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INTRODUCTION

The global initiative by the Convention on Biological Diversity (CBD) to achieve a significant reduction in the rate of biodiversity loss by 2010 was not achieved. Of primary concern is the continuing loss of forests and associated species. New biodiversity targets for 2020 were approved at the Conference of the Parties in Nagoya in 2010 and include the need to 'prevent the extinction of known threatened species and improve and sustain their conservation status.' The symposium on 'The Preservation of Forest Species' addressed how cryopreservation can support the conservation of species and thus impact on this target. The 'Free Communications'session covered fundamental aspects of cryoprotection.

The meeting was organised by Prof. Hugh W. Pritchard, Prof. Florent Engelmann, Dr Maurizio Lambardi, Dr Jon Green and Dr Martin Schumacher.

ABSTRACTS

CRYOPRESERVATION STRATEGIES FOR FOREST TREES NATIVE TO THE SOUTH ATLANTIC FOREST OF BRAZIL

A.M. Viana[1]*, E da Costa Nunes[1], T.C. Higa[1], T. Simões[1], T. Aguiar[1], F.K. Pilatti[1], C. Rescarolli[1] E. Pedrotti[1], M.T.S. Paulilo[1] and E.E. Benson[2]

[1]*Departamento de Botânica & Departamento de Fitotecnia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, 88049-070, Brazil;
[2]Damar Research Scientists, Drum Road, Cupar Muir, Fife, Scotland, UK, KY155RJ.
**Author for correspondence*: amarna@mbox1.ufsc.br.

The Atlantic Forest is one of the most endangered ecosystems on the planet; 70% of Brazil's population lives in this territory and it is the country's most endangered biome (4). Non-sustainable practices and habitat destruction have caused a drastic reduction in the forest's biodiversity and conservation is hampered by a limited knowledge of tree life cycles and germplasm storage behaviour. The *in vitro* conservation and cryopreservation of Atlantic Forest tree species can: (i) facilitate conservation in cases where traditional methods are problematic; (ii) offset sampling from wild populations; and (iii) support the sustainable use of forest products. Criteria for applying *in vitro* conservation are: (a) dispersed, fragmented, small populations; (b) limited sexual reproduction; (c) infrequent, low seed production/viability (d) idiosyncratic and/or recalcitrant storage behaviour and (e) a restricted capacity to proliferate *in situ*. *Cedrela fissilis* (2, 3) a valuable, endangered timber tree of the

Atlantic Forest has been conserved using medium-term storage of alginate-encapsulated vegetative propagules (viability 96-100 %) and the cryogenic storage of orthodox seeds (viability 100%) (4). Thereafter, seeds of species representing medicinal, ornamental and timber trees of the Bignoniaceae, Cecropiaceae and Meliaceae were also cryopreserved resulting in post-storage germination that ranged from 33-85%. Although the seed storage characteristics of certain south Atlantic Forest tree species are classified as orthodox they can exhibit idiosyncratic behaviour regarding their longevity in conventional seed banks. This is the case for the endangered tree *Tabebuia heptaphylla*, the seeds of which have been placed in cryostorage and recovered achieving a germination range of 54-67% (1). This presentation considers the potential role of cryostorage and *in vitro* conservation in safeguarding the genetic resources of endangered trees native to Brazil's South Atlantic Forest and highlights some of the technical challenges and logistical strategies involved in supporting cryobank end users and field conservationists.

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COMPARATIVE THERMAL ANALYSIS OF SITKA SPRUCE (*Picea sitchensis*) SOMATIC EMBRYOS AND SHOOT MERISTEMS EXPOSED TO AN ENCAPSULATION-DEHYDRATION CRYOPRESERVATION PROTOCOL

Samantha Gale[1], Erica E Benson[2] and Keith Harding[2]

[1]*(current address) Cawthron Institute, 98 Halifax Street East, Private Bag 2, Nelson 7042, New Zealand;

[2] Damar Research Scientists, Damar, Drum Road, Cuparmuir, Fife, KY15 5RJ, UK.

