these structures automatically showed contraction that suggested MSCs differentiation into cardiomyocytes or other cell types. The stem cells were successfully cultured on top of a layer of fibroblasts, using ablated varicose vein as the source of fibroblast.

(6) Three of 90 containers ruptured during thawing, but there were neither bacterial contamination nor obstacles in the culture results afterwards.
(7) Movements of cisternae of Golgi apparatus and vesicular transport in colony forming cells were very fast rotation and vibration which were recorded by video for 3 h or more.
(8) The cryopreservation of stem cells and also cord blood cells at −60 to −80°C in the mechanical freezer and in the vapour phase of liquid nitrogen, did not constantly succeed in the formation of colonies, probably due to long-term duration of unstable temperatures caused by the repetitive opening and shutting of the door, as previously reported.
(9) The cryopreserved autologous stem cells or progenitor cells will play an indispensable role of therapeutic procedures not only for patients, but also for special workers who have had accidental radiation injury.

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*23. Comparison of vitrification-based techniques in the efficacy of cryopreservation of Passiflora suberosa L. and P. foetida L. shoot tips.* M.G. Vianna, A.L. Ferreira, R.O. Garcia, E. Falcão, G. Pacheco, E. Mansur, Núcleo de Biotecnologia Vegetal, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier nº 524 PHLC sala 505, 20550-013, Maracanã, Rio de Janeiro, RJ, Brazil

*Passiflora* are used as ornamentals and in popular medicine. *Passiflora suberosa* is commonly used to treat hypertension, diabetes, skin diseases and as a sedative, whereas *P. foetida* shows analgesic and antibacterial activities. The goal of this study was the development of *in vitro* conservation strategies for long-term storage for both species through cryopreservation using vitrification and encapsulation-vitrification techniques. The efficacy of vitrification and encapsulation-vitrification protocols was compared, and several parameters were evaluated, including pregrowth on medium with high sucrose concentrations, type of vitrification solution (PVS2 and PVS3), exposure time to vitrification solutions, and recovery conditions. Shoot tips (0.3 cm) excised from *in vitro*-grown plants were precultured for 24 h on solid 1/2 MSM medium supplemented with different sucrose concentrations (0.3, 0.5 or 0.7 M), at 25 °C. Explants precultured on medium supplemented with 0.3 M sucrose were incubated in the cryoprotectant solutions PVS2 or PVS3 for different periods (0–240 min) at room temperature, and immersed into liquid nitrogen (LN) for 72 h. In the encapsulation-vitrification protocol, precultured shoot tips were encapsulated in calcium alginate beads, followed by incubation in liquid 1/2 MSM medium containing 0.75 M sucrose for 24 h, on a rotary shaker (100 rpm) at 25 ± 2°C. After this period, beads were maintained in cryovials containing PVS2 or PVS3 for 30, 60, 120 or 180 min, before immersion into LN. The materials were rapidly thawed in a warm water bath (38-40 °C) for 2 minutes and transferred to the recovery medium (1/2 MSM or MSM supplemented with 0.44, 2.2 or 4.4 μM BA). Alternatively, explants were kept in the dark for 30 or 60 days before transfer to the presence of light. Plant recovery from the shoot tips before and after cooling in LN was evaluated by assessing the percentage of shoot formation. The shoots were cultured on 1/2 MSM medium for 60–90 days for root induction, and whole plants were transferred to *ex vitro* conditions. The highest recovery rates for both *P. suberosa* and *P. foetida* (28% and 60%) were obtained with the encapsulation-vitrification protocol, after a pre-treatment with 0.3 M sucrose, followed by exposure to PVS2 for 60 or 120 min, respectively, and post-freezing incubation in the absence of light for 30 days on MSM medium supplemented with 0.44 μM BA. These results suggest that alginate beads provided additional protection, reducing the toxicity of the cryoprotectant solution. In addition, they demonstrate that different species from genus *Passiflora* display distinct responses to cryopreservation treatments.

