previously marked and by zones (to facilitate the subsequent nematodes counting). 24 hours after rewarming, we counted the specimens that were crawling. In adults, we achieved an average survival rate of 43.9% with maximum of 84.5% and warming rates around 100 °C/min with thermal gradients lower than 10 °C.
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P63
CHANGES OF CRYOTOLERANCE DURING SEED DEVELOPMENT IN POMELO AND GRAPEFRUIT

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Pomelo and grapefruit are commercially important tropical and subtropical fruit crops and both produce intermediate seeds. Their seeds can be desiccated to below 10% moisture content (fresh weight basis) safely, but significant seed viability losses (≥15%) were detected after only 12-months' low-temperature storage at both 4°C and -18°C, thus cannot be stored in conventional seed bank for long-term germplasm conservation, and cryopreservation is the only method to ensure this purpose, necessitating development of cryopreservation protocol. To develop cryopreservation protocol, there is a critical question to be answered firstly: *When to harvest the seeds?* Our studies revealed changes in cryotolerance of pomelo and grapefruit seeds during development. Although no seeds survived cryo-exposure at early stages of their development, 20% postthaw seeds emerged around 150 days after flowering; cryotolerance increased with seeds development, and maximized around 250 days after flowering, with only a small fall in cryotolerance at the last stage of seed development. This cryotolerance development pattern differs from those of orthodox and recalcitrant seeds previously reported. Mature pomelo and grapefruit seeds had cytological traits characterized by orthodox seeds, i.e., organelles concerning metabolism were rare except sporadic mitochondria, embryonic cells arrested at an inactively metabolic status, but poor in accumulation of soluble and heat-stable proteins, indicating cytological and biochemical basis of cryotolerance. This knowledge can be a help for seed collection when developing cryopreservation protocol for pomelo and grapefruit seeds.
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P64
BIOTECHNOLOGY FOR VIRUS-FREE PLANTING STOCK OF APPLE AND TREE NUTS USING CRYOTHERAPY

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Improved fruit and nut production is dependent on healthy planting stocks. Many traditionally propagated cultivars have accumulated a heavy virus load, making them less productive. The goal of this project was to use cryotherapy to eliminate viruses from important cultivars. *In vitro* apple cultures infected with one or more viruses, *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* and *Apple mosaic virus*, were subjected to cryotherapy. For cryotherapy shoot apices (0.8 mm) were aseptically isolated from 3-week cold acclimated shoots (8 hours at 22°C, light intensity 10 $\mu E \bullet m^{-2} \bullet s^{-1}$/16 hours at -1°C in the dark). Apices were cultured on Murashige-Skoog medium with 0.3 M sucrose for 2 days under cold acclimation and cryopreserved using a PVS2 vitrification protocol. After immersing in liquid nitrogen, shoot tips were rewarmed and placed on multiplication medium for regrowth. On average, 40.1% of the tested materials were virus-free after one cryotherapy procedure. Virus elimination varied from 12% to 100% among the genotypes. Cryotherapy efficiency depended on apple cultivar studied and virus (or viruses) infected. 'Rennet Landsbergskyi' mix-infected by ACLSV and ASPV retained both viruses in all shoots after cryotherapy whereas 'Arm 18' infected by ACLSV was totally virus-free following cryotherapy. Since apple is a clonally propagated crop, even a relatively low percentage of virus elimination can provide virus-free cultures for producing stock plants. Recovered shoot tips were micropropagated, rooted, and transferred to containers with sterile soil and covered with plastic caps. Virus-free apple stocks were obtained, and a similar procedure was used for virus elimination in hazelnut and walnut shoots. The resulting *in vitro* collection will serve as the basis for the creation of a germplasm cryobank, and for obtainining super elite plant stocks for distribution to nurseries.
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P65
DEVELOPMENT OF THE D CRYO-PLATE METHOD FOR CRYOPRESERVATION OF GARLIC (*ALLIUM SATIVUM*)

