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Review

Portuguese *Castanea sativa* Genetic Resources: Characterization, Productive Challenges and Breeding Efforts

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Abstract: Chestnuts are multipurpose trees that grow mainly in the Northern Hemisphere due to their aptitude for fruit and wood production. These trees are vastly significant for the economy and wildlife. The widespread distribution of these trees demonstrates their genetic adaptability to many environmental conditions. The main varieties of European chestnut (*Castanea sativa* Miller) cultivated in Portugal, their productive challenges and breeding and biotechnological efforts developed over the last decades are described. This paper highlights the efforts focused on the improvement of varieties and rootstocks through selection and hybridization of European chestnut with the Asian species *Castanea crenata* Siebold and Zuccarini and *Castanea mollissima* Blume, which are resistant to ink disease, which have been the foundation of the Portuguese chestnut breeding programs. Breeding and biotechnological efforts developed over the last decades, focused on ink disease and chestnut blight resistance, are described. The potentialities of this research to stimulate the competitiveness of bioeconomy-based knowledge and innovation in the productive chestnut sector is also discussed.

Keywords: bioeconomy; chestnut; *Cryphonectria parasitica*; genetic resources; genetic variability; genomics; *Phytophthora cinnamomi*

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1. Introduction

Chestnuts, genus *Castanea*, are multipurpose trees that grow mainly in the Northern Hemisphere due to their aptitude for fruit and wood production [1,2]. These are vastly significant for the economy and wildlife [1,2]. The name of the genus, *Castanea*, derived from "Kastah", which means "dried fruit, seed" in eastern Asia. It is suspected that chestnuts were introduced in Europe by Greeks, where the Latin word *Castanea* is derived from the Greek "Kastanon" [3,4]. The chestnut is incorporated in the *Fagaceae* family, in the *Castaneoidiae* subfamily and in the genus *Castanea*, which includes *sativa* and 12 other species, all diploid ($2n = 24$) [3]. The species were distributed mainly in three regions of the world: Europe and the Mediterranean basin (*C. sativa* Miller), Asia (China (*C. mollissima* Blume)), North Korea (*C. crenata* Siebold and Zuccarini), Republic of Korea (*C. seguinii* Dode), Japan (*C. davidii* Dode), Vietnam (*C. henryi* Rehder and Wilson), and in North America (*C. dentata* (Marsh.) Borkh, *C. pumila* Miller, *C. floridiana* Ashe, *C. ashei* Sudworth, *C. alnifolia* Nuttal, *C. paucispina* Ashe and *C. azarkensis*) [5,6]. Each species clearly differs from one another in terms of vegetative habits, fruit and wood characteristics, sizes, resistance to biotic and abiotic factors and adaptability. Altogether, these factors have influenced their distribution throughout time.

As result of its dispersal and use in Europe, the species name acquired the epithet *sativa* (derived from the Latin "*sativus*"), which means "cultivated" [4]. The introduction and dispersion of the chestnut tree in Portugal, as in other European countries, was made by the Romans during their colonization [7]. *C. sativa*, which has hundreds of varieties with significant economic potential for fruit production, is one of the most-planted *Castanea* species in Europe [1]. According to official data made available by FAO [8], over the past few years, harvesting and production of chestnuts have expanded substantially around the world.

Although *Castanea* species have adapted to different environmental conditions, their production in Europe has been continuously threatened by biotic and abiotic stresses, presently exacerbated by climate change. To tackle this problem, the scientific community has been focusing on identifying the cellular, molecular and genetic interactions underlying all biotic and abiotic stresses in the chestnut tree [2]. Most efforts on this topic have been toward breeding and biotechnology to address the main chestnut's biotic stresses. Numerous biotic stressors affect *Castanea* species, but the two most damaging pathogens are *Cryphonectria parasitica* (Murr.) Barr. (*CP*) and *Phytophthora cinnamomi* Rands (*PC*) [9,10]. *C. parasitica* causes chestnut blight or chestnut canker, while *P. cinnamomi* causes the ink disease, also known as root rot. Despite the availability of chemical treatments, they have proven to be ineffective and harmful for the environment [9,10]. Biological treatments based on hypovirulence have successfully controlled chestnut blight in some locations in Europe [10]. Other diseases affect chestnuts, such as the leaf spot (*Marssonina ochroleuca*), twig canker (*Cryptodiaporthe castanea*) and chestnut mosaic virus (*ChMV*), although the damage they inflict is not as severe as that from the above-mentioned diseases [9]. The Asian chestnut gall wasp *Dryocosmus kuriphilus* is another pest that caused serious impacts on chestnut production and orchard health, but the introduction of the biological control agent *Torymus sinensis* seems to have had positive results on the issue [10]. Other approaches include, as an example, the use of *Castanea crenata* (Sieb and Zucc), a rootstock for *C. sativa* that created hybrid rootstock plants that are advantageously used today due to their growth and disease resistance [11,12], which will be discussed in more detail in the following sections.

