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Review

Conifer Biotechnology: An Overview

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Abstract: The peculiar characteristics of conifers determine the difficulty of their study and their great importance from various points of view. However, their study faces numerous important scientific, methodological, cultural, economic, social, and legal challenges. This paper presents an approach to several of those challenges and proposes a multidisciplinary scientific perspective that leads to a holistic understanding of conifers from the perspective of the latest technical, computer, and scientific advances. This review highlights the deep connection that all scientific contributions to conifers can have in each other as fully interrelated communicating vessels.

Keywords: biotechnological research in conifers; genomics; cell biology and biochemistry research in conifers; micropropagation techniques; transgenesis; CRISPR/Cas9

1. General Traits, Distribution, and Diversity

Conifers are a group of plants that encompasses the oldest living trees and shrubs on our planet. They have existed for more than 300 million years [1,2], starting from a common ancestor of gymnosperms and angiosperms. Conifers comprise two-thirds of gymnosperms [3] and include species of high forest interest, such as pines, spruces, cypresses, or sequoias [4]. Conifers constitute the largest and most diverse group of gymnosperms (for a complete review, see [5]). Conifers and other gymnosperms were the dominant trees during the Mesozoic Era, which is also known as the Age of the Conifers, although they posteriorly declined and were replaced by angiosperms as the dominant group.

It is very complex to gather all the characteristics of conifers into one definition; they typically have simple needle-shaped or scale-shaped evergreen leaves, even though some deciduous species have been described. In general, conifers are large woody plants with strong apical dominance, although there are shrubby species too. Its main characteristic is to develop cones or strobil es, which are primitive reproductive structures. The highly variable fruiting structures reflect strong selective pressures associated with modes of seed dispersal [5]. Regarding the mating system, conifers are predominantly allogamous. This fact, together with the long-distance pollen dispersal by wind, is responsible for the high gene flow among distant populations leading to the low levels of genetic differentiation between them and the great genetic diversity observed in multiple species [6].

Like many other green plants, they have a diplohaplonic life cycle with the particularity that the dominant diploid sporophyte phase and the annual gametophyte phase occur on the same plant (for a complete revision, see [7]). Three phases can be distinguished in the sporophyte: the juvenile stage, during which they are not reproductively competent; the reproductive onset stage, where cones are only produced in response to certain external stimuli; and the reproductive competence stage, during which cones develop annually under almost any conditions. Most conifers produce woody cones, and seeds are dispersed mainly by wind and gravity, although some species have developed edible fleshy structures for favoring animal seed dispersal.

Citation: Rodríguez, S.M.; Ordás, R.J.; Alvarez, J.M. Conifer Biotechnology: An Overview. *Forests* **2022**, *13*, 1061. https://doi.org/10.3390/f13071061

Academic Editor: Carol Loopstra

Received: 30 May 2022
Accepted: 2 July 2022
Published: 5 July 2022

Conifers are distributed worldwide in a great variety of ecosystems, especially in the boreal and temperate forests of North America and Eurasia. This fact reveals great adaptability to variable environmental conditions, although they are practically absent in deserts, steppes, the Arctic tundra, and some tropical rainforests [5].

Although there are some discrepancies about their taxonomic classification, it is currently accepted based on morphological and molecular studies that conifers include the Class Coniferae or Pinosida, the subclass Pinidae, and three different taxa at the level of order: the Pinales, which only includes the family Pinaceae; the Araucariales, which includes the Araucariaceae and Podocarpaceae families; and the Cupressales, which comprises the Sciadopityaceae, Cupressaceae, and Taxaceae families, although some authors include in this order the two additional families Cephalotaxaceae and Phyllocladaceae [2,8].

The family Pinaceae is the largest in terms of species, as it includes about 232 species distributed in 11 genera (*Abies*, *Cathaya*, *Cedrus*, *Keteleeria*, *Larix*, *Nothotsuga*, *Picea*, *Pinus*, *Pseudolarix*, *Pseudotsuga*, and *Tsuga*). *Pinus* is the largest genus in the family, with about 119 recognized species (The Gymnosperm Database: https://www.conifers.org/) (accessed on 28 May 2022).

Conifers comprise a probably monophyletic group of highly branched trees or shrubs with simple leaves, this being a possible apomorphy of the group. Phylogenetically they are a paraphyletic group with respect to Gnetales (taxon Pinidae, Coniferophyta or others). Different genera such as *Picea* are believed to have originated in North America and then dispersed across the Bering land bridge, showing how the place of origin does not determine the center of diversity [3]. Some results even suggest that, before the division between angiosperms and gymnosperms occurred, a functional specialization had already taken place. The current conifer species map is not yet fully known. It has been reported that the discovery of new genuine conifer species is unsettled, especially in varied and inaccessible ecosystems in the southern hemisphere [2].

Scientific analysis of phylogenetic relationships at various taxonomic levels, mechanisms of evolution at the molecular level of lineages and genomes, and biogeographic dispersal in the development of intercontinental disjunctions or patterns of species diversification [3] are challenges at this point. Understanding the genetic basis of biological processes, evolution, or variation in certain traits is also key to the molecular improvement of specimens [9]. Comparative transcriptomics and genomics of conifer developmental studies are currently being used for these purposes [10], and a database of research related to the study of conifer genera and species [1,2] will be useful for further studies on a more comprehensive and solid basis. These aspects are not unique to conifer studies, and they also have great importance in other fields [11]. However, in conifers, which have such a varied global distribution in disparate environments, and which maintain enormous adaptability and diversification, the challenge is even greater. In conclusion, the use of new technologies is key to scientific innovation in the field of conifer research.

