DOCTOR OF PHILOSOPHY

Identification, Ecology and Function of Armillaria species on oak trees in the Forest of Dean

Diggens, Mallory

Award date:
2020

Awarding institution:
Bangor University

Identification, Ecology and Function of *Armillaria* species on oak trees in the Forest of Dean

by

Mallory Diggens

PRIFYSGOL
BANGOR
UNIVERSITY

School of Natural Sciences
College of Environmental Sciences and Engineering

A thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Ecology.

Supervisors Prof. James McDonald, Dr. Sandra Denman *and* Dr. Mike Hale

21st August 2020

Declaration and Consent

Details of the Work

I hereby agree to deposit the following item in the digital repository maintained by Bangor University and/or in any other repository authorized for use by Bangor University.

Author Name: .....
Title: .....
Supervisor/Department: .....
Funding Body: .....
Qualification/Degree Obtained: .....

This item is a product of my own research endeavours and is covered by the agreement below in which the item is referred to as "the Work". It is identical in content to that deposited in the Library, subject to point 4 below.

Non-exclusive Rights

Rights granted to the digital repository through this agreement are entirely non-exclusive. I am free to publish the Work in its present version or future versions elsewhere.

I agree that Bangor University may electronically store, copy or translate the Work to any approved medium or format for the purpose of future preservation and accessibility. Bangor University is not under any obligation to reproduce or display the Work in the same formats or resolutions in which it was originally deposited.

Bangor University Digital Repository

I understand that work deposited in the digital repository will be accessible to a wide variety of people and institutions, including automated agents and search engines via the World Wide Web.

I understand that once the Work is deposited, the item and its metadata may be incorporated into public access catalogues or services, national databases of electronic theses and dissertations such as the British Library's EThOS or any service provided by the National Library of Wales.

Statement 1

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless as agreed by the University for approved dual awards..

Signed: . . . . . (Mallory Diggens)
Date: . . . . . .

Statement 2

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

All other sources are acknowledged by footnotes and/or a bibliography.

Signed: . . . . . (Mallory Diggens)
Date: . . . . . .

Statement 3

I hereby give consent for my thesis, if accepted, to be available for photocopying, for inter-library loan and for electronic repositories, and for the title and summary to be made available to outside organisations.

Signed: . . . . . (Mallory Diggens)
Date: . . . . . .
NB: Candidates on whose behalf a bar on access has been approved by the Academic Registry should use the following version of **Statement 3:**

Statement 3 (Bar)

I hereby give consent for my thesis, if accepted, to be available for photocopying, for inter-library loans and for electronic repositories after expiry of a bar on access.

Signed: . . . . . (Mallory Diggens)
Date: . . . . . .

Statement 4

Choose **one** of the following options

I agree to deposit an electronic copy of my thesis (the Work) in the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University and where necessary have gained the required permissions for the use of third party material.	
I agree to deposit an electronic copy of my thesis (the Work) in the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University when the approved **bar on access** has been lifted.	
I agree to submit my thesis (the Work) electronically via Bangor University's e-submission system, however I **opt-out** of the electronic deposit to the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University, due to lack of permissions for use of third party material.	

Options B should only be used if a bar on access has been approved by the University.

In addition to the above, I also agree to the following:

1. That I am the author or have the authority of the author(s) to make this agreement and do hereby give Bangor University the right to make available the Work in the way described above.
2. That the electronic copy of the Work deposited in the digital repository and covered by this agreement, is identical in content to the paper copy of the Work deposited in the Bangor University Library, subject to point 4 below.
3. That I have exercised reasonable care to ensure that the Work is original and, to the best of my knowledge, does not breach any laws – including those relating to defamation, libel and copyright.
4. That I have, in instances where the intellectual property of other authors or copyright holders is included in the Work, and where appropriate, gained explicit permission for the inclusion of that material in the Work, and in the electronic form of the Work as accessed through the open access digital repository, or that I have identified and removed that material for which adequate and appropriate permission has not been obtained and which will be inaccessible via the digital repository.
5. That Bangor University does not hold any obligation to take legal action on behalf of the Depositor, or other rights holders, in the event of a breach of intellectual property rights, or any other right, in the material deposited.
6. That I will indemnify and keep indemnified Bangor University and the National Library of Wales from and against any loss, liability, claim or damage, including without limitation any related legal fees and court costs (on a full indemnity bases), related to any breach by myself of any term of this agreement.

Signed: . . . . . **Date:** . . . . . .

Acknowledgements

I would like to thank my supervisors James McDonald, Sandra Denman and Mike Hale for there continued support throughout the project, and to my supervisory committee Gary Carvalho as my chair and Andy Smith as my internal examiner for all your feedback and guidance.

This work would not of been possible with out the tireless work and fundraising done at Woodland Heritage that ultimately funded this project. Special thanks to the late Peter Goodwin for his dedication to Woodland heritage. Also to Forest Research that provided me with my first introduction into the work of mycology and forest ecology.

I would also like to thank everyone in the MPEG laboratory group who had to deal with me and all my samples hogging the incubators on a daily basis. Much of this work required in house training from James Doonan, the PhD would not of happened if it was not for his continued support and the vast amount of patience required to teach me code. A thanks goes to Beth, Deborah and Bashaer for their contributions to this PhD during their own projects, your help was greatly received and good luck for the future.

A big thanks goes to my family giving all the support through those years, and especially Connor, for keeping me relatively human and my moral high throughout this time, we started PhD together and now we are submitting together.

'action, not words' - Peter Goodwin

Abstract

Armillaria is a genus of filamentous fungi within the phylum Basidiomycota, and comprises 40 species. Species are found globally in both temperate and tropical regions, with some species having a limited geographical range whist others are more widespread. As as soil-borne fungus, *Armillaria* spp. can act as saprophytes/parasites or pathogens, depending on host species and substrate availability. As a phytopathogen, *Armillaria* is a facultative necrotroph, causing necrosis of the living tree tissue of a variety of host species. Tree declines arise from a combination of biotic and abiotic factors. In oak, *Armillaria* species are one of those biotic factors, acting either as primary or secondary pathogens. The events that lead to decline act sequentially, or in parallel, and are often unknown, and *Armillaria* infection is assumed to occur after a primary pathogen or predisposing factor. However, the function of *Armillaria* species within oak declines is poorly understood, and in particular, it's activity as a saprophyte or pathogen of oak has barely been studied.

In this work, a combination of isolation studies, molecular identification, rapid diagnostic development, field-based analyses on the ecology and interactions of *Armillaria* with oak, and contemporary metatranscriptome sequencing was applied to characterise the role of *Armillaria* spp. in oak declines. First, methodological approaches were optimised for the rapid isolation and growth of *Armillaria* spp., and subsequent DNA extraction, inhibitor removal and PCR-based analysis of *Armillaria* phylogenetic marker genes. These approaches formed the basis for the development and validation of a High Resolution Melt curve (HRM)-based rapid diagnostic for *Armillaria* spp.

Subsequently, these approaches were applied in an ecological study of the interactions of *Armillaria* spp. with native oak in Chestnuts Wood, Forest of Dean, where *Armillaria* spp. are having a significant impact on tree health. *A. gallica* was the only species isolated from the study site and was observed as both a pathogen and saprophyte of oak trees, colonising both asymptomatic and symptomatic oak trees. Symptomatic trees were shown to have less buttress roots than asymptomatic trees, which is a potential predisposing factor to disease that could represent a quick tool to identify at risk trees. Soils within 1 m of symptomatic tree were shown to have significantly greater moisture content, suggesting that *Armillaria*

infection induced root malfunction and reduced the trees ability for water uptake.

RNAseq analysis identified significantly up-regulated *Armillaria* genes associated with fungal pathogenesis in symptomatic tree lesions; CP protein, serpin, and Ubiquitin (although one ubiquitin homologue was down-regulated). This work has highlighted that *Armillaria* acts as a pathogen on trees at this site, and the severity of infection varies. Manion (1981) described the decline disease model, where the cumulative effect of biotic and abiotic factors causes a decline spiral that amplifies disease. The data from this thesis support this model, highlighting a continuum of tree health status from asymptomatic to symptomatic. The presence of *A. gallica* on asymptomatic trees also indicates that it is broadly distributed and associated with oak at this site, as both saprophytes and pathogens. It is unclear whether there are separate saprophytic and pathogenic *Armillaria* strains present on the site, or if the switch to pathogenesis is triggered by abiotic and biotic cues.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

The genus *Armillaria* is comprised of 40 species, including biological species and morphological species (Watling et al., 1991; Metaliaj et al., 2006; Baumgartner et al., 2011). Representatives of this genus can be found all over the world, in both temperate and tropical regions, with some species having a limited geographical range whist others are more widespread (Watling et al., 1991; Pérez-Sierra et al., 1999; Coetzee et al., 2001; Sicoli et al., 2003; Baumgartner et al., 2011; Mulholland et al., 2012; Haavik et al., 2015). All species of *Armillaria* have the ability to act as a saprophyte on dead tree tissue to obtain nutrients but some species are phytopathogens.

As a phytopathogen, *Armillaria* is a soil-borne, and facultative necrotrophic plant pathogen, causing necrosis of the living tree tissue of a variety of host species. *Armillaria* species have been associated with past and present oak declines in Europe (Guillauim et al., 1985; Marçais and Caël, 2006). However, it is difficult to assess the role of *Armillaria* within those oak declines, as this is confounded by species variations in host specificity and virulence. In Britain four *Armillaria* species are associated with disease in oak: *A. mellea*, *A. gallica*, *A. ostoyae*, and *A. tabescens*. The influence of *Armillaria* species on the tree root system and the potential contribution of these species to other tree declines is not fully understood, although tree root health has been highlighed as an significant factor within tree declines (Denman et al., 2014).

Root rot pathogens such as *Armillaria* are thought to represent some of the most destructive within the forest environment, and are a major influencing factor in spatial and temporal diversity within forest ecosystems (Garbelotto, 2004; Oliva et al., 2012). Soil-borne pathogenic *Armillaria* cause root rot disease and the decay of tree tissues predominately root and cambial tissues) , potentially resulting in tree mortaility; tree mortality can range from 3% to 50% in monoculture plantations, having significant economic and ecological effects (Baumgartner et al., 2011; Collins et al., 2013; Guillaumin et al., 1991; Guillaumin et al., 1993; Hock, 2006;

Metaliaj et al., 2006; Morrison, 2004; Robene-Soustrade et al., 1992; Scholthof, 2007). In developing countries, the loss of agronomic crops through severe *Armillaria* infection can threaten food security (Hock, 2006). *Armillaria* infection is often described as 'shoestring root rot', due to the appearance of the rhizomorphs (a root-like thread of fungal hyphae, often black in colour) within the soil, and 'honey fungus' that refers to the appearance of the mushrooms (Cooley, 1946; Garbelotto, 2004). *Armillaria* are long-living basidiomycete fungal species, with three main structures important for their lifestyle. The mycelium is a branching network of hyphae found in soils and white fans found underneath the bark of infected trees (Figure 1.1) (Cooley, 1946; Esser, 2007; Garbelotto, 2004; Rizzo and Harrington, 1992). *Armillaria* species exhibit three contrasting morphological forms, which include; basidiocarps (the fruiting body), mycelium and rhizomorphs (Figure 1.1). The rhizomorphs of *Armillaria* are a complex and organised thread of mycelium with a high accumulation of hyphae, forming a dark cord-like structure, covered in a tough dark hydrophobic melanin outer layer, with specialised pores used for gas exchange (Garbelotto, 2004; Pareek et al 2006). The rhizomorphs display apical growth (Motta 1969) and grow through the soil to find the next food resource, ultimately spreading the *Armillaria* infection from tree to tree (Baumgartner et al., 2011). The fruiting bodies of this fungus are mushrooms, commonly found in the autumn at the edges of the mycelium extent and usually after a period of wet weather, its purpose is to release spores through the gilled hymenium on underside of the cap (Baumgartner et al., 2011; Garbelotto, 2004; Rishbeth, 1985).

Armillaria may attack both dead and living tree tissue. All species of *Armillaria* have the ability to act as a saprophyte on dead tree tissue to obtain nutrients, colonising dead wood, or colonising dead or dying trees (Baumgartner et al., 2011; Travadon et al., 2012). Once the *Armillaria* has colonised a tree, mycelial fans develop and can be found within the decaying wood, surviving for decades if sufficient nutrients are available. Some species within the *Armillaria* genus are pathogenic with various levels of virulence. In 1973, the Federation of British plant pathologists outlined definitions of pathogenicity and virulence; pathogenicity is described as the ability of a species or group of species to cause disease, and virulence refers to the degree of infection caused by a pathogenic species (Federation of British plant pathologists 1973; Gregory et al 1991). As a pathogen, *Armillaria* is a facultative necrotroph, firstly attacking the tree at the roots, followed by decay of the tree tissues (i.e. the stem) which appears white and stringy (Figure 1.1) (Baumgartner et al., 2011; Coetzee et al., 2001;

Figure 1.1 – *Armillaria* infection, a-c) Are oaks effect by *A. tabescens* on a site in Suffolk, a) Crown defoliation following *A. tabescens* infection. b) External bleeds indicating *Armillaria* mycelium underneath, c) Wood panel removed to expose mycelial fans within the cambial layer of the trunk. d-e) Macrophotography of oak panels taken infected with a unknown species of *Armillaria* in the Forest of Dean, d) Wood panel with rhizomorphs, the wood tissue red with tree defence compounds, e) Wood panel with rhizomorphs growing on the bark, f) *A. gallica* mushrooms growing on a stump at a site in Bangor, North Wales.

Garbelotto, 2004; Morrison, 2004; Oliva et al., 2014). *Armillaria* infection will spread to the living tissue within the cambial layer of the tree trunk, spreading laterally and radially while feeding off the nutrients (Aguileta et al., 2009; Baumgartner et al., 2011; Deacon, 2006; Prospero, 2003; Prospero et al., 2004). This negatively affects water and carbon consumption and economy within the tree, potentially leading to defoliation of the crown, reduced growth rates and tree death (Baumgartner et al., 2011; Oliva et al., 2014).

1.0.1 Tree declines

Forest declines or die-off events are a natural process within forest stands, with documented events reported globally (Millers et al., 1989; Manion and Lachance 1992; Tomiczek 1993). Such die-off events can affect one species or a group of closely related tree species (Sinclair 1965; Mueller-Dombois 1986; Manion 1991). Oak tree declines have been reported across Europe and globally over the 19th and 20th centuries (Marçais and Caël, 2006; Haavik et al., 2015). However, it has been suggested that the occurrence of decline effects are increasing with instances of mega disturbance set to increase, although this is difficult to determine due the advancement in diagnostic techniques and higher resolution of reporting during this time (Haavik et al., 2015; Millar and Stephenson, 2015).
The tree disease triangle, first described by McNew in the 1960s (McNew 1960), was created to improve the overall understanding of biotic and abiotic factors involved with epidemics, to aid the prediction of epidemics, and to control or limit the overall impact. The tree disease triangle (Figure 1.2) includes three main variables; 1) severity and influence of the environment, 2) pathogen virulence or inoculum potential, and 3) host susceptibility (Manion 1991; Scholthof, 2007).

Tree declines result from complex combinations of abiotic and biotic agents that potentially result in reduced growth, defoliation, degradation of the roots, and tree death (Manion, 1981; Thomas et al., 2002; Deman et al., 2014; Haavik et al., 2015; Marçais et al., 2011). Abiotic and biotic agents fit into three main categories of factors. Firstly, predisposing factors are usually long-term characteristics including overall age and genetics of the tree, forest history, site characteristics and environmental parameters (i.e. climate and soils characteristics) (Manion, 1991;Worrall et al., 2010; Haavik et al., 2015). Secondly, inciting factors that are characterised as events that cause stress; those events are usually short-term or recurring and often occur alongside other factors, such as drought and air pollution (Manion 1991). The overall effect of inciting factors is to compromise the tree health, leading to increased susceptibility to other abiotic or biotic factors. The tree has the potential for recovery from inciting factors can be reduced by the severity of predisposing factors (Worrall et al., 2010). Finally, contributing factors are usually secondary biotic agents that attack weakened trees and are not usually potent enough to kill the tree alone, although, in conjunction with severe inciting factors, the combination can be enough to kill a weakened tree (Figure 1.3) (Sinclair 1965; Manion

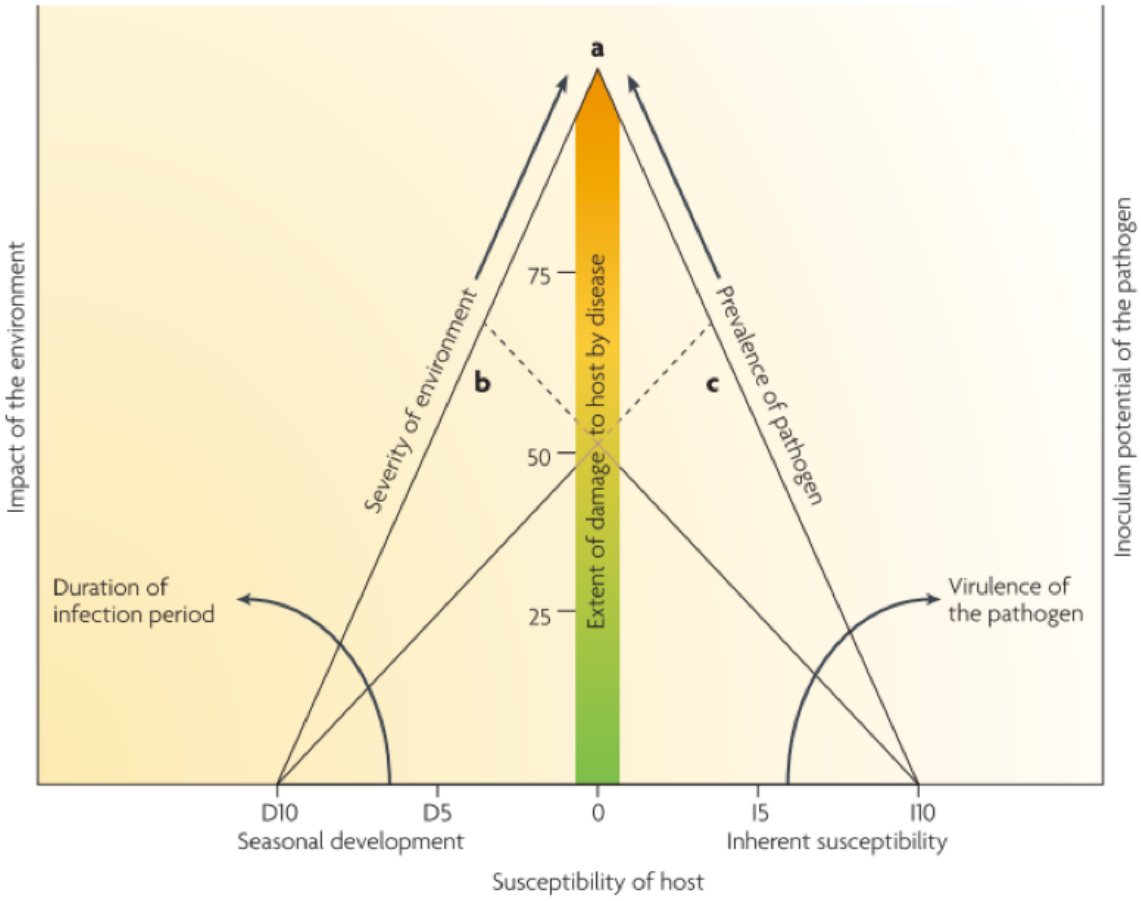


Figure 1.2 – The Tree disease triangle. The extent of damage to the host by disease increases with increased severity of the environment, prevalence and inoculum potential of the pathogen. This is influenced by the seasonal or inherent characteristics of the host at the time of infection, as well as the duration of the infection and virulence of the pathogen (Scholthof, 2007)

1981; 1991; Mueller-Dombois 1992; Haavik et al., 2015). In the literature, *Armillaria* spp. are usually thought of as a contributing factors within tree declines, however, this view may underestimate the role that some *Armillaria* species play within tree declines (Figure 1.3) (Haavik et al., 2015).

Armillaria species have been associated with past and present tree declines in Europe and consequently, the impetus to understand the role of *Armillaria* species in oak tree declines has grown in importance (Guillauim et al., 1985; Marçais and Caël, 2006). However, there is difficulty in assessing the role that *Armillaria* species have within those oak declines, due to variations between species and population host specificity, pathogenicity and virulence. In Britain, four *Armillaria* species are associated with disease in oak: *A. mellea*, *A. gallica*, *A. ostoyae*, and *A. tabescens*. The most commonly observed species within oak declines are *A. mellea* and *A. gallica*, with occasional reports of *A. tabescens* (Guillaumin and Legrand 2013).

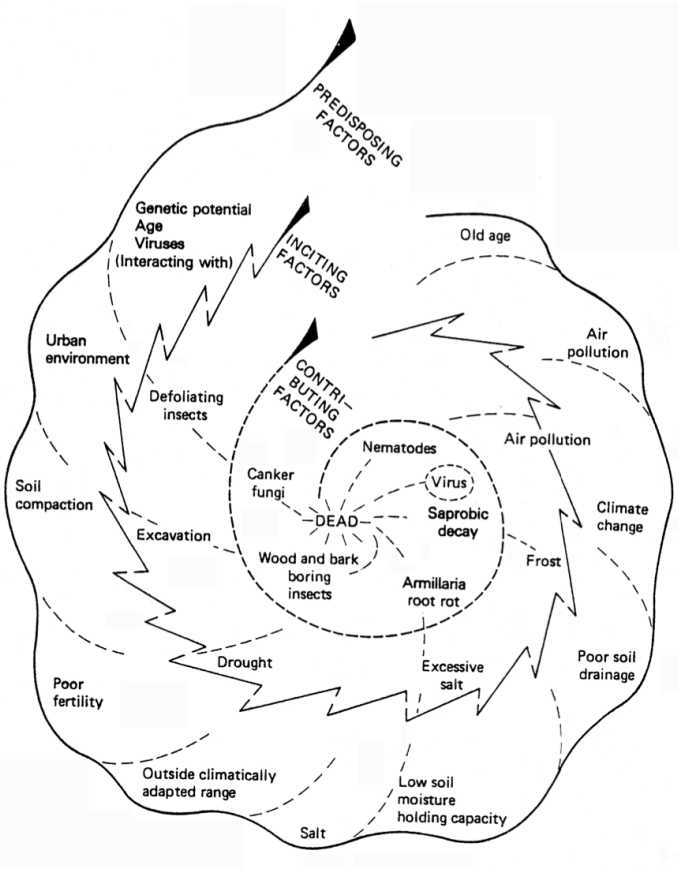


Figure 1.3 – Tree disease spiral concept, relating abiotic and biotic factors that contribute to the overall disease of a tree or stand (Manion 1991).

In addition, isolates of these species have been found on oak trees that are symptomatic of Acute Oak Decline (AOD) (Denman pers. comns.). AOD is a relatively new oak decline syndrome found on sessile oak (Quercus petraea (Matt.) Liebl.) and pedunculate oak (Quercus robur L.), cause rapid decline over 3 to 5 year period (Denman et al 2014). The symptoms associated with AOD are weeping patches on the oak stem, cracks between the bark plates, necrosis of the sapwood and inner bark, and finally larval galleries of the *Agrilus biguttatus* beetle (Denman et al 2014). Consequently, the influence of *Armillaria* species on the tree root system of AOD affected trees and the potential contribution of *Armillaria* infection to the overal syndrome of AOD is not fully understood. However, tree root health has been highlighed as an significant factor within tree declines (Denman et al., 2014).

1.1 Conclusions

In Britain there has been increasing focus on detecting new emerging tree diseases, although oak declines are not new tree disease in the UK. There are, however, variations of oak declines

detected within the wider concept of tree disease. The primary causal agents of tree disease are often difficult to distinguish, as the disease itself is a combination of abiotic and biotic factors (Denman et al., 2014; Thomas et al., 2002; Wargo and Carey, 2001). In Britain currently, there are two syndromes that are causing decline of oak, chronic oak decline COD and acute oak decline (AOD). AOD is an above ground disease causing rapid decline usually over 3-5 year period, bacteria have been found to be key species in the formation of necrotic tissue on the trunk and galleries formed by the larvae of the *Agrilus biguttatus* beetle have been found in close proximity to those necrotic legions (Denman et al., 2014). *Armillaria* species may have an influential role within AOD at the root level, causing disruption of the carbon and water economy within the tree. COD is a below ground syndrome, that is slower to progress, it has a strong connection with tree root health and *Armillaria* has been strongly associated with this syndrome. COD and AOD can occur simultaneously on the same tree (Denman et al., 2017). The occurrence of *Armillaria* infection within tree declines is thought to be on the increase, however, this increase is confounded by better reporting (Haavik et al., 2015). Increases in general tree declines are uncertain, although there have been some suggestions, such as climate change or a change in weather patterns or the introduction of new pests and pathogens (Haavik et al., 2015). The sequence of events that lead to decline are usually unknown, and *Armillaria* infection is assumed to occur after a primary pathogen or predisposing factor. The function of *Armillaria* species within those tree declines are predominately associated as a secondary pathogen (Chandelier et al., 2016; Denman et al., 2014; Guillaumin et al., 1993; Marçais and Caël, 2006; Ross-Davis et al., 2013; Wargo, 1996). This assumption of *Armillaria* as a secondary pathogen may underestimate the importance of some species, such as *A. gallica*. Current research into tree declines have found *A. gallica* and *A. ostoyae* co-existing on the same infected tree; there is little information of the purpose of the co-occurrence, or if this poses any benefits to either species in causing disease (Denman et al., 2017). This highlights gaps in our knowledge of how the relationship between the two *Armillaria* species, for instance what is the effect on growth and pathogenicity of the other, or can each species occupy the same area on the tree as one infection or remain separate infections. Current knowledge gaps regarding the role of *Armillaria* in tree health include the mechanism for how different *Armillaria* species change from saprophyte to pathogen (Baumgartner et al., 2011), the nature of the triggers that cause this change, or if the change is in response to nutritional availability i.e. cellulose or glucose, or environmental cues. For

those species that do seemingly undertake a shift from saprophyte to pathogen, transcriptome analysis may reveal how this change or response occurs, and which factors or cues can be used as predictors for this change.

1.2 Aims and Objectives of the PhD

The overall aims of the PhD is to access the identity, ecology and function of *Armillaria* species on oak species within Chestnut Woods, Forest of Dean. To address those aims a mixture of tradition culture methods will be used alongside the molecular tools, PCR and next generation sequencing.

1.3 Chapter 3 - Method optimisation to improve biomass production and DNA yield of *Armillaria* species

The first set of aims and objectives of the PhD are to firstly isolate and characterise textitArmillaria isolates from UK oak woodlands where COD and AOD is both present and absent. Isolates are to be obtained or collected, and identified to species level. The majority of the unknown *Armillaria* isolates were isolated from 3 sites across the UK, Treborth Gardens (Bangor), Bigwood (Suffolk), and Chestnut wood (Forest of Dean). The unknown isolates underwent DNA extraction and PCR for taxonomic marker genes, focusing on 3 loci; ITS, IGS and EF1-α (Mulholland et al., 2012). The sequences obtained from the PCR were sent for Sanger sequencing and phylogenetic analysis completed to provide species identification and taxonomic information.

1.4 Chapter 4 – Phylogenetic analysis of UK *Armillaria* species present on oaks, and development of a High Resolution Melt curve assay (HRM) – based rapid diagnostic tool

The objective for chapter 3 are to develop a rapid diagnostic tool for species-specific *Armillaria* detection, leading to rapid detection of *Armillaria* spp. across the UK, with the hope to provide additional ecological information and species distribution across the UK. This rapid diagnostic tool is a PCR based method, using species-specific primers for rapid diagnostics. Using this

PCR based method, tissue, wood and soils samples taken from an infected site to give an immediate indication to what species are present. This method speeds up the diagnostic procedure to potentially a few days, rather than weeks using traditional culture methods.

1.5 Chapter 5 – Identification and ecology of *Armillaria* spp. across Chestnut wood, Forest of Dean

This chapters aim is to understand the ecology and function of *Armillaria* species at Chestnuts Wood, Forest of Dean, where it it has been observed to play a major role in oak decline. It has been found in previous studies, that more than one species can be present on a site, even colonising the same tree (Denman et al., 2017; Prospero, 2003). A field study of a site with established pathogenic and saprophytic *Armillaria* infection was selected, with the aim to collect observational and environmental data from asymptomatic and symptomatic trees and to obtain *Armillaria* isolates from trees across the site. Soil samples were collected and analysed for soil chemistry, moisture and pH, to understand if any localised differences between the asymptomatic and symptomatic trees. The *Armillaria* isolates collected were grown in culture and DNA extracted for phylogenetic analysis, spatial analysis using GIS software was completed see the distribution of isolates across the site.

1.6 Chapter 6 - Meta transcriptomic analysis on oak tree lesions caused by *Armillaria* spp.

The aim of this chapter is to understand the role of *Armillaria* within oak declines and to characterise its interaction with oak trees via transcriptome analysis of host:*Armillaria* interactions. This may also provide information regarding the interactions with other pest or pathogenic species present, such as the bacterial species associated with AOD *Brenneria goodwinii* or *Gibbsiella quercinecans* (Denman et al., 2014). Previously *Armillaria* inoculation trials found little impact when inoculated alone, however, within a natural or planted forest, trees would almost never be exposed to one factor at the time. The oaks are more likely to face the combination of abiotic and biotic agents which cause predisposition to disease or overall decline (Manion 1981; 1991; Marçais and Caël, 2006; Haavik et al., 2015). Current work on *Armillaria* as pathogen uses *Armillaria* in culture, where it is in its saprophytic phase and therefore

much of the information could be missed regarding host:pathogen interactions. Therefore, an *in situ* transcriptome approach was used to collect the first datasets regarding *Armillaria* interactions within a live tree during infection in a forest stand environment. Combinations of pathogens and environmental parameters may be more indicative of a tree's natural response to infection and informative for disease predictions. Garrett (1960) recommended the use of living trees to complete inoculation trials to artificially reproduce the infection process within the laboratory. An inoculation study is not viable for this study, as it could take years or even decades to establish disease artificially, if possible at all. It would be difficult to reproduce the natural predisposition of the trees and local environmental factors leading to infection seen in woodland environments. Therefore, a site with a long history of *Armillaria* infection (Chestnuts Wood, Forest of Dean) was chosen as the study site. In order to gain information on the role of *Armillaria* during infection, oak tissue samples symptomatic of *Armillaria* were used as a source material for gene expression analysis using RNA extraction and analysis techniques. This will provide information on the genes used by *Armillaria* during colonisation and disease progression, as well as enabling understanding of the influence of other pathogenic species if present.

Chapter 2

Background of UK *Armillaria* species

2.1 *Armillaria* Taxonomy

2.1.1 Historical and cultural taxonomy of *Armillaria* species present on oaks in Britain

Armillaria mellea was first described as a fungal species in 1767 by Vahl, and was assigned the name *Agaricus melleus* (Baumgartner et al., 2011). In 1821, Fries published the Systema Mycologicum, wherein the *Armillaria* tribe was described, including 12 species described by Fries in 1819; *A. robustus, A. persoonii, A. guttatus, A. bulbiger, A. constrictus, A. subcavus, A. mucidus, A. vagans, A. griseo-fuscus, A. denigratus, A. rhagadiosus, A. mellus* (previously *Agaricus melleus*). By 1825 Fries had merged the genus *Armillaria* with *Lepiota*, but this was later revised in 1838 with some species being re-described within the *Armillaria* genus (Fries 1838; Watling 1991; Ellis 2007). In 1857, Staude upgraded *Armillaria* from tribe to validated genus (Burdsall and Volk, 1993; Staude 1857; Watling et al., 1988).

The nomenclature of *Armillaria* species is complex, with many species being re-described and re classified numerous times (Guillaumin et al., 1993). *Armillaria* species names have changed over time, with the advent of new techniques for greater resolution of taxonomic relationships and the description of new species (Table2.1). At the beginning of *Armillaria* taxonomy only one morphological observation, the mushroom, aided distinction between species; the annulate species have a veil on the stipe, whereas exannulate species have no veil present (Watling et al., 1991). Until the late 1970's, all annulate isolates were thought to be polymorphisms of *A. mellea*. However, the subsequent introduction of pairing tests in culture helped to identify that *A. mellea* is a complex made up of numerous species, now referred to as *A. mellea senso lato* (Guillaumin et al., 1993; Matsushita and Suzuki, 2005).

The use of pairing tests, such as mating incompatibility, for species identification help to determine *A. mellea* senso lato as a complex group of species, comprising of biological species and inter-sterile groups (Baumgartner et al., 2011; Coetzee et al., 2000). More recently, molecular techniques alongside basidiome morphology has been used to identify *A. mellea senso stricto* as a separate species within the *Armillaria* genus (Coetzee et al., 2000). Additionally, Anderson (et al 1989) characterised *A. mellea s. s.* by identifying a 4 KB insertion within the ribosomal RNA operon. Later, Miller et al (1994) used DNA-DNA hybridisation methods to demonstrate that *A. mellea s.s.* formed a homogeneous cluster separating them from the other species within the *Armillaria* genus (Coetzee et al., 2000). The most recent change is the addition of the subgenus *Desarmillaria*, this includes the exannulate armillarloid species previously named *A. tabescens* and *A. ectypa* (Table2.1) (Koch et al., 2017).

Table 2.1 – A list of synonymous species names for 4 species of *Armillaria*; *A. mellea*, *Desarmillaria. tabescens*, *A. gallica*, *A. ostoyae* (All data collected from MycoBank February 2019)

Modern nomenclature	**Synonymous nomenclature**	**Reference**
Armillaria mellea (Vahl) Kummer 1871	*Agaricus melleus*	Vahl 1790
	Armillariella mellea	Karsten 1881
	Omphalia mellea	Quélet 1886
	Agaricites melleus	Meschinelli 1891
	Clitocybe mellea	Rigken 1915
	Lepiota mellea	Lange 1915
Desarmillaria tabescens (Scop.) R. A. Koch & Aime 2017	*Armillaria tabescens*	Emel 1921
	Agaricus tabescens	Scopoli 1772
	Clitocybe tabescens	Bresadola 1900
	Collybia tabescens	Saccardo 1887
	Fungus tabescens	Kuntze 1898
	Lentinus caespitosus	Berkeley 1847
	Agaricus monadelphus	Morgan 1883
	Armillaria mellea var. exannulata	Peck 1893
	Armillaria mellea var. tabescens	Rea & Ramsbottom 1917
Armillaria gallica (Marxm) H. Marxmüller 1987	*Armillaria lutea*	Gillet 1878
	Armillaria mellea var. bulbosa	Barla 1887
	Armillaria inflata	Velenovský 1920
Armillaria ostoyae (Romagn.) Herink 1973	*Agaricus congregatus*	Bolton 1791
	Armillaria mellea var. obscura	Gillet 1878
	Armillaria solidipes	Peck 1900
	Armillariella ostoyae	Romagnesi 1970

2.1.2 The Morphological and Biological species concept in *Armillaria* spp

Prior to the 1980s, the morphological species concept was influential in the taxonomy of *Armillaria* (Baumgartner et al., 2011). This concept used specific characteristics to identify *Armillaria* spp.. This system uses a range of morphological characteristics include; structure of the mushroom, pileus, stipe, location of pigmentation, basidiospore morphology, the presence of clamp connections within the mycelium, the release of white spores, diploid mycelium that appear crustose ranging from black to red-brown in colour, and habitation in areas dominated by woody plants and trees (Baumgartner et al., 2011; Volk et al., 1996; Watling et al., 1991). The biological species concept emerged in the 1970s and utilises compatibility trails or molecular techniques to identify species. This approach was first used to identify species within the *A. mellea* senso lato complex by growing two isolates within the together, if there was fusing of the colonies they were deemed compatible, if not compatible a visible clearing would be present between the two isolates (Baumgartner et al., 2011). Researchers from Europe, identified species within Europe as a biological group named the European biological species (EBS) (Korhonen, 1978) and North America, species identified as the NABS North American biological species (Anderson and Ullrich, 1979; Baumgartner et al., 2011). However, over the past 20 years, the development of molecular techniques has revolutionised our ability to characterise microbial phylogeny and taxonomy, with PCR-based amplification and sequencing methods allowing analysis of *Armillaria* IGS, ITS and EF1-α gene regions for species-level resolution (Baumgartner et al., 2010; Maphosa et al., 2006; Matsushita and Suzuki, 2005; Mulholland et al., 2012; Pérez-Sierra et al., 1999; Volk et al., 1996)

2.2 *Armillaria* body forms and reproduction

2.2.1 Mycelial fans

The mycelia appear as white fans or cords, colonising dead wood and host trees by entering the cambial layer of the collar and root system first, causing localised necrotic legions visible on the outer bark and killing the living tissue of the tree, and are able to colonise up to 1.5

meters or more up the trunk of the tree, typically in an 'A-frame' shape, although this pattern is not always observed (Guillaumin and Legrand 2013; Garbelotto, 2004; Baumgartner et al., 2011). Within the wood tissue the mycelium excretes extracellular enzymes, obtaining the nutrients and water from the tree, effecting the tree by reduced growth of the tree and potential death. The gene expression profile of mycelial fans shows the most extensive arsenal of plant cell wall degrading enzymes (PCWDE) compared to other body forms of *Armillaria* species (Sipos et al., 2018).

Armillaria mycelium has been found growing vast distances within the forest floor, spreading the disease across forest stands, however, this is not generally the case for *Armillaria* (Travadon et al., 2012). Viable *Armillaria* mycelium can survive within colonised tree stumps and soils for decades, and can be used a inoculum source to cause disease in surrounding trees (Baumgartner et al., 2011). In culture the mycelium appearance can vary from white and fluffy to a darker crustose layer with the white fluffy mycelium only present on the outer edges. Haploid and diploid *Armillaria* forms can produce mycelium (Figure 2.1), however, the haploid only produces the fluffy white phenotype and is rarely found in wild conditions (Figure 2.1) (Baumgartner et al., 2011; Carvalho et al., 1995; Rizzo and Harrington, 1993, 1992).

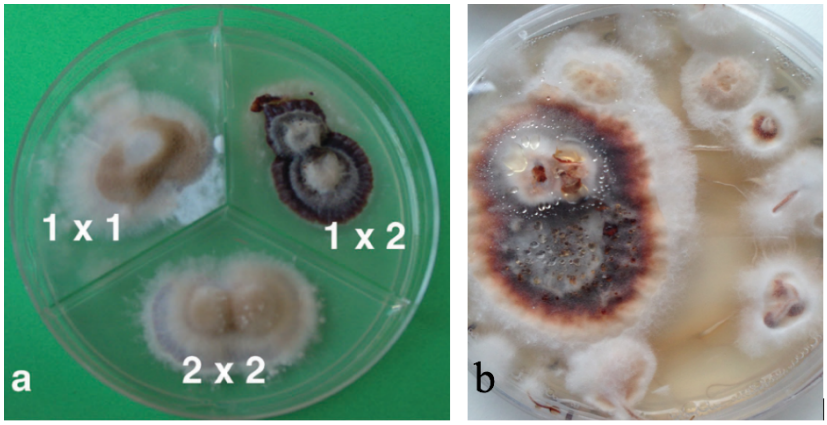


Figure 2.1 – *Armillaria* haploid and diploid morphologies (a) haploid mating strain pairings, same strains keeping white fluffy haploid morphology (1 x 1, 2 x 2), compatible mating of 1 x 2 shows formation of dark crustose diploid mycelium, taken from (Baumgartner et al., 2011). (b) Shows dark crustose mycelium of *Armillaria mellea* isolate W6

2.2.2 Production of rhizomorphs

Rhizomorphs produced by *Armillaria* species, in either the pathogenic or saprophytic phase, grow within the soil in search of host plants or organic matter within the soil, and are

used as primary mode of disease spread through direct contact and in the translocation of nutrients (Guillaumin et al., 1993; Rishbeth 1985; Aguileta et al., 2009; Sipos et al., 2017). Rhizomorphs are a rare feature associated with *Armillaria* species and are an organised concentrated cord of hyphae encased in a dark layer of melanised cells, thought to increase longevity within the soil and survival in harsh environmental conditions (Koch et al., 2017). These structures have the ability to grow approximately 1 metre per year, in multiple directions simultaneously, and can be up to 4 mm in diameter depending on species (Sipos et al., 2018). It is thought rhizomorphs are used to deliver the maximum inoculation potential either to invade a tree root system, colonising organic matter or attacking a new tree (Garbelotto, 2004; Sipos et al., 2017). The production and growth rate of rhizomorphs predominately depends of species; i.e *A. gallica* rhizomorphs grow larger and quicker than those produced by *A. ostoyae*, whilst *A. tabescens* rhizomorphs are rarely found outside the laboratory (Guillaumin et al., 1993; Wargo and Carey, 2001). The growth rate also depended on the original size of the *Armillaria* colony present; equally, growth rate is reduced with increased distance from the original nutrient resource, due to the consumption of nutrients and increased competition for resources (Garrett 1960). Some *Armillaria* species express a limited ability to grow rhizomorphs; for example, *A. gallica* produces vast amounts of rhizomorphs, in comparison *A. mellea* are found to produce smaller and far fewer rhizomorphs within the soil (Redfern and Filip 1991; Guillaumin et al., 1993; Aguileta et al., 2009; Travadon et al., 2012). There are also phenotypic variation in production, growth patterns and formation of the rhizomorphs in response to temperature or compounds within the soil, such as tannic acid; however, those differences may not be the same across all species or isolate (Rishbeth, 1986; Rizzo and Harrington, 1993). Sipos et al., (2017) found that rhizomorphs gene expression profiles indicate that of a transitional phase between vegetative mycelium and fruiting bodies, with the stipe being the most similar body form. Rhizomorphs show the capability for plant cell wall degradation and pathogenicity, such as expansins, cell wall proteins important for plant-pathogen interactions, used in loosening cell walls (Marowa et al., 2016), are more similar to vegetative mycelium. The genes involved with morphogenesis were more similar to mushroom body form than vegetative mycelium (Sipos et al., 2017). The rhizomorphs contain a wide range of genes involved in secondary metabolisms, defence, plant cell wall degradation, typical of soil borne organisms used for resource competition and defence against other microorganisms. Sipos et al., 2017 found six bacterial extracellular solute-binding

proteins (SBPs) were found expressed within the *Armillaria* with rhizomorphs having the highest expression levels compared to any other form, SBPs are required for ABC transporters in ion transport, essential survival within host organisms, indicating a pathogenic aspect to the production of rhizomorphs. The gene expression for the PCWDE repertoire of the rhizomorphs was moderate, although less extensive than vegetative mycelium, it does still however have the capacity to invade hosts or assimilative agent for finding food resources in soils and host plants (Sipos et al., 2017).

2.2.3 Fruiting bodies and spore dispersal

Armillaria species produce a yellow to brown basidiocarps (Siu et al., 2016). Reproduction is completed via releasing haploid sexual spores from those basidiocarps through the hymenium and via the gills or pores on the underside of the mushroom cap (Oliva et al., 2014; Rishbeth, 1985). Spore dispersal is less important in disease spread rhizomorph extension and mycelial growth, with rhizomorphs being the primary mode (Baumgartner et al., 2011; Cooley, 1946; Prospero, 2003; Travadon et al., 2012). The mushrooms release haploid basidiospores through the gills, on the underside of the cap (Garbelotto, 2004; Rishbeth, 1985). The fundamental aim of the spores are to colonise woody tissues, such as stumps or tree roots, exploiting those tissues for nutrients before further colonisation of stumps or trees via mycelium or rhizomorphs (Rishbeth, 1985). The spores use the wind for dispersal, to either spread the *Armillaria* infection further across a site, or to colonise new sites, and in pathogenic species is used for persistence (Baumgartner et al., 2011; Coetzee et al., 2018; Travadon et al., 2012). However, the importance of spores in *Armillaria* infection is unclear and the traditional concept is that spore dispersal is relative less important regarding disease spread compared to mycelium and rhizomorphs growth (Travadon et al., 2012). The distance in which spore dispersal is effective remains to be further quantified, however, many studies found the majority of the spores are found within a few meters of the basidiocarps, with very few spores traveling beyond 150 m (Travadon et al., 2012). The spatial distribution of diploid individuals within a site can be assessed by completing somatic incompatibility trials. These trials can give information regarding the dispersal patterns within a disease centre (Baumgartner et al., 2011). The disease centre in hardwood trees is usually colonised by one or several isolates or species of *Armillaria* (Travadon et al., 2012). Haploid mycelia are rare within the natural forest environment, and the methods for measuring the spatial distribution of spores, such

as spore trapping and following the extent of the disease spread from the disease centre, are limited by their detection levels and lack information regarding the whole landscape (Travadon et al., 2012). The germinated haploid spores are capable of undertaking plasmogamy with the parent isolate, where the parent isolate will replace the haploid nucleus with its own diploid nucleus. The benefit of this is a rapid increase in the amount of the parent diploid *Armillaria* and the ability to gain the nutrient source occupied by the haploid mycelium (Travadon et al., 2012)

2.2.4 Reproduction

For most *Armillaria* species, successful mating between haploid/haploid and haploid/diploid individuals is controlled by bifactorial heterothallic mating systems (Figure 2.2) (Anderson and Ullrich, 1979; 1982; Baumgartner et al., 2011). The process begins with basidia cells that contain a diploid nucleus, this nucleus splits into four haploid nuclei through meiosis and the haploid nuclei then migrate to separate basidiospores (Figure 2.2) (Baumgartner et al., 2011). The haploid spores can germinate without reproduction occuring and the resulting mycelium appears white and fluffy in culture consisting of uninucleate cells (Guillaumin et al., 1991).

