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*Review*

# The Cryobiotechnology of Oaks: An Integration of Approaches for the Long-Term Ex Situ Conservation of *Quercus* Species

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**Abstract:** Conventional dry seed storage is unlikely for about one third of all tree species (and nearly half of evergreen rain forest trees) as they probably produce desiccation sensitive (recalcitrant) seeds. Consequently, international ex situ conservation targets for threatened trees will be difficult to achieve without innovation, especially in cryobiotechnology. We assessed progress in the development of various cryobiotechnology approaches for the preservation of oaks (*Quercus*), which are keystone species of functioning landscapes, important to the bioeconomy and under increasing threats from the spread of pests and diseases under a changing climate. Various tissues of oaks can be used for banking, from pollen grains to embryo axes. Pollen from five oak species have been shown to be highly desiccation tolerant, making dry pollen storage at low temperatures (including in liquid nitrogen) a valuable technology to support conservation and breeding programs. Somatic embryo (SE) technology and/or shoot tip in vitro technology is available for 39 species, and SE cryopreservation is routinely performed on three commercial species and shoot tips cryopreservation successful in two more species. Seed embryonic axes are the preferred explants for oak ex situ conservation, with tissue survival and regeneration of plants after cryopreservation recorded for 14 and seven species respectively; although differential responses between the shoot and root meristems in the axes are known. Dormant bud preservation seems promising, but is under-researched. Overall, these results indicate the possibility of establishing an integrated platform for the ex situ conservation of oak species based on cryobiotechnology. Challenges of explant choice, optimization of methodologies and large-scale application do remain. However, multiple approaches for the cryopreservation of oak genetic resources are available and implementation programmes should not be delayed, particularly in the centres of species diversity.

**Keywords:** cryopreservation; DOE; dormant buds; embryonic axes; in vitro culture; pollen; shoot tips; tree genetic resources; recalcitrant seeds

## 1. Introduction

Trees are anchors of the forest, providing crucial resources for shelter, food and medicines and supporting livelihoods. They are directly or indirectly essential for the survival of many wild ecosystems and agrobiodiverse landscapes, and have immense cultural importance. However, it is estimated that ca. 10,000 of the world's tree species are threatened with extinction [1] and the target of renewed efforts for their ex situ conservation. For example, Target 8 of the Global Strategy for Plant Conservation (GSPC), of the Convention on Biological Diversity (CBD), calls for "At least 75% of threatened plant species in ex situ collections, preferably within the country of origin, and at least 20% available for recovery and restoration programmes" by 2020 [2]. However, only 26% of threatened trees are reported

as conserved in ex situ collections (by means of cultivated plants and seed stored in seed banks) and the majority of trees are represented in a single or small number of collections [1]. Moreover, the CBD's Aichi Biodiversity Target 12, on improving the conservation status of known threatened species, has not been achieved, according to the Global Biodiversity Outlook 5 [3]. Such findings confirm the urgent need to redirect resources from identifying threatened species to actioning conservation intervention.

The most applied strategy for the ex situ conservation of plant species is the storage of dry seeds in biobanks operating at −20 °C [4–6]. However, such conventional seed banking is not suitable for a large proportion of tree species. For example, it is estimated that 33% of tree species might produce desiccation sensitive (recalcitrant) seeds that die on drying; this value could be ca. 47% for evergreen rain forest trees [7,8]. This seed physiology trait then explains the low proportion of threatened trees conserved in ex situ collections [1,9]. The wider implication is that many tropical, subtropical and temperate trees that are canopy-forming or landscape dominants, need innovative solutions for their conservation; preferable a range of options that enable the integration of ex situ with in situ approaches.

For species with recalcitrant seeds, cryopreservation has been shown to be the most promising technology for their long-term conservation [5,10,11]. Unlike conventional seed banking, numerous tissues of trees can be used for banking at ultra-low temperature, including winter dormant buds, shoot tips, embryogenic calli, somatic embryos, embryonic axes, plumules and pollen [11–13]. With respect to the preservation of the genetic resource, the different tissues provide various options for the haploid and diploid genome, and the paternal and maternal lines. This has made the large-scale long-term ex situ conservation of tree genetic resources a reality for a wide range species with economic and food value, such as fruit trees, and forestry and plantation species [12,14–16]. However, standard long-term conservation strategies for wild, particularly threatened, species are far less developed [1,11,17]. Moreover, no single method of preservation is appropriate for all propagules or tissues. Rather, multiple approaches are needed to bring a wider range of tree species into long-term storage [11,18,19].

We have focused this review on *Quercus* sp. for two reasons: the importance of oaks in most of the north hemisphere forests [20], and the lack of large-scale long-term conservation initiatives for the conservation of the c. 600 wild oak species, particularly those that are threatened [17]. The conclusions we make about progress on the cryobiotechnology of oaks should help with the development of appropriate strategies for the long-term ex situ conservation of other taxa, particularly those with low or nil representation in ex situ conservation programmes, whether threatened and/or recalcitrant seeded [1,9].

## 2. Oaks, the Case Study

Oaks (*Quercus* sp.) are dominant trees in most European, North African, American and Asian forests, from cool temperate to tropical environments, on which thousands of other species depend on. They are an important source of timber and coppice wood, but also are used to produce fuelwood, charcoal, medicinal products, dyes, cork and bark used in tanning [20]. Acorns provide animal fodder in important food industries such as the Spanish Iberico ham [21], and many species of oak are grown ornamentally around the world [20]. There are at least 600 species of *Quercus* globally [22], however, 45% of the species evaluated by IUCN are considered threatened [20], being affected by habitat destruction or diseases and pests, such as, sudden oak death, acute oak decline or chronic oak dieback [14,17]. Consequently, the conservation of oaks is increasingly important in many countries [1,14,17,20]. As most species of *Quercus* have desiccation sensitive (i.e., recalcitrant) seeds [23], their long-term conservation ex situ, beyond limited individuals in living collections in botanic gardens, depends on the development and application of cryobiotechnology [19]. In the first instance, progress is needed to support the few initiatives there are on the conservation of elite *Quercus* genotypes, i.e., a few species with economical interest [14,17].