The micropropagation of elite clonal genotypes is an important part of the modern forestry industry; cryopreservation has the advantage of conserving *in vitro* juvenile tissues whilst progeny are being tested for the selection of desirable ('Plus tree') characteristics (4). Phenotype validation of Sitka Spruce can take up to 6 years for maturing seedlings and cryostorage allows the preservation of cultures whilst field-testing is undertaken. Sitka Spruce tissue culture can involve two different morphogenetic pathways: somatic embryogenesis or shoot micropropagation. Although both are effective, only somatic embryos have thus far proved amenable to cryopreservation (1, 2). *Picea sitchensis* somatic embryos survive and develop after cryopreservation (1, 4) whereas *in vitro* shoot meristems are incapable of sustaining recovery following exposure to liquid nitrogen. The objective of this study was to ascertain if the differential responses of Sitka Spruce somatic embryos and shoot meristems can be attributed to cryogenic (biophysical) factors by using differential scanning calorimetry

(DSC) to profile the responses of each type of germplasm. The biophysical basis of disparate survival responses between somatic embryos and shoot meristems of Sitka Spruce was investigated by constructing thermal profiles of different pretreatments at each stage of the encapsulation/dehydration protocol. Sodium-alginate encapsulated somatic embryos and shoot meristems showed similar thermal profiles throughout progressive steps of dehydration and desiccation; glass stability correlated with viability (triphenyl tetrazolium chloride [TTC] vital staining) and recovery (resumption of regrowth and development) of somatic embryos (1, 2, 4). Although thermal profiles also confirmed glass stabilization on cooling and rewarming in Sitka Spruce shoot meristems and some initial survival was indicated, their recovery was not sustained. This is indicative of delayed-onset cryopreservation induced cell death (3). As ice nucleation and devitrification were eliminated as causal factors in cryoinjury this study implies that other non-biophysical parameters are responsible for the differential survival responses between the two types of Sitka Spruce germplasm investigated.

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MORPHOMETRIC AND GENETIC CHANGES IN CHRYSANTHEMUM PLANTS DERIVED FROM CRYOPRESERVATION

Y. Cifuentes, J.B. Martínez-Laborde, C. Martin and M.E. González-Benito *

Departamento de Biología Vegetal, Universidad Politécnica de Madrid, Madrid 28040, Spain.
*E-mail: me.gonzalezbenito@upm.es

There is some concern about the possible occurrence of somaclonal variation induced by cryopreservation techniques in plants. Morphological variations have been reported after *in vitro* culture and after cryopreservation. Recent genetic stability studies in cryopreserved chrysanthemum shoot apices have revealed that variation in the regenerated cryopreserved material is not a rare phenomenon and also that the instability can be observed in regenerants from certain pre-culture steps (1). Sixteen plants derived from several steps of an encapsulation-dehydration protocol were potted, grown in a green house for two years and used to study their morphological characters and genetic profiles by RAPD markers. Leaf (six), stem (one) and inflorescence/flowers (sixteen) morphometric characters were studied and twelve RAPD primers were employed. Two of the cryopreserved samples showed morphometric differences with respect to other samples according to the principal component and cluster analyses performed, and differences in the RAPD profiles were found in some samples, although these two types of variations were not related.

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FORESTS IN A TANK! THE OPTIONS OF CRYOPRESERVATION

A. De Carlo[1*], A.E. Ozudogru[1], M. Capuana[2], F. Engelmann[3,4] and M. Lambardi[1]

[1] IVALSA/Trees and Timber Institute, National Research Council (CNR), 50019 Sesto Fiorentino (Firenze), Italy. *corresponding author.
[2] IGV/Institute of Plant Genetics, National Research Council (CNR), 50019 Sesto Fiorentino(Firenze), Italy.
[3] IRD, 34394 Montpellier cedex 5, France.
[4] Bioversity International, 00057 Maccarese (Fiumicino), Rome, Italy.