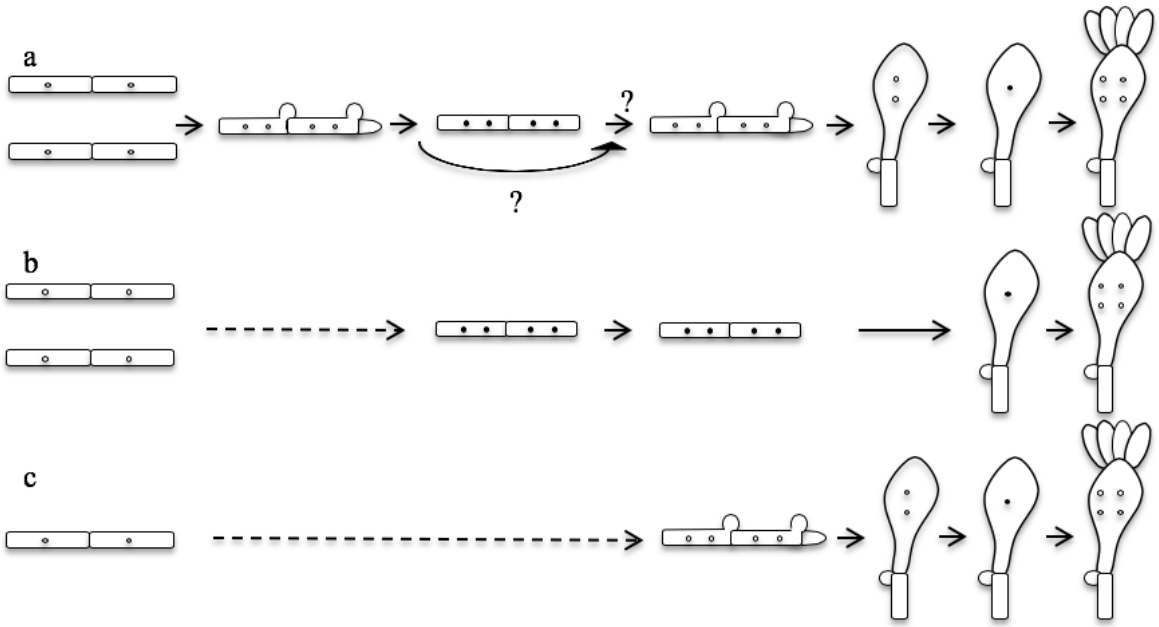


Figure 2.2 – Modes of reproduction within the *Armillaria* genus. a) Heterothallic reproduction system b) Heterothallic reproduction system with diploid stage c) Homothallic reproduction. Black filled circles represent diploid nuclei and white filled circles represent haploid nuclei (Modified from Guillaumin et al., 1991).

For heterothallic *Armillaria* species, mating can only occur with compatibile isolates, whereby mating is controlled by a bifactorial or tetrapolar mating system (Ullrich and Anderson 1978; Baumgartner et al., 2011). The mating between two haploid isolates occurs at the interaction region of the hyphae. For a short time after successful mating the nucleus is dikaryotic, containg both haploid nuclei, and the nucleus then diploidises to form one complete diploid nucleus (Figure 2.2). The bifactorial (or tetrapolar) mating systems uses two mating type alleles Ax and Bx within the haploid cells, therefore mating attempts can have 4 separate outcomes and 25% compatibiliy with siblings (Guillaumin et al., 1991). Firstly, compatible mating could occur between the two haploid cells, that have different mating-type alleles present i.e. A1B1 x A2B2 (Guillaumin et al., 1991; Raper, 1953; Ullrich and Anderson, 1978).

The isolates then grow and begin to merge into one colony, the phenotype will change from the fluffy white to a dark and crutose, usually one diploid isolate will become dominant (Guillaumin et al., 1991; Rishbeth, 1985; Ullrich and Anderson, 1978). Nuclear migration is indicated by the presence of partly disintegrated septa within the hyphae (Guillaumin et al., 1991). The second outcome is incompatibile mating between two haploid cells which have the same mating-type alleles A1B1 x A1B1, the colony will grow alongside each other without integrating, and no changes to the phenotype (Guillaumin et al., 1991). A hemi compatible outcome occurs when only one allele out of four is different. There are two forms of this: common-A (A1B1 x A1B2) and the common-B (A1B1 x A2B1). There are two potential outcomes; primarily there is no change in phenotype from the haploid fluffy white mycelium (Ullrich and Anderson, 1978); alternatively, there is the formation of a barrage zone, in pairings of common-A (A1B1 x A1B2) can show signs of nuclear migrations or in instances of common-B (A1B1 x A2B1) the phenotype changes to a crustose mycelium (Guillaumin et al., 1991).

The most frequent interaction between compatible diploid and haploid isolates is a displacement of the nucleus of the compatible haploid isolate with the diploid nucleus, with no genetic exchange (Carvalho et al., 1995; Guillaumin et al., 1991; Rizzo and Harrington, 1992). The haploid mycelium then changes to the dark and crustose phenotype of the diploid mycelium, further indicating the change from haploid to diploid (Baumgartner et al., 2011; Guillaumin et al., 1991). A form of mating can occur between the compatible diploid and haploid *Armillaria* species, however, the mechanisms for this are poorly understood (Guillaumin et al.,

1991). This form of mating probably involves nuclear fusion, loss and exchange of genetic information and potentially recombination (Baumgartner et al., 2011; Carvalho et al., 1995).

There are some populations of *A. mellea* that exhibit primary homothallic mating system; this system uses recombination of the chromosomes, occurring within the basidiocarps (Baumgartner et al., 2011; Guillaumin and Legrand 2013; Coetzee et al., 2018). This recombination reduces requirement of finding a compatible mate, within the homothallic mating system there is 100% compatibility between sibling basidiospores (Ota et al., 1998). This allows the haploid nucleus to self-fertilise immediately within the sibling basidia after meiosis, producing 100% fertile diploid basidiospores (Baumgartner et al., 2011; Ota et al., 1998).

Somatic incompatibility is the prevention of nuclei fusion within the mycelium of two isolates that are genetically distant from one another, this system is non-reproductive and separate from sexual incompatibility (Worrall, 1997). It can only be used by diploid or dikaryotic fungal species (Garbelotto, 2004). If two isolates from the same species meet whilst colonising a substrate, a chemical reaction prevents the growth of both isolates, forming a border between the two isolates that limits fungal growth (Garbelotto, 2004). There are two forms of somatic incompatibility; during intraspecific somatic incompatibility there is a space between the two isolates are occupied by faint sterile mycelium, and for interspecific somatic incompatibility (Abomo-Ndongo and Guillaumin, 1997). It is also thought that this somatic incompatibility plays a role in the protection from viruses and other pathogens, however, this role and its mechanisms are poorly understood (Garbelotto, 2004). For heterothallic *Armillaria* species, mating can only occur with compatible isolates, whereby mating is controlled by a bifactorial or tetrapolar mating system (Ullrich and Anderson 1978; Baumgartner et al., 2011). The mating between two haploid isolates occurs at the interaction region of the hyphae. For a short time after successful mating the nucleus is dikaryotic, containing both haploid nuclei, and the nucleus then diploidises to form one complete diploid nucleus (Figure 2.2). The bifactorial (or tetrapolar) mating systems uses two mating type alleles Ax and Bx within the haploid cells, therefore mating attempts can have 4 separate outcomes and 25% compatibility with siblings (Guillaumin et al., 1991). Firstly, compatible mating could occur between the two haploid cells, that have different mating-type alleles present i.e. A1B1 x A2B2 (Guillaumin et al., 1991; Raper, 1953; Ullrich and Anderson, 1978).

2.3 *Armillaria* spp. as pathogens

Saprotrophic fungal species are organisms that use dead organic matter to obtain nutrients. Those fungal species are essential in the forest environment for the breakdown of nutrients and are essential for carbon cycling (Baumgartner et al., 2011; Deacon, 2006; Travadon et al., 2012). Cellulose degradation is probably the most important process, as cellulose comprises approx. 40% of plant cell walls (Deacon, 2006). All *Armillaria* species have the ability to act as saprophytes, surviving within the soil for years using dead roots or organic matter for nutrition (Risbeth 1985 ;Baumgartner et al., 2011). *Armillaria* species that are predominantly pathogenic kill the host tree, and will then revert to, or begin to act as a saprophyte consuming the nutrients and carbon, and can survive as a saprophyte for long periods of time (Burdon and Silk, 1997; Travadon et al., 2012). The ability of *Armillaria* species to survive within dead trees, stumps and soil, can make the eradication or prevention of *Armillaria* root disease from a stand or woodland difficult (Baumgartner et al., 2011). It has been suggested for over a century that *Armillaria* species can cause disease, however, understanding the role of *Armillaria* can be difficult, as these fungi can be present within the same forest environment, with some areas affected by the disease with mass mortality, whilst other areas show no signs of infection (Gregory et al., 1991). The pathogenicity of *Armillaria* species is known to vary within species and between species, and is dependent on abiotic factors such as drought or defoliation. *Armillaria* colonise the living tissues of trees to obtain nutrients, particularly structural carbon such as cellulose and hemicellulose (Kües and Rühl, 2011; Oliva et al., 2014; Ross-Davis et al., 2013; Stamets and Chilton, 1983). The growth of *Armillaria* is enhanced with the availability of glucose and glucose polymers; glucose also encourages *Armillaria* growth when high levels of inhibitory phenolic compounds are present (Wargo, 1996). *Armillaria* will then use the oxidised phenols as carbons sources encouraging further and more vigorous growth (Wargo, 1996).

Pathogenic species are very diverse, with examples present within all major groups of microorganisms; the ability to be pathogenic has developed as a consequence of specificity either to a host, from environmental cues, or species interactions (Vayssier-Taussat et al., 2014). Saprophytic and pathogenic fungi are found intermingled within groups that exhibit various different types of life history characteristics (Berbee 2001). This suggests that the genes associated with life history strategies evolved independently and repeatedly though periods of

lineage-specific evolution within the fungal kingdom (Aguileta et al., 2009; Ross-Davis et al., 2013). Some fungal species that appear to be pathogenic exhibit a reduced genomic size (Yuen et al., 2003). An explanation for this is loss of genes or the entire loss of metabolic pathways that are not in use or necessary. Pathogenic fungal genomes exhibit adaptations through the presences of lineage specific genes, and development of gene groupings, associated in causing disease of the host (Aguileta et al., 2009). Co-evolution may also be a driving factor in genetic divergence; in *Armillaria* the fungi live alongside their host within the surrounding soils, with some species able to colonise healthy trees and weakened trees, whilst others attack trees weakened by predisposing factors.

2.3.1 *Armillaria* virulence and pathogenicity

A. mellea

In the northern hemisphere, *A. mellea* is the most significant and aggressive pathogenic species within the *Armillaria* genus, with a broad host range of up to 500 host species, causing serious disease on many deciduous and evergreen hardwood trees (Guillaumin et al., 1991; Rishbeth, 1985; Pérez-Sierra et al., 1999). Many *Armillaria* species cause disease on predisposed or weakened trees, however, some species are able to overcome the host resistance and cause disease on resistant trees (Gregory et al., 1991; Rishbeth, 1985). *Armillaria mellea* is a virulent pathogen, and has been reported as a primary pathogen of hardwood tree species, such as oak or beech (Wargo and Harrinton 1991; Marçais and Caël, 2006). It is able to colonise healthy trees, and is the most efficient *Armillaria* species to penetrate intact bark (Rishbeth, 1985). In 1982, Rishbeth completed inoculation trials on a variety of host tree species with *Armillaria* to compare virulence, and found that *A. mellea* is able to kill trees, as *A. mellea* was isolated from 88.5% of dead broad-leaved trees, compared to 8.5% for *A. gallica*. Inoculation trials have suggested that the European isolates of *A. mellea* are most virulent when compared to North American isolates (Gregory et al., 1991). These species are found on oak species in Europe, and are thought to be a major factor in oak decline syndromes, either as a predisposing or contributing factors that cause the final demise of the tree (Manion 1981; Bruhn et al., 2000; Guillaumin and Legrand 2013). Although this species is the most virulent, their rhizomorphs are brittle and are less effective in spreading the disease across the woodland. The maximum distance *A. mellea* can usually spread disease is approximately 60

m by rhizomorphs and 100m for spore dispersal (Rishbeth, 1985; Travadon et al., 2012).
In general, the *Armillaria* genus has received little attention with respect to its role in tree diseases and is has often been dismissed as a biotic factor within tree declines. The attention received by *A. mellea* is likely due to the increased virulence of *A. mellea* compared to other *Armillaria* species, and the capability to produce unusual substances and secondary metabolites (Collins et al., 2013). *Armillaria* species secrete enzymes, proteins and other substances important for virulence and pathogenicity either within the mycelium, or as external secretions. Collins et al., (2013) completed a genomic and proteomic study on *A. mellea* and compared the secretions between the mycelium and secretome; the proteins were classified into 3 main categories; biological processes, molecular function and cellular components. The amount of proteins secreted as part of catabolic processes and carbohydrate metabolism in the external secretome were found to be higher than those found in the mycelium, suggesting that the external secretions play an important role in how *Armillaria* obtain nutrients during infection (Collins et al., 2013). The genome revealed 226 glycoside hydrolases (GH) belonging to 43 gene families. Fifty two of these genes were expressed as proteins (Collins et al., 2013). The quantity of the GH proteins GH76, GH28, and AA9 (previously GH61) found in the *A. mellea* genome are higher than those found in other root rotting fungi (Collins et al., 2013). The GH76 protein family are a group of a-mannanase enzymes that hydrolyse the plant polysaccharide α 1-6-mannan (Lombard et al., 2014 http://www.cazy.org/; Nakajima et al., 1976). The GH28 protein family is a group of enzymes that hydrolyse glycosidic bonds in pectin, and represent an essential extracellular enzyme within the secretome of saprophytic and pathogenic fungi, with necrotrophic fungal genomes known to code for several GH28 proteins (Lombard et al., 2014; Markovic and Janecek, 2001; Sprockett et al., 2011). The AA9 protein family are a group of copper dependent lytic polysaccharide monooxygenases that are significant enzymes used for the degradation of crystalline polysaccharides (Lombard et al., 2014; Morgenstern et al., 2014). Collins et al., (2013) found that *A. mellea* has similar GH proteins present to those species found within the ascomycetes, as well as one protein family group GH109 that is more often found in prokaryotes and chitinases (GH18).

Amongst the other carbohydrate active enzymes classes found in the *A. mellea* genome 72 were glycosyltransferases (GT), with the highest number (29) found within GT2 family that include enzymes such as cellulose synthase and chitin synthase (Collins et al., 2013; Coutinho

et al., 2003). In addition, 16 polysaccharide lyase (PL), 93 carbohydrate esterase (CE) and 18 carbohydrate-binding module (CBM) were found (Collins et al., 2013). Chitin deacetylases (CDA) belonging to the CE family which are secreted by the *Armillaria* hyphae as it penetrates the tree tissue, and the CDA modifies the chitinases released by tree, it also catalyses the conversion of chitin to chitosan, further preventing the trees defences and allowing the fungi to grow and colonise (Collins et al., 2013). Collins et al., (2013) found multicopper oxidases (MCOs) within the *A. mellea* genome, 6 of which code for laccases. The laccases have a varety of functions, and in plant pathogens are essential for lignocellulose degradation (Hoegger et al., 2006; Kües and Rühl, 2011). Perioxidases were also located within the *A. mellea* genome, with 9 enzymes encoded; these enzymes are also thought to be important for lignin degradation, mostly though manganese peroxidas (MnP) activity and phylogenetic analysis indicates that those enzymes arise from species-specific expansion (Collins et al., 2013).

A. ostoyae

A. ostoyae is thought to be a primary pathogen, displaying some host specificity on soft wood trees (Morrison and Pellow, 2002; Rishbeth, 1985), and are able to cause major damage within coniferous forests (Robene-Soustrade et al., 1992; Guillaumin et al., 1993; Travadon et al., 2012; Guillaumin and Legrand 2013). It has also been found to be a secondary pathogen on hardwood trees, although less virulent (Gregory et al., 1991; Rishbeth, 1985). *A. ostoyae* has the ability to colonise healthy trees when host resistance is low, and this species is likely to use conifers as a nutrient base before attempting to attack other trees (Rishbeth, 1985). Once the disease has established it will then spread aggressively using the rhizomorphs within the soils, although the presence of rhizomorphs is inconsistent (Wargo and Carey 2001; Morrison and Pellow 2002; Rishbeth, 1985). It can be found to grow up to 500 m from the disease centre, with the population structure typical of a clonal reproductive strategy, with many infected areas infected by one or a few genets (Dettman and van der Kamp, 2001; Baumgartner et al., 2011)

Desarmillaria. tabescens, previously *Armillaria tabescens*

A. tabescens is mostly defined as a saprophytic species and occasional or opportunistic weak pathogen; it is thought to cause substantial root decay, increasing the potential of wind-throw in oaks (Rishbeth, 1985). In Europe the distribution of *A. tabescens* is more prominent

in southern warmer regions, with a higher ability to be fatal in those areas (Lushaj et al., 2010; Rishbeth, 1985; Pérez-Sierra et al., 1999). However, this species can only colonise tree species with a low threshold for resistance, or those already weakened by predisposing factors (Rishbeth, 1985). Despite the low virulence, this species grows slowly and can cover vast areas, as isolates have been found to cover up to 100 m (Rishbeth, 1985, 1982). The basidiocarp morphology of *A. tabescens* is different to other species, as it lacks an annulus on the stipe of the basidiocarp, enabling rudimentary species identification (Guillaumin et al., 1993; Tsopelas and Tjamos, 1997; Guillaumin and Legrand 2013). This species produces thin rhizomorphs, which are difficult to find within the soil and are sometimes missed (Rishbeth, 1985).

A. gallica

A. gallica, is a long-lived species and is predominantly described as a saprophyte or weak pathogen, showing variation in virulence, with some isolates rarely causing disease and others causing significant damage (Gregory et al., 1991; Rishbeth, 1985, 1982). It is commonly found within natural woodland communities, growing networks of mycelium and rhizomorphs that take advantage when a tree becomes weakened or stressed (Rishbeth, 1985). Infection with *A. gallica* is less likely to kill a healthy tree, however, as a secondary pathogen is able to kill trees that have been weakened by predisposing factors or a primary disease (Rishbeth, 1985, 1982; Gregory et al., 1991). This species is less effectual against natural tree resistance and struggles to penetrate intact bark (Rishbeth, 1985). Inoculation trials indicated that broad-leaved trees with severe symptoms and extensive decayed regions of root rot were associated with *A. gallica* (Rishbeth, 1982). The extensive decayed areas on broad-leaved trees were found to reach 3-4 m above ground level, whereas on conifers those legions reach to approximately 0.6 m (Greig, 1962; Rishbeth, 1982). *A. gallica* is commonly found as a benign epiphytic rhizomorph within the hardwood forest floor, and can be found up to 800m from the disease centre (Baumgartner et al., 2011; Marcais and Cael, 2006). It is known to become more virulent on trees, can be seen through the production of highly dense epiphytic rhizomorphs present on the tree collar, those trees are show less vigour potentially weakened by predisposing factors or previous infection (Guillaumin et al., 1993; Pérez-Sierra et al., 1999; Wargo and Carey, 2001; Guillaumin and Legrand 2013). Metaliaj et al., (2006) inoculated seedlings with *A. gallica*, and compared the effects of various watering regimes; it

was found that the pathogenicity of *A. gallica* could be almost as virulent as that seen in *A. mellea*, attributed to the increased water stress. The variation in virulence within *A. gallica* species could suggest that it is a more important root rot disease in overall tree declines than previously thought (Rishbeth, 1985). In culture *A. gallica* produces a dark brown staining on the agar, this also is seen in the species *A. cepistipes* (Pérez-Sierra et al., 1999).

2.3.2 Role of *Armillaria* species in tree declines

Wargo (1977) suggested that during a decline event a pathogenic species or pest may infect the tree as a secondary pathogen, however, the importance of that infection may not necessarily be a secondary effect. Tree mortality occurs due to a disruption of water and carbon sources, with the pest and pathogenic species involved affecting all areas of the tree, suggesting that all the organisms involved are as important as each other in the decline (Wargo, 1977). This concept highlights that it is difficult to assign *Armillaria* species a specific role as either primary or secondary pathogens; therefore, declines should be seen as complete syndromes including all of the observed symptoms (Garbelotto, 2004; Wargo, 1977). In Europe, *Armillaria* species have repeatedly been highlighted as the biotic factor in previous oak decline events (Marçais and Caël, 2006; Guillaumin et al. 1985; Sicoli et al. 1998). The role of *Armillaria* species in overall tree declines are usually assumed to be a contributing or secondary factor, however, this may not fully explain the role of *Armillaria* spp. (Wargo, 1977). Variations in species host, virulence and site characteristics may influence individual *Armillaria* species role in tree declines. Within the decline syndrome, *A. mellea*, *A. gallica* and occasionally *A. tabescens* have been associated with tree declines of oak tree species in Europe, and are commonly referred as the predisposing or contributing factors (Manion 1981; Metaliaj et al., 2006; Guillaumin and Legrand 2013).

2.3.3 The relationship between *Armillaria* spp. and other tree diseases

Acute oak decline (AOD) is an oak dieback syndrome found in Britain. It is causing rapid decline of affected oaks in a relatively short time, usually within approximately 3 to 5 years (Denman et al., 2014). AOD is thought to be a combination of abiotic and biotic agents, the latter of which include the bacterial species *Brenneria goodwinii* and *Gibbsiella quercinecans*, and activity of the larvae of the European bark-boring bettle, *Agrilus biguttatus*. Symptoms of

AOD are easily confused with pathogenic *Armillaria* infection, as both result in stem bleeds. However, there are small differences in how the bleeds occur, as *Armillaria* bleeds appear in an 'A frame' formation, or via the tracking of mycelium growth in the cambrial layers of the tree with previous bleeds drying out, whereas AOD lesions have a vertical orientation starting high on the tree trunk with continuous bleeding from all bleeds (Denman et al., 2014). The fluid eminating from bleeds caused by *Armillaria* come from the natural spaces between the bark plates, whereas during AOD bleeds those spaces between the bark plates open or bulge. In addition, the fluid from bleeds differ slightly in colour; with *Armillaria* infection the liquid is clear light brown in colour, whereas in AOD bacterial lesions, the fluid is a transulent liquid, dark brown and almost black in colour (Denman pers. comns). ; Denman et al., 2014). However, *Armillaria* species have been found infecting trees that are also showing symptoms of AOD. The influence of *Armillaria* species on the root system of trees symptomatic of AOD is not fully understood, although tree root health has been highlighted as a significant factor within tree declines, and therefore the presents of root rot pathogens such as *Armillaria* species are important factors to consider (Denman et al., 2014; Haavik et al., 2015). Root health may also be important for the tree's potential for recovery after a decline event, to prevent general infection and withstand adverse environmental conditions (Haavik et al., 2015). Denman et al (2014) recommended that root condition, and the soil environment should be considered in future research into acute oak decline and other decline events (Denman et al., 2014). It is therefore important to understand the state of *Armillaria* infection on the tree root system.

Phytophthora species represent another group of pathogens that affect oak tree health; symptoms include fine root death when studied in the field. However, Phytophthora spp., do not cause seedling mortality or an overall reduction in growth of oak trees. Two species within the Phytophthora genus have been associated with oak declines; *P. ramorum*, *P. cinnamomi* and *P. quercina* (Robin et al., 1998; Jung et al., 2000; Johnson et al., 2003; Balci et al., 2008). *Armillaria* spp. have been reported to occur alongside Phytophthora species on oak trees. The weakening effect of one pathogenic species may cause an advantageous environment for the other to thrive, with examples of both pathogens strongly influenced by the overall condition of the host tree (Camy et al., 2003b; Blaker and MacDonald, 1981; Wargo and Harrington, 1991; Marçais et al., 1993; Maurel et al., 2001; Marçais and Breda 2006; Marçais et al., 2011). Previous field studies by Marçais et al., (2011) on 2-year-old oak seedlings have shown that interaction between *Phytophthora* spp. and root rot pathogens *A. mellea*

and *Collybia fusipes* cause greater damage when they co-occur as a complex of pathogens rather than as independent infections on oak trees, and the damage was particularly notable for lesions on the taproot (Marçais et al., 2011).

2.3.4 Pathogenic *Armillaria* root rot disease infection

External symptoms

Infection with pathogenic *Armillaria* species can be observed through a range of above ground symptoms, however they are not specific to this pathogen, *Armillaria* infection is often more severe on younger oak trees than older trees (Morrison et al., 1991). The effects on the crown can also vary between host and symptoms include; reduced foliage growth and size, reduced overall yield or vigour, reduced crown or premature defoliation, wilting and tree death (Baumgartner et al., 2011; Kowalski, 1991; Morrison et al., 1991). On older trees, the first visible symptom would be overall smaller leaves than expected, followed by them progressively turning yellow in colour with premature defoliation, resulting in weakening and death of the leaves and branches above the *Armillaria* infection on the root collar (Kowalski, 1991; Morrison et al., 1991; Mueller-Dombois, 1992). Due to the number of potential of host species, *Armillaria* infections can show variations of these described symptoms, or affected trees may be asymptomatic. One of the most visually distinctive symptoms of pathogenic *Armillaria* infection is the presents of dark coloured bleeds on the tree trunk: those bleeds are produced by the host tree excreting resin or gum (Baumgartner et al., 2011). In hardwood trees infected with *Armillaria*, bleeds can appear on the root collar and trunk. The bleeds are produced by a dark fluid being coming from between the bark plates, and it is usually found at the extent of the mycelial fans within the cambial layer (Kowalski, 1991; Morrison et al., 1991). *Armillaria* infection on oak tree species can result in necrotic legions, causing decay of the bark and phloem, and the occasional decay of the sapwood (Kowalski, 1991). The outer bark at the site of the necrotic areas can loosen as the mycelium grows within the layers of the living tissue (Kowalski, 1991). Extensive necrotic legions can develop, covering one fifth to two thirds of the tree's circumference (Kowalski, 1991). The decaying wood appears to be wet, ranging from white to yellow in colour, with the texture changing to sponge-like form and finally, gelatinous (Baumgartner et al., 2011; Kowalski, 1991). Zone-lines or pseudosclerotial plates are visible in the decaying wood; these zone lines are dark thin sheets of hyphae that

enclose the mycelium decaying a section of wood (Lopez-Real, 1975).

Armillaria infection is able to kill some host tree species within one season, or after years of infection, depending upon the host species (Baumgartner et al., 2011). If or when the external symptoms become visible, this indicates that the *Armillaria* infection has spread to the sub cortex layer within the root system and root collar via mycelial fans (Baumgartner et al., 2011). The tree can survive after *Armillaria* infection if there is sufficient living tissue remaining within the root system and root collar, and if the water and carbon economy within the tree is not completely destroyed.

Internal symptoms

The cambial region experience the greatest chemical changes during pathogen colonisation, with changes to the sugars and amino acid composition (Wargo, 1972). Necrotrophic pathogens such as *Armillaria* obtain the required nutrients from structural carbon sources, causing areas of tissue death within the cambial layer and vascular tissues of the tree (Oliva et al., 2014). For *Armillaria*, the decay of such tissues is predominately based within the root system and root collar of the host tree, and occasionally on the trunk (Baumgartner et al., 2011; Garbelotto, 2004; Marçais and Bréda, 2006; Wargo, 1977). The destruction of those structures causes disruption of the carbon and water flows within the tree, causing the host to trigger its defence systems or root repair, adding a further strain on carbon resources (Oliva et al., 2014). External factors can also place a strain on the water and carbon sources of the tree, such as drought or defoliation, adding further demand for cellular resources and providing less for defence (Oliva et al., 2014, 2012). Defoliation alongside *Armillaria* infection or other necrotrophic pathogens negatively affect the levels of starch in the root system, and reducing sugar concentrations are considerably higher than normal (4-5 times greater than the level in trees unaffected by defoliation or naturally high levels used for growth in the spring) (Wargo, 1996). The survival of the tree relies on the amount of carbon in the form of starch that is available during tree stress (Wargo, 1996). If the abiotic or biotic factors are improved the original resistance of the tree can be resorted (Oliva et al., 2014). *Armillaria* disease and other root rot infections can cause a strain on water resources within the tree, effecting the availability of water and the hydraulic conductivity, that subsequently effect the overall growth and sapwood depth of the tree (Aguade et al., 2015).

2.3.5 Susceptibility to infection

External factors can influence the tree's susceptibility to infection; if the tree becomes stressed due to those external or predisposing factors the mechanisms for resistance and pathogen response will become less efficient (Oliva et al., 2014). To mitigate the effects of predisposing factors, a tree has to make adjustments to the amino acids, fatty acids and carbohydrates (Wargo, 1972). For example, during drought or defoliation the tree degrades starch from the roots into sugars, such as glucose and fructose, that are easily mobilised to restore the crown, in doing this making the carbon more accessible to the occupying *Armillaria*. However, the role of starch in this context has been disputed (Oliva et al., 2014; Wargo, 1972). The advanced ages of a tree stand or its density are also predisposing factor in oak declines and to *Armillaria* infection, however, some species of *Armillaria* able to kill tree of all ages (Fan et al., 2008; Garbelotto, 2004; Lushaj et al., 2010). Older trees are more susceptible to fungal root pathogens due to a greater availability of substrates and a loss of structure in older trees allows greater access for *Armillaria* with a greater susceptible to decay (Garbelotto, 2004). Older trees are usually also large, with extensive root systems, thus the *Armillaria* can use those extensive roots to colonise new substrates (Garbelotto, 2004). From a management perspective, thinning of areas with high oak density may make managing the spread of secondary *Armillaria* easier (Garbelotto, 2004). Areas of greater species diversity within a forest have beneficial effects in the ability to prevent the spread of the infection and reduce overall mortality caused by pathogenic *Armillaria* (Gerlach et al., 1997). A reduction in tree resistance or pathogen response can allow *Armillaria* spp. to colonise the tree and gain access to the nutrients. Using the carbon and water sources within the tree the *Armillaria* will increase its biomass (hyphae and mycelium), developing a higher inoculum potential (Oliva et al., 2014). The necrotic action of the *Armillaria* species on the living tissue physically disrupts the carbon and water regimes within the tree and causes carbon sources to be used for carbon expensive pathogen-host response mechanisms, therefore depleting the carbon sources quicker, giving an overall result of reduced growth and potential tree death (Oliva et al., 2014). Some insect species, such as bark beetles, find trees with a previous root infection more desirable to colonise and can significantly increase the mortality rate within a stand (Garbelotto, 2004; Oliva et al., 2014). There is the potentially that the activity of *Armillaria*

also may provide gaps within the soil and root systems, allowing other opportunistic parasitic or pathogenic species to colonise the tree and continue on the tree disease spiral.

Oak tree host defences against Armillaria spp

The interaction between *Armillaria* and its host varies depends on species, with environmental factors having an influential role (Garraway et al., 1991). The mechanisms used by the hosts can be; (1) resistance, that limit or prevent fungal attack by using chemical and physical barriers, or (2) tolerance strategies that are compensatory mechanisms against the damaged and negative impacts caused by the disease (Lattanzio et al., 2006). For plants there is a choice to be made between the defences systems to protect the tree from an invading pathogen or the growth rate and competitiveness (Herms and Mattson, 1992; Oliva et al., 2012). The primary obstacle for *Armillaria* species is to penetrate is the tree bark; this physical barrier provides little protection against some *Armillaria* species, such as *A. mellea* that is still able to penetrate the bark of health trees with no apparent vector (Oliva et al., 2014; Prospero, 2003; Rishbeth, 1985; Thomas, 1934). The capability of *Armillaria* to penetrate tree bark varies between species, and it may not be possible for some members of the genus. The bark may slow the growth of Armillaria and provide some delay in colonisation. However, there is little variation between bark from *Armillaria* resistant or susceptible trees in the lack of ability to prevent *Armillaria* penetration (Garraway et al., 1991). The major approach used by trees to contain the advancement of *Armillaria* and other pathogenic fungi is to compartmentalise the mycelium using costly carbon boundaries. This approach, however, sacrifices part of the living tissue, for example the sacrificing infected parts of the cambial layer covered by cankers (Oliva et al., 2014). The carbon required for compartmentalising is expensive for tree and it can reduce radial growth (Oliva et al., 2014, 2012). The tree's defences can identify the attacking pathogen by recognising the cell-wall chitin present in fungal species; this activates the chitinase enzyme within tree defences, triggering the release of chitin oligomers that can be used as elicitors for the formation of lignin and callus (Collins et al., 2013; Tsigos and Bouriotis, 1995). Tree defence systems are stimulated when the pathogen makes contact with living host cells (Lattanzio et al., 2006). This indicates that tree resistance to *Armillaria* infection must use a chemical response to prevent *Armillaria* colonisation. Many secondary metabolites produced by plant hosts in response to biotic and abiotic factors or as part of the normal tree growth, are toxic to pathogenic species (Lattanzio et al., 2006). Phenolic

compounds are secondary metabolites produced by the host's phenylpropanoids pathway (Lattanzio et al., 2006; Vogt, 2010), and play an important role in the chemical resistance against *Armillaria* infection. The phenolic compounds inhibit the hydrolytic enzymes used by the *Armillaria* therefore acting as a chemical barrier preventing further colonisation (Garraway et al., 1991). *Armillaria*, however, secretes extracellular enzymes capable of degrading phenols: perioxidase and laccases, and intracellular enzymes tyrosinase. Those enzymes cause oxidation and polymerization of the toxic phenol compounds and are important enzymes for *Armillaria* species in wood degradation. This process can result in the successful breakdown of the tree's chemical barriers, leading to further colonization into the living tissue of the tree (Garraway et al., 1991).

2.4 Identification: method comparison

2.4.1 Basidiocarp /Mushroom morphology

Basidiocarps are the vegetative mushroom of *Armillaria* spp. and the macro and microscopic morphological characteristics of basidiocarps are still used for identification (Coetzee et al., 2000; Maphosa et al., 2006). However, using basidiocarp morphology for identification has its limitations; firstly, basidiocarps are difficult to obtain due to high seasonality (they are usually only found in the late summer to early autumn), they are short-lived, and their pattern of fruiting is difficult to predict (Maphosa et al., 2006; Rishbeth, 1985). Basidiocarps normally grow in clusters in either stumps, or at the base of an infected tree, or at the fringes of the mycelium and rhizomorphs growth within the soil (Prospero, 2003). Furthermore, in natural systems, the environment can influence the morphology of the basidiocarps produced, and some species of *Armillaria* produce identical basidiocarps, making it impossible to separate between them (Watling 1991; Bérubé and Dessureault, 1989; Maphosa et al., 2006; Baumgartner et al., 2011). It is challenging to induce fruiting of the basidiocarps in artificial culture, however, those produced are often not representative of those found in natural populations (Baumgartner et al., 2011). Due to the limitations of using basidiocarp morphology, it is usually only used as an alternate method of identification or used in combination with other methodologies (Baumgartner et al., 2011).

2.4.2 Mating incompatibility trials

Mating incompatibility trials can also be used for *Armillaria* species identification. They can be used to identify species by pairing unknown haploid isolates with a known tester haploid isolate. In successful mating the haploid nuclei will form a diploid nucleus, causing a change in morphology from white fluffy mycelium to dark crustose mycelium (Hintikka, 1973; Baumgartner et al., 2011). Mating incompatibility tests can be done between known haploid tester strains and unknown diploid strains, however the results are sometimes ambiguous (Baumgartner et al., 2011). The diploid nucleus invades the haploid mycelium, displacing the haploid nucleus, however, occasionally there is recombination of the nuclei and genetic material from both diploid and haploid nucleus are retained and lost (Guillaumin et al., 1991; Rizzo and Harrington, 1992; Worrall, 1997). Mating trials are a time consuming method, as time is needed for the isolates to be grown up and for the mating to occur, with haploid-diploid the interactions are particularly slow, with ambiguous and unreliable results (Korhonen, 1978, 1983: Siepmann, 1987).

2.4.3 DNA based methods

DNA sequencing has become the dominant method of species identification since the early 1990s. DNA is usually extracted from rhizomorphs and mycelium from wood panels or artificial culture, and less commonly from mushrooms. Commonly used DNA regions used for identification and phylogenetic analysis of *Armillaria* species are; ITS, IGS and EF-1α (Anderson and Stasovski, 1992; Coetzee et al., 2001a; Maphosa et al., 2006; Baumgartner et al., 2011; Denman et al., 2016). This methodology has proven very useful when taking samples from previously un-surveyed sites, where other methodologies are unsuitable: such as mating incompatibility trials due to a lack of known tester strains, or a lack of identifiable structures. Phylogenetic analysis of gene sequences form such sites has unearthed distinct and previously unknown taxonomic groups (Baumgartner et al., 2011).

A study by Pérez-Sierra et al. (1999) developed a PCR-RFLP for *Armillaria* species identification, this used tradition PCR based on IGS gene region. The PCR generated amplicons which were then exposed to restriction digestion enzymes *Alu I, Nde 1 and Bsm I*. The restriction enzymes cut the DNA strands into fragments of different sizes, which show different and specific patterns between species. The digested DNA regions are then visualised on a gel

electrophoresis, the different fragment patterns are compared and different species can be identified by those patterns. The *Alu I* effectively distinguished between *A. mellea* and *A. gallica*, for other species further digestion is needed using the *Nde I* or *Bsm I*.
DNA-based methods have also been used in taxonomic research to find similarities between taxonomic linkages and the morphological characteristics of the mushrooms from species identified using the morphological species concept (Pildain et al., 2009, 2010; Baumgartner et al., 2011).

2.5 Site background

During this project the work will focus on one site; Chestnuts wood, located within the located with the Forest of Dean, Gloucestershire. This woodland is a 65-year-old oak (*Quercus robur*) plantation (Figure 5.1). Previously, this site was predominately a coniferous forest of pine tree species, which were all felled and replanted as an oak plantation (Forestry commission, pers. comns. 2015). Many of the oaks at the site were planted between 1948-1950 and in 1960 in high densities, approximately 2-3 meters apart, with some areas of ancient oaks planted much earlier in 1814 and 1850, which are not part of this study here. This site has an identified history of *Armillaria* infection. Previous samples collected by Forest research have isolated both *A. gallica* and *A. ostoyae* colonising separate trees and also the same tree (Pers. comns, Denman 2017). The area containing oaks with symptoms of *Armillaria* infection correlates with the high-density planting of oak trees, planted in the mid-1900s, and now approximately 65 years old. The area west of the track is approximately 5.72 ha in size, and part of a long-term monitoring program undertaken by Nathan Brown at Rothamsted Research in collaboration with Forest Research (Personal communication)(Figure 5.1).
A site visit was completed on the 20th April 2016 with the Forestry Commission, Sandra Denman (Forest Research) and James McDonald (Bangor University), where two previously sampled trees were identified (FOD4 and FOD6), both trees were located west the track within the long-term monitoring site. Wood panel samples were taken from potential lesion areas on the trunk, and isolations were completed at Bangor University where only *A.gallica* was isolated from both trees.

2.5.1 Sample material collected at Chestnuts Wood

During this project all sample material will be collected from this site. An ecological study will focus on a small subplot detailed in chapter 5, were soils and rhizomorphs from asymptomatic and symptomatic trees and wood panels from symptomatic trees only are collected. The RNA analysis in chapter 6 will use wood panel collected from asymptomatic symptomatic trees which are immediately flash frozen in liquid nitrogen.

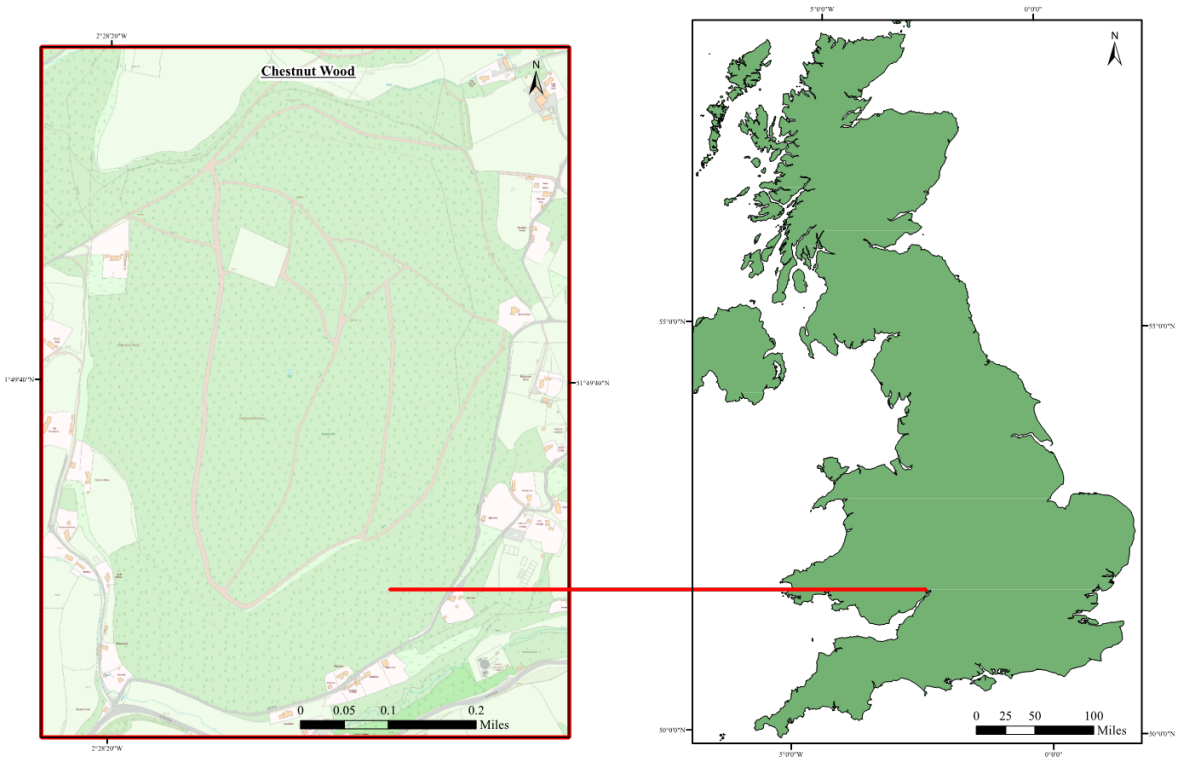


Figure 2.3 – Location of the Chestnuts wood sampling site.

Chapter 3

Method optimisation to improve biomass production and DNA yield of *Armillaria* species

3.1 Introduction

The genus *Armillaria* comprises 40 species, including biological species described using molecular techniques and compatibility trials, in addition to morphological species described using phenotypic characteristics (Watling et al., 1991; Metaliaj et al., 2006; Baumgartner et al., 2011). *Armillaria* spp. can be found globally in both temperate and tropical regions, with some species having a limited geographical range whist others are more widespread (Watling et al., 1991; Pérez-Sierra et al., 1999; Coetzee et al., 2001; Sicoli et al., 2003; Baumgartner et al., 2011; Mulholland et al., 2012; Haavik et al., 2015). In addition to its saprotrophic role in the decomposition of dead tree tissue, *Armillaria* is also a soil borne plant pathogen that causes necrosis of live root and stem tissue in a variety of host species. *Armillaria* species have been associated with numerous past and present oak declines in Europe (Guillauim et al., 1985; Marçais and Caël, 2006), however it is difficult to assess the role of *Armillaria* within these oak declines, as progress in this area is confounded by species variation in host specificity and virulence, and their ability to act as both a pathogen and a saprotroph. In Britain, there are seven species of *Armillaria* , with four *Armillaria* species are associated with disease in oak: *A. mellea*, *A. gallica*, *A. ostoyae*, and *A. tabescens* (Drakulic et al., 2017). These species have also been found on oak trees that are symptomatic of different disease syndromes, most commonly associated with Chronic Oak Decline (COD), which effects the below ground part of the tree, but also as secondary pathogens on trees effected by Acute Oak Decline (AOD). AOD effects the above parts of the tree characterised by bacterial lesion formation and the presence of the European bark-boring bettle, *Agrilus biguttatus* (Denman et al., 2014). The

influence of *Armillaria* species on the tree root system is potentially an important biotic factor in overall tree declines, such as COD, as root health has been highlighed as a significant factor in disease susceptibility (Denman et al., 2014).

