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Getting to the root of the problem

Understanding and Controlling Armillaria

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**Getting to the root of the problem:
Understanding and Controlling *Armillaria***

Helen J. Rees

A dissertation submitted to the University of Bristol in accordance with the requirements for
award of the degree of Doctor of Philosophy in the Faculty of Life Sciences.

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Abstract

The Agaricomycete fungal root rot pathogen, *Armillaria*, is important worldwide causing serious losses in forestry, horticulture and agriculture. Historically, soil fumigants were used to control the spread of *Armillaria*, however, due to negative environmental impacts these have been banned. Current methods of control are laborious and involve removing the entire infected plant including the root system. To explore an environmentally-friendly option for control, this thesis will consider the potential of *Trichoderma* spp. as biocontrol agents of *Armillaria mellea*, the most virulent species of *Armillaria* in the UK.

A collection of 40 endophytic *Trichoderma* spp. were isolated from the roots of healthy plants in areas where *A. mellea* infection was endemic. All *Trichoderma* isolates were assessed for antagonism against *A. mellea* in challenge assays, where almost unanimously, *Trichoderma* overgrew *Armillaria* colonies. In dual-culture assays five out of eight *Trichoderma* isolates eliminated *Armillaria* growth and in wood, two *Trichoderma* isolates eliminated *Armillaria* growth completely. Seven *Trichoderma* isolates protected plants from *Armillaria* Root Rot (ARR) when screened in strawberry plants. These isolates were further tested in privet plants, where two isolates of *Trichoderma atrobrunneum* were identified as potential biocontrol agents against ARR.

Although a number of *Armillaria* species genomes have been published, we have a poor understanding of gene function in *Armillaria*, particularly regarding virulence. Seven candidate genes with homologs to virulence genes in model fungal plant pathogens were chosen for study. Promoter:GFP fusion transgenic lines of *A. mellea* were created, however poor rate of fluorescence combined with autofluorescence made gene function assessment difficult. RNA expression profiles were assessed for candidate genes on mycelial tissue from cultures and mature fruiting bodies. Results suggest that signalling-related genes, effectors and some SSPs were constitutively expressed.

Together this shows that whilst *Armillaria* infection is poorly understood, the future prospects to control ARR are positive.

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Authors Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed:Helen Rees..... Date:.....

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List of abbreviations

| Abbreviations | Full name |
|---|---|
| % | Percent |
| (Ha)SSP47 | (<i>Heterobasidion annosum</i>) small secreted protein 47 |
| (NH ₄) ₂ SO ₄ | Ammonium sulphate |
| μg | Microgram |
| μl | Microlitre |
| μm | Micrometre |
| μmol m ⁻² s ⁻¹ | Micromoles of photons per second per square meter |
| χ ² | Chi-squared |
| 1KFG | 1000 Fungal Genomes |
| AIM | <i>Agrobacterium</i> induction medium |
| AMT | <i>Agrobacterium</i> mediated transformation |
| ARR | <i>Armillaria</i> Root Rot |
| BB | Backbone |
| BDS | Benomyl, dicloran and streptomycin MEA |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| BSMA | Benomyl and streptomycin MEA |
| BSPP | British Society for Plant Pathology |
| CA | Carrot agar |
| CaCl ₂ .2H ₂ O | Calcium chloride dihydrate |
| CaMV | Cauliflower mosaic virus |
| cDNA | Complimentary DNA |
| <i>chi</i> 18-5 | Chitinase 18-5 |
| cm | Centimetre |
| CMC | Carboxymethylcellulose |
| CP | Cerato-platanin |
| CTAB | Cetyl trimethylammonium bromide |
| DEPC | Diethylpyrocarbonate |
| DF | Degrees of freedom |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dpi | Days post inoculation |
| DSI | Disease severity index |
| dsRED | Red fluorescent protein |
| dT | Deoxythymine |
| DW | Deionised water |
| Ecp | Extracellular proteins |
| EDTA | Ethylenediaminetetraacetic acid |
| eGFP | Enhanced green fluorescent protein |
| EI | Enzymatic index |
| FRF | Field Research Facility |
| <i>g</i> | Centrifugal force |

| | |
|--------------------------------------|--|
| g | Gram |
| GAPDH | Glyceraldehyde-3-Phosphate dehydrogenase |
| gDNA | Genomic DNA |
| GFP | Green fluorescent protein |
| gpd | Glyceraldehyde-3-phosphate dehydrogenase |
| GTP | Guanosine triphosphate |
| ha | Hectare |
| hph | Hygromycin phosphotransferase |
| hr(s) | Hour(s) |
| IAA | Isoamyl alcohol |
| ID | Identification |
| IDT | Integrated DNA Technologies |
| IMS | Industrial methylated spirits |
| ITS | Internal transcribed spacers |
| JA | Jasmonic acid |
| JGI | Joint Genome Institute |
| JJG | J. J. Guillaumin Medium |
| K ₂ HPO ₄ | Dipotassium phosphate |
| KAc | Potassium acetate |
| kb | Kilobase |
| kg | Kilogram |
| KH ₂ PO ₄ | Monopotassium phosphate |
| KOH | Potassium hydroxide |
| kPa | Kilopascal |
| kV | Kilovolt |
| L | Litre |
| L : D | Light : dark |
| LB | Left border |
| LB | Lennox broth |
| LBA | Lennox broth agar |
| LysM | Lysin Motif |
| M | Molar |
| m ³ | Cubic metre |
| MAP | Mitogen-activated protein |
| MAPK | Mitogen-activated protein kinase |
| MAPKK | Mitogen-activated protein kinase kinase |
| MAPKKK | Mitogen-activated protein kinase kinase kinase |
| Mb | Million base pairs |
| MEA | Malt extract agar |
| MEB | Malt extract broth |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| mg | Milligram |
| MgSO ₄ .7H ₂ O | Magnesium sulphate |
| MGW | Molecular grade water |
| min(s) | Minute(s) |

| | |
|-----------------|---|
| MJ | Megajoule |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| MM | Minimal medium |
| mm ² | Millimetre squared |
| mpi | Months post inoculation |
| MRB | Malt Rose Bengal Agar |
| mRFP | Monomeric red fluorescent protein |
| mRNA | Messenger RNA |
| ms | Milliseconds |
| N | North |
| n | Number (n = x in graphs) |
| N ₂ | Nitrogen |
| NA | Nutrient agar |
| NCBI | National Centre for Biotechnology Information |
| NEB | New England Biolabs |
| nm | Nanometre |
| NTC | No template control |
| °C | Degrees Celsius |
| OPH | Old Park Hill Greenhouse |
| PAMP | Pathogen associated molecular pattern |
| PCR | Polymerase chain reaction |
| PCWDE | Plant cell wall degrading enzyme |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| PEG | Polyethylene glycol |
| PIPES | 1,4-Piperazinediethanesulfonic acid |
| PMK1 | Mitogen-activated protein kinase gene |
| pt | Point |
| qPCR | Quantitative PCR |
| RB | Right border |
| RHS | Royal Horticultural Society |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| <i>rpb2</i> | RNA polymerase II subunit |
| rpm | Revolutions per minute |
| RST | Rice Sawdust Tomato |
| RT | Room Temperature |
| RT- | Reverse transcriptase minus' |
| RT+ | Reverse transcriptase plus |
| RT-PCR | Reverse-transcription PCR |
| s | Seconds |
| SA | Salicylic acid |
| SDS | Sodium dodecyl sulphate |

| | |
|------------------|--|
| SDW | Sterile deionised water |
| SEM | Scanning electron microscopy |
| Slp1 | Secreted LysM protein 1 |
| SM | Secondary metabolite |
| SOB | Super Optimal Broth |
| sp. | Unknown species |
| SPAD | Soil Plant Analysis Development |
| spp. | Species (plural) |
| SS-DNA | Salmon sperm DNA |
| SSP | Small secreted proteins |
| syn. | Synonym |
| TE | Tris-EDTA |
| <i>tef1</i> | Translation elongation factor 1 alpha |
| Tris-HCl | Tris hydrochloride |
| trpC | Tryptophan synthetase |
| U | Unit(s) |
| UCD | University of California, Davis |
| UK | United Kingdom |
| USA | United States of America |
| v/v | Volume / volume |
| VOC(s) | Volatile organic compound(s) |
| W | West |
| W/m ² | Watts per metre squared |
| w/w | Weight / weight |
| WA | Water agar |
| wpi | Weeks post inoculation |
| WT | Wild type |
| YENB | Yeast extract nutrient broth |
| YPD(A) | Yeast extract peptone dextrose (agar) |
| YSDM(A) | Yeast synthetic drop out medium (agar) |

Publications

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A review was written for the RHS Science magazine, *The Plant Review*:

Rees, H. J., Drakulic, J., Cromey, M. G., Bailey A. M., & Foster G. D. (2020) Fungi for a healthy plant. *The Plant Review*. December, p.50-53.

Copies of this work can be found in the appendices.

| Chapter 1: Introduction

1.1 Tree health

The importance of tree health and the threats posed by invasive pests and diseases was highlighted by the arrival of ash dieback (*Hymenoscyphus fraxineus*) to the UK in 2012. Widely reported in the media and drawing comparisons with Dutch Elm Disease, ash dieback brought tree health to the attention of the general public (Woodward and Boa, 2013). Dutch Elm Disease, caused by the fungal pathogens *Ophiostoma ulmi* and *O. novo-ulmi*, resulted in two pandemics at the start and end of the 1900's which resulted in extensive loss of mature elms (*Ulmus* spp.) in areas of Europe, North America and Western Asia (Santini and Faccoli, 2015). Today, the bacterial pathogen *Xylella fastidiosa*, which affects over 500 plant species (Schneider *et al.*, 2020), poses a serious threat to plant health in Europe and the UK. Already found in Italy, France, Spain and Portugal, the economic loss caused by *X. fastidiosa* in olives alone could reach billions of Euros (Schneider *et al.*, 2020). The UK is on high alert for the arrival of *Xylella* which could have serious implications in the horticultural industry. Climate change and global trade are important factors which could result in invasions of new pathogens threatening our forests and trees (Ramsfield *et al.*, 2016). In 2011, chestnut blight, caused by the fungal pathogen *Cryphonectria parasitica* was identified on sweet chestnut trees (*Castanea sativa*) in the UK and subsequently eradicated (Hunter *et al.*, 2013). New infections of *C. parasitica* were found in mature chestnut trees in 2016, and through wider surveys, it was established that multiple introductions of *C. parasitica* had occurred in the UK over the last 20 years (Pérez-Sierra *et al.*, 2019). In addition, *Gnomoniopsis smithogilyyi* was found in the UK in 2016 causing cankers on sweet chestnut trees (Lewis *et al.*, 2017). In the UK, *Phytophthora ramorum* and *P. austrocedrae* are serious pathogens of larch (*Larix* spp.) (Brasier and Webber, 2010) and juniper (*Juniperus* spp.) (Green *et al.*, 2015), respectively which can result in statutory plant health notices and removal of a large numbers of trees. The most important pathogen to UK pine plantations is *Dothistroma septosporum* (Fraser *et al.*, 2015) and *Sirococcus tsugae* is an emerging shoot pathogen of cedars (Perez-Sierra *et al.*, 2015).

Armillaria species are a group of Agaricomycete root rot pathogens affecting a range of over 500 species of plants (Raabe, 1962), predominantly trees and are considered to be important fungal plant pathogens (Greig *et al.*, 1991; Baumgartner *et al.*, 2011). Yet, controlling *Armillaria* root rot (ARR) is challenging. A lack of effective chemicals available means the most viable option of control is physical removal of all infected plant material. Consequently, this thesis will focus primarily on biological control as a method to control ARR.

1.2 *Armillaria*

1.2.1 Taxonomy and ecology

The genus *Armillaria* is estimated to have evolved 21 - 33 million years ago after diverging from *Guyanagaster* 42 million years ago (Koch *et al.*, 2017; Sipos *et al.*, 2017). *Armillaria* species have long been of interest to mycologists with the earliest recordings thought to be by Micheli in 1729 or Battarra in 1755 (Watling *et al.*, 1991). *Agaricus melleus* was first taxonomically described by Danish-Norwegian botanist Martin Vahl in *Flora Danica* (1787). Swedish mycologist Elias Fries introduced the name *Armillaria* in reference to the characteristic ring ('*armilla*') on the stipe which later became the genus name for modern-day *Armillaria* species (Fries, 1821; Watling *et al.*, 1991). There are over 40 species of *Armillaria* (division: Basidiomycota, family: Physalacriaceae, order: Agaricales) (Baumgartner *et al.*, 2011) and two species were recently moved from the genus *Armillaria* to the new genus *Desarmillaria* (Koch *et al.*, 2017). In the UK and Europe there are seven species of *Armillaria* / *Desarmillaria*. *Armillaria mellea* (Vahl) P.Kumm., *A. ostoyae* (Romagnesi) Herink., *A. gallica* Marxm. & Romagn., *A. borealis* Marxm. & Korhonen., *A. cepistipes* Velen., and *Desarmillaria tabescens* (syn. *A. tabescens*) (Scop.) Emel. are all species found in the UK (Perez Sierra *et al.*, 1999; Koch *et al.*, 2017; Heinzelmann *et al.*, 2019); *D. ectypa* (syn. *A. ectypa*) (Fr.) Lamoure is the seventh European species (Burdall and Volk, 1993; Guillaumin *et al.*, 1993; Koch *et al.*, 2017; Heinzelmann *et al.*, 2019). Some of the common names for *Armillaria* spp. include honey fungus and bootlace fungus after their honey-coloured mushrooms and distinctive rhizomorph structures, respectively.

While some species of *Armillaria* are saprophytic on wood and dead plant material, others are primary plant pathogens (Fox, 2000c). In the UK, *A. gallica* is commonly an opportunistic pathogen, whereas *A. mellea* is mainly a primary pathogen (Rishbeth, 1982) and more likely to spread between hosts in garden situations than *A. gallica* (Drakulic *et al.*, 2017). Although generally regarded as a pathogen of coniferous trees (Rishbeth, 1982; Blodgett and Worrall, 1992), recent evidence shows that in garden settings, *A. ostoyae* has no preference for gymnosperms (Cromeley *et al.*, 2019). In England, *D. tabescens* is considered non-pathogenic and found mainly on broadleaved tree stumps (Rishbeth, 1982). *Desarmillaria ectypa*, commonly known as the marsh honey fungus, grows in wetland areas (Ohenoja, 2006; Stasińska, 2015) and is classified as near threatened by the IUCN Red List of threatened species (Svetasheva, 2015). Recently, a study in disused iron mines found abundant growth of *Armillaria sinapina* rhizomorphs in many locations in the mines in MN, USA (Held *et al.*, 2020). While *Armillaria* species are usually considered parasites, roles are reversed in the achlorophyllous orchid, *Gastrodia elata* Blume found in Central Asia, where *Armillaria* spp. are the host and *G. elata* the parasite (Cha and Igarashi, 1995; Kikuchi *et al.*, 2008).

In the fungal kingdom there are four distinct lineages of bioluminescent fungi, all found in the Agaricales group (Oliveira *et al.*, 2012). Recent evidence suggests fungal bioluminescence evolved at least 160 million years ago (Ke *et al.*, 2020). The phenomenon of bioluminescence was first described by Aristotle (384–322 BC), but was not linked to fungi and bacteria until the mid-19th century (Desjardin *et al.*, 2007). Bioluminescence is the result of a catalytic oxidation by a luciferase enzyme of a luciferin (Weitz, 2004; Oliveira *et al.*, 2012). A number of *Armillaria* spp. are bioluminescent (Figure 1.1), a phenomenon known for millennia as ‘foxfire’ (Desjardin *et al.*, 2007; Oliveira *et al.*, 2012). *Armillaria* species are constitutively luminescent in the dark; under environmental illumination *A. gallica* luminescence is enhanced but luminescence in *A. mellea* and *D. tabescens* is reduced (Mihail and Bruhn, 2007). It is thought that bioluminescent fungi attract fungivorous invertebrates to aid spore dispersal (Weitz, 2004; Desjardin *et al.*, 2007).

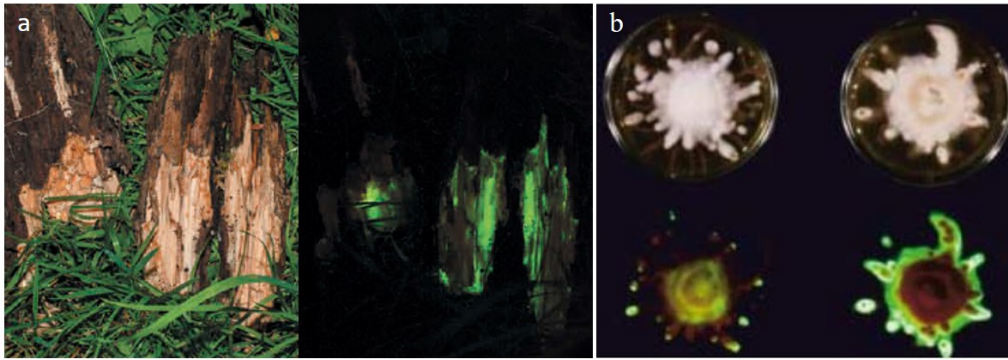


Figure 1.1: Example of bioluminescence in *Armillaria* spp.

a) *Armillaria* spp. on colonised wood (Heinzelmann *et al.*, 2019); b) *A. mellea* grown on agar based medium (Weitz, 2004).

1.2.2 Life cycle

The life cycle of *Armillaria* spp. is illustrated in five stages within Figure 1.2 (Heinzelmann *et al.*, 2019). Stage one describes the process whereby infection occurs by spore dispersal (Figure 1.2) which is regarded as the least frequent mode of infection by *Armillaria* spp. (Baumgartner *et al.*, 2011). The long-lived vegetative state of *Armillaria* is diploid, as opposed to dikaryotic which is usually the case for Agaricomycetes (Korhonen and Hintikka, 1974) as shown in stage 2 of Figure 1.2. In culture, haploid, single spore isolates and diploid isolates taken from mycelial tissue or fruiting bodies are morphologically distinct. After crossing isolates from single spores with diploid isolates, the single spore isolates morphologically change becoming more representative of diploid isolates, providing evidence for a bifactorial mating system in *Armillaria* (Hintikka, 1973; Guillaumin *et al.*, 1991). Traditionally, *Armillaria* species identification was determined using infertility tests between a known, haploid strain and the unknown isolate (Guillaumin *et al.*, 1991), but is now confirmed through sequencing. The virulence of haploid *Armillaria* spp. is unknown. In a pot-based experiment, Norway spruce (*Picea abies*) seedling and saplings were inoculated with haploid *A. ostoyae* isolates. All *A. ostoyae* re-isolated were diploid, presumably caused by *Armillaria* contamination (Heinzelmann *et al.*, 2018). In autoclaved *P. abies* stems, haploid *A. ostoyae* isolates produced mycelial fans which grew slower than diploid *A. ostoyae* isolates (Heinzelmann and Rigling, 2016) but the behaviour in non-sterile stems remains unknown.

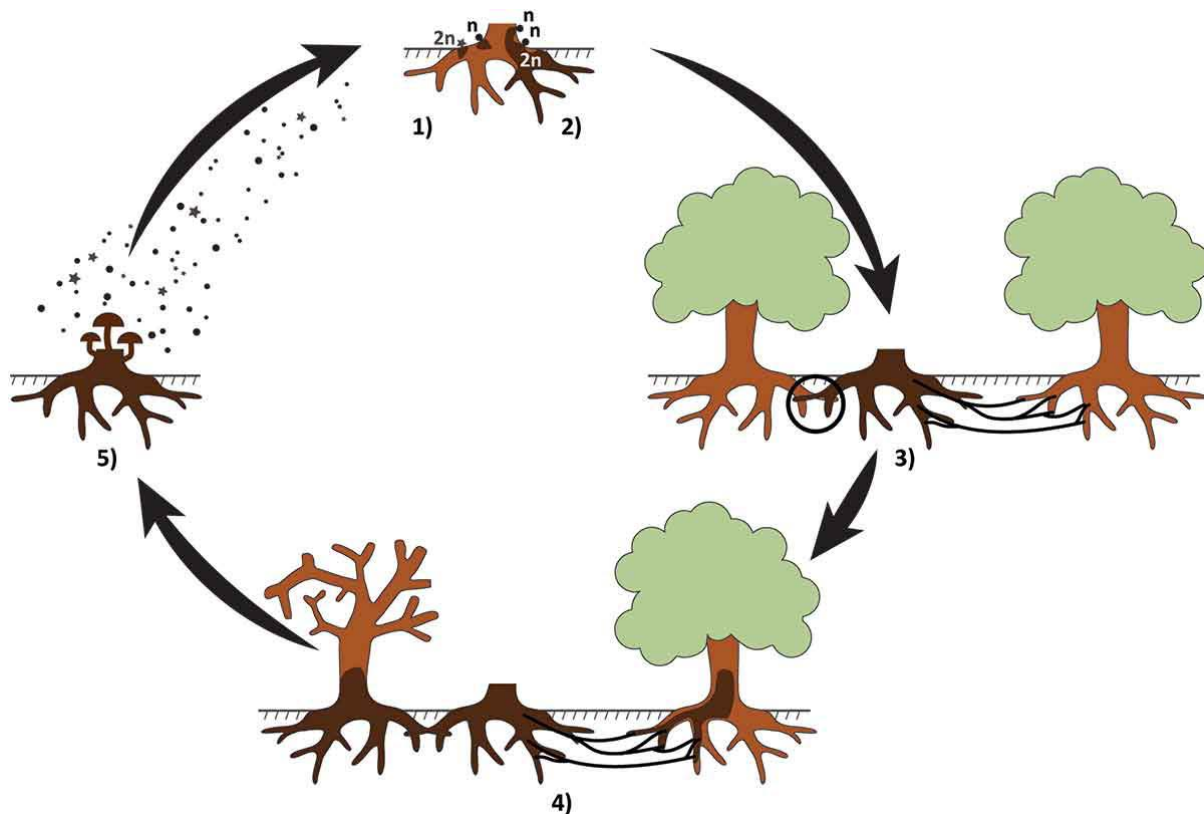


Figure 1.2: Life cycle of *Armillaria* spp. (Heinzlmann et al., 2019) split into five main stages

Stage three of Figure 1.2 represents the spread of *Armillaria* by root to root contact, which combined with spread through rhizomorphs (stage four), is the predominant mode by which *Armillaria* spp. spreads (Garraway *et al.*, 1991; Baumgartner *et al.*, 2011). It is this asexual growth pattern of *Armillaria* which allowed *A. gallica* to become known as the ‘humungous fungus’. First recorded as the ‘largest and oldest living organism’ in 1992, a recent study found that the clonal individual of *A. gallica* covered 75 hectares and was estimated to be at least 2,500 years old (Smith *et al.*, 1992; Anderson *et al.*, 2018).

Rhizomorphs are a characteristic feature of *Armillaria* spp. (Fox, 2000a; Baumgartner *et al.*, 2011) (Figure 1.3a) and can grow up to one metre a year (Redfern, 1973). Although closely related genera to *Armillaria* produce rhizomorphs in culture, they are unmelanised and have not been recorded in nature (Koch *et al.*, 2017). Rhizomorphs allow translocation of resources and nutrients (Fox, 2000a) and can grow into substrate which may not support their growth (Garraway *et al.*, 1991). The rhizomorph is made up of highly differentiated mycelial chords, which can grow apically and are fully autonomous

(Garraway *et al.*, 1991). The hyphae on the surface of rhizomorphs form a branched web (Figure 1.3b) (Yafetto *et al.*, 2009). The structure of a rhizomorph can be broken into four distinct zones based on hyphal differentiation (Figure 1.3c), these are the peripheral layer of thin hyphae, the cortex, the outer and the inner medulla (Yafetto *et al.*, 2009). Redfern (1973) suggested that rhizomorphs with diameters over 2 mm hold sufficient resources to continue growing without a food base. This was later confirmed by Perez Sierra and Gorton (2005) who found rhizomorphs severed from their food sources could continue to grow in a layer of wood chip mulch.

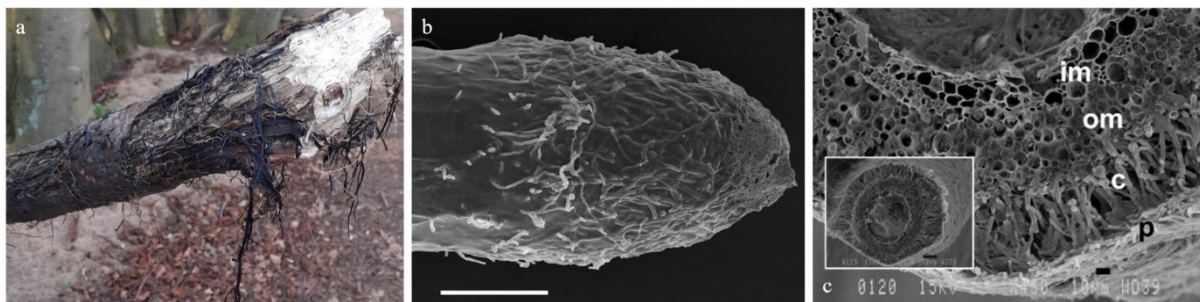


Figure 1.3 Rhizomorphs of *Armillaria* spp.

a) Rhizomorphs from *Armillaria* sp. growing on deadwood; b) SEM of the rhizomorph tip from *A. gallica*, scale = 100 μm (Yafetto *et al.*, 2009); c) Transverse section of a rhizomorph (inset where scale = 100 μm) and the anatomical structure of a rhizomorph where p = peripheral layer, c = cortex, om = outer medulla and im = inner medulla. scale = 10 μm (Yafetto *et al.*, 2009).

Symptoms of ARR are manifested as typical signs of root disease such as wilting, stunted growth, defoliation and resinous bleeds from the trunk of a tree. In heavily infected plants a white mycelial mat formed by *Armillaria*, known as a mycelial fan (Figure 1.4a), is visible when the outer layer of bark is removed (Baumgartner *et al.*, 2011). Fruiting bodies of the fungus (Figure 1.4b) can only be found for a short period in the autumn (Fox, 2000a), and if *Armillaria* spp. are fruiting then it is likely to be well established on the host.



Figure 1.4: Signs of *Armillaria* Root Rot

a) Characteristic mycelial fan caused by *A. ostoyae* adapted from Sipos et al. (2018); b) fruiting bodies of *A. mellea*

1.2.3 Infection process of *Armillaria* spp.

A recent review by Devkota and Hammerschmidt (2020) described what we understand of the *A. mellea* and *A. solidipes* infection process, as depicted in Figure 1.5. *Armillaria* infection of a plant begins when a piece of inoculum comes into contact with a plant, however a number of questions in the infection process of *Armillaria* spp. remain.

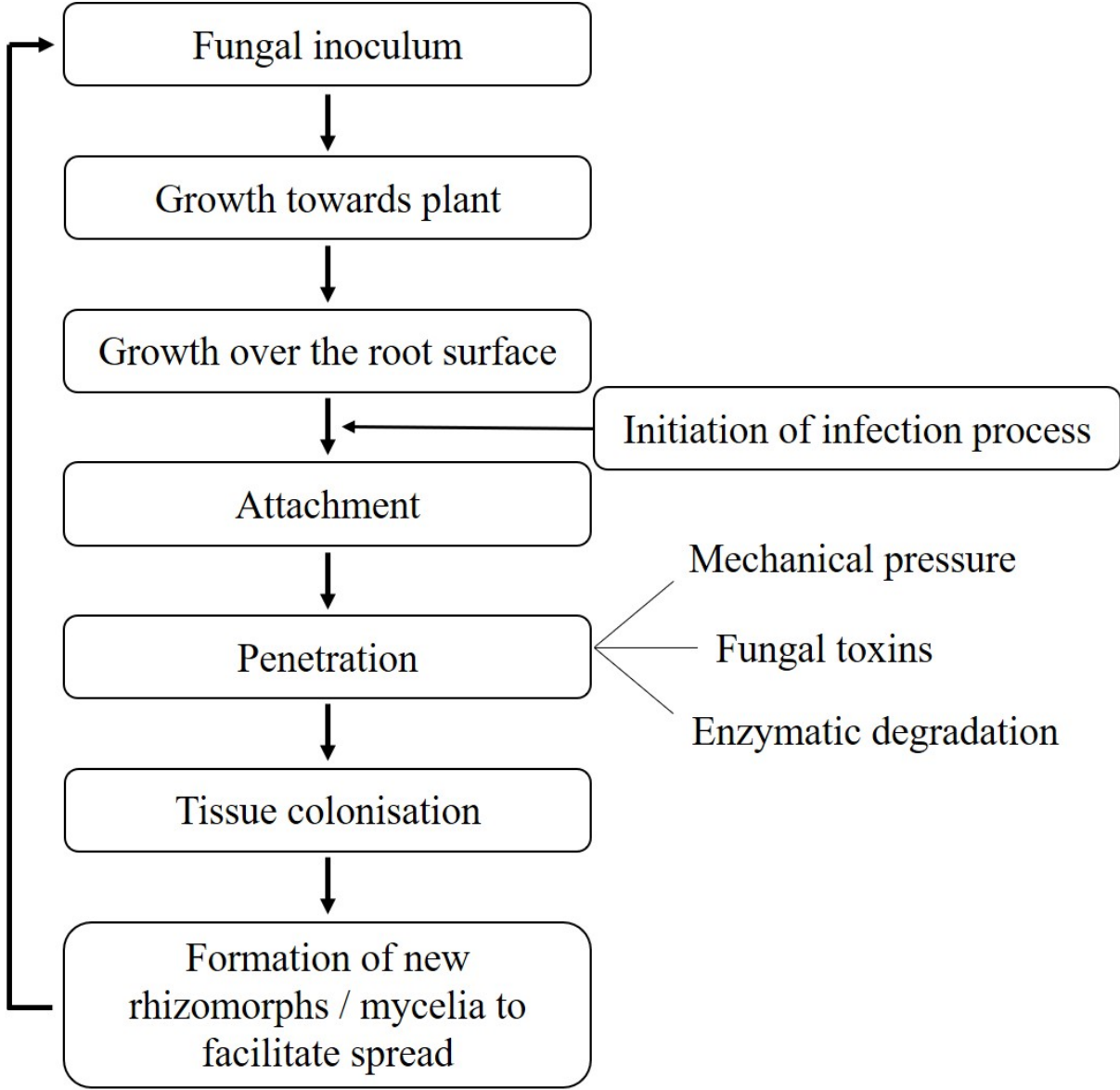


Figure 1.5: Infection process of *A. mellea* and *A. solidipes* adapted from Devkota and Hammerschmidt (2020).

The first stage of plant infection by *Armillaria* spp. is the attachment of the fungus to the plant. It is thought that attachment to the surface of the host is aided by a mucilaginous substance produced by rhizomorphs (Powell and Rayner, 1983; Yafetto *et al.*, 2009). The mechanism by which penetration of host roots occurs is not fully understood, although a combination of factors are likely to play a role (Devkota and Hammerschmidt, 2020). A wounded host is not required by *Armillaria* spp. to cause infection (Solla *et al.*, 2002; Cleary *et al.*, 2012). Field experiments in 20 – 30 year old conifer plantations challenged trees with *A. ostoyae* to find that early stages of infection took a minimum of

five weeks to appear and did not require wounding to infect roots (Cleary *et al.*, 2012). A more detailed study of mature *Picea sitchensis* trees considered the difference in colonisation of unwounded, superficially wounded and deeply wounded roots (Solla *et al.*, 2002). Solla *et al.* (2002) found that outer bark of unwounded roots could be penetrated by *A. mellea* within five days and after 25 days decay had reached secondary phloem. Mechanical pressure by rhizomorphs is likely to play a role in penetration of host roots. The pressure exerted by rhizomorphs was measured to be 40 - 300 kPa and the turgor pressure estimated at 760 kPa (Yafetto *et al.*, 2009), far lower than that in other plant pathogenic fungi, such as *Magnaporthe grisea*, where turgor pressure prior to penetration reaches 8000 kPa (Howard *et al.*, 1991; Devkota and Hammerschmidt, 2020). *Armillaria* spp. encode a number of secondary metabolites (Sipos *et al.*, 2017) and proteomics show that levels in abundance differ when grown in agar and liquid culture (Collins *et al.*, 2013). It has been shown *in vitro* that toxic secondary metabolites are released by *A. ostoyae* and can cause necrotic signs on *Picea abies* callus tissue before contact is made by the fungus (Peipp and Sonnenbichler, 1992). Degradation of host tissues is another factor which aids *Armillaria* penetration by production of plant cell wall degrading enzymes (PCWDE) such as lignin-, cellulose-, hemicellulose- and pectin-degrading enzymes which are all encoded in the *Armillaria* spp. genomes (Sipos *et al.*, 2017). A recent study has found that PCWDEs were upregulated in the saprophytic *A. cepistipes* more so than the pathogenic *A. ostoyae*, and compared to rhizomorphs, invasive mycelium had greater upregulation of PCWDEs (Sahu *et al.*, 2020), however, PCWDE are still likely to perform an important role in attacking host plants. After penetration of plant roots, Solla *et al.* (2002) found that after 48 days *A. ostoyae* and *A. mellea* had colonised 39 and 25 cell layers respectively on unwounded roots. As colonisation of *Armillaria* becomes established, symptoms of root disease begin to occur which can eventually lead to the death of the host and any piece of infected tissue can become a source of inoculum for infection of a new host to continue the disease cycle. Inoculum can survive in the soil for long periods of time which is one reason why ARR is such a serious disease.

1.3 The scale of problems caused by *Armillaria* root rot

Serious losses from *Armillaria* occur in forestry (Wargo and Shaw, 1985), vineyards (Baumgartner and Rizzo, 2001; Aguin-Casal *et al.*, 2004) and in stone-fruit production (Baumgartner and Rizzo, 2001). *Armillaria* root rot has consistently been the most common enquiry related to garden diseases at the Royal Horticultural society (RHS) since records began 24 years ago (RHS, 2020a). A study was conducted to determine the effect of *Armillaria* infections in Canadian forests. In Douglas fir stands, non-lethal *Armillaria* infections can cause 0 – 27 % reduction in volume per tree and an average reduction in yield of 15 m³ / ha (Cruickshank *et al.*, 2011). The effect of climate change is likely to increase losses caused by *Armillaria* infection as a result of weather anomalies such as long, regular periods of drought and storms (Kubiak *et al.*, 2017).

The biology of *Armillaria* as described in sections 1.2.2 and 1.2.3 make controlling ARR incredibly difficult. When infected plant material is removed in order to control spread of ARR, it is important to remove as much inoculum as possible because *Armillaria* can survive for long periods of time which risks future infection on the site. Indeed, Reaves *et al.* (1993) reported that residual inoculum of *A. ostoyae* on a regenerated Ponderosa pine (*Pinus ponderosa*) stand was still viable after 12 years. Another reason that *Armillaria* spp. can cause such devastation is due to the number of susceptible hosts. In 1962, Raabe reported > 500 hosts susceptible to *Armillaria* spp. infection, and additional susceptible species have since been found (Drakulic *et al.*, 2017; Ford *et al.*, 2017). As Raabe (1962) stated, it is almost impossible to create a complete host list for any pathogen, particularly one such as *Armillaria* which has a large host and geographical range.

1.4 Controlling *Armillaria*

1.4.1 Chemical control

In the past, chemicals were widely used in attempts to control ARR. Research focused on three main soil fumigants used against *Armillaria* infection: methyl bromide (CH₃Br), carbon disulphide (CS₂) and Sodium tetrathiocarbonate (STTC). Until soil fumigants were banned, where high value crops, such as

grapes or walnuts were to be planted, and the site had a history of forestry and/or *Armillaria* infection, soil fumigation was routinely carried out to reduce risk of disease (Baumgartner *et al.*, 2011). Use of soil fumigants was widespread because they provided good penetration of soil where *Armillaria* was present, however, fumigation of soil was not target-specific (Hagle and Shaw, 1991), thus due to negative environmental impacts and safety of workers, use of soil fumigants were phased out (Ristaino and Thomas, 1996; Baumgartner *et al.*, 2011). Other chemicals have been trialled including azoles (Aguín *et al.*, 2006), phenolic compounds (West and Fox, 2002) and natural extracts such as allicin (Beal *et al.*, 2015), from garlic in attempt to control *Armillaria* infections and spread.

Methyl bromide was once a common soil fumigant used for many soil borne diseases and to control *Armillaria* infections (Kolbezen *et al.*, 1974; Baumgartner *et al.*, 2011). *In vitro* assays found that CH₃Br could reduce the survival of *Armillaria* by up to 95 % in infected stems treated with high concentrations of CH₃Br and left in soil for three weeks (Munnecke *et al.*, 1970). Penetration of soils by CH₃Br is more effective in dry soils opposed to wet soils, thus to reduce loss of CH₃Br after fumigation, soil surfaces were wetted or covered by a tarpaulin to create an impervious layer against gas exchange (Kolbezen *et al.*, 1974). Adaskaveg *et al.* (1999) found that in soils covered with tarpaulin and fumigated with CH₃Br, no *A. mellea* could be recovered from colonised segments of almond roots after one year at depths of 0.3 and 1.2 m. Where soils were not covered, *A. mellea* recovery at 0.3 m depth was not significantly different to untreated controls but was significantly reduced to ~30 % recovery at 1.2 m depth (Adaskaveg *et al.*, 1999). Sherman and Beckman (1999) planted grafted peach trees on a site after *A. mellea* infected trees along with the roots had been removed and the site treated with 0.45 or 0.9 kg of CH₃Br over 9.3 m² which was found to prevent tree loss from ARR. A major issue with CH₃Br is the extremely damaging effect on the environment. During the late 1990's, agriculture was the largest source of anthropogenic CH₃Br in the atmosphere with an estimated 16 – 47.3 Gg per year produced from soil fumigation alone (Ristaino and Thomas, 1996). CH₃Br breaks down to form bromine which reacts with atmospheric ozone and is a major contributor to ozone depletion. Thus, in the early 2000's the use of CH₃Br was phased out (Ristaino and Thomas, 1996) and was completely banned by the EU commission in 2011 (EU Commission, 2020).

In 1951, Bliss suggested that after soil fumigation by CS₂ *Trichoderma viride* killed *Armillaria*. Fumigation of soil by CS₂ lead to *T. viride*, which has some tolerance to CS₂, to become a dominant species. Garrett (1957) showed that while CS₂ had direct fungicidal action on *A. mellea*, the combination with *T. viride* made control more effective. However, vapour of CS₂ is highly flammable (Bliss, 1944; Adaskaveg *et al.*, 1999). In attempt to make a safer form of control, Adaskaveg *et al.* (1999) trialled the use of STTC which degrades to produce CS₂. In order to reduce the recovery of *A. mellea* from almond billets a high concentration (2000 and 4000 mg / L) of STTC over long periods (120 hours) was required to produce enough CS₂ (Adaskaveg *et al.*, 1999). The growth of *A. mellea* was assessed after STTC was added to agar Petri dishes and found that a high dose of STTC (877 mg / L) was required to reduce the growth of *A. mellea* by only 50 % (Aguín *et al.*, 2006). STTC was trialled as a fumigant in a field experiment by Adaskaveg *et al.* (1999) and was found to significantly reduce the recovery of *A. mellea* from 0.3 m and 1.2 m depths when applied both before and after planting. A liquid and gel formulation of STTC was used to treat stumps of trees cut down due to *Armillaria* infection. Both were successful in eradicating *A. mellea* from the stump, but the gel also eradicated the fungus from the primary roots. Neither eradicated *A. mellea* from the secondary roots (Adaskaveg *et al.*, 1999). This highlights that to reduce the risk of future infection to trees infected by *Armillaria*, treating the stump alone is not sufficient and an effort to remove of as much of the roots as possible should made.

Azoles are often used in agriculture because of their systemic action which can prevent and cure plant fungal infections (Aguín *et al.*, 2006). Aguín *et al.* (2006) studied the effect of four azoles (hexaconazole, cyproconazole, propiconazole and tetraconazole) on *A. mellea* growth *in vitro* and found that cyproconazole and hexaconazole were most effective with 67 – 72 % inhibition of mycelial growth after the addition of 1 mg / L in the medium. At a higher concentration, Adaskaveg *et al.* (1999) reported that 10 mg / L of propiconazole *in vitro* completely inhibited mycelial growth. When hazel discs were soaked in the aforementioned azoles and introduced to *Armillaria* cultures, hexaconazole and cyproconazole soaked disks protected the wood from infection by *Armillaria*. In field studies, two applications of cyproconazole were injected at five points (total of five litres) into the soil surrounding grapevines infected with *Armillaria*. Of four different doses tested (10, 50, 500 and 1000 mg / L), 50

mg / L was sufficient to stop the death of plants from ARR after symptoms were noted in the vineyard (Adaskaveg *et al.*, 1999). However, the effectiveness of the cyproconazole to control *Armillaria* was only studied for two years and results thereafter were not published.

West and Fox (2002) investigated the use of three phenol-based fungicides: Armillatox (1500 mg & 150 mg cresylic acid / L), Brays emulsion (2400 mg & 240 mg cresylic acid / L) and phenyl phenol (5000 mg & 500 mg / L). Hazel billets, pre-colonised by *A. mellea*, were exposed to high concentrations (1500 mg / L or more) of phenolic fungicides and after three months were found to have more fungal growth than the water control. Field experiments tested these fungicides by watering into the soil of inoculated privet plants. No treatment was significantly different to the untreated control (West and Fox, 2002), suggesting that these phenol based fungicides are ineffective as control against ARR.

Allicin (diallyl thiosulfinate), a compound produced by garlic plants, with known antibiotic and antifungal properties was investigated for control of *A. gallica* and *A. mellea* by Beal *et al.* (2015). *In vitro* experiments found that both *Armillaria* species had slower growth when allicin was present, however, at lower concentrations (20 and 30 mg / L allicin), *A. mellea* growth and production of rhizomorphs was stimulated. The use of allicin in the field is unpredictable as it degrades quickly and may form antifungal properties, dependant on field conditions. Use of injections to apply the allicin could be used to penetrate woody roots but further research is required to determine success *in situ* (Beal *et al.*, 2015).

1.4.2 Mechanical control

Mechanical control of *Armillaria* focuses on removal of inoculum sources to control the spread of infections. The main methods by which this is achieved are through removal of infected stumps and creation of physical barriers.

Removal of infected stumps is frequently recommended as an effective method to control *Armillaria* (Hagle and Shaw, 1991; Vasaitis *et al.*, 2008; RHS, 2020c). In forestry, stump removal is considered more effective than chemicals, although it is more expensive (Vasaitis *et al.*, 2008). While stump removal cannot completely eradicate disease (Fox, 2000a) it can prevent a build-up of inoculum (Cleary

et al., 2013) and in Canada and the US, forestry machinery can remove between 83 and 94 % of the estimated belowground biomass of a tree (Vasaitis *et al.*, 2008). There are concerns machinery can cause damage and disturbance to tree roots, and put remaining trees at risk of infection, however this largely depends on the method of extraction and soil type (Vasaitis *et al.*, 2008). *Armillaria* can survive on small pieces of root or woody material as a source of inoculum for up to 12 years (Reaves *et al.*, 1993), but removal of most belowground biomass will reduce the energy available to rhizomorphs and therefore reduce the lifespan and incidence of disease (Fox, 2000b). A long-term trial of stump removal in Canada, Sweden and Denmark found that incidence of *A. ostoyae* was reduced where stumps had been removed. After stump removal in Sweden, *A. ostoyae* only caused 2.8 - 4.6 % mortality after 40 years compared to 23 – 38 % mortality where stumps were left (Cleary *et al.*, 2013). At some sites stumps are left upturned on site after removal (Vasaitis *et al.*, 2008) and, given that *Armillaria* is killed if it dries out or is exposed to high temperatures (Baumgartner, 2004) this effectively reduces the risk of *Armillaria* infection. This approach is beneficial on sites where the removal of the stumps is impractical, for example, due to steep slopes (Vasaitis *et al.*, 2008). A further benefit to stump removal is that stumps could be used in energy production (Vasaitis *et al.*, 2008), which is beneficial to foresters who could make money from selling stumps which are not normally sold in timber producing forests. Conversely, in a garden setting, stump removal is labour intensive, often only targeting a small number of plants and gardeners do not usually have access to stump removal machinery (Fox, 2000b). Removal of stumps in a garden setting can also be aesthetically unpleasant. Nevertheless, the (RHS, 2020b) recommends removal of stumps and of as much of the root as possible on their honey fungus advice websites. Once the stumps are removed, the site may still carry a risk from secondary inoculum for example, stumps from hardwood trees cut 40 years ago could still produce a low number of rhizomorphs (Fox, 2000b). Thus a fallow period of one year is occasionally recommended (Hagle and Shaw, 1991; Fox, 2000b; RHS, 2020b), which should allow small pieces of secondary inoculum to decay. During a fallow period a cash crop such as cereal (dependant on land quality) could be grown for a season so the land will still be profitable (Hagle and Shaw, 1991).

Outbreaks of *Armillaria* infection in vineyards can be contained by digging trenches to control the spread of disease without removing individual vines (Hagle and Shaw, 1991; Fox, 2000b). In French vineyards and African coffee plantations, trenches are dug 1.1 m deep to isolate infected plants from healthy plants, whereas in cocoa plantations trenches are only dug to 0.6 m depth. Trenches dug in kiwi orchards in New Zealand are lined with plastic before being filled in again (Hagle and Shaw, 1991; Fox, 2000b). A study in vineyards found that excavating root collars of grapes during the early stages of *Armillaria* infection increased the yield from plants resulting in a similar yield to healthy plants (Baumgartner, 2004). Trenching around a disease centre of *Armillaria* can help to control the spread of disease, but is labour intensive and needs to be managed regularly (Fox, 2000b), nevertheless high value crops could benefit in small areas of disease outbreaks. In a garden setting, the RHS (2020b) recommend digging a trench 45 cm deep lined with thick plastic and refilled or ‘regular deep cultivation’ to reduce the spread of rhizomorphs.

1.4.3 Silvicultural control

Silvicultural practices can be important factors in mitigating incidence of ARR. Factors include, site choice and preparation, careful consideration of species planted and management of sites.

Site choice is an important factor to consider when planting species susceptible to *Armillaria*. Where plants, such as oak (*Quercus* spp.), which are susceptible to *Armillaria* were previously grown, sites should be avoided where possible due to the potential of established inoculum present in the soil (Sherman and Beckman, 1999). The use of local provenance seeds for trees grown in natural species mixes may have higher resistance and tolerance to *Armillaria* (Fox, 2000b) and is recommended on sites where *Armillaria* is present in North West America and Western Canada (Hagle and Shaw, 1991). In New Zealand, radiata pine (*Pinus radiata*) is more susceptible to *Armillaria* infection compared to its natural range in Western America, which could be due to differences in the environment or *Armillaria* populations (Hagle and Shaw, 1991). Planting trees with a bare-root stock significantly increases the chances of *Armillaria* infection compared to planting with seeds (Hagle and Shaw, 1991; Fox, 2000b) which is potentially caused by damage during transplantation and exposure to a new environment. Management of forests by thinning where *Armillaria* is a potential risk is not

recommended as it increases the incidence of disease by increasing the food base available to the fungus (Hagle and Shaw, 1991; Fox, 2000b; Robinson, 2003; Cleary *et al.*, 2013). Robinson (2003) reported significant increases in *Armillaria* incidence 15 years after thinning karri (*Eucalyptus diversicolor*) forests. In some cases use of fertilisers during planting might be considered beneficial, for example, chronically infected Scots pine (*Pinus sylvestris*) trees have shown to benefit more from fertilisers than young Scots pine (Fox, 2000b).

In addition to site choice, research has also addressed whether prescribed burning might reduce incidence of ARR. Filip and Yang-Erve (1997) found that at shallow depths of eight cm *Armillaria* was significantly decreased after prescribed burning in autumn, but at a depth of 30 cm there was no significant difference as a result of burning. Reaves *et al.* (1990) found that *Trichoderma* populations shifted from predominantly *T. harzianum* to predominantly *T. citrinoviride* after burning and that *Trichoderma* spp. isolates were more antagonistic towards *A. ostoyae* after burning. After 10 years, Whitney and Irwin (2005) found that there was no significant difference in the number of saplings killed by *Armillaria* in sites which had or had not been burned.

Another form of prevention against *Armillaria* is through use of resistant, or at least partially resistant species or cultivars. In forest settings, Douglas fir (*Pseudotsuga menziesii*) has some resistance to *A. ostoyae* thus is recommended for sites in the UK where Scots pine and Sitka spruce (*Picea sitchensis*) are at risk of infection (Hagle and Shaw, 1991). In fruit production of *Prunus* species, resistant rootstocks from plums are commonly used because they are generally more resistant to *Armillaria* attack than species such as cherry, almond and peach (Fox, 2000b). A recent study by Cromey *et al.* (2019) assessed the susceptibility of garden plants to *Armillaria* infection, which will allow gardeners to choose species suitable to their garden. This study was based upon records submitted to the RHS advisory service giving a bias towards popular garden plants. Species with high susceptibility to ARR included privet (*Ligustrum* spp.) and a number of gymnosperm species while *Buxus* was considered to have some resistance to ARR (Cromey *et al.*, 2019) however, box are highly susceptible to box blight (*Cylindrocarpon buxicola*) (Henricot *et al.*, 2000) and box tree caterpillar (*Cydalima perspectalis*) (Plant *et al.*, 2019) thus would be inappropriate alternatives. Other species considered to be resistant

include box elder maple (*Acer negundo*), black walnut (*Juglans nigra*) and yew (*Taxus baccata*) (Greig *et al.*, 1991).

Mulches are frequently used in gardens to suppress weeds and regulate soil moisture and temperature. Perez Sierra and Gorton (2005) conducted an experiment into the survival of *Armillaria* in different mulching treatments. It was found that *A. mellea* can use wood and bark chip mulching as a food base to survive, and although risk was low, plants could be infected by *A. mellea* in mulches. Where infected mulch pieces were smaller, the chance of *A. mellea* survival was smaller. Finally, where *A. mellea* was already present, the type of mulching treatment (pine wood chip or bark chip) did not prevent *A. mellea* infection (Perez Sierra and Gorton, 2005). Mulching trees will not protect them from ARR, but if gardeners are chipping wood to create their own mulch, it is important that this is done with clean material to prevent the spread of ARR.

1.4.4 Biological control

Owing to the laborious cost of mechanical controls of *Armillaria*, a lack of resistant plants and the negative environmental impact of chemical control there is an interest in the use of biological control to control ARR.

Saprobic fungi including *Hypholoma fasciculare*, *Ganoderma lucidum*, *Schizophyllum commune*, *Phanerochaete velutina* and *Xylaria hypoxylon* were considered as potential biocontrol agents against *A. mellea* and *D. tabescens* by Cox and Scherm (2006). *In vitro* interactions showed that all five fungi could damage hyphae of both species (*A. mellea* and *D. tabescens*) and reduced viability on wood blocks. In root sections, antagonistic fungi were able to colonise the surface of roots pre-colonised with *Armillaria* to some extent, but they were unable to colonise them internally. *Armillaria/Desarmillaria* species were unable to colonise root segments pre-colonised with antagonists. The results from Cox and Scherm (2006) indicate that some fungal species are able to act antagonistically towards *Armillaria* spp., however Kwaśna *et al.* (2004) found that a number of fungi (including *Cylindrocarpon destructans*, *Nectria grammicospora*, *Penicillium lanosum* and *P. notatum*) were stimulants of *A. ostoyae*.

An alternative method of controlling *A. luteobubalina* in karri forests in Australia considered the use of stump treatment with a biological control agent. Stump treatment against the root rot *Heterobasidion annosum* with the biocontrol agent *Phlebiopsis gigantea*, is considered an effective method of control frequently used in forests (Cleary *et al.*, 2013). Pearce and Malajczuk (1990) tested three fungi (*Stereum hirsutum*, *Coriolus versicolor* and *Xylaria hypoxylon*) as potential biocontrol agents for stump treatment against *A. luteobubalina*. Although all three fungi significantly reduced the ability of *A. luteobubalina* to colonise the stump it was not prevented, and therefore not considered an effective control.

Baumgartner and Warnock (2006) investigated the potential of soil inoculants, which are produced by composting and contain bacteria, as potential biocontrol agents with *in vitro* and field experiments. Growth of *A. mellea* was significantly inhibited where cultures were challenged with the soil inoculant, which was soaked onto filter paper. From these assays 12 bacteria were isolated and grown in dual culture with *A. mellea*, five of which (*Comamonas testosteroni*, *Bacillus subtilis*, *B. lentimorbus*, *Pseudomonas aeruginosa* and *P. mendocina*) produced the same inhibitory effect on *A. mellea* as the soil inoculant. A field trial took place in a commercial vineyard where the soil inoculant was added to the irrigation system. The addition of the inoculant had no effect on the healthy controls but was found to significantly increase the yield of symptomatic vines making them equal to healthy vines. Although the soil inoculant can significantly improve the yield from vines, the rate of *Armillaria* symptom development and mortality of vines was not reduced. The *in vitro* results hint at the potential of soil inoculants by certain bacteria to provide a biocontrol against *Armillaria*, and further research may improve this possibility.

Nematodes, which are often used to protect pines from pine weevils (Evans *et al.*, 2004) could be considered for control of *Armillaria*. The mycophagous nematode, *Aphelenchus avenae*, has shown to reduce the mortality of ponderosa pine (*Pinus ponderosa*) seedlings which were inoculated with *Armillaria* (Fox, 2003). In Australian karri forests termites have been suggested as a potential biocontrol agent as they feed on decayed wood (Robinson and Smith, 2001), but there has been no further research into this subject.

Trichoderma species are most frequently referred to as potential biocontrol agents of *Armillaria* (Fox, 2003; Otieno *et al.*, 2003; Raziq and Fox, 2003; Kwaśna *et al.*, 2004; Baumgartner and Warnock, 2006; Baumgartner *et al.*, 2011; Chen *et al.*, 2019). *Trichoderma* were first proposed as an antagonist of *Armillaria* by Bliss in 1951 where soil fumigation was thought to weaken *Armillaria*, allowing attack by antagonists such as *Trichoderma* (Bliss, 1951; Garrett, 1957).

Trichoderma hamatum and *T. viride* have been found to reduce the production of rhizomorphs by *A. ostoyae* in *in vitro* studies using wood segments incubated in soil but antagonism by *Trichoderma* spp. was dependant on *A. ostoyae* isolates (Kwaśna *et al.*, 2004). A further study by Otieno *et al.* (2003) looked at the use of *Trichoderma* as antagonists to *Armillaria* sp. in woody segments of tea plants. *Trichoderma longibrachiatum*, *T. harzianum* and *T. koningii* could colonise tea stem sections, even if *Armillaria* was present and were able to deter the growth of *Armillaria* where they were already established (Otieno *et al.*, 2003). Antagonism towards *Armillaria* spp. by *Trichoderma* spp. has been studied *in vitro* (Raziq and Fox, 2003; Chen *et al.*, 2019) and *in planta* (Raziq and Fox, 2003, 2006a, 2006b; Chen *et al.*, 2019) where culture growth was inhibited and ARR disease incidence in plants was reduced by *Trichoderma* spp..

1.5 Endophytes

1.5.1 Fungal endophytes as biocontrol agents

Endophytes are microbes that live within (*endo*) a plant (*phyte*) without causing any harm. Indeed, fungal endophytes can promote plant growth (Varma *et al.*, 1999), improve growth in drought and salt stressed environments (Azad and Kaminskyj, 2016; Sánchez-Montesinos *et al.*, 2019) and induce tolerance to insect herbivory (Cosme *et al.*, 2016). Fungal endophytes use a range of mechanisms to antagonise pathogens. Mechanisms for biological control are split into four groups: competition (for space and/or nutrients), inhibition of growth by production of secondary metabolites or extracellular enzymes, mycoparasitism and induced plant defence systems (Latz *et al.*, 2018). The predominant benefit of the use of endophytic fungi in biological control is the ability to provide protection to the plant from within.

The soilborne pathogen *Rhizoctonia solani* is an important disease of potatoes. The endophytic fungus *Epicoccum nigrum* produces secondary metabolites to inhibit growth of *R. solani* *in vitro* and can significantly increase the yield of potato plants inoculated with disease compared to those without the presence of endophytic antagonistic fungi (Lahlali and Hijri, 2010). *In vitro* studies with the cocoa pathogens *Moniliophthora roreri*, *M. pernicioso* and *Phytophthora palmivora* with a number of endophytes found the primary mode of antagonism to be by competition followed by antibiosis. In field trials, endophytic *Colletotrichum gloeosporioides* could reduce the number of cocoa fruits infected by *P. palmivora* (Mejía *et al.*, 2008). Endophytic fungi are commercially available as biocontrol agents including examples such as *Ampelomyces quisqualis*, marketed for a variety of agricultural crops such as wheat, barley and grapevines to protect against powdery mildews, and *Candida oleophila* to target *Botrytis* spp. and *Penicillium* spp. in citrus (de Silva *et al.*, 2019).

1.5.2 Biocontrol of phytopathogens using *Trichoderma* spp.

Trichoderma species have been demonstrated as effective biocontrol agents against many diseases, examples of which include *Phytophthora* root rot (Park *et al.*, 2019; Sanchez *et al.*, 2019), *Botrytis cinerea*, *Cylindrocarpon destructans* (Park *et al.*, 2019), *Pythium ultimum* (Sánchez-Montesinos *et al.*, 2019) and *Fusarium oxysporum* (Bunbury-Blanchette and Walker, 2019; Yu and Luo, 2020).

Antagonism by *Trichoderma* is complex and achieved through various mechanisms (Howell, 2003; Benítez *et al.*, 2004). *Trichoderma* spp. are frequently found to parasitise the hyphae of fungal plant pathogens, examples of which include *Sclerotinia sclerotiorum*, *S. rolfsii* (Rajani *et al.*, 2021), *Phytophthora* root rot (Sanchez *et al.*, 2019), *Rhizoctonia solani* and *Botrytis cinerea* (Park *et al.*, 2019). In addition *Trichoderma* spp. can produce extracellular enzymes such as chitinase (Loc *et al.*, 2020), cellulase and protease (Tchameni *et al.*, 2020), secondary metabolites including polyphenolic compounds and flavonoids (Tchameni *et al.*, 2020) and a variety of volatile organic compounds (VOCs) (Chen *et al.*, 2016; Rajani *et al.*, 2021) which can inhibit the growth of fungal pathogens. *Trichoderma* species can also induce systemic resistance in plants against fungal pathogens, such as *Sclerotinia sclerotiorum* (Alkooranee *et al.*, 2019), *Sclerospora graminicola* (Siddaiah *et al.*, 2017), and *Curvularia lunata* (Saravanakumar *et al.*, 2016).

1.5.3 Endophytic biocontrol of *Armillaria* spp.

Although *Trichoderma* spp. have been reported as potential biological control agents against *Armillaria* spp. (Dumas and Boyonoski, 1992; Cheruiyot *et al.*, 1997; Kwaśna *et al.*, 2004; Raziq and Fox, 2006b, 2006a; Asef *et al.*, 2008; Baldi *et al.*, 2016; Kwaśna and Szykiewicz-Wronek, 2018; Chen *et al.*, 2019), very few studies focus on the use of endophytic *Trichoderma* spp. to achieve control against ARR. Reports have been made using *Trichoderma* spp. pathogenic to commercial mushrooms (Raziq and Fox, 2003, 2006a, 2006b), from soils (Dumas and Boyonoski, 1992; Chen *et al.*, 2019), the rhizosphere of roots (Kwaśna and Szykiewicz-Wronek, 2018; Chen *et al.*, 2019) and soils associated with *Armillaria* rhizomorphs (Chen *et al.*, 2019). Endophytic isolates of *Trichoderma* spp. are rarely utilised. Taken from root samples in oak (Kwaśna *et al.*, 2004) and beach (Kwaśna and Szykiewicz-Wronek, 2018) endophytic *Trichoderma* spp. were found to be antagonistic against *Armillaria* spp. *in vitro*, but no plant based studies were made. Inoculation of *Trichoderma* spp. in plant based studies use colonised substrates, such as wheat bran, (Raziq and Fox, 2003, 2004c, 2006a, 2006b) or direct application of spores to roots prior to planting (Chen *et al.*, 2019). While Chen *et al.* (2019) found *Trichoderma* spp. could reduce ARR in Turkey oaks (*Quercus cerris*), the *Trichoderma* spp. isolates were taken from soils associated with rhizomorphs and might not have used isolates able to form endophytic associations. The work in this thesis focuses on using endophytic *Trichoderma* isolates to control *A. mellea in vitro* and, to my knowledge, is first report of endophytic *Trichoderma* spp. isolates used to protect host plants from ARR.

1.6 Genetic understanding and pathogenicity of *Armillaria* spp.

The genomes of several *Armillaria* species have been published in the last decade (Collins *et al.*, 2013; Wingfield *et al.*, 2016; Sipos *et al.*, 2017; JGI, 2020) including two isolates of *A. mellea* (Collins *et al.*, 2013; JGI, 2020). *Armillaria* spp. genomes are between 53 Mb and 97 Mb in size comprising of ~14,400 to 25,700 genes and show significant expansion compared to related fungal species (Collins *et al.*, 2013;

Wingfield *et al.*, 2016; Sipos *et al.*, 2017; JGI, 2020). A genetic map and chromosome assembly of *A. ostoyae* have been recently published (Heinzelmann *et al.*, 2017, 2020) which will aid future genome-wide analyses of *Armillaria* spp.. The *Armillaria* genome encodes an array of PCWDE; like other white rot fungi, *Armillaria* encodes several cellulose and xylan degrading genes, ligninolytic gene families are underrepresented, however, unusually in white rot fungi pectinolytic families are overrepresented in *Armillaria* (Collins *et al.*, 2013; Sipos *et al.*, 2017). The rhizomorphs transcriptome has significant overexpression in cerato-platanins, carboxylesterases and secondary metabolite related genes and are found to express several cell-wall proteins. Putative pathogenicity genes including expansins, carboxylesterases and salicylate hydroxylases are enriched in *Armillaria* spp.. Secondary metabolites in *Armillaria* are overrepresented and the Major Facilitator Superfamily 1 and cytochrome p450 families, both related to pathogenicity are expanded in *Armillaria* spp. (Sipos *et al.*, 2017).

Gene function studies in *Armillaria* spp. have been hampered by a lack of suitable genetic tools. In many pathogenic fungal species, gene function is studied using genetic manipulation tools such as gene silencing, overexpression and gene knockouts, none of which have been developed for use in *Armillaria* species. *Agrobacterium* mediated transformation has recently been developed in *Armillaria* (Baumgartner *et al.*, 2010b; Ford, 2015; Ford *et al.*, 2016) which will allow gene function to be studied through visualisation. The prospect of gene function studies could be enhanced still further when the CRISPR-Cas9 system is developed for *Armillaria*, work towards which is currently underway at the University of Bristol (Ford and Bourquin, 2018).

1.7 Aims:

- To determine whether *Trichoderma* species are antagonistic against *A. mellea* using *in vitro* assays.
- To assess the levels of biological control *Trichoderma* spp. offer to plants against *A. mellea* infection.
- To study the role of selected virulence genes in *A. mellea*.

| Chapter 2: Materials and Methods

This chapter details the experimental methods used and considerations made to achieve results described in chapters three - five. All media and solutions were prepared using deionised water (DW) in Pyrex Duran bottles and autoclaved prior to use. Unless otherwise stated all autoclaving cycles were carried out at 121 °C for 15 mins at 15 psi. Temperature sensitive solutions, such as antibiotics, were filter-sterilised through a 0.22 µm Millipore filter. All tools required for aseptic techniques were flame sterilized with 100 % ethanol in a Bunsen burner. Chemicals and media were of molecular biology standard and purchased from the following suppliers: Alfa Aesar, Formedium, Melford, Sigma Aldrich and Thermo Fisher Scientific. Restriction enzymes were purchased from New England Biolabs (NEB). Primers were obtained from Integrated DNA Technologies (IDT) and sequencing was carried out by Source Bioscience. Plasmid miniprep and yeast miniprep kits were purchased from Macherey-Nagel and Cambridge BioScience, respectively.

2.1 Media

Media were either prepared on the day of use or in advance and stored at room temperature. Any solid media were microwaved to melt entirely prior to use. Media were cooled to 50 °C before any antimicrobial amendments required were added at the specified concentration and 25 ml molten medium was aseptically poured into nine cm diameter Petri dishes under a laminar flow hood. Preparation of the various media is described in Table 2.1 with details of additional media for *Armillaria* inoculum, induction of *Armillaria* basidiocarps and *Agrobacterium*-mediated transformations given below.

Table 2.1: Media preparation for culturing fungi and bacteria.