2. Ecological and Economic Importance of Conifers

From an ecological and economic point of view, conifers are the most important group of gymnosperms [3]. Coniferous forests account for 31% of the world's total forest plantation area (FAOSTAT), covering vast areas in the Northern hemisphere, and constitute one of the largest terrestrial carbon sinks and play an important role in climate change mitigation. Conifers, in addition to being widely used for ornamental purposes, have an enormous economic importance, as they are a renewable source of timber, both for the elaboration of manufactured product, and to produce energy (50% of the global timber obtained is supplied by conifers, mainly by *Pinus*, as they generate higher and faster economic yield than angiosperms [2]), paper pulp, and other non-wood products such as resins, natural oils, edible seeds, and products with medical use (for example, the anti-cancer drug Taxol).

It has been reiterated that coniferous forests have a relevant role as carbon sinks [12,13] and are expected to increase their prevalence in the current century [13]. However, soil

respiration in coniferous forest systems also releases greenhouse gases. Understanding gas release will allow scientific and efficient management of coniferous forests and maintaining forest reserves [14] without neglecting care in urban environments [15]. In addition, being able to accurately monitor the intensity of conifer burning on a large scale would be key to a good analysis of climatic and biological changes in ecosystems [16]. The challenge of understanding all the mechanisms governing the plant biomass and organic carbon stocks behavior will subsequently allow for improved soil organic carbon projections [17] and for combating air pollution and its consequences on health and the economy. The results of this research will, in turn, lead to the sustainability and efficiency of biotechnological conifer forestry.

Habitat deterioration, particularly fires, is depleting conifer ecosystems, a situation aggravated by ineffective conservation methods [18]. Certain conifer groups are already seriously threatened with extinction due to this combination of factors [2]. For example, *Araucaria angustifolia* is listed by the International Union for Conservation of Nature as a critically endangered species [18]. Not only this, but the preservation of conifers has a direct impact on other species so that a decrease in their numbers can have repercussions on the global functionality of the ecosystem, causing changes in trophic cascades and even the loss of biodiversity [2]. For all these reasons, the discipline of restoration ecology has arisen, which, due to the growing importance of the above, is requiring great efforts in current research [2]. Knowing the mechanisms that allow the fastest and most efficient reconfiguration of each deteriorated ecosystem, in our case, coniferous forests, will require a very complex diagnostic analysis that will involve different fields: genetic, molecular, tissue, organic, etc. It is a great challenge for the international scientific community to coordinate its efforts in ecological restoration processes, in which bioinformatics and technological innovations will play a crucial role.

Globally, conifers are currently suffering increased mortality due to recent droughts [19,20]. Understanding their ability to adjust their physiology to adapt to drought is another research challenge. Studies in *Pinus sylvestis*, *Pinus halepensis*, or *Pinus pinaster* have shown that in drier and warmer conditions, there is reduced growth and a higher mortality rate, leading to a loss of tree productivity. This can also be used in order to identify forest regions, such as those under Mediterranean conditions, with increased vulnerability so that the responses of different forest ecosystems to ongoing aridification can be monitored [19]. One of the first studies addressing the physiological and biochemical dynamics under extreme drought stress, and subsequent recovery of a gymnosperm species, *Pinus massoniana*, has now been carried out [20]. It is highly likely that, as the climate continues to warm, forest ecosystems will increase their susceptibility to severe drought, leading to community deterioration, death, or reduced growth of individuals. It is, therefore, essential to conduct different studies that assess the response peculiarities of different conifer species [21].

To face the new challenges brought about by climate change and increased pressure due to land use for agriculture, livestock, or urban development, actions must be taken to improve the use and conservation of genetic resources [22]. The seeds have great importance at this point. In this regard, experiments in *Pinus sylvestrys* have been carried out to evaluate the effect of humidity reduction on the quality of seeds obtained from cones in order to observe a faster and more intensive scale opening of cone scales [23]. Research has also been initiated on the performance of seedlings from color-sorted seeds of Scots pine [24]. In addition, the impacts of seed source in pine, and the possibility of selecting provenances to improve growth rates and physical and anatomical wood quality attributes related to tracheid dimensions, have also been analyzed in *Pinus banksiana* [25].

On the other hand, the sustainability of forest ecosystems is seriously altered by pine plantation forestry [13], this being one of the most interesting challenges in the immediate future. Furthermore, understanding ecological relationships in forestry could also prevent the proliferation of diseases in conifers in the face of increasing pests [14] and facilitate research on the suitability of seed and plant production systems, leading to better use of their breeding programs. Due to the increasing wood demand, the establishment of

high-yield plantations with enhanced biomass production is necessary. For that purpose, breeding programs for the identification and selection of superior genotypes with improved production traits, such as growth rate, wood quality, and tolerance to biotic and abiotic stresses, have been developed. However, the domestication of conifer species through traditional genetic improvement techniques is much more difficult than in other crops due to their long generation times and the fact that some traits that are important for production cannot be evaluated during the juvenile stage.

