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Review

Threatened Woody Plants of Georgia and Micropropagation as a Tool for In Vitro Conservation

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Abstract: Georgia is the major part of the Caucasus; it is considered as one of the distinguished regions of the world with respect to biodiversity. The majority of Georgia's biodiversity is connected with forest ecosystems, which cover about 38% of the country's territories. In Georgia, as in other countries, many unique species of forest phytocenosis are threatened by extinction and/or genetic pollution due to the negative impact of various environmental and anthropogenic factors. Implementation of biotechnological approaches in practice for in vitro conservation of species can significantly speed up the processes of protection, thus guaranteeing the sustainability of the phytogenetic pool of the country. The present review summarizes the current status of several threatened woody perennials of the Red List of Georgia belonging to the genera *Castanea*, *Quercus,* and *Betula*, which are the dominant or edificatory species of forest phytocenosis. The feasibility for their in vitro propagation for conservation purposes is discussed.

Keywords: *Betula*; biodiversity; *Castanea*; conservation; hardwood; in vitro propagation; *Quercus*

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1. Introduction

Georgia is one of the countries with various types of climates from the agronomic point of view due to its uneven climate, altitude variations, and the existence of ecological habitats. The major part of the country is the Caucasus, which is considered by international organizations as one of the distinguished regions of the world with respect to biodiversity. It is within one of the World Wide Fund for Nature's (WWF) 35 'priority places' (Greater Black Sea Basin), and is also part of two of 36 'biodiversity hotspots' (the Caucasus and Irano-Anatolian hotspots) identified by Conservation International as being simultaneously the richest and most threatened reservoirs of plant and animal life [1–4].

The natural habitats of Georgia represent heterogeneous ecosystems, and the country's dominant flora is frequently presented by rare and endemic species. Over 2000 species of Georgian flora have direct economic value as timber resources and food for humans and animals; 1200 species of vascular plants are used as medicinal herbs [3,5]. The unique phyto-genetic pool of Georgia is the constituent part of the natural and cultural heritage of the country [3]. According to the National Report to the Convention on Biological Diversity, Georgian flora is one of the richest among countries with moderate climates, with 4130 vascular plant species, including around 900 species (approximately 21%) that are either Caucasian or Georgian endemics [5]: 275 species of vascular plants are endemic to Georgia, and 52 species of Georgian endemic flora (approximately 60% of endemic species) are categorized as endangered due to disturbance to their habitats, excessive use, pathogens, and other pressures [2,3].

Forests are the most important habitats of the Caucasus Region. The majority of Georgia's biodiversity is directly connected with forest ecosystems. Approximately 2.66 million ha, or 38.3% of the country's territories, are covered by forest, and about 95–98% of these are of natural origin [4,6]. Forests are especially valuable natural resources in Georgia. Forest massifs surviving in the Georgian mountains are the last untouched forests in the moderate climate zone of the Earth, and thus have a global importance. They also have an exceptional importance at the national and regional levels. Georgian forests not only conserve the unique biological diversity of Georgia, but ensure continuous delivery of vital direct and indirect benefits and resources to the population. This in turn facilitates the functioning of the field of economy, the growth of human welfare, and poverty elimination, and creates a favorable environment for the sustainable development of the country. Their composition, structure, growth, development, and other characteristics determine a rich biological diversity—up to 400 tree and shrub species grow in Georgian forests. The large number of endemic timber tree species points to the high diversity of dendroflora. Among endemic species, 61 species are endemic to Georgia and 43 are endemic to the Caucasus [7]. The main wood-formative species of forests in Georgia from deciduous species are: beech tree (*Fagus orientalis* Lipsky), with 51.8% of the total growing wood stock; oak tree (mostly *Quercus iberica* Steven ex M.Bieb)—5.5%; hornbeam (*Carpinus* L.)—5.7%; and edible chestnut (*Castanea sativa* Mill.)—2.9%. From coniferous species, they are: fir tree (*Abies nordmanniana* (Steven) Spach)—17.2%; spruce (*Picea orientalis* L.)—7.5%; and pine tree (*Pinus sosnowskyi* Nakai, *Pinus eldarica* Medw.)—3.4% [8].

Georgian forest hardwoods are required to meet different demands of the national economy and the population. They provide timber for industrial and household use and thus can generate significant value and work possibilities, as well as increase incomes and prosperity of the rural population. Forest ecosystem health determines the clean water supply for the major part of the Georgian population, as well as supply for agriculture. Besides the other useful functions of great significance to the State, Georgian forest ecosystems also have an exceptional aesthetic and recreational importance. The existence and development of a number of resorts largely increases tourism potential and incomes from tourism activities [7].

After the 1990s, the forests of Georgia became intensively exploited for economic purposes, while for the part of rural communities, firewood has remained one of the sources of energy. Significant demand exists for timber. As a consequence, the introduction of sustainable forestry principles plays a significant role in the conservation of the country's biodiversity. The protection and restoration of forest ecosystems are important from the viewpoint of biodiversity preservation, along with the social–economic point of view, including sustainable development of agriculture and provision of food safety.

The "National Forest Concept of Georgia" (2013) [7] is the main policy document that defines forest management principles and establishes priority directions in the development of forestry, and also defines the State's approach to forests, taking into account the main functional purpose of forests and their values. A considerable portion of forests with high conservation values have been assigned the status of Protected Areas (PAs), an effective tool for protecting species and habitats. Currently, the area covered by forests that is under the Agency of Protected Areas of Georgia is estimated at 303,000 ha, which is about 11.3% out of the 2.7 million ha of total forest area covered by forests [4]. There are 14 Strict Nature Reserves, 11 National Parks, 19 Managed Nature Reserves, 41 Natural Monuments, and 2 Protected Landscapes [4,8].