The purpose of this review is to provide a description of the main varieties of chestnut cultivated in Portugal, their productive challenges and a description of the most recent accomplishments, as well as challenges underlying their improvement regarding biotic stresses.

2. Chestnut Varieties in Portugal

Portugal occupies the seventh position worldwide in chestnut production, with a production of 37,876 tons, as result of the expansion of chestnut grove planting areas [8]. Portuguese chestnuts have an enhanced quality recognized nationally and internationally. To protect the specificity of this product, and due to its economic and social importance, protected designations of origin (PDO) (EU Regulation no. 1151/2012, article 5) for Portuguese chestnuts were created. This agrarian policy of the European Union outlines territorial quality products as components of local development, whose primary objective is the improvement and preservation of the genetic heritage of the product, setting guidelines, parameters and rules to distinguish the production and market in the various regions [13,14]. Four PDO for chestnut were established: two of them in Trás-os-Montes ("Castanha da Padrela" and "Castanha da Terra Fria"), one in the region of Portalegre ("Castanha de Marvão-Portalegre") and another in Beira Alta ("Castanha dos Soutos da Lapa") [10]. A schematic representation of the 4 PDOs in Portugal is depicted in Figure 1, highlighting the municipalities included. Each PDO for chestnut production has its own history, predominant varieties and production area. The most popular and representative varieties in Portugal are "Boa ventura", "Judia", "Longal" and "Martaínha", but many more were identified and cultivated, namely "Amarelal", "Aveleira", "Bebim", "Benfeita", "Côta", "Lada", "Lamela", "Negral" and "Trigueira", among others [7,15].

In geographical terms, the main production areas are located in the most mountainous part of the North Centre "Soutos da Lapa" and Northeast region in Trás-os-Montes, which have optimal climatic characteristics to produce chestnuts [4]. Trás-os-Montes is the region with the highest national production, and two PDOs can be found there [15]. The presence of the chestnut tree in Trás-os-Montes is millenary, having been one of the main sources of food in the region where the Padrela PDO is found [16]. The Judia is the principal variety of the "Castanha da Padrela PDO", but contributions from the "Lada", "Negral", "Côta", "Longal" and "Preta" varieties are also included [15]. The geographical production area (production, processing and packaging) is circumscribed to the municipality of Chaves, Murça, Valpaços and Vila Pouca de Aguiar [16]. The typical "Castanha da Padrela PDO" is very versatile and may be in peeled, frozen, candied or syrup form and have some special features, such as striped rind and a good peeling aptitude [16]. Similarly, to the previous PDO, the local rural community of the "Terra Fria PDO" has long depended on the chestnut trees that contribute significantly to the local economy and serve as a source of food [16]. The "Castanha da Terra Fria PDO" includes contribuitions of several varieties, such as the "Amarelal", "Aveleira", "Boa ventura", "Côta", "Judia", "Lamela", "Longal", "Martaínha", "Negral" and "Trigueira", among others. Its production is concentrated in the municipalities of Alfândega da Fé, Bragança, Chaves, Macedo de Cavaleiros, Mirandela, Valpaços and Vimioso e Vinhais [15,16].

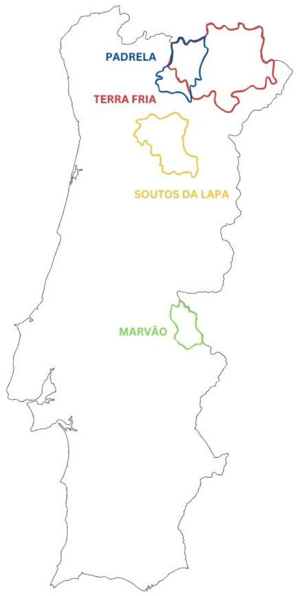

Figure 1. Chestnut protected designations of origin (PDO) in Portugal. In each PDO, the municipalities included in the PDO production area are depicted. Legend: PADRELA PDO (blue) (Murça, Vila Pouca de Aguiar, Chaves and Valpaços municipalities), TERRA FRIA PDO (red) (Alfândega da Fé, Bragança, Chaves, Macedo de Cavaleiros, Mirandela, Valpaços, Vimioso and Vinhais municipalities), SOUTOS DA LAPA PDO (yellow) (Lamego, Trancoso, Tarouca, Tabuaço, Armamar, Aguiar da Beira, S.João da Pesqueira, Moimenta da Beira, Sernancelhe and Penedono municipalities) and MARVÃO PDO (green) (Castelo de Vide, Marvão and Portalegre municipalities).

