HYPOTHERMIC ORGAN PRESERVATION : HOW WE GOT HERE, WHAT WE LEARNED FROM NATURE AND WHERE WE GO IN THE 2000'S.

Barry J Fuller

Royal Free & UCL Medical School; University Department of Surgery; Hampstead Campus; UCL; London NW3 2QG; UK.

Organ transplantation has become the treatment of choice in many countries for end-stage diseases, but now demand for donor organs outpaces the supply, and improved organ cold preservation is again a focus of attention. Over the past 30 years, organ cold preservation has developed via two main strategies, based on laboratory experimentation but which overlap many of the natural strategies evolved in organisms living in extreme environments. These are (1) hypometabolism in cold hypoxic flush (HCF) and (2) residual metabolic support by hypothermic perfusion preservation (HPP). In HCF, solutions were developed empirically which reduce the energy demand required for ongoing homeostatic controls, and suppress metabolism over and above the reduction resulting from cooling alone. On the contrary, in HPP, a low continuous perfusion of the vascular bed of the organ is performed at low temperatures, supplying small amounts of oxygen which support ongoing aerobic metabolism and maintain mitochondrial integrity. The development and current practices for both modalities of organ preservation will be discussed, and parallel observations to natural cold tolerance will be drawn. The balance between HCF and HPP has waxed and waned in the intervening period, with HCF seen as logistically-simpler and predominating worldwide over the past 20 years. However, the continuing organ shortage has driven the need to access organs from donors previously considered unsuitable (the so-called expanded criteria donors and non-heart beating donors) where organ function may already be compromised before the cold preservation step, and here HPP has a role to play. Gradual improvements in organ preservation have been achieved, and further advances, in both HCF and HPP are being proposed. Beyond these, a much greater understanding of molecular mechanisms involved in signalling for cytoprotective strategies in non-adapted mammals will be required and we can still learn much from studies in natural cold tolerance.

CHALLENGES OF CRYOPRESERVATION OF TISSUE ENGINEERED CONSTRUCTS

Lilia Kuleshova

Low Temperature Preservation Unit, National University Medical Institutes, National University of Singapore , #03-01C Block MD11, 10 Medical Drive, Singapore 117597

Tissue engineering (TE) is an emerging approach in regenerative medicine. There are several levels in the complexity of TE constructs (TECs). Self-assembled cell aggregates can be considered as the initial concept in TECs. Along with self-assembled aggregates, living cells combined with scaffold/matrix, which are expected to support cells, are grown in laboratory conditions. The ability to cryogenically preserve these TECs, so that they can be physically distributed to patients in need, is the ultimate goal. There are several challenges of cryopreservation of TECs:

- · preservation of integrity of constructs.
- · maintaining high cell viability after cryopreservation
- · cell functions should be also maintained
- · cell proliferation and differentiation ability (if stem cells involved)
- · cell-cell interaction should be preserved

There are several specific challenges. The matrix which allowed migration of cells should retain its original properties after cryopreservation. Maintaining cell attachment ability to

scaffold is essential for cell-scaffold systems. Implementation of vitrification strategy allowed us to successfully preserve viability and functions of self-assembled cell aggregates and microencapsulated hepatocytes. In regards to more complex TECs (nanofiber scaffolds and multicomponent biomaterial beads) involving MSCs studied by us, vitrification does not impair integrity of TECs while preserving MSCs viability and metabolic activity, proliferation and differentiation ability. Additionally, products of human and animal origin are not safe to use since they could be derived from infected donors/sources. Currently available "freezing" protocols use a large amount of serum which supports cells during gradual decrease of temperature. We have shown using neuronal SCs that effective vitrification protocols can be established without inclusion of serum or protein. There is a need to develop customized vitrification protocols for constructs of varying configuration as well as for cells from different species. For instance, constructs of different sizes require specific holders to contain during the vitrification process, besides that, cooling/warming rate during immersion into liquid nitrogen/water bath may depend on the dimensions of the constructs or the quantity of cells that need to be cryopreserved simultaneously. With respect to cell biology, an optimized vitrification procedure, for example, for red blood cells from one species, may not be optimal for the same type of cells from another species. Furthermore, SCs after phenotype induction have different characteristics and properties, therefore the protocol must be re-developed.

CRYOPRESERVATION OF MAMMALIAN REPRODUCTIVE CELLS

G. J. Morris

Asymptote Ltd., St John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, UK

Cryopreservation of cattle sperm was one of the first breakthroughs in cryopreservation and remains the largest single application of cryobiology. Reproductive cells are preserved for a variety of applications:

1) Human and veterinary artificial insemination (AI) and *in vitro* fertilisation (IVF)
2) As a source of embryonic stem cells
3) Preservation of endangered species ("The Frozen Zoo")
4) Maintenance of stocks of transgenic animals

Conventional technology is adequate for some cell-types, for example human sperm and embryo cryopreservation is routine in IVF, however human oocyte cryopreservation is problematical. Also, gametes and embryos from many primates, pigs and rat are difficult to cryopreserve. This talk will focus on three of the topics that need to be resolved in the field of reproductive cell cryopreservation.

