





Review

Epigenetic and Genetic Integrity, Metabolic Stability, and Field Performance of Cryopreserved Plants

Min-Rui Wang^{1,2}, Wenlu Bi³ , Mukund R. Shukla³ , Li Ren⁴, Zhibo Hamborg⁵ , Dag-Ragnar Blystad⁵, Praveen K. Saxena³  and Qiao-Chun Wang^{2,*}

- ¹ State Key Laboratory of Crop Stress Biology for Arid Areas, College of Life Science, Northwest A&F University, Yangling District, Xianyang 712100, China; wangmr@nwsuaf.edu.cn
- ² State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A&F University, Yangling District, Xianyang 712100, China
- ³ Department of Plant Agriculture, Gosling Research Institute for Plant Preservation, University of Guelph, Guelph, ON N1G 2W1, Canada; wenlubi@uoguelph.ca (W.B.); mshukla@uoguelph.ca (M.R.S.); psaxena@uoguelph.ca (P.K.S.)
- ⁴ Institute for Agri-Food Standards and Testing Technology, Shanghai Academy of Agricultural Sciences, Shanghai 201403, China; renliq@163.com
- ⁵ Division of Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), 1431 Ås, Norway; zhibo.hamborg@nibio.no (Z.H.); dag-ragnar.blystad@nibio.no (D.-R.B.)
- * Correspondence: qiaochunwang@nwsuaf.edu.cn

Abstract: Cryopreservation is considered an ideal strategy for the long-term preservation of plant genetic resources. Significant progress was achieved over the past several decades, resulting in the successful cryopreservation of the genetic resources of diverse plant species. Cryopreservation procedures often employ in vitro culture techniques and require the precise control of several steps, such as the excision of explants, preculture, osmo- and cryoprotection, dehydration, freeze-thaw cycle, unloading, and post-culture for the recovery of plants. These processes create a stressful environment and cause reactive oxygen species (ROS)-induced oxidative stress, which is detrimental to the growth and regeneration of tissues and plants from cryopreserved tissues. ROS-induced oxidative stresses were documented to induce (epi)genetic and somatic variations. Therefore, the development of true-to-type regenerants of the source germplasm is of primary concern in the application of plant cryopreservation technology. The present article provides a comprehensive assessment of epigenetic and genetic integrity, metabolic stability, and field performance of cryopreserved plants developed in the past decade. Potential areas and the directions of future research in plant cryopreservation are also proposed.

Keywords: cryopreservation; (epi)genetic integrity; field performance; metabolic stability; reactive oxygen species; shoot tips; tissue culture



Citation: Wang, M.-R.; Bi, W.; Shukla, M.R.; Ren, L.; Hamborg, Z.; Blystad, D.-R.; Saxena, P.K.; Wang, Q.-C. Epigenetic and Genetic Integrity, Metabolic Stability, and Field Performance of Cryopreserved Plants. *Plants* **2021**, *10*, 1889. <https://doi.org/10.3390/plants10091889>

Academic Editor: Panayiotis Dimitrakopoulos

Received: 12 August 2021
Accepted: 7 September 2021
Published: 13 September 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Overall Developments and Progresses in Plant Cryopreservation

Cryopreservation refers to the storage of biological samples, such as cells, tissues, and organs, in liquid nitrogen (LN) at extremely low temperatures, usually $-196\text{ }^{\circ}\text{C}$. The motive of initial studies was to establish cryopreservation methods for the long-term preservation of plant genetic resources [1,2]. Since the first success of plant cryopreservation using the two-step freezing of mulberry (*Morus alba*) by Sakai [1], considerable progress has been made in the field over the past 65 years [3–6]. Successful cryopreservation studies resulted in a number of technical advancements, including the development of various cryopreservation methods, such as vitrification [7–9], encapsulation-vitrification [9,10], droplet-vitrification [11], encapsulation-dehydration [10,12] and the use of cryo-plates [13,14]. Of these, the droplet-vitrification method, described by Panis et al. [11], is noteworthy as it has largely helped to remove the genotype-specific barriers, which had impeded the

potential application of cryopreservation technology across diverse species. These technical refinements led to the successful cryopreservation of a wide range of genotypes in many plant species, such as *Malus* [15], *Vitis* [16,17], and *Lilium* [18]. The cryopreservation technology is considered an ideal strategy for the long-term preservation of plant genetic resources [3–6].

Diverse plant species have been successfully cryopreserved, including tuber crops [19,20], fruit crops [15,21], ornamental species [22], medicinal herbs [23], and forest species [24], as well as endangered and endemic plants [25–29]. Cryo-banks using shoot tips have been set up in several countries for economically important plant species [3,4,20,23,30,31]. Studies have also advanced in the evaluation of the performance of cryo-derived plants when they are re-introduced from laboratories to their natural habitat [25,28,32].

Cryopreservation has been shown to retain or even promote the regenerative capacity of embryogenic tissues, which were widely used in genetic transformation in various plant species [31,33]. The use of cryopreserved embryogenic tissues for genetic transformation improved the transformation efficiency and regeneration frequency of transformed plant tissues [34]. Cryopreservation was shown to maintain the transgenes in transformed materials, providing a safe and reliable strategy for the long-term preservation of transgenes [31,33,34]. These studies demonstrated the usefulness of cryobiotechnology in plant genetic engineering.

Shoot tip cryotherapy, which was refined based on shoot tip cryopreservation, has been established as a novel method for the efficient eradication of plant pathogens, including viruses, phytoplasma, and bacteria [31,35,36]. Cryopreservation has also been successfully applied to cryopreservation of plant viruses and viroids in cryopreserved shoot tips [37–39], opening a new avenue for the long-term preservation of plant obligate pathogens, which are otherwise difficult-to-preserve for long periods with the traditional methods. Thus, cryobiotechnology offers the dual advantage in the preservation as well as eradication of plant pathogens [38,40].

A number of plant species have been threatened and are currently facing extinction, mainly by anthropogenic processes, such as deforestation, land clearing for agricultural, industrialization, and urbanization, as well as through plant diseases and pests [41,42]. Global warming has further worsened the situation. A recent report warned that about 21% of total global plant species (approximately 390,900 plants) are at risk of extinction [43]. Therefore, special attention should be paid to the cryopreservation of these endangered and rare species [41,42,44,45]. However, studies on cryopreservation of endangered and rare plant species are far behind those of horticultural species, ornamental plants, tuber crops, and forest species [6,15,19–29,41,42].

2. Major Concerns in Recovery of Plants from Cryopreserved Tissues

For the preservation of plant genetic resources, it is necessary to not only ensure viability and quality of the preserved plants, but to also ensure the true-to-type status of the regenerants (Figure 1). Shoot tips or buds are able to develop into plants that are generally genetically stable and identical to the source plants, and are therefore preferred over other tissues, such as cell suspensions, embryogenic tissues, and callus for preserving plant genetic resources [6,31,46–48].

In vitro tissue culture has become an integral part of the cryopreservation technology currently used for the establishment and maintenance of stock cultures, and the post-culture process for the recovery of shoot tips, cell suspensions, and embryogenic tissues, in a number of plant species [6,31,46,48]. However, in vitro tissue cultures are prone to somaclonal and (epi)genetic variations [49–52]. It is well documented that in vitro tissue culture imposes stressful conditions and induces the generations of reactive oxygen species (ROS), thus resulting in ROS-induced oxidative stress [49–51]. Cryopreservation requires the use of plant tissue culture methods in several essential steps, including the excision of explants, preculture, osmo- and cryoprotection, dehydration, freeze-thaw cycle, unloading,

and the re-initiation of cultures for plant recovery. All of these steps have been shown to cause ROS-induced oxidative stress [4,26,47,53–55].

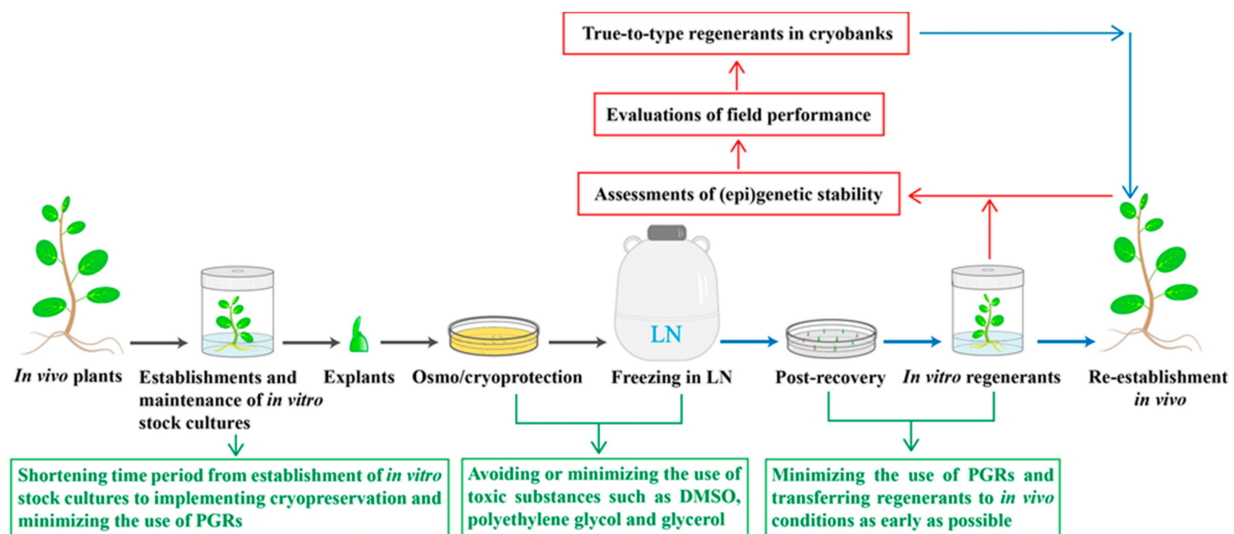


Figure 1. General cryopreservation procedures (black arrows), post-culture for recovery and re-establishment of plants *in vivo* (blue arrows), assessments of (epi)genetic stability and evaluations of field performance in cryo-derived regenerants/plants (red arrows), and measures taken to ensure (epi)genetic stability and true-to-type regenerants/plants recovered after cryopreservation (green arrows). DMSO, dimethyl sulfoxide; LN, liquid nitrogen; PGRs, plant growth regulators.

