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FUNDAMENTAL ASPECTS OF PLANT CRYOPRESERVATION

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For further details of COST Action 871 on the 'Cryopreservation of crop species in Europe' see http://www.biw.kuleuven.be/dtp/tro/cost871/Home.htm.

Abstracts

EXPLORING CRYOPRESERVATION STRESS, TOLERANCE AND RECALCITRANCE IN NATURE'S LABORATORY

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COST Action 871 'CRYOPLANET' comprises two Working Groups: (WG1) fundamental aspects of cryopreservation, cryoprotection and genetic stability and (WG2) technology application and validation of plant cryopreservation. The project COST Action 871 proposal explains: "*Currently for each species and tissue type, a cryopreservation protocol needs to be developed/adapted to the natural, cold, freezing and desiccation of the species, explant size and type*". The development of new and improved cryostorage protocols and their optimization and efficient application across a diverse spectrum of genetic resources thus requires connectivity between WG1 and WG2. In particular, feedback of fundamental knowledge can assist the cryopreservation of storage recalcitrant species and germplasm types. With a view to supporting the interplay of activities between these Working Groups, this presentation explores the sentiment of Harry Meryman[1], who suggested, that in the absence of a complete understanding of basic events, it may be fruitful, to build upon what nature has already discovered, rather than taking trial and error approaches to cryobiological problem solving. Using the concept (2) of '*Nature's Laboratory*' the problem of cryostorage recalcitrance will thus be considered in terms of seed, tissue culture and cryostorage recalcitrance. How these different storage practices interface (1) will also be examined with respect to: (a) applying fundamental knowledge to help elucidate and circumvent different types of recalcitrant behaviour and (b) improving cryobank operations. Taking a more holistic approach to addressing recalcitrance may be particularly important when tissue culture manipulations are used to overcome problematic seed storage behaviour. In this

respect, the identification of extrinsic and intrinsic stress and tolerance factors will also be explored as a means improving cryostorage outcomes.

1. Benson EE (2008) *Critical Reviews in Plant Science* **27**, 141-219.
2. Meryman H (2004) Foreword to "*Life in the Frozen State*" Fuller BJ, Lane N, Benson EE (eds), CRC Press, Boca Raton, Florida, USA.

FUNDAMENTAL ASPECTS AND ECONOMIC NEEDS OF PLANT CELL CRYOPRESERVATION

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In the 70s efforts have been made for the production of secondary metabolites by dedifferentiated plant cell cultures. For a long time the production of shikonin remained to be the only case commercial application and plant cell line fermentation was considered to be too costly. In the meantime without causing sensation different commercial applications have been realized and furthermore the production of recombinant proteins came into view.

For different reasons the safe long term preservation of the production source is essential for any commercial application: 1 it secures the investments for the production facilities of the producer of raw material 2 it secures the investment for product development for the dealer of a specific brand 3 it is an essential requirement of regulatory aspects like approval and patent protection.

Although it is an economic need even basic aspects of cryopreservation are not solved to a level which guarantees the safe application of this technology in any case. It is still not predictable if a cell line can be cryopreserved or not. Even cell lines of closely related species require different parameter. Also physiological traits cannot always be used to make predictions about the applicability of cryopreservation. Examples are given for different cell lines of the Solanaceae.

Another problem is the reproducibility of results. Even the same cell line (demonstrated by BY2) reacts different in different laboratories.

Finally systematic studies about the storage temperature are missing. While cryopreserved animal cell lines can be easily transported in dry ice, this seems to be impossible for plant cells. This causes problems for patent deposition. Even if a cryopreservation method has been worked out the problems of transporting the cultures or reproducing the same method in a different laboratory remain.

REACTIVE OXYGEN SPECIES PRODUCTION FROM THE INITIAL STAGES OF CRYOPRESERVING *Castanea sativa* EMBRYONIC AXES

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Mechanisms of extracellular superoxide ($O_2^{\bullet-}$) production were studied in seeds of the sweet chestnut (*Castanea sativa*) in response to embryonic axis excision and desiccation. Axis excision led to an immediate burst of $O_2^{\bullet-}$ production, demonstrated by a colorimetric assay using epinephrine, electron spin resonance and staining with nitroblue tetrazolium. Isolated axes subjected to variable levels of desiccation stress showed a decrease in viability and vigour. Superoxide production displayed a bell-shaped pattern in response to increasing desiccation stress. The production of $O_2^{\bullet-}$ was sensitive to sodium azide, and could be stimulated by H_2O_2 and NADH, suggesting that the apoplastic enzymes involved in $O_2^{\bullet-}$production include peroxidases. Cell wall fractionation of embryonic axes revealed that the peroxidases potentially involved in $O_2^{\bullet-}$ production were mainly bound to the cell wall by strong electrostatic forces. Hypothesising that the stimulated production of $O_2^{\bullet-}$ during non-critical seed desiccation was beneficial, mildly desiccated seeds and axes were treated with various concentrations of hydrogen peroxide (H_2O_2). Improvements in total germination were seen beyond 8 d and embryonic axes showed greater vigour than those just treated with H_2O. Initial results with *Castanea sativa* suggest that H_2O_2 may be a useful tool in the conservation of recalcitrant germplasm.