Cryopreservation of *in vitro* cultured organs and tissues offers an important option for long-term conservation of woody plant germplasm. For vegetatively-propagated forest species, storage in liquid nitrogen (-196°C) of shoot tips represents a complementary approach to in-field conservation of clonal collections, reducing the need for large land areas and periodic monitoring of trees to prevent the spread of pest and diseases. In seed-propagated species, cryopreservation of embryogenic lines can play an important role for the conservation of species with non-orthodox seeds and of elite trees. Moreover, the maintenance of valuable embryogenic lines utilized in bioengineering is facilitated, thus avoiding the risks of contamination and somaclonal variation due to repeated subculturing. This report presents the various techniques available for cryopreservation of shoot tips and embryogenic lines of forest species, such as PVS-based vitrification, encapsulation-vitrification and slow cooling. Examples from the studies carried out at the CNR-IVALSA Institute of Florence on poplar [*Populus* spp.] (2, 5), horse chestnut [*Aesculus hippocastanum*] (1), ash [*Fraxinus excelsior*] (3) and redwood [*Sequoia sempervirens*] (4) are reported and critically analysed.

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CRYOPRESERVATION OF QUINCE (*Cydonia obonga* MILL.)

P.T. Lynch[1], A. Siddika [1], A. Mehra[1], M. Lambardi [2] and C. Benelli[2]

[1]Biological Sciences Research Group, University of Derby, Kedleston Road, Derby DE22 1GB, UK
[2]National Research Council of Italy (CNR), Ivalsa / Instuto per la Valorizzazione del Legno e delle Specie Arboree (Trees and Timber Institute) Polo Scientifico, via Madonna del Piano, 50019 Sesto Fiorentino (Firenze), Italy.

Quince (*Cydonia oblonga* Mill) is a temperate fruit species. In the past quince fruits were widely eaten and also used to make marmalades, jellies and liqueurs. In Europe quince has been used as a rootstock for pears, but this has declined due to problems with graft incompatibility and disease susceptibility. However, as a result of its use in Chinese medicine and strong fragrance its greatest potential for the future utilisation of quince is in the

pharmaceutical and food industries. Conservation of both improved varieties and wild type of this currently under utilised crop are essential if its future potential is to be realised. Shoots and nodal sections of *in vitro* shoot cultures of quince have been successfully cryopreserved using encapsulation-osmoprotection/dehydration, vitrification or encapsulation-dehydration protocols. The highest frequency of regrowth after rewarming (80%) was obtained from cold hardened shoots following encapsulation-osmoprotection/dehydration or vitrification protocols. Callus growth on rewarmed nodal sections was reduced by incubation on auxin free medium. The morphology of cryopreserved and non-cryopreserved shoots was comparable.

THE POTENTIAL OF DORMANT BUD CRYOPRESERVATION TO CONTRIBUTE TO THE CONSERVATION OF FOREST SPECIES

Brian Grout [1] and Erik Dahl Kjær[2]

[1]Department of Agriculture and Ecology, University of Copenhagen, Højbakkegård Allé 13, 2630 Taastrup, Denmark
[2]Department of Forest and Landscape, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark

Cryopreservation provides a valuable conservation tool for forest genetic resources, in that it provides both security for rescued germplasm and effectively stops the evolutionary process. In this respect it can be seen as complementary to evolutionary conservation, where the evolutionary process itself is an important conservation goal, as long as it supports local adaptation of target species. Static preservation, such as cryopreservation, in a forest tree conservation programme can provide an indeterminate window of time in which to handle a large number of genotypes, from rescue to repatriation in restored ecosystems. It can also provide the time, and phasing of resources, to allow for their establishment as seed sources for planting programmes, e.g. in integrated use and conservation programmes.