Reforestation and conifer plantations sometimes have to deal with poor, eroded, or degraded soils; it is, therefore, necessary to understand the factors that facilitate the reabsorption of nutrients despite the existence of a shortage or limitation of nutrients [26], which could open interesting perspectives for the articulation of the productive system, and the obtaining of resilient conifers. Soil fertilization and the type of containers used in nurseries have been shown to improve yield and crop quality [27,28]. Future studies should address the different macronutrients present in soils at different depths and their variations in order to understand their relationship with conifer growth and development, which will contribute to sustainable *Cunninghamia lanceolata* plantations [26]. One of the factors that has allowed conifers to survive in suboptimal conditions has been the establishment of symbiotic relationships with fungi [2]. In this way, they manage to increase up to ten thousand times the area that allows them to absorb water and nutrients. The effect of inoculated native ectomycorrhizal strains and compatible fungus–conifer combinations for inoculation in seedling nurseries should be increased, even under real field conditions. This would ensure root colonization before transplanting to the field, thus, reducing seedling mortality due to water stress in *Pinus hartwegii* and *Abies religiosa* [29]. Current studies also address the usefulness of mycorrhizae to biologically control different diseases, such as pine wilt [30]. Alternatively, to improve sustainable pest management in the field of conifer bioprotection, and obtaining safe and highly effective insecticides, numerous benefits of soil fumigation for forest conifer seedling production have been described [31,32]. It is foreseeable that new and promising lines of research in this field will open up in the near future. This holistic study will require multidisciplinary and integrative research in order to encompass all interacting microbial communities [33].

At the same time, it is essential to preserve the genetic diversity of native conifer forests, which is essential to conserve the capacity for stress resilience and adaptation to variable environmental conditions of an ecosystem [12,34]. Thus, sustainable forest management requires the development of efficient breeding programs and alternative strategies for the conservation of conifer's genetic diversity.

A summary of the main ecological problems faced by conifers is listed in Table 1.

Table 1. Summary of the main ecological problems faced by conifers.

Problem	References
Diseases and pests	[14]
Habitat deterioration	[18]
Drought	[19–21]
Climate change and human pressure	[22]

Biotechnology would have a strong impact on conifer breeding, propagation programs, and their adaptation to the environmental settings that support their development, such as soils, light, or temperature. New biotechnological tools, such as genomic, micropropagation, and genetic engineering, would also offer the possibility to overcome these problems. Nevertheless, the application of these techniques requires a better knowledge of conifer biology. For that purpose, it is necessary to better understand the molecular basis of traits and processes that are important for production and adaptation and the development of reliable experimental systems for their study.

3. Genomic Research in Conifers

The availability of full genomes is key to the identification and characterization of gene networks controlling multiple processes, as well as to elucidating the relationship between genotypic and phenotypic diversity in populations. The flowering plant *Arabidopsis thaliana* (*Arabidopsis*), the model plant species par excellence, was the first plant genome sequenced (The Arabidopsis Genome Initiative, 2000). Since then, several angiosperm genomes with high economic importance, as well as model species from other plant groups, have been sequenced. The first tree genome sequenced was the *Populus trichocarpa* (black cottonwood) in 2006 [35].

Conifers are characterized by extraordinarily large genomes [36] with high heterozygosity levels and high repetitive DNA content [37,38]; that is why full genome sequencing of conifers was not technically or economically viable before 2013 (Table 2). The development of next-generation DNA sequencing (NGS) technologies and powerful bioinformatics methods for the assembly and annotation of the genome sequence allowing the obtaining of the full genome and/or transcriptome from several conifer species (for a complete review, see [39,40]).

Table 2. A comparison between the genome size of different species.

Species	Genome Size	Released in
Arabidopsis thaliana	119.1 Mb	2000
Populus thrichocarpa	434.1 Mb	2006
Picea glauca	26.6 Gb	2013
Picea abies	12.0 Gb	2013
Pinus taeda	22.1 Gb	2014
Pinus lambertiana	27.6 Gb	2016
Pseudotsuga menziesii	14.7 Gb	2017

The genome drafts from several conifers such as white spruce (*Picea glauca*) [41], Norway spruce (*Picea abies*) [42], loblolly pine (*Pinus taeda*) [43,44], sugar pine (*Pinus lambertiana* Dougl.) [45], and Douglas fir (*Pseudotsuga menziesii*) [46] are already available (for a complete review, see [39]). *Pinus pinaster* and *P. sylvestris* genomes have also been sequenced. A reference transcriptome was also obtained by RNA sequencing (RNA-seq) in maritime pine (*P. pinaster*) [47] and sugar pine (*P. lambertiana*) [48]. There is also a lot of transcriptome data in public databases, such as CONGENIE (https://congenie.org accessed on 28 May 2022) or Gymnoplaza (https://bioinformatics.psb.ugent.be/plaza/versions/gymno-plaza/ accessed on 28 May 2022).

Annotated transcripts of *Pinus elliotti* using third-generation technologies are key information for phylogenetic research and breeding of other non-referenced species [9]. Diverse algorithms have also been developed to process data related to different processes of plant development, such as caulogenesis and rhizogenesis, which can be extrapolated to conifers in a consistent and compatible way; this has a high impact on the research of naturogenic processes and anthropogenic influences on tree growth and development [49], e.g., to refine selection criteria for germination-competent mature embryos in conifers [4,50].

To promote the reproduction, biodiversity, and conservation of conifers, different comparative genome analyses are carried out with an emphasis on the evolution of key traits. This might help to understand why the genomes of these organisms are so large [42]. This is another pending challenge deeply linked to the development of bioinformatics and new technologies. First, comparative analyses show that the estimated number of coding genes in conifers is similar to or slightly higher than the one from model angiosperms [39]. Furthermore, it has been observed that there is considerable conservation of gene families among seed plants, although there are genes that are unique in conifers, and there are notable differences in relative abundances.

