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The potential impacts of climate change on *ex situ* conservation options for recalcitrant-seeded species

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Recalcitrant seeds are characterized by desiccation and freezing sensitivity, and short storage longevity. These physiological attributes obviate their *ex situ* conservation in conventional seed banks, where seeds are stored dry at sub-zero temperatures (typically, 15% relative humidity and -20°C) for extended periods of time. Propagation of plants for field collections (e.g., botanical gardens, nurseries, and arboretums) is a valuable *ex situ* conservation option. However, these collections are relatively costly, require high maintenance, preserve limited genetic diversity and/or are directly exposed to biotic (e.g., pests) and abiotic (e.g., climatic) threats. Therefore, recalcitrant-seeded (RS) species are dependent on cryopreservation for their safe and long-term *ex situ* conservation. Different explant sources such as whole seeds, zygotic embryos, dormant buds, shoot tips, and pollen, can be used for plant propagation of RS species in field collections as well as for their cryopreservation. The success of the propagation or the cryopreservation of these explants often depends on their developmental status, vigor, and/or tolerance to desiccation and chilling/freezing. These attributes are modulated by the environment where the donor plant grows and we hypothesize that climate change, by affecting these biological attributes, would impact the success of explant propagation and cryopreservation. To support this hypothesis, we have reviewed how temperature changes and drought, the two main climate change scenarios, affect the main biological attributes that are directly involved in the success of *ex situ* conservation of tropical and temperate RS species. In general, increases in temperature and drought will negatively affect plant development in field collections and the quality of the explants used in cryopreservation. Consequently, field collections of RS species may need to be moved to more suitable places (e.g., higher latitudes/altitudes). Additionally, we may find a reduction in the success of cryopreservation of RS species germplasm directly harvested from field collections. However, we cannot always generalize these effects for all species since they often depend on the origin of the species (e.g., tropical and temperate species tend to respond to climate change differently), the genotype, the adaptive genetic potential of each

population, and the severity of the environmental change. On the other hand, the increase in temperatures and water stress in donor plants at high-latitude areas and also some tropical environments may favor the production of seeds and seedlings better adapted to drying, and hence, increase the success of plant propagation and zygotic embryo cryopreservation.

KEYWORDS

cryopreservation, living collections, seeds, zygotic embryos, pollen, dormant buds, temperature, drought

1. Introduction

The *ex situ* conservation of plant genetic diversity is mainly achieved by the storage of seeds in conventional seed banks, where seeds are stored dry at sub-zero temperatures (e.g., ~15% relative humidity and -20°C) for decades (FAO, 2014; Breman et al., 2021; Walters and Pence, 2021). Despite the low number of species that have been characterized in terms of seed storage behavior to date [$<5\%$ of seed-bearing plant species; 18,147 species (Wyse and Dickie, 2017) over 376,366 species of the world vascular flora (Qian et al., 2022)], nearly 92% of seeded plants globally are predicted to be tolerant to desiccation (Wyse and Dickie, 2017). This explains why conventional seed banking is a widespread methodology, with potentially over 2,100 seed banks established globally (Breman et al., 2021). However, conventional seed banking is not possible for recalcitrant seeds (Roberts, 1973), as they are characterized by physiological attributes that obviate their conventional storage in seed banks (Walters et al., 2013; Pence et al., 2020). In a broad sense, recalcitrant seeds are sensitive to drying below a certain threshold (ca. 0.2–0.3 g $\text{H}_2\text{O}/\text{g}$ DW or relative humidity $<90\%$; FAO, 2014; Walters, 2015). If stored at -20°C at such high moisture contents, lethal intracellular ice crystals are formed (FAO, 2014; Walters, 2015). In addition, recalcitrant seeds tend to be chilling sensitive and lose viability relatively fast (days to months) when stored hydrated (FAO, 2014). Other “non-orthodox” species with “intermediate” storage behaviors in terms of drying and freezing sensitivity, or very short longevity (e.g., Coffea, Citrus, etc.) also present challenges during their conventional storage in seed banks (Walters et al., 2013; Pence et al., 2020) and are collectively considered as exceptional plant species (Pence et al., 2022).

To overcome the limitation imposed by seed recalcitrance and other non-orthodox storage behaviors, *ex situ* conservation has mainly been achieved through the propagation and cultivation of a small number of plants in field collections in botanical gardens, nurseries, arboretums, or GenBank field collections (Engelmann and Engels, 2002; Breman et al., 2021). Here, the correct propagation and horticultural management of plants are indispensable (Rae, 2011; Cibrian-Jaramillo et al., 2013; Ensslin and Godefroid, 2019). However, this is a relatively costly option that requires high maintenance and a considerable amount of space, where minimal genetic diversity is preserved and is directly exposed to biotic (e.g., pests) and abiotic (e.g., climatic) threats (Li and Pritchard, 2009; Pence, 2011). In addition to field collections, other valuable resources for the *ex situ* conservation of RS species are *in vitro* collections. Here, whole plants or explants, represented as

shoot tips or embryonic lines, are cultured under aseptic conditions and preserved under slow (minimal) growth conditions, with sub-culturing when needed (Cha-um and Kirdmanee, 2007). While this option is still costly and requires high maintenance, the genetic diversity conserved in terms of the number of genotypes per species can be increased compared with field collections (Pence, 2011). Nonetheless, in this option, the preserved species and genotypes are potentially exposed to somaclonal variation that could affect the genetic identity of the species/genotypes preserved. To overcome the limits of field and *in vitro* collections in terms of costs and long-term maintenance, cryopreservation of diverse plant explants is proposed for the safe and long-term *ex situ* conservation of RS species, especially those that are endangered (Pence, 2011; Walters et al., 2013; Pence et al., 2020; Breman et al., 2021; Philpott et al., 2022).

The main effects of climate change on plants have been extensively investigated and reviewed (e.g., Parmesan and Hanley, 2015, and articles within the same special issue on “Plants and Climate Change”; Fahad et al., 2021; Baskin and Baskin, 2022), and include exposure to increased temperatures, increased drought, catastrophic climate events, and/or increased sea levels. The average global temperature rise is expected to reach or exceed 1.5°C within the next few decades, affecting all regions of Earth (IPCC, 2021). The effects of climate change on RS species have often been studied from an ecological perspective (Ramírez-Valiente et al., 2009; Joët et al., 2013; Amimi et al., 2020; Badano and Sánchez-Montes de Oca, 2022; Magni et al., 2022) and there are recent reports on how climate change may affect their propagation from seeds as well (Pritchard et al., 2022). However, the effect of climate change on the available *ex situ* conservation options for RS species has been largely overlooked.

Sexual, vegetative and *in vitro* propagation of RS species for field and *in vitro* collections, as well as for cryopreservation, can be achieved using different explants such as zygotic embryos, embryonic axes, dormant buds, shoot tips, somatic embryos, embryogenic cell lines, undifferentiated calli, and pollen (Reed, 2008; Engelmann, 2011; Pence et al., 2020). The success of these propagation options and the cryopreservation of plant explants, often depends on their developmental status (Goveia et al., 2004), vigor, and/or tolerance to desiccation (Pammenter and Berjak, 2014), and chilling/freezing (Popova et al., 2012; Xia et al., 2014; Ballesteros and Pence, 2017; Bharuth and Naidoo, 2020). These attributes are typically modulated by the environment where the donor plants grow (Gutterman, 2000). We hypothesize that climate change, by affecting these biological attributes, will impact the

success of explant cryopreservation limiting the success of *ex situ* conservation methods such as cryopreservation for RS species.

To support this hypothesis, we have reviewed and discussed the current knowledge on how the expected climatic drivers induced by climate change (mainly changes in temperature and rainfall) can impact the successful propagation-based *ex situ* conservation of terrestrial tropical and temperate RS species by modulating the main biological attributes of propagules. Two case studies on plants of significant conservation interest, namely, Cycads (tropical) and *Quercus* spp. (temperate) are included to illustrate these findings. Finally, a set of recommendations to guide future strategies for the *ex situ* conservation of RS species in the face of climate change are provided.