ROS are highly reactive and toxic by-products of aerobic metabolism. ROS include the superoxide anion, hydrogen peroxide, and hydroxyl radicals, all of which have inherent reactivity to different biological targets, playing a dual role of inducing cellular toxicity, while also serving as signaling transduction stimuli in plant responses [56,57]. The ROS-induced oxidative stresses have proven to induce the somaclonal and (epi)genetic variations in *in vitro*-derived regenerants [49–51,58] and in cryo-derived regenerants [4,26,47,53–55]. Therefore, it is necessary to assess and monitor the (epi)genetic integrity and field performance of cryo-derived regenerants. Literature (up to 2010) on relevant aspects of such genetic variations in plants originating from *in vitro* and cryopreserved cultures was comprehensively reviewed previously [31,41,42,47,53–55].

The present review focuses on the advances made in the past decade in the assessment and improvement of the (epi)genetic integrity, metabolic efficiency, and field performance of cryo-derived plants. Research areas and applications that may benefit from further studies on plant cryopreservation are also discussed. These efforts would certainly promote further studies on cryopreservation of endangered and rare plant species.

3. Assessments of Epigenetic and Genetic Integrity

3.1. Epigenetic Integrity

Epigenetics refers to heritable changes in gene expression that is not associated with changes in the underlying DNA sequence [59]. Major types of epigenetic changes include DNA methylation, histone modification, and changes in chromatin structure [60]. DNA methylation has been widely used for the assessments of epigenetic integrity in cryo-derived plants [47,53–55] and methylation-sensitive amplified polymorphism (MSAP) is the most frequently used marker for the assessments of epigenetic stability in plant populations of cryogenic origin.

Hao et al. [61–63] were the first to assess epigenetic stability in cryo-derived regenerants. Changes in DNA methylation were detected in plants recovered from the cryopreserved shoot tips of *Malus pumila* “M2” and *Fragaria gracilis* “Joho” [61,63], and somatic embryogenic calli of *Citrus sinensis* “Newhall” [62]. Some representative examples of the

use of DNA methylation-induced changes in cryopreservation research in the past decade are listed in Table 1.

Peredo et al. [64] assessed epigenetic integrity in *Humulus lupulus* plants recovered from shoot tips cryopreserved for three years. Changes in DNA methylation were detected in 36% of the loci. Further analysis showed that only 2.6–9.8% of the changes were induced by cryopreservation procedure and the rest were attributed to the in vitro culture processes. In vitro culture-induced DNA methylation was also found in the regenerants recovered from cryopreserved *Carica papaya* shoot tips [65] and *Theobroma cacao* somatic embryos [66]. DNA methylation levels were higher in in vitro stock somatic embryos of *Theobroma cacao* than those regenerated after cryopreservation [66]. Therefore, the manipulations of in vitro culture procedures, particularly for regeneration via somatic embryogenic, are needed to reduce the probability of alternations in DNA methylation.

Alternations in DNA methylation varied with cryogenic steps and cryopreservation durations: 0.12% following PVS2 treatment, 0.12% after 2 h of cryo-storage, and 5.5% after 10 years of cryo-storage in *Wasabia japonica* [67]. Variations in DNA methylation varied among three *Ribes* species, including *R. ciliatum*, *R. sanguineum* and *R. nigrum* [68], and *Humulus lupulus* [64], and *Carica papaya* genotypes [65] subjected to cryopreservation. The assessment of epigenetic stability in the regenerants of the same *Carica papaya* genotype Z6 following cryopreservation detected higher levels of DNA methylation in DNA extracts pooled from mixed leaves of several plants [65] than in those from single leaves of single plants [69]. These data indicated that variations in DNA methylation differed among clones resulted from the same cryopreservation experiment.

Johnston et al. [68] reported DNA methylation in regenerants recovered from *Ribes ciliatum* shoot tips cryopreserved by encapsulation-dehydration. They found that DNA methylation initially occurred during sucrose preculture and progressively increased during successive steps of the encapsulation-dehydration protocol. However, DNA methylation values were similar between the controls and the regenerants recovered from cryopreserved shoots after 18–20 weeks of post-recovery, including two subculture cycles. These data indicate that cryopreservation-mediated DNA methylation is a reversible epigenetic mechanism [68]. Such reversible epigenetic mechanism has also been noted in the regenerants recovered from cryopreserved shoot tips of *Carica papaya* [65], *Mentha × piperita* [70], and *Actinidia chinensis* var. *deliciosa* [71], and from the cryopreserved somatic embryos of *Bactris gasipaes* [72] and *Theobroma cacao* [66]. As speculated by Harding et al. [54], changes in DNA methylation may be an adaptive response to oxidative stress induced during cryopreservation. Therefore, once the stress is removed, DNA demethylation occurs and the regenerants can gradually revert to the normal DNA status.

In contrast with the studies addressed above, no marked alternations in DNA methylation were found in the regenerants recovered from *Quercus robur* plumules cryopreserved by desiccation [73], *Solanum tuberosum* plants from shoot tips preserved with dimethyl sulfoxide (DMSO)-droplet method and cryo-stored for nearly 7 years [74], *Wasabia japonica* plants from vitrified shoot tips cryo-stored for 10 years [68], and *Gentiana cruciata* regenerants from embryogenic cell suspensions cryopreserved by encapsulation-dehydration [75].

Table 1. Some examples from the past decade of epigenetic integrity assessments by DNA methylation in regenerants recovered after cryopreservation.

Plant Species	Explants	Cryopreservation Method *	Molecular Methods **	DNA Methylation (%)	Causes	Reference
<i>Actinidia chinensis</i> var. <i>deliciosa</i>	Shoot tips	Drop-vitri	MSAP	1.6 and 12.8	Cryoprotocols and in vitro cultures	[71]
<i>Bactris gasipaes</i>	Somatic embryos	Drop-vitri	The global DNA methylation	25.2–29.7	Cryoprotocols	[72]
<i>Carica papaya</i>	Shoot tips	Vitri	AMP	0–0.22	Genotypes and cryoprotocols	[65]
<i>Gentiana</i>	Shoot tips	Encap-dehy	MSAP	16.61–16.88	in vitro culture	[75]
<i>Mentha</i> × <i>piperita</i>	Shoot tips	Encap-dehy	MSAP	17.1–32	Cryoprotocols	[70]
<i>Quercus robur</i>	Seed plumules	Desiccation	The global DNA methylation	8.7–11	Cryoprotocols	[73]
<i>Solanum tuberosum</i>	Shoot tips	DMSO droplet	MSAP	0.9	Cryoprotocols and in vitro cultures	[74]
<i>Theobroma cacao</i>	Somatic embryos	Encap-dehy	MSAP	3.6	Cryoprotocols	[66]
<i>Wasabia japonica</i>	Shoot tips	Vitri	MSAP	0.12–5.5	Cryoprotocols	[67]

* Dehy, dehydration; DMSO, dimethyl sulfoxide; Drop, droplet; Encap, encapsulation; Vitri, vitrification. ** AMP, amplified DNA methylation polymorphism; MSAP, methylation sensitive amplified polymorphism.

3.2. Genetic Integrity

Molecular markers were widely used for assessments of genetic stability in cryo-derived plants, including random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), and single sequence repeats (SSR) [53–55]. The RAPD amplifies some areas of the genome and screens a low fraction of the genome, whereas the ISSR amplifies DNA segments between two microsatellite regions [54]. The AFLP technique is based on the selective amplification of restriction fragments from a total digest of genomic DNA [76], and the SSR can detect differences in the length of a particular locus [77,78]. Since different DNA markers detect polymorphisms in different genomic regions, use of more than one molecular marker would provide more reliable results of genetic integrity assessments [31,79]. Therefore, most of the studies conducted in the past decade employed more than one molecular marker for assessments of genetic stability in the regenerants recovered from cryopreservation. Flow cytometry (FCM) was usually used for assessments of DNA ploidy levels in cryopreserved plants [80–82].

Krajnáková et al. [83] reported some changes in the RAPD profiles of *Abies cephalonica* embryogenic cells after six years of cryopreservation. However, proliferation and maturation abilities were maintained in the cryopreserved cells. Applying microsatellite and sequence-related amplified polymorphism (SRAP) for assessments of the genetic stability in the cryo-derived plants of *Hedeoma todsenii* after 13 years of cryo-storage, Pence et al. [84] did not find any DNA variations in the same genotypes, but found an average of 10.4% variation between the replicate samples. Genetic variations were observed in the regenerants recovered from cryopreserved shoot tips of *Chrysanthemum morifolium*, with 40% and 6% of polymorphic bands detected by AFLP and RAPD, respectively [85]. Further analysis found that the genetic variations detected by RAPD were induced in the sucrose preculture step (0.3 M sucrose at 5 °C for 3 days), while those detected by AFLP were in the cold-hardening of the in vitro stock shoots (10 °C for 3 weeks). Freezing in LN induced the highest levels of genetic variations analyzed by both RAPD and AFLP [85]. Applying RAPD to assessments of genetic stability in the regenerants recovered from cryopreserved shoot tips of *Mentha × piperita*, Martín et al. [86] reported that genetic stability varied with genotypes and cryoprotocols: 97% by droplet-vitrification and 87% by encapsulation-dehydration in “MEN 198” (stable genotype), and 80% by droplet-vitrification and 24% by encapsulation-dehydration in “MEN 186” (sensitive genotype). In the analysis of the genetic stability by FCM, RAPD, and ISSR in the cryo-derived plants of three *Chrysanthemum chimeric* cultivars grown in greenhouse conditions, Kulus et al. [80] reported that FCM did not detect any differences in DNA ploidy levels among the three cultivars. RAPD detected no polymorphic bands in “Richmond” (a solid mutant), but detected 7.8% and 3.2% polymorphic bands in “Lady Orange” and “Lady Salmon” (periclinal chimeras). ISSR markers detected 15% polymorphic bands in “Lady Orange” and no polymorphic bands in “Lady Salmon” and “Richmond” [80].

González-Benito et al. [87] tested the effects of adding vitamin E in the pretreatment medium on cryopreservation and genetic stability in “MEN 186” and “MEN 198”. They found that although it did not have significant effects on recovery, vitamin E improved the genetic stability in the regenerants recovered after cryopreservation, particularly in the sensitive genotype “MEN 186”.

In the cryopreservation of *Rubus grabowskii* shoot tips, Castillo et al. [88] reported that the SSR did not detect any polymorphic bands in the cryo-derived regenerants immediately after cryopreservation and those that were subcultured in vitro for seven months after the recovery. The AFLP did not detect any polymorphic bands in the cryo-derived regenerants immediately after cryopreservation, but detected polymorphic bands in the cryo-derived shoots that were subcultured in vitro for seven months. However, when these in vitro cultured shoots were re-established in the field conditions, polymorphic bands were no longer detected [88]. These results indicated that there might be a transitory phase of the polymorphism when the cryopreserved plants were transferred to the field conditions.