Currently, many unique species in natural habitats across the country are threatened by extinction and/or genetic pollution due to the negative impact of various environmental and anthropogenic factors. Due to habitat destruction and extensive, unregulated exploitation, many plant species have become endangered. Local temperature and edaphic factors of each zone, such as soil moisture and depth, are all of importance in determining the composition of vegetation and ecological biodiversity that leads to the establishment of different subcommunities. These and other questions concerning biodiversity of forests of Georgia and the threat of extinction of some species were discussed by Patarkalashvili [8].

At present, the Red List of Georgia [9] contains 56 wooded plant species; 36 have been identified as vulnerable (VU), 18 taxa as endangered (EN), and 2 species as critically endangered (CR) according to the International Union for Conservation of Nature (IUCN) Red List Categories and Criteria (2012) [10]. It is noteworthy that several species from the Red List are also catalogued in the globally threatened trees and shrubs of the Caucasus [11,12]. Populations with limited geographical distribution or fragmentary and decreasing species are referred to the above categories, which are not threatened by extinction at this stage, but are under a certain risk and are defined as rare species. They usually occur only in specific locations and are localized in limited geographical areas, or are narrowly scattered in a wider area. Approximately 60% of the total numbers of endemic plant species are classified as endangered due to disturbance to their habitats, excessive use, pathogens, and other pressures [3,4]. Irregular climatic conditions and nonsustainable management of natural resources create a real danger for the flora biodiversity of Georgia. The main threats for biodiversity in Georgia are the destruction/degradation of habitats and the extensive use of biological resources. The threats to the forest ecosystems in Georgia include unsustainable utilization of forest resources, which is mainly caused by lack of access to alternative energy sources; overgrazing by livestock, which results in degradation of the forests' natural regeneration capabilities; forest pests and diseases; alien invasive species; frequent forest fires; and legislative gaps and shortcomings in management [2,6,13]. Unsustainable utilization of forest resources has inflicted damage on beech forests in mountainous regions of Georgia and chestnut forests in the Colchis foothills; oak forests are only preserved in distant canyons and relatively meager soils. Several threatened tree species included in the Red List of Georgia belonging to genera of *Castanea*, *Quercus,* and *Betula* are especially noteworthy, as they are dominant or edificatory species of forest phytocenosis. Among them are European chestnut (*Castanea sativa* Mill.), Imeretian oak (*Quercus imeretina* Steven ex Woronow), and endemic and relict species of birch (*Betula medwediewii* Regel, *Betula megrelica* Sosn.). The Caucasian oak (*Quercus macranthera* Fisch. & C.A.Mey. ex Hohen.) and birch (*Betula* spp.) are the dominants of subalpine sparse and crook-stem forests (1800–2500 m) and play a key role in the restoration of the high-mountainous forest belt (by elevation of the upper subalpine border, which is already lowered by 100–150 m). The above condition will create serious problems for Bakhmaro and Bakuriani forests in the near future. An alert should be focused on the local provenance of European chestnut (*Castanea sativa*). Along with the relict Colchic oak (*Quercus hartwissiana* Steven), chestnut is the dominant of mountainous forests of Western Georgia, generally extending from 100 m up to 900–1000 m above sea level, having the absolute upper limit at 1400 m in sporadic locations of West and East Georgia, which contain the highest percentage of areas covered with forests (approximately 75%) [14,15]. At present, due to low self-renewal and diseases, the big massifs of chestnut forests in the Tkibuli region are on the verge of destruction. Despite the recent tendencies of reduction of illegal harvesting of timber resources, the logging of timber and fuel wood is still considered as one the most important pressures for country biodiversity, as well as unsustainable utilization of natural resources for obtaining energy, food, and financial benefits.

2. Country Policies and Networks for Conservation of Biodiversity

The Law on Red List and Red Book adopted by the Parliament of Georgia [16] gives the legal definitions (relevant recommendations and methodological issues) of endangered species. It also regulates the issues related to planning and financial matters connected with the protection, rehabilitation, and conservation of endangered species. Alongside this, the participation of the country in all global conventions for biodiversity protection provides aspects of security and regulation of exploitation of species. Ratified in 1994, the Convention on Biological Diversity (1992) [17] is the main international guiding document for the protection and conservation of national biodiversity. Hereafter, the country accepted the responsibility to safeguard the nation's rich diversity of plant, animal, and microbial life, to begin sustainably using biological resources, and to ensure equitable sharing of benefits from biodiversity. Later, the country joined other conventions, including the United Nations Framework Convention on Climate Change (1992) [18], Convention on International

Trade in Endangered Species of Wild Fauna and Flora (CITES) [19], Convention on the Conservation of European Wildlife and Natural Habitats [20], and the European Landscape Convention [21]. As a signatory to these important international environmental treaties, Georgia entered the world scene with the potential for joining the most advanced nations in the field of environmental protection.

Realizing the importance of protection and rational use of biodiversity resources for the conservation of national heritage, 'The Biodiversity Strategy and Action Plan of Georgia' (NBSAP) was first approved in 2005 [22]. NBSAP-Georgia defined the strategies of protection and balanced use of natural resources for a 10-year period and more tangible actions for five-year intervals. According to this article, the ex situ and on-farm conservation of endemic and threatened species of flora of Georgia and cultural breeds/varieties is believed to be one of the key achievements of implementation of NBSAP, and considered as the best way of preservation of biodiversity. Alongside this, NBSAP emphasizes that loss of biodiversity may be avoided only as a result of synchronization of in situ and ex situ conservation measures. Updated in 2014, NBSAP includes the vision and the overall national targets for safeguarding Georgia's biodiversity for 2014–2020, followed by thematic chapters describing the situation for Georgia's biodiversity in more detail under the headings covering species and habitats, protected areas, forest ecosystems, agricultural biodiversity, natural grasslands, etc. [2]. Strategic goals and national targets of NBSAP (Target C.2) imply a considerable improvement of 75% of Red List species through effective conservation measures and sustainable use by 2020. It is in agreement with the Target 8 of the Global Strategy for Plant Conservation, which determines the new and important role of the ex situ conservation in the conservation strategy aimed at least 75% of threatened plant species in ex situ collections, preferably in the country of origin, and at least 20% available for recovery and restoration programs [23,24]. The Convention on Aichi Biodiversity Targets (2011–2020) statement says that by 2020, the rate of loss of all-natural habitats, including forests, should at least be halved, and where feasible, brought close to zero (Target 5), and that areas under agriculture and forestry should be managed sustainably, ensuring biodiversity conservation (Target 7) [25]. The 2020 edition of the Ecoregional Conservation Plan (ECP) for the Caucasus explains the role of ECP as the regional instrument for implementing international agreements related to biodiversity, and presents the plan itself with its targets and actions [13]. According to this document, sustainable management, restoration, and maintenance of the ecological connectivity of forest ecosystems require following targets by 2035: C1—illegal logging must be eliminated, forests must be managed sustainably, and management policies and practices must take into consideration the potential impact of climate change and goals of biodiversity conservation; and C2—connectivity of forest ecosystems are restored and enhanced within CLs and BLs through application of the FLR approach and taking into account the potential impact of climate change.

