Final Report

Knowledge gaps of nut rot of chestnuts

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Public summary

Nut rot of chestnuts is an emerging disease becoming of worldwide importance. In Australia up to 70% of chestnuts may be affected, while in Europe losses may be as high as 90%. The disease is mainly expressed after harvest and it is cryptic, as healthy-looking nuts on the surface are rotten internally. It is a disease of great economic importance as infected nuts cannot be consumed, is not easily detected and hence reduce consumer confidence when unknowingly purchasing infected chestnuts. The overall objective of this study is to provide a literature review by collating all available scientific literature on chestnut nut rot to have a better understanding of the disease biology, control and management. From the literature, it is now well established that the pathogen, *Gnomoniopsis smithogilvyi*, is also an endophyte that can be found in symptomless chestnut tissue. However, the triggers allowing the fungus to "switch" from an endophyte to a pathogen is still unclear. A modelling study identified high temperatures and wind in the months prior to harvesting as associated with disease development, but narrowing this down to specific months/periods would be helpful in developing a disease forecast model. Various options for disease control have been investigated, both biocontrol and chemical. Biocontrol options seems largely ineffective as a control option in orchards or as a post-harvest option, while chemical options may provide some, but perhaps not adequate protection. Their efficacy under Australian conditions needs further investigation. The effect of chemical applications on the endophyte community is unexplored and may actually be detrimental to chestnut health in the long term. Reducing the number of diseased chestnuts in storage and on the market, still poses challenges even though a number of disease-reducing options have been investigated, mostly with limited success. Removing plant debris and floating chestnuts will assist in getting rid of the worst affected chestnuts, but not all. Options such as a hot water treatment (*curatura*) seems largely ineffective, while an ozone treatment may offer some protection, although it is unclear how long that protection lasts. An integrated management strategy is suggested by removing litter on the orchard floor to reduce inoculum, combined with possible chemical applications. Further investigating the factors associated with infection will help in establishing a disease forecast model to inform growers when chemical applications will be most effective based on environmental conditions.

Keywords

Chestnut; nut rot; *Gnomoniopsis*; endophyte; infection and disease biology; integrated disease management.

Literature review

Introduction

Nut rot, also called brown rot of chestnuts, is a disease attributed to an emerging fungal pathogen *Gnomoniopsis smithogilvyi* L.A. Shuttlew, E.C.Y. Liew & D.I. Guest (syn. *Gnomoniopsis castaneae*). *Gnomoniopsis smithogilvyi* infects the nut kernel, developing into browning and necrosis of the endosperm and embryo (Crous et al. 2012). The disease is mainly expressed after harvest and it is cryptic, as healthy-looking nuts on the surface are rotten internally. It is a disease of great economic importance as infected nuts cannot be consumed, is not easily detected and hence reduce consumer confidence when unknowingly purchasing infected chestnuts. The infection is initially asymptomatic, with disease expression as the nuts ripen. At early infection stages, the slightly moldy or parasitized nuts are not easily differentiated from healthy nuts, and infection is detected only when they are processed or consumed. The incidence of nut rot of chestnuts has increased sharply in the last decade in Australia, New Zealand, Europe and the UK (Visentin et al. 2012, Maresi et al. 2013, Shuttleworth et al. 2013), with disease incidences of more than 90% reported in some regions.

The nut rot fungus was initially identified independently by Shuttleworth et al. (Crous et al. 2012) and Visentin et al. (2012) as *G. smithogilvyi* and *G. castaneae* (originally called *G. castanea*), respectively. However, morphological and phylogenetic analyses (based on DNA sequencing) showed the synonymy between the two taxons (Maresi et al. 2013). Currently, *G. smithogilvyi* is the name adopted for this fungus by most authors, with a notable exception from mostly the Italian authors.

G. smithogilvyi is a pathogen causing rot of chestnut fruits, but can also cause cankers, as first reported in India (Dar and Rai 2015), but also in the UK (Lewis et al. 2017), Spain (Trapiello et al. 2018), and Ireland (O'Loinsigh et al. 2022). In fact, the bark canker caused by *G. smithogilvyi* shows a symptomatology very similar to that caused by the chestnut blight pathogen *Cryphonectria parasitica*, which is the causal agent of chestnut canker, but may not be as severe. The fungus can also cause necrosis of leaves and reported blight symptoms on inoculated chestnut twigs (Magro et al. 2010). Pasche and co-workers (2016a) reported infection of wood and graft points as well as asymptomatic scions, demonstrating that it can develop as a canker agent on chestnut wood. In Switzerland, mortality of up to 40% of infected 3-year-old grafted trees were reported (Pasche et al. 2016a).

It has been reported as a pathogen of several other host species, affecting mainly nuts (hazelnuts (*Corylus avellana* L.), manna ash (*Fraxinus ornus* L.), holm oak (*Quercus ilex* L.), Turkey oak (*Quercus cerris* L.), and maritime pine (*Pinus pinaster* Aiton), causing fruit rot, cankers, and necrosis in branches and leaves (reviewed in (Dobry and Campbell 2023)). The role these hosts play in inoculum production and subsequent disease development in chestnuts has not been investigated. These hosts may further have contributed significantly to the spread of the pathogen and incidence of the disease on a global scale.

