University of Reading

Integrated Management of Honey Fungus, *Armillaria mellea*: Biological Control, Culture, and Early Detection.

Submitted for the degree of Doctorate of Philosophy

School of Biological Sciences

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Luke Eric Hailey

In memory of Joy Hailey,

my grandmother,

who gifted me a little of her passion for plants.

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Abstract

Worldwide, trees face increased levels of stress both abiotic and biotic, e.g., climate change, insect and disease invasions associated with trade. Impacts are especially prominent on high value cultivated trees, typically already stressed through human oversight. Such stress increases susceptibility to attack by *Armillaria* fungi; root and stem phytopathogens with a wide host range. Saprobic ability allows their survival following host/tissue mortality; persistence on buried woody inoculum extends into decades. Local inoculum volume is the other key factor in infection risk. *A. mellea* is a highly aggressive species and is a key focus for control efforts: this study aims to further knowledge that can be used within, or in the development of, its integrated management.

Current *Armillaria* management relies on laborious physical removal. Chemical controls are ineffective or banned due to environmental concerns. Biological controls, e.g., single *Trichoderma* strains, have shown much potential experimentally but failed to provide a control for widespread use. Endophytes are a potential source of targeted biological control agents, but their development is challenging. The use of well-characterised bacteria and compounds that stimulate host immunity are under-investigated. *In vitro*, combination of a sub-lethal potassium phosphite dose, 750-1000mg/L agar, and antagonism by *Bacillus subtilis* QST713 reduced *A. mellea* growth by around 80%, meriting further *in planta* testing.

Early *Armillaria* symptoms are ambiguous/cryptic, hindering timely detection and compounding existing management issues. While studies utilize leaf physiology measurements to monitor infection, there has been little investigation of their application for early detection. Stomatal conductance and chlorophyll responded 35-40 days after *Armillaria mellea* inoculations in *Ligustrum vulgare*, a sensitive woody host. This occurred prior to conclusive visual symptoms and faster than the duration of typical host infection assays on *Fragaria × ananassa*; a herbaceous host which demonstrates comparatively rapid onset of symptoms and mortality. Chlorophyll fluorescence measures also reacted. These infection signatures can be masked by drought and other physiological stresses. Combination of measurements can improve classification of infected and uninfected plants.

Armillaria research methodologies, i.e. for fungal culture or host inoculation, face little comparison and vary greatly between studies. As inoculum substrates, the large seeds of *Aesculus hippocastanum* increased the extent and speed of disease symptoms and mortality compared to conventional *Corylus avellana* billets, on *Fragaria* and *Ligustrum* hosts. *Quercus robur* seeds had the lowest performance. The differences reflect resources provided to the fungus. On agar, subculture by homogenized mycelium halved variability in comparison to colony fragments. These findings can increase research efficiency.

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3. Preliminary Investigations into the Integrated Control of *Armillaria mellea* using biological controls and plant defence activators	Original Ideas: LH Design: LH Analysis: LH Writing: LH **LH estimated contribution: 100%**
4. *Armillaria* inoculation and culture: an exploration of novel methodologies	Original Idea: LH, further developed with JD & HR. Design: *In vitro* by LH. *In planta* developed by LH & JD. Data collection: LH, JD, HR. Analysis: LH with some input on non-parametric testing from HR Writing: LH. Suggested edits and inclusions were given by JD, HR, GP, SG, GB, & RJ. **LH estimated contribution: 85%**
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6. General Discussion	**LH estimated contribution: 100%**

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1. General Introduction: Opportunities in the management & control of *Armillaria* root rot of trees, with special reference to the UK & Europe

1.1 Trees and Humans

Trees are generally defined as large woody plants with a perennial lifecycle, although there are multiple exceptions (Hirons & Thomas, 2018); the grouping is non-taxonomic as woodiness has evolved multiple times independently (Carlquist, 2013; Groover & Cronk, 2013). Being the largest of all plants, trees form a great proportion of the global biomass and support much of the remainder, therefore they are of fundamental importance for the functioning of ecosystems worldwide (Petit & Hampe, 2006; Rabiey *et al.*, 2019). Accordingly, trees provide resources to many species of animal, including humans. Trees provide oxygen, food and fodder, shelter, timber, fuel, as well as substances for ritual and medical use (Percival *et al.*, 2014). While some uses have reduced as technology develops, timber and food provided by trees remain widely valued resources. Alongside their historic value, trees often also have strong emotional, spiritual, and cultural value to human individuals and groups (Cloke & Jones, 2020).

Due to the resources they offer, be they material or psychological, trees are commonly cultivated by humans. These trees, from ornamental plantings to food and timber crops, are likely to suffer increased stress compared to those arising naturally, due to oversights in their selection, cultivation, and planting. For example, choice of species on an aesthetic basis rather than adaptation to the local environment (Percival *et al.*, 2006; Sjöman & Nielsen, 2010), low diversity of planted species (Sjöman *et al.*, 2012), or incorrect cultivation, transplantation, and aftercare (Aldhous & Mason, 1994; Ferrini & Nicese, 2002; Grossnickle, 2005; Gilman *et al.*, 2015; Percival & Schaffert, 2015).

Trees are receiving increasing recognition as ‘green infrastructure’ in urban environments: providing habitat to urban wildlife, promoting physical and mental well-being, reducing air pollution, intercepting rainfall and reducing flooding, shading and reducing heat absorption by man-made surfaces (Xiao & McPherson, 2002; Nowak, 2004; Tyrväinen *et al.*, 2005; Rabiey *et al.*, 2019). As such, these trees can be of high individual and collective value: for example, trees in London, UK, are estimated to have a mean ‘CAVAT’ amenity value of over £5000 per tree and collectively give annual benefits worth over one hundred million pounds (Rogers *et al.*, 2015). However, the urban environment also has its own

particular set of abiotic stress factors, which are widely recognized as particularly challenging for many tree species e.g., the urban heat island effect, high cover of impermeable surfaces, flooding, compaction, and high salinity (Tattar, 1981; Ballach *et al.*, 1998; Pauleit, 2003; Tubby & Webber, 2010; Sjöman *et al.*, 2012; Hailey & Percival, 2015).

In tandem, and often more severely in urban environments, climate change may be increasing abiotic stress levels for many trees (Hailey & Percival, 2015; Rabiey *et al.*, 2019). It is also playing a role in altering pest and disease distribution and severity, aided by poor biosecurity practices as well as causing potential shifts to pathogenicity in endophytes (Guillaumin *et al.*, 2005a; Brasier, 2008; Tubby & Webber, 2010; Moricca & Ragazzi, 2011; Delaye *et al.*, 2013; Rabiey *et al.*, 2019).

As is typical worldwide, a number of serious tree pest and disease invasions have happened in the UK during the past few decades. For example, oak processionary moth (*Thaumetopoea processionea*), oriental chestnut gall wasp (*Dryocosmus kuriphilus*), horse chestnut leafminer (*Cameraria ohridella*), larger eight-toothed European spruce bark beetle (*Ips typographus*), bleeding canker of horse chestnut (*Pseudomonas syringae* pv. *aesculi*), ash dieback (*Hymenoscyphus fraxineus*) and various *Phytophthora* species. There are also significant threats posed by other potential invasive species such as bacterial leaf scorch (*Xylella fastidiosa*), emerald ash borer (*Agrilus planipennis*), canker stain of plane (*Ceratocystis platani*), citrus and Asian longhorn beetle (*Anoplophora chinensis, A. glabripennis*) (Rabiey *et al.*, 2019; Forest Research, 2021). As demonstrated in these examples, there are multiple invasive species which are hosted on a single species or genus e.g. horse chestnut (*Aesculus hippocastaneum*) and ash (*Fraxinus* spp.), which is likely to exacerbate the losses in their populations when these pests and diseases co-occur.

As such, trees are currently facing increased levels of stress within and outside of cultivation. This is likely to make them more susceptible to native pathogens.

1.2 *Armillaria* Root Rot Disease

Armillaria is a globally distributed genus of fungus, in the phylum Basidiomycota. Many of its species are root phytopathogens, typically of woody species such as trees, shrubs, and vines (Baumgartner *et al.*, 2011), but also of herbaceous hosts (Ford *et al.*, 2017). Cumulatively the species of this genus are capable of infecting a very wide range of hosts, with a high economic impact (Raabe, 1962; Guillaumin & Botton, 2005; Baumgartner *et al.*, 2011; Ford *et al.*, 2017; Cromey *et al.*, 2020). *Armillaria* infection, primary or otherwise, is associated with abiotic stresses such as root damage, waterlogging, and drought. Changes in root nutrient and secondary metabolite status under such conditions enhance *Armillaria* growth (Popoola & Fox, 1996, 2003; Lung-Escarmant *et al.*, 2005). In Britain, it has long been

recognized that *Armillaria* is mainly an issue for amenity/ornamental trees (Rishbeth, 1983; Laflamme & Guillaumin, 2005). It was the most diagnosed disease on samples submitted to the Royal Horticultural Society by its members between 2001 and 2018, being present on 18% of samples (Cromey *et al.*, 2020). As discussed above, cultivated trees often suffer stress due to adverse environmental conditions and mistakes in their care. Root deformities and deep burial in particular have been linked to increased *Armillaria* susceptibility (Ouellette *et al.*, 1971; Singh & Richardson, 1973; Livingston, 1990; Legrand & Lung-Escarmant, 2005; Day *et al.*, 2009; Percival *et al.*, 2011). Biotic stress also increases susceptibility e.g. defoliation, ash dieback, and acute oak decline (Wargo, 1981; Chandelier *et al.*, 2016; Denman *et al.*, 2016). Aside from host stress, the other main factor driving infection risk aside from stress is the volume and viability of inoculum, i.e. colonized material, present within the soil of a site.

Ecologically, *Armillaria* spp. are generalists with high plasticity and, as a group, exhibit multiple strategies in their acquisition of nutrition from varied sources (Heinzelmann *et al.*, 2019). In relation to plants, these strategies occur on a spectrum ranging from saprophytic to pathogenic, via parasitism. All three of these strategies are utilized in the disease cycle. As saprobes, *Armillaria* spp. play an important ecological role by degrading deadwood in the environment, breaking down both cellulose and lignin. Any infested material, from saprophytic or pathogenic colonization, can provide a reservoir of infection, remaining viable up to decades after colonization and the death of hosts (Guillaumin & Legrand, 2005; Baumgartner *et al.*, 2011). Therefore, sites as a whole are often considered 'infected'.

Armillaria spp. can spread through soil and between food sources, including host root systems, as rhizomorphs (Plate 1) which melanize, resembling black bootlaces. *Armillaria* rhizomorphs demonstrate high complexity and unique adaptations in comparison to those of other fungi (Baumgartner *et al.*, 2011; Koch *et al.*, 2017; Sipos *et al.*, 2017). Their spread can cover large areas; *Armillaria* individuals are some of the largest recorded organisms in the world (Sipos *et al.*, 2017). *Armillaria* spp. also spread between hosts via direct root-root or root-inoculum contact, while dispersal of typically a few hundred metres can be achieved via spores released from basidiocarps (Beal *et al.*, 2015; Heinzelmann *et al.*, 2019). Transcriptomic data indicates rhizomorphs are an adaptation of fruiting body stipes (stalks) (Sipos *et al.*, 2017). The outer sheath of rhizomorphs is melanised, and can accumulate metal ions, protecting hyphae inside from antagonistic soil microbiota (Rizzo *et al.*, 1992). The melanised sheath also forms a physical barrier and facilitates the transport of oxygen, water, and nutrients for growth and host invasion (Smith & Griffin, 1971; Anderson & Ullrich, 1982; Morrison, 1982; Cairney, 1992; Pareek *et al.*, 2006; Yafetto, 2008). Rhizomorphs are a key route of host infection by *Armillaria* species, attaching to woody roots and physically penetrating them using a combination of toxins, secreted enzymes and mechanical pressure (Sipos *et al.*, 2017; Devkota & Hammerschmidt, 2020). However, infection in the absence of rhizomorphs

is also reported (Thomas, 1934; Solla *et al.*, 2002). There is an apparent correlation between higher pathogenicity and a greater dependence on spreading via root-root contacts, whereas the more saprobic/opportunistically pathogenic species form wide networks of rhizomorphs through soil and between food sources (Heinzelmann *et al.*, 2019).

Complimenting their saprobic role in natural ecosystems, as pathogens *Armillaria* spp. remove and recycle stressed and dying individuals from tree populations. *In planta Armillaria* spp. are facultative necrotrophs: first parasitizing live cambium, then killing the tissue and forming a lesion which spreads through the cambium and wood. Initial infection typically occurs on the roots and can then spread into the trunk/stem. Initial root infections that do not succeed in spreading into the host may persist on colonized material as 'latent lesions' until host susceptibility increases, or the tree is felled, and spread can proceed (Guillaumin & Legrand, 2005).

Root collar infection is key to tree mortality, as once this area is infected the fungus can girdle the tree and/or move into the trunk (Baumgartner, 2004; Guillaumin & Legrand, 2005; Percival *et al.*, 2011). Infections typically spread into this area from lesions on the roots. Characteristic symptoms such as *Armillaria* fruiting bodies at the base of the tree or peeling bark with mycelial fans spreading beneath it, are often only present in the time around root collar infection. Prior symptoms are a general decline in health, e.g. yellowing, wilting, and/or dieback, which can be wrongly attributed to other factors such as environmental stresses. Susceptible experimental hosts have appeared healthy despite significant infections below-ground (West & Fox, 2002). Infections are not always discovered prior to host mortality. Trees with advanced infections may suffer unpredictable structural failure e.g. windthrow in a storm (Cromey *et al.*, 2020) or die suddenly in adverse weather such as drought.

Armillaria pathogenicity varies in its constituent factors by species and strain, e.g. propensity to be a primary or secondary pathogen, virulence, host mortality and health impact (Gregory *et al.*, 1991; Guillaumin & Legrand, 2005). Comparisons of virulence show wide variation between strains, even in species considered to be highly pathogenic e.g. 3-49% infection in *A. ostoyae* (Prospero *et al.*, 2004).

Plate 1. Growth of unmelanized *Armillaria mellea* rhizomorphs in culture. *On malt agar, shown from below. Small squares in scale = 1mm².*

1.3 *Armillaria mellea*

Armillaria mellea is the type species for the genus, and was also the taxonomic classification of many *Armillaria* species before the application of the biological species concept in the late 1970's (Baumgartner *et al.*, 2011). This information took time to disseminate to researchers. Therefore, studies prior to around 1990 on '*Armillaria mellea*' require extra scrutiny and may be best considered in generalised terms, although there are likely biases in which biological species were used in particular types of experiments (Guillaumin & Legrand, 2005). The taxonomy of *Armillaria* continues to develop at the time of writing (Heinzelmann *et al.*, 2019).

A. mellea commonly attacks cultivated plants worldwide and is regarded one of the most aggressive primary pathogens in the genus, attacking healthy plants and is a significant pest of orchards and vineyards. Therefore, it is an important focus of research for *Armillaria* root rot control (Morrison, 1989; Gregory *et al.*, 1991; Guillaumin & Legrand, 2005). The species is associated with angiosperm hosts but will also attack gymnosperms and even monocots (Guillaumin & Legrand, 2005; Ford *et al.*, 2017).

Molecular studies have demonstrated a comparatively large distance between *A. mellea* and its congeneric species, which may relate to its pathogenicity, and geographical isolates of the species may also be in the process of further speciation (Coetzee *et al.*, 2000). Another unique trait is that the rhizomorphs of *A. mellea*, whilst produced in large numbers, are of a transient nature, being short and fragile, indicating that they do not play a role in long-distance spread of the disease; instead the species relies primarily on root-root and root-inoculum contact to move between hosts (Guillaumin & Legrand, 2005). In a recent survey of infections in British gardens, the most common species of *Armillaria* was *A.*

mellea (83.1%) followed by *A. gallica* (15.8%) (Beal *et al.*, 2015; Drakulic *et al.*, 2017). This fits the general pattern for the majority of Europe as well as other areas in the world (Mihail & Bruhn, 2005).

1.4 Chemical Control

Control methods for *Armillaria* are relevant to any situation where trees are valued, because of the pathogen's wide host-range, and especially where trees are of high individual value e.g. urban/amenity trees or orchards (Legrand et al. 2006).

Historic *Armillaria* control was based on chemical soil sterilants such as methyl bromide gas or the phenolic formulation 'Armillatox' applied as an aqueous soil drench (Baumgartner *et al.*, 2011; Beal *et al.*, 2015). Both had issues with toxicity of various forms and Armillatox may have stimulated pathogen growth in the long term (Pawsey & Rahman, 1976; West, 1994; West & Fox, 2002; Martin, 2003). As such they are now withdrawn and there are no approved chemical controls in the UK or EU (Commission Decision, 2008; Chemicals Regulation Directorate, 2021).

Environmental concerns may be a factor as legislative shifts are being made towards the reduction of synthetic pesticide usage (Lamichhane *et al.*, 2017); of particular concern is the environmental fate of soil applied compounds which may have greatly increased persistence once bound to soil particles. A number of potential controls applied to soil or by trunk injection have been studied (Heaton & Dullahide, 1990; Turner, 1991; West, 1994; Adaskaveg *et al.*, 1999; Raziq & Fox, 2003a; Aguín *et al.*, 2006; Amiri *et al.*, 2008; Amiri & Schnabel, 2012; Thomidis & Exadaktylou, 2012; Beal *et al.*, 2015), although many proved ineffective in field application (Raziq, 1998; Amiri & Schnabel, 2012).

1.5 Cultural Control

In the absence of chemical controls, contemporary practices are based on cultural methods and are mostly aimed at reducing the inoculum volume on a site. Infected trees and stumps are removed as far as possible via a range of methods, dependent on context, e.g. pulling, digging, grinding, or chipping (Vasaitis *et al.*, 2008; Percival *et al.*, 2011; Bogdanski *et al.*, 2018). Fragmenting inoculum can expose it to fatal drying or antagonism by soil flora (Munnecke *et al.*, 1976; Tsoumou-Gavouka, 1982).

Compressed air excavation tools are used to disrupt the fungus in the root system and around the root collar. This technology has shown potential for physically breaking up inoculum, reducing its viability, and slowing early stage infections (Baumgartner, 2004; Percival *et al.*, 2011; Miller *et al.*, 2020). However, these tools are laborious to use for treating large areas such as the root zone of a mature tree. They must also disturb soil, which is not compatible with many situations e.g. being restricted by hard surfaces or for aesthetic reasons (Beal *et al.*, 2015). Regardless of the labour input, efforts to remove infected

material are unlikely to remove or disrupt all inoculum present. Barriers are sometimes placed into the soil in an attempt to contain infections but are also of limited impact (Wallace, 1935; Hagle & Shaw, 1991; Raziq, 1998; Legrand *et al.*, 2005).

Replanting species known for a higher tolerance to *Armillaria* (Gerlach *et al.*, 1997) is another common technique and diversifying planting is also used to reduce infection rates (Modi *et al.*, 2021). However, it would appear that there is no species entirely immune to the pathogen, especially under physiological stress; environmental factors may also alter species resistance (Hagle & Shaw, 1991; Raziq, 1998).

While practices such as stump removal provide economic levels of control in forestry, i.e. population level, they do not provide levels of control desired for situations where trees are of a higher individual value e.g. private gardens and parks. Reflecting this uncertainty, cultural controls are often referred to as 'management' instead by arborists and other plant health professionals.

1.6 Detection

All problems in the control of *Armillaria* are compounded by the issues in its detection. As described earlier, characteristic symptoms of *Armillaria* are often only present or obvious when there is little chance of the individual tree recovering. Often the first case identified on a site is of a late stage due to this. This appears to be a typical characteristic of root rot pathogens (Agustini *et al.*, 2015). The delay in detection allows the fungus to cause more damage to trees and form greater inoculum reservoirs to infect new hosts from. It also limits efforts to control or manage it, especially on an individual host basis. Early detection is a recognised area of weakness by other authors, although there is promise from advanced technologies e.g. 'electronic noses' used in the root zone (Navaei, 2015; Loulier *et al.*, 2020), or from leaf physiological measurements. Technologies are most developed for the latter and a number of available devices have been used successfully in studies monitoring infection (Loreto *et al.*, 1993; Nogales *et al.*, 2008; Percival *et al.*, 2011; Agustini *et al.*, 2015; Nowakowska *et al.*, 2020).

1.7 Biological Controls

Biological control agents (BCAs) provide a potential alternative to chemical controls and may complement or reduce the need for cultural controls. Soils may be 'suppressive' to particular pathogens due to their biological constituents and this is of specific relevance to soil-borne examples (Weller *et al.*, 2002) such as *Armillaria*. The influence of tree species mixtures and stump removal on *Armillaria* infection rates may be mediated via populations of such bacteria and fungi to varying degrees (DeLong *et al.*, 2002; Modi *et al.*, 2020, 2021).

Worldwide, the majority of BCAs applied against phytopathogens are based on bacteria and fungi. These are antagonistic to phytopathogens via a number of mechanisms, with BCA strains often using these in combination. These include direct action by antibiosis, competition, parasitism, and suppression of pathogenicity e.g. via enzyme inhibition, or indirect action by stimulating other beneficial organisms, and the alteration of host metabolism, defences, and/or resistance (Xu *et al.*, 2011; Pellegrini *et al.*, 2012). The colonization of the host e.g. via mycorrhizal association or the formation of biofilms, is also an important, although understudied, feature. The formation of biofilms may also radically alter BCA efficacy: via differences in metabolites produced, orchestrated antagonism by multiple cells, and increased resilience to antagonism and environmental factors in comparison to planktonic cells (Triveni *et al.*, 2015; Pandin *et al.*, 2017).

The species selected for commercialisation are generally ubiquitous in the natural world, being originally isolated from soil. This is especially advantageous for applications against soil-borne pathogens, although BCAs often also demonstrate survival elsewhere on the plant. Another trend is that they also form mutualistic relationships with plants, an added benefit to their application. However, this also complicates measuring their impact on pathogens by quantifying plant health; any increases may be unrelated to direct control of the pathogen, being a reduction in symptoms instead. For example *Glomus interadices* (Syn. *Rhizophagus irregularis*) has been shown to slow the progress of *Armillaria* infection of grapevines via stimulation of the host, rather than through direct antagonism or antibiosis (Nogales *et al.*, 2008, 2010). Similar effects on grape yield were produced by application of a mix of bacteria (Baumgartner & Warnock, 2006).

Pesticide legislation, designed for synthetic compounds, has historically been ill-fitted to the unique characteristics of BCAs. It is now far more amenable and there have been a number of governmental efforts to stimulate BCA development in Europe as an increasing number of synthetic pesticides are withdrawn (Gwynn & Dale, 2010; Chemicals Regulation Directorate, 2013; Lamichhane *et al.*, 2017).

BCAs have also faced practical issues in the past with variable performance and quality, often relating to viability of the living material (Corkidi *et al.*, 2004, 2005; Bashan *et al.*, 2013). In addition, to utilize a BCA is to attempt to harness an ecological interaction for a specific goal. This by its very nature involves a large number of variables e.g. soil type, organic matter levels, nutrient availability, climate, native soil biota, root exudates (Weller, 1988; Ongena & Jacques, 2008; Niu *et al.*, 2020) and so often yields variable results. However, there are now a number of well recognized commercial BCAs, examples of which will be discussed in the following sections. Direct antagonists provide the most desirable form of *Armillaria*

control for valued trees, protecting roots and inducing a soil suppressive to the primary spread of the pathogen (Pellegrini *et al.*, 2012).

Perhaps the most innovative example of potential BCAs for *Armillaria* are mycophagous nematodes (Riffle, 1973; Cayrol *et al.*, 1978; Tomalak, 2017). Biological interactions can also be the source of candidate chemicals that may be produced outside of the original organism. For example, species of *Gastrodia* orchids are dependent on *Armillaria* to grow, with a relationship that is typically referred to as symbiotic, although it is parasitic at some points of the shared lifecycle (Xu & Guo, 2000; Baumgartner *et al.*, 2011; Guo *et al.*, 2016); the benefits for the plant appear to be much better defined than those for the fungus. An antifungal protein produced by the orchid has shown potential for control of *Armillaria* (Hu *et al.*, 1999). In addition, there is also evidence of parasitism by other myco-heterotrophs, *Galeola* spp. (Baumgartner *et al.*, 2011) and *Monotropa uniflora* (Campbell, 1971). Unfortunately, both nematodes and parasitic plants fall outside the scope of this project.

1.8 Fungal Antagonists

The fungal antagonist of *Armillaria* which has received the most research attention is *Trichoderma* (Rees *et al.*, 2020). Although other fungi or their antibiotic metabolites have shown potential for control of *Armillaria*, alone or in combination with *Trichoderma* strains, e.g. *Scytalidium lignicola* (Cusson & Lachance, 1974; Highley, 1990), *Penicillium adametzii* (Kwaśna, 2001; Szwajkowska-Michalek *et al.*, 2012), *Hypomyces rosellus*, *Chaetoinium olivaceum* (Raziq, 1998), and the entomopathogenic fungi *Beauveria bassiana* (Reisenzein & Tiefenbrunner, 1997), of which one strain is already registered for commercial use as a bio-insecticide within the UK (Chemicals Regulation Directorate, 2021). Saprobic fungi have also shown potential use for reducing *Armillaria* inoculum pressure on hosts, by excluding and removing the pathogen from deadwood (Cox & Scherm, 2006). Perhaps the most surprising potential BCAs for the most pathogenic *Armillaria* species are more saprobic sympatric species of the genus (Warwell *et al.*, 2019; Heinzelmann *et al.*, 2019; Kedves *et al.*, 2021); although their utilization, relevance to highly valued individual trees, and applicability outside of forest ecosystems is unexplored.

The interaction between *Armillaria* and *Trichoderma* species has a long history of study, since around seventy years ago when *Trichoderma* was identified as the causal agent of long term *Armillaria* control following soil fumigation (Bliss, 1951). The action of methyl bromide and other soil fumigant treatments against *Armillaria* is believed to be indirect, having greater detriment to *Armillaria* than to its natural antagonists such as *Trichoderma* spp., allowing them to attack the weakened pathogen (Bliss, 1951; Munnecke & Wilbur, 1973; Lung-Escarmant *et al.*, 1985).

Armillaria produces multiple antibiotic compounds, e.g. sesquiterpene aryl esters, as part of its competition with soil flora, including antagonistic organisms, other saprophytes, root rot fungi, and even between different species of the genus (Obuchi *et al.*, 1990; Peipp & Sonnenbichler, 1992; Sonnenbichler *et al.*, 1997; Cremin *et al.*, 2000; Misiek & Hoffmeister, 2012). However, the growth of *Trichoderma harzianum* is not inhibited by any sesquiterpene aryl esters produced (Misiek & Hoffmeister, 2012). This species is mycoparasitic and attacks and parasitizes *Armillaria* hyphae by attaching, coiling and then penetrating them, after which it releases cell wall degrading enzymes and antibiotics to aid feeding (Dumas & Boyonoski, 1992; dos Reis Almeida *et al.*, 2007). This ultimately results in the destruction of the hyphae. *Trichoderma* will also penetrate rhizomorphs to reach the accumulated hyphae within, fragmenting the fungus and disrupting transport. Three strains of *Trichoderma* (*T. asperellum* T34, *T. harzianum* T22, *T. atroviride* SC1) are registered for use within the UK at the time of writing, although none for use against *Armillaria* or on amenity trees (Chemicals Regulation Directorate, 2021). *Trichoderma atroviride* SC1, has shown strong antagonism of *Armillaria* but is only registered in the EU & UK as a wound, trunk, or pre-planting treatment for viticulture, although treatment for *Armillaria* is 'envisioned' as a future use (Prodorutti *et al.*, 2009; Savazzini *et al.*, 2009; EFSA, 2015; Chemicals Regulation Directorate, 2021).

Despite strong *in vitro* evidence of *Trichoderma*'s antagonism of *Armillaria*, studies have produced mixed results as to the efficacy of treatments on hosts, varying by species and strain (Raziq, 1998; Prodorutti *et al.*, 2010). Problems in field applications cited include treating soil to a sufficient depth and, like other BCAs tested, maintaining effective populations for control (Shaw & Roth, 1978; Otieno *et al.*, 2003). This is commonly observed for BCAs applied for soil-borne phytopathogens (Niu *et al.*, 2020). Despite these issues, the great potential of *Trichoderma* strains as BCAs of *Armillaria* means their study continues (Rees *et al.*, 2020).

1.9 Bacterial Antagonists

With the historic focus on developing *Trichoderma* as a BCA for *Armillaria*, bacteria have received comparatively little research attention. While antibiotics produced by *A. mellea* and *A. novae-zelandiae* have shown activity against *B. subtilis* ATCC6633 and another strain (Obuchi *et al.*, 1990; Cremin *et al.*, 2000), multiple authors have demonstrated bacterial antagonism of *Armillaria* by various isolates. These include *Bacillus*, *Pseudomonas* and *Streptomycetes* species; however bacterial antagonism appears to vary by strain with only a small number tested showing inhibition of mycelial and rhizomorph growth (Hutchins, 1980; Fox *et al.*, 1991; Dumas, 1992; DeLong *et al.*, 2002; Baumgartner & Warnock, 2006; de Vasconcellos & Cardoso, 2009; Prodorutti *et al.*, 2010; Pellegrini *et al.*, 2013; Mesanza *et al.*, 2016).

Bacteria inhabit *Armillaria* rhizomorphs with involvement in attacks on hosts (Przemieniecki *et al.*, 2021). This group could provide potential sources, or targets for, *Armillaria* BCAs.

In a survey study, *Bacillus* and *Pseudomonas* were the most effective groups of bacterial antagonists of *Armillaria* tested *in vitro* (Pellegrini *et al.*, 2013), agreeing with prior research and opinion (Dumas, 1992; Dumas & Strunz, 1998). In a later study, isolates of *B. simplex* and *P. fluorescens* were also selected amongst the most effective bacterial antagonists against *A. mellea in vitro* (Mesanza *et al.*, 2016). Applied preventatively, seven days before host inoculation, they reduced mortality in *A. mellea* inoculated *Pinus radiata* by 87% and 76% respectively. The *B. simplex* strain gave the most significant reductions in infection of all strains tested. *Erwinia billingae*, a potential BCA of the pathogenic species of its own genus, was also tested and appeared to reduce mortality further but showed mixed results in terms of reducing *A. mellea* lesion occurrence. Results from this study support using *in vitro* growth inhibition for screening bacteria for use against *A. mellea in planta*, although the correlation is not consistent (1.7).

Despite their widely-regarded antagonistic qualities, no studies have surveyed interactions of *A. mellea* with a number of well characterised *Pseudomonas* strains, aside from the Pf-5 strain of *P. protegens* (Pellegrini *et al.*, 2012), previously *P. fluorescens* (Ramette *et al.*, 2011; Mercado-Blanco, 2015).

Bacteria may antagonise phytopathogenic fungi through a number of different mechanisms as described above. Arguably the most significant of the direct mechanisms is the production of antibiotics (Cawoy *et al.*, 2015). Both *Pseudomonas* and *Bacillus* strains produce a wide range of antifungal compounds in their antagonistic efforts (Raaijmakers *et al.*, 2010; Cawoy *et al.*, 2015; Mercado-Blanco, 2015). Accordingly, *P. protegens* Pf-5 antagonises *Armillaria* via the release of antibiotic substances rather than direct parasitism, demonstrated in antagonism experiments against ^{13}C-labelled *Armillaria mellea* 05BV (Pellegrini *et al.*, 2012). Soil bacteria that gave the greatest growth inhibition of this *Armillaria* strain visually demonstrated high antibiosis, or alteration of morphology also putatively caused by antibiotics (Pellegrini *et al.*, 2013).

There are two *Pseudomonas* spp. currently registered for use in the UK, but only as seed treatments (Chemicals Regulation Directorate, 2021). In contrast to *Pseudomonas, Bacillus* species form highly resilient endospores which gives them greater potential as commercial products. When compared to synthetic products, biological products typically have a reduced shelf-life and more complex storage and handling (Bashan *et al.*, 2013): the resilience of endospores lessens these issues. Therefore, there are a greater number of registered *Bacillus* products in the UK, including insecticides, fungicides and a nematicide. *B. subtilis* QST713 is registered as a biofungicide with off-label approval for other soil-borne

diseases i.e. *Phytophthora*, *Pythium*, *Rhizoctonia*, Damping off, and *Streptomyces scabies* (Chemicals Regulation Directorate, 2021). The approval for *Phytophthora* is especially relevant as it may easily be extended to cover applications against *Armillaria*. Firstly, it covers the 'Amenity Vegetation' field of use, allowing application to a wide range of ornamental trees and shrubs, reflecting *Armillaria*'s wide host range; secondly, it also permits the product's application as a soil drench in comparison to on-label spray, which if activity is shown against *Armillaria* may allow more targeted applications.

1.10 Combination and selection of Antagonists

Combined application of biological control agents (BCAs) can give a synergistic effect, increasing overall efficacy to a greater degree than the sum of additive effects. The use of radically different organisms e.g. bacteria and fungi, or those with varied mechanisms of control or ecological niches, may be especially promising (Guetsky *et al.*, 2002; Schmidt *et al.*, 2004). Potential improvements offered by combinations are enhanced host colonization, establishment, biofilm formation, competitive ability, synergy in antibiosis, exploitation of nutritional resources, and spatial spread. Indeed there are a number of recorded successful multi-strain BCAs for other soil-borne diseases, yielding apparent increases in efficacy over single-strain examples (Niu *et al.*, 2020). However a prior 'meta-analysis' style review indicates that such cases are rare, occurring in 2% of 465 studies, and that antagonistic interactions between combined BCAs are common (Xu *et al.*, 2011). Therefore, selection of constituent BCAs may need to be a sophisticated process, e.g. a 'reductionist synthetic community approach', to increase the chances of success (Niu *et al.*, 2020). There is only one example found of research into combinations of antagonists against *Armillaria*; here a single *Trichoderma* strain outperformed combinations and there was an antagonistic effect when it was combined (Raziq & Fox, 2005). This area of research is so small that it did not feature in a recent review on *Armillaria* BCAs (Kedves *et al.*, 2021)

However, combinations of previously described potential BCA candidates have been trialled against other pathogens. For example, *Beauveria bassiana* has potential in combined applications with *Trichoderma*: dual inoculations between various strains were established in cabbage plants, including the UK commercialized strain (Zhang, 2014). There were signs of antagonism of the *Beauveria* by some *Trichoderma* strains, but this may relate to *Beauveria* demonstrating higher establishment in stems than roots. This preference and mode of application may be exploited; if effects are systematic within the plant for one or more antagonists, they may be applied to and colonize different parts of the plant e.g. roots and foliage, avoiding any direct negative effects on one another.

Against various soil-borne plant pathogens or for general plant health and vitality, mixtures of *Trichoderma* and *Bacillus* spp. have shown varied, although often reduced, impacts in comparison to

singular applications of either (Hervas *et al.*, 1998; Rudresh *et al.*, 2005; Abeysinghe, 2009; Yobo *et al.*, 2009, 2011; Izquierdo-García *et al.*, 2020). The overall nature of interactions between these two often commercialized genera is unclear (Li *et al.*, 2005). The two can form biofilms together, which may improve host colonization; man-made formulations have also shown promise in control of soil-borne ascomycete *Macrophomina phaseolina* on cotton (Triveni *et al.*, 2012, 2013, 2015). Additionally, association of bacteria and fungi may increase the spatial spread of both parties e.g. bacteria travelling on mycelium or fungal spores being moved by bacteria (Niu *et al.*, 2020).

As described for BCAs in general, there are a large number of variables and interactions involved in overall efficacy and combinations have an added degree of complexity. As such, inferring potential results of BCA combinations for a specific case from prior research may be particularly unreliable. Therefore, direct experimental study may be the only way to examine the efficacy of particular BCA combinations in controlling particular pathogens on specific hosts.

Additionally, some authors suggest that strains selected for high antagonism *in vitro* may not provide the highest efficacy *in planta* (Izquierdo-García *et al.*, 2020). Similar inconsistencies in performance were demonstrated by fungal antagonists of *A. mellea* between interactions on agar and in hosts (Raziq & Fox, 2003b). Regardless, *in vitro* antagonism is widely used to screen potential BCAs. Further testing on host plants is often absent (Dumas, 1992; DeLong *et al.*, 2002; Pellegrini *et al.*, 2013), perhaps due to the comparative complexity of *in planta* experiments, potential commercialisation efforts and intellectual property issues, or positive-results publication bias.

1.11 Plant Defence Activators (PDAs)

Plant defence activators (PDAs) are an eclectic group of biological and chemical compounds/polymers related by function, which is that they stimulate plant immune systems, priming them for pathogen attack and reducing disease severity (Reddy, 2013). This response includes the accumulation of defensive compounds and enzymes, defensive alterations in cell wall structure, and increased hypersensitive cell death response (Hailey & Percival, 2014).

1.11.1 Chitin compounds

Chitin is a biopolymer present in both fungal cell walls, insect exoskeletons, and nematode eggshells (Gortari & Hours, 2008). *In planta* the polymer and its derivatives chitosan, oligochitosan, etc., act as PDAs, more specifically pathogen-associated molecular patterns (PAMPs), which are naturally released by the invading hyphae of fungal pathogens (de Jonge *et al.*, 2010; Hadwiger, 2013). In field trials against *Armillaria*, spent mushroom compost, containing large amounts of chitin, appears to have caused a reduction in the size of rotted areas on roots (Raziq & Fox, 2006a).

Chitosan is a derivative that has improved solubility over chitin and has shown anti-microbial effects on a range of microorganisms (El Hadrami *et al.*, 2010). *In vitro* growth reductions caused by chitosan have been shown for a number of other tree root pathogens (Laflamme *et al.*, 2000). Oligochitosans are further altered chitosans, of lower molecular weight and water-soluble at neutral pHs which vastly improves their ease of use (Kim & Rajapakse, 2005; Yin *et al.*, 2009). The mode of action of chitin derivatives upon bacteria and fungi appears to be by multiple mechanisms. Evidence suggests one mechanism is by altering the permeability of the cell membrane via interactions with macromolecules at the cell surface (Xu *et al.*, 2007a; Raafat *et al.*, 2008; Yang *et al.*, 2012). This increased permeability and release of cell contents might presumably influence phytopathogen evasion of host defences. Oligochitosan may also pass into cells, binding to DNA and RNA, as has been demonstrated on *Phytophthora capsici* (Xu *et al.*, 2007b). Both derivatives vary in direct and plant-mediated activity by chemical structure, with indications of links to efficacy against certain groups or species of pathogen (Kim & Rajapakse, 2005; Yin *et al.*, 2010). Due to their ubiquity in the natural world and use in medicine, there are few toxicological concerns with the compounds and their use in plant disease control (Hadwiger, 2013). Phytotoxicity of chitin soil amendments are recorded, due to breakdown products, but only at high concentrations and this can be avoided via limiting dosage (Tian *et al.*, 2000).

Chitin and its derivatives show potential for combination with BCAs. They can act as a food source and simulate the production of anti-fungal compounds by *Bacillus* (San-Lang *et al.*, 2002), as well as enhancing biocontrol activity of *Trichoderma*, *Bacillus*, and other antagonistic chitinolytic bacteria (Benhamou *et al.*, 1998; Sid Ahmed *et al.*, 2003; Kim *et al.*, 2008). A chitosan and *Trichoderma* strain applied together has shown promise for the control of a *Fusarium* sp. (El-Mohamedy *et al.*, 2014). Applied to soil, chitin also stimulates natural bacterial populations, e.g. actinomycetes, as well as reducing the incidence of soil-borne pathogen attack (Bell *et al.*, 1998; Hallmann *et al.*, 1999). In BCA fungi, tolerance to chitosan has been related to the ability to degrade the biopolymer as part of acting as antagonists towards phytopathogenic fungi or nematode eggs (Palma-Guerrero *et al.*, 2008). The case may be similar for BCA bacteria such as *Pseudomonas*, which produce chitinolytic enzymes even when cultured in the absence of the polymer (Nielsen & Sørensen, 1999).

1.11.2 Phosphites

Phosphites, phosphorous acid salts, commonly potassium phosphite, are now widely used in agriculture and horticulture as PDAs (Achary *et al.*, 2017). Phosphite has a complex mode of action via multiple mechanisms, stimulating plant defences, but also includes direct effects against pathogens (Han *et al.*, 2021), including slowing their rate of development and growth (Grant *et al.*, 1990; Deliopoulos *et al.*, 2010). Directly, phosphites can interfere with the molecular perception of phosphate deficiency, as has

been shown in plants and yeast (McDonald *et al.*, 2001) and potentially reduce production of suppressors used by pathogens to evade host immune responses (Grant *et al.*, 1990). The direct effects of phosphites may play a role in their PDA properties, stimulating the release of stress metabolites from pathogens and also revealing their presence to host immune systems. Remaining undetected in this way is of paramount importance for phytopathogens to infect and proliferate within hosts (Bent & Mackey, 2007). Phosphites indirectly affect pathogens via plant mediated effects, with application inducing upregulation of numerous genes related to plant defences (Han *et al.*, 2021).

Phosphites are also often sold labelled as fertilizers, as in time they break down into phosphate, a source of the essential plant nutrient phosphorous; however this breakdown is relatively slow and phosphites exacerbate existing phosphate deficiency, damaging afflicted plants (McDonald *et al.*, 2001; Thao & Yamakawa, 2009). Phosphites are also a significant phytotoxicity risk if applied at over 5g/L (Deliopoulos *et al.*, 2010), and dosages over this rate also appear to impact the establishment of mycorrhizae (Howard *et al.*, 2000).

Early research into the impact of phosphites on *Armillaria* was promising, with injections of potassium phosphite into the xylem of peach trees controlling already present *A. luteobubalina* infections (Heaton & Dullahide, 1990). *In vitro* testing has showed fungistatic effects with 1 mg/L potassium phosphite reducing mycelial growth on host material by 41.1% and 1000 mg/L totally inhibiting growth of a single *A. mellea* isolate (Aguín *et al.*, 2006). This demonstrates potential for using phosphites in integrated control even when their PDA effects are ignored.

Most work with the related, and more commercialized, compound fosetyl-aluminium has conversely shown only a small impact on *Armillaria* growth (West, 1994; Raziq & Fox, 2003a; Guillaumin *et al.*, 2005b; Aguín *et al.*, 2006), although other studies demonstrated significant benefits (Raziq & Fox, 2006b,a). The compound is believed to be converted to phosphite within the plant (Deliopoulos *et al.*, 2010), which may mean it has less direct activity against *Armillaria* than a similarly applied phosphite drench; cations present may also alter the efficacy of phosphorous acid derivative compounds against plant pathogens significantly (Hailey & Percival, 2014).

Hagle & Shaw (1991) name stress of *Armillaria* as a critical factor to allow antagonistic organisms to attack the pathogen successfully. Phosphites show great potential for causing such stress to the pathogen and therefore potential for enhancing control by antagonists, if it does not adversely affect them as well. These compounds have shown activity against phytopathogenic *Pseudomonas* strains (Moragrega *et al.*, 1998; Percival & Banks, 2014), which may indicate activity against BCA strains. Accordingly, mangesium phosphite reduced the antagonistic activity of a biocontrol strain of

Pseudomonas fluorescens but increased efficacy in combination with a *Bacillus subtilis* strain, when applied as a combined treatment against an ascomycete phytopathogen (Simonetti *et al.*, 2015). Experiments in the glasshouse with strawberries as model hosts or in the field with apple trees, showed varying effects of fostyl-aluminium application in combination with fungal antagonists, including *Trichoderma* spp. against *A. mellea*, with the sequence of application having significant interactions (Raziq & Fox, 2006b,a).

1.12 Recent *Armillaria* Research

As detailed above, there is little to no new research into using combinations of fungi, bacteria, and/or plant defence activators against *Armillaria*, despite their potential for enhanced antagonism. It is also apparent that the typical techniques currently utilized in researching *Armillaria* interactions *in vitro* and *in planta* have not advanced much past those used 50 or more years ago e.g. host inoculations (Gregory *et al.*, 1991). As described below, advanced techniques have arisen but received little attention so far.

Inherent difficulties arise in *Armillaria* research due to the biology of the genus and act as potentially unavoidable bottlenecks, although some mitigation is possible. In comparison to many other phytopathogens, such as ascomycete fungi or bacteria, *Armillaria* is slow to culture and infect hosts; conventional host inoculation experiments are complex and time consuming, often requiring years of monitoring with significant failure rates and inconstancy of infection and mortality (Ford *et al.*, 2017; Baumgartner *et al.*, 2018). Similar to the ambiguities in above-ground symptoms of infection, growth measures are often unreliable in reflecting the infection status of hosts, and plants with significant infections may appear healthy above-ground (West & Fox, 2002; Ford *et al.*, 2017). Destructive harvests can be used to elucidate infections which are not yet symptomatic above-ground. However, they have a high labour cost in comparison to the data yielded when compared to non-destructive monitoring. Destructive harvests still require significant experimental run times, as they are typically made when some above ground symptoms are present to avoid premature destruction of the trial. Mortality is typically an important dependent variable in *in planta* studies, e.g. of potential controls. However, for *Armillaria* its occurrence is slow, and the long duration of experiments increases the chances of other factors, e.g. abiotic stresses, causing plant deaths.

Herbaceous models such as strawberry, *Fragaria* × *ananassa*, are rapidly infected and may show quicker mortalities decreasing trial duration, especially in controlled environments; still, even these ‘rapid’ trials last three months or more (Fox & Popoola, 1990; Raziq & Fox, 2003b; Ford *et al.*, 2017). Alternative hosts or genotypes could further reduce experimental duration; within strawberries, there has been some selection of cultivars, i.e. genotypes, for susceptibility (Percival *et al.*, 2011) and variation between

genotypes has been demonstrated on a woody host (Solla *et al.*, 2011). Another factor is the appreciable difference in herbaceous and woody host physiology. This is a potential issue in inferring experimental results from herbaceous hosts to woody hosts, where the majority of economic damage from *Armillaria* takes place. Experimental woody hosts in greenhouse/pot-based *Armillaria* assays are typically young trees (Mesanza *et al.*, 2016; Ford *et al.*, 2017; Elias-Roman *et al.*, 2019; Camprubi *et al.*, 2020). Shrubby species also have potential, for example highly susceptible *Ligustrum* spp. (West & Fox, 2002; Cromey *et al.*, 2020), being amenable for producing uniform and small plantlets via cuttings. Smaller plants may allow greater replication within experimental designs than saplings and increase inoculum pressure from economically sized inocula. Use of clonal material to produce hosts may be used to increase comparability between experiments.

Improving non-destructive methods for the early detection of *Armillaria* infection, such as volatiles in soil (Navaei, 2015; Loulier *et al.*, 2020), or changes in above-ground physiology (Loreto *et al.*, 1993; Nowakowska *et al.*, 2020), is desirable. Such measures could provide an indirect measurement of infection, which could increase the data yields by reducing the need for destructive sampling and reduce the duration of trials if they occur prior to visible above-ground symptoms.

Another under investigated factor is the influence of the inoculum parent material, which is typically sections of woody stems (Gregory *et al.*, 1991; Nowakowska *et al.*, 2020) but may be large seeds instead (Elias-Roman *et al.*, 2019; Camprubi *et al.*, 2020). These different inoculum substrates may provide different nutritional and structural resources to the fungus and, if so, could be used to modulate the inoculum potential in experiments e.g. increasing it to speed host infections.

Host assay techniques using Quantitative Real-Time Polymerase Chain Reaction (qPCR) and confocal fluorescence microscopy have been developed (Baumgartner *et al.*, 2010). Such measures offer unique benefits: quantifying infection directly from the amount of pathogen present rather than an indirect measurement, such as mortality or vitality, can reduce the influence of confounding plant health benefits from BCAs (Nogales *et al.*, 2008, 2010). These increases in health may only be short term and of little relevance to infection and long term mortality (Baumgartner & Warnock, 2006; Baumgartner *et al.*, 2010). Plant defence activators or soil amendments which are of general benefit to host plants may also cause similar conflicts.

The qPCR method is the most promising as the primers are more specific than the dyes used in the microscopy, which dye any fungi present; this may allow the method to be used for *in planta* or *in natura* experiments, such as testing BCAs or other treatments (Diguta *et al.*, 2010; Sanzani *et al.*, 2014). qPCR may also be less affected by inhibitors than conventional PCR measurements. qPCR may also be used to

monitor populations of specific BCA strains e.g. using sequence characterised amplified regions (Sanzani *et al.*, 2014; Rotolo *et al.*, 2016). One limitation is discerning between live and dead cells but, if required, this can be overcome using pre-treatments to reduce the amplification of dead template DNA, such as propidium monoazide, or by amplifying mRNA instead (Vesper *et al.*, 2008; Nocker *et al.*, 2010; Crespo-Sempere *et al.*, 2013; Sanzani *et al.*, 2014). In terms of early detection, comparison of qPCR results from inoculated non-symptomatic, inoculated symptomatic, and un-inoculated plants, could yield thresholds valuable for monitoring plants at risk of infection. A search of citing literature reveals only two studies which have utilized or adapted the qPCR protocol since its publication (Lovato *et al.*, 2014; Calvet *et al.*, 2015). This may be due to lack of direct statistical significance in the original publication (Baumgartner *et al.*, 2010), although one derived methodology yielded statistically significant differences in *Armillaria* DNA quantity between treatments (Calvet *et al.*, 2015).

Historically *in vitro* cultures are grown on agar from fragments of previous cultures. Some authors have integrated submerged culture techniques into their studies, where *Armillaria* is grown in liquid media under agitation (Baumgartner *et al.*, 2010, 2018; Pellegrini *et al.*, 2012, 2013). Submerged cultures have improved the uniformity and growth rates of other basidiomycetes (Zweck *et al.*, 1978; Tan & Moore, 1992). Despite these potential benefits, no comparative study could be found.

1.13 Summary, Aims and Objectives

To summarize, there is considerable scope for new investigations concerning the integrated management of *Armillaria*, with *A. mellea* as a focus of particular value. Such management is of great importance to many trees valued by humans because of the ubiquitous threat posed to them by *Armillaria*.

Bacterial BCAs of *Armillaria* and their combination with PDAs and/or fungal BCAs are under-investigated, despite offering significant potential for direct control. Historically, BCAs have not provided adequate control, likely due to inherent issues in their selection, handling, and application. However, given advances in BCA technologies, and in the light of the lack of alternative controls and the desirability of reduced pesticide usage, this is still a worthy path of investigation.

Lack of early detection methods compounds current difficulties in the control of *Armillaria*. Leaf physiological measurements have been used to monitor infection in a number of studies but there is little research in using these for disease detection, therefore further studies are required to assess this. They may be of use for guiding *Armillaria* management practices including the application of novel BCAs or current cultural controls.

To support investigations in these areas, there are potential improvements to be made in *Armillaria* research practice. Comparisons between historic and novel methodologies, e.g. different inoculation substrates or *in vitro* culture techniques, are required. The results could help to increase the efficiency and ease of research on this somewhat uncooperative organism.

1.13.1 Overall Aim

The primary aim of this study is to further knowledge that can be used within, or in the development of, integrated management of *Armillaria mellea*.

1.13.2 Hypotheses

1. PDAs will have direct effects on the growth of *Armillaria in vitro*.
2. Previously characterized *Pseudomonas* and *Bacillus* BCA bacteria isolates will demonstrate potential as controls of *Armillaria mellea*.
3. Combination of different BCAs will yield improved control of *Armillaria mellea* or reduced control/no improvement due to antagonism between the BCAs.
4. PDAs active against *Armillaria mellea*, especially phosphites, will have negative impacts on BCA growth and/or antagonism.
5. *Bacillus* and *Pseudomonas* isolates will respond differently to the presence of phosphite during antagonism of *Armillaria mellea*.
6. BCA & PDA controls for *Armillaria mellea* will have different efficacies *in vitro* and *in planta*.
7. Submerged and agar-based *in vitro* culture techniques will yield differences in the variability of resultant *Armillaria mellea* growth on agar.
8. Wood and seed based *Armillaria mellea* inoculum will yield different host infection rates.
9. Herbaceous and woody hosts of *Armillaria mellea* will vary in their reaction to infection.
10. Significant changes in leaf physiology will occur in an *Armillaria mellea* infected susceptible host prior to conclusive visual symptoms.
11. Multiple leaf physiological measures will increase the classification of infected and uninfected susceptible hosts.
12. Drought stress will influence the detectability of *Armillaria mellea* infection from leaf physiology of a susceptible host.

1.13.3 Objectives

- Use *in vitro* experiments to select bacterial BCAs and PDAs for combination with a *Trichoderma* strain against *Armillaria mellea in planta*.
- Comparison of the various methods of *in vitro Armillaria mellea* culture by variation in growth of the colonies produced.

- Comparison of wood and seed based *Armillaria mellea* inoculum by host infection efficacy, on strawberry and a woody host.
- Elucidation of multiple leaf physiological measures for the detection of *Armillaria mellea* infection on susceptible hosts. Including comparison to visual symptoms, investigation of the impact of drought and the potential of combinations of measures to improve the classification of infected and uninfected plants.

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1.15 References

Abeysinghe S. **2009**. Effect of Combined Use of *Bacillus subtilis* CA32 and *Trichoderma harzianum* RU01 on Biological Control of *Rhizoctonia solani* on *Solanum melongena* and *Capsicum annuum*. *Plant Pathology Journal* **8**: 9–16.

Achary VMM, Ram B, Manna M, Datta D, Bhatt A, Reddy MK, Agrawal PK. **2017**. Phosphite: a novel P fertilizer for weed management and pathogen control. *Plant Biotechnology Journal* **15**: 1493–1508.

Adaskaveg JE, Förster H, Wade L, Thompson DF, Connell JH. **1999**. Efficacy of Sodium Tetrathiocarbonate and Propiconazole in Managing *Armillaria* Root Rot of Almond on Peach Rootstock. *Plant Disease* **83**: 240–246.

Aguín O, Mansilla JP, Sainz MJ. **2006**. *In vitro* selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapevine in the field. *Pest management science* **62**: 223–8.

Agustini L, Beadle C, Barry K, Mohammed C. **2015**. Photosynthetic Responses of *Eucalyptus nitens* Maiden at Initial Stages of Root-Rot Infection. *Indonesian Journal of Forestry Research* **2**: 9–20.

Aldhous JR, Mason WL. **1994**. *Forest nursery practice, Forestry Commission Bulletin No.111*. London: HMSO, Great Britain.

Amiri A, Bussey KE, Riley MB, Schnabel G. **2008**. Propiconazole inhibits *Armillaria tabescens in vitro* and translocates into peach roots following trunk infusion. *Plant Disease* **92**: 1293–1298.

Amiri A, Schnabel G. **2012**. Persistence of Propiconazole in Peach Roots and Efficacy of Trunk Infusions for *Armillaria* Root Rot Control. *International Journal of Fruit Science* **12**: 437–449.

Anderson JB, Ullrich RC. **1982**. Translocation in rhizomorphs of *Armillaria mellea*. *Experimental Mycology* **6**: 31–40.

Ballach H-J, Goevert J, Kohlmann S, Wittig R. **1998**. Comparative Studies on the Size of Annual Rings, Leaf Growth and the Structure of Treetops of Urban Trees in Frankfurt/Main. In: Urban Ecology. Springer, 699–701.

Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J-P. **2013**. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil* **378**: 1–33.

Baumgartner K. **2004**. Root Collar Excavation for Postinfection Control of *Armillaria* Root Disease of Grapevine. *Plant Disease* **88**: 1235–1240.

Baumgartner K, Bhat R, Fujiyoshi P. **2010**. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal biology* **114**: 107–19.

Baumgartner K, Coetzee MPA, Hoffmeister D. **2011**. Secrets of the subterranean pathosystem of *Armillaria*. *Molecular plant pathology* **12**: 515–534.

Baumgartner K, Fujiyoshi P, Ledbetter C, Duncan R, Kluepfel DA. **2018**. Screening almond rootstocks for sources of resistance to *Armillaria* root disease. *HortScience* **53**: 4–8.

Baumgartner K, Warnock AE. **2006**. A soil inoculant inhibits *Armillaria mellea in vitro* and improves productivity of grapevines with root disease. *Plant Disease* **90**: 439–444.

Beal EJ, Henricot B, Peace AJ, Waghorn IAG, Denton JO. **2015**. The action of allicin against *Armillaria* spp. *in vitro*. *Forest Pathology* **45**: 450–458.

Bell AA, Hubbard JC, Liu L, Davis RM, Subbarao KV. **1998**. Effects of Chitin and Chitosan on the Incidence and Severity of *Fusarium* Yellows of Celery. *Plant Disease* **82**: 322–328.

Benhamou N, Kloepper JW, Tuzun S. **1998**. Induction of resistance against Fusarium wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. *Planta* **204:** 153-168.

Bent AF, Mackey D. **2007**. Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annual review of phytopathology* **45**: 399–436.

Bliss DE. **1951**. The destruction of *Armillaria mellea* in citrus soils. *Phytopathology* **41**: 665–683.

Bogdanski BEC, Cruickshank M, Mario Di Lucca C, Becker E. **2018**. Stumping out tree root disease – An economic analysis of controlling root disease, including its effects on carbon storage in southern British Columbia. *Forest Ecology and Management* **409**: 129–147.

Brasier CM. **2008**. The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* **57**: 792–808.

Cairney JWG. **1992**. Translocation of solutes in ectomycorrhizal and saprotrophic rhizomorphs. *Mycological Research* **96**: 135–141.

Calvet C, Garcia-figueres F, Lovato P, Camprubi A. **2015**. Role of the arbuscular mycorrhizal symbiosis in tolerance response against *Armillaria mellea* in lavender. *Spanish Journal of Agricultural Research* **13**: 1–8.

Campbell EO. **1971**. Notes on the fungal associations to two *Monotropa* sp. in Michigan. *Michigan Botany* **10**: 63–67.

Camprubi A, Solari J, Bonini P, Garcia-Figueres F, Colosimo F, Cirino V, Lucini L, Calvet C. **2020**. Plant performance and metabolomic profile of loquat in response to mycorrhizal inoculation, *Armillaria mellea* and their interaction. *Agronomy* **10**: 899.

Carlquist S. **2013**. More Woodiness/Less Woodiness: Evolutionary Avenues, Ontogenetic Mechanisms. *International Journal of Plant Sciences* **174**: 964–991.

Cawoy H, Debois D, Franzil L, De Pauw E, Thonart P, Ongena M. **2015**. Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microbial Biotechnology* **8**: 281–295.

Cayrol JC, Dubos B, Guillaumin J-J. **1978**. Etude preliminaire in vitro de l'agressivite de quelques nematodes mycophages vis-a-vis de *Trichoderma viride* Pers., *Trichoderma polysporum* (Link. ex Pers.) Rifai et *Armillariella mellea* (Vahl.) Karst. *Annales de Phytopathologie* 10: 177-185.

Chandelier A, Gerarts F, San Martin G, Herman M, Delahaye L. **2016**. Temporal evolution of collar lesions associated with ash dieback and the occurrence of *Armillaria* in Belgian forests. *Forest Pathology* **46:** 289-297.

Chemicals Regulation Directorate. **2013**. Biopesticides Scheme Review - Conclusions. *Regulatory Update 11/2013*. http://www.pesticides.gov.uk/guidance/industries/pesticides/News/Collected-Updates/Reg-Updates-2013/July/Biopesticides-Scheme-Review-Conclusions

Chemicals Regulation Directorate. **2021**. Pesticides - Databases Home Page. https://www.hse.gov.uk/pesticides/databases/index.htm

Cloke P, Jones O. **2020**. *Tree cultures: the place of trees and trees in their place*. Abingdon: Routledge.

Coetzee MPA, Wingfield BD, Harrington TC, Dalevi D, Coutinho TA, Wingfield MJ. **2000**. Geographical diversity of *Armillaria mellea* s.s. based on phylogenetic analysis. *Mycologia* **92**: 105–113.

Commission Decision. **2008**. of 18 September 2008 concerning the non-inclusion of methyl bromide in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance. *Official Journal of the European Union* **/753/EC**: L258/6.

Corkidi L, Allen EB, Merhaut D, Allen MF, Downer J, Bohn J, Evans M. **2004**. Assessing the infectivity of commercial mycorrhizal inoculants in plant nursery conditions. *Journal of Environmental Horticulture* **22**: 149–154.

Corkidi L, Allen EB, Merhaut D, Allen MF, Downer J, Bohn J, Evans M. **2005**. Effectiveness of Commercial Mycorrhizal Inoculants on the Growth of *Liquidambar styraciflua* in Plant Nursery. *Journal of Environmental Horticulture* **23:** 72-76.

Cox KD, Scherm H. **2006**. Interaction dynamics between saprobic lignicolous fungi and *Armillaria* in controlled environments: Exploring the potential for competitive exclusion of *Armillaria* on peach. *Biological Control* **37**: 291–300.

Cremin P, Guiry PJ, Wolfender J-L, Hostettmann K, Donnelly DMX. **2000**. A liquid chromatography–thermospray ionisation–mass spectrometry guided isolation of a new sesquiterpene aryl ester from *Armillaria novae-zelandiae*. *Journal of the Chemical Society, Perkin Transactions 1*: 2325–2329.

Crespo-Sempere A, Estiarte N, Marín S, Sanchis V, Ramos AJ. **2013**. Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. contamination in tomato products. *International Journal of Food Microbiology* **165**: 214–220.

Cromey MG, Drakulic J, Beal EJ, Waghorn IAGG, Perry JN, Clover GRGG. **2020**. Susceptibility of garden trees and shrubs to *Armillaria* root rot. *Plant Disease* **104**: 483–492.

Cusson Y, Lachance D. **1974**. Antagonisme de *Scytalidium lignicola* Pesante envers deux champignons de carie. *Phytoprotection* **55**: 17–28.

Day SD, Watson G, Wiseman PE, Harris JR. **2009**. Causes and consequences of deep structural roots in urban trees: from nursery production to landscape establishment. *Arboriculture & Urban Forestry* **35**: 182–191.

Delaye L, García-Guzmán G, Heil M. **2013**. Endophytes versus biotrophic and necrotrophic pathogens-are fungal lifestyles evolutionarily stable traits? *Fungal Diversity* **60**: 125–135.

Deliopoulos T, Kettlewell PS, Hare MC. **2010**. Fungal disease suppression by inorganic salts: A review. *Crop Protection* **29**: 1059–1075.

DeLong RL, Lewis KJ, Simard SW, Gibson S. **2002**. Fluorescent pseudomonad population sizes baited from soils under pure birch, pure Douglas-fir, and mixed forest stands and their antagonism toward *Armillaria ostoyae in vitro*. *Canadian Journal of Forest Research* **32**: 2146–2159.

Denman S, Barrett G, Kirk SA, McDonald JE, Coetzee MPA. **2016**. Identification of *Armillaria* species on declined oak in Britain: implications for oak health. *Forestry*: 1–14.

Devkota P, Hammerschmidt R. **2020**. The infection process of *Armillaria mellea* and *Armillaria solidipes*. *Physiological and Molecular Plant Pathology* **112**: 101543.

Diguta CF, Rousseaux S, Weidmann S, Bretin N, Vincent B, Guilloux-Benatier M, Alexandre H. **2010**. Development of a qPCR assay for specific quantification of *Botrytis cinerea* on grapes. *FEMS Microbiology Letters* **313**: 81–87.

Drakulic J, Gorton C, Perez-Sierra A, Clover G, Beal L. **2017**. Associations between *Armillaria* species and host plants in U.K. gardens. *Plant Disease* **101**: 1903–1909.

Dumas MT. **1992**. Inhibition of *Armillaria* by bacteria isolated from soils of the boreal mixedwood forest of Ontario. *European Journal of Forest Pathology* **22**: 11–18.

Dumas MT, Boyonoski NW. **1992**. Scanning electron microscopy of mycoparasitism of *Armillaria* rhizomorphs by species of *Trichoderma*. *European journal of forest pathology* **22**: 379–383.

Dumas MT, Strunz GM. **1998**. Modes of action of antagonistic microbes to *Heterobasidion annosum* and *Armillaria ostoyae*. In: Delatour C, Guillaumin J-J, Lung-Escarmant B, Marçais B, eds. Proceedings 9th International Conference on Root and Butt Rots, IUFRO Working Party, Carcans-Maubuisson, France, September 1-7, 1997. Les Colloques de l'INRA. 448.

EFSA. **2015**. Conclusion on the peer review of the pesticide risk assessment of the active substance *Trichoderma atroviride* strain SC1. *EFSA Journal* **13**: 4092.

El-Mohamedy RSR, Abdel-Kareem F, Daami-Remadi M. **2014**. Chitosan and *Trichoderma harzianum* as fungicide alternatives for controlling fusarium crown and root rot of tomato. *Tunisian Journal of Plant Protection* **9**: 31–43.

Elias-Roman RD, Calderon-Zavala G, Guzman-Mendoza R, Vallejo-Perez MR, Klopfenstein NB, Mora-Aguilera JA. **2019**. 'Mondragon': A clonal plum rootstock to enhance management of *Armillaria* root disease in peach orchards of Mexico. *Crop Protection* **121**: 89–95.

Ferrini F, Nicese FP. **2002**. Response of English oak (Quercus robur L.) trees to biostimulants application in the urban environment. *Journal of Arboriculture* **28**: 70–75.

Ford KL, Henricot B, Baumgartner K, Bailey AM, Foster GD. **2017**. A faster inoculation assay for *Armillaria* using herbaceous plants. *The Journal of Horticultural Science and Biotechnology* **92**: 39–47.

Forest Research. **2021**. Pest and disease resources. https://www.forestresearch.gov.uk/tools-andresources/pest-and-disease-resources/

Fox RTV, McQue AM, Obore JO. **1991**. Prospects for the integrated control of *Armillaria* root rot of trees. In: Biotic Interactions and Soil-borne Diseases. Amsterdam: Elsevier Science Publishers, 154–159.

Fox RTV, Popoola TOS. **1990**. Induction of fertile basidiocarps in *Armillaria bulbosa*. *Mycologist* **4**: 70–72.

Gerlach JP, Reich PB, Puettmann K, Baker T. **1997**. Species, diversity, and density affect tree seedling mortality from *Armillaria* root rot. *Canadian Journal of Forest Research* **27**: 1509–1512.

Gilman EF, Paz M, Harchick C. **2015**. Container and Landscape Planting Depth and Root Ball Shaving Affects Magnolia grandiflora Root Architecture and Landscape Performance. *Arboriculture & Urban Forestry* **41**.

Gortari MC, Hours RA. **2008**. Fungal chitinases and their biological role in the antagonism onto nematode eggs. A review. *Mycological Progress* **7**: 221–238.

Grant B, Dunstan R, Griffith J, Niere J, Smillie R. **1990**. The Mechanism of Phosphonic (Phosphorous)

Acid Action in *Phytophthora*. *Australasian Plant Pathology* **19**: 115.

Gregory SC, Rishbeth J, Shaw CG. **1991**. Pathogenicity and Virulence. In: Shaw CG, Kile GA, eds. *Armillaria* Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 76–87.

Groover A, Cronk Q. **2013**. From Nehemiah Grew to Genomics : The Emerging Field of Evo-Devo Research for Woody Plants. *International Journal of Plant Sciences* **174**: 959–963.

Grossnickle SC. **2005**. Importance of root growth in overcoming planting stress. *New Forests* **30**: 273–294.

Guetsky R, Shtienberg D, Elad Y, Fischer E, Dinoor A. **2002**. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**: 976–85.

Guillaumin J-J, Botton B. **2005**. Place de l'armillaire parmi les agents de pourridiés. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 23–29.

Guillaumin J-J, Legrand P. **2005**. Cycle infectieux de l'armillaire – Stratégie biologique des espèces européennes. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 177–201.

Guillaumin J-J, Legrand P, Lung-Escarmant B, Botton B. **2005a**. Les armillaires : acquis, problèmes et prospectives. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 409–418.

Guillaumin J-J, Lung-Escarmant B, Dubos B. **2005b**. Lutte chimique. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 365–375.

Guo T, Wang HC, Xue WQ, Zhao J, Yang ZL. **2016**. Phylogenetic analyses of *Armillaria* reveal at least 15 phylogenetic lineages in China, seven of which are associated with cultivated *Gastrodia elata*. *Plos One* **11**.

Gwynn RL, Dale J. **2010**. European Union with special reference to the United Kingdom. In: Kabaluk JT, Svircev AM, Goettel MS, Woo SG, eds. The Use and Regulation of Microbial Pesticides in Representative Jurisdictions Worldwide. IOBC Global, 24–34.

El Hadrami A, Adam LR, El Hadrami I, Daayf F. **2010**. Chitosan in plant protection. *Marine drugs* **8**: 968–87.

Hadwiger L a. 2013. Multiple effects of chitosan on plant systems: Solid science or hype. *Plant Science* **208**: 42–49.

Hagle SK, Shaw CG. **1991**. Avoiding and reducing losses from *Armillaria* root disease. In: Shaw CG, Kile GA, eds. *Armillaria* Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 157–173.

Hailey LE, Percival GC. **2014**. Comparative assessment of phosphite formulations for apple scab (*Venturia inaequalis*) control. *Arboriculture & Urban Forestry* **40**.

Hailey LE, Percival GC. **2015**. The Influence of long term flooding on tree biology and approaches to flood stress alleviation and management. *Arboricultural Journal* **37**: 135–149.

Hallmann J, Rodríguez-Kábana R, Kloepper JW. **1999**. Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biology and Biochemistry* **31**: 551–560.

Han X, Xi Y, Zhang Z, Mohammadi MA, Joshi J, Borza T, Wang-Pruski G. **2021**. Effects of phosphite as a plant biostimulant on metabolism and stress response for better plant performance in *Solanum tuberosum*. *Ecotoxicology and Environmental Safety* **210**: 111873.

Heaton JB, Dullahide SR. **1990**. Efficacy of phosphonic acid in other host pathogen systems. *Australasian Plant Pathology* **19**: 133–134.

Heinzelmann R, Dutech C, Tsykun T, Labbé F, Soularue J-P, Prospero S. **2019**. Latest advances and future perspectives in *Armillaria* research. *Canadian Journal of Plant Pathology* **41**: 1–23.

Hervas a, Landa B, Datnoff LE, Jimenez-Diaz RM. **1998**. Effects of commercial and indigenous microorganisms on *Fusarium* wilt development in chickpea. *Biological Control* **13**: 166–176.

Highley TL. **1990**. Laboratory studies on antagonism of *Scytalidium lignicola* to wood decay fungi. *Material und Organismen* **25**: 181–192.

Hirons AD, Thomas PA. **2018**. *Applied tree biology*. Wiley Online Library.

Howard K, Dell B, Hardy GE. **2000**. Phosphite and mycorrhizal formation in seedlings of three Australian Myrtaceae. *Australian Journal of Botany* **48**: 725–729.

Hu Z, Huang Q-Z, Liu X-Z, Yang J-B. **1999**. Primary structure and cDNA cloning of the antifungal protein GAFP-I from *Gastrodia elata*. *Acta Botanica Yunnanica* **21**: 131–138.

Hutchins AS. **1980**. *In vitro* inhibition of root-rot pathogens *Phellinus weirii*, *Armillariella mellea*, *Fomes annosus*, and *Phytophthora cinnamomi* by a newly isolated *Bacillus* sp. *Microbial Ecology* **6**: 253–259.

Izquierdo-García LF, González-Almario A, Cotes AM, Moreno-Velandia CA. **2020**. *Trichoderma virens* Gl006 and *Bacillus velezensis* Bs006: a compatible interaction controlling *Fusarium* wilt of cape gooseberry. *Scientific reports* **10**: 1–13.

de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MHAJ, Thomma BPHJ. **2010**. Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science (New York, N.Y.)* **329**: 953–955.

Kedves O, Shahab D, Champramary S, Chen L, Indic B, Bóka B, Nagy VD, Vágvölgyi C, Kredics L, Sipos G. **2021**. Epidemiology, biotic interactions and biological control of armillarioids in the northern Hemisphere. *Pathogens* **10**: 1–33.

Kim YC, Jung H, Kim KY, Park SK. **2008**. An effective biocontrol bioformulation against *Phytophthora* blight of pepper using growth mixtures of combined chitinolytic bacteria under different field conditions. *European Journal of Plant Pathology* **120**: 373–382.

Kim SK, Rajapakse N. **2005**. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers* **62**: 357–368.

Koch RA, Wilson AW, Séné O, Henkel TW, Aime MC. **2017**. Resolved phylogeny and biogeography of the root pathogen *Armillaria* and its gasteroid relative, *Guyanagaster*. *BMC Evolutionary Biology* **17**: 33.

Kwaśna H. **2001**. Fungi in the rhizosphere of common oak and its stumps and their possible effect on

infection by *Armillaria*. *Applied Soil Ecology* **17**: 215–227.

Laflamme P, Benhamou N, Bussières G, Dessureault M. **2000**. Differential effect of chitosan on root rot fungal pathogens in forest nurseries. *Canadian Journal of Botany* **77**: 1460–1468.

Laflamme G, Guillaumin J-J. **2005**. L'armillaire, agent pathogène mondial : répartition et dégâts. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 273–289.

Lamichhane JR, Bischoff-Schaefer M, Bluemel S, Dachbrodt-Saaydeh S, Dreux L, Jansen JP, Kiss J, K??hl J, Kudsk P, Malausa T, *et al.* 2017. Identifying obstacles and ranking common biological control research priorities for Europe to manage most economically important pests in arable, vegetable and perennial crops. *Pest Management Science* **73**: 14–21.

Legrand P, Lung-Escarmant B. **2005**. L'armillaire en plantations forestières en Europe. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 307–318.

Legrand P, Lung-Escarmant B, Guillaumin J-J. **2005**. Lutte contre l'armillaire en forêts: méthodes sylvicoles et culturales. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 349–363.

Li L, Qu Q, Tian B, Zhang KQ. **2005**. Induction of chlamydospores in *Trichoderma harzianum* and *Gliocladium roseum* by antifungal compounds produced by *Bacillus subtilis* C2. *Journal of Phytopathology* **153**: 686–693.

Livingston WH. **1990**. *Armillaria ostoyae* in young spruce plantations. *Canadian journal of forest research* **20**: 1773–1778.

Loreto F, Burdsall HH, Tirro A. **1993**. *Armillaria* infection and water stress influence gas-exchange properties of Mediterranean trees. *HortScience* **28**: 222–224.

Loulier J, Lefort F, Stocki M, Asztemborska M, Szmigielski R, Siwek K, Grzywacz T, Hsiang T, Ślusarski S, Oszako T, *et al.* 2020. Detection of fungi and oomycetes by volatiles using E-Nose and SPME-GC/MS platforms. *Molecules (Basel, Switzerland)* **25**.

Lovato PE, Garcia-Figueres F, Camprubí a., Parladé J, Calvet C. **2014**. A semiaxenic phototrophic system to study interactions between arbuscular mycorrhizal and pathogenic fungi in woody plants. *European Journal of Plant Pathology* **140**: 207–212.

Lung-Escarmant B, Legrand P, Guillaumin J-J, Guyon D. **2005**. Épidémiologie. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 203–219.

Lung-Escarmant B, Taris B, Ducom P, Lanusse M, Tarlet GP, Malato G. **1985**. Action et comportement in situ du bromure de méthyle sur le développement d'*Armillaria obscura*, parasite du pin maritime dans le sud-ouest de la France. *EPPO Bulletin* **15**: 97–104.

Martin FN. **2003**. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology* **41**: 325–350.

McDonald A, Grant B, Plaxton W. **2001**. Phosphite (phosphorous acid): its relevance in the environment and agriculture and influence on plant phosphate starvation response. *Journal of Plant Nutrition* **24**: 1505–1519.

Mercado-Blanco J. **2015**. Pseudomonas strains that exert biocontrol of plant pathogens. In: Pseudomonas. Dordrecht: Springer Netherlands, 121–172.

Mesanza N, Iturritxa E, Patten CL. **2016**. Native rhizobacteria as biocontrol agents of *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*. *Biological Control* **101**: 8–16.

Mihail JD, Bruhn JN. **2005**. Foraging behaviour of *Armillaria* rhizomorph systems. *Mycological research* **109**: 1195–1207.

Miller SB, Gasic K, Reighard GL, Henderson WG, Rollins PA, Vassalos M, Schnabel G. **2020**. Preventative root-collar excavation reduces peach tree mortality caused by *Armillaria* root rot on replant sites. *Plant Disease* **104**.

Misiek M, Hoffmeister D. **2012**. Sesquiterpene aryl ester natural products in North American *Armillaria* species. *Mycological Progress* **11**: 7–15.

Modi D, Simard S, Bérubé J, Lavkulich L, Hamelin R, Grayston SJ. **2020**. Long-term effects of stump removal and tree species composition on the diversity and structure of soil fungal communities. *FEMS Microbiology Ecology* **96**: 1–12.

Modi D, Simard S, Lavkulich L, Hamelin RC, Grayston SJ. **2021**. Stump removal and tree species composition promote a bacterial microbiome that may be beneficial in the suppression of root disease. *FEMS Microbiology Ecology* **97**: 1–25.

Moragrega C, Manceau C, Montesinos E. **1998**. Evaluation of drench treatments with phosphonate derivatives against *Pseudomonas syringae* pv. *syringae* on pear under controlled environment conditions. *European Journal of Plant Pathology* **104**: 171–180.

Moricca S, Ragazzi A. **2011**. The holomorph *Apiognomonia quercina/Discula quercina* as a pathogen/endophyte in oak. In: Endophytes of Forest Trees. Springer, 47–66.

Morrison DJ. **1982**. Effects of soil organic matter on rhizomorph growth by *Armillaria mellea*. *Transactions of the British Mycological Society* **78**: 201–207.

Morrison DJ. **1989**. Pathogenicity of *Armillaria* species is related to rhizomorph growth habit. In: Morrison DJ, ed. Proceedings of the Seventh International Conference on Root and Butt Rots, August 9-16, 1988. Vernon and Victoria, British Columbia.: International Union of Forestry Research Organizations, co-published by Forestry Canada, Victoria, BC., 584–589.

Munnecke DE, Wilbur WD. **1973**. Effect of methyl bromide or carbon disulfide on *Armillaria* and *Trichoderma* growing on agar medium and relation to survival of *Armillaria* in soil following fumigation. *Phytopathology* **63**: 1352–1357.

Munnecke DE, Wilbur W, Darley EF. **1976**. Effect of heating or drying on *Armillaria mellea* or *Trichoderma viride* and relation to survival of *Armillaria mellea* in soil. *Phytopathology* **66**: 1363–1368.

Navaei M. **2015**. Integration of micro-gas chromatography system for detection of volatile organic compounds. Doctoral Thesis. Georgia Institute of Technology.

Nielsen MN, Sørensen J. **1999**. Chitinolytic activity of *Pseudomonas fluorescens* isolates from barley and sugar beet rhizosphere. *FEMS microbiology ecology* **30**: 217–227.

Niu B, Wang W, Yuan Z, Sederoff RR, Sederoff H, Chiang VL, Borriss R. **2020**. Microbial interactions within multiple-strain biological control agents impact soil-borne plant disease. *Frontiers in Microbiology*

11: 1–16.

Nocker A, Richter-Heitmann T, Montijn R, Schuren F, Kort R. **2010**. Discrimination between live and dead cells in bacterial communities from environmental water samples analyzed by 454 pyrosequencing. *International Microbiology* **13**: 59–65.

Nogales A, Aguirreolea J, Santa María E, Camprubí A, Calvet C. **2008**. Response of mycorrhizal grapevine to *Armillaria mellea* inoculation: disease development and polyamines. *Plant and Soil* **317**: 177–187.

Nogales A, Camprubí A, Estaún V, Marfà V, Calvet C. **2010**. *In vitro* interaction studies between *Glomus intraradices* and *Armillaria mellea* in vines. *Spanish Journal of Agricultural Research* **8**: S62–S68.

Nowak DJ. **2004**. Assessing environmental functions and values of veteran trees. In: Nicolotti G, Gonthier P, eds. Proceedings of the International Congress on the Protection and Exploitation of Veteran Trees. Torino, Italy, 45–49.

Nowakowska JA, Stocki M, Stocka N, Ślusarski S, Tkaczyk M, Caetano JM, Tulik M, Hsiang T, Oszako T. **2020**. Interactions between *Phytophthora cactorum*, *Armillaria gallica* and *Betula pendula* Roth. Seedlings Subjected to Defoliation. *Forests* **11**: 1107.

Obuchi T, Kondoh H, Watanabe N, Tamai M, Omura S, Yang JS, Liang XT. **1990**. Armillaric acid, a new antibiotic produced by *Armillaria mellea*. *Planta medica* **56**: 198–201.

Ongena M, Jacques P. **2008**. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology* **16**: 115–125.

Otieno W, Termorshuizen A, Jeger M, Othieno CO. **2003**. Efficacy of soil solarization, *Trichoderma harzianum*, and coffee pulp amendment against *Armillaria* sp. *Crop Protection* **22**: 325–331.

Ouellette GB, Bard G, Cauchon R. **1971**. Self-strangulation of roots: points of entry of root-rot fungi in the Grand'Mère white spruce plantations. *Phytoprotection* **52:** 119-124.

Palma-Guerrero J, Jansson HB, Salinas J, Lopez-Llorca L V. 2008. Effect of chitosan on hyphal growth and spore germination of plant pathogenic and biocontrol fungi. *Journal of Applied Microbiology* **104**: 541–553.

Pandin C, Le Coq D, Canette A, Aymerich S, Briandet R. **2017**. Should the biofilm mode of life be taken into consideration for microbial biocontrol agents? *Microbial Biotechnology* **10**: 719–734.

Pareek M, Allaway WG, Ashford AE. **2006**. *Armillaria luteobubalina* mycelium develops air pores that conduct oxygen to rhizomorph clusters. *Mycological research* **110**: 38–50.

Pauleit S. **2003**. Urban street tree plantings: identifying the key requirements. *Proceedings of the ICE-Municipal Engineer* **156**: 43–50.

Pawsey RG, Rahman MA. **1976**. Field trials with Armillatox against *Armillariella mellea*. *PANS* **22**: 49–56.

Peipp H, Sonnenbichler J. **1992**. Secondary fungal metabolites and their biological activities, ii. occurrance of antibiotic compounds in cultures of *Armillaria ostoyae* growing in the presence of an antagonistic fungus or host plant cells. *Biological Chemistry Hoppe-Seyler* **373**: 675–684.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2012**. Studying trophic interactions between a plant pathogen and two different antagonistic microorganisms using a 13C-labeled compound and isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **26**: 510–516.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2013**. Isotope ratio mass spectrometry identifies soil microbial biocontrol agents having trophic relations with the plant pathogen *Armillaria mellea*. *Applied Soil Ecology* **64**: 142–151.

Percival GC, Banks JM. **2014**. Evaluation of plant defence activators for the potential control of *Pseudomonas syringae* pv. *aesculi*. *Arboricultural Journal* **36**: 76–88.

Percival GC, Keary IP, Al-Habsi S. **2006**. An assessment of the drought tolerance of *Fraxinus* genotypes for urban landscape plantings. *Urban Forestry and Urban Greening* **5**: 17–27.

Percival GC, Schaffert E. **2015**. Evaluation of water-retaining polymers on transplant survival of bare-rooted silver birch (*Betula pendula* Roth.) and European beech (*Fagus sylvatica* L.). *Arboricultural Journal* **36**: 185–196.

Percival GC, Schaffert E, Hailey L. **2014**. Trees in the rural landscape. In: Horticulture: Plants for People and Places, Volume 2. Springer, 713–730.