The effective population size of threatened tree populations (Ne) may be small because of, for example, reproductive fragmentation resulting from scattered distribution or the breakdown of the pollination chain in disturbed ecosystems. Collection, grafting and the establishment and management of *ex situ* clonal archives can be used in order to mitigate against the effects of such fragmentation, counteract the effect of a small Ne and rescue germplasm of highly threatened trees. However, such programmes with field plantings on any significant scale are heavily resource-consuming and therefore difficult to implement simultaneously for a large number of species. A complicating, practical factor can be that species that may hybridise have to be kept separately. Therefore, any simple approach to preservation is of interest if it has the potential to buy time for investigation and resource allocation, is relatively low in terms of resource demand and can be used to secure effective genetic security.

If the reproductive capacity of the target species has been maintained, and it produces orthodox seed, then frozen storage at -20°C is ideal. If the seed is recalcitrant then rescue of zygotic embryos, or their apices, can be employed, followed by cryopreservation at -196°C. This will, necessarily, involve a significant amount of *in vitro* culture and will be resource-demanding. These demands can limit, for example, the number of individual trees that it is feasible to conserve and the number of institutions capable of undertaking the conservation.

As an alternative, cryopreservation of winter-dormant buds for the *ex situ* conservation of woody species provides significant resource-based advantages, for field-harvested material is taken directly into the preservation protocol without additional pre-treatment and, after

recovery, direct grafting replaces regrowth via *in vitro* culture. The technique has the benefits of high genetic security and allows an easy re-mobilisation of the genotypes for their use, demonstrating a significant potential for the conservation of forest species. Each tree considered for preservation can be considered as a clonal collection of buds and the target for conservation is a small segment of branch bearing a single bud, easily collected.

Studies with *Malus*, and other fruit trees, indicate it is necessary that winter-dormant buds are gathered at a point where natural hardiness will ensure ecodormancy. This raises an interesting question as to whether dormancy imposed by arid conditions might provide the necessary amount, and quality, of cellular hardening. No additional pretreatment is applied to the harvested material, and variation in post-cryopreservation survival between collection years must be expected. Growing season conditions will also have an effect as they influence the physiological condition of the trees, their yield and their response to winter hardening conditions

The critical steps are concerned with the induced reduction, and eventual restoration, of cellular water content, as in any other cryopreservation protocol An initial, prolonged incubation at -4°C, during which some tissue water freezes, providing an opportunity for tissue water content to be reduced by both evaporative loss and cryodehydration. Subsequent slow cooling (1°C/h) to -30°C provides an extended opportunity for further cellular cryodehydration and relocation of water prior to direct immersion in liquid nitrogen.

Ongoing studies are aimed both at understanding the mechanism that influence survival of dormant bud cryopreservation to improve success, and achieving the transfer of the system to forest trees. The nature of the required dormancy for successful entire bud cryopreservation is also an urgent topic for further investigation

RAPID THAWING (>5°C/MIN) OF CRYOPRESERVED DORMANT BUDS OF *Malus* CAN SIGNIFICANTLY REDUCE RECOVERY OF GRAFTED EXPLANTS

Brian Grout [1], Christina Vogiatzi[1] and Andy Wetten [2]

[1]Department of Agriculture and Ecology, University of Copenhagen, Højbakkegård Allé 13, 2630 Taastrup, Denmark
[2] School of Biological Sciences, University of Reading, Harborne Building 132, RG6 6AS, UK

Cryopreservation of winter dormant buds is of significant value for the long-term, *ex situ* conservation of fruit crops as it is effective, technically simple and avoids resource-costly *in vitro* technology. Recovery of viable buds is by grafting a thawed explant comprising of a bud and associated cortical tissues, normally following relatively slow thawing from liquid nitrogen (>4°C/min). Faster thawing rates, investigated in an attempt to improve survival of *Malus* buds, had a disadvantageous effect on survival, very quickly reducing bud outgrowth to zero for many cultivars. Typically, the cortical tissues made a successful graft union but the buds did not develop. This may be due to accumulation of ice crystals disrupting physical connections between the bud and the graft issues, an explanation supported by ultrastructural investigation of the bud tissues, previous studies with *Ribes* and reports from the literature.

