Further repetitions of experiments need to be done to strengthen the received results. This is possible with the protocol newly established here for potato shoot tips. With improvement of the potato EST database also better results can be expected.

4.4. Ultrastructural changes associated with cryopreservation of shoot tips of S. tuberosum *'Désirée'*

The ultrastructural analysis of potato shoot tips was made in the consecutive steps of the DMSO droplet-freezing protocol and subsequent rewarming. Ultrastructural characteristics of preservation, damage, and regeneration of cells were analyzed to find the most critical steps in the protocol and the origin of shoot regeneration. The infiltration with 10 % DMSO solution proved critical in the DMSO droplet method, since after this step damages appeared in the cellular ultrastructure for the first time. This result is similar to that of Mikula et al. (2005) where proembryogenic cells of *Gentiana tibetica* were damaged in the ultrastructure after treatment with sorbitol and DMSO. The swelling of organelles could be due to increased membrane permeability caused by DMSO, or it might be the first indication of cell damage since similar ultrastructural changes were also found after chilling (Huang et al., 1995; Kratsch and Wise, 2000) or water stress (Lopez-Carbonell et al., 1994). Besides the swelling of organelles formation of vesicles was observed. The origin of these small vesicles is not clear. Since most of these vesicles were found in the proximity of the cell wall it is assumed that they have been derived from invaginations of the plasma membrane (Diekmann et al., 1993). It can not be excluded, however, that parts of the vesicles were the result of fragmentation of the central vacuole.

DMSO is most widely employed as a cryoprotectant for animal, human and plant tissues (Kim et al., 2004). It is known for its ability to penetrate cellular membranes (Jacob and Herschler, 1986; Kim et al., 2004) and for its effectual binding of toxic radicals (Jacob and Herschler, 1986). It can act as cryoprotectant either alone, like in the method described here, or as part of a complex cryoprotectant solution like in the vitrification method (Sakai, 1999). DMSO is thought to produce highly structured water clusters, thus reducing the concentration of free water molecules and lowering the freezing point (Szmant, 1975). When shoot tips are cooled rapidly, the free cellular water becomes vitrified (Grout and Henshaw, 1978), thus preventing the formation of ice crystals which are lethal for cell survival. In the vitreous or so-called 'glassy state', no further physical or ultrastructural changes should happen in the plant tissues regardless of storage time (Finkle et al., 1985). During rewarming recrystallization of ice is possible, which can be avoided through high rewarming speeds, so that there is no time for formation of damaging ice crystals.

In this study, however, ice crystallisation was found in *S. tuberosum* 'Désirée' shoot tips during cooling and melting of ice during rewarming by DSC. Directly after rapid rewarming

the ultrastructure of plant tissues appeared basically unaltered when compared to the condition just prior to cooling. These results show that ice crystals might not damage cells directly during the process of cooling and rewarming. Ultrastructural changes became apparent within 1 h after rewarming and, 2 d later; it was evident that most cells of the meristematic dome had died. Consequently, in this period processes should have occurred which would be critical for the further fate of the cells. The factors which contribute to these processes can only be speculated so far. The cellular metabolism is arrested during cryopreservation. Immediately after rewarming structures, which are visible by ultramicroscopic observation so far used, seem to be the same as before cooling. Therefore, the delayed decay of cellular structures must have another background. It can be hypothesized that cells may die through programmed cell death, perhaps in the sense of apoptosis. It is assumed, that structural damages, e.g. such of the plasma membrane, could be very small and, therefore, not be visible. The cell could either compensate them ore not be able to do so.

Kratsch and Wise (2000) found that disintegration of chloroplasts, vesiculation of cellular membranes, shrinkage of cytoplasm and nuclear DNA condensation are activators for programmed cell death in plants. This theory would be confirmed through the accumulation of nuclear heterochromatin and survival of cells directly in the neighbourhood of dead cells within the tissue. This has been observed in this study as well. Another hypothesis is that the cytoskeleton might be damaged during the cryopreservation process. The cytoskeleton is mainly responsible for cellular shape and mitosis. Cells would not be able to divide and it would not be possible for the shoot tip to regrow, if actin filaments would be severely damaged. So far for plant cryopreservation only few studies were published in this field. Only Morisset et al. (1993) showed that the structure and distribution of actin filaments were stable for successfully cryopreserved carrot cell suspensions. Further investigations on the behaviour of the cytoskeleton are, therefore, desirable.

The ultrastructural damages could partly be due to DMSO influence. As is known, cryoprotective agents are not able to allow 100 % survival after freezing and thawing (Fahy, 1986). As for all cryoprotectants, the concentration and incubation times are very critical in the experiment. Too long incubation periods or too high concentrations are toxic for plant tissue. That is why cryoprotective agents can directly be related to cryoinjury (Fahy, 1986). Also DMSO can play some damaging role during freezing (Fahy, 1986). So, e.g., it can destroy cells, when the initial concentrations were too high (Fahy and Karow, 1977). During cooling of biological tissue, damages take place primarily on the membranes (Gonzalez-Arnao et al., 2008; Li et al., 1979; Muldrew et al., 2004). These damages could lead to cell

breakdown and cell death. Small vesicles close to the plasma membrane are a typical feature of membrane alteration and damage during freezing and rewarming (Fujikawa, 1994; Steponkus, 1984). These vesicles were also observed in this study. They could be the result of material deleted from the plasma membrane (Dowgert and Steponkus, 1984) or broken intracellular organelles, which had formed after ice crystal formation (Fujikawa, 1994). The formation of ice crystals could also be the reason for cell death. Ice crystal formation can occur during cooling or as recrystallisation during warming. If crystals are big in size, they destroy membranes, and cells die. Small ice crystals can be tolerated by cells as demonstrated by Gonzalez-Arnao et al. (2008) and Mazur (1977). Shoot tips consist of inhomogeneous tissue. Therefore, it is possible, that some parts contain ice crystals during cryopreservation and other parts do not. Depending on the relative size of undamaged parts and amount of ice formation, the tissue dies or can survive.

The DMSO droplet method was found successful, because some primordial cell groups survived to form the nucleus of plant regeneration. This result was also found by other authors (Fukai and Oe, 1990; Grout and Henshaw, 1980; Haskins and Kartha, 1980; Helliot et al., 2003). Thus, it seems that these small areas are sufficient for plant regeneration. A similar result was found by Sussex (1952), who showed that only a small number of cells next to the apical meristem are necessary for complete new shoot formation of potato. These areas should be genetically stable, because primordial and adjacent cells are located directly next to the apical meristem (Sitte, 1998). If the apical meristem is damaged, the leaf primordium and adjacent cells can generate an axillary meristem, which can be stimulated through cytokinins in the regeneration medium. This axillary meristem will take over the function of the apical meristem, and new shoot regeneration can take place. In this case, shoots would be newly formed, but because the mother tissue is never unstructured like callus tissue, it should be genetically stable. Also, like mentioned in the introduction, genetic stability was proved for this method by Schäfer-Menuhr (1997). Further confirmation of genetic stability of cryopreserved potato shoot tips was done by other studies (Benson et al., 1996b; Harding, 1991; Harding and Benson, 2000). Direct regeneration, i.e. outgrowth of the existing meristems without callus formation, was only rarely observed. In these cases, most cells of the shoot tips must have withstood cryopreservation. Both ways of shoot tip regeneration, direct outgrowth and further development of leaf primordia structures, were also found in *S. goniocalyx* by Henshaw et al. (1985a). The formation of multiple shoots could be due to the presence of zeatin riboside in the regeneration medium (Towill, 1983). This phytohormone, belonging to the group of cytokinins, stimulates adventitious shoot formation, cell division,

and outgrowth of axillary buds (Salisbury and Ross, 1992). Because only small tissue areas survived cryopreservation it is necessary to apply phytohormones into the regeneration medium to stimulate cell division and growth (Schäfer-Menuhr et al., 1994). Without these hormones, callus formation can be avoided, but regeneration percentages would be less (Henshaw et al., 1985b).

AT of donor plants before shoot tip isolation improved the cryopreservation results significantly as compared to plants grown under constantly high temperatures. The oscillation between low and high temperatures could enhance the viability of the donor plants, because this temperature regime is more similar to natural conditions. Cultivated potatoes are not able to cold-acclimate (Li, 1977), but low temperatures can induce increase in soluble sugars (Chen and Li, 1980b). Addition of sugars to the maintenance medium of *in vitro* plants or pretreatment of explants in media rich in sucrose is widely used in cryopreservation of plants (Helliot et al., 2003; Hitmi et al., 1999; Huang et al., 1995; Kim et al., 2004; Mikula et al., 2005; Zhu et al., 2006). Sugars are, therefore, cryoprotectants, which facilitate the formation of the glassy state and can improve cryopreservation results.