Percival GC, Smiley ET, Fox RTV. **2011**. Root collar excavation with *Trichoderma* inoculations as a potential management strategy for honey fungus (*Armillaria mellea*). *Arboricultural Journal* **33**: 267–280.

Petit RJ, Hampe A. **2006**. Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution, and Systematics* **37**: 187–214.

Popoola TOS, Fox RT V. **1996**. Effect of root damage on honey fungus. *Arboricultural Journal* **20**: 329–337.

Popoola TOS, Fox RTV. **2003**. Effect of water stress on infection by species of honey fungus (*Armillaria mellea* and *A. gallica*). *Arboricultural Journal* **27**: 139–154.

Prodorutti D, Pellegrini A, Gobbin D, Vanblaere T, Pertot I. **2010**. *Armillaria* root rot on highbush blueberry in northern Italy: monitoring, identification and inoculum sources. *IOBC/WPRS bulletin* **54**: 187–190.

Prodorutti D, Pellegrini A, Pertot I. **2009**. Biocontrol of *Armillaria* root rot on highbush blueberry in Italy. *IOBC WPRS bulletin.* **43**: 207–210.

Prospero S, Holdenrieder O, Rigling D. **2004**. Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances. *Forest Pathology* **34**: 1–14.

Przemieniecki SW, Damszel M, Ciesielski S, Kubiak K, Mastalerz J, Sierota Z, Gorczyca A. **2021**. Bacterial microbiome in *Armillaria ostoyae* rhizomorphs inhabiting the root zone during progressively dying Scots pine. *Applied Soil Ecology* **164**: 103929.

Raabe RD. **1962**. Host list of the root rot fungus, *Armillaria mellea*. *Hilgardia* **33**: 24–89.

Raafat D, Von Bargen K, Haas A, Sahl HG. **2008**. Insights into the mode of action of chitosan as an antibacterial compound. *Applied and Environmental Microbiology* **74**: 3764–3773.

Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M. **2010**. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiology Reviews* **34**: 1037–1062.

Rabiey M, Hailey LE, Roy SR, Grenz K, Al-Zadjali MAS, Barrett GA, Jackson RW. **2019**. Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health? *European Journal of Plant Pathology* **155**: 711–729.

Ramette A, Frapolli M, Saux MF Le, Gruffaz C, Meyer JM, Défago G, Sutra L, Moënne-Loccoz Y. **2011**. *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Systematic and Applied Microbiology* **34**: 180–188.

Raziq F. **1998**. Biological and integrated control of the root rot caused by *Armillaria mellea*. Doctoral Thesis. University of Reading.

Raziq F, Fox RT V. **2003a**. Integrated control of *Armillaria mellea* by *Trichoderma harzianum* and fosetyl-Al. In: Laflamme G, Bérubé JA, Bussières G, eds. Proceedings 10th International Conference on Root and Butt Rots, IUFRO Working Party, September 16-22, 2001. Québec, Canada, 133–138.

Raziq F, Fox RT V. **2003b**. Comparisons between the *in vitro* and *in vivo* efficacies of potential fungal antagonists of *Armillaria mellea*. *Biological Agriculture & Horticulture* **21**: 263–276.

Raziq F, Fox RTV. **2005**. Combinations of fungal antagonists for biological control of *Armillaria* root rot of strawberry plants. *Biological Agriculture & Horticulture* **23**: 45–57.

Raziq F, Fox RT V. **2006a**. The integrated control of *Armillaria mellea* 2. field experiments. *Biological Agriculture & Horticulture* **23**: 235–249.

Raziq F, Fox RT V. **2006b**. The integrated control of *Armillaria mellea* 1. glasshouse experiments. *Biological Agriculture & Horticulture* **23**: 225–234.

Reddy PP. **2013**. Plant defence activators. In: Recent advances in crop protection SE - 9. Springer India, 121–129.

Rees HJ, Bashir N, Drakulic J, Cromey MG, Bailey AM, Foster GD. **2020**. Identification of native endophytic *Trichoderma* spp. for investigation of *in vitro* antagonism towards *Armillaria mellea* using synthetic- and plant-based substrates. *Journal of Applied* Microbiology **131**: 392-403.

dos Reis Almeida FB, Cerqueira FM, do Nascimento S, Ulhoa CJ, Lima AL. **2007**. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnology Letters* **29**: 1189–1193.

Reisenzein H, Tiefenbrunner W. **1997**. Growth inhibiting effect of different isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. to the plant parasitic fungi of the genera *Fusarium*, *Armillaria* and *Rosselinia*. *Pflanzenschutz Berichte* **57**: 15–24.

Riffle JW. **1973**. Effect of two mycophagous nematodes on *Armillaria mellea* root rot of *Pinus ponderosa* seedlings. *Plant disease reporter* **57**: 355–357.

Rishbeth J. **1983**. The importance of honey fungus (*Armillaria*) in urban forestry. *Arboricultural Journal* **7**: 217–225.

Rizzo DM, Blanchette RA, Palmer MA. **1992**. Biosorption of metal ions by *Armillaria* rhizomorphs. *Canadian Journal of Botany* **70**: 1515–1520.

Rogers K, Sacre K, Goodenough J, Doick K. **2015**. *Valuing London's urban forest: results of the London i-Tree eco project*. London: Treeconomics.

Rotolo C, De Miccolis Angelini RM, Pollastro S, Faretra F. **2016**. A TaqMan-based qPCR assay for quantitative detection of the biocontrol agents *Bacillus subtilis* strain QST713 and *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747. *BioControl* **61**: 91–101.

Rudresh DL, Shivaprakash MK, Prasad RD. **2005**. Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer aritenium* L.). *Applied Soil Ecology* **28**: 139–146.

San-Lang W, Shih IL, Wang CH, Tseng KC, Chang WT, Twu YK, Ro JJ, Wang CL. **2002**. Production of antifungal compounds from chitin by *Bacillus subtilis*. *Enzyme and Microbial Technology* **31**: 321–328.

Sanzani SM, Li Destri Nicosia MG, Faedda R, Cacciola SO, Schena L. **2014**. Use of quantitative PCR detection methods to study biocontrol agents and phytopathogenic fungi and oomycetes in environmental samples. *Journal of Phytopathology* **162**: 1–13.

Savazzini F, Longa CMO, Pertot I. **2009**. Impact of the biocontrol agent *Trichoderma atroviride* SC1 on soil microbial communities of a vineyard in northern Italy. *Soil Biology and Biochemistry* **41**: 1457–1465.

Schmidt CS, Agostini F, Leifert C, Killham K, Mullins CE. **2004**. Influence of soil temperature and matric potential on sugar beet seedling colonization and suppression of *Pythium* damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. *Phytopathology* **94**: 351–363.

Shaw CG, Roth LF. **1978**. Control of *Armillaria* root rot in managed coniferous forests. *European Journal of Forest Pathology* **8**: 163–174.

Sid Ahmed A, Ezziyyani M, Pérez Sánchez C, Candela ME. **2003**. Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *European Journal of Plant Pathology* **109**: 633–637.

Simonetti E, Viso NP, Montecchia M, Zilli C, Balestrasse K, Carmona M. **2015**. Evaluation of native bacteria and manganese phosphite for alternative control of charcoal root rot of soybean. *Microbiological Research* **180**: 40–48.

Singh P, Richardson J. **1973**. Armillaria root rot in seeded and planted areas in Newfoundland. *The Forestry Chronicle* **49**: 180–182.

Sipos G, Prasanna AN, Walter MC, O'Connor E, Bálint B, Krizsán K, Kiss B, Hess J, Varga T, Slot J, *et al.* 2017. Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungi *Armillaria*. *Nature Ecology & Evolution* **1**: 1931–1941.

Sjöman H, Nielsen AB. **2010**. Urban Forestry & Urban Greening Selecting trees for urban paved sites in Scandinavia – A review of information on stress tolerance and its relation to the requirements of tree planners. *Urban Forestry & Urban Greening* **9**: 281–293.

Sjöman H, Östberg J, Bühler O. **2012**. Diversity and distribution of the urban tree population in ten major Nordic cities. *Urban Forestry & Urban Greening* **11**: 31–39.

Smith AM, Griffin DM. **1971**. Oxygen and the ecology of *Armillariella elegans* Heim. *Australian Journal of Biological Sciences* **24**: 231–262.

Solla A, Aguín O, Cubera E, Sampedro L, Mansilla JP, Zas R. **2011**. Survival time analysis of *Pinus pinaster* inoculated with *Armillaria ostoyae*: Genetic variation and relevance of seed and root traits. *European Journal of Plant Pathology* **130**: 477–488.

Solla A, Tomlinson F, Woodward S. **2002**. Penetration of *Picea sitchensis* root bark by *Armillaria mellea*, *Armillaria ostoyae* and *Heterobasidion annosum*. *Forest Pathology* **32**: 55–70.

Sonnenbichler J, Guillaumin J-J, Peipp H, Schwarz D. **1997**. Secondary metabolites from dual cultures of

genetically different *Armillaria* isolates. *European Journal of Forest Pathology* **27**: 241–249.

Szwajkowska-Michalek L, Kwaśna H, Lakomy P, Perkowski J. **2012**. Inhibition of *Armillaria* and *Heterobasidion* growth by *Penicillium adametzii* isolated from *Pinus sylvestris* forest soil. *Forest Pathology* **42**: 454–466.

Tan YH, Moore D. **1992**. Convenient and effective methods for *in vitro* cultivation of mycelium and fruiting bodies of *Lentinus edodes*. *Mycological Research* **96**: 1077–1084.

Tattar TA. **1981**. Stress models for trees in the urban environment. *Arboricultural Journal* **5**: 55–56.

Thao HTB, Yamakawa T. **2009**. Phosphite (phosphorous acid): Fungicide, fertilizer or bio-stimulator? *Soil Science and Plant Nutrition* **55**: 228–234.

Thomas HE. **1934**. Studies on *Armillaria mellea* (Vahl) Quel., infection, parasitism, and host resistance. *Journal of Agricultural Research* **48**: 187–218.

Thomidis T, Exadaktylou E. **2012**. Effectiveness of cyproconazole to control *Armillaria* root rot of apple, walnut and kiwifruit. *Crop Protection* **36**: 49–51.

Tian H, Riggs RD, Crippen DL. **2000**. Control of soybean cyst nematode by chitinolytic bacteria with chitin substrate. *Journal of nematology* **32**: 370–376.

Tomalak M. **2017**. Parasitic association of the mycetophagous wood nematode, *Bursaphelenchus fraudulentus* with the honey fungus *Armillaria ostoyae*. *Forest Pathology*: 1–10.

Triveni S, Prasanna R, Kumar A, Bidyarani N, Singh R, Saxena AK. **2015**. Evaluating the promise of *Trichoderma* and *Anabaena* based biofilms as multifunctional agents in *Macrophomina phaseolina*-infected cotton crop. *Biocontrol Science and Technology* **25**: 656–670.

Triveni S, Prasanna R, Saxena AK. **2012**. Optimization of conditions for *in vitro* development of *Trichoderma viride*-based biofilms as potential inoculants. *Folia Microbiologica* **57**: 431–437.

Triveni S, Prasanna R, Shukla L, Saxena AK. **2013**. Evaluating the biochemical traits of novel *Trichoderma*-based biofilms for use as plant growth-promoting inoculants. *Annals of Microbiology* **63**: 1147–1156.

Tsoumou-Gavouka A. **1982**. Contribution à l'étude de la morphogenèse du rhizomorphe D'*Armillaria mellea* (Vahl) Kümmer sensu stricto et *Armillaria bulbosa* (Barla) Romagn. Thèse de 3e Cycle. Université Clermont-Ferrand II.

Tubby K V., Webber JF. **2010**. Pests and diseases threatening urban trees under a changing climate. *Forestry* **83**: 451–459.

Turner JA. **1991**. Biology and control of *Armillaria*. Doctoral Thesis. University of Reading.

Tyrväinen L, Pauleit S, Seeland K, De Vries S. **2005**. Benefits and uses of urban forests and trees. In: Konijnendijk C, Nilsson K, Randrup T, Schipperijn J, eds. Urban Forests and Trees: A Reference Book. Springer Berlin / Heidelberg, 81–114.

Vasaitis R, Stenlid J, Thomsen IM, Barklund P, Dahlberg A. **2008**. Stump removal to control root rot in forest stands: a literature study. *Silva Fennica* **42**: 457–483.

de Vasconcellos R, Cardoso E. **2009**. Rhizospheric streptomycetes as potential biocontrol agents of

Fusarium and *Armillaria* pine rot and as PGPR for *Pinus taeda*. *BioControl* **54**: 807–816.

Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S, Vesper A. **2008**. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). *Journal of Microbiological Methods* **72**: 180–184.

Wallace GB. **1935**. *Armillaria* root rot in East Africa: *Armillaria Mellea* (Vahl.) Quél. *The East African Agricultural Journal* **1**: 182–192.

Wargo PM. **1981**. Defoliation and secondary-action organism attack: with emphasis on *Armillaria mellea*. *Journal of Arboriculture* **7**: 64–69.

Warwell M V., McDonald GI, Hanna JW, Kim MS, Lalande BM, Stewart JE, Hudak AT, Klopfenstein NB. **2019**. *Armillaria altimontana* Is associated with healthy western white pine (*Pinus monticola*): potential *in situ* biological control of the *Armillaria* root disease pathogen, *A. solidipes*. *Forests* **10**.

Weller DM. **1988**. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual review of phytopathology* **26**: 379–407.

Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS. **2002**. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual review of phytopathology* **40**: 309–48.

West J. **1994**. Chemical control of Armillaria root rot. Doctoral Thesis. University of Reading.

West JS, Fox RT V. **2002**. Stimulation of *Armillaria mellea* by phenolic fungicides. *Annals of Applied Biology* **140**: 291–295.

Xiao Q, McPherson EG. **2002**. Rainfall interception by Santa Monica's municipal urban forest. *Urban Ecosystems* **6**: 291–302.

Xu J, Guo S. **2000**. Retrospect on the research of the cultivation of *Gastrodia elata* Bl., a rare traditional Chinese medicine. *Chinese medical journal* **113**: 686–92.

Xu X-M, Jeffries P, Pautasso M, Jeger MJ. **2011**. Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology* **101**: 1024–1031.

Xu J, Zhao X, Han X, Du Y. **2007a**. Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi *in vitro*. *Pesticide Biochemistry and Physiology* **87**: 220–228.

Xu J, Zhao X, Wang X, Zhao Z, Du Y. **2007b**. Oligochitosan inhibits *Phytophthora capsici* by penetrating the cell membrane and putative binding to intracellular targets. *Pesticide Biochemistry and Physiology* **88**: 167–175.

Yafetto L. **2008**. Biomechanics of rhizomorph development in *Armillaria mellea*. Doctoral Thesis. Miami University.

Yang LY, Zhang JL, Bassett CL, Meng XH. **2012**. Difference between chitosan and oligochitosan in growth of *Monilinia fructicola* and control of brown rot in peach fruit. *LWT - Food Science and Technology* **46**: 254–259.

Yin H, Du Y, Zhang J. **2009**. Low molecular weight and oligomeric chitosans and their bioactivities. *Current topics in medicinal chemistry* **9**: 1546–1559.

Yin H, Zhao X, Du Y. **2010**. Oligochitosan: a plant diseases vaccine - a review. *Carbohydrate Polymers* **82**:

1–8.

Yobo KS, Laing MD, Hunter CH. **2009**. Effects of single and dual applications of selected *Trichoderma* and *Bacillus* isolates on performance of dry bean seedlings grown in composted pine bark growth medium under shadehouse conditions. *Journal of Plant Nutrition* **32**: 1271–1289.

Yobo KS, Laing MD, Hunter CH. **2011**. Effects of single and combined inoculations of selected *Trichoderma* and *Bacillus* isolates on growth of dry bean and biological control of *Rhizoctonia solani* damping-off. *African Journal of Biotechnology* **10**: 8746–8756.

Zhang L. **2014**. Colonization pattern of crop plants by endophytic fungi. Doctoral Thesis. University of Göttingen.

Zweck S, Hüttermann A, Chet I. **1978**. A convenient method for preparing inocula of homogenized mycelia. *Experimental Mycology* **2**: 377–378.

2. Review: Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health?

Preface

There are many considerations specific to the development, utilization, and commercialization of biological controls in comparison to synthetic plant protection products (pesticides). This collaborative paper examines some of these considerations through the lens of the potential of endophytes as biological controls. Endophytes are an area of study which offers much potential for biological control. The paper provides greater context to the discussion of biological control agents for *Armillaria* root rot, for example detailing other major pests and diseases of trees and the technologies used to apply plant protection products.

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REVIEWS

Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health?

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Abstract Like all other plants, trees are vulnerable to attack by a multitude of pests and pathogens. Current control measures for many of these diseases are limited and relatively ineffective. Several methods, including the use of conventional synthetic agro-chemicals, are employed to reduce the impact of pests and diseases. However, because of mounting concerns about adverse effects on the environment and a variety of economic reasons, this limited management of tree diseases by chemical methods is losing ground. The use of biological control, as a more environmentally friendly alternative, is becoming increasingly popular in plant protection. This can include the deployment of soil inoculants and foliar sprays, but the increased knowledge of microbial ecology in the phytosphere, in particular phylloplane microbes and endophytes, has stimulated new thinking for biocontrol approaches. Endophytes are microbes that live within plant tissues. As such, they hold potential as biocontrol agents against plant diseases because they are able to colonize the same ecological niche favoured by many invading pathogens. However, the development and exploitation of endophytes as biocontrol agents will have to overcome numerous challenges. The optimization and improvement of strategies employed in endophyte research can contribute towards discovering effective and competent biocontrol agents. The impact of environment and plant genotype on selecting potentially beneficial and exploitable endophytes for biocontrol is poorly understood. How endophytes synergise or antagonise one another is also an important factor. This review focusses on recent research addressing the biocontrol of plant diseases and pests using endophytic fungi and bacteria, alongside the challenges and limitations encountered and how these can be overcome. We frame this review in the context of tree pests and diseases, since trees are arguably the most difficult plant species to study, work on and manage, yet they represent one of the most important organisms on Earth.

Keywords Endophytes · Biological control · Trees · Pathogen · Pest · Disease

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Introduction

Importance of trees and their diseases

Being some of the largest organisms on Earth, trees in forest and woodland settings cover 40% of the Earth's terrestrial surface (Fao 2010). This forms a major part of the global biomass and provides habitat for large numbers of animal and plant species with varying levels of association. To humans, the importance of trees for food, timber and non-timber resources has been historically and widely identified (Cazorla and Mercado-Blanco 2016). Carbon sequestration is one of the most significant ecosystem services provided by trees, with

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total carbon stocks in UK forests (including soil) alone calculated at 800 megatons (Quine et al. 2011).

The economic value of trees and shrubs in urban landscapes has been increasingly recognised since the turn of the millennium, with the popularisation of electronic tools for estimating this value, e.g. i-Tree software, and research revealing a suite of functional benefits; providing habitat to urban wildlife, reducing air pollution, intercepting rainfall, shading and reducing heat absorption by man-made surfaces (Binner et al. 2017; Nowak 2004; Tyrväinen et al. 2005; Xiao and Mcpherson 2002). The cultural services provided by trees are also significant, offering both physical and mental health benefits. The capitalised value of the social and environmental benefits provided by woodlands and forests in the UK alone was estimated to be over £29 billion (Lawrence et al. 2009; Willis et al. 2003).

However, trees in particular can be susceptible to attack from pests and diseases (Table 1), especially if invading from other geographical locations (Hansbrough 1965; Tubby and Webber 2010). Most diseases are caused by microbial pathogens (fungi, bacteria and viruses), the effects of which are amplified during periods of unfavourable environmental conditions including unseasonal temperature shifts and extremes in rainfall patterns (Cazorla and Mercado-Blanco 2016) and anthropogenic climate change (Dukes et al. 2009; La Porta et al. 2008; Sturrock 2012; Sturrock et al. 2011; Tubby and Webber 2010).

Tree pest invasions are also increasing alongside climate change and expanded global trade and may act in tandem with native or invasive diseases, as vectors or co-occurring on hosts, to greatly reduce the populations of particular tree species (Brasier 2008; Tubby and Webber 2010), with the potential to ultimately cause their local extinction.

Plant susceptibility to pests and diseases is often related to the stress level of the individual. Unfortunately, trees in urban areas, which have a particularly high value to humans, often face high stress levels. In urban areas, stress can arise from mismatching of the planting stock's ecological traits to the planting site, root deformities, damage and desiccation, planting at improper depths in unsuitable soils, poor nutrient and water availability, and increased exposure to pollutants, xenobiotics and contaminants (Aldhous and Mason 1994; Ferrini and Nicese 2002; Gilman et al. 2015; Grossnickle 2005; Pauleit 2003; Percival et al. 2006; Pfeiffer et al. 2014; Sjöman and Busse Nielsen 2010). Monocultures also pose a specific problem, as plants grown in monoculture are more susceptible to pest and disease outbreaks and are sensitive to changes in climate, which are less likely with polycultures (Sjöman et al. 2012). Lax biosecurity, including the importation of planting stock and tree products, can also drive biological invasions by tree pests and diseases, as has been demonstrated in Europe (Brasier 2008; Epanchin-Niell 2017; Potter et al. 2011). Some non-native pests are highly destructive and can cause substantial damage to forests and urban/suburban trees (Aukema et al. 2011). Such invasions often lead to significant changes in forest structure and species composition, which in turn lead to changes in ecosystem functions (Lovett et al. 2016). Given the range of pests and diseases that trees are facing, the long generation time of trees, the practical difficulty of working with many of them, and also the speed with which the environment is changing, we are faced with a very difficult challenge – how do we improve our disease and pest management to help trees survive?

Classical control approaches for tree pests and diseases

The application of plant protection products (PPPs) for the control of tree pests and diseases is already often limited by ecological concerns and modulated by the particular local context, as exemplified by the varied management of oak processionary moth, *Thaumetopoea processionea*., in Europe (Tomlinson et al. 2015). However, PPPs are well accepted within commercial tree fruit production and the tree care industry of North America.

Presently, PPPs are generally synthetic chemicals that disrupt the cellular function, or life cycle of the target organism. Other PPPs work on a physical basis e.g. killing insect or acarid targets on contact via suffocation, or abrasion of the exoskeleton and subsequent desiccation. These products are typically those formulated for use in agriculture. Aboveground and external tree pests and diseases are often controlled with aqueous sprays of PPPs to the foliage and bark. Specialised high-pressure spray systems can be used for such applications to large trees (Hirons and Thomas 2018).

Internalised pests and diseases, such as nematodes, are more difficult to reach due to their physical concealment within the host; adjuvants (additives) may improve the penetration of externally applied PPPs for such targets e.g. through bark (Garbelotto et al. 2007),

Table 1 Examples of some current major pathogens and pests of trees

Pathogen	Host	Symptoms	Reference
Oomycete *Phytophthora ramorum*	*Larix* spp. and *Quercus* spp. (sudden death)	Shoots and foliage can be affected. Visible as wilted, withered shoot tips with blackened needles. Trees with branch dieback can have numerous resinous cankers on the branches and upper trunk. It has killed millions of native oak and tan-oak trees in the USA	Davidson et al. (2003)
Fungus *Ceratocystis fagacearum*	*Quercus* spp. (wilt)	Symptoms vary between oak species. White oaks may suffer from scattered dieback in the crown to the death of a single limb of major fork. Red oak succomb to the disease usually within a month. Early foliar symptoms start as vein banding whch later develop to foliar necrosis. Thus far only recorded cases in the USA.	Juzwik et al. (2008) Yang and Juzwik (2017)
Fungus *Ceratocystis platani*	*Platanus* spp.	Wound coloniser causing cankers, xylem staining and restriction of water flow throughout the tree resulting in eventual death of the tree. In oriental plane, *Platanus orientalis*, parts of the crown can suddenly die. Can be identified by cankers on the trunk, defined by bluish-black to reddish-brown discolouration of sap-wood and necrosis of the inner bark. Found in the United states and across Europe, such as in Greece, France and Turkey.	Ocasio-Morales et al. (2007) Lehtijarvi et al. (2018)
Fungus *Hymenoscyphus fraxineus*	*Fraxinus* spp. (Chalara ash dieback)	*Dark brown/orange lesions on leaves, diamond-shaped lesions may occur on stems which, if girdled, can cause wilting. The wood beneath lesions usually is strongly stained. Dieback can be seen throughout the crown, with dieback shoots and twigs at the edges of crowns. Originating in Asia but a serious pathogen across Europe.*	*Landolt* et al. *(*2016*)* *McMullan* et al. *(*2018*)*
Fungus *Cryphonectria parasitica*	*Castanea* spp. (blight)	Attacks the bark by entering cracks or wounds which may lead to crown dieback. Discolouration of the bark and dead bark forms sunken cankers. Pin-head sized, yellow-orange pustules develop on the infected bark and exude long, orange-yellow tendrils of spores in moist conditions. Pale brown mycelial fans may be found in the inner bark. Discolouration of the bark may be more visible in younger trees. Widespread thoughout the eastern US, China, Japan and many European countries with an abundance of sweet chestnut.	Rigling and Prospero (2017)
Fungus *Ophiostoma ulmi* and *O. novo-ulmi*	*Ulmus* spp. (Dutch Elm Disease)	Symptoms emerge in early summer as clusters of wilting/yellowing leaves that turn brown and fall. A mixture of healthy and suffering foliage may be seen as the disease progresses throughout the tree. Affected shoots die back from the tip and twigs may turn down-wards. Exposing the outer wood on symptomatic twigs should reveal dark brown or purple streaks. When cut across, a dark brown stain may be present in the outer wood. Common across Europe, North America and Western Asia.	Brasier and Buck (2001)
Fungus *Rigidoporus microporus*	*Hevea* spp. (white root rot)	Fungal mycelium can be found on the tree collar. Multi-layered fruiting bodies form at the base of the tree and white/white-brown rhizomorphs can be seen on the root surface. Off season flowering may occur as well as yellow-brown discolouration of the foliage. Significant funal pathogen to timber and rubber industry in Indonesia, Malaysia, Sri Lanka and the Ivory coast.	Siri-udom et al. (2017) Khairuzzaman et al. (2017) Hamidson and Naito (2004)
Fungus *Colletotrichum acutatum*	*Olea* spp. (anthracnose)	Fruit rot. Soft to dark brown rot that produces an orange, gelatinous matrix in moist conditions and	Talhinhas et al. (2011)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		mummification in dry as the fruit loses moisture. In spring there may be extensive yellowing of the leaf blade which in summer leads to premature fall of infected leaves. Found in the majority of olive growing countries.	Cacciola et al. (2012)
Fungus *Cytospora chrysosperma, Phomopsis macrospora,* and *Fusicoccum aesculi*	*Populus* spp. (canker)	Young twigs form brown, sunken, rough circle areas in the bark which may spread to the larger branches. Large cankers may form on the branches an trunk. Orange/orange-brown discolouration of bark is often seen exuding orange-brown viscouse liquid. Fruiting bodies in the bark make the canker appear pimpled. In later stages of infection, perithecial stroma form in the dead cankered areas. Widespread across North America, Europe and China.	Ren et al. (2013)
Fungus *Heterobasidion spp.*	Conifers and some deciduous trees	Symptoms may vary depending on the pathogen involved and host plant. White root rot fungus that in early stages of growth causes staining and discolouration of the host wood. Initial decay is usually pale yellow, developing to light brown and resulting in a white pocket rot with black flecks. Eventually results in tree death. Widespread across the Northern Hemisphere and cases in Australia.	Asiegbu et al. (2005) Garbelotto and Gonthier (2013)
Fungus *Dothistroma septosporum* and *Dothistroma pini*	Conifers (Dothistroma (red band) needle blight)	Yellow bands on needles develop into red bands, where small, black fruiting bodies can occur. Can cause needle dieback, defoliation and eventual tree death. Occurs worldwide. Severe cases in Southern hemisphere plantations of New Zealand, Australia, Chile and Kenya. Also found in North America, Canada and Europe.	Schneider et al. (2019) Barnes et al. (2004) Bradshaw (2004)
Bacterium *Xylella fastidiosa*	*Vitis* spp., *Citrus* spp., *Olea* spp. and several species of broadleaf trees	Leaf scorch/browning, wilting foliage and withering of branches. In extreme cases can result in dieback and stunted growth. Cases found in the Americas, Taiwan, Italy, France and Spain.	Simpson et al. (2000) Araújo et al. (2002) Almeida et al. (2019)
Most likely a decline syndrome with possible Bacterial pathogen components: *Brenneria goodwinii*, *Gibbsiella quercinecans, Rahnella victoriana*	*Quercus* spp. (Acute oak decline)	Stem bleeds occur on the trunk, weeping dark, translucent liquid. Bark cracks, which may reveal underlying dark, necrotic tissue. Lesions and 'D' shaped exit holes of *Agrilus biguttatus* may be present in the bark.	Denman et al. (2014)
Chronic oak dieback – Complex disorder or syndrome (also referred to as oak decline, dieback-decline)	*Quercus* spp. (particularly *Q. robur*)	Results from a combination of abiotic and biotic factors. Early foliage deterioration, gradual branch death and dieback in the crown. Abiotic stressors and weakening of trees allows for opportunistic attack from insects and disease which can result in tree death. Seen in the UK and across Europe.	Thomas et al. (2002) Gagen et al. (2019) Mitchell et al. (2019)
Bacterium *Xanthomonas citri* subsp. *citri*	Citrus cultivars (canker)	Distinct raised, necrotic lesions on fruits, stems and leaves. As the disease progresses, lesions on the stem can appear as corky, rough, dead tissue with a yellow halo. Present in South America, Africa, Middle East, India, Asia and South Pacific.	Graham and Leite (2004) Ference et al. (2018)
Bacterium *Erwinia amylovora*	Pome trees and rosaceous plants (fireblight)	Affects all above ground parts of the plant. The floral recepticle, ovary and peduncles turn a greyish green, eventually whithering to black. Creamy white bacterial droplets may emerge from affected tissues in humid conditions. Shoots wilt rapidly, forming 'Shepard's crooks', that turn necrotic. In later stages, bark becomes	Mohan and Thomson (1996) Johnson (2015) Schropfer et al. (2018)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		cracked, sunken and may leak amber bacterial ooze. Found across North America, Central Europe, Israel, Turkey Lebanon and Iran.	
Bacterium *Candidatus Liberibacter* spp.	Citrus trees (Huanglongbing disease)	Blotchy, asymmetric mottling of newly mature leaf blades. Fruit may exhibit stunted growth, premature drop and low soluble acid content. Found across Asia, America and Africa.	Kalyebi et al. (2015)
Bacterium *Pseudomonas syringae* pv *aesculi* (*Phytophthora cactorum* and *Ph. plurivora*)	*Aesculus hippocastanum* (Bleeding canker of Horse Chestnut)	Rusty-red/brown/black gummy ooze found on the bark. Dead phloem under the bleeds which may appear mottled orange-brown. In extensive cases where affected areas encircle the trunk or branch, leaf yellowing and defoliation may occur and eventual crown death. Fungal bodies may also be seen in areas of dead bark. Found across the UK and Europe, including France, Netherlands, Belgium and Germany.	Webber et al. (2008) Green et al. (2009) Green et al. (2010)
Asian longhorn beetle *Anoplophora glabripennis*	Wide range of broadleaved trees	Adults are about 20-40mm long, black with white makings and long, black and white antennae. Oval shaped pits on scraoed into the bark where eggs are laid, occasional sap may be visible bleeding from the damaged areas. Galleries in bark may be up to 10mm in diamteter and several cm long. Wood shavings may be found in distinctive chambers where pupation occurs. Large, circular exit holes from emerging adult beetles in the upper trunk and branches, usually 10mm in diameter. Piles of sawdust may be found at the base of infested trees. Originally from China and the Korean peninsula, now found in the USA, Italy and across the EU.	McKenna et al. (2016) Haack et al. (2010)
Beetle *Dendroctonus micans*	*Picea* spp.	Resin bleeding on stems with resin tubes coloured purple-brown with bark particles where the female enters the trunk. Attacks may occur anywhere on tree. Found across Europe.	Yaman et al. (2010) Wainhouse et al. (1990)
Leaf miner *Cameraria ohridella*	*Aesculus* spp.	In early summer, elongated blotches appear white at first but turn brown throughout the foliage. Caterpillars or pupal cocoons may be seen within mined areas. Heavily infested trees may drop their leaves prematurely. Spread throughout central and eastern Europe.	Pocock and Evans (2014) Gilbert and Tekauz (2011)
Lappet moth *Dendrolimus pini*	*Pinus* spp.	Needle defoliation and subsequent tree death. Prescence of cocoons on trunk. Native to Europe, Russia and Asia.	Ray et al. (2016)
Oak processionary moth *Thaumetopoea processionea*	*Quercus* spp.	Voraciously feed on the foliage of oak trees. Large populations lead to significant defoliation, making the tree susceptible to other threats. Found in Central and Southern Europe. In the UK, outbreaks are localised to London and a few neighbouring counties.	Freer-Smith et al. (2017) Quero et al. (2003)
Ambrosia beetle (Black timber bark beetle), *Xylosandrus germanus*	Wide range of hardwood host species	Infestations can be indentified by entry holes into the bark and distinctive, compact cylindrical frass about 3-4cm in length. Other indications of their presence include wilting and yellowing of the leaves, defensive sap production and dieback. Native to East Asia but has spread across North America, Europe and the Caucasus region.	Agnello et al. (2015)
Citrus longhorn beetle *Anoplophora chinensis*	Deciduous and shrub species	Adult males are about 21mm long, females 37mm. They are black with white markings, with distinctive, long antennae. Symptoms include feeding damage from adult beetles on bark and twigs, circular exit holes in bark and 'T' shaped oviposition slits where eggs are laid within the bark tissue. Tunnelling in bark and larval galleries may cause structural weaknesses, disrupt the	Eschen et al. (2015) Eyre et al. (2010) Haack et al. (2010)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		vascular system and result in eventual plant death. Native ranges of China, Japan and Sout East Asia although incidences have occurred in Europe, such as in the Netherlands, Germany, France, Italy, Croatia, Switzerland and the UK.	
Oak Pinhole Borer *Platypus cylindrus*	*Quercus* and other hardwood species	Adult beetles are blackish in colour, 5-7mm long. Usually establishing in stressed trees, galleries about 1.6mm wide are made in the bark with bore dust appearing pale and fibrous. The beetles introduce ambrosia fungi for their nourishment, principly *Raffaelea* spp., which stains the surrounding wood blacky-brown. Found across Europe and North Africa with some incidences occuring in healthy Portuguese trees.	Belhoucine et al. (2011) Bellahirech et al. (2016) Inácio et al. (2011)
Pine wood nematode *Bursaphelenchus xylophilus*	*Pinus* spp. (wilt disease)	Discolouration of some/many branches from green to yellow. Rapid loss of resin flow occurs in 48 hours. Found in Japan, China, Korea, Taiwan, Portugal and Spain.	Futai (2013) Odani et al. (1985)

although many products will still not be transported significantly within the tree. Some PPPs can be injected into the vascular system at the base of the tree and transported upwards e.g. emamectin benzoate used in the control of Emerald Ash Borer larvae in North America (Smitley et al. 2010). The neonicotinoid compound imidacloprid is a soil applied insecticide that is taken up through roots and into the whole plant. However, neonicotinoids face severe restrictions on their use in many countries due to associations with negative impacts on bees (Goulson 2013). Control of fully internalised diseases of trees are also a particular issue, for instance, one of the difficulties in controlling *Verticillium dahliae* and *Xylella fastidiosa* in olive (*Olea europaea*) and grapevine (Baccari and Lindow 2011) is due to the inaccessible location of the pathogen within the vascular system (Cazorla and Mercado-Blanco 2016). Similar difficulties are faced in the control of Huanglongbing disease, *Candidatus liberibacter* spp., which causes citrus greening and is a phloem-limited phytoplasma spread by insect vectors (Abdullah et al. 2009).

Root and soil-borne pathogens have been treated by injections into the soil of PPPs or sterilizing agents such as phenolic compounds or methyl bromide gas (Martin 2003; West and Fox 2002). While many synthetic PPPs break down quickly when exposed on stems or foliage, soil applied compounds may persist for extended periods once bound to soil particles (Edwards 1975).

Stump treatments, e.g. urea, sodium borate, or the saprobic fungus *Phlebiopsis gigantea*, have also been applied to exclude and reduce the build-up of fungal pathogens that can also utilize buried dead wood saprobically, often *Heterobasidion* spp., but may also exclude *Armillaria* spp. and other basidiomycetes, while allowing non-pathogenic species to proliferate (Nicolotti and Gonthier 2005; Nicolotti et al. 1994; Vasiliauskas et al. 2004).

In Europe, and elsewhere, environmental concerns have fuelled a movement away from synthetic "chemical" PPPs or those based on toxic heavy metals e.g. copper (Lamichhane et al. 2018). In the absence of other effective controls this reduction in authorised pesticides may conflict with protecting vital resources such as food and timber.

Biocontrol agents (BCAs)

An area that is gaining much more attention in recent years is biological control (or biocontrol) – the use of biological agents to counter a pest or disease. The desired outcome of a biological control application is to reduce the pathogen or pest population below a threshold of ecological and economic impact, ideally enabling the host to regain health and eventually restoring the invaded community to the pre-invaded state (Bale et al. 2008). This approach is highly favourable because most BCA source species are already present in the host's environment, and in some cases provide a narrow range

of target specificity, so are less likely to be harmful to non-target organisms. BCAs can come in many forms, from viruses or bacteriophage, to bacteria or fungi, and even higher organisms like nematodes, mites or insects (Lenteren et al. 2018).

As PPPs, BCAs are generally applied in similar ways to synthetic compounds and the selected application method typically aims to maximise contact with the target organism. *Bacillus subtilis* QST 713 is a commercialized bacterial strain used in biocontrol programmes around the world (Abbasi and Weselowski 2014). For foliar pathogens like *Botrytis* of fruit or nut trees, *B. subtilis* QST 713 is applied as an aqueous spray, whereas for protection against *Phytophthora* root rots it is applied as an aqueous drench, e.g. via pressurized soil injection systems or irrigation. BCAs may also be physically incorporated into soils (Abbasi and Weselowski 2015). For example, *Trichoderma* strains, often grown on a solid food source such as grain, but also as spore powders, are variously mixed into the soil around roots or placed in cores in close proximity to roots for the treatment of root diseases (Srivastava et al. 2016). One study demonstrated that trunk injections of various *Bacillus* strains into the vascular system of Avocado trees, *Persea americana*, reduced the disease severity of *Phytophthora cinnamomi* infections (Darvas and Bezuidenhout 1987). However, as with the majority of studies discussed in this review, this control method does not appear to have been commercialized or widely utilized to date.

Nematodes, which are used against slugs and snails or insect larvae feeding on roots within the soil, may be dispersed in water and applied to the target area as a drench. Although relatively understudied, nematodes and other soil microfauna e.g. springtails, also have potential in the integrated control of soil borne fungal plant pathogens (McGonigle and Hyakumachi 2001, Riffle 1973, Tomalak 2017). Control of stem boring *Zeuzera pyrina* larvae has been demonstrated by injecting nematode suspensions into the stem cavities created by the larvae (Ashtari et al. 2011). The spores of *Verticillium* strain WCS850 have been applied to Elm trees (*Ulmus* spp.) via punctures in the bark of the tree to induce host resistance to Dutch Elm Disease, caused by the pathogens *Ophiostoma ulmi* and *O. novo ulmi*. The BCA itself does not move far from these sites and the disease is controlled via plant-mediated effects (Scheffer et al. 2008).

Natural enemies are also a popular option for biological control of insect pests in agroforestry settings (Dix et al. 1995). Insects as BCAs have shown great applicability for controlling pests of woody plants, forming around 55% of such introductions up until 2010. The establishment rates of natural enemies and success rates were higher when targeting pests of woody plants than other pests (Kenis et al. 2017). Aphids cause extensive economic losses around the world, as one of the major pest groups of crops plants but are also problematic for trees. To control and counter this, aphid predators, including ladybird larvae, lacewings and gall midges as well as adult spiders, carabids (Carabidae) and rove beetles (Staphylinidae) are used in integrated pest management strategies (Evans 2009; Gardiner & Landis 2007; Messelink et al. 2013; Schmidt et al. 2004; Snyder & Ives 2003). However, the efficiency of control is limited due to insufficient post-application persistence, slow kill rate and high host specificity, in combination with generally high production and maintenance costs, and thus contribute to restricted use in pest control.

The greatest challenges of using BCAs with trees, however, relate to the scales associated with trees – many are very large, thus restricting access to the whole tree and canopy, and woodlands can occupy great areas. Arguably, there is also a dearth of information on many tree diseases and pests, especially for newly emergent outbreaks where monitoring endeavours are struggling to keep up with the incidence and speed of outbreaks (Boyd et al. 2013).

Other major challenges in developing BCAs are the identification, characterisation, formulation and application of the agents. Laboratory analyses may not be reliable predictors of the protective capability of biocontrol agents. For example, the modes of action for most BCAs are still not fully understood, and there is no efficient and effective screening method for identifying field-competent BCAs by laboratory tests (Parnell et al. 2016). The development of appropriate screening methods for BCAs may therefore rely on studies of their interaction with plants, which would slow the screening process. Factors affecting production and delivery of a BCA from laboratory to field include loss of viability, storage stability, environmental conditions, compatibility with other microorganisms, and consistent efficacy over multiple time periods including seasonal variations (Bashan et al. 2013; Slininger et al. 2003). In comparison to synthetic PPPs, storage requirements for preserving BCA product efficacy can be far more varied and

particular, which has been a major issue historically (Bashan et al. 2013; Corkidi et al. 2005).

In this review, we will examine the options for using BCAs for tree diseases and consider how they might be used. In particular, we will focus on endophytes, which are a relatively understudied group. Presented here are examples of endophytes reported as biological control agents in the literature, and most have not been commercialized, but have proven effects under laboratory conditions.

Endophytes as BCAs

Endophytes are defined as microorganisms that accomplish part of their life cycle within living host tissues without causing apparent damage to the plant (Schulz and Boyle 2005; Sun et al. 2014). In all ecosystems, many plant parts are colonized by endophytes (Brundrett 2002; Mandyam and Jumpponen 2005). Depending on the species and the interaction, endophytes may be located in roots, leaves or needles, shoots, or adapted to growth within the bark (Grünig et al. 2008; Rodriguez-Cabal et al. 2013; Sokolski et al. 2007; Verma et al. 2007). Endophytes may grow inter– and intra– cellularly as well as endo– and epi– phytically (Schulz and Boyle 2005; Zhang et al. 2006). However, endophytes can switch their behaviour depending on a set of abiotic and biotic factors, including the genotypes of plants and microbes, environmental conditions, and the dynamic network of interactions within the plant biome (Hardoim et al. 2015; Schulz and Boyle 2005).

Plant ecosystems rely heavily on their microbial communities to optimise health (Pfeiffer et al. 2014), though this intimate association can be a fine balance between mutualism and disease (Knief 2014). Microbes (as epiphytes) can colonise the surfaces of roots (rhizoplane) and leaf/shoot (phylloplane) as well as the internal spaces of plants (as endophytes), with overall abundance being higher for epiphytes compared to endophytes, and rhizosphere compared to phyllosphere (Lindow and Brandl 2003). These differences may reflect the short life span of leaves, nutrient richness in the rhizosphere, and the ability of microorganisms to survive in soil in a dormant state for long periods of time (Vorholt 2012) or due to the physiochemical variations between these two respective environments (Lindow and Brandl 2003).

Endophytes can act in defence against pathogens and disease (Ownley et al. 2004), as well as provide protection or act as deterrents to insect herbivores and nematodes (Breen 1994; Slippers and Wingfield 2007; Vega et al. 2008). However, these defensive properties may not be unanimous to every endophyte-host-pathogen interaction, as shown by Gonthier et al. (2019) where investigations into the protective benefits of ectomycorrhizal fungus *Suillus luteus* in Scots pine (*Pinus sylvestris*) against the fungal pathogens *Heterobasidion irregular* and *Heterobasidion annosum* found that it only reduced host tree susceptibility to *H. annosum*, not both pathogens.

The roles of endophytes in disease and pest resistance are comparatively understudied, but recent work has started to highlight the importance of endophytes, in particular, as an increasingly popular biological control option (Dutta et al. 2014; Gao et al. 2010). Endophytes are also being increasingly recognised as potential controls of significant economic threats such as the invasive spotted lanternfly in North America (Eric et al. 2019).

Isolation and identification of endophytes

Traditionally, the research of endophytes has focussed on identification of culturable fungi and bacteria from plants has involved culturing them from plant tissue on different media. Although successful, it is apparent from the use of culture independent approaches (e.g. metagenomics), that the true diversity and abundance of the endophytic community has not been fully represented or utilized (Bisseling et al. 2009). As a result, it is highly likely that a range of potential candidate organisms with beneficial and exploitable biocontrol capabilities are being overlooked (Moricca et al. 2012; Ragazzi et al. 2001). Slower growing endophytic species are likely to be outcompeted or inhibited in the medium by more rapidly growing species. Other species may be as yet unculturable due to lack of a key growth component, because of an obligate relationship with their host plant for survival or due to a range of environmental parameters. Culture-dependent methods tend to favour the dominant endophytic species, so rarer species that have an irregular existence, are likely to be missed in any sampling effort (Moricca and Ragazzi 2008). However, methods used to isolate, and study endophytes have continued to be improved in light of developments in genetics and genomics. The advancements in next generation sequencing (NGS) has greatly improved the study of endophytes by allowing enormous amounts of genetic sequence data to be processed in parallel at a

fraction of the cost of traditional methods (Knief 2014; Rastogi et al. 2013). Metagenomic analysis employing NGS of whole microbial communities allows much deeper and more accurate DNA sequencing, thus providing insight into the composition and physiological potential of plant-associated microorganisms. NGS reveals both culturable and unculturable endophytes that may be beneficial microbes and appropriate isolation media can then be developed to further study these species of interest (Akinsanya et al. 2015). For example, the presence of endophytic fungi in roots of different plant species in a temperate forest in Japan were identified using NGS (Toju et al. 2013), while whole genome analysis of endophytic microbes has revealed the genetic features that directly or indirectly influence the various bioactivities and colonisation preferences (Kaul et al. 2016). Identification, isolation and characterisation of genes involved in beneficial endophyte-host interactions is critically important for the effective manipulation of the mutualistic association between the two. Endophyte genomic analysis has provided a new tool to pick apart the mechanisms of endophytic associations and to reveal the requisite features needed to inhabit plants. Studies have revealed a wide range of specific genes commonly found across genomes that are important for endophytic lifestyles and symbioses. These include genes coding for nitrogen fixation, phytohormone production, mineral acquisition, stress tolerance, adhesion and other colonization related genes (Firrincieli et al. 2015, Fouts et al. 2008, Kaul et al. 2016, Martínez-García et al. 2015).

Examples of tree endophytes as BCAs

As BCAs, endophytes have diverse mechanisms of action, categorised into direct, indirect or ecological effects (Gao et al. 2010). Endophytes may possess the ability to directly inhibit pathogens by producing antifungal or antibacterial compounds. For example, the endophytic bacterium *Bacillus pumilus* (JK-SX001) is particularly efficient at reducing the infection rate and severity of canker caused by three pathogens (*Cytospora chrysosperma*, *Phomopsis macrospora* and *Fusicoccum aesculi*) in Poplar cuttings. This *Bacillus* strain produces a combination of extracellular enzymes (including cellulases and proteases) and other secondary metabolites that are thought to inhibit the mycelial growth of the pathogen (Ren et al. 2013). When *B. pumilus* (JK-SX001) was applied as a root drench, the bacterial cells migrated from the roots up to the leaves and were reported to also increase host photosynthetic activity and ultimately increase biomass production in the saplings, while suppressing pathogenic activities. These results were promising, but the experiments were performed under greenhouse conditions using fast growing, young cuttings that were sensitive to the canker pathogens. These young trees were easier to inoculate and probably more likely to respond to pathogens and colonisation by endophytes than mature trees.

In another study, the pathogen *Phytophthora meadii*, which causes abnormal leaf fall of rubber trees (*Hevea brasiliensis*), was suppressed using the endophytic bacterium *Alcaligenes* sp. (EIL-2) isolated from healthy rubber tree leaves. In dual cultures, *Alcaligenes* sp. (EIL-2) produced a substance that inhibited hyphal growth of the pathogen. When the endophyte was applied as a foliar and soil drench to one-year old greenhouse plants prior to infection by the pathogen, infection rates were reduced by more than 50% (Abraham et al. 2013). Whilst promising in scope, trials need to be conducted in natural systems to ascertain effectiveness *in situ*.

Species of *Pseudomonas* were the most commonly isolated endophytes antagonistic to the oak wilt fungus, *Ceratocystis fagacearum* (Brooks et al. 1994). When grown in dual culture, these endophytes produced siderophores and/or antibiotic compounds in response to the pathogen. Injecting trees with *Pseudomonas denitrificans* reduced crown loss to *C. fagacearum*, but the response varied depending on what time of year the inoculum was introduced. Seasonal changes affect the physiological state of trees and therefore the availability of nutrients in their vascular system to the introduced endophytes (Brooks et al. 1994), which is likely to influence efficacy of the endophytic BCA.

Berger et al. (2015) compared foliar applications of phosphite, and the endophytes *Trichoderma aureoviride* UASWS and *T. harzianum* B100 on reducing the necrotic area of *Phytophthora plurivora* lesions on oak leaves (*Quercus robur)*. Results showed that given the diffusable nature of phosphite it was able to reduce necrosis on both treated and untreated leaves. However, with UASWS and B100, only untreated leaves showed reduced necrosis suggesting that the interaction was affected by a number of fungal secondary metabolites. However, when applied via trunk injections (endotherapy) a similar endophyte,

T. atroviride ITEC was able to significantly reduce the necrosis size, compared to the control and the phosphite treatment, on 30-year-old beech trees (*Fagus sylvatica*) artificially inoculated with *P. plurivora*. It is clear from this example that the effectiveness of an endophytic BCA is likely to be influenced by the mode of application.

Endophytes may induce such delocalized plant defence reactions, called induced systemic resistance, leading to a higher level of host tolerance toward pathogens (Robert-Seilaniantz et al. 2011; Zamioudis and Pieterse 2012). There is increasing evidence that in the initial stages of endophyte colonisation, interactions between beneficial microorganisms and plants trigger an immune response in plants similar to that against pathogens, but that, later on in the plant growth stage and/or interaction stage, mutualists escape host defence responses and are able to successfully colonize plants (Zamioudis and Pieterse 2012). The shoot endophyte *Methylobacterium* sp. strain IMBG290 was observed to induce resistance against the pathogen *Pectobacterium atrosepticum* in potato, in an inoculum density-dependent manner (Pavlo et al. 2011). The observed resistance was accompanied by changes in the structure of the innate endophytic community. Endophytic community changes were shown to correlate with disease resistance, indicating that the endophytic community as a whole can play a role in disease suppression (Pavlo et al. 2011). Inoculation of white pine (*Pinus monticola*) seedlings with native fungal endophytes reduced disease severity caused by *Cronartium ribicola,* the causal agent of white pine blister rust. The results were temporally persistent suggesting a form of induced resistance. However, the authors did not measure any gene expression or defence pathways to confirm this hypothesis (Ganley et al. 2008).

Inoculation of part of a plant with an endophyte may benefit plants via the production or suppression of phytohormones; for example, genes encoding proteins for biosynthesis of indole acetic acid (IAA) (Zúñiga et al. 2013), cytokinins (CKs) (Bhore et al. 2010) and gibberellins (GAs) (Shahzad et al. 2016) are often present in the metagenome of plant endophytic bacterial communities (Liu et al. 2017). Induction of jasmonic acid biosynthesis enhances localized resistance to biotic agents such as *Hylobius abietis* (large pine weevil) (Heijari et al. 2005), *Ceratocystis polonica* (bluestain fungus) (Krokene et al. 2008; Zeneli et al. 2006) and *Pythium ultimum* (white root rot) (Kozlowski et al. 1999). Mycorrhizae can influence tree susceptibility and tolerance to economically important root pathogens such as *Heterobasidion* spp. and *Armillaria mellea*, even in the absence of direct antagonism of the pathogen by the endophyte (Gonthier et al. 2019; Nogales et al. 2010). Mycorrhizae are well recognized for their positive influence on tree growth and health so may antagonise pathogens via plant-mediated responses or ecologically through inhabiting the same niche, as is seen in other endophytes. The economically important tropical tree, *Theobroma cacao,* is a natural host to endophytes that can significantly reduce the foliar damage caused by a *Phytophthora* species (Arnold et al. 2003). Leaves inoculated with endophytes showed reduced leaf necrosis and mortality when exposed to the foliar pathogen compared to endophyte-free leaf controls. The method of defence appears to be either direct or ecological and not one of induced plant resistance. Only leaves inoculated with the endophytes were resistant to *Phytophthora* infection. This may pose a problem for feasible endophyte application as a BCA if effective disease control is dependent on each individual leaf being sprayed with the endophyte inoculum.

Host-associated microbes can colonize the host horizontally via the environment, vertically from within the parent to the offspring, or by mixed transmission modes (Bright and Bulgheresi 2010). Ecological and evolutionary relationships affect transmission mode and vice versa (Frank et al. 2017). Theory predicts that vertical transmission evolves when symbiotic partners are mutualistic, as a way to ensure faithful transmission of the beneficial symbiont from one generation to the next (Herre et al. 1999). Vertical transmission of bacterial symbionts from parent to offspring is, indeed, common in systems where the symbiont provides an indispensable function, as in the extensively studied nutritional symbioses between bacteria and insects (Moran 2006). Vertical transmission via seeds is also well documented for certain groups of fungal endophytes, e.g., the well-studied *Epichloë* fungal endophytes of grasses (Schardl 2001).

Entomopathogens including fungi, nematodes and bacteria, naturally play important roles in regulating insect populations and are being exploited in biocontrol strategies (Lacey et al. 2015). Miller et al. (2002) investigated the effects of endophytic organisms in white spruce trees (*Picea glauca*) on the pest spruce budworm. They observed that larval growth was significantly

affected by the presence of the endophytes, with some strains proving toxic to the insects. Production of endophytic metabolites is thought to have been the antagonistic factor and in a follow-up study conducted by Miller (2008), the presence of rugulosin toxin produced by the needle endophyte in nursery grown *P. glauca* significantly reduced budworm (*Choristoneura fumiferana*) growth. Decreased palatability for insects and antagonism towards pathogens of needles might be possible benefits for the host trees. Sieber (2007) also found that the colonization of elm bark by *Phomopsis velata* had significant effects on two beetle pests of bark, *Scoltus scolytus* and *Scolytus multistriatus*. These beetles are known vectors of the Dutch Elm disease pathogen *Ophiostoma ulmi* and on introduction of *P. oblonga*, there was a noticeable reduction both in beetle galleries as well as larval success rate thus providing evidence in support of an effective biocontrol agent. In addition to the discovery of more effective isolates and toxins, an increase in the use of entomopathogens will rely on innovations in formulation and better delivery systems.

Challenges in biocontrol of tree pathogens and pests with endophytes

Climate change has and will continue to alter the ranges of pests and diseases and aid their establishment by subjecting plants to stress (Shaw and Osborne 2011). Occurrence of extreme temperatures and weather events, such as heatwaves and flooding, are increasing in frequency worldwide as CO_2 levels increase and thus our natural capital may require active management to protect its current condition (Fischer and Knutti 2015; Hailey and Percival 2015).

Climate change is predicted to have a profound impact on the distribution, abundance, physiology, productivity, phenology, behaviour and ecology of all plant species (Hughes 2000; Nooten et al. 2014). Forest species are particularly susceptible to climate change as the higher longevity of trees hinders rapid adaptation (Broadmeadow et al. 2005; Lindner et al. 2010). Moreover, climate change is known to impact plant-associated microbes some of which play critical, mutualistic roles in maintaining healthy environments. For example, climate change is likely to impact the dispersal of mycorrhizal fungi, key symbionts of trees, which may in turn limit tree migration and colonisation of novel habitats (Pickles et al. 2015). Warming may induce a decrease in arbuscular mycorrhizal fungal colonisation, as has been demonstrated in a manipulated Mediterranean climate, with a likelihood of significant impacts on plant communities and ecosystem function (Wilson et al. 2016).

It has also been recognised that changes in the environment or host can alter the nature of the host-endophyte interaction (Schulz and Boyle 2005). When a tree is subjected to physiological or environmental stress the intimate plant-endophyte relationship is altered and the endophyte may become pathogenic. For example, the fungal endophyte *Discula quercina,* which inhabits healthy *Quercus cerris* trees, causes damage to host structure and function when the tree experiences drought stress (Moricca and Ragazzi 2008; Ragazzi et al. 2001). *Picea abies* (Norway spruce) and other conifers are predicted to become unsuitable for forestry in the central regions of Europe due to rises in temperature (Breymeyer 1996; Fanta 1992), especially at lower elevations (Lexer et al. 2002), which may subject the present large spruce forests to severe stress in the future. Similarly, altered climates may affect BCA function and efficacy. Climate change may also change the lifecycles and feeding behaviour of phytophagous insects, with vector-mediated impacts on tree disease spread (Battisti 2008). It is therefore possible that we will observe an increasing incidence of disease in trees caused by endemic endophytic species, in addition to and potentially interacting with highly destructive pest invasions.

Numerous factors play a role in the under implementation of BCAs for control of diseases in trees and woody plants. These include the size, area, complex root system, inoculum size, and impact of release on the associated ecological system. Many of the difficulties are shared with conventional PPPs, such as reaching internal pests and diseases. Arthropod pests of crops and trees are extremely diverse like their hosts and thus can be notoriously hard to control. Rapid reproduction rates of some of these species (e.g. aphids, gypsy moth, and spruce budworm) mean dense infestations can arise rapidly, reaching levels damaging the plants that then leads to losses that impact both the environment and local and regional economies. Furthermore, different life cycle stages mean that one method of control may not be adequate to manage a pest population effectively, such as in the case of scale insects (Mansour et al. 2017). There is a general consensus that the detrimental effects of insect pests on crops and woody plants are set to worsen with accelerated climate change and control of these will be required for agriculture to keep up with the demands of a growing world population (Dukes et al. 2009).

Effects of introduction of 'alien' species. What are the consequences?

It is as yet unclear as to whether endophytes introduced as BCAs on plants may be effective in reducing disease, but another important aspect is to understand if they have adverse effects on the natural microbial community of the host when the plant is under environmental stress. The introduction of endophytes that have not co-evolved with the host plant may result in the loss of beneficial organisms and so negatively impact the host plant (Whipps 2001). Furthermore, it is important to consider whether the gains provided by the endophyte outweigh the costs associated with it. For example, gall wasps are a problematic species to trees. However, *Apiognomonia errabunda*, the dominant endophyte in beech leaves, has been found to cause abscission of galls by forming necrotic tissue around the affected area, but this may, in time, prove to be more harmful to the host than the gall would have been (Sieber 2007). Furthermore, there is also a risk that some endophytes may not be as useful as hoped in integrated pest management systems as they may affect the efficacy of other BCAs employed to combat pest species. Bultman et al. (2017) found that although endophytes proved effective against plant herbivores, they had repercussions higher up the trophic chain, significantly affecting the performance of parasitoids by reducing pupal mass, which would reduce the parasitoids' success as a BCA.

Pros of using endophytes as BCAs

1. No known adverse environmental effects of native endophytic biological control agents as they are already present in the plant ecosystem.
2. Ability to colonize internal host tissues, the same ecological niche as pathogens, allowing persistence and competition for resources in addition to antagonism. The internal tissue also protects the biological control agent from dangerous UV rays and temperature fluctuations therefore they can persist for longer periods of time.
3. As well as controlling infection, endophytes may simultaneously promote plant growth, for example by increasing photosynthetic activity (Ren et al. 2013).
4. Narrow range of target specificity, less likely to be harmful to non-target organisms.
5. Endophytes may induce systemic resistance in the host and may consequently induce resistance against other pathogens and/or pests (Zamioudis and Pieterse 2012).
6. Pathogens may be less likely to acquire resistance to endophytic BCAs than they are to pesticides due to dynamic interactions.

Cons of using endophytes as BCAs

1. Most research to date has taken place in laboratory conditions, but it is unknown how the endophyte-pathogen interaction will alter in the presence of changing environmental conditions and competition with other organisms in the tree ecosystem.
2. More research must be conducted to find the optimum time for delivery of biocontrol agent inoculum, as seasonal changes in weather and tree physiology could alter efficacy (Brooks et al. 1994).
3. In some cases, resistance to pathogens is isolated only to the plant part that is inoculated with endophytic control. Delivery, and systemic transmission, of BCA to whole tree is likely to be difficult in many cases.
4. Possible changes in host-endophyte-pathogen interaction with climate change, could the endophyte itself become a pathogen? (Moricca and Ragazzi 2008; Ragazzi et al. 2001).
5. Endophytic BCA may alter the microbial community of the host tree, which may adversely affect the host or may have consequences at higher trophic levels.

Conclusion

With growing concern about environmental pollution and the harmful effects of chemicals, the use of biological control as an alternative environmentally friendly option is becoming necessary. The traditional breeding of trees for resistance remains one potential route, but it is a strategy that might be outpaced by the spread and introduction of pests and diseases, as well as being a time consuming and sometimes difficult task. Despite the challenges confronting biocontrol of tree diseases and

pests, research shows that endophyte treatments can be successfully implemented and there is clear potential for endophytes to be applied to trees as BCA in the future. However, it is unclear how the endophyte enters the plant tissues and disperses throughout the plant. The efficacy of the biocontrol method can be enhanced by integrating it with complimentary cultural and environmental conditions to stimulate plant health and enhance inhibition of the pathogen or pest, but this still requires more attention in the future. Advancements in molecular techniques, such as NGS, are revealing more accurate community structures and, as new environments are studied, it is very likely that new bacterial and fungal species will be discovered and enable the dissection of community effects of individual organisms. Application of community analysis and metagenomics technologies in future studies will advance understanding in both plant-microbe associations and biological control science, with endophytes being prime candidates for use as BCAs.

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Compliance with Ethical Standards

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References

Abbasi, P. A., & Weselowski, B. (2014). Influence of foliar sprays of Bacillus subtilis QST 713 on development of early blight disease and yield of field tomatoes in Ontario. *Canadian Journal of Plant Pathology, 36*, 170–178.

Abbasi, P. A., & Weselowski, B. (2015). Efficacy of *Bacillus subtilis* QST 713 formulations, copper hydroxide, and their tank mixes on bacterial spot of tomato. *Crop Protection, 74*, 70–76.

Abdullah, T. L., Shokrollah, H., Sijam, K., & Abdullah, S. N. A. (2009). Control of Huanglongbing (HLB) disease with reference to its occurrence in Malaysia. *African Journal of Biotechnology, 8*, 4007–4015.

Abraham, A., Philip, S., Kuruvilla Jacob, C., & Jayachandran, K. (2013). Novel bacterial endophytes from *Hevea brasiliensis* as biocontrol agent against Phytophthora leaf fall disease. *Biological Control, 58*, 675–684.

Agnello, A., Breth, D., Tee, E., Cox, K., & Warren, H. R. (2015). Ambrosia beetle–an emergent apple pest. *New York Fruit quality, 23*, 25–28.

Akinsanya, M. A., Goh, J. K., Lim, S. P., & Ting, A. S. Y. (2015). Metagenomics study of endophytic bacteria in Aloe vera using next-generation technology. *Genomics Data, 6*, 159–163.

Aldhous, J. R., & Mason, W. L. (1994). *Forest nursery practice, Forestry Commission Bulletin No.111*. (2nd ed.). *London*: *HMSO, Great Britain*.

Almeida, R. P. P., De La Fuente, L., Koebnik, R., Lopes, J. R. S., Parnell, S., & Scherm, H. (2019). Addressing the new global threat of *Xylella fastidiosa*. *Phytopathology, 109*, 172–174.

Araújo, W. L., Marcon, J., Maccheroni, W., Jr., Van Elsas, J. D., Van Vuurde, J. W. L., & Azevedo, J. L. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and Environmental Microbiology, 68*, 4906–4914.

Arnold, A. E., Mejia, L. C., Kyllo, D., et al. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences, 100*, 15649–15654.

Ashtari, M., Karimi, J., Rezapanah, M. R., & Hassani, M. (2011). Biocontrol of leopard moth , *Zeuzera pyrina* L . (Lep .: Cossidae) using entomopathogenic nematodes in Iran. *Agriculture, 66*, 333–335.

Asiegbu, F. O., Adomas, A., & Stenlid, J. (2005). Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. S.l. *Molecular Plant Pathology*, 6, 395–409.

Aukema, J. E., Leung, B., Kovacs, K., et al. (2011). Economic impacts of Non-Native forest insects in the continental United States. *PLoS ONE, 6*, 1–7.

Baccari, C., & Lindow, S. E. (2011). Assessment of the process of movement of *Xylella fastidiosa* within susceptible and resistant grape cultivars. *Phytopathology, 101*, 77–84.

Bale, J. S., Lenteren, J. C. V., & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions of the Royal Society, Biological Sciences, 363*, 761–776.

Barnes, I., Crous, P. W., Wingfield, B. D., & Wingfield, M. J. (2004). Multigene phylogenies reveal that red band needle blight of Pinus is caused by two distinct species of Dothistroma, *D. septosporum* and *D. pini*. *Studies in Mycology*, 551–565.

Bashan, Y., De-Bashan, L. E., Prabhu, S. R., & Hernandez, J.-P. (2013). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil, 378*, 1–33.

Battisti, A. (2008). Forests and climate change - lessons from insects. *Iforest-Biogeosciences and Forestry, 1*, 1–5.

Belhoucine, L., Bouhraoua, R. T., Meijer, M., et al. (2011). Mycobiota associated with *Platypus cylindrus* (Coleoptera: Curculionidae, Platypodidae) in cork oak stands of North West Algeria, Africa. *African Journal of Microbiology Research, 5*, 4411–4423.

Bellahirech, A., Inacio, M. L., Nobrega, F., et al. (2016). Can behavioural differences in Platypus cylindrus (Coleoptera: Platypodinae) from Portugal and Tunisia be explained by genetic and morphological traits? *Bulletin of Entomological Research, 106*, 1–8.

Berger, G., Czarnocka, K., Cochard, B., Oszako, T., & Lefort, F. (2015). Biocontrol endotherapy with *Trichoderma* spp. and *Bacillus amyloliquefaciens* against *Phytophthora* spp.: A comparative study with phosphite treatment on *Quercus robur* and *Fagus sylvatica*. *Journal of Agricultural Science and Technology, 5*, 428–439.

Bhore, S. J., Ravichantar, N., & Loh, C. Y. (2010). Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [*Gynura procumbens* (Lour.) Merr.] for cytokinin-like compounds. *Bioinformation, 5*, 191–197.

Binner, A., Smith, G., Bateman, I., Day, B., Agarwala, M., & Harwood, A. (2017). *Valuing the social and environmental contribution of woodlands and trees in England, Scotland and Wales*. Edinburgh: In. Forestry Commission https://ore.exeter.ac.uk/repository/bitstream/handle/10871/25958/FCRP027.pdf.

Bisseling, T., Dangl, J. L., & Schulze-Lefert, P. (2009). Next-generation communication. *Science, 324*, 691.

Boyd, I. L., Freer-Smith, P. H., Gilligan, C. A., & Godfray, H. C. (2013). The consequence of tree pests and diseases for ecosystem services. *Science, 342*, 1235773.

Bradshaw, R. E. (2004). Dothistroma (red-band) needle blight of pines and the dothistromin toxin: a review. *Forest Pathology, 34*, 163–185.

Brasier, C. M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology, 57*, 792–808.

Brasier, C. M., & Buck, K. W. (2001). Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biological Invasions, 3*, 223–233.

Breen, J. P. (1994). Acremonium endophyte interaction with plant resistance to insects. *Annual Review of Entomology, 39*, 401–423.

Breymeyer AI, 1996. *Global change: effects on coniferous forests and grasslands*. International Council of Scientific Unions. Scientific Committee on Problems of the Environment: Wiley.

Bright M, Bulgheresi S (2010. A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology* 8, 218-.

Broadmeadow, M. S. J., Ray, D., & Samuel, C. J. A. (2005). Climate change and the future for broadleaved tree species in Britain. *Forestry: An International Journal of Forest Research, 78*, 145–161.

Brooks, D. S., Gonzalez, C. F., Appel, D., & Filer, T. H. (1994). Evaluation of endophytic bacteria as potential biological-control agents for Oak Wilt. *Biological Control, 4*, 373–381.

Brundrett, M. (2002). Coevolution of roots and mycorrhizas of land plants. *New Phytologist, 154*, 275–304.

Bultman, T. L., Borowicz, K. L., Schneble, R. M., et al. (2017). Nordic society oikos effect of a fungal endophyte on the growth and survival of two euplectrus parasitoids Coudron and Lowell P. Bush. *Wiley on behalf of Nordic Society Oikos Stable, 78*, 170–176.

Cacciola, S., Faedda, R., Sinatra, F., Agosteo, G., & Schena, L. (2012). Olive anthracnose. *Journal of Plant Pathology, 94*, 29–44.

Cazorla, F. M., & Mercado-Blanco, J. (2016). Biological control of tree and woody plant diseases: an impossible task? *BioControl, 61*, 233–242.

Corkidi, L., Allen, E. B., Merhaut, D., et al. (2005). Effectiveness of commercial mycorrhizal inoculants on the growth of *Liquidambar styraciflua* in plant nursery conditions. *Journal of Environmental Horticulture, 23*, 72–76.

Darvas, J. M., & Bezuidenhout, J. J. (1987). Control of Phytophthora root rot of avocados by trunk injection. *South African Avocado Growers' Association Yearbook, 10*, 91–93.

Davidson JM, Werres S, Garbelotto M, Hansen EM, Rizzo DM, 2003. Sudden Oak Death and associated diseases caused by *Phytophthora ramorum*. *Plant Health Progress*.

Denman, S., Brown, N., Kirk, S., Jeger, M., & Webber, J. (2014). A description of the symptoms of Acute Oak Decline in Britain and a comparative review on causes of similar disorders on oak in Europe. *Forestry, 87*, 535–551.

Dix, M. E., Johnson, R. J., Harrell, M. O., Case, R. M., Wright, R. J., Hodges, L., Brandle, J. R., Schoeneberger, M. M., Sunderman, N. J., Fitzmaurice, R. L., Young, L. J., & Hubbard, K. G. (1995). Influences of trees on abundance of natural enemies of insect pests: A review. *Agroforestry Systems, 29*(3), 303–311.

Dukes, J. S., Pontius, J., Orwig, D., et al. (2009). Responses of insect pests, pathogens, and invasive plant species to climate change in the forests of northeastern North America: What can we predict?This article is one of a selection of papers from NE Forests 2100: A Synthesis of Climate Change Impacts on Forests of the Northeastern US and Eastern Canada. *Canadian Journal of Forest Research, 39*, 231–248.

Dutta, D., Puzari, K. C., Gogoi, R., & Dutta, P. (2014). Endophytes: Exploitation as a tool in plant protection. *Brazilian Archives of Biology and Technology, 57*, 621–629.

Edwards CA, 1975. Factors that affect the persistence of pesticides in plants and soils. In: Varo P, ed. *Pesticide Chemistry–3*. Butterworth-Heinemann, 39-56.

Epanchin-Niell, R. (2017). Economics of invasive species policy and management. *Biological Invasions*, 1–22.

Eric, C. H., Castrillo, L. A., Gryganskyi, A., & Hajek, A. E. (2019). A pair of native fungal pathogens drives decline of a new invasive herbivore. *Proceedings of the National Academy of Sciences, 116*, 9178.

Eschen, R., Gregoire, J. C., Hengeveld, G. M., De Hoop, B. M., Rigaux, L., & Potting, R. P. J. (2015). Trade patterns of the tree nursery industry in Europe and changes following findings of citrus longhorn beetle, Anoplophora chinensis Forster. *Neobiota, 26*, 1–20.

Evans, E. W, (2009). Lady beetles as predators of insects other than Hemiptera. Biological Control 51, 255–67.

Eyre, D., Cannon, R., Mccann, D., & Whittaker, R. (2010). Citrus longhorn beetle, *Anoplophora chinensis*: an invasive pest in Europe. *Outlooks on Pest Management, 21*, 195–198.

Fanta, J. (1992). Possible impact of climatic change on forested landscapes in central Europe: a review. *Catena, 22*, 133–151.

Fao. (2010). Global forest resources assessment 2010: global tables.

Ference, C. M., Gochez, A. M., Behlau, F., Wang, N., Graham, J. H., & Jones, J. B. (2018). Recent advances in the understanding of *Xanthomonas citri* ssp. citri pathogenesis and citrus canker disease management. *Molocular Plant Pathology, 19*, 1302–1318.

Ferrini, F., & Nicese, F. P. (2002). Response of english oak (Quercus robur L.) trees to biostimulants application in the urban environment trees to biostimulants application in the urban environment. *Journal of Arboriculture, 28*, 70–75.

Firrincieli A, Otillar R, Salamov A, et al. (2015) Genome sequence of the plant growth promoting endophytic yeast Rhodotorula graminis WP1. *Frontiers in Microbiology* 6, 978-.

Fischer, E. M., & Knutti, R. (2015). Anthropogenic contribution to global occurrence of heavy-precipitation and high-temperature extremes. *Nature Climate Change, 5*, 560–564.

Fouts, D. E., Tyler, H. L., Deboy, R. T., et al. (2008). Complete Genome Sequence of the N(2)-Fixing Broad Host Range Endophyte Klebsiella pneumoniae 342 and Virulence Predictions Verified in Mice. *PLoS Genetics, 4*, e1000141–e100014e.

Frank, A., Saldierna Guzmán, J., & Shay, J. (2017). Transmission of bacterial endophytes. *Microorganisms, 5*, e70.

Freer-Smith, P. H., & Webber, J. F. (2017). Tree pests and diseases: The threat to biodiversity and the delivery of ecosystem services. *Biodiversity and Conservation, 26*, 3167–3181.

Futai, K. (2013). Pine wood nematode, *Bursaphelenchus xylophilus*. *Annual Review of Phytopathology, 51*, 61–83.

Gagen, M., Matthews, N., Denman, S., et al. (2019). The tree ring growth histories of UK native oaks as a tool for investigating Chronic Oak Decline: An example from the Forest of Dean. *Dendrochronogia, 55*, 50–59.

Ganley, R. J., Sniezko, R. A., & Newcombe, G. (2008). Endophyte-mediated resistance against white pine blister rust in *Pinus monticola*. *Forest Ecology and Management, 255*, 2751–2760.

Gao, F. K., Dai, C. C., & Liu, X. Z. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research, 4*, 1346–1351.

Garbelotto, M., & Gonthier, P. (2013). Biology, epidemiology, and control of Heterobasidion species worldwide. *Annual Review of Phytopathology, 51*, 39–59.

Garbelotto, M., Schmidt, D. J., & Hamik, T. Y. (2007). Phosphite injections and bark application of phosphite + Pentrabark™ control sudden oak death in coast live oak. *Arboriculture and Urban Forestry, 33*, 309–317.

Gardiner, M. M., & Landis, D. A. (2007). Impact of intraguild predation by adult Harmonia axyridis (Coleoptera: Coccinellidae) on Aphis glycines (Hemiptera: Aphididae) biological control in cage studies. *Biological Control, 40*, 386–395.

Gilbert, J., & Tekauz, A. (2011). Strategies for management of fusarium head blight (FHB) in cereals. *Prairie Soils Crops, 4*, 97–104.

Gilman, E. F., Paz, M., & Harchick, C. (2015). Container and landscape planting depth and root ball shaving affects magnolia grandiflora root architecture and landscape performance. *Arboriculture and Urban Forestry, 41*, 260–269.

Gonthier, P., Giordano, L., Zampieri, E., et al. (2019). An ectomycorrhizal symbiosis differently affects host susceptibility to two congeneric fungal pathogens. *Fungal Ecology, 39*, 250–256.

Goulson, D. (2013). REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology, 50*, 977–987.

Graham, J. H., & Leite, R. P. (2004). Lack of control of citrus canker by induced systemic resistance compounds. *Plant Disease, 88*, 745–750.

Green, C., Laue, B., Fossdal, C. J., A'hara, S. W., & Cottrell, J. E. (2009). Infection of horse chestnut (*Aesculus hippocastanum*) by *Pseudomonas syringae* pv. *aesculi* and its detection by quantitative real-time PCR. *Plant Pathology, 58*, 731–744.

Green S, Studholme DJ, Laue BE, *et al.* (2010. Comparative genome analysis provides insights into the evolution and adaptation of *Pseudomonas syringae* pv. *aesculi* on *Aesculus hippocastanum*. *PLoS ONE* 5.

Grossnickle, S. C. (2005). Importance of root growth in overcoming planting stress. *New Forests, 30*, 273–294.

Grünig, C. R., Queloz, V., Sieber, T. N., & Holdenrieder, O. (2008). Dark septate endophytes (DSE) of the Phialocephala fortinii s.l. – Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany-Botanique, 86*, 1355–1369.

Haack RA, Herard F, Sun JH, Turgeon JJ, (2010. Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: a worldwide perspective. *Annual Review of Entomology* 55.

Hailey, L. E., & Percival, G. C. (2015). The Influence of long term flooding on tree biology and approaches to flood stress alleviation and management. *Arboricultural Journal, 37*, 135–149.

Hamidson, S. H., & Naito, S. (2004). Distribution of *Rigidoporus lignosus* genotypes in a rubber plantation, as revealed by somatic compatibility. *Mycoscience, 45*, 72–75.

Hansbrough, J. R. (1965). Biological control of forest tree diseases. *Journal of the Washington Academy of Sciences, 55*, 41–44.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., et al. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews, 79*, 293–320.

Heijari, J., Nerg, A. M., Kainulainen, P., Viiri, H., Vuorinen, M., & Holopainen, J. K. (2005). Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in Scots pine seedlings. *Entomologia Experimentalis et Applicata, 115*, 117–124.

Herre, E. A., Knowlton, N., Mueller, U. G., & Rehner, S. A. (1999). The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution, 14*, 49–53.

Hirons A D, Thomas P. A. 2018. *Applied tree biology* Wiley, UK

Hughes, L. (2000). Biological consequences of global warming: is the signal already apparent? *Trends in Ecology and Evolution, 15*, 56–61.

Inácio, M. E., Henriques, J., Guerra-Guimaraes, L., Gil Azinheira, H., Lima, A., & Sousa, E. (2011). *Platypus cylindrus* Fab. (Coleoptera: Platypodidae) transports *Biscogniauxia mediterranea*, agent of cork oak charcoal canker. *Boletin de Sanidad Vegetal Plagas, 37*, 181–186.

Johnson KB, 2015. Fire blight of apple and pear. *The Plant Health Instructor*.

Juzwik, J., Harrington, T. C., Macdonald, W. L., & Appel, D. N. (2008). The origin of *Ceratocystis fagacearum*, the oak wilt fungus. *Annual Review of Phytopathology, 46*, 13–26.

Kalyebi A, Aisu G, Ramathani J, Ogwang J, Mcowen N, Russell P, 2015. Detection and identification of etiological agents (*Liberibacter* spp.) associated with citrus greening disease in Uganda. *Uganda Journal of Agricultural Sciences* 16, 43-.

Kaul, S., Sharma, T., & Dhar, K. M. (2016). "Omics" tools for better understanding the plant–endophyte interactions. *Frontiers in Plant Science, 7*, 1–9.

Kenis, M., Hurley, B. P., Hajek, A. E., & Cock, M. J. W. (2017). Classical biological control of insect pests of trees: Facts and figures. *Biological Invasions, 19*(11), 3401–3417.

Khairuzzaman, N. A., Hashim, H., Kamal, M. M., Sampian, A. F. M., & Yussuf, U. R. (2017). White root disease infected rubber tree detection through statistical inference of Dry Rubber sheet Near Infrared (NIR) spectrum measurement method. *Journal of Mechanical Engineering, 2*, 167–177.

Knief, C. (2014). Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Frontiers in Plant Science, 5*.

Kozlowski, G., Buchala, A., & Metraux, J. P. (1999). Methyl jasmonate protects Norway spruce [Picea abies (L.) Karst.] seedlings against *Pythium ultimum* Trow. *Physiological and Molecular Plant Pathology, 55*, 53–58.

Krokene, P., Nagy, N. E., & Solheim, H. (2008). Methyl jasmonate and oxalic acid treatment of Norway spruce: anatomically based defense responses and increased resistance against fungal infection. *Tree Physiology, 28*, 29–35.

La Porta, N., Capretti, P., Thomsen, I. M., Kasanen, R., Hietala, A. M., & Von Weissenberg, K. (2008). Forest pathogens with higher damage potential due to climate change in Europe. *Canadian Journal of Plant Pathology, 30*, 177–195.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *Journal of Invertebrate Pathology, 132*, 1–41.

Lamichhane, J. R., Osdaghi, E., Behlau, F., Köhl, J., Jones, J. B., & Aubertot, J. N. (2018). Thirteen decades of antimicrobial copper compounds applied in agriculture. *A review. Agronomy for Sustainable Development, 38*, 28.

Landolt, J., Gross, A., Holdenrieder, O., & Pautasso, M. (2016). Ash dieback due to *Hymenoscyphus fraxineus*: what can be learnt from evolutionary ecology? *Plant Pathology, 65*, 1056–1070.

Lawrence A, Carter C, O'brien L, Lovell R, 2009. Social benefits from the Forestry Commission public forest estate in England: review of current evidence. In. The Research Agency of the Forestry Commission.

Lehtijarvi, A., Oskay F., Lehtijarvi H.T.D. et al. 2018. Ceratocystis platani is killing plane trees in Istanbul (Turkey). *Forest Pathology, 48*.

Lenteren, J. C. V., Bolckmans, K., Köhl, J., Ravensberg, W. J., & Urbaneja, A. (2018). Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl, 63*, 39–59.

Lexer, M. J., Honninger, K., Scheifinger, H., et al. (2002). The sensitivity of Austrian forests to scenarios of climatic change: a large-scale risk assessment based on a modified gap model and forest inventory data. *Forest Ecology and Managment, 162*, 53–72.

Lindner, M., Maroschek, M., Netherer, S., et al. (2010). Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. *Forest Ecology and Management, 259*, 698–709.

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Applied and Environmental Microbiology, 69*, 1875–1883.

Liu, H., Carvalhais, L. C., Crawford, M., et al. (2017). Inner plant values: Diversity, colonization and benefits from endophytic bacteria. *Frontiers in Microbiology, 8*, 1–17.

Lovett, G. M., Weiss, M., Liebhold, A. M., et al. (2016). Nonnative forest insects and pathogens in the United States: Impacts and policy options. *Ecological Applications, 26*, 1437–1455.

Mandyam, K., & Jumpponen, A. (2005). Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology, 53*, 173–189.

Mansour, R., Grissa-Lebdi, K., Suma, P., Mazzeo, G., & Russo, A. (2017). Key scale insects (Hemiptera: Coccoidea) of high economic importance in a Mediterranean area: Host plants, bio-ecological characteristics, natural enemies and pest management strategies - a review. *Plant Protection Science, 53*, 1–14.

Martin, F. N. (2003). Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annual Review of Phytopathology, 41*, 325–350.

Martínez-García, P. M., Ruano-Rosa, D., Schilirò, E., et al. (2015). Complete genome sequence of *Pseudomonas fluorescens* strain PICF7, an indigenous root endophyte from olive (*Olea europaea* L.) and effective biocontrol agent against *Verticillium dahliae*. *Standards in Genomic Sciences, 10*, 10.