2. Ex situ conservation of RS species as field collections and the effects of climate change on their production and culture

2.1. Effects of climate change on RS species growth in field collections

Plants propagated in botanical gardens, arboretums, field GeneBanks, or plant nurseries represent a valuable option for *ex situ* conservation of RS species (Li and Pritchard, 2009; Primack and Miller-Rushing, 2009; Breman et al., 2021; Primack et al., 2021). However, climate change projections introduce large uncertainties around the sustainable use of field collections due to the increase in the frequency of extreme climate events (flooding, late frosts, intensive summer droughts, heat waves, and amongst other events). Severe weather events in the past decade have already impacted global coconut (the RS *Cocos nucifera*) production, with crop changes to different genotypes and a shift in cultivation areas predicted for India, the world's third biggest producer of coconut (Hebbar et al., 2022). A shift in production regions is also predicted for cacao (*Theobroma indica*), another RS species, in West Africa and Colombia (Schroth et al., 2016; González-Orozco et al., 2022). Similarly, Asare-Nuamah et al. (2022) showed that climate events resulted in stunted growth, poor germination, and increased attack by pests and diseases on Mango (*Mangifera indica*) in Ghana. Extreme climate events can affect germination strategies, initial seedling establishment, mature plant growth rate (Primack and Miller-Rushing, 2009; Pritchard et al., 2022), and/or the timing of key phenological processes for the development of fruits and seeds (Santos et al., 2017; Ma et al., 2022). For example, in both temperate and tropical tree species, early flowering induced by warmer winters may result in low fruit set due to the lack of pollinators or the negative effect of low night temperatures on fruit development (Dinesh and Reddy, 2012; Ma et al., 2022). On the other hand, rising temperatures also appear to reduce fruit set by inducing flower drops, affecting the synchronization of flowering or inducing sex changes in hermaphrodite and male plants, promoting stigma and stamen sterility, or reducing the functionality of pollen grains and causing poor pollinator activity (Dinesh and Reddy, 2012; Nepi et al., 2001). All these alterations in the reproductive biology of RS

species could ultimately limit their conservation value (Lobdell and Thompson, 2017).

From a perspective of water-stress physiology, some RS species like *Quercus ilex* and *Q. faginea* are anisohydric, displaying less strict stomatal control and more negative water potential during drought and heat waves. It has been hypothesized that these plants would be more likely to suffer extensive embolism and, ultimately, hydraulic failure during an intense drought (McDowell et al., 2008; Resco de Dios et al., 2020). Therefore, the geographical location of field collections housing RS species should consider the potential impact of climate change, especially in terms of the increased frequency of heatwaves and hot droughts (Williams and Dumroese, 2013).

2.2. Technological and horticultural solutions to overcome the negative effects of climate change on field collections

For short-term (from months up to 2 years) applications such as plant production for ecological restoration or distribution, technological solutions can be used to overcome the impacts of climate change. For example, a slow-growth method in a nursery where seedlings are maintained at 16°C, 90% relative humidity and under 4 h of low light intensity (i.e., 400 lux; Krishnapillay et al., 1999) can be employed.

Long term horticultural practices, specifically breeding, can contribute to the production of phenotypes with desirable morphological and physiological attributes that improve plant responses to stressors associated with climate change (Andivia et al., 2021). Other horticultural practices that could be of use include preconditioning and enhancement of root and stem growth by nutrient supplementation (Davis and Jacobs, 2005; Oliet et al., 2009; Ovalle et al., 2016a; Acevedo et al., 2020). Root and stem architecture play a critical role in plant hydraulics and in turn drought response (McDowell et al., 2008). Furthermore, an adequate volume container increases root mass and improves the root absorptive ability conferring a greater seedling drought avoidance capability (Villar-Salvador et al., 2004; Ovalle et al., 2016b). Fall nutrient loading can also enhance stress resistance (Timmer, 1997). Another effective horticultural practice for RS species growing in field collections is nutrient loading that consists of applying a nutritional concentration during the fall season. This will increase the availability of nutrient reserves that are rapidly remobilized to support the nutrient demand of new growth once the seedlings are under stress (Oliet et al., 2013; Andivia et al., 2014).

Finally, for fully grown trees for which correct flowering and fruit set are dependent on a certain degree of chilling (which may not be adequate in temperate tree species due to climate change; see section "5. Climate change and *ex situ* conservation of RS species through dormant buds") to induce and release dormancy, the use of agricultural/horticultural practices have been suggested (Luedeling, 2012). These treatments or practices include tree defoliation to induce dormancy (Salama et al., 2021), microclimate manipulation by shading and special irrigation practices to increase chill requirements of plants and break dormancy, and the application of dormancy-breaking chemicals to promote budbreak when chill

accumulation is not sufficient due to climate change (Luedeling et al., 2009; Luedeling, 2012). However, for some RS species in field collections, these procedures may not be effective, and field collections may have to be established/moved to other locations at higher altitudes or latitudes where temperature regimes are more suitable for growth (Luedeling et al., 2009; Luedeling, 2012).

3. Implications of climate change-induced temperature and water stress on storability of recalcitrant seeds

3.1. Climatic effects on seed development and quality

Temperature and water availability are critical drivers during the different stages of seed development, including maturation (Guterman, 2000), dormancy (initiation, break), and germination (Probert, 2000). Global climate change, in altering environmental cues related to temperature and rainfall, could therefore preclude, delay, or enhance regeneration from seeds (Walck et al., 2011; Sentinella et al., 2020; Pritchard et al., 2022). There is also ample direct evidence to suggest that changes in temperature and rainfall will affect the yield and quality of seeds in orthodox crops (Hatzig et al., 2018; Abdul Rahman and Ellis, 2019; Wijewardana et al., 2019; Ben Mariem et al., 2021; Poggi et al., 2022) and native plant species (Buechling et al., 2016; Delgado-Vargas et al., 2018). More recent evidence suggests that these effects may extend to the regulation of dormancy and germination (Bernareggi et al., 2016; Klupczyńska and Pawłowski, 2021) as well viability retention (Ooi et al., 2009; Ooi, 2012) and seed longevity (White et al., 2022).

In orthodox seeds, quality attributes such as germ inability, desiccation tolerance, and longevity are acquired during the three phases of their development: (i) histodifferentiation and cell expansion, (ii) reserve accumulation, and (iii) maturation drying (Bewley et al., 2013). Acquisition of desiccation tolerance is associated with events that mostly occur during the reserve accumulation phase (Bewley et al., 2013). In the case of recalcitrant seeds, although they have a similar pattern of development regarding the first two phases, they do not fully develop desiccation tolerance, are dispersed relatively wet (usually > 40% water content, fresh weight), and do not present the maturation drying phase (Finch-Savage and Blake, 1994; Kermod and Finch-Savage, 2002). However, there are differences in the level of desiccation tolerated across RS species (Sun, 1999), within genera (Xia et al., 2012, 2014; Ganatsas and Tsakalidimi, 2013; León-Lobos and Ellis, 2018; de Almeida Garcia Rodrigues et al., 2022), genotypes of the same species (Lamarca and Barbedo, 2015; Ganatsas et al., 2016), and even among seeds from the same genotype (Finch-Savage and Blake, 1994). Differences in the level of desiccation tolerance have also been detected in intermediate type (i.e., sub-orthodox) seeded species at genera (Dussert et al., 2000) and species level (Ellis et al., 1991). In recalcitrant seeds, the level of desiccation tolerance can also vary according to the desiccation rate (Farrant et al., 1985; Ntuli et al., 2011), equilibrium dehydration method, and temperature (Sun and Liang, 2001). Because seeds increase their tolerance to

desiccation as they advance in their development and lose water, the degree of maturity that they reach at the time of harvest or dispersal is one of the factors that affect their tolerance to desiccation (Berjak and Pammenter, 1997; Kermod and Finch-Savage, 2002).

In general, a stress condition during anthesis and seed development, such as drought or extreme temperatures, will affect the number of seeds that are formed, the quality and seed size in orthodox seeds (Delouche, 1980; Singh et al., 2013). It can be assumed that similar effects would be seen in recalcitrant seeds (see Llanderal-Mendoza et al., 2017 for evidence of this). However, the effects are less evident on seed quality. Although higher temperatures and lower water availability commonly result in the production of smaller seeds in orthodox-seeded species, seed quality is not necessarily affected (Pieta Filho and Ellis, 1991; Sinniah et al., 1998; Contreras et al., 2009).

3.2. Potentials impacts of climatic change on recalcitrant seed storage

In the case of recalcitrant seeds, there is limited information on the effects of the maternal environment on seed quality, which directly influences storability, with practically no direct or experimental evidence on the effect of temperatures and water availability on seed development and germinative potential. Additionally, specific attributes of recalcitrant seed post-harvest physiology such as desiccation tolerance and sensitivity to low temperatures, which in general have been under-studied, become very important for predicting the potential impacts of climate on the storability, and hence, germplasm conservation of RS species.

Recalcitrant-seeded species are naturally in greatest abundance in sub-tropical and tropical regions (Berjak and Pammenter, 2008; Wyse and Dickie, 2017), and to a lesser extent in temperate and Mediterranean ecosystems (Wyse and Dickie, 2017), which do, however, host some highly representative genera, with unique and endangered genera, such as *Quercus* (Denk et al., 2017). The regions that harbor RS species have already experienced a significant rise in atmospheric temperature (IPCC, 2021) and alterations in rainfall patterns (Tabari, 2020). As discussed earlier, environmental conditions (temperature and water deficit) have a direct consequence on seed quality and subsequent offspring performance (Fenner, 1991; Guterman, 2000). Following this logic, and based on data now emerging on the potential responses of recalcitrant seeds to abiotic stress [water stress (Varghese et al., 2011), warming (Sershen et al., 2014), and acid rain (Ramlall et al., 2015)], climate change will likely affect the viability of stored RS seeds as well as the response of explants derived from these seeds to biotechnologies.