In the Beira Alta region, where the "Castanha de Soutos da Lapa PDO" is located, the chestnut tree stands out for its numerous applications in construction and handicraft, with the use of its wood and in the food sector, as well as for fruit consumption [16]. The geographical production area of this PDO is limited to the municipalities of Armamar, Aguiar da Beira, Lamego, Moimenta da Beira, Penedono, S. João da Pesqueira, Tabuaço, Tarouca, Trancoso and Sernancelhe [16]. The main varieties are the "Longal" and "Martaínha" [16].

The "Castanha de Marvão-Portalegre PDO", in the inner south of Portugal, includes the municipalities of Marvão, Castelo de Vide and Portalegre [16]. Similarly, the Alentejo chestnut orchards represent a considerable source of subsistence for the populations of this area, which have limited economic resources [16]. The color of the "Castanha de Marvão-Portalegre PDO" ranges from dark dull brown in the "Bárea" variety to lighter brown in the "Clarinha" and "Enxerta" varieties and brilliant reddish-brown in the "Bravo" variety [16]. Additionally, the ability to peel varies from good to very good in the main varieties—"Clarinha", "Enxerta" and "Bravo"—and regular in the "Bárea" variety [16].

3. Ecophysiology and Fruit Characterization

3.1. Plant Growth Requirements

The chestnut (*C. sativa*) tree is a species of great interest due to its ability to be associated with different agricultural and forestry crops systems [5,17]. This species has great variability in morphological and ecological traits, wide biodiversity and the capacity to adapt to different ecosystems due to its high frugality, reproductive and vegetative growth habits, production (fruit size and wood characteristics), adaptability and resistance to biotic and abiotic stresses [5,17]. However, it is vital to understand the most advantageous environmental conditions to grow them to extract the full productive potential of this crop [5,17].

All chestnut varieties are heliophytic, needing sun exposure [18], with hypogeous germination—deciduous, megaphanerophyte and mesothermic—a characteristic that makes them quite suitable for establishment in mountainous areas and valleys [1]. It is considered a species also adapted to semi-arid to extremely humid climates, tolerating some aridity levels if they last no more than two months [19].

The chestnut tree prefers sub-Atlantic climates with temperatures below minus 15 °C, between 400 to 1000 m altitude, on slightly acidic soils [20]. However, it adapts to many regions with different climates [20]. The optimal conditions for the growth and production of the chestnut tree are found in the fresh, light and well-drained soils based on granitic gravel and siliceous sand or from the decomposition of gneisses, schists, volcanic soils, sandstone and alluvium with moderately high molybdenum rates with the pH below 6–6.5 [20]. It also requires at least 180 days of cold exposure and a growth temperature of −6 °C [1]. The root system adjusts, encouraging extensive horizontal root development rather than deep and strong roots, if penetration challenges occur [19].

3.2. Morphological Characterization

Numerous studies have shown that leaves can adapt, in terms of their morphological and photochemical characteristics, in changing environments. A relationship between temperature variations and changes in the anatomy, chemistry and morphology of chestnut varieties' leaves has been described [21]. The European chestnut tree has a long lifespan and displays a tremendous stature (it may grow to a height of 30–35 m) [22,23]. The deciduous leaves of the chestnut tree are characterized by their petiolate morphology, with a crenate-serrated or serrated marginal cut and an oblong-lanceolate limb [22,23]. On the extension of the secondary veins, they also have spatulate-aristate teeth that range in size from 5 to 8 cm in width and 10 to 25 cm in length and are subcordate to subtruncate at the base of the lamina [22,23]. This plant is monoicous, meaning that it has separate male and female flowers on the same plant [24]. The staminate flowers are arranged in glomerules of 5–6 flowers with yellow anthesis, from which emerge 10–20 stamens with yellow-greenish ovoid anthers [1]. The male flowers, usually gathered in catkins, either erect or subpendent, may be about 1 cm in diameter and 15–30 cm long and are inserted near the base of their leaves or in the terminal part of the year's branches [1]. In contrast, the androgynous female flowers are protected by a protective coriaceous envelope of green spinescent bracts with 2–3 flowers, which will give rise to the cupule [25]. A single viable seed-containing ovule (monospermic) or many fertile ovules (polyspermic) from each female flower are used to create fruit [25]. The pollen is viscous, and pollination can be anemophilous or entomophilous [25]. The *C. sativa* species has a single reddish-brown dry pseudo-fruit usually consisting of three ovoid to subglobose achenes with a small and irregular insertion scar at the base [1,12,25]. Aside from the embryo, each seed contains two cotyledons, the part of the fruit that is edible [25]. The following table represents the morphological characterization of the principal chestnut varieties (Table 1).