Similar results were also found in the cryo-derived plants of *Abies cephalonica* [89] and *Carica papaya* [65]. Nevertheless, further studies are needed to verify these findings of varied polymorphism in plants from cryopreserved tissues.

A number of studies showed that cryopreservation did not cause or caused minor changes in genetic stability of the regenerants. Genetic stability assessments in the regenerants of *Wasabia japonica* following shoot tip cryopreservation detected only 0.27%, 0.95%, and 2.2% variations in AFLP profiles in the samples following exposure to PVS2, cryo-storage in LN for 2 h, and for 10 years, respectively [67]. RAPD and ISSR did not detect any polymorphic bands in the plants recovered from cryopreservation of *Passiflora pohlii* nodal segments [90]. The maintenance of genetic stability was reported in the regenerants recovered after shoot tip cryopreservation in various plant species, including tuber crops such as *Solanum tuberosum*, analyzed by ISSR and FCM [91] and by AFLP and ISSR [79]. The ornamental plant analyses included *Oncidium flexuosum* by FCM [92], *Chrysanthemum morifolium* by SSR and FCM [82] and RAPD and ISSR [93], *Argyranthemum* by AFLP and ISSR [94], *Torenia fournieri* by FCM and ISSR [95], and *Pleione bulbocodioides* (protocorm-like bodies) by ISSR [96]. Similar results were obtained in vegetable crops, such as *Phaseolus vulgaris* (seeds) by SSR [97], *Asparagus officinalis* (rhizome buds) by EST-SSR and FCM [98], and *Allium* by SSR [81], AFLP and ISSR [99]. Examples of analyses of other crops included fruit trees, such as *Musa* (suck meristem) by SSR [100], *Malus* by FCM and ISSR [101,102], *Vaccinium corymbosum* by RAPD and ISSR [103,104], *Vitis* by RAPD and ISSR [17], and *Actinidia chinensis* by AFLP and ISSR [71], and medicinal species, such as *Rabdosia rubescens* by FCM and SRAP [105] and *Bacopa monnieri* by RAPD [106].

Thus far, there were only a few studies that assessed the genetic stability in pathogen-free plants recovered after shoot tip cryotherapy. FCM did not detect any variations in ploidy levels in Chinese jujube plants (*Ziziphus jujuba*), free of Jujube witches' broom phytoplasma [107], and artichoke plants (*Cynara scolymus*), free of artichoke latent virus, produced by shoot tip cryotherapy [108]. The SSR and AFLP did not detect any polymorphic bands in cryo-derived potato plants (*Solanum tuberosum*) free of potato leafroll virus, potato virus S, and potato virus Y [109].

Cryopreservation was reported to maintain the *NPTII* and *GUS* genes in transgenic *Oryza sativa* protoplasts [110], *hCT1A4Ig* in the transgenic cell suspensions of *O. sativa* [111], *Escherichia coli* heat labile enterotoxin (LT) protein in the transgenic cells of *Nicotiana tabacum* [112], and *npt II* and *Gus* genes in the transgenic sweetgum (*Liquidambar*) embryogenic cultures [113]. Similar examples of genes maintained in other species included the *GUS* gene in the transgenic *Citrus* callus [114], *npt II* gene in the transgenic shoots of *Betula pendula* [115] and *Populus tremula* × *P. tremuloides* [116], human serum albumin in the transgenic BY-2 cell cultures [117], *uidA* gene in the transgenic plants of *Castanea sativa* [118], and the Cry 1Ab in the transgenic plantlets of *Torenia fournieri* [95]. The successful cryopreservation of transgenes provides a safe and reliable strategy for long-term preservation of the transgenic plant materials, which otherwise may be lost by preservation through in vitro cultures and environmental contamination or gene flow by preservation in vivo [33,34]. Some examples of genetic integrity assessments in cryo-derived regenerants are listed in Table 2.

Table 2. Some examples from the past decade of genetic integrity assessments by molecular markers and FCM in regenerants recovered after cryopreservation.

Plant Species	Explants	Cryopreservation Method *	Molecular Markers **	Polymorphism (%)	Causes	Reference
<i>Abies</i>	Embryogenic cells	Vitri	RAPD	Not specified	Cryoprotocols and in vitro culture	[83]
<i>Actinidia chinensis</i> var. <i>deliciosa</i>	Shoot tips	Drop-vitri	AFLP and ISSR	None		[71]
<i>Allium cepa</i> var. <i>aggregatum</i>	Shoot tips	Drop-vitri	AFLP and ISSR	None		[99]
<i>Allium sativum</i>	Shoot tips	Vitri	SSR and FCM	None		[71]
<i>Arachis glabrata</i>	Leaflets	Drop-vitri	RAPD	0–3.4	Cryoprotocols	[119]
<i>Asparagus officinalis</i>	Rhizome buds	Encap-dehy	EST-SSR and FCM	None		[98]
<i>Bacopa monnieri</i>	Shoot tips	Vitri	RAPD	None		[106]
<i>Carica papaya</i>	Shoot tips	Vitri	RAF	0–0.7	Genotypes and cryoprotocols	[66]
<i>Chrysanthemum</i> × <i>grandiflorum</i>	Shoot tips	Encap-dehy	ISSR	0–2	Genotypes and cryoprotocols	[80]
			RAPD	0–7.8		
			FCM	None		
<i>Chrysanthemum</i> × <i>morifolium</i>	Shoot tips	Encap-dehy	AFLP	40.1	Sucrose preculture	[85]
			RAPD	5.78		
		Drop-vitri	SSR	None	[82]	
			FCM	None		
<i>Cynara scolymus</i>	Shoot tips	Vitri	ISSR and RAPD	None	[93]	
			FCM	None		
<i>Hedeoma todsenii</i>	Shoot tips	Encap-dehy and Encap-vitri	Microsatellite	5.36–13.04	Genotypes and cryoprotocols	[84]
			SRAP	4.55–20.45	Genotypes and cryoprotocols	
<i>Lotus tenuis</i>	Adventitious buds clusters	Vitri	ISSR	63	Cryoprotocols	[120]

Table 2. Cont.

Plant Species	Explants	Cryopreservation Method *	Molecular Markers **	Polymorphism (%)	Causes	Reference
<i>Malus</i> spp.	Shoot tips	Encap-dehy	ISSR	None		[101]
		Drop-vitri or Encap-dehy	ISSR and RAPD	None		[102]
<i>Mentha × piperita</i>	Shoot tips	Drop-vitri	RAPD	30–40	Genotypes and cryoprotocols	[121]
			RAPD	1–20	Genotypes and cryoprotocols	[86]
		Encap-dehy	RAPD	13–76	Genotypes and cryoprotocols	
			AFLP	0–85.7	Genotypes, cryoprotocols, and in vitro culture	[87]
			RAPD	0–62		
			AFLP	2.65	Sucrose preculture and encapsulation	[70]
RAPD	None					
<i>Musa</i> spp.	Sucker meristems	Vitri	SSR	None		[122]
<i>Passiflora pohlii</i>	Nodal segments	Encap-vitri	ISSR and RAPD	None		[90]
		Vitri	ISSR and RAPD	None		
<i>Phaseolus vulgaris</i>	Seeds	Direct immersion into LN	SSR	None		[123]
<i>Picea abies</i>	Embryogenic tissues	Vitri	SSR	None		[124]
<i>Pinus nigra</i>	Embryogenic tissues	Slow-freezing	RAPD	None		[125]
<i>Pistacia vera</i>	Shoot tips	Vitri	RAPD	5.4	Cryoprotants and post-culture	[126]
<i>Pleione bulbocodioides</i>	Protocorm-like bodies	Vitri	ISSR	None		[96]
<i>Rabdosia rubescens</i>	Shoot tips	Encap-dehy	SRAP	0.01	Cryoprotocols	[105]
			FCM	None		

Table 2. Cont.

Plant Species	Explants	Cryopreservation Method *	Molecular Markers **	Polymorphism (%)	Causes	Reference
<i>Saccharum</i> spp.	Shoot tips	Drop-vitri	ISSR	1.5	Cryoprotection	[127]
		Vitri	AFLP and ISSR	None		[79]
<i>Solanum tuberosum</i>	Shoot tips	Drop-vitri	ISSR and RAPD	None		[91]
		Encap-vitri				
<i>Thymus lotocephalus</i>	Shoot tips	Drop-vitri	RAPD	0.06	Cryoprocedures	[128]
<i>Torenia fournieri</i>	Shoot tips	Drop-vitri	ISSRFCM	None		[95]
<i>Triticum aestivum</i>	Calli	Dehy	ISSR	None	Cryoprocedures	[129]
			REMAP	0.3		
<i>Vaccinium corymbosum</i>	Shoot tips	Drop-vitri	ISSR and RAPD	None		[103]
	Adventitious buds	Drop-vitri	ISSR and RAPD	None		[104]
<i>Vitis</i> spp.	Shoot tips	Drop-vitri	ISSR and RAPD	None		[17]
<i>Wasabia japonica</i>	Shoot tips	Vitri	AFLP	0.27–2.2	Cryoprocedures	[67]
<i>Ziziphus jujuba</i>	Shoot tips	Drop-vitri	FCM	None		[107]

* Dehy, dehydration; Drop, droplet; Encap, encapsulation; LN, liquid nitrogen; Vitri, vitrification. ** AFLP, amplified fragment length polymorphism; EST-SSR, expressed sequence tags-simple sequence repeats; FCM, flow cytometry; ISSR, inter-simple sequence repeats; SRAP, sequence-related amplified polymorphism; SSR, simple sequence repeats; RAF, randomly amplified DNA fingerprinting; RAPD, random amplified polymorphic DNA; REMAP, retrotransposon-microsatellite amplified polymorphism.

4. Metabolic Stability

For plant species that contain special biochemical compounds, the assessment of metabolic abilities is an important issue in cryo-derived plants. *Chrysanthemum morifolium* “Hangju” contains valuable biochemical compounds, such as anthocyanins and carotenoids, and has long been used as a medicine in China [93]. Applying high performance liquid chromatography (HPLC) for quantitative analyses of biochemical compounds in the cryo-derived plants grown in greenhouse conditions, Bi et al. observed no differences in the levels of the five selected biochemical compounds produced between the cryo-derived plants and in vitro-derived plants in the control [93]. No differences were found in the contents of anthocyanins and carotenoids in the inflorescences of the cryo-derived plants and the control of three *Chrysanthemum chimeric* cultivars grown in greenhouse conditions, except for reduced chlorophyll contents found in the cryo-derived plants [80]. In *Bacopa monnieri*, HPLC analysis detected no differences in the level of bacoside A, a functional biocompound in *Bacopa monnieri*, in the cryo- and in vitro-derived (control) plants [106]. More recently, Wang et al. [99] observed no significant differences in the levels of carbohydrates and flavanols in bulbs produced by cryo- and in vitro-derived shallots plants grown in greenhouse conditions. These results indicate that metabolic stability can be maintained in the plants derived from cryopreserved tissues.