Biology and distribution

Gnomoniopsis smithogilvyi is an ascomycete, ie. it produces ascospores as its sexual spores and conidia as asexual spores. Fungi are peculiar in that they usually reproduce sexually (producing ascospores) only when growing conditions deteriorate, and reproduce asexually (producing conidia) when ample resources are available. A recent population genetic study using European isolates (Sillo et al. 2017) showed it indeed having a mixed reproduction system in Europe, reproducing both sexually and asexually. The absence of linkage disequilibrium in European populations suggest frequent sexual reproduction (Sillo et al. 2017). The mating types of the fungus has, however, not been identified yet and it is unclear whether it is a homothallic (both mating type genes located in one individual, hence can self – sexually reproduce with itself) or heterothallic fungus (need to mate with another individual of different mating type). It has a wide distribution currently occurring in several countries and continents including Europe, Asia, Australia and America (Visentin et al. 2012, Lione et al. 2019, Cisterna-Oyarce et al. 2022, Dobry and Campbell 2023), although is absent from the largest chestnut-producing nations of China, Korea or Japan. In the latter regions, two other *Gnomoniopsis* species, *G. daii* and *G. chinensis*, have been identified as agents of nut rot and cankering in chestnuts, respectively (Jiang and Tian 2019, Jiang et al. 2020). The latter two *Gnomoniopsis* species were identified on *Castanea mollissima*, not *Castanea sativa* (sweet chestnut), with China being the largest producer of Chinese chestnuts (*C. mollissima*).

Dobry and Cambbell (2023) suggested that the origin of the pathogen is unknown, and hence an introduced fungus wherever it has been observed as a pathogen. However, the population genetic structure of the pathogen in Europe as measured with high-resolution melting analyses (HRM) (Sillo et al. 2017) suggests that the levels of diversity is higher than expected for a recently introduced pathogen. These high levels of diversity can also be attributed to multiple introductions, a possible scenario given that *G. smithogilvyi* is a ubiquitous endophyte with many plant species as hosts. Similarly, another endophyte *Hymenoscyphus fraxineus*, also the causal agent of ash dieback in Europe and introduced from Asia, showed a higher than expected genetic diversity for an introduced (founder population) pathogen (Orton et al. 2018). A clear population structure of *G. smithogilvyi* in Europe as shown both with the HRM study (Sillo et al. 2017) and a ß-tubulin sequencing study using European and US isolates (Seddaiu et al. 2023), showing two subpopulations/haplotypes, both represented by isolates from Europe and the US, does indeed suggest the pathogen has been introduced to Europe from an as yet unknown origin.

There is also evidence of the expansion of the range of the pathogen as it continues to be identified in new regions, further suggesting some degree of introduction is occurring. Its recent discovery in North and South America (Sakalidis et al. 2019, Cisterna-Oyarce et al. 2022), means there is a need to re-examine assumptions regarding the epidemiology and future implications this fungus may have on chestnut production. Even though the pathogen has been present in Europe since at least 2005 (Visentin et al. 2012, Maresi et al. 2013), it was reported for the first time in Portugal in 2020 by Possamai (2023) and then by Coelho and Gouveia (2021), providing anecdotal evidence of a pathogen being introduced to new areas. Taking its population genetic biology into account, I consider it a fungus which has always been present as an endophyte, however certain conditions favoured its emergence as a pathogen. Its low genetic diversity suggests that only a selected number of strains evolved this pathogenicity (eg. The same sequence genotype is present in Australia and Portugal (Possamai et al. 2023) and are dispersed around the world. This does assume that the endophyte population has a higher genetic diversity than the pathogen population, considering the endophyte population has a high host range, and can sexually reproduce which promotes high levels of genetic diversity. However, apart from HRM analyses, all the genetic markers applied to this fungus is based around ITS sequencing, which is a good diagnostic marker, but not sufficiently variable within and among populations to make assumptions about its population structure. Thus, more informative genetic markers need to be applied to show migration patterns of the fungus. Furthermore, conditions favouring the emergence of this pathogen should be investigated. **Is the genetic make-up of the endophytic population similar to that of the pathogen population? Are endophytic fungal strains able to cause nut rot? Is the genetic diversity of endophytic populations equal to that of the pathogenic populations? Investigations into such issues will shed light on the epidemiology and evolution of this emergent pathogen.**

Gnomoniopsis as endophytes

Members of the genus *Gnomoniopsis* are often encountered as endophytes (fungi and bacteria living in healthy, symptomless plants) (Walker et al. 2010). In Australia, it has been isolated as an endophyte from asymptomatic chestnut flowers, nuts, burrs, leaves and stems, and from rotten chestnut kernels (Shuttleworth 2012). This was followed by reports as an endophyte from symptomless chestnut material elsewhere, even in 1-2 year old shoots (Visentin et al. 2012, Maresi et al. 2013). In Switzerland, the pathogen could be isolated from asymptomatic scions, with 49% of bark tissue and 17% of woody tissue colonised (Pasche et al. 2016a). The fungus can also grow saprophytically on dead organic material, in particular in the burs and other residues on the ground, which is posited to represent the main reservoir for the formation of perithecia and subsequent release of infectious ascospores spores (Crous et al. 2012, Visentin et al. 2012, Dar and Rai 2015).