Many important milestones have already been achieved, but there is still a long way to go in the area of genetic information. Their big data pose challenges such as storage, management, integration, security, and confidentiality [51]. Efficient improvements and developments will be needed in the computational methods and technologies used in research. The projection of bioinformatics in conifer science seems undeniable. An important and encouraging factor is that most of these biotechnological innovations are trying to be made accessible to the scientific community in appropriate databases, which, in turn, are key for subsequent studies [52]. One factor that must be taken into account in the immediate future is that these technological resources, in addition to being accessible to researchers globally, must be affordable [38]. Universal and real accessibility (simple and inexpensive) to the latest technological, scientific innovations will give a formidable boost to conifer research in all its facets and, above all, will open up a feedback loop that will allow a constant progression of knowledge. Consolidating and progressing the use of technological innovations is one of the cornerstones of the forthcoming development of conifer research.

4. Breeding Programs and Biotechnological Alternatives

Pines show, in general, a great variation among individuals for productivity of forests traits. This aspect provides an interesting opportunity for the establishment of breeding programs to obtain an increase in yield for these traits. The development of breeding plans should combine three general objectives: (1) Conserve genetic resources in forestry and manage naturally regenerated forests; (2) improve the production in the most productive areas through specific treatments; and (3) define a breeding line oriented to obtain and propagate highly productive genotypes for use in new plantations and forest crops.

Nowadays, pine genetic improvement programs are aimed at increasing productivity. These programs are based on field identification of outstanding specimens, their establishment in clonal banks to evaluate their behavior under different environmental conditions and to select the most productive specimens [53]. Once the best individuals have been determined, their vegetative propagation is planned in order to establish high-production plantations or seed orchards [53]. Vegetative propagation is an optimal method to capture all the genetic gain within a given generation since it allows exploiting all the components of genetic variance (dominant, additive, and epistatic) without the need to carry out crossing and selection procedures [54].

Unfortunately, the conventional breeding of trees is not as straightforward as for herbaceous plants [55]. Trees have long life cycles, are self-incompatible, highly heterozygous, and many relevant traits of interest in conifers cannot be adequately assessed until a mature stage is reached [56]. This makes the fixation of an allele of commercial interest very difficult and time-consuming [57]. Maturation induces changes in the meristem behavior, reducing the propagation potential of the tree [56,58]. For this reason, it would be desirable to develop a more efficient and economic technology to facilitate the propagation of selected adult trees for the establishment of clonal banks and plantations. In vitro cultures can restore this regenerative competence, either through a transient increase in vigor (reinvigoration) or through a rejuvenation that allows the recovery of characteristics of juvenile individuals, such as rapid growth and rooting capacity [58]. Therefore, breeders and biotechnologists should work together and focus on traits that improve productivity, sustainability, and wood quality [59]. Nowadays, forest regeneration after harvest is often left to natural processes, although prompt artificial regeneration with selected genotypes provides the most effective means to increase forest yield [59–61].

Biotechnological tools, such as in vitro asexual propagation, are suitable procedures for mass and commercial clonal production of trees in both coniferous and hardwood species. Micropropagation is the in vitro multiplication and/or regeneration of plant material under aseptic and controlled environmental conditions. Micropropagation techniques are often used successfully in most species. However, it is complex in conifers if tissues from adult individuals are used due to their recalcitrance [62]. If it were possible to use material

from mature trees, the time needed to obtain improved varieties would be reduced [12,63]. This is why their use for forest breeding is currently limited [22], and new avenues are opening up.

However, the success of in vitro culture applied to adult conifers is variable. In most cases, regeneration of new plants from adult material is either impossible or too difficult to be practically applied. In any case, there are species where promising results have been obtained, such as *Taxus mairei* [64], *Larix* [65], and *Pinus pinaster* [66]. Moreover, in the case of *Pinus radiata*, research has led to a practical application, with the establishment of a company that offers micropropagation of adult individuals among its services (The Tree Lab, Rotorua, New Zealand).

There are three micropropagation procedures for regenerating a plant; the production of plantlets via axillary shoots growth with the least somaclonal variation, adventitious shoot induction via direct or indirect organogenesis, and somatic embryogenesis [67]. Organogenesis is the initiation of a unipolar structure, shoots, or roots in response to a treatment or to an appropriate culture conditions. Somatic embryogenesis (SE) can be described as an asexual process where somatic cells develop bipolar structures similar to zygotic embryos to form a whole plant without a vascular connection with the parental tissue.

Micropropagation is carried out by taking small sections of tree tissue called primary explants and growing them under artificial conditions. Then, the different types of explants begin a process of growth stimulation of axillary preformed buds, or morphogenesis that will produce adventitious buds—through de novo organogenesis (DNO)—or somatic embryos—through SE [12,68].

For the clonal propagation of trees considered recalcitrant, factors such as the culture medium, the time of year, and the position of the primary explant on the mother tree (topophysis and cyclophysis) must be considered because they have a great influence on the regeneration response [69,70]. Apart from successful cases, most clonal propagation protocols present difficulties, often in the culture media. Problems may include changes in morphology accompanied by hyperhydricity (previously known as vitrification), lack of elongation, or occurrence of necrosis, poor rooting efficiency, poor regeneration, and excessive phenolic exudation.