All recipes made up to 1 L with DW and autoclaved.

| Media | Abbreviation | Recipe | Use |
|--|--------------|---|---|
| Malt extract broth | MEB | 15 g malt extract broth (Sigma) | General fungal liquid medium |
| Malt extract agar | MEA | 50 g malt extract agar powder (Formedium) | General fungal soil medium |
| MEA+SA | | MEA supplemented with 100 µg / ml each of streptomycin and ampicillin | Fungal medium to prevent bacterial contamination |
| Potato dextrose broth | PDB | 24 g potato dextrose broth (Formedium) | General fungal liquid medium |
| Potato dextrose agar | PDA | 41 g potato dextrose agar powder (Formedium) | General fungal soil medium |
| PDA+HT | | PDA supplemented with appropriate concentration of hygromycin B (30 µg / ml for <i>Armillaria</i>) and 200 µg / ml of timentin | Medium to select for AMT fungal transformants |
| J. J. Guillaumin Medium | JJG | MEA supplemented with ampicillin (100 µg / ml), streptomycin (100 µg / ml), polymyxin B (25 µg / ml) and 23 % thiabendazole after autoclaving | <i>Armillaria</i> selective medium described by Drakulic <i>et al.</i> , 2017 |
| Rose Bengal agar | MRB | 10 g malt extract, 1 g yeast extract, 0.2 g pentachloronitrobenzene, 3 ml (50 mg / ml) rose Bengal and 1 ml chloramphenicol (100 mg / ml) | <i>Trichoderma</i> selective medium described by Hill <i>et al.</i> , 2016 |
| Water agar | WA | 15 g granulated agar, bacteriological grade (Formedium) | A basal medium for veneer to visualise <i>A. mellea</i> transformants |
| Minimal medium | MM | 0.5 g KCl, 0.5 g MgSO ₄ ·7H ₂ O, 0.01 g FeSO ₄ ·7H ₂ O, 1 g K ₂ HPO ₄ , 2 g (NH ₄) ₂ SO ₄ and 16 g agar | For enzyme activity assays |
| Nutrient agar | NA | 5 g NaCl, 5 g peptone, 3 g micro-granulated yeast extract and 16 g agar | For enzyme activity assays |
| Benomyl and streptomycin MEA | BSMA | MEA supplemented with 40 µg / ml of benomyl and 100 µg / ml of streptomycin. | Adapted from Worrall, 1991 to test for <i>Armillaria</i> selective medium |
| Benomyl, dicloran and streptomycin MEA | BDS | BSMA supplemented with 2 mg / L of dicloran | Adapted from Worrall, 1991 to test for <i>Armillaria</i> selective medium |

| | | | |
|---------------------------------------|---------|--|--|
| Yeast synthetic drop out media (agar) | YSDM(A) | 1.7 g yeast nitrogen base, 5 g ammonium sulphate, 20 g glucose and 0.77 g yeast dropout mix (-ura3). For solid media, 20 g agar was added. | For homologous yeast recombination |
| Yeast extract peptone dextrose (agar) | YPD(A) | 10 g yeast extract, 20 g peptone, 20 g glucose and 50 mg adenine hemisulphate. For solid media, 20 g agar was added. | For homologous yeast recombination |
| Lennox broth | LB | 20 g LB-Broth Lennox' powder (Formedium) | General bacterial liquid medium |
| Lennox broth agar | LBA | 35 g 'LB-Agar Lennox' powder (Formedium) | General bacterial soil medium |
| Yeast extract nutrient broth | YENB | 7.5 g yeast extract and 8 g nutrient broth | For preparation of <i>E. coli</i> electrocompetent cells |
| Super Optimal Broth | SOB | 20 g bacto tryptone, 5 g yeast extract, 2 ml NaCl (5 M), 2.5 ml KCl (1 M), 10 ml MgCl ₂ (1M), 10 ml MgSO ₄ (1 M) | For preparation of <i>E. coli</i> chemically competent cells |

Carrot agar (CA)

Carrot agar was prepared by blending 200 g of washed carrots with 500 ml tap water. The macerated carrot was filtered through a muslin and squeezed to extract all juice. The juice was made up to 1 L with tap water and 15 g agar was added. The CA was autoclaved, left to set overnight and autoclaved again. CA was used to prepare hazel billets arranged vertically in 500 ml Nalgene wide mouth jars.

Rice, sawdust and tomato (RST)

RST was prepared in 1 L clear Nalgene wide mouth jars as described by Ford *et al.* (2015). In each pot, 30 g rice and 15 g sawdust were mixed together with enough water to wet mixture without excess water. One roughly blended tomato was poured on top of rice and sawdust mix which was then autoclaved. RST was used to induce fruiting of *A. mellea* under laboratory conditions.

Agrobacterium induction medium

For *Agrobacterium* induction medium, stock solutions 'A' and 'B', 70 mM CaCl₂.2H₂O, 9 mM FeSO₄.7H₂O, 1 M D-glucose, 1 M MES (adjusted to pH 5.3 with 5 M KOH) and 50% glycerol were prepared separately, autoclaved and stored at room temperature until required to make induction medium.

Stock solution A contained of 34.84 g K₂HPO₄, 27.22 g KH₂PO₄ and 2.93 g NaCl in 1 L DW. **Stock solution B** contained of 9.86 g MgSO₄.7H₂O and 10.58 g (NH₄)₂SO₄ in 1 L DW. Acetosyringone (3', 5'-dimethoxy-4'-hydroxyacetophenone) was made fresh on the day of use at a concentration of 200 mM, dissolved in DMSO.

Agrobacterium induction medium (AIM) contained 50 ml stock solution A, 50 ml stock solution B, 10 ml 70 mM CaCl₂.2H₂O, 1 ml 9 mM FeSO₄.7H₂O, 10 ml 1 M D-glucose, 40 ml 1 M MES and 10 ml 50% glycerol made up to 1 L DW and autoclaved. In addition, AIM medium contained 15 g agar. *Agrobacterium* induction medium was used in *Agrobacterium*-mediated transformations.

Transformation Buffer (TB)

0.1 M PIPES (1,4-Piperazinediethanesulfonic acid.), 0.015 M calcium chloride, 0.25 M potassium chloride adjusted to pH 6.7 with potassium hydroxide and 0.055 M manganese (II) chloride (tetrahydrate) added. The buffer was filter sterilised and cooled on ice prior to use.

2.1.1 Long-term storage in – 70 °C freezer

All cultures were snap frozen in liquid nitrogen with 30 % glycerol (autoclaved) and stored at -70 °C. Mycelial plugs (5 mm diameter) of *Armillaria* were taken from the actively growing margin of four week-old cultures and flooded in 30 % glycerol for long term storage. *Trichoderma* cultures were grown on PDA or MEA for 10 days under L : D conditions to induce sporulation. petri dishes were flooded with 5 % Tween 20 and gently scraped to release spores then gently mixed with an equal volume of 30 % glycerol (500 – 800 µl) in a 2 ml cryovial. Overnight bacterial cultures were mixed with an equal volume of 30 % glycerol (500 – 800 µl) in 2 ml cryovials.

Stock cultures were revived by partial thawing on ice in a Cat II biosafety cabinet. *Armillaria* mycelial plugs were removed from glycerol using a sterile needle and incubated on PDA or MEA. A loop of bacterial suspension or *Trichoderma* spore suspension was streaked onto appropriate media with antibiotics where required.

2.1.2 Antibiotic concentrations

All antibiotics and chemicals were filter-sterilised through a 0.22 µm Millipore filter. Details of preparation and stock and working concentrations for all antibiotics and chemicals can be found in Table 2.2.

Table 2.2: Concentrations and preparation of antibiotics and chemicals used in this study.

| Antibiotic/Chemical | Dissolved in | Stock Concentration | Working Concentration |
|----------------------------|---------------------|----------------------------|------------------------------|
| Acetosyringone | DMSO | 200 mg / ml | 200 µg / ml |
| Ampicillin | Water | 100 mg / ml | 100 µg / ml |
| Benomyl | Water | 40 mg / ml | 40 µg / ml |
| Carbenicillin | Water | 75 mg / ml | 75 µg / ml |
| Chloramphenicol | Ethanol | 100 mg / ml | 100 µg / ml |
| Hygromycin | Water | 500 mg / ml | 30 - 100 µg / ml |
| Kanamycin | Water | 50 mg / ml | 50 µg / ml |
| Polymyxin B | Water | 25 mg / ml | 25 µg / ml |
| Rifampicin | Methanol | 20 mg / ml | 20 µg / ml |
| Rose Bengal | Water | 50 mg / ml | 50 µg / ml |
| Streptomycin | Water | 100 mg / ml | 100 µg / ml |
| Thiabendazole | Lactic acid | - | 23 % |
| Timentin | Water | 200 mg / ml | 200 µg / ml |

2.2 Culture collections

2.2.1 Fungal cultures

2.2.1.1 *Armillaria* species

Armillaria cultures were obtained from the Royal Horticultural Society (RHS) Wisley, Surrey, UK and University of California, Davis (UCD), USA. Details of each *Armillaria* isolate can be found in Table 2.3. Laboratory experiments primarily used the *A. mellea* isolate, CG440 as it is a UK isolate and known to be a virulent strain (Beal *et al.*, 2015; Ford *et al.*, 2015, 2017). Isolates were maintained on MEA or PDA and grown at 25 °C in the dark for four weeks. A pure culture of *A. mellea* CG440 was recovered from inoculated

plants and was maintained for future experiments, as described by Percival *et al.* (2011). Subcultures were made by cutting a 5 mm diameter piece of agar from the actively growing edge of *Armillaria* colonies and transferring to a fresh Petri dish.

Table 2.3: Details of *Armillaria* spp. isolates.

| Isolate | <i>Armillaria</i> species | Host Plant | Year Isolated | Origin |
|---------|---------------------------|--|---------------|--|
| CG440 | <i>A. mellea</i> | <i>Ligustrum</i> sp. | 2006 | Guildford, UK - RHS |
| CG675 | <i>A. mellea</i> | <i>Salix</i> sp. | | Portsmouth, UK - RHS |
| NAPA187 | <i>A. mellea</i> | <i>Quercus kelloggii</i> | 2007 | Napa Valley, California, USA - UCD |
| ELDO17 | <i>A. mellea</i> | <i>Vitis</i> sp. | 2000 | El Dorado, California, USA - UCD |
| ELDO19 | <i>A. mellea</i> | <i>Vitis</i> sp. | 2000 | El Dorado, California, USA - UCD |
| DSM3731 | <i>A. mellea</i> | <i>Prunus persica</i> | Pre-1986 | Vaucluse - France |
| RGF1 | <i>A. mellea</i> | From grass surrounding deciduous trees | 2019 | Royal Fort Gardens, UK University of Bristol |
| CG510 | <i>A. gallica</i> | <i>Salix</i> sp. | | Portsmouth, UK - RHS |
| CG259 | <i>A. ostoyae</i> | <i>Larix</i> sp. | | Kensington, UK - RHS |
| CG425 | <i>A. ostoyae</i> | <i>Aesculus</i> sp. | | Carlisle, UK - RHS |
| CG522 | <i>A. ostoyae</i> | <i>Daphniphyllum</i> sp. | | RHS Wisley, UK - RHS |

2.2.1.2 *Trichoderma* species

2.2.1.2.1 *Trichoderma* isolation method

Trichoderma isolates were collected by Niamah Bashir, British Society for Plant Pathology (BSPP) funded summer student between April and August 2017 from RHS Garden Wisley. Forty *Trichoderma* isolates were obtained from root material of healthy herbaceous or woody plant species (Table 2.4) in close proximity to plants which has succumbed to ARR. Samples were taken from three sites: ‘Seven Acres’ and the ‘Entrance Terraces’ were chosen based on the presence of woody species close to sites with known *A. mellea* infection, and ‘Battleston Hill’ was included to increase the range of *Trichoderma* sampling sites. Feeder roots were collected from vigorous and healthy host plants in zip-lock bags and stored at 4 °C before

isolation. Soil debris were removed from roots by rinsing under cold water then cut into segments approximately 25 mm long and 2 – 5 mm diameter. Roots were immersed in 1% Virkon for 2 – 10 mins with regular inversion then rinsed in sterile water for two mins, drained and dried on sterile filter paper. Two samples were surface sterilized with 5 % bleach (T17/17 & T17/18). Five samples were not surface sterilized but soaked in sterile water for 5 minutes (see * in Table 2.4). Isolations were made onto MRB from root pieces 3 – 6 mm in length and stored at room temperature under natural light conditions. Cultures were observed daily for putative *Trichoderma* isolates which were sub-cultured onto PDA. The isolate of *T. harzianum* T22 was kindly donated by James Fortune, University of Hertfordshire.

2.2.1.2.2 *Trichoderma* spp. maintenance and isolate identification

A collection of 40 *Trichoderma* cultures was obtained from sampling at the RHS Garden Wisley. Isolates were maintained on MEA or PDA and grown at 20 °C in 8 : 16 hr L / D conditions ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 3 – 10 days unless stated otherwise. All Petri dishes were wrapped in Parafilm to avoid cross contamination via spores. Subcultures were made cutting a 6 mm diameter plug of the actively growing hyphal edge or taking a sterile loop and transferring spores of *Trichoderma* to a fresh Petri dish.

Trichoderma species identity was confirmed morphologically and identified to species level through sequencing. DNA was extracted using the rapid fungal DNA extraction protocol as described in section 2.3.2. The internal transcribed spacers (ITS) region 1 and ITS4 – ITS6 regions, the translation elongation factor 1 alpha (*tef1*), chitinase18-5 (*chi18-5*) and RNA polymerase II subunit (*rpb2*) genes were PCR-amplified and sequenced to identify *Trichoderma* isolates to species level. Details for which combinations of genes were used for sequence ID can be found in Table 2.4. All sequences which amplified the ITS1 region were cloned into pJET (Thermo Fisher Scientific) (section 2.4.6) and plasmids were subsequently screened and purified prior to sequencing.

Table 2.4: Details of *Trichoderma* spp. isolate collection.

Additional information regarding host plant, RHS Wisley location information and genes/regions used for *Trichoderma* spp. ID.

| Isolate | Host | BGBASE | Location | ITS primer sets and amplified regions |
|----------------------|--------------------------------|-------------|---------------------------------|--|
| T17/01 | <i>Viburnum carlesii</i> | W20012329-A | WE0313 | ITS 1 & 2 |
| T17/02 ⁺ | Soil/debris | | Outside Plant Health laboratory | ITS 1 & 2 |
| T17/03 ⁺ | Soil/debris | | Outside Plant Health laboratory | <i>chi18-5</i> ITS 4 & 6 <i>rpb2</i> <i>tefl</i> |
| T17/04 | <i>Viburnum carlesii</i> | W20012329-A | WE0313 | ITS 4 & 6 <i>rpb2</i> <i>tefl</i> |
| T17/05 | <i>Citrus trifoliata</i> | | | ITS 1 & 2 |
| T17/06 | <i>Quercus</i> sp. | | WE0201 | ITS 1 & 2 |
| T17/07 ⁺ | <i>Quercus</i> sp. | | WE0201 | ITS 1 & 2 |
| T17/08 ⁺ | <i>Quercus</i> sp. | | WE0201 | ITS 1 & 2 |
| T17/09 | <i>Sorbus aria</i> 'Lutescens' | W961285-A | WB0222 | ITS 1 & 2 |
| T17/10 ^{*+} | <i>Sorbus aria</i> 'Lutescens' | W961285-A | WB0222 | ITS 1 & 2 ITS 4 & 6 <i>tefl</i> |
| T17/11 ⁺ | <i>Quercus</i> sp. | | WE0201 | ITS 1 & 2 ITS 4 & 6 <i>tefl</i> |
| T17/12 [*] | <i>Quercus</i> sp. | | WE0201 | ITS 4 & 6 <i>rpb2</i> <i>tefl</i> |
| T17/13 | <i>Quercus</i> sp. | | WE0201 | ITS 1 & 2 |
| T17/14 [*] | <i>Sorbus aria</i> 'Lutescens' | W961285-A | WB0222 | ITS 1 & 2 ITS 4 & 6 <i>tefl</i> |
| T17/15 ⁺ | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tefl</i> |
| T17/16 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tefl</i> |
| T17/17 | <i>Betula pendula</i> | | WS0111 | <i>RPB2</i> <i>Tefl</i> |
| T17/18 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tefl</i> |
| T17/19 [*] | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> <i>rpb2</i> <i>tefl</i> |
| T17/20 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> <i>rpb2</i> <i>tefl</i> |

| | | | | |
|----------|--|-----------|--------|--|
| T17/21 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/22 | <i>Betula pendula</i> | | WS0111 | - |
| T17/23 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/24 | <i>Betula pendula</i> | | WS0111 | ITS 1 & 2 |
| T17/25 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/26 | <i>Betula pendula</i> | | WS0111 | <i>tef1</i> |
| T17/27 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> <i>rpb2</i> <i>tef1</i> |
| T17/28 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/29 | <i>Betula pendula</i> | | WS0111 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/30 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> <i>rpb2</i> <i>tef1</i> |
| T17/31 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | - |
| T17/32 | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' | W915086-A | WW0102 | <i>chi18-5</i> <i>rpb2</i> <i>tef1</i> |
| T17/33 * | <i>Rhododendron</i> 'moonstone' | W915105-B | WW0101 | <i>chi18-5</i> <i>rpb2</i> <i>tef1</i> |
| T17/34 | <i>Rhododendron</i> 'moonstone' | W915105-B | WW0101 | <i>chi18-5</i> <i>rpb2</i> <i>tef1</i> |
| T17/35 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/36 | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' | W915086-A | WW0102 | <i>rpb2</i> |
| T17/37 | <i>Viburnum bodnantense</i> | W933122-A | WS0318 | <i>chi18-5</i> ITS 1 & 2 <i>rpb2</i> <i>tef1</i> |
| T17/38 | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' | W915086-A | WW0102 | ITS 1 & 2 |
| T17/39 | <i>Conifer</i> sp. | | WL0504 | ITS 1 & 2 |
| T17/40 | <i>Conifer</i> sp. | | WL0504 | <i>rpb2</i> |
| T17/41 | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' | W915086-A | WW0102 | <i>rpb2</i> |
| T17/42 † | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' | W915086-A | WW0102 | <i>rpb2</i> |
| T22 | <i>Quercus</i> sp. | N/A | N/A | <i>rpb2</i> |

* Samples which were not surface sterilised for isolation. † Isolates selected for further assessment in strawberry and privet experiments.

2.2.1.3 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, strain Y10000, was used for plasmid construction by homologous yeast recombination. Cultures were stored at -70 °C and maintained on YPDA Petri dishes incubated for two days at 28 °C. Liquid cultures were grown in YSDM and incubated for two days at 28 °C with shaking at 200 rpm.

2.2.2 Bacterial cultures

2.2.2.1 *Escherichia coli*

The *E. coli* strains Top10 and DH5 α (Thermo Fisher Scientific) were used for cloning, heat shock and electroporation transformations. Cultures were stored at -70 °C and grown in LB or LBA, amended with appropriate antibiotics for selection, overnight at 37 °C with shaking (220 rpm) for liquid cultures.

2.2.2.2 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strains AGL-1 (Lazo *et al.*, 1991) and LBA1126 (Bundock and Hooykaas, 1996) were used for *Agrobacterium*-Mediated Transformation of *Armillaria mellea* and *Trichoderma* spp.. Cultures were stored at -70 °C and grown on LBA or LB with appropriate antibiotics for selection and incubated for two days at 28 °C with shaking (220 rpm) for liquid cultures.

2.3 Nucleic acid extraction

All mycelium was harvested in a Cat II biosafety cabinet. Rapid DNA extractions were carried out on a benchtop and phenol chloroform extractions were carried out under a flow hood.

2.3.1 Buffers and stocks for nucleic acid extraction

Lysis buffer

400 mM Tris-HCL (pH 8.0), 60 mM EDTA (pH 8), 1 % SDS and 150 mM NaCl made up to required volume with DW and autoclaved.

3 M Potassium acetate (pH 4.8)

60 ml 5 M potassium acetate and 11.5 ml glacial acetic acid made to 100 ml with DW, adjusted to pH 4.8 and autoclaved.

Tris-EDTA (TE) buffer

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA made up to required volume with DW and autoclaved.

Phenol extraction buffer

0.1 M Tris (pH 8.0), 0.1 M LiCl, 10 mM EDTA (pH 8.0), 1 % SDS made up to required volume with DW and autoclaved.

RNase A

10 mg / ml of pancreatic RNase A was dissolved in 10 mM sodium acetate (pH 5.2), heated to 100 °C for 15 mins and slowly cooled to room temperature. The pH was adjusted to 7.4 with 0.1 vol 1 M Tris-HCl. The RNase A was aliquoted into 1.5 ml Eppendorf tubes and stored at -20 °C. RNase A was used at a concentration of 1 mg/ml.

2.3.2 Rapid fungal DNA extraction

A rapid fungal DNA extraction based on that described by Liu *et al.* (2000) produced DNA sufficient for PCR analysis of *Armillaria* spp. and sequencing of *Trichoderma* spp. Mycelium from *Armillaria* spp. was harvested after one to four weeks. Mycelium from *Trichoderma* spp. was harvested from two-day old cultures to reduce risk of cross-contamination from spores. Aerial mycelia (from one colony) was scraped from Petri dishes using a flame sterilised mini-spatula and added to a 1.5 ml Eppendorf tube containing 500 µl lysis buffer. A sterilised (10 % bleach for 10 minutes) plastic pestle was used to disrupt the mycelium which was then incubated at room temperature for 10 mins. Next, 150 µl of potassium acetate (pH 4.8) was added, vortexed for 15 s and centrifuged at 13,000 g for 1 min. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and centrifuged as before. At this point, an optional step to incubate samples overnight with an equal volume of 4 M LiCl at 4 °C was included. Samples were centrifuged at 13,000 g for 1 min and the supernatant was transferred to a fresh 1.5 ml

Eppendorf tube. A 0.1 vol. of potassium acetate and 0.7 vol. of isopropanol was added and centrifuged again as before. The supernatant was discarded and the pellet was washed with 300 μ l of cold 80 % ethanol and centrifuged again. The supernatant was discarded, the pellet air dried and then DNA was resuspended in 30 μ l TE buffer and stored at -20 °C.

2.3.3 Phenol chloroform DNA extraction

A phenol chloroform extraction protocol, described for RNA by Verwoerd *et al.* (1989) was modified for high-yielding DNA extraction. Fungal tissue (~ 1 g) was harvested in a Cat II biosafety hood and either used directly for extraction or snap frozen and stored at -70 °C. Using 4 ml phenol extraction buffer : phenol (water saturated, pH 8.0) (1 : 1 v/v) amended with 200 μ l RNase A, fungal tissue was ground with a pre-cooled, autoclaved mortar and pestle, transferred into a 15 ml Falcon tube and vortexed. Two ml of chloroform : isoamyl alcohol (IAA) (25 : 1 v/v) was added, vortexed and centrifuged at 10,000 g for 10 mins. The upper aqueous phase was transferred to a fresh Falcon tube, an equal volume of LiCl was added and the sample was incubated overnight at 4 °C. The DNA was propanol precipitated with 0.1 vol 3 M potassium acetate (KAc) and 0.7 vol isopropanol, gently mixed and centrifuged at 10,000 g for 10 mins and the supernatant was discarded. The DNA pellet was washed with cold 80 % ethanol, centrifuged at 12,000 g for 5 mins and the supernatant discarded. The pellet was air dried and the DNA resuspended in 100 μ l TE buffer and transferred into a fresh 1.5 ml Eppendorf tube. DNA was stored at -20 °C.

2.3.4 Phenol chloroform RNA extraction

RNA was extracted for RT-PCR using a phenol chloroform extraction based on that by Verwoerd *et al.* (1989). *Armillaria* tissue was harvested in a Cat II Biosafety hood, snap frozen in liquid nitrogen and stored at -70 °C until required. Fungal tissue (~2 g) was ground in a pre-cooled, autoclaved mortar with 8 ml phenol extraction buffer : phenol (1 : 1 v/v), transferred to a 15 ml Falcon tube and vortexed for 30 s. Four ml of chloroform : IAA (25 : 1 v/v) was added and vortexed again for 30 s then centrifuged at 10,000 g for 10 mins. The upper aqueous phase was transferred to a clean Falcon tube and an equal volume of 4 M LiCl was added. Samples were incubated at 4 °C overnight and the RNA was pelleted

at 12,000 g for 10 mins. The RNA pellet was washed in 80 % ethanol and centrifuged at 12,000 for 5 mins. The supernatant was discarded, the resulting pellet was dried and resuspended in 250 μ l TE buffer. The RNA was precipitated with 0.1 vol 3M KAc and 2.5 volumes of ethanol for 15 mins on ice. The precipitated RNA was centrifuged for 10 mins at 12,000 g and the supernatant removed. The resulting pellet was washed twice in 1 ml cold 80 % ethanol and centrifuged for 5 min at 12,000 g. The supernatant was removed, and the pellet was dried in a SpeedVac (Eppendorf Ltd) for 5 mins. The RNA was resuspended in 250 μ l TE buffer and transferred into a fresh 1.5 ml Eppendorf tube. RNA was stored at - 70 °C.

2.4 Nucleic acid manipulation

2.4.1 Gel electrophoresis

Nucleic acids were visualised by gel electrophoresis. Agarose gels were prepared with 1 x TAE buffer (Tris-acetate-EDTA) containing Midori Green (Nippon Genetics Europe) stain at a concentration of 1 % for gDNA and PCR products or 2 % for cDNA and small DNA fragments. The appropriate amount of agarose was melted in 1 x TAE buffer, cooled and poured into a gel tray with a well comb. Once agarose was set, the comb was removed, and the gel was placed into a gel tank containing sufficient 1 x TAE buffer with Midori Green stain to submerge the gel. The agarose gel was run at 90 – 120 v for 30 – 45 mins (Bio-Rad PowerPac Basic). Nucleic acids were visualised with a transilluminator (Bio-Rad Gel Doc TM EZ Imager) and size was measured using a 1 kb plus (NEB) or 100 bp (Promega) reference ladder (Figure 2.1).

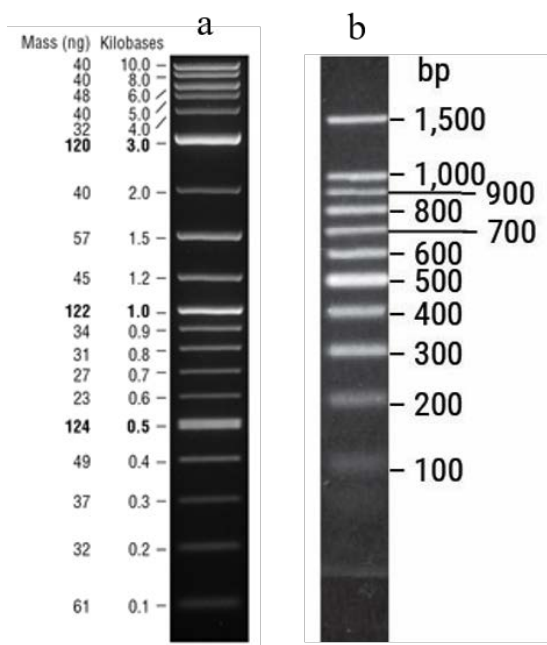


Figure 2.1: DNA Reference ladders to measure nucleic acid bands on agarose gels.

a) 1 kb plus DNA ladder (NEB) on 1 % agarose gel; b) 100 bp DNA ladder (Promega) on a 2 % gel.

2.4.2 Primers

All primers were supplied by Integrated DNA Technologies (IDT) at 100 mM and diluted to 10 mM in MGW (molecular grade water) for use in PCR. Details of taxonomy primer sequences can be found in Table 2.5. Primers were designed using serial cloner v 2.6.1 and the online software, Primer3 (<http://primer3.ut.ee/>). Annealing temperature was calculated using an online T_m calculator (<https://tmcaculator.neb.com/#!/main>). Details of primers designed in this thesis can be found in chapter five and the relevant appendices.

Table 2.5: Details of taxonomy primers used in this study

Additional information including primer sequence, annealing temperature and reference for ITS (internal transcribe spacer), *tef1* (Elongation factor 1 alpha), *rpb2* (RNA polymerase II subunit) and *chi18-5* (chitinase 18-5) primers.

| Amplification region | Primer name | Primer Sequence | Annealing temp. (°C) | Reference |
|----------------------|--------------------------|--------------------------------|----------------------|--|
| ITS | ITS1 | TCCGTAGGTGAACCTGCGG | 65 | White <i>et al.</i> (1990) |
| ITS | ITS2 | GCTGCGTTCTTCATCGATGC | 62 | White <i>et al.</i> (1990) |
| ITS | ITS4 | TCC TCC GCT TAT TGA TAT GC | 58 | White <i>et al.</i> (1990) |
| ITS | ITS6 | GAAGGTGAAGTCGTAACAAGG | 63 | Cooke and Duncan (1997) |
| <i>tef1</i> | <i>tef1</i> α - EF1-983F | GCY CCY GGH CAY GGT GAY TTY AT | 55 | Chaverri and Samuels, (2003) |
| <i>tef1</i> | Tef 1α - EF1-2218R | ATA CRT GRG CRA CRG TYT G | 55 | Chaverri and Samuels, (2003) |
| <i>rpb2</i> | <i>RPB2</i> -5F | GAY GAY RGW GAT CAY TTY GG | 50 | Chaverri and Samuels, (2003) |
| <i>rpb2</i> | <i>RPB2</i> -7cR | CCC ATR GCT TGY TTR CCC AT | 50 | Chaverri and Samuels, (2003) |
| <i>chi18-5</i> | chit42-1af | AGCWAGCACSGATGCCAAC | 62 | Kullnig-Gradinger <i>et al.</i> (2002) |
| <i>chi18-5</i> | chit42-2ar | AGGTTCTGAGTYGWTCCA | 62 | Kullnig-Gradinger <i>et al.</i> (2002) |

2.4.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify fragments of DNA for sequencing, confirmation of putative transformants and amplification of DNA fragments for yeast recombination. DreamTaq DNA polymerase was used for colony PCR and standard fungal PCR. Phusion DNA polymerase was used for sequencing or cloning and KAPA was used for amplification of fragments for yeast recombination. A 2x master mix for DreamTaq DNA polymerase was prepared with 1000 µl 10x buffer, 200 µl 10 mM dNTPs, 3750 µl MGW and 50 µl DreamTaq and aliquoted into 100 µl. The Phusion DNA polymerase 2x master mix was prepared using 2000 µl 5x Phusion green buffer, 200 µl 10 mM dNTPs, 2750 µl water and 50 µl Phusion and aliquoted into 100 µl. A 20 µl PCR reaction was prepared with 10 µl 2x master mix, 7 µl MGW, 1 µl of forward and reverse primer (10 mM) and 1 µl template

DNA (10 - 100 ng). KAPA PCR reactions were set up in 25 μ l volumes with 1.5 μ l 10x KAPA Taq buffer, 0.5 μ l 10 mM dNTPs, 1 μ l of forwards and reverse primers (10 mM), 0.1 μ l KAPA Taq DNA Polymerase (5 U / μ l), 20 μ l MGW and 1 μ l of template DNA.

For amplification of DNA using ITS primers with DreamTaq, thermocycler conditions were as follows: an initial denaturing at 95 °C for 5 mins, followed by 32 cycles of 95 °C for 30 s, 55 °C for 30 s and 70 °C for 60 s and a final extension of 72 °C for 10 mins. Reactions using Phusion were amplified under the following thermocycler conditions: initial denaturing at 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s / kb and a final extension of 10 mins at 72 °C. For amplification of DNA with KAPA thermocycler conditions were as follows: initial denaturing at 95 °C for 3 mins followed by 35 cycles of 98 °C for 20 s, 60 °C for 15 s, 72 °C for 1 min / kb and a final extension at 72 °C for 1 min.

Colony PCR was used to confirm successful *E. coli* transformants. A single colony was scraped from the transformation Petri dish and directly mixed with DreamTaq PCR reaction mix made up to 20 μ l with SDW. Thermocycler conditions for colony PCR with DreamTaq were as follows: 95 °C for 3 mins, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 mins and a final extension of 72 °C for 5 mins.

All PCR reactions were performed on Eppendorf (Master Cycler Nexus gradient) or PCR max (alpha thermocycler) thermocycler machines. All PCR products were visualised by gel electrophoresis.

2.4.4 Candidate virulence gene selection

To understand how *Armillaria* spp. attack their hosts, genes from model fungal plant pathogens with homologues in *Armillaria* spp. were considered. Sipos *et al.* (2017) identified putative pathogenicity-related genes, SM and PCWDE in four *Armillaria* species: *A. ostoyae*, *A. cepistipes*, *A. gallica* and *A. solidipes*. Similar analysis of putative genes was performed on the available *A. mellea* DSM 3731 genome sequence in this study since the genome sequence for *A. mellea* ELDO17 was not published until June 2019 (JGI, 2019). This work used gene or protein sequences for candidate virulence genes identified from model fungal plant pathogens in the NCBI database and appropriate publications.

Selection predominantly focused on specific genes involved in pathogenicity as opposed to complex gene families such as SM and PCWDE. The BLAST function on the JGI *A. mellea* DSM 3731 portal (JGI, 2013) was used to search for homologs to virulence gene or protein sequences from model organisms.

2.4.5 Cloning

Amplified PCR products were cloned into pJet using the 'CloneJET PCR Cloning Kit' (Thermo Fisher Scientific) following manufacturer's instructions. Controls used pUC19 to confirm successful transformations.

Blunt-end cloning was performed using PCR products amplified by Phusion. The ligation reaction was set up on ice with 10 µl 2 x reaction buffer, 1 µl PCR product (non-purified), 1 µl pJET 1.2 / blunt cloning vector (50 ng / µl), 1 µl T4 DNA ligase (5 U/µL) and made up to 20 µl with MGW. The ligation mixture was incubated at room temperature for 5 mins and either used directly for transformation or stored at -20 °C until required.

2.4.6 Plasmid restriction digest

Restriction digests were performed to confirm plasmid construction by homologous recombination in yeast and extraction of plasmids from *E. coli*. FastDigest® restriction enzymes (Thermo Fisher Scientific) were used with FastDigest® Green buffer following the manufacturer's instructions: 2 µl FastDigest® Green buffer, 0.5 µl FastDigest® enzyme and 2 µl (up to 1 µg) DNA were made up to 20 µl with MGW, mixed gently and incubated at 37 °C for up to 1 hour. Analysis of banding patterns was performed by gel electrophoresis on a 1 % agarose gel.

2.4.7 Sequencing

Sequencing of DNA was carried out by Source Bioscience, Nottingham, England. Samples were prepared according to the Source Bioscience sample preparation guidelines. Sequence analysis was carried out using Sequencher (v 5.0). Pairwise alignments of *Trichoderma* spp. were made with BioEdit (v 7.2.6.1). Phylogenetic trees were produced using MEGA (v 7.0.26). All *Trichoderma* spp. trees were

built using maximum likelihood phylogenetic tree with 1000 bootstrap replications and the Jukes-Cantor model was applied. DNA sequence identity was confirmed using NCBI BLAST.

2.4.8 Reverse-transcription PCR

To determine the expression of candidate virulence genes in *A. mellea* reverse-transcription PCR (RT-PCR) was performed on cDNA. Firstly, residual gDNA was removed with DNase I (Thermo Fisher Scientific) following manufacturer's instructions: 5 µg of RNA, 1 µl of 10 x reaction buffer and 1 µl of DNase I (1 U) was made up to 10 µl with diethylpyrocarbonate (DEPC) water and incubated at 37 °C for 30 min. The reaction was terminated with 1 µl 50 mM EDTA at 65 °C for 10 min. The quantity of RNA was measured using a nanophotometer (Implen GeneFlow) and the quality was visually assessed by gel electrophoresis.

First-strand cDNA synthesis (Thermo Fisher Scientific) was performed on RNA following manufacturer's instructions. Control reactions for cDNA synthesis included a no template control (NTC) and a positive control for RNA based on the human GAPDH control supplied with the kit. For every cDNA synthesis reaction a 'reverse transcriptase minus' (RT-) control was included to assess for gDNA contamination. All reactions were prepared on ice as follows: 5 µg RNA and oligo (dT) primer (1µl) were made up to 12 µl with DEPC water and incubated at 65 °C for 5 mins, centrifuged and chilled on ice. The subsequent components were added in the following order:

- 4 µl 5 x reaction buffer
- 1 µl RiboLock RNase inhibitor (20 U / µl)
- 2 µl 10 mM dNTP mix
- 1 µl Revert aid M-MuL V RT (200 U / µl)

Reactions were made up to 20 µl with DEPC water, gently mixed and briefly centrifuged before incubation at 42 °C for 60 mins. The reaction was terminated at 70 °C for 5 mins. RT-PCR was performed with 2 µl cDNA (1 : 5 dilution) under the following conditions: an initial denaturation at 95 °C for 5 mins followed by 40 cycles of 95 °C for 30 s, 49 °C for 30 s 70 °C for 30 s and a final extension at 70 °C for 1 min.

2.4.9 Quantitative PCR

Quantitative PCR (qPCR) was performed in chapter five to determine expression levels of candidate virulence genes in *A. mellea*. Design and optimisation of qPCR are described in chapter five.

qPCR was performed with iTaqTM Universal SYBRTM Green Supermix (Bio-Rad) in 10 µl volumes for each reaction. The qPCR master mix was prepared with 5 µl 2 x iTaqTM Universal SYBRTM Green master mix, 0.5 µl each of forwards and reverse primers made up to 9 µl with DEPC water. The master mix was mixed thoroughly, and aliquoted into wells of an optical plate and 1 µl cDNA template was added to the master mix in triplicate. Plates were sealed with an optical adhesive cover and briefly centrifuged to eliminate air bubbles. qPCR was performed using a Bio-Rad CFX Real-Time System under the following conditions: an initial denaturation at 95 °C for 2 mins followed by 40 cycles of 95 °C for 5 s, 59 °C for 30 s. Melt Curve analysis was performed at 65 °C to 95 °C in 0.5 °C increments for 5 s.

2.5 Transformations

2.5.1 *E. coli* transformation

2.5.1.1 Preparation of electrocompetent *E. coli* cells

A single colony from a freshly streaked *E. coli* Top10 culture, grown on LBA with no antibiotics, was inoculated into YENB and incubated overnight at 37 °C with shaking (220 rpm). A portion of the starter culture (500 µl) was added into 50 ml fresh YENB in a sterile conical flask and incubated as before for a further 3 – 3.5 h to reach an OD⁶⁰⁰ of 0.5 – 0.6. The culture was transferred into a falcon tube and centrifuged at 4000 g for 20 min at 4 °C after which the remaining steps were carried out on ice. The supernatant was discarded and the pellet was resuspended in 10 ml ice cold SDW before centrifugation at 4000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 20 ml ice cold SDW and centrifugation was repeated. The supernatant was again discarded and the pellet resuspended in 20 ml ice cold glycerol (10 %) and centrifuged as before. The supernatant was again discarded and the pellet resuspended in 1 ml of ice cold glycerol (10 %). Electrocompetent cells were

aliquoted into 1.5 ml Eppendorf tubes in 50 μ l volumes and snap frozen in liquid nitrogen then stored at -70 °C.

2.5.1.2 Electroporation of *E. coli*

Electroporation cuvettes were sterilised with 10 % bleach for 10 minutes after each use and stored under 70 % ethanol. Prior to use, cuvettes were rinsed with SDW and dried in a laminar flow cabinet. Electrocompetent Top 10 *E. coli* cells were used for transformations. On ice, 50 μ l of electrocompetent cells were mixed with 2 μ l DNA (low salt concentration) and transferred to a sterile 2 mm electroporation cuvette which was placed in an electroporation machine (Bio-Rad GenePulser) and samples were electroporated at 2.5 kV and pulsed for 5 ms. The cuvette was promptly returned to ice and 1 ml of LB was added to stabilise cells. The LB was transferred to a 1.5 ml Eppendorf tube and incubated at 37 °C with shaking (220 rpm) for 1 hr (30 mins for ampicillin selection). The transformation mix was plated onto LBA with appropriate antibiotics and incubated at 37 °C overnight. Transformants were confirmed with colony PCR and plasmid restriction digestion.

2.5.1.3 Preparation of chemically competent *E. coli* cells

Chemical transformations of *E. coli* were based on methods by Hanahan (1983) and Inoue *et al.* (1990). A single colony of freshly streaked DH5 α was inoculated in 10 ml LB and incubated overnight with shaking (220 rpm) at 37 °C. In a 1 L flask 2.5 ml of starter culture was inoculated into 250 ml SOB and incubated with shaking (225 rpm) at 19 °C for 24 – 36 h to reach an OD⁶⁰⁰ of 0.5 – 0.6. The flask was incubated on ice for 10 mins and cells were centrifuged at 2,500 g for 10 mins at 4 °C. Pelleted cells were resuspended in 80 ml cold TB and incubated on ice for a further 10 mins. Cells were centrifuged again and resuspended in 20 ml cold TB with 1.4 ml DMSO. Competent cells were aliquoted in 50 μ l, snap frozen in liquid nitrogen and stored at -70 °C until required.

2.5.1.4 Heat shock transformation of *E. coli*

Chemically competent cells were thawed slowly on ice. One μ l DNA (or 5 μ l of ligation mix) was mixed with 50 μ l competent cells and incubated on ice for 30 mins. Cells were heat shocked in a water bath at 42 °C for 45 s and quickly returned to ice for 2 mins after which cells were stabilised with 1 ml

LB. Cells were incubated for 1 hr (30 mins for ampicillin resistance) at 37 °C with shaking (220 rpm). Transformation mix (100 µl) was plated onto LBA with appropriate antibiotics and incubated overnight at 37 °C. Transformants were confirmed by colony PCR and plasmid restriction digestion.

2.5.2 *Agrobacterium* transformation

2.5.2.1 Preparation of electrocompetent *A. tumefaciens*

Electrocompetent *A. tumefaciens* cells were prepared with strains AGL-1 and LBA1126. AGL-1 is resistant to rifampicin (20 mg / ml) and carbenicillin (75 mg / ml), LBA1126 is also resistant to streptomycin (50 mg / ml). Single colonies of *A. tumefaciens* were inoculated into 5 ml LB containing appropriate antibiotics and shaken at 220 rpm for 48 h at 28 °C. A sterile 250 ml conical flask was inoculated with 500 µl of starter culture in 50 ml LB containing appropriate antibiotics and incubated overnight at 28 °C with shaking at 220 rpm until the OD⁶⁰⁰ was > 0.5. The culture was transferred into a 50 ml Falcon tube and centrifuged at 4,000 g for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 50 ml of ice cold glycerol (10 %) and centrifuged again. This step was repeated twice with 25 ml and 2 ml of ice cold glycerol (10 %). The final cell pellet was resuspended in 1 ml ice cold glycerol (10 %). Electrocompetent cells were aliquoted into 100 µl volumes, snap frozen and stored at - 70 °C.

2.5.2.2 Electroporation of *A. tumefaciens*

Electroporation cuvettes were sterilised with 10 % bleach for 10 minutes after each use and stored under 70 % ethanol. Prior to use cuvettes were rinsed with SDW and dried in a laminar flow cabinet. On ice, 100 µl of electrocompetent *A. tumefaciens* cells were mixed with 2 µl DNA (low salt concentration) and transferred to a sterile 2 mm electroporation cuvette which was placed in an electroporation machine (Bio-Rad) and samples were electroporated at 2.5 kV for 9 ms (Bio-Rad GenePulser). The cuvette was promptly returned to ice and 900 µl of LB was added to stabilise cells. The cells were transferred to a 1.5 ml Eppendorf tube and incubated at 37 °C with shaking (220 rpm) for 3 h. The transformation mix was plated onto LB Petri dishes with appropriate antibiotics and incubated at 37 °C for 48 h. Transformants were confirmed with colony PCR and plasmid restriction digestion.

2.5.3 Homologous recombination in yeast

Homologous recombination was used to build plasmids in *Saccharomyces cerevisiae* for *Agrobacterium-mediated* transformation. Plasmid and primer design are detailed in Chapter five.

Homologous recombination in yeast was performed as described by Gietz and Woods (2002). A single colony of *S. cerevisiae* Y1000 was inoculated in 10 ml YPD and incubated overnight at 28 °C with shaking (200 rpm). The starter culture was mixed into 40 ml YPD in a sterile conical flask and incubated at 28 °C with shaking for 5 h. The culture was transferred to a 50 ml Falcon tube and centrifuged at 4,000 g for 5 min. The supernatant was discarded and the pellet was resuspended in 25 ml SDW. The culture was centrifuged as before and the supernatant was discarded. The pellet was resuspended in 1 ml 0.1 M LiOAc (filter sterilised) and transferred into a 1.5 ml Eppendorf tube. The cells were centrifuged at 13,000 g for 15 s and the supernatant removed. The pellet was resuspended in 400 µl 0.1 M LiOAc and 50 µl was aliquoted per transformation. Each aliquot was centrifuged for 15 s to pellet cells and remove supernatant. The subsequent solutions were added in the following order:

- 240 µl 50 % PEG 3350 (filter sterilised)
- 36 µl 1 M LiOAc (filter sterilised)
- 50 µl salmon sperm single-stranded DNA (SS-ssDNA) (2 mg / ml dissolved in SDW for 4 h at 25 °C with shaking at 100 rpm and stored at -20 °C. Prior to use SS-ssDNA was thawed and denatured at 100 °C for 5 mins and chilled on ice)
- 5 µl DNA fragments, including plasmid BB, made up to 35 µl with SDW

Transformation reactions were vortexed for 1 min to resuspend cells and incubated for 30 mins at 30 °C followed by a further 30 min incubation at 42 °C. The cells were pelleted at 6,000 g for 15 s. The supernatant was removed and the pellet resuspended in 500 µl of SDW by gentle pipetting. The transformation mixture (100 µl) was spread onto four or five SMA Petri dishes and incubated upside down at 28 °C for three days. For each transformation a linearised plasmid (without DNA fragments), circular plasmid and water control was included.

Plasmids were recovered from yeast transformation Petri dishes using the Zymoprep™ II Yeast plasmid mini-prep kit following the manufacturer's instructions. Recovered plasmids were transformed into *E. coli* Top10 electrocompetent cells and putative transformants were confirmed by colony PCR. Plasmids were extracted from successful transformants using the Macherey-Nagel NucleoSpin Plasmid, Mini kit for plasmid DNA following the manufacturer's instructions and stored at -20 °C until required.

2.5.4 *Agrobacterium*-mediated transformation (AMT)

2.5.4.1 *Armillaria* fruiting production

Armillaria mellea was fruited *in vitro* using the protocol developed by Ford *et al.* (2015). Jars (1 L wide mouth jars, VWR) were prepared with a minimum of four replicates using *A. mellea* ELDO17. RST medium was prepared in clear Nalgene jars. Four to five 10 mm diameter *A. mellea* plugs were inoculated onto the autoclaved jars and incubated in the dark for 4 - 6 weeks, or until rhizomorphs had fully colonised the medium. Jars were then moved into a growth cabinet at 23 °C with a 16 : 8 h L / D cycle at 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 60 % humidity. After six weeks the temperature was reduced to 15 °C with 10 : 14 h L/D cycle with light levels reduced to 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and humidity raised to 70 %. Primordia production occurred within three weeks after which fruiting bodies developed. Once mature, the cap was removed with a sterile scalpel, placed onto sterile black paper and left for two days to collect spores. Spores were harvested under a Cat II biosafety cabinet with a sterile loop and suspended in SDW. The number of spores was adjusted to 10^8 spores / ml with a haemocytometer, aliquoted into 2 ml Eppendorf tubes and stored at 4 °C.

Wild-harvested spores were collected from Royal Fort Gardens, University of Bristol. *Armillaria mellea* species identification was confirmed morphologically and with species specific primers described by Mulholland *et al.* (2012). Mature fruiting bodies of *A. mellea* were collected and the spores harvested as described above.

2.5.4.2 *Agrobacterium*-mediated transformation (AMT) of *Armillaria*

Agrobacterium-mediated transformation (AMT) of *Armillaria* was based on the protocol described by Baumgartner *et al.* (2010). Freshly propagated *Agrobacterium tumefaciens* containing the required plasmid was grown on LBA supplemented with 20 µg / ml rifampicin, 75 µg / ml carbenicillin and 50 µg / ml kanamycin for 2 – 4 days. A starter culture was inoculated into 5 ml LB with kanamycin (50 mg / ml) and incubated for 48 h at 28 °C with shaking (220 rpm). In a sterile 250 ml conical flask, 50 µl of starter culture was inoculated into 50 ml LB with kanamycin (50 mg / ml) and incubated at 28 °C with shaking (220 rpm). After 24 h the OD⁶⁰⁰ was measured to calculate the volume of culture required to reach a final OD⁶⁰⁰ of 0.15 in 20 ml using the formula:

$$\frac{0.15 \times 20}{\text{OD}^{600}} = \text{required volume}$$

The required volume of culture was centrifuged for 10 mins at 4,000 g and the supernatant discarded. The pellet was resuspended in 20 ml AIM supplemented with acetosyringone (at a final concentration of 200 µM) and incubated for 4 – 5 h until the OD⁶⁰⁰ reached 0.2 – 0.3. An equal volume of induced *Agrobacterium tumefaciens* was mixed with *Armillaria mellea* basidiospores (10⁸ basidiospores / ml) and 100 µl was spread onto AIM agar (supplemented with acetosyringone at a final concentration of 200 µM), kanamycin (50 µg / ml) and rifampicin (20 µg / ml)) with a sterile cellophane overlay. Co-cultivation Petri dishes were prepared in triplicate and incubated upright at 25 °C for 3 – 5 days.

After initial co-cultivation cellophane disks were moved onto freshly prepared PDA containing hygromycin B (30 mg / ml) and timentin (200 mg / ml) (PDA+HT). For wild *Armillaria mellea* spores thiabendazole (final concentration 0.023 %) was added to prevent growth of environmental contaminants. Transformation Petri dishes were further incubated at 25 °C until putative transformant colonies appeared after 10 – 21 days. Putative transformant colonies were subcultured onto PDA+HT and confirmed by PCR of the *hph* cassette.

2.5.4.3 *Agrobacterium*-mediated transformation of *Trichoderma*

Red and green fluorescing strains of *Trichoderma* were created by AMT using *Agrobacterium tumefaciens* strains LBA1126 and AGL-1. The dsRED plasmid pCAMdsRED was kindly donated by Dr. M. Urban (Rothamstead Research). The dsRED plasmid contained a Hygromycin B resistance gene controlled by *Aspergillus nidulans* trpC promoter and a dsRED gene under control of *A. nidulans* gpdA promoter with a trpC terminator. dsRED plasmids were grown on LBA amended with 50 µg / ml kanamycin. The GFP plasmid which was kindly donated by Dr. S. Sarrocco (Università di Pisa) contains a hygromycin B resistance gene and a synthetic GFP gene, both of which are constitutively controlled by the *A. nidulans* gpd promoter (Moar *et al.*, 1998). GFP plasmids were grown on LBA amended with 100 µg / ml ampicillin.

The AMT of *Trichoderma* followed the same protocol developed for *Armillaria* with some modifications. *Agrobacterium* cultures in induction medium grew to a final OD⁶⁰⁰ of 0.5 - 0.6, as opposed to 0.2 – 0.3. In the transformation 10⁶ *Trichoderma* conidia / ml were used rather than 10⁸ basidiospores / ml required for *Armillaria*.

2.6 Microscopy

Microscopy analysis of fungal morphology and to determine spore density of *Trichoderma* spp. and *A. mellea* was performed using a Leica DM LB microscope. Analysis of fluorescence by *A. mellea* and *Trichoderma* spp. was carried out using mycelium cultured on agar and transferred onto a microscope slide, and in the case of *A. mellea* grown on walnut veneer, this was also visualised at low power *in situ* on agar Petri dishes. An epifluorescent filter was used for visualisation of fluorescence where the excitation filter for GFP was 450 – 490 nm with an emission filter of 515 nm and the dichroic filter was 510 nm. The excitation filter for mRFP was 545 nm with an emission filter of 610 nm. All images were captured with a Samsung mobile phone.

2.7 Statistical analysis

All statistical analysis was carried out in RStudio (V 1.2.5042) (RStudio Team, 2020) using the packages ‘tidyverse’ (Wickham *et al.*, 2019) and ‘multcomp’ (Hothorn *et al.*, 2008) for linear models and ‘emmeans’ (Lenth, 2020) for repeated measures ANOVA. Graphs were created using the packages ‘ggplot2’ (Wickham, 2009), ‘ggrepel’ (Slowikowski, 2020) and ‘extrafonts’ (Chang, 2014).

2.8 *In vitro* assays

2.8.1 Selective medium for *A. mellea*

To develop a selective medium for *Armillaria*, where *Trichoderma* growth was reduced or eliminated, different antifungal and chemical supplements were assessed. Four media were tested including the *Armillaria* selective medium, JJG (Table 2.1) and the *Trichoderma* selective medium, MRB (Table 2.1). Two hymenomycete selective media, BDS and BSMA (Table 2.1), were described by Worrall (1991). Three isolates of *A. mellea* (AM240518, CG440 and CG675) and *Trichoderma* spp. (*T. hamatum* T17/10, *T. atrobrunneum* T17/11 and T17/15) were tested. Fungal plugs (6 mm diameter) from the actively growing margin of cultures were placed in the centre of individual Petri-dishes and colony diameter was measured after two days for *Trichoderma* and ten days for *Armillaria*. The effect of various test media was determined using a one-way ANOVA to compare fungal growth between selective media and MEA on which all media were based. The null hypothesis assumes there is no difference in colony diameter when fungi are grown on MEA and test media.

2.8.2 Agar-based dual culture assays

To determine the interaction between *Trichoderma* spp. and *A. mellea* *in vitro*, challenge assays were set up with both fungi on MEA. *Armillaria mellea* GC440 plugs (6 mm diameter) were incubated for one week prior to the addition of *Trichoderma* spp. plugs (6 mm diameter) 50 mm apart and grown at 20 °C in 8:16 hr L : D cycle for six weeks. Control treatments were only inoculated with *A. mellea*. Colony diameter and morphology was recorded every two days for the first 10 days and then weekly

for the next four weeks. This assay was conducted with all 40 *Trichoderma* isolates and all pairings were performed in triplicate. The effect of *Trichoderma* isolates on the inhibition of radial growth of *Armillaria* colonies was assessed using a linear model carried out at eight days and six weeks post-inoculation. The assumed null hypothesis was that there would be no significant difference of *Armillaria* growth in the presence of *Trichoderma* compared to *Armillaria*-only controls.

To assess the viability of *A. mellea*, the assay was repeated with a selection of eight *Trichoderma* isolates (Table 2.4⁺) and two *A. mellea* isolates (CG440 and CG675). After six weeks isolations were made from *A. mellea* colonies onto JYG to determine viability.

2.8.3 Dual culture assays in woody stems

Disks of hazel (*Corylus avellana*) (~15 mm diameter, 10 mm thick) were prepared by cutting to size and washing before being placed in a beaker of water and autoclaved twice at 121 °C for 45 mins, replenishing the water each time. Hazel disks were laid flat in Tupperware boxes and a final dry autoclave cycle was carried out at 121 °C for 45 mins. Once dry, hazel discs were covered with MEA, and to ensure there was no contamination, incubated for one week. *Armillaria mellea* isolates CG440 or CG675 were inoculated onto the MEA-covered hazel disks and incubated at 20 °C in the dark for one month until the wood was colonised. The colonised discs were recovered from the culture medium, residual agar was removed and the discs were placed onto MEA Petri dishes as in the agar-based dual culture assay. A selection of eight *Trichoderma* isolates (Table 2.4⁺) were inoculated one week after *Armillaria* and isolations were taken from internal *Armillaria* colonised wood at various locations to assess viability of the *Armillaria* after six weeks.

2.8.4 Rhizomorph assay

The effect of *Trichoderma* spp. on *A. mellea* growth in woody substrates was studied using a modified method based upon that developed by Kwaśna *et al.* (2004). Hazel stems (13 – 17 mm diameter) were cut to either 10 mm or 50 mm lengths and arranged into magenta pots with seven stems per pot. Hazel stems were autoclaved as described for section 2.8.3 before stems were covered with MEA. *Armillaria* isolates CG440 or CG675 were inoculated onto 10 mm hazel stems and incubated in the dark for one

month at 25 °C. Magenta pots containing 50 mm long hazel billets were inoculated with one of eight *Trichoderma* isolates (Table 2.4⁺) and incubated under L : D conditions at 20 °C for one month. After one month, agar was removed from hazel disks and stems. One *Armillaria* colonised disk was nailed (20 mm length nail, Wilko, UK) to a *Trichoderma* colonised stem and incubated in individual foil trays (150 x 125 x 65 mm, Wilko, UK) of autoclaved silver sand. Positive controls were inoculated with *Armillaria* alone and negative controls were prepared by nailing together an uncolonised hazel disk and stem. Each treatment was replicated seven times. After seven months the woody segments were removed from the sand and the presence of rhizomorphs or other signs of *Armillaria* and *Trichoderma* was recorded.

2.8.5 Enzyme assays

Petri dish based assays were used to determine whether *Trichoderma* isolates produced extracellular enzymes. Enzymes selected for investigation included amylase, cellulase, laccase, pectinase and protease. A selection of *Trichoderma* isolates were grown in the centre of a minimal medium (MM) Petri dish for all enzyme assays except laccase which was grown on nutrient agar (NA). All media was supplemented with 0.2 % sodium deoxycholate to slow *Trichoderma* spp. growth for optimal visualisation of enzyme activity. For each enzyme assay, appropriate amendments were made to the media as detailed in Table 2.6. Enzymatic activity was measured at four dpi for all assays, except laccase which was measured at six dpi. Each assay was carried out in triplicate. Radial diameter of *Trichoderma* colonies was measured prior to staining with appropriate solutions (Table 2.6). After staining, the radial diameter of the halo produced was measured for amylase, cellulase, pectinase and protease assays. *Trichoderma* colony size and the brown halo produced by laccase activity was measured in laccase assays. The enzymatic index (EI) (Florencio *et al.*, 2012; Chen *et al.*, 2018) was calculated as a ratio between the radial diameter of the *Trichoderma* colony and the associated halo with the following formula:

$$EI = \frac{\text{Diameter of clearing zone}}{\text{Trichoderma colony diameter}}$$

Statistical analysis of the EI was conducted using one-way ANOVA with Tukey's post hoc test for normally distributed data or Kruskal-Wallis with the Bonferroni correction applied for non-normal data.

Table 2.6: Media and stains used to determine production of extracellular enzymes by *Trichoderma* spp.

Enzyme assays used a basal medium of minimal medium (MM) or nutrient agar (NA) which was amended with supplements. Where necessary, pH was adjusted with 2 M HCl. Production of extracellular enzymes was assessed by staining media.

| Enzyme | Agar | pH | Supplements | Staining | Reference |
|-----------|------|-----|---|---|---|
| Amylase | MM | 6.8 | 0.2 % soluble starch | <ul style="list-style-type: none"> 5 ml Grams iodine (1 % KI and 0.5 % I₂). Stain removed after 30 mins | Sunitha <i>et al.</i> , 2013 |
| Cellulase | MM | - | 1% high viscosity carboxymethylcellulose (CMC) Medium was blended prior to autoclaving | <ul style="list-style-type: none"> 5 ml of 0.2 % aqueous Congo-red Stain was removed after 15 mins by washing with 10 ml 1 M NaCl | Florencio <i>et al.</i> , 2012 |
| Laccase | NA | 7.4 | 2 % tannic acid (filter sterilised) added to cooled, autoclaved medium | n/a | Kumar <i>et al.</i> , 2010 |
| Pectinase | MM | 7 | 10 g citrus pectin powder | <ul style="list-style-type: none"> 5 ml 1 % CTAB Stain was removed after 30 mins with 10 ml DW | Sunitha <i>et al.</i> , 2013; Cherkupally <i>et al.</i> , 2017 |
| Protease | MM | - | 10 % skimmed milk (sterilised) was added to cooled, autoclaved medium. | <ul style="list-style-type: none"> 5 ml 0.1 % amido black (in 7 % acetic acid) Stain was removed after 3 h with 10 ml 7 % acetic acid | Shaikh, 2003; Mallikharjuna Rao <i>et al.</i> , 2016 |

2.9 *In planta* assays

All strawberry (*Fragaria × ananassa* 'Elsanta' (F)) plants were sourced from R W Walpole Ltd (Kings Lynn, UK). The variety Elsanta was chosen based on its susceptibility to fungal diseases. Privet cuttings were collected from a privet (*Ligustrum vulgare*) hedge located in Wisley Village, UK (51.322620, -0.474560). Hazel billets colonised vertically for three months were used only for the strawberry screening experiment and RST inoculum experiments, after which hazel billets colonised horizontally for one month were prepared for *in planta* experimentation.

All experiments carried out using strawberry plants were located at the University of Bristol greenhouses at Old Park Hill (OPH) (51.456310, -2.599110). A consistent temperature of 15 °C was maintained with 16 h day length. Colonisation of *Trichoderma* spp. in privet assays were carried out at the University of Bristol, Life Sciences Building GroDome facility. All other experiments carried out using privet plants were located at the RHS Wisley Field Research Facility (FRF). All privet plants were maintained at 18 °C day and 15 °C night with 16 h day length.

2.9.1 Propagating privet plants

All privet plants were propagated from material collected from the same privet hedge located in Wisley Village on the day of propagation. Using a sharp pair of secateurs cleaned with IMS, actively growing stems were cut down to the old wood and wrapped in a damp paper towel then placed in a larger plastic sample bag. In the laboratory, new growth from privet stems was cut into 50 – 100 mm lengths including a leaf node at the base where leaves were removed, a leaf node in the middle where leaves were kept, and the top cut was made just below a third leaf node (Figure 2.2). Stems were placed into a Hydropod (Greenhouse Sensation, UK) and propagated following the manufacturer's instructions. Large leaves which crowded neighbouring cuttings were trimmed by up to one half. A water temperature of 18 °C was maintained in the Hydropod which continuously sprayed the base of the cuttings to encourage root growth. Within four to six, weeks privet cuttings were sufficiently rooted and *Trichoderma* inoculation occurred at this point as per methods described in section 2.9.4.

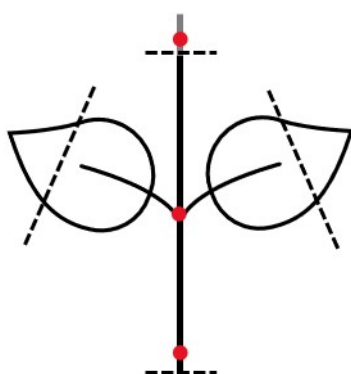


Figure 2.2: Schematic of privet stems prepared for propagating.

Red spots indicate leaf nodes and dotted lines indicate where stems and leaves were cut.

2.9.2 *Armillaria* inoculum preparation

2.9.2.1 Vertically arranged hazel billet colonisation

Armillaria mellea infected hazel billets for use as plant inoculum were prepared as per methods described by Desray *et al.* (1998). One to two meter stems of hazel (*Corylus avellana*) measuring between 13 mm and 17 mm diameter were harvested on the day of inoculum preparation from a hazel coppice at RHS Garden Wisley or by the University of Bristol Halls of Residence, Durdham Hall. Any side branches were removed, and the hazel was cut into 50 mm segments then washed twice in warm soapy water before rinsing twice in tap water. Billets were arranged vertically in 500 ml Nalgene jars and packed tightly with ca. 30 billets per jars. Billets were covered in DW with a piece of foil covering the pot and the lids resting on top. Jars were autoclaved twice at 121 °C for 30 mins replacing water between autoclave cycles. After a third autoclave cycle with fresh foil and no water covering the billets, they were left in a laminar flow hood overnight to dry. Billets were submerged in carrot agar and left for one week to allow detection of any contamination. Four plugs of *Armillaria* from young cultures were used to inoculate each pot, negative control jars were set up in the same way without the inoculation of *A. mellea*. Lids were sealed tightly and wrapped in clingfilm for protection against contamination and stored in a dark incubator at 20 – 25 °C for use between three to six months. Prior to use agar was scrapped off inoculum with a flame-sterilised scalpel.

2.9.2.2 Horizontally arranged hazel billet colonisation

Hazel billets were harvested and prepared for autoclaving as described above. Billets were arranged horizontally in a single layer in rectangular plastic containers (Wilko, UK) and submerged in DW. Containers were covered with foil and autoclaved twice at 121 °C for 30 mins replenishing water between cycles. Billets were autoclaved for a third time at 121 °C for 30 mins without water and left to dry in a laminar flow hood overnight. The hazel billets were submerged in MEA and once set incubated for one week in the dark to allow for detection of contaminants. Six plugs of *A. mellea* were used to inoculate each container. Negative control billets were set up in the same way, but without inoculation of *A. mellea*. Lids were sealed and containers wrapped in clingfilm to avoid contamination then stored

in a dark incubator at 20 – 25 °C for use after one month. Prior to use agar was scrapped off inoculum with a flame-sterilised scalpel.

2.9.2.3 Rice sawdust & tomato inoculum

Armillaria inoculum is time consuming to make so in an effort to produce inoculum more quickly, an alternative method was trialled. *Armillaria mellea* CG440 plugs were inoculated on the RST medium as per methods to induce *Armillaria* fruiting (Ford *et al.*, 2015; section 1.5.4.1). After four to six weeks of colonisation in the dark, a 10 mm diameter cork-borer or scalpel was used to make an inoculum billet. The cork borer represented a diameter slightly smaller than the hazel billet and three pieces together made up 50 mm length. This produced the ‘cored’ piece of inoculum. A second treatment was also trialled using a wedge of colonised inoculum cut with a sterile scalpel to 3 cm x 1 cm x 4.5 cm in size. An *Armillaria*-free negative control was prepared using RST uninoculated with *Armillaria* using the cork-borer.

2.9.2.4 Hazel, horse chestnut and acorn substrates for alternative *Armillaria* inoculum

Hazel billets were collected from hazel coppices and cut to a standard size as described in section 2.9.2.1 with the option of freezing at this point. Horse chestnuts (*Aesculus hippocastanum*) and acorns (*Quercus* sp.) obtained from Tree Seeds Online LTD (www.treeseedonline.com) were stored at -20 °C before use and defrosted in a drying oven. Once deforested, seeds were sorted by submerging in water and removing any which floated or showed signs of damage. All inoculum was weighed to achieve a standard weight of 9 – 11 g for hazel, 9 – 14.5 g for horse chestnuts and 2.4 – 4.5 g for acorns. Acorns were cracked once underfoot and horse chestnuts pierced once with a sterile scalpel to avoid bursting during autoclaving, then washed as per section 2.9.2.1. Substrates were arranged horizontally in a single layer into rectangular plastic containers then submerged in DW and covered with foil. Trays were autoclaved twice at 121 °C for 30 mins replacing water between autoclaving cycles. To complete the setup, MEA was prepared, and melted rather than autoclaved to ensure agar was equally distributed then used to cover inoculum before a final autoclave at 121 °C for 30 mins. Once agar was set, containers were sealed, left in the dark at 20 °C and checked after one week to ensure there was no contamination.

Six agar plugs from seven-day old *A. mellea* (CG440) cultures were used per container for inoculation, negative controls were uninoculated. Inoculum was incubated in the dark for a minimum of one month at 20 – 25 °C prior to use when external agar was removed.

2.9.3 Alternative *Armillaria* inoculum *in planta* experiments

2.9.3.1 RST based *Armillaria* inoculum

To determine the efficacy of RST as a substrate for *A. mellea* inoculum, strawberry plants were used as a host system. Due to limited plants available, and the pilot nature of the experiment, each treatment was set up in duplicate. Healthy bare-rooted strawberry plants were potted into Levington compost and inoculated with *Armillaria* one week after potting. Inoculum was prepared as described in section 2.9.2.3 with four treatments: *A. mellea* CG440 colonised ‘cored RST’ and ‘RST wedge’, and two controls with an uninoculated RST wedge and a hazel billet colonised vertically for three months.

Plants were monitored for three months at which point they were destructively sampled to assess *Armillaria* root rot (ARR) using a 1 – 4 pt. disease severity index (DSI) (

Table 2.7). Inoculum was retrieved from harvested jars and isolations were made onto PDA.

