CryoLetters 44 (1), 1 – 12 (2023)

https://doi.org/10.54680/fr23110110112

PERSPECTIVE

UNIFYING PRINCIPLES OF CRYOPRESERVATION PROTOCOLS FOR NEW PLANT MATERIALS BASED ON ALTERNATIVE CRYOPROTECTIVE AGENTS (CPAS) AND A SYSTEMATIC APPROACH

Haeng-Hoon Kim[1*] and Elena Popova[2*]

[1] Department of Agricultural Life Science, Sunchon National University, Suncheon, 57922, Korea.
[2] K.A. Timiryazev Institute of Plant Physiology of Russian Academy of Sciences, Moscow, 127276, Russia
* Corresponding authors' E-mails: cryohkim@scnu.ac.kr; elena_aygol@hotmail.com
Both authors contributed equally to this paper.

Abstract

This review addresses a frequently encountered problem of designing an effective cryopreservation procedure for new (not previously cryopreserved) or difficult plant materials. This problem hinders worldwide efforts of applying cryopreservation across a wide genetic base of wild and a number of cultivated plants. We review recent advances in modifications of routinely applied cryoprotective solutions (CPAs) and suggest a practical approach to protocol development which embraces the physiological complexity of plant tissues as well as a wide spectrum of behaviours under CPA treatment. We suggest that vegetative plant materials are classified into four categories based on their size, structure, and the response to osmotic and chemical stresses provoked by CPA mixtures of varied composition and concentration, including alternative osmoprotection and vitrification solutions. A number of up to 15 preset protocols designed specifically for each category is then applied to the material. The protocols resulting in the best regrowth are then combined into the optimized procedure. The main advantage of this system over a conventional "trial-and-error" search for working cryopreservation protocol is a minimal amount of starting materials required for the tests and a relatively accurate prediction of material behaviour under cryopreservation stress provided by the relatively few CPAs treatments. The unifying principles revealed by this approach could broaden a spectrum of wild species and materials which can be safely conserved by cryopreservation. Also anticipated is application of this approach to plant materials of biotechnological value as well as cultivars of agricultural and horticultural crops which do not respond well to standard protocols developed for their kind.

Keywords: alternative vitrification solutions; droplet-vitrification; endangered species; osmoprotection; systematic approach.

INTRODUCTION

Back in 1970-80s, cryopreservation was envisioned as the most reliable method for the long-term conservation of plant genetic resources (PGR) (1, 2, 3). Since then, it was tested, with greater or lesser success, on hundreds of species of agricultural, horticultural and medicinal importance (4, 5, 6, 7, 8). At the time of this review, over 20 world PGR genebanks have routinely operating cryobanking facilities with priorities given to staple crops that are vegetatively propagated or have recalcitrant seeds (9). By 2017, over 15,000 crop varieties were

permanently frozen in cryobanks worldwide (10), most as in vitro propagules cryopreserved through a droplet-vitrification (DV) or other vitrification-based methods (11, 12, 13, 14, 15, 16, 17), others as dormant buds (11, 18, 19, 20, 21), pollen, or embryonic tissues (22, 23, 24, 25, 26). However, these cryopreserved materials represent less than 16% of the global genebank collections. In addition, it was estimated that over 100,000 unique accessions remain potentially vulnerable in the field, in need for a safety cryo back-up (10). Transferability and a wider use of cryopreservation is largely hampered by the absence of a universal protocol applicable across the wide genetic base (27). Even within one crop, individual accessions or accession groups may return zero or unacceptably low regrowth when cryopreserved via a seemingly well-developed procedure (12, 28).

The use of cryopreservation to securely conserve wild flora is even of greater concern. In 2020, the Global Strategy for Plant Conservation reported that Target 8, which aimed to have 75% of threatened plants in ex situ collections, was not feasible, partially due to limited information on threatened species and species that produce non-bankable seeds (29). This is particularly true for species with recalcitrant, short-lived or strongly dormant seeds as well as for species at point of extinction with few individuals left. A number of studies demonstrated that cryopreservation combined with in vitro seed germination and plant multiplication might give exceptional species a new hope (30, 31, 32, 33). Yet, both national and international efforts in this direction are hindered by often unknown chilling and dehydration tolerance of wild species and genotype-specific responses to cryoprotective treatments. In addition, slow growth and low multiplication rate, frequently observed for wild species in vitro (25, 34, 35), limit the quantity of material available for multi-factorial cryo-experiments. When considered together with restricted funds often granted to a time-limited program (e.g. MSc or PhD student project), the results may not satisfy the effort and the researchers, after several unsuccessful attempts to develop a working cryopreservation protocol for a new species, would be forced to switch to a more "fruitful" topic.

There is, therefore, a great emerging need for a strategic approach to design a working cryopreservation protocol: (i) in the shortest possible time period; (ii) for any of the most commonly used types of plant material; and (iii) with limited number of experiments and minimum sufficient number of explants.

This approach, while based on fundamental principles of cryobiology, should step beyond the limitations of currently available methodology, and particularly two common cryoprotectant mixtures, PVS2 and PVS3, which have now been applied for over 30 years (36).

This review highlights recent research towards the development of such an approach with the emphasis on in vitro grown material – shoot apices, axillary shoot tips, hairy and adventitious roots, and embryogenic tissues.

CRYOPROTECTIVE AGENTS

CPAs development to cope with freezing injuries

The advances in PGR cryopreservation, including discovery and implementation of cryoprotective agents (CPAs), is a result of cross-disciplinary empirical and fundamental research (37). Consequently, basic principles of cryoprotection and the most effective CPAs are shared between medical, animal, and plant cryo-worlds. Following Maximov's pioneer works on cryoprotective properties of sugars and glycerol during plant freezing (38) and classical works of Polge (39) and Lovelock (40, 41) demonstrating cryoprotective actions of glycerol and dimethylsulfoxide (DMSO) on animal and plant tissues, multiple CPAs have been identified and employed as individual compounds or, more frequently, in mixtures (42). Glycerol, sucrose, DMSO and ethylene glycol (EG) remain the most common components of CPA formulations (43, 44).