UNRAVELLING SUGAR PRECULTURE: NEW NUTRIENTS, EXCISION AND OSMOTIC STRESS

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We (KU Leuven) host the *Musa* Bioversity International collection as an *in vitro* and a cryopreserved collection. We developed a cryopreservation protocol based on an osmotic pretreatment with 0.4M sucrose. The osmotic pretreatment with 0.4M sucrose consists of different important aspects. To initiate the pretreatment, the meristems are excised and put on a new medium containing 0,4M sucrose. This pretreatment is thus a mild stress consisting of wounding stress provoked by excision and osmotic stress provoked by the high sucrose content. Via a kinetic proteome study, we discovered that the acclimation triggered by the pretreatment consisted of different aspects showing different responses and we wanted hence to calculate the impact of each aspect. Therefore we have set up additional cryopreservation experiments over time with a good acclimating variety. The average regeneration rate of the control (no pretreatment) was 30,6%. The pretreatment (no osmotic stress, no new nutrients and no excision) resulted in a regeneration rate of 19,6% and 0% after respectively 4 and 14 days. This illustrates that nutrient depletion has a seriously negative influence on the regeneration rate. The pretreatment (no osmotic stress, no new nutrients and excision stress) resulted in a regeneration rate of 47,2 % and 1,7% after respectively 4 and 14 days. This illustrates that the excision triggers an acclimation and confirms that nutrient depletion has a negative influence on the regeneration rate. The pretreatment (osmotic stress, no new nutrients and no excision) resulted in a regeneration rate of 3,3% and 8,3% after respectively 4 and 14 days. This illustrates that osmotic stress is not beneficial in absence of excision and confirms that excision has a beneficial effect and that nutrient depletion has a negative influence on the regeneration rate. The pretreatment (osmotic stress, no new nutrients and

excision) resulted in a regeneration rate of 11,7% and 23,3% after respectively 4 and 14 days. This confirms that excision has a beneficial effect and that osmotic stress is not beneficial in absence of new nutrients. The reference pretreatment (osmotic stress, new nutrients and excision stress) resulted in a regeneration rate of 38,3 % and 46,0% after respectively 4 and 14 days. This confirms that the osmotic pretreatment is only beneficial when accompanied by new nutrients and excision.

We conclude that excision is essential to (i) trigger a specific stress response and hence acclimation and (ii) to take up fresh nutrients and sucrose. An additional kinetic experiment with a good and a bad responding variety confirms that excision is essential and that an osmotic sucrose pretreatment is not essential for a good acclimating variety but it reduces variability and it increases the regeneration rate of a bad acclimating variety.

CRYOPRESERVATION OF *IN VITRO* SHOOT TIPS OF *Fragaria* x *ananassa* DUCH.

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The development and wider availability of an effective, reliable cryopreservation method with concomitant explant regeneration without incurring genetic change are fundamental requirements for *in vitro* germplasm conservation. Although, a number of disparate protocols for the successful cryopreservation of *in vitro* strawberry shoot tips of *Fragaria* x *ananassa* Duch. have been reported to include: controlled rate freezing, PVS2, droplet freezing, encapsulation-dehydration and modified encapsulation-dehydration. Determinations of the relatively efficacy of the reported protocols is compounded making protocol comparisons problematic by the use of a variety of different strawberry cultivars with a diverse range of *in vitro* culture regimes. While, these strawberry genotype-specific cryopreservation procedures are effective, they also provide essential information to develop a more generic protocol for wider *in vitro* germplasm applications.

To determine the most practical and applicable procedure to cryopreserve strawberry *in vitro* derived meristems, an evaluation of the effectiveness of six protocols: controlled rate freezing, PVS2, encapsulation-PVS2, droplet freezing encapsulation-dehydration and modified encapsulation-dehydration was assessed. The cold acclimation of *in vitro* strawberry stock cultures has been reported by several of authors to significantly enhance the post-thaw recovery of cryopreserved meristems. However, despite this methodological improvement, there is no general uniformity in the approach to achieve cold acclimation of *in vitro* material. It is important to explicate the requirement for this critical conditioning stage. Therefore, as part of this assessment, a direct comparison of the post-thaw response for each cryopreservation protocol between the non-cold hardened and cold-hardened meristems, which were derived from *in vitro* cultures that were maintained in the dark for two weeks at 4°C. Throughout this evaluation of the six cryopreservation protocols, the same standard method for the post-thaw culture of the meristems of the day neutral strawberry cultivar Selva was used.

Following cryopreservation, the post-thaw meristem regrowth after 4 weeks, defined as both recovery and regeneration, was observed from several protocols as follows: 96 ± 4.0% with PVS2 based vitrification; 32 ± 12% with encapsulation-dehydration and 33 ± 4.4%

following the modified encapsulation-dehydration. In contrast to these protocols, post-thaw regrowth was not observed after controlled rate freezing, droplet freezing or encapsulation-PVS2. Overall, regarding the conditioning factor, post-thaw regrowth was consistently greater from meristems derived from cold hardened *in vitro* cultures hence the above figures relate to the use of cold hardened tissue. Given the specific conditions within a protocol, the frequency of post-treatment and post-thaw growth of meristems was shown similarly to be affected, as would be expected, by a number of fundamental factors such as sucrose concentration in preculture treatment, time of incubation in loading solution, cryoprotection treatments and PVS2 incubation time. The summary evaluation of the six cryopreservation protocols enabled a further investigative assessment to focus on the application PVS2 based vitrification and encapsulation-dehydration approaches to cryopreserve strawberry meristems.