The axillary bud multiplication involves the development of the axillary buds. The primary explants are usually isolated from the tip of young shoots that develop axillary bud under the effect of cytokinins. The role of cytokinin is to suppress apical dominance and promote the development of axillary buds. The axillary bud method is often combined with the single node method. A concrete example is the micropropagation protocol for *Juniperus thurifera* L., using microcuts with axillary buds from young shoots. This is relevant as this plant is endangered precisely because of the lack of viable seeds [71]. There have been several reports dealing with in vitro culture of mature conifers in the last twenty years [64–66,72–76].

However, in most cases, plant regeneration from adult trees showed severe problems that limit its practical application. The authors of [77] presented a plant regeneration method for producing clonal plants from mature trees of *P. pinea* via shoot development from winter-dormant buds. The low rooting percentage and the lack of axillary bud proliferation despite the juvenile appearance of the shoots indicate that in vitro culture induced reinvigoration (transient appearance of juvenile characteristics) in the brachyblast meristems rather than the desirable rejuvenation [58]. Nevertheless, the results showed that it is possible to obtain rooted shoots from a mature origin, encouraging further investigation into the elongation and rooting phases of the protocol.

Micropropagation of conifers is usually limited to juvenile materials [69], being the in vitro amplification of progeny common by DNO or SE from seeds selected in seed orchards [78]. Mass vegetative propagation of selected families is a useful adjunct to improve programs based on recurrent and non-recurrent selection [79]. Experimentally, statistical efficiency is increased when treatments are applied to clones instead of families due to the absence of genetic variance within the clones. In breeding, clonal banks are

mainly used in populations with large economic (or ecological) value. The purpose of using clonal propagation is to capitalize on both the time saving of large-scale implementation and the relatively larger genetic gains available through clonal tree improvement programs [80].

De novo organogenesis generally begins using juvenile or embryonic primary explants, embryonic axes, and cotyledons [81–83]. The DNO process consists of four or five different stages: initiation, proliferation, elongation/rooting, and acclimation. During initiation, usually in the presence of cytokinins (CKs), explants acquire morphogenetic competence, cell identity is determined, and meristemoids are induced and differentiated, which later, in the absence of the stimulus, develop shoots [81]. The multiplication phase, carried out with shoots separated from the cotyledonary explants and elongated by sequential subculturing on hormone-free medium and preferably with activated charcoal, led to the production of axillary shoots, which were excised and subcultured. Axillary buds and brachyblasts formed during the elongation phase can be used to produce new shoots [83]. One of the main bottlenecks of this method is the rooting of micro-shoots already developed and preferably elongated; the efficiency of the process is usually not high and depends on the auxin treatment, degree of juvenility of the primary explants used, the species, and even the genotype of the seed [12]. More roots imply a better performance upon outplanting, but the need still exists to understand what effect root system quality plays in long-term growth and development [83]. The goal is that this procedure can be used in full-sib family forestry with high predicted performance for deployment to forestry. The performance of a full-sib family can be predicted either based on the performance of the family itself and/or on the performance of its parents in crosses with other genotypes.

As commented above, superior genotypes can be propagated by vegetative multiplication using in vitro techniques, such as organogenesis [83–86] and somatic embryogenesis [63,87–90], both considered to have greater potential than traditional rooting of cuttings [91]. Somatic embryogenesis enables clonal propagation for forestry and forest research and is a key tool for genetic transformation [92]. It allows the production of plants with known, uniform, and desirable characteristics [62]. In addition, the resulting plants closely resemble those from seed due to the development of zygotic embryos with a strong root–shoot connection [4]. All this has a great impact on species of great economic interest, such as Norway spruce (*Picea abies* L. Karst.) and Scots pine (*Pinus sylvestris* L.), as this would ensure their stable production even in situations of climate stress or biodiversity crisis [93]. Another advantage of the method is that it facilitates automation and amplification, thereby reducing the personnel costs required and increasing the reliability of the entire process as a whole [4]. Somatic embryogenesis offers, in short, significant advantages and reduced difficulties and costs.

Somatic embryogenesis involves the formation of proembryogenic masses (PEM) at the early stage, which later gives rise to plants [68,94]. Such PEMs are usually initiated from immature zygotic embryos but have also been studied from shoot explants and mature embryos in *Picea abies* and *Picea glauca*. The SE technique in conifers is multi-stage: embryogenic cultures are initiated from explants, then somatic embryo induction occurs; later, embryogenic masses proliferate and multiply; then, somatic embryos mature and develop from the previous embryogenic masses. Somatic embryo maturation is a critical process that affects the subsequent germination ability of embryos [12,95]. Maturation and conversion of somatic embryos in plants are two crucial steps that hamper the development of efficient somatic embryogenesis systems.

The successful induction of embryonic tissues and SE depends on the genotypes, explant types, date of seed collection, and the media compositions at each step of production. Phytohormones play a key role in embryo formation, particularly auxins; these enhance regenerative responses in vitro because they facilitate the activation of specific developmental programs, which could also be induced by stress factors (temperature, osmotic stress, starvation, heavy metal ions) or wounding [96].

Somatic embryo maturation in pine species is stimulated by the transfer of proliferating tissue to a medium devoid of auxins and cytokinins and supplemented by abscisic

acid (ABA). The optimum concentration of ABA varies depending on species [97,98] and may differ between two cell lines of the same species [99]. Despite the potential offered by SE and despite the progress made in the last few years, the main bottleneck of the technique continues to be the progression from immature embryogenic cultures into mature cotyledonary embryos able to develop properly into well-growing plants [89].