Accordingly, Smith & Ogilvy (2008) found that ascospores released from dead chestnut litter on the orchard floor was the primary inoculum initiating chestnut rot. Similarly, Shuttleworth and Guest (2017) found that the primary infection is caused by ascospores that spend the winter on the infected plant debris lying on the soil surface of chestnut orchards. Litter on the orchard floor as a primary source of ascospore inoculum has been contested in Europe where management practices recommend removal of litter following harvest (Vannini et al. 2017). However, litter removal is not always performed, particularly traditionally managed orchards (Lione et al. 2021). Thus, the Europeans consider the association with litter posited but not yet demonstrated.

During the flowering period, ascospores infect flowers (predominantly female flowers) (Shuttleworth and Guest 2017), leaves, and branches, and spores may be dispersed by wind and wind-driven rain, as well as insects (Lione et al. 2019). Confirmation that flowers need to be infected for nut rot development is provided by Visentin et al. (2012) who reported

that 25% of artificially infected chestnut flowers developed chestnut rot. The fungus infects only the female part of the flower (pistil) through the stigma–style and not through the ovary wall (Shuttleworth and Guest 2017). The appearance of multiple embryos in the fruit, where one is rotted and the other healthy, suggests that once the fungus infected the flower, the pellicle surrounding each embryo is preventing the spread of the fungus to other embryos (Shuttleworth and Guest 2017). Additionally, it is hypothesised that the infection of female flowers may also occur via *G. smithogilvyi*-infected pollen (Lione et al. 2019).

Abiotic and biotic factors favouring infection

Ascospore infection of the chestnut flowers is affected by several abiotic and biotic factors. These factors vary at scales of individual trees and even individual flowers. Biotic factors include timing of flowering of each chestnut variety (ie. whether it coincides with eg. ascospore release, or has natural resistance), when and whether fungal spores are released, and whether spores are able to be transmitted to flowers. Transmission occur likely through wind but may also be assisted by vectors such as bees, beetles and earwigs (Shuttleworth et al. 2013).

The biotic factors include rain, temperature, relative humidity, and wind. Ascospore release occur after rain (approx. 1 week) during the day and at night with peak periods at sunset and the hours following sunset peaking at 10 pm, and the hours following sunrise peaking at 8 am (Shuttleworth and Guest 2017). Rainfall during the flowering period has been reported to increase the incidence of chestnut rot in Australia and New Zealand, (Ogilvy 1998, Smith and Ogilvy 2008).

Although rainfall is critical for ascospore formation, temperature is reported to play the more important role in disease development. Based on modelling various climatic factors, an increase in temperature in the months before nut harvesting was reported to lead to a higher incidence of nut rot in northwest Italy (Lione et al. 2015). They specifically modelled temperatures in the months of January to October (July to April for the southern hemisphere), thus a fairly wide period. Based on a spore trapping experiment over 2 years, the authors also showed that spore (both ascospores and conidia) deposition occur all year round, with significantly higher incidences in summer and autumn, followed by spring. The lowest spore deposition occurs in winter. Spore deposition was positively correlated with mean, maximum, and minimum temperature, growing degree days at 0 and 5 °C thresholds, and wind gusts, for the months June to October (summer to autumn). This suggests that warmer temperatures in the second half of the vegetative season are associated with increased nut rot incidence. Thus, it is expected that rising temperatures and strong winds due to climate change may increase the incidence of the disease in the future. However, although there is no correlation between disease incidence and rainfall, the authors do not preclude the possible role of drought, which often occur because of multiple interacting factors not limited to reduced rainfall (Lione et al. 2015, Lione et al. 2021). In fact, warm, dry periods (drought stress) as predisposing factors to disease development were anecdotally linked to nut rot in Italy (Maresi et al. 2013). However, the spore-trapping study could only identify temperature and wind as the main factors in disease occurrence (Lione et al. 2015, Lione et al. 2021).

The mechanism whereby high temperatures seem to favour nut rot development is unclear. Possamai et al (2023) conducted fungal growth experiments to determine its optimum growing temperatures. They found that high temperatures indeed favour fungal growth, with optimum growing temperatures of 25 - 30 ºC recorded. On a medium made from chestnuts, the fungus is outperforming growth on two other tested media, with optimal growth at a high of 30 ºC. Furthermore, conidial production was highest at 30 ºC, also on the chestnut medium (Possamai et al. 2023). Their experiments thus show that *G. smithogilvyi* prefers chestnut as a medium, but also that higher temperatures in chestnut medium stimulate conidial production most. This is indicative of an adaptation to high temperatures in chestnut, which may further increase under climate change. High temperatures may also induce physiological changes in the host which predisposes it to infection. Warm temperatures associated with tree stress was also suggested by Maresi et al (Maresi et al. 2013) to be involved in higher nut rot incidence. Tree stress induced by high temperatures and/or drought is a common mechanism whereby plants are rendered susceptible to pathogens. It is most often benign pathogens that benefit from altered host defences induced by climate change.

The role of the asexual phase in disease biology

The role of the asexual phase (conidial infection) in chestnut endophytic infection has not been studied. The repeated

occurrence of haplotypes in some locations of Italy, Switzerland and France, as determined with a HRM analyses, suggests that asexual reproduction does play a role in the reproduction and dissemination of the fungus (Sillo et al. 2017). Additionally, abundant asexual fruiting bodies can be found in association with cankers and grafted scions (Pasche et al. 2016a) and naturally infected nuts as well as galls formed by chestnut wasps (Maresi et al. 2013).