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*24. Willow seeds viability after imbibition, freezing and freeze-drying treatments.* Helena Ott [1,2], Patricio R. Santagapita [2], M. Pilar Buera [2], [1] Student of Food Engineering of AGROSUP DIJON, Dijon, France, [2] Departamento de Industrias y Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (FCEyN-UBA) & Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina

The conservation of labile biomolecules/structures in biological, pharmaceutical and food sciences is generally performed in frozen or dehydrated systems. Vegetal germplasm is usually conserved as seeds, which are naturally dehydrated systems. Orthodox seeds (the most common) tolerate dehydration at low water contents (wc), and remain viable for several years upon conservation at 20 °C or at 4–8 °C. Willow seeds are orthodox but show some recalcitrant characteristics: the longevity does not follow the dehydration tolerance as is shown for orthodox seeds, and they lose viability in a few weeks at 20 °C. In practice, collected seeds are usually dehydrated at 20 °C for 3 h from wc 70% to 10–12% (dry basis) and then kept at −70°C, which highly increases storage time-dependant costs. The purpose of the present work was to analyze the conservation of willow (*Salix nigra L.*) seeds through freezing and freeze-drying. Seed imbibition with 45%w/v trehalose or polyethylene glycol 400 – PEG – or water were conducted for 16 h at 4 °C prior to freezing or freeze-drying, in order to introduce cryo- or dehydro-protective agents in the seeds by imbibition. A comparison with vacuum drying seeds was also performed. The germination rate was measured after each treatment. Water content, water activity ($a_w$), and differential scanning calorimetry (DSC) determinations were conducted. The control seed pool ($a_w$ of 0.745; 13%db wc) retained 77% of its original germination capacity.

Priming effect (Chojnowski et al., Seed Science Research 7 (1997) 323–331) was observed during seed imbibition with water and trehalose, but only 70% of the imbibed seeds with PEG germinated after imbibition, which can be related to a certain degree of PEG toxicity toward the seeds. After freezing, a great germination capability loss was observed in trehalose and water imbibed seeds; instead, PEG seeds maintained their ability to germinate.

Water crystallization was observed in all the imbibed seeds ($a_w \sim 0.97$), and followed the increasing order: PEG < trehalose < water. Even though water crystallization was observed in PEG imbibed seeds, their lower water content (40%wb) with respect to those imbibed with trehalose or water (wc of 45 and 65%wb) had a major impact on the germination capability of imbibed seeds.

Water crystallization was not observed in control seeds. Their thermograms showed a broad lipid melting (between −40 and 10 °C) and protein denaturation (between 55 and 110 °C). Since there are no major changes in protein denaturation after freezing of imbibed seeds, membrane damage could be the major responsible of the lower germination capacity observed after freezing and freeze-drying.

After freeze-drying, a 30% decrease of the germination capability was observed in the PEG imbibed seeds in comparison to the frozen ones, arriving at $a_w$ of 0.269 and wc of 5%db. Water and especially trehalose imbibed seeds subjected to vacuum drying showed better germination capability with respect to the freeze-dried ones, revealing that both the amount of crystallized water and the presence of adequate protective agents were the main critical factors involved in willow seed conservation.

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*25. The influence of desiccation, cryopreservation and rehydration on the survival of polyembryonic Citrus seeds.* Natalia Graiver [1], Alicia Califano [1], Noemí Zaritzky [1,2], [1] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET, Facultad de Ciencias Exactas, UNLP. 47 y 116, La Plata (1900), Argentina, [2] Depto. de Ingeniería Química, Facultad de Ingeniería, UNLP, Argentina

Three categories of seed storage behavior are generally recognized among plant species: orthodox, intermediate and recalcitrant. Intermediate seeds, such as Citrus, can withstand partial dehydration, but they cannot be stored under conventional genebank conditions because they are cold-sensitive and desiccation does not increase their longevity. Citrus seeds that are immersed in liquid nitrogen (LN) without a previous dehydration process show a very low viability; therefore the water content when they are immersed in LN is considered to be the most critical factor in cryopreservation process.

The purpose of this study was to investigate the basis of the optimal hydration status for cryopreservation of intermediate oily Citrus seeds: *Citrus sinensis* (sweet orange) and *Citrus paradise* (grapefruit).

In the present work two conditions were tested: (a) seed desiccation, (b) seed desiccation followed by LN treatment with thawing under controlled conditions. In addition two pre-sowing procedures (preheating and prehumidification prior to the

germinability test) were applied and their influences on the viability of the seeds were analyzed.