Table 2.7: Disease Severity Index (1 – 4 pt. scale) descriptions.

Assessment is based on health observations from above and belowground symptoms.

| DSI | Above-ground | Below-ground |
|-----|-----------------------------------|--|
| 1 | No aerial symptoms | No visible <i>A. mellea</i> mycelium |
| 2 | Some aerial symptoms | No visible <i>A. mellea</i> mycelium |
| 3 | May show signs of aerial symptoms | Visible <i>A. mellea</i> mycelium colonisation |
| 4 | Dead Plant | Heavy <i>A. mellea</i> mycelial colonisation |

2.9.3.2 Hazel, horse chestnut and acorn based inoculum

The efficiency of vertically colonised hazel billets, horse chestnuts and acorns as substrates for *A. mellea* was assessed in strawberry plants over three months. Dormant bare-rooted strawberry plants were potted and grown for one month

prior to inoculation in Levington compost with a 5 ml pipet tip inserted at planting to preserve space free-from growing roots for inoculum to be inserted. Each treatment was replicated ten times. Negative control plants received one uncolonised hazel billet each. The remaining three treatments comprised of material colonised with *A. mellea* CG440: one hazel billet, one horse chestnut, or three acorns were inoculated per plant. Plants were monitored for three months before disease assessment using the 1 – 4 pt. DSI (

Table 2.7). Inoculum was visually inspected upon destructive harvesting of the plants, but no isolations were made to confirm viability. Isolations for *Armillaria* were made onto JJG from root material with ARR symptoms where possible.

Data were not normally distributed, thus a Kruskal-Wallis test was carried out on DSI values with a Bonferroni correction applied to determine the effect of *A. mellea* inoculum. The null hypothesis assumed no significant difference between the treatment and controls on the DSI of plants.

2.9.3.2.1 Substrate analysis

Chemical analyses of hazel billets, horse chestnuts and acorns were conducted. A selection of defrosted substrates, within the standardised weight range described were assessed. Substrates were analysed for calorific content, nitrogen : carbon ratio, nutrient and trace element content by NRM laboratories (Cawood Scientific Ltd, Berkshire, UK).

2.9.4 *Trichoderma* spp. inoculation of plants

The method of *Trichoderma* spp. inoculation of plants was consistent for all studies. All plants were inoculated with a single *Trichoderma* spp. isolate. *Trichoderma* isolates were subcultured onto fresh MEA and grown for 10 days with a L : D cycle of 16 : 8 h at 20 °C to induce sporulation. In a class II biosafety cabinet *Trichoderma* spp. dishes were flooded with 5 % Tween 20 (autoclaved) and gently scraped with a sterile loop to release spores which were collected in sterile universals by pipette. Once collected, *Trichoderma* spore suspensions were stored at 4 °C for a maximum of two days. Using a haemocytometer, the average number of spores in five squares was counted to determine the number of conidia per ml. The required volume of SDW was allocated per plant (50 – 65 ml) with the addition of sufficient spore suspension to reach a final concentration of 10^5 conidia / ml. Plants were selected for uniformity and roots of plants were dipped into the *Trichoderma* spore suspension for two minutes in

Magenta pots. Sufficient volumes of growing medium (Levingtons compost : Sterile Sand, 3 : 1) were prepared to pot up the number of plants required into 9 cm pots (ca. 0.3 L per plant) and mixed with the remaining spore suspension. Compost mixed with *Trichoderma* spp. was prepared in a plastic tray lined with a plastic bag which was discarded between each treatment to avoid cross-contamination. Fungus-free plants were treated in the same manner with the addition of SDW, free from *Trichoderma* spp..

2.9.5 Determination of *Trichoderma* colonisation in host plant systems

To ensure that host systems selected for ARR assays were suitable, a series of studies were conducted to prove *Trichoderma* spp. could colonise plants. Virkon efficiency to sterilise roots of *Trichoderma* spp. was assessed to ensure endophytic *Trichoderma* spp. isolations were made. Previous studies have reported *Trichoderma* spp. colonisation of strawberry plants (Porrás *et al.*, 2007) and, coupled with isolation of *Trichoderma* spp. after five months inoculation in strawberry plants (reported in chapter four (4.4.4.1)), strawberry colonisation was not assessed. The time for colonisation of *Trichoderma* spp. to occur and the longevity of endophytic colonisation of *Trichoderma* spp. was assessed in freshly rooted privet cuttings.

2.9.5.1 Determining efficiency of 1 % Virkon for sterilisation of *Trichoderma* spp.

To ensure endophytic *Trichoderma* species were isolated, roots were surface sterilised with 1 % Virkon. In order to determine whether 1 % Virkon solution was sufficient for surface sterilisation, an *in vitro* test was performed using *Trichoderma* spore suspensions prepared to a concentration of 4×10^4 conidia / ml. Five, 10-fold serial dilutions of *T. atrobrunneum* T17/11 were prepared in 2 ml aliquots. Each suspension was shaken gently with an equal volume of SDW or 1 % Virkon (1 ml) for 2 mins. Twenty μ l of each suspension was spread onto MEA and incubated at 20 °C in L : D (16 : 8 h) conditions and the number of *Trichoderma* colonies were counted 2 dpi. The efficiency of 1 % Virkon was calculated as the percentage of *Trichoderma* colonies grown after Virkon treatment compared to the water treated control for each dilution series.

2.9.5.2 Colonisation of *Trichoderma* – efficiency

To determine the efficiency by which *Trichoderma* spp. colonised plant roots, privet plants were inoculated with *T. atrobrunneum* T17/11 then re-isolations were attempted from roots between one and seven days after inoculation. Inoculations with *T. atrobrunneum* T17/11 were performed via root dipping followed by mixing the remaining spore suspension into silver sand with a sterile piece of filter paper lining the base of a 10 cm pot to prevent loss of sand and placed on a saucer. Seven privet plants were treated and destructively sampled daily over a seven-day period so isolations could be made from the roots. Sand was washed from plant roots with tap water and sections of root tips (25 mm) were cut to 5 mm lengths using a sterile scalpel before plants were discarded. Pieces of root were surface sterilised in 1 % Virkon for two mins then washed twice in SDW for two mins. Ten root pieces were plated onto two dishes of MRB which were incubated at 20 °C for four weeks in 16 : 8 hr L : D conditions and checked at weekly intervals for growth of *Trichoderma* spp..

2.9.5.3 Colonisation of *Trichoderma* – longevity

The longevity of *Trichoderma* colonisation in privet plants was tested with *Trichoderma* isolates *T. hamatum* T17/10 and *T. atrobrunneum* T17/11 and T17/15. Freshly rooted privet cuttings were inoculated with *Trichoderma* spp. isolates as per section 2.9.4 with six replicates per treatment. Two control groups (with six replicates each) were mock inoculated with SDW. Plants were grown in medium composed of a 3:1 ratio of compost (Levingtons) : sand (Silver sand) and placed in individual saucers. *Trichoderma* spp. isolations were made as described in section 2.9.5.2 from one plant in each treatment on a weekly basis for six weeks, however here, plants were re-potted after isolations were made.

2.9.5.3.1 Pilot study to assess *Armillaria* inoculation in privet

To ensure young privet plants grown from rooted cuttings could be successfully infected with *Armillaria*, plants retained from the longevity of *Trichoderma* spp. colonisation assay (section 2.9.5.3) were inoculated with *A. mellea* at nine weeks post inoculation (wpi) with *Trichoderma* spp.. A control treatment was set up with *Armillaria*-only and fungus-free plants. Plants were monitored for signs of

infection over a six month period where colonisation of roots was checked, non-destructively on a monthly basis. Isolations to confirm *A. mellea* presence were made onto JYG where ARR symptoms were found.

2.9.6 Screening of *Trichoderma* spp. for growth promotion and biocontrol potential

2.9.6.1 Growth promotion in strawberry plants

To determine whether *Trichoderma* promoted growth in strawberry plants, a screening test was set up with all *Trichoderma* isolates detailed in Table 2.4, except *T. atrobrunneum* T17/20 which did not grow. *Trichoderma* isolates were grown, harvested and inoculated onto plant roots as detailed in section 2.9.4. To ensure use of 5 % Tween 20 for the suspension of *Trichoderma* spores had no effect on experimental treatments, controls were set up with fungus-free 5% Tween 20 in one treatment and SDW in another, but otherwise following the same procedure as with *Trichoderma* treatments. Strawberry plants were potted into 10 cm plant pots, placed on saucers and arranged in a non-randomized block design with three replicates per treatment. Measurements of leaf size (determined as length of leaf blade multiplied by the width at its widest point) were taken at 10 day intervals for 60 days at which point plant height was also measured.

A one-way repeated measures ANOVA with pairwise comparisons between each treatment was used to determine the effect of *Trichoderma* isolate on leaf size. To determine the effect of *Trichoderma* on plant height after 60 days a one-way ANOVA with Tukey's post hoc test was applied. The null hypothesis assumed no significant difference in leaf size or plant height between *Trichoderma* treatments and controls.

2.9.6.2 *Trichoderma* recovery from soil

After two months, soil samples were collected using a 10 mm diameter cork-borer from each plant and mixed with replicates from the same treatment in a plastic bag which was stored at 4°C overnight. One gram of soil was suspended in 10 ml SDW, thoroughly mixed and 100 µl was spread onto MEA+SA.

Potential *Trichoderma* colonies were sub-cultured onto fresh MEA+SA after two days. Original cultures and sub-cultured colonies were assessed after one week for *Trichoderma* colony growth and spores, which were confirmed under a light microscope.

2.9.6.3 Disease Assessment in strawberry plants

After two months of inoculation with *Trichoderma* spp. there were no signs of growth promotion in strawberry plants. Thus plants were inoculated with *A. mellea* to determine whether *Trichoderma* spp. offered protection from disease. As described in section 2.9.6.2, a cork-borer was used to collect soil samples leaving a hole into which plants could be inoculated with *A. mellea* colonised hazel billets. *Trichoderma*-free control treatments included mock-inoculated fungus-free plants using an uncolonised hazel billet and *Armillaria*-only controls where strawberry plants were treated to root-dipping with 5 % Tween20 or SDW. Roots were permitted to be damaged to encourage *Armillaria* infection and highlight any protection offered by *Trichoderma* spp..

Plants were monitored fortnightly and any fruits or flowers were removed. After three months plant height was measured and plants were destructively harvested and assessed for disease using a disease severity index from 0 – 6 (healthy – dead) as detailed in

Table 2.8. Inoculum billets were recovered and visually assessed for *A. mellea* colonisation, identified by the presence of mycelial fans under the bark or rhizomorphs growing from the wood. Isolations were made for *A. mellea* and *Trichoderma* spp. onto selective media (JJG or MRB, respectively). *Trichoderma* spp. isolations were made primarily from root tissue which appeared healthy. *Armillaria mellea* isolations were made from root and crown tissue with symptoms of infection such as presence of dark, water-soaked lesions or pale mycelial fans. Isolations were also made from inoculum billets. Pieces of root tissue or the outer layers of hazel billets were cut into small pieces (< 0.5 cm²) and surface sterilised in 10 % bleach (*A. mellea*) or 1 % Virkon (*Trichoderma* spp.) for two mins then rinsed in two washes of SDW for a further two minutes each. Petri dishes were incubated at 20 °C under light/dark conditions for *Trichoderma* or 25 °C in the dark for *Armillaria* and all Petri dishes were assessed for fungal growth after one and three weeks.

Table 2.8: Disease Severity Index (0 – 6 pt. scale) descriptions.

Where *Armillaria* mycelial fans were visible in the roots, isolation confirmation was optional because re-isolation is expected to be successful.

| DSI | Above-ground Symptoms | <i>Armillaria mellea</i> mycelium under bark of roots or crown | Re-isolation success of <i>A. mellea</i> |
|-----|-------------------------------|--|--|
| 0 | No aerial symptoms | Not visible | Unsuccessful |
| 1 | Some aerial symptoms | Not visible | Unsuccessful |
| 2 | Some signs of aerial symptoms | Not visible | Successful |
| 3 | No aerial symptoms | Visible | Successful |
| 4 | Some signs of aerial symptoms | Visible | Successful |
| 5 | Progressed aerial symptoms | Visible/heavy | Successful |
| 6 | Dead Plant | Visible/heavy | Successful |

A one-way ANOVA with Tukey's post hoc test was used to determine whether *Trichoderma* treatment or *Armillaria* infection had a significant effect on plant height at the point of disease assessment. As DSI data was not normally distributed and could not be transformed to achieve normality, the Kruskal-Wallis test with a Bonferroni correction was used to determine whether *Trichoderma* isolates had an effect on the DSI of strawberry plants. The null hypothesis states that treatment with any *Trichoderma* spp. tested will not have a significant effect on plant height or disease severity of strawberry plants.

2.9.7 Assessment of protection from ARR in strawberry plants with a selection of *Trichoderma* spp.

Based on the seven best-performing *Trichoderma* spp. isolates, with the addition of the poorly performing isolate *T. olivascens* T17/42 from the strawberry screening experiment, a second experiment was set up to establish the level of protection offered to strawberry plants from *A. mellea* infection when pre-inoculated with *Trichoderma* spp.. *Trichoderma* spp. isolates were chosen based on those with an average DSI lower than that of *Armillaria*-only controls (indicated by ⁺ in Table 2.4). The experiment

was set up in the same manner as the first strawberry experiment with some important differences. Firstly, after inoculation with *Trichoderma* spp., plants were left to establish endophytic associations for one month before *A. mellea* was introduced. Secondly, *A. mellea* billets were colonised horizontally for one month as opposed to vertically for three months. All treatments were set up in a randomised block design with ten replicates each. Controls comprised of a fungus-free and an *Armillaria*-only treatment. Plants were monitored fortnightly and any flowers or fruits were removed. Dead plants were assessed for *A. mellea* infection and the date of death was recorded.

Trichoderma spp. colonisation was assessed in five strawberry plants inoculated with *T. atrobrunneum* T17/10 which had died from *A. mellea* infection at five mpi. Isolations were made from ARR infected root balls, where possible in regions showing little or no infection. Root pieces were surface sterilised as described above and plated onto MRB. After one and three weeks incubation at 20 °C under 16 : 8 hr L : D conditions, Petri dishes were checked for *Trichoderma* spp. colonies. Plants that were still alive were assessed visually for *Armillaria* infection after eight months. No isolations were attempted and severity of disease was not measured.

2.9.8 Assessment of *Trichoderma* spp. for biocontrol of ARR using privet plants as a host system

To ensure potential protection offered by root-associated or endophytic *Trichoderma* spp. against ARR was possible in a range of hosts, and particularly in woody hosts, privet (*Ligustrum vulgare*) plants were selected for assessment due to their high susceptibility to infection (Drakulic *et al.*, 2017). The selection of eight *Trichoderma* spp. isolates highlighted by a + in Table 2.4, which were also used in the second strawberry experiment, were inoculated into privet plants as previously described, with nine replicates per treatment. Plants were grown for one month prior to inoculation with *Armillaria* using hazel billets colonised horizontally by *A. mellea* CG440. The isolate of CG440 had recently been re-isolated from infected strawberry plants to prevent a loss of pathogenicity due to repeated subculturing. An *Armillaria*-only and a fungus-free treatment were included as controls. Plants were measured every two months for plant height, leaf size and chlorophyll content. Leaf size was measured from a randomly

selected mature leaf (determined as length of leaf blade multiplied by the width at its widest point). Greenness of plants was measured using a SPAD metre (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc.) on two fully expanded leaves.

Privet plants were assessed for vitality once a fortnight and the date was recorded for any plant deaths. Dead plants were harvested and the roots dissected. If no *A. mellea* colonisation was visible at this point, isolations were made onto JJG after surface sterilisation as described in 2.9.6.3 using 10% bleach. After nine months, when no new deaths were recorded, final measurements of plant height, leaf size and chlorophyll content were made before plants were destructively assessed for disease. Plant roots and stem bases were inspected for *A. mellea* colonisation and isolations for *A. mellea* were made from plants without signs of *Armillaria* infection. All plants were scored according to a DSI on a 0 – 4 pt. scale (Table 2.9). Presence of rhizomorphs in the soil was recorded and billets were visually assessed for viability as detected by presence of soft, white mycelial fans under the bark or rhizomorphs growing from the material. Statistical analysis of the DSI was performed with a one-way ANOVA and Tukey's post hoc test. The null hypothesis assumed *Trichoderma* spp. would not affect the disease severity of *A. mellea* infection.

Table 2.9: Disease Severity Index (0 – 4 pt. scale) descriptions.

Armillaria mellea isolations were only made from roots of plants not showing *Armillaria* mycelial colonisation.

| DSI | Above-ground aerial symptoms | <i>Armillaria mellea</i> mycelium under bark of roots or crown |
|------------|-------------------------------------|---|
| 0 | None | Not visible |
| 1 | Some | Not visible |
| 2 | None | Visible |
| 3 | Some | Visible <i>Armillaria</i> |
| 4 | Dead Plant | Visible/heavy |

2.9.9 Assessment of *Trichoderma* spp. for biocontrol of ARR in soils with microbial communities

Trichoderma spp. showed potential as a biocontrol agents of ARR using both strawberry and privet plants as a model host systems based on a compost : sand growing medium. Therefore, to determine the effects of microbial communities on the protection offered by *Trichoderma* spp. three soils with varying microbial communities were investigated. Compost : sand (3 : 1 ratio) used in previous experiments was considered to have a relatively low number and diversity of microbes present. The use of compost based growing medium allowed a control treatment that would enable results to be comparative to previous plant-based experiments. Soil was collected from RHS Wisley Gardens Trials Field (51.310297, -0.473523). This was divided to form two treatments: 1) Fresh soil which was untreated and considered to have a rich microbial community representative of garden soils. 2) Baked soil which was heated at 60 °C in a drying oven for three days. Although not killing 100 % of the microbial communities, this reduced the soil microflora and fauna by killing living invertebrate pests and some microbes, but not spores. Three *Trichoderma* isolates were selected for this study: *T. atrobrunneum* T17/11, *T. atrobrunneum* T17/15 and the commercially available *T. harzianum* T22. Privet plants were inoculated with *Trichoderma* spp. as previously described. Control treatments with *Armillaria*-only and fungus-free plants were included. Each treatment was only replicated seven times (due to poor rooting of privet plants) for each soil type; there were 105 plants in total. One month post inoculation with *Trichoderma* spp., horizontally colonised *A. mellea* GC440 hazel billets were inoculated to each plant. Fungus-free plants received an uncolonised hazel billet.

Due to travel restrictions imposed as a result of COVID-19 and low numbers of plants showing infections, plants were not ready to be harvested prior to completion of this thesis. The experiment continues to be monitored by staff at the RHS.

| Chapter 3: *In vitro* interaction assays between *Armillaria mellea* and *Trichoderma* spp.

3.1 Introduction

Trichoderma species exist freely in the soil and within leaf or root tissues of plants as fungal endophytes and various *Trichoderma* species are used for biological control in different situations (Harman *et al.*, 2004a). In order to understand the potential control of ARR by *Trichoderma*, *in vitro* assays can provide the option to visualise and investigate aspects of individual fungal interactions and discover antagonistic behaviours of fungi, such as mycoparasitism and antibiosis in an artificial environment. Understanding of biological processes by identifying key morphological and biochemical attributes could allow rapid screening of isolates to eliminate poor performing antagonists from time-consuming *in planta* trials.

3.1.1 *Trichoderma* spp. selection for biological control of ARR

Existing studies investigating the biological control of *Armillaria* are limited and can have conflicting findings. Studies have focussed on strains isolated from diseased commercial mushrooms (Raziq and Fox, 2003, 2004b) and free-living soil isolates (Cheruiyot *et al.*, 1997) although sometimes the isolate selection process is not fully described (Ohr and Munnecke, 1974). Chen *et al.* (2019) collected *Trichoderma* isolates from the rhizosphere of oak and spruce trees, soil samples and soil around *Armillaria* rhizomorphs. Kwańska *et al.* (2004) and Kwańska and Szykiewicz-Wronek (2018) conducted studies investigating soil fungal communities which were isolated from the rhizosphere and roots of beech and oak to determine inhibition against *A. ostoyae* and *A. gallica*. *Trichoderma* spp. collected which were associated with the rhizosphere of beech, could reduce rhizomorph length of *A. ostoyae* and *A. gallica* (Kwańska and Szykiewicz-Wronek, 2018) while *Trichoderma* spp. isolated from the oak rhizosphere could reduce *A. ostoyae* growth in some *A. ostoyae* isolates and yet stimulate growth in others (Kwańska *et al.*, 2004). Raziq and Fox (2003) report that *Trichoderma* quickly covered *Armillaria* cultures and stopped any growth in dual culture assays, a characteristic also noted by Chen *et al.* (2019).

Crucially, control by *Trichoderma* tends to be isolate specific (Cheruiyot *et al.*, 1997; Kwańska *et al.*, 2004).

3.1.2 Using artificial media to investigate fungus-fungus interactions

The use of artificial media to study fungal ecology *in vitro* has a number of limitations, the most important being a lack of complexity in substrates which is not representative of the real world (Crowther *et al.*, 2018), however, it can provide a simplified insight into understanding the potential antagonistic properties of fungal interactions, allowing observations to be made. In addition a large number of fungal isolates can rapidly be screened. In order to understand whether *Trichoderma* spp. are mycoparasitic, a characteristic which has been shown by *Trichoderma* spp. towards *Phytophthora* spp. (Sanchez *et al.*, 2019), *Pythium ultimum* (Sánchez-Montesinos *et al.*, 2019), *Alternaria panax*, *Botrytis cinerea* and *Rhizoctonia solani* (Park *et al.*, 2019), agar cultures provide a simple system by which the fungal interaction between two species of interest can be observed. Extracellular enzyme activity of *Trichoderma* spp. can be studied using *in vitro* assays in pure cultures using staining assays (Gochev and Krastanov, 2007; Colonia and Junior, 2014) or to determine antagonistic potential in assays with phytopathogens (Yan and Qian, 2009; Qualhato *et al.*, 2013; Saravanakumar *et al.*, 2018). Application of purified extracellular enzymes of *Trichoderma* spp. has been used to investigate the direct effect on pathogens (De Marco and Felix, 2002; De Marco *et al.*, 2003).

3.1.2.1 Using agar-based assays to study the interaction between *Armillaria* and *Trichoderma* spp.

Trichoderma spp. have been shown to have potential as biocontrol agents of *Armillaria* spp. in agar based assays. Reports of fast *Trichoderma* growth over *Armillaria* have been reported in the literature (Raziq and Fox, 2003; Asef *et al.*, 2008; Chen *et al.*, 2019) where *Trichoderma* is able to sporulate profusely (Chen *et al.*, 2019). *Trichoderma* species which offer potential control of *Armillaria* in agar-based interactions include *T. virens*, *T. atrobrunneum*, *T. atroviride*, *T. hamatum*, *T. tomentosum*, *T. harzianum* and *T. viride*, where *T. harzianum* and *T. virens* are frequently the best performers (Raziq and Fox, 2003, 2004a; Asef *et al.*, 2008; Chen *et al.*, 2019). Some *Trichoderma* species which have

limited capability to antagonise *A. mellea* include *T. koningii*, *T. asperellum*, *T. paraviridescens* and *T. longipile* (Chen *et al.*, 2019). *Trichoderma harzianum* and *T. viride* have been shown to degrade rhizomorphs of *Armillaria* after prolonged co-incubation (Raziq and Fox, 2003). Using SEM, *T. harzianum* and *T. virens* have been found to penetrate and colonise rhizomorphs of *A. mellea* (Asef *et al.*, 2008). Thus, *Trichoderma* species seemingly offer varying levels of control to *Armillaria* spp. where *T. harzianum* and *T. virens* are most commonly reported as good biocontrol agents in agar-based assays. Additionally, the mechanism by which *Trichoderma* is able to antagonise *Armillaria* can be clearly studied using *in vitro* assays.

3.1.2.2 Alternative methods to study interactions between *Armillaria* and *Trichoderma* spp. *in vitro*

Since *Armillaria* is primarily a coloniser of woody tissues, investigations into the interaction with *Trichoderma* spp. have occasionally been carried out using wood-based substrates under controlled conditions. Stems colonised by *Armillaria* spp. and *Trichoderma* spp., and incubated together in sterile soils have provided an insight into how both fungi interact in woody substrates. *Armillaria novae-zelandiae* and *A. limonea* were significantly reduced in pine stems co-inoculated with *Trichoderma* sp. (Li and Hood, 1992). Similarly, growth of *Armillaria* sp. on tea stems was reduced by *T. harzianum* (Otieno *et al.*, 2003) and inoculation of soil with *T. hamatum* or *T. harzianum* significantly reduced the number of hazel stems colonised by *A. mellea* (Raziq and Fox, 2004a). This highlights the potential of *Trichoderma* spp. as successful biocontrol agents of *Armillaria*, whereby antagonism can be observed between fungi in woody substrates which mimic a natural environment for *Armillaria*. *Trichoderma hamatum* and *T. viride* have been found to reduce or stimulate the growth of *A. ostoyae* rhizomorphs dependant on individual *A. ostoyae* isolates (Kwańska *et al.*, 2004). In addition, *T. aureoviride*, *T. koningii* and *T. viride* have been reported to reduce the production of rhizomorphs by *A. ostoyae* and *A. gallica* in woody tissues (Kwańska and Szykiewicz-Wronek, 2018). This suggests that as well as antagonising *Armillaria* in colonised wood, *Trichoderma* spp. could impair the ability of *Armillaria* to spread to new food sources.

3.1.3 Extracellular enzymes produced by *Trichoderma* spp.

Trichoderma species are known to produce a range of extracellular enzymes, for example *T. reesei* are well known producers of cellulase and are used in industry for production of renewable biofuels (Bischof *et al.*, 2016). Laccase produced by *T. asperellum* has the potential for use in bioremediation of the toxic dye, malachite green (Shanmugam *et al.*, 2017) or phenolic sytan, a waste product in leather production (Lawrance *et al.*, 2019). The production of extracellular enzymes by *Trichoderma* spp. are likely to be important for biological control of fungal plant pathogens. For example, laccases produced by *Trichoderma* are known to degrade the sclerotia of *Botrytis cinerea* (Catalano *et al.*, 2011). Pectinases and proteases are known to be produced by *Trichoderma* and have some, potentially advantageous, biological control properties against fungal plant pathogens (Calistru *et al.*, 1997; Qualhato *et al.*, 2013).

3.2 Statement of collaboration

The results from sections 3.4.1, 3.4.3 and 3.4.4 have recently been published. I was the lead author, I designed and conducted experiments, analysed the data and wrote the manuscript. Work presented in this chapter is my own unless otherwise stated.

- Rees, H. J., Bashir, N., Drakulic, J., Cromey, M. G., Bailey A. M., & Foster G. D. (2020) Identification of native endophytic *Trichoderma* spp. for investigation of *in vitro* antagonism towards *Armillaria mellea* using synthetic and plant based substrates. *Journal of Applied Microbiology*.

All *Trichoderma* isolates were collected by Niamah Bashir prior to the start of my PhD. Sequences for Chi18-5, ITS1 & ITS4, some *tef1* and *rpb2* sequences from *Trichoderma* were provided by Niamah. Ellie Murphy obtained some *Trichoderma* sequences during a summer project under my close supervision. Additional *Trichoderma* sequences were generated by Maya Ambasana and Morgan Millen. I conducted all sequence analysis and *Trichoderma* ID analysis myself.

The results from section 3.4.2 are a result of a project I devised and supervised for Anisha Uppal and Kerstin Lieu during their final year undergraduate dissertation project in 2018. The project aimed to develop a selective medium for *Armillaria*, from which *Trichoderma* cannot grow. The data from section 3.4.6 was a result of supervising Maya Ambasana, a MSci student during her final year dissertation project. This project was an investigation into enzyme activity by *Trichoderma* spp.. In both cases I guided the direction of the project, and provided training required. The students conducted the routine laboratory work and I analysed the data.

3.3 Aims:

To understand the interactions between individual *Trichoderma* spp. and *A. mellea* isolates and to investigate potential antagonistic mechanisms utilised by *Trichoderma* through *in vitro* assays.

Specifically:

- To determine the species composition of endophytic *Trichoderma* isolates collected at RHS Wisley Gardens.
- To screen *Trichoderma* isolates for biological control potential against *A. mellea* using agar and wood based interaction assays.
- To visualise how *Trichoderma* spp. interact with *A. mellea* using microscopy.
- To determine whether endophytic *Trichoderma* isolates produce extracellular enzymes which might antagonise or degrade *A. mellea* hyphae.

3.4 Results

3.4.1 Isolation and characterisation of endophytic *Trichoderma* spp.

A collection of 40 *Trichoderma* isolates was established from endophytes growing within the roots of healthy plants from areas where *A. mellea* was endemic. This collection was made from isolations taken from nine different host species and two individual soil samples (Table 3.1). *Trichoderma* was not isolated from a sample in two instances taken from *Betula pendula* and *Viburnum bodnantense* (Table 3.1). Isolates were stored at -70 °C to avoid unnecessary subculture since the routine subculture might result in loss of bioactivity as attenuation is often seen in pathogens if sub-cultured.

Trichoderma spp. ID was confirmed using the Chi18-5, ITS1 & ITS2, ITS4 & ITS6, *rpb2* and *tef1* primers (Table 2.4) to PCR-amplify DNA. The amplified products were sequenced and identification was confirmed using BLAST. The genes *rpb2* and *tef1* along with the ITS1 region (ITS1 & ITS2 primer pair) which are common for *Trichoderma* species identification, provided the most informative data for *Trichoderma* species identification in GenBank and were used to create phylogenetic trees (Appendices: Figure A.1, Figure A.2, Figure A.3).

The collection of *Trichoderma* isolates included 12 different species. The greatest diversity of *Trichoderma* species was found on *Rhododendron × obtusum* 'amoenum' and *Viburnum bodnantense* which were associated with four *Trichoderma* species each. The most common species identified was *T. cerinum* with 12 isolates identified as *T. cerinum*. Other *Trichoderma* species included *T. atrobrunneum* (9), *T. harzianum* (6), *T. hamatum* (4), *T. olivascens* (2), *T. deliquescens* (1), *T. fertile* (1), *T. hirsutum* (1), *T. koningiopsis* (1), *T. spirale* (1), *T. viridescens* complex (1), and *T. virens* (1). All ITS, *rpb2* and *tef1* sequences were deposited in GenBank, accession numbers can be found in Appendix Table A.1.

Table 3.1: Details of *Trichoderma* spp. ID including host plant.

| Isolate | <i>Trichoderma</i> ID | Host |
|---------------------|--------------------------------|--|
| T17/01 | <i>T. harzianum</i> | <i>Viburnum carlesii</i> |
| T17/02 ⁺ | <i>T. virens</i> | Soil/debris |
| T17/03 ⁺ | <i>T. harzianum</i> | Soil/debris |
| T17/04 | <i>T. atrobrunneum</i> | <i>Viburnum carlesii</i> |
| T17/05 | <i>T. cerinum</i> | <i>Citrus trifoliata</i> |
| T17/06 | <i>T. harzianum</i> | <i>Quercus</i> sp. |
| T17/07 ⁺ | <i>T. harzianum</i> | <i>Quercus</i> sp. |
| T17/08 ⁺ | <i>T. harzianum</i> | <i>Quercus</i> sp. |
| T17/09 | <i>T. hamatum</i> | <i>Sorbus aria</i> 'Lutescens' |
| T17/10 ⁺ | <i>T. hamatum</i> | <i>Sorbus aria</i> 'Lutescens' |
| T17/11 ⁺ | <i>T. atrobrunneum</i> | <i>Quercus</i> sp. |
| T17/12 | <i>T. atrobrunneum</i> | <i>Quercus</i> sp. |
| T17/13 | <i>T. cerinum</i> | <i>Quercus</i> sp. |
| T17/14 | <i>T. cerinum</i> | <i>Sorbus aria</i> 'Lutescens' |
| T17/15 ⁺ | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> |
| T17/16 | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> |
| T17/17 | <i>T. viridescens complex</i> | <i>Betula pendula</i> |
| T17/18 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/19 | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> |
| T17/20 | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> |
| T17/21 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/22 | No <i>Trichoderma</i> isolated | <i>Betula pendula</i> |
| T17/23 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/24 | <i>T. spirale</i> | <i>Betula pendula</i> |
| T17/25 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/26 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/27 | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> |
| T17/28 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/29 | <i>T. cerinum</i> | <i>Betula pendula</i> |
| T17/30 | <i>T. cerinum</i> | <i>Viburnum bodnantense</i> |
| T17/31 | No <i>Trichoderma</i> isolated | <i>Viburnum bodnantense</i> |
| T17/32 | <i>T. atrobrunneum</i> | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' |
| T17/33 | <i>T. hamatum</i> | <i>Rhododendron</i> 'moonstone' |
| T17/34 | <i>T. hamatum</i> | <i>Rhododendron</i> 'moonstone' |
| T17/35 | <i>T. deliquescens</i> | <i>Viburnum bodnantense</i> |
| T17/36 | <i>T. hirsutum</i> | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' |
| T17/37 | <i>T. fertile</i> | <i>Viburnum bodnantense</i> |
| T17/38 | <i>T. koningiopsis</i> | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' |
| T17/39 | <i>T. cerinum</i> | <i>Conifer</i> sp. |
| T17/40 | <i>T. harzianum</i> | <i>Conifer</i> sp. |
| T17/41 | <i>T. olivascens</i> | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' |
| T17/42 ⁺ | <i>T. olivascens</i> | <i>Rhododendron</i> × <i>obtusum</i> 'amoenum' |
| T22 | <i>T. harzianum</i> | Commercial strain |

⁺ Indicates the refined selection of *Trichoderma* spp. used for *in vitro* assays.

3.4.2 Development of a selective medium for *A. mellea*

A selective medium was required for *in vitro* interaction assays which supported growth of *A. mellea* whilst preventing the growth of *Trichoderma* spp.. Four media using MEA as a base with various chemical and antibiotic supplements were trialled. Colony diameters of *Armillaria mellea* (CG440, CG675 and DSM3731) and *Trichoderma* spp. (*T. hamatum* T17/10, *T. atrobrunneum* T17/11, *T. atrobrunneum* T17/15) were measured at ten dpi and two dpi, respectively to account for different fungal growth rates (Figure 3.1). Growth of *Trichoderma* spp. was completely prevented on JYG (0 % of MEA) and reduced compared to MEA by BDS, MRB and BSMA (13.7 %, 41.8 % and 75.6 % of MEA colonies, respectively) (Figure 3.1). The colony diameter of *Trichoderma* spp. was significantly reduced on all media compared to the MEA control (Kruskal-Wallis with Bonferroni correction: JYG, BDS and MRB, $p < 0.001$, BSMA, $p < 0.01$). *Armillaria mellea* could grow on all media tested; growth was significantly increased on BDS (169.4 % compared to MEA colony. Kruskal-Wallis with Bonferroni correction $p < 0.001$) and significantly reduced on MRB and JYG (47.7 % and 69.3 % of MEA colonies, respectively. Kruskal-Wallis with Bonferroni correction $p < 0.001$) compared to the MEA control. On BSMA there was no significant difference in colony diameter (125.1 % of MEA colony) from the MEA control (Kruskal-Wallis with Bonferroni correction $p > 0.05$). Thus, JYG can be considered an appropriate selective medium for recovery of *A. mellea* from dual culture interaction assays since *Trichoderma* spp. are eliminated. A combination of BDS and JYG was not trialled but might allow elimination of *Trichoderma* without reduced growth of *A. mellea*.

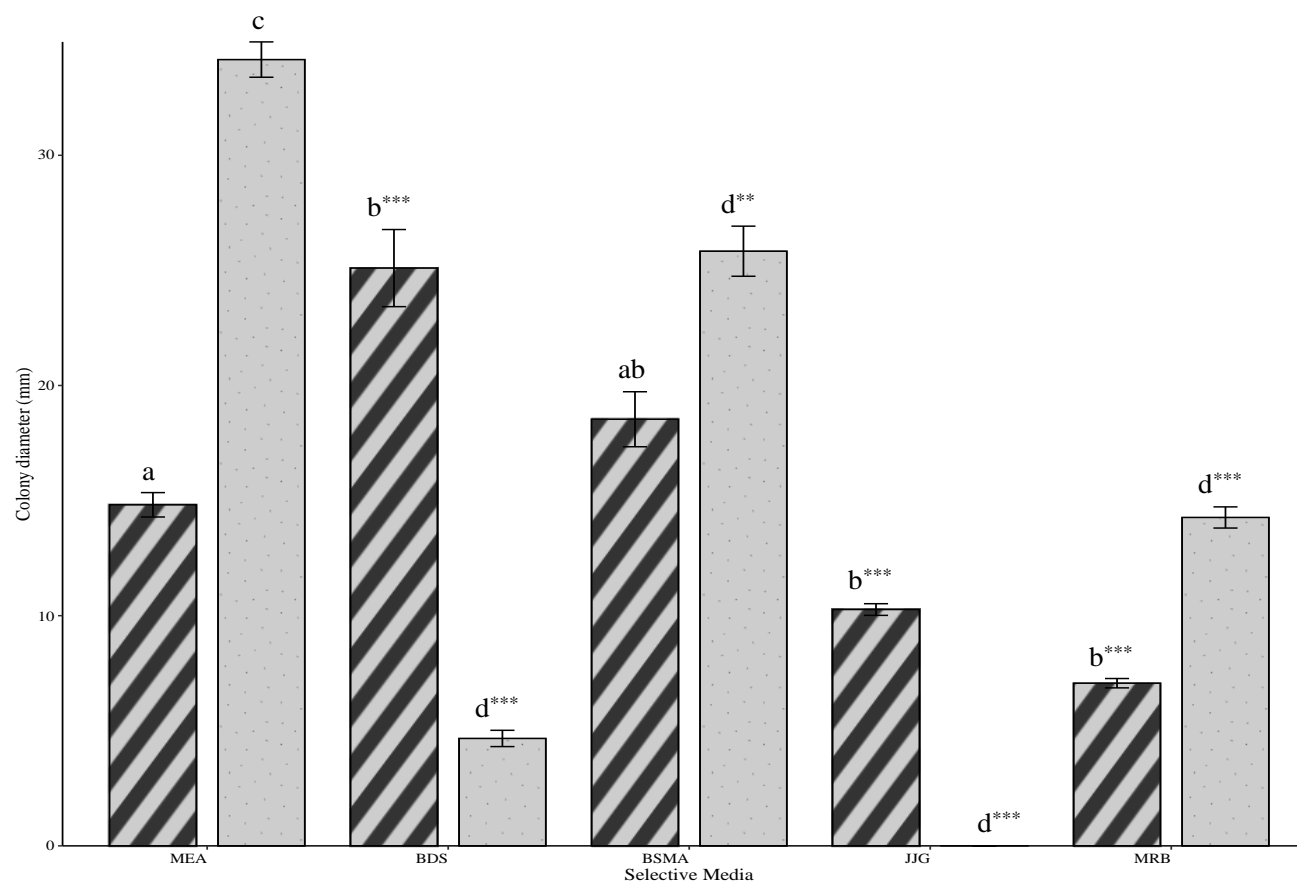


Figure 3.1: Growth of *A. mellea* and *Trichoderma* spp. on selective media

Colony diameter of *A. mellea* (diagonal stripe) 10 dpi and *Trichoderma* spp. (spotted) 2 dpi on selective media: MEA (control), BDS, BSMA, JYG, MRB. Measurements based on average diameter for three *A. mellea* (CG440, CG675 and DSM3731) or *Trichoderma* spp. (*T. hamatum* T17/10, *T. atrobrunneum* T17/11 and *T. atrobrunneum* T17/15) isolates. Error bars represent the standard error of the mean (n = 15). a,b and c,d represent statistical groupings for *Armillaria* and *Trichoderma* spp., respectively (Kruskal-Wallis with Bonferroni correction). * p < 0.05, ** p < 0.01, *** p < 0.001

3.4.3 Controlling *A. mellea* with *Trichoderma* spp. in agar cultures

3.4.3.1 Growth of *A. mellea* in agar based dual cultures

Dual culture interaction assays between *A. mellea* CG440 and all *Trichoderma* isolates (Table 3.1) were used to screen potential biocontrol activity *in vitro* against *A. mellea*. *Trichoderma* isolates were inoculated 50 mm apart from a seven day pre-cultured *A. mellea* colony. After inoculation with *Trichoderma* spp., measurements of the *A. mellea* colony were made every two days for one week and then weekly for six weeks. No *Trichoderma* spp. were inoculated onto *A. mellea* control assays. The majority of *Trichoderma* spp. cultures overgrew *A. mellea* colonies within four dpi, at which point the *Armillaria* growth stopped (Figure 3.2). *Trichoderma fertile* T17/37 was the poorest performing isolate and was slow growing. *Trichoderma olivascens* T17/41 and T17/42 were the next poorest performing isolates which was also a result of slow growth. *Trichoderma cerinum* T17/13 was weakly antagonistic towards *A. mellea*. All remaining *Trichoderma* isolates performed with a similar ability, halting *A. mellea* growth by day four. At the point of *Trichoderma* spp. inoculation, *Armillaria* colonies (seven days pre-culture) had an average diameter of 11.7 mm. In the first four dpi, *Armillaria*-only controls grew and additional 6.2 mm, however in the presence of *Trichoderma* spp., *A. mellea* colonies only grew an average of 4.7 mm and typically did not expand after this time point (except T17/37, T17/41 and T17/42 where growth continued for another six days). The size of *A. mellea* colonies when grown in the presence of *Trichoderma* (averaged for all *Trichoderma* isolates) decreased in size by an average of 1.4 mm over the duration of the experiment. The *Armillaria*-only control grew steadily over the duration of the experiment to reach an average diameter of 65 mm (Figure 3.2). There were no signs of defence or antagonism from *Armillaria* towards *Trichoderma*. No exclusion zones, defence barrages or pseudosclerotial plates were observed.

By 8 dpi, presence of all *Trichoderma* isolates (except slow growing isolates T17/37 & T17/42) significantly reduced the growth of *Armillaria* compared to the *Armillaria*-only control ($P < 0.01$). After six weeks, all *Trichoderma* isolates tested significantly reduced *Armillaria* colony size compared to *Armillaria*-only controls ($P < 0.001$).

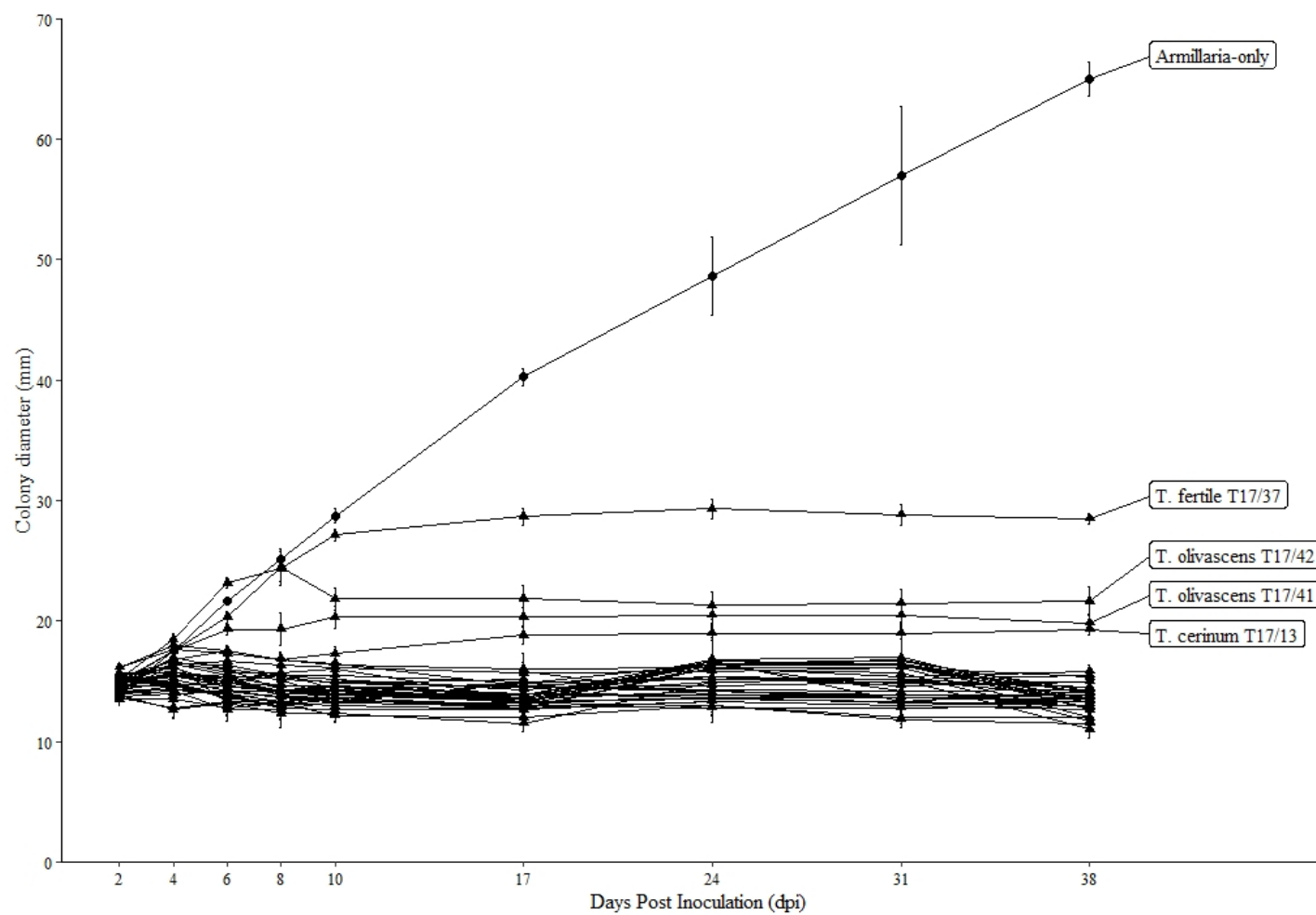


Figure 3.2: Inhibition of *A. mellea* growth by *Trichoderma* spp..

Trichoderma spp. (triangle) were introduced after seven days pre-culture of *A. mellea* CG440. The *Armillaria*-only control (circle) had no *Trichoderma* spp. added. All 40 *Trichoderma* isolates are represented, only those where *A. mellea* colony size is > 20 mm at 38 dpi are labelled. Error bars represent the standard error of the mean (n = 3).

3.4.3.2 Viability of *A. mellea* after agar based dual cultures

A subset of eight *Trichoderma* isolates (*T. virens* T17/02, *T. harzianum* T17/03, *T. harzianum* T17/07, *T. harzianum* T17/08, *T. hamatum* T17/10, *T. atrobrunneum* T17/11, *T. atrobrunneum* T17/15 and the slower growing isolate *T. olivascens* T17/42; see ⁺ in Table 3.1) were selected to repeat the interaction assay and determine the viability of *A. mellea* after six weeks, evaluating two different *A. mellea* isolates CG675 & CG440. The *Armillaria*-selective medium, JJG, identified in section 3.4.2 allowed for isolation of *A. mellea* from co-cultures without becoming overrun by *Trichoderma* spp. to enable assessment of *A. mellea* viability.

Isolations were made from *A. mellea* colonies six wpi with *Trichoderma* spp. to determine the viability of *A. mellea*. Each co-culture was replicated three times, and three isolations were taken from each, giving a total of nine isolations per *Trichoderma* isolate. When re-isolation of *A. mellea* was performed from the *Trichoderma* spp. co-cultures, in five of the *Trichoderma* co-cultures (*T. virens* T17/02, *T. harzianum* T17/03 & T17/08, *T. hamatum* T17/10 and *T. olivascens* T17/42), no viable *A. mellea* was recovered (CG675 & CG440; Table 3.2). *Armillaria mellea* could only be recovered from three of the interactions with *Trichoderma* spp. (*T. harzianum* T17/07, *T. atrobrunneum* T1711 & T17/15). In all cases, *A. mellea* was readily isolated from the *Trichoderma*-free controls (100% recovery). There was some variation between *A. mellea* isolates; CG675 was re-isolated from only 25% of the *Trichoderma* treatments (two of eight) compared to re-isolation of CG440 in 62 % of the *Trichoderma* treatments. In all cases where *A. mellea* could be re-isolated from *Trichoderma* co-cultures, recovery of *Armillaria* colonies was at a lower rate than *Armillaria*-free controls. This demonstrated that some *Trichoderma* isolates could eliminate *A. mellea* in agar culture, although this was with varying efficiency between isolates.

Table 3.2: Viability of *A. mellea* from agar based dual culture assays with *Trichoderma* spp..

Re-isolation of *A. mellea* CG440 & CG675 (%) following six weeks of dual culture on agar with *Trichoderma* spp. isolates. Re-isolation was performed using JIG agar to minimise growth of *Trichoderma*. Isolation was deemed successful where *A. mellea* colonies were apparent within two weeks. +/- represent whether *A. mellea* could be eliminated.

| <i>Trichoderma</i> spp. isolates | <i>A. mellea</i> CG440 recovery % | <i>A. mellea</i> CG675 recovery % | Elimination by both <i>A. mellea</i> strains (CG440 & CG675) |
|----------------------------------|-----------------------------------|-----------------------------------|--|
| <i>Armillaria</i> -only | 100 | 100 | n/a |
| <i>T. virens</i> T17/02 | 0 | 0 | + |
| <i>T. harzianum</i> T17/03 | 0 | 0 | + |
| <i>T. harzianum</i> T17/07 | 77.7 | 22.2 | - |
| <i>T. harzianum</i> T17/08 | 0 | 0 | + |
| <i>T. hamatum</i> T17/10 | 0 | 0 | + |
| <i>T. atrobrunneum</i> T17/11 | 22.2 | 55.5 | - |
| <i>T. atrobrunneum</i> T17/15 | 11.1 | 0 | - |
| <i>T. olivascens</i> T17/42 | 0 | 0 | + |

3.4.3.3 Visualisation of *Trichoderma* spp. displaying antagonism towards *A. mellea*

To assess whether *Trichoderma* spp. displayed mycoparasitic behaviour towards *A. mellea*, interactions were visualised using microscopy. *Trichoderma atrobrunneum* T17/04 and *T. harzianum* T17/08 were inoculated onto Petri dishes with 25-day old *A. mellea* CG440 cultures grown on a cellophane overlay. Within three dpi *Trichoderma* spp. reached the *A. mellea* colonies and the interaction zone was visualised. The predominant observation made was apparent degradation of *A. mellea* hyphae, seen as vacuolation and increased transparency of hyphae (Figure 3.3). The apparent hyphal degradation was frequently observed on *A. mellea* co-inoculated with both *T. atrobrunneum* T17/04 and *T. harzianum* T17/08. The ability for *Trichoderma* spp. to degrade the hyphae of *A. mellea* makes it a strong candidate as a biological control agent and suggests potential enzyme activity. When *A. mellea* was co-inoculated with *T. atrobrunneum* T17/04 formation of crystalline structures on *A. mellea* hyphae were also observed (Figure 3.4) often but less frequently than apparent hyphal degradation. Crystalline structures were not observed on *A. mellea* CG440 grown without *Trichoderma* spp..

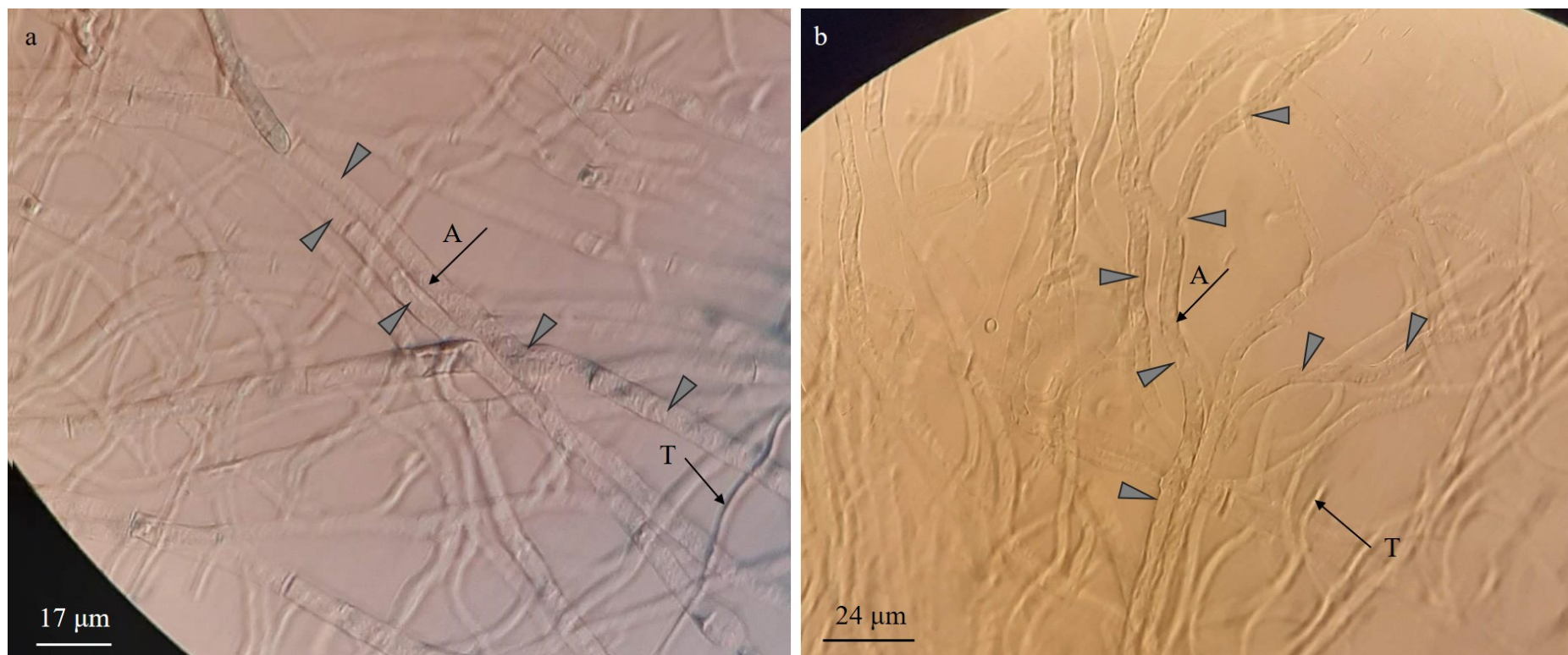


Figure 3.3: Signs of hyphal degradation in *A. mellea* after dual culture with *Trichoderma* spp..

Signs of hyphal degradation as indicated by grey arrows were observed after three days dual culture between *A. mellea* CG440 (A) by *Trichoderma* spp. (T). *Armillaria* colonies were inoculated with *Trichoderma* spp. after 25 days pre-culture and visualised at 400 x magnification under brightfield light. a) Dual interaction between *T. atrobrunneum* T17/04 and *A. mellea* CG440; b) Dual interaction between *T. harzianum* T17/08 and *A. mellea* CG440. Scale bars are represented on individual images.

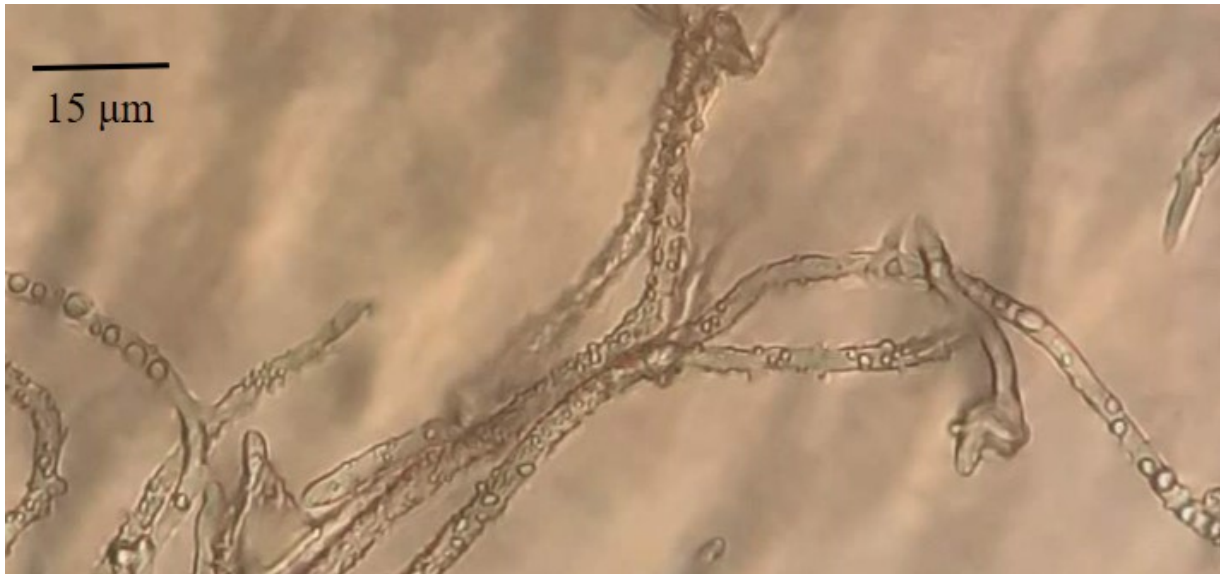


Figure 3.4: Crystalline structures observed on *A. mellea* hyphae after dual culture with *Trichoderma* spp..

During co-inoculation with *T. atrobrunneum* T17/04, 25-day old *A. mellea* CG440 produced crystalline structures on hyphae. Interactions were visualised after three dpi with *T. atrobrunneum* T17/04 at 400 x magnification under brightfield light. Scale bars are represented on individual images.

To further visualise the interaction between *A. mellea* and *Trichoderma* spp., in particular to visualise potential hyphal coiling by *Trichoderma* spp., *Trichoderma* spp. was transformed with green and red fluorescent markers. Green fluorescent strains of *Trichoderma* spp. were transformed with pGFP using *Agrobacterium tumefaciens* AGL-1 (Figure 3.5) and showed good fluorescence in hyphae. No spores were produced to assess fluorescence. Red fluorescing strains of *Trichoderma* spp. were successfully generated with pCAMdsRED using *A. tumefaciens* AGL-1 and LBA1126 (Figure 3.6) where both hyphae and spores readily fluoresced. *Armillaria mellea* CG440 was challenged with red fluorescent strains of *Trichoderma* spp. since this produced low autofluorescence and could be visualised in both hyphae and spores. Red fluorescent *T. harzianum* were visible against *A. mellea* under red fluorescence (Figure 3.7), however, no hyphal coiling or other signs of microparasitic activity by *T. harzianum* was visible when viewed under a light microscope.

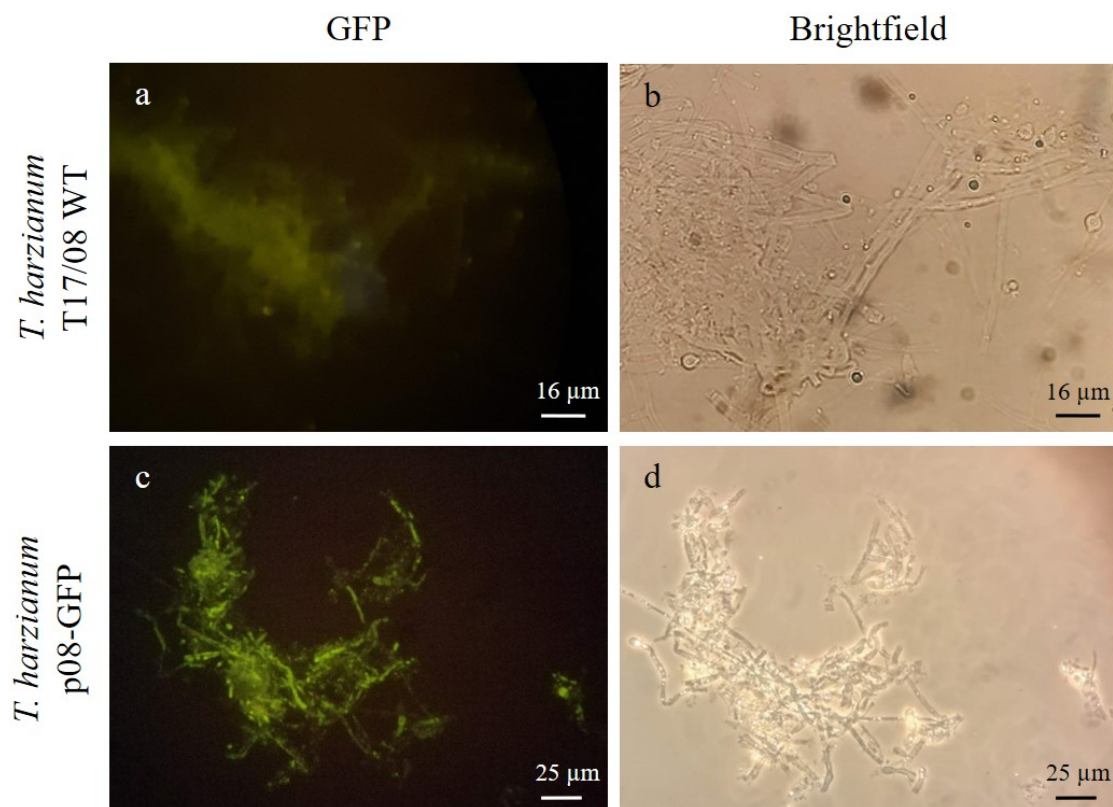


Figure 3.5: GFP *T. harzianum* T17/08 transformants.

Trichoderma harzianum visualised under GFP epifluorescent (a, c) and brightfield (b, d) filters at 400 x magnification. a) Wild type (WT) *T. harzianum* T17/08 showed some autofluorescence under the GFP epifluorescent filter, b) hyphae and spores of WT *T. harzianum* T17/08 are visible under the brightfield filter; c – d) *T. harzianum* T17/08 transformed with *A. tumefaciens* AGL-1 showed clear fluorescence in hyphae. Scale bars are represented on individual images.

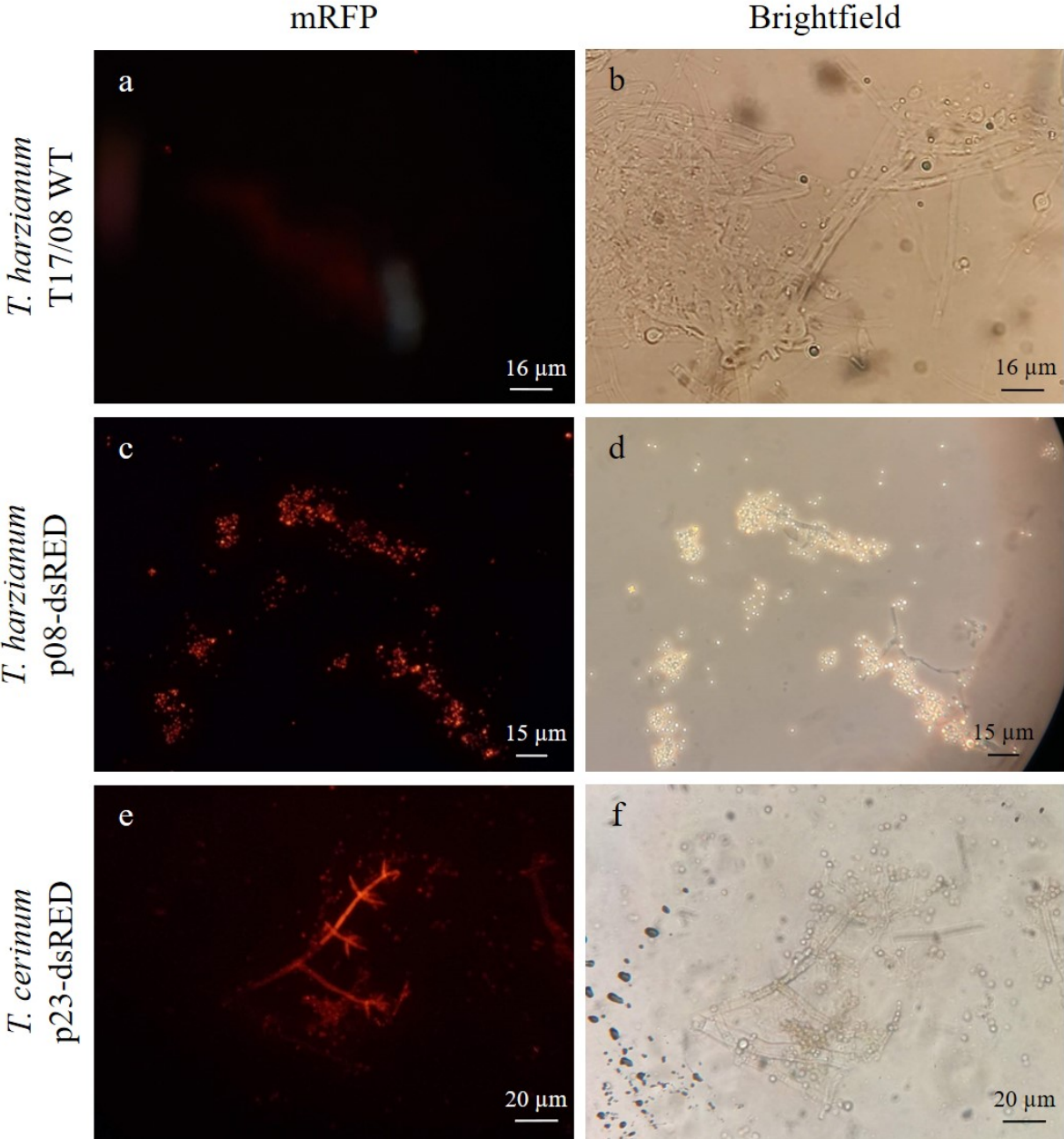


Figure 3.6: mRFP *Trichoderma* spp. transformants.

Trichoderma spp. visualised under mRFP epifluorescent (a, c, e) and brightfield (b, d, f) filters at 400 x magnification. a) Wild type (WT) *T. harzianum* T17/08 showed no fluorescence under the mRFP epifluorescent filter; b) hyphae and spores of WT *T. harzianum* T17/08 are visible under the brightfield filter; c – d) *T. harzianum* T17/08 transformed with pCAMdsRED using *A. tumefaciens* AGL-1 showed clear fluorescence in spores; e – f) *T. cerinum* T17/23 transformed with pCAMdsRED using *A. tumefaciens* LBA1126 showed good fluorescence in spores and hyphae. Scale bars are represented on individual images.

