

Review

Application of Biotechnology in the Conservation of the Genus *Castanea*

Elena Corredoira, M^a Teresa Martínez, M^a José Cernadas and M^a Carmen San José *

Group of Biotechnology and Forestry Improvement, Department of Plant Physiology, Instituto de Investigaciones Agrobiológicas de Galicia, CSIC. Avda. de Vigo s/n, 15705 Santiago de Compostela, Spain; elenac@iiag.csic.es (E.C.); temar@iiag.csic.es (M.T.M.); cernadas@iiag.csic.es (M.J.C.)

* Correspondence: sanjose@iiag.csic.es; Tel.: +34-981-590-958

Received: 5 September 2017; Accepted: 12 October 2017; Published: 17 October 2017

Abstract: *Castanea* is a hardwood forest genus of considerable agro-economic importance for both timber and nut production. Chestnuts are one of the most significant nut crops in the temperate zone. However, this species is threatened by pollution, social factors, economical changes, and two major fungal diseases: ink disease (*Phytophthora* spp.), and chestnut blight canker (*Cryphonectria parasitica*). Similar to other wood species, chestnuts are difficult to propagate both generatively by seed and vegetatively by means of grafting or cuttings. Biotechnological methods such as in vitro culture have been developed in the last few years as an alternative to conventional vegetative propagation. Biotechnology plays a very important role not only in the propagation of selected individuals (being used at a commercial level), but also in its short-term preservation, and offers the possibility of preserving the propagated material in the medium-term (cold storage) or long-term using cryopreservation.

Keywords: chestnut; cold storage; cryopreservation; micropropagation

1. Introduction

The Fagaceae family is a large angiosperm family that consists of seven genera and around 1000 species that are spread throughout the whole of the northern hemisphere, from tropical zones to the boreal regions. Fagaceae produce an enormous amount of biomass, which possibly exceeds even that produced by conifers [1]. Among the most prominent genera of this family are the *Fagus*, *Quercus*, and *Castanea*. The species of these three genera are distributed throughout Asia, Europe, and North America, where they cover, or used to cover, large forestry areas, and have a wide range of uses (biomass, fiber, wood products, and food) [2].

Castanea is a small genus within the Fagaceae family that is a native of the temperate zones of Asia, Europe, and the eastern United States (US) [3,4]. The genus *Castanea* consists of three sections: Eucastanon (chestnuts), Balanocastanon (chinkapins), and Hypocastanon (the Henry chestnut). The exact number of species that make up this species is difficult to calculate, due to the wide use of synonyms and the lack of a reliable characterization for some species of chinkapins. The species that are more representative and of greater economic importance within this genus are: the European chestnut (*C. sativa* Mill.), the American chestnut (*C. dentata* (Marshall) Borkh.), the Chinese chestnut (*C. mollissima* Blume), and the Japanese chestnut (*C. crenata* Sieb. and Zucc.). The other species are small trees or bushes of importance only for breeding, as rootstocks, or for special uses [5].

The European chestnut (*C. sativa*) is widely distributed, and has an important economic role in Europe. It is present in 25 countries, covering an area of around 2 million hectares, and is considered the only chestnut species native to Europe. It extends from the Iberian Peninsula to the Caspian Sea, from Germany to Morocco, Algeria, and Tunisia. The western limit reaches the Canary Islands, the

Azores, and Madeira, while the eastern limit is in Asia Minor, which is considered by some authors to be the homeland of the European chestnut. There are isolated populations in Lebanon, Iran, the Taurus Mountains, and western Anatolia. The American chestnut (*C. dentata*) is the largest native chestnut species of North America, from the state of Maine to the 43° parallel in New Hampshire and the southern shores of Lake Ontario in New York state, going down to the states of Georgia, Alabama, and Mississippi in the south. It also extends into the states of North and South Carolina, Virginia, Maryland, Pennsylvania, Kentucky, and Tennessee. It crosses into Canada, where it occupies a small geographic area between lakes Erie, Huron, Saint Clair, and Ontario. Like the European chestnut, it is used for timber, tannins, and chestnut production. China is considered to be one of the centers of origin of the genus *Castanea*. The Chinese chestnut (*C. mollissima*) geographic area extends from Korea to Laos, where there are a large number of individuals in China. *Castanea crenata*, is of Japanese origin, and is widely distributed in Korea and in several Chinese provinces. It is particularly abundant in the central provinces of Japan [6]. Most genetic and biotechnology studies on resistance to diseases have been carried out using these four species.

The chestnuts are deciduous trees that have single alternate leaves 20–30 cm long, oblong to lanceolate leaves, straight-veined leaves, a serrated edge, and an acute to acuminate apex. They are a monoecious species, and their flowers appear in two types of catkins in the same season as the shoots. The staminate flowers sprout into long, slender male catkins that are situated in the lower part of the shoot, while the female flowers are clustered in the base of the bisexual catkins situated around the terminal part of the shoot. They are annual bearers, and normally begin to bear fruit before other nut trees. The nuts are large and brown-colored, and are enclosed in a prickly hard involucre, or bur. They mature between September and October, and most of them fall free from the burs, which makes them easy to collect [5].

They are very long-living trees that are able to reach 500–1000 years, with examples of European chestnuts even being found that are estimated to be more than 2000 years-old [7].

The chestnut tree has traditionally been cultivated in China, Korea, Japan, and in the Mediterranean area. In China, it has been cultivated for around 6000 years, and in Europe, the Greeks are among the first cultivators of this species. In Europe and Asia, the chestnut trees are mainly appreciated for the chestnut production, particularly the European tree. In general, chestnuts are between 40% and 44% water, 49% carbohydrate, 5% protein, and 1% of fat, making it an excellent nutritive product [4]. The chestnuts from this species are used to make purees, stuffings, stews, the French delicacy *marrons glacés*, and even beer and liquors [8]. When they reach maturity, a tree can produce up to 100 kg of chestnuts per year, and chestnuts currently constitute the only commercially important nut crop within the family. However, in North America, it is a multipurpose forest tree that provides a wide range of products to the local populations.

The chestnut trees in Europe and the USA have experienced a massive dieback caused by two main diseases: ink disease, also known as *Phytophthora* root rot, and chestnut blight canker. Ink disease is caused by *P. cambivora* (Petri) Buism. This disease was first discovered in Portugal in the 1830s [9], and has caused the death of thousands of European chestnut trees. In the USA, the ink disease caused by *P. cinnamomi* Rands has affected the American chestnut tree, and destroyed entire populations of chinkapin species [5]. Chestnut blight canker, produced by *Cryphonectria parasitica* (Murr.) Barr, entered the US in the late 1800s, and was detected for the first time in 1904 [10,11]. The disease spread rapidly, causing the death of millions of examples of American chestnut trees [12]. In the US, it is considered as a great ecological disaster that almost exterminated the species. *Castanea sativa* is less susceptible to this disease, but it has also caused the death of European chestnuts. The different methods used to eradicate or control these diseases up until now have not been successful, and the strategies to obtain chestnut trees tolerant/resistant to blight and/or ink disease are based on interspecific crossings with Asian species. In some countries, natural remission is due to the natural occurrence of debilitated strains of *C. parasitica* that contain a virus known as *C. hypovirus CHV1*, which acts by reducing the virulence of the fungus and enables the chestnut to overcome the infection. The Asiatic chestnuts *C. mollissima* and

C. crenata are resistant to both diseases, although the levels of the resistance vary. The resistance to ink disease seems to be determined by two recessive genes, and that of blight resistance also seems to be polygenic [13]. However, the Asiatic chestnut populations have also suffered losses due to repeated infections by chestnut gall wasp (*Dryocosmus kuriphilus* Yamumatsu), which is endemic to China, and naturalized in Korea and Japan. This insect was introduced accidentally into the US and Europe, and caused significant damages in growing and fruit production [5].