## 3. Whole Seed (Acorn) Storage

Desiccation sensitivity in the acorns (fruits) and seeds of *Quercus* sp. [23] precludes storage in conventional seed banks [6] and contributes to their death after non-dry storage in liquid nitrogen (LN) [24]. However, such seeds can be stored briefly in hydrated storage at, typically, close to 0 °C (FAO, 2014); for example, storage at near-full hydration (30–50% moisture contents) at low temperatures (−3 to +4 °C) [25–28]. Under such conditions acorn viability can be maintained for 6–24 months, and in some temperate species up to a few years. This is of benefit to the provision of planting stock for reintroduction and restoration programmes. However, extending storage for this period remains a challenge, particularly for warm-temperate/tropical oaks; these may be sensitive to chilling injury, thus precluding hydrated storage at close to 0 °C. Understanding the interspecies variability in the response to hydrated storage at low temperatures may be important to find stress tolerance biomarkers (e.g., ecological, structural, genetic and molecular) that may hold some lessons for the optimization of the design of cryopreservation strategies.

## 4. Tissue Culture and Preservation by In Vitro Collections

Tissue culture (in vitro culture or micropropagation) is an important applied tool within cryobiotechnology for the conservation of many plant species [4,29–31]. Within plant genetic resources conservation, the four main applications are: (1) maintaining germplasm collections in vitro for medium-term storage [4,14,31,32]; (2) increasing the number of individuals in a population for use in reintroductions or reforestation, both outside and inside native habitat [4,31]; (3) supplying shoot tips or somatic embryos for cryopreservation [14,29–31] and (4) recovering growth in isolated embryo axes following the application of a cryopreservation protocol [33–36].

Overall, tissue culture research on mainly shoots or somatic embryos has extended to at least 39 *Quercus* sp. (Table 1). Of these, over a third (14 species) are listed as being near threatened or under greater risk of extinction based on IUCN criteria.

Juvenile and mature tissue material has been used, usually from shoots or buds but also from leaves [14,17]. Mature material tends to be more difficult to establish and grow in culture due to the high contamination rates and the low regeneration potential of their cells [14,37]. This problem requires deep morphogenetical studies to understand why some cells/parts of the tree are more competent than others and what leads the differences in response to diverse oak species [14]. In addition, it requires the appropriate balance of minerals (e.g., N and S) and phytohormones, and extra preparation to produce clean cultures with high shoot regeneration response [37]. However, these challenges need to be addressed when dealing with threatened species [37].

Tissue culture can be used to create and maintain in vitro germplasm collections of *Quercus* sp. for intermediate-term storage [14,32]. For example, somatic embryos are multiplied by secondary embryogenesis and cultured at about 20–25 °C in media with varying doses of plant growth regulators depending on the species [14]. As soon as the embryogenic capacity through secondary embryogenesis is maintained, cultures can be subcultured for several (e.g., over 4) years [14]. In addition, *Q. suber* shoot tips have been maintained in culture at 4 °C in the dark for at least 6 months with WP medium enriched with 0.1 mg/L BA [32]. However, the genetic representation in vitro can be as limited as living collections, i.e., based on a few individuals. For longer-term preservation and better genetic representation it is preferable to use propagules, or parts thereof, and cryopreservation [4,5,31,44]. Such an approach is a cost-effective option when considered over the long term, i.e., several decades [5,10,11,39].

**Table 1.** Example of *Quercus* species for which tissue culture have been investigated and developed.

| Species [1] | Range of Distribution [2] | Threatened Species [3] | Tissue Cultured | References |
|---|---|---|---|---|
| *Q. acutissima* Carruth. | Native to China (including Tibet), Korea, Japan, Indochina (Vietnam, Thailand, Myanmar, Cambodia) and the Himalayas (Nepal, Bhutan, Northeastern India) | NO | Somatic embryos | [14,38] |
| *Q. acerifolia* (E.J.Palmer) Stoynoff and W.J.Hess ex R.J.Jensen | United States (Arkansas) | YES | Somatic embryos; Shoot cultures | [17] |
| *Q. alba* L. | Eastern half of the United States, extending north into southern Ontario and Quebec in Canada | NO | Shoot cultures | [14,38] |
| *Q. arbutifolia* Hickel and A. Camus | China, Vietnam | YES | Shoot cultures | [39] |
| *Q. arkansana* Sarg. | United States (Alabama, Arkansas, Florida, Georgia, Louisiana, Texas) | YES | Somatic embryos; Shoot cultures | [17,37] |
| *Q. bicolor* Willd. | North-central and eastern parts of the United States, extending into parts of south Quebec and Southeastern Ontario in Canada | NO | Shoot cultures | [14,38] |
| *Q. boyntonii* Beadle | United States (Alabama, Texas - Regionally Extinct) | YES | Somatic embryos; Shoot cultures | [17,37] |
| *Q. canbyi* Trel. | United States (Texas) | YES | Shoot cultures | [37] |
| *Q. canariensis* Willd. | Native to southern Portugal, Spain, Tunisia, Algeria and Morocco | YES | Somatic embryos | [38] |
| *Q. castanea* Née | Native to El Salvador, Guatemala, Honduras and Mexico | NO | Shoot cultures | [40] |
| *Q. cerris* L. | South-eastern Europe and Asia Minor | NO | Somatic embryos; Shoot cultures | [14,38] |
| *Q. chrysolepis* Liebm. | Mexico (Baja California); United States (Arizona, California, Nevada, New Mexico, Oregon) | NO | Shoot cultures | [37] |
| *Q. dumosa* Nutt. | Mexico (Baja California); United States (California) | YES | Shoot cultures | [37] |
| *Q. eduardii* Trel. | Mexico | NO | Shoot cultures | [40] |
| *Q. engelmannii* Greene | Mexico (Baja California); United States (California) | YES | Shoot cultures | [37] |
| *Q. euboica* (syn. of *Q. trojana* subsp. *euboica* (Papaioannou)) | Italy and the Balkan peninsula from Croatia, south to northern Greece. The species is found in both European and Asian Turkey | NO | Shoot cultures | [37,39] |
| *Q. gambelii* Liebm. | Mexico (Chihuahua, Coahuila, Sonora); United States (Arizona, Colorado, Nevada, New Mexico, Texas, Utah, Wyoming) | NO | Shoot cultures | [37,39] |