Table 1. Morphological characterization of the principal Portuguese chestnut varieties. Information in this table has been retrieved from [14].

Name	Port	Cup	Leaves	Catkins	Blooming	Fruit	Maturation
"Aveleira"	Open	Spherical-oval	Petiolate, mucronate, obtuse base, oblong-lanceolate, acute on some leaves, slightly asymmetric and slightly concave, light green on the abaxial surface and glabrous on the adaxial surface	Unisex male astaminates inserted very close to each other, in the axilla of the leaves, and androgynics at the base of the catkin	Early, 2nd week of June	Medium fruit, ovoid to broad ovoid, shiny strong brown, red tint with well-spaced dark streaks. Apex with sericeous star-shaped pubescence. Ventral surface predominantly planoconcave and convex dorsal surface, almost semispherical	Early, fall between the 2nd and 3rd decade of September
"Martaínha"	Semi-erect to open, branch insertion angles of branches 45° to 60°	Round-oval	Petiolate, base predominantly obtuse, oblong-lanceolate, toothed, straight to slightly concave and slightly asymmetric, glabrous only on the adaxial surface, light green on the abaxial surface, shiny medium green on the adaxial surface	Unisex male brachyaminates, inserted in the leaf axil of the shoots and androgynics	Early, 2nd week of June	Medium to large fruit, ovoid, brown, satiny luster, with dark bordeaux dissipated longitudinal stripes. Triangular pubescence sericeous at the apex on the dorsal side. Flat concave on the ventral surface and predominantly convex on the dorsal surface	Early, fall in the 3rd decade of September
"Longal"	Erect to semi-erect, branch insertion angles of branches $\leq 45°$	Pyramidal	Petiolate, obtuse base oblong-lanceolate, dentate, slightly asymmetric and straight to slightly concave, glabrous only on the adaxial surface, light green on the abaxial surface and shiny light green on the adaxial surface	Unisex male mesostaminates, inserted in the leaf axil and androgynics	Average, 4th week of June	Medium to large fruit, ovoid, shiny light brown, reddish tint, with well-defined longitudinal stripes, forming slightly prominent, slightly protruding edges on the integument. Small, silky white pubescence at the apex, involving the stipes up to the stigmas. Planoconvex on the ventral surface and convex on the dorsal surface	Late, fall in the 2nd decade of October.
"Judia"	Erect to semi-erect, big insertion angles, small branches < 45° to more open angles (about 70°)	Spherical-pyramidal	Petiolate, obtuse base, oblong-lanceolate, toothed, slightly asymmetric and slightly concave, glabrous on the adaxial surface, shiny and more faded green on the abaxial surface and medium green on the adaxial surface	Unisex male astaminated with fillets and anthers not visible on the outside of the flower and androgynics	Average, 3rd week of June	Medium fruit, large ovoid, intense brightness brick-brown, lighter and dark brown tint. Apex with reduced sericeous pubescence. Ventral surface concave-plane to slightly convex in some fruits and convex on the dorsal surface	Average, fall in the 2nd decade of October

