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Impact of chestnut 'brown rot' by *Gnomoniopsis castaneae* and mycotoxin producing fungi on fruit production and quality: mitigation measures in open field and post-harvest.

Settore scientifico disciplinare

(**AGR/12 PATOLOGIA VEGETALE**)

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Abstract

Chestnut (*Castanea sativa*, Miller) is a seasonal fruit found in many parts of the world, although most of commercial production is in Europe and Asia. Chestnut rot is a significant problem facing the chestnut industry (Sieber et al., 2007) and the highly perishable nature of the nut and the need of long-lasting storage create significant pre- and post-harvest challenges for industry. The predominant factors which reduce fresh-chestnut quality are microbiological internal decays, which are primarily related to rot and insect damage (Moscetti et al., 2014). The main storage problems with chestnuts are the presence of insect worms (*Cydia splendana* Hb, *C. fagliglandana* Zel. and *Curculio elephas* Gyll.) and fungi development, mainly *Cyboria*, which clackens the flesh, but also *Rhizopus*, *Fusarium*, *Collectotrichum* and *Phomopsis* (Breisch, 1993; Washington et al., 1997). Since 2005, in Italy, France and Switzerland, chestnut growers have noticed an abnormal increase in the number of rotten nuts, locally affecting more than 80% of nuts (Maresi et al., 2013). Most of these nut rots were associated with an emerging fungal pathogen recently described as *Gnomoniopsis castaneae*, an ascomycete belonging to the family of *Gnomoniaceae* (Visentin et al., 2012). It is present most frequently in female flowers during the flowering period, the ascospores infect chestnut trees and particularly the flowers. The fungus then exists as a latent pathogen in reproductive and vegetative tissues, leading to the development of the disease during nut maturity (Shuttleworth & Guest, 2017). Furthemore, a broad number of organisms have been identified colonizing the product after harvest and causing chestnut decay. Among these, *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., *Phomopsis castanea*, *Acrosperia mirabilis* and *Sclerotinia pseudotuberosa* have been repeatedly identified. Improved mold control strategies are required for commercial chestnuts. Consequently, various pre-storage treatments have been adopted to reduce the likelihood of mold contamination, such as warm and cold baths (or "water curing"), which were developed in Italy during the 1930s. Thermo-hydrotherapy prior to cold storage, frequently paired with floating, is nowadays the standard method used for its proven ability to control insect infestations without influencing nut quality (Sieber et al., 2007). This treatment consists of placing nuts in steel cages and then pouring them in baths containing hot water at 50°C for 45 minutes and then pouring them in cold water to lower the temperature. The temperature for sterilization is the maximum that the nut protein can withstand without the risk of denaturing, while the duration of the treatment is determined according to the survival capacity of the insects. Furthermore, most of the control strategies are applied post-harvest, and little is known about the use of fungicides to reduce nut infection in orchards early in the season, beside the results obtained by Silva-Campos et al. (2022), using conventional fungicides. The research activities carried out during the PhD project aimed to solve the most problematic aspects of the chestnut industry, following the whole chestnut production chain, to find critical phases from the point of view of the healthiness of fruits.

For this reason, the first step was focused on pre-harvest, doing field treatments in a chestnut orchard in the Monti Cimini area to understand when and how brown rot can develop on chestnut trees. The focus

was to block the disease spread during flowering season, using two different treatment's methodologies (crown spray and endotherapy) and innovative products with the minimum impact on the environment (such as Phosphonate salts). Although a still ongoing debate on phosphonate salts use and efficacy in agriculture, they can be considered an optimal fungicide in chestnut orchards because of the low environmental impact when used at the recommended doses, the high translocability and stability, and the multiple mechanisms of actions. Phosphonate salts, and at first Zn-phosphonate, resulted highly effective to protect chestnut fruits from the "brown rot" fungus *G. castaneae*.

In addition, during post-harvest, chestnuts were sampled at different phases of the industrial storage process, to observe how brown rot can be reduced, for instance thanks to thermo-hydrotherapy. In a more detailed way, the present study demonstrated that application of strict conditions at the sterilization phase of chestnut handling can definitively inactivate *G. castaneae* in non-symptomatic and rotting fruits. Sterilization at 50 °C and over was not effective in inactivating the complex of fungal taxa responsible for contamination and development of moulds, many of which produce mycotoxins. The efficacy of the warm bath strictly depends on the quality of the fruit stocks entering the handling plant and the conditions to which they are exposed before the thermo-hydrotherapy. Always in each phase of chestnut handling, beside *G. castaneae* presence on fruits, also fungal population associated with chestnuts fruit, both in pulp and shell, was investigated; the methodology chosen was the Next-Generation Sequencing (NGS) technology and specifically metabarcoding based on Illumina *de novo* sequencing, able to identify the presence of a large number of distinct taxa in a single sample (Ruiz-Gomez et al, 2019). Simultaneously, mycotoxins' contamination was evaluated through ELISA test for Aflatoxins, Ocratoxin A, Fumonisins and T-2/HT-2.

The positive results obtained, albeit preliminary, represent a promising step forward for harvest technologies employed in chestnut handling facilities. However, further studies are needed, both at laboratory and at the industrial plant level, to develop a better understanding of the critical points of fungal and toxin contamination in the chestnut treatment chain. In conclusion, the use of Phosphonate salts, highly effective against the "brown rot" fungus *G. castaneae*, in orchard management may complement the post-harvest processing of fruits by "heat treatments" in water that resulted efficient in completely inactivating *G. castaneae* in fruits. Indeed, the adoption of the appropriate management from the orchard to the processing plant will assure the minimal impact of the disease on fruit stocks for the market. In this sense, these studies, regarding pre-harvest and post-harvest, must be intended as synergic methodologies. An efficient strategy for the control of 'brown rot' must include mitigation protocols applied in the open field, followed by effective physical treatments in the handling plant. This combination of methods will ensure a safe and edible chestnut fruit, free from fungal and mycotoxin contaminations. This work will allow to extend the knowledge on the diversity of the fungal population that colonizes the chestnut fruit and at the same time, along the entire supply chain, to improve the conditions of treatment and

conservation of the chestnut, in order to reduce the risk of contamination. The results obtained will be functional to application as target of control methods with low impact products to limit the population of fungi producer of mycotoxins.

Keywords: *Gnomoniopsis castaneae* G. Tamietti, chestnut brown rot, field treatment, post-harvest, mycotoxin contamination, High Throughput Sequencing (HTS).

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CHAPTER I

State of the art

1.1 Chestnut (*Castanea* spp.)

1.1.1 Geographic distribution

Chestnut (*Castanea* spp.) is a tree with an invaluable historical and cultural heritage and provides an important economic and environmental resource (Bounous et al., 2005). It's one of the most well-liked nut-bearing trees in the Mediterranean region and several Asian nations, such as China, Japan, and Korea (Ridley, 1999). More than 70% of the world's chestnut production is supplied by Europe and Asia, with production currently rising in Australia, New Zealand, and Chile (Fulbright and Mandujano, 2000; Grau and France, 1999; Klinac et al., 1999; Ridley, 1999; Mencarelli, 2001).

Due to their natural dissemination and human cultivation, the genus *Castanea* includes 13 woody species that are extensively scattered throughout both hemispheres (Mellano et al., 2012). The natural distribution of the chestnut includes three major geographical areas: Europe, where the sweet chestnut (*Castanea sativa* Mill.) grows; Asia, where *Castanea crenata* Sieb. and Zucc. (in Japan), *C. mollissima* Bl. (in China and Korea) prospers; North America, original distribution area of *C. dentata* Borkh. Chestnut plantations of both Asian and European species have lately been established throughout Europe, North America, Australia, and New Zealand. Many countries have potential areas with soil and climate suitable to cultivate chestnuts. East Asian production keeps rising, thanks to China and South Korea both establishing additional plantings, expanding their export markets, and continuing to be the world's top producers of chestnuts (Bounous, 2005).

1.1.2 European chestnut (*Castanea sativa* Mill.)

European chestnut, *Castanea sativa* Mill., is a multipurpose tree species in the *Fagaceae* family found in all the nations surrounding the Mediterranean sea. It also grows as enormous, unaltered natural forests in Portugal, Germany, and the southern United Kingdom (Figure 1.1). Sometimes these forests are maintained in good condition to increase fruit production, or chestnut is grown in orchards specifically designed for that purpose.

It is a moderately thermophilic species, typically mesophyte with respect to the temperature and moisture, moderately heliophilous, sensitive to late frosts, demanding in very loose, deep, light, fresh soils rich in potassium and phosphorus; chestnut is considered a calcifuge species as it shuns from soils rich in calcium. It is a typical species able to grow on acidic and basic sulfur substrates (pH ranging from 4.5 to 6.8), yet it can also an excellent producer on carbonatic soil; the chestnut requires fertile soil for healthy growth and fruit production. In order to achieve positive technical-economic results it is

essential to cultivate the chestnut in environments that respect its specific pedoclimatic needs and in conditions that allow suitable cultivation techniques. The chestnut grows between 200 and 1200 m slm and prefers environments characterized by annual temperatures with an average between 8 and 15°C. The crop prefers environments with rainfall generally higher than 600-700 mm/year, distributed evenly throughout the growing season (Nicese and Ferrini, 1999). During flowering and fertilization, the thermal factor and the rain are important, as for the germination of the pollen requires temperatures of 27-30° C. In these stages are harmful prolonged rains that hinder the transport of pollen, reduce fruit set and increase the incidence of vacuum (Bolvansky, 1989).

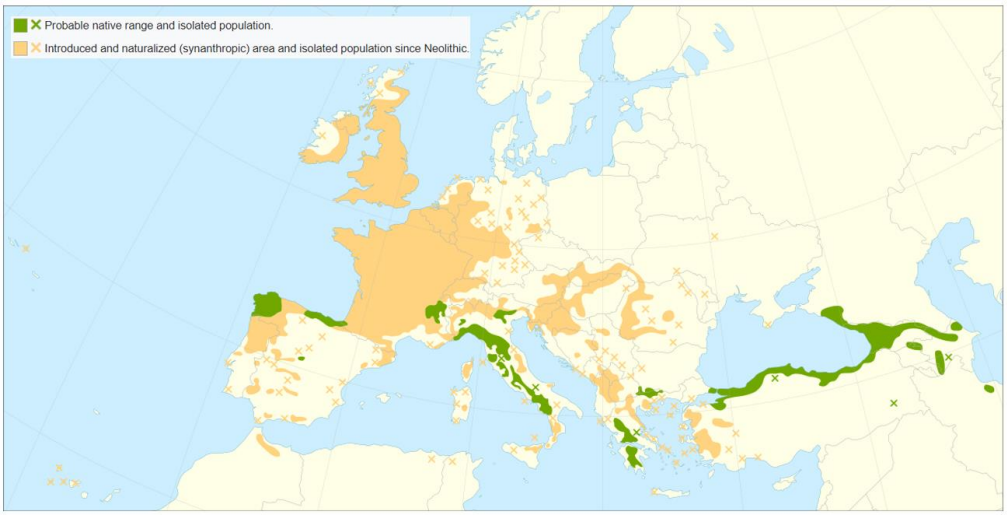

Figure 1.1. Distribution map of *Castanea sativa* (sweet chestnut). Source: Caudullo, G., Welk, E., San-Miguel-Ayanz, J., (2017). Chorological maps for the main European woody species. Data in Brief 12, 662-666.

The success of chestnut is due to its multifunctionality, i.e. it is both a fruit species, a source of timber, and a valuable landscape element, moreover it is also an environmental asset that must be valued and protected. European chestnuts are the most consumed because of their interesting nutritional characteristics. They are rich in carbohydrates (around 40%), mostly starch, and present minerals, vitamins, and appreciable levels of fibre, but low amounts of protein (2–4%) and, unlike typical nuts, low amounts of fat (1.5–5%). They are also an interesting source of essential fatty acids (Barreira et al., 2009). Chestnut tree has represented for centuries an important food resource for mountain populations, integrating the scarce availability of cereals. This led to chestnut cultivation being extended to the biological limits of the species, colonizing even the most difficult environments. Chestnut intake was common in rural places in the past, particularly in Europe and China, and it was seen as a food for the poor. Chestnuts are currently widely consumed and employed as a cooking component with health advantages because of their distinctive nutritional content (De la Montana Miguelez et al., 2004; Borges et al., 2007; Xu, 2005). These fruits are currently widely consumed and used as a cooking ingredient

with health advantages because of their distinctive nutritional content (De la Montana Miguelez et al., 2004; Borges et al., 2007; Xu, 2005). Certainly, today chestnut is no longer a food of sustenance, but continues to play an important role in many agroforestry systems, especially in recent years when chestnut cultivation has once again become an object of interest, probably thanks to the current prices of chestnuts and marrons on national and foreign markets. Due of the significant changes in socioeconomic situations, this species is now acknowledged to play several roles in addition to its original productive one, including cultural, erosion protection, landscape, and tourism-related functions (Bellini, 2004).

The chestnut, a long-time staple food, is now fulfilling the demands of new customers who are tending more and more toward organic food, required for a balanced diet. Chestnuts' dietary and nutritional qualities can make them more useful in our regular diets. The chestnuts are a real, energizing, digestible food low in fat, high in essential fatty acids, moderately but very high in protein quality, low in sodium, and high in potassium; chestnuts are also very useful in the form of gluten-free flour for coeliacs (Bounous et al., 2000).

1.1.3 Chestnut distribution and cultivation in Italy

In Italy, chestnut trees grow throughout the mid-mountain plain of the Apennines and the islands and in the basal plane of the Pre-Alps and the Alps; it is obviously the “leading” species of the phytoclimatic area of *Castanetum*.

Its diffusion is limited by low temperatures and drought: any change in oceanic or temperate-humid climate has favored the diffusion of the chestnut tree while any modification towards aridity restricted this expansion.

Data on the evolution of number of chestnut farms and area invested in Italy from 1970 to 2016 show a drastic decrease in both variables (ISTAT). Between 1970 and 2000 the farms were reduced by 75% and the area dedicated to chestnut trees by 62%. In particular, between 2000 and 2003 there was a phenomenon of restructuring of the cultivated chestnut groves which led to a 50% reduction in the number of farms and a 30% reduction in surfaces. Despite the strong contraction, a hard core of chestnut growers (around 34,000 units) remains in this period, according to the data relating to 2007. The reduction of farms and surfaces continued in the following decade: the data for 2016 indicate 18,000 farms and 43,000 hectares of land planted with chestnut trees. In 2020, ISTAT resumed the survey of production and surfaces invested in fruit chestnut groves. According to the survey relating to 2021, the latest data available, the planted area decreased further, reaching 34,273 ha, equal to a reduction of 20.3% compared to 2016. In 2021, production was just under 43,000 tonnes, with a national average yield of 1.2 tons/ha.

Despite the progressive reduction of chestnut farms and orchards, the production of chestnuts in Italy is still concentrated in 5 regions, with small-medium sized farms: Campania, Calabria, Lazio, Piedmont and Tuscany. According to ISTAT (2021) concerning the survey of surfaces and crop production, Campania is the first region by area invested, with a weight on the national figure of 44% and an increase of 60% compared to 2016, followed by Calabria (20%), Lazio (12%), Tuscany (9%) and Emilia Romagna (7%). The regional production is proportional to the area invested. Also, Italian chestnut exports are strongly concentrated at a territorial level: according to data relating to 2020, 46% of the quantity of exported chestnuts comes from Campania and 15% from Piedmont. In 2020, imports from Campania stood at around 26,000 tons while those from Piedmont around 6,000 tons. On the export side, Campania registers just over 7,000 tons of quantities sold and Piedmont just over 2,000 tons (ISTAT).

Lazio is the fourth Italian region for chestnut production. In this region, chestnut tree is present in over 6,000 farms covering an area of 5,567 ha. Chestnut production is mainly concentrated in the province of Viterbo, where the invested surface has shown a consistent increase, settling at 50%, followed by the provinces of Rieti and Rome, both with 20% of the regional chestnut area (ISTAT, 2000). Monti Cimini area, in the Province of Viterbo, represents one of the most important areas for chestnut cultivation in Italy. This area stands out notably for its annual average production of over 56,000 quintals of chestnuts with a corresponding average value of approximately 7,3 million euros per year. Plant productivity and the prices of chestnuts and marrons help to determine a good profitability of Monti Cimini chestnut farms. Furthermore, these have a better structural set-up than that of the other regional territories involved in this production; their size is, in fact, greater than that found in the chestnut-growing areas of the provinces of Rieti and Rome

From a national point of view, this area occupies a position of great importance as regards the consistent surface area and chestnut production, whether it be fruit or timber (Perone Pacifico et al., 2004). Soil physical and chemical properties are highly favorable to host chestnut forests and plantations, such as the presence of a basically acidic soil of volcanic origin, good fertility and a high rainfall regime (Perone Pacifico et al., 2004). The most widespread fruit varieties are early *marrone* (early or prismatic), *Marrone Fiorentino* (*Viterbese* or *marrone*) and chestnut (*maschia* or *velletrana*) (Perone Pacifico et al., 2004).

Chestnut growing in the province of Viterbo, with its continuous increasing, is in contrast with the national context, in which a progressive contraction of the production and a strong increase in imports are nowadays occurring. This fact can be attributed to the technical characteristics, qualitative and commercial aspects of the Viterbo area. These peculiarities essentially concern three aspects: the efficiency of production techniques, in particular the widespread adoption of mechanized harvesting, the

excellent quality of the productions (both chestnuts and marrons) and the presence of a dynamic business sector. Moreover, the province of Viterbo represents a good area not only for chestnut production but also for product treatment and transformation in post-harvest. Several companies around Monti Cimini use the local production for chestnut-based foodstuff to meet the needs of consumers.

1.2 Fungal community associated to chestnut fruits

The highly perishable nature of chestnuts creates significant postharvest challenges for the industry. The predominant factors which reduce fresh-chestnut quality are microbiological internal decays, which are primarily related to rot and insect damage. Some fungi infect at various stages during fruit development, while others grow during storage (Jermini et al., 2006; Sieber et al., 2007).

Several fungal taxa can colonize chestnut fruits both on tree and after storage, causing severe losses and leading to completely unmarketable chestnuts. Some fungal species are known to cause challenging postharvest decay and disease problems in chestnuts worldwide. Among these, *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Phomopsis castanea*, *Sclerotinia pseudotuberosa* (syn. *Ciboria batschiana, S. batschiana*; anamorphic form *Rhacodiella castanea*, syn. *Myrioconium castanea*) *Rhizopus* spp., *Collectotrichum* spp., *Alternaria* spp., *Trichothecium* spp., *Botrytis* spp., *Fusicoccum* spp., *Phoma* spp. have been repeatedly identified in France, Italy, Australia, Chile and the USA (Wright, 1960; Wells and Payne, 1980; Breisch, 1993; Washington et al., 1997; Xiao-qing et al., 2009; Marinelli et al., 2009; Donis-Gonzalez et al., 2009a). Also, species such as *Acrospeira mirabilis* and *Amphiporthe castanea*, found on chestnut nuts as endophytes or pathogens, were isolated from harvested nuts by Sieber et al. (2007).

Despite damage increases with the amount of fungi causing fruit decay, even a very small infection compromises fruit quality. Therefore, chestnut fungal contamination can cause significant financial losses for farmers, traders, and food handlers. In fact, fungal disease can cause direct loss of product or reduced value due to the lower-quality grade of the lot. Moreover, the presence of fungal infection is a food safety issue due to the potential for subsequent mycotoxin development (Tallada et al., 2011). For these reasons, a detailed investigation on fungal contamination on chestnuts is provided in the next paragraphs.

1.2.1 *Gnomoniopsis castaneae* G. Tamietti

Chestnut growers in several parts of Europe and Australasia have noticed a significant increase in the prevalence of rotten nuts during the mid-2000s (Smith and Agri 2008; Smith and Ogilvy 2008; Gentile et al., 2009; Visentin et al., 2012). The symptoms of spoiled kernels were not entirely consistent with any typical disease of chestnut fruits. In 2012, a new fungal species *Gnomoniopsis castaneae* G. Tamietti (syn *Gnomoniopsis smithogilvyi*) was identified as the outbreak's causative agent (Visentin et

al., 2012; Tamietti, 2016; Shuttleworth, 2012). Since those identifications, *G. castaneae* may currently be recognized as a major emergent plant disease that threatening to the production of chestnut and presents a challenge to scientists and chestnut growers globally.

1.2.1.1 Taxonomy

All around the world, chestnut species have been known to develop fruit rot and mummification. However, brown rot of chestnut fruits is probably the most notorious, worrying chestnut growers in Europe and Oceania since the turn of the twenty-first century. Exactly how many fungi are agents of brown rot, and, of those, how many are currently described is a matter of debate. Fungi identified as *Phoma endogena*, *Phomopsis viterbensis*, *P. endogena*, *P. castanea*, *Dendrostoma castaneum* (syn. *Amphiporthe castanea*), *Gnomonia pascoe* sp. nov. and its anamorph *Discula pascoe* sp. nov., *G. castaneae* and *G. smithogilvyi* have all been described as causal agents of the disease. While *P. castanea* and *A. castanea* (syn. *D. castaneum*) have been identified as genetically distinct (Jaklitsch & Voglmayr, 2019; Maresi et al., 2013), other relationships are less clear due to a lack of genetic material and extant cultures necessary for definitive comparison with newly described fungi.

Nut rots epidemics reported in Europe and Australasia since the mid-2000s were firstly attributed to *Gnomonia pascoe* sp. nov. or to its anamorphic stage *Discula pascoe*, although both binomials were not formally and validly assigned (Smith and Agri, 2008; Smith and Ogilvy, 2008; Gentile et al., 2009; Shuttleworth et al., 2015). The fungus, which was isolated from fruits and young seedlings, produced pycnidium-like fruiting bodies that were ellipsoid in shape and bore single-celled, ovoid-oblong, biguttulate conidia (Gentile et al., 2009).

The fungi responsible for the above epidemics were validly described only in 2012 by two independent laboratories as *Gnomoniopsis castaneae* G. Tamietti sp. nov. (Visentin et al., 2012) and *G. smithogilvyi* L.A. Shuttlew., E.C.Y. Liew & D.I. Guest sp. nov. (Shuttleworth et al., 2012), in Europe and Australasia, respectively. These descriptions appeared in scientific publications within weeks of one another, which has contributed to confusion regarding which was first to be validly published, thus taking naming priority. Later, morphological observations, DNA sequencing and phylogenetic analyses demonstrated the synonymy between the two taxa (Shuttleworth et al., 2015), *G. castaneae* having priority over *G. smithogilvyi* (Tamietti, 2016). *Gnomoniopsis castaneae* is argued to be the first to be validly published as a full digital manuscript in May 2012 (Gonthier et al., 2017; Tamietti, 2016) and is now generally accepted throughout Europe as the valid name and published as such. However, *G. smithogilvyi* was first to appear in print in the June 2012 *Persoonia* publication, while *G. castaneae* appeared in print in the July 2012 *Journal of Plant Pathology*. To the author's knowledge, no official determination regarding legitimacy has been made and each name is frequently used as a synonym of the other. Given the uncertainty surrounding the status of the name, the frequency with which supporters

of each binomial cite contradictory personal communications (Gonthier et al., 2017; Shuttleworth et al., 2015, 2018; Tamietti, 2016) and the argument for first valid publication occurring in May 2012 (Tamietti, 2016), the name *G. castaneae* will be considered the legitimate name and *G. smithogilvyi* its synonym for the purposes of this thesis.

The fungus is known in both the teleomorphic and anamorphic stages, producing ascomata (i.e. perithecia) and conidiomata (i.e. acervuli), respectively (Visentin et al., 2012). Although clearly defined as a species, some ambiguities related to the taxonomy of *G. castaneae* still need to be elucidated. For instance, Meyer et al. (2015) and Ibrahim et al. (2017) listed *Amphiporthe castanea* (Tul. & C. Tul.) M.E. Barr as a synonym of *G. castaneae*. However, *Gnomoniopsis* and *Amphiporthe* are indicated as clearly distinct within the *Gnomoniaceae* according to the list of accepted genera of *Diaporthales* (Senanayake et al., 2017). Preliminary observations suggest that isolates of *A. castanea* display both morphological traits and sequences of the internal transcribed spacers (ITS) of ribosomal DNA identical to those of *G. castaneae*, although the possible synonymy could be unravelled only through more detailed analyses conducted by sequencing and comparing conserved DNA loci between the holotypes of the two species (T. Sieber, ETH Zürich, Switzerland, pers. comm.).

In 2013, Maresi et al. hypothesized that this species may be synonymous with *P. endogena*, suggesting that the emergence of brown rot occurred much earlier and had a larger distribution than previously recognized. If such speculations were proven, the emergence of the nut rots caused by *G. castaneae* might predate the 2000s and the known geographic distribution of the pathogen might be broader. However, further studies are required to confirm or reject the above hypotheses. However, *P. endogena* has been described as displaying α-and β-conidia (Camici, 1948; Ciferri, 1950), which are characteristics of *Phomopsis* spp. (Rosskopf et al., 2000). Such characteristics have never been described for *G. castaneae* isolates. Therefore, they are probably separate species that produce similar symptoms. Because brown rot isolates have historically been identified through morphological analysis of culture and spore characteristics (Camici, 1948; Osmonalieva et al., 2000; Sieber et al., 2007; Spegazzini, 1879; Wadia et al., 2000; Washington et al., 1997), it is probable *G. castaneae* emerged earlier than currently documented and was misidentified as a different, previously described agent following visual assessment. This is supported by the work of Cisterna-Oyarce et al. (2022), which confirmed through molecular identification that the pathogen had been mistakenly identified in Chile as *P. castanea* in the mid-1980s. Unfortunately, unless more preserved specimens are uncovered for use in molecular identification, it is unlikely the question of how long *G. castaneae* has been associated with brown rot will ever truly be resolved.

1.2.1.2 Host range and world distribution

Gnomoniopsis castaneae has been observed on a variety of tree and shrub species in the families Betulaceae, Fagaceae, Oleaceae, and Pinaceae, including both domesticated and wild plants.

All members of the genus *Castanea*, including the American chestnut (*C. dentata*; Campbell et al., 2019), Chinese chestnut (*C. mollissima*; Sakalidis et al., 2019), Japanese chestnut (*C. crenata*; Shuttleworth et al., 2012), and European sweet chestnut (*C. sativa*; Visentin et al., 2012; Chandelier et al., 2018; Magro et al., 2010; Gentile et al., 2009; Meyer et al., 2015; Lione et al., 2015; Vinale et al., 2014) are the main hosts associated with *G. castaneae*, including also their hybrids (Sakalidis et al., 2019; Shuttleworth et al., 2012; Smith and Agri, 2008; Dennert et al., 2015;). Beside *Castanea* spp, *G. castaneae* has been isolated also on other species such as hazelnut (*Corylus avellana* L.; Linaldeddu et al., 2016), manna ash (*Fraxinus ornus* L.; Ibrahim et al., 2017), maritime pine (*Pinus pinaster* Aiton; Fernandez-Conradi, 2017), Turkey oak (*Quercus cerris* L.; Fernandez-Conradi, 2017), common box (*Buxus sempervirens*; Şimşek et al., 2019) and holm oak (*Quercus ilex* L.; Shuttleworth et al., 2012). Therefore, although though *Castanea* species are thought to be *G. castaneae* main hosts, it is obvious that other hardwoods are also impacted by the fungus, and a wider investigation of alternative hosts is necessary. To date, *G. castaneae* has been considered an emerging ubiquitous endophyte and pathogen that is host-genus specific to *Castanea* species (Dobry & Campbell, 2022).

Gnomoniopsis castaneae current geographic distribution includes countries in three continents, including Europe, Asia, and Australasia (Figure 1.2). The origin of the fungus is still unknown, despite numerous explanations has been proposed for its current distribution and potential intra- and intercontinental expansion (Pasche et al., 2016a; Seddaiu et al., 2017; Sillo et al., 2017). Since the first identification of the new species, *G. castaneae* has been reported in eighteen different countries all over the world, beside Italy: New Zealand (Smith and Agri, 2008); Australia (Shuttleworth ang Guest, 2017; Smith and Ogilvyi, 2008); Michigan (Sakalidis et al., 2019); India (Mudasir Ahmad Dar & Mahendra Rai, 2015); Chile (Cisterna-Oyarce et al., 2022; Morales-Rodriguez et al., 2021); Switzerland (Pasche et al., 2016a; Meyer et al., 2017); Spain (Trapiello et al., 2017); Portugal (Coelho and Gouveia, 2021); France (Sillo et al., 2017; Visentin et al., 2012); Belgium (Chandelier et al., 2018); Netherlands (Pvan Rijswick, unpublished); United Kingdom (Lewis et al., 2017); Ireland (O'Loinsigh et al., 2022); Slovenia (EPPO, 2017); Croatia (Ivic and Novak, 2018); Czech Republic (Gonthier & Jankovsky, unpublished); Greece (Tziros, 2018); Turkey (Şimşek et al., 2019; Çakar and Şimşek, 2022).

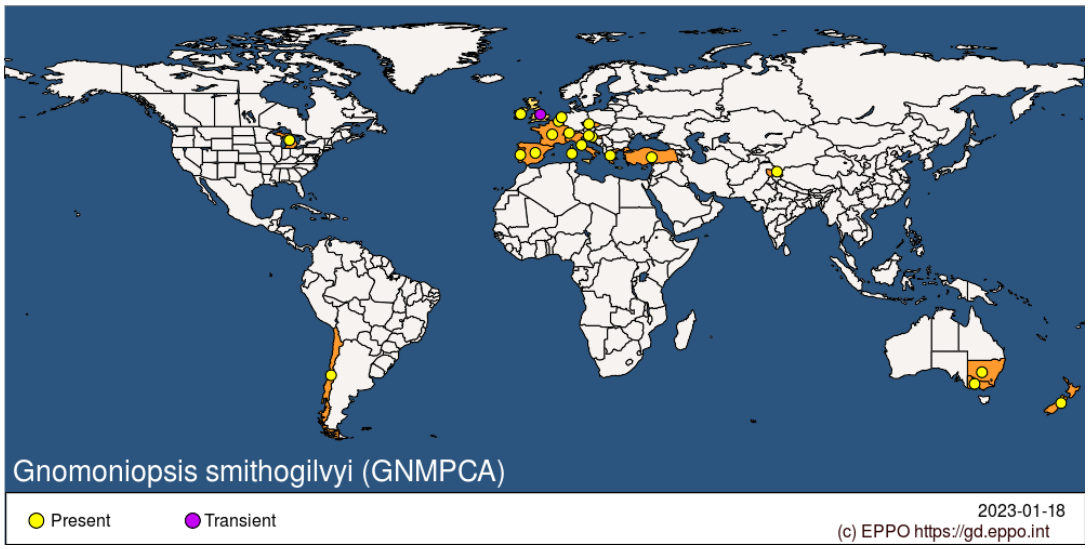

Figure 1.2 *Gnomoniopsis castaneae* world distribution. Source: EPPO Global Database. https://gd.eppo.int/

In Italy, since the early 2000s, *G. castaneae* has been reported as the causal agent of brown rot in different regions: Piedmont (Gentile et al., 2009; Visentin et al., 2012); Trentino and South Tyrol (Maresi et al., 2013; Ibrahim et al., 2017); Ticino (Ibrahim et al., 2017); Aosta Valley (Lione et al., 2015; Lione et al., 2016; Sillo et al., 2017); Lazio (Magro et al., 2010; Vannini et al., 2017; Vannini et al., 2018); Campania (Vinale et al., 2014; Ruocco et al., 2016); Sardinia (Linaldeddu et al., 2016; Seddaiu et al., 2017); Calabria (Bonsignore & Bernardo, 2018).