However, plants are much more "demanding" for the sufficient dehydration and cryoprotection compared to animal or microorganism specimens. The main problem is high (80-99%) initial water content (WC) in vegetative plant materials and the concomitant danger of ice crystallization during cooling and rewarming (45, 46). Sufficient dehydration of plant samples is required to achieve vitrification of the protoplast upon liquid nitrogen (LN) exposure, i.e. transition of water from the liquid phase directly into an amorphous or glassy state, thereby avoiding the lethal formation of intracellular ice (47, 48, 49). Nonetheless, while escaping from a wide range of ice-induced injuries, plant cells may be pushed too close towards extensive, and harmful, dehydration (46, 50). The most harmful effects of the latter include, among others, irreversible phase

transition in the plasmalemma and organelle membranes causing the loss of semi-permeability and other vital functionalities, destabilization of macromolecule structures, increasing cytoplasm viscosity and concentration of potentially toxic electrolytes in dehydrated protoplasts (51, 52, 53, 54). Hence, the effecive cryoprotection treatment is built on achieving a fine balance between avoiding the lethal freezing of intracellular water and minimizing the detrimental effects of its removal (55, 56).

CPAs and water content in plant tissues

Thermal analysis suggested that the safe upper WC level for cryopreserving vegetative plant propagules (shoot tips, axillary or dormant buds) can be vaguely defined as 25-33% on a fresh weight (FW) basis, or ca. 0.33 to 0.4 gH_2O/g DW (57, 58, 59), although it varies depending on the material type and species. A small portion of this moisture (up to 0.25 gH_2O/gDW, or 20% FW) is usually represented by "non-freezable" water which is bound by membranes and macromolecules and is crucial to maintain their conformation and functionality (60, 61).

In modern cryopreservation methods, dehydration is achieved osmotically when bathing plant material in concentrated CPA mixtures, or vitrification solutions (VS), followed by direct quick immersion in LN (36, 47). The most commonly used CPAs are four-component PVS2 which consists of, w/v, 30 % glycerol, 15 % ethylene glycol (EG), 15 % DMSO and 0.4 M sucrose (62), and a two-component PVS3 comprising, w/v, 50 % glycerol and 50 % sucrose (63). Various modifications of these two basic solutions have also been tested on plant materials with promising results (44, 64, 65).

The time required to reach a safe dehydration level depends upon a number of factors, e.g. osmotic pressure of the CPA mixture, sample size and structure, and its permeability for individual CPAs. The dehydration process seems to be nonlineal. For example, WC reduction of garlic shoot tips during exposure to PVS3 was bi-phasic: the WC fell from the initial 80% to 47% during the first 30 min of treatment then declined slowly during the next 90 min until reaching 33%, compared to the maximum (80%) regrowth after cryopreservation (64).

There are evidences, however, that in some cases, plant explants are cable of surviving cryopreservation at WC above the 'average' safe level and tolerate crystallization of some of their water. Bilavčík et al. reported that apple dormant bud segments could withstand crystallization of 8.9–12.4% of water in their bud tissues (57). Moreover, bud regrowth after cryopreservation showed no obvious correlation with their remaining WC. Apices excised from cloves of *Allium sativum* showed 87-99% regrowth after being dehydrated with PVS3 to 37-45% WC (64). Even after dehydration to 25-33% WC, crystallization was still detected in apex tissues during rewarming, but the explants were able to recover (66).

These data suggest that plant tissue survival during cryopreservation involves a complex cellular adjustment, and CPA mixtures act beyond simple osmotic dehydration.

"Standard" CPAs mode of action and the quest for alternatives

The effective CPAs are thought to act through reducing the temperatures of freezing and glass transition (*Tg*) in cell solutes, inhibit ice nucleation and growth and affect the shape of the ice crystals (46, 67). They are known to mitigate toxic effects of concentrating electrolytes and to stabilize cell membranes and macromolecules during dehydration and cooling-rewarming events (42). Sugars and alcohols, e.g. glycerol, are thought to replace water in the hydration "shells" of macromolecules during dehydration (61). At severe dehydration, they may interact with proteins and polar head groups of membrane phospholipids through direct hydrogen bonding, thereby stabilizing their conformation and preventing liquid crystalline-to-gel phase transition in membranes (42, 60, 68, 69, 70). Volk & Walters (56) suggested multifaceted action of PVS2 on garlic and mint shoot tips which included osmotic dehydration, replacing the water by CPA components and changing water behavior during cooling-rewarming. In *Magnolia officinalis* embryogenic culture, PVS2 treatment induces membrane lipid remodeling and increases in total lipid concentration (70). Free radical scavenging and potent antioxidant activities are also reported for some CPAs (37, 42).

It is logical to assume that regrowth of organized plant tissues, such as shoot tips, after cryopreservation is higher when more cell clusters survive in the explant. Hence, the protective action of CPAs is likely to be enhanced through their penetration inside the samples. However, data on CPA accumulation in plant tissues is scarce. In garlic shoot tips, sucrose concentration raised from 1.1 to 117.4 mg/g FW

during the initial 30 min of PVS3 exposure followed by a slower increase to 128.0 mg/g during the next 150 min (71). In a similar way, glycerol concentration in shoot tips increased rapidly (from 14.0 to 100.2 mg/gFW) within the first 30 min of PVS3 treatment, and to 128.0 mg/gFW during the next 30 min. Concentration of both compounds reached a plateau after 2 h of exposure, coinciding with maximum regrowth. There was a negative correlation between the accumulation of both compounds and WC of shoot tips. The kinetics of DMSO penetration into shoot tips also displayed a bi-phasic curve, but with much faster rates compared to sucrose or glycerol: more than half of the total DMSO (75 mg/gFW) entered shoot tips within 5 min of exposure to PVS2 while the maximum concentration of 85 mg/gFW was reached after 30 min (72).

Similar to WC loss, penetration of CPAs is affected by explant size and cryoprotectant chemical structure. Very similar DMSO concentrations (38-41 mg/g FW) were found in shoot tips of different sizes (1.5 to 3.5 mm) after 20 min treatment with PVS2 (72). By contrast, much longer (150 min) treatment with a more concentrated PVS3 resulted in very variable content of glycerol and sucrose in shoot tips: in 1.5 mm explants, glycerol and sucrose reached 171 and 130 mg/gFW, respectively, compared to 121 and 96 mg/g in 3.5 mm shoot tips (71). Both pace and the amount of PVS2 components accumulation was different between garlic and mint shoot tips. This was likely due to their different size and structure, with highest accumulation of "volatile compounds" (determined as the difference between total volatile mass and water fraction after heating the shoot tips) achieved within 60 min (56).