**Table 1.** *Cont.*

| Species [1] | Range of Distribution [2] | Threatened Species [3] | Tissue Cultured | References |
|---|---|---|---|---|
| *Q. georgiana* M.A. Curtis | United States (Alabama, Georgia, North Carolina - Possibly Extinct, South Carolina—Possibly Extinct) | YES | Somatic embryos; Shoot cultures | [17] |
| *Q. glauca* Thunb | Native to Assam, China, East and West Himalaya, Hainan, Japan, Korea, Laos, Myanmar, Nansei-shoto, Nepal, Taiwan, Tibet and Vietnam | NO | Shoot cultures | [41] |
| *Q. graciliformis* C.H. Mull. (syn. of *Q. canbyi* Trel.) | United States (Texas) | YES | Shoot cultures | [37] |
| *Q. hinckleyi* C.H. Mull. | Mexico (North Chihuahua); United States (Texas) | YES | Shoot cultures | [39] |
| *Q. ilex* L. | Widespread across the Mediterranean and Balkan regions of Europe, in North Africa and in Turkey | NO | Somatic embryos | [14,38] |
| *Q. leucotrichophora* A.Camus ex Bahadur [syn of *Q. oblongata* D.Don] | Native to Assam, Bangladesh, India, Myanmar, Nepal, Pakistan, Thailand, Vietnam, West Himalaya | NO | Shoot cultures | [41] |
| *Q. libani* G. Olivier | Eastern Mediterranean and western Asia (including Lebanon, western Syria, northeastern Israel, eastern Turkey, and northern Iraq and Iran). | NO | Somatic embryos | [38] |
| *Q. lusitanica* Lam. | Iberian Peninsula (north-west), Morocco | YES | Shoot cultures | [39] |
| *Quercus palmeri* (Engelm.) Engelm. | United States (S. California, Arizona) and Mexico (N. Baja Califonia). | YES | Shoot cultures | [37] |
| *Q. petraea* (Matt.) Liebl. | Widespread species found in Europe, Russia, the Caucasus and west Asia | NO | Shoot cultures | [14,38] |
| *Q. pubescens* Brot. (syn. of *Q. pyrenaica* Willd.) | From northern Spain (Pyrenees) east to the Crimea and the Caucasus. It is also found in France and parts of central Europe. | NO | Somatic embryos | [38] |
| *Q. resinosa* Liebm. | Mexico | NO | Shoot cultures | [40] |
| *Q. robur* L. | Very widespread species, found in most countries in Europe and Russia, The Caucasus, Iran, Kazakhstan and Turkey | NO | Somatic embryos; Shoot cultures | [14,38] |
| *Q. rubra* L. | Occurs widely throughout eastern North America, across the eastern US and southeastern Canada | NO | Somatic embryos; Shoot cultures | [14,38] |

**Table 1.** *Cont.*

| Species [1] | Range of Distribution [2] | Threatened Species [3] | Tissue Cultured | References |
|---|---|---|---|---|
| *Q. rugosa* Née | United States (Arizona, New Mexico, Texas), Mexico, Guatemala, Honduras | NO | Shoot cultures | [40] |
| *Q. semecarpifolia* Sm. | Native to the Himalayas and nearby mountains in Tibet, Afghanistan, India, Nepal, and Pakistan | YES | Shoot cultures | [37] |
| *Q. serrata* Murray | China, Taiwan, Japan, and Korea | NO | Somatic embryos | [38,41] |
| *Q. suber* L. | Found in Europe and Africa, within Mediterranean regions. Within Europe the species is found from Portugal to Sicily and in Africa it is found in Morocco, Algeria and Tunisia | NO | Somatic embryos; Shoot cultures | [14,38,39] |
| *Q. texana* Buckley | United States (Alabama, Arkansas, Illinois, Kentucky, Louisiana, Mississippi, Missouri, Oklahoma, Tennessee, Texas) | NO | Shoot cultures | [37] |
| *Q. tomentella* Engelm. | Mexico (Guadalupe I.); United States (California) | YES | Shoot cultures | [37] |
| *Q. vacciniifolia* Hittell | United States (California, Nevada, Oregon) | NO | Shoot cultures | [37] |
| *Q. virginiana* Mill. | United States (Southeaster's Coastal Plain, from Virginia to Florida and Texas) | NO | Shoot cultures | [39] |

[1] Names authorities follow [42]. [2] [43]. [3] Refers to species considered "near threatened" and above by [43] (except for *Q. semecarpifolia* and *Q. accutissima,* non-assessed by IUCN, information was obtained in [37]).

## 5. Cryopreservation

Cryopreservation is recognised as the only option for the long-term banking of all plant species [11]. The cryobiotechnological approach (as defined by [45]) depends on the determination of the natural level of stress tolerance, combined with a mechanistic understanding of survival stability and the implementation of appropriate protocols. Necessarily, cryobiotechnology is demanding and precise and a single option is not always available for each tissue. Thus, scientific and wide-scale-implementation challenges must be faced [11,18,19]. In this section we reviewed the main tissues of *Quercus* sp. that can be cryopreserved, the main approaches used and their associated challenges. Tissues are presented in order of tissue complexity, from pollen to embryos and embryonic axes.

### 5.1. Pollen

Whilst the seeds of *Quercus* sp. are desiccation sensitive and require cryopreservation of their axes for long-term storage (see Section 5.6), the pollen seems to have much greater drying tolerance [46,47]. This indicates that the pollen could be routinely dried and preserved at low temperatures with relatively simple methods, akin to those used for conventional seed banking. For example, *Q. coccinea* and *Q. alba* pollen survived for at least a year when dried at 25–35% RH and stored at 2 °C [46,48], germinability of dry pollen of *Q. robur* was preserved up to two years at −20 °C [49], dry pollen of *Q. petraea* and *Q. robur* was stored for at least 1 year at −18 °C and used in breeding programmes for acorn production [50], and freeze-dried pollen retained some viability after 300 days at −5 °C [46]. The pollen of some *Quercus* sp. is also known to tolerate cryopreservation; for example, *Q. petraea* and *Q. robur* pollen had high survival after exposure to LN [51].