Concerning ultrastructure, there were only minor differences between CT and AT plants. Control explants after both precultures showed no significant differences. In this study, biochemical analysis revealed higher soluble sugar contents after AT. This could not be recognized in the ultrastructure. However, Helliot et al. (2003) and Mikula et al. (2005) found fragmentation of vacuoles from one big to many small ones, due to sucrose addition. After rewarming, CT explants showed slightly stronger damages in the nuclei in comparison to AT explants. Therefore, the AT seems to make cells less susceptible to the stress during DMSO incubation, cooling and rewarming. Nevertheless in this study, increase in soluble sugars after AT was found in *S. tuberosum* 'Désirée', which would strengthen the discussed hypothesis. Further, low temperatures can increase or induce the production and accumulation of other biochemical components like abscisic acid and soluble proteins (Chen and Li, 1980a; Sakai and Larcher, 1987). These substances can enhance protective functions within the plant tissue and, therefore, regeneration after cryopreservation (Suzuki et al., 2006; Urbanova et al., 2006).

More microscopical studies should be done in order to analyse the structural position of ice formation during cooling, e.g. by cryo-electron microscopy or freeze-fracture technique. Fluorescent dyes as used by Morisset et al. (1993) can be applied to find out, if the cytoskeleton is involved into the damage occurring after rewarming. The first steps of ultrastructural changes after rewarming should be detected. This could be done by analysing

the period from directly after rewarming to 1 h after rewarming in more detail. Thus, major improvements of the tolerance of tissues to cryopreservation could be found, if the reasons for cellular damage after rewarming could be better understood in detail.

4.5. Use of thermal analysis in cryopreservation of Solanum sp. *shoot tips*

DSC can be successfully applied as a tool to optimize cryopreservation protocols based on evaporative or osmotic cell dehydration before cooling (CRYMCEPT Report, 2002 - 2005). Vitrification using PVS belongs to this type of protocols. In this study, shoot tips of *S. tuberosum* 'Désirée' were incubated in PVS2 for different durations. Thermograms revealed that, after 60 min incubation, no ice crystallization and melting were observed, but two stable glass transitions were found. In contrast to this, incubation times of 40 min and shorter caused ice crystallization and melting. It can be concluded that, for optimal cryoprotection of *S. tuberosum* 'Désirée', incubation in PVS2 should be about 60 min. This result should be applied in cryopreservation experiments to prove the optimal incubation time. Also further DSC measurements could be done to test incubation times between 40 and 60 min, to find the exact span of time, from where on no crystallization of water occurs. This would optimize the vitrification protocol, because too long incubation times in PVS2 can be damaging to shoot tips since the PVS2 is toxic (Kim et al., 2006b). Like in this study, DSC measurements were also used in coffee, *Citrus*, banana, and *Ribes* cryopreservation for determination of dehydration times to optimize protocols (CRYMCEPT Report, 2002 - 2005).

The DMSO droplet protocol is, however, not based on cell dehydration before cooling, but on fast cooling and rewarming speeds. Thermal behaviour of potato shoot tips was also investigated by means of DSC in order to characterize the physical state of water within the tissue during cooling and rewarming.

Shoot tips of *S. tuberosum* 'Désirée' and *S. demissum,* freshly isolated after CT and AT, were measured. The fresh shoot tips showed the highest melt-endotherms during rewarming of the samples independent of the preculture. This result was expected, because fresh shoot tips contain higher amounts of freezable water in comparison to cryoprotected ones. The comparison between accessions showed, that 'Désirée' had a higher portion of crystallized water than *S. demissum*. Also in 'Désirée', the onset temperature was higher compared to *S. demissum*. This means that crystallized water was melting later at higher temperatures in 'Désirée'. This result would correlate to the better cryopreservation results in *S. demissum* in

comparison to 'Désirée' independent of preculture. The differences in thermal behaviour between the two analyzed species, measured within the freshly isolated shoot tips, disappeared after cryoprotection. Differences between species and genotypes were, therefore, only detected in fresh shoot tips in this study. More repetitions with other genotypes and species are necessary to prove if there is really a correlation between the amount of crystallized water and cryopreservation results.

A small Tg at -115 °C after CT and at -117 °C after AT was detected in shoot tips in *S. tuberosum* 'Désirée'. This Tg was not significantly different between precultures. Although the Tg heat capacity was very low with 0.03 J/(g °C) for shoot tips after CT and 0.05 J/(g °C) after AT, it was detected in all repetitions and, therefore, assumed to be stable. Bilavčík et al. (2007) found Tg in apple and pear shoot tips in the range of -88 °C and -45 °C with water contents of 0.72 g/g dry matter and 0.24 g/g dry matter. They found that the higher the water content within the measured material is, the lower is the Tg. In this study, shoot tips of *S. tuberosum* 'Désirée' had an average water content of 0.9 g/g dry matter. The low Tg at around -116 °C is consistent with the result of Bilavčík et al. (2007). The reason, why this Tg was only found in *S. tuberosum* 'Désirée', and not in *S. demissum,* remains unclear.

After the Tg was found in cryoprotected shoot tips of *S. tuberosum* 'Désirée', it was interesting to analyse its causes. Therefore, assessment of DMSO cryoprotectant solution proper was made. It showed peaks during cooling and warming, indicating ice crystallization and melting of the solution. Parallel to crystallization and melting glass transition was found at about -122 °C. The cryoprotectant solution consists, besides water, of DMSO, sucrose, plant hormones and MS salts. To find the compound responsible for the Tg, pure DMSO and MSH solution were measured. With the MSH solution small Tg was found at -40 °C. Pure DMSO (99.5 %) also showed Tg, but at lower temperatures of -125 °C. When these curves are compared with the cryoprotectant solution, it can be concluded, that DMSO is responsible for the Tg in the cryoprotectant solution, because the temperature of Tg is similar to that in pure DMSO. Interesting is the specific heat capacity of Tg, which is less in pure DMSO and higher for the cryoprotectant solution. Similar results were given by Murthy (1998), who found that aqueous solution of DMSO are good glass formers, even though the components within the mixture are known to be bad glass formers on their own.

Cryopreservation studies applying the DMSO droplet method showed that DMSO is absolutely necessary for the success of the method (Grübe, personal communication). The effect of the cryoprotectant solution was visible in the comparison of DSC-thermograms of fresh and cryoprotected shoot tips. In both accessions tested in this study a clear suppression

of the freezing point and lowering of free water content was determined after cryoprotection. Murthy (1998) also found depression of the freezing point in aqueous DMSO solutions, which resulted from complex formation between water and DMSO. As discussed in the ultrastructure section, DMSO is thought to produce highly structured water clusters, thus reducing the concentration of free water molecules and lowering the freezing point (Szmant, 1975). Freezing point depression and lowering of free water content was also found in shoot tips of potato and *Ribes* after encapsulation and subsequent dehydration (Grospietsch and Zámečník, 2001; Sherlock et al., 2005). In the present study, the lowering of free water contents was small, because shoot tips were incubated in DMSO solution for 2 h only. It was, therefore, not as low as in and comparable to protocols using evaporative or osmotic cell dehydration before cooling.

The action of the DMSO droplet method during cooling and rewarming is not completely elucidated yet. There are two explanations about the mode of action. On one side, it is thought that vitrification occurs and the water within samples is transferred to the 'glassy state' (Benson et al., 2006). On the other side, it is possible that very small intracellular ice crystals are formed, which are not lethal for samples. In this study, during cooling of shoot tips on the aluminium foils, however, opaque droplets were observed, which indicate ice crystallization. If the droplets would have been vitrified, they would have looked transparent. With the here presented DSC results it was proven, that in most cases ice crystallization occurs. In *S. tuberosum* 'Désirée' also some vitrified state was observed, but only to a small extent. Therefore, this study is a confirmation that, besides vitrification, also cryopreservation using rapid cooling and rewarming rates with ice crystallization is successful. The extent of crystallization and vitrification could not be determined absolutely, because the DSC does not allow cooling and warming speeds higher than 20 °C/min. During experiments true cooling and rewarming rates are much faster with speed rates of 12000 °C/min for cooling and 8000 °C/min for warming (as found in thermocouple measurements during thermal analysis workshop in Prague, 2007). Considering this fact, some shoot tips of *S. tuberosum* 'Désirée' were cryoprotected, sealed in pans, and cooled outside the DSC device directly in LN. The cooling speed was, therefore, increased to a similar extent as in the experiments. Cooled pans were transferred quickly to the precooled measuring chamber of the DSC, and warming curve was measured. This experiment was done only with three repetitions, because it is dangerous to open the measuring chamber of the DSC, while it is cooled to -180 °C. For experiments, it is advantageous to cool the sample within the DSC, because during the transfer of the pan from the LN outside to the measuring chamber inside the DSC, air humidity can condense