1.2.1.3 Life cycle and reproduction

Gnomoniopsis castaneae exists as a systemic endophyte, saprophyte, and pathogen, associated with all above-ground tissue of *Castanea* spp. The most accredited hypothesis on *G. castaneae* life cycle is shown in Figure 1.3. The sexual phase (teleomorph) is probably the primary reproductive driver, even if it has been associated with damaging infections of nut and stem tissues together with the asexual phase (anamorph) (Gentile et al., 2009; Pasche et al., 2016; Shuttleworth et al., 2012; Visentin et al., 2012). Sillo et al. (2017) suggested the sexual reproduction as a dominant phase, since the research group identified high differentiation within the populations and minimal linkage disequilibrium, using High-resolution melting analysis (HRM) of isolates obtained from multiple locations in Italy, France, and Switzerland. In Australia, Shuttleworth & Guest (2017) found the aerial ascospores as the primary agent of infection in *Castanea* female floral tissues. However, the asexual phase may still be crucial for the pathogen's ability to reproduce. Repeated haplotypes were found in several areas during HRM analysis, which may be a sign that asexual reproduction occurs there (Sillo et al., 2017). Moreover, a large presence of pycnidia has been observed in association with cankers of grafted scions (Pasche et al., 2016) and naturally infected nuts and Asian Chestnut Gall Wasp galls

(Maresi et al., 2013). These differences in dominant reproductive phases may be the result of geographical and temporal variability, where the circumstances at a certain location at a given moment favour one reproductive phase over the other. It's possible that *Dryocosmus kuriphilus*, the Asian chestnut gall wasp (ACGW), has a significant role on promoting asexual reproduction. The pest has caused severe damage to *Castanea* spp. in most European countries, likely raising the stress levels of the trees and favouring the anamorph to colonize galls and damaged tissues. This has led to the pest being a major concern throughout much of Europe. However, the pest's presence in Australia has not yet been confirmed, which may explain why sexual reproduction predominates there whereas both phases are found in Europe (Dobry and Campbell, 2022). Further research is needed to determine whether environmental factors affect which sexual phase dominates and, if so, which factors have the biggest influence. Such knowledge would help us better understand the pathogen's reproductive modes and would also enable us to predict how those modes might change as environmental conditions continue to evolve.

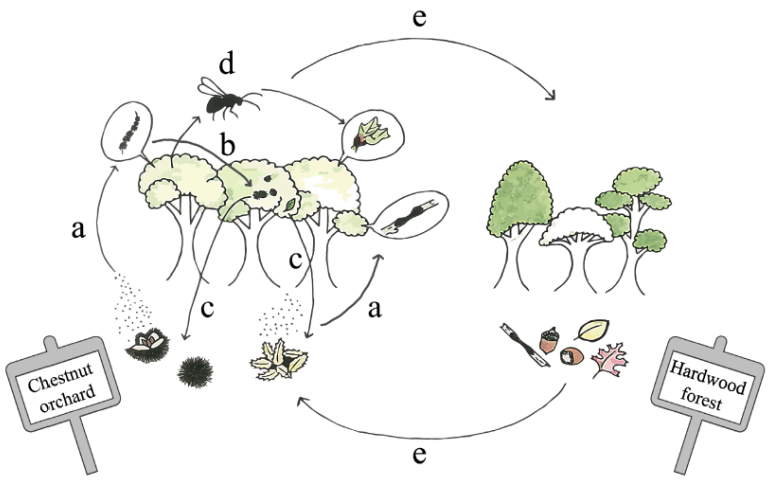

Figure 1.3 Proposed disease cycle of *Gnomoniopsis castaneae*. (a) Release of spores from previous season litter composed of burrs and leaves on the chestnut orchard floor infects developing chestnut flowers and wounded stems and branches via wind transmission. (b) Infected flowers develop into infected burrs. (c) Infected nuts, burrs and leaves fall and become a source of infection for the following season. (d) Spores are carried by the ACGW from infected chestnuts to uninfected chestnuts, resulting in infected galls following oviposition. Infected galls may be a result of the transmission of viable inoculum or oviposition creating a conducive environment for fungal colonization. (e) The probable transmission of spores back and forth between chestnut orchards and other hardwoods, probably through wind transmission. Although hardwoods other than chestnut are known to become infected with *G. castaneae*, it is unknown whether they act as reservoirs for the pathogen. Source: Dobry and Campbell, 2022.

1.2.1.4 Epidemiology

The origin of *G. castaneae* is currently unknown, and the pathogen is frequently believed to be ubiquitous. Although some evidence suggest that it is spreading or being introduced to other areas, it is unclear how its biology works on a local and global scale. It is also unclear if these recently discovered populations resulted from an introduced species or from the emergence caused by climate change. Lione et al. (2019) thoroughly described the epidemiology of the disease, and only one study published since

that time has provided further insight, evidencing the lack of the state of the art on this topic. The earliest descriptions of *G. castaneae* both noted that it overwintered on burrs, and in fruits and branches of *Castanea* species in Italy and Australia (Shuttleworth et al., 2012; Visentin et al., 2012). The assumption that litter on the orchard floor is the primary source of inoculum was made because of this study and an ascospore trapping experiment conducted in Australia (Shuttleworth & Guest, 2017), albeit this has been contested in Europe where management practices already recommend removal of litter after harvest (Vannini et al., 2017). But removal doesn't always happen, especially in traditionally maintained orchards where minimal maintenance is performed (Lione et al., 2021). Furthermore, even if association with litter is hypothesized, it is not yet demonstrated. According to Shuttleworth & Guest (2017), ascospore frequency was highest during sunrise and sunset, and release did not seem to be dependent on rainfall, although it was hypothesized that release would be delayed after a rainfall event. While some study from Australia (Shuttleworth et al., 2013) reveals a positive correlation between rainfall and rot symptoms, data from Europe suggests a bigger impact for drought (Maresi et al., 2013) or temperature (Lione et al., 2015, 2021). Lione et al. (2015), studying the role of different climatic conditions on rot incidence, reported also that warmer temperatures later in the season in Italy were associated with an increase in rot, but there was no association between rainfall and rot frequency. It was pointed out that the absence of correlation between incidence and rainfall does not rule out that the function of drought, which is caused by a few interrelated variables other than a decrease in rainfall, may play a part. A recent spore-trapping experiment that was carried out during a 2-year period at numerous sites validated temperature and wind as the main factors influencing incidence and, once more, showed no association between rot and rainfall or humidity. Year-round inoculum was present, and environmental load varied by site but was positively linked with increased degree days (Lione et al., 2021). Litter removal was not a management technique used at the investigated locations, which may support litter as a primary source of inoculum. However, *G. castaneae* endophytic and pathogenic activity is not restricted to *Castanea* species. As a result, additional hosts or reservoirs may contribute to the spread of the fungus. The prevalence of the pathogen in other woods has not been thoroughly investigated, but it has been found in manna ash as a leaf endophyte and in holm oak leaves with unknown activity. The first is a tree with deciduous leaves that fall off during autumn. The latter tree, an evergreen, gives nuts that fall in autumn as well. When temperature warm up in the spring and the tissues that have been dropped start to decompose, releasing fungal spores, both may act as sources of inoculum. Further studies are required to determine how much the occurrence of rot is influenced by sources of inoculum other than *Castanea* species. It would be interesting to investigate whether an increase in rot occurrence is seen after mast years since mast-producing trees like oak operate as significant reservoirs. The ability to forecast the pathogen's global spread and the occurrence of the disease may be significantly impacted by this.

1.2.1.5 Symptoms

Chestnut brown rot

The most well-known effect of *G. castaneae* is the brown rot of chestnuts (Figure 1.4c), which can be seen in nuts and burrs still attached to the trees, even if it is usually most frequently noticed during postharvest processing (Maresi et al., 2013; Shuttleworth et al., 2013; Vettraino et al., 2019; Visentin et al., 2012; Bedini et al., 2020). Chestnut brown rot, as its name suggests, is a disease that often causes the endosperm to turn brown, symptom that can only be detected by removing the pericarp. On the outside of the kernel, nuts may look healthy, but the soft tissues inside may be rotten (Shuttleworth et al., 2013). In addition to the discoloration, the endosperm is sometimes shown to be dry, spongy, or chalky in appearance (Lione et al., 2015; Maresi et al., 2013; Shuttleworth et al., 2012). Iconographic tables showing the main symptoms on nuts are available (Smith and Agri 2008; Gentile et al., 2009; Shuttleworth et al., 2012; Visentin et al., 2012; Maresi et al., 2013; Shuttleworth and Guest 2017). Additionally, depending on how the disease evolves, it is possible that it may be mistaken for symptoms caused by moulds or other fungi, including *P. endogena*.

It is crucial to remember that *G. castaneae* has been isolated from nuts that do not exhibit any symptoms (Dennert et al., 2015); thus the absence of nut rot does not exclude the presence of the pathogen. The fact that *G. castaneae* can also exist as an endophyte inside of asymptomatic nuts adds further complication, making it more difficult to spot the disease visually (Dennert et al., 2015; Ruocco et al., 2016). For instance, when the diagnosis was made based solely on visual inspection rather than isolation, Dennert et al., (2015) observed a significant underestimating of the incidence of *G. castaneae* (approximately 30%).

Figure 1.4 *Gnomoniopsis castaneae* symptoms. (a) Yellow-orange pycnidia containing asexual spores (conidia) extrude from a naturally infected American chestnut (*Castanea dentata*) tree. (b) Canker induced by live *G. castaneae* on artificially inoculated 1-year- old American chestnut seedling. (c) Diseased (black arrows) and healthy (white arrows) endosperm of Chinese chestnut (*C. mollissima*). Source: Dobry and Campbell, 2022.

Stem and branch cankers

In 2010, artificial twig infection assays that produced symptoms similar to those caused by *Cryphonectria parasitica* (chestnut blight) infection were the first to suggest a relationship with damaging cankers of stems, sprouts, or branches of chestnut species (Magro et al., 2010). It may be challenging to assess the impact of *G. castaneae* as a canker agent in the field, given that the chestnut blight pathogen *C. parasitica* also causes bark cankers on young chestnut branches and scions (Pasche et al., 2016a). Not surprisingly, during routine surveys targeting *C. parasitica*, *G. castaneae* was frequently found, almost accidentally, in conjunction with cankers (Dar and Rai, 2015; Pasche et al., 2016a; Lewis et al., 2017; Trapiello et al., 2017). It's interesting to note that Shuttleworth et al., (2012) reported perithecia on branches but did not describe any damaging lesions. No more associations with cankers or fructifications on bark in naturally or artificially diseased branches were made for a long time (Maresi et al., 2013; Visentin et al., 2012). It was not until 2015 that *G. castaneae* was again associated with chestnut blight-like lesions, when the pathogen was identified in multiple stands of cultivated *C. sativa* (sweet chestnut) distributed throughout the Kashmir valley of India (Dar & Rai, 2015). Dar and Rai did complete Koch's postulates to demonstrate *G. castaneae* as the causal agent of the observed cankers. *Gnomoniopsis castaneae* was clearly identified as the causative agent of damaging chestnut cankers by the pathogen's production of large lesions that manifested as a darkening of the stem extending through the phloem (Figure 1.4b). *Gnomoniopsis castaneae* was also observed in association with cankers on hazelnut, albeit in this case the fungus was described as a weak pathogen (Linaldeddu et al., 2016). In fact, tests for pathogenicity showed that *G. castaneae* could qualitatively replicate hazelnut cankers, but their severity did not reach levels that were noticeably higher than those demonstrated by untreated controls (Linaldeddu et al., 2016).

Leaf necrosis

Several studies have proven the connection between the infection of *G. castaneae* and the development of necrosis on chestnut galls and leaves (Magro et al., 2010; Vinale et al., 2014; Seddaiu et al., 2017; Vannini et al., 2017). It has occasionally been noted that the infection is associated with leaf yellowing and necrosis. Recent findings pointed out that some secondary metabolites produced by strains of *G. castaneae*, namely the abscisic acid (ABA) and the 1,4-trans-diol ABA, display phytotoxic effects on chestnut leaves and could be involved in galls necrosis (Vinale et al. 2014). When Magro et al. (2010) noticed that leaves on sweet chestnut trees highly infested by ACGW had spots amber in colour with green borders and irregularly shaped necrotic lesions, they became the first to record this. A few years later, Pasche et al. (2016) showed that after artificial inoculation, the pathogen could enter the vascular tissue of sweet chestnut leaves; they did not observe any necrosis or yellowing of the treated leaves, though. In a multiyear study of managed sweet chestnut coppices and orchards in Monti Cimini

(Italy), where early summer leaf discoloration on ACGW-infested trees occurred, a strong correlation with yellowing was found. The leaves gradually withered late in the season after turning yellow but keeping their turgor (Vannini et al., 2018). Although *G. castaneae* was the most frequently isolated fungus attributed to the yellowing, the pathogen was also highly present in healthy control tissues, making it impossible to determine if it was the cause of the yellowing. It is important to remember that Vinale et al. (2014) documented the phytotoxic effects of the pathogen's metabolites, which manifested as necrotic and chlorotic signs on treated leaves. Only a couple of studies have linked *G. castaneae* symptoms to those seen in chestnut leaves. When assessing more severe symptoms elsewhere on the tree's body, signs like yellowing and necrosis may be overlooked because they can be caused by a variety of biotic and abiotic sources. Although it is yet unclear if these symptoms represent a switch in *G. castaneae* activity from endophytic to pathogenic, it does seem that infected leaf litter from the previous season contributes to infection in the current season.

Endophytic lifestyle

In nut, branch, stem, leaf, and gall tissue, the fungus has been found to exist as pathogen, usually at very high levels of incidence. However, *G. castaneae* appears to mainly exist as an endophyte in all above-ground tissues of chestnut trees, including those in which it may become pathogenic, without giving any symptoms. *Gnomoniopsis castaneae* has been associated to woody tissue, however it is much more often isolated from tissues from the current or previous year (Pasche et al., 2016; Shuttleworth & Guest, 2017; Vannini et al., 2017, 2018). Vannini et al. 2017 reported that also in the case of gall necrosis, symptoms may occur on galls following endophytic colonization rather than from an external source of inoculum. *Gnomoniopsis castaneae* presence in green tissues of chestnut trees may have considerable effects on long-term crown health and growth, since the mechanisms underlying the transition from endophyte to pathogen for this organism are still not fully understood (Maresi et al., 2013; Lione et al., 2016; Pasche et al., 2016a,b; Shuttleworth and Guest 2017; Vannini et al., 2017). The endophytic presence of *G. castaneae* within asymptomatic plant tissues, as well as the difficulties in the diagnosis of the pathogen in symptomatic plants, might have led to a substantial underestimation of both its host range and geographic distribution.

1.2.1.6 Diagnosis

Nowadays, there are a variety of methods available for pathogen identification in different contexts, and the choice depends on the question being asked. Researchers can select from a variety of methodologies to find the one that best suits their objectives, whether they are only looking to assess the quality of a product, trying to determine whether one pathogen is present, or trying to quantify the environmental load.

Regardless of the disease incidence, the most reliable diagnostic methods for *G. castaneae* rely on field samplings, followed by isolation on substrates such as MEA (Malt Extract Agar), MYA (Malt Yeast Agar) and PDA (Potato Dextrose Agar), and subsequent identification of isolates through morphometric and/or biomolecular assays (Shuttleworth et al., 2012; Visentin et al., 2012). Some observations can be performed directly in planta, possibly after incubation of infected host tissues in a damp chamber (Vannini et al., 2017), while others need to be conducted *in vitro*. Nonetheless, the correct identification of *G. castaneae* might not be successfully accomplished through the mere morphological characterization of the fungal isolates, since colonies of other fungi inhabiting the same hosts can display similar morphological traits, as remarked by Meyer et al. (2017) for isolates of *Sirococcus castaneae* comb. nov. J.B. Meyer & B. Senn-Irlet & T.N. Sieber (syn. *Diplodina castaneae* Prill. & Delacr.), just to cite an example. A taxon-specific molecular assay was designed, tested, and validated for the identification of *G. castaneae* through a Polymerase Chain Reaction (PCR) based on a set of specific primers (Lione et al., 2015). Alternatively, the identification of the fungus may be achieved by a multilocus phylogenetic analysis of the internal transcribed spacers (ITS) of ribosomal DNA, the translation elongation factor 1-alpha (TEF1-α) and the β-tubulin genes (Visentin et al., 2012; Linaldeddu et al., 2016; Pasche et al., 2016a).

Morphological and molecular identification is a highly specific process, but it is time consuming, and its sensitivity is low, particularly when the pathogen is not dominant within the sampled tissue. Additionally, this process often occurs after the observation of a related symptom, such as bark cankers, necrotic galls, necrotic leaves, or chestnut brown rot, or when general observations of endophytes are carried out. Thus, rapid innovations in detection and quantification techniques may allow rapid diagnoses in field, laboratory, and commercial settings, that could help to mitigate the spread of the disease and reduce associated losses. The commercial chestnut industry has investigated a few such non-destructive imaging techniques, including terahertz (Di Girolamo et al., 2021), Fourier-transform near-infrared (FT-NIR), and Vis/NIR hyperspectral imaging (HSI; Bedini et al., 2020). While these methods show promise for sorting sound and unsound nuts, they do not specifically identify *G. castaneae*, making it unable to monitor changes in the pathogen's incidence.

Indeed, two highly specific methods of detection already exist, which would be useful in multiple settings including both field and commercial: real-time PCR and loop-mediated isothermal amplification (LAMP). With both approaches, tissue can be examined directly for the presence of the pathogen, avoiding the clean cultures procedure. Even in the presence of DNA from other plants and fungi, these tests have been demonstrated to be extremely accurate, sensitive, and specific (Turco et al., 2021; Vettraino et al., 2021). Although the pathogen can be quickly identified and quantified using real-time quantitative PCR (qPCR), LAMP may be the most effective method for field-based detection efforts. This tool may be useful to growers who wish to detect early stages of infection within their

orchards. Quantitative PCR can indeed detect the pathogen in very low quantities, despite the presence of other fungal or plant DNA, and this demonstrates the significant advantages and great diagnostic value of this assay. Quantitative PCR assay is also a significant tool in tracking the presence and the amount of pathogen infection in order to evaluate the effectiveness of control measures in field, such as in tracking infected plant material, helping to reduce the spread of the pathogen on regional, national and international scales (Turco et al., 2021). Moreover, Turco et al. (2021) reported HTS as a possible detection method for *G. castaneae*, since they found a correlation between qPCR results and the number of reads due to the pathogen obtained in parallel metagenomic HTS analysis of bulk fruit samples. In this case, results demonstrated that the number of reads of the same taxon between different samples was consistent with the quantitative presence of that taxon measured in qPCR. This fact suggests that HTS methods can be able to detect and quantify the pathogen's presence in samples, even in low quantities.

1.2.1.7 Biotic interactions

Interspecific interactions, particularly when native hosts and plant microbiomes are threatened by foreign or new threats, such as insect pests and plant pathogenic fungi, may influence the dynamics of epidemics and promote the development of plant diseases (Quacchia et al., 2008; Sillo et al., 2015; Garbelotto et al., 2017; Zampieri et al., 2017). The research on potential connections between *G. castaneae* and ACGW has been triggered by the regional and chronological overlap between the outbreak of *G. castaneae* and the invasion by ACGW in Europe (Brussino et al. 2002; Visentin et al. 2012). The plant parasites known as cynipids, commonly referred to as gall wasps (*Hymenoptera: Cynipidae: Cynipinae*), almost exclusively colonize a single species or group of closely related species. The vast majority of described cynipid species are *Quercus* genus tree parasitoids. A major exception to this rule is the ACGW (Maldonado-López et al., 2015; Stone et al., 2002).

Originally native to China, the species was identified in Europe for the first time in the northern Italian region of Piedmont in 2002 (Brussino et al., 2002), although it was probably introduced 2 to 3 years earlier (Quacchia et al., 2008). The ACGW is thought to have been introduced to countries all over the world through the importation of infected plant material (Avtzis et al., 2019; EPPO, 2021; Martinez-Sañudo et al., 2019; Payne et al., 1983; Quacchia et al., 2008). According to the EPPO (2021), the ACGW has been identified in 28 countries worldwide. An association between *G. castaneae* and *D. kuriphilus* was suspected because of the increasing of chestnut brown rot throughout or near the same regions known to be infested with Gall Wasp. The association between the two species was first observed by Magro et al. (2010) that isolated an undescribed species of *Gnomoniopsis* from necrotic galls in Italy (Magro et al., 2010). Subsequent studies attempted to evaluate and describe the relationship between pest and pathogen (Lione et al., 2016; Muñoz-Adalia et al., 2019; Seddaiu et al.,

2017; Vannini et al., 2017, 2018). The coincident occurrence of pest and disease symptoms in or close to the same Italian regions raised the possibility that ACGW served as a vector for the pathogen's spread. Through genetic identification, Lione et al. (2016) assessed the ecological interactions between the two species and found that the pathogen was present in or on 40% of the adults emerging from infected galls but was never found in adults emerging from noninfected galls. Even though *G. castaneae* was found in the infected gall adults, researchers were unable to reisolate live fungal cultures from the wasps, indicating that the insects didn't contain a viable inoculum. The wasp's role as a vector is further questioned by a different study that found varied fungal communities related to developing adults; while *G. castaneae* was present in these communities, it was not a dominating species (Morales-Rodrigues et al., 2019).

The respective ranges of the Asian Chestnut Gall Wasp and *G. castaneae* may be the clearest argument against the role of the insect as a vector for the fungus. The pathogen has only recently been discovered in India (Dar & Rai, 2015), Ireland (O'Loinsigh et al., 2022), Chile (Morales-Rodriguez et al., 2021) and in the United States (Campbell et al., 2019; Sakalidis et al., 2019), where the Gall Wasp has had an established and spreading population since the early 1970s (EPPO, 2021; Payne et al., 1983). Moreover, the wasp cannot or is not likely to be operating as a vector in these areas, further proving that the interaction between pathogen and pest is different. One such interaction could originate from a tissue's increased attraction to one pest due to the other's presence or activity. Lione et al. (2016) tested two scenarios to determine whether ACGW would prefer oviposition in *Castanea* buds infected with *G. castaneae* over uninfected buds and whether the pathogen would more readily colonize buds in which the ACGW had oviposited or those in which oviposition had not occurred. The models in both situations supported the attractiveness of the pathogen to the pest and the pest to the pathogen. In other words, they discovered that the ACGW was more likely to oviposit in buds already colonized by the pathogen and the pathogen was more likely to colonize oviposited buds. Different research groups investigated on this topic and demonstrated that either or both have potential to be valid. Vinale et al. (2014) reported that *G. castaneae* produced abscisic acid (ABA) and 1′,4′-*trans*-diol ABA inside of necrotic *D. kuriphilus* galls, which may act as a negative regulator of plant disease resistance, thereby favouring colonization of the pathogen in oviposited buds. In another study by Xu et al. (2018), increased production of ABA by a mangrove plant contributed to a decrease in mycotoxins, which have insect resistance and antipathogen activity. As a result, a plant may become more susceptible to disease and/or pest attack if its ABA level increases. In the same way, enhanced cytokinin synthesis in response to pathogen infection may act as an attractive signal for oviposition in contaminated tissues. Many insects also synthetize cytokinins while feeding or ovulating, which may allow them to further control the host's defensive response (Andreas et al., 2020). While plants have extensive defensive networks to protect themselves, pathogens may manipulate these responses to favour fungal colonization and promote

performance of a transmitting vector (Chanclud & Morel, 2016; Dhar et al., 2020; Ye et al., 2021). Similarly, oviposited eggs may actively suppress plant defences and diminish larval mortality (Reymond, 2013). There is some evidence to suggest that *G. castaneae*-infected non-necrotized galls promote in the development of viable *D. kuriphilus* adults, possibly by providing additional nutrients, softening the gall tissue, and improving the tissue's digestibility (Lione et al., 2016). However, the pathogen in necrotic galls has been linked to an increase in mortality of emerging adults, which is thought to be a consequence of the tissues in the necrotic galls becoming dry and so hard to obstruct or block adults' emergence (Magro et al., 2010; Vannini et al., 2014, 2017). Instead of acting as an entomopathogen, in this instance, the pathogen might be acting as an inquiline, infecting the growing gall and indirectly killing the wasp (Wilson, 1995). Many other investigations have linked the Gall Wasp's mortality with other fungal species, supporting a role as a possible inquiline. One study, which took place about 8 years after the first known incidence of *D. kuriphilus* in Italy, evaluated parasitic fungus in Piedmont galls. Addario & Turchetti (2011) identified *Fusarium incarnatum-equiseti*, *Alternaria alternata* and two *Botrytis* spp. associated with 60% mortality of ACGW in necrotic galls. In this study, no *Gnomoniopsis* species were associated with or isolated from the necrotic galls. Beside brown rot, Meyer et al. (2015) investigate the occurrence of chestnut blight colonization in the Gall Wasp galls occurring on sweet chestnut; in this case, *G. castaneae* resulted to be the most frequently isolated colonizing fungus and a negative correlation between the two pathogens was proven. Similarly, a negative correlation between the pathogens in abandoned galls and dormant buds has also been observed. A preference for green tissues such as leaves, galls and buds has been noted for *G. castaneae* while a preference for bark has been noted for chestnut blight (Vannini et al., 2018). Even while *G. castaneae* is frequently characterized as having a low pathogenic potential, its considerable presence as an endophyte in all tissues of above-ground chestnuts may provide a competitive advantage in the early colonization of galls and other green tissues. Further research is necessary to determine if the observed negative correlations are the result of direct antagonistic activity or competition during establishment.

1.2.1.8 Impact

Brown rot caused by *G. castaneae* can harm nuts still on the tree, lying on the ground, or stored before being processed, indiscriminately before and after harvest. Although *G. castaneae* incidence on nuts has been shown to vary in time and space, it is frequently linked to substantial yield losses yield reductions. Postharvest problems in sweet chestnut changed in the first decade of the 21st century, when European and Australian chestnut growers first noticed a severe increase in internal rot in the fruits, locally affecting up to 80% of production in particular seasons (Visentin et al., 2012; Maresi et al., 2013; Shuttleworth et al., 2013). Depending on the season, in north-western Italy peaks of incidence between 71.4 and 93.5% were reported in chestnut orchards (Visentin et al., 2012; Lione et al., 2015; Lione and Gonthier, 2016), while a peak up to 49% was reported in north-eastern Italy (Maresi et al.,

2013). Similar levels were observed in Australasia and Switzerland, 72 and 91% respectively (Shuttleworth et al., 2013; Dennert et al., 2015). Not surprisingly, brown rot caused by *G. castaneae* is currently acknowledged as a major threat affecting chestnut nuts (Shuttleworth et al., 2013; Dennert et al., 2015). The incidence of cankers caused by *G. castaneae* may be locally relevant as well. For instance, Dar and Rai (2015) showed that the average incidence of *G. castaneae* reached 39% in symptomatic branches. While there is a lack of information regarding the pathogen's frequency and the severity of symptoms on leaves, throughout investigations have been carried out on galls induced by *D. kuriphilus*. Here, *G. castaneae* incidence was reported in Switzerland, Sardinia, and central Italy at rates of up to 53.8%, 68%, and over 80%, respectively (Meyer et al., 2015; Seddaiu et al., 2017; Vannini et al., 2017).