For example, Sershen et al. (2014) showed that even though exposure of *Trichilia emetica* seeds to elevated temperatures (~ 5 to 6°C above ambient) did not disrupt metabolic and ultrastructural integrity in embryonic axes, it hastened germinative development possibly due to an earlier burst of reactive oxygen species (ROS) that triggers germination (based on the findings in orthodox seeds by El-Maarouf-Bouteau and Bailly (2008) and Varghese et al. (2011) for recalcitrant seeds). This has implications for the short- to medium-term storage of recalcitrant seed germplasm that involves maintenance at water contents close to that at

shedding and at ambient or slightly lower temperatures (termed “hydrated-storage”; Berjak et al., 1989). The duration of storage under these conditions is curtailed by in-storage germination-associated events, culminating in seed death (Berjak et al., 1989; Pammenter et al., 1994). If seeds were to enter this storage at an advanced stage of germinative development and/or heightened levels of ROS production, storage lifespan could be further reduced through germination or loss of viability. This rationale is supported by the fact that storage at temperatures lower than ambient has been shown to post-pone the onset of germination in recalcitrant seeds by slowing down the metabolic rate (e.g., Pritchard et al., 1995), provided the seeds are not chilling-sensitive. Additionally, ROS have also been implicated in cell wall loosening and radical protrusion during germinative development (Bailly, 2004; Müller et al., 2009), and if climate change were to result in water stress in recalcitrant seeds during or shortly after shedding, then these seeds could experience desiccation induced oxidative stress (Varghese et al., 2011), compromising their viability even before they enter storage.

Furthermore, exposing recalcitrant seeds to elevated temperatures (above ambient) promotes the proliferation of a spectrum of endogenous microbes that can start immediately after shedding and can curtail the lifespan of recalcitrant seeds in hydrated storage (Mycock and Berjak, 1990; Sutherland et al., 2002). Even when seeds are treated with fungicidal agents and stored at low temperatures these microbes are almost impossible to suppress (Mycock and Berjak, 1995; Moothoo-Padayachie et al., 2018).

As alluded to above, there is evidence that exposure of recalcitrant seeds to abiotic stress such as elevated temperature (Pukacka and Ratajczak, 2005; Sershen et al., 2014) and water stress (Varghese et al., 2011) can lead to the enhanced production of potentially harmful ROS [reviewed by Pritchard et al. (2022)]. This implies that if the recalcitrant seeds to be used as a source of the embryonic axes/zygotic embryos (explants) for cryopreservation have been exposed to climate-induced temperature or water stress during development and/or after shedding, then the explants from them may already possess heightened levels of ROS production upon harvest. This will predispose them to oxidative stress induced viability loss during the excision and partial dehydration steps which in themselves lead to increased ROS production during cryopreservation (Berjak and Pammenter, 2014).

The *Quercus* case study (subsection “8. Case study–*Quercus* spp”) details the potential impacts of climate change on the long-term storability of typical recalcitrant seeds. These impacts could emerge as a consequence of the effects of climate change on the morpho-physiological characteristics of the seeds of this genus in various parts of the world. Further, Daws et al. (2004, 2006) found that recalcitrant seeds of *Aesculus hippocastanum* and *Acer pseudoplatanus* (Sapindaceae) from the northern limit of this species in Europe (Scotland) were less developed when shed because of cooler temperatures during development. These seeds were smaller (fresh mass), presented embryonic axes with higher water content, and had lower tolerance to desiccation than seeds produced in southern locations (Greece in the case of *A. hippocastanum*; France and Italy in the case of *A. pseudoplatanus*). These studies suggest that for certain RS temperate trees, an increase in air temperature during seed development should favor

the production of more developed and desiccation tolerant seeds, which could benefit their germplasm conservation.

Despite the importance of temperature in seed production and quality, their effects would depend on the species and other environmental factors during plant growth and seed development. For instance, Xia et al. (2014) found that the embryo of *Quercus gambelii*, a species adapted to the desert environment of North America, displayed intermediate desiccation tolerance, and embryos from temperate desert populations were more tolerant than those from the warm desert. In the case of species from tropical environments, different studies have reported that seed maturation and quality would be more affected by precipitation during the growing season than by temperatures. For instance, in *Inga vera* (Lamarca and Barbedo, 2015), *Eugenia pyriformis* (Lamarca et al., 2016), and *Araucaria angustifolia* (Shibata et al., 2021) seeds produced under low precipitation matured in less time, had lower water content at shedding and were more tolerant to desiccation than seeds produced under higher precipitation.

Sensitivity to chilling injury is another important attribute for recalcitrant seed conservation that may be affected by the environment. Bharuth et al. (2020), in evaluating the effect of chilling on *Ekebergia capensis* (Meliaceae) from southern Africa, found that when stored at 3°C seeds from the temperate environment [Port Elizabeth, South Africa (SA)] were able to retain full viability (100%) for 8 weeks, while seeds from subtropical (St. Lucia, SA) and tropical (Tanzania) environments were susceptible to chilling and lost all viability after 38 and 9 days, respectively. These differences in viability status were reflected at the ultrastructural level in embryonic axes, in the form of increased vacuolation, and mitochondrial and nuclear degeneration (Bharuth et al., 2020). Furthermore, embryonic axes of *E. capensis* seeds from the temperate environment (Port Elizabeth, SA, USA) exhibited 30% survival after cryopreservation, while those from the warmer environment (St. Lucia, SA, USA) did not survive cryopreservation (Bharuth and Naidoo, 2020).

Chilling sensitivity appears to be intimately linked to cryo-survival and if climate change does bring about changes in localized populations of RS species, this could influence the success achieved in conserving the germplasm of these seeds via cryopreservation.

4. Climate change and *ex situ* conservation of RS species through pollen

4.1. Storage physiology and *ex situ* conservation of pollen

Ex situ pollen conservation is a valuable alternative to overcoming the critical limitation of seed banking for safeguarding the germplasm of RS species (Pence and Bruns, 2022). By containing all the information of the haploid genome of the species, it is a way of conserving genes more than genotypes, and in this sense, its use should be thought of as a complementary way to the storage of other plant propagules (Grout and Roberts, 1995; Berthaud, 1997; Ren et al., 2019). The most used *ex situ* pollen conservation method involves the grains’ dehydration before

storage in a fridge (4°C), freezer (−20°C), or in liquid nitrogen (Dinato et al., 2020). However, not all pollen types survive the initial desiccation needed for successful cryopreservation. As in seeds, we can find “orthodox” or “recalcitrant” pollen, depending on their tolerance or sensitivity to dehydration (Pacini et al., 2006). Nevertheless, most species studied to date produce orthodox pollen (Franchi et al., 2011). Although more commonly applied to crop species, hand-pollination and pollen storage have been used as part of an integrated conservation strategy for a few endangered RS tree species. For example, pollen conservation of *Castanea dentata* (American chestnut) provides options to restore genetic diversity of lines that are resistant to chestnut blight (Fernando et al., 2006; Walters and Pence, 2021). Similarly, the long-term storage of pollen has been found as part of the integrated conservation strategy for *Metrosideros bartlettii*, a critically endangered tree from New Zealand (van der Walt et al., 2022). Interestingly, there are several examples of RS species with desiccation tolerant (i.e., orthodox) pollen that allows the relatively easy *ex situ* storage of dry pollen in species with recalcitrant seeds. Examples include *Acer pseudoplatanus*, *Aesculus hippocastanum*, *Camellia sinensis*, *Castanea sativa*, *Eriobotrya japonica*, *Fagus sylvatica*, *Litchi chinensis*, *Quercus* spp., *Salix caprea*, *Cocos nucifera*, among others (Franchi et al., 2011; Ballesteros and Pritchard, 2020).

Pollen can be stored in liquid nitrogen for many years without losing its ability to pollinate, fertilize, or bring about fruit production (Xu et al., 2014; Ren et al., 2019). However, certain factors have been recognized as responsible for the success of pollen cryopreservation: its initial hydration state, the type of pollen or morphology (bi or trinucleate), the maturity of the pollen grain, the genotype and taxonomy, the physiological state of the donor plant, ecological adaptations, pollen grain size, and protocol/methodology used (Xu et al., 2014; Ren et al., 2019; Vishwakarma et al., 2021). Some of these factors are modulated by climate change.

4.2. Effects of water stress on pollen

Various studies show that the proportion of RS species decreases as the habitat becomes drier (Tweddle et al., 2003; Probert et al., 2009). Unfortunately, all projected climate change scenarios predict with likely/high confidence that extreme events associated with droughts, either in intensity or frequency, will increase in the future in more regions of the world (IPCC, 2021). For example, in Chile, the decline of precipitation has been accentuated since 2010, with annual rainfall deficits ranging between 25 and 45% (i.e., “Central Chile Mega Drought”; CR2, 2015) due to natural and anthropogenic forces (Garreaud et al., 2020).