outside the pan. Little water drops can accumulate, which can give wrong results during the measurement. To prevent the accumulation of water through air humidity, the pan transfer should be done under a nitrogen flushed chamber. However, this was not possible in the present study, because of technical difficulties. Nevertheless, the samples cooled in LN gave highly reproducible results. A first Tg at -117 °C was found similar to slowly cooled samples. But in contrast to this, a second Tg was detected at -33 °C, which could not be found in samples cooled with 10 °C/min. This Tg could be due to the sugars or other compounds in MSH, because Tg of MSH was -43 °C. Besides Tg's also melting was found during rewarming with heat flow of 190 J/g, which was lower in comparison to slowly cooled samples. The second Tg at -43 °C passed directly into the melting peak. Further measurements should be done using temperature modulated DSC (TMDSC) to separate the two thermodynamic events. TMDSC can separate glass transitions from kinetic events like melting by a sinusoidal temperature oscillation over the traditional linear ramp (Höhne et al., 2003). In this study, differences in properties of DSC warming thermograms were found after fast cooling of samples directly in LN in comparison to slow cooling with 10 °C/min. In contrast to this, Volk and Walters (2006) did not find differences in warming thermograms of mint shoot tips treated with PVS2 and applying fast cooling rates in LN and slow cooling rates with 10 °C/min. Also Sun (1999) did not find differences in thermal behaviour of seed tissues of *Quercus rubra* cooled fast (LN) or slowly (10 °C/min). It can be concluded that the fast cooling rates applied in the DMSO droplet method are an important factor for shoot tip survival. Summarizing, it was found that faster cooling rates revealed two Tg's and lower heat flow during melting, which stands for less ice crystallization during cooling. It is suggested, that the ice crystallization in cryopreservation experiments is less in comparison to DSC results, because shoot tips are exposed directly to LN on aluminium foils. Another method to improve the cooling rates would be to get additional equipment for the DSC, which allows direct cooling with LN in the measuring chamber.

DSC analysis revealed Tg and melting of ice in shoot tips during warming applying the DMSO droplet method. Therefore, it can be concluded that crystallization of water takes place during cooling. But, as can be seen by positive cryopreservation results, shoot tips survive the ice crystallization and melting process. Some authors (Gonzalez-Arnao et al., 2008; Mazur, 1977; Santos and Stushnoff, 2003) also confirmed survival of cryopreserved tissues with intracellular ice formation, if those ice crystals were small in size, so that cells are not destroyed. It can be assumed, that in this study ice crystals were small, because crystal size is inversely related to cooling rates (Mazur, 1977). Because of the application of high cooling

rates (12000 °C/min) it is more likely that intracellular ice formation occurs using the DMSO droplet method. It can not be excluded however, that extracellular ice crystal formation is the reason for thermal events in DSC during cooling and rewarming. Extracellular ice could have formed around the shoot tips or between cells. It was tried however, to avoid extracellular ice formation on the surface of shoot tips through drying the shoot tips on filter paper, before putting them in the pan for DSC analysis. Also extracellular ice formation could explain the opaque droplets formed on the aluminium foil during cooling. Further microscopical studies need to be done in order to prove conclusively if ice crystallization is intra- or extracellular located when using the DMSO droplet method for cryopreservation.

4.6. Conclusions

The aim of this study was to get deeper insight into the function of the DMSO droplet cryopreservation method and the changes in biochemical characteristics which occur during preculture with constant and alternating temperatures. Cold tolerances were determined by electrolyte leakage tests and revealed, that no change in cold tolerances and, therefore, no cold acclimation takes place in wild and cultivated potato cultures after AT. The better regeneration results after AT of donor plants and cryopreservation can, therefore, not be directly related to cold acclimation. After AT biochemical changes in shoot tips consisted of slight increase in soluble sugars, slight decrease in starch and amino acid concentration. The increase in soluble sugars could explain the better cryopreservation results. Slight decrease in starch and amino acid concentration confirm the fact that no cold acclimation occurs. The proteome analysis does not give clearly interpretable results, because of the low number of proteins identified. But the change in protein expression between shoot tips of plants treated with CT and AT, respectively, was small. This is in accord with the other biochemical compounds, which do not show strong changes after AT in comparison to CT. Also in the ultrastructure there were no significant changes between shoot tips isolated directly after CT and AT, which is in concordance with the biochemical results. However, cryopreserved and rewarmed shoot tips were slightly less damaged when precultured with AT in contrast to CT. TEM revealed that the DMSO cryoprotectant incubation is the step, after which ultrastructure is changed strongly. DSC analysis showed that incubation in DMSO solution is necessary to depress the freezing point and to lower the amount of freezable water. These analyses made clear, that the incubation of shoot tips in DMSO is the critical step of the method. Immediately after rewarming, shoot tips showed similar structure as before cooling. The

influence of LN on ultrastructure can, therefore, not be seen directly after this stage. Microscopic studies revealed that survival was, however, detectable 2 days later. Only small areas of the original shoot tips survived, mainly in leaf primordia and sometimes in the meristematic dome. Here cells showed mitotic activity and regrowth of shoot tips. Donor plants subjected to AT had mainly significant higher shoot regeneration than plants precultured in CT.

DSC analysis was used to analyse the water status of fresh and cryoprotected shoot tips as well as for different solutions applied during cryopreservation. The influence of preculture was also determined. In all measurements ice crystallization was found during cooling and melting of ice during warming. Differences in heat flow during melting of *S. tuberosum* 'Désirée' were only found in freshly isolated shoot tips. Cryoprotected shoot tips revealed no differences after CT or AT in *S. tuberosum* 'Désirée' as well as in *S. demissum*. This is consistent with the results of the cold tolerance determination and biochemical analysis.

The comparison of DSC thermograms cooled at different speeds showed the importance of cooling rates using the DMSO droplet method. Cryoprotected shoot tips cooled fast directly in LN (ca. 12000 °C/min) showed a lower heat flow during melting, a higher specific heat capacity for the first Tg at -117 °C and an additional second Tg at -33 °C, which was not detected at slow cooling rates with 10 °C/min. This study showed that the amount of ice melted during warming is directly related to cooling speed. From the outcome of the DSC analysis it is concluded, that ice crystallization as well as glass transitions take place during cooling in shoot tips applying the DMSO droplet method. But ice crystals must be small, because shoot tips survive the method or ice crystallization is sterically located in different areas in comparison to Tg within the tissue.

The outcome of this work resulted in an improvement of the practical DMSO droplet method including that AT and solid regeneration medium are already applied in the routine work of cryopreservation of potato in the IPK.

Further studies with lower oscillation temperatures in preculture could improve the increase in soluble sugars and regeneration results. Also addition of sucrose in preculture could lead to better results. Further microscopical studies need to be done to reveal clarification about the area of ice crystallization as well as the process after rewarming and reasons of later occurring ultrastructural damages. Higher cooling rates, e.g. using of LN-slush (24000 °C/min; Pennycooke and Towill, 2000) for cooling, could improve cryopreservation results. The use of direct LN cooling equipment on top of the measuring chamber of the DSC would allow fast

cooling for all measurements. TMDSC, which separates Tg's from crystallization/melting events, can be used to get clarification about the properties of Tg at -33 °C.

Chapter 5. Literature

Amme, S., A. Matros, B. Schlesier and H.P. Mock. 2006. Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. Journal of Experimental Botany 57:1537-1546.

Anchordoguy, T.J., A.S. Rudolph, J.F. Carpenter and J.H. Crowe. 1987. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. Cryobiology 24:324-331.

Bachiri, Y., C. Bajon, A. Sauvanet, C. Gazeau and C. Morisset. 2000. Effect of osmotic stress on tolerance of air-drying and cryopreservation of *Arabidopsis thaliana* suspension cells. Protoplasma 214:227-243.

Bae, W., Y.J. Lee, D.H. Kim, J. Lee, S. Kim, E.J. Sohn and I. Hwang. 2008. AKr2A-mediated import of chloroplast outer membrane proteins is essential for chloroplast biogenesis. Nature Cell Biology 10:220-227.

Bajaj, Y.P.S. 1977. Initiation of shoots and callus from potato-tuber sprouts and axillary buds frozen at -196°C. Crop Improvement 4:48-53.

Bajaj, Y.P.S. 1981. Regeneration of plants from potato meristems freeze-preserved for 24 months. Euphytica 30:141-145.

Barandalla, L., I. Sánchez, E. Ritter and J.I. Ruiz de Galarreta. 2003. Conservation of potato (*Solanum tuberosum* L.) cultivars by cryopreservation. Spanish Journal of Agricultural Research 1:9-13.

Bauw, G., H.V. Nielsen, J. Emmersen, K.L. Nielsen, M. Jorgensen and K.G. Welinder. 2006. Patatins, Kunitz protease inhibitors and other major proteins in tuber of potato cv. Kuras. FEBS Journal 273:3569-3584.

Benson, E.E. 2008. Cryopreservation theory. In: Reed, B.M. (ed) Plant Cryopreservation. A Practical Guide. Springer, New York. 15-32.

Benson, E.E., K. Harding and H. Smith. 1989. Variation in recovery of cryopreserved shoot-tips of *Solanum tuberosum* exposed to different pre- and post-freeze light regimes. CryoLetters 10:323-344.