Mckenna, D. D., Scully, E. D., Pauchet, Y., et al. (2016). Genome of the Asian longhorned beetle (*Anoplophora glabripennis*), a globally significant invasive species, reveals key functional and evolutionary innovations at the beetle–plant interface. *Genome Biology, 17*, 227.

McGonigle T, Hyakumachi M, 2001. Feeding on plant-pathogenic fungi by invertebrates: comparison with saprotrophic and mycorrhizal systems In: Jeger MJ, Spence Nj, Ed, eds. Biotic interactions in plant-pathogen associations. CABI, 63–85.

Mcmullan, M., Rafiqi, M., Kaithakottil, G., et al. (2018). The ash dieback invasion of Europe was founded by two genetically divergent individuals. *Nature Ecology and Evolution, 2*, 1000–1008.

Messelink, G. J., Bloemhard, C. M. J., Sabelis, M. W., & Janssen, A. (2013). Biological control of aphids in the presence of thrips and their enemies. *Biological Control, 58*, 45–55.

Miller, J. D. (2008). Mycotoxins in small grains and maize: Old problems, new challenges. *Food Additives and Contaminants, 25*, 219–230.

Miller, J. D., Mackenzie, S., Foto, M., Adams, G. W., & Findlay, J. A. (2002). Needles of white spruce inoculated with rugulosin-producing endophytes contain rugulosin reducing spruce budworm growth rate. *Mycological Research, 106*, 471–479.

Mitchell, R. J., Bellamy, P. E., Ellis, C. J., et al. (2019). Collapsing foundations: The ecology of the British oak, implications of its decline and mitigation options. *Biological Conservation, 233*, 316–327.

Mohan, S. K., & Thomson, S. V. (1996). An outbreak of fire blight in plums. *Acta Horticulturae*, (411), 73–76.

Moran, N. A. (2006). *Symbiosis. Current Biology, 16*, R866–RR71.

Moricca, S., & Ragazzi, A. (2008). Fungal endophytes in Mediterranean oak forests: a lesson from *Discula quercina*. *Phytopathology, 98*, 380–386.

Moricca, S., Ginetti, B., & Ragazzi, A. (2012). Species- and organ-specificity in endophytes colonizing healthy and declining Mediterranean oaks. *Phytopathologia Mediterranea, 51*, 587–598.

Nicolotti, G., & Gonthier, P. (2005). Stump treatment against Heterobasidion with *Phlebiopsis gigantea* and some chemicals in Picea abies stands in the western Alps. *Forest Pathology, 35*, 365–374.

Nicolotti G, Gangemi D, Lanata F, Anselmi N, 1994. Antagonistic activity of wood decay basidiomycetes against european *Armillaria* species. In: Johansson M, Stenlid J, eds. *Proceedings 8th International Conference on Root and Butt Rots* IUFRO Working Party, Wik, Sweden, and Haikko, Finland, 725-735.

Nogales, A., Camprubi, A., Estaun, V., Marfa, V., & Calvet, C. (2010). In vitro interaction studies between Glomus intraradices and *Armillaria mellea* in vines. *Spanish Journal of Agricultural Research, 8*, S62–SS8.

Nooten, S. S., Andrew, N. R., & Hughes, L. (2014). Potential impacts of climate change on insect communities: A transplant experiment. *PLoS ONE, 9*, e85987–e8598e.

Nowak DJ (2004) Assessing environmental functions and values of veteran trees. In: Nicolotti G, Gonthier P, eds. *Proceedings of the International Congress on the Protection and Exploitation of Veteran Trees* Torino, Italy, pp. 45–49.

Ocasio-Morales, R. G., Tsopelas, P., & Harrington, T. C. (2007). Origin of *Ceratocystis platani* on native Platanus orientalis in Greece and its impact on natural forests. *Plant Disease, 91*, 901–904.

Odani, K., Sasaki, S., Nishiyama, Y., & Yamamoto, N. (1985). Differences in dispersal and multiplication of two associated nematodes, *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* in pine seedlings in relation to the pine wilt disease development. *Journal of the Japasnese Forestry Society, 67*, 398–403.

Ownley, B., Pereira, R., Klingeman, W. E., Quigley, N. B., & Leckie, B. (2004). *Beauveria bassiana*, a dual purpose biological control with activity against insect pests and plant pathogens. *Emerging concepts in plant health management*, 255–269.

Parnell, J. J., Berka, R., Young, H. A., et al. (2016). From the lab to the farm: An industrial perspective of plant beneficial microorganisms. *Frontiers in Plant Science, 7*, 1110.

Pauleit, S. (2003). Urban street tree plantings: identifying the key requirements. *Municipal Engineer, 156*, 43–50.

Pavlo, A., Leonid, O., Iryna, Z., Natalia, K., & Maria, P. A. (2011). Endophytic bacteria enhancing growth and disease resistance of potato (*Solanum tuberosum* L.). *Biological Control, 56*, 43–49.

Percival, G. C., Keary, I. P., & Al-Habsi, S. (2006). An assessment of the drought tolerance of Fraxinus genotypes for urban landscape plantings. *Urban Forestry & Urban Greening, 5*, 17–27.

Pfeiffer, S., Pastar, M., Mitter, B., et al. (2014). Improved group-specific primers based on the full SILVA 16S rRNA gene reference database. *Environmental Microbiology, 16*, 2389–2407.

Pickles, B. J., Twieg, B. D., O'neill, G. A., Mohn, W. W., & Simard, S. W. (2015). Local adaptation in migrated interior Douglas-fir seedlings is mediated by ectomycorrhizas and other soil factors. *New Phytologist, 207*, 858–871.

Pocock, M. J. O., & Evans, D. M. (2014). The Success of the Horse-Chestnut Leaf-Miner, Cameraria ohridella, in the UK Revealed with Hypothesis-Led Citizen Science. *PLoS ONE, 9*, e86226.

Potter, C., Harwood, T., Knight, J., & Tomlinson, I. (2011). Learning from history, predicting the future: the UK Dutch elm disease outbreak in relation to contemporary tree disease threats. *Philosophical Transactions of the Royal Society B: Biological Sciences, 366*, 1966–1974.

Quero, C., Bau, J., Guerrero, A., et al. (2003). Sex pheromone of the oak processionary moth *Thaumetopoea processionea*. Identification and biological activity. *Journal of Agricultural and Food Chemistry, 51*, 2987–2991.

Quine CP, Cahalan C, Hester A*, et al.* (2011). Woodlands. *UK National Ecosystem Assessment: Technical Report*, 241-295.

Ragazzi, A., Mancini, F., Dellavalle, I., Capretti, P., & Moricca, S. (2001). Endophytic fungi in *Quercus cerris* : isolation frequency in relation to phenological phase, tree health and the organ affected. *Phytopathologia Mediterranea, 40*, 165–171.

Rastogi, G., Coaker, G. L., & Leveau, J. H. J. (2013). New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. *FEMS Microbiology Letters, 348*, 1–10.

Ray, D., Peace, A., Moore, R., et al. (2016). Improved prediction of the climate-driven outbreaks of *Dendrolimus pini* in *Pinus sylvestris* forests. *Forestry: An International Journal of Forest Research, 89*, 230–244.

Ren, J. H., Li, H., Wang, Y. F., Ye, J. R., Yan, A. Q., & Wu, X. Q. (2013). Biocontrol potential of an endophytic *Bacillus pumilus* JK-SX001 against poplar canker. *Biological Control, 67*, 421–430.

Riffle, J. (1973). Effect of two mycophagous nematodes on *Armillaria mellea* root rot of *Pinus ponderosa* seedlings. *Plant Disease Report*, 355–357.

Rigling, D., & Prospero, S. (2017). Cryphonectria parasitica, the causal agent of chestnut blight: invasion history, population biology and disease control. *Molocular Plant Pathology, 19*, 7–20.

Robert-Seilaniantz, A., Grant, M., & Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology, 49*, 317–343.

Rodriguez-Cabal, M. A., Barrios-Garcia, M. N., Amico, G. C., Aizen, M. A., & Sanders, N. J. (2013). Node-by-node disassembly of a mutualistic interaction web driven by species introductions. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 16503–16507.

Schardl, C. L. (2001). Epichloë festucae and Related Mutualistic Symbionts of Grasses. *Fungal Genetics and Biology, 33*, 69–82.

Scheffer, R. J., Voeten, J. G. W. F., & Guries, R. P. (2008). Biological Control of Dutch Elm Disease. *Plant Disease, 92*, 192–200.

Schmidt, M. H., Thewes, U., Thies, C., & Tscharntke, T. (2004). Aphid suppression by natural enemies in mulched cereals. *Entomologia Experimentalis et Applicata, 113*, 87–93.

Schneider, S., Jung, E., Queloz, V., Meyer, J. B., & Rigling, D. (2019). Detection of pine needle diseases caused by *Dothistroma septosporum*, *Dothistroma pini* and *Lecanosticta acicola* using different methodologies. *Forest Pathology, 49*, e12495.

Schropfer, S., Bottcher, C., Wohner, T., et al. (2018). A single effector protein, AvrRpt2(EA), from *Erwinia amylovora* can cause fire blight disease symptoms and induces a salicylic acid-dependent defense response. *Molecular Plant-Microbe Interactions, 31*, 1179–1191.

Schulz, B., & Boyle, C. (2005). The endophytic continuum. *Mycological Research, 109*, 661–686.

Shahzad, R., Waqas, M., Khan, A. L., et al. (2016). Seed-borne endophytic *Bacillus amyloliquefaciens* RWL-1 produces gibberellins and regulates endogenous phytohormones of Oryza sativa. *Plant Physiology and Biochemistry, 106*, 236–243.

Shaw, M. W., & Osborne, T. M. (2011). Geographic distribution of plant pathogens in response to climate change. *Plant Pathology, 60*, 31–43.

Sieber, T. N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biology Reviews, 21*, 75–89.

Simpson, A. J. G., Reinach, F. C., Arruda, P., et al. (2000). The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature, 406*, 151.

Siri-Udom, S., Suwannarach, N., & Lumyong, S. (2017). Applications of volatile compounds acquired from *Muscodor heveae* against white root rot disease in rubber trees (*Hevea brasiliensis* Müll. Arg.) and relevant allelopathy effects. *Fungal Biology, 121*, 573–581.

Sjöman, H., & Busse Nielsen, A. (2010). Selecting trees for urban paved sites in Scandinavia – A review of information on stress tolerance and its relation to the requirements of tree planners. *Urban Forestry & Urban Greening, 9*, 281–293.

Sjöman, H., Östberg, J., & Bühler, O. (2012). Diversity and distribution of the urban tree population in ten major Nordic cities. *Urban Forestry and Urban Greening, 11*, 31–39.

Slininger, P. J., Behle, R. W., Jackson, M. A., & Schisler, D. A. (2003). Discovery and development of biological agents to control crop pests. *Neotropical Entomology, 32*, 183–195.

Slippers, B., & Wingfield, M. J. (2007). Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews, 21*, 90–106.

Smitley, D. R., Rebek, E. J., Royalty, R. N., Davis, T. W., & Newhouse, K. F. (2010). Protection of individual Ash trees from Emerald Ash Borer (Coleoptera: Buprestidae) with basal soil applications of imidacloprid. *Journal of Economic Entomology, 103*, 119–126.

Snyder, W. E., & Ives, A. R. (2003). Interaction between specialist and generalist natural enemies: Parasitiods, predators, and pea aphid biocontrol. *Ecology, 84*, 91–107.

Sokolski, S., Bernier-Cardou, M., Piché, Y., & Bérubé, J. A. (2007). Black spruce (*Picea mariana*) foliage hosts numerous and potentially endemic fungal endophytes. *Canadian Journal of Forest Research, 37*, 1737–1747.

Srivastava, M., Kumar, V., Shahid, M., Pandey, S., & Singh, A. (2016). Trichoderma- a potential and effective bio fungicide and alternative source against notable phytopathogens: A review. *African Journal of Agricultural Research, 11*, 310–316.

Sturrock, R. N. (2012). Climate change and forest diseases: using today's knowledge to address future challenges. *Forest Systems, 21*, 329–336.

Sturrock, R. N., Frankel, S. J., Brown, A. V., et al. (2011). Climate change and forest diseases. *Plant Pathology, 60*, 133–149.

Sun, S.-S., Chen, X.-M., & Guo, S.-X. (2014). Analysis of endophytic fungi in roots of *Santalum album* Linn. and its host plant *Kuhnia rosmarinifolia* Vent. *Journal of Zhejiang University. Science, 15*, 109–115.

Talhinhas, P., Mota-Capitão, C., Martins, S., et al. (2011). Epidemiology, histopathology and aetiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. *Plant Pathology, 60*, 483–495.

Thomas, F. M., Blank, R., & Hartmann, G. (2002). Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. *Forest Pathology, 32*, 277–307.

Toju, H., Yamamoto, S., Sato, H., Tanabe, A. S., Gilbert, G. S., & Kadowaki, K. (2013). Community composition of root-associated fungi in a Quercus-dominated temperate forest: "codominance" of mycorrhizal and root-endophytic fungi. *Ecology and Evolution, 3*, 1281–1293.

Tomalak, M. (2017). Parasitic association of the mycetophagous wood nematode, *Bursaphelenchus fraudulentus* with the honey fungus *Armillaria ostoyae*. *Forest Pathology, 47*, e12325.

Tomlinson, I., Potter, C., & Bayliss, H. (2015). Managing tree pests and diseases in urban settings: The case of Oak Processionary Moth in London, 2006-2012. *Urban Forestry and Urban Greening, 14*, 286–292.

Tubby, K. V., & Webber, J. F. (2010). Pests and diseases threatening urban trees under a changing climate. *Forestry: An International Journal of Forest Research, 83*, 451–459.

Tyrväinen, L., Pauleit, S., Seeland, K., & De Vries, S. (2005). Benefits and Uses of Urban Forests and Trees. In C. Konijnendijk, K. Nilsson, T. Randrup, & J. Schipperijn (Eds.), *Urban Forests and Trees: A Reference Book* (pp. 81–114). Berlin, Heidelberg: Springer Berlin Heidelberg.

Vasiliauskas, R., Lygis, V., Thor, M., & Stenlid, J. (2004). Impact of biological (Rotstop) and chemical (urea) treatments on fungal community structure in freshly cut Picea abies stumps. *Biological Control, 31*, 405–413.

Vega, F. E., Posada, F., Aime, M. C., & Pava-Ripoll, M. (2008). Entomopathogenic fungal endophytes. *Biological Control, 46*, 72–82.

Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., & Strobel, G. (2007). The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (neem) from Varanasi (India). *Microbial Ecology, 54*, 119–125.

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nature Reviews Microbiology, 10*, 828–840.

Wainhouse, D., Cross, D. J., & Howell, R. S. (1990). The role of lignin as a defence against the spruce bark beetle *Dendroctonus micans*: effect on larvae and adults. *Oecologia, 85*, 257–265.

Webber, J. F., Parkinson, N. M., Rose, J., Stanford, H., Cook, R. T. A., & Elphinstone, J. G. (2008). Isolation and identification of *Pseudomonas syringae* pv. *aesculi* causing bleeding canker of horse chestnut in the UK. *Plant Pathology, 57*, 368.

West, J. S., & Fox, R. T. V. (2002). Stimulation of *Armillaria mellea* by phenolic fungicides. *Annals of Applied Biology, 140*, 291–295.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany, 52*, 487–511.

Willis, K. G., Garrod, G., Scarpa, R., et al. (2003). The Social and Environmental Benefits of Forests in Great Britain. *Social & Environmental Benefits of Forestry Phase, 2*, 36.

Wilson, H., Johnson, B. R., Bohannan, B., Pfeifer-Meister, L., Mueller, R., & Bridgham, S. D. (2016). Experimental warming decreases arbuscular mycorrhizal fungal colonization in prairie plants along a Mediterranean climate gradient. *Peer Journal, 4*, e2083–e208e.

Xiao, Q., & Mcpherson, E. G. (2002). Rainfall interception by Santa Monica's municipal urban forest. *Urban Ecosystems, 6*, 291–302.

Yaman, M., Erturk, O., & Aslan, I. (2010). Isolation of some pathogenic bacteria from the great spruce bark beetle, Dendroctonus micans and its specific predator, Rhizophagus grandis. *Folia Microbiol (Praha), 55*, 35–38.

Yang, A., & Juzwik, J. (2017). Use of nested and real-time PCR for the detection of *Ceratocystis fagacearum* in the sapwood of diseased Oak species in Minnesota. *Plant Disease, 101*, 480–486.

Zamioudis, C., & Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions, 25*, 139–150.

Zeneli, G., Krokene, P., Christiansen, E., Krekling, T., & Gershenzon, J. (2006). Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiology, 26*, 977–988.

Zhang, H. W., Song, Y. C., & Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural Product Reports, 23*, 753–771.

Zúñiga, A., Poupin, M. J., Donoso, R., et al. (2013). Quorum sensing and indole-3-acetic acid degradation play a role in colonization and plant growth promotion of Arabidopsis thaliana by Burkholderia phytofirmans PsJN. *Molecular Plant-Microbe Interactions, 26*, 546–553.

3. Preliminary Investigations into the Integrated Control of *Armillaria mellea* using biological controls and plant defence activators

Preface

This chapter of the thesis details the earliest work in the project: towards a combination of biological control agents and plant defence activators which could be used for the control of *A. mellea*. The bulk of this work was only replicated once and is therefore considered preliminary. However, it records a number of significant findings which may provide a foundation for future work. It is presented here as it was the earliest work chronologically and informed the work in the later chapters/papers. The work is presented in a more 'classical' thesis style, which was felt to be more appropriate due to the preliminary nature of the results and the numerous variations in techniques between the experiments as the project evolved.

This chapter is not intended for submission to a journal.

3.1 Introduction

As described in the General Introduction (1), a biological control for *Armillaria* root rot, especially that caused by *A. mellea*, is a desirable tool for its management, as cultural and chemical controls are lacking in multiple aspects. While *Trichoderma* fungi have been a focus of the majority of investigations into this area (Rees *et al.*, 2020), bacteria from the genera *Bacillus* and *Pseudomonas* have also shown promise in a number of studies (Dumas, 1992; Pellegrini *et al.*, 2013; Mesanza *et al.*, 2016). These include *P. protegens* Pf-5 (Pellegrini *et al.*, 2012), a strain widely recognized for its biological control capabilities. *Bacillus subtilis* QST713 is approved for soil application in the UK (Chemicals Regulation Directorate, 2021) and is used as a biological control agent (BCA) worldwide, therefore is worthy of further investigation. The strain has been re-classified as *B. velezensis* (Pandin *et al.*, 2018), but is referred to *B. subtilis* in this text due to wider historical usage.

Plant defence activators (PDAs) are another under-investigated area. Potassium phosphite has shown activity against *Armillaria* infections *in planta* and direct activity against the fungus *in vitro* (Heaton & Dullahide, 1990; Aguín *et al.*, 2006). Chitin is a widespread biopolymer which is strongly associated with plant pests and pathogens, being found in fungal cell walls, the exoskeletons of insects, and nematode eggs (Gortari & Hours, 2008). Mushroom compost, which is high in chitin, can potentially reduce *A. mellea* lesion size (Raziq & Fox, 2006a). Chitin derivatives are water soluble at neutral pHs which increases the ease of their application and reduces the risk of chemical burn on hosts (Kim & Rajapakse, 2005; Yin *et al.*, 2009). Chitin derivatives, e.g. chitosan, oligochitosan, can stimulate host defences as well as act directly to control fungal pathogens (Yang *et al.*, 2012). Another material suggested to stimulate plant immune systems is biochar (Zwart & Kim, 2012; Graber *et al.*, 2014), which is fundamentally charcoal applied to soil in the root zone. A multitude of factors influence biochar's composition and effects on plants and soil (Frenkel *et al.*, 2017; Al-Wabel *et al.*, 2018): they vary greatly by feedstock e.g. woody material, animal wastes, sewage sludge, with the end product further altered by pyrolysis conditions and treatment post-production such as composting. Biochars have recorded impacts on root diseases, prospectively via multiple mechanisms, e.g. improved rooting conditions for the host, the release of fungitoxic substances, absorption of phytotoxic substances, or benefits to native soil microbiota (Hailey & Percival, 2015; de Medeiros *et al.*, 2021).

Combinations of BCAs and/or PDAs have received little attention in *Armillaria* rot root control, and even alone they have apparently not yielded any commercialised controls for this express purpose. However, it is probable that antagonistic effects will be observed and may hinder control of *A. mellea* (Xu *et al.*, 2011; Simonetti *et al.*, 2015; Niu *et al.*, 2020).

Therefore the experiments detailed here attempt to identify potential BCA and PDA treatments for use against *A. mellea*, investigating the various interactions *in vitro*, followed by testing *in planta* or *in natura*. *In vitro* experiments may elucidate positive/negative interactions between various BCA and/or PDA treatment observed *in planta*. While results are expected to vary between *in vitro* and *in planta* experiments to due radically different environments in each impacting PDAs, BCAs, and hosts, *in vitro* trials are an accepted method of screening treatments prior to large scale trials. The individual experiments and their aims are outlined below.

3.1.1 Experiments & Aims

Heading	Experiment	Aim
3.3.1	*In vitro* inhibition of *A. mellea* CG440 growth by commercially available plant defence activator compounds	Selection of PDAs which could inhibit growth of *A. mellea*, and their sublethal dosages for later experiments.
3.3.2	*In vitro* inhibition of *A. mellea* CG440, CG447, & CG581 growth by effective doses of the plant defence activators potassium phosphite and oligochitosan and their combination	Assessing variation in *A. mellea* growth inhibition from the selected PDAs between strains of the species, as well as the effect of their combination.
3.3.3	*In vitro* inhibition of *A. mellea* CG440 by *Pseudomonas* bio-control strains	Assessing antagonism of CG440 by a range of well characterised *Pseudomonas* BCA bacteria strains for use in later experiments.
3.3.4	*In vitro* inhibition of *A. mellea* strains by combinations of BCA bacteria	Assessing variation in *A. mellea* growth inhibition from selected *Pseudomonas* & *Bacillus* BCA bacteria strains between strains of the fungus. Assessing if BCA strains with varied antagonism of the fungus individually can be combined to enhance growth inhibition.
3.3.5	Interactions between *Pseudomonas* bio-control strains and plant defence activators effective against *A. mellea* CG440	Assessing interactions between PDAs and BCA bacteria effective against *A. mellea*, in the absence of the fungus.
3.3.6	*In vitro* inhibition of *A. mellea* strain CG440 growth by effective doses of the plant defence activators potassium phosphite and oligochitosan, bacterial antagonists, and combination treatments	Assessing the interaction of PDAs and BCA bacteria in their antagonism of a strain of *A. mellea*.
3.3.7	*In vitro inhibition* of *A. mellea* CG440, CG447, & CG581 growth by a combination of potassium phosphite and *Bacillus subtilis* QST713.	Assessing the variation between *A. mellea* strains in their inhibition by a BCA & PDA combination that was effective on CG440.
3.3.8	Comparison of the *in vitro* effects of pure potassium phosphite product and 'Phusion' product with silicone-based surfactant/wetting agent on *A. mellea* CG440, *Pseudomonas protegens* Pf-5, *Bacillus subtilis* QST713, and interactions between each bacteria and the fungus.	Contextualizing the results of previous experiments using the phosphite product containing the silicone-based surfactant/wetting agent. Expanded replication of previous experiments.
3.3.9	*In vitro* growth inhibition of *A. mellea* CG440 by a range of concentrations of pure potassium phosphite.	Assessment of *in vitro* effect of pure aqueous potassium phosphite product prior to *in planta* testing.
3.3.10	*In planta* testing of potassium phosphite, *Bacillus* subtilis *QST713, and* Trichoderma atroviride T-15603.1 *combinations for* prevention *of* A. *mellea* CG440	*In planta* testing of treatment combinations with direct effects *in vitro* and combination with a fungal antagonist.
3.3.11	*In natura* testing of 'biochar' and chitin soil amendments as preventative treatments against *Armillaria* infection in a woodland	Assessment of biochar and chitin soil amendments against *Armillaria* infection in a natural infection context.
3.3.12	*In planta* testing of 'biochar' and chitin soil amendments as preventative treatments against *Armillaria* infection	Controlled replication of woodland experiment.

3.2 Materials & Methods

All experiments to date were undertaken using conventional aseptic technique: using sterile equipment, surfaces and media and working in sterile environments e.g. within laminar flow cabinets or in close proximity to lit Bunsen burners burning a blue flame.

3.2.1 *Armillaria mellea* Culture Maintenance & Incubation

3.2.1.1 Stock Cultures

Cultures were obtained from the Royal Horticultural Society Plant Pathology Laboratory (Table 1), as slants on a carrot-amended potato dextrose agar. Each year they were transferred to fresh slants on 1ml malt extract agar (MEA, Oxoid Ltd., Basingstoke, Hampshire, UK) in a 1.5ml Eppendorf tube. Slants were stored at 4°C.

Table 1. Details of *Armillaria mellea* cultures received from the Royal Horticultural Society Plant Pathology Laboratory.

RHS isolate	Genbank Accession	Geographic Location	Original Host	Date isolated
CG440	none	South East England, Surrey, UK	*Ligustrum*	22-Nov-06
CG447	KP288445	East Midlands, Northamptonshire, UK	*Hydrangea*	29-Nov-06
CG427	KP288444	East Midlands, Lincolnshire, UK	*Prunus*	09-Nov-06
CG333	KP288437	East Midlands, Lincolnshire, UK	*Salix babylonica* var. *pekinensis*	07-Apr-06
CG581	KP288448	South West England, Dorset, UK	*Forsythia*	23-Jul-07

3.2.1.2 Incubation & Culture Conditions

Material taken from slants was used to grow further cultures on MEA. These were 20ml MEA plates in 9cm Petri dishes. Inoculated plates were placed in an incubator at 21°C in darkness. Where possible an incubator contained within a 4°C cold store or climate controlled room was used to provide stabilized temperatures, exceptions will be noted. In light of the long incubation times for the fungus, plates were individually sealed with 'parafilm' after the various inoculations were completed, to reduce contamination risks and the spread of infection should any *Ascomycete* contamination occur. Each plate had a unique ID within each experiment.

3.2.1.3 Plug Inoculation

A uniform 6mm diameter agar plug was taken from the margin of an actively growing *Armillaria* colony on MEA (Fox *et al.*, 1991; Raziq, 1998), using a flame sterilised cork-borer or punch tool.

These were applied to agar plates either:

a) Mycelium down onto the surface.
b) Mycelium up into an identically sized hole in the agar.

3.2.1.4 Liquid Culture

Potato dextrose broth (PDB) (Neogen Europe Ltd., Auchincruive, Ayr, UK) was amended with 2.5mM sodium acetate (APDB) (Baumgartner *et al.*, 2010): solid sodium acetate was weighed and added prior to autoclaving the broth (holding at 121°C for 30 minutes). Aseptic handling of APDB was performed using large-volume sterile pipettes to avoid contamination risks inherent in pouring.

Homogenized mycelium was prepared by two different methods:

a) Mycelium from MEA slant cultures was homogenized by placing approximately 0.5g into approximately 5ml APDB in a 50ml conical based 'Falcon' tube with glass beads of 2-3mm diameter and shaking by hand until homogenized, approximately 1 minute. Twenty microliters of homogenized mycelium/APDB was then added to 10ml APDB in 40ml 'Falcon' tubes and incubated at 27°C, in low light, shaking at 200rpm, for 1 week. Incubated mycelium was then homogenized, as above, before use.
b) Fragments of mycelium under $1mm^2$ were collected from a young culture or a derived a long-term slant. Using a pipette tip, mycelium was transferred to a 50ml conical based 'Falcon' tube containing 10ml of APDB. The resultant culture was incubated in an orbital shaker at 27°C, 200rpm, in low light, for one week. This produced spherical colonies of mycelium, these were poured into a Petri dish and were handled with sterile needles or tweezers. Approximately 0.5g of the colonies were added to 1ml APDB in an MP Biomedicals 'lyzing matrix M' tube with a ¼ inch (6.35mm) ceramic bead. Mycelium was homogenized by shaking at $4m\ s^{-1}$ for 10 seconds in a FastPrep-24™ 5G lysis system (MP Biomedicals, USA).

Inoculation consisted of applying 20µL of this homogenized mycelium to agar plates (Pellegrini *et al.*, 2012), plates were then left with the agar facing upwards for a short time until the liquid media had soaked into the agar or dried, leaving a more uniform area of inoculum.

These liquid culture techniques appear to increase the uniformity and establishment of inoculated *Armillaria*. The plug techniques detailed above (3.2.1.3) often yielded repeats where the fungus did not establish to the same degree as the other plates.

3.2.2 Antagonistic *Pseudomonas* and *Bacillus* Culture Maintenance & Incubation

3.2.2.1 Overnight cultures (O/Ns)

Streak plates were made on King's Agar (KA) from frozen stocks and incubated for around 24 hours, until single colonies were visible. Pipette tips were used to sample single colonies and placed into 10ml of King's Broth (KB) in vials and incubated at 27°C in an orbital shaker at 200rpm for 12-18 hours to ensure exponential growth, with longer incubation until cell density was visually apparent if required. A separate overnight was used for each repeat in a treatment.

3.2.2.2 Stock Cultures

Stock cultures were produced by making O/Ns (3.2.2.1), diluting and mixing these with equal parts 40% glycerol solution in cryotubes. These stock cultures were then stored at -80°C, kept on ice during handling and returned to storage as soon as possible. Initial cultures were sourced from the collections of the Molecular Microbiology and Plant Pathology group at The University of Reading, Whiteknights Campus, Knight Building, Reading, Berkshire, RG6 6AR, UK.

3.2.2.3 Adjusted cultures

Optical Density at 600nm wavelength (OD_{600}) of cultures was measured using a spectrophotometer and adjusted to the desired figure by pelleting the appropriate volume, calculation below, by centrifugation (5000rpm, 5 minutes), removing the supernatant then re-suspending cells in 1ml of fresh KB by pipetting up and down gently a few times. Combined cultures were made by mixing equal parts of these adjusted cultures.

Volume to use (ml) = desired OD ÷ current OD

3.2.3 Digital measurement of *Armillaria* growth

Digital images of the bases of petri-dishes were taken on a black background, either from a high-quality flat-bed scanner (HP Scanjet G4010) at ≥300dpi resolution or a DSLR camera (Canon EOS 650D) at 5184x3456 pixels resolution, alongside a $1mm^2$ grid scale mounted in an identical petri-dish. Growth diameter was measured along four axis, approximately 45° from each other and was, as for any other linear measurements, taken in millimetres using the straight line and measurement tools in in 'ImageJ' software (National Institutes of Health, USA), and adjusting the scale for each separate image. Area was also measured in this program by setting the scale, changing image type to 8-bit,

adjusting thresholds and filling to highlight the fungus, then encircling via freehand selection and measuring. Rhizomorphs made up most of the growth diameter and mycelial growth was hard to discern from them: the rhizomorphs spread densely and moved in and out of the agar haphazardly, with mycelium spreading from the protuberances and merging with that spreading out from the plug, this can be seen in Plate 1.

Generally *Armillaria* growth was only just visibly moving onto the agar from the agar plug or initial area of inoculation at 7 days from inoculation (3.2.1), so measurements were taken after this point.

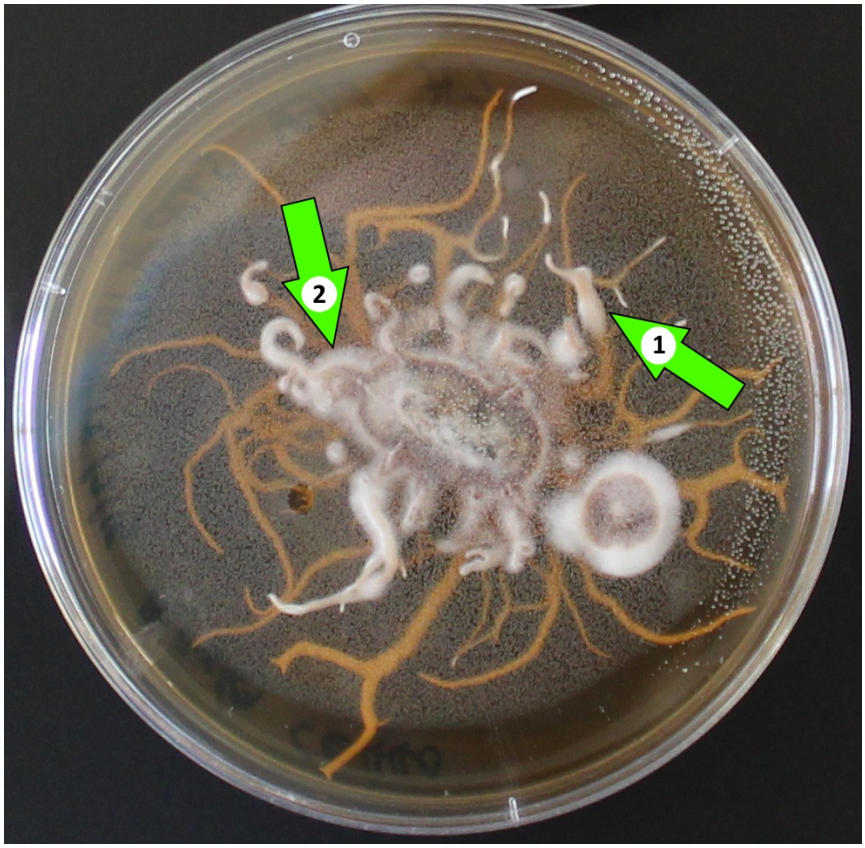

Plate 1. *Armillaria mellea* rhizomorph and mycelial spread through agar. Arrow 1 = Rhizomorphs move out of agar and mycelium spreads from them. Arrow 2 = Mycelium spreading from rhizomorph merges into original mycelial spread.

3.2.4 Extraction of *Bacillus subtilis* QST713 from 'Serenade' commercial product

Serenade (Serenade ASO, Bayer CropScience Ltd, Cambridge, UK) is a bio-fungicide product containing *Bacillus subtilis* QST713 as its active ingredient and marketed for use against foliar and root diseases. The commercial product was sampled aseptically and used to produce O/Ns, which were used to make a 1:10 dilution series (100µl in 900µl KB media). This was then spread as a lawn onto King's Agar plates and the dishes incubated at 27°C overnight, with the 10^{-4} and 10^{-5} plates producing significant numbers of isolated single colonies. Colony morphology was visually confirmed to be *Bacillus* and four of these single colonies used to produce O/Ns, which were used to produce

frozen stocks and prepared for sequencing. Other authors have extracted the strain from the product in similar ways (Lahlali *et al.*, 2012; Meng *et al.*, 2016; Rotolo *et al.*, 2016).

The 16S RNA region was sequenced from each isolate. DNA was extracted with a Qiagen QIAamp DNA Micro Kit and amplified using 8F primers (James, 2010), then run on a gel and visualized to check for amplification. Once this was confirmed, it was purified using a Qiagen QIAprep Spin Miniprep Kit and sent for sequencing by Source Bioscience (Nottingham, UK). Sequences received were then run through a megaBLAST search (National Center for Biotechnology Information, Bethesda MD, USA; https://blast.ncbi.nlm.nih.gov/). All matched strongly (99.75-100%) with *Bacillus* strains from the appropriate group (Fan *et al.*, 2017). Sequences were aligned, using megaBLAST, with a published 16S region from the strain (Genbank Accession CP025079, region 9761-11313) (Pandin *et al.*, 2018) and all strongly aligned (99.6-99.8%) with it.

3.2.5 Sterile stock solutions

Stock solutions of 10g AI L^{-1} of plant defence activators were prepared by adding 400mg of either chitosan hydrochloride (Degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China) or oligochitosan (molecular weight 2300 & 3740, G.T.C. Bio Corporation) or 440mg of potassium phosphite and silicon solution (Phusion, Orion Future Technologies Ltd., Kent, UK: 80-90% volume potassium phosphite, 3% polyether modified trisiloxane) to 40ml nanopure water. This was then sterile filtered through Millex Millipore filters into a sterile 50ml 'Falcon' tube, using a sterile syringe, inside of a laminar flow cabinet. It is acknowledged that the variable concentration given for 'Phusion' means that is likely that the actual concentration of potassium phosphite was lower than 10g AI L^{-1} and is henceforth referred to as an estimate.

3.2.6 Amended Medias

Concentrated malt extract agar (MEA, Oxoid), was made by using only 90% of water in the standard recipe. This was melted, then cooled and held at 55°C using a thermostatically controlled water bath. The still liquid agar was then amended with stock solutions of plant defence activators, neat products, and/or sterile nanopure water to give variously amended growths media of desired concentrations. Aside from one experiment, potassium phosphite products, 'Phusion' and 'PolyPhosphite 30' (56% w/w, 838.89 g/L, aqueous potassium phosphite, Plant Food Company Inc., Cranbury, NJ 08512, USA), were added directly to the media with no resultant contamination.

3.2.7 Antagonism Assays

Plates were inoculated with *Armillaria* at approximately 2cm from the edge of each dish (Pellegrini *et al.*, 2013), which was allowed to establish for 7 days prior to antagonist inoculation (Fox *et al.*, 1991).

Bacterial cultures adjusted to OD_{600} = 0.5 were made as per 3.2.2. Two microliters of culture were applied either directly to the agar and allowed to dry, or to a sterile filter paper disk of 5mm circumference that had previously been placed opposite the fungus on the agar. This point of bacterial inoculation was also approximately 2cm in from the edge of each dish. Sterile bacterial culture media was used as a control. Plates were then incubated and monitored over time.

3.2.8 Plate Reader Interaction Tests

a) A concentration gradient was set up in a 96-well plate by directly amending KB to 1000ppm 'Phusion' (equalling an estimated 800-900ppm potassium phosphite, 31ppm polyether modified trisiloxane, Orion Future Technologies Ltd.) or oligochitosan stock solution to 1000mg/L and filling the first row of wells with 135µl of the resultant media, using a multichannel pipette. The rest of the wells were filled with 135µl unamended media. Fifteen microliters of the original stock solution were added to the KB in the second row of wells and mixed by pipetting up and down 10 times. 15µl of this amended media was taken and used in a sequential dilution repeating the same actions down the plate, stopping at the penultimate row of wells, where the excess 15µl was discarded after mixing. The final row of wells was left un-amended as a control.

   Adjusted antagonist cultures of OD_{600} = 0.5 were made as per 3.2.2. Wells of the prepared plate were then inoculated with 5µl of culture, each column using a different O/N culture, and 5µl of un-amended KB added to the wells of final column as a non-inoculated control. The plate was then placed into a microplate reader (Tecan) at 28°C, measuring OD_{600} every 20 minutes with 20 seconds of orbital shaking before readings, for at least 22 hours.

b) The columns of a 96 well-plate were alternately filled with 135µl KB, KB amended to 1000ppm 'Phusion', or with 1000ppm 'PolyPhosphite 30'. Adjusted antagonist cultures of OD_{600} = 0.5 were made as per 3.2.2. Wells of the prepared plate were then inoculated with 5µl of culture, each column using a different O/N culture, and 5µl of un-amended KB added to the wells of final column as a non-inoculated control. The top three rows of the plate were inoculated with Pf-5, the fourth with KB as a control, and the last 4 rows with QST713. The plate was then placed into a microplate reader (Tecan) at 28°C, measuring OD_{600} every 20 minutes with 20 seconds of orbital shaking before readings, for at least 22 hours.

3.2.9 *In Planta* Pot Trials

3.2.9.1 Plant Material, Growing Conditions, Experimental Design

Pot trials took place in a protected polytunnel at the R.A. Bartlett Tree Research Laboratory, Cutbush Lane East, Shinfield, Reading, Berkshire, UK.

Plants were purchased as cell grown plants, 20-40cm high from Alba Trees, East Lothian, UK. The original compost was kept moist and plants were planted into 5L pots of a 50:50 mix of a peat-based Multi-Purpose Compost (Clover Peat, Dungannon, Co. Tyrone, Northern Ireland) and top soil. Soil amendments were added at this stage if appropriate, being well mixed into the soil. The roots were gently teased apart prior to planting to promote rooting. All plants were potted within a week of reception, by the 19/09/2017. Plants were placed on automated irrigation, with the potting medium kept moist throughout the year.

Each experiment was split into four blocks, spread north-west to south-east. Treatments were completely randomized within blocks but with the same numbers of plants under each treatment within each block. All plants were given unique identification numbers using tree tags secured on loose loops of wire around the base of the stem.

3.2.9.2 Inoculum and Inoculation

Inoculum was created from acorns of *Quercus robur* (Beckman & Pusey, 2001), which had been pierced once with a 1mm needle. These were then autoclaved twice in water, holding at 121°C for 45 minutes. Resultant intact acorns were then arranged in a single layer (horizontally) in polypropylene 'takeaway' tubs of 170mm x 120mm x 50mm, and nearly covered with molten autoclaved MEA. The lids were then put on the tubs loosely before the final autoclaving, holding at 121°C for 30 minutes. The tubs were then closed and the agar allowed to solidify prior to inoculation. The MEA-acorn matrix was inoculated by applying 0.5ml of *A. mellea* CG440 homogenized mycelium (3.2.1.4b), alongside 4.5ml of APDB as a carrier, prepared as described above. The tub was then closed and gently tilted back and forth in multiple directions, for approximately 10 seconds, to mix and spread the homogenized mycelium. Control acorns were prepared with 5ml APDB. All tubs were sealed with parafilm and incubated in the dark at 21°C for 28-31 days. Tubs were checked weekly for visual signs of contamination, such as bacterial colonies or uncharacteristic fungal growth, and any contaminated tubs removed. No control tubs were contaminated.

To inoculate plants, a single prepared acorn was placed into a slit made into the soil and roots, within 5cm of the root collar and 10cm deep. Uninfected control treatments received the non-inoculated acorns.

3.2.9.3 Monitoring

Plants were monitored monthly for mortality in the growing season and any dead plants dissected to check for mycelial fans of *Armillaria*. At the conclusion of the experiment, all plants were dissected in this manner.

3.2.10 *In natura* testing of 'biochar' and chitin soil amendments as preventative treatments against *Armillaria* infection in a woodland

The experiment took place in Sandhurst Copse & Sheepwalk Forest, located west of Farley Heath Road, Albury, Guildford, UK. The site is a woodland spread across located on large slopes. The soils have a thin organic horizon of around 10cm depth with almost pure sand below.

Two areas of this woodland had shown symptoms of infection with *Armillaria*. 'Site 1' was an area with six silver birch stumps, *Betula pendula* Roth, which have become infected by *Armillaria*. Scots pine, *Pinus sylvestris* L., planted in this area (c. 6 years old) were observed being attacked by the fungus, causing canopy dieback and mortality with growth of fruiting bodies at the root collars. 'Site 2' was another area with ten infected silver birch stumps. Here, reportedly, bracken was cleared from around self-sown alder buckthorn, *Frangula alnus* Mill., saplings and following this, large amounts of *Armillaria* fruiting bodies grew in the area. Despite this there have been no recorded tree mortalities to the fungus in this area so far. In neither area were the felled silver birch known to have *Armillaria* infections, suggesting invasion of the dead or dying wood. Fruiting bodies found during the course of the experiment match the morphology of *A. ostoyae* (Romagnesi) Herink (Kibby, 2021). This is an aggressively pathogenic species of *Armillaria*, like *A. mellea* (Heinzelmann *et al.*, 2019).

Both sites were mown 2-3 times a year to maintain clear ground and suppress bracken growth. Each area was located in a clearing and had space available for new planting. Prior research has indicated that mortality is concentrated around infected stumps, although not necessarily directly correlated to distance from them (van der Pas, 1981).

Soils were amended on the 23rd of May 2017. Seventy-two individual planting plots were made in each area with stumps throughout. Planting plots were set out through each area and uniquely numbered with metal tree tags on small softwood batons as they were selected. A spacing of approximately 90cm was left between each, as well as any other obstacles such as stumps or small trees already present, to allow mowing. In each planting plot, an approximately 4 litre hole was dug to approximately 20cm deep using a posthole spade. During this step, many *Armillaria* infected roots were found. Turf, large stones, root material, and bracken rhizomes were removed from the excavated soil. The remaining soil, approximately 1 litre, was amended with 3 litres of a commercial pest-based compost and either 4g of chitin (approximately 1g^{-1} L soil, Tidal Vision, Alaska, USA), 200ml volume of biochar equalling approximately 5% soil volume, which has shown promise for other pathogens (Zwart & Kim, 2012), a combination of the two, or left un-amended as a control. The biochar was a British softwood & hardwood mix with an approximately 2-10mm^3 particle size, supplied by Carbon Gold, Bristol, UK. The feedstock was a mix of woodchip from UK grown *Quercus*

robur L., *Q. palustris* Münchh., *Fagus sylvatica* L., *Fraxinus excelsior* L., and *Pinus sylvestris*. During pyrolysis the highest treatment temperature was 350°C. Nutrient breakdown of final biochar in mg/kg: <10 nitrate N, <10 ammonium N,47 P, 541 K, 77 Mg, 1 Cu, 12 Zn, 29 S, 734 Ca, 124 Na. pH 5.11. It had previously been pre-soaked in tap water for 48 hours and drained. Amendments were mixed into the soil with a hand fork until distributed homogenously, then firmly packed into the hole. Each site had 18 repeats (36 over both sites) of each of the 4 treatments, which were allocated to planting sites via their numbers using a randomized list and giving a completely randomized design in each area.

The planting material selected was Scots pine, due to the prior mortality of the species from *Armillaria* on the site. Younger trees, especially conifers, generally appear to have higher susceptibility and less resilience to infection (Hood *et al.*, 1991): therefore, trees were germinated at the start of May 2017 from commercially sourced seed (RPseeds, Lancashire, United Kingdom) in a peat-based potting compost amended with sand, following two weeks at 4°C in a moistened small amount of this growing medium. Planting was postponed until a significant period of rain allowed planting of the young trees without the need for continued watering: the 3rd of October 2017. Trees were protected from animal damage by small plastic guards, with larger metal wire guards added as they grew.

Plant height was measured to the nearest 0.5cm from the base of the stem using a measuring tape. Mortality was judged visually and by checking for green cambium below the bark, it was coded as a binary with 1 for dead and 0 for live. Measurements were taken on days 0, 238, 345, 598, and 996 following planting.

3.2.11 Statistical analysis

For all tests, a significance level of 5% (α=0.05) was used. Unless otherwise stated, statistical analysis was conducted in the R statistical programming language (R Core Team, 2020). First, an appropriate linear model was applied i.e. a linear model for non-longitudinal data and a linear mixed model for longitudinal data of more than two time points. Growth measurements were typically log transformed as appropriate. *Armillaria* and bacterial strains and time were used as fixed factors. Interactions were used between all factors. Subject, typically a single colony or plant, was used as a random factor, nested within block within experimental replicate where appropriate. In factorial designs, variables were coded as binary factors. Analysis of variance testing (ANOVA) was then conducted on the linear model as an omnibus test. This was followed by pairwise testing using Tukey's Honest Significant Difference Test (Tukey's test) to elucidate differences between treatments. All estimates are back-transformed to the arithmetic scale where appropriate. For continuous variable datasets without time points, linear models ('lm' function) were fitted to dependent variables. Linear mixed models ('lme' function, 'nlme' package) were fitted to longitudinal data. Marginal and conditional R2 were calculated from models where appropriate ('r.squaredGLMM' function, 'MuMIn' package). ANOVA tables were calculated from the models as an omnibus test ('Anova' function, 'car' package). Tukey's test was performed, treatment means and/or trends were estimated ('emmeans' and 'emtrends' functions, 'emmeans' package), and also separated ('cld', 'multcomp' package).

Graphs were created in the 'ggplot2' package or with built-in graphics functions.

3.3 Results

In this section, each experiment is described under its own heading, firstly the methodology referring to the Materials and Methods section (3.2), followed by the results and a focussed discussion.

3.3.1 *In vitro* inhibition of *A. mellea* CG440 growth by commercially available plant defence activator compounds

Aside from their plant mediated effects, a number of plant defence activator (PDA) compounds, i.e. chitin derivatives, phosphite salts, show direct activity against phytopathogens (Heaton & Dullahide, 1990; Xu *et al.*, 2007; El Hadrami *et al.*, 2010). As such, a primary investigation is to measure responses in *Armillaria mellea* growth to varying concentrations of each *in vitro*. This experiment tested *in vitro* growth inhibition of a single *A. mellea* strain, CG440, by available forms of chitosan hydrochloride, oligochitosan, and potassium phosphite. Fungistatic effects of potassium phosphite have been demonstrated on one isolate of *A. mellea* (Aguín *et al.*, 2006). Strain CG440 is in use by contemporary UK researchers with potential to become a model strain (Beal *et al.*, 2015; Ford *et al.*, 2015, 2017; Rees *et al.*, 2020). The aims were to compare the activity of the different compounds in inhibiting the pathogen's growth, as well as to find sub-lethal dosages to be used in further experiments addressing combination treatments.

Specific Methodology

Armillaria mellea strains: CG440

Workflow:

- Sterile stock solutions (3.2.5)
- Amended Medias: 0, 1, 10, 100, 1000 mg/L of Chitosan, Oligochitosan, & Potassium Phosphite [as 'Phusion', approximate concentrations] (3.2.6)
- Plug inoculation at centre of dish (3.2.1.3a)
- Typical incubation (3.2.1.2)
- Digital images at days 14 & 21, Camera (3.2.3)
- Measure: Mean Growth Diameter
- ANOVA & Tukey's test, per PDA (3.2.11), dosage as categorical factor.

Repeats: 4

Effective sub-lethal dosages of two plant defence activators were found, of potassium phosphite and oligochitosan, both at 1000mg/L (Table 2). Potassium phosphite had an effect at both time points, while oligochitosan only significantly inhibited growth at 21 days after inoculation. Chitosan had an impact at day 14 but this was not sustained, suggesting that *A. mellea* CG440 can

detoxify/metabolize the compound between 14 & 21 days. Inhibition by potassium phosphite (65.2% inhibition, day 21) was far stronger than oligochitosan (9.0% inhibition, day 21) (Figure 1). This indicates potassium phosphite will be a stronger control of *Armillaria* if applied *in planta* in terms of direct effects. The effective dosage of both potassium phosphite and oligochitosan for reducing *A. mellea* CG440 growth *in vitro* is somewhere between 0.1-1mg/L.

The next approach will be to test compounds & doses which actively inhibit growth of *A. mellea* CG440 against multiple strains of the pathogen and also in combination to detect additive/synergistic effects.

Table 2. Direct effect of various plant defence activators (PDAs) on *Armillaria mellea* CG440 mean growth diameter (mm) at a logarithmic range of concentrations, 14 & 21 days after inoculation.

PDA	**Chitosan HCl**		**Oligochitosan**		**KPhi**	
Day	**14**	**21**	**14**	**21**	**14**	**21**
0 mg/L	60.6ab (44.0, 77.1)	87.6a (80.5, 94.7)	59.2a (39.5, 79.0)	86.7a (84.2, 89.2)	38.4ab (23.84, 53.0)	83.6a (79.5, 87.6)
1 mg/L	38.7a (24.4, 53.0)	84.3a (78.1, 90.4)	50.1a (30.4, 69.9)	86.9a (84.4, 89.4)	39.5ab (24.86, 54.1)	81.6a (77.6, 85.6)
10 mg/L	51.3ab (36.9, 65.6)	85.2a (79.1, 91.3)	44.1a (24.3, 63.8)	85.9a (83.4, 88.4)	56.7a (42.07, 71.3)	86.5a (82.5, 90.6)
100 mg/L	43.5ab (29.1, 57.8)	86.0a (79.8, 92.1)	38.0a (18.2, 57.7)	85.1a (82.6, 87.6)	50.6ab (33.77, 67.5)	82.0a (77.3, 86.6)
1000 mg/L	68.2b (53.9, 82.6)	87.9a (81.8, 94.0)	50.7a (27.9, 73.5)	78.4b (75.5, 81.2)	18.6b (1.73, 35.4)	29.1b (24.5, 33.8)
ANOVA p-value	0.047	0.887	0.58	0.002	0.029	<0.001
Tukey significant p-value/s	0.049	-	-	0.006-0.015	0.019	<0.001
adjusted R^2	0.327	-0.191	-0.061	0.589	0.400	0.969
***F*-statistic**	3.183	0.278	0.740	7.439	3.832	133.7
Tukey significant t-ratios	-3.124	-	-	3.781-4.789	3.689	17.330-20.139

Estimated marginal means with 95% confidence intervals in brackets. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Grown on amended malt extract agar. *n*=4.

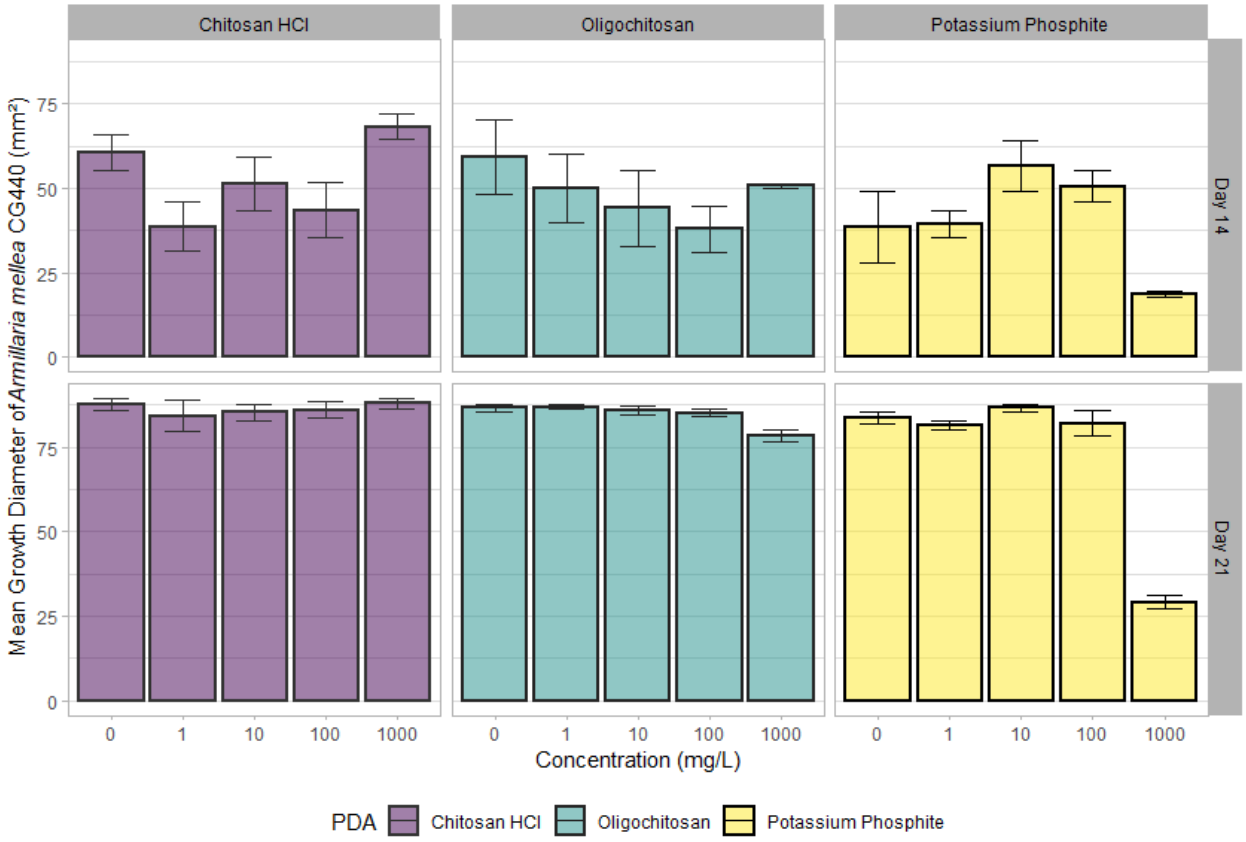

Figure 1. Direct effect of various plant defence activators (PDAs) on *Armillaria mellea* CG440 mean growth diameter (mm) at a logarithmic range of concentrations, 14 & 21 days after inoculation.
Estimated marginal means, small bars represent standard error range. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Grown on amended malt extract agar. *n*=4.

3.3.2 *In vitro* inhibition of *A. mellea* CG440, CG447, & CG581 growth by effective doses of the plant defence activators potassium phosphite and oligochitosan and their combination

Armillaria strains have shown varying sensitivity *in vitro* to antifungal compounds such as allicin and culture filtrates from potential antagonists (Szwajkowska-Michalek *et al.*, 2012; Beal *et al.*, 2015). Many other studies on antifungals examine effects on just a single strain of *Armillaria* (West & Fox, 2002; Raziq & Fox, 2006b,a; Aguín *et al.*, 2006). These studies include those on potassium phosphite, which reduced the growth of strain CG440 in a prior experiment (3.3.1), and the related compound 'Fostyl-Al' (1.11.2).

This experiment aimed to study the activity of doses of potassium phosphite and oligochitosan similar to those found active against *A. mellea* strain CG440 (3.3.1), and their combination, against three other strains. To compare and contrast inhibition and growth rates of all three strains, as well as potential interactions between the two. This was to give insight into variability in susceptibility and resistance of *Armillaria mellea* strains to these PDAs, and a component of the variability one may expect from applying the compounds against *Armillaria mellea* in the field.

Liquid culture techniques (3.2.1.4), were used in an attempt to increase uniformity of inoculations in comparison to the typical 'plug' method (3.2.1.3), where establishment of the fungus was variable. Semi-automated measurement of area (3.2.3) was used to simplify the measurement process and reduce the chance of unconscious bias during measurement.

Specific Methodology

Armillaria mellea strains: CG440, CG447, CG581

Workflow:

- Sterile stock solution, Oligochitosan (3.2.5)
- Amended Medias: Control, 800-900ppm [estimated] Potassium Phosphite, 1000mg/L Oligochitosan, combination (3.2.6)
- Liquid inoculation at centre of dish (3.2.1.4a)
- Typical incubation (3.2.1.2)
- Digital images at days 10, 12, & 14, Scanner (3.2.3)
- Measure: Mean Growth Area, using thresholding method
- ANOVA & Tukey's, by PDA and *Armillaria* strain (3.2.11), factorial design with PDAs coded as binaries, growth area log transformed.

Repeats: 8

There was a significant interaction between time, presence of each PDA, and *Armillaria* strain (ANOVA P=0.032, Wald X^2 = 6.856, marginal R^2 = 0.852, conditional R^2 = 0.968). The different PDA treatments yielded significant differences within each strain and timepoint (P<0.001-0.029, Table 3).

In general, the oligochitosan and combination treatments produced earlier impacts on *Armillaria* growth (Table 3), while the phosphite and combination treatments gave the largest reductions in growth by day 14. *A. mellea* CG581 overcame the impact of oligochitosan alone by day 14. Given the small numbers of strains tested in this experiment, the results found could imply that this resistance is relatively common in strains of *A. mellea*. Though, it is worth noting that this may only be true for this specific form of oligochitosan: the impact of other forms, of differing degree of acetylation and molecular weight, may vary (Kim & Rajapakse, 2005; Yin *et al.*, 2010).

Table 3. Mean growth area (mm^2) and growth trend of selected *Armillaria mellea* strains grown on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 1000mg/L oligochitosan (OC) or a combination, 10, 12 & 14 days after inoculation.

Strain	Treatment	Growth Area (mm^2), Day 10	Growth Area (mm^2), Day 12	Growth Area (mm^2), Day 14	Trend/Growth Rate
CG440	**Control**	346a (300, 400)	620a (542, 710)	1111a (963, 1281)	0.291a (0.268, 0.315)
	OC	247b (214, 285)	421b (368, 482)	716b (621, 826)	0.266a (0.242, 0.290)
	KPhi	368a (319, 424)	424b (370, 485)	489c (424, 564)	0.071b (0.048, 0.095)
	OC+KPhi	297ab (258, 343)	353b (308, 404)	418c (363, 483)	0.085b (0.062, 0.109)
CG447	**Control**	346a (300, 399)	543a (474, 621)	852a (739, 983)	0.226a (0.202, 0.249)
	OC	259b (224, 298)	412b (360, 471)	655ab (568, 756)	0.232a (0.209, 0.256)
	KPhi	383a (332, 442)	471ab (411, 539)	579bc (502, 667)	0.103b (0.080, 0.127)
	OC+KPhi	322ab (279, 371)	386b (337, 441)	462c (401, 534)	0.091b (0.067, 0.114)
CG581	**Control**	375a (325, 433)	627a (548, 717)	1047a (908, 1208)	0.257b (0.233, 0.280)
	OC	316ab (274, 365)	599ab (523, 685)	1134a (983, 1308)	0.319a (0.296, 0.343)
	KPhi	393a (340, 453)	473b (414, 542)	571b (495, 658)	0.094c (0.070, 0.117)
	OC+KPhi	268b (233, 310)	386c (292, 382)	416c (361, 480)	0.110c (0.086, 0.133)

Marginal R^2 = 0.852, conditional R^2 = 0.968, ANOVA Day:OC:KPhi:Strain effect P=0.032. Tukey significant p-values of means by strain and day <0.001-0.029, significant t ratios = -3.803 to -6.826 & +2.886 to +9.972. Tukey significant p-values of trends by strain <0.002, significant t-ratios = -3.731 to -13.435 & +7.287 to +13.107. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Letters indicate grouping of means, 95% confidence interval in brackets. n=8.

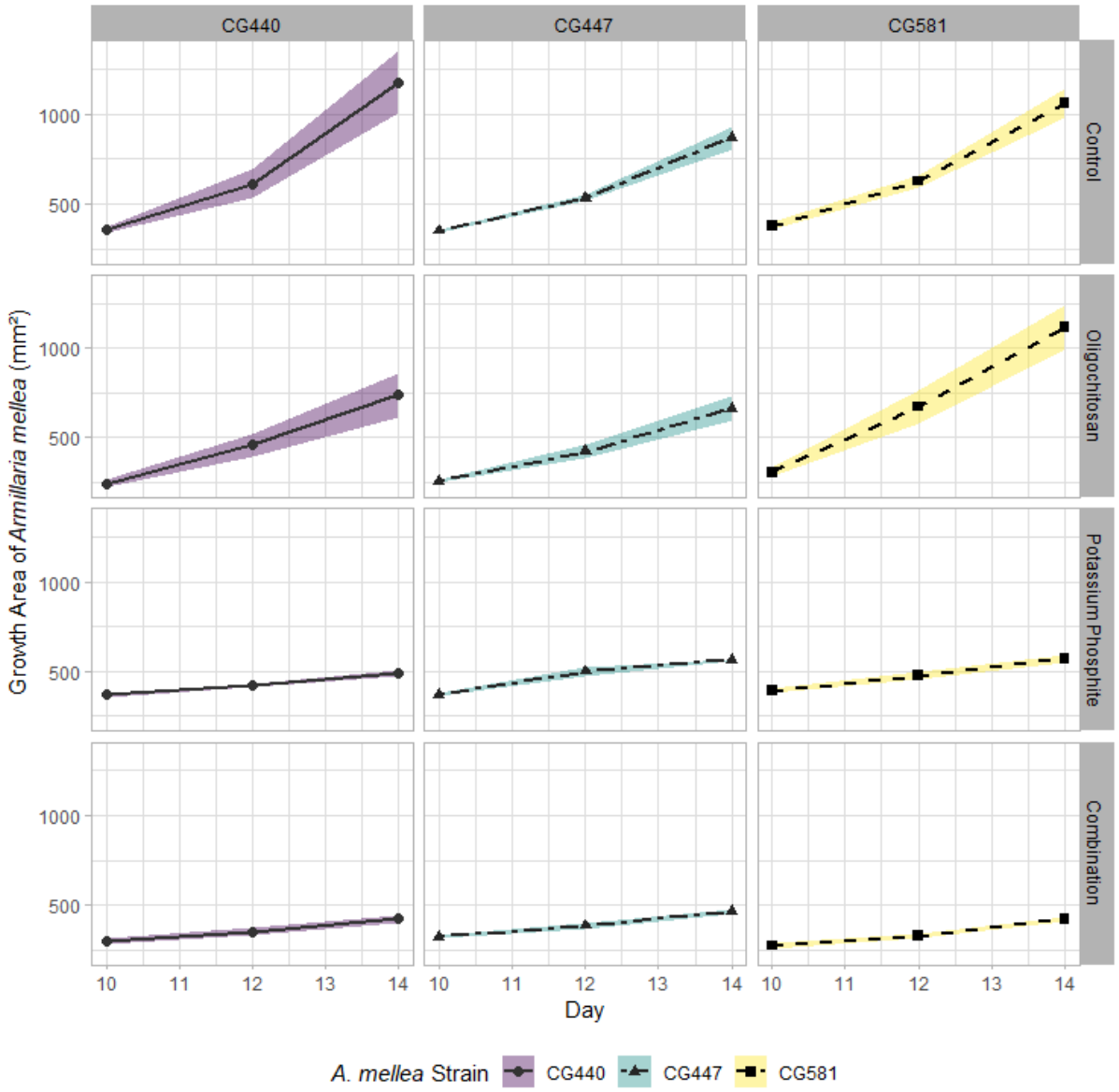

Figure 2. Mean growth area of selected *Armillaria mellea* strains (mm^2), days 10-14, on malt extract agar amended with 800-900ppm [estimated] potassium phosphite, 1000mg/L oligochitosan or a combination of the two. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Ribbons represent standard error ranges. *n*=8.

Corresponding with the previous experiment (3.3.1), potassium phosphite caused greater reductions in the growth area of these strains (31-56%, oligochitosan 8-36%) and growth rate than oligochitosan (Table 3). The combination treatment mean separated significantly from that of potassium phosphite in *A. mellea* strains CG447 and CG581, within an increase of 14-15% growth inhibition at day 14, but not CG440. CG440 may be more sensitive to potassium phosphite than the other strains. There is a

general benefit of the combination treatment over potassium phosphite alone. Plant mediated effects or interactions with antagonists could increase this effect *in planta*.

Differences in the growth rates of each strain between PDA treatments were significant ($P<0.002$). All strains had significantly slower growth rates (2-3 fold reduction) in the presence of an estimated 800-900ppm potassium phosphite (Table 3). The combination treatment was not significantly different from potassium phosphite alone. Oligochitosan alone significantly increased the growth rate over the control, by around 25% (Figure 2, Table 3).

The use of liquid culture and area measurements appear to be comparable with prior techniques of plug inoculation and linear measurements of growth diameter, making them appropriate for use in following experiments. Each has potential advantages: liquid culture and inoculation appears to increase the uniformity of *Armillaria* growth and as such the statistical power of experiments. Due to being semi-automated and measuring the whole colony rather than multiple diameters, measuring fungal growth area using ImageJ may also increase the replicability of measurements and reduce the chance of unconscious bias. The new techniques proved acceptable for use in further experiments.

The next experimental concern was to investigate combinations and interactions of these plant defence activator compounds and dosages with biological antagonists of *Armillaria mellea*, as well as their plant mediated effects against the pathogen by applying them *in vivo*.

3.3.3 *In vitro* inhibition of *A. mellea* CG440 by *Pseudomonas* bio-control strains

As described previously (General Introduction, 1) there is a lack of investigation into antagonism of *Armillaria* by well characterised strains of *Pseudomonas* despite a theorised main role in natural suppression of the disease by some authors. This experiment set out to challenge *Armillaria mellea* strain CG440 with a variety of known bio-control strains of *Pseudomonas*. Only *P. protegens* Pf-5 is well characterised with previously recorded activity against *A. mellea* (Pellegrini *et al.*, 2012). The production of known antifungal metabolites varies between strains and species of *Pseudomonas* (Calderón *et al.*, 2015) and the sensitivity of *A. mellea* to each is likely to differ. Therefore, classic dual culture experiments were performed to gain insight into the antagonistic potential of these strains.

Specific Methodology

Armillaria mellea strains: CG440

Antagonists: Seven strains from two species were used: *P. protegens* Pf-5; *P. fluorescens* strain ATCC17400, F113, Pf-01, SBW25; plus a novel strain of *P. fluorescens* that is a pathogen of aphids.

Workflow:

- Antagonism Assay, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4a, 3.2.2, & 3.2.7)
- Digital images at days 0, 4, 8, 11, & 13 after bacterial inoculation, Scanner (3.2.3)
- Measure: Mean Growth Area of pathogen & antagonist colonies (thresholding method) & Mean radial growth towards antagonist
- ANOVA & Tukey's test (3.2.11), growth areas log transformed.

Repeats: 5

Pseudomonas strain had a significant effect on the growth area of *A. mellea* CG440 over time (Figure 3, ANOVA *Pseudomonas* strain & day interaction $P<0.001$, Wald $X^2 = 31.037$, marginal $R^2 = 0.958$, conditional $R^2 = 0.963$). Only *P. protegens* Pf-5 caused a significant reduction in CG440 growth area in comparison to the control of 43% (Tukey $P<0.020$, significant t-ratios -3.701 to -4.454 & +3.918 to +5.636, Table 4). Although some of the other strains appeared to be diverging from the control, i.e. F113 and the aphid pathogen strain.

Also, in terms of CG440 growth towards the bacteria, only Pf-5 caused a significant reduction in comparison to the control here as well from day 8 onwards (ANOVA *Pseudomonas* strain & day interaction $P=<0.001$, Wald $X^2 = 49.151$, marginal $R^2 = 0.892$, conditional $R^2 = 0.893$. Tukey day 8-13 $P<0.001$, significant t-ratios = -5.781 to -6.836 & +5.413 to +8.664). At day 13 this was 58% lower than the control (control mean 47.98a, CI 41.11, 56.01 & Pf-5 mean 20.29, CI 17.38, 23.69).

The impact of Pf-5 on the whole of the pathogen's growth, not just the radius facing the colony, and its earlier occurrence, suggest that Pf-5 produces an antifungal which is either unique amongst the tested strains or produced in a higher volume. This antifungal may be more effective, of a smaller molecular weight than antifungals produced by the other strains therefore diffusing through the agar faster, or be volatile, leading to its earlier impact.

Only *P. fluorescens* F113 and *P. protegens* Pf-5 were observed to have distinct zones of inhibition at the final measurement. However this was not as consistent for Pf-5, while it was not significant for F113 and this strain did not inhibit the overall growth of CG440. It is known that these strains each produce differing ranges of antimicrobials, overlapping in the production of certain compounds and not for others (Calderón *et al.*, 2015). This could mean it is a specific mix of antimicrobials that Pf-5 produces which gives it high *in vitro* antagonism of CG440, rather than a single compound.

Table 4. Mean growth area (mm^2) of *Armillaria mellea* CG440 confronted with different *Pseudomonas* bio-control strains 0 to 13 days after antagonist inoculation.

Strain	Day 0	Day 4	Day 8	Day 11	Day 13
CON	152a (126, 183)	388a (338, 445)	994ab (879, 1120)	2010a (1740, 2320)	3220a (2720, 3810)
PF-01	160a (133, 194)	411a (359, 472)	1050a (933, 1190)	2140a (1850, 2470)	3420a (2890, 4050)
F113	166a (138, 201)	383a (334, 439)	882ab (779, 997)	1650ab (1430, 1900)	2500ab (2110, 2960)
17400	169a (140, 204)	430a (375, 493)	1090a (965, 1230)	2200a (1900, 2530)	3500a (2960, 4140)
APH	181a (150, 218)	404a (352, 464)	906ab (801, 1020)	1660ab (1440, 1910)	2480ab (2100, 2940)
SBW	184a (152, 222)	436a (380, 501)	1040a (916, 1170)	1980a (1720, 2290)	3060a (2580, 3620)
Pf-5	185a (154, 224)	375a (327, 431)	760b (672, 860)	1290b (1120, 1490)	1840b (1550, 2170)

CON = Control (King's Broth on paper disc), PF-01 = *P. fluorescens* PF-01, F113 = *P. fluorescens* F113, 17400 = *P. fluorescens* ATCC17400, APH = *P. fluorescens* Aphid Pathogen, SBW = *P. fluorescens* SBW25 Pf-5 = *P. protegens* Pf-5. Marginal R^2 = 0.958, conditional R^2 = 0.963. ANOVA *Pseudomonas* strain & day interaction $P<0.001$, Wald X^2 = 31.037. In Tukey's test on means, significant p-values by strain and day <0.02, significant t-ratios -3.701 to -4.454 & +3.918 to +5.636. Letters indicate grouping of means from Tukey's test, 95% confidence interval in brackets. Antagonists applied after 7 days CG440 growth. Malt extract agar. n=5.

The *P. fluorescens* aphid pathogen strain grew large colonies in comparison to the other *Pseudomonas* strains from day 4 onwards (ANOVA *Pseudomonas* strain & day interaction $P<0.001$, Wald X^2 = 189.852, marginal R^2 = 0.932, conditional R^2 = 0.933. Tukey day 4-13 significant $P<0.020$, significant t-ratios = -3.799 to -22.404 & +3.828 to +25.040. Table 5), while Pf-5 produced the third smallest colonies at day 13 and yet provided the greatest inhibition of CG440 growth, as described above. The aphid pathogen was observed to be in contact with the pathogen in all repeats by day 13. It also caused splitting of the media which was un-observed for any other strain. This suggests that the aphid pathogen strain may be competing with CG440 via general competition for space, whereas

Pf-5 is producing an antifungal compound which is more successful given the day 13 results. The provider of the aphid pathogen strain also noted its rapid growth in comparison to other strains of *P. fluorescens* (Personal comm., Dr Deepa Paliwal, 2017).

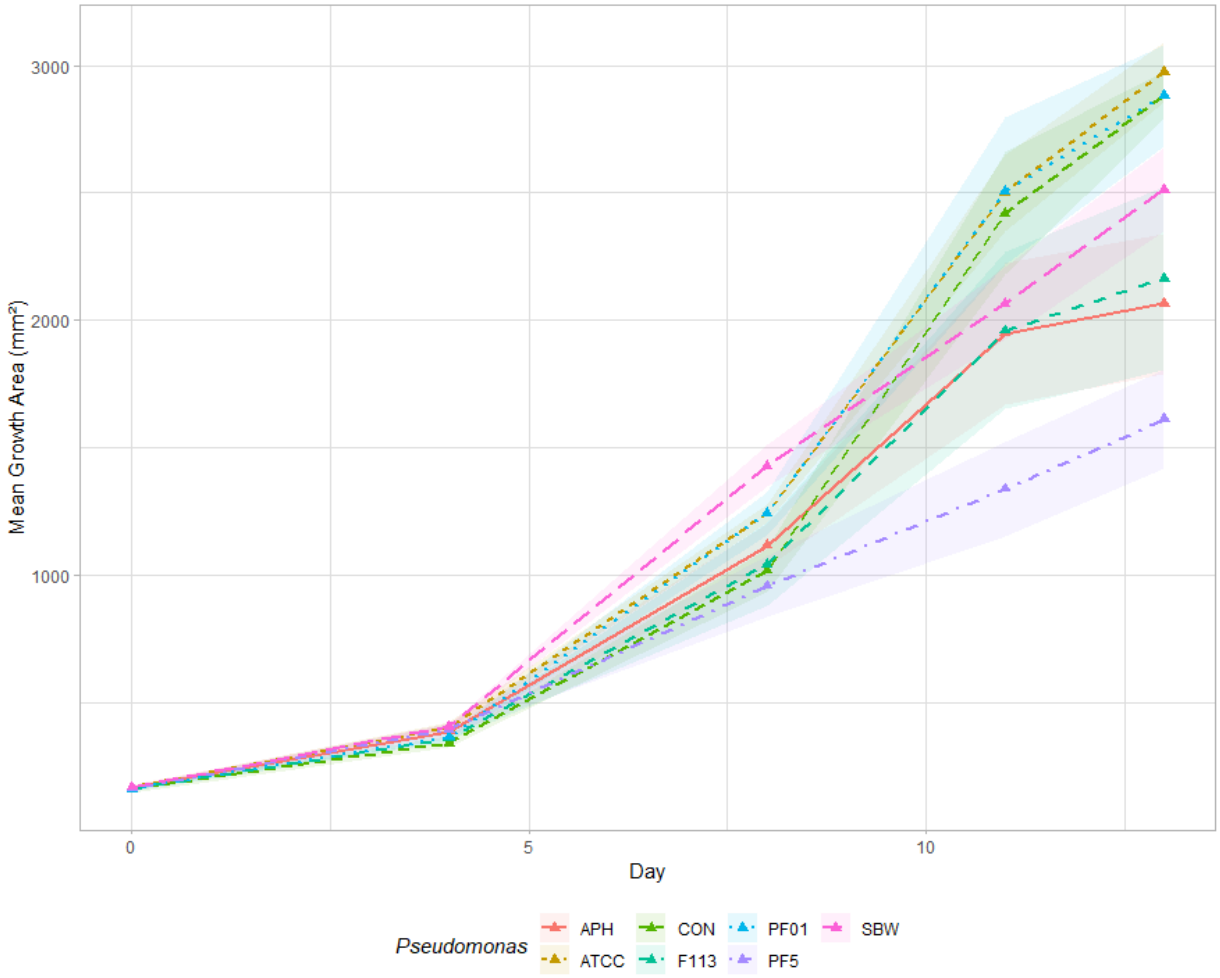

Figure 3. Mean growth area (mm^2) of *Armillaria mellea* CG440 confronted with different *Pseudomonas* bio-control strains 0 to 13 days after antagonist inoculation. CON = Control (King's Broth on paper disc), PF-01 = *P. fluorescens* PF-01, F113 = *P. fluorescens* F113, 17400 = *P. fluorescens* ATCC17400, APH = *P. fluorescens* Aphid Pathogen, SBW = *P. fluorescens* SBW25 Pf-5 = *P. protegens* Pf-5. Ribbon behind line represents standard error range. Antagonists applied after 7 days CG440 growth. *n*=5.

Table 5. Mean growth area (mm^2) of different *Pseudomonas* bio-control strains growing in the presence of *Armillaria mellea* CG440 between 0 and 13 days after their inoculation on Malt Extract Agar.

Strain	Day 0	Day 4	Day 8	Day 11	Day 13
CON	25.7a (18.8, 35.2)	26.3a (21.4, 32.5)	27a (22.7, 32.2)	27.5a (22.1, 34.4)	27.9a (21.2, 36.7)
PF5	33.4a (24.4, 45.7)	83.8bc (68, 103)	210bc (176, 250)	419bc (335, 523)	663bc (505, 871)
F113	33.6a (24.5, 45.9)	71.9b (58.4, 88.6)	154b (129, 184)	273b (219, 341)	399b (304, 525)
ATCC	35a (25.5, 47.8)	96.9bcd (78.6, 119)	269cd (225, 320)	577cd (462, 720)	961cd (731, 1260)
SBW	38.7a (28.2, 52.9)	106bcd (86.2, 131)	292cd (245, 348)	623cd (499, 777)	1030cd (786, 1360)
PF01	42.2a (30.8, 57.7)	123cd (100, 152)	361de (303, 430)	807de (647, 1010)	1380de (1050, 1810)
APH	43a (31.4, 58.8)	144d (117, 178)	484e (406, 577)	1200e (962, 1500)	2200e (1680, 2890)

CON = Control (King's Broth on paper disc), PF-01 = *P. fluorescens* PF-01, F113 = *P. fluorescens* F113, 17400 = *P. fluorescens* ATCC17400, APH = *P. fluorescens* Aphid Pathogen, SBW = *P. fluorescens* SBW25 Pf-5 = *P. protegens* Pf-5. Marginal R^2 = 0.932, conditional R^2 = 0.933. ANOVA *Pseudomonas* strain & day interaction $P<0.001$, Wald X^2 = 189.852. In Tukey's test on means, significant p values by strain and day <0.020, significant t-ratios = -3.799 to -22.404 & +3.828 to +25.040. Letters indicate grouping of means from Tukey's test, 95% confidence interval in brackets. Antagonists applied after 7 days CG440 growth. *n*=5.

The next approach was to address combination of antagonists to look for synergy, as well as testing the antagonistic Pf-5 strain against a range of different strains of *A. mellea* to ensure this antagonism is not specific to CG440.

3.3.4 *In vitro* inhibition of *A. mellea* strains by combinations of BCA bacteria

There have been a number of studies looking at one-on-one antagonism between various bacteria and *Armillaria* strains as detailed in the General Introduction (1), however none can be found studying the impact of combinations of BCA strains. *Armillaria* strain is also often overlooked. Therefore these experiments took BCA bacteria and combined them within various combinations in dual-culture antagonism assays against multiple *A. mellea* strains to assess if this yielded an enhanced/synergistic impact on the pathogen. These consisted of *Pseudomonas* strains (*P. fluorescens* SBW25 and ATCC17400, *P. protegens* Pf-5) which showed a mixed range of antagonistic potential against *Armillaria mellea* CG440 in a previous experiment (3.3.3), as well as the previously best performing *Pseudomonas* strain, *P. protegens* Pf-5, and a *Bacillus* strain, QST713, isolated from a commercial product. The three *Pseudomonas* strains are known to produce varying antimicrobial compounds as discussed previously (3.3.3), as is the *Bacillus* strain (Pandin *et al.*, 2018).

Specific Methodology

Armillaria mellea strains: CG440, CG447, CG581

Antagonists:

1. *P. protegens* Pf-5, *P. fluorescens* SBW25, *P. fluorescens* ATCC17400, plus equal volume combinations of pairs and all three strains.
2. *P. protegens* Pf-5 and *Bacillus subtilis* QST713 plus equal volume combination of both.

Workflow:

- Antagonism Assay, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4a, & 3.2.7)
- Digital images at days 0, 8, 10, 12, & 14 after bacterial inoculation, Scanner (3.2.3)
- Measure: Mean Growth Area of pathogen (thresholding method)
- ANOVA & Tukey's test (3.2.11) non-factorial design, due to variable amounts of bacteria between treatments and bacterial isolates. Growth area log transformed.

Repeats: *Pseudomonas* strains = 6, Pf-5 & QST713 = 4

3.3.4.1 Pseudomonas *strains*

There was a significant interaction effect between *A. mellea* strain and *Pseudomonas* strain/s on the pathogen's growth over time (ANOVA *A. mellea* strain, *Pseudomonas* strain, time interaction $P<0.001$, Wald $X^2 = 30.428$, marginal $R^2 = 0.923$, conditional $R^2 = 0.952$. Tukey significant p-values <0.050, significant t-ratios = -3.365 to -4.578 & +3.106 to +6.612. Table 6). Focussing on day 14, typically *P. protegens* Pf-5 was the best performing strain, with 45-63% growth inhibition of the *A. mellea* strains (Table 6, Figure 4). Combination with either of the other strains, *P. fluorescens* SBW25

or ATCC17400, did not significantly increase the growth inhibition Pf-5 caused. Combinations only yielded lower mean growth of CG581, again non-significantly. Results from CG440 only show significantly higher growth inhibition from the combination of SBW25 and ATCC17400.

This experiment indicates that Pf-5 is the most effective *Pseudomonas* strain of those tested and efficacy is not improved through combination. This is probably due to antagonism between the different *Pseudomonas* strains applied together.

Table 6. Mean growth area (mm^2) of *Armillaria mellea* strains in antagonism assays with various combinations of *Pseudomonas* strains, over time since inoculation of the bacteria.