The impacts on Caucasus ecosystems are already apparent and will become stronger. During the last 50-year period, an average annual temperature in the whole country was manifesting a growth trend, with maximum increments observed in the east Georgia semi-arid zone (+0.7 °C) and the Black Sea coastal zone (+0.6 °C). According to the forecast, by 2100, precipitation reduction by 10–20% is expected throughout the whole country [3]. The named trend of climate change is already affecting forest ecosystems: in the mountainous zone, birch is being replaced by pine and fir trees. As for abiotic disorders, there was an increase in the number of forest fire incidents, and at the same time biotic disorders were manifesting in several catchments through growing establishment of various pests and diseases [3]. The World Economic Forum's 2020–21 Global Risk Report ranks biodiversity loss and ecosystem collapse as one of the top five threats humanity will face in the 10 years leading to 2030, and "climate action failure" was recognized as the most impactful and second most likely long-term risk identified in the Global Risks Perception Survey (GRPS) [26,27]. Furthermore, because of the strong interconnection between biodiversity loss and climate change, conserving biodiversity and preserving ecosystem health are a top priority [13]. Delayed response can put large areas of forest at risk of catastrophic degradation. This will lead to a large reduction in the quantity and quality of the forest resources and useful functions, on which many people in the country depend. Among the strategies and implemented activities elaborated by The National Forest Concept for Georgia [7] aimed at mitigating and/or adapting to the impacts of climate change on forests the following actions draws attention are: (i) expansion of protected areas; and (ii) reforestation—forest planting, greening urban areas, and forest plantations. These activities are largely focused on the reinforcement of in situ and ex situ conservation means.

Since 1990, when the WWF country office was established in Georgia, large projects have been launched to assist the protected-area system in Georgia and build an environmental education program. More projects have been funded, including ones regarding species conservation and sustainable forest management under the WWF/World Bank Alliance for Forest Conservation and Sustainable Use. Since then, in a 10-year period, the overall area of strictly protected areas, national parks, and sanctuaries (IUNC I to IV) have been increased from 2.4% to 4.4% (307,246 ha). In 1999, a Forest Code of Georgia was adopted [28], which created different categories of protected forests over an area of 1,113,130 ha (IUCN categories V and VI) [11,29]. As a result of these actions, the total percent of protected areas in Georgia increased permanently [3,6], and today it counts more than 670,978 ha, which is about 10% of the country [4].

Plant genetic resources are vulnerable; they can reduce and disappear, placing the continuity of the species in serious danger. The use and loss of plant genetic resources depend on human intervention; in fact, the population increase, industrialization, and expansion of the agricultural frontier contribute to genetic erosion, and to these are added climate changes that contribute to the modification and/or destruction of centers of genetic diversity [30]. To avoid this loss of plant genetic resources, they must be conserved and used sustainably, and the conservation strategy adopted should be applied while considering the targeted species.

Plant genetic resources can be conserved in their natural habitats (in situ), in conditions different from their natural habitats (ex situ), or in complementarity. Conservation objectives should be defined in terms of logical, scientific, and economic criteria, such as the need for and the value and use of a given species, and the feasibility of preservation [31]. Programs for ex situ conservation of plant genetic resources by different methods, such as seed banks [32–34], in vitro collections [35,36], and cryobanks [37,38], are established in various countries. The main objective is to collect and maintain the genetic diversity in order to ensure its continued availability and to meet the needs of different users.

Despite the extensive in situ and ex situ conservation measures practiced (including the renewal of seed banks, maintenance of field collections, and botanical gardens) less attention is paid to recovery measures. As a consequence, rehabilitation of reducing habitats largely relies on natural restoration processes. In this regard, implementation of in vitro conservation of species in practice can significantly speed up the processes of protection, thus guaranteeing the sustainability of the phytogenetic pool of the country.

3. Micropropagation and In Vitro Approaches to Conservation

Traditionally, in situ conservation implies the continuation of the evolution process in the natural area of existence of species, whereas ex situ conservation is determined as the protection of plants' diversity beyond the natural habitat. It provides a better degree of protection of a gene pool with respect to the in situ conditions; in fact, the natural protected areas are not always safe and are subject to destruction. The ex situ conservation can be considered a complementary process of the in situ conservation, and it provides the safety strategy of 'safe copying' against the extinction of the wilderness. Ex situ conservation has received international recognition after its inclusion in the 'Convention on Biological Diversity' (Article 9) [39]. An approach to ex situ conservation methods like seed storage in seed banks remains the most convenient and low-cost plant conservation means in many countries. Within the scope of the Millennium Seed Bank Partnership

(MSBP) program [40] implemented by many botanical gardens in the world, the ex situ conservation of rare and endemic species has been conducted by the Seed Bank of Georgia since 2006 [41]. Concerning tree species, the preservation of genetic resources is traditionally implemented in seed banks. Indeed, conservation of seeds is an acceptable method in the case of plants with 'normal' seeds, by drying and placing them in low-temperature storage for a long-term preservation. Although the fundamental role of seed banks and clonal collections is undeniable, these traditional approaches to the storage and conservation of tree germplasms are not always acceptable, and have some important limitations and drawbacks. These limitations include: (i) some plants do not produce seeds, and their reproduction is possible only by vegetative reproduction; (ii) some seeds have germinability only during a certain period, or germinate with difficulty (so-called 'recalcitrant seeds'); (iii) some seeds do not store in dry form or low-temperature conditions; (iv) seeds are heterozygotic and are not suitable for obtaining of the genotype of identical type; and (v) seeds of some species quickly decay under the influence of seed pathogens. These drawbacks are especially relevant to numerous forest angiosperms having nonorthodox seeds that quickly lose their viability or are subject to a rapid decrease in germinability during storage [42].