The establishment of new plantations is difficult, due to not only the lack of improved genotypes, but also the difficulty with its vegetative propagation. As with the majority of the large seeded hardwoods, chestnuts are very difficult to propagate vegetatively using conventional techniques. Among the different propagation methods used on chestnuts, grafting gives the best results. It is especially used for the propagation of varieties of fruit, as it has the advantage of being able to be combined with rootstock resistant to *Phytophthora* [14]. However, the cost of this method makes it unfeasible for its mass propagation. The rooting of cuttings, which is in principle more practical and cheaper, is considered very difficult. In 1992, Vieitez [15] reported that cuttings coming from adult trees contain chemicals that inhibited rooting, while in cuttings of juvenile origin, which are much easier to root, these (chemical) compounds were not detected. Stool bed layering has been used for the propagation of *C. sativa*, *C. crenata*, and *C. dentata*, but its success varies a lot depending on the clone, and is relatively expensive [14].

2. Biotechnological Approaches Used for the Conservation of Plant Species

The ex situ conservation has been a widely used and internationally accepted strategy by numerous biodiversity conservation organizations [16]. Several recent reviews have pointed out the possibilities of forest biotechnology in relation to tree improvement [14,17–20].

Tree biotechnology includes methods of mass and clonal/vegetative propagation of superior tree genotypes, molecular breeding, and genetic transformation. Within these methods, the in vitro culture techniques of plant tissue are considered very effective for the conservation of germplasm, especially in the case of rare or threatened species [21]. These techniques have been developed as an alternative to the conventional propagation methods that, as we have already mentioned previously, are costly, long, and in many cases, not very effective. The use of in vitro tools can help obtain genotypes selected for their wood and/or fruit quality, as well as for their tolerance/resistance to diseases, and provide alternative methods not just for propagation, but also for the conservation of the species [22]. However, conservation programs must not only be confined to natural populations, but also should include new genotypes identified and produced on a global scale using gene transformation.

In the chestnut, a very important line of investigation has focused on the development of propagation systems capable of satisfying the demand by elite genotypes, with good quality wood and/or fruits, and resistance to the most important diseases that affect the chestnut (ink disease and chestnut blight canker).

The aim of this review is to provide a summary of the works carried out in the micropropagation of the different species of the *Castanea* genus. This technique is considered to be an effective method for the short-term conservation of genetic material, and as a necessary step for the application of medium and long-term conservation techniques (cryopreservation). Other recent reviews on this genus that have sought disease-resistant examples have dealt with silvics [23], the biological control with mycoviruses [24], integrating technological, ecological, and social factors in restoration [25], plant propagation [5,26], genetic modification, and breeding [4,14,27].

2.1. Short-Term Conservation: Micropropagation

In vitro propagation methods in the chestnut include the differentiation of adventitious buds, somatic embryogenesis, and the proliferation of axillary buds, with this latter technique being the most important from the conservation point of view.

2.1.1. Proliferation by Axillary Buds

Micropropagation by means of axillary bud proliferation is the preferred method for the commercial propagation of woody species. It is considered to be the easiest method to apply, the most suitable for ensuring the genetic stability of the regenerated plant, and one of the most effective methods of ex situ short-term conservation [28–30]. This technique has been successfully used in different species to achieve a rapid production and regeneration of trees with desired characteristics, thus achieving genetically superior traits without gene segregation. In the *Castanea* genus, most of the works on micropropagation have been carried out on *C. sativa* (European chestnut) (Figure 1A) and *C. dentata* (American chestnut).

European Chestnut: *Castanea sativa*

Since Jacquot [31] published the first work on the in vitro culture of the chestnut in 1947, many researchers have focused their efforts on the in vitro propagation of the different species of this genus, and those on the European chestnut are the most numerous.

Vieitez and Vieitez [32] explored the possibility of cultivating chestnut buds as a propagation medium of this species, using lateral buds of plantlets three to four months old. The addition of 1 mg/L of benzyladenine (BA) to the culture medium helped in the development of the buds. Similar results were obtained through cultivating embryonic axes isolated in medium with BA [33]. In this latter case, they mentioned a limited rooting of the shoots on adding 3 mg/L indole-3-butyric acid (IBA) to the culture medium for 12 days. The rooting percentages improved using the basal immersion of the shoots in concentrated solutions of IBA (0.5–1 mg/mL) for short periods of time (2–15 min). Rodríguez [34] also achieved the regeneration of European chestnut plants using the culture of apical buds of juvenile material. Chevre et al. [35] studied the nutritional requirements for the micropropagation of the juvenile and adult material of this species. The authors achieved the rooting of the material of juvenile origin, but not from adult material. From these first works, there have been numerous studies to establish the best culture conditions for this important species.

Different micropropagation works have managed to develop axillary buds, and the subsequent rooting of the shoots, through using material of juvenile origin [15,36–50] (Figure 1B). The results obtained with this type of material may be useful for carrying out studies that investigate pathogenicity, nutrition, the multiplication of the plus-tree progeny, genotype selection, and above all, serve to establish the conditions suitable for the in vitro multiplication of material from selected adult trees.

In the last few years, research has focused on micropropagation using explants from selected adult material, but propagation on a large scale is currently not possible, mainly because the majority of genotypes require specific adjustments to the protocols. However, companies have been formed that produce thousands of chestnut plants per year.

When the material of origin is taken directly from the crown of selected adult trees, there are considerable difficulties in micropropagation, and limited percentages of success obtained [51]. However, the reactivity of this material has been able to be increased through pre-harvest etiolation, grafting on to seedling rootstocks, serial grafting, and the repeated cytokinin spraying of stock plants [51–58]. In general, the micropropagation of adult trees is more feasible when tissues that retain physiologically juvenile characteristics, such as basal shoots and stump sprouts, are used [18,59,60]. Several different authors have achieved the micropropagation of chestnut using this type of material [26,39,51,55,56,61–67]. Mert and Soylu [68] studied the use of dormant buds, with the extending of the explant collection period, although rooting of the shoots was not achieved in this work. Schwarz [69] and Vieitez et al. [26,70] reviewed the protocols for the micropropagation of different chestnut species from material of both juvenile and adult origin. In these works, they define the different stages of the micropropagation process, as well as the media and requirements necessary for their development. It should be mentioned that the culture medium most used in the in vitro propagation of the European chestnut is that defined by Gresshoff and Doy [71], normally supplemented with BA (Figure 1C). This medium is usually employed half- or one third-strength in the

rooting stage, and supplemented with auxin (Figure 1D). Plants are later transferred to the greenhouse for acclimatization (Figure 1E).