Benson, E.E., N. Chabrillange and F. Engelmann. 1992. Mise au point de méthodes de cryoconservation de méristemes pour la conservation à long terme des ressources génétiques du manioc (*Manihot spp*.) Rapport de fin d'etude, Laboratoire de Ressources Génétiques et Amélioration des Plantes Tropicale. ORSTOM. Montpellier. France.

Benson, E.E., K. Harding and J.W. Johnston. 2007. Cryopreservation of shoot tips and meristems. In: Stacey, G.N. and J.G. Day (eds) Methods in Molecular Biology, Vol. 368: Cryopreservation and Freeze-Drying Protocols. Humana Press Inc., Totowa, NJ. 163-183.

Benson, E.E., J. Johnston, J.A. Muthusamy and K. Harding. 2006. Physical and engineering perspectives of *in vitro* plant cryopreservation. In: Gupta, S.D. and Y. Ibaraki (eds) Physical and Engineering Perspectives of *in vitro* Plant Cryopreservation, Springer, Dordrecht, The Netherlands. 441-476.

Benson, E.E., M. Wilkinson, A. Todd, U. Ekuere and J. Lyon. 1996a. Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot-tips. CryoLetters 17:119-128.

Benson, E.E., B.M. Reed, R.M. Brennan, K.A. Clacher and D.A. Ross. 1996b. Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L. Germplasm. CryoLetters 17:347-362.

Bevan, M., I. Bancroft, E. Bent, K. Love, H. Goodman, C. Dean, R. Bergkamp, W. Dirkse, M. Van Staveren, W. Stiekema, L. Drost, P. Ridley, S.A. Hudson, K. Patel, G. Murphy, P. Piffanelli, H. Wedler, E. Wedler, R. Wambutt, T. Weitzenegger, T.M. Pohl, N. Terryn, J. Gielen, R. Villarroel, R. De Clerck, M. Van Montagu, A. Lecharny, S. Auborg, I. Gy, M. Kreis, N. Lao, T. Kavanagh, S. Hempel, P. Kotter, K.D. Entian, M. Rieger, M. Schaeffer, B. Funk, S. Mueller-Auer, M. Silvey, R. James, A. Montfort, A. Pons, P. Puigdomenech, A. Douka, E. Voukelatou, D. Milioni, P. Hatzopoulos, E. Piravandi, B. Obermaier, H. Hilbert, A. Dusterhoft, T. Moores, J.D.G. Jones, T. Eneva, K. Palme, V. Benes, S. Rechman, W. Ansorge, R. Cooke, C. Berger, M. Delseny, M. Voet, G. Volckaert, H.W. Mewes, S. Klosterman, C. Schueller and N. Chalwatzis. 1998. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature 391:485-488.

Bilavčík, A., J. Zámečník and M. Faltus. 2007. The use of differential scanning calorimetric analysis of plant shoot tips in cryopreservation. Oviedo Spain 13-14 April:26-27.

Bork, P. 1993. Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally. Proteins: Structure, Function, and Genetics 17:363-374.

Bouafia, S., N. Jelti, G. Lairy, A. Blanc, E. Bonnel and J. Dereuddre. 1996. Cryopreservation of potato shoot tips by encapsulation-dehydration. Potato Research 39:69-78.

Canovas, F.M., E. Dumas-Gaudot, G. Recorbet, J. Jorrin, H.P. Mock and M. Rossignol. 2004. Plant proteome analysis. Proteomics 4:285-298.

Carpentier, S.C., E. Witters, K. Laukens, P. Deckers, R. Swennen and B. Panis. 2005. Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. Proteomics 5:2497-2507.

Carpentier, S.C., E. Witters, K. Laukens, H. Van Onckelen, R. Swennen and B. Panis. 2007. Banana (*Musa spp*.) as a model to study the meristem proteome: acclimation to osmotic stress. Proteomics 7:92-105.

Chang, Y. and B.M. Reed. 2000. Extended alternating-temperature cold acclimation and culture duration improve pear shoot cryopreservation. Cryobiology 40:311-322.

Chang, Y., R.E. Barker and B.M. Reed. 2000. Cold acclimation improves recovery of cryopreserved grass (*Zoysia* and *Lolium sp*.). CryoLetters 21:107-116.

Channuntapipat, C., M. Sedgley and G. Collins. 2003. Changes in methylation and structure of DNA from almond tissues during *in vitro* culture and cryopreservation. Journal of the American Society of Horticultural Science 128:890-897.

Chen, H.-H. and P.H. Li. 1980a. Characteristics of cold acclimation and deacclimation in tuberbearing *Solanum* species. Plant Physiology 65:146-148.

Chen, H.H. and P.H. Li. 1980b. Biochemical changes in tuber-bearing *Solanum* species in relation to frost hardiness during cold acclimation. Plant Physiology 66:414-421.

Coulthurst, S.J., K.S. Lilley and G.P.C. Salmond. 2006. Genetic and proteomic analysis of the role of luxS in the enteric phytopathogen, *Erwinia carotovora*. Molecular Plant Pathology 7:31-45.

CRYMCEPT Report. 2002 - 2005. Establishing Cryopreservation Methods For Conserving European PlanT Germplasm Collections. Project final report:176.

Delaplace, P., J.F. Dierick, M.L. Fauconnier, F. Van Der Wal, J.G. Cordewener, T.A. America and P. du Jardin. 2005. Characterization of potato (*Solanum tuberosum* L.) tuber ageing using physiological and proteomic markers (2D-PAGE). FEBS Journal 272:71-71.

Dexter, S.T., W.E. Tottingham and L.F. Graber. 1932. Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiology 7:63-78.

Diekmann, W., R. Hedrich, K. Raschke and D.G. Robinson. 1993. Osmocytosis and vacuolar fragmentation in guard-cell protoplasts: their relevance to osmotically-induced volume changes in guard-cells. Journal of Experimental Botany 44:1569-1577.

Dodds, J.H., Z. Huaman and R. Lizarraga. 1991. Potato germplasm conservation. In: Dodds, J.H. (ed) *In Vitro* Methodes for Conservation of Plant Genetic Resources, Chapman and Hall, London. 93-109.

Dowgert, M.F. and P.L. Steponkus. 1984. Behavior of the plasma-membrane of isolated protoplasts during a freeze-thaw cycle. Plant Physiology 75:1139-1151.

Dumet, D. and E.E. Benson. 2000. The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 43-56.

Dumet, D., Y. Chang, B.M. Reed and E.E. Benson. 2000. Replacement of cold acclimation with high sucrose pretreatment in black currant cryopreservation. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 385-387.

Engelmann, F. 1991. *In vitro* conservation of tropical plant germplasm - a review. Euphytica 57:227-243.

Fabre, J. and J. Dereuddre. 1990. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot-tips. CryoLetters 11:423-426.

Fahy, G.M. 1986. The relevance of cryoprotectant toxicity to cryobiology. Cryobiology 23:1-13.

Fahy, G.M. and A.M. Karow. 1977. Ultrastructure-function correlative studies for cardiac cryopreservation. V. Absence of a correlation between electrolyte toxicity and cryoinjury in slowly frozen, cryoprotected rat heart. Cryobiology 14:418-427.

Falbe, J. and M. Regitz (eds.) 1997. Römpp-Lexikon Chemie. Band 2. Georg Thieme Verlag, Stuttgart, Deutschland. 781-1636.

Falbe, J. and M. Regitz (eds.) 1998. Römpp-Lexikon Chemie. Band 5. Georg Thieme Verlag, Stuttgart, Deutschland. 3369-4363.

Faltus, M., A. Bilavčík, J. Zámečník, P. Svoboda and J. Domkarova. 2008. Establishment of cryobank of potato and hop apices in the Czech Republic. Agrifood Research Working Papers 153:65-66.

FAO. 2007. http://www.potato2008.org/en/potato/. Access date: 8th May 2008.

FAOSTAT. 2007. http://faostat.fao.org. Access date: 8th May 2008.

Fernie, A.R., L. Willmitzer and R.N. Trethewey. 2002. Sucrose to starch: a transition in molecular plant physiology. Trends in Plant Science 7:35-41.

Finkle, B.J., M.E. Zavala and J.M. Ulrich. 1985. Cryoprotective compounds in the viable freezing of plant tissues. In: Kartha, K.K. (ed) Cryopreservation of Plant Cells and Organs. CRC Press, Florida. 75-113.

Frickey, T. and A.N. Lupas. 2004. Phylogenetic analysis of AAA proteins. Journal of Structural Biology 146:2-10.

Fujikawa, S. 1994. Ultrastructural studies of intracellular freezing: diversity of alterations in the plasma membrane upon freezing, thawing and recooling. CryoLetters 15:223 - 234.

Fukai, S. and M. Oe. 1990. Morphological observations of *Chrysanthemum* shoot tips cultured after cryoprotection and freezing. Journal of the Japanese Society for Horticultural Science 59:383-387.

Gavrilenko, T. 2008. Ex situ conservation of plant genetic resources in Russia: history, current status and persectives. Agrifood Research Working Papers 153:65-66.