In Italy, spore production is highest during spring, autumn and summer, although it was variable within and among sites, with a site average ranging from 173 to 765 spores $.m^{-2} \cdot h^{-1}$ (Lione et al. 2021). However, the type of spore produced (conidia vs ascospores) cannot be distinguished here. High spore loads for most of the year, does suggest that infection can occur almost any time, although flowers are only infected by ascospores. In Europe, conidial infection is thought to be associated with the attacks of the wasp *Dryocosmus kuriphilus*, known as the chestnut gall wasp, and responsible for forming galls in the branches and leaves of chestnut trees (Maresi et al. 2013). However, Vannini et al. (2017) suggested that the association with galls are from resident endophytic inoculum. Furthermore, in Switzerland, the asexual form of *G. smithogilvyi* has been reported from cankers of *Cryphonectria sativa* (Pasche et al. 2016a). Therefore, the galls and cankers may promote sporulation, which will allow conidia to be transmitted by eg. insects, promoting fungal dispersal for further endophytic colonisation. Some suggest that the stress induced by the galls/cankers may allow a transition from endophyte to pathogen for the fungus (Turco et al. 2021, Lema et al. 2023), although there is no direct evidence for such a switch in trophic mode facilitated by galls/cankers in orchards.

The absence of the chestnut gall wasp from Australia may explain the apparent dominance of sexual reproduction in Australia, compared to Europe (Dobry and Campbell 2023). Whether and which environmental factors may contribute to the dominance of either reproduction phase needs further investigation. Such knowledge may allow us to make predictions as to which mode of reproduction the fungus may favour under continuously changing environmental conditions.

Apart from nut infection, Pasche et al (2016a) also showed that the fungus as an endophytic infection can develop into a pathogenic infection of the wood and xylem, even causing mortality in 3-year-old grafted trees, albeit under glasshouse conditions. The authors suggested that to control the pathogen which started as an endophytic colonisation, biological control would be the method of choice for sanitation and grafting material, although no evidence has been provided of biological control as effective in reducing mortality in grafted seedlings (Pasche et al. 2016b).

Endophytic infection which presumably is asexual infection, has been shown in graft material in Europe, resulting in 12.5% mortality in the first 2 years after grafting, and up to 40% mortality after 3 years (Pasche et al. 2016a). A noticeable difference between European and Australian grafting practices is the use of bud grafting in Australia versus stem grafting in Europe, providing fewer opportunities for buds to carry the pathogen to scions. Hence chestnut mortality associated with grafting is not a major issue in Australia.

Endophyte communities

Endophytes (fungi and bacteria) are critical components of forest ecosystems, and are ubiquitous microbes that live within seemingly healthy plant tissues for part of their life cycle, without causing any disease symptoms on their plant hosts (Carroll 1995). These microbial symbionts are important as they can improve plant growth, enhance stress resistance, potentially play a role in plant defense, shape phytochemical profiles, and mediate plant functional trait expression. However, the endophytic relationship with host plants can also turn to parasitism, which is triggered by environmental stressors and can depend on the genetics or physiology of the host plant or endophyte.

Dispersal ability of endophytes is associated with a number of factors. Unlike the clavicipitaceous endophytes (such as ergot) of grasses, which are vertically (through seed, thus plant sexually reproductive material) transmitted, endophytes associated with the foliar tissue of woody plants appear to be horizontally (from tree to tree) transmitted, and thus show strong spatial scale structure, dispersal limitation and ecological drift. However chestnut rot is an exception since it is both horizontally (from tree to tree) and vertically (via nut fall) transmitted. Thus, perhaps not unexpected that limited spatial structure was identified for *G. smithogilvyi* in a small-scale endophyte study on chestnuts (Fernandez-Conradi et al. 2019) in Italy. However, endophyte communities naturally differ across plant species and even host tree genotypes, and are expected to differ in stressed versus healthy trees.

Endophytic fungi have diverse roles, sometimes influencing plant fitness negatively as pathogens (Newton et al. 2010). For example, *Hymenoscyphus fraxineus*, the causal agent of ash dieback in Europe, can be found as an asymptomatic endophyte living inside leaves of several *Fraxinus* species in its native East Asian range (Cleary et al. 2016). Endophytes may also confer benefits to their hosts by increasing plant tolerance to stress (Rodriguez and Redman 2007), sometimes reducing herbivory through the production of toxic alkaloids (Wilkinson et al. 2000) and via antagonistic effects that reduce infection of plant tissues by pathogens (Arnold et al. 2003). Such roles are of course dependent on any large number of variables including the affected host plant species and environmental parameters that condition the host for infection.

