



ROS-induced oxidative stress in plant cryopreservation: occurrence and alleviation

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Abstract

Main conclusion Reactive oxygen species (ROS)-induced oxidative stress results in low success or even total failure of cryopreservation. Better understanding of how the plant establishes resistance/tolerance to ROS-induced oxidative stress facilitates developments of robust cryopreservation procedures.

Abstract Cryopreservation provides a safe and efficient strategy for long-term preservation of plant genetic resources. ROS-induced oxidative stress caused damage to cells and reduced the ability of the plant to survive following cryopreservation, eventually resulting in low success or even total failure. This paper provides updated and comprehensive information obtained in the past decade, including the following: (1) ROS generations and adaptive responses of antioxidant systems during cryopreservation; (2) expressions of oxidative stress-associated genes and proteins during cryopreservation; (3) ROS-triggered programmed cell death (PCD) during cryopreservation; and (4) exogenous applications of enzymatic and non-enzymatic antioxidants in improving success of cryopreservation. Prospects for further studies are proposed. The goal of the present study was to facilitate better understanding of the mechanisms by which the plant establishes resistance/tolerance to oxidative stress during cryopreservation and promote further studies toward the developments of robust cryopreservation procedures and wider application of plant cryobiotechnology.

Keywords Antioxidants · Cryopreservation · Gene expression · Oxidative stress · Programmed cell death · Reactive oxygen species

Introduction

Plant cryopreservation refers to storage of plant cells, tissue and organs in liquid nitrogen (LN, $-196\text{ }^{\circ}\text{C}$) or liquid nitrogen vapor (LNV, approx. -165 to $-190\text{ }^{\circ}\text{C}$) (Kaczmarczyk et al. 2012; Wang et al. 2021a). Cryopreservation

is considered at present time an ideal means for long-term preservation of plant genetic resources (Kaczmarczyk et al. 2012; Bettoni et al. 2021; Wang et al. 2014, 2021a, b). Cryo-banks using shoot tips have been set up in several countries for economically important plant species (Kulus and Zalewska 2014; Wang et al. 2014; Vollmer et al. 2016; Jenderek and Reed 2017). Cryobiotechnology has also been extended to long-term preservation of transgenes in transgenic cells (Wang et al. 2012, 2014), production of pathogen-free plants (Wang and Valkonen 2009; Wang et al. 2014; Zhao et al. 2019) and long-term preservation of obligate pathogens such as viruses and viroids (Zhao et al. 2019). Studies have also advanced in the evaluation of the performance of cryo-derived plants when they were re-introduced from laboratories to their natural habitats (Salama et al. 2018; Bi et al. 2021). However, ROS-induced oxidative stress is recognized to be a major constraint for further developments of plant cryopreservation, particularly endangered, endemic and

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tropical species, which are still recalcitrant to cryopreservation (Kaczmarczyk et al. 2012; Reed 2014; Funnekotter et al. 2017; Normah et al. 2019; Streczynski et al. 2019; Coelho et al. 2020).

As signaling molecules, ROS supports cellular proliferation and physiological function, and therefore, maintenance of a basal level of ROS is essential for life (Mittler 2017). However, overproduction of ROS is highly reactive and toxic and causes damage to DNA and protein, as well as membrane oxidation (Gill and Tuteja 2010; Mittler 2017). Under normal circumstances, there is a balance between ROS production and clearance in plants. However, under stress conditions, excessive ROS are produced, resulting in oxidative stress (Gill and Tuteja 2010). Oxidative stress refers to a state of imbalance between oxidation and anti-oxidation, which is caused by excessive generations of ROS (Halliwell 2006; Mittler 2017). $O_2\cdot^-$, H_2O_2 , and $OH\cdot$ are the three major ROS and H_2O_2 is the main ROS component that causes membrane lipid peroxidation in plant cryopreservation (Chen et al. 2015; Ren et al. 2015; Zhang et al. 2015). Detailed information on generation of $O_2\cdot^-$, H_2O_2 and $OH\cdot$ can be found in the articles of Mittler (2002) and Vranová et al. (2002).

In response to oxidative stress, plants develop antioxidant defense systems via enzymatic and non-enzymatic antioxidant reactions to maintain normal metabolisms and functions in the cell, thus protecting themselves against oxidative stress (Gill and Tuteja 2010). An antioxidant is defined as a molecule or compound that can delay, prevent, or remove damage caused by oxidative stress (Halliwell 2006). Plant antioxidants include two types: enzymatic and non-enzymatic, both of which can regulate ROS production (Halder et al. 2018). Enzymatic antioxidants mainly include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR) (Gill and Tuteja 2010). Non-enzymatic antioxidants consist of many members, for example, glutathione (GSH), ascorbic acid (AsA), abscisic acid (ABA), tocopherol (VE) and melatonin (Gill and Tuteja 2010; Nawaz et al. 2016; Sah et al. 2016). Among these antioxidants, SOD functions in scavenging $O_2\cdot^-$ and lowering the possibility of $OH\cdot$ production (Scandalios 1990). POD, CAT and APX play an essential protective role in scavenging H_2O_2 when coordinating with SOD (Chaitanya et al. 2002). Melatonin fortifies plants against abiotic and biotic stress, mainly by scavenging ROS and reactive nitrogen species (Nawaz et al. 2016). AsA is the most effective water-soluble antioxidant in plants, which can provide electrons to a large number of antioxidants (Gill and Tuteja 2010) and scavenge $O_2\cdot^-$ and H_2O_2 (Noctor and Foyer 1998; Smirnoff 2000). GSH helps to scavenge ROS via AsA-GSH cycle and a balance between

GSH and oxidized glutathione is important for maintaining the redox status of cells (Pastori et al. 2000; Gill and Tuteja 2010).

Plant cryopreservation requires several necessary steps such as the establishment of stock cultures, excision of explants, preculture, osmoprotection and cryoprotection, dehydration, freeze–thaw cycle, unloading and post-culture for recovery (Fig. 1A). All these steps induce ROS generation (Fig. 1B, C, E, F). ROS-induced oxidative stress reduces the ability of the explant to survive following cryopreservation, eventually resulting in poor recovery or even total failure (Benson and Bremner 2004; Kaczmarczyk et al. 2012; Reed 2014; Funnekotter et al. 2017; Normah et al. 2019; Streczynski et al. 2019; Coelho et al. 2020; Fig. 1B, C, F–H).

This review focuses on advances over the past decade in analyses of ROS generation and adaptive responses of the antioxidant system during cryopreservation procedures. ROS-induced programmed cell death (PCD), and expressions of oxidative stress-associated genes and proteins in cryopreservation are also presented. Finally, applications of enzymatic and non-enzymatic antioxidants to improvements of recovery of cryopreserved explants are presented in detail.

ROS generation, programmed cell death (PCD) and adaptive responses of antioxidant system in plant cryopreservation

ROS generation

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 cryopreserved tissues (Funnekotter et al. 2017; Wang et al. 2021b; Fig. 1A). It is well documented that in vitro tissue culture imposes stressful conditions and induces the generations of ROS, thus resulting in ROS-induced oxidative stress (Kaczmarczyk et al. 2012; Funnekotter et al. 2017; Bednarek and Orłowska 2020; Wang et al. 2021b; Fig. 1B, F). Cold-hardening of the in vitro stock cultures was frequently used for improving plant cryopreservation (Kaczmarczyk et al. 2012; Funnekotter et al. 2017). Cold-hardening of the in vitro stock cultures was proven to induce expressions of antioxidant enzymes, such as SOD and catalase, increase antioxidant levels, and maintain membrane stability, eventually enhancing recovery of cryopreserved plants (Kaczmarczyk et al. 2012; Funnekotter et al. 2017).