These and other literature data suggest that 30-60 min exposure to PVS2/3 may be an optimum timeframe required to achieve an acceptable dehydration level in medium-sized plant explants and allow glycerol and sucrose time enough to penetrate into the samples. This time window is also close to the empirical findings reported for many crops and wild species (15, 73, 74, 75, 76, 77, 78). However, this time may be too short for sufficient dehydration and cryoprotection of large and hard-structured explants (12, 79) and too long for materials that are small and dehydration-sensitive (80, 81, 82). These differences in optimum CPA exposure windows for different materials are illustrated in Figure 1 using representative data for the materials of different types.

As displayed in Figure 1, plant materials can be classified into four groups depending on their optimum CPA treatment window:

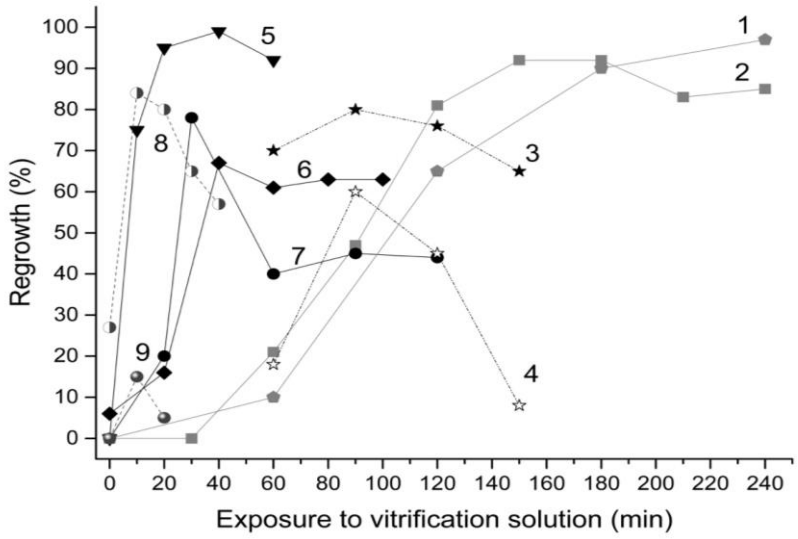

Figure 1. Representative regrowth curves of different plant materials cryopreserved after various duration of exposure to concentrated CPAs (plant vitrification solutions): lines 1, 4 – PVS2 (0°C); 2, 6 – PVS3; 3 – PVS3 (0°C); 5 – A3-80% (0°C); and 7, 8, 9 – B5.
Explants: 1 – *Lilium* hybrid, shoot tips from adventitious shoots (93); 2 – *Allium sativum*, clove shoot tips (66, 71); 3 – *Chrysanthemum morifolium*, shoot tips (86); 4 - *Lilium* × *siberia*, shoot tips (79); 5 – *Kalopanax septemlobus*, embryogenic culture (77); 6 – *Castilleja levisecta*, shoot tips (76); 7 – *Hypericum perforatum*, root segments (92); 8 – *Rubia akane*, hairy root tips (80); 9 - *Panax ginseng*, adventitious root tips (81).

Group 1 (lines 1 and 2 in Fig. 1) is represented by physically hard and large samples that are very tolerant to both osmotic and chemical action of CPAs, e.g. garlic clove apices or lily shoot tips or bulbils, with slow dehydration and optimum time of exposure above 120 min.

Group 2 (lines 3 and 4 in Fig. 1). Moderately-tolerant explants with a relatively large safe window of exposure to concentrated CPAs and optimum exposure times around 90 min.

Group 3 (lines 5, 6 and 7 in Fig. 1). Moderately sensitive explants with rather narrow CPA treatment window and optimum exposure times around 30-40 min. Sometimes these explants have the ability to tolerate longer exposure while retaining acceptable (40-60%) regrowth level.

Group 4 (lines 8 and 9 in Fig. 1). Explants that are very sensitive to both chemical and osmotic stresses with a sharp peak of optimum CPA exposure duration below 20-25 min.

In all groups, shorter than optimum exposure duration results in insufficient cryoprotection and dehydration while with longer exposure cytotoxic effects of CPAs prevail and reduce the regrowth.

Not surprisingly, the vast majority of cryopreservation studies are focused on experimenting with CPA treatment duration, sometimes combined with low temperature to reduce toxicity and prolong the exposure (78). Standard CPA mixtures PVS2 and PVS3 have been successfully tested across a wide range of genotypes (36, 43, 73). However, it became evident that they do not always provide optimum dehydration-cryoprotection balance. PVS3 has a very strong osmotic action while high concentrations of DMSO and EG in PVS2 are toxic to living tissues (83). Experiments with alternative two- and four-component CPA mixtures with altered concentrations of individual components demonstrated that some materials are more sensitive to the chemical rather than the osmotic toxicity of CPAs (25, 64, 84). For example, in chrysanthemum shoot tips, elevating DMSO+EG concentrations by only 10% caused a 50% dropdown in regrowth after CPA treatment (64).

Multiple experiments including regrowth tests and thermal analysis of enthalpies and onset temperatures of cryoprotected explants led to a selection of the most promising alternative CPA formulations (Table 1). This formulations provide balance between dehydration and cryoprotective properties of permeating and non-permeating CPAs. These solutions and their combinations have been shown to be more effective than standard PVS2 and PVS3 with a number of plant species, including *Allium sativum, Chrysanthemum morifolium, Aster altaicus, Prunus* and *Malus x domestica* cultivars, *Rubia akane, Kalopanax septemlobus, Pogostemon yatabeanus* and some others, providing on average a 20-65% increase in post-cryopreservation regrowth compared to standard PVS2 and PVS3 treatments (64, 74, 77, 85, 86, 87, 88).

SYSTEMATIC APPROACH TO CPA DESIGN AND PROTOCOL DEVELOPMENT

Understanding the multi-step cryopreservation protocol

The frequently encountered problem in plant cryopreservation is that samples are not

Table 1. Composition of osmoprotectant and vitrification solutions and their promising alternatives.