Assay[1]	Day 0	Day 8	Day 10	Day 12	Day 14
CG440, C	188.5b (146.7, 242.3)	1226b (1008, 1492)	1958a (1612, 2379)	3127a (2563, 3814)	4993a (4054, 6150)
CG440, A	183.2b (146.1, 229.8)	962.2b (804.8, 1150)	1457ab (1215, 1745)	2205ab (1824, 2665)	3338ab (2724, 4090)
CG440, S	173.8b (136.4, 221.3)	924.2b (764.4, 1117)	1404ab (1160, 1698)	2131ab (1751, 2595)	3237ab (2627, 3988)
CG440, P	343.4a (273.1, 431.8)	1028b (857.1, 1234)	1353ab (1125, 1627)	1779b (1467, 2158)	2341b (1904, 2878)
CG440, AS	191.6b (151.2, 242.8)	943.2b (780.5, 1140)	1405ab (1161, 1700)	2093ab (1719, 2548)	3117b (2530, 3841)
CG440, PA	186.6b (147.8, 235.8)	882b (736.3, 1057)	1301ab (1084, 1560)	1918b (1587, 2317)	2827b (2310, 3461)
CG440, PS	221ab (172.7, 282.9)	888.6b (745, 1060)	1258b (1055, 1502)	1782b (1480, 2145)	2523b (2063, 3086)
CG440, SAP	165.3b (130.5, 209.4)	849.5b (703.7, 1026)	1279b (1059, 1545)	1926b (1585, 2340)	2900b (2361, 3562)
CG447, C	103.4b (80.57, 132.6)	624.1b (515.5, 755.5)	978.3b (809.3, 1182)	1533a (1263, 1862)	2404a (1960, 2948)
CG447, A	167.5b (131.1, 214)	672.5b (557.7, 810.8)	951.8b (789.2, 1148)	1347ab (1110, 1636)	1907ab (1551, 2345)
CG447, S	105.9b (84.36, 133)	519.8b (435.8, 620.1)	773.7b (648.1, 923.8)	1152ab (957.8, 1385)	1714ab (1408, 2087)
CG447, P	137.2b (108.9, 172.9)	501.2b (415.6, 604.5)	692.9b (574.2, 836.1)	957.9b (789.5, 1162)	1324b (1080, 1623)
CG447, AS	116.1b (91.5, 147.2)	525.7b (434.9, 635.5)	766.9b (634.7, 926.7)	1119ab (921.5, 1358)	1632ab (1331, 2001)
CG447, PA	134.2b (105.9, 170)	514.6b (426.5, 620.8)	720.1b (596.3, 869.7)	1008ab (828.8, 1225)	1410b (1146, 1735)
CG447, PS	108.6b (86.69, 136)	515.4b (431.7, 615.4)	760.8b (636.6, 909.2)	1123ab (933.5, 1351)	1658ab (1362, 2017)
CG447, SAP	131.2b (103.5, 166.3)	585.7b (483.2, 709.9)	851.3b (702.1, 1032)	1238ab (1015, 1509)	1799ab (1461, 2215)
CG581, C	252.7b (201.6, 316.7)	1175a (993.4, 1391)	1726a (1458, 2044)	2535a (2125, 3024)	3723a (3079, 4501)
CG581, A	175.8b (137.4, 224.9)	876.5ab (716, 1073)	1310ab (1069, 1604)	1957ab (1589, 2410)	2925ab (2350, 3639)
CG581, S	168.2b (133.8, 211.5)	844.5ab (707.7, 1008)	1264ab (1059, 1509)	1892ab (1575, 2273)	2832ab (2330, 3442)
CG581, P	194.9b (154, 246.8)	817.2ab (683.5, 977.1)	1169b (977.9, 1398)	1673b (1390, 2014)	2394b (1964, 2918)
CG581, AS	194.9b (154.1, 246.4)	836ab (698, 1001)	1203ab (1006, 1440)	1732ab (1440, 2082)	2492ab (2052, 3027)
CG581, PA	207.4b (165.9, 259.3)	801.5b (671.3, 956.9)	1124b (940.3, 1343)	1575b (1310, 1895)	2209b (1816, 2686)
CG581, PS	206.6b (163.4, 261.4)	798.2ab (661.3, 963.4)	1119b (926.8, 1351)	1569b (1292, 1905)	2199b (1792, 2699)
CG581, SAP	162.5b (127.4, 207.2)	737.1b (617.3, 880.1)	1076b (901, 1284)	1570b (1305, 1888)	2291b (1878, 2794)

[1] *Armillaria mellea* strain followed by *Pseudomonas* bacteria used in antagonism assay on malt extract agar. C = control (king's broth), P = *P. protegens* Pf-5, S = *P. fluorescens* SBW25, A = *P. fluorescens* ATCC17400. Combinations of capital letters represent mixes of equal volumes of prepared strains. Marginal R^2 = 0.923, conditional R^2 = 0.952. ANOVA *A. mellea* strain, *Pseudomonas* strain & day interaction $P<0.001$, Wald X^2 = 30.428. In Tukey's test on means, significant p-values by *A. mellea* strain, antagonist combination and day <0.050, significant t-ratios = -3.365 to -4.578 & +3.106 to +6.612. Lower case letters indicate grouping of means from Tukey's test, 95% confidence interval in brackets. Antagonists applied after 7 days CG440 growth. *n*=6.

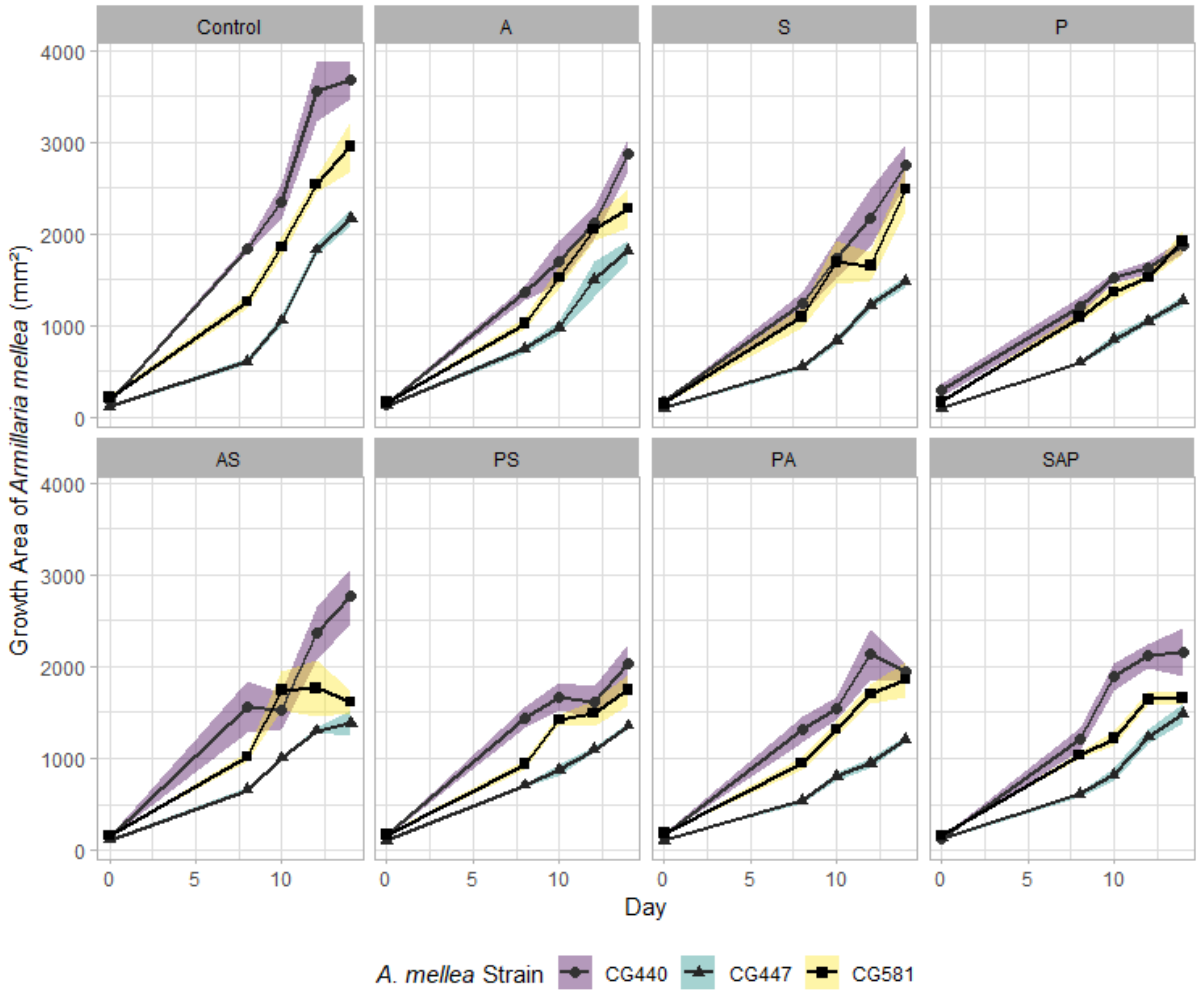

Figure 4. Mean growth area (mm²) of *Armillaria mellea* strains in antagonism assays with various combinations of *Pseudomonas* strains, over time since inoculation of the bacteria. Antagonism assay on malt extract agar. C = control (king's broth), P = *P. protegens* Pf-5, S = *P. fluorescens* SBW25, A = *P. fluorescens* ATCC17400. Combinations of capital letters represent mixes of equal volumes of prepared strains. Ribbons represent standard error ranges. Antagonists applied after 7 days CG440 growth. *n*=6.

3.3.4.2 Bacillus & Pseudomonas

There was a significant interaction of antagonist/s and A. mellea strain on the growth of the fungus over time (ANOVA *P*=0.001, Wald X^2 = 23.105. Tukey significant p-values <0.040, significant t-ratios = -4.805 & +2.281 to +6.559. Marginal R^2 = 0.921, conditional R^2 = 0.948. Table 7). *A. mellea* strain CG581 appears to be less susceptible to antagonism by QST713 than the other *A. mellea* strains (Table 7, Figure 5). Those strains, CG440 and CG447, were the most inhibited by *B. subtilis* QST713 (60% & 48% respectively), although this was not significantly different from growth inhibition from *P. protegens* Pf-5 or the combination.

This experiment suggests that combination of these two bacteria does not increase growth inhibition of *A. mellea*, and that in these conditions the bacteria inhibit its growth to a similar degree.

Table 7. Mean growth area (mm^2) of *Armillaria mellea* strains in antagonism assays with *Pseudomonas protegens* Pf-5, *Bacillus subtilis* QST713 and a combination of the two, over time following inoculation of the bacteria.

***A. m*ellea**	**Bacteria**	**Day 0**	**Day 8**	**Day 10**	**Day 12**	**Day 14**
CG440	Control	233.1b (178.9, 303.6)	1358a (1149, 1607)	2111a (1783, 2499)	3280a (2733, 3935)	5096a (4149, 6258)
CG440	Pf-5	297.4b (227.1, 389.6)	978.1b (831.7, 1150)	1317b (1122, 1547)	1774b (1492, 2108)	2389b (1964, 2905)
CG440	QST713	215.3b (167.9, 276)	773.8b (655.4, 913.6)	1065b (902.4, 1258)	1467b (1230, 1749)	2020b (1663, 2453)
CG440	Combo'	219.8b (167.8, 287.8)	959.8b (813.7, 1132)	1388b (1177, 1636)	2006b (1679, 2396)	2900b (2371, 3546)
CG447	Control	112.6b (87.9, 144.2)	658.5b (558.4, 776.5)	1024a (864.5, 1213)	1593a (1324, 1916)	2477a (2009, 3053)
CG447	Pf-5	174.6ab (135.1, 225.6)	589b (496.5, 698.8)	798.3ab (670.3, 950.9)	1082b (894.7, 1308)	1466b (1184, 1817)
CG447	QST713	253.4a (192.7, 333.3)	644.1b (523.7, 792.2)	813.3ab (660.8, 1001)	1027b (827.1, 1275)	1297b (1028, 1635)
CG447	Combo'	131.6b (99.92, 173.4)	504.2b (417.6, 608.7)	705.3b (582.5, 854)	986.7b (803.6, 1212)	1380b (1099, 1735)
CG581	Control	197.2b (153.2, 253.8)	1101b (933.9, 1298)	1692a (1435, 1996)	2602a (2182, 3102)	3999a (3287, 4866)
CG581	Pf-5	202.4b (158.9, 257.9)	844.4b (722.4, 986.9)	1207b (1032, 1411)	1725b (1458, 2040)	2465b (2042, 2974)
CG581	QST713	171b (131.5, 222.3)	839b (690.6, 1019)	1249ab (1026, 1520)	1858ab (1512, 2284)	2766ab (2212, 3459)
CG581	Combo'	206.5b (159.6, 267.1)	834b (692.5, 1004)	1182b (980.2, 1426)	1676b (1375, 2043)	2376b (1915, 2949)

Control = king's broth, Combo' = equal volume combination of both bacteria. Antagonism assay on malt extract agar. Marginal R^2 = 0.921, conditional R^2 = 0.948. ANOVA *A. mellea* strain, bacteria & day interaction *P*<0.001, Wald X^2 = 23.105. In Tukey's test on means, significant *P*-values by *A. mellea* strain, antagonist combination and day <0.040, significant t-ratios = -4.805 & +2.281 to +6.559. Lower case letters indicate grouping of means from Tukey's test, 95% confidence interval in brackets. Antagonists applied after 7 days CG440 growth. *n*=4.

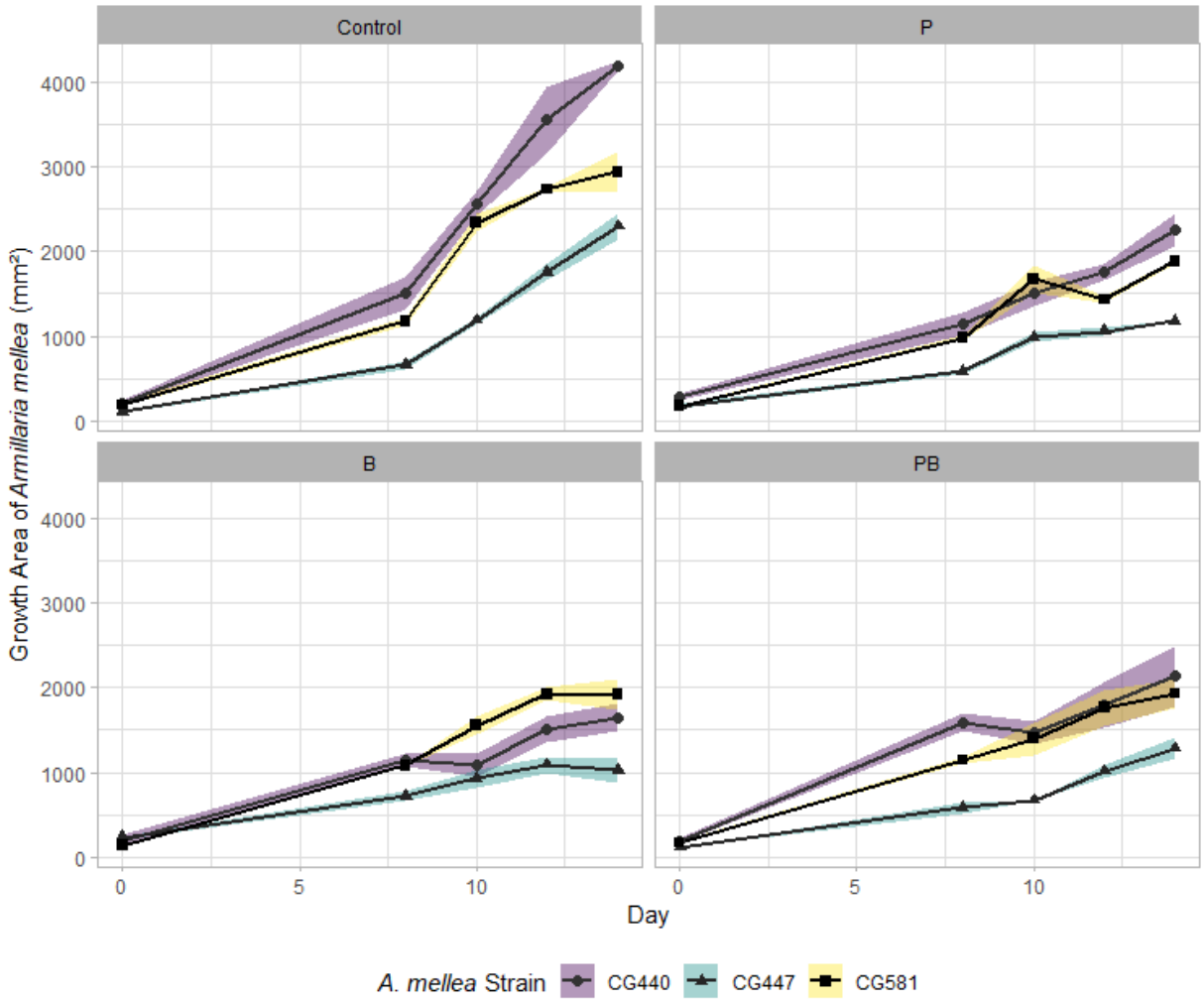

Figure 5. Mean growth area (mm^2) of *Armillaria mellea* strains in antagonism assays with *Pseudomonas protegens* Pf-5 (P), *Bacillus subtilis* QST713 (B) and a combination (PB) of the two, over time following inoculation of the bacteria. Control = king's broth, P = *P. protegens* Pf-5, B = *Bacillus subtilis* QST713, PB = equal volume combination of both bacteria. Antagonism assay on malt extract agar. Antagonists applied after 7 days CG440 growth. *n*=4.

3.3.4.3 Closing comments

In general, combination of different BCA bacteria did not increase growth inhibition. The next course of action was to investigate the combination of BCAs and PDAs, starting with checking for direct impacts of PDAs on the BCAs alone.

3.3.5 Interactions between *Pseudomonas* bio-control strains and plant defence activators effective against *A. mellea* CG440

As described previously, there is potential for increased efficacy of phytopathogen control by combining plant defence activators and bio-control organisms, as detailed in the General Introduction (1). However simultaneously there is potential for negative impacts of plant defence activators on bio-control organisms, as has been recorded for phytopathogens, and this could impact on the efficacy of combinations. For example, negative impacts were seen when applying manganese phosphite with a *Pseudomonas* biocontrol (Simonetti *et al.*, 2015).

Given the recorded direct impacts of various PDAs, this effect could be direct rather than mediated via the target pathogen or the host plant. This experiment set out to investigate if dosages of PDAs effective on *A. mellea* have direct effects on two *Pseudomonas* BCAs, one effective against *A. mellea* and one not. This was achieved by growing two strains and their combination on a dilution gradient of two PDAs, with the maximum dosages corresponding to dosages found effective, but sublethal, against *Armillaria mellea* in previous experiments.

Specific Methodology

Antagonists: *P. protegens* Pf-5, *P. fluorescens* ATCC17400, & a 50:50 volume combination of both strains

Workflow:

- Adjusted cultures (3.2.2.3)
- Plate Reader Interaction Test (3.2.8a)
- Measure: Optical density
- Compare exponential growth phase of bacteria with maximum dosage (~30,000 to 40,000 seconds) to control, using Repeated Measures Analysis of Variance (ANOVA) in Genstat 17th edition software
- Graphs and assess interaction type i.e. growth increase or reduction

Repeats: *P. protegens* Pf-5 = 4, *P. fluorescens* ATCC17400 = 4, combination = 3

The highest doses of potassium phosphite and oligochitosan, PDAs previously found to be effective against *Armillaria*, either caused small decreases or increases in growth to the different strains tested (Table 8, Figures 6 to 11). However, only potassium phosphite caused changes which were significant at a 95% confidence level.

Table 8. Mean percentage difference in OD_{600} between highest PDA dosage and control in the exponential growth phase (30-40k sec), accounting for differences caused by the PDA alone, for different *Pseudomonas* strains.

Strain	Potassium phosphite 800-900ppm [estimated] (1000ppm 'Phusion')	Oligochitosan 1g L^{-1}
ATCC17400	-0.30%	+22.32%
Pf-5	+12.92%	+2.83%
Mix	+1.34%	-4.99%
Significance	** (*P*=0.003, *F*=5.63, R^2 = 0.998)	N/A (*P*=0.583, *F*=0.80, R^2 = 0.975)

Pf-5 = *P. protegens* Pf-5, ATCC17400 = *P. fluorescens* ATCC17400, Mix = combination of the two, initial inoculum adjusted to OD_{600} = 0.5. Based on results generated from growth in King's Broth at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Significance based on interaction of treatment and strain over time, in repeated measures ANOVA testing, 95% confidence level. Significance codes: < 0.001 = ***, ≤ 0.01 = **, ≤ 0.05 = *. *n*=3 minimum.

3.3.5.1 Potassium Phosphite 'Phusion'

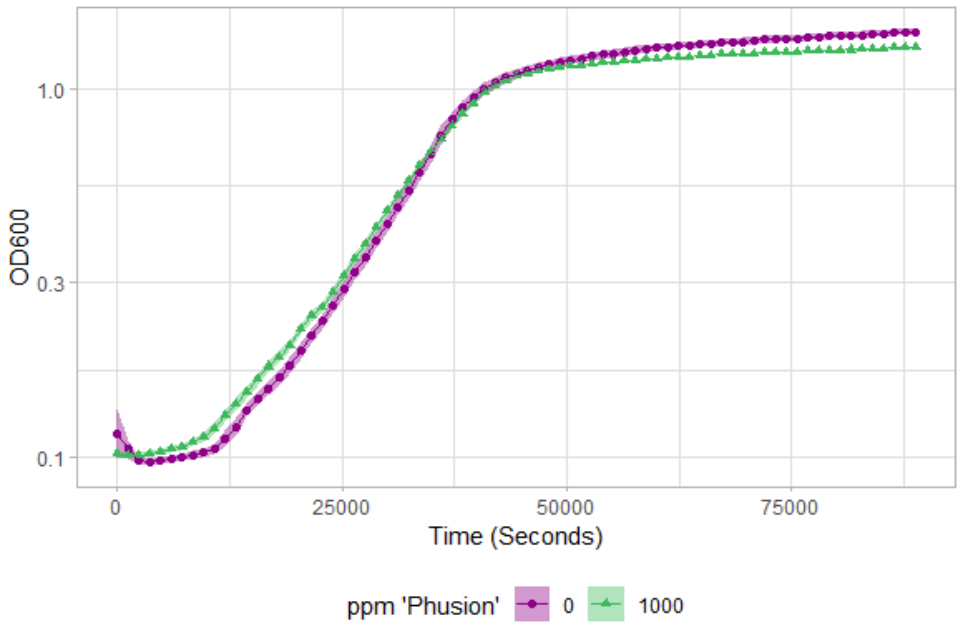

Figure 6. Comparison of growth of *Pseudomonas fluorescens* ATCC17400 in King's Broth control and King's Broth amended with 'Phusion', 80-90% potassium phosphite, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Ribbons around lines represent standard error ranges. *n*=3 minimum.

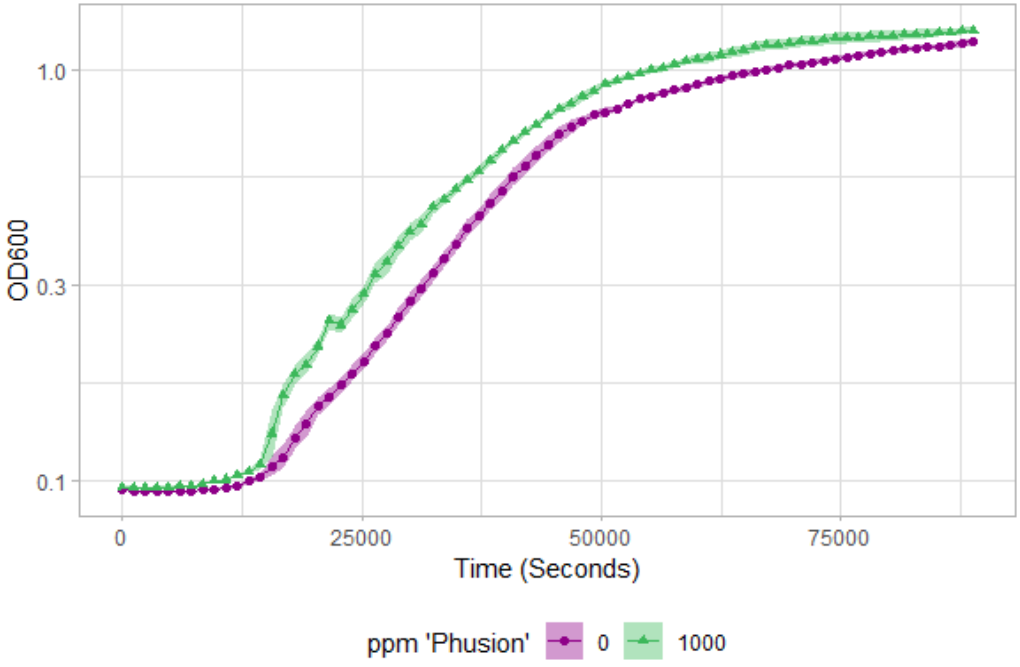

Figure 7. Comparison of growth of *Pseudomonas protegens* Pf-5 in King's Broth control and King's Broth amended with 'Phusion', 80-90% potassium phosphite, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Ribbons around lines represent standard error ranges. *n*=3 minimum.

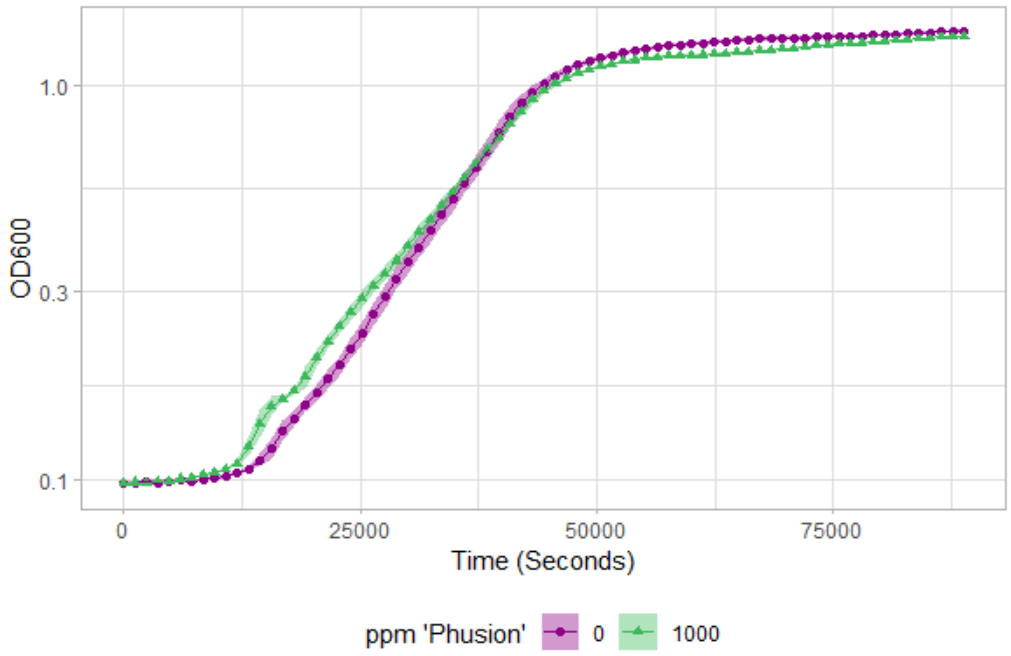

Figure 8. Comparison of growth of *Pseudomonas fluorescens* ATCC17400 and *P. protegens* Pf-5 combination, 50-50 mix of equalized OD cultures, in King's Broth control and King's Broth amended with 'Phusion', 80-90% potassium phosphite, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Ribbons around lines represent standard error ranges. *n*=3 minimum.

3.3.5.2 Oligochitosan

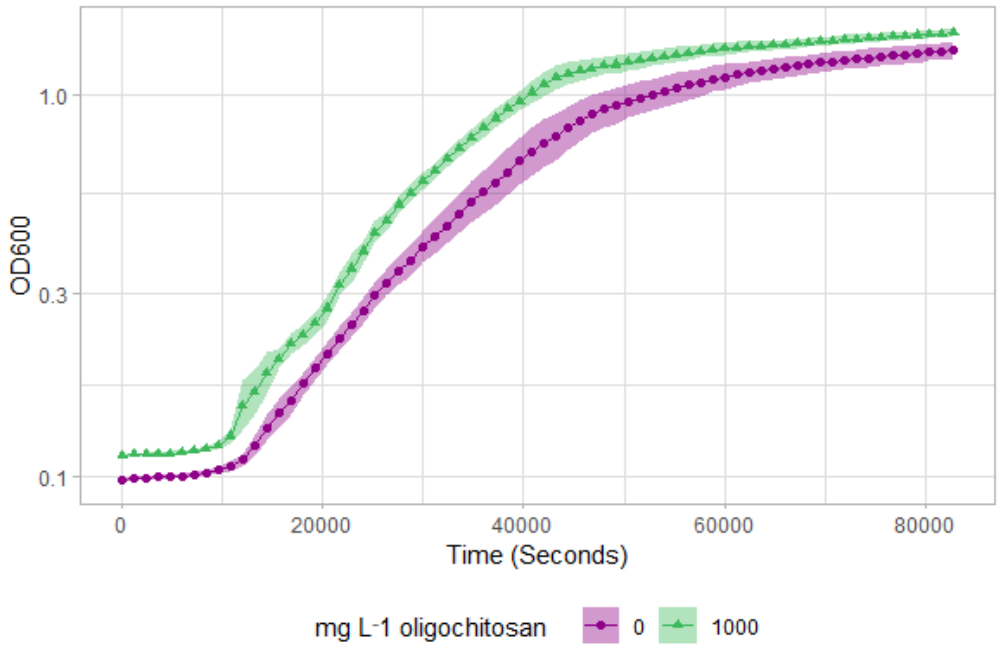

Figure 9. Comparison of growth of *Pseudomonas fluorescens* ATCC17400 in King's Broth control and King's Broth amended with 1000mg L^{-1} oligochitosan, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to *each reading.* Ribbons around lines represent standard error ranges. *n*=3 minimum.

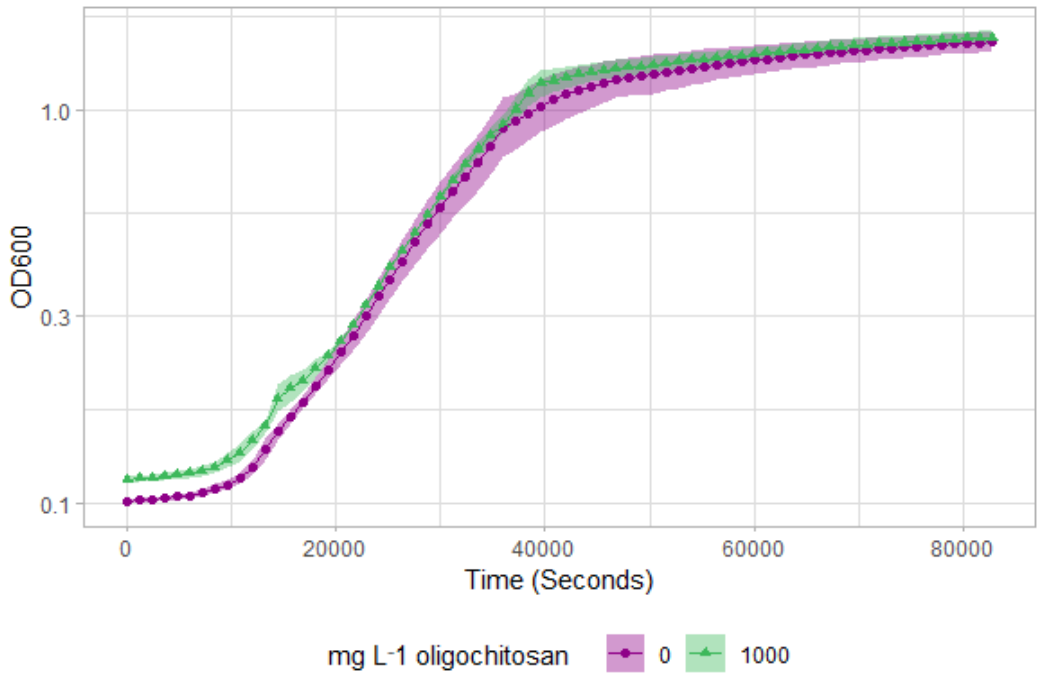

Figure 10. Comparison of growth of *Pseudomonas protegens* Pf-5 in King's Broth control and King's Broth amended with 1000mg L^{-1} oligochitosan, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Ribbons around lines represent standard error ranges. *n*=3 minimum.

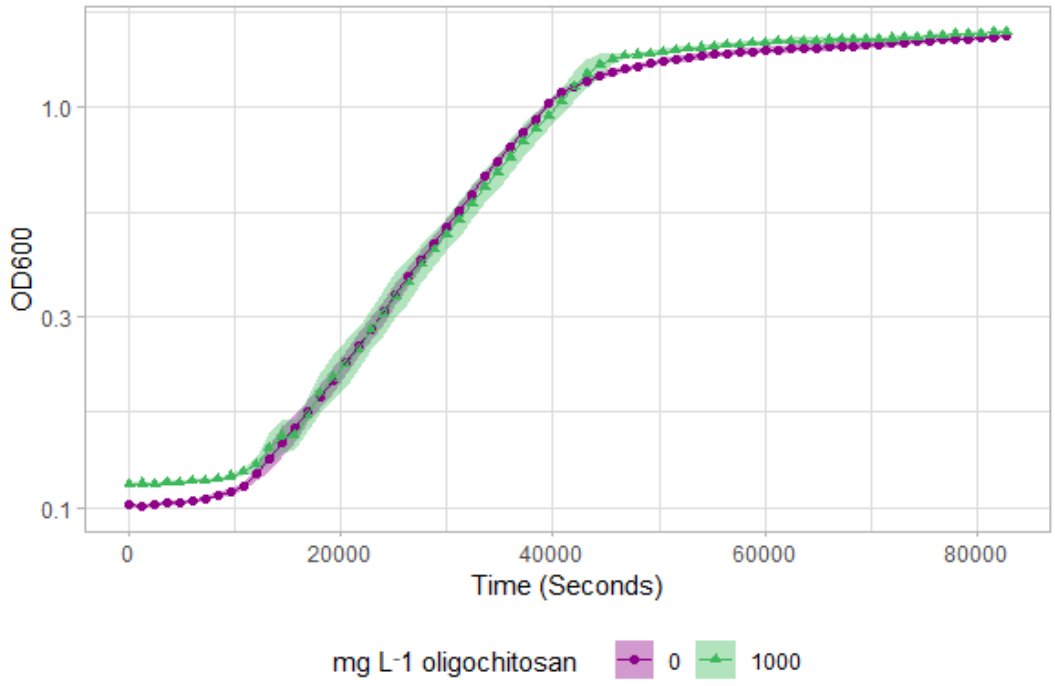

Figure 11. Comparison of growth of *Pseudomonas fluorescens* ATCC17400 and *P. protegens* Pf-5 combination, 50-50 mix of equalized OD cultures, in King's Broth control and King's Broth amended with 1000mg L^{-1} oligochitosan, at 28°C in an automated Tecan microplate reader, shaking for 20s prior to each reading. Ribbons around lines represent standard error ranges. *n*=3 minimum.

3.3.5.3 Closing comments

The results from this plate reader assay suggest there is little negative impact of these plant defence activators and dosages on these particular strains. The next approach was to look at the impact of combinations of PDAs and BCAs against *Armillaria mellea*, to see if results differed on solid media, over a larger scale, and in the presence of the pathogen, and also if there were impacts on antagonism unrelated to growth.

3.3.6 *In vitro* inhibition of *A. mellea* strain CG440 growth by effective doses of the plant defence activators potassium phosphite and oligochitosan, bacterial antagonists, and combination treatments

A prior experiment (3.3.5) indicates that there will be little impact of potassium phosphite and oligochitosan on the growth of *Pseudomonas* Pf-5. However, direct impacts of these plant defence activators may differ for other potential antagonists, may not have been evident in the short duration or small size of the prior experiment, could differ when combined with antagonism from the pathogen, or may not be reflected in growth but in antagonism instead.

Other researchers have shown reductions in antagonism towards *Macrophomina phaseolina* by a *Pseudomonas fluorescens* strain when manganese phosphite was applied in combination. In the same study, in contrast, a *Bacillus* strain in combination with this phosphite salt showed increased efficacy, over both the phosphite and the *Bacillus* strain alone, indicating no reduction in antagonism (Simonetti *et al.*, 2015). This difference indicates that *Bacillus* species may have this advantage over those of *Pseudomonas* when applied in combination with phosphite salts.

In light of these results, this experiment set out to investigate impacts of effective dosages of oligochitosan and potassium phosphite, and their combination, on antagonism of *Armillaria mellea* CG440 by effective *in vitro* antagonists *Pseudomonas protegens* Pf-5 and *Bacillus subtilis* QST713.

Specific Methodology

Armillaria mellea strains: CG440

Antagonists: *P. protegens* Pf-5 & *B. subtilis* QST713

Workflow:

- Sterile stock solution, Oligochitosan (3.2.5)
- Amended Medias: Control, 800-900ppm [estimated] Potassium Phosphite, 1000mg/L Oligochitosan, combination (3.2.6)
- Antagonism Assay, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4a, 3.2.2, & 3.2.7)
- Digital images at days 0, 4, 6, 8, 10, 12, & 14 after bacterial inoculation, Scanner (3.2.3)
- Measure: Mean Growth Area of both pathogen and antagonists, using thresholding method
- ANOVA & Tukey's test, by PDA and also antagonist strain (3.2.11), growth areas log transformed.

Repeats: 5

In the analysis of *A. mellea* CG440 growth area (Marginal R^2 = 0.886, conditional R^2 = 0.890), there were significant interactions with time for bacteria (ANOVA P<0.002, Wald X^2 = 13.032) and media (ANOVA *P*<0.001, Wald X^2 = 422.63) but not between media, bacteria, and time together (ANOVA *P*=0.351, Wald X^2 = 6.684). This suggests that more subjects were required to elucidate this interaction effect, likely due to the strong interaction effect of media and time. However, differences were apparent when means were compared within media and day combinations (Table 9, Tukey's test significant p-values < 0.05, significant t-ratios = -2.695 to -7.335 & +2.695 to +14.588). The PDA combination, potassium phosphite and oligochitosan, significantly inhibited CG440 growth earlier than potassium phosphite alone in all cases (Table 9, Figure 12). The combination treatment also significantly increased CG440 growth inhibition when combined with *B. subtilis* QST713, by 12% over potassium phosphite alone when both were compared to the QST713 on control media. The potassium phosphite and combination media did not yield significantly different growth inhibition when combined with *P. protegens* Pf-5. The QST713 and two PDA combination gave the greatest growth inhibition in comparison to the control: 83%.

There was also a significant interaction effect of treatment combination and time on antagonist colony size (ANOVA bacteria, media, and day interaction *P*=1.24e^{-12}, Wald X^2 =67.641, marginal R^2 = 0.886, conditional R^2 = 0.936). There were significant differences within media and day combinations (Table 10, Tukey's test significant p-values < 0.013). From this analysis it is apparent that the increase in CG440 growth inhibition from QST713 and the PDA combination does not relate to QST713 colony size, which was not significantly larger in this treatment (Table 10, Figure 13).

Oligochitosan alone caused sporadic establishment of both bacteria, which caused increased CG440 growth due to a lack of antagonism (38-96%). There was also increased growth on the 'no antagonist' control albeit non-significant, and a significant increase in growth was recorded for strain CG581 in a previous experiment (3.3.2). Therefore there is possible interference to bacterial growth by this single amendment, which the addition of the 'Phusion' phosphite product in the combination treatment alleviates. Potentially this is due to interaction between oligochitosan and potassium phosphite, or another constituent of the product, such as the polyether modified trisiloxane wetting agent/surfactant (Hill, 1997). This alleviation may not be total however, for example whilst not significant, Pf-5 had consistently lower growth on the combination media (Table 10).

Despite such potential differences for both the *Pseudomonas* and *Bacillus*, mean colony area grouped consistently on the different media from day 4 onwards (Table 10). This indicates that the media was a greater influence on bacterial growth than the growth of the pathogen, which varied over the different media in the absence of bacteria.

Removing the strong influence of oligochitosan from the analysis, QST713 grows significantly larger on the phosphite containing medias than the un-amended control (Tukey's test day 14 *P*=0.0001-0.0003, significant t-ratios = -4.312 to -4.673), while Pf-5 does not (*P*=0.4-0.6, t-ratios -0.3324 to +1.299). As CG440 grew less on these media, this gives some indication that Pf-5 is resilient to phosphite and *A. mellea* antagonism in terms of growth. Despite this, Pf-5 did not yield significantly increased inhibition of CG440 in combination with phosphite. This suggests a negative impact or overpowering effect of phosphite. In contrast, QST713 grew larger when the fungus was inhibited by phosphite suggesting it is more sensitive to *A. mellea* antagonism but can antagonize the pathogen in the presence of phosphite. Replication of the whole experiment is required with additional controls of bacteria without CG440 and higher a sample number, to further elucidate these possibilities. Such a difference in antagonism could correspond with the findings of other authors, as described above (Simonetti *et al.*, 2015).

As an aside, it was noted that CG440 colonies on potassium phosphite media were larger at the beginning of the measurements (Table 9), although this trend had resolved by day 6: this is due to the plates being inoculated one after the other and this group in particular being placed on an uneven surface after so the drops of homogenized mycelium ran slightly before absorbing, meaning the initial colonies were wider.

Given the mixed performance of oligochitosan alone and in combination, it was decided to remove it from further studies. Research continues with potassium phosphite and QST713 alone which have similar impacts of on the pathogen. QST713 and potassium phosphite products are already commercialized and widely available, giving them practical advantages over Pf-5.

Testing with host plants is required to examine if a combination of QST713 and potassium phosphite has similar impacts on host infections compared to *in vitro*.

Testing with other strains of *A. mellea* would also be prudent. The *A. mellea* strain used in this experiment, CG440, has shown increased susceptibility to growth inhibition by potassium phosphite in comparison to other strains (3.3.2), which may mean that if this experiment or a similar one is repeated on other strains, results will probably vary to some degree. Therefore this was the next course of action.

Table 9. Mean growth area (mm^2) of *Armillaria mellea* CG440 in antagonism assays with *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 1000mg/L oligochitosan (OC) or a combination, over time since inoculation of the bacteria.

Bacteria	Media	Day 0	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Control	Control	96.94b (77.63, 121)	268.4b (230.8, 312.1)	446.6a (392.8, 507.7)	743.1b (658, 839.3)	1237b (1082, 1413)	2058b (1754, 2413)	3424a (2819, 4157)
Control	OC	115.8b (92.73, 144.6)	334.2ab (287.4, 388.6)	567.7a (499.4, 645.5)	964.5a (854, 1089)	1639a (1434, 1872)	2784a (2373, 3265)	4729a (3894, 5743)
Control	KPhi	260.4a (208.5, 325.1)	389.9a (335.3, 453.4)	477.1a (419.7, 542.5)	583.9c (517, 659.4)	714.5c (625.3, 816.5)	874.4c (745.5, 1026)	1070b (881.2, 1299)
Control	KPhi+OC	140.9b (112.9, 176)	255.6b (219.8, 297.3)	344.2b (302.8, 391.4)	463.6d (410.5, 523.6)	624.4c (546.4, 713.5)	840.9c (716.9, 986.2)	1132b (932.5, 1375)
Pf-5	Control	118.3b (94.72, 147.7)	267.4b (230, 311)	402.2b (353.7, 457.2)	604.8b (535.5, 683)	909.4b (795.9, 1039)	1368b (1166, 1604)	2056b (1693, 2497)
Pf-5	OC	111.1b (89.01, 138.8)	309.9b (266.5, 360.4)	517.4a (455.1, 588.3)	864a (765, 975.8)	1443a (1263, 1649)	2409a (2054, 2825)	4022a (3312, 4885)
Pf-5	KPhi	319.3a (255.7, 398.7)	440.9a (379.2, 512.7)	518.2a (455.8, 589.1)	609b (539.2, 687.8)	715.7b (626.3, 817.8)	841c (717.1, 986.5)	988.4c (813.9, 1200)
Pf-5	KPhi+OC	140.5b (112.5, 175.4)	239.1b (205.6, 278.1)	312c (274.4, 354.7)	407c (360.4, 459.7)	531c (464.7, 606.8)	692.8c (590.7, 812.6)	904c (744.4, 1098)
QST713	Control	117.3b (93.91, 146.4)	275.7b (237.1, 320.6)	422.8a (371.9, 480.7)	648.3b (574, 732.1)	994b (869.9, 1136)	1524b (1300, 1788)	2337b (1925, 2838)
QST713	OC	103.8b (83.16, 129.7)	298b (256.3, 346.5)	504.8a (444, 574)	855.2a (757.3, 965.9)	1449a (1268, 1656)	2454a (2093, 2879)	4158a (3424, 5049)
QST713	KPhi	326.6a (261.6, 407.8)	432.4a (371.8, 502.9)	497.6a (437.6, 565.7)	572.5b (506.9, 646.6)	658.7c (576.5, 752.7)	758c (646.2, 889)	872.1c (718.2, 1059)
QST713	KPhi+OC	131.1b (105, 163.7)	201.8c (173.5, 234.6)	250.3b (220.2, 284.6)	310.5c (275, 350.7)	385.2d (337.1, 440.2)	477.9d (407.5, 560.5)	592.9d (488.2, 719.9)

ANOVA bacteria and day interaction p=0.0015, media and day interaction $p < 2.2e^{-16}$. Tukey significant p-values of means by bacteria, media, and day < 0.05. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% silicone, concentration estimated. Letters indicate grouping of means, 95% confidence interval in brackets. $n = 5$.

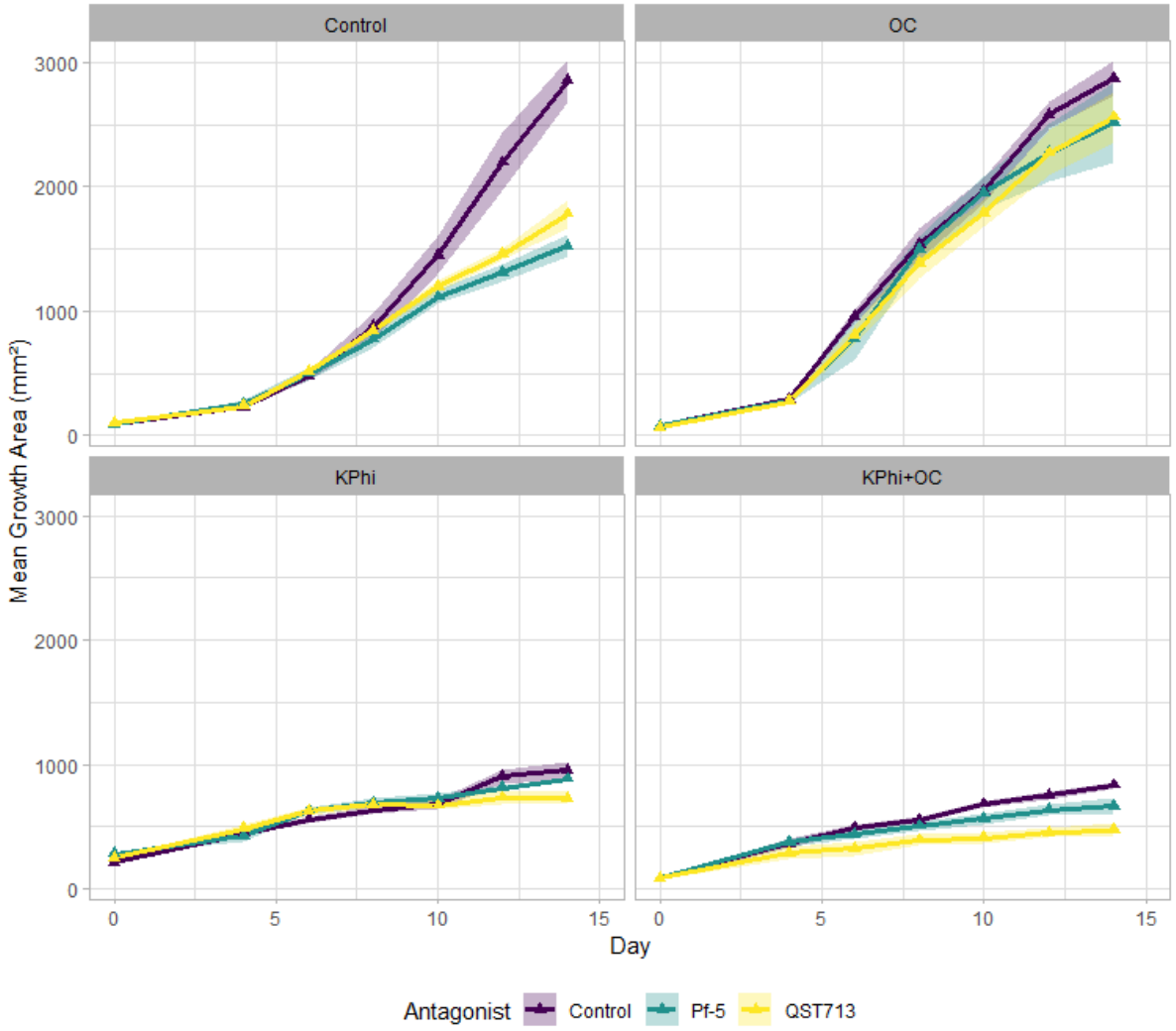

Figure 12. Mean growth area (mm^2) of *Armillaria mellea* CG440 in antagonism assays with *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 1000mg/L oligochitosan (OC) or a combination, over time since inoculation of the bacteria. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Ribbons around lines represent standard error ranges. *n*=5.

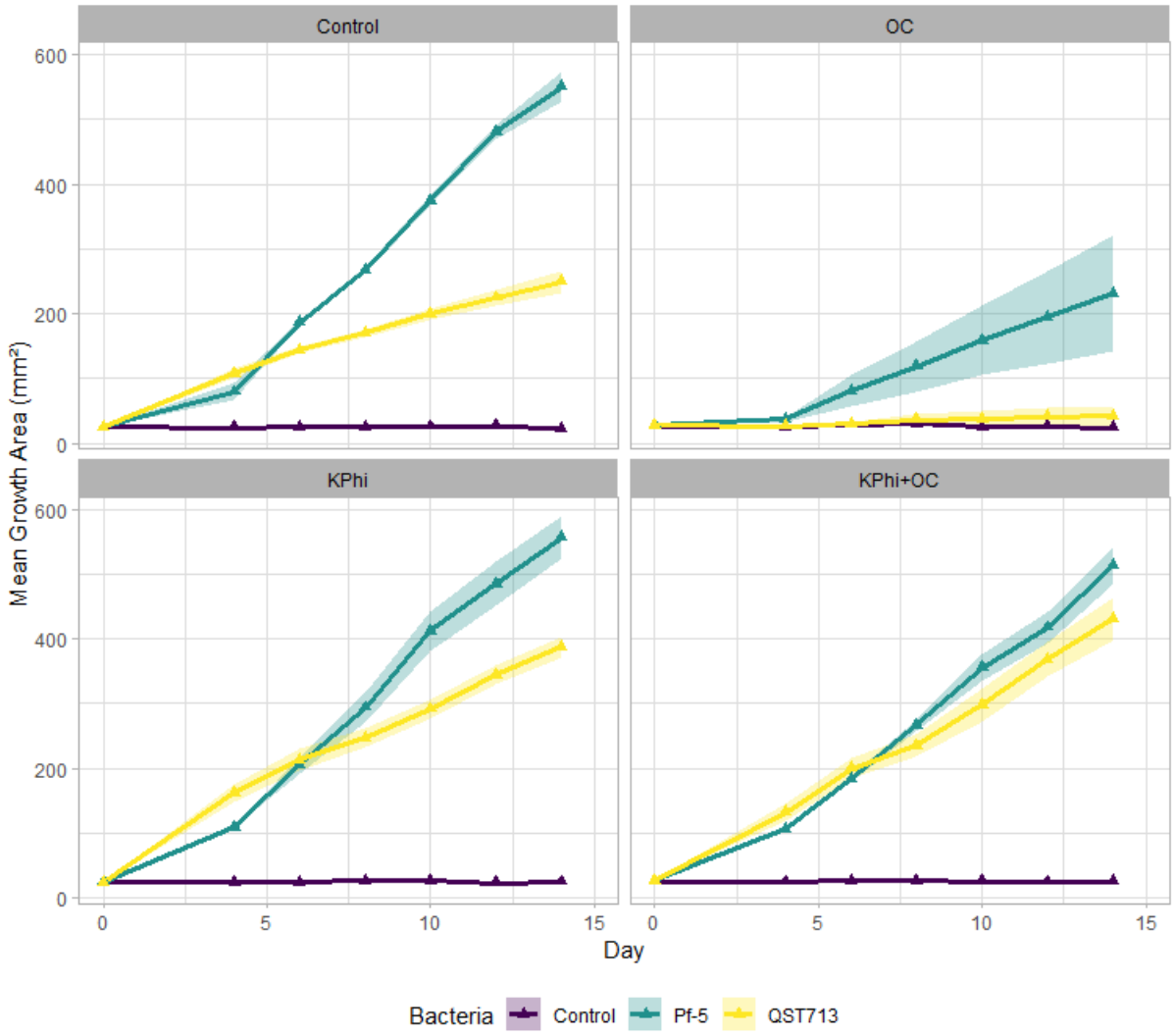

Figure 13. Mean growth area (mm²) of *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 in antagonism assays with *Armillaria mellea* CG440 on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 1000mg/L oligochitosan (OC) or a combination, over time since inoculation of the bacteria. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Ribbons around lines represent standard error ranges. *n*=5.

Table 10. Mean growth area (mm^2) of *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 in antagonism assays with *Armillaria mellea* CG440 on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 1000mg/L oligochitosan (OC) or a combination, over time since inoculation of the bacteria.

Bacteria	Media	Day 0	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Control	Control	26.18a (19.17, 35.75)	25.71a (19.61, 33.71)	25.48a (19.64, 33.06)	25.25a (19.52, 32.67)	25.03a (19.25, 32.54)	24.8a (18.84, 32.66)	24.58a (18.31, 33)
Control	OC	27.82a (20.37, 37.99)	27.35a (20.86, 35.85)	27.11a (20.9, 35.17)	26.88a (20.78, 34.77)	26.65a (20.5, 34.65)	26.42a (20.07, 34.8)	26.2a (19.51, 35.17)
Control	KPhi	25.24a (18.48, 34.47)	25.38a (19.36, 33.28)	25.45a (19.62, 33.02)	25.52a (19.73, 33.02)	25.59a (19.68, 33.28)	25.67a (19.49, 33.8)	25.74a (19.17, 34.55)
Control	KPhi+OC	26.41a (19.34, 36.07)	26.28a (20.04, 34.46)	26.22a (20.21, 34.01)	26.15a (20.22, 33.83)	26.09a (20.07, 33.92)	26.03a (19.76, 34.27)	25.96a (19.34, 34.86)
Pf-5	Control	34.13a (24.99, 46.62)	83.45a (63.65, 109.4)	130.5a (100.6, 169.3)	204a (157.7, 263.9)	319a (245.4, 414.8)	498.8a (378.8, 656.9)	780a (581, 1047)
Pf-5	OC	28.65a (20.98, 39.14)	45.56b (34.75, 59.74)	57.45b (44.28, 74.53)	72.44b (56, 93.71)	91.34b (70.25, 118.8)	115.2b (87.46, 151.7)	145.2b (108.2, 195)
Pf-5	KPhi	39.93a (29.23, 54.53)	94.35a (71.96, 123.7)	145a (111.8, 188.2)	223a (172.4, 288.4)	342.8a (263.6, 445.7)	526.9a (400.1, 693.9)	810a (603.3, 1087)
Pf-5	KPhi+OC	41.03a (30.04, 56.03)	92.13a (70.27, 120.8)	138.1a (106.4, 179.1)	206.9a (159.9, 267.6)	310.1a (238.5, 403.1)	464.6a (352.8, 611.9)	696.3a (518.6, 934.8)
QST713	Control	42.51ab (31.12, 58.05)	76.72a (58.51, 100.6)	103.1a (79.45, 133.7)	138.5a (107, 179.1)	186a (143.1, 241.9)	249.9a (189.8, 329.1)	335.8a (250.1, 450.8)
QST713	OC	26.55b (19.44, 36.27)	28.93b (22.07, 37.93)	30.2b (23.28, 39.18)	31.52b (24.37, 40.78)	32.9b (25.3, 42.78)	34.34b (26.08, 45.23)	35.85b (26.7, 48.13)
QST713	KPhi	49.36a (36.14, 67.41)	98.53a (75.15, 129.2)	139.2a (107.3, 180.6)	196.7a (152.1, 254.5)	277.9a (213.8, 361.4)	392.7a (298.2, 517.2)	554.9a (413.3, 745)
QST713	KPhi+OC	45.82ab (33.55, 62.57)	94.57a (72.12, 124)	135.9a (104.7, 176.2)	195.2a (150.9, 252.5)	280.4a (215.7, 364.6)	402.9a (305.9, 530.5)	578.8a (431.1, 777)

ANOVA bacteria, media, and day interaction p= $1.24e^{-12}$. Tukey significant p-values of means by bacteria, media, and day < 0.013. Chitosan hydrochloride, degree of acetylation 95%, molecular weight ~10-15kDa, G.T.C. Bio Corporation, Qingdao, China. Oligochitosan: molecular weight 2300 & 3740, G.T.C. Bio Corporation, Qingdao, China. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% silicone, concentration estimated. Letters indicate grouping of means, 95% confidence interval in brackets. n = 5.

3.3.7 *In vitro inhibition* of *A. mellea* CG440, CG447, & CG581 growth by a combination of potassium phosphite and *Bacillus subtilis* QST713.

The previous experiment (3.3.6) indicated that a combination of *B. subtilis* QST713 and potassium phosphite could provide increased *in vitro* growth inhibition of *A. mellea* CG440 over treatment either alone. This experiment compares the effect on three different strains to see if this also the case for other strains. A comparison of bacteria growth was also made to investigate any potential effect of the potassium phosphite and antagonism from the different strains.

Specific Methodology

Armillaria mellea strains: CG440, CG447, CG581

Antagonists: *B. subtilis* QST713

Workflow:

- Amended Medias: Control, 800-900ppm [estimated] Potassium Phosphite (3.2.6)
- Antagonism Assay with paper discs, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4a, 3.2.2, & 3.2.7)
- Digital images at day 14 after bacterial inoculation, Scanner (3.2.3)
- Measure: Mean Growth Area of both pathogen and antagonists, using thresholding method
- ANOVA & Tukey's test, by treatment as combination of factors (3.2.11). Factorial design. Estimated marginal means calculated are adjusted for unbalanced design. *Armillaria* growth area log transformed.

Treatments and sample numbers:

***Armillaria mellea* strain**	**Treatment**	***n***
None	QST713	10
None	KPhi+ QST713	10
CG440	Control	13
CG440	QST713	12
CG440	KPhi	12
CG440	KPhi+ QST713	14
CG447	Control	13
CG447	QST713	13
CG447	KPhi	12
CG447	KPhi+ QST713	12
CG581	Control	13
CG581	QST713	13
CG581	KPhi	12
CG581	KPhi+ QST713	12

The growth area of *A. mellea* was significantly affected by its strain (ANOVA P=4.574e^{-4}, F= 8.132. Adjusted R^2=0.853) but more strongly impacted by the presence of *B. subtilis* QST713 and potassium

phosphite (ANOVA $P<2.2e^{-16}$, $F= 28.913$). *A. mellea* CG447 growth area was significantly smaller than that of strains CG440 and CG581 (Table 11, Figure 14, Tukey's test of *Armillaria* growth area by *A. mellea* strain only: significant p-values ≤0.002, significant t-ratios -3.520 & 3.469). This corresponds with previous observations (3.3.2) regarding the comparative growth of these strains, i.e. that CG447 is slower growing.

Table 11. Mean growth area (mm^2) of *Armillaria mellea* strains in antagonism assays with *Bacillus subtilis* QST713 on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 14 days after inoculation of the bacteria.

Strain	Treatment	Mean growth area (mm^2) estimate and 95% confidence interval
CG440	Mean	1682a (1565, 1809)
CG447	Mean	1404b (1305, 1511)
CG581	Mean	1688a (1569, 1816)
Mean	Control	3868a (3561, 4201)
Mean	QST713	2055b (1889, 2235)
Mean	KPhi	973.3c (893, 1061)
Mean	KPhi+ QST713	817.5d (751.6, 889.2)
CG440	Control	4021a (3484, 4641)
CG440	QST713	2115b (1822, 2456)
CG440	KPhi	1113c (958.5, 1292)
CG440	KPhi+ QST713	846.3d (737.1, 971.6)
CG447	Control	3469a (3006, 4003)
CG447	QST713	1749b (1516, 2019)
CG447	KPhi	880c (758.1, 1022)
CG447	KPhi+ QST713	728.1c (627.2, 845.2)
CG581	Control	4148a (3594, 4787)
CG581	QST713	2345b (2032, 2706)
CG581	KPhi	941.7c (811.2, 1093)
CG581	KPhi+ QST713	886.8c (763.9, 1029)

ANOVA strain effect $P=4.574e^{-4}$, $F= 8.132$, Adjusted $R^2=0.853$. ANOVA KPhi & QST713 interaction effect $P<2.2e^{-16}$, $F= 28.913$. Tukey's test by phosphite and QST713 presence p-values <0.025, t-ratios +2.866 to +10.612. Tukey's test phosphite alone vs. combination by strain p-values: CG440 = 0.043, CG447 = 0.289, CG581 = 0.943, all other p-values <0.001, significant t-ratios = -6.021 to -8.719 & +2.662 to +15.482. KPhi: Potassium phosphite, as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Letters indicate grouping of means, 95% confidence interval in brackets. n ≥ 12.

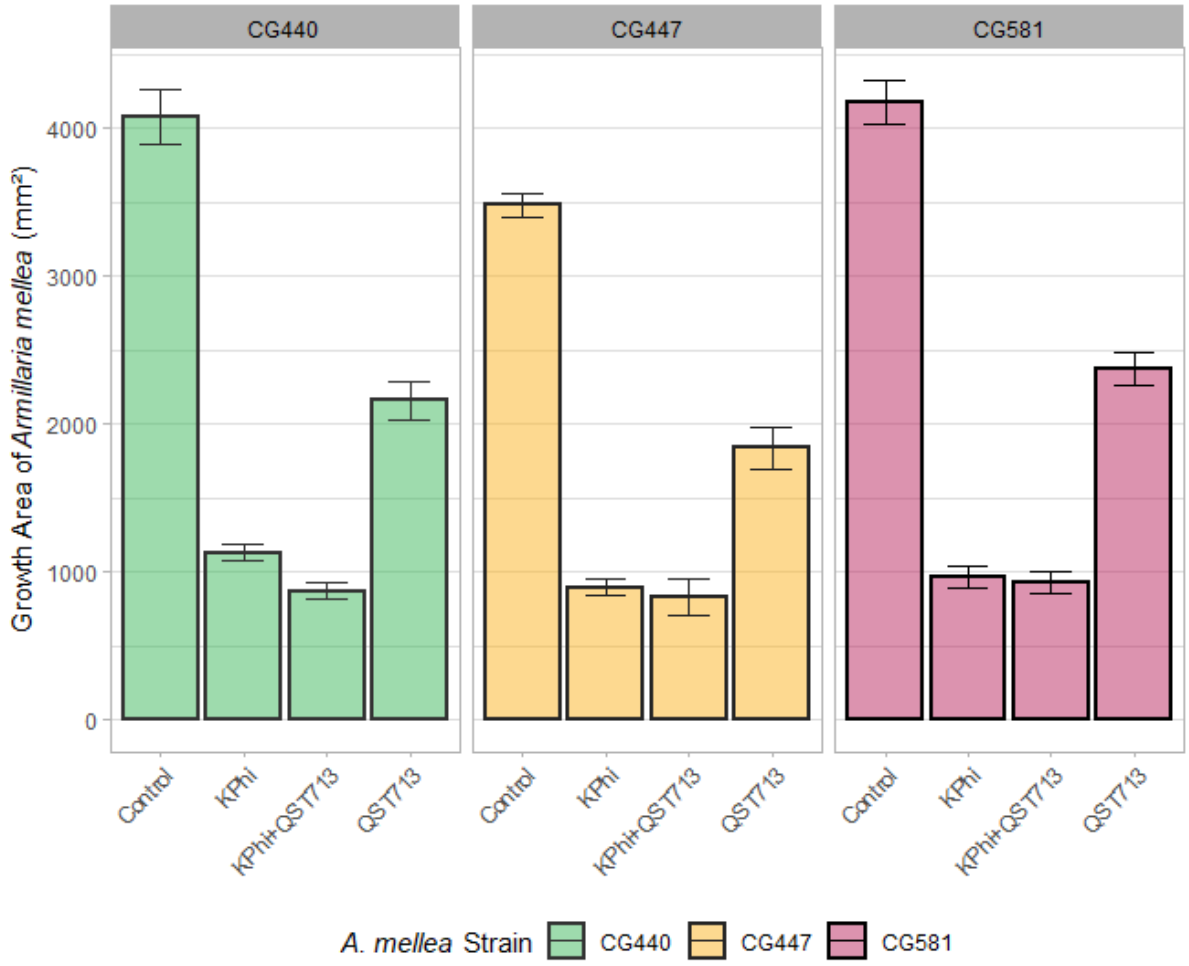

Figure 14. Mean growth area (mm^2) of *Armillaria mellea* strains in antagonism assays with *Bacillus subtilis* QST713, on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 14 days after inoculation of the bacteria. Potassium phosphite (KPhi), as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Error bars represent standard error ranges. Antagonists applied after 7 days *A. mellea* growth. $n \geq 12$.

Averaging over *A. mellea* strains, all treatments were significantly different (Table 11, Tukey's test by phosphite and QST713 presence p-values < 0.025, t-ratios +2.866 to +10.612): *B. subtilis* QST713 caused 47% growth inhibition, potassium phosphite 75% and the combination 79%. As observed previously, potassium phosphite had the greatest effect alone (3.3.2, 3.3.6), and causes the majority of *A. mellea* growth inhibition when combined with antagonists (3.3.6). When treatments were compared within each strain alone, only CG440 was more significantly impacted by the combination than potassium phosphite alone (Table 11, Tukey's test phosphite alone vs. combination by strain p-values: CG440 = 0.043, CG447 = 0.289, CG581 = 0.943, all other p-values < 0.001, significant t-ratios = -6.021 to -8.719 & +2.662 to +15.482). This could relate to the potential sensitivity of CG440 to

potassium phosphite discussed previously, which could allow QST713 to antagonise CG440 to a greater degree than the other strains. However, based on p-values, the impact of strain was far weaker than the treatment combinations, which may be why there was not a significant QST713-Phosphite-Strain interaction in the ANOVA despite significant differences in the pairwise comparison.

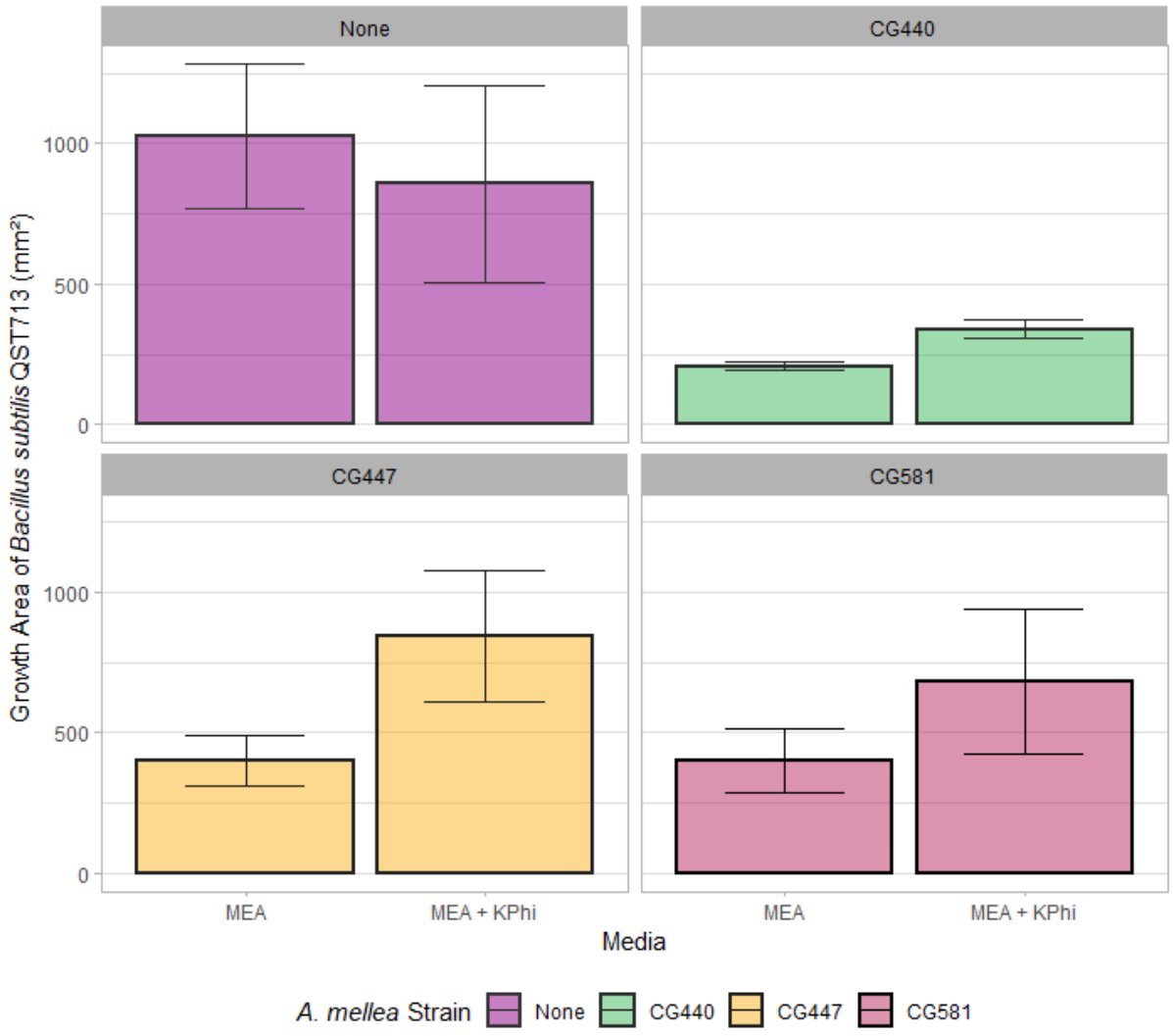

Figure 15. Mean growth area (mm²) of *Bacillus subtilis* QST713 in antagonism assays with *Armillaria mellea* strains, on malt extract agar amended with 800-900ppm [estimated] potassium phosphite (KPhi), 14 days after inoculation of the bacteria. Potassium phosphite (KPhi), as Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. Error bars represent standard error ranges. Antagonists applied after 7 days *A. mellea* growth. $n \geq 10$.

In the absence of the fungus, *B. subtilis* QST713 growth area was not significantly different on media with and without phosphite (Figure 15, ANOVA *P*=0.707, *F*=0.146. Adjusted R^2=-0.047), suggesting no negative impact on growth, although the mean was lower. In the presence of the fungus, QST713 growth area was significantly higher in the presence of phosphite (Figure 15, ANOVA *P*=0.045, *F*= 4.107. Adjusted R^2= 0.023) increasing by 82% (mean of all strains), with no effect of *A. mellea* strain (ANOVA *P*=0.136, *F*=2.025). This corresponds with the previously observed results (3.3.6): suggesting that the impact of phosphite on *A. mellea* reduces its antagonism of QST713, and that the bacteria is more susceptible to antagonism from the fungus than by the presence of phosphite.

The results of this experiment suggest that a combination of potassium phosphite and QST713 can yield increased growth inhibition of *A. mellea* via direct effects alone. Therefore testing of this combination *in planta* is required to elucidate if this performance is maintained in the heterogeneous environment of the host root zone and if plant mediated effects enhance it. Combination with a *Trichoderma* strain would also be a valuable addition to such a trial. This could produce insight into if the impact of *Trichoderma*'s biocontrol of *A. mellea* is enhanced or hindered by each component of the combination.

Given the imprecise concentration in the currently used phosphite product ('Phusion') and also the presence of polyether modified trisiloxane surfactant/wetting agent within it, a pure phosphite product would reduce any factors that might call the results of further trials into question. One was found prior to the following experiment, which therefore assesses and compares results from the new product with those from 'Phusion'.

3.3.8 Comparison of the *in vitro* effects of pure potassium phosphite product and 'Phusion' product with silicone-based surfactant/wetting agent on *A. mellea* CG440, *Pseudomonas protegens* Pf-5, *Bacillus subtilis* QST713, and interactions between each bacteria and the fungus.

Prior experiments used a potassium phosphite product (Phusion) which contains a polyether modified trisiloxane wetting agent/surfactant and had an imprecisely defined concentration of potassium phosphite. A pure aqueous potassium phosphite product with a precise concentration was found (Polyphosphite30). Therefore this experiment compares the impact of the two on *A. mellea* CG440 and previously studied biological control agent (BCA) strains to investigate any differences in the performance of these products and contextualise previous results.

Specific Methodology

Armillaria mellea strains: CG440

Antagonists: *P. protegens* Pf-5 & *B. subtilis* QST713

Workflow:

- Amended Medias: MEA control, 1000ppm 'Phusion' = 800-900ppm [estimated] Potassium Phosphite, 'Polyphosphite30' 1000ppm = 839ppm Potassium Phosphite (3.2.6)
- Antagonism Assay with paper discs, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4a, 3.2.2, & 3.2.7)
- Digital images at day 14 after bacterial inoculation, Scanner (3.2.3)
- Measure: Mean Growth Area of both pathogen and antagonists, using thresholding method
- ANOVA & Tukey's test, by treatment as various combinations of factors (3.2.11). Estimated marginal means calculated are adjusted for unbalanced design. All dependent variables log transformed.

Repeats: *n*=6, aside from QST713 on control media where *n*=4 and bacteria only plates where *n*=3

Followed by:

- Adjusted cultures (3.2.2.3)
- Plate Reader Interaction Test (3.2.8b)
- Measure: Optical density
- Compare exponential growth phase of bacteria (~20,000 to 40,000 seconds) using ANOVA & Tukey's test, by treatment as various combinations of factors (3.2.11). Estimated marginal means calculated are adjusted for unbalanced design. All dependent variables log transformed. Optical densities adjusted using minimum of each well of plate.
- Graphs and assess interaction type i.e. growth increase or reduction

Repeats: Pf-5 *n*=9, QST713 *n*=12

3.3.8.1 *Armillaria mellea CG440*

A previous experiment (3.3.6) demonstrated significant differences in *A. mellea* CG440 antagonism when *P. protegens* Pf-5 and *B. subtilis* QST713 were applied in combination with potassium phosphite. Therefore, CG440 growth data was split by bacteria for analysis (Marginal R^2 = 0.675-0.924, conditional R^2 = 0.974-0.985). There was a significant effect of media or interaction effect of media and day in each analysis (ANOVA P<0.001-0.031, Wald X^2 = 6.323-53.819). Pairwise comparisons showed significant CG440 growth inhibition on the phosphite amended medias compared to the unamended control media (Tukey's test P=<0.001-0.002, significant t-ratios = +4.360 to +13.846), but not between the different products (Tukey's test P=0.107-0.991, t-ratios = -0.130 to -2.180). This suggests the large difference in CG440 growth, between the control and amended media, may mask differences between growth impacts from the two products. Such a difference between the products is likely to be comparatively small.

Therefore, the control media was excluded from the analysis to elucidate the more subtle differences between the phosphite product formulations. Only in the absence of either BCA bacteria was there a significant difference in CG440 growth area between media amended with either product (Figure 16, ANOVA media & day interaction effect, no bacteria P=0.016, Wald X^2 = 5.828. Marginal R^2 = 0.831, conditional R^2 = 0.932). In pairwise testing by day, CG440 growth was significantly higher, by 18-26%, on the Phusion amended media on days 9-12 (Tukey's test P=0.001-0.007, t-ratios = -3.415 to -4.462), becoming non-significant at day 14 (Tukey's test P=0.053, t-ratio = -2.193, 13% higher). This increase could be due to a number of possibilities. Firstly, a higher concentration of potassium phosphite in Polyphosphite30 than Phusion, which caused greater growth inhibition. Secondly, a positive impact on CG440 growth by the siloxane surfactant/wetting agent present. This is perhaps via releasing surface tension of the media and making it easier for the fungus to spread and/or penetrate the media. Another possibility is reducing the impact of phosphite or a direct benefit to growth of the fungus. To elucidate any such mechanism, experiments with treatments containing the siloxane alone are required, which are out of the scope of this study. Further experiments should proceed with Polyphosphite30 rather than Phusion to avoid extraneous and superfluous complications. Prior experiments should also be repeated with Polyphosphite30 in place of Phusion for publication.

Regardless of the mechanisms involved, these results suggest that the previously observed growth inhibition from 'Phusion' at 1000ppm is smaller than would have been observed if Polyphosphite30 was used at 1000ppm.

Analysing CG440 growth area in the presence of each bacterial strain separately, and including the control media, no combination of bacteria and phosphite product yielded significantly increase CG440 growth inhibition compared to phosphite products alone (Tukey's test P>0.180, t-ratios = -0.931 to -2.066).

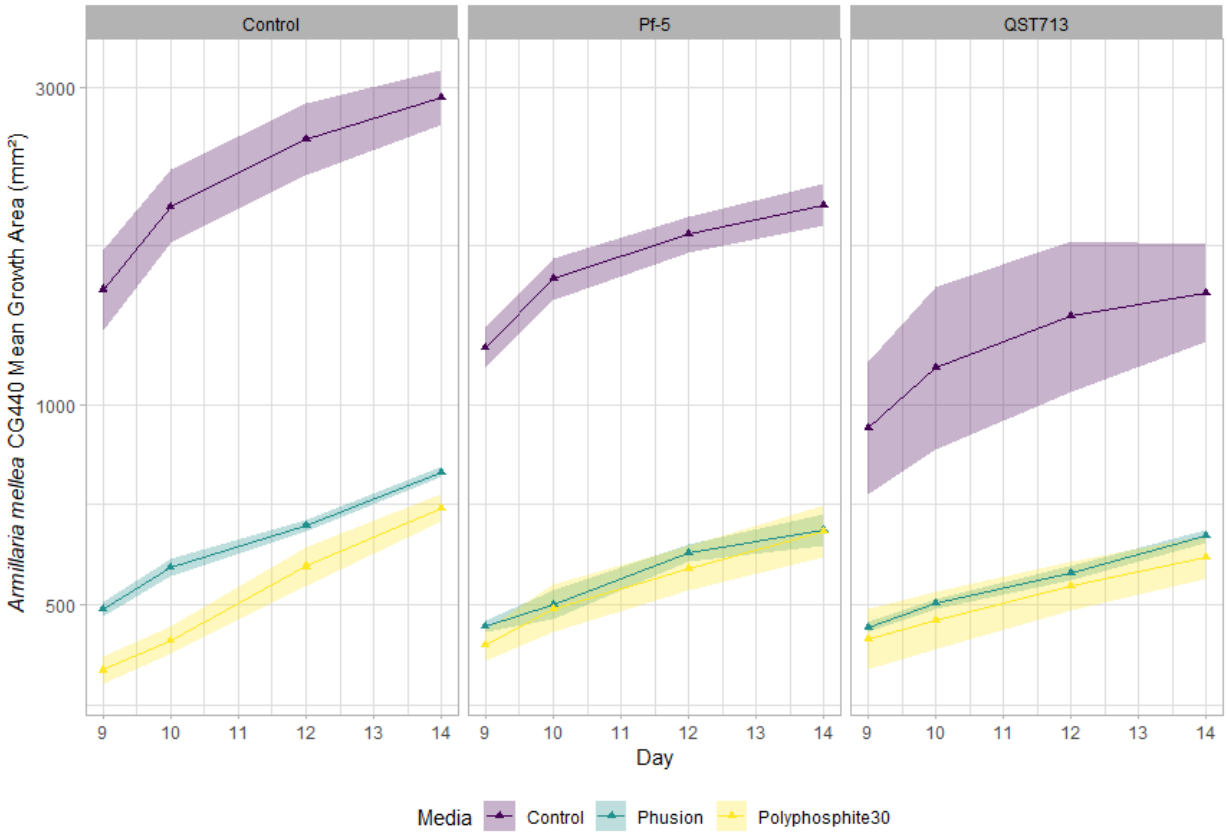

Figure 16. Mean growth area (mm²) of *Armillaria mellea* CG440 in antagonism assays with *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 on malt extract agar amended with 1000ppm Phusion or Polyphosphite30, over time since inoculation of the bacteria. Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Ribbons represent standard error ranges. Antagonists applied after 7 days *A. mellea* growth. $n \geq 4$.

3.3.8.2 BCA bacteria under antagonism

In terms of bacteria growth area, there was a significant interaction between all factors in the experiment, i.e. the presence of *A. mellea* CG440, media, bacterial strain and day (ANOVA, with bacteria paper-disc & KB control removed, interaction effect P=0.018, Wald X^2 = 8.008. Marginal R^2 = 0.697, conditional R^2 = 0.991). In pairwise comparisons of growth by media within each combination

of bacteria and presence/absence of CG440, *B. subtilis* QST713 under antagonism from CG440 had a significantly smaller growth area in the presence of Polyphosphite30 at all time-points (Tukey's test significant p-values <0.001, all other *P*>0.329, significant t-ratios = -4.336 & +3.905, Table 12, Figure 17). However, this did not significantly impact its antagonism of CG440 (3.3.8.1). This reduction in growth area could relate the reduced growth inhibition of CG440 by Phusion, as discussed above, and therefore increased pressure on QST713. Another possibility is an impact on the spreading/motility of QST713 from potassium phosphite e.g. the production of surfactin (Ghelardi *et al.*, 2012; Pandin *et al.*, 2018; Molina-Santiago *et al.*, 2019), which could be mitigated by the presence of the siloxane wetting agent/surfactant in Phusion. *P. protegens* Pf-5 growth area was not significantly affected by either phosphite product, corresponding with observations in earlier experiments (3.3.5, 3.3.6). Removal of the control media from the analysis did not significantly alter the results. A higher sample number may increase the confidence in these initial results.

Table 12. Mean growth area (mm^2) of *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 bacteria in antagonism assays with *Armillaria mellea* CG440 on malt extract agar amended with 1000ppm Phusion or Polyphosphite30, averaged days 9-14 since bacterial inoculation.

Bacteria	*Armillaria* CG440 Present	Media	Mean growth area (mm^2)
Pf-5	0	Control	262.6a (188.1, 366.6)
		Phusion	323.9a (232, 452.2)
		Polyphosphite30	354.6a (254, 495.1)
	1	Control	281.4a (222.3, 356.3)
		Phusion	319.4a (252.2, 404.3)
		Polyphosphite30	303.7a (239.9, 384.5)
QST713	0	Control	384.1a (275.2, 536.3)
		Phusion	424.9a (304.3, 593.1)
		Polyphosphite30	154.4b (110.6, 215.5)
	1	Control	156.4a (117.1, 208.8)
		Phusion	155.8a (123.1, 197.3)
		Polyphosphite30	122.8a (97.01, 155.5)

Presence of CG440, media, bacteria and day ANOVA interaction effect (bacteria control removed) *P*=0.018, Wald X^2 = 8.008. Marginal R^2 = 0.697, conditional R^2 = 0.991. Tukey's test significant p-values ≤0.001, all other *P*>0.329, significant t-ratios = -4.336 & +3.905. Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Letters indicate grouping of means, 95% confidence interval in brackets. Antagonists applied after 7 days *A. mellea* growth. $n \geq 3$.