The reproductive stage of plants is the phase most sensitive to drought, and although this stage involves many processes, anther and pollen development are the most sensitive among them (Saini and Lalonde, 1997; Guo et al., 2016). Drought generates changes in internal cellular homeostasis, altering intracellular sugar content, hormonal balance, and the presence of ROS (Yu et al., 2019). We, therefore, hypothesize that drought stress in plants could indirectly affect the use of pollen in cryopreservation by decreasing the quantity and quality of pollen, reducing its tolerance to desiccation and/or storage longevity in terms of germination ability (Figure 1).

Sucrose confers desiccation tolerance in ways reminiscent of orthodox seeds, that is, by forming a glassy state that limits cell mobility and protects membranes by replacing water in chemical bonds (Buitink and Leprince, 2004). Although the amount of soluble sugars is higher in anthers during a drought, the transfer of these sugars to the pollen grains is blocked due to a dysfunction of the tapetum and the suppression of enzymes of sugar metabolism and starch biosynthesis (Sheoran and Saini, 1996; Yu et al., 2019), which ultimately causes sterility and pollen abortion (Jin et al., 2013). An explanation of the above is the hormonal imbalance derived from drought stress, which affects not only the functionality of the tapetum and sugar metabolism (De Storme and Geelen, 2014), but also ROS detoxification system (Hu et al., 2011; Jin et al., 2013; Dong et al., 2017; Zhang et al., 2021), anther dehiscence, pollen maturation, induction of pollen germination (Kinoshita-Tsujimura and Kakimoto, 2011), and defects in the exine and Ubisch bodies (Aya et al., 2009). Since the latter provides resistance to the internal pressure of pollen, environmental injury, and desiccation damage (Edlund et al., 2004; Mach, 2012; Hu et al., 2022), such defects could also affect the ability of pollen to tolerate the decrease in water contents necessary for cryopreservation.

An illustrative example of these potential effects on pollen can be found in studies on pollen flows of the RS species *Theobroma cacao* in Ecuador, which shows a significant correlation between pollen production and climatic factors, mainly precipitation. Interestingly, the months with the highest pollen production and rainfall also have the highest air temperatures. In this sense, it is important to understand the interactions between air temperature and soil moisture since an adequate water supply becomes critical to ensure adequate pollen production when temperatures rise (García-Cruzatty et al., 2020; Mena-Montoya et al., 2020).

4.3. Effects of increased temperatures on pollen

An increase in temperature also has a significant impact on the performance of male gametophytes, and thus, has a qualitative and quantitative impact on seed production (Hedhly, 2011). Poor fertilization and fruit production in coconut, for example, have been linked to above average temperatures impacting the germination of pollen on the stigma as well as pollen tube growth through the style (Hebbar et al., 2018, 2022). In fact, Zinn et al. (2010) found that even a single day of extreme weather conditions had a detrimental impact on the reproductive success of several plant species. Early stages of pollen development, including anther wall development, microsporogenesis and microgametogenesis are particularly vulnerable, and the application of heat stress during this developmental window can lead to pollen abortion (Raja et al., 2019; Chaturvedi et al., 2021).

Environmental conditions during another development also influence the chemical composition of pollen (see Delph et al., 1997 and references within). Investigations into the impact of heat stress on crop species showed that an accumulation of ROS in the anthers resulted in an imbalance in ROS and ROS-quenching enzymes (Zhao et al., 2018), which, as with drought, leads to sterile phenotypes. It should also be noted that heat stress often coincides with drought and higher light intensities, and the combined effect

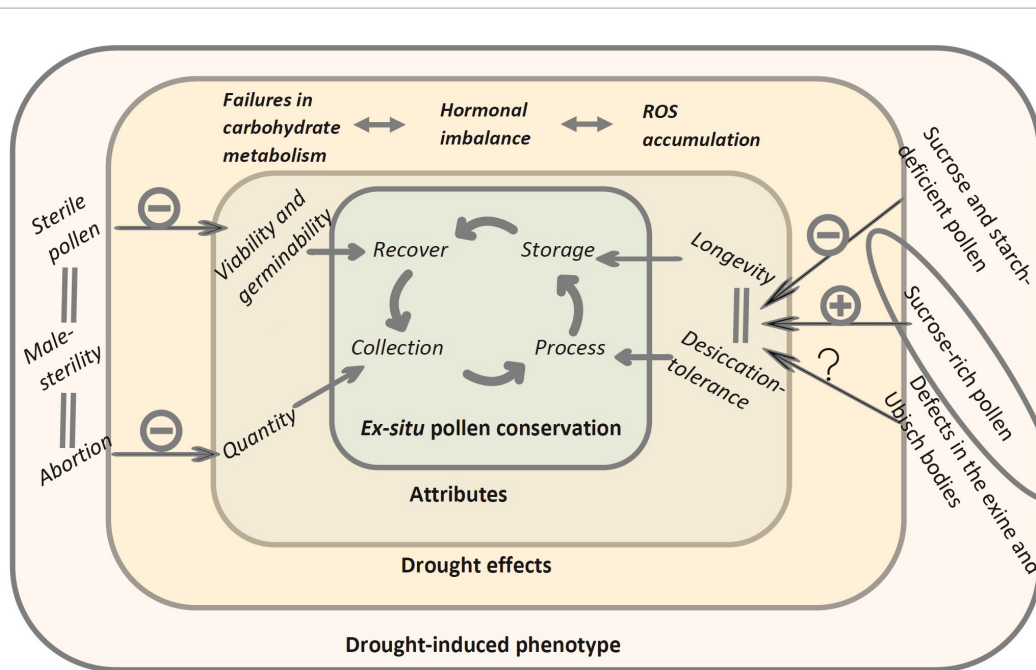


FIGURE 1

Potential effects of drought-induced phenotypes on *ex situ* pollen conservation stages. Failures in the metabolism and delivery of assimilates and carbohydrates, hormonal imbalance, and collapse in maintaining homeostatic balance to control ROS, result in (i) increased pollen abortion rates, affecting the amount available for collections, (ii) pollen with low sucrose and starch content, which has been related to lower longevity and tolerance to desiccation, directly affecting the suitability of pollen to resist the drying process and storage, (iii) defects in the exine and Ubisch bodies, which is hypothesized could affect the resistance of the pollen grain to desiccation and changes in internal pressure, as well decreasing its aptitude as an explant to be cryopreserved, and (iv) increased pollen sterility, impacting its germination ability and ultimately, being unable to recover for fertilization. In a separate case within an elliptical figure, drought stress within tolerable ranges for the plant, which it is capable of coping with, could be beneficial by increasing the sucrose content of the pollen, increasing its longevity, and working as a precondition for its desiccation. (–) Decrease suitability; (+), increase suitability.

of these stresses on pollen are not well studied (Raja et al., 2019). However, there is evidence that mature pollen grains can respond to high-temperature stress through acclimatization of their physiological and biochemical mechanisms and by synthesizing heat shock proteins (Rosbakh et al., 2018; Zhang et al., 2021).

Pollen from the highly recalcitrant tropical species *Mangifera indica* has been successfully cryopreserved (Chaudhury et al., 2010). However, recent studies indicate that its flowering is extremely sensitive to temperature changes, which would decrease the rate of pollen germination and the length of the pollen tube. Moreover, in all the genotypes evaluated, pollen tubes were more tolerant to low-temperature than high-temperature stress (Liu et al., 2023). Similarly, in another highly RS fruit species, *Litchi chinensis*, it has been shown that high temperatures and reduced relative humidity affect pollen by increasing dehiscence and decreasing the germination rate; however, the severity of the affection is strongly influenced by the genotype (Matsuda and Higuchi, 2017). This species also has an *ex situ* conservation strategy that includes pollen cryopreservation (Chaudhury et al., 2010).

Therefore, climate change, particularly heat stress, reduced relative humidity, and drought is likely to modulate pollen development, morphology and biochemistry in both recalcitrant-seeded and recalcitrant-pollen species adversely affecting pollen conservation. An increase in the frequency of intense and severe tropical cyclones is also expected, with extreme winds, rainfalls, and floods (Walsh et al., 2016; Wu et al., 2020; Chand et al., 2022).

Although there are no specific studies regarding the effect of these phenomena on pollen of RS species, research on pollen from orthodox species suggests that microsporogenesis could be more sensitive to flooding than to drought, affecting its quantity, viability, germination and size (Yamburov et al., 2014). The effect on size is of particular importance since some studies suggest that the size of the pollen grain is a variable that could affect the tolerance to cryopreservation (Ren et al., 2019).