PECULIARITIES OF FUNCTIONAL STATE MODULATION OF GENETIC APPARATUS OF FETAL LIVER CELLS WITH STEMNESS CHARACTERISTICS AFTER CRYOPRESERVATION

A.N. Goltsev, E.D. Lutsenko, A.Yu. Dimitrov, E.Ye. Yampolskaya, T.G. Dubrava, L.V. Ostankova, M.V. Ostankov, O.V. Chelombitko, N.N. Babenko and Yu. A. Gaevskaya.

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkov, Ukraine, e-mail:cryopato@rambler.ru

Fetal liver cells (FLCs) are now important components of the widely used cell and tissue therapy. They are efficiently used for many pathologies, including treatment of autoimmune diseases. Demand for FLCs is determined by the necessity of correction at a given type of immune status and hemopoiesis disease. Hemopoietic (HSCs) and mesenchymal stem cells (MSCs) in heterogenic populations of FLCs are the main components manifesting such an activity. Re-population potential of HSCs is implemented with gene expression and production of mRNA, accordingly, of such transcriptional factors as Oct-4, Rex-1, Nanog and GATA-2. In implementation of MSCs immunomodulating activity *ido* gene takes an active part. Its expression is accompanied by production of a potentially unique enzyme, indoleamine 2, 3-dioxygenase (IDO), capable of activating suppressor link immunity.

In clinical practice, FLCs are used from different gestation terms, which characteristics such as expression repertoire and level of phenotypic markers, re-population potential, spectrum of produced mediators are significantly changed. Herewith it is known that the character of the cryopreservation effect on the bio-object is determined by its initial status. Actually, it is shown that the same cryopreservation regimens change differently the hemopoietic and immunomodulating potentials of FLCs of various gestation terms. The research aim is to comparatively assess the expression level of *gata-2* and *ido* genes in FLCs of different gestation terms after cryopreservation.

The research objects were unfractioned FLCs of CBA/H mice of the 14th (FLC-14) and 18th (FLC-18) gestation days and, derived from them, cell fractions with HSCs and MSCs. The FLCs were disintegrated with Potter homogenizer in Medium 199, with 10% fetal bovine serum and 2% sodium citrate (handling medium), followed by filtration through a capronic filter. The fractions of HSC-like and MSC-like cells of fetal liver were isolated with a magnetic sorter (BDTM Imagnet). To derive HSC-like fractions, the method used relied on negative selection with Biotinylated Mouse Lineage Depletion Cocktail (Lin^-) and Streptovidin Magnetic Particles-DM and the following positive selection with using CD117. MSC-like cells were isolated by the method of positive selection with CD105 MicroBeads (Miltenyi Biotec). Phenotypic characteristics of FLCs were assessed with flow cytofluorimeter FACS Calibur (Becton Dickinson, USA) on expression of surface membranous markers with a kit of monoclonal anti-murine antibodies to HSC-like: CD34 (PE)/ CD38 (FITC); MSC-like: CD73 (FITC)/CD105 (PE) (BD). The cryopreserving solution was prepared using a base of handling medium with 20% dimethyl sulphoxide (DMSO). Cryopreserving solution, in 1:1 ratio at room temperature (final concentration of cryoprotectant of 10%), was added into the suspension of FLCs derived in handling medium and stemness-like cell fractions isolated with the magnetic sorter. Cell exposure in cryopreserving solution was carried-out for 10 min at the same temperature.

Whole suspension of FLCs was frozen in plastic ampoules (1.8 ml Nunc, Germany) with a cell concentration of $5x10^6$ cells/ml; isolated cell fractions were placed in vials (minitubes d - 0.25 mm, 15 μl, Germany) at a $2x10^6$ cells/ml concentration using the device UOP6 of

IPC&C of NASU production, according to two-stage program.