The developed methodology has been transferred to private companies for the large-scale propagation of ink-resistant genotypes [70]. In addition, plantations of micropropagated chestnut plants have been evaluated in the field by private companies (B. Cuenca, Tragsa SA, Maceda, Spain).



Figure 1. (A) Adult example of *Castanea sativa* × *C. crenata* cv HV (Vieitez hybrid) resistant to ink disease. (B) In vitro multiplication by proliferation of axillary buds in chestnut material of juvenile origin. (C) In vitro multiplication of *C. sativa* × *C. crenata* cultures selected for the size of their fruits (cv. Marigoule, left) and the quality of the wood (cv 431, the two on the right). (D) In vitro rooting of axillary shoots of *C. sativa* in Gresshoff and Doy medium with 25 mg/L indole butyric acid for 24 h. (E) Chestnut plants from in vitro culture, subsequently acclimatized in the greenhouse. (F) Chestnut shoots after one year of storage at 4 °C. (G) The same shoots stored in cold (F) after four weeks in multiplication medium (Gresshoff and Doy supplemented with 0.1 mg/L BA).

American Chestnut: *Castanea dentata*

As with the European chestnut, the first micropropagation works on the American chestnut (*C. dentata*) used material originating from zygotic embryos and nodal explants of plantlets that were cultivated in media supplemented with BA. The axillary shoots that subsequently developed in culture were rooted to obtain complete plants [72–74]. In adult material, Read et al. [75] used nodal sections of softwood shoots forced from suckers and branches harvested during winter. The explants were cultivated in the medium by Lloyd and McCown (WPM) [76] supplemented with 0.5 mg/L of BA. The shoots were subsequently rooted by dipping in a solution of IBA (3 g/L) and stuck in WPM supplemented with activated carbon or in sterilized sand, and achieved rooting percentages of between 40–50%. Xing et al. [77] used a similar process for obtaining axillary bud cultures from an adult tree of the same species, and developed a three-step medium sequence for the rooting of the micro-cuttings, with rooting percentages between 57–73%. Yang et al. [78] investigated the role of auxins and cytokinins in the proliferation of shoots, callus production, and rhizogenesis of the American chestnut. More recently, Oakes et al. [79] managed to increase the rooting and survival percentages of the plants by means of changes in the light intensity, activated carbon, and the time spent in the rooting medium. In 2016, Oakes et al. [80] published the results of their study for the improvement in the rooting of micropropagated transgenic American chestnut shoots, which contained a wheat oxalate oxidase gene, conferring resistance to the chestnut blight. According to these authors, the improvement in the survival rates will benefit the American chestnut recovery project by providing more plants for ecological studies and possible reforestation, although the achievement of a non-regulated status for these transgenic trees will require extensive field testing.

Asian Chestnut Trees: *C. crenata* and *C. mollissima*

The proliferation of axillary buds and the subsequent rooting of the in vitro developed shoots of *C. mollissima* has been reported by McPheeters et al. [72] and Qiguang et al. [81], who used material of juvenile origin. Micropropagation from adult material has not yet been achieved in this species, but there are works that mention the micropropagation of individual adults of *C. crenata* [39,66], and of the hybrid of *C. crenata* × *C. sativa* [39].

Micropropagation offers the opportunity to preserve the genetic material. However, the maintenance of in vitro cultures for long periods of time requires carrying out numerous sub-cultures, which increases the maintenance costs and the risk of contamination and somaclonal variation. These risks can be reduced using medium-term preservation under slow growth conditions (cold storage) or in liquid nitrogen (cryopreservation) for long-term maintenance.

2.2. Medium-Term Preservation (Slow Growth)

Short-term preservation techniques are used routinely for the conservation of numerous species, and are of particular interest for the conservation of hardwood species with recalcitrant seeds, selected genotypes, and those obtained by genetic transformation [82,83]. The main aim of this technique is to reduce the number of sub-cultures without affecting the viability of the explants, or their capacity to re-start their development once normal growth conditions return. Different methods have been employed to achieve this, such as reductions in temperature and/or lighting, the induction of osmotic stress, the reduction in the partial pressure of oxygen, the drying of the plant material, or the change in the composition of the culture media. The media modifications would include a reduction in mineral elements or the source of carbon, the addition of osmotically active compounds such as chlorocholine chloride (CCC) or abscisic acid, or growth retardants such as mannitol, as well as changes in the nature and/or concentration of growth regulators [84,85].

Of all these methods, the most widely used is low temperature preservation, combined with darkness or low light intensity [86]. This technique has been used due to its simplicity, flexibility, and practicality, and is the simplest way to reduce the growth of in vitro cultures [87,88]. The reduction in

temperature produces a reduction in metabolic activity (respiration, water loss, and wilting). In woody species, the temperature ranges for storing the cultures vary between -3°C and 12°C , although most of the species are stored between 2°C and 5°C [89,90]. The type and physiological state of the explants is an important factor in cold preservation, and although different explants have been used, the best results have been obtained with apices and nodal segments [91].

One of the advantages of this type of preservation is that it allows the use of the same basic in vitro facilities as required for micropropagation. This technique is based on the modification of the processes that are normally used for micropropagation, whereby the cultures are easily changed to the multiplication method [92].

These techniques can be used for germplasm collections, as they avoid genetic changes. They also considerably reduce staff costs, energy, and material that could limit the number of genotypes that can be managed, thus improving conservation plans.

The first works on the medium-term preservation of the chestnut were carried out by Janeiro [93] and Janeiro et al. [94]. These authors report on the storage of shoot cultures of *C. sativa* \times *C. crenata* for one year at $2\text{--}4^{\circ}\text{C}$, with a survival percentage rate of 96%. The survival and proliferation were strongly influenced by the time elapsed between the sub-culture and the cold storage. The physiological state of the explants at the time of storage capacity was the key to their survival, with better results being obtained when the explants were stored in cold for 10 days after sub-culture. According to these authors, this time would be necessary in order for the explants to recover from the stress suffered during the sub-culture process. The protocol for the preservation of these sub-cultures was published by Vieitez et al. [70]. Corredoira et al. [95] increased the storage temperature of the chestnut cultures (*C. sativa* and *C. sativa* \times *C. crenata*) up to 18 months at 4°C , and in conditions of darkness. They also pointed out the importance of a pre-culture period of 10–12 days prior to storing in the cold. In *C. sativa*, Lambardi et al. [96] achieved culture survival after storing at 4°C for five months, but they observed a reduction in the re-growth potential after transferring them to standard conditions. Better results were obtained by Capuana and Di Lonardo [21] in the storage of shoot cultures of *C. sativa*; they achieved survival percentages greater than 80% after 48 months at 8°C . A storage temperature of 4°C significantly decreases the survival percentages, only recovering 56% of the cultures after 12 months storage, and 0% survival after 24 months. Gomes et al. [97] compared two techniques (synthetic seeds and slow growth storage) for the preservation of hybrid clones produced from controlled crosses between *C. sativa* \times *C. crenata* and *C. sativa* \times *C. mollissima*. The best treatment was achieved with synseeds stored for six months with sterile distilled water, and with slow growth storage with 0.22 M sucrose.