Generally, it may be found that experimental protocols tested on one genotype may not necessarily be applicable across the diversity of germplasm of a given plant species. Indeed, this is the case regarding the published reports of strawberry cryopreservation do indicate there are significant genotype effects but there appears to have been little or no systematic attempt to assess the effect of several genotypes against a single cryopreservation protocol. Hence, regarding the requirement to develop a generic protocol, the effectiveness of the optimised PVS2 based vitrification was assessed on 12 different strawberry cultivars, selected to include several significant European and America contemporary varieties. In this comparison, standardisation of experimental detail is essential, therefore all the *in vitro* shoot cultures from which the meristems were derived were maintained by a same culture procedure prior to cryopreservation. The frequency of post-thaw regrowth of strawberry meristems was significantly ($P<0.05$) dependent on the cultivar. This response could be divided into two groups: group A, where nine cultivars exhibited high (>50%) frequencies of post-thaw regrowth and group B, where three cultivars exhibited lower (<50%) post-thaw regrowth frequencies. It was also noted, that the cultivars in group B all exhibited significantly lower rates of *in vitro* proliferation between subcultures. This observation improved, by changing the frequency of subculture of the *in vitro* strawberry shoots from four to two weeks, which significantly increased the intrinsic rates of proliferation for these group B cultivars. Consequently, in altering the *in vitro* culture conditions, the frequency of post-thaw regrowth of meristems derived from *in vitro* cultures based on a two week subculture cycle, all significantly increased so that meristems from all but one of the cultivars exhibited an overall greater frequency of 50% for post-thaw regrowth.

Regarding the issues highlighted in this case study; the problems associated with the development of reliable and effective cryopreservation protocol for strawberry germplasm will be discussed.

CRYOPRESERVATION OF DIFFICULT-TO-HANDLE PALM SEEDS

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This project was aimed at developing cryopreservation protocols for one orthodox (*Washingtonia filifera)* and two recalcitrant palm species namely, *Arenga westerhoutii* and *Thrinax radiata* respectively. The experimental strategies involved *in vitro* tissue manipulation, embryo rescue techniques, application of dehydration and encapsulation-dehydration cryopreservation techniques and use of differential scanning calorimetry (DSC).

A comparative morphological study was also conducted on these seeds to investigate the embryo structure, locality and their affect on cryo-recovery. Seed germination, seedling development patterns and seed desiccation sensitivity were investigated for each species.

W. filifera seeds were successfully cryopreserved with a maximum germination after cryopreservation of 89% at moisture content of 5.3%. Excised zygotic embryos of *W. filifera*, showed 100% survival after one day and 97% after one week in liquid nitrogen when desiccated to 6.5 - 7.7% moisture content. Compared to the whole seeds, the zygotic embryos showed higher recovery after cryopreservation. Whole seed of *T. radiata* were successfully cryopreserved with 77% germination at 16% moisture content. *In vitro* culture establishment of excised embryos for *T. radiata* and *A. westerhoutii* was quite challenging as there were many problems associated with this. These include; embryo browning or oxidation, fungal contamination and manipulation of recovery medium. Fungal contamination inhibited *T. radiata* embryos development. Embryo oxidation was overcome for both *A. westerhoutii* and *T. radiata* embryos imbibing seed in water or on agar before excision and addition of 2% charcoal to the *in vitro* germination medium. Embryo survival was increased from 0% to 77% and 33% for *A. westerhoutii* and *T. radiata* after water imbibition for 7 days and agar imbibition for 3 days respectively. Morphological studies were conducted on all three species to investigate any correlation between embryo structure and position with response to cryopreservation. It is concluded that no correlation was noticed in all the species studied. Further understanding of recalcitrance, including the use of different cryoprotectants, is required to enable the further development of cryopreservation protocols for embryos of *T. radiata* and *A. westerhoutii*.

FUNDAMENTAL ASPECTS IN CRYOPRESERVATION OF MEDICINAL PLANTS: THE *Hypericum* STORY

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Plant cryobiology as multidisciplinary field of science comprises diverse approaches and analyses of possible particular interrelated involvements of low temperature physics and chemistry, plant physiology and plant biochemistry, all directed to cell viability and somatic cell genetics and focused on the genome itself with its unique features. The genome “quality” reflects its organisation and structure, the genome “flexibility” reveals the complex functionality including capability to response to intracellular and exogenous signals.

The measure of tolerance of the genome to exogenous factors depends on the genome “flexibility” generating genetic variation. To assess the genome functionality it is necessary to know a lot or at least something about the particular genotype which is not the case of *Hypericum* spp. In the GenBank database more than 200 gene sequences from about 90 *Hypericum* species are available recently. However, less than 30 percent of them represent structural genes. Moreover, many representatives of the genus possess different genome size due to different basic chromosome numbers and occurrence of polyploidy.