Pollen can retain viability for at least 10–15 years (and potentially for decades) when stored at LN temperatures as reported for diverse agricultural and ornamental species [52–54]. Therefore, cryopreservation of pollen could be used as a complementary technology to support conservation and breeding programmes of *Quercus* sp., as currently implemented for diverse fruit tree species [12,16]. For example, pollen preservation is a useful tool when researching and implementing disease resistance breeding in diverse threatened species within *Fagaceae* (the oak family). For instance, pollen preserved dry at 4 °C was the source for controlled cross-pollinations with extant American beech trees that are resistant to beech bark disease [55]. Furthermore, long term cryopreservation of pollen can play a vital role when resurrecting genotypes that have been lost to disease, as it was the case of *Castanea dentata* trees, which pollen was stored for over 20 years in LN and successfully used to produce seeds in extant American chestnut trees [11].

Pollen viability after preservation experiments is often tested with in vitro germination assays that test the ability of pollen to produce and elongate the pollen tube. The levels of nutrients, sucrose and agar influence pollen germination and need to be determined and adjusted per species. In the case of *Quercus* sp., pollen germination media usually contains 10–20% sucrose and is often solidified with 0.75–1% agar ([48,49,51,56] for *Q. coccinea*, *Q. petraea* and *Q. robur*). Sometimes mineral nutrients have been added to the basic sucrose solutions, such as 20 ppm boron for *Q. alba*, *Q. coccínea*, and *Q. ilicifolia* [46] or MS salts for *Q. robur* [56].

### 5.2. Embryogenic Calli and Somatic Embryos

Somatic embryogenesis is a powerful tool for conservation of forest trees. It is considered to be the most appropriate means of in vitro regeneration of woody plants and may be the only method of regenerating truly juvenile propagules of difficult-to-propagate species (reviewed in [14] for *Quercus* sp.). Cryopreservation of somatic embryos of *Q. robur* and *Q. suber* [57–59], embryogenic lines initiated from mature selected trees of *Q. suber* [60], and embryogenic tissues from mature *Q. ilex* [61] has been studied and developed. All these approaches allowed the regeneration of whole plants, regardless of the method used. However, research may be needed to optimize and increase the differentiation ability of the cryopreserved embryogenic lines in some species (e.g., [61]).

Cryopreservation is routinely applied to embryogenic lines of some conifers [62] and the first approaches undertaken on the application of cryopreservation to hardwood forest tree species [63,64]. In addition, the feasibility of constructing a large cryo-bank for cork oak (*Q. suber*) genotypes has been proposed on the basis of the successful proliferation of 51 embryogenic lines after immersion in LN [14]. Research to determine the field productivity and heritable characters of the recovered is ongoing [64]. These pioneer studies have laid the groundwork for the development of large-scale propagation and cryopreservation initiatives of threatened woody species for which micropropagation procedures have already been developed (Table 1).

### 5.3. Shoot Tips

Shoot tips grown in vitro are a common source of plant material for the cryopreservation of plant species of interest for agriculture [16,44], forestry [64] and conservation, including threatened species [65]. It appears there is only one report on the successful cryopreservation of *Quercus* species' shoot tips [39]. The shoot tips cultures used were initiated from either seedlings (*Q. virginiana* and *Q. suber*) or shoot cuttings of new growth (*Q. gambelii* and *Q. hinckleyii*). *Q. virginiana* shoot tips had the best survival level (56%), compared with *Q. hinckleyi* (20%), *Q. suber* (12%) and *Q. gambelii* (0%) [39]. Considering the wide range of *Quercus* species for which shoot tip cultures can be initiative (Table 1), these results significantly expand the options for ex situ conservation of the threatened species within this genus. Further research on a wider range of species and on the in vitro conditions necessary for the rooting of shoot tips is necessary as part of the implementation of cryobiotechnology programs for oak conservation.

### 5.4. Plumules

The cryopreservation of oak germplasm as plumules (i.e., shoot apical meristems of embryos) may offer a potential approach for the conservation in gene banks of some *Quercus* sp., particularly when the cryopreservation of the whole embryo is challenging [35]. This is a promising approach for two reasons: (1) the plumule has a great potential for the development of a whole plant in vitro in contrast to the root axes [35,66,67]; and (2) high genetic diversity could be stored from a population by excising the plumules of the embryos of individual seeds, similarly to that achieved by preserving the whole seed or the embryo axes. Cryopreservation of plumules has been performed routinely on *Q. robur* and *Q. petraea* at the Kostrzyca Forest Gene Bank, Poland [68]. Regrowth is between 8 and 20%, suggesting the need to optimize further the cryobiotechnology protocols [35].

### 5.5. Dormant Buds

For woody species of temperate regions, there is the option of cryopreserving vegetative buds, particularly dormant buds after their natural adaptation to cold conditions. This approach has been used for the large-scale cryopreservation of many woody crop species, such as apple, pear, mulberry, sour cherry, silver birch, aspen, willow and persimmon (reviewed in [16,69]). Briefly, dormant buds are harvested from the field in mid-winter, and after partial desiccation (if needed) to 25–41% moisture content, depending on species, they are usually cooled in a two-steps: controlled, relatively slow cooling to −30 or −40 °C; followed by transfer to LN or LN vapour. The rate of control cooling depends on the species, and sometimes the use of cryoprotectants is needed. Dormant buds are later recovered by direct grafting of the bud on the tree, micrografting of the dissected shoot tip, micropropagation of the bud or the dissected shoot tip or direct rooting of the cryopreserved branch section, depending on the species [69]. Cryopreservation of dormant vegetative buds depends on the cold-hardiness level of the collected material. Hence, the level of cold acclimation that the species can attain and the extent of acclimation that the material possesses when collection are the most critical factors for success [16,69].