Gnanapragasam, S. and I.K. Vasil. 1992. Ultrastructural changes in suspension culture cells of *Panicum maximum* during cryopreservation. Plant Cell Reports 11:169-174.

Golmirzaie, A.M. and A. Panta. 1997. Advances in potato cryopreservation by vitrification. CIP Program Report:71-76.

Golmirzaie, A.M. and A. Panta. 2000. Advances in potato cryopreservation at CIP. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 250-254.

Golmirzaie, A.M., A. Panta and C. Delgado. 2000. Structural observations on potato shoot-tips after thawing from liquid nitrogen. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 388-389.

Gonzalez-Arnao, M.T. and F. Engelmann. 2006. Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. CryoLetters 27:155-168.

Gonzalez-Arnao, M.T., A. Panta, W.M. Roca, R.H. Escobar and F. Engelmann. 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tissue and Organ Culture 92:1-13.

Grospietsch, M. and J. Zámečník. 2001. The real water content of encapsulated potato shoot tips after desiccation. Cryobiology 43:379-380.

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

Grout, B.W.W. and G.G. Henshaw. 1978. Freeze preservation of potato shoot-tip cultures. Annals of Botany 42:1227-1229.

Grout, B.W.W. and G.G. Henshaw. 1980. Structural observations on the growth of potato shoot-tip cultures after thawing from liquid nitrogen. Annals of Botany 46:243-248.

Hajirezaei, M.-R., S. Biemelt, M. Peisker, A. Lytovchenko, A.R. Fernie and U. Sonnewald. 2006. The influence of cytosolic phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPC) on potato tuber metabolism. Journal of Experimental Botany 57:2363-2377.

Halmagyi, A., C. Deliu and A. Coste. 2005. Plant regrowth from potato shoot tips cryopreserved by a combined vitrification-droplet method. CryoLetters 26:313-322.

Hannah, M.A., A.G. Heyer and D.K. Hincha. 2005. A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. PLoS Genetics 1:e26.

Hao, Y.J., C.X. You and X.X. Deng. 2002. Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus. CryoLetters 23:27-35.

Harding, K. 1991. Molecular stability of the ribosomal RNA genes in *Solanum tuberosum* plants recovered from slow growth and cryopreservation. Euphytica 55:141-146.

Harding, K. 1994. The methylation status of DNA derived from potato plants recovered from slow growth. Plant Cell Tissue and Organ Culture 37:31-38.

Harding, K. 1997. Stability of the ribosomal RNA genes in *Solanum tuberosum* L plants recovered from cryopreservation. CryoLetters 18:217-230.

Harding, K. 2004. Genetic integrity of cryopreserved plant cells: a review. CryoLetters 25:3-22.

Harding, K. and E.E. Benson. 1994. A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot-tips: implications for *in vitro* germplasm collections. CryoLetters 15:59-66.

Harding, K. and E.E. Benson. 2000. Analysis of nuclear and chloroplast DNA in plants regenerated from cryopreserved shoot-tips of potato. CryoLetters 21:279-288.

Harding, K. and E.E. Benson. 2001. The use of microsatellite analysis in *Solanum tuberosum* L. *in vitro* plantlets derived from cryopreserved germplasm. CryoLetters 22:199-208.

Haskins, R.H. and K.K. Kartha. 1980. Freeze preservation of pea meristems: cell survival. Canadian Journal of Botany 58:833-840.

Hawkes, J.G. 1978. Biosystematics of the potato. In: Harris, P.M. (ed) The Potato Crop. Chapman & Hall, London. 15-69.

Hawkes, J.G. (ed) 1990. The Potato: Evolution, Biodiversity and Genetic Resources. Belhaven Press, London. 259.

Helliot, B., R. Swennen, Y. Poumay, E. Frison, P. Lepoivre and B. Panis. 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa spp*.) highly proliferating meristems. Plant Cell Reports 21:690-698.

Henshaw, G.G., J.F. O`Hara and J.A. Stamp. 1985a. Cryopreservation of potato meristems. In: Kartha, K.K. (ed) Cryopreservation of Plant Cells and Organs. CRC Press, Inc., Boca Raton, Florida. 159-170.

Henshaw, G.G., Keefe, D.P. and O`Hara, J.F. 1985b. Cryopreservation of potato meristems. In: Schäfer-Menuhr, A. (ed) *In Vitro* Techniques: Propagation and Long Term Storage. Martinus Nijoff/Junk Publishers, Dordrecht for CEC. 155-160.

Hils, U. and L. Pieterse (eds) 2005. World Catalogue of Potato Varieties 2005. AGRIMEDIA, Bergen/Dumme, Germany. 243.

Hincha, D.K. 1990. Differential effects of galactose containing saccharides on mechanical freeze-thaw damage to isolated thylakoid membranes. CryoLetters 11:437-444.

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

Hirai, D. and A. Sakai. 2000. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 205-211.

Hirsh, A.G., R.J. Williams and H.T. Meryman. 1985. A novel method of natural cryoprotection - intracellular glass formation in deeply frozen *Populus*. Plant Physiology 79:41-56.

Hitmi, A., C. Barthomeuf and H. Sallanon. 1999. Cryopreservation of *Chrysanthemum cinerariaefolium* shoot tips. Effects of pretreatment conditions and retention of biosynthetic capacity. CryoLetters 20:113-120.

Höhne, G.W.H., W.F. Hemminger and H.-J. Flammersheim (eds). 2003. Differential Scanning Calorimetry. Springer-Verlag, Berlin Heidelberg. 298.

Huang, C.N., J.H. Wang, Q.S. Yan, X.Q. Zhang and Q.F. Yan. 1995. Plant-regeneration from rice (*Oryza-sativa* L.) embryogenic suspension cells cryopreserved by vitrification. Plant Cell Reports 14:730-734.

Huecas, S., M. Villalba and R. Rodriguez. 2001. Ole e 9, a major olive pollen allergen is a 1,3-beta-glucanase - isolation, characterization, amino acid sequence, and tissue specificity. Journal of Biological Chemistry 276:27959-27966.

IPK. 2005. Annual Report.

IPK. 2007. Annual Report.

Ishikawa, H.A. 1996. Ultrastructural features of chilling injury: injured cells and the early events during chilling of suspension-cultured mung bean cells. American Journal of Botany 83:825-835.

Iyer, L.M., D.D. Leipe, E.V. Koonin and L. Aravind. 2004. Evolutionary history and higher order classification of AAA plus ATPases. Journal of Structural Biology 146:11-31.

Jacob, S.W. and R. Herschler. 1986. Pharmacology of DMSO. Cryobiology 23:14-27.

Kaczmarczyk, A., T. Rutten, M. Melzer and E.R.J. Keller. 2008. Ultrastructural changes associated with cryopreservation of potato (*Solanum tuberosum* L.) shoot tips. CryoLetters 29:145-156.

Kaeppler, S.M., H.F. Kaeppler and Y. Rhee. 2000. Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43: 179-188.

Kartha, K.K. and F. Engelmann. 1994. Cryopreservation and germplasm storage. In: Vasil, I.K. and T.A. Thorpe (eds) Plant Cell and Tissue Culture. Springer. 195-230.

Kartha, K.K., N.L. Leung and L.A. Mroginski. 1982. *In vitro* growth responses and plant regeneration from cryopreserved meristems of cassava (*Manihot esculenta* Crantz). Zeitschrift für Pflanzenphysiologie 107:133-140.

Keller, E.R.J. and M. Dreiling. 2003. Potato cryopreservation in Germany - using the droplet method for the establishment of a new large collection. Acta Horticulturae 623:193-200.

Keller, E.R.J. 2005. Improvement of cryopreservation results in garlic using low temperature preculture and high-quality *in vitro* plantlets. CryoLetters 26:357-366.

Keller, E.R.J., A. Senula, S. Leunufna, and M. Grübe. 2006. Slow growth storage and cryopreservation - tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. International Journal of Refrigeration 29:411-417.

Keller, E.R.J., A. Senula and A. Kaczmarczyk. 2008a. Cryopreservation of herbaceous dicots. In: Reed, B.M. (ed) Plant Cryopreservation. A Practical Guide. Springer, New York. 281-332.

Keller, E.R.J., A. Kaczmarczyk and A. Senula. 2008b. Cryopreservation for plant genebanks - a matter between high expectations and cautious reservation. CryoLetters 29:53-62.

Kim, H.-H., J.-B. Kim, H.-J. Baek, E.-G. Cho, Y.-A. Chae and F. Engelmann. 2004. Evolution of DMSO concentration in garlic shoot tips during a vitrification procedure. CryoLetters 25:91-100.

Kim, H.H., J.K. Lee, J.W. Yoon, J.J. Ji, S.S. Nam, H.S. Hwang, E.G. Cho and F. Engelmann. 2006a. Cryopreservation of garlic bulbil primordia by the droplet-vitrification procedure. CryoLetters 27:143-153.

Kim, H.H., J.W. Yoon, Y.E. Park, E.G. Cho, J.K. Sohn, T.S. Kim and F. Engelmann. 2006b. Cryopreservation of potato cultivated varieties and wild species: critical factors in droplet vitrification. CryoLetters 27:223-234.