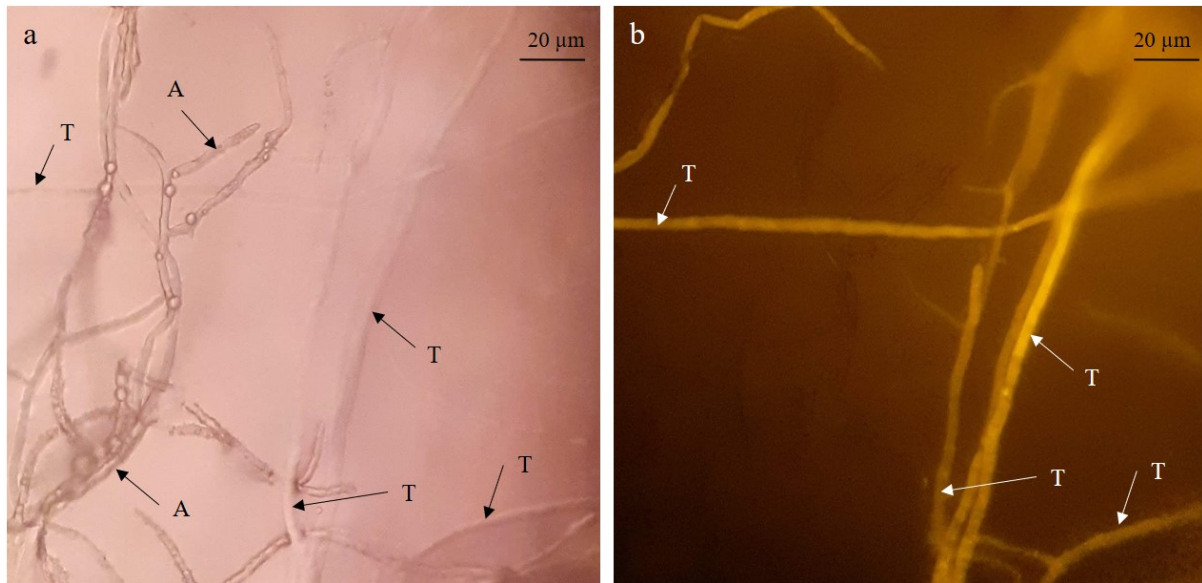


Figure 3.7: *Armillaria mellea* challenged with fluorescent *T. harzianum* T17/08.

Fifteen-day old *A. mellea* CG440 colony challenged with red fluorescent *T. harzianum* T17/08. The interaction between *A. mellea* (A) and *Trichoderma* spp. (T) was visualised 3 dpi under 400 x magnification with brightfield showing both fungi (a) and a red epifluorescent filter showing just *T. harzianum* T17/08 (b). No visible signs of mycoparasitism were observed. Scale bars are presented on individual images.

3.4.4 Controlling *A. mellea* with *Trichoderma* in woody stems

3.4.4.1 Growth of *A. mellea* in woody stem based dual cultures

Since *Armillaria* naturally grows in a woody substrate, the interaction was assessed using pre-colonised sections of woody hazel stems. These were selected because such hazel billets are commonly used as the inoculum for *in planta* bioassays so known to be easily colonised by *Armillaria* and as a woody substrate represents the natural food source of *Armillaria*. The same refined selection of *Trichoderma* isolates were studied as those used for viability tests in agar-based interactions. *Trichoderma* spp. covered *A. mellea* colonised hazel disks and at a similar rate to agar based assays appeared to stop *A. mellea* out-growth. There were no instances of *A. mellea* inhibiting the growth of *Trichoderma* spp. before being covered. Rhizomorphs were inconsistently produced by *A. mellea*. Where rhizomorphs were produced, after 1.5 months in dual culture, in almost all cases they had become blackened. Only in *A. mellea* CG440 in dual culture with *T. hamatum* T17/10 were some rhizomorphs still white.

3.4.4.2 Viability of *A. mellea* in woody stem dual cultures

To assess the viability of *A. mellea* from colonised hazel billets after co-culture with *Trichoderma* spp. the same procedure with agar-based interactions was followed. In all *Armillaria*-only controls, *A. mellea* was still viable from internal wood of hazel disks after six weeks in culture (100%). Two *Trichoderma* isolates (*T. virens* T17/02 & *T. hamatum* T17/10) eliminated *Armillaria* growth for both *A. mellea* isolates tested (Table 3.3). *Trichoderma* isolates *T. harzianum* T17/08, *T. atrobrunneum* T17/11 & T17/15 and *T. olivascens* T17/42 were able to stop *Armillaria* recovery in isolate CG675, and whilst CG440 growth was not eliminated, it was always with reduced frequency of re-isolation success (22%, 67%, 44% and 11% respectively). Only two isolates of *Trichoderma* did not eliminate *A. mellea* recovery from both isolates CG440 & CG675; these were isolates *T. harzianum* T17/03 & T17/07 which allowed re-isolation of *A. mellea* with 11 & 56 % success for CG440 and 22 & 33 % success for CG675 respectively. *Trichoderma* spp. isolates were less likely to eliminate growth of *A. mellea* CG440 than *A. mellea* CG675 in both assays. *Trichoderma* isolates *T. harzianum* T17/07, *T. atrobrunneum* T17/11 and T17/15 were the worst performers in both assays.

Table 3.3: Viability of *A. mellea* from plant based dual culture with *Trichoderma* spp..

Re-isolation of *A. mellea* CG440 & CG675 (%) following six weeks of dual culture with *Trichoderma* spp. isolates using hazel billets pre-colonised with *Armillaria*. Re-isolation was performed using JYG agar to minimise growth of *Trichoderma*. Isolation was deemed successful where *A. mellea* colonies were apparent within two weeks. +/- represent whether *A. mellea* could be eliminated.

| <i>Trichoderma</i> spp. ID | <i>A. mellea</i> CG440 recovery % | <i>A. mellea</i> CG675 recovery % | Elimination by both <i>A. mellea</i> strains (CG440) & (CG675) |
|-------------------------------|-----------------------------------|-----------------------------------|--|
| <i>Armillaria</i> -only | 100 | 100 | |
| <i>T. virens</i> T17/02 | 0 | 0 | + |
| <i>T. harzianum</i> T17/03 | 11.1 | 22.2 | - |
| <i>T. harzianum</i> T17/07 | 55.5 | 33.3 | - |
| <i>T. harzianum</i> T17/08 | 22.2 | 0 | - |
| <i>T. hamatum</i> T17/10 | 0 | 0 | + |
| <i>T. atrobrunneum</i> T17/11 | 66.6 | 0 | - |
| <i>T. atrobrunneum</i> T17/15 | 44.4 | 0 | - |
| <i>T. olivascens</i> T17/42 | 11.1 | 0 | - |

3.4.5 Investigation into rhizomorph production by *A. mellea* in the presence of *Trichoderma* spp.

To investigate the interaction between *A. mellea* and *Trichoderma* spp., colonised hazel stems were nailed together and incubated in sand for seven months. Stems 50 mm in length were colonised by *Trichoderma* spp. (subset used in section 3.4.3) and shorter pieces of hazel, 10 mm in length, were colonised by *A. mellea* CG440 or CG675. The positive control treatment comprised of sterile hazel stems nailed to *A. mellea* CG440 or CG675 segments. Negative controls were comprised of sterile hazel stems nailed together. The experiment contained seven replicates in each treatment. It was expected that in the presence of *Trichoderma* spp., production of rhizomorphs by *Armillaria* would be reduced compared to controls where only *A. mellea* was present.

After one month a random sample from a positive *A. mellea* CG675 control and *A. mellea* CG657 challenged with *T. harzianum* T17/08 was selected for isolation. In the control, *A. mellea* grew from

both the hazel colonised by *A. mellea* and the sterile stem. Where *A. mellea* was challenged by *T. harzianum* T17/08, no *A. mellea* grew from either stem section but *Trichoderma* could be recovered from both. After three months a random selection of hazel billets from *Armillaria*-only controls were visually assessed where *A. mellea* had colonised the adjoining piece of wood as visualised by white mycelial fans under the bark (Figure 3.8a), however, no rhizomorphs were noted. After seven months all treatments were assessed by visual observation. Rhizomorphs were only observed from three *Armillaria*-only controls inoculated with *A. mellea* CG440 (Figure 3.8b). No rhizomorphs were observed in hazel inoculated with *A. mellea* CG675 control treatments or any *Trichoderma* spp. challenge tests. *Armillaria mellea* could not be re-isolated from *Armillaria*-only controls after seven months. During the early stages of the experiment the incubator overheated to 37 °C for a prolonged period at which temperature *A. mellea* cannot grow Rishbeth (1968). In addition, the sand was not regularly moistened to stimulate growth as was previously described by Mihail and Bruhn (2005). A combination of underwatering and overheating is likely to have led to poor growth by *Armillaria* which dried out. This experiment was partially successful at three months where *Armillaria* could be re-isolated from controls without *Trichoderma* spp., but not where *Trichoderma* spp. were present.



Figure 3.8: Hazel billets colonised by *Armillaria mellea*.

Examples of *A. mellea* control billets without the addition of *Trichoderma* spp.. The dashed line highlights the point where 10 mm (*A. mellea*) and 50 mm (sterile) hazel stems were joined; a) *A. mellea* CG675 has colonised the sterile hazel billet and white mycelial growth can be seen under the bark as indicated by the grey arrow at 3 mpi; b) Production of rhizomorphs by *A. mellea* CG440 at 7 mpi.

3.4.6 Enzyme activity of *Trichoderma* spp. with the potential for antagonism

Petri dish based enzyme assays were used to determine whether *Trichoderma* spp. produced extracellular enzymes which may antagonise or degrade *A. mellea*. Production of amylase, cellulose, laccase, pectinase and protease was investigated in the following *Trichoderma* isolates: *T. harzianum* T17/01, *T. virens* T17/02, *T. harzianum* T17/03, *T. atrobrunneum* T17/04, *T. hamatum* T17/10, *T. atrobrunneum* T17/11, *T. atrobrunneum* T17/15, *T. cerinum* T17/30, *T. hamatum* T17/33, *T. hamatum* T17/34 and *T. harzianum* T22 (Figure 3.9). The enzymatic index (EI) was calculated by determining

the relationship between the *Trichoderma* colony and the halo produced after staining in the enzyme assays (section 2.8.5).

All *Trichoderma* isolates tested produced amylase, as indicated by the clear zone around colonies after starch amended Petri dishes were stained with iodine (Figure 3.10a, f, k). The starch was degraded by amylase and so the iodine stain could not bind to the starch. All isolates showed amylase production and whilst most isolates were similar, *T. harzianum* T17/01 was significantly lower (one-way ANOVA $p > 0.05$) than all except for *T. harzianum* T17/03 and *T. cerinum* T17/30.

Cellulase was produced by all isolates except *T. virens* T17/02 as indicated by the yellow halo produced when Petri dishes containing carboxymethylcellulose (CMC) were stained with congo-red dye (Figure 3.10 b, g, l). In some areas the stain did not penetrate hyphae and resulted in uneven staining (Figure 3.10 g, l). There was no significant difference in cellulase EI between *Trichoderma* spp. isolates tested (one-way ANOVA $p > 0.05$) except for *T. virens* T17/02 which was significantly lower (one-way ANOVA $p < 0.001$).

All *Trichoderma* isolates produced laccase activity which showed significant variation in EI between isolates (One-way ANOVA $p < 0.01$). Laccase activity was investigated using nutrient agar amended with tannic acid, which resulted in a colour change in the medium from beige to brown (Figure 3.10 c, h, m). In five *Trichoderma* isolates (*T. harzianum* T17/01, *T. virens* T17/02, *T. atrobrunneum* T17/11, *T. atrobrunneum* T17/15 and *T. harzianum* T22) concentric rings below the colony were produced which decreased in colour intensity with distance from the inoculation point (Figure 3.10 c, h). The number of rings varied between isolates after six days; four rings were produced by *T. atrobrunneum* T17/15, three by *T. virens* T17/02 and *T. harzianum* T22 and two by *T. harzianum* T17/01 and *T. atrobrunneum* T17/11. When incubated in foil to determine whether rings produced were a result of the incubation light cycle, T17/02 did not produce rings but T17/15 continued to produce rings.

The pectinase EI of *Trichoderma* isolates varied significantly (Kruskal-Wallis $p < 0.05$); five *Trichoderma* isolates did not produce pectinase enzymatic activity, and in the six isolates which did, variation in EI was noted. Pectinase activity was indicated by a clear halo produced when Petri dishes

amended with citrus pectin were stained with CTAB, however, the halo produced was often indistinct or irregular (Figure 3.10 i, n). A clear, thin halo was produced by *T. harzianum* T17/01 (Figure 3.10 d) and *T. harzianum* T17/03.

All *Trichoderma* isolates showed protease activity as indicated by a clear area corresponding to *Trichoderma* growth on MM Petri dishes amended with skimmed milk and stained with Amido black, which produces a blue stain when reacting with intact proteins (Figure 3.10 e, j, o). There was no significant difference in protease EI between *Trichoderma* isolates (one-way ANOVA $p > 0.05$).

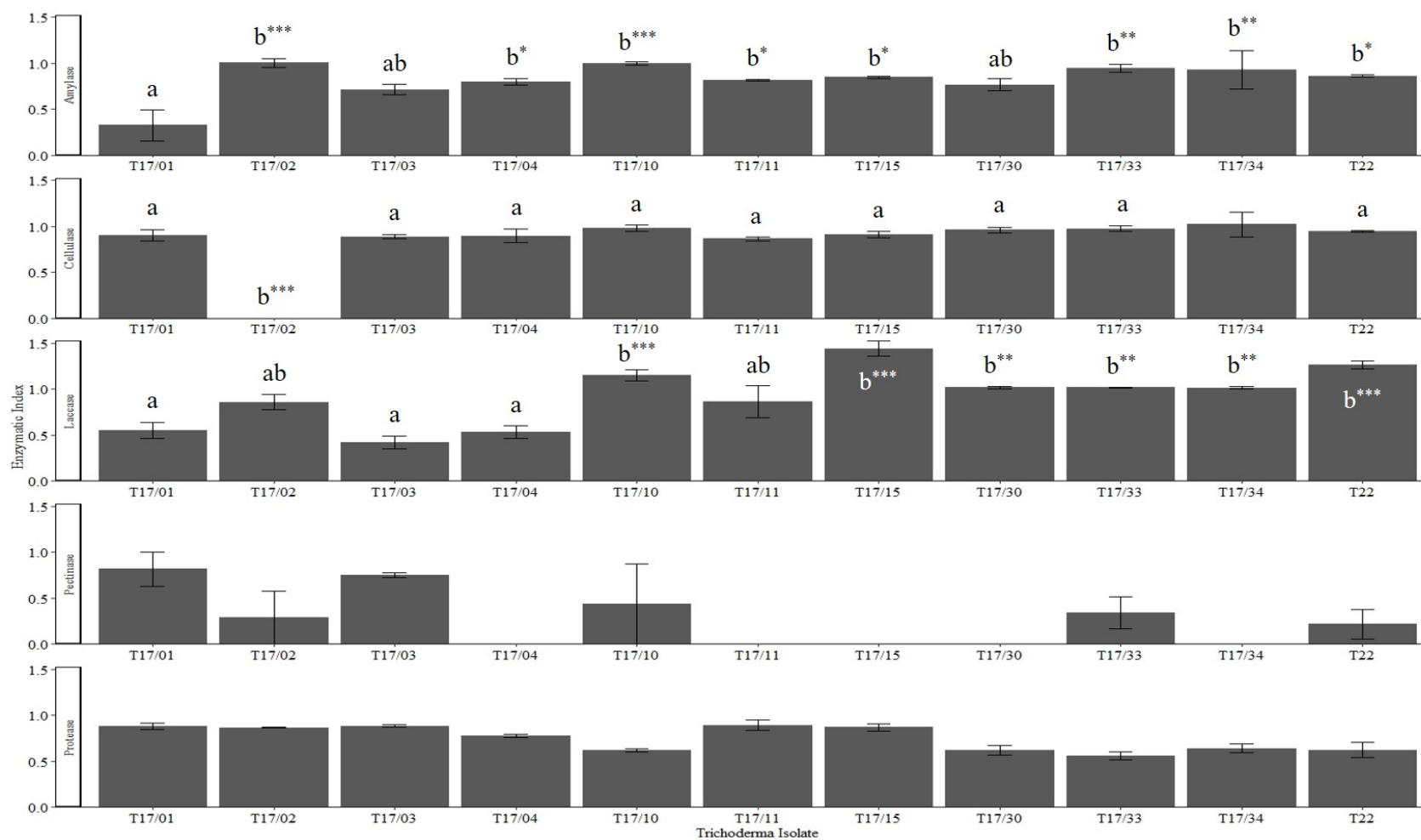


Figure 3.9: Enzymatic index of extracellular enzyme production by *Trichoderma* spp.

Enzymatic index of each *Trichoderma* spp. (n = 3, calculated as halo/colony) for production of amylase, cellulase, laccase, pectinase and protease (top – bottom). Error bars represent the standard error. a,b represent statistical groupings (One-Way ANOVA, Tukey's post hoc test) * p < 0.05, ** p < 0.01, *** p < 0.001.

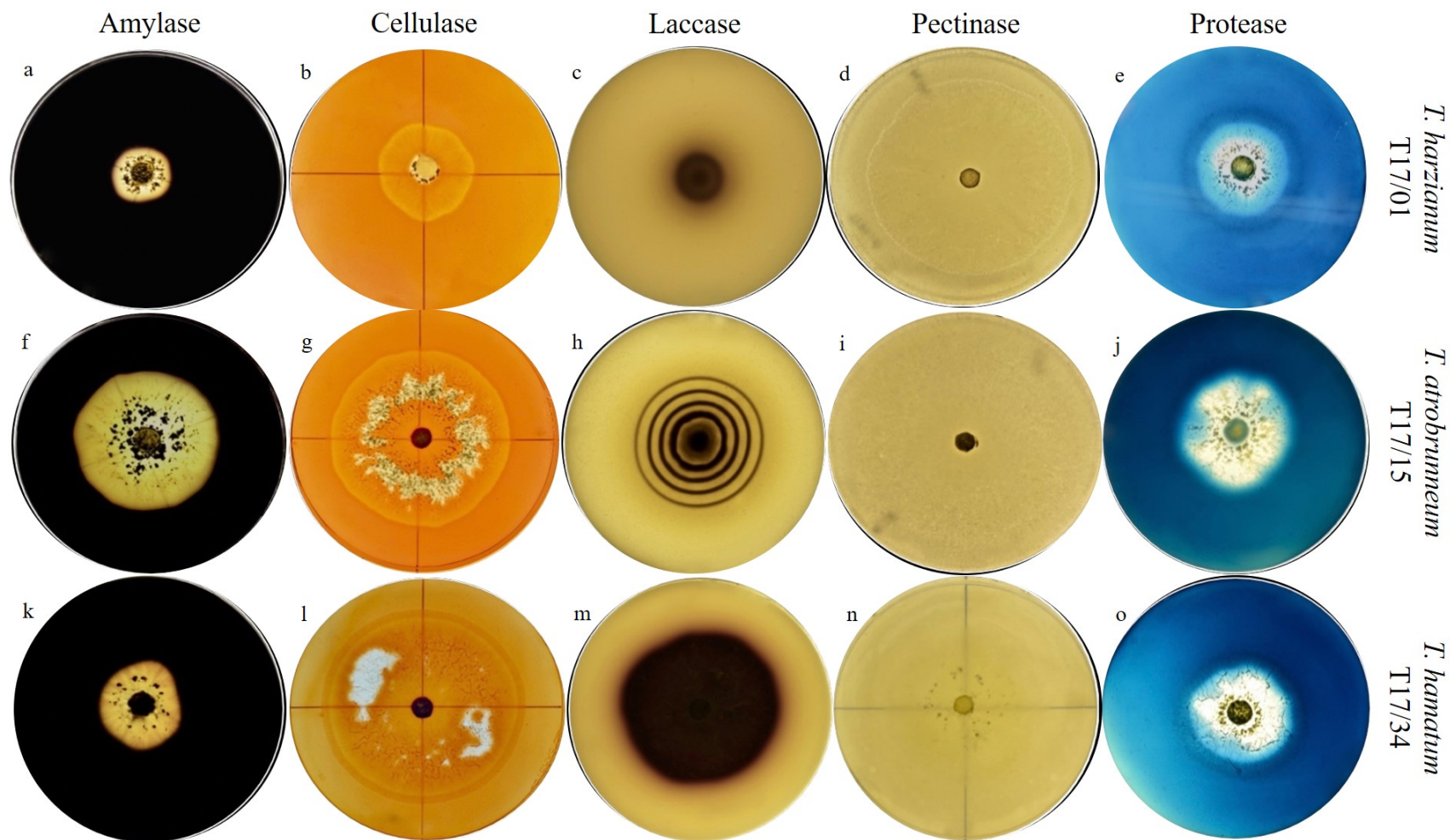


Figure 3.10: Extracellular enzyme production by *Trichoderma* spp. on agar.

Examples of Petri dish-based assays to investigate production of amylase, cellulase, laccase, pectinase and protease in *T. harzianum* T17/01 (a – e), *T. atrobrunneum* T17/15 (f – j) and *T. hamatum* T17/34 (k – o). Images shown are at 4 dpi, except laccase which is 6 dpi.

3.5 Discussion

3.5.1 Culture collection and taxonomy

Population studies of endophytic *Trichoderma* species in Europe represent some similarities in *Trichoderma* species composition as those isolated from RHS Gardens Wisley (UK). In Poland, four *Trichoderma* species (*T. koningii*, *T. polysporum*, *Trichoderma* sp. and *T. viride*) were isolated from roots of Scots pine and oak trees (Kwaśna, 2002, 2004). Another study in Poland isolated six species of *Trichoderma* from beech trees (*T. hamatum*, *T. aureoviride*, *T. harzianum*, *T. strictipile*, *T. koningii*, and *T. viride*) (Kwaśna and Szykiewicz-Wronek, 2018). At the sample sites in the UK (RHS Wisley Gardens) *T. hamatum* and *T. harzianum* were found but there was no other overlap with species found in Poland. This could be due to differences in geographical location, host species sampled or habitat preference of *Trichoderma* spp. as UK isolates were taken from a managed garden as opposed to a forest stand. In a study of *Trichoderma* species isolated from rhizosphere soils of spruce and oak trees, soil samples and *Trichoderma* species associated with *Armillaria* rhizomorphs in Hungary and Austria, Chen *et al.* (2019) isolated 14 species of *Trichoderma* comprised of 64 strains (including *T. hamatum*, *T. atrobrunneum* and *T. virens*). In the present study, *T. atrobrunneum* was the second most isolated species but recorded as fifth by Chen *et al.* (2019) who recorded *T. virens* as the third most isolated species whereas in the UK isolates only one isolate of *T. virens* was confirmed. *Trichoderma hamatum* was isolated four times in this study and three times by Chen *et al.* (2019). In contrast to this study, none of the *Trichoderma* species isolated by Chen *et al.* (2019) were endophytic which could be another factor influencing the variation in *Trichoderma* species identified. Many of the *Trichoderma* species identified from the present study are considered biological control agents of phytopathogens, including *T. atrobrunneum* (Chen *et al.*, 2019), *T. koningiopsis* (Yu and Luo, 2020), *T. hamatum* (Bunbury-Blanchette and Walker, 2019), *T. harzianum* (Bunbury-Blanchette and Walker, 2019) and *T. virens* (Chen *et al.*, 2019). *Trichoderma cerium* is infrequently tested as a biological control agent and has been found to show little biocontrol potential to avocado white root rot (Ruano Rosa and López Herrera,

2009). *Trichoderma olivascens*, *T. hirsutum* and *T. deliquescens* have rarely been tested as biological control agents of phytopathogens and were all infrequently isolated in this study.

3.5.2 Antagonism of *Trichoderma* spp. against *A. mellea* in agar cultures

All *Trichoderma* spp. were very successful at controlling *A. mellea* growth when using agar in Petri dishes. Within eight dpi *Trichoderma* spp. were able to overgrow *A. mellea* and inhibit any further growth. Within six weeks, *Trichoderma* spp. could eliminate *A. mellea* growth, stopping it from being re-isolated from dual culture assays, thus it is presumed *Trichoderma* spp. can probably kill *A. mellea* when assessed using *in vitro* assays. The ability of *Trichoderma* spp. to grow over *Armillaria* colonies has been noted in the literature (Raziq and Fox, 2003; Asef *et al.*, 2008; Chen *et al.*, 2019) where dual culture interaction assays are predominantly conducted using agar-based, artificial substrates. This study has shown with *in vitro* agar-based dual culture interactions that *Trichoderma* spp. can significantly reduce growth and viability of *A. mellea* consistently across a broad range of *Trichoderma* species. A study looking at *T. harzianum* and *T. viride* interactions with *A. mellea* found that after two months, *Armillaria* could not be re-isolated from interaction assays (Raziq and Fox, 2003). The authors also reported that *Armillaria* rhizomorphs did not melanise and appeared to disintegrate after two months co-inoculation. Similarly, in this study *A. mellea* could not be re-isolated from interaction assays with *T. hamatum*, *T. harzianum*, *T. olivascens* and *T. virens* after 1.5 months. While the isolates used in this study were endophytic, the reduced viability of *A. mellea* indicates that *Trichoderma* species do not simply occupy a niche to prevent *A. mellea* colonisation but also actively parasitise the *Armillaria* fungus.

Armillaria mellea was unable to inhibit the growth of any of the *Trichoderma* spp. isolates tested. There were no observations of a deadlock or zones of inhibition from *Armillaria* towards *Trichoderma* spp. which improves the potential of *Trichoderma* spp. to be a successful biocontrol agent. Interaction studies between *A. bulbosa* with other Agaricomycete fungi found that *A. bulbosa* formed a deadlock interaction with five species (*Coriolus versicolor*, *Phallus impudicus*, *Stereum hirsutum*, *Tricholomopsis platyphylla* and *Psathyrella hydrophilum*). The Ascomycota fungus, *Lopadostoma*

turgidum, also formed a deadlock interaction (Chapela *et al.*, 1988). In the present study, however, no deadlock interactions were noted, including between *A. mellea* and the *Trichoderma* isolates which were slow growing (*Trichoderma* sp. T17/13, *T. fertile* T17/37, *T. olivascens* T17/41 and T17/42) highlighting that *Trichoderma* spp. are a potential option for biological control.

The interactions between *Trichoderma* and other plant pathogens *in vitro* have been widely studied. *Trichoderma* is reported to reduce the mycelial growth of *Phytophthora* spp. (Sanchez *et al.*, 2019) and overgrow phytopathogens (Raziq and Fox, 2003; Asef *et al.*, 2008; Troian *et al.*, 2014; Park *et al.*, 2019) as shown in this study. Interactions between *Trichoderma* and fungal or fungus-like plant pathogens have been studied using light microscopy and scanning electron microscopy (SEM). It has been shown that *T. citrinoviride* uses multiple antagonistic mechanisms. It can penetrate the hyphae of *Alternaria panax* and *Phytophthora cactorum*, and in other species, such as *Botrytis cinerea* and *Rhizoctonia solani*, the *Trichoderma* hyphae can coil around the pathogen hypha (Park *et al.*, 2019). A third interaction was recorded by Park *et al.* (2019) where appressoria were formed by *T. citrinoviride* in association with *Cylindrocarpon destructans* and *Pythium* sp.. Multiple antagonistic mechanisms were found by Sanchez *et al.* (2019) from several *Trichoderma* species co-cultured with *Phytophthora cactorum*, *P. inundata* and *P. rosacearum* under both light microscopy and SEM. *Trichoderma* spp. hyphae adhered to and coiled around the *Phytophthora* hyphae which, in some cases, became vacuolated (Sanchez *et al.*, 2019). The majority of published studies explore the interaction between *Trichoderma* and fungal or oomycete hyphae, but *Trichoderma* can parasitise other tissues including apothecia and sclerotia, as seen by *T. harzianum* against *Sclerotinia sclerotiorum* (Troian *et al.*, 2014). Very few studies have looked at the interaction between *Trichoderma* spp. and *Armillaria* spp. at a microscopic level, and what little literature there is examines the effects on rhizomorphs. Using SEM, one study found *T. virens* and *T. harzianum* can coil around, penetrate and sporulate within rhizomorphs of *A. mellea* (Asef *et al.*, 2008) similarly to the interaction between *Trichoderma* spp. and hyphae of other phytopathogens. Another study using SEM showed *Trichoderma* spp. (*T. polysporum*, *T. harzianum* and *T. viride*) could penetrate and coil around rhizomorph hyphae of *A. gallica*. After one week *Trichoderma* spp. conidia germinated on and penetrated *A. gallica* rhizomorphs. Internal hyphae

of rhizomorphs were parasitised by *Trichoderma* spp. and after one week, were devoid of hyphae (Dumas and Boyonoski, 1992). The agar based dual culture assays carried out in this study show that *Armillaria* was no longer viable after co-culture with five *Trichoderma* isolates. Using microscopy, no hyphal coiling by *Trichoderma* was observed, however, after three days dual interaction with *T. atrobrunneum* or *T. harzianum*, *A. mellea* hyphae showed signs of degradation. The degradation might be a result of internal colonisation by *Trichoderma* spp., or by production of antifungal agents causing hyphal degradation. Potential parasitism by *Trichoderma* spp. could be confirmed in the future by freeze fracture to visualise a cross section of *A. mellea* hyphae and any internal colonisation by *Trichoderma* spp. using SEM and/or red fluorescent *Trichoderma* strains.

In co-culture with *T. atrobrunneum* T17/04, crystalline structures were noted on *A. mellea* CG440 hyphae which were most similar in appearance to crystals found on *Suillus luteus* (Böllmann *et al.*, 2010). Crystalline hyphal structures are considered a defence strategy used by fungal species including *Piloderma croceum*, *S. collinitus*, *S. flavus* and *S. luteus* against the Collembola species *Folsomia candida*. *Armillaria ostoyae* was included as a control group where no crystalline structures on hyphae were present (Böllmann *et al.*, 2010). Although observations of potentially defensive crystalline structures on *A. mellea* hyphae were made, no defensive interactions, such as defence barrages, were visually observed in agar dual culture assays. The microscopic interaction should be further studied to determine the frequency and cause of crystalline structures produced by *A. mellea*.

3.5.3 Antagonism of *Trichoderma* spp. against *A. mellea* in woody stems

Interaction assays between *A. mellea* and *Trichoderma* spp. on agar gave an indication into the antagonism offered by *Trichoderma* spp. and how both fungi may interact on a woody substrate. While interactions between two fungi can easily be studied through *in vitro* dual culture assays with artificial media, these systems fail to reflect the complexity of natural fungal systems (Crowther *et al.*, 2018). Sections of hazel colonised by *A. mellea* were grown on agar in Petri dishes and challenged with *Trichoderma* spp. in attempt to increase substrate complexity to study the interaction between *A. mellea* and *Trichoderma* spp.. After 1.5 months *Armillaria* was eliminated from the hazel in the presence of

two *Trichoderma* spp. (*T. virens* T17/02 and *T. hamatum* T17/10). As noted in agar based interactions, the elimination of *A. mellea* meant it was probably killed by *Trichoderma* spp., either by mycoparasitism or production of antifungal agents. Cox and Scherm (2006) studied the antagonism of saprobic fungi towards *Armillaria* spp. in wood blocks. Like with *Trichoderma* spp., *Desarmillaria tabescens* and *A. mellea* were overgrown by fungi including *Ganoderma lucidum*, *Hypholoma fasciculare*, *Phanerochaete velutina* and *Xylaria hypoxylon* and for all five species tested (including *Schizophyllum commune*) the viability of *Armillaria* spp. was less than 30 % (Cox and Scherm, 2006). Another study also observed the elimination of *Armillaria* by *Trichoderma* spp. in tea stems. After 12 weeks co-incubation, *Trichoderma* spp. could be isolated from stem segments colonised by both *Armillaria* sp. and *Trichoderma* spp. and *T. harzianum* could eliminate *Armillaria* sp. from tea stems (Otieno *et al.*, 2003). A series of studies used oak segments to investigate the effect of fungi from the rhizosphere of oak and beech trees on the growth of rhizomorphs by *Armillaria* spp. (Kwańska *et al.*, 2004; Kwańska and Szykiewicz-Wronek, 2018, respectively). Six of nine species of oak rhizosphere fungi were found to stimulate the growth of *A. ostoyae* rhizomorphs, however, two *Trichoderma* species were found to have antagonistic properties towards *A. ostoyae* where rhizomorph growth was inhibited (Kwańska *et al.*, 2004). In contrast, only two out of 26 fungal species isolated from beech rhizospheric fungi were found to have stimulatory effects on *A. gallica* and *A. ostoyae* (Kwańska and Szykiewicz-Wronek, 2018). In both *A. gallica* and *A. ostoyae*, growth of *Armillaria* rhizomorphs was inhibited by *T. aureoviride*, *T. koningii* and *T. viride* where inhibition was much greater in *A. ostoyae* (100 %) than *A. gallica* (28 - 75 %).

The viability of *Armillaria* colonies challenged by *Trichoderma* using agar plugs or hazel as a food source was isolate-dependent. Isolate *A. mellea* CG440, which is generally regarded to be highly virulent and is commonly used in the UK for *A. mellea* based experiments (Beal *et al.*, 2015; Ford *et al.*, 2015, 2017), was less susceptible to *Trichoderma* spp. and remained viable after dual culture more often than isolate CG675 in both agar- and wood- based food sources. Similarly, differences in the effect of *Trichoderma* spp. on *A. ostoyae* isolates was previously reported by Kwańska *et al.*, (2004). Isolates *T. harzianum* T17/07 and *T. atrobrunneum* T17/11 were consistently less effective against *A.*

mellea CG440 than other isolates including *T. virens* T17/02 and *T. hamatum* T17/10, which killed *A. mellea* in all conditions. Because isolates of *Trichoderma* spp. used in this study are endophytic, they may not all provide antagonism towards fungi, and this could account for the differences noted. The variability in effectiveness of *Trichoderma* spp. and isolates to reduce the viability of *A. mellea* colonies suggests that a combination of *Trichoderma* isolates might be required to achieve a broad control on a population wide scale of *A. mellea* infection. Additionally, the use of local *Trichoderma* isolates is possibly important since these isolates are likely to be acclimatised to local conditions and therefore could be more effective in a biological control situation than other commercial isolates. These *Trichoderma* isolates should also be tested *in planta* for protection from ARR because of differences between *Trichoderma* spp. and *A. mellea* interactions based upon different food-sources in controlled *in vitro* environments so their effectiveness *in planta* cannot be predicted.

3.5.4 Use of *in vitro* assays to screen for biocontrol agents

Whilst agar based challenge interactions are simple and informative assays for understanding fungus-fungus interactions, they do not fully represent the complex environments found in nature (Crowther *et al.*, 2018). In attempt to bridge this gap, segments of wood were placed onto malt agar which kept the immediate environment damp but gave increased heterogeneity in which to observe the interaction between *A. mellea* and *Trichoderma* spp. under controlled conditions. When *A. mellea* used a woody substrate as its food source, two *Trichoderma* isolates were able to control its growth and prevent viable recovery. These two isolates, *T. virens* T17/02 and *T. hamatum* T17/10 were successful in controlling *A. mellea* in a situation that more closely resembles its natural growth conditions, and warrants further investigation *in planta*. Although all 40 *Trichoderma* spp. isolates tested had a fairly similar ability to control *A. mellea* in dual culture assays with agar as a food source, when the woody substrate was present, some *Trichoderma* spp., such as *T. virens* T17/02 and *T. hamatum* T17/10 appear to have an advantage.

In choosing potential isolates for biological control trials it can be useful to carry out *in vitro* tests first (Pearce, 1990; Sanchez *et al.*, 2019) before carrying out plant based experiments to select the best

isolates. This, however, only highlights isolates which directly affect the pathogen through mechanisms such as antibiosis and mycoparasitism and not those which might change the host in its reaction to attack by fungal pathogens. Studies comparing the potential of biological control agents against root rot pathogens *in vitro* and *in planta* have had mixed results. *Trichoderma* spp. isolates were screened for antagonism and siderophore production *in vitro* and two isolates, which performed well *in vitro*, were tested for their ability to control ARR in bare rooted Turkey oak seedlings (Chen *et al.*, 2019). Both isolates (*T. virens* and *T. atrobrunneum*) were isolated from local soil samples associated with *Armillaria* rhizomorphs (the *Armillaria* could not be re-isolated), and showed good antagonism *in vitro* and when applied together, reduced ARR in field trials (Chen *et al.*, 2019). Park *et al.* (2018) found that endophytic *T. citrinoviride* isolated from ginseng roots could reduce the mycelial growth of four fungal and two oomycete pathogens *in vitro* (*Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria panax*, *Cylindrocarpon destructans*, *Phytophthora cactorum* and *Pythium* spp.). When inoculated onto ginseng roots to test against *B. cinerea* or *C. destructans*, *T. citrinoviride* reduced incidence of both pathogens. The aforementioned studies suggest that use of *in vitro* assays to select potential biological control isolates of *Trichoderma* spp. is a reliable method to reduce numbers of isolates tested for biological control *in planta*. In contrast, Raziq and Fox (2003) found that one isolate of *T. harzianum* which was highly antagonistic against *A. mellea* *in vitro* was not effective in strawberry plants. In this case, the *Trichoderma* sp. used were isolated as pathogens from commercial mushrooms thus may not have the ability to form beneficial endophytic associations with host plants. In the present study, *Trichoderma* spp. were isolates known to form endophytic associations, thus could have a greater chance as a biological control agent against *A. mellea* if they are able to form endophytic associations in host plants.

3.5.5 Production of enzymes by *Trichoderma* spp.

To understand the mechanism by which *Trichoderma* species antagonise *A. mellea*, the production of extracellular enzymes by *Trichoderma* spp. was assessed. Little or no variation in production of amylase, cellulase or protease was found between most isolates (representing five *Trichoderma* spp.) tested. Purified amylase from a *T. harzianum* isolate has been shown to have no effect on the Agaricomycete *Moniliophthora perniciosa* using SEM, suggesting that amylase used by *T. harzianum*

hydrolyses starches from the surrounding environment (De Azevedo *et al.*, 2000; De Marco *et al.*, 2003). This suggests that perhaps the amylase produced by *Trichoderma* spp. has no effect on *A. mellea* and a similar study could determine if amylase had any direct influences on *A. mellea*.

Trichoderma reesei, is a major species for production of a range of cellulase enzymes by the genus *Trichoderma* and is widely used in industry for production of renewable biofuels (Bischof *et al.*, 2016). The cellulase activity of *Trichoderma* spp. for isolates with biocontrol potential has been reported, although the enzyme is not directly linked to biological control activity. For example, it was found that *T. asperellum*, which can control the mango anthracnose fungus, *Colletotrichum gloeosporioides*, though competition produced cellulases (de los Santos-Villalobos *et al.*, 2013) which potentially aided the competitive advantage of *T. asperellum*. Cellulase producing isolates of *T. viride* and *T. harzianum* have shown antagonism towards *Aspergillus flavus* and *Fusarium moniliforme in vitro*, however, these isolates were also found to produce other extracellular enzymes (Calistru *et al.*, 1997) which are likely to act in combination to achieve biocontrol through various mechanisms other than direct degradation of fungal cells. In this study *Trichoderma* spp. isolates antagonistic towards *A. mellea in vitro* were found to be good producers of cellulase, although little variation was noted between isolates.

All *Trichoderma* isolates produced protease, with some, insignificant variation between isolates. Protease from *T. harzianum* has been shown to have antagonistic effects against *F. oxysporum*, *S. sclerotiorum*, *R. solani*, *Cytospora chrysosperma* and *Alternaria alternata*. Using enzyme assays, inhibition zones and abnormal mycelial growth were observed for each of these pathogens (Yan and Qian, 2009). Proteases from *T. harzianum* have been also shown to hydrolyse the cell wall of the Agaricomycetes *M. perniciosus* (De Marco and Felix, 2002), which suggests that *Trichoderma* isolates with protease activity may have an advantage as potential strains for biological control of *Armillaria mellea*.

Laccase production was variable between *Trichoderma* species and isolates. One study found when the laccase gene (*lcc1*) was deleted from *T. virens* the ability to degrade the sclerotia of *Botrytis cinerea* was significantly reduced but in *Sclerotinia sclerotiorum* degradation was enhanced. The difference in

sclerotial formation of these fungi is thought to be the reason for variation in degradation ability of sclerotia by *T. virens* (Catalano *et al.*, 2011). Since rhizomorphs of *Armillaria* spp. are hyphal aggregations with a melanised outer cortex (Garraway *et al.*, 1991), the direct function of laccase on rhizomorphs of *Armillaria* spp. should be further investigated.

Pectinase activity was variable between *Trichoderma* isolates. None of the *T. atrobrunneum* isolates tested produced pectinase, whereas the *T. harzianum* isolates tested generally had the highest production of pectinase. As pectinases are plant cell wall degrading enzymes (Mojsov, 2016), it is perhaps expected that low levels of pectinases will be found in *Trichoderma* spp.. In a study on bean leaves infected with *B. cinerea* and inoculated with a biocontrol strain of *T. harzianum*, a reduction in pectinases (polygalacturonases, pectin methyl esterase and pectate lyase) from the pathogen *B. cinerea* was noted, suggesting that *T. harzianum* is able to reduce the ability of *B. cinerea* to cause plant infection (Zimand *et al.*, 1996; Kapat *et al.*, 1998). The ability to limit the enzyme activity of pathogens during infection stage could be a key method of antagonism against *A. mellea* in plants and should be investigated further.

Trichoderma isolates exhibited good extracellular enzyme activity for amylase, laccase, protease and cellulase *in vitro* and are likely to be involved in antagonism towards *A. mellea*. The degradation of *A. mellea* hyphae when grown in the presence of *Trichoderma* spp. could be a result of enzyme production from *Trichoderma* spp.. The direct effect of extracellular enzymes produced by *Trichoderma* spp. should be further investigated. While the enzymes assessed in this study were produced *in vitro*, the production of extracellular enzymes in the presence of plants could vary and therefore should also be investigated to fully understand the role of enzymes produced by *Trichoderma* spp. to antagonise fungal pathogens such as *A. mellea*. This work would be further enhanced with the inclusion of chitinases and glucanases which are both important extracellular enzymes produced by *Trichoderma* spp.. Chitinases and glucanases can break down β -glucans, chitin and polysaccharides in fungal cell walls and impact cell wall integrity (Howell, 2003) an important feature for a biological control agent against fungal plant pathogens.

In conclusion, the *in vitro* assays presented in this chapter have demonstrated the potential of *Trichoderma* spp. as a biological control agent against *A. mellea*. Using a range of *in vitro* assays has enabled the interaction between *A. mellea* and *Trichoderma* spp. to be studied in detail. The use of hazel billets successfully created an artificial assay whereby the interaction could be studied in a medium which more closely resembled the habitat of *A. mellea* in the natural environment. Microscopy studies of both fungi in dual culture and investigating the production of enzymes by *Trichoderma* spp. have begun to consider the mechanism by which *Trichoderma* spp. might be antagonising *A. mellea*.

3.6 Summary

- A collection of 40 endophytic *Trichoderma* species was created comprising of 12 species; the most common were *T. cerinum*, *T. atrobrunneum*, *T. harzianum* and *T. hamatum*.
- Dual culture assays on agar in Petri dishes showed that growth of *A. mellea* was suppressed within four days for almost all *Trichoderma* isolates.
- In agar-based assays, *A. mellea* was killed by five of eight *Trichoderma* isolates tested. Observation of *A. mellea* hyphal degradation was frequently noted when interaction zones were visualised by microscopy.
- In dual culture assays on wood, *A. mellea* growth was suppressed by *Trichoderma* spp. and two isolates (from a selection of eight) could kill *A. mellea* in internally colonised wood for both *A. mellea* strains tested.
- Eleven *Trichoderma* strains were tested for enzyme production. All isolates showed amylase, laccase and protease activity. Ten isolates showed cellulase activity and activity of pectinase was shown in six isolates.

| Chapter 4: Investigation into the potential biological control offered by *Trichoderma* spp. against *Armillaria* root rot *in planta*

4.1 Introduction

Whilst *in vitro* assays provide an informative basis for the interaction between antagonists and pathogens, the tendency is to explore these relationships in homogeneous environments which leaves questions of complex environmental interactions unanswered (Crowther *et al.*, 2018). *In planta* assays fill this gap by adding relevant complexity to investigate the interactions in pathogen-host-antagonist systems with the additional opportunity to identify growth promotion by antagonists such as *Trichoderma* spp.. For studies using pervasive pathogens such as *Armillaria*, field-based experiments are limited, to avoid creating new sites of infection. Therefore, pot-based experiments were relied upon which also allowed suitable replication within experiments and use of controlled environmental conditions. To carry out *Armillaria* infection assays a new and faster inoculation technique needed to be developed to reduce the time-frame required to prepare inoculum for plant based experiments. The basis of this chapter is to investigate how *Trichoderma* and *Armillaria* interact *in planta* with prior knowledge of their interactions based on *in vitro* studies.

4.1.1 *Armillaria* inoculum for plant infection

Current methods used to inoculate plants with *Armillaria* to study infection and biology of the pathogen are laborious time consuming. *Armillaria* inoculum is primarily prepared using woody billets, usually hazel, cut to 15 – 20 mm diameter and 50 – 100 mm in length (Fox and Popoola, 1990; Mansilla *et al.*, 2002; West and Fox, 2002; Popoola and Fox, 2003; Raziq and Fox, 2003, 2005; Ford *et al.*, 2017) which are colonised with *Armillaria* over one – six months before being ready to use in experiments. Other studies have used different substrates to prepare inoculum in a similar time-frame including, billets of alder (*Alnus rubra*) (Entry *et al.*, 1992), aspen (*Populus tremuloides*) (Klein-Gebbinck *et al.*, 1993), beech (*Fagus sylvatica*) (Solla *et al.*, 2002), eucalyptus (*E. diversicolor*) (Pearce and Malajczuk, 1990;

Nelson *et al.*, 1995; Mansilla *et al.*, 2002), oak (*Quercus* spp.) (Tsopelas and Tjamos, 1999) and nuts such as acorns (*Quercus* spp.) (Beckman) and sweet chestnuts (*Castanea sativa*) (Calvet *et al.*, 2015). Woody inoculum is either placed in direct contact with roots (Entry *et al.*, 1992; Klein-Gebbinck *et al.*, 1993; Beckman and Pusey, 2001; Mansilla *et al.*, 2002; West and Fox, 2002; Popoola and Fox, 2003; Raziq and Fox, 2003) sometimes being sealed with a layer of parafilm, or similar (Beckman and Pusey, 2001; Solla *et al.*, 2002; Mesanza *et al.*, 2017; Elias-Roman *et al.*, 2019) or in other instances inoculum is placed at a distance from host roots (Pearce and Malajczuk, 1990; Tsopelas, 1999; Calvet *et al.*, 2015; Ford *et al.*, 2017). Rather than using a woody inoculum Percival *et al.* (2011) made an ‘*Armillaria* slurry’ from blended lab grown *Armillaria* agar cultures. The ‘*Armillaria* slurry’ was spread over a raised bed containing a general potting compost: John Innes No 2: wood chip (40:40:20) which *Armillaria* could colonise, this was left for one year and then used as a planting medium for experiments. *Armillaria* colonisation of strawberry plants was successful, however the preparation time increased from one – six months to a year when most are trying to reduce inoculum preparation time. Ford *et al.* (2017) found that using *Armillaria* agar plugs was not an effective inoculum source when tested on a range of herbaceous and a woody hosts including strawberries for pot plant experiments. The agar plugs dried out and so the *Armillaria* could not effectively colonise host plants, whereas with woody inoculum this was not an issue. Re-isolation of *Armillaria* from inoculum at the end of the experiment was only possible with woody inoculum. Under lab conditions however, tissue cultures of grapes were successfully infected with *Armillaria* agar plugs (Baumgartner *et al.*, 2010a) which required a short preparation time of seven days. While the inoculum preparation time is short, the tissue cultures were rooted for two months prior to inoculation. Additionally plants were grown in agar medium and so they do not represent the soil environment. A method developed by Mansilla *et al.* (2002) reduced the colonisation time of woody inoculum to two weeks using 50 mm lengths of hazel or eucalyptus which were incubated horizontally rather than vertically which is the traditional method. This is a dramatic reduction in the time required to prepare inoculum but the hazel or eucalyptus still has to be collected and cut to size which is the most laborious section of the protocol. Individual seeds of acorns (Beckman and Pusey, 2001) and sweet chestnuts (Calvet *et al.*, 2015) have successfully been used as substrates

for *Armillaria* inoculum requiring two – three months or five months colonisation, respectively. Here I evaluated the effectiveness of using rice sawdust tomato (RST) medium (used to induce fruiting of *Armillaria in vitro*), acorns (*Quercus sp*) or horse chestnuts (*Aesculus hippocastanum*) each colonised for one month as alternative substrate for *Armillaria* inoculum. These alternative inoculum substrates do not require a laborious preparation stage which is an added benefit. Horse chestnuts and acorns can be readily collected in season or brought from commercial seed producers and only require weighing to achieve a standard size and disregarding of damaged seeds before autoclaving three times. RST is prepared in the lab as per any other routine medium. Within one – two months the inoculum was fully colonised and ready to use for pot-based assays.

4.1.2 Using *Trichoderma* as a biological control agent in plants

4.1.2.1 Colonisation of plant roots by endophytic *Trichoderma*

Knowledge of the ability for *Trichoderma* to colonise particular plant hosts is important before studies reliant on endophytic colonisation can take place, to investigate both growth promotion and biological control. Using spore suspensions to inoculate plants with *Trichoderma* has been used in numerous plant systems including *Pinus radiata* (Hill *et al.*, 2016; Amaral *et al.*, 2019), maize (*Zea mays*), ryegrass (*Lolium perenne*) (Cripps-Guazzone *et al.*, 2016), olives (*Olea europaea*) (Ruano-Rosa *et al.*, 2016), tomato (*Solanum lycopersicum*) seeds (Sanchez *et al.*, 2019) and oak (*Quercus cerris*) seedlings (Chen *et al.*, 2019). Other studies have used *Trichoderma*-inoculated rice grains to inoculate plants in vineyard settings (Savazzini *et al.*, 2009) or used granules of the commercial strain *T. harzianum* T22 on red clover (*Trifolium sp.*) (Vitale *et al.*, 2012). Crucially strawberry (*Fragaria × ananassa*) roots can be inoculated with *Trichoderma* spp. via root dipping and drip irrigation (Porrás *et al.*, 2007). To date, there have been no reports of *Trichoderma* colonisation in privet roots.

4.1.2.2 *Trichoderma* as a plant growth promoter

Trichoderma species are widely regarded as plant growth promoting fungi. Studies have found *T. harzianum* to promote growth in *P. sylvestris* (Halifu *et al.*, 2019) and maize plants (Akladios and

Abbas, 2014). *Trichoderma hamatum* has been found to promote growth of *P. radiata* (Hohmann *et al.*, 2011). *Trichoderma virens* has been reported to promote plant growth on a range of plants including Japanese mustard spinach (*Brassica rapa* var. *perviridis*), tomato (*Solanum esculentum*), radish (*Raphanus sativus*) (Zaw and Matsumoto, 2020), and *P. sylvestris* (Halifu *et al.*, 2019). A number of parameters are frequently measured for plant growth promotion including seedling emergence (Hohmann *et al.*, 2011; Kandula *et al.*, 2015), stem or plant height (Hohmann *et al.*, 2011; Akladios and Abbas, 2014; Halifu *et al.*, 2019; Shang *et al.*, 2020; Zaw and Matsumoto, 2020) fresh shoot and / or root weight (Akladios and Abbas, 2014; Halifu *et al.*, 2019; Shang *et al.*, 2020; Zaw and Matsumoto, 2020), dry shoot and / or root weight (Hohmann *et al.*, 2011; Akladios and Abbas, 2014; Halifu *et al.*, 2019; Shang *et al.*, 2020) with some including number of leaves or leaf size (Rabeendran *et al.*, 2000; Akladios and Abbas, 2014). In some cases chlorophyll content and leaf pigments were measured (Akladios and Abbas, 2014; Wonglom *et al.*, 2020). There are numerous and complex means by which *Trichoderma* can promote plant growth. One study demonstrated, *in vitro*, that *T. harzianum* could dissolve insoluble elements such as manganese dioxide, zinc, phosphate, and iron (III) oxide (Altomare *et al.*, 1999). Fungal volatile organic compounds (VOCs) have been shown to enhance growth promotion of lettuce by *T. asperellum*. Lettuce plants were exposed to VOCs produced by *Trichoderma* for two weeks and resulted in significant increases in the number of leaves and roots and the fresh and dry weight of plants as well as the chlorophyll content (Wonglom *et al.*, 2020). Upregulation of indole-3-acetic acid (IAA) genes suggest that *T. asperellum* growth promotion in tea (*Camellia sinensis*) (Shang *et al.*, 2020) is a result of production of growth inducing hormones. *Trichoderma* has also been credited with the ability to increase photosynthetic potential in plants (Harman *et al.*, 2019) which in turn results in increased plant growth.

4.1.2.3 *Trichoderma* as a biocontrol agent of fungal plant pathogens

Although *Trichoderma* is able to promote the growth of plants, increase nutrient uptake and reduce the effect of drought, it is of particular interest in this study for its potential as a biological control agent. *Trichoderma* has frequently been cited as a successful biological control agent against fungal plant pathogens. *Trichoderma asperellum* has been found to reduce disease incidence of anthracnose

(*Colletotrichum gloeosporioides*) by 58.37 % in tea plants (Shang *et al.*, 2020). A reduction in disease severity by *T. virens* was noted for *Sclerotinia trifoliorum* on red clover (Kandula *et al.*, 2015) and *Fusarium oxysporum* f. sp. *lycopersici* on Japanese mustard spinach, tomato and radish (Zaw and Matsumoto, 2020). *Trichoderma atroviride*, *T. hamatum*, *T. koningiopsis* and *T. viride* were also noted to significantly reduce disease incidence of *Sclerotinia trifoliorum* on red clover (Kandula *et al.*, 2015). Studies with strawberry and beans plants have shown *T. harzianum* to reduce plant death or disease incidence by *Rhizoctonia solani* (Elad *et al.*, 1981) and commercial *T. harzianum* strains including T22 have been shown to reduce *F. oxysporum* f. sp. *radicis-cucumerinum* on cucumber (Javanshir Javid *et al.*, 2016) and *Calonectria pauciramosa* disease on red clover (Vitale *et al.*, 2012).

As with plant growth promotion, *Trichoderma* works in many complex ways to protect host plants from disease. When tea plants were inoculated with *T. asperellum*, up-regulation of disease resistance genes was enhanced compared to *Trichoderma*-free controls. Expression of jasmonic acid and ethylene responsive genes (Shang *et al.*, 2020) suggests *T. asperellum* protects plants from disease through induced systemic resistance. Additionally a study looking at VOC's produced by *T. asperellum* in lettuce found that exposure to VOCs increased the levels of plant defence enzymes β -1,3-glucanase and chitinase. The study found 22 VOCs in total which showed antifungal activity, induced disease defence or promoted plant growth (Wonglom *et al.*, 2020).

4.1.2.4 *Trichoderma* as a biocontrol agent of *Armillaria*

Trichoderma has been tested for biological control of *Armillaria* with some signs of success. Most recently, Chen *et al.* (2019) found a mix of two *Trichoderma* species, *T. atroviride* and *T. virens*, isolated from decaying rhizomorphs could reduce the severity of ARR. In strawberries, *T. harzianum* could reduce *Armillaria* disease severity (Raziq and Fox, 2003, 2004b, 2005) and in apple seedlings *T. harzianum*, *T. viride* and *T. hamatum* could reduce ARR infection (Raziq and Fox, 2006b). One study found ARR disease was reduced in strawberry plants which were mulched with bark (from pine, larch and fir) pre-inoculated with *T. atroviride*. In pot based studies the *Trichoderma* mulch reduced disease severity of *Armillaria* infection to by 50 % compared to controls (Pellegrini *et al.*, 2014). *Trichoderma*

has been trialled as a preventative treatment in eucalyptus stumps. A study found that inoculation of a *Trichoderma* spore suspension including (*T. hamatum*, *T. virens* and *T. harzianum*) could limit the root colonisation of eucalyptus by *A. luteobubalina* (Nelson *et al.*, 1995).

The potential of *Trichoderma* spp. to reduce *Armillaria* infection in plants has been considered above. The selection of *Trichoderma* isolates has mainly focused on isolates pathogenic to commercial mushrooms (Raziq and Fox, 2003, 2004c, 2005) and those isolated from degrading *Armillaria* rhizomorphs (Chen *et al.*, 2019). Use of truly endophytic *Trichoderma* isolates, such as those used here, is relatively rare. Endophytes provide multiple benefits to plants, including growth promotion, enhancing tolerance to stress and protection from diseases (Latz *et al.*, 2018), thus were considered likely to provide greater benefits to the plant overall, compared to soil-dwelling biological control agents. The long-term goal of this project is to develop a selection of plant hosts pre-colonised by *Trichoderma* spp. with protection from *Armillaria* infection. Thus, protected plants would be marketed for gardeners and professionals as safe to plant where *Armillaria* is present in the soil. The potential of using host plants pre-colonised with endophytic *Trichoderma* isolates to offer protection from ARR is considered in this chapter.

4.2 Statement of collaboration

The results from section 4.4.1.2 come from a collaboration with Dr. Jassy Drakulic, PhD supervisor and Plant Pathologist at RHS Wisley and Luke Hailey, PhD student with Bartlett Trees and The University of Reading. The aim of the study was to investigate the use of alternative substrates for *Armillaria* inoculation which is due to be published as a collaborative methods paper. In this section only the results I collected on strawberry plants will be reported which form part of a wider experiment including biological replicates and comparisons between host systems using strawberry and privet.

4.3 Aims:

To use plant based systems to improve techniques for *Armillaria* inoculation and to investigate plant responses to *Trichoderma* and the potential for biological control of *Armillaria*. Specifically:

- To trial different inoculum substrates for *Armillaria* to develop a faster inoculation method in plants.
- To use freshly rooted privet plants inoculated with *Trichoderma* to determine the rate and longevity of colonisation in roots.
- To screen *Trichoderma* isolates in strawberry plants for growth promotion and potential protection from ARR.
- To confirm protection from ARR in strawberry and privet plants from high performing *Trichoderma* isolates.
- To determine the effects of microbial communities in soil on the efficiency of ARR protection offered by *Trichoderma*.

4.4 Results

4.4.1 Alternative *Armillaria* inoculum trials

Development of a reliable *Armillaria* inoculum which could be produced in one month with reduced effort compared to current methods was considered. Strawberry plants were used as a host system because they are readily available from commercial suppliers and show symptoms of *Armillaria* infection within three months. Firstly, a medium using Rice Sawdust and Tomato (RST) was trialled which could be colonised with *Armillaria* in one – two months. Hazel billets were colonised horizontally as opposed to the traditional vertical method to increase surface area and reduce time required for colonisation. Further to this, substrates including horse chestnuts and acorns were trialled as alternative inocula because they are readily available from commercial seed suppliers and little preparation is required before inoculation with *Armillaria*.

4.4.1.1 Rice Sawdust Tomato (RST) as an alternative *Armillaria* inoculum

In attempt to reduce the time required to colonise *Armillaria* inoculum the RST medium which is used to fruit *Armillaria* was trialled. After four to eight weeks of incubation with *A. mellea* CG440, RST had been fully colonised and formed a solid block of substrate (Figure 4.1a). The uninoculated RST crumbled easily, not having mycelium holding it together (Figure 4.1b, c). The inoculated RST medium was fully colonised by *A. mellea* which could be successfully be re-isolated at the time of inoculation. To test the potential of the inoculum a 10 mm diameter cork-borer was used to cut pieces of inoculum to a standard size similar in shape to hazel billets, three of which were used to inoculate one strawberry plant. The *A. mellea* colonised RST inoculum was crumbly in texture when cut with a cork-borer and did not hold together, but it could be compacted into the soil. A roughly 33 x 10 x 45 mm wedge (Figure 4.1d) was cut using a sterile scalpel which held together without crumbling and could easily be compacted into the soil. A pilot study was set up in duplicate to test the potential of RST as inoculum for plants. Plants were mock-inoculated with uncolonised RST or inoculated with *A. mellea* CG440

RST cored- or wedged-inoculum or a three month *A. mellea* CG440 vertically inoculated hazel billet (Figure 4.1e) as a positive control.



Figure 4.1: Alternative *Armillaria* inoculum using Rice Sawdust and Tomato (RST) medium and traditional hazel billets.

a) RST medium after 6 weeks colonisation by *A. mellea* CG440; b) negative RST inoculum after 6 weeks; c) crumbly texture of negative RST inoculum; d) *A. mellea* CG440 RST inoculum wedge; e) *A. mellea* CG440 vertically colonised hazel billet after three months.

Three mpi (months post inoculation) strawberry plants were destructively sampled to determine inoculum efficiency. Plants were assessed for aerial symptoms classed as good, moderate or poor. Leaves were not removed during the experiment which increased the appearance of stress, so symptoms were based on the health of the freshest growth. The severity of disease was scored using a 1 - 4 point Disease Severity Index (DSI) (Table 4.1) for each inoculum source (Figure 4.2).

Table 4.1: Disease Severity Index (1 – 4 pt. scale) descriptions.

Assessment is based on health observations from above and belowground symptoms.

| DSI | Above-ground | Below-ground |
|-----|-----------------------------------|---|
| 1 | No aerial symptoms | No visible <i>Armillaria</i> mycelium |
| 2 | Some aerial symptoms | No visible <i>Armillaria</i> mycelium |
| 3 | May show signs of aerial symptoms | Visible <i>Armillaria</i> mycelium colonisation |
| 4 | Dead Plant | Heavy <i>Armillaria</i> mycelium colonisation |

Armillaria-free RST plants were healthy with an average DSI of 1.5 due to some slight leaf chlorosis but otherwise there were no signs of *Armillaria* and *Armillaria* was not re-isolated. Slight aerial symptoms were noted on some plants from the cored *Armillaria* RST inoculum although there were no signs of *Armillaria* on the plant roots which was further supported by lack of isolation from the plants. These plants had an average DSI of 1.5, the same as the mock-inoculated plants. Both plants inoculated with a wedge of RST were in good health with a DSI of 1. There were some signs of lesions in the roots of both RST wedge inoculated plants but *Armillaria* was not visible and could not be isolated from the plant roots. The strawberry plants inoculated with *Armillaria*-colonised hazel billets showed signs of aerial symptoms and *Armillaria* mycelium was visible in the plant roots; the plants had an average DSI of 3 and *Armillaria* could be re-isolated. The *Armillaria*-free RST inoculum had become mixed into the soil at the end of the experiment and attempts at isolation of *Armillaria* were unsuccessful. The *Armillaria*-colonised RST cored inoculum could be recovered from the soil but there were no signs of *Armillaria* or any outgrowth and *Armillaria* could not be recovered. Small lesions were observed on pieces of sawdust sampled from the RST inoculum wedge and outgrowth of three small rhizomorphs were observed, however, *Armillaria* could not be re-isolated from lesions or rhizomorphs. *Armillaria* mycelium was visible on the hazel billets and could be successfully re-isolated.

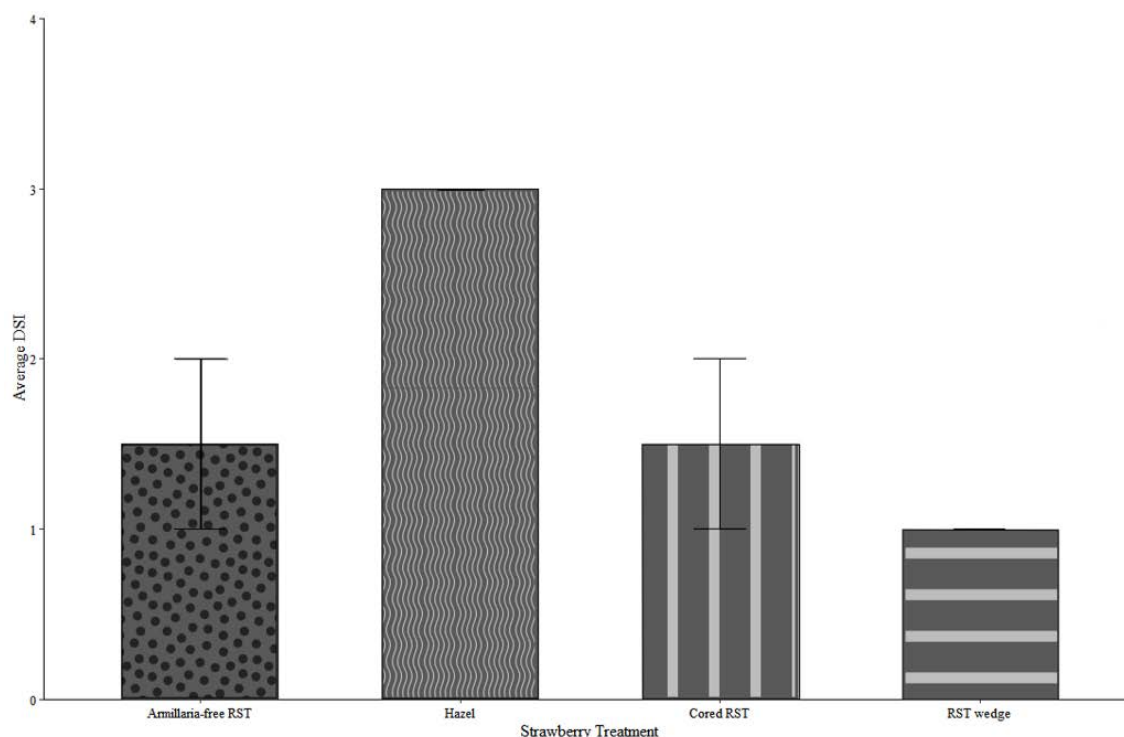


Figure 4.2: Disease severity index for strawberry plants inoculated with conventional and RST based substrates.

DSI (1 – 4 pt. scale) for strawberry plants inoculated with RST based substrates: *Armillaria*-free RST, hazel billets, *Armillaria* colonised RST core or wedge. Error bars represent the standard error (n = 2).