1) *Sperm cryopreservation*. Surprisingly the mechanisms of freezing injury to mammalian sperm are not well understood and relatively poor recovery rates are tolerated with many species. Advances in sperm cryopreservation will only be possible when the biophysics of cryoinjury to sperm are understood.
2) *Human oocytes.* There is controversy over the relative benefits of vitrification over conventional slow cooling, especially for the cryopreservation of human oocytes. Recent data will be reviewed.
3) *High throughput cryopreservation*. The practical problems associated with the cryopreservation of mutant strains of laboratory animals (mouse, rat, pig) have become acute. It is estimated that over the next decade some 5000 new strains of mice will be created annually –this is equivalent to the number of mouse strains cryopreserved over the last 30 years. Strategies to deal with these large numbers need to be developed, current technologies are not appropriate. The most relevant stage for cryopreservation – embryo, spermatozoa or spermatogonial stem cells needs to be agreed and equipment for robotic freezing, storage and retrieval should be developed.

DELIVERING STEM CELLS TO THE CLINIC: CHALLENGES IN BANKING OF HUMAN EMBRYONIC STEM CELLS FOR FUTURE THERAPY

C J Hunt

UK Stem Cell Bank, National Institute for Biological Standards and Control, Blanch Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

The successful derivation of an embryonic stem cell (hESC) line from an *in vitro* fertilized human embryo was first reported in 1998. Whilst the use of hESC in improving our understanding of human developmental biology and mechanism of neurodegenerative and other diseases was the primary motivation behind this development, there has nevertheless been a rapid drive (and much hype) towards the use of these cells as precursors in the development of novel therapies in the field of regenerative medicine.

The expansion of hESC in culture continues to pose significant problems; mechanisms governing the maintenance of the undifferentiated state have still to be fully elucidated and controlled differentiation of these cells to produce consistently high yields of specific somatic cell types remains difficult. It is therefore of little surprise that methods for cryopreservation too have so far yielded significant variation in post-thaw recovery.

An essential prerequisite to widespread clinical application is the ability to provide consistent banks of early passage cells for use as seed stocks for clinical trials and eventual commercial therapies. For both the cell bank producing these stocks and others involved in their clinical application, effective cryopreservation protocols that preserve cellular viability whilst maintaining both the undifferentiated state and pluripotency are essential. Within this environment an added level of complexity is supplied by the need to take account of both microbiological safety and the regulatory environment surrounding the use of human cells and tissues.

As research groups move toward clinical trials, the need to develop facilities and culture systems that are compatible with the regulatory systems has been recognised but cryopreservation methods, storage and delivery systems which are both effective and compatible with such regulation have largely been overlooked. This paper will review current cryopreservation methods, discuss some approaches to optimising cryopreservation protocols and examine some aspects of the regulatory environment that impact directly on the banking of hESC lines for therapy

CRYOPRESERVATION PROGRESS, CHALLENGES AND APPLICATIONS IN AQUATIC SPECIES

D. Rawson and T. Zhang

LIRANS Institute of Research in the Applied Natural Sciences,250 Butterfield, Great Marlings, Luton, LU2 8DL, UK

Aquatic species are increasingly being the subjects in cryo-biological studies, either as model species or because of the value successful protocols would be to the organisms in their own right. Research in this area has focused mainly on gametes and embryo cryopreservation, and whilst considerable success has been made for sperm, the story for oocytes and embryos is less impressive. The diversity of species studied is small in comparison with the vast number of aquatic organisms, with the main representatives coming from the molluscs (mussels, oysters), echinoderms (sea urchins, starfish), crustaceans (brine and freshwater shrimps, horseshoe crab) and bony fish (zebrafish, gilthead seabream).

Successful cryopreservation of male and female gametes would have significant benefits in many areas of aquaculture, and in conservation of endangered species through cryo-conservation and the establishment of cryobanks.

Whilst the barriers to cryopreservation of oocytes and early stage embryos are becoming better understood, only limited progress has been made to overcome them. As one would expect, the focus of cryopreservation studies on these recalcitrant problems is the achievement of post-thaw survival, and development. Indeed once this has been achieved, as in material such as sperm, the cryobiology is seen to be complete and other possible impacts of cryo-protocols on the cells/tissues are not explored. However, there is growing evidence that subtle changes can be induced in the material, even when the survival criterion has been achieved. This paper will review the 'state of play' in the cryopreservation of gametes and embryos of aquatic species, and explore the potential opportunities that this and related research can bring to aquaculture and conservation. The nature of subtle, non-fatal, changes resulting from cryo-procedures that are beginning to be recognised in fish oocytes and embryos will also be explored.

CRYOBIOLOGICAL CHALLENGES FOR 21ST CENTURY SCIENCE: THE CONSERVATION OF TYPE SPECIMENS

JG Day

Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, PA37 1QA, UK.

Recent developments in molecular biology and the application of a polyphasic approach, combining genotypic, phenotypic and mating-type characters, have revealed that protistan biodiversity is significantly greater than was traditionally thought. This has presented a range of new challenges to protistan Biological Resource Centres (BRCs), not least in the need to re-categorise/rename their historic holdings. What is rapidly becoming apparent is that these holdings are more diverse than was thought previously. Furthermore, an increased number of new taxa are being identified, primarily by molecular techniques, to which the scientific community need access.