Cryopreservation of oak dormant buds could be a very valuable approach to explore based on success with other forestry species, including *Juglans cinerea*, *Ulmus* sp., *Populus* sp., *Salix* sp. and *Pinus sylvestris* [69–71]. Many *Quercus* sp. inhabit very cold environments, and these could be good

candidates for dormant buds' cryopreservation [69]. For example, mean cold hardiness values in December–January are about −56, −45 and −27 °C for European *Q. robur*, *Q. pubescens* and *Q. ilex*, respectively [72], and between −30 and −50 °C for *Q. rubra* from diverse US provenances [73]. Twelve other *Quercus* sp. grown in the US (*Q. acutissima, Q. alba, Q. bicolor, Q. coccinea, Q. imbricaria, Q. macrocarpa, Q. montana, Q. muehlenbergii, Q. palustris, Q. phellos, Q. shumardii* and *Q. velutina*) can tolerate winter temperatures between −20 and −40 °C [74]. For comparison, *Malus* sp., a genus for which dormant buds' cryopreservation is quite successful, are considered "quite cold hardy" [69]. For example, dormant buds' cryopreservation with >60% regrowth is known for trees with cold hardiness values around −12 °C to −15 °C (e.g., *M. domestica* and *M. sieversii* [75]). Similarly, species native to areas with winter lower temperatures below −15 °C (e.g., *M. ioensis* from Central North America and *M. coronaria* from NE North America [76]) are amenable to cryopreservation.

In terms of explant recovery and growth after cryopreservation, one of the requirements is the availability of suitable micropropagation or grafting protocols. Importantly, micropropagation protocols have been developed already for dormant buds of *Q. alba, Q. bicolor, Q. cerris, Q. falcata, Q. imbricaria, Q. macrocarpa, Q. pagoda, Q. palustris, Q. robur, Q. rubra, Q. texana, Q. variabilis* and *Q. virgiliana* [77–79]. Grafting of *Quercus* species is a common horticulture practice [80,81], and micrografting has been successfully used, for example, on *Q. robur* [82].

However, to our knowledge, cryopreservation of dormant vegetative buds has not been properly explored for *Quercus* sp. Yet, although pilot studies have been implemented in a few species [83].

### *5.6. Seed Embryos*

Seed embryonic axes or zygotic embryos (i.e., explants with both shoot and root meristems) are the preferred explants for the ex situ conservation of recalcitrant seeds [84,85], as they can be grown into full plants with relatively simple micropropagation procedures and its preservation could capture high genetic diversity (similarly to that captured with whole seed storage) [11]. Cryopreservation has been attempted in about 16 species of oaks (summarized in Table 2). Success has been relatively low, with only four species (*Q. faginea, Q. gambellii, Q. rubra* and *Q. schottkyana*) showing root and shoot recovery (5–60%) after exposure to liquid nitrogen. High (>60%) explant survival (i.e., axes expanded, greened, formed callus or showed normal development of roots or shoots) was found in only six of the species tested. Most species showing good survival and plantlet growth were dried from initial water contents of >1 $gH_2O\ g^{-1}DW$ (>50% FWB) to between 0.27 and 0.40 $gH_2O\ g^{-1}DW$ (21–29% FWB), except for *Q. gambellii* that tolerated drying down to 0.10 $gH_2O\ g^{-1}DW$ (9% FWB; Table 2). In addition, all species showing good survival and plantlet growth, were cooled and warmed relatively fast, with cooling rates above 3 °C $s^{-1}$ (Table 2). WPM was the recovery media used in all species showing good survival and plantlet growth (Table 2).

**Table 2.** Cryopreservation successes with embryonic axes of *Quercus* sp.

| Species # | Desiccation | Moisture Content [1] (% FWB) [($gH_2O$/gDW)] | Cooling | Warming | Basic Salts and Organics [2] | Plant Growth Regulators | Survival [3] (%) | Plantlet Formation [4] (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *Q. alba* L. | air stream (laminar flow hood) | N/A | in aluminium foil packet or cryovials, submerged into LN. | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 25 | 0 | [86] |
| *Q. faginea* Lam. | air stream (laminar flow hood) | 21% [0.27] | in cryovials and submerged into LN | 40 °C water bath (1 min) | WPM + MS vitamins | 1.5 mg/L BAP | 75 | 60 [a] | [87] |
| *Q. falcata* Michx. | air stream (laminar flow hood) | 36% [0.56] | in cryovials and submerged into LN | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 65 | 0 | [88] |
| *Q. franchetii* Skan | Flash drying 2 | diverse MC | diverse cooling rates | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 0 | 0 | [36] |
| *Q. gambelii* * Liebm. | Flash drying 2 | 38% [0.6] | in cryovials and submerged into LN (3–8 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 60 | 0 | [36] |
| *Q. gambelii* ** Liebm. | Flash drying 2 | 9% [0.1] | in aluminium foil packets and plunged into $N_2$ slush (30–80 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 90 | 3% | [36] |
| *Q. gambelii* ** Liebm. | Flash drying 2 | 17% [0.2] | in cryovials and submerged into LN (3–8 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 90 | 3% | [36] |
| *Q. ilex* L. | air stream (laminar flow hood) | 13% [0.15] | in cryovials and submerged into LN | 40 °C water bath (1 min) | WPM | 0.1 mg/L BA | 85 | 0 [b] | [33] |
| *Q. ilex* L. | air stream (laminar flow hood) | 18% [0.22] | in plastic mesh bags and immersed in sub-cooled LN. | In WPM liquid medium at room temperature. | WPM | 1 mg/L BA | 94 | 0 [b] | [33] |
| *Q. leucotrichophora* ex Bahadur (syn of *Q. oblongata* D.Don) | air stream (laminar flow hood) | 13–14% [0.15–0.16] | in cryovials and submerged into LN | 37 °C water bath (15 min) | MS + 0.17 g/L $NaH_2P0_4$ + 2 g/L charcoal | 1 mg/L NAA or IAA + 1 mg/L Kinetin, BAP or 2iP | 15–25 | Non available | [89] |
| *Q. macrocarpa* Michx. | air stream (laminar flow hood) | N/A | in aluminium foil packet or cryovials, submerged into LN. | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 20 | 0 | [86] |
| *Q. macrocarpa* Michx. | air stream (laminar flow hood) | 36% [0.56] | in cryovials and submerged into LN | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | N/A | 0 | [88] |
| *Q. marilandica* (L.) Müncch. | air stream (laminar flow hood) | N/A | in aluminium foil packet or cryovials, submerged into LN. | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 0 | 0 | [86] |
| *Q. muhlembergii* Engelm. | air stream (laminar flow hood) | N/A | in aluminium foil packet or cryovials, submerged into LN. | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 40 | 0 | [86] |
| *Q. nigra* L. | air stream (laminar flow hood) | 25% [0.33] | in cryovials and submerged into LN | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 50 | 0 | [88] |
| *Q. palustris* Regel ex A.DC. (syn *Q. coccinea* Münchh) | air stream (laminar flow hood) | 20% [0.25] | in cryovials and submerged into LN | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 12 | 0 | [88] |
| *Q. robur* L. | Flash drying 1 | 21% [0.27] | 'naked' axes plunged into isopentane held in a LN reservoir. | axes immersed in solution containing Ca2+ and Mg2+ | N/A | N/A | 100 | 0 [d] | [90] |