1.2.1.9 Control strategies

In terms of management and control of the disease, there are no proven options available to growers. Studies focused on testing if the management practices could influence the incidence of spoiling fungi are notably few for chestnut (Sieber et al. 2007). It may be possible to prevent the disease in new plantations by screening and evaluating host varieties or cultivars that are resistant to *G. castaneae* or at least more tolerant to it. In this context, a pioneering effort was made in Australia using some of the most significant chestnut varieties cultivated there for nut production (Shuttleworth et al. 2013; Shuttleworth and Guest 2017). The biogeographical origin of the fungal strains employed for the pathogenicity testing revealed variations in the severity of symptoms despite all *G. castaneae* isolates were pathogenic and all tested cultivars susceptible (Shuttleworth and Guest 2017). The susceptibility profiles to nut rot caused by *G. castaneae* are comparable between the *C. sativa* wild-type and various chestnut cultivars of local or global relevance, according to preliminary data from Lione (2016). However, additional research is required before making a conclusion. The lack of association between the plantation density and the spatial pattern of nut rot caused by *G. castaneae* suggests that the attempt of controlling this pathogen by fine-tuning the orchard plantation density is likely to fail (Lione and Gonthier, 2016). On the other hand, considering the prevalence of sexual reproduction in *G. castaneae* (Sillo et al., 2017), the removal of the fallen burrs on which the teleomorph stage develops could be an effective strategy (Visentin et al., 2012; Shuttleworth et al. 2013; Shuttleworth and Guest 2017; Sillo et al., 2017). However, even if this and other similar practices are proposed in the literature (Shuttleworth et al., 2013) to prevent ascospores release, they might not lead to the expected outcomes because of the potential long-distance dispersal of the pathogen and of the local relevance of asexual reproduction (Sillo et al., 2017). Moreover, as evidenced previously there are other potential sources of inoculum. Hence, additional studies are needed to determine which management strategies could be successful in containing *G. castaneae* in the field. To date, management suggestions have focused almost exclusively on clean-up of autumn litter to eliminate potential sources of inoculum. Moreover, different treatments

on tree included only conventional fungicides (Silva-Campos et al., 2022), but not specifically targeting *G. castaneae*. However, one study used artificial inoculation of 1-year-old sweet chestnut to examine the efficacy of common endophytes *Trichoderma atroviride* and *Bacillus amyloliquefaciens* as antagonists to *G. castaneae* in young seedlings (Pasche, Crovadore, et al., 2016). Their results suggested that the presence of endophytic antagonists delayed the disease's development but did not eliminate it. Eventually, *G. castaneae* overcame over its competitors and acquired control of the environment. They speculated that all growth would finally cease if the antagonists completely colonized the plant. However, two separate studies contradict this conclusion. Meyer et al. (2015) observed that *Gnomoniopsis* species were by far the dominant fungi at nearly every site, even when *Trichoderma* species were present with moderate incidence levels. Similarly, Muñoz-Adalia et al. (2019) found *T. atroviride* to be the second most abundant fungus at one study site but entirely absent at another. *Gnomoniopsis castaneae* was the dominant fungus at both sites, but its incidence was greater at the site where *T. atroviride* was also identified, suggesting the potential antagonist did not influence growth of the pathogen. Management strategies will be more crucial since *G. castaneae* has been discovered in new places and within new plant species; moreover, controlling its spread and reducing damage will be difficult because it is a constantly present as endophyte. Since brown rot symptoms develop when fruits are still in the burrs on the trees, appropriate protocols to control brown rot in chestnut orchards are required to reduce the fungus in fruit tissues and the consequent development of brown rot symptoms in the field. Besides the homologation of some molecules for their use on chestnut, such as Tebuconazole, Boscalid, and Pyraclostrobin, a specific protocol to control the disease does not exist. Recently, in field trials, Silva-Campos et al. (2022) reported that the use of pyraclostrobin and difenoconazole-based fungicides combined were the most effective in suppressing the level of nut infection caused by *G. castaneae*. Moreover, a modern approach to control this disease in a peculiar agroforestry ecosystem such as chestnut groves, cannot rely on the use of molecules with relevant environmental impact. Even if *G. castaneae* spread currently occurs in field, incidence of nut rot may significantly rise during post-harvest storage (Maresi et al., 2013; Shuttleworth et al., 2013; Dennert et al., 2015). Ruocco et al. (2016) first tried to test a post-harvest control strategy with the aim to reduce the incidence of the disease on chestnut nuts. In this study, a traditional method based on the treatment of nuts in water (i.e., *curatura*) was customized by adding to the water a cell-wall degrading enzyme mixture gathered from cultures of the fungus *Trichoderma harzianum* strain T22. The improved treatment resulted in a significant reduction of brown rot incidence (Ruocco et al. 2016), hence providing new and intriguing perspectives to reduce the post-harvest losses caused by the pathogen. As a postharvest method, also controlled atmosphere storage was proposed with a high carbon dioxide concentration (Mignani and Vercesi, 2003) also to avoid flesh hardening which occurs during cold storage, and which is very detrimental to transformation products (Estévez et al., 2005). A very high concentration of carbon

dioxide has been proposed as an alternative to water curing, but water curing gave better results in controlling fungal decay, if the high concentration of carbon dioxide treatment was not followed by controlled atmosphere storage (Anelli et al., 1984). Among the many technologies available to extend the fruit storage, ozone technology has proven to be an effective sterilization technique, both using gaseous ozone and ozonated water treatments (Vettraino et al., 2020; Botondi et al., 2021).

All the control strategies reported here are suitable for reducing *G. castaneae* inoculum in field and brown rot symptoms in harvesting, but the methods, especially those carried in field, not only are not specific for *G. castaneae*, but cannot also ensure that contamination is completely avoided. For this reason, further attempts are needed to efficiently block *G. castaneae* spread both on tree and during the harvesting.

1.3 Mycotoxins producer fungi on chestnut fruits

A few fungi associated to chestnut fruits are well-known mycotoxin producers. There is a vast literature on fungi and mycotoxins associated with different types of foods and food commodities, and numerous reviews have been published (Bennett & Klich, 2003; CAST, 2003; Magan, 2006; Murphy et al., 2006; Venâncio & Paterson, 2007). In recent years, investigations have been conducted on the contamination level of food and the possibility of efficient mycotoxin control, particularly in cereals, nuts, and wine. Regulatory bodies continuously monitor the presence of mycotoxins during the various production phases, from the farm to the fork since it is nearly impossible to evaluate their occurrence in final products. Therefore, knowledge of the essential elements of microbial and mycotoxin contamination in food commodities is essential for all phases of manufacturing. Pre-harvest conditions, post-harvest storage, transport and processing are all significant stages in the food chain that need to be monitored since fungal contamination and mycotoxin production vary substantially with the environmental circumstances in which they develop. Given various production, storage, and processing conditions, the incidence and diversity such as mycotoxigenic potential of the fungal population provide an indication of the probable safety of the products. Mycotoxins are produced by an array of diverse fungal species that are generally saprophytic and opportunistic or weak pathogens. Most of the mycotoxins which are considered important food contaminants are produced primarily by three genera of fungi, namely *Aspergillus*, *Penicillium* and *Fusarium* (CAST, 2003; Reddy et al., 2009; Venâncio & Paterson, 2007). Fungi of the genera *Penicillium* and *Aspergillus* have been associated with toxin production on many agricultural commodities (Wilson, B. J., 1971; Yates, S. G., 1971). Other fungi, especially those of the genera *Alternaria* and *Fusarium*, are also reported as toxin producers (Detroy et al., 1971; Doupnik and Sobers, 1968; Escrivá et al., 2017). The genus *Aspergillus* represents a large group of fungi that occupies very diverse ecological niches. Generally regarded as saprophytes, *Aspergillus* spp. grow on an enormous number of substrates; their ability to thrive in high temperatures

and with relatively low available water makes them well suited to colonise several grain and nut crops. Mycotoxins associated with *Aspergillus* species include aflatoxins, ochratoxins, versicolorins, sterigmatocystin, gliotoxin, citrinin, patulin, citreoviridin, cyclopiazonic acid, penicillic acid and tremorgenic mycotoxins (CAST, 2003). More recently, fumonisins have been added to this group after they have been confirmed to be produced by *Aspergillus* section *Nigri* (Nielsen, Mogensen, Johansen, Larsen, & Frisvad, 2009). Anyway, the most concerning mycotoxin produced by *Aspergillus* genus is the Ochratoxin A, released by *Aspergillus ochraceus*, *A. carbonarius*, *A. niger*, and *Penicillium verrucosum* (Bui-Klimke et al., 2015). *Penicillium* spp. cause various decays on fruit (Washington et al., 1997), and act as a contaminant in the post-harvest phase (Nielsen, 2003). The most frequent species reported in nuts belong to *Penicillium* (Frisvad and Samson, 2004) and a few papers have reported *Penicillium* species isolated from chestnuts. Overy et al. (2003) stated that *P. crustosum*, *P. glabrum*-clade and *P. discolor* were the dominant species in fresh chestnuts. Sieber et al. (2007) isolated *P. expansum* and *P. crustosum* in particular, while Donis-González et al. (2016) stated that *P. expansum*, *P. griseofulvum* and *P. chrysogenum* were the main species. *Penicillium* spp. (about 104 CFU/g) have also been reported on dried chestnuts and chestnut flour (Pietri et al., 2012). Mycotoxins associated to *Penicillium* spp. include patulin (the most common, produced by *P. expansum*), ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid, citreovirdin, penitrem, PR-toxin. Furthermore, several studies have reported the production of different secondary metabolites, such as alkaloids, antibiotics, and allergens, by *Penicillium*, with noxious effects on human health (Barkai-Golan, 2008). Members of the genus *Penicillium* generally grow and can produce mycotoxins over a wider range of temperatures than those of the genus *Aspergillus* but are not generally as adapted to hot and dry conditions, being more abundant in temperate climates. Some of the most important secondary metabolites produced by these fungi are common to *Aspergillus*: OTA, citrinin, patulin, penicillic acid, penitrem A, cyclopiazonic acid.

Fusarium is a large complex genus with species adapted to a wide range of habitats. They are cosmopolitan soil borne or saprophytes fungi and many are important plant pathogens. Nowadays at least 15 different *Fusarium* species are known as significant mycotoxin producers (Rheeder et al., 2002) and more than 150 compounds are under investigation (EFSA, 2017). Some of the most important mycotoxins related to this genus are trichothecenes, zearalenone and fumonisins. Fumonisins, most common mycotoxins produced by *Fusarium* spp., are usually released by *F. verticillioides* (Sacc.) Nirenberg (previously known as *Fusarium moniliforme* Sheldon) and *F. proliferatum* (Tosi et al., 2015). Thus, the species *F. sporotrichioides* and *F. poae* are responsible for T-2 and HT-2 toxins production (EFSA, 2017).

It's clear that mycotoxins have a wide range of chemical and physical properties due to their enormous structural diversity. According to the European Union's Commission Regulation from 2006, the most significant mycotoxins in food and feed include aflatoxins (AFB1, AFB2, AFG1, AFG2), ochratoxin A

(OTA), type A and B trichothecenes (such as HT-2 toxin, T-2 toxin, and deoxynivalenol (DON)), Fumonisins (FBs), and zearalenone. Recent years have brought attention to the potential presence of aflatoxins (AFs: AFB1, AFB2, AFG1, AFG2) in chestnut products. In 2010 and 2011, the European Rapid Alert System for Food and Feed (RASFF, 2011) shared several Food Safety Notifications regarding high aflatoxin contamination in dried chestnuts, flours, and flakes produced in Italy (EU 2010.0995, 2010.1614, 2010.1615, 2010.1616 and 2011.0550). The International Agency for Research on Cancer (IARC) has categorized AFs as a class 1 carcinogen (Castegnaro & Wild, 1995) and the OTA and FBs in Group 2 carcinogens (WHO and IARC, 1993); T2 and HT2 are indeed not considered carcinogenic according to recent knowledge, classified as group 3 (not classifiable as to its carcinogenicity to humans) by IARC (Ostry et al., 2017).

Although the concern about mycotoxins regards in general all food commodities, a few studies were carried on mycotoxin levels in chestnut fruits. The occurrence of toxigenic species, as well as their associated mycotoxin contaminations on chestnuts and derived commercial products, have been reported in different countries (Abdel-Gawad and Zohri, 1993; Bertuzzi et al., 2015; Jermini et al., 2006; Overy et al., 2003; Pietri et al., 2012; Prencipe et al., 2018; Rodrigues et al., 2013; Wells and Payne, 1975). Moreover, mycotoxins limits have only been established in chestnuts for AFs, and they are specified by Commission Regulation (EU) No. 165/2010 for dried fruits. There are no indications concerning the safety of chestnut consumptions in terms of maximum levels for the other mycotoxins indicated above, besides AFs. The information available about the occurrence of these mycotoxin producer species and their food-borne mycotoxins contamination is still incomplete, thus underlining the need to set up chestnut management procedures from the orchard to the commercial product, since these species could represent a serious human health risk and cause significant economic losses. This, together with the almost total absence of maximum levels for mycotoxins in chestnut fruits, demonstrates the urgent need for further investigations and regulations.

1.4 Chestnut fruits post-harvest

A high-quality product that can fully satisfy the needs of modern Italian consumers and associated with wholesomeness and naturalness is what the chestnut market currently desires. Size, shape, color, flavor, and texture are all factors that affect chestnut quality and are crucial for both fresh consumption and processing (Korel & Balaban, 2008), as well as for ensuring that growers receive a profit. Miller (2009) stated that, quality of chestnut has two major components: characteristics and condition. Condition is mainly determined by environmental factors, especially temperature and humidity in postharvest handling, and includes attributes like moisture content, insect infestation, fungal decay, and sugar content. These factors directly affect the quality of chestnut kernels. The quality and quantity of nut production are essential for the economic profitability of chestnut cultivation, but quality

and quantity are increasingly threatened by various factors, mainly due to post-harvest conditions (Hulcr and Dunn, 2011).

Chestnuts are a common seasonal fruit that only briefly retains its best commercial quality, turgescence, and health. The product's high perishability is one of the main challenges (Sieber et al., 2007; Bounous, 2004). Mould, rotting and insect damages are the main causes of postharvest deterioration (Wells and Payne, 1980; Breisch, 1993; Bassi et al., 2001), which lowers nut quality and can result in significant financial losses (Jermini et al., 2006; McCarter et al., 1980; Narayanasamy, 2006). In Italy, postharvest decay has been identified as one of the major problems that negatively impacts fresh chestnuts, accounting for up to 90 percent of losses after harvest in certain season, e.g. 2016 (Bedini et al., 2020).

Before the nuts are processed or consumed, at early infection stages, it is difficult to distinguish between the healthy nuts and slightly moldy or parasitized ones (Rutter et al., 1990). Furthermore, parasitized fruits, especially on the shell, if stored together with the healthy one, could cross-contaminate other nuts. This usually occurs since notably, fruits that appear healthy have latent infections of the pathogen that, in conditions of optimal temperature and presence of water, rapidly colonize the endosperm in postharvest conditions. As a result, numerous post-harvest nut-treatment methods have been created. In the past, the *curatura* (water curing) was a traditional technique used for its demonstrated ability to control insect infestation, killing the larvae of pests and molds without affecting nut quality (Botondi et al., 2009). Fruits were typically kept in water for 9 days during the *curatura*, giving it the old name "*novena*" (Botondi et al., 2009; Jermini et al., 2006; Migliorini et al., 2010). Nowadays, the most popular treatment technique is thermo-hydrotherapy, which was developed in Italy in the 1930s with *curatura*. In thermo-hydrotherapy, the nuts are immersed in steel bins filled with warm water that should be between 48° and 52°C for 35 to 45 minutes in order to sterilize them, then the nuts are quickly cooled in tap water (about 15 minutes) (Jermini et al., 2006; Payne and Wells, 1978; Wells and Payne, 1980). This approach was suggested to decrease, but not eliminate, the mycoflora and insect larvae contaminating them more quickly than the *curatura*; hydrothermal treatment before cold storage significantly decreased moulding from 60% to 30% (Delatour, 1979; Jermini et al., 2006).

According to current production methods, the most common strategy for identifying unhealthy chestnuts includes floating, *curatura* frequently paired with thermo-hydrotherapy (Sieber et al., 2007) and followed by manual sorting, even if in some case manual sorting precedes the floating. The conventional approach, however, is problematic since it frequently discards too much good material. Additionally, manual sorting takes a lot of time, is arbitrary, labor-intensive, and misses chestnuts with hidden damage. For these reasons, thermo-hydrotherapy is the go-to technique, also thanks to its effectiveness at reducing insect infestations without affecting the quality of nuts (Jermini et al., 2006).

In more recent research, hot air-assisted radiofrequency treatments, which specifically target *Penicillium* spp., were used as a physical way to lower the rate of mold infection of chestnut fruits during storage (Hou et al., 2018). Moreover, other studies have reported the use of a mix of biological and physical methods (Ruocco et al., 2015). These techniques decreased nut rot and spoilage caused by a variety of fungal taxa in the postharvest period (Jermini et al., 2006; Bounous and Paglietta, 1979; Lione et al., 2019). Donis González et al. (2009) reported also a variety of chemical treatments targeting fungal contamination. In most cases, sanitizing agents are added to processing water to reduce microbial populations and prevent cross-contamination of the product. Of these, chlorine has been used for several decades and is still the most widely used sanitizer in the food industry (IAFP, 2002). However, chlorine does not always reduce microbial populations, including foodborne pathogens and may be harmful due to the formation of toxic chlorine by-products (Foley et al., 2004). Other technologies, *i.e.*, food irradiation, which have been reported to achieve greater than a 5-log reduction of pathogens and other microorganisms (pasteurization treatments), have the potential to greatly improve the microbiological quality and safety of fruits (Niemira, 2003). Fortunately, a medium-long (4–12 months) storage period is possible using both conventional (drying and curing in water) and modern techniques like freezing and refrigeration in a normal and controlled atmosphere (Bounous, 2005).

Although a few studies have examined the effects of treatments and different conservation techniques on the quality of the fruit (Wells and Payne, 1980; Fadanelli et al., 1995; Tian and Bertolini, 1997; Washington et al., 1997; Bassi et al., 2005; Jermini et al., 2006), none have in-depth examined the effect of post-harvesting techniques on fungal postharvest deterioration. Furthermore, the spread of brown rot all over the world clearly demonstrated the urgent need for appropriate treatment parameters, in order to ensure a good chestnut quality during storage. Fruit-handling procedures are nowadays unable to mitigate the disease's severe postharvest effects and this fact affects the sustainability of the chestnut market across Europe.

1.5 References

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1.6 Aims and objectives

Italy is the fourth-largest producer of chestnuts in the world, with over 53,000 Mg, even though the chestnut supply chain is a specialist industry at the national level (FAOSTAT, 2020). The chestnut industry has considerable pre- and post-harvest challenges because of the extreme fruit perishability. Rot and insect damage are the main factors that affect the quality of fresh chestnuts. To reduce the latent fungal population in fruit tissues and the consequent development of brown rot symptoms in the post-harvest handling process, it is necessary to apply the appropriate protocols to control "brown rot" in chestnut orchards. Control methods with low impact products are also needed to limit the population of fungi producer of mycotoxins. Moreover, traditional storage practices by growers and cooperatives have been unable to efficiently maintain chestnuts quality after harvest. This demonstrates that better post-harvest chestnut handling and storage procedures are urgently required.

This thesis has the general aim of contributing to the understanding of the critical phases that the chestnut fruits are passing through from the field to the consumption; this project is to mitigate the impact and effect of harmful microorganisms on pre- and post-harvest chestnut production chain affecting the product quantity and quality. This work aims to extend the knowledge on the diversity of the fungal population that colonizes the fruit and at the same time, along the entire supply chain, to improve the conditions of treatment and conservation of the chestnut, to reduce the risk of contamination.

For these reasons, the principal objectives of this thesis were the following:

- the mitigation of the infestation and damage caused by *Gnomoniopsis castaneae* during the pre-harvest period, specifically during the flowering of the chestnut tree, using new strategies and low impact products, in comparison to the conventional ones;
- to assess the efficacy of postharvest physical treatments to reduce microbial decay of fresh chestnuts during conditioning phases in a commercial plant, emphasizing critical treatment parameters and proposing new solutions;
- to evaluate the presence of mycotoxins and to study the microbial populations in chestnut fruits sampled at the end of different conditioning phases and after storage in a commercial plant, taking into account the risk for human health.

CHAPTER II

This chapter includes data obtained thanks to the development of a TaqMan qPCR assay for the detection and quantification of *Gnomoniopsis castaneae* in chestnut tissues (Turco et al., 2021). Further information about this innovative tool is reported in Chapter V.

Use of Phosphonate Salts to Control Chestnut 'Brown Rot' by *Gnomoniopsis castaneae* in Fruit Orchards of *Castanea sativa*

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Abstract: The fungus *Gnomoniopsis castaneae* is the causal agent of the "brown rot" of sweet chestnut fruits. These days, this pathogen represents one of the main limiting factors for the sustainability of fruit production worldwide. Although heat treatment post-harvest is efficient in completely inactivating the pathogen, the application of appropriate protocols to control "brown rot" in chestnut orchards is required to help in reducing the latent population of the fungus in fruit tissues, and the consequent development of "brown rot" symptoms in the field before the post-harvest handling process. The present study aims to evaluate and compare the efficiency of products at a minimum environmental impact in experimental trials conducted in chestnut orchards in Central Italy for two consecutive years in 2019 and 2020. Phosphonate-based salts and, specifically, Zn- phosphonate were efficient in reducing the impact of the disease and the pathogen inoculum in fruits with an efficacy comparable to the fungicide Tebuconazole. A unique treatment at the blooming time produced the best

results for both Zn-phosphonate and Tebuconazole, also giving indirect evidence of female flowers as a main site of infection. Phosphonate salts, and at first Zn- phosphonate, are highly effective to protect chestnut fruits from the 'brown rot' fungus *G. castaneae*. Its use in orchard management may complement the post-harvest heat treatment during the processing of fruits. Although a still ongoing debate on phosphonate salts use and efficacy in agriculture, they can be considered an optimal fungicide in chestnut orchards because of the low environmental impact when used at the recommended doses, the high translocability and stability, and the multiple mechanisms of action.

Keywords: *Gnomoniopsis smithogilvyi*; fungicide; low-impact commercial molecules; agroforestry; chestnut orchards; control disease

2.1 Introduction

The fungus *Gnomoniopsis castaneae* G. Tamietti (syn. *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest) [1–3] is the causal agent of the "brown rot" of sweet chestnut fruits. These days, this pathogen represents one of the main limiting factors for the sustainability of fruit production. The disease outbreak can be dated to the first decade of the 21st century, when European and Australian chestnut growers first noticed a severe increase in internal rot in the fruits, locally affecting up to 80% of production in particular seasons [2,4,5]. The name "brown rot" refers to the typical symptomatology, which includes a progressive rot and browning of the endosperm and embryo [1]. The symptoms are mainly expressed postharvest, although rotting fruits can be found in burrs on the tree before harvest [4]. Notably, fruits that appear healthy could have latent infections of the pathogen [6] that rapidly colonize the endosperm during the postharvest period in case of conditions of optimal temperature and the presence of water. Indirect evidence supports the hypothesis that female flowers are the main site of infection of *G. castaneae* [7]. The severe impact of the pathogen in the last decade was associated with a massive presence of inoculum in the environment boosted by climate change [8] and in synergy with infestation by the Chinese gall wasp *Dryocosmus kuriphilus* Yasumatsu [9–11]. Quantifying the impact suggests that incidence may be as high as 93.5% in northwest Italy, [2,8], 72% in Australia [5], and 21% in Switzerland [12]; the disease was more recently recorded in North America [13] and Chile [14]. Recent studies demonstrated that the pathogen can be easily controlled in post-harvest fruit handling by physical methods, indicating that the water-bath phase is the critical step. A strict parametrization of this phase was required to effectively inactivate *G. castaneae* in fruits. Specifically, treatment of fruits at 50 °C for a maximum of 45 min provided optimal conditions to completely inactivate *G. castaneae* inoculum during postharvest handling [6]. However, the control of "brown rot" in post-harvest fruit handling only partially solves the problem since, in particular seasons, the fruit stocks enter the post-harvest handling process at a rather high proportion of fruits with "brown rot" symptoms developed when they were still in the burrs on the trees, and during the harvest phase.

Thus, the application of appropriate protocols to control “brown rot” in chestnut orchards is required to help reduce the fungus in fruit tissues, and the consequent development of “brown rot” symptoms in the field. Besides the homologation of some fungicide molecules for their use on chestnut, Tebuconazole, Boscalid, and Pyraclostrobin, a specific protocol to control the disease does not exist. Recently, in field trials, Silva-Campos et al.

[15] reported that the use of pyraclostrobin and difenoconazole-based fungicides combined were the most effective in suppressing the level of nut infection caused by *G. castaneae*. Moreover, a modern approach to control this disease in a peculiar agroforestry ecosystem such as chestnut groves, cannot rely on the use of molecules with relevant environmental impact. The present study aims to evaluate and compare the efficiency of products at minimum environmental impact between them and a currently homologated fungicide as a positive control. The results presented are the results of experimental trials conducted in chestnut orchards in Central Italy for 2 consecutive years in 2019 and 2020.

2.2 Materials and Methods

2.2.1 Evaluated Products

Three different products were evaluated for their efficacy in mitigating the impact of brown rot: treatment Kalex® (AlbaMilagro International Ltd., Parabiago, Italy) containing 50% *w*/*w* potassium-phosphite (KH_2PO_3), is a liquid fertilizer and enhancer of the natural resistance of plants against external pathogenic agents; Kalex Zn® (AlbaMilagro International Ltd., Parabiago, Italy) containing 4% *w*/*w* Ureic nitrogen, 36% *w*/*w* zinc- phosphonate ($O_6P_2Zn_3$), is an innovative mineral fluid fertilizer containing, in the form of zinc phosphonates, high quantities of phosphorus and zinc, with its known fungicidal activity; Mystic® 430 SC (Nufarm Italia Ltd., Milano, Italy) containing 40.18% (*w*/*v*) Tebuconazole ($C_{16}H_{22}ClN_3O$), is used as conventional chemical treatment against fungal contamination (Table 2.1). Doses used were those recommended by producers: Kalex®, 200 mL/L, endotherapy; Kalex Zn®, 300 mL hL−1, crown spray; Mystic® 430 SC, 35 mL hL−1, crown spray.

2.2.2 In Vitro Tests

Before application in open field conditions, the products were tested for their fungistatic and fungicide efficacy “*in vitro*” against mycelium and conidia of *G. castaneae*. The inhibition tests were carried out on a potato dextrose agar (PDA) medium amended with each product separately. For each product, eight concentrations (0.1 and 0.5, 1, 2, 5, 7.5, 10 and 20 mL L−1) for mycelial radial growth assessment and fourteen concentrations (0.001, 0.01, 0.1, 1, 10 and 100 µL L−1, 1, 2, 5, 7.5, 10, 20, 50 and 100 mL L−1) for conidia germination assessment were compared. Plates with PDA only were used as control. For each of the tests, there were five replicate plates per treatment dose. All the *in vitro* experiments were repeated twice.

Inhibition of Mycelial Growth

Mycelial discs (6 mm) from actively growing colonies of the two *G. castaneae* isolates from the collection (GN01 and GNAm, GeneBank accession number MW494885 and OM818661, respectively) were placed in the center of a PDA plate. All the plates were incubated at 22 ± 2 °C. To calculate the percent inhibition, radial growth (mean of two perpendicular diameters) was expressed as the mean percentage of the growth in the control plates [16]. The mycelium plugs of the plates with 100% inhibition were removed at the end of the experiment and were plated on PDA without the fungicide and the plates were maintained for another 30 days at 22 ± 2 °C, to evaluate the fungicidal or fungistatic effect of the products.

Inhibition of Conidia Germination

Gnomoniopsis castaneae isolates (GN01 and GNAm) were grown on malt extract agar (MEA) and incubated for 15 days at 22 ± 2 °C. Conidial suspensions from each isolate were prepared by flooding the agar surface with approximately 15 mL of sterile distilled water (SDW) and scraping with a sterile spatula. The suspension was filtered through two layers of cheesecloth and adjusted with SDW to 100 conidia mL−1, and 0.5 mL aliquot spread over a Petri plate containing medium with the product's addition. The plates with PDA only were used as controls. All the plates were incubated at 22 ± 2 °C to support the conidia germination. The number of colonies forming units (CFUs) from the conidial suspensions was assessed after four, eight, and 30 days. To calculate the percentage of inhibition, conidia germination was expressed as the mean percentage of germination in control plates [16].

2.2.3 Treatment Trials

Trials were established in 2019 and 2020 in a young (15 years old; an average of 25 cm in trunk diameter and 3 m in height) sweet chestnut (*Castanea sativa*) plantation for fruit production located in Central Italy in the Province of Viterbo (42°17′35 N 12°08′37 E). Chestnut trees of the cultivar "Marrone Fiorentino" were grown in the orchard. A Randomized Complete Design (RCD) was adopted with a statistical unit of 3 adjacent trees along the row, and four replications per treatment. The units of replication were separated from each other by one tree along the row. Four and six treatments were considered in 2019 and 2020, respectively (Table 2.1). In 2019, two treatments were done with Kalex Zn® and Mystic® 430 SC (June and July); in 2020, a third repetition of the treatment with these products in August was also included. For each treatment, product doses (according to manufacturers' recommendation), application method, and data are summarized in Table 2.1. Endotherapy treatments were carried out using the Chemjet® Tree Injectors (Banyo Queensland, 4014 Australia), which are reloadable syringes for micro-injection and infusion systems developed for trees. Each syringe loads 20 mL of product. Each tree received two injections at breast height and on the opposite side. Crown spray was carried out with a Trailer Mist-Blower Sprayer fitted with a "Top-Fan" with an instantaneous

variable pitch fan unit, Model Trend by Caffini (Verona, Italy), with a tank of 2000 L (120–170 L min−1).

2.2.4 Samples Processing

At the end of the treatment campaigns, the clusters of burrs on branches were en- closed in a plastic net to avoid ripened fruits to fall on the ground. At harvest time, fruits were collected from the nets and transferred to the laboratory. In 2019, the fruits were stored at 20 ± 1 °C in moist chambers and the presence of *G. castaneae* conidiomata on the kernel surface was annotated after 14 days, through assessment of the characteristic cirri emerging from conidiomata [10]. Following that, 10% of fruits were randomly sorted from each sample tree, surface sterilized, split in half, and assessed for the presence of "brown rot" symptoms and *G. castaneae*, upon isolation in pure culture. In 2020, after harvest, it was decided to proceed immediately with visual scoring and isolation in a pure culture: all fruits were surface sterilized, then split in half; one half was assessed for the presence of "brown rot" symptoms and *G. castaneae* by isolation in the pure culture; the other half was stored at −20 °C for embryo DNA extraction. Isolation in pure was carried out as follows: after shell removal, each kernel was split in half with a sterilized razor blade. Half of the embryo of each healthy and symptomatic fruit was placed onto Petri dishes containing potato dextrose agar (PDA, Oxoid, Basingstoke, UK, 39 g/L) amended with streptomycin sulfate (0.06 g L−1) (PDAs) and incubated at 22 ± 2 °C [6,17]. After 7 days of incubation, the plates were scored for the presence of *G. castaneae* colonies. The plates were re-scored after 30 days to confirm the absence of growth on fragments that scored negative on day 7. Identification was based on the colony and reproductive structure morphology, and confirmed by molecular barcoding according to the protocol reported by Morales- Rodríguez et al. [11].

2.2.5 DNA Extractions and qPCR Reactions

In the 2020 trials, the detection and quantification of *G. castaneae* were done by newly developed molecular methods according to the protocols reported by Turco et al. [18]. DNA was extracted from six randomly sorted chestnuts from each of the twelve trees (4 blocks) of each thesis, using the NucleoSpin Plant II mini kit (Macherey Nagel, Düren, Germany), following the manufacturer's instructions. DNA concentration was measured with Qubit (Thermo Fisher, Waltham, MA, USA) using the High Sensitivity dsDNA Assay kit. DNAs were stored at −20 °C until further analysis. The qPCR reactions were per- formed as previously described in Turco et al. [18]. Briefly, 2 µL of DNA was used as a template in a reaction composed of 1× GoTaq Probe qPCR Master Mix (Promega, Madison, WI, USA), 0.5 µM of each primer, 0.3 µM probe, and ultrapure water to reach a final volume of 20 µL. Amplifications were performed in a RotorGeneQ (Qiagen, Hilden, Ger- many) with the first step at 95 °C for 4 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 45 s. Fluorescence was measured once per cycle at the end of the 60 °C segment and the Ct values were automatically calculated by the device. Two replicates per sample were tested and the Ct values

obtained by qPCR amplification were used for the data analysis described below.