Klessig, D.F., J. Durner, R. Noad, D.A. Navarre, D. Wendehenne, D. Kumar, J.M. Zhou, J. Shah, S.Q. Zhang, P. Kachroo, Y. Trifa, D. Pontier, E. Lam and H. Silva. 2000. Nitric oxide and salicylic acid signaling in plant defense. Proceedings of the National Academy of Sciences of the United States of America 97:8849-8855.

Kratsch, H.A. and R.R. Wise. 2000. The ultrastructure of chilling stress. Plant, Cell and Environment 23:337-350.

Kryszczuk, A., J. Keller, M. Grübe and E. Zimnoch-Guzowska. 2006. Cryopreservation of potato (*Solanum tuberosum* L.) shoot tips using vitrification and droplet method. Food, Agriculture & Environment 4:196-200.

Kumar, A. 1994. Somaclonal variation. In: Bradshaw, J.E. and G.R. Mackay (eds) Potato Genetics. CAB International, Wallingford, UK. 197-212.

Lehesranta, S.J., H.V. Davies, L.V.T. Shepherd, K.M. Koistinen, N. Massat, N. Nunan, J.W. McNicol and S.O. Karenlampi. 2006. Proteomic analysis of the potato tuber life cycle. Proteomics 6:6042-6052.

Lehesranta, S.J., H.V. Davies, L.V.T. Shepherd, N. Nunan, J.W. McNicol, S. Auriola, K.M. Koistinen, S. Suomalainen, H.I. Kokko and S.O. Karenlampi. 2005. Comparison of tuber proteomes of potato varieties, landraces, and genetically modified lines. Plant Physiology 138:1690-1699.

Leunufna, S. and E.R.J. Keller. 2005. Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose. CryoLetters 26:93-102.

Levitt, J. (ed) 1972. Responses of Plants to Environmental Stress, Academic Press, New York. 697.

Li, H.P. and A. Fennel. 1985. Potato frost hardiness. In: Li, H.P. (ed) Potato Physiology. Academic Press, Orlando, Florida. 457-479.

Li, P.H. 1977. Frost killing temperatures of 60 tuber-bearing *Solanum* species. American Potato Journal 54:542-456.

Li, P.H., J.P. Palta and H.H. Chen. 1979. Freezing stress in potato. In: Lyons, J.M., Graham, D. and J.K. Raison (eds) Low Temperature Stress in Crop Plants. The Role of the Membrane. Academic Press. 291-303.

Lohaus, G., M. Buker, M. Hussmann, C. Soave and H.W. Heldt. 1998. Transport of amino acids with special emphasis on the synthesis and transport of asparagine in the Illinois low protein and Illinois high protein strains of maize. Planta 205:181-188.

Lopez-Carbonell, M., L. Alegre and H.v. Onckelen. 1994. Changes in cell ultrastructure and endogenous abscisic acid and indole-3-acetic acid concentrations in *Fatsia japonica* leaves under polyethylene glycolinduced water stress. Plant Growth Regulation 15:165-174.

Martín, M.C. and M.E. González-Benito. 2006. Sequence comparison in somaclonal variant of cryopreserved *Dendranthema grandiflora* shoot apices. Cryobiology 53:424.

Mastenbroek, C. 1956. Some experiences in breeding frost-tolerant potatoes. Euphytica 5:289-297.

Mattinen, L., R. Nissinen, T. Riipi, N. Kalkkinen and M. Pirhonen. 2007. Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. Proteomics 7:3527-3537.

Mazur, P. 1970. Cryobiology: the freezing of biological systems. Science 168:939-949.

Mazur, P. 1977. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology 14:251-272.

Messer, E. 2000. II.B.3. – Potatoes. In: Kiple, K.F. and K.C. Ornelas (eds) The Cambridge World History of Food. Cambridge University Press.

Mikula, A., T. Tykarska and M. Kuras. 2005. Ultrastructure of *Gentiana tibetica* proembryogenic cells before and after cooling treatments. CryoLetters 26:367-378.

Mix-Wagner, G., H.M. Schumacher and R.J. Cross. 2002. Recovery of potato apices after several years of storage in liquid nitrogen. CryoLetters 24:33-41.

Morisset, C., C. Gazeau, J. Hansz and J. Dereuddre. 1993. Importance of actin cytoskeleton behaviour during preservation of carrot cell suspensions in liquid nitrogen. Protoplasma 173:35-47.

Mroginski, L. and H. Rey. 2007. Cryopreservation of plant germplasm in Argentina. Advances in Horticultural Science 21:270-273.

Muldrew, K., J.P. Acker, J.A.W. Elliott and L.E. McGann. 2004. The water to ice transition: implications for living cells. In: Fuller, B.J., Lane, N. and E.E. Benson (eds) Life in the Frozen State. CRC Press LLC, Florida. 67-108.

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

Murthy, S.S.N. 1998. Some insight into the physical basis of the cryoprotective action of dimethyl sulfoxide and ethylene glycol. Cryobiology 36:84-96.

Nagl, W. and H.-P. Fusenig. 1979. Types of chromatin organization in plant nuclei. Plant Systematics and Evolution Supplement 2:221-235.

Nguyen, T.T.A., D. Michaud and C. Cloutier. 2007. Proteomic profiling of aphid *Macrosiphum euphorbiae* responses to host-plant-mediated stress induced by defoliation and water deficit. Journal of Insect Physiology 53:601-611.

Niino, T. and A. Sakai. 1992. Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear, and mulberry. Plant Science 87:199-206.

Palta, J.P. and H.P. Li. 1978. Cell membrane properties in relation to freeze injury. In: Li, H.P. (ed) Plant cold hardiness and freezing stress, Academic Press, Inc. 93-115.

Panis, B. and M. Lambardi. 2005. Status of cryopreservation technologies in plants (crops and forest trees). The role of biotechnology. Villa Gualino, Turin, Italy 5.-7.March 2005:43-54.

Panis, B., R. Swennen and F. Engelmann. 2001. Cryopreservation of plant germplasm. Acta Horticulturae 560:79-86.

Panis, B., B. Piette and R. Swennen. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Science 168:45-55.

Panta, A., B. Panis, C. Ynouye, B. Criel, R. Swennen and W. Roca. 2006. Improvement of potato cryopreservation for the long-term conservation of Andean landraces at the International Potato Center (CIP). Cryobiology 53:401.

Pennycooke, J.C. and L.E. Towill. 2000. Cryopreservation of shoot tips from *in vitro* plants of sweet potato [*Ipomoea batatas* (L.) Lam.] by vitrification. Plant Cell Reports 19:733-737.

Pozueta-Romero, J., M. Klein, G. Houlne, M.L. Schantz, B. Meyer and R. Schantz. 1995. Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (*Capsicum annuum*): identification of a new family of transposable elements. Plant Molecular Biology 28:1011-1025.

Reed, B.M. 2002. Implementing cryopreservation for long-term germplasm preservation in vegetatively propagated species. In: Towill, L.E. and Y.P.S. Bajaj (eds) Biotechnology in Agricultue and Forestry, Cryopreservation of Plant Germplasm II. Springer-Verlag, Berlin, Heidelberg, New York. 23-33.

Reed, B.M., N. Okut, J. D'Achino, L. Narver and J. DeNoma. 2003. Cold storage and cryopreservation of hops (*Humulus* L.) shoot cultures through application of standard protocols. CryoLetters 24:389-396.

Revilla, M.A. and D. Martínez. 2002. Cryopreservation of *Humulus lupulus* L. (Hop) In: Towill, L.E. and Y.P.S. Bajaj (eds) Biotechnology in Agricultue and Forestry, Cryopreservation of Plant Germplasm II. Springer-Verlag, Berlin, Heidelberg, New York. 136-150.

Rolletschek, H., M.-R. Hajirezaei, U. Wobus and H. Weber. 2002. Antisense-inhibition of ADP-glucose pyrophosphorylase in *Vicia narbonensis* seeds increases soluble sugars and leads to higher water and nitrogen uptake. Planta 214:954-964.

Sakai, A. 1999. The potential of vitrification technique for cryopreservation of *in vitro* grown apices of temperate and tropical plants. Cryobiology 39:307.

Sakai, A. and W. Larcher (eds). 1987. Frost Survival of Plants. Responses and Adaptation to Freezing Stress. Springer-Verlag, Berlin Heidelberg. 321.

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

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

Salisbury, F.B. and C.W. Ross (eds) 1992. Plant Physiology. Fourth ed. Wadsworth Publishing Company, Belmont, California. 682.

Sant, R., B. Panis, M. Taylor and A. Tyagi. 2008. Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (*Colocasia esculenta* var. *esculenta*) accessions. Plant Cell Tissue and Organ Culture 92:107-111.

Santos, I.R.I. and C. Stushnoff. 2003. Desiccation and freezing tolerance of embryonic axes from *Citrus sinensis* (L.) Osb. pretreated with sucrose. CryoLetters 24:281-292.