5. Climate change and *ex situ* conservation of RS species through dormant buds

5.1. Climate change derived changes in cold acclimation and the cryopreservation of dormant buds

In the autumn, vegetative and floral buds of some species from temperate climates move through a set of natural adaptations permitting their dormancy and subsequent cold acclimation (i.e., cold hardiness) (Hänninen and Tanino, 2011; Cooke et al., 2012; Wisniewski et al., 2018; Fadón et al., 2020; Chang et al., 2021). The entry into this dormant and cold-acclimated stage allows vegetative and floral buds to survive

the chilling and freezing cold conditions of winter. This natural adaptation to cold/freezing environments was ingeniously used in the 1960s to develop a particular set of cryopreservation technologies (Sakai, 1960) that are currently the base for the long-term preservation of genetic resources of many clonal temperate fruit and nut trees and shrubs (Tanner et al., 2021). These cryobiotechnologies are important for the long-term *ex situ* preservation of species with orthodox seeds that present extremely short longevity when banked, such as those from the genera *Salix* and *Populus* (Sakai, 1960; Bonnart et al., 2014; Ballesteros and Pence, 2017; Tanner et al., 2021). In addition, dormant bud cryopreservation has a great potential for the *ex situ* conservation of RS species such as oaks (*Quercus* spp.), chestnuts (*Castanea* spp.), and horse-chestnuts (*Aesculus* spp.) (Ballesteros and Pritchard, 2020).

The effects of climate change on dormant buds' cryopreservation could be significant. Buds used for cryopreservation are collected during mid-winter, when they have the high levels of dormancy needed to achieve the greatest freezing tolerance (Tanner et al., 2021). If collected earlier, at the onset of cold acclimation during the fall season, buds have lower freezing tolerance (Tanner et al., 2021). If collected after buds' de-acclimation in spring, buds become cold tender and are susceptible to freeze damage (Arora and Taulavuori, 2016). Hence, twigs collected outside of mid-winter dormancy can be expected to have very low survival using the standard cryopreservation method (Sakai, 1960; Tanner et al., 2021). It is furthermore possible that the time range for sample collection will be reduced if species and populations experience late autumns and early springs due to climate change (Arora and Taulavuori, 2016; Vyse et al., 2019). In addition, due to the shorter time in the dormancy stage, they may acquire lower cold-acclimation (Cooke et al., 2012; Wisniewski et al., 2018; Fadón et al., 2020; Chang et al., 2021), producing buds in the mid-winter with lower cold-hardiness than desired. In this sense, it is important to note that the geographical origin of source trees seems to have a strong effect on cryopreservation success, with species from colder locations or years (so with higher cold hardiness) showing higher recovery after cryopreservation (Stushnoff and Junttila, 1986; Towill et al., 2004; Toldam-Andersen et al., 2007; Jenderek et al., 2011).

Despite the challenges encountered for dormant bud cryopreservation in the context of climate change, there are some mitigatory measures. In terms of winter dormant bud cryopreservation, twigs collected outside of mid-winter dormancy may present better cryopreservation success using shoot tip vitrification-based protocols (Tanner et al., 2021). Alternatively, in some cases, dormant buds could be artificially acclimated by exposure to gradually cooler temperature under controlled laboratory conditions after twig harvest (Tanner et al., 2021).

5.2. Climate change derived changes in cold acclimation and the effects on field collections

Dormancy and cold acclimation of buds are also very important for healthy tree growth, pollen development and fruit set. The level of chill accumulated during this stage is critical to coordinate

the subsequent floral development and vegetative growth at the climatically favorable time of the year (Cooke et al., 2012; Fadón et al., 2020; Chang et al., 2021). In recent years, regulation and release of bud dormancy and cold acclimation have received a lot of interest from the perspective of global climate change. For instance, high autumn and/or winter temperatures, as well as early spring induced by climate change have been shown to have profound effects on phenology (Cooke et al., 2012; Fadón et al., 2020; Chang et al., 2021), causing dramatic and unpredictable effects on deciduous fruit tree orchards by advancing or delaying dormancy and leaf senescence, flowering, or fruit ripening (Luedeling, 2012; Fadón et al., 2020). In addition, the ability of some species to tolerate low temperature or freezing is being modified (Chang et al., 2021), while warmer autumn temperatures have been related to late dormancy induction and late leaf senescence (Beil et al., 2021). Late dormancy induction can have a negative impact on plants, particularly when early autumn frosts co-occur (Cooper et al., 2019; Vyse et al., 2019). In addition, it has been shown that warmer autumn temperatures result in a shortening of winter, with a delayed spring leaf-out (Beil et al., 2021). Warmer winters do not appear to affect the spring phenology (Beil et al., 2021), but seem to have unpredictable effects. For example, mild temperatures during winter may lead to an erratic bud burst and blooming due to the lack of accumulated cold temperatures during winter dormancy (Fadón and Rodrigo, 2018). Finally, an advance in the arrival of the warm temperatures of late winter and spring is causing early de-acclimation of buds (Arora and Taulavuori, 2016; Vyse et al., 2019). Since de-acclimated buds are no longer tolerant to freezing, early de-acclimation is exposing buds of many trees to late frost events, leading to devastating effects on whole ecosystems and significantly reducing the yield of crop plants (Arora and Taulavuori, 2016; Vyse et al., 2019).

Due to this variety of effects of climate change on the phenology of woody perennials, the suitability of orchard locations for some species and cultivars has considerably shifted, leading growers to modify the array of fruit tree species and varieties (Luedeling et al., 2009; Fadón et al., 2020; Dinu et al., 2021). This is particularly relevant for field collections of RS species with temperate origins (see section "2. *Ex situ* conservation of RS species as field collections and the effects of climate change on their production and culture"), although the impact will vary across locations. For example, temperate trees grown in warmer regions of the Mediterranean or in tropical and subtropical regions may face insufficient winter chill, which has a crucial role in dormancy and productivity (Luedeling, 2012; Salama et al., 2021). Temperate regions will experience relatively little change in relation to chill but may suffer stronger de-acclimation and late frost effects (Vyse et al., 2019). On the other hand, cold regions, as winters warm up, may even see chill increases, which may favor the migration of species toward northern regions where certain fast-growing deciduous species can outcompete the slow-growth cold acclimated deciduous trees or conifers (Sykes and Prentice, 1996; Luedeling, 2012; Chang et al., 2021). In addition, as bud dormancy and tolerance to cold winter temperatures are required for proper flowering and fruit set (Fadón et al., 2020), shifts in the suitability of orchard locations for some species will affect pollen and seed production and quality, affecting not only breeding but also seed collections for ecological restoration (detailed in the pollen and seed subsections).

6. Climate change and *ex situ* conservation of RS species through shoot tips

6.1. Importance of cold acclimation on shoot tips cryopreservation

Shoot tips are one of the most used explants for cryopreservation of plants, particularly clonal crop species or genotypes of interest, but also exceptional wild plants, including many RS species (Nadarajan et al., 2008; Varghese et al., 2009; Normah et al., 2019; Streczynski et al., 2019; Kulus, 2020; Pence et al., 2020; Pence and Chaiken, 2021). Generally, shoot tips are excised from plants cultured *in vitro*, and then processed for their cryopreservation. Sometimes, shoot tips are directly excised from winter dormant buds (Tanner et al., 2021), from seedlings of seeds germinated *in vitro* or from young shoots developed in spring (Pence and Chaiken, 2021). Most shoot tips also go through a process of *in vitro* multiplication prior to their cryopreservation (Benson et al., 2007; Uchendu et al., 2013; Normah et al., 2019; Pence and Chaiken, 2021).

The developmental and physiological status of the shoot tips is critical for their cryopreservation, and as with seeds and dormant buds, differences in response to cryopreservation are found across species and genotypes adapted to different environments. The response of shoot tips to cryopreservation can be significantly different between temperate and tropical species (Normah et al., 2019). For example, temperate genotypes, due to their natural adaptation to winter low temperatures (see section “5. Climate change and *ex situ* conservation of RS species through dormant buds”), present positive responses to some degree of desiccation, cold hardiness, acclimation, and dormancy, and these are important factors used to cryoprotect the shoot tips in cryopreservation protocols (Benson et al., 2007; Normah et al., 2019). In contrast, tropical and warm temperate species are more sensitive to chilling and desiccation stresses, and their cryopreservation protocols must be developed accordingly (Benson et al., 2007; Normah et al., 2019). Despite this general trend related to the potential of a species to cold acclimate, there are not many publications clarifying why shoot tip cryopreservation differs across species, genotypes, or geographical regions. Some authors indicate a better performance of cryopreservation in genotypes that present average to high field cold hardiness in contrast to those with low field cold hardiness (Kushnarenko et al., 2009), but this trend is not always clear (Reed, 1990).