2.2.6 Assessment of Disease and Infection Indexes

Disease Indexes Based on Symptoms Expression

The assessment of brown rot symptoms was carried out according to an arbitrary visual scale in 5 classes: class 0, healthy fruits (0% brown rot), class 1 (10–30% brown rot), class 2 (30–50% brown rot), class 3 (>50% brown rot) and class 4 (100% brown rot) [6]. The incidence of brown rot (Ibr) was calculated as the ratio

$$\mathrm{Ibr} = \frac{n}{N}$$

where *n* is the number of symptomatic fruits and *N* is the total number of fruits scored.

The severity of brown rot (Sbr) was calculated by the equation

$$\mathrm{Sbr} = \frac{\sum(xi\ ni)}{n}$$

where *xi* represents the disease class, in the number of diseased fruits in class *xi*, and *n* the total number of diseased fruits [19].

Infection Index Based on qPCR Results

Given that qPCR is a qualitative and quantitative detection method, both aspects were in the definition of an Infection index (I_i) suitable for statistical analysis. The limit of quantification (LOQ) of this assay was estimated at cycle threshold (*Ct*) 37, corresponding to about two pathogen cells in the analyzed sample [18]. Here, to avoid borderline misinterpretations of data, this limit was prudentially set to *Ct* 35, thus assuming the null presence of the pathogen for any *Ct* value beyond this threshold. Consequently, if N_p is the number of samples positive for pathogen's infection ($Ct < 35$) and N_t is the total number of chestnut samples analyzed for each treatment (i.e., 6 chestnuts per 12 plants, 2 reps, 144 in total), αt represents the proportion of infected samples per treatment as follow:

$$\alpha_t = \frac{N_p}{N_t}$$

Additionally, detracting the actual *Ct* measured in each sample (Ct_i) from the thresh- old 35, it is possible to define the coefficient ΔCt_i that will return positive values only for infected samples ($Ct < 35$), increasing proportionally with the infection level.

$$\Delta Ct_i = 35 - Ct_i$$

Combining both the values, it is possible to calculate an Infection index I_i as:

$$I_i = \Delta Ct \cdot \alpha t$$

This index, pondering both the ratio of infected samples and their level of infection, summarizes in a single value the infection in each sample and thus, indirectly, the efficiency of each treatment under

evaluation.

2.2.7 Residues in Fruits

Three ripen fruits were sorted from each Kalex Zn®, Kalex® treated, and control trees. The residue of phosphonate was assessed using the official EURL-SRM (QuPPe) method by an external laboratory licensed for residues analysis in agriculture (AGRO-BIO-ECO Laboratori Riuniti s.r.l., Pomezia, Italy).

2.2.8 Statistical Analyses

Statistics were carried out with GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA) (http://www.graphpad.com/ (accessed on 8 August 2022)). For the calculation of half-maximal effective concentration (EC50) for mycelial and conidia inhibitions, the non-linear regression module "agonist vs. response" of the GraphPad Prism version 8.01 was used where the "dose" was the "agonist", and the "inhibition" was the "response". The model comparison was carried out with the extra sum of squares F-test function of the "non-linear regression (curve fit)" module of GraphPad Prism version 8.01. Parametric, ANOVA, and Dunnett's multiple comparations were used to analyze the result from isolation in the 2019 trial and incidence in the 2020 trial. Due to the lack of homoscedasticity of the data, the severity data were analyzed by Brown–Forsythe and Welch ANOVA test and Holm–Sidek's multiple comparations. Non-parametric, Kruskal– Wallis tests were used for the statistical significance of differences in the Infection index followed by Dunn Multiple comparison tests. An ANOVA test for the equality of group variances and the D'Agostino and Pearson test for normality were carried out for the choice of ANOVA or Kruskal–Wallis tests.

2.3 Results

2.3.1 In Vitro Test

The EC50 values of Kalex Zn®, Kalex®, and Mystic® 430 SC are shown in Table 2.2 for both mycelium and conidia of *G. castaneae* isolates GN01 and GNAm. EC50 values of Mystic® 430 SC were by far the lowest, being 0.06 µL/L for mycelium of both isolates, and 0.017 and 0.01 for conidia of isolates GN01 and GNAm, respectively. The EC50 values for Kalex Zn® were 3.5 and 2.4 times lower than Kalex® for mycelium of GN01 and GNAm, respectively, and five times for the conidia of the two isolates. Mystic® 430 SC at 1 µL L−1 completely inhibited the conidia germination and mycelial growth of both isolates. Kalex Zn® at 5 mL L−1 completely inhibited the conidia germination and mycelial growth of both isolates. Finally, Kalex® at 5 mL L−1 completely inhibited the mycelial growth of both isolates, while 100% inhibition of conidia germination was obtained only between 50 and 100 mL L−1. The 100% inhibition of the mycelium resulted in a fungicidal effect of the products tested, with no fungistatic effect observed when mycelium plugs were plated on PDA without the fungicide.

2.3.2 Treatment Trials

Production of conidiomata on fruits of the 2019 season was very low and irregularly distributed within the thesis; their assessment did not produce any reliable results. A total of 24 fruits per thesis (6

per block) were processed in isolation trials in the laboratory. In Figure 2.1, the percentage of isolation of *G. castaneae* for each thesis is shown as the average of the four blocks. Besides the low number of fruits processed for isolation, ANOVA was significant ($F = 6.104$; $p = 0.0025$). Dunnett's multiple comparison tests showed significant differences between each treatment and the control. After incubation in moist chambers (14 days), 94.6% of fruits were still asymptomatic, with no significant differences in Ibr and Sbr between theses.

In the season of 2020, a total of 864 were collected, 144 for each of the six theses (36 for each of the four blocks). In Figure 2.2, the Ibr and Sbr for each treatment are shown as the average of the values of the four blocks for each thesis. ANOVA was significant ($F = 5.782$; $p = 0.0002$). Dunnett's multiple comparison tests showed significant differences be- tween each treatment and the control except for the treatment with Kalex® by endotherapy. No significant differences were found both for Kalex Zn® and Mystic® between the double treatments in June and July and the triple treatment in June, July, and August.

In the case of tests carried out in 2020; the DNA of sampled fruits from each thesis (72) was analyzed by qPCR with a specific TaqMan probe [18], and the Ii was calculated. In Figure 2.3, the Ii is shown for each treatment. Again, significant differences were high- lighted between each treatment and the control at the Kruskal–Wallis test.

2.3.3 Residues in Fruits

The residues of phosphonate expressed as phosphonic acid and its salts are shown in Table 2.3.

2.4 Discussion

The present paper provides for the first time a comparative study on the efficacy of chemical treatments for the control of the "brown rot" of chestnut fruits incited by *G. castaneae* in orchards. Specifically, the efficacy of phosphonate salts to control a no-oomycete plant pathogen was demonstrated in comparison with the use of Tebuconazole (Mystic®).

Phosphonate salts have been widely used since the 1970s in agriculture, agroforestry, and forestry to control oomycetes and more recently fungal driven diseases [20]. Indeed, their application in the management of non-oomycete diseases, along with other functionalities, demonstrates their versatility in agriculture and more broadly. Guest and Bompeix [21] suggested phosphonate-based products as the optimal protectant for annual and perennial crops, being "systemically translocated in both the xylem and phloem, have protective and therapeutic activity, a complex mode of action involving several biochemical mechanisms, are persistent in the plant but ephemeral in the environment, leave no toxic residues and be cheap enough to provide economic returns to the grower". Furthermore, the lack in plants of an efficient enzymatic system that promotes the oxidation of phosphonate to phosphate [22,23]

makes these products highly persistent in plants con- tributing to their success as protectants [20]. Their activity as crop protectants is related to three different mechanisms of action that have been studied mostly against oomycetes plant pathogens (i) indirect stimulation of host plant defense responses; (ii) changes in the production of compounds produced by pathogens that affect the plant's defense; and (iii) direct fungistatic effect towards pathogens [20,24]. Whether these mechanisms are all in- volved in protection against non-oomycetes plant pathogens still has yet to be clarified.

Kalex Zn® at the recommended doses (3 L/ ha, crown spray) was efficient in control- ling the disease with no statistical differences with Mystic® (recommended dose, 350 mL/ha, crown spray). The efficacy of Kalex Zn® was demonstrated in 2019 and 2020 experimental trials in orchards employing different methods of assessment, such as isolation of the pathogen in pure culture, assessment of symptoms, and detection of the pathogen by qPCR. In *in vitro* experiments, Kalex Zn® required a lower dose to inhibit radial growth and conidia germination of *G. castaneae* than Kalex®. In addition to phosphonate, zinc metal is also reported to have a sensible protective activity against plant pathogens and to represent a potential substitute for copper in commercial products [25]. Moreover, the fungistatic potential of nanoparticles of ZnO against fungal pathogens such as *Fusarium* sp., *Botrytis cinerea*, *Penicillium expansum*, *Aspergillus niger* and *Rhizopus stolonifer* has been reported [26–28].

The results of the use of Kalex® (K-phosphonate, 0.4 mL/trunk injection) were controversial. In the 2019 experimental trial, it was efficient in controlling the pathogen and its presence in fruits. However, in 2020, it did not significantly reduce the expression of symptoms, although it was associated with detection of the pathogen in fruits significantly lower than the untreated control. It must be pointed out that in 2019, trunk injections were applied immediately after the budburst, while in 2020, it was during blooming. One possible reason for failure in protection in 2020 could be related to the date of treat- ment corresponding to the blooming phase and the time needed to translocate the product to flowers to assure protection from infection. The time of translocation of trunk injected K-phosphonate in perennial crops was studied in avocado to protect against *Phytophthora cinnamomi*. In these studies, translocation in leaves was evident after 24 h, while a longer time was needed for detection in roots [29]. It must be clarified that the choice of trunk injection in the present study was determined by the fact this method (at the same doses) is widely used to rapidly translocate K-phosphonate to protect chestnut trees against the ink disease causal agents *P. cinnamomi* and *P. cambivora* [11]. Whether a single treatment at the budburst phase might protect trees from both "ink disease" and "brown rot" must be further studied in experimental trials. Potassium phosphonate salts have been reported as effective in the infection caused by other plant pathogens such as *Hymenoscyphus fraxineus* [30], *Venturia inaequalis* [31], *Phytophthora infestans* [32] or *Plasmopara viticola* [33].

In the present study, the *in vitro* dose-dependent response of *G. castaneae* to Kalex Zn® and Kalex® (direct effect) was investigated, evidencing a fungicidal effect of both products, although at different doses. The doses showing a fungicidal effect *in vitro* are in the range of the concentration found in fruits. Thus, we can speculate that protection from brown rot is due to a direct fungicidal effect of the products instead of a resistance induction mechanism. However, a correlation between *in vitro* and *in planta* doses of phosphonate salts efficient for pathogen suppression is not always possible. Indeed, a suppression dose might depend on several factors including the media composition and pathogen development stage for *in vitro* tests, while the nutritional status of the plant can make the difference for *in planta* efficacy [20]. For instance, in the present study, the EC50 for inhibition of radial growth or conidia germination was different for both Kalex Zn® and Kalex®.

The results of the present study also support the epidemiological evidence given by Shuttleworth and Guest [7] about female flowers as the main site of infection. Indeed, the August treatment in addition to the spring one with both Mystic® and Kalex Zn® did not result in any significant reduction of disease expression or detection of *G. castaneae* in fruits compared to a single spring treatment at the blooming stage. This provides indirect evidence of the relevance of flowers as the site of infection [7] and the role of airborne inoculum as demonstrated by Lione et al. [34].

The use of phosphonate salts is differently regulated on a global scale. It is considered a systemic fungicide in Australia, the USA, and South Africa, where it is widely used in cultivated and natural ecosystems. In these areas, it is homologated on a wide range of plant hosts, including tree nuts and fruit trees, by trunk injection and/or crown spray. In South America, phosphonate salts (either K or Zn salts) are still homologated as fertilizers [35], thus with almost no restrictions. In Europe, with the entry in force on July 2022 of the new regulation on fertilizers (REGULATION (EU) 2019/1009 OF THE EUROPEAN PAR- LIAMENT AND OF THE COUNCIL of 5 June 2019), any molecule based on phosphonates will be definitively banned from fertilizers and will be used only if homologated as a pesticide on target crops/plants. Now, in Europe, K-phosphonate is homologated as a systemic fungicide under the commercial name of Century® SL (BASF Italia Spa, Cesano Maderno, Italy) and is usable only on grapes by crown spray. Thus, the possibility of using phosphonate salts to protect against "brown rot" of chestnut fruits depends on country- based regulations and, in addition, i) on their categorization as a fertilizer or fungicide; ii) for those homologated as systemic fungicides, on the plant host range for which the product is registered; and iii) on the modality of treatment for which they are authorized, basically crown spray and/or trunk injection.

A further aspect related to the use of phosphonate salts refers to the residues in the final product and the Maximum Residue Limit (MRL) according to local regulations. In the present study, according to the current bibliography [20], residues after crown spray treatments were lower than those after

endotherapy. However, both values were far lower than, for instance, the MRLs recently issued by the EU regulation 2021/1807 that increase the limit for chestnut fruits to 1500 mg/kg (https://eur-lex.europa.eu/legal-con-tent/IT/TXT/?uri=uris-erv%3AOJ.L_.2021.365.01.0001.01.ITA&toc=OJ%3AL%3A2021%3A365%3ATOC) (accessed on 8 August 2022).

2.5 Conclusions

Most of the control strategies are applied post-harvest, and little is known about the use of fungicides to reduce nut infection in orchards early in the season. This is the first study showing the efficiency of products with the minimum environmental impact. Phosphonate salts, and at first Zn-phosphonate, are highly effective to protect chestnut fruits from the "brown rot" fungus *G. castaneae*. Its use in orchard management may complement the post-harvest processing of fruits by "heat treatments" in water that resulted efficient in completely inactivating *G. castaneae* in fruits [6]. Indeed, the adoption of the ap- propriate management from the orchard to the processing plant will assure the minimal impact of the disease on fruit stocks for the market. Although a still ongoing debate on phosphonate salts use and efficacy in agriculture [20,24,36], they can be considered an optimal fungicide in chestnut orchards because of the low environmental impact when used at the recommended doses, the high translocability and stability, and the multiple mechanisms of actions. Unfortunately, their availability on the market can be locally un- certain depending on the differences in categorization and authorization for their use. Further efforts should then be put into reinforcing the evidence of their efficacy also for no-oomycetes plant pathogens such as *G. castaneae*.

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

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2.7 List of figures

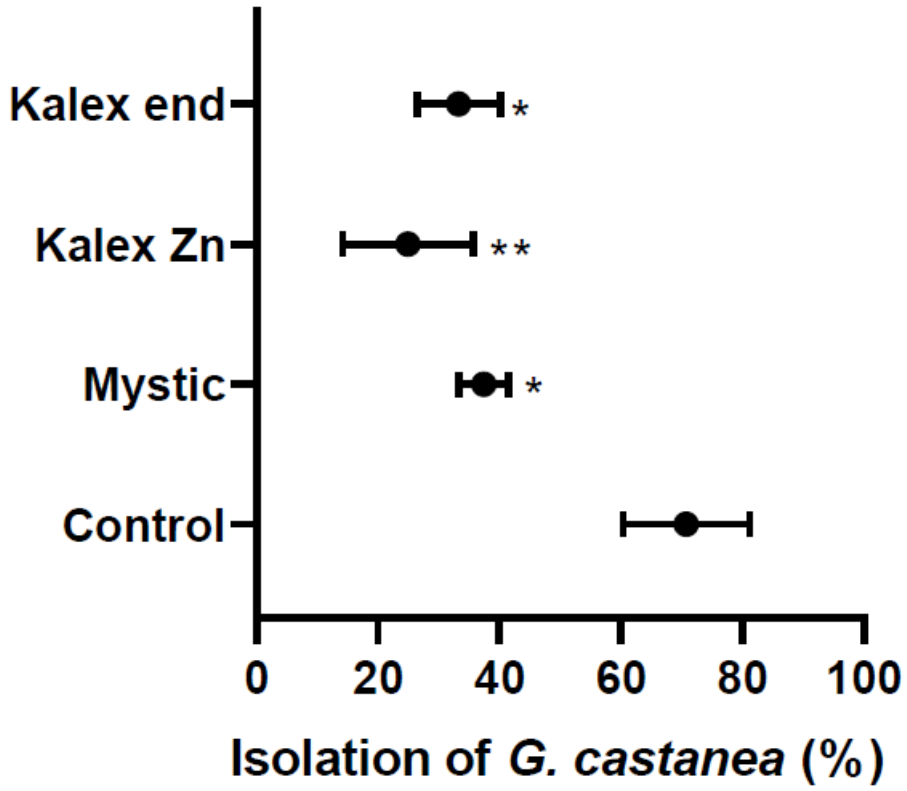

Figure 2.1 Percent of isolation of *Gnomoniopsis castaneae* from chestnut fruits of trees in the four treatments of 2019. Bars represent the SEM. Statistical differences with the untreated control at the Dunnett's Multiple comparison tests are evidenced by * = $p < 0.05$, and ** = $p < 0.01$.

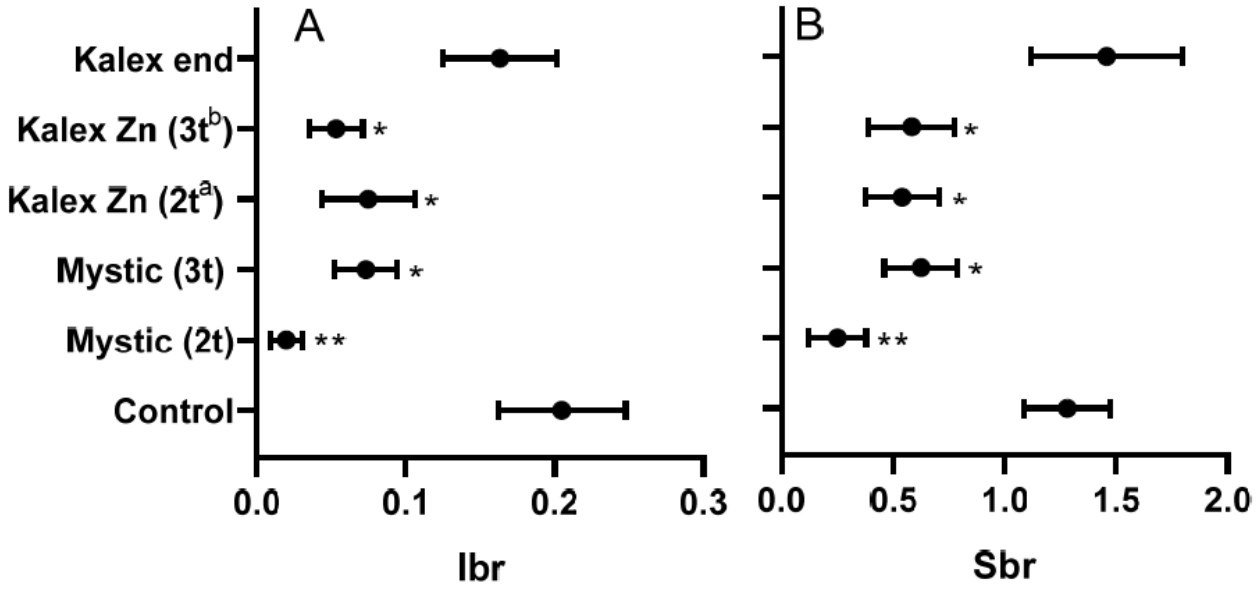

Figure 2.2 Incidence (**A**) and severity (**B**) of brown rot (Ibr and Sbr, respectively) of chestnut fruits collected from trees in the six treatment theses in 2020. Bars represent the SEM. Statistical differences with the untreated control at Dunnett's multiple comparisons (**A**) and Holm–Sidek's multiple comparison (**B**) tests are evidenced by * = $p < 0.05$, ** = $p < 0.01$. a two treatments (June and July), b three treatments (June, July and August).

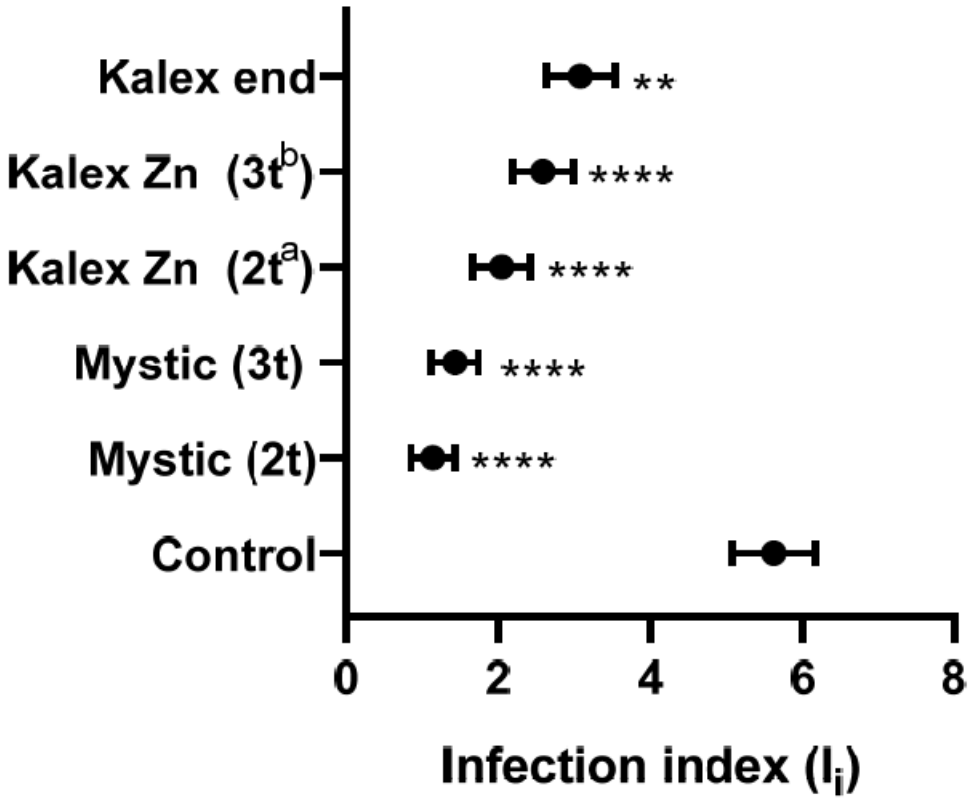

Figure 2.3 Infection index (Ii) based on qPCR-specific detection of *Gnomoniopsis castaneae* fromchestnut fruits of trees in the six treatments in 2020. Bars represent the SEM. Statistical differences with the untreated control at the Dunn multiple comparison tests are evidenced by ** = $p < 0.01$, and **** = $p < 0.0001$. a: two treatments (June and July), b: three treatment (June, July and August).

2.8 List of tables

Table 2.1 Details of treatments, rate, application date, and phenological stage for field trials conducted at the chestnut orchard.

Year	Product	Application	Rate	Date	Phenological Stage
2019	Kalex®	endotherapy	0.8 mL/tree	13 June	After bud burst
2019	Kalex Zn®	crown spray	3 L/ha	25 June and 6 July	Blooming and burr formation
2019	Mystic® 430 SC	crown spray	350 mL/ha	25 June and 6 July	Blooming and burr formation
2019	Control	-	-	-	-
2020	Kalex®	endotherapy	0.8 mL/tree	23 June	Blooming
2020	Kalex Zn®	crown spray	3 L/ha	23 June and 9 July	Blooming
2020	Kalex Zn®	crown spray	3 L/ha	23 June; 9 July and 27 August	Blooming; burr development and kernel development
2020	Mystic® 430 SC	crown spray	350 mL/ha	23 June and 9 July	Blooming
2020	Mystic® 430 SC	crown spray	350 mL/ha	23 June; 9 July and 27 August	Blooming; burr development and kernel development
2020	Control	-	-	-	-

Table 2.2 Values of 50% effective concentration (EC50) for inhibition of radial growth and conidia germination of 2 isolates (GN01 and GNAm) of *Gnomoniopsis castaneae* by Mystic® 430 SC, Kalex Zn® and Kalex®.

	EC50 (μL L^{-1})			
	GN01		GNAm	
	Mycelium	Conidia	Mycelium	Conidia
Mystic® 430 SC	0.06	0.017	0.06	0.01
Kalex Zn®	8×10^2	2×10^3	1×10^3	1.8×10^3
Kalex®	2.8×10^3	1×10^4	2.4×10^3	1×10^4

Table 2.3 Residues of phosphonic acid and its salts in fruits treated with Kalex Zn® by crown spray, Kalex® by endotherapy, and untreated control. SEM, standard error.

	Phosphonic Acid and Its Salts (mg/Kg ± SEM)
Kalex Zn®	7.05 ± 0.2
Kalex®	19 ± 4.2
Untreated control	0.19 ± 0.03

CHAPTER III

Impact of 'brown rot' caused by *Gnomoniopsis castaneae* on chestnut fruits during the post-harvest process: critical phases and proposed solutions

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Abstract

BACKGROUND: The brown rot fungus, *Gnomoniopsis castaneae*, is the main organism responsible for the outbreak of chestnut postharvest decay that is threatening the sustainability of the chestnut market in Europe. Currently, no specific strategy is available to mitigate the impact and remediate the high losses of fruits in postharvest storage. In the present study, the different phases of chestnut handling in a standard facility plant were analysed by evaluating the amount of fruit rot and infection by *G. castaneae* at each phase.

RESULTS: The warm bath (48 °C) was identified as the critical phase, requiring strict parametrization to effectively inactivate *G. castaneae* in fruits. Laboratory tests indicated that maintaining fruits at 50 °C for a maximum of 45 min provided optimal conditions to completely inactivate *G. castaneae* inoculum during postharvest handling. However, the warm bath at 50 °C and over was not effective in inactivating the complex of fungal taxa responsible for contamination and development of molds. Higher temperatures and extended treatment times caused significant losses in fruit quality, as indicated by taste panel evaluation. Upscaling of postharvest facilities is discussed and critically evaluated.

CONCLUSION: The warm bath (50 °C for 45 min) is effective in completely inactivating *G. castaneae* in fruits but did not reduce the impacts of the complex of molds responsible for external contamination and mycotoxin production.

Supporting information may be found in the online version of this article.

Keywords: *Castanea sativa*; internal decay; sterilization; fungal contamination; sensory qualification

3.1 Introduction

Before the outbreak of the brown rot fungus *Gnomoniopsis castaneae*, G. Tamietti (syn. *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew 26 & D.I. Guest),[1,2] fruit spoilage caused by insects, fungi, bacteria, and abiotic factors was considered to be one of the problems of the sweet chestnut industry and market in different production regions worldwide, but generally affecting a low percentage of fruits in storage. As a consequence, various nut- treatment techniques were developed. The most common treatments are water curing (*curatura* in Italian) and thermo- hydrotherapy (warm bath), developed in Italy during the 1930s.3 The *curatura*, known in the past as 'novena', because fruits were usually kept in water for 9 days,[4] is a classical method used for its proven ability to control insect infestation, killing the larvae of pests (mainly *Curculio* spp. and *Cydia* spp.) and molds without influencing nut quality.[5-7] In contrast, sterilization consists of sub- merging the nuts in steel bins containing a warm water at a temperature recommended to be in the range of 48–52 °C for 35–45 min followed by rapid cooling in tap water (c.a. 15 min).[5,8,9] This method was proposed to kill insect larvae in a shorter time than the *curatura*, although it could also reduce, but not eliminate, the mycoflora contaminating the fruits.[5,8,9] More recent studies introduced the use of hot air-assisted radiofrequency treatments as a physical method to reduce the rate of mold contamination of chestnut fruits in storage, specifically targeting *Penicillium* spp.[10] Studies were published employing a combination of biological and physical methods,[11] or the use of a range of chemical treatments[12] targeting contaminating microorganisms. These methods reduced contamination by a range of fungal taxa associated with the spoilage and rot of the product in postharvest, including *Acrospeira mirabilis*, *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Sclerotinia pseudotuberosa*, *Colletotrichum acuta- tum.*, *Fusarium* spp., *Mucor* spp., *Penicillium* spp., *Phoma castanea*, *Phomopsis endogena*, *Phomopsis viterbensis*, *Rhizopus* spp., *Trichoderma* spp., and *Trichothecium roseum*.[5,13-19]

Postharvest problems in sweet chestnut changed in the first decade of the 21st century, when European and Australian chest- nut growers first noticed a severe increase in internal rot in the fruits, locally affecting up to

80% of production in particular sea- sons.[2,20-22] This new disease was named brown rot for its typical symptomatology and was demonstrated to be caused by *G. castaneae*, a fungus commonly found as an endophyte in most chestnut tissues and known to cause a range of different symptoms in the genus *Castanea* and other tree species.[19] The severe impact of the pathogen in the last decade was associated with a massive presence of inoculum in the environment boosted by cli- mate change,[23] and in synergy with infestation by the Chinese gall wasp *Dryocosmus kuriphilus* Yasumatsu.[24-26] Chestnut rot results in browning and necrosis of the endosperm and embryo.[1] The symptoms are mainly expressed postharvest, although rot- ting fruits can be found in burrs on the tree before harvest.[20] Notably, fruits that appear healthy have latent infections of the pathogen that, in conditions of optimal temperature and presence of water, rapidly colonize the endosperm in postharvest conditions. Quantifying the impact suggests that incidence may be as high as 93.5% in north-west Italy,[2,23] 72% in Australia,[22] and 21% in Switzerland;[27] the disease was more recently recorded in North America[28] and Chile (Vannini, unpublished).

The sudden outbreak of chestnut rot exacerbated the problems of fruit spoilage, putting the sustainability of the chestnut market at serious risk in different regions of Europe: 'business-as-usual' fruit-handling methods were not able to remediate the strong impact of the disease postharvest. Thus, efforts are required to analyze the commonly employed postharvest handling chain, deter- mining the critical phases that exacerbate the problem, and those that may best contribute to a reduction in brown rot incidence.

The aims of the present study were (i) to evaluate the impact of *G. castaneae* and associated brown-rot symptoms at each phase of handling under standard conditions, and to highlight critical phases in postharvest handling; and (ii) to determine the optimum parameters for sterilization to assure complete and durable inactivation of *G. castaneae* inoculum, without affecting the organoleptic characteristics of the fruit.