**Table 2.** *Cont.*

| Species # | Desiccation | Moisture Content [1] (% FWB) [($gH_2O$/gDW)] | Cooling | Warming | Basic Salts and Organics [2] | Plant Growth Regulators | Survival [3] (%) | Plantlet Formation [4] (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *Q. rubra* L. | air stream (laminar flow hood) | 20% [0.25] | in cryovials and submerged into LN | ambient temperature (20 min) | MS | 2 mg/L BAP + 2 mg/mL IAPhe | 85 | 0 | [88] |
| *Q. rubra* L. | Flash drying 2 | 23% [0.3] | in aluminium foil packets and plunged into $N_2$ slush (30–80 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 90 | 60 | [36] |
| *Q. rubra* L. | Flash drying 2 | 23% [0.3] | in cryovials and submerged into LN (3–8 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 90 | 40 | [36] |
| *Q. schottkyana* Rehder and E.H. Wilson | Flash drying 2 | 29% [0.4] | in cryovials and submerged into LN (3–8 °C $s^{-1}$) | axes immersed in 0.5 M sucrose heated to 42 °C | WPM with 0.3% charcoal | none | 30 | 5 | [36] |
| *Q. suber* L. | air stream (laminar flow hood) | 18% [0.22] | in cryovials and submerged into LN | 40 °C water bath (1 min) | WPM | 0.1 mg/L BA | 30 | 0 [c] | [33] |

# Names authorities follow [42]. * from a population collected in Nevada, USA; ** from a population collected in Wyoming, USA. Flash drying 1: computer fan and silica-gel device; Flash drying 2: axes rapidly dried over a stream of nitrogen gas (technical specifications can be found in [91]). [1] when moisture contents were available only in fresh weight basis (FWB) or dry weight basis (DWB), the following formula was used to calculate moisture content: FWB = DWB/(DWB + 1) [92]; [2] all media enriched with 3% sucrose and generally solidified with 0.8% agar; [3] axes had expanded, greened, formed callus or showed normal development of roots and shoots; [4]normal development of roots and shoots (often considered as doubling of radicle length or greening of shoots). BAP: 6-benzylaminopurine; BA: N6-benzyladenine; IAPhe: indoleacetylphenylalanine. [a] plants showing normal development of roots and shoots or only shoots; [b] 15% shoot development (no plantlet); [c] 5% shoot development (no plantlet); [d] 70% organised shoot development after a few days (no actual growth measured in vitro).

## 6. Challenges in Oak Cryobiotechnology

Plant cryobiotechnology has a relatively recent past and a favourable present [15,45]. However, plant cryobiotechnology does not consist simply of plunging plant tissues into LN and growing them after melting, i.e., cryopreservation. Rather, cryobiotechnology provides a conceptual framework for the integration of thinking about the evolution of and natural adaptation to low temperatures with advances in fundamental understanding of stress tolerance and survival, and the design of enabling cryopreservation techniques and infrastructure [45]. Only by integrating these components of cryobiotechnology will it be possible for the preservation of tissues of all plant species to become a reality [11]. In this section we explored some of the research challenges associated with two aspects of cryobiotechnology: embryonic axis cryopreservation and in vitro plant growth.

### *6.1. Cryobiotechnology of Embryonic Axes and Zygotic Embryos*

Cryopreservation of embryonic axes and zygotic embryos of recalcitrant seeds can be limited by different responses between species and populations to desiccation and LN exposure, physical damage to the embryo during isolation, oxidative stress associated to excision and cryopreservation procedures, differential stress tolerances of shoot and root meristems and the need to improve in vitro growth and acclimation procedures (e.g., Table 3). Many of these challenges have been investigated for *Quercus* sp. (references highlighted in bold letters in Table 3) and the findings translated into some cryopreservation successes. Indeed, three out of the four oak species for which shoot and root growth have been obtained (*Q. gambellii, Q. rubra* and *Q. schottkyana*; [36]) were cryopreserved after the application of three cryobiotechnological advances pioneered by [86,88]. These advances include the application of antioxidants during axis excision and the use of fast ("flash") drying and cooling procedures (Table 3, [36]).

**Table 3.** Research challenges and innovations in the cryopreservation of embryonic axes and zygotic embryos of recalcitrant seeds. References in bold indicate that the work is related to *Quercus* sp.