In conifers, maturation into cotyledonary embryos is stimulated by the exogenous application of abscisic acid (ABA) and osmotic stress through the use of PEG [100] or by reduced water availability through the use of a gelling agent [101]. Changes in the composition of the maturation medium have been reported as a significant improvement in mature embryos in *P. radiata* [98]. In conifers, there is an inverse correlation between maturation capacity and subculture number; in the case of *P. pinaster*, this loss of capacity occurs in less than 10 months [102]. Another problem is the passage from immature embryogenic cultures to mature cotyledonary embryos, their acclimatization, and their conversion into plants [90,103].

An important objective for improvement is to overcome the conversion of PEMs into plants due to low maturation rate, low germination frequencies, and poor quality of somatic embryos [12]. Solutions have been investigated to overcome disadvantages related to the low production rate of somatic embryos, not to mention the reduction in yields that occur at each step during further development up to conversion [68]. Research on the incidence of the initiation environment and the effect on subsequent conversion to somatic seedlings should be further investigated [104].

Another unknown is the knowledge of the mechanisms responsible for periderm establishment and formation, which, despite being so relevant, remain largely unknown [105], or the implications of telomere shortening in explants on the frequency of SE induction [106]. The effect of different types of auxins on the physiological reaction of plant materials during SE has been studied. This is a starting point for further studies on the mechanisms of SE induction [107].

A key solution and challenge have been the automation of the process in order to reduce labor, which is still required in these processes today, and which is costly in areas where conifer forestry is of high relevance [4]. The development of universal protocols for coniferous species is very difficult, as the efficiency of the SE procedure varies greatly, and it remains a challenge [93].

Factors that until recently seemed of little relevance are now showing great promise for advancing research. One example is the case of temperatures during the different stages of the SE of conifers. It has recently been reported that high temperature in SE alters the subsequent stages of the process and the ex vitro behavior of the resulting somatic plants [108].

Another example is the importance of light, its intensity, and spectra on the particular stages of SE. It has recently been reported that somatic embryos germinate differentially under exposure to different light spectra. The differences lie in the shoot, root growth, and their survival [93,109]. Here, too, the origin of responses to different temperatures in SE in conifers will have to be analyzed [110]. The effects of the timing of sample collection, its family components, and the means of induction of embryogenic lines have now been successfully demonstrated [111]. It is a challenge for researchers to permanently question all solutions that might seem definitively adopted only a short time ago.

Somatic embryogenesis technology is usually associated with cryopreservation, which offers an appropriate tool to overcome these problems since all the metabolic and physical processes are arrested and require minimal equipment and maintenance [112]. Additionally, cryopreservation can establish dormancy and help enable massive clonal propagation [18,95]. During the juvenile stage, embryogenic tissues can be stored for long periods of time (up to hundreds of years) in liquid nitrogen containers [93]. This method allows the greatest genetic gains to be made, as cryopreservation enables long-term field trials to be carried out [113], and maintenance costs can be reduced [104,114]. This enables the establishment of field tests to evaluate the different lines during their adult stage. Only the best

performing clones are massively propagated from the cryopreserved stock of embryogenic tissue [115–117]. Thus, more studies developing a cryopreservation protocol that ensures a continuous supply of juvenile mass are desirable.

In addition, when the cultures are maintained for longer durations, the frequency of mutation will be higher in in vitro regenerated plants [118,119]. That is a significant advantage of SE in rapport to DNO, which lacks cryopreservation methods to maintain the long-term juvenility of the material [12,113], contrasting with the possibility of cryopreservation that does occur when using SE [110]. The development of effective cryopreservation protocols and appropriate genetic markers would make DNO as promising as SE. This is another challenge that remains open to the scientific community. An example of this is the vegetative propagation of Norway spruce, formerly using rooted cuttings and more currently by SE [114]. This possibility is key, given the scarcity of high-quality forest regeneration materials, as the flowering of the species is irregular, and there have also been pest problems that hinder seed generation [62].

Somatic embriogenesis has been shown to be very successful in other genera of the Pinaceae family: *Abies*, *Larix*, *Picea*, *Pinus*, and *Pseudotsuga*, and others of the families Cupressaceae, Taxaceae, Cephalotaxaceae, and Araucariaceae [68,120]. Currently, SE has been achieved for almost 30 pine species [111].

Although the SE system has been developed for a large number of conifers, such as spruce or pine, some desirable adult trees with known characteristics cannot be propagated through SE and can only be initiated from juvenile plants [62,69]. Its extensive use in practice is limited as it can only be applied to certain genotypes. Furthermore, a potential link between biotic defense and SE induction recalcitrance has been observed. In addition, a relevant problem is that production management costs are very high in this case, which also hinders its use [93].

In order to provide an efficient and abundant supply of somatic embryos for industrial applications, the molecular mechanisms of SE will need to be further studied [121], and protocols for the efficient induction of embryogenic cell lines will need to be improved [122]. The most pressing challenge is to understand the molecular regulation of embryogenesis in conifers, the knowledge of which remains very limited [123], largely due to the lack of identified embryonic defective mutants [124]. In conclusion, it would be desirable to further develop clear, detailed, and reliable protocols, which are essential for the mechanization and homogenization of experimental systems and, to this end, all mechanisms and resources for vegetative propagation and plant regeneration should be investigated [125].

Somatic embryogenesis is suitable for studying the molecular basis of embryogenesis processes in conifers. Primarily, the model species under study are angiosperms; therefore, the identification of key proteins in the control and regulation of SE processes in conifers is limited for now, and the study of their structural domains will be very relevant for the understanding and monitoring of the process. Much more research is needed on the role of different genes [126,127], particularly homeoboxes, in conifer development [124,128–131].