It has been known for a long time that age of stock cultures from which explants were excised and used for cryopreservation considerably affected success of cryopreservation (Wang and Perl 2006). Using vitrification cryopreservation for different ages of *Arabidopsis thaliana*

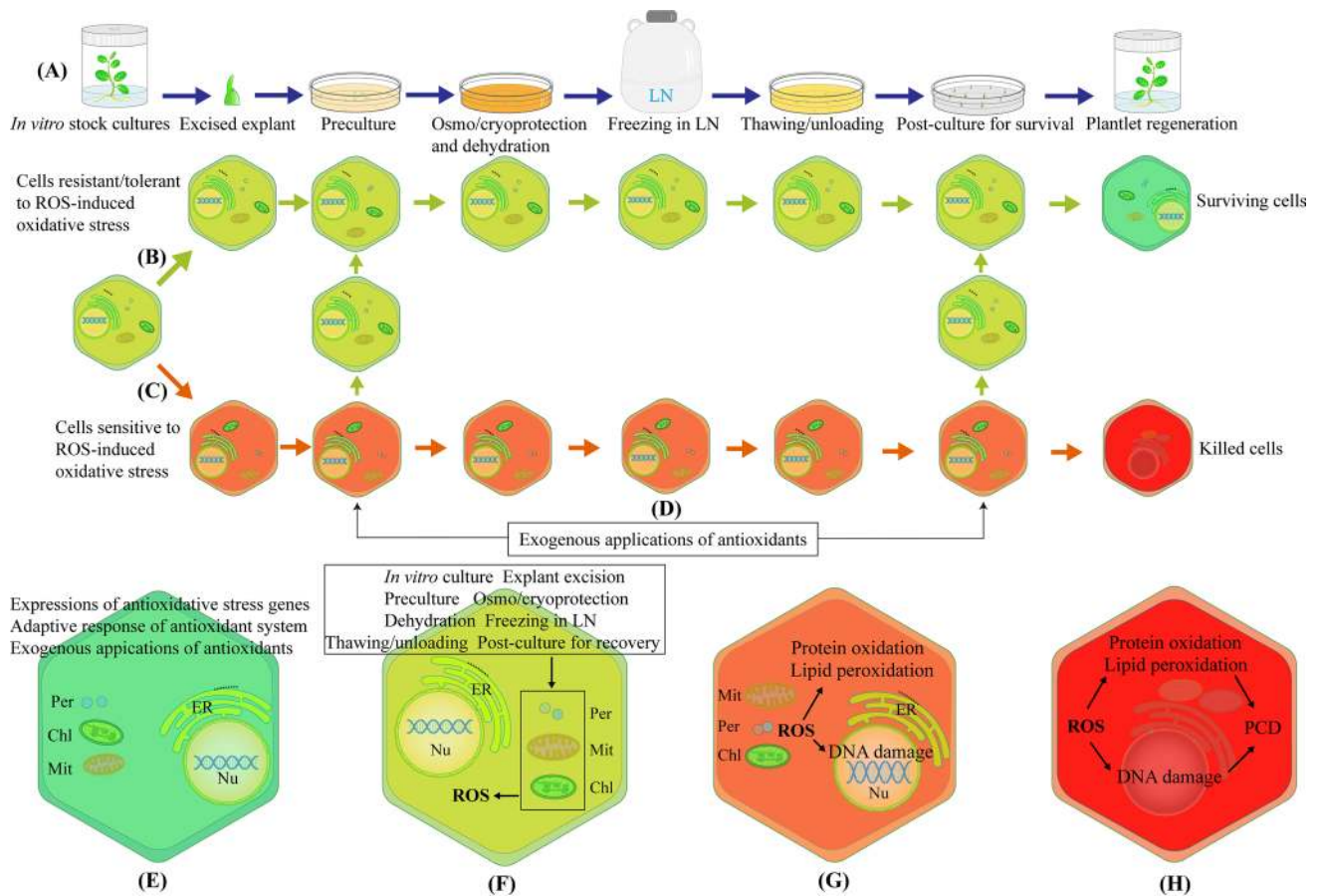


Fig. 1 Hypothetical illustration of ROS-induced oxidative stress, oxidative stress-caused cell damage and PCD, adaptive responses of antioxidant system, expressions of antioxidative stress genes, and exogenous applications of antioxidants for improving recovery of cryopreserved shoot tips. **A** Major steps of cryopreservation procedure. **B** Responses of cells that are resistant/tolerant to oxidative stress by expressions of antioxidative genes and proteins to alleviate the oxidative stress during cryopreservation, thus helping cells to survive after cryopreservation. **C** Responses of cells that are sensitive to oxidative stress during cryopreservation. Oxidative stress causes membrane lipid peroxidation, protein oxidation and DNA damage, and induced programmed cell death (PCD), eventually killing the cells after cryopreservation. **D** Exogenous applications of antioxidants to preculture and/or post-culture media for improving recovery of cryopreserved plants. **E** A representative cell (green color) that is resistant/tolerant

to oxidative stress, in which antioxidative stress genes are expressed, and adaptive responses of antioxidant system are established to respond to oxidative stress. Exogenous applications of enzymatic and non-enzymatic antioxidants alleviate ROS-induced oxidative stress and improve recovery of cryopreserved plants. Such cells are most likely to survive after cryopreservation. **F** A representative cell (yellow color), in which ROS generation is induced in in vitro culture and the major steps of cryopreservation procedure. **G** A representative cell (light red color) that is sensitive to oxidative stress, in which ROS causes protein oxidation, lipid peroxidation and DNA damage. **H** A representative cell (red color) that is sensitive to oxidative stress, in which PCD is induced. *Chl* chloroplasts, *ER* endoplasmic reticulum, *Mit* mitochondria, *Nu* nucleus, *PCD* programmed cell death, *Per* peroxisomes, *ROS* reactive oxygen species

seedlings, Ren et al. (2013) found that survival of cryopreserved seedlings significantly decreased with an increase in age of stock seedlings: 97% and 0% for 48-h and 72-h seedlings, respectively. MDA was much higher in the 72-h stock seedlings than in the 48-h ones following osmoprotection and dehydration. There was a significant negative correlation between MDA content and survival levels following cryopreservation. A further study of the same group found that during the whole vitrification procedure, $O_2\cdot^-$ activities were much higher and H_2O_2 levels were much lower in the 48-h seedlings than in the 72-h ones. Chen et al. (2015)

reported that although $OH\cdot$ activities continuously increased during the whole cryopreservation procedure, the increased $OH\cdot$ activities were much more pronounced in 72-h seedlings than in 48-h ones. These findings provide new insights on better understanding of the mechanism as to how the age of stock cultures influences plant cryopreservation.

Excision of the explant is a necessary step, particularly in cryopreservation of shoot tips and embryogenic tissues. Excision of the explant is a wounding process, which has been documented to induce accumulations of ROS (Savatini et al. 2014). Roach et al. (2008) reported that the excision

of embryonic axes from the seeds of a recalcitrant sweet chestnut (*Castanea sativa*) induced an oxidative burst of $O_2^{\cdot-}$, resulting in the reduced viability of the axes following desiccation and subsequent freezing in LN. More examples that explant excision-mediated oxidative stress were responsible for the reduced recoveries of cryopreserved explants are listed in Table 1.

Using a vitrification cryo-plate (V cryo-plate) for cryopreservation of *Paphiopedilum niveum* somatic embryos, Soonthornkalump et al. (2020) found that the highest level of the total ROS was induced in the preculture step. MDA level started to significantly increase from the preculture and reached the highest level in the osmoprotection step. These results indicated that preculture step was a critical step for successful cryopreservation of *Paphiopedilum niveum* somatic embryos.