| Steps during Cryopreservation of Embryonic Axes | Research/Technical Challenges | Methodological/Conceptual Innovations that Can Be Used | Some References, and Context |
|---|---|---|---|
| Collection | • Selection of seeds with optimal quality.<br>• How seed (and embryo) maturity affects desiccation sensitivity level and response to cryogenic temperatures.<br>• How plant tolerance to drought or cold influences seed (and embryo) tolerance to cryo. | • Fruit, seed and embryo maturity are not necessarily synchronous and propagule development times vary amongst species. | • ***Quercus* seed biology [25].**<br>• Excised axes [101].<br>• Seed developmental age and environment across Europe [102].<br>• Narrowing the hydration window for cryo and risk of ultra-drying [103].<br>• ***Quercus* sp. or populations from colder environments show higher tolerance to cryo [36].** |
| Excision | • Physical (mechanical) damage of the axes (shoot or root tips) during excision.<br>• How excision affects to ROS generation in diverse tissues of the explants.<br>• Protection against ROS generation and oxidation. | • Excision of axes with small pieces of cotyledon still attached (to avoid physical damage and oxidation on shoot tip).<br>• Protection of explants with "cathodic water" or antioxidants (DMSO, ascorbic acid), $N_2$ (anoxic) atmosphere, catalase, MDH, glutathione (GSH), lipoic acid (LA), glycine betaine (GB), polyvinylpyrrolidone (PVP), etc.) | • Excised axes [101].<br>• Superoxide burst on axis excision [104].<br>• ROS production and cryopreservation [105].<br>• Cathodic amelioration oxidative stress in axes [106]. |
| Pretreatments/ Cryoprotection | • Reduction of toxicity.<br>• Optimization of infiltration.<br>• Protection against ROS generation and oxidation. | • Use of single cryoprotectant substances (e.g., only glycerol) instead of PVS2, which contains three (plus sucrose in the medium).<br>• New PVS recipes, including variations on PVS2 and PVS3.<br>• Other biological or non-biological substances (?)<br>• Vacuum-induced permeation of viscous cryoprotectants.<br>• Protection of explants with antioxidants and anti-stress substances (e.g., DMSO, ascorbic acid, vitamins, MDH, glutathione (GSH), lipoic acid (LA), glycine betaine (GB), polyvinylpyrrolidone (PVP), etc.) | • Cryopreservation of amaryllid axes [107].<br>• Personalisation of plant vitrifications [97,98].<br>• Vitamins C and E and lipid peroxidation of blackberry shoot tips [108].<br>• Antioxidants and *Rubus* shoot tips [109].<br>• Vacuum infiltration vitrification of tropical oilseed embryos [96].<br>• **Glycerol cryoprotection increase shoot survival in *Q. robur* embryonic axes [95].**<br>• **Effects of PVS2 in *Q. imbrica* [99].**<br>• **Effects of PVS3 in *Q. imbrica* [100].** |

**Table 3.** *Cont.*

| Steps during Cryopreservation of Embryonic Axes | Research/Technical Challenges | Methodological/Conceptual Innovations that Can Be Used | Some References, and Context |
|---|---|---|---|
| Desiccation | • Reduction of drying times.<br>• Homogenization of drying between and within tissues (shoot tips use to be more sensitive in *Quercus* sp.).<br>• Protection against ROS generation and oxidation.<br>• Preservation of structural integrity. | • Rapid airflow drying reduces critical WC of zygotic embryos compared to when within whole "seeds".<br>• Flash (fast) drying (can reduce critical WC of explants from 0.80 to about 0.40 g $H_2O$/g DW).<br>• Drying down quickly to close to or below the unfrozen water content (c. 0.3 g $H_2O$/g DW).<br>• Use of cryoprotectants such as glycerol as structural-protectants. | • *Araucaria* recalcitrant seed [110].<br>• Homoiohydrous seeds [111].<br>• **Desiccation and cryopreservation *Quercus* sp. [86,88].**<br>• **Rapidly dried axes of *Q. rubra* [112].**<br>• **Rapid dried axes of *Q. robur* [113].**<br>• Dehydration, freezing rate and thermal properties of tea axes [114].<br>• Water distribution in *Araucaria* embryo tissues [115].<br>• **Desiccation and cryopreservation *Quercus* sp. [33,87,116].**<br>• Drying rate and jackfruit seeds [94].<br>• Uneven drying of zygotic embryos during cryopreservation [84].<br>• **Stress tolerance in *Quercus* sp. embryonic axes [36].** |
| Cooling | • Avoid ice nucleation and growth.<br>• Induce vitrification.<br>• Preservation of structural integrity. | • Fast cooling (e.g., LN slush) of naked embryos<br>• Use of cryo-protectants | • Dehydration, freezing rate and thermal properties of tea axes [114].<br>• **Freezing rate and cryopreservation in *Quercus* sp. [33].**<br>• Intracellular ice and cell structure [93].<br>• Cryopreservation of Amaryllid embryonic axes [107].<br>• **Freezing rate and cryopreservation in *Quercus* sp. embryonic axes [36].** |
| Storage | • Avoid changes in temperature that induce devitrification and ice formation.<br>• Longevity of cryopreserved axes. | • Reduce pre-storage times. | • Long-term cryopreservation of seeds [117].<br>• **Long term cryostorage embryo axes [118].**<br>• Long term cryostorage embryo axes [119]. |
| Warming | • Avoid ice nucleation and growth.<br>• Protection against ROS generation and oxidation. | • Fast warming (e.g., warming at 40 °C of naked embryos vs. warming cryovial).<br>• Protection of explants with Ca/Mg solution alone or as cathodic water. | • *Q. robur* warming on Ca/Mg solution [90].<br>• Influence of warming rate on survival [120].<br>• Cathodic amelioration oxidative stress in axes [106]. |

**Table 3.** *Cont.*

| Steps during Cryopreservation of Embryonic Axes | Research/Technical Challenges | Methodological/Conceptual Innovations that Can Be Used | Some References, and Context |
| --- | --- | --- | --- |
| Regeneration/ In vitro culture | • Culture media (mineral and hormone composition).<br>• Culture conditions (light and temperature). | • Design of Experiments (DOE) approach to find optimal nutrient composition.<br>• Initial recovery in dark conditions to avoid photo-oxidation. | • Design of Experiments (DOE) approach for plant in vitro culture [121].<br>• ***Quercus* in vitro after cryo [33].**<br>• **DOE *Quercus* in vitro growth [122].** |
| Plant growth and acclimation | • Acclimation.<br>• Tolerance to stress of acclimated plants. | • Venting lids.<br>• Bottom cooling.<br>• Decreased osmotic potential of the medium.<br>• Application of ABA and/or elevated $CO_2$ concentration. | • ***Quercus* sp. acclimation from in vitro [123].**<br>• Acclimation of plantlets to ex vitro conditions [124].<br>• ***Quercus* sp. acclimation from in vitro [125].**<br>• Stress tolerance of acclimated plants after cryopreservation [126]. |

The general low success in oak embryonic axes cryopreservation (Table 2) in early works could be related to the methods then available and practiced. For example, all (eight) species studied by Pence [86,88] were thawed slowly at ambient temperature for 20 min (Table 2). This slow thawing in axes containing water contents >0.20 g $H_2O$/g dry weight (Table 2) could have enabled lethal ice formation during thawing (Table 3 [3]), reducing overall survival. In addition, embryos of most species used by Pence [88] were cooled relatively slowly within cryovials (Table 2), whereas there is more recent evidence that faster cooling (and warming) is beneficial for the survival and growth of recalcitrant seed embryo axes, including those of oak species (Table 3, [36,93]). Furthermore, greatest success after cryopreservation has been obtained in oak species when dried very fast, i.e., "flash drying" (Table 3, [94]) [36], which was not the drying method used in early works (Table 2).