Cold preservation has also been used for the maintenance of transgenic chestnut axillary shoots that were transformed with genes that coded pathogenesis-related proteins with survival percentages of 100% after 6–9 months of cold storage [95].

In no case has morphological differences been observed between the stored material and the cultures maintained in a normal system (Figure 1F,G).

2.3. Long-Term Preservation (Cryopreservation)

The advances in biotechnology have led to opportunities for the conservation of genetic resources, and the use and maintenance of plant material at cryogenic temperatures is an option for long-term preservation [98,99]. This method is based on the total arrest of cell division and metabolic processes as a result of storing at ultra-low temperatures [100].

Cryopreservation or storage in liquid nitrogen is a particularly effective technique in the case of species with recalcitrant seeds that have been vegetatively propagated, and in the case of products obtained by the biotechnology, such as somatic embryos, cell lines, and genetically transformed material. Its use in the preservation of woody species has increased considerably, especially in the case of conifers [101,102]. In hardwoods, the cryopreservation of a collection of elm trees (*Ulmus* spp.) that

had survived the epidemics of Dutch elm disease should be mentioned, as they are potentially resistant to this disease [103,104].

The storage in liquid nitrogen (cryopreservation) at -196°C currently constitutes one of the most efficient and low cost methods for the long-term storage of biological material. The main advantages of this technique are its simplicity and ease of application to a wide range of species [84,105]. Theoretically, the material thus stored may be maintained without changes for indefinite periods of time [87]. This method has been used for the preservation of different types of material: seeds, isolated embryonic axes, and vegetatively propagated material, among which include apical or axillary buds, somatic embryos, and embryogenic tissues [98,106].

Pence [107,108] showed the possibility of cryopreserving the isolated embryonic axes of *C. mollissima* and *C. sativa* using the drying method, although the percentages of regenerated plants were low. Subsequently, Holliday and Merkle [109] managed to recover embryogenic cultures of *C. dentata* using 0.4 M sorbitol for the pre-treatment and 5% dimethylsulphoxide (DMSO) for the cryoprotection. Samples were placed into a pre-chilled “Mr. Frosty” freezing container for 1.5 h and then transferred to a Cryosafe freezer, where they were held in liquid nitrogen at -196°C for 48 h. Corredoira et al. [110] and San José et al. [111] achieved survival percentages greater than 93% and a plant recovery of 63% with isolated embryonic axes of mature seeds of *C. sativa*. The axes were dried in a laminar flow chamber until reaching a water content of 20–24%. Vieitez et al. [112] published the protocol for the preservation of embryonic axes and somatic embryos of the European chestnut using cryopreservation. Drying is one of the simplest methods, and although there are other ways to do it (silica gel or with sterile compressed air), drying in a laminar flow chamber is the most widely used. This technique is mainly used with pollen, seeds, zygotic embryos, and embryonic axes.

Most of the new cryopreservation techniques use the vitrification process, by means of which the internal solutes pass directly from the liquid phase to an amorphous or metastable phase, thus avoiding the formation of ice crystals that can damage the cells [113]. This technique involves the treatment of samples with concentrated cryoprotectants, dehydration with a concentrated vitrification solution, and immersion in liquid nitrogen. One of the most widely used vitrification solutions is that known as “plant vitrification solution 2” (PVS2), with a composition consisting of 30% (*w/v*) glycerol, 15% (*w/v*) ethylene glycol, and 15% (*w/v*) DMSO in a liquid medium with 0.4 M of saccharose [114]. The optimization of time and temperature are essential in order to avoid producing osmotic stress or toxicity.

In *C. sativa*, Vidal et al. [115] and Jorquera et al. [116] investigated the possibility of cryopreserving shoot tips from material of juvenile and adult origin excised from *in vitro* cultures using the vitrification process. The shoot recovery percentages varied between 33–54% depending on the genotype, although these differences did not appear to be related to the juvenile or adult status of the material. According to these authors, the most important factors for the success of cryopreservation are: the source of the shoot tips, their size, the length of exposure to the cryoprotectant solution, and the composition of the post-cryostorage recovery medium. Vidal et al. [117] subsequently performed a study for the development of an applied germplasm bank of selected chestnut genotypes as a backup to the field collection gene banks, with the survival of 63% of the 46 genotypes selected for their resistance to ink disease.

Vitrification has also been applied with success in the cryopreservation of somatic embryos of the European chestnut, with percentages of embryogenic resumption rates of 68%. Embryo clumps of globular and/or heart-stage embryos were treated with PVS2 for 60 min at 0°C . The same process has been used successfully for the cryopreservation of transgenic embryogenic lines [118]. Genetic transformation experiments in woody species are increasingly more numerous, with the vast majority of cases using somatic embryos as target material. The cryopreservation of the transgenic lines produced in these experiments is of great importance for the conservation of this material, particularly while molecular studies are performed and the results are verified in the field. Once identified, the best genotypes can be recovered from cryostorage and multiplied *in vitro*. The genetic stability of the

cryopreserved material must be confirmed; however, in general, there is no evidence of morphological or genetic alterations in forest species that have been cryopreserved [102].

3. Conclusions and Future Prospects

There are species of great ecological and commercial value within the genus *Castanea* that are spread throughout wide areas of the northern hemisphere. These species are seriously threatened by two main diseases (ink disease and blight canker), and also have a series of difficulties for their propagation using conventional methods, particularly when selected examples are desired in their adult stage. The biotechnology applied to the genus *Castanea* has mainly been focused on the identification and development and resistance to these two diseases [14]. The application of these techniques offers the possibility of developing tolerant/resistant genotypes that could subsequently be propagated in vitro on a large scale for the re-introduction of these species in their natural medium, and permit the regeneration of the ecosystems. In vitro culture methods not only offer the possibility of cloning these genotypes, but also preserving them for an indefinite time while field trials are being carried out. The storage of cultures at low temperatures (medium-term storage) reduces maintenance costs and the risks of contamination, as well as somaclonal variation. On the other hand, cryopreservation enables storing the material for long periods without altering its conditions, thus enabling field trials to be carried out. This point is especially important in the case of genetically transformed material.

Acknowledgments: To all those that have formed part of the *Grupo de Biotecnología y Mejora Forestal*, and who have contributed in way or another to the development of its projects. With a special mention to E. Vieitez and A.M. Vieitez for their works on the propagation and conservation of the European chestnut.