At the CCAP the majority of the strains held are eukaryotic microalgae. These are currently classified under the rules for Botanical Nomenclature (The Vienna Code 2006). Under these rules there is no requirement for the deposition of living specimens in any depository. In fact, the code states that the name should be attached to a designated type specimen in herbarium, or an image. The code also states that any type material must be in a metabolically inactive state. One could debate whether this approach is truly suitable for science in the 21st century, where access to live specimens, DNA and/or bioinformatics data is of increasing importance. The protistan service culture collections are positioned to take on the role of conserving "live", but metabolically inactive, type specimens and in addition to holding them as reference material, providing cultures to the scientific community on request. They already hold significant numbers of authentic strains, derived from the original type material, e.g. CCAP holds >290, and most protistan service BRCs have the capability to cryopreserve microalgae. This paper discusses the suggestion that (algal) protist type specimens should be held as cryopreserved cultures and investigates the challenges that this would bring in the following areas: ensuring phenotypic/ genotypic stability; quality standards, validation of methodologies and the logistics/ organisation that would be required to achieve this objective.

TOWARDS UNDERSTANDING THE MECHANISTIC BASIS OF PLANT COLD ACCLIMATION

DK Hincha

Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Potsdam, Germany

Considerable effort has been directed towards understanding how plants adapt to low temperature. In common with many plants, the model plant *Arabidopsis thaliana* is able to increase its freezing tolerance when exposed to low, non-freezing temperatures. Both the non-acclimated and cold acclimated freezing tolerance of Arabidopsis is strongly influenced by the geographical origin of the investigated genotype (ecotype). In general, freezing tolerance increases with increasing latitude of origin and with decreasing habitat temperature during the growth season.

Plant freezing tolerance is a multigenic trait. Recently, gene expression studies with microarrays and metabolite profiling experiments using gas chromatography-mass spectrometry have revealed thousands of changes in gene expression and hundreds of changes in metabolite levels in response to cold acclimation. These changes show significant differences in different *Arabidopsis* ecotypes, opening the possibility of characterizing the functional significance of such changes through correlation with the freezing tolerance phenotype. Through such analyses we are able to identify candidate molecules with a high probability of being functionally important for plant freezing tolerance.

We are interested in two types of molecular changes: those responsible for low temperature signal transduction and regulation of gene expression (mainly transcription factors that regulate the expression of many other genes) and molecules that directly protect cellular structures during freezing and/or severe dehydration. To better understand the regulation of gene expression we are currently investigating the interplay of low temperature and circadian clock regulation of gene expression during cold acclimation and the regulation and function of transcription factors during both cold acclimation and sub-zero acclimation. To identify molecules that may directly affect cellular stability, we use metabolite profiling by mass spectrometry based techniques. This allows us to search for correlations between the cellular content of many compounds and the freezing tolerance of the tissues. The function of compounds of specific interest (e.g. particular membrane components, LEA proteins) is investigated in detail using biophysical approaches such as fluorescence spectroscopy and infrared spectroscopy to determine their exact mechanisms of action.

OIL, WATER, AND THE BIOPHYSICS OF SEED CRYOBIOLOGY

HW Pritchard, T Roach, MI Daws, J Nadarajan and I Kranner

Seed Conservation Dept., Royal Botanic Gardens Kew, Wakehurst Place, Ardingly, West Sussex RH17 6TN, UK. E-mail: h.pritchard@kew.org

The world's plants are under increasing threat of extinction due to human population growth and the accelerating rate of consumption of natural resources and habitats. With *in situ* conservation action plans under continuing pressure, efforts are underway to conserve species *ex situ* to safeguard resources that could hold future cures for numerous diseases and provide a range of other ecosystem services, including regulatory, provisioning and supporting. Whilst about 80% of species may have desiccation tolerant, 'orthodox' seeds that are suited to conventional banking at –20°C, a quarter of these (perhaps 60,000 species) may be inherently short-lived and require cryopreservation to achieve long-term storage objectives. In addition,

the other 60,000 species (20% of the world's flora) may have varying levels of desiccation tolerance: from high levels of sensitivity to drying (the 'recalcitrants') to moderate tolerance of dehydration, but sensitivity to cold temperatures (the so-called 'intermediates'). Such cold sensitivity was originally linked to poor performance at –20°C, but more recently it has been shown to be temperature- and species-specific in relation to oil composition and transformation events. There are still challenges in cryopreserving and recovering such relatively 'dry' seeds. For the 'recalcitrant' species, knowledge of oil content is crucial as it determines the high moisture freezing limit (≈ unfrozen water content) below which tissues should be dried before cooling. Thus for both desiccation-tolerant and –intolerant seeds an understanding of the biophysics of water and oil is important for cryopreservation success.

SOME RECENT DEVELOPMENTS IN PLANT CRYOPRESERVATION

F Engelmann

Institut de Recherche pour le Développement (IRD), UMR DIA-PC, 911 Avenue Agropolis, BP 64501, 34394 Montpellier cedex 5, France.