Solution	Composition (%, w/v)*	Total concentration (%, w/v)	Endothermic enthalpies, (J g^{-1} FW)**	Ref.
	Osmoprotectant solutions			
LS	G 18.4 + S 13.7	32.1	-139.9 ± 7.0	63
C4	G 1.9 + S 0.5	35.0	-134.2 ± 5.9	25, 64
	Vitrification solutions			
PVS2	G 30.0 + DMSO 15 + EG 15 + S 13.7	73.7	-35.9 ± 1.8	62
VS A3	G 37.5 + DMSO 15 + EG 15 + S 22.5	90.0	-0.3 ± 0.1	64
PVS3	G 50 + S 50	100.0	-6.9 ± 1.5	63
VS B5	G 40 + S 40	80.0	-48.3 ± 8.9	64

*DMSO, dimethyl sulfoxide; EG, ethylene glycol; G, glycerol; S, sucrose

** Endothermic enthalpies of osmoprotectant and vitrification solutions diluted to 50% and 80%, respectively; FW – fresh weight.

sufficiently dehydrated and cryoprotected, even following the optimum VS exposure. This problem is partially solved by use of the droplet-vitrification (DV) method, which applies two complementary strategies. First, samples are cryopreserved on aluminum foil strips in microliter drops of concentrated CPAs which increases cooling and rewarming rates and hence leaves less chances/time for ice (re)crystallization in insufficiently dehydrated samples compared to freezing/rewarming in vials (78). Second, the use of a series of sequential CPA treatments to slowly prepare material for dehydration reduces the damaging action of CPAs in the pre-LN steps, and 'buffers' the extensive rehydration during rewarming and CPA removal (post-LN steps) (36).

The standard DV protocol consists of material excision, its preculture on high-sucrose containing medium, osmoprotection (otherwise known as "loading") in CPA mixtures of moderate concentrations (35–45%), cryoprotection/dehydration with highly concentrated CPAs (VS, up to 100%), cooling, rewarming, washing off cryoprotectants (unloading) and recovery (Fig. 2). The total CPA concentration increases steadily in the course of the pre-LN treatments, reaches a maximum at the cryoprotection step and then decreases again during unloading and reculture on standard medium (Figs. 2, 3).

The impact of each protocol stage to the success of cryopreservation has been previously reviewed (14, 36, 78). Thermal analysis of cryoprotected apices during cooling and rewarming (Fig. 3) suggested that preculture had little effect on thermal behavior of explant water and, in general, serves to induce metabolic and structural changes in plant material that enables greater tolerance of exposure to highly concentrated CPAs (70, 89, 90). During pre-LN steps, CPAs penetrate into samples while causing their WC to decrease (Fig. 3). A notable shift in sample enthalpies and onset temperatures is usually recorded at the dehydration/cryoprotection steps.

The concentration and composition of CPAs and duration of each step play a vital role in plant material regrowth. Standard conditions include preculture with 0.3-0.5 M sucrose for 1-3 days, 20 min osmoprotection with 2 M glycerol + 0.4 M sucrose, treatment with PVS2 (or PVS3) for varied durations, and unloading with 1.2 M sucrose for up to 40 min. This scheme with several modifications is usually applied to newly researched plant material.

However, the success or failure of this approach may be a matter of pure luck. In the case of misfortune, the researcher is forced to test multiple combinations of these treatments via one-factor or orthogonal design with a hope to find a combination that shows enhanced (or at least some) regrowth. Also referred to as the *experimental* or *trial-and-error* approach, this blind screening of multiple conditions is time- and labour-consuming and may not be feasible for wild species due to limited number of samples.

Benson (47) suggested that the potential way to cope with unknown sensitivity of plant material in multi-factor procedures is to have an arsenal of different methods (DV, encapsulation-dehydration, encapsulation-vitrification, cryo-plate, preculture-desiccation) ready to be applied. However, this requires staff to be experienced in professionally implementing more than one cryopreservation methodology, which is not the case in many emerging genebanks or botanic gardens. An alternative to consider is having a series of treatments based on one standard DV procedure which could be shifted in a predictable way to test new material to reveal its sensitivity barriers. This is where alternative CPAs comes into view.

Having new CPA mixtures introduced into the already complicated procedure may seem illogical. However, when combined with the existing treatment sequence and traditional PVS2 or PVS3, these alternative solutions equip the DV protocol with the previously lacking adaptation flexibility achieved by manipulation with a wider range of osmotic and chemical action of CPAs at both pre-LN and post-LN stages (Fig. 2).

The systematic approach is based on the assumption that plant materials of different sensitivity groups (as shown in Fig. 1) require different strategies in both pre- and post-LN treatment. In general, the more sensitive the explant is, the more gradual should be the change in CPA concentrations at both preculture and osmoprotection steps. Except for Group 1, the application of CPAs containing DMSO and EG is recommended at 0°C to reduce their toxic effects.

Table 2 summarizes the series of standard treatments and their variations proposed for each material group.

Group 1 samples require long exposure to concentrated CPAs (e.g. PVS3) to be properly dehydrated. Preculture with 0.3-0.5 M sucrose for

1-2 days as well as osmoprotection for 20 min may improve the regrowth but these steps are not always necessary.

For Groups 2 and 3, the strategy is focused on material adaptation to the upcoming dehydration and toxic actions of CPAs. Therefore, both preculture and osmoprotection treatments are necessary and need to be carefully designed. In most cases, preculture with progressively increasing sucrose concentrations

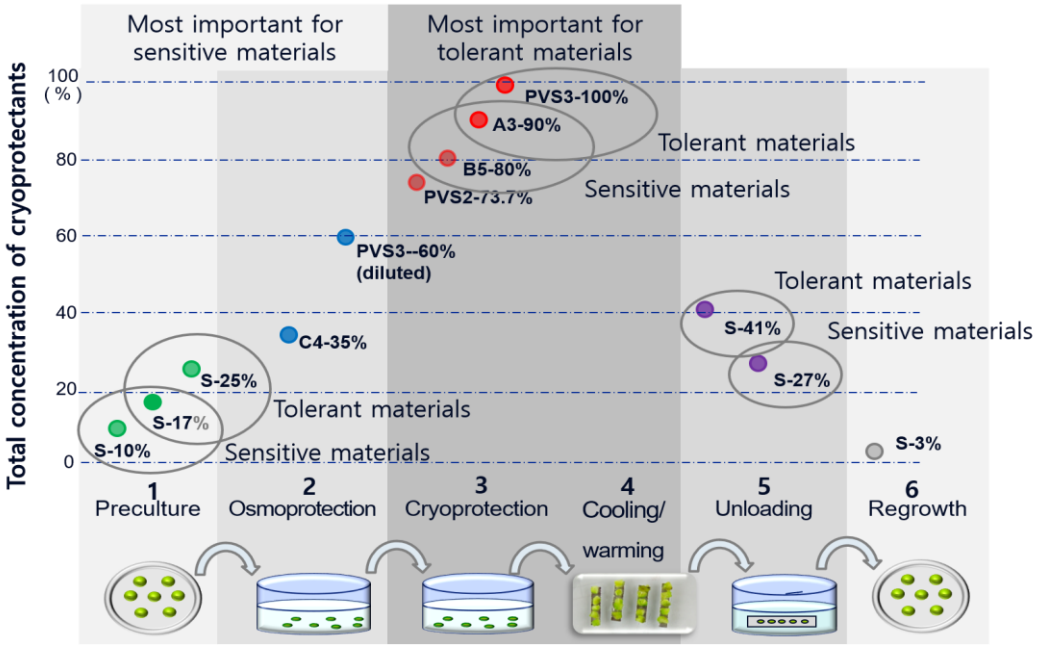

Figure 2. Evolution of CPA concentrations in the course of a droplet-vitrification method and its importance based on material sensitivity. S – sucrose. For solution composition see Table 1. Sucrose concentration is presented in % for consistency.