The main value of an in vitro technique for conservation purposes is an innovative approach to the protection of biodiversity. In vitro techniques have a clear role within ex situ conservation strategies, including for trees and endangered species, particularly where it is important to conserve specific genotypes or where normal propagules, such as recalcitrant seeds, may not be suitable for long-term storage. Standard in vitro propagation methods, in vitro seed germination, micropropagation, somatic embryogenesis, zygotic embryo culture, and callus culture systems have been developed successfully for a large number of woody species with basic protocols [43–45]. During the last decades, research efforts have been made to develop and refine in vitro techniques and culture medium for large-scale woody plant multiplication; however, many woody species still remain recalcitrant to in vitro culture and require specific conditions such as medium and hormones for growth and development [44,46].

In vitro conservation, based on plant tissue culture technology, is considered as one of the most efficient methods of ex situ conservation, and it is especially important for the plants propagated by vegetative methods and for nonorthodox seed plant species (e.g., *Acer* spp., *Quercus* spp., chestnut, horse chestnut, and many tropical species) [47–51]. Cell and tissue culture techniques ensure the rapid multiplication and production of plant material under aseptic conditions, allowing the propagation and the preservation of endangered plant species for the short-, medium- and long-term. Up to now, in vitro techniques have been practiced for the conservation of a large number of threatened plants, and became the reliable option for long-term germplasm preservation [51–53]. In vitro conservation involves the use of conventional micropropagation systems, slow growth techniques, and cryopreservation [53]. Collecting efficiency can be significantly increased through the use of in vitro collecting. The first in vitro collecting systems were developed for cocoa (*Theobroma cacao* L.) and coconut (*Cocos nucifera* L.), generating two in vitro collecting methods that were used as a model to develop other protocols [12]. In vitro collecting represents an alternative for rare and endangered species, since usually this material is limited in supply, and seed collection may be restricted.

Plant micropropagation and the establishment of the in vitro collections can be considered the first approaches toward in vitro recovery and in vitro preservation of germplasms [52,54]. However, frequently subcultured material experiences the risk of somaclonal variation, which can compromise the genetic fidelity of the multiplied material, making micropropagation effective only for short-term preservation of germplasms [50,55]. Even so, micropropagation is a fundamental step for the stored explants under in vitro slow-growth conditions or preserved by cooling to very low temperatures (cryopreservation).

Reliable in vitro reproduction methods are important for the creation of alternative sources of rare and endangered plants to prevent the loss of biodiversity, as well as the large-scale production of commercially valuable species, thus decreasing pressure on wilderness. This technique is useful especially when the population number of species is critically low in the wilderness, or when populations of rare plants decrease as a result of their unreasonable harvesting and intensive exploitation of natural resources. The method has prospects for obtaining in vitro conditions of genetically pure elite populations, free from systematic bacterial, fungal, and viral diseases, thus guaranteeing the emergence of quality specimens. At the same time, it provides the large-scale production of young seedlings within a short period and with limited space. For many rare species, the method of tissue culture is the only reliable system of propagation, in comparison with vegetative reproduction or reproduction by seeds [56,57]. However, the results of the cloning are accompanied by plants with an identical genome, which can cause problems in terms of genetic variability. On the contrary, the majority of plants that emerged from seeds are heterozygotic and manifest high variability within the species, which is expressed in differing growth, viability, and reproduction. Hence, to ensure the protection of biodiversity of species, it is expedient to carry out reproduction by traditional methods (by seed or vegetative means) in parallel with the in vitro reproduction.

In vitro propagation can be carried out via organogenesis, a process by which cells and tissues are forced to undergo changes that lead to the production of a unipolar structure—a shoot or root primordium—whose vascular system is often connected to the parent tissues [58]. Using apical meristems (shoot tips) as initial explants for in vitro propagation, virus-free plants can be obtained through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying procedures for the international exchange of germplasms [49]. Moreover, these explants give a higher guarantee for clonal stability [59,60]. The risk of somaclonal variation increases together with the number of subcultures, particularly after the second year of subculturing [50]; therefore, medium-term conservation in slow-growth storage conditions is desirable for maintenance of in vitro collecting, avoiding the handling of plant material, and also preventing its contamination.

Propagation by somatic embryogenesis, on the contrary, leads to the production of a bipolar structure containing a root/shoot axis, with a closed independent vascular system [58]. Plant regeneration via somatic embryogenesis may offer many advantages over organogenesis, such as the feasibility of single-cell origin and the possibilities of automating the large-scale production of embryos in bioreactors and field planting with synthetic seeds [61]. The bipolar nature of embryos allows for direct development into plantlets without the need for the rooting stage required for plant regeneration organogenesis [62]. However, the application of somatic embryogenesis in a wide range of woody plants is limited by genotypic influences, poor germination of somatic embryos, and limited numbers of explants [63].

