Fungi and their potential as biological control agents of Beech Bark Disease

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DECLARATION OF AUTHORSHIP

I, Sarah Elizabeth Thomas, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

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Date: 4th May 2014

ABSTRACT

Beech bark disease (BBD) is an invasive insect and pathogen disease complex that is currently devastating American beech (*Fagus grandifolia*) in North America. The disease complex consists of the sap-sucking scale insect, *Cryptococcus fagisuga* and sequential attack by Neonectria fungi (principally *Neonectria faginata*). The scale insect is not native to North America and is thought to have been introduced there on seedlings of *F. sylvatica* from Europe. Conventional control strategies are of limited efficacy in forestry systems and removal of heavily infested trees is the only successful method to reduce the spread of the disease. However, an alternative strategy could be the use of biological control, using fungi. Fungal endophytes and/or entomopathogenic fungi (EPF) could have potential for both the insect and fungal components of this highly invasive disease.

Over 600 endophytes were isolated from healthy stems of *F. sylvatica* and 13 EPF were isolated from *C. fagisuga* cadavers in its centre of origin. A selection of these isolates was screened *in vitro* for their suitability as biological control agents. Two *Beauveria* and two *Lecanicillium* isolates were assessed for their suitability as biological control agents for *C. fagisuga* and nine *Trichoderma* isolates were screened for their antagonistic ability against *Neonectria* spp. Colonisation of beech saplings with *Beauveria*, *Lecanicillium* and *Trichoderma* isolates was attempted using three inoculation techniques.

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GLOSSARY

Ancient tree   Ancient trees are those that are either of interest biologically, aesthetically or culturally because of their age, in the ancient stage of their life or are old relative to others of the same species see: http://frontpage.woodland-trust.org.uk/ancient-tree-forum/atfheritage/faq.htm

Invasive species   A species that is:
1) non-native (or alien) to the ecosystem under consideration and
2) whose introduction causes or is likely to cause economic or environmental harm or harm to human health.
http://www.invasivespeciesinfo.gov/whatis.shtml

Koch's postulates   Koch's postulates are four criteria designed to establish a causal relationship between a causative microbe and a disease.
http://en.wikipedia.org/wiki/Koch's_postulates

1. GENERAL INTRODUCTION

1.1 INVASIVE SPECIES

Non-indigenous invasive species including plants, mammals, birds, reptiles and amphibians, fish, arthropods, molluscs and microbes are a global threat to biodiversity. In the USA it has been estimated that 50,000 invasive species have been introduced and account for losses costing $120 billion per year (Pimentel *et al.*, 2005) and they cause significant environmental, ecological and economic damage in agriculture and forestry (Pimentel *et al.*, 2001). Insect pests and pathogens pose the greatest threat to forest ecosystems (Lovett *et al.*, 2006) and some examples of invasive insect pests include: gypsy moth (*Lymantria dispar dispar)*, balsam woolly adelgid *(Adelges piceae)*, hemlock woolly adelgid (*Adelges tsugae)*, emerald ash borer *(Agrilus planipennis)* and the Asian long-horned beetle *(Anoplophora glabripennis)*. Examples of invasive pathogens include Dutch elm disease (*Ophiostoma ulmi*), chestnut blight (*Cryphonectria parasitica*), dogwood anthracnose *(Discula destructiva),* sudden oak death (*Phytophthora ramorum*) and beech bark disease (Lovett *et al.*, 2006).

This thesis will focus on beech bark disease (BBD) that affects both American Beech (*Fagus grandifolia*) and European Beech (*F. sylvatica*) in North America and will investigate the potential of using endophytic fungi and entomopathogenic fungi (EPF) from Europe as biological control agents for this highly invasive disease complex.

1.2 THE GENUS *FAGUS* (FAGACEAE) BEECH

The genus *Fagus*, was named by Linnaeus in 1735. All members of the genus are tall monoecious (reproductive organs or flowers of both sexes), deciduous trees belonging to the family Fagaceae. According to Shen (1992), a total of thirteen *Fagus* species exist worldwide: *F. bijiensis, F. brevipetiolata, F. chienii, F. crenata* (Japanese beech), *F. engleriana* (Chinese beech), *F. japonica* (Japanese blue beech), *F. grandifolia* (American Beech), *F. hayatae* (Taiwan beech), *F. longipetilata* (South Chinese beech), *F. lucida* (Shining beech), *F. okamotoi, F. sylvatica* (European or Common beech) and *F. tientatiensis* and are native to temperate Europe, Asia and North America (see Figure 1.1). Other subspecies and many ornamental varieties also exist. The origin of the genus *Fagus* probably lays in east Asia as this has the greatest diversity of species and each has a restricted geographical range. In western Eurasia and North America, only one species exists in each; *F. sylvatica* and *F. grandifolia* respectively. Peters (1997) provides an excellent review of beech forests worldwide, including in-depth chapters on topics such as tree growth, climate and beech forest structure.