5. Field Performance

5.1. Seed Germination and Seedling Growth

Seed conservation provides a useful and relatively easy strategy for preserving genetic resources in seeded plants [41,130]. Three categories of seed storage behavior are generally recognized among species: orthodox, intermediate, and recalcitrant [41,130]. Seeds of most species belong to the orthodox category, and can be dried to low water contents and thus stored at low temperature for extensive periods [4,26,29,130]. Intermediate seeds can withstand partial dehydration, while recalcitrant seeds are sensitive to dehydration. Therefore, they cannot be stored under the desiccation and low temperature conditions for a long period of time [4,26,29,41,130]. The intermediate and recalcitrant seeds contain numerous important tropical and tropical rain forest species [26,29,130]. Cryopreservation is the only technique available for long-term germplasm preservation of these two categories of seeds [4,26,29,130]. There were several studies conducted over the past decade on seed germination and seedling growth in cryopreserved seeds.

No significant differences in seed germinations were obtained between cryopreserved and non-cryopreserved (control) seeds in *Phaseolus vulgaris* [97,131], *Solanum lycopersicum* [132], and *Zea mays* [133]. However, reduced seed germinations were reported in the cryopreserved seeds of *Zea mays* and *Glycine max* [133,134]. In the study of cryopreservation of *Solanum lycopersicum* seeds, Zevallos et al. [135] reported that cryopreservation increased the germination percentage of cryopreserved seeds at day 5 of germination, albeit with no significant differences at day 7. Increased seed germinations were also observed in the cryopreserved seeds of *Teramnus labialis* at 7 and 28 days of germination [136]. The increased seed germinations were attributed to the breaking of physical dormancy by increased malondialdehyde levels induced during cryopreservation [97,132,135,136].

More than 90% and 94% seedlings from the cryopreserved seeds of *Oncidium flexuosum* [92] and *Hibiscus sabdariffa* [137], respectively, survived after transfer to greenhouse conditions. The morphology of the seedlings developed from cryopreserved seeds were similar to those from the control in *Phaseolus vulgaris* [97,131], *Solanum lycopersicum* [135], *Zea mays* [134], *Glycine max* [134], and *Hibiscus sabdariffa* [137]. Seedling growth, measured by the fresh weight of roots, stem, and leaves, was markedly delayed in cryopreserved seeds of *Zea mays* [133]. Seedling growth, including plant height and fresh and dry mass, was greater in seedlings recovered from cryopreserved seeds than in those from the control during the four-week growth [136]. No differences were found in vegetative growth, including shoot length, number of leaves, number and length of roots, and fresh and dry weight, between the cryo-derived and the control seedlings in *Oncidium flexuosum* [92].

Evaluating vegetative growth and grain production in cryo-seed-derived plants of *Phaseolus vulgaris*, Cejas et al. [123] did not find significant differences in all parameters tested, including the number of stem internodes, plant height, fruit number, grain number per plant, and weight per grain between the cryopreserved and the control seeds. In addition, Cejas et al. [131] reported that seed cryopreservation decreased Cu, Cd, and Na uptake, and increased the absorption of B and Al in the cryo-derived seedling (10 days old) of *Phaseolus vulgaris*.

5.2. Field Performance of Cryopreserved Plants

Field performance is critical for evaluating the true-to-type cryopreserved plants in comparison to the source plants (Figure 1). Similar survival percentages and vegetative growth were obtained for the cryo- and in vitro-derived (control) plants of *Actinidia chinensis* var. *deliciosa* after their re-establishment in greenhouse conditions [71]. Agrawal et al. [100] compared the field performance of cryo-derived, micropropagated, and field sucker-propagated *Musa* plants and found that greater than 90% of the cryo-derived and in vitro micropropagated plants survived and were established in field conditions. Vegetative growth (plant height and leaf number) and reproductive growth (flowering and fruit production) were similar among the three sources of plants [100]. Vegetative growth patterns, and morphologies of leaves and flower production, were identical in the cryo- and in vitro-derived plants of *Torenia fournieri* [95] and *Chrysanthemum morifolium* [88] when grown under greenhouse conditions. Wang et al. [99] reported that there were no significant differences in rooting, vegetative growth, and bulb production between the cryo- and in vitro-derived plants of *Allium cepa* var. *aggregatum* when grown in the greenhouse.

Zhang et al. [94] reported that, although root formation and vegetative growth in cryo-derived plants was reduced to a certain degree, the quantity and quality of the flowers were similar in both the cryo- and in vitro-derived plants of perennial ornamental species *Argyranthemum* grown in greenhouse conditions. Vegetative regrowth at the early stage was lower in cryo-derived plants of *Chrysanthemum morifolium* grown in the greenhouse [93] and *Solanum tuberosum* cultured in vitro [91] than their corresponding controls. Vegetative growth markedly increased, however, in the cryo-derived plants of *Solanum tuberosum* after 6 months of in vitro culture [91]. Furthermore, in vitro microtuber production in *Solanum tuberosum* was significantly greater in cryo-derived shoots than in the control [91].

Evaluating the field performance of cryo-derived plants of three *Chrysanthemum* × *grandiflorum* chimeric cultivars Lady Orange, Lady Salmon, and Richmond, Kulus et al. [80] found that some cryo-derived plants had shorter internodes and shorter and/or narrower leaves than the control plants. The inflorescences of Lady Salmon opened slower, but faded faster than the control. However, flower traits, including color, diameter, fresh weight, and length of ray florets, were similar between the cryo-derived plants and the control in all three cultivars.

5.3. Reintroduction of Cryo-Derived Plants to Nature

Castilleja lewisii, a hemiparasitic herbaceous plant, naturally inhabits British Columbia, Canada and the USA. This species is currently listed as an endangered plant in Canada and the USA. Recently, a cryopreservation method was described for this plant [32]. Cryopreserved plants were successfully acclimated in greenhouse conditions. Acclimatized plants were reintroduced to their natural habitats in Canada and 21% of the reintroduced plants survived the transit from lab to the natural habitat and also showed flower development [32].

Hill's thistle (*Cirsium hillii*) is also listed as a threatened species in Canada and its populations are restricted to alvars of Southern Ontario. Cryopreservation was applied for preserving germplasm of Hill's thistle and cryopreserved plants were reintroduced to their natural habitats [25]. Field performances, including survival, vegetative growth, and plant developments, over 10 months were comparable or even better than the micropropagated

plants (the control), although site-specific differences in the percent flowering and amount of indole amines compounds were observed among the plants [25].

These two studies provide a paradigm for the use of cryopreservation for the long-term preservation of plants at risk and their reintroduction in natural habitats.

6. Conclusions and Perspectives

Significant progress was made in the development of plant cryopreservation technology. A wide range of genetically diverse plants that are propagated sexually through seeds and vegetatively through shoot tips have now been successfully cryopreserved. The reintroduction of plants from cryobanks into natural habitats and assessments of field performance, including growth, reproduction, and adaptation to natural environments, was also achieved in many species. The plants from cryopreserved sources survived as well as those from non-cryopreserved tissues when established *in vivo* conditions. Overall, morphologies, vegetative growth, and reproduction of the cryopreserved plants were comparable to those of the non-cryopreserved ones. However, certain changes in DNA methylation were detected in the regenerants recovered after cryopreservation in some plant species. Some of these changes were attributed to *in vitro* culture processes, particularly in the cases of embryogenic tissues. The reversible epigenetic mechanism indicates that DNA methylation is temporary and plants can revert to normal DNA status when *in vitro* cryopreserved plants are established in the field conditions. Therefore, DNA methylation was not closely related to genetic variations in cryopreserved plants. Although the genetic variation was detected in the regenerants recovered after cryopreservation in some plant species, such variations were small in locus and low in frequency, and some of the variations were attributed to *in vitro* culture processes. Analyses by FCM and molecular markers proved that overall genetic stability was maintained in the regenerants recovered after cryopreservation in many of the plant species. Therefore, cryopreservation can maximally maintain genetic stability of cryo-derived plants compared to other traditional methods. Cryopreservation of transgenes provides a safe and reliable strategy for the long-term preservation of transgenic plant materials. Cryopreservation processes do not adversely affect the metabolic stability of plants of cryogenic origin and biochemical profiles are also maintained in the regenerated progeny. In addition, cryopreservation can preserve transgenes in the transformed materials.

Nevertheless, manipulations of *in vitro* stock cultures and cryogenic procedures are still needed to ensure genetic stability in the cryo-derived plants, and assessments of epigenetic and genetic stability in cryo-derived plants are necessary before any cryopreservation protocols are used for establishing cryobanks (Figure 1). Manipulations of *in vitro* stock cultures can be performed by shortening the time periods from establishing *in vitro* stock cultures to implementing cryopreservation as much as possible, and minimizing the use of plant growth regulators for the maintenance of *in vitro* stock cultures and post-culture of cryopreserved samples for plant recovery (Figure 1). Manipulations of cryoprotocols can be performed by avoiding or minimizing the use of toxic substances, such as DMSO, polyethylene glycol, and glycerol (Figure 1). Due to the low fraction of the genome screened in the analysis of genetic fidelity using available molecular markers, new methods are needed for broader screening of the genome in the regenerants recovered after cryopreservation. Next generation sequencing provides an ultra-high throughput platform by which the sequence of the entire genome of DNA or RNA can be determined and epigenetic variations can be detected, thus providing a solid evidence of epigenetic and genetic fidelity in the regenerants recovered after cryopreservation.

In conclusion, the process of cryopreservation seems to preserve the genetic make-up of plants and the plants regenerated from cryopreserved tissues demonstrate the ability to adapt through transient adaptation and through DNA methylation. It would be interesting to investigate the impact of stress ameliorating treatments before, during, and after cryotreatments at the biochemical and molecular level. Stress mitigation during

the culture process prior and after vitrification may further improve the cryopreservation technology and reduce incidences of genetic and epigenetic variations.