Nevertheless, *Hypericum perforatum* may serve as a model for the genus and, possibly, also for other plant species containing bioactive substances.

Temperature as a major triggering variable in low temperature biology affects all structures and processes in the living matter with no exception. Using the *Hypericum peforatum* model the following aspects were investigated:

Temperature and physical processes*:* The way how the temperature is decreasing affects the course of phase transition, dehydration profile, state of solutes within the cell, etc.
Application of slow cooling approach and vitrification resulted in 2.7times higher recovery in favour of slow cooling process. The cooling rate in the phase transition interval showed significant influence on cell viability.
Temperature and plant physiology*:* The choice of explant and its physiological features, endogenous circadian and/or seasonal rhythms revealed possible effects on survival rate. The pre-culture conditioning does not bring ambiguous conclusions (duration of pregrowth period, type and concentration of the pre-culture additives has to be further examined). Estimation of the level of oxidative stress effect was based on the MDA and hydrogen peroxide tests. A question rose whether oxidative stress induces an increased activity of the antioxidant system. Another important question concerns the relevance measure of these results performed after longer time period when the plants are regenerated.

EVALUATION OF THERMOGRAMS FROM DIFFERENTIAL SCANNING CALORIMETER

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Two basic methods, the differential thermal analysis (DTA) and differential scanning calorimetry (DSC) were used to discover the principal thermal events connected with the plant cryopreservation procedures. The differences in principles of both methods predestine their application for ultra-low temperature plant behaviour study. DSC thermograms are used mainly for evaluation of glass transition, melting temperature and frozen water volume in plant samples at ultra-low temperatures. Overlapping thermal events in biological objects are possible to separate by modification of standard DSC method. Temperature modulated DSC (MDSC) and quasi-isothermal temperature modulated DSC (QITMDSC) are used for glass transition and endothermic ice crystal thawing separation. The artefacts caused by nonstandard preparation of samples or by measurement are summarized. For practical purposes, the method of measurement is chosen according to the application with respect to the main specifity of plant samples, to keep their regeneration ability after warming from ultra-low temperatures. Different conditions less than optimum or errors occurring during measurement leading to the misinterpretation of measured data are discussed. Some procedures and solutions are recommended to improve the overall quality and interpretation of standard DSC, MDSC and QITMDSC results. (This work was supported by project OC08062 MSMT CR)

COMPLEXITY AND A REGULATORY ROLE FOR SUGARS IN THE ACCLIMATION TO COLD AND FREEZING OF PLANTS

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There are a number of sensors of cold and several pathways regulating acclimation to cold and freezing. There are differences in the acclimation of different tissues to cold. The

talk will outline the extent of current molecular knowledge and give insights into tissue differences in acclimation and evidence for a role for sucrose (in addition to well-known direct protective effects) in regulating cold-responsive gene expression.

THERMAL CHARACTERISTICS OF SOME VITRIFICATION SOLUTIONS

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Different vitrification solutions are used for plant material dehydration and/or cryoprotection by different cryopreservation methods. Differential Scanning Calorimetry (DSC) was used to define thermal characteristics of some vitrification solutions and their components. A first-order phase transitions (melting and crystallization) and a second-order phase transition (glass transition) of sucrose as a basic component of most vitrification solutions was measured at different concentrations. Exothermic events during cooling of sucrose solution were observed at low or medium concentrated solutions up to the eutectic point (63 % w/w sucrose). Two glass transitions at -31°C and -46°C were detected during warming at low sucrose concentrations up to 50 % (w/w). No exothermic event was observed during cooling when the sucrose concentration increased above the eutectic point. Glass transition of highly concentrated sucrose solution (78 % w/w) was -43°C. Glycerol at low concentrations decreased freezing point of solutions more effectively in comparison with sucrose. No exothermic or endothermic events were found in PVS3 solution. No exothermic event was found in the modification of PVS3 solution (40 % w/v glycerol, 40 % w/v sucrose) during cooling but small exothermic and endothermic events were observed during warming in this solution. Lower concentrations of the PVS3 components resulted in exothermic event presence already during cooling. Similar differences in thermal characteristics were found in PVS2 solution and its modifications with lower concentration of basic components.
(Project OC08062 - Thermal analysis - a tool for cryopreservation efficiency improvement)

PRACTICAL BENEFITS OF DORMANT BUD CRYOPRESERVATION FOR GENETIC RESOURCE CONSERVATION

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Many woody plants offer the possibility of direct cryopreservation of dormant, winter buds following natural, or supplemented hardening regimes. Collections working as a genebank e.g University of Copenhagen with 1700 accessions of fruit crops) can optimize the use of human and material resources by devolving collection, cryopreservation and recovery of accessions (by grafting onto an appropriate rootstock) to field staff during the winter season. This reduces significantly pressure on gene bank laboratory staff and the micropropagation facility. The absence of any extended period of *in vitro* culture also limits risks to genetic stability. A further benefit is that recovered whole buds can be grafted directly on to the required rootstocks, whereas recovered in vitro apices, for example, would have to be raised to independent plants of a significant size before grafting, otherwise micrografting

would have to be employed. The technical requirement is to ensure survival of both bud and cambial tissue, to provide the graft union, in a single protocol and, where this is not currently successful, viable buds can be excised from the explants and recovered in a single cycle of *in vitro* whole bud culture. Conventionally cryopreserved, *in vitro* material will be tested for disease status at an appropriate point in the production cycle before distribution and field establishment, and the same can be done for post-recovery, grafted material.