1.2.1 *Fagus sylvatica* (L.), European beech

F. sylvatica has a wide geographical range, from northern Iran to north western Europe and exists as two subspecies: *F. sylvatica* subsp. *sylvatica* in the western part of its range and as *F. sylvatica* subsp. *orientalis* in the east. Often, these subspecies are referred to as separate species: *F. sylvatica* and *F. orientalis* respectively. *F. sylvatica* (subsp. *sylvatica*) is native to parts of the UK; a boundary from King's Lynn to Swansea and Weymouth (see Figure 1:2). Outside of this range, beech is believed to have been planted (Rackham, 2003). *F. orientalis* is found from northern Greece and Bulgaria to northern Iran, including most of the Black Sea drainage basin and the Caucasus Mountains (Gwiazdowski *et al.*, 2006).
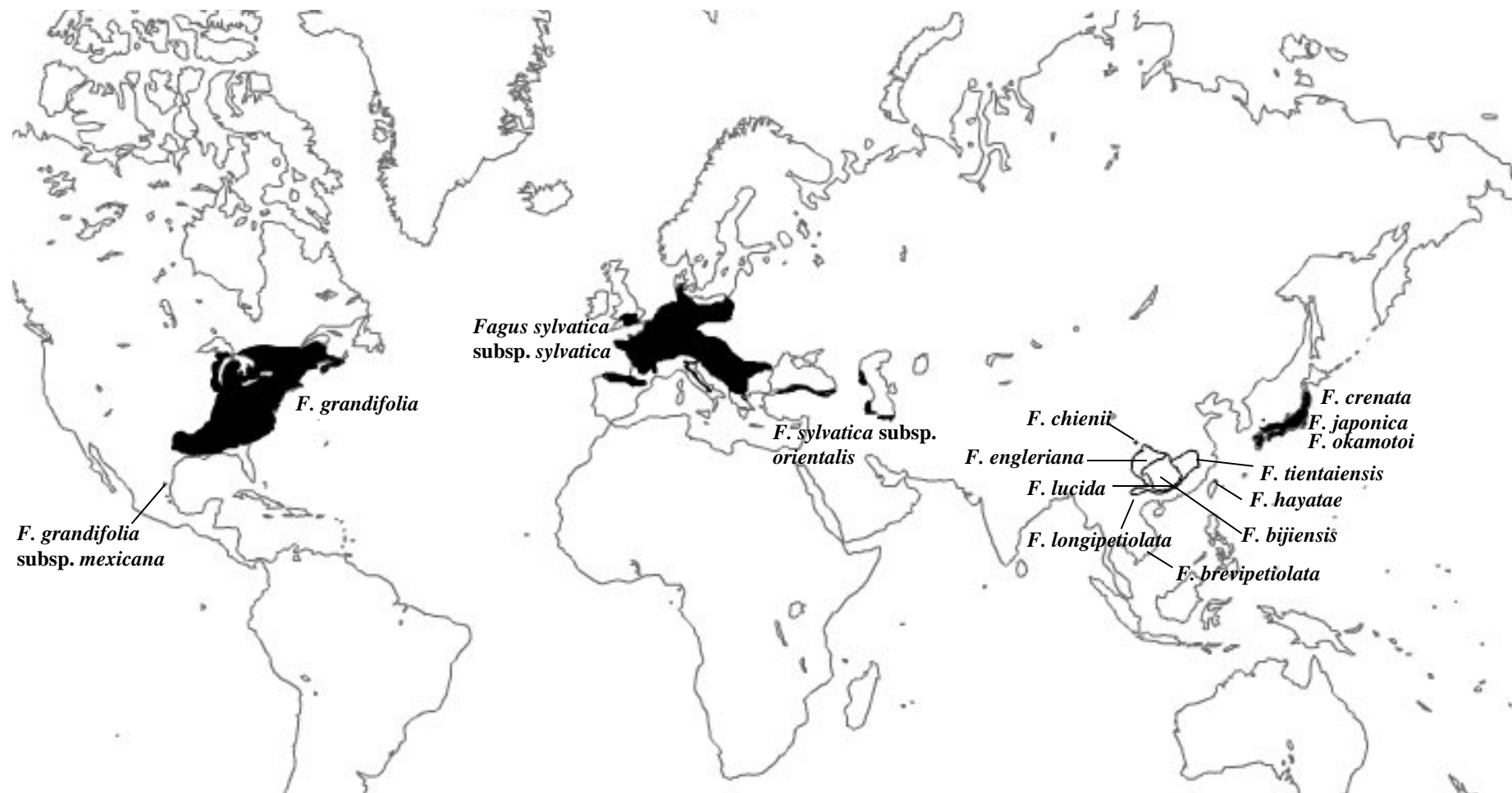
Figure 1:1: Worldwide distribution of *Fagus* species, re-drawn from Peters (1997).
