CryoLetters **34** (2), SLTB Annual Meeting 2012;174-216 (2013)

"ADVANCES IN LOW TEMPERATURE BIOLOGY" SELECTED ABSTRACTS

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SESSION 1. HUMAN CELL AND TISSUE PRESERVATION AND BIOBANKING

CRYOBIOLOGY IN TISSUE BANKING

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BRIDGING THE GAP BETWEEN TISSUE BANKS AND BIOBANKS

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Biobanking is an exciting tool for furthering our understanding of the causes and treatment of human disease, leading to the development of new therapies and translation into clinics. Tissue banks that supply tissue for transplantation are well established and support the treatment of substantial numbers of patients. For example, approximately 100,000 corneal transplants are carried out annually worldwide. The primary purpose of these tissue banks is to maximize the provision of tissue for transplantation. However, there is also a major need for tissue for research and surgical training and tissue that cannot currently be transplanted or indeed turns out to be unsuitable for transplantation is an extremely valuable resource that is insufficiently exploited. Tissue banks have access to large numbers of donors, often with known pathology. The CTS Bristol Eye Bank receives eyes from more than 1000 donors a year. Many of the eyes have known pathology, such as retinal disease, which does not affect the suitability of corneal tissue for transplantation. This tissue is invaluable for the study of ocular disease, such as diabetic retinopathy and age-related macular generation. The purpose of this talk will be to explore how tissue banks can further support research through collaboration with biobanks and research tissue banks. A major question that needs to be addressed concerns preservation, be it for histopathology and immunohistochemistry (especially when integrated with clinical imaging), extraction of DNA for genetic studies, cellular-based functional studies, or a source of cells for advanced therapeutic medicinal products. The two latter objectives will most likely depend on cryopreservation for the long-term storage and archiving of tissues and cells. An integrated and co-ordinated approach is therefore needed to realize the potential contributions that tissue banks can make to biobanking.

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CRYOPRESERVATION IN THE FUTURE

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The clinical and commercial success of various modern therapies relies upon the ability to cryopreserve products of regenerative medicine of interest. While progress has been made in creating tissue engineered implants, there was little known about their cryobiology. Research on cryopreservation of engineered neo-tissues involving primary cells and stem cells derived from variety of sources was undertaken by my group with aim to complete these vital steps (1-11). Particularly, we developed a vitrification strategy that is highly effective for microencapsulated hepatocytes for hepatic support of mesenchymal stem cells in collagen, matrix/scaffold systems for cartilage and bone regeneration and other neo-tissues. It was demonstrated that vitrification provides intactness of cell membrane, attachment ability of cells to carrier, and insure integrity and quality of material involved and cell-cell interaction. It was also established that vitrification is superior in the maintenance of viability and metabolic function of biological material and proliferation and differentiation of stem cells in tissue-like arrangements.

Over the years we provide evidence that not only vitrification strategy is vital for cell engineered constructs successful preservation, but tissue engineering is vital for success of cryopreservation itself (8). We have used the same developed by us vitrification solution in application studies of cryopreservation of attached hepatocytes in tissue-engineered constructs, with great success in terms of viability and cell metabolic function. However, results of our experimental studies on primary hepatocyte in suspension were less effective using the same vitrification approach. The reason is in the fact that hepatocyte functions are linked to attachment status of cells during cryopreservation in addition to the preservation protocol itself. We developed and reported success in vitrification strategy for neuronal stem cells mainly due to the fact that cells were preserved as 3D cultured, i.e. neurospheres (9, 10). This ensures rapid restoration of cell-cell signalling post-warming leading to further cell proliferation and differentiation rates identical to those of controls (10). Additionally, the protocol allowed the structural integrity of neurospheres to be maintained, suggesting that it may be applicable to other structured cultures for clinical applications such as nerve bridges or matrixes for filling lesion cavities. In acknowledgement of importance our discipline, CRYOBIOLOGY became a part of Tissue Engineering text book (8). To conclude, in the future cryopreservation linked to tissue engineering will be vital in the improvement of human quality of life but also promises to bring fruitful outcomes with principles and results presented here.

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A NOVEL TECHNIQUE FOR LONG-TERM STORAGE OF HUMAN CARTILAGE ALLOGRAFTS

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Approximately 60,000 knee replacements are performed in the UK every year. Joint replacement can be delayed by grafting articular cartilage to the damaged region. Both autografts and allografts can be used in surgical repair and these grafts are successful at 5 – 10 years (1). Osteochondral allografting techniques are limited by restricted availability of living donor tissue at the required time (2). Cartilage allografts can be stored for up to 3 – 4 weeks at ~4°C however cell function decreases over time (3). Standard cryopreservation techniques give poor post-thaw functional cell survival rates due to extracellular and intracellular crystallisation of ice in the chondrons (4, 5). Cryoprotective agents (CPAs) reduce the amount of ice formed at any given subzero temperature and in sufficient concentration prevent the crystallization of ice altogether. CPA toxicity may severely limit successful cryopreservation (6). In our process, increasing concentrations of CPA are supplied during cooling, sufficient to prevent freezing. Toxicity is minimised by supplying only the concentration of CPA required to inhibit crystallisation at the actual temperature reached. This novel 'liquidus tracking' technique builds on a procedure that was highly effective at maintaining cell functionality levels at 75 – 95% in ovine articular cartilage (7). Our aim is to achieve the long-term banking of whole

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femoral condyles from deceased donor human knee joints and to provide them as living allografts to surgeons on demand.

Knee joints were obtained from donors with full consent for research and development work. Dimethyl sulphoxide (Me_2SO) was applied to femoral condyles in progressively increasing concentrations up to 72% w/w in CPTes2 whilst tissue cools to ultra-low temperatures. Tissue CPA content was measured using an HPLC method and residual water content measured by Karl Fischer titration, permitting the calculation of CPA concentration in the aqueous compartment of the tissue. Data was collected to ascertain the kinetics of CPA transport into whole femoral condyles. Measurements of tissue CPA were progressively taken at 0°C, -15°C, -25°C, -30°C, -40°C, -45°C and -80°C during cooling. CPA concentration adjustments were made to ensure sufficient cryoprotectant was present to protect the tissue from ice formation. A reversed two stage warming procedure and structural and functional testing is currently underway to ensure the tissue is not affected by these processes. In collaboration with Planer plc this study will develop the first safe, effective and practical automated 'liquidus tracking' process to achieve the long-term banking of human articular cartilage.

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SESSION 2. CONTRIBUTION OF LOW TEMPERATURE MEDICINE TO THE FIELD OF CARDIOVASCULAR SURGERY

NOVEL SUPERCOOLANT, KAEMPFEROL 7-O-BETA-D GLYCOPYRAMOSIDE (KF7G), ENABLED 30-HOUR DONOR HEART PRESERVATION-2 IN A RAT HEART TRANSPLANTATION MODEL

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Background: Donor shortage is one of the major issues in heart transplantation. To solve this problem, longer preservation of the donor heart is essential. If the cold ischemic time could be longer than 24 hours, more donor hearts could be effectively utilized. In this study, a novel supercooling substance, kaempferol 7-O-beta-D-glucopyranoside (KF7G), a kind of flavonoid extracted from Siberian hardwoods, was used for subzero non-freezing heart

preservation. Kaempferol has been approved as a food additive and is considered to be harmless to the human body.

Methods: Fifty-four heterotopic rat heart transplantations were performed between syngeneic Lewis rats. Donor hearts were perfused antegradely and preserved under the following 3 solution conditions. University of Wisconsin (UW) solution at +4°C was used as a control, and UW+0.01%KF7G solution at -2 or -5°C for supercooled donor hearts. These were preserved for 18, 24, or 30 hours (6 experiments/group), and were transplanted into the abdomens of the recipient rats. Posttransplant cardiac function was assessed according to the degree of visible cardiac movement and was graded semi-quantitatively on a scale of 0 (no contraction) to 4 (good contraction).

Results: No supercooled hearts froze. In all preservation periods, the -2°C groups showed significantly better contractility than the control groups (3.7 vs. 2.0, p=0.006, in 18 hours; 2.7 vs. 1.3, p=0.041 in 24 hours; 2.5 vs. 1.3, p<0.001 in 30 hours). On the other hand, contractility in the -5°C groups was no better than that in the control groups in any preservation period. (1.8 vs. 2.0, p=0.753 in 18 hours; 1.7 vs. 1.3, p=0.584 in 24 hours; 0.0 vs. 1.3, p<0.001 in 30 hours).

Conclusion: Supercooling at -2°C with the novel supercoolant (kaempherol) increased the rat heart preservation period up to 30 hours. This supercooling methodology may become a potent strategy for expanding the donor pool for cardiac transplantation.

POTENTIATING CRYOABLATION: COMBINATORIAL STRATEGIES IN CANCER THERAPY

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Clinically-based cryoablation, grounded on well-recognized scientific principles along with the use of multiprobe devices and advanced imaging techniques, has experienced a rebirth in physician-managed destruction of diverse cancers. Cryoablative techniques have beneficially evolved over the past forty years with the development of successive generations of devices including cryoneedles, cryoballoons, intraoperative ultrasound and an expanded knowledge of the mechanisms by which cancer cells are challenged by low temperatures.

A key discovery in 1998 identified the putative role of gene regulated cell death (apoptosis) in the management of the freeze zone. We now recognize three modes of cell death following a freezing insult: ice-dependent cell rupture in the tumor core, necrosis (primary and secondary) throughout the tumor and apoptosis. The AUA 2008 Best Practice Policy Statement on Cryosurgery for the Treatment of Localized Prostate Cancer recognizes that "prostate cancer cells experiencing multiple molecular-targeted stressors (cytotoxic agents) succumb more readily to low temperature exposure and that with the adoption of appropriately paired combinations, even freezing at -1°C can be totally lethal."

This presentation will focus on the evolution of recent developments supportive of the use of combinatorial cryoablative strategies that may raise the ablative temperature to near -1°C. Data will demonstrate the existence of an "apoptotic continuum" whereby the more

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severe cryogenic stress activates the extrinsic (membrane mediated) apoptotic pathway while less severe freezing activates the intrinsic (mitochondrial mediated) path. Ultimately, it is our aim to decipher the events and signaling pathways that are specifically involved in triggering these diverse cryo-induced mechanisms of cell death. Once known, cryosurgical procedures might be modified such that rapid-onset and delayed programmed cell death pathways would be selectively and preferentially induced in an effort to improve the overall efficacy of cryoablation. Early evidence suggesting that select cryo-sensitizers offer the potential to improve freeze sensitivity will be presented.

CRYOPRESERVED AORTIC VALVE ALLOGRAFT FOR CRITICAL INFECTIVE ENDOCARDITIS – 13-YEAR FOLLOW-UP RESULT

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Background: Infective endocarditis (IE) with annular abscess, structural destruction in the aortic root, or prosthetic valve endocarditis is often very critical, and often leads to unfavorable results. Surgery is the sole option for cure in such cases and cryopreserved heart valve allograft is favored as conduit option, because it gives resistance against infection and provide superior clinical outcome over the other prosthetic conduit. In this research, we presented long-term clinical results of cryopreserved aortic allograft which was provided by the University of Tokyo Tissue Bank (UTTB) for critical IE cases.

Patients and Methods: The patients who received aortic root replacement with cryopreserved allograft (provided by UTTB) for critical IE from December 1998 through July 2012 were involved in the analysis. Short-term results and long-term outcome including survival, freedom from reoepration, and freedom from recurrence of infection were calculated using Kaplan-Meier method.