The isolation, cultivation and identification (by morphological or molecular approaches) of *Armillaria* isolates can be a slow and laborious process, with 3 - 4 weeks incubation at 22 - 25°C often required for growth, depending upon the species identity (Robene-Soustrade et al., 1992; Guillaumin et al., 1993; Coetzee et al., 2001; Maphosa et al., 2006; Guillaumin and Legrand, 2013). A malt-based growth medium is the mostly commonly used media for laboratory isolation and cultivation of many different types of fungal isolates, for *Armillaria* growth, media can range between 1 - 3% malt agar and left to grow for 2 - 4 weeks (Anderson, Korhonen and Ullrich, 1980; Rishbeth, 1986; Rizzo and Harrington, 1993; Guillaumin et al., 1993; Hsiau, 1996; Pérez-Sierra et al., 1999; Coetzee et al., 2001; Brang et al., 2003; Maphosa et al., 2006; Intini et al., 2010; Mulholland et al., 2012; Collins et al., 2013; Elías-Román et al., 2013; Keča et al., 2015; Lee, Dey and Muzika, 2016; Denman et al., 2017; Sipos et al., 2018; Heinzelmann, Prospero and Rigling, 2018). A commercial vegetable juice (V8 juice) can be used to make a vegetable based media, commonly used for Phytophthora species, but also has been used as in a 1:1 (v/v) with tomato juice as a liquid medium for *A. mellea* and *A. ostoyae* (Englander and Roth, 1979; Robene-Soustrade et al., 1992; Angay et al., 2014; Englander, Browning and Tooley, 2017). Previous studies used the V8 to grown *Armillaria*, using a liquid V8 media inoculated from colonies grown on malt-based agar, this was then macerated and used to inoculate the liquid broth, after 12 days this liquid broth was used as an inoculation source and added to sawdust for the production of fruiting bodies (Robene-Soustrade et al., 1992). In addition, tomato- or carrot-based media have also been suggested as alternative bases for *Armillaria* growth (Pérez-Sierra et al., 1999; Ford et al. 2015; Denman, personal communications 2015). Ford et al, (2015) compared the effectiveness of different *Armillaria* growth media to create reliable fruiting systems of *Armillaria mellea* basidiocarps using 15 g sawdust 30 g rice mixed with 150 ml water, covering with a 1 cm layer of either homogenised carrot and tomato. The addition of the tomato or carrots is to encourage extensive mycelial growth with the main objective to increase production of the basidiocarps (Ford et al., 2015). Previous studies that have used macerated carrots as an addition in fruiting media have shown a rapid increase in biomass and that primodial formation was encouraged (Shim et al., 2006; Ford et al., 2015). Carrots have also been suggested as a suitable addition to fruiting media for

the mass production of *Armillaria* mushrooms (Shim et al., 2006). Some *Armillaria* species are difficult to produce fruiting bodies in culture, depending on species and if fruiting is possible to induce some isolates will produce basidiocarps multiple times a year whilst others will only fruit once regardless of laboratory conditions. This can also be a time consuming process taking approximately 10 weeks (Shim et al., 2006; Ford et al., 2015).

The mass production of basidiocarps for medicinal proposes and the food industry has received much of the attention (Siu et al., 2016), a comparison of growth media types for the rapid growth of *Armillaria* mycelium is absent. All species produce hyphae and mycelium in culture within 3-4 weeks, those long incubation times can cause problems of contamination and mite infestations, and act a barrier to understanding *Armillaria* epidemiology and ecology as a saprophyte, parasite and pathogen.

An effective media to increase fungal biomass in the laboratory is essential for increasing the speed of diagnosis of horticultural and forest disease caused by *Armillaria* in the UK and globally; as well as to further understand the ecology and function of *Armillaria* within gardens and forests. The increased biomass is also vital for DNA extractions used in downstream molecular techniques such as end-point PCR for species identification and phylogenetic analysis of isolates and species but also in the development of a rapid diagnostic.

3.1.1 Aims and objectives

The key overarching aims of this thesis involve; (1) the collection and phylogenetic analysis of *Armillaria* isolates associated with UK oaks, (2) the development of a rapid molecular diagnostic test for *Armillaria* spp. and (3) an ecological study of *Armillaria* spp. associated with COD in Chestnuts Wood, Forest of Dean. In order to achieve these aims, it is critical that effective and rapid isolation procedures are available for the growth of all species of oak-associated *Armillaria* and to identify a growth medium that increases *Armillaria* biomass on agar plates for DNA extraction and analysis. A second requirement is the ability to extract suitable quantities of DNA from *Armillaria* biomass obtained from culture plates to enable multi-locus sequence-based phylogenetic analysis and development of a rapid molecular diagnostic for *Armillaria* species.

Consequently, the aims of this chapter were to; conduct a growth media trial to identify the optimum medium for *Armillaria* growth and biomass production. The purpose of the

experiment was to decrease the incubation time for *Armillaria* growth and increase the overall biomass available for effective DNA extraction. The medium must also be suitable for the growth of isolates of all *Armillaria* species, with comparable growth rate and biomass. DNA extracted from the mycelium is generally of a better quality than DNA extracted from rhizomorphs or fruiting bodies, therefore it was preferable to find a medium that promoted white mycelial growth, rather than crustose mycelium or rhizomorphs forms. All medium types used within the trial are previously suggested within the literature used for the growth of all the *Armillaria* species and isolates. It was hypothesised that the vegetable-based growth medium will provide greater *Armillaria* colony area and biomass than malt-based medium.
Molecular analysis of *Armillaria* spp. identity and ecology is reliant on the effective extraction of suitable quantities of DNA of high purity for PCR-based analyses. Consequently, to identify the optimum DNA extraction method for *Armillaria* isolates, several DNA extraction methods were tested on *Armillaria* isolate biomass obtained from the growth medium trial in order to identify the best combination of growth medium and DNA extraction method for culture-based and molecular characterisation of *Armillaria* spp. Lastly, DNA extracted from *Armillaria* isolates was used to complete end-point PCR and sanger sequencing of three loci; EF1-α, ITS and IGS, and to analyse the sequencing data using multi-locus sequence-based phylogenetic analysis of all three loci.

3.2 Materials and Methods

3.2.1 Preparation of *Armillaria* growth media

Four different agar growth media were selected for the growth trial; carrot, commercial V8 juice, tomato, and malt (1.6 %). The carrot medium was prepared using 350 g l^{-1} washed carrots, blended and passed through a muslin cloth; 15 g l^{-1} of agar was added to the blended carrot liquid and the medium was made up to 1 L using deionised water (Pérez-Sierra et al., 1999). All vegetable media were autoclaved twice, 24 hours apart.
The tomato medium was modified from Ford et al. (2015), using 500 g l^{-1} tomatoes blended and passed through a sieve to remove skin and seeds, followed by straining through a muslin cloth. Subsequently, 6 g l^{-1} dextrose, 6 gl^{-1} malt extract, 1.2 g l^{-1} yeast extract, 1.8 g l^{-1} maltose and 20 g l^{-1} of agar was added and the final volume was adjusted to 1 L using

deionised water. The V8 medium was prepared using a 1:1 ratio of 500 mL V8 juice and 15 g l^{-1} of agar, final volume was adjusted to 1 L using deionised water.

The malt medium comprised of 16 g l^{-1} malt extract and 15 g l^{-1} agar, final volume was adjusted to 1 L using deionised water. The pH of carrot and malt media were pH 5.6 – 5.7, the tomato and V8 were pH 3.5 and 4 respectively, therefore the tomato and the V8 were adjusted to pH 5.1 - 5.5 at 19°C, with 1 M NaOH. All vegetable media were autoclaved twice, 24 hours apart. After the second autoclave step, the V8 based medium exhibited a variable consistency when compared to the other media types, this media failed to set solid combined with the dark colouring made it difficult distinguish the edge of the mycelium extent and there for difficult to measure the mycelial growth accurately (Figure 3.1). As a result, the V8 medium was deemed unsuitable as a growth medium and therefore excluded from the trial.

3.2.2 *Armillaria* growth medium trial

To identify the optimal growth medium for rapid growth and maximum biomass production, isolates of each of the four species associated with oak species in the UK were selected for testing. Three isolates of *A. gallica* and *A.ostoyae* were selected, whereas only two *A. mellea* isolates and one *A. tabescens* were included due to a lack of available isolates for the latter two species at the outset of the project. A previously identified *A. cepistipes* isolate was also included in the study; however, this was later identified as an *A. gallica* isolate.

To establish the growth trial, from a malt agar plate a flame sterilised scalpel was used to remove a plug of mycelium from the outer edges of the mycelium of an established colony approximately 1 - 2 mm in diameter, the plug transferred onto three replicate agar plates of each growth medium, the plates were sealed using parafilm. The inoculated agar plates were placed at 24°C for 28 days. The position of each plate in the incubator was altered every 2 - 3 days to account for temperature variability within the incubator. Using the agar plug used to inoculate the plate as the central point an X and Y axis was drawn on the underside of each petri dish, and a image was taken and this was used to measure diameter of *Armillaria* colony growth (Figure 3.1). A photograph of each plate was taken using a camera set at a fixed height using clamp stands, an image taken at days 0, 3, 5, 7, 10, 14 and 21, using IMAGE J software each image had colony growth measured to determine colony radius, diameter, rhizomorph presences, and colony area, (Rasband., 1997-2018). Grow rate was determined by mm of mycelium growth per week.

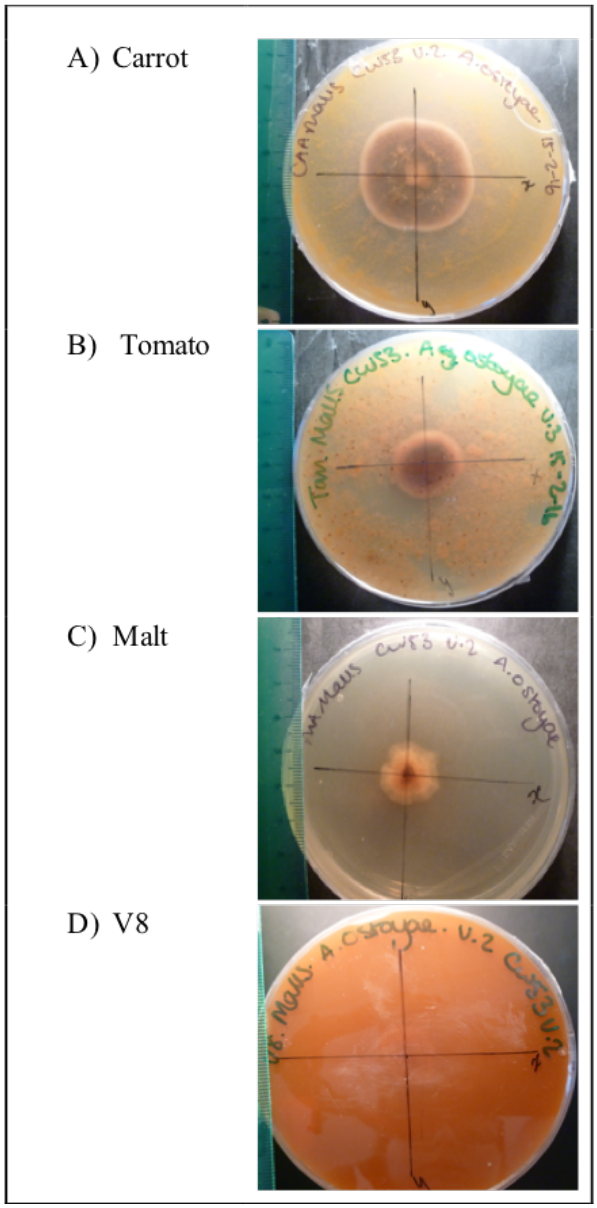


Figure 3.1 – *Armillaria* isolate CW53 (*A. ostoyae* growth on (A) carrot, (B) malt, (C) malt, and (D) V8-based agar growth medium after 21 days. A growth trial was conducted to identify the best growth medium for the cultivation of *Armillaria* species.

3.2.3 Statistical analysis of *Armillaria* colony growth and rhizomorph presences

Colony growth data was analysed using R studio (R Development Core Team, 2009). Due to the non-normal distribution of the data, the colony area after 21 days was statistically analysed using a general linear mixed model using the R function glmmPQL from the MASS library (Venables and Ripley, 2002). Medium type and number of days incubation (0, 3, 5, 7, 10, 14, and 21 days) were selected as the main factors, whereas isolate ID was selected as a random factor, using a gamma error family distribution. Due to non-normal distribution of the data and presence of non-positive values, a non-parametric Kruskal Wallis (KW) test was ran using dunns posthoc test, from the dunn test library, to statistically analyse growth rate over 21 days and the number of rhizomorphs observed between different media types (R Development Core Team, 2009).

3.2.4 Optimisation of DNA extraction from *Armillaria* spp.

Ten *Armillaria* isolates were propagated on carrot-based agar for 21 days as described above. DNA was extracted from colony biomass using three different methods; (i) DNeasy Plant Mini Kit from Qiagen, (ii) FastDNA^{TM} Spin Kit from mpbio (Denman et al., 2017) and (iii) a CTAB-based DNA/RNA co-extraction method described by (Griffiths et al., 2000) following the manufacturer's protocol. Using a flame sterilised scalpel the mycelium and rhizomorphs were scraped from the top of the agar, and the appropriate amount of fungal tissue was added according to manufacturer's protocol; 100 mg for the DNeasy Plant Mini Kit, 200 mg for the FastDNA^{TM} Spin Kit, and 0.5 g for the CTAB-based DNA/RNA co-extraction.

The concentration of extracted DNA obtained for each strain/kit combination was determined using the Qubit^{TM} dsDNA HS Assay Kit, and DNA quality was determined via 2% agarose gel electrophoresis and NanoDrop^{T}M Spectrophotometer. The DNeasy Plant Mini Kit was not taken forward for further analysis, as the DNA yield was too low to visualise using gel electrophoresis and DNA concentrations readings were low to be read by the Qubit^{TM} dsDNA HS Assay Kit.

3.2.5 PCR amplification of extracted *Armillaria* DNA using phylogenentic marker gene primer pairs

In order to test the efficacy of PCR amplification with DNA extracted using the methods described above. PCR reactions were completed using 12.5 μL GoTaq$_{®}$ G2 green mastermix, 2X (Promega, Madison, WI, USA), $MgCl_2$ 1.5mM, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 2 μL DNA template (1-40 ng/μL) and 8.5 μL PCR water, total volume of 25 μL. The primers used to amplify the EF1-α gene region were; EF595F and EF1160R (Kauserud and Schumacher, 2001) and, ITS; ITS1 and ITS4 (White et al., 1990) and IGS; IGS01 (Duchesne and Anderson, 1990)and IGS P1 (Hsiau, 1996). (Table. 3.1) (Denman et al., 2017). PCR cycling conditions were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturing at 95°C for 1 minute, 30 s annealing at 55°C for the ITS or IGS primer pairs, or 48°C for EF1-α primers, and extension at 72°C for 1 minute, with a final at 72°C for 5 minute extension step. All of the amplicons were ran out on a 2 % gel.

Table 3.1 – Primer sequences used in endpoint PCR.

Primer	**Sequence** (5'-3')	**Temperature** (°C)
ITS1	TCCGTAGGTGAACCTGCGG	55
ITS4	GCTGCGTTCTTCATCGATGC	
EF1-α_F	CGTGACTTCATCAAGAACATG	48
EF1-α_R	CCGATCTTGTAGCGTCCTG	
IGS01	AGTCCTATGGCCGTGGAT	55
IGSP1	TTGCAGACGACTTGAATGG	

From the literature an additional DNA clean-up step was completed to reduce polysaccharide contamination and to purify the extracted genomic DNA (gDNA) further; this was done using the DNA Clean & Concentrator kit from Zymo research prior to PCR (Denman et al., 2017). Subsequently, PCR reactions containing both the unpurified and purified gDNA were prepared to assess the benefit of the additional clean-up step.

3.3 Results

3.3.1 Growth medium trial for optimal *Armillaria* growth rate and biomass production

Armillaria colony size and growth rate

Armillaria colony area was significantly larger after 21 days when grown on the carrot (glmmPQL; t= 24.00, p< 0.00), and tomato (glmmPQL; t = 3.15, p < 0.00) based agar compared to colony area when grown on malt-based media (Figure 3.2). The growth rate was significantly greater on the carrot media when compared to malt (KW; chi squared = 3.92, p $<$ 0.00, df = 2) and tomato (KW; chi squared = 2.67, p = 0.004, df = 2) (Table 3.2).

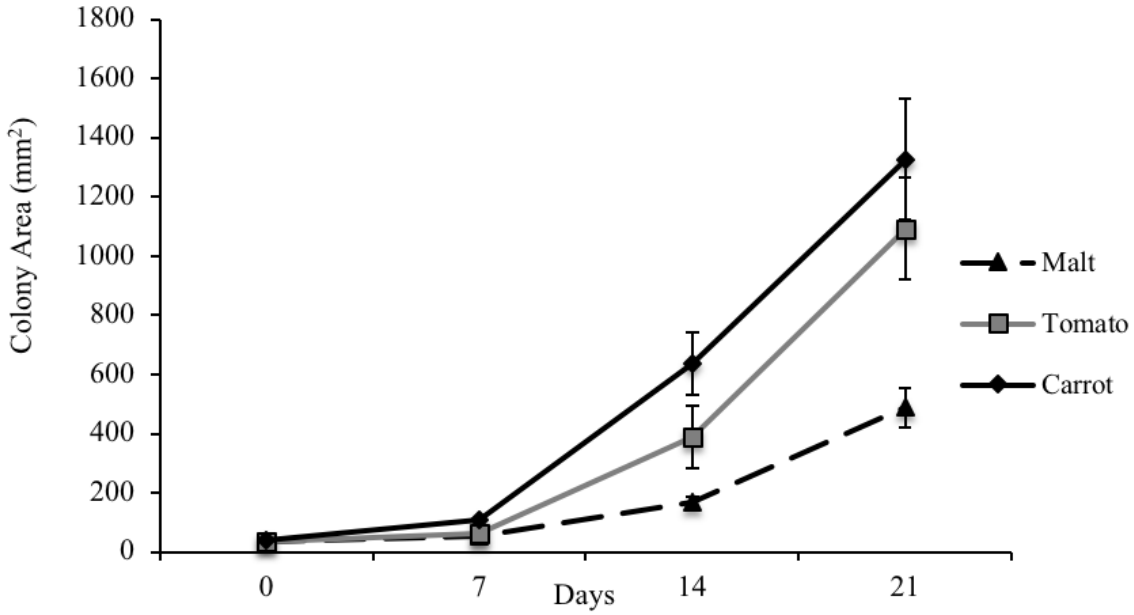


Figure 3.2 – *Armillaria* growth on malt, tomato and carrot based media, error bars represent standard error (±SE)

Observations on rhizomorph formation and phenotype on different growth media

The tomato medium demonstrated the highest number of rhizomorphs growing through the agar (Table 3.3). However, there was no significant difference (KW; chi squared = 4.92, df = 2, p = 0.11). The production of rhizomorphs did not appear to be consistent between

Table 3.2 – *Armillaria* average growth rate per week (mm/wk) on tomato, malt and carrot based media (± SE).

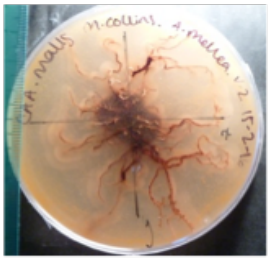
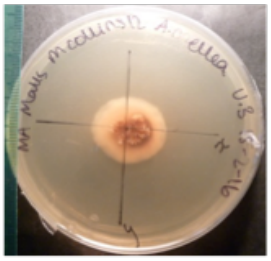
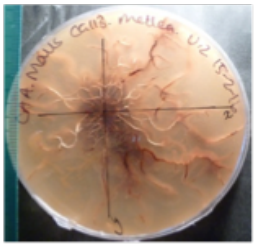
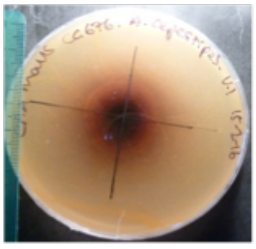
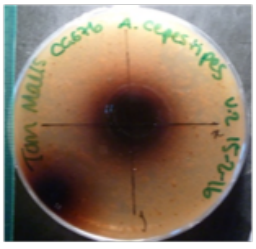
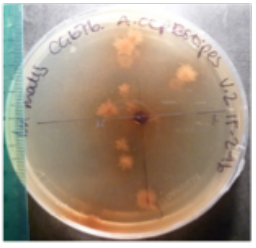
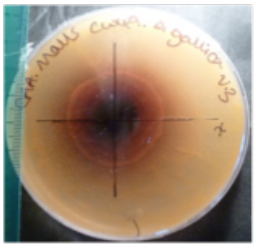
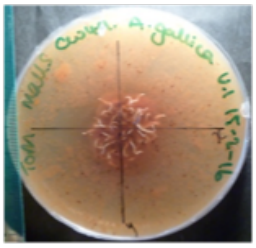
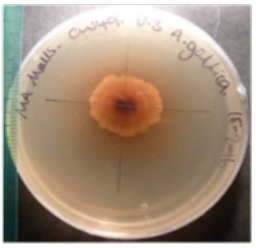
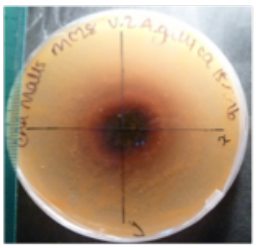
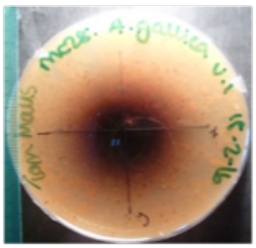
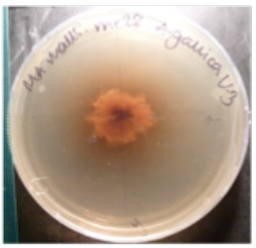
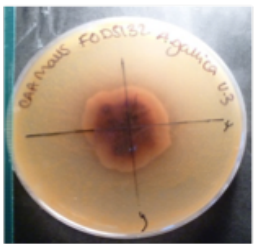
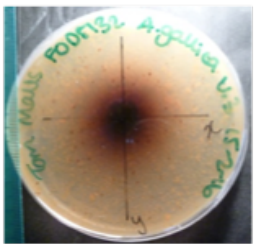
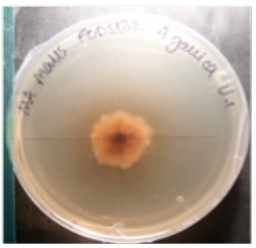
	N^o. of Isolates	**Tomato**	**Malt**	*Carrot*
Growth rate/week (mm/wk)	10	8.90(±1.0)	5.71(±0.5)	10.76 (±0.7)**
Species				
A. gallica	4	6.45(±0.69)	5.5(±m0.42)	9.19(±0.56)
A. mellea	2	17.64(±1.26)	8.89(±1.06)	17.79(±0.69)
A. tabescens	1	12.18(±2.91)	2.88(±0.85)	9.59(±0.51)
A. ostoyae	3	4.60(±0.46)	4.63(±0.65)	8.07(±0.91)

Table 3.3 – Number of rhizomorphs grown on each media type (± SE).

		Tomato	**Malt**	**Carrot**
Average N^o of rhizomorphs		9.34(± 2.32)	1.97(± 0.76)	3.33 (± 1.09)
Isolate	**Species**			
CG113	*A. mellea*	34.0	11.33	13.33
CG522	*A. ostoyae*	2.0	0	1.0
CG676	*A. gallica*	3.67	0	0
CW48	*A. ostoyae*	0	0	0.33
CW49	*A. gallica*	9.67	0	2.33
CW53	*A. ostoyae*	0	0	0.33
FOD5-32	*A. gallica*	0	0	0.33
MC28	*A. gallica*	0	0.67	0
MCOLLINS12	*A. mellea*	24.33	7.0	14.67
OH87	*A. tabescens*	17.33	0.67	1.0

isolate and species in this study. Three of the *Armillaria* isolates shown rhizomorph growth regardless of media type (Figure 3.3).

In addition, there were detectable variations in the phenotype across treatments; the mycelium and rhizomorphs appear to have a darker crustose layer when grown on the carrot-based medium, when compared to the malt and tomato-based media. The isolates that did produce rhizomorphs on the tomato and malt media types were paler in colour and appeared to grow tighter together than those grown on the carrot agar (Figure 3.3). Some isolates propagated on tomato-based medium appear to have more consistent and darker extracellular excretions, seen as a halo around the fungal colony; however, those were less visible in the carrot and malt media (Figure 3.3).

	CARROT	TOMATO	MALT	
MCOLLINS1				
2				
CG113				***A. mellea***
CG676				
CW49				
MC28				
FOD5-32				***A. gallica***

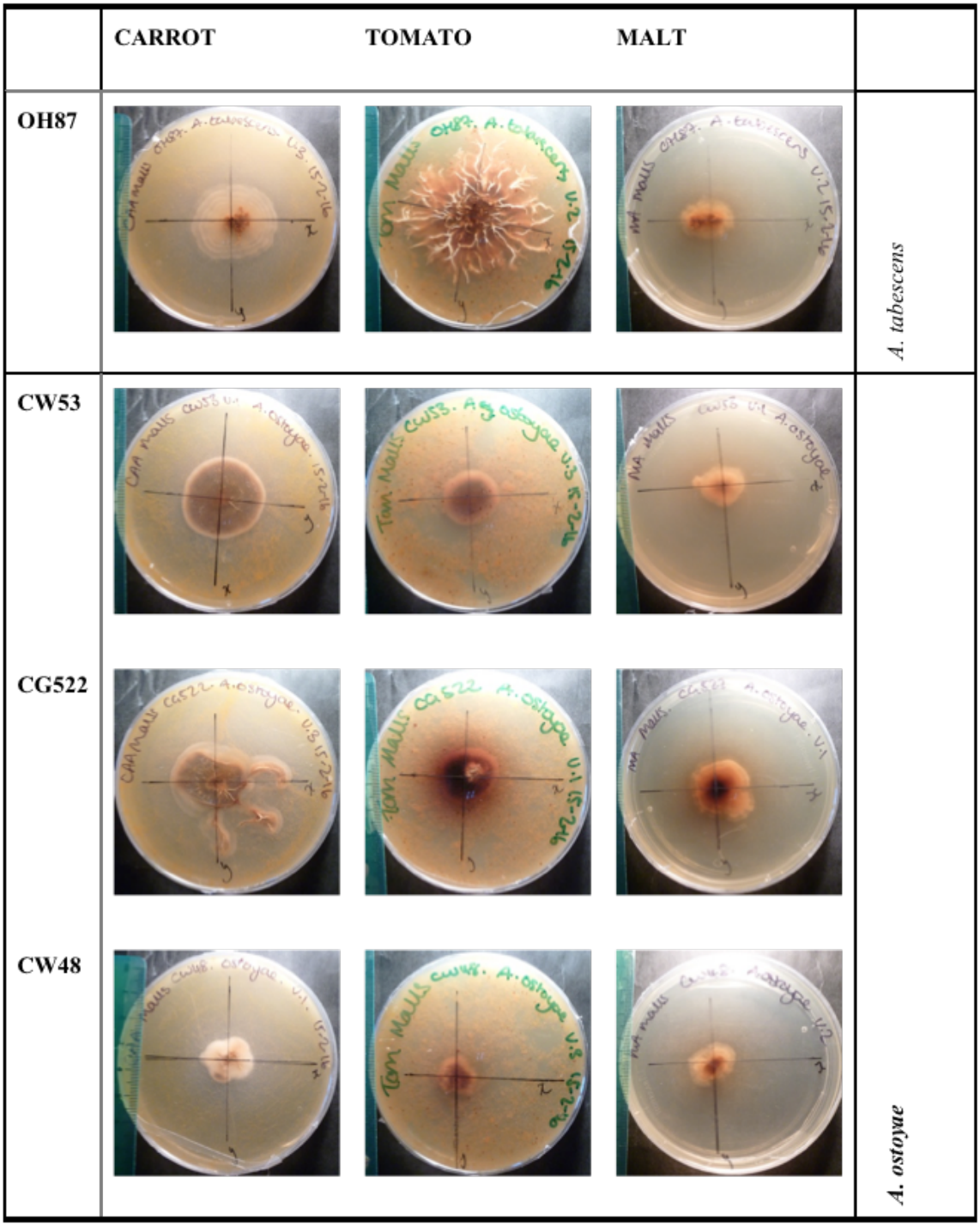


Figure 3.3 – Comparison of *Armillaria* isolate phenotype and growth on carrot, tomato and malt based medium

3.3.2 DNA extraction and PCR optimisation

The FastDNA^{TM} Spin Kit extracted produced a good DNA yield, average concentration of 18.4 ng/μl(Table 3.4)., and produced a higher DNA quality across all isolates used in the study when visualised on the gel electrophoresis. Griffiths DNA/RNA co-extraction method did produce the highest yield out of the three methods, average concentration of 24.47 ng/μl (Table 3.4), however, this DNA was of a lesser quality when compared to the FastDNA^{TM} Spin Kit when visualised on the gel electrophoresis (Figure 3.4).

Table 3.4 – DNA yields from 10 *Armillaria* isolates using different DNA extraction kits (± SE), measured using Qubit^{TM} dsDNA HS Assay Kit.

		Griffiths DNA/RNA co extraction	**FastDNA^{TM} Spin Kit**	**Qiagen mini plant kit**
Average DNA conc. (ug/μl)		24.47(±2.16)	18.4(±0.92)	1.77(±0.23)
Isolate	**Species**			
TG1	*A. gallica*	13.4	11	0.9
OH87	*A. tabescens*	19.8	6.3	0.8
FOD5-32	*A. gallica*	13.7	5.72	1.4
MCOLLINS12	*A. mellea*	10.9	24	1.2
MC28	*A. gallica*	34.8	19.3	0.9
BW6	*A. gallica*	11.3	18.3	0
BW7AC	*A. gallica*	12.8	5.54	1.9
CW53	*A. ostoyae*	*	46.6	2.6
CW49	*A. gallica*	21.3	35.8	8
CW48	*A. ostoyae*	27.6	72.2	0

* No data

When visualised using gel electrophoresis, the gDNA extracted using the FastDNA^{TM} Spin Kit was of good quality seen as smear of approximately 10 kb, and DNA is a lower quality when extracted using the Griffiths DNA/RNA coextraction method, observed as smearing within the lane, indicating the presence of RNA and degraded gDNA (Figure 3.4 A, B). Amplicons for isolates were only achieved during PCR amplification after the DNA extractions were first purified using the zymo clean and concentrate kit from using both DNA extraction methods (Figure 3.4 C, D), except CW49 which produced an amplicon using unpurified DNA for EF1-α and ITS only.

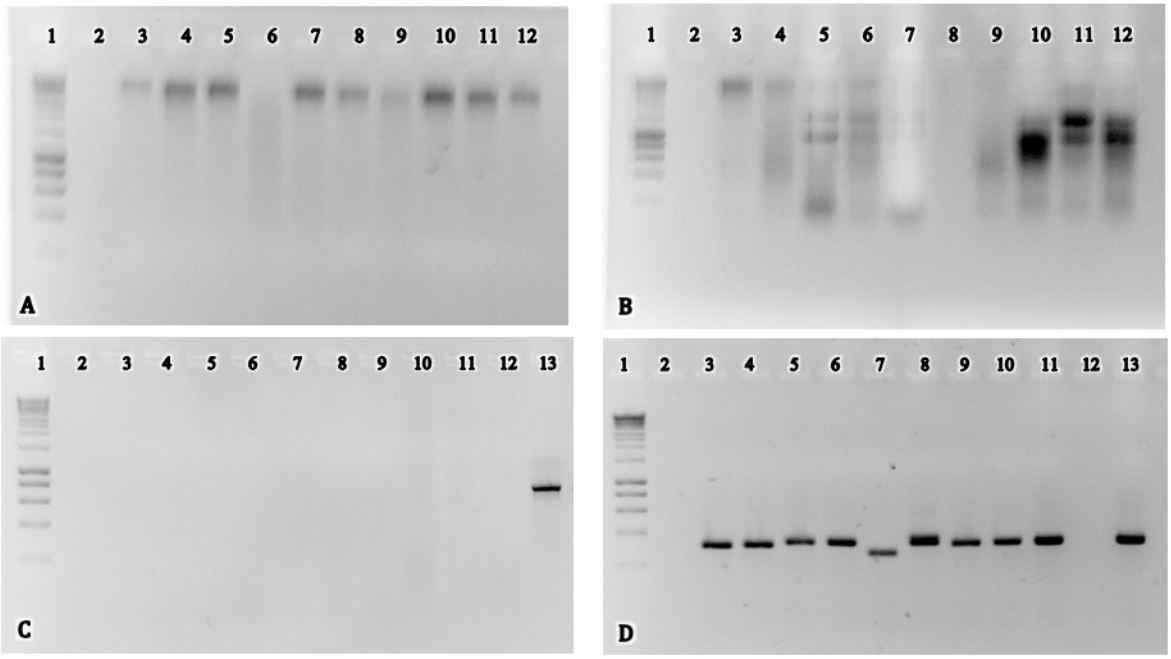


Figure 3.4 – Gel electrophoresis of genomic DNA (gDNA) extracted from 10 *Armillaria* isolates using different methods, lane 1 1KB ladder, lane 2 - blank lane, 3-12 genomic DNA, A) FastDNA^{TM} Spin Kit, B) Griffiths DNA/RNA co extraction method. C) PCR productions without clean up step, lane 1 1KB ladder, lanes 3-12 failed PCR amplification using unpurified gDNA (using ITS1/4 primers), lane 13 PCR positive control. D) Amplicons produced from PCR amplification, using purified gDNA using the zymo clean and concentrate kit (using EF1-α primers), lane 1 1KB ladder, lanes 3 -11 using purified gDNA, lane 13 PCR positive control.

3.4 Discussion

3.4.1 Media Trial

This study found that the addition of macerated tomato and carrot did significantly increase the mycelial biomass of the *Armillaria* isolates when compared to a basic sugar based medium, such as malt. After 14 days, both carrot and tomato agar generated higher *Armillaria* biomass than the malt-based medium, which took 21 days to reach the same level of biomass production. A shorter time spent in culture reduces the potential for contamination with secondary organisms, and is therefore beneficial. The main benefit however is that an increase in grow rate allows for quicker species identification either via morphologicalor subsequent molecular identification.

Active substances present by the carrot agar are thought to encourage *Armillaria* growth and the formation of the darker mycelium, also observed herein (Figure 3.3). These substances, however, have yet to be identified (Iwao and Namio, 1994, 1995; Shim et al., 2006). As carrots

are root vegetables it could be that *Armillaria* can grow in a more similar fashion to how it would behave when infecting roots of plants and crops in the forest, garden or agricultural fields, where rhizomorphs are a common indicator of *Armillaria* species presence as either a pathogen or saprophyte (Guillaumin et al., 1993; Denman et al., 2017). In this study, the formation of rhizomorphs between species was not attributed to medium type. There were species and isolate variations in the formation of rhizomorphs which are not always seen in culture or in the field, and the reasons for such variation are unclear. Ford et al. (2015), found that some isolates of *Armillaria* did grow significantly faster when tomato was added to the fruiting medium (Ford et al., 2015). This study found that *A. tabescens* had the largest growth rate on the tomato media when compared to carrot and malt, however, there is a low sample size for this species and more isolates would need to be tested to determine if this was due to isolate variations or whether *A. tabescens* grow preferentially on the tomato-based medium (Table 3.2).

3.4.2 DNA extraction and PCR optimisation

Polysaccharides from fungal cell walls are the largest contaminant in fungal DNA extractions (Kuhad, Kapoor and Lal, 2004; Niu et al., 2008; Möller et al., 2016). Here, the FastDNA^{TM} Spin Kit was determined to be the most appropriate DNA extraction method to be used throughout the project due to good quality DNA and the consistency in the DNA yield between the isolates and species (Figure 3.4). This kit is also preferred due to the use of safer chemicals, and thus not requiring a fume hood. Nevertheless, this kit did show signs of polysaccharide contamination and therefore the DNA extraction still needed to be purified before any downstream processes, such as PCR, could be completed. The Griffiths DNA/RNA co extraction appears to have less polysaccharide carry over in the final extraction, probably precipitated out into the CTAB buffer during the cell lysis step (Kuhad, Kapoor and Lal, 2004). Although this method is cheaper and quicker, it was not selected as the most appropriate due to fragmented DNA and inconsistent yield across all the isolates and species, alongside phenol contamination in a few extractions. Further testing using cellophane or filter paper placed onto the surface of an agar plate and inoculated with a plug of *Armillaria* isolate, will help to avoid contamination from the growth media (Elías-Román et al., 2013). The good quality DNA extracted from the isolates allows for future testing using phylogenetic marker genes; this analysis would give more understanding of isolates in the collection, but also the

wider ecology of *Armillaria* present on oaks. *Armillaria* taxonomy is complex, therefore having an extraction method that is reliable and allows for high throughput processing of the *Armillaria* isolates will help to widen the current understanding of this genus. Greater fungal biomass also allows for more tissue to be available for DNA extractions, allowing for more isolates to be analysed phylogenetically and potential for future studies to use next generation sequencing to provide more detailed insights into *Armillaria* taxonomy and ecology.

Chapter 4

Phylogenetic analysis of UK *Armillaria* species present on oaks, and development of a High Resolution Melting (HRM) – based rapid diagnostic tool

4.1 Introduction

Armillaria is a genus of basidiomycete fungi, with three body forms; mycelium, rhizomorphs and fruiting bodies, and the production of those different body forms varies greatly between species and growing conditions. Historically all *Armillaria* strains that caused root rot were all described as *A. mellea*. Early identification of *Armillaria* relied upon application of the morphological species concept, using predominately basidiocarp morphology, comparing a combination of macro and micro morphological characteristics of the fruiting body (Herink, 1973 in Watling et al., 1991; Baumgartner et al., 2011; Denman et al., 2017). Identification using basidiocarps is difficult, as either the basidiocarps must be collected from the environment, or in the absence of basidiocarps, suspected *Armillaria* mycelium from infected tissue would have to be collected to induce the production of fruiting bodies which takes approximately 40+ days, and some species it is impossible to induce mushroom formation in culture outside of natural cycles (Ford et al., 2015; Shim et al., 2006). Later identification on European and Australasian isolates used mycelium and rhizomorph morphology in combination with basidiocarp morphology (Watling et al., 1991).

In the 1970s, through discovery of the bifactorial sexual incompatible mating system, identification of *Armillaria* species could be determined by taking advantage of the sexual mating interaction. This is done by the use of haploid mating strains, those strains represent each species and if compatible mating occurs the mycelium of the haploid strain will change morphology from a white fluffy appearance to a darker crustose, indicating diploidisation of

the nucleus within the mycelium of the haploid strain (Baumgartner et al., 2011; Carvalho et al., 1995; Heinzelmann et al., 2018; Ullrich and Anderson, 1978). The biological species concept, states that interfertile groups are the same species, and interfertile groups represent different species. Using this method, *Armillaria* species are divided by continents North American biological species NABS I-V, European biological species groups EBS (A-E), Japanese Nagasawa groups NAG (A-D, F-H and T) and Chinese biological species CBS A-P (Anderson et al., 1980; Baumgartner et al., 2011; Coetzee et al., 2000; Denman et al., 2017; Guo et al., 2016; Watling et al., 1991). Those *Armillaria* species groups (NABS, EBS and CBS), show complex interfertile and intersterile interactions across the groups, i.e Anderson et al., (1980) demonstrated that NABS IV and X groups that are intersterile between each other, on the other hand, both show interfertile interactions with EBS group B (*A. cepistipes*). EBS group C (*A. ostoyae*) is found to be interferile with both NABS I (*A. ostoyae*) and VII (*A. gallica*) groups (Anderson et al., 1980; Coetzee et al., 2018).

DNA-based methods have also been used in taxonomic research to find similarities between taxonomic linkages and the morphological characteristics of the mushrooms from species identified using the morphological species concept (Pildain et al., 2009, 2010; Baumgartner et al., 2011)

4.1.1 Molecular techniques for *Armillaria* species specific identification

DNA-based species identification methods combining PCR amplification and sequencing of phylogenetic marker genes, such as ITS (internal transcribed spacer), IGS (intergenic spacer) and EF1-α (translation elongation factor) are the most commonly applied method for species identification, and other methods are based on morphology and incompatibility mating tests (Antonín et al., 2009; Baumgartner et al., 2010; Coetzee et al., 2001; Denman et al., 2017; Guo et al., 2016; Klopfenstein et al., 2017; Koch et al., 2017; Maphosa et al., 2006; Mulholland et al., 2012; Park et al., 2018; Pérez-Sierra et al., 1999; Volk et al., 1996). Phylogenetic marker gene sequences provided by this approach are subsequently searched against a chosen databased to give a species ID, but this process assumes that all sequences submitted to the public databases have the correct species ID. These data can also be used to generate phylogenies by comparing sequences corresponding to the same gene/region across different isolates or species. The phylogenetic analysis will identify differences in nucleotide

composition within the sequences, and phylogenetic trees can then be constructed for each gene region or combined to create a species tree.

A DNA based method using PCR-RFLP technique can also be used to determine *Armillaria species*. This PCR-RFLP uses tradition PCR based on IGS gene region, the amplicons were then exposed to restriction digestion enzymes *Alu I, Nde 1 and Bsm I*. Those enzymes cut the DNA strands into fragments of differences sizes between species. The digested DNA regions are then visualised on a gel electrophoresis, each species have a different and specific pattern of fragments. The different fragments patterns are compared and species can be identified. The *Alu I* effectively distinguished between *A. mellea* and *A. gallica*, for other species further digestion is needed using the *Nde I* or *Bsm I* (Pérez-Sierra et al., 1999).

Both DNA based molecular method is quicker and more accurate when compared to traditional methods for species identification, nevertheless, this method still requires a starting material of DNA extracted from pure fungi cultures isolated from the infected material. This isolation process to species identification can take a number of weeks to get to from isolate in to pure culture and to get a large enough biomass for DNA extraction and PCR.

Rapid identification of *Armillaria* species is therefore problematic and currently a barrier to understanding the ecology of *Armillaria* species on plant and tree species globally. The taxonomy of *Armillaria* is complex, and potentially not fully resolved, causing issues for the phylogenetic analysis and rapid diagnostic development. The genus has different groups based geographical locations; NABS, EBS and CBS groups, and many of those species groups demonstrate complex intersterile and interfertile interactions. The *A. gallica* species is a large and complex group, with *A. cepistipes* as a sister clade, with the presents of distinct polyphyletic clades or cryptic species (Baumgartner et al., 2011; Coetzee et al., 2018; Keča et al., 2015).For example, during this PhD project *Armillaria* tabescens was reclassified into a new sub genus *Desarmillaria tabescens* (Heinzelmann et al., 2019; Park et al., 2018). However, in this project, it will be referred to as *Armillaria* tabescens.

Current molecular techniques still rely on isolating and growing the fungus in pure culture or pore prints from fruiting bodies for DNA extraction and subsequent PCR, PCR-RFLP and sequencing. When pure cultures are used this often requires 4-6 weeks before species identification can be determined (Ford et al., 2015). This also requires space in incubators and laboratories for isolating and then growing the *Armillaria* isolates limiting the number of samples which can be process at one time. The aim of this project is to firstly to compete

phylogenetic analysis on the panel of isolates collected from across the UK. Secondly, to develop a rapid diagnostic tool using the panel of *Armillaria* isolates collected to reduce the time it takes to give a species identification on samples from infected sites. The ideal tool would be able to give a species identification on isolates and environmental samples within hours to a few days. This would be beneficial by reducing time to species identification and increasing the number of samples that can be analysed, therefore allowing high throughput of samples, generating more data on the ecology of *Armillaria* disease spread across a site.

4.1.2 Rapid diagnostic development - High Resolution Melt (HRM) curve analysis

High resolution melt curve analysis is a molecular diagnostic tool used identify species and DNA sequence variants within a group of isolates. It is an inexpensive and simple closed tube method, relatively fast technique for high sensitivity genotyping, mutation scanning and gene scanning (Alnuaimi et al., 2014; Słomka et al., 2017). It is a PCR-based method, where fluorescence is generated using an intercalating dye as double stranded PCR amplification products are generated. Subsequently, the DNA PCR amplicons are gradually heated and will denature at a specific temperature based on their nucleotide composition, at which stage the fluorescent dye is released and can be measured as a melt curve. The temperature of DNA denaturation varies with nucleotide composition, with nucleotide A/T affecting the denaturation temperature by 0.2-0.3°C and nucleotides C, G affecting the temperature by 0.5°C (Biosystems, n.d.). The denaturation and fluorescence released from this process creates a melt curve and the profile of this melt curve depends on the nucleotides within the amplicon, using the melt curve profiles the variants are then assigned based on those differences.

4.2 Materials and methods

4.2.1 Panel collection

A panel of 22 isolates representing all species found on UK oak were collected from across the UK including; *A. gallica*, *A. mellea*, *A. ostoyae* and *A. tabescens*. These isolates came from fungal collections at Forest research, Farnham and Dr Mike Hale, Bangor University and were previously isolated from a variety of sites, such as, oak plantations, private and

public gardens (Table 4.1). Those isolates were grown onto carrot-based agar media for 3 - 4 weeks 25°C.

Table 4.1 – The panel of isolates collected from Bangor University and Forest Research, UK (Denman et al., 2017).

Collection	Species	Isolate Code	Host	Host location
Bangor University Isolates	*A. mellea*	CG113	Unknown	Unknown, UK
	A. mellea	106H	Unknown	Unknown, UK
	A. ostoyae	CG522	Unknown	Unknown, UK
	A. gallica	FR07006 – NSG66	Unknown	Unknown, UK
	A. cepistipes	CG676	Unknown	Unknown, UK
	Unknown	W1	Oak log	Treborth, North Wales, UK
	Unknown	W6	Oak log	Treborth, North Wales, UK
	Unknown	W4	Oak log	Treborth, North Wales, UK
	Unknown	W7	Oak log	Treborth, North Wales, UK
Forest research isolates	*A. gallica*	CW21	Oak tree	Plantation, Chestnut wood tree 2, Chesnuts Wood, UK
	A. ostoyae	CW48	Oak tree	Plantation, Chestnut wood tree 4, Chesnuts Wood, UK
	A. gallica	CW49	Oak tree	Plantation, Chestnut wood tree 4, Chesnuts Wood, UK
	A. ostoyae	CW53	Oak tree	Plantation, Chestnut wood tree 4, Chesnuts Wood, UK
	A. gallica	FOD5/32	Oak tree	Plantation, Speculation tree 5, Speculation Woods, UK
	A. gallica	GW20	Oak tree	Woodland, Grafton wood tree 6, UK
	A. tabescens	H21	Oak tree	Parkland, Hatchlands tree 1, UK
	A. gallica	MC27	Oak tree	Woodland, Morrend common tree 1, UK
	A. gallica	MC28	Oak tree	Woodland, Morrend common tree 2, UK
	A. mellea	MC12	Oak tree	Garden, Mrs Collins, Kent, UK
	A. mellea	MK7	Oak tree	Garden, Mr Keast, Reading, UK
	A. tabescens	OH87	Oak tree	Garden, Oak Hill, Kent, UK
	A. gallica	RW4	Oak tree	Woodland, Runswood, Wattlington, Norfolk, UK

4.2.2 Species identification and phylogeny

Fresh mycelial growth, approximately 200 mg, of each *Armillaria* isolate was scraped from the agar surface using a flame sterilised scalpel and DNA was extracted using FastDNA spin kit, according to the manufacturer's instructions, and stored at -20°C. End-point PCR was used to generate amplicons EF1-α, ITS and IGS using the primer sets; EF1-α gene region were; EF595F and EF1160R (Kauserud and Schumacher, 2001) and, ITS; ITS1 and ITS4 (White et al., 1990) and IGS; IGS01 (Duchesne and Anderson, 1990)and IGS P1 (Hsiau, 1996) (Table 3.1). PCR reactions comprised 12.5 μl GoTaq® G2 green mastermix, 2X (Promega, Madison, WI, USA), $MgCl_2$ 1.5mM, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 2 μl DNA template (1-40 ng/μl) and 8.5 μl PCR water, total volume of 25μl. PCR cycling conditions were as follows; 30 cycles of denaturing at 95°C for 2 minutes, annealing at 55°C using ITS IGS or 48°C using EF1-α for 30 s and extension at 72°C for 1 minute, with a final 5 minutes extension step at 72°C, in a total volume of 25μl. PCR amplification products were visualised on a 2 % electrophoresis gel, concentration was determined using the Qubit^{TM} dsDNA HS Assay Kit, and sequenced using a sanger sequencing.