Author Contributions: M.-R.W.: data collection and analysis, preparation of tables, and manuscript writing and revisions; W.B.: data collection and analysis, and manuscript revision; M.R.S.: manuscript revision; L.R.: manuscript revision; Z.H. and D.-R.B.: valuable discussions; P.K.S.: manuscript proposal and manuscript revision; Q.-C.W.: manuscript proposal, manuscript writing and revision, and financial supports. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge funding from the National Key R&D Program of China (project no. 2019YFD1001803) for M.-R.W. and Q.-C.W., the National Natural Science Foundation of China (project no. 31870686) for L.R., and the Research Council of Norway (project No. 255032/E50) and NIBIO for Z.H. and D.-R.B.

Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations

AFLP	Amplified fragment length polymorphism
DMSO	Dimethyl sulfoxide
FCM	Flow cytometry
HPLC	High performance liquid chromatography
ISSR	Inter-simple sequence repeats
LN	Liquid nitrogen
MSAP	Methylation sensitive amplified polymorphism
RAPD	Random amplified polymorphic DNA
ROS	Reactive oxygen species
SSR	Single sequence repeats
SRAP	Sequence-related amplified polymorphism

References

1. Sakai, A. Survival of plant tissue of super-low temperature. *Contrib. Inst. Temp. Sci. Haikaido Univ. Ser. B* **1956**, *14*, 17.
2. Sakai, A. Survival of the twigs of woody plants at -196°C . *Nature* **1960**, *185*, 392–394. [[CrossRef](#)]
3. Bettoni, J.C.; Bonnart, R.; Volk, G.M. Challenges in implementing plant shoot tip cryopreservation technologies. *Plant Cell Tissue Org. Cult.* **2021**, *144*, 21–34. [[CrossRef](#)]
4. Pence, V.C.; Ballesteros, D.; Walters, C.; Reed, B.M.; Philpott, M.; Dixon, K.W.; Pritchard, H.W.; Culley, T.M.; Vanhove, A.-C. Cryobiotechnologies: Tools for expanding long-term ex situ conservation to all plant species. *Biol. Conserv.* **2020**, *250*, 108736. [[CrossRef](#)]
5. Tanner, J.D.; Chen, K.Y.; Bonnart, R.M.; Minas, I.S.; Volk, G.M. Considerations for large-scale implementation of dormant budwood cryopreservation. *Plant Cell Tissue Org. Cult.* **2021**, *144*, 35–48. [[CrossRef](#)]
6. Wang, M.-R.; Lambardi, M.; Engelmann, F.; Pathirana, R.; Panis, B.; Volk, G.M.; Wang, Q.-C. Advances in cryopreservation of *in vitro*-derived propagules: Technologies and explant resources. *Plant Cell Tissue Org. Cult.* **2021**, *144*, 7–20. [[CrossRef](#)]
7. Langis, R.; Schnabel-Preikstas, B.J.; Earle, E.D.; Steponkus, P.L. Cryopreservation of *Brassica campestris* L. suspensions by vitrification. *CryoLetters* **1989**, *10*, 421–428.
8. Sakai, A.; Hirai, D.; Niino, T. Development of PVS-based vitrification and encapsulation-vitrification protocols. In *Plant Cryopreservation: A Practical Guide*; Reed, B.M., Ed.; Springer: New York, NY, USA, 2008; pp. 33–58.
9. Uragami, M.; Sakai, A.; Nagai, M.; Takahashi, T. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. *Plant Cell Rep.* **1989**, *8*, 418–421. [[CrossRef](#)]
10. Fabre, J.; Dereuddre, J. Encapsulation-dehydration, a new approach to cryopreservation of *Solanum* shoot tips. *CryoLetters* **1990**, *11*, 413–426.
11. Panis, B.; Piette, B.; Swennen, R. Droplet vitrification of apical meristems: A cryopreservation protocol applicable to all Musaceae. *Plant Sci.* **2005**, *168*, 45–55. [[CrossRef](#)]
12. Engelmann, F.; Arnao, M.T.; Wu, Y.; Escobar, R. Development of encapsulation dehydration. In *Plant Cryopreservation: A Practical Guide*; Reed, B.M., Ed.; Springer: New York, NY, USA, 2008; pp. 59–75.
13. Niino, T.; Yamamoto, S.; Fukui, K.; Martínez, C.R.C.; Arizaga, M.V.; Matsumoto, T.; Engelmann, F. Dehydration improves cryopreservation of mat rush (*Juncus decipiens* Nakai) basal stem buds on cryo-plates. *CryoLetters* **2013**, *34*, 549–560.
14. Yamamoto, S.-I.; Rafque, T.; Priyantha, W.S.; Fukui, K.; Matsumoto, T.; Niino, T. Development of a cryopreservation procedure using aluminum cryo-plates. *CryoLetters* **2011**, *32*, 256–265.

15. Wang, M.-R.; Chen, L.; Teixeira da Silva, J.A.; Volk, G.M.; Wang, Q.-C. Cryobiotechnology of apple (*Malus* spp.): Development, progress and future prospects. *Plant Cell Rep.* **2018**, *37*, 689–709. [[CrossRef](#)]
16. Bi, W.-L.; Pan, C.; Hao, X.-Y.; Cui, Z.-H.; Kher, M.M.; Marković, Z.; Wang, Q.-C.; Teixeira da Silva, J.A. Cryopreservation of grapevine (*Vitis* spp.)—A review. *In Vitro Cell. Dev. Biol.-Plant* **2017**, *53*, 449–460. [[CrossRef](#)]
17. Bi, W.-L.; Hao, X.-Y.; Cui, Z.-H.; Volk, G.M.; Wang, Q.-C. Droplet-vitrification cryopreservation of in vitro-grown shoot tips of grapevine (*Vitis* spp.). *In Vitro Cell. Dev. Biol.-Plant* **2018**, *54*, 590–599. [[CrossRef](#)]
18. Li, J.-W.; Zhang, X.-C.; Wang, M.-R.; Bi, W.-L.; Faisal, M.; Teixeira da Silva, J.A.; Volk, G.M.; Wang, Q.-C. Development, progress and future prospects in cryobiotechnology of *Lilium* spp. *Plant Meth.* **2019**, *15*, 125. [[CrossRef](#)] [[PubMed](#)]
19. Feng, C.-H.; Yin, Z.-F.; Ma, Y.-L.; Chen, L.; Zhang, Z.-B.; Wang, B.; Li, B.-Q.; Huang, Y.-S.; Wang, Q.-C. Cryopreservation of sweetpotato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. *Biotechnol. Adv.* **2011**, *29*, 84–93. [[CrossRef](#)]
20. Vollmer, R.; Villagaray, R.; Egúsqüiza, V.; Espirilla, J.; García, M.; Torres, A.; Rojas, E.; Panta, A.; Barkley, N.; Ellis, D. The potato cryobank at the International Potato Center (CIP): A model for long term conservation of clonal plant genetic resources collections of the future. *CryoLetters* **2016**, *37*, 318–329.
21. Benelli, C.; De Carlo, A.; Engelmann, F. Recent advances in the cryopreservation of shoot-derived germplasm of economically important fruit trees of *Actinidia*, *Diospyros*, *Malus*, *Olea*, *Prunus*. *Biotechnol. Adv.* **2013**, *31*, 175–185. [[CrossRef](#)]
22. Kulus, D.; Zalewska, M. Cryopreservation as a tool used in long-term storage of ornamental species—A review. *Sci. Hortic.* **2014**, *168*, 88–107. [[CrossRef](#)]
23. Yang, X.X.; Popova, E.; Shukla, M.R.; Saxena, P.K. Root cryopreservation to biobank medicinal plants: A case study for *Hypericum perforatum* L. *In Vitro Cell. Dev. Biol.-Plant* **2019**, *55*, 392–402. [[CrossRef](#)]
24. Li, J.-W.; Ozudogru, E.A.; Li, J.; Wang, M.-R.; Bi, W.-L.; Lambardi, M.; Wang, Q.-C. Cryobiotechnology of forest trees: Recent advances and future prospects. *Biodivers. Conserv.* **2017**, *27*, 795–814. [[CrossRef](#)]
25. Bi, W.; Saxena, A.; Ayyanath, M.M.; Harpur, C.; Shukla, M.R.; Saxena, P.K. Conservation, propagation, and redistribution (CPR) of Hill’s thistle: Paradigm for plant species at risk. *Plant Cell Tissue Org. Cult.* **2021**, *145*, 75–88. [[CrossRef](#)]
26. Coelho, N.; Gonçalves, S.; Romano, A. Endemic plant species conservation: Biotechnological approaches. *Plants* **2020**, *9*, 345. [[CrossRef](#)]
27. Normah, M.N.; Sulong, N.; Reed, B.M. Cryopreservation of shoot tips of recalcitrant and tropical species: Advances and strategies. *Cryobiology* **2019**, *87*, 1–14. [[CrossRef](#)]
28. Popova, E.V.; Shukla, M.R.; McIntosh, T.; Saxena, P.K. In vitro and cryobiotechnology approaches to safeguard *Lupinus rivularis* Douglas ex Lindl., an endangered plant in Canada. *Agronomy* **2021**, *11*, 37. [[CrossRef](#)]
29. Streczynski, R.; Clark, H.; Whelehan, L.M.; Ang, S.-T.; Hardstaff, L.K.; Funnekotter, B.; Bunn, E.; Offord, C.A.; Sommerville, K.D.; Mancera, R.L. Current issues in plant cryopreservation and importance for ex situ conservation of threatened Australian native species. *Aust. J. Bot.* **2019**, *67*, 1–15. [[CrossRef](#)]
30. Jenderek, M.M.; Reed, B.M. Cryopreserved storage of clonal germplasm in the USDA National Plant Germplasm System. *In Vitro Cell. Dev. Biol.-Plant* **2017**, *53*, 299–308. [[CrossRef](#)]
31. Wang, B.; Wang, R.-R.; Cui, Z.-H.; Bi, W.-L.; Li, J.-W.; Li, B.-Q.; Ozudogru, E.A.; Volk, G.M.; Wang, Q.-C. Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. *Biotechnol. Adv.* **2014**, *32*, 583–595. [[CrossRef](#)]
32. Salama, A.; Popova, E.; Jones, M.P.; Shukla, M.R.; Fisk, N.S.; Saxena, P.K. Cryopreservation of the critically endangered golden paintbrush (*Castilleja levisecta* Greenm.): From nature to cryobank to nature. *In Vitro Cell. Dev. Biol.-Plant* **2018**, *54*, 69–78. [[CrossRef](#)]
33. Wang, B.; Zhang, Z.; Yin, Z.; Feng, C.; Wang, Q.C. Novel and potential application of cryopreservation to plant genetic transformation. *Biotechnol. Adv.* **2012**, *30*, 604–612. [[CrossRef](#)]
34. Wang, Q.C.; Wang, R.R.; Li, B.Q.; Cui, Z.H. Cryopreservation: A strategy for safe preservation of genetically transformed plant materials. *Adv. Genet. Eng. Biotechnol.* **2012**, *1*, 1–2. [[CrossRef](#)]
35. Wang, Q.C.; Panis, B.; Engelmann, F.; Lambardi, M.; Valkonen, J.P.T. Cryotherapy of shoot tips: A technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. *Ann. Appl. Biol.* **2009**, *154*, 351–363. [[CrossRef](#)]
36. Wang, Q.C.; Valkonen, J.P.T. Cryotherapy of shoot tips: Novel pathogen eradication method. *Trends Plant Sci.* **2009**, *14*, 119–122. [[CrossRef](#)] [[PubMed](#)]
37. Li, J.-W.; Wang, M.-R.; Chen, H.-Y.; Zhao, L.; Cui, Z.-H.; Zhang, Z.; Blystad, D.-R.; Wang, Q.-C. Long-term preservation of potato leafroll virus, potato virus S, and potato spindle tuber viroid in cryopreserved shoot tips. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 10743–10754. [[CrossRef](#)] [[PubMed](#)]
38. Wang, M.-R.; Hamborg, Z.; Ma, X.-Y.; Blystad, D.-R.; Wang, Q.-C. Double-edged effects of cryogenic technique for virus eradication and preservation in shallot shoot tips. *Plant Pathol.* **2021**, accepted.
39. Wang, M.-R.; Yang, W.; Zhao, L.; Li, J.-W.; Liu, K.; Yu, J.-W.; Wu, Y.-F.; Wang, Q.-C. Cryopreservation of virus: A novel biotechnology for long-term preservation of virus in shoot tips. *Plant Meth.* **2018**, *14*, 47. [[CrossRef](#)] [[PubMed](#)]
40. Zhao, L.; Wang, M.-R.; Li, J.-W.; Volk, G.M.; Wang, Q.-C. Cryobiotechnology: A double-edged sword for plant obligate pathogens. *Plant Dis.* **2019**, *103*, 1058–1067. [[CrossRef](#)]
41. Engelmann, F. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cell. Dev. Biol.-Plant* **2011**, *47*, 5–16. [[CrossRef](#)]