Consequently, this technique can provide significant, and welcome, operational resource benefits to many woody plant conservation programmes, particularly where temperate fruits are concerned. The involved labour force is significantly enhanced, costs of preparation and preservation drastically reduced and the time to field establishment of the required, grafted plant shortened. The usage of the micropropagation facility is also significantly reduced, reducing cost to the field facility and liberating valuable laboratory time that would otherwise be used for routine, not research-level, procedures. The merits, and disadvantages, of this system will be discussed.

EPIGENETIC CHANGES ASSOCIATED WITH THE CRYOPRESERVATION OF CLONAL CROPS

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Cognate with the implementation of COST Action 871 'CRYOPLANET' is Objective 5: to assure the genetic stability and 'true to typeness' of plants after cryopreservation as described within the fundamental aspects of the WG1 package. As tissue culture continues to play a vital role in the development of cryopreservation techniques, there remain challenges regarding the detection of genomic change. Therefore, it is important to assess whether plant germplasm surviving cryogenic storage is genetically identical to the material prior to cryostorage. Consequentially, there is an increasing requirement to determine whether plants derived from cryopreservation are 'true to type' and to measure the extent of the 'normal phenotype' in cryopreserved plants and estimate the degree of closeness to the 'true' parental genotype. These determinations may be achieved through the application of a range of analytical techniques to examine changes at the phenotypic, histological, cytological, biochemical and molecular levels. Regarding the use of investigative tools, the 'state-of-the-art' analytical technology of the most widely used methods is not without criticism (1). Technical limitations exist based on the 'PCR-type' analysis of the primary DNA sequence as these reflect only a small fraction of the plant genome analysed. Therefore, there is a requirement for the presently available techniques to become more genomically widespread in their analysis, also particular PCR-based approaches are unlikely to reveal other changes mediated by epigenetic mechanisms potentially creating a 'gap' in fundamental knowledge. This presentation will consider three clonally propagated crops, blackcurrant (*Ribes*), garlic (*Allium sativum*) and potato (*Solanum tuberosum* L.) and examine the application of different cryopreservation protocols in relation to changes in their epigenetic status. The role of DNA methylation changes during the acclimation and cryopreservation of *Ribes* shoot meristems using genotypes with differential recoveries will also be presented (2). These studies showed DNA methylation was induced in tolerant genotypes and demethylation was evident in the

sensitive genotypes following acclimation and cryopreservation. These differential methylation patterns exist during the initial vegetative cycle but regress to control values following subculture, indicating DNA methylation to be a reversible epigenetic mechanism (3). The significance of these changes for cryo-conservation will be discussed.

1. Harding K (2004) *CryoLetters* **25**, 3-22.
2. Harding K, Johnston JW & Benson EE (2008). In: *Cryopreservation of Crop Species in Europe*. Laamanen J, Uosukainen M, H¨aggman H, Nukari A, Rantala S (eds.), Proceedings of CRYOPLANET COST Action 871, Oulu, 20th–23rd February 2008. Agrifood Research Working Papers 153. MTT Agrifood Research, Finland. pp. 10–11.
3. Johnston J W, Benson E E & K. Harding (2009). *Plant Physiology & Biochemistry* **47**, 123–131.

APPLICATIONS OF MOLECULAR MARKERS PRIOR CONSERVATION ACTIONS ON THE SPANISH ENDEMIC *Senecio boissieri*

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Randomly Amplified Polymorphic DNA (RAPD) and chloroplast microsatellite (cpSSR) variation was surveyed in the Spanish alpine endemic, *Senecio boissieri*, to define the genetic variation present in the species, to help resolve the the causes of its disjunct distribution in the southern Sierra Nevada and Sierra de Baza, centrally located Sierra de Guadarrama, and northern Cordillera Cantábrica and to know whether differentiated groups are present within the species as populations within species may be sufficiently differenciated that they deserve management as separate units. RAPD analysis detected a high genetic diversity in each of the analysed localities. Also, two divergent genetic groups were identified, one containing individuals from the Cordillera Cantábrica and another comprising individuals from the three other mountain ranges. In contrast, chloroplast DNA variation was much more limited with only one of 42 cpSSR loci examined showing polymorphism. At this locus the same allele occurred at high frequency in material from each mountain range. One possible reason for Cantabrian material showing RAPD divergence from other material is that it is derived from plants that survived the Last Glacial Maximum in a northern refugium isolated from the main distribution of the species which spanned the area between the southern and central Spanish sierras. Postglacial fragmentation of the species' main distribution in southern and central Iberia would have resulted in the current disjunction of genetically similar populations in southern and centrally located mountains being this fact a key issue in the conservation of the global variability within the specie.