Table 1. *Cont.*

Name	Port	Cup	Leaves	Catkins	Blooming	Fruit	Maturation
"Côta"	Semi-erect	Pyramidal	Mucronate, lamina obtuse base, very concave, slightly asymmetrical cross-section leaf, whitish on the abaxial surface and medium green on the adaxial surface	Unisex male brachyaminates	Male flowering (1st week of July) and female flowering (2nd week of July)	Small fruit, large ovoid, dark brown	Average, fall between the 1st and 2nd decades of October
"Negral"	Erect	Rounded conic	Petiolate, cordiform limb base, oblong-lanceolate, asymmetrical leave, toothed, slightly asymmetrical cross-section, whitish abaxial surface and dark green adaxial surface	Unisex male brachyaminates	Male flowering (1st week of July) and female flowering (3rd week of July)	Large fruit, globular, dark brown	Average, fall in the 2nd decade of October
"Amarelal"	Semi-erect to open, branch insertion angles of branches 45° to 60°	Pyramidal	Petiolate, obtuse base, oblong-lanceolate, mucronate, slightly asymmetric, slightly concave in cross section. Glabrous light green on the abaxial surface and bright green on the adaxial surface	Unisex male mesostaminates, inserted in the axillae of the leaves and androgynics	Average, occurring in the 2nd/3rd weeks of June	Large to medium fruit, globular, light brown with clearly visible dark and shiny longitudinal well, occasionally with a slight concavity. Silky white pubescence on the apex. Almost flat ventral surface and convex on the dorsal surface	Early, occurring in the last week of September/1st week of October
"Lamela"	Open, the insertion angles of most of the lower branches > 45°	Rounded	Petiolate, limb with acute base, oblong-lanceolate, toothed, symmetrical, lighter green color on the abaxial surface and glabrous medium green on the adaxial surface	Unisex male brachyaminates to mesostaminates inserted in the leaf axils and androgynics	Average	Large, globular, light brown color, dull, near the hilum shows a dark brown color. Apex with reduced sericeous pubescence. Large hilum. Flat concave on ventral side	Average, fall in the 2nd decade of October

3.3. Fruit Nutritional Characterization

Many works characterize the nutritional qualities and technological aptitude of each chestnut variety. Chestnut is a gluten free fruit, mostly composed of complex carbs, but it also contains a considerable number of proteins (with high biological value) and fibers, which help to control insulin response and cholesterol levels [26]. Compared to other nuts and dried fruits, it is a fruit with a low-fat content [26,27] and an excellent source of vitamins, namely B6, vitamin C and folic acid [28]. It also has a relevant quantity of minerals such as calcium, copper, iron, manganese, magnesium, potassium, phosphorus, selenium and zinc [29]. Indeed, the phenolic compounds it contains further contribute to its nutritional importance [30]. Given its nutritional value and beneficial effects on health, it is a fruit that can be chosen as part of a balanced diet throughout the year. In addition, it can be used in both meat meals and sweets [13,31].

3.4. Commercialization and Uses

In Portugal, chestnut trees have a dual purpose, being planted and cultivated for their fruit as well as for their high-quality wood, which makes them a relevant income source for rural communities' economies [13]. The selection of the best varieties and the best trees was made through successive generations, choosing those that produced the best nuts and those that gave the best wood. The wood has a good density and is quite appreciated for furniture, cooperage and basketry [13]. Currently, the chestnut's main markets in Portugal are its commercialization in fresh form for traditional (street roasters) and domestic consumption (boiled, raw or roasted), as well as exportation to the international market in either fresh or frozen form, which has been increasing, particularly to Canada, France, Italy, Spain, Switzerland and the United States [13,32,33]. According to Portuguese data available in [32,33], for July 2022, the trade balance was positive (14,299€ (2010) vs. 20,461€ (2021)), with average export prices higher (24,461€) than import prices (3934€).

4. The *Castanea* Genetic Resources, Genetic Diversity and Biotechnology Based Approaches for Improvement

The foundation of agricultural progress is represented by plant genetic resources, which also serve as a genetic adaption reserve that serves as a biodiversity repository for defense against environmental changes [34,35]. Due to environmental disruptions, intense parasite infestations and cultivation in recent decades, the genetic variability of the chestnut tree has been significantly diminished in both natural populations and cultivated stands [34,35]. When handled properly, the plant genetic resources supply the raw material that results in new and improved varieties and are an unreplaceable source of features including greater yields, environmental adaptation and pest and disease resistance [34,35]. A huge number of genetic resources [36] has been developed for the American chestnut (*Castanea dentata* L.) and the Chinese chestnut (*Castanea mollissima*) throughout several research projects in the scope of the American Chestnut Foundation [37]. Importantly, the genome sequence for these two species is already available at Phytozome [38]. These resources were a relevant basis for comparative genetic and genomic studies in *C. sativa*, supporting the development of marker-assisted breeding programs for this species [38].