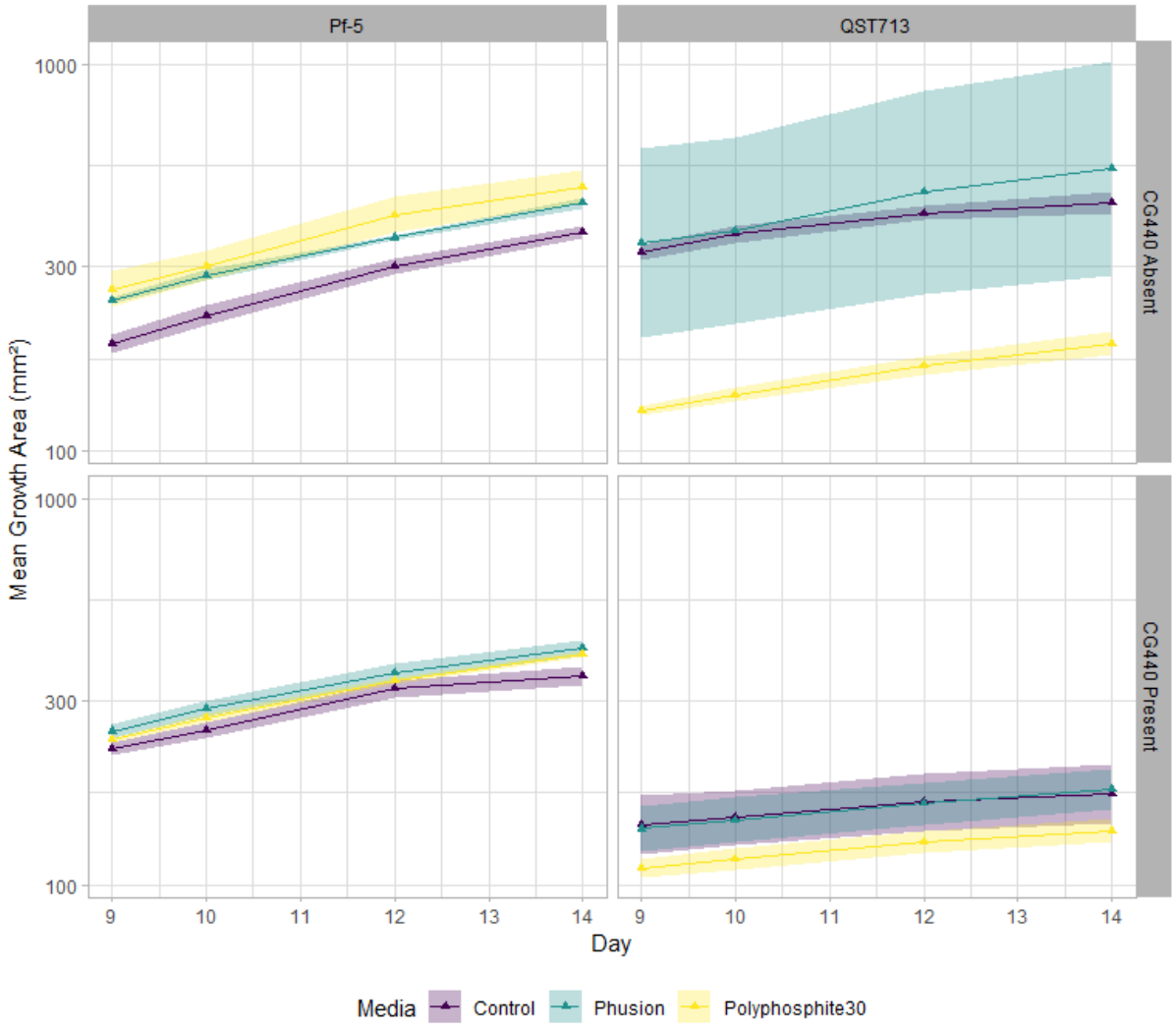

Figure 17. Mean growth area (mm^2) of *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 bacteria in antagonism assays with *Armillaria mellea* CG440 on malt extract agar amended with 1000ppm Phusion or Polyphosphite30, over time since inoculation. Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Ribbons represent standard error ranges. Antagonists applied after 7 days *A. mellea* growth. $n \geq 3$.

3.3.8.3 BCA bacteria planktonic growth

In the plate reader assay of the bacteria and phosphite products alone, there was a significant interaction effect of bacterial strain and phosphite product over time (ANOVA interaction effect $P<0.001$, Wald $X^2 = 21.446$. Marginal $R^2 = 0.576$, conditional $R^2 = 0.970$). For *B. subtilis* QST713, growth rates were significantly slower in the phosphite amended media. They were also significantly different between the products, with Phusion amended media being the slowest (Table 13, Tukey's test all p-values <0.003, t-ratios = +3.339 to +7.028). There were no such effects on the *P. protegens*

Pf-5 growth rate (Tukey's test all p-values >0.500, t-ratios = -0.977 to +1.092). In contrast to the results for the trends/growth rates, when means were compared over time there were no significant differences (Figure 18, Tukey's test all p-values >0.250, t-ratios = -0.966 to +1.592), suggesting a weak effect of the different phosphite products on QST713 growth overall. It is possible that under antagonism from *A. mellea*, the effect could be more pronounced, as was observed above (3.3.8.2). Phusion showed the greatest effect on planktonic growth rate, while it did not show an effect on colony growth. In contrast, Polyphosphite30 showed an impact on colony growth and a lesser impact on planktonic growth. This conflict between the two experiments is potentially due to the differences in product composition, i.e. the interaction of the siloxane surfactant/wetting agent with QST713 in the different culture environments. The results of this assay also reinforce previous observations that Pf-5 growth is not significantly impacted by potassium phosphite.

Table 13. Mean exponential growth rate (trend) of *Pseudomonas protegens* Pf-5 or *Bacillus subtilis* QST713 growing in king's broth amended with 1000ppm Phusion or Polyphosphite30

Strain	Media	Growth trend
Pf-5	Control	$4.664e^{-5}$a ($4.227e^{-5}$, $5.102e^{-5}$)
Pf-5	Phusion	$4.628e^{-5}$a ($4.19e^{-5}$, $5.066e^{-5}$)
Pf-5	Polyphosphite30	$4.973e^{-5}$a ($4.535e^{-5}$, $5.41e^{-5}$)
QST713	Control	$7.357e^{-5}$a ($6.978e^{-5}$, $7.736e^{-5}$)
QST713	Phusion	$5.436e^{-5}$c ($5.057e^{-5}$, $5.815e^{-5}$)
QST713	Polyphosphite30	$6.349e^{-5}$b ($5.97e^{-5}$, $6.728e^{-5}$)

Results from plate-reader assay (Tecan) at 28°C, 20000-40000 seconds growth, measuring every 20 seconds. Media, bacteria and time ANOVA interaction effect $P<0.001$, Wald $X^2 = 21.446$. Marginal $R^2 = 0.576$, conditional $R^2 = 0.970$. Tukey's test significant p-values <0.003, t-ratios = +3.339 to +7.028. Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Letters indicate grouping of means, 95% confidence interval in brackets. $n \geq 9$.

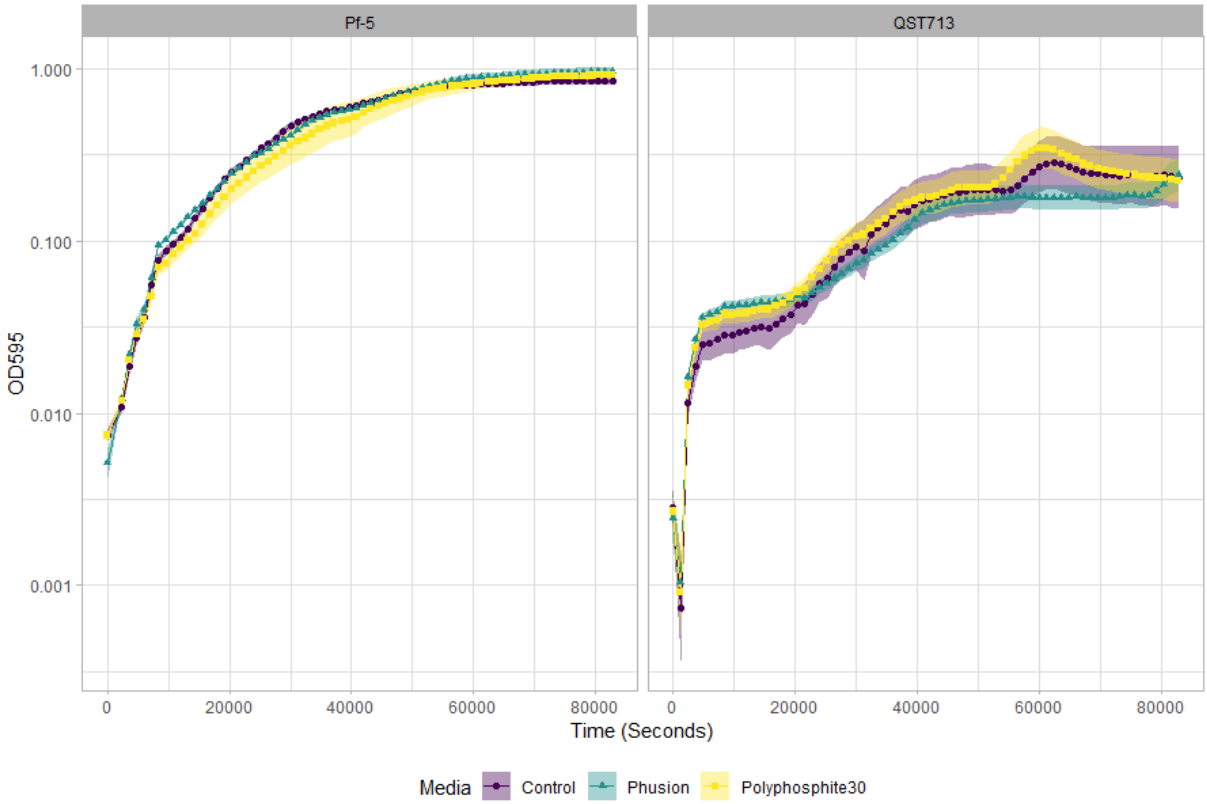

Figure 18. Optical density at 595nm of *Pseudomonas protegens* Pf-5 and *Bacillus subtilis* QST713 growing in king's broth amended with 1000ppm Phusion or Polyphosphite30, over time. Results from plate-reader assay (Tecan) at 28°C, 20000-40000 seconds growth, measuring every 20 seconds. Adjusted by minimum density of each well. Phusion, Orion Future Technologies Ltd., 80-90% volume potassium phosphite, 3% polyether modified trisiloxane, concentration estimated. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Ribbons represent standard error range. $n \geq 9$.

3.3.8.4 Closing comments

These experiments suggest that the use of Phusion in previous experiments may have modulated the impacts of potassium phosphite treatments on *A. mellea* and *B. subtilis* QST713 in comparison to the pure product. The next course of action was to prepare for *in planta* experiments, firstly by re-examining the dose-response of CG440 to the pure potassium phosphite.

3.3.9 *In vitro* growth inhibition of *A. mellea* CG440 by a range of concentrations of pure potassium phosphite.

This experiment aimed to investigate the impact of pure potassium phosphite on *A. mellea* CG440 *in vitro* in preparation for its use against this strain *in planta*, including at the concentration that would be applied to plants.

Specific Methodology

Armillaria mellea strains: CG440

Workflow:

- Amended Medias: Control, Potassium Phosphite (Polyphosphite30) 250, 500, 750, 100, 5000 mg/L (3.2.6).
- Antagonism Assay, Liquid inoculation, Typical incubation (3.2.1.2, 3.2.1.4b, 3.2.2, & 3.2.7)
- Digital images at days 0, 8, 10, 12, & 14, Scanner (3.2.3)
- Measure: Mean Growth Area using thresholding method
- ANOVA & Tukey's test, by concentration and day (3.2.11), concentration as categorical factor, growth areas log transformed.

Repeats: *n*=5

There were significantly different *A. mellea* CG440 growth areas between treatments (Figure 19) at all days aside from day 0 (Table 14, ANOVA interaction of concentration and day $P<0.001$, Wald $X^2 = 111.052$. Marginal $R^2 = 0.956$, conditional $R^2 = 0.969$. Tukey's test on means by day and concentration, and trend by concentration, significant p-values <0.037, non-significant p-values >0.140, significant t-ratios = +3.015 to + 12.280). A potassium phosphite concentration below 500mg/L did not significantly inhibit growth and concentrations of 750 and 1000mg/L produced similar results, suggesting a non-linear dose response (Table 14).

While 100% *in vitro* growth inhibition of *A. mellea* by potassium phosphite has been reported at 1000mg/L (Aguín *et al.*, 2006), it appears that this does not occur for CG440, even at 5000mg/L despite previous experiments indicating it is more sensitive to potassium phosphite than other strains tested (3.3.2, 3.3.7). 5000mg/L is a typical concentration for a potassium phosphite solution applied as a root drench to woody plants (Howard *et al.*, 2000). Therefore it would be expected that such an application would slow the growth of the fungi in treated soil/tissues but not inhibit it completely, especially as the concentration would decrease on infiltration through materials and with rainfall.

The next experiment will apply this recommended rate of potassium phosphite in combination with *B. subtilis* QST713 and *Trichoderma* treatments.

Table 14. *Armillaria mellea* CG440 mean growth area (mm²) on malt extract agar amended with a range of concentrations of potassium phosphite, days 0-14.

mg/L	Day 0	Day 8	Day 10	Day 12	Day 14	Overall Growth Rate/Trend
0	35.74a (28.69, 44.52)	321.2a (276.6, 372.9)	556a (478.9, 645.6)	962.7a (822.2, 1127)	1667a (1401, 1982)	0.2744a (0.2569, 0.292)
250	33.56a (27.22, 41.38)	305.3a (266.7, 349.4)	530.2a (461.6, 608.9)	920.7a (791.1, 1072)	1599a (1345, 1900)	0.2760a (0.2573, 0.2947)
500	31.07a (24.99, 38.62)	218.5b (187.7, 254.4)	355.8b (304.9, 415.1)	579.4b (491.4, 683.1)	943.5b (786.5, 1132)	0.2438ab (0.2259, 0.2617)
750	33.48a (26.93, 41.63)	209.7b (180.6, 243.4)	331.7b (285.7, 385.2)	524.7b (448, 614.6)	830.1bc (697.7, 987.7)	0.2293bc (0.2119, 0.2468)
1000	36.73a (29.49, 45.74)	189bc (162, 220.5)	284.6 b (243.7, 332.3)	428.6b (363.8, 504.9)	645.5c (539.5, 772.3)	0.2047c (0.1873, 0.2221)
5000	37.47a (30, 46.81)	142.3c (122.7, 165.1)	198.7c (171.5, 230.1)	277.3c (237.8, 323.4)	387.1d (327.3, 457.9)	0.1668d (0.1494, 0.1842)

ANOVA interaction of concentration and day P=<2e^{-16}, Wald X^2 = 111.052. Marginal R^2 = 0.956, conditional R^2 = 0.969. Tukey's test on means by day and concentration, and trend by concentration, significant p-values <0.037, non-significant p-values >0.140, significant t-ratios = +3.015 to + 12.280. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Letters indicate grouping of means, 95% confidence interval in brackets. *n*=5.

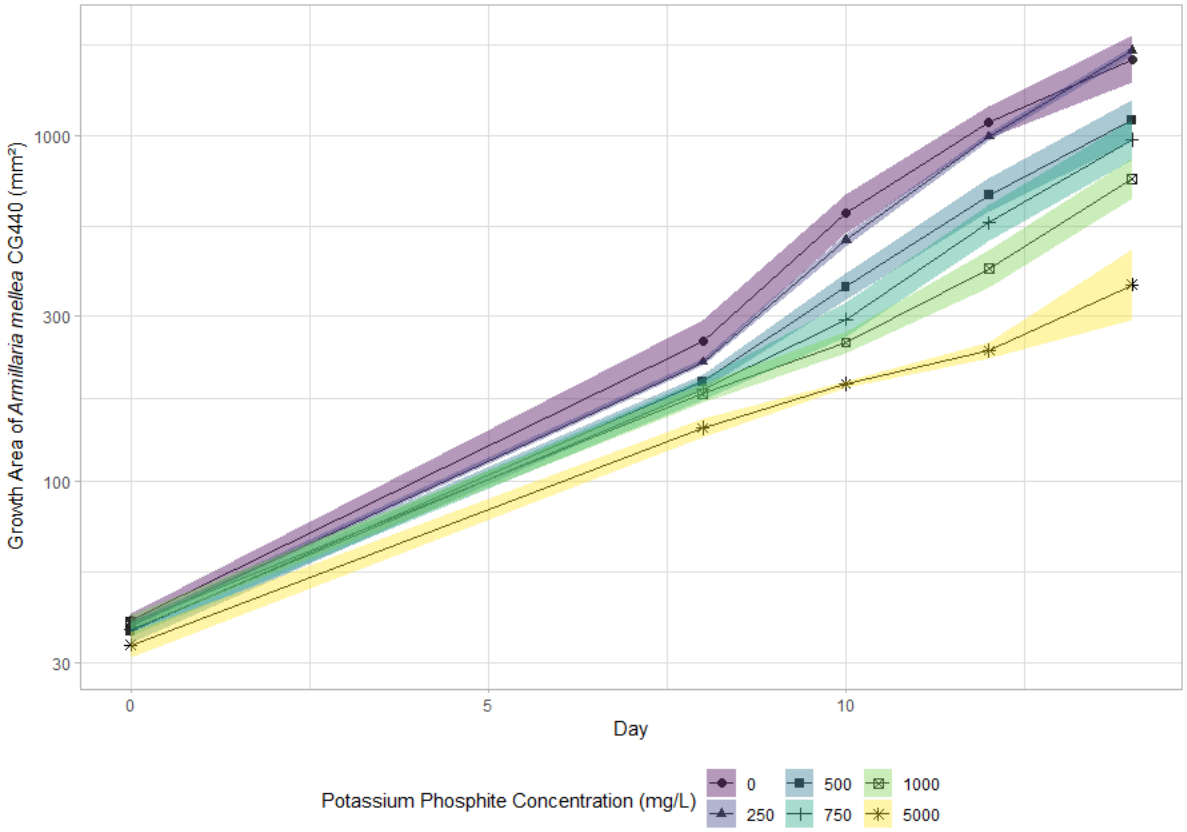

Figure 19. Mean growth area (mm²) of *Armillaria mellea* CG440 on malt extract agar amended with a range of concentrations of potassium phosphite over time. PolyPhosphite 30, Plant Food Company Inc., Cranbury, NJ 08512, USA, 56% w/w, 838.89 g/L, aqueous potassium phosphite. Ribbons represent standard error ranges. *n*=5.

3.3.10 *In planta* testing of potassium phosphite, *Bacillus* subtilis QST713, and *Trichoderma atroviride* T-15603.1 combinations for prevention of *A. mellea* CG440 infection

This large experiment was designed to test combinations of treatments studied *in vitro* above and those already established to have an impact on *Armillaria* infection, i.e. *Trichoderma* strains, as preventative treatments against the pathogen *in planta*. Infected and uninfected plants were included to control for potential health benefits of the treatments which might delay infection by increasing host resistance. Root collar infection was used as the only measure as this is a unique characteristic of *Armillaria* and mortality due to the fungus would be a key metric for assessing the efficacy of treatments against infection. The scale of the experiment, 448 plants, also made collecting any other measures very time consuming.

Specific Methodology

Armillaria mellea strains: CG440

Workflow:

- *In planta* pot trial, wild privet (*Ligustrum vulgare* L.) plants (3.2.9)
    - 28/6/2018 treatments applied.
        - Treatments consisted of all possible combinations (single treatments to all four, 16 treatments in total) of CG440 inoculum or dummy inoculation, 1g *Trichoderma atroviride* T-720 wheat grain inoculum (provided by Carbon Gold, Bristol, UK) buried 3cm from the stem and 3cm below the soil level, 100ml of 5000mg/L potassium phosphite as Polyphosphite30 diluted in tap water, 100ml of 10ml/L Serenade ASO (*Bacillus subtilis* QST713) diluted in tap water. Aqueous treatments were combined before application, and control treatments were also watered, so each plant received 100ml tap water.
- 19/7/2018 plants inoculated, on the opposite side of the pot to the applied *Trichoderma*.
- Measure: occurrence of root collar infection as plants died, destructive harvest in mid-August 2019.

Repeats: *n*=28

Unfortunately, root collar infection rates were so low as to prevent statistical analysis: only 2.2% of the inoculated plants were infected, with half of the inoculated treatments, including the control having no root collar infections at all. The amount of inoculum applied, a single acorn, may have been too small for successful infection. No root collar infections occurred in dummy inoculated treatments. There were 5 root collar infections in total, two occurred in the inoculated *Trichoderma atroviride* T-720 & *B. subtilis* QST713 treatment, and one in each of the inoculated potassium phosphite alone, QST713 alone, and potassium phosphite and QST713 combination treatments.

3.3.11 *In natura* testing of 'biochar' and chitin soil amendments as preventative treatments against *Armillaria* infection in a woodland

As described in the introduction (3.1), biochar and chitin have potential to stimulate host immune systems and therefore could reduce infection rates by *Armillaria* species. This experiment amended the planting pits of Scots pine (*Pinus sylvestris* L.) seedlings with these amendments, and a combination of the two, at typically recommended rates.

Specific Methodology

Armillaria strains: wild strain/s

Workflow:

- Woodland experiment using biochar and chitin soil amendments on Scots pine (*Pinus sylvestris* L.) seedlings (3.2.10)
- Measure: plant height and mortality at days 0, 238, 345, 598, and 996 following planting.
- Plant Height: ANOVA & Tukey's test, by biochar, chitin, site, and day (3.2.11), factorial design.
- Mortality: Kruskal-Wallis Rank Sum Test ('kruskal.test' function in R) on day 996 (final) mortality data by treatment.

Repeats: *n*=36

There were no significant impacts of biochar or chitin amendment on plant growth (ANOVA $P>0.200$, Wald $X^2 > 1.478$. Marginal $R^2 = 0.887$, conditional $R^2 = 0.910$). However, the interaction of chitin application, site and time may warrant further experimental investigation (ANOVA $P=0.063$, Wald $X^2 = 3.451$), potentially indicating a small and site-specific benefit of chitin application on plant growth (Figure 20, Site 1). If present, this could be from the release of nitrogen from the chitin as it degraded and may have been site specific due to different pre-existing soil conditions, i.e. lower nitrogen availability on Site 1.

There were no significant differences in mortality over both sites at day 996 (Kruskal-Wallis Rank Sum Test $P=0.882$, $X^2 = 0.662$). Plant deaths were associated with a period of drought in 2018 (Figure 21) and no mycelial fans were found when dead plants were inspected.

The results indicate that there were no significant differences in measurements between the different soil amendments, possibly because the level of natural infection was low. The heavy fruiting of *Armillaria*, including *A. ostoyae*, observed on both sites prior to planting does not seem to correlate with the fungus being highly active in the duration of the experiment. Reductions in carbohydrate and nitrogen levels, i.e. available nutrition, are associated with the induction of fruiting bodies in many mushroom forming fungi (Sakamoto, 2018). Although for *Armillaria in vitro* culture techniques for fruiting bodies suggest temperature reductions and changes in light levels are of

greater importance, e.g. a temperature reduction from 23°C to 14°C for *A. ostoyae* (Guillaumin & Botton, 2005; Ford *et al.*, 2015). Assuming a role of decreased nutrient availability in inducing the observed fruiting, it would suggest a reduction in resources for future host infections. Changes in environmental conditions following fruiting, such as drought, may have also adversely affected the fungus, e.g. there are some indications of a negative impact of drought on *Armillaria* inoculum and rhizomorphs (Heinzelmann *et al.*, 2019).

This experiment was mirrored in a controlled replication which is discussed in the following section.

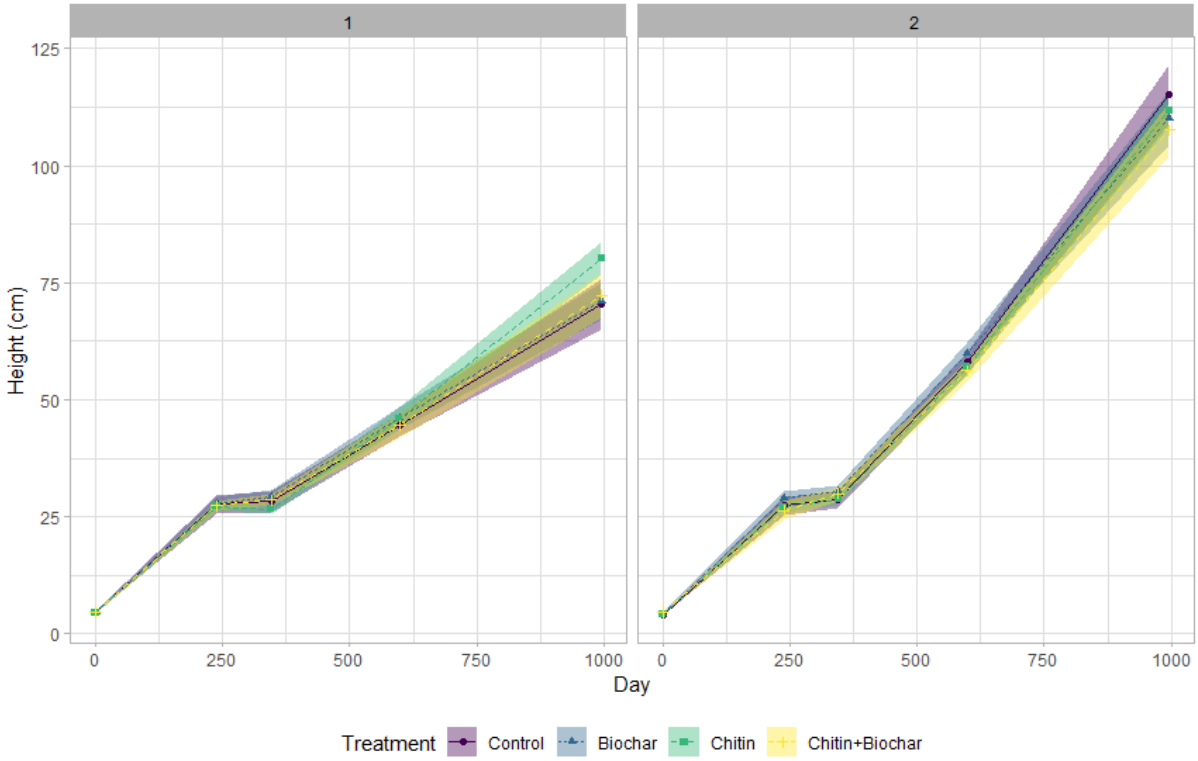

Figure 20. Mean height of Scots pine (*Pinus sylvestris* L.) seedlings growing in an *Armillaria* infected woodland in soils amended with different amendments at planting. Chitin, 1g/L soil, Tidal Vision, Alaska, USA. Biochar 5% soil volume, British softwood & hardwood mix, previously pre-soaked in tap water for 48 hours and drained, approximately 2-10mm^3 particle size, Carbon Gold, Bristol, UK. *n*=18.

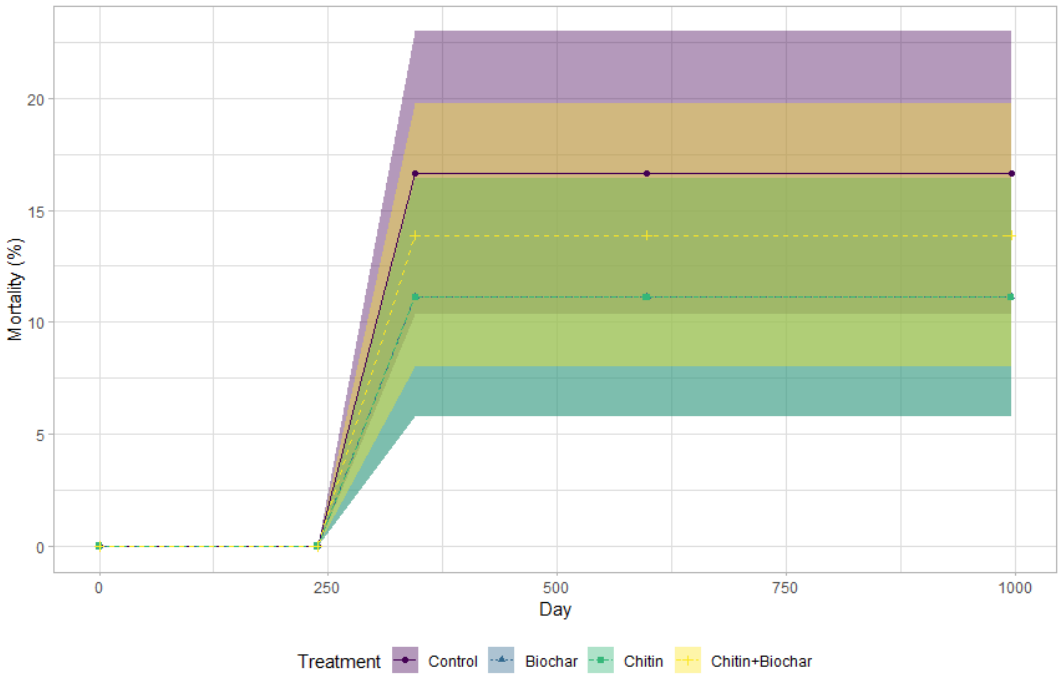

Figure 21. Mortality of Scots pine (*Pinus sylvestris* L.) seedlings growing in an *Armillaria* infected woodland in soils amended with different amendments at planting. Chitin, 1g/L soil, Tidal Vision, Alaska, USA. Biochar 5% soil volume, British softwood & hardwood mix, previously pre-soaked in tap water for 48 hours and drained, approximately 2-10mm^3 particle size, Carbon Gold, Bristol, UK. *n*=36.

3.3.12 *In planta* testing of 'biochar' and chitin soil amendments as preventative treatments against *Armillaria* infection

This experiment was intended to act as a controlled replication of the woodland trial (3.3.11), so comparisons could be made between the treatments in both natural and more tightly controlled conditions. In addition, dummy inoculated treated plants would provide control for any general health benefits outside of impacts on *Armillaria* infection.

Specific Methodology

Armillaria mellea strains: CG440

Workflow:

- In planta pot trial, Scots pine (*Pinus sylvestris* L.) plants (3.2.9)
- Treatments applied when plants potted:
    - Control
    - Chitin, 1g/L soil (Tidal Vision, Alaska, USA).
    - Biochar 5% soil volume (British softwood & hardwood mix, previously pre-soaked in tap water for 48 hours and drained, approximately 2-10mm^3 particle size, Carbon Gold, Bristol, UK: see 3.2.10).
    - Combination of both chitin and biochar, as above.
- 22/5/2018 plants inoculated, half inoculated with CG440 and the other half dummy inoculated (8 treatments total).
- Measure: occurrence of root collar infection as plants died, destructive harvest in mid-August 2019.

Repeats: *n*=24

As found in the previously described pot trial (3.3.10), the levels of *A. mellea* CG440 root collar infection occurring were very low and only three occurrences were recorded, equalling 3.1% of all inoculated plants. Again, this is likely due to low inoculum pressure or low performance of the inoculum. None of the dummy inoculated plants were infected. One root collar infection occurred in the infected control and two in the infected chitin alone treatment. This small number means there was not enough statistical power for analysis and any inferences drawn e.g. that the biochar amendment reduced infection, would be highly tenuous.

3.4 Discussion

3.4.1 Plant Defence Activators

Of those tested in this study, potassium phosphite is the most effective single treatment against *Armillaria mellea*. This has been demonstrated in multiple experiments, (3.3.1, 3.3.2, 3.3.6, 3.3.7, 3.3.8), and on multiple strains of *A. mellea*: CG440, CG447, & CG581 (3.3.2, 3.3.7). Over the experiments and strains, growth inhibition was around 70-77% after 21 days of growth on agar amended with 750-1000mg/L potassium phosphite. Other authors have also shown impacts on other strains *in vitro* and another species *in vivo* via trunk injection (Heaton & Dullahide, 1990; Aguín *et al.*, 2006). The active dosage at which potassium phosphite impacts *A. mellea* growth, is above 250mg/L and well below the 5000 mg/L dose which is regarded as being widely phytotoxic (Deliopoulos *et al.*, 2010). Application rate is however unlikely to reflect the actual soil concentration following treatment, as the phosphite solution would be distributed through the soil. As suggested by the results of these experiments, even at such lower concentrations, potassium phosphite can reduce *A. mellea* growth and may still directly provoke a stress reaction in the pathogen which could reduce its infective capacity by increasing its detection by host defences (1.11.2).

Oligochitosan also showed some inhibition of *Armillaria* growth in the experiments, but far less than potassium phosphite. *A. mellea* CG581 also appeared tolerant of the compound alone (3.3.2). Oligochitosan may also have earlier activity than potassium phosphite (3.3.2, 3.3.6). The combination of these two plant defence activators consistently gives slightly increased growth inhibition in comparison to potassium phosphite alone but is not significantly different. There may also be earlier effects as for oligochitosan alone. Due to the comparatively small and mixed performance of oligochitosan and its combination with potassium phosphite (3.3.1, 3.3.2, 3.3.6), as well as potential interference in the establishment of BCA bacteria (3.3.6), it was decided to halt studies using the compound.

Strain of *A. mellea* has an impact on the response to potassium phosphite and oligochitosan *in vitro*, albeit with general trends (3.3.2, 3.3.7). This is a level of detail that few other authors have considered. It is of potential value given the apparent total resistance of *A. mellea* CG581 to oligochitosan and the increased sensitivity of CG440 to potassium phosphite in comparison to the other two strains tested. This indicates that such tolerances and sensitivities commonly vary strain to strain given the small amount of strains surveyed. Further strengthening this reasoning, other authors have also seen variable growth inhibition/stimulation from single antimicrobial compounds depending on *Armillaria* strain/isolate (Beal *et al.*, 2015). Differences in sensitivity may explain some of the difference in results from potassium phosphite these experiments and that of Aguín et al.

(2006) where growth was totally inhibited. However, even apparently phosphite sensitive CG440 was not inhibited at 5000mg/L potassium phosphite, a concentration five times that used by Aguín et al. (2006).

3.4.2 Bacterial antagonists

Both *P. protegens* Pf-5 and *Bacillus subtilis* QST713 consistently reduced *A. mellea* colony size *in vitro* on multiple strains, with either having a similar impact (3.3.3, 3.3.4, 3.3.7, 3.3.8). In these experiments, *in vitro* growth inhibition from either isolate was typically around 40-45%, although there were apparent variations in sensitivity to each by between *A. mellea* strains. While a minimum number of different strains were used in the experiments of this study, they do provide initial evidence that *A. mellea* strain is a significant factor in its antagonism by BCA bacteria. A wider study is therefore advisable in the development of any BCA bacteria for use against *A. mellea*, given the pathogen's aforementioned recorded variability in response to antifungal compounds (Beal *et al.*, 2015).

For Pf-5 the typically observed growth inhibition is greater than previously reported for *A. mellea* 05BV: 15-31% (Pellegrini *et al.*, 2012). This suggests a wider natural variation by *A. mellea* strain in sensitivity to antagonism by Pf-5 than observed here. *In vitro A. mellea* growth inhibition twice that typically observed in this study or more, around 80-90%, has been recorded from other species and strains of *Bacillus* and *Pseudomonas* (Mesanza *et al.*, 2016). They also went on to reduce mortality and infection in inoculated plants. These strains were selected from a healthy tree in an infected area and suggests that this bioprospecting technique may yield highly antagonistic strains in comparison to those selected elsewhere e.g. Pf-5 and QST713, corresponding with prior discussion (2).

Bacillus subtilis QST713 may have a large practical advantage over Pf-5 and many other strains used experimentally: this strain is already commercialised and registered for use in many places, e.g. being licenced in the UK for application as a soil drench for *Phytophthora* (1.9). It also readily forms biofilms which are likely to enhance root colonization and persistence (Pandin *et al.*, 2018). In addition, Pf-5 has recorded control activity for insects (Kupferschmied *et al.*, 2013), therefore has potential non-target/ecological risks.

Combinations of *Pseudomonas* strains including with Pf-5 did not provide enhanced control over Pf-5 strain alone (3.3.4), although this may differ on a host plant due to colonization ability etc. Increased growth inhibition was also not apparent when Pf-5 and QST713 were combined. This could be due to competition between strains, e.g. Pf-5 outcompeted the others, or that its impacts were still similar at a reduced population level. Both Pf-5 and QST713 produce a multitude of antibiotic compounds (Mercado-Blanco, 2015; Pandin *et al.*, 2018) indicating that this characteristic is important for

successful antagonism of *A. mellea*. Specific mixtures of antibiotic compounds may be more important than any one compound, especially given the generalist nature of *Armillaria* species.

A number of the *Pseudomonas* strains used have had their genetic sequences analysed for genes coding for the production of antagonistic compounds (Table 15)(Calderón *et al.*, 2015). Again, the wide range of antimicrobials produced by Pf-5 is the most striking feature of the only significant antagonist. 2,4-diacetylphloroglucinol (DAPG) or hydrogen cyanide (HCN) production are potential causes of the zone of inhibition observed from both Pf-5 and F113 (3.3.3). Assuming no differences in production levels of these antagonistic compounds and that one specific compound was effective, then there are a number of potential candidates: rhizoxins, pyoluteorin, pyrrolnitrin, pyochelin, or orfamide. The antimicrobial antibiotic volatiles, rhizoxins, pyoluteorin, and pyrrolnitrin, may be more likely candidates, given the overall reduction in growth rather than just facing the bacterial colony, as these compounds may have spread through the media/air within the petri dishes more quickly/easily in comparison to larger and non-volatile compounds. If spreading through the air, air-tight dishes may show quicker impacts, or gas samples could be analysed. Further testing with purified antifungals or knock-out mutants could be to used confirm the pathogen's sensitivity to particular compounds or mixtures of them. Multiple isolates, and perhaps other species, would need to be used to demonstrate the robustness of control as *Armillaria* strains can show great variation in reaction to antimicrobial compounds (Beal *et al.*, 2015). Phytotoxicity has been shown for DAPG, pyoluteorin, phenazines, and viscosinamide and so should be a factor monitored for in field applications (Haas & Keel, 2003). QST713 produces a very different range of antimicrobials (Pandin *et al.*, 2018) and the similar impact of Pf-5 and QST713 on *A. mellea* growth is testament to the resistance of the fungus to many different antibiotic compounds.

Table 15. Detection of genes for production of antagonistic compounds present in the analyzed sequences of selected *Pseudomonas* spp. Adapted from Calderón *et al.* 2015.

	Antimicrobial antibiotic volatiles							Cyclic-lipopeptides		Siderophores			
Strain	2-hexyl, 5-propyl resorcinol	2,4-diacetylphloroglucinol	Pyrrolnitrin	Hydrogen Cyanide	Phenazine	Pyoluteorin	Rhizoxins	Orfamide	Viscosin	Pyoverdine	Pyochelin	Achromobactin	Hemophore
Pf-5	–	+	+	+	–	+	+	+	–	+	+	–	+
Pf0-1	–	–	–	+	–	–	–	–	–	+	–	–	–
F113	–	+	–	+	–	–	–	–	+	+	–	–	+
SBW25	–	–	–	–	–	–	–	–	+	+	–	–	+

A caveat of the *in vitro* results of this study is the temperature they were carried out at. Temperature has a strong influence on antagonism by and growth of *Pseudomonas* (Nüske & Fritsche, 1989; Rainey *et al.*, 1991; Ullrich *et al.*, 1995; Schmidt *et al.*, 2004; Humair *et al.*, 2009) and *Bacillus subtilis* (Gupta & Utkhede, 1986; Fiddaman & Rossall, 1993; Ongena & Jacques, 2008). Temperate trees generally root within the top 5m of soils, with 50-60% of root biomass in the top 30cm of soil and the top 15cm being a key zone for the formation of essential small absorbing roots, in covered soils (Harris, 1992; Canadell *et al.*, 1996; Jackson *et al.*, 1996). Therefore, it may be appropriate to repeat experiments at a typical soil temperature. The majority of UK annual mean soil temperatures, at 30cm depth, 1981-2010, were 10-11°C (Met Office *et al.*, 2017). A more sophisticated study might investigate the impact of a fluctuating temperature regime on antagonist by mimicking the daily fluctuations in rhizosphere temperature within the apparent optimal infection windows for *Armillaria*: early spring and autumn (Perez-Sierra, 2004).

3.4.3 Combination of bacterial antagonists and plant defence activators

The most effective combination of plant defence activator and antagonist against the CG440 model strain *in vitro* was *Bacillus subtilis* QST713 and potassium phosphite (3.3.6, 3.3.7). Similar significant effects were apparent averaging over three *A. mellea* strains (CG440, CG447, CG581) with 79% inhibition compared to 75% from potassium phosphite alone (3.3.7). As discussed previously, CG440

may be more sensitive to potassium phosphite which could influence its interaction with QST713. Also, the treatment combination did not always show significantly greater control on CG440 in experiments (3.3.8.1), potentially due to the more subtle effect of the combination in comparison to potassium phosphite alone.

Plant mediated effects are presumed to be a large influence upon efficacy in control of the pathogen. Therefore, the 4% increase in *A. mellea* direct growth inhibition yielded by the combination over potassium phosphite alone may be amplified. However, there will also be many other factors involved such as establishment of antagonist populations. Other authors note frequent disparity in results when moving from *in vivo* growth inhibition to working with host plants (Raziq & Fox, 2003; Mesanza *et al.*, 2016). Although, some isolates have shown similar *in vitro* and *in planta* control of *A. mellea* (Mesanza *et al.*, 2016). It is possible that the efficacy of phosphite salts against the pathogen will be even greater on host plants in comparison to *in vitro* testing, as has been found by other authors (Simonetti *et al.*, 2015).

Although inferring results outside of the specific interaction of strains is unreliable (1.10), there are some indications from other studies that the combined treatments may have provided increased control. *Bacillus*-phosphite salt combinations, including *B. subtilis* QST713 and potassium phosphite have shown increased *in planta* control of other diseases over their constituents alone (Simonetti *et al.*, 2015; Bahadou *et al.*, 2017), as have *Trichoderma*-phosphite (Figueira *et al.*, 2020), and *Bacillus*-*Trichoderma* combinations (Triveni *et al.*, 2015; Zaim *et al.*, 2018; Wang *et al.*, 2019; Karuppiah *et al.*, 2020). Although the impact of phosphite salts on the interaction of the two species is unknown.

In terms of direct interaction between potassium phosphite and antagonists, experiments using a plate reader showed either relatively subtle or no significant impacts of potassium phosphite on *P. protegens* Pf-5 or *B. subtilis* QST713 growth (3.3.5, 3.3.8.3). Results on agar for Pf-5 were similar (3.3.8.2): in general this strain's growth does not appear to be impacted by potassium phosphite at around 1000 mg/L or antagonism from *A. mellea*. There is some evidence to suggest that potassium phosphite can reduce the growth of QST713 on agar in the absence of *A. mellea* antagonism (3.3.8.2). However, under *A. mellea* antagonism QST713 growth was not significantly affected by the presence of potassium phosphite, which suggests *A. mellea* antagonism alone causes a greater reduction. BCA antagonism of *A. mellea* CG440 was also not significantly different between the products (3.3.8.1). If potassium phosphite does impact surfactin production as previously discussed (3.3.8.2), then it could be of wider scientific merit to investigate its impact on QST713 and Pf-5 combinations, as surfactin may be important in ecological interactions between *Pseudomonas* and *Bacillus* strains (Molina-Santiago *et al.*, 2019).

Potassium phosphite, while not significantly influencing growth of Pf-5 did appear to reduce its antagonistic capacity, or at least totally negate its impact as no increase in control was shown when the two were combined. Reduction in antagonistic capability was shown for another *Pseudomonas* bio-control strain applied in combination with manganese phosphite (Simonetti *et al.*, 2015). That study found that the *Bacillus* and phosphite combination performed equally as well as the *Pseudomonas* alone, while the results found in this study show the potential for the QST713 and potassium phosphite combination to be a more effective control than Pf-5 alone. Phosphite salts have shown a control effect on *Pseudomonas* phytopathogens e.g. horse chestnut bleeding canker (Percival & Banks, 2014), therefore these results (3.3.6) could reflect a facet of their mode of action i.e. reduction of antagonistic potential either against the host plant or its endophytes.

The imprecise concentration and surfactant/wetting agent in the Phusion product used in many of the experiments described poses an issue in the significance of the results in this study. This is due to the differences observed in the responses of *A. mellea* CG440 and QST713 between Phusion and Polyphosphite30, an aqueous potassium phosphite product. As previously stated, it would be prudent to repeat experiments prior to publication with Polyphosphite30 or an equivalent. However, results were similar enough between the two products to assume that these differences are minor and the observations with Phusion will, in general, reflect those with a pure potassium phosphite. Conversely, it is worth acknowledging the potential influence of adjuvants in PDA and BCA combinations.

Repetition of whole experiments would also help bring much of the data to a publishable standard and enhance the statistical output, as typically they were only carried out once. It would also be appropriate to use the liquid culture method with weighing (3.2.1.4b) within the work as this appears to give very regular growth of *Armillaria* colonies.

3.4.4 *In planta* experiments

The prior experiments yielded a potential PDA and BCA combination, *B. subtilis* QST713 and potassium phosphite, for trials on host plants. When this was attempted the inoculation failed on the majority of plants. Similar issues with low infection rates negated results from the trials of biochar and chitin soil amendments (3.3.11, 3.3.12). Therefore work is required to improve protocols for the successful artificial inoculation of host plants. This is the next step in this project and is required before any further *in planta* testing can take place.

3.4.5 Improvements in technique

During the course of the project so far, a number of improvements were made to the methodologies used. The use of incubators located within cold stores or climate controlled rooms stabilized temperatures and increased replicability between experiments.

Liquid culture techniques (3.2.1.4) based on those used by other authors (Baumgartner *et al.*, 2010; Pellegrini *et al.*, 2012), especially when weighing was used (3.2.1.4b), have proved highly reliable in comparison to more conventional techniques. Traditional techniques are based on using plugs cut from mycelium growing on agar. Selecting these from similar sections of *Armillaria* colonies, e.g. in terms of thickness and therefore initial inoculum volume, was often not possible. Initial experiments were plagued by repeats which did not establish as well as others despite no evident contamination. The equipment used in inoculating plates is also more reliably sterilised and easily handled with the liquid technique: disposable pipette tips are easily sterilized and changed between plates whereas cork-borers and tweezers etc. must be repeatedly flamed using ethanol. Over time such tools became sooty and rusted, which may increase the risk of contamination.

It would appear that 'liquid' inoculations establish without fail in typical experimental use. Growth of colonies is also more even and there is no time period waiting for the fungus to move onto the media from the plug. Despite some visual variation in the density of inoculum applied, colonies are of a relatively uniform area with treatments. The culture conditions used for the submerged culture are somewhat different to those used by other authors: use of smaller culture vessels, higher rpms and a higher temperature, 27°C in comparison to 25°C, still provides a very useable level of fungal growth but means that bacterial antagonist cultures can also be produced in the same conditions. These techniques also offer convenient methods of producing fungal lawn plates and of producing inoculum, where homogenized mycelium can be spread over the entire surface of solid media or prepared woody tissues.

A comparison of traditional and liquid culture techniques could not be found in the published literature, with few papers using the submerged culture technique at all. Therefore a comparison of these techniques is required and will be carried out in the following section of this project.

Digital measurements from scanned images have yielded good quality results. Those taken with cameras are likely to have suffered artefacts from differing angles between images. Measurements of length and area are easily collected and processed in comparison to using calipers to measure growth diameters. Transcription of data between notes and digital datasets is avoided and so reduces the chance of error.

Semi-automated measurement of area via 'thresholding' in 'ImageJ' software works very well in the majority of cases. A dark background is used during image collection as this contrasts with healthy white mycelium. However, *Armillaria* releases dark stress metabolites, which in severe cases can make measurement difficult from the images, especially as they must be converted to greyscale images in the current method. It may be possible to use specific colour ranges for automated thresholding, although this might take such a degree of development as to be outside the scope of this project.

3.4.6 Closing Comments

Time constraints mean that no further work could be carried out on PDA & BCAs for *A. mellea* control. However, the work in this chapter and the issues faced within it inspired the studies following it. As mentioned above, the next steps were to compare the traditional agar culture and submerged culture methodologies empirically and to improve protocols for the artificial inoculation of host plants.

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3.6 References

Aguín O, Mansilla JP, Sainz MJ. **2006**. In vitro selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapevine in the field. *Pest management science* **62**: 223–8.

Al-Wabel MI, Hussain Q, Usman ARA, Ahmad M, Abduljabbar A, Sallam AS, Ok YS. **2018**. Impact of biochar properties on soil conditions and agricultural sustainability: A review. *Land Degradation and Development* **29**: 2124–2161.

Bahadou SA, Ouijja A, Boukhari M, Tahiri A. **2017**. Development of field strategies for fire blight control integrating biocontrol agents and plant defense activators in Morocco. *Journal of Plant Pathology* **99**: 51–58.

Baumgartner K, Bhat R, Fujiyoshi P. **2010**. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal biology* **114**: 107–19.

Beal EJ, Henricot B, Peace AJ, Waghorn IAG, Denton JO. **2015**. The action of allicin against *Armillaria* spp. *in vitro*. *Forest Pathology* **45**: 450–458.

Beckman TG, Pusey PL. **2001**. Field testing peach rootstocks for resistance to *Armillaria* root rot. *HortScience* **36**: 101–103.

Calderón CE, Ramos C, de Vicente A, Cazorla FM. **2015**. Comparative genomic analysis of Pseudomonas chlororaphis PCL1606 reveals new insight into antifungal compounds involved in biocontrol. *Molecular Plant-Microbe Interactions* **28**: 249–260.

Canadell J, Jackson R, Ehleringer J, Mooney HA, Sala OE, Schulze E-D. **1996**. Maximum rooting depth of vegetation types at the global scale. *Oecologia* **108**: 583–595.

Chemicals Regulation Directorate. **2021**. Pesticides - Databases Home Page. https://www.hse.gov.uk/pesticides/databases/index.htm

Deliopoulos T, Kettlewell PS, Hare MC. **2010**. Fungal disease suppression by inorganic salts: A review. *Crop Protection* **29**: 1059–1075.

Dumas MT. **1992**. Inhibition of *Armillaria* by bacteria isolated from soils of the boreal mixedwood forest of Ontario. *European Journal of Forest Pathology* **22**: 11–18.

Fan B, Blom J, Klenk HP, Borriss R. **2017**. *Bacillus amyloliquefaciens, Bacillus velezensis,* and *Bacillus siamensis* Form an 'Operational Group *B. amyloliquefaciens*' within the *B. subtilis* species complex. *Frontiers in Microbiology* **8**: 1–15.

Fiddaman PJ, Rossall S. **1993**. The production of antifungal volatiles by *Bacillus subtilis*. *Journal of Applied Bacteriology* **74**: 119–126.

Figueira EPP, Kuhn OJ, Martinazzo-Portz T, Stangarlin JR, Pereira MDP, Lampugnani C. **2020**. Histochemical changes induced by *Trichoderma* spp. and potassium phosphite in common bean (*Phaseolus vulgaris*) in response to the attack by *Colletotrichum lindemuthianum*. *Semina: Ciências Agrárias* **41**: 811.

Ford KL, Baumgartner K, Henricot B, Bailey AM, Foster GD. **2015**. A reliable *in vitro* fruiting system for *Armillaria mellea* for evaluation of *Agrobacterium tumefaciens* transformation vectors. *Fungal Biology* **119**: 859–869.

Ford KL, Henricot B, Baumgartner K, Bailey AM, Foster GD. **2017**. A faster inoculation assay for *Armillaria* using herbaceous plants. *The Journal of Horticultural Science and Biotechnology* **92**: 39–47.

Fox RTV, McQue AM, Obore JO. **1991**. Prospects for the integrated control of *Armillaria* root rot of trees. In: Biotic Interactions and Soil-borne Diseases. Amsterdam: Elsevier Science Publishers, 154–159.

Frenkel O, Jaiswal AK, Elad Y, Lew B, Kammann C, Graber ER. **2017**. The effect of biochar on plant diseases: what should we learn while designing biochar substrates? *Journal of Environmental Engineering and Landscape Management* **25**: 105–113.

Ghelardi E, Salvetti S, Ceragioli M, Gueye SA, Celandroni F, Senesi S. **2012**. Contribution of surfactin and SwrA to flagellin expression, swimming, and surface motility in *Bacillus subtilis*. *Applied and Environmental Microbiology* **78**: 6540–6544.

Gortari MC, Hours RA. **2008**. Fungal chitinases and their biological role in the antagonism onto nematode eggs. A review. *Mycological Progress* **7**: 221–238.

Graber ER, Frenkel O, Jaiswal a. K, Elad Y. **2014**. How may biochar influence severity of diseases caused by soilborne pathogens? *Carbon Management* **5**: 169–183.

Guillaumin J-J, Botton B. **2005**. Ontogénie des armillaires. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 151–175.

Gupta VK, Utkhede RS. **1986**. Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. *Journal of Phytopathology* **117**: 9–16.

Haas D, Keel C. **2003**. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual review of phytopathology* **41**: 117–153.

El Hadrami A, Adam LR, El Hadrami I, Daayf F. **2010**. Chitosan in plant protection. *Marine drugs* **8**: 968–87.

Hailey LE, Percival GC. **2015**. The Influence of long term flooding on tree biology and approaches to flood stress alleviation and management. *Arboricultural Journal* **37**: 135–149.

Harris RW. **1992**. *Arboriculture: integrated management of landscape trees, shrubs, and vines.* Prentice-Hall International.

Heaton JB, Dullahide SR. **1990**. Efficacy of phosphonic acid in other host pathogen systems. *Australasian Plant Pathology* **19**: 133–134.

Heinzelmann R, Dutech C, Tsykun T, Labbé F, Soularue J-P, Prospero S. **2019**. Latest advances and future perspectives in *Armillaria* research. *Canadian Journal of Plant Pathology* **41**: 1–23.

Hill RM. **1997**. Siloxane surfactants. In: Robb ID, ed. Specialist Surfactants. Dordrecht: Springer Netherlands, 143–168.

Hood IA, Redfern DB, Kile GA. **1991**. *Armillaria* in Planted Hosts. In: Shaw CG, Kile GA, eds. *Armillaria* Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 122–149.

Howard K, Dell B, Hardy GE. **2000**. Phosphite and mycorrhizal formation in seedlings of three Australian Myrtaceae. *Australian Journal of Botany* **48**: 725–729.

Humair B, González N, Mossialos D, Reimmann C, Haas D. **2009**. Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0. *The ISME journal* **3**: 955–965.

Jackson RB, Canadell J, Ehleringer JR, Mooney HA, Sala OE, Schulze ED. **1996**. A global analysis of root distributions for terrestrial biomes. *Oecologia* **108**: 389–411.

James G. **2010**. Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. In: Schuller M, Sloots TP, James GS, Halliday CL, Carter IWJ, eds. PCR for Clinical Microbiology: An Australian and International Perspective. Dordrecht: Springer Netherlands, 209–214.

Karuppiah V, Li Y, Sun J, Vallikkannu M, Chen J. **2020**. Vel1 regulates the growth of *Trichoderma atroviride* during co-cultivation with *Bacillus amyloliquefaciens* and is essential for wheat root rot control. *Biological Control* **151**: 104374.

Kibby G. **2021**. The genera *Armillaria* and *Desarmillaria* in Britain. *Field Mycology* **22**: 29–32.

Kim SK, Rajapakse N. **2005**. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers* **62**: 357–368.

Kupferschmied P, Maurhofer M, Keel C. **2013**. Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Frontiers in Plant Science* **4**: 1–17.

Lahlali R, Peng G, Gossen BD, Mcgregor L, Yu F, Hynes R, Hwang S-F, McDonald MR, Boyetchko S. **2012**. Evidence that the biofungicide Serenade® (*Bacillus subtilis*) suppresses clubroot on canola via antibiosis and induced host resistance. *Phytopathology* **103**: 121031093352002.

de Medeiros EV, Lima NT, de Sousa Lima JR, Pinto KMS, da Costa DP, Franco Junior CL, Souza RMS, Hammecker C. **2021**. Biochar as a strategy to manage plant diseases caused by pathogens inhabiting the soil: a critical review. *Phytoparasitica*.

Meng Q, Jiang H, Hao JJ. **2016**. Effects of *Bacillus velezensis* strain BAC03 in promoting plant growth. *Biological Control* **98**: 18–26.

Mercado-Blanco J. **2015**. *Pseudomonas* strains that exert biocontrol of plant pathogens. In: *Pseudomonas*. Dordrecht: Springer Netherlands, 121–172.

Mesanza N, Iturritxa E, Patten CL. **2016**. Native rhizobacteria as biocontrol agents of *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*. *Biological Control* **101**: 8–16.

Met Office, Hollis D, McCathy M. **2017**. *UKCP09: Met Office gridded and regional land surface climate observation datasets. Centre for Environmental Data Analysis.*

Molina-Santiago C, Pearson JR, Navarro Y, Berlanga-Clavero MV, Caraballo-Rodriguez AM, Petras D, García-Martín ML, Lamon G, Haberstein B, Cazorla FM, *et al.* 2019. The extracellular matrix protects *Bacillus subtilis* colonies from *Pseudomonas* invasion and modulates plant co-colonization. *Nature Communications* **10**.

Niu B, Wang W, Yuan Z, Sederoff RR, Sederoff H, Chiang VL, Borriss R. **2020**. Microbial interactions within multiple-strain biological control agents impact soil-borne plant disease. *Frontiers in Microbiology* **11**: 1–16.

Nüske J, Fritsche W. **1989**. Phaseolotoxin production by *Pseudomonas syringae* pv. *phaseolicola*: The influence of temperature. *Journal of Basic Microbiology* **29**: 441–447.

Ongena M, Jacques P. **2008**. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology* **16**: 115–125.

Pandin C, Le Coq D, Deschamps J, Védie R, Rousseau T, Aymerich S, Briandet R. **2018**. Complete genome sequence of *Bacillus velezensis* QST713: A biocontrol agent that protects *Agaricus bisporus* crops against the green mould disease. *Journal of Biotechnology* **278**: 10–19.

van der Pas JB. **1981**. A statistical appraisal of *Armillaria* root rot in New Zealand plantations of *Pinus radiata*. *New Zealand Journal of Forestry Science* **11**: 23–36.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2012**. Studying trophic interactions between a plant pathogen and two different antagonistic microorganisms using a 13C-labeled compound and isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **26**: 510–516.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2013**. Isotope ratio mass spectrometry

identifies soil microbial biocontrol agents having trophic relations with the plant pathogen *Armillaria mellea*. *Applied Soil Ecology* **64**: 142–151.

Percival GC, Banks JM. **2014**. Evaluation of plant defence activators for the potential control of *Pseudomonas syringae* pv. *aesculi*. *Arboricultural Journal* **36**: 76–88.

Perez-Sierra A. **2004**. Systematics, diagnostics and epidemiology of the fungal genus *Armillaria*. Doctoral Thesis. University of London.

R Core Team. **2020**. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rainey PB, Brodey CL, Johnstone K. **1991**. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiological and Molecular Plant Pathology* **39**: 57–70.

Raziq F. **1998**. Biological and integrated control of the root rot caused by *Armillaria mellea*. Doctoral Thesis. University of Reading.

Raziq F, Fox RTV. **2003**. Comparisons between the *in vitro* and *in vivo* efficacies of potential fungal antagonists of *Armillaria mellea*. *Biological Agriculture & Horticulture* **21**: 263–276.

Raziq F, Fox RTV. **2006a**. the integrated control of *Armillaria mellea* 2. field experiments. *Biological Agriculture & Horticulture* **23**: 235–249.

Raziq F, Fox RTV. **2006b**. The integrated control of *Armillaria mellea* 1. glasshouse experiments. *Biological Agriculture & Horticulture* **23**: 225–234.

Rees HJ, Bashir N, Drakulic J, Cromey MG, Bailey AM, Foster GD. **2020**. Identification of native endophytic *Trichoderma* spp. for investigation of *in vitro* antagonism towards *Armillaria mellea* using synthetic- and plant-based substrates. *Journal of Applied Microbiology* **131**: 392-403.

Rotolo C, De Miccolis Angelini RM, Pollastro S, Faretra F. **2016**. A TaqMan-based qPCR assay for quantitative detection of the biocontrol agents *Bacillus subtilis* strain QST713 and *Bacillus amyloliquefaciens* subsp. *plantarum* strain D747. *BioControl* **61**: 91–101.

Sakamoto Y. **2018**. Influences of environmental factors on fruiting body induction, development and maturation in mushroom-forming fungi. *Fungal Biology Reviews* **32**: 236–248.

Schmidt CS, Agostini F, Leifert C, Killham K, Mullins CE. **2004**. Influence of soil temperature and matric potential on sugar beet seedling colonization and suppression of *Pythium* damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. *Phytopathology* **94**: 351–363.

Simonetti E, Viso NP, Montecchia M, Zilli C, Balestrasse K, Carmona M. **2015**. Evaluation of native bacteria and manganese phosphite for alternative control of charcoal root rot of soybean. *Microbiological Research* **180**: 40–48.

Szwajkowska-Michalek L, Kwaśna H, Lakomy P, Perkowski J. **2012**. Inhibition of *Armillaria* and *Heterobasidion* growth by *Penicillium adametzii* isolated from *Pinus sylvestris* forest soil. *Forest Pathology* **42**: 454–466.

Triveni S, Prasanna R, Kumar A, Bidyarani N, Singh R, Saxena AK. **2015**. Evaluating the promise of *Trichoderma* and *Anabaena* based biofilms as multifunctional agents in *Macrophomina phaseolina*-infected cotton crop. *Biocontrol Science and Technology* **25**: 656–670.

Ullrich M, Pen A, Bailey A, Bender CL. **1995**. A modified two-component regulatory system is involved in temperature-dependent biosynthesis of the *Pseudomonas syringae* phytotoxin coronatine. *Journal of Bacteriology* **177**: 6160–6169.

Wang Z, Li Y, Zhuang L, Yu Y, Liu J, Zhang L, Gao Z, Wu Y, Gao W, Ding G, *et al.* 2019. A Rhizosphere-

derived consortium of *Bacillus subtilis* and *Trichoderma harzianum* suppresses common scab of potato and increases yield. *Computational and Structural Biotechnology Journal* **17**: 645–653.

West JS, Fox RT V. **2002**. Stimulation of *Armillaria mellea* by phenolic fungicides. *Annals of Applied Biology* **140**: 291–295.

Xu X-M, Jeffries P, Pautasso M, Jeger MJ. **2011**. Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology* **101**: 1024–1031.

Xu J, Zhao X, Wang X, Zhao Z, Du Y. **2007**. Oligochitosan inhibits *Phytophthora capsici* by penetrating the cell membrane and putative binding to intracellular targets. *Pesticide Biochemistry and Physiology* **88**: 167–175.

Yang LY, Zhang JL, Bassett CL, Meng XH. **2012**. Difference between chitosan and oligochitosan in growth of *Monilinia fructicola* and control of brown rot in peach fruit. *LWT - Food Science and Technology* **46**: 254–259.

Yin H, Du Y, Zhang J. **2009**. Low molecular weight and oligomeric chitosans and their bioactivities. *Current topics in medicinal chemistry* **9**: 1546–1559.

Yin H, Zhao X, Du Y. **2010**. Oligochitosan: A plant diseases vaccine - A review. *Carbohydrate Polymers* **82**: 1–8.

Zaim S, Bekkar AA, Belabid L. **2018**. Efficacy of *Bacillus subtilis* and *Trichoderma harzianum* combination on chickpea *Fusarium* wilt caused by *F. oxysporum* f. sp. *ciceris*. *Archives of Phytopathology and Plant Protection* **51**: 217–226.

Zwart DC, Kim SH. **2012**. Biochar amendment increases resistance to stem lesions caused by *Phytophthora* spp. in tree seedlings. *HortScience* **47**: 1736–1740.

4. *Armillaria* inoculation and culture: an exploration of novel methodologies

Preface

Given the failure of the prior attempts at the artificial inoculation of host plants with *Armillaria mellea* (3.3.10-3.3.12), further development of the techniques was needed, as was a comparison of the traditional and novel methods of culturing the fungus on agar, especially given the reductions in the variability of colony growth observed when homogenized mycelium was used. A published study examining both topics could potentially help increase the efficiency of future *Armillaria* research. In addition, I had attempted rooting privet cuttings for use as small experimental hosts in infection assays. I discussed this with Dr Jassy Drakulic, Royal Horticultural Society, who was also trialling *Ligustrum* plants as experimental hosts due to their high susceptibility. Following our discussion, Dr Drakulic developed successful practices for rooting privet cuttings using a misting propagator. I was interested if choice of inoculum substrate, which varied significantly in the literature, could improve the host inoculation success rate. Helen Rees, Bristol University, was already collaborating with Dr Drakulic on research *Armillaria* research, using strawberries as a host. As such, we decided to combine our various research techniques and questions in the following collaborative study. This paper has not yet been submitted to a journal.

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4.1 Abstract

Many species of the genus *Armillaria* are phytopathogens, causing significant economic damage to a wide range of cultivated hosts. *In vitro* culture and experiments involving *Armillaria*-host interactions are required for research into disease management and ecology. Inoculation relies on placing colonized woody substrates into root systems. Methodologies vary significantly between groups of researchers with little standardisation. Even for factors regarded as controlled, e.g. substrate stem diameter, there is potential for significant effects on, and variation in, the performance of applied inoculum. Studies have utilized different substrates, typically stem sections (billets), often *Corylus*, or tree seeds of varying size and genotype, e.g. *Quercus* or *Castanea*, with no published comparison of relative efficacies.

To assess this, *A. mellea* CG440 inoculum was prepared from *Corylus avellana* billets, *Quercus robur* and *Aesculus hippocastanum* seeds. Crowns of the herbaceous model host, strawberry, *Fragaria × ananassa*, and cuttings of woody wild privet, *Ligustrum vulgare*, were inoculated. Strawberries were grown for the conventional three months duration before quantification of symptoms and infection by destructive harvest. As the timescale of symptoms for wild privet are less documented, symptoms were monitored for a ten-month period before harvest. Physical and nutritional characteristics of substrates were quantified. Comparison was also made of variation in growth of the fungus when applied to agar as colony fragments or homogenized mycelium from submerged culture.

Despite differences in the speed of visual symptoms and mortality occurring, each substrate demonstrated similar performance between hosts. *Aesculus* seeds had the highest infection efficacy and *Corylus* billets also performed well, while *Quercus* seeds performed poorly in comparison. Results correspond to the resources and habitat provided by the inocula. Use of submerged culture techniques halved the variation in growth of the fungus on agar in comparison to traditional subculture by colony fragments, which could increase research output.

4.2 Introduction

Armillaria is a globally distributed genus of fungus in the phylum Basidiomycota. Many of its species are root phytopathogens, typically of woody species (trees, shrubs, vines), but also of herbaceous hosts (Ford *et al.*, 2017). Collectively, the species of this genus are capable of infecting a wide range of hosts, and its economic impact is high (Raabe, 1962; Guillaumin & Botton, 2005a; Baumgartner *et al.*, 2011; Ford *et al.*, 2017). *Armillaria mellea* is recognized as a highly pathogenic species and attacks a wide range of cultivated hosts, therefore being an important focus for research into control of *Armillaria* root rot (Morrison, 1989; Gregory *et al.*, 1991; Guillaumin & Legrand, 2005; Drakulic *et al.*, 2017).

Studies involving *Armillaria* interactions with host plants are vital if viable control methods are to be developed (Ford *et al.*, 2017). This requires methods for the inoculation of the pathogen which are efficient and that simulate natural infection as closely as is practical. In the past 20 years, inoculation methodologies have typically relied on the use of woody substrates colonized by the fungus and placed into the root zone, with variable success rates (Table 1). This approach arguably replicates typical natural infection situations more faithfully (Baumgartner *et al.*, 2018): 'Less natural' methodologies, which may exaggerate infection rates, place mycelium or colonized woody substrates into artificial wounds (Loreto *et al.*, 1993; Solla *et al.*, 2002; Cruickshank *et al.*, 2006; Aslam & Magel, 2018), or wrap inoculum in contact with the roots/root collar using self-sealing film (Beckman & Pusey, 2001; Solla *et al.*, 2002).

Table 1. Details of selected *Armillaria* infection assays on live unwounded hosts using woody inocula, published from 2000 onwards.

Group	Study (stated origin)	Substrate tissue, species & dimensions (cm)[a]	Leaching, sterilisation, antibiotics[b]	Incubation media and substrate placement	Incubation temp. (°C)	Approx. incubation time (days)	Placement or distance from root (cm)	No. per plant	Estimated vol. per host (cm^3)	Host genus	*Armillaria sp.* & infection + mortality rate at conclusion
A	West et al. 2000 (West 2000)	Stem, *Corylus avellana* , 6 x 2.5 Ø	A	-	-	-	"adjacent"	-	-	*Rumex*	*mellea* (*m*) & *ostoyae* (*o*) >70%
A	West & Fox 2002 (West 2000)	Stem, *Corylus avellana* , 10 x 2.5 Ø	A	-	-	90	"touching"	1	49	*Ligustrum*	*m* 71-100%
A	Solla et al. 2002	Stem, *Fagus sylvatica* , 2 x 0.7 Ø	L, A	on MEA	20-24	90	0	1	1	*Picea*	*m* , *o* (no % data)
A	Raziq & Fox 2003 (Raziq 1998)	Stem, *Corylus avellana* , 5 x 1.5-2 Ø	A	on MEA	25[c]	21-28	"beside"	1[c]	9-16	*Fragaria*	*m* 75%
A	Raziq & Fox 2004a (Raziq 1998)	Stem, *Corylus avellana* , 5 x 1.5-2 Ø	A	on MEA	25[c]	21-28	"beside"[c]	1[c]	9-16	*Fragaria*	*m* 100%
A	Raziq & Fox 2004b (Raziq 1998)	Stem, *Corylus avellana* , 5 x 1.5-2 Ø	A	on MEA	21	21	"beside"	1	9-16	*Fragaria*	*m* 83-100%
A	Prodorutti et al. 2009	Stem, *Malus pumila*	A	-	-	28-42	"between"	-	-	*Fragaria* *Vaccinium*	*gallica* (*g*) 80%, *m* 70% *g* 60-70%, *m* 40-80%
A	Pellegrini et al. 2014	Stem, *Malus pumila* , 5 x 0.7 Ø	A	on MEA	25	30	2-3	2	4	*Fragaria*	*g* 70%
A	Nowakowska et al. 2020	Stem, *Corylus avellana* , 10 x 1.5-2 Ø	A	with MEA	25	90	in pot	2	35-63	*Betula*	*g* 100%
B1	Beckman & Pusey 2001	Bark, *Prunus persica* & *Carya illinoinensis* , "6 x 1.5cm"	A	in MEA	"room"	30-60	0	1	11-14	*Prunus*	tabescens +6%
B1	Mansilla et al. 2001	Stem, *Corylus avellana* , 5-6 x 1.5-2 Ø	L, A, AB	in MEA	24-25	15	"close"	1	9-19	*Vitis*	*m* 100%
B1	Mansilla et al. 2001	Stem, *Eucalyptus globulus* , 5-6 x 1.5-2 Ø	L, A, AB	in MEA	24-25	30	NA	1	9-19	-	*m* discarded
B1	Solla et al. 2011 (Mansilla et al. 2001)	Stem, *Corylus avellana* , 5-6 x 1.5-2 Ø + stem, *Pinus pinaster* , 5-6 x 1.5-2 Ø	L, A	in PDA	25	14	in pot	1	18-39	*Pinus*	*o* 60-93%
B1	Agustini et al. 2015 (Mansilla et al. 2001)	Stem, *Eucalyptus globulus* , 5-6 x 1-2 Ø	L, A, AB	in MEA	21-22	90	0	3[d]	12-57	*Eucalyptus*	*luteobubalina* 70-100%
B1	Perez-Sierra 2004, Method C	Stem, *Corylus avellana* , 5 x 1.5-2 Ø	L, A	in carrot agar	23-24	30	-	1	9-16	*Rosa*	*m* 3-53%
B1	Ford et al. 2017 (Perez-Sierra 2004)	Stem, *Corylus avellana* , 10 x 1.5-2 Ø	L, A	in carrot agar	"room"	180	"adjacent"	2	35-63	*Fragaria*	*g* 50%, *m* 50%
B2	Rigling et al. 2003 (Shaw 1977)	Stem, *Corylus avellana* , 10 x 2 Ø	A	in wood-chips	25	120	-	1	31	*Picea*	*m* % unclear, *o* 5-27%
B2	Prospero et al. 2004 (Rigling et al. 2003)	Stem, *Corylus avellana* , 10 x 3-4 Ø	A	in wood-chips	25	120	<1.5	1	71-126	*Picea*	*cepistipes* (*c*) 1-3%, *o* 3-49%
B2	Heinzelmann et al. 2017 (Rigling et al. 2003)	Stem, *Corylus avellana* , 10 x 3-4 Ø	A	in wood-chips	25	120	<1.5	1	71-126	*Picea*	*borealis* 26-42%, *c* 36-38%, *o* 2%
B2	Heinzelmann et al. 2018 (Rigling et al. 2003)	Stem, *Corylus avellana* , 10 x 3-4 Ø	A	in wood-chips	25	120	root zone	1	71-126	*Picea*	*o* 49-54%
B/C1	Szwajkowska-Michalek et al. 2012	Stem, *Quercus robur* , 5 x 2-2.5 Ø + infected 1 x 2-2.5 Ø	A	in wet sand (sterile)	22	120	<12.5	-	(19-30)	*Pinus*	*g* , *o* both possibly 100%
C1	Cleary et al. 2012	Stem, live *Q. garryana* , 8 x 2-3 Ø + infected *F. grandifolia* , 0.5 x 1 Ø	S (*Quercus*) A (*Fagus*)	in moist sand (non-sterile)	"room"	56-84	0	-	(26-57)	*Pseudotsuga* *Thuja* *Tsuga*	*o* 72%[e] *o* 50%[e] *o* 66%[e]
C2	Cruickshank et al. 2006	Stem, live *Q. garryana* , 15 x 12-15 Ø + wood, *Betula papyrifera* , 1500g	A (wood)	in moist sand	"room"	>730	0	1	>1696 -2650	*Pseudotsuga*	*o* 70%
C2	Cruickshank et al. 2010 (Sturrock & Reynolds 1998)	Stem, live *Q. garryana* , 15 x 12-15 Ø + wood with bark, *B. papyrifera* , 255g	S (stem) A (wood)	in moist sand	19	820 -1245	<2	1	>1696 -2650	*Pseudotsuga*	*o* 93%
C2	Cruickshank & Jaquish 2014 Cruickshank & Filipescu 2017 (above)	Stem, live *Q. garryana* , 10 x 15-19 Ø + wood with bark, *B. papyrifera* , 1500g	S (stem) A (wood)	in moist sand	17	730 -1215	- -	2	>3534 -5670	*Pseudotsuga*	*o* 88% *o* 45%
D	Beckman & Pusey 2001	Seed, *Q. prinus* & *Q. acutissima*	A	in MEA	"room"	30-60	-	1	-	*Prunus*	(*Desarmillaria*) *tabescens* +21%
D	Nogales et al. 2008 (Beckman & Pusey 2001)	Seed, *Quercus suber*	A	in MEA	"room"	30	"near"	2	-	*Vitis*	*m* 71%
D	Mesanza et al. 2016 (Beckman & Pusey 2001)	Seed, *Quercus* spp.	A	in MEA	"room"	30	0	1	-	*Pinus*	*m* ca. 65%
D	Elias-Roman et al. 2019 (Beckman & Pusey 2001)	Seed, *Quercus* spp.	L, A	3 x 1cm Ø MEA	ca. 20	60	0	2	-	*Prunus*	*m* 25%, *mexicana* 49%
D	Calvet et al. 2015 Camprubi et al. 2020 (above)	Seed, *Castanea sativa*	A, AB	in MEA	25	150	<10 "near"	1	-	*Lavandula* *Eriobotrya*	*m* 63% *m* ca. 42%

- = not detailed. MEA = malt extract agar. PDA = potato dextrose agar. [a] Ø = diameter. [b] L = leaching, A= autoclaved, S = surface sterilised, AB = antibiotics. [c] from Raziq 1998 (thesis). [d] from Figure 2B. [e] local root infection.

In one study, woodchips were mixed into the soil and homogenized mycelium mixed in afterwards. This was followed by a period to allow colonization before planting strawberry hosts which were successfully infected (Percival *et al.*, 2011). Researchers have also attempted *Armillaria* inoculation by applying colonized agar plugs into the root zone but failed, likely due to desiccation (Sitienei *et al.*, 2015; Ford *et al.*, 2017) and/or attack from natural antagonists. However, plug/mycelium inoculations are successful in axenic systems (Nogales *et al.*, 2010; Baumgartner *et al.*, 2010, 2013, 2018; Lovato *et al.*, 2014). Other non-woody substrates have also performed poorly (Redfern & Filip, 1991). In comparison to agar plugs, intact woody substrates provide greater physical protection from desiccation (Ford *et al.*, 2017) and antagonists. Early infection of woody material is less ligninolytic (Guillaumin & Botton, 2005a; Sahu *et al.*, 2021), with potential benefits from the retention of this resilient hydrophobic and structural polymer. *Armillaria* is well known for its ability to survive extended periods on colonized woody substrates (Baumgartner *et al.*, 2011, 2018), which is perhaps owed to the capacity to produce protective pseudosclerotial plates within or around such materials (West & Fox, 2002; Heinzelmann *et al.*, 2019). These melanised barriers are a defence against desiccation and antagonism (Guillaumin & Botton, 2005b). As such, lower surface area to volume ratio of inoculum could be a benefit for infection, i.e. a lower ratio of surface requiring defence in proportion to the volume of nutritional resources and habitat provided. However, decomposition rates and gaseous exchange requirements are likely to be limiting factors (Fukasawa *et al.*, 2020).

Multiple methods of artificial inoculation and inoculum production have been developed by different groups of researchers. These vary significantly in multiple aspects that could influence infection rates such as preparation of substrates, incubation conditions and duration, substrate size and number per plant, and distance from roots (Table 1). Virulence of the particular strain/s and resistance of the host genotype/s used are also major factors (Prospero *et al.*, 2004; Solla *et al.*, 2011). Examples of large variation between studies include the volume of applied inoculum, with estimates ranging from 1 to >5000 cm^3, and incubation duration, ranging from 14 to >1000 days. Other factors with potentially high significance such as placement of inoculum in relation to roots and incubation temperature are sometimes unreported or reported without sufficient detail (Table 1).

Previous authors have used different types of woody substrates, including root/stem material (Table 1, ABC) and large seeds (Table 1, D). Hazel (*Corylus avellana* L.) stem material is favoured in general, while acorns (*Quercus* L. spp. seed) are the seed of preference, although used to a lesser degree. Some authors have used sweet chestnuts (*Castanea sativa* Mill. seed) (Calvet *et al.*, 2015; Camprubi *et al.*, 2020). While there is ample experimental evidence of *Armillaria* utilising such small and discrete woody substrates,

and seeds, there appears to be no available evidence of this occurring in nature. Presumably this does occur to some degree, especially with the more saprobic *Armillaria* species. The seeds and wood of different plant species vary in calorific density (Hough, 1969; Grodziński & Sawicka-Kapusta, 1970; Smith & Follmer, 1972), size, structure, and composition: factors which could prospectively influence inoculum efficacy (Garrett, 1956; Gregory *et al.*, 1991).

Even within the same study, variation between substrates can be large. Many studies utilise billets, selecting small ranges of diameter and cutting them to length. However, due to the relationship between diameter and volume, an apparently small change in diameter can cause large changes in volumes (Table 1) and this effect becomes larger as diameters increase, a potentially underappreciated source of variation in many studies. The efficiency of inoculum production is another consideration, as the authors agree that trimming billets to length is a somewhat impractical and time-consuming process, whereas seed selection and preparation may be more rapid. Taking the whole canopy into consideration, diameter of above-ground woody tissue is arguably more heterogeneous than seed weight and, therefore, billets within a small diameter range may require accessing more trees in comparison. Seeds can often be collected in large amounts from below a single mature tree, or purchased more easily, while billets are more easily collected from multiple younger plants with low canopies or coppice.

Details provided regarding the conditions of substrate colonization are generally brief, despite their probable influence on the resulting inoculum. Royal Horticultural Society researchers have previously produced inoculum using vertically arranged hazel billets in agar, inoculated from above with pieces of colonized agar, yielding slow colonization (Perez-Sierra, 2004). Many other authors also submerge woody substrates in agar during colonization (Table 1, B1) presumably with the aim of increasing food resources and maintaining moisture levels to support the growth of the fungus. Moist woodchips (Table 1, B2) or sand (Table 1, C) have also been used. Conversely, *Armillaria* requires a “good oxygen supply” for initiation and growth of rhizomorphs (Pareek *et al.*, 2006), which are the primary means of spread in agar and substrates: therefore a dense aggregate of substrates and media may slow colonization rates. Oxygen availability may also influence the formation of protective pseudosclerotial plates (Lopez-Real & Swift, 1977) and therefore inoculum longevity. Authors have demonstrated rapid colonization of smaller substrates, in 15-30 days (Mansilla *et al.*, 2001), producing viable inoculum either unsubmerged (Table 1, B1) or submerged in conservative amounts of media (Table 1, B2). Meanwhile, recent publications are still utilizing methods of inoculum production which take more than 2 years (Table 1, C2) (Cruickshank & Filipescu, 2017), which may limit the scope and flexibility of trials in comparison to shorter durations.

In general, there is little available information to compare the efficiency of various methods. For example, infection rates might provide a point of comparison between trials but differences in host and inoculum species, strain, size, and culture (Table 1) make this unfeasible. Assessment of substrate colonization is generally visual, and data is unrecorded. Quantitative polymerase chain reaction (qPCR) has been used to quantify *Armillaria* root colonization (Baumgartner *et al.*, 2010; Lovato *et al.*, 2014; Calvet *et al.*, 2015) and may be an appropriate molecular method to quantify the colonization yielded by various substrates and/or methods of inoculum production. However, a lack of comparison between inoculation methodologies undermines inference and synthesis from the resultant data. Similarly, a number of authors have looked towards herbaceous hosts to speed up *Armillaria*-host assays (Raziq & Fox, 2006; Pellegrini *et al.*, 2014; Ford *et al.*, 2017), with strawberries being a common choice (table 1). However, the significant differences between herbaceous and woody plant physiology may have ramifications for how the wider implications of such results can be interpreted.