Key words: alpine plant, cpDNA, genetic diversity, plant dispersal, population fragmentation, RAPD**.**

DYNAMIC STUDY OF CELLULAR EVENTS DURING CRYOPRESERVATION

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Conventional microscopy techniques present some limits for studying cellular events occurring through the various steps of a cryopreservation process. It is particularly difficult to quantify and topologically identify cellular changes.

These last years, new imaging techniques were developed, mainly in animal sciences, creating real time or 3D images. The Real-Time Microscopy (RTM) permit to observe living cells without any stain or contrast agent. Combined with a perfused disposable chamber it is possible to change the cellular environment during the observation. Confocal Laser Scanning Microscopy (CLSM), combined with special software, allows three-dimensional reconstructions of cells, tissues or organs.

We tried to adapt these two techniques in order to better understand *Pelargonium* apex evolution during some steps of a droplet-vitrification protocol. The first step was to determine the degree of feasibility of the project. For practical reasons, we first focus on the structural modifications due to LS and PVS2 addition. We demonstrated that it is possible to have a sequence of the events in real time and to quantify them.

THE CONSERVATION OF VEGETATIVELY PROPAGATED *Allium* COLLECTION IN THE PORTUGUESE GENEBANK

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The Portuguese Genebank (BPGV) started to collect and establish the Portuguese field collection of vegetative propagated *Allium* in 1994. Presently the genebank conserves in *ex situ* conditions, more than 300 garlic accessions in field and *in vitro* collection. The *in vitro* conservation is not considered to be a basic method for the maintenance of garlic accessions, because this species is subject to alteration in quality after 1-2 years in culture. The cryoconservation is considered to be the method for conservation in long-term conditions. Shoot tips excised from garlic cloves can be successfully cryopreserved using vitrification methods.

The Portuguese genebank is represented in the ECPGR *Allium* Working Group since 1996 and because of the important collection conserved and scientific work done since then, was invited to participate in the AEGIS model crops curators and database managers meeting, that took place in Poland, July 2008.

Under the ECPGR Working group a project EU GENRES was proposed, the EURALLIVEG. The project objectives are: define a unique clonal material, the use of in vitro culture to remove viral infection and to develop a European collection of garlic cryopreserved. The EURALLIVEG partner collections (Czech Republic, France, Germany, Italy, NGB and Poland) contained 1549 garlic accessions. In order to amplify this group, it has been proposed a project for ECPGR Phase VIII to fingerprint material from Portugal and

Spain. Currently in EURALLIVEG there are three institutions involved in the study of garlic cryopreservation, namely the Czech Republic, Germany and Poland. During the AEGIS meeting both an interest in and importance of developing cryopreservation capabilities were demonstrated. During the year 2004 in collaboration with Oporto University, we have tested different plant vitrification solutions. The diverse garlic accessions tested were successfully cryopreserved using the vitrification solution: 15% v/v DMSO, 15% v/v ethylene glycol, 70% v/w glycerol. At least 38% of explants exhibit regrowth after liquid nitrogen exposure in the total.

The Portuguese genebank is also interested in developing cryopreservation capabilities for other species vegetative propagated, like *Mentha* spp. and *Humulus lupulus* L.

CHANCES FOR UTILIZATION OF CRYOPRESERVATION IN COMPLEX BOTANICAL COLLECTIONS – THE *Alliaceae/Amaryllidaceae* CLADE

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The families *Alliaceae* and *Amaryllidaceae* form a distinct clade within the order Asparagales. Many species of this group are used by man as vegetables, spices and medicinal plants. Overexploitation and habitat destruction are endangering many of these species. Members of these families have in common many morphological and physiological characters and interesting distribution patterns on one side and sufficiently high diversity on the other one. Thus, they represent a well suitable group for comparative investigation on cryopreservation using the well-established results in garlic as a model case. The main aim of these studies is the establishment of new strategies to conserve a complex botanical collection as it is present within the *Allium* collection of IPK Gatersleben and the transfer of the results to endangered wild species of both families. These strategies will extent the spectrum of target organs for cryopreservation from shoot bases (bulb basal plates) which are already used routinely to recalcitrant seeds (*Amaryllidaceae*), pollen and other organs to keep the mother bulb intact (root tips, bases of leaves and inflorescences). As most of the species in regard are outbreeders, comparisons of the conserved diversity should be done when combining the storage of a certain number of mother plants together with a corresponding pollen population. This should enable the collection holders to avoid the tremendous problems of properly propagating a highly diverse collection at a limited space.

APPLICATIONS OF DIFFERENTIAL SCANNING CALORIMETRY IN DEVELOPING CRYOPRESERVATION STRATEGIES FOR *Parkia speciosa,* A TROPICAL TREE SPECIES