With the changing climate, endophytes have reported capabilities to alleviate the plant responses to several adverse environmental conditions such as drought, elevated temperatures, nutrient scarcity, flooding, sedimentation, and many more (Ryan et al. 2008, Wani et al. 2015, Santoyo et al. 2016). Endophytes, particularly endophytic bacteria, also improve plant health and longevity under harsh conditions and these effects are universal for different plant taxa and stressors (Franco-Franklin et al. 2021). The genetics or physiology of the host plant or endophyte and/or ecological conditions influence the characteristics of the relationship between host plant and endophytes (Wu et al. 2021), however, many features of endophytic biology are unexplored. Therefore, analytical research is required for specific plant species under specific stress to recognize the endophyte-plant relationship and yield the most productive consequences. It is important to note that endophytes may also play a role in plant health, and altering those communities may result in disease (Arnold et al. 2003), such as dieback in *Parkinsonia* trees which is associated with altered endophyte communities (Steinrucken et al. 2016).

Since endophytes may confer benefits to plant growth and health, a central question to management of chestnut nut rot is, should we attempt to control endophytes? By applying eg. chemical applications to control *G. smithogilvyi*, inadvertently the endophyte community will be affected too. How will the indiscriminate eradication of endophytes affect plant health? From the literature it is evident that endophytes play a crucial role in plant health and adaptability, thus managing tree health/vigour rather than the endophytic community may be a more sensible option.

Microbiomes (really just endophytes, but microbiomes refer to the endophyte community as determined with DNA sequencing)

Microbiomes are the living (fungi and bacteria) endophytic component of plants. Fungal community studies using DNA barcoding have shown that highly diverse fungal communities are associated with healthy tissue of angiosperm trees (eg. (Jumpponen and Jones 2009, Cordier et al. 2012, Bálint et al. 2013)). The only microbiome study in chestnuts, a leaf endophyte study in southern Italy, showed that the fungal microbiome community differs among different tissue types, but were independent of the forest stand composition (pure vs mixed stands) (Fernandez-Conradi et al. 2019). They also showed that the microbiome composition differs, and richness is lower in gall tissue compared to leaf tissue. Although it is impossible to assign cause and effect with the microbiome community and gall formation, it is clear that the microbiome community is altered in diseased tissue. Gall tissue are rapidly growing plant cells, where *G. smithogilvyi* may have a competitive advantage over other endophytes. The fact that fungal endophyte communities in both galls and associated tissues were independent of tree species composition of forest stands is probably due to the large variability in endophytes species traits, particularly their host specificity (Fernandez-Conradi et al. 2019). It is likely though that endophyte communities differ among chestnut cultivars, as is the case for many other crops. More research on functional diversity of leaf-inhabiting fungi is clearly needed to better disentangle the mechanisms underlying plant endophytic fungi interactions in forests. For disease management, it may thus be important to maintain a healthy microbiome, as it may play a role in *G. smithogilvyi* disease development/prevention.

Control of chestnut nut rot

Unfortunately, there are no one magic bullet for management and control of the disease. To date, management advice has focused almost exclusively on clean-up of autumn litter to eliminate potential sources of inoculum. However, typically fungal ascospores disperse over long distances (10-100 km), coupled with other hosts potentially providing inoculum, and the unknown proposed role of asexual reproduction (Sillo et al. 2017), removal of litter is unlikely to provide complete disease protection, but will assist in reducing the inoculum potential. Furthermore, no association could be found between orchard density and the spatial pattern of nut rot (Lione and Gonthier 2016), suggesting nut rot control by altering the orchard plantation density is likely to fail.

Biological control

The addition of biological control agents to control *G. smithogilvyi* have been investigated extensively in the last decade. For example, Pasche et al. (2016b) artificially inoculated 1-year-old chestnut to examine the efficacy of common endophytes *Trichoderma atroviride* and *Bacillus amyloliquefaciens* as antagonists to *G. smithogilvyi*. Dobry and Cambell (2023) provided a good summary of their findings: "Their results indicated that the presence of endophytic antagonists slowed the progression of the disease but did not eliminate it entirely. Eventually, *G. smithogilvyi* overcame the antagonists and became the dominant fungus present. They hypothesized that total colonization of the plant by the antagonists would cause all growth to eventually cease. However, two separate studies that described the fungal communities of chestnut wasp galls in multiple locations over several years 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. *G. smithogilvyi* 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 sufficiently."

Recently, the use of biological control agents to suppress nut rot has also been investigated in an *in vitro* study in Australia (Silva-Campos et al. 2022a). Three commercially available biocontrol products (TRI, SUP and D25) were tested for their ability to reduce the *in vitro* culture growth of *G. smithogilvyi*. Although the commercial products tested by Silva-Campos et al (2022a) were labelled as containing a number of species of *Trichoderma* and/or *Bacillus* and *Pseudomonas*, only *Trichoderma* (TRI) and *Bacillus* (SUP) species were found to be present. Thus, first of all, this study demonstrated the perils of commercial biological control formulations, which do not contain all the biological agents they are claimed to have present. This is true for commercial biocontrol products worldwide, where only 10% of products were found to contain the biocontrol agents specified on the packaging. The authors did not identify the biocontrol agents in D25, although reported it as non-complex formulae, suggesting few species present. *Trichoderma* was most effective and significantly suppressed growth of *G. smithogilvyi* in a dual culture assay, although it did not completely suppress growth. Similarly, extracted non-volatile compounds from the biocontrol products SUP followed by TRI, significantly reduced *G. smithogilvyi* growth at 20 mg/mL, as tested on two cultures of *G. smithogilvyi.*

Furthermore, metabolites from TRI followed by SUP, but not D25, was able to significantly suppress *G. smithogilvyi* mycelial growth and conidial germination (Silva-Campos et al. 2022a). These products show some promise, although their efficacy in natural environments still have to be validated. However, at best, these products only suppressed *G. smithogilvyi* growth by approximately 50%. Furthermore, the effect these products may have on other fungi and bacteria that may be beneficial to chestnut trees, either in promoting growth/health, or as providing competition to pathogens such as *G. smithogilvyi* has to be assessed. Unfortunately, based on the work in Switzerland (Pasche et al. 2016b), it is unlikely that these biocontrol agents will be able to outcompete *G. smithogilvyi*, hence will not provide sufficient protection against the disease.