3.2 Materials and Methods

This work was carried out during the 2018 harvest season in one of the largest chestnut-producing districts in Central Italy, the Monti Cimini area (province of Viterbo, Italy), hitherto characterized by highly productive managed orchards and coppices. The chestnut fruits analyzed in this study were harvested during the 2018 pro- duction season and were provided by the Mastrogregori Ltd processing facility (www.mastrogregori.it/, Vallerano, Italy). Chestnut fruits at this facility are passed through the following phases: (P1) delivery to the plant when a rough estimation of overall dam- age is carried out by visual assessment of 0.1% per batch with a minimum of 1 kg; a first grading is carried out, and the product is separated from impurities by forced air; (P2) curatura when the product is immersed in tap water (10–15 °C) for 72–96 h in large bins (ratio fruits: water = 2:1 v/v); (P3) soaking phase, where the product is rapidly immersed in tap water in a large bin – floating fruits and debris are removed; (P4) the sterilization phase (warm bath) at a temperature of 48 °C5 for 35–40 min in continuous flow (ratio fruits: water = 1:1 v/v) followed by a cooling phase in tap water (12–17 min) and drying with forced air.

The equipment can handle up to 5000 kg h −1 of chestnuts. The water in the warm bath is changed daily and the tank is cleaned. The water in the cooling tank is changed continuously to maintain a low temperature (15 ° C); (P5) grading and manual selection before storage in large refrigeration cells at 0 ± 2 °C and 90% ± 1% RH.

3.2.1 Impact and presence of Gnomoniopsis castaneae and total fungal community during chestnut handling phases in a chestnut warehouse

Evaluation of the presence of G. castaneae in chestnut fruits

Three fruit samples (500 ± 30 g; ca. 30 fruits per sample) were collected randomly at the end of phases 1 to 5; half of the sample was analyzed at time 0 (T0) and the other half was stored for 30 days (T30) at 0 °C. Fruits were opened, the presence of 'brown rot' recorded, and symptomatic fruits were analyzed to check for the presence of *G. castaneae*. Isolation in pure culture was carried out as follows: after shell removal, each kernel was split in half with a sterilized razor blade. Five fragments of each symptomatic fruit were placed onto Petri dishes containing potato dextrose agar (PDA) (Oxoid, Basingstoke, UK, 39 g L−1) amended with streptomycin sulfate (0.06 g L−1) (PDAs) and incubated at 20 ± 1 °C.[29] After 7 days of incubation, cultures were scored for the presence of *G. castaneae* colonies. Cultures were re-scored after 30 days to con- firm the absence of growth on fragments that were scored negative at day 7. Identification was based on the morphology of the colony and reproductive structures and was ultimately confirmed by molecular barcoding using the protocol reported by Morales-Rodriguez et al.[30] DNA was extracted from fresh mycelium grown in PDA with the NucleoSpin Plant II mini kit (Mackery Nagel, Düren, Germany) following the manufacturers' instructions. The nuclear Internal Trascribed Spacer (ITS) of rDNA was amplified and sequenced from a subset of each sample using the primers ITS4 and ITS1F.[31] Amplicons were purified with NucleoSpin Gel and PCR Clean-up (Mackery Nagel, Düren, Germany). Sequencing reactions were performed by Eurofins Genomics Laboratory (Ebersberg, Germany). Forward and reverse sequences were assembled and edited using BioEdit (Ibis Bioscience, Carlsbad, CA, USA) and com- pared to accessions in the National Center for Biotechnology Information (NCBI) database. Data were expressed based on the percentage of rotted fruits and the percentage of fruit where *G. castaneae* was isolated. The experiment was repeated twice.

Evaluation of the total fungal community in water during the sterilization and cooling phase

One-and-a-half liters of water were collected from each the sterilization tank (48 °C) and the cooling tank in phase 4. Serial dilutions were prepared using the protocol described by Hirte;[32] 500 µL of each dilution was plated on PDA; ten dishes per dilution were prepared and the experiment was repeated twice. Fungal colony-forming units (CFU) were counted after 72 h of incubation at 20 ± 2 °C. Fungal morphotypes

were transferred on fresh PDA, incubated for 7 days at 20 ± 2 °C and identified by sequencing the ITS region, as described in the previous subsection.

3.2.2 Laboratory trials to evaluate the efficacy of warm bath treatment on the survival of G. castanea in chestnut fruits

To investigate the impact of the warm bath treatment on the survival of *G. castaneae* inoculum, the experimental design included temperature and time as factors. Thus, three temperatures (45, 50, and 54 °C) plus control (20 °C), and three times (30, 45, and 60 min) were tested, for a total of 12 treatments. A circulating water bath was used (TSCIR35, ThermoFisher Scientific, Waltham, MA, USA) with temperature stability of ±0.1 °C and temperature uniformity of ±0.05 °C. There were two replicates of 1 kg (ca. 60 fruits) per treatment. After water treatment, each sample was cooled in cold water (4 °C) for 1 h and air dried until the weight before treatment was attained. After 15 days at 0 °C in a cold room (90% ± 1% RH and airflow 12 $m^3 h^{-1}$), the rot class from all fruits treated was assessed. The internal rot class was estimated according to a visual scale where 0 = healthy fruit; 1 = rot up to 50%; 2 = rot >50%. For each treatment, six chestnut fruits were randomly sorted from each rot class (0, 1 and 2) and five fragments of each fruit were placed onto Petri dishes containing PDA before incubation at 20 ± 2 °C. After 7 and 30 days of incubation, plates were scored for the presence of *G. castaneae* as described above. Data were expressed as the number of *G. castaneae* positive fragments.

Furthermore, 10 mL of water used on each treatment was analyzed for the presence of *G. castaneae* by serial dilutions, as described above.

3.2.3 Sensory evaluation of chestnut fruits after warm bath treatment: laboratory trials

A sensory evaluation was carried out to evaluate changes in the main organoleptic traits of fruit after hot-water treatment. Four temperature treatments (50, 54, 58, and 60 °C) effective in inactivating *G. castaneae* inoculum, three times of exposure (30, 45, 60 min) and two storage times (no storage, 2 weeks of storage at 0 °C in cold room) were combined in a randomized block design to obtain 48 different treatments (1 kg of chestnuts per treatment). The quantitative descriptive analysis (QDA) method[33] was used by a group of ten trained panelists as an analytical-descriptive method. The members of the panel were similar in experience, and training background. A sensory evaluation environment was set up with white booths and plastic containers to hold each test sample and water. The containers held five fruits and the tasters were invited to eat as many fruits as they wished from each treatment and then to fill in the evaluation. The samples were evaluated from left to right and the order was randomized in each booth. The sensory evaluation took place in a single session and was recorded manually using report forms especially prepared for the test.[34]

Four sensory attributes considered important for consumer acceptance and potentially affected by warm bath treatment[35] were chosen for evaluation: crispness, bitterness, shell color, and pulp color. Sweetness was also considered as the indication of a higher risk of perishability due to the release of free sugar as a substrate for contamination by saprotrophic micro- organisms. Qualitative variables of the descriptors were trans-

formed into quantitative variables using a numerical four- degree scale (Table 3.1). The evaluation was carried out using untreated chestnuts as controls, for which all descriptors were scored 1.

3.2.4 Statistical analysis

Data normality and equal variances were tested using the Kolmogorov–Smirnov and Bartlett tests, respectively. Percentages of brown rot and *G. castaneae* isolation in the chestnut-handling phases fulfilled these assumptions and were further analyzed by a one-way ANOVA, followed by Tukey's honest significant difference (HSD) post-hoc tests at the 95% family-wise confidence level ($P < 0.05$). To compare between two sets as the percentages of brown rot and *G. castaneae* isolation at T0 and T30, and CFUs from water collected from the sterilization tank and the cooling tank in phase 4, an unpaired Student's *t*-test was performed. All analyses were carried in Prism version 8.00 (GraphPad Software, San Diego, California, USA).

Data from laboratory trials to evaluate the efficacy of hot-water treatments were not normally distributed; therefore, a general linear model (GLM) was employed[36] using Statgraphics Centurion XVI, v16.1.18 (Statgraphics.Net, Madrid, Spain). A Welch ANOVA and Games-Howell's multiple comparison tests were applied to analyze CFUs from water samples in laboratory trials because of significant differences in Bartlett's tests for equal variances.[37] Analyses were carried out in Prism version 8.00.

Past version 4.0 (Paleontological Statistics Software, Oslo, Nor- way) was used to obtain a radar chart of the panel test data.

3.3 Results

3.3.1 Impact and presence of Gnomoniopsis castaneae and total fungal community during chestnut handling phases in a chestnut warehouse

Evaluation of the presence and impact of G. castaneae in chestnutfruits from standard treatment

The one-way ANOVA showed significant differences ($P < 0.001$) in the percentage of rotted fruits among phases at both storage times (T0; F = 16.87; T30; F = 7.14). The percentage of fruits with rot increased during the phases of the postharvest handling: treatments carried out in phases 2 and 4 (*curatura* and sterilization, respectively) presented the lower percentage of rot (16%) but this increased to 25% at phase 5 (manual sorting). Tukey's HSD post-hoc test results are shown in Fig. 3.1(A). After 30 days of storage, the percentages of brown rot doubled in all phases. In the case of the last phase (manual sorting) corresponding to a commercially ready product, the percentage increased from 25% to 54%. An unpaired *t*-test between T0 and T30 showed significant differences at each phase, with the percentage of brown rot always higher after 30 days (Fig. 3.1(A)). In the same way, for the isolation of *G. castaneae*, a one-way ANOVA showed a significant reduction ($P < 0.001$) in the percentage of isolation during the postharvest handling phases analyzed at both times (T0; F = 35.45; T30; F = 5.16). The percentage of isolation was between 82% and 78% for the first three phases of the industrial chain (respectively, P1, P2 and P3) subsequently decreased to 20% for P4 (sterilization) and 5% for P5 (manual sorting) (Fig. 3.1 (B)). The effect of the treatments carried out in the

handling chain were not permanent and at T30 the percentage of *G. castaneae* isolation increased. An unpaired *t*-test between T0 and T30 at each phase showed a significant ($P < 0.001$) increase in isolation of *G. castaneae* after storage at P4 (F = 40) and P5 (F = 47.79).

Evaluation of the total fungal community in water during phase4 (sterilization and cooling)

Sterilization water contained a significantly higher number of fun-gal CFUs than cooling water (unpaired *t*-test, $P < 0.0001$), with values of 926 and 340 CFU mL^{-1}, respectively (Fig. 3.2). The same fungal taxa (8) were identified from both sterilization and cooling water, including *Penicillium echinulatum, P. glandicola, P. glabrum, Trichoderma orientale,* then *Cladosporium cladosporoides, Geotrichum candidum, Talaromyces amestolkiae,* and several Mucora-ceae. *Gnomoniopsis castaneae* was never detected.

3.3.2 Laboratory trials to evaluate the efficacy of hot-water treatment on the survival of G. castaneae in chestnut fruits

Treatments at 50 and 54 °C resulted in the total and permanent inactivation of *G. castaneae*: the pathogen was not isolated from any fruits that were analyzed, regardless of the storage time and rot class. Thus, these two treatments were not considered in the following statistical analysis. The GLM, with *G. castaneae* isolation as dependent variable and temperature, time (30, 45, 60 min), and rot class (0, 1, 2) as categorical factors, was significant ($F = 9.56$; $P < 0.0001$) (Table 3.2).

However, because of the significant temperature × rot class interaction ($P = 0.0005$), GLMs were performed for each temperature (20 and 45 °C) with time and rot class as categorical factors. At 20 °C, the GLM was significant ($F = 4.30$; $P < 0.001$). Rot class was a significant factor ($F = 16.24$; $P < 0.001$), whereas time and the interaction rot class × time interaction were not ($F = 0.03$ and $F = 0.52$ respectively; $P > 0.05$). Tukey's HSD post-hoc test showed significant differences between rot class 0 (healthy fruits) and rot classes 1 and 2 (Fig. 3(A)). At 45 °C, the GLM was also significant ($F = 2.20$; $P < 0.05$). The rot class was significant ($F = 2.21$; $P < 0.005$), but time and the interaction rot class × time were not ($F = 1.03$ and $F = 1.21$ respectively; $P > 0.05$). Tukey's HSD post- hoc test showed significant differences between the rot class0 and classes1 and 2 (Fig. 3.3(B)).

A presence of fungal CFUs was detected in all warm bath treatments although in decreasing numbers from 45 °C to 54 °C (Fig. 3.4). For each time (30, 45, 60 min) significant differences were found between temperatures: 30 min ($W = 13.89$; $P = 0.001$); 45 min ($W = 13.89$; $P = 0.0018$), and 60 min ($W = 72.04$; $P < 0.0001$).

A total of seven morphotypes were identified, three of which (*Penicillium echinulatum*, *Galactomyces geotrichum*, and members of the *Mucoraceae*) represented 79.3, 13.5, and 6.9% of the total CFU. *Gnomoniopsis castaneae* was not detected from sterilization water in any temperature, time, or rot class.

3.3.3 Sensory evaluation of chestnut

The values for each descriptor are shown in a radar chart at T0 (a) and T15 (b) (Fig. 3.5). At T0, differences compared with the control (fruits in water at 20 °C, T0, descriptors reference value = 1) were evident but within

40% deviation, except for the treatments at 58 °C for 45 and 60 min, where differences were over 50%. At T15, the fruits treated at 50 °C for 30 and 45 min deviated from the controls (fruits in water at 20 °C, T15, descriptors reference value = 1) by 4% and 6% respectively, whereas deviations were much higher for the other treatments, ranging from 30% to 154%. The results of the sensory evaluation of chestnut fruits are reported as a single descriptor and the total score for each temperature and time of treatment are available in the supplementary information Table S3.1.

3.4 Discussion

In the present study, an assessment of the impact of *G. castaneae* on the quality of the fruits in postharvest during each of the phases of handling was carried out in a facility in central Italy. This analysis provided very useful, detailed information on the importance of specific phases in mitigating or exacerbating the impact of the disease. It is important to highlight that halting the progression of the disease relies on irreversible inactivation of the patho gen inoculum. Assuming efficient and continuous sanitation of the handling plant, active inoculum is mostly introduced with sweet chestnuts in the delivery phase (P1). On delivery, there is a rather high proportion of rotted fruits, as determined by destructive mycological analyses of fruit samples; nevertheless, some rotten fruits enter the plant and pass through all the post- harvest handling phases, thereby introducing active inoculum of *G. castaneae*. In the second phase (*curatura*), the sanitation mechanism is attributed to the partial lactic and alcoholic fermentation taking place during the treatment, which results in a reduction in pH, and an increase in alcohol and acetaldehyde content, coupled with a probable diffusion of phenol compounds from the pericarp to the seed.[4] The present study suggested that the cold bath did not inactivate the *G. castaneae* inoculum. Moreover, there was no fungistatic effect either, as the percentage of isola tion of *G. castaneae* was not significantly different from that of the previous phase. The soaking phase (P3) also did not affect the proportions of rotten fruits or *G. castaneae* isolation frequency.

The soaking phase enables the detection and removal of debris and floating chestnuts, commonly those damaged by insects. Chestnuts affected by brown rot, even at an advanced stage of rot, do not float on water. In contrast, P4 drastically reduced the percentage isolation of *G. castaneae*, although it did not result in total inactivation of inoculum in the running conditions at the handling plant (48 °C; 40 min). The effect was more fungistatic than fungicidal because after 30 days storage isolation frequency returned to the values obtained immediately after phase 1. As also reported by Jermini et al.,[5] the warm bath (45–48 °C) did not sup- press the complexity of mold species present in the chestnuts, some of which also produce mycotoxins; the molds were easily recovered from both the warm bath and the cold bath of the cool- ing phase. These taxa were the same species or co-generic with those responsible for causing molds in chestnuts, including *Peni- cillium* spp.[38] Several previous publications reported work aiming to develop sustainable chemical and biological treatments of chestnuts for postharvest treatments to reduce fruit spoil- age.[11,12,38,39] The most effective techniques must be considered in the near future to control the complex communities of organ- isms other

than *G. castaneae* responsible for fruit spoilage.

The fungistatic effect resulting from P4 continued into the grading and manual selection phase. This phase did not significantly affect both the percentage of rotted fruits or the frequency of isolation of *G. castaneae*. The whole process in the facility plant did not reduce the impact of *G. castaneae*, as demonstrated by the severe and significant increase in the percentage of rotted fruits after 30 days of storage at 0 °C. The *sterilization* phase is the crucial stage for further work to optimize the process and thereby reduce the impact of *G. castaneae* in chestnuts postharvest, thus increasing storability, safety, and shelf-life of the fruit. Laboratory tests suggested that a warm bath at 50 °C for 30 or 45 min was tomography.[43] In fact, recent studies on the feasibility of Fourier transform near infrared (FT-NIR) spectroscopy or visible-near infra- red hyperspectral imaging in detecting 'brown rot' of chestnut fruits demonstrated the feasibility of the approach[44] and the need for an upscale from prototype to commercial equipment. A molecular tool for the sensitive detection and quantification of *G. castaneae* in symptomatic and asymptomatic fruits has been developed to quantify the *G. castaneae* inoculum.[45] Furthermore, a recent study by Vettraino et al.,[46] provided promising evidence on the use of ozone in storage conditions, specifically as a fungistatic option to avoid increasing chestnut rot in stored stocks. It is clear that an integrated protocol must be developed including the most promising methodologies and technologies to be applied in postharvest handling.

3.5 Conclusions

In conclusion, the present study demonstrated that application of strict conditions at the *sterilization* phase of chestnut handling can definitively inactivate *G. castaneae* in non-symptomatic and rotting fruits. Sterilization at 50 °C and over was not effective in inactivating the complex of fungal taxa responsible for contamination and development of molds, many of which produce mycotoxins. The efficacy of the warm bath strictly depends on the quality of the fruit stocks entering the handling plant and the conditions to which they are exposed before the P4 phase. An efficient strategy for the control of 'brown rot' must include mitigation protocols applied in the open field, followed by effective physical treatments in the handling plant.

The positive results obtained in this work, albeit preliminary, were practical to a targeted application of low-impact containment methods for *G. castaneae* and represent a promising step for- ward for harvest technologies employed in chestnut handling facilities. However, further studies are needed, both at laboratory and at industrial plant level, to develop a better understanding of the critical points of fungal contamination in the chestnut treatment chain.

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3.7 List of figures

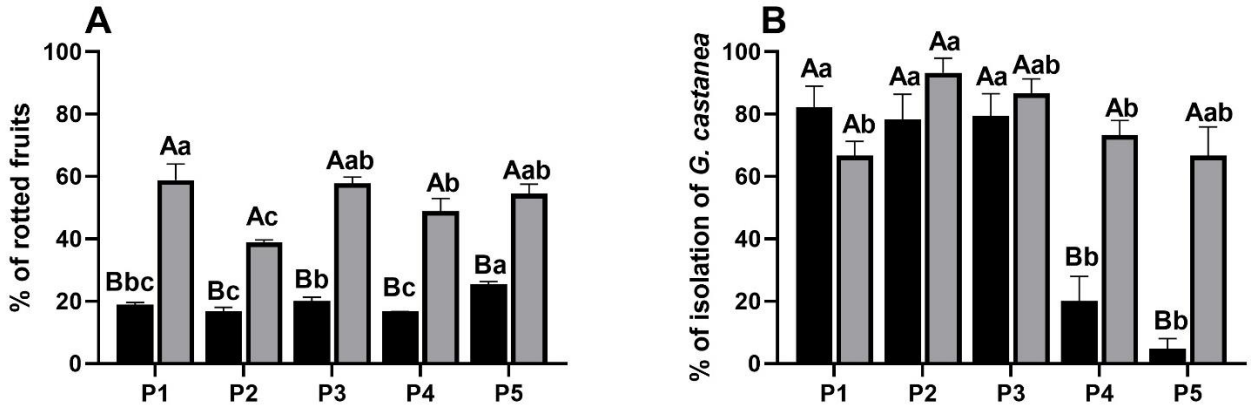

Figure 3.1. Percentage of brown rot (± SEM) (A) and percentage of isolation of *G. castaneae* from rotten fruits (± SEM) of *Castanea sativa* (B) in the five defined phases of postharvest, at time 0 (T0; black bars) and after 30 days of storage at 0 °C (T30, grey bars). The same lower case letters indicate no significant differences among phases (Tukey's HSD post-hoc test). The same upper case letters indicate no significant differences at each phase between T0 and T30 (unpaired *t*-test; $P < 0.001$).

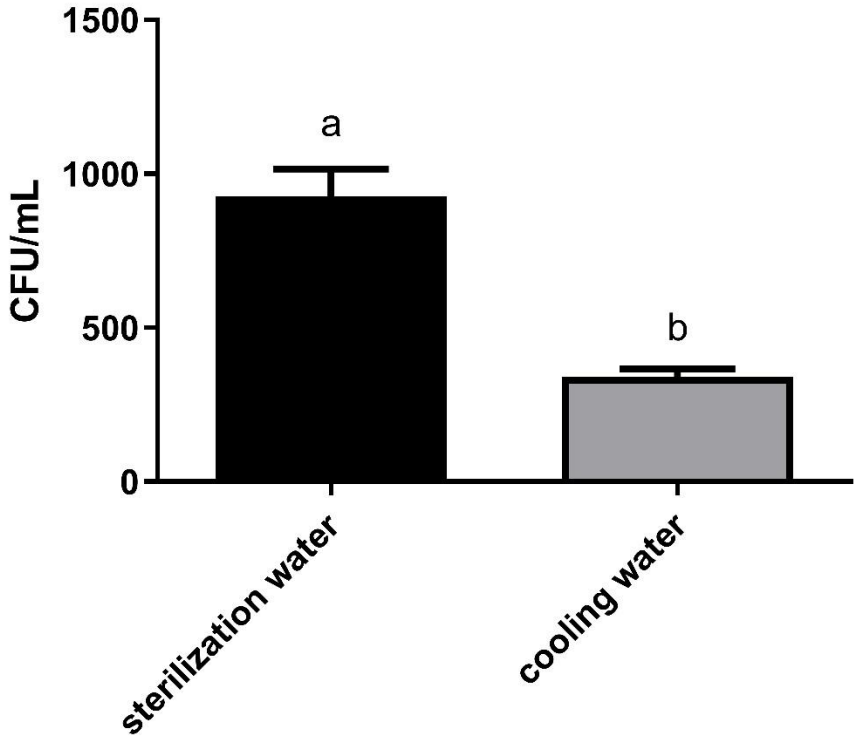

Figure 3.2 Fungal colony-forming units (CFU mL−1 ± SEM) in 50 °C sterilization water (black bar) and cooling water (gray bar). Values followed by different letters differ significantly (unpaired t-test, P < 0.0001).

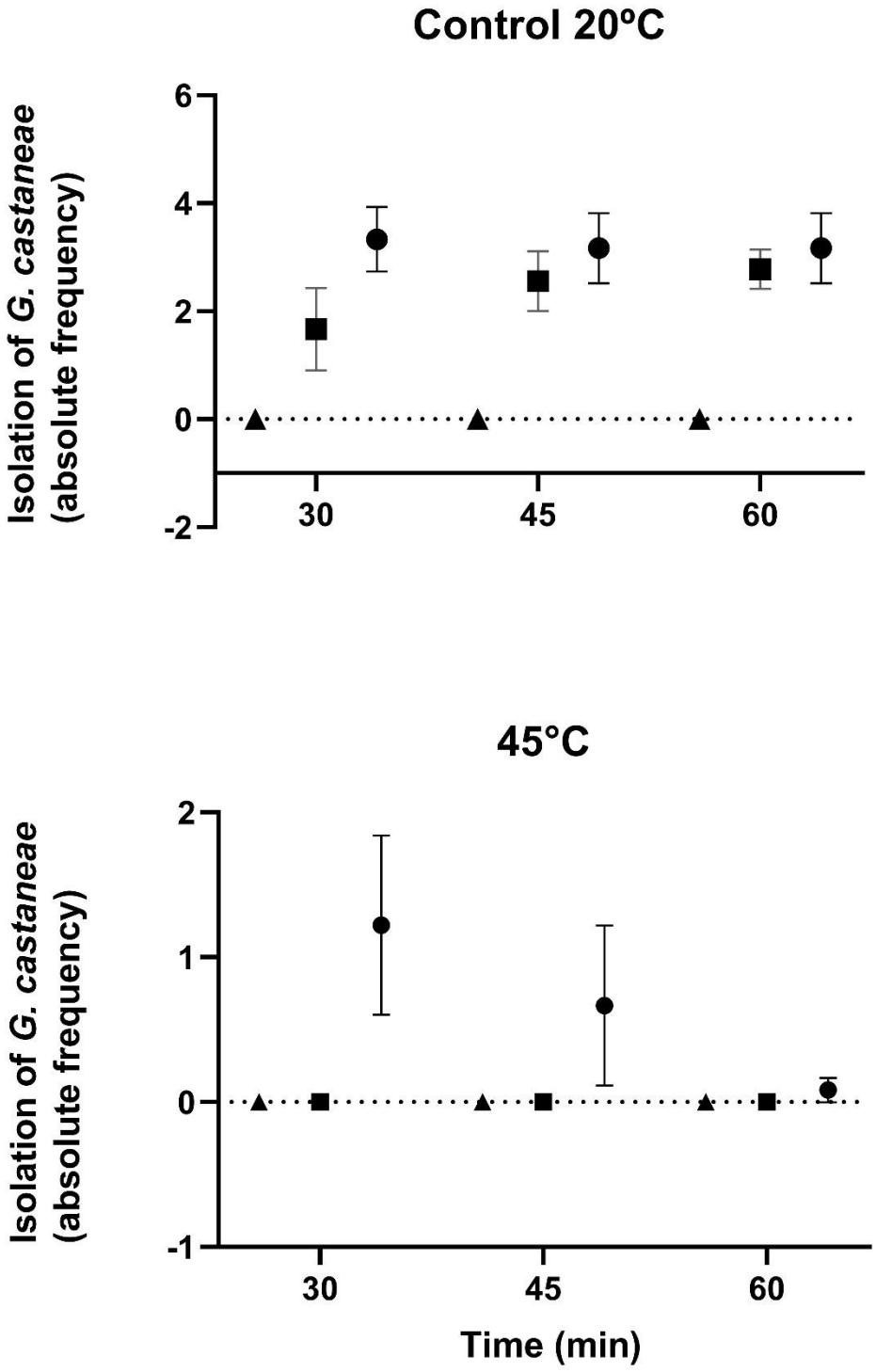

Figure 3.3 Absolute frequency of *G. castaneae* isolation from fruits in rot classes 0 (no-symptomatic, triangle), 1 (up to 50% of rotted pulp, square), 2 (rotten pulp over 50%, circle) at control temperature (20 °C) (A) and 45°C (B). For each time of treatment, points with the same letter were not significantly different (P > 0.05) according to Tukey's test. Error bars indicate SEM.

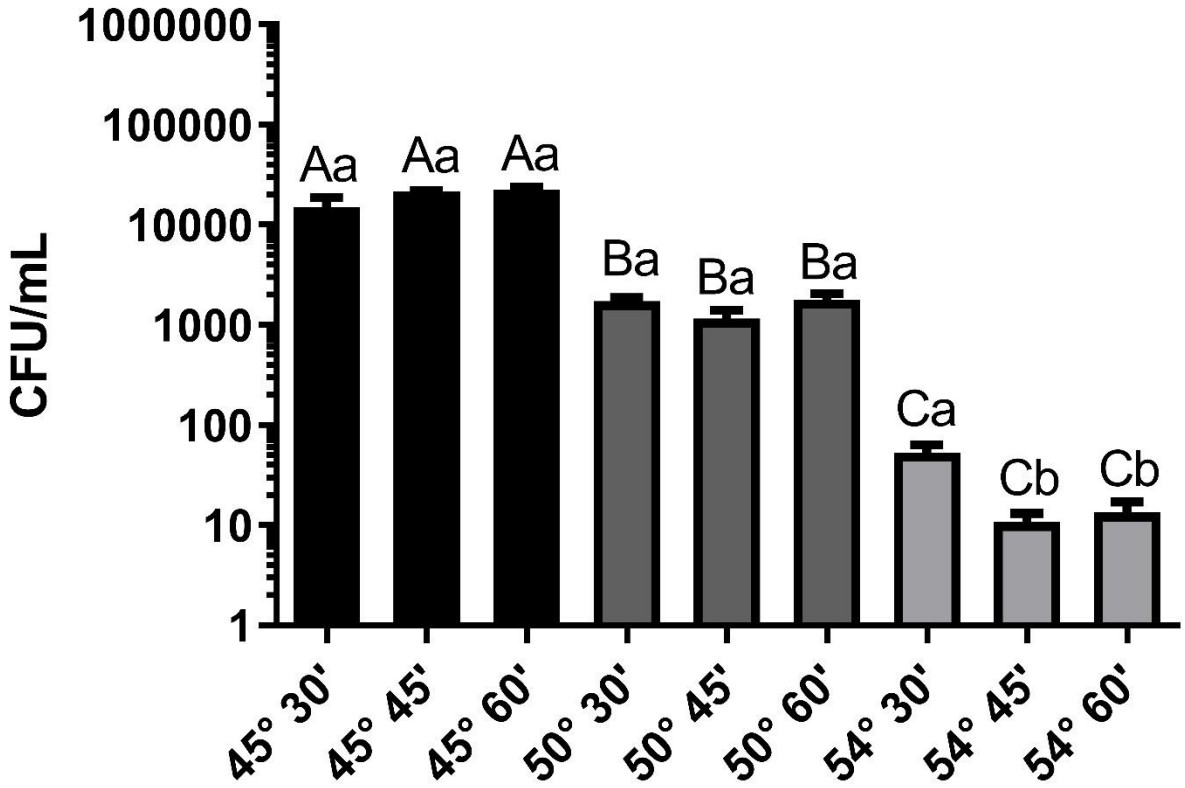

Figure 3.4 Fungal CFUs from warm-bath treatments (45°, 50° and 54 °C) of chestnut fruits. Different upper case letters indicate significant differences between temperatures at each time (Games–Howell multiple comparison test). Different lowercase letters indicate significant differences between time at each temperature (Games–Howell multiple comparison test). Error bars indicate SEM.