Results: There were 34 patients, 28 males and average age of 57 year-old. Critical prosthetic endocarditis (PVE) were seen in 32 cases (94.1%), among whom 25 cases (73.5%) had periannular abscess with severe aortic root destruction. Microorganisms were isolated from 21 cases, and 4 of them were Methicillin-Resistant Staphylococcus Aureus. There were 6 in-hospital death (17.6%), and late survival for the remaining 28 cases were 92.1/73.7%/54.6/54.6% at 1/5/10/13 year, respectively. Freedom from recurrence of infection were 92.0/87.4/87.4/58.2%, and freedom from any reoperation were 84.0/69.5/69.5/69.5%. Required procedures were repair of pseudoaneursym at the aortic root, aortic valve reoperation, Bentall procedure, coronary artery bypass grafting, and coronary angioplasty, and 5 of them tolerated well after those additional procedures.

Conclusion: Clinical results of cryopreserved allograft for critical IE were satisfactory in overcoming refractory infection. Cardiac or graft related procedures were often required in long-term follow up period, which were safely completed and did not have impact on late survival.

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SESSION 3. ANIMAL CELL AND TISSUE PRESERVATION AND BIOBANKING

THE DIVERSITY AND OBJECTIVES OF GENETIC RESOURCE BANKS

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Genetic resource banks (GRBs) have been widely discussed as measures for the conservation and protection of genetic diversity across many species and taxon groups. To be useful they (i) must have clearly defined objectives, and (ii) must be capable of integration into animal management and breeding plans. These qualities are often lacking, leading to the establishment of inadequately focused GRBs. Several GRBs have been established for wildlife protection but only one, for black-footed ferrets in the USA, has played a significant role in supporting population management. Conversely, GRBs (mainly semen and embryo banks) are also being established as support measures for agricultural breeds in many countries throughout the world. Some of these provide genetic support for the maintenance of threatened local breeds (preservation of genotype), while others concentrate more specifically on the preservation of allelic diversity within breeds. Among developed countries the United Kingdom government is exceptional in actively refusing to support the establishment and maintenance of GRBs for threatened agricultural species. GRBs are not only being established for mammalian species; notable progress has recently been achieved for the cryopreservation and successful recovery of coral somatic cells, and a major new initiative for the creation of a major coral cryobank is in progress (see http://www.coralrepository.org/).

Genetic resource banks are not only established with conservation and breeding in mind. The preservation of viable somatic tissues and cells will provide a valuable source of research materials for future investigators who may be interested in evolutionary biology, phylogenetics and epidemiology. Recent progress in this area includes the establishment of a major new cryofacility at the Natural History Museum in London, and The Frozen Ark (http://www.frozenark.org/), an international consortium of centres that hold frozen tissues, cells and DNA samples.

Interestingly, the successful cryopreservation of somatic cells from various endangered mammalian species and breeds has raised unforeseen and controversial ethical issues in some countries, because it means that these materials are thereby available for cloning. Although there is no imperative to use these materials for cloning, some GRB managers deliberately avoid collecting viable cells such as fibroblasts, specifically to prevent them ever being available for such use. From a scientific point of view this is incredible as it implies the cells have no other value.

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CRYOPRESERVATION OF A HUMAN CORNEA EQUIVALENT: PRESERVATION OF THE BARRIER FUNCTION FOR TRANSCORNEAL DRUG ADSORPTION STUDIES

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Currently, cryopreservation is the only method for long-term storage of living cells and tissues. However effective cooling and thawing of native and engineered tissue pose a specific challenge. So far no successful cryopreservation strategy for human cornea is established. Aim of this project is to develop an effective transport and storage protocol for human hemi-cornea equivalents (HCC) used as in vitro model for transcorneal drug absorption studies (1).

Effective cryopreservation requires optimization of freezing parameters for every cell type. We used a systematic approach (2) to optimize these conditions for the two cell types of the HCC. For suspended human corneal epithelial cells (HCE-T cells) (1) a cooling rate of 0.2 K/min led to the highest survival rates, whereas human keratocytes (HCK-Ca) [3] showed optimal survival with 5 to 10 K/min. Despite this discrepancy acceptable cell survival could be achieved by adaption the cryoprotective agent (CPA) both composition and CPA concentrations. The findings are transferred to the cryopreservation protocol of the 3D HCC. With the applied protocol the main properties with respect to the barrier characteristics of the in vitro model could be maintained. This is evaluated by measuring the transepithelial electrical resistance (TEER) and of Na-fluorescein permeation. This data showed promising results to develop an effective transport and storage protocol for HCC. Furthermore this will offer new options for corneal graft banking in the future.

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INVOLVEMENT OF MICROTUBULES AND p38 IN THE COLD-INDUCED LOSS OF ENDOTHELIAL BARRIER INTEGRITY

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Hypothermia is widely used for the protection of tissue and organ grafts, but several studies, including our own, have shown that hypothermic preservation induces severe damage and gross morphological alterations of endothelial cells. The aim of this study was to elucidate the complex mechanisms of cold-induced loss of endothelial barrier integrity.

For this purpose porcine aortic endothelial cells were incubated in Krebs-Henseleit buffer at 4°C for up to 72 hours. Afterwards, cell morphology was assessed by phase contrast microscopy or the cells were fixed with paraformaldehyde and stained for F-actin (TRITC-phalloidin), G-actin (Alexa Fluor-DNAse I) or the junctional proteins ZO-1 and VE-cadherin and examined by fluorescence microscopy. In addition, we examined endothelial permeability for Evans Blue in cold-stored aortic segments.

Hypothermia caused pronounced endothelial cell injury, which was completely abolished by the addition of the iron chelator deferoxamine. Independent of cell injury there was a strong cell retraction and gap formation in the endothelial monolayer after cold incubation. A massive degradation of F-actin with a simultaneous increase in G-actin was observed and microtubules were disintegrated completely. All these processes were reversible during rewarming when cells were protected by an iron chelator. The microtubule stabilizer paclitaxel not only prevented disintegration of microtubules, but surprisingly also gap formation and actin decomposition. An activation of p38 was shown and inhibitors of p38 (SB202190, SB203580) inhibited gap formation and part of the actin decomposition (i.e. the peripheral band remained intact), without any effect on microtubule disintegration. Both, paclitaxel and the p38 inhibitor SB202190 preserved the peripheral distribution of the junctional proteins ZO-1 and VE-cadherin. The protection of barrier integrity by SB202190 could be confirmed in porcine aortic segments.

In conclusion, our study demonstrates an involvement of microtubules and p38 in cold-induced loss of endothelial barrier integrity, and suggests new ways for the prevention of endothelial disruption in organ preservation.

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CRYOPRESERVATION OF BUCK (CAPRA HIRCUS) EPIDIDYMAL SPERMATOZOA AS A TOOL TO IMPROVE GENEBANKING IN LOCAL BREEDS: EFFECT OF TESTICLES STORAGE TEMPERATURE AND POST-MORTEM TIME.

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The interest in developing assisted reproductive technologies (ARTs) and cryobanking for the conservation of farm animal genetic resources is recently increased. However, cryopreservation for ex-situ management of genetic diversity sometimes is not routinely feasible, due to the lack of facilities (AI centres, laboratories) and expertise near the farming area of local breeds. In these cases epididymal semen obtained from slaughtered or castrated animals, associated to the possibility of managing rather long periods between animal death, sperm recovery and freezing would increase the opportunities to create semen storages. This investigation addresses the pre-freeze/post-thaw quality of epididymal spermatozoa as function of testicles storage temperature (environment or 5°C) and time elapsed between animal's death and sperm recovery (0, 24, 48, 72 h) in order to establish the optimal protocols for the recovery and cryopreservation of epididymal sperm. Testicles of 51 Alpine bucks collected at the abattoir, were divided in two groups: the testicles of the first group of bucks (n = 25) were transported to the laboratory at environment (E) temperature while in the second group (n = 26) the testicles were transported at a refrigeration (R) temperature of 5°C. In the two groups (E) and (R) one testicle from each pair was processed within 4 h forming the control group (T0E) and (T0R). The contra-lateral testicle was processed after 24, 48 or 72 hof storage. Sperm motility, viability and morphology were assessed in pre-freeze and post-thaw samples. Until 48 h post mortem, both E and R temperatures are able to maintain good pre-freeze epididymal sperm quality. After 48 h of post mortem storage, R temperature is fundamental to reduce epididymal sperm quality decay both in pre-freeze and post-thaw samples. A significant reduction of viability after cryopreservation was observed, this trend was less evident in the refrigerated samples. Moreover testicles refrigeration has prevented the increase of sperm abnormalities after freezing/thawing. Therefore when sperm cryopreservation is not immediately practicable goat testicles should be transported and stored at 5°C up to a maximum of 48 h post mortem to ensure an acceptable sperm quality.

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BIO-PRESERVATION & BIOBANKING FOR NEMATODE SPECIES BY ULTRA-RAPID COOLING

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The availability of high quality biological and environmental specimens for research purposes requires the development of standardized methods for collection, long-term storage, retrieval and distribution of specimens that will enable their future use. Cryopreservation methods has enabled stocks of organisms to be stably maintained free from the hazards of contamination, genetic variation and laboratory accidents. At present, the Nematode and Molecular (NeMo) collection at CRA-ABP, Italy (1; 2; 3) contains a number of the most relevant nematode genera and species stored as cryo-samples and genomic DNAs, as well. Among these, several plant-parasitic nematodes as Bursaphelenchus, Globodera, Heterodera and Meloidogyne spp., the free-living Caenorhabditis elegans (N2) an exceptional model-organism for doing research and experimental applications. Biological tissues when exposed to ultra-low temperatures form ice crystals that disrupt membranes resulting in semi-permeability loss and tissue death. Individual cell and tissues types show different levels of tolerance. Through vitrification, a no-crystalline solidification of water, we can prevent ice crystal formation at ultra-low temperatures without an extreme reduction in moisture. To succeed in a vitrified solution and thus to avoid ice crystals, (i) cooling rates need to be very high (1), (ii) the solution needs to be concentrated enough and meantime no-toxic. The cryoprotective property of low ethylene glycol solution have been studied as function of nematode-survivals and correlated to the strategic physical parameter of cooling-rate. In this study the ultra-rapid cooling-rate (URC, approx. 10^5 °C/min) was assessed using a thin-thermocouple probe-system (T-type, 0.6 mm Ø) in conjunction with a data acquisition board (ATTEN ADS 1022CL, 0.8 K S/sec rate). It is now demonstrated that the key for successful cryopreservation lies in the ability of the organism/tissue to reduce its water content, or by its tolerance towards the loading of cryoprotective compounds or dehydration, in combinantion to a flash-cooling technique.