To study the tolerance to dehydration and LN exposure, seeds were equilibrated at relative humidities (ERH) between 11% and 95% using saturated salts (LiCl, $MgCl_2$, $K_2CO_3$, $NaNO_2$, NaCl, KCl, $KNO_3$). Sorption isotherms were determined and modeled. Seed viability was analyzed by germination experiments; seeds were sown in hermetic controlled germination conditions and the percentage of normal seedling was evaluated 4–6 weeks after sowing. Seed desiccation sensitivity was quantified by the quantal response model. Differential scanning calorimetry thermograms between −120 and 100 °C were determined on cotyledon tissue that was previously dehydrated reaching different moisture contents; ice melting enthalpies and unfrozen water contents were measured from these thermograms. In order to analyze the effect of pre-sowing treatment on seed viability, experiments were carried out in a narrower range of equilibrium relative humidities (ERH: 59–85%). All the seeds subjected to conditions of seed desiccation, or seed desiccation followed by LN treatment were either directly sown under germination conditions or subjected to pre-sowing rehydration procedures; the tested procedures were preheating (40 °C during 4 h) and pre-humidification (equilibrium at 100% RH, 20 °C).

In the cases of seeds that were only dried and in seeds that were dried and treated with LN, the tested pre-sowing treatments (pre-humidification or heating) did not significantly improve the viability of the seeds with respect to the control samples ($P < 0.05$); in fact preheating significantly deteriorated the viability of the LN treated seeds.

The survival of *C. sinenis* and *C. paradise* seeds, subjected to cryopreservation in LN was examined and seed desiccation sensitivity following rehydration procedures was quantified. Results showed that in order to reach the maximum viability, the seeds exposed to LN, must be first dehydrated to a range of ERH 69–81%(0.16–0.31 $gH_2O\ g^{-1}$dry basis) for *C. sinensis* and 69–75% (0.09–0.11 $gH_2O\ g^{-1}$dry basis) for *C. paradise.* The limit of hydration for LN *Citrus* seeds treatment corresponded to the unfrozen water content in the tissue, confirming that seed moisture should be reduced to such an extent to avoid the formation of intra-cellular ice crystals during ultra-rapid freezing, thus preserving the integrity of seed tissues.

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*26. Invariance of the glass transition temperature of plant vitrification solutions with cooling rate.* Aline Schneider Teixeira [1], Miloš Faltus [2], Jiří Zámečník [2], Renata Kotková [2], Maria Elena González-Benito [3], Antonio Diego Molina-García [1], [1] ICTAN (CSIC), Jose Antonio Novais 10, 28040 Madrid, Spain, [2] Crop Research Institute, Prague, Czech Republic, [3] Dept. Biología Vegetal, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid, Spain

Glass, the state of matter where molecular mobility is so reduced that most physicochemical processes are virtually detained (including ice formation), is basic for cryopreservation. The glass transition temperature ($T_g$), a temperature range at which supercoooled liquid becomes glass, is characterized by a change in heat capacity ($C_p$), evaluated at its inflection point.

Vitrification in cryopreservation protocols is achieved, without sophisticated cooling equipment, simply plunging specimens into liquid nitrogen (LN) after a set of physicochemical treatments increasing cytoplasmatic microviscosity and enhancing tissue resistance to cold and dehydration. Quick cooling is required to achieve vitrification avoiding ice formation. Both $T_g$ and $C_p$ are generally considered dependent on cooling rate (e.g. Angell et al. 82 (1978) J. Phys. Chem., 2622; Debenedetti et al. 410 (2001) Nature 259). The present work endeavors to increase knowledge in this area characterizing the calorimetric glass transition of the most common plant vitrification solutions, under a wide range of cooling rates.

The solutions studied were Plant Vitrification Solutions 1, 2 and 3: (Uragami et al. 8 (1989) Plant Cell Rep. 418; Sakai et al. 9 (1990) Plant Cell Rep. 30; Nishizawa et al. 91 (1993) Plant Sci. 67). Cooling was performed using the calorimeter control (5, 10 and 20 °C $min^{-1}$), or for higher rates, by quenching closed pans with PVS in LN, either naked (− 5580 °C $min^{-1}$) or inside cryovials (360 °C $min^{-1}$). Quenched pans were then transferred to the pre-cooled sample chamber. Glass transition temperature was observed by DSC with a TA 2920 instrument, upon warming pans with solution samples from −145 °C to room temperature, at standard warming rate: 10 °C $min^{-1}$.