The molecular basis and signaling events in conifers SE need to be understood [12,132]. In addition, detailed genetic research is needed for conifers because genes involved in SE suppression (such as PICKLE) are unknown [68,133]. In the near future, genetics and transcriptomics research will be boosted by single-cell 'omics' [134].

Every step forward in the SE process will contribute to the final success rate [93]. Improved robotization and automation will even lead to the identification of weak regulatory interactions, the identification of rare intermediate cell states, the understanding of histone modifications or methylation, and many other advances [134]. It is very likely that we will witness, in a few years, a new scenario in research and knowledge of the conifer genome and its underlying mechanisms and an unprecedented advance of knowledge in these fields. We will foreseeably witness a shortening of phases and an improvement in the quality and yield of wood by bringing advances to conventional breeding [135]. There will also be a strengthening in the combined use of methods, as has already occurred in conifers with the combination of SE with reverse genetics as a model for studying the regulation of

embryonic development. If the system is well-coordinated, it can lead to abundant somatic embryos at different stages of development [136].

Genetic engineering (discussed below) has been reported recently as a valuable tool to overcome some tissue culture limitations. The ectopic expression of growth regulator genes may bypass tissue culture-based regeneration and allow direct regeneration in a wide range of species [137–139].

Thanks to advances in molecular research and technology, genomics, cell biology, and biochemistry will also converge [134,140] in the quantitative analysis of the whole genome of individual conifer cells. This convergence will be part of a new era in biotechnological research.

5. Genetic Transformation

Genetic transformation, genetic modification, genetic engineering, or transgenesis is defined as 'the use of recombinant DNA and asexual gene transfer methods to alter the structure or expression of specific genes and traits' (FAO, 2004). The genes from an organism that are inserted into another are called transgenes and have the ability to confer to the latter a particular trait. Often, but not always, the transgene is obtained from a different species than that of the recipient. Successful genetic transformation depends on the stable incorporation of the novel gene in the genome of the recipient, leading to the transmission of the input gene (transgene) to successive generations.

Genetic improvement of conifers by traditional breeding is time-consuming due to the long juvenile phase and genome complexity. The ability to rapidly transfer new traits from one species to another has the potential to enhance traditional tree breeding and improvement since generation times for forest tree species are rather prolonged. Plant transformation technology has become a versatile platform for tree improvement, as well as for studying gene function in plants.

In general, the introduction of new genes via genetic transformation is fully justified if there is a difficulty in transferring a trait from one species or variety to another without the risk of altering the rest of the phenotypic characteristics or an excessive complexity and duration of crossing, backcrossing, and selection programs. It is evident that a large part of these conditions are present in the improvement of species with long reproductive cycles, as well as in species with vegetative propagation, in which there is generally a high degree of heterozygosis, which, associated with the need to maintain the characteristics of the variety unaltered, limit the breeding programs.

Therefore, from the above, we can deduce the possible potential of this methodology in agroforestry and woody species in general, which would perfectly complement existing breeding programs. Genetic engineering methods increase the diversity of genes and germplasm available for incorporation into a given species and reduce the time required to produce new varieties and hybrids [141,142]. Progress in the development of genetic engineering protocols for conifers has been rapid at the end of the last century, and there are numerous reports of conifers that have been transformed using biolistic and *Agrobacterium*-mediated techniques.

Agrobacterium tumefaciens, now named *Rhizobium radiobacter*, is a gram-negative soil bacterium that belongs to the family *Rhizobiaceae* and is the causal agent of crown gall disease (the formation of tumors) in dicots. Tumorigenesis is caused by the insertion of a small segment of DNA (known as the T-DNA, for 'transfer DNA') from a plasmid called Ti (for 'Tmour-inducing'), which is incorporated at a semi-random location into the plant genome (resulting in genetic manipulation of the host). The genes within the T-DNA region responsible for tumorous growth can be removed and replaced by DNA segments of interest. Strains are considered 'disarmed' if they do not contain oncogenes that could be transferred to a plant. This capacity to transfer genes into plants has been used to develop *A. tumefaciens* as a vector for genetic manipulation. *Agrobacterium* transformation has been demonstrated in several conifers [143–147]. *Larix decidua* was the first conifer from which transgenic plants regenerated and transformed with *A. tumefaciens* were obtained [148].

In early transformation studies, conifers appeared to be recalcitrant and less susceptible to *Agrobacterium* infection, and, therefore, direct gene transfer protocols such as polyethylene glycol-based methods, electroporation, and particle bombardment were also developed [149–152].

The biolistic method consists of bombarding competent cells and tissues with metal microparticles, preferably gold, which is less toxic, coated with DNA. Biolistics, also named the gun gen method, can be used for the routine transformation of many conifers. Regenerated transgenic *P. radiata* plants were obtained from bombarded embryogenic calli explants [153]. It has certain disadvantages, such as the integration of many gene copies, which can lead to undesirable effects, such as gene fragmentation or silencing of gene expression [154,155]. *Agrobacterium tumefaciens*-mediated transformation results in transgenic lines with lower transgene copy number compared to particle bombardment but show more stable transgene expression in subsequent generations and fewer cases of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). In general, as mentioned above, the biolistic techniques are estimated to be less efficient than *Agrobacterium*-mediated in the case of conifers.

Modifications introduced and demonstrated in conifers include insect resistance [156,157], herbicide tolerance [158,159], wood pulp efficiency [160,161], stress tolerance [162], and sterility [163]. These technologies that allow the design of modified conifers to produce biochemicals and biomass for specific purposes [164], however, have not yet been commercialized.