Expression of *gata-2* and *ido* genes in unfractioned FLCs and isolated fractions Lin^-117^+ and $CD73^+105^+$ cells was determined by RT-PCR; amplification products were detected with Agilent 2100 bioanalyzer (USA). Primers of the studied genes were designed based on information on the database of GenBank website (NCBI BLAST, USA): *gata2* - NM_008090.5 (fragment length 272), *ido* - NM_008324.1 (fragment length 342) and synthesized in CJSC Medbioservis (Kiev). A comparison of transcript number of the studied targets was carried-out on the basis of relative quantitative assessment of amplification products. Dilution logarithm (lg) of cDNA was the index of expression level of the studied genes. The results were normalized as for the index of gene expression beta-actin (housekeeping gene) (NM_007393.3).

The findings demonstrated a significant decrease in expression level of *gata-2* and *ido* genes with the prolongation of gestation terms. After cryopreservation, the expression level of the studied genes in FLC-14 was reduced; meanwhile in FLC-18 expression level significantly increased if compared with native FLC-14. The increase of expression level of *ido* gene in cryopreserved FLC-18 was greater than in *gata-2*.

The obtained results may testify to re-programming under the effects of cryopreservation via an epigenomic cascade affecting functioning in stemness-like FLCs with activation of some transcription factors. This fact may explain our previous observation of an increase of immunocorrective and hemopoietic activities of FLCs of the late gestation terms.

TECHNICAL PROBLEMS EVALUATING CRYOPRESERVATION SUCCESS IN BASIC RESEARCH

E. Heine-Dobbernack, L. Vaas and H.M. Schumacher

Leibniz Institute DSMZ – Deutsche Sammlung von Mikroorgansimen und Zellkulturen (DSMZ) GmbH, 38124 Braunschweig, Germany

To develop a cryopreservation method for long term preservation of plants, regrowth of intact shoots is the ultimate criterion for cryopreservation success. For the investigation of the influence of a single physiological trait on cryopreservation success a more precise and more sensitive criterion may be needed. Undifferentiated cell cultures may be a tool to achieve a better correlation of cell survival with a physiological trait than shoots. All the methods of viability testing have certain pitfalls and cannot be used under certain conditions. The FDA test does not easily give quantitative results. Widholm et al. had shown that the results obtained for the TTC test are influenced by the osmotic value of the culture medium. For such tests cell damage measured and regrowth does not always correlate. Quantitative measurement of dry weight accumulation during regrowth is an alternative to obtain quantitative results for cryopreservation success, cell survival or cell damage but even this approach has to be applied with care.

At DSMZ we tried to investigate the influence of mechanisms of drought and salt tolerance on cryopreservation success. In a first approach, the comparison of cell lines derived from plant cultivars with different osmotic tolerance turned out to be complicated because the cell cultures of these cultivars consisted of cells differing not only in osmotic tolerance but also in size, shape and growth characteristics. In regrowth experiments, it was impossible to inoculate a plate with an equal number of cells on the basis of fresh weight. Furthermore after thawing the cells cultures differed in regrowth rate depending on cell damage or number of surviving cells. Using a transgenic approach solved the problem of comparing cells of

different size but the problem of comparing cell cultures with different growth rates depending on the degree of damage persisted. Growth of a cell line is always a logarithmic process and therefore cell harvest for determination of accumulated dry weight after a standardized regrowth period may result in measurement under nonlinear conditions either in the early lag or in the late stationary phase. Nevertheless measurement of dry weight accumulation is probably the best way to characterize cryopreservation success for undifferentiated cells provided the specific weight and size of cells and the characteristics of the regrowth curve is taken into account. To measure an exact relationship between the number of cells surviving cryopreservation and accumulated dry weight, recording of the complete growth curve is necessary. A workable approximation can be obtained by "3 point measurement".