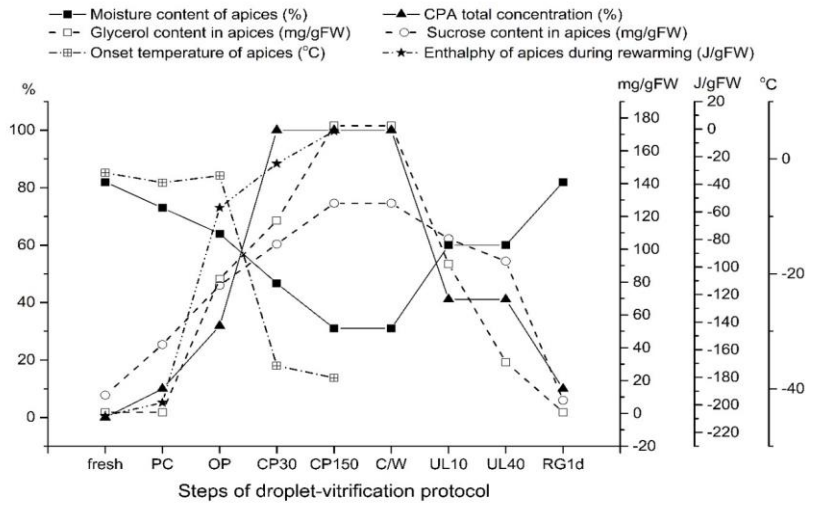

Figure 3. Relationship between moisture content, glycerol and sucrose concentration in *Allium sativum* clove apices, their enthalpies and onset temperatures during rewarming and total concentrations of CPAs in the course of the droplet-vitrification method. Data from (64, 66, 71).
Fresh – fresh apices, PC – preculture, OP – osmoprotection, CP30 and CP150 – cryoprotection with PVS3 for 30 min and 150 min, respectively, C/W – cooling and rewarming on Al foil strips, UL10 and UL40 – unloading for 10 min and 40 min, respectively, RG1d – regrowth on standard medium (3% sucrose) for 1 day, FW – fresh weight.

(up to 0.7 M in Group 2 and 0.5 M in Group 3) and one-step osmoprotection with classic or alternative CPA solutions for 30-40 min are beneficial. Explants of these groups may be too sensitive to PVS2 hence, its four-component alternative VS A3 with increased sucrose and glycerol concentrations (full-strength or diluted to 80%) is recommended. The treatment time may vary from 30 to 90 min. To cope with the potential sensitivity of explants to DMSO and EG in VS A3, PVS3 and its less concentrated alternative VS B5 are worth testing for 30-60 min at room temperature.

The most sensitive explants of Group 4 require longer (up to several days) preculture with mild (0.3 M) sucrose concentrations followed by very gentle (sometimes two-step) osmoprotection with alternative CPAs. Both VS B5 and diluted A3 are worth testing for a short time (up to 20 min).

The whole scheme takes into account several critical factors affecting the response of plant materials:

1) Total concentration of CPAs (higher glycerol and sucrose for better dehydration of tolerant materials, low to moderate concentrations for sensitive materials).
2) Concentrations of individual components in CPA mixtures (most toxic and easy-to-penetrate DMSO and EG are balanced by highly osmotically active glycerol and sucrose).
3) Toxic CPAs (DMSO, EG) effect is minimized for most sensitive materials by

Table 2. Four categories of plant materials with tentative standard conditions (bold) and their variations at different steps of the droplet-vitrification method which may be applied for initial testing of the tolerance/sensitivity of new plant materials (25).

Material group	**Group 1**	**Groups 2-3**		**Group 4**
Examples of material type	Bulbil primordia, 1.5 - 3.0 mm	Shoot tips, axillary buds 1.0 – 2.5 mm		Root apices, 7–10 mm
Osmotic	Very tolerant	Tolerant	Moderately tolerant	Very sensitive
Chemical stress	Very tolerant	Moderately tolerant	Sensitive	Very sensitive
Proposed standard conditions at each step (bold) and their variations*				
Preculture	No 0.3 M (17 h) **0.5 M (17 h)** 0.7 M (17 h)	0.3 M suc (24 h) 0.3 M suc (48 h) 0.3 M suc (24 h) → 0.5 M suc (5 h) **0.3 M suc (24 h) → 0.5 M suc (17 h)** 0.3 M suc (24 h) → 0.5 M suc (17 h) → 0.7 M suc (5 h)		0.3 M suc (24 h) **0.3 M suc (48 h) → 0.5 M (5 h)** 0.3 M suc (54 h) → 0.5 M (17 h)
Osmo-protection	No **C4-35% (20 min)**	No C4-35% (20 min) **C4-35% (40 min)**	**C4-35% (30 min)** C4-35% (15 min) → PVS3-60% (15 min)	**C4-35% (20min)** C4-35% (40min)
Cryo-protection	PVS3-100%(120 min) **PVS3**-100%**(150 min)** PVS3-100% (180 min) A3-90%(150min) 0°C	PVS3-100% (40 min) **PVS3**-100% **(60 min)** PVS3-100% (90 min) A3-90% (40min) 0°C A3-90% (60min) 0°C A3-90% (90min) 0°C	**B5-80% (30-40 min)** B5-80% (60 min) PVS3-100% (30 min) A3-80% (40min) 0°C A3-80% (60min) 0°C A3-90% (40min) 0°C	**B5-80% (15 min)** A3-70% (20min) 0°C A3-90% (10min) 0°C A3-90% (20min) 0°C
Unloading	0.8 M suc (40 min) **1.2 M suc (40 min)**	0.8 suc (20 min) **0.8 M suc (40 min)**	**0.8 M suc (30 min)**	0.8 M suc (15 min) **0.8 M suc (30 min)**

* PVS3-100%, C4-35%, etc., CPA solutions are presented as abbreviations with total % concentrations of cryoprotectants (see also Table 1). Abbreviations A3-70% and A3-80% mean then the original solution A3-90% (Table 1) is diluted to achieve 70% or 80% total concentration of cryoprotectants.

applying alternative VSs, reducing the temperature and time of exposure.