The forward and reverse sanger sequences from EF1-α, ITS, IGS regions from the isolates

were quality clipped using FourPeaks (2004-2015, Mekentosj, Amsterdam). Quality clipped Sanger sequences from this study were combined with publicly available *Armillaria* sequences obtained from Forest Research (EF1-α and IGS only) (Sayers et al., 2019). The sequences were imported into Mega7, and trimmed to equal coverage (Kumar et al., 2016), aligned using MUSCLE. In Mega7, maximum likelihood phylogenetic trees were generated using; the model TN93, with gamma and evolutionary invariant, and 1000 bootstrap values (Kumar et al., 2016). The subsequent phylogenetic trees were then edited using interactive tree of life (ITOL) (Letunic and Bork, 2019), and branches with a bootstrap value below 0.4 were collapsed, branches with a bootstrap above 0.60 were labelled with black dots (Keča et al., 2015). A species tree was produced using BEAST analysis v2.4.5, using EF1-α and IGS gene regions, the ITS gene region was omitted from the BEAST analysis due to incomplete data set.

4.2.3 HRM Rapid diagnostic development

Following the phylogenetic analysis, EF1-α region had the greatest resolution between all four species and was therefore chosen as the candidate gene region for development of primer sets for the rapid diagnostic development. A HRM primer set was also designed using IGS gene region. The HRM analysis is dependent on differences between amplicons,and as such, amplicon size can greatly affect the sensitivity of HRM reactions, with larger amplicons showing lower resolution. Consequently, amplicon sizes can vary between 54 - 250 bps (Słomka et al., 2017; Yamagata et al., 2018). During this study, various primer sets were designed to generate PCR amplicons that fitted within this range. For HRM analysis, Genus-specific primers were designed using the alignment files generated by the earlier phylogenetic analysis and the addition of fungal out-group sequences from NCBI (Table 4.2. The primers were developed to amplify marker gene areas with species-specific variable regions to maximise differentiation between the species (Table 4.3).

All newly designed primer combinations were initially screened using end point PCR. These end point PCR products were used to verify that the primer sets only produce single amplicons of the correct size at 60°C, the optimal annealing temperature for HRM, with no, or limited, primer-dimer present and were visualised using gel electrophoresis. PCR reactions were completed using 12.5 μl GoTaq® G2 green mastermix, 2X (Promega, Madison, WI, USA), $MgCl_2$ 1.5mM, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 2 μl DNA

Table 4.2 – Fungal sequences from NCBI (downloaded October 2016) used as out-groups to develop *Armillaria* genus species primers

Genbank reference	Species	Strain ID
DQ029194.1	*Hymenopellis radicata*	AFT0L-ID 561
KU289105.1	*Oudemansiella cubensis*	MCA 5434
KU170936.1	*Dactylosporina glutinosa*	MCA 1775
KU289106.1	*Cyptotrama spp.*	MCA 2483
GU187732.1	*Physalacria bambusae*	CBS 712.83
KU289110.1	*Guyanagaster necrorhizus*	G31.4
JN796056.1	*Flammulina velutipes*	CBS 771.81

template (1-40 ng/μl) and 8.5 μl PCR water, total volume of 25 μl. Using; initial denaturation 95°C 2 minutes, 40 cycles of; denaturation 95°C 1 minutes, Annealing 60°C 30 seconds, extension 72°C 1 minute, and followed by final extension 72°C 5 minutes and hold 4°C. Primer combinations that passed this initial screening process were taken forward for HRM validation. The thermofisher MeltDoctor^{TM} HRM Master Mix and bioline SensiFAST^{TM} HRM mastermix were trialled again each other, and bioline SensiFAST^{TM} HRM mastermix shown better amplification during the PCR section of the HRM run and so the results were more consistent therefore the SensiFAST^{TM} HRM mastermix was chosen for the final development and validation. The primer combinations were tested using the SensiFAST^{TM} HRM master mix 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl DNA template (1-25 ng/μl) and 6.6 μl PCR water, total volume of 20 μl. PCR step: initial denaturation 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, at 60°C data collected on amplification efficiency of the assay, this was followed by the HRM stage: 95°C for 10 seconds, 60°C for 1 minute, and the dissociation step to generate the melt curve profiles for each sample, data collected every 0.025°C from 60°C to 95°C, and to finish the assay 95°C for 15 seconds and then 60°C for 15 seconds. The primers were tested using representative of each species, 12 isolates taken out of a pool of 21, depending on availability of good quality DNA. The DNA quality and DNA concentration is thought to also effect the variant calling capabilities of fungal isolates (Yamagata et al., 2018), initial tests were done using genomic DNA, however due to the amount of good quality DNA required, and to improve efficiency of the assay this was changed to control DNA in the form of EF1-α PCR amplicons. The specificity of the HRM assay at genus-level was tested using primer combination 'P134' against DNA extracted from basidiocarps from three species *Lentinula edodes, Agrocybe aegerita* and *Agaricus bisporus*.

Table 4.3 – Primer combinations designed and screened using endpoint PCR for HRM analysis.

Target gene	Primer set	Primer name	Sequence	Amplicon length
EF1-α	EF1ARMb	EF1ARMF2Y	ACATGTTGGAGGAGTCYGCC	77
		EF1ARMR2B	ATGCTWYMRASAACGTTAAGV	
EF1-α	EF1ARMt	EF1ARMF2T	ACATGTTGGAGGAGTCTGCC	77
		EF1ARMR2T	ATGCTWYMRAGAACGTTAAGA	
IGS	IGS101	IGS101	CTTGTTCTAAAGATTTGTTCAAC	101
		IGS2R	CTGTCGCGAGTGCACA	
IGS	IGS110	IGS110	CTTGTTCTAAAGATTTGTTCAAC	110
		IGS1R	AGTAACATGCTGTCGCG	
IGS	IGS107	IGS107F	GTTCTAAAGATTTGTTCAACTTTG	107
		IGS107R	AGTAACATGCTGTCGCG	
EF1-α	P267	P267F	CGTCGCCGTCAACAAGATGG	267
		P267R	AGAACGTTAAGGGACAGCACTGA	
		P267TR	TTCAACAACGTTAAGCCGCC	
EF1-α	P145	P145F	TGCTGTCCCTTAACGTTCTCTGT	145
		P145TF	GGCGGCTTAACGTTGTTGAA	
		P145R	GAGAGGCTTGTCGGAGGGAC	
EF1-α	P120	P145F	TGCTGTCCCTTAACGTTCTCTGT	120
		P145TF	GGCGGCTTAACGTTGTTGAA	
		P120R	GACTCTCCTCGATGCCATTGA	
EF1-α	P134	P134F	TTAACGTTCTCTGTAGCAT	134
		P145TF	GGCGGCTTAACGTTGTTGAA	
		P145R	GAGAGGCTTGTCGGAGGGAC	
EF1-α	103A	P134F	TTAACGTTCTCTGTAGCAT	103
		P145TF	GGCGGCTTAACGTTGTTGAA	
		P120R	GACTCTCCTCGATGCCATTGA	

4.3 Results

4.3.1 *Armillaria* species identification and phylogeny

The phylogenetic tree generated from EF1-α, and IGS sequence data, respectively, split all the isolates into distinct species clusters with bootstrap value of 0.95 or above representing *A. mellea*, *A. tabescens*, *A. ostoyae* (Figure 4.1), Figure 4.2). A cluster was also present for *A. gallica*, the bootstrap value for EF1-α is only 0.8 (Figure 4.1) and for the IGS data lacks resolution within the *A. gallica* group, as branches collapsed as bootstrap values > 0.4, Figure 4.2. The *A. gallica* cluster in EF1-α indicates a potential split into two sub clades, with bootstrap 0.66 (Figure 4.1). The phylogenetic tree generated from the ITS lacks support for all 4 species groups, only showing 2 distinct groups; *A. mellea* and *A. tabescens*, supported with bootstrap values 0.92 and 0.75 respectively (Figure 4.3). The isolates that represent *A. ostoyae* (isolate CW48 and CW53) and *A. gallica* grouped together supported with 0.82 bootstrap values (Figure 4.3). The BEAST analysis was completed using two loci, EF1-α

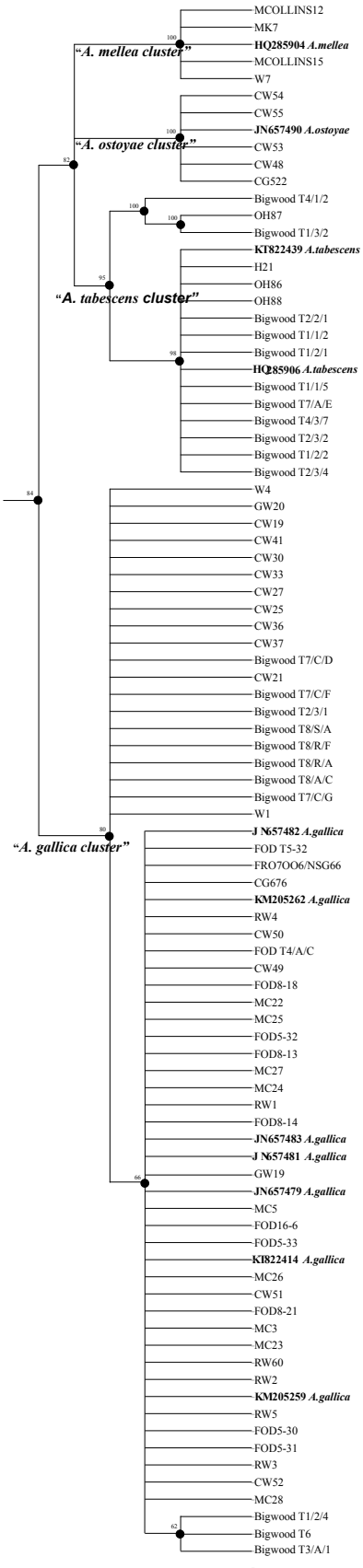


Figure 4.1 – Phylogenetic tree generated using EF1-α sequences, based on the maximum likelihood. Filled black circles on the nodes indicate bootstrap ≥ 60 based on the maximum likelihood. Isolates in bold indicate reference sequences.

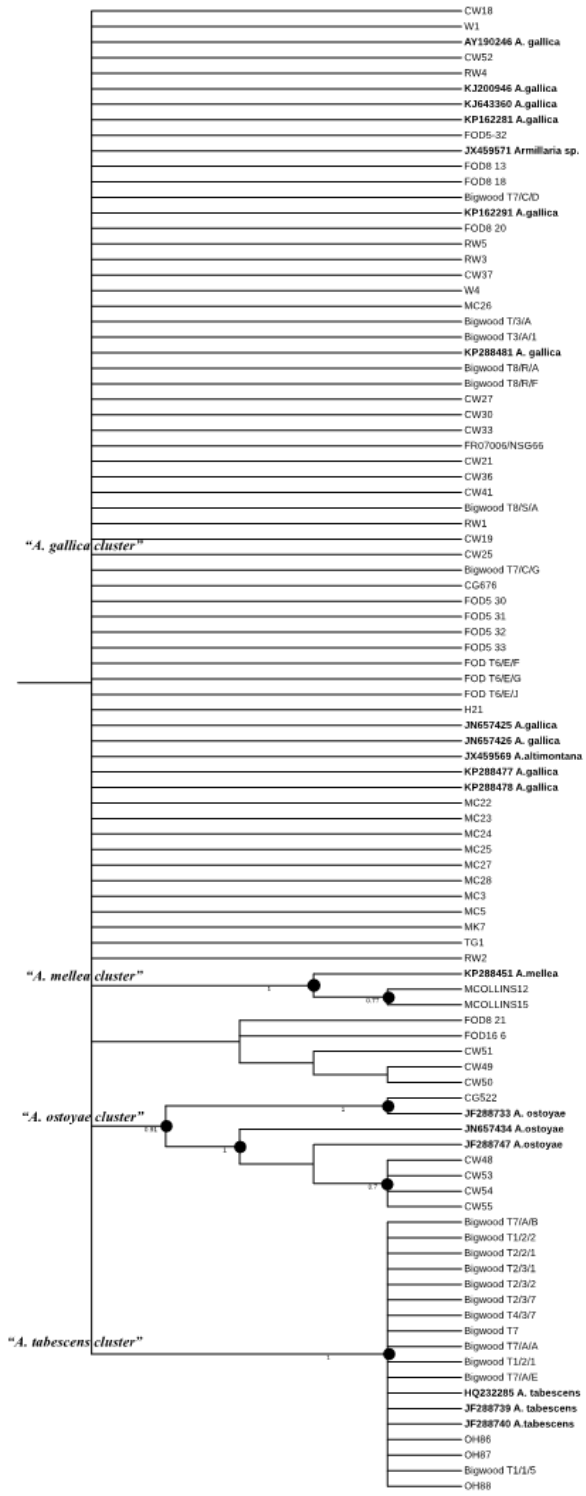


Figure 4.2 – Phylogenetic tree generated using IGS sequences, based on the maximum likelihood. Filled black circles on the nodes indicate bootstrap ≥ 60 based on the maximum likelihood. Isolates in bold indicate reference sequences.

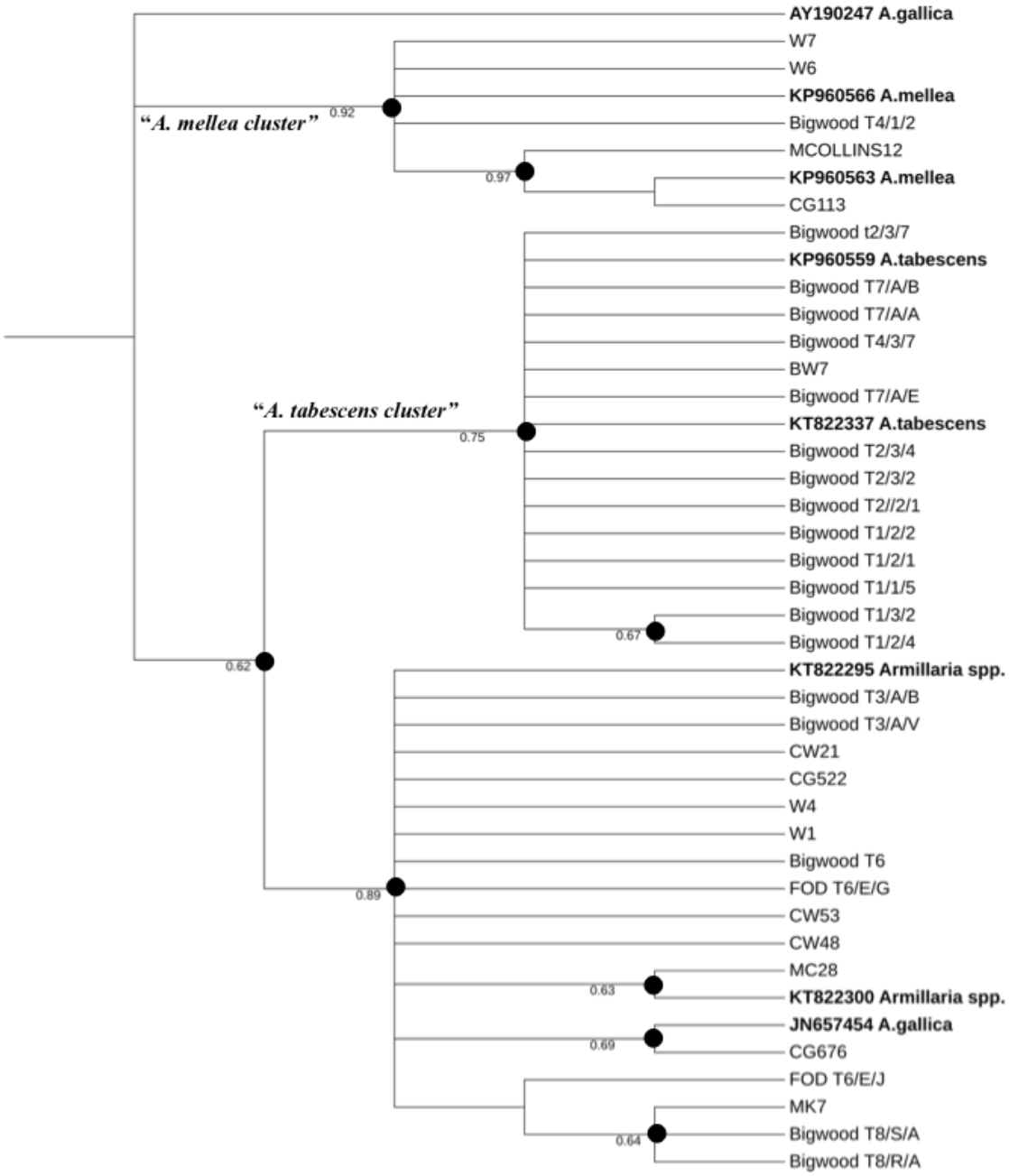


Figure 4.3 – Phylogenetic tree generated using ITS sequences, based on the maximum likelihood. Filled black circles on the nodes indicate bootstrap ≥ 60 based on the maximum likelihood. Isolates in bold indicate reference sequences.

and IGS, and demonstrated 3 distinct groups, of which one contained representatives of *A. mellea* and *A. ostoyae*, the next contained only *A. tabescens* (posterior probability limit value 0.85), and the final contained only *A. gallica* (Figure 4.4). Although the support for the *A. gallica* group is low, with a posterior probability limit value of >0.60, there is support for 3 sub clades within the *A. gallica* group, supported by posterior probability limit values; 0.84, 0.84 and 0.97 (Figure 4.4).

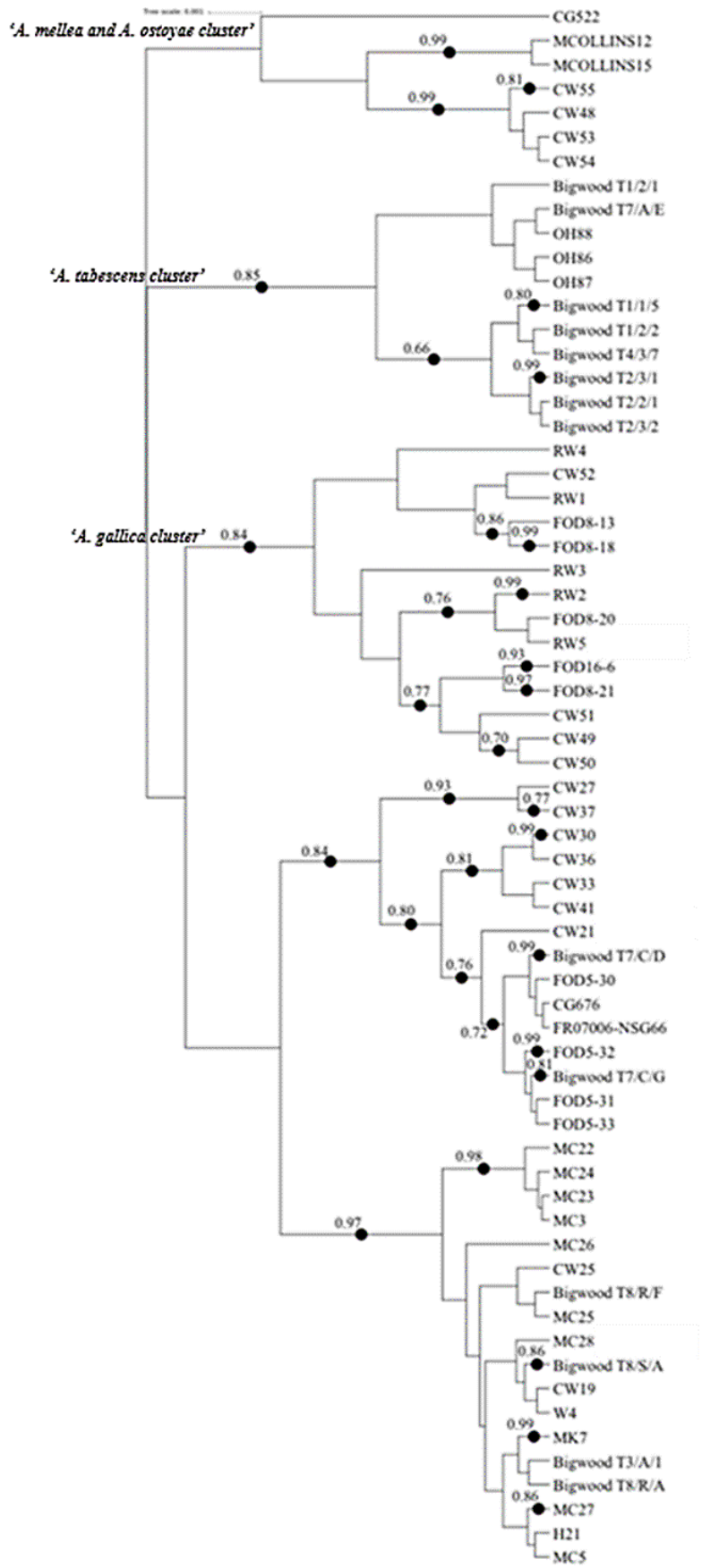


Figure 4.4 – Phylogenetic tree based on BEAST analysis of EF1-α and IGS sequence data only. Filled black circles on the nodes indicate posterior probability limit ≥ 0.60 based on the maximum likelihood. Isolates in bold indicate reference sequences.

4.3.2 High Resolution Melt curve analysis (HRM) development

Primers EF1ARM and IGS110. 101, 107

Primers EF1ARMb, EF1ARMt based on EF1-α gene region and IGS110, IGS101, IGS107 based on IGS gene region were designed, Table 1. During the end point PCR screening, the EF1ARM, IGS101 and IGS107 combinations did show unintended amplicons on the electrophoresis gel (Figure 4.5B), so were discounted from further HRM analysis and the combination IGS110 passed screening and was taken forward for HRM analysis. During HRM analysis however IGS110did not generate PCR amplification products at the PCR stage, leading to no results from the the HRM stage, and therefore IGS110 was discounted from further testing.

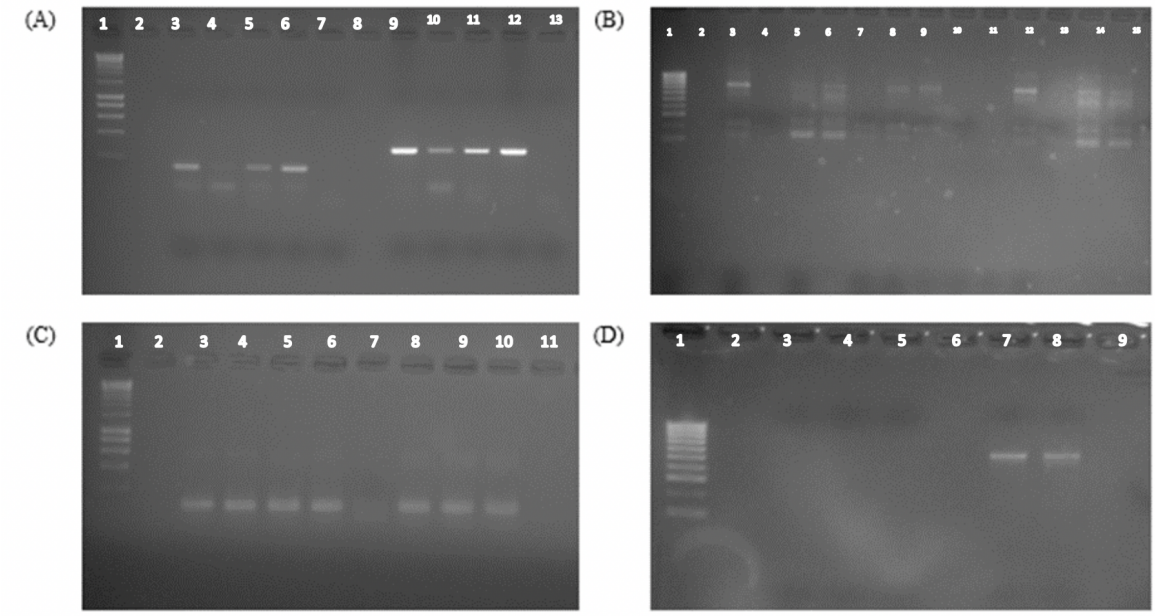


Figure 4.5 – Gel electrophoresis, using *Armillaria gallica* DNA as template. for all gels lane 1 DNA ladder, lane 2 blank lane. (A) End-point PCR; Lane 3-6, primer set P145 and lane 8-11, primer set P267, lanes 7 and 12 were no template controls. (B) End-point PCR primer set IGS110. (C) End-point PCR primer set P134. (D) End-point PCR primer P134 (lanes 3-6: *Lentinula edodes*, *Agrocybe aegerita* and *Agaricus bisporus* and lanes 5-6 *Armillaria gallica* isolates as positive control.

Primer set P145 and P267

Primer sets P145 and P267 were designed based on EF1-α gene regions Table 1. During end point PCR screening, both primers generated a single amplicon band of the correct size with no visible unintended amplicons, visualised using a gel electrophoresis (Figure 4.5A).

Table 4.4 – Species to species comparisons of the number of SNPs and approximate melt temperature differences of amplicons for HRM analysis in silica.

Primer Set		***A. gallica***				*A. tabescens*		*A. ostoyae*
		A. tabescens	*A. ostoyae*	*A. mellea*	*A. gallica*	*A. ostoyae*	*A. mellea*	*A. mellea*
P267	SNPs	32	14	17	12	37	39	24
	Temperature differences (Δ°C)	14.4	6	7	4.4	16.3	15	11.1
P145	SNPs	6	3	9	12	19	19	10
	Temperature differences (Δ°C)	2.5	0.8	3.3	4.4	8.1	8.1	3.9
P134	SNPs	7	3	5	2	6	14	6
	Temperature differences (Δ°C)	2.8	1.2	2.3	1	2.7	6.1	2.6
103A	SNPs	11	1	8	4	10	16	8
	Temperature differences (Δ°C)	5	0.5	3.8	2	5	7.3	3.6

However, primer set P267 failed HRM analysis due to inconsistency of amplification during the PCR stage of the cycle (Figure 4.6A). Primer set P145 passed HRM with 76% efficiency on calling correct variant to species, however, there was some issues with consistency, as some isolates failed to amplify or there was mis-identification of *A. tabescens* and *A. ostoyae* from *A. gallica* isolates or *A. mellea* from *A. gallica* (Figure 4.6B).

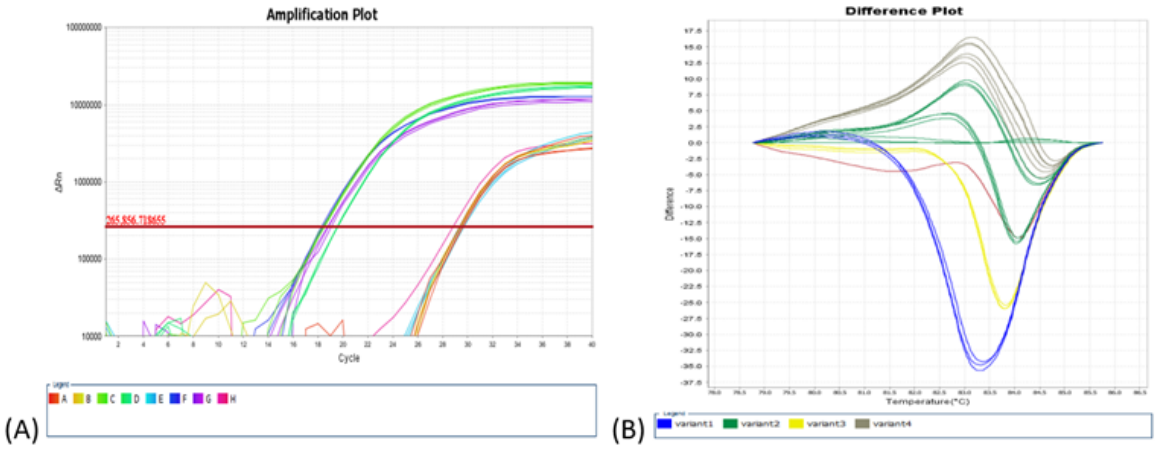


Figure 4.6 – HRM software outputs; (A) Amplification plot from primer set P267, showing inconsistent amplification during PCR, curves at cycle 14-16 is desired P267 amplification, curves shown earlier are failed amplification of the amplicon and curves later in the cycle is primer dimer amplification. (B) HRM difference plot, shows the differences between melt curve profiles from the HRM for primer set P145. *A. gallica* variant 2, *A. gallica* and *A. mellea* variant 4, *A. tabescens* variant 1 and *A. ostoyae* variant 3.

The other outcome from this work is that genus specific primers P267 and P145 can be used on samples using end-point PCR with sanger sequence of those amplicons to give species identification (Figure 4.5A). Blast searches using P145 and P267 give the correct species identification and the phylogenetic analysis of amplicons produced from using P145 show similar clustering found in EF1-α, however, *A. tabescens* is more closely clustered with *A. gallica* 2 and *A. ostoyae* more closely clustered with *A. gallica* 1 (Figure 4.7).

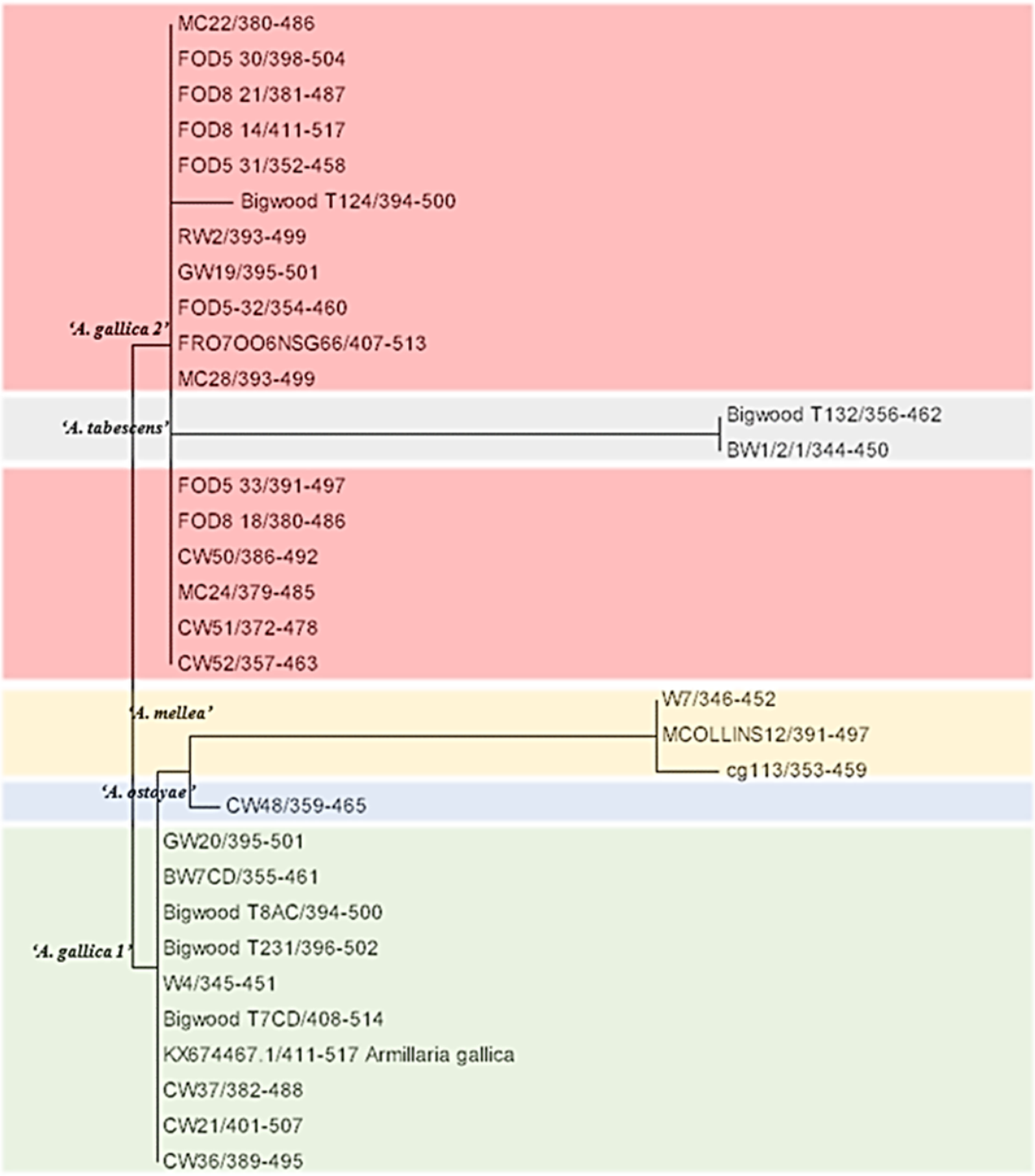


Figure 4.7 – A maximum likelihood tree to show the clustering of amplicons produced from P145 primer set using the control panel isolates.

Using P145 as a base P120, 103A and P134 combinations were developed Table 1. Those combinations were designed to reduce the effect of differences between the *A. gallica* isolates by reducing the number of SNPs present between *A. gallica* isolates but keep the differences between different species (Table 4.4).

Primers P120 and 103A

P120 demonstrated primer-dimer production in end point PCR reactions and was discounted after screening. Primer combination 103A passed end point PCR screening, however, shown inconsistencies between replicates during HRM analysis, with 43 - 57 % efficiency in calling correct variants to species between replicates and runs respectively.

Primer P134

Primer combination P134 passed the end point PCR screening and shown no amplification when using DNA from *Lentinula edodes*, *Agrocybe Aegerita* and *Agaricus bisporus* (Figure 4.5C, D). Therefore, P134 was taken forward for HRM analysis, in calling correct variant to species it had between 76 - 82% efficiency, this was calculated by total number of correct isolates and replicates and mis-identified isolates and replicates on each run, P134 and had no isolates that failed to amplify (Figure 4.8). P134 however, did show the same issue as P145, as the software was still not able to efficiently separate *A. tabescens* and *A. ostoyae* species from within the *A. gallica* group (Table 4.5). This issue of variant calling, however was not consistent, and only occurred with an increased number of *A. gallica* isolates were used. When a small number of 4 - 5 isolates was used the species identification was correct on all samples, although it was inconsistent (Table 4.5). When using 17 isolates (measured in triplicate) were analysed, up to 4 isolates were incorrectly identified this dropped the efficiency to 76 % (Table 4.5).

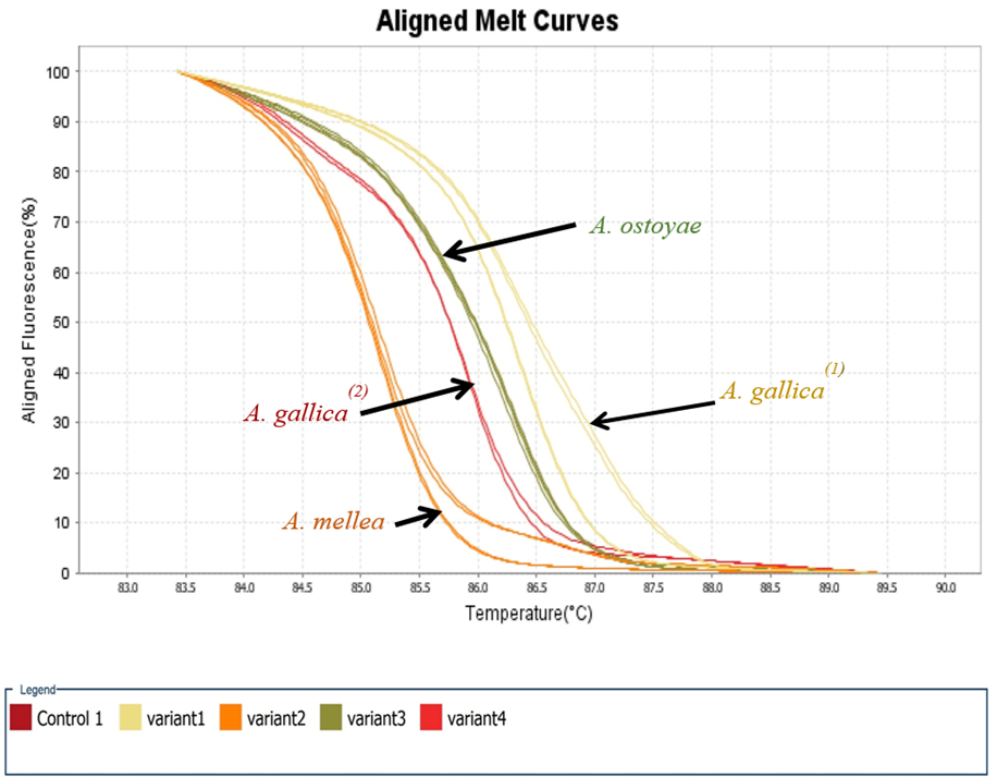


Figure 4.8 – HRM analysis aligned melt curve plot using P134, used to variant call isolates by temperature using melt curve profiles, species ID labels added. Two variant groups of *A. gallica* were identified labelled 1, 2. *A. tabescens* isolates were omitted from this run.

Table 4.5 – HRM analysis variant calls, using P134 primer combination, compared with EF1-α species ID. Y - correct variant to species identification, N - incorrect variant to species identification

Isolate ID	Species ID	P134 Amplification	Variant call	HRM correct ID
BW221	*A. tabescens*	Y	*A. gallica*	N
CW53	*A. ostoyae*	Y	*A. ostoyae*	Y
CG676	*A. gallica*	Y	*A. gallica*	Y
MC28	*A. gallica*	Y	*A. gallica*	Y
W6	*A. mellea*	Y	*A. mellea*	Y
W1	*A. gallica*	Y	*A. gallica*	Y
FOD4AF	*A. gallica*	Y	*A. gallica*	Y
CW21	*A. gallica*	Y	*A. gallica / A. ostoyae*	Y/N
FOD5-32	*A. gallica*	Y	*A. gallica*	Y
CW48	*A. ostoyae*	Y	*A. ostoyae*	Y
FOD4B	*A. gallica*	Y	*A. gallica*	Y
BW132	*A. tabescens*	Y	*A. tabescens/ A. gallica*	Y/N
W7	*A. mellea*	Y	*A. mellea*	Y
MC12	*A. mellea*	Y	*A. mellea*	Y
BW7CF	*A. gallica*	Y	*A. gallica*	Y
W4	*A. gallica*	Y	*A. gallica*	Y
RW4	*A. gallica*	Y	*A. gallica*	Y

4.4 Discussion

4.4.1 Phylogenetic analysis

The taxonomy of the genus *Armillaria* is complex. Using the available panel of Armillaria isolates, the gene regions EF1-α, IGS and ITS were able to distinguish between the major groups. However, resolution between these gene regions did vary. The phylogenetic analysis indicated that EF1-α and IGS loci have support for four species groups representing each species used in the study, with EF1-α showing the greatest resolution, as also found in previous studies of *Armillaria* species (Denman et al., 2017). However, the ITS locus had insufficient resolution to distinguish between *A. gallica* and *A. ostoyae* species. This dataset did have the least number of sequences submitted for the analysis, so therefore future work should seek to improve ITS sequence representation to see if this could resolve this issue. Nevertheless, the ITS gene region is known as a good locus for identification of a broad range fungal groups, but shows poor species level resolution (Schoch et al., 2012). The general fungal ITS database is thought to have approximately 10 % of incorrectly assigned sequences and 39 % not identified to species level (Kõljalg et al., 2013; Nilsson et al., 2009). The effects of low species-level resolution within the ITS database can been seen in Figure 3, where two references sequences used are only identified to genus level. To improve the multilocus sequence analysis more loci that provide better species-level resolution within basidiomycetes could be used, such as; β–tubulin (Guo et al., 2016), and rpb2 (Brandon Matheny et al., 2007). The inclusion of more loci and sequences into the BEAST analysis may provide more detail and support for the lineages.

The phylogenetic analysis in this study agrees with the wider suggestion that *A. tabescens* is a monophyletic group , with IGS, ITS and BEAST analysis showing the most support (Coetzee et al., 2018). During this research the sister clade containing *A. tabescens*, and the only other exannulated species *A. ectypa* within the genus, were reclassified into sub genus *Desarmillaria* (Coetzee et al., 2018; Heinzelmann et al., 2019; Park et al., 2018).

4.4.2 Rapid diagnostic development

The primer combinations P145 and P134 used to develop the HRM analysis were 76 % accurate in determining species,caluated by the number of correct identification to mis-

identification and failed samples. This reduces the time from 4 - 6 weeks to a 1-2 days depending on starting material and DNA extraction method. The resolution of this method for species specific identification does however need further development, as the current primer set P134 can call species identification when good species standards are being used and a balanced number of unknown samples are used. HRM variant/species calling is based on differences present within the samples during each run, therefore controls that represent each species is required during each run. If too many unknown samples are present within the run, the software reduces the differences between the controls and favours any differences between the unknowns, this skews the results and changes the overall grouping of the species/variants in the controls and unknowns leading to mis-identification unknowns and controls. As a result, this can be used as a rapid diagnostic if limited samples of are used; as seen in this study with the introduction of too many *A. gallica* analysed at the same time, when 17 isolates (measured in triplicate) were analysed, up to 4 isolates were incorrectly identified, compared to previous analysis where a smaller number of 4 - 5 isolates was used and species identification was correct. This may be an effect of the complex taxonomy of *A. gallica*, the HRM primers were designed to keep intraspecies differences to a minimum but this was impossible in the case of *A. gallica* (Table 4.4) . Some primer combinations shown larger differences between isolates of *A. gallica* that between differences species, especially that of *A. ostoyae* (Table 4.4). The EF1-α gene is known to be heterozygous in some cases, and was seen in this study by some sanger sequences when visualised, indicated by double peaks during quality control and were omitted from the phylogenetic analysis (Coetzee et al., 2018; Guo et al., 2016). This heterozygous nature of some *Armillaria* isolates is not thought to effect species identification during phylogenetic analysis, however, due to the sensitivity of the HRM analysis the heterozygous sequences could complicate the analysis making identifying between species more difficult, especially between those with limited SNPs.

As an alternative to HRM method the genus specific primers P267 and P145 can be used in end-point PCR and sanger sequencing to give a species identification. The benefit of using end-point PCR is that it does not require specialised equipment such as a qPCR machine and can be deployed easily into many laboratories that need rapid detection of *Armillaria* species within samples and cultures. The PCR data will provide *Armillaria* present/absent data if visualised on a gel, and species identification if sequencing completed. The end-point PCR method only requires DNA extraction, PCR and sequencing of the amplicon is required, so

isolation or culturing of mycelium can be avoided. Although this method is not as fast as HRM method it will give species ID within few days to a week. There are potential limitations to those primer combinations, the primers were tested on limited environmental samples and no isolates were included from outside the UK in the analysis. Further work would be needed to full validate those primers using a range of environmental samples, as well as, using isolates from Africa, Australasia, China and North America to determine if the primers truly are genus specific to all *Armillaria* species.

Armillaria taxonomy is complex, with differences between biological, phylogenetic and morphological species. Some biological and phylogenetic species do not match up to a morphological species, and there are examples of biological species which appear in more than one phylogenetic group (Coetzee et al., 2018). During this phylogenetic analysis of EF1-α loci, two groups emerged within the *A. gallica* lineage, suggesting that *A. gallica* may represent a species complex. Other studies have also suggested that *A. gallica* is a species complex or with potentially cryptic species, it has also been referred to as a polyphyletic group, where *A. gallica* has more than one common ancestor not present within the clade (Coetzee et al., 2018; Park et al., 2018). The phylogenetic analysis of EF1-α in this study could suggest that *A. gallica* is a paraphyletic group, where one clade contains the common ancestor, however, the bootstrap value for this second clade is 0.66 this is not high enough to fully support the second clade separation and the poly or paraphyletic clade hypothesis (Figure 4.1). Park et al., (2018) suggested that *A. gallica* group separates into 5 clades; *A. gallica* C1, *A. gallica* C2, *A. gallica* C3, European *A. gallica* and North American *A. gallica*. Whereas Guo et al., (2016) suggests that the species within each CBS, NABS and EBS should be identified as different species (Coetzee et al., 2018; Guo et al., 2016). This division in *A. gallica* has implication for the phylogenetic analysis, and so also has an impact on the outcome of the HRM analysis; *A. gallica* has as many or more intra-specific differences within the amplicons as it does with between other species.