Author Contributions: All the authors have contributed equally in the carrying out of this work, and have read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Heywood, V.H.; Brummitt, R.K.; Culham, A.; Seberg, O. *Flowering Plant. Families of the World*; Royal Botanic Gardens: Kew, UK, 2007.
- Kremer, A.; Abbott, A.G.; Carlson, J.E.; Manos, P.S.; Plomion, C.; Sisco, P.; Staton, M.E.; Ueno, S.; Vendramin, G.G. Genomics of Fagaceae. *Tree Genet. Genomes* **2012**, *8*, 563–610. [[CrossRef](#)]
- Camus, A. Les chataigniers. Monographie des genres *Castanea* et *Castanopsis*. In *Encyclopédie Economique de Sylviculture*; Le Chevalier, P., Ed.; Paul Lechevalier: Paris, France, 1929.
- Maynard, C.A.; Powell, W.A.; Polin-McGuigan, L.D.; Vieitez, A.M.; Ballester, A.; Corredoira, E.; Merkle, S.A.; Andrade, G.M. Chestnut. In *Compendium of Transgenic Crop Plants: Transgenic Forest Tree Species*; Kole, C., Hall, T.C., Eds.; Wiley Blackwell: West Sussexcity, UK, 2008; pp. 169–192.
- Vieitez, F.J.; Merkle, S.A. Fagaceae. In *Biotechnology of Fruit and Nut Crops*; Litz, R.E., Ed.; CAB International: Wallingford, UK, 2005; pp. 263–296.
- Vieitez, E.; Vieitez, A.M.; Vieitez, F.J. *El Castaño*; Edilesa: León, Spain, 1996.
- Vieitez, F.J. Somatic embryogenesis in chestnut. In *Somatic Embryogenesis in Woody Plants. Angiosperms*; Jain, S.M., Gupta, P.K., Newton, R.J., Eds.; Kluwer: Dordrecht, The Netherlands, 1995; Volume 2, pp. 375–407.
- Bounous, G. The chestnut: A multipurpose resource for the new millenium. *Acta Hort.* **2005**, *693*, 33–40. [[CrossRef](#)]
- Taveira Fernandes, C. *A doença da Tinta dos Castanheiros. Parasitas do Género Phytophthora*. De Bary; Direcção-Geral dos Servicios Florestais e Aquícolas, Tipografia Alcobacense: Alcobaca, Portugal, 1966.
- Merkel, H.W. A deadly fungus on the American chestnut. *N. Y. Zool. Soc.* **1905**, *10*, 97–103.
- Murrill, W.A. A new chestnut disease. *Torreya* **1906**, *6*, 186–189.
- Connors, B.J.; Miller, M.; Maynard, C.A.; Powell, W.A. Cloning and characterization of promoters from American chestnut capable of directing reporter gene expression in transgenic *Arabidopsis* plants. *Plant Sci.* **2002**, *163*, 771–781. [[CrossRef](#)]

13. Jaynes, R.A. *Genetics of Chestnut*; USDA Forest Service: Washington, DC, USA, 1974.
14. Nelson, C.D.; Powell, W.A.; Merkle, S.A.; Carlson, J.E.; Hebard, F.V.; Islam-Faridi, N.; Staton, M.E.; Georgi, L. Biotechnology of trees: Chestnut. In *Tree Biotechnology*; Ramawat, K.G., Mérillon, J.M., Ahuja, M.R., Eds.; CRC News: Boca Raton, FL, USA, 2014; pp. 3–35.
15. Vieitez, E. The lack of rootability of chestnut cuttings. In *Proceedings International Chestnut Conference*; Double, M.L., MacDonald, W.L., Eds.; West Virginia University Press: Morgantown, WV, USA, 1992; pp. 82–88.
16. Sarasan, V.; Cripps, R.; Ramsay, M.M.; Atherton, C.; McMichen, M.; Prendergast, G.; Rowntree, J.K. Conservation in vitro of threatened plants-progress in the past decade. *In Vitro Cell. Dev. Biol. Plant* **2006**, *42*, 206–214. [[CrossRef](#)]
17. Vieitez, A.M.; Corredoira, E.; Martínez, M.T.; San José, M.C.; Sánchez, M.C.; Valladares, S.; Vidal, N.; Ballester, A. Application of biotechnological tools to *Quercus* improvement. *Eur. J. For. Res.* **2012**, *131*, 519–539. [[CrossRef](#)]
18. Ballester, A.; Corredoira, E.; Vieitez, A.M. Limitations of somatic embryogenesis in hardwoods trees. In *Vegetative Propagation of Forest Trees*; Park, Y.-S., Bonga, J.M., Moon, H.-K., Eds.; NIFoS: Seoul, Korea, 2016; pp. 56–74.
19. Corredoira, E.; Vieitez, A.M.; San José, M.C.; Vieitez, F.J.; Ballester, A. Advances in somatic embryogenesis and genetic transformation of European chestnut: Development of transgenic resistance to ink and blight disease. In *Vegetative Propagation of Forest Trees*; Park, Y.-S., Bonga, J.M., Moon, H.-K., Eds.; NIFoS: Seoul, Korea, 2016; pp. 279–301.
20. Monteuiis, O. Micropropagation and production of forest trees. In *Vegetative Propagation of Forest Trees*; Park, Y.-S., Bonga, J.M., Moon, H.-K., Eds.; NIFoS: Seoul, Korea, 2016; pp. 32–55.
21. Capuana, M.; Di Lonardo, S. In vitro conservation of chestnut (*Castanea sativa*) by slow growth. *In Vitro Cell. Dev. Biol. Plant* **2013**, *49*, 605–610. [[CrossRef](#)]
22. Reed, B.M.; Sarasan, V.; Kane, M.; Bunna, E.; Pence, V.C. Biodiversity conservation and conservation biotechnology tools. *In Vitro Cell. Dev. Biol. Plant* **2011**, *47*, 1–4. [[CrossRef](#)]
23. Wang, G.G.; Knapp, B.O.; Clark, S.L.; Mudder, B.T. *The Silvics of Castanea dentata (Marsh.) Borkh., American Chestnut, Fagaceae (Beech Family)*; GTR-SRS-173; USDA Forest Service, Southern Research Station: Asheville, NC, USA, 2013.
24. Milgroom, M.G.; Hillman, B.I. The ecology and evolution of funga viruses. In *Studies in Viral Ecology: Microbial and Botanical Host Systems*; Christon, J.H., Ed.; John Wiley and Sons Inc.: New York, NY, USA, 2011; Volume 1, pp. 217–253.
25. Jacobs, D.F.; Dalgeish, H.J.; Nelson, C.D. A conceptual framework for restoration of threatened plants: The effective model of American chestnut (*Castanea dentata*) reintroduction. *New Phytol.* **2013**, *197*, 378–393. [[CrossRef](#)] [[PubMed](#)]
26. Vieitez, A.M.; Vieitez, M.L.; Vieitez, E. Chestnut (*Castanea* spp.). In *Biotechnology in Agriculture and Forestry*; Bajaj, Y.P.S., Ed.; Springer: Berlin, Germany, 1986; Volume 1, pp. 393–414.
27. Worthen, L.M.; Woeste, K.E.; Michler, C.H. Breeding American chestnut for blight resistance. *Plant Breed. Rev.* **2010**, *33*, 305–339.
28. Bonga, J.M.; von Aderkas, P. *In Vitro Culture of Trees*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1992.
29. Monteuiis, O.; Doulebeau, S.; Verdeil, J.L. DNA methylation in different original clonal offspring from mature *Sequoiadendron giganteum* genotype. *Trees* **2008**, *22*, 779–784. [[CrossRef](#)]
30. Gomes, F.; Canhoto, J.M. Micropropagation of strawberry tree (*Arbustus unedo* L.) from adult plants. *In Vitro Cell. Dev. Biol. Plant* **2009**, *45*, 72–82. [[CrossRef](#)]
31. Jacquot, T.C. Effect inhibiteur des tannins sur le développement des cultures in vitro du cambium de certains arbres fruitiers. *CR Hebd. Sci. D Natur.* **1947**, *225*, 434–436.
32. Vieitez, A.M.; Vieitez, M.L. Culture of chestnut shoots from buds in vitro. *J. Hortic. Sci.* **1980**, *55*, 83–84. [[CrossRef](#)]
33. Vieitez, A.M.; Vieitez, E. Plantlet formation from embryonic tissue of chestnut grown in vitro. *Physiol. Plant* **1980**, *50*, 127–130. [[CrossRef](#)]
34. Rodríguez, R. In vitro propagation of *Castanea sativa* Mill. through meristem tip culture. *HortScience* **1982**, *17*, 888–889.