4.4.1.2 Horse chestnut and acorns as alternative substrates for *Armillaria* inoculum

The efficiency of using horse chestnuts and acorns as an alternative *Armillaria* inoculum substrate to hazel was assessed in strawberry plants. Horse chestnuts and acorns weighed between 2.4 – 4.5 g and 9 – 14.5 g, respectively. Hazel billets were cut to 50 mm lengths with a 15 – 20 mm diameter and weighed between 9 – 11 g. Each inoculum type was laid out in plastic Tupperware, autoclaved three times and covered in MEA. Substrates were inoculated with *Armillaria* and left for one month after which all were suitably colonised for use as inoculum (Figure 4.3). Hazel billets were arranged horizontally (Figure 4.3a – d) to maximise surface area for *Armillaria* colonisation within one month compared to the slower colonised (three – six months) traditional hazel billets which are arranged vertically (Figure 4.4). One hazel billet or horse chestnut and three acorns were used per plant for inoculation to make the substrate mass comparable. Uninoculated hazel billets were used as a negative control and ten replicates were made per substrate.



Figure 4.3: Colonisation by *A. mellea* of inoculum substrates.

Alternative *Armillaria* inoculum substrates in MEA freshly inoculated and viewed from above (left) and after one-month colonisation viewed from below (right) with *A. mellea* CG440. Substrates tested as alternative *Armillaria* inoculum include a-b) hazel based *Armillaria*-free control; c-d) horizontally positioned hazel; e-f) horse chestnut, g-h) acorn.

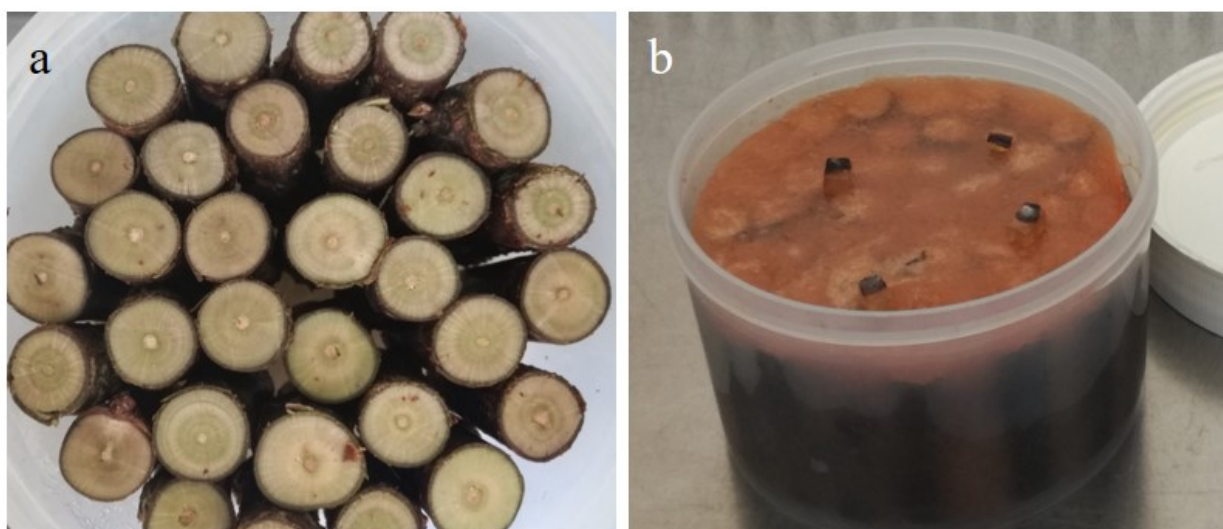


Figure 4.4: 'Traditional' hazel billets.

Billets require three – six months colonisation. a) Billets positioned vertically and b) submerged in carrot agar for colonisation with *Armillaria*.

After three months, strawberry plants were assessed using a 1 - 4 pt. DSI (Table 4.1) to measure health and *Armillaria* infection (Figure 4.5 and Figure 4.6). There was a significant difference in disease severity (Kruskal-Wallis: $\chi^2 = 19.954$, DF = 3) for *Armillaria*-free controls compared with hazel and horse chestnut based substrates (Bonferroni correction $p < 0.01$ and $p < 0.001$, respectively). Plants inoculated with *Armillaria*-free hazel billets showed no signs of *Armillaria* infection. Plants were healthy, with a healthy average DSI of 1 (Figure 4.6), there were no signs of rhizomorphs in the soil or on the mock-inoculum substrate and no *Armillaria* could be isolated from the plant. *Armillaria*-infected hazel billets gave an average DSI of 2.9 (Figure 4.6) with only two of ten plants still healthy at the end of the experiment. Eight were infected with *Armillaria*, three of which died. Although only six plants inoculated using horse chestnuts were infected with *Armillaria*, five of these died. The average DSI for horse chestnut based inoculum was higher than hazel infected plants at 3.1 (Figure 4.6) and rhizomorphs were reliably produced by *Armillaria*. Only three plants inoculated with acorn based substrates developed *Armillaria* infections where one plant died. The remaining plants were healthy. The average DSI for acorn based inoculum was 1.9 (Figure 4.6).



Figure 4.5: Alternative *Armillaria* inoculum trial with *A. mellea* CG440.

Inoculum substrates comprised of horizontally colonised hazel billets, horse chestnuts and acorns. Examples are shown of five strawberry plants, the recovered inoculum and the strawberry root ball of plants inoculated with a - c) *Armillaria*-free hazel; d - f) *Armillaria* colonised hazel; g - j) horse chestnut; k - m) acorn. See 30 cm ruler for scale on strawberry plants and in photos of strawberry roots one square represents 10 mm.

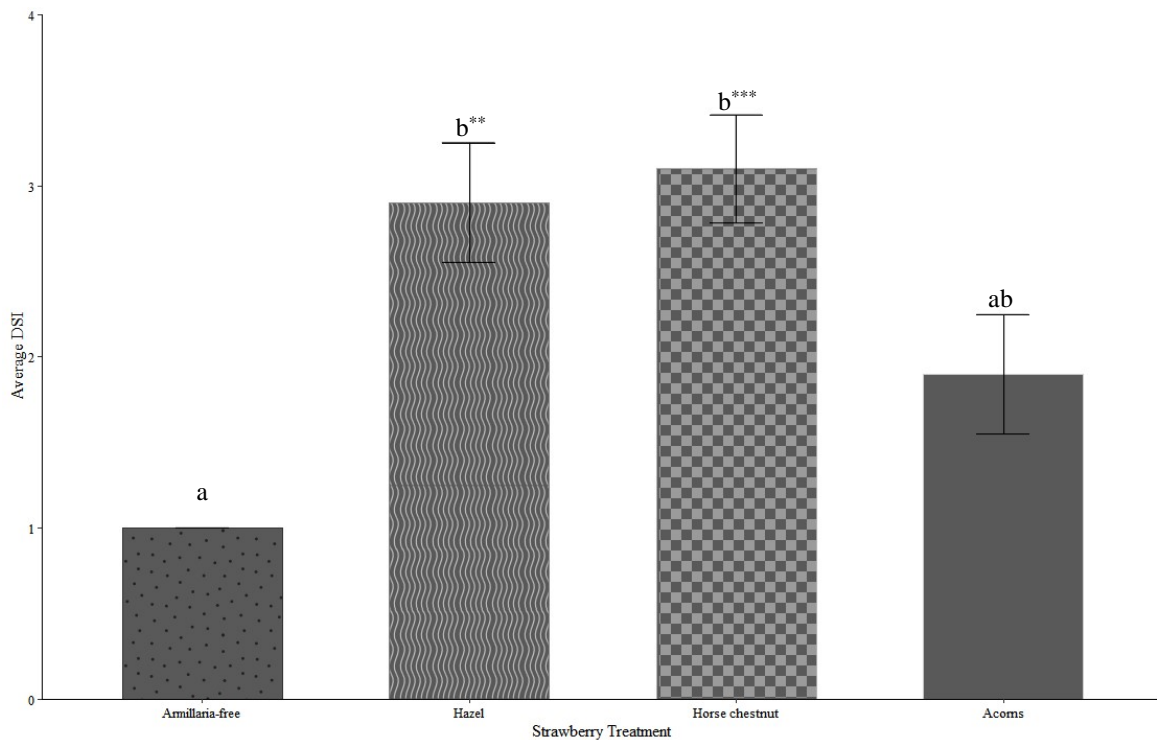


Figure 4.6: Disease severity index for strawberry plants inoculated with different *Armillaria* inoculum substrates.

DSI (1 – 4 pt. scale) for strawberry plants inoculated with *Armillaria*-free hazel billets, or *A. mellea* colonised hazel, horse chestnut and acorn based inoculum. Error bars represent the standard error (n = 10). a, b represent statistical groupings (Kruskal Wallis with Bonferroni corrections) * p < 0.05, ** p < 0.01, *** p < 0.001.

To confirm the presence of *Armillaria*, isolations were made from the roots of each plant and the percentage recovery of *Armillaria* was calculated (Figure 4.7). Although horse chestnut and acorn based inoculum infected fewer plants than hazel inoculum sources, where plants were infected the re-isolation success was higher for these substrates than hazel. In plants infected with hazel inoculum sources the minimum recovery was 8.3 % compared to 91.7 % in horse chestnut and 75 % in acorn infected plants. *Armillaria* re-isolation reached 100 % for four horse chestnut, three hazel and one acorn infected plants (Figure 4.7). Viability of inoculum was assessed visually at the end of the experiment. Eight *Armillaria* infected hazel billets had mycelium present. Four horse chestnuts and three acorns did not have visible mycelium at the end of the experiment and no signs of *Armillaria* infection were recorded in plants. Hazel billets remained intact, however the shell of one horse chestnut and the acorns from three plants were broken open and hollow, in each case with unsuccessful colonisation of the plant.

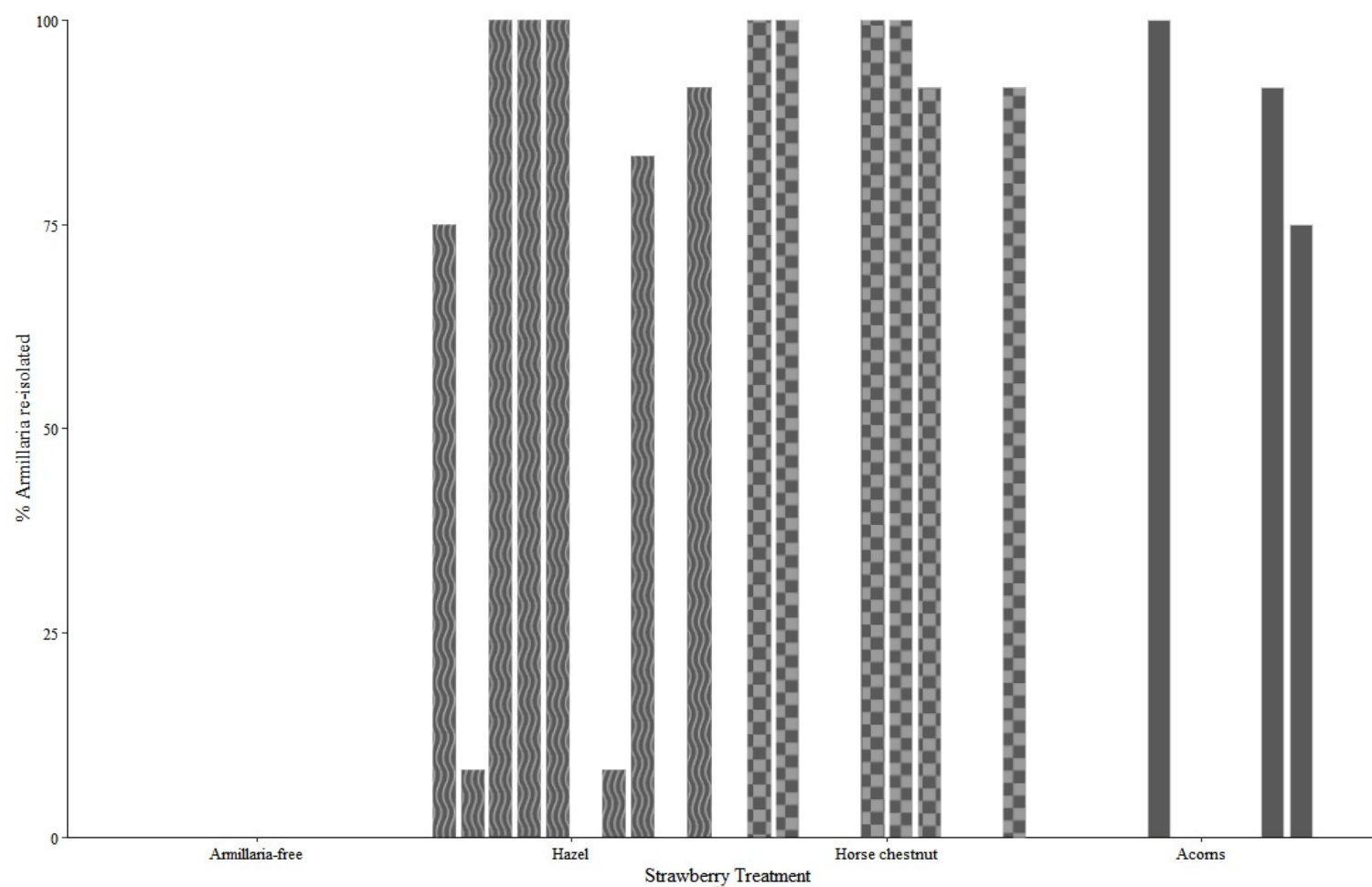


Figure 4.7: Recovery of *Armillaria* from strawberry plants

Re-isolation of *Armillaria* (%) from plants inoculated with *Armillaria*-free hazel billets, or *A. mellea* CG440 colonised hazel, horse chestnuts and acorns. Seven attempted isolations were made per plant.

4.4.1.2.1 Substrate analysis

To investigate how chemical composition of substrates effected their success as *Armillaria* inoculum, uncolonised hazel, horse chestnuts and acorns were analysed for various compounds (Figure 4.8). An equal mass of each substrate (400 g) was used to standardise analysis, since this was the determinant for the number of hazel billets, horse chestnuts or acorns to used as inoculum. All three substrates had a similar gross calorific value and total C. The hazel based substrate had a higher C: N ratio and higher weight for weight of sodium and calcium. Hazel had lower levels of total nitrogen, potassium, magnesium, and sulphur. The lignin content of substrates was not measured.

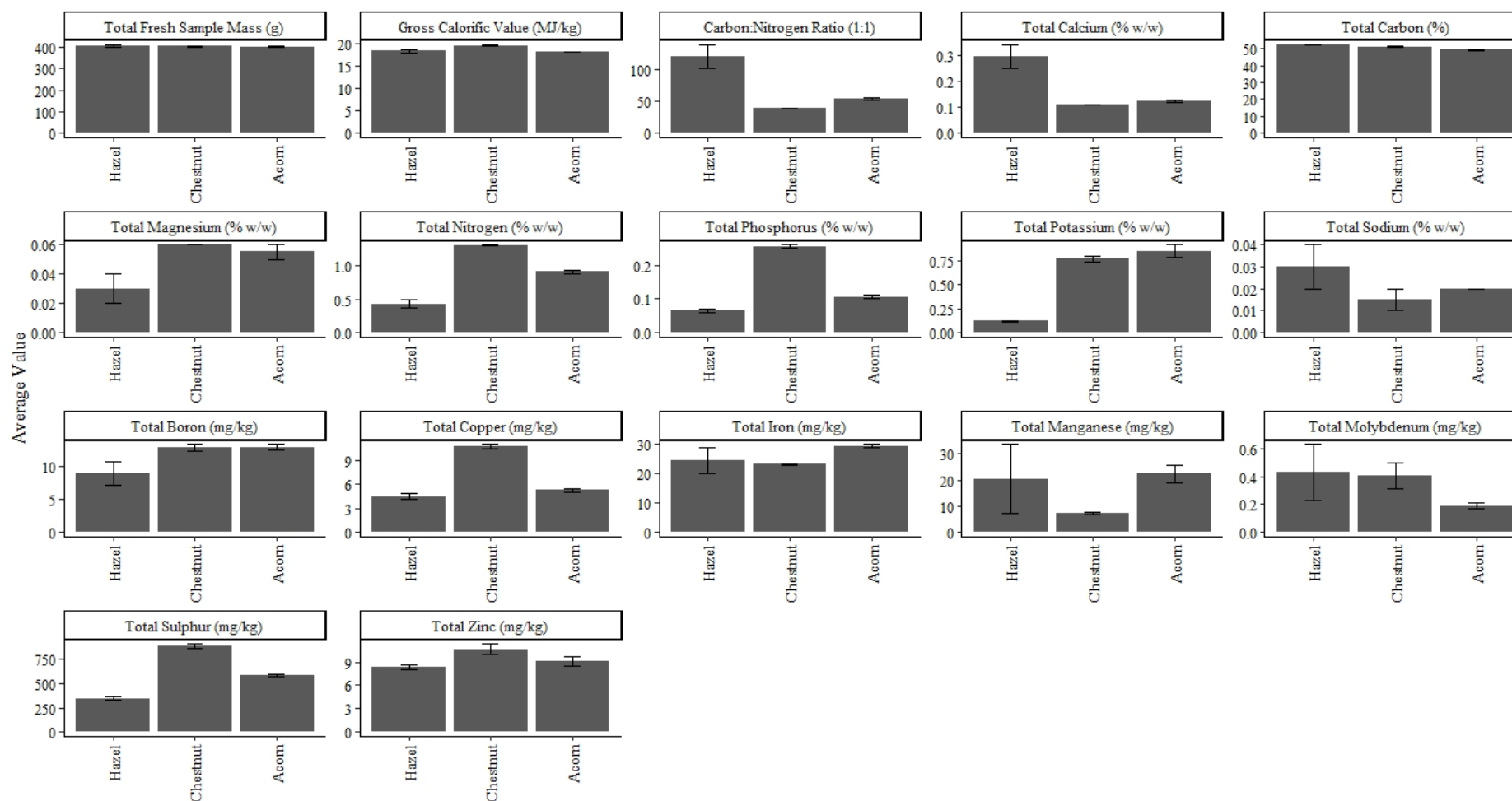


Figure 4.8: Chemical composition of substrates for *Armillaria* inoculum

Chemical composition was based on averages from two replicates taken from a 400 g sample of hazel, horse chestnut and acorn. Units for value of each chemical is labelled in titles.

4.4.2 *Trichoderma* Colonisation of Privet

To determine whether *Trichoderma* colonised the plant roots and if the colonisation was long-lasting, a series of bioassays were conducted. Privet plants were propagated as cuttings from a privet hedge to ensure minimal genetic variation and uniform cuttings. Firstly, plants were inoculated with *Trichoderma* spp. and daily isolations were made to find out how quickly *Trichoderma* colonisation occurred. Secondly, weekly isolations were made to confirm the longevity of *Trichoderma* colonisation over six weeks.

4.4.2.1 Privet propagation

Privet cuttings were collected from a hedge at RHS Wisley (Figure 4.9a) cut into 50 - 100 mm lengths to include a leaf node and propagated in a Hydropod (Figure 4.9b). Within four – six weeks rooted cuttings (Figure 4.9c, d) were ready for *in planta* assays.



Figure 4.9: Privet collection and propagation.

a) Privet hedge at RHS Wisley from which cuttings were collected; b) Privet cuttings cut into 50 – 100 mm lengths including a leaf node and arranged in the Hydropod which sprays water to hydroponically root plants; c) privet cuttings extensively rooted in Hydropod after four – six weeks; d) Privet plants laid out for inoculation assay with *Trichoderma*. Ruler represents 30 cm scale bar.

4.4.2.2 Virkon efficiency for surface sterilisation of plants

To ensure *Trichoderma* surface sterilisation methods were effective the efficiency of 1 % Virkon was tested. Five serial dilutions of *T. atrobrunneum* T17/11 (4×10^4 conidia / ml) were mixed with an equal volume of 1 % Virkon or SDW, shaken for two minutes and spread onto fresh PDA. Virkon effectively killed 83 - 100 % of *Trichoderma* colonies from serial dilutions 2 - 5. *Trichoderma* colony size was smaller after sterilisation with 1 % Virkon for two minutes. From the fourth serial dilution *Trichoderma* colonies were measured after sterilisation with Virkon or water. The average *Trichoderma* colony size was 3.00 μm after shaking with water or 1.02 μm after shaking with 1 % Virkon treatment.

4.4.2.3 Colonisation efficiency of *Trichoderma*

To determine the efficiency of *Trichoderma* colonisation in plant roots, freshly propagated privet plants were used. Six plants were inoculated with *T. atrobrunneum* T17/11 and grown in sterile sand (Figure 4.10a). Isolations onto MRB (*Trichoderma* selective medium) were made from one plant daily (Figure 4.10b) for one week, discarding the plant after isolation. Isolations on 'Day 0' from an uninoculated propagated root were made to control for potential background presence of *Trichoderma*. *Trichoderma* did not grow from isolations made from uninoculated privet at 'Day 0'. *Trichoderma* colonisation after inoculation was highly efficient with 100 % *Trichoderma* re-isolation from each daily isolation over one week.



Figure 4.10: Privet plants grown in sand to assess efficiency of *Trichoderma* colonisation.

Privet plants were inoculated with *T. atrobrunneum* T17/11 and planted in sand for easy removal to determine the efficiency of *Trichoderma* colonisation. a) Freshly inoculated privet plant in sand; b) privet plant prepared for isolation by washing sand from roots. One green square represents 10 mm.

4.4.2.4 Longevity of *Trichoderma* colonisation in roots

To ensure that *Trichoderma* colonisation was long-term, freshly propagated privet plants were inoculated with three *Trichoderma* isolates (*T. hamatum* T17/10, *T. atrobrunneum* T17/11 and T17/15) or mock inoculated as a control. Six privet plants were inoculated per treatment and grown in compost : sand (3 : 1) mix (Figure 4.11a). Isolations were made from roots of one plant per treatment on a weekly basis (Figure 4.11b). Plants were only selected for *Trichoderma* sampling once and after isolation, plants were re-potted in the original soil.

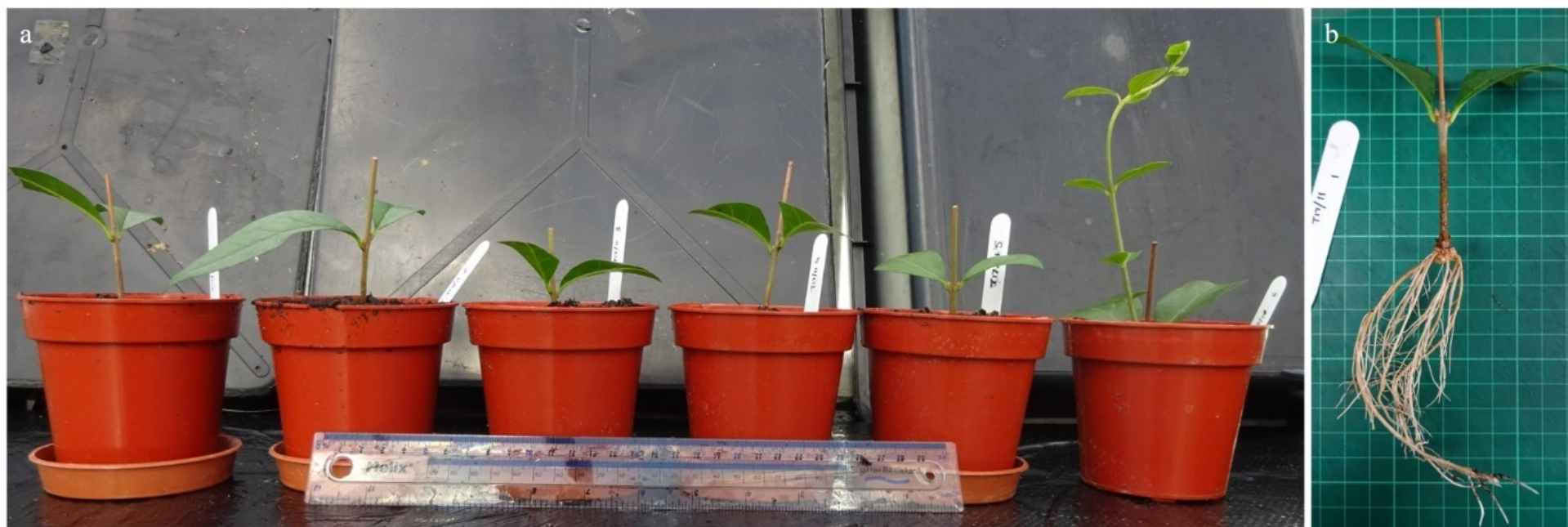


Figure 4.11: Privet plants grown in soil were inoculated with *Trichoderma* spp. to assess longevity of colonisation over six weeks.

a) Privet plants (n = 6) one week post inoculation with *T. atrobrunneum* T17/11; b) a privet plant prepared for isolation by washing soil from roots at one week. Ruler represents 30 cm scale and one green square represents 10 mm.

Only one *Trichoderma* colony was isolated from any *Trichoderma*-free control over six weeks, suggesting low background presence of *Trichoderma*. The isolation efficiency was generally > 80 % for *Trichoderma* inoculated plants (Figure 4.12). By week six there was a slight reduction in isolation efficiency, but *Trichoderma* was still present in all inoculated plants. During week four the incubator in which Petri dishes were kept overheated perhaps providing a reason for apparent low success rates of *Trichoderma* isolation for *T. hamatum* T17/10 and *T. atrobrunneum* T17/15.

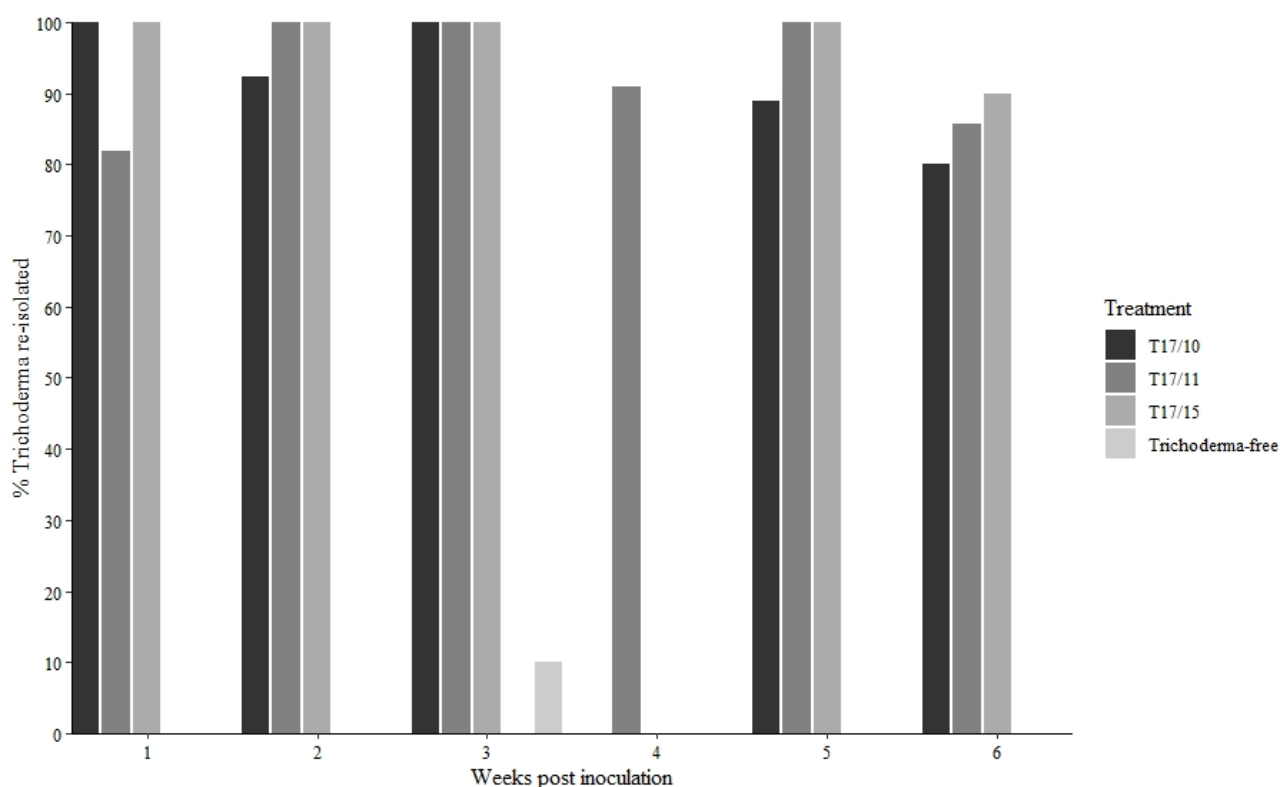


Figure 4.12: Isolation success of *Trichoderma* spp. from plants grown in soil

Percentage (%) recovery of *Trichoderma* over six weeks on freshly propagated privet plants with *Trichoderma*-free controls and three *Trichoderma* treatments: *T. hamatum* T17/10, *T. atrobrunneum* T17/11 and *T. atrobrunneum* T17/15.

4.4.2.4.1 *Armillaria* inoculation of privet

A pilot study was set up to inoculate plants from section 4.4.2.4 (Longevity of *Trichoderma* colonisation) with *Armillaria* to assess efficiency of infection. Severity of *Armillaria* infection was not measured. Numerous *Armillaria* rhizomorphs were produced which wound amongst roots of plants inoculated with and without *Trichoderma* spp. but in most cases did not visibly adhere to privet roots (Figure 4.13a - c). However, in some cases infection did occur at the base of the plant pot where roots became pot-bound (Figure 4.13d), although protection by *Trichoderma* spp. was not analysed. *Armillaria*-only controls were visibly colonised by *Armillaria* mycelium and rhizomorphs (Figure 4.13e - f). Attempts to isolate *Armillaria* from *Trichoderma*-inoculated and *Trichoderma*-free plants were made from a random selection of plants after three months. One potential *Armillaria* colony grew from each *T. hamatum* T17/10 and *T. atrobrunneum* T17/11. *Armillaria*-only isolations were contaminated and no *Armillaria* grew. *Trichoderma* spp. were isolated from plants inoculated with *T. atrobrunneum* T17/11 & T17/15.



Figure 4.13: Privet plants inoculated with *Armillaria* after five months.

Rhizomorphs are a dark reddish brown and privet roots are a pale brown – cream colour. a) Rhizomorphs growing alongside roots of privet inoculated with *T. atrobrunneum* T17/11; b) Rhizomorphs intertwined with privet roots at the root collar of privet inoculated with *T. hamatum* T17/10; c) Rhizomorphs heavily intertwined in roots of privet inoculated with *T. atrobrunneum* T17/15; d) Pot-bound roots with signs of *Armillaria* attack on plants inoculated with *T. atrobrunneum* T17/11; e) *Armillaria*-only control showing *Armillaria* infection; f) *Armillaria* control heavily infected with blackened rhizomorphs.

4.4.3 Potential growth promotion and protection from ARR by *Trichoderma* spp. in strawberry plants

This project began with a collection of 40 *Trichoderma* isolates (Table 3.1) each of which were screened for growth promotion and protection against *Armillaria* in strawberry plants to identify *Trichoderma* isolates of interest for future studies. Strawberry plants were inoculated (via root dipping and pouring spore suspension into soil) with *Trichoderma* spore suspensions (10^5 Conidia / ml) in triplicate for each *Trichoderma* isolate to allow for mass screening of *Trichoderma* isolates.

4.4.3.1 Assessing growth promotion by *Trichoderma* in strawberries

Leaf size was measured at 10 day intervals for two months to assess potential growth promotion. There was no visible difference between strawberry plants inoculated with *Trichoderma* and *Trichoderma*-free plants after two months (Figure 4.14). As expected, with time leaf size significantly increased (one-way repeat measure ANOVA: $F_{5, 40} = 3.0684$, $p < 0.001$) over 60 days after inoculation with *Trichoderma*, but *Trichoderma* treatment had no significant effect on leaf size (pairwise comparisons $p > 0.9$) compared to *Trichoderma*-free controls (Figure 4.14c). *Trichoderma* conidia were suspended in 5% Tween 20 for root application. To determine effect of Tween 20 on strawberry plant growth plants were mock-inoculated with *Trichoderma*-free 5% Tween 20 ('+Tween') or SDW ('- Tween'). No significant difference ($p = 1.0$) in leaf size of strawberry plants was noted in Tween 20 treatments. One plant from *T. virens* T17/02 and *T. atrobrunneum* T17/11 died and both were removed from experiment.

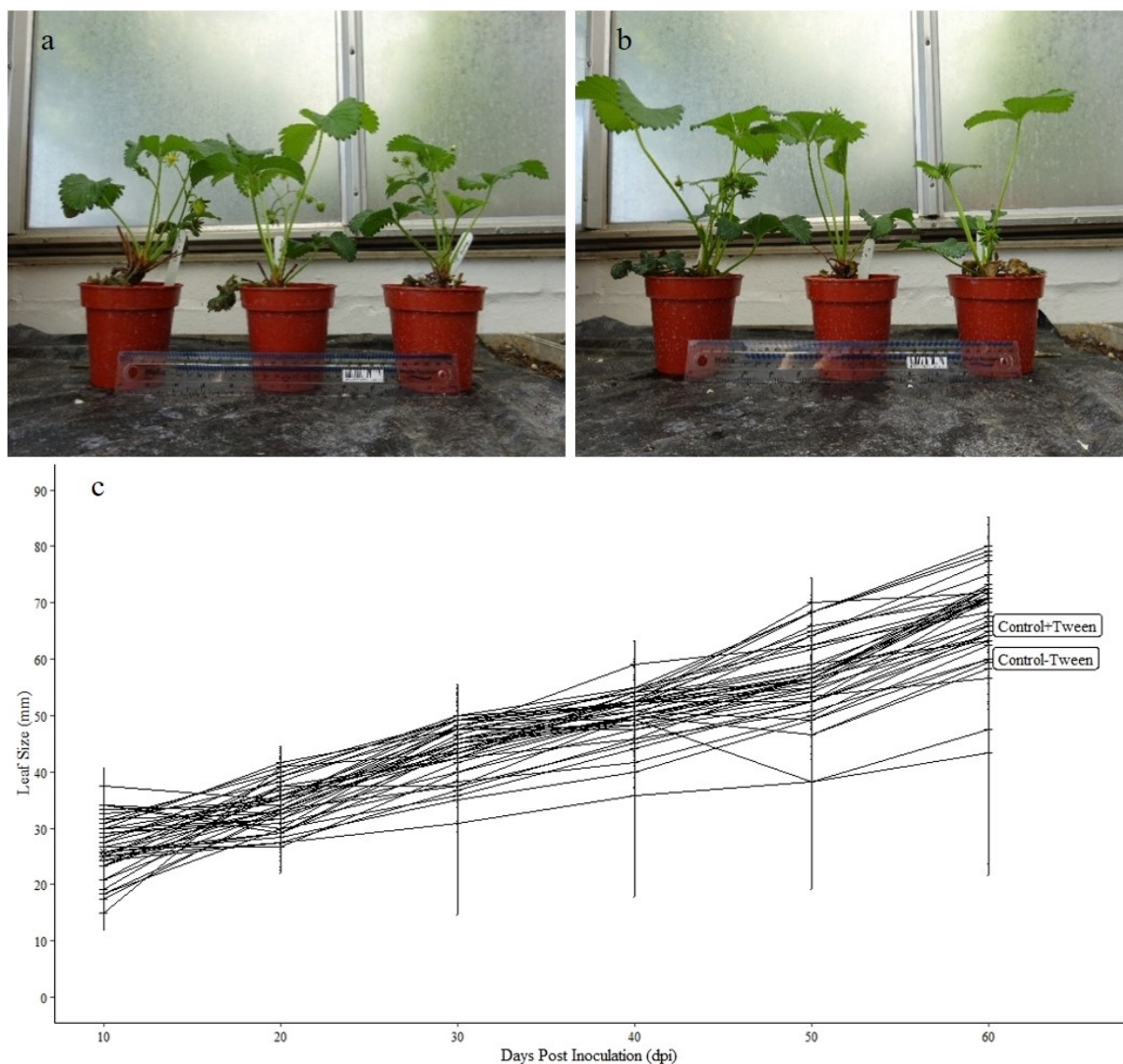


Figure 4.14: Screening of *Trichoderma* isolates in strawberry plants for growth promotion.

a) Control strawberry plant at 2 mpi; b) *Trichoderma* isolate *T. atrobrunneum* T17/04 inoculated strawberry plant at 2 mpi; c) Growth of leaf size (average, $n = 3$) over 60 days in strawberry plants with controls labelled (with and without 5 % Tween 20) compared to all 40 *Trichoderma* isolates (unlabelled).

4.4.3.2 *Trichoderma* recovery from compost

To determine whether *Trichoderma* had colonised strawberry plants after two months, soil samples were taken from each plant for isolation when *Armillaria* was added. Strawberry plants had been inoculated with a suspension of 10^5 conidia / ml which were presumed to colonise host plants readily. Of 40 *Trichoderma* isolates tested, only 14 were successfully re-isolated from the compost at two

months. Since many isolations were overgrown with other fungi, confirmation of *Trichoderma* was based on spore morphology and compared to a pure culture of each isolate.

4.4.3.3 Protection by *Trichoderma* against ARR in strawberry hosts plants

As there was no significant difference between *Trichoderma* treatments on plant growth, plants were inoculated with *Armillaria* to test whether different *Trichoderma* treatments were able to protect strawberries or reduce disease severity of ARR. Plants were monitored over three months, before being destructively sampled to assess *Armillaria* infection. A 0 - 6 pt. DSI (Table 4.2) was used to assess severity of infection and attempts to re-isolate *Armillaria* and *Trichoderma* were made using an appropriate selective medium for each. To ensure that Tween 20 had no effect on *Armillaria* infection, three controls were included. Extra plants mock-inoculated with SDW (as with ‘-Tween’) at the start of the experiment were included as *Armillaria*-free controls (in triplicate). *Armillaria* inoculum was added to the strawberry growth promotion controls ‘+Tween’ and ‘-Tween’.

Table 4.2: Disease Severity Index 0 – 6 descriptions.

Where *Armillaria* is visible in the roots isolation confirmation is optional because re-isolation is expected to be successful.

| DSI | Above-ground Symptoms | Below-ground Symptoms | Re-isolation |
|-----|-------------------------------|---|--------------|
| 0 | No aerial symptoms | No visible <i>Armillaria</i> mycelium | Unsuccessful |
| 1 | Some aerial symptoms | No visible <i>Armillaria</i> mycelium | Unsuccessful |
| 2 | Some signs of aerial symptoms | No visible <i>Armillaria</i> mycelium | Successful |
| 3 | No aerial symptoms | Visible <i>Armillaria</i> mycelium colonisation | Successful |
| 4 | Some signs of aerial symptoms | Visible <i>Armillaria</i> mycelium colonisation | Successful |
| 5 | Progressed aerial symptoms | Visible/heavy <i>Armillaria</i> mycelium colonisation | Successful |
| 6 | Dead Plant | Visible/heavy <i>Armillaria</i> mycelium colonisation | Successful |

Strawberry plant height was measured at the point of *Armillaria* inoculation and termination of the experiment. At the point of *Armillaria* inoculation there was no significant effect on plant height (one-

way ANOVA: $F_{41, 82} = 1.725$, Tukey $p > 0.9$) for *Trichoderma* treatments compared to *Trichoderma*-free controls. At the time of plant destruction there was still no significant effect on strawberry plant height (one-way ANOVA: $F_{41, 82} = 1.8546$, Tukey $p > 0.9$) between treatments and *Armillaria*-free controls.

After three months inoculation with *Armillaria*, plants were visually assessed for infection and disease severity. Isolations were made to detect the presence of *Armillaria* and *Trichoderma* from the root ball of strawberry plants. Hazel billets were assessed for viability of *Armillaria* including the presence of rhizomorphs (Figure 4.15a) and mycelial fans (Figure 4.15b). *Trichoderma* sporulation was prolific on the hazel billet of one plant inoculated with *T. harzianum* T17/06 and two inoculated with *T. hamatum* T17/10 (Figure 4.15c - d). A small amount of *Trichoderma* sporulation was observed on billets from a plant inoculated with *T. virens* T17/02, *T. spirale* T17/24 and *T. atrobrunneum* T17/11. In one case *Trichoderma* was found sporulating in the soil of plants inoculated with *T. atrobrunneum* T17/12. At the point of sampling, 36 strawberry plants had died from a total of 126 plants. Of the *Armillaria*-only control plants, one ('-Tween') and two ('+Tween') of three plants died. Of the *Trichoderma* treatments, 16 resulted in no plant deaths, 14 in one death and seven in two deaths. There were two *Trichoderma* treatments where all three plants died (*T. hamatum* T17/33 & *T. olivascens* T17/42).



Figure 4.15: Hazel billets colonised by *Armillaria* after three months inoculation in strawberry plants.

a) hazel billet with a rhizomorph; b) outer bark peeled away to reveal *Armillaria* mycelial fan on a hazel billet; c - d) *T. hamatum* T17/10 sporulating profusely on the *Armillaria*-colonised hazel billet.

The presence of *Armillaria* was assessed visually and through isolation with typical images of plants shown in Figure 4.16 and data summarised in Figure 4.17. Mycelial colonisation of *Armillaria* was found in many plants ($DSI \geq 3$) when outer epidermis of roots was removed (Figure 4.16b - c). Severity

of infection was assessed for each plant; generally there was high variation in DSI among treatments. As expected no infection was found in *Armillaria*-free controls (Figure 4.16a) where plants had a healthy DSI of 0. In one *Trichoderma* treatment with *T. atrobrunneum* T17/11 both plants inoculated with *Armillaria* remained healthy over the experimental period; only two replicates were assessed for this treatment because one died prior to *Armillaria* inoculation and was not replaced. Of three replicates, two plants remained healthy (DSI 0 - 1) for nine *Trichoderma* treatments (*T. virens* T17/02, *T. atrobrunneum* T17/04, *T. harzianum* T17/07, *T. harzianum* T17/08, *T. hamatum* T17/10, *T. atrobrunneum* T17/11, *T. cerinum* T17/14, *T. atrobrunneum* T17/15, *T. atrobrunneum* T17/16). Some *Trichoderma* isolates seemingly stimulated the rate of *Armillaria* infection with all plants in treatments *T. hamatum* T17/33 and *T. olivascens* T17/42 dying. There was a significant difference in the DSI of *Armillaria* infection in strawberry plants between the *Trichoderma* treatments and *Armillaria*-only controls (Kruskal-Wallis: $\chi^2 = 73.15$, DF = 41, $p < 0.01$). Disease severity in *Trichoderma* treatments *T. cerinum* T17/23, *T. hamatum* T17/33, *T. cerinum* T17/39 and *T. olivascens* T17/42 was significantly higher than that from *Armillaria* free controls (-Tween 20) at $p < 0.05$. Consideration for the effect of Tween 20 as a spore suspension for *Trichoderma* showed no significant difference between DSI of *Armillaria* infected plants with and without tween (Kruskal-Wallis: $\chi^2 = 73.38$, DF = 41, $p = 1$).

The average DSI of *Armillaria*-only infected plants was and 4.7 and 2.3 for '+Tween' and '-Tween' controls (Figure 4.17). Using the lower DSI (2.3) value as a baseline, eight isolates were considered of interest for further study (Table 4.3). Using this baseline *T. atrobrunneum* T17/04 and *T. cerinum* T17/14 with two healthy plants remaining at the point of assessment were not included because the third plant had died from ARR. *Trichoderma atrobrunneum* T17/16 was considered a potentially clonal isolate of *T. atrobrunneum* T17/15 from early sequence analysis and was excluded for further assessment. Although all plants for *T. olivascens* T17/42 died, it was included in selection for further study as an example of an isolate potentially stimulating ARR.



Figure 4.16: *Armillaria mellea* infection on strawberry plants after three months.

a) washed roots of *Armillaria*-free control; b) washed roots of strawberry plant colonised with *Trichoderma* sp. T17/09; c) outer root epidermis removed to show severe *Armillaria* colonisation of roots.

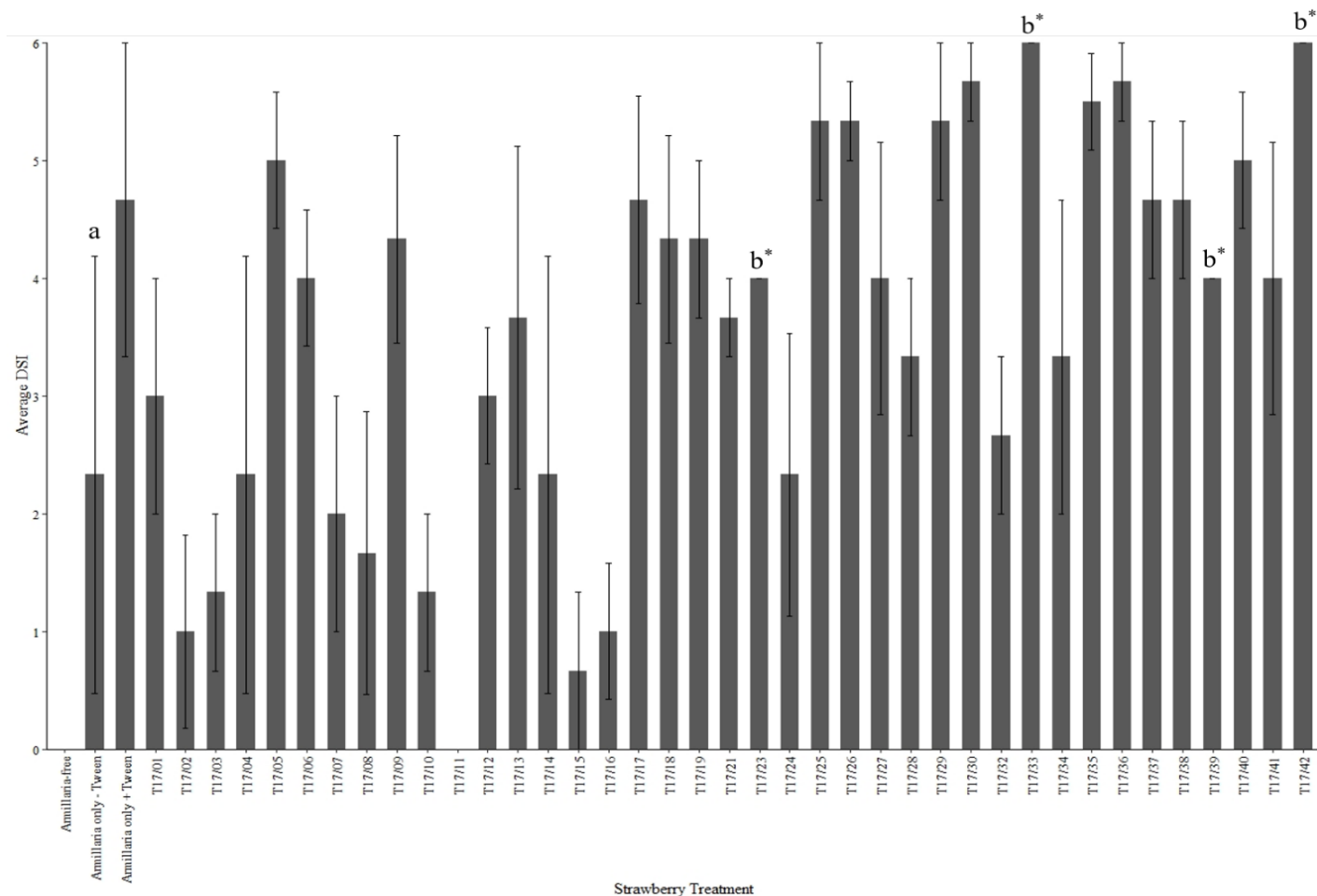


Figure 4.17: DSI of *Armillaria* infection in strawberry plants pre-colonised with *Trichoderma* spp.

Strawberry plants were pre-colonised with *Trichoderma* spp. for two months prior to *A. mellea* CG440 inoculation. The average DSI for strawberry plants identified potential *Trichoderma* isolates which offer protection from ARR. The error bars represent the standard error of the mean (n = 3). a, b represent statistical groupings (Kruskal Wallis). * p < 0.05, ** p < 0.01, *** p < 0.001

Table 4.3: Details of *Trichoderma* isolates selected for further assessment based on average DSI (n = 3) of strawberry plants.

| <i>Trichoderma</i> isolate | <i>Trichoderma</i> species ID | Host species | DSI (average) |
|----------------------------|-------------------------------|--------------------------------|---------------|
| T17/02 | <i>T. virens</i> | Soil/debris | 1.0 |
| T17/03 | <i>T. harzianum</i> | Soil/debris | 1.2 |
| T17/07 | <i>T. harzianum</i> | <i>Quercus</i> sp. | 2.0 |
| T17/08 | <i>T. harzianum</i> | <i>Quercus</i> sp. | 1.7 |
| T17/10 | <i>T. hamatum</i> | <i>Sorbus aria</i> 'Lutescens' | 1.0 |
| T17/11 | <i>T. atrobrunneum</i> | <i>Quercus</i> sp. | 0.0 |
| T17/15 | <i>T. atrobrunneum</i> | <i>Viburnum bodnantense</i> | 0.7 |
| T17/42 | <i>T. olivascens</i> | <i>Rhododendron</i> 'amoenum' | 6.0 |

Armillaria recovery from strawberry plants was variable (Figure 4.18). In *Armillaria*-only controls recovery varied between 33.5 % and 64.9 %. Where plants were considered healthy, no *Armillaria* could be isolated, however where plants had died from ARR only 60 – 70 % of isolations yielded positive *Armillaria* confirmation. *Trichoderma* was isolated from all plants including *Trichoderma*-free plants (Figure 4.18). Often, where there was high incidence of *Armillaria* recovery, there was a lower incidence of *Trichoderma* recovery (e.g. T17/35, T17/37 & T17/42, Figure 4.18). In contrast, with some *Trichoderma* spp. isolates (e.g. T17/02, T17/11, T17/30, Figure 4.18), where *Trichoderma* recovery was high, *Armillaria* recovery was low, suggesting one fungus might replace the other in strawberry plant roots. When recovery of either *Trichoderma* or *Armillaria* was assessed by *Trichoderma* species groups there was much variation (Figure 4.19). *Armillaria* recovery was highest in the presence of *T. hirsutum* and *T. olivascens* and lowest from plants inoculated with *T. spirale* or *T. atrobrunneum*. In contrast *Trichoderma* re-isolation was lowest for *T. olivascens*, *T. hamatum* and *T. hirsutum*, and highest from plants inoculated with *T. atrobrunneum* and *T. spirale* (Figure 4.19).

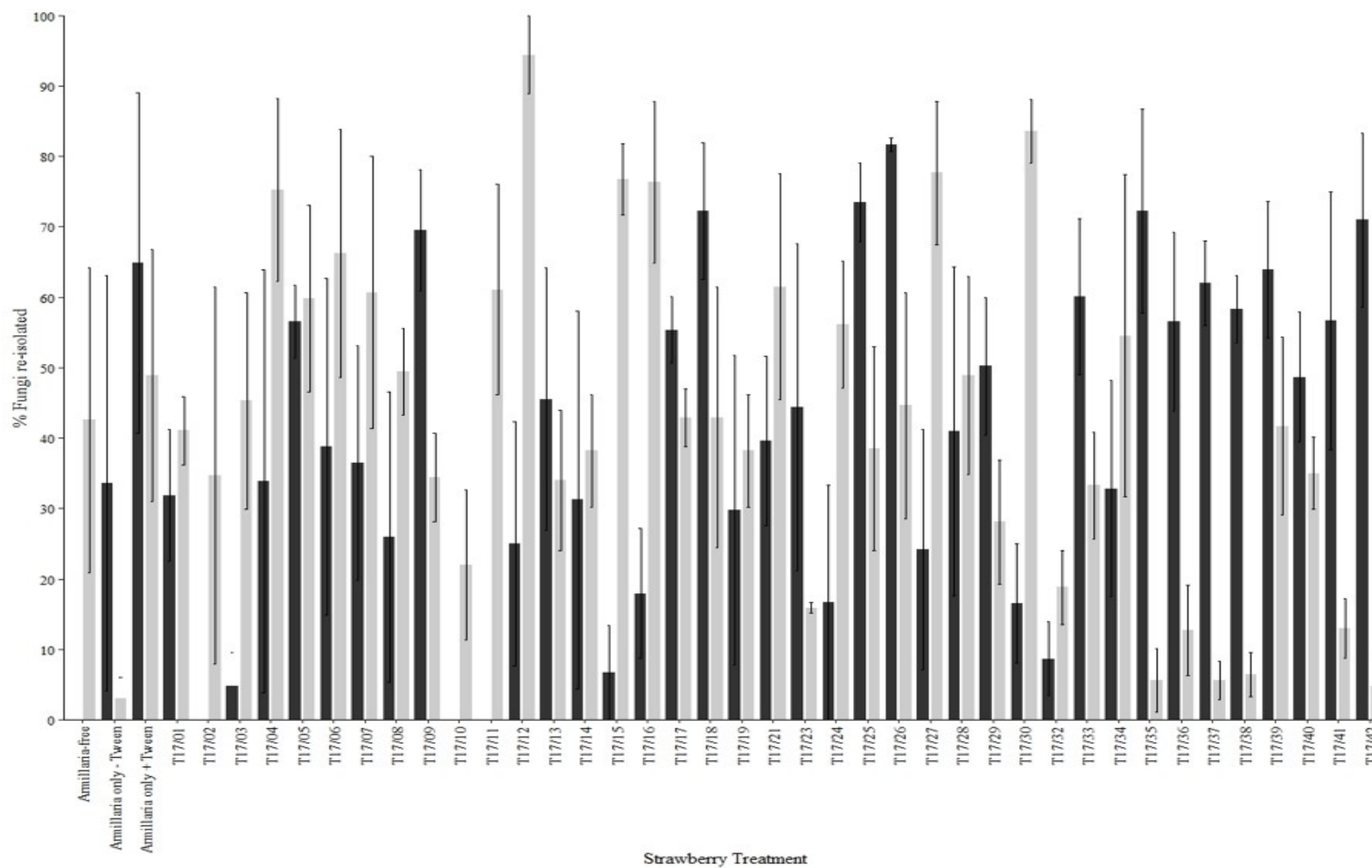


Figure 4.18: Percentage recovery of *Armillaria* (black) and *Trichoderma* (grey) from strawberry plants 3 mpi with *Armillaria*.

Isolations were made from strawberry root balls onto *Armillaria* or *Trichoderma* selective media (JJG and MRB respectively). Isolations were assessed for *Armillaria* or *Trichoderma* colonies after one and three weeks. Error bars represent the standard error of the mean (n = 3).

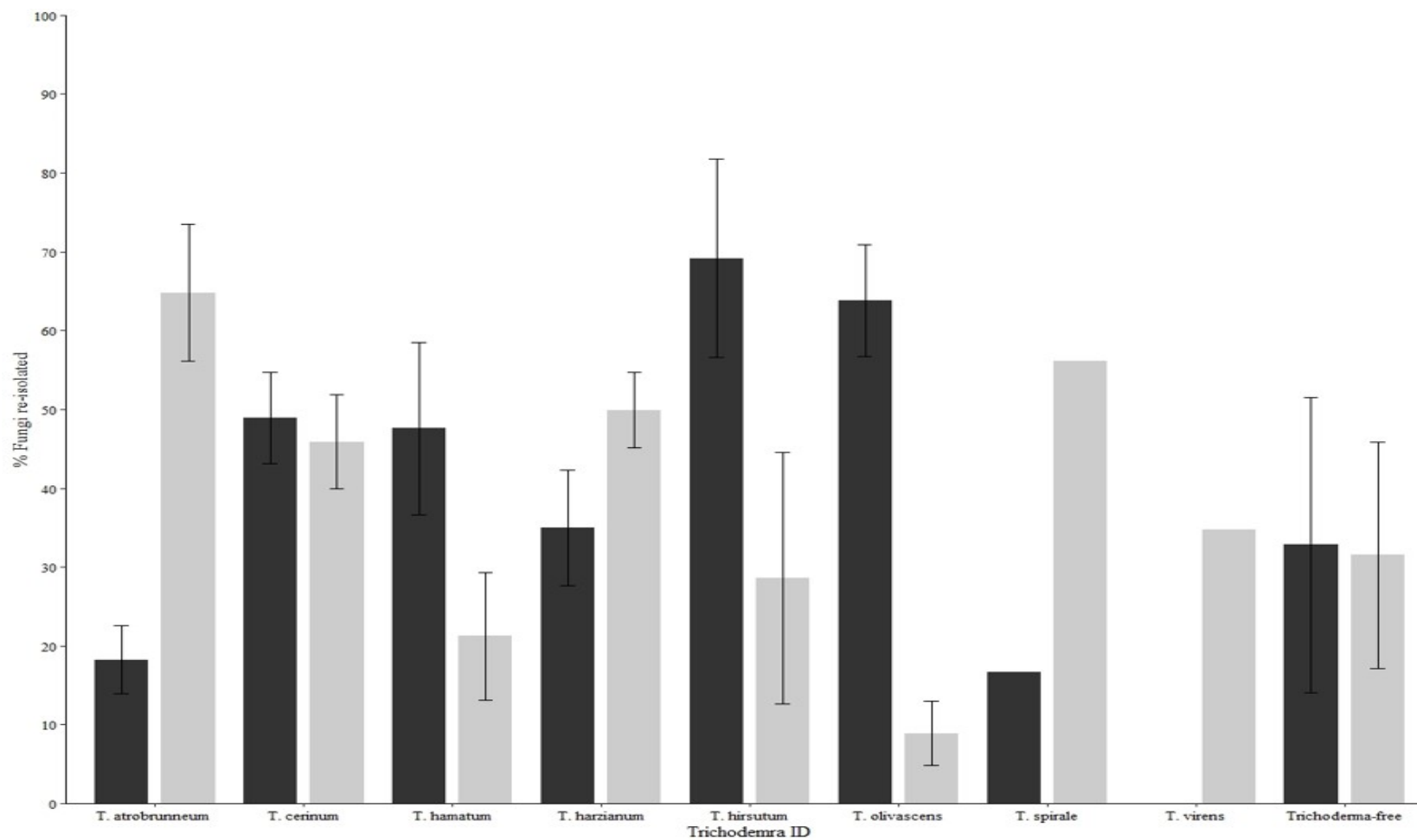


Figure 4.19: Percentage recovery of *Armillaria* (black) and *Trichoderma* (grey) from strawberry plants after 3 months infection with *Armillaria* for each *Trichoderma* species.

Trichoderma atrobrunneum (n = 8), *T. cerinum* (n = 10), *T. hamatum* (n = 6), *T. harzianum* (n = 6), *T. hirsutum* (n = 2), *T. olivascens* (n = 2), *T. virens* (n = 1), *T. spirale* (n = 1) and *Trichoderma*-free plants (n = 3). Error bars represent the standard error of the mean.

4.4.4 Assessment of ARR protection by selected *Trichoderma* spp.

To confirm the effectiveness of the eight selected *Trichoderma* isolates from the screening experiment in 4.4.3 (Table 4.3) a second *in planta* assay was designed using ten replicates per treatment with two major alterations. Firstly, strawberry plants were colonised with *Trichoderma* for one month prior to *Armillaria* inoculation because growth promotion was not being measured. Inoculation with *Trichoderma* was carried out in the same manner as before. Secondly, *Armillaria* inoculum, in the form of hazel billets, was colonised horizontally for one month as opposed to vertically for three – six months.

4.4.4.1 Level of *Armillaria* infection in strawberry plants over experimental duration

Within one mpi of *Armillaria* two plants died, one each from *Trichoderma* treatments T17/02 and T17/07 (Figure 4.20), with visible *Armillaria* in the root ball. In the *Armillaria*-only controls there was a lower than expected death rate among plants, therefore plants were observed for eight months when 90 % of control plants had died. The first *Armillaria*-only death occurred at four months and a total of nine plants died overall. No new deaths were reported between seven and eight months, so the experiment was terminated and dead plants were visually assessed to confirm *Armillaria* infection. Five *Trichoderma* treatments resulted in the death of all strawberry plants (*T. hamatum* T17/10, *T. atrobrunneum* T17/15, *T. harzianum* T17/08, *T. harzianum* T17/03 and *T. virens* T17/02), two resulted in the death of nine plants (*T. atrobrunneum* T17/11 and *T. harzianum* T17/07) and one *Trichoderma* resulted in the death of eight plants (*T. olivascens* T17/42). *Trichoderma olivascens* T17/42 was included in the subset of selected isolates due to the potential stimulating effect of the *Trichoderma*, however in this case it resulted in the lowest number of deaths from ARR. As expected, there were no deaths recorded from *Armillaria*-free strawberry plants. The severity of *Armillaria* infection was not measured.

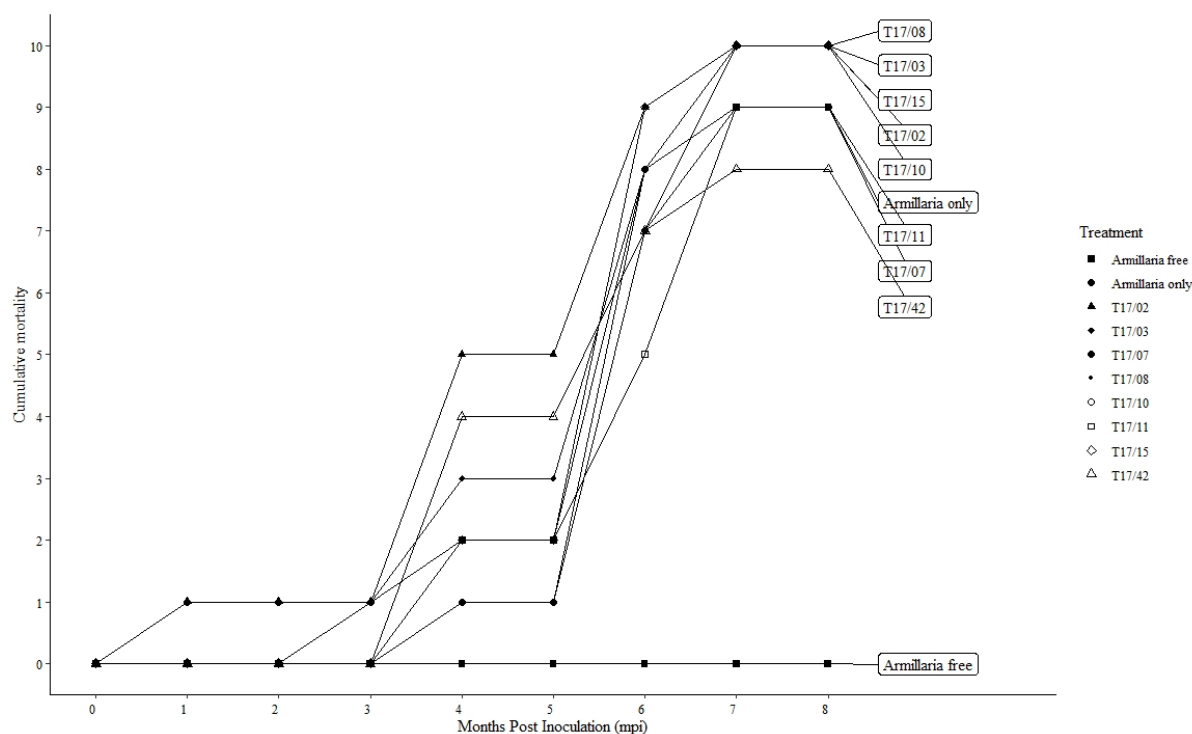


Figure 4.20: Number of strawberry plant deaths due to ARR over eight months.

Treatments included two *Trichoderma*-free controls (*Armillaria*-only and *Armillaria*-free) and eight *Trichoderma* isolates. *Trichoderma* species included *T. virens* (T17/02), *T. harzianum* (T17/03, T17/07, T17/08), *T. hamatum* (T17/10), *T. atrobrunneum* (T17/11 and T17/15) and *T. olivascens* (T17/42). There were ten plant replicates per treatment.

To determine whether *Trichoderma* had colonised plants which were increasingly dying from *Armillaria* infection compared to controls at five mpi, all five dead plants were selected from *T. hamatum* T17/10 inoculated plants. Isolations were made onto *Trichoderma* selective medium (MRB). Only one colony of *Trichoderma* was recovered from one plant (8% recovery success) of the five tested. Isolations were also made for *Armillaria* which was recovered from all plants which suggests that for unknown reasons, protection failed. This could be caused by a number of factors such as the batch of plants used.

4.4.5 Potential of protection from ARR in privet plants by *Trichoderma* spp.

To establish whether plant host impacted the effectiveness of *Trichoderma* as a potential protectant against ARR, privet (family: Oleaceae) was used as an alternative to strawberry (family: Rosaceae) host plant systems. Privet plants were chosen based on high susceptibility to *A. mellea* infection. Privet

cuttings were collected from the same privet hedge at RHS Wisley to reduce genetic variation in plants which were propagated to provide uniform plants in experiments. Nine replicate plants per treatment were used in this experiment.

4.4.5.1 Health of privet plants

Generally, the growth of privet plants over nine months inoculation with *Armillaria* was good. Although plants appeared visually stressed (pale or chlorotic appearance of leaves) at six months from both controls and *Trichoderma* treated plants, they recovered by nine months when the experiment was terminated (Figure 4.21). Between six and nine months many plants lost the parent leaves from original cuttings which increased the apparent chlorosis caused by fresh leaves and lead to apparent improved visual health at nine months as new leaves matured. Most plants produced new auxiliary buds (Figure 4.22a), some of which gave rise to leaves (Figure 4.22b) which grew slowly as can be seen in plants pictured in Figure 4.21c - d. Those plants which had produced a new stem showed healthy growth of fresh leaves (Figure 4.22c). One plant, inoculated with *T. hamatum* T17/10, flowered (Figure 4.22d).



Figure 4.21: Privet plants pre-inoculated with *T. harzianum* T17/08, imaged at four time points post inoculation with *A. mellea*.

a) 2 mpi (n = 9); b) 4 mpi (n = 8); c) 6 mpi (n = 7) where many plants have lost their parent leaves; d) 9mpi (n = 7) where fresh leaf growth from 6 mpi has matured. Note visual health of remaining plants improved at nine mpi. Ruler represents 30 cm scale.