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CELL ENCAPSULATION INTO ALGINATE MICRO-CAPSULES PROVIDES LIVING CELLS WITH A MILD ENVIRONMENT DURING CRYOPRESERVATION

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Cell encapsulation technology has been proposed to treat a variety of chronic disorders. However, the availability of some cell types such as human stem cells, in turn, promotes the need for such cells to be preserved for longer periods of time. For this, cryopreservation procedures are commonly used, which can cause injuries to the cells. A semi-permeable membrane might protect the cells during cryopreservation, serving as reservoir for cryoprotective agents (CPAs) and further additives [1]. Furthermore, smaller alginate-based micro-capsules (≤250μm) offer additional advantages over larger ones for transplantation and cryopreservation owing to higher specific surface area, less water content as well as improved heat and mass transfer [2]. In this work we indicate on the possibility to encapsulate living cells into semi-permeable alginate micro-capsules via electrospraying in order to improve viability of living cells post-cryopreservation as well as to support their normal proliferation rate after thawing. NIH 3T3 fibroblasts ($5*10^6$ cells/ml) were encapsulated into 1,5% or 2,0% (w/v) alginate micro-capsules (diameter ≈ 250μm) under sterile conditions using high-voltage processes based on previously optimized parameters. Cryopreservation was conducted under 2K/min to -30°C and 5K/min from -30°C to -80°C freezing protocol using DMEM, 20% FCS, 10% DMSO as freezing medium. Thawing was performed at 20°C using standardized equipment. Proliferation and viability of encapsulated cells before cryopreservation and after thawing was measured using MTT and Calcein AM/Ethd assays respectively. The change in morphology of alginate micro-capsules was observed under Carl Zeiss Axiovert 200M microscope using 5x or 10x magnifications and AxioVision V 4.8.2.0 built-in software. Alginate micro-capsules have been entrapped with NIH 3T3 fibroblasts cells at a density of 130 ± 24 cells per capsule. Preliminary results on cell survival after encapsulation showed that electrospraying is a suitable technique for living cells entrapment. Microscopic observations revealed that low temperature treatment and further thawing did not significantly affect the morphology of alginate capsules – they appeared to be stable and round in shape. The results of cell viability assays indicated an increase in viability of encapsulated cells post-cryopreservation as compared to non-encapsulated by 10%. The MTT proliferation assay showed that cells proliferate well after thawing. In order to further improve the viability of cells, the most suitable freezing and thawing protocol as well as the finding of alternative and less-harmful for the cells CPAs are of necessary and will supplement this study.

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ELECTROSPRAYING AS A PROMISING TECHNIQUE FOR CELL ENCAPSULATION IN SEMI-PERMEABLE MICRO-CAPSULES FOR IMMUNOISOLATION

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Cell-based therapies have been tested for the treatment of diseases, including anemia, liver failure, Parkinson disease and tumors. Encapsulation of living cells in semi-permeable membranes has been proposed as a means to avoid undesired host immune response, to achieve controlled delivery of therapeutic products produced by encapsulated cells and to continuously release therapeutic products for longer durations (1). Fast degradable alginate-based micro-capsules containing stem cells are also of great interest in bone-tissue engineering (2). Size of alginate micro-capsules, their permeability and degradation rate should be previously matched with the type of living cell and their proliferation rate being used for encapsulation. Alginate (Sigma Aldrich) polymer solution is kept inside the syringe and pumped through the nozzle applied high-strength electric field into the grounded bath with $CaCl_2$ cross-linking solution. When being dropped into the cross-linking solution, alginate becomes immediately gelled due to the fast physic-chemical interaction between cross-linkable agents. To provide small micro-capsules with controlled size distribution the process parameters (alginate concentration, flow rate, spraying distance, applied voltage etc.) were optimized. NIH 3T3 fibroblasts cells were encapsulated at different densities (10^6, $5*10^6$, 10^7 cells/ml) under sterile conditions. Size distribution of micro-capsules as well as cell encapsulation efficiency was studied using Carl Zeiss Axiovert 200M microscope with 5x or 10x magnifications and AxioVision V 4.8.2.0 built-in software. The size of alginate micro-capsules produced by electrospraing can be controlled by adjusting one or a couple of optimized parameters. The precise control over the bead diameter is only possible when spraying in jet flow regime (ranging from 15-17 kV). No significant effect of flow rate on bead diameter (within strength of electric field 2 kV/cm) has been observed. Voltage and alginate concentration were found to be the most important parameters that determine the micro-capsules size, while $CaCl_2$ concentration affects mainly their morphology and homogeneity. Cell encapsulation efficiency as well as bead diameter can be controlled by cell density, flow rate, applied voltage, concentration cross-linking solution. It has also been observed that an increase in initial cell concentration causes a decrease in micro-capsules diameter. Previous experiments on cell survival post encapsulation process (viability 75±17% with respect to control) have proven the effectiveness of cell encapsulation technology into alginate semi-permeable polymer matrices using high-voltage processes.

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IMPROVED COLD STORAGE AND CRYOPRESERVATION OF RAT HEPATOCYTES IN A CUSTOMIZED SOLUTION

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The demand for primary hepatocytes for pharmacological/toxicological research and clinical applications necessitates the development of effective storage options. For medium-term storage (hours to days), cells are usually stored at 0-4°C. Various (adherent) cell types display an iron-dependent cold-induced cell injury in this temperature range, and, depending on cell type, prefer different ion compositions in the cold storage solution, mainly with respect to chloride concentration. For long-term storage (weeks to years), cryopreservation is the only option so far. However, primary hepatocytes are particularly sensitive to cryopreservation injury and lose their attachment ability after thawing. We here aimed to improve cold storage and cryopreservation of hepatocyte suspensions by using customized preservation solutions.

Rat hepatocytes were isolated from male Wistar rats by collagenase digestion. For cold storage, cells were stored in suspension at 4°C in cell culture medium or variants of the vascular storage solution TiProtec® at a density of 10^6 cells/ml. For cryopreservation, all solutions were supplemented with 10% DMSO and cell suspensions were frozen at -1°C/min in a controlled rate freezer. After cold storage/thawing, viability was assessed by flow cytometry (propidium iodide staining) or cells were seeded on collagen-coated 6-well-plates (10^6 cells/well) in supplemented L-15 culture medium. 24 h after seeding, cell attachment (microscopy, intracellular LDH) and metabolic activity (resazurin conversion, forskolin-induced glucose release, urea production) were assessed.

Cell viability of rat hepatocytes was virtually unchanged after one week of cold storage in a TiProtec modification and cell attachment was significantly higher than in cell culture medium (77 ± 24% vs. 1 ± 2% compared to non-stored control cells). In frozen cell suspensions, viability immediately after thawing was equal to non-frozen control cells in both media (TiProtec modification + 10% DMSO: 70 ± 14%, cell culture medium + 10% DMSO: 71 ± 6%), but cell attachment (37 ± 15% vs. 9 ± 7%) and metabolic activity (resazurin conversion: 47 ± 23% vs. 25 ± 8%, glucose release: 44 ± 6% vs. 15 ± 7%, urea production: 31 ± 16% vs. 5 ± 8%) were higher for cells frozen in the TiProtec modification.

In summary, primary rat hepatocyte attachment and metabolic function after cold storage and after freezing/thawing could be greatly improved by using modified TiProtec solution. Currently, the transferability of these results to human hepatocytes is under investigation.

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SESSION 4. PRESERVATION OF MICROBES AND ALGAE

GLOBAL MICROBIAL DIVERSITY: MICROALGAE, THE CONSERVATION OF RECALCITRANT STRAINS

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The magnitude of biodiversity on Earth is frequently quantified by the total number of species. There are ≤ 2 million that have been formally described and given official names which include animals, plants and algae, with many more unknown extant species. The Earth's varied and diverse environments are teeming with microscopic life which composes the planet's 'micro' biological resources. Global microbial distributions pervade all domains of the marine, terrestrial and atmospheric environments comprising abundant and diverse communities of bacteria, archaea, protists and fungi. Oceanic waters contain huge microbial cell counts which represent ≥ 90% of the total marine biomass. The biodiversity of microalgae is enormous, and includes huge populations of picoplankton: diatoms, dinoflagellates, picoflagellates and cyanobacteria, collectively they are the primary catalysts of carbon fixation. Soil is a complex microhabitat, its microflora is very diverse containing thousands of cells with only 1–10% being culturable microbes. Bacteria and fungi are ubiquitous in the atmosphere dispersing through airborne dust and moisture particles and contributing to atmospheric clouds which contain copious biological ice nuclei. Numerous global initiatives are concerned with capturing biodiversity from the air-land-sea and understanding, conserving and protecting species and their associated habitats (1). Sharing the benefits of biological diversity is part of the Convention on Biological Diversity regarding plants, animals and microorganisms and their ecosystems.

This presentation highlights the global significance of microbial diversity as an introduction to opening the Society for Low Temperature Biology "Preservation of Microbes and Algae" symposium. It focuses on issues and concerns of cryostorage recalcitrance in microalgae, exemplified by the difficult-to-freeze strains of the Euglenophyta that exhibit low or zero viability. The limitation of the widely accepted 'traditional' 2-step colligative controlled rate methodology has proven to be insufficiently reliable and/or robust for preserving recalcitrant strains. This cryopreservation protocol is not universally applicable to all microalgae necessitating the need for alternatives. The exposure of cryoprotected Euglenoid cells to vitrification-based techniques that use the alginate bead encapsulation/dehydration protocol also appears to effect viability severely. Whereas, the reproducible growth of E. gracilis cells following the combined encapsulation/dehydration, colligative and controlled cooling cryoprotective strategies has shown to be an effective approach to algal recalcitrance (2,3). The integration of a controlled, multi-step cooling programme will be highlighted by exploring a refined, combined cryoprotective strategy to expedite the cryo-conservation of microalgae.

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SURVIVAL STRATEGIES IN PSYCHROPHILIC SNOW ALGAE - ICE STRUCTURING PROTEINS (ISP)

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Ice structuring proteins (ISPs), formerly also termed antifreeze proteins (AFPs), are known from a number or microorganisms, fungi, plants and animals. Only recently they have been reported within microalgae from a marine diatom and also from a unicellular green alga from the marine high intertidal in Antarctica. ISPs have the ability to bind to ice crystal surfaces and to modify these in size and shape. At suitable concentrations the have a strong recrystallisation inhibition (RI) activity.

When screening our Culture Collection of Cryophilic Algae CCCryo (cccryo.fraunhofer.de) we found ISPs in 15 psychrophilic green algae strains from different evolutionary lines within the Chlamydomonadaceae, Chlorophyta (Chloromonas spp., Chlamydomonas spp. and cf. Desmotetra spp.). Non-obligate cryophilic (= psychrotrophic) algae do not produce such ISPs. Under our standard culture conditions at 2-4°C the psychrophilic, unicellular algae excrete the ISPs into the culture medium, from which these easily can be isolated and purified. For a mass production this can be done by ultrafiltration through a 50/10 kDa membrane combination, as the molecular size of the different ISPs ranges between 14 and 27 kDa. Further purification is not necessary for use. Ice crystal growth and recrystallisation was significantly inhibited at protein concentrations of 20-50 $\mu g\ ml^{-1}$ in the culture supernatant and 2-2.5 $\mu g\ ml^{-1}$ of purified ISP respectively. Storage of ISPs is unproblematic. They could be kept at room temperature or 4°C for 7 days without losing their activity, even after heating to 75°C for 10 min. their RI-activity was not lost. Though obviously equal in their ability to bind to ice surfaces and shape the crystals, homologies between the ISPs from different evolutionary clades on nucleotide or amino acid level are hardly detectable. Even within clades a high diversity seems to exist. So far we could determine sequences of 5 ISP isoforms from our strains CCCryo 257-06 of cf. Desmotetra sp. and CCCryo 273-06 (Chloromonas sp.). Striking in the primary structures were the multiple threonine motifs (TxT) all lying on beta strands, making up beta sheets in the secondary structure. Homology predictions of the tertiary structure led to a tunnel-like structure with the Txt-motifs presented on the outside of the beta sheets. Putatively these are responsible for the binding to ice crystal surfaces.

ISPs are interesting for biological (cryopreservation, hypothermal perfusion), but also for industrial applications, such as frozen food.