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Shoot-tips of *Parkia speciosa*, a recalcitrant seed producing tropical leguminous tree withstood cryopreservation using encapsulation-vitrification in combination with trehalose preculture. Differential Scanning Calorimetry (DSC) revealed that trehalose moderated the thermal characteristics of the shoot-tips. A 30 min PVS2 treatment had the lowest glass transition temperature (Tg) (-50.21 ± 1.07ºC) when applied in combination with 5% (w/v) trehalose. The Tg increased to -40.22 ± 0.95ºC as the sugar's concentration was decreased to 2.5% (w/v). Tg heat capacity for shoot-tips treated with 2.5% and 5% (w/v) trehalose and exposed to PVS2 for 30 min increased from 0.17 ± 0.05 to 0.23 ± 0.01 $J.g^{-1}$ respectively. Enthalpies of the melt-endotherm varied in proportion to trehalose concentration, for the 30 min PVS2 treatment, whereas the melt enthalpy for control shoots was >150 $J.g^{-1}$ and decreased to ca. 60 $J.g^{-1}$ with 2.5% (w/v) trehalose. For 5% and 10% (w/v) trehalose treatments, enthalpy declined to ca. 24 and 12 $J.g^{-1}$ respectively and freezing points were depressed to –75ºC and –85ºC with 2.5% and 5% trehalose (w/v), respectively. DSC elucidated the critical points at which vitrification occurred in germplasm exposed to trehalose and PVS2. A 60 min PVS2 treatment supporting ca. 70% survival was found optimal for stable glass formation during cooling and on rewarming.

CRYOPRESERVATION OF SHOOT TIPS FROM DORMANT BUDS OF *Diospyros kaki* BY ONE-STEP VITRIFICATION

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The safeguard genetic resources of persimmon in the Mediterranean basin is limited. Some European institutions have contributed, through EC GENRES29 Project 'Conservation of minor fruit tree species', to the safeguarding and conservation of a number of persimmon accessions collected in clonal orchards. Cryopreservation could be an advanced technique to reduce serious implications and risks in the management of germplasm collections and preserve *Diospyros kaki* cultivars. To optimize cryopreservation procedure for shoot tips from dormant buds of persimmon, mainly used in the Mediterranean area, several experiments were carried out by one-step vitrification method. Vegetative dormant buds were taken, during the winter, from twigs of persimmon cultivars ('Kaki Tipo', 'Jiro', 'Triumph', 'Hiratanenashi' and 'Rojo Brillante'). Shoot tips (2-3 mm) were dissected from sterilized buds and precultured on MS medium (modified with half-strength NH_4NO_3 and KNO_3) with 0.3 M sucrose for 48h. To induce osmodehydration, the shoot tips were loaded in Plant Vitrification Solution 2 (PVS2), for different times (20 min in the first experiment; 30, 60, 90 min in the following experiments) and two treatment temperatures (0°C and 25°C). The explants were rapidly frozen to –196°C by direct immersion in liquid nitrogen (LN). For recovery, they were quickly rewarmed in a waterbath at 40°C for 1 min, then the shoot tips were transferred on the regrowth medium (MS modified with 0.06 M sucrose, 22.2 μM BA, 2 g/l gerlite) and maintained at 24°C with 16h photoperiod. Survival of cryopreserved shoot tips was assessed after 3 weeks. By applying a treatment with PVS2 for 20 min at 25°C on shoots tips from dormant buds, the survival rate observed in cv 'Triumph' (25%), 3 weeks after

cryopreservation, is lower than those reported by Matsumoto *et al.* (2001). In a subsequent experiment on cv 'Kaki Tipo', where we applied a longer exposure time to PVS2, a higher shoot tip survival rate was obtained, in particular with PVS2 treatment for 90 min (73%). The same protocol applied on other cultivars confirmed the effectiveness of this vitrification procedure and with 90 min PVS2 treatment, the survival percentage in three cultivars ('Jiro', 'Triumph' and 'Rojo Brillante') was recorded between 56-80%. Whereas, in cv 'Hiratanenashi', the best survival (86%) was observed with 60 min PVS2 treatment before explant immersion in LN. Molecular assays were performed with fresh material, *in vitro*-cultured and cryopreserved shoots tips of three persimmon cultivars ('Kaki Tipo', 'Jiro', 'Triumph') in order to validate both the micropropagation procedure and the optimized cryopreservation technique. In our experiments, no morphological deviation was observed in the development of persimmon cryopreserved plantlets.

IMPROVEMENT OF THE CRYOPRESERVATION OF APPLE THROUGH THE DROPLET VITRIFICATION METHOD

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The Fruit Research Center of Rome hosts an *in field* fruit germoplasm collection and its Propagation Group has been gradually establishing an *in vitro* collection to be conserved through "slow growth". Cryopreservation protocols for various fruit species have been also established and recently a National Project was financed to support the establishment of protocols for old pear and apple varieties. Till now the encapsulation-dehydration has been applied in the Centre to cryopreservation of shoot tips of genotypes of species such as *Pyrus*, *Prunus*, *Malus*, *Actinidia*, *Fragraria*, but vitrification protocols could simplify explant handling, compared to encapsulation-dehydration, and their results are reported to be less genotype dependent (2). In particular, the droplet vitrification technique gives rise to cooling rates considerably higher compared to other vitrification methods, reducing the lethal effects of intracellular ice crystals formation during freezing and thawing and, when previously applied to *Musaceae*, was shown to give higher and more reproducible post-thaw regeneration rates than normal cryo-vial freezing protocols (1).

In the framework of a STSM of COST 871 spent in K.U. Leuven-Laboratory of Tropical Crop Improvement, Leuven (Belgium), with the supervision of Dr Bart Panis, the droplet vitrification method was applied to *in vitro* grown apple cultivars (Pinova and Jonagold), kindly supplied by Dr Philippe Druart from the Department of Biotechnology of the CRA-W, Gembloux (Belgium).