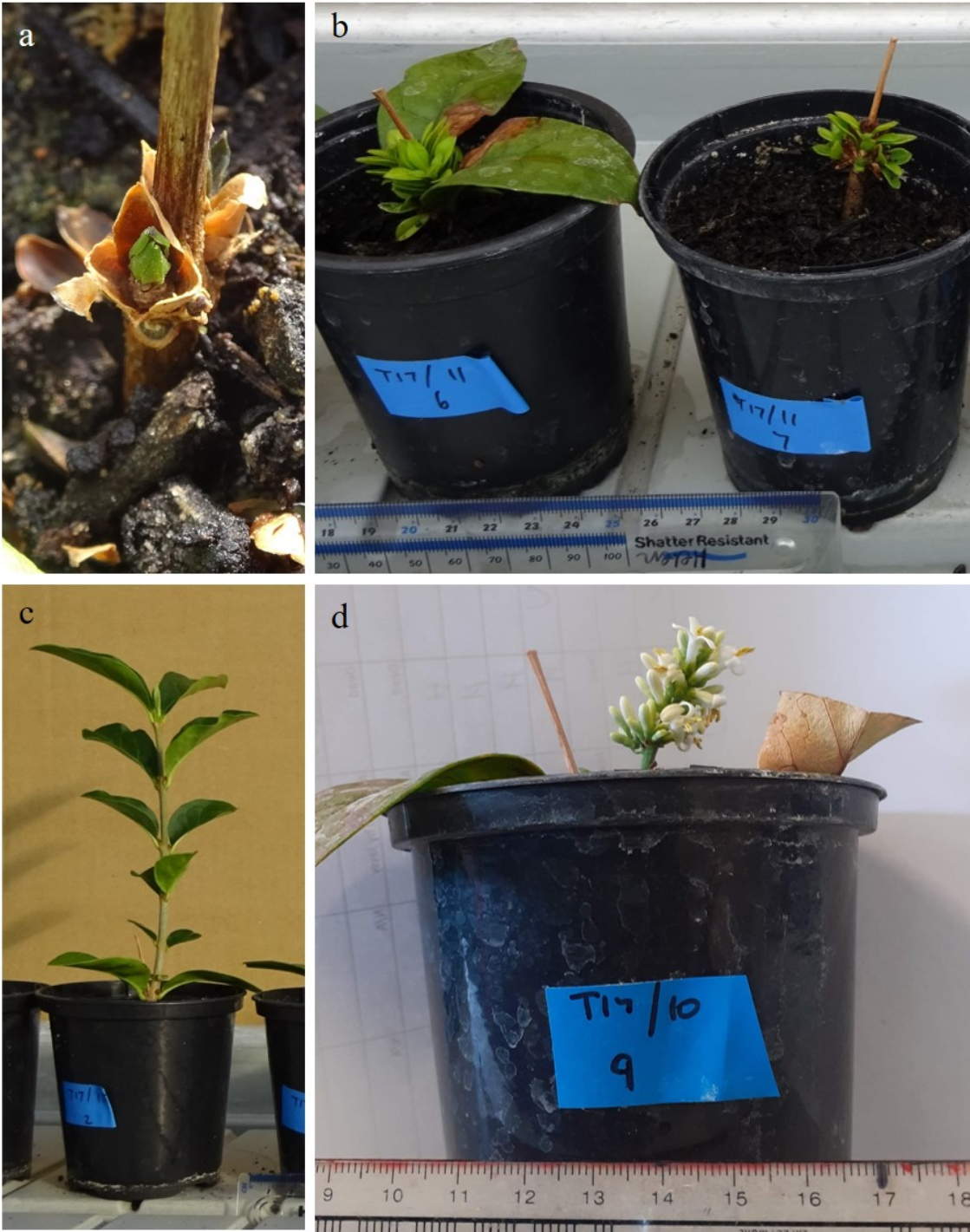


Figure 4.22: Examples of privet plant growth features noted over the nine-month observation period post inoculation with *Armillaria*.

a) example of healthy axillary buds; b) Axillary buds which have produced small leaves where original parent leaves are still present (left) and have dropped off (right) from plants inoculated with *T. atrobrunneum* T17/11 at six mpi with *Armillaria*; c) a new shoot produced by privet plants inoculated with *T. atrobrunneum* T17/15 at two mpi with *Armillaria*; d) a flower stem produced by privet inoculated with *T. hamatum* T17/10 after nine mpi with *Armillaria*. Ruler represents scale in cm.

Chlorophyll content, or greenness, of plants was measured on each plant using a SPAD (Soil Plant Analysis Development) meter to determine whether *Armillaria* infection or inoculation with *Trichoderma* affected plants. SPAD metres measure the difference between red and infrared transmittance in a leaf which is related to the chlorophyll content (Minolta 1989). The SPAD was measured for all plants every two months over the course of the experiment and the average was calculated (Figure 4.23). SPAD measurements should be taken from fresh leaf material, however since privet plants rarely produced large enough leaves for sampling, parent leaves were used where possible which could account for the general decrease in SPAD recorded. At six months many parent leaves were senescing (Figure 4.21) which could account for the low SPAD values reported. Statistical analysis was not performed on SPAD measurements due to a lack of suitable data. New stems of privet plants were produced irregularly, and measurements had an increasing proportion of ‘missing’ data points towards the end of the experiment as plants died.

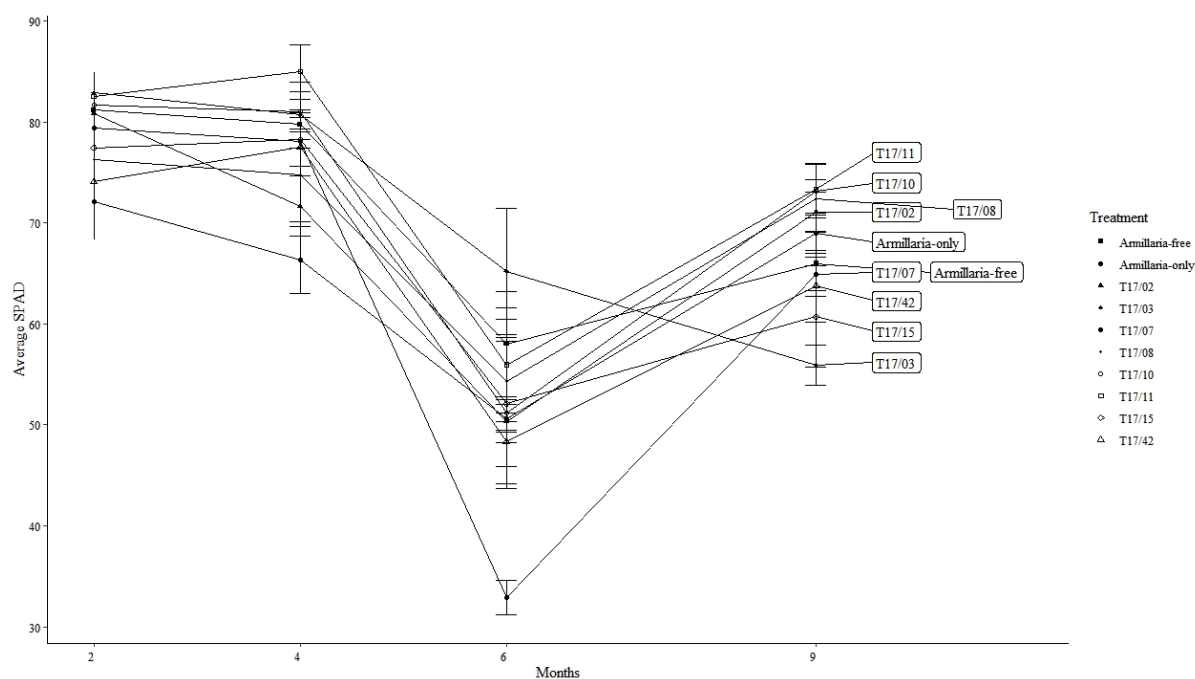


Figure 4.23: Chlorophyll content, or greenness, measurements of privet plants over time.

Average SPAD (unitless) of privet plants for each *Trichoderma* treatment (with 9 replicates, excluding some dead plants) over time. Treatments included two *Trichoderma*-free controls (*Armillaria*-only and *Armillaria*-free) and eight *Trichoderma* isolates. *Trichoderma* species included *T. virens* (T17/02), *T. harzianum* (T17/03, T17/07, T17/08), *T. hamatum* (T17/10), *T. atrobrunneum* (T17/11 and T17/15) and *T. olivascens* (T17/42). The error bars represent the standard error of the mean.

4.4.5.2 Level of *Armillaria* infection in privet plants over experimental duration

The first deaths in privet plants occurred four mpi with *Armillaria* where four plants died from confirmed *Armillaria* infection in treatments *T. virens* T17/02 (two plants) and *T. harzianum* T17/03 (two plants) (Figure 4.24). The highest number of deaths (six plants) was recorded from *Armillaria*-only inoculated plants and no new deaths were recorded between eight and nine months, when the experiment was terminated. One *Trichoderma* treatment resulted in the death of five (*T. virens* T17/02) and four (*T. harzianum* T17/03) plants. Two *Trichoderma* treatments resulted in the death of three (*T. olivascens* T17/42 & *T. harzianum* T17/07) and two (*T. hamatum* T17/10 & *T. harzianum* T17/08) plants. Only one plant died with inoculation from *T. atrobrunneum* T17/11 or *T. atrobrunneum* T17/15. As expected, no *Armillaria* was confirmed in *Armillaria*-free plants.

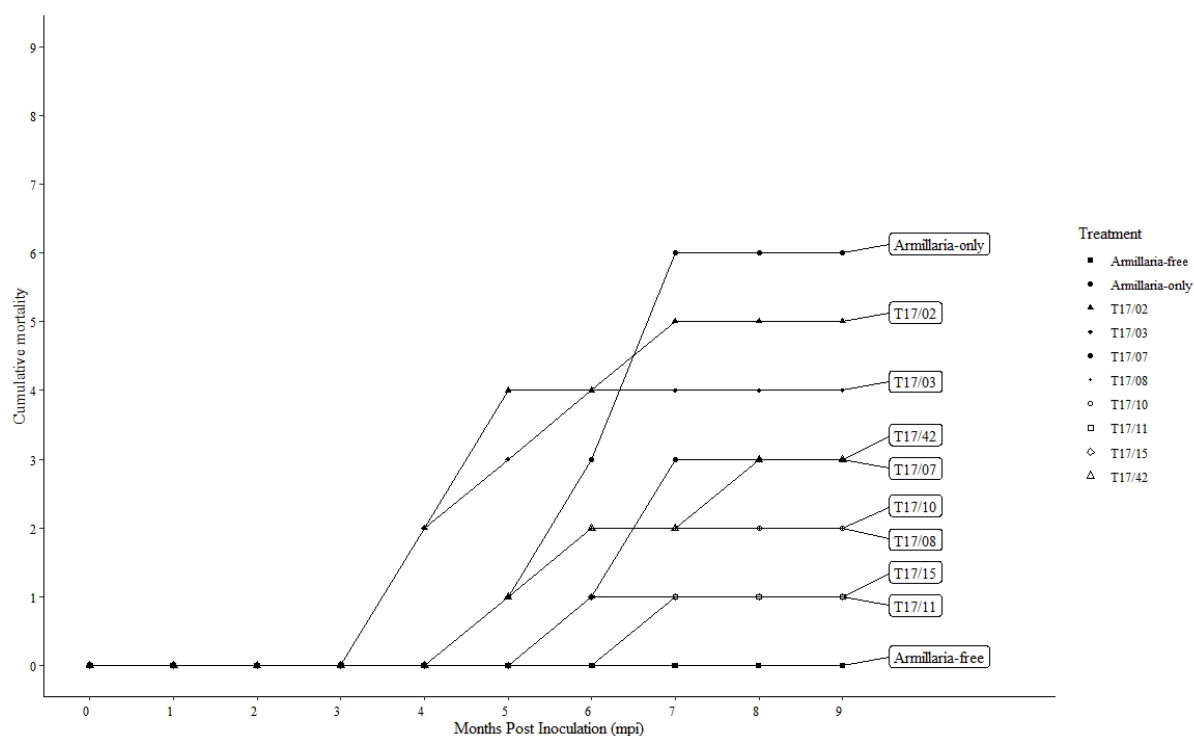


Figure 4.24: Number of privet plant deaths from ARR over nine months.

Treatments included two *Trichoderma*-free controls (*Armillaria*-only and *Armillaria*-free) and eight *Trichoderma* isolates. *Trichoderma* species included *T. virens* (T17/02), *T. harzianum* (T17/03, T17/07, T17/08), *T. hamatum* (T17/10), *T. atrobrunneum* (T17/11 and T17/15) and *T. olivascens* (T17/42). There were nine plant replicates per treatment.

4.4.5.3 Disease severity

After nine months, at the time of destructive sampling 27 of 90 plants had died. Visual assessment of all plants inoculated with *Armillaria* found rhizomorphs present in the soil (Figure 4.25a) of 90 % of plants. Symptoms of *Armillaria* infection were visually assessed and severity of disease was recorded on a 0 – 4 pt. scale (Table 4.4). In one case a rhizomorph was found adhered to a side root of a privet plant inoculated with *T. harzianum* T17/03 (Figure 4.25b – c). Some plants had a healthy root collar but with *Armillaria* mycelial colonisation at the tips of lateral roots and in other cases plants had died from extensive colonisation of *Armillaria* throughout the roots including in the root collar (Figure 4.25; d - e).

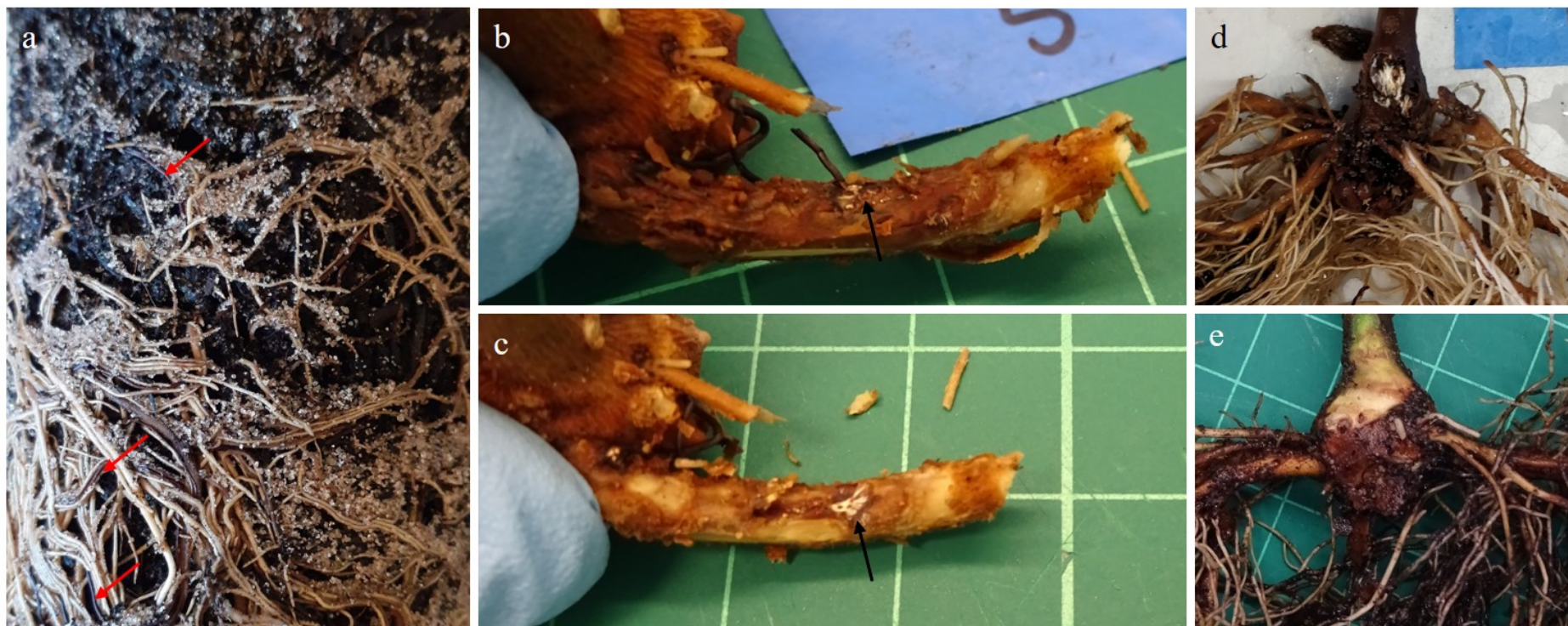


Figure 4.25: *Armillaria* infection in privet plants.

a) Brown rhizomorphs (red arrow) growing amongst cream coloured privet roots; b) Privet plant inoculated with *T. harzianum* T17/03 showing *Armillaria* infection on a lateral root (black arrow) with an adhered rhizomorph; c) Uncovered lesion (black arrow) of the same plant; d) Privet plant inoculated with *T. harzianum* T17/03 killed by *Armillaria*; e) advanced *Armillaria* lesion on privet inoculated with *T. atrobrunneum* T17/15.

Table 4.4: Disease Severity Index 0 – 4 descriptions.

Armillaria mellea isolations were only made from roots of plants not showing *Armillaria* mycelial colonisation.

| DSI | Above-ground | Below-ground |
|-----|--------------------------|---|
| 0 | No aerial symptoms | No visible <i>Armillaria</i> mycelium |
| 1 | Some aerial symptoms | No visible <i>Armillaria</i> mycelium |
| 2 | No aerial symptoms | Visible <i>Armillaria</i> mycelium colonisation |
| 3 | Signs of aerial symptoms | Visible <i>Armillaria</i> mycelium colonisation |
| 4 | Dead Plant | Visible/heavy <i>Armillaria</i> mycelium colonisation |

The DSI (0 – 4 pt. scale) of *Armillaria*-infected plants was lowest for plants inoculated with *T. atrobrunneum* T17/11 (Figure 4.26). Only one plant died from ARR with *T. atrobrunneum* T17/11 or *T. atrobrunneum* T17/15 inoculation and two plants without symptoms had small mycelial fans in the roots. As expected the highest DSI was for *Armillaria*-only plants where six died, two showed symptoms without visible *Armillaria* infection and one plant was healthy. The DSI was similar for *T. virens* T17/02 where five plants died, three showed symptoms without visible ARR and one was healthy. The average DSI for *Armillaria*-free plants was close to healthy (DSI 0.33) and highest for *Armillaria*-only plants (DSI 3.11) as expected (Figure 4.26). *Trichoderma virens* T17/02 (DSI 2.89) had an average DSI close to *Armillaria*-only control and one point higher than the next highest *Trichoderma* treatment, *T. harzianum* T17/03 (DSI 1.89). Statistical analysis showed that the *Armillaria*-only control and *T. virens* T17/02 were significantly different to the *Armillaria*-free controls (One-Way ANOVA with Tukeys post hoc test: $F_{9, 80} = 2.4285$, $df = 9$, $p < 0.5$).

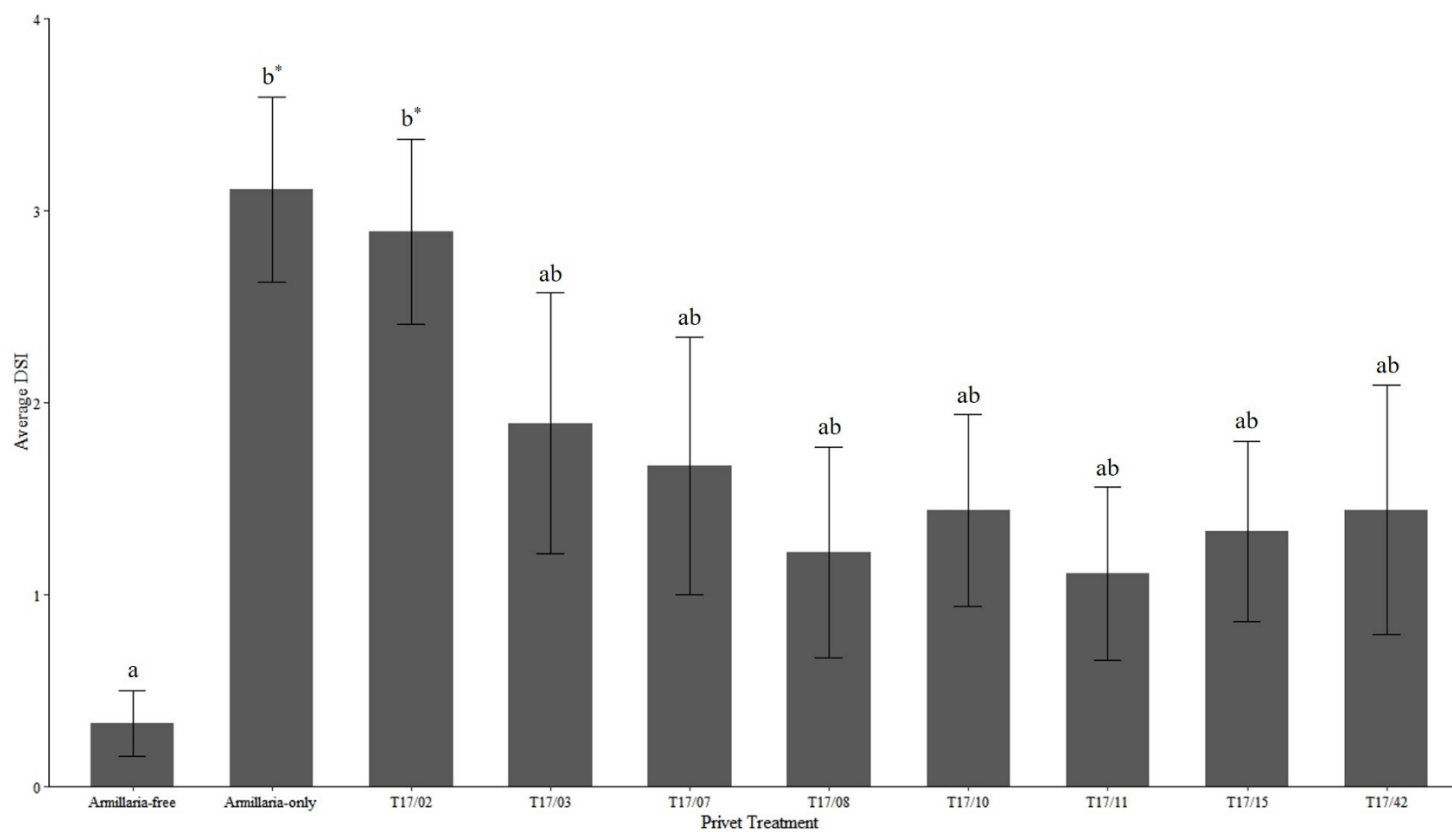


Figure 4.26: DSI of *Armillaria* infection in privet plants pre-colonised with *Trichoderma* spp..

Freshly rooted privet cuttings were pre-colonised with *Trichoderma* spp. for one month prior to the addition of *A. mellea* CG440. The average DSI (0 – 4 pt scale) for privet plants assessed the potential for *Trichoderma* treatments to offer protection from ARR. The error bars represent the standard error of the mean (n = 9). a, b represent statistical groupings (One-way ANOVA with Tukey's post hoc test). * p < 0.05, ** p < 0.01, *** p < 0.001

4.4.6 Effect of soil microbial communities on the biocontrol potential of *Trichoderma* spp.

The results from previous *in planta* studies used low-nutrient compost : sand (3 : 1) based mediums which were considered relatively free from microbial communities. To further test potentially protective *Trichoderma* isolates, in ‘sterile’ and microbially rich substrates three soil types were tested. A control was based on the compost : sand (3 : 1) mix previously used. Soil was collected from RHS Wisley Gardens Trial plots to represent microbial communities in garden soils. The same was soil baked at 60 °C for three days to reduce microbial community and act as a ‘sterile’ substrate. Three *Trichoderma* isolates were used based on the two best performing isolates from the first experiment in privet detailed in section 4.4.5 (*T. atrobrunneum* T17/11 and T17/15) and the commercially available *Trichoderma* strain, *T. harzianum* T22. An *Armillaria*-free and *Armillaria*-only control was set up free from *Trichoderma*. Plants were inoculated with *Trichoderma* (or mock inoculated for *Trichoderma*-free controls) with seven replicates and left for one month before addition of *Armillaria mellea* CG440.

After one year, only two plants had died. These were an *Armillaria*-only control (after nine months) and a plant inoculated with *T. harzianum* T22 (after seven months) both from baked soils. It is possible poor infection might have been caused by a disrupted watering regime due to skeleton staff at RHS Wisley as a result of COVID-19 or a ‘bad batch’ of *A. mellea* inoculum. Given the low mortality in *Armillaria*-only control plants at the point of thesis writing, the experiment is being continued and data will not be reported here.

4.5 Discussion

4.5.1 The most efficient *Armillaria* inoculation method

The RST inoculum trial proved that using this crumbly textured medium was an unsuccessful alternative to the traditional woody billets used for *Armillaria* inoculum. Since *Armillaria* infection of plants requires a minimum of three months for herbaceous species and longer for larger woody plants a substantial piece of inoculum is required as the food-reserve for *Armillaria* to prevent it from drying out before infection occurs. Ford *et al.* (2017) found that agar plugs were an unsuccessful alternative to a woody inoculum because they were completely desiccated before infection occurred. It was thought that using RST medium would have a higher moisture content than agar plugs and sufficient mycelial growth and compaction would prevent desiccation and provide enough substrate to enable *Armillaria* infection, but this was not the case. Acorns and horse chestnuts proved a more successful alternative to woody billets than RST inoculum. The preparation of these inoculum required less time since seeds could be brought from commercial suppliers and only had to be assessed for damage and weighed to achieve a standard size. Hazel billets still required collection from a hazel coppice and being cut to size before they were ready for colonisation. Both acorns and horse chestnuts were easily colonised by *Armillaria* and usually held shape while being used as inoculum, although in some cases the substrate disintegrated during inoculation, more often with acorns than horse chestnut. When arranged horizontally, colonisation of hazel was faster than traditional vertical methods, reducing preparation time by two months whilst still giving good infection.

Fungi require a carbon, nitrogen, and oxygen source together with other nutrients and some trace elements. Carbon and nitrogen are required for structural growth. Carbon, usually in the form of sugars, provides fungi with an energy supply and nitrogen, typically as ammonium salts, is involved in protein production (Walker and White, 2017). The higher level of carbon : nitrogen in hazel billets compared to horse chestnuts and acorns provide the *Armillaria* with more of its primary food source, suggesting a potential reason for the high survival rate of *Armillaria* in hazel. Calcium levels were again higher in hazel and are potentially required for signal transduction (Walker and White, 2017). Lower levels of

potassium, magnesium and sulphur were reported for hazel compared to horse chestnut and acorns although survival of *Armillaria* in those substrates was lower than in hazel. Enzyme activity requires potassium and magnesium among other elements (Walker and White, 2017) which was provided in greater quantities to horse chestnuts and acorns. A study looking at *in vitro* growth of *A. borealis* and *A. gallica* found various chemical compounds including potassium sulphate, manganese sulphate, ferrous sulphate, potassium chloride and sodium chloride could increase the number of rhizomorphs (Przybył and Mańka, 2004) suggesting that they are involved in growth and infection of *Armillaria* sp.. Although the chemical analysis of substrates provides some understanding between the differences of nutrient availability to *Armillaria* within different substrates, it has not provided a conclusive reason for the varying levels of *Armillaria* survival in inoculum and severity of ARR in plants.

In the present study, the skin of horse chestnuts and acorns was pierced prior to autoclaving which could make seeds more prone to desiccation than hazel. Tannins were not measured in this study but acorns and horse chestnuts have been reported to contain large amounts of tannins (7 – 11 %) (Shimada, 2001) which may stimulate *Armillaria* spp. growth as reported in *in vitro* studies by Shaw (1985). Extent of substrate lignification was not measured, however, Saura-Calixto *et al.* (1983) reported that lignin composition of hulls of chestnuts and acorns was lower than for hazelnuts. A study has shown that there is a correlation between potential lignin degradation by *Armillaria* and available sugars (Entry *et al.*, 1992), thus *Armillaria* in hazel billets might have had less available sugars than the starchy acorns and chestnuts leading to slower degradation of hazel. Another study found that *Armillaria* preferentially degraded cell walls with lower lignin content (Schwarze *et al.*, 2000). Hulls of chestnuts and acorns were found to have a lower lignin content by Saura-Calixto *et al.* (1983), therefore if cell walls with a low content of lignin were preferentially targeted, these substrates might undergo a faster structural change than hazel billets as noted in this study. Additionally, if increased degradation of horse chestnuts and acorns occurred, it may promote the fungus to find a new food source sooner. This could account for a higher severity of ARR in horse chestnut based substrates where infection might have occurred earlier as a result of *Armillaria* searching for another food-source. Furthermore, where ARR infection occurred, a higher rate of recovery of *Armillaria* from plants inoculated with horse chestnuts and acorns

could be supported by this hypothesis; if *Armillaria* infection were to occur more quickly, ARR infection would be heavier or further advanced hence a greater recovery of *A. mellea* from horse chestnut and acorn based substrates compared to hazel based substrates.

Sweet chestnut seeds have successfully been used to infect plants with *Armillaria* in the literature (Calvet *et al.*, 2015) with no report of inoculum degradation at the point of disease assessment after ten months. Chestnuts are rarely used as inoculum, and as far as I am aware there have been no cases of horse chestnuts used as inoculum sources for *Armillaria* in the literature. *Armillaria* colonised acorns have been used as inoculum where they were secured to roots using parafilm with one acorn per plant on peach (*Prunus persica*) (Beckman and Pusey, 2001) or *Pinus radiata* plants (Mesanza *et al.*, 2016) achieving mortality rate of 48 % and 54 %, respectively. Elias-Roman *et al.* (2019) used two acorns per plant and reported ARR infection of ~ 50 %. Published data using acorns as *Armillaria* inoculum sources report a higher infection rate than that in the present study (30 %) which could be due to the use of parafilm preventing desiccation of inoculum. In addition, the inoculum made direct contact with roots in aforementioned studies whereas here inoculum was placed 50 mm from the root collar of the plant. The purposeful direct contact between inoculum and roots will likely aid quick infection. Reports in the literature place the *Armillaria* inoculum in close proximity or direct contact with host plant roots (Entry *et al.*, 1992; Klein-Gebbinck *et al.*, 1993; Beckman and Pusey, 2001; Mansilla *et al.*, 2002; Solla *et al.*, 2002; West and Fox, 2002; Popoola and Fox, 2003; Raziq and Fox, 2003, 2005) more frequently than with spacing between roots and inoculum (Pearce and Malajczuk, 1990; Tsopelas and Tjamos, 1999; Calvet *et al.*, 2015; Ford *et al.*, 2017). The efficiency of *Armillaria* inoculum when in direct contact with plant roots versus separating inoculum and roots needs to be studied in further detail.

Studies of alternative substrates for *Armillaria* inoculation have shown that time required to colonise hazel billets can be reduced from a minimum of three months to only one month. By incubating billets horizontally, surface area of inoculum available to *Armillaria* is increased and duration of colonisation can be reduced. Using alternative substrates such as RST was unsuccessful, likely due to desiccation during plant inoculation period. Although a firm mycelial block of RST was used, after three months inoculum could be rescued from plants where a larger piece was used but had clearly dried out before

Armillaria could infect plants. Where acorn inoculum was able to infect plants, ARR severity was high, however the number of plants with signs of ARR was only 30 %. Horse chestnuts have proven to be a suitable alternative to traditional hazel billets. The ready availability of horse chestnuts reduces the laborious collection time and cutting steps required to prepare hazel billets. Based on the data from this study (not including data from the wider study this work was part of) horse chestnuts are a good source for producing *Armillaria* inoculum, however, the number of plants in trials should be increased to account for the lower level of ARR infection than hazel provided (60 % vs 80 %). If the number of plants needs to be reduced in trials, hazel billets might be recommended because a greater number of plants can be infected, although severity of infection is seemingly reduced. Finally, if the aim of a trial is to study severity of disease, horse chestnuts should be used since data here shows infection is more aggressive within the time periods used in this study. Further analysis of the whole data set is required to confirm this conclusion.

4.5.2 *Trichoderma* colonisation efficiency

There are two main methods for plant inoculation with *Trichoderma*. Some studies use substrates such as boiled rice (Savazzini *et al.*, 2009), rice bran (Chen *et al.*, 2016), wheat bran (Raziq and Fox, 2004c, 2004b; Kandula *et al.*, 2015) or mushroom compost containing mushroom mycelium (Raziq and Fox, 2003, 2004a, 2004b, 2005) to grow *Trichoderma* before the addition to soil. Others use spore suspensions of *Trichoderma* varying from $2 \times 10^5 - 10^8$ (Cripps-Guazzone *et al.*, 2016; Hill *et al.*, 2016; Ruano-Rosa *et al.*, 2016; Chen *et al.*, 2019) which are either directly inoculated onto plant roots via dipping (Ruano-Rosa *et al.*, 2016; Chen *et al.*, 2019), coated onto seeds (Cripps-Guazzone *et al.*, 2016) or added as a suspension to the soil (Hill *et al.*, 2016; Amaral *et al.*, 2019). In this study, where *Trichoderma* isolates were chosen on the basis of their endophytic nature, a spore suspension of 10^5 conidia / ml was inoculated directly onto roots and any remaining suspension was mixed into the soil to maximise opportunity for endophytic colonisation.

To confirm *Trichoderma* colonisation of plant roots, freshly propagated privet plants were inoculated with *Trichoderma*. Surface sterilisation with 1% Virkon solution and isolation onto selective medium

allowed confirmation of endophytic *Trichoderma* isolations. Efficiency of Virkon to kill *Trichoderma* spp. was found to be up to 100 % and so surface sterilisation was concluded to be sufficient to confirm isolates as endophytic. In the first week of colonisation, *Trichoderma* was isolated from all privet roots. In a study with eleven *Trichoderma* isolates (species not reported) inoculated into soil of *Pinus radiata* endophytic colonisation at three days prior to seedling emergence, one and seven days post emergence was investigated (Hill *et al.*, 2016). Isolation and visualisation of fluorescent *Trichoderma* isolates confirmed the internal colonisation of *Trichoderma* in all seedlings at all time points, with some isolates providing a greater degree of colonisation (Hill *et al.*, 2016). This supports data found in the present study of rapid colonisation by *Trichoderma* with variation in the degree of colonisation. *Trichoderma* seed-coated ryegrass and maize plants were shown to be internally colonised by *T. atroviride* from maize and ryegrass at seven and 21 dpi, respectively (Cripps-Guazzone *et al.*, 2016). Visual colonisation of *Trichoderma* was assessed in olive roots using GFP fluorescing *T. harzianum* (Ruano-Rosa *et al.*, 2016). Within one dpi *Trichoderma* hyphae from pre-germinated conidia could be observed on the root surface but not within the root. On the second dpi chlamydospores began to develop with the first fully developed chlamydospores being observed three dpi. It was presumed that pre-germinated conidia and mature hyphae were present in this study since spore suspensions were unfiltered after collection from Petri dishes as described by Ruano-Rosa *et al.* (2016), which potentially allowed faster colonisation of roots although this was not described by the authors. Results reported in the present study are in line with those reported from Hill *et al.* (2016) where *Trichoderma* colonisation of plant roots occurs quickly.

Over time, recovery of *Trichoderma* spp. reduced slightly but could be recovered from internal plant tissues for six weeks. Observations by Ruano-Rosa *et al.* (2016) suggest *Trichoderma* mycelium covered roots at three dpi but by seven dpi had almost completely disappeared. The last observation of mycelium was made 21 dpi. After 100 dpi *T. harzianum* was predominantly visualised in the form of chlamydospores (Ruano-Rosa *et al.*, 2016). Their visualisation study suggests that *T. harzianum* is unable to endophytically colonise roots of olive plants. However, results from this study have confirmed internal colonisation of *Trichoderma* spp. over the duration of a six week colonisation assay where

privet was the host plant. Colonisation of roots and hyphal networks were noted intracellularly in rhizodermal cells and colonisation of young differentiated cells were reported in an example of endophytic colonisation of *P. radiata* roots (Hill *et al.*, 2016). Although visualisation of *Trichoderma* was not assessed in this report, the latter study (Hill *et al.*, 2016) reports visual and isolation success to show endophytic colonisation of plant roots by *Trichoderma* similarly to those found here. Thus *Trichoderma* is able to quickly colonise privet roots with long-lasting success.

Studies investigating *Trichoderma* spp. colonisation have made some reports of *Trichoderma* colonisation of uninoculated plants. A small amount of *Trichoderma* was found in controls, presumed to originate from airborne greenhouse contamination or unsterilized potting mix by Hill *et al.* (2016). Cripps-Guazzone *et al.* (2016) reported *Trichoderma* isolation at a low level (20 %) from one control plant. Presence of *Trichoderma* in *Trichoderma*-free controls was also noted here where ~ 10 % re-isolation of *Trichoderma* which occurred from one privet plant in the colonisation study. In order to reduce occurrence of *Trichoderma* movement between pots, all plants were kept in individual saucers for all experiments. In the strawberry screening experiment more *Trichoderma* was re-isolated from negative controls compared to that reported in privet plants. Strawberry plants were not assessed for *Trichoderma* colonisation prior to inoculation with *Trichoderma*, therefore the background level of *Trichoderma* colonisation in bare-rooted plants is unknown. In addition, the *Trichoderma* species ID was not confirmed from *Trichoderma*-free plants and airborne contamination from the greenhouse over five months might have contributed to background levels of colonisation by *Trichoderma*.

Few studies measure long-term colonisation of *Trichoderma* (more than two weeks) although there are some suggesting that *Trichoderma* colonisation is short term. Ruano-Rosa *et al.* (2016) found that hyphae lasted a maximum of three weeks on root surfaces and did not colonise internal tissue and at 100 dpi only chlamydospores could be observed. In contrast, colonisation of *Trichoderma* spp. (*T. atroviride* and *T. hamatum*) from the rhizosphere and endorhizosphere of *Trichoderma*-inoculated *P. radiata* seedling was recorded over seven months of sampling (Hohmann *et al.*, 2011). In line with Hohmann *et al.* (2011), here recovery of *Trichoderma* spp. was possible after five months in strawberry plants.

4.5.3 *Trichoderma* growth promotion

Strawberry plants were grown in a low nutrient compost : sand mix with no added fertiliser so that any potential growth promotion by *Trichoderma* might be enhanced. Parameters of growth promotion by *Trichoderma* typically assess fresh and dry weight of plant roots and shoots (Rabeendran *et al.*, 2000; Hohmann *et al.*, 2011; Mwangi *et al.*, 2011; Dehariya *et al.*, 2015; Marín-Guirao *et al.*, 2016). Assessing these parameters of plant growth promotion in this study would have been preferential, however, this was not done in order to keep inoculated plants and introduce *Armillaria*. Leaf size, also measured in cabbages by Rabeendran *et al.* (2000), was selected for assessment of growth promotion in strawberry plants but showed no significant variation between treatments. To further assess growth promotion, plant height was measured at the final time point where again, no significant differences were found between treatments. To confirm that change in plant height was not significantly different for *Trichoderma* treatment, measurements should have been made from the start of the experiment, but plants were selected for uniformity so were visually comparable. These findings suggest that *Trichoderma* did not provide growth promotion to strawberry plants. In contrast to this, a study using a mix of *T. harzianum* and *T. viride* inoculated on strawberry plant roots found increased strawberry yield and root weight (Porrás *et al.*, 2007) although this was over a longer time-frame of one year. Another study found *T. harzianum* could increase the shoot growth of strawberry plants (Vestberg *et al.*, 2004). There are numerous reports of growth promotion by *Trichoderma* in the literature for a number of plant hosts. For example, in *Pinus radiata*, *T. hamatum* and *T. atroviride* were able to promote the growth of seedling height, stem diameter and root biomass (Hohmann *et al.*, 2011). Since bare-rooted strawberry plants have been cultivated to burst into vigorous growth, they are likely to have a considerable internal nutrient reserve, limiting the chance of detecting growth promotion by *Trichoderma* spp. in the initial growth stages of the plant. In addition, strawberry plants have a bushy growth form which makes height a poor indicator of growth promotion in this host compared to tree seedlings such as *P. radiata*. It has been suggested that where plants are grown under suboptimal conditions, growth promotion is likely to be more noticeable (Rabeendran *et al.*, 2000). Although referring to solar radiation (Rabeendran *et al.*, 2000), this hypothesis was applied to the current project, whereby plants were grown in low-nutrient

soils without the addition of fertilisers or nutrients, however, no growth promotion was noted in leaf size or stem height of strawberry plants after two months growth.

4.5.4 *Trichoderma* protective ability in strawberry plants

After two months inoculation with *Trichoderma*, strawberry plants showed no signs of growth promotion. Therefore, *Armillaria* was introduced to investigate the potential of *Trichoderma* spp. to protect strawberry plants from ARR. *Trichoderma atrobrunneum* T17/11 resulted in no signs of *Armillaria* infection on strawberry plants, displaying promising signs of protection against ARR. There were nine isolates from *Trichoderma* species including *T. atrobrunneum* (four), *T. harzianum* (two), *T. hamatum* (one), *T. cerinum* (one) and *T. virens* (one) where two thirds of the plants remained healthy. *Trichoderma atrobrunneum*, which sits in the *T. harzianum* species complex (Chaverri *et al.*, 2015), showed the greatest potential as a biological control agent followed by *T. harzianum* and *T. hamatum*. A study by Raziq and Fox (2003) investigated the effect of *T. harzianum* inoculated into the soil of strawberry plants for control of ARR. After nine months, only two of five *T. harzianum* isolates protected strawberry plants from *Armillaria* indicating that the potential of *Trichoderma* as a biocontrol agent for ARR is isolate specific. The results from this first *in planta* experiment identified seven *Trichoderma* isolates with the potential to reduce ARR on strawberry. The selection criteria assessed the average DSI of all plants and those below the average DSI of control plants were considered putative biocontrol agents for further assessment. One isolate where all plants died was included as an example of a potentially disease-stimulating *Trichoderma* isolate.

4.5.4.1 Timing of *Trichoderma* and pathogen application

A second experiment was set up with the refined selection of *Trichoderma* spp. isolates chosen from the previous experiment and assessed in strawberry plants with ten replicates per treatment. There were two important differences in the experimental set up. Firstly, plants were inoculated with *Trichoderma* for only one month prior to infection with *Armillaria*. Secondly, *A. mellea* inoculum used hazel billets which were colonised horizontally for one month as opposed to vertically for three – six months. It is

unknown whether this change in preparation makes the inoculum more aggressive since comparative assessments were not made.

After seven months, 90 % of *Armillaria*-only control plants had died and the experiment was terminated. Only one treatment resulted in a lower number of deaths than the *Armillaria*-only control which was found for the isolate *T. olivascens* T17/42, originally suspected of stimulating ARR. The remaining seven *Trichoderma* isolates resulted in the death of 100 % (*T. virens* T17/02, *T. harzianum* T17/03, *T. harzianum* T17/08, *T. hamatum* T17/10 and *T. atrobrunneum* T17/15) or 90 % (*T. harzianum* T17/07 and *T. atrobrunneum* T17/11) of plants. The results from this second experiment to assess biocontrol potential of *Trichoderma* spp. suggests that ARR was stimulated by five *Trichoderma* spp. isolates (*T. virens* T17/02, *T. harzianum* T17/03, *T. harzianum* T17/08, *T. hamatum* T17/10 and *T. atrobrunneum* T17/15).

A study by Amaral *et al.* (2019) essentially proposed that if plants being assessed for biological control are inoculated with the pathogen too soon after the addition of *Trichoderma*, plant death could be exacerbated. This finding could explain the increased death of *Trichoderma*-inoculated plants compared to the previous study. Amaral *et al.* (2019) inoculated five month old *P. radiata* seedlings with a *T. viride* conidial suspension (1 ml of 10^6 spores / ml added to soil) and after two weeks, infected plants with *Fusarium circinatum*. Symptom development occurred sooner in plants inoculated with both fungi and increased necrosis was reported in comparison to plants inoculated with only the pathogen. Furthermore, pre-inoculation with *T. viride* before introduction of the pathogen increased the salicylic acid concentration compared to other plant control groups (Amaral *et al.*, 2019). Salicylic acid is a key hormone in plant defence against microbes (Pieterse *et al.*, 2012) and an increase in levels suggests increased activity against microbial infection or colonisation. Although in the current study none of these factors were measured, the similarity of findings by Amaral *et al.* (2019) whereby *Trichoderma* seemingly increased disease symptoms would suggest, if measured, similar results would be found. The authors suggest that a greater length of time should be allowed between fungal inoculations so the plant can acclimatise to inoculation with *Trichoderma* spp. which may be recognised by plants as an invading organism (Amaral *et al.*, 2019). The short time period does not allow the plant microbiome to adapt

before the pathogen is introduced and, with a weakened immune response, the disease severity is increased (Amaral *et al.*, 2019). In the present study, strawberry plants were in a dormant stage after prolonged cold-storage when inoculated with *Trichoderma*. Therefore, although left for one month prior to *Armillaria* introduction, plants would potentially require much longer return to normal growth with a strong immune response before a pathogen can be introduced. This is perhaps why *Trichoderma* inoculated plants had increased disease in the second strawberry experiment where *Armillaria* was added after one month pre-inoculation with *Trichoderma* which was not noted in the previous experiment where two months passed before introduction of a pathogen.

4.5.5 *Trichoderma* protective ability in privet plants

Armillaria infection of privet was expected, since privet is a known and susceptible host (Raabe, 1962; Drakulic *et al.*, 2017; Cromey *et al.*, 2019). Privet plants were readily colonised by *Armillaria* which produced numerous rhizomorphs within a few months. A pilot study inoculated plants with *Armillaria* and assessed the roots before visible symptoms developed where rhizomorphs could be seen growing alongside roots, apparently not yet causing infection. A visual system using SEM, and fluorescent *Trichoderma* isolates inoculated into plant roots would allow a more detailed understanding of whether *Armillaria* is attempting or being prevented from infecting privet roots during early infection stages.

Privet plants were inoculated with *Trichoderma* and infected with *Armillaria* one month later in greenhouse experiments conducted at RHS Wisley's FRF facility. In the case of dormant strawberry plants inoculated with *Trichoderma*, it is suspected that inoculation of *Armillaria* after only one month can lead to an increase in *Armillaria* infection. This phenomenon was not noted in privet plants where cuttings were taken and rooted in a hydroponic system thus, it is suspected that *Trichoderma* colonisation occurred more quickly and plants were acclimatised within one month prior to the addition of *Armillaria*.

There was not sufficient data from chlorophyll content of leaves for statistical analysis due to data gaps towards the end of the experiment where plants died. The SPAD metre measures chlorophyll content or 'greenness' (Spectrum Technologies, 2020) of plants and is designed for use by farmers to assess

nutrient levels or early signs of disease. Measurements were included in this study to determine whether *Trichoderma* could increase the relative chlorophyll content in plants and, whether signs of *Armillaria* could be detected before symptoms developed. A study of *Armillaria* infection in strawberry plants by Percival *et al.* (2011) measured chlorophyll content with a SPAD meter and showed significant reductions of chlorophyll content in *Armillaria* infected plants compared to the control. Although these results suggest that in strawberry plants, SPAD readings can indicate various stresses or disease in plants, no variation was noted in privet plants which have a thick waxy cuticle.

Investigations into potential plant protection offered by *Trichoderma* using privet as a host system found *T. atrobrunneum* isolates T17/11 and T17/15 gave good protection. On the other hand, plants inoculated with isolates *T. virens* T17/02 and *T. harzianum* T17/03 gave the poorest protection to privet plants. *Trichoderma harzianum* T17/07 resulted in moderate protection of privet plants. No new deaths were recorded from plants between eight and nine mpi with *Armillaria* at which point plants were assessed for *Armillaria* infection. No plant had a healthy DSI average. Some *Armillaria*-free control plants displayed aerial symptoms, although no *Armillaria* was confirmed giving an average DSI < 1. The lowest average DSI (0 – 4 pt. scale) for plants infected with *Armillaria* were from privet inoculated with *T. atrobrunneum* T17/11 (DSI 1.1), followed by *T. atrobrunneum* T17/15 (DSI 1.3) and *T. hamatum* T17/10 (DSI 1.4) all of which had a DSI < 1 in strawberry plants (based on a 0 – 6 pt. scale). These findings suggest that protection of privet plants, a highly susceptible species to ARR, is possible. Although privet is a common host plant for *Armillaria*, very few studies use it as a plant species for studies on ARR. West and Fox (2002) used privet plants 50 cm tall with a 15 mm stem diameter to study the impact of phenolic compounds on *Armillaria* monitoring plants for 2.5 years before destructive assessment. Another study looked at the impact of water stress on *Armillaria* infection. ‘Privet bushes’ were inoculated with *Armillaria* and subjected to various watering regimes for almost two years before destructive assessment (Popoola and Fox, 2003). Both these studies highlight that ARR can be monitored for multiple growing seasons before privet dies from infection where medium-sized but established plants are used. In the current study, small (< 20 cm), young privet plants were used which subsequently gave rise to a faster infection rate of control plants (< 1 year).

4.5.6 *Trichoderma* as a potential biocontrol of *Armillaria*

Using strawberry and privet plants as host systems for *Armillaria* was found to be reasonably successful. Strawberry plants are readily available as dormant, cold stored plants and enable complete experimental assessment of ARR within three to eight months. Privet plants could be easily rooted from cuttings in a Hydropod within ca. one month allowing for selection of uniform plants which were considered to be clonal since they were collected from the same hedge and were consistent over experiments. Greenhouse experiments using *Armillaria* could be carried out within nine months when using privet. Although strawberry plants are frequently used as host systems for *Armillaria* assays (Fox and Popoola, 1990; Popoola and Fox, 2003; Raziq and Fox, 2003, 2005; Percival *et al.*, 2011; Ford *et al.*, 2017), experiments using privet are far fewer (West and Fox, 2002; Popoola and Fox, 2003) and used larger plants compared to the freshly rooted privet in this study. Privet plants were less susceptible to general greenhouse pests and diseases compared to strawberries in the studies reported here. Strawberry plants had incidences of red spider mites, aphids and powdery mildew, none of which occurred on privet. Although privet plants were younger than strawberries when planted, infection of the softer root tissue of strawberries generally occurred faster. Overall, both plant systems are considered suitable for *Armillaria* infection assays, depending on experimental requirements such as root tissue composition (soft or lignified) or duration.

The severity of disease for strawberry and privet plants was often comparable for *Trichoderma* isolates tested. In both privet and strawberry host plants *T. atrobrunneum* T1711 and T17/15 were best performers with little or no signs of *A. mellea* infection suggesting that *T. atrobrunneum* has a good potential as a biocontrol agent against ARR which can be replicated in two plant species from different families. Chen *et al.* (2019) obtained *Trichoderma* isolates from decaying *Armillaria* rhizomorphs to investigate the biological control potential of a mix with both *T. virens* and *T. atrobrunneum* against ARR. Inoculated Turkey oak seedlings had a higher survival rate after six months than *Trichoderma*-free seedlings. The present study found both strawberry plants and privet plants had a much higher survival rate when inoculated with *T. atrobrunneum*. Survival in strawberry plants was 91.3 % (23

plants, eight isolates) which reduced only to 89 % (18 plants, two isolates) survival in privet plants indicating that *T. atrobrunneum* may have a role as an antagonist towards *Armillaria*.

The commercially available *T. harzianum* 'Trianum' was found to have some potential to reduce *Armillaria* infection by 13 % - 67 % based on visual assessment of above ground plant health (Percival *et al.*, 2011). Another study looked at protection offered to strawberry plants from ARR by *T. harzianum* strains pathogenic to commercial mushrooms. Between *T. harzianum* isolates the survival rate of plants varied from 25 % to 83 % (Raziq and Fox, 2003) suggesting that, like *in vitro* studies (Cheruiyot *et al.*, 1997; Kwańska *et al.*, 2004; Kwańska and Szykiewicz-Wronek, 2018), antagonism varies greatly between isolates. The different levels of protection reported between *Trichoderma* isolates from this study and in the literature highlight the importance of robust screening of isolates. In the present studies a screening test was carried out for 40 *Trichoderma* isolates to select potential biocontrol agents of ARR for further testing resulting in fewer replicates for each isolate in favour of testing greater number of *Trichoderma* isolates. Other studies have favoured large numbers of replicates for fewer *Trichoderma* isolates. Greater replication of plants for the chosen *Trichoderma* isolates would be a recommendation for future experiments to certify the effectiveness to protect plants from ARR.

Protection from ARR by *Trichoderma* can vary, but by using a mix of the most effective *Trichoderma* isolates protection might be increased. This was trialled by Raziq and Fox (2005) where two *T. harzianum* isolates were inoculated in strawberry plants to protect against ARR, however, where two *T. harzianum* isolates were present, all plants died. The findings suggest that combinations of isolates may not always be successful as biocontrol agents and should be carefully considered. A study investigated a combination of *Trichoderma* (*T. harzianum*, *T. virens* or *T. viride*) with arbuscular mycorrhizal fungi (AMF) (*Funneliformis mosseae*, *Glomus cerebriiform* and *Rhizophagus irregularis*) to control *Fusarium udum* in pigeon pea (*Cajanus cajan*) plants found *T. harzianum* in combination with AMF species could reduce the severity of *F. udum* disease (Dehariya *et al.*, 2015). Another study found that a mix of *Trichoderma* spp. (*T. atrobrunneum* and *T. virens*) could offer protection against ARR in Turkey oak seedlings (Chen *et al.*, 2019). In the present study *Trichoderma* isolates from different plant species have shown the potential to reduce *A. mellea* disease with individual

Trichoderma isolates. To further investigate the potential of a biological control agent of *Armillaria*, combinations of *Trichoderma* isolates could be assessed, however, compatibility of isolates and spatial competition must be considered.

Armillaria killed all plants inoculated with *T. olivascens* T17/42 in the first strawberry screening experiment and was included as a control for the potential stimulation of ARR by *Trichoderma*. Yet, in the second strawberry experiment and in privet, ARR infection was not as severe and the lowest number of deaths was reported for *T. olivascens* T17/42 in the second strawberry experiment. To my knowledge, *T. olivascens* has not been trialled as a growth promoter or biological control agent to date for any plants. The presence of pests and diseases such as mildew or red spider mite may have influenced the severity of infection in the first strawberry experiment, weakening the plants and making them more vulnerable to ARR, hence exacerbated disease.

Finally, although these studies have determined that there is real potential for pre-inoculation of plants with *Trichoderma* to reduce the severity or infection of *Armillaria* root rot, the consequence of introducing a ubiquitous fungus into the soil must be considered. In recent years there has been an increased concern about human-induced environmental damage over many spectrums, from the use of chemicals such as neonicotinoids to plastic pollution in the oceans. The impact of adding new *Trichoderma* isolates into an environment could change the pre-existing microbial communities. On one hand, *Trichoderma* is a ubiquitous fungus found in soils, plant foliage and roots (Harman *et al.*, 2004a) thus introducing a new isolate should not overtly affect the microbial community. However as discussed previously (Chapter 3) *Trichoderma* spp. display a range of mechanisms pathogenic towards other fungi. One study has looked into the effect of *Trichoderma atroviride* on microbial communities using sensitive real-time PCR assay developed for soil microcosms where soils at varying depths could be tested over several months (Savazzini *et al.*, 2009). The study found that after initial *Trichoderma* inoculation, and in the first weeks for both fungi and bacteria, differences were noted between inoculated and non-inoculated soils particularly from the soil surface at the point of inoculation. However, no long-lasting effect of *Trichoderma atroviride* was noted on the fungal communities of the soil (Savazzini *et al.*, 2009). Before the novel *Trichoderma* isolates in this study are enrolled on a large

scale to protect plants from ARR, investigations should consider how aggressive the isolates are towards the general microbial community to ensure no lasting damage is caused.

4.6 Summary

The studies carried out in this chapter have developed a faster inoculation system for *Armillaria*, proven that endophytic *Trichoderma* spp. can rapidly colonise privet plants which are a suitable host for *Armillaria* assays and found *Trichoderma* spp. to be a positive candidate to control ARR.

- Horse chestnuts and hazel billets, arranged horizontally, can be sufficiently colonised by *A. mellea* within one month for use as *Armillaria* inoculum. Horse chestnut-based inoculum results in slightly increased plant death compared to hazel and could be used as an alternative to hazel for plant inoculation with *Armillaria*.
- Colonisation of privet roots by endophytic *Trichoderma* spp. can occur rapidly and is long-lasting. This is the first case of endophytic *Trichoderma* colonisation reported in privet plants.
- *Trichoderma* is a promising biological control agent of *A. mellea*. Isolates of *T. atrobrunneum*, and *T. hamatum* have shown reduced severity of ARR and increased survival in infected privet and strawberry plants. A greater variation in protection from ARR was found by individual isolates as opposed to particular *Trichoderma* species, although two *T. atrobrunneum* isolates were consistently the best performing biological control agents.
- Timing of *Armillaria* infection after *Trichoderma* inoculation is important. If the pathogen is introduced too soon after *Trichoderma* inoculation, infection can be increased.

| Chapter 5: An investigation into *Armillaria mellea* virulence genes and their expression

5.1 Introduction

Advances for -omics studies in *Armillaria* have been slow. The first transformation system for *A. mellea* was only described in 2010 (Baumgartner *et al.*, 2010b) and there are no gene disruption systems developed as yet. Only in the last eight years have the genomes of *Armillaria* species been published (Collins *et al.*, 2013; Wingfield *et al.*, 2016; Sipos *et al.*, 2017), and we still know relatively little about the molecular genetics of *Armillaria* spp.. This chapter looks at potential gene function of virulence-related genes known from other model fungal plant pathogens that are encoded in the *A. mellea* DSM 3731 genome (JGI, 2013).

5.1.1 The *Armillaria* Genome

The first *Armillaria* genome to be published was that of *A. mellea* DSM3731 in 2013 (Collins *et al.*, 2013). Thereafter others followed including genomes of *A. fuscipes*, *A. gallica*, *A. ostoyae*, *A. cepistipes* and *A. solidipes* (Wingfield *et al.*, 2016; Sipos *et al.*, 2017). The 1000 Fungal Genomes Project (1KFG) aims to sequence genomes representing recognised fungal families, providing a tool to enhance our understanding (JGI, 2020) and has sequenced genomes of several *Armillaria* species (Table 5.1) including a second *A. mellea* isolate, ELDO17 (JGI, 2019), the strain which is amenable to *in vitro* fruiting and transformation. There is a lot of variation between and within the genomes of *Armillaria/Desarmillaria* species (Table 5.1). For instance, the *D. ectypa* genome is only 40.6 Mb whilst *A. luteobubalina* is > 97 Mb. Even within *A. mellea* there is 10 % variation in genome size between ELDO17 and DSM 3731. Many species of *Armillaria* show significant genome expansion compared to other Agaricales species (Sipos *et al.*, 2017). *Armillaria* species generally have a larger number of genes and genome size in comparison to other species within the same family. Both *Flammulina velutipes* and *Cylindrobasidium torrendii* have almost half the number of predicted genes

(12,218 and 13,940 respectively, (Sipos *et al.*, 2017)) and less than half the genome size (35.6 Mb and 31.5 Mb respectively, (Sipos *et al.*, 2017)) compared to some *Armillaria* species (e.g. *A. cepistipes*, *A. gallica* and *A. mellea*). The genome of *Guyanagaster necrorhiza*, a species in the sister genus of *Armillaria*, is similar in size to *A. fuscipes* with 14,276 genes and 53.6 Mb long (Sipos *et al.*, 2017) compared to *A. fuscipes* which is 53 Mb long containing 14,515 genes (Wingfield *et al.*, 2016) although this is much smaller than many *Armillaria* species (Table 5.1).

Table 5.1 *Armillaria* / *Desarmillaria* species genome statistics.

Data was adapted from respective JGI portals (except *A. fuscipes*) and includes reference to individual genome publications.

| <i>Armillaria</i> species and isolate | Reference | Genome Assembly size (Mb) | Number of genes | Average Gene length (bp) | Exons / gene |
|---------------------------------------|--------------------------------|---------------------------|-----------------|--------------------------|--------------|
| <i>A. borealis</i> FPL87.14 | 1KFG, (JGI, 2020) | 71.69 | 19,984 | 1671 | 6.05 |
| <i>A. cepistipes</i> B5 | Sipos <i>et al.</i> , 2017 | 75.83 | 23,460 | 1,658 | 5.5 |
| <i>A. fuscipes</i> CMW 2740 | Wingfield <i>et al.</i> , 2016 | 53 | 14,515 | 1,350 | 6 |
| <i>A. gallica</i> 21-2 | Sipos <i>et al.</i> , 2017 | 85.34 | 25,704 | 1,459 | 5.4 |
| <i>A. luteobubalina</i> HWK02 | 1KFG, (JGI, 2020) | 97.11 | 20,318 | 1,676 | 6.21 |
| <i>A. mellea</i> ELDO17 | 1KFG, (JGI, 2020) | 70.86 | 15,646 | 1,728 | 6.49 |
| <i>A. mellea</i> DSM 3731 | Collins <i>et al.</i> , 2013 | 79.55 | 14,473 | 1,613 | 6.02 |
| <i>A. nabsnona</i> CMW6904 | 1KFG, (JGI, 2020) | 62.72 | 19,015 | 1,701 | 6.11 |
| <i>A. ostoyae</i> C18/19 | Sipos <i>et al.</i> , 2017 | 60.11 | 22,705 | 1,526 | 5.51 |
| <i>A. solidipes</i> 28-4 | Sipos <i>et al.</i> , 2017 | 58.01 | 20,811 | 1,580 | 5.69 |
| <i>D. ectypa</i> FPL83.16 | 1KFG, (JGI, 2020) | 40.6 | 12,228 | 1,809 | 6.62 |
| <i>D. tabescens</i> CCBAS 213 | 1KFG, (JGI, 2020) | 74.88 | 19,032 | 1,685 | 6.19 |

5.1.2 Pathogenicity genes in *Armillaria*

Using genomics, proteomics and transcriptomics, Sipos *et al.* (2017) described some putative pathogenicity genes in *Armillaria* spp. identified as homologues of genes found in model plant pathogens which were known to be involved in virulence, induced host-cell death and plant defence responses in those species. The genes identified by Sipos *et al.* (2017) and from other literature on plant pathogenic fungi were used to make a proposed set of candidate genes of interest including genes involved in signalling pathways, small secreted proteins and effectors. Details of selected candidate genes are outlined below.

5.1.2.1 Genes involved in signalling pathways

Mitogen-activated protein kinase (MAPK) cascades are found in eukaryotes and are widely conserved in fungi. The MAPK are protein kinases which function in a succession to activate cell signals. The progression of the MAPK cascade occurs when MAPK kinase kinase (MAPKKK) phosphorylates MAPK kinase (MAPKK) which results in activation of MAPK after dual phosphorylation of amino acid residues threonine and tyrosine (Turrà *et al.*, 2014). There are five MAPK cascades found in the yeast *Saccharomyces cerevisiae* which are involved in mating, filament invasion, cell integrity, high-osmolarity growth and assembling the spore wall (Gustin *et al.*, 1998). The Ascomycota rice blast pathogen, *Magnaporthe grisea* (and most other Ascomycota) contains three MAPK cascades PMK1, MPS1 and OSM1 (Dean *et al.*, 2005) which are homologues to FUS3/KSS1 (Xu and Hamer, 1996), SLT2 (Xu *et al.*, 1998) and HOG1 (Dixon *et al.*, 1999), respectively in *Saccharomyces cerevisiae*. The function of PMK1 is involved in the formation of appressoria and developing conidia of *M. grisea* (Bruno *et al.*, 2004) which are both involved in the infectious stages of the pathogens life cycle. The MPS1 gene in *M. grisea* is involved in appressoria penetration and sporulation (Xu *et al.*, 1998) and the OSM1 gene in osmoregulation (Dixon *et al.*, 1999). Study of MAPK genes in Basidiomycota is limited to a few examples. In *Ustilago maydis*, the gene *kpp2* which is related to *pmk1*, is involved in mating and development of pathogenic infection (Müller *et al.*, 1999). Cell fusion in *kpp2* deletion mutants was reduced and it was found that the deletion mutants produced less pheromone and were unable to react

to pheromone stimulation. Furthermore, *kpp2* deletion mutants were found to significantly reduce tumours on plants (Müller *et al.*, 1999). The decay fungus *Ganoderma lucidum* encodes a homolog of the MAPK gene *SLT2* from *Saccharomyces cerevisiae* named *GLSt2* which has a variety of functions including fungal growth and sexual development. *GLSt2* knockout strains had reduced hyphal growth and impacted hyphal branching. In fruiting body development *GLSt2* transcription was induced and in knockout strains, primordia and fruiting bodies could not develop (Zhang *et al.*, 2017). Furthermore, *GLSt2* was also shown to be involved in cell wall integrity. In knockout strains, production of chitin and β -1,3-D-glucan were decreased, which was confirmed by downregulated transcription of related genes. *GLSt2* was also shown to be involved in regulation of cellular ROS and ganoderic acid biosynthesis (Zhang *et al.*, 2017).

Ras GTPases are small GTP (guanosine triphosphate) binding proteins (Kou and Naqvi, 2016) required for signalling pathways including the MAPK cascade (Ramezani-Rad, 2003; Zhao *et al.*, 2007). A number of fungi encode Ras GTPases including *Fusarium graminearum* (Bluhm *et al.*, 2007) *M. oryzae* (Kershaw *et al.*, 2019), *Trichoderma reesei* (Zhang *et al.*, 2012) and *Ustilago maydis* (Lee and Kronstad, 2002). In *F. graminearum* the *RAS2* gene is required for female fertility, fungal growth and pathogenicity of both maize and wheat (Bluhm *et al.*, 2007). In *M. oryzae* the protein SMO₁ which regulates Ras signalling, is required for conidial morphogenesis and SMO₁ deletion mutants produce small appressoria suggesting partial involvement in their development (Kershaw *et al.*, 2019). In *U. maydis*, *ras2* deletion mutants produced shorter and rounder cells and *RAS2* was shown to be required for filamentous growth and induction of disease symptoms (Lee and Kronstad, 2002). In *T. reesei*, Ras GTPases are required for hyphal growth, branching and sporulation (Zhang *et al.*, 2012).

5.1.2.2 Small secreted proteins

The *Armillaria* spp. genome encodes a number of putative small secreted proteins (SSPs). In other fungi these can act as effectors in both mutualistic and pathogenic interactions (Sipos *et al.*, 2017). In the model ectomycorrhizal Agaricomycete *Laccaria bicolor* the SSP, MiSSP7, is an important effector in the establishment of mutualistic relationships with host plant roots and formation of the Hartig net (Plett *et al.*, 2011). Furthermore an arbuscular mycorrhiza effector protein known as SP7 was shown to

interact with ethylene response factors, dampening the ethylene signalling pathway (Plett and Martin, 2018). The root and butt rot Agaricomycete pathogen, *Heterobasidion annosum* produces a number of SSPs which were studied for their potential to increase host-cell death by Raffaello and Asiegbu (2017). Two SSPs (HaSSP30 and HaSSP47) from *H. annosum* were transiently expressed in *N. benthamiana* and were found to induce rapid cell death (within 2 days) and loss of turgidity. In *N. benthamiana* defence genes stimulated by JA were induced (Raffaello and Asiegbu, 2017).