Major advances in the mass propagation of woody plants have been made over the past decade [64,65]. The number of woody plant species that have been clonally propagated through tissue culture is increasing at a rapid rate, and in fact, most commercially important species have been studied [46,50,51,64]. However, numerous forest trees and bushes are still recalcitrant toward in vitro establishment. Due to the rather variable response of species and cultivars to an in vitro environment, much research still is needed to define the cultural conditions required by woody species. The main problems that may occur during the micropropagation of woody perennials and their successful solutions are: (i) obtaining a suitable vegetation material for isolation of explants may become complicated, and in an optimal case, the material must be in an actively grown phase and must be obtained from seeds, shoots, or a newly reproduced plant; and (ii) a tissue may show low susceptibility with respect to the cultivation process. In such cases, the various stages of micropropagation must be carried out in parallel. These are: isolation, proliferation, and optimization of already-developed shoots, rooting of micro shoots, and acclimatization. However, micropropagation techniques have contributed to the conservation of a large number of endangered plants [52]. For example, the Micropropagation Unit at the Royal

Botanical Garden Kew alone has helped conserve at least 3000 species from around the world [66].

3.1. The Genus Castanea

European chestnut or sweet chestnut (*Castanea sativa* Mill.), representative of the genus *Castanea,* is dominant in mountainous forests of West Georgia (150–1800 m). It occupies the highest percentage of areas covered with forests (approximately 75%). The 'Dendroflora of Caucasus', issued earlier in 1961, lists 14 species within the genus *Castanea;* however, only *Castanea sativa* Mill. occurs in Georgia [67]. Chestnut forests are distributed continuously along the southern slope of the Caucasus Mountains near the Black Sea, and are found in isolated populations on the north side of the Caucasus, at elevations ranging from 200 to 1300 m [68]. Chestnut-dominated forests comprise only a few percent of total forest cover in the Caucasus Biosphere Preserve, and usually occur in mountain valleys or coves with deep brown soil. *Castanea sativa* forests are developed in both West and East Georgia, but to the west of the country, they occupy larger areas. In some localities, pure stands of *C. sativa* can be found, but they mainly occur as a component of oligodominant beech–sweet chestnut and hornbeam–beech–sweet chestnut forests [69]. Chestnut trees generally extend from 100 m (West Georgia) up to 900–1000 m above sea level, having an absolute upper limit of 1400 m in sporadic locations of West and East Georgia [14,15]. At present, due to low self-renewal and diseases, the large massifs of chestnut forests in the Caucasus are on the verge of destruction. Chestnut blight was apparently introduced into the region after 1880, and continues to destroy chestnut forests now. In addition, sweet chestnut in the Caucasus is infested by several fungal and bacterial parasites [70,71]. *Castanea sativa* Mill. is filed in the Red List of Georgia (2006) [9] due to the tendency to decrease the distribution range and habitat fragmentation. The IUCN category for this taxon was evaluated as Vulnerable (VU), according to the IUCN Red List Categories and Criteria (2012) [10]. According to the official IUCN list (2019) [72], *Castanea sativa* has been assessed as Least Concern [73].

Chestnuts are very difficult to propagate by vegetative reproduction using conventional techniques, resulting in difficulties in establishing new plantations. Among the different propagation methods used for chestnuts, grafting and rooting of cuttings give the best results, but are considered very difficult [74]. Like other hardwoods, the in vitro culture techniques developed as an alternative to the conventional propagation appear to be more effective for the mass propagation of chestnut [75,76]. In this sense, much effort is focused on improving the chestnut genotypes, thus increasing both the economic value of fruit and building materials [76,77]. Another target in chestnut micropropagation is the species' resistance to major fungal pathogens such as *Phytophthora* spp. and *Cryphonectria parasitica.*

In the genus *Castanea,* most of the research on micropropagation has been carried out on *Castanea sativa* (European chestnut) [78], *Castanea dentata* (American chestnut) [79,80], and chestnut hybrids [81–83], and there also have been numerous studies to establish the best culture conditions for the important Asian species such as: *Castanea crenata* [84] and *Castanea mollissima* [85]. In vitro propagation methods for the chestnut include the differentiation of adventitious buds [86], somatic embryogenesis [87–89], and the proliferation of axillary buds [90,91], apical buds [92], and nodal segments [93]. Different micropropagation approaches have managed to develop axillary buds and the subsequent rooting of the shoots through using juvenile [94,95] and adult material of this species [96–99], partially focusing on the axillary shoot culture proliferation for short- and medium-term conservation [80,100]. A temporary immersion bioreactor (TIB) has been also suggested as an efficient and economic tool for mass clonal propagation of chestnut [75,101]. This system, with its immersion/dry-period cycle, allows the plants to have greater accessibility to the medium components, allowing greater gains in biomass and a time reduction for propagation; moreover, there are also factors such as ease of handling and the possibility to automate, which would reduce labor costs [102,103].

From the conservation point of view, proliferation of axillary buds is considered to be the most important method [104]. Nevertheless, despite some success in the proliferation and rooting of mature plant material [98], attempts to propagate on a large scale from adult material have not been effective, mainly because the majority of genotypes require specific adjustments to the protocols. It seems the micropropagation of adult trees is more achievable from tissues retaining physiologically adolescent characteristics, such as basal shoots and stump sprouts, or when gradual reinvigoration of adult chestnut is used [105]. In some cases, problems arise in the rooting phase depending on the origin (genotype) of the chestnut [99,106,107]. However, new combinations of adapted modified nutrient media applied in different stages of the propagation process resulted in high rates of rooting, survival, and continuous growth in the field [95,107]. Generally, the genotype and environmental influence are considered as the most important factors in all traits related to multiplication rates in clonal propagation [82,108]. The suggestion was given to obtain the individual analysis while trying to multiply a concrete clone, and to leave aside the results of the total population when multiplying a concrete clone, because the best culture media for one clone are not necessarily the best for other clones. Some authors reported that *Castanea sativa* clones had medium or low multiplication rates in comparison with hybrid clones. During the past years, many studies have been carried out using in vitro culture of *Castanea sativa* [76]. The main research on in vitro propagation and conservation during the last decade are reported in Table 1.