Sarkar, D. and P.S. Naik. 1998. Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. Annals of Botany 82:455-461.

Schäfer-Menuhr, A., H.M. Schumacher and G. Mix-Wagner. 1994. Langzeitlagerung alter Kartoffelsorten durch Kryokonservierung der Meristeme in flüssigem Stickstoff. Landbauforschung Völkenrode 44:301-313.

Schäfer-Menuhr, A. 1996. Refinement of cryopreservation techniques for potato. Final report for the period September 1, 1991 - August 31, 1996. International Plant Genetic Resources Institute, Rome, Italy:1-41.

Schäfer-Menuhr, A., E. Müller and G. Mix-Wagner. 1996. Cryopreservation: an alternative for the long-term storage of old potato varieties. Potato Research 39:507-513.

Schäfer-Menuhr, A. and H.M. Schumacher. 1997. Cryopreservation of potato cultivars - Design of a method for routine application in genebanks. Acta Horticulturae 447:477-482.

Schäfer-Menuhr, A., H.M. Schumacher and G. Mix-Wagner. 1997. Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid nitrogen. Plant Genetic Resources Newsletter 111:19-24.

Schlesier, B. and H.-P. Mock. 2006. Protein isolation and second-dimension electrophoretic separation. In: Salina, J. and J.J. Sanchez-Serrano (eds) Methods in Molecular Biology, vol. 323: Arabidopsis Protocols, second ed. Humana Press, Inc., Totowa, NJ. 381-391.

Schlesier, B., A. Berna, F. Bernier and H.P. Mock. 2004. Proteome analysis differentiates between two highly homologues germin-like proteins in *Arabidopsis thaliana* ecotypes Col-0 and Ws-2. Phytochemistry 65:1565-1574.

Seibert, M. and P.J. Wetherbee. 1977. Increased survival and differentiation of frozen herbaceous plant organ cultures through cold treatment. Plant Physiology 59:1043-1046.

Senula, A., E.R.J. Keller, T. Sanduijav and T. Yohannes. 2007. Cryopreservation of cold-acclimated mint (*Mentha spp*.) shoot tips using a simple vitrification protocol. CryoLetters 28:1-12.

Seong, E.S., D. Choi, H.S. Cho, C.K. Lim, H.J. Cho and M.H. Wang. 2007a. Characterization of a stress-responsive ankyrin repeat-containing zinc finger protein of *Capsicum annuum* (CaKR1). Journal of Biochemistry and Molecular Biology 40:952-958.

Seong, E.S., H.S. Cho, D. Choi, Y.H. Joung, C.K. Lim, J.H. Hur and M.H. Wang. 2007b. Tomato plants overexpressing CaKR1 enhanced tolerance to salt and oxidative stress. Biochemical and Biophysical Research Communications 363:983-988.

Sherlock, G., W. Block and E.E. Benson. 2005. Thermal analysis of the plant encapsulation-dehydration cryopreservation protocol using silica gel as the desiccant. CryoLetters 26:45-54.

Shibli, R.A., S.S. Al-Ababneh and M.A.L. Smith. 2004. Cryopreservation of plant germplasm: a review (scientific review). Dirasat, Agricultural Sciences 31:60-73.

Sitte, P. (1998). Erster Teil: Morphologie. In: Sitte, P., H. Ziegler, F. Ehrendorfer, and A. Bresinsky (eds). Lehrbuch der Botanik für Hochschulen. begr. von E. Strasburger. 34 ed. Gustav Fischer Verlag, Stuttgart. 117-142.

Smallwood, M. and D.J. Bowles. 2002. Plants in a cold climate. Philosophical Transactions of the Royal Society of London 357:831-847.

Spooner, D.M. and J.B. Bamberg. 1994. Potato genetic resources: sources of resistance and systematics. American Potato Journal 71:325-337.

Steponkus, P., R. Langis and S. Fujikawa. 1992. Cryopreservation of plant tissues by vitrification. Advances in Low Temperature Biology 1:1-16.

Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annual Review of Plant Physiology 35:543-584.

Stewart, G.R. and F. Larher. 1980. Accumulation of amino acids and related compounds in relation to environmental stress. In: Miflin, B.J. (ed) The Biochemistry of Plants. Volume 5. Amino Acids and Derivatives. Academic Press, New York. 609-635.

Stone, J.M., J.P. Palta, J.B. Bamberg, L.S. Weiss and J.F. Harbage. 1993. Inheritance of freezing resistance in tuber-bearing *Solanum* species: evidence for independent genetic control of nonacclimated freezing tolerance and cold acclimation capacity. Proceedings of the National Academy of Science of the United States of America 90: 7869-7873.

Sun, W.Q. 1999. State and phase transition behaviors of *Quercus rubra* seed axes and cotyledonary tissues: relevance to the desiccation sensitivity and cryopreservation of recalcitrant seeds. Cryobiology 38:372-385.

Sussex, I.M. 1952. Regeneration of the potato shoot apex. Nature 170:755-757.

Suzuki, M., M. Ishikawa, H. Okuda, K. Noda, T. Kishimoto, T. Nakamura, I. Ogiwara, I. Shimura and T. Akihama. 2006. Physiological changes in gentian axillary buds during two-step preculturing with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. Annals of Botany 97:1073-1081.

Swaaij, A.C., E. Jacobsen and W.J. Feenstra. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. Physiologia Plantarum 64:230-236.

Szmant, H.H. 1975. Physical properties of dimethyl sulfoxide and its function in biological systems. Annals of the New York Academy of Sciences 243:20-23.

Takagi, H. 2000. Recent developments in cryopreservation of shoot apices of tropical species. In: Engelmann, F. and Takagi, H. (eds) Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan. 178-193.

Tao, D. and P.H. Li. 1986. Classification of plant cell cryoprotectants. Journal of Theoretical Biology 123: 305-310.

Taylor, M.J., Y.C. Song and K.G.M. Brockbank. 2004. Vitrification in tissue preservation: New developments. In: Fuller, B., Lane, N. and E.E. Benson (eds) Life in the Frozen State. CRC Press, London, New York. 603-644.

Teichert, S., B. Schonig, S. Richter and B. Tudzynski. 2004. Deletion of the *Gibberella fujikuroi* glutamine synthetase gene has significant impact on transcriptional control of primary and secondary metabolism. Molecular Microbiology 53:1661-1675.

Thieme, R. 1992. An *in vitro* potato cultivar collection: microtuberization and storage of microtubers. Plant Genetic Resources Newsletter 88/89:17-19.

Thomashow, M.F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology 50:571-599.

Towill, L.E. 1981. Survival at low temperatures of shoot-tips from cultivars of *Solanum tuberosum* group *tuberosum.* CryoLetters 2:373-382.

Towill, L.E. 1983. Improved survival after cryogenic exposure of shoot tips derived from *in vitro* plantlet cultures of potato. Cryobiology 20:567-573.

Urbanova, M., J. Kosuth and E. Cellarova. 2006. Genetic and biochemical analysis of *Hypericum perforatum* L. plants regenerated after cryopreservation. Plant Cell Reports 25:140-147.

Urquhart, A.A. and K.W. Joy. 1982. Transport, metabolism, and redistribution of xylem-borne amino-acids in developing pea shoots. Plant Physiology 69:1226-1232.

Vandenbussche, B., S. Leuridan, V. Verdoodt, M. Gysemberg and M. De Proft. 1999. Changes in sugar content and fatty acid composition of *in vitro* sugar beet shoots after cold acclimation: influence on survival after cryopreservation. Plant Growth Regulation 28:157-163.

Volk, G.M. and C. Walters. 2006. Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection. Cryobiology 52:48-61.

Ward, A.C.W., E.E. Benson, N.W. Blackhall, S. Cooperbland, W. Powell, J.B. Power and M.R. Davey. 1993. Flow-cytometric assessments of ploidy stability in cryopreserved dihaploid *Solanum tuberosum* and wild *Solanum* species. CryoLetters 14:145-152.

Weckwerth, W. 2008. Integration of metabolomics and proteomics in molecular plant physiology - coping with the complexity by data-dimensionality reduction. Physiologia Plantarum 132:176-189.

Weiler, E.W. 2002. Zweiter Teil: Physiologie. In: Sitte, P., E. W. Weiler, J. W. Kadereit, A. Bresinsky, and C. Körner (eds) 2002. Lehrbuch der Botanik für Hochschulen. begr. von E. Strasburger. 35 ed. Spektrum, Akademischer Verlag Heidelberg, Berlin. 221-359.

Withers, L.A. 1985. Long-term storage of *in vitro* cultures. In: Schäfer-Menuhr, A. (ed) *In vitro* Techniques: Propagation and Long Term Storage, Martinus Nijhoff / Dr. W. Junk Publishers, Brussels-Luxembourg. 137-148.

Witzel, K., Surabhi, G.-K., Jyothsnakumari, G., Sudhakar, C., Matros, A. and H.-P. Mock. 2007. Quantitative proteome analysis of barley seeds using ruthenium(II)-tris-(bathophenanthroline-disulphonate) staining. Journal of Proteome Research 6:1325-1333.