Cerato-platanin (*CP*) genes, enriched in *Armillaria* spp. (Sipos *et al.*, 2017), were first identified in *Ceratocystis fimbriata* f. sp. *platani* (Pazzagli *et al.*, 1999) where they play a role in virulence, induced cell death (Yang *et al.*, 2018; Liu *et al.*, 2019), have the potential to trigger the salicylic acid (SA) signalling pathway (Yang *et al.*, 2018) and are involved in penetration of banana roots (Liu *et al.*, 2019). In the Agaricomycete *Heterobasidion annosum*, a plant root-rot pathogen, CP-like proteins have been shown to induce cell death and phytoalexin production with significant increases in upregulation of defence genes in *Nicotiana tabacum* and stunted growth and cell death in the natural host, *Pinus sylvestris* (Chen *et al.*, 2015).

Expansins are thought to have evolved in fungi and bacteria after a horizontal gene transfer event from plants (Nikolaidis *et al.*, 2014) and have recently been found to be structurally and functionally similar to *CP* genes (Luti *et al.*, 2020). In the genome of *Armillaria* spp., expansins are also enriched (Sipos *et al.*, 2017). The expansin *ExII* in the phytopathogenic bacterium *Pectobacterium brasiliense* has been shown to increase virulence and is detected by plants to induce defence mechanisms by increasing reactive oxygen species (ROS) and activating jasmonic acid (JA) and SA defence pathways (Narváez-Barragán *et al.*, 2020). In 2015 the first report of an expansin from a white-rot Agaricomycete was made in *Schizophyllum commune* (Tovar-Herrera *et al.*, 2015). The expansin protein, ScExlx1, can bind cellulose, birchwood, xylan and chitin and can enhance the fungi's ability to hydrolyse cellulose (Tovar-Herrera *et al.*, 2015).

5.1.2.3 Effectors

Effectors are produced in many fungi to suppress the pathogen associated molecular patterns (PAMP) recognised by plants. Lysin motif (LysM) effectors are widely conserved in a number of fungi (Bolton *et al.*, 2008). In *Cladosporium fulvum*, Ecp6 is required for virulence and contains LysM domains of a carbohydrate binding molecule. Ecp6, is induced in compatible interactions in *C. fulvum* and when overexpressed in *F. oxysporum*, disease symptoms occur faster and result in more severe infections than wild type (WT) isolates (Bolton *et al.*, 2008) indicating involvement in disease. Ecp6 is a specific chitin-binding effector which inhibits activation of chitin-triggered immunity in hosts (de Jonge *et al.*, 2010). In *M. oryzae* the LysM effector, Slp1, suppresses chitin triggered immune responses and in *SLP* deletion mutants, infection is much reduced (Mentlak *et al.*, 2012). The LysM effectors in *Mycosphaerella graminicola* (Mg1LysM & Mg3LysM) can prevent lysis of fungal hyphae by plant chitinases, which is not something seen in Ecp6 or Slp1 (Kombrink and Thomma, 2013). The biological control fungus, *Trichoderma atroviride* also uses LysM effectors. A study suggests Tal6 is involved in the establishment of fungus-plant interactions, and protecting hyphae from chitinases by binding to chitin oligomers (Romero-Contreras *et al.*, 2019). LysM effectors have recently been identified in the entomopathogenic *Beauveria bassiana*, two of which are required for fungal virulence which also offered protection to fungal hyphae from chitinase hydrolysis (Cen *et al.*, 2017).

5.1.3 Secondary metabolites and plant cell wall degrading enzymes

Secondary Metabolites (SM) are not essential for fungal development, growth or reproduction, however, SM can have a role in establishing specific ecological niches or enhancing virulence (Rodriguez-Moreno *et al.*, 2018). Secondary metabolites which are involved in virulence produce either host-specific toxins (HST) or non-HST which can target a range of hosts (Rodriguez-Moreno *et al.*, 2018). Homologs of enzymes involved in secondary metabolism from *Heterobasidion* can be found in the *Armillaria* genome (Sipos *et al.*, 2017) including examples such as polyketide synthases, non-ribosomal peptide synthase-like enzymes and terpene cyclases which are produced during plant and

fungal interactions (Olson *et al.*, 2012). Any SMs with non-HST activity from *Armillaria* spp. may help to explain why a large number of hosts can become infected.

Ross-Davis *et al.* (2013) published a transcriptome for *A. solidipes* which listed a number of enzymes related to the degradation of plant cell walls. Enzymes included ligninolytic enzymes which act on lignin; pectinolytic enzymes, carbohydrate active enzymes and cellulolytic enzymes were described with roles related to the degradation of cellulose. Glycoside Hydrolase (GH) families GH6 and GH7 contain cellulases which are related to pathogenicity of *Magnaporthe oryzae* and are involved in invasion and penetration of the host epidermis (Van Vu *et al.*, 2012). *Armillaria* encodes GH75 Chitinases and a number of other GH families: GH1, GH28, GH78 and GH88 (Sipos *et al.*, 2017). The GH families GH28, GH78 and GH88 are known to be involved in the degradation of pectin (Van Den Brink and De Vries, 2011) potentially lending themselves to degradation of *Armillaria* host plants. The glycoside hydrolase (GH) families PL1, PL3 and PL9 which are found in *Armillaria* (Sipos *et al.*, 2017) are known to be involved in the degradation of pectin, alongside GH families (Van Den Brink and De Vries, 2011).

The selection of genes, secondary metabolites and plant cell wall degrading enzymes (PCWDE) introduced above are involved in fungal growth, various pathogenicity and virulence factors or inducing plant defence responses. Homologues for many of them can be found in the *Armillaria* genome. The investigation of a selection of these genes in this chapter aims to lead to a greater understanding of the infection system of *Armillaria*, an important fungal pathogen found worldwide.

5.2 Aims:

To examine candidate virulence genes found in *A. mellea*. Specifically:

- To choose a selection of candidate virulence genes within the *A. mellea* genome with homologs to model fungal plant pathogens.
- To create *A. mellea* transformants with GFP fused to the promoter region of candidate virulence genes.
- To visualise GFP expression to determine the localisation and timing of expression of the candidate virulence genes in *A. mellea* transformants.
- To determine expression profile of candidate virulence genes in different *A. mellea* tissue using GFP reporter and RT-PCR.

5.3 Results

5.3.1 Choosing candidate virulence genes in *A. mellea*

To identify potential candidate virulence genes, gene and protein sequences from model fungal plant pathogens in the NCBI database and appropriate publications were identified (Table 5.2). Three sequence matches were made with homologs of *PMK1* from *Schizosaccharomyces pombe* (NCBI: CAA17923) (named *AmPMK1a*, *AmPMK1b* and *AmPMK1c*) and *RAS2* from *Ustilago maydis* (NCBI: AAO19639) (named *AmRAS2a*, *AmRAS2b* and *AmRAS2c*). Matches were made with a SSP from *Heterobasidion annosum* (*AmSSP47*), an effector secreted LysM protein (*AmSLP1*) from *M. oryzae* (NCBI: EHA54333) and a *CP* gene (*AmCP*) from *Hypocrea virens* (NCBI: DQ121133) which can also be found in *Magnaporthe grisea* (Table 5.2). No significant matches were generated with four sequences including a LysM effector from *Cladosporium fulvum* and a SSP (MiSSP7) from the ectomycorrhizal fungi *Laccaria bicolor* (Table 5.2).

Table 5.2: Virulence genes in model plant pathogens and *A. mellea*

Candidate virulence genes from model plant pathogens were searched for in the *Armillaria mellea* DSM 3731 genome (JGI, 2013). Where homologs to candidate genes were found, the *A. mellea* ID is given; n/a indicates that no significant homologues were identified.

| Name | Description | Species | Function | Gene sequence Source | <i>A. mellea</i> ID (JGI) | Reference |
|-------------|---|---|---|--|----------------------------|--|
| <i>PMK1</i> | Mitogen-activated protein kinases | <i>Schizosaccharomyces pombe</i> | Appressorium formation and conidial development in <i>M. grisea</i> | NCBI: CAA17923 | g16430 g12999 g13528 | Wood <i>et al.</i> , 2002; Bruno <i>et al.</i> , 2004 |
| <i>RAS2</i> | Small GTPase | <i>Ustilago maydis</i> | Formation of perithecia, hyphal growth & regulation of virulence in <i>Fusarium graminearum</i> | NCBI: AAO19639 | g265 g18617 g15709 | Müller <i>et al.</i> , 2003; Bluhm <i>et al.</i> , 2007 |
| HaSSP47 | Small secreted protein | <i>Heterobasidion annosum</i> | Induced cell death | JGI <i>Heterobasidion</i> Protein ID: 447006 | g10712 | Raffaello and Asiegbu, 2017 |
| Slp1 | Effector secreted LysM protein | <i>Pyricularia oryzae</i> (syn. <i>Magnaporthe oryzae</i>) | Overcomes plant defences which target chitin in fungal cell walls | NCBI: EHA54333 | g18812 | Dean <i>et al.</i> , 2005; Mentlak <i>et al.</i> , 2012 |
| <i>SM1</i> | Cerato-platanin (CP) gene | <i>Hypocrea virens</i> | Activates defence responses against fungi & bacteria | NCBI: DQ121133 | g18393 | Djonović <i>et al.</i> , 2006; Yang <i>et al.</i> , 2009 |
| Ecp6 | LysM effector | <i>Cladosporium fulvum</i> | Increased virulence | Bolton <i>et al.</i> 2008 | n/a | Bolton <i>et al.</i> , 2008 |
| MiSSP7 | Small secreted protein in ECM | <i>Laccaria bicolor</i> | Establishment of mutualistic symbiosis | NCBI: EDR02836 | n/a | Martin <i>et al.</i> , 2008; Plett <i>et al.</i> , 2011 |
| NEP1 | Necrosis inducing extracellular protein | <i>Fusarium oxysporum</i> | Induced cell death and plant stress responses | NCBI: AF036580 | n/a | Nelson <i>et al.</i> , 1998; Keates <i>et al.</i> , 2003; Bae <i>et al.</i> , 2006 |

5.3.2 Constructing GFP-fused promoters of candidate virulence genes to visualise gene function and localisation in *A. mellea*

Nine candidate virulence genes which were found in the *A. mellea* genome were selected for further study (Table 5.2). Gene function studies in *Armillaria* spp. are limited, and with no gene disruption systems available, alternative methods are required. Thus, a promoter:GFP fusion was used to visualise promoter activity of candidate virulence genes, constructed by homologous yeast recombination and transformed into *A. mellea* through *Agrobacterium* mediated transformation (AMT).

5.3.2.1 Plasmid design for candidate virulence genes fused to GFP

The aim was to amplify a promoter region of 1 kb, and clone this upstream of an intron-containing GFP, in the t-DNA region of an *Agrobacterium* vector along with a suitable hygromycin resistance cassette for selection of transformants (Figure 5.1). Plasmids were designed using Serial Cloner (version 2.6) to fuse the promoter region of candidate virulence genes to an eGFP cassette to visualise gene activity and localisation. All plasmids used the hygromycin phosphotransferase (*hph*) cassette for transformant selection with Hygromycin B (Figure 5.1) and were constructed by homologous recombination. The *A. tumefaciens* vector (pCAMBIA0380) was adapted by adding the 2 μ origin of replication and *URA3* gene to a *ura3* mutant *S. cerevisiae* Y10,000 (Ford *et al.*, 2015). The yeast-adapted vector, pCAMBIA0380, formed the backbone of all plasmids and was linearised with *Bam*HI for direct use in yeast recombination (Ford *et al.*, 2015). The full length *hph* cassette had a cauliflower mosaic virus (CaMV) 35S terminator from pBGgHg (Chen *et al.*, 2000; Ford *et al.*, 2016) and was under regulatory control of the *A. mellea* *gpd* (Glyceraldehyde-3-phosphate dehydrogenase I) promoter (Figure 5.1). The *hph* cassette was amplified from pCAM-Amgpdihph-Amtrp1iGFP (Ford, unpublished) (Figure 5.2). Primers were designed to have a 30 bp overlap with the left border (LB) of the backbone for homologous yeast recombination (Appendix Table A.2). The eGFP cassette was amplified from pCAM-hph-Amgpd-xiGFP (Ford *et al.*, 2016) (Figure 5.2) which has a one kb *A. mellea* *gpd* promoter with an intron and exon region from *Phanerochaete chrysosporium* and an *Aspergillus nidulans* *trpC* terminator (Ford *et*

al., 2016) (Figure 5.1). As with the *hph* cassette, primers were designed with a 30 bp overlap with the right border (RB) of the backbone (Appendix Table A.2).

Appropriate promoter regions of candidate virulence genes were amplified from gDNA extracted from the genome sequenced strain *A. mellea* DSM 3731 using primers designed to have a 30 bp overlap with the *hph* and eGFP cassettes (Appendix Table A.2). All plasmids were named using the same format, for example the plasmid containing the promoter for the *AmCP* gene was named pChy-AmCP-eGFP. This identifies the pCAMBIA0380 backbone (C) with the *hph* cassette (h) which is yeast adapted (y) and determines that it contains the *AmCP* promoter gene fused to the eGFP cassette.

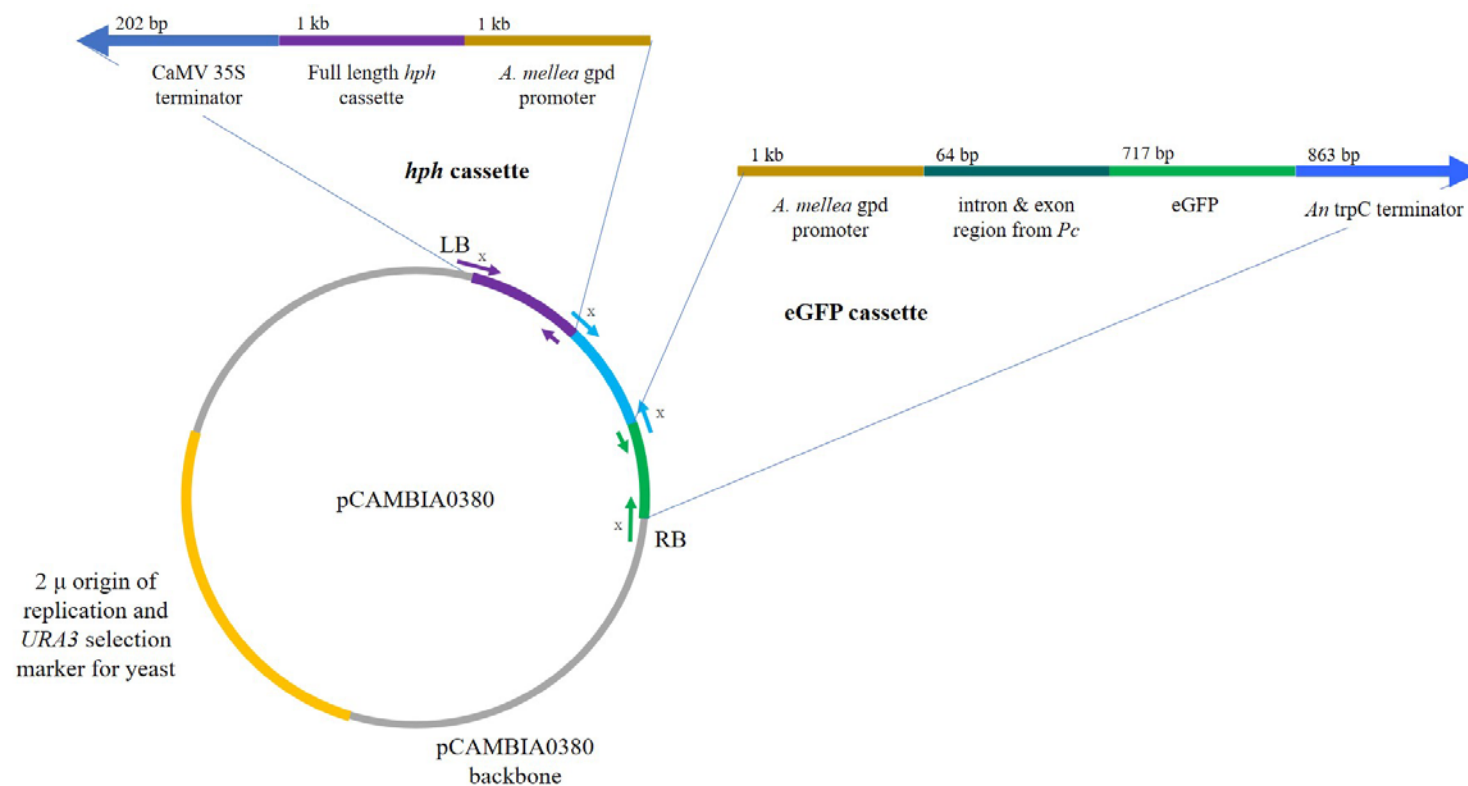


Figure 5.1: Schematic of plasmid design for promoter:GFP reporter cassettes.

The yeast adapted pCAMBIA0380 vector formed the backbone (grey) which was linearized with *Bam*HI and had a 2 μ origin of replication and *URA3* yeast selection gene (yellow) (Ford, 2015). The purple, blue and green fragments represent the *hph* cassette, 1kb promoter region of candidate virulence genes and eGFP cassette, respectively. The primer sets (coloured by fragment) are labelled with arrows showing the direction, 'X' indicates where a 30 bp overlap between fragments exists for homologous yeast recombination. Inserts show the full length *hph* cassette which is under regulatory control of the *A. mellea* gpd promoter with a CaMV 35S terminator. The eGFP cassette has an *A. mellea* gpd promoter with an intron and exon region from the *Phanerochaete chrysosporium* (*Pc*) gpd and an *Aspergillus nidulans* (*An*) trpC terminator.

5.3.2.2 Amplification of candidate virulence genes from *A. mellea*

Armillaria mellea DNA was extracted using the fast fungal DNA protocol from the genome sequenced isolate DSM 3731. Confirmation that DNA was of amplifiable quality was made using ITS1 & ITS4 primers (Table 2.5) which amplified ~1 kb over the ITS 1 & 2 region as expected (Figure 5.2). Primers were designed to amplify 1 kb of the promoter region for each candidate virulence gene with a 30 bp overhang for the eGFP and *hph* cassettes to construct plasmids by homologous yeast recombination (Appendix Table A.2). Using the KAPA *Taq* DNA polymerase enzyme and gene-specific primers amplified DNA under the following conditions: 95 °C for 3 mins, followed by 35 cycles of 98 °C for 20 s, 60 °C for 15 s and 72 °C for 30 s and a final extension of 72 °C 1 min. Good quality amplification of expected sizes was achieved for all candidate virulence gene promoters, except *AmRas2b* which was weakly amplified, as seen in Figure 5.2.

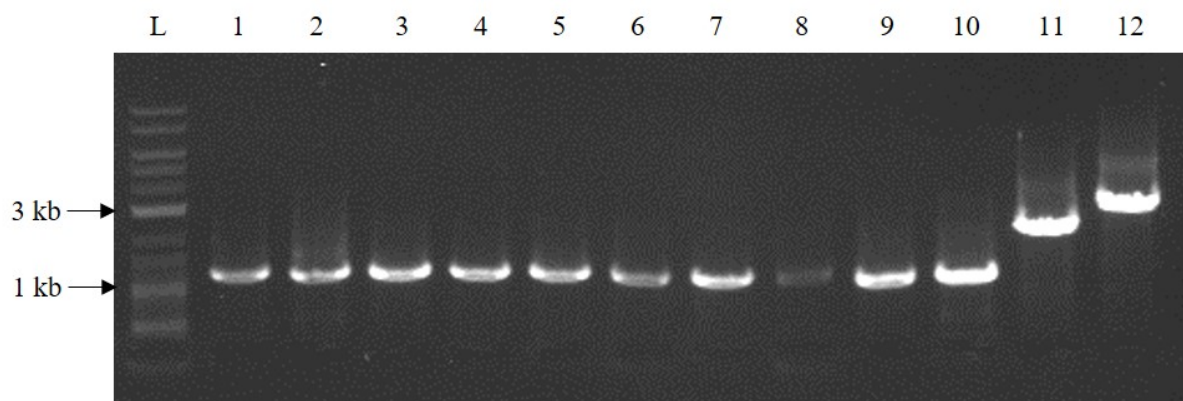


Figure 5.2: Amplification of *A. mellea* DSM 3731 gDNA for homologous recombination in yeast.

Amplifiable quality of gDNA assessed with ITS1 & ITS4 primers (amplicon size is 932 bp) in lane 10. The eGFP (in lane 11, amplicon size 1680 bp) and *hph* (in lane 12, amplicon size 2330 bp) cassettes, were amplified for yeast recombination from pCAM-*hph*-*Amgpd*-*xiGFP* and pCAM-*Amgpdihph*-*Amtrp1iGFP*, respectively. Gene-specific primers amplified the ~1 kb promoter region of candidate virulence genes from *A. mellea* in lanes 1 – 9: *AmPMK1a*, *AmPMK1b*, *AmPMK1c*, *AmCP*, *AmSLP1*, *AmSSP47*, *AmRAS2a*, *AmRAS2b* and *AmRAS2c*. DNA was measured with a 1 kb plus DNA maker (L).

5.3.2.3 Homologous recombination in yeast to construct Promoter:GFP fusion plasmids

Homologous recombination in yeast was used to construct plasmids containing three fragments: the *hph* cassette, the one kb promoter region of candidate virulence gene promoter and the eGFP cassette. Competent cells for yeast recombination were prepared on the day of transformation. Each transformation reaction contained 10 µl of appropriate amplified fragments and 5 µl of linearized yeast adapted pCAMBIA0380 backbone. Control transformations were made up to 35 µl with SDW and included a control for a linearized or circular backbone and a SDW only control. No growth occurred on water controls (SDW only) since without the pCAMBIA0380 *URA3* insertion, yeast cells could not grow on selective medium. Circular pCAMBIA0380 backbone controls produced a lawn of transformed yeast cells and the linear control produced a low number of yeast colonies (~10's – 100's). Successful recombination resulted in a higher number of colonies than linear controls typically with > 10 x increase. There were no colonies produced by yeast recombination for constructs pChy-AmPMK1c-eGFP and pChy-AmRAS2b-eGFP. Yeast colonies from putatively successful recombination events were harvested for plasmid extraction using the Zymoprep Yeast Plasmid Miniprep II kit and transformed into electrocompetent *E. coli* Top10 cells. Colony PCR confirmed presence of the inserted DNA (data not shown) and *E. coli* transformants were grown overnight in LB broth amended with Kanamycin (50 µg / ml). Cells were harvested by centrifugation and the plasmids were extracted with the Macherey-Nagel NucleoSpin Plasmid, Mini kit for plasmid DNA. To further confirm successful recombination by yeast, banding patterns of digested plasmids were predicted by Serial Cloner. Confirmation was carried out using two restriction enzymes for each construct containing candidate promoter:GFP cassettes (Figure 5.3). Digestions of selected constructs with *EcoRI* are shown in Figure 5.3. Confirmed constructs were transformed into *Agrobacterium tumefaciens* AGL1 by electroporation and verified by colony PCR (data not shown). Successful promoter:GFP fusion plasmids were constructed for the following candidate virulence genes: *AmPMK1a*, *AmPMK1b*, *AmRAS2a*, *AmRAS2c*, *AmCP*, *AmSlp1* and *AmSSP47*.

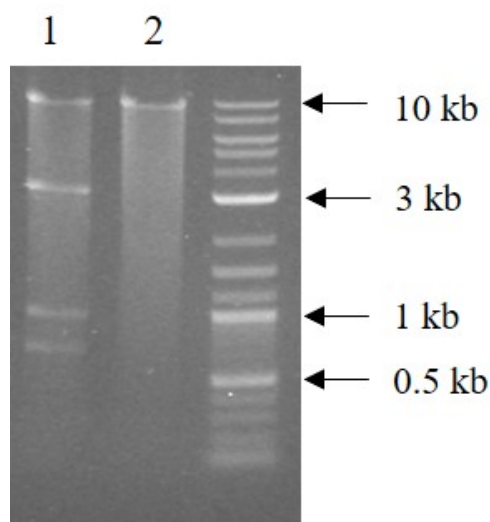


Figure 5.3: Candidate promoter:GFP constructs digested by *EcoRI*.

Lane 1: pChy-AmRAS2c-eGFP (expected sizes 9642, 3223, 1006, 688 bp) lane 2: empty backbone (expected size 9204 bp) using a 10 kb plus DNA marker.

Table 5.3: Restriction enzymes used to digest constructs transformed into *E. coli*.

| Plasmid | Restriction enzyme 1 | Restriction enzyme 2 |
|-------------------|----------------------|----------------------|
| pChy-AmPMK1a-eGFP | <i>EcoRI</i> | <i>PstI</i> |
| pChy-AmPMK1b-eGFP | <i>PstI</i> | <i>AccII</i> |
| pChy-AmRAS2a-eGFP | <i>EcoRI</i> | <i>NcoI</i> |
| pChy-AmRAS2c-eGFP | <i>EcoRI</i> | <i>PstI</i> |
| pChy-AmCP-eGFP | <i>EcoRI</i> | <i>HindIII</i> |
| pChy-AmSlp1-eGFP | <i>EcoRI</i> | <i>HpaI</i> |
| pChy-AmSSP47-eGFP | <i>PstI</i> | <i>ScaI</i> |

5.3.2.4 Transformation of constructs into *A. mellea*

5.3.2.4.1 Production of *A. mellea* basidiospores

Armillaria mellea ELDO17 was fruited *in vitro*. Colonisation of fruiting medium by *A. mellea* ELDO17 typically required four weeks after which fruiting jars were incubated under 16 : 8 h L : D conditions at 23 °C for six weeks. Subsequently fruiting was induced by moving jars into cooler and darker conditions (15 °C with shorter days (10 : 14 h L : D) and lower light intensity). Primordia (Figure 5.4 a, b) typically

began to form within one month but the time to full maturation varied with only ~ 60 % of fruiting jars reaching full maturity (Figure 5.4 c, d). Attempts were made to fruit other *A. mellea* isolates including GC440, GC657 and DSM 3731 but no fruiting bodies or primordia were produced. Where mature fruiting bodies were produced for ELDO17, basidiospores were collected by removing the cap which was placed onto sterile black paper with a piece of filter paper underneath to absorb excess moisture. A Tupperware box was placed over the cap to reduce contamination and airflow. After two days basidiospores were collected from the spore print (Figure 5.4 e, f), suspended in SDW, aliquoted into 1.5 ml Eppendorf tubes and stored at 4 °C.



Figure 5.4: *In vitro* production of basidiocarps from *A. mellea* ELDO17.

a-b) Production of primordia; c) Immature fruiting bodies; d) Mature fruiting bodies; e – f) *A. mellea* spore print.

Fruiting bodies of *Armillaria* were found in the grounds of Royal Fort Gardens at the University of Bristol (Figure 5.5 a) and were PCR-confirmed as *A. mellea* using *Armillaria* species specific primers. Mature basidiocarps were collected and a spore print (Figure 5.5 b, c) was used to collect basidiospores as previously described for use in AMT.



Figure 5.5 *Armillaria mellea* growing at Royal Fort Gardens.

a) Clump of *A. mellea* fruiting bodies; b) Excised cap of *A. mellea*; c) *A. mellea* spore print to collect basidiospores

5.3.2.4.2 *Agrobacterium* Mediated Transformation of *Armillaria*

Seven plasmids were successfully constructed by yeast recombination and transformed into *A. tumefaciens* in preparation for AMT transformation into *A. mellea* to visualise gene expression. Basidiospores (10^8 basidiospores / ml) were co-cultured with equal volumes of plasmid-containing *A. tumefaciens* on induction medium with cellophane discs for three days. Cellophane discs were moved onto selection medium (PDA amended with hygromycin (30 $\mu\text{g/ml}$) and timentin (200 $\mu\text{g/ml}$);

PDA+HT) to eliminate *Agrobacterium* and to select for *A. mellea* transformants which grew within 10 – 21 days. Putative transformants were sub-cultured onto PDA+HT and confirmed through PCR amplification of the *hph* gene. Transformations were predominantly performed using *A. mellea* basidiospores produced *in vitro*. Basidiospores from a natural fruiting body clump of *A. mellea* (Figure 5.5) were included for some transformations. Plasmids pCAM-Amgpdihph-Amtrp1iGFP and pCAM-hph-Amgpd-xiGFP were included as a positive control for each AMT.

Putative *A. mellea* transformants were obtained from each plasmid (Table 5.4). Transformation of *A. mellea* was most successful with pChy-AmRAS2a-eGFP (Table 5.4) where 52 putative transformants were produced with basidiospores from *A. mellea* ELDO17 and a further 32 putative transformants were produced from wild-collected basidiospores *A. mellea* RFG1.

Table 5.4: Number of putative *A. mellea* transformants transformed using basidiospores from ELDO17 or RFG1.

| Plasmid | <i>A. mellea</i> ELDO17 | <i>A. mellea</i> RFG1 |
|---------------------------|-------------------------|-----------------------|
| pCAM-Amgpdihph-Amtrp1iGFP | 32 ^d | 19 |
| pCAM-hph-Amgpd-xiGFP | 16 ^c | 7 |
| pChy-AmPMK1a-eGFP | 27 ^a | 0 |
| pChy-AmPMK1b-eGFP | 6 ^a | n/a |
| pChy-AmRAS2a-eGFP | 52 ^b | 32 |
| pChy-AmRAS2c-eGFP | 49 ^a | n/a |
| pChy-AmCP-eGFP | 14 ^a | 0 |
| pChy-AmSlp1-eGFP | 42 ^b | 7 |
| pChy-AmSSP47-eGFP | 12 ^a | n/a |

Only one AMT was performed with RFG1 spores, transformants from ELDO17 were produced over two (^a), three (^b), four (^c) or five (^d) transformations.

5.3.3 Visualising GFP in putative *A. mellea* transformants

Putative *A. mellea* transformants were visualised using fluorescence microscopy. GFP fluorescence from transformants on agar was difficult to determine due to yellow autofluorescence of selective medium (PDA+HT) (Figure 5.6 a) and fungal ‘fibre optics’ meant occasionally hyphal tips would show intense yellow fluorescence (Figure 5.6 b). No GFP was readily apparent, however, transformants were

designed to fluoresce only when candidate pathogenicity genes were active, thus growth on agar-based substrates would not necessarily activate genes. Several plant-based substrates were trialled for visualising fluorescence from putative transformants which might activate pathogenicity related genes *in vitro*. First, 10 mm thick slices of carrots and potatoes were surface sterilised in 10 % bleach for five minutes then placed into empty Petri dishes with or without sterile filter paper dampened with SDW. Mycelial plugs of putative transformants were placed onto carrots or potatoes and incubated at 20 °C in the dark. Within two – three days contaminants had grown from some potatoes (Figure 5.7; a) and within one week the mycelial plugs were desiccated and *A. mellea* transformants failed to grow. Agar based Petri dishes containing rice and sawdust (Figure 5.7; b) were trialled where putative *A. mellea* transformants were able to grow, however autofluorescence from the medium was too strong to visualise potential green fluorescence.

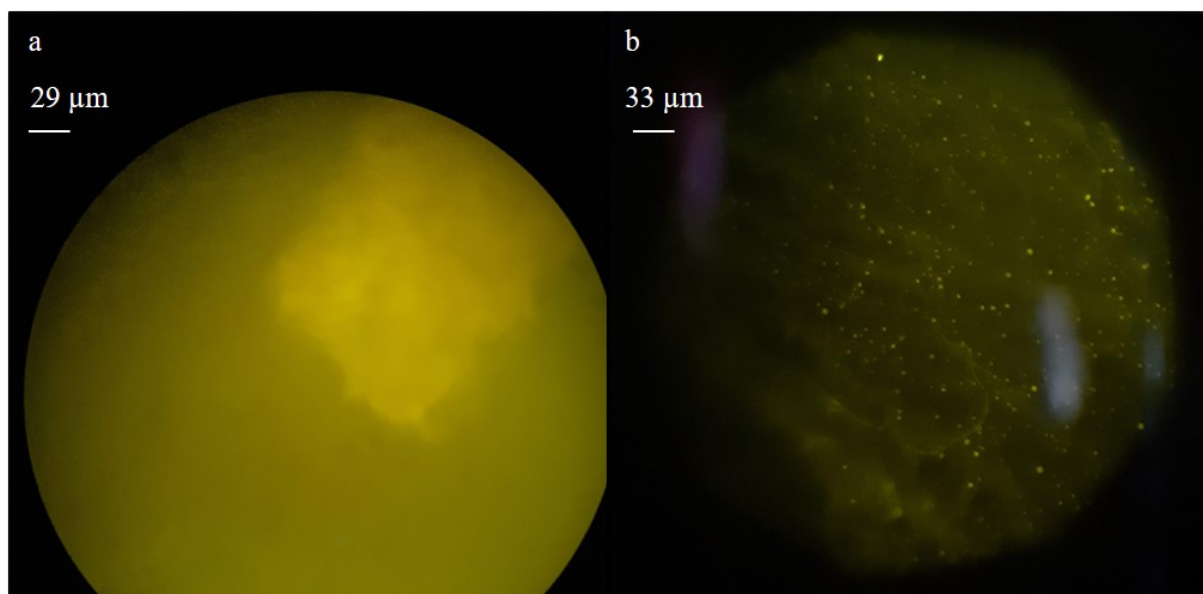


Figure 5.6: Visualising fluorescence in *A. mellea* putative transformants.

Armillaria mellea observed under 400 x magnification with a GFP epifluorescent filter. a) WT ELDO17 on PDA+HT; b) fibre optics observed from pChy-AmPMK1b-eGFP seen as bright spots corresponding with tips of aerial hyphae.

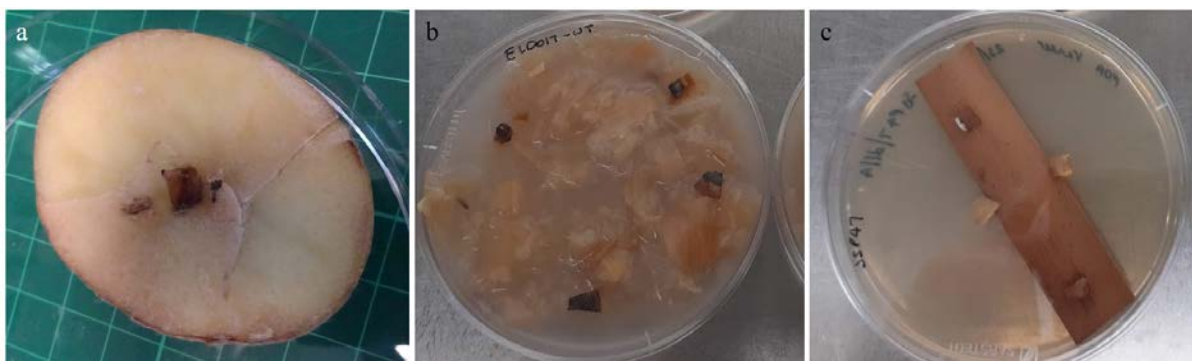


Figure 5.7: Plant based substrates used to assess candidate gene expression of *A. mellea* *in vitro*.

a) Potato slice inoculated with *A. mellea* transformant 3 dpi showing early signs of contaminant growth; b) Rice and sawdust agar inoculated with WT *A. mellea* ELDO17; c) Water agar with walnut veneer strip inoculated with pChy-AmSSP47-eGFP.

Strips of sterile walnut veneer cut into ~80 mm lengths and placed on water agar (WA) without antimicrobial amendments provided a successful plant based medium. Mycelial plugs were positioned to lie over agar and veneer to provide the fungi with moisture to prevent desiccation but allow access to a plant based substrate (Figure 5.7; c). There was some autofluorescence from WA (Figure 5.8) and veneer, but potential fluorescence was visible from fungal hypha. A preliminary test was carried out on veneer using transformants carrying the plasmids pChy-AmPMK1b-eGFP, pChy-AmRAS2c-eGFP and pChy-AmSSP47-eGFP along with the WT ELDO17 isolate from which basidiospores were collected for transformations. Only one transformant for each plasmid was used. Potential hyphal fluorescence was more yellow than expected, and recorded on pChy-AmRAS2c-eGFP (Figure 5.9) and pChy-AmPMK1b-eGFP (Figure 5.10) transformants where it was visible on veneer and but not agar which is particularly noticeable in Figure 5.10 d. No green fluorescence was observed from WT ELDO17 colonies, controls without promoter sequences or pChy-AmSSP47-eGFP. Confocal microscopy was attempted with both potentially fluorescing transformants to confirm fluorescence, with the aid of a microscopy expert from the Wolfson imaging facility at the University of Bristol. Due to the high autofluorescence and thickness of veneer, confocal microscopy was not possible. A selection of putative transformants from each construct were due to be grown on veneer and WA to assess whether candidate virulence genes were activated in the presence of plant substrates, however, due to COVID-19 lab

closures this work was unable to proceed.

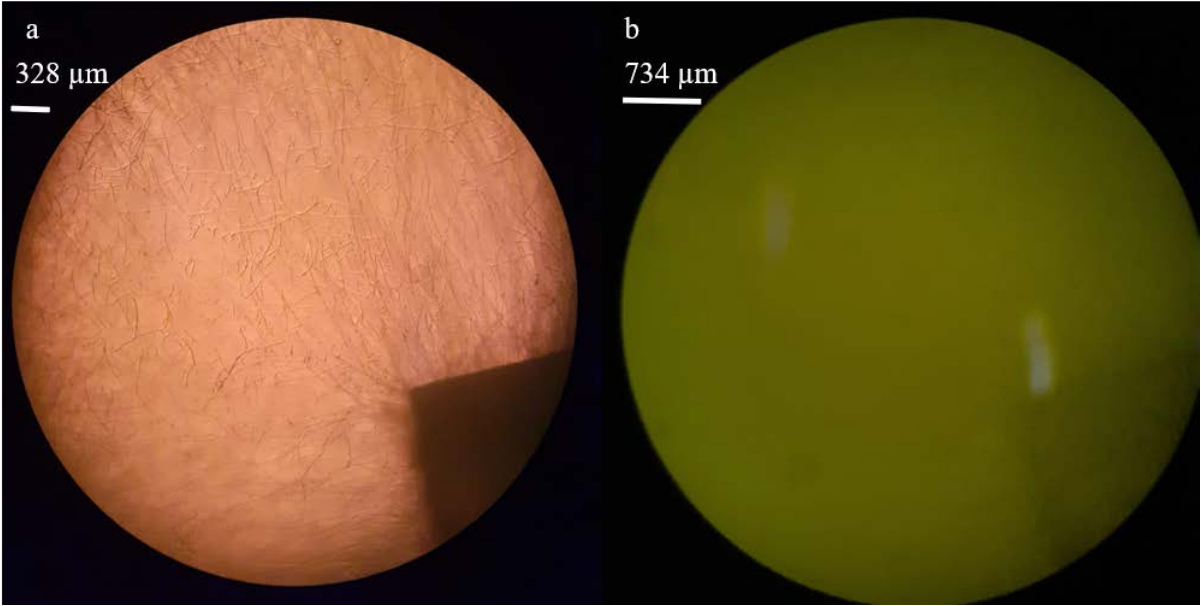


Figure 5.8: *Armillaria mellea* ELDO17 (WT) on water agar visualised under fluorescent microscopy.

Cultures visualised at 400 x magnification under a) brightfield light; b) with a GFP epifluorescent filter. The mycelial inoculation plug inoculation can be seen in the bottom right corner of both images.

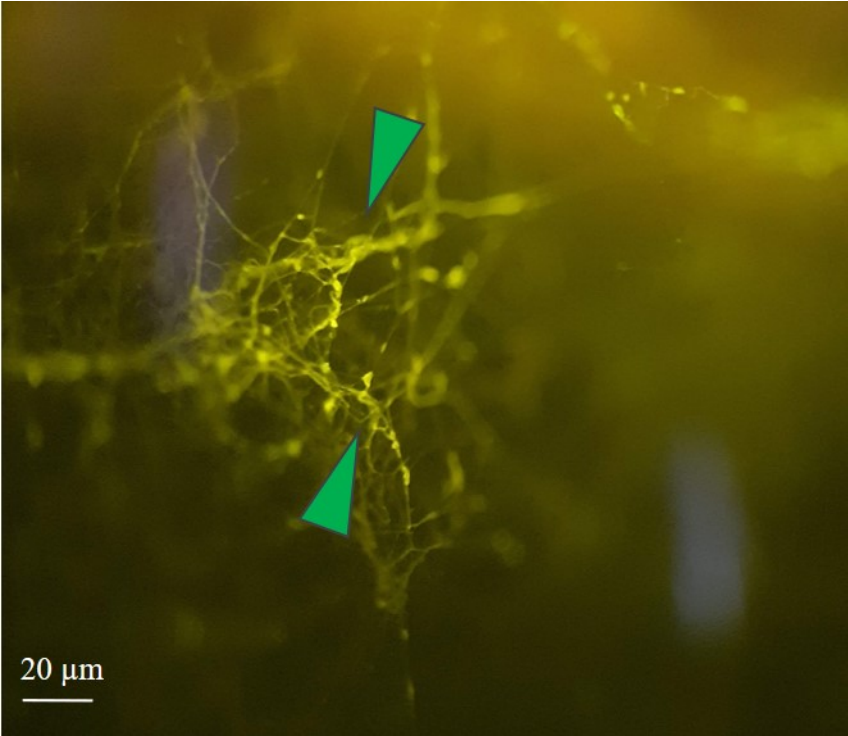


Figure 5.9: Putative green fluorescing pChy-AmRAS2c-eGFP.

Putative *A. mellea* transformant growing on veneer viewed under 400 x magnification with a GFP epifluorescent filter. The green arrows indicate potentially fluorescing hyphae.

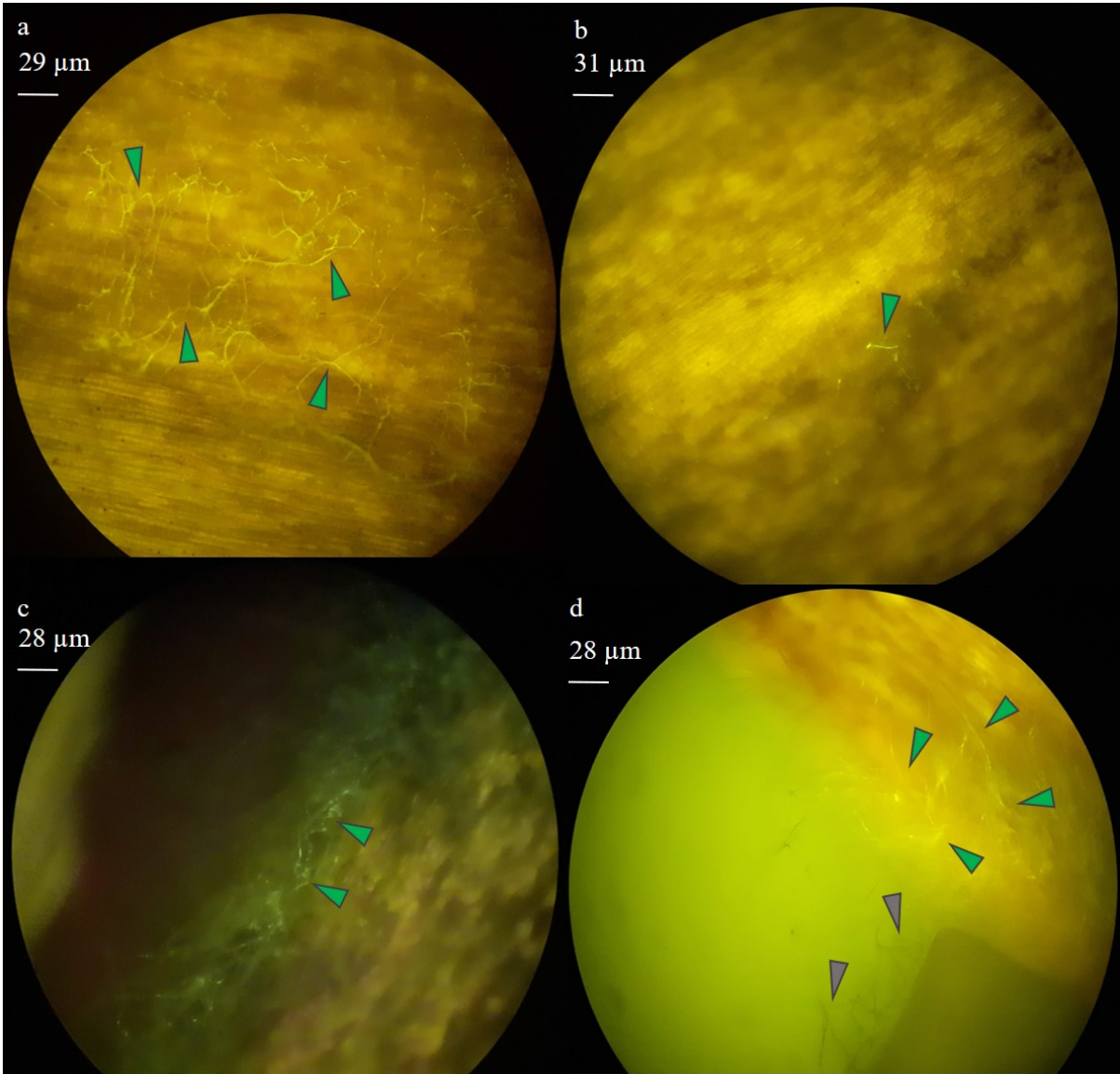


Figure 5.10: Putative green fluorescing p-Chy-AmPMK1b-eGFP.

Putative *A. mellea* transformant growing on veneer viewed under 400 x magnification with a GFP epifluorescent filter. The green arrows indicate potentially fluorescing hyphae. a – c) hyphal growth on veneer showing some potential fluorescence. d) potential fluorescence can be seen as indicated by green arrows where *A. mellea* hyphae are in contact with veneer. Darker hyphae showing no fluorescence (grey arrows) can be seen growing on the agar.

5.3.4 Determining presence and localisation of candidate virulence genes

Given the difficulties of microscopy-visualisation of candidate virulence gene expression, gene expression was also studied using reverse-transcriptase PCR (RT-PCR). Expression of genes from a variety of fungal materials could be used to deduce potential function in relation to virulence. Further study of gene expression was planned using qPCR but could not be completed because of laboratory closures due to COVID-19.

5.3.4.1 RNA isolation from *A. mellea* tissue

Candidate virulence genes in *A. mellea* were studied in a range of tissues from ELDO17 cultures to determine whether expression was localised. Selected tissues included mycelium of laboratory-grown cultures (on Petri dishes), the *A. mellea* stipe, immature and mature caps, primordia, and rhizomorphs from *in vitro* produced fruiting bodies. Fungal material from *A. mellea* GC440 infected hazel billets was collected but could not be taken from infected plant roots because no infected plants were available. The range of tissues selected for RNA isolation allowed localisation of gene expression to be studied at different life stages of *Armillaria*. Material for RNA isolation was harvested, weighed and immediately frozen in liquid nitrogen (N₂) then stored at -70 °C, if required. The Qiagen 'RNeasy Plant Mini Kit' and Zymo Research 'YeaStar™ RNA Kit' were trialled, according to the manufacturer's instructions, however insufficient quantities of RNA were isolated for cDNA synthesis. A phenol chloroform RNA extraction was performed using ~2 g of starting material and yielded a minimum of ~1 µg / µl. Quality and concentration of RNA was determined by spectrometry using a nanodrop and gel electrophoresis (Figure 5.11) prior to cDNA synthesis. RNA was isolated from *A. mellea* ELDO17 cultures, stipes, mature fruiting body caps, and mycelium from *A. mellea* CG440 infected hazel billets, however, isolation of good quality RNA was variable, as seen in Figure 5.11. Quality of RNA was assessed by visualising the 28S and 18S rRNA and small RNAs using gel electrophoresis, these can be clearly seen in samples extracted from laboratory-grown *A. mellea* ELDO17 cultures and from mycelium taken from *A. mellea* colonised hazel billets in Figure 5.11. Degraded RNA can be seen in samples taken from

unopened caps and putative immature fruiting bodies of *A. mellea* ELDO17, however, because these tissues did not develop promptly they might have undergone senescence.

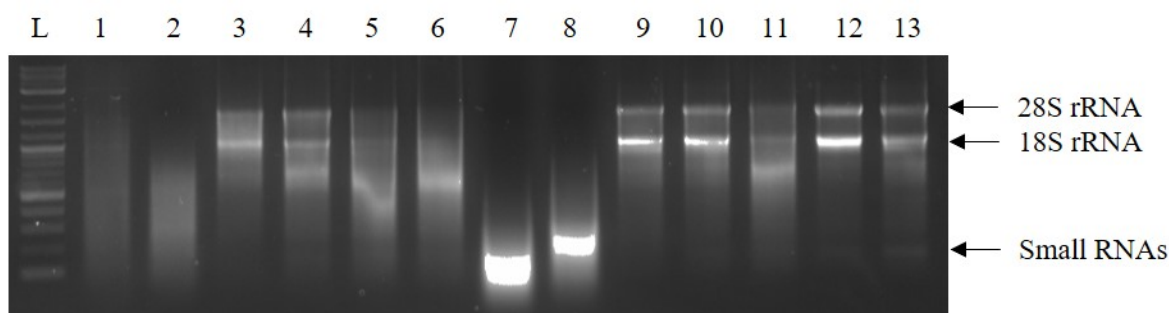


Figure 5.11 Visualisation of RNA by gel electrophoresis.

RNA quality was visualised by gel electrophoresis. Crisp bands of the 28S and 18S ribosomal RNA and presence of small RNAs indicate undegraded RNA. *Armillaria mellea* RNA was isolated from: ELDO17 Stipe (lanes 1 – 3), ELDO17 mature caps (lanes 4 – 6), ELDO17 unopened cap (lane 7), ELDO17 immature cap (lane 8), CG440 mycelium colonising hazel billets (lane 9), ELDO17 mycelium from RST (lanes 10 - 11), mycelium from lab grown cultures of ELDO17 (lane 12 -13). RNA was measured with a 1 kb plus bp maker (L).

5.3.4.2 End-point RT-PCR to determine candidate virulence gene expression

To ensure cDNA contained no gDNA contamination, RNA was treated with DNase (Thermo Fisher Scientific). Five μg of RNA were treated following the manufacturer's instructions and ethanol precipitated to purify RNA. Quantity of RNA was again determined via spectrometry (Nanodrop) and gel electrophoresis. First Strand cDNA synthesis (Thermo Fisher Scientific) was carried out following the manufacturer's instructions using 5 μg of template DNase treated RNA. A control with no reverse transcriptase added ('RT -') was included for each sample and acted as confirmation of successful cDNA synthesis. To further confirm samples did not contain gDNA contamination, primers were designed to span introns of all genes except *AmCP* and *AmSSP47* which did not contain introns (Appendix Table A.3). Presence of gDNA would amplify the intron resulting in a fragment larger than that expected from pure cDNA.

Synthesis of cDNA from good quality *A. mellea* RNA was variable, and after considerable effort, was synthesised from a culture of *A. mellea* CG440 and from the mature, sporulating cap of laboratory-grown *A. mellea* ELD017 fruiting body (Figure 5.12; Table 5.5). End-point RT-PCR was used to assess the RNA expression of candidate virulence genes. In both tissues, the housekeeping genes *tef1* and α -*Tub* were expressed, while cDNA from β -*Act* was only amplified from laboratory-grown cultures. All candidate virulence genes tested (*AmCP*, *AmPMK1a*, *AmRAS2c*, *AmSLP1* and *AmSSP47*) were expressed in laboratory-grown cultures (Table 5.5) although *AmCP* and *AmRAS2c* produced faint bands, possibly indicating low expression. In mature caps of ELD017 fruiting bodies, β -*Act* was not expressed suggesting that cDNA synthesis was not fully reliable as this is a housekeeping gene so expression would always be expected. Like in laboratory-grown cultures, RNA expression of *AmCP* from caps of ELD017 fruiting bodies was low and *AmRAS2c* was not expressed (Table 5.5).

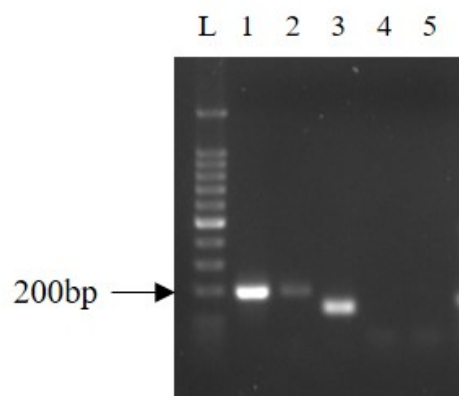


Figure 5.12: End-point RT-PCR for RNA expression in *A. mellea*.

Example of end-point RT-PCR visualised by gel electrophoresis for the β -*Act* gene in *A. mellea* using RNA extracted from laboratory-grown cultures. gDNA = 193 bp, cDNA = 141 bp. Lanes 1 – 5: gDNA from ELD017, ‘RT-’ amplified gDNA, ‘RT+’ amplified cDNA, no template control (NTC) without RT, NTC with RT. A 100 bp marker (L) was included.

Table 5.5: End-point RT-PCR results.

End-point RT-PCR to assess expression of *A. mellea* candidate virulence genes in laboratory-grown cultures (culture tissue) of CG440 and the mature cap from ELDO17 fruited *in vitro*. ± indicates whether genes were expressed.

| Gene | Culture tissue (CG440) | ELDO17 cap |
|----------------|------------------------|----------------|
| <i>β-Act</i> | + | - |
| <i>tef1</i> | + | + |
| <i>A-Tub</i> | + | + |
| <i>AmCP</i> | + (very faint) | + (very faint) |
| <i>AmPMK1a</i> | + | + |
| <i>AmRAS2c</i> | + (very faint) | - |
| <i>AmSLP1</i> | + | + |
| <i>AmSSP47</i> | + | + |

5.3.4.3 qPCR design

Originally it was planned that qPCR would be used to quantify gene expression of candidate virulence genes in *A. mellea*, however optimisation was not completed before lab closures due to COVID-19. This section details primer design, and initial optimisation results achieved.

Reference or housekeeping genes are an important consideration when performing gene expression using qPCR and act as positive controls in a qPCR assay. Fungal reference genes from Pathan *et al.* (2017) were searched for using the BLAST DNA:DNA function in the *A. mellea* DSM 3731 genome (JGI, 2013) to find suitable housekeeping genes. Five housekeeping genes were identified in *A. mellea* DSM 3731: *β-Act* (β - actin), *Tef1* (translation elongation factor), *α-Tub* (α -tubulin), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) and *Ubc* (Ubiquitin-conjugating enzyme).

Primers for qPCR were designed for all five housekeeping genes and all candidate virulence genes found in *A. mellea* as detailed in Table 5.2 using primer3 (<http://primer3.ut.ee/>) (Appendix Table A.4). Amplicon size was between 115 – 200 bp, favouring longer amplicons with primer length ~ 20 bp. A T_M of 59 °C was achieved for all primer sets. To test primers, gDNA was amplified for each primer set. Four housekeeping genes (*αTub*, *βAct*, *eEF1* and *UBC*) were amplified at the correct size (Figure

5.13). GAPDH was not amplified from *A. mellea* gDNA and therefore was not selected for optimisation.

All candidate virulence genes were successfully amplified from gDNA (data not shown).

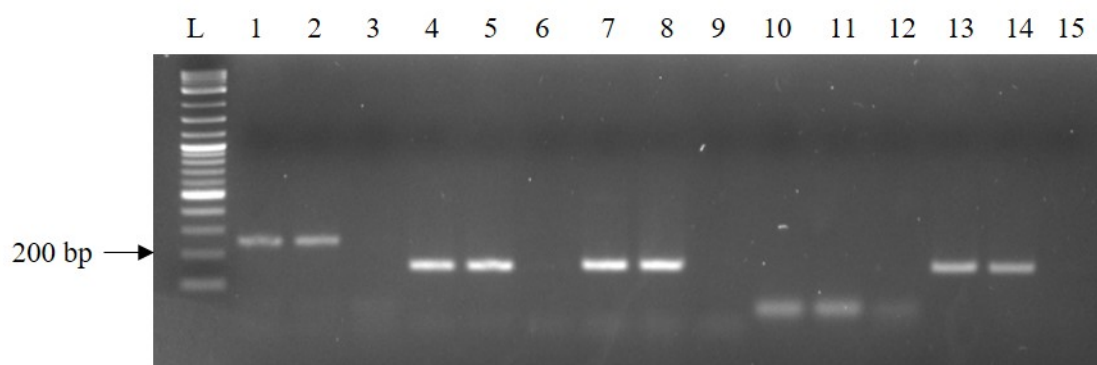


Figure 5.13: Amplification of gDNA using housekeeping primers for qPCR.

Amplification from DSM 3731 and CG440 with a negative control for the housekeeping genes *βAct* (161 bp) (lanes 1 – 3), *α-Tub* (196 bp) (lanes 4 – 6), *tef1* (115 bp) (lanes 7 – 9), *GAPDH* (143 bp) (lanes 10 – 12) and *Ubc* (152 bp) (lanes 13 – 15) with a 100 bp DNA ladder.

To begin qPCR optimisation three housekeeping genes were tested in a serial dilution assay. Housekeeping genes *β-Act*, *Tef1* and *Ubc* were tested for primer efficiency with *A. mellea* DSM 3731 cDNA in triplicate with four dilution series. In an optimised qPCR assay the efficiency should be close to 100 % and have an R^2 value close to one (Bustin *et al.*, 2009), therefore, the results from serial dilutions of cDNA suggested primers were not optimised (Table 5.6). To optimise primer concentration, nine primer combinations were tested (Table 5.7) with the *β-Act* primer set. Primers with a concentration of 500 : 400 nM (forwards and reverse, respectively) had the lowest Ct value thus considered most efficient (Table 5.7). The next step would be to optimise the annealing temperature before efficiency is reassessed in another serial dilution assay. To fully optimise the qPCR assay for gene expression of candidate genes this must be completed for each primer set.

Table 5.6: qPCR efficiency of housekeeping genes.

The efficiency and standard curve of a qPCR assay based on four serial dilutions with three housekeeping genes (*βAct*, *Tef1* and *Ubc*) using *A. mellea* DSM 3731 cDNA from two RNA extractions.

| Gene | Extraction number | Efficiency (%) | Standard Curve (R ²) |
|--------------|-------------------|----------------|----------------------------------|
| <i>B-Act</i> | 1 | 234 | 0.81 |
| <i>β-Act</i> | 2 | 147 | 0.84 |
| <i>tef1</i> | 1 | 134.3 | 0.99 |
| <i>tef1</i> | 2 | 112.2 | 0.99 |
| <i>Ubc</i> | 1 | 139.6 | 0.95 |
| <i>Ubc</i> | 2 | 118.4 | 1.00 |

Table 5.7: Optimisation of *β-Act* primer concentrations for qPCR.

Average Ct values (n =2) from qPCR assay to optimise primer concentration of *β-Act* using a combination of nine concentrations.

| Primer F / Primer R | 300 nM | 400 nM | 500 nM |
|---------------------|--------|--------|--------|
| 300 nM | 29.84 | 30.31 | 30.26 |
| 400 nM | 30.37 | 30.23 | 30.17 |
| 500 nM | 29.86 | 29.80 | 30.14 |

5.4 Discussion

5.4.1 Visualisation of GFP in *A. mellea* transformants

Homologous recombination in yeast was successfully used to construct plasmids designed to fuse candidate virulence gene promoters with GFP to visualise expression. Using *A. mellea* basidiospores fruited *in vitro* and collected from the wild, AMT yielded several putative transformants. The use of AMT for *A. mellea* was first described by Baumgartner *et al.* (2010) who used basidiospores collected from wild *A. mellea* mushrooms in California, USA. To aid AMT of *Armillaria* spp., Ford *et al.* (2015) developed an *in vitro* fruiting system to harvest basidiospores. Ford *et al.* (2016) then used AMT of the American *A. mellea* isolate ELDO17 to construct constitutive GFP and mRFP fluorescing transformants. The use of WT basidiospores from Royal Fort Gardens in this chapter is the first time *A. mellea* has been transformed using an isolate from the UK, and is probably also the first European isolate of *A. mellea* to be transformed. The eGFP cassette used in this study was from the published eGFP plasmid, pCAM-hph-Amgpd-xiGFP (Ford *et al.*, 2016). When transformed into *Clitopilus passeckerianus* 60 % of transformants showed fluorescence, however when transformed into *A. mellea* the number of transformants fluorescing halved (30 %). The low rate of *A. mellea* transformants showing fluorescence as reported by Ford *et al.* (2016) may explain why no GFP was visualised in controls with constitutive GFP fluorescence when grown on agar cultures.

The lack of *A. mellea* fluorescence in transformants with plasmids containing promoter sequences grown on agar-based medium indicated that genes may not be expressed on agar. To determine whether this was the case, transformants were grown in the presence of plant material. Potatoes and carrots were ineffective because of contamination or desiccation of agar plugs. Rice and sawdust agar had high levels of background autofluorescence although transformants grew well. Walnut veneer was a successful substrate for growth of transformants, although incubation on WA was necessary to prevent desiccation of agar plugs. Any fluorescence visualised was found to be weak and more yellow than green in colour. In WT cultures, there was a strong yellow background and sometimes intense yellow bursts on hyphae as a result of fibre optics. Where putative fluorescence was visualised, although often yellow, it was

observed along the length of visible hyphae. Potential green fluorescence of pChy-AmPMK1b-eGFP and pChy-AmRAS2c-eGFP could be visualised with fluorescent microscopy. Since this was a preliminary investigation limited transformants were assessed. No fluorescence was recorded for pChy-AmSSP47-eGFP, although as noted with constitutively fluorescing controls this could be due to the low proportion of transformants showing fluorescence. To increase the reliability of fluorescence by *A. mellea* different constructs should be trialled. Ford *et al.* (2016) found that the proportion of *A. mellea* transformants showing fluorescence with mRFP was much higher at 50 or 75 %. In future, construction of plasmids with mRFP or a different eGFP cassette should be considered to yield more informative results for fluorescent analysis.

5.4.2 Expression of candidate virulence genes in *A. mellea*

Data from RT-PCR was not in agreement with the promoter:reporter data. RT-PCR indicated that the genes *AmPMK1a*, *AmSLP1* and *AmSSP47* were expressed in both laboratory-grown culture tissue and mature caps of *A. mellea* basidiocarps whilst *AmCP* was only faintly expressed in both. The only difference noted between laboratory-grown culture tissue and mature *A. mellea* fruiting body caps was in the expression of *AmRAS2c* which was expressed faintly in the former but not the latter. Promoter:GFP fusion transformants suggest that *AmRAS2c* and *AmPMK1b* are expressed in the presence of plant tissue but unfortunately, without data on expression levels *in planta*, putative function of candidate virulence genes cannot be reliably assessed.

Variable extraction of good quality RNA for downstream applications and inconsistent cDNA synthesis limited the ability for expression of candidate virulence genes selected in this chapter to be studied. Firstly, RNA of sufficient quantity could not be reliably extracted using kits frequently used for RNA extraction including Qiagen's 'RNeasy Plant Mini Kit' or the Zymo Research 'YeaStar™ RNA Kit'. A phenol chloroform extraction based on a scaled-up version of that published by Verwoerd *et al.* (1989) yielded high concentrations of RNA, although degradation of RNA still occurred which Verwoerd *et al.* (1989) warned was a risk in large scale extractions. Furthermore, the RNA extraction protocol included a LiCl overnight incubation and an ethanol wash which is considered to be important

for isolation of good quality RNA (Jaakola *et al.*, 2001) but can interfere with downstream applications such as RT-PCR due to interference by chloride ions (Fort *et al.*, 2008). To counter issues of RNA purification by LiCl incubation, an additional precipitation step and ethanol wash was included in the RNA isolation protocol. While this did allow cDNA synthesis of some samples, cDNA synthesis was unreliable. Further optimisation of the RNA and cDNA synthesis protocols was not continued due to time limitations caused by disrupted access to laboratories as a result of COVID-19 restrictions.

In *Beauveria bassiana* (Xie *et al.*, 2013), *Fusarium graminearum* and *Ustilago maydis* *RAS2* is involved in regulation of fungal or filamentous growth (Lee and Kronstad, 2002; Müller *et al.*, 2003; Bluhm *et al.*, 2007; Xie *et al.*, 2013). *RAS2* is also involved in other gene functions such as conidial morphology in *M. oryzae* (Kershaw *et al.*, 2019), germination in *Penicillium marneffei* (Boyce *et al.*, 2005) and infection of wheat and maize plants by *F. graminearum* and *U. maydis* (Lee and Kronstad, 2002; Bluhm *et al.*, 2007). In *A. mellea* the *AmRAS2c* gene was faintly expressed in laboratory-grown cultures but not in caps of mature fruiting bodies suggesting it may have a role in fungal growth but not in spore production. Signs of putative green fluorescence were noted on *A. mellea* transformants with pChy-RAS2c-eGFP grown on wood veneer but not agar suggesting some regulated gene expression when growing on plant substrates, but this was not confirmed with RT-PCR. In some fungi with more than one Ras GTPase, one is considered essential for survival (Bluhm *et al.*, 2007; Zhou *et al.*, 2014). Since three were found in *Armillaria*, it is possible that a least one *RAS* gene is essential. This has been supported in other fungi through failed attempts to delete the essential *RAS* gene (Boyce *et al.*, 2005; Bluhm *et al.*, 2007; Zhou *et al.*, 2014) a technique not yet developed for *Armillaria*.