4.4.3 Future work

For further rapid diagnostic development, the taxonomy should be considered and investigated further to try and identify the potential polyphyletic nature of the clade *A. gallica* and identify and cryptic species present. Future work should considering using restriction-site associated DNA (RAD seq) sequencing method or similar, this technique is used for SNP analysis it uses

more of DNA than single or multilocus analysis (MLSA), with potentially x10 more SNPs available for analysis (Ye et al., 2016). RAD seq is a cost-effective way for high-throughput analysis, when compared to whole genome sequencing or increasing more loci within the MLSA (Ye et al., 2016). This method uses more of the genome would be beneficial for analysing *Armillaria* isolates; it would reduce the effect of loci with bad resolution, relying on incomplete or inaccurate databases, and does not require a reference genome to complete the analysis RADseq is a cost-effective way for high-throughput analysis, when compared to whole genome sequencing or increasing more loci within the MLSA (Ye et al., 2016).

Chapter 5

Ecology of *Armillaria* spp. across Chestnut wood, Forest of Dean

5.1 Introduction

Armillaria species are one of the most important pathogens within gardens, with 17.8 % of plant tissue samples submitted to the Royal Horticultural Society (RHS) between 2001 - 2016 identified as being infected with *Armillaria* species (Drakulic et al., 2017). While the importance of *Armillaria* species as pathogens in horticulture is well-established, there is a paucity of information on their role in tree disease. In Britain, four *Armillaria* species are associated with oak: *A. mellea*, *A. gallica*, *A. ostoyae*, and *A. tabescens*. These species have been found on oak trees that are symptomatic of chronic oak decline (COD), which is a below ground syndrome effecting the tree root system, causing a slow decline in tree health that can ultimately be fatal (Denman et al., 2017, 2014). Temperature, soil moisture and host-type are the main drivers of the distribution of different *Armillaria* species (Drakulic et al., 2017). Increased nitrogen levels within soils surrounding oak species can result in nutrient imbalances and impact soil acidification; however, the effects of increased soil nitrogen levels on trees and its effect on disease predisposition and progression is not fully understood (Thomas et al., 2002). Previous studies have suggest that increased nitrogen levels can affect the foliar chemistry within oak trees leading to an increased risk of insect defoliation (Thomas et al., 2002). Phosphorous is also an important nutrient for plant growth in natural forest soils systems. However, phosphorous availability in its usable form is inherently low, and the microbial community play an important role in converting phosphorous into its plant usable form, and is strongly influenced by the mycorrhizal fungi present (Zavišić et al., 2018). In previous studies, carbon assimilation and photosynthesis in plants were both found to be negatively effected by low or poor soil phosphorous availability (Zavišić et al., 2018). Reduction in carbon assimilation can result in a reduced defence response to pathogens, such

as *Armillaria*, as trees compartmentalise infected tissues using carbon rich barriers (Oliva et al., 2014). A tree root system in poor health affects how quickly a tree can respond to environmental conditions, such as rain and drought events, ultimately affecting the ability of the tree to compete for resources and reduce defence responses (Burgess et al., 1998). The relative influence of *Armillaria* species in tree declines is not fully understood, although tree root health has been highlighted as an significant factor (Denman et al., 2014).

A. tabescens is found in warmer climates most commonly found in southern England and is thought to be the least virulent pathogen of the four species found on trees (Rishbeth, 1986). This species can be found within oak forests and plantations, mostly in the saprophytic phase on stumps, and can cause fatal infections on young oaks (Lushaj et al., 2010).

A. ostoyae as a pathogen displays some host specificity to conifer, but is able to colonise and cause disease in other tree species, including oak species (Rishbeth, 1985; Robene-Soustrade et al 1992; Guillaumin et al., 1993; Morrison and Pellow, 2002; Propero et al., 2004; Travadon et al., 2012; Guillaumin & Legrand, 2013). *A. ostoyae* has the ability to colonise trees that have been weakened by predisposing factors, and it can also be a primary and/or secondary pathogen colonising a vast area of forest stands agricultural land and gardens (Guillaumin et al., 1993; Heinzelmann et al., 2018; Mulholland et al., 2012; Rizzo and Harrington, 1993). Often *A. ostoyae* infection on oaks can be traced back to previous infection on conifers or that the site was previsouly a coniferous forest (Rishbeth, 1985). Similar to other *Armillaria* species, *A. ostoyae* can spread through rhizomorph growth; however, the production of rhizomorphs between infections are inconsistent. In previous studies, *A. ostoyae* has been found to colonise the same tree as another *Armillaria* species such as *A. gallica* and *A. cepistipes*, however it is not fully understood if this relationship has any benefits to either species in colonising and causing infection, or whether the relationship between the two species is antagonistic (Denman et al., 2017; Prospero, 2003). It is known that *A. mellea* is the most common species infecting garden plant and tree species, causing 87% of infections, and *A. gallica* causing 9.7% of total *Armillaria* infections within gardens (Drakulic et al., 2017; Intini et al., 2010). Previous studies have been mostly based within gardens, vineyards and orchards, and identified *A. mellea* as the most virulent *Armillaria* pathogen, however, the majority of this data refers to its pathogenicity on garden plants. More recently, studies have isolated *A. mellea* less often than other *Armillaria* species within woodland environments, suggesting that *A. mellea* is a less important pathogen within forest stands and UK tree declines (Denman

et al., 2017; Drakulic et al., 2017).

Armillaria gallica is a long-lived species, more commonly considered as a saprophyte or parasite on trees and garden plants, causing superficial or low impact on growth and production, but recent studies have suggested that *A. gallica* is emerging as the most important forest phytopathogen, alongisde *A. ostoyae*, in forest stands (Denman et al., 2017; Elías-Román et al., 2013; Guillaumin et al., 1993; Keča et al., 2015; Lushaj et al., 2010; Marçais and Bréda, 2006; Pérez-Sierra et al., 1999). As as pathogen, *A. gallica* has some isolates that rarely cause disease and others cause significant damage (Gregory et al., 1991; Rishbeth, 1985, 1982). These observations highlight the need to understand the *A. gallica* clade generally, but also to determine if phylogenetic differences amongst *A. gallica* strains do translate to differences in virulence as a pathogen, its roles as a saprophyte or parasite, or host specificity. This variability in ecological strategies between virulent pathogen, saprophyte and parasite within *A. gallica* species complex suggests that this species plays a more important role in root rot disease and overall tree declines than previously thought (Rishbeth, 1985). It is not clear whether there are separate isolates of *A. gallica* where one strain is a virulent pathogen, one being a weak pathogen and one a saprophyte, or if every isolate within this species has the ability to select their ecological strategy depending on environmental triggers.

Currently, the taxonomy of the *Armillaria gallica* complex is poorly understood and can vary depending on which gene region is being analysed, many of the patterns seen in this group are predominately found using EF1 $-\alpha$ (Martin P A Coetzee et al., 2018; Keča et al., 2015; Klopfenstein et al., 2017). It is the largest group within the holarctic lineage, showing vast complexity as a polyphyletic group, possibly containing several cryptic species, with *Armillaria solidipes* and *A. ostoyae* lineages as sister groups and potentially containing up to eight different clades (Coetzee et al., 2018; Keča et al., 2015; Klopfenstein et al., 2017). This group has representatives of biological and phylogenetic species that have no corresponding morphological species identified (Coetzee et al., 2018). There are also vast differences in geography, with some species showing a restricted distribution whilst others are found globally, with distant groups shown by inter-sterile groups and genetic variations within the wider *A. gallica* clade, with European groups different from both North American and East-Asian groups (Coetzee et al 2018). Mulholland et al., (2012) designed species-specific primers for rapid identification of *Armillaria* species; *A. mellea*, *A. gallica*, *A. ostoyae*, *A tabesens*, *A cepistipes*, and *A. borealis*. Those species specific primers were designed using end-point PCR

primer sets, and validated on species isolated from the UK and across Europe. This method uses PCR and gel electrophoresis to give a quick and low technological method for species specific detection. Infection is predominately spread by rhizomorphs, and *A. gallica* has the ability to produce vast amounts, growing through the soils in search of a nutrient source, such as a tree stump or predisposed host tree. The rhizomorphs are able colonise the tree at the collar and root systems, with rhizomorph attachment ranging from small rhizomorphs to large mats of rhizomorphs attached to the tree and attempting to penetrate the outer bark and cause infection. *Armillaria gallica* has been found colonising trees as primary or secondary pathogens, as well as colonising the same tree as other *Armillaria* species (Denman et al., 2017). Denman et al., (2017) isolated *A. gallica* from trees weakened or dying trees, with active lesions on some trees indicating the predominate pathogen or the primary cause of disease. Rhizomorphs were also found on the buttress roots of asymptomatic trees. On the diseased trees, A. gallica and *A. ostoyae* were isolated co-occurring within the same active lesion, potentially with some interaction (either cooperative, or antagonistic) between the two isolates (Denman et al., 2017). It is unclear if the co-occurrence of species is present on all trees and if other species within the genus can also co-occur, and if it is a beneficial interaction or are they competitors on the same lesion driving forward disease through competition for resources.

The site chosen for this study is Chestnuts Wood, a 65 year old oak plantation in the Forest of Dean, with a history of *Armillaria* infection. It is a small woodland area maintained by the Forestry Commission. Previous studies by Forest Research and Rothamsted Research have highlighted areas of *Armillaria* infection, primarily by *A. gallica* with occasional isolates of *A. ostoyae* isolated from symptomatic trees on the site, suggesting a growing importance of *A. gallica* within forest systems (Denman et al., 2017). The aim of this chapter was to undertake an ecological study of Chestnuts Wood, combining utilising the methodological approaches developed in Chapters 2 and 3 to identify and characterise *Armillaria* species found across the site characterised by *Armillaria* infection. This chapter also aimed to determine if *Armillaria* is the main biotic contributor to the severe declines found on the site, and to identify which species is the most abundant, i.e. the virulent pathogen *A. ostoyae* or if the saprophyte and weak pathogen *A. gallica* is causing this site wide infection. This was achieved by isolation and molecular detection to determine if *Armillaria* spp. were present within soils and trees of different health statuses (asymptomatic and symptomatic) from across the site, alongside

isolating and characterising those *Armillaria* species colonising buttress roots and trunks of asymptomatic and symptomatic from across Chestnuts Wood. For *Armillaria* isolates found on the site, their species distribution across the site was established, to identify any differences between species and isolates colonising trees,and assess their interaction and correlation with other environmental factors such as soil moisture and nutrient status. Species identification of isolates was determined using DNA-based molecular methods developed in Chapter 3; HRM and PCR and sequencing of amplicons, and species specific primer sets for the species; *A. mellea*, *A. gallica*, *A. ostoyae*, *A tabescens*, will be used to validate if this method can be used on all UK species.

5.2 Materials and Methods

5.2.1 Site or Sampling Area Description

Chestnuts wood is a 65-year-old oak (*Quercus robur*) plantation, located with the Forest of Dean, Gloucestershire (Figure 5.1). Many of the oaks were planted between 1948-1950 and in 1960 in high densities, approximately 2-3 meters apart, with some areas of ancient oaks planted much earlier in 1814 and 1850, which are not part of the plantation studied here. Previously, this site was predominately a coniferous forest of pine tree species, which were all felled and replanted as an oak plantation (Forestry Commission, personal communication, 2015). The area containing oaks with symptoms of *Armillaria* infection correlates with the high-density planting of oak trees, planted in the mid-1900s, and now approximately 65 years old. The area below the track is approximately 5.72 ha in size, and part of a long-term monitoring program undertaken by Nathan Brown at Rothamsted Research in collaboration with Forest Research (Personal communication, 2015); from this monitoring area, 151 dead trees were identified and 64 trees were showing symptoms of tree decline in 2017 (personal communication, Brown 2017). Previous samples collected by Forest Research have isolated both *A. gallica* and *A. ostoyae* colonising separate trees and also the same tree (personal communication, Denman 2017). A site visit was completed on the 20th April 2016 with the Forestry Commission, Sandra Denman (Forest Research) and James McDonald (Bangor University), where two previously sampled trees were identified (FOD4 and FOD6), both trees were located below the track within the long-term monitoring site. Wood panel samples

were taken from potential lesion areas on the trunk, and isolations were completed at Bangor University where only *A.gallica* was isolated from both trees.

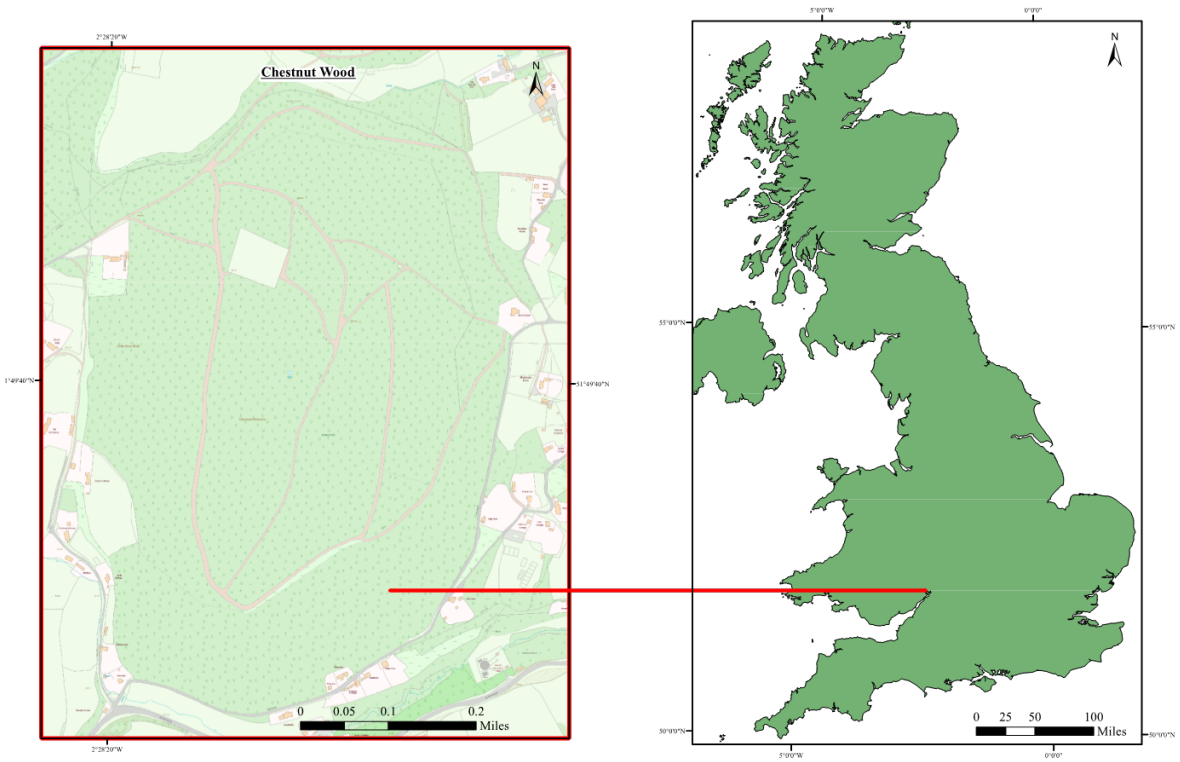


Figure 5.1 – Location of the Chestnuts wood sampling site.

5.2.2 Tree sampling

A sub plot in an area of the site where the work for this chapter could be conducted was preselected, based on previous sampling, and to ensure that it was outside of the long-term monitoring area, Figure 5.2; within the subplot, 15 GPS points were selected 25 m apart, and at each point a symptomatic tree and asymptomatic tree were selected based on the presence of lesions and crown condition using both the Hessain guide: graded 1 - 9 of oak crown morphology, and ARCHI guide: winter health 'ARCHI' types of pedunculate oak (*Q. robur*) categories; SAIN - healthy tree, R - resilient, I - irreversible decline, S - stressed and D - natural crown retrenchment (Hessian Forest Research Institute Hahn, Münden, Germany, Drénou et al., 2015). In total, 24 trees were sampled and comprised 12 symptomatic trees and 12 asymptomatic trees of *Armillaria* infection. For each tree sampled, observational data was collected including; the number of buttress roots, presence of stem insect holes, and the presence of fruiting bodies, mycelium and rhizomorphs, Figure 5.3. Lesions were identified

on symptomatic trees by the presence of tissue necrosis or black liquid seeping from the bark plates on the tree collar or trunk. At each lesion site, a wood panel approximately 2" x 2" inches and comprising the outer and inner bark tissues was collected; only 10 of the 12 symptomatic trees selected had a wood panel taken, as the two remaining trees had lesions that appeared dried and inactive. Separate sampling equipment was used for asymptomatic trees and symptomatic trees, and each piece of equipment was cleaned using chemgene and 70 % IMS, to reduce contamination between trees and samples. The top soil surrounding the buttress roots of all the sampled trees was removed to expose the buttress roots, and the presence of rhizomorphs was recorded and collected where present, a soil sample was taken from within 1 metre of the north side of the tree.

5.2.3 Soil sampling

In the sub-plot (orange box in Figure 5.2) soil samples were collected every 25 m, and every 50 m outside the sub-plot grid format using GPS (black circles in Figure 5.2), soil samples were taken within a 5 m radius of the GPS point, using either a hand trowel or auger. Approximately 1 L of soil was collected and placed into sample bags. Once back to the laboratory, the soils were sieved using a 5 mm sieve and were stored at 4°C until analysis for a maximum of 3 weeks.

5.2.4 Soil Chemistry

The pH of the soil was determined by adding 1 g of soil to 5 mL water in a 15 mL falcon tube with shaking at 200 rpm for 30 minutes at room temperature. The soil mix was then centirfuged at 3000 rpm for 5 minutes, and the pH was measured using a Jenway 3520 Standard Digital pH meter. The moisture of the soil was calculated by drying 3 g of soil at 105°C overnight, and the difference in weight after drying was determined to be the moisture content. The dried soil was then placed in a 450°C for 16 hours, the difference in weight was used to determine the organic matter (OM) content. Soil nitrate (NO_3) and phosphates were determined as described by (Miranda et al., 2001; Mulvaney, 1996; Murphy and Riley, 1962); firstly a soil acetic acid mix and a potassium chloride (KCl) mix were made up using a 1:5 ratio to create soil extracts to be used for soil chemistry analysis. The extracts were shaken at 200 rpm for 30 minutes, then centrifuged at 3000 rpm for 5 minutes, a 3 mL aliquot of each extract was frozen for later analysis. To calculate the nitrate content of the soil, 100

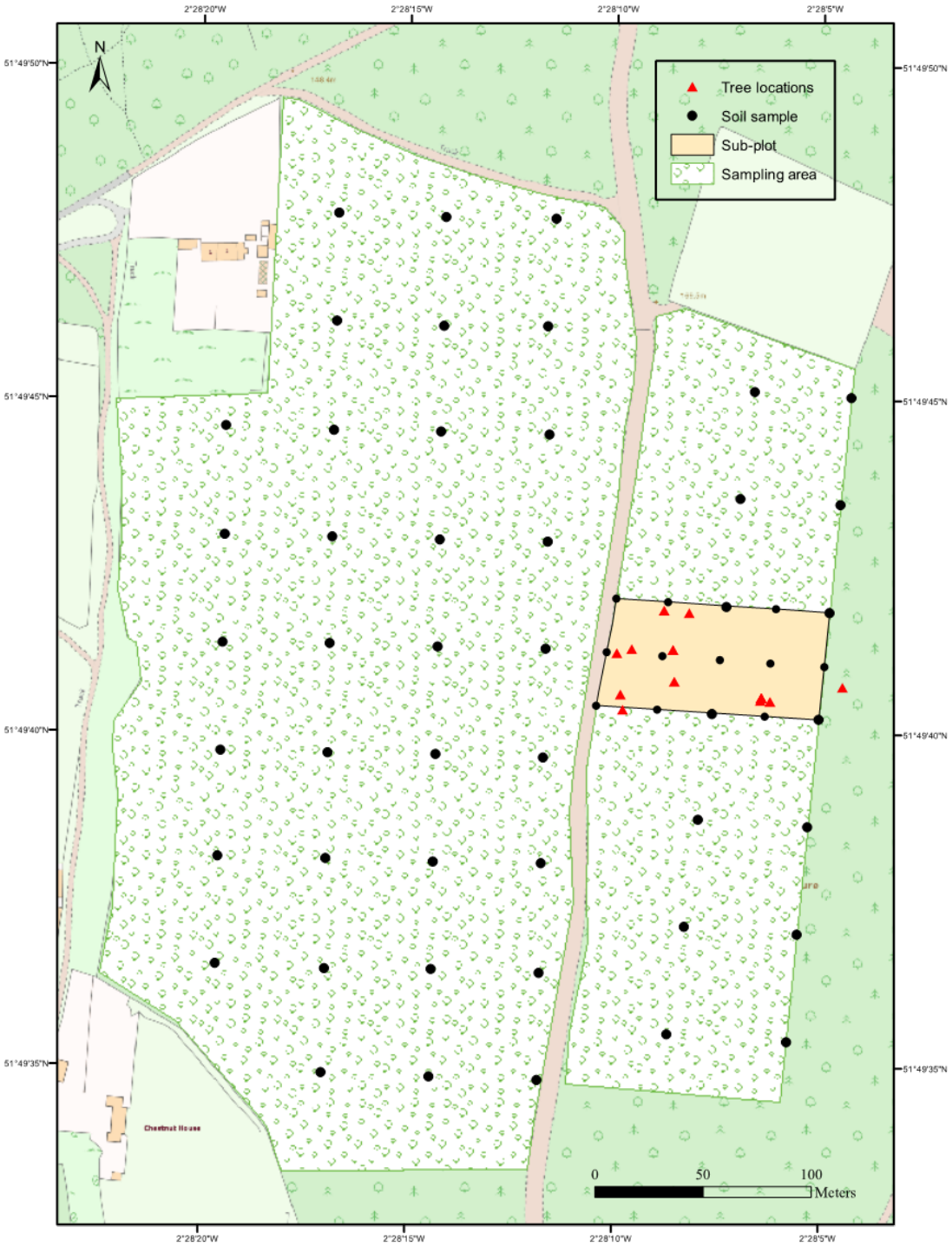


Figure 5.2 – A map to show the targeted soil sampling points and actual GPS points of sampled trees within the subplot (orange box) areas of Chestnuts Wood sampling on the $25^{t}h$ - $28^{t}h$ April 2017. Tree samples were taken from the sub plot; soil samples were taken from the whole site every 50 m and more intensely within the subplots every 25 m (indicated by the black circles).

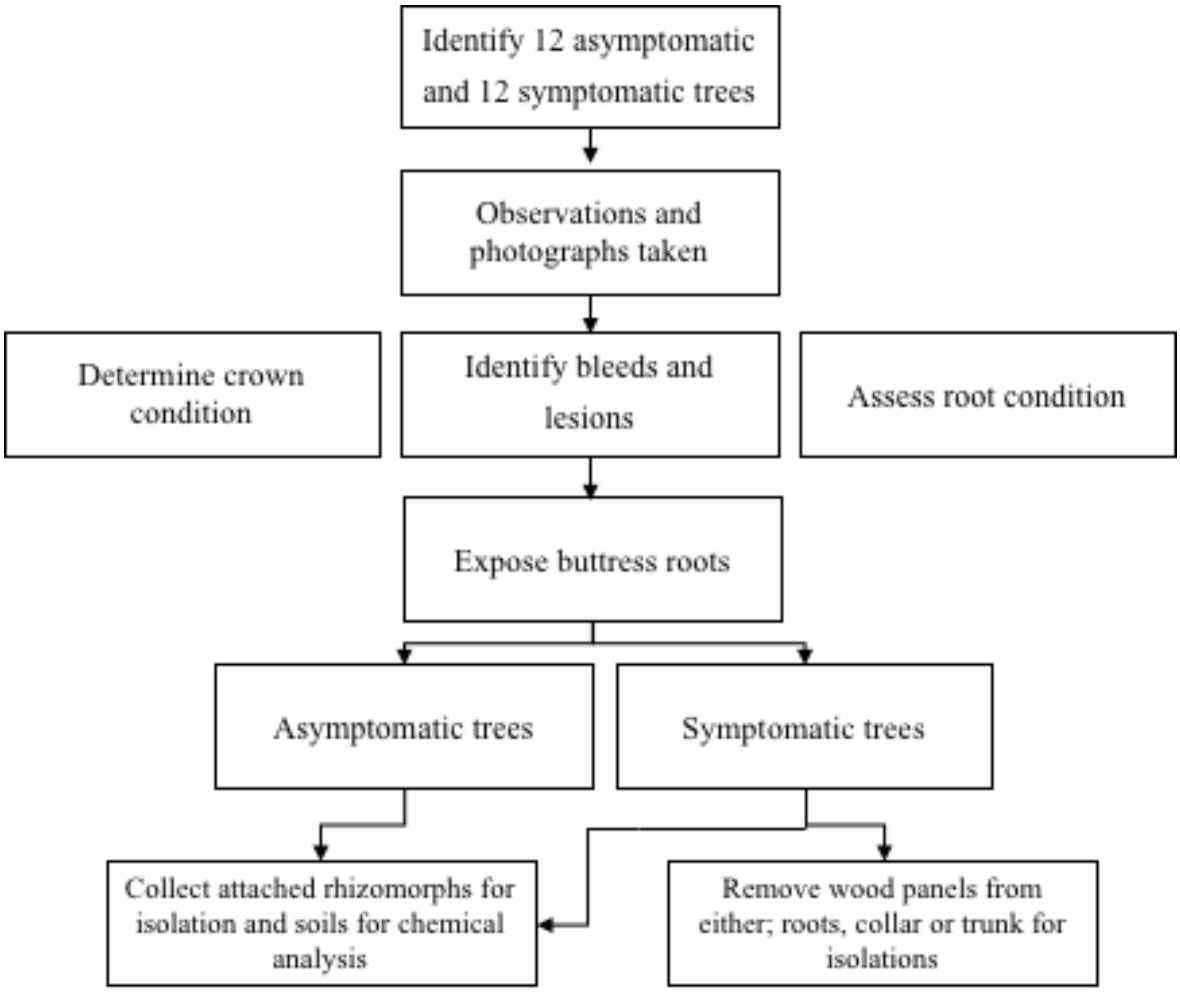


Figure 5.3 – Schematic of tree and soil sampling.

μL of Vanadium (III) Chloride (VCl_3) was added to 100 μL of KCl soil extract, alongside standards, then 100 μL of a 1:1 mixture of N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) Sulfanilamide was added and mixed. The mixture was incubated for 15 minutes at room temperature to allow the pink colour to develop, using a spectrometer the colour was measured at 540 nm (Mulvaney 1996, Miranda et al 2001). The phosphate content of the soil was determined by adding 180 μL of Ames reagent (0.42 % Ammonium Molybdate (NH_4M_o) in 0.5 M Sulphuric Acid (H_2SO_4) to 80 μL of each acetic acid soil extract and standards, 30 μL of 10 % ascorbic acid was added and mixed and incubated at room temperature for 20 minutes to allow the colour to develop, using a spectrometer the colour was measured at 820 nm (Murphy and Riley, 1962). The ammonia content was determined as follows; 15 μL of EDTA was added to 150 μL of each KCl soil extract, 60 μL of Na-salicylate-nitroprusside reagent and 30 μL of hypochlorite reagent was added and mixed. The mixture was incubated at room temperature for 15 minutes to allow the colour to develop, using a spectrometer the colour was measured at 667 nm (Mulvaney, 1996).

Calculations

The concentrations were calculated using standard concentrations minus the blank values for each plate to create a linear regression, each of the soil extract absorbance values from the phosphate, nitrate or ammonia assays were plotted against the corresponding standard linear regression. To calculate the concentration per gram of soil the following equations were used, with phosphate as an example;

$$PO_4 - P\ moist\ soil(\mu g mg^{-1}) = \frac{PO_4 - P\ in\ extract(\mu g mg^{-1})\ X\ extract\ volume(mL)}{weight\ of\ moist\ soil(g)}$$

$$MoistureFactor = \frac{Moistsoil(g)}{Oven\ dried\ soil(g)}$$

$$PO_4 - P\ dry\ soil(\mu g g^{-1}) = PO_4 - P\ moist\ soil(\mu g g^{-1})\ X\ Moisture\ Factor$$

5.2.5 Isolation of *Armillaria* spp. from wood panels and rhizomorphs

After 3-4 weeks incubation at 4°C the wood panels and rhizomorphs were surface sterilised using the steps below. Each step was done for 30 seconds, and all the steps were repeated for wood panels if necessary.

Step 1. 70 % Alcohol (IMS)

Step 2. 2 % Bleach

Step 3. 70 % Alcohol (IMS)

Step 4. Autoclaved distilled water

Step 5. Wood panels or rhizomorphs left to dry

A flame sterilised scalpel was taken to cut away the outer surface of the wood panel at the dead live junction of the lesion, the scalpel was flame sterilised again and another layer of wood tissue was removed, the scalpel was sterilised a final time and 10 small pieces of wood tissue (labelled A-J) were plated onto MAT media. The MAT medium comprised of 15 g l^{-1} malt extract and 15 g l^{-1} agar then autoclaved, antibiotics were made up using 10 mL deionised water then filter sterilised; 50 mg of Penicillin-G, streptomycin sulphate, and 25 mg polymycin, and finally 1 mL^{-1} 23% thaibendazole lactate was also added (Forest Research, personal communication, 2015). The final volume was adjusted to 1 L using deionised water. For isolation from rhizomorphs, the rhizomorph was cut open with a sterile scalpel and a sterile needle was used to remove mycelia from the inside of the surface sterilised rhizomorph, and 10 pieces (A-J) were plated onto MAT media and incubated at 24°C. The agar plates were checked daily to assess growth, identify any potemtial contamination and any potential *Armillaria* growth was immediately sub cultured onto clean MAT plates. Long term storage of cleaned isolated was achieved by sub culturing onto 4 % malt extract agar slants and stored at 4°C. The isolates were numbered corresponding to the tree number that they were isolated from, followed by the buttress root number and if they were derived from a rhizomorph or wood panel plated out i.e. P111 5 A. A sterile sample bag was filled with soil from each soil sample and baited with autoclaved apple twigs and irradiated sterilised oak sapwood and incubated for four weeks at room temperature. The apple twigs and wooden blocks were removed from soil and inspected for colonisation by mycelium or rhizomorphs. Any tissue with potential *Armillaria* colonisation were plated on MAT plates and incubated at 24°C.

5.2.6 Species Identification

The *Armillaria* isolates were inoculated onto a 0.45 μm cellulose nitrate filter membrane and placed on to carrot agar, the filter membrane was used to reduce agar contamination for downstream processing. The filter paper with the *Armillaria* colony growth on the surface was removed from the agar and the DNA was extracted using the Griffiths DNA-RNA co-extraction method (Griffiths et al., 2000), and stored at -20°C. Sequences of EF1-α (translation elongation factor 1- alpha) EF595F and EF1160R (Kauserud and Schumacher, 2001) , ITS (internal transcribed spacers) ITS1 and ITS4 (White et al., 1990), IGS (intergenic spacer) IGS; IGS01 (Duchesne and Anderson, 1990) and IGS P1 (Hsiau, 1996). PCR reactions were completed using 12.5 μl GoTaq G2 green mastermix, 2X (Promega, Madison, WI, USA), $MgCl_2$ 1.5mM, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μ), 2 μl DNA template (1-40 ng/μl) and 8.5 μl PCR water, total volume of 25 μl. PCR cycling conditions were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturing at 95°C for 1 minute, 30 seconds annealing at 55°C for the ITS or IGS primer pairs, or 48°C for EF1-α primers, and extension at 72°C for 1 minute, with a final 5 minutes at 72°C extension step. All of the amplicons were ran out on a 2 % gel. Excess primers and dNTPs the amplicons were removed using ExoSAP-IT *express* kit (Thermofisher). Sanger sequencing of EF1-α, ITS, IGS regions and BLAST searches were used to confirm species identification (Altschul et al., 1990).

The species-specific primers, were also amplified using PCR, using the conditions 95°C for 5 min, followed by, 35 cycles of; 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, followed by 7 minutes at 72°C, the products were measured using the Qubit^{TM} dsDNA HS Assay Kit and ran out on a 2 % gel electrophoresis to determine positive identifications (Mulholland et al., 2012). Any PCR products that produced a single positive band of the correct size were sent for sanger sequencing to confirm species identification (Table 5.1). The DNA extracted from the *Armillaria* isolates were also used to validate the use of High resolution melt curve analysis (HRM) in the field, using primer set P134 (Table 5.1).This was only completed on 5 samples that were available at the time of HRM development. Spatial mapping was used to show areas of unknown *Armillaria* isolates and isolates that the HRM had determined species identification. HRM was completed using the SensiFAST^{TM} HRM master mix 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1 μl DNA template

Table 5.1 – Species specific primers (Mulholland et al., 2012) and HRM genus specific primers used as species ID in this study. All based on EF1-α

Species/Primer set	Primer name	Sequence (5'-3')	Amplicon size (bps)
Species specific primers			
A. gallica	Agal-F123	YTG CTT TGC CTT KTG TTT AGC C	220
	Agal-R342	CGT TAA GRG RCA RCA CTG ATC ATA TGG	
A. mellea	Amel-F165	TGG AGC GAG GAC CGA TT GRC	236
	Amel-R401	GRC GAC ACC RGC CTT ABT C	
A. ostoyae	Aost-F102	CCA CCA AGG TAC GAG ATC TAT CG	240
	Aost-R341	GTT AAG AGG CAG CAC TGG	
A. tabescens	Atab-F147	GTT CGA CAT TTA TCT TAG TGG AGT G	329
	Arm EF1-a-REV	AGA CGG AGA GGC TTG TCG GAG	
Genus Specific primers			
P134	P134F	TTAACGTTCTCTGTAGCAT	134
	P145TF	GGCGGCTTAACGTTGTTGAAP	
	145R	GAGAGGCTTGTCGGAGGGAC	

(1-25 ng/μl) and 6.6 μl PCR water, total volume of 20 μl. PCR step: initial denaturation 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, at 60°C data collected on amplification efficiency of the assay, this was followed by the HRM stage: 95°C for 10 seconds, 60°C for 1 minute, and the dissociation step to generate the melt curve profiles for each sample, data collected every 0.025°C from 60°C to 95°C, and to finish the assay 95°C for 15 seconds and then 60°C for 15 seconds

5.2.7 Statistical analysis of tree sampling and soil chemistry

All statistical analysis used R studio (R development Core Team, 2009). The number of buttress roots between asymptomatic and symptomatic was analysed using a one-way ANOVA. To analyse the soil chemistry a non-parametic option was used as this data did not fit within normal distribution, so a kruskal-wallis test (KW) was selected (Kruskal-Wallis Test, 2008) and were significant the non-parametric post hoc test Dunns test was also completed.

5.2.8 Phylogeny

Sanger sequencing reads of the PCR amplification products of the EF1-α, ITS, IGS regions of the isolates were quality clipped using FourPeaks (2004-2015, Mekentosj, Amsterdam) and combined with NCBI reference sequences from *Armillaria* genus and *Fusarium oxysporum* as a outgroup (Altschul et al., 1990; Sayers et al., 2019), then imported into Mega7 (Kumar et al.,

2016), and aligned using MUSCLE. The model TN93, with gamma and evolutionary invariant was selected to generate maximum likelihood phylogenetic trees, with 1000 bootstrapping (Kumar et al., 2016). The trees were then edited using ITOL (Letunic and Bork, 2019), branches with a bootstrap value below 0.4 was collapsed, branches with a bootstrap above 0.62 were labelled with the bootstrap value (Keca et al., 2015).

5.2.9 Spatial mapping of *Armillaria* isolates

The spatial mapping was completed using ESRI's ArcMap v10.6, the coordinates for the actual trees sampled were imported into ArcMap and categorised based on isolates falling into one of two *A. gallica* clades present in the EF1- α DNA amplicon sequence data. The sampled tree points were then grouped by the presence of isolates within each clade using the spatial mapping toolbox.

5.3 Results

5.3.1 Observational data from tree sampling

Observational data of tree sampled (Table 5.2), using ARCH and Hessain tree health status categories, an ANOVA shown no significant difference in number of trees with tree diameter at breast height and rhizomorph, insect or mycelium presence.

5.3.2 Tree Sampling and Fungal Isolations

The overall isolation efficiency of *Armillaria* spp. from rhizomorphs and wood panels varied from 8-13 %, with isolations from rhizomorphs (113/1010 tissue pieces, 11 %) having the highest efficiency and wood panels having the lowest (17/210 tissue pieces, 8 %). Isolation efficiencies from the rhizomorphs of symptomatic trees (47/520 tissue pieces, 9 %) was generally lower efficiency compared to those of asymptomatic trees (66/490 tissue pieces, 13 %) (Table 5.3).

Interestingly, the symptomatic trees sampled within the subplot were shown to have significantly fewer buttress roots than asymptomatic trees (ANOVA, df = 1, p = 0.03), with 91 % of

Table 5.2 – Observational data of all trees sampled. Diameter at breast height (DBH) was measured at 120 cm for each tree. ARCHI and Hessain guides (categories 1-9) were used to determine tree health. ARCHI guide categories; SAIN - healthy tree, R - resilient, I - irreversible decline, S - stressed and D - natural crown retrenchment. Present of insect holes, mycelium and rhizomorphs; Y present, N is absent.

Tree number	Health status	Circumference (cm)	Diameter at breast height (cm)	Archi	Hessain	Number of buttress roots	Number of bleeds	Insect holes (Y/N)	Mycelium (Y/N)	Rhizomorphs (Y/N)
B101	Asymptomatic	94	29.94	S	6	7	0	N	N	Y
B102	Asymptomatic	72	22.93	SAIN	5	6	0	N	N	Y
B103	Asymptomatic	97	30.89	R	5	9	0	N	N	Y
B104	Asymptomatic	130	41.40	SAIN	3	9	0	N	N	Y
B105	Asymptomatic	109	34.71	SAIN	5	5	0	N	N	Y
B106	Asymptomatic	104	33.12	S	6	7	0	N	N	Y
B107	Asymptomatic	140	44.59	SAIN	3	7	0	N	N	N
B108	Asymptomatic	Missing data	Missing data	SAIN	5	7	0	N	Y	N
B109	Asymptomatic	108	34.39	SAIN	3	8	0	N	Y	Y
B111	Asymptomatic	88	28.03	SAIN	6	5	0	N	N	Y
B110	Asymptomatic	103	32.80	R	6	5	0	N	N	Y
B40	Asymptomatic	95	30.25	SAIN	4	8	0	N	N	
P101	Symptomatic	77	24.52	S	7	6	0	Y	N	Y
P102	Symptomatic	120	38.22	I	7	5	0	N	N	N
P103	Symptomatic	80	25.48	S	7	6	0	N	N	Y
P104	Symptomatic	92	29.30	S/I	6	7	1	Y	N	Y
P105	Symptomatic	108	34.39	S	5	4	1	Y	N	Y
P106	Symptomatic	104	33.12	S	7	5	1	Y	N	Y
P107	Symptomatic	107	34.08	S	8	6	1	Y	N	Y
P109	Symptomatic	110	35.03	S	8	5	1	N	Y	Y
P110	Symptomatic	98	31.21	I	8	6	0	Y	N	Y
P111	Symptomatic	127	40.45	Resilient	5	7	1	N	Y	Y

Table 5.3 – Isolation efficiency of *Armillaria* spp. from different tissue types; rhizomorphs from asymptomatic and symptomatic trees and wood panels from only symptomatic trees.

	Pieces of tissues isolated	Positive *Armillaria* isolations	Isolation efficiency
Rhizomorphs	1010	113	11%
Asymptomatic trees	490	66	13%
Symptomatic trees	520	47	9%
Wood panels (symptomatic only)	210	17	8%
Total	1220	130	11%

buttress roots on symptomatic trees having *Armillaria* visible rhizomorphs present, compared to 61 % of the asymptomatic trees, (Table 5.4).

Table 5.4 – The number of buttress roots and percentage of those colonised with *Armillaria* and those with positive *Armillaria* isolations (± Standard Error).

	Average number of buttress roots (±SE)	Average number of buttress roots with rhizomorphs	Rhizomorphs found on buttress roots (%)	Buttress roots with positive *Armillaria* isolates (%)
Asymptomatic trees	6.9 (±0.4)	4.1	61 %	61 %
Symptomatic trees	5.7 (±0.3)	5.2	91 %	58 %
Total	6.4	4.6	75 %	60 %

For the soil baiting, 10 pieces of tissues were plated out after the 4 weeks, however, after incubation no successful *Armillaria* isolates were identified.

5.3.3 Analysis of soil moisture and nutrient content in relation to tree health status across the site

The soil moisture data indicates greater moisture levels in the rhizosphere soils of symptomatic trees 24.6 %, (KW; p = 0.06) compared to soils surrounding asymptomatic trees 21.4 %, (Dunns test; p = 0.03) and were more in line with the moisture content of soils found across the sample site that were not within the direct vicinity of the trees 25.2 %, (Dunns test; p < 0.00) (Figure 5.4A). Organic content was found to be lower in soils surrounding symptomatic trees when compared with asymptomatic trees; however this was not significant (ANOVA, df = 1, p = 0.40) (Figure 5.4B).

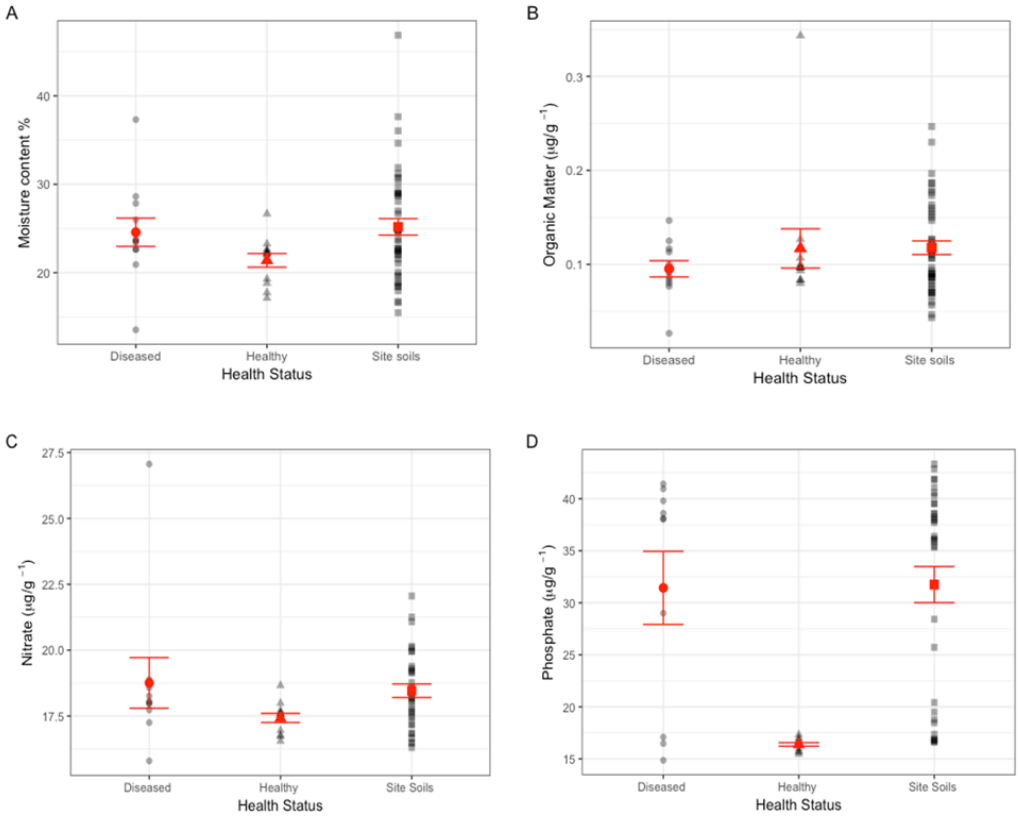


Figure 5.4 – Soil sample chemistry from soils surrounding diseased (symptomatic) and healthy (asymptomatic) trees and soils from across the site. A) Moisture content (%), B) Organic Matter, C) Nitrate, and D) Phosphate

The nitrate levels were shown to be lower in soils surrounding asymptomatic trees (KW; p = 0.05) than soils surrounding symptomatic (Dunns test; p = 0.04) and site soils (Dunns test; p < 0.00) (Figure 5.4C). The phosphate levels were lower in soils surrounding asymptomatic trees (KW; p < 0.00) when compared to symptomatic trees (Dunns test; p < 0.00) and site soils (Dunns test; p < 0.00) (Figure 5.4D).

5.3.4 Species Identification

Armillaria isolates were identified using three approaches; (i) EF1$-\alpha$, ITS, IGS gene sequencing and BLAST searches (ii) using the species-specific primers (Mulholland et al., 2012), and (iii) using the HRM rapid diagnostic developed in Chapter 3. Sanger sequencing of EF1$-\alpha$, ITS, IGS regions confirmed, by BLAST (Basic Local Alignment Search Tool) search (Altschul et al., 1990), that all isolates from wood panel and rhizomorphs obtained from Chestnuts Wood were *A. gallica*. When using the species-specific primers, designed by Mulholland (et al., 2012) to identify specific *Armillaria* species on the site, an agarose gel and Sanger sequencing was used to determine species ID (Figure 5.1). A band, a faint band or smear at 200-300 bps was determined as a positive and sent for sequencing for confirmation (Figure 5.1). Out of a possible 85 isolates, 33 isolates were identified correctly as *A. gallica*, showing 39 % method efficiency in calling the correct species identification on this panel of isolates. Inconsistencies in the ability of the specific specific primers (Mulholland et al., 2012) to identifying the correct species were seen when compared to EF1$-\alpha$, ITS and IGS gene sequencing; from a total of 85 isolates, 45 isolates produced no band on the gel, which indicates no species tested were present. However those isolates were previously confirmed as *A. gallica*, therefore 53 % of the isolates had type II error: false negative result. Those species specific primers also misidentified 10 isolates; 5 (6 %) were misidentified as *A. ostoyae*, 4 (5 %) were misidentified as *A. tabescens* and 1 (1 %) was misidentified as *A. mellea*.