With plants, cryopreservation is used for long-term storage of recalcitrant and intermediate seed species, vegetatively propagated plants, rare and endangered species and products from biotechnology such as metabolite-producing cell lines and genetically engineered material. New vitrification-based techniques have been developed over the last 15 years including vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet vitrification. Cryopreservation protocols have been established for a large range of species from temperate and tropical origin. The development and application of cryopreservation is much more advanced for vegetatively propagated species than for recalcitrant seed species. A growing number of examples of large-scale, routine applications of cryopreservation is found with orthodox seeds of short-lived and endangered species, pollen of horticultural crops, non-orthodox seed species, dormant buds of fruit and forestry species, *in vitro* shoot tips of vegetatively propagated plants and biotechnology products. Large-scale, international projects focusing on the development and application of cryopreservation for plant species are underway, with funding coming from various sources including e.g. the EU (COST Action 871 "Cryopreservation of crop species in Europe") or the Global Diversity Trust (Development and refinement of cryopreservation protocols for the long-term conservation of vegetatively–propagated crops. Fundamental research aimed at improving the understanding of the physical and biological mechanisms related to tolerance of plants to dehydration and cryopreservation is being carried out by several groups world-wide. Additional uses of cryopreservation such as its utilization for eliminating viruses from plants have been under investigation for several years. In conclusion, dramatic progress has been made during the last 15 years in the development of cryopreservation techniques and there is a steadily increasing number of situations where cryopreservation is being used routinely for long-term storage of plant genetic resources.

PRESENT STATE OF POTATO CRYOPRESERVATION – NEW ASPECTS REVIEWED

A. Kaczmarczyk, M. Grübe and E.R.J. Keller

Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany

Potato is one of the main crops, whose breeding is based on clonal propagation. Maintaining vegetatively propagated germplasm is a challenge for genebanks because of very high labour input and phytosanitary requirements. Since 1992 maintenance of potato

germplasm has been successfully performed by cryopreservation in Germany. It is carried out in IPK by applying the DMSO droplet method. Initially developed mainly empirically, it is now more and more important to improve the causal insight into this method. The present contribution aims at summarizing the work done recently at IPK. In this study, the application of alternating temperature preculture of *in vitro* donor plants at 22/8 °C day/night temperature for 7 d (AT) before cryopreservation improved regeneration results significantly in comparison to plants grown under 22 °C constant temperature (CT). Shoot tips of plants from AT had higher total soluble sugar, lower starch and lower proline concentrations. As the preculture regime used here did not induce cold acclimation in potato the observed improvement of regeneration is not based on cold-stress reactions. This was confirmed by a newly developed protocol for 2D gel analysis. Results showed a low change in protein expression between differently precultured shoot tips. Protein expression was mainly down-regulated after AT in comparison to CT. Ultrastructural analysis revealed less damage of the nuclei after AT in comparison to CT plants after rewarming from cryopreservation. Regeneration of shoot tips started from leaf primordia and seldom from the meristematic dome proper. Thermal analysis showed that when applying the DMSO droplet method a glass transition and small ice crystal formation take place in the tissues. No significant differences were observed by this method between explants from AT and CT. Altogether AT revealed no strong changes in biochemical compounds, ultrastructure and thermal properties of shoot tips, but better cryopreservation results accompanied by an increase in soluble sugars in the tissue. This would mean that alternating temperatures simulating natural conditions are more favourable than constant ones for cryopreservation.

IMPROVED CRYOPRESERVATION OF HUMAN PULMONARY ENDOTHELIAL CELLS (HPMEC-ST1.6R): METHODS OF ANALYSIS INFLUENCE CELL SURVIVAL RATES

N. Hofmann, C. Stoll, I. Bernemann, B. Glasmacher

Institute of Multiphase Processes and Centre for Biomedical Engineering, Leibniz Universität Hannover, Callinstr. 36, 30167 Hannover, Germany

Optimisation of cell specific freezing and thawing parameters must precede the effective long-term storage of cells by cryopreservation. For estimation of vitality after thawing it is a common practice to use Trypanblue staining as viability test. Sustained success of cryopreservation however depends on functional, proliferating cells. Thus the results of optimised cryopreservation protocols were verified considering particular recultivation for testing the proliferating capability of the cells and MTT-assay as test with relation to their metabolic activity vs. Trypanblue staining.

Based on a previous parametric study [1], the parameter optimisation was conducted for a human pulmonary endothelial cell line (HPMEC-ST1.6R). In this study two step cooling protocols with various cooling rates (1, 3, 5, 7,5 and 10 K/min for the first step from 1°C to –30°C in all possible combinations with 3, 5, 7,5 and 10 K/min for the second step from -30°C to –80°C) were used as well as four different concentrations of Me_2SO as cryoprotectant (2.5, 5, 7.5 and 10 % v/v Me_2SO). Cell survival rates of the thawed samples were estimated by Trypanblue staining and MTT-assay directly after thawing and counting of the adhered cells after recultivation for 24 hours.

For HPMEC a concentration of 5 and 7.5 % Me_2SO resulted in survival rates of 90% proliferating cells, whereas with a lower or higher concentration, survival rates are up to 10 % lower. Within a wide variation of cooling rates an inappropriate combination of cooling rates (P5/3, P10/10) yields up to 16 % lower survival rates.