DESICCATION TOLERANCE AND STORAGE RESPONSE OF MATURE TEA (*Camellia sinensis* L.) SEED FROM THREE SITES IN YUNNAN, CHINA

Hongying Chen [1,2,3], Hugh W. Pritchard [1,2], Charlotte E. Seal [2], Jayanthi Nadarajan [2], Weiqi Li [1], Shixiong Yang [1] and Ilse Kranner [2]

[1] Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei Road, Kunming 650201, China.
[2] Seed Conservation Department, Royal Botanic Gardens, Kew, Wakehurst Place, West Sussex RH17 6TN, United Kingdom
[3] Graduate University of the Chinese Academy of Sciences, 19A Yuquanlu, Beijing, 100049, China

The maximal potential desiccation tolerance (MPDT) of tea (*Camellia sinensis*) seeds has been the subject of scientific debate for decades. Here we assessed the ability of tea seeds from three sites in Yunnan Province, Southwest China to germinate after desiccation. Collections were made from all three sites in two separate years. Tolerance of drying was greatest for seeds of Kunming > Puer > Lincang. Kunming seeds had a mid-point for viability loss on dehydration at a moisture content equivalent to c. 50% RH, based on the water sorption isotherm for the material. MPDT in Puer seeds was c. 70% RH. However, for Lincang seeds this level of tolerance was observed only when the pro-oxidant hydrogen peroxide (H_2O_2) was used as a short-term post-drying treatment. A similar simulatory role for exogenously applied H_2O_2 in post-desiccation germination has been observed previously in *Castanea sativa*; it is presumed that such treatment mimics the transient ROS burst at the onset of desiccation, serving as a key signalling component of regeneration and growth (3). Two predictive models for seed storage responses of woody species were applied to tea seeds: the long (many months) dry season after seed shedding (2) and the presence of a relatively 'thin' (based on mass) seed coat (1). Both validated the observations of MPDT in tea seeds being greater than those of recalcitrant seeds. Although the seeds survived drying to MCs equivalent to c. 70% RH, death occurred within one month of storage at -20°C. Possible causes of such viability loss include devitrification of the cytoplasmic glass and physical / structural rearrangements as a result of lipid transitions, as shown to occur at this temperature by differential scanning calorimetry.

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UNDERSTANDING THE CRYOPROTECTIVE ACTION OF GLYCEROL

J.J. Towey[1], A.K. Soper[2] and L. Dougan[1]
[1] School of Physics and Astronomy, University of Leeds, Leeds, LS2 9JT, United Kingdom
[2] ISIS Facility, Rutherford Appleton Laboratory, Chilton, Didcot, Oxon, OX11 OQX, United Kingdom

Glycerol is a sugar alcohol and has been of great scientific interest for many years. This attention is due, in part, to its ability to act as a cryoprotective agent (CPA). A long-standing hypothesis for the action of CPAs is that they modify the structure of the water around biological molecules. A necessary first step toward determining the stabilising mechanism of CPAs (such as glycerol) is to test this hypothesis by directly examining glycerol's impact on water structure. To this end, we have completed a series of neutron diffraction experiments combined with computational modelling to obtain atomistic level structural information on aqueous glycerol across the concentration range. Our studies of a dilute glycerol solution demonstrate that, to the first neighbour level, water-water interactions are not perturbed by the addition of glycerol. There is, however, an inward shift of the second neighbour distance. This movement indicates that the tetrahedral structure of water is modified by the addition of glycerol. Interestingly, evidence is found for more glycerol monomers than would be expected in the solution. This prevalence of isolated glycerol molecules indicates a preference for glycerol-water hydrogen bonding leading to a very well mixed solution. This mixing may diminish the ability of water to form an extended hydrogen bonded network. Our results demonstrate that efforts to explain the mechanism of cryoprotection should focus on not just local water structure but the extended hydrogen bonded network of the system.