The EF1- α phylogenetic tree shows the Chestnuts Wood *A gallica* isolates do appear to fall into two distant groups, (Figure 5.6). There are isolates present multiple times on the same tree, but also isolates from the same tree are present across both clades, indicating differences with those isolates. Most of the *Armillaria* isolates from wood panels are present in one clade separated with a bootstrap value of 0.92; however, there was one representative from a wood panel present in the adjacent clade, separated with bootstrap value of 0.88 (Figure 5.6).

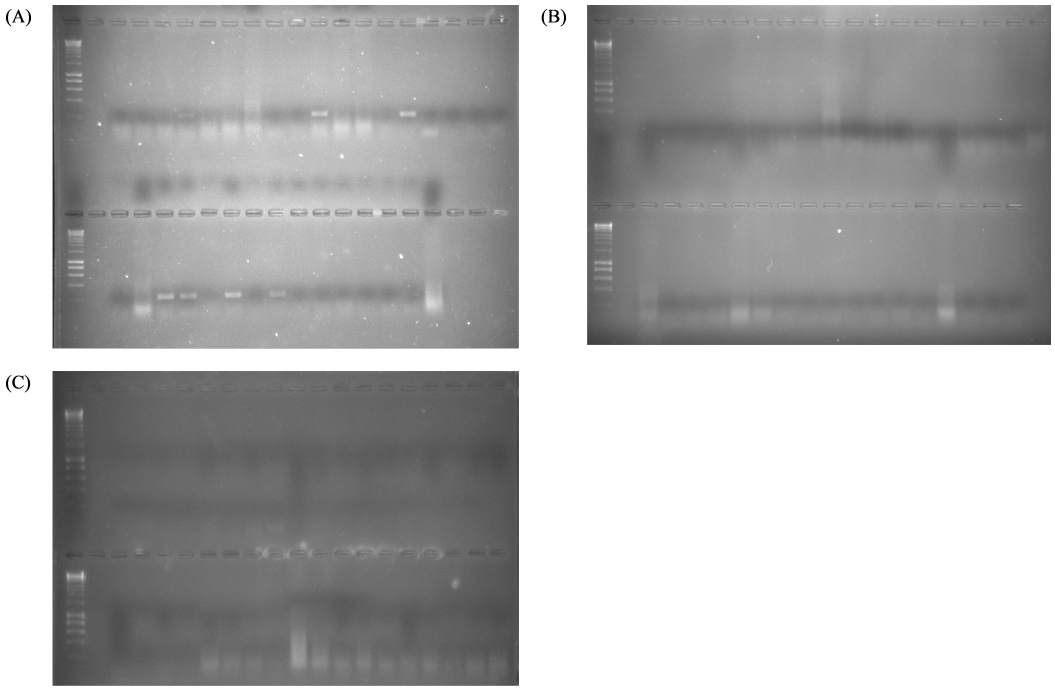


Figure 5.5 – Species specific primer sets for species identification, band or smear at 200 - 300 bps range was determined a positive and sent for sequencing for confirmation. (A) Isolates 59-90, *A. gallica* species ID primer set: Agal-F123 Agal-R342; (B) Isolates 47-81, *A. mellea* primer set: Amel-F165 Amel-R401; (C) Isolates 83-90 top row *A. ostoyae* primer set Aost-F102 Aost-R341: , Bottom row *A. tabenscens*, primer set: Atab-F147 Arm EF1-a-REV. All isolates confirmed as *A. gallica* using EF1$-\alpha$, ITS, and IGS.

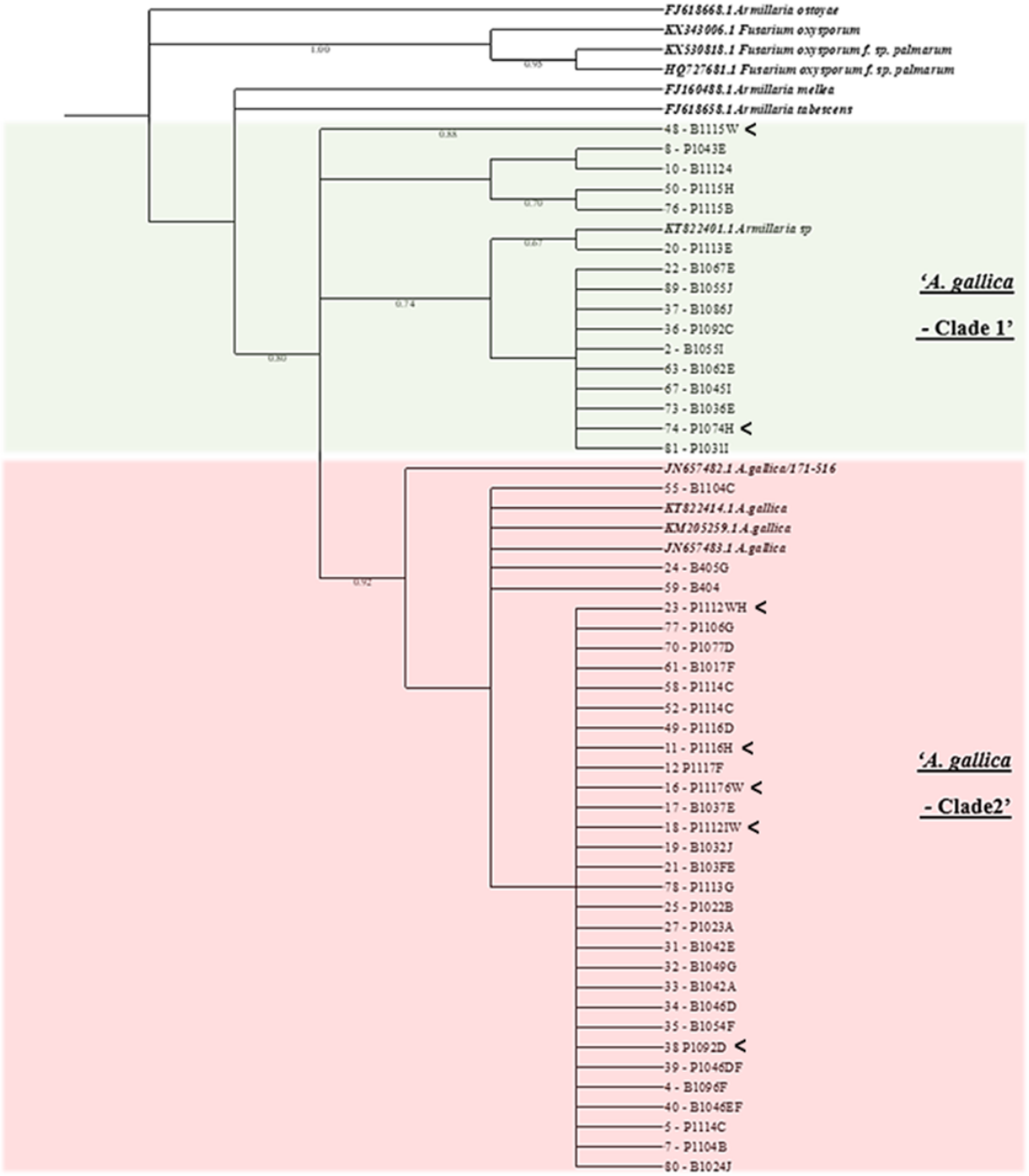


Figure 5.6 – A maximum likelihood phylogenetic tree generated from EF1−α DNA sequence data showing the *Armillaria* isolates from Chestnuts Wood, with *Armillaria* reference sequences and *Fusarium oxysporum* outgroup (rooted) highlighted in bold. Branches were collapsed with bootstrap values bellow 0.50, nodes with bootstrap values between 0.62 - 1.00 are shown by the presence of a value. Isolate labels indicate isolates from; asymptomatic trees are B, symptomatic trees are P, isolates came from rhizomorphs, isolates from wood panels are indicated with a <.

5.3.5 Rapid diagnostic of *Armillaria* isolates using High resolution melt curve analysis (HRM)

The high resolution melt analysis developed in the previous chapter of this thesis was tested on 5 isolates and identified 3 unknown isolates as *A. gallica* and 2 samples of *A. ostoyae* (Figure 5.7). All isolates were confirmed by EF1-α, ITS and IGS amplicon sequencing. Therefore these data suggest that shows that the on the limited samples the HRM shows a 60 % efficiency on calling the correct *Armillaria* identification when using isolates from this site.

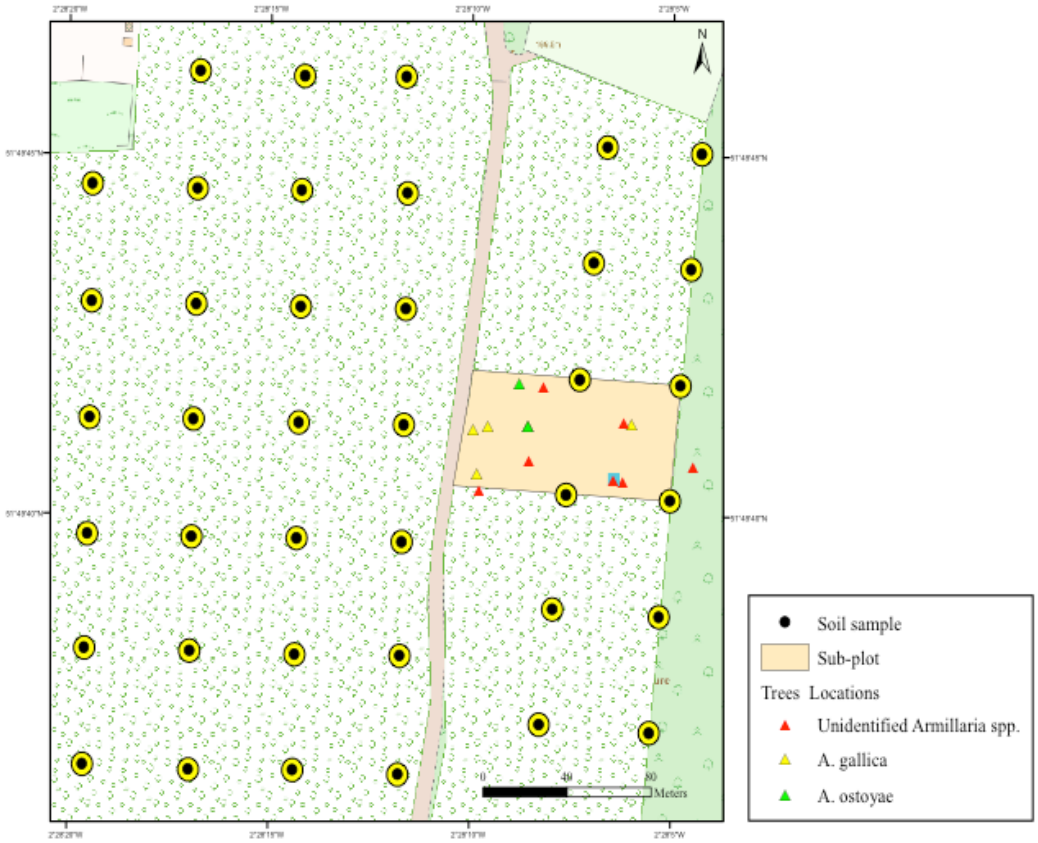


Figure 5.7 – Chestnuts Wood subplot area, red triangles: trees/rhizomorphs isolates HRM unknown species ID, yellow triangles: identified as *A. gallica*, and green triangles: identified as *A. ostoyae*. Blue square: tree B108 with no *Armillaria* isolated

5.3.6 Spatial mapping of *A. gallica* isolates

The spatial mapping data shows there are three different areas where *A. gallica* is present within the subplot, using the EF1$-\alpha$ DNA sequence data those *A. gallica* groups are split into two subclades (Figure 5.8). Those isolates are a from rhizomorphs and wood panels. There is one area of *A. gallica* clade 1 and *A. gallica* clade 2 occurred in a separate location within the subplot. However, the largest affected area had both *A. gallica* clades present, and in some cases those isolates were occupying the same tree (Figure 5.8). The sub plot area had four disease centres identified, and this was confirmed by the presence of 2 - 3 dead trees with rhizomorphs growing up the trunk, no bark present, and trees symptomatic of *Armillaria* infection surrounding the dead trees (Figure 5.8).

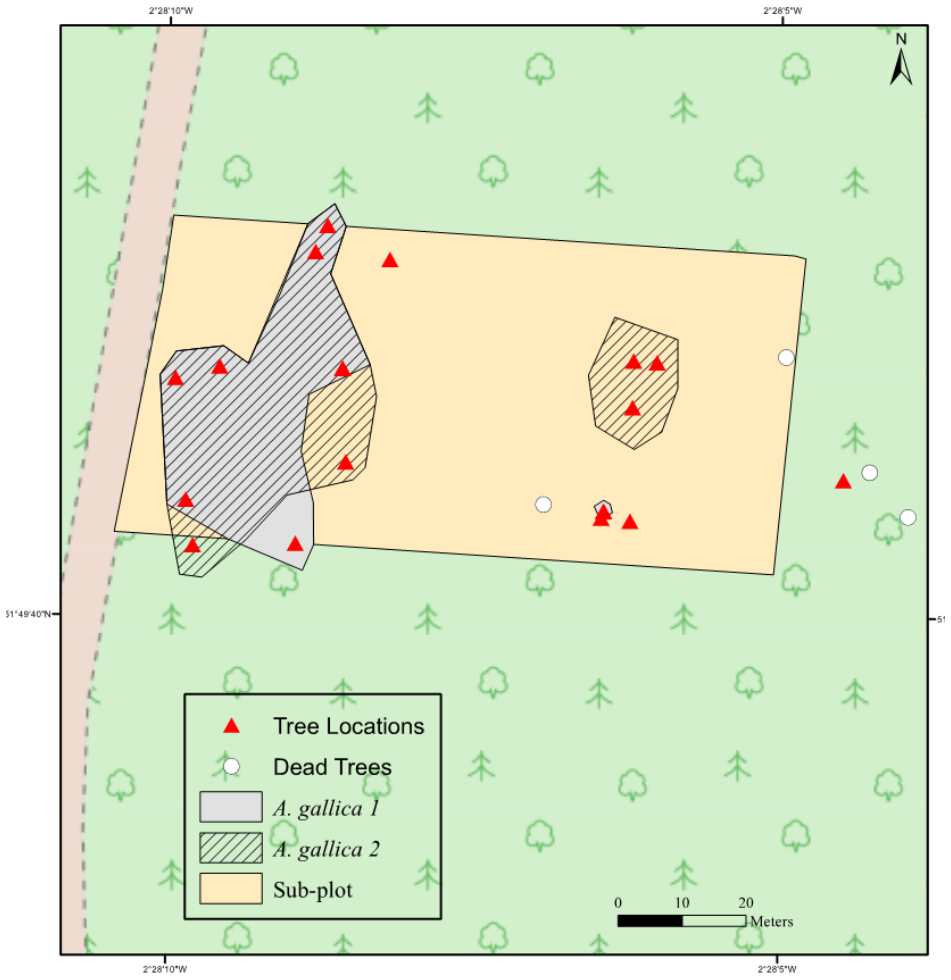


Figure 5.8 – Chestnuts Wood subplot area, red triangles indicating trees sampled, white circles indicate disease centres, which are areas with 2 of more dead trees and *Armillaria* rhizomorphs present. Solid grey area shows area of only isolates that fall into the subclades, identified by the EF1-α DNA sequence data; *A. gallica* 1 (separated by bootstrap value of 0.80), hashed area shows area of only isolates that fall into the subclade *A. gallica* 2 (separated by bootstrap value 0.92). Where both grey and hash areas are present representatives of both subclades are present within that area. The presences of sub clades of *A. gallica* were identified by the EF1-α DNA sequence data, isolates are a from rhizomorphs and wood panels.

5.4 Discussion

Previous studies from this site isolated both *A. gallica* and *A. ostoyae* from wood tissue (Denman et al., 2017), however, this project only *A. gallica* isolates were successful cultured from wood tissue, and new to this site are isolates directly isolated from rhizomorphs. From the culture work, *A. gallica* is the dominant *Armillaria* species present colonising and causing disease. Although the absence of *A. ostoyae* from this culture work should not suggest this pathogen is not present and not an issue, as it has been isolated from other areas of the woodland, alone and colonising trees alongside *A. gallica* (Denman et al., 2017). Potentially *A. ostoyae* is not present within this area of the woodland stand, or the techniques used missed potential isolates of *A. ostoyae*.

The species-specific primers developed by Mulholland (et al 2012) used in this study did not reliably identify the species of *Armillaria* present within this study, suggesting that those primers are not suitable for identification of *A. gallica* present on this site. This unreliability of the primers could be due to the complications in the taxonomy of *A. gallica* previously discussed.

Symptomatic trees were found to have significantly fewer buttress roots than those asymptomatic of *Armillaria* infection, less buttress roots could indicate a reduced capacity for water and nutrient uptake suggesting an inherent predisposition for disease within those symptomatic trees. Although the lower number of buttress roots of symptomatic trees could be a result of the trees growing in a high dense area of the woodland leading to a higher competition for resources, which could have implications for trees predisposition (Brang et al., 2003). The lower number of buttress roots on symptomatic trees is an unexpected result and should be tested further with a larger sample size and across different forest stands to ascertain if this could be informative for disease management. It could be implemented as a quick way to identify trees at risk of being infected with *Armillaria* below ground or at risk of *Armillaria* colonisation or other forest pathogens. This information could also be implemented in the selection process for the thinning of future sites affected with *Armillaria* species, to reduce the number of predisposing trees available for *Armillaria* infection and therefore reduce the spread of the infection across a site.

Armillaria is a root rot pathogen, known to cause necrosis of tree tissues of the root, collar and trunk of the tree, leading to disruptions of the water and carbon cycles (Oliva et al.,

2014). This destruction of cambial tissues negatively impacts the carbon storage within tree tissues, as carbon is an important element; it has a vital role in defence responses to compartmentalise the pathogen using carbon rich barriers, used in cell structures such as cellulose and hemicellulose and used for cell repair (Oliva et al., 2014). The redistribution of carbon resources can therefore affect tree storage systems and water transport (Oliva et al., 2014). The effects on the water cycle can be seen within this study site, with as higher moisture levels in the rhizosphere soils surrounding symptomatic trees when compared to soils surrounding asymptomatic trees and the wider site soils. The increase water levels in surrounding soils of symptomatic trees indicates root malfunction as a result of necrotrophic *Armillaria* infection. The infection destroys cambial and vascular tree tissues negatively effecting the hydraulic conductivity and phloem transport capabilities within the tree, resulting in overall less water uptake (Brang et al., 2003; Oliva et al., 2014).

After periods of rain tree roots are used to redistribute water and nutrients from surface soils to deeper drier soils, this redistribution of water and nutrients during wetter times keeps the trees access to nutrients and water by the taproot in drier periods to avoid drought stress. This is important adaptation in the competition for resources by reducing reliance on surrounding soils occupied by shallow rooted plants (Burgess et al., 2001, 1998). This redistribution can also be used to counteract periods of waterlogging, by redistributing the water into deeper soils for later use. The manipulation of water and nutrients within soils has wider implications for the rhizosphere and will impact the composition of rhizophere microbiome of the tree and soils (Burgess et al., 2001, 1998). The trees present on this study site were obtained from a nursery, common forestry practices involve removal of the taproot of the nursery trees. The loss of this taproot marks a large physiological change in how the trees function compared to trees with a taproot. This has potential implications for tree vigour in drier soils or in drier weeks and months, potentially predisposing those trees to drought stress sooner or more often than those trees with a taproot, increasing pathogen susceptibility during those times (Burgess et al., 1998). As well as reactions to drought, the redistribution of water and nutrients with the soils will potentially alter the natural microbiome, favouring organisms more adapted to drought stress. Further research into how the removal of the taproot and how this effects plantation trees should be considered, to enhance the understanding of the physiological impacts and also the wider changes to the microbiome of rhizophere soils and trees, and if this increases predisposition to pathogenic colonisation.

Soils that have a healthy microbiome usually have the ability to suppress pathogen growth, this effect is attributed to the total microbial activity (Berendsen et al., 2012). The phosphate and nitrate levels were found significantly lower in asymptomatic bulk soils when compared to the wider site soils and soils form around symptomatic trees. This suggests that the roots are taking these nutrients up and using them in healthy trees, but this is not as effective in diseased trees. This increase higher phosphate and nitrate could again indicating root malfunction as described above or it could be attributed to suppressed microbial activity. The lower microbial activity of those soils surrounding symptomatic soils could have a lower suppressing effect on *Armillaria* growth within the soils resulting in increased severity of attack on the tree and chance of colonisation (Berendsen et al., 2012).

A. gallica isolates cultured from both rhizomorphs or wood panels, are present in the same clade, as well as, colonising the same area of the site. Isolates within the same clade could therefore be the same isolates across soils and between trees, however this would need to be confirmed. To give more information on how many of those isolates within each clade are truly different isolates rather than copies of one isolate from across the colony extent, mating trials should be completed this would identify separate vegetative incompatibility groups of *A. gallica* isolates (Anderson et al., 1980; Rizzo and Harrington, 1993; Heinzelmann et al., 2018).

From the subplot area it appears that either *A. gallica* isolates that once infected the dead trees has moved on towards the living trees at the bottom of the subplot or there was previously were two areas of *Armillaria* causing infection and one has reduced back after the trees had died. To understand if the *A. gallica* has progressed from an area of dying or dead trees to an area of living trees, areas of disease centres with the presences of dead trees should be identified and the *A. gallica* should then be sampled radially or in transects to see if there is a pattern of the isolates moving away from the areas of dead trees to living ones or whether the *A. gallica* stays and colonises the dead wood and the colony extent expands to include more living trees.

A. gallica rhizomorphs were able to grow attached to both asymptomatic and symptomatic trees and were connected across the site, and colonising the soils around the trees as a saprophyte. It is unclear if the same isolates are able to infect the tree in all cases. Trees P111, P107 and P109 have confirmed *A. gallica* isolates present from wood panels and isolates, suggesting that in those isolates were saprophyte in the soils until it reach a tree in which it

could over come the host defences and colonising tree tissues causing disease. Tree P111, does have 14 isolates: 3 isolates from wood panels and 9 isolates from rhizomorphs. The rhizomorph isolates for tree P111 and are present in both clades of *A. gallica*, indicating this tree has more than one isolate with rhizomorphs present around the tree.

Previous phylogeny studies have identified isolates within the *A. gallica* complex at very different positions within the clade compared to other studies using different gene regions, and it is suggested that within the *A. gallica* complex there are between 3 and 8 different distinct *A.gallica* clades (Coetzee et al., 2018; Keča et al., 2015; Klopfenstein et al., 2017). The *A. gallica* taxonomy is poorly understood, various previous studies found a range of clades present depending on geographical location of the studies as well as type and number of isolates used (Keča et al., 2015). The results from this field site survey show that two types of *A. gallica* present on the EF1$-\alpha$ phylogenetic tree, however this complexity of the *A. gallica* clade makes understanding the true ecological value of those differences difficult. Further work is needed to determine if the differences in saprophytic and pathogenic isolates found on this site are due to natural variability in virulence between isolates, or if there are different strains that express different ecologically strategies responses, depending in environmental conditions. Those differences in strategies and virulence potentially could translate further into the taxonomy, with the *A. gallica* splitting into more than one species within this super clade.

Chapter 6

Meta transcriptomic analysis on oak tree lesions caused by *Armillaria* spp. in Chesnuts Wood, Forest of Dean

6.1 Introduction

Pathogenic fungal species are diverse and have adapted to pathogenic lifestyles via expansion of gene families encoding plant cell wall degrading enzymes (PCWDE) in pathogenic fungal species, and deletions in whole pathways associated with saprophytic living (Aguileta et al 2009). This diversification has driven specialisation and speciation amongst some species, with some species exhibiting small host ranges and specialised lifestyles, to more generalised pathogens with wide host ranges, geographical locales and a lifestyle that is more adaptable (Hu et al., 2014). The gene expression profile and subsequent secretome (the set of proteins expressed by an organism and secreted into the extracellular space) of a fungal species indicates the type of lifestyle; pathogen, saprophyte/parasite or symbiont, with the objective to extract as much nutrients from the environment as possible (Muszewska et al., 2017). Many fungal phytopathogens act as competitive saprophytes with the ability to survive within the dead woody tissue of the host species until a new living host is located (King et al., 2011). *Armillaria* is a genus of basidiomycete fungus with a global distribution found in natural and planted forests, gardens and agricultural land; it uses rhizomorphs and mycelia to create a network of hyphae within tree tissues and forest soils, also producing mushroom fruiting bodies to produce spores for reproduction (Ford et al., 2015; Ross-Davis et al., 2013). As a white-rot pathogen, *Armillaria* is a facultative necrotroph causing decay of the cambial tissue of the trunk and roots. *Armillaria* are important plant pathogens, but in comparison to other phytopathogens are relatively understudied. Many *Armillaria* species obtain nutrients from living and dead tissue across a wide variety of host species by displaying pathogenic, parasitic

and saprophytic ecological strategies at different times and to a varying extent throughout their life cycle (Muszewska et al., 2017). This complex lifestyle makes this genus an interesting candidate to search novel genes responsible for virulence, plant cell wall degrading enzymes (PCWDE), overcoming host defences, and network signalling and translocating nutrients across the three body forms.

Within plant cell walls cellulose is the most abundant polysaccharide; in *Quercus robur* cell walls comprise approximately 41 - 46 % dry weight cellulose (Le Floch et al., 2015; Sista Kameshwar and Qin, 2018). Cellulose is made of linear chains of repeated β-D-glucose units providing rigidity to the cells (Le Floch et al., 2015). Plant cell walls contain up to 30 % hemicellulose, with *Q. robur* cell walls comprising approximately 18 – 22 % dry weight hemicellulose (Herrera et al., 2014a; Le Floch et al., 2015). In hardwood species, such as oak, hemicellulose is made up of relatively large molecules containing between 150 - 200 residues, constituting of predominately xylans with many containing O-acetyl groups, there are also lesser amounts of galacturonic acid, arabinose, rhamnose, and glucomannan (Le Floch et al., 2015; Martínez et al., 2005; Moreira and Filho, 2008; Puls, 1997; Sista Kameshwar and Qin, 2018). Lignin is the third most abundant component of wood, it makes up approximately 16-29 % of plant cell walls in hardwood tree species, with *Q. robur* having 29 % dry weight lignin (Herrera et al., 2014b). It is made up of Guaiacyl-syringyl lignin with different types of linkages between the monomers with lots of branching making it three dimensional instead of linear, and is very resistant to degradation chemically or biologically (Le Floch et al., 2015; Martínez et al., 2005).

Armillaria is a white rot fungus known to have an arsenal of plant cell wall degrading enzymes to penetrate the woody tissue and degrade cellulose, hemicellulose, lignin and pectin, in order to obtain access to nutrients. Ross-Davis et al., (2013) applied transcriptome approaches to characterise plant cell wall degrading enzymes in *A. solidipes* isolated from a natural grand fir (*Abies grandis*), identifying 23 homologs for ligninolytic (and related) activity, 28 homologs for pectinolytic activity, 41 homologs combined for cellulolytic and hemicellulolytic (and related) activity. *A. solidipes* genes associated with overcoming host defences were also represented within this transcriptome, however to a much lesser extent, with only one GST (Glutathione S-transferases) and 39 fungal ABC (ATP- binding cassette) transporters identified (Ross-Davis et al., 2013). Sipos et al., (2018) sequenced the genome of four *Armillaria* species, *A. gallica*, *A. ostoyae*, *A. cepistipes* and *A. solidipes*, and through transcriptomic

and quantitative proteomic analysis on the invasive and reproductive developmental stages of *A. ostoyae*, identified 20 genes related to pathogenesis, causing degradation of plant well walls and cell death. There was also an enrichment for expansins and cerato-platanin genes. Salicylate hydroxylases were found to be moderately enriched, those enzymes are used combat the host defences, and Glycoside Hydrolase group 75 (GH75) and SM (secondary metabolite) genes homologues were also found within the *Armillaria* genome. Homologues of PCWDE; ligninolytic enzyme families were underrepresented and enzyme Carbohydrate Esterase 5 CE5 (cutinases) is completely missing within the *Armillaria* genome, whereas cellulolytic and pectinolytic enzymes were over represented, notably GH1, PL3, CE8 and CMB67 (Sipos et al., 2018).

Transcriptome-based approaches therefore represent a promising strategy to identify gene expression patterns associated with *Armillaria* pathogenicity and function. However, the only previous studies have focused on *A. ostoyae* grown in laboratory conditions (Sipos et al., 2018), or a single *A. solidipes* mycelial fan from a natural grand fir (Ross-Davis et al., 2013). Consequently, there is a paucity of information on the transcriptome profile of *A. gallica* as a pathogen, and specifically it's interaction with oak. The overarching aim of this chapter was therefore to utilise contemporary transcriptome and computational approaches to characterise the gene expression profile of lesion tissue from symptomatic oaks with *A. gallica* infection with inner bark tissue from asymptomatic trees. By characterising the metatranscriptome of lesion tissue from active *Armillaria* infections, this work provides the first in situ metatranscriptome of an *Armillaria* infection in oak. Firstly will help determine if *Armillaria* is active and functioning as a pathogen within the tree lesion and identify other pathogenic fungal species which may be contributing to the necrosis. It will address important knowledge gaps regarding gene expression and activity of *Armillaria* in oak lesions, their co-occurrence and interactions with other members of the oak microbiota, and the oak host. Furthermore, comparison of gene expression profiles between symptomatic and asymptomatic oak tissue provided insights into the enzyme profile of *Armillaria* as a saprophyte or a pathogen which is capable of causing necrosis and decay of the wood tissue of roots and stems.

6.2 Materials and Methods

6.2.1 Sampling Area

Chestnuts wood is located within the Forest of Dean, Gloucestershire. It is a small woodland area maintained by the Forestry Commission (Figure 5.1). Many of the oaks were planted between 1948-1950 and 1960 in high densities (approximately 2-3 meters apart), with some areas of oak planted in 1814 and 1850. The *Armillaria* infected areas are within the high-density areas with steeper gradients that were planted in the mid-1900s.

6.2.2 Previous Detection of *Armillaria* spp. in Chesnuts Wood

Previous isolation studies conducted by Forest Research has isolated two *Armillaria* species from the trees on this site; *A. gallica* and *A. ostoyae*, with both isolated separately and co-occurring on the same tree (Denman et al., 2017). In Chapter 5, 12 asymptomatic and 10 symptomatic oak trees were identified and sampled for rhizomorphs and disease status using Hessain and ARCHI guides (Drénou et al., 2015). Visible lesions on the symptomatic trees were sampled. *Armillaria* gallica was isolated from rhizomorphs and wood panels, but *A. ostoyae* was not isolated. Soil samples were also collected every 50 metres across the site and approximately 1 metre away from each tree sampled, and organic matter, moisture and soil chemistry data was collected shown in chapter 5.

6.2.3 Sampling design: Tree sampling for Meta-transcriptome analysis

Trees were selected using the ARCHI guide; trees categorised as healthy were designated as asymptomatic, and symptomatic trees were trees in the ARCHI irreversible category and with visible rhizomorphs colonising the roots (Drénou et al., 2015). A mallet and sterilised chisel was used to remove a panel of tissue from the root collar or trunk, approximately 2 inches by 2 inches, from 6 asymptomatic trees and from lesion areas in 6 symptomatic trees. Each panel was immediately placed into a sterile falcon tube and frozen in liquid nitrogen on site and transferred to -80°C once back at the laboratory.

Table 6.1 – List of tree asymptomatic and symptomatic trees sampled with sample ID, tree health statue according to ARCHI guide (Drénou et al., 2015) and rhizomorph presence.

Health status	Tree ID	Sample ID	ARCHI category	Rhizomorphs present
Asymptomatic	403	403A	Healthy	No
	403	403B	Healthy	No
	404	404C	Healthy	No
	405	405A	Healthy	No
	406	406A	Healthy	No
Symptomatic	3101	3101B	Irreversible	Yes
	306	306A	Irreversible	Yes
	P105	P105A	Irreversible	Yes

6.2.4 Laboratory analysis

RNA isolation and sequencing

The wood tissue samples were stored at -80°C until RNA extraction. The wood panels were macerated in liquid nitrogen using a pestle and mortar, and whilst still frozen, 5 mL of RNA extraction buffer was added (4 M guanidine thiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5 % (w/v) polyvinylpyrrolidone and 1 % (v/v) β-mercaptoethanol) and mixed until thawed, another 2.5 mL of RNA extraction buffer was added alongside 500 μL 20 % sodium lauroyl sarcosinate. The sample and buffer mixture was then incubated whilst shaking (200 rpm) at room temperature for 15 minutes. The RNeasy Plant Mini kit (Qiagen) was used for RNA extraction, with one modification to the manufacturer kit instructions; after centrifuging the mixture through the QIAshredder column 350 μL, the supernatant was mixed with 0.9 mL 100 % ethanol (instead of 0.5 mL 100 % ethanol) and transferred to the RNeasy mini column. The extracted RNA was then treated with DNase 1 amplification grade (Sigma) to remove any genomic DNA carry over. To reduce freeze thaw cycles, samples were immediately transferred into single use 20-50 μL aliquots in fresh Eppendorf tubes for sequencing and quality control and immediately frozen in liquid nitrogen and stored at -80°C. The RNA concentration was determined using Qubit dsDNA HS Assay Kit and RNA quality determined using 2 % gel electrophoresis and NanoDrop^{TM} spectrophotometer (LabTech). The RNA extracts with the best quality RNA (8 extracted samples out of 38 attempted extracts) were sent to the Centre for Genomics Research, University of Liverpool, for rRNA removal using the Ribo-Zero rRNA removal kit (Illumina) for low RNA input, to generate clone DNA

libraries and sequenced using one lane on the Illumina HiSeq® 4000 with; paired-end reads, 2 x 150 bps sequencing, using 280 M clusters.

6.2.5 RNA data processing and analysis.

The RNA sequencing data was initially processed at CGR Liverpool using Cutadapt v.1.2.1 to remove Illumina sequencing adapters (Martin, 2011), the reads were also trimmed using Sickle v1.200 (Josh and Fass, 2011). The forward and reverse reads were interleaved. To assign taxonomy to transcript sequences, the interleaved files were uploaded to the online software tool OneCodex (https://onecodex.com/) and using R studio (R Development Core Team, 2009) a one-way ANOVA was completed to compare phylum, families and *Armillaria* reads between symptomatic and asymptomatic trees. Sample 403B was selected to represent asymptomatic tree 403, 403A was not used in further analysis (Table 6.2).

Table 6.2 – Meta-transcriptome sequencing statistics.

Sample	Total Number of Reads (bps)	Average read length	Average GC content (%)
P105A	78412196	141.8	49.6
306A	79555886	140.9	49.4
3101B	71864026	141.8	48.3
403A	63106723	145.1	47.9
403B	87919614	143.2	47.9
405A	84014761	140.3	47.1
406A	73937881	142.1	47.2
404C	74913548	143.0	46.6

The interleaved files were then used to create a *de novo* transcriptome assembly using the RNAspades option within SPADES analyses (Bankevich et al., 2012). The alignments were completed against a custom fungal database downloaded from NCBI (O'Leary et al., 2016) using DIAMOND (Buchfink et al., 2014), this generated total count data for *Armillaria* transcripts. The sequences were also aligned using bowtie2 (Langmead and Steven L Salzberg, 2013) against a custom *Armillaria* only database, and a fungal only database, to generate sam files; those files were then converted to bam files using samtools (Li et al., 2009). From those bam files analysis of the reads and differential expression analysis were done using against the same custom *Armillaria* and fungal databases (Buchfink et al., 2014). To visualise and statistically analyse the transcripts per million data from the eXpress analysis and enzyme data searches from the DIAMOND analysis were both put through R packages ANOVA, DESeq2 (Love et al., 2014) and EdgeR (Chen et al., 2010). The DESeq2 analysis was

used to generate a MA plot to show log2 fold changes differentially expressed genes only, and EdgeR data was used to generate MSD plot using the BCV (biological coefficient of variation) method to determine distance (Chen et al., 2010). The EdgeR analysis initially used the exacttest function to statistically analyse differential expression assuming negative binomial distribution, due to the lack of fit to the model and low expression within samples, the data was further analysed using generalised linear models (glm); glmOLFiT which uses quasi-Likelihood Tests and glmTreat which tests differential expression relative to a threshold (Chen et al., 2016, 2010). All heatmaps generated from EdgeR analysis using eXpress or DIAMOND, outputs were compiled with hierarchical clustering and normalised by library size.

6.3 Results

6.3.1 Phylum-level composition of transcripts between asymptomatic and symptomatic lesion tissue in *Armillaria* infected oak

Preliminary data analysis using the OnecodeX online tool classified between 7-14 % of all reads. Protobacteria had the highest percentage of the total read counts in all samples; in asymptomatic trees ranging between 93-69 %, with an average of 86 z%, and in symptomatic trees ranging between 5-74% with an average of 57 %, Figure 6.1 and Figure 6.2. Asymptomatic samples 403A, 404C and 405A show a similar pattern of phyla present, whereas asymptomatic tree 406A shows less phyla present compared to other samples. All symptomatic tree samples 306A, 3101B and P105A appear to have different profiles. Symptomatic sample 306A had the highest percentage of reads associated to Ascomycota (10.6 %), symptomatic 3101B and P105 had the lowest overall classified read counts, for 3101B the phylum with the highest percentage (after Proteobacteria) was Candidatus Margulisbacteria 0.2 %, Basidiomycota 0.2 % and Firmicutes and 0.3 %. P105 had highest read count for Basidiomycota 7 %, followed by Firmicutes 6.7 %, Ascomycota 5.1 %, Acidiobacteria 4.7 %, Actinobacteria 3.4%, and Cyanobacteria 3 %. The asymptomatic and symptomatic trees were then grouped to produce mean values (Figure 3). Between asymptomatic and symptomatic trees the ANOVA results show no significant difference in phylum-level composition. Asymptomatic tree 406A had consistently low numbers of classified reads during the analysis, there no indication that this sample was of low quality after sequencing, it did show lower diversity

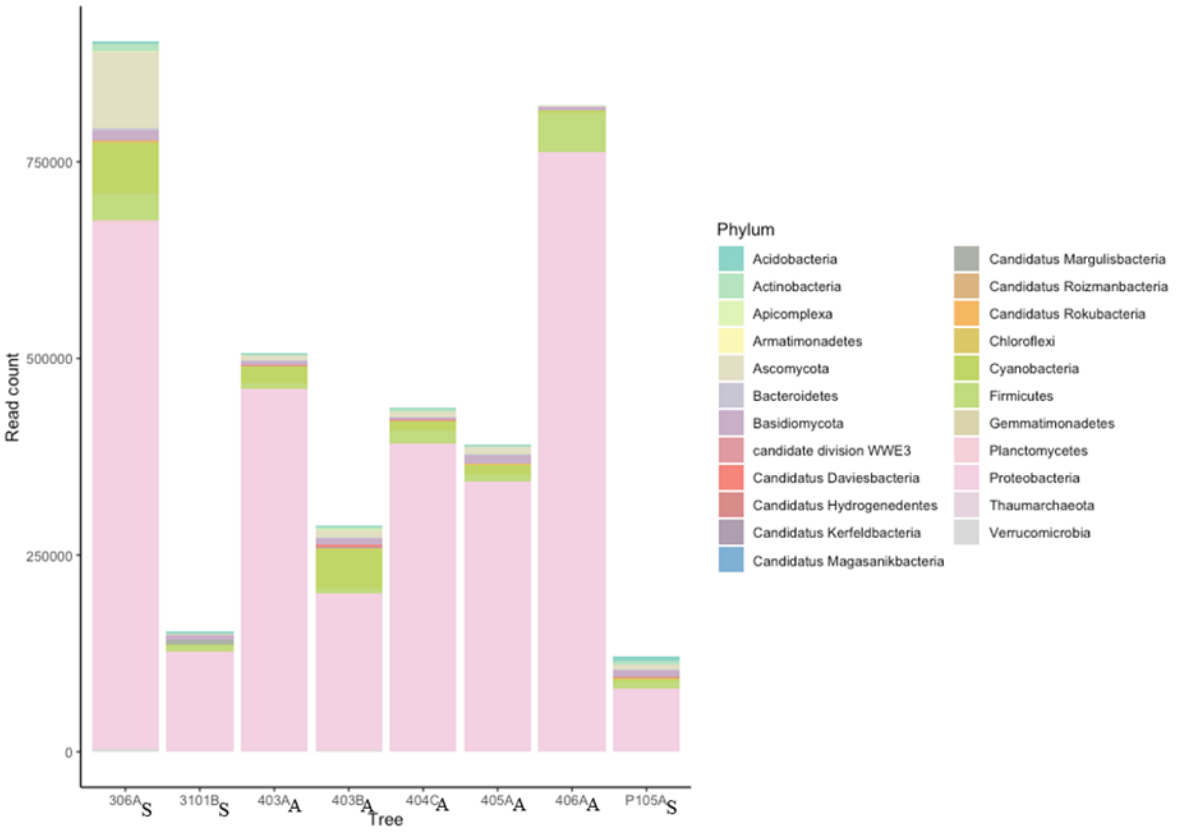


Figure 6.1 – Read count of each phylum present by tree. Data generated using OnecodeX database. Asymptomatic trees labelled with A, samples; 403A, 403B, 404C, 405A and 406A. Symptomatic tree labelled with S, samples; 306A, 3101B and P105A.

compared to other samples. Symptomatic tree P105 shows the highest percentage read count for *Armillaria* species, where *Armillaria* reads account for 85 % of all reads assigned to Basidiomycota, all other symptomatic and asymptomatic samples the classified *Armillaria* reads account for between 0.21 – 0.78 % of all Basidiomycota reads.

6.3.2 *Armillaria* composition of transcripts between asymptomatic and symptomatic lesion tissue in infected oak

The OnecodeX data shows more *Armillaria* sequences present in symptomatic trees 14.3 % total read count (Table 6.3), however this was not significant using an (ANOVA, df = 3, p = 0.09), with homologs to *A. gallica* and *A. cepistipes* being the most abundant. Asymptomatic trees do have low numbers of *Armillaria* reads present, with homologs of *A. cepistipes* and *A. gallica* reads as the most abundant. For sample P105 *A. gallica* is the most abundant with 6.52 % (322834) of the reads, followed by *A. cepistipes* with 3.60 % (165245) classified reads.

Trees 404 and 3101 lack reads classified to *Armillaria* species, and 306 and 403 shows an intermediary number of reads classified to *Armillaria* species, (Table 6.3).

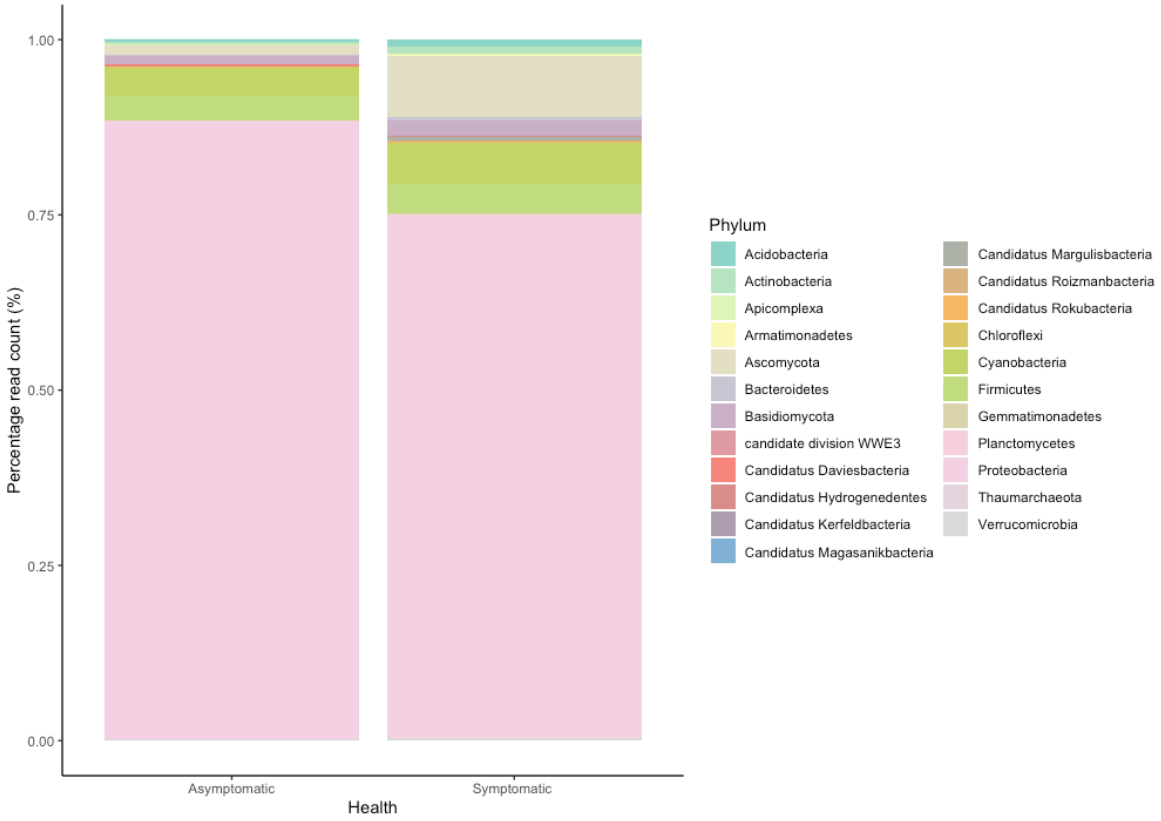


Figure 6.2 – Percentage (%) of reads classified to phylum present by tree health status; Asymptomatic (403A, 403B, 404C, 405A) and Symptomatic trees (306A, 3101B and P105A). Data generated by OnecodeX database.