In situ and ex situ gene bank repositories play a major role in the conservation of genetic resources. Some studies about the *Castanea* species highlight the value of cryopreservation as an approach to conserve genetic resources [39,40]. Throughout cryopreservation, several plant materials/explants, such as recalcitrant seeds, somatic embryos, cell lines, genetically transformed material and vegetatively propagated species, could be safely and long-term conserved [39]. These repositories include the collection and cataloguing of germplasm information, identification, application, description of accessions, molecular research and preservation as part of the backing-up strategy for germplasm [40]. According to Costa [41], Columbano Taveira Fernandes, a member of Vieira Natividade's team, pioneered the controlled hybridization of chestnut trees in Portugal in the 1950s at Alcobaça. Consequently, a collection of clones that Columbano Fernandes identified has been handpicked and was made available at the Polytechnic Institute of Bragança's Escola Superior Agrária (ESA-IPB). Both Centro Nacional de Sementes Florestais (CENASEF, Amarante) and the University of Trás-os-Montes and Alto Douro (UTAD, Vila Real) received the same collection as a gift from IPB [41]. A collection of clones of the "Aveleira", "Bária", "Colarinha", "Côta", "Judia", "Lada", "Longal", "Martaínha", "Negral" and "Verdeal" varieties are maintained at the Agricultural Colony by Martim Rei. Clones of the "Martaínha", "Longal", "Judia" and "Verdeal" varieties and the hybrid "Marigoule" are maintained at the Agricultural Station of Viseu, while "Amarelal", "Martaínha" and "Longal" varieties are maintained at the "Quinta de Sergude" [14].

4.1. Intra- and Inter-Varietal Variability

The genetic variability of the regional chestnut varieties differs greatly, with certain varieties being more stable than others [14]. The results of a preliminary study using single sequence repeats (SSR) has been successfully used for typing Portuguese traditional chestnut varieties [42]. These results evidenced a low genetic variability among the varieties, explained to some extent by reduced variability for fruit orchard populations compared to the populations explored for wood or mixed purposes [42]. Despite being mainly propagated by grafting, Portuguese chestnut varieties are polyclonal, which means that a single variety might have many genotypes, indicating the existence of multiple clones of the same variety, possibly due to mutations and cross-pollination between varieties [14]. According to the literature, there are varieties with highly repeated genotypes that may thus be regarded as the primary clone from which additional clones within the same variety have been descended or even having given rise to other varieties through seed [14]. The genetic variability and stability of the varieties are directly related to the common practice of exchanging plant material for grafting [14]. This practice is regularly used in the Northern regions for production purposes [14].

Intra- and inter-varietal diversity studies have been conducted in Portuguese chestnut populations. Dinis et al. [43] used SSR markers to assess the heterogeneity of the genotype of the cultivar "Judia" in Trás-os-Montes, and they describe some intra-varietal differences within the studied accessions. In another approach, seven different types of isoenzyme markers were utilized by Pereira and colleagues [44], allowing for the differentiation of 32 genotype classes in Portuguese varieties. Each class has a matching cultivar or varieties, which have various names (synonymy) and may be genetically extremely similar or genetically different and have the same name but belong to separate classes (homonymy) [44].

According to [42] and [44]'s molecular characterization investigations of Portuguese chestnut varieties, a heteronymy between the varieties "Martaínha" and "Verdeal" can exists, which is supported by the 82% similarity value observed between the two varieties. The varieties "Longal" and "Martaínha" are also mentioned in these articles as being genotypically unstable, meaning that they exhibit high intra-varietal variability. The sets of varieties that emphasize this quality can only be alternative names for the same genotype:

"Cancela" (Lamego) and "Negral" (Valpaços); "Cota" (Murça) and "Negral" (Moimenta da Beira); "Cota" (Valpaços) and "Demanda" (Tarouca); "Lamela" (Vinhais) and "Pelada" (Moimenta da Beira); "Pelada" (Boticas) and "Redonda" (Montalegre); "Riscal" and "Verdeal" (from Armamar).

4.2. Portuguese Breeding Programs and Candidate Gene Identification

Chestnut breeding in Portugal has been focused on the improvement of varieties and rootstocks through selection and hybridization with Asian species resistant to ink disease. Interspecific crosses between the European chestnut and the Asian species of *C. crenata* and *C. mollissima* have been the foundation of the Portuguese chestnut breeding programs [45]. The first interspecific crosses in Portugal were made in 1948 by Bernardino Gomes using *C. crenata* (Tamba variety) as the pollen donor [46–48]. At the University Trás-os-Montes e Alto Douro, in the 1990s, Professor Lopes Gomes launched a breeding program that resulted in the development of 53 genotypes resistant to ink disease [49,50].