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ENDOPHYTES IN CRYOPRESERVED PLANT GERMPLASM SHOOT CULTURES

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Recently endophytes have been found ubiquitously colonizing plant tissues. Concomitantly, random microbial contaminations are common in long-term plant in vitro cultures. In cryopreservation experiments performed at IPK microbial contaminations also occurred in samples of different plant species rewarmed after cryopreservation. Often in samples of the same cryopreservation series outbreaks of contamination appear only in several explants of one Petri dish. In the present study the problem was addressed whether these contaminations had been latently present already in the initial plant material or whether some samples had been contaminated by handling or unsterile components during cryopreservation and/or in the regrowth phase.

Microorganisms from initial plant material of species of the genera Solanum, Allium, Mentha, Sechium and Artemisia have been isolated, cultivated and characterized by 16S and 18S rDNA phylogeny, fatty acid analysis and physiological tests. The same has been done with microorganisms isolated from samples showing contamination after regrowth in Solanum, Allium and Mentha.

It turned out that some of the identified organisms, surviving cryopreservation, are likely to represent a class of bacteria which can be addressed as plant endophytes like Pseudomonas or Methylobacterium. Some others seem to be airborne or handling contaminations, like Staphylococcus. It is notable that not only bacteria but also yeasts, (e.g. Rhodotorula, Pichia and Candida.) have been found among the contaminants after cryopreservation.

CHARCOAL FILTER PAPER IMPROVES THE VIABILITY OF CRYOPRESERVED FILAMENTOUS FUNGI

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We assessed viability of 18 strains of filamentous ectomycorrhizal and saprotrophic basidiomycetes and ascomycetes after cryopreservation with a novel technique based on charcoal filter paper strips (CFS). The results indicate that axenic fungal cultures grown on CFS recovered from freezing within a few days, even though none survived cryopreservation by the conventional straw method. Fungal growth on CFS was more vigorous, with morphological differentiations such as rhizomorphs and an increased amount of aerial mycelia compared to the unamended culture media. Accordingly CFS allows the cryopreservation of a wide range of rare and important ectomycorrhizal and saprotrophic fungi, which hitherto were

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difficult to revive from liquid nitrogen storage with the conventional and widely applied straw technique.

The application of CFS as a second surface carrier for the cryopreservation of fungal mycelia turned out to be well suited for preserving ectomycorrhizal strains that do not tolerate preservation by conventional methods such as the straw technique. Among the 18 strains tested almost 100% viable mycelia (counted as the number of thawed CFS for each strain with at least one emerged viable colony per CFS) were obtained for 13 (of 18) strains when using the Schleicher & Schuell type 508 or C. Roth HC18.1 filter paper. However when using the standard quantitative filter paper AP40.1 only a small number of emerging colonies was revived. These results indicate that the combination of filter paper and the charcoal used for impregnation, but neither part alone were responsible for the higher survival rates.

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CRYOPRESERVATION AT CCAP: CONSERVATION OF THE MODEL ALGA ECTOCARPUS (PHAEOPHYCEAE)

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The Culture Collection of Algae and Protozoa (CCAP) is one of the largest and most diverse protistan culture collections world-wide and employs cryopreservation routinely for long-term maintenance with in excess of 35% of the holdings being held in a cryopreserved state. To date, the approach has most successfully been used for a diverse range of unicellular taxa, but a small number of multicellular green algae including Ulva (Enteromorpha) intestinalis CCAP 320/1, as well as the majority of the filamentous, prokaryotic cyanobacteria held at CCAP have been successfully cryopreserved.

Over the past 5 years there has been a significant shift in phycological research, with the development, and increased application, of modern genomic and metabolomics approaches. This has reemphasised the importance of model strains, with much of the work being focused on the ever-expanding group of algae where full genome sequences are available. The CCAP holdings include around 300 strains of the multicellular brown algal genus Ectocarpus, which has recently become the first fully-sequenced macroalga (1). With the large and growing number of Ectocarpus strains isolated, as well as the development of mutant strains for specific studies, the research community has an urgent need for the development of reliable, cost-effective long-term maintenance techniques to ensure their genotypic stability and security of access to biological materials for future users.

We present here a robust two-step cryopreservation protocol employing combined DMSO 10% (v/v) and sorbitol 9% (w/v) as cryoprotectants. Microscopic observations of the post-cryo recovery demonstrated that despite their relatively large size, vegetative Ectocarpus

cells can survive the freezing process. Recovery has been observed in all strains tested in this study (2), showing that cryopreservation constitutes an attractive option for reliable, cost-effective long-term maintenance of this model organism. Furthermore, reliable methodologies now exist for most of the eukaryotic genomic model algae including: Chlamydomonas reinhardtii, Chlorella variabilis, Ostreococcus tauri, Phaeodactylum tricornutum and Thalassiosira pseudonana (3).

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CRYOPRESERVATION OF YEASTS AND A NOVEL RHODOTORULA SPECIES FROM THE ANTARCTIC

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The National Collection of Yeast Cultures (NCYC), housed at the Institute of Food Research is the UK's largest collection of non-pathogenic yeasts. At present, the collection comprises of over 4,000 different yeast strains, isolated from a wide variety of different sources and geographic locations, and assembled over the course of the last 50+ years. Methods currently used for cryopreservation (1) will be described and their usefulness for a range of purposes will be discussed. We will also report on a collaborative project with colleagues from Ecuador which has resulted in the recent discovery of a novel pigmented yeast species from a low temperature environment (Greenwich Island, Antarctica). This species may be of potential academic and industrial interest through its application in low temperature biochemistry and also in its ability to synthesise photo-protective compounds (e.g. carotenoids).

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ASSESSMENT, DEVELOPMENT AND VALIDATION OF CRYOPRESERVATION REGIMES

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Successful cryopreservation of fungi relies on the application of optimised preservation protocols that do not compromise the genomic integrity of the organism. Most major European BRCs use lyophilisation and cryopreservation as the methods of choice. Although based on generic principals, protocols can vary between institutions and do not always result in successful recovery. This is particularly the case for more recalcitrant organisms such as Basidiomycete fungi and the heterotrophic Chromists.

In order to evaluate the efficacy of the methods, a range of fungal strains were circulated around partner collections in the EMbaRC project and the organisms preserved using the standard methods used in each collection. In addition, two alternative techniques were evaluated. An encapsulation vilification (EV) method based on a protocol originally developed through the EU Cobra project was modified for use with fungi and a controlled rate cooling approach using a 'N2 free' Stirling Cycle controlled rate freezer were applied.

The effectiveness of existing cryopreservation regimes was assessed using a series of techniques including DNA fingerprinting and sequencing, analysis of culture characteristics, viability assessments and the use of MALDI-TOF. The results showed that when viable cultures were obtained after cryopreservation, they appeared to retain their genomic integrity, but there was evidence of delayed growth and attenuation in some cultures. Not all fungi were successfully preserved by all methods. However researchers at the MUCL collection in Belgium, found that a cryopreservation protocol that involved 'pre-growth' in cryovials was particularly effective in preserving some of the more delicate fungi and the use of this method has been evaluated and validated by the EMbaRC project partners.

Use of the Stirling Cycle controlled rate freezer for the cryopreservation of fungi was encouraging. In total, 80 fungi were frozen using a cooling rate of $-1^{o}C\ min^{-1}$, only a recalcitrant Basidiomycete and filamentous Chromist failed to survive. Of these, 15 strains were also cryopreserved using a traditional 'N2 gas chamber' controlled rate cooler and a comparison of culture morphology and genomic stability against non-cryopreserved starter cultures was undertaken. No changes were detected in genomic profile after preservation, suggesting that genomic function is not adversely compromised as a result of using 'N2 free' cooling. Application of the EV cryopreservation method has also been effective, with several recalcitrant fungi and Chromist's being successfully preserved without adverse effects on culture characteristics. Research into this method is on-going and our most recent findings will be presented.

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ALGAL PRESERVATION AT CULTURE COLLECTIONS: ARE WE READY TO MEET BIOTECHNOLOGY DEMANDS?

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Algae and cyanobacteria play a major role in global biogeochemical cycles and have a major influence on climate for example through the production of DMS, which nucleates water droplets thus producing rain and reflecting light. As phototrophic primary producers they can be found in almost all euphotic habitats and as many taxa can metabolise fixed carbon some inhabit unexpected niches such as in the roots of cycads. This level of genotypic and phenotypic diversity, along with new developments in genomics, proteomics and biotechnology has widened the traditional focus of phycological research. These new tools as well as growing political and economic interest in using algae as energy source, food-stock etc has raised the scientific, economic and political profiles of these often largely overlooked group of organisms.

To ensure the quality and reliability of fundamental and applied phycological research the biotechnological exploitation of algae needs reliable and highly reproducible strain material. European culture collections such as SAG and CCAP are well positioned to underpin the recent developments with a high variety and number of strains. Over the past 10 years cryopreservation has become a standard procedure to ensure the safe holding of material in algal culture collections. However despite progress, for example during the EU funded COBRA project (1), a significant proportion of the holdings are still maintained solely by serial transfer, as they have proven to be recalcitrant to the known cryopreservation methods, or have not yet been tested. Furthermore, in common with most other conserved microbes, only very few of the strains successfully cryopreserved have been tested thoroughly for phenotypic and genotypic stability. In the opinion of the authors our current inability to successfully cryopreserve a number of species and even whole algae groups is one of the bottlenecks in algal biotechnology. We discuss in this paper, where we are now and what is still needed to meet the increasing demands of both, fundamental research and algal biotechnology. Furthermore, we discuss the potential of traditional and alternative cryopreservation approaches for the development of novel protocols and the likely future requirements for effective validation of methods.

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CRYOPRESERVATION OF FUNGI USING AN ENCAPSULATION – VITRIFICATION APPROACH

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The use of Encapsulation-Vitrification (EV) cryopreservation methodology has been applied to many different cell types, notably plant cells and algae. Although EV has been used to preserve orchidaceous mycorrhizal fungi, it has not been widely used for filamentous fungi or for the heterotrophic filamentous chromists. An encapsulation vilification (EV) method, based on a protocol originally developed through the EU Cobra project was modified for use with fungi. A suite of fungi were selected for study and included fungi from the Zygomycota, Basidiomycota, Ascomycota and some heterotrophic chromists. Before cryopreservation fungi were grown under optimal growth conditions and an assessment of characters (morphological and molecular) was undertaken before preservation. Strains were cryopreserved using the EV technique which involved encapsulation of mycelium into calcium alginate, stepwise dehydration using sucrose gradient solutions and air drying. As a means of comparison samples were also cryopreserved using traditional controlled rate cryopreservation techniques.

The fungi Mucor racemosus f. sphaerosporus, Cryphonectria sp., Phomopsis sp. and the yeast Pichia fermentans have been successfully recovered after encapsulation with no adverse effects on culture morphology. There were no significant differences in post preservation recovery rates when compared to cultures stored by traditional cryopreservation methodologies. For the more preservation recalcitrant fungi results have been mixed, but the fact that some replicates retained viability is an improvement over existing methodologies. Good recovery rates (93%, 26 out of 28 beads viable) were evident with the dry-rot fungus Serpula lacrymans although on recovery growth rate was slower and there were some minor morphological changes. After poor initial results, success was achieved after a minor adaption to the methodology and viable cultures were obtained with Phytophthora citrophthora and Saprolegnia diclina. A genomic assessment of stability post cryopreservation is currently being undertaken.

The results indicate the potential of using an encapsulation-vitrification approach for the cryopreservation of fungi. Advantages include the use of plunge cooling which negates the need for expensive controlled rate cooling equipment, which may be useful when financial resources are limited. A summary of the results and a critique of the method for possible long term use will be presented and discussed.