Cold acclimation is commonly stimulated in naturally acclimating species or genotypes to increase the cold hardiness of the shoot tips and achieve enhanced shoot tip survival after cryopreservation (Scott et al., 1992; Vandenbussche et al., 1999; Benson et al., 2007; Uchendu et al., 2013; Folgado et al., 2015; Funnekotter et al., 2017; Mathew et al., 2018; Normah et al., 2019; Roque-Borda et al., 2021). Additionally, it can also be used for some chill-sensitive species (Folgado and Panis, 2019; Normah et al., 2019). Cold acclimation of shoot tips is typically applied to full plants growing *in vitro* before shoot tips are excised, to *in vitro* rejuvenated buds, or to *in vitro* shoot cultures. Cold-hardened dormant buds, which have undergone natural seasonal acclimation

(see section “5. Climate change and *ex situ* conservation of RS species through dormant buds” of this article), may also be used as a source of cold-acclimated shoot tips (Uchendu et al., 2013; Tanner et al., 2021).

6.2. Potential effects of climate change on shoot tips cryopreservation

Tropical species generally do not possess natural adaptations to low temperatures, and cryopreservation protocols developed in these species rely on the addition of sucrose and other compounds to overcome the lack of natural cold hardiness present in temperate species (Benson et al., 2007; Folgado et al., 2015; Normah et al., 2019). This suggests that climate change alterations should not affect the current cryopreservation protocols developed for tropical species. On the other hand, temperate species and genotypes, as well as chilling tolerant tropical species, tend to rely on their natural adaptation to low temperatures for the successful development of cryopreservation protocols. Therefore, cryopreservation protocols currently used for these RS species may be less successful as they will potentially suffer the physiological changes against natural cold hardiness induced by climate change (see section “5. Climate change and *ex situ* conservation of RS species through dormant buds”). These effects will be considerable for shoot tips directly excised from winter dormant buds (Uchendu et al., 2013; Tanner et al., 2021) or could be significant for shoot tips obtained directly from spring sprouts. However, most shoot tips used for cryopreservation are obtained after a step of *in vitro* shoot multiplication with multiple passes. These shoot tips cultured *in vitro* do not retain the direct cold hardiness achieved outdoors as is the case in winter dormant buds, but they have the potential to adapt to cold, as this is genetically controlled (Volk, 2010; Zeng et al., 2014).

However, will warmer scenarios induced by climate change modify the genetic composition of temperate and chilling tolerant tropical RS species making them less adapted to low temperatures? (Ramantha Rao and Sthapit, 2012). The study of the evolution of temperate species through climate changes in the past has shown that some species that adapted well to warmer climates lost their genetic and physiological ability to tolerate cold and increased their susceptibility to freezing (Chang et al., 2021). These changes, though, occurred slowly over evolutionary time scales, which differ from the fast rate of changes induced by the current climate change. However, certain genetic plasticity has been observed in some tree species to adapt to new climate change scenarios, mainly through epigenetic modifications (Alberto et al., 2013; Chang et al., 2021). The extent of these adaptations and their reversibility during cold acclimation *in vitro* are not fully understood (Chang et al., 2021), hence, it is difficult to predict how potential genetic changes in temperate species when adapting to new climate change scenarios will affect their potential to adapt to cold *in vitro*. There have been no published attempts to clarify this in the case of tropical tree species (Ramantha Rao and Sthapit, 2012).

In addition to temperature changes, climate change is affecting water availability and increasing drought in many environments (see introduction). In this regard, Pence and Chaiken (2021) observed in their experiments that the successful cryopreservation

of *Quercus* spp. was achieved in species from wet environments with poor success associated with species from dry environments. If this pattern can be generalized for *Quercus* or other RS genera from both temperate and tropical areas, drier environments due to climate change could potentially have a negative effect on cryopreservation of the species living and adapting to these drier environments. But more comparative studies are needed to confirm this suggestion.

7. Case study—Cycads

7.1. Background

Cycads are distributed in over 60 countries across five continents; Central and Latin America, Australia, Asia, and Africa (Okubamichael et al., 2016). Cycads are also the most threatened group of plants on earth with more than 62% of species threatened with extinction and urgent integrated conservation efforts are required to prevent extinction (Wade et al., 2016). Globally botanical gardens hold significant cycad conservation collections, which due to their recalcitrant-seeded nature is almost exclusively represented as field collections (Griffith et al., 2014; Cousins and Witkowski, 2017). For example, the Lowveld National Botanical Garden in South Africa has been collecting seed from native Encephalartos species since the early 1980's and now has extensive field gene banks from which thousands of seedlings are generated each year (van der Walt, 2010). Similarly, the Montgomery Botanical Center cycad collection spans over 5 ha and represents cycads from Asia, Africa, America, and Australia (Griffith et al., 2014). Plants held in field collections, seed orchards or field GeneBanks, are however, vulnerable to abiotic and biotic impacts and the physical proximity of plants can increase the risk of infestation by plant pathogens (Volis, 2017).

7.2. Pathogens

Evidence shows that climate change will aggravate the impacts of invasive species into novel communities and ecosystems (Wei et al., 2018). One of these pathogens is the Cycad Aulacaspis Scale (CAS) (*Aulacaspis yasumatsui*) Takagi, which was first discovered in 1972 in Thailand on *Cycas revoluta* (Takagi, 1977). Since its discovery, CAS has spread to 39 countries and is now considered the single most important threat to natural cycad populations by the International Union for the Conservation of Nature Cycad Specialist Group (IUCN) (IUCN, 2005; Marler et al., 2021). Although CAS naturally occurs on *Cycas revoluta*, it has been recorded on 21 different cycad species from eight genera (Bowenia, Ceratozamia, Dioon, Encephalartos, Macrozamia, Microcycas, Stangeria, and Zamia). At least two species, *Cycas micronesica* and *C. taitungensis* now face imminent extinction due to CAS (Muniappan et al., 2012) and the Cycad Specialist Group has been working on ways to mitigate the impact of CAS on both wild and cultivated cycad collections since 2006 (IUCN, 2005). By Nesamari et al. (2015) CAS was found to be widespread in three provinces in South Africa, namely, Gauteng, KwaZulu-Natal and Limpopo, where it established in

botanical gardens on *Cycas thouarsii*, *C. revoluta* and seven native Encephalartos species. It is furthermore predicted that favorable conditions for CAS establishment exist under current and future climate scenarios in an additional five provinces of South Africa (Sathishchandra and Geerts, 2020). These areas include localities of botanical gardens with significant Encephalartos conservation collections (i.e., Lowveld National Botanical Garden in Mpumalanga and Kirstenbosch National Botanical Garden in Cape Town). Cycads infected with CAS have shown a drastic decline in non-structural carbohydrates and the ongoing depletion of carbohydrates eventually kills the host plant (Marler et al., 2021). In addition to the impact on adult plants, the gametophyte starch pool of CAS infected seed has been found to be 63% smaller compared to healthy seed resulting in a six-fold decrease in germination of seed infected with CAS (Marler et al., 2021). An increase of CAS, as predicted for various climate change scenarios, will likely impact adult plants, the production of new leaf flushes, and development of gametophytes. This may result in low quality shoots, seed and pollen which are the main propagules used for cryopreservation of cycads.

7.3. Reproduction

Thermogenesis functions as an attractant or reward for insect pollinators in basal angiosperms and cycads, and pollination periods in cycad cones have been found to have daily patterns of heat production (Suinyuy and Johnson, 2018). The impact of climate change on cycad pollinators is unknown but ambient temperatures are crucial in the initiation, magnitude, and timing of cone temperature in Encephalartos and *Macrozamia* species (Laidlaw and Forster, 2012; Suinyuy and Johnson, 2018). An increase in temperatures may therefore result in earlier flowering, which will require pollinators to adapt, thereby impacting reproduction (Laidlaw and Forster, 2012).

7.4. Seed and pollen

Cycads produce recalcitrant seeds and while tissue culture methods via somatic embryogenesis have been developed for some species, establishing plantlets *ex vitro* has been mostly unsuccessful (Litz et al., 2004; Griffith et al., 2014; Nadarajan et al., 2018) although research is ongoing (Berjak and Pammenter, 2014). Cycads are deciduous and the storage of pollen has been identified as an essential conservation tool for many species (Nadarajan et al., 2018). Although pollen cryopreservation has been successful in some *Cycas* and Encephalartos species (Osborne et al., 1992; Mostert et al., 2017), limited research and baseline data on pollen biology and storage is hampering effective application for *ex situ* conservation (Nadarajan et al., 2018). Although there are no studies on the predicted impacts of climate change on cycad pollen quality and quantity specifically, the impact of increased temperatures on the development of male gametophytes has been extensively described, especially in crop species (see pollen section above).

Cycads, as a flagship group for *ex situ* conservation, clearly illustrate the detrimental impact of climate change on the quality of explants (vegetative buds, pollen, seed, and zygotic embryos) obtained from adult plants in *ex situ* collections.