Table 1. Conservation status and in vitro propagation of some threatened woody species of Georgia.

Genera/Species	Family	Red List Georgia	IUCN	Explant Used	References
Betula medwdewii Regel	Betulaceae	VU [1] B1b(i,ii,iii)	DD [2]	Shoot tips, buds	[109–111]
Betula megrelica Regel	Betulaceae	VU B1a	EN [3]	Shoot tips, buds	[109–111]
Betula raddeana Trautv.	Betulaceae	VU B2a(i)b	LC [4]	Shoot tips, buds; Leaves, buds	[109–112]
Castanea sativa Mill.	Fagaceae	VU A2	LC	In vitro nodal explants Buds Embryonic axis Single-node segments Root, nodal, and internodal segments Cotyledons of immature seeds	[95] [99,109] [109,113–115] [98] [116] [89]
Quercus pedunculiflora C. Koch.	Fagaceae	VU B1b(I,ii,iii)	LC	Nodal segments, green and mature acorns Nodal segments, immature and mature acorns	[117,118]

Note: [1] VU, Vulnerable; [2] DD, Data Deficient; [3] EN, Endangered; [4] LC, Least Concern.

Micropropagation of hardwoods for large-scale reproduction and/or for in vitro conservation is not commonly practiced in Georgia. Therefore, in vitro culture research on the Georgian origin of *Castanea sativa* plays a role of high importance, and it currently is carried out in our faculty's Plant Tissue Culture Laboratory. Particularly, the most interesting areas in terms of conservation priority considered are located in Italy, Georgia, and eastern Turkey [108]. Our research demonstrated the high feasibility of the in vitro propagation protocol of *Castanea sativa* using embryonic axes as initial explants (Figure 1a) [109,113].

Plantlets, from embryonic axes cultivated in vitro, showed considerable rates of initiation and rooting rates in woody plant medium (WPM) [119] supplemented with low concentration (0.4 µM) of N6-6-benzylaminopurine (BAP) and 15 µM of indole-3-butyric acid (IBA), which is in agreement with general consideration on superiority of WPM, BAP, and IBA on organogenesis and root formation (Figure 1b). Kinetin was not as effective for multiplication of shoots; however, it promoted the rapid elongation of apical shoots. Many buds sprouted on the initiation media without growth regulators, but the shoots did not elongate (Figure 1c) [109]. Phenol oxidation is another challenge in chestnut micropropagation in initiation stages. Mostly, it occurs with buds turning brown after a few minutes in a nutrient medium. Implementation of an ascorbic acid or polyvinylpyrrolidone (PVP) in the nutrient media did not prevent the browning. Nevertheless, the addition of 0.3% activated charcoal (AC) was effective in eliminating browning and acceleration of rooting (Figure 1d) [120]. Other our research was directed toward the long-term preservation of Castanea sativa, and reported the feasibility of in vitro embryonic axes when applying different cryogenic techniques such as dehydration, PVS2-vitrification, and encapsulation–vitrification followed by 'one-step freezing' in liquid nitrogen [120,121]. The valid protocol developed for the Georgian provenance of Castanea sativa can now be tested on a wide range of chestnut cultivars and hybrid clones to achieve the practical long-term cryopreservation of the Castanea genus germplasm. Hopefully, the work done on the Georgian provenance of Castanea sativa offers a useful platform for further studies toward the improvement of micropropagation strategies applied to the other threatened species of genus Castanea.

Figure 1. Micropropagation of selected woody plants. (**a**) Embryonic axes of *Castanea sativa*; (**b**) rooted *Castanea sativa* shoots after 6 weeks of culture in WPM supplemented with 0.4µM N6-6-benzylaminopurine (BAP) and 15 µM indole-3-butyric acid (IBA); (**c**) *Castanea sativa* bud culture in WPM; (**d**) embryonic axes of *Castanea sativa* cultured in WPM supplemented with 0.3% AC; (**e**) in vitro development of *Quercus hartwissiana* on WPM supplemented with 2.5 µM BAP after 28 days of culture; (**f**) buds of *Betula megrelica*; (**g**) multiplication stage of *Betula* spp. initiated in MS medium supplemented with 4.4 µM BAP; and (**h**) potted plantlets of *Betula* spp. 6 weeks after potting and acclimatization in a greenhouse.

3.2. The Genus Quercus

The genus *Quercus* is one of the most diverse and economically important tree genera within the Fagaceae family, and is widely distributed in temperate and subtropical areas of the Northern hemisphere [122]. Based on large-scale collections and the use of canonical and discriminant analysis, a complete revision of the Caucasian species and the related species of Europe and Asia Minor was made, and a key for the eight recognized species of Caucasian Oaks was presented in [123]. Two species, *Quercus hartwissiana* Stev. and *Quercus macranthera* Fisch. and C.A.Mey. ex Hohen., are currently included in the Red List of Georgia [9]. *Quercus hartwissiana* (vernacular name Colchic oak, or oak of Hartwiss) is a tertiary relict [69]. *Quercus hartwissiana* is an element of Colchic forests. This is a light-demanding species occurring in the lowlands and lower montane zone of West Georgia, together with *Quercus imeretina*, *Quercus iberica,* and other broad-leaved groupings. *Quercus hartwissiana* creates forests in the lowlands and lower montane belts. *Quercus hartwissiana* mainly resides in the western part of Georgia (Abkhazia, Racha-Lechkhumi, Samegrelo, Imereti, Guria, Ajara); to the East, middle Kakheti is the only distribution area. *Quercus hartwissiana* Stev. is included in the Red List of Georgia [9] as Vulnerable (VU) based on the IUCN categories and criteria [10], and it also is listed in the checklist of Vascular Plants of Georgia [124]. This taxon is therefore considered to be facing a high risk of extinction in the wild. *Quercus hartwissiana* is included in IUCN Red List under the status DD (Data Deficient) [125]. *Quercus macranthera* Fisch. and C.A.Mey. ex Hohen., known as high mountain oak or oriental oak, is the oldest in of Southwestern Asia, and has great importance in the study of the flora history [67,69]. *Quercus macranthera* is distributed in almost all high mountain regions of Georgia and mountainous areas of East Georgia at 1700–2400 m a.s.l. In West Georgia, high mountain oak forests are comparatively widely distributed only in the Racha and Zemo Svaneti regions. The total area of mountain oak forests attains approximately 10,000–12,000 ha. Oak forests created by high mountain oak are mostly distributed in the subalpine belt, above 1750–1800 m, although in some localities (slopes of Trialeti range), *Q. macrantehra* comes down to even lower altitudes of 1400–1500 m, and together with *Quercus iberica,* creates the oak forest [126]. *Quercus macranthera* is listed in the Red List of Georgia as Vulnerable (VU) [9].