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CRYOPRESERVATION OF MICROALGAE: IS AXENICITY A MUST?

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Cryopreservation is considered the best method for saving microalgae in large culture collections such as the ACOI culture collection of algae (http://acoi.ci.uc.pt/), that holds more than 4000 freshwater microalgae and cyanobacteria. It's a less expensive method for long-term storing since many resources are needed for routine activities, from field sampling to the study and preservation of the unialgal isolates. Since cryopreservation was first applied to microalgae (1), improvements have been achieved for freezing and thawing of samples but no universal method is applicable to all species. The ACOI cultures are non-axenic, as decontaminating and keeping them free from partner organisms would require a demanding resource management (2). Furthermore, current methods to achieve a pure culture may cause the selection of a resistant population of algae, thus increasing the risk of eroding its genetic diversity. Non-axenicity implies the presence of organisms other than algae, usually bacteria and fungi, whose proliferation is disadvantaged by the use of algal-specific culture media. The choice of purposefully hold non-axenic cultures is further supported by the recent findings that the presence of bacteria may be crucial for vitamin uptake by some microalgae (3). Nevertheless, when stress is imposed to the cultures, such as cryoprotectant toxicity and extreme cold exposure, an increase of partner organism proliferation occurs (4), possibly due to the extra source of nutrients released from algal cell lysis caused by cryoinjury (5). Nine mucilaginous ACOI microalgae were cryopreserved by standard two-step methods and the consistent observation of covert organisms proliferation after thawing raised questions about when and how this affects microalgal cells recovery. We found that the choice of the right cryoprotectant may prevent this effect. Our results suggest that the cryoprotection step triggers covert organism proliferation to higher levels when MeOH is used. On the contrary, DMSO was not as efficient to prevent cell loss during freezing/thawing but showed a compensatory effect of keeping the algal culture stable regarding the presence of partner organisms. The use of either MeOH or DMSO led to similar final viability of the cryopreserved cultures but opposite effects regarding covert organism proliferation. When cold stress is imposed to non-axenic cultures the choice of the right cryoprotectant may keep the thawed cultures free from partner organism proliferation. Research to understand how MeOH triggers the proliferation effect and/or how DMSO prevents it is needed, in order to develop a better cryoprotectant strategy combining efficiency on preventing cell loss with keeping covert organisms controlled

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SESSION 5. STEM CELL CRYOPRESERVATION

PRESERVATION OF FISH GAMETES, EMBRYOS AND STEM CELLS: A REVIEW

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Initially, interest in the cryopreservation of fish gametes and embryos lay in their potential applications in conservation, fish farming and genetic improvement of brood stock. The difficulties in achieving successful cryopreservation of eggs and embryos have meant that there have been relatively modest gains for cryopreservation applications in these areas. Only fish sperm cryopreservation has proven successful, with sperm from over 200 species having been reported as successfully cryopreserved. The main applications of milt cryopreservation have been with freshwater species - salmonids, sturgeons and carps – but more recently marine species have benefitted. However, the level of exploitation of the technique has been limited.

Fish oocyte and embryo cryopreservation has proven to be a major challenge. Three model species have dominated the research in this area – zebrafish, sea bream and medaka – and in all cases the same barriers to successful cryopreservation are found – low membrane permeability to water and cryoprotectants, chilling sensitivity, and large yolk component.

To date only one claim has been made for the successful cryopreservation of a fish embryo, Atlantic cod, but the full technical details of this are still awaited. However, dechorionization and removal of the yolk component have been shown to allow successful vitrification and recovery of viable cells in the remaining embryo mass of blastula stage embryos of zebrafish. Whilst this does not provide a route to embryo cryopreservation, it does enable viable primordial germ cell preservation.

Fish are seen as excellent experimental models for understanding the development and genetic expression in higher vertebrates. As a result there has been increased research interest in fish stem cell and somatic cell line production. Embryonic stem cell cultures have been obtained from both marine (gilthead sea bream, herring, sole, sea bass, turbot) and freshwater species (zebrafish, medaka, carp, Nile tilapia). Other stem cell lines have been established, including mesenchymal SC (spiny dogfish), neural SC from the brain of sea bass, and haploid embryonic SC from medaka. Often these cell lines are simply held in culture and the use of cryopreservation is less frequently reported, but where cryopreservation has been adopted a number of successful protocols have been reported enabling the successful cryopreservation of embryonic stem cells of sea perch, turbot, rohu and Leopard danio amongst others. The increased success in both the establishment of stem cell lines and their cryopreservation now opens up alternative routes for the achievement of the goals hoped for from egg and embryo cryopreservation. Germ cell transplantation into host embryos provides a method of obtaining the desired oocyte and sperm genotypes that can be exploited in conservation and fish farming.

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COMPARISON OF CRYOPRESERVATION AND VITRIFICATION METHODS FOR PRESERVATION OF HUMAN EMBRYONIC STEM CELL LINES

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National Institute for Biological Standards and Control (NIBSC) has a primary remit to develop, establish and perform biological assays for the control and standardisation of biological medicines. To fulfil this activity, NIBSC has a number of programmes where Human embryonic stem cell lines (hES) are being adapted for use in functional cell based bioassays. The availability and suitability of hES lines distributed from the UK Stem cell Bank (UKSCB) located at NIBSC, for use in these assays underpins much of this work.

Successful cell banking must address challenges associated with; receipt of new cell lines (possibly requiring non-standard culture conditions), their expansion (to generate sufficient material for generation of master and working cell banks), cell banking with appropriate characterisation and implementation of suitable procedures for further onward distribution of cells. Human ES lines condense a number of these variables into their banking. In addition hES cell lines commonly require complex "specialist" culture regimes employing manual cutting of "blocks" of cells from feeder layers. Alternative, enzymatic subculture strategies may be employed, however, have not been traditionally used by academic or commercial groups deriving hES lines. The UKSCB has expertise in the expansion of a wide range of hES lines from both commercial and academic depositors. This experience has allowed UKSCB to overcome many of the challenges associated with culturing hES lines, whilst maintaining genetic stability and pluripotency. The UKSCB is able to maintain their deposits within the stringent controls imposed by numerous end user & regulatory demands whilst maintaining cells consistent with the characteristics of the depositor's original material.

Here we review post-thaw viability and the retention of pluripotency from 40 hES lines cryopreserved by either vitrification or two step cryopreservation (10% DMSO, -1°Cmin^{-1} to -80°C followed by LN plunge), using data from two standard operating procedures used for routine cell banking procedures at the UK Stem Cell Bank.m Cells were assessed for viability, pluripotency, time to first passage and karyology. In addition, batch to batch variation along with possible freeze-recalcitrance were assessed following recovery from original depositors cryopreserved material and following in house cryopreservation of expanded hES lines produced as part of the banking activities of the UKSCB.m Key observations were a high degree of variability in vitrified material (0–100% post thaw viability as measured by a colony establishing and expanding as a pluripotent hES line). That initial poor vitrification survival (<70%) of hES lines by "non-cryospecialist" depositors was not due to inherent freeze-recalcitrance on the part of the hES line. Instead, cryopreservation post thaw viability (with maintenance of pluripotency) was readily improved following cryopreservation by experienced UKSCB staff (>80%). Post thaw viability, as a measure of expansion and retention of pluripotency was less variable when two step cryopreservation was employed. That adaptation of hES lines to a TrypLE (trypsinisation) passaging had no adverse impact on post thaw viability or on maintenance of pluripotency. Instead trypsinisation of hES lines allowed robust two-step cryopreservation of a wide range of hES with high >85% post thaw viability.

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OPTIMISATION OF CURRENT GOOD MANUFACTURING PRACTICE (CGMP) COMPLIANT CONTROLLED RATE FREEZING FOR HUMAN EMBRYONIC STEM CELLS

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The generation of clinical grade embryonic stem cell lines to a level of current Good Manufacturing Process (cGMP) requires a validated, reproducible and safe method of freezing cells for the creation of a Master Cell Bank. Here we describe the optimisation of cGMP compliant controlled rate freezing for human embryonic stem cells (hESC) which can be applied within a grade B clean room. Controlled nucleation of ice within the cryovial is essential for high cell recovery on thawing. At a linear rate of cooling of 1°C min^{-1} the viability on thawing increased from 39.9% ± 2% in non nucleated samples to 65.9% ± 4.5% followed controlled ice nucleation and a consequent increase in hESC colony survival following 48 hr culture. Electron micrographs (CryoSEM and freeze-substitution) of hESC in the frozen state are presented to examine the beneficial effects of ice nucleation. CryoSEM of straws demonstrate that following controlled ice nucleation large ice crystals form, whilst in straws in which spontaneous nucleation occurred the ice crystals were smaller. CryoSEM of control-nucleated samples revealed cell structures which are extensively distorted compared to those in spontaneously nucleated samples. When examined by freeze substitution these observations are confirmed, controlled ice nucleation results in extensive cell shrinkage compared with non-nucleated samples, but intracellular ice nucleation was not observed. Specific non-linear temperature profiles were also employed, which further increased recovery.

CRYOPRESERVATION OF LARGE VOLUMES OF CELLS FOR REGENERATIVE MEDICINE: ACTIVE CONTROL OF THE FREEZING PROCESS

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We have demonstrated previously that a small volume liquid nitrogen-free controlled rate freezer based on Stirling cryocoolers is effective the cryopreservation of a range of cell types including human embryonic stem cells and encapsulated liver cells. In this study we address the issues in scaling up small scale cryopreservation methods and equipment to the

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development of a large volume GMP compatible process. The cell types that are currently cryopreserved at large volume could be considered to be "freezing resistant" as they do not required controlled ice nucleation during cooling and some loss of viability and cell function upon thawing may be tolerated. This is not the case with cells and cell constructs for application in regenerative medicine: control of ice nucleation is essential for high recovery on thawing. The feedback control of the cryocoolers allows ice nucleation in the sample to be detected by a rapid increase in the voltage to compensate for the heat release following nucleation. This feature facilitates active control of the freezing process allowing the cooling profile to be modified automatically following nucleation of ice in the sample to achieve beneficial non linear profiles. Encapsulated liver spheroids (ELS) are used as a model system for equipment development and testing. These provide a complex multicellular test system which can be investigated both for immediate post-thaw viabilities and longer-term functional assays. It is anticipated that the equipment and methods developed could be transferred to other regenerative medicine therapies which require large cell volumes for clinical treatment.

APOPTOSIS INDUCED BY COLD STORAGE: IMPLICATIONS FOR REVIVAL, GROWTH AND FUNCTION OF MAMMALIAN CELL POPULATIONS

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Exposure of mammalian cells to sub-physiological temperatures causes stress responses that can induce the activation of the cell death programme, apoptosis, through ill-defined mechanisms (1). Such responses, often termed 'cold-shock-induced apoptosis' or 'delayed-onset cell death' (DOCD) can have devastating consequences: cell populations displaying high viability at the point of revival may undergo dramatic and synchronous apoptosis over relatively short time frames following their return to physiological conditions, resulting in poor or altered quality or quantity of cells for research applications, screening, expansion, bioprocessing or therapy. In general terms, apoptotic (dying) and necrotic (dead) cells exert differential effects on their microenvironments. While necrotic cells have toxic and pro-inflammatory properties, apoptotic cells engender reparatory and anti-inflammatory properties, in part through their ability, in vivo, to activate mononuclear phagocytes, which engage rapidly with the dying cells, phagocytosing them and providing the milieu with a range of anti-inflammatory and trophic mediators (2). In vitro, in the absence of professional phagocytes, apoptotic cells persist and ultimately exert inhibitory effects over their viable neighbours (3). These observations indicate that, upon revival from cold storage, persistent apoptotic cells arising as a consequence of DOCD could limit the usefulness of the stored cell populations. We reasoned that selective removal of dead cells following revival from cold storage could enhance cell-population quality. Here we demonstrate that substantial DOCD via apoptosis can occur following hypothermic storage or cryopreservation and that such responses were only partially inhibited by the anti-apoptosis protein, Bcl-2. Selective removal of dead cells was found to improve the activity of revived cell populations in vitro, including the rate of re-establishment and growth of embryonic stem-cell lines and the antibody productivity of hybridoma cells. These results demonstrate that dead-cell removal can profoundly improve the

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quality of revived cell populations undergoing DOCD. We describe the development of a novel means to remove dead cells selectively from cell populationsin vitro and our current activities in developing an automated instrument for revival of high-viability, high-quality cells for a wide variety of applications, including effective delivery of therapeutic cells to patients.