35. Chevre, A.M.; Gill, S.S.; Mouras, A.; Salelles, G. In vitro vegetative multiplication of chestnut. *J. Hortic. Sci.* **1983**, *58*, 23–29. [[CrossRef](#)]
36. Vieitez, A.M.; Vieitez, M.L. *Castanea sativa* plantlets proliferated from axillary buds cultured in vitro. *Sci. Hortic.* **1982**, *18*, 343–353. [[CrossRef](#)]
37. Vieitez, A.M.; Vieitez, M.L. Secuencia de cambios anatómicos durante la rizogénesis in vitro del castaño. *Phyton* **1983**, *43*, 185–191.
38. Chevre, A.M.; Salelles, G. Choice of explants for chestnut micropropagation. *Acta Hortic.* **1984**, *212*, 517–523. [[CrossRef](#)]
39. Chevre, A.M.; Salelles, G. Micropropagation du châtaignier. Problèmes et perspectives. In *5ème Colloque. sur les Recherches. Fruitières*; Ponte de la Maye: Bordeaux, France, 1985; pp. 215–218.
40. Strullu, D.G.; Grellier, B.; Marciniak, D.; Letouze, R. Micropropagation of chestnut and conditions of mycorrhizal synthesis in vitro. *New Phytol.* **1986**, *102*, 95–101. [[CrossRef](#)]
41. Mato, M.C.; Vieitez, A.M. Changes in auxin protectors and IAA oxidases during rooting of chestnuts shoots in vitro. *Physiol. Plant* **1986**, *66*, 491–494. [[CrossRef](#)]
42. Mulins, V.V. Micropropagation of chestnut (*Castanea sativa* Mill.). *Acta Hortic.* **1987**, *212*, 525–530. [[CrossRef](#)]
43. Ivanova, E.; Erdelsky, K. Cultivation of chestnut *Castanea sativa* Mill. embryos isolated at various stages of development. *Biologia* **1989**, *44*, 393–400.
44. Piagnini, C.; Eccher, T. Factors affecting the proliferation and rooting of chestnut in vitro. *Acta Hortic.* **1988**, *227*, 384–386. [[CrossRef](#)]
45. Wilhem, E.; Rodkachane, P. Micropropagation of juvenile and adult *Castanea sativa* by using thidiazuron. In *Proceedings International Chestnut Conference*; Double, M.L., MacDonald, W.L., Eds.; West Virginia University Press: Morgantown, WV, USA, 1992; p. 129.
46. Vidal, N.; Ballester, A.; Vieitez, A.M.; Kevers, C.; Gaspar, T. Biochemical characteristics of chestnut shoots related to in vitro multiplication and rooting capacities. *Adv. Hortic. Sci.* **1994**, *8*, 19–24.
47. Ballester, A.; San José, M.C.; Vidal, N.; Fernández-Lorenzo, J.L.; Vieitez, A.M. Anatomical and biochemical events during in vitro rooting of microcuttings from juvenile and mature phases of chestnut. *Ann. Bot.* **1999**, *83*, 619–629. [[CrossRef](#)]
48. Fernández-Lorenzo, J.L.; Rigueiro, A.; Ballester, A. Polyphenols as potential markers to differentiate juvenile and mature chestnut shoot cultures. *Tree Physiol.* **1999**, *19*, 461–466. [[CrossRef](#)] [[PubMed](#)]
49. Soyly, A.; Ertük, Ü. Researches on micropropagation of chestnut. *Acta Hortic.* **1999**, *494*, 247–253. [[CrossRef](#)]
50. Roussos, P.A.; Archimandriti, A.; Beldekou, I. Improving in vitro multiplication of juvenile European chestnut (*Castanea sativa* Mill.) explants by the use of growth retardants. *Sci. Hortic.* **2016**, *198*, 254–256. [[CrossRef](#)]
51. Sánchez, M.C.; Vieitez, A.M. In vitro morphogenetic competence of basal sprouts and crown branches of mature chestnut. *Tree Physiol.* **1991**, *8*, 59–70. [[CrossRef](#)] [[PubMed](#)]
52. Ballester, A.; Sánchez, M.C.; Vieitez, A.M. Etiolation as a pretreatment for in vitro establishment and multiplication of mature chestnut. *Physiol. Plant* **1989**, *77*, 395–400. [[CrossRef](#)]
53. Ballester, A.; Sánchez, M.C.; San José, M.C.; Vieitez, F.J.; Vieitez, A.M. Development of rejuvenation methods for the in vitro establishment, multiplication and rooting of mature trees. In *Plant Aging: Basic and Applied Approaches*; Rodríguez, R., Sánchez-Tamés, R., Durzan, D.J., Eds.; Plenum Press: New York, NY, USA, 1990; pp. 43–49.
54. Feijo, J.A.; Pais, M.S. Rejuvenation of adult specimens of *Castanea sativa* Mill. through in vitro micropropagation. In *Plant Aging: Basic and Applied Approaches*; Rodríguez, R., Tamés, S.R., Durzan, D.J., Eds.; Plenum Press: New York, NY, USA, 1990; pp. 43–49.
55. Sánchez, M.C. Propagación de Material Adulto de Castaño y Roble. Métodos de Rejuvenecimiento. Ph.D. Dissertation, University of Santiago de Compostela, Santiago de Compostela, Spain, 1991.
56. Sánchez, M.C.; Ballester, A.; Vieitez, A.M. Reinvigoration treatments for the micropropagation of mature chestnut. *Ann. Sci. For.* **1997**, *54*, 359–370. [[CrossRef](#)]
57. Sánchez, M.C.; San José, M.C.; Ferro, E.; Ballester, A.; Vieitez, A.M. Improving micropropagation conditions for adult-phase shoots of chestnut. *J. Hortic. Sci.* **1997**, *72*, 433–443. [[CrossRef](#)]
58. Giovanelli, A.; Gianini, R. Reinvigoration of mature chestnut (*Castanea sativa*) by repeated grafting and micropropagation. *Tree Physiol.* **2000**, *20*, 1243–1248. [[CrossRef](#)]