Alongside the dependence on varied woody inoculum, the majority of *Armillaria* studies are still reliant on 'traditional' culture methodologies: namely the growth of the fungus on solid media and sub-culturing via transferring fragments of actively growing mycelium to fresh media, typically cutting 'plugs' from within actively growing colonies using a scalpel or cork borer. Older growth on agar often forms a melanised crust (Guillaumin & Botton, 2005b) and colonies transition to spreading through agar unevenly via rhizomorphs. This interferes with 'colony plug' collection and use, so typically plugs are collected only from the younger colony margin (Aguín *et al.*, 2006). Methodologies for submerged culture have been used by some authors (Baumgartner *et al.*, 2010, 2018; Pellegrini *et al.*, 2012, 2013), consisting of growing the fungus in broth and shaking during incubation in otherwise typical conditions. This produces spherical colonies of mycelium which remain comparatively undifferentiated: in older cultures rhizomorphs may be initialised all over the surface but do not expand significantly. Colonies are weighed out and homogenized in a standard volume of broth. All fungal material produced can be used, reducing waste in comparison to agar cultures. For other basidiomycetes, submerged culture has been demonstrated to be more controlled than culture on agar and may be advantageous in terms of uniformity and speed of growth (Zweck *et al.*, 1978; Tan & Moore, 1992). Avoiding the cutting and handling of individual plugs reduces the required time and dexterity to prepare cultures. There is also an intermediate technique, where lawns are grown using homogenized mycelium and plugs are cut from them (Baumgartner *et al.*, 2010). However, there does not appear to be any recorded comparison between the three techniques described above: homogenized mycelium, colony plugs, and lawn plugs.

We have carried out multiple *Armillaria* experiments and seen large variations in the efficacy of infection between trials of similar design. Reported infection rates vary greatly and are often around 50% or less

(Table 1). The significant rate of inoculation failure (escape), slow infection, and inconsistent infection and mortality between experimental replicates, have been identified as key bottlenecks in 'greenhouse' *Armillaria* infection assays (Baumgartner *et al.*, 2018). Therefore, there is significant scope for improving the reliability and efficacy of inoculations and thus the efficiency of *Armillaria* host bioassays.

The primary aim of this study was to compare wood- and seed-based *Armillaria* inoculum efficacy for the first time. Our hypothesis is that the material from which the fungal growth substrate is made influences its reliability as an inoculum source. Woody hazel billets were compared against tree seeds (acorns and the larger 'conkers' from *Aesculus hippocastanum* L.) as a means to transfer infection *in planta*. A further aim was to explore the suitability for these infection methods in a range of hosts. To achieve this, we compared infection of the widely used herbaceous model host, *Fragaria × ananassa* Duchesne, and a new potential model woody host, *Ligustrum vulgare* L.. We also aim to understand any differences in infectivity of the different substrates, by quantifying nutritional and physical characteristics. One final aspect to consider in creating optimised infection assays, both *in planta* and *in vitro*, is how the fungus is most reliably inoculated onto the substrate. To investigate this, we compare the speed and consistency of agar colonisation following inoculation by homogenised liquid-grown cultures and cultures from agar plates. Overall, our aim is to provide data to inform best working practices in *in vitro* and host bioassay work on *Armillaria* root rot, to increase productivity and reliability within *Armillaria* research.

4.3 Methodology

4.3.1 *Armillaria* Culture

4.3.1.1 Isolate

The isolate used in all trials was *Armillaria mellea* CG440 from the culture collection of the Royal Horticultural Society, originally isolated from a *Ligustrum* spp., Surrey, United Kingdom, 22nd November 2006 (Beal *et al.*, 2015). The isolate had recently been inoculated into a live host (*Fragaria × ananassa*) and re-isolated from the resulting infection in an effort to ensure pathogenicity. This isolate has been used in a number of other studies (Beal *et al.*, 2015; Ford *et al.*, 2015, 2017), with potential as a model strain.

4.3.1.2 Homogenized Mycelium

Under aseptic conditions, fragments of *A. mellea* CG440 mycelium under 1mm^2 were collected from a young culture or a derived a long-term slant (malt extract agar (MEA), Oxoid Ltd., Basingstoke, Hampshire, UK) in storage at 4°C. Using a pipette tip, mycelium was transferred to a 50ml conical based 'Falcon' tube containing 10ml of potato dextrose broth (PDB) (Neogen Europe Ltd., Auchincruive, Ayr, UK) which had been amended with solid sodium acetate to a final concentration of 2.5mM (Amended

PDB, APDB) prior to autoclaving (holding at 121°C for 30 minutes) (Baumgartner *et al.*, 2010). The resultant culture was incubated in an orbital shaker at 27°C, 200rpm, in low light, for one week. This produced spherical colonies of mycelium, approximately 0.5g of which were added to 1ml APDB in an MP Biomedicals 'lyzing matrix M' tube with a ¼ inch (6.35mm) ceramic bead. Mycelium was homogenized by shaking at 4m s^{-1} for 10 seconds in a FastPrep-24™ 5G lysis system (MP Biomedicals, USA).

4.3.2 Colony and Lawn Plugs

MEA plates were produced using 20ml agar in 9cm Petri dishes. Colony 'plugs' were produced by pipetting 20µl volumes of the homogenized mycelium (Pellegrini *et al.*, 2012) onto MEA plates. Colonies were evenly spaced with seven on each plate. Lawn 'plugs' were produced from lawns of mycelium created by spreading 100µl onto MEA plates (Baumgartner *et al.*, 2010). The homogenized mycelium was allowed to dry sufficiently for adherence onto the MEA surface prior to sealing the plate with parafilm. After incubating for one week in the dark at 21°C, plugs of approximately 5mm x 5mm were cut from the colonies or lawns using a scalpel. Generally, these plugs covered a whole quarter of a colony, being cut from just within its margins, and one was taken per colony.

4.3.3 Comparison of *In Vitro* Culture Techniques

MEA plates were inoculated at the centre by applying a colony 'plug', lawn 'plug', or a 20µl drop of homogenized mycelium and allowing it to dry/absorb into the agar prior to any significant movement. All plates were sealed with parafilm and incubated at 21°C in the dark.

At 0, 6, 8, 10, 12, 14, & 16 days post inoculation, digital images of the base of plates on a black background were taken using a flat-bed scanner (HP Scanjet G4010) at 300dpi resolution, alongside a $1mm^2$ grid scale mounted inside an identical Petri-dish. Growth area was measured from the digital images, semi-automatically, in 'ImageJ' (Rasband, 2020). The pixel to mm scale was set from that featured in the image. Images were converted to greyscale, and 'thresholding' was used to select areas with greyscale values corresponding to fungal growth which were measured in mm^2. This process was supplemented by manual selection/cropping if required. There were three complete replicates of the experiment, with 10 repeats for each method.

To quantify the variability of *A. mellea* CG440 growth yielded by each method, the exponential growth period (*ca.* 4 long days in all cases) was identified by graphing the area of CG440 against time. The three time points of exponential growth were then isolated and the relative standard deviation (aka co-efficient of variation) calculated for each time point in each replicate experiment, with the time points forming repeats for statistical analysis:

$$relative\ standard\ deviation = \frac{standard\ deviation}{mean} \times 100$$

The first day at which rhizomorphs began to spread beyond mycelial growth was also recorded for each individual plate.

4.3.4 Comparison of Inoculum Properties

4.3.4.1 Substrate Preparation

Inoculation substrates used were either: sections of *Corylus avellana* (hazel) stems (billets) (Mansilla *et al.*, 2001) of 50 x 15±2 mm diameter (weighing approximately 7.5-12.0g), acorns (Beckman & Pusey, 2001) of *Quercus robur* weighing 2.4-4.5g, or 'conkers' of *Aesculus hippocastanum* L. weighing 9.0-14.5g. The seeds were obtained from treeseedonline.com, and further selected by fresh weight to increase uniformity. Hazel billets were made from material local to the authors (Reading, Bristol, or Wisley, UK), to the dimensions described. The substrates were bagged and frozen at -20°C, being defrosted before inoculum was prepared, or used in 2-3 days following receipt of seeds or billet collection. The fresh weight of 35 individual 'used' substrates of each type, per three 'selectors' (scientists conducting this work), was recorded prior to use.

Seeds were pierced once with a 1mm needle to avoid rupturing during autoclaving. Substrates were autoclaved twice in water, holding at 121°C for 45 minutes. Resultant whole substrates were placed in single layer in polypropylene 'takeaway' tubs of 170mm x 120mm x 50mm, and almost entirely covered with molten sterile MEA. Lids were put loosely on the tubs before a final autoclaving, holding at 121°C for 30 minutes. The lids were then closed, and the agar allowed to solidify before inoculation.

Prepared tubs of substrates were inoculated with six colony plugs evenly distributed across the MEA surface. Corresponding controls were prepared without the addition of colony plugs. All tubs were sealed with parafilm. Incubation took place in the dark at 21°C for 28-31 days. Weekly checks were made for visual signs of contamination, i.e. bacterial colonies or uncharacteristic fungal growth. Any contaminated tubs were discarded. No control tubs were contaminated.

4.3.4.2 Physical Characteristics

Individual fresh substrates were chosen by two selectors (14 each). These 'typical' substrates were given unique identifiers, weighed, and measured in mm for length and two diameters at 90° to each other using calipers. Volume was measured in millilitres using water displacement in a measuring cylinder. The fresh substrates were then dried in an oven (*ca.* 80°C), until the weights of a monitored subset plateaued, and dry weights recorded for the calculation of density. Surface areas of acorns and conkers were estimated by using target dimensions in a cylinder surface area calculation for hazel billets and

measurements of elliptic radii in an ellipsoid surface area calculation known as 'Knud Thomson's Formula' (Mwasame *et al.*, 2017):

$$estimated\ surface\ area\ of\ cylinder = 2\pi rh + 2\pi r^2$$

$$estimated\ surface\ area\ of\ ellipsoid = 4\pi\left(\frac{a^p b^p + a^p c^p + b^p c^p}{3}\right)^{\frac{p}{1}}$$

Where 'r' = radius, 'h' = height/length, 'p' = 1.6, 'a' = widest radius, 'b' & 'c' = radii at 90° from 'a' and one another.

4.3.4.3 Nutritional Characteristics

Two samples of each substrate (400-410g fresh weight) were prepared to the point prior to inoculation as above (autoclaving in water and media), then dried in an oven (*ca.* 80°C). Nutritional analysis was then provided by NRM Laboratories (Coopers Bridge, Braziers Lane, Winkfield Row, Bracknell, Berkshire, RG42 6NS, UK).

The whole sample was ground prior to subsampling for the various tests. Gross calorific value was determined using a bomb calorimeter. Total carbon and nitrogen were determined using the 'Dumas Technique' (AOAC International, 1990). Elemental make-up of the sample was determined by digestion in a concentrated nitric and hydrochloric acid solution ("reverse aqua-regia") followed by Inductively Coupled Plasma Mass Spectrometery (ICP-MS) or Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), as appropriate, to quantify Boron, Calcium, Copper, Iron, Magnesium, Manganese, Molybdenum, Phosphorus, Potassium, Sodium, Sulphur, and Zinc (MAFF, 1986). Results were averaged over the two samples.

4.3.5 Efficacy of different *Armillaria* substrates to initiate disease *in planta*

4.3.5.1 Plant Material

Strawberry crowns (*Fragaria × ananassa* cv. Elsanta) were obtained from R W Walpole Ltd. (King's Lynn, Norfolk, PE34 4PX, UK) and selected visually for uniform root systems. Strawberries are a commonly used herbaceous experimental host for *Armillaria* (Raziq & Fox, 2004a; Pellegrini *et al.*, 2014; Ford *et al.*, 2017) and cv. Elsanta was chosen for its susceptibility to fungal diseases. These were potted into Sinclair All Purpose Multi Purpose Growing Medium Compost (Sinclair Pro, Ellesmere Port, Cheshire, UK) in 1L pots.

Rooted cuttings of wild privet (*Ligustrum vulgare* L.) were selected to serve as a woody model host because this species/genus is highly susceptible to *Armillaria* root rot infection (West & Fox, 2002; Guillaumin *et al.*, 2005; Cromey *et al.*, 2020), although above-ground symptoms may not be present

despite significant root infestations (West and Fox, 2002). Parent material was collected from a hedge in Wisley, Surrey, United Kingdom on the 25th October 2018. The parent material was assumed to be one individual, or clonal material of such, in all cases. Cuttings were made from 6-8cm sections of semi-woody stems, each bearing a single leaf node. A straight cut was made approximately 1.5cm above the node and a 45° cut made just above the proceeding node. Moist tissue paper was used to stop the bases drying before propagation. Leaves were trimmed or removed to produce uniform cuttings with one or two mature leaves. These were placed into a misting propagator (Hydropod, Greenhouse Sensation, Lancashire, UK), under grow-lights with a 16h photoperiod, for 5-6 weeks. The basal ends of the cuttings were continuously misted with tap-water during this period. The rooted cuttings were then planted in moist Sylvagrow Sustainable Growing Medium (Melcourt Industries Ltd., Tetbury, Gloucestershire, UK) in 9cm pots with the roots 2-3cm below the surface.

A solid 2cm diameter plastic spacer was added during potting-up of host plants, in close proximity (1-2cm) to the stem or crown. The removal of this rod, prior to inoculation, created a void of approximately 2.5 cm by 7cm: large enough for the woody infection substrate to be buried with minimal root disturbance. Privet cuttings were allowed to acclimatise to the potted conditions for 1-2 weeks, and strawberries for 4 weeks, before inoculum was added.

4.3.5.2 Plant Inoculation

Plug-inoculated substrates were used as *A. mellea* inoculum sources. Inoculum per plant consisted of a single billet, conker, or three acorns (as a single unit), all of comparable weights, which had been cleaned of excess agar and/or externally growing mycelium. Prepared inoculum was placed into the void in the root zone formed by removing the spacer. It was then covered with surrounding compost. Controls received the non-inoculated hazel billets.

4.3.5.3 Experimental Design

For each host, two identical rounds of experiments were set up in a randomized block design. Within each round there were four treatments, consisting of each of the three substrates colonized by CG440 and a control treatment inoculated with uncolonized hazel billets. There were 10 replicates per treatment, totalling 20 per host over both rounds.

All privet cuttings were propagated on the 25th October 2018 from material collected from a hedge in Wisley, Surrey, United Kingdom. Plants for the first experimental round were potted on the 30th November 2018 and inoculated on the 7th December 2018, and for the second round on the 6th December 2018 and 14th December 2018. Both were harvested on the 11th October 2019. The first strawberry replicate was potted on the 15th November 2018, inoculated on the 15th of December 2018,

and harvested on the 10th March 2019; the second on the 28th November 2018, 18th December 2018 and 1st April 2019.

4.3.5.4 Growing Conditions

Both experimental rounds of privet and one of strawberry took place in a controlled environment chamber at the Field Research Station, Royal Horticultural Society Garden Wisley, (Woking, Surrey, UK), under a temperature regime of 23°C daytime & 15°C night, humidity 50%, and daytime light levels supplemented to 36 µmol m^{-2} s^{-1} minimum for a 16h day length. The privet experiments were located approximately 5m apart, on opposite sides of the chamber. The second round of the strawberry experiment took place at the University of Bristol Old Park Hill greenhouses, at a constant 15°C temperature, mean humidity 54%, 16 hour day length and daytime light levels supplemented to 160 µmol m^{-2} s^{-1} minimum. Plants were monitored and watered as needed to maintain moist soil, but not fed or re-potted for the duration of the experiment.

4.3.5.5 Host Condition Assessment

Measurements from strawberry plants were taken three months after inoculation during destructive harvest, while privet plants were measured monthly for ten months and then destructively harvested.

The symptoms and infection of strawberry plants were scored on a scale of 1-4, based on Aguin et al. 2006 (Aguín *et al.*, 2006):

1. Plant appears healthy.
2. Plant appears unhealthy but no sign of *Armillaria* mycelial fans.
3. Host showing signs of *Armillaria* infection including above ground symptoms and/or mycelial fans.
4. Host plant dead.

The visual health of privet plants was scored on a scale of 1-5:

1. Plant visually healthy.
2. Leaves yellowing.
3. Darkening or necrosis of leaves.
4. Visible wilting or extensive leaf loss.
5. Plant visibly dead.

Relative chlorophyll content readings were taken from leaves using a Konica Minolta Soil Plant Analysis Development (SPAD) 502 meter. On privet, readings were taken from the two original leaves present at the start of the experiment and, if present, from two hardened leaves grown during the experiment.

These were then averaged per plant at each time point. On strawberry, readings were taken from three randomly selected younger, but fully expanded, leaves. Extension growth measurements of privet focused on any shoots arising from the original cuttings. Shoots were measured from the base to the bottom of the terminal bud and summed per individual at each time point. Measurements were not taken from dead plants.

4.3.5.6 Root and Root Collar Examination and Sampling

At the end of the trial duration, or following the death of plants, root systems were inspected for the presence of rhizomorphs. Roots and shoots were then removed, leaving just the root collar, and a clean knife was used to check for the presence of mycelial fans under the bark. The root collar was then split into two approximately even halves and all pieces retained. This allowed a final check for mycelium within. Presence/absence of mycelial fans was recorded as a binary factor: 1 reflecting the presence of a fan and 0 the absence.

Under aseptic conditions, root collar pieces were surface sterilised by rinsing in a <5% sodium hypochlorite solution for 1 minute under agitation, then rinsed in a similar manner three times in sterile distilled water and allowed to drain in a laminar flow cabinet.

4.3.5.7 Re-isolation

Armillaria selective media, attributed to Jean-Jacques Guillaume, (JJG) was prepared by amending molten MEA, cooled to 55°C, with 50mg/L Penicillin, 50mg/L Streptomycin sulphate, and 25mg/L Polymyxin, each in sterile distilled water, and 1ml/L Thiabendazole lactate solution (Beal *et al.*, 2015). The thiabendazole lactate solution was prepared previously by dissolving 23g thiabendazole in 100ml of 100% lactic acid.

Under aseptic conditions, the root collar was sampled in twelve areas, targeting mycelial growth if present, and plated on JJG, six to a plate, close to the edge of the plate and incubated at 21°C in the dark. After three days the plates were checked for fast growing contaminants and samples were moved to fresh plates if required. Plates were then incubated for a further four days and checked for characteristic growth of *Armillaria* i.e. white, densely arranged aerial mycelium or rhizomorph formation. If growth was ambiguous, samples were moved onto another plate which was incubated for a further seven days and then checked for *Armillaria* growth. The success of re-isolation was coded as a binary factor, with 1 representing one or more successful re-isolations and 0 representing none.

4.3.6 Statistical Analysis

The R statistical programming language (R Core Team, 2020) was used for all data analysis. For all tests, a significance level of 5% (α=0.05) was used.

Datasets were balanced by random sampling where appropriate. In the longitudinal privet data, month 8 data was incomplete and removed from the dataset as a precaution.

For continuous variable datasets without time points, linear models were fitted to dependent variables, with substrate, methodology, selector, and/or experimental round, with block nested within it where appropriate, as fixed factors. An interaction effect was coded between substrate and selector only. Linear mixed models ('lme' function, 'nlme' package) were fitted to longitudinal data, with an interaction between treatment and time fixed factors, and random factors consisting of subject (individual plant or culture) nested within block nested within experimental replicate. Relative standard deviation was log-transformed to normalise the data. Marginal and conditional R^2 were calculated from models where appropriate ('r.squaredGLMM' function, 'MuMIn' package). Analysis of Variance (ANOVA) tables were calculated from the models as an omnibus test ('Anova' function, 'car' package). Tukey's honest significance test (Tukey) was performed, and treatment means and/or trends estimated ('emmeans' and 'emtrends' functions, 'emmeans' package), estimates are back-transformed to the arithmetic scale where appropriate. Mean/trend separation was also calculated ('cld', 'multcomp' package).

For binary variables or where residuals from parametric testing were non-normal, data was analysed using the Kruskal-Wallis Rank Sum Test (KW) as an omnibus test, followed by Pairwise Wilcoxon Rank Sum Tests with a Bonferroni adjustment (Wilcox).

The package 'ggplot2' was used for graphical interpretation of the data.

4.4 Results

Salient results of all statistical analyses are presented in supplementary data (P-values & CIs, Appendix A): *In vitro* (Table SM1), substrate selection (Table SM2), substrate characteristics (Table SM3), and *In planta* (Table SM4).

4.4.1 Comparison of Culture Techniques

There is no recorded direct comparison of the different techniques for growing *Armillaria* on agar. Therefore, we compared the growth of *Armillaria mellea* CG440 on agar inoculated using pieces cut from actively growing colonies or lawns grown from homogenized mycelium, or by directly applying homogenized mycelium.

Culture technique caused significant differences in growth area of *A. mellea* CG440 over time (ANOVA interaction P=0.002, Wald X^2 = 12.919, marginal R^2 = 0.962, conditional R^2 = 0.963, figure 1A). Growth area mean was significantly lower in the homogenized mycelium treatment for the duration of the

experiment (e.g. at day 6, Tukey *P*<.001, t-ratios = -8.629 & -10.588). While the colony plug treatment mean growth area was significantly higher than that of the lawn plug treatment from day 8 (Tukey *P*=0.025, t-ratio = +2.655, Table SM1).

Reflecting the differences in growth area, the homogenized mycelium treatment entered exponential growth around two days later than the two 'plug' treatments (figure 1A). Corresponding significant differences (ANOVA *P*<0.001, *F* = 0.788, adjusted R^2 = 0.639, Tukey significant *P*<0.001 & t-ratios = +10.464 to +11.215) were also present in the time point at which rhizomorphs spread beyond the area of surface growth: plug methods mean day 6.07a-6.21a, homogenized mycelium 8.53b. Exponential growth rate was significantly higher in the two 'plug' treatments than the homogenized mycelium treatment (ANOVA *P*<0.001, Wald X^2 = 24.595, marginal R^2 = 0.931, conditional R^2 = 0.979, Tukey trends significant *P*<0.001 & t-ratios = -4.139 & -4.425, Table SM1).

Estimated means for the variation (relative standard deviation) of the 'plug' treatments were more than double that of homogenized mycelium (19.6-20.0a vs 9.4b respectively), reflecting significantly lower variability in the spread of CG440 when applied directly to agar as a homogenised slurry (ANOVA *P*<0.001, *F* = 16.660, adjusted R^2 = 0.539, Tukey significant *P*<0.001 & t-ratios = -4.919 & -5.075, figure 1B).

The two 'plug' methods did not differ significantly in the time point at which rhizomorphs spread beyond the area of surface growth or variability (Tukey respective *P*=0.791 & 0.987, t-ratios = -0.654 & -0.155).

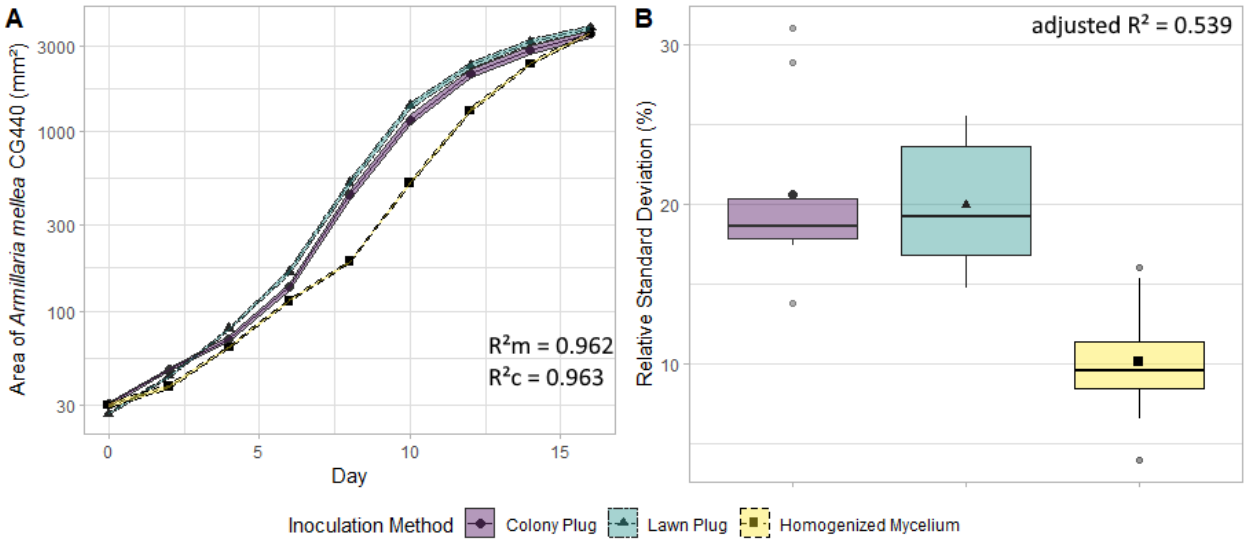

Figure 1. Growth curves of *Armillaria mellea* CG440 on agar inoculated with various methods (A), *n*=30, and the spread in variation between subjects of each method during exponential growth (B) *n*=9. Ribbons in the line graph express standard error ranges. Shaped points in box-plots express treatment means.

4.4.2 Comparison of Inoculum Properties

4.4.2.1 Physical Characteristics

To provide context for any significant differences in substrate efficacy, the physical characteristics of the selected materials must be compared. For saliency, the results discussed in this paragraph are averaged over selector: substrate had a more significant impact, i.e. lower ANOVA *P*, than selector and the interaction effect in all cases (Table SM3). Omnibus tests for fresh and dry mass, volume, dry density, and estimated surface area to volume ratio showed a significant effect of substrate (Table 2, ANOVA P<0.001, KWs *P*<0.001-0.006). Fresh and dry mass were significantly different between all substrates (Table 2, Tukey *P*<0.001, t-ratios = -7.439 to -37.103 & +6.590 to +12.192). Hazel billet and conker substrates were not significantly different in individual volume (Wilcox *P*=1). Multiplying the individual volume of acorns by three, as a proxy for the applied volume, indicates that acorns may have a higher applied volume than hazel billets (Wilcox *P*=0.007). Doing the same for dry mass indicates that a similar dry mass of both seeds was applied (Wilcox *P*=1). Hazel billets had a significantly lower dry density (Tukey *P*<0.001, t-ratio = 5.686 & 8.013) than the seeds, which were not significantly different (Tukey *P*=0.058, t-ratio = -2.327). Conkers had the lowest surface area to volume ratio (Tukey *P*<0.001-0.036, t-ratios = -5.852, +2.522, & +8.374).

Table 2. Used and typical physical characteristics of various substrates used for the inoculation of *Armillaria mellea* CG440, expressed as means, their separation, and, varying by statistical test[1,2], their 95% confidence interval in brackets or ± standard deviations ('used' n=28, 'typical' n=35).

Substrate	*Corylus avellana* stem	*Quercus robur* seed	*Aesculus hippocastanum* seed	Adj' R^2	Substrate F / X^2
Used Fresh Mass (g)[1]	9.6b (9.3, 9.9)	3.7a (3.4, 4.0)	11.6c (11.3, 11.9)	0.829	745.373
Typical Fresh mass (g)[1]	9.4b (9.0, 9.9)	3.9a (3.4, 4.4)	11.6c (11.1, 12.1)	0.881	289.170
Typical Dry mass (g)[1]	4.2b (3.9, 4.6)	2.4a (2.1, 2.7)	7.2c (6.9, 7.6)	0.830	196.450
Typical Estimated Applied Dry mass (g)[2]	4.4a ± 0.7	7.2b ± 1.6	7.2b ± 1.4	-	44.183
Typical Dry density (g^{-1} cm^3)[1]	0.45a (0.40, 0.50)	0.66b (0.61, 0.71)	0.75b (0.61, 0.80)	0.488	33.982
Typical Estimated surface area to volume ratio[1]	2.9b (2.8, 3.0)	3.1a (3.0, 3.3)	2.3c (2.2, 2.5)	0.479	36.908
Typical Individual Volume (cm^3)[2]	9.5b ± 1.3	3.6a ± 0.5	10.2b ± 1.9	-	57.848
Typical Estimated Applied Volume (cm^3)[2]	9.5a ± 1.3	10.7b ± 1.6	10.2ab ± 1.9	-	10.335

Used substrates were the group used in the *in planta* comparison and typical substrates were substrates from the same source, chosen in the same way. [1] normal data analysed by Tukey's Honest Significant Difference Test, following a significant effect of substrate in Analysis of Variance omnibus test. Means are estimates from the test. [2] non-normal data analysed by Pairwise Wilcoxon Rank Sum Tests with a Bonferroni adjustment, following a significant effect of substrate in Kruskal-Wallis Rank Sum Test omnibus test. Means and standard deviations from raw data.

4.4.2.1.1 Impact of 'selector'

Significant differences between substrates selected by different individuals (selectors) may influence variation between rounds of the comparison of inoculum substrate efficacy. They may also indicate where substrate selection targets e.g. weight ranges, can be improved. Comparison of the fresh weight of substrates selected for the *in planta* experiment indicates that conkers selected by one author of three were significantly lighter, by around 1.6g (ANOVA interaction P=0.007, F = 3.619, adjusted R^2 = 0.828, Tukey significant P<0.001 & t-ratios = -4.396 & +4.549). Dry weight of 'typical' conkers was also different between the two selectors, by around 1.2g (ANOVA selector P=0.001, F = 11.668, Tukey P=0.002 & t-ratio = -3.276). In both analyses all other comparisons, including between selectors for other substrates, were non-significant (Tukey P=0.065-0.913 & t-ratios = -0.408 to -1.536 & +0.099 to +2.245).

4.4.2.2 Nutritional Characteristics

Nutritional analysis indicates potential differences in the nutrition which different substrates can provide to *Armillaria* (Table 3), which may play a role in any differences in their efficacy. Conkers may provide more calories than hazel billets or acorns. Seeds may provide greater amounts of nitrogen, phosphorous, potassium, magnesium, sulphur and boron than hazel billets.

Table 3. Typical Nutritional Values of Inoculum Substrates Prepared as Those Made Available to *Armillaria mellea* CG440 (means, *n*=2).

Determinand	**Unit**	***Corylus avellana* stem**	***Quercus robur* seed**	***Aesculus hippocastanum* seed**
Sample Size	count	51	110	34
Gross Calorific Value	MJ/kg	18.50	18.30	19.65
Total Carbon	%	51.950	49.100	50.950
Total Nitrogen	% w/w	0.435	0.915	1.315
Total Phosphorus	% w/w	0.065	0.105	0.255
Total Potassium	% w/w	0.120	0.855	0.770
Total Calcium	% w/w	0.295	0.125	0.110
Total Magnesium	% w/w	0.030	0.055	0.060
Total Sodium	% w/w	0.030	0.020	0.015
Total Sulphur	mg/kg	345	580	887
Total Manganese	mg/kg	20.400	22.400	7.300
Total Copper	mg/kg	4.500	5.250	10.700
Total Zinc	mg/kg	8.350	9.100	10.650
Total Iron	mg/kg	24.400	29.400	23.300
Total Boron	mg/kg	8.950	13.000	12.850
Total Molybdenum	mg/kg	0.430	0.190	0.405

4.4.3 Efficacy of different *Armillaria* substrates to initiate disease *in planta*

To assess the efficacy of inoculation by the three substrates, a range of measurements were taken from inoculated and dummy-inoculated strawberry and privet plants and compared between the groups within hosts.

Where there was a significant effect of experimental round, the groupings of means were the same between rounds in all cases. Therefore, all results in this section are shown as averages over the rounds. All unmarked *P*-values in this section are from Tukey's test.

4.4.3.1 Strawberry

There was a significant effect of inoculum substrate on strawberry symptom and infection score (1-4 healthy-dead) (ANOVA $P<0.001$, $F = 11.501$, adjusted $R^2 = 0.263$, Table SM4). Higher scores represent greater visual symptoms and extent of infection present. The mean score of strawberry plants at harvest (3 months after inoculation) was significantly higher than the control when conker inoculum was used, 2.90c ($P<0.001$, t-ratio = 5.742), followed by hazel billet inoculum, 2.30bc ($P=0.002$, t-ratio = +3.773), which scored 1.15a (figure 2A). The acorn inoculum mean was not significantly different from that of hazel billet inoculum ($P=0.661$, t-ratio = -1.148) or the control ($P=0.053$, t-ratio = +2.625). These differences in scores correspond with mortality rates observed over both rounds: 0% for the control, 5% for acorn inoculum, 15% for hazel billet inoculum, and 40% for conker inoculum (figure 2B).

4.4.3.2 Privet

In longitudinal analysis, there was a significant interaction effect of time and inoculum substrate on privet visual symptom score (1-5, healthy-dead) (figure 2C, ANOVA $P=0.036$, marginal $R^2 = 0.309$, conditional $R^2 = 0.526$, table SM4). One month after inoculation, mean scores separated significantly ($P=0.023$ & t-ratio = -2.958) between the control, 0.793a, and conker inoculum, 1.600b, while hazel billet and acorn inoculum means did not separate from either ($P=0.290$-0.999 & t-ratios = -0.137 to -1.787), being 1.243ab and 1.280ab respectively. Means maintained this separation in month 2. Three months after inoculation, all *Armillaria* inocula host scores, 1.826-2.110b, were significantly different from the control, 1.191a, ($P=0.001$-0.042 & t-ratios = -1.050 to -2.715) and this grouping of means was maintained until the conclusion of the experiment.

Over time, hazel billet inoculum showed a significantly ($P=0.022$ & t-ratio = -2.873) faster increase in mean host score, +0.311b score $month^{-1}$, from the control, +0.199a, while acorn and conker inoculum, +0.255-0.273ab, were not significantly different from hazel billet inoculum or the control ($P=0.228$-0.969 & t-ratios = -1.448 to -1.903 & +0.455 to +1.425). This appears to be linked to later mortality observed in the hazel billet inoculum treatment, with the first observation occurring two months later than the other substrates (month 5, figure 2D). At three months, mortality rate was 5% for both seed inocula and 0% in

the control and hazel billet inoculum treatments. At the conclusion of the experiment, privet mortality rates were 5% for the control, 30% for acorn inoculum, and 40% for both hazel billet and conker inocula.

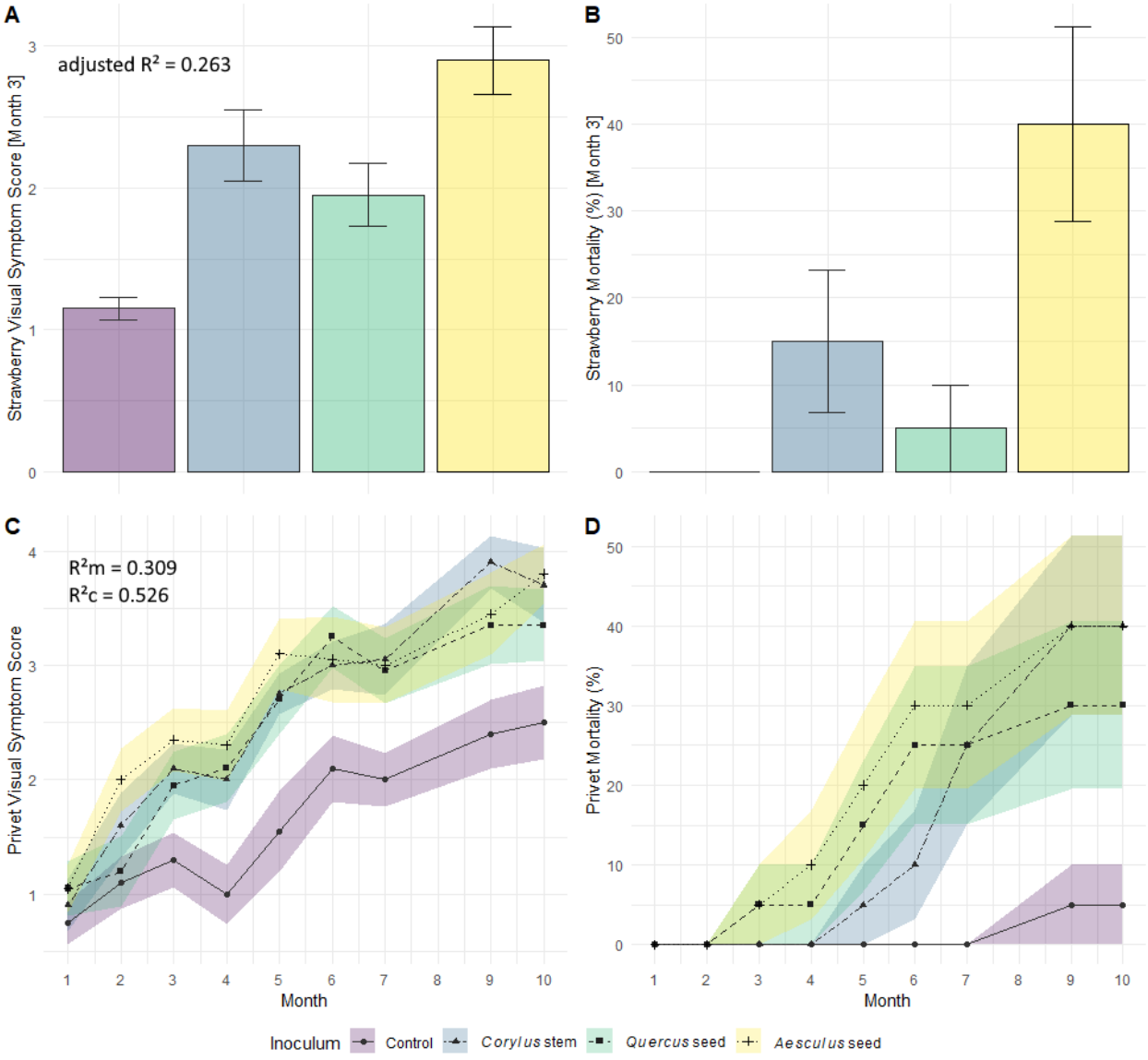

Figure 2. Mean visual symptom score and mortality at 3 months in strawberry plants, *Fragaria × ananassa* Duchesne, (A, B) and over time between wild privet plants, *Ligustrum vulgare* L., (C, D) inoculated with *Armillaria mellea* CG440 grown on various woody substrates (*n*=20). Scores healthy-dead = 1-4 for strawberry and 1-5 for privet. Ribbons and bars in the graphs express standard error ranges.

For comparison to the strawberry scores, privet visual symptom scores were analysed independently at identical time points (month 1, 2, 3, 10). The only notable differences in the analysis results were that there were no significant differences in month 1 and acorn inoculum means did not separate significantly (P>0.05) from those of the control at any time point. Increased significance in the longitudinal analysis is interpreted to relate to increased statistical power and replication.

There was a significant effect of inoculum substrate on privet relative chlorophyll content under longitudinal analysis (ANOVA *P*=0.042, Wald X^2 = 8.191, marginal R^2 = 0.223, conditional R^2 = 0.601): there was a significant difference between conker inoculum and the control 1 month after inoculation only (Tukey *P*=0.042 & t-ratio = +2.721), while independent analysis of data at this time point showed no significant differences (P>0.05). Independent analysis of month 3 shows a significant effect of inoculum substrate on privet relative chlorophyll content (ANOVA *P*=0.008, *F* = 4.397, adjusted R^2 = 0.342). The control mean, 62.1a, separated significantly from that of hazel billet inoculum, 53.1b, and conker inoculum, 50.6b (*P*=0.007-0.043 & t-ratios= +2.711 to + 3.402), while the acorn inoculum treatment mean was not significantly different from either group, 54.1ab (*P*=0.095-0.992 & t-ratios = -0.288 to +2.371).

4.4.3.3 Non-significant measures

There were no significant differences between infected substrates, on either host, in any other measurements recorded. Strawberry relative chlorophyll content, and privet extension growth, showed no significant differences (*P*>0.05). Presence of mycelial fans and re-isolation success only showed significant differences (*P*<0.05) between *Armillaria* inoculated and dummy-inoculated plants.

4.5 Discussion

This study aimed to compare wood- and seed-based *Armillaria* inoculum efficacy on a woody and a herbaceous host, as well as different *in vitro* culture techniques, to inform and improve working practices within *Armillaria* research. All have received little research attention in comparison to their potential impact on research outputs. The expectations were that seed-based inocula would provide improved nutrition and resources to *Armillaria mellea* for host infection and that reactions to infection would differ between the two hosts/physiologies. Culture of the species on agar from homogenized mycelium was also expected to yield lower variation between repeated cultures, due to the more controlled production and application of the source mycelium.

4.5.1 *In vitro* culture

In the assessment of agar culture methods, inoculating agar with homogenized mycelium produced *Armillaria* colonies which grew more consistently than either of the 'plug' methods, halving the relative standard deviation. This large impact may have substantial benefits for studies by lowering the sample size required to obtain statistical significance, i.e. increasing statistical power. Accordingly, there are potential savings in set-up time, materials consumed, and incubation space required. Additionally, as described above, there may also be beneficial decreases in required time, materials, and dexterity from the inherent differences of homogenized mycelium protocols to the other assessed methodologies.

Replicability of experiments, e.g. repeated by an individual or between multiple researchers, should also be increased by use of these submerged culture techniques.

Differences in variation between homogenized mycelium and colony plugs may be influenced by condition of the source colony which plugs are cut from. As demonstrated, variation in growth is significantly influenced by the condition of the source tissue. In this study, colony plugs were cut from small colonies of a specific young age (7 days), these were grown from homogenized mycelium and therefore less variable than had they been from colony fragments. In the literature, source colony material may sometimes only be described as having been "taken from the margin of an actively growing culture" or similar, and age not mentioned (Rishbeth, 1968; Pareek *et al.*, 2006; Aguín *et al.*, 2006; Keca, 2009; Thomidis & Exadaktylou, 2012) and details may be even more scant (Dumas, 1992; Popoola & Fox, 2003; Szwajkowska-Michalek *et al.*, 2012). This is despite the original colony age having potential impacts on the density and morphology of the tissue present (Guillaumin & Botton, 2005b). In addition, when age is recorded, source cultures grown for 7 days, or more, prior to inoculation are often used (Raziq & Fox, 2003; Beal *et al.*, 2015; Ford *et al.*, 2017; Chen *et al.*, 2019). In this study, on average 7 days was after plug grown colonies had started to spread unevenly via rhizomorphs, while homogenized mycelium colonies had not. Therefore, the plug colonies measured in this study, with source material from young, less rhizomorphic, homogenized mycelium colonies, may be less variable than those in other studies.

In contrast to the reductions in variation offered by use of homogenized mycelium, exponential growth was slower in this treatment and, along with the transition to spreading by rhizomorphs, delayed by around two days in comparison to the 'plug' methods. This may lead to a trade-off between required incubation time and the number of experimental subjects, depending on which factor is greater in a particular context. There is potential that this difference in growth rate, and that seen between the two 'plug' methods in the later part of the experiment, are related to differences in the initial amount of inoculum. This was not controlled in this study and therefore it may be appropriate for further work to address the role of this factor. Time required for recovery from the apparently greater physical damage of homogenization is another potential source of differences in growth observed between the methods. The cutting of plugs is likely to damage a smaller proportion of the original cells. Retained homogenized mycelium was observed to take on a red hue within an hour of processing. It may be that applying more concentrated or larger volumes of homogenized mycelium can mitigate the observed slower growth.

4.5.2 Efficacy of inoculation substrates

Conker was the most effective inoculum substrate for the infection of both hosts. In strawberry, this was reflected in significantly higher visual symptoms and infection, plus high mortality at harvest. In wild privet, it was evident from the significant difference in visual symptoms compared to the control in

month 1, occurring two months prior to that from the hazel substrate. This was supported by similar differences in early (month 1 & 3) wild privet relative chlorophyll concentration. Due to their earlier destructive harvest, in line with typical practices, it is not possible to know if strawberries would also move towards no significant differences between the infected substrates over time, as seen in wild privet, but it is assumed likely. Recorded increases in the visual symptom score and mortality of control treatment privets (figure 2C, 2D) are interpreted to relate to increasing environmental stress, i.e. nutrient and rooting volume availability, and the ambiguity of visual *Armillaria* symptoms.

Hazel billet inoculum also performed well, being significantly different from the control at month 3 in wild privet. Its mean score grouped with conker in strawberries, but it also grouped with acorn which did not separate clearly from the control. At the end of the wild privet experiment, hazel billet inoculum yielded an identical overall mortality rate to the conker inoculum (40%).

Acorns grouped cleanly with the other CG440 inocula from month 3 in the long term analysis of the privet data, but not in the independent analyses of time points (month 3 & 10), perhaps due to the increased statistical power of the longitudinal test. Acorn inoculum performance was noticeably poor in comparison to the conker and hazel billet inocula, yielding lower mortality, especially considering the possibly larger applied volume (Table 2). As proposed above, surface area to volume ratio may also be a factor in inoculum efficacy, due to apparent benefits in defence of the inoculum unit, which may go some way to explaining the poor performance of this granular inoculum. On an individual basis, the acorns were also significantly smaller meaning they may have provided less habitat and resources to CG440. Acorn size and mass are underappreciated factors in the literature. The acorns of *Q. acutissima*, *Q. prinus* & *Q. suber* have all been used and can vary in their comparative size, mass, nutritional value and level of defensive compounds e.g. phenols (Gilman & Watson, 1994a,b; Shimada & Saitoh, 2006; Ramírez-Valiente *et al.*, 2009). Sometimes different species are used in different rounds of the same experiment (Beckman & Pusey, 2001), or no species is given (Mesanza *et al.*, 2016; Elias-Roman *et al.*, 2019), while in all cases size and mass are not quantified. The number applied per plant also varies, although use of additional acorns in this study does not appear to have increased efficacy (Table 1D).

On an individual basis, the conker inoculum was the largest and densest, with the lowest surface area to volume ratio (Table 2). Possessing a combination of high density and applied mass, the highest calorific value measured, as well as the generally higher nutrient contents of seeds (Table 3) suggests the conker substrate would have provided the greatest nutrition to the fungus. There has apparently been little use of conkers in *Armillaria* research, although both conkers and acorns increased the infection success of an agar-based inoculum (Guyot, 1927). The nutritional reserves of inoculum units have long been linked to

infection success of *Armillaria* (Garrett, 1956; Gregory *et al.*, 1991). Larger inoculum also increases basidiomycete performance in other forms of competition (Fukasawa *et al.*, 2020). Therefore, while the least used inoculum substrate found in our survey of the literature (Table 1), larger chestnut type seeds, e.g. *Castanea* or *Aesculus* spp., may be optimum inoculation substrates for *Armillaria* in terms of infection success. They may have other practical benefits, as *Q. robur* acorns were often found to have germinated which may affect their nutrition. Conkers may suffer lower amounts of seed pests, a factor which can necessitate extra processing of acorn substrates (Elias-Roman *et al.*, 2019): in Europe *Quercus* spp. have higher numbers of associated insects in general, owing to their ecological dominance (Kennedy & Southwood, 1984; Brändle & Brandl, 2001). The significant differences observed between selectors in conker weight, in both the substrates used in experiments and the 'typical' substrates, indicates that the selection criteria may be too wide, i.e. a smaller weight range should be used to select conkers for use as inoculum in future. Regardless, this factor appeared to be of little detriment to the observed inoculum efficacy.

The nutritional analysis of the various substrates presented only quantified the gross calorific value and elemental nutritional profile, however the release of nutrients to the fungus would have been mediated by the varied compounds they constituted. For example, the tannins present in the acorn inoculum (Łuczaj *et al.*, 2014) may have taken more energy or time to detoxify, despite being known to stimulate *Armillaria* growth once broken down (Cheo, 1982; West & Fox, 2002), therefore reducing the performance of acorn inoculum. In comparison, the saponins or other defensive compounds present in conker inoculum do not appear to have posed a significant issue to *A. mellea* CG440, although this could relate to their activity, concentration, and solubility in comparison to the acorn tannins. Longitudinal analysis of the privet visual health scores, indicates visual decline may accelerate more over time in plants receiving hazel inoculum, despite evidence suggesting it is somewhat slower to kill hosts (Figure 2). This could relate to slower breakdown of this substrate by the fungus in comparison to seeds and therefore energy release, correspondingly initial *Armillaria* infections are less ligninolytic (Guillaumin & Botton, 2005a; Sahu *et al.*, 2021).

It is of note that the performance of the different inoculum substrates ranked similarly between hosts, as described above. The influence of inoculum substrates on host infection was strong enough to be apparent despite differences in timescale and host species/physiology.

4.5.3 Design of *Armillaria*-host experiments

At three months, the overall mortality rate was much higher in strawberries than in wild privet. The mortality rate caused by conker inoculum in strawberry hosts was equal to that observed six months later in privet, while hazel billet inoculum caused mortality occurred two months earlier (Figure 2). Privet

mortalities plateaued around the time that mortality was observed in the control, presumably due to root restriction or nutrient deficiency, which could suggest a greater role of host stress. The large differences in response to infection between the two hosts highlight potential differences between woody and herbaceous hosts, as well as a current and candidate model host. The difference is even more dramatic in the context of the lower ratio of inoculum to host biomass in strawberries, as the privet plants were visually far smaller. While the prompt reaction of strawberries may reduce experimental durations, the comparative *Armillaria* resistance of wild privet, a woody host considered highly susceptible, demonstrates that use of such a sensitive host in bioassays may be a disadvantage. For example, a slower reaction may elucidate more subtle or distinct differences between the treatments. It could also improve inferences regarding larger, and therefore more resistant, hosts, increasing applicability.

Infection success should not be considered in isolation when designing *Armillaria*-host interaction studies. As suggested for host susceptibility above, artificially high inoculum pressure has the potential to inappropriately eliminate effective *Armillaria* management measures in trials. It may be controlled for by the inclusion of saprobic *Armillaria* spp. in experimental designs (Baumgartner *et al.*, 2018). For *Armillaria mellea, A. gallica* may be the most suited for this use, given their common co-occurrence (Heinzelmann *et al.*, 2019), while less virulent strains of the same species might also be considered (Prospero *et al.*, 2004).

Selection of inocula by efficacy, or application of varying numbers of inoculum units, may also allow experimental designs to be adjusted to specific inoculum pressures on hosts or to yield data within particular timeframes. Varying inoculum efficacy and volume may also appropriately challenge hosts of varying susceptibility to similar degrees e.g. one comparable to infection events *in natura*. This study provides a starting point for developing host assays for particular areas of *Armillaria* management.

As an aside, it is apparent this is the first study to use small cuttings of *Ligustrum vulgare* in an *Armillaria*-host assay. A previous study inoculated larger plants of a member of the same genus (West & Fox, 2002). Fans in the root collar, a key diagnostic symptom, still occurred on these small plants. Use of a misting propagator made generating uniform small clonal plants low-maintenance and more successful than other methods attempted. Use of clonal material can help reduce variation in host responses (Solla *et al.*, 2011) and emphasise differences between treatments. Conversely, varied genotypes may be used to make stronger inferences about effects on the species as a whole. As for many of the factors discussed here, the level of experimental control applied may limit inferences regarding wider contexts i.e. *in natura* infections.

4.5.4 Concluding Remarks

This paper demonstrates for the first time that there are advantages for using homogenized mycelium for agar culture and that the choice of wood- or seed-based inoculum has a significant effect on host infection and mortality. A large seed, of *Aesculus hippocastanum*, was the inoculum substrate which yielded the highest infection/mortality rate on both a woody and a herbaceous host.

It is clear that, whatever the methods are used for *Armillaria* inoculation and culture, standardization offers enhanced comparability between studies. It is acknowledged that there are of course practical and financial limits on the degree to which this can be performed. To increase comparability, authors and journals should aim to provide clear and full descriptions of the materials and techniques used, even if supplied as supplementary materials. It may be prudent to provide data on inoculum substrates e.g. species, mass, volume, and density. The use of strawberries in host assays by multiple authors has increased comparability between trials and potential for synthesis. However, our trial highlights the potential differences between this herbaceous host and a woody host, although general inferences were similar between the two.

In a wider context, the findings on substrates for fungal growth may have ecological implications. Presumably seeds or pieces of wood are often present beneath host species, such as oaks or horse chestnuts, and could provide nutrition to invading *Armillaria* species. As discussed above, the differences in performance observed between inoculum substrates, including changes over time, may relate to their constituents, e.g. lignin. This could also provide an ecological role for observations of delayed ligninolytic actvity in recent –omics data (Sahu *et al.*, 2021), i.e. retention of pre-existing protective structures in host material. Further studies are required examining the colonization, metabolism, longevity, and temporal variation in infection potential of inoculum grown on substrates where macromolecules/biopolymers, including lignin, have been characterised. Further studies on the formation of pseudosclerotial plates, their interaction with pre-existing materials, and the impact of submerging substrates in media during culture upon them may compliment this. The extreme sensitivity of strawberries has led some to suggest them as indicator species for monitoring *Armillaria* inoculum pressure in parks and gardens. As demonstrated in this study, the speed of their reaction to infection lends evidence towards this use, although other factors such as differences in rooting depth compared to larger hosts could limit it.

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4.7 References

Aguín O, Mansilla JP, Sainz MJ. **2006**. *In vitro* selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapevine in the field. *Pest management science* **62**: 223–8.

Agustini L, Beadle C, Barry K, Mohammed C. **2015**. Photosynthetic responses of *Eucalyptus nitens* Maiden at initial stages of root-rot infection. *Indonesian Journal of Forestry Research* **2**: 9–20.

AOAC International. **1990**. Method 949.12. In: Anon, ed. Official Methods of Soil Analysis. Arlington, VA.

Aslam AJ, Magel EA. **2018**. Influence of drought and season on compartmentalization of black locust (*Robinia pseudoacacia* L.) inoculated with *Armillaria mellea*. *European Journal of Plant Pathology* **152**: 21–31.

Baumgartner K, Bhat R, Fujiyoshi P. **2010**. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal biology* **114**: 107–19.

Baumgartner K, Coetzee MPA, Hoffmeister D. **2011**. Secrets of the subterranean pathosystem of *Armillaria*. *Molecular plant pathology* **12**: 515–534.

Baumgartner K, Fujiyoshi P, Browne GT, Leslie C, Kluepfel DA. **2013**. Evaluating paradox walnut rootstocks for resistance to *Armillaria* root disease. *HortScience* **48**: 68–72.

Baumgartner K, Fujiyoshi P, Ledbetter C, Duncan R, Kluepfel DA. **2018**. Screening almond rootstocks for sources of resistance to *Armillaria* root disease. *HortScience* **53**: 4–8.

Beal EJ, Henricot B, Peace AJ, Waghorn IAG, Denton JO. **2015**. The action of allicin against *Armillaria* spp. *in vitro*. *Forest Pathology* **45**: 450–458.

Beckman TG, Pusey PL. **2001**. Field testing peach rootstocks for resistance to *Armillaria* root rot. *HortScience* **36**: 101–103.

Brändle M, Brandl R. **2001**. Species richness of insects and mites on trees: Expanding Southwood. *Journal of Animal Ecology* **70**: 491–502.

Calvet C, Garcia-figueres F, Lovato P, Camprubi A. **2015**. Role of the arbuscular mycorrhizal symbiosis in tolerance response against *Armillaria mellea* in lavender. *Spanish Journal of Agricultural Research* **13**: 1–8.

Camprubi A, Solari J, Bonini P, Garcia-Figueres F, Colosimo F, Cirino V, Lucini L, Calvet C. **2020**. Plant performance and metabolomic profile of loquat in response to mycorrhizal inoculation, *Armillaria mellea* and their interaction. *Agronomy* **10**: 899.

Chen L, Bóka B, Kedves O, Nagy VD, Szűcs A, Champramary S, Roszik R, Patocskai Z, Münsterkötter M, Huynh T, *et al.* 2019. Towards the biological control of devastating forest pathogens from the genus *Armillaria*. *Forests* **10**: 1013.

Cheo PC. **1982**. Effects of tannic acid on rhizomorph production by *Armillaria mellea*. *Phytopathology* **72**: 676–679.

Cleary MR, van der Kamp BJ, Morrison DJ. **2012**. Effects of wounding and fungal infection with *Armillaria ostoyae* in three conifer species. II. Host response to the pathogen. *Forest Pathology* **42**: 109–123.

Cromey MG, Drakulic J, Beal EJ, Waghorn IAGG, Perry JN, Clover GRGG. **2020**. Susceptibility of garden trees and shrubs to *Armillaria* root rot. *Plant Disease* **104**: 483–492.

Cruickshank MG, Filipescu CN. **2017**. The interactive effect of root disease and climate on wood

properties in halfsibling Douglas-fir families. *Forest Ecology and Management* **392**: 58–67.

Cruickshank MG, Jaquish B. **2014**. Resistance and tolerance in juvenile interior Douglas-fir trees *Pseudotsuga menziesii* var. glauca artificially inoculated with *Armillaria ostoyae*. *Forest Pathology* **44**: 362–371.

Cruickshank MG, Lejour D, Morrison DJ. **2006**. Traumatic resin canals as markers of infection events in Douglas-fir roots infected with *Armillaria* root disease. *Forest Pathology* **36**: 372–384.

Drakulic J, Gorton C, Perez-Sierra A, Clover G, Beal L. **2017**. Associations between *Armillaria* species and host plants in U.K. gardens. *Plant Disease* **101**: 1903–1909.

Dumas MT. **1992**. Inhibition of *Armillaria* by Bacteria Isolated from Soils of the Boreal Mixedwood Forest of Ontario. *European Journal of Forest Pathology* **22**: 11–18.

Elias-Roman RD, Calderon-Zavala G, Guzman-Mendoza R, Vallejo-Perez MR, Klopfenstein NB, Mora-Aguilera JA. **2019**. 'Mondragon': A clonal plum rootstock to enhance management of *Armillaria* root disease in peach orchards of Mexico. *Crop Protection* **121**: 89–95.

Ford KL, Baumgartner K, Henricot B, Bailey AM, Foster GD. **2015**. A reliable *in vitro* fruiting system for *Armillaria mellea* for evaluation of *Agrobacterium tumefaciens* transformation vectors. *Fungal Biology* **119**: 859–869.

Ford KL, Henricot B, Baumgartner K, Bailey AM, Foster GD. **2017**. A faster inoculation assay for *Armillaria* using herbaceous plants. *The Journal of Horticultural Science and Biotechnology* **92**: 39–47.

Fukasawa Y, Gilmartin EC, Savoury M, Boddy L. **2020**. Inoculum volume effects on competitive outcome and wood decay rate of brown- and white-rot basidiomycetes. *Fungal Ecology* **45**: 100938.

Garrett SD. **1956**. Rhizomorph behaviour in *Armillaria* (Vahl) Quél. II. logistics of infection. *Annals of Botany* **20**: 193–209.

Gilman EF, Watson DG. **1994a**. Fact Sheet ST-557, *Quercus prinus*, Chestnut Oak. *Environmental Horticulture Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida*.

Gilman EF, Watson DG. **1994b**. Fact Sheet ST-540, *Quercus acutissima*, Sawtooth Oak. *Environmental Horticulture Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida*.

Gregory SC, Rishbeth J, Shaw CG. **1991**. Pathogenicity and virulence. In: Shaw CG, Kile GA, eds. Armillaria Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 76–87.

Grodziński W, Sawicka-Kapusta K. **1970**. Energy Values of Tree-Seeds Eaten by Small Mammals. *Oikos* **21**: 52–58.

Guillaumin J-J, Botton B. **2005a**. Place de l'armillaire parmi les agents de pourridiés. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 23–29.

Guillaumin J-J, Botton B. **2005b**. Ontogénie des armillaires. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 151–175.

Guillaumin J-J, Legrand P. **2005**. Cycle infectieux de l'armillaire – Stratégie biologique des espèces européennes. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 177–201.

Guillaumin J-J, Lung-Escarmant B, Legrand P. **2005**. Sensibilité des hôtes ligneux à l'armillaire et sélection pour la tolérance. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 377–388.

Guyot R. **1927**. Mycelium lumineux de l'Armillaire. *Compte - Rendus de la Société de Biologie* **96**: 114–116.

Heinzelmann R, Dutech C, Tsykun T, Labbé F, Soularue J-P, Prospero S. **2019**. Latest advances and future perspectives in *Armillaria* research. *Canadian Journal of Plant Pathology* **41**: 1–23.

Heinzelmann R, Prospero S, Rigling D. **2017**. Virulence and stump colonization ability of *Armillaria borealis* on norway spruce seedlings in comparison to sympatric *Armillaria* species. *Plant Disease* **101**: 470–479.

Heinzelmann R, Prospero S, Rigling D. **2018**. Frequent diploidisation of haploid *Armillaria ostoyae* strains in an outdoor inoculation experiment. *Fungal Biology* **122**: 147–155.

Hough WA. **1969**. *Calorific value of some forest fuels of the Southern United States (Vol. 120)*. Southeastern Forest Experiment Station, US Department of Agriculture, Forest Service.

Keca N. **2009**. *In vitro* interactions between *Armillaria* species and potential biocontrol fungi. *Glasnik Sumarskog fakulteta*: 129–142.

Kennedy CEJ, Southwood TRE. **1984**. The number of species of insects associated with British trees: a re-analysis. *Journal of Animal Ecology* **53**: 455–478.

Lopez-Real JM, Swift M. **1977**. Formation of pseudosclerotia ('zone lines') in wood decayed by *Armillaria mellea* and *Stereum hirsutum*: III. Formation in relation to composition of gaseous atmosphere in wood. *Transactions of the British Mycological Society* **64**: 465-IN7.

Loreto F, Burdsall HH, Tirro A. **1993**. *Armillaria* infection and water stress influence gas-exchange properties of Mediterranean trees. *HortScience* **28**: 222–224.

Lovato PE, Garcia-Figueres F, Camprubí A, Parladé J, Calvet C. **2014**. A semiaxenic phototrophic system to study interactions between arbuscular mycorrhizal and pathogenic fungi in woody plants. *European Journal of Plant Pathology* **140**: 207–212.

Łuczaj Ł, Adamczak A, Duda M. **2014**. Tannin content in acorns (*Quercus* spp.) from Poland. *Dendrobiology* **72**: 103–111.

Mackenzie M, Shaw III CG. **1977**. Spatial relationships between *Armillaria* root rot of *Pinus radiata* seedlings and the stumps of indigenous trees. *New Zealand Journal of Forestry Science* **7**: 374–383.

MAFF. **1986**. *Reference Book RB427: Analysis of Agricultural Materials*. London: HMSO, Great Britain.

Mansilla PJ, Aguín O, Sainz MJ. **2001**. A fast method for production of *Armillaria* inoculum. *Mycologia* **93**: 612–615.

Mesanza N, Iturritxa E, Patten CL. **2016**. Native rhizobacteria as biocontrol agents of *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*. *Biological Control* **101**: 8–16.

Morrison DJ. **1989**. Pathogenicity of *Armillaria* species is related to rhizomorph growth habit. In: Morrison DJ, ed. Proceedings of the Seventh International Conference on Root and Butt Rots, August 9-16, 1988. Vernon and Victoria, British Columbia.: International Union of Forestry Research Organizations, co-published by Forestry Canada, Victoria, BC., 584–589.

Mwasame PM, Wagner NJ, Beris AN. **2017**. On the macroscopic modelling of dilute emulsions under

flow. *Journal of Fluid Mechanics* **831**: 433–473.

Nogales A, Camprubí A, Estaún V, Marfà V, Calvet C. **2010**. *In vitro* interaction studies between *Glomus intraradices* and *Armillaria mellea* in vines. *Spanish Journal of Agricultural Research* **8**: S62–S68.

Nowakowska JA, Stocki M, Stocka N, Ślusarski S, Tkaczyk M, Caetano JM, Tulik M, Hsiang T, Oszako T. **2020**. Interactions between *Phytophthora cactorum*, *Armillaria gallica* and *Betula pendula* Roth. seedlings subjected to defoliation. *Forests* **11**: 1107.

Pareek M, Allaway WG, Ashford AE. **2006**. *Armillaria luteobubalina* mycelium develops air pores that conduct oxygen to rhizomorph clusters. *Mycological research* **110**: 38–50.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2012**. Studying trophic interactions between a plant pathogen and two different antagonistic microorganisms using a 13C-labeled compound and isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **26**: 510–516.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2013**. Isotope ratio mass spectrometry identifies soil microbial biocontrol agents having trophic relations with the plant pathogen *Armillaria* mellea. *Applied Soil Ecology* **64**: 142–151.

Pellegrini A, Prodorutti D, Pertot I. **2014**. Use of bark mulch pre-inoculated with *Trichoderma atroviride* to control *Armillaria* root rot. *Crop Protection* **64**: 104–109.

Percival GC, Smiley ET, Fox RTV. **2011**. Root collar excavation with *Trichoderma* inoculations as a potential management strategy for honey fungus (*Armillaria mellea*). *Arboricultural Journal* **33**: 267–280.

Perez-Sierra A. **2004**. Systematics, diagnostics and epidemiology of the fungal genus *Armillaria*. Doctoral Thesis. University of London.

Popoola TOS, Fox RTV. **2003**. Effect of Water Stress on Infection by Species of Honey Fungus (*Armillaria mellea* and *A. gallica*). *Arboricultural Journal* **27**: 139–154.

Prodorutti D, Pellegrini A, Pertot I. **2009**. Biocontrol of *Armillaria* root rot on highbush blueberry in Italy. *OBC WPRS bulletin* **43**: 207–210.

Prospero S, Holdenrieder O, Rigling D. **2004**. Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances. *Forest Pathology* **34**: 1–14.

R Core Team. **2020**. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raabe RD. **1962**. Host list of the root rot fungus, *Armillaria mellea*. *Hilgardia* **33**: 24–89.

Ramírez-Valiente JA, Valladares F, Gil L, Aranda I. **2009**. Population differences in juvenile survival under increasing drought are mediated by seed size in cork oak (*Quercus suber* L.). *Forest Ecology and Management* **257**: 1676–1683.

Rasband M. **2020**. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA.

Raziq F, Fox RTV. **2003**. Comparisons between the *in vitro* and *in vivo* efficacies of potential fungal antagonists of *Armillaria mellea*. *Biological Agriculture & Horticulture* **21**: 263–276.

Raziq F, Fox RTV. **2004a**. Cultural techniques for improvement in biocontrol potential of fungal antagonists against *Armillaria* root rot of strawberry plants under glasshouse conditions. *Biological Agriculture & Horticulture* **22**: 271–287.

Raziq F, Fox RTV. **2004b**. The effect of carrier substrate, dose rate and time of application on biocontrol

efficacy of fungal antagonists against *Armillaria* root rot of strawberry plants. *Biological Agriculture & Horticulture* **22**: 157–172.

Raziq F, Fox RTV. **2006**. The integrated control of *Armillaria mellea* 1. glasshouse experiments. *Biological Agriculture & Horticulture* **23**: 225–234.

Redfern DB, Filip GM. **1991**. Inoculum and infection. In: Shaw CG, Kile GA, eds. Armillaria Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 48–60.

Rigling D, Lawrenz P, Blauenstein H, Heiniger U. **2003**. An experimental study of the effects of ozone on tree–*Armillaria* interactions. In: Laflamme G, Bérubé JA, Bussières G, eds. Root and Butt Rots of Forest Trees. Proceedings of the IUFRO Working Party 7.02.01. Quebec City, Canada, September 16–22, 2001. Information Report LAU-X-126. Québec, Canada: Laurentian Forestry Centre, 122–126.

Rishbeth J. **1968**. The growth rate of *Armillaria mellea*. *Transactions of the British Mycological Society* **51**: 575–586.

Sahu N, Merényi Z, Bálint B, Kiss B, Sipos G, Owens RA, Nagy LG. **2021**. Hallmarks of basidiomycete soft- and white-rot in wood-decay-omics data of two *Armillaria* species. *Microorganisms* **9**: 1–20.

Shimada T, Saitoh T. **2006**. Re-evaluation of the relationship between rodent populations and acorn masting: a review from the aspect of nutrients and defensive chemicals in acorns. *Population Ecology* **48**: 341–352.

Sitienei PC, Wagara IN, Kariuki ST, Jefwa J. **2015**. Evaluation of arbuscular mycorrhizal fungus and *Trichoderma harzianum* against *Armillaria* species and growth response of *Dombeya torrida* seedlings. *Global Journal of Wood Science, Forestry and Wildlife* **3**: 101–107.

Smith CC, Follmer D. **1972**. Food preferences of squirrels. *Ecology* **53**: 82–91.

Solla A, Aguín O, Cubera E, Sampedro L, Mansilla JP, Zas R. **2011**. Survival time analysis of *Pinus pinaster* inoculated with *Armillaria ostoyae*: genetic variation and relevance of seed and root traits. *European Journal of Plant Pathology* **130**: 477–488.

Solla A, Tomlinson F, Woodward S. **2002**. Penetration of *Picea sitchensis* root bark by *Armillaria mellea*, *Armillaria ostoyae* and *Heterobasidion annosum*. *Forest Pathology* **32**: 55–70.

Sturrock RN, Reynolds G. **1998**. A new technique for inoculation of conifer seedling roots with the laminated root rot pathogen, *Phellinus weirii*. *Canadian Journal of Plant Pathology* **20**: 324–330.

Szwajkowska-Michalek L, Kwaśna H, Lakomy P, Perkowski J. **2012**. Inhibition of *Armillaria* and *Heterobasidion* growth by *Penicillium adametzii* isolated from *Pinus sylvestris* forest soil. *Forest Pathology* **42**: 454–466.

Tan YH, Moore D. **1992**. Convenient and effective methods for in vitro cultivation of mycelium and fruiting bodies of *Lentinus edodes*. *Mycological Research* **96**: 1077–1084.

Thomidis T, Exadaktylou E. **2012**. Effectiveness of cyproconazole to control *Armillaria* root rot of apple, walnut and kiwifruit. *Crop Protection* **36**: 49–51.

West JS. **2000**. Chemical control of *Armillaria*. In: Fox RTV, ed. Armillaria. Root Rot: Biology and Control of Honey Fungus. Andover, UK: Intercept, 173–182.

West JS, Fox RTV. **2002**. Stimulation of *Armillaria mellea* by phenolic fungicides. *Annals of Applied Biology* **140**: 291–295.

West JS, Hughes C, Fox RTV. **2000**. *Armillaria mellea* can infect the perennial weed, *Rumex obtusifolius*,

in the UK. *Plant Pathology* **49**: 808.

Zweck S, Hüttermann A, Chet I. **1978**. A convenient method for preparing inocula of homogenized mycelia. *Experimental Mycology* **2**: 377–378.

5. Changes in leaf physiology of *Ligustrum vulgare* associated with infection by the root pathogenic fungus *Armillaria mellea*, with comparison to drought stress

Preface

Within my work at the UK Bartlett Tree Research Laboratory, there has been a strong focus on non-destructive assessments of plant physiological vitality and stress e.g. chlorophyll fluorescence, stomatal conductance, leaf water potential, and chlorophyll content. This work was initiated by Dr Glynn Percival and has been expanded by Dr Jon Banks. From the beginning of my studies it had occurred to me that physiological measurements might provide a solution to the issue of timely detection of Armillaria infection. Studies had already been published using some physiological measurements to monitor infection during experiments. Later, Dr Banks published a paper where such measurements were used to quantify the tolerance of various *Acer* cultivars to drought. Discussing this, we wondered if there would be an overlap in physiological responses to drought stress and the root damage caused by *Armillaria*. Investigating the literature I discovered initial research showing similarities. Therefore it was decided to collect physiological measurements from a subset of the collaborative work with Dr Drakulic (Chapter 4) and expand this with an experiment including drought stress.

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5.1 Abstract

Armillaria root rot attacks a wide range of host plants, often producing no conclusive visual symptoms until extensive infection. Previous research indicates infection causes drought-like changes in the above-ground physiology of susceptible hosts. However, measurements vary between studies and time points are minimal. Measurements of leaf physiology may provide simple, rapid, and non-destructive elucidation of infection in susceptible hosts. To assess practical use, higher resolution data from multiple parameters is required to clarify the succession of responses and interactions with drought. This study investigates changes in selected physiological functions of a highly susceptible woody host (wild privet: *Ligustrum vulgare* L.) following *Armillaria mellea* inoculation.

To assess whether it was possible to reliably distinguish between infected and uninfected plants prior to conclusive visual symptoms, rooted cuttings were inoculated and their visual condition, stomatal conductance (g_S), chlorophyll fluorescence, and relative chlorophyll content measured for 308 days. A follow-up experiment examined these physiological responses during early infection and under drought stress.

Without drought stress, *Armillaria* infection on wild privet was characterised after 35-40 days by a 20-35% reduction in g_S and a 5-9% reduction in chlorophyll concentration. Chlorophyll fluorescence parameters (Area, $V_0(B_0)$, PI_{ABS}, F_V/F_M) also showed significant reactivity. Differential chlorophyll fluorescence kinetics also showed varying reactions to infection, drought, and their combination. Multivariate analysis suggests combining these measurements could improve classification of infection and drought status.

Results demonstrate that *Armillaria* infection can be detected from *Ligustrum vulgare* leaf physiology. However this can be masked by environmental stresses e.g. drought, nutrient, and/or root restriction. Leaf physiological measurements may detect infection in non-experimental contexts if there is confidence in the absence of other root pathogens, drought or other conflicting environmental factors. In controlled conditions, g_S of woody wild privet responds to infection in less than half the time required to complete trials on herbaceous hosts.

5.2 Introduction

Armillaria (Fr.) Staude species are economically important soil-borne fungal root pathogens. While widely recognized as pathogens of woody plants, most significantly those cultivated by humans, there are over 200 known host genera including herbaceous plants (Raabe 1962; Ford *et al.* 2017; Cromey *et al.* 2020). *Armillaria mellea* (Vahl) P.Kumm. is recognized as the most pathogenic species worldwide, while all species vary in their relative propensity for acting as pathogens, parasites, and/or saprobes (Gregory *et al.* 1991; Guillaumin & Legrand 2005). In the UK, *Armillaria* Root Rot disease is mostly known for killing horticulturally important trees, especially those in parks and gardens (Rishbeth 1983; Laflamme & Guillaumin 2005), including specimens of personal, cultural, and historic human significance. In UK gardens, *A. mellea* is the most commonly found species in infection surveys (Drakulic *et al.* 2017; Cromey *et al.* 2020). While *Armillaria* Root Rot may serve an important ecological role in natural ecosystems, by removing and recycling stressed and dying trees from the population, infections on cultivated plants are an anthropocentric issue due to high economic impacts on artificial plantings (Heinzelmann *et al.* 2019). Errors are often made in the selection and/or preparation of the site and planting stock, and also in the following treatment of trees, which can increase physiological stress (Rabiey *et al.* 2019) and therefore host susceptibility (Hadfield *et al.* 1986; Legrand & Lung-Escarmant 2005). For example, root deformities originating from cultivation practices and deep burial in particular have been linked to increased *Armillaria* susceptibility (Ouellette *et al.* 1971; Singh & Richardson 1973; Livingston 1990; Legrand & Lung-Escarmant 2005; Day *et al.* 2009; Percival *et al.* 2011). Climate change is also acknowledged as a factor in host susceptibility (Cromey *et al.* 2020; Heinzelmann *et al.* 2019).

Sanitation via basic stump removal ('stumping') can provide economic *Armillaria* control in forestry (Vasaitis *et al.* 2008; Bogdanski *et al.* 2018; Modi *et al.* 2020) and regular tillage of root zones with compressed air can provide economically viable reductions in disease incidence in infected orchards (Miller *et al.* 2020). However for trees of a higher individual value, i.e. those on amenity sites where major soil disturbance is generally undesirable, no economic and ecologically viable methods for direct treatment and control of *Armillaria* Root Rot have been developed/adopted outside of research so far (Guillaumin *et al.* 2005a; Baumgartner *et al.* 2011; Amiri & Schnabel 2012; Heinzelmann *et al.* 2019). While direct treatment deservedly receives much research attention, techniques aimed at detection and diagnosis of infection at an appropriate stage for treatment application, i.e. as early as possible, have received comparatively little attention (Agustini *et al.* 2015).

Early symptoms of *Armillaria* root rot disease are that of a general host decline, with the cause often falsely attributed to factors such as the environmental stresses increasingly commonplace under climate

change (Hailey & Percival 2015; Nunes *et al.* 2020). Visible symptoms may not always be present above ground, even when root damage is severe, e.g. as recorded on highly susceptible *Ligustrum ovalifolium* Hassk. (West & Fox 2002): this is a typical characteristic of root rot pathogens (Agustini *et al.* 2015). Infections may also persist in a latent or parasitic fashion for an extended period (Guillaumin & Legrand 2005), potentially for many years, which ultimately leads to host mortality unless the infection is compartmentalised and remains so. If present at the host's natural death, latent lesions can be released from compartmentalisation to colonize the remaining tissues and serve as a potential source of infection to other plants in the locality (Guillaumin & Legrand 2005).

Late-stage infection is characterized by invasion of the fungus into the tree root collar where it can girdle and kill the tree. This above-ground stage is typically the first instance when the infection becomes evident to the owner or arborist: peeling bark reveals characteristic white mycelial fans beneath, or fruiting bodies are discovered growing from the root collar area. In the absence of direct discovery, other potential outcomes are hosts succumbing to structural issues in a sudden failure, e.g. windthrow in a storm due to physically weakened root systems (Cromey *et al.* 2020), or suddenly dying in adverse weather conditions e.g. drought.

Early detection would provide an opportunity for intervention techniques prior to root collar invasion. This could include therapeutic measures such as the application of plant protection products such as fungicide trunk injections (Amiri & Schnabel 2012) or other plant healthcare measures e.g. root collar excavation (Baumgartner 2004), with an earlier intervention likely to improve host prognosis. Sanitation, one of the most utilized control strategies (Heinzelmann *et al.* 2019), is also likely to increase in efficacy if infected individuals can be identified and removed earlier. Doing so will reduce localised inoculum accumulation. Optimally this would be prior to the pathogen overwhelming/suppressing host resistance mechanisms and gaining unbridled access to the host root system. Rapid detection may also contribute to ecological surveys of the pathogen in natural situations.

Table 1. Previous literature with data addressing the impact of *Armillaria* spp. infection on physiological measurements collected from host foliage.

Susceptible Host/s & time points after inoculation (months)	*Armillaria* species	Measurement, Technique, percentage change of infected [control] plants in comparison to uninfected control (Δ%)	Reported severity of infection, final time point	Reference
***Olea europaea* L. (Oe), 2** ***Quercus rubra* L. (Qr), 2**	*mellea* (*ostoyae* similar in Qr)	Net Photosynthesis, IRGA, Oe -21.6%, Qr -20.3% Stomatal Conductance g_s, IRGA, Oe -26.7%, Qr -21.7% Leaf Water Potential, PB, Oe +73.3%, Qr +49.6%	Rhizomorphs in root system	Loreto et al. 1993
***Vitis berlandieri* Planch. x *Vitis rupestris* L., 13**	*mellea*	Chlorophyll Content, SPAD, -12.5%	56-68% root collar infection	Nogales et al. 2008
***Fragaria* × *ananassa* Duchesne cv. 'Cambridge Favourite', 6, 12, 18, 24**	*mellea*	Chlorophyll Content[1], SPAD, -67.1% to -79.2% F_V/F_M: Photosystem II Efficiency, CF, -45.9 to -61.9%	Severe foliar symptoms, *A. mellea* re-isolated from roots or soil	Percival et al. 2011
***Eucalyptus nitens* (H.Deane & Maiden) Maiden, 4 & 7**	*luteobubalina*	F_V/F_M: Photosystem II Efficiency, CF, -1.8% to -9.3% A_{max}: Photosynthetic Rate, IRGA, -4.7% Chlorophyll Content, SPAD, -22.0%	Mean 71.4% of plants with mycelial fans present in roots	Agustini et al. 2015
***Betula pendula* Roth., 12**	*gallica*	F_0: Fluorescence Origin, CF, +26.1% (non-significant) F_M: Fluorescence Maximum, CF, -20.0% F_0/F_M: an alternate scaling of F_V/F_M, CF, +57.4% = -33.1% F_V/F_M, see Strasser *et al.* 2000 DI_0/CS_0: "Photosynthetic Efficiency Measured as Energy Dissipation in Form of Heat", CF, +43.0% PI_{TOT}: Total Performance Index, CF, -41.0%	Rhizomorphs in root system	Nowakowska et al. 2020

IRGA = Infra-Red Gas Analysis, PB = Pressure Bomb, SPAD = Soil Plant Analysis Development chlorophyll meter, CF = Chlorophyll Fluorescence

[1]Published control values erroneous; SPAD values should be month 6 = 40.0, 12 = 42.3, 18 = 41.8, 24 = 42.9 (G. C. Percival, Personal Communication, 2020).

In previous studies, conventional measures of plant growth have demonstrated a lack of consistency in reflecting infection status (Ford *et al.* 2017) and may only indicate infections after the window of opportunity for treatment. Promising initial work has been made recently towards direct detection of the fungus using 'electronic noses', although it is not yet known if this technique would be effective in assessing disease incidence when the fungus is obscured underground (Navaei 2015; Loulier *et al.* 2020) and technologies remain in active development (Azzouz & Bachari 2018). Various imaging technologies have also shown promise for the early detection of other root rots (Calderón *et al.* 2013; Salgadoe *et al.* 2018; 2019; Pérez-Bueno *et al.* 2019). However, indirect detection may also be possible via physiological measurements of the host with an array of commercially available and widely used devices. These measurements include relative chlorophyll content and fluorescence, stomatal conductance (g_s), and leaf water potential, which a small number of studies have utilized (Table 1). Although available data is limited, it appears that host responses are comparable between varying combinations of (susceptible) host and *Armillaria* species (Loreto *et al.* 1993; Agustini *et al.* 2015). This may even extend to other distinct genera of root rot pathogens due to the fundamental roles of these parameters in plant physiological function, with recorded reactions and reactivity to various infections e.g. those by *Ganoderma* species (Goh *et al.* 2016; Rakib *et al.* 2019), *Phytophthora* species (Fleischmann *et al.* 2005; Clemenz *et al.* 2008), *Rosellinia necatrix* (Martínez-Ferri *et al.* 2016), and *Verticillium dahliae* (Calderón *et al.* 2014). For the most part, these foliar measurements can be collected non-destructively or, in the case of leaf water potential, at the expense of a small number of leaves. This allows employment of the techniques with minimal disturbance to plants and sites. Some are relatively fast to conduct and require minimal training, which could aid their adoption outside of the scientific community. Indeed, cases using these technologies already exist: relative chlorophyll fluorescence (SPAD) is utilized by farmers to guide fertilizer applications (Xiong *et al.* 2015) and continuous excitation chlorophyll fluorescence has been adapted for use by tree care professionals with the 'Arborcheck' device (Hansatech Instruments Ltd., King's Lynn, UK).

The reactivity of these physiological measurements (Table 1) may relate to host susceptibility as found by Loreto *et al.* (1993). If so, a species may be checked against an existing list of susceptibility ratings, such as that provided by Cromey *et al.* (2020), prior to physiological assessments to determine if they are appropriate to monitor for the disease. Also of some concern is that atypical inoculation methods were used in two of these studies: inoculation into a wound (Loreto *et al.* 1993) and fungal culture incorporation directly into the plant growth medium (Percival *et al.* 2011), whereas more typical inoculation protocols place infested woody substrates into the root zone. These atypical inoculations

may yield differences in results to typical natural infection scenarios which consist of contact between roots and inoculum, rhizomorphs, or other roots (Solla *et al.* 2002; Guillaumin & Legrand 2005). In fact, results from one of the previous studies suggests significant interactions of wounding and inoculation on photosynthetic parameters (Agustini *et al.* 2015).

Of the studies detailed above (Table 1), only Percival *et al.* (2011) provided longitudinal data with more than two time points, and no authors provided data collected prior to 60 days after infection. From a review of available literature, it does not appear that there have been any more in depth studies of the potential usefulness of these physiological measurements in detecting *Armillaria* infection. In their study, Agustini *et al.* (2015) named F_V/F_M, which is believed to reflect the efficiency of photosystem II, as the most sensitive photosynthetic parameter to detect disease. Conversely, the current consensus is that F_V/F_M is a more stable measure of plant health in comparison to continuous excitation chlorophyll fluorescence parameters such as the performance index (PI) (Živčák *et al.* 2008; Banks 2018): therefore other parameters may be more reactive and sensitive to the presence of infection. Indeed, one PI 'form', PI_{TOT}, showed higher sensitivity to *A. gallica* infection than F_0/F_M (Nowakowska *et al.* 2020), which yields F_V/F_M when subtracted from 1 (Strasser *et al.* 2000).