Table 6.3 – Read count and percentage (%) for *Armillaria* species, *A. gallica*, *A. cepistipes*, *A. solidipes* and *A. ostoyae* from Onecodex analysis.

	Asymptomatic					**Symptomatic**			
Sample	404	403	405	406	Total	306	3101	P105	Total
Total read count	62334992	87462498	83425598	73587830	306810918	79200054	71538982	77955770	228694806
Total *Armillaria* spp .	347	1459	160	182	2148	1488	215	706656	707143
% *Armillaria* spp	0.04 %	0.04 %	0.01 %	0.01 %	0.11 %	0.06 %	0.01 %	14.2 %	14.3 %
A. gallica	79	337	21	27	464	175	27	322834	323119
A. cepistipes	95	367	10	73	526	278	67	165245	165590
A. solidipes	14	59	6	4	83	4	0	18225	18229
A. ostoyae	0	0	0	0	0	0	0	33958	33958

The diamond analysis classified more reads to *Armillaria* species compared to the OnecodeX database. Symptomatic tree P105 had the most *Armillaria* reads identified (18468), 0.4 % of total counts (Table 6.4). The DIAMOND analysis did not classify any reads belonging to *A. cepistipes* or *A. ostoyae* (Table 6.4).

Table 6.4 – Total number and percentage (%) of reads from DIAMOND analysis filtered for *Armillaria* only

	Asymptomatic					**Symptomatic**			
Sample	403	404	405	406	Total	P105	306	3101	Total
Total read count	8329799	2218972	887318	318723	11754812	4995666	2790870	2781732	10568268
Armillaria spp. reads	9858	2426	1220	463	13967	18468	7805	443	26716
% *Armillaria* reads	0.1 %	0.1 %	0.1 %	0.2 %	0.1 %	0.4 %	0.3 %	0.02 %	0.3 %
A. gallica read count	4954	1217	616	236	7023	9726	3902	224	13852
% reads	0.05 %	0.05 %	0.07 %	0.07 %	0.06 %	0.20 %	0.14 %	0.008 %	0.315 %
A. cepistipes read count	0	0	0	0	0	0	0	0	0
% reads	0	0	0	0	0	0	0	0	0
A. solidipes read count	4904	1209	604	227	6944	8742	3903	219	12864
% reads	0.05 %	0.05 %	0.07 %	0.07 %	0.06 %	0.17 %	0.14 %	0.008 %	0.12 %
A. ostoyae read count	0	0	0	0	0	0	0	0	0
% reads	0	0	0	0	0	0	0	0	0

6.3.3 Enzyme composition of transcripts between asymptomatic and symptomatic lesion tissue in *Armillaria* infected oak

Symptomatic trees have more homologues for all ligninolytic, pectinolytic, chitinolytic and cellulose/hemicellulose enzymes (for the full list, see appendix 1), however this difference is not significant (ANOVA, df =1, p = 0.09) (Table 6.5). P105 has the most reads classified for each enzyme searched for, as seen by the yellow colour of the heatmap and dendrogram (Figure 6.3). This analysis grouped symptomatic tree 3101 and asymptomatic tree 406 together, both have low read counts for all the enzymes investigated. Asymptomatic trees 403, 405 and 406 are clustered together by read abundance, with symptomatic tree 306 read count was closer related to this cluster of asymptomatic trees than P105. The extacttest shown that when symptomatic trees were compared to asymptomatic trees xylosidase and GH5 was significantly up regulated (extacttest, p = 0.04, extacttest, p = 0.04, respectively) (Figure 6.3).

Table 6.5 – Number of reads classified to enzyme homologues using DIAMOND outputs.

	Symptomatic		**Asymptomatic**	
Substrate	Number of homologues	Read count	Number of homologues	Read count
Lignin	6	142	3	24
Pectin	6	377	3	314
Chitin	3	16	2	4
Cellulose, hemicellulose	18	312	12	118

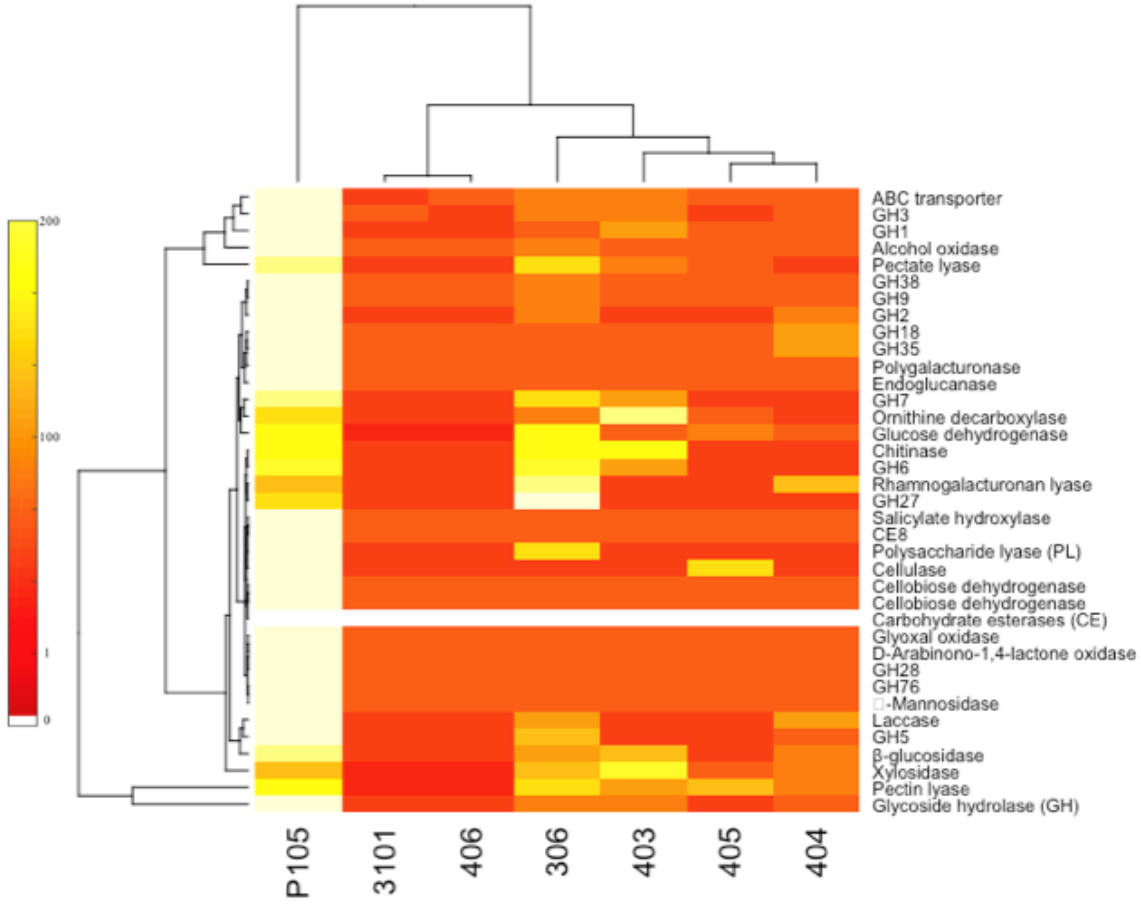


Figure 6.3 – Enzyme read count, normalised by library size. Symptomatic trees: P105, 3101, and 306. Asymptomatic trees; 406, 403, 405, 404. Data produced from Diamond outputs.

6.3.4 Differential gene expression of *Armillaria* transcripts between asymptomatic and symptomatic lesion tissue in infected oak

Differential expression analysis using DESeq2

The DESeq2 analysis of *Armillaria* only eXpress outputs transcripts per million counts shown that there was differential expression with more genes upregulated to 1-1.5 log fold change Figure 6.4, however, due to the lack of fit to the model no statistical analysis was performed.

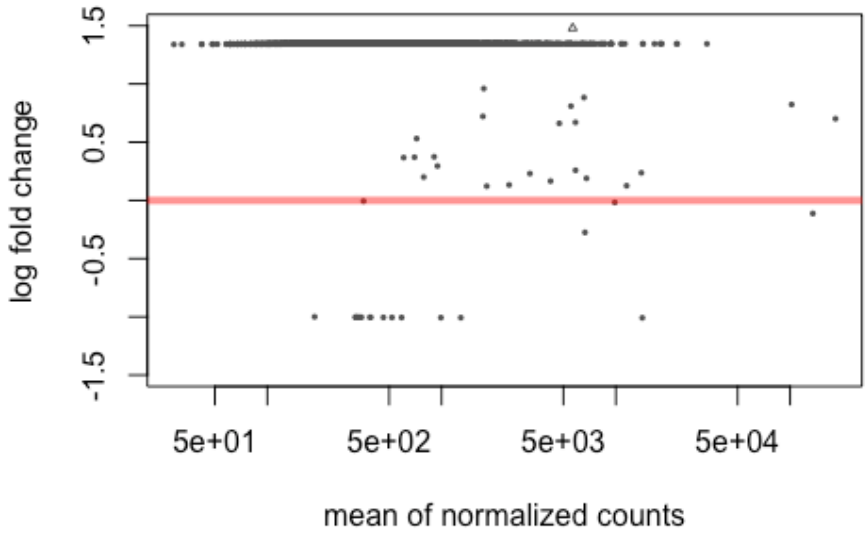


Figure 6.4 – MA plot generated by the DESeq2 analysis, showing the log fold change of *Armillaria* genes present using transcripts per million, normalised by library size factor.

Differential expression analysis using EdgeR

The EdgeR analysis of *Armillaria* eXpress transcripts per million (tpm) outputs did cluster asymptomatic trees 405, 403 and 404 together, with 406 separated from other trees shown in the MDS plot (Figure 6.5). The symptomatic trees 306, P105 and 3101, were separated from asymptomatic trees and each other in the MDS plot (Figure 6.5). Further analysis of normalised tpm using dendrogram within heatmap R package clustered the symptomatic tree 3101 as a separate group, and P105 and 306 were clustered together, asymptomatic trees; 403, 405, 406 and 404 clustered together (Figure 6.6).

Genes identified by the EdgeR analysis as being significantly up-regulated in the gene expression; Hypothetical proteins, Snodprot1, Serine proteinase inhibitor 1A-1, Ubiquitin (although one homologue was down-regulated) and Histone H4 (Table 6.6).

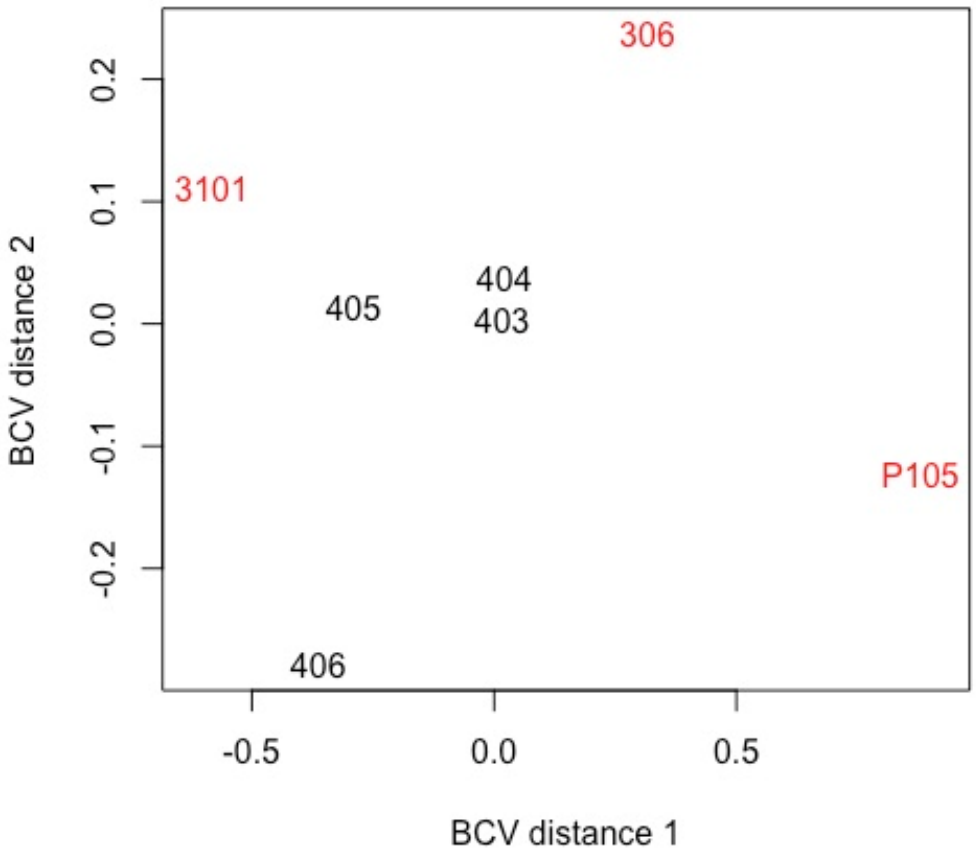


Figure 6.5 – MDS plot using read data generated by eXpress and analysed using EdgeR R package, using BCV (biological coefficient of variation) method. Symptomatic trees are in red 3101, 306 and P105 and asymptomatic trees are in black; 404, 403, 405 and 406.

Table 6.6 – EdgeR statistical analysis of *Armillaria* species eXpress outputs (transcripts per million normalised by library size) using exacttest and glmQLFTest, glmLRT and glmTreat. Up gene regulation ↑ and down gene regulation ↓. Hypothetical proteins and uncharacterised proteins removed, for full table see appendix 1, Table 9.2

			EdgeR statistical test p-value		
GenBank accession	**GenBank definition**	**Gene regulation (↑ / ↓)**	**Exacttest**	**glmQLFTest**	**glmTreat**
SJL04025.1	Probable ubiquitin - ribosomal protein S27a fusion protein	↑	***	-	-
PBL01295.1	Eukaryotic ADP/ATP carrier	↑	*	-	-
SJL03400.1	Histone H4	↑	***	-	*
PBL00157.1	Ubiquitin	↑	-	-	**
PBK95666.1	Ribosomal protein s30	↑	-	-	*
PBK76382.1	Ubiquitin	↓	-	-	*
PBK91638.1	Serine proteinase inhibitor IA-1	↑	-	-	*
PBK74990.1	SnodProt1	↑	-	-	*
PBK85573.1	LysM-domain-containing protein	↑	-	-	*
PBK73105.1	Ribosomal protein s25	↑	-	-	*
PBK74393.1	Serine proteinase inhibitor IA-1	↑	-	-	*
		p < 0.000 ***	No sig -		
		p = 0.02 **			
		p = 0.05 *			

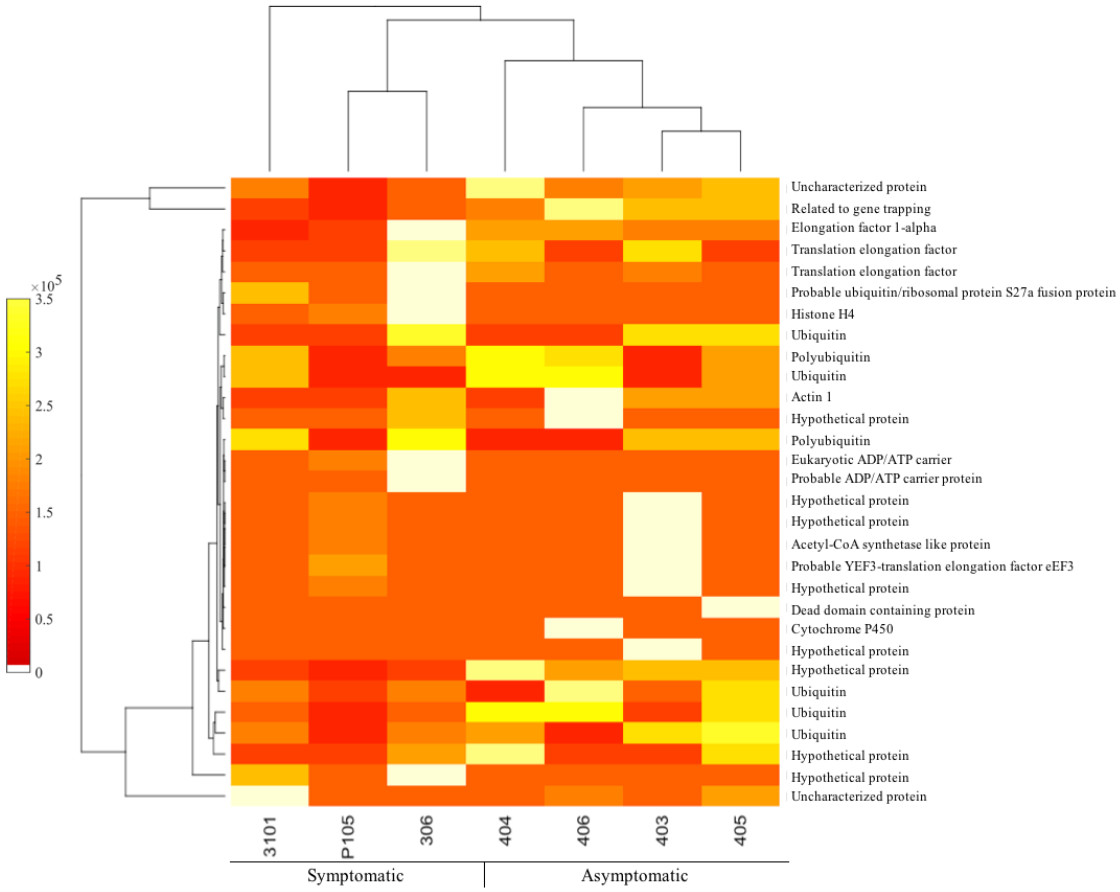


Figure 6.6 – Heatmap of *Armillaria* species transcripts per million from EgdeR package, genes present in each sample only and normalised by library size. Symptomatic trees; 3101, P105, 306 and asymptomatic trees; 404, 406, 403, 405.

Pathogen composition of transcripts between asymptomatic and symptomatic lesion tissue in *Armillaria* infected oak

Using the OnecodeX and DIAMOND analysis outputs other tree pathogen species were searched for. However, no reads were classified to *Phytophthora, Klebsiella, Gibbsiella* and *Brenneria* species. There were between 100-840 reads classified to *Clostridium* in each sample. *Pseudomondas* genus had reads classified in all samples only in the OnecodeX database, with 30663 (0.03 %) in 403B, 73993 (0.09 %) in 306A and 15333 (0.02 %) in P105A: however, no reads classified to *P. syringae*. *Gymnopus* (or *Colyibia*) has no reads classified in OnecodeX outputs, although in DIAMOND output there were 3247 (0.004 %) in P105, 4039 (0.005 %) in 306, 5113 (0.006 %) in 403 and 1280 (0.002 %) in 404.

6.4 Discussion

6.4.1 *Armillaria* composition of transcripts

The analysis of percentage read count and tpm (transcripts per million) data confirms that *Armillaria* is present on all trees sampled, asymptomatic and symptomatic. The analysis of RNAseq by the online OnecodeX tool is not designed to determine abundance of each organisms, therefore this analysis cannot indicate that there is a larger biomass of *Armillaria* hyphae or is more active on trees with high read counts. Previously, *A. gallica* and *A. ostoyae* have been isolated from this site colonising the same tree (Denman et al., 2017), however, only *A. gallica* was isolated from previous sampling. Reads from *A. gallica* were found using OnecodeX, DIAMOND and eXpress on all symptomatic and asymptomatic trees, indicating that *A. gallica* is always present on trees when there are incidences of *Armillaria* infection on a site. *Armillaria* species have the ability to survive within the soils and dead wood, therefore on sites of *Armillaria* infection there will be mycelium and rhizomorphs surviving, growing and spreading infection within the soils, with the *Armillaria* either acting as a saprophyte or the translocation of nutrients (Baumgartner et al., 2011; Keĉa and Solheium, 2006; Koch et al., 2017; Oliva et al., 2012; Sipos et al., 2018). The presence of *Armillaria* on all trees suggests that either none of the trees on this site are truly asymptomatic or that *A. gallica* is acting as a saprophyte and can change strategy to a pathogen under the right conditions and predisposing factors. *A. gallica* has also been found to colonise the same tree as other

Armillaria species, further research into interactions between those species which can occur; whether *A. gallica* colonises the tree first as a primary pathogen or as a saprophyte making more favourable conditions for another *Armillaria* species to colonise cause disease or and the combination of both species cause overall disease in the tree.

Reads were classified to both *A. cepistipes* and *A. solidipes*, both of which have not been isolated from this site before, both species are closely related to *A. gallica*, the presents of reads classified to those species are likely to be homologous genes of *A. gallica* therefore this does not infer presence and is potentially due to the database used in the analysis. *A. cepistipes* is thought to be a weak pathogen or parasite, rarely colonising hardwoods and more commonly isolated from coniferous trees (Guillaumin et al., 1993; Keĉa and Solheium, 2006; Sicoli et al., 2002). This site was a coniferous forest previous to the oak plantation, there is no knowledge if any *Armillaria* species were present at that time (pers. comn., Denman 2018), however, it is possible that *A. cepistipes* was present and remained in the soil and was able to colonise the oak species. Further microbiome analysis would be needed to confirm the presences of different *Armillaria* species, as the read counts used in OnecodeX and DIAMOND are not normalised against the *Armillaria* genome.

6.4.2 Composition of fungal enzymes

Ayyappa et al., (2018) found that the GH5 enzyme family in wood rot fungi was the most represented enzyme within the genome, alongside GH3. GH5 was significantly up-regulated in symptomatic trees, this is a vital group of enzymes used in the degradation of mannan, which is a constituent of hemicellulose in plant cell walls (Marchetti et al., 2015; Moreira and Filho, 2008). GH38 enzyme family is used in processing N-/O-glycans, which are complex glycomolecules found in cell walls, N-/O- glycosylation is an important process in growth and defence signalling as well as response signally to environmental factors (Nguema-Ona et al., 2014). In this project CE5 (cutinases) was found to have no reads classified, this was also found by Sipos et al. (2018) and reduced copy numbers is common in white rot fungi (Ayyappa et al., 2017). GH28 is an important PCDWEs for saprophytic and pathogenic fungi, and are thought to have high copy numbers in necrotrophic fungi in comparison to biotrophic and non-pathogenic fungi (Figure 6.3 and appendix 1, Table 9.1). This enzyme is important in the breakdown of pectin by hydrolysing the glycosidic bonds present (Sipos et al., 2018; Sprockett et al., 2011). Although GH28 is not identified as significantly upregulated is does

have a higher read count present in P105, all other samples produce lower GH28 read counts (Figure 6.3). Further analysis of the enzymes within sample is needed, the data used for this analysis was aligned against the NCBI fungal database, to get a more comprehensive overview of the enzymatic activity within the samples the reads need to be aligned using the CAZy database (Carbohydrate-Active enZYmes database: http://www.cazy.org/).

6.4.3 *Armillaria* gene differential expression

There were four genes identified by the EdgeR analysis as being significantly up-regulated in the gene expression; Snodprot1, Serine proteinase inhibitor 1A-1, Ubiquitin (although one homologue was down-regulated) and Histone H4. Snodprot1 is a cerato-platanin (CP) protein that are released during early developmental stages, it can induce cell death and stimulate the plants immune response (Gomes et al., 2015; Luti et al., 2019). Serine proteinase inhibitor or serpin are used to bind to the target proteinases causing a conformational change rendering it useless, this process destroys both the serpin and proteinase (Huntington et al., 2000; Soukup et al., 2009). This suggests that this serpin is produced by the *Armillaria* in response to proteinases released by either the oak as an immune response or by other competitors within the lesion microbiome. To determine this repeating the eXpress analysis against oak genomes or bacterial database may identify where the proteinases are up-regulated. Ubiquitin is a common protein found within cells, and maintained by recycling, it is thought to have a vital function in host-pathogen interactions, although that role is not understood. Oh et al., (2012) created ubiquitin knock-out mutants of *Magnaporthe oryzae* and found a loss of vigour, problems will reproduction and development of infection structures alongside a decrease in overall virulence (Oh et al., 2012).

Previous studies have compared transcriptome analysis of mycelium from one tree or using laboratory cultured *Armillaria* mycelium, rhizomorphs and fruiting bodies (Ross-Davis et al., 2013; Sipos et al., 2018). The process of extracting RNA from woody tissue for metatranscriptome analysis is difficult, to produce 8 RNA samples of a high enough quality for NGS the RNA extractions were repeated 38 times. Precautions were taken to preserve the integrity of the RNA such as, reducing freeze thaw cycles by using single-use aliquots, flash freezing samples in liquid nitrogen and storing samples at -80°C.

The use of statistical packages within this analysis posed difficulty, EdgeR and DESeq2 packages found low replication of gene expression and was therefore unable to fit the standard

models, DESeq2 was unable to identify any genes. Differential expression analysis was possible using the EdgeR package, it was more flexible in allowing manipulation of the glm used, which negated some of the previous assumptions. Further studies should consider enumerating pathogen markers, such as serine inhibitor proteins or cerato-platanin protein highlighted in this work through qPCR, to identify samples with high *Armillaria* expression before sequencing to ensure replication and allow for thorough gene expression analysis.

The sample 3101 also appears to be different the other symptomatic trees sampled P105 and 306, potentially indicating low quality sequencing. Sample 3101 was called as irreversible according to the ARCHI guide, however the fungal gene expression data does not highlight a fungal species that could be responsible for this disease status, further research into other potential causes of disease such as a bacteria should be considered. This continuum of healthy to disease, variability in disease response, or the potential of another pathogen, has made analysing this data difficult with not many genes being significantly up or down regulated. The determination of a tree health is difficult, the use of health guides can only give an indication, the trees in this study were all classed as irreversible according to the ARCHI guide (Drénou et al., 2015). The RNAseq profiles, do indicate a continuum of health status between asymptomatic and symptomatic trees. Symptomatic trees samples P105 and 306, seem to be similar but potentially in different stages of decline and infection although both classified as irreversible on site. Further research of metagenomics and transcriptomics is needed of trees from healthy to diseased, to determine natural variability between trees health status, and the profile of a truly healthy tree and if the spiral of disease, indicated by Manion (1981), is reflected within the gene expression.

The gene expression of the tree tissue from this site did show that *Armillaria* is present on both symptomatic and asymptomatic trees. For symptomatic trees P105 and 306 *Armillaria* species were the main pathogenic fungi present, indicating that this is the dominant pathogen present in those lesions. Differential gene expression analysis indicated that the *Armillaria* is expressing more genes, 8 genes homologues significantly up-regulated with 4 of those genes actively involved with pathogenesis.

Chapter 7

Synthesis

The genus *Armillaria* is a complex group of fungi comprising representatives of parasitic, pathogenic and saprophytic lifestyles, and with many are able to express all three ecological strategies. As a pathogen, *Armillaria* can be responsible for between 3 - 50 % of crop and plant losses, depending on geographical location, host plant species and *Armillaria* species, as well as severity of infection (Baumgartner et al., 2011; Collins et al., 2013; Guillaumin et al., 1991; Guillaumin et al., 1993; Hock, 2006; Metaliaj et al., 2006; Morrison, 2004; Robene-Soustrade et al., 1992; Scholthof, 2007). It is impossible to determine if *Armillaria* can be eradicated from a site once established, due to its ability to form rhizomorphs and mycelial growth within the soils and to stay dormant for decades. Once *Armillaria* is present on an affected site it is important to determine which species is causing infection in order to assess the potential impacts on future crops and plantings (Baumgartner et al., 2011; Burdon and Silk, 1997; Travadon et al., 2012). *Armillaria* species have been associated with past and present tree declines in Europe and consequently, the impetus to understand the role of *Armillaria* species in oak tree declines has grown in importance (Guillauim et al., 1985; Marçais and Caël, 2006). Although, there is difficulty in assessing the role that *Armillaria* species have within those oak declines, due to variations between species and population host specificity and virulence. The assumption that some species of *Armillaria* are secondary pathogens may underestimate the importance of this fungi within woodlands, forest stands and overall tree declines (Rishbeth, 1985, 1982; Gregory et al., 1991; Oliva et al., 2014; Denman et al., 2017).

The overall aim of this project was to assess the identity, ecology and function of *Armillaria* species on native oak species in Chestnuts woods, Forest of Dean. Initially, several agar-based growth media were tested for the rapid growth of *Armillaria* species, generating biomass for optimisation of DNA extraction and PCR protocols for the detection and identification of *Armillaria* species (Chapter 2). These approaches enabled multi-locus phylogenetic analysis

of a panel of *Armillaria* isolates, and in combination with other *Armillaria* sequences from public DNA sequence repositories, facilitated the development of a High Resolution Melt curve assay (HRM): a DNA-based rapid diagnostic for the detection of *Armillaria* species. (Chapter 3). Using these approaches, a combination of *Armillaria* isolations and molecular identification was applied to characterise the *Armillaria* species affecting oak health in Chestnuts Wood, Forest of Dean, and to link the epidemiology of *Armillaria* infection to host and environmental factors (Chapter 4). Finally, metatranscriptome analysis of asymptomatic oak stem tissue and lesion tissue for symptomatic trees with *Armillaria* infection provided the first insights into the role of *Armillaria* as a pathogen or saprophyte in oak, its co-occurrence with other microbiota in tree stem tissue, and gene expression profiles that reveal its potential mechanisms of pathogenicity (Chapter 5).

7.1 Chapter 3 - Method optimisation to improve biomass production and DNA yield of *Armillaria* species

7.1.1 Aims, Key findings and Synthesis

Firstly, a panel of isolates representing all *Armillaria* species found on UK oak were collected from across the UK including; *A. gallica*, *A. mellea*, *A. ostoyae* and *A. tabescens*. These isolates came from fungal collections held at Forest research and Bangor University, and were previously isolated from a variety of sites, such as, oak plantations, woodlands, private and public gardens. The isolates were used to determine the most effective agar-based growth medium for *Armillaria* isolates, in order to obtain the greatest amount of biomass in the shortest period of time. This was achieved using comparisons of malt extract, V8, tomato and carrot-based media. The isolates were also used to test DNA extraction methods to obtain the highest yield of good quality DNA, vital for downstream molecular tools, such as PCR. It was found that *Armillaria* isolates grown on tomato and carrot-based media grew to larger colony sizes after 14 days, when compared to those isolates grown on malt-based medium. The carrot-based medium was selected to be used throughout the project as it produced the highest biomass, alongside the tomato-based medium, but it was easier and cheaper to produce. Three DNA extraction methods were tested; the Qiagen mini plant kit (Qiagen, UK), the FASTDNA® Spin Kit system (MP Biomedicals, USA) and a CTAB/Phenol-based

DNA/RNA co-extraction method (Griffiths et al., 2000). The FastDNA kit was chosen to be used throughout the project due to the similar yields to the CTAB/Phenol-based co-extraction method, producing a better quality and purity of DNA and obviating the requirement for phenol. Although a Zymo clean and concentrate kit was still needed to purify the DNA for use in PCR and other downstream processes. During the project, the manufacturers of the FastDNA kit changed the kit from a spin column-based method to a nano particle method, rendering it ineffective for *Armillaria* DNA extraction. As such, the DNA extraction method had to be changed to the CTAB/Phenol-based DNA/RNA co-extraction method (Griffiths et al., 2000). *Armillaria* colonies were inoculated onto and grown on sterile 0.2 μM cellulose nitrate filter membrane placed on top of the agar to avoid contamination, and the CTAB/Phenol-based co-extraction was the retested. The addition of the filter paper on the solid media during *Armillaria* growth and CTAB/Phenol-based co-extraction lead to the purification step no longer being needed and therefore this was used for the remainder of the project.

7.2 Chapter 4 – Phylogenetic analysis of UK *Armillaria* species present on oaks, and development of a High Resolution Melt curve assay (HRM) – based rapid diagnostic tool

7.2.1 Aims and objectives

The aim of this chapter was to characterise the phylogeny of representative *Armillaria* isolates collected from the UK, and use the resultant DNA sequence alignments to generate new *Armillaria* genus-specific primers sets that could be used to identify isolates to species level (based on species-specific sequence variation within the amplicon region) using the HRM approach. This was achieved by amplifying the ITS, IGS and EF1-α gene regions using end point PCR and sanger sequencing. This data was then used to design a rapid diagnostic tool in the form of a high-resolution melt curve or HRM assay. This uses genus-specific end point PCR primers alongside an intercalating fluorescent dye. The HRM software measures the amount of intercalating dye released as the amplicons denature with slowly increasing temperatures. This generates a melt curve with a profile dependant on the nucleotides present within the amplicon. Therefore, any differences present between groups will produce slightly different profiles and the HRM software can determine different variants based on those

different melt curve profiles (Alnuaimi et al., 2014; Biosystems, n.d.; Słomka et al., 2017; Wittwer, 2009; Yamagata et al., 2018). Current rapid diagnostics use species specific primer sets, using pure culture samples and confirmed using gel electrophoresis, or PCR-RFLP of the IGS region. (Antonín et al., 2009; Coetzee et al., 2000; Mulholland et al., 2012; Volk et al., 1996). The main benefit of the HRM technique compared with other diagnostic tools is that it does not require fungal isolation, which reduces the time to species identification from a few weeks to 1 - 2 days. The only specialist equipment requirement is a qPCR machine with HRM capacity and DNA extracted from fungal biomass or from environmental samples such as soil, tree or fungal tissue.

7.2.2 Key findings and synthesis

The analysis of the EF1-α gene shows two clades of *Armillaria* within the species *A. gallica*, that is not present within ITS and IGS data. The EF1-α amplicon was subsequently used to design the genus-specific primers to be used for the HRM rapid diagnostic. For HRM analysis required a smaller amplicon length is required, between 80 – 300 bps, smaller amplicons are better to have more resolution for A/T SNP differences (Biosystems, n.d.). The genus specific primers show specificity to the *Armillaria* genus, and when sequenced and analysed phylogenetically shows the same arrangement of species as an EF1-α phylogenetic tree. The primers P145 and P267 can be also used without the HRM analysis and give the same results but require Sanger sequencing. Benefits of using genus-specific primers in end-point PCR are that the method uses a PCR machine and gel electrophoresis, it can give an initial confirmation of *Armillaria* presence within a sample if a band is present on a gel, and then when sequenced will give a species ID. This will give species identification within a few days to a week, which is still faster than tradition identification methods. The HRM method does require a qPCR machine and specialist mastermix with an intercalating dye, but can give identification results within a day. When using the genus specific primers for the HRM as a rapid diagnostic tool on the samples collected as part of this project, the HRM was 76 % efficient at identifying the species correctly. There are, however, limitations to the HRM method when used for species identification. According to the manufacturer, the HRM software is designed to identify up to 6 nucleotide differences and identify variants between strains based on those differences, when using this method between species there is usually more nucleotide differences present (Biosystems, n.d.). The software calls variant identifications relative to the other samples

in each run, therefore a set of controls that represent all potential species is required to be present, the software uses those controls to identify the differences between the unknown isolates. If too many unknown isolates are present the software reduces the variations between the controls and favours variations between the unknown samples present, even if the controls are strictly specified. Therefore, this method for variant/species calling creates a limit of how many unknown samples can be used per run to avoid incorrect species identification, when 17 samples were used the efficiency was 76 %, if a smaller number of unknown isolates (4 - 5) the efficiency did improve but not consistently.

7.3 Chapter 5 – Identification and ecology of *Armillaria* spp. across Chestnut wood, Forest of Dean

7.3.1 Aims and objectives

The aim of this chapter was to isolate *Armillaria* species colonising asymptomatic and symptomatic oaks with *Armillaria* infection. Isolations were completed to determine if *Armillaria* was the predominant pathogen, and if species colonising asymptomatic and symptomatic trees and within soils are different. Soil samples were also taken and analysed to assess soil moisture, organic content and nutrient availability surrounding the tree and around the site.

7.3.2 Key findings and synthesis

After soil analysis it was found that the soil surrounding symptomatic trees contained significantly more moisture when compared to asymptomatic trees, suggesting that the tree roots are taking up less water, indicating root malfunction caused by *Armillaria* decaying the vascular tissues within the roots and trunk cambial layer. It is not clear if this was a predisposing factor or as a result of the infection. Symptomatic trees were found to have significantly less buttress roots than asymptomatic trees, which could potentially be a predisposing factor to infection and a contributing factor to the increased soil moisture. *Armillaria* rhizomorphs were found to be attached to buttress roots of both asymptomatic (accept B108) and symptomatic trees across the site. The isolates were mostly obtained from rhizomorphs attached to trunk and roots with some isolates from wood panels of the symptomatic trees. All isolates obtained were *A. gallica*, and this was confirmed by EF1-α, ITS and IGS gene sequencing and BLAST

searches. Phylogenetic analysis was completed on EF1-α. The *A. gallica* species is complex and previous studies have found up to eight clades (Coetzee et al., 2018; Elías-Román et al., 2013; Klopfenstein et al., 2017). Of the isolates obtained from the site in this work, two clades were seen within the EF1-α data. The majority of the wood panel isolates were found in clade 2 within EF1-α data, isolates from rhizomorphs were distributed between both clades.
The species-specific primers designed by Mulholland et al., (2012) were not appropriate as a rapid diagnostic tool on the isolates used in this chapter, with only 35 % of isolates identified as the correct species. The HRM developed in the previous chapter was used to determine species identification on a small subset of samples from the site, it shown 60 % efficiency at calling the correct species identification. This is an improvement on the species specific primers, however, more samples would need to be tested to fully compare those two methods. *A. gallica* was the dominant *Armillaria* species on the site with rhizomorphs found colonising the roots of asymptomatic and symptomatic trees. Previously this species has been considered a saprophyte to weak pathogen causing disease less often than other members within the *Armillaria* genus (Gregory et al., 1991; Rishbeth, 1985, 1982). This work has highlighted the importance of *A. gallica* as a pathogen within forest systems and how this species interacts with other species, such as *A. ostoyae* previously found co-occurring on the site, although *A. ostoyae* was not isolated during this project (Denman et al., 2017). Previous studies and this work suggest that *A. gallica* use different ecological strategies when colonising a site. It will be important to understand if all species can act as both saprophyte and pathogen, depending on ecological cues, creating a continuum of colonisation and disease from saprophyte to pathogen, or if different strains or isolates are capable of different strategies and potentially work separately or together and with other species to cause overall disease (Baumgartner et al., 2011; Gregory et al., 1991; Guillaumin et al., 1993; Lushaj et al., 2010; Rishbeth, 1985).

7.4 Chapter 6 - Meta transcriptomic analysis on oak tree lesions caused by *Armillaria* spp.

7.4.1 Aims and objectives

The aim of this chapter is to extract good quality RNA from tree tissue, asymptomatic and symptomatic of *Armillaria* colonisation, for metatranscriptome analysis. This work provides

the first in situ metatranscriptome of an *Armillaria* infection in oak. It will help determine if *Armillaria* is active and functioning as a pathogen within the tree lesion and identify other pathogenic fungal species which may be contributing to the necrosis. It will address important knowledge gaps regarding gene expression and activity of *Armillaria* within oak lesions. Furthermore, comparison of gene expression profiles between symptomatic and asymptomatic oak tissue provided insights into the enzyme profile of *Armillaria* as a potential saprophyte or a pathogen which is capable of causing necrosis and decay of the wood tissue of roots and stems.

7.4.2 Key findings and Synthesis

The sequencing data showed that there are RNA transcripts reads mapping to *Armillaria* genes from all tree samples both asymptomatic and symptomatic. Most *Armillaria* reads were classified as *A. gallica*, however, reads were also classified to *A. cepistipes* and *A. ostoyae* to various degrees depending on the database used. This does not infer abundance or ascertain the presence of all those species, and could be related to the homologous genes within the database, as only *A. gallica* and *A. ostoyae* have been previously isolated from this site. The analysis identified four genes as significantly upregulated in *Armillaria* present on the symptomatic trees compared to asymptomatic trees; cerato-platanin (CP) protein, Serine proteinase inhibitor 1A-1 (serpin), Ubiquitin (although one homologue was downregulated) and Histone H4. CP-protein, serpin and ubiquitin, are all relevant for pathogenesis in fungi and host-pathogen interactions (Baccelli, 2015; Gomes et al., 2015; Huntington et al., 2000; Oh et al., 2012). CP proteins are involved in inducing cell death and plant immune response (Baccelli, 2015; Gomes et al., 2015), serpins cause a conformational change in proteinases to inhibit function. The role of ubiquitin is not fully understood (Huntington et al., 2000), however, knock-out studies of other fungi show it is vital for virulence, growth, and vigour as pathogen (Oh et al., 2012). The presence of these three genes suggest a pathogenic function of the *A. gallica* present on the site infecting symptomatic trees and the lower expression on asymptomatic suggests a more saprophytic role of the *Armillaria*. Previous studies have focused on *Armillaria* grown in culture, whole genome studies have highlighted genes within the genome that can be used in pathogenesis (Collins et al., 2017; Sipos et al., 2017). Inoculation trials can take years to decades to get colonisation, many of those studies

have relied on drought stress with *Armillaria* inoculations to achieved colonisation (Metaliaj et al., 2006; Sicoli et al., 2002).

7.5 Future work

The observation that a lower number of buttress roots occur on symptomatic oak trees was an unexpected finding. This simple observation could potentially be introduced as a quickly identifiable feature of trees that are more at risk to infection. Those trees identified could then be selected for closer monitoring or could be used in the selection process for the future thinning of the woodland. The RNAseq data highlighted the potential of more than one sequence present on each of the trees sampled, although this could be due to homologous genes between the species. To confirm which species are present further metagenomic analysis to identify different species and give more accurate data on abundance using the rapid diagnostic developed or through qPCR of virulence genes highlighted in this project. The ecological significance of the two clades seen within the EF1-α data is still not fully understood, other studies with more isolates from across the world found up to eight clades (Coetzee et al., 2018; Klopfenstein et al., 2017). To assess if the differences found in EF1-α are present within the wider genome and have impacts on ecological strategy, further analysis using more gene regions or molecular tools such as dd-RAD sequencing that uses x10 more SNPs available for analysis (Ye et al., 2016). Identifying these differences genetically could help to determine if the ecological differences are due to natural intra species variations in response to environmental cues or if there are pathogenic and saprophytic strains within the *A. gallica* species complex. It could also indicate if these differences are wider reaching within the genome and indicate the presence of cryptic species or multiple species within the complex. It would be important to compare other woodland sites infected with *A. gallica* to see if the isolates there follow the split between the clades or if this is an artefact of this sampling site.

7.6 Overall synthesis

In this work, *Armillaria* rhizomorphs were found colonising symptomatic and asymptomatic trees within the oak plantation in Chestnuts wood, on symptomatic trees the colonising *Armillaria* acts as a pathogen, and surrounding asymptomatic trees it acts as a saprophyte

surviving within soils not causing disease on the tree. The *Armillaria* colonising the outside of the asymptomatic trees is potentially lying in wait to penetrate the tree defences once weakened by other pathogens or abiotic factors. Symptomatic trees are shown to have less buttress roots than asymptomatic, which is potentially a predisposing factor to disease that could be used as a quick tool to identify at risk trees. The soils surrounding symptomatic trees were shown to have significantly more moisture, suggesting *Armillaria* infection is reducing the trees ability for water uptake indicating root malfunction.

Only *A. gallica* was isolated from the site, however RNAseq data indicate reads from other species are present too, however, this could be due to homologous genes within the database. The RNAseq data, indicated genes are significantly upregulated in symptomatic trees; CP protein, serpin, Ubiquitin (although one homologue was downregulated) and Histone H4. The ubiquitin has an unknown function in *Armillaria* but for pathogenic fungi it is vital for virulence, growth and general vigour of the pathogen (Oh et al., 2012). The serine proteinase inhibitors are used by pathogenic fungi to inhibit proteinases released by either host immune response or by other microbes present within the tree lesion (Huntington et al., 2000). The CP protein are released during early developmental stages, and it is used to induce cell death and stimulates the plants immune response (Gomes et al., 2015; Luti et al., 2019

The work on this project has highlighted that *Armillaria* acts as a pathogen on trees at this site, and the severity of infection varies. Manion (1981) described the tree disease model, where the cumulative effect of factors coming together to cause a spiral effect to disease. The gene expression data from this sites indicates there is a continuum of tree health from asymptomatic to symptomatic fitting the spiral to disease as described in the model.

The presence of *Armillaria* on asymptomatic trees on this site indicates that *Armillaria* is widespread at this site and has isolates that are both saprophytes and pathogens, examples of both present in both clades found in the EF1-α data. It is unclear whether there are separate saprophytic and pathogenic *Armillaria* strains present on the site. The alternative is the isolates are triggered into becoming a virulent pathogen by environmental cues or the presence of another pathogenic strain or species. This would assume that all the strains potentially start as saprophyte and biotic or abiotic cue triggers pathogenic genes, thus leading to the overall diseased seen on symptomatic trees on this site.

The work within this thesis explored the impact of *Armillaria* species on native UK oak within an oak plantation, generating new methodological approaches in the form of HRM and

providing the first insights into the ecology, ecological strategy (saprophyte vs pathogen) and infection biology of *A. gallica* in the Forest of Dean. Therefore this research improves our overall understanding of *Armillaria* interactions with the oak host, and provides a platform for future progress in detecting and managing *Armillaria* infection of oak using molecular techniques.

Chapter 8

References

Abomo-Ndongo, S., Guillaumin, J.-J., 1997. Somatic incompatibility among African *Armillaria* isolates. Eur. J. For. Pathol. 27, 201–206.