Chemical control

Investigation into chemical control for nut rot is limited. One study was conducted in Australia, where six fungicides were first tested *in vitro* against two isolates, then applied in the orchards (Silva-Campos et al. 2022b). The fungicides tested *in vitro* included pyraclostrobin, difenoconazole, Iprodione, fludioxonil, prochloraz, and cyprodinil+fludioxonil. The experiments showed that of those tested fungicides, pyraclostrobin followed by difenoconazole-based fungicides inhibited

conidial germination and mycelial growth most. The two isolates tested differed in their sensitivity to the fungicides, suggesting that a much larger range of isolates need to be tested. Based on the *in vitro* study, pyraclostrobin and difenoconazole were selected for field trials and applied during flower anthesis. Active ingredients combined were more effective than single applications in suppressing the level of nut infection caused by *G. smithogilvyi.* Furthermore, pyraclostrobin and difenoconazole were more effective at reducing infection of the stigmas than of styles and nuts. The authors suggest that "the stigmas are continuously exposed to the external environment, which facilitates their contact with the fungicides, whereas styles and nuts are rapidly enclosed by the burr (30 dpi in their experiment). The physical barrier of the burrs suggests that a critical time for fungicide application to hamper *G. smithogilvyi* infection is before the burr encloses the styles and nuts. However, further studies should be performed to clarify this and determine if fungicide applications delivered between flower anthesis, and burr enclosure reduce nut infection and thus chestnut rot" (Silva-Campos et al. 2022b). Although these fungicides showed promise in reducing *G. smithogilvyi* infection, the majority of treatments did not do so significantly and no statistical difference from control infections could be found 110 days after inoculation, suggesting fungicides not a viable option for control.

Using the selected fungicides above may though provide an additional tool for growers to complement their current practices in the control of chestnut rot, however, the effectiveness of the fungicides tested in suppressing growth of the fungus is a little underwhelming, given that even the most effective combined fungicide treatment, irrespective of time of inoculation, did not result in adequate protection (at most it halved the infection occurrence). Fine tuning eg. time of fungicide application and perhaps applying a follow up application could be investigated. Investigating fungicides with other active ingredients and those with multi-site activity will further provide alternatives, especially to rotate fungicides to prevent build-up of fungicide resistance. The effect of fungicides on the chestnut microbiome should also be investigated as fungicides may prove detrimental to the microbiome community in the long run, eventually perhaps requiring an increased number of fungicide applications to combat the disease if the microbiome community is altered by the fungicide applications.

In central Italy, another study focused on chemical applications, this time using chemicals which are proposed to have minimal environmental impacts, to control nut rot both *in vitro* and in chestnut orchards (Bastianelli et al. 2022a). A detailed explanation of this experiment will be provided below, as it shows some promise for nut rot control. Products evaluated in this experiment included Kalex® (AlbaMilagro International Ltd., Parabiago, Italy) containing 50% w/w potassium-phosphite (KH_2PO_3), this is a liquid fertilizer and enhancer of the plant resistance; 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; and Mystic® 430 SC (Nufarm Italia Ltd., Milano, Italy) containing 40.18% (w/v) Tebuconazole ($C_{16}H_{22}ClN_3O$), a conventional chemical treatment. All three products significantly reduced *G. smithogilvyi in vitro* growth.

Trials in the field were conducted on 15-year-old "Marrone Fiorentino" trees. Two trials were conducted, in the first trial in 2019, products were sprayed once onto trees during blooming and burr formation. In the second trial in the following year, products were spray applied either twice or three times, approximately 1 month apart; during blooming; during burr development; and during kernel development. A treatment where Kalex® applied as a once off stem injection with syringes (endotherapy) was also conducted, similar to *Phytophthora* control in avocados in Australia. A once off application of all the tested products significantly reduced the percentage of chestnuts from which *G. smithogilvyi* could be isolated. In 2020, all treatments, except the Kalex injection (endotherapy), significantly reduced nut rot incidence and severity. The different results for the Kalex injections in the two years is likely because of the differences in timing of the application (after bud burst in 2019; blooming in 2020), thus application before blooming commences is more effective as it takes time for the active ingredient to be translocated to the flowers (Bastianelli et al. 2022b).

Application of Kalex Zn significantly reduced disease, however whether Kalex Zn was applied once, twice or three times per year did not affect the disease incidence or severity, suggesting infection is during flowering and Kalex Zn application at burr and kernel development had no effect on disease development. Interestingly though, the fungicide application of Mystic® (tebuconazole), was most effective at reducing disease incidence and severity when applied twice (Bastianelli et al. 2022b), although it is unclear whether this is an artefact as there is no explanation why two applications should be more effective than three applications.