How to classify new material for CPA optimisation

The obvious challenge in the proposed scheme (Table 2) is how to classify the new material to the Group that matches its sensitivity to CPA action.

At the first stage, material might be categorizing by its type, size and structure and by comparing with published data on relative species, if available. In general, thin and fragile materials prone to desiccation such as hairy and adventitious roots usually fit into Group 4, apices and axillary shoot tips usually comprise Groups 2 and 3, bulbils, cloves and other structurally solid and hard-surfaced samples may be considered for Group 1. Once the tentative group is selected, the standard protocol recommended for the group (Table 2) is applied in parallel with its one-factor variations.

The regrowth of both cryoprotected (CPA only, no cryopreservation) and cryopreserved (CPA+LN) materials should be recorded to understand the effectiveness of cryoprotection and material response to CPA toxicity. Notable difference between CPA and CPA+LN will imply insufficient cryoprotection and the need to increase CPA concentration or exposure time; low regrowth after both CPA and CPA+LN treatments will indicate high CPA toxicity and the need to reduce exposure duration or switching to a more sensitive material group.

Protocol sets proposed in Table 2 are comprised of 10 to 13 treatments that can be performed by one technician in 1-2 days with the minimum required number of explants. The treatments resulting in best regrowth can be incorporated into one protocol which can be further adapted for specific genotypes.

Systematic approach application for endangered species and different material types

While the systematic approach was developed and initially tested with plants of agricultural and horticultural importance, we found it to be particularly useful for designing cryopreservation protocols for wild species with unknown chilling and desiccation tolerance. During the past decade, this scheme has been applied to cryopreserve Korean and Canadian endangered species of Asteraceae, Betulaceae, Leguminosae / Fabaceae, Lamiaceae, Orchidaceae, and Orobanchaceae. None of the researched species were previously cryopreserved, their sensitivity barriers were unknown, and tissue culture protocols needed to be developed de novo.

One of the most appealing examples is the conservation, propagation and restoration of *Castilleja levisecta* (Orobanchaceae), a globally critically imperiled species with two populations left in Canada totalling only 3361 flowering plants in 2006 (76). The plant responded well to in vitro conditions and showed moderate dehydration tolerance with steadily high (60-70%) regrowth within a wide window of CPA exposure (40-100 min). After cryopreservation, plants were acclimatized and reintroduced in their region of habitat where they survived for several years and produced flowers.

By contrast, *Betula lenta* (Betulaceae), an endangered Canadian tree species, was very sensitive to the toxic effects of all tested CPAs with regrowth below 10% irrespective of CPA composition and time of exposure. The critical factor for its successful cryopreservation was modification of unloading step with best regrowth (52%) achieved with unloading in 0.8 M sucrose for 30 min (75).

Cryopreserving *Lupinus rivularis* (Fabaceae), an endangered species in Canada, first seemed undoable due to its very slow growth and low multiplication index (2.8) in vitro (63). After a 1.5-month-long subculture cycle, only 60-90 apical shoot tips could be selected for the experiments. In this particular case, having a single standard protocol pre-selected for species was critically important and resulted in 62% regrowth during the very first run with only 30 shoot tips (35).

Cryopreservation of endangered wild Korean species *Aster altaicus* var. *uchiyamae* (Asteraceae) and *Pogostemon yatabeanus* (Lamiaceae) using shoot tips required 60 min osmoprotection and moderately concentrated VSs A3-80% or B5-80% applied for 60 min for effective cryoprotection (74). Application of the systematic approach to hydrated seeds of *Dendrobium moniliforme* (Orchidaceae), a critically endangered Korean orchid, revealed that 41 h preculture with 10% sucrose followed by 40 min osmoprotection and A3-90% (30 min) on ice produced highest germination (80%) after cryopreservation (91). A similar approach was used to successfully cryopreserve adventitious root segments of *Panax ginseng* (Araliaceae) and hairy roots of five medicinal plant species, the

materials most sensitive to both osmotic and chemical toxicity of CPAs (80, 81).

In all the abovementioned studies, the acceptable regrowth (>50%) of new materials was achieved by comparing the CPA and CPA+LN data for a total of 12-18 treatments. In all cases, regrowth was counted as formation of whole plants which could be further acclimated and rooted ex vitro.

CONCLUSION

Organized plant tissues are unique living systems with limited natural ability to tolerate thermal, osmotic and chemical stresses. CPAs play a critical role in material adaptation and protection during the whole cryopreservation process. Hence, to develop successful cryopreservation protocols, it is critically important to understand the barriers of material tolerance and susceptibility to chemical and osmotic toxicity provoked by CPA mixtures. Alternative vitrification solutions equip the standard droplet-vitrification method with higher flexibility and multiple combinations of osmotic and cryoprotection conditions, thus boosting its adaptability to a larger spectrum of genotypes and to very different plant materials including bulbils, shoot tips, axillary buds, embryogenic tissues, hairy and adventitious roots.

Consequently, a rationalized systematic approach to protocol development is built on sample differentiation according to their size, type and regrowth responses in a limited number of standardized treatments with progressively increasing concentration of CPAs. Using this approach as an alternative to blind condition screening showed great effectiveness when developing cryopreservation protocols for wild species with tissues of unknown chilling and desiccation tolerance. Limited number of explants required for the trial runs makes the system suitable for cryopreserving problem genotypes, plants that are not well adapted to propagation in vitro and species at high risk of extinction. The concept is continuously evolving with more species from different families been tested and classified.

Acknowledgments: E. Popova acknowledges the financial support of the Ministry of Science and Higher Education of the Russian Federation (themes No. 122042700045-3 and 122042600086-7).