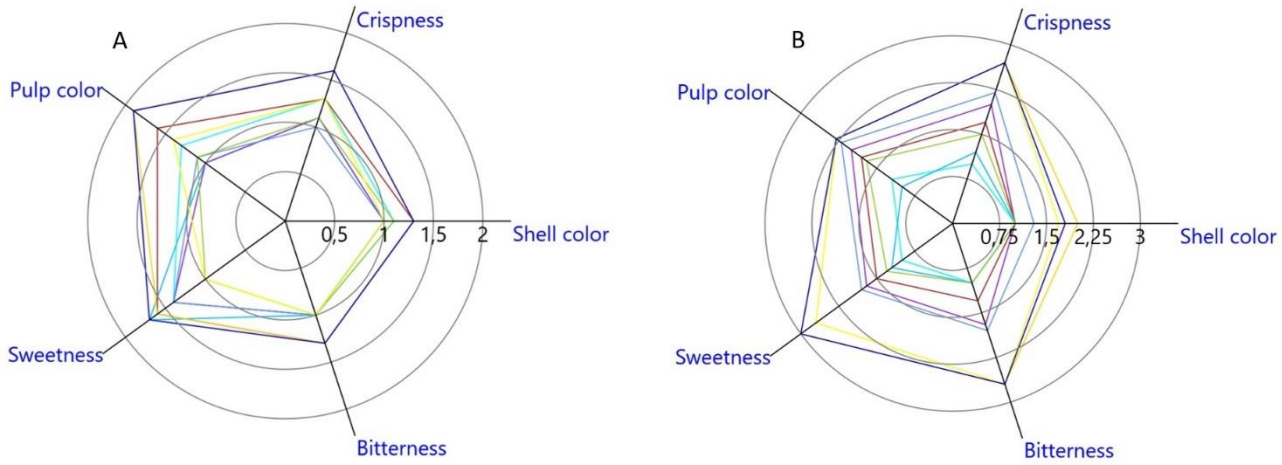

Figure 3.5 Radar chart of the sensory evaluation of chestnut fruits for each descriptor at T0 (no storage; A) and T15 (after 15 days of storage at 0 °C; B).

3.8 List of tables

Table 3.1 Qualitative descriptors used in for sensory analysis.

Table 3.1 Qualitative descriptors used in for sensory analysis

Descriptors	1	2	3	4
Crispness	normal	slightly altered	altered	absent
Bitterness	absent	tolerable	bitter	very bitter
Sweetness	normal	Slightly altered	sweet	very sweet
Shell color	normal	slightly altered	altered	very altered
Pulp color	normal	slightly altered	altered	very altered

Table 3.2 General linear model (GLM) for *G. castaneae* isolation with water temperature, time of treatment, and rot class, as categorical factors.

Table 3.2 General linear model (GLM) for *G. castanea* isolation with water temperature, time of treatment, and rot class, as categorical factors

Source	*Sum of squares*	*df*	*Mean sum of squares*	*F-test*	*P-value*
Temperature	100.463	1	100.463	48.05	**<0.0001**
Rot class	75.6594	2	37.8297	18.09	**<0.0001**
Time	0.366072	2	0.183036	0.09	0.9162
Temperature * rot class	33.6612	2	16.8306	8.05	**<0.001**
Temperature *time	1.47767	2	0.738 837	0.35	0.7030
Rot class * time	6.36837	4	1,59209	0.76	0.5521
Temperature * rot class * time	4.49737	4	1.12434	0.54	0.7082
Residual	275.982	132	2.09077		
Total	54.824	149			

Supplementary Table 3.1 Individual score for each descriptor and total score of the sensorial evaluation of chestnut fruits following hot-water treatments for different times. The last column expresses the deviation in percentage from the control whose score for each descriptor was fixed at 1.

Storage (days)	Temp (°C)	Time (min)	Individual score					Total score	Deviation from control (%)
			Shell color	Crispness	Pulp color	Sweetness	Bitterness		
none	50	30	1,1	1,3	1,3	1,4	1,0	6.1	22
		45	1,0	1,1	1,0	1,7	1,0	5,9	18
		60	1,0	1,1	1,0	1,4	1,0	5,6	12
	54	30	1,3	1,3	1,6	1,6	1,3	7,0	40
		45	1,0	1,0	1,1	1,4	1,0	5,6	12
		60	1,1	1,1	1,1	1,0	1,0	5,4	8
	58	30	1,0	1,3	1,4	1,0	1,0	5,7	14
		45	1,3	1,6	1,9	1,6	1,3	7,6	52
		60	1,3	1,6	1,9	1,7	1,3	7,7	54
15	50	30	1,0	1,0	1,2	1,0	1,0	5,2	4
		45	1,0	1,2	1,0	1,2	1,0	5,3	6
		60	1,0	2,0	2,0	1,7	1,7	8,3	66
	54	30	1,0	1,7	1,8	1,5	1,3	7,3	46
		45	1,3	2,2	2,2	1,8	1,8	9,3	86
		60	1,0	1,5	1,7	1,3	1,0	6,5	30
	58	30	1,7	2,7	2,3	2,7	2,7	12,0	140
		45	2,0	2,7	2,3	3,0	2,7	12,7	154
		60	1,8	2,7	2,3	3,0	2,7	12,5	150

CHAPTER IV

Fungal community and mycotoxins contamination in chestnut fruits during post-harvest conditioning and storage

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ABSTRACT

Chestnut quality is greatly affected by postharvest fungal colonization and mycotoxin contamination. In order to understand how fungal colonization varies on fruits, the composition of mycobiota was investigated along different phases of post-harvest handling and in different fruit parts (shell and kernel). Using a high-throughput sequencing (HTS), identified fungi were clustered into 314 operational taxonomic units (OTUs). Within these OTUs, specifically those known as main mycotoxins producer species were investigated; 47 OTUs were identified as belonging to *Penicillium* genus, 8 OTUs belonging to *Fusarium* genus and 2 OTUs belonging to *Aspergillus* genus. Two *Penicillium* OTUs represented up to 40% of total OTUs, two *Fusarium* OTUs up to 10% and the only two *Aspergillus* OTUs barely represented 0,2% Mycotoxins detection was performed separately for shell and kernel samples by ELISA test for total Aflatoxin (B1, B2, G1, G2), Ochratoxin A, Fumonisins and T-2/HT-2. The results showed a generally higher mycotoxin contamination on shell than in kernel samples. Mycotoxins detection was always within the limits recommended by EU, confirming the fruit healthiness for human consumption. For the first time, FBs and T-2/HT-2 contamination was found on chestnuts, even if under the limits. FBs and T-2/HT-2 detection resulted positively correlated with a single *Fusarium* OTU, while no correlations were found between AFs and OTA and the respective producer taxa. Investigating the circumstances and causes of fungi and mycotoxins contamination on post-harvest chestnuts is crucial to determine whether these contaminations occur in fresh chestnuts, throughout the industrial chain, or because of gradually accumulation.

KEYWORDS: post-harvest, chestnut handling, fungal contamination, High-Throughput Sequencing, mycotoxins.

4.1 Introduction

The quality of fresh chestnuts is limited due to microbial decay after harvest and during storage, leading to unmarketable chestnuts (Donis-Gonzalez et al., 2008). Microbiological parameters, including the presence of fungi, are of fundamental importance, since some of these fungi may produce carcinogenic mycotoxins (Jardim & Caldas, 2009). Depending on how they are processed and stored, chestnuts can potentially promote the growth of a wide variety of fungi due to their high starch and moisture levels (Wareing et al., 2000; Barreira et al., 2010). Molds can taint chestnuts before they are harvested as well as during shipping, storage, and processing. In addition, several fungi that have been isolated from chestnuts are well-known producers of mycotoxin (Prencipe et al., 2018). During post-harvest, other opportunistic fungi that can contribute to chestnut contamination and rot include *Phomopsis endogena* Speg. (cif. *Phoma endogena* Speg.), *Phomopsis viterbenis* Camici, *Diplodia castanea* Prill and Del., *Alternaria* sp, *Aspergillus* sp., *Penicillium* spp., *Botrytis cinerea* Pers., *Gloesporium* sp., *Fusarium* spp., *Dothiorella* sp., *Cytodiplospora castanea* Oud., *Pestalotia* spp., *Diplodia* spp., *Acrospeira mirabilis* Berk. and Broome and *Rhizopus* spp (Breisch, 1993; McCarter et al., 1980; Montealegre and Gonzalez, 1986; Paglietta and Bonous, 1979; Puttoo et al., 1988; Ridé and Gudin, 1960; Washington et al., 1997; Wright, 1960). *Fusarium* spp., *Penicillium* spp., and *Aspergillus* spp., are well known as mycotoxin producers of chestnuts and goods made from them (Abdel-Gawad and Zohri, 1993; Bertuzzi et al., 2015; Jermini et al., 2006; Overy et al., 2003; Pietri et al., 2012; Prencipe et al., 2018; Rodrigues et al., 2013; Wells and Payne, 1975). Despite all attempts to prevent fungal contamination, toxic fungi are common in nature and are frequently found in the world's food sources. Only under favourable conditions—which differ for each species based on adaptability—do fungi grow and produce mycotoxin. Mycotoxins are secondary metabolites that are found in a broad range of food and feed, such as cereals, spices, coffee, nuts or dried fruits (Zöllner et al., 2006). They contaminate the diet of a large proportion of the world’s population and represent a global public health issue (Wambacq et al., 2016), with the highest exposure reported in developing countries (Shephard, 2008). Consumption of dried fruits tainted with mycotoxins might have negative effects on human health (Trucksess et al., 2008; Fernández-Cruz et al., 2010; Ozer et al., 2012). Mycotoxins have a wide range of chemical and physical properties due to their enormous structural diversity. According to the European Union's Commission Regulation from 2006, the most significant mycotoxins in food and feed include aflatoxins (AFB1, AFB2, AFG1, AFG2), ochratoxin A (OTA), type A and B trichothecenes (such as HT-2 toxin, T-2 toxin, and deoxynivalenol (DON)), Fumonisins (FBs), and zearalenone. Recent years have brought attention to the potential presence of aflatoxins (AFs: AFB1, AFB2, AFG1, AFG2) in chestnut products (Liang et al., 2019; Prencipe et al., 2018). In 2010 and 2011, the European Rapid Alert System for Food and Feed (RASFF, 2011) shared several Food Safety Notifications regarding high aflatoxin contamination in dried chestnuts, flours, and flakes produced in Italy (EU

2010.0995, 2010.1614, 2010.1615, 2010.1616 and 2011.0550). The International Agency for Research on Cancer (IARC) has categorized AFs as a class 1 carcinogen (Castegnaro & Wild, 1995). Some researchers have shown that fresh and stored chestnuts contain ochratoxin A (OTA) (Donis-Gonzalez et al., 2009; Overy, Seifert, Savard, & Frisvad, 2003). Ochratoxin A (OTA) is a mycotoxin generated by several fungi, including *Aspergillus ochraceus, A. carbonarius, A. niger*, and *Penicillium verrucosum* (Bui-Klimke et al., 2015). The International Agency for Research on Cancer has included OTA in its Group 2 carcinogens (WHO and IARC, 1993). Moreover, produced by cultures of *Fusarium verticillioides* (Sacc.) Nirenberg (previously known as *Fusarium moniliforme* Sheldon), the FBs are a class of food-borne carcinogenic mycotoxins produced by 15 different *Fusarium* species (Rheeder et al., 2002) and classified as "Group 2B carcinogens," or probably carcinogenic to humans, (WHO and IARC, 1993). One of these *Fusarium* species, *F. proliferatum*, shows natural occurrence on chestnuts (Tosi et al., 2015). Trichothecenes, which are a subset of *Fusarium* mycotoxins with more than 150 compounds, include T-2 and HT-2 toxins (EFSA, 2017). These mycotoxins are produced by species *Fusarium sporotrichioides* and *Fusarium poae*, found on dried fruits but without reference to the maximum levels in nuts.

Although mycotoxin contamination may be an important risk for consumer health and global trade, few studies have addressed the problem; therefore, the influence of storage conditions during chestnut post-harvest on mycotoxin contamination has scarcely been investigated. Our previous study (Morales-Rodriguez et al., 2022) reported the impact of brown-rot caused by *G. castaneae* at each phase of handling under standard conditions, to highlight critical phases in chestnut postharvest; other fungal contaminations were not considered, even if fungal taxa responsible for contamination and development of moulds were isolated from chestnuts. A new methodology able to identify the presence of many distinct taxa in a single sample is Next-Generation Sequencing (NGS) technology and metabarcoding based on Illumina de novo sequencing is a successful example (Ruiz-Gomez et al., 2019). To gain a better knowledge of how microorganisms may affect postharvest chestnut quality in Italy, our objectives were to determine the microorganisms responsible for shell mould and kernel decay on chestnuts using this technique. The aim of this work was to determine during different industrial phases and storage of chestnuts (1) the associated mycobiota, focusing on mycotoxins producer fungi, and (2) the presence of the main mycotoxins. These results should contribute to a better understanding of the influence of fungal community on chestnuts and to the identification of important fungal taxa related to mycotoxin production in post-harvest.

4.2 Materials and methods

The Monti Cimini area (province of Viterbo, Italy), one of the largest chestnut-producing districts in Central Italy, was the site of this study during the 2018 harvest season. The region has

historically been known for its extremely productive maintained orchards and coppices. Chestnut fruits used in this study were obtained from the Mastrogregori Ltd processing plant (www.mastrogregori.it, Vallerano, Italy). Chestnut at this facility are passed through 5 storage phases as reported by Morales-Rodriguez et al (2022): (P1) delivery to the plant when a rough estimation of overall damage is carried out by visual assessment of 0.1% per batch with a minimum of 1kg; a first grading is carried out, and the product is separated from impurities by forced air; (P2) *curatura* when the product is immersed in tap water (10-15 °C) for 72-96 h in large bins (ratio fruits: water = 2:1 v/v); (P3) soaking phase, where the product is rapidly immersed in tap water in a large bin – floating fruits and debris are removed; (P4) the sterilization phase (warm bath) at a temperature of 48 °C 5 for 35-40 min in continuous flow (ratio fruits: water = 1:1 v/v) followed by a cooling phase in tap water (12-17 min) and drying with forced air. The equipment can handle up to 5000 kg h −1 of chestnuts; water in the warm bath is changed daily and the tank cleaned; water in the cooling tank is continuously changed to maintain a low temperature (15 °C); (P5) grading and manual selection before storage in large refrigeration cells at 0 ± 2°C and 90% ±1% RH.

4.2.1 Collection of chestnut samples and storage

Five kg chestnuts samples were collected randomly at the end of phases P1 to P5. For all the samples, chestnuts were stored in plastic bags at -20°C at time 0 (T0) in triplicate (500 g, ± 30 fruits); for P5 samples, a half was stored in plastic bags at -20°C at time 0 (T0) in triplicate (500 g, ± 30 fruits) while the other half was stored at 4°C for 30 days (T30) in cold chamber and then at -20°C in triplicate samples. Fruits were opened and the percentage of 'brown rot' recorded, then chestnuts were ground, keeping the shell and kernel separate.

4.2.2 Mycotoxins detection in chestnut kernel and shell samples

Five g of kernels and shells were placed each in a 50ml falcon, with 25ml of methanol (70%), vortexed for 3 minutes and centrifuged for 10 minutes at 5000 rpm at 20°C. 100 µL of sample surnatant were stored at -20°C for the ELISA test for mycotoxins detection, using the VERATOX® HS Quantitative High Sensitivity Test (Neogen, Lansing, MI, USA) kits for: Aflatoxin HS (B1, B2, G1, G2); Ochratoxin HS; Fumonisin; T-2/HT-2. ELISA test was carried out according to manufacturer's instructions: hundred µL of the coniugate was placed in each mixing well; hundred µL of control and sample surnatant were transferred in each mixing well and mixed with the conjugate by pipetting it up and down 3 times. Hundred µL of the liquid of each mixing well were transferred to the antibody-coated wells and incubated 10 minutes. Contents of the antibody wells were shake out and the wells were filled and washed with distilled water 5 times. Hundred µL of the substrate were put into the wells and incubated 10 minutes, then substrates were discarded, and the plates were blocked with 100 µL of stop

solution, added in each well. The plates were read within 20 minutes in the spectrophotometer Neogen 4700 Reader, designed for use with Neogen's Veratox® microwell test kits.

4.2.3 DNA extraction and High Throughput Sequencing (HTS)

Total DNA was extracted from kernel and shell samples from each phase of the storage process, previously ground with a blender, with the NucleoSpin Plant II mini kit (Mackery Nagel, Düren, Germany) following the manufacturers' instructions. The DNA concentration was quantified, with a Qubit dsDNA BR Assay kit (Thermo Fisher Scientific Inc., MA, USA), prior to amplification. Additionally, two mock communities were created by pooling DNA from a pure culture of 10 different species of fungi; these were used as controls and were processed in the same way as the samples, following the methodology of Morales Rodriguez (Unpublished). The internal transcribed spacer 1 (ITS1) was amplified in a multiplexing PCR using a set of barcoded primers ITS1F and ITS2 for fungi. The primers were tagged with different barcodes to distinguish different samples. Moreover, 3 replicates of each control community were amplified with different barcoded primers of the ITS1F/ITS2 primer pairs. Negative controls did not originate any PCR product. The PCR reaction mixture consisted of 25 μl of Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, USA), 2 μM of each primer, 4 μl of 2 mg ml−1 BSA and 3 μl of DNA template diluted to 50 ng μl−1, in a total volume of 50 μl. The thermal cycle was an initial 5-min step at 95 °C, followed by 30 cycles of 40 s of denaturation at 95 °C, 2 min of annealing at 58 °C and 1 min of extension at 72 °C, with a final extension step of 7 min at 72 °C. For each sample and the controls, three replicates of PCR for fungal ITS were carried out and the products were pooled. Amplicons were purified using the MagJet NGS Cleanup and Size Selection kit (Thermo Fisher Scientific Inc., MA, USA) and the final concentration of purified PCR product was quantified using a Qubit dsDNA HS Assay kit (Thermo Fisher Scientific Inc., MA, USA). The resulting amplicons were pooled in equal amounts to give a total volume of 40 μL, with a concentration of 200 ng μL−1, and were sent to Eurofins Genomics AT GmbH (Vienna, Austria), to be sequenced in an Illumina MiSeq 2 × 300 bp platform.

4.2.4 Statistical and bioinformatic analyses

For the HTS dataset, to decrease the occurrence of false assignments and cross-contamination, only the reads having the combination of 5' barcode and forward primer and the expected 3' barcode and reverse primer were paired and considered for further analysis. The use of CLC Genomic Work bench Version 8.5.1 (QIAGEN bioinformatics, Aarhus, Denmark) allowed for quality filtration (limit = 0.05) and trimming, excluding all sequences with "N"s. Following this, the paired-end reads were merged. If some mismatches were recorded between the overlapping fragments of the forward and reverse reads, these were adjusted according to the base call with the higher sequencer-assigned quality score. After quality filtering, paired-end assembly, and demultiplexing, the sequences were processed,

and similarity clustering was performed based on the UPARSE pipeline of USEARCH v8 (Edgar, 2010) using a 98 % clustering threshold. Sequences failing alignment, or identified as chimeric, were removed before downstream analysis. To identify the resulting OTUs, they were uploaded as FASTA files in the BLAST tool of the Genbank database with the following algorithm parameters: max target sequence = 50; expect threshold = 0,05; word size = 28; max matches in a query range = 0; match/mismatch scores = 1, -2; gap cost = linear. The resulting xml file was imported together with the FASTA file into MEGAN (Huson et al., 2007) to organize and adjust the results. To support this process was employed the NCBI taxonomy. The lowest common ancestor (LCA) parameters were Min score = 150; Max expected = 0,01; Top percent = 5; Min support percent = 0,05; Min support = 1, and Percent to cover = 40. At the same time, the minimum requirements of similarity to accept the taxonomy were: Species 99 %; Genus 97 %; Family 95 %; Order 90 %; Class 85 %; and Phylum 80 %. A LEfSe analysis was performed to find specific taxa that could significantly affect sample structure at different levels as the basis of a strategy on the LDA threshold. (http://huttenhower.sph.harvard.edu/galaxy). Venn diagrams were developed to illustrate the number of OTUs shared between the industrial phases. These graphs were performed using an online application at http://bioinformatics.psb.ugent.be/webtools/Venn. Mycotoxins datasets were checked for normality distribution with the D'Agostino & Pearson test, Anderson-Darling test, and Shapiro-Wilk tests (α=0,05). Normalized data were analyzed by one-way ANOVA using Tukey's post hoc test ($P<0.05$). All the analyses were carried out with GraphPad Prism version 8.00 (GraphPad Software, San Diego, CA, USA) (http://www.graphpad.com/). A correlation matrix was calculated to analyse the relationships between mycotoxins detections and % of OUTs in each phase of the industrial chain. Spearman's rank analysis was performed with GraphPad Prism version 8.00, since the two variables were not distributed normally; null hypotheses were rejected in all cases when $p \leq 0.05$.

4.3 Results

4.3.1 OTUs clustering and taxonomy associated to chestnuts

From the 36 samples, after filtration and trimming, 314 OTUs were detected. Each of them was defined at the highest taxonomic level possible: Phylum, Class, Order, Family, Genus, and Species. LEfSe analysis showed that from Phylum to Species level, fungi exhibited significant differences among different chestnut tissues (LDA score > 3.5). The non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance for fungi among shell and kernel samples was statistically significant ($p < 0,05$). On chestnut shell samples (in red, Fig. 4.1A and 4.1B), the four significantly most abundant taxa were the species *Aureobasidium pullulans,* the order *Dothideales,* the genus *Aureobasidium* (same order) and the family *Saccotheciaceae* (same order). On chestnut kernel samples (in green, Fig. 4.1A and 4.1B), the species *Fusarium fujikuroi* and the two

genera *Papilliotrema* and *Tausonia* were the taxa that were significantly predominant with respect to shell samples.

The total number of OTUs detected for each category was plotted in the Venn diagrams for fungi (Table 4.1C). Between chestnut kernel and shell samples, the fungal OTUs shared were 109; the most representative and shared fungal OTUs were the genus *Penicillium*, the species *G. smithogilvyi* and *F. fujikuroi*, the genera *Didymella, Cladosporium* and *Papiliotrema* and the species *Aureobasidium pullulans*. The shell had the highest number of unique OTUs (52) such as *Pilidium* and *Coniochaeta*, while the kernel had a lower number of unique OTUs (34), including *Tomentella* and *Acremonium*.

LEfSe analysis showed that from Phylum to species level, fungi exhibited significant differences also among different phases of the chestnut industrial chain (LDA score > 3.5) (Fig 4.2A and 4.2B). On phase 1, no one of taxa was significantly predominant on the others. On phase 2 (*curatura*), the three significantly most abundant taxa were the order *Cladosporiales*, the family *Cladosporiaceae*, and the species *Cladosporium allicinum*, followed by the genus *Debaryomyces* and the family *Debaryomycetaceae*. On phase 3 (soaking phase), the three significantly most abundant taxa were the class *Saccharomycetes*, the order *Saccharomycetales* and the species *Cyberlindnera fabianii* and genus *Candida*, always belonging both to *Saccharomycetales* order. On phase 4 (thermo-hydrotherapy), only the order *Diaporthales* was the significantly most abundant taxon. On phase 5 (grading and manual selection), the three significantly most abundant taxa were the class *Tremellomycetes*, the family *Rhynchogastremataceae* and the genus *Papiliotrema* (Fig. 4.2A and 4.2B).

The total number of OTUs detected for each category was plotted in the Venn diagrams for fungi (Fig.4.2C). Between all the 5 phases, the fungal OTUs shared were 53, including the classes *Eurotiomycetes, Sordariomycetes, Dothideomycetes, Microbotryomycetes* and *Tremellomycetes*. The phase 2 had the highest number of unique OTUs (32), followed by P1 and P5 (7), P3 (6) and P4 (3). Furthermore, P1 and P2 shared the higher number of OTUs. However, each industrial phase shared fungal members. For example, P1 and P2 shared OTU of the *Fusarium* genera; P1, P2 and P3 shared OTU of the genera *Cladosporium, Alternaria* and *Phoma*; P1, P3, P4 and P5 shared more the species *Penicillium paneum*; P1, P2, P3 and P5 OTU of the genera *Rhodotorula* and *Trichoderma*; P2, P3 and P5 shared more the genus *Curvularia;* P2, P3 and P4 shared members of the order *Agaricales* and the genus *Gnomoniopisis*.

4.3.2 Analysis of fungal OTUs known as mycotoxin producers: Penicillium spp., Fusarium spp., and Aspergillus spp.

Total OTUs were investigated to evaluate mycotoxins producer species. Many studies reported, as the main mycotoxins producer, three different genera: *Aspergillus* sp., *Fusarium* sp. and *Penicillium*

sp. (Abdel-Gawad and Zohri, 1993; Bertuzzi et al., 2015; Jermini et al., 2006; Overy et al., 2003; Pietri et al., 2012; Prencipe et al., 2018; Rodrigues et al., 2013; Wells and Payne, 1975). Within the 314 OTUs detected in these studies, 47 OTUs were identified as belonging to *Penicillium* genus, 8 OTUs belonging to *Fusarium* genus and 2 OTUs belonging to *Aspergillus* genus; no one of these OTUs was identified up to the species level. In any case, the traditional molecular identification allowed to identify different species of *Penicillium* and *Fusarium* genera, such as *P. echinolatum* and *F. proliferatum*.

Focusing on these three genera of mycotoxin producers, reads were counted to evaluate their distribution along the industrial phases and reported as percentage of total reads for each phase. Percentage of each OUT's reads were summed separately for each phase of the storage chain; for *Penicillium* genus, since the number of OTUs was high and just 7 OTUs were the most abundant in number of reads, the remaining 40 OTUs were summed together and renamed generally as OTHERS. At the same time, % reads counting was done also for kernel and shell sample separately for each phase. Results are shown in Figure 4.3, 4.4 and 4.5.

Concerning *Penicillium* genus, its reads represented 6.72 % of total reads in P1, 26.42 % in P2, 7.18 % in P3, 6.28 % in P4, 7.19 % in P5 and and 7.28 % after 30 days of storage in chestnut samples, independently from the tissue (Fig. 4.3A). Looking at the 7 most representative OTUs, the highest % of reads in each phase was registered for OTU_1 and OTU_3, especially in P5 and after storage (Fig. 4.3A). Furthermore, % reads counting was done separately for kernel and shell chestnut for *Penicillium* genus (Fig. 4.3B and 4.3C). Also in this case, in both shell and kernel samples such as in all the phases, OTU_1 and OTU_3 had the highest % of reads; moreover, shell and kernel samples resulted contaminated in a similar way by OTU_1 and OTU_3, while shell samples resulted generally more contaminated than kernel samples by the other OTUs belonging to *Penicillium* genus (Fig. 4.3B and 4.3C). Significant differences were found in kernel samples for OTU_1 (Test statistic = 10.9181; $P \leq 0,05$) and OTU_3 (Test statistic = 13.7485; $P \leq 0,01$). For peel samples, significant differences were found for OTU_257 (Test statistic = 14.1363; $P \leq 0,01$) and OUT_43 (Test statistic = 13.0234; $P \leq 0,05$).

Also, the reads % of the 8 OTUs belonging to *Fusarium* genus were calculated to evaluate their distribution along the industrial phases (Fig. 4.4). *Fusarium* genus represented 1.78 % of the total reads in P1, 1.12 % in P2, 2.12 % in P3, 1.61 % in P4, 1.13 % in P5 and 0.21 % after 30 days of storage in chestnut samples, independently from the tissue. For this genus, the OTU_5 showed the highest reads % in all the phases such as in chestnut kernel and shell samples (Fig. 4.4), followed by OTU_40. Also for *Fusarium* genus, shell samples resulted generally more contaminated than kernel samples by the 8 OTUs. Differences were not statistically significant for *Fusarium* OTUs.

Regarding *Aspergillus* genus, only 2 OTUs belong to it and showed significantly lower % of reads in comparison to % of reads counted for *Fusarium* and *Penicillium* genera. *Aspergillus* genus represented 0.02 % of the total reads in P1, 0.11 % in P2, 0.03 % in P3, 0.004 after 30 days of storage, while in P5 reads of *Aspergillus* sp. were not found (Fig. 4.5A). Even if the reads % was negligible, *Aspergillus* sp. contamination was higher in kernel samples than in shell samples; moreover, in shell samples the number of reads decreased through the industrial chain. Differences were not statistically significant for *Aspergillus* OTUs.

4.3.3 Mycotoxins detection in chestnut kernel and shell samples

In total, four mycotoxins associated with the more prolific mycotoxigenic fungi detected by HTS analysis in this survey, were evaluated from chestnut tissues: AFs produced by *Aspergillus* sp., OTA produced mainly by *Aspergillus* sp. and *Penicillium* sp., and FBs and T2/HT-2 produced by *Fusarium* sp..

The results showed a widespread and not negligible contamination for all the mycotoxins detected in this work (Fig. 4.6, 4.7, 4.8 and 4.9). As regards the AFs contamination in kernel (Fig. 4.6B), there were no significant differences along the chestnut phases or in storage. Concerning the AFs contamination in shell (Fig. 4.6A), the amount in storage was significantly higher than in phase 5 at time 0 ($F = 4.206$; $P < 0,01$), with medium level of 3.1 ppb; significant differences were detected also between phase 1 and phase 5 at time 0, which was the significantly lower medium level detected for AFs (1.4 ppb). All the shell and kernel samples didn't exceed the value of 4.0 ppb (maximum limit fixed by EC Regulation 165/2010 in dried fruits for AFs).

Results showed that also OTA detection in chestnut kernel (Fig. 4.7B) had no significative differences between phases and/or times of storage and it was always lower than the maximum limit fixed by EC Regulation 165/2010 (3, 10 and 15 ppb respectively for cereals, dried vine fruit and spices; for OTA, on the basis of the information available, it does not appear necessary for the protection of public health to set a maximum level of OTA in dried fruit other than dried vine fruit, cocoa products, meat products, as they are not a significant contributor to OTA exposure and high levels of OTA); only kernel samples in P4 barely exceed the limit of 3 ppb, with a detected medium level of 3.1 ppb. Indeed, the presence of OTA in chestnut shell showed significative differences between storage (medium level of 10.6 ppb) and all the phases at time 0 ($F = 7.702$; $P < 0,01$). Significant differences were found also between phase 2 and phase 5 at time 0 (medium level of 8.4 and 6.3 ppb respectively) (Fig. 4.7A). The detected medium levels for OTA in chestnut shell samples always exceeded the EU limit, with a range of 6.2-10.6 ppb.