Previous studies suggest that *Armillaria* spp. infection, at least on susceptible species (Loreto *et al.* 1993), shows similar physiological signatures to drought stress, e.g. closure of stomata, reductions in leaf water potential and photosynthetic activity. Paralleling this, there are potential links between drought tolerance from wood anatomy and the resistance to *Armillaria* sp. infection (Cruickshank & Filipescu, 2017; Heinzelmann *et al.* 2019). Previous work by Banks and others, has found unique differences in continuous excitation chlorophyll fluorescence readings between droughted and non-droughted plants (Živčák *et al.* 2008; Kalaji *et al.* 2016), including such sensitivity as to be able to discern drought and desiccation stresses in *Acer* trees (Banks 2018).

The aim of this study was to investigate changes in selected physiological functions of *Ligustrum vulgare* L. (wild privet) after inoculation with *Armillaria mellea* using typical inoculation methods. This was also compared to changes caused by drought or combined drought and infection. Higher temporal resolutions were used than previous studies. The earliest stages of infection were a particular focus. The purpose of this was to assess whether it is possible to reliably distinguish between infected and uninfected plants at a stage when no conclusive visual symptoms are present and, furthermore, to determine the influence of drought on this detection.

5.3 Method

5.3.1 Outline & Aims

The study consisted of two experiments. In the first (hereafter referred to as the *Long-term experiment or* LTE) we took physiological measurements from inoculated and uninoculated host plants over a 308 day period: a time period sufficient for some of the hosts to die. This experiment aimed to identify physiological changes triggered by infection and the time points at which they occurred.

In the second experiment (hereafter referred to as the *Short-term experiment* or STE), the leaf physiology of inoculated and uninoculated plants was measured in a similar way, but at a higher temporal resolution than in the LTE. The STE also included a drought treatment in which watering was withheld from a subset of plants until severe drought symptoms were evident and then watering was resumed. This component of the experiment aimed to examine if physiological changes caused by *Armillaria mellea* infection can be discerned from those caused by drought.

5.3.2 Plant Material

Both experiments were performed on rooted wild privet (*Ligustrum vulgare* L.) cuttings. This species/genus is highly susceptible to *Armillaria* root rot infection (West & Fox 2002; Guillaumin *et al.* 2005b; Cromey *et al.* 2020). Above-ground symptoms may not be present despite significant root infestations (West & Fox, 2002). Parent material for the LTE was collected from a hedge in Wisley, Surrey, United Kingdom on the 25th October 2018 and for the STE from commercially sourced parent plants (Best4hedging, Euxton, Lancashire, UK) on the 28th of May 2019. The parent material was assumed to be one individual, or clonal material of such, in all cases. Cuttings were 6-8cm sections of semi-hardened stems of the current year's growth, bearing a single leaf node. These were cut straight approximately 1.5cm above the node and a 45° cut just above the next node below. During this process the bases of the freshly made cuttings were kept in moist tissue paper. Leaves were trimmed or removed to give uniform cuttings with one or two mature leaves. These were placed into a propagator (Hydropod, Greenhouse Sensation, Lancashire, UK) which continuously misted the basal ends with tap-water for 5-6 weeks under grow-lights. The cuttings were then planted in moist compost (see below for specifics) with the roots 2-3cm below the surface, alongside a solid 2cm diameter plastic or glass rod spacer which was in close proximity (1-2cm) to the stem. The removal of this rod, prior to inoculation, created a void of approximately 2.5 cm by 7cm, 1-2cm from the bottom of the pot: large enough for the woody infection substrate to be buried with minimal root disturbance (Prospero *et al.* 2004; Cruickshank *et al.* 2010). Cuttings were then allowed to root before inoculum was added: for 1 week in the LTE and 5 weeks in the STE.

5.3.3 Growing Conditions

The LTE took place in a controlled environment chamber at the Field Research Station, Royal Horticultural Society Garden Wisley, (Woking, Surrey, UK), under a temperature regime of 23°C daytime & 15°C night, humidity 50%, and daytime light levels supplemented to 36 µmol m^{-2} s^{-1} minimum. Plants were potted into Sylvagrow Peat Free Sustainable Growing Medium (Melcourt Industries Ltd., Tetbury, Gloucestershire, UK). The STE took place in a covered open-ended polytunnel at The R A Bartlett Tree Research Laboratory, Shinfield, Reading, Berkshire, UK. Plants were potted into a peat-based Multi-Purpose Compost (Clover Peat, Dungannon, Co. Tyrone, Northern Ireland).

In all experiments, unless undergoing drought treatment, plants were monitored and watered as needed to maintain moist soil. Watering was always applied by adding tap water to the tray, i.e. watering from the base. In both experiments, the plants were not fertilised or re-potted for the duration of the experiment.

5.3.4 *Armillaria* culture, Inoculum and Inoculation

The isolate used in all trials was *Armillaria mellea* CG440 from the culture collection of the Royal Horticultural Society, originally isolated from a *Ligustrum* spp. host, Surrey, United Kingdom, 22nd November 2006 (Beal *et al.* 2015). The isolate had been recently inoculated into a live host (*Fragaria × ananassa*) and re-isolated from the resulting infection in an effort to ensure pathogenicity, by Helen Rees of Bristol University. This isolate has been used in a number of other studies (Beal *et al.* 2015; Ford *et al.* 2015; 2017), with potential as a model strain.

Under aseptic conditions, fragments of *Armillaria mellea* CG440 mycelium under 1mm^2 were collected from the young culture provided or a derived from a long-term slant (malt extract agar (MEA), Oxoid Ltd., Basingstoke, Hampshire, UK) stored at 4°C. A pipette tip was used to transfer the fragments to a 50ml conical-bottom tube containing 10ml of potato dextrose broth (PDB) (Neogen Europe Ltd., Auchincruive, Ayr, UK) amended with sodium acetate to 2.5mM (Amended PDB: APDB) prior to autoclaving (holding at 121°C for 30 minutes) (Baumgartner *et al.* 2010). The resultant culture was incubated in an orbital shaker at 27°C, 200rpm, in low light, for one week.

Approximately 0.5g of the resulting spherical colonies of mycelium were added to 1ml APDB in an MP Biomedicals 'lyzing matrix M' tube with a ¼ inch (6.35mm) ceramic bead and shaken at 4m s^{-1} for 10 seconds in a FastPrep-24™ 5G lysis system (MP Biomedicals, USA). Colony plugs were produced by pipetting 20µl volumes of this homogenized mycelium (Pellegrini *et al.* 2012) to the surface of 20ml MEA in 9cm Petri dishes, evenly spaced, seven on each plate, allowing it to dry sufficiently to adhere onto the

MEA surface and then sealing the plate with parafilm. After incubating for one week in the dark at 21°C, plugs of approximately 5mm x 5mm were cut from the colonies using a scalpel. Generally, these sections covered a whole quarter of a colony and one was taken per colony.

Inoculation substrates used were either sections of *Corylus avellana* stem (billets) (Mansilla *et al.* 2001) of 5.00 x 1.5±0.2cm diameter in the LTE, or acorns (Beckman & Pusey 2001) of *Quercus robur* weighing 2.4 - 4.5g, which had been pierced once with a 1mm needle, in the STE. Substrates were prepared by autoclaving twice in water, holding at 121°C for 45 minutes. Following this, intact substrates were arranged in a single layer (horizontally) in polypropylene 'takeaway' tubs of 170mm x 120mm x 50mm, and nearly covered with molten autoclaved MEA. The lids were then put on the tubs partly loose and the tubs autoclaved, holding at 121°C for 30 minutes. The tubs were then closed and the agar allowed to solidify prior to inoculation.

Tubs of acorns were inoculated by applying 0.5ml of homogenized mycelium, alongside 4.5ml of APDB as a carrier, prepared as described above. The tub was then closed and gently tilted back and forth in multiple directions, for approximately 10 seconds, to mix and spread the homogenized mycelium. Tubs of billets were inoculated using 6 colony plugs applied to the MEA surface in as even a spread as possible. Corresponding controls were prepared for each, either having 5ml APDB for acorns or no additions for the billets. All tubs were sealed with parafilm and incubated in the dark at 21°C for 28-31 days. Tubs were checked weekly for visual signs of contamination, such as bacterial colonies or uncharacteristic fungal growth, and any contaminated tubs removed. No control tubs were contaminated. Inoculum per plant consisted of a single billet in the LTE or three acorns (as a single unit) in the STE, which had been cleaned of excess agar and/or externally growing mycelium. Spacers were removed and prepared inoculum was placed into the void in the root zone. The surrounding compost was then used to cover the inoculum. Controls received uncolonized facsimile substrates.

5.3.5 Experimental Design

5.3.5.1 Long-term experiment (LTE)

The LTE was conducted in two experimental rounds ('replicates'). In each replicate, plants were placed into a randomized block pattern within a single tray and situated on opposite sides of a spacious growth chamber. Plants for the first replicate were potted/inoculated on the 30th of November 2018/7th December 2018 and the second on the 6th December 2018/14th December 2018. In each replicate of the experiment, 10 plants were subject to the *Armillaria* infection treatment (see above), and 10 plants were control plants. Visual health and physiological measurement data (see below) were collected from the plants at days 34, 75, 89, 104, 116, 144, 175, 224, 273 and 308: although, due to adverse weather

conditions and atypical fluctuations in light levels, it was not possible to collect stomatal conductance readings on day 224. At the end of the experiment, or following the death of plants, root systems were dissected and inspected for the presence of rhizomorphs in the root system and the presence of mycelial fans in the root collar.

5.3.5.2 Short-term experiment (STE)

In the STE, plants were potted on the 27th of June 2019, and inoculated on the 1st of August 2019. The experiment was timed to coincide with a natural infection window for *Armillaria* spp. in the early autumn (Perez-Sierra 2004). There were four treatment combinations: uninfected-watered (UW), infected-watered (IW), uninfected-droughted (UD), & infected-droughted (ID). Plants were assigned to treatment groups prior to inoculation. Plants were placed into a randomized block design, with blocks formed from a plant of each of the four treatments in a randomized order. To account for variation in plant size, plants were ordered by size and arranged so that each block contained plants of a similar size. This reduced the variation in plant size between the treatments. There were three trays, each containing 6 blocks i.e. 24 plants, giving 72 plants in total. All treatments originally had 18 replicates. Following the conclusion of the experiment, the root systems were dissected for inspection and the data was consolidated to infected plants with rhizomorphs visible in their root systems/soil and the uninfected controls neighbouring them. This avoided data being skewed by the large proportion of apparently failed inoculations. Eight repeats remained for each treatment, aside from ID which had seven.

For plants allocated to the drought treatment, drought was imposed by placing the pots into small plastic bags attached with tape. Drought was imposed when chlorophyll fluorescence indicated initial effects of infection and removed once visually severe: days 40-65. Watered treatments had an open-bottomed 'dummy' bag secured to their pots. Physiological measurements were taken at multiple time points (detailed below) and halted when the control plants appeared to have entered dormancy, following day 79. Given the timing of the drought treatment, there were three 'stages' to the experiment, 'Pre-Drought' (days 0-40: no differences expected between plants assigned to different watering regimes), 'Drought' (days 40-65), and 'Recovery' (days 65-79). The subset of plants that received continuous watering could also be analysed as a continuous period (days 0-79), referred to as 'STE Watered'.

Measurements consisted of stomatal conductance (g_s), chlorophyll content, continuous excitation chlorophyll fluorescence parameters and differential kinetics, as well as visual condition. Fluorescence readings were collected at days 0 (the day before inoculation), 2, 9, 15, 22, 29, 36, 40, 41, 42, 43, 45, 44, 46, 47, 49, 51, 54, 55, 56, 57, 58, 61, 62, 63, 64, 65, 70, 71, 78 & 79. Stomatal conductance (g_s), relative chlorophyll concentration, and visual health index measurements were taken at days 0, 2, 9, 15, 22, 29,

36, 40, 41, 43, 51, 57, 64, 71, & 79. Due to adverse weather conditions and atypical fluctuations in relative humidity, it was not possible to collect g_S readings on day 40. Following the aforementioned dissection of the root system, shoots and roots/root collar were separated and dried in an oven at 85°C for 24 hours, then removed and immediately weighed. Root:shoot ratio was calculated by dividing root and total root collar dry weight by that of the shoots.

5.3.6 Physiological Measurements

The condition of the plants in the experiments was assessed using the following qualitative indices and quantitative measurements:

5.3.6.1 Visual Health Index (VHI)

The visual health of plants was scored on a scale of 1-4:

1. Plant visually healthy
2. Visible symptoms of ill health (leaves yellowing or dying)
3. Visible wilting or extensive leaf loss
4. Plant visibly dead

5.3.6.2 Mortality

A binary mortality indicator was derived from the VHI score, with dead plants = 1 and live plants = 0.

5.3.6.3 Extension Growth

Extension growth was measured for plants in the LTE and focused on any stems arising from the original cutting. Stems were measured from the base to the bottom of the terminal bud and summed per individual at each time point. When plants died they were recorded as NA values.

5.3.6.4 Stomatal Conductance (g_S)

Stomatal conductance (g_S) in mmol m^{-2} s^{-1} was measured from leaves using an AP4 Porometer (Delta-T Devices Ltd, Cambridge, UK), which was calibrated prior to data collection at each time point. If a measurement took longer than approximately 5 minutes, it was assumed the stomata were closed and the reading was recorded as 1.00 mmol m^2 s^{-1}: this took place in 2 of 433 total STE readings, with the lowest recorded conductance being 2.56 mmol m^2 s^{-1}, and did not occur in the LTE. In the LTE, readings were taken from the two oldest leaves present on a plant. In the STE, a reading was taken from each plant's healthiest looking leaf at the time.

5.3.6.5 Relative Chlorophyll Content

Readings were taken from leaves using a Konica Minolta Soil Plant Analysis Development (SPAD) 502DL data-logging meter, yielding chlorophyll content in SPAD arbitrary units. In the LTE, a reading was taken

from the two original leaves present at the start of the experiment and, when present, from two hardened leaves which had grown during the course of the experiment. In the STE, three readings were taken randomly from the healthiest leaves present.

5.3.6.6 Continuous Excitation Chlorophyll Fluorescence

Measurements utilised the Handy PEA device (Hansatech Instruments Ltd, King's Lynn, UK). All leaves were dark adapted (~15 min) before a fluorescence response was induced by a one second flash of 650 nm light at an intensity of 1500 μmol m^{-2} s^{-1}, provided by an array of three light-emitting diodes covering a 4mm diameter circle of leaf surface. Chlorophyll fluorescence parameters were calculated according to the calculations described by Banks (2017). F_V/F_M is the ratio of variable (F_V) to maximal chlorophyll fluorescence (F_M) expressing the efficiency of photosystem II. PI_{ABS} (Performance Index) is the product of multiple ratiometric chlorophyll fluorescence calculations as an overall plant vitality indicator. Area is the fluorescence-time area above the fluorescence curve (relative to F_M) expressing the size of the electron acceptor pool on the reducing side of photosystem II (PSII). A programmed second flash was used to collect light adapted measurements, allowing the calculation of $V_0(B_0)$ as described by (Mehta *et al.* 2010). $V_0(B_0)$ is a ratio, from comparison of light and dark adapted F_V/F_M readings, expressing the fraction of 'closed' PSII reaction centres (non-reducing plastoquinone B).

$$\frac{F_v/F_m\ (dark) - \ light\ adapted\ F_v/F_m}{F_v/F_m\ (dark)}$$

Equation 1. Calculation of $V_0(B_0)$ from Mehta et al. (2010), where F_V/F_M is the ratio of variable (F_V) to maximal chlorophyll fluorescence (F_M).

In the LTE, readings were taken from the two oldest leaves present. In the LTE, replicate readings were performed using a Pocket PEA device (Hansatech instruments Ltd., Norfolk), after ensuring no difference between measurement device these were combined with the Handy PEA readings for double-normalised differential kinetics only. In the STE, readings were taken randomly from three visually typical leaves from day 0 to day 40, increasing to six readings afterwards. Double-normalised differential kinetics (L-band and K-band, or ΔW_{OK} and ΔW_{OJ} respectively) were calculated in accordance with Oukarroum *et al.* (2007). Raw data was processed in a modified fashion, means for the control group (uninfected in the LTE, UW in the STE) were calculated for each time point and all readings were compared to their respective time point's control average, including those of the control itself, allowing the visualisation of variation (as standard error) for all treatment groups.

$$\Delta W_{0X} = \frac{F_t - F_{\hat{0}}}{F_x - F_{\hat{0}}}(treated) - \frac{F_t - F_{\hat{0}}}{F_x - F_{\hat{0}}}(control)$$

Equation 2. Calculation of ΔW_{ox} from Banks (2018), where F_t is the fluorescence at time (t) across the induction curve, $F_{\hat{0}}$ = fluorescence at time zero, and F_x is replaced with either F_J (F_{2ms}) or F_K ($F_{300\mu s}$) for W_{0J} or W_{ok} respectively.

For all fluorescence measures, and derived values, infinite values were recoded as missing data. Zero values of the Area parameter, and any V_0(B_0) values of exact 0 or 1, were also recoded as missing data.

Relative variable florescence (V_t) was also calculated in accordance with Oukarroum *et al.* (2007) and Banks (2018). Using ΔV_t better displays differences present between the central regions of OJIP transients (graphed continuous excitation fluorescence over one second) because V_t is normalised to F_0 and F_M from raw OJIP transients.

$$V_t = \frac{F_t - F_{\hat{0}}}{F_M - F_{\hat{0}}} \times 10$$

Equation 3. Calculation of relative variable fluorescence V_t from Banks (2018), where F_t is the fluorescence at time (t) across the induction curve, $F_{\hat{0}}$ = fluorescence at time zero, and F_M = maximal fluorescence.

5.3.7 Statistical Analyses

All data analysis was conducted in the R statistical programming language (R Core Team 2020). A significance level of 5% (α=0.05) was used for all tests unless otherwise stated. In brief, each response variable was analysed as a function of infection, alone or in combination with drought, and time (Table 2). Where possible, linear mixed modelling was used to account for non-independence of measurements from the experimental design and individual values for all leaves were used for response modelling.

Type III ANOVA analysis was performed on each fitted model as an omnibus test, using the 'Anova' function from the 'car' package. Contrast coding was used for data where drought was a factor, due to the factorial nature of this part of the experimental design, thus all ANOVA effects reported are main effects. Marginal and conditional R^2 ('r.squaredGLMM' function, 'MuMIn' package) or Nagelkerke's pseudo R^2 ('r.squaredLR' function, 'MuMIn' package) were calculated from models where appropriate.

Infection Hazard Ratio was interpreted from the mixed-effect Cox Proportional Hazard model via exponentials of the coefficient and its standard error in the model summary. For the visual scale data, the effect of treatment group on probabilities for each level of the scale were visualized using the 'effects' package and interpreted. For all other data, pairwise comparisons between treatment groups were tested: means at each time point and trends over time (slopes) were calculated from the model for each treatment group, where appropriate using estimated marginal means (aka least-square means),

using the ‘emmeans’ and/or ‘emtrends’ functions from the ‘emmeans’ package. This package used Tukey’s honest significant difference test (Tukey’s test) to adjust p-values for multiple comparisons and provided groupings alongside back-transformed mean values. The ‘effects’ and ‘ggplot2’ packages, respectively, were used to generate predicted values for modelled effects and interactions and plot them for further interpretation. Y-axes for transformed dependent variables were back-transformed with appropriate inverse functions (Table 2) and plotted on the arithmetic scale. To plot mean data over time and stage for each measurement, ‘ggplot2’ was used.

Linear discriminant analysis (LDA) was used for multivariate analysis of a combined dataset of LTE & STE pre-mortality physiological and fluorescence parameters (Table 2). Data was combined from the two experiments in an effort to find responses which were less sensitive to differences in host and environment. Data was averaged to give one observation per individual at each time point. Transformations (Table 2) were applied prior to analysis and any records with missing observations removed. Using the ‘caret’ package, each dataset was scaled and centred about the mean, then split into training and testing datasets of 80% and 20% respectively. Assumptions were checked on the training dataset using boxplots, Q-Q plots, Levene’s test (‘car’ package), and Box’s M Test (‘heplots’ package). LDA was performed and the predictive power of the model was used to optimise the variables selected. Quadratic Discriminant Analysis (QDA) was used in place of LDA for analysis of both STE drought stage fluorescence parameters and time points (Table 2), which failed Box’s M Test breaking the LDA assumption of equal covariance. Stomatal conductance (g_S) and relative chlorophyll concentration data were excluded from the drought stage analysis due to their substantially lower numbers of observations in comparison to the fluorescence data. Drought time point and parameter training datasets both consisted of 496 observations, combined LTE & STE parameters 337 observations, and combined LTE & STE time points 572 observations.

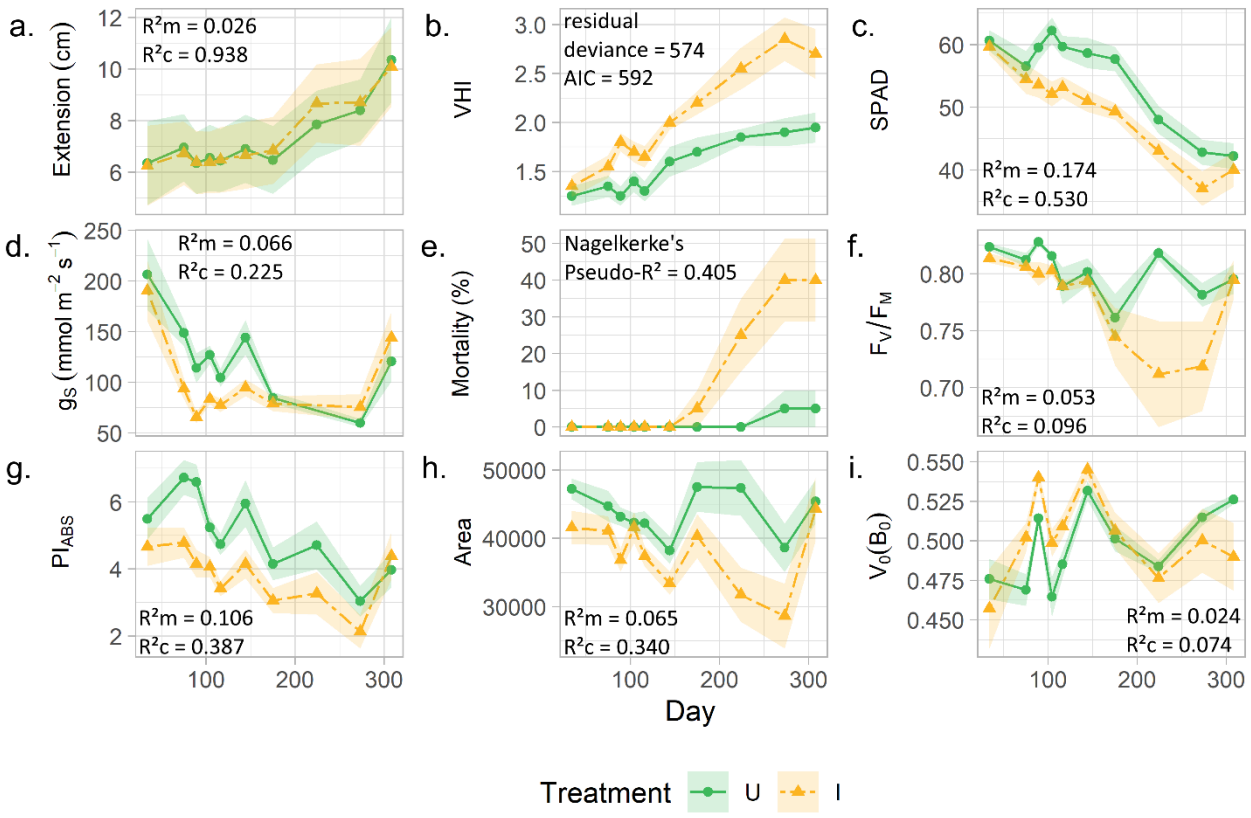
Table 2: Details of statistical models

Measurement	Model[1]	Dataset[2]	Transformation/Backtransformation[3]	Dependent Variable ~ Fixed Factors	Random Factors
Extension Growth	LME	LTE	-	Extension~Infection*Time	1|Replicate/Block/Subject
Root:Shoot Ratio	LME	STE	-	logit of Root:Shoot Ratio~Drought*Infection	1|Block
Mortality	Cox-ME	LTE	-	Survival(Time,Mortality)~Infection	1|Replicate/Block/Subject
Stomatal Conductance (g_s)	LME	LTE	log/exp	log of g_s ~Infection*Time	1|Replicate/Block/Subject
		STE - watered	log/exp	log of g_s ~Infection*Time	1|Block/Subject
		STE - each stage	log/exp	log of g_s ~Drought*Infection*Time	1|Block/Subject
Visual	OLR	LTE	-	Visual Index Score~Infection*Time+Replicate/Block	-
		STE - watered	-	Visual Index Score~Infection*Time+Block	-
		STE - each stage	-	Visual Index Score~Drought*Infection*Time+Block	-
Relative Chlorophyll Content	LME	LTE	-	SPAD~Infection*Time	1|Replicate/Block/Subject
		STE - watered	-	SPAD~Infection*Time	1|Block/Subject
		STE - each stage	-	SPAD~Drought*Infection*Time	1|Block/Subject
Area	LME	LTE	log/exp	log of Area~Infection*Time	1|Replicate/Block/Subject
		STE - watered	-	Area~Infection*Time	1|Block/Subject
		STE - each stage	-	Area~Drought*Infection*Time	1|Block/Subject
F_V/F_M	LME	LTE	logit/inverse	logit of F_V/F_M~Infection*Time	1|Replicate/Block/Subject
		STE - watered	logit/inverse	logit of F_V/F_M ~Infection*Time	1|Block/Subject
		STE - each stage	logit/inverse	logit of F_V/F_M ~Drought*Infection*Time	1|Block/Subject
PI_{ABS}	LME	LTE	-	PI_{ABS} ~Infection*Time	1|Replicate/Block/Subject
		STE - watered	-	PI_{ABS} ~Infection*Time	1|Block/Subject
		STE - each stage	-	PI_{ABS} ~Drought*Infection*Time	1|Block/Subject
$V_0(B_0)$	LME	LTE	logit/inverse	logit of Vo(Bo)~Infection*Time	1|Replicate/Block/Subject
		STE - watered	logit/inverse	logit of Vo(Bo)~Infection*Time	1|Block/Subject
		STE - each stage	logit/inverse	logit of Vo(Bo)~Drought*Infection*Time	1|Block/Subject
Multivariate	LDA	LTE Pre-Mortality & STE watered	log/exp on PI abs & g_s, logit/inverse on $V_0(B_0)$, then scale & centre on all	Infection ~ log of PI_{ABS} + logit of $V_0(B_0)$ + log of g_s	-
		STE Drought	log/exp on g_s, logit/inverse on & $V_0(B_0)$ then scale & centre	Treatment Group ~ Area + logit of $V_0(B_0)$ + Log of PI_{ABS}	-
	QDA	STE Drought	log/exp then scale & centre	Treatment Group ~ log of 0.0006 + log of 0.001 + log of 0.0015 + log of 0.0026 + log of 0.013 (chlorophyll fluorescence at time point in seconds)	-

[1]Model Types (R package::function): LME = Linear Mixed Model (nlme::lme), Cox-ME = Cox Proportional Hazards Mixed Model (coxme::coxme), OLR = Ordinal Logistic Regression (MASS::polr), LDA= Linear Discriminant Analysis (MASS::lda) QDA= Quadratic Discriminant Analysis (MASS::qda). [2]LTE = long term experiment, STE = short term experiment. [3]Transformations (R package::function): log/exp = base::log/base::exp, logit/inverse = boot::logit/boot::inv.logit, scale & centre = caret::preProcess(method = c("center", "scale")).

5.4 Results

Two experiments, the LTE and STE, were set up to examine how *A. mellea* influences the leaf physiology of *Ligustrum vulgare* in the long term, short term and presence of drought. A range of measures were used: stomatal conductance, chlorophyll content and fluorescence, with the experiments taking place in varied environments with differing inoculum sources. Salient results of all statistical analyses are presented in supplementary data (*P*-values & CIs, Appendix B): ANOVA (Table SD1), LTE and STE watered pairwise comparisons (Table SD2), and STE pairwise comparisons of trends (Table SD3) and means (Table SD4).



$V_0(B_0)$ is a ratio

from comparison of light and dark adapted F_V/F_M readings ((F_V/F_M [dark] – light adapted F_V/F_M) ÷ F_V/F_M [dark]) expressing the fraction of 'closed' PSII reaction centres (non-reducing plastoquinone B). n = 20. Generated in the R statistical programming environment using the package 'ggplot2'. See Tables S1 & S2 for statistical analyses.

5.4.1 Mortality & Extent of Infection

Host mortality and extent of infection, from destructive harvests of root systems, was used to assess the success of inoculation and provide context for any significant impacts of infection on leaf physiology.

At the conclusion of the LTE, mortality of infected and control plants was 40% and 5% respectively (Figure 1e). The first plants died at day 175 in infected plants, and subsequent plant deaths occurred up until day 273. Only a single uninfected control plant died, and this happened towards the end of the experiment (between days 224 and 273). The hazard ratio for mortality was 14.2 times greater for infected plants than uninfected, with a 95% confidence interval (CI) of 1.33, 152.01 (Cox mixed-effects model/ANOVA effect of treatment P=0.028, model z = 2.19, ANOVA Wald X^2 = 4.807, Nagelkerke's pseudo R^2 = 0.405). Following death, or at the conclusion of the LTE, all but one of the infected plants (95%) had visible rhizomorphs in their root systems and 40% had mycelial fans within the root collar: neither of these diagnostic symptoms occurred in the uninfected control.

No mortality or mycelial fans within root collars were recorded in the STE. All plants in the infected treatment (IW, ID) retained in the dataset had visible rhizomorphs in their root systems. As described above this was to avoid the data being skewed by the large proportion of apparently failed inoculations.

Observations of mortality and rhizomorphs within root systems clearly indicated that inoculations were a success and inoculated hosts were exposed to infection stress. Therefore the influence of infection, and its combination with drought, on previously collected measurements of plant physiology can be investigated. By comparing a wide range of such measurements, it is possible to indicate which have the greatest potential, alone and in combination, for the detection of *A. mellea* infection.

5.4.2 Visual Health Index (VHI)

Indices of visual health are a commonly used and subjective measure of plant disease. Plant custodians, e.g. gardeners, arborists, are often completely dependent on visual symptoms for the initial detection of pests and diseases. Typically visual symptoms of *Armillaria* are generalised symptoms of ill health, with characteristic symptoms found too late for the action on an individual plant basis. Therefore a visual index of plant health is an important point of comparison for any indicators of infection arising from leaf physiology.

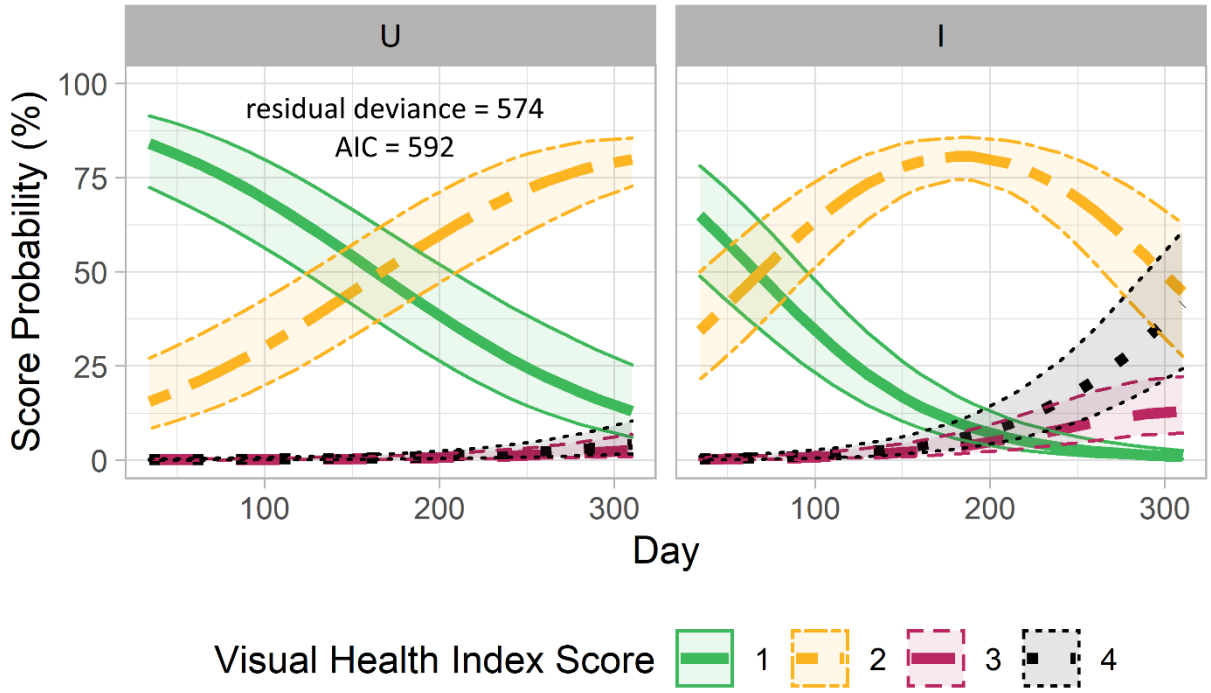


Figure 2. Modelled Long-term impact of *Armillaria mellea* infection on probabilities of Visual Health Index Score of *Ligustrum vulgare* over time. Ordered logistic regression model. Heavy lines represent fitted probability values for each score, bordered ribbon around line represents 95% confidence interval. U = Uninfected (watered). I = Infected (watered). Score: 1 = plant visually healthy, green solid line & ribbon; 2 = visible symptoms of ill health (leaves yellowing or dying), yellow alternate dashed line & ribbon; 3 = visible wilting or extensive leaf loss, hibiscus red dashed line & ribbon; 4 = plant visibly dead, black dotted line & ribbon. *n* = 20. Generated in the R statistical programming environment using the packages 'MASS', 'effects', & 'ggplot2'. See Table SD1 for statistical analysis.

In analyses of the LTE and STE watered treatments, there was a significant interaction effect of infection and time on VHI (LTE ANOVA *P*=0.028, likelihood-ratio $X^2 = 4.850$, residual deviance = 574.341, AIC = 592.341. STE watered *P*=0.190, likelihood-ratio $X^2 = 5.537$, residual deviance = 302.940, AIC = 314.940). All plants were more likely to visually decline over time (figure 2, 3D). However, infected plants were more likely to transition to higher scores than uninfected, e.g. modelled LTE data predicts the probability of a visually healthy plant at day 310 as 0.9% (CI 0.3, 2.6) for infected plants and 13.0% (CI 6.2, 25.4) for uninfected. In the STE watered treatments, UW outscored IW until day 45 i.e. having worse visual health (figure 3D).

Significant drought-infection-time interaction effects on VHI were present prior to and during the drought period (figure 3A, 3B. Pre-drought *P*=0.045, likelihood-ratio $X^2 = 4.012$, residual deviance = 286.801, AIC = 306.801. Drought *P*=0.039, likelihood-ratio $X^2 = 4.258$, residual deviance = 286.972, AIC =

306.972). Just before drought application, uninfected plants were more likely to exhibit symptoms of ill health (score = 2), than infected plants, and had transitioned to higher scores more rapidly (figure 3A). Visual health of UW improved in the drought period, in contrast to all other treatments (figure 3B). Droughted treatments declined rapidly in comparison to IW (figure 3B). Wilting and/or extensive leaf loss (score = 3) was also more probable in these groups: after 25 days of drought (day 64) modelled data predicts its probabilities for each treatment as IW 12.4% (CI 4.1, 32.1), UW 1.6% (CI 0.4, 59.2), ID 39.1% (CI 14.7, 70.6), and UD 44.1% (CI 19.9, 71.4). Significant drought-time interaction effects on VHI were present during the recovery from drought, while infection had no significant effects (P=0.208-0.387). The visual health of droughted treatments improved faster than watered treatments (figure 3C).

The visual health of plants was influenced by factors outside of infection, warranting investigation into more objective continuous measurements of plant physiology and health.

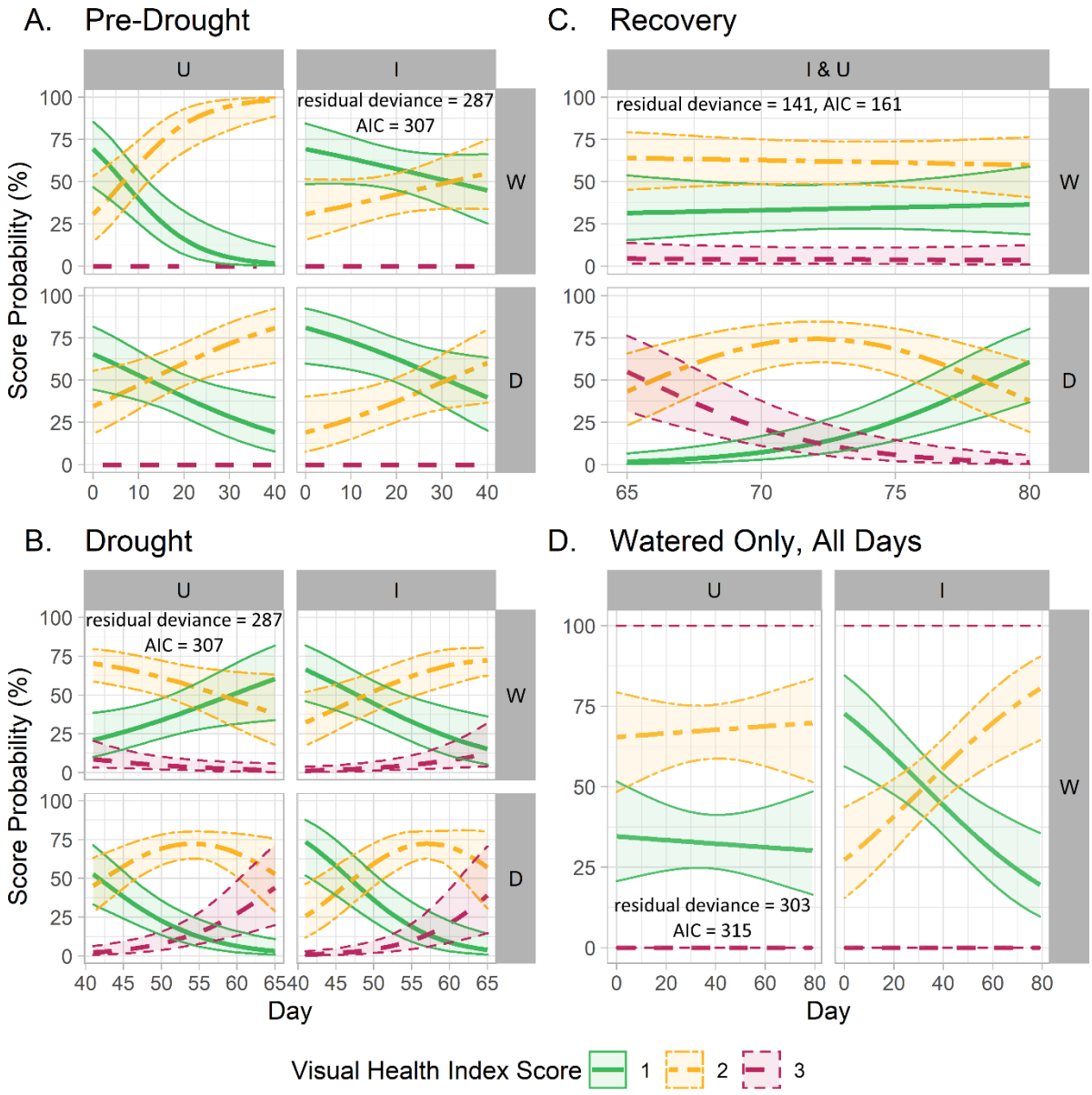


Figure 3. Modelled impact of *Armillaria mellea* infection and imposed drought on probabilities of Visual Health Index Score of *Ligustrum vulgare* over time. Ordered logistic regression models. Heavy lines represent fitted probability values for each score, bordered ribbon around line represents 95% confidence interval. Treatments: U = Uninfected, I = Infected, W = Watered, D = Droughted. Score: 1 = plant visually healthy, green solid line & ribbon; 2 = visible symptoms of ill health (leaves yellowing or dying), yellow alternate dashed line & ribbon; 3 = visible wilting or extensive leaf loss, hibiscus red dashed line & ribbon (this ribbon is removed in panel D for clarity). n = 7-8. Generated in the R statistical programming environment using the packages 'MASS', 'effects', 'ggplot2', & 'ggpubr'. See Table SD1 for statistical analysis.

5.4.3 Extension Growth

Extension growth is a typical general indicator of plant health. Measures of plant growth have been suggested to be unreliable indicators of *Armillaria* infection. In the LTE, there was a significant interaction effect of infection and time on extension growth (ANOVA P=0.002, Wald X^2 =9.715, marginal R^2 = 0.026, conditional R^2 = 0.938). Only the modelled trend (slope) of extension growth was significantly slower (59.8%) in infected plants (I) than uninfected plants (U) (P=0.002, t-ratio = 3.117, figure 2a). I & U means did not separate significantly at any time point. This measurement reacts weakly to *Armillaria* infection and other more specific physiological measurements may be more appropriate.

5.4.4 Root:Shoot Ratio

Root:shoot ratio is a common destructive measure of a plant's physiological health and decreases may reflect damage to the root system by *Armillaria*. In the STE, there was a significant effect of drought on root:shoot ratio (ANOVA P=0.052, Wald X^2 = 12.770, marginal R^2 = 0.228, conditional R^2 = 0.554). Root:shoot ratio was significantly higher in UW than the droughted treatments (P=0.012-0.030, t-ratios = -3.043 to -3.483), IW was not significantly different from either (P=0.209-0.449, t-ratios = -0.566 to -2.033). Means in comparison to UW were: IW -5.9%, UD -12.4%, and ID -15.1%. This destructive measure could not classify infected and uninfected plants, non-destructive measures may be able to.

5.4.5 Stomatal Conductance (g_S)

Stomatal conductance (g_S) quantifies the degree of stomatal opening, with strong links to plant water status. Therefore it can be influenced by both root damage i.e. from *Armillaria* infection (Table 1) and drought stress.

There were significant infection-time interaction effects on g_S (ANOVA: LTE P=0.037, Wald X^2 = 4.354, marginal R^2 = 0.066, conditional R^2 = 0.225. STE watered P=0.003, Wald X^2 = 8.993, marginal R^2 = 0.171, conditional R^2 = 0.227): on infected plants it was significantly lower in the LTE days 34-175 (-35.6% to -19.3% respectively, P=0.001-0.027, t-ratios = +2.404 to +3.932), and in the watered treatments of the STE from day 41 (-21.8%, P=0.008-0.041, t-ratios = -2.500 to -3.686).

During drought and recovery, there was a significant drought-time interaction effect on g_S (ANOVA: drought P<0.001, Wald X^2 = 104.613, marginal R^2 = 0.595, conditional R^2 = 0.701. Recovery P<0.001, Wald X^2 = 46.606, marginal R^2 = 0.629, conditional R^2 = 0.629. Figure 4d). After 12 days of drought (day 51), it was significantly lower in ID (-75.6%) and UD (-76.2%) (P<0.001-0.012, t-ratios = -3.468 to -5.128 & +3.693). In recovery, ID and UD showed significant increases in g_S and all treatments grouped together by day 79 (P=0.174-0.922, t-ratios = -2.144 to +0.773). In drought and recovery analyses IW and UW did not separate significantly (day 51-71, P=0.220-0.866, t-ratios = -0.833 to -2.002).

Physiological measurements from chlorophyll may complement the differences found in g_s.

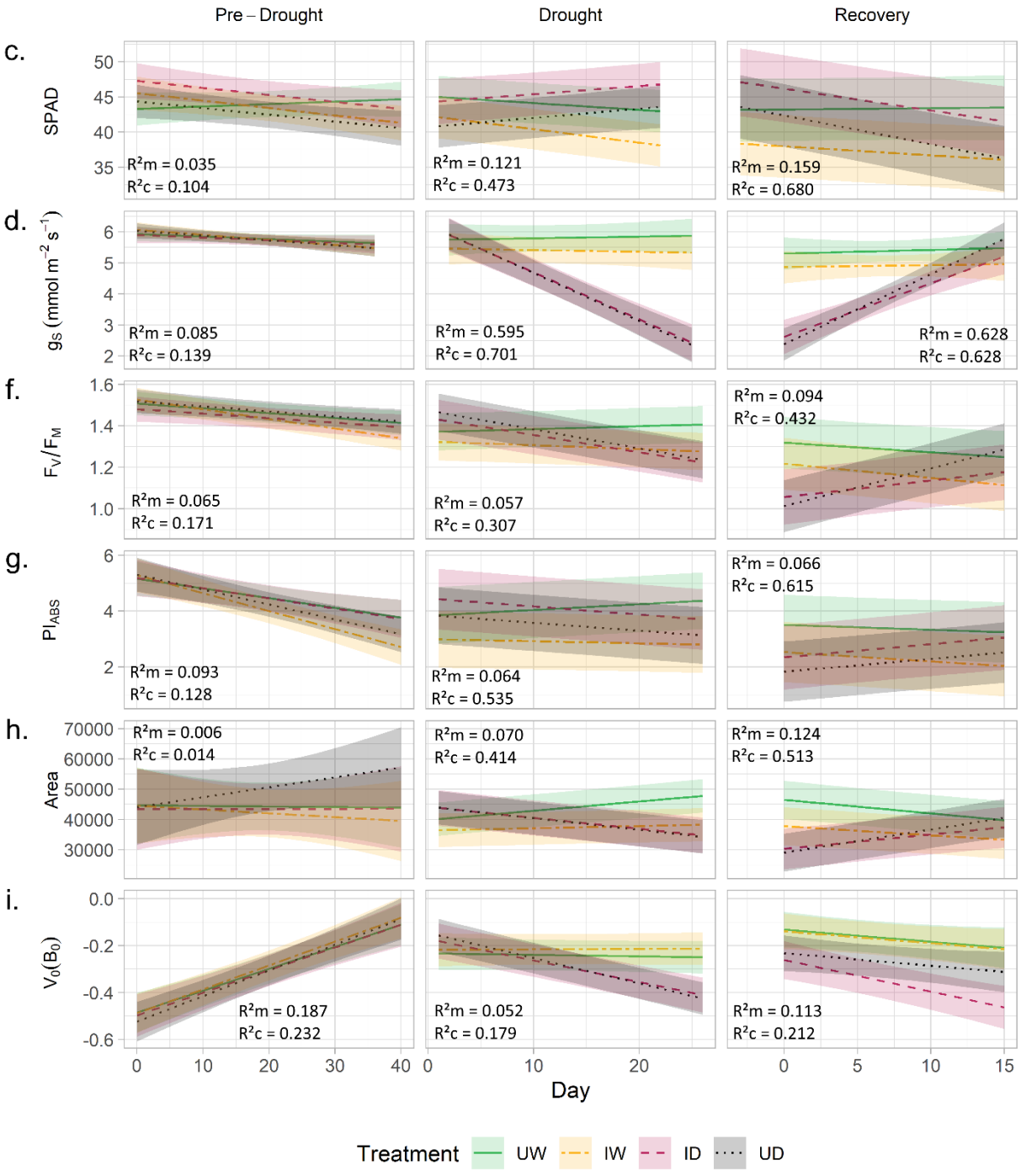


Figure 4. Modelled impact of *Armillaria mellea* infection and drought on various physiological measures of *Ligustrum vulgare*. Linear mixed effects models. Lines represent fitted values, ribbon around line represents 95% confidence interval. UW = Uninfected Watered treatment: solid line & ribbon in yellow. IW = Infected Watered treatment: alternate dashed line & ribbon in green. ID = Infected Droughted treatment: dashed line & ribbon in hibiscus red. UD = Uninfected Droughted treatment: dotted line & ribbon in black/grey. Chlorophyll content shown in SPAD (Soil Plant Analysis Development) arbitrary units. Visual health index scores: 1 = plant visually healthy, 2 = visible symptoms of ill health (leaves yellowing or dying), 3 = visible wilting or extensive leaf

loss evident, 4 = plant visibly dead. Stomatal conductance (g_S) presented in mmol m^2 s^{-1}, F_V/F_M is the ratio of variable (F_V) to maximal chlorophyll fluorescence (F_M) expressing the efficiency of photosystem II, PI_{ABS} (Performance Index) is the product of multiple ratiometric chlorophyll fluorescence calculations as an overall plant vitality indicator (Banks 2017), Area is the fluorescence-time area above the fluorescence curve (relative to F_M) expressing the size of the electron acceptor pool on the reducing side of photosystem II (PSII), and $V_0(B_0)$ is a ratio from comparison of light and dark adapted F_V/F_M readings ((F_V/F_M [dark] – light adapted F_V/F_M) ÷ F_V/F_M [dark]) expressing the fraction of 'closed' PSII reaction centres (non-reducing plastoquinone B). n=7-8. Generated in the R statistical programming environment using the packages 'nlme', 'effects', & 'ggplot2'. See Tables S1 & S3 for statistical analyses.

5.4.6 Relative Chlorophyll Content

Chlorophyll content, estimated non-destructively from leaf absorbance of various wavelengths, is a commonly used measure of relative plant health: a decline in response to *Armillaria* infection has been observed in other studies (Table 1).

There was a significant infection effect on relative chlorophyll content in the LTE (ANOVA P=0.015, Wald X^2 = 5.887, marginal R^2 = 0.174, conditional R^2 = 0.530) and a significant infection-time interaction effect in the STE watered (ANOVA P<0.001, Wald X^2 = 38.587, marginal R^2 = 0.101, conditional R^2 = 0.273): on infected plants it was significantly lower in the LTE, days 34-116 (-9.2% to -9.9% respectively, P=0.027-0.042, t-ratios = +2.177 to +2.395), and in the watered treatments of the STE from day 36 (-5.3%, P<0.001-0.042, t-ratios = -2.483 to -6.008).

Pre-drought, there were significant separate effects of infection and time on relative chlorophyll content (ANOVA: infection P=0.035, Wald X^2 = 4.450. Time P=0.001, Wald X^2 = 10.719. Marginal R^2 = 0.035, conditional R^2 = 0.104), neither means nor trends separated significantly under Tukey's test, suggesting weak effects (trends P=0.061-0.999 & t-ratios = -2.497 to +0.190, means P=0.130-0.999 & t-ratios = -2.305 to +2.264). Averaging over treatment groups, relative chlorophyll content was higher in infected plants (IW & ID mean 44.4 SPAD units, UW & UD mean 43.3), but when plotted over time it started higher in infected plants then decreased (figure 4c). This correlates with trends in visual health (5.4.2, figure 3A).

ANOVA analyses of drought and recovery data (drought marginal R^2 = 0.121 & conditional R^2 = 0.473, recovery marginal R^2 = 0.160 & conditional R^2 = 0.680) indicate that the dataset was too small to elucidate an infection-drought-time interaction effect on relative chlorophyll content: in both periods it was not significant (drought P=0.385 & Wald X^2 = 0.755, recovery P=0.067 & Wald X^2 = 3.353). Significant drought-infection (drought P=0.044 & Wald X^2 = 4.060, recovery P=0.048 & Wald X^2 = 3.904) and drought-time interaction effects were present (drought P<0.001 & Wald X^2 = 35.857, recovery P<0.001 & Wald X^2 = 20.560). Applying Tukey's test, IW mean was significantly lower than that of ID after 18 days of

drought (-16.0% at day 57, *P*=0.013, t-ratio= +3.422) and no means were significantly different at any recovery time point (*P*=0.072-1.000, t-ratios = -2.233 to +2.617). Averaging over time points to elucidate the drought-infection interaction, in drought and recovery, only infected plants had significantly different means between watering regimes, with IW being lower than ID (drought -12.1%, *P*=0.019, t-ratio = 2.563 & recovery -16.5%, *P*=0.033, t-ratio = 2.285). During drought there were only significant differences in relative chlorophyll content trends between watering regimes (*P*<0.001-0.006, t-ratios = -5.212 & +3.290 to +4.767): it decreased in watered treatments and increased in drought treatments (figure 4c). In the recovery trends, relative chlorophyll content increased only in UW over time and was significantly different from the UD trend (*P*<0.001, t-ratio = -4.581). IW and ID trends were negative and not significantly different from each other (*P*=0.239, t-ratio = -1.879) or from their respective watering regime controls (ID-UD *P*=0.727, t-ratio = +1.038 & IW-UW *P*=0.403, t-ratio = -1.561).

Given these reactions of relative chlorophyll concentration to *A. mellea* infection, it would be appropriate to investigate other chlorophyll-based measurements such as selected chlorophyll fluorescence parameters. This group of measurements has scope for more nuanced reactions to stresses than chlorophyll concentration.

5.4.7 Continuous Excitation Chlorophyll Fluorescence

Parameters derived from graphed chlorophyll fluorescence have recorded responses to *Armillaria* infection and as have related measurements such as chlorophyll content and infra-red gas analyser parameters (Table 1). Responses to drought have also been recorded, but the impacts of these stresses have not been compared. Therefore the reactions of a select range of parameters to *A. mellea* infection, drought, and their combination were investigated.

5.4.7.1 PI_{ABS}

There were significant infection-time interaction effects on PI_{ABS} (ANOVA: LTE & STE watered *P*<0.001. LTE: Wald X^2 = 7.780, marginal R^2 = 0.106, conditional R^2 = 0.387. STE watered: Wald X^2 = 24.044, marginal R^2 = 0.105, conditional R^2 = 0.482), on infected plants it was significantly lower in the LTE, days 34-175 (-32.2% to -24.2% respectively, *P*=0.001-0.036, t-ratios = +3.055 to +4.018). In the watered treatments of the STE, there was a significantly faster decline in PI_{ABS} of infected plants (IW +109%, *P*<0.001, t-ratio = -4.903). Applying Tukey's test, differences in PI_{ABS} means were close to α at days 78 & 79, with *P*=0.052 and *P*=0.050, respectively (t-ratios = -2.338 & -2.362): at day 79, the IW mean was -43.4% of the UW mean (Appendix B, Table SD2).

Pre-drought, PI_{ABS} had a significant infection-watering regime-time interaction (*P*=0.042 & Wald X^2 = 1100.649), although Tukey's test did not detect differences in means or trends (*P*=0.132-1.000, t-ratios = -2.298 to +2.130). This would appear to relate to similar initial means and slopes between pairs of treatments: UW & ID and IW & UD (figure 4g). During drought there was a significant infection-drought-time interaction for PI_{ABS} (*P*=0.025, Wald X^2 = 5.022, marginal R^2 = 0.064, conditional R^2 = 0.535). The trend for UW separated from IW, UD, & ID (*P*<0.001-0.005, t-ratio = -3.315 to -5.774), being the only treatment with a positive trend in PI_{ABS} (figure 4g). During recovery, there was a significant drought-time interaction effect for PI_{ABS} (ANOVA *P*<0.001, Wald X^2 =29.478, marginal R^2 = 0.066, conditional R^2 = 0.615, figure 4g): PI_{ABS} trends were significantly different between watered and droughted treatments (*P*<0.001-0.004, t-ratios = -4.289 & +3.405 to +4.233), increasing and decreasing respectively. In drought and recovery, means did not separate significantly (both stages, all factors, *P*=0.132-1.000, t-ratio = -2.298 to +2.130).

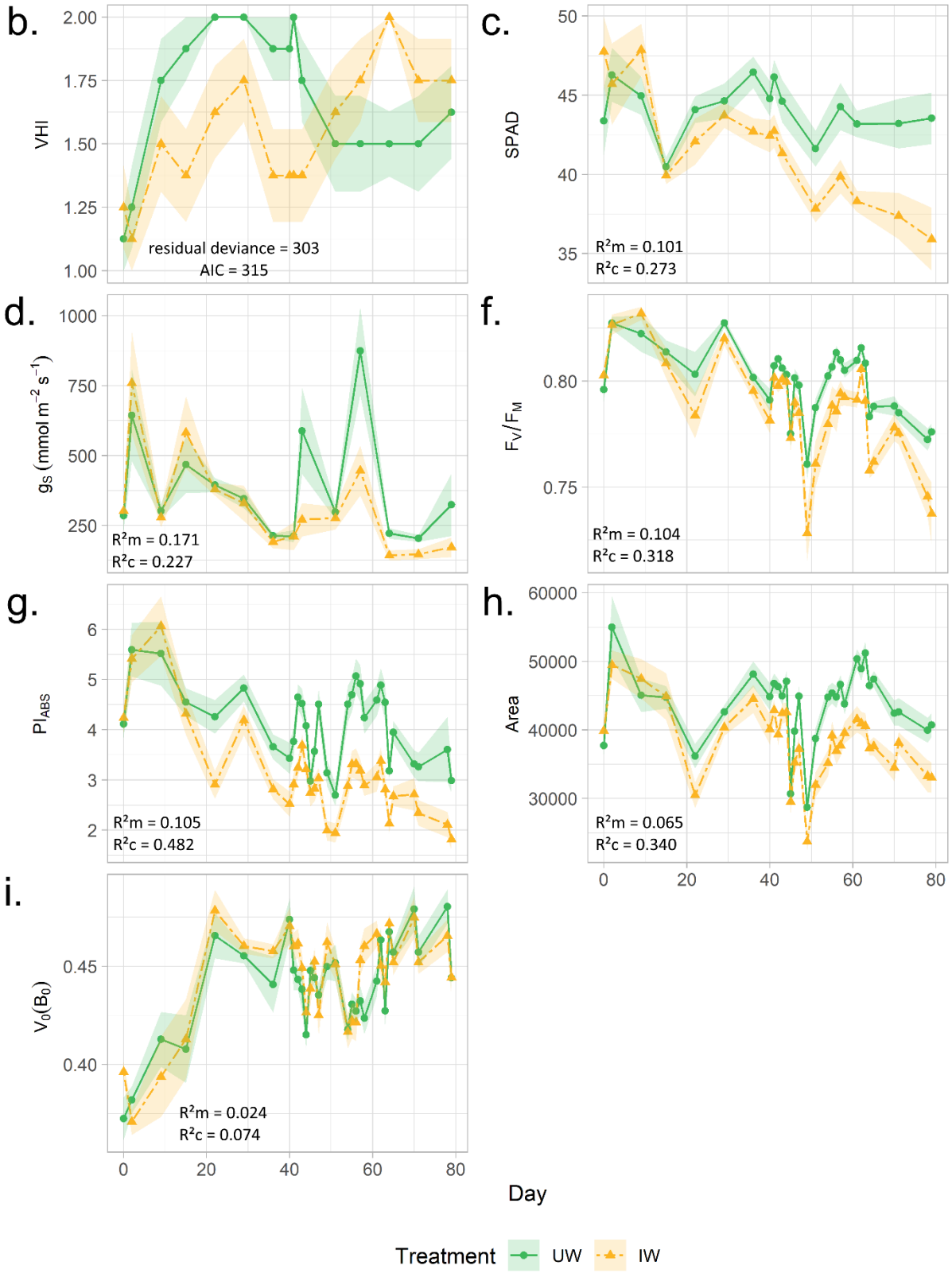


Figure 5. Impact of *Armillaria mellea* infection on various physiological measures of *Ligustrum vulgare*, days 0-80. Line and points represent mean values of 8 replicates (plants), with points located at each data

collection time point. Ribbon around line represents standard error range. UW = Uninfected Watered treatment: solid line, circles & ribbon in yellow. IW = Infected Watered treatment: alternate dashed line, triangles & ribbon in green. Visual health index scores: 1 = plant visually healthy, 2 = visible symptoms of ill health (leaves yellowing or dying), 3 = visible wilting or extensive leaf loss evident, 4 = plant visibly dead. Chlorophyll content shown in SPAD (Soil Plant Analysis Development) arbitrary units, stomatal conductance (g_S) in mmol m^2 s^{-1}, F_V/F_M is the ratio of variable (F_V) to maximal chlorophyll fluorescence (F_M) expressing the efficiency of photosystem II, PI_{ABS} (Performance Index) is the product of multiple ratiometric chlorophyll fluorescence calculations as an overall plant vitality indicator (Banks 2017), Area is the fluorescence-time area above the fluorescence curve (relative to F_M) expressing the size of the electron acceptor pool on the reducing side of photosystem II (PSII), and $V_0(B_0)$ is a ratio from comparison of light and dark adapted F_V/F_M readings ((F_V/F_M [dark] – light adapted F_V/F_M) ÷ F_V/F_M [dark]) expressing the fraction of 'closed' PSII reaction centres (non-reducing plastoquinone B). n = 8. Generated in the R statistical programming environment using the package 'ggplot2'. See Tables S1 & S2 for statistical analyses.

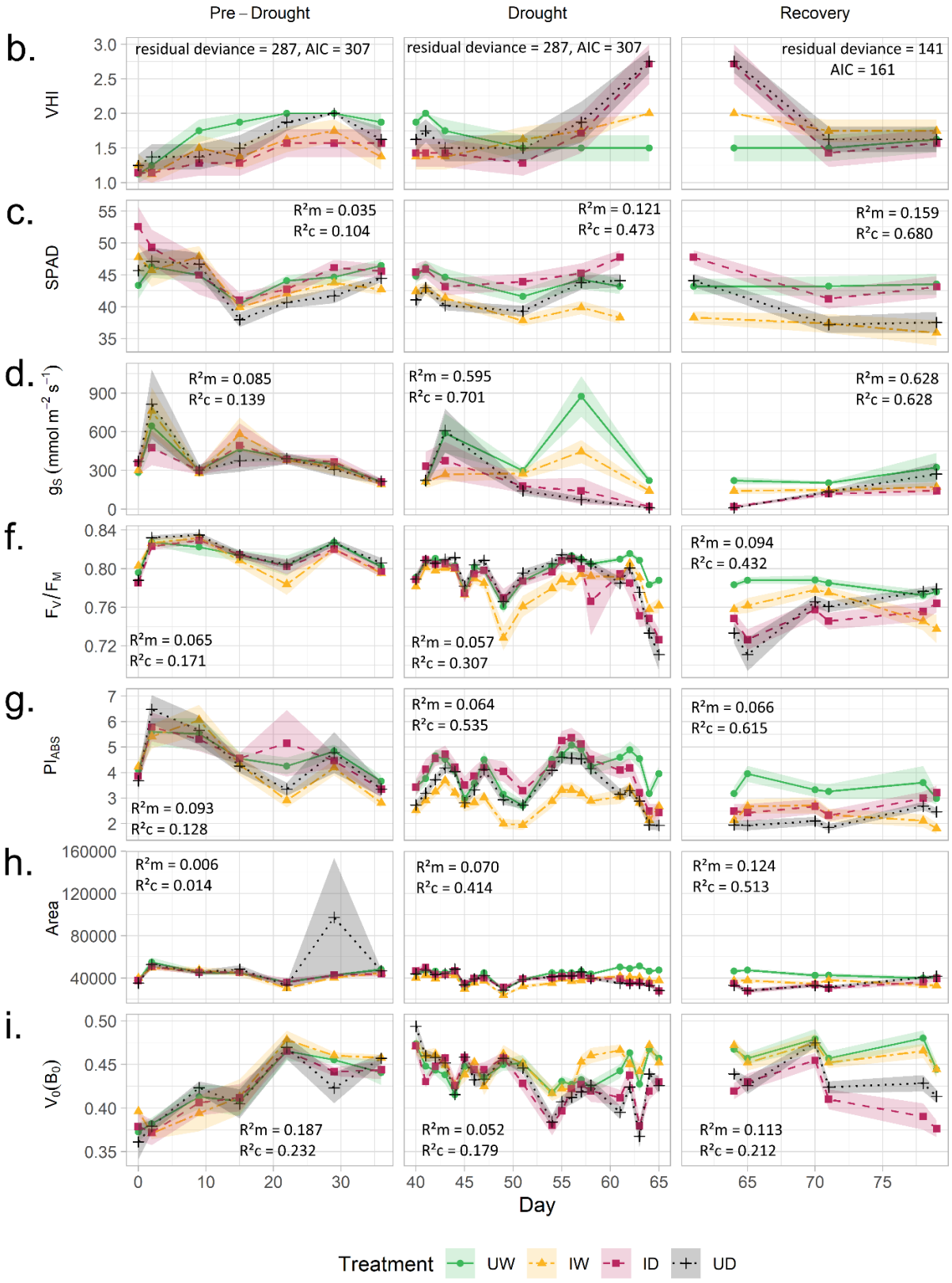


Figure 6. Impact of *Armillaria mellea* infection and drought on various physiological measures of *Ligustrum vulgare*. Line and points represent mean values of 8 replicates (plants) in UW, IW, UD, and 7 replicates

in ID, with points located at each data collection time point, ribbon around line represents standard error range. UW = Uninfected Watered treatment: solid line, circles & ribbon in yellow. IW = Infected Watered treatment: alternate dashed line, triangles & ribbon in green. ID = Infected Droughted treatment: dashed line, squares & ribbon in hibiscus red. UD = Uninfected Droughted treatment: dotted line, crosses & ribbon in black/grey. Visual health index scores: 1 = plant visually healthy, 2 = visible symptoms of ill health (leaves yellowing or dying), 3 = visible wilting or extensive leaf loss evident, 4 = plant visibly dead. Chlorophyll content shown in SPAD (Soil Plant Analysis Development) arbitrary units, stomatal conductance (g_S) in mmol m^2 s^{-1}, F_V/F_M is the ratio of variable (F_V) to maximal chlorophyll fluorescence (F_M) expressing the efficiency of photosystem II, PI_{ABS} (Performance Index) is the product of multiple ratiometric chlorophyll fluorescence calculations as an overall plant vitality indicator (Banks 2017), Area is the fluorescence-time area above the fluorescence curve (relative to F_M) expressing the size of the electron acceptor pool on the reducing side of photosystem II (PSII), and $V_0(B_0)$ is a ratio from comparison of light and dark adapted F_V/F_M readings ((F_V/F_M [dark] – light adapted F_V/F_M) ÷ F_V/F_M [dark]) expressing the fraction of 'closed' PSII reaction centres (non-reducing plastoquinone B). n=7-8. Generated in the R statistical programming environment using the package 'ggplot2'. See Tables S1 & S3 for statistical analyses.

5.4.7.2 $V_0(B_0)$

There was a significant infection-time interaction effect on $V_0(B_0)$ in the LTE (ANOVA P<0.001, Wald X^2 = 13.491, marginal R^2 = 0.024, conditional R^2 = 0.074). On infected plants it was significantly (P=0.002-0.036, t-ratios = -2.435 to -3.661 & +2.257 to +2.596) higher between days 34-116 (+8.0% to +3.5% respectively), prior to the period of mortalities, and significantly lower afterwards, from day 273 (-4.8%). Significantly, it declined over time in infected plants and increased in uninfected (P<0.001, t-ratio = +3.673). In contrast, infection had no significant impacts in the STE watered analysis (ANOVA P=0.521-0.599, Wald X^2 = 0.342-0.412, marginal R^2 = 0.043, conditional R^2 =0.136), increasing in all plants over time (figure 5i).

During drought, there was a significant drought-time interaction effect on $V_0(B_0)$ (P<0.001, Wald X^2 = 89.808, marginal R^2 = 0.052, conditional R^2 = 0.179). IW $V_0(B_0)$ mean was significantly higher than that of UD at 22 days of drought (day 61, P=0.015, t-ratio = +3.355). Two days later both watered treatments had significantly higher $V_0(B_0)$ than the drought treatments (day 24, P=0.007-0.047, t-ratios = -2.828 to -3.277 & +3.717, figure 6i).

During recovery, there were significant drought-time and infection-time interaction effects on $V_0(B_0)$ (P=0.003, Wald X^2 = 8.847 & P=0.034, Wald X^2 = 4.496 respectively, marginal R^2 =0.113, conditional R^2 =0.212). It is interpreted that there were not enough replicates to determine the infection-drought-time interaction (P=0.148, Wald X^2 = 2.088). When interactions were examined separately by averaging over each type of host stress, $V_0(B_0)$ means were significantly lower for each stress, at every time point (day 64-79) for drought (P<0.001-0.018, t-ratios = -2.581 to -5.385) and from 14 days recovery (day 78) onwards for infection (P=0.026-0.030, t-ratios = -2.333 & -2.397).

5.4.7.3 F_V/F_M

In the LTE ANOVA analysis, the F_V/F_M infection-time interaction p-value was very close to α at *P*=0.051 (Wald X^2 = 3.802, marginal R^2 = 0.053, conditional R^2 = 0.096): F_V/F_M of infected plants was significantly lower from day 104 (-2.2%, *P*=0.001-0.004, t-ratios = +2.241 to +3.907). In the STE watered analysis, there was a significant infection-time interaction effect on F_V/F_M (ANOVA *P*<0.001, Wald X^2 = 19.635, marginal R^2 = 0.104, conditional R^2 = 0.318): it was significantly lower in infected plants from day 78 (-3.0%, *P*=0.046 & 0.048, t-ratios = -2.388 & -2.418).

During drought, there was a significant infection-drought-time interaction effect on F_V/F_M (ANOVA *P*=0.019, Wald X^2 = 5.504, marginal R^2 = 0.057, conditional R^2 = 0.307). Trends for UW, IW, and droughted treatments were all significantly different (*P*<0.001-0.040, t-ratios = -2.654 to -8.622 & 5.971), only UW had a positive trend, while the IW trend was less negative than the droughted treatments. UW and ID F_V/F_M means separated significantly after 26 days of drought (day 65), being respectively higher and lower (-3.7%, *P*=0.046, t-ratio = -2.845), while UD & IW were not significantly different from either (P=0.057-0.997, t-ratios = -0.209 to +0.648).

In recovery, there were significant drought-time and infection-time interaction effects on F_V/F_M (ANOVA *P*<0.001 & Wald X^2 = 66.976, *P*=0.007 & Wald X^2 = 7.349, respectively): it is interpreted that the dataset was too small to elucidate the infection-drought-time interaction effect (*P*=0.082 & Wald X^2 = 3.032). F_V/F_M means were significantly lower in droughted treatments F_V/F_M at days 0-1 of recovery (respective *P*=0.001 & 0.003, t-ratios = -3.713 & -3.444). F_V/F_M increased significantly faster in uninfected plants (trend *P*=0.007, t-ratio = -2.711) but means did not differ significantly by infection at any time point after one day's recovery (*P*=0.068-0.654, t-ratios = -0.456 to -1.933).

5.4.7.4 Area

There were significant infection-time interaction effects on Area (ANOVA LTE & STE watered *P*<0.001. LTE: Wald X^2 = 12.550, marginal R^2 = 0.063, conditional R^2 = 0.145. STE: Wald X^2 = 23.370, marginal R^2 = 0.065, conditional R^2 = 0.340): on infected plants it was significantly lower in the LTE, from day 144 onward (-23.7%, *P*<0.001-0.022, t-ratios = +2.488 to +4.402), and in the watered treatments of the STE from day 70 (-18.6%, *P*=0.031-0.046, t-ratios = -2.423 to -2.699).

During drought and recovery, there were significant infection-drought-time interaction effects on Area (ANOVA: drought *P*<0.001, Wald X^2 = 8.784, marginal R^2 = 0.070, conditional R^2 = 0.414, & recovery *P*<0.001, Wald X^2 = 4.780, marginal R^2 = 0.124, conditional R^2 = 0.513). Droughted treatment means were significantly lower than that of UW from 24 days of drought to 1 day of recovery (day 63-65, *P*=0.005-

0.041, t-ratios = -2.862 to -3.862), with IW not being significantly different from either. UW and UD means were the first to separate significantly, from 23 days of drought (day 62, *P*=0.041, t-ratio = -2.901).

The varied reactions of the chlorophyll fluorescence parameters to *A. mellea* infection recorded above suggests it may be advantageous to combine physiological variables in some way for infection detection.

5.4.8 Multivariate Analysis

A combination of physiological measurements may be more powerful for detecting infection and excluding the influence of drought stress, discriminant analyses provide an initial way of assessing and comparing such combinations. Results described here reflect the greatest classification accuracies found from multiple assessments of the various datasets.

Linear discriminant analysis (LDA) of pre-mortality physiological measurements from watered treatments, from both experiments, LTE & STE, combined, indicates that a combination of transformed PI_{ABS}, $V_0(B_0)$, and g_S readings can discern infected and uninfected plants with an accuracy of 69.9%, i.e. somewhat better than at random (50%). The analysis yielded a single linear discriminant, with the following coefficients: log of PI_{ABS} = +0.87, logit of $V_0(B_0)$ = -0.18, log of g_S = +0.30. Relative chlorophyll concentration data broke the LDA normality assumption, including under transformation, and did not increase classification accuracy if included. Fluorescence time point data from this dataset violated the normality or variance assumptions of LDA and QDA under various transformations and therefore could not be tested. This may relate to the long timespan and therefore increased variability of the LTE data or inherent differences in raw fluorescence between the experiments e.g. due to the different environments or individual hosts.

QDA of STE drought stage physiological measurements indicates that a combination of Area and transformed $V_0(B_0)$ readings can discern between the four treatment groups with an accuracy of 41.1%, in comparison to a random guess accuracy of 25%. Training dataset means of parameters indicate that Area is lowest in IW, and highest in UW, with droughted treatments, ID & UD, intermediately low, while $V_0(B_0)$ is lower in droughted treatments and slightly higher in IW (Table 3A). Addition of PI_{ABS} to the analysis did not increase accuracy, however analysis of PI_{ABS} & $V_0(B_0)$ alone had a similar accuracy: 40.3%. PI_{ABS} means were lower in the singly stressed treatments (Table 3A). QDA of STE drought stage log transformed fluorescence readings from time points of 0.0006, 0.001, 0.0015, 0.0026, 0.013 seconds had a treatment classification accuracy of 49.2%. Fluorescence treatment means at these time points indicate that the combined stress, ID, had fluorescence 2-3% lower than UW (all time points) and its

fluorescence at 0.013 seconds was uniquely 2-3% lower than the other treatments. IW was more likely to show increases in fluorescence of 1-7%, with UD showing smaller increases of 1-2% (Table 3B).

Table 3. Treatment means ± standard deviation of chlorophyll fluorescence parameters and raw fluorescence at selected time points from training datasets used in quantitative discriminant analyses (QDA) to discern between treatment combinations of *Armillaria mellea* infection and drought stress of *Ligustrum vulgare*.

	A. Fluorescence Parameter Analysis			**B.** Fluorescence Time Point Analysis				
Treatment	Area	$V_0(B_0)$	PI_{ABS}	0.0006 s	0.0010 s	0.0015 s	0.0026 s	0.0130 s
UW	44004 ± 8656	0.4429 ± 0.0347	4.04 ± 1.71	671 ± 85	798 ± 92	881 ± 94	941 ± 95	1149 ± 80
IW	37699 ± 10236	0.4441 ± 0.0454	2.96 ± 1.54	721 ± 80	843 ± 82	917 ± 83	963 ± 87	1156 ± 83
ID	39153 ± 12726	0.4308 ± 0.0447	4.00 ± 2.04	652 ± 72	772 ± 82	853 ± 89	913 ± 94	1129 ± 103
UD	38762 ± 9714	0.4310 ± 0.0438	3.37 ± 1.35	683 ± 64	810 ± 77	891 ± 86	946 ± 96	1149 ± 112

n = 7-8.

In comparison to univariate analyses, the results of the multivariate analysis of pre-mortality physiological measurements from watered treatments suggests that PI_{ABS}, g_s, and $V_0(B_0)$ have a higher predictive ability for infection when combined. Comparing the two fluorescence parameter discriminant analyses suggests that higher $V_0(B_0)$ is associated with infection, whereas lower $V_0(B_0)$ is more strongly associated with drought. However the STE drought data contains data from fewer plants and experiments than the combined dataset, which may reduce confidence in the results.

The sensitivity of chlorophyll fluorescence, and derived parameters, to *A. mellea* infection and drought justifies a deeper comparative investigation of the differences between OJIP transients between treatments i.e. differential kinetics.

5.4.9 Differential Kinetics

Differential kinetics are comparisons of raw chlorophyll fluorescence graphs (OJIP transients), using various normalisation techniques to elucidate subtle differences within different regions of the OJIP transient. This allows comparison to previous observations for plants under other abiotic stresses and may indicate the origins of differences within the photosynthetic system and/or host physiology.

5.4.9.1 ΔV_t

For *Armillaria mellea* alone, in both the LTE (figure 7) and STE (figure 8A), ΔV_t showed a bimodal arrangement with the largest of the two peaks occurring between F_K and F_J ($F_{300\mu s} – F_{2ms}$), the second and smaller peak or plateau occurs after F_J up to ~F_I ($F_{2ms} – F_{30ms}$). Drought stress alone (UD in STE) reduced the intensity of ΔV_t in comparison to *A. mellea* infection alone and flattens the response to a single wide peak from F_0 to F_M. Combined, *A. mellea* and drought caused a further reduction to ΔV_t until F_I where a small peak was observed between F_I and F_{300ms}.

5.4.9.2 L- and K-band fluorescence.

K-band (W_{OJ}) fluorescence occurred in response to singular drought (UD) or infection (IW) but no peak was present when these stressors were applied in combination (ID) (figure 8B). The maximal ΔW_{OJ} peak occurred between F_K (0.3ms) and F_{1ms}.

No L-band (W_{OK}) peak was present in response to drought, *Armillaria* or a combination. All ΔW_{OK} were <0.01 (data not shown).

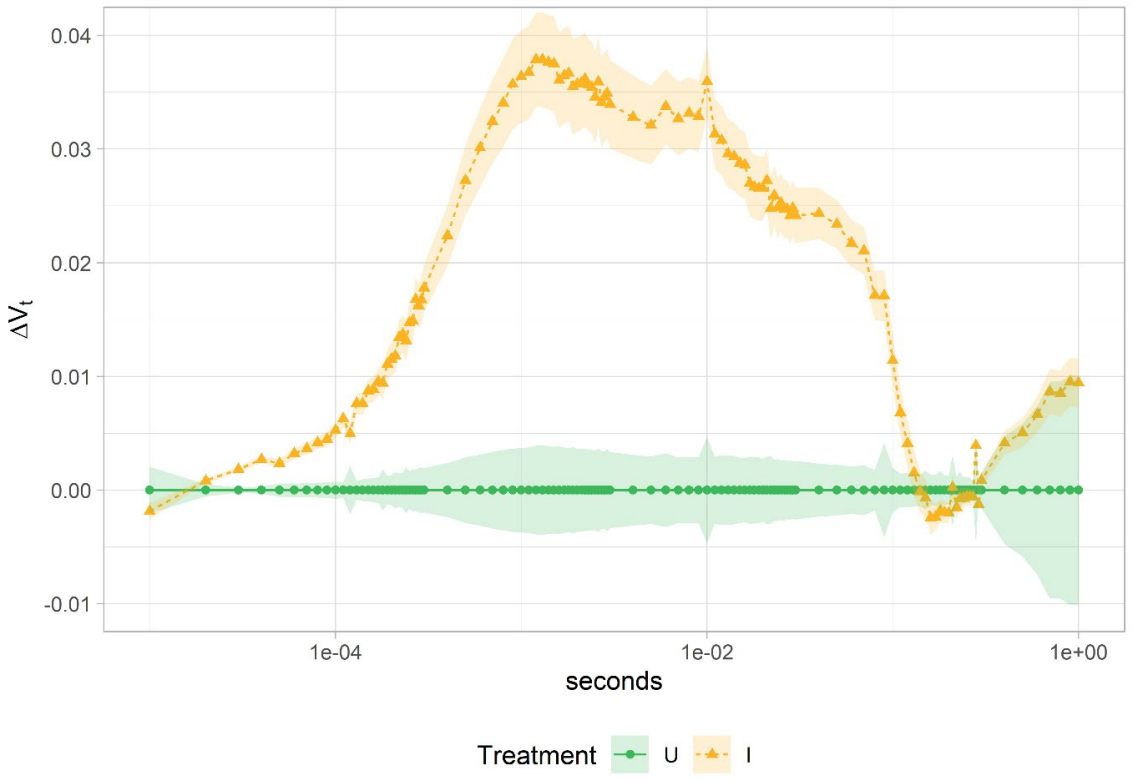


Figure 7. Pre-mortality ΔV_t chlorophyll fluorescence kinetics of *Armillaria mellea* infected and uninfected *Ligustrum vulgare* plants, in comparison to mean uninfected fluorescence, mean of days 34-144 after inoculation. *n*=20. Generated in the R statistical programming environment using the package 'ggplot2'.

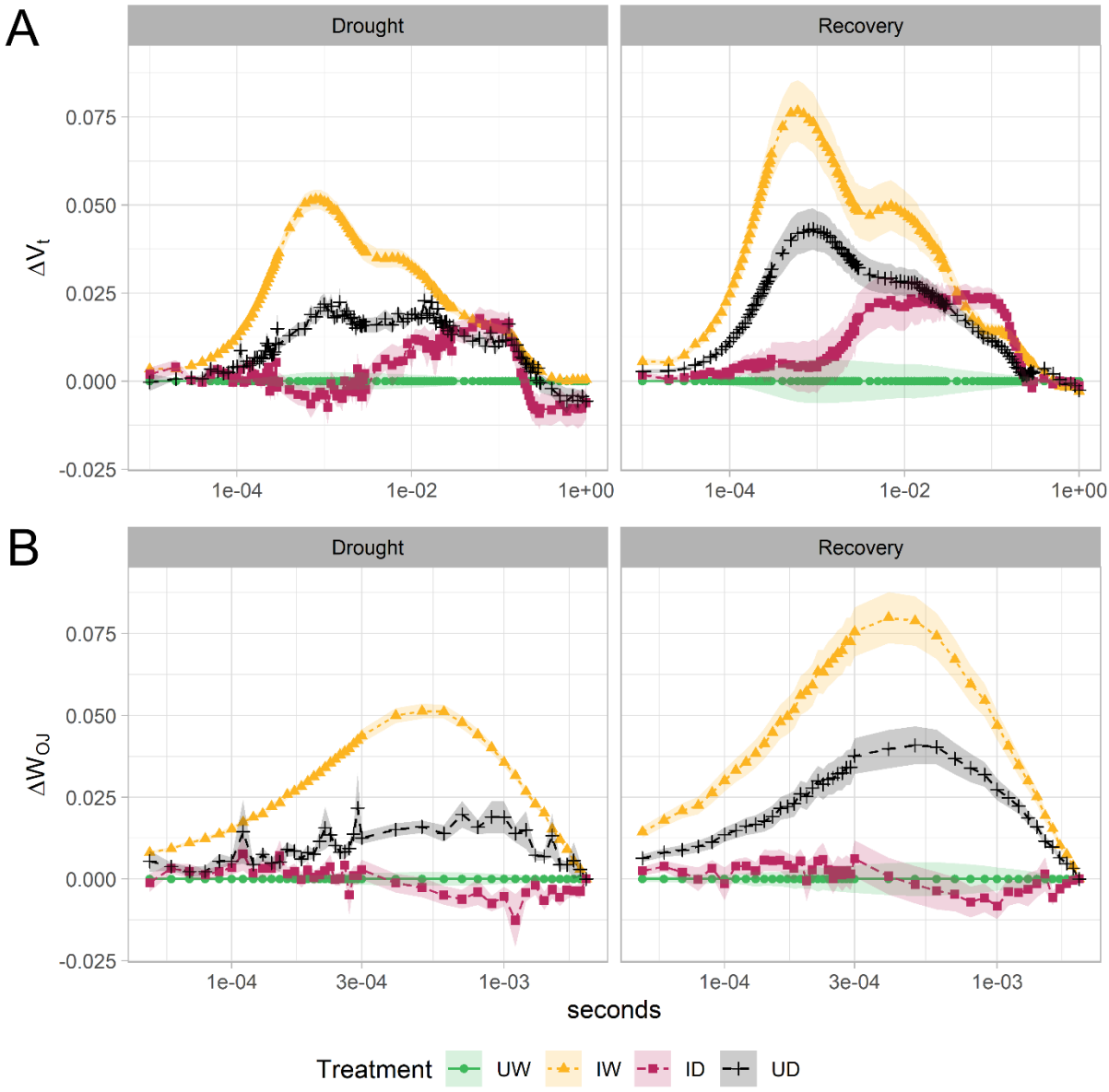


Figure 8. Mean ΔV_t (A) and K-band ΔW_{OJ} (B) chlorophyll fluorescence kinetics impact of *Armillaria mellea* infection and drought on *Ligustrum vulgare*, during and following drought, in comparison to mean uninfected watered fluorescence. *n*=7-8. Generated in the R statistical programming environment using the packages 'ggplot2' & 'ggpubr'.