Working on dehydration cryopreservation of recalcitrant seeds of *Ekebergia capensis*, Bharuth and Naidoo (2020) found that the reduced survival levels were closely associated with the increased levels of $O_2^{\cdot-}$ and H_2O_2 in each step of explant excision, cryoprotection, dehydration and freezing in LN. In the study of vitrification cryopreservation for *Oryza sativa* zygotic embryos, Huang et al. (2018) found that the major steps significantly affected levels of $O_2^{\cdot-}$ and MDA, which were negatively correlated with survival of the embryos following cryopreservation. Analyzing ROS production in zygotic embryos of recalcitrant *Haemanthus montanus* seeds subjected to cryopreservation, Sershen et al. (2012) found that although dehydration and cryoprotection increased $O_2^{\cdot-}$ levels, freezing in LN induced the highest level of $O_2^{\cdot-}$, and was responsible for considerable or complete viability loss of the zygotic embryos following cryopreservation.

In vitrification cryopreservation of *Arabidopsis* seedlings, H_2O_2 and OH^{\cdot} content continuously increased during the cryopreservation procedure and reached the highest level after rapid warming (Ren et al. 2015). Further analysis found that a large amount of ROS induced in the freeze–thaw cycle altered the redox state in the cell, caused membrane lipid peroxidation, and destroyed plant photosynthetic phosphorylation and oxidative phosphorylation systems (Ren et al. 2013, 2015). All these negative effects of excessive accumulations of ROS resulted in the reduced recovery of cryopreserved *Arabidopsis* seedlings.

It is worth noting that the production of ROS was found to increase the viability of pollen after cryopreservation in *Lilium* × *siberia* (Xu et al. 2014). In this study, ROS generation was analyzed by a flow cytometry with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe. The fluorescence levels at 488 nm (excitation) and 525 nm (emission) were expressed as mean green intensity fluorescence units for index of ROS level. ROS increased from 16 in the control to 26 and 50 in cryopreserved pollen by rapid

cooling and vitrification, respectively. Pollen viability significantly increased from 47% in the control (fresh pollen) to 59% and 70% in cryopreserved pollen by rapid cooling and vitrification, respectively (Xu et al. 2014). These results indicate that ROS may have positive effects on cryopreservation, and the positive effects may depend on its concentration, plant species, types of explant and cryogenic procedures used.

ROS-induced oxidative stress and cell damage are illustrated in Fig. 1C, F and some examples of ROS generation and cell damage induced in plant cryogenic procedures are listed in Table 1.

ROS-induced PCD

PCD refers to an ordered and energy-required physiological process that is genetically programmed, eventually leading to the cell death (Reape and McCabe 2008). There are three main forms in PCD, including apoptosis, necrosis and autophagic cell death (Reape and McCabe 2008). It has been well documented that the generation and accumulation of ROS under oxidative stress induced PCD, and apoptosis and necrosis were considered the major forms of PCD in cryopreservation (Reape and McCabe 2008; Bissoyi et al. 2014).

Harding et al. (2009) were the first to propose that cryopreservation may induce PCD in plants. Since then, oxidative stress-mediated ROS has been proven to be involved in induction of PCD in plant cryopreservation. Working on *Eucalyptus grandis* axillary buds, Risenga et al. (2013) found that bud excision and drying induced high ROS levels, which reduced viability of the explant analyzed by caspase-3-like protease activity. Caspases have been demonstrated to be responsible for the stress-triggered PCD processes (Reape and McCabe 2008). Therefore, Risenga et al. (2013) believed that dehydration-induced ROS generation triggered PCD. Wesley-Smith et al. (2015) found that exposure to LN resulted in autophagic degradation and ultimately autolysis, and formation of small intracellular ice crystals in cryopreserved embryonic axes of recalcitrant *Acer saccharinum* seeds. These results indicated that freezing stress induced PCD. In cryopreservation of *Dendrobium* PLBs by vitrification, Jiang et al. (2019) found that the preculture and freeze–thaw cycle induced expression of the autophagy-related protein 8C gene (*Atg 8C*) and reticulon-like protein B8 gene (*Rtnl B8*). Caspase-3-like activity increased following the osmoprotection treatment and dehydration and reached the highest level following freeze–thaw cycle. Levels of H_2O_2 and NO started to increase in the preculture stage and reached the highest level in the osmoprotection treatment. These results provided experimental concrete evidence that ROS generations triggered PCD in cryopreservation (Jiang et al. 2019).

Table 1 Some examples of ROS, cell damage and antioxidants induced in plant cryopreservation

Plant species	Explant	Cryopreservation method	ROS, cell damage and antioxidants detected	Steps of cryoprotocol, in which ROS, cell damage and antioxidants were detected	Reference
<i>Agapanthus praecox</i>	Embryogenic callus	Vitrification	O ₂ ^{·-} , H ₂ O ₂ and OH [·] ; MDA; SOD, POD, CAT, AsA and GSH	PC, DH, FTC, UL, PCR	Zhang et al. (2015)
<i>Amaryllis belladonna</i> and <i>Hae-manthus montanus</i>	Zygotic embryos	Vitrification	O ₂ ^{·-} , H ₂ O ₂ and OH [·] ; MDA; SOD, POD, CAT, AsA and GSH	PC, DH, FTC, UL, PCR	Chen et al. (2016)
<i>Arabidopsis thaliana</i>	Seedlings	Vitrification	O ₂ ^{·-} , H ₂ O ₂ and OH [·] ; MDA; SOD, POD, CAT, AsA and GSH	PC, DH, FTC, UL	Ren et al. (2020a)
			H ₂ O ₂ and OH [·] ; MDA; SOD, POD and CAT	PC, DH, UL, PCR	Chen et al. (2021a)
			O ₂ ^{·-} and H ₂ O ₂	DH, UL, PCR	Chen et al. (2021b)
<i>Amaryllis belladonna</i> and <i>Hae-manthus montanus</i>	Zygotic embryos	Vitrification	O ₂ ^{·-} ; MDA; SOD, CAT, GR and APX	DH, FTC	Sershen et al. (2012)
<i>Arabidopsis thaliana</i>	Seedlings	Vitrification	MDA	DH	Ren et al. (2013)
			O ₂ ^{·-} , H ₂ O ₂ and OH [·] ; MDA; SOD, POD, CAT, AsA and GSH	OP, DH, FTC, UL, PCR	Ren et al. (2015)
			O ₂ ^{·-} , H ₂ O ₂ and OH [·] ; MDA; SOD, POD, CAT, AsA and GSH	OP, DH, FTC, UL, PCR	Chen et al. (2015)
<i>Dendrobium nobile</i>	Protocorm-like bodies	Vitrification	H ₂ O ₂ ; MDA; CAT, AsA and GSH	OP, DH, FTC	Zhang et al. (2021a)
			O ₂ ^{·-} and H ₂ O ₂ ; MDA and PCO; SOD, CAT and AsA	PC, OP, DH, FTC, UL	Jia et al. (2016)
			H ₂ O ₂	PC, OP, DH, FTC, UL	Jiang et al. (2019)
			H ₂ O ₂ and NADPH; SOD, CAT, APX, GR, AsA and GSH	PC, OP, UL	Zhang et al. (2021b)
<i>Ekebergia capensis</i>	Seeds	Vitrification	O ₂ ^{·-} and H ₂ O ₂ ; total aqueous anti-oxidant (types not specified)	DH, FTC	Bharuth and Naidoo (2020)
<i>Eucalyptus grandis</i>	Axillary buds	Dehydration	O ₂ ^{·-}	EE, PC, RE	Risenga et al. (2013)
<i>Hypericum perforatum</i>	Shoot tips	Vitrification	ROS and H ₂ O ₂ ; SOD and CAT	EE, DH, PCR	Skyba et al. (2012)
<i>Lilium x siberia</i>	Pollen	Vitrification	ROS (types not specified)	OP, DH, FTC, UL	Xu et al. (2014)
<i>Magnolia denudata</i> and <i>Paeonia lactiflora</i>	Pollen	Direct immersion in LN	ROS (types not specified); MDA; SOD, CAT and AsA	FTC	Jia et al. (2018)
<i>Malus sylvestris</i> , <i>Prunus avium</i> and <i>Prunus padus</i>	Seeds	Dehydration	H ₂ O ₂ ; MDA; AsA	PCR	Wawrzyniak et al. (2020)
<i>Oryza sativa</i>	Zygotic embryos	Vitrification	O ₂ ^{·-} and H ₂ O ₂ ; MDA; SOD, CAT, APX, DHAR, MDHAR, GR, AsA and GSH	PC, OP, DH	Huang et al. (2018)
<i>Paeonia lactiflora</i>	Pollen	Direct immersion in LN	ROS (types not specified); MDA; SOD, CAT and AsA	FTC	Ren et al. (2019)
			H ₂ O ₂ and OH [·] ; SOD, POD, CAT, GR, APX, AsA and GSH	FTC	Ren et al. (2020b)