Regards to FBs detection, there were no significant differences in chestnut shell samples during the phases and/or in storage (Fig. 4.8A); all the samples had a detected level of FBs between 814 ppb (in

P1) and 889 ppb (in P4). Chestnut kernel samples showed indeed significant differences in contamination by FBs ($F = 53.01$; $P < 0.01$) (Fig. 4.8B). The FBs amount in P2 and P1 (medium level of 299 and 227 ppb respectively) were statistically higher than all the other phases at time 0 and during storage, followed by P3 (medium level of 110 ppb); indeed, FBs detection in P5 after storage, with a medium level of 34 ppb) was significantly lower than in all the other phases at time 0. No one of shell and kernel samples exceeded the limits set by the EC Regulation n. 1126/2007 for non-transformed corn and corn for direct human consumption, respectively 4000 and 1000 ppb.

Concerning the presence of T-2/HT-2, results showed significant differences both in chestnut shell ($F = 138.7$; $P < 0.01$) and kernel ($F = 26.12$; $P < 0.01$) (Fig. 4.9). For kernel samples, in P1 and P2, T-2/HT-2 contamination was significantly higher than the other phases and after storage, with a medium level of 82 and 68 ppb respectively; from P3 to P5 after storage, T-2/HT-2 contamination was progressively lower, up to 27 ppb. In all the phases and time of storage, levels were always lower than the indicative limit of 100 ppb set by The European Recommendation n. 165/2013, as a sum of T-2 and HT-2 mycotoxins for non-transformed cereals for human consumption (Fig. 4.9B). As regards T-2/HT-2 contamination in chestnut shell, P5 was significantly higher than the other phases (medium level of 350 ppb), followed by P2 and P3, with a medium level of 319 and 315 ppb respectively, P5 after storage (289 ppb) and P1 (221 ppb) (Fig. 4.9A); P4 contamination was significantly lower than the other phases, with a level of 198 ppb, and was the only phase to not exceed the limit recommended by EU.

4.3.4 Correlation analysis of fugal taxa distribution and mycotoxins detection in chestnut kernel and shell samples

Pearson correlation test was performed to evaluate correlations between fugal taxa and mycotoxin detections through the industrial phases. Each OTU of *Penicillium*, *Fusarium* and *Aspergillus* genera was compared to the relative produced mycotoxins: OTU belonging to *Penicillium* genus with OTA, OTU belonging to *Fusarium* genus with FBs and T-2/HT-2, and OTU belonging to *Aspergillus* genus with AFs and OTA. Based on single OTU of each genus considered, the measured mycotoxins were generally not correlated with reads %. Only OTU_123 (*Fusarium* sp.) in kernel samples resulted positively correlated with FBs and T-2/HT-2 detection ($\rho = 0.639$, $P < 0.01$ and $\rho = 0.5645$, $P < 0.01$ respectively for FBs and T-2/HT-2). Also considering the sum of OTUs of each genus considered, there was no correlation between mycotoxins detection and reads %.

4.4 Discussion

Fungal contamination on chestnut during post-harvest have been amply investigated (Wells and Payne, 1980; Breisch, 1993; Washington et al., 1997; Vettraino et al., 2005; Marinelli et al., 2009; Wright, 1960; Donis-González et al., 2016; Xiao-qing et al., 2009). In this study, mycobiota associated

to chestnuts shell and kernel in different industrial phases and storage was analysed, to focus on mycotoxin producer fungi.

Differences between kernel and shell tissues demonstrated that fungi and mycotoxin contamination on shell occurs more often than on kernel. As expected, different biochemical characteristics in different fruit parts and postharvest treatments have been reported to influence the structure of the resident microbial communities during fruit storage. In postharvest chestnuts, Donis-Gonzalez et al. (2016) reported much higher microbial counts on shells than on kernels; in the same study, the predominant pathogens caused the shell decay were *Penicillium* and *Coniophora*, but more than five mold genera were associated with infected kernels including *Penicillium*, *Acrospeira*, *Botryosphaeria*, *Fusarium* and *Sclerotinia* (Donis-Gonzalez et al., 2016).

In this survey, most of the taxa revelated by Illumina analysis are not known as mycotoxins producers and this is probably the main reason for which mycotoxins levels were mostly safe for human consumption. Despite these interesting results, other taxa resulted belonging to *Penicillium*, *Fusarium* and *Aspergillus* genera, which are capable of producing a variety of mycotoxins. Typically, these fungi have been identified as significant contaminants in almonds and chestnuts (Rodrigues et al. 2012b for review). A high presence of some *Penicillium* species is reported by Overy et al., (2003), who explained the relative high frequencies of *P. crustosum*, *P. discolor* and *P. glabrum/spinulosum* thanks to their ability to grow under the refrigeration conditions that are commonly used for chestnut storage. Indeed, other *Penicillium* species isolated at low frequencies (*P. chermesinum*, *P. glandicola*, *P. echinulatum* and *A. ochraceus*) are less tolerant to cold and will not grow at refrigeration temperatures (Domsch et al., 1980; Payne et al., 1983; Pitt, 1988; Frisvad et al., 1997). Also, Donis-Gonzales et al. (2016) reported *P. expansum*, *P. griseofulvum*, *P. chrysogenum* causing shell mold on fresh chestnuts. As *Penicillium* genus, also *Fusarium* genus, and in particular *F. verticillioides*, *F. proliferatum*, *F. tricinctum* and *F. oxysporum*, has been reported as fungal contamination on chestnut shell and kernel by several studies (Ruocco et al., 2016; Dai et al., 2001; Zhu et al., 2003; Wang et al., 2006). On the contrary, if compared to the total fungal contamination, the low representativeness of *Aspergillus* in chestnut samples in post-harvest showed that these environments condition (mainly temperature) and this fruit matrix are probably not favourable for *Aspergillus* establishment. On the other hand, *Aspergillus* was the most dominant genus isolated from nuts and dried fruit, as reported by Alhussaini (2012); in this survey, the genus *Aspergillus* was represented by 8 species of which *A. flavus* and *A. niger* were the commonest.

OTUs distribution along the phases was different for the three genera analysed and within each genus, considering single OTUs. *Penicillium* OTU_1 and OTU_3 represented up to 50% and 20% of total OTUs respectively, especially in P5 and after storage, and *Fusarium* OUT_5 represented generally up to

10% of total OTUs. Differently, other OTUs of these two genera represented less than 1% in all the phases. The only two *Aspergillus* OTUs represented barely 0,2 % of total OTUs. The low % of *Aspergillus* sp. was in line with AFs detections in chestnut kernel, always lower than the EU limits. Even if from the Illumina sequencing *Aspergillus* OTUs were not identified to species level, the AFs results could suggest that these two taxa are not those mainly responsible for AFs production (*A. flavus* and *A. parasiticus*). Results of this survey were different from those reported in the previous years. Pietri et al. (2012) found some contamination of dried chestnuts and chestnut flour by AFs: 24.3% of chestnut flour samples and 7.1% of dried chestnut samples overcame the maximum limit for AFB1 set by EC Regulation 165/2010 in dried fruits (2.0 mg kg 1) and the same samples also exceeded the limit of 4.0 mg kg 1 for total AFs. Abdel-Gawad and Zohri (1993) in Saudi Arabia reported AFs contamination in 3/5 dried chestnut samples. Differently, shell samples in this survey resulted more contaminated by AFs, especially after storage for 30 days. Despite the optimum conditions for aflatoxins production by *A. flavus* and *A. parasiticus* are at 33 °C (Milani, 2013), cold storage didn't inhibit the synthesis of these secondary metabolites, even if in P5 a significant reduction of AFs was detected. Mycotoxins detection along the chestnut chain in this case could be influenced by the accumulation of this secondary metabolites. Gbashi et al (2019) demonstrated the thermal stability of mycotoxins, suggesting that the low temperature applied during thermo-hydrotherapy cannot deactivate mycotoxins, even if it can deactivate mycotoxins producer fungi. Consequently, the amount of mycotoxins detected can increase phase by phase because of the accumulation, non-necessarily indicating that mycotoxin production increases.

On the other hand, even if the taxa linked to two main genera responsible for OTA productions (*Aspergillus* e *Penicillium*) were not identified to species level, the OTA levels detected in this study were often higher than the EU limits for products with a similar intake (cereal, spices and dried vine fruit). Generally, shell samples resulted more contaminated than kernel samples, and contamination was higher after storage, as occurred for AFs. Similar results for the presence of OTA has been reported in fresh and stored chestnuts by some studies. Pietri et al (2012) reported a worrying contamination for OTA in dried chestnut and chestnut flour: 94.6, 64.9 and 21.6% for chestnut flour samples and 71.4, 42.8 and 35.7% for dried chestnuts samples exceeded respectively the limits of 3, 10 and 15 mg kg_1 (EU limits for cereals dried vine fruit and spices -from 1.7.2012). OTA occurrence in chestnuts was reported also by Overy, Seifert, Savard, & Frisvad (2003), who isolated *A. ochraceus* and detected the presence of OTA in commercially marketed chestnuts, and by Donis-Gonzales et al. (2009), who showed how OTA occurred in fresh chestnuts and concentration increased each month after storage at 4°C. To date, no data were found about the occurrence of OTA in dried chestnuts and chestnut flour. A high presence of OTA even after thermo-hydrotherapy could be due to the fact that *Aspergillus ochraceous*, the most important OTA producer, can grow over the temperature range 8–37 °C with an

optimum of about 30°C (Ramos et al. 1998); thermo-hydrotherapy in this survey was probably able to deactivate *Aspergillus* taxa only temporarily.

In this survey safe levels of FBs detection were found in kernel samples and the amount of FBs decreased significantly along the industrial chain and after 30 days of storage. On the contrary, shell samples resulted more and constantly contaminated than shell samples, almost reaching the limit of 1000 ppb set by the EC Regulation n. 1126/2007 for corn. Sanchis and Magan (2004) reported temperatures of 15–30°C as optimum conditions for toxin secretion by *F. verticillioides* and *F. proliferatum*, the main species responsible for FBs production. In this work, the 8 taxa belonging to *Fusarium* genera, resulted more abundant in shell than in kernel samples, especially in the first phases of the industrial chain, in accordance with the FBs detection results. Similar results were found for T-2/HT-2 contamination, with significant higher contamination on shell samples than on kernel. Shell samples always exceeded limit of 100 ppb recommended by EU (n. 165/2013) and were significantly higher in P5 and after storage. Safer levels were found on kernel samples, decreasing more and more during the industrial chain. Even if *F. sporotrichioides*, the main *Fusarium* species able to produce these types of mycotoxins, has an optimum temperature of 20–25°C (Nazari et al., 2014), in this case thermo-hydrotherapy succeeded to inhibit T-2/HT-2 production only inside the kernel. Despite *Fusarium* is a complex group of ubiquitous soil fungi with saprotrophic and/or pathogenic properties (Booth, 1971), this is the first report of FBs and T-2/HT-2 contamination on chestnut, according to Liang et al (2019), who excluded FBs contamination on these fruits. Moreover, FBs and T-2/HT-2 detections showed positive correlations with. OTU_123 in chestnut kernel. This OTU, belonging to *Fusarium* genus, was not identified to species level, even if it showed 100% identity on BLAST with *F. proliferatum*, which was isolated from chestnut samples of this survey.

Concerning the production of mycotoxins by these three genera, results showed generally a low and safe concentration of these secondary metabolites, in comparison with limits and/or recommendations provided by EU. In general terms, mycotoxins detection in this work resulted always higher in shell samples than in kernel; moreover, contamination on shell increased along the chain for all the secondary metabolites investigated, while on chestnut kernels was constant (e.g. AFs and OTA) or decreased (FBs and T-2/HT-2). On the basis of these results, it is important to investigate the causes and conditions of contamination by fungi and mycotoxins. In particular, it's essential to verify if these contaminations occur in fresh chestnuts, during the industrial chain (where producer could not be eliminated by thermo-hydrotherapy) or are a result of progressively accumulation. A more detailed survey, regarding also the other major producing countries (e.g. China, Spain, Turkey), may be opportune in order to evaluate if contamination concerns only Italian products and if it may be appropriate to set a maximum admissible level for each mycotoxin investigated in this work. Yet, mycotoxins occurrence in chestnuts shell and kernel in different times of storage before P4 can indicate that those phases were not suitable to block

the fungal contamination on fruits; in P5, differences between times of storage can suggest that the storage temperature could be decrease to 0-2°C to avoid fungal and mycotoxin development.

4.5 Conclusions

Fungal and mycotoxin contamination is a major problem in various nuts and nut products and is mostly related to improper post-harvest storage conditions. The range of contaminating and dominant mycobiota on chestnuts is caused by food intrinsic parameters linked to environmental conditions (Rodrigues et al., 2013). These complications with postharvest mold, decay, deterioration of chestnuts after processing, and safety concerns all point to the need for more efficient microbial reduction techniques post-harvest and processing (Donis-Gonzalez et al., 2008). The complete elimination of contamination is an impossible task and, for that reason, the knowledge on the fungal population incidence and diversity and on their mycotoxigenic potential, as well as on environmental and storage conditions, is of major help in trying to reduce the risk of contamination. Nonetheless, information on chestnut contamination is still scarce and further studies are needed. Results from this survey showed a high diversity of fungal communities on postharvest chestnuts, and how fruit part can impact the composition of the fungal communities. Despite the low presence of mycotoxins in kernel chestnuts indicated that the contamination likely occurred during post-harvest, fungal colonization and mycotoxin production may have occurred at any stage from flowering to harvest, storage, and sorting. An efficient strategy for the control of fungi and mycotoxins contamination must include mitigation protocols applied in the open field, followed by effective physical treatments in the handling plant, able to reduce contaminations on shell during storage. This perspective must be intended to guarantee a product (fresh chestnuts, flour or dried chestnuts) with quality standards and healthiness that protects the consumer and are accepted by large retailers.

4.6 References

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4.7 List of figures

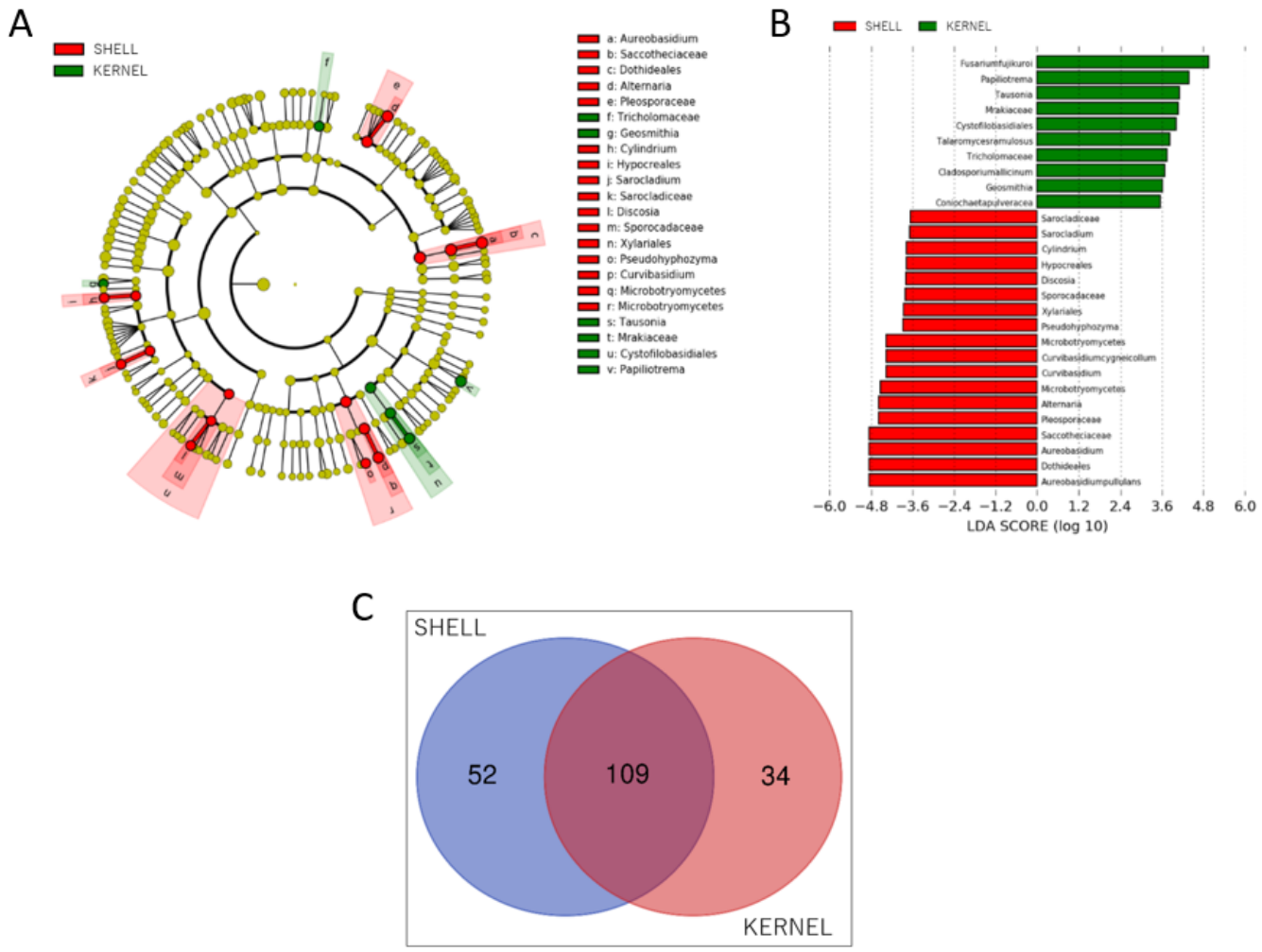


Figure 4.1 A) Cladogram of different fungal taxa from phylum to species identified between two different chestnut tissues (Shell and Kernel). Different colour nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference between different categories. B) LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have. Alpha value <0.05. C) Venn diagram built on the distribution of each fungal OTUs group of the chestnut tissue, shell and kernel.

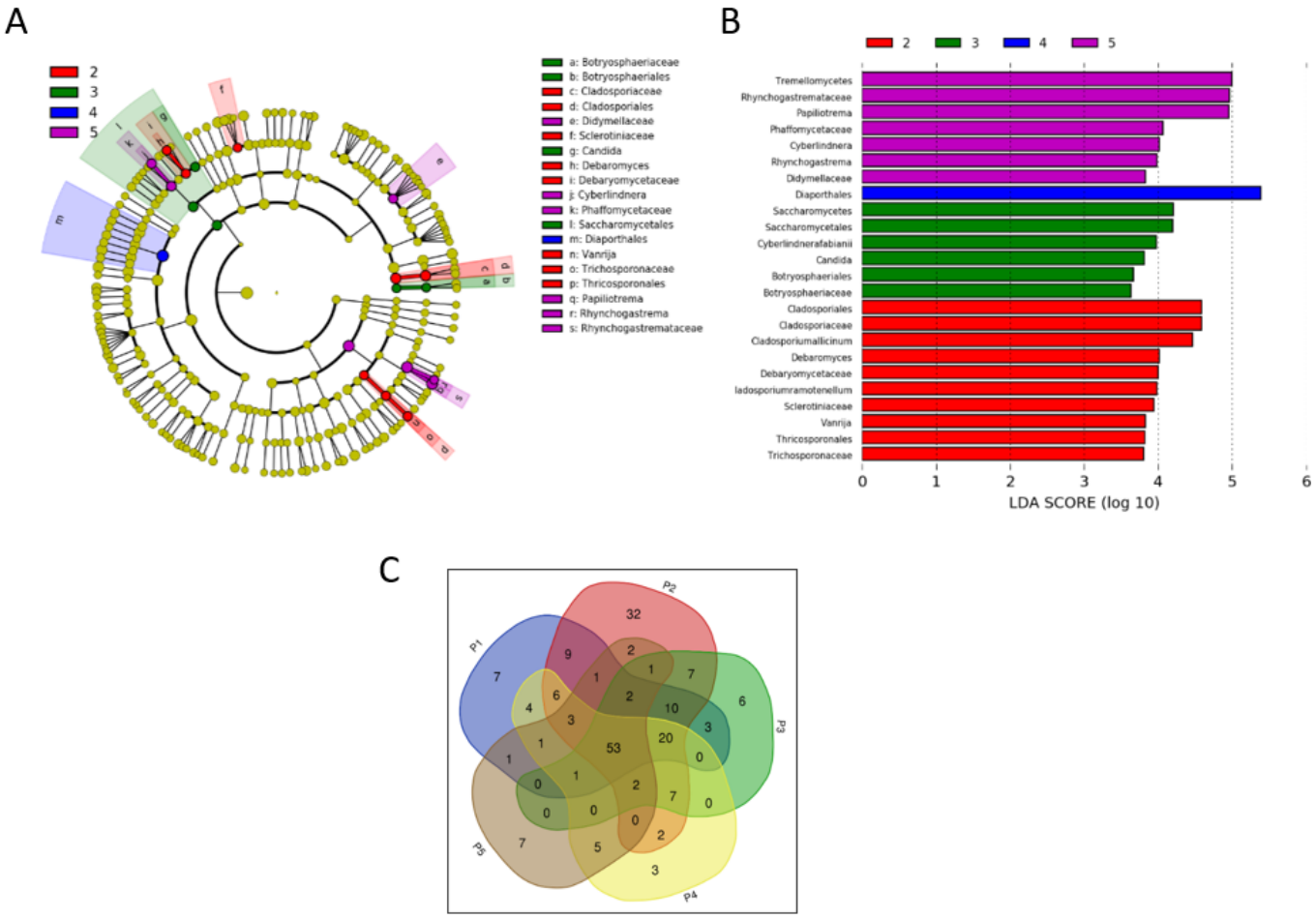


Figure 4.2 A) Cladogram of different fungal taxa from phylum to species identified between five different chestnut phases of the industrial chain: (P1) delivery to the plant; (P2) *curatura*; (P3) soaking phase; (P4) the sterilization phase (warm bath); (P5) grading and manual selection before storage in large refrigeration cells. Different colour nodes represent significantly enriched and important taxa among samples, except for yellow which means no significant difference between different categories. B) LDA graph; detected taxa ranked by effect size, the higher LDA scores the taxa are, the more effect the taxa have. C) Venn diagram built on the distribution of each fungal OTUs group of the industrial phases.

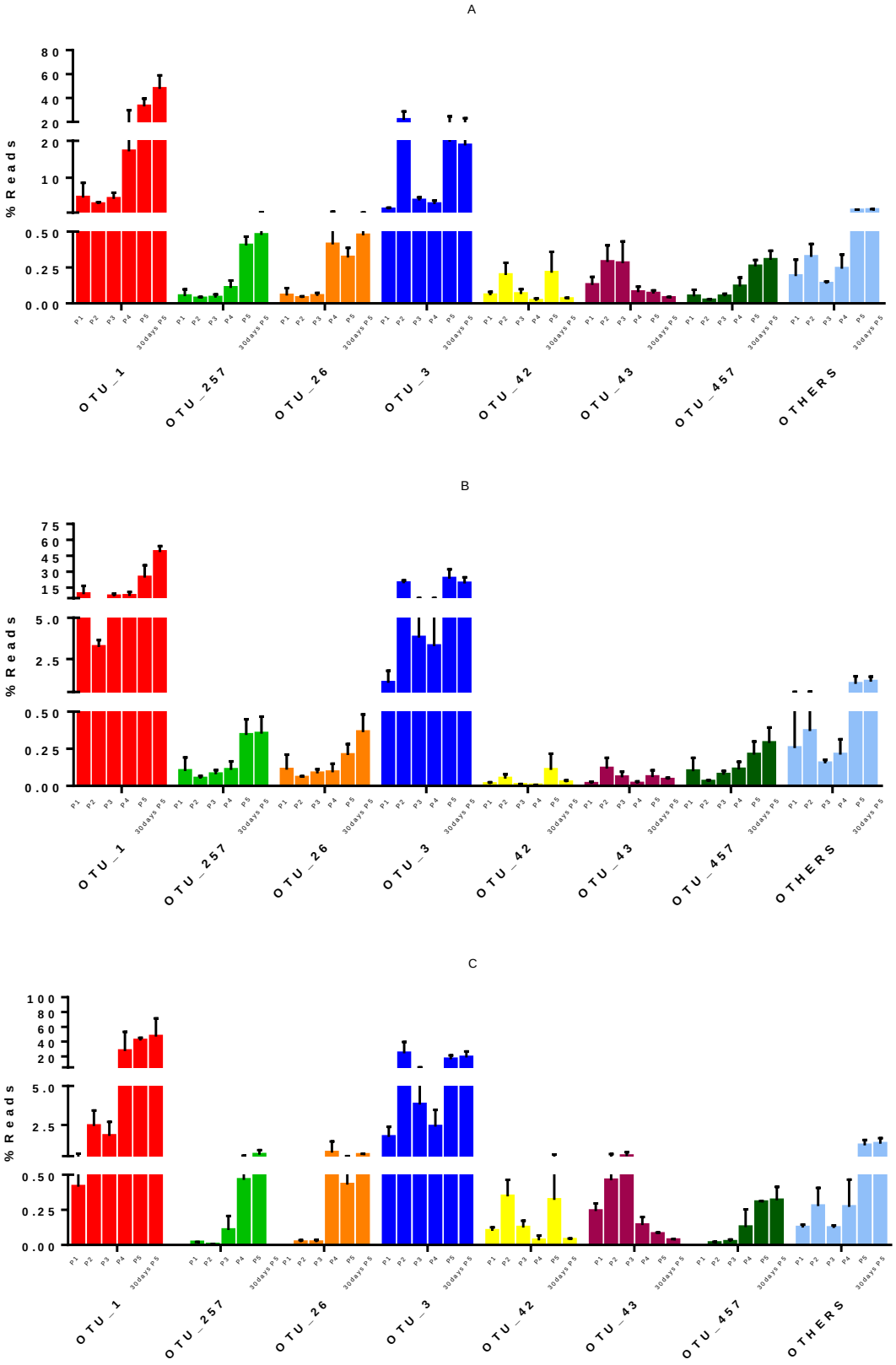


Figure 4.3 Percentage (± SEM) of reads related to *Penicillium* genus. A) Total reads % (± SEM) of *Penicillium* sp.; B) *Penicillium* sp. reads % (± SEM) in kernel samples; C) *Penicillium* sp. reads % (± SEM) in shell samples.

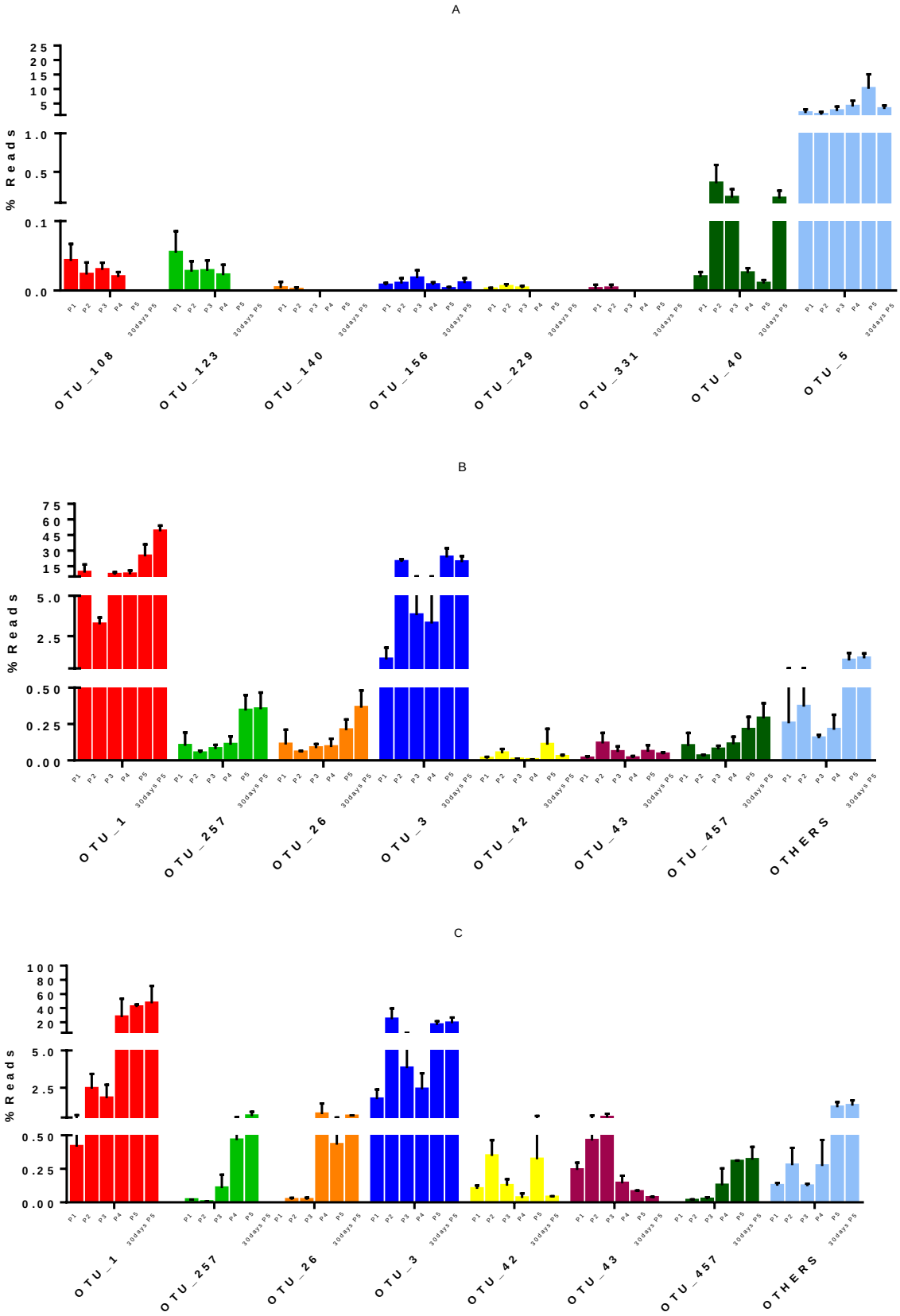


Figure 4.4 Percentage (± SEM) of reads related to *Fusarium* genus. A) Total reads % (± SEM) of *Fusarium* sp.; B) *Fusarium* sp. reads % (± SEM) in kernel samples; C) *Fusarium* sp. reads % (± SEM) in shell samples.