42. Kaczmarczyk, A.; Turner, S.R.; Bunn, E.; Mancera, R.L.; Dixon, K.W. Cryopreservation of threatened native Australian species—What have we learned and where to from here? *In Vitro Cell. Dev. Biol.-Plant* **2011**, *47*, 17–25. [CrossRef]
43. RBG Kew. *The State of the World's Plants Report—2016*; Royal Botanic Gardens, Kew: Richmond, UK, 2016. Available online: <https://stateoftheworldsplants.org/2016/> (accessed on 30 August 2021).
44. Edesi, J.; Tolonen, J.; Ruotsalainen, A.L.; Aspi, J.; Häggman, H. Cryopreservation enables long-term conservation of critically endangered species *Rubus humulifolius*. *Biodivers. Conserv.* **2020**, *29*, 303–314. [CrossRef]
45. Sharma, N.; Gowthami, R.; Devi, S.V.; Malhotra, E.V.; Pandey, R.; Agrawal, A. Cryopreservation of shoot tips of *Gentiana kurroo* Royle—A critically endangered medicinal plant of India. *Plant Cell Tissue Org. Cult.* **2021**, *144*, 67–72. [CrossRef]
46. Grout, B.W.W. Cryopreservation of plant cell suspensions. In *Cryopreservation and Freeze-Drying Protocols*; Methods in Molecular Biology™; Day, J.G., Stacey, G.N., Eds.; Human Press: Totowa, NJ, USA, 2007; Volume 368, pp. 153–161.
47. Kaczmarczyk, A.; Funnekotter, B.; Menon, A.; Phang, P.Y.; Al-Hanbali, A.; Bunn, E.; Mancera, R.L. Current issues in plant cryopreservation. In *Current Frontiers in Cryobiology*; Katkov, I.I., Ed.; InTech: Rijeka, Croatia, 2012; pp. 417–438.
48. Kulus, D. Application of cryogenic technologies and somatic embryogenesis in the storage and protection of valuable genetic resources of ornamental plants. In *Somatic Embryogenesis in Ornamental and Its Applications*; Mujib, A., Ed.; Springer: New Delhi, India, 2016; pp. 1–26.
49. Bednarek, P.T.; Orłowska, R. Plant tissue culture environment as a switch-key of (epi)genetic changes. *Plant Cell Tissue Org. Cult.* **2020**, *140*, 245–257. [CrossRef]
50. Miguel, C.; Marum, L. An epigenetic view of plant cells cultured in vitro: Somaclonal variation and beyond. *J. Exp. Bot.* **2011**, *62*, 3713–3725. [CrossRef]
51. Us-Camas, R.; Rivera-Solís, G.; Duarte-Aké, F.; De-la-Peña, C. In vitro culture: An epigenetic challenge for plants. *Plant Cell Tissue Org. Cult.* **2014**, *118*, 187–201. [CrossRef]
52. Zhang, D.; Wang, Z.; Wang, N.; Yang, G.; Ying, L. Tissue culture-induced heritable genomic variation in rice, and their phenotypic implications. *PLoS ONE* **2014**, *9*, e96879.
53. Benson, E.E. Cryopreservation of phytodiversity: A critical appraisal of theory & practice. *Crit. Rev. Plant Sci.* **2008**, *27*, 141–219.
54. Harding, K. Genetic integrity of cryopreserved plant cells: A review. *CryoLetters* **2004**, *25*, 3–22.
55. Harding, K.; Johnson, J.W.; Benson, E.E. Exploring the physiological basis of cryopreservation and failure in clonally propagated *in vitro* crop plant germplasm. *Agric. Food Sci.* **2009**, *18*, 103–116. [CrossRef]
56. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48*, 909–930. [CrossRef]
57. Mittler, R. ROS are good. *Trends Plant Sci.* **2017**, *22*, 11–19. [CrossRef] [PubMed]
58. Smulders, M.J.M.; de Klerk, J.R. Epigenetics in plant tissue culture. *Plant Growth Regul.* **2011**, *63*, 137–146. [CrossRef]
59. Ito, A.; Yoshida, M. Epigenetics. In *Biopros: Biochemical Tools for Investigating Cell Functions*, 2nd ed.; Osada, H., Ed.; Springer: Tokyo, Japan, 2017; pp. 47–65.
60. Boyko, A.; Kovalchuk, I. Epigenetic control of plant stress response. *Environ. Mol. Mutagen.* **2008**, *49*, 61–72. [CrossRef] [PubMed]
61. Hao, Y.J.; Liu, Q.L.; Deng, X.X. Effect of cryopreservation on apple genetic resources at morphological, chromosomal, and molecular levels. *Cryobiology* **2001**, *43*, 46–53. [CrossRef]
62. Hao, Y.J.; You, C.X.; Deng, X.X. Analysis of ploidy and the patterns of amplified fragment length polymorphism and methylation sensitive amplified polymorphism in strawberry plants recovered from cryopreservation. *CryoLetters* **2002**, *23*, 37–46.
63. Hao, Y.J.; You, C.X.; Deng, X.X. Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus. *CryoLetters* **2002**, *23*, 27–35.
64. Peredo, E.L.; Arroyo-García, R.; Reed, B.M.; Revilla, M.A. Genetic stability of in vitro conserved germplasm of *Humulu lupulus* L. *Agric. Food Sci.* **2009**, *18*, 144–151. [CrossRef]
65. Kaity, A.; Drew, R.A.; Ashmore, S.E. Genetic and epigenetic integrity assessment of acclimatised papaya plants regenerated directly from shoot-tips following short- and long-term cryopreservation. *Plant Cell Tissue Org. Cult.* **2013**, *112*, 75–86. [CrossRef]
66. Adu-Gyamfi, R.; Wetten, A.; Rodriguez, L.C.M. Effect of cryopreservation and post-cryopreservation somatic embryogenesis on the epigenetic fidelity of cocoa (*Theobroma cacao* L.). *PLoS ONE* **2016**, *11*, e0158857.
67. Maki, S.; Hirai, Y.; Niino, T.; Matsumoto, T. Assessment of molecular genetic stability between long term cryopreservation and tissue cultured wasabi (*Wasabia japonica*) plants. *CryoLetters* **2015**, *36*, 318–324.
68. Johnston, J.W.; Benson, E.E.; Harding, K. Cryopreservation induces temporal DNA methylation epigenetic changes and differential transcriptional activity in *Ribes* germplasm. *Plant Physiol. Biochem.* **2009**, *47*, 123–131. [CrossRef]
69. Kaity, A.; Ashmore, S.E.; Drew, R.A. Assessment of genetic and epigenetic changes following cryopreservation in papaya. *Plant Cell Rep.* **2008**, *2*, 1529–1539. [CrossRef]
70. Ibáñez, M.A.; Alvarez-Mari, A.; Rodríguez-Sanz, H.; Kremer, C.; González-Benito, M.E.; Martín, C. Genetic and epigenetic stability of recovered mint apices after several steps of a cryopreservation protocol by encapsulation-dehydration. A new approach for epigenetic analysis. *Plant Physiol. Biochem.* **2019**, *143*, 299–307. [CrossRef]
71. Zhang, X.-C.; Bao, W.-W.; Zhang, A.-L.; Pathirana, R.; Wang, Q.-C.; Liu, Z.-D. Cryopreservation of shoot tips, evaluations of vegetative growth, and assessments of genetic and epigenetic changes in cryo-derived plants of *Actinidia* spp. *Cryobiology* **2020**, *94*, 18–25. [CrossRef]