Like other forest angiosperms, *Quercus* spp. has nonorthodox seeds that are no longer viable or are subject to a rapid decrease in germinability during storage [42]. Propagation by vegetative reproduction is also very difficult for most oaks [127]. Their rooting ability decreases significantly with the age of the oak tree, although grafting, cutting back, and etiolation are usually adopted to improve rooting [95]. Since both in vivo and in vitro methodologies were suggested in the early 1980s for the genus *Quercus* [127], in vitro propagation systems have been further developed for *Q. petraea* [128], *Q. robur* [128,129], Himalayan oaks *Q. leicotrichophora* and *Q. glauca* [130,131], and endangered and rare oak species such as *Q. euboica* [132] and *Q. lusitanica* [133]. Studies on micropropagation through axillary shoot proliferation and somatic embryogenesis in different species have been summarized in several reviews [134,135]. Standardized procedures have been developed for micropropagation of the most important European (*Q. robur*, *Q. petarea*, *Q. suber*) and American (*Q. alba*, *Q. bicolor*, *Q. rubra*) oaks by axillary shoot growth [128,136]. Nevertheless, similar to many woody perennials oaks, their characteristic strong episodic seasonal shoot growth is highly problematic for clonal micropropagation, resulting in the inability to achieve a stabilized shoot multiplication stage [136]. Tissue culture of oak species, especially using mature-phase plant material, is still a challenge, often resulting in low growth and a low survival ratio [136–139], which requires rejuvenation of plant material [140]. Most commonly, micropropagation from shoot tips or nodal explants is an efficient way to produce uniform quality oak clones [141,142]. In most cases, the micropropagation systems are based on the proliferation of axillary shoots, and juvenile seedling material is used [143].

Studies on micropropagation through axillary shoot proliferation in oak species of economic relevance have been carried out in last 25 years [136]. Various culture media were used for *Quercus* spp. micropropagation; Veitez et al. [144] showed that, out of the eight media tested on *Q. robur,* the best results were achieved with Gresshoff and Doy (GD) (1972) medium [145], while MS Murashige and Skoog (1962) (MS) [146] medium showed poorly developed axillary buds, small leaves, and necrosis that spread from the shoot tips to the whole culture, but without basal callus formation [144]. Moreover, the cultures had thick succulent or hyperhydric shoots. However, woody plant medium (WPM) [119] supplemented with BA in the range of 0.44–4.44 µM for shoot proliferation and IBA in the range 0.98–4.9 µM is most commonly used for the micropropagation of many *Quercus* species [127]. The ratios and concentrations of plant growth regulators are greatly varied among the species, even in different regional populations and genotypes of the same species. This is mainly due to the influence of the genotype, and the different responses to micropropagation of oak genotypes are large enough to explain the lack of repeatability in culture establishment, subculture, and rooting [136,147].

However, in general, adventitious shoots and axillary buds have been widely applied to in vitro propagation, and the research is still largely focused on using somatic embryogenesis (SE). In woody plants, SE is widely recognized as the most efficient system of regeneration [77,136,148–150]. Different explants from juvenile and mature plant material were used to establish embryogenic cultures, most frequently using leaf explants from mature trees [134,151] and zygotic embryos [152]. Embryogenic cultures and embryo axes were also used in *Quercus* spp. cryopreservation [153].

Attempts to establish cultures of *Quercus hartwissiana* and *Quercus macranthera* from the field-grown adult trees using axillary buds as explants have been made in our faculty laboratory. The buds collected from the adult maternity trees and cultured in vitro sprouted (Figure 1e), but did not produce fully developed plantlets (unpublished work). From the *Quercus* species included in the Red List of Georgia, only *Q. pedunculiflora* can be found micropropagated from the nodal segments, and immature and mature acorns (Table 1) [117,118].

3.3. The Genus Betula

The birch family (Betulaceae) includes taxa distributed across a large proportion of the northern hemisphere, from Canada in the west to China, Japan, and Siberia in the east. The genus *Betula* is presented in Georgia by five species: (i) *Betula medwediewii* Regel., (ii) *Betula megrelica* Sosn., (iii) *Betula raddeana* Trautv., (iv) *Betula litwinowii* Doluch., and (v) *Betula pendula* Roth. [154,155]. Three of these, *B. medwediewii*, *B. megrelica*, and *B. raddeana*, are included in the Red List of Georgia [9] based on assessments made earlier and according to the requirements of IUCN Red List Categories and Criteria [10], and guidelines for the application of IUCN Red List criteria at regional and national levels [156].