The establishment of the protocol was performed evaluating the effect of various factors, such as length of application of the PVS2 solution, type of explant (shoot tips or nodal segments) and age of plant material, application of high sugar or cold pre-treatments. The best result, 46.7% of surviving buds after liquid nitrogen immersion, 64% of which developed plants, was obtained in cultivar Pinova, treating axillary buds, without pretreatment, for 30 min in PVS2 solution. To our knowledge, in the results of cryopreservation on *in vitro* grown apple plantlets, the explants have been previously cold acclimated and/or precultured on high sugar media. With this method no cold acclimation and preculture were required. Moreover, the employment of axillary buds allows to obtain large amount of material starting from a

little number of shoots. Other experiments are now in progress to apply the protocol to other apple cultivars and to pear.
1. Panis B, Piette B & Swennen R (2005). *Plant Science* **168**, 45-55.
2. Sakai A (1997) in Lazdan MK, Cocking EC (eds), *Conservation of Plant Genetic Resources* In Vitro. *Volume 1, General Aspects*, Science Publishers, USA, pp 53-66.

MORPHOGENIC POTENTIAL OF GENTIANA KURROO (ROYLE) CELL SUSPENSION AFTER CRYOTREATMENT

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Embryogenic suspension is the best source of getting unlimited number of cells ready to be used for any somatic cell genetic manipulation. Cryopreservation is the method helping to carry on the uniformity of cell characters during long-term period. At present, our progress in biotechnological studies of gentianas required to develop proper system to preserve the cells which high morphogenic potential could be repeatedly employed for somatic cell genetics manipultion.

The aim of the presentation is to show the effect of the cryotreatment on somatic embryogenesis of cell suspension and plant regeneration in post-thawing culture. Experiments were carried out on embryogenic cell suspensions of *Gentiana kurroo.* For experiments only cell suspension aggregates closed in alginate beads were used. In post-thawing culture beads were transferred to agar medium at 14 days and later into liquid medium. After 3-4 weeks new cell suspension were formed. Cell aggregates from frozen and non-frozen culture were transferred on agar regeneration medium MS + 0.5 mg/l GA_3 + 1.0 mg/l Kin + 80.0 mg/l AS, to complete somatic embryogenesis. Later, somatic embryos converted into germlings on hormone-free 0.5MS medium. The quality and uniformity of regenerants to initial plant material were assessed with application of the following methods: flow cytometry and DNA molecular analysis. Conclusion: cryopreservation stimulated yielding of somatic embryo production, cryotretment did not change the genome size and very similar level of sequence variation of regenerants derived from non- and cryopreserved tissue may suggest that differences are due to selection pressure than mutation processes induced by cryopreservation.

WHICH ROLE PLAYS OSMOTIC STRESS ON (EPI-GENETIC) INSTABILITY?

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The genetic analysis of shoots regenerated from cryopreserved apices of chrysanthemum (*Dendranthema grandiflora*), using the encapsulation-dehydration method, showed important differences compared with the control plants. Two different genetic markers (RAPDs and AFLPs) were used to analyze the whole process from the first step of the protocol (sucrose culture) to the recovery of the cryopreserved material. Analyses were carried out through the whole process. Data obtained with RAPDs markers showed always the same mutation in all

the analyzed samples, from the high sucrose concentration treatment onwards. On the other hand, differences revealed with the AFLPs markers seemed quite untargeted through the whole-genome level, although the number of differences increased through the process, being the cryopreservd samples those with higher amount of differences within each line.

These results suggest that the genetic instability detected with these analyses could be correlated to the degree of stress suffered by the samples during the cryopreservation protocol. Similar conclusions have been obtained when studying the genetic and epigenetic modification in rapeseed induced by salt stress (3). After the analysis of AFLPs data, they observed a higher accumulation of mutations correlated to the salt concentration. A higher salinity level leads to more severe genetic damage. Likewise, the DNA damage induced by salt stress is randomly distributed over the genome, as it was observed in our results of AFLPs in chrysanthemum.

The mechanism that could explain these results, and also our results in chrysanthemum, is the theory that water deficit caused by salinity stress (or osmotic stress in general) will lead to the production of reactive oxygen species (ROS) including superoxides, hydrogen peroxide and hydroxyl radicals (4). These ROS are highly reactive and can disturb the normal cellular activity through oxidative damage to membrane lipids, proteins and nucleic acids (1, 2). We try to evaluate the relationship between DNA modifications and stress in plants, since the cryopreservation protocols imply a high stress effect on the plant tissues.

1. Aust AE, Eveleigh JF (1999) *Proc. Soc. Exp. Boil. Med*. **222**, 246-252.
2. Imlay JA (2003) Pathways of oxidative damage. *Ann. Rev. Microbiol*. **57**, 395-418.
3. Lu G, Wu X, Chen B, Gao G & Xu K (2007) *J. Integr. Plant Biol* **49**, 1599-1607.
4. Mittler R (2002) *Trends Plant Sci*. **7**, 405-410.