72. Heringer, A.S.; Steinmacher, D.A.; Fraga, H.P.F.; Vieira, L.N.; Ree, J.F.; Guerra, M.P. Global DNA methylation profiles of somatic embryos of peach palm (*Bactris gasipaes* Kunth) are influenced by cryoprotectants and droplet-vitrification cryopreservation. *Plant Cell Tissue Org. Cult.* **2013**, *114*, 365–372. [[CrossRef](#)]
73. Plitta, B.P.; Michalak, M.; Naskret-Barciszewska, M.Z.; Barciszewski, J.; Chmielarz, P. DNA methylation of *Quercus robur* L. plumules following cryo-pretreatment and cryopreservation. *Plant Cell Tissue Org. Cult.* **2014**, *117*, 31–37. [[CrossRef](#)]
74. Kaczmarczyk, A.; Houben, A.; Keller, E.R.J.; Mette, M.F. Influence of cryopreservation on the cytosine methylation state of potato genomic DNA. *CryoLetters* **2010**, *31*, 380–391.
75. Miłkuła, A.; Tomiczak, K.; Rybczyński, J.J. Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi)genetic uniformity of regenerants. *Plant Cell Rep.* **2011**, *30*, 565–574. [[CrossRef](#)]
76. Vos, P.; Hogers, R.; Bleeker, M.; Reijmans, M.; van de Lee, T.; Hornes, M.; Frijters, A.; Pot, J.; Peleman, J.; Kuiper, M.; et al. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* **1995**, *23*, 4407–4414. [[CrossRef](#)] [[PubMed](#)]
77. Jacob, H.J.; Lindpainter, K.; Lincoln, S.E.; Kusumi, K.; Bunker, R.K.; Mao, Y.-P.; Ganten, D.; Dzau, V.J.; Lander, E.S. Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. *Cell* **1991**, *67*, 213–224. [[CrossRef](#)]
78. Akkaya, M.S.; Bhagwat, A.A.; Cregan, P.B. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* **1992**, *132*, 1131–1139. [[CrossRef](#)]
79. Wang, B.; Li, J.-W.; Zhang, Z.-B.; Wang, R.-R.; Ma, Y.-L.; Blystad, D.-R.; Keller, E.R.J.; Wang, Q.-C. Three vitrification-based cryopreservation procedures cause different cryo-injuries to potato shoot tips while all maintain genetic integrity in regenerants. *J. Biotechnol.* **2014**, *184*, 47–55. [[CrossRef](#)]
80. Kulus, D.; Rewers, M.; Serocka, M. Cryopreservation by encapsulation-dehydration affects the vegetative growth of chrysanthemum but does not disturb its chimeric structure. *Plant Cell Tissue Org. Cult.* **2019**, *138*, 153–166. [[CrossRef](#)]
81. Liu, X.-X.; Wen, Y.-B.; Cheng, Z.-H.; Mou, S.-W. Establishment of a garlic cryopreservation protocol for shoot apices from adventitious buds in vitro. *Sci. Hortic.* **2017**, *226*, 10–18. [[CrossRef](#)]
82. Wang, R.-R.; Gao, X.-X.; Chen, L.; Huo, L.-Q.; Li, M.-F.; Wang, Q.-C. Shoot recovery and genetic integrity of *Chrysanthemum morifolium* shoot tips following cryopreservation by droplet-vitrification. *Sci. Hortic.* **2014**, *176*, 330–339. [[CrossRef](#)]
83. Krajňáková, J.; Sutela, S.; Aronen, T.; Gömöry, D.; Vianello, A.; Häggman, H. Long-term cryopreservation of Greek fir embryogenic cell lines: Recovery, maturation and genetic fidelity. *Cryobiology* **2011**, *63*, 17–25. [[CrossRef](#)]
84. Pence, V.C.; Philpott, M.; Culley, T.M.; Clair, B.; Yorke, S.R.; Lindsey, K.; Vanhove, A.C.; Ballesteros, D. Survival and genetic stability of shoot tips of *Hedeoma todsenii* R.S. Irving after long-term cryostorage. *In Vitro Cell. Dev. Biol.-Plant* **2017**, *53*, 28–338.
85. Martín, C.; Cervera, M.T.; González-Benito, M.E. Genetic stability analysis of chrysanthemum (*Chrysanthemum x morifolium* Ramat) after different stages of an encapsulation–dehydration cryopreservation protocol. *J. Plant Physiol.* **2011**, *168*, 158–166. [[CrossRef](#)] [[PubMed](#)]
86. Martín, C.; Kremer, C.; González, I.; González-Benito, M.E. Influence of the cryopreservation technique, recovery medium and genotype on genetic stability of mint cryopreserved shoot tips. *Plant Cell Tissue Org. Cult.* **2015**, *122*, 185–195. [[CrossRef](#)]
87. González-Benito, M.E.; Kremer, C.; Ibáñez, M.A.; Martín, C. Effect of antioxidants on the genetic stability of cryopreserved mint shoot tips by encapsulation–dehydration. *Plant Cell Tissue Org. Cult.* **2016**, *127*, 359–368. [[CrossRef](#)]
88. Castillo, N.R.F.; Bassil, N.V.; Wada, S.; Reed, B.M. Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. *In Vitro Cell. Dev. Biol.-Plant* **2010**, *46*, 246–256. [[CrossRef](#)]
89. Aronen, T.S.; Krajňakov, J.; Häggman, H.M.; Ryyänen, L.A. Genetic fidelity of cryopreserved embryogenic cultures of open-pollinated *Abies cephalonica*. *Plant Sci.* **1999**, *142*, 163–172. [[CrossRef](#)]
90. Merhy, T.S.M.; Vianna, M.G.; Garcia, R.O.; Pacheco, G.; Mansur, E. Cryopreservation of *Passiflora pohllii* nodal segments and assessment of genetic stability of regenerated plants. *CryoLetters* **2014**, *35*, 204–215.
91. Li, J.-W.; Chen, H.-Y.; Li, X.-Y.; Zhang, Z.; Blystad, D.-R.; Wang, Q.-C. Cryopreservation and evaluations of vegetative growth, microtuber production and genetic stability in regenerants of purple-fleshed potato. *Plant Cell Tissue Org. Cult.* **2017**, *128*, 641–653. [[CrossRef](#)]
92. Galdiano, R.F., Jr.; de Macedo Lemos, E.G.; Vendrame, W.A. Cryopreservation, early seedling development, and genetic stability of *Oncidium flexuosum* Sims. *Plant Cell Tissue Org. Cult.* **2013**, *114*, 139–148. [[CrossRef](#)]
93. Bi, W.-L.; Pan, C.; Liu, J.; Wang, Q.-C. Greenhouse performance, genetic stability and biochemical compounds in *Chrysanthemum morifolium* ‘Hangju’ plants regenerated from cryopreserved shoot tips. *Acta Physiol. Plant.* **2016**, *38*, 268. [[CrossRef](#)]
94. Zhang, Z.; Skjeseth, G.; Elameen, A.; Haugslie, S.; Sivertsen, A.; Wang, Q.-C.; Blystad, D.R. Field performance evaluation and genetic integrity assessment in *Argyranthemum maderense* plants recovered from cryopreserved shoot tips. *In Vitro Cell. Dev. Biol.-Plant* **2015**, *51*, 505–513. [[CrossRef](#)]
95. Li, J.-W.; Li, H.-H.; Wang, R.-R.; Gao, X.-X.; Wang, Q.-C. Cryopreservation for retaining morphology, genetic integrity, and foreign genes in transgenic plants of *Torenia fournieri*. *Acta Physiol. Plant.* **2016**, *38*, 8. [[CrossRef](#)]
96. Cheng, W.; Li, H.; Zhou, F.; Zhu, B.; Yu, J.; Ding, Z. Cryopreservation of *Pleione bulbocodioides* (Franch.) Rolfe protocorm-like bodies by vitrification. *Acta Physiol. Plant.* **2020**, *42*, 82. [[CrossRef](#)]
97. Cejas, I.; Vives, K.; Laudat, T.; González-Olmedo, J.; Engelmann, F.; Martínez-Montero, M.E.; Lorenzo, J.C. Effects of cryopreservation of *Phaseolus vulgaris* L. seeds on early stages of germination. *Plant Cell Rep.* **2012**, *31*, 2065–2073. [[CrossRef](#)]
98. Carmona-Martín, E.; Regalado, J.J.; Perán-Quesada, R. Cryopreservation of rhizome buds of *Asparagus officinalis* L. (cv. Morado de Huétor) and evaluation of their genetic stability. *Plant Cell Tissue Org. Cult.* **2018**, *133*, 395–403. [[CrossRef](#)]