8. Case study—*Quercus* spp.

8.1. Background

Quercus (Oak) is a large genus within the family Fagaceae comprising of around 500 taxa (Carrero et al., 2020), predominantly distributed in the temperate regions of the Northern Hemisphere, ranging from the Mediterranean in Europe to North America and the subtropical forests in Asia and Central-South America (Nixon, 2006). Oak forests are facing a sustained decline due to various driving factors including climate change, pests, pathogens and anthropogenic forest degradation (Thomas et al., 2003; Haavik et al., 2015; Tiberi et al., 2016; Carrero et al., 2020). As a consequence, thirty-one percent of oaks are considered threatened by extinction (Carrero et al., 2020).

There is an urgent need for conservation of oak genetic diversity, which is at present mainly conducted *ex situ* in field collections due to the recalcitrant nature of the seeds (that impedes its dry storage in seed banks). There are 296 oak species reported in *ex situ* collections globally, while nearly one-third, including 58 threatened species, of oak species (134) not reported in any *ex situ* collection (Carrero et al., 2020). This means that more than half of the Critically Endangered or Endangered taxa face imminent extinction if current threats are not addressed (Carrero et al., 2020) and the *ex situ* conservation of oak genetic resources is not expanded into more efficient germplasm that can be long-term stored and preserved (Ballesteros and Pritchard, 2020).

8.2. Reproduction

Quercus species are predominantly monoecism, wind pollinated and highly self-incompatible (Ducouso et al., 1993; references included). As in most flowering plants, reproductive biology in oaks, in particular flowering phenology, is strongly affected by climatic conditions (Gómez-Casero et al., 2007; Gerst et al., 2017). This is particularly relevant for deciduous oak species producing winter dormant buds, as flowering phenology is directly related to environmental cues occurring mainly during winter and spring and detected by the dormant buds (see dormant buds' subsection; Fadón et al., 2020; Chang et al., 2021). For example, winter precipitation and temperature have been found to be the main climate drivers of vegetative growth and reproductive potential for native California oaks, with high winter and spring temperatures advancing bud burst onsets (Armstrong-Herniman and Greenwood, 2021). Similarly, shorter *Quercus* phenological ranges were obtained in years with warmer springs, and longer ranges in colder years in the Iberian Peninsula (Gómez-Casero et al., 2007). All these phenological changes are due to buds' de-acclimation processes, which are increasingly being detected due to climate change in oaks and many other temperate tree species (Arora and Taulavuori, 2016; Vyse et al., 2019; Vogel, 2022). Recent climatic change is leading to changes in the timing and intensity of the pollen season in members of this genus (López-Orozco et al., 2021; Adams-Groom et al., 2022). Other effects on flowering strongly related to climate change, but not necessarily negative are, for example, the triggering of a secondary *Quercus* flowering in autumn, as found in some species in the Iberian Peninsula after

dry summers and warm autumns (García-Mozo et al., 2022). This seems to be an unusual event, but it has been related to a relict capacity of some Mediterranean *Quercus* spp. to adapt to climatic changes, as it has occurred frequently during the evolutionary history of this area (García-Mozo et al., 2022).

In addition to flowering phenology, there is some evidence that climate change could affect pollen quality and production in *Quercus* species, as in *Q. ilex* (Bykova et al., 2018). Hence, smaller quantities of lower quality pollen produced due to climate change, could reduce the reproductive potential of the species (Bykova et al., 2018; Schermer et al., 2019).

Climatic conditions also drive mast seeding and interannual variation in seed production in *Quercus* species, as found for most long-lived and wind-pollinated plants (Abrahamson and Layne, 2003; Koenig et al., 2016). Here, we can find indirect and direct evidence that the change in environmental conditions due to climate change is altering seed production in oaks globally. For example, Pérez-Ramos et al. (2015) detected that mast seeding in Mediterranean oaks was strongly and positively correlated with water availability and air temperature, mainly in the spring and summer. Warmer springs increased seed production in deciduous species, but this effect was variable for the evergreen species (Pérez-Ramos et al., 2015). In this regard, a marked increase in seed production due to increased spring temperature had been detected in diverse deciduous oaks (Caignard et al., 2017; Shibata et al., 2020). On the other side, Shibata et al. (2020) found evidence of decadal changes in the masting behavior of the Japanese oak *Q. crispula* showing that the shortening of the masting interval was associated with rising temperature. In addition, Sánchez-Humanes and Espelta (2011) detected (experimentally) that increased drought reduced seed production on *Q. ilex* in Spain. The negative effects of increased water deficit during summer have also been detected on female flowers and total fruit biomass production (Liu et al., 2015; Gavinet et al., 2019) and fruit abortion rate in *Q. ilex* (Le Roncé et al., 2021).

8.3. Seed and pollen

Climate variables affect the seed morphophysiological traits in *Quercus* species suggesting a potential impact of climate change on seed quality. Evidence of this includes the fact that acorn length increased significantly with mean temperature of the warmest month, decreased with precipitation, and displayed negative linear relationships with equivalent latitude in *Q. variabilis* from China, (Zhou et al., 2013; Gao et al., 2021). A similar finding was detected by Llanderal-Mendoza et al. (2017) for *Q. rugosa* from Mexico. Moreover, larger acorns, in general, have higher germination (Bonito et al., 2011; Llanderal-Mendoza et al., 2017; Sánchez-Montes de Oca et al., 2018; Gavranović Markić et al., 2022) and result in larger seedlings (Lloret et al., 1999; Alfonso-Corrado et al., 2014; Shi et al., 2019), and also seedling survival under greenhouse conditions (Alfonso-Corrado et al., 2014) and *in situ* (Aizen and Woodcock, 1996; Ramírez-Valiente et al., 2009). Ramírez-Valiente et al. (2009) found that *Q. suber* populations originating from the site with the driest summer in Spain produced bigger acorns that also showed the higher seedling survival rate under dry conditions. A similar finding was obtained by Zhang et al. (2017)

for *Q. acutissima* from China while Badano and Sánchez-Montes de Oca (2022) illustrated it in the Mexican species *Q. viminea* and *Q. eduardii* by placing seeds under a simulated increase of 2°C with a 17–18% reduction in rainfall.

In terms of seed storage, all oaks studied to date have recalcitrant seeds (Xia et al., 2012, 2022; Royal Botanic Gardens Kew, 2022); consequently, traditional long-term *ex situ* conservation in seed banks is not possible and thus require cryobiotechnological approaches (Walters et al., 2013; Ballesteros and Pritchard, 2020). Seed embryonic axes (with shoot and root meristems) are the preferred explants for oak cryopreservation as, analogously to conventional seed banking, high genetic diversity can be captured and axes can be grown into full plants with relatively simple micropropagation procedures (Ballesteros and Pritchard, 2020). However, the successful cryopreservation of embryonic axes is often species-specific and appears to be influenced by the environmental conditions at which the seed producing trees grow (Xia et al., 2012). According to these authors, the embryonic axes from two North American species adapted to cold, wet winters, were more tolerant to desiccation and freezing (the stresses inferred by cryopreservation) than the embryonic axes from Chinese subtropical species from a semi-humid habitat. Similarly, Amimi et al. (2020) found that *Q. ilex* and *Q. canariensis* seeds from Tunisia originating from high elevation, where frost events are frequent, showed the lowest freezing sensitivity compared with *Q. coccifera* and *Q. suber*. These results suggest that the warmer and drier conditions expected by climate change could negatively impact the cryopreservation output of oak embryonic axis cryopreservation. However, this environmentally-modulated response to cryopreservation of the oak seeds may not be shared among all species. For example, another study indicated that seed of subalpine oak species from China were most desiccation sensitive and died faster when dried than subtropical and temperate oak from the same country (Xia et al., 2022), suggesting the opposite trend found for North American and Mediterranean species (Xia et al., 2012; Amimi et al., 2020).

Pollen cryopreservation could also be used as a complementary technology to support conservation and breeding programs in oaks. Interestingly, whilst the seeds of oak are recalcitrant it appears that the pollen has much greater desiccation tolerance (Ballesteros and Pritchard, 2020). However, climate change could impact the potential longevity of pollen stored in germplasm banks (Volk, 2011), as this tends to be produced in smaller quantities and with lower quality when trees experience long-term drought stress during the summer (Bykova et al., 2018).

As an alternative to seeds (embryonic axes) and pollen, cryopreservation of clonal tissues cultured *in vitro* has been feasible for some *Quercus* species using somatic embryos and shoot tips (Valladares et al., 2004; Vidal et al., 2010; Pence and Chaiken, 2021; Martínez et al., 2022), so the establishment of long-term cryopreserved collections of these species is feasible through these clonal tissues (Vidal et al., 2010). In addition, cryopreservation of winter dormant buds is suggested as a promising cryobiotechnology method (Ballesteros and Pritchard, 2020). While the effects of climate change on the cryopreservation of *in vitro* cultured somatic embryos and shoot tips are not clear (see, e.g., section “6. Climate change and *ex situ* conservation of RS species through shoot tips”), oak winter dormant bud cryopreservation options may be reduced due to the negative effects

of climate change on their cold hardiness (see section “5. Climate change and *ex situ* conservation of RS species through dormant buds”).