Glass transitions showed clear and consistent temperature differences among vitrification solutions, related to composition and water content. Roughly, two sets of $T_g$ values were obtained, for PVS1 and 2, at −112 °C and −114 °C, respectively, and for PSV3, at −90 °C. The observed $T_g$ did not significantly change within a wide range of cooling rates (from 5 to 20 °C $min^{-1}$). The highest cooling rate (5580 °C $min^{-1}$) increased glass transition temperature significantly, compared to the values at the slowest cooling rates (5–20°C $min^{-1}$). This change in $T_g$ inflexion (by 1.2 °C $min^{-1}$) did not influence considerably the glass transition region because the whole transition interval was, on average, 7 °C. However, no significant differences were found between $T_g$ obtained with the highest cooling rate and that with the middle cooling rate (360 °C $min^{-1}$). In conclusion, $T_g$ of plant vitrification solutions did not significantly change when the cryopreservation methods based on either direct plunging samples into liquid nitrogen or plunging of samples in closed cryovials were used. We can conclude that the $T_g$ of commonly used PVSs did not change with the cooling rates tested.

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*27. Physiological–biochemical characteristics of Pisum sativum seedlings after long-term storage of seeds in the permafrost conditions.* I.A. Prokopiev, G.V. Filippova, A.A. Shein, E.S. Khlebnyy, Institute of Biological Problems of Cryolithozone SB RAS, Yakutsk, Russia

Loss of seed viability cannot be prevented, this process is irreversible, but it is quite possible to slow the process down if the necessary storage conditions are created. In this context it is very important to develop and use methods of long-term storage of seeds. It is known that some of the main factors affecting the duration of seed storage are temperature and humidity (moisture content). Long-term storage of plant material in the form of seeds is one of the most popular and effective methods to the preservation of most species of the world. Creation of seed banks has significant advantages over other methods for plant preservation *ex-situ*: storage of a large number of samples is simplified, space saving and has relatively low labour costs.

The material for the study were the seeds and seedlings of three cultivars ("Imposant", "Latores" and "Rosol") of pea (*Pisum sativum* L.). Seeds of 1977 harvest were laid down in long-term storage in permafrost in the underground laboratory of the Institute of Permafrost, SB RAS (Yakutsk) to a depth of 12 m. Storage for 34 years was carried out in hermetical glass vessels of 100 $cm^3$ at a constant temperature of −6.5 ± 0.5 °C. Before laying down the seeds for storage their moisture content did not exceed 5–7%. As a control seeds and seedlings were used from the same cultivars of *P. sativum* 2007–2009 from the collection of N. I. Vavilov Research Institute of Plant Industry.

It is shown that after 34 years of low temperature (−6.5 ± 0.5 °C) storage of three cultivars seeds ("Imposant", "Latores" and "Rosol") of pea (*Pisum sativum* L.) under permafrost conditions, the physiological parameters of seed and seedlings (germination, root length, dry weight), as well as the mitotic activity of roots cells did not differ from control. However, a small increase in the number of abnormal ana-telophase of mitosis in root meristem cells of cultivars "Imposant" and "Latores" was shown in comparison to the control. After long-term storage of three *P. sativum* cultivar seeds balanced work prooxidant-antioxidant systems in a simple compensation regime was observed in the tissues of seedlings. It was noted that long-term storage of seed cultivars "Imposant" and "Latores" caused a decrease of photosynthetic pigment content in the seedling tissue that along with a small increase of the number of abnormal mitoses may be evidence of the beginning of the process of aging of seeds.

In general, it is shown that long-term storage of seeds of three cultivars of *P. sativum* in permafrost conditions enabled the preservation of their viability and can be offered as prospective way to store seeds.

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*28. Using Synchroton infrared microspectroscopy to better understand the freezing-resistance of lactic acid bacteria.* J. Gautier [1], S. Passot [2], F. Jamme [3,4], S. Cenard [1], F. Fonseca [1], [1] INRA, UMR 782, 78850 Thiverval Grignon, France, [2] AgroParisTech, UMR 782, 78850 Thiverval Grignon, France, [3] Synchrotron SOLEIL, SMIS Beamline, 91192 Gif-sur-Yvette, France, [4] INRA, CEPIA, 44026 Nantes, France

Freezing is commonly applied to preserve the functionalities of concentrates of lactic acid bacteria (LAB). However, it is still a critical step in the production of LAB concentrates, as it affects both the viability and acidifying activity upon thawing. Several environmental factors influence the resistance to freezing of LAB (strain, medium composition, temperature, etc.) but the mechanisms of cell injury during