6. Genome Editing with CRISPR/Cas9

Functional gene research in gymnosperms lags behind that in angiosperms. As already mentioned, the absence of an efficient transformation system and a genome-wide mutant library, together with the complexity of conifer life cycles, has hindered progress in its plant biology knowledge. Furthermore, the extrapolation of research and data based on angiosperm model systems, such as *Arabidopsis*, to conifers is often less informative and confusing, as gymnosperms and angiosperms started to diverge 300 million years ago [165]. Therefore, other approaches and molecular methodologies suitable for conifer species are needed.

Genome editing is an effective technology for functional gene research and trait improvement and has opened up a promising alternative. To date, various tools have been applied successfully in genome editing, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas); of which, CRISPR/Cas is the most widely used tool owing to its high mutagenesis efficiency and easy application [166].

The CRISPR/Cas system can be categorized into different classes depending on the effector proteins. Among the different CRISPR/Cas systems, CRISPR/Cas9 was the first to be used for plant genome editing [167]. Thanks to this technology, it is possible to generate site-specific mutations and obtain a theoretically very agile route to study processes or improve and introduce traits in conifers [168]. Since it was released in 2012 that CRISPR/Cas9 could be used for targeted genome editing and that it allowed the introduction of targeted modifications at a locus in the genome of any living entity [169], these technologies have boosted the genomics of forest trees and made the scientific determination of gene function possible [170]. In addition, the availability of RNA seq and NGS methods, together with other directions in genome editing, such as CRISPR/Cas9 double cutting, will contribute substantially to shortening the breeding period of conifers [168].

On the other hand, in the last decade, the CRISPR/Cas9 system has been used for both fundamental research and precision breeding [171–173]. Novel traits that are difficult to achieve through breeding, such as resistance to biotic and abiotic stresses [174–176] and sterility [177], can be generated through knockout-mediated trait improvement. Desirable traits can be fine-tuned by generating a range of alleles through genome editing or base editing [178–181].

To date, this technology has already been used in more than 45 genera from 24 plant families, such as *Arabidopsis*, economically relevant crop plants or plants with medical uses [170,182]. Genome editing has also been employed in trees such as poplar and eucalyptus [183–186]. However, scarce applications in gymnosperms have been published, so the use of this technique in conifers remains a challenge [168]. For example, using larch (*Larix gmelinii*) protoplasts, a Cas9 variant without PAM SpRY, has been found to possess genome editing capacity, but no plants were regenerated [187]. An efficient CRISPR/Cas9 system based on SE suitable for conifers has also been published [188].

The possibility of applying the CRISPR/Cas9 technique to conifers to rapidly modify key traits of interest has recently been demonstrated in *Pinus radiata* [168]. Very recently, the pioneering case of targeted mutagenesis using CRISPR/Cas in a conifer species, *Cryptomeria japonica*, has been described, and genome editing studies using the improved vector and producing edited male sterile lines have been announced [189]. However, these publications, while interesting, still showed a high occurrence of chimeras. Based on prior reports, the application of transgenic conifers still requires a lot of development, but the technology is progressing.

A summary of biotechnological alternatives to traditional breeding programs in conifers, their effectiveness, and their emerging problems can be found in Table 3.

Table 3. Biotechnological alternatives to traditional breeding programs in conifers.

Alternative	Effectiveness	Emerging Problems
Organogenesis	High in juvenile explants Difficult in adult material in most cases	Some recalcitrant species Somaclonal variation Rooting
Somatic embryogenesis	High in juvenile explants Difficult in adult material in most cases	Some recalcitrant species Somaclonal variation Maturation and germination are a bottleneck
Genetic transformation (including gene editing)	Insect resistance Herbicide tolerance Wood pulp efficiency Stress tolerance Sterility	Some recalcitrant species Genotype-dependent Chimeras in some cases Gene silencing

The challenges facing genetic modification and genome editing technologies are not only scientific. As mentioned above, genetic modification (GM) offers the opportunity to make transformational changes in shorter time frames but is challenged by current genetically modified organism (GMO) regulations. Legislation and social consideration can be and are, in many cases, barriers as strong as the scientific and technical ones. The important discrepancy is whether the process or the product is focused [190]. Thus far, there is no complete assent on the regulation of gene editing that develop after the current regulatory frameworks were established.

The time and cost of developing and obtaining regulatory approval to commercialize GMOs are usually prohibitive. The global social and regulatory landscape around GM crops is complex, with many different regulatory systems in different countries [191,192]. Several nations, including the United States, Canada, and Argentina, have resolved that gene-editing technologies where the cultivated or commercialized plant does not contain introduced DNA will not be regulated [190,193]. In contrast, the European Union has recently decided that all gene editing methodologies will be regulated in the same way as conventional transgenic organisms [194,195]. Others, such as China and Australia, have not yet decided on their regulatory approach [172].

7. Conclusions

Conifers have always fascinated researchers, but the complexity of their genomes and their peculiar characteristics determine the difficulty of their study. This review tries to make an enunciative approach to some of the innumerable challenges that conifers suggest in current research. It seeks to offer a synthesis of some of the most urgent challenges and refers to some of the latest biotechnological advances from a multidisciplinary perspective.

Author Contributions: All authors contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: We thank all collaborators and former members for their contribution in the knowledge here presented. We apologize to colleagues whose work is not cited in this review owing to space limitations.

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

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