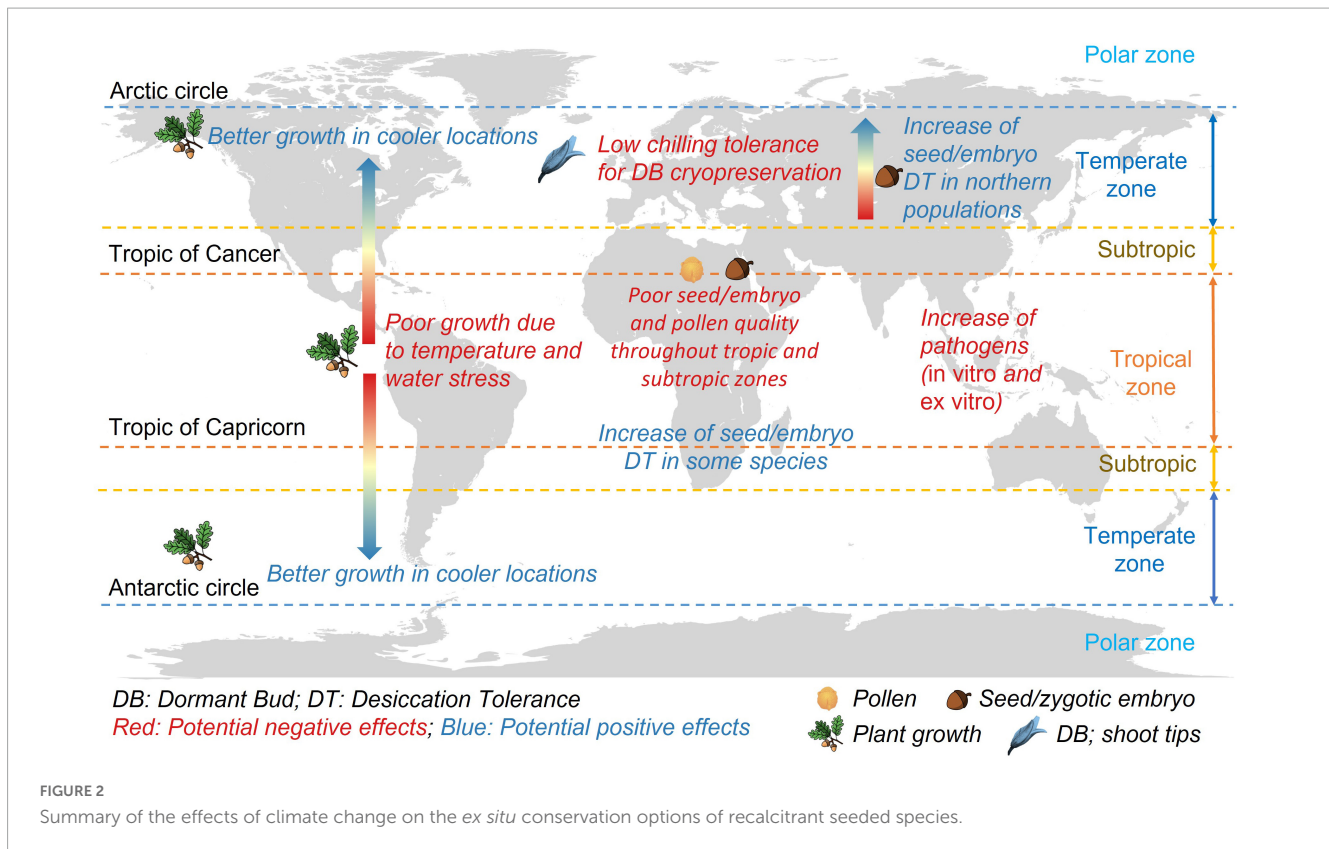
9. Concluding remarks and recommendations

One of our main conclusions is that climate change will have significant effects on the cryopreservation of germplasm (e.g., pollen, seed/seed embryos, dormant buds) directly harvested from field collections of RS species. Germplasm quality and physiology are fundamental for a successful cryopreservation and these attributes will likely be affected by climate change in diverse ways and intensities depending on species and locations. Climate change may not affect the genotypes conserved *in vitro* for many generations, but may affect new genotypes introduced in such collections. However, we cannot always generalize whether these effects are negative or positive for all species since, in most cases, they depend on the origin of the species (often tropical and temperate species are predicted to respond to climate change differently), the genotype, the adaptive genetic potential of each population, the severity of the environmental change, and the *ex situ* conservation option and/or explant in question (Figure 2).

In terms of field collections, RS species grown in tropical and subtropical habitats may encounter serious developmental problems related to high temperatures and water deficit (Figure 2). These problems can also affect temperate RS species growing in warm climates for which a certain level of chilling is required, but may favor the growth of these plants in northern locations where it is currently too cold for their proper development (Figure 2). Literature suggests that some of the limitations of field collections, i.e., the nursery techniques that become a maladaptive selective force on critical traits to respond to abiotic stress, can be intensified by climate change. However, we can also use these selective forces in our favor. For instance, some innovative nursery and horticultural techniques are being developed to improve desirable quality attributes concerning the response of plants to different types of stress imposed by a changing environment, which would allow us to grow these species in their current locations without the need to move field collections to higher latitudes/altitudes.

When focusing on seeds and seed tissues, increases in temperature or decreases in rainfall could cause effects on seeds related to their *ex situ* conservation in the short or long (hydrated storage or cryopreservation, respectively) term (Figure 2). For example, recalcitrant seeds can increase their pathogenic load, present an early germination triggered by an earlier burst of ROS, and/or low viability/longevity in storage due to the increased oxidative damage accumulated. Similarly, when embryonic axes/zygotic embryos are excised for cryopreservation, these explants may be more predisposed to contamination, or more prone to oxidative damage during excision, desiccation and recovery, which has been related to a low success of cryopreservation protocols.

Finally, we must consider the environmental effects on the mother plant regarding the seed tolerance to the stresses inferred by desiccation and low temperatures, which are key to predict the



response of the embryonic axes to cryopreservation. There are two current hypotheses, one suggesting that a drier/warmer climate could favor the acquisition of desiccation tolerance and hence a better response to cryopreservation (Figure 2), while the second suggests that this tolerance is determined by the influence of cold environments. However, more data is needed to support these hypotheses and understand the potential effects of climate change on the cryopreservation of the embryonic axes of recalcitrant seeds from both tropical and temperate environments.

In terms of pollen, there is strong evidence that drought events and high temperatures affect pollen development by altering hormonal and ROS balance and sugar metabolism, ultimately limiting the amount of pollen produced and fertility attributes (Figure 1). However, there is also the possibility that better-adapted genotypes or cases of less severe water stress may generate certain acclimatization or preconditioning of the pollen as an explant for cryopreservation by making it more tolerant to desiccation due to its higher sucrose content. Nevertheless, these specific hypotheses require more evidence to ensure a beneficial effect for cryopreservation protocols due to decreased rainfall as a result of climate change.

Regarding dormant buds, significant research has emerged from a climate change perspective to assess effects on phenology in terms of regulation and release of dormancy and cold acclimation. These traits are important not only for the maintenance of field collections but also for the cryopreservation of these explants and shoot tips. Generally, dormant buds used for cryopreservation are collected during mid-winter, when they have the required levels of dormancy and cold acclimation. However,

climate change could disturb these attributes concerning the season's progress and cause the collection of dormant buds that are not acclimatized or less suitable for successfully tolerating cryopreservation protocols (Figure 2). Similarly, this could apply to shoot tips of temperate or cold-tolerant tropical species in future climate change scenarios in terms of a potential loss of cold acclimatization that would impair their performance with cryopreservation protocols.

This review is mainly based on analysis of successes and failures in applying *ex situ* conservation and cryopreservation protocols of RS species from contrasting environments and inferences from evidence of stress physiology and ecophysiology of orthodox-seeded species, for which much more information is available. These approaches allow us to highlight hypotheses that require more stringent testing than possible here, given the challenges and urgencies that seed recalcitrance imposes on *ex situ* conservation. In this sense, it is suggested to focus research efforts on the following general lines: (i) ecophysiological considerations that determine the boundary between a (beneficial) plastic acclimatization response and potentially irreversible fitness damage on propagules, mainly for species prioritized by their vulnerability in the face of climate change, (ii) possibilities of adaptive management in artificial acclimatization protocols (new or updated) in plant biotechnology, particularly *in vitro* culture and cryopreservation, if *in situ* conditions affect such suitability. Likewise, possibilities for innovation in field collections and nursery practices could yield a source of better artificially acclimatized plants, seedlings, and propagules, as long as genetic diversity is conserved.

Author contributions

PL-L and DB conceived the initial idea for this manuscript, which was further developed by the AF, PL-L, SC, JO, KvdW, S, and DB. All authors contributed to write the manuscript and revision, read, and approved the submitted version.

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