Table 1 (continued)

Plant species	Explant	Cryopreservation method	ROS, cell damage and antioxidants detected	ROS, cell damage and antioxidants detected	Steps of cryoprotocol, in which ROS, cell damage and antioxidants were detected	Reference
<i>Paeonia suffruticosa</i>	Pollen	Direct immersion in LN	O ₂ ⁻ , H ₂ O ₂ and OH [·] ; MDA and PCO; SOD, POD, CAT, GR, APX, AsA and GSH	FTC	FTC	Ren et al. (2021)
<i>Paphiopedilum niveum</i>	Somatic embryos	Cryo-plate	ROS (types not specified)	FTC	FTC	Ren et al. (2020c)
<i>Passiflora ligularis</i>	Seed embryos	Vitrification	ROS (types not specified); MDA	PC, DH	PC, DH	Soonthornkalump et al. (2020)
<i>Strychnos gerrardii</i> and <i>Boophae disticha</i>	Zygotic axes	Dehydration	H ₂ O ₂ ; MDA; SOD, CAT and APX	PCR	PCR	Prudente et al. (2019)
<i>Trichilia dregeana</i>	Embryonic axes	Dehydration	O ₂ ⁻ ; total antioxidant activity	EE, DH, RH	EE, DH, RH	Berjak et al. (2011)
<i>Amygdalus davidiana</i> , <i>Kerria japonica</i> , <i>Jasminum nudiflorum</i> , <i>Orychophragmus violaceus</i> and <i>Paulownia tomentosa</i>	Pollen	Direct immersion in LN	O ₂ ⁻ ; POD	EE, RH	EE, RH	Whitaker et al. (2010)
<i>Cercis chinensis</i> , <i>Magnolia biondii</i> , <i>Robinia pseudoacacia</i> and <i>Rosa primula</i>	Pollen	Direct immersion in LN	ROS (types not specified)	FTC	FTC	Jia et al. (2017)
<i>Malus spectabilis</i> , <i>Paeonia suffruticosa</i> , <i>Philadelphus pekinensis</i> , <i>Syringa oblata</i> and <i>Xanthoceras sorbifolium</i>	Pollen	Direct immersion in LN	ROS (types not specified) and MDA	FTC	FTC	

LN liquid nitrogen, APX ascorbate peroxidase, AsA ascorbic acid, CAT catalase, DHAR dehydroascorbate reductase, GR glutathione reductase, GSH glutathione, H₂O₂ hydrogen peroxide, MDA malondialdehyde, MDHAR monodehydroascorbate reductase, NADPH nicotinamide adenine dinucleotide phosphate, O₂⁻ superoxide anion, OH[·] hydroxyl radicals, PCO protein carbonyl, POD peroxidase, ROS reactive oxygen species, SOD superoxide dismutase, DH dehydration, EE explant excision, FTG freeze-thaw cycle, OP osmoprotection, PC preculture, PCR post-culture for recovery, DH rehydration, UL unloading

Fluorescein diacetate (FDA) staining is generally used as a marker for apoptosis-like events (Burbridge et al. 2006). Ren et al. (2013) found that the 72-h *Arabidopsis* seedlings completely failed to recover following cryopreservation, compared with 97% survivals in the 48-h seedlings. The 72-h seedlings showed negative responses to FDA staining during the whole cryopreservation procedure. The defender against apoptotic death 1 (*DADI*), which encodes for the protein involved in suppression of apoptosis (Gallois et al. 1997), was found to be upregulated in the 48-h seedlings, while its expression levels maintained unchanged in the 72-h ones after dehydration (Ren et al. 2013). These results supported that apoptosis-like events were induced in the 72-h *Arabidopsis* seedlings during cryopreservation.

In cryopreservation of *Agapanthus praecox* embryogenic callus (EC), Zhang et al. (2015) found that the burst outbreak of ROS induced the occurrence of PCD. Further analysis of PCD showed that some EC cells exhibited autophagy and apoptosis-like events, and a few cells underwent necrosis during cryopreservation. Autophagy appeared at the dehydration and unloading steps, and apoptosis-like events occurred through the cryopreservation procedure. Exogenous applications of PCD modulators like caspase inhibitor and cinnamtannin B-1 significantly improved cell viability following cryopreservation (Zhang et al. 2015).

In addition, ROS-triggered PCD has also been reported in cryopreservations of pollen of *Paeonia lactiflora* (Ren et al. 2020b), and *Paeonia suffruticosa* (Ren et al. 2020c), and *Agapanthus praecox* embryogenic callus (Chen et al. 2021a, b). ROS-induced PCD is illustrated in Fig. 1F.

Adaptive enzymatic and non-enzymatic responses of antioxidant system

For enzymatic antioxidants, dehydration and freezing in LN were reported to reduce GR activity and aggravate cell membrane lipid peroxidation in zygotic embryos of *Zea mays* (Wen et al. 2010) and *Livistona chinensis* (Wen et al. 2012). Manipulations of pretreatments enhanced activities of antioxidants, especially GR, and partly increased tolerance to dehydration and freezing in LN of *Olea europaea* somatic embryos, resulting in increased survivals following cryopreservation (Lynch et al. 2011). Using droplet-vitrification cryopreservation of *Lomandra sonderi* shoot tips, Funnekotter et al. (2016) found that SOD activity was positively correlated to the recovery following cryopreservation, among the other three antioxidant enzymes CAT, GR and GPX.

In vitrification cryopreservation of *Dendrobium sonia*-28 protocorm-like bodies (PLBs), Poobathy et al. (2013) reported that drastic increases in CAT activities were produced following the treatments of preculture, thawing and unloading, and in the initial stage of shoot regrowth following cryopreservation. In the study of droplet-vitrification for

cryopreservation of *Brassidium* PLBs, Rahmah et al. (2015) found that the activity of APX and CAT reached the highest level in dehydration and freezing in LN. SOD activities maintained an overall increase in different steps of the cryopreservation, and reached the highest level in the late shoot regrowth stage (Poobathy et al. 2013). Huang et al. (2018) showed that the preculture treatment stimulated $O_2^{\cdot-}$ generation and activated the antioxidative response system in the apical meristems of germinated embryos. Treatment of osmoprotection motivated the activity of CAT and APX. In the study on vitrification cryopreservation of *Dendrobium* Sabin Blue PLBs, Antony et al. (2019) found that the lowest activity of CAT was obtained in the explant after exposure to the PVS2 dehydration; the lowest activity of POX enzyme following the dehydration and post-culture for shoot regrowth; and the lowest activity of APX in the thaw and unloading steps. These changes in antioxidant enzymes resulted in the low level of shoot regrowth in the explant following cryopreservation (Antony et al. 2019). In a V cryo-plate cryopreservation for *Passiflora suberosa* shoot tips, Vianna et al. (2019) reported that the highest SOD activity was detected in the preculture step, and continuously decreased during the cryopreservation procedure. Significantly higher activities of CAT and APX were obtained after the osmoprotection treatment. CAT activity gradually decreased after osmoprotection, whereas the APX activity decreased after 10 days of post-culture. All these results indicated that the elevated activities of enzymatic antioxidants enhanced cell resistance/tolerance to cryoinjury, and control of oxidative damage via ROS homeostasis can lead to the high recovery in cryopreservation.