One of the most well-documented outcomes of this breeding program was the selection of the COLUTAD (COLumbano + UTAD), a hybrid between *Castanea sativa* × *Castanea crenata* found resistant to *PC* and broadly used as rootstock [49].

Another breeding program was started more recently by Costa et al. [50], focused on development of *C. sativa* × *C. crenata* and *C. sativa* × *C. mollissima* hybrids. Four F1 hybrids were chosen for extensive propagation according to their potential to proliferate and root in vitro, ink disease resistance levels and field development [51–53]. The selection process began with the identification of genotypes, in vitro establishment, large-scale propagation and characterization of molecular basis behind susceptible and resistant genotypes [45,54]. The elucidation of the genetic basis and molecular mechanism of *P. cinnamomi* resistance is a need for the implementation of marker-assisted selection in the breeding programs for resistance, since they will allow the selection of molecular markers and candidate genes to support breeding purposes. Costa et al. [50] performed a DNA marker:trait association analysis to identify quantitative trait loci (QTLs) related to ink disease and also to identify putative resistance genes to *P. cinnamomi* using a transcriptomic approach. Since the genome of *C. sativa* is still not available, the authors took advantage of the genetic resources developed and genome sequence available for *C. molissima.* The genetic linkage map and QTLs developed in an interspecific cross between *C. sativa* and *C. crenata* by Santos et al. [51] constituted the first effort to map genomic regions (QTLs) associated with *P. cinnamomi* resistance. Serrazina et al. [55] compared the root transcriptome of the susceptible species *C. sativa* and the resistant species *C. crenata* after *P. cinnamomi* inoculation in an approach to elucidate chestnut defense mechanisms. These results evidenced that the *C. crenata* response triggered more relevant changes in expressed genes related with biotic stress upon pathogen inoculation than the same situation in *C. sativa*, suggesting that despite both species recognizing the pathogen attack, the resistant species (*C. crenata*) may involve more genes in the defense response than the susceptible species (*C. sativa*) [55].

Two distinct strategies, genetic mapping and transcriptomics, were combined to better understand the molecular and genetic pathways and to find molecular markers that enable the early selection of resistant genotypes from the ongoing breeding program. Indeed, the genetic linkage maps created allowed the identification of regions of the genome associated with resistance (Table 2) [45].

Table 2. Summary of efforts to control *Cryphonectria parasitica* (*CP*) and *Phytophthora cinnamomi* (*PC*) using mapping and identification of Quantitative Trait Loci (QTL) in *Castanea*.

Plant Material	**Approach for Improvement**	**Resources**	**Description**	**References**
C. molissima × *C. dentata* F2 hybrids	Disease resistance (chestnut blight)	Study genetic architecture of *CP* resistance; future MAS	3 QTLs: *Cbr1* (LG B), *Cbr2* (LG F), *Cbr3* (LG G)	[56]
BC1, BC4 *C. dentata* × *C. dentata molíssima* " Nanking" and "Mahogany"	Disease resistance (chestnut blight and ink disease)	Study genetic architecture of *CP* and *PC* resistance; future MAS	1 QTL: LG E	[57]
BC1F1 *BC3F1* *C. dentata* × *C. dentata molíssima* " Nanking" and "Mahogany"	Disease resistance (chestnut blight and ink disease)	Study genetic architecture of *CP* and *PC* resistance; future MAS	22 QTLs: hb52208, hb39959 (LG A), nk12394 (LG C), h25723, h54539, hb54410, jb79599, jb32342, jb13258, nk29352, nk35044, nk19473 (LG E), h31744, hb7824, hb27106 (LG K)	[58]

Table 2. *Cont.*

Plant Material	Approach for Improvement	Resources	Description	References
C. sativa × *C. crenata* F1 hybrids	Disease resistance (ink disease)	Study genetic architecture of *PC* resistance; future MAS	17 QTLs: CC_3129_774, CmSNP00773E, CC_48142_849_ CmSNP00522E, AC_32934_470, AC_36335_960 (LG E), CC_46475_1222, CC_6279_2669, AC_14650_453 (LG K)	[59]

Legend: *CP* (*Cryphonectria parasitica*); MAS (marker-assisted selection); *PC* (*Phytophthora cinnamomi)*; QTL (quantitative trait loci); LG (linkage groups)

4.3. The Potential of Genetic Transformation and Genome Editing as a Tool for Pathogen Control

Genetic transformation and breeding have been combined by researchers due to their ability to speed up restoration when compared to standard backcross breeding. Chestnut genetic transformation systems have made tremendous advances, and, today, it is possible to evaluate genes for their capacity to confer pathogen resistance. In the 1990s, Carraway et al. [60] carried out the first attempt on *Castanea* spp. genetic transformation through embryogenic regeneration systems using microprojectile bombardment; however, they only obtained transgenic *calli*. Later, Seabra and Pais [61] successfully transformed and regenerated *C. sativa* shoots expressing the *uidA* gene encoding for the β-*GLUCURONIDASE* by *Agrobacterium*-mediated transformation and regeneration. Nevertheless, and despite many woody crops, the regeneration of transformed plants and their ex vitro acclimation remains a challenge to be addressed.