In *M. grisea*, *PMK1* is expressed during the development of conidia and in the formation of appressoria and infection hyphae (Bruno *et al.*, 2004). *PMK1* in *M. oryzae* is involved in appressorium penetration and the hyphal movement into new plant cells (Sakulkoo *et al.*, 2018). In this study, *AmPMK1b* was expressed in laboratory-grown cultures and mature caps of *A. mellea* fruiting bodies, which suggests that it is involved in hyphal growth and possibly in the development of basidiospores. *PMK1* homologs in other fungal pathogens including *Corynespora cassiicola*, *Colletotrichum truncatum*, *C. fructicola* and *Claviceps purpurea*, are involved in fungal growth, appressoria formation, perithecia formation and

pathogenicity (Mey *et al.*, 2002; Xiong *et al.*, 2015; Liu *et al.*, 2017; Liang *et al.*, 2019). The *PMK1* homolog in *F. graminearum* has been shown to be involved in fungal growth, mating, and pathogenicity (Jenczmionka *et al.*, 2003) and is also required for colonisation of root tissue or penetration and spread in wheat ears (Urban *et al.*, 2003). In *A. mellea* pChy-PMK1b-eGFP promoter:GFP fusion transformants there were putative signs of green fluorescence in the presence of wood veneer but not on agar suggesting that there might be a role linked to saprophytic growth on woody substrates, however this was not confirmed by RT-PCR.

AmSLP1 is a homologue of the secreted LysM effector protein (Slp1) from *M. oryzae* which has significant homology to the Ecp6 effector from *Cladosporium fulvum* (Mentlak *et al.*, 2012). A study into the function of Slp1 found that it is expressed during intracellular growth of *M. oryzae* within the host and is involved in pathogenicity. *Magnaporthe oryzae* mutants with a *SLP1* gene deletion have shown a significantly lower lesion size and density than the WT strain. The mutants were still able to produce appressoria, but comparisons with the number of cells with *M. oryzae* growth after 48 hours were significantly lower in mutants than in the WT (Mentlak *et al.*, 2012). This suggest that although *SLP1* is not directly involved in formation of invasion structures, it is important for fungal growth inside cells and production of lesions (Mentlak *et al.*, 2012). In this study, *AmSLP1* was expressed in both laboratory-grown cultures and in caps of mature fruiting bodies suggesting it might be involved in fungal growth. Additionally in *M. oryzae* *SLP1* has been shown to bind to chitin and suppress chitin triggered immunity, a significant factor for infection of plants (Mentlak *et al.*, 2012). Should expression of *AmSLP1* in *A. mellea* tissues infecting plants be tested, it is likely that expression will be noted. In contrast the core LysM effectors of *Verticillium dahlia* do not seem to play a role in virulence or host colonisation of tomato or *Nicotiana benthamiana* plants (Kombrink, 2014), which might be similar to what was found in *A. mellea* in this study.

In *A. mellea*, *AmSSP47* was expressed in laboratory-grown cultures and in caps of mature fruiting bodies. Using transient gene expression, the function of 58 SSPs in *Heterobasidion annosum* was investigated and found eight SSPs could induce cell death in *N. benthamiana*. Two SSPs, (HaSSP30 & HaSSP47) were found to be gene isomorphs and could induce rapid cell death and loss of leaf turgidity

in *N. benthamiana* and HaSSP30 was shown to induce plant defence genes (Raffaello and Asiegbu, 2017). *AmHASSP47* is a homolog of the SSP in *H. annosum*, *HaSSP47*, therefore it was expected to have a similar gene function in *A. mellea*, however. RT-PCR results from material tested suggest that *AmSSP47* could be involved in fungal growth, reproduction or the gene might be constitutively active. There were no results available for expression of *AmSSP47* by RT-PCR or from pChy-*AmSSP47*-eGFP transformants for expression in the presence of plant tissue.

Cerato-platanin proteins are well studied in numerous fungi and are largely associated with increased virulence and induction of plant defences, however the biological control fungi, *Trichoderma* spp. also use CP proteins. The *AmCP* gene in this study is homologous to the *MgSM1* (small protein 1) in *M. grisea* which is constitutively expressed during different stages of fungal growth, including mycelial growth on PDA, and during infection of rice plants (Yang *et al.*, 2009). The results of the RT-PCR in this study suggest that *AmCP* might also be constitutively expressed during these stages, although where expression was measured (laboratory-grown cultures and caps of mature fruiting bodies) it was faint. The CP protein, FocCP1, from *F. oxysporum* f. sp. *cubense* tropical race 4 was expressed during conidia germination and early stages of infection and was shown to also be involved in infection, virulence, penetration of and growth within banana roots (Liu *et al.*, 2019). *MgSM1* from *M. grisea* results in induced disease resistance in plants; in *Arabidopsis*, transient expression resulted in a hypersensitive response and upregulation of defence genes (Yang *et al.*, 2009). The CP protein MSP1 in *M. oryzae* (Wang *et al.*, 2016), FocCP1 in *F. oxysporum* (Li *et al.*, 2019) and SsCP1 in *Sclerotium sclerotinia* (Yang *et al.*, 2018) have been shown to result in induced disease resistance in plants. Given the varying roles of CP proteins in fungi, further work is required to determine the role of *AmCP* in *A. mellea*. For example, use of *Agrobacterium* infiltration assays together with tobacco should be used to further investigate the potential induction of a hypersensitive response and defence related genes by *AmCP*.

5.4.3 Limitations of studying gene function in *A. mellea*

Development of AMT in *Armillaria* (Baumgartner *et al.*, 2010b; Ford *et al.*, 2015, 2016) provided a significant step forward in our ability to study the genetics of *Armillaria* spp.. Expression of candidate

genes can be assessed with RT-PCR and qPCR, but visual assessment of the effect of individual genes on plant infection and fungal biology of *Armillaria* spp. is limited.

To further study the function of candidate virulence genes in *A. mellea* a combination of alternative techniques are required. Many studies utilise gene disruption or transgenic expression of candidate genes in tobacco, for example, in studies investigating the function of CP proteins (Yang *et al.*, 2009, 2018; Pan *et al.*, 2018; Li *et al.*, 2019; Liu *et al.*, 2019; Quarantin *et al.*, 2019), this is a method which would be beneficial in deciphering gene function of candidate virulence genes from this study. In *Puccinia striiformis* f. sp. *tritici* the fungal plant pathogens *F. graminearum* and *M. oryzae* were used as ‘surrogate systems’ to study the function of highly conserved MAPK genes (Guo *et al.*, 2011), a system which could provide an option for further research in *A. mellea*. Due to the predominantly diploid nature of *Armillaria*, such methods are difficult, however, a CRISPR system might make it possible for both alleles to be disrupted simultaneously. CRISPR has not yet been developed for *Armillaria* spp., but there are CRISPR systems available for other Basidiomycota fungi (Schuster *et al.*, 2016; Waltz, 2016; Lu *et al.*, 2017; Qin *et al.*, 2017; Sugano *et al.*, 2017; Jan Vonk *et al.*, 2019; Otoupal *et al.*, 2019; Lin *et al.*, 2020). A CRISPR system for *Armillaria* spp. is being developed at the University of Bristol (Ford and Bourquin, 2018) and would provide a significant breakthrough in availability of techniques to study gene function. Gene silencing in *A. mellea* has been described but resulted in low levels of silencing (Ford, 2015). A combination of these techniques for *Armillaria* spp. will provide the necessary advancements to functionally characterise candidate genes in this species.

5.5 Summary

- Nine candidate virulence genes with homologues to fungal plant pathogens were found in the *A. mellea* genome including a *CP* gene, three small RAS GTPases, and three MAP kinases, a LysM effector and a SSP from *H. annosum*.
- Transgenic *Armillaria mellea* promoter:GFP lines were successfully produced for seven candidate virulence genes.
- Preliminary data shows that promoter:GFP transformants were able to fluoresce when grown on strips of wood veneer incubated on WA.
- Putative green fluorescence was observed in transformants containing promoter regions of *PMK1b* and *RAS2c*.
- In laboratory-grown cultures and mature caps of *A. mellea* fruiting bodies, the genes *AmPMK1a*, *AmSLP1*, *AmSSP47* were expressed. *AmCP* was faintly expressed and *AmRAS2c* showed faint expression only in laboratory-grown cultures.

| Chapter 6: General discussion

6.1 Understanding pathogenicity of *A. mellea*

One aim in this thesis was to use available molecular tools to elucidate gene expression and potential function of candidate virulence genes in *A. mellea* to better understand the infection process. Molecular tools for use in *Armillaria* are limited. An *Agrobacterium* mediated transformation system in *Armillaria* was developed in 2010 and vectors with GFP or mRFP expression have since been described (Baumgartner *et al.*, 2010b; Ford *et al.*, 2015, 2016). Unfortunately there are no gene disruption techniques available yet, although work to develop CRISPR in *Armillaria* is underway (Ford and Bourquin, 2018).

A selection of candidate virulence genes were chosen based on genes from model fungal plant pathogens with homologous genes within the *A. mellea* genome. Candidate virulence genes included homologues of PMK1 from *M. grisea* and Ras GTPases from *Ustilago maydis*, both involved in cell signalling, as well as small secreted proteins, expansins and effectors (Bruno *et al.*, 2004; Djonović *et al.*, 2006; Bluhm *et al.*, 2007; Mentlak *et al.*, 2012; Raffaello and Asiegbu, 2017; Sipos *et al.*, 2017). While cell-signalling genes were found without difficulty, homologues of other genes such as *Ecp6*, the LysM effector in *Cladosporium fulvum* (Bolton *et al.*, 2008), or Nep1-like proteins (Nelson *et al.*, 1998) could not be found. This suggests that these are pathogen-specific processes and that a comparative-based approach such as that used in this study is not suitable to discover these genes in *A. mellea*. To visualise gene activation, promoter:GFP plasmids were designed and transformed into *A. mellea*. Successful promoter:GFP transgenic lines were created for each of seven candidate virulence genes chosen. Attempts to visualise when candidate virulence genes were activated were made by growing transformant cultures on agar and plant-based tissue (i.e. veneer), however, visualisation of putative green fluorescence was poor. On agar, background autofluorescence made detection of fluorescent hyphae difficult. Coupled with this, Ford *et al.* (2016) reported a low rate of *A. mellea* transformants showing green fluorescence using the same GFP cassette as that used in this thesis. When transformants

were inoculated onto veneer strips, the wood exhibited strong autofluorescence. Still, putative green fluorescence was recorded in *AmRas2c* and *AmPMK1b* transformants grown on veneer indicating that these genes are either constitutively expressed or potentially involved in pathogenicity of plant tissue.

To further determine the role of selected candidate virulence genes, RNA expression was assessed. The aim here was to use reverse-transcriptase PCR on a variety of *A. mellea* tissues to assess expression of candidate virulence genes. A phenol chloroform based RNA extraction was optimised for isolation of RNA, yet the successful isolation of RNA from *A. mellea* tissue was unreliable. Furthermore, synthesis of cDNA from RNA was unreliable, perhaps due to residual LiCl ions (Fort *et al.*, 2008). Thus, expression of RNA was only assessed in two tissue types: mycelium from MEA-grown cultures and a cap from a mature fruiting body of *A. mellea* CG440 and ELDO17, respectively. Although genes *AmCP* and *AmRAS2c* showed faint bands, potentially indicating low levels of expression, all candidate virulence genes were expressed in mycelium from MEA-grown cultures. In mature fruiting body caps, *AmCP* again showed potentially low levels of expression, and no expression of *AmRAS2c*. In the caps of fruiting bodies the actin gene did not show expression, further indicating the unreliability of this method since actin was chosen as a housekeeping gene because it is constitutively expressed in Agaricomycetes (Luis *et al.*, 2005). Quantitative analysis of gene expression was originally planned, but due to several limiting factors was not completed. Poor isolation of RNA and synthesis of cDNA meant limited materials were available for qPCR and optimisation. Additionally, lab closures due to COVID and subsequent reduced capacity limited progress in the available timeframe.

Overall, the limited availability of molecular tools, and difficulties in achieving reliable cDNA synthesis meant that elucidating the expression and function of candidate virulence genes was not possible, and only tentative conclusions could be drawn from results. Future studies might investigate induction of plant defences to further study the function of candidate virulence genes. The technical challenges encountered in this study reinforce the difficulty of making progress in understanding this complex host-microbe interaction, particularly given the diploid nature of *Armillaria* infection which is unusual for fungi. Ultimately, progression in our understanding of *Armillaria* genetics and virulence factors is limited by the genomic tools available and more robust molecular genetic techniques need to be

developed. Therefore, the option of biological control through use of endophytic *Trichoderma* spp. was explored using *in vitro* and pot-based experiments to determine the potential of controlling ARR.

6.1.1 Future work to understand pathogenicity genes in *A. mellea*

- Design new plasmids with mRFP to potentially increase the number of transformants showing fluorescence.
- Further investigate gene function of candidate virulence genes for potential induction of defence genes and hypersensitive responses in plants.
- Develop more robust genetic manipulation tools for *Armillaria*.

6.2 *Trichoderma* spp. as a biocontrol agent of *Armillaria* Root

Rot

Chapters three and four of this thesis have demonstrated that endophytic *Trichoderma* spp. have the potential as biological control agents against the root rot pathogen, *Armillaria mellea*. *In vitro* agar-based dual culture assays found such endophytic *Trichoderma* spp. could quickly overgrow *A. mellea*. This has been reported in other studies (Raziq and Fox, 2003; Chen *et al.*, 2019), however, *Trichoderma* isolates used in other studies were not endophytic. Further dual culture assays using hazel disks pre-colonised with *A. mellea* showed a subsection of endophytic *Trichoderma* spp. were again able to overgrow *A. mellea* and prevent outgrowth. Few similar studies have been conducted in the literature, however, there are reports that *Trichoderma* spp. are able to prevent growth of *Armillaria* spp. in woody substrates to a varying degree (Li and Hood, 1992; Otieno *et al.*, 2003; Kwańska and Szykiewicz-Wronek, 2018) which can be dependent on the isolate of *Armillaria*, for example, *A. ostoyae* (Kwańska *et al.*, 2004). In the work presented in chapter three, the viability of *A. mellea* after dual culture with *Trichoderma* isolates (*T. atrobrunneum*, *T. harzianum*, *T. hamatum* and *T. olivascens* and *T. virens*) was variable between isolates for both *A. mellea* and *Trichoderma* spp.. Lack of *A. mellea* re-isolation from dual cultures suggested that some *Trichoderma* spp. were able to kill *A. mellea in vitro*. Using microscopy to elucidate the method by which *Trichoderma* spp. antagonised *A. mellea*, examples of

degraded *A. mellea* hyphae in the presence of *T. harzianum* and *T. atrobrunneum* were found but no evidence for specialised infection strategies were observed. Since *Trichoderma* spp. are well known producers of extracellular enzymes (Harman *et al.*, 2004a), a selection of enzymes were chosen to indicate whether enzyme production by *Trichoderma* spp. was related to an increased ability to act as biocontrol agents. All *Trichoderma* spp. isolates tested were shown to produce extracellular amylases, cellulases, laccases, pectinases and proteases to varying degrees which might offer a mode by which *A. mellea* hyphae were degraded.

All *Trichoderma* spp. isolates were screened in strawberry plants to identify those with increased biocontrol potential against ARR. Seven *Trichoderma* spp. isolates reduced the severity of ARR below that of the *Armillaria*-only control. *Trichoderma harzianum* has been found to reduce *A. mellea* infection of strawberry plants (Raziq and Fox, 2003), however, the isolates used were from commercial mushroom pathogens in contrast to this research which focused on using endophytic isolates of *Trichoderma* spp. to offer protection. There have been numerous reports of enhanced growth promotion by *Trichoderma* spp. (Hohmann *et al.*, 2011; Akladios and Abbas, 2014; Halifu *et al.*, 2019; Zaw and Matsumoto, 2020), however, in this study there were no signs of growth promotion recorded in the strawberry plants. The lack of growth promotion might have been a result of using dormant, bare-rooted strawberry plants, cultivated for vigorous growth, therefore, limiting the chance of detecting growth promotion in the first two months. The seven best performing *Trichoderma* spp. isolates along with one poorly performing isolate were selected to determine the control offered against ARR in a privet host system. Endophytic colonisation of strawberry and privet hosts was verified through isolation to ensure long-term protection by *Trichoderma* spp. was offered against ARR. In both strawberry and privet plants *T. atrobrunneum* T17/11 and T17/15 performed well, suggesting protection could be offered to several hosts in different families.

6.2.1 Comparisons between *in vitro* and *in planta* antagonism against ARR

6.2.1.1 Antagonism of *Armillaria* spp. by *Trichoderma* spp.

Although the screening of all 40 *Trichoderma* isolates in dual culture assays showed very little variation between isolates, the variation in strawberry plants was more pronounced. Seven *Trichoderma* spp. isolates increased the rate of survival and reduced the level of disease in strawberry plants infected with *A. mellea* compared to those free from *Trichoderma* spp.. The remaining *Trichoderma* spp. isolates resulted in similar or increased levels of ARR compared to *Trichoderma*-free plants inoculated with *A. mellea*. The three *Trichoderma* isolates which were slow growing *in vitro* (*T. fertile* T17/37, *T. olivascens* T17/41 and *T. olivascens* T17/42) did not stop *A. mellea* growth after four days and showed limited or no protection to strawberry plants, suggesting that these are not viable candidates for biocontrol. In contrast, Chen *et al.* (2019) found large variation in inhibition of *Armillaria* spp. in a screening with *Trichoderma* spp. using dual culture assays. Siderophore production by *Trichoderma* spp. was also tested by Chen *et al.* (2019) which showed that all *Trichoderma* isolates tested could produce siderophores. Siderophores, which are produced by most fungi, are compounds used to facilitate iron transport from the surrounding environment which could increase the competitive advantage of particular organisms (Renshaw *et al.*, 2002). In the case of fast-growing *Trichoderma* spp., production of siderophores could give them a competitive advantage over *Armillaria* spp. which grow slower, provided *Armillaria* spp. are not able to access the iron chelated by *Trichoderma* spp. siderophores, a phenomenon noted in *Saccharomyces cerevisiae*, among other fungal species (Haas, 2014).

Hazel stems inoculated with *A. mellea* and *Trichoderma* spp. were nailed together and incubated for seven months to assess the level of antagonism offered by *Trichoderma* spp.. Prolonged overheating of incubators meant that antagonism of *Trichoderma* spp. towards *A. mellea* could not be assessed, however, some similar studies have found that *Trichoderma* spp. can reduce the number of *A. ostoyae* and *A. gallica* rhizomorphs, while others can stimulate growth (Kwaśna *et al.*, 2004; Kwaśna and Szykiewicz-Wronek, 2018). Drakulic *et al.* (2017) reported that isolation of both *A. mellea* and *A.*

gallica from the same garden site was infrequent and only one case of a host (x *Cupressocyparis leylandii*) from which both species were isolated was reported. While little is understood of the interaction between *A. mellea* and *A. gallica*, the suggestion was made that if the less pathogenic species (*A. gallica*) could occupy the same niche as the more pathogenic species (*A. mellea*) it could prevent the invasion of the pathogenic species (Drakulic *et al.*, 2017). In a situation where this might occur, the addition of a *Trichoderma* spp. which stimulates *A. gallica*, growth could increase the chances of the less virulent species occupying an ecological niche before the more pathogenic *A. mellea* can become established. However caution must be taken as recently *A. gallica* isolates in Mexico have been reported to be primary pathogens in *Malus*, *Pinus* and *Quercus* species (Duarte-Mata *et al.*, 2021) and in Europe (Prospero *et al.*, 2004) and the USA (Robinson and Morrison, 2001) *A. ostoyae*, which can also be stimulated by *Trichoderma* spp. (Kwańska *et al.*, 2004), is an important pathogen, particularly towards coniferous hosts.

6.2.1.2 Viability studies of *Armillaria* spp. after co-cultivation with *Trichoderma* spp.

A limited number studies have directly compared the viability of *Armillaria* spp. after dual inoculation with *Trichoderma* spp. *in vitro*, with the level of protection offered to host plants from ARR. Chen *et al.* (2019) selected two *Trichoderma* isolates (*T. virens* and *T. atrobrunneum*) which were good antagonists *in vitro* to create a *Trichoderma*-mix which was inoculated into Turkey oak seedlings. Plants inoculated with *Trichoderma* had reduced levels of ARR, suggesting in this case, dual culture interactions and observations of siderophores were a good indicator of biocontrol potential. Raziq and Fox (2003) noted differences in levels of antagonism by *Trichoderma* spp. using *in vitro* assays compared to strawberry plants. One isolate of *T. harzianum* was effective against *A. mellea* *in vitro* and *in planta*, however, a different *T. harzianum* isolate was effective *in vitro* but did not result in protection of strawberry plants. In their study Raziq and Fox (2003) attempted re-isolation of *A. mellea* from dual interaction assays with *Trichoderma* spp., however could not successfully recover *A. mellea* which the authors suggest was due to 'lethal effects of the antagonists' where the isolations for *A. mellea* were made onto 3 % MEA without supplements. In the present study recovery of *A. mellea* was made with a selective medium to prevent growth of faster growing *Trichoderma* spp. outcompeting potential *A.*

mellea growth. It is possible that re-isolation of *A. mellea* was inhibited by antagonists in the study by Raziq and Fox (2003) thus hinting that all *Trichoderma* spp. had the same level of 'lethal' effect. In addition Raziq and Fox (2003) were not using endophytic *Trichoderma* isolates, but pathogenic mushroom fungi, nor was *Trichoderma* directly applied to roots, both of which are factors which could impact the biocontrol potential of *Trichoderma* spp., particularly in plants. Furthermore, consideration must be given to the microbial communities present in plant based experiments but absent from dual-culture *in vitro* studies. In the present study, a selection of *Trichoderma* spp. isolates were tested in dual-culture with hazel disks pre-colonised by *A. mellea*. Here there was variation in the levels of antagonism offered by *Trichoderma* isolates to two isolates of *A. mellea* which did not reflect the levels of protection offered to plants from ARR. *Trichoderma* isolates *T. virens* T17/02 and *T. hamatum* T17/10 were able to kill multiple *A. mellea* isolates in agar- and wood- based *in vitro* assays. When inoculated into strawberry plants, both isolates offered a similar level of protection against *Armillaria* infection in strawberries, however, in privet *T. virens* T17/02 did not reduce *A. mellea* infection, and while *T. hamatum* T17/10 could offer some protection to privet plants, it ranked fourth in the level of protection offered. *Trichoderma atrobrunneum* demonstrated limited ability to kill *A. mellea* in hazel disks but had strong protective ability against ARR in both privet and strawberry hosts. It is therefore possible that the presence of a microbial community from the soil and rhizosphere of plants can affect the outcome of *Trichoderma* spp. as a biocontrol agent. Pearce (1990) studied the antagonistic effects of various fungi towards *A. luteobubalina* using agar-based assays, sterile and non-sterile eucalyptus stems. Some fungi were reported to be more antagonistic in dual culture and in sterile stems compared to non-sterile stems. For example, *Poria medullaris*, was strongly antagonistic in agar-based dual culture and showed strong-moderate antagonism when sterile eucalyptus stems inoculated with *A. luteobubalina* and *P. medullaris* were incubated together, however, when non-sterile stems were used no antagonism was reported (Pearce, 1990). Another study similarly reported that in fungus-colonised tea stem segments, the antagonism offered by *T. harzianum* towards *Armillaria* sp. was reduced when incubated in non-sterile soils compared to sterile soils (Otieno *et al.*, 2003). On the other hand, a study has reported the increase in beneficial microbial communities after application of *Trichoderma* spp. (Saravanakumar *et al.*, 2017) which could result in greater biological control potential. These studies

highlight how the interaction between antagonistic fungi (i.e. *Trichoderma* spp.) and *A. mellea* can change as a microbial community is introduced and might explain the variation between *in vitro* and *in planta* studies noted in this thesis and in the literature by (Raziq and Fox, 2003).

6.2.1.3 Protection by *Trichoderma* spp. against ARR across multiple plant families

In this study seven *Trichoderma* spp. isolates which offered enhanced protection from ARR in strawberry plants were tested in privet plants, an additional poor-performing isolate in strawberry plants was included which performed better in privet plants. Two isolates of *T. atrobrunneum* (T17/11 & T17/15) which resulted in the rate of greatest protection against *A. mellea* in strawberry plants and also showed the greatest level of protection in privet plants, demonstrating protection against ARR in multiple plant families (Rosaceae and Oleaceae) by *T. atrobrunneum*. On the other hand, *Trichoderma virens* T17/02 and *T. harzianum* T17/03 showed good protection against ARR in strawberries but resulted in poor protection against *Armillaria* infection in privet. In dual culture with pre-colonised hazel disks, *T. virens* T17/02 was able to eliminate *A. mellea* growth showing strong antagonism *in vitro* whereas *T. harzianum* T17/03 did not eliminate *A. mellea* from pre-colonised hazel disks. A series of studies by Raziq and Fox (2003, 2006b, 2006a) show that the reliability of *Trichoderma* spp. for protection against ARR may not always be repeatable and protection can vary between hosts, as noted in chapter four. The *Trichoderma* isolates used in the current study were taken from a range of host families including woody species such as viburnum (Adoxaceae), oak (Fagaceae) and silver birch (Betulaceae) (Table 3.1), but the screening was carried out using herbaceous strawberry plants from the Rosaceae family. Original selection of hosts for *Trichoderma* isolation was based upon healthy plants in close proximation to ARR infected plants. It is therefore possible that some *Trichoderma* isolates have a species-specific symbiosis that could offer the original host protection from ARR but would not be recorded in plants from other species or families. Although not directly assessed in this work, if *Trichoderma* isolates were found to have a species-specific symbiosis protecting limited hosts they would be of less interest as a biocontrol agent of ARR due to the a number of susceptible hosts spanning a range of plant families which *Armillaria* can infect. Isolates from *T. atrobrunneum* (T17/11 and T17/15) demonstrated strong biocontrol potential in both strawberry and privet host systems. It is

possible that these isolates, originally from *Quercus* sp. and *Viburnum bodnantense* respectively, may protect numerous host plants from ARR. In contrast, isolates such as *T. virens* T17/02 and *T. harzianum* T17/03 (both originally isolated from soil/debris) may have a smaller range of hosts with which a beneficial symbiosis can be formed. *Trichoderma* spp. as biocontrol agents of multiple hosts have been reported in the past. For example, *T. harzianum* strain T22 is commercially available for a number of hosts such as bean, cucumber, lettuce, maize and tomato (Harman *et al.*, 2004a, 2004b; Innocenti *et al.*, 2015; Javanshir Javid *et al.*, 2016). Therefore, it is possible that the *Trichoderma* isolates from this study have the potential to act as biocontrol agents of *Armillaria* across multiple hosts. In addition, *T. harzianum* T22 is reported to have biocontrol properties against a number of fungal plant pathogens such as *Alternaria solani*, *Botrytis cinerea*, *Colletotrichum graminicola*, *Fusarium oxysporum* f. sp. *lactucae* and *F. oxysporum* f. sp. *radicis-cucumerinum* (Harman *et al.*, 2004a, 2004b; Innocenti *et al.*, 2015; Javanshir Javid *et al.*, 2016). This was further noted in another study where the biocontrol potential of an isolate of *T. citrinoviride* was tested against six ginseng pathogens (*Rhizoctonia solani*, *B. cinerea*, *A. panax*, *Cylindrocarpon destructans*, *Phytophthora cactorum*, and *Pythium* spp.). *Trichoderma citrinoviride* parasitised all pathogens *in vitro* and reduced infection of *B. cinerea* and *C. destructans* in ginseng plants (Park *et al.*, 2019). The biological control potential for *Trichoderma* spp. used in this work was only tested against *Armillaria mellea*. Given the range of host and pathogen combinations *Trichoderma* spp. can be effective against and the mode of action by which *Trichoderma* spp. acts as a biological control agent (e.g. mycoparasitism, antibiosis, competition and induced defence responses in plants), it is possible that the *Trichoderma* isolates protective against ARR could also reduce root-infections from other plant pathogens. Pathogens of particular interest to for further study would be horticultural diseases since these *Trichoderma* spp. were isolated from a garden setting.

6.2.1.4 Understanding *Trichoderma* spp. as a biocontrol agent

Consideration of a combination of *Trichoderma* spp. isolates which offer protection to hosts from ARR in different settings (e.g. host family or pathogen) might be considered to increase the commercial viability of a biocontrol product. Using a combination of two *Trichoderma* isolates (*T. virens* and *T. atrobrunneum*), Chen *et al.* (2019) reported a reduction of ARR in Turkey oak seedlings. In contrast,

two *T. harzianum* isolates selected by Raziq and Fox (2005) resulted in the death of all strawberry plants during the course of an experiment compared to one of the *T. harzianum* isolates alone which offered protection. Evidently a detailed understanding of how *Trichoderma* isolates interact together, and whether more than one *Trichoderma* species or isolates could colonise the same host tissue to offer effective biocontrol against ARR is important. Furthermore, *Trichoderma* spp. are well known for antagonism by competition (Harman *et al.*, 2004a), thus further studies should determine whether *Trichoderma* spp. can be displaced after time by other endophytes and whether colonisation of plants by *Trichoderma* spp. can be limited by fungal endophytes already colonised in a plant. The longevity of soil and root colonisation by *Trichoderma* spp. reported in the literature is usually short-term, although some studies cite long-term colonisation by *Trichoderma* spp., mainly from the soil rhizosphere. *Trichoderma* spp. were reported to colonise the rhizosphere of *P. radiata* seedlings for up to seven months (Hohmann *et al.*, 2011), while *T. asperellum* and *T. atroviride* have been shown to colonise the soil for one year (Savazzini *et al.*, 2009; Xian *et al.*, 2020). One study reported the rhizospheric and endophytic colonisation of *T. virens* with biocontrol potential against *Fusarium oxysporum* f. sp. *lycopersici* in tomato. The amount of *T. virens* DNA in the rhizosphere did not obviously change over one month, however endophytic colonisation of the *T. virens* isolate increased four times in tomato plants over the same time period (Zaw and Matsumoto, 2020). This highlights the importance of assessing the endophytic colonisation of plants given the variation in behaviour of *Trichoderma* spp. in the rhizosphere and as endophytes for biological control. Colonisation of cocoa trees by some strains of *T. stromaticum* was reported for up to four months (De Souza *et al.*, 2008) and in this study endophytic *Trichoderma* spp. associations were recorded in strawberry plants after five months. Further research into the longevity of endophytic *Trichoderma* spp. associations with plants is required to determine the potential for long term protection from plant diseases. In part, poor understanding of long-term colonisation of *Trichoderma* spp. as endophytes is driven by research into pathogens which shorter life cycles than *Armillaria*. Much work on biocontrol agents focuses on pathogens with short life cycles such as Ascomycota and *Phytophthora* spp.. *Trichoderma* spp. with biocontrol potential have been reported to induce disease resistance of plants from one – 14 days post inoculation (Harman *et al.*, 2004a) where assays can often be completed within three weeks. This

highlights the difficulties in finding a long-lived biocontrol option for persistent Agaricomycete pathogens such as *Armillaria* where plant infection and pathogenicity assays in pot-based experiments require a minimum of three months.

The research presented in this thesis found that the timing of *Trichoderma* spp. application is important, and where applied too early could lead to increased levels of *A. mellea* infection, a phenomenon reported in *Pinus radiata* seedlings inoculated with *Trichoderma viride* and infected with *Fusarium circinatum* (Amaral *et al.*, 2019). Further research must be undertaken to gain an understanding of the timing and method of *Trichoderma* spp. application for successful control of ARR if it is to be considered as a potential biocontrol agent of *A. mellea*, available commercially.

6.2.2 Extracellular enzyme production by *Trichoderma* spp. in relation to performance against ARR in plants

To determine whether the extracellular enzyme activity by *Trichoderma* spp. reported in chapter three enhanced biocontrol ability, comparisons were made with the level of *A. mellea* infection in pot-based experiments from chapter four. Of *Trichoderma* isolates assessed for extracellular enzyme activity, five did not offer protective ability against ARR in strawberry plants (*T. harzianum* T17/01, *T. atrobrunneum* T17/04, *T. cerinum* T17/30, *T. hamatum* T17/33 and *T. hamatum* T17/34), two offered good protection against strawberry, but less so to privet plants (*T. virens* T17/02 and *T. harzianum* T17/03) and three offered good protection against both strawberry and privet plants (*T. atrobrunneum* T17/11 & T17/15 and *T. hamatum* T17/10).

Very little difference was noted between the enzyme production of *Trichoderma* isolates in most cases which suggests that there is a low connection between enzyme production and biocontrol potential. Amylase production was similar in all isolates except in *T. harzianum* T17/01 (poor biocontrol potential) which had low production of amylase. A study found amylase production by *T. harzianum* to have no effect on the Agaricomycete fungus *Moniliophthora perniciosa* (De Azevedo *et al.*, 2000; De Marco *et al.*, 2003), but is thought to encourage fast growth of *Trichoderma* spp. on potato dextrose agar (Barbosa *et al.*, 2001). There was very little variation in production of cellulase and protease in the

Trichoderma spp. isolates tested, regardless of performance in plants. Cellulose production by *Trichoderma* spp. has been linked to antagonism against phytopathogens including *Colletotrichum gloeosporioides*, *Aspergillus flavus*, *Fusarium moniliforme* and *F. oxysporum* f. sp. *lentis* *in vitro* (Calistru *et al.*, 1997; de los Santos-Villalobos *et al.*, 2013; El-Hassan *et al.*, 2013). The lack of variation produced by isolates in this study in relation to performance in plants, suggests while cellulose might cause some antagonism *in vitro* (due to faster growth of *Trichoderma* spp.), other factors are likely to be important. Likewise in production of protease which can be antagonistic towards a range of phytopathogens (*F. oxysporum*, *S. sclerotiorum*, *R. solani*, *Cytospora chrysosperma* and *Alternaria alternata*) *in vitro* (Yan and Qian, 2009), other factors are likely to be important for effective antagonism by *Trichoderma* spp. in plants.

While there was significant variation in laccase production between *Trichoderma* isolates, this was not linked to the level of protection given to plants. A link between production of laccase by *T. virens* and antagonism of *Botrytis cinerea* sclerotia has been made (Catalano *et al.*, 2011) warranting further investigation into the direct effects on *A. mellea* rhizomorphs. Pectinase production was highly variable between *Trichoderma* isolates tested, with some isolates, including all *T. atrobrunneum* isolates, producing no pectinase but there was no comparison in levels of pectinase produced and biological control potential in plants.

Overall, while extracellular enzyme production by *Trichoderma* spp. is widely reported (Calistru *et al.*, 1997; De Azevedo *et al.*, 2000; De Marco *et al.*, 2003; Yan and Qian, 2009; Catalano *et al.*, 2011; de los Santos-Villalobos *et al.*, 2013), in this study there was no direct link found between specific enzyme production and improved performance in plants. Degradation of *Armillaria* hyphae was reported when challenged by *T. atrobrunneum* T17/04 and *T. harzianum* T17/08 in this thesis. The direct effects of enzyme production were not assessed on *A. mellea* hyphae, however it is possible that the hyphal degradation reported was a result of enzyme production or secondary metabolite activity from *Trichoderma* spp.. It is most likely that a combination of factors are involved in antagonism and production of cellulases, proteases and laccases might improve the ability of *Trichoderma* spp. as antagonists alongside production of other enzymes and secondary metabolites. Factors such as enzyme

regulation in free-living *Trichoderma* spp. compared with endophytic-living *Trichoderma* spp. as well as induced disease resistance and mycoparasitism are likely to be other influencing factors. To further elucidate the role of enzymes produced by *Trichoderma* spp. and apparent antagonism, the direct effect on *A. mellea* should be investigated further.

6.2.3 Risks and environmental impact of using *Trichoderma* spp. as biocontrol agents

Cases where introduced biological control agents have been unsuccessful and themselves become pests are well known. Examples include the introduction of the cane toad to Australia to control cane beetles (Shanmuganathan *et al.*, 2010) or introduction of the harlequin ladybird to control aphids (Camacho-Cervantes *et al.*, 2017). Therefore it is important to assess and carefully consider the necessity of the introduction of biological control agents to a new location. The present study has focused on the use of native *Trichoderma* spp. which are considered to be a ubiquitous fungi (Harman *et al.*, 2004a). Since individual *Trichoderma* isolates in this study provided varying degrees of protection against ARR which is a persistent pathogen in the UK, introduction of *Trichoderma* isolates with strong biocontrol potential to sites to control ARR was deemed a viable option to consider. The effect of introducing *Trichoderma* spp. on the existing microbial communities has been assessed in the past. Studies have reported *Trichoderma* spp. colonisation in soils to be present after one year (Savazzini *et al.*, 2009; Xian *et al.*, 2020), which while beneficial for biological control could have negative consequences for the species diversity in the microbiome. Savazzini *et al.* (2009) showed that *T. atroviride* did not have a long-term affect (over three months) on the fungal and bacterial communities in the rhizospheric soils of vineyards. The effect of *T. harzianum* on the microbial community in the rhizospheric soil of maize was assessed in tandem with biological control potential of *Fusarium graminearum*. An increase in beneficial microbial communities was reported which included acidobacteria, and there was no significant difference reported in the fungal communities (Saravanakumar *et al.*, 2017). In addition the combination of *T. harzianum* with mycorrhizal fungi has been found to improve growth and reduce disease caused by *Fusarium udum* in pigeon pea (*Cajanus cajan*) (Dehariya *et al.*, 2015). This means

that introduced *Trichoderma* spp. could form a beneficial association with mycorrhizae and increase the biocontrol potential. While studies highlight the relatively low and short-term effects of introducing *Trichoderma* spp. on the native microbial community, caution should be taken when introducing a new *Trichoderma* spp. to limit genetic pollution and potential negative impacts on delicate soil microbial systems. Where a beneficial *Trichoderma* spp. is found, at least to begin with, thorough trials should occur in the local area to its original isolation to monitor for any negative impacts. Subsequently, the *Trichoderma* isolate could be distributed further, but should not be taken overseas due to potential phytosanitary risks. In addition, differences in climate, and provenance of pathogens and hosts might change the biocontrol potential, making it ineffective if taken outwith the native range. Finally, plants treated with *Trichoderma* spp. to control ARR should be monitored in case selection pressure causes *Armillaria* to develop resistance against the biological control agent.

6.2.4 Commercial opportunity for *Trichoderma* spp. as a biocontrol agent of ARR.

The overarching aim of this project was to find a *Trichoderma* isolate, or isolates, capable of preventing ARR. To reach the point of commercialisation of the *Trichoderma* spp. used in this study, rigorous tests must be conducted to determine suitability of *Trichoderma* isolates (i.e. environmental impact and host range ect.). Initial investigations suggest that isolates *T. atrobrunneum* T17/11 or T17/15 have the potential to be used as a biocontrol agents of ARR across multiple hosts. The *Trichoderma* spp. should be pre-inoculated into plants which could be marketed to gardeners as ‘*Armillaria* safe’. This would allow any gardener with *Armillaria* present in their garden to buy and plant a *Trichoderma*-protected plant which would not succumb to *Armillaria* infection after planting, giving the plant a chance to become established and healthy.

6.2.5 Limitations in studying biocontrol potential of *Trichoderma* spp.

Initial assessment of the antagonistic effects towards *A. mellea* by *Trichoderma* spp. was studied using classical dual culture assays with agar plugs. While these assays provide an important understating of fungus-fungus interactions in pure culture, the homogeneous environment fails to represent the

complexity of interactions in a real-world situation (Crowther *et al.*, 2018). To further our understanding of the interaction between *A. mellea* and *Trichoderma* spp., wood segments pre-colonised with *A. mellea* were challenged with *Trichoderma* spp.. The introduction of wood segments was able to introduce a level of heterogeneity to the assay, and as expected, *Trichoderma* spp. were less efficient antagonists when *A. mellea* was pre-colonised in wood segments, although some isolates of *Trichoderma* spp. could still eliminate *A. mellea* from hazel disks. A third experiment aimed to look at the effect of *Trichoderma* spp. on rhizomorph production by *A. mellea* in woody billets incubated in sand. Due to technical issues, this assay failed. The use of woody billets in sand was designed to assess the *in vitro* potential of *Trichoderma* spp. as a biocontrol agent of *A. mellea* in controlled conditions more closely representing natural growth conditions for *A. mellea*.

The infection process of *Armillaria* has recently been reviewed by Devkota and Hammerschmidt (2020) to highlight our understanding to date alongside key, unanswered questions. A major limitation in controlling *A. mellea* is due to our poor understanding of the *A. mellea* infection process. An *in vitro* study into the infection of *A. mellea* on culture tissue of grape rootstocks found that *A. mellea* could be detected in plant roots after two weeks (Baumgartner *et al.*, 2010a). Since *Armillaria* infection is often not noticed until symptoms have progressed, the speed at which infection can occur is important information to guide assays conducted in plants. While Baumgartner *et al.* (2010) noted rapid infection, the assay was conducted in a small scale system using magenta pots (75 x 75 x 100 mm) and plants were heavily inoculated with four agar plugs of *A. mellea*. Through the use of clear soils such as those described by Downie *et al.* (2012), an experiment might be designed to investigate further the infection rate by *Armillaria* in a volume of substrate more closely representing that used in plant-based trials. Furthermore, such an experiment could identify whether *A. mellea* can grow directionally towards a host plant. This was investigated *in vitro* by Mihail and Bruhn (2005). In the presence of an uncolonised woody substrate, there was no directional lateral growth towards the target by *A. gallica* rhizomorphs which grew in an asymmetric fashion. In contrast, when an uncolonised stem of *Quercus velutina* was placed above or below a source of *A. gallica* directional growth was observed (Mihail and Bruhn, 2005).

A limiting factor in the experimental design for plant infection based experiments with *A. mellea* was the use of the 'disease severity index'. While this was a useful scale to assess the level of *A. mellea* infection in plants there were major drawbacks. Since the scale changed in each plant-based assay comparisons between experiments could not easily be made based on the DSI which was particularly evident when comparing the efficiency of *Trichoderma* spp. as biocontrol agents against *A. mellea* in privet and strawberry plants. In the first plant based experiment, where *Trichoderma* isolates were screened in strawberry plants a six-point scale was used which was found to be too objective and was narrowed to a five-point scale for future use. In the collaborative work investigating a new inoculation method for *Armillaria*, the DSI was based on the four-point scale, adapted to fit the aims of the project and remove any potential subjectivity. To overcome this problem in future, a robust scale should be detailed, with limited subjectivity and used throughout experimentation.

6.2.6 Future prospects for ARR control

To further understand the potential of *Trichoderma* spp. as biocontrol agents of *A. mellea* the following suggestions are made:

- Determine the mechanisms by which *Trichoderma* spp. acts upon *A. mellea* *in vitro* and in plant roots using SEM/microscopy.
- Determine the effect of purified enzymes from *Trichoderma* spp. on *A. mellea* to further understand mechanisms of biocontrol.
- Investigate *A. mellea* movement towards plants during initial infection to understand the mechanism utilised and rate at which *A. mellea* infects plants.
- Determine the most efficient timing and method of *Trichoderma* spp. application for protection against ARR.
- Determine whether protection against ARR by *Trichoderma* spp. is offered across a range of different host plant families.
- Conduct field-based trials for the protection of plants by *Trichoderma* spp. from ARR to gain an applied understanding of the efficiency of biocontrol offered by *Trichoderma* spp.
- Determine whether *Trichoderma* spp. impacts soil microbial communities, and if so, to what degree.

6.3 Conclusion

The work presented in this thesis has made progress towards finding a biological control for *Armillaria* root rot. The ability to further our understanding of how *Armillaria* causes disease through investigations into functions of selected virulence genes was limited by available tools. To improve plant-based assays, a method to reduce the time required to prepare inoculum for *in planta* assays was developed, and strawberry and privet plants were successfully used as host plant systems for *Armillaria* with relatively fast disease development. This thesis highlights that *Trichoderma* spp. have the potential as biological control agents of *Armillaria*. Endophytic *Trichoderma* spp. were shown to eradicate *A. mellea in vitro* and were found to provide protection against *Armillaria* root rot in two plant hosts: strawberry and privet. Thus, work presented in this thesis suggests that the future prospects to control *Armillaria* root rot are positive and that *Trichoderma* spp. are viable biological control agents against *Armillaria*.

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Appendices

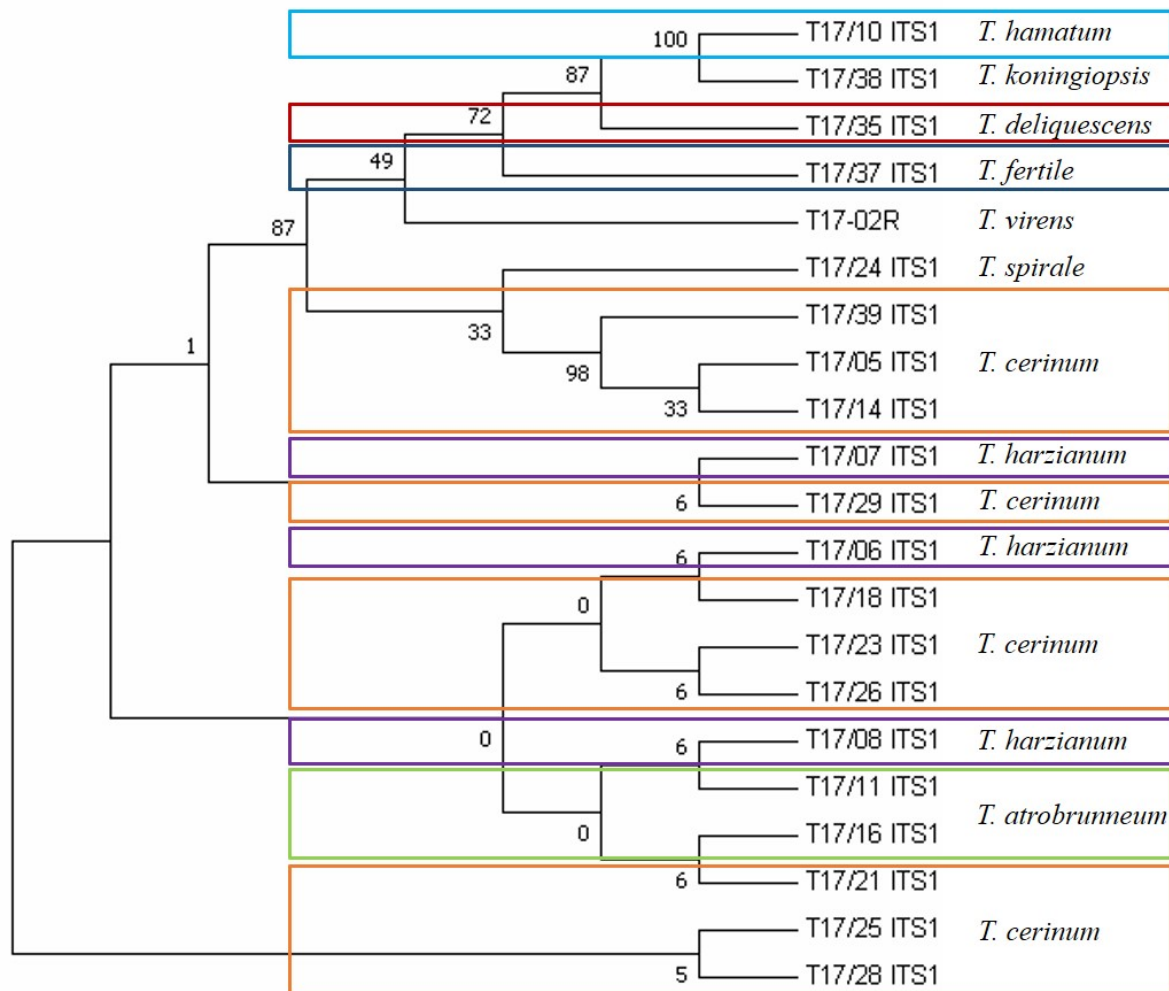


Figure A.1: Phylogenetic groupings of the *Trichoderma* ITS1 region.

Tree is built based on the maximum likelihood model with 1000 bootstraps. Sequence data for T17/02 is only available for the reverse fragment, with primer ITS2.

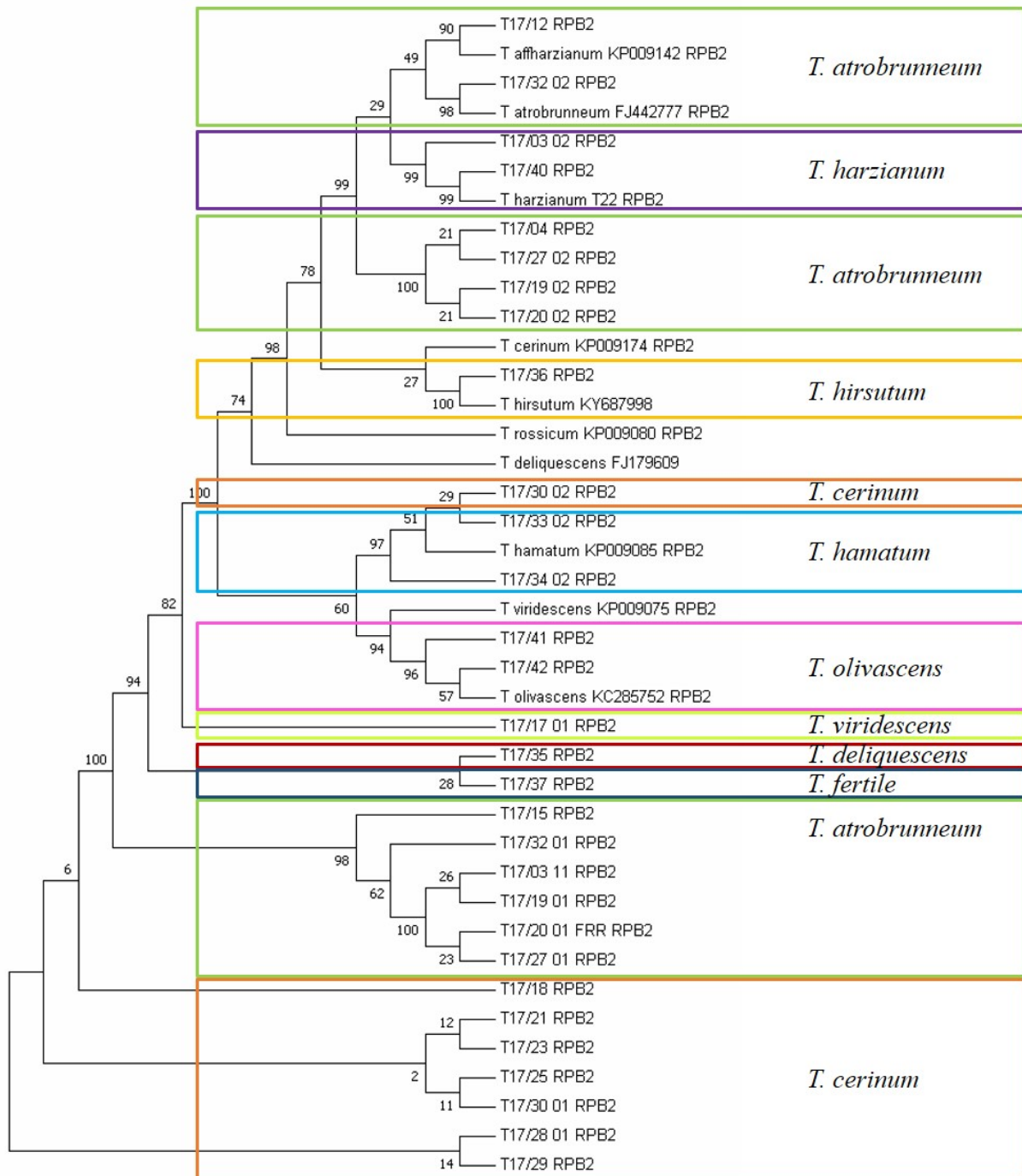


Figure A.2: *rpb2* phylogenetic groupings of *Trichoderma* isolates.

Tree is built based on the maximum likelihood model with 1000 bootstraps. GenBank reference sequences for *Trichoderma* spp. are included.

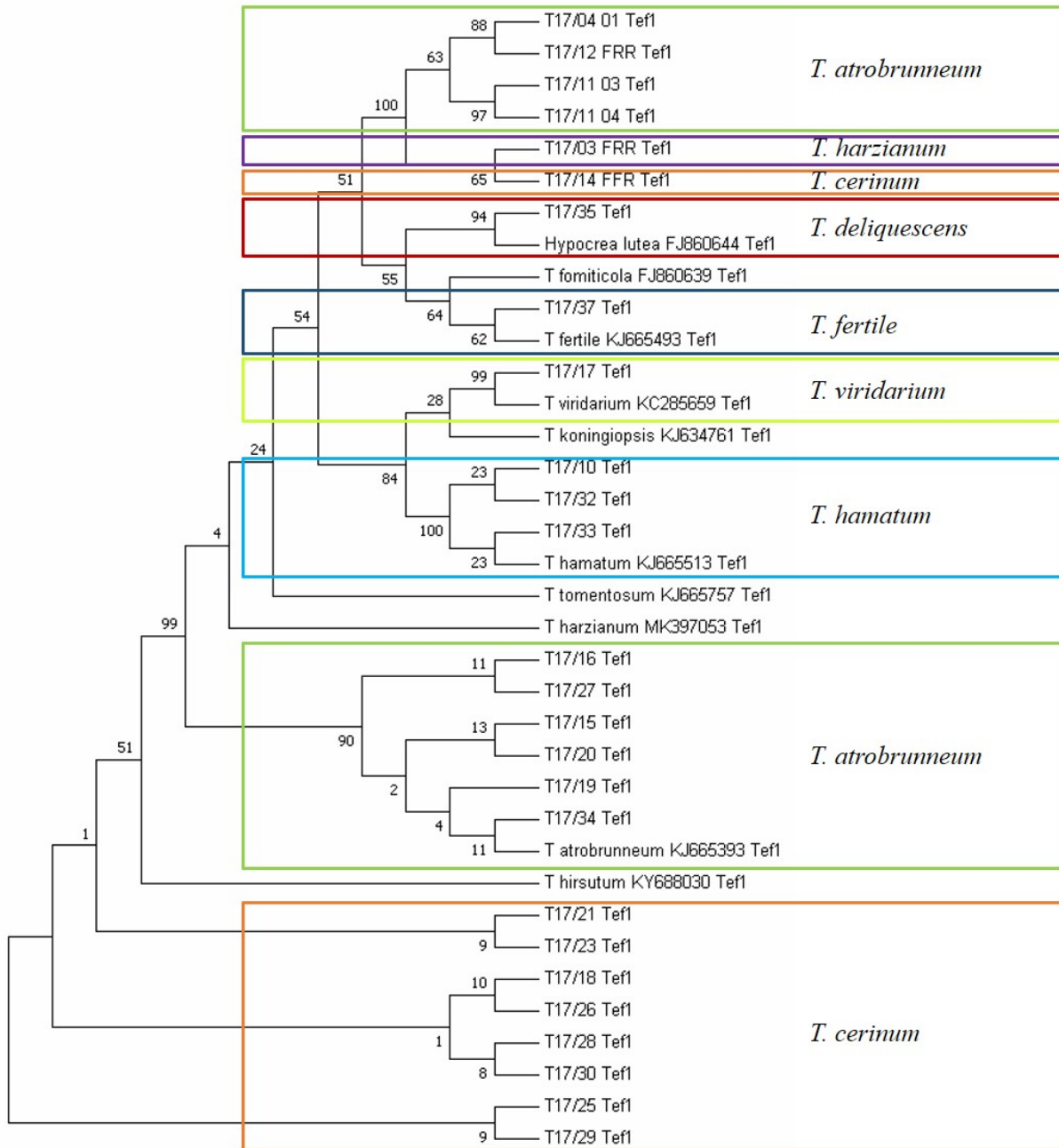


Figure A.3: *tef1* phylogenetic groupings of *Trichoderma* isolates.

Tree is built based on the maximum likelihood model with 1000 bootstraps. GenBank reference sequences for *Trichoderma* spp. are included.

Table A.1: GenBank accession numbers of *Trichoderma* spp. sequences.

| Isolate | ITS1 | <i>rpb2</i> | <i>tef1</i> |
|---------|----------|-------------|-------------|
| T17/01 | MW241136 | | |
| T17/02 | MW241137 | | |
| T17/03 | | MW232497 | MW232521 |
| T17/04 | | MW232498 | MW232522 |
| T17/05 | MW241138 | | |
| T17/06 | MW241139 | | |
| T17/07 | MW241140 | | |
| T17/08 | MW241141 | | |
| T17/09 | MW241142 | | |
| T17/10 | MW241143 | | MW232523 |
| T17/11 | MW241144 | | MW232524 |
| T17/12 | | MW232499 | MW232525 |
| T17/13 | MW241145 | | |
| T17/14 | MW241146 | | MW232526 |
| T17/15 | | MW232500 | MW232527 |
| T17/16 | MW241147 | | MW232528 |
| T17/17 | | MW232501 | MW232529 |
| T17/18 | MW241148 | MW232502 | MW232530 |
| T17/19 | | MW232503 | MW232531 |
| T17/20 | | MW232504 | MW232532 |
| T17/21 | MW241149 | MW232505 | MW232533 |
| T17/23 | MW241150 | MW232506 | MW232534 |
| T17/24 | MW241151 | | |
| T17/25 | MW241152 | MW232507 | MW232535 |
| T17/26 | MW241153 | | MW232536 |
| T17/27 | | MW232508 | MW232537 |
| T17/28 | MW241154 | MW232509 | MW232538 |
| T17/29 | MW241155 | MW232510 | MW232539 |
| T17/30 | | MW232511 | MW232540 |
| T17/32 | | MW232512 | MW232541 |
| T17/33 | | MW232513 | MW232542 |
| T17/34 | | MW232514 | MW232543 |
| T17/35 | MW241156 | MW232515 | MW232544 |
| T17/36 | | MW232516 | |
| T17/37 | MW241157 | MW232517 | MW232545 |
| T17/38 | MW241158 | | |
| T17/39 | MW241159 | | |
| T17/40 | | MW232518 | |
| T17/41 | | MW232519 | |
| T17/42 | | MW232520 | |

Table A.2 Primers used for homologous yeast recombination.

Primers were designed as detailed in the plasmid schematic in Figure 5.1. All primers amplified 30 bp of the required fragment. The promoter genes were designed to amplify an additional 30 bp to overlap with the *hph* cassette (forwards) and the eGFP cassette (reverse). The *hph* cassette overlapped with the LB (forwards) and the eGFP cassette overlapped with the RB (reverse).

| Amplified region | Name | Sequence | Primer length (bp) |
|-----------------------|----------|---|--------------------|
| <i>PMK1a</i> Promoter | PMK1a-F | CGTGAGCACTTACAGGACATCTACCCAATTAGGACCCTCTTGTC AAGCCCTGGGGAAGTC | 60 |
| <i>PMK1a</i> Promoter | PMK1a-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATGGTGGCCACCAAACGAAGTCTTGGGAGGGT | 60 |
| <i>PMK1b</i> Promoter | PMK1b-F | CGTGAGCACTTACAGGACATCTACCCAATTATGGACCTCCGTTTCACCTATCGTTTCGAGG | 60 |
| <i>PMK1b</i> Promoter | PMK1b-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATTAGAGAAAAGAAAGGGAGCGGTGGTGGTAA | 60 |
| <i>PMK1c</i> Promoter | PMK1c-F | CGTGAGCACTTACAGGACATCTACCCAATTATACTTGCTGCTTCGTCCTCGTAATTCTC | 60 |
| <i>PMK1c</i> Promoter | PMK1c-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATGAAGTGGGCCATTTTCTCTTCCAGCCCATT | 60 |
| <i>SSP47</i> Promoter | SSP47-F | CGTGAGCACTTACAGGACATCTACCCAATTTTAGAAAAATGTATTGTATGTTCTGTAGCC | 60 |
| <i>SSP47</i> Promoter | SSP47-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATTGTAGCTGTTGCAACGGCAGGGTGAGAAA | 60 |
| <i>SLP1</i> Promoter | Slp-F | CGTGAGCACTTACAGGACATCTACCCAATTCCTCGGGCCTGGGGTCTTCCCCTTCCAGA | 60 |
| <i>SLP</i> Promoter | Slp-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATGGCGCACGATGAAAAGAGCAGTCACACTTG | 60 |
| <i>CP</i> Promoter | CP-F | CGTGAGCACTTACAGGACATCTACCCAATTCAAGCTTATAGCCGACACTAAGTTTATCTA | 60 |
| <i>CP</i> Promoter | CP-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATTATTGTAGTATATCGGCGGTTTATCAACGA | 60 |
| <i>RAS2a</i> Promoter | RAS2a-F | CGTGAGCACTTACAGGACATCTACCCAATTCAATGTTGATCAACATACATAGCCTTACTA | 60 |
| <i>RAS2a</i> Promoter | RAS2a-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATTCTGCCTTTCAAAGTTTGACTGGGATCTAG | 60 |
| <i>RAS2b</i> Promoter | RAS2b-F | CGTGAGCACTTACAGGACATCTACCCAATTGATCACGCTTTGAGGCTTGACACGACGTGA | 60 |
| <i>RAS2b</i> Promoter | RAS2b-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATTGACGCGCCCTCGACGCCCTGTGCGTCGC | 60 |
| <i>RAS2c</i> Promoter | RAS2c-F | CGTGAGCACTTACAGGACATCTACCCAATTGATGGCCACGTCGAGATATACCACCAACGA | 60 |
| <i>RAS2c</i> Promoter | RAS2c-R | GGTCGGGCTGTGTGGTGTACTGACCGGCATGCTGTAGGTGGGGCAAGATATGTCACAGTG | 60 |
| <i>hph</i> cassette | LB-hph-F | ATGTTGGGCCCCGGCGCGCCGAATTCGCGGACTGGATTTTGGTTTTAGGAATTAGAAATT | 60 |
| <i>hph</i> cassette | hph-R | AATTGGGTAGATGTCCTGTAAGTGCTCACG | 30 |
| eGFP cassette | GFP-F | ATGCCGGTCAGTACACCACAGCCCCGACC | 30 |
| eGFP cassette | GFP-RB-R | TCTTAAAGCTTGGCTGCAGGTCGACGGATCGCGGCCCGCCAGTGTGATGGATATCTGCAGA | 60 |

Table A.3: Intron spanning primers to confirm cDNA samples have no gDNA contamination.

| Gene | Gene ID | Primer | Size with intron | Size without intron | Sequence |
|---------|---------|------------------|------------------|---------------------|-----------------------|
| β-Actin | g18706 | aACT_intron_F | 193 | 141 | CCTGAAGCCTTGTTCCAACC |
| | | RC_aACT_intron_R | | | TGTTGTGCCACCAGAGAGAA |
| Tef1 | g19873 | eEF1_intron_F | 167 | 115 | TAAAGTCTGGCGATGCTTGC |
| | | RC_eEF1_intron_R | | | GTCTGCCTCATGTCACGAAC |
| α-Tub | g19743 | bTub_intron_F | 220 | 172 | TGCTCTACCGTGGTGATGTT |
| | | RC_bTub_intron_R | | | CTCCGAGTCGTCCTTTGCAAG |
| PMK1 | g16430 | PMK1_F_Intron | 193 | 140 | GGAACGCCATCTGAAGACAC |
| | | RC_PMK1_R_Intron | | | GAGAGCAAGTCAATGGCGAG |
| PMK2 | g16430 | PMK2_F_Intron | 250 | 197 | CATAGCCATGCATCGTCGAC |
| | | RC_PMK2_R_Intron | | | CGCAGACAGAACATGGAGTG |
| RAS1 | g265 | RAS1_F_Intron | 209 | 159 | ACTTGTCGTTGTTGGTGGTG |
| | | RC_RAS1_R_Intron | | | CGGTAAGAATCTACGCGCTG |
| RAS3 | g15709 | RAS3_F_Intron | 240 | 185 | TCTACGACACAGCTGGACAG |
| | | RC_RAS3_R_Intron | | | TGTACCCACTATCACGCAGG |
| Slp1 | g18812 | Slp1_F_Intron | 221 | 167 | CGCGCCTGAAGATAAACCAA |
| | | RC_Slp1_R_Intron | | | TCATCCCGCATTTGAAGCAG |
| CP | g14461 | CP_F | No introns | 183 | TGAAGTTCTTCTCCGCCCTC |
| | | CP_R | | | ATGGGAAGTTGGGAAGGGAG |
| SSP47 | g10712 | SSP47_F | No introns | 118 | TTCCCTCCCTGCGATATGTC |
| | | SSP47_R | | | CCTCCACAGCTATCCCAGAC |

Table A.4: Primer design of housekeeping and candidate virulence genes for qPCR.

| Gene | Gene type | Primer F | Primer R | Amplicon size (bp) |
|---|------------------|------------------------|----------------------|---------------------------|
| <i>βAct</i> (β- actin) | Housekeeping | GCTCTTCCTCACGCTATCCT | GGGTATCCTCGCTCCATCAA | 89 |
| eEF1α (translation elongation factor) | Housekeeping | TAAAGTCTGGCGATGCTTGC | GTCTGCCTCATGTACGAAC | 115 |
| <i>Tub-α</i> (α-tubulin) | Housekeeping | AGGACGCTGCAAACAACACTAC | CCATAGTCGGTTGAAAGGCG | 196 |
| <i>Ubc</i> (Ubiquitin-conjugating enzyme) | Housekeeping | TGACCCGAACCCTAATAGCC | GATGTACTCTCCTGCCCTC | 152 |
| <i>CP</i> | Candidate | TGAAGTTCTTCTCCGCCCTC | ATGGGAAGTTGGGAAGGGAG | 183 |
| <i>SSP47</i> | Candidate | TTCCCTCCCTGCGATATGTC | CCTCCACAGCTATCCCAGAC | 118 |
| <i>PMK1a</i> | Candidate | CACCGCGATCTTAAACCTGG | GATCTCTGGAGCTCGGTACC | 174 |
| <i>PMK1b</i> | Candidate | CATAGCCATGCATCGTCGAC | CGCAGACAGAACATGGAGTG | 197 |
| <i>PMK1c</i> | Candidate | CTTCAAGGAATACACCCGCG | CGATCTGCTGGACGAAATGG | 178 |
| <i>RAS2a</i> | Candidate | AACAGTATATGCGAACGGGC | TTCATGCCGACTTGTCGTTC | 181 |
| <i>RAS2b</i> | Candidate | CCTTCTTTGCGCCTAGAACG | TCAGACACATGCCAGTTTCG | 188 |
| <i>RAS2c</i> | Candidate | TCTACGACACAGCTGGACAG | TGTACCCACTATCACGCAGG | 185 |
| <i>Slp1</i> | Candidate | CAGAACTGCGTACGATGGG | CTCCCCATCACTCCTCCATG | 192 |