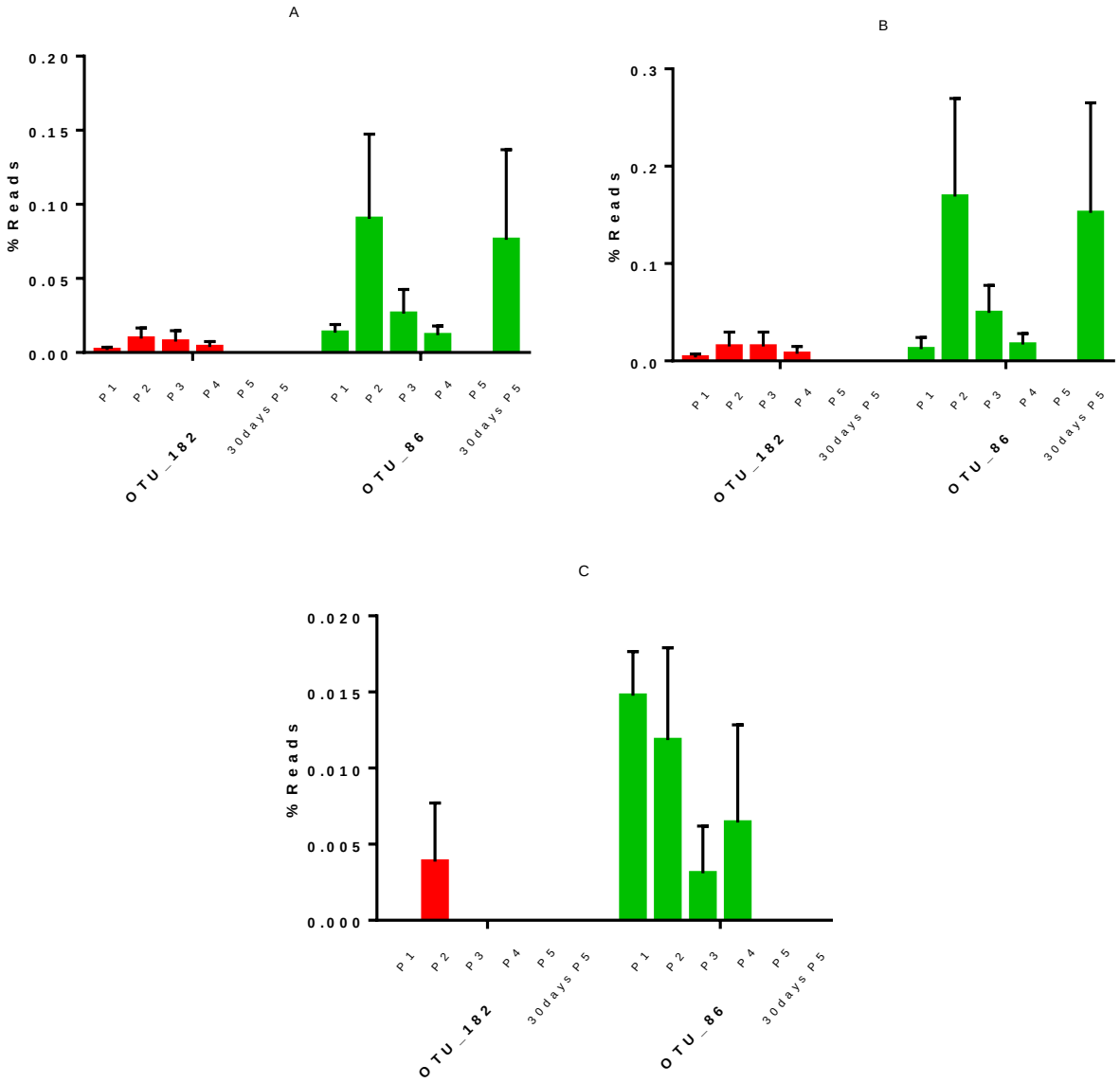


Figure 4.5 Percentage (± SEM) of reads related to *Aspergillus* genus. A) Total reads % (± SEM) of *Aspergillus* sp.; B) *Aspergillus* sp. reads % (± SEM) in kernel samples; C) *Fusarium* sp. reads % (± SEM) in shell samples.

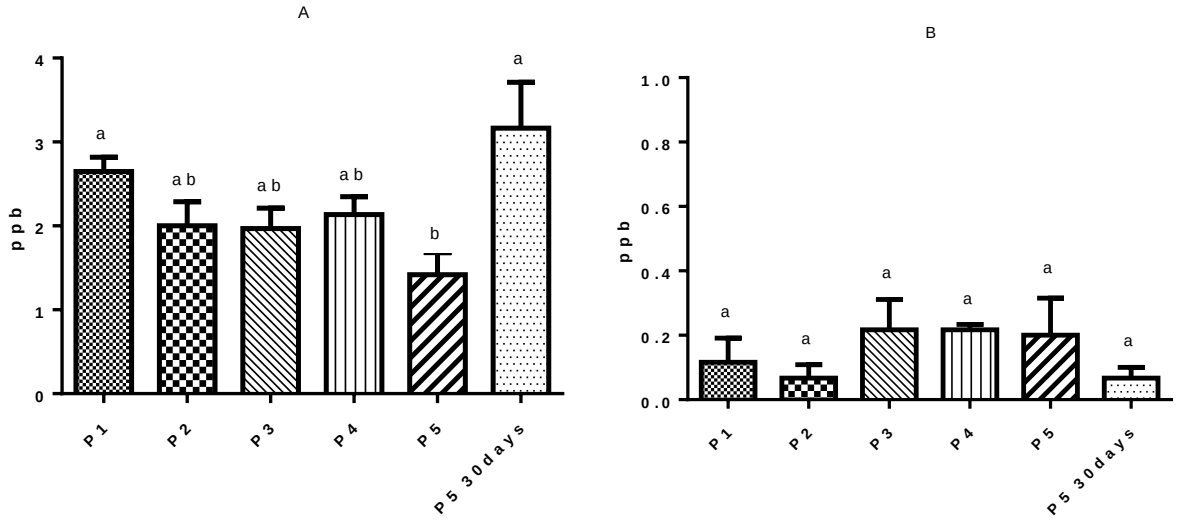


Figure 4.6 Results of AFs detection in chestnut shell (A) and kernel (B) between five different chestnut phases of the industrial chain and after 30 days of storage; (P1) delivery to the plant; (P2) *curatura*; (P3) soaking phase; (P4) the sterilization phase (warm bath); (P5) grading and manual selection before storage in large refrigeration cells; (P5 30days) after 30 days of storage. Different lowercase letters indicate significant differences between phases and time of storage (P>0,05) according to Tukey's test. Error bars indicate SEM.

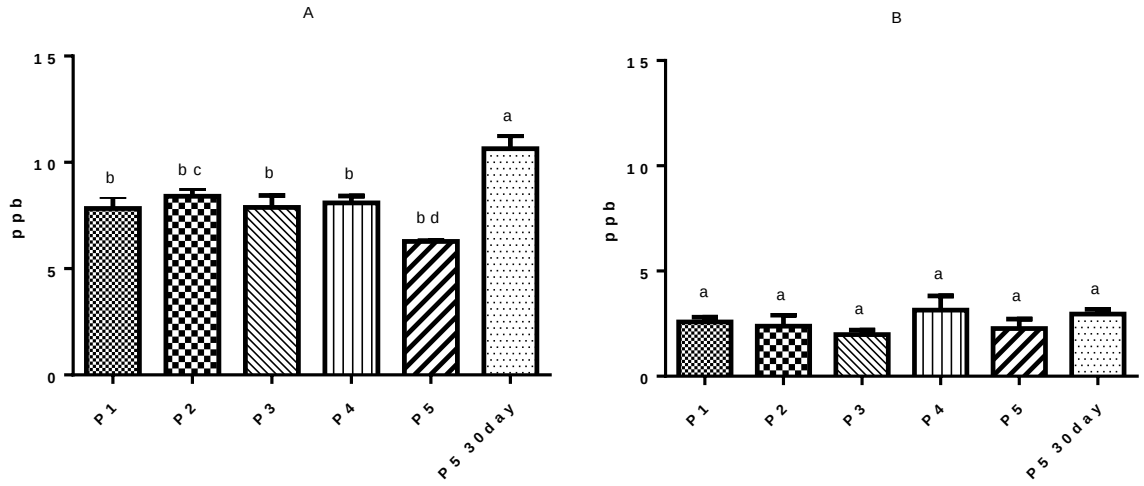
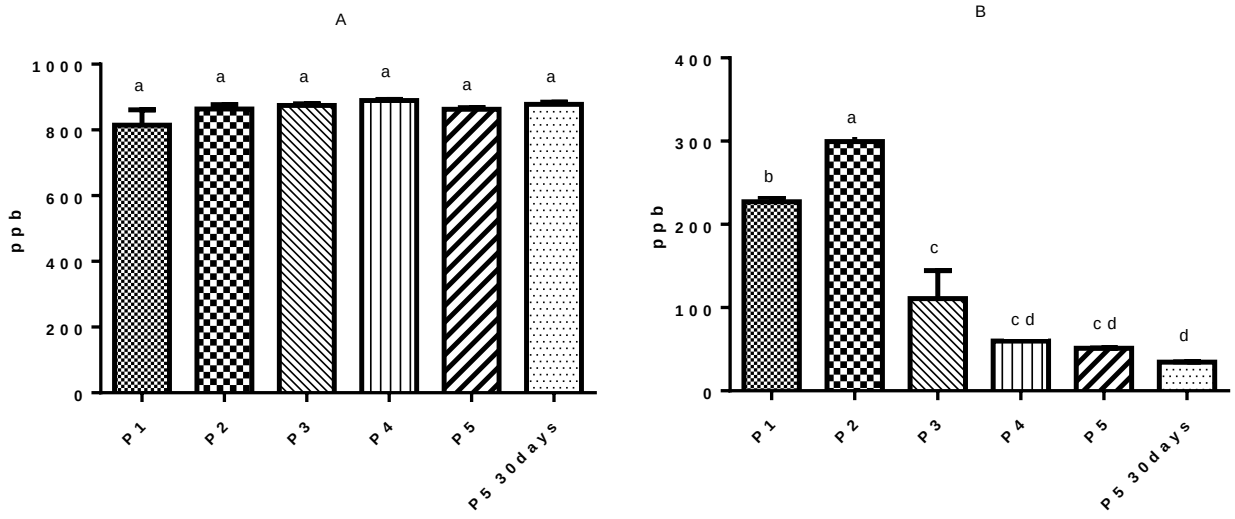


Figure 4.7 Results of OTA detection in chestnut shell (A) and kernel (B) between five different chestnut phases of the industrial chain and after 30 days of storage; (P1) delivery to the plant; (P2) *curatura*; (P3) soaking phase; (P4) the sterilization phase (warm bath); (P5) grading and manual selection before storage in large refrigeration cells; (P5 30days) after 30 days of storage. Different lowercase letters indicate significant differences between phases and time of storage (P>0,05) according to Tukey's test. Error bars indicate SEM.



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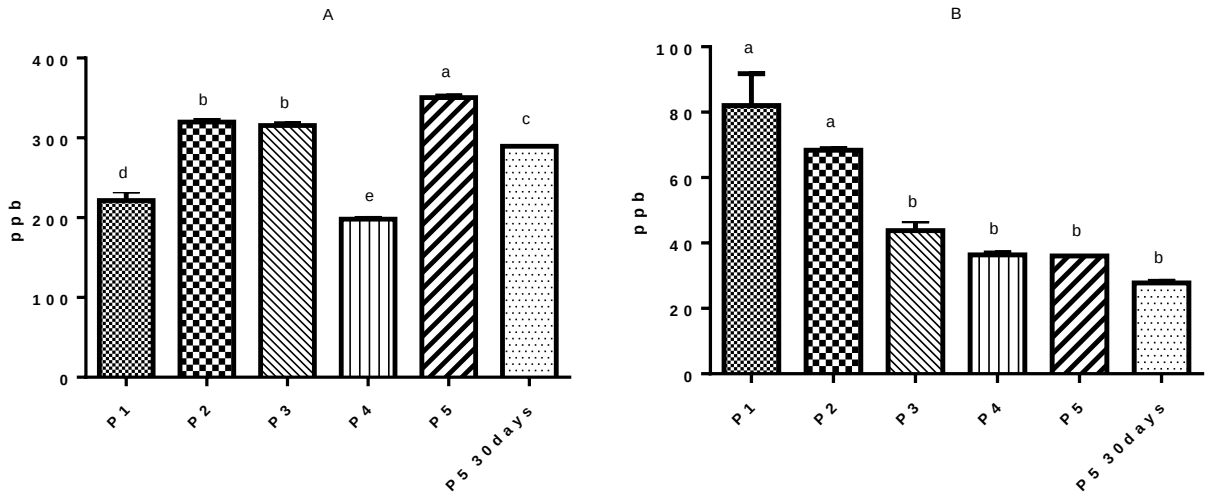

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CHAPTER V

Collaborations to further research

The project of this thesis allowed several collaborations to related research. In the following paragraphs, a brief review on what was done is presented.

5.1 Development of a TaqMan qPCR assay for the detection and quantification of *Gnomoniopsis castaneae* in chestnut tissues

This project has been already introduced in Chapter III, concerning the development of a TaqMan qPCR assay for the detection and quantification of *G. castaneae*. This development was carried out in collaboration with Professor Mazzaglia Angelo and Dr Turco Silvia, from the research group on Plant Pathology at the "Department of Agriculture and Forest Sciences" (DAFNE) - University of Tuscia, Viterbo; the relative results have been published in "Development of a TaqMan qPCR assay for the detection and quantification of *Gnomoniopsis castaneae* in chestnut tissues", (2021) on *Forest Pathology*. 2021;00:e12701. and are available at https://doi.org/10.1111/efp.12701 . This novel real-time PCR assay based on the TaqMan probe was created for the identification of *G. castaneae*, the causal agent of brown rot of chestnut kernels as well as leaf necrosis, shoot blight, and bark canker. There are evidences to suggest that its epidemiology includes an endophytic life cycle, during which the pathogen colonize all of the tissues of chestnut and other hosts. The biology of the fungus and its epidemiology may be greatly influenced by *G. castaneae*'s endophytic behaviour. Understanding the biology of the fungus and how this organism can shift from an endophytic to a pathogenic phase requires the ability to monitor the distribution of the inoculum in the various tissues and the patterns of endophytic inoculum accumulation as a function of host phenology and environmental parameters. There are currently no techniques available to monitor the presence of *G. castaneae* in chestnut tissues (Manias et al., 2020), other than the traditional biological detection method of isolation in pure culture, which is highly specific but has a limited sensitivity. Thus, a molecular tool for sensitive detection and quantification of *G. castaneae* in symptomatic and asymptomatic host tissues was developed with the aim to better understand *G. castaneae* ecology, biology and epidemiology, even in the endophytic state, and in different host tissues. Quantitative PCR (qPCR) is a powerful, practical tool for solving this problem. The use of qPCR assay was adopted to overcome not only the issues concerning the traditional morphological analysis, usually used for fungal isolation, but also the concern about the endophytic presence of the pathogen in the hosts.

The TaqMan probe turned out to be incredibly sensitive and showed a high proficiency in detecting the pathogen in very low quantities, irrespective of the presence of other fungal or plant DNA,

demonstrating the significant advantages and great diagnostic value of this assay. The assay successfully distinguished *G. castaneae* from other fungi that are taxonomically related to it as well as from other fungi that are frequently identified as endophytes or saprophytes of chestnut tissues and may putatively contaminate field samples. The EF1α-gene's selection as the target for amplification directly affected the assay's specificity. As reported in different assays (Guinet et al., 2016; Klymus et al., 2020; LaSalle et al., 2011) EF1α-gene increases both the accuracy and affordability of quantifying fungal infection in plant tissue, since there is a clear correlation between the amount of fluorescence measured and the number of fungal cells present. The ability to meticulously counting the quantity of pathogen cells is of considerable importance for epidemiological investigations including the creation of natural *G. castaneae* inoculum and dispersal, as well as its role and impact on the emergence of floral infection and nut rot (Lione et al., 2015). The pathogen was detected definitively, without any obvious effect imputable to DNA of the host plant, *C. sativa*, or other inhibitory compounds. The assay clearly exceeded the detection threshold of isolation methods by detecting pathogen presence in asymptomatic kernels, twigs and leaves. This aspect is significative, especially thinking about further studies on the fungus's endophytic/pathogenic life strategy. It is possible to identify the infection threshold that causes the fungus to go from an endophytic to a pathogenic lifestyle by comparing the amount of the pathogen that is latently present in host tissues or when symptoms become apparent.

This assay will enable the movement of infected plant material and its level of infection, conceivably helping to reduce the spread of the pathogen on regional, national and international scales. Since its ability to detect a latent infection of the pathogen, the TaqMan qPCR can also have a crucial impact in postharvest fruit storage to reduce the impact of fruit rot and the subsequent economic losses, (Maresi et al., 2013; Ruocco et al., 2016).

5.2 Hybrid *de novo* genome assembly and comparative genomics of three different isolates of *Gnomoniopsis castaneae*

This project was carried out in collaboration with Professor Mazzaglia Angelo, Dr Turco Silvia and Dr Mounira Inas Drais, from the research group on Plant Pathology at the "Department of Agriculture and Forest Sciences" (DAFNE) - University of Tuscia, Viterbo; the relative results have been published in "Hybrid *de novo* genome assembly and comparative genomics of three different isolates of *Gnomoniopsis castaneae*", (2023) on *Scientific Reports* (2023) 13:3356 and are available at https://doi.org/10.1038/s41598-023-30496-0. The fungus *Gnomoniopsis castaneae* G. Tamietti is mainly known as the cause of the brown rot of chestnuts, and one of the limiting factors affecting the fruit production industry. The disease is present in the main chestnut production areas worldwide including Australia, New Zealand, Asia, North America, South America, and Europe, affecting *Castanea sativa*, *C. mollissima* and *C. sativa* x *C. crenata* hybrids 8 (Shuttleworth et al., 2013; Cisterna-

Oyarce, V. et al., 2022). The similarity in symptoms, and the subsequent synonymy, to previously identified chestnut fruit pathogens like *Phomopsis castanea*, *Phoma endogena*, or *Amphiportae castaneae* seems highly probable and would indicate a long-standing global distribution (Lione et al., 2019). Beside the specific TaqMan qPCR assay reported in the previous paragraph (Turco et al., 2021), other molecular analysis like MultiLocus Sequence Typing (Visentin et al., 2012; Pasche et al., 2016; Linaldeddu et al., 2016), as well as SSR and HRM techniques (Dennert, Broggini, Gessler & Storari, 2015; Sillo et al., 2017) have been extensively carried out to detect the presence of the pathogen. To promote omics investigations that attempt to clarify many facets of *G. castaneae* biology and lifestyle, whole genome sequencing and de novo assembly, however, offer a more potent method. In fact, this acquired knowledge serves as the basis for further deeper investigations into how the pathogen interacts with its host and cause disease, as well as phylogenetic analysis and genetic population studies on a global scale.

To better understand the genetics behind the endophytic behavior and the pathogenic mechanisms of this fungus, to provide tools for local and global population studies, the first genome sequences of three different isolates: the Italian ex-type MUT-401 assembled at the chromosomal level, a second Italian isolate MW494885 (GNO1) and the ICMP 14040 isolate from New Zealand (NZ), assembled at contigs level. Using a hybrid assembly approach with both short Illumina reads and Oxford Nanopore Technologies (ONT) long reads and comparing different assembler performance, it was possible to assemble almost completely the genomes of the three isolates, with an average N50 of 4Mb and average length of 39.5 MB. The choice of combining different approaches was done in order to take the advantages of the Illumina short reads sequencing method, providing high depth coverage, and of the Oxford Nanopore long reads (ONT) sequencing technology, providing longer scaffolds.
As a result, this characterisation of the *G. castaneae* genome represents the foundation for further molecular investigations. In fact, these findings serve as the first step for future research focused on better identifying the fungal populations worldwide, their epidemiology, genetic diversity, and interactions with hosts, providing us the ability to develop targeted control measures.

5.3 SANCAST and FORECAST Projects

The chestnut supply chain, despite being one of the excellent Italian agri-food sectors of and a high quality territorial brand of quality that contributes to the notoriety of our country, is characterized by a historically modest technological level (Moscetti et al., 2014), which has not been able to respond effectively to the collapse of production (about 90% of losses in 2017) and the increase in damage products caused by the proliferation of fruit rot and disease agents associated with changing climatic conditions . Compared to these currently problems, strictly related to a serious more and more reduced productivity in post-harvest - for instance contamination by fungi producing mycotoxins (*Aspergillus*,

Penicillum, Fusarium) (CAST, 2003; Reddy et al., 2009; Venâncio & Paterson, 2007), high incidence of fruit rot, poor ability to identify and select the damage product, spread of rot and surface contamination undergoing conditioning and conservation and too high incidence of contamination for large-scale distribution - the methods traditionally adopted, not only they show little efficacy, but also can become risk factors amplification. For these reasons, the Plant Pathology Lab and other research groups from DIBAF Department were involved in two different projects with the common aim to provide an answer to the technological and process critical phases of the supply chain and guarantee a healthy and quality product. It is correct to assume that these projects, in the short term, will have positive effects on: consumers (increasing the quality and wholesomeness of the products), the chestnut industry (providing useful tools to reduce losses due to attack by insects and moulds), the legislators and the scientific community (representing a step forward in the field of the application of hyperspectral image analysis in the agri-food sector). Therefore, the potential results will contribute to the growth of the competitiveness of the Central Italy chestnut production.

In 2019 and 2020, SANCAST project involved 150.000 Euros and 26 month working project on mitigation of impact of kernel brown rot in Central Italy by mean of novel assessment technologies and accompanying measures. Similarly, between 2020 and 2021, with the same aims, FORECAST project allocated 3.000 Euros and 32 month working project in Tuscany region. Both activities were multi-actor projects involving different competences in post-harvest technology, plant pathology and entomology, and agroindustry. The projects included physical and biological methods with zero impact in the chestnut production chain to guarantee product quality and marketing of fruits with damage/rotten percentages compatible with the standards of the Large Organized Distribution. The objectives were gained through: the transfer to the chestnut supply chain of good practices in the field capable to mitigate damage and fruit rot; the test and development of discriminating models for the online sorting of chestnuts based on the use of Fourier transformed spectrometry in the near infrared (FT-NIR) and of hyperspectral image analysis (HSI) in the visible/near infrared (Vis/NIR). Both projects put pressure on the study and introduction of "good practices" for the management of the fruit in all its phases, from harvesting to conservation and pre-marketing storage.

Concerning the methods with zero impact in the chestnut production chain, the trials were conducted on several plots, specially selected, to confirm the statistical validity of the results obtained, in two different chestnut orchards, one situated at Monte Cimini (Natural Reserve of Vico Lake – Province of Viterbo, Lazio Region) and one at Monte Amiata (Province of Grosseto, Tuscany Region). The elaboration of phytoiatric protocols based on zero impact methodologies already identified in previous research activities was carried out. In order to guarantee a low incidence of rot and insect damage in fruits entering the post-harvest plant, and to ensure wholesomeness and quality of the fruit up to the stages of

marketing and consumption protocols for the treatment of plants in open field and in the post-selection fruit were developed, using zero-impact strategies of proven efficacy such as bio-fumigation with products based on *Brassicaceae* or endotherapic treatments on plants with resistance inducers such as potassium phosphite (Vig et al., 2009).

Concerning the trials about sorting, in general, the results showed a greater effectiveness of Vis/NIR hyperspectral image analysis in product selection, with respect to the traditional one. The feasibility of using FT-NIR point spectroscopy and Vis/NIR hyperspectral image analysis for chestnut sorting was further confirmed by the investigation conducted during the trials. In comparison to traditional sorting methods, the performance of the techniques tested in the present study was slightly better. The good results obtained through these innovative methods were published on Italus Hortus Vol. 27 (2020), Pages 3-18 - Feasibility of FT-NIR spectroscopy and Vis/NIR hyperspectral imaging for sorting unsound chestnuts (Bedini et al., 2020). However, further research will be needed to test the effectiveness of new analysis algorithms discriminant, filters and image analysis methods, including emerging and promising Deep Learning techniques, specifically the Deep Chemometrics for spectra analysis and the Convolutional Neural Network (CNN) for the development of classification models.

The projects' goal is to support, modernize and make the regional and national chestnut supply chain effective, providing it with technological and biotechnological tools that guarantee its economic and environmental sustainability and allow certification of the quantity, quality and healthiness of the productions. Moreover, the projects aim to obtain a production with a degree of reliability such as to make it compatible with introduction into large-scale distribution channels. The combination of the planned actions will have a synergistic effect on the production system. The guarantee of a lower incidence of fruit rot and insect damage on the plant, an accurate selection of the product before packaging and a lower possibility of contamination after packaging, will lead to an optimization of production costs and will allow to reconquer lost markets, with positive effects on all the actors in the supply chain. Furthermore, consumers will have qualitatively more controlled and healthier products, distributors will be able to count on stable production with reduced storage problems and the chestnut industry will be able to benefit from tools that will increase the productivity and profitability of the sector.

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CHAPTER VI

Conclusions

Fungal contamination, and correlated mycotoxin contamination, is the major problem facing the chestnut industry. The reduced quality of nuts and the economic losses caused by brown rot and other fungal contamination made the chestnut owners lose interest in chestnut production in recent years. Most causes of this problem depend on numerous variables attributable to the methods of field treatment and storage which nuts must pass through, from the collection to the consumption. This thesis aimed to propose a new approach in solving these issues related to chestnut pre- and post-harvest, by looking at them from a different perspective.

So far, research has mainly tended to focus on post-harvest control strategies rather than the pre-harvest ones. For this reason, to make up for the lack of knowledge on pre-harvest control strategies, the first objective of this study was to develop new strategies for *G. castaneae* control in field. Different products with the minimum environmental impact were tested in efficacy in comparison to the conventional ones. The use of phosphonate salts, and in particular Zn-phosponate, demonstrated a high effectiveness against the "brown rot" fungus *G. castaneae*. The reducing in nut infection in orchards early in the season was similar to the one obtained by conventional chemical treatments. The efficacy of phosphonate salts, together with their low environmental impact, provide a good alternative to the conventional chemical treatments applied in field so far. Moreover, the development of the TaqMan probe for Real Time PCR, highly specific for *G. castaneae*, demonstrates the significant advantages and great diagnostic value of this assay. From an industrial chain viewpoint, the adoption of a suitable management from the orchard to the processing facility will ensure that the disease has the least possible impact on market-ready fruit supplies. In other words, a reduced presence of brown rot at the collection phase in orchard, paired with appropriate post-harvest strategies, can guarantee good quality nuts, free from contaminations.

Concerning the post-harvest strategies, the second objective of this thesis aimed to reduce microbial decay of fresh chestnuts in a private company, intervening on treatment parameters and proposing new solutions. In this sense, the efficacy of postharvest thermo-hydrotherapy was evaluated both in industrial plant level and in laboratory. Results demonstrated that the first phases of the industrial chain (delivery and *curatura*) had no effects on *G. castaneae* isolation and brown rot incidence, while the thermo-hydrotherapy showed a good effectiveness against the brown rot causal agent. Unfortunately, an increase of brown rot was observed after storage, confirming that the chestnut handling was not able to stop the disease progression.

On the other hand, the application of strict conditions (in terms of temperature and time of treatment) at the sterilization phase in laboratory could definitively inactivate *G. castaneae* both in non-symptomatic and rotting fruits. The sterilization parameters were accepted as the best temperature/time combination for chestnut treatment in correlation with getting high quality fruits, according to the panel test results. Additionally, the sterilization parameters such as time and temperature have been intended as effective, concerning the flow-process chestnut were passing through; in this sense, time of sterilization started when the effective water temperature in the tank was restored, and not in the moment chestnut were submerged in the tank. Always from a supply chain point of view, the feasibility of using FT-NIR spectroscopy and Vis/NIR hyperspectral imaging for chestnut sorting was confirmed. This technology, preceded by an efficient thermo-hydrotherapy, can ensure a good quality of chestnut during the storage, avoiding the rot spread from occult or latent contaminations.

Unfortunately, sterilization at 50 °C and over was not effective in inactivating the complex of fungal taxa responsible for contamination and development of molds, many of which produce mycotoxins. For this reason, the third objective of this thesis was to study the microbial populations associated with chestnut along the processing and storage in a private company. This trial was performed using Next-Generation Sequencing (NGS) technology and metabarcoding based on Illumina de novo sequencing, which allow a better knowledge of how total microorganisms community may affect postharvest chestnut quality. At the same time, the evaluation of mycotoxins contamination in chestnut fruits sampled at the end of different phases of the processing and after storage was performed, taking into account the risk for human health.

Since fungal and mycotoxin contamination is a major problem in chestnuts and chestnut products and is mostly related to improper post-harvest storage conditions, the last objective of this thesis aimed to determine, during different industrial phases and storage, the associated mycobiota and the presence of the main mycotoxins in chestnuts. High-Throughput Sequencing allowed to track the three main responsible genera for mycotoxins production (*Penicillium* sp., *Fusarium* sp. and *Aspergillus* sp.) along the phases of a chestnut handling. Mycotoxins associated with these three genera resulted stable and always under the limits recommended by EU, ensuring a safe consumption for humans. However, for the first time FBs and T-2/HT-2 contamination was found on chestnut, underlining how limits for these mycotoxins are needed also for dried fruit such as chestnuts, like occurs for AFs and OTA.

In conclusion, the main objectives of this thesis were achieved separately but together they aimed to provide a new approach to solve problems regarding chestnut quality in Italy. The results obtained by treatments in open field and in chestnut handling must be designed from the point of view of the supply chain. In fact, treatments in the open field were able to decrease *G. castaneae* inoculum and brown rot damage “on tree” but did not eliminate completely the pathogen. Sterilization in the chestnut handling

was able to eliminate the brown rot causal agent but its effectiveness depended on the severity of the disease in the fruit delivered from the field and in any case was not able to reduce the damage already present in field. The efficacy of the warm bath strictly depended on the quality of the fruit stocks entering the handling plant and the conditions to which they were exposed before the thermo-hydrotherapy. An efficient strategy for the control of ‘brown rot’ and fungal contamination in general must include mitigation protocols applied in the open field, followed by effective physical treatments in the handling plant. For these reasons, the treatments in pre- and post-harvest must be understood as synergistic and functional one to each other. In a perspective of chestnut supply chain, the methodologies provided in this work represent a promising step forward for pre-harvest treatment and post-harvest technologies employed in chestnut production. However, to acquire a better understanding of the critical points of fungal contamination in the chestnut treatment chain, additional laboratory and industrial plant investigations are required.