5.5 Discussion

Armillaria infections are often not visually evident until the most severe stages of infection. Early detection may provide an opportunity for accelerated treatment or sanitation practices, especially for individual trees. Physiological measurements from host foliage may elucidate below-ground symptoms and can be collected quickly and non-destructively. Previous studies have been of low temporal resolution and there has been little comparison of the measures. One study has suggested the chlorophyll fluorescence parameter F_V/F_M as the most sensitive indicator of *Armillaria* infection (Agustini *et al.* 2015). The present study recorded significant differences in physiological measurements caused by *Armillaria* infection on a susceptible host at earlier time points and at a higher resolution than previous studies (Table 4), comparing them to those caused by, or in combination with, drought.

Table 4. Influence of *Armillaria mellea* infection on visual symptoms and physiological measurements of artificially inoculated and watered *Ligustrum vulgare*, in comparison to un-inoculated control.

Experiment Infection symptoms at conclusion or death (% of population)	Variable	Impact of infection, minimum significant difference of infected from control mean (Δ%)	Period of significant difference between infected and control means (days)
Long Term **95% rhizomorphs in soil, 40% mycelial fans in root collar, 40% mortality**	Visual Index	Infected plants progress to death via ill health and then wilting. Mortalities occurred day 144-273.	
	Chlorophyll Concentration	-9.2%	34-116
	$V_0(B_0)$	+3.5%	34-116
	PI_{ABS}	-24.2%	34-175
	Stomatal Conductance	-19.3%	34-175
	Extension	n.s.	n.s.
	F_V/F_M	-2.2%	104 onward
	Area	-23.7%	144 onward
Short Term **100% rhizomorphs in soil**	Visual Index	Cross-over with uninfected starting worse (higher) (random variation, due to environmental factors). Infected plants more likely to decline visually. Infected plants worse from around day 50 onward.	
	Chlorophyll Concentration	-5.3%	36 onward
	Stomatal Conductance	-21.8%	41 onward
	Area	-18.6%	70 onward
	F_V/F_M	-3.0%	78 onward
	PI_{ABS}	-43.4%	78 onward (*P*=0.0502-0.0520)
	$V_0(B_0)$	n.s.	n.s.

Δ% = percentage difference, infected vs uninfected, n.s. = non-significant, α = 0.05.

5.5.1 Impact of abiotic stress

In both experiments, the lack of external nutrient additions and repotting are presumed to have led to an increasing amount of abiotic stress over time, i.e. nutrient deficiencies and the restriction of rooting volume. In general, studies demonstrate that photosynthesis and transpiration decrease with increased root restriction because of impacts on water availability, with variable impacts on chlorophyll

concentration (Fascella & Rouphael 2017; Sinclair *et al.* 2017; Wang *et al.* 2019; Turner 2019; Zakaria *et al.* 2020; Espinoza *et al.* 2020). Osmotic stress could arise from root restriction and therefore reduced water availability or the build-up of salts from the water supply in the growth media: this and deficiencies in nitrogen, phosphorous, potassium, calcium, magnesium, and iron may affect continuous excitation fluorescence measurements (Kalaji *et al.* 2016). Nitrogen and iron deficiencies may lead to reduced chlorophyll concentrations (SPAD). Therefore, this increasing abiotic stress is a key part of the context of this study.

5.5.2 Physiological and visual signatures of infection

Visual symptoms of *Armillaria* root rot are of a general nature and are ambiguous, often appearing similar to those caused by environmental stresses, this is exemplified by: increasing visual symptoms on all plants in both experiments (figure 2, figure 3) presumably due to increasing abiotic stress; the visual overlap between *Armillaria* root rot and drought symptoms (figure 3); and UW outscoring IW in visual health index (VHI) for the majority of the STE (figure 3D, figure 5b). Data from both experiments suggest visible declines over time, i.e. increasing VHI, rather than the presence of symptoms of ill health may be more indicative of infection. Wilting or leaf loss in the absence of drought, or another environmental cause, is perhaps the most definitive visual symptom observed on wild privet, albeit of late-stage infection.

Physiological signatures of infection elucidated in this study may demonstrate a succession of detectable symptoms which are separate from visual symptoms and less tenuous. Conversely, they are all impacted by factors outside of *Armillaria* infection to some degree which may reduce their reliability outside of experimental situations or where other factors are present at significant levels e.g. other root pathogens, drought, root restriction, compaction etc. These physiological measurements may also vary in their specificity to wild privet and to *Armillaria mellea*.

Stomatal conductance (g_S) reacted consistently, promptly, and strongly to infection in comparison to other physiological measures, with around a 20-35% reduction in infected plants 35-40 days after infection (Table 4). This is similar to the reaction in g_S recorded by Loreto *et al.* (1993) on *Quercus rubra* and *Olea europaea* 60 days after *Armillaria mellea* infection, with a larger response on *Q. rubra* infected with *A. ostoyae* (Table 1). However, this was only observed in susceptible species. Therefore, in the absence of drought or other significant root stresses, a 20% or greater reduction in g_S is potentially the best physiological indicator of *Armillaria* infection in susceptible species. In addition, g_S was one of two parameters which retained a significant difference between infected and uninfected plants for 175 days

from infection in the LTE (the other being PI_{ABS}), suggesting it was less impacted by root restriction and nutrient stresses which are assumed to have been increasing in all plants over time.

Chlorophyll concentration also showed a significant 5-9% reduction around 35 days after infection (Table 4). In the LTE, a reduction of around 9% was maintained at each significant time point, whereas the difference continued to increase in the STE. In the STE, chlorophyll concentration reacted to infection earlier than g_S (Table 4). Conversely, in the LTE it stopped separating significantly earlier than g_S and PI_{ABS}, likely reflecting its strong relationship to nutrient availability which was decreasing during the long duration of the experiment. In previous studies where plants were fertilized, or conducted in raised beds, and therefore nutrients were less limited, chlorophyll concentration continued to decrease in infected plants of various hosts (Nogales *et al.* 2008; Percival *et al.* 2011; Agustini *et al.* 2015). This apparent higher abiotic sensitivity of chlorophyll content and the larger difference in g_S between infected and control plants suggests g_S is the more reliable measure of the two.

PI_{ABS} showed an early response in the LTE but responded later in the STE. Other authors also recorded a significant response in the PI_{ABS} derivative PI_{TOT} (Nowakowska *et al.* 2020). $V_0(B_0)$ also showed an early response in the LTE but did not respond significantly in the STE, despite some periods where it was higher (figure 5i). This is unfortunate as PI_{ABS} maintained a significant difference until day 175 in the LTE and because $V_0(B_0)$ is a relatively understudied fluorescence parameter. Given the less regulated and therefore heterogeneous environment in which the STE took place, these variations in response to infection could relate to PI_{ABS} and $V_0(B_0)$ being more strongly influenced by environmental factors. Interactions between such stresses and root damage caused by the fungus may also explain the cross-over interaction of infection and time on $V_0(B_0)$ in the LTE. In the STE, plants were given much more time to root into the pots prior to inoculation than in the LTE, which may have influenced the interactions between infection, environmental stresses, and physiological parameters. Another potential source of this difference is variability in the rate of infection, which was likely influenced by variations in inoculation technique and environmental conditions between the experiments. Direct comparison between the two experiments in this regard, i.e. inferring infection levels present on plants in the LTE at around 80 days, is not possible. This is also an inherent difficulty in comparing results of this study to those previous to it, and which is further complicated by differences in host species, *Armillaria* species, and methodologies.

Contrary to the univariate analyses, the multivariate analysis suggests that when combined, g_S, PI_{ABS} and $V_0(B_0)$ are the parameters of the greatest utility for classifying infected and uninfected plants. Here, PI_{ABS}

contributes the majority of discriminatory power, while Area and $V_0(B_0)$ have the greatest potential utility for discerning drought stress from infection (although raw fluorescence from a combination of selected time points may have greater classification accuracy). Therefore Area, PI_{ABS} and $V_0(B_0)$ may be best utilised when taken into consideration with g_S. Further studies are required to clarify the consistency of these parameters reacting to *Armillaria* infection. It may be that they only react under certain environmental conditions and should therefore be regarded as additional indicators of infection.

Area showed a later but sizeable reaction in both experiments, being around 20% lower in infected plants. In the STE the significant reaction was much earlier, at day 70, whereas in the LTE it was first recorded at day 144. In the LTE its divergence between infected and uninfected plants appeared to be associated with late-stage infections and impending mortality, with means diverging significantly from day 144 onward. It was not possible to discern if this was the case in the STE.

While F_V/F_M was named as the most sensitive parameter studied by Agustini *et al.* (2015), in this study F_V/F_M reacted to infection relatively late in both experiments, around days 80-100, and by a small amount, 2-3%. The aforementioned authors recorded around a 9% reduction in F_V/F_M (unwounded treatments) at 210 days since infection of *Eucalyptus nitens* with *A. luteobubalina*. The observations of mycelial fans were much higher in this previous study, indicating more advanced infection and/or increased host persistence under it (Table 1). Although significant, the small size of the changes in F_V/F_M may allow the infection response to be masked easily by changes due to other factors e.g. environmental stresses.

Extension growth, only measured in the LTE, showed a weak response to infection (figures 1a & 4a), which corresponds with other authors who investigated their utility in infection assays with a wide range of hosts (Ford *et al.* 2017).

To summarize, in this study and in the absence of drought stress, *Armillaria* infection on wild privet was characterised by a 20-35% reduction in g_S and a 5-9% reduction in chlorophyll concentration after 35-40 days. Area also reduced by around 20% after 80-100 days. Increases in $V_0(B_0)$ around 5% or more and 25% reductions in PI_{ABS} are also indicative of infection but less reliable, varying in their occurrence and timing. Small but significant reductions in F_V/F_M, around 2-3%, may also occur after 80-100 days. On comparison with the existing data, there is some evidence that these responses are similar between varied hosts and species of *Armillaria*.

5.5.3 Differential kinetics

In differential kinetics analysis, ΔV_t kinetics identify specific differences between infected and uninfected plants, being normalised against both F_0 and F_M (figure 7). Bussotti *et al.* (2011) and Banks (2018) both identified a peak at the J (F_{2ms}) and I (F_{30ms}) time points from abiotic stresses: in both cases, however, the I peak amplitude was greater than J peak in response to individual ozone and drought stress respectively. In this study, infection alone (IW) yielded a J peak larger than the I peak but little bimodal response was observed under drought (UD) or combined drought and infection (ID) (figure 8A). This could suggest ΔV_t responds distinctly to *Armillaria* infection, but further work is necessary to determine the lack of corroboration between the ΔV_t response observed in this study and those previously recorded in response to abiotic stresses (Bussotti *et al.* 2011; Banks, 2018).

K-band (ΔW_{OJ}) peaks occurred in response to singular stresses (UD, IW), however, no peak was present when the two were combined (ID) (figure 8B). Banks (2018) reported a similar K-band peak, and no L-band (ΔW_{OK}) peak in response to drought alone in *Acer* trees. A K-band peak is indicative of inactivation, breakdown or reduced efficiency of the oxygen-evolving complex (Srivastava *et al.* 1997; Bussotti *et al.* 2011; Li *et al.* 2014) and/or an increase in the PSII antenna size (Strasser *et al.* 2004; Kalaji *et al.* 2014; Li *et al.* 2014) and is also widely reported to occur in response to heat stress (Srivastava *et al.* 1997; Oukarroum *et al.* 2016). *Armillaria* root rot is known to damage root and root collar tissue, therefore an exacerbation of drought stress response is expected. However, the K-band response was greater than that observed when drought occurred alone (UD), and no response occurred with their combination (ID) (figure 8B).

It could be suggested that this combination (ID) overloaded PSII resulting in energy overflow into fluorescence and a lower raw fluorescence signal in ID than IW or UD. However, Banks (2018) identified a peak after severe desiccation with almost zero fluorescence rise, while a strong fluorescence rise occurred in this study. It could be postulated that the lack of a K-band peak in ID relates to host responses to infection priming pre-drought stress. Heat shock proteins, produced in response to drought stress (Grigorova *et al.* 2011) and defence compounds (such as ROS-detoxifying enzymes & glycinebetaine), which are produced in response to *Armillaria* root rot (Torres *et al.* 2006; Perazzolli *et al.* 2010), have been shown to protect the oxygen-evolving complex (OEC) (Papageorgiou *et al.* 1991; Downs *et al.* 1999). Drought alone is not thought to produce ROS until photodamage has occurred (Henmi *et al.* 2004) therefore, a combination of biotic and abiotic priming may be necessary for OEC protection.

5.5.4 Influence of drought on elucidation of infection

In general, results from the drought period indicate that drought stress and *Armillaria* infection share similar effects on VHI and plant physiological functions. This correlates with the observations from differential kinetics. This masking of infection may be particularly significant for g_S which showed the earliest significant response to drought when other symptoms were less severe. Indeed, for g_S and PI_{ABS} analysis of watered treatments alone showed a significant impact of infection, but this was not present in the drought analyses. Severe drought stress caused greater changes in physiological measurements than early infection alone. This suggests the larger differences caused by drought can mask those caused by infection, at least within the first 40-65 days of infection.

Corresponding with this study, other authors recorded slightly lower g_S, but not significantly so, under the infection-drought stress combination in comparison to infection alone on two the susceptible species *Olea europaea* L. and *Quercus rubra* L. (Loreto *et al.* 1993). If such a difference is present in early infection it is likely very small and may require further replication to detect. However, under the greater root damage caused by later stage infections, it would be expected that the combined impact on g_s with drought would be amplified. Visual symptoms give some indication that early infection may modulate drought response: at the end of the drought period UD displayed a 5.0% higher probability of wilting or leaf loss than ID. Reaction to drought may have been delayed to a small degree in ID compared to UD, which may be caused by reduced root function and transpiration or another physiological impact of infection. Area and F_V/F_M showed potential for discerning infection from drought stress but only under severe drought stress when symptoms like wilting and/or leaf loss are already visually prominent. In the drought and recovery periods, significant increases in chlorophyll concentration, particularly in ID, appear to have been highly influenced by leaf loss (unrecorded), with either a compensatory response or selective senescence counterintuitively increasing mean chlorophyll content. Leaf loss may have also influenced decreases in $V_0(B_0)$ observed under drought: previously this parameter has only been recorded to increase in response to stressors (Lu & Zhang 1999; Mehta *et al.* 2010; Banks 2018). Artificial defoliation may also mask photosynthetic infection signatures (Nowakowska *et al.* 2020), suggesting this aspect of drought stress could complicate interactions.

It is worth noting that towards the end of the drought period symptoms were most severe, and differences between treatments were larger in general (figure 6). Typically the statistical models reduced these latter differences (figure 4), though this 'focus' on earlier, more cryptic, effects may be of benefit to practical users.

Significant differences in VHI and physiological measurements pre-drought in the STE appeared to be of a random origin, i.e. watered uninfected plants initially looked less healthy than infected plants and the significant differences in PI_{ABS} between all treatment groups over time. General declines in this period potentially arose from environmental disturbance as plants adjusted to their new locations. Correlated significant infection-time interaction effects on VHI and chlorophyll content (figure 3A, 4c) may reflect a decreased response to environmental stress under initial infection.

As is apparent for drought itself, recovery from drought stress may mask the visual and physiological symptoms of *Armillaria* infection. This is evident for g_S, where significant differences between IW and UW were present when analysed alone, but not in the full analysis including drought.

A significant question remains: does drought aid or hamper the progress of a pre-existing infection? Experimental data examining Armillaria-drought interactions is rare in general and the majority is focussed on infection rates, i.e. population level impacts, rather than the prognosis of individual hosts (Legrand *et al.* 2005; Holuša *et al.* 2018; Aslam & Magel 2018). While research on the impact of drought on inoculum and rhizomorphs is also insufficient, there are suggestions of a negative impact (Heinzelmann *et al.* 2019). From this study, root:shoot ratio was slightly lower on ID than UD, although not significantly different, suggesting that the combination of infection and drought stress could cause a slightly larger amount of root damage, which would place wild privet plants at an increased risk of mortality after a drought event. The cumulative effect of *Armillaria* root rot and drought has been suggested by previous authors (Guillaumin *et al.* 1985; 1988; Anselmi & Puccinelli 1992; Loreto *et al.* 1993). However, there are several recorded exceptions from this pattern and the relationship between *Armillaria* infection and drought may be further complicated due to potential associations between drought tolerance and resistance to *Armillaria* (Wargo & Harrington 1991; Legrand *et al.* 2005; Cruickshank & Filipescu 2017; Heinzelmann *et al.* 2019).

5.5.5 Conclusion: Potential Utilisation of physiological measures for detection of *Armillaria* infection

Given the masking of physiological infection signatures by environmental stresses, leaf physiological measures are of practical use for infection detection if there is confidence in the absence of other root pathogens, drought or other conflicting environmental factors, and there are plants which are healthy to measure against. Watering plants prior to measurement may be prudent. As a horticultural example, if there have been mortalities within a linear privet hedge, g_S and chlorophyll measurements from the visually healthy plants furthest from the mortalities could be used as a baseline. These 'control' plants should fall within generalized benchmarks of plant health in these parameters. Once this is established,

measurements can then be made along the hedge for comparison to this 'control' group, to elucidate infection likelihood for individual plants and guide sanitation and treatment efforts.

However, the physiological measurements assessed in this study are most suitable for screening for infection in experiments, where general environmental factors can be controlled. Stomatal conductance (g_s) in particular, can be used to expedite experiments on a susceptible woody host, wild privet, as infection success could be determined within 35-45 days: significantly shorter than assays on herbaceous hosts, which are regarded as being rapid at around 90 days length (Ford *et al.* 2017). Utilising these methods may also reduce the time required to perform host screening e.g. reducing the need for plant dissections or provide a proxy for infection removing the need for time-consuming destructive harvests and therefore increasing data yields. Further work is required to indicate appropriate experimental durations and reactivity in other woody hosts. However, wild privet may provide a suitable model host due to its high susceptibility to *Armillaria* root rot. A woody model host is more appropriate for research developing control measures than a herbaceous one, as the majority of economically or otherwise important hosts of *Armillaria* spp. are woody plants.

While physiological measures may be more reliable than visual symptoms of infection, this is not to say they are without their caveats. Stomatal conductance (g_s) is the parameter of the most merit, but it is also the least temporally stable. It was the only measure which was not possible to take due to ambient conditions in both experiments. Additionally, in environments where humidity and light levels fluctuate, readings must be taken in as short a time as possible following calibration to ensure their relevance. These factors may also influence the ease of calibration and the length of time required to take readings, especially from infected plants which have reduced conductance.

Further research should address the applicability of these measurements to larger plants and other susceptible species. This study cannot separate the effect of physical root damage by the fungus from the effect of the many varied phytotoxic substances produced by the fungus (Peipp & Sonnenbichler 1992; Kobori *et al.* 2013; 2015; Sipos *et al.* 2017; Zhelifonova *et al.* 2019; Devkota & Hammerschmidt 2020). The study of their phytotoxic effects has so far focussed on impacts on the germination and growth of seedlings. These compounds alone could cause a physiological response which occurs prior to that from physical root damage. The immediate and delayed physiological responses to such substances applied to the root system should be examined and may elucidate finer details of the pathogenesis of *Armillaria* species. Comparison to physiological responses caused by other species of root rot are also required.

This study significantly advances the knowledge of physiological responses to *Armillaria* infection in a susceptible candidate model woody host, records new parameters, compares their responsiveness, and suggests that physiological signatures of *Armillaria* infection can be masked by environmental stresses from various sources e.g. drought, nutrient, and/or root restriction stresses.

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5.7 Conflict of Interest

Hailey, Banks, & Percival are employees of Bartlett Tree Experts: the company (not the individuals) receives royalties from the sale of the 'Arborcheck' system (Hansatech Instruments Ltd., King's Lynn, UK). This mention of the product can be removed if required, although we believe it is justified as a unique example of chlorophyll fluorescence measurements being used outside of the scientific realm.

5.8 References

Agustini L, Beadle C, Barry K, Mohammed C. **2015**. Photosynthetic Responses of *Eucalyptus nitens* Maiden at Initial Stages of Root-Rot Infection. *Indonesian Journal of Forestry Research* **2**: 9–20.

Amiri A, Schnabel G. **2012**. Persistence of Propiconazole in Peach Roots and Efficacy of Trunk Infusions for *Armillaria* Root Rot Control. *International Journal of Fruit Science* **12**: 437–449.

Anselmi N, Puccinelli P. **1992**. Studies on *Armillaria* attacks on declining oak trees. In: Luisi N, Lerario P, Vannini A, eds. Recent advances in studies on oak decline. Bari, Italia: Universita degli studi, 23–29.

Aslam AJ, Magel EA. **2018**. Influence of drought and season on compartmentalization of black locust (*Robinia pseudoacacia* L.) inoculated with *Armillaria mellea*. *European Journal of Plant Pathology* **152**: 21–31.

Azzouz I, Bachari K. **2018**. MEMS Devices for Miniaturized Gas Chromatography. In: Yellampalli KBE-S, ed. MEMS Sensors - Design and Application. Rijeka: InTech, Ch. 7.

Banks JM. **2018**. Chlorophyll fluorescence as a tool to identify drought stress in *Acer* genotypes. *Environmental and Experimental Botany* **155**: 118–127.

Baumgartner K. **2004**. Root Collar Excavation for Postinfection Control of *Armillaria* Root Disease of Grapevine. *Plant Disease* **88**: 1235–1240.

Baumgartner K, Bhat R, Fujiyoshi P. **2010**. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal biology* **114**: 107–19.

Baumgartner K, Coetzee MPA, Hoffmeister D. **2011**. Secrets of the subterranean pathosystem of *Armillaria*. *Molecular Plant Pathology* **12**: 515–534.

Beal EJ, Henricot B, Peace AJ, Waghorn IAG, Denton JO. **2015**. The action of allicin against *Armillaria* spp. *in vitro* (M-S Kim, Ed.). *Forest Pathology* **45**: 450–458.

Beckman TG, Pusey PL. **2001**. Field testing peach rootstocks for resistance to *Armillaria* root rot. *HortScience* **36**: 101–103.

Bogdanski BEC, Cruickshank M, Mario Di Lucca C, Becker E. **2018**. Stumping out tree root disease – An economic analysis of controlling root disease, including its effects on carbon storage in southern British Columbia. *Forest Ecology and Management* **409**: 129–147.

Bussotti F, Desotgiu R, Cascio C, Pollastrini M, Gravano E, Gerosa G, Marzuoli R, Nali C, Lorenzini G, Salvatori E, *et al.* 2011. Ozone stress in woody plants assessed with chlorophyll a fluorescence. A critical reassessment of existing data. *Environmental and Experimental Botany* **73**: 19–30.

Calderón R, Lucena C, Trapero-Casas JL, Zarco-Tejada PJ, Navas-Cortés JA. **2014**. Soil temperature determines the reaction of olive cultivars to *Verticillium dahliae* pathotypes. *PLoS ONE* **9**.

Calderón R, Navas-Cortés JA, Lucena C, Zarco-Tejada PJ. **2013**. High-resolution airborne hyperspectral and thermal imagery for early detection of *Verticillium* wilt of olive using fluorescence, temperature and narrow-band spectral indices. *Remote Sensing of Environment* **139**: 231–245.

Clemenz C, Fleischmann F, Häberle KH, Matyssek R, Oßwald W. **2008**. Photosynthetic and leaf water potential responses of *Alnus glutinosa* saplings to stem-base inoculaton with *Phytophthora alni* subsp. *alni*. *Tree Physiology* **28**: 1703–1711.

Cromey MG, Drakulic J, Beal EJ, Waghorn IAG, Perry JN, Clover GRG. **2020**. Susceptibility of Garden

Trees and Shrubs to *Armillaria* Root Rot. *Plant Disease* **104**: 483–492.

Cruickshank MG, Filipescu CN. **2017**. The interactive effect of root disease and climate on wood properties in halfsibling Douglas-fir families. *Forest Ecology and Management* **392**: 58–67.

Cruickshank MG, Jaquish B, Nemec AFL. **2010**. Resistance of half-sib interior Douglas-fir families to *Armillaria ostoyae* in British Columbia following artificial inoculation. *Canadian Journal of Forest Research* **40**: 155–166.

Day SD, Watson G, Wiseman PE, Harris JR. **2009**. Causes and Consequences of Deep Structural Roots in Urban Trees: From Nursery Production to Landscape Establishment. *Arboriculture & Urban Forestry* **35**: 182–191.

Devkota P, Hammerschmidt R. **2020**. The infection process of *Armillaria mellea* and *Armillaria solidipes*. *Physiological and Molecular Plant Pathology* **112**: 101543.

Downs CA, Coleman JS, Heckathorn SA. **1999**. The chloroplast 22-Ku heat-shock protein: A lumenal protein that associates with the Oxygen Evolving Complex and protects Photosystem II during heat stress. *Journal of Plant Physiology* **155**: 477–487.

Drakulic J, Gorton C, Perez-Sierra A, Clover G, Beal L. **2017**. Associations Between *Armillaria* Species and Host Plants in U.K. Gardens. *Plant Disease* **101**: 1903–1909.

Espinoza S, Yañez M, Magni C, Santelices R, Cabrera A. **2020**. Outplanting performance of three provenances of *Quillaja saponaria* Mol. established in a Mediterranean drought-prone site and grown in different container size. *iForest - Biogeosciences and Forestry* **13**: 33–40.

Fascella G, Rouphael Y. **2017**. Influence of container volume and irrigation system on photosynthesis, water productivity and growth of potted *Euphorbia × lomi*. *Acta Scientiarum Polonorum, Hortorum Cultus* **16**: 163–171.

Fleischmann F, Koehl J, Portz R, Beltrame AB, Oßwald W. **2005**. Physiological changes of *Fagus sylvatica* seedlings infected with *Phytophthora citricola* and the contribution of its elicitin ' Citricolin' to pathogenesis. *Plant Biology* **7**: 650–658.

Ford KL, Baumgartner K, Henricot B, Bailey AM, Foster GD. **2015**. A reliable *in vitro* fruiting system for *Armillaria mellea* for evaluation of *Agrobacterium tumefaciens* transformation vectors. *Fungal Biology* **119**: 859–869.

Ford KL, Henricot B, Baumgartner K, Bailey AM, Foster GD. **2017**. A faster inoculation assay for *Armillaria* using herbaceous plants. *The Journal of Horticultural Science and Biotechnology* **92**: 39–47.

Goh KM, Dickinson M, Alderson P, Yap LV, Supramaniam CV. **2016**. Development of an *in planta* infection system for the early detection of *Ganoderma* spp. in oil palm. *Journal of Plant Pathology* **98**.

Gregory SC, Rishbeth J, Shaw CG. **1991**. Pathogenicity and Virulence. In: Shaw CG, Kile GA, eds. *Armillaria* Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 76–87.

Grigorova B, Vaseva I, Demirevska K, Feller U. **2011**. Combined drought and heat stress in wheat: Changes in some heat shock proteins. *Biologia Plantarum* **55**: 105–111.

Guillaumin J-J, Bernard C, Delatour C, Belgrand M. **1985**. Contribution à l'étude du dépérissement du chêne : pathologie racinaire en forêt de Tronçais. *Annales des Sciences Forestières* **42**: 1–22.

Guillaumin J-J, Legrand P. **2005**. Cycle infectieux de l'armillaire – Stratégie biologique des espèces

européennes. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 177–201.

Guillaumin J-J, Lung-Escarmant B, Dubos B. **2005a**. Lutte chimique. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 365–375.

Guillaumin J-J, Lung-Escarmant B, Legrand P. **2005b**. Sensibilité des hôtes ligneux à l'armillaire et sélection pour la tolérance. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 377–388.

Guillaumin JJ, Mohammed C, Berthelay S. **1988**. *Armillaria* species in the northern temperate hemisphere. In: Morrison DJ, ed. Proceedings of the Seventh International Conference on Root and Butt Rots of Forest Trees. Victoria, Canada: Forestry Canada, 27–43.

Hadfield JS, Goheen DJ, Filip GM, Schmitt CL, Harvey RD. **1986**. *Root diseases in Oregon and Washington conifers (R6-FPM-250-86)*. USDA Forest Service, Pacific Northwest Region.

Hailey LE, Percival GC. **2015**. The Influence of long term flooding on tree biology and approaches to flood stress alleviation and management. *Arboricultural Journal* **37**: 135–149.

Heinzelmann R, Dutech C, Tsykun T, Labbé F, Soularue J-P, Prospero S. **2019**. Latest advances and future perspectives in *Armillaria* research. *Canadian Journal of Plant Pathology* **41**: 1–23.

Henmi T, Miyao M, Yamamoto Y. **2004**. Release and Reactive-Oxygen-Mediated Damage of the Oxygen-Evolving Complex Subunits of PSII during Photoinhibition. *Plant and Cell Physiology* **45**: 243–250.

Holuša J, Lubojacký J, Čurn V, Tonka T, Lukášová K, Horák J. **2018**. Combined effects of drought stress and *Armillaria* infection on tree mortality in Norway spruce plantations. *Forest Ecology and Management* **427**: 434–445.

Kalaji HM, Jajoo A, Oukarroum A, Brestic M, Zivcak M, Samborska IA, Cetner MD, Łukasik I, Goltsev V, Ladle RJ. **2016**. Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. *Acta Physiologiae Plantarum* **38**: 102.

Kalaji HM, Schansker G, Ladle RJ, Goltsev V, Bosa K, Allakhverdiev SI, Brestic M, Bussotti F, Calatayud A, Dąbrowski P, *et al.* 2014. Frequently asked questions about *in vivo* chlorophyll fluorescence: Practical issues. *Photosynthesis Research* **122**: 121–158.

Kobori H, Sekiya A, Suzuki T, Choi J-H, Hirai H, Kawagishi H. **2015**. Bioactive Sesquiterpene Aryl Esters from the Culture Broth of Armillaria sp. *Journal of Natural Products* **78**: 163–167.

Kobori H, Sekiya A, Yasuda N, Noguchi K, Suzuki T, Choi J-H, Hirai H, Kawagishi H. **2013**. Armillariols A to C from the culture broth of *Armillaria* sp. *Tetrahedron Letters* **54**: 5481–5483.

Laflamme G, Guillaumin J-J. **2005**. L'armillaire, agent pathogène mondial : répartition et dégâts. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 273–289.

Legrand P, Lung-Escarmant B. **2005**. L'armillaire en plantations forestières en Europe. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des végétaux ligneux. Versailles, France: Editions Quae, 307–318.

Legrand P, Marçais B, Guillaumin J-J, Lung-Escarmant B. **2005**. Prédisposition des arbres et sensibilité à l'armillaire. In: Guillaumin J-J, Inra, Cemagref, Cirad, Ifremer, eds. L'armillaire et le pourridié-agaric des

végétaux ligneux. Versailles, France: Editions Quae, 221–237.

Li XM, Chen MJ, Li J, Ma LJ, Bu N, Li YY, Zhang LH. **2014**. Effect of endophyte infection on chlorophyll a fluorescence in salinity stressed rice. *Biologia Plantarum* **58**: 589–594.

Livingston WH. **1990**. *Armillaria ostoyae* in young spruce plantations. *Canadian journal of forest research* **20**: 1773–1778.

Loreto F, Burdsall HH, Tirro A. **1993**. *Armillaria* infection and water stress influence gas-exchange properties of Mediterranean trees. *HortScience* **28**: 222–224.

Loulier J, Lefort F, Stocki M, Asztemborska M, Szmigielski R, Siwek K, Grzywacz T, Hsiang T, Ślusarski S, Oszako T, *et al.* 2020. Detection of Fungi and Oomycetes by Volatiles Using E-Nose and SPME-GC/MS Platforms. *Molecules (Basel, Switzerland)* **25**.

Lu C, Zhang J. **1999**. Effects of water stress on photosynthesis, chlorophyll fluorescence and photoinhibition in wheat plants. *Functional Plant Biology* **25**: 883–892.

Mansilla PJ, Aguín O, Sainz MJ. **2001**. A Fast Method for Production of *Armillaria* Inoculum. *Mycologia* **93**: 612–615.

Martínez-Ferri E, Zumaquero A, Ariza MT, Barceló A, Pliego C. **2016**. Nondestructive detection of white root rot disease in avocado rootstocks by leaf chlorophyll fluorescence. *Plant Disease* **100**: 49–58.

Mehta P, Allakhverdiev SI, Jajoo A. **2010**. Characterization of photosystem II heterogeneity in response to high salt stress in wheat leaves (*Triticum aestivum*). *Photosynthesis Research* **105**: 249–255.

Miller SB, Gasic K, Reighard GL, Henderson WG, Rollins PA, Vassalos M, Schnabel G. **2020**. Preventative Root-Collar Excavation Reduces Peach Tree Mortality Caused By *Armillaria* Root Rot On Replant Sites. *Plant Disease* **104**.

Modi D, Simard S, Bérubé J, Lavkulich L, Hamelin R, Grayston SJ. **2020**. Long-term effects of stump removal and tree species composition on the diversity and structure of soil fungal communities. *FEMS Microbiology Ecology* **96**: 1–12.

Navaei M. **2015**. Integration of Micro-Gas Chromatography System for Detection of Volatile Organic Compounds. Doctoral Thesis. Georgia Institute of Technology.

Nogales A, Aguirreolea J, Santa María E, Camprubí A, Calvet C. **2008**. Response of mycorrhizal grapevine to *Armillaria mellea* inoculation: disease development and polyamines. *Plant and Soil* **317**: 177–187.

Nowakowska JA, Stocki M, Stocka N, Ślusarski S, Tkaczyk M, Caetano JM, Tulik M, Hsiang T, Oszako T. **2020**. Interactions between *Phytophthora cactorum*, *Armillaria gallica* and *Betula pendula* Roth. Seedlings Subjected to Defoliation. *Forests* **11**: 1107.

Nunes LJR, Meireles CIR, Gomes CJP, de Almeida Ribeiro NMC. **2020**. Global Climate Change Outlook. In: Climate Change Impact on Environmental Variability in the Forest. Springer, 9–17.

Ouellette GB, Bard G, Cauchon R. **1971**. Self-strangulation of roots: points of entry of root-rot fungi in the Grand'Mère white spruce plantations. *Phytoprotection* **52:** 119-124.

Oukarroum A, El Gharous M, Goltsev V, Strasser RJ. **2016**. Delayed fluorescence emission as a probe for the response of photosynthetic organisms to high temperature exposure: A comparative study. *Journal of Luminescence* **180**: 321–327.

Oukarroum A, Madidi S El, Schansker G, Strasser RJ. **2007**. Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll a fluorescence OLKJIP under drought stress and re-watering. *Environmental and Experimental Botany* **60**: 438–446.

Papageorgiou GC, Fujimura Y, Murata N. **1991**. Protection of the oxygen-evolving photosystem II complex by glycinebetaine. *BBA - Bioenergetics* **1057**: 361–366.

Peipp H, Sonnenbichler J. **1992**. Secondary Fungal Metabolites and Their Biological Activities, II. Occurrance of Antibiotic Compounds in Cultures of *Armillaria ostoyae* Growing in the Presence of an Antagonistic Fungus or Host Plant Cells. *Biological Chemistry Hoppe-Seyler* **373**: 675–684.

Pellegrini A, Corneo PE, Camin F, Ziller L, Tosi S, Pertot I. **2012**. Studying trophic interactions between a plant pathogen and two different antagonistic microorganisms using a 13C-labeled compound and isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **26**: 510–516.

Perazzolli M, Bampi F, Faccin S, Moser M, De Luca F, Ciccotti AM, Velasco R, Gessler C, Pertot I, Moser C. **2010**. *Armillaria mellea* induces a set of defense genes in grapevine roots and one of them codifies a protein with antifungal activity. *Molecular Plant-Microbe Interactions* **23**: 485–496.

Percival GC, Smiley ET, Fox RTV. **2011**. Root Collar Excavation with *Trichoderma Inoculations* as a Potential Management Strategy for Honey Fungus (*Armillaria mellea*). *Arboricultural Journal* **33**: 267–280.

Pérez-Bueno ML, Pineda M, Vida C, Fernández-Ortuño D, Torés JA, de Vicente A, Cazorla FM, Barón M. **2019**. Detection of white root rot in avocado trees by remote sensing. *Plant Disease* **103**: 1119–1125.

Perez-Sierra A. **2004**. Systematics, diagnostics and epidemiology of the fungal genus *Armillaria*. Doctoral Thesis. University of London.

Prospero S, Holdenrieder O, Rigling D. **2004**. Comparison of the virulence of *Armillaria cepistipes* and *Armillaria ostoyae* on four Norway spruce provenances. *Forest Pathology* **34**: 1–14.

R Core Team. **2020**. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raabe RD. **1962**. Host list of the root rot fungus, *Armillaria mellea*. *Hilgardia* **33**: 24–89.

Rabiey M, Hailey LE, Roy SR, Grenz K, Al-Zadjali MAS, Barrett GA, Jackson RW. **2019**. Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health? *European Journal of Plant Pathology* **155**: 711–729.

Rakib MRM, Borhan AH, Jawahir AN. **2019**. The relationship between SPAD chlorophyll and disease severity index in Ganoderma-infected oil palm seedlings. *Journal of the Bangladesh Agricultural University* **17**: 355–358.

Rishbeth J. **1983**. The importance of honey fungus (*Armillaria*) in urban forestry. *Arboricultural Journal* **7**: 217–225.

Salgadoe ASA, Robson AJ, Lamb DW, Dann EK. **2019**. Assessment of canopy porosity in avocado trees as a surrogate for restricted transpiration emanating from *Phytophthora* root rot. *Remote Sensing* **11**.

Salgadoe ASA, Robson AJ, Lamb DW, Dann EK, Searle C. **2018**. Quantifying the severity of *Phytophthora* root rot disease in avocado trees using image analysis. *Remote Sensing* **10**.

Sinclair TR, Manandhar A, Shekoofa A, Rosas-Anderson P, Bagherzadi L, Schoppach R, Sadok W, Rufty

TW. **2017**. Pot binding as a variable confounding plant phenotype: theoretical derivation and experimental observations. *Planta* **245**: 729–735.

Singh P, Richardson J. **1973**. *Armillaria* root rot in seeded and planted areas in Newfoundland. *The Forestry Chronicle* **49**: 180–182.

Sipos G, Prasanna AN, Walter MC, O'Connor E, Bálint B, Krizsán K, Kiss B, Hess J, Varga T, Slot J, *et al.* 2017. Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungi *Armillaria*. *Nature Ecology & Evolution* **1**: 1931–1941.

Solla A, Tomlinson F, Woodward S. **2002**. Penetration of *Picea sitchensis* root bark by *Armillaria mellea*, Armillaria ostoyae and Heterobasidion annosum. *Forest Pathology* **32**: 55–70.

Srivastava A, Guissé B, Greppin H, Strasser RJ. **1997**. Regulation of antenna structure and electron transport in Photosystem II of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll a fluorescence transient: OKJIP. *Biochimica et Biophysica Acta - Bioenergetics* **1320**: 95–106.

Strasser RJ, Srivastava A, Tsimilli-Michael M. **2000**. The fluorescence transient as a tool to characterize and screen photosynthetic samples. *Probing Photosynthesis: Mechanism, Regulation & Adaptation*: 443–480.

Strasser RJ, Tsimilli-Michael M, Srivastava A. **2004**. Analysis of the Chlorophyll a Fluorescence Transient. In: Chlorophyll Fluorescence: A Signature of Photosynthesis. 321–362.

Torres MA, Jones JDG, Dangl JL. **2006**. Reactive oxygen species signaling in response to pathogens. *Plant Physiology* **141**: 373–378.

Turner NC. **2019**. Imposing and maintaining soil water deficits in drought studies in pots. *Plant and Soil* **439**: 45–55.

Vasaitis R, Stenlid J, Thomsen IM, Barklund P, Dahlberg A. **2008**. Stump removal to control root rot in forest stands: a literature study. *Silva Fennica* **42**: 457–483.

Wang H, Wang C, Wang F, Niu R, Zhu W, Wu X. **2019**. Root restriction effects of nectarines grown in a non-arable land greenhouse. *Scientia Horticulturae* **250**: 399–404.

Wargo PM, Harrington TC. **1991**. Host Stress and Susceptbility. In: Shaw CGI, Kile GA, eds. Armillaria Root Disease, Agriculture Handbook No. 691. Washington, D.C.: USDA Forest Service, 88–101.

West JS, Fox RTV. **2002**. Stimulation of *Armillaria mellea* by phenolic fungicides. *Annals of Applied Biology* **140**: 291–295.

Xiong D, Chen J, Yu T, Gao W, Ling X, Li Y, Peng S, Huang J. **2015**. SPAD-based leaf nitrogen estimation is impacted by environmental factors and crop leaf characteristics. *Scientific Reports* **5**: 1–12.

Zakaria NI, Ismail MR, Awang Y, Megat Wahab PE, Berahim Z. **2020**. Effect of Root Restriction on the Growth, Photosynthesis Rate, and Source and Sink Relationship of Chilli (*Capsicum annuum* L.) Grown in Soilless Culture (L-SP Tran, Ed.). *BioMed Research International* **2020**.

Zhelifonova VP, Antipova TV, Litvinova EA, Baskunov BP, Litovka YA, Pavlov IN, Kozlovsky AG. **2019**. Biosynthesis of Protoilludene Sesquiterpene Aryl Esters by Siberian Strains of the Genus *Armillaria* Fungi. *Applied Biochemistry and Microbiology* **55**: 277–283.

Živčák M, Brestič M, Olšovská K, Slamka P. **2008**. Performance index as a sensitive indicator of water stress in *Triticum aestivum* L. *Plant, Soil and Environment* **54**: 133–139.

6. General Discussion

6.1 Main findings

Together, the studies which form this thesis fulfil the primary aim of furthering knowledge that can be used towards the integrated management of *Armillaria mellea*. The main areas of this research are comprised of *Armillaria* chemical and biological control (Chapter: 3), monitoring and detection (5), and research techniques (4).

Respectively, the main findings were that:

- Potassium phosphite at 750-1000mg/L agar can directly inhibit *A. mellea* growth *in vitro* by around 70-77% after 21 days. While *Pseudomonas protegens* Pf-5 and *Bacillus subtilis* QST713 both inhibited *A. mellea* growth *in vitro* by around 40-45%, only QST713 showed promise for increased inhibition when combined with potassium phosphite, yielding around 80% inhibition.
- It was possible to use stomatal conductance and chlorophyll content to detect *Armillaria mellea* infections on a sensitive woody host (wild privet; *Ligustrum vulgare*), after 35-40 days; prior to conclusive visual symptoms and more rapidly than typical host infection assays. Chlorophyll fluorescence measures also showed sensitivity. Combinations of measurements can improve classification of infected and uninfected plants. Drought stress and other physiological stresses can mask physiological signatures of infection.
- On both strawberry (*Fragaria × ananassa*) and wild privet hosts, inoculum based on *Aesculus hippocastanum* seeds had the highest infection efficacy and *Corylus avellana* stem billets also performed well; *Quercus robur* seeds performed poorly in comparison. Results correspond with the resources and habitat provided by the inocula. The strawberry model host reacted severely to *A. mellea* infection in comparison to the privet cuttings, which are considered highly susceptible. Privet mortalities equivalent to those observed in strawberry took nine months in comparison to three. Use of submerged culture techniques halved the variation in growth of the fungus on agar in comparison to traditional subculture by colony fragments.

6.2 Evolution of the project & motivations

At its inception, this project was solely aimed at development of a liquid applied biological control agent (BCA) and plant defence activator (PDA) combination for use in the control of *A. mellea*, be it preventative or therapeutic. Over time it became apparent that this was an undertaking of such a scale that it was more appropriate as a goal of researcher's career rather than of a PhD thesis. Further inspiration was provided by difficulties in the earlier work which were reflected in the field as a whole.

Therefore, later research focussed on the interfaces between *Armillaria* studies and areas of personal prior experience, alongside collaboration with other researchers with varied specialisms; namely the work on *Armillaria* research methodology (4) and early detection (5).

All studies focussed on issues which are under-investigated in some form, e.g. antagonism of *A. mellea* by well characterized BCA strains or the combination of BCAs and PDAs (3). The later studies (4, 5) were motivated by problems faced in the earlier work (3) that are significant generalized issues within *Armillaria* research, i.e. inoculation and culture methodology. However, these issues had not been approached by other researchers for some time or in enough depth, i.e. early detection. The results of these issues can still be seen within the work, especially as the detection (5) and methodology studies (4) were carried out concurrently. If the results on the comparatively poor performance of the *Quercus* seed inoculum were available, then *Aesculus* seed inoculum might have been used instead in the short-term experiment of the detection work. This would have likely increased the number of plants adequately challenged by the fungus and therefore the sample number. The differences in inoculum pressure between the two substrates may go some way to explaining the differences seen between the long- and short-term experiments of the detection paper. For example, the non-significant reaction in $V_0(B_0)$ and later reaction of PI_{ABS} in the short-term experiment may reflect a lower inoculum pressure, as suggested by the inoculum comparison results.

The long history of prior *Armillaria* research is immensely valuable to contemporary researchers and historical techniques, especially culture by colony fragments, are well proven. However, it is valuable to regularly reassess methodologies, ensuring that innovation and high research efficiency, and quality, are maintained. This is especially true in the light of the continuously developing taxonomy of the genus (1.3). Older studies may have experienced variation in results due to unknown taxonomic differences and methods trialled may have been discarded erroneously. Presumably this issue has decreased with increasing taxonomic knowledge and will continue to do so until the taxonomy is resolved, if possible. As discussed in the methodology paper (4), a lack of standardization of methodology, especially for host inoculation, is another key issue that limits the comparisons between, and synthesis from, the results of different studies. The development of more standardized methodologies e.g. model host plants, inoculum substrates and production, which are utilized by different groups of researchers would be the optimum way of ensuring comparability. This is a valuable goal, however it would be slow to come to fruition and complete uptake is unlikely. Therefore, increasing the details provided in these areas was suggested in the methodology discussion (4.5) as a viable intermediate aim for the *Armillaria* research community. Leading by example may be one way of encouraging the use of more standardized and

completely described methodologies; publications resulting from this thesis and continuing the work will aim to do so.

6.3 Synthesis & significance of the studies

In comparison to the other studies, the work on combined application of BCAs and PDAs requires a significant amount of research before any practical use, i.e. testing *in planta*, with high probability of environmental factors influencing results. The other studies provide techniques to successfully test the combination of potassium phosphite and *Bacillus subtilis* QST713 *in planta*. This also concisely demonstrates how the different themes of research in this thesis might interact. For example, any further *in vitro* work on agar should be carried out using the submerged culture/homogenized mycelium methodology. Following this, infection assays on hosts could be carried out using the inoculation techniques tested, with well characterised inoculum. *A. gallica* should be used as a control to ensure appropriate inoculum pressure (Baumgartner *et al.*, 2018). Results may begin on strawberry hosts for preliminary assessment, then move to privet or start with privet and monitor leaf physiology for infection signatures. This could be followed by a wider range of woody hosts and plant sizes to assess how widely applicable the treatment is. Assays on hosts could be carried out in controlled and/or natural conditions depending on the aims the particular experiment i.e. applied or pure science. If the treatment proves successful in the field, then the physiological indicators of infection could be used to guide application of the treatment, as well as concurrent sanitation efforts. Further specialised trials might also be required, e.g. ecotoxicology, before the treatment is granted regulatory approval for use in plant protection i.e. commercially.

The improved research techniques developed or tested can be put to use by other researchers almost immediately, with minimal prior testing, improving research efficiency. However, their benefit can only indirectly influence *Armillaria* management practices and is likely to take a significant period of time to do so due to the required uptake by other researchers and subsequent publishing timescales. Although not exhaustive, this paper also appears to be the first in the field to gather, present, and compare inoculation methodologies from a large number of prior studies (Chapter 4: Table 1). Providing a convenient resource for other researchers.

In contrast, the work on the early detection of *Armillaria* infection has the most potential to directly improve the monitoring of *Armillaria* infections in multiple contexts ranging from experimental use to industrial cultivation to private gardens. The techniques are not overly complex and the equipment is comparable to, or less expensive than, that used within tree decay surveys, e.g. resistograph drills or sonic tomography; the total price of a porometer, chlorophyll content meter, and chlorophyll fluorescence meter together is ~£10-15k. Therefore, utilisation by those in the tree care industry, namely

arboricultural consultants, is viable. Although, while previous studies indicate that physiological reactions of other susceptible species will be similar to privet (Loreto *et al.*, 1993), testing a wider range of hosts is required to confirm this.

6.4 Further work

The methodology paper (4) was originally planned to include the use of qPCR to quantify and compare the colonization of various materials by *A. mellea* CG440 (Baumgartner *et al.*, 2010; Calvet *et al.*, 2015). This included analysis of host root collars/crowns, as a measure of infection from the different substrates and between hosts, and may have elucidated asymptomatic infection levels. The colonization of the various substrates by CG440 was also to be compared, including substrates inoculated using homogenized mycelium and those inoculated by colony fragments. This work was to link the *in vitro* culture and host infection assay aspects of the study, while also increasing cohesion in general and allowing further synthesis from the various results. Unfortunately there were significant issues with PCR inhibitors. Plant material is notorious for having high amounts of inhibitors such as polyphenols, e.g. tannins, or polysaccharides which can block amplification. Amplification failed in many cases despite the use of a chloroform-phenol extraction method (Porebski *et al.*, 1997), the use of proteinase-K, and increased dilution of samples prior to amplification to reduce inhibitors. Work continues to develop a successful extraction and amplification protocol, as this would be a valuable addition to this paper prior to its submission to a journal. If, as would be expected, a correlation between substrate colonization and host infection is demonstrated, this information would be useful in comparing host infection rates between studies.

Aside from this, the work extending this study is focussed on further investigating the use of leaf physiology measurements for the early detection and monitoring of *Armillaria* infections. Firstly, other susceptible species need to be tested. Listed as susceptible in a recent ranking (Cromey *et al.*, 2020), species of the genera *Cotoneaster, Weigela, Laburnum, Syringa,* and *Salix* have been selected as susceptible hosts which readily form cuttings. Cuttings of a similar biomass will be made if possible. Alongside *Ligustrum* species, these will be inoculated and leaf physiological measurements recorded weekly. Results of this trial will indicate if the reactions to infection recorded in wild privet are general to susceptible hosts or how much they vary between susceptible hosts. The hypotheses being that susceptible species have similar physiological reactions to infection, as previously suggested (Loreto *et al.*, 1993), but that genotype would influence this. A further extension of this work would be to test a range of species with differing resistances to *Armillaria*.

Larger plants also need to be tested. *Armillaria* infections are typical in *Ligustrum* hedges and spread along from plant to plant in a linear fashion. Therefore, tests on larger plants could be carried out using

containerised privet hedges, applying infection at one end and monitoring the changes in leaf physiology over time. There is scope for testing sanitation interventions in this fashion, in terms of their timing, i.e. at what stomatal conductance in comparison to the non-inoculated end of the hedge, and number of plants removed/distance.

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6.6 References

Baumgartner K, Bhat R, Fujiyoshi P. **2010**. A rapid infection assay for *Armillaria* and real-time PCR quantitation of the fungal biomass *in planta*. *Fungal biology* **114**: 107–19.

Baumgartner K, Fujiyoshi P, Ledbetter C, Duncan R, Kluepfel DA. **2018**. Screening almond rootstocks for sources of resistance to *Armillaria* root disease. *HortScience* **53**: 4–8.

Calvet C, Garcia-figueres F, Lovato P, Camprubi A. **2015**. Role of the arbuscular mycorrhizal symbiosis in tolerance response against *Armillaria mellea* in lavender. *Spanish Journal of Agricultural Research* **13**: 1–8.

Cromey MG, Drakulic J, Beal EJ, Waghorn IAG, Perry JN, Clover GRG. **2020**. Susceptibility of Garden Trees and Shrubs to *Armillaria* Root Rot. *Plant Disease* **104**: 483–492.

Loreto F, Burdsall HH, Tirro A. **1993**. *Armillaria* infection and water stress influence gas-exchange properties of Mediterranean trees. *HortScience* **28**: 222–224.

Porebski S, Bailey LG, Baum BR. **1997**. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter* **15**: 8–15.

Appendix A: Chapter 4 'Methods' supplementary data tables

Table SM1. *In vitro* statistical analysis results

Variable	ANOVA type	Treatment ANOVA p-value	Colony Plug Mean (CI)	Lawn Plug Mean (CI)	Homogenized Mycelium Mean (CI)	Significant pairwise p-values	Non-significant pairwise p-values
Growth Area Co-efficient of Variation in Exponential Growth i.e. variability of growth between individuals with compensation for size	both (II & III)	3.94E-05	20.0a (16.10, 24.9)	19.6a (15.73, 24.4)	9.4b (7.55, 11.7)	0.0001-0.0002	0.9868
Day at which rhizomorphs overtake surface spread	both (II & III)	<2e-16	6.21a (5.90, 6.53)	6.07a (5.76, 6.38)	8.53b (8.22, 8.84)	<.0001	0.7906
exponential growth rate (trend)	III (LME)	4.56E-06	0.537a (0.520, 0.553)	0.533a (0.516, 0.549)	0.484b (0.468, 0.501)	0.0001-0.0002	0.9446
growth means day 6	III (LME)	0.00000681 treatment 0.001565 interaction	191.1a (172.1, 212.1)	203.6a (183.8, 225.7)	142.4b (128.5, 157.8)	<.0001	0.154
growth means day 8	III (LME)	0.00000681 treatment 0.001565 interaction	373.6a (338.6, 412.2)	406.8b (369.3, 448.1)	273.2c (248.0, 300.9)	<.0001-0.0254	-

Table SM2. Substrate selection statistical analysis results

Variable	Omnibus Test type	Omnibus p-value	Pairwise Comparison Test	Selector 1 Mean (CI)	Selector 2 Mean (CI)	Selector 3 Mean (CI)	Significant pairwise p-values	Non-significant pairwise p-values
Aesculus seed used fresh weight (g)	ANOVA, Type III	Substrate:Selector 0.006715	Tukey's Honest Significant Difference	10.49a (9.98, 11.00)	12.10b (11.59, 12.61)	12.16b (11.65, 12.67)	<.0001	0.9871
Aesculus seed typical dry weight (g)	ANOVA, Type III	Substrate <2.2e-16 Selector 0.001012	Tukey's Honest Significant Difference	NA	7.79a (7.30, 8.27)	6.65b (6.16, 7.14)	0.0016	-

Table SM3. Substrate characteristics statistical analysis results

Variable	Omnibus Test type	Omnibus p-value	Pairwise Comparison Test	Hazel Mean (CI)	Acorn Mean (CI)	Conker Mean (CI)	Significant pairwise p-values	Non-significant pairwise p-values
Typical fresh weight (g)	ANOVA, Type III	Substrate <2.2e-16 Selector 2.35e-06 Substrate:Selector 0.0007636	Tukey's Honest Significant Difference	9.42b (8.96, 9.89)	3.88a (3.42, 4.35)	11.61c (11.14, 12.07)	<.0001	-
Typical dry weight (g)	ANOVA, Type III	Substrate <2.2e-16 Selector 0.001012 Substrate:Selector 0.272627	Tukey's Honest Significant Difference	4.22b (3.88, 4.57)	2.39a (2.05, 2.74)	7.22c (6.87, 7.56)	<.0001	-
Typical estimated applied dry mass (g)	Kruskal-Wallis Rank Sum	Substrate 2.546e-10	Pairwise Wilcoxon Rank Sum, Bonferroni adjustment	4.4a ± 0.7 SD	7.2b ± 1.6 SD	7.2b ± 1.4 SD	7.3e-07 - 8.9e-10	1
Typical dry density (g^{-1} cm^3)	ANOVA, Type III	Substrate 2.433e-11 Selector 0.0004354 Substrate:Selector 0.2844323	Tukey's Honest Significant Difference	0.450a (0.398, 0.502)	0.660b (0.608, 0.712)	0.746b (0.694, 0.797)	<.0001	0.0579
Typical estimated surface area to volume ratio	ANOVA, Type III	Substrate 5.252e-12 Selector 0.03729 Substrate:Selector 0.23454	Tukey's Honest Significant Difference	2.90b (2.76, 3.03)	3.14c (3.00, 3.27)	2.34c (2.21, 2.47)	<.0001	0.0361
Typical individual volume (cm^3)	Kruskal-Wallis Rank Sum	Substrate 2.745e-13	Pairwise Wilcoxon Rank Sum, Bonferroni adjustment	9.5b ± 1.3	3.6a ± 0.5	10.2b ± 1.9	1.4e-10 - 1.6e-10	1
Typical estimated applied volume (cm^3)	Kruskal-Wallis Rank Sum	Substrate 0.005698	Pairwise Wilcoxon Rank Sum, Bonferroni adjustment	9.5a ± 1.3	10.7b ± 1.6	10.2ab ± 1.9	0.0066	0.0581-1

Table SM4. *In planta* statistical analysis results

Host	Variable	Month	Treatment ANOVA p-value	Control Mean (CI)	Hazel Mean (CI)	Acorn Mean (CI)	Conker Mean (CI)	Significant pairwise p-values	Non-significant pairwise p-values
Strawberry	Visual Symptoms and Infection Mean	3	5.34E-06	1.15a (0.718, 1.58)	2.30bc (1.868, 2.73)	1.95ab (1.518, 2.38)	2.90c (2.468, 3.33)	<.0001-0.0148	0.0527-0.6614
Privet	Visual Mean	2	0.02739	1.1a (0.645, 1.55)	1.6ab (1.145, 2.05)	1.2ab (0.745, 1.65)	2.0b (1.545, 2.45)	0.0341	0.0723-0.9894
Privet	Visual Mean	3	0.002122	1.30a (0.918, 1.68)	2.10b (1.718, 2.48)	1.95ab (1.568, 2.33)	2.35b (1.968, 2.73)	0.0015-0.0222	0.0865-0.9445
Privet	Visual Mean	10	0.02122	2.50a (1.87, 3.13)	3.70b (3.07, 4.33)	3.35ab (2.72, 3.98)	3.80b (3.17, 4.43)	0.0258-0.0455	0.2386-0.9960
Privet	Visual Longitudinal Analysis Mean	1	0.035463 (time interaction)	0.793a (-3.361, 4.95)	1.243ab (-2.911, 5.40)	1.280ab (-2.874, 5.43)	1.600b (-2.554, 5.75)	0.0227	0.2901-0.9991
Privet	Visual Longitudinal Analysis Mean	2	0.035463 (time interaction)	0.992a (-3.050, 5.03)	1.554ab (-2.489, 5.60)	1.553ab (-2.489, 5.60)	1.855b (-2.188, 5.90)	0.0059	0.1255-1
Privet	Visual Longitudinal Analysis Mean	3	0.035463 (time interaction)	1.191a (-2.768, 5.15)	1.865b (-2.095, 5.82)	1.826b (-2.133, 5.79)	2.110b (-1.849, 6.07)	0.0013-0.0422	0.6212-0.9984
Privet	Visual Longitudinal Analysis Mean	10	0.035463 (time interaction)	2.584a (-1.643, 6.81)	4.041b (-0.186, 8.27)	3.738b (-0.489, 7.97)	3.898b (-0.329, 8.12)	<.0001-0.0009	0.7160-0.9588
Privet	Visual Longitudinal Analysis **Trend**	1-10	0.035463 (time interaction)	0.199a (0.145, 0.253)	0.311b (0.257, 0.365)	0.273ab (0.219, 0.327)	0.255ab (0.201, 0.309)	0.0218	0.2277-0.9686
Privet	SPAD Mean	3	0.007634	62.1a (57.4, 66.8)	53.1b (48.5, 57.8)	54.1ab (49.2, 59.0)	50.6b (45.8, 55.5)	0.0067-0.043	0.0948-0.9916
Privet	SPAD Longitudinal Analysis Mean	1	0.04223 (treatment)	64.4a (24.54, 104.3)	58.8ab (18.757, 98.9)	58.5ab (18.459, 98.6)	56.0b (15.883, 96.2)	0.0416	0.2322-0.9997

NB. All Type-III ANOVA

Appendix B: Chapter 5 'Detection' supplementary data tables

Table SD1. ANOVA p-values

Variable	Term	Long Term	Short Term Watered	Pre-Drought	Drought	Recovery
Stomatal Conductance	(Intercept)	<2.2e-16	<2.2e-16	<2.2e-16	<2e-16	<2.2e-16
Stomatal Conductance	drought	-	-	0.7702	0.02516	<2.2e-16
Stomatal Conductance	infection	1.99e-04	0.369429	0.9974	0.59393	0.7129
Stomatal Conductance	time	1.06e-05	1.046e-08	1.66e-05	<2e-16	7.34e-14
Stomatal Conductance	drought-infection	-	-	0.2481	0.64413	0.2041
Stomatal Conductance	drought-time	-	-	0.9629	<2e-16	8.68e-12
Stomatal Conductance	infection-time	0.0369255	2.71e-03	0.9252	0.80912	0.2856
Stomatal Conductance	drought-infection-time	-	-	0.1825	0.62167	0.3872
Visual	drought	-	-	0.63788	0.025494	8.69e-06
Visual	infection	0.06822	0.001276	0.39949	6.38e-04	0.2078
Visual	time	<2e-16	5.16e-03	7.437e-09	1.291e-06	2.42e-05
Visual	drought-infection	-	-	0.38953	0.1864	0.1465
Visual	drought-time	-	-	0.28234	3.351e-05	8.55e-05
Visual	infection-time	0.02765	0.018617	0.02478	3.31e-03	0.387
Visual	drought-infection-time	-	-	0.04518	0.03906	0.6002
Visual	replicate	0.09952	-	-	-	-
Visual	block	-	0.51266	0.74259	0.822756	0.4365
Visual	replicate-block	0.45047	-	-	-	-
Extension Growth	(Intercept)	2.814e-09	-	-	-	-
Extension Growth	infection	0.621	-	-	-	-
Extension Growth	time	<2.2e-16	-	-	-	-
Extension Growth	infection-time	7.58e-06	-	-	-	-
Root:Shoot Ratio	(Intercept)	-	8.4e-13	-	-	-
Root:Shoot Ratio	drought	-	3.52e-04	-	-	-
Root:Shoot Ratio	infection	-	0.1455537	-	-	-
Root:Shoot Ratio	drought-infection	-	0.5234218	-	-	-
Mortality	infection	0.02834	-	-	-	-
Chlorophyll Content	(Intercept)	<2.2e-16	<2.2e-16	<2e-16	<2e-16	<2.2e-16
Chlorophyll Content	drought	-	-	0.25156	0.43188	0.10842
Chlorophyll Content	infection	0.01525	0.1085	0.0349	0.80342	0.75612
Chlorophyll Content	time	<2e-16	3.65e-11	1.06e-03	0.65492	6.38e-10
Chlorophyll Content	drought-infection	-	-	0.78647	0.04391	0.04816

Chlorophyll Content	drought-time	-	-	0.12836	2.124e-09	5.78e-06
Chlorophyll Content	infection-time	0.17325	5.236e-10	0.07435	0.20944	0.73558
Chlorophyll Content	drought-infection-time	-	-	0.09187	0.3848	0.0671
Area	(Intercept)	<2.2e-16	<2.2e-16	<2e-16	<2e-16	<2.2e-16
Area	drought	-	-	0.8922	0.03314	1.27e-04
Area	infection	0.2979435	0.9505	0.9505	0.5356	0.251074
Area	time	5.164e-07	2.85e-05	0.7242	2.209e-05	0.015237
Area	drought-infection	-	-	0.9636	0.56005	0.124986
Area	drought-time	-	-	0.3676	<2e-16	<2.2e-16
Area	infection-time	3.96e-04	1.34e-06	0.4122	0.01415	0.490491
Area	drought-infection-time	-	-	0.6781	3.04e-03	0.0288
Fv/Fm	(Intercept)	<2.2e-16	<2.2e-16	<2.2e-16	<2e-16	<2.2e-16
Fv/Fm	drought	-	-	0.5115	0.01599	2.05e-04
Fv/Fm	infection	0.8722	0.7854	0.728	0.36499	0.648578
Fv/Fm	time	1.15e-06	<2.2e-16	1.47e-11	<2e-16	1.45e-03
Fv/Fm	drought-infection	-	-	0.2981	0.92229	0.247877
Fv/Fm	drought-time	-	-	0.1622	<2e-16	2.75e-16
Fv/Fm	infection-time	0.0512	9.38e-06	0.2292	0.17946	6.71e-03
Fv/Fm	drought-infection-time	-	-	0.137	0.01897	0.081645
PI abs	(Intercept)	3.583e-13	<2.2e-16	<2e-16	<2e-16	<2.2e-16
PI abs	drought	-	-	0.94576	0.1590626	0.09547
PI abs	infection	4.42e-05	0.7742	0.99353	0.8176472	0.68127
PI abs	time	3.208e-14	<2.2e-16	<2e-16	2.96e-04	0.11874
PI abs	drought-infection	-	-	0.6924	0.1751991	0.18424
PI abs	drought-time	-	-	0.68625	7.379e-09	5.66e-08
PI abs	infection-time	5.28e-03	9.41e-07	0.58238	0.0181237	0.59302
PI abs	drought-infection-time	-	-	0.04222	0.0250289	0.51118
Vo(Bo)	(Intercept)	0.5458657	<2e-16	<2e-16	<2.2e-16	<2.2e-16
Vo(Bo)	drought	-	-	0.5681	0.08672	9.85e-03
Vo(Bo)	infection	1.52e-04	0.521	0.7743	0.9479	0.71229
Vo(Bo)	time	0.2927251	<2e-16	<2e-16	2.91e-14	1.21e-05
Vo(Bo)	drought-infection	-	-	0.7356	0.5403	0.766755
Vo(Bo)	drought-time	-	-	0.7212	<2.2e-16	2.94e-03
Vo(Bo)	infection-time	2.40e-04	0.559	0.8778	0.47507	0.033973
Vo(Bo)	drought-infection-time	-	-	0.4891	0.42678	0.14849

Table SD2. LTE and STE watered pairwise comparisons results

Variable	Dataset	Metric	Period of significant difference	Significant day p-values	Non-significant day p-values	time point	pairwise p-value	Uninfected value (CI)	Infected value (CI)	trend scale
Visual Index	LTE	Mean	-	-	-	-	-	-	-	-
Extension	LTE	Mean	No significant timepoints	-	0.3630-0.9893	Mean	0.8766	7.11a (-8.48, 22.7)	6.85a (-8.75, 22.5)	-
Stomatal Conductance	LTE	Mean	Day 34-175	0.0010-0.0266	0.6794-0.9971	Day 34	0.0010	122.1a (36.8, 405)	78.6b (23.3, 265)	-
						Day 175	0.0266	85.9a (31.3, 235)	69.3b (24.4, 197)	
Chlorophyll Content	LTE	Mean	Day 34-116	0.0271-0.0423	0.0545-0.3177	Day 34	0.0271	65.6a (4.793, 126)	59.1b (-1.738, 120)	-
						Day 116	0.0423	58.6a (-1.532, 119)	53.2b (-6.949, 113)	
Area	LTE	Mean	Day 144-308	0.0003-0.0223	0.1185-0.6602	Day 144	0.0223	39747a (13053, 121037)	30342b (9792, 94021)	-
Fv/Fm	LTE	Mean	Day 104-308	0.0009-0.0035	0.0836-0.5301	Day 104	0.0371	0.813a (0.657, 0.908)	0.795b (0.631, 0.898)	-
PI abs	LTE	Mean	Day 34-175	0.0007-0.0193	0.1062-0.6799	Day 34	0.0007	6.70a (-4.11, 17.5)	4.54b (-6.30, 15.4)	-
						Day 175	0.0193	4.87a (-5.64, 15.4)	3.69b (-6.87, 14.2)	
Vo(Bo)	LTE	Mean	Days 34-116 & 273-308	0.0017-0.0360	0.1660-0.8740	Day 34	0.0017	0.476a (0.333, 0.622)	0.514b (0.367, 0.658)	-
						Day 116	0.0249	0.489a (0.362, 0.619)	0.506b (0.377, 0.634)	
						Day 273	0.0360	0.516a (0.372, 0.657)	0.491b (0.339, 0.645)	
Visual Index	STE Watered	Mean	-	-	-	-	-	-	-	-
Stomatal Conductance	STE Watered	Mean	Day 41-79	0.0078-0.0410	0.0805-0.9733	Day 41	0.041	308a (257, 370)	241b (201, 289)	-
Chlorophyll Content	STE Watered	Mean	Day 36-79	0.0005-0.0420	0.1525-0.9067	Day 36	0.042	43.8a (41.2, 46.5)	41.5b (38.9, 44.2)	-
Area	STE Watered	Mean	Day 70-79	0.0307-0.0459	0.0580-0.9523	Day 70	0.0459	43814a (38063, 49565)	35654b (29903, 41405)	-
Fv/Fm	STE Watered	Mean	Day 78-79	0.0462-0.0483	0.0667-0.9842	Day 78	0.0483	0.789a (0.772, 0.804)	0.765b (0.748, 0.782)	-
PI abs	STE Watered	Mean	*Near significance Day 78-79	0.0502-0.0520*	0.0672-0.7825	Day 79	0.0502	3.62a (2.477, 4.77)	2.05a (0.910, 3.20)	-
Vo(Bo)	STE Watered	Mean	No significant timepoints	-	0.5414-0.8811	Mean	0.7154	0.440a (0.423, 0.457)	0.444a (0.427, 0.461)	-
Visual Index	LTE	Trend/Slope	-	-	-	-	-	-	comparatively increased symptoms of ill health & subsequent mortality, via wilting and defoliation	-
Extension	LTE	Trend/Slope	-	-	-	-	0.002	+0.01365a (0.01112, 0.0162)	+0.00747b (0.00449, 0.0104)	arithmatic
Stomatal Conductance	LTE	Trend/Slope	-	-	-	-	0.0374	-0.00250a (-0.00349, -0.001507)	-0.00089b (-0.00203, 0.000252)	log
Chlorophyll Content	LTE	Trend/Slope	-	-	-	-	0.1673	-0.0857a (-0.0977, -0.0737)	-0.0729a (-0.0869, -0.0588)	arithmatic
Area	LTE	Trend/Slope	-	-	-	-	0.0004	-0.000647a (-0.00177, 0.000476)	-0.003744b (-0.00504, -0.002445)	log
Fv/Fm	LTE	Trend/Slope	-	-	-	-	0.0516	-0.000696a (-0.00131, -7.99e-05)	-0.001624a (-0.00233, -9.20e-04)	logit
PI abs	LTE	Trend/Slope	-	-	-	-	0.0054	-0.01295a (-0.01614, -0.00976)	-0.00599b (-0.00971, -0.00227)	arithmatic
Vo(Bo)	LTE	Trend/Slope	-	-	-	-	0.0003	+0.000678a (0.000308, 1.05e-03)	-0.000375b (-0.000800, 4.95e-05)	logit
Visual Index	STE Watered	Trend/Slope	-	-	-	-	-	-	comparatively visually healthier at start of the experiment, higher probability of exhibiting symptoms of ill health over time	-
Stomatal Conductance	STE Watered	Trend/Slope	-	-	-	-	0.003	-0.00422 (-0.00854, 9.89e-05)	-0.01351b (-0.01783, -9.19e-03)	log
Chlorophyll Content	STE Watered	Trend/Slope	-	-	-	-	<.0001	-0.00379a (-0.0297, 0.0221)	-0.11978b (-0.1457, -0.0939)	arithmatic
Area	STE Watered	Trend/Slope	-	-	-	-	<.0001	+7.61 (-24.9, 40.2)	-105.81b (-138.3, -73.3)	arithmatic
Fv/Fm	STE Watered	Trend/Slope	-	-	-	-	<.0001	-0.00252a (-0.00313, -0.00192)	-0.00447b (-0.00508, -0.00386)	logit
PI abs	STE Watered	Trend/Slope	-	-	-	-	<.0001	-0.0159a (-0.0208, -0.0110)	-0.0333b (-0.0382, -0.0284)	arithmatic
Vo(Bo)	STE Watered	Trend/Slope	-	-	-	-	0.559	+ 0.00317a (0.00241, 0.00392)	+0.00285a (0.00210, 0.00360)	logit

Table SD3. STE trends pairwise comparisons results

Variable	Stage	Metric	significant pairwise p-values [test subset]	Non-significant pairwise p-values	Value of Mean/Trend by day [test subset] (CI)				
					UW	IW	ID	UD	trend scale
Relative Chlorophyll Content	Prior to Drought	Trend/Slope	-	0.0611-0.9999	+0.0348a (-0.0434, +0.1130)	-0.1059a (-0.1841, -0.0277)	-0.0992a (-0.1828, -0.0156)	-0.0952a (-0.1734, -0.0170)	arithmatic
PI abs	Prior to Drought	Trend/Slope	-	0.2475-0.9996	-0.0348a (-0.0568, -0.0127)	-0.0643a (-0.0863, -0.0422)	-0.0364a (-0.0600, -0.0129)	-0.0534a (-0.0754, -0.0313)	arithmatic
Stomatal Conductance	Drought	Trend/Slope	<.0001	0.9519-0.9981	+0.00519a (-0.0239, 0.0342)	-0.00578a (-0.0348, +0.0233)	-0.15110b (-0.1821, -0.1200)	-0.15486b (-0.1839, -0.1258)	log
Relative Chlorophyll Content	Drought	Trend/Slope	<.0001-0.0058	0.4209-0.9932	-0.0961a (-0.1821, -0.0101)	-0.1908a (-0.2768, -0.1047)	+0.1149b (+0.0229, +0.2068)	+0.1321b (+0.0461, +0.2181)	arithmatic
Vo(Bo)	Drought	Trend/Slope	<.0001	0.7212-0.9999	+0.000951a (-0.000922, +0.00282)	+0.000873a (-0.000999, +0.00274)	-0.007575b (-0.009582, -0.00557)	-0.009043b (-0.010920, -0.00717)	logit
PI abs	Drought	Trend/Slope	<.0001-0.0051	0.0662-0.9998	+0.02065a (0.00889, 0.0324)	-0.00745b (-0.01919, 0.0043)	-0.02906b (-0.04164, -0.0165)	-0.02832b (-0.04008, -0.0166)	arithmatic
Fv/Fm	Drought	Trend/Slope	<.0001-0.0399	0.8979	+0.00140a (-0.000296, +0.00310)	-0.00185b (-0.003546, -0.00015)	-0.00828c (-0.010091, -0.00646)	-0.00916c (-0.010858, -0.00746)	logit
Area	Drought	Trend/Slope	<.0001-0.0006	0.9847	+306.8a (+223.69, +390)	+73.2b (-9.85, +156)	-361.9c (-450.77, -273)	-383.9c (-467.05, -301)	arithmatic
Stomatal Conductance	Recovery	Trend/Slope	<.0001-0.0010	0.5395-0.9989	+0.0120a (-0.0429, +0.0668)	+0.0063a (-0.0486, +0.0611)	+0.1726b (+0.1139, +0.2312)	+0.2265b (+0.1716, +0.2813)	log
Relative Chlorophyll Content	Recovery	Trend/Slope	<.0001-0.0144	0.2389-0.7271	+0.0175a (-0.112, +0.14692)	-0.1277ab (-0.257, +0.00169)	-0.3088bc (-0.447, -0.17040)	-0.4088c (-0.538, -0.27934)	arithmatic
Area	Recovery	Trend/Slope	<.0001-0.0071	0.0975-0.7539	+7.62a (-60.7, +75.9)	-105.81ab (-174.1, -37.5)	-156.40b (-229.4, -83.4)	-214.19b (-282.5, -145.9)	arithmatic
Vo(Bo)	Recovery	Trend/Slope	0.003	-	All Continously Watered -0.00183a (-0.0054, +0.00173)		All Previously Droughted -0.00963b (-0.0133, -0.00593)		logit
			0.0342	-	All Uninfected -0.00295a (-0.00653, 0.000623)		All Infected -0.00851b (-0.01220, -0.004815)		
Fv/Fm	Recovery	Trend/Slope	<.0001	-	All Continously Watered -0.00576a (-0.00890, -0.00262)		All Previously Droughted +0.01310b (+0.00985, +0.01635)		logit
			0.0068	-	All Uninfected +0.006794a (+0.00365, +0.00994)		All Infected +0.000546b (-0.00271, +0.00380)		
PI abs	Recovery	Trend/Slope	0.0001-0.0038	0.8265-0.9998	-0.0178a (-0.0433, +0.00781)	-0.0336a (-0.0592, -0.00801)	+0.0472b (+0.0199, +0.07454)	+0.0456b (+0.0199, +0.07120)	arithmatic

Table SD4. STE means pairwise comparisons results

Variable	Stage	Metric	Period of significance difference in days (stage)	Significant p-values, all timepoints, all comparisons	Non-significant p-values, all timepoints, all comparisons	time point in days (stage)	significant pairwise p-values [test subset]	Non-significant pairwise p-values	Value of Mean/Trend by day [test subset] (CI)			
									UW	IW	ID	UD
Relative Chlorophyll Content	Prior to Drought	Mean	-	-	0.1303-0.9999	40 (40)	-	0.1303-0.9750	44.7a (41.7, 47.7)	41.3a (38.3, 44.3)	43.3a (40.1, 46.5)	40.6a (37.6, 43.6)
PI abs	Prior to Drought	Mean	-	-	0.1320-1.0000	40 (40)	-	0.1320-0.9997	3.77a (3.00, 4.53)	2.72a (1.95, 3.48)	3.73a (2.91, 4.55)	3.17a (2.40, 3.94)
Stomatal Conductance	Drought	Mean	51-64 (12-25)	<.0001-0.0072	0.4364-1.0000	51 (12)	0.0003-0.0119	0.4931-0.9997	331.7a (202.06, 544.5)	221.8a (135.14, 364.2)	80.9b (47.65, 137.2)	78.8b (48.01, 129.4)
Relative Chlorophyll Content	Drought	Mean	57-61 (18-22)	0.0048-0.0132	0.0710-1.0000	61 (22)	0.0048	0.0840-0.9913	43.0ab (39.4, 46.6)	38.1a (34.5, 41.7)	46.8b (42.9, 50.6)	43.6ab (40.0, 47.2)
						Mean	0.0185 [I]	0.4041 [U]	44.0a [U] (40.5, 47.4)	40.1a [I] (36.7, 43.6)	45.6b [I] (41.9, 49.3)	42.2a [U] (38.8, 45.7)
Vo(Bo)	Drought	Mean	61-65 (24-26)	0.0032-0.0471	0.0639-1.0000	61 (22)	0.0153-0.0423	0.0866-0.9937	0.442ab (0.422, 0.461)	0.447a (0.428, 0.467)	0.411bc (0.391, 0.432)	0.408c (0.389, 0.428)
						63 (24)	0.0032-0.0258	0.9682-0.9827	0.443a (0.422, 0.463)	0.448a (0.428, 0.468)	0.404b (0.383, 0.425)	0.400b (0.380, 0.419)
PI abs	Drought	Mean	-	-	0.1739-1.0000	65 (26)	-	0.1739-0.9706	4.37a (3.15, 5.60)	2.80a (1.58, 4.03)	3.70a (2.39, 5.01)	3.13a (1.90, 4.35)
Fv/Fm	Drought	Mean	65 (26)	0.0455	0.0570-1.0000	65 (26)	0.0455	0.0570-0.9966	0.803a (0.786, 0.820)	0.782ab (0.763, 0.800)	0.773b (0.752, 0.792)	0.775ab (0.755, 0.793)
Area	Drought	Mean	62-65 (23-26)	0.0138-0.0405	0.0573-1.0000	62 (23)	0.0405	0.0605-0.9996	46872a (40278, 53466)	38046ab (31453, 44640)	35830ab (28781, 42880)	35430b (28836, 42024)
						63 (24)	0.0284-0.0439	0.1335-0.9996	47179a (40570, 53787)	38120ab (31511, 44728)	35468b (28403, 42534)	35046b (28437, 41654)
Stomatal Conductance	Recovery	Mean	64-71 (0-7)	<.0001-0.0038	0.1737-0.9438	71 (7)	<.0001-0.0038	0.2204-0.9438	218.0a (146.47, 324.4)	135.4a (90.98, 201.5)	45.9b (29.98, 70.2)	52.6b (35.33, 78.3)
Relative Chlorophyll Content	Recovery	Mean	-	-	0.1485-1.0000	Mean	0.0334 [I]	0.4283 [U]	43.3a [U] (38.0, 48.6)	37.5a [I] (32.1, 42.8)	44.9b [I] (39.3, 50.6)	40.7a [U] (35.4, 46.0)
Area	Recovery	Mean	64-65 (0-1)	0.0049-0.0178	0.1238-0.9997	64 (1)	0.0086-0.0178	0.2560-0.9967	46060a (38545, 53576)	37481ab (29965, 44996)	30774b (22740, 38809)	29807b (22292, 37323)
Vo(Bo)	Recovery	Mean	64-79 (0-15)	<.0001-0.0178	-	79 (15)	<.0001	-	All Continously Watered 0.458a (0.441, 0.474)		All Previously Droughted 0.404b (0.387, 0.420)	
			78-79 (14-15)	0.0264-0.0302	0.1454-0.7162	79 (15)	0.0264	-	All Uninfected 0.442a (0.426, 0.459)		All Infected 0.418b (0.402, 0.435)	
Fv/Fm	Recovery	Mean	64-65 (0-1)	0.0014-0.0026	0.0615-0.6305	64 (1)	0.0026	-	All Continously Watered 0.779a (0.760, 0.798)		All Previously Droughted 0.740b (0.718, 0.761)	
			-	-	0.0675-0.6535	79 (15)	-	0.0675	All Uninfected 0.780a (0.761, 0.799)		All Infected 0.759a (0.737, 0.779)	
PI abs	Recovery	Mean	-	-	0.1702-0.9991	79 (15)	-	0.4272-0.9953	3.24a (1.939, 4.55)	2.03a (0.729, 3.34)	3.06a (1.660, 4.45)	2.52a (1.212, 3.82)
Root:Shoot Ratio	Recovery	Mean	-	-	-	Conclusion	0.0115-0.0301	0.2094-0.9410	0.724a (0.660, 0.779)	0.681ab (0.613, 0.742)	0.615b (0.539, 0.686)	0.634b (0.562, 0.700)