REFERENCES

1. Bajaj YPS (1979) *Euphytica* **28**, 267-285.
2. Kartha KK (1985) *Cryopreservation of Plant Cells and Organs*, CRC Press Inc., Boca Raton, Florida, 276 p.
3. Withers LA (1980) *CryoLetters* **1**, 239–250.
4. Engelmann F (2004) *In Vitro Cellular & Developmental Biology - Plant* **40**, 427–433.
5. Kaviani B & Kulus D (2022) *Biology* **11**, 847.
6. Kundu S, Salma U & Gantait S (2018) in *Biotechnological Approaches for Medicinal and Aromatic Plants*, (ed) Kumar N, Springer, Singapore, pp. 353–381.
7. Reed BM (2001*) CryoLetters* **22**, 97-104.
8. Reed BM (2008) *Plant Cryopreservation: a Practical Guide*. Springer, New York.
9. Panis B, Nagel M & Van den Houwe I (2020) *Plants* **9**, 1634.
10. Acker JP, Adkins S, Alves A, Horna D & Toll J (2017) *Feasibility Study for a Safety Back-up Cryopreservation Facility*, Independent Expert Report, July 2017, Biodiversity International, Rome, 100 pp. https://hdl.handle.net/10568/91009
11. Jenderek MM & Reed BM (2017) *In Vitro Cellular & Developmental Biology - Plant* **53,** 299–308.
12. Kim HH, Popova E, Shin DJ, Yi JY, Kim CH, Lee JS, Yoon MK & Engelmann F (2012) *CryoLetters* **33**, 45–57.
13. Kushnarenko S, Kovalchuk I, Turdiev T, Romadanova N & Reed B (2018) *Cryobiology* **80**, 162.
14. Panis B & Lambardi M (2005) in *International Workshop on The Role of Biotechnology for the Characterisation and Conservation of Crop, Forestry, Animal and Fishery Genetic Resources*, Turin, Italy, pp. 43-54.
15. Panis B, Piette B & Swennen R (2005) *Plant Science* **168**, 45–55.
16. Volk GM, Bonnart R, Shepherd A, Yin Z, Lee R, Polek M & Krueger R (2017) *Plant Cell, Tissue and Organ Culture* **28**, 327–334.
17. Vollmer R, Villagaray R, Egusquiza V, Espirilla J, García M, Torres A, Rojas E, Panta A, Barkley NA & Ellis D (2016) *CryoLetters* **37**, 318–329.
18. Benelli C, De Carlo A & Engelmann F (2013) *Biotechnology Advances* **31**, 175–185.
19. Fukui K, Shirata K, Niino T & Kashif IM (2011) *Acta Horticulturae* **908**, 483-488.

20. Höfer M (2015) *Plant Cell, Tissue and Organ Culture* **121,** 647–656.
21. Towill LE, Forsline PL, Walters C, Waddell JW & Laufmann J (2004) *CryoLetters* **25**, 323-334.
22. Cyr DR (1999) in *Somatic Embryogenesis in Woody Plants*, *Forestry Sciences* 55, (eds) Jain SM, Gupta PK & Newton RJ, Springer, Dordrecht, pp. 239–261.
23. Häggman H, Aronen T & Ryyänen LA (2000) in *Somatic Embryogenesis in Woody Plants* 6, (eds) Jain SM, Gupta PK & Newton RJ, Kluwer Academic Publishers, the Netherlands, pp. 707-728
24. Lambardi M, Ozudogru EA & Benelli C (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) Reed BM, Springer, New York, USA, pp. 177–210.
25. Popova E, Shukla M, Kim HH & Saxena P (2015) in *Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools*, (eds) Al-Khayri J, Jain S & Johnson D, Springer International Publishing, Cham, Switzerland, pp. 63–93.
26. Ren R, Li Z, Li B, Xu J, Jiang X, Liu Y & Zhang K (2019) *Cryobiology* **89**, 14-20.
27. Bettoni JC, Bonnart R & Volk GM (2021) *Plant Cell, Tissue and Organ Culture* **144,** 21–34.
28. Vollmer R, Villagaray R, Cárdenas J, Casro M, Chávez O, Anglin NL & Ellis D (2017) *In Vitro Cellular & Developmental Biology - Plant* **53,** 309–317.
29. Sharrock S (2020) *Plant Conservation Report 2020: A Review of Progress in Implementation of the Global Strategy for Plant Conservation 2011-2022,* **Technical Series No. 95**, Secretariat of the Convention on Biological Diversity: Montréal, Canada and Botanic Gardens Conservation International, Richmond, UK, 68 pp.
30. Funnekotter B, Mancera RL & Bunn EH (2017) *In Vitro Cellular & Developmental Biology - Plant* **53**, 289-298.
31. Pence VC, Ballesteros D, Walters C, Reed BM, Philpott M, Dixon KW, Pritchard HW, Culley TM & Vanhove AC (2020) *Biological Conservation* **250**, 108736.
32. Touchell D & Dixon K (1993) *Biodiversity Conservation* **2**, 594–602.
33. Walters C & Pence VC (2020) *Plants, People, Planet* **3**, 83– 91.
34. Popova E & Kim HH (2020) in *Orchid Biology: Recent Trends & Challenges*, (eds) Khasim S, Hegde S, González-Arnao M & Thammasiri K, Springer, Singapore, pp. 119-135.
35. Popova EV, Shukla MR, McIntosh T & Saxena PK (2021) *Agronomy* **11**, 37.
36. Sakai A & Engelmann F (2007) *CryoLetters* **28**, 151–172.
37. Benson EE (2004) in *Life in the Frozen State*, (eds) Fuller BJ, Lane N & Benson EE, CRC Press, Boca Raton, FL, pp. 299-328.
38. Maximov NA (1912) *Berichte Der Deutschen Botanischen Gesellschaft* **30**, 52–65.
39. Polge C, Smith AU & Parkes AS (1949) *Nature* **164**, 666.
40. Lovelock JE (1953) *Biochimica et Biophysica Acta* **11**, 28-36.
41. Lovelock JE & Bishop MWH (1959) *Nature (Lond.)* **183**, 1394.
42. Fuller BJ (2004) *CryoLetters* **25**, 375-388.
43. Sakai A, Hirai D & Niino T (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) Reed BM, Springer, Singapore, pp. 33–57.
44. Zamecnik J, Faltus M & Bilavcik A (2021) *Plants* **10**, 2623.
45. Mazur P, Leibo S & Chu E (1972) *Experimental Cell Research* **71**, 345-355.
46. Samygin GA (1994) *Russian Journal of Plant Physiology* **41**, 539-549.
47. Benson EE (2008) in *Plant Cryopreservation: A Practical Guide*, (ed) Reed BM, Springer, New York, NY, pp. 15–32.
48. Fahy GM, MacFarlane DR, Angell CA & Meryman HT (1984) *Cryobiology* **21**, 407-426.
49. Steponkus PL, Langis R & Fujikawa S (1992) in *Advances in Low Temperature Biology Volume 1*, (ed) Steponkus PL, JAI Press Ltd, London, UK, pp. 1-61.
50. Meryman HT & Williams RJ (1985) in *Cryopreservation of Plant Cells and Organs*, (ed) Kartha KK, CRC Press, Boca Raton, FL, pp. 13-47.
51. Crowe JH, Crowe LM, Carpenter JF & Aurell Wistrom C (1987) *Biochemical Journal* **242**, 1–10.
52. Fujikawa S & Jitsuyama Y (2000) in *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Applications*, (eds) Engelmann F & Takagi H, JIRCAS and IPGRI, Rome, pp. 36-42.
53. Mazur P (2004) in *Life in the Frozen State*, (eds) Fuller BJ, Lane N & Benson EE, CRC Press, Boca Raton, FL, pp. 3-65.