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The D cryo-plate method was successfully adapted for the cryopreservation of garlic plants (*Allium sativum*). The optimal D cryo-plate method for garlic is as follows. Shoot tips were collected from harvested bulbs and precultured for 1-3 days at 25°C on solidified MS medium containing 0.3 M sucrose. Precultured shoot tips were placed in wells on an aluminum cryo-plate and embedded in calcium alginate gel. Osmoprotection was performed by immersing the cryo-plates for 30 min at 25°C in LS (2.0 M glycerol and 1.0 M sucrose). The optimal dehydration time of shoot tips by air dehydration under the air current of a laminar flow cabinet for 30 to 180 min at 25°C. Cooling was performed by transferring the samples in uncapped 2 ml cryotubes held on a cryo-cane which was directly plunged into liquid nitrogen. Regrowth rate of cryopreserved shoot tips was 100%. Furthermore, shoot tips treated as described above but then air-dried for 120-180 min can be stored at -80°C for at least 1 week without a significant decrease in survival rate, which is convenient for most laboratories that have a -80°C freezer but not a liquid nitrogen container for long-term storage. These preservation techniques for *Allium* spp. should increase their availability in the breeding community.
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Conflict of Interest: None to disclose

P66
IN VITRO STORAGE AND CRYOPRESERVATION OF CLONALLY PROPAGATED PLANT GERMPLASM IN KAZAKHSTAN

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In vitro storage and cryopreservation are now considered reliable ways to provide medium and long-term *ex situ* conservation of clonally propagated plant germplasm. The Institute of Plant Biology and Biotechnology is developing techniques for cryopreservation of shoot tips for several fruit, berry, tuber and nut-bearing crops. Aseptic *in vitro* cultures were established by testing for endophytic infection on the 523 detection medium. The PVS2-vitrification protocol optimized for *Malus* germplasm was successful with few modifications for diverse germplasm cryopreservation. The modifications included variations of *in vitro* shoot cold acclimation (temperature regime, constant or variable temperatures, duration of cold

acclimation), pretreatment media composition for isolated shoot tips, and PVS2 exposure time and temperature. For apple and barberry genotypes tested three or four weeks cold acclimation of shoot cultures at alternating temperature (22°C with 8 h light (10 μmol / m^2 / s)/ -1°C 16 h darkness), preculture of isolated shoot tips on Murashige and Skoog (MS) medium with 0.3 M sucrose for two days at cold acclimation conditions, and PVS2 treatment of shoot tips on ice for 80 min were required for successful cryopreservation. Cold acclimation of potato shoot cultures was not needed. Preculture of shoot tips on MS medium with 0.3 M sucrose at 24°C and PVS2 treatment of shoot tips for 30 min at 24°C were effective for potato. Recovery of shoot tips of 15 apple cultivars ranged from 55.6 to 84.6%, 10 rootstoks from 68.2 to 95.4% and 14 wild forms of *Malus* from 70.8 to 92.1%, 10 wild forms of *Berberis* from 50.6 to 83.2% and 20 potato cultivars and hybrids form 41.2 to 68.5% following liquid nitrogen exposure. The first cryogenic bank at -196°C for preservation of valuable clonally propagated plant germplasm has been established in Kazakhstan.
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P67
CRYOPRESERVATION OF HUMULUS GERMPLASM

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Cryopreservation is a process of plant conservation under ultra-low temperatures of 196 °C under zero in a liquid nitrogen. It is used for conservation of plant germplasms, which are multiplied in a vegetative way. This method contributes to the conservation of genetic stability and prevent from ageing. It is used for conservation of genetic resources of cultural and wild plants in such virus free material, which is endangered by depreciation caused by biotic and non-biotic stresses if multiplied in ex vitro conditions.The hop plant, *Humulus lupulus*, L., is a dioecious perennial species, and only female clones are used for beer brewing. Hops are used to impart bitterness, flavour and preservation properties to modern beers.*In vitro* cultures were derived from extracted meristems tips. Nodal cuttings were acclimated by low temperature and sucrose treatment. Isolated shoot tips were loaded with 0.7M sucrose for overnight and subsequently dehydrated above silicagel for approximately 100 minutes on aluminium plates. Shoot tips were plunged directly into liquid nitrogen. Control explants were thawed at 40 °C water bath and regenerated on medium for 8 weeks. Altogether 45 hop genotypes have been cryopreserved with average recovery rate of 40%. 79% of accession showed higher plant recovery than 30%. The minimal number of plants to recover for each cultivar was calculated as a sum of minimal numbers of viable plants in particular cryopreservation procedures. The methods used and results are presented on Czech variety Kazbek and new breeding material of hop number 13966 and 14516, 13971.
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P68
EFFECT OF BLANCHING, FREEZING AND THAWING ON OLIVE FRUITS QUALITY