For non-enzymatic antioxidants, Chen et al. (2015) found that the AsA content of 48-h *Arabidopsis* seedlings was significantly higher than in 72-h ones from the dehydration step to post-culture for recovery. Exogenous application of AsA or SK₃-type dehydrin protein to PVS2 significantly elevated the content of endogenous AsA in the dehydration step, and increased the regrowth levels of *Arabidopsis* seedlings after cryopreservation (Ren et al. 2015; Zhang et al. 2021a). Significantly higher contents of AsA and GSH were induced in *A. praecox* EC following treatments of unloading and rewarming (Zhang et al. 2015). Chen et al. (2016) found that adding exogenous GSH to PVS2 increased endogenous AsA and GSH contents after dehydration step and improved cell viability following cryopreservation. Improved viability of cryopreserved pollen of *Paeonia suffruticosa* was attributed to sufficient maintenance of the internal balance of oxidative metabolism by SOD, AsA and GSH (Ren et al. 2021).

A recent study clearly demonstrated that the activities of antioxidants were closely related to success of cryopreservation (Ren et al. 2021). In this study, pollen viability following cryopreservation differed among three *P. suffruticosa* cultivars: one decreased, one was stable and one increased.

Contents of ROS, MDA and protein carbonyl (PCO) were significantly lower in the cultivar with increased viability than in the other two cultivars, while SOD activity was higher in the former than in the latter two. SOD activity was negatively correlated with the MDA and POD contents, and positively correlated with pollen viability following cryopreservation. AsA was lower, while GSH was higher, in the cultivar with increased viability than in the other two cultivars. AsA significantly increased, while GSH significantly decreased, the MDA and PCO contents. The membrane lipid oxidation and protein oxidative damage caused by ROS were responsible for the decrease in pollen viability after cryopreservation. SOD, AsA and GSH effectively maintained the internal balance of oxidative metabolism and reduced the levels of oxidative damage, thus improving pollen viability (Ren et al. 2021).

Some examples are presented in Table 1 and adaptive responses of antioxidant system induced in plant cryopreservation are illustrated in Fig. 1G.

Expressions of oxidative stress-related genes and proteins in plant cryopreservation

Over the past decade, efforts have been invested to study expressions of oxidative stress-related genes and proteins in plant cryopreservation. Some examples are presented in Table 2, and alleviation of ROS-induced oxidative stress by expressions of oxidative stress-related genes and proteins in plant cryopreservation is illustrated in Fig. 1G.

Huang et al. (2018) reported expressions of antioxidant enzyme genes *Cu/Zn SOD*, *CAT1*, *APX7*, *GR2*, *GR3*, *MDHAR1* and *DHAR1* in *Oryza sativa* zygotic embryos in vitrification cryopreservation. The authors suggested that these genes might serve as potential indicators of oxidative stress-induced genes in cryopreservation. Zhang et al. (2015) found that expressions of genes, particularly SOD and BAG, were positively related with cell viability of *Agapanthus praecox* EC following cryopreservation. Using high-throughput omics technology to screen two dehydrins of *A. praecox* EC subjected to cryopreservation, Yang et al. (2019) reported that their expression levels were specifically upregulated at the transcription and protein levels. Working on *A. praecox* EC, Chen et al. (2016) reported that inclusion of 0.08 mM GSH in PVS2 markedly increased recovery of cryopreserved EC and enhanced expressions of stress-responsive genes, including *POD*, *APX*, *MDHAR* and *GPX* in the treated samples during cryopreservation procedures. Gene expression patterns provided molecular mechanisms that the application of GSH to PVS2 solution effectively improved recovery of *A. praecox* EC.

Volk et al. (2011) used the *Arabidopsis* genome chip to analyze the differential expression genes of shoot tips during

cryopreservation and found that dehydration-related genes were specifically upregulated after vitrification solution treatment. Application of 1 mM AsA in PVS2 significantly increased recovery in 60-h *Arabidopsis* seedlings following cryopreservation (Ren et al. 2014). DREBs/CBFs assisted establishments of tolerance cryoinjury, and calcium-binding protein, OXI1, WRKY and MYB family members served as key factors in ROS signal transduction and activated the ROS-producing and -scavenging networks. Increased expressions of these genes contributed to improvements of recovery of the 60-h seedlings following cryopreservation (Ren et al. 2015). Further studies by Chen et al. (2015) and Zhang et al. (2021a) both found that upregulations of *Cu/Zn-SOD*, *APX* and *CAT* increased tolerance to oxidative stress and improved the recovery of *Arabidopsis* seedlings after cryopreservation.

PVS2 and plant vitrification solution 3 (PVS3) are the most frequently used plant vitrification solutions in plant cryopreservation. Gross et al. (2017) found that expression levels of 180 transcripts differed in *Arabidopsis* shoot tips following exposures to PVS2 and PVS3. The transcripts induced by the treatment of PVS2 dehydration and freezing in LN induced oxidative responses, whereas the treatment of PVS3 dehydration and freezing in LN invoked more metabolic responses. These results provided molecular insights into varying levels of post-cryopreservation recoveries produced by PVS2 and PVS3 dehydration.

In the study of the translation level following treatments of sucrose or cold preculture of *Solanum tuberosum* and *S. commersonii* shoot tips, Folgado et al. (2014) found that the oxidative homeostasis-related proteins were associated with the improved tolerance to cryopreservation. In the study of cryopreservation of *Dendrobium nobile* PLBs, Di et al. (2018) found that protein synthesis, processing and degradation might be the main strategies to re-establish cell balance in the PLBs following cryopreservation. The production of ROS and the decline in energy production, signaling transduction, and membrane transport during LN exposure might be responsible for the viability loss. Studying microRNA (miRNA)-based post-transcriptional regulations in 48-h and 72-h *Arabidopsis* seedlings subjected to cryopreservation, Ekinici et al. (2021) demonstrated that the alteration of expression levels of cold-induced genes related-miRNAs played a key role in successful cryopreservation.

Exogenous applications of antioxidants to alleviate oxidative stress for improving plant cryopreservation

A number of studies have been conducted over the past decade on exogenous applications of enzymatic and non-enzymatic antioxidants to alleviate oxidative stress for improving

Table 2 Expressions of oxidative stress-related genes and proteins induced in plant cryopreservation

Plant species	Explant	Cryopreservation method	Major molecular experiments	Differentially expressed genes, proteins or pathways	Reference
<i>Agapanthus praecox</i>	Embryogenic callus	Vitrification	qRT-PCR	Eight oxidative stress related-genes and seven PCD related-genes	Zhang et al. (2015)
				Eight oxidative stress related-genes and two PCD related-genes	Chen et al. (2016)
				Twelve oxidative stress related-genes	Ren et al. (2020a)
<i>Arabidopsis thaliana</i>	Shoot tips	Vitrification	Fully sequenced <i>Arabidopsis</i> genome and readily available microarray slides	Ten cell death related genes	Chen et al. (2021b)
			Array gene expression, qRT-PCR	Genes involved in cold, desiccation and oxidation responses	Volk et al. (2011)
			cDNA-AFLP, qRT-PCR	Transcripts related to abiotic stress, oxidation, and wounding	Gross et al. (2017)
<i>Dendrobium</i>	Seedlings	Vitrification	Transcriptome microarray, qRT-PCR	Genes involved in stress response, protein synthesis and metabolism, and metabolism and energy cofactor and vitamin metabolism, especially <i>DREBs/CBFs</i> , <i>calcium-binding protein</i> , <i>OXII</i> , <i>WRKY</i> and <i>MYB</i> family members	Ren et al. (2013)
			qRT-PCR	Six PCD-regulating genes	Jiang et al. (2019)
			iTRAQ proteomic analysis, qRT-PCR	Protein synthesis, processing and degradation, production of ROS, energy production, signaling transduction, and membrane transport	Di et al. (2018)
<i>Oryza sativa</i>	Zygotic embryos	Vitrification	qRT-PCR	Twenty antioxidant enzyme genes	Huang et al. (2018)
			Two-dimensional electrophoresis, qRT-PCR	Proteins related to carbohydrate metabolism, stress response, oxidative metabolism, and carbohydrate metabolism, especially HSP and 14-3-3-like protein	Lei et al. (2021)
<i>Prunus mume</i>	Pollen	Direct immersion in LN	Two-dimensional electrophoresis	Some protein spots between 12.6–72.8 and 5.6–7.3 kDa	Zhang et al. (2012)
			Two-dimensional electrophoresis	Proteins related to carbon fixation and mechanisms, oxidative homeostasis	Folgado et al. (2014)