Xin, Z. and J. Browse. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. Plant, Cell and Environment 23:893-902.

Yoon, J.-W., H.-H. Kim, H.-C. Ko, H.-S. Hwang, E.-S. Hong, E.-G. Cho and F. Engelmann. 2006. Cryopreservation of cultivated and wild potato varieties by droplet vitrification: effect of subculture of mother-plants and of preculture of shoot tips. CryoLetters 27:211-222.

Zhao, M.A., Y.Z. Xhu, S.P. Dhital, D.M. Khu, Y.S. Song, M.Y. Wang and H.T. Lim. 2005a. An efficient cryopreservation procedure for potato (*Solanum tuberosum* L.) utilizing the new ice blocking agent, Supercool X1000. Plant Cell Reports 24:477-481.

Zhao, M.A., S.P. Dhital, Y.L. Fang, D.M. Khu, Y.S. Song, E.J. Park, C.W. Kang and H.T. Lim. 2005b. Application of slow-freezing cryopreservation method for the conservation of diverse potato (*Solanum tuberosum* L.) genotypes. Journal of Plant Biotechnology 7:1-4.

Zhu, G.Y., J.M.C. Geuns, S. Dussert, R. Swennen and B. Panis. 2006. Change in sugar, sterol and fatty acid composition in banana meristems caused by sucrose-induced acclimation and its effects on cryopreservation. Physiologia Plantarum 128:80-94.

6. References

6.1. Curriculum vitae

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Date and place of birth:	April 30th 1979, Altenburg, Germany
Sex:	female
Nationality:	German

Education:

07/1997	Abitur, final secondary-school examinations, Leipzig, Germany
08/1997 - 08/1998	Au Pair, Yaphank, New York, USA
10/1998 - 10/2004	biology studies at the University of Leipzig, Germany
07/2001 - 08/2001	project work in La Gamba ("Regenwald der Österreicher"), Costa Rica
09/2003 – 03/2004	practical works for diploma thesis in the National Park Serra dos Órgãos, Rio de Janeiro, Brazil
10/2004	diploma degree in biology from the University of Leipzig, Germany

Work Experience

since 02/2005	PhD student at the Leibniz Institute of Plant Genetics and Crop Plant research (IPK), Gatersleben, Germany
Title of PhD Thesis:	'Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot tips'
10.-21.10.2005	International Training Workshop on plant cryopreservation, Montpellier, France, organized through the EU-Project CRYMCEPT
12.-15.02.2007	operator training on Differential Scanning Calorimetry (DSC) Q2000, IPK Gatersleben, Germany, through the company TA Instruments
17.-21.09.2007	Thermal Analysis training school, Crop Research Institute, Prague, Czech Republic
28.-29.11.2007	advances training course in 'Gene Technology, Biosafety and Biosecurity' through the state commercial regulatory authority of Hanover, IPK Gatersleben, Germany

Gatersleben, 2nd June 2008

6. 2. List of Publications

6.2.1. Publications in scientific journals and books

Kaczmarczyk, A., Shvachko, N., Lupysheva, Y., Hajirezaei, M-R. and E.R.J. Keller (2008) Influence of alternating temperature preculture on cryopreservation results for potato shoot tips. Plant Cell Reports 27: 1551-1558.

Kaczmarczyk, A., Rutten, T., Melzer, M. and E.R.J. Keller (2008) Ultrastructural changes associated with cryopreservation of potato (*Solanum tuberosum* L.) shoot tips. CryoLetters 29:145-156.

Keller, E.R.J., Senula, A. and **A. Kaczmarczyk** (2008) Cryopreservation of herbaceous dicots. In: Reed, B.M. (ed.) Plant Cryopreservation: A Practical guide. Springer. New York. 281-332.

Keller, E.R.J., **Kaczmarczyk, A**. and A. Senula (2008) Cryopreservation for plant genebanks – a matter between high expectations and cautious reservation. CryoLetters 29: 53-62.

6.2.2. Contributions to conferences

Kaczmarczyk, A., Keller, E.R.J., Melzer, M. and T. Rutten (2006) Ultrastructural analysis of potato cryopreservation using the droplet method. Cryobiology 53: 426.

Keller, E.R.J., Senula, A. and **A. Kaczmarczyk** (2006) Development and organization of the Gatersleben cryobank of potato, garlic and mint – maintenance, safety and logistics. Cryobiology 53: 401.

Kaczmarczyk, A., Grübe, M. and E.R.J. Keller (2007) Cryopreservation of potato: New Results from the IPK Gatersleben. Germany, Memories of the International Congress of Plant Biotechnology and Agriculture (BioVeg2007) CD ROM. Centro de Bioplantas, Ciego de Avila, Cuba, 7-11 May.

Keller, E.R.J., **Kaczmarczyk, A**. and K.J. Dehmer (2007) On the impact of cryopreservation on genetic resources conservation of the two most advanced temperate crops – potato and garlic. Proc. 18th EUCARPIA Genetic Resources Section Meeting, 23-26 May, 2007, Piestany, Slovak Republic, 39.

Kaczmarczyk, A., Keller, E.R.J., Shvachko, N.A. and Y.V. Lupysheva (2007) Optimization of cryopreservation conditions for two potato accessions (*Solanum tuberosum* 'Desiree' and *S. acaule*). II Vavilov International Conference. Genetic Resources of cultivated plants in XXI century, St. Petersburg, Russia, 26.-30 November. 195-197.

Keller, E.R.J. Senula, A., **Kaczmarczyk, A**., Büchner, D. and C. Zanke (2008) Different protocols – different situations – different genotypes from the research laboratory to application in genebanks. Some subjects of discussion. Agrifood Research Working Papers 153: 61.

Kaczmarczyk, A., Grübe, M. and E.R.J. Keller (2008) Influence of alternating cold preculture on cryopreservation results for potato shoot tips. CryoLetters 29: 79.

6.2.3. Presentations and Posters

Presentations

Kaczmarczyk, A. (2005) Physiological, biochemical, histological and ultra structural aspects of the development of cryopreservation tolerance in meristematic tissue of potato shoots tips. 1st ISC IPK student conference Gatersleben, 22.-25. June.

Kaczmarczyk, A. (2005) Cryopreservation of genetic resources in a large plant collection: Does cold acclimation of donor plants improve success in potato? – Annual Scientific Meeting Society of Low Temperature Biology, York/UK, 16. September.

Kaczmarczyk, A. und M. Grübe (2007) Kartoffel – Kryokonservierung im IPK. Minisymposium Kryokonservierung, Genbank, IPK, 25.–26. January.

Kaczmarczyk, A., Grübe, M. and E.R.J. Keller (2007) Thermal analysis of the water status in explants of potato (*Solanum tuberosum* L.) comparing two cryopreservation methods. 3rd Plant Science Student Conference, Leibniz Institute of Plant Biochemistry Halle, 06. June.

Kaczmarczyk, A., Grübe, M. and E.R.J. Keller (2007) Cryopreservation of potato: New Results from the IPK Gatersleben. N.I. Vavilov All-Russian Research Institute of Plant Industry (VIR), Puschkin, Russia. 28. June.

Kaczmarczyk, A., Grübe, M. and E.R.J. Keller (2007) Influence of alternating cold preculture on cryopreservation results for potato shoot tips. Annual Scientific Meeting Society of Low Temperature Biology, Derby/UK, 12.-14. September.

Kaczmarczyk, A., Senula, A. and E.R.J. Keller (2008) DSC - Anwendung in der Kryokonservierung vegetativer pflanzengenetischer Ressourcen. Würzburger Tage – Anwendungen der Thermischen Analyse, Mikrokalorimetrie & Rheologie im Bereich der Pharmazie, Biotechnologie, Lebensmitteltechnologie & Kosmetik. Würzburg, 10.-11. April.

Posters

Kaczmarczyk, A., Keller, E.R.J., Melzer, M. and T. Rutten (2006) Ultrastructural analysis of potato cryopreservation using the droplet method. Annual Scientific Meeting Society of Low Temperature Biology, Hamburg, 24.-27. June.

Kaczmarczyk, A., Hincha, D.K. and E.R.J. Keller (2006) Determination of cold tolerance of wild- and cultivated potatoes to explore presumable links to cryopreservation results. 2nd ISC IPK student conference Gatersleben, 29.May-1. June.

Kaczmarczyk, A., Witzel, K., Matros, A. and H.-P. Mock (2007) Investigations on cryopreservation of potato shoot tips – new results using a proteomics approach. Institut's Day IPK, Gatersleben, Germany, 22.-23. October.

6.3. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher weder der Mathematisch-Narturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Ich erkläre, dass ich mich bisher noch nie um den Doktorgrad beworben habe.

Ferner erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst sowie keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und diese auch als solche kenntlich gemacht habe.

Halle (Saale), den 02.06.2008

Anja Kaczmarczyk

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