54. Steponkus PL (1984) *Annual Review of Plant Physiology* **35**, 543-584.
55. Fahy GM, Wowk B, Wu J & Paynter S (2004) *Cryobiology* **48**, 22–35.
56. Volk GM & Walters C (2006) *Cryobiology* **52**, 48–61.
57. Bilavčík A, Zámečník J, Faltus M & Jadrná P (2019) *Horticultural Science (Prague)* **46**, 163–170.
58. Lynch PT, Souch GR, Zamecnik J & Harding K (2016) *CryoLetters **37***, 308–317.
59. Sherlock G, Block W & Benson EE (2005) *CryoLetters* **26**, 45–54.
60. Crowe JH, Carpenter JF, Crowe LM & Anchordoguy TJ (1990) *Cryobiology* **27**, 219-231.
61. Hoekstra FA, Golovina EA & Buitink J (2001) *Trends in Plant Science* **6**, 431-438.
62. Sakai A, Kobayashi S & Oiyama I (1990) *Plant Cell Reports* **9**, 30–33.
63. Nishizawa S, Sakai A, Amano Y & Matsuzawa T (1993) *Plant Science* **91**, 67–73.
64. Kim HH, Lee YG, Shin DJ, Ko HC, Gwag JG, Cho EG & Engelmann F (2009) *CryoLetters* **30**, 320–334.
65. Turner SR, Senaratna T, Touchell DH, Bunn E, Dixon KW & Tan B (2001) *Plant Science* **160**, 489-497.
66. Kim HH, Yoon JW, Kim JB, Engelmann F & Cho EG (2005) *CryoLetters* **26**, 33-44.
67. Han Z & Bischof JC (2020) *CryoLetters* **41**, 185-193.
68. Anchordoguy TJ, Rudolph AS, Carpenter JF & Crowe JH (1987) *Cryobiology* **24**, 324-331.
69. Crowe JH, Oliver AE & Tablin F (2002) *Integrative and Comparative Biology* **42**, 497-503.
70. Lin L, Ma J, Ai Q, Pritchard HW, Li W & Chen H (2021) *International Journal of Molecular Sciences* **22**, 2174.
71. Kim JB, Kim HH, Baek HJ, Cho EG, Kim YH & Engelmann F (2005) *CryoLetters* **26**, 103-112.
72. Kim HH, Kim JB, Baek HJ, Cho EG, Chae YA & Engelmann F (2004) *CryoLetters* **25**, 91-100.
73. Kaczmarczyk A, Rokka VM & Keller ERJ (2011) *Potato Research* **54**, 45–79.
74. Lee HE, Popova E, Park HN, Park SU & Kim HH (2021) *Plants* **10**, 2018.
75. Rathwell R, Popova E, Shukla MR & Saxena PK (2016) *Canadian Journal of Forest Research* **46**, 1284–1292.
76. Salama A, Popova E, Jones MP, Shukla MR, Fisk NS & Saxena PK (2018) *In Vitro Cellular & Developmental Biology - Plant* **54**, 69–78.
77. Shin DJ, Kong H, Popova EV, Moon HK, Park SY, Park SU, Lee SC & Kim HH (2012) *CryoLetters* **33**, 402–410.
78. Wang MR, Lambardi M, Engelmann F, Pathirana R, Panis B, Volk GM & Wang QC (2020) *Plant Cell, Tissue and Organ Culture* **144**, 7-20.
79. Chen XL, Li JH, Xin X, Zhang ZE, Xin PP & Lu XX (2011) *South African Journal of Botany* **77**, 397-403.
80. Kim HH, Popova EV, Shin DJ, Bae CH, Baek HJ, Park SU & Engelmann F (2012) *CryoLetters* **33**, 506–517.
81. Le KC, Kim HH & Park SY (2019) *Horticulture, Environment and Biotechnology* **60**, 501-510.
82. Oh SY, Wu CH, Popova E, Hahn EJ & Paek KY (2009) *Journal of Plant Biology* **52**, 348–354.
83. Fahy GM, Lilley TH, Linsdell H, Douglas MS & Meryman HT (1990) *Cryobiology* **27**, 247–268.
84. Choi CH, Popova E, Lee H, Park SU, Ku J, Kang JH & Kim HH (2019) *CryoLetters* **40**, 113–122.
85. Kim HH, Popova EV, Yi JY, Cho GT, Park SU, Lee SC & Engelmann F (2010) *CryoLetters* **31**, 473-484.
86. Lee YG, Popova E, Cui HY, Kim HH, Park SU, Bae CH, Lee SC & Engelmann F (2011) *CryoLetters* **32**, 487-497
87. Ružić D, Vujović T & Cerović R (2014) *Horticultural Science (Prague)* **41**, 55-63.
88. Vujović T, Ružić Đ, Vranić D & Marjanović T (2020) *Acta Horticulturae* **1289**, 1-8.
89. Carpentier SC, Witters E, Laukens K, Van Onckelen H, Swennen R & Panis B (2007) *Proteomics* **7**, 92–105
90. Ramon M, Geuns JMC, Swennen R & Panis B (2002) *CryoLetters* **23**, 345–352
91. Popova E & Kim HH (2019) *Acta Horticulturae* **1262**, 43-52.
92 Yang X, Popova E, Shukla MR & Saxena P (2019) *In Vitro Cellular & Developmental Biology - Plant* **55**, 392–402.
93 Yin ZF, Bi WL, Chen L, Zhao B, Volk GM & Wang QC (2014) *Acta Physiologiae Plantarum* **36,** 1683–1692