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USING A CONTROLLED VITRIFICATION MACHINE (LIQUIDUS TRACKER) AND DEVELOPING A LOW TOXIC CPA SOLUTION TO CRYOPRESERVE CELLS FOR A BIOARTIFICIAL LIVER DEVICE

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Acute liver failure (ALF) is defined as the loss of 80-90% of liver function within a short period of time, with a high risk of significant morbidity and mortality. Despite a number of treatments available, liver transplantation is often necessary as a long-term cure. However, there are not sufficient donor organs available to meet this urgent clinical need. A bioartificial liver (BAL) device could "buy" time until a donor liver is available or until the liver has spontaneously undergone self-repair.

For the clinical application of a BAL large quantities of cells should be available immediately necessitating cryo-banking. However, cryopreserving large volumes results in increased ice formation and increased cell death. By using high concentrations of cryoprotectants (CPAs) at low temperatures ice formation can be prevented; this is known as vitrification. However, high CPA concentrations are normally toxic to mammalian cells, and therefore the exposure time should be minimised. This can be achieved in small samples (few tens of microlitres) where fast cooling rates can be reached (in the order of 500 degrees / min) but this is impractical for bulky samples.

To reduce CPA toxicity and to avoid ice formation during the preservation process of encapsulated liver cells (volume 300ml) a vitrification machine designed by Planer was used. This automatic pumping and stirring device (Liquidus Tracker) provides the lowest toxic effect that can be established for a given CPA concentration by decreasing the sample temperature to just above the melting point of that particular mix. The CPA concentration is then gradually increased as temperature is decreased along the liquidus curve [1-5]. To further increase cell viability a low-toxicity CPA solution was developed with the requirement of low viscosity so that it may be used within the Liquidus Tracker (LT). The liquidus curve of the low toxic CPA solution was established by measuring the melting point at increasing CPA concentrations. This was done by two methods; differential scanning calorimetry and the release of latent heat.

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ICE BINDING PROTEINS AND THEIR DYNAMIC INTERACTION WITH ICE

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Adaptation to cold climates have led a variety of organisms to evolve specialized ice-binding proteins (IBPs), which protect from freezing damage in several ways. Antifreeze proteins (AFPs) depress the freezing point of the body fluids below the melting point, resulting in a thermal hysteresis (TH) that prevents freezing of the organism. Ice recrystallization inhibition proteins inhibit growth of ice crystals in frozen tissues, and ice nucleating proteins initiate freezing at moderate supercooling temperatures. The potential of these proteins in the medical sector, in cryopreservation, in the frozen food industry, and in agriculture is enormous. Although these proteins have been studied for over 50 years, the mechanisms by which IBPs interact with ice surfaces are not completely understood and their potential as cryoprotecting agents has not yet been realized. We are investigating the mechanism by which IBPs interact with ice surfaces and inhibit ice crystallization. We have developed novel methods, including fluorescence microscopy techniques combined with temperature-controlled microfluidic devices (1-4). These techniques have enabled the replacement of the IBP solution surrounding an IBP-bound ice crystal by buffer, without losing the bound IBP or the TH activity. These results show the irreversibility of the protein:ice interactions and the indirect dependence of TH activity on the protein concentration in solution. For the investigation of the dynamic nature of the protein:ice interactions, the TH gap was measured as a function of the time that the proteins are allowed to accumulate on the ice surfaces. We found that the dynamics of the interactions with ice vary dramatically between different types of IBPs. In a study of ice shaping during growth and melting we have demonstrated a correlation between ice crystal shapes, the shaping process, and the affinity of the IBPs for the basal plane (1).
Our results contribute to an understanding of the mechanisms by which various IBPs act that is critical for the successful use of IBP in cryobiological applications.

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DRY PRESERVATION OF ANIMAL CELLS: STATE OF THE ART IN MICROWAVE PROCESSING

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Currently, cryopreservation using slow-cooling or vitrification methods, with storage at cryogenic temperatures, remains the standard approach for long-term preservation of cells and tissues. Recent research has shown that anhydrous preservation may be an innovative approach to achieve long-term storage of biological samples. Specifically, isothermal vitrification of sugar-based preservation solutions has emerged as an alternative to traditional rapid cooling vitrification techniques requiring high concentrations of toxic cryoprotective agents. A range of static and passive drying techniques have been used to dehydrate samples, often-times leading to considerable inhomogeneity and inconsistency in the sample water content. Recent studies from our laboratory have shown that microwave processing can be effective in solving some of the problems associated with current drying methods including high variability in processing time and sample non-homogeneity (1). An optimized microwave-based drying process has been developed that enables continuous drying at variable low power settings, with automatic feedback of average temperature. A new turntable was manufactured from ultra-high molecular weight polyethylene to allow drying of up to 12 samples at a time. The new process enabled rapid and simultaneous drying of multiple samples in containment devices suitable for long-term storage and improved rehydration of the sample. Samples were dried on polycarbonate Isopore track-etched membranes positioned within one side of a syringe filter holder (MilliporeTM, Billerica, MA). At the end of the drying period, the syringe filter was assembled for aseptic storage in a humidity-controlled environment or vacuum-packing for shipping and/or long-term storage. The drying time could be altered by changing the power level, and equilibrium moisture contents could be achieved within 30 minutes of drying.

We have recently applied this drying technique to cat germinal vesicle preparations. The ability to compact and inject the cat germinal vesicle (GV) into a recipient cytoplast provides a new fertility preservation strategy that could avoid the need to preserve the whole oocyte. Previous studies demonstrated that as many as 60% of GVs can survive simple air-drying and 4 weeks of storage at 4°C in the presence of trehalose and 10% of reconstructed oocytes with rehydrated GV can achieve nuclear maturation (2). However, air-drying is an uncontrolled process that often results in non-uniform distributions of water within and between samples. To explore the feasibility of microwave drying of GV, the structural integrity

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(chromatin configuration and incidence of DNA damage) was assessed following microwave processing to various endpoints, and it was observed that the GV samples were highly resistant to processing conditions and cumulative osmotic stresses. Even though intermediate moisture levels (~ 8 g H20/g dw) could be reached with no impact on the GV structural integrity, the detrimental effect of lower moisture content (< 5 g H2O/g dw) on DNA will need to be mitigated for future storage conditions at supra-zero temperatures.

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SESSION 6. COLD ADAPTATION AND CRYOPRESERVATION OF PLANTS

THE FUNCTION OF COR15 PROTEINS IN PLANT FREEZING TOLERANCE

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Late embryogenesis abundant (LEA) proteins were first described about 30 years ago as accumulating in plant seeds during late stages of embryogenesis. Later they were also found in vegetative plant tissues, especially after exposure to abiotic stresses and in several desiccation tolerant bacteria and invertebrates. Most LEA proteins are intrinsically disordered proteins (IDPs), i.e. they have no stable secondary structure under physiological conditions, but many fold upon drying. the two highly homologous, strongly cold induced Arabidopsis thaliana LEA proteins COR15A and COR15B are localized in the chloroplasts, as shown by expressing the GFP-tagged proteins in protoplasts. The recombinant proteins are disordered in solution, but mostly α-helical in the dry state, as shown by CD spectroscopy. This gain of structure also takes places under conditions of low water availability mimicking partially frozen systems and is enhanced in the presence of lipid membranes. In silico analysis indicates folding into amphipathic α-helices. Matching this structure, FTIR spectroscopy showed that both proteins interact with liposomes in the dry state, presumably through the helices' hydrophobic face resulting in liposome stabilization during freezing and drying. Simultaneous RNAi silencing of both COR15A and COR15B results in plants with impaired freezing tolerance after cold acclimation, measured with an electrolyte leakage assay, while overexpression of either gene leads to increased freezing tolerance in nonacclimated plants. Collectively, these findings suggest that the COR15 proteins protect plants during freezing by associating with labile membranes during dehydration accompanied by protein folding. However, both COR15 proteins are also able to protect the enzyme lactate dehydrogenase

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(LDH) during freeze-thawing in vitro. Therefore, we evaluated a possible protective effect of COR15 proteins on different chloroplast enzymes in vivo using the COR15 RNAi silencing lines. The results clearly show that the loss of both COR15 proteins has no negative effects on the stability of freezing-labile chloroplast enzymes in leaves, confirming the role of the proteins as membrane stabilizers.

IMPACT OF THE pr-10a GENE ON CRYOPRESERVATION SUCCESS VIA REGULATION OF STRESS RESPONSE

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Exogenous application of substances like desferrioxamine or vitamin C may help plants to overcome cryopreservation stress for example caused by ROS. To investigate how endogenous anti-stress mechanisms improve survival after cryopreservation we established a transgenic cell line of Solanum tubersosum cv. Desiree homologously overexpressing the pr-10a gene. This cell line showed increased salt and osmotic tolerance as well as an increased production of prolin and oxidized glutathione under stress conditions. In a controlled rate freezing procedure consisting of osmotic preculture, cryoprotection and programmed freezing, cell survival after cryopreservation in relation to pr-10a expression was compared for the wild type and the transgenic cell line.

When exposing this transgenic cell line to different sorbitol preculture concentrations for 48h it showed better cryopreservation success over the whole range of concentrations compared to the wild type. Although a stronger influence of pr-10a overexpression was expected for higher concentrations, both genotypes showed the same shape of the curve with an optimum at 0.6M sorbitol, which was only shifted upwards in case of the GMO. Real Time PCR measurement of thepr-10a induction showed that the endogenous pr-10a gene was still normally responding. In addition at nearly all sorbitol concentrations the absolute expression level of pr-10 was substantially higher in the GMO than in the wild type, although the relative increase of pr-10a was much higher in the wild type.

Investigating the effect of pretreatment duration at the optimal concentration of 0.6M sorbitol alone without freezing, no considerable negative effect of the osmotic treatment on the wild type could be detected compared to the GMO. However when performing the whole cryopreservation procedure using different pretreatment durations a coincidence of pr-10a induction and a measurable increase survival rates could be shown.

With increasing pretreatment duration the ability to survive and regrow after cryopreservation increased in both genotypes. Up to 24h in both genotypes the amount of pr-10a transcript as well as the cryopreservation success increased. Thereafter in the wild type the pr-10a expression decreased again and the cryopreservation success did not increase further. In contrast in the GMO the pr-10a expression level remained constantly high and the cryopreservation success further increased. These results indicate that pr-10a overexpression leads to a better adaptation to osmotic stress if the stress lasts for a minimum of 24h.

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DROPLET VITRIFICATION: APPLICATION TO A WIDE RANGE OF PLANT SPECIES

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The droplet vitrification protocol for plants was established because it combines the application of highly concentrated vitrification solutions with ultra-fast freezing and thawing rates. As such the chance to obtain a vitrified cytoplasm upon liquid nitrogen exposure increases and the chance for lethal ice crystal formation reduces.