Correlations between combinations of cooling rates and survival rate of the cells could be shown by counting adhered cells after recultivation or MTT-assay but not by Trypanblue staining. Generally results for cell survival obtained by Trypanblue staining were up to 20% higher than the actual percentage of proliferating cells. This implies that this test of membrane

integrity after thawing leads to an overestimation of cell survival rates after cryopreservation. MTT-assay however showed similar results as recultivation studies, suggesting that this testing method can be used in following studies instead of the time and cost consuming recultivation procedure.

Acknowledgement: The project was supported by the Cluster of Excellence "REBIRTH" (DFG).

References

[1] Bernemann I. et al.: Strategy to improve cryopreservation protocols: A systematic parametric analysis for suspended cells. Cryobiology 55 (3), 338 (2007)

DESIGNING AN OPTIMAL CRYOPRESERVATION PROTOCOL FOR HUMAN EMBRYONIC STEM CELLS: A METHODOLOGICAL APPROACH

O Olarewaju[1], DE Pegg[1], PG Genever[1], CJ Hunt[2]

[1]Biology Department, University of York, Heslington, York YO10 5DD, UK. [2]UK Stem Cell Bank, Potters Bar EN6 3QG, UK.

Human embryonic stem cells (hESCs) are derived from the inner cell mass of the human blastocyst. They are pluripotent cells which can differentiate into a wide range of cell types, making them potentially useful for the treatment of diseases such as diabetes, Alzheimer's disease and heart disease. Currently, the methods employed for cryopreserving hESCs are suboptimal. Post-thaw cell survival is very variable and maximum recovery ranges widely from 5-90%. As a result, generating a large number of cells that can be successfully frozen for future use is difficult. Current methods have been derived empirically; protocols have been adopted from embryo storage (vitrification) and mouse embryonic cells (conventional freezing in dimethyl sulfoxide, Me_2SO, in culture medium). In this work, a methodological approach was used to determine the fundamental physical properties important for cryopreservation including the hydraulic conductivity (L_p), solute permeability (P_s) and the non-osmotic volume (V_b). The hESC lines, RH1 and SHEF3, had V_b values of 0.21 and 0.19, respectively. This is comparable to that of human hematopoietic progenitor cells from bone marrow. The L_p values for RH1 at +20°C and +2°C were $1.3x10^{-6}$ cm/atm/s and $1.52x10^{-7}$cm/atm/s, respectively. The P_s values for DMSO at +20°C and +2°C were $1.9x10^{-5}$cm/s and $6.48x10^{-6}$cm/s, respectively. These permeability values are one order of magnitude higher than those of $CD34^+$ cells from umbilical cord blood at both temperatures. Further studies are underway to determine L_P and P_s for SHEF3. This data will be used to model protocols for the addition and elution of cryoprotectant that minimise osmotic damage during the storage of human embryonic stem cells.

A SIMPLE ASSAY SYSTEM TO MONITOR THE POTENTIAL FOR CONTAMINATION DURING DIFFERENT STAGES OF CRYOPRESERVATION

G. J. Morris

Asymptote Ltd., St John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, UK

The potential of contamination of cryopreserved samples during controlled rate freezing, vitrification, shipping and long term storage is a major issue for the development of long term preservation strategies, and is especially important in the storage of stem cells and other therapeutic cells for subsequent clinical application. Contamination of samples from liquid nitrogen has been examined using a number of biological indicators, but this experimental approach requires sophisticated assay methods and is time consuming.

We describe here a simple assay that can be used to monitor the potential contamination of samples during different stages of cryopreservation. The basis of the assay is to introduce a harmless, inert material into the nitrogen storage system as an analogue of harmful contaminants that are likely to be present, and then monitor the transfer of this marker to cryocontainers and the environment. The assay exploits the crystal growth of sucrose hemi-heptahydrate, the cause of sugar bloom of certain high sucrose foodstuffs (icings and fruit preserved in sugar syrup) when preserved at -20°C. Crystals of sucrose hemi-heptahydrate are stable within liquid nitrogen, in nitrogen vapour and ambient air and can be monitored by a simple assay which allows contamination risks to be evaluated in a direct, rapid manner

LOW TEMPERATURE STORED *Botrytis cinerea* IN CRYOBANK® MAINTAINS PATHOGENICITY IN GRAPEVINE

R Pathirana[1], L-H Cheah[1], F Carimi[2] and A Carra[2]

[1] New Zealand Institute for Crop & Food Research Limited, Private Bag 11600, Palmerston North, New Zealand; [2] Istituto di Genetica Vegetale, Sezione di Palermo, CNR Corso Calatafimi 414, Palermo, Italia.

Grey mould or bunch rot caused by the fungus *Botrytis cinerea* is the major cause of fruit and wine losses for the fast-growing New Zealand grapevine industry. The cost of Botrytis rot to the industry worldwide is also enormous. In a collaborative project we have developed protocols for somatic embryogenesis in grapevine and this system is now in use for inducing mutations and selecting for fungal pathogen resistance using toxins from Botrytis (Pathirana et al. 2008). The availability of pathogenic cultures of *Botrytis* throughout the year facilitates rapid screening and evaluation of genotypes. For long-term maintenance, we suspended fresh *Botrytis* cultures from potato dextrose agar (PDA) plates in Cryobank® beads in glycerol and maintained them at -20°C. The stored cultures were retrieved after more than one month and their pathogenicity was tested on leaves from *in vitro*-grown susceptible cultivars. Results indicate that the strains maintain the same pathogenicity as the original cultures. This method of cold storage is therefore more effective for maintaining *B. cinerea* cultures than on PDA plates at low temperatures. The latter method requires frequent subculture and often results in loss of pathogenicity of the original isolates.