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Freezing of olives includes an initial blanching step, followed by freezing itself and thawing. Fresh olive fruits (*Olea europaea* L. vars. *Manzanilla* and *Hojiblanca*), collected at the appropriated ripening stage, were immersed in a water bath at 95 °C for 2.5 min, subjected to calcium treatment, frozen by forced convection with air at -30 °C, packed under slight vacuum, stored at -24 °C (48 h) and thawed in sodium hydroxide (NaOH) solution at 1%. Olive firmness was determined through a texture analyzer equipped with a 2-mm diameter cylinder probe. Blanching operation had the most negative effect on alterations in drupe consistency. Compared to fresh samples, significant decreases in the cuticle hardness and pulp consistency were observed varying from 8.98 to 5.55 N and 9.66 to 6.19 Nmm, respectively in *Manzanilla* fruit and from 12.6 to 7.53 N and 11.3 to 6.48 Nmm, respectively in *Hojiblanca* one. However, subsequent freezing and thawing only cause an additional loss of cuticle hardness and pulp consistency, with respect to mechanical properties of fresh fruits, of 6.9 and 9.2% in *Manzanilla* and 8.0 and 12.8%, in *Hojiblanca* olives, respectively. Total chlorophylls content (TCC) and total carotenoids (TC) were determined by a spectrophotometric assay. TCC was similar in both fresh fruits (49.0±5.57 and 55.6±5.29 μg/g fw in *Manzanilla* and *Hojiblanca*, respectively). Blanching did not significantly change the TCC in *Hojiblanca* olive, but significantly increased (23%) in *Manzanilla*. TC was significantly higher in *Hojiblanca* than in *Manzanilla* (90.5±6.68 and 68.4±4.34 μg/g fw, respectively), and blanching increased ($p < 0.05$) TC by 68% and 14% in both cultivars, respectively. Complete processing maintained the TCC of both fresh drupes, but increased by 70% their initial TC. Results were dependent on cultivar and can help in designing blanching and freezing operations which can be applied in olive industry.
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Conflict of Interest: None to disclose

P69
LONGEVITY OF SEEDS OF SIX BRITISH ORCHID SPECIES IN CRYOPRESERVATION AND CONVENTIONAL STORAGE AT THE MILLENNIUM SEED BANK (ROYAL BOTANIC GARDENS, KEW)

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Seeds ≤ 0.2 mm are known as micro-seeds. Long-term survival of micro-seed collections stored in ex-situ seed banks is not clearly understood and data is lacking to inform an optimal strategy for seed conservation. Orchids (family Orchidaceae) are economically and culturally valuable plants which produce micro-seeds. Although orchid's seeds are desiccation tolerant (and so able to be stored using conventional storage methods at ~15% eRH and -20°C), it is thought that they may have short life spans at such conditions and cryopreservation (storage at ~-197°C) may be needed to extend seed survival rates in long-term storage. As such, since 2012, the protocol for the curation of micro-seeded species at the Royal Botanic Gardens, Kew's Millennium Seed Bank (MSB) is to duplicate the collection in both conventional storage and cryopreservation. We have analysed routine viability test results of six collections of British orchid species (*Dactylorhiza fuchsii, D. viridis, Gymnadenia borealis, Liparis loeselii, Ophrys apifera* and *Orchis mascula*) to compare longevity between storage methods following two to three years storage. At the point of storage, each collection was duplicated to both conventional and cryo-storage at ~15% eRH. Where germination data is unavailable, a Fluorescein Diacetate (FDA) test is used to indicate seed viability. All germination tests were performed using the standard MSB protocol for temperate, terrestrial orchids on BM1 medium. Preliminary results from on-going tests indicate that seeds stored in conventional storage are retaining comparable viability when compared to those stored in cryo-preservation over this two to three year period. More time may be needed to assess whether storage conditions have impacted on seed longevity for these collections in the long-term and hence the suitability of the current protocol for the ex-situ conservation of orchid seed in the MSB.
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