Aguade, D., Poyatos, R., Gomez, M., Oliva, J., Martinez-Vilalta, J., 2015. The role of defoliation and root rot pathogen infection in driving the mode of drought-related physiological decline in Scots pine (*Pinus sylvestris* L.). Tree Physiol. 35, 229–242.

Aguileta, G., Hood, M.E., Refrégier, G., Giraud, T., 2009. Chapter 3 Genome Evolution in Plant Pathogenic and Symbiotic Fungi. Adv. Bot. Res. 49, 151–193.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Alnuaimi, A.D., Wiesenfeld, D., O'Brien-Simpson, N.M., Reynolds, E.C., Peng, B., Mccullough, M.J., 2014. The development and validation of a rapid genetic method for species identification and genotyping of medically important fungal pathogens using high-resolution melting curve analysis. Mol. Oral Microbiol. 29, 117–130. https://doi.org/10.1111/omi.12050

Anderson, J. B., Korhonen, K. and Ullrich, R. C. (1980) 'Relationships between European and North American biological species of *Armillaria mellea*', Experimental Mycology, 4(1), 78–86.

Antonín, V., Tomšovský, M., Sedlák, P., Májek, T., Jankovský, L., 2009. Morphological and molecular characterization of the *Armillaria cepistipes - A. gallica* complex in the Czech Republic and Slovakia. Mycol. Prog. 8, 259–271. https://doi.org/10.1007/s11557-009-0597-1

Angay, O., Fleischmann, K., Recht, S., Herrmann, S., Matyssek, R., 2014. 'Sweets for the foe – effects of nonstructural carbohydrates on the susceptibility of *Quercus robur* against *Phytophthora quercina*', 1282–1290.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477.

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 Biol. 114, 107–119.

Baumgartner, K., Coetzee, M.P. a., Hoffmeister, D., 2011. Secrets of the subterranean pathosystem of *Armillaria*. Mol. Plant Pathol. 12, 515–534.

Berendsen, R.L., Pieterse, C.M.J., Bakker, P.A.H.M., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci.

Biosystems, A., n.d. A Guide to High Resolution Melting (HRM) Analysis.

Brandon Matheny, P., Wang, Z., Binder, M., Curtis, J.M., Lim, Y.W., Henrik Nilsson, R., Hughes, K.W., Hofstetter, V., Ammirati, J.F., Schoch, C.L., Langer, E., Langer, G., McLaughlin, D.J., Wilson, A.W., Frøslev, T., Ge, Z.W., Kerrigan, R.W., Slot, J.C., Yang, Z.L., Baroni, T.J., Fischer, M., Hosaka, K., Matsuura, K., Seidl, M.T., Vauras, J., Hibbett, D.S., 2007. Contributions of rpb2 and tef1 to the phylogeny of mushrooms and allies (Basidiomycota, Fungi). Mol. Phylogenet. Evol. 43, 430–451.

Brang, P., Innes, J.L., Cherubini, P., Dobbertin, M., Fontana, G., Rigling, D., 2003. Tree-life history prior to death: two fungal root pathogens affect tree-ring growth differently. J. Ecol. 90, 839–850.

Buchfink, B., Xie, C., Huson, D.H., 2014. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60.

Bruhn, J.N., Wetteroff, J., Mihail, J.D., Kabrick, J., Pickens, J.B., 2000. Distribution of *Armillaria* species in upland Ozark Mountain forests with respect to site, overstory species composition and oak decline. For. Pathol. 30, 43–60.

Burdon, J.J., Silk, J., 1997. Sources and patterns of diversity in plant-pathogenic fungi. Phytopathology 87, 664–669.

Burdsall, H.H., Volk, T.J., 1993. The state of taxonomy of the genus *Armillaria*. McIlvainea 11, 4–12.

Burgess, S.S.O., Adams, M.A., Turner, N.C., Ong, C.K., 1998. The redistribution of soil water by tree root systems. Oecologia 115, 306–311.

Burgess, S.S.O., Adams, M.A., Turner, N.C., White, D.A., Ong, C.K., 2001. Tree roots: Conduits for deep recharge of soil water. Oecologia 126, 158–165.

Carvalho, D.B., Smith, M.L., Anderson, J.B., 1995. Genetic exchange between diploid and haploid mycelia of *Armillaria gallica*. Mycol. Res. 99, 641–647.

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. For. Pathol. n/a-n/a.

Chen, Y., Lun, A.T.L., Smyth, G.K., 2016. From reads to genes to pathways: Differential expression analysis of RNA-Seq experiments using Rsubread and the EdgeR quasi-likelihood pipeline. F1000Research 5, 1–48.

Chen, Y., Ritchie, M., Smyth, G.K., Robinson, M., McCarthy, D., 2010. edgeR: differential expression analysis of digital gene expression data. Bioconductor.Fhcrc.Org 1–76.

Coetzee, M. P. A., Wingfield M. D., Bloomer P., Ridley G.S., Kile G.A., Wingfield M.J., 2001. 'Phylogenetic relationships of Australian and New Zealand *Armillaria* species.', Mycologia, 93(5), 887–896.

Coetzee, M. P.A., Musasira, N.Y., Roux, J., Roets, F., van der Merwe, N.A., Wingfield, M.J., 2018. *Armillaria* root rot spreading into a natural woody ecosystem in South Africa. Plant Pathol. 67, 883–891.

Coetzee, M.P. a, Wingfield, B.D., Bloomer, P., Ridley, G.S., Kile, G. a, Wingfield, M.J., 2001. Phylogenetic relationships of Australian and New Zealand *Armillaria* species. Mycologia 93, 887–896.

Coetzee, M.P.A., Musasira, N.Y., Roux, J., Roets, F., Merwe, N.A. Van Der, Wingfield, M.J., 2018. *Armillaria* root rot spreading into a natural woody ecosystem in South Africa 883–891.

Coetzee, M.P.A., Wingfield, B.D., Coutinho, T.A., Wingfield, M.J., 2000. Identification of the Causal Agent of *Armillaria* Root Rot of Pinus Species in South Africa. Mycologia 92, 777–785.

Coetzee, Martin P A, Wingfield, B.D., Wingfield, M.J., 2018. *Armillaria* Root-Rot Pathogens: Species Boundaries and Global Distribution 1–18.

Coetzee, Wingfield, B.D., Harrington, T.C., Dalevi, D., Coutinho, T.A., Wingfield, M.J., 2000. Geographical diversity of *Armillaria mellea* s. s. based on phylogenetic analysis. Mycologia 92, 105–113.

Collins, C., Keane, T.T.M., Turner, D.J., O'Keeffe, G., Fitzpatrick, D. a, Doyle, S., 2013. Genomic and proteomic dissection of the ubiquitous plant pathogen, *Armillaria mellea*: toward a new infection model system. J. Proteome Res. 12, 2552–70.

Cooley, J., 1946. Root Diseases of Deciduous Fruit Trees. Bot. Rev. 12, 83–100.

Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. 328, 307–317.

Deacon, J., 2006. Fungal Biology, Fourth edi. ed. Blackwell Publishing, Oxford.

Denman, S., Barrett, G., Kirk, S.A., McDonald, J.E., Coetzee, M.P.A., 2017. Identification of *Armillaria* species on declined oak in Britain: Implications for oak health. Forestry 90, 148–161.

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.

Drakulic, J., Gorton, C., Pèrez-Sierra, A., Clover, G., Beal, L., Society, R.H., Wisley, R.H.S., 2017. Associations Between *Armillaria* Species and Host Plants in U . K . Gardens 1903–1909.

Drénou, C., Bouvier, M., Lemaire, J., 2015. The diagnostic method ARCHI applied on declining pedunculate oaks. Arboric. J. 37, 166–179.

Duchesne, L.C. and Anderson, J.B. 1990 Location and direction of tran- scription of the 5S rRNA gene in Armillaria. Mycol. Res. 94, 266–269

Elías-Román, R.D., Guzmán-Plazola, R.A., Klopfenstein, N.B., Alvarado-Rosales, D., Calderón-Zavala, G., Mora-Aguilera, J.A., Kim, M.S., García-Espinosa, R., 2013. Incidence and phylogenetic analyses of *Armillaria* s associated with root disease in peach orchards in the State of Mexico, Mexico. For. Pathol. 43, 390–401.

Englander, L. and Roth, L. F. (1979) ‘Interaction of Light and Sterol on Sporangium and Chlamydospore Production by *Phytophthora lateralis* V agar’.

Englander, L., Browning, M. and Tooley, P. W. 2017. 'in vitro in response to temperature and light', 5514.

Fan, Z., Kabrick, J.M., Spetich, M.A., Shifley, S.R., Jensen, R.G., 2008. Oak mortality associated with crown dieback and oak borer attack in the Ozark Highlands. For. Ecol. Manage. 255, 2297–2305.

Ford, K. L., Baumgartner K., Henricot B., Bailey A.M., Foster G.D. 2015. 'A reliable in vitro fruiting system for *Armillaria mellea* for evaluation of *Agrobacterium tumefaciens* transformation vectors', Fungal Biology, 119(10), 859–869.

Garbelotto, M., 2004. Root and butt rot diseases. Encycl. For. Sci. 750–758.

Gerlach, J.P., Reich, P.B., Puettmann, K., Baker, T., 1997. Species, diversity, and density affect tree seedling mortality from *Armillaria* root rot. Can. J. For. Res. 27, 1509–1512.

Gomes, E.V., Costa, M.D.N., De Paula, R.G., Ricci De Azevedo, R., Da Silva, F.L., Noronha, E.F., José Ulhoa, C., Neves Monteiro, V., Elena Cardoza, R., Gutiérrez, S., Nascimento Silva, R., 2015. The Cerato-Platanin protein Epl-1 from *Trichoderma harzianum* is involved in mycoparasitism, plant resistance induction and self cell wall protection. Sci. Rep. 5, 1–13.

Gregory, S., Risbeth, J., Shaw, C.., 1991. Pathogenicity and Virulence, Agricultur. ed. Forest Service United States Department of Agriculture.

Greig, B., 1962. *Fomes annosus* (Fr .) eke . and other Root-rotting Fungi in Conifers on ex-Hardwood Sites. Forestry 35, 164–182.

Griffiths, R.I., Whiteley, A.S., Anthony, G., Donnell, O., Bailey, M.J., Griffiths, R.I., Whiteley, A.S., Donnell, A.G.O., 2000. Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA and rRNA-Based Microbial Community Composition Rapid Method for Coextraction of DNA and RNA from Natural Environments

for Analysis of Ribosomal DNA and 66, 1–5.

Guillauim, JJ. Bernard, CH. Delatour, C. Belgrand, M., 1985. Contribution à l'etude du deperissement du chene, pathologie racinaire en foret de Troncais. Ann. For. Sci. 42, 1–22. Guillaumin, J. and Legrand, P. 2013. 'No Title', in Gonthier, P. and G, N. (eds) Infectious forest diseases. Wallingford, UK; Boston, M: CABI, 159–177.

Guillaumin, J. -J, Mohammed, C., Anselmi, N., Courtecuisse, R., Gregory, S.C., Holdenrieder, O., Intini, M., Lung, B., Marxmüller, H., Morrison, D., Rishbeth, J., Termorshuizen, A.J., Tirró, A., van Dam, B., 1993. Geographical distribution and ecology of the *Armillaria* species in western Europe. Eur. J. For. Pathol. 23, 321–341.

Guillaumin, J.., Anderson, J.B., Korhonen, K., 1991. Life Cycle, Interfertility, and Biological Species, Agricultur. ed. Forest Service United States Department of Agriculture, Washington, D.C.

Guo, T., Wang, H.C., Xue, W.Q., Zhao, J., Yang, Z.L., 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, 1–21.

Haavik, L.J., Billings, S.A., Guldin, J.M., Stephen, F.M., 2015. Emergent insects, pathogens and drought shape changing patterns in oak decline in North America and Europe. For. Ecol. Manage. 354, 190–205.

Heinzelmann, R., Prospero, S., Rigling, D., 2018. Frequent diploidisation of haploid *Armillaria* ostoyae strains in an outdoor inoculation experiment. Fungal Biol. 122, 147–155.

Heinzelmann, R., Dutech, C., Tsykun, T., Labbé, F., Soularue, J.P., Prospero, S., 2019. Latest advances and future perspectives in *Armillaria* research. Can. J. Plant Pathol. 41, 1–23.

Herms, D. a., Mattson, W.J., 1992. The Dilemma of Plants: To Grow or Defend. Q. Rev. Biol. 67, 283.

Herrera, R., Erdocia, X., Llano-Ponte, R., Labidi, J., 2014, b. Characterization of hydrothermally treated wood in relation to changes on its chemical composition and physical properties. J. Anal. Appl. Pyrolysis 107, 256–266.

Hock, B., 2006. Fungal Genomics, 1st ed, The Mycota. Springer-Verlag, Berlin/Heidelberg.

Hoegger, P., Kilaru, S., James, T., Thacker, J., 2006. Identification of fungal multi-copper oxidase gene families: Overexpression and characterization of *Coprinopsis cinerea* laccases for applications in biotechnology., in: Kilaru, S. (Ed.), . Cuvillier Verlag, 165–188.

Hsiau, P. T. 1996 'The taxonomy and phylogeny of the mycangial fungi from *Dendroctonus brevicomis* and *D frontalis* (Coleoptera: Scolytidae)'.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., St. Leger, R.J., Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl. Acad. Sci. U. S. A. 111, 16796–16801. https://doi.org/10.1073/pnas.1412662111

Huntington, J.A., Read, R.J., Carrell, R.W., 2000. Structure of a serpin-protease complex shows inhibition by deformation. Nature 407, 923–926. https://doi.org/10.1038/35038119

Iwao, T. and Namio, T. (1994) 'Effect of adding carrot, Daucus carota variety sativa, to media in the rhizomorph production of *Armillaria* species.', Mokuzai gakkaishi, 40(2), 213–219.

Iwao, T. and Namio, T. 1995 'Effects of primordium formation method and moisture content of medium on fruiting body production of *Armillaria* species. T', Mokuzai gakkaishi, 21(2), 211–217.

Josh, N., Fass, J., 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) [Software].

Kauserud, H. and Schumacher, T. 2001 Outcrossing or inbreeding: DNA markers provide evidence for type of reproductive mode in Phellinus nigrolimitatus (Basidiomycota). Mycol. Res. 105, 676–683.

Keca, N.; Klopfenstein, N.B.; Kim, M.S.; Solheim, H.; Woodward, S., 2015. 'Initial characterization of an unidentified *Armillaria* isolate from Serbia using LSU-IGS1 and TEF-1-α genes', Forest Pathology, 45(2), 120–126.

Keca, N., Solheium, H., 2006. Hosts and distribution of *Armillaria* species in Serbia, in: Alterations of Scots Pine Needle Characteristics after Severe Weather Conditions in South-Eastern Estonia. 28–31.

King, B.C., Waxman, K.D., Nenni, N. V., Walker, L.P., Bergstrom, G.C., Gibson, D.M., 2011. Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. Biotechnol. Biofuels 4, 1–14.

Klopfenstein, N.B., Stewart, J.E., Ota, Y., Hanna, J.W., Richardson, B.A., Ross-Davis, A.L., Elías-Román, R.D., Korhonen, K., Keca, N., Iturritxa, E., Alvarado-Rosales, D., Solheim, H., Brazee, N.J., Łakomy, P., Cleary, M.R., Hasegawa, E., Kikuchi, T., Garza-Ocañas, F., Tsopelas, P., Rigling, D., Prospero, S., Tsykun, T., Bérubé, J.A., Stefani, F.O.P., Jafarpour, S., Antonín, V., Tomšovský, M., McDonald, G.I., Woodward, S., Kim, M.S., 2017. Insights into the phylogeny of northern hemisphere *Armillaria*: Neighbor-net and bayesian analyses of translation elongation factor $1-\alpha$ gene sequences. Mycologia 109, 75–91.

Koch, R.A., Wilson, A.W., Sene, O., Henkel, T.W., Aime, M.C., 2017. Resolved phylogeny and biogeography of the root pathogen *Armillaria* and its gasteroid relative , Guyanagaster. BMC Evol. Biol. 1–16.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martín, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja,

J., Peay, K.G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J.A., Senés, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M., Larsson, K.H., 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol. Ecol. 22, 5271–5277.

Kowalski, T., 1991. Oak decline: I. Fungi associated with various disease symptoms on overground portions of middle-aged and old oak (*Quercus robur* L.). For. Pathol. 21, 136–151.

Kues, U., Ruhl, M., 2011. Multiple multi-copper oxidase gene families in basidiomycetes - what for? Curr. Genomics 12, 72–94.

Kruskal-Wallis Test (2008). In: The Concise Encyclopedia of Statistics. Springer, New York, NY.

Kuhad, R. C., Kapoor, R. K. and Lal, R. 2004. 'Improving the yield and quality of DNA isolated from white-rot fungi', Folia Microbiologica, 49(2), 112–116.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874.

Langmead and Steven L Salzberg, 2013. Bowtie2. Nat. Methods 9, 357–359.

Lattanzio, V., Lattanzio, V.M.T., Cardinali, A., Amendola, V., 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects, Phytochemistry.

Lee, C. A., Dey, D. C. and Muzika, R. 2016. 'Forest Ecology and Management Oak stump-sprout vigor and *Armillaria* infection after clear cutting in southeastern Missouri , USA', Forest Ecology and Management. Elsevier B.V., 374, 211–219.

Le Floch, A., Jourdes, M., Teissedre, P.L., 2015. Polysaccharides and lignin from oak

wood used in cooperage: Composition, interest, assays: A review. Carbohydr. Res. 417, 94–102.

Letunic, I., Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 2–5.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, 490–495.

Lopez-Real, J.M., 1975. Formation of pseudosclerotia ('zone lines') in wood decayed by *Armillaria mellea* and *Stereum hirsutum*. Trans. Br. Mycol. Soc. 64, 465-IN7.

Luisi, N., Sicoli, G., Lerario, P., Studi, U., Amendola, G., 1995. Observations on *Armillaria* occurrence.

Lushaj, B.M., Woodward, S., Kećа, N., Intini, M., 2010. Distribution, ecology and host range of *Armillaria* species in Albania. For. Pathol. 40, 485–499.

Lushaj, B.M., Woodward, S., Kećа, N., Intini, M., 2010. Distribution, ecology and host range of *Armillaria* species in Albania. For. Pathol. 40, 485–499.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 1–21.

Luti, S., Sella, L., Quarantin, A., Pazzagli, L., Baccelli, I., 2019. Twenty years of research on cerato-platanin family proteins: clues, conclusions, and unsolved issues. Fungal Biol. Rev. 1–12.

Maphosa, L., Wingfield, B.D., Coetzee, M.P.A., Mwenje, E., Wingfield, M.J., 2006. Phylogenetic relationships among *Armillaria* species inferred from partial elongation factor 1-alpha DNA sequence data. Australas. Plant Pathol. 35, 513–520.

Marcais, B., Bréda, N., 2006. Role of an opportunistic pathogen in the decline of stressed oak trees. J. Ecol. 94, 1214–1223.

Marcais, B., Cael, O., 2006. Spatial pattern of the density of *Armillaria* epiphytic rhizomorphs on tree collar in an oak stand. For. Pathol. 36, 32–40.

Marcais, B., Cael, O., Delatour, C., 2011. Interaction between root rot basidiomycetes and *Phytophthora* species on pedunculate oak. Plant Pathol. 60, 296–303.

Marchetti, R., Berrin, J.G., Couturier, M., Ul Qader, S.A., Molinaro, A., Silipo, A., 2015. NMR analysis of the binding mode of two fungal endo-β-1,4-mannanases from GH5 and GH26 families. Org. Biomol. Chem. 14, 314–322.

Markovic, O., Janecek, S., 2001. Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution. Protein Eng. 14, 615–631.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.J 17, 10–12.

Martínez, Á.T., Speranza, M., Ruiz-Duenas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A., Del Río, J.C., 2005. Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int. Microbiol. 8, 195–204.

Marowa, P., Ding, A., Kong, Y., 2016. Expansins: roles in plant growth and potential applications in crop improvement. Plant Cell Rep. 35, 949–965.

Matsushita, N., Suzuki, K., 2005. Identification of *Armillaria* species in Japan using PCR-

RFLP analysis of rDNA intergenic spacer region and comparisons of *Armillaria* species in the world. J. For. Res. 10, 173–179.

Metaliaj, R., Sicoli, G. and Luisi, N. (2006) 'Pathogenicity of *Armillaria* isolates inoculated on five Quercus species at different watering regimes', Phytopathologia Mediterranea, 45(1), 3–9.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide - Biol. Chem.

Moreira, L.R.S., Filho, E.X.F., 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. 79, 165–178.

Morgenstern, I., Powlowski, J., Tsang, A., 2014. Fungal cellulose degradation by oxidative enzymes: Fromdysfunctional GH61 family to powerful lytic polysaccharide monooxygenase family. Brief. Funct. Genomics 13, 471–481.

Morrison, D., Williams, R., Whitney, R., 1991. Infection, Disease Development, Diagnosis, Detection, in: Shaw, C.., Kile, G.A. (Eds.), *Armillaria* Root Disease. Forest Service United States Department of Agriculture, 62–75.

Morrison, D.J., 2004. Rhizomorph growth habit, saprophytic ability and virulence of 15 *Armillaria* species. For. Pathol. 34, 15–26.

Morrison, D.J., Pellow, K.W., 2002. Variation in virulence among isolates of *Armillaria* ostoyae. For. Pathol. 32, 99–107.

Mueller-Dombois, D., 1992. A natural dieback theory, cohort senescence as an alternative to the decline disease theory, 1st ed, Forest decline concepts. The American Phytopathology Society, Minnesota.

Mulholland, V., MacAskill, G.A., Laue, B.E., Steele, H., Kenyon, D., Green, S., 2012.

Development and verification of a diagnostic assay based on EF-1α for the identification of *Armillaria* species in Northern Europe. For. Pathol. 42, 229–238.

Mulvaney, R.L., 1996. Chapter 38 5371.

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta.

Muszewska, A., Stepniewska-Dziubinska, M.M., Steczkiewicz, K., Pawlowska, J., Dziedzic, A., Ginalski, K., 2017. Fungal lifestyle reflected in serine protease repertoire. Sci. Rep. 7, 1–12.

Nakajima, T., Maitra, S.K., Clinton, B., 1976. An Endo-cul + 6-D-mannanase from a Soil Bacterium 251.

Nguema-Ona, E., Vicré-Gibouin, M., Gotté, M., Plancot, B., Lerouge, P., Bardor, M., Driouich, A., 2014. Cell wall O-glycoproteins and N-glycoproteins: Aspects of biosynthesis and function. Front. Plant Sci. 5, 1–12.

Nilsson, R.H., Bok, G., Ryberg, M., Kristiansson, E., Hallenberg, N., 2009. A software pipeline for processing and identification of fungal ITS sequences. Source Code Biol. Med. 4, 1–6. https://doi.org/10.1186/1751-0473-4-1

Niu, C., Kebede, H., Auld, D.E., Woodward, J. E., Burow, G., Wright, R. J., 2008. 'A safe inexpensive method to isolate high quality plant and fungal DNA in an open laboratory environment', African Journal of Biotechnology, 7(16), 2818–2822.

O'Leary, N., Wright, M., Brister, J., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C., Goldfarb, T., Gupta, T., Haft, D., Hatcher, E., Hlavina, W., Joardar, V., Kodali, V., Li, W., Maglott, D., Masterson, P., McGarvey, K., Murphy, M., O'Neill, K., Pujar, S., Rangwala, S., Rausch,

D., Riddick, L., Schoch, C., Shkeda, A., Storz, S., Sun, H., Thibaud-Nissen, F., Tolstoy, I., Tully, R., Vatsan, A., Wallin, C., Webb, D., Wu, W., Landrum, M., Kimchi, A., Tatusova, T., DiCuccio, M., P, K., Murphy, T., Pruitt, K., 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 4, (D1):D733-45 PubMed.

Oliva, J., Julio Camarero, J., Stenlid, J., 2012. Understanding the role of sapwood loss and reaction zone formation on radial growth of Norway spruce (*Picea abies*) trees decayed by *Heterobasidion annosum* s.l. For. Ecol. Manage. 274, 201–209.

Oliva, J., Stenlid, J., Martínez-Vilalta, J., 2014. Letters The effect of fungal pathogens on the water and carbon economy of trees, implications for drought-induced mortality included in theoretical models for drought induced mortality.

Oh, Y., Franck, W.L., Han, S.O., Shows, A., Gokce, E., Muddiman, D.C., Dean, R.A., 2012. Polyubiquitin is required for growth, development and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. PLoS One 7.

Ota, Y., Fukuda, K., Suzuki, K., 1998. Mycological Society of America The Nonheterothallic Life Cycle of Japanese *Armillaria mellea* 90, 396–405.

Park, K.H., Oh, S.Y., Park, M.S., Kim, M.S., Klopfenstein, N.B., Kim, N.K., Park, J.Y., Kim, J.J., Han, S.K., Lee, J.K., Lim, Y.W., 2018. Re-evaluation of *Armillaria* and Des*Armillaria* in South Korea based on ITS/tef1 sequences and morphological characteristics. For. Pathol. 48, 1–15.

Pèrez-Sierra, A., Whitehead, D.S., Whitehead, M.P., 1999. Investigation of a PCR-based method for the routine identification of British *Armillaria* species. Mycol. Res. 103, 1631–1636. https://doi.org/10.1017/S0953756299001148

Prospero, S., 2003. Ecology of *Armillaria cepistipes*: population structure, niches, pathogenicity and interactions with *Armillaria* ostoyae. Swiss Federal Institute of Technology Zurich.

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

Puls, J., 1997. Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. Macromol. Symp. 120, 183–196.

R Development Core Team, R. (2009) 'R: A Language and Environment for Statistical Computing'.

Raper, J., 1953. Tetrapolar Sexuality. Q. Rev. Biol. 28, 233–259.

Rishbeth, J. 1986 'Some characteristics of English *Armillaria* species in culture.', Transactions of the British Mycological Society. British Mycological Society, 86(2), 213–218.

Rishbeth, J., 1982. Species of *Armillaria* in southern England. Plant Pathol. 31, 9–17.

Rishbeth, J., 1985. Infection cycle of *Armillaria* and host response. Eur. J. For. Pathol. 15, 332–341.

Rishbeth, J., 1986. Some characteristics of English *Armillaria* species in culture. Trans. Br. Mycol. Soc. 86, 213–218.

Rizzo, D.M., Harrington, T.C., 1992. Migration in Diploid-Haploid Pairings of *Armillaria* ostoyae Author (s): 84, 863–869.

Rizzo, D.M., Harrington, T.C., 1993. Delineation and biology of clones of *Armillaria* ostoyae, *Armillaria* gemina and *Armillaria* calvescens. Mycologia 85, 164–174.

Robene-Soustrade, I., Lung-Escarmant, B., Bono, J.J., Taris, B., 1992. Identification and partial characterization of an extracellular manganese-dependent peroxidase in *Armillaria* ostoyae and *Armillaria mellea*. Eur. J. For. Pathol. 22, 227–236.

Ross-Davis, A.L., Stewart, J.E., Hanna, J.W., Kim, M.S., Knaus, B.J., Cronn, R., Rai, H., Richardson, B.A., Mcdonald, G.I., Klopfenstein, N.B., 2013. Transcriptome of an *Armillaria* root disease pathogen reveals candidate genes involved in host substrate utilization at the host-pathogen interface. For. Pathol. 43, 468–477.

Sayers, E.W., Cavanaugh, M., Clark, K., Ostell, J., Pruitt, K.D., Karsch-Mizrachi, I., 2019. GenBank. Nucleic Acids Res. 47, D94–D99.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Bolchacova, E., Voigt, K., Crous, P.W., Miller, A.N., Wingfield, M.J., Aime, M.C., An, K.D., Bai, F.Y., Barreto, R.W., Begerow, D., Bergeron, M.J., Blackwell, M., Boekhout, T., Bogale, M., Boonyuen, N., Burgaz, A.R., Buyck, B., Cai, L., Cai, Q., Cardinali, G., Chaverri, P., Coppins, B.J., Crespo, A., Cubas, P., Cummings, C., Damm, U., de Beer, Z.W., de Hoog, G.S., Del-Prado, R., Dentinger, B., Diéguez-Uribeondo, J., Divakar, P.K., Douglas, B., Dueñas, M., Duong, T.A., Eberhardt, U., Edwards, J.E., Elshahed, M.S., Fliegerova, K., Furtado, M., García, M.A., Ge, Z.W., Griffith, G.W., Griffiths, K., Groenewald, J.Z., Groenewald, M., Grube, M., Gryzenhout, M., Guo, L.D., Hagen, F., Hambleton, S., Hamelin, R.C., Hansen, K., Harrold, P., Heller, G., Herrera, C., Hirayama, K., Hirooka, Y., Ho, H.M., Hoffmann, K., Hofstetter, V., Högnabba, F., Hollingsworth, P.M., Hong, S.B., Hosaka, K., Houbraken, J., Hughes, K., Huhtinen, S., Hyde, K.D., James, T., Johnson, E.M., Johnson, J.E., Johnston, P.R., Jones, E.B.G., Kelly, L.J., Kirk, P.M., Knapp, D.G., Kõljalg, U., Kovács, G.M., Kurtzman, C.P., Landvik, S., Leavitt, S.D., Liggenstoffer, A.S., Liimatainen, K., Lombard, L., Luangsa-ard, J.J., Lumbsch, H.T., Maganti, H., Maharachchikumbura, S.S.N., Martin, M.P., May, T.W., McTaggart, A.R., Methven, A.S., Meyer, W., Moncalvo, J.M., Mongkolsamrit, S., Nagy, L.G., Nilsson, R.H., Niskanen, T., Nyilasi, I., Okada, G., Okane, I., Olariaga, I., Otte, J., Papp, T., Park, D., Petkovits, T., Pino-Bodas, R., Quaedvlieg, W., Raja, H.A., Redecker, D., Rintoul, T.L., Ruibal, C., Sarmiento-Ramírez, J.M., Schmitt, I., Schüßler, A., Shearer, C., Sotome, K., Stefani, F.O.P., Stenroos, S., Stielow, B., Stockinger, H., Suetrong, S., Suh, S.O., Sung, G.H., Suzuki, M., Tanaka, K., Tedersoo, L., Telleria, M.T., Tretter, E., Untereiner, W.A., Urbina, H., Vágvölgyi, C., Vialle, A., Vu, T.D., Walther, G., Wang, Q.M., Wang, Y., Weir, B.S., Weiß, M., White, M.M., Xu, J., Yahr, R., Yang, Z.L., Yurkov, A., Zamora, J.C.,

Zhang, N., Zhuang, W.Y., Schindel, D., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. U. S. A. 109, 6241–6246. https://doi.org/10.1073/pnas.1117018109

Scholthof, K.G., 2007. The disease triangle, pathogens , the environment and society 5, 152–157.

Shim, J.-O. Shim JO, Chang KC, Lee YS, Park CH, Kim HY, Lee UY, Lee TS, Lee MW, 2006. The fruiting body formation of Armillaria mellea on oak sawdust medium covered with ground raw carrots. The Korean society of mycology 34: 206-208.

Sicoli, G., Fatehi, J. and Stenlid, J. 2003 'Development of species-specific PCR primers on rDNA for the identification of European *Armillaria* species', Forest Pathology, 33(5), 287–297.

Sierra, A. P., Whitehead, D. S. and Whitehead, M. P. (1999) 'Investigation of a PCR-based method for the routine identification of British *Armillaria* species', Mycological Research, 103(12), 1631–1636.

Sipos, G., Prasanna, A.N., Walter, M.C., Connor, E.O., Bálint, B., Krizsán, K., Kiss, B., Hess, J., Varga, T., Slot, J., Riley, R., Bóka, B., Rigling, D., Barry, K., Lee, J., Mihaltcheva, S., Labutti, K., Lipzen, A., Waldron, R., Moloney, N.M., Sperisen, C., Kredics, L., Vágvölgyi, C., Patrignani, A., Fitzpatrick, D., Nagy, I., Doyle, S., Anderson, J.B., Grigoriev, I. V, Güldener, U., Münsterkötter, M., Nagy, L.G., 2018. Genome expansion and lineage-specific genetic innovations in the forest pathogenic fungi *Armillaria*. Nat. Ecol. Evol. 1.

Sista Kameshwar, A.K., Qin, W., 2018. Comparative study of genome-wide plant biomass-degrading CAZymes in white rot, brown rot and soft rot fungi. Mycology 9, 93–105.

Siu, K., Xu, L., Chen, X., Wu, J., 2016. 'Molecular properties and antioxidant activities of polysaccharides isolated from alkaline extract of wild *Armillaria ostoyae* mushrooms', Carbohydrate Polymers. Elsevier Ltd., 137, 739–746.

Słomka, M., Sobalska-Kwapis, M., Wachulec, M., Bartosz, G., Strapagiel, D., 2017. High resolution melting (HRM) for high-throughput genotyping-limitations and caveats in practical case studies. Int. J. Mol. Sci. 18.

Soukup, S.F., Culi, J., Gubb, D., 2009. Uptake of the necrotic serpin in Drosophila melanogastervia the lipophorin receptor-1. PLoS Genet. 5.

Sprockett, D.D., Piontkivska, H., Blackwood, C.B., 2011. Evolutionary analysis of glycosyl hydrolase family 28 (GH28) suggests lineage-specific expansions in necrotrophic fungal pathogens. Gene 479, 29–36.

Stamets, P., Chilton, J.S., 1983. The Mushroom Cultivator: A Practical Guide to Growing Mushrooms at Home 416.

Thomas, F.M., Blank, R., Hartmann, G., 2002. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. 32, 277–307.

Thomas, H., 1934. Studies on *Armillaria mellea* (Vahl) Quel., infection, parasitism, and host resistance. J. Agric. Res. 48, 187–218.

Travadon, R., Smith, M.E., Fujiyoshi, P., Douhan, G.W., Rizzo, D.M., Baumgartner, K., 2012. Inferring dispersal patterns of the generalist root fungus *Armillaria mellea*. New Phytol. 193, 959–969.

Tsigos, I., Bouriotis, V., 1995. Purification and Characterization of Extracellular Chitin Deacetylase from Colletotrichum lindemuthianum. J. Biol. Chem. 270, 26286–26291.

Tsopelas, P., Tjamos, E.C., 1997. Occurrence and pathogenicity of *Armillaria tabescens* on almond in Greece. EPPO Bull. 27, 455–461.

Ullrich, R.C., Anderson, J.B., 1978. Sex and diploidy in *Armillaria mellea*. Exp. Mycol. 2,

119–129.

Vayssier-Taussat, M., Albina, E., Citti, C., Cosson, J.-F., Jacques, M.-A., Lebrun, M.-H., Le Loir, Y., Ogliastro, M., Petit, M.-A., Roumagnac, P., Candresse, T., 2014. Shifting the paradigm from pathogens to pathobiome: new concepts in the light of meta-omics. Front. Cell. Infect. Microbiol. 4, 29.

Venables, W. N. and Ripley, B. D. 2002 Modern Applied Statistics with S. 4th edn. New York, NY: Springer New York (Statistics and Computing).

Vogt, T., 2010. Phenylpropanoid biosynthesis. Mol. Plant 3, 2–20.

Volk, T.J., Burdsall, H.H., Banik, M.T., 1996. *Armillaria* nabsnona, a new species from western North America. Mycologia 88, 484–491.

Wargo, P.M., 1972. Defoliation-induced chemical changes in sugar maple roots stimulate growth of *Armillaria mellea*. Phytopathology 62, 1278–1283.

Wargo, P.M., 1977. *Armillariella mellea* and *Agrilus dilineatus* and mortality of defoliated oak trees. For. Sci. 23, 485–492.

Wargo, P.M., 1996. Consequences of environmental stress on oak: predisposition to pathogens. Ann. des Sci. For. 53, 359–368.

Wargo, P.M., Carey, A.C., 2001. Effects of metals and pH on in vitro growth of *Armillaria ostoyae* and other root and butt rot fungi of red spruce. For. Pathol. 31, 5–24.

Watling, R., Kile, G.A., Burdsall, H.H., 1988. Nomenclature, Taxonomy and Identification 51, 1–9.

Watling, R., Kile, G.A., Burdsall, H.H., 1991. Nomenclature, Taxonomy and Identification, in: Shaw, C.., Kile, G.A. (Eds.), *Armillaria* Root Disease. Forest Service United States

Department of Agriculture, 2–9.

Westbrook, A., Moo-Young, M., Chou, C. P., 2016. Applied and Environmental Microbiology Jul 2016, 82 (16) 4876-4895;

White, T. J., Bruns, T. D., Lee, S. B., Taylor, J. W., 1990. 'Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes', PCR protocols: A Guide to Methods and Applications, (2016), 315–322.

Worrall, J.J., 1997. Somatic incompatibility in Basidiomycetes 89, 24–36.

Worrall, J.J., Marchetti, S.B., Egeland, L., Mask, R.A., Eager, T., Howell, B., 2010. Effects and etiology of sudden aspen decline in southwestern Colorado, USA. For. Ecol. Manage. 260, 638–648.

Yamagata, Y., Yoshimura, A., Anai, T., Watanabe, S., 2018. Selection criteria for SNP loci to maximize robustness of high-resolution melting analysis for plant breeding. Breed. Sci. 68, 488–498. https://doi.org/10.1270/jsbbs.18048

Ye, F., Yu, X.D., Wang, Q., Zhao, P., 2016. Identification of SNPs in a nonmodel macrofungus (*Lepista nuda*, Basidiomycota) through RAD sequencing. Springerplus 5. https://doi.org/10.1186/s40064-016-3459-8

Zavišić, A., Yang, N., Marhan, S., Kandeler, E., Polle, A., 2018. Forest Soil Phosphorus Resources and Fertilization Affect Ectomycorrhizal Community Composition, Beech P Uptake Efficiency, and Photosynthesis. Front. Plant Sci. 9, 1–13.

Chapter 9

Appendix 1

9.1 RNA data processing and analysis.

9.2 Enzyme composition

Table 9.1 – List of all enzymes searched for within the DIAMOND outputs using grep function.

		Symptomatic			**Asymptomatic**			
Sample		306A	3101B	P105	403B	404C	405A	406A
	Glycoside hydrolase (GH)	46	3	183	38	14	7	4
	Carbohydrate esterases (CE)	0	0	0	0	0	0	0
	Polysaccharide lyase (PL)	1	0	2	0	0	0	0
Ligin								
	Laccase	7	0	20	0	7	0	0
	Manganese peroxidase	0	0	0	0	0	0	0
	Versatile peroxidase	0	0	0	0	0	0	0
	Alcohol oxidase	12	0	78	0	4	3	0
	D-Arabinono-1,4-lactone oxidase	0	0	4	0	0	0	0
	Cytochrome P450s	0	0	0	0	0	0	0
	Glyoxal oxidase	0	0	4	0	0	0	0
	Cellobiose dehydrogenase	0	0	1	0	0	0	0
	Glucose dehydrogenase	8	0	8	3	3	4	0
	Total	27	0	115	3	14	7	0
Pectin								
	CE8	0	0	2	0	0	0	0

		Symptomatic			Asymptomatic			
Sample		306A	3101B	P105	403B	404C	405A	406A
	Pectinesterase	0	0	0	0	0	0	0
	Feruloyl esterase	0	0	0	0	0	0	0
	Rhamnogalacturonan lyase	2	0	1	0	1	0	0
	Polygalacturonase	0	0	7	0	0	0	0
	Pectin lyase	100	13	113	84	68	90	20
	Pectate lyase	54	4	81	21	10	16	4
	Total	156	17	204	105	79	106	24
Chitin								
	Chitinase	2	0	2	2	0	0	0
	Copper-dependent lytic polysaccharide monooxygenase	0	0	0	0	0	0	0
GH18		0	0	8	0	2	0	0
GH75		0	0	0	0	0	0	0
AA10		0	0	0	0	0	0	0
GH28		0	0	4	0	0	0	0
	Total	2	0	14	2	2	0	0
Cellulose and hemicellulose								
GH38	GH38	2	0	13	0	0	0	0

		Symptomatic			Asymptomatic			
Sample		306A	3101B	P105	403B	404C	405A	406A
	α-Mannosidase	0	0	3	0	0	0	0
GH43	Xylosidase	12	0	12	18	9	7	2
GH42	β-galactosidase	0	0	0	1	0	0	0
GH5	GH5	8	0	21	0	1	0	0
	β-glucosidase	6	0	17	8	5	0	0
	Cellulose 1,4-β-cellobiosidase	0	0	0	0	0	0	0
	Cellulase	0	0	2	0	0	1	0
	Mannan endo-1,4-β-mannosidase	0	0	0	0	0	0	0
GH10		0	0	2	0	0	0	0
GH51	Endoglucanase	0	0	10	0	0	0	0
	Xylan 1,4-β-xylosidase	0	0	0	0	0	0	0
	α-N-Arabinofuranosidase	0	0	0	0	0	0	0
GH11	Xyloglucan-specific exo-β-1,4-glucanase	0	0	0	0	0	0	0
AA3_1	Cellobiose dehydrogenase	0	0	1	0	0	0	0
GH1		11	1	64	24	5	7	4
GH2		3	0	13	0	2	0	0
GH3		11	2	56	10	6	0	0

		Symptomatic			Asymptomatic			
Sample		306A	3101B	P105	403B	404C	405A	406A
GH5 subfamily 4		0	0	0	0	0	0	0
GH6		2	0	2	1	0	0	0
GH7		5	0	7	3	0	0	0
GH9		2	0	12	0	0	0	0
GH12		0	0	0	0	0	0	0
GH29		0	0	0	0	2	0	0
GH30 subfamily 7		0	0	0	0	0	0	0
GH45		0	0	0	0	0	0	0
GH27		2	0	1	0	0	0	0
GH35		0	0	6	0	2	0	0
GH74		0	0	0	0	0	0	0
GH76		0	0	3	0	0	0	0
	Total	64	3	245	65	32	15	6
Pathogenicity genes								
	Salicylate hydroxylase	0	0	2	0	0	0	0
	ABC transporter	9	0	62	12	1	1	1
	Ornithine decarboxylase	2	0	4	6	0	1	0
	Total	11	0	68	18	1	2	1

9.3 EdgeR statistics

Table 9.2 – Complete list of genes with differential expression from EdgeR analysis.

			EdgeR statistical test p-value			
GenBank accession	**GenBank definition**	**Gene regulation Up or down**	**Exacttest**	**glmQLFTest**	**glmLRT**	glmTreat
PBK92864.1	Hypothetical proteins	↑	***	***	**	***
SJL04025.1	Probable ubiquitin/ Ribosomal protein S27a fusion protein	↑	***	-	-	-
PBL01295.1	Eukaryotic ADP/ATP carrier	↑	*	-	-	-
PBK97893.1	Hypothetical proteins	↑	-	***	*	-
SJK97647.1	Uncharacterized protein	↑	-	***	*	*
SJL03400.1	Histone H4	↑	***	-	-	*
PBK92819.1	Hypothetical proteins	↑	-	-	-	**
PBK77756.1	Hypothetical proteins	↑	-	-	-	**
PBK90160.1	Hypothetical proteins	↑	-	-	-	**
PBK92875.1	Hypothetical proteins	↑	-	-	-	**
PBL00157.1	Ubiquitin	↑	-	-	*	**
PBK78440.1	Hypothetical proteins	↑	-	-	-	*
PBK75871.1	Hypothetical proteins	↑	-	-	-	*
PBK95666.1	Ribosomal protein s30	↑	-	-	-	*

Table 9.2 – Complete list of genes with differential expression from EdgeR analysis.

			EdgeR statistical test p-value			
PBK92818.1	Hypothetical proteins	↑	-	-	-	*
PBK85428.1	Hypothetical proteins	↑	-	-	-	*
PBL02972.1	Hypothetical proteins	↑	-	-	-	*
PBK74653.1	Hypothetical proteins	↑	-	-	-	*
PBK97894.1	Hypothetical proteins	↑	-	-	-	*
PBK76382.1	Ubiquitin	↓	-	-	-	*
PBK61601.1	Hypothetical proteins	↑	-	-	-	*
PBK97893.1	Hypothetical proteins	↑	-	-	-	*
PBK91638.1	Serine proteinase inhibitor	↑	-	-	-	*
PBK89543.1	Hypothetical proteins	↑	-	-	-	*
PBK74990.1	SnodProt1	↑	-	-	-	*
PBK85293.1	Hypothetical proteins	↑	-	-	-	*
PBK59000.1	Hypothetical proteins	↑	-	-	-	*
PBK85573.1	LysM-domain-containing protein	↑	-	-	-	*
PBK84867.1	Hypothetical proteins	↑	-	-	-	*
PBK73105.1	Ribosomal protein s25	↑	-	-	-	*
PBK77130.1	Hypothetical proteins	↑	-	-	-	*

Table 9.2 – Complete list of genes with differential expression from EdgeR analysis.

			EdgeR statistical test p-value			
PBL03475.1	Hypothetical proteins	↑	-	-	-	*
PBK89810.1	Mismatch	↑	-	-	-	*
PBK93295.1	Hypothetical proteins	↑	-	-	-	*
PBK69190.1	Hypothetical proteins	↑	-	-	-	*
SJK98805.1	Uncharacterized protein	↑	-	-	-	*
PBK90167.1	Hypothetical proteins	↑	-	-	-	*
SJK98589.1	Uncharacterized protein	↑	-	-	-	*
PBL03772.1	Hypothetical proteins	↑	-	-	-	*
PBK74393.1	Serine proteinase inhibitor	↑	-	-	-	*
PBL00277.1	Hypothetical proteins	↑	-	-	-	*
	$p < 0.000$		***	No sig		
	$p = 0.02$		**			
	$p = 0.05$		*			