Acknowledgements: This work is funded in New Zealand by grants from the Sustainable Farming Fund of the Ministry of Agriculture and Forestry and the ISAT Linkages Fund of the Royal Society of New Zealand, and in Italy by the Regione Siciliana, Assessorato Agricoltura e Foreste project "Coltura in vitro per la conservazione del germoplasma vegetale siciliano minacciato da erosione genetica." We thank Mr Tony Baker of the Marlborough Grapevine Improvement Group for supplying plant and pathogen samples.

Reference: Pathirana R, Carimi F, Carra A and Cheah L-H (2008) Integrating biotechnological advancements with induced mutagenesis: new opportunities for viticulture. Proceedings, International Symposium on Induced Mutations in Plants. IAEA, Vienna. 12-15 August 2008. P82.

CRYOPRESERVATION OF COCOA (*THEOBROMA CACAO* L.) SOMATIC EMBRYOS – IS SOMACLONAL VARIATION AN ISSUE?

Andy Wetten[1], Raphael Adu-Gyamfi[1], Jong-Yi Fang[2] and Carlos Rodriguez-Lopez[3]

[1]Reading University, School of Biological Sciences, Plant Science Laboratories, Whiteknights, PO Box 221, RG6 6AS, UK. [2]National Pingtung University of Science and Technology, Department of Tropical Agriculture and International Cooperation, Neipu, Pingtung, Taiwan 91201. [3]University of Wales Aberystwyth, Institute of Biological Sciences, Edward Llwyd Building, Penglais, Ceredigion SY23 3DA, UK.

Increasing demand for improved yield, quality and stress and disease resistance in cocoa (*Theobroma cacao* L.) highlight the importance of securely preserving the diversity of the species for future breeding goals. Though vital, field collections are prone to losses through climatic catastrophes and the action of pests and diseases and the recalcitrant nature of cocoa seed with regard to storage make the establishment of a cryopreserved collection of key cocoa germplasm a sensible precaution. To this end approximately 600 accessions of cocoa are being cryopreserved at Reading University through the encapsulation-dehydration of floral-derived somatic embryos (SEs) (1) and a newly-developed PVS2-based approach. These vitrification-based procedures involve the rapid cooling of the prolific secondary SEs obtained from cultured cotyledonary explants of primary SEs. With a view to maintaining the genetic fidelity of cryopreserved plant germplasm, shoot tip culture has become the propagation system of choice. In the case of cocoa *in vitro* shoot regeneration has proved problematic though rapid clonal multiplication can be achieved via somatic embryos derived from the floral tissues. Primary SEs can be induced from petal bases and staminodes and multiplication rates can be further increased through the production of secondary SEs from cotyledonary explants of primary embryos. Due to concern about somaclonal variation arising through the protracted callus phase involved in the generation of these propagules, their genetic fidelity has been tested and primary SEs have been found to exhibit a significant number of mutations (2). In this study nuclear microsatellite-based screening has been applied to each of the cocoa linkage groups in SEs sampled from sequential stages of the cryopreservation procedure (i. e. following culture, sucrose pretreatment, dehydration over silica and thawing after storage in liquid nitrogen) and compared with profiles for the donor tree. For all 48 regenerants tested in duplicate none exhibited aberrant profiles with respect to the donor tree for any of the 12 microsatellites screened. Furthermore, the use of microscopy techniques involving only minimal sample preparation (i. e. confocal and environmental scanning EM) have provided convincing evidence that post-cryo' regenerants are derived from epidermal rather than callus cells, minimising any additional mutation risk. We conclude that, within the limits of this test population, no gross chromosomal changes occurred during cryopreservation and given the recent demonstration of virus elimination as a result of somatic embryogenesis [3], secondary SEs constitute an acceptable target tissue for cocoa germplasm conservation.

1. Fang, J-Y, Wetten, A. and Hadley, P. (2004) Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. Plant Science 166: 669-675.

2. Rodriguez-Lopez, CM, Wetten, AC. and Wilkinson, MJ. (2004) Detection and quantification of in vitro-culture induced chimerism using simple sequence repeat (SSR) analysis in *Theobroma cacao*(L.). *Theor Appl Genet* 110: 157–166.

3. Quainoo AK, Wetten AC, Allainguillaume J (2008) The effectiveness of somatic embryogenesis in eliminating swollen shoot virus from infected cocoa trees. *Journal of Virological Methods* 149(1): 91-96

TEXTURAL, HISTOLOGICAL AND CELL WALL COMPOSITION CHANGES IN APPLE TISSUE AFFECTED BY FREEZING

S Chassagne-Berces[1], Fabienne Guillon[2], Marie-Françoise Devaux[2], Marc Lahaye[2], Giuseppe Pigorini[3], Christel Girault[3], Michèle Marin[1], Fernanda Fonseca1[1]

[1]UMR 782 GMPA, INRA, AgroParisTech F-78850 Thiverval-Grignon, France. [2]INRA-UR1268 Biopolymères Interactions Assemblages, F-44300 Nantes, France. [3]AIR LIQUIDE, Centre de Recherche Claude Delorme, Paris, France.