However, the low success in oak embryonic axes cryopreservation (see Section 5.6, Table 2) could also have been due to the natural sensitivity of the axes of the different oak species to the stresses of partial desiccation and LN exposure [36] in relation to the species' environmental cues. This seems to contribute to both inter- and intraspecific variability in success (Table 3, [36]), and might, in future, be used to better predict species/populations responses. With this in mind, we are studying the cryopreservation of 12 oak species from diverse provenances in Europe and the Mediterranean region. In our protocol [91] we are using antioxidants at the collection of excised embryos, a flash drying method, a range of fast cooling rates and fast warming. We observed the relatively successful cryopreservation of *Q. ilex*, *Q. coccifera* and *Q. pyrenaica*, with root and shoot recovery around 25% and 50% for *Q. ilex* and *Q. pyrenaica*, respectively [95]. These results raise the overall success of oak embryonic axis cryopreservation from four to seven species, just by improving the cryobiotechnology approach (Table 3). Our interests extend to innovations around cryoprotection, e.g., using vacuum infiltration vitrification [96] and the development of bespoke cryoprotectant mixtures [97,98]. Our overall aim is to generate accurate predictive tools for the optimal application of cryopreservation methods to a wide range of oak species from in the world. We are not alone in this ambition [36,99,100] and anticipate greater opportunities for a global collaborative effort in the future.

### *6.2. Overcoming Challenges of In Vitro Plant Growth*

In vitro growth after cryopreservation of embryo axes of several recalcitrant seeded species from tropical and temperate origin has been performed generally on media without plant growth regulators [36,93,94,106,127,128]. However, the presence of some growth regulators, such as 6-benzyladenine (BAP), may favour shoot development after cryopreservation in some *Quercus*, and other, species of temperate origin (e.g., [33,89,129], Table 3). These contrasting results suggest that more research is needed to ascertain the positive, neutral or negative role of plant growth regulators on the recovery of embryo axes after cryopreservation.

Vanhove et al. [122] have provided guidance for an improved medium for the recovery of *Q. palustris* embryonic axes pre-cryo, i.e., a control mainly subjected to excision stress. However, it is possible that the optimal conditions for axes growth pre- and post-cryo (i.e., subjected to excision, desiccation and low temperature stresses) are not the same [93,130]. Clearly, improvements are needed to help overcome the low success of shoot growth from cryopreserved embryo axes (Table 3).

One challenge for the optimisation of media for in vitro plant growth or organogenesis from embryo axes, pre- and post-cryo, is the seemingly infinite number of potential phytohormone combinations, generally resolved through rigorous research [66]. Other factor combinations (e.g., diverse salts and pH) might also be important and interact [121]. Vanhove et al. [122] addressed this challenge using the DOE (design of experiments) approach. Design of Experiments (DOE) is a statistical approach to determine the relationship between factors affecting a process and the output of that process. DOE is much more rigorous than traditional methods of experimentation such as one-factor-at-a-time and expert trial-and-error. This rigour allows researchers to model the relationships among the numerous variables in a system and reach better solutions sooner, without using a fully factorial design. For example, of the six factors tested ($NO_3$, $PO_4$, BAP, IAA, MS vitamins and the proportion of $NH_4$:K), *Q. palustris* in vitro

root growth, shoot appearance and leaf expansion benefitted from lower BAP, IAA and N levels, but a higher $NH_4$:K ratio was beneficial for root growth [122]. DOE could be a valuable tool also in designing more efficient cryoprotectant combinations, e.g., to balance toxicity risks with acceptable osmotic stress level [97,98].

## 7. Final Remarks and Conclusions

Cryobiotechnology is "the use of modern technologies to understanding the response of biological systems to low temperature environments, whether natural or imposed, and leading to the production of knowledge, goods and services, including the preservation of cells and tissues for industry, agriculture, medical science and conservation agencies" [45]. This definition goes beyond the concept of cryopreservation per se, which is directed primarily at the development of protocols for preservation, and does not articulate clearly the importance of a range of innovative technologies being used, including plant tissue culture, functional genomics, structural biology, etc. Whilst the literature on plant cryo research has increased exponentially over the last few decades [15], progress on the cryobiotechnology of *Quercus* and other tree species has been slowed by the need for empirical approaches, e.g., on in vitro shoot micropropagation and somatic embryogenesis [14,34]. Nonetheless, it is now known that the cryopreservation of elite genotypes over several years does not seem to change their genetic integrity after regeneration [12,14,58,65]. Similarly, large-scale propagation and cryopreservation of elite genotypes of *Q. suber* is being pursued [14,64], opening the window to the propagation and cryopreservation of threatened species [17]. However, to accelerate progress in the development and implementation of plant cryobiotechnology to support the bioeconomy and conservation, a conceptual framework is needed. Such a framework should take into account the challenges associated with variation in tissue complexity, provenance of material, genome and explant size, etc., based on fundamental principles of ensuring viability and structural stability.

We show here that considerable progress was made in preserving diverse propagules of oak using cryobiotechnology. We also affirmed the opportunities for establishing large-scale cryobiobanks, using pollen, embryogenic calli, somatic embryos, shoot tips, plumules and seed embryonic axes. Consequently, we believe that ex situ programmes for the conservation of tree, and other, species should not be limited to considerations of conventional seed banking but fully embrace cryobiotechnology [11,17,18]. In this regard, it is important to use research (Table 3) to establish standards that are acceptable to the community. For clonal crops, a successful cryopreservation is defined by a regrowth level of 20–40% [131,132]. Such a standardization of success should be applied equally to wild and threatened tree species, whether for pollen, somatic embryos, shoot tips, plumules, dormant buds or embryonic axes.

Finally, this perspective on the cryobiotechnology of *Quercus* species should encourage the development and implementation of similar programmes on targeted groups of species that are not easily banked conventionally and are of high conservation concern, such as within the families Arecaceae, Brunelliaceae, Chrysobalanaceae, Lauraceae, Meliaceae, Myrtaceae and Rubiaceae [1,133].

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