LN liquid nitrogen, *cDNA-AFLP* cDNA amplified fragment length polymorphism, *iTRAQ* isobaric tags for relative and absolute quantification, *qRT-PCR* quantitative reverse transcription-polymerase chain reaction, *DREBs/CBFs* dehydration-responsive element-binding proteins/C-repeat binding factors, *MYB* v-Myb avian myeloblastosis viral oncogene homolog, *PCD* programmed cell death, *HSP* heat shock proteins, *miRNAs* microRNAs, *OXII* oxidative signal-inducible 1.*ROS* reactive oxygen species, *WRKY* tryptophan-arginine-lysine-tyrosine

Table 3 Exogenous applications of enzymatic and non-enzymatic antioxidants for improving recovery of cryopreserved plants

Plant species	Explant	Cryopreservation method	Antioxidants (concentrations), application steps	Increases in recovery from control to treatment (survival, regrowth or germination)	Reference
Enzymatic antioxidants					
<i>Dendrobium nobile</i>	Protocorm-like bodies	Vitrification	CAT (400 U ml ⁻¹), UL	From 6 to 21% (regrowth)	Di et al. (2017)
<i>Euonymus fortunei</i>	Shoot tips	Vitrification	ETH (200–400 mg l ⁻¹), PC	From 67 to 73% (survival)	Zhang et al. (2021b)
<i>Magnolia denudata</i> and <i>Paeonia lactiflora</i>	Pollen	Direct immersion in LN	CAT (200 U ml ⁻¹), UL CAT (400 IU ml ⁻¹) and MDH (100 IU ml ⁻¹), FTC	From 27 to 58% (survival) From 24–46 to 64–70% for CAT and 30–84% for MDH (germination)	Xu et al. (2017) Jia et al. (2018)
Non-enzymatic antioxidants					
<i>Actinidia chinensis</i> var. <i>chinensis</i>	Shoot tips	Vitrification	AsA (0.4 mM), PT	From 0 to 40% (regrowth)	Mathew et al. (2019)
<i>Agapanthus praecox</i>	Embryogenic callus	Vitrification	Cinnamtannin B-1 (50 µg ml ⁻¹), DH GSH (0.08 mM), DH Nanomaterials SWCNTs (0.1 g l ⁻¹), DH ApSerp-ZX protein (1.2 mg l ⁻¹), DH	From 49.14 to 86.85% (survival) From 49 to 83% (survival) From 53 to 85% (survival)	Zhang et al. (2015) Chen et al. (2016) Ren et al. (2020a)
	Callus	Vitrification	Nanomaterials SWCNTs (0.1 g l ⁻¹) or C ₆₀ (0.3 g l ⁻¹), DH	From 24 to 49% for SWCNTs and 61% for C ₆₀ (survival)	Chen et al. (2017)
<i>Amaryllis belladonna</i>	Zygotic embryos	Dehydration	Glycerol (5–10%), PT	From 8 to 20–72%	Sershen et al. (2012)
<i>Arabidopsis thaliana</i>	60-h seedlings	Vitrification	ABA (1 µM), GB (10 mM), GSH (0.16 mM), AsA (1 mM), DH	From 18–29 to more than 40% (regrowth)	Ren et al. (2014)
<i>Chrysanthemum grandiflorum</i>	Shoot tips	Vitrification	ApDHN protein (2 µM), DH Y ₂ SK ₂ - and SK ₃ -type dehydrins (5 µM), DH AFP (500–1000 µg l ⁻¹), DH	From 23 to 50% (regrowth) From 25 to 46% (Y ₂ SK ₂) and 52% (SK ₃) (regrowth) From 55–80 to 65–95% (regrowth)	Yang et al. (2019) Zhang et al. (2021a) Jeon et al. (2015)
<i>Dioscorea alata</i> and <i>D. cayenensis</i>	Shoot tips	Desiccation	Melatonin (0.05 and 0.1 µM), DH	From 15 to 35% (regrowth)	Uchendu and Keller (2016)
<i>Eucalyptus grandis</i>	Axillary buds		ABA (5 mg l ⁻¹), PT	From 55 to 70% (survival)	Risenga et al. (2013)
<i>Haemanthus montanus</i>	Zygotic embryos	Dehydration	Glycerol (5–10%), PT	From 10 to 15–55%	Sershen et al. (2012)
<i>Hancornia speciosa</i>	Lateral buds	Vitrification	Proline (0.1–0.2 M), PC	From 11 to >40% (regrowth)	Prudente et al. (2017)
<i>Hypericum perforatum</i>	Shoot tips	Vitrification	ABA (0.076 µM), PT	From 59 to 71% (regrowth)	Bruňáková et al. (2011)
<i>Hypericum perforatum</i> and <i>Nicotiana tabacum</i>		Encapsulation-vitrification and vitrification	Melatonin (0.1–0.5 µM), PC and PCR	From 50–63 to 80–100% (regrowth)	Uchendu et al. (2014)

Table 3 (continued)

Plant species	Explant	Cryopreservation method	Antioxidants (concentrations), application steps	Increases in recovery from control to treatment (survival, regrowth or germination)	Reference
<i>Lamprocapnos spectabilis</i>	Shoot tips	Encapsulation-vitrification	Gold nanoparticles (10 ppm), EnCap	From 51 to 70% (regrowth)	Kulus and Tymosuk (2021)
<i>Nephetium ramboutan-ake</i>			AsA (0.28 mM), OP	From 0 to 3.3% (regrowth)	Chua and Normah (2011)
<i>Oncidium flexuosum</i>	Mature seed	Vitrification	PG (1%), DH	From 47 to 78% (germination)	Galdiano et al. (2013)
<i>Paeonia lactiflora</i>	Pollen	Direct freezing	Supercool X-1000 (1%), DH HSP70 (0.5–10 µg ml ⁻¹), FTC	From 47 to 59% (germination) From about 21–28 to 26–36% (germination)	Ren et al. (2019)
<i>Paphiopedilum insignne</i>	Protocorm	Encapsulation-vitrification	Glutathione (30 µM), PC and PCR	From 37 to 63% (regrowth)	Diengdoh et al. (2019)
<i>Paphiopedilum niveum</i>	Somatic embryos	V cryo-plate	AsA (0.1 mM), PT	From 9 to 39% (regrowth)	Soonthornkalump et al. (2020)
<i>Picea abies</i>	Embryogenic tissue	Vitrification	ABA (10 µM), PT and DH	From 20 to 54% (survival)	Hazubska-Przybył et al. (2013)
<i>Picea glauca</i> × <i>P. engelmannii</i> and <i>Pseudotsuga menziesii</i>	Immature somatic embryo	Direct immersion in LN	ABA (50 µM), PT	From 10–40 to 100% for spruce genotype ISP 11 (survival)	Kong and von Aderkas (2011)
<i>Rhodiola crenulata</i>	Callus	Vitrification	Melatonin (0.1 µM), PC	From 62 to 72% (survival)	Zhao et al. (2011)
<i>Rubus</i>	Shoot tips	Vitrification	Vitamin E (11 mM), AsA (0.28 mM), PT, OP, UL and PCR	From 40 to 70% (VE) and from 40 to 90% (AsA) (regrowth)	Uchendu et al. (2010a)
<i>Strychnos gerrardii</i>	Zygotic axes	Drying and direct freezing	LA (4–8 mM), GSH (0.16 mM) and GB (10 mM), PT, OP, UL and PCR	From 40–50 to > 80% (regrowth)	Uchendu et al. (2010b)
<i>Ulmus americana</i>	Shoot tips	Vitrification and encapsulation-vitrification	Cathodic water, RD Melatonin (0.1 µM), PC and PCR	From 6 to 70% (regrowth) From 50–63 to 80–100% (regrowth)	Berjak et al. (2011) Uchendu et al. (2013)
<i>Vitis</i> spp.		Droplet-vitrification	SA (0.1 mM), PT of stock cultures	From 0–13 to 7–45% (regrowth)	Pathirana et al. (2016)