Texture is one of the primary quality attributes of fruit. Many studies described the texture modifications after freezing, but few combined such different approaches as mechanical, histological, cytological and chemical measurements, and among microscopic methods even fewer quantify the structural modifications visualized. The aim of this study was to quantify

the effect of freezing on the texture of apple fruit at the histological, microscopic cellular and biochemical levels and to relate them to the mechanical properties in order to better understand the mechanisms involved in apple tissue degradation during freezing. Studies were carried out on fresh apple (Granny Smith) and thawed apple after three different freezing protocols. The firmness of fresh and processed apple cylinders was investigated by penetrometry. Histological changes in apple tissues after thawing were evaluated from microtome sections. Macroscopic images were acquired using a CCD monochrome camera. Cell integrity was assessed by confocal laser-scanning microscope. Ice morphology in frozen samples was observed on a cold stage scanning electron microscope. In images, the structure observed can be described in terms of image texture. Morphological grey level granulometries using erosion, dilation and closing are proposed to extract size information from these textures. The global composition of the cell wall was studied using biochemical techniques. Loss of firmness following freezing varied according to the freezing protocol applied. Slow freezing rate caused severe changes in product macrostructure: fewer small cells, more large intercellular spaces and an important cell collapse. The presence of a tissue breakage was observed after immersion in liquid nitrogen. Whatever the freezing rate applied, the tonoplasts were ruptured. Modification of the neutral sugar and pectin composition of the cell wall were observed. These changes were ascribed to the apparition of ice crystals which size decreases with increasing freezing rates.

CRYOPRESERVATION OF BY2 CELL LINE – APPLICABILITY AND REPRODUCIBILITY OF DIFFERENT APPROACHES

E Heine-Dobbernack, H Kiesecker and HM Schumacher

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7b, 38124 Braunschweig, Germany

The so called BY-2 tobacco cell line has been applied worldwide for various research purposes as well as for biotechnological applications. At DSMZ a sample of the BY-2 cell line is maintained by continuous sub-culturing since 2004.

A cryopreservation method based on classical controlled rate freezing has been published by Schmale et al. 2006. At DSMZ the same method was applied successfully for the preservation of BY-2 cells although slight modifications led to better survival rates.

For the public collection at DSMZ not only preservation but also delivery is a concern. Delivery in dry ice is not working and delivery in a liquid nitrogen dry shipper is too costly. Therefore delivery in the living state is practically unavoidable.

A very simple and efficient approach for cryopreservation, published by Kobayashi et al. 2005, is based on a combination of encapsulation and controlled rate freezing. Cryopreservation of immobilized cells would allow subsequent delivery of thawed but still immobilized cells. Unfortunately it was not possible to achieve survival with the BY-2 cell line maintained at DSMZ.

To find an alternative method which allows freezing of immobilized cells a combination of encapsulation and vitrification according to Hirai and Sakai (2003) was tested to cryopreserve BY-2 cells. The method turned out to be successful. Nevertheless a disadvantage is that the alginate beads tend to disintegrate during exposure to liquid nitrogen. Therefore it was tested whether the method of Schmale could be successfully applied also with immobilized BY-2 cells. No survival could be achieved although all other parameters for freezing were kept constant. Why controlled rate freezing failed in combination with encapsulation is unclear.

Hirai D, Sakai A (2003) Plant Cell Rep. 21:961

Kobayashi T, Niino T, Kobayashi M (2005) Plant Biotechnology 22:105-112

Schmale K, Rademacher T, Fischer R, Hellwig S (2006) J. Biotechnol. 124(1): 302-311

SOMACLONAL VARIATION AFTER CRYOPRESERVATION. HOW TO DETECT IT?: AFLPS VS. RAPDS

Carmen Martín[1], Mª Teresa Cervera[2] & Mª Elena González-Benito[1]

[1] Departamento de Biología Vegetal, Universidad Politécnica de Madrid, Spain.
[2]Departamento de Sistemas y recursos Forestales. Centro de Investigación Forestal, INIA, Madrid, Spain.

Cryopreservation techniques are useful tools for genotype conservation and also as an intermediate step for several processes employed in biotechnology. Although cryopreservation was supposed to be a guarantee for genetic stability in comparison with other *in vitro* long term storage procedures, genetic variation in cryopreserved material has been found (Harding, 2004). However, the cause of these variations is usually attributed to the *in vitro* proliferation or regeneration processes instead of the cryopreservation itself (Harding, 1997). In a previous work on the genetic stability of cryopreserved shoot apices of chrysanthemum using two different methods (Martín & González-Benito, 2005), we detected a somaclonal variant, employing RAPD markers, in the cryopreserved material. This variant derived from the encapsulation-dehydration method, which is the least suspected method to increase the frequency of mutagenesis, compared with riskier methods as vitrification. Considering these results, we have studied the stability along the encapsulation-dehydration cryopreservation process (sucrose pre-culture, encapsulation, dehydration, freeze-treatment and recovery) in order to distinguish the frequency of variation for each step, if this variation was again detected. In this new study we have incorporated another technique: AFLP, which is able to assess a higher proportion of the genome. The results show clear differences between the recovery material after cryopreservation and the control samples, using both techniques. Variations in different steps along the process have also been detected.