99. Wang, M.-R.; Hamborg, Z.; Slimestad, R.; Elameen, A.; Blystad, D.-R.; Haugslie, S.; Skjeseth, G.; Wang, Q.-C. Assessments of rooting, vegetative growth, bulb production, genetic integrity and biochemical compounds in cryopreserved plants of shallot. *Plant Cell Tissue Org. Cult.* **2021**, *144*, 123–131. [[CrossRef](#)]
100. Agrawal, A.; Sanayaima, R.; Singh, R.; Tandon, R.; Verma, S.; Tyagi, R.K. Phenotypic and molecular studies for genetic stability assessment of cryopreserved banana meristems derived from field and in vitro explant sources. *In Vitro Cell. Dev. Biol.-Plant* **2014**, *50*, 345–356. [[CrossRef](#)]
101. Li, B.-Q.; Feng, C.-H.; Hu, L.-Y.; Wang, M.-R.; Chen, L.; Wang, Q.-C. Shoot regeneration and cryopreservation of shoot tips of apple (*Malus*) by encapsulation–dehydration. *In Vitro Cell. Dev. Biol.-Plant* **2014**, *50*, 357–368. [[CrossRef](#)]
102. Li, B.-Q.; Feng, C.-H.; Wang, M.-R.; Hu, L.-Y.; Volk, G.M.; Wang, Q.-C. Recovery patterns, histological observations and genetic integrity in *Malus* shoot tips cryopreserved using droplet-vitrification and encapsulation-dehydration procedures. *J. Biotechnol.* **2015**, *214*, 182–191. [[CrossRef](#)] [[PubMed](#)]
103. Wang, L.-Y.; Li, Y.-D.; Sun, H.-Y.; Liu, H.-G.; Tang, X.-D.; Wang, Q.-C.; Zhang, Z.-D. An efficient droplet-vitrification cryopreservation for valuable blueberry germplasm. *Sci. Hortic.* **2017**, *219*, 60–69. [[CrossRef](#)]
104. Chen, H.-Y.; Liu, J.; Pan, C.; Yu, J.-W.; Wang, Q.-C. In vitro regeneration of adventitious buds from leaf explants and their subsequent cryopreservation in highbush blueberry. *Plant Cell Tissue Org. Cult.* **2018**, *134*, 193–204. [[CrossRef](#)]
105. Ai, P.-F.; Lu, L.-P.; Song, J.-J. Cryopreservation of in vitro-grown shoot-tips of *Rabdosia rubescens* by encapsulation-dehydration and evaluation of their genetic stability. *Plant Cell Tissue Org. Cult.* **2012**, *108*, 381–387. [[CrossRef](#)]
106. Sharma, N.; Singh, R.; Pandey, R.; Kaushik, N. Genetic and biochemical stability assessment of plants regenerated from cryopreserved shoot tips of a commercially valuable medicinal herb *Bacopa monnieri* (L.) Wettst. *In Vitro Cell. Dev. Biol.-Plant* **2017**, *53*, 346–351. [[CrossRef](#)]
107. Wang, R.-R.; Mou, H.-Q.; Gao, X.-X.; Chen, L.; Li, M.-F.; Wang, Q.-C. Cryopreservation for eradication of Jujube witches' broom phytoplasma from Chinese jujube (*Ziziphus jujuba*). *Ann. Appl. Biol.* **2015**, *166*, 218–228. [[CrossRef](#)]
108. Tavazza, R.; Luciola, A.; Benelli, C.; Giorgi, D.; D'Aloisio, E.; Papacchioli, V. Cryopreservation in artichoke: Towards a phytosanitary qualified germplasm collection. *Ann. Appl. Biol.* **2013**, *163*, 231–241. [[CrossRef](#)]
109. Li, J.-W.; Chen, H.-Y.; Li, J.; Zhang, Z.; Blystad, D.-R.; Wang, Q.-C. Growth, microtuber production and physiological metabolism in virus-free and virus-infected potato in vitro plantlets grown under NaCl-induced salt stress. *Eur. J. Plant Pathol.* **2018**, *152*, 417–432. [[CrossRef](#)]
110. Meijer, E.G.M.; Iren, E.; Schrijnemakers, E.; Hensgens, L.A.M.; van Zijderveld, M.; Schilperoort, R.A. Retention of the capacity to produce plants from protoplasts in cryopreserved cell lines of rice (*Oryza sativa* L.). *Plant Cell Rep.* **1991**, *10*, 171–174. [[CrossRef](#)]
111. Cho, J.S.; Hong, S.M.; Joo, S.Y.; Yoo, J.S.; Kim, D.I. Cryopreservation of transgenic rice suspension cells producing recombinant hCTLA4Ig. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1470–1476. [[CrossRef](#)]
112. Van Eck, J.; Keen, P. Continued expression of plant-made vaccines following long-term cryopreservation of antigen-expressing tobacco cell cultures. *In Vitro Cell. Dev. Biol.-Plant* **2009**, *45*, 750–757. [[CrossRef](#)]
113. Vendrame, W.A.; Holliday, C.P.; Montello, P.M.; Smith, D.R.; Merkle, S.A. Cryopreservation of yellow-poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar* spp.) embryogenic cultures. *New For.* **2001**, *21*, 283–292. [[CrossRef](#)]
114. Hao, Y.J.; Deng, X.X. GUS gene remains stable in transgenic *Citrus* callus recovered from cryopreservation. *CryoLetters* **2003**, *24*, 375–380.
115. Ryyänen, L.; Sillanpää, M.; Kontunen-Soppela, S.; Tiimonen, H.; Kangasjärvi, J.; Vapaavuori, E.; Häggman, H. Preservation of transgenic silver birch (*Betula pendula* Roth) lines by means of cryopreservation. *Mol. Breed.* **2002**, *10*, 143–152. [[CrossRef](#)]
116. Jokipii, S.; Ryyänen, L.; Kallio, P.T.; Aronen, T.; Häggman, H. A cryopreservation method maintaining the genetic fidelity of a model forest tree, *Populus tremula* L. × *Populus tremuloides* Michx. *Plant Sci.* **2004**, *166*, 799–806. [[CrossRef](#)]
117. Schmale, K.; Rademacher, T.H.; Fischer, R.; Hellwig, S. Towards industrial usefulness—Cryocell banking of transgenic BY-2 cell cultures. *J. Biotechnol.* **2006**, *124*, 302–311. [[CrossRef](#)]
118. Corredoira, E.; San-Jose, M.C.; Vieitez, A.M.; Ballester, A. Improving genetic transformation of European chestnut and cryopreservation of transgenic lines. *Plant Cell Tissue Org. Cult.* **2007**, *91*, 281–288. [[CrossRef](#)]
119. Dolce, N.R.; Faloci, M.M.; Gonzalez, A.M. In vitro plant regeneration and cryopreservation of *Arachis glabrata* (Fabaceae) using leaflet explants. *In Vitro Cell. Dev. Biol.-Plant* **2018**, *54*, 133–144. [[CrossRef](#)]
120. Espasandin, F.D.; Brugnoli, E.A.; Ayala, P.G.; Ayala, L.P.; Ruiz, O.A. Long-term preservation of *Lotus tenuis* adventitious buds. *Plant Cell Tissue Org. Cult.* **2019**, *136*, 373–382. [[CrossRef](#)]
121. Martín, C.; Senula, A.; González, E.; Acosta, A.; Keller, E.R.J.; González-Benito, M.E. Genetic identity of three mint accessions stored by different conservation procedures: Field collection, in vitro and cryopreservation. *Genet. Resour. Crop Evol.* **2013**, *60*, 243–249. [[CrossRef](#)]
122. Agrawal, A.; Tyagi, R.K.; Goswami, R. Cryobanking of Banana (*Musa* sp.) germplasm in India: Evaluation of agronomic and molecular traits of cryopreserved plants. *Acta Hortic.* **2011**, *908*, 129–138. [[CrossRef](#)]
123. Cejas, I.; Méndez, R.; Villalobos, A.; Palau, F.; Aragón, C.; Engelmann, F.; Carputo, D.; Aversano, R.; Martínez, M.E.; Lorenzo, J.C. Phenotypic and molecular characterization of *Phaseolus vulgaris* plants from non-cryopreserved and cryopreserved seeds. *Am. J. Plant Sci.* **2013**, *4*, 844–849. [[CrossRef](#)]

124. Hazubska-Przybył, T.; Chmielarz, P.; Michalak, M.; Dering, M.; Bojarczuk, K. Survival and genetic stability of *Picea abies* embryogenic cultures after cryopreservation using a pregrowth-dehydration method. *Plant Cell Tissue Org. Cult.* **2013**, *113*, 303–313. [[CrossRef](#)]
125. Salaj, T.; Matušíková, I.; Fráterová, L.; Piršelová, B.; Salaj, J. Regrowth of embryogenic tissues of *Pinus nigra* following Cryopreservation. *Plant Cell Tissue Org. Cult.* **2013**, *106*, 55–61. [[CrossRef](#)]
126. Akdemir, H.; Süzerer, V.; Tilkat, E.; Yildirim, H.; Onay, A.; Çiftçi, Y.O. In vitro conservation and cryopreservation of mature pistachio (*Pistacia vera* L.) germplasm. *J. Plant Biochem. Biotechnol.* **2013**, *22*, 43–51. [[CrossRef](#)]
127. Kaya, E.; Souza, F.V.D. Comparison of two PVS2-based procedures for cryopreservation of commercial sugarcane (*Saccharum* spp.) germplasm and confirmation of genetic stability after cryopreservation using ISSR markers. *In Vitro Cell. Dev. Biol.-Plant* **2017**, *53*, 410–417. [[CrossRef](#)]
128. Coelho, N.; González-Benito, M.E.; Martín, C.; Romano, A. Cryopreservation of *Thymus lotocephalus* shoot tips and assessment of genetic stability. *CryoLetters* **2014**, *35*, 119–128.
129. Solov'eva, A.I.; Dolgikh, Y.I.; Vysotskaya, O.N.; Popov, A.S. Patterns of ISSR and REMAP DNA markers after cryogenic preservation of spring wheat calli by dehydration method. *Russ. J. Plant Physiol.* **2011**, *58*, 423–430. [[CrossRef](#)]
130. Hirano, T.; Godo, T.; Miyoshi, K.; Ishikawa, K.; Ishikawa, M.; Mii, M. Cryopreservation and low-temperature storage of seeds of *Phaius tankervilleae*. *Plant Biotechnol. Rep.* **2009**, *3*, 103–109. [[CrossRef](#)]
131. Cejas, I.; Rumlow, A.; Turcios, A.; Engelmann, F.; Martínez, M.E.; Yabor, L.; Papenbrock, J.; Lorenzo, J.C. Exposure of common bean seeds to liquid nitrogen modifies mineral composition of young plantlet leaves. *Am. J. Plant Sci.* **2016**, *7*, 1612–1617. [[CrossRef](#)]
132. Zevallos, B.; Cejas, I.; Valle, B.; Yabor, L.; Aragón, C.; Engelmann, F.; Martínez, M.E.; Lorenzo, J.C. Short-term liquid nitrogen storage of wild tomato (*Solanum lycopersicum* Mill.) seeds modifies the levels of phenolics in 7 day-old seedlings. *Sci. Hort.* **2013**, *160*, 264–267. [[CrossRef](#)]
133. Arguedas, M.; Gómez, D.; Hernández, L.; Engelmann, F.; Garramone, R.; Cejas, I.; Yabor, L.; Martínez-Monter, M.E. Maize seed cryo-storage modifies chlorophyll, carotenoid, protein, aldehyde and phenolics levels during early stages of germination. *Acta Physiol. Plants.* **2018**, *40*, 118. [[CrossRef](#)]
134. Arguedas, M.; Perez, A.; Abdelnour, A.; Hernandez, M.; Engelmann, F.; Martínez, M.E.; Yabor, L.; Lorenzo, J.C. Short-term liquid nitrogen storage of maize, common bean and soybean seeds modifies their biochemical composition. *Agric. Sci.* **2016**, *4*, 6–12. [[CrossRef](#)]
135. Zevallos, B.; Cejas, I.; Rodríguez, R.C.; Yabor, L.; Aragón, C.; González, J.; Engelmann, F.; Martínez, M.E.; Lorenzo, J.C. Biochemical characterization of Ecuadorian wild *Solanum lycopersicum* Mill. Plants produced from non-cryopreserved and cryopreserved seeds. *CryoLetters* **2013**, *34*, 413–421.
136. Acosta, Y.; Pérez, L.; Linares, C.; Hernández, L.; Escalante, D.; Pérez, A.; Zevallos, B.E.; Yabor, L.; Martínez-Montero, M.E.; Cejas, I.; et al. Effects of *Terammus labialis* (L.f.) Spreng seed cryopreservation on subsequent seed and seedling growth and biochemistry. *Acta Physiol. Plant.* **2020**, *42*, 7. [[CrossRef](#)]
137. Moraes, R.M.; Souza, L.B.; Nery, F.C.; Paiva, R.; Barbosa, S. Seed cryopreservation as an alternative for the conservation of *H. sabdariffa* L. (Malvaceae) germplasm. *Acta Hort.* **2018**, *1224*, 165–174. [[CrossRef](#)]