LN liquid nitrogen, ABA abscisic acid, AsA ascorbic acid, AFP antifreezing protein, C₆₀ spherical carbon nanomaterial, CAT catalase, DH dehydration, EnCap encapsulation, ETH ethephon, FTC freeze–thaw cycle, GB glycine betaine, GSH glutathione, HSP heat shock protein, LA lipoic acid, MDH malate dehydrogenase, OP osmoprotection, PC preculture, PCR post-culture for recovery, PG phloroglucinol, PT pretreatment, RD rehydration, SA salicylic acid, SWCNTs single walled carbon nanotubes, UL unloading

plant cryopreservation. Some examples are presented in Table 3. Improvement of recovery of cryopreserved plants by exogenous applications of the antioxidants is illustrated in Fig. 1D, G.

Enzymatic antioxidants

Testing effects of application of CAT or PDH into osmoprotection solution, PVS2 and unloading solution on vitrification cryopreservation of *Dendrobium nobile* PLBs, Di et al. (2017) found that adding of 400 IU ml⁻¹ CAT to the unloading solution produced about 50% of survival and 21% of shoot regrowth in cryopreserved PLBs, which were significantly higher than 17% and 6% produced in the control. Another study from the same group found that only adding of 400 IU ml⁻¹ CAT to osmoprotection solution significantly improved survival of *Euonymus fortunei* shoot tips (Xu et al. 2017). Jia et al. (2018) found that inclusion of 400 IU ml⁻¹ CAT in the germination solution produced much higher pollen germination rates of cryopreserved pollen than the control in *Magnolia denudata* and *Paeonia lactiflora*. The addition of malate dehydrogenase (MDH) in the germination solution was also found to improve pollen germination following cryopreservation, but the optimal concentrations varied with plant species: 100 IU ml⁻¹ for *Magnolia denudata* and 200 IU ml⁻¹ for *Paeonia lactiflora* (Jia et al. 2018). These results indicated that effects of the antioxidant enzymes on cryopreservation vary with types and concentrations of antioxidant enzymes, steps in which antioxidant enzymes are added, and plant species and explant types. Inclusion of CAT and 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide in the preculture medium was reported to significantly increase survival in *Dendrobium* PLBs following cryopreservation (Jiang et al. 2019).

Exogenous application of CAT significantly reduced H₂O₂ and MDA contents, increased AsA content and induced higher activity of endogenous CAT in the treated PLBs of *Dendrobium nobile* (Di et al. 2017). Jia et al. (2018) found that the use of MDH significantly reduced ROS and MDA levels, but increased SOD activity in cryopreserved pollen of *M. denudata*. Application of CAT significantly reduced levels of ROS and MDA, but increased activity of CAT and SOD in *P. lactiflora*. These effects of CAT and MDH were believed to contribute to the improvement of recovery in cryopreserved explants (Di et al. 2017; Jia et al. 2018).

Non-enzymatic antioxidants

Over the past decade, there have been increasing interests in exogenous applications of melatonin and nanoparticles to improvements of plant cryopreservation. Non-enzymatic

antioxidants such as AsA, ABA and GSH were also used as usual in plant cryopreservation.

Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolic compound, which is naturally biosynthesized in diverse plant species including annual and perennial species, and woody and herbaceous species (Nawaz et al. 2016). It has been well documented that melatonin is involved in regulations of all plant development processes and enhancements of resistance and/or tolerance of plants to abiotic and biotic stress.

Zhao et al. (2011) were the first to demonstrate that exogenous application of melatonin significantly improved survival of *Rhodiola crenulata* callus following vitrification cryopreservation. In this study, the callus was pretreated with 0.1 μM melatonin at 25 °C in the dark for 5 days, followed by osmoprotection, exposure to PVS2 and freezing in LN. The melatonin pretreatment resulted in significantly higher survival level (72%) than other treatments. The melatonin pretreatment was found to significantly reduce MDA levels and increase POD and CAT activities during the whole cryopreservation procedure. Uchendu et al. (2013) reported that exogenous application of melatonin improved recovery levels of cryopreserved shoot tips of American elm (*Ulmus americana*). In this study, adding 0.1–0.5 μM melatonin into preculture and recovery medium resulted in 100% shoot regrowth in both in vitro-grown shoot tips and dormant buds following PVS2-vitrification and encapsulation-vitrification. More examples of the improved recovery of cryopreserved plants by exogenous application of melatonin can be found in Table 3.

Nanoparticles

Nanoparticles are defined as atomic or molecular aggregates, with their size smaller than 100 nm in at least one dimension (Preetha and Balakrishnan 2017). Applying gold nanoparticles (AuNPs) to preculture medium, alginate solution and recovery medium, Kulus and Tymoszuk (2021) reported that 10 ppm AuNPs added into the alginate solution produced much higher recovery of *Lamprocapnos spectabilis* shoot tips following cryopreservation. AuNPs treatment significantly increased the activities of antioxidant enzyme in the LN-derived plantlets. Adding of AuNPs at 10–30 ppm in the preculture medium or at 30 ppm in the alginate solution increased SOD activity. Much higher APX activities were obtained when 10–30 ppm AuNPs were added to the recovery medium, while adding 30 ppm AuNPs in the recovery medium significantly increased CAT activity.

Chen et al. (2017) reported that application of 0.3 g l⁻¹ fullerene (C₆₀) as a cryoprotectant significantly improved

survival of *Agapanthus praecox* callus following cryopreservation. Application of C_{60} significantly reduced the relative conductivity, the MDA content and ROS activity in the treated callus. This datum indicates that the use of C_{60} can alleviate oxidative stress-induced in cryopreservation by protecting the cell membrane from damage and preventing the membrane lipid peroxidation. In addition, single-wall carbon nanotubes (SWCNTs) were reported to improve cryopreservation of *Agapanthus praecox* EC (Ren et al. 2020a). SWCNTs (0.1 g l^{-1}) added in PVS2 entered EC, and most of the SWCNTs moved out at the unloading step. Analysis of the antioxidant system and oxidative stress-related gene expression found that the AsA-GSH and GPX cycle were responsible for scavenging H_2O_2 produced in the control, but the CAT cycle was essential for scavenging H_2O_2 produced in the SWCNTs-treated EC, thus reducing levels of H_2O_2 and MDA. Adding of SWCNTs in PVS2 increased the antioxidant levels during dehydration, thus enhancing the resistance of the SWCNTs-treated EC to oxidative stress and improving recovery of the SWCNT-treated EC.

Abscisic acid (ABA)

ABA has been well demonstrated to increase the tolerance and/or resistance of plants to abiotic stress including freezing (Sah et al. 2016). Kong and Aderkas (2011) described a novel method for efficient cryopreservation of somatic embryogenic tissues (SETs) of interior spruce (*Picea glauca* × *engelmannii*) and Douglas-fir (*Pseudotsuga menziesii* subsp. *menziesii*). Pretreatment of SETs with $50 \mu\text{M}$ ABA at 5°C for 4–8 weeks produced the highest rate of survival and all survivors retained their embryogenic ability to regenerate whole plantlets in cryopreserved SETs.