The percentage of polymorphic bands obtained with AFLP markers was higher than with RAPD, showing that AFLP technique is more powerful and effective in discrimination genetic variation. The effectiveness of these techniques in the analysis of genetic stability is compared. Likewise, a study of the percentage of variation found in each step of the cryopreservation process has been carried out.

References:

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

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

Martín C & González-Benito ME (2005) Survival and genetic stability of *Dendranthema grandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydration. Cryobiology 51: 281-289.

CRYOPRESERVATION FOR THE SAFEGUARD OF TURKISH PEANUT GERMPLASM

EA Ozudogru[1], Y Ozden-Tokatli[1], F Gumusel[1], C Benelli[2] and M Lambardi[2]

[1]Gebze Institute of Technology, Faculty of Science, Biology Department, Plant Tissue Culture and Molecular Biology Laboratory, Istanbul cad. No: 101, 41400, Gebze, Kocaeli, Turkey. [2]IVALSA/Istituto per la Valorizzazione del Legno e delle Specie Arboree, National Research Council (CNR), via Madonna del Piano 10, 50019 Sesto Fiorentino (Firenze), Italy.

Among the edible legumes and nuts, peanut (*Arachis hypogaea* L.) seeds contain the highest vegetable oil and protein levels, thus providing an excellent source for human nutrition and animal consumption. In Turkey, as well as in the rest of the world, the American

cultivar 'Virginia' has found a dominant distribution, replacing the local germplasm of valuable organoleptic and agronomic characteristics. Hence, the aim of this study was the development of an effective cryopreservation procedure for the safe long-term preservation of this valuable germplasm, thus ensuring an access to peanut genetic diversity of strategic importance to valorise local productions and to face climatic changes. Due to the scarce quantity of seeds available from local germplasm, one-step freezing methods were first developed on 'Virginia' embryonic axes by means of procedures based on PVS2 treatment or dehydration in the sterile air flow of a laminar flow hood. Application times for both the procedures (0, 1, 1.5, 2, 2.5 or 3 h) were based on the information obtained by the moisture content evaluation of the embryonic axes at various dehydration times (i.e., 30 minute intervals up to 24 hour). The optimized procedures in terms of embryo viability and germinability (2-h PVS2 treatment or 2.5-h dehydration) were then applied to three local cultivars, namely 'NC.7', 'Com' and '7x77'. With the dehydration procedure, 100% viability and from 75% ('Com', '7x77') up to 91% ('NC.7') germinability of the cryopreserved embryos were achieved. Also the vitrification procedure produced 100% viability after the storage in liquid nitrogen, but the subsequent embryo germinability was slightly lower (50%-80%). One significant difference between the two procedures was the presence of considerable callus proliferation at the basal ends of the germinating shoots and the poor root development observed in vitrification method. On the contrary, dehydration procedure confirmed its superiority in terms of quality of the germinating seedlings.

ANDEAN FOOD TUBER CROPS: CRYOPRESERVATION AND LINKS WITH THEIR RESPONSE TO ABIOTIC STRESS

Ana Panta[1], Bart Panis[1], Dino Sanchez[2], Cecilia Ynouye[2], Jan Geuns[3], Rony Swennen[1], David Tay[2], and William Roca[2]

[1] Laboratory of Tropical Crop Improvement. Katholic University of Leuven. Kasteelpark Arenberg 13, B-3001 Leuven, Belgium. [2] International Potato Center. Apartado 1558, Lima 12, Perú. [3] Laboratory of Plant Physiology. Katholic University of Leuven. Kasteelpark Arenberg 31, 3001 Heverlee, Leuven, Belgium.

Thousands of potato landraces and two other Andean tuber crops (oca, *Oxalis tuberose*, and ulluco, *Ullucus tuberosus*) are grown by Andean farmers from southern Venezuela to north-western Argentina. These crops are extremely important in the Andean population's diet, and are staple crops for over 20 million people. These crops grown in high altitude with extreme climate, have attracted scientists' attention for their adaptation to frost and water stress conditions. The International Potato Center (CIP) maintains and studies the largest collections of genetic resources for these crops worldwide. Currently, there are 4601 potato, 457 oca and 422 ulluco landraces held in vitro in the CIP genebank in Lima. Long-term conservation using cryopreservation protocols based on PVS2 droplet vitrification is now routinely applied for potato and optimized for oca and ulluco. Since cryopreservation is strongly linked with dehydration and freezing processes, potato genotypes with known abiotic stress responses were utilized for measuring polyamine, phospholipid, and glycolipid contents in correlation with regeneration rates from cryopreservation.

More than 200 potato landraces are now successfully cryopreserved using an improved methodology that utilizes a 3-week cold acclimation of donor plants at 6°C. Data are demonstrating a positive correlation between the content of linoleic acid and cryoability. Both, oca and ulluco also showed a positive response to the droplet PVS2 vitrification method. Results of plant recovery following cryopreservation are presented in this study.