Since then, chestnut researchers have been dedicated to improving genetic *Agrobacterium*-mediated transformation of this recalcitrant species. The method involves co-culturing somatic embryos with liquid *Agrobacterium* suspension, while still in semi-solid multiplication media, and micropropagation of the plants. Several works were published on the *Agrobacterium*-mediated transformation in *C. sativa* with pathogen resistance genes as genes of interest, either as an attempt to validate gene function or modulate their resistance levels. Nowadays, cisgenes are the focus of increased investigation [2]. Corredoira et al. [62,63] obtained cisgene overexpressing lines with a thaumatin-like protein (*CsTL1*) gene and an *endochitinase* gene (*CsCH3*) (Table 3).

Table 3. Genetic transformation studies performed in European and American chestnuts with the goal of developing pathogen control strategies.

Explant	Gene of Interest	Gene Function	Target Pathogen	References
C. sativa somatic embryos	*CsTL1*	Thaumatin-like protein; promotes osmotic rupture in the pathogen	*Phytophthora cinnamomi*	[62]
C. sativa somatic embryos	*CsCH3*	Chitinase-like protein; hydrolyses chitin from pathogen's cell wall	*Cryphonectria parasitica*	[63]
C. dentata somatic embryos	*OxO*	Detoxifying enzyme; degrades oxalic acid	*Cryphonectria parasitica*	[64–67]
C. dentata somatic embryos	*Cast_Gnk2-like*	Antifungal	*Phytophthora cinnamomi*	[67]

Legend: *CsTL1* (CYSTATIN-LIKE 1 gene); *CsCH3* (ENDOCHITINASE gene); *OxO* (OXALATE OXIDASE gene); *Cast_Gnk2-like* (GINKBILOBIN-2 HOMOLOGOUS DOMAIN gene).

The functional characterization of genes involved in plant–pathogen interaction now has new directions thanks to genome editing [68]. Two S-genes, *POWDERY MILDEW*

RESISTANCE 4 (*pmr4*) and *DOWNY MILDEW RESISTANCE* 6 (*dmr6*), which are likely candidates for functional validation via *CRISPR/Cas9* knockdown, were identified and chosen by the same research team in *C. sativa* after *PC* and *CP* infection [69]. The potentialities of the genome editing tools and the research on S-genes may enable us to determine if *PC* has been adapted to the vulnerable chestnuts, possibly inducing effector-triggered susceptibility, and how it is interfering with their immunity [69].

5. Conclusions

Chestnuts are under threat from several biotic pressures, yet numerous initiatives are being developed to overcome the obstacles and conserve this valuable species. In the context of climate change, the prevalence of *Phytophthora cinnamomi* and *Cryphonectria parasitica* infections might increase due to the potential emergence of new strains. Several breeding programs have been implemented to improve chestnut resistance, particularly to ink disease. The next phase of chestnut breeding may involve strengthening resistance to both pathogens and searching for long-lasting resistance. Exploring the pathogen's virulence/avirulence variables specific to chestnut interactions may be beneficial for the future *Castanea*–pathogen study plan. The resistant genotypes, as a result of the successful approach to develop *C. sativa* × *C. crenata* and *C. sativa* × *C. mollissima*, are being mass-propagated using micropropagation and tested in the field conditions under various edaphoclimatic conditions, as well as for graft compatibility with Portuguese varieties for fruit production. Still, to overcome the lack of improved germplasm for plantation in Portugal and Europe, several biotechnology-based or conventional breeding approaches are being tested. Understanding the genetic basis of disease resistance, as well as the identification of candidate genes, will support the development of new tools and genotypes capable of coping with the threating scenarios. The use of modern techniques such as *CRISPR/Cas* systems in trees is opening up new avenues for the functional investigation and characterization of genes. Applying these technical advancements, along with biotic stressors, will enhance our capacity to respond to chestnut challenges and are expected to bring innovation and competitiveness to the productive chestnut sector.

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