59. Evers, P.; Haanstra, L.; Vermeer, E.; Van Eeden, S. Influence of reverse phase change on micropropagation of *Quercus robur*. *Plant Tissue Cult. Biotechnol.* **1996**, *2*, 148–153.
60. Bonga, J.M. Can explant choice help to resolve recalcitrance problems in in vitro propagation, a problem still acute especially for adult conifers? *Trees* **2017**, *31*, 781–789. [[CrossRef](#)]
61. Biondi, S.; Canciani, L.; de Paoli, G.; Bagni, N. Shoot formation from bud cultures of mature chestnut. In *Colloque International sur la Culture In Vitro des Essences Forestières*; AFOCEL: Fontainebleau, France, 1981; pp. 181–185.
62. Vieitez, A.M.; Ballester, A.; Vieitez, M.L.; Vieitez, E. In vitro plantlet regeneration of mature chestnut. *J. Hortic. Sci.* **1983**, *58*, 457–463. [[CrossRef](#)]
63. Read, P.; Szendrak, E. Generating new plants through tissue culture methods. *J. Am. Chestnut Found.* **1995**, *9*, 50–53.
64. Read, P.E.; Hosier, M.A.; Qiguan, Y. Forcing solutions: A novel plant growth regulator delivery method for in vitro culture of woody species. *HortScience* **1985**, *20*, 592.
65. Chauvin, J.E.; Saleses, G. Effect du fructose sur la micropropagation du châtaignier *Castanea* spp. *CR Acad. Sci. Paris (Ser. D)* **1988**, *206*, 207–212.
66. Chauvin, J.E.; Saleses, G. Advances in chestnut micropropagation (*Castanea* spp.). *Acta Hortic.* **1988**, *227*, 340–345. [[CrossRef](#)]
67. Miranda, M.E.; Fernández, J. Genotypic and environment variation of *Castanea crenata* x *C. sativa* and *Castanea sativa* clones in aptitude to micropropagation. *Silvae Genet.* **2001**, *50*, 153–162.
68. Mert, C.; Soyulu, A. Shoot apex culture of dormant buds in chestnut. *Acta Hortic.* **2009**, *844*, 145–152. [[CrossRef](#)]
69. Schwarz, O.J. Plant growth regulator effects in the in vitro propagation of three hardwood tree genera: *Castanea*, *Juglans*, and *Quercus*. *Plant Growth Regul.* **1987**, *6*, 113–135. [[CrossRef](#)]
70. Vieitez, A.M.; Sánchez, M.L.; García-Nimo, M.L.; Ballester, A. Protocol for micropropagation of *Castanea sativa*. In *Protocols for Micropropagation of Woody Trees and Fruits*; Jain, S.M., Häggman, H., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 299–312.
71. Gresshoff, P.M.; Doy, C.H. Development and differentiation of haploid *Lycopersicon esculentum*. *Planta* **1972**, *107*, 161–170. [[CrossRef](#)] [[PubMed](#)]
72. McPheeters, K.; Skirvin, R.M.; Bly-Monnen, C.A. Culture of chestnut (*Castanea* sp.) in vitro. *HortScience* **1980**, *15*, 417–418.
73. Keys, R.N.; Cech, F.C. Propagation of American chestnut in vitro. In *Proceedings USDA Forest Service American Chestnut Cooperators Meeting*; Smith, H.V., MacDonald, W.L., Eds.; West Virginia University Press: Morgantown, WV, USA, 1982; pp. 106–110.
74. Serres, R.; Read, P.; Hackett, W.; Nissen, P. Rooting of American chestnut microcuttings. *J. Environ. Hortic.* **1990**, *8*, 86–88.
75. Read, P.E.; Hosier, M.A.; Qiquang, Y. Tissue culture of chestnuts. *Ann. Rep. North. Nut Growers Assoc.* **1985**, *76*, 142–145.
76. Lloyd, G.; McCown, B.H. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Comb. Proc. Int. Plant Propagators Soc.* **1980**, *30*, 421–427.
77. Xing, Z.; Satchwell, M.; Powell, W.A.; Maynard, C.A. Micropropagation of American chestnut: Increasing rooting rate and preventing shoot-tip necrosis. *In Vitro Cell. Dev. Biol. Plant* **1997**, *33*, 43–48. [[CrossRef](#)]
78. Yang, G.; Lu, Z.; Asante, T.M.; Read, P.E. In vitro responses of American chestnut to plant growth regulators in culture medium. *Acta Hortic.* **2009**, *844*, 229–334. [[CrossRef](#)]
79. Oakes, A.D.; Powell, W.A.; McGuigan, L.D.; Maynard, C.A. Darkness and activated charcoal increase micropropagated American chestnut (*Castanea dentata*) rooting and acclimatization success. *Acta Hortic.* **2013**, *988*, 85–92. [[CrossRef](#)]
80. Oakes, A.D.; Desmarais, T.; Powell, W.A.; Maynard, C.A. Improving rooting and shoot tip survival of micropropagated transgenic American chestnut shoots. *HortScience* **2016**, *51*, 171–176.
81. Qiguang, Y.; Read, P.E.; Fellman, C.D.; Hosier, M.A. Effect of cytokinin, IBA, and rooting regime on Chinese chestnut cultured in vitro. *HortScience* **1986**, *21*, 133–134.
82. Engelmann, F. Management of field and in vitro germplasm collections. In *Proceedings of the Consulting Meeting, CIAT Cali, Colombia, 15–20 January 1996*; International Plant Genetic Resources Institute: Rome, Italy, 1999.