Phosphonate salts such as Kalex Zn thus provide a promising option for *G. smithogilvyi* nut rot control. It is though, still no magic cure, as even in the best treatments, infected nuts were reduced from 70 to 25% only. Although a significant reduction in infection, 25% infection is still high. Fortunately, phosphonate salts have a low environmental impact when used at the recommended doses, are effectively translocated systemically in the phloem and xylem within the tree, are

relatively stable in the tree, and pose multiple mechanisms of actions. Phosphonates have a complex mode of action whereby it both improves the resilience of the tree and affect pathogen growth, leaves no toxic residues, and are relatively inexpensive (Bastianelli et al. 2022b).

Gnomoniopsis smithogilvyi is an endophyte poses unique challenges for its control and management. Recently Dobry and Campbell (2023) suggested that "Perhaps the greatest potential to combat this pathogen lies in the development of transgenic trees such as those presently being developed in the United States to combat chestnut blight infection in American chestnuts through introduction of the oxalate oxidase gene into the chestnut genome (Steiner et al. 2017). Presently, it is unknown whether *G. smithogilvyi* induces damage through the production of oxalate, a trait often associated with pathogenic fungi including chestnut blight, that increases acidification and promotes degradation of host tissues (Chen et al. 2010). However, should *G. smithogilvyi* be an oxalate-producing pathogen, there is hope in the development of transgenic trees to diminish or neutralize its destructive capabilities."

Storage and handling

Reducing the number of infected chestnuts harvested appears to be the main strategy to minimize losses in storage. During mechanical harvesting, infected chestnuts are separated from healthy fruit by forced air. In Italy, this may be followed up by a *curatora* step immersing chestnuts in hot water (45-50 ºC; 40-45 min), followed by immersing chestnuts in cold (10-15 ºC) tap water for 72-96h (*curatura*) in large bins (ratio fruits: water 2:1 v/v). Water curing treatments permit especially insect-infected nuts to be separated. Regardless of whether the *curatura* stage occurs, fruits are rapidly immersed in tap water in large bins to remove floating fruits and debris (Morales-Rodriguez et al. 2022).

A hot water treatment was investigated in Italy to sterilize *Gnomoniopsis* infected chestnut fruit (Morales-Rodriguez et al. 2022). The most effective hot water treatment (50 ºC; 45 min) was able to drastically reduce the percentage of fruit from which *G. smithogilvyi* could be isolated immediately after treatment. However, the heat treatment was more fungistatic than fungicidal because after 30 days of storage, isolation frequency of *G. smithogilvyi* was similar to that of untreated fruit. Higher temperatures (> 50 ºC) were more effective at sterilising the fruit, but caused unacceptable changes to the sensory quality of the fruit (Morales-Rodriguez et al. 2022).

One of the earliest experiments to improve post-harvest storage, included adding cell-wall degrading enzymes from *Trichoderma harzianum* to chestnuts during a hot water treatment (45-50 ºC for 50 min; then in 15-18 ºC for 50 min) at a ratio of 3:1 (Ruocco et al. 2016). After 2 months the disease incidence was 50% in treated nuts compared to 85% in untreated chestnuts. Unfortunately, in this experiment, disease was caused by a plethora of fungi, not only *G. smithogilvyi*. Furthermore, disease still occurred at approximately 50% incidence after treatment, albeit reduced, and a large volume of enzymes needed to be added, rendering this not a particularly useful method of control.

An ozone (O_3) gaseous treatment has also been proposed to control *G. smithogilvyi* in storage (Vettraino et al. 2019). Ozone inhibited *in vitro* growth of *G. smithogilvyi* cultures. Ozone was also applied to chestnut fruit in storage. Chestnuts were maintained under a continuous flow of ozone in the air (150 ppb during the day, 300 ppb during the night), at 2.0 ± 0.5 ºC; relative humidity at 95 ± 2.0%. Chestnuts were kept in perforated trays and turned every 3 days. The incidence of nuts with decay after 24 days was 25% (from a baseline of 18% nuts infected), compared to 87% in storage without an ozone treatment. However, after 150 days of storage, incidence of infected nuts increased to 75% for ozone treated nuts, and disease incidence was not different to control treatments of chestnuts (Vettraino et al. 2019), suggesting ozone does not kill the fungus and thus nuts cannot be reliably stored for extensively long periods, but may provide an option for short term storage (24 days). Unfortunately the authors did not test the effect of ozone on the fungus for periods slightly longer than 24 days

Investigating methods to detect and remove infected chestnuts fruit prior to storage will assist in improving the quality of the saleable product. Such methods may include non-destructive near infrared (NIR) spectroscopy (Moscetti et al. 2014). Fourier transform near infrared (FT-NIR) spectroscopy or visible-near infrared hyperspectral imaging has been shown to successfully detect "unsound" chestnuts (Bedini et al. 2020), although most of the chestnuts in their study appear to have been insect-damaged and not infected with *G. smithogilvyi*. Recently, a molecular quantitative PCR assay was developed to detect *G. smithogilvyi* in asymptomatic and symptomatic tissue (Turco et al. 2021). Further, in Australia, a multiplex PCR was developed to detect *G. smithogilvyi* in chestnut tissue (Silva-Campos et al. 2022c). Although useful techniques to study

eg. the epidemiology of the pathogen, all these proposed methods are unfortunately currently impractical at large scale detection of chestnuts after harvest as a way of reducing diseased fruit on the market.