Betula megrelica Sosn., or Megrelian birch, is identified as Endangered (EN) in the IUCN Red List of Threatened Species [72,155]. It is endemic to Georgia (local endemic of West Georgia), belongs to a very rare species, and is often regarded as a synonym to *Betula medwediewii*. *B. megrelica* is gathered on Migaria Mountain, and can spread up to 1800 m a.s.l., with an upper elevation limit of 2800 m a.s.l. It is scattered throughout pine, mixed, or beech forests, and forms a tree line together with sallow (*Salix*) and *Picea orientalis*. In the IUCN Red List of Threatened Species, *Betula raddeana* Trautv. or Radde's birch, has a status of Least Concern (LC) [72,157]. *Betula raddeana* belongs to the relic species; it is endemic to Caucasus, and occurs in Azerbaijan, Georgia, and Russia. In the Red List of Georgia, *Betula raddeana* is identified as Vulnerable (VU). *Betula medwediewii* Regel, or Medwedew's birch, has not yet been assessed for the IUCN Red List of Threatened Species [158], but it is included in the Catalogue of Life Annual Checklist [159]. *Betula medwedewii* occurs in the western part of the country, in the zone of the subalpine crook-stem forest, limestone slopes, and in the upper and subalpine belt at 1900–2250 m a.s.l., in some cases reaching 2350 m elevations. *B. medwedewii* grows in small groups and creates peculiar complexes with Pontic oak. Like *B. raddeana,* in the Red List of Georgia, *Betula medwedewii* is identified as Vulnerable (VU) [9]. The species is protected in the Kintrishi State Reserve, and is of considerable conservation and evolutionary interest [160]. Overexploitation of wild populations can pose a threat to the Betulaceae family. A 'small fragmented distribution range' is named as a reason for including these species in the Red List [9]. The following factors are considered to be responsible for the declining population of these three species: for *Betula medwediewii*—forest logging and grazing, which damages seedlings; for *Betula megrelica*—its small stands are heavily threatened by excess grazing and habitat destruction; and for *Betula raddeana*—cutting of subalpine birch-woods, and use of twigs for feeding the livestock, mostly horses [160,161].

Several reports are available on the micropropagation of various *Betula* spp. from both juvenile and adult plant material [162]. Earlier studies include clonal propagation from young trees using shoot tips of *B. schmidtii, B. costata,* and *B. davurica* [163]; apical and axillary buds of *B. pendula* [164,165]; and nodal and internodal segments of *B. platyphylla* [166], and *B. pendula* [167]. Adventitious shoot induction from leaf callus, as well as directly on the explant, has also been reported for *B. pendula* [168] and *B. platyfylla* [169]. The feasibility of micropropagation of a mature colchicine-polyploid and irradiation-mutant of *Betula pendula* Roth. was reported by Särkilahti (1988), where tetraploid plantlets were regenerated from cultured apical and axillary buds of a 23-year-old tree [165]. The regenerated plantlets had a tetraploid chromosome set (4n = 56) and an altered leaf morphology typical of colchicine-polyploid birches. The mutant nature of the parent tree was also evident in the light-green color of the leaves of the plantlets.

Like other hardwoods, various species and genotypes of birch respond differently to in vitro conditions and explant sources. Successful plant regeneration has been reported using several basal culture media and growth regulators during the different growth phases. In general, WPM [119] or MS [146] supplemented with N6-6-benzylaminopurine (BAP) in a range 0.5–5 mg/L and 1-Naphthaleneacetic acid (NAA) in a range 0.01–2 mg/L is recommended for shoot induction and multiplication [159]. Low salt concentration in the medium, as well as the addition of low auxin in concentrations of 0.02–0.5 mg/L, improves rooting [156,164].

An interesting study applied the TIB for micropropagation of *B. pubescens* Ehrh and *B. pendula* var. *carelica*, with good results in the proliferation, rooting, and acclimatization for both species [170]. In particular, *B. pendula* explants exhibited a 1.75-fold increase in their multiplication rate with the TIB system compared to a solid culture medium.

Micropropagation of threatened *Betula* species distributed in Georgia was successfully initiated and grown in vitro, resulting in multiple shoot formation and rooting through axillary shoot proliferation derived from mature-phase plant material (Figure 1f) [109] and seedlings grown in vitro (Figure 1g,h) [110,111]. These are the only reports on in vitro propagation of *Betula* spp. listed in the Red List of Georgia (Table 1). Different responses to culture media and concentration of growth regulators, including bud-sprouting and shoot proliferation, were observed in general for all specimens from Georgian *Betula* spp. Proliferated shoots of all *Betula* spp. exhibited rapid elongation either in WPM or MS medium supplemented with 4.4 µM BAP, with an average of 3–4 shoots greater than 2.5–3 cm long. Low concentration (2.5 µM) of BAP was not effective for shoot proliferation. Kinetin at 0.5 or 1.16 µM applied in MS or WPM culture media had a significant effect on shoot elongation, but did not affect the proliferation [109]. In other studies, shoot elongation showed different responses when different concentrations of BAP (2.5, 7.5, or 10 µM) were used in combination with 1 µM gibberellic acid (GA3) [110,111]. Considerable elongation of shoots was achieved in MS medium supplemented with 7.5 µM BA and 1 µM gibberellin for all *Betula* taxa. The fastest shoot growth response was achieved for the *Betula megrelica,* forming shoots consistent in size. Remarkably, the lowest response to proliferation was shown by *B. medwedewii,* while the fastest shoot growth was achieved by *Betula megrelica.* The optimal medium for in vitro rooting of shoots was WPM medium containing 1 µM IBA, which showed the highest (93–98%) rooting frequency and survival rate (95–100%). WPM prevailed over $\frac{1}{2}$ MS for rooting efficiency. Severe problems with contamination occurred when establishing bud cultures initiated from the field-grown adult trees. Many twigs of *Betulaceae* were severely contaminated because of the high levels of micro-organisms existing in nature during the collection period, and disinfestation was unsuccessful.

4. Conclusions

The micropropagation of Georgian indigenous threatened hardwood species is significant, requiring the development of effective in vitro conservation strategies for rare and endangered species. In many countries, it allowed storage in in vitro gene banks of several species, and it can have a crucial role in the conservation of genetic resources of forest ecosystems of Georgia and in global biodiversity. The work done on the threatened woody species of Georgia offers a useful platform for further work, including the application of new biotechnological tools such as temporary immersion systems and cryopreservation, toward the establishment of comprehensive in vitro conservation programs for other plant species of commercial importance that are not possible to propagate and preserve by conventional ways.

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