83. Engelmann, F. Use of biotechnology for the conservation of plant biodiversity. *In Vitro Cell. Dev. Biol. Plant* **2011**, *47*, 5–16. [[CrossRef](#)]
84. Kaviani, B. Conservation of plant genetic resources by cryopreservation. *Aust. J. Crop. Sci.* **2011**, *5*, 778–800.
85. Cruz-Cruz, C.A.; González-Arno, M.T.; Engelmann, F. Biotechnology and conservation of plant biodiversity. *Resources* **2013**, *2*, 73–95. [[CrossRef](#)]
86. Marco-Medina, A.; Casas, J.L. Polyamine content during minimal growth storage of *Thymus moroderi* explants. *Biol. Plant.* **2012**, *56*, 590–594. [[CrossRef](#)]
87. Engelmann, F. In vitro conservation methods. In *Biotechnology and Plant Genetic Resources: Conservation and Use*; Callow, J.A., Ford-Lloyd, B.V., Newbury, H.J., Eds.; School of Biological Sciences: Wallingford, CT, USA, 1997; pp. 119–161.
88. Turner, S.; Krauss, S.L.; Bunn, E.; Senaratna, T.; Dixon, K.; Tan, B.; Touchell, D. Genetic fidelity of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. *Plant Sci.* **2001**, *161*, 1099–1106. [[CrossRef](#)]
89. Watt, M.P.; Thokoane, N.L.; Mycock, D.; Blackeway, F. In vitro storage of *Eucalyptus grandis* germplasm under minimal growth conditions. *Plant Cell Tissue Organ Cult.* **2000**, *61*, 161–164. [[CrossRef](#)]
90. Lambardi, M.; De Carlo, A. Application of tissue culture to germplasm conservation of temperate broad-leaf trees. In *Micropropagation of Woody Trees and Fruits*; Jain, S.M., Ishii, K., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2003; pp. 815–840.
91. Corredoira, E.; Martínez, M.T.; San José, M.C.; Ballester, A. Conservation of hardwood species. In *Biodiversity and Conservation of Woody Plants*; Ahuja, M.R., Jain, S.M., Eds.; Springer: Berlin, Germany, 2017; in press.
92. Blakesley, D.; Pask, N.; Henshaw, G.G.; Fay, M.F. Biotechnology and the conservation of forest genetic resources: In vitro strategies and cryopreservation. *Plant Growth Regul.* **1996**, *20*, 11–16. [[CrossRef](#)]
93. Janeiro, L.V. Almacenamiento en frío y Criopreservación de Especies Leñosas Propagadas In Vitro. Ph.D. Dissertation, University of Santiago de Compostela, Santiago de Compostela, Spain, 1993.
94. Janeiro, L.V.; Vieitez, A.M.; Ballester, A. Cold storage of the in vitro cultures of wild cherry, chestnut and oak. *Ann. Sci. For.* **1995**, *52*, 287–293. [[CrossRef](#)]
95. Corredoira, E.; Valladares, S.; Martínez, M.T.; Couselo, J.L.; San José, M.C.; Ballester, A.; Vieitez, A.M. Conservación de germoplasma en especies leñosas con técnicas de cultivo in vitro y almacenamiento en frío. *Span. J. Rural Dev.* **2011**, *1*, 15–24. [[CrossRef](#)]
96. Lambardi, M.; Benelli, C.; De Paoli, G.; Battistini, A. Biotechnologie per la conservazione del castagno. In Proceedings of the “Convegno. Nazionale. Castagno. 2001”, Firenze, Italy, 25–27 October 2001; pp. 86–91.
97. Gomes, F.; Clemente, M.; Figueiredo, P.; Plácito, F.; Machado, H.; Santos, C.; Costa, R. *Castanea* spp. hybrid clones in vitro conservation: Synthetic seeds vs. slow growth storage. *Acta Hort.* **2017**, *1155*, 37–44. [[CrossRef](#)]
98. Reed, B.M. Cryopreservation-practical consideration. In *Plant Cryopreservation: A Practical Guide*; Springer: New York, NY, USA, 2008; pp. 3–13.
99. Kong, L.; von Aderkas, P. A novel method of cryopreservation without a cryoprotectant for immature somatic embryos of conifer. *Plant Cell Tissue Organ Cult.* **2010**, *106*, 115–125. [[CrossRef](#)]
100. Niino, T.; Sakai, A. Cryopreservation of alginate-coated in vitro-grown shoot tips of apple, pear and mulberry. *Plant Sci.* **1992**, *87*, 199–206. [[CrossRef](#)]
101. Reed, B.M.; Paynter, C.L.; DeNoma, J.; Chang, Y. Techniques for medium- and long-term storage of *Pyrus* L. genetic resources. *Plant Genet. Resour. Newsl.* **1998**, *115*, 1–4.
102. Häggman, H.; Rusanen, M.; Jokipii, S. Cryopreservation of in vitro tissues of deciduous trees. In *Plant Cryopreservation: A Practical Guide*; Reed, B., Ed.; Springer: New York, NY, USA, 2008; pp. 365–386.
103. Harvengt, L.; Meier-Dinkel, A.; Dumas, E.; Collin, E. Establishment of a cryopreserved gene bank of European elms. *Can. J. For. Res.* **2004**, *34*, 43–55. [[CrossRef](#)]
104. Shukla, M.R.; Jones, A.M.P.; Sullivan, J.A.; Liu, C.; Gosling, S.; Saxena, P.K. In vitro conservation of American elm (*Ulmus americana*): Potential role of auxin metabolism in sustained plant proliferation. *Can. J. For. Res.* **2012**, *42*, 686–697. [[CrossRef](#)]
105. Pence, V.C. Tissue cryopreservation for plant conservation: Potential and challenges. *Int. J. Plant Sci.* **2014**, *175*, 40–45. [[CrossRef](#)]
106. Sakai, A.; Engelmann, E. Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *CryoLetters* **2007**, *28*, 151–172. [[PubMed](#)]

107. Pence, V.C. Cryostorage of embryo axes of several large-seeded temperate tree species. *Cryobiology* **1990**, *27*, 212–218. [[CrossRef](#)]
108. Pence, V.C. Desiccation and the survival of *Aesculus*, *Castanea*, and *Quercus*. embryo axes through cryopreservation. *Cryobiology* **1992**, *29*, 391–399. [[CrossRef](#)]
109. Holliday, C.; Merkle, S.A. Preservation of American chestnut germplasm by cryostorage of embryogenic cultures. *J. Am. Chestnut Found.* **2000**, *14*, 46–52.
110. Corredoira, E.; San José, M.C.; Ballester, A.; Vieitez, A.M. Cryopreservation of zygotic embryo axes and somatic embryos of European chestnut. *CryoLetters* **2004**, *25*, 33–42. [[PubMed](#)]
111. San José, M.C.; Jorquera, L.; Vidal, N.; Corredoira, E.; Sánchez, C. Cryopreservation of European chestnut germplasm. *Acta Hort.* **2005**, *693*, 225–232. [[CrossRef](#)]
112. Vieitez, A.M.; San José, M.C.; Corredoira, E. Cryopreservation of zygotic embryonic axes and somatic embryos of European chestnut. In *Plant Embryo Cultures: Methods and Protocols, Methods in Molecular Biology*; Thorpe, T.A., Yeung, E.C., Eds.; Springer: New York, NY, USA, 2011; Volume 710, pp. 201–213.
113. Fahy, G.M.; MacFarlane, D.R.; Angell, C.A.; Meryman, H.T. Vitrification as an approach to cryopreservation. *Cryobiology* **1984**, *21*, 407–426. [[CrossRef](#)]
114. Sakai, A.; Kobayashi, S.; Oiyama, I. Cryopreservation of nucellar cell of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tnaka) by vitrification. *Plant Cell Rep.* **1990**, *9*, 30–33.
115. Vidal, N.; Sánchez, C.; Jorquera, L.; Ballester, A.; Vieitez, A.M. Cryopreservation of chestnut by vitrification of in vitro-grown shoot tips. *In Vitro Cell. Dev. Biol. Plant* **2005**, *41*, 63–68. [[CrossRef](#)]
116. Jorquera, L.; Vidal, N.; Sánchez, C.; Vieitez, A.M. Optimizing conditions for successful plant regeneration from cryopreserved *Castanea sativa* shoot tips. *Acta Hort.* **2005**, *693*, 511–518. [[CrossRef](#)]
117. Vidal, N.; Vieitez, A.M.; Fernández, M.R.; Cuenca, B.; Ballester, A. establishment of cryopreserved gene banks of European chestnut and cork oak. *Eur. J. For. Res.* **2010**, *129*, 635–643. [[CrossRef](#)]
118. Corredoira, E.; San José, M.C.; Vieitez, A.M.; Ballester, A. Improving genetic transformation of European chestnut and cryopreservation of transgenic lines. *Plant Cell Tissue Organ Cult.* **2007**, *91*, 281–288. [[CrossRef](#)]



© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).