Hazubska-Przybył et al. (2013) found that adding $10 \mu\text{M}$ ABA to the preculture medium improved recovery and normal plantlet regeneration in the cryopreserved SETs of Norway spruce (*Picea abies*). Assessments of genetic stability by simple sequence repeat (SSR) did not find any polymorphic bands in the plantlets regenerated from ABA-assisted cryopreservation. These results indicate that ABA can be considered safe for use in plant cryopreservation in terms of genetic stability of cryostored plants. More examples of the improved recovery of cryopreserved plants by exogenous application of ABA can be found in Table 3.

It is worth noting that Edesi et al. (2020) reported that preculture of the explant with $2\text{--}4 \text{ mg l}^{-1}$ ABA at room temperature significantly decreased shoot regrowth in cryopreserved buds of *Rubus humulifolius*. There existed a synergistic effect of cold and ABA treatment in many plant species (Vandenbussche and Proft 1998; Chang and Reed 2001).

Therefore, ABA-preculture at room temperature failed to produce positive effects or even exerted negative effects on cryopreservation, as reported by Edesi et al. (2020).

Ascorbic acid (AsA)

AsA (vitamin C) is the most effective water-soluble antioxidant in plants and plays an important role in protecting plants against oxidative stress (Smirnoff 2000; Gill and Tuteja 2010). In the study of V cryo-plate cryopreservation of *Paphiopedilum niveum* SEs, adding 0.1 mM AsA into the preconditioning medium 1 day before the 1st preculture produced much higher recovery (39%) than that (8.5%) of the control (Soonthornkalump et al. 2020). AsA treatments considerably reduced ROS and MDA levels in the cryopreservation steps including 1st preculture, 2nd preculture, osmoprotection and dehydration. More examples of the improved recovery of cryopreserved plants by exogenous application of AsA are presented in Table 3.

More recently, Khor et al. (2020) reported that the inclusion of AsA ($50\text{--}150 \text{ mg l}^{-1}$) in the medium of four steps, including preculture, osmoprotection, PVS2 dehydration and post-culture, reduced the shoot regrowth percentage of cryopreserved *Aranda broga* PLBs. Similar results were also obtained in cryopreserved *Rubus* shoot tips (Uchendu et al. 2010a). High concentrations of the antioxidant disturb the redox balance and result in cellular dysfunction (Bouayed and Bohn 2010). Application of AsA (2 and 8 mM) increased ROS contents and inhibited growth of *Arabidopsis* seedlings (Qian et al. 2014). These results indicated that AsA might also work as a stress factor. Therefore, beneficial effects of antioxidants including AsA on cryopreservation are dose- and plant species-specific.

Glutathione (GSH)

GSH is one of the most abundant low molecular weight thiols, which is naturally biosynthesized in plants. Uchendu et al. (2010b) reported that exogenous application of GSH exerted positive effects on recovery of cryopreserved shoot tips of *Rubus*. Optimal GSH concentrations for the best results of cryopreservation varied with different steps in which GSH was added. Diengdoh et al. (2019) reported that adding of GSH in the preculture and recovery medium promoted recovery of *Paphiopedilum insigne* protocorms in vitrification and encapsulation-vitrification cryopreservation. In vitrification cryopreservation, $10\text{--}40 \mu\text{M}$ GSH enhanced shoot regrowth, with the best results obtained at $20 \mu\text{M}$ GSH; in encapsulation-vitrification, $10\text{--}50 \mu\text{M}$ GSH enhanced shoot regrowth, with the highest shoot regrowth obtained at $30 \mu\text{M}$ GSH.

Inclusion of $8 \mu\text{M}$ GSH in the cryoprotectant solution significantly improved survival of *Agapanthus praecox*

EC (Chen et al. 2016). The application of GSH was found to reduce $\text{OH}\cdot$ and $\text{O}_2\cdot^-$ production, as well as H_2O_2 and MDA contents, while increasing endogenous AsA and GSH contents in PVS2-dehydrated EC. GSH-treatment was also found to promote expression of oxidative stress-responsive genes, including *POD*, *APX*, *MDHAR* and *GPX* during cryopreservation processes, and enhance the expression of *DAD1*, a defender against apoptotic cell death, while suppressing cell death-related protease SBT. All these changes contributed to improvements of cryopreservation of *A. praecox* EC.

Others

Several other non-enzymatic antioxidants were reported to improve cryopreservation (Table 3), such as lipoic acid (LA), vitamin E and glycine betaine (GB) for *Rubus* shoot tips (Uchendu et al. 2010b), cathodic water for *Strychnos gerrardii* zygotic embryonic axes (Berjak et al. 2011), glycerol for zygotic embryos of *Amaryllis belladonna* and *Haemanthus montanus* (Serksen et al. 2012), phloroglucinol (PG) for *Oncidium flexuosum* seeds (Galdiano et al. 2013) and *Paphiopedilum insigne* protocorms (Diengdoh et al. 2019), salicylic acid for *Vitis* buds (Pathirana et al. 2016), Supercool X-1000^R for *Oncidium flexuosum* seeds (Galdiano et al. 2013), antifreeze protein (AFP) for *Chrysanthemum grandiflorum* shoot tips (Jeon et al. 2015), proline for *Hancornia speciosa* buds (Prudente et al. 2017), ApSerpine-ZX protein for *Agapanthus praecox* embryogenic callus (Chen et al. 2021a), heat shock protein (HSP) for *Paeonia lactiflora* pollen (Ren et al. 2019), and the LEA family recombinant dehydrin (DHN) proteins for *Arabidopsis thaliana* seedlings (Zhang et al. 2021a).

Conclusion and perspectives

A number of the studies conducted in the past decade have proven that ROS unavoidably generated in the cryopreservation process and was responsible for the low success or even total failure of plant cryopreservation. ROS generation was identified to be the trigger for PCD. Adaptive responses of the antioxidant system and expressions of the oxidative stress-associated genes and proteins helped the plant to establish resistance and/or tolerance to the oxidative stress induced during cryopreservation. Applications of enzymatic and non-enzymatic antioxidants considerably improved recovery of various explants in different cryopreservation procedures and thus provided alternative strategies for efficient cryopreservation of plants. All these results are helpful for further developments and wider applications of plant cryobiotechnology.

Oxidative stress has been identified to be a major constraint for further developments of plant cryopreservation. To alleviate oxidative stress for improving recovery of cryopreserved plants, further studies should be strengthened in the following aspects: (1) to better understand how the explants respond to ROS-induced oxidative stress; (2) to elucidate the mechanism as to how the explants establish resistance and/or tolerance to ROS-induced oxidative stress; and (3) to apply both enzymatic and non-enzymatic antioxidants for improving cryopreservation of plants, particularly endangered, endemic and tropical species, which are still recalcitrant to cryopreservation; (4) various methods for quantitative analysis and visual observations have been used on the studies of ROS generations in plant cryopreservation. Novel and advanced techniques should be considered for use in the said subject. For example, fluorescence probes for monitoring ROS generations offer a potentially powerful tool for studying the chemistry and biology of ROS with high spatial and temporal resolution (Choi et al. 2012). High-throughput omics technology can be used to identify functions of the specific genes and proteins in protecting the cells against ROS-induced oxidative stress (Yang et al. 2019). These studies are expected to develop robust cryopreservation protocols, which can be used as routine methods for setting-up cryobanks of genetic resources of diverse plant species, and facilitate wider applications of cryobiotechnology.

Author contribution statement LR: data collection and analysis, writing of original manuscript; preparation of Tables 1, 2 and 3, and manuscript revision; M-RW: data collection and analysis, preparation of Fig. 1 and manuscript revision; Q-CW: proposal of the present study, manuscript revision and financial supports.

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Data availability The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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