TOWARDS THE CONSERVATION OF CYCADS THROUGH CRYOPRESERVATION

Jayanthi Nadarajan[1], Satya R Dandugula[2] and Hugh W. Pritchard[1]

[1]Seed Conservation Department, Royal Botanic Gardens Kew, Wakehurst Place, Ardingly, West Sussex RH17 6TN, UK
[2]Faculty of Creative Arts, Technologies & Science, University of Bedfordshire, Luton LU1 3JU, UK

The cycads are remnants of the most ancient group of higher plants. Cycads have been referred to as living fossils as this group of plants are acknowledged as the oldest seed plants still in existence with origins dating back to the late Carboniferous period 300 million years ago. The cycad flora consists of c. 300 species and subspecies grouped into three families, Cycadaceae, Stangeriaceae and Zamiaceae. These families are distributed throughout the warmer areas of North and South America, Africa, Asia and Australia. Many species exist as relatively small isolated populations, often in transformed habitats. Cycad populations have declined in nature and face risk of extinction because of habitat destruction and removal of

mature plant specimens from the wild by plant traders. Twenty three percent of the cycad species are either critically endangered or endangered, and 15% are vulnerable and the other 62 % are in the least concern or near threatened category (IUCN, 2010). All cycads are listed in either Appendix I or II of the Convention on International Trade in Endangered Species (CITES; http://www.cites.org/). Cycads need urgent conservation action, through both *in situ* and *ex situ* interventions. Little is known about the desiccation tolerance of cycad seeds. Partial drying combined with cryopreservation may be the only viable long-term *ex situ* conservation method for this group of plants.

The seed biology of three cycad species was studied: *Cycas revoluta*, *Dioon edule* and *Zamia furfuracea*. The experimental strategies involved assessment of seed and embryo desiccation sensitivity, *in vitro* establishment of cycad embryos and assessing different cryopreservation protocols. Differential scanning calorimeter (DSC) was used to elucidate the critical moisture content for successful cryopreservation of the embryos. Seeds of all three species exhibited sensitivity to desiccation. Whilst isolated embryos of *D. edule* and *Z. furfuraceae* had LD50s for recovery growth *in vitro* of c. 20 % moisture content, *C. revoluta* embryos were more desiccation tolerant. Current studies are assessing the effects of cryoprotection on the cryopreservation of *C. revoluta* embryos.

Reference:

IUCN (2010) *Red List of Threatened Species*. http://www.iucnredlist.org/

EFFECT OF THE SUCCESSIVE STEPS OF A CRYOPRESERVATION PROTOCOL ON STRUCTURAL INTEGRITY OF *Rubia akane* HAIRY ROOTS

M. Salma[1], I. Sylvestre[1], M. Colin[1], J. Escoute[2], M. Lartaud[2], J.L. Verdeil[2] and F. Engelmann[1],

[1]IRD, UMR DIADE, Montpellier, France
[2]CIRAD-BIOS, UMR DAP, PHIV-MRI, Montpellier, France
[3]Bioversity International, Maccarese, Rome, Italy

In this work we studied the impact of the successive steps of a cryopreservation protocol developed for *R. akane* hairy roots on tissue and cell structural integrity, using classical histology, with qualitative and quantitative observations, and real time miscroscopy (RTM). Pretreatment in sucrose enriched media (0.3/0.5 M) induced the largest plasmolysis, of approximately 40% compared with their original state. Treatment with loading solution C4 induced an additional, albeit lower, plasmolysis (0-27%). Treatment with vitrification solution B5 did not induce any additional plasmolysis. Treatment with unloading solution brought cells back to a state close to their original plasmolysis and revealed differences between the categories of cells observed. Plasmolysis was lower (9-17%) in cortical cells, whereas it was higher in pericycle and endoderm cells. This work confirmed the interest of image analysis techniques, which allow quantification of precise information on the qualitative observations performed with classical histology. The preliminary trials performed with RTM showed that the root sections treated displayed a low contraction percentage (3.9-4.6%), in response to the injection of vitrification solutions, without any difference between the experimental conditions tested. However, it seems interesting to use RTM on individual cells or groups of cells to compare the dynamics of reaction to the injection of loading and vitrification solutions of the different categories of cells studied in our work, viz. cortex, endoderm and pericycle cells.