Outputs

Table 1. Output summary

Output	Description	Detail
Literature review	A collation of all available scientific literature on the subject of nut rot of chestnuts. Specific attention is given to the biology of the pathogen, infection, factors associated with disease development, its endophytic nature, as well as control and management options from the literature. The intended audience is growers as members of Chestnut Australia Inc.	Findings of this literature review will be presented to Chestnut growers at their next meeting on 18 November, Bright. Discussion on the literature review content occurred with a Project Reference Group on the 7th of August. 2023. The literature review will be uploaded to the Delivery Partner Portal.
Set up a Project Reference Group	A group consisting of growers representing multiple chestnut growing areas of Australia	The PRG was set up with meeting held on the 7th of August 2023

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Critically evaluated scientific knowledge of the disease biology, epidemiology, and control and management options for chestnut nut rot	Critically evaluate scientific knowledge of the disease biology, epidemiology, and control and management options	Information in scientific papers were summarised and critically evaluated, to provide an easy to understand summary of the disease.	Scientific literature
Develop a research plan to manage and control nut rot of chestnuts	Develop a research plan	Identify key areas that require further investigation for adoption/adaptation to Australian conditions	In collaboration with the Project Reference Group
Coordinate with a chestnut research group (Project Reference Group) and growers	Coordinate with a chestnut research group (Project Reference Group)	The Project Reference group provided valuable feedback and direction for the literature review	Feedback from the Project Reference Group and chestnut growers

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
Which nut rot management strategies are available in the literature	All available literature on control options were critically evaluated. Most over-claimed the efficacy of control options, and did not take into account the effect of such methods on tree endophyte health	Promising control options posited in the literature should be evaluated for efficacy in Australia, as well as their long-term effect on eg endophyte communities
What is the current knowledge on the disease biology and infection	Literature addressing these issues were critically examined. Although some progress has been made worldwide in understanding the disease, critical gaps were identified in knowledge	Having narrowed down critical aspects for disease development, a targeted approach is recommended to identify factors in disease development, such as timing of high temperatures experienced by the plant in favouring disease development

Recommendations

A recent insurgence in papers on chestnut rot, especially from Europe, highlights the current and future threat *G. smithogilvyi* poses to chestnut production. It is an emergent pathogen that is continuously spreading to new areas of the world. Much is still to learn about its biology, but it is now generally accepted that the pathogen is also an endophyte. It is also reasonably well established that infection is with ascospores through the female flowers, both from direct evidence from research done by Shuttleworth and co-workers, but also from a number of anecdotal evidences. Many questions remain though eg. 1. **Do the endophytic and pathogenic populations differ in their population structure**? ie. are the two populations genetically isolated; 2. Are all isolates able to cause disease, regardless of whether they were obtained from symptomless material or diseased tissue?; 3. Are *G. smithogilvyi* from other hosts able to cause disease in chestnuts, ie. what is the real threat of having other hosts near chestnut orchards?; 4. What causes the "switch" from being an endophyte to becoming pathogenic?; 5. What is the role of the endophyte community (diversity and abundance) in disease development?; 6. What is the mating structure of the pathogen (homo- or heterothallic)?; and 7. We need to better understand environmental and ecological factors favouring disease development, eg. when and for how long do high temperatures favour disease development? Answers to these questions will help us understand the biology of the fungus and inform us on control and management of the disease.

For control and management of the disease, there is no silver bullet, **rather an integrated control strategy is suggested**. This includes management by **removing litter** from the orchard floor (to reduce ascospore load). Biological control options seem largely ineffective in the orchard, but some promise is offered with **fungicide and phosphonate** salt applications. There is however still much to learn about these options, not only to establish whether they are effective under Australian conditions, but also what their optimal timing of application is, as well as their effect on the microbiome (endophyte) community, both in the short and long term. Less so for phosphonate salts, but fungi often develop resistance against fungicides, thus long-term use of a fungicide further poses additional risks.

The association of the disease with high temperature stress, coupled with rainfall required for ascospore release prior to chestnut flowering, suggest that occurrence of the disease could be forecasted based on the environmental variables to inform the necessity of chemical applications. However, for forecast models to work best, more details are required as to exactly when eg. temperatures need to be higher than usual, for disease to develop. More details on other environmental triggers such as wind, are also required.

Reducing the number of diseased chestnuts in storage and on the market, still poses challenges even though a number of disease-reducing options are described. Removing plant debris and floating chestnuts will assist in getting rid of the worst affected chestnuts, but not all. Options such as a hot water treatment (*curatura*) seems largely ineffective, while an ozone treatment may offer some protection for up to 24 days, which in many cases will not be sufficient to get healthy chestnuts to the customer. Investigations into whether visible-near infrared hyperspectral imaging (FT-NIR) could be effective in identifying diseased nuts, and whether it could be feasible and cost effective to apply at the farm gate, could be investigated.

In conclusion, as with most diseases, we have much to learn about the disease. It is unfortunately a disease that is likely to have even more impact under current climate predictions given that high temperatures are linked to disease occurrence.

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Intellectual property

No project IP or commercialisation to report

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