Practically the method consists of the 10 following steps:

1. pregrowing plants from which meristems are excised under cold or osmotic stress conditions
2. careful excision of the explant (often an apical, sometimes and axillary meristem)
3. preculture of meristems for 1 to 2 days on normal or osmotic stress medium
4. loading of explants for at least 20 min with 2 M glycerol and 0.4 M sucrose dissolved in MS medium at room temperature
5. dehydration with PVS2 (30 % (3.26.M) glycerol, 15 % (2.42 M) ethylene glycol (EG) and 15 % (1.9.M) DMSO dissolved in MS medium that contained 0.4 M sucrose) at 0°C for periods varying between 10 minutes and 2 hours.
6. transfer of explants to a droplet of PVS2 (of about 15 µl) on a strip of aluminium foil and rapid plunge in liquid nitrogen.
7. transfer of frozen strip to 2ml Cryovial filled with liquid nitrogen for storage
8. after storage, rapid thawing by rinse the aluminum foil with explants in recovery solution that 1.2 M sucrose dissolved in MS medium at room temperature and keep for 15 minutes
9. transfer of explants to regeneration medium with elevated sucrose concentration (0.3) for 1 to 2 days
10. regeneration on suitable medium (first week of post-thaw culture always takes place in the dark).

In collaboration with the Laboratory for Tropical Crop Improvement, droplet vitrification was thus far successfully applied to meristem tips of Musa spp. (6), taro (9), pelargonium (3), potato (7), thyme (5), apple (1), hop, ulluco, sweet potato, cassava, strawberry, photinia, rose and daffodil (unpublished results). Also embryogenic cultures of olive (8), date palm (2), avocado (4), chicory, and grape (unpublished results) could be successfully cryopreserved with this method.

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Our experience has shown that depending on the plant species, it takes 4 months up to 2 years before a suitable protocol can be developed. This depends on whether (i) there is already information available for the species under investigation related to in vitro culture and cryopreservation, (ii) it is a tropical or temperate species, (iii) it is woody or herbaceous and (iv) it is a monocot or a dicot.

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FEASIBILITY OF CRYOSTORAGE OF SEEDS, EMBRYOGENIC TISSUE AND ISOLATED PLUMULES OF FOREST TREE SPECIES

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The seeds are classified as orthodox (tolerant to desiccation), suborthodox(intermediate) recalcitrant (sensitive). Not all of these seed categories can be stored in gene banks using classical methods (storage at -18^{o}C), because seeds of many species quickly lose viability in such conditions. Cryopreservation can be the only way for long-term storage of suborthodox seeds (e.g. of beech) and recalcitrant seeds (e.g. of oaks). Besides, cryopreservation is a backup for conventional storage of orthodox seeds. We have investigated the potential to store seeds in liquid nitrogen of more than 20 forest tree species successfully. Seeds were desiccated to the safe water content and then directly frozen through fast plunging into LN. To break seed dormancy, an appropriate combination of cold or warm/cold stratification was used for each species. We can conclude that investigated orthodox andsuborthodox seeds are tolerant to freezing in LN within species-specific safe ranges of water content, for example: 3.0-16.5% in Carpinus betulus (nuts), 5.2-19.5% in Fraxinus excelsior (germination test) (or 7.2-19.5% if determined in the seedling emergence test) (samaras), 5.2-20.1% in Tilia cordata (seeds), 2.7-19.2% in Alnus glutinosa (nuts), 2.0-23.2% in Betula pendula (nuts), 3.3-17.7% in Ulmus glabra (nuts), 8.5-13.2% in Fagus sylvatica (nuts), 6.2-19.4% in Malus sylvestris, 6.7-20.5% in Pyrus communis, and 7.3% inCorylus avellana (nuts).Effective cryopreservation of embryogenic tissue (ET) of Picea omorica and Quercus robur was ensured by ET cryoprotection on solid medium (Woody Plant Medium) with increasing sucrose concentrations (0.25-1,0M), followed by fast drying over silica gel and direct freezing in liquid nitrogen. From rewarmed ET somatic embryos developed, which were next converted into

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plantlets. For the recalcitrant seeds of Quercus robur, plumules (apical meristems of embryo axes) were isolated from the acorns and cryopreserved. Satisfactory effects were achieved when plumules were first subjected to cryoprotection in sucrose and then in glycerol solutions. Next plumules were dried to about 30% water content and frozen. After thawing from –196°C, plumules were cultured in vitro on agar Woody Plant Medium to produce properly growing seedlings. In Poland, the Kostrzyca Forest Gene Bank has been using above cryopreservation techniques to preserve genetic resources of Polish forests.

THE EFFECT OF THE CRYOPRESERVATION TECHNIQUE, REGENERATION MEDIUM AND GENOTYPE ON THE GENETIC STABILITY OF MINT (MENTHA X PIPERITA) CRYOPRESERVED APICES

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One of the most reliable methods for long-term conservation of vegetatively propagated plants is cryopreservation, mainly due to its capability to guarantee the genetic stability of the preserved material during the process. However, the stresses suffered due to the treatments applied together with in vitro culture required for the regeneration of the plant material may result in some degree of genetic instability (1, 2, 3). Mint (Mentha x piperita) is an unfertile hybrid with clonal propagation. Two cryopreservation techniques (droplet-vitrification vs. encapsulation dehydration) were compared with the mint genotype MEN 186. The genetic analysis, using RAPD markers, revealed a higher variation in the regenerated plants from the encapsulation-dehydration protocol (4). In this work the genetic and epigenetic stability analyses of mint apices from a different genotype, MEN 198, cryopreserved under the same techniques and regenerated using three different media composition were carried out. Comparisons of the different treatments on the two mint genotypes are evaluated in order to obtain a protocol of cryopreservation technique and regeneration medium that could optimized the quality of the regenerated plants.

Acknowledgment: This research was supported by the Spanish Government project AGL2010-21989-C02-01.

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INVESTIGATIONS ON OXIDATIVE METABOLISM DURING PROCEDURAL STAGES WITH CRYOPRECERVATION OF EMBRYONIC AXES OF RECALCITRANT SEEDED SPECIES

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The relationship between stresses associated with preservation at low temperatures, oxidative metabolism and viability of potentially photosynthetic material, specifically recalcitrant germplasm, is pertinent to successful cryopreservation. Free radicals or reactive oxygen species (ROS) are extremely reactive, self-propagating species that are ubiquitous in production and volatile in behaviour. Cryopreservation may promote various events leading to ROS-mediated reactions, some of which are important to normal cellular functioning while others perturb intracellular integrity if not controlled. Thus, normal cellular status calls for either the avoidance of oxidative stress and/or efficient functioning of antioxidant and repair systems. Cryopreservation of zygotic embryonic axes of tropical recalcitrant-seeded species remains difficult since shoot development is generally precluded once cotyledons have been removed. It is probable that each manipulation constituting the cryopreservation process is accompanied by a surge of uncontrolled oxidative activity within the tissue, in response to stress. The primary aim of the study was to investigate the possible underlying causes of failure of shoot development after procedures necessary for cryopreservation and to focus on ways to ameliorate the consequences of unbalanced oxidative metabolism. Changes in ROS, in particular superoxide, the hydroxyl radical and hydrogen peroxide, were assessed after axes were exposed to various treatments. Concomitantly, the elicited responses of endogenous antioxidant systems accompanying these treatments were investigated. Changes in the levels of ROS and antioxidant activity were determined using various biochemical assays, and these parameters were assessed in conjunction with shoot development/survival after exposure to processing procedures, with and without the provision of exogenous antioxidants, *viz.* DMSO, ascorbic acid and cathodic water (the catholic fraction of electrolysed calcium- & magnesium chloride solution), to determine the efficacy of selected ROS scavengers. Results showed that excessive ROS activity was assuaged in those treatments incorporating cathodic water in comparison to those treatments that did not. Furthermore, endogenous antioxidant activity was shown to be enhanced in material treated with cathodic water after exposure to stresses induced by procedures involved in cryopreservation. There was a significant increase in survival of explants when exposed to cathodic water compared with those that were not. It is therefore evident that the reducing power of cathodic water is beneficial in balancing oxidative metabolism that could be disrupted during cryopreservation

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UNRAVELLING THE LOW TEMPERATURE AND FREEZING STRESS RESPONSE STRATEGIES IN EUCALYPTUS

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Eucalyptus species have commercial significance in several parts of the world, including South Africa. However, climate change concerns have emphasized the need to identify species with the ability to tolerate extreme climatic conditions and to understand the mechanisms involved in stress tolerance (1). This will facilitate more informed selection choices in breeding programmes and will consequently allow breeders to meet future challenges. Of particular concern to South African tree breeders is the occurrence of frost and its adverse effects on eucalypt survival and productivity (1). The ability to assess for frost tolerance is therefore, strategically important (2). Special emphasis is placed on the evaluation of electrolyte leakage as a frost tolerance indicator and the determination of biochemical and physiological responses that characterise low temperature and freezing stress (including cryopreservation) in Eucalyptus (3, 4). Investigations in our laboratory are conducted on seedlings of various commercially important Eucalyptus species and with in vitro shoots and axillary buds of the sub-tropical E. grandis. Preliminary studies indicate that electrolyte leakage is a potentially good indicator of different levels of tolerance and that greater differentiation may be achieved after gradual cold acclimation of seedlings over 3 weeks. This ability of eucalypts to "acclimate" to low temperature was also demonstrated in cryopreservation studies on E. grandis in vitro material. Low temperature exposure (10^{o}C, 3 days) of E. grandis in vitro shoots elicited a water conservation strategy as it resulted in the maintenance of a significantly higher water content (~20% higher) and higher viability after drying over silica gel than un-pretreated explants dried for the same period. This was also associated with significantly higher levels of total soluble sugars (TSS) in pretreated, dried samples than in unpretreated, dried material (which, in turn was higher than in undried control material). An analogous response was found in shoots pretreated with abscisic acid (5 mg l^{-1}ABA, 5 days, 25^{o}C) (3), suggesting the existence of cross-talk in the responses to low temperature and drying in E.grandis, and the likely involvement of ABA in both. This presentation will discuss these findings, amongst others, in more detail.

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DEVELOPMENT OF A SIMPLE AND UNIVERSAL CRYO-PROCEDURE FOR LILIUM BY DROPLET VITRIFICATION

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Lilium is one of the most economically important ornamental crops worldwide. Availability of genetic resources is a prerequisite for breeding of novel cultivars by both classic and biotechnological programme. Information on cryopreservation of Lilium has been quite limited. Lacking of simple and universal cryopreservation severally limited wide application to long-term preservation of Lilium. In the present study a simple and universal cryopreservation has been successfully described for Lilium by droplet-vitrification. Shoot tips (2 mm in length) including 2-3 leaf primordia were excised from 4-week-old adventitious shoots directly regenerated from basal leaf segments and precultured on MS containing 0.5 M sucrose for 1 day. The precultured shoot tips were treated with a loading solution composed of solid MS containing 0.4 M sucrose and 2 M glycerol for 20 min at room temperature and dehydrated for 4 h by PVS2 at 0°C. Dehydrated shoot tips were transferred onto droplets made on sterile aluminium foil (7 x 20 mm), each droplet containing 3.5 µl PVS2 and single shoot tip, prior to a direct immersion into liquid nitrogen for 1 h. Following cryostorage, frozen foil strips with shoot tips were incubated for 20 min in an unloading solution composed of MS containing 1.2 M sucrose at room temperature, followed by post-culture on a recovery medium made of MS supplemented with 0.2 mg/l TDZ and 1.0 mg/l NAA. The cultures were kept in the dark for 3 days, and then transferred to light conditions for recovery. With the optimized parameters developed in this protocol, the highest shoot regrowth rate (86.7%) and lowest (43.7%) were obtained in Lilium Oriental hybrid 'Siberia' and L. longiflorum × Oriental 'Triumphator', respectively, with an average shoot regrowth at 66.7% obtained for the 5 Liliumspecies or hybrids tested. Successful development of the simple and universal cryopreservation protocol will provide a technical support for setting-up Lilium cryo-banking and is being tested for lily virus elimination.

DETERMINATION OF MALONDIALDEHYDE IN GARLIC CRYOPRESERVATION INDICATING STRESS INFLUENCE

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The concentration of malondialdehyde is proven for denoting the level of lipid peroxidation in biological tissues. MDA is a highly reactive and toxic molecule and its attachment to nucleic acids and proteins disrupts biological functions. This study investigates the production of MDA at each step of garlic cryopreservation and explores the relation between MDA levels and regrowth rate. Shoot tips of Allium sativum were cryopreserved using plant vitrification solution 3 (PVS3; glycerol 50% + sucrose 50% in MS medium).

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During cryosteps I (explant preparation), II (preculture), III (PVS 3 treatment) and IV (storage in liquid nitrogen + rewarming), explants undergo a sequence of abiotic stress conditions including mechanical, osmotic and freezing. Here, a simple and rapid method was used for determination of MDA which excludes additional mechanical stress to explants. MDA production was observed in all cryosteps. Overall results suggest that there is increasing production of MDA with subsequent steps of cryopreservation, although there were no statistically concordant values within variants tested. Interestingly, among 'step IV' explants, some had MDA levels similar to explants of 'step I'. Since explants of 'step I' attain 98 % survival, it is chosen as control. It is expected that the success rate of garlic cryopreservation may correlate with the percentage of explants showing MDA levels in 'step IV' similar to those of 'step I'. Hence, MDA production during the course of cryopreservation process may significantly reflect the regrowth rate of garlic cryopreservation.

THE ROLE OF ANTIOXIDANTS ON SURVIVAL AND GENETIC STABILITY OF CRYOPRESERVED MINT APICES

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Cryopreservation protocols impose oxidative stresses to tissues that may affect plant regeneration and also to the genetic stability of cultures. The use of antioxidants at different steps of a cryopreservation protocol may reduce those negative effects. We have studied the use of antioxidants in an encapsulation-dehydration protocol with shoot apices of two different mint (Mentha xpiperita) genotypes (MEN 186 and MEN 198). The antioxidants were added in a preculture step, in which 0.3 M sucrose was also employed: 0.28 or 0.43 mM ascorbic acid, 0.16 or 0.24 mM glutathione (both supplemented to the preculture medium), or α-tocopherol (spread on filter paper and placed on the medium). This treatment was applied to the apices previous to the encapsulation step of the cryopreservation protocol.

After a 8-week recovery period, the regeneration response (organized growth) of apices not treated with antioxidants differed between genotypes: 37% in MEN 198 vs 13% in MEN 186. The use of α-tocopherol increased the regeneration percentage in MEN 198 to 52%, although that increase was not so significant in MEN 186 (20%). The response of these two genotypes to the other antioxidant treatments was variable. The genetic stability of the recovered cultures was studied by RAPDs markers. Genetic differences among samples were detected in both genotypes. Near 50% of the studied samples showed the same genotype in both cases. A clear relationship between the quantity of variation accumulated and the type of sample was observed.

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CRYOPRESERVATION OF *THYMUS LOTOCEPHALUS* BY DROPLET VITRIFICATION: EFFECT OF COLD-HARDENING

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Thymus lotocephalus G. López & R. Morales is a rare aromatic species endemic to the Algarve region (South of Portugal). In order to fight the extinction of this species and contribute to its conservation, our group has been developing *ex situ* strategies, such as *in vitro* propagation and seed cryopreservation (1, 2). Now we intend to implement a cryopreservation protocol for shoot tips of *T. lotocephalus* as an alternative method for long-term preservation.

Droplet vitrification was the method tested for shoot tip cryopreservation and in this study we set out to evaluate the response of shoot tips after cold-hardening of *in vitro* donor plants.

Apices were excised from *in vitro*-grown shoots of *T. lotocephalus* maintained at a constant temperature of 25ºC during the first two weeks and then transferred to constant 10ºC or alternate 25/-1ºC for further two weeks. After excision, shoot tips were pre-cultured on semi-solid Murashige and Skoog medium containing 0.3 M sucrose for one day. Shoot tips were placed in loading solution (0.4 M sucrose + 2 M glycerol) for 20 min, followed by dehydration in PVS2 solution at 0ºC for 60 min, placed on aluminium foil strips and then immersed in liquid nitrogen. Rewarming took place in unloading solution (1.2 M sucrose) for 20 min at room temperature.

Four weeks after rewarming, cryopreserved apices excised from cold-hardened shoots presented higher survival percentages, 96.7 ± 3.3 and 93.3 ± 4.2% for 10ºC and 25/-1ºC, respectively, than the control, 86.7 ± 6.7%. This tendency was maintained eight weeks after rewarming, as the survival and regrowth percentages were higher for cold-hardened shoot tips. The best result, 73.3 ± 9.9%, for both survival and regrowth percentages, was obtained in cryopreserved shoot tips excised from *in vitro*-grown shoots maintained at 10ºC.
According to these results, cold-hardening of *in vitro* donor plants improves the survival and regrowth of cryopreserved shoot tips of *T. lotocephalus* by droplet-vitrification.

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MORPHOLOGICAL AND GENETIC CHARACTERISTICS IN ACCLIMATIZED CHRYSANTHEMUM PLANTS DERIVED FROM ENCAPSULATION-DEHYDRATION CRYOCONSERVED APICES

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The occurrence of genetic instability in regenerated chrysanthemum plants from encapsulation-dehydration cryopreservation protocol has been detected using RAPD and AFLP markers (1). Beside, previous analyses have indicated that some morphological variations, mainly related to the leave shape, can be observed in regenerated plants, although no correlation could be established between those samples with genetic variation and morphological alterations.

In this study, 30 acclimatized plants derived from different steps of an encapsulation-dehydration cryopreservation protocol have been analyzed to assess their genetic (using RAPD markers) and epigenetic stability (through CRED-RA markers). Different morphological parameters (18) have been measured. The three different approaches have been compared in order to establish the relationship between (epi)-genetic changes and morphological characteristics.

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CRYOPRESERVATION OF A POTATO SOMATIC HYBRID (CN2) SHOOT TIPS USING VITRIFICATION PROCEDURES

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Our research work is aimed at cryopreserving elite genotypes which have been regenerated by biotechnological methods. This is of paramount importance for all agro-economical, genetical, ecological and toxicological evaluations before starting commercialization process. The first part of this research focused on the optimization of in vitro culture conditions enabling the production of a high number of apical buds of a potato somatic hybrid CN2 (1) from axillary buds. The use of non-hermetically sealed jars as culture vessel and Phytagel as a gelling agent promoted the regeneration of vigorous shoots from which apical buds can be excised for medium and long term conservation. Bioencapsulation of

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apical buds using 3% sodium alginate and 75 mM calcium chloride produced spherical artificial seeds without affecting the viability of plant tissues. At 8°C, encapsulated apical buds were able to stay alive for 5 months. Cryopreservation of such buds was found to be more efficient when a droplet-vitrification protocol was followed, if compared to simple vitrification and encapsulation-vitrification protocols. Sucrose preculture (180 g L^{-1}) or cold hardening of the meristems, LS and PVS2 treatments were necessary for the maintenance of viability at the temperature of liquid nitrogen. Viability rates were relatively low (23 %); this is why an additional optimization of these techniques and/or testing other cryopreservation protocols is essential. A preliminary study was implemented in order to understand the mechanism of tolerance to severe osmotic stress. Biochemical analyzes show that sucrose preculture and cold hardening were able to change the protein profiles of tissues.

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EFFECTS OF POST-HARVEST STORAGE OF ALLIUM SATIVUM BULBS ON THE CRYOPRESERVATION OF STEM-DISCS

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In situ and ex situ conservation is important for the long-term conservation and sustainable utilisation of Allium germplasm (1). In vitro culture can reduce some of the difficulties associated with these conservation approaches, but in the longer term there are cost implications and an increasing risk of culture instability as a result of somaclonal variation (1). However, these short comings can be alleviated by cryostorage. Hence the long-term conservation of vegetatively propagated Allium germplasm is dependent upon the development of reproducible cryopreservation protocols which can be routinely applied to diverse Allium species and cultivars (2). Such protocol development requires the assessment of both cryogenic and non-cryogenic factors which can influence the storage outcome. The aim of this study was to determine the effect of the duration of post-harvest storage of garlic bulbs on the survival and regrowth of shoots from stem disks, a novel explant for garlic micopropagation (3), following cryopreservation. Survival was defined following the criteria established by Keller (2). Significant increases were observed in the survival and the mean number of shoots regrown per stem-disc quarter after three to six months storage (December-March) as compared with shorter (September-November) and longer (April-June) storage periods ($p < 0.05$). The highest frequency of regrowth (55%) was observed after five months storage (January), while survival was greatest (75%) following a storage period of six months (February). A minimum post-harvest storage period at 10^{o}C is necessary to break garlic bulb dormancy for the effective induction of in vitro shoots following cryopreservation of stem-disc quarters. While the reduction in the frequency of regrowth after extended post-harvest storage relates to bulb quality, which is known to decline during extended storage (4). This study identifies the need to standardise the harvesting, drying and storage parameters of Allium

material which is to be cryopreserved to facilitate the effective implementation of cryostorage within genebanks and between different institutions.

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OPTIMIZING CRYOPRESERVATION OF ENDEMIC HLADNIKIA PASTINACIFOLIA BY ENCAPSULATION–DEHYDRATION

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Hladnikia pastinacifolia is monotypic endemic genus with an extremely narrow distribution area (Slovenia). Cryopreservation of this endemic species, using encapsulation-dehydration, would be one of several conservation activities for this endangered species. The final steps in the encapsulation-dehydration procedure were optimized, and the survival rate and regrowth of different genotypes after the encapsulation-dehydration procedure was explored. The culture of in vitro micropropagated shoots in an MS multiplication medium was pre-cultivated at 4°C in the dark for 7-14 days. Shoot tips from 1 to 2.5 mm of different genotypes were excised, incubated in 3% Na-alginate with 0.4 M sucrose and encapsulated in the MS medium with 0.25-0.4 M sucrose with or without 1 M glycerol, without $CaCl_2$ for 30-60 minutes. They were then polymerized in the MS medium with 100 mM $CaCl_2$, treated with an osmoprotectant for 10-12 hours in MS medium with 0.4 M sucrose with or without 1 M glycerol, air-dried in laminar flow and frozen by direct immersion in liquid nitrogen. The cryopreserved shoots were quickly rewarmed, rehydrated in liquid MS medium with 0.4 M sucrose and transferred to an MS or MS multiplication medium, both with 0.1 M sucrose in a growth chamber. Only shoots transferred to the MS medium were viable. They regenerated only when these clearly viable shoots were soon placed on the MS multiplication medium. The procedure was suitable for only two genotypes from the five tested. Regrowth was obtained on 20% to 27% of shoots. Further research is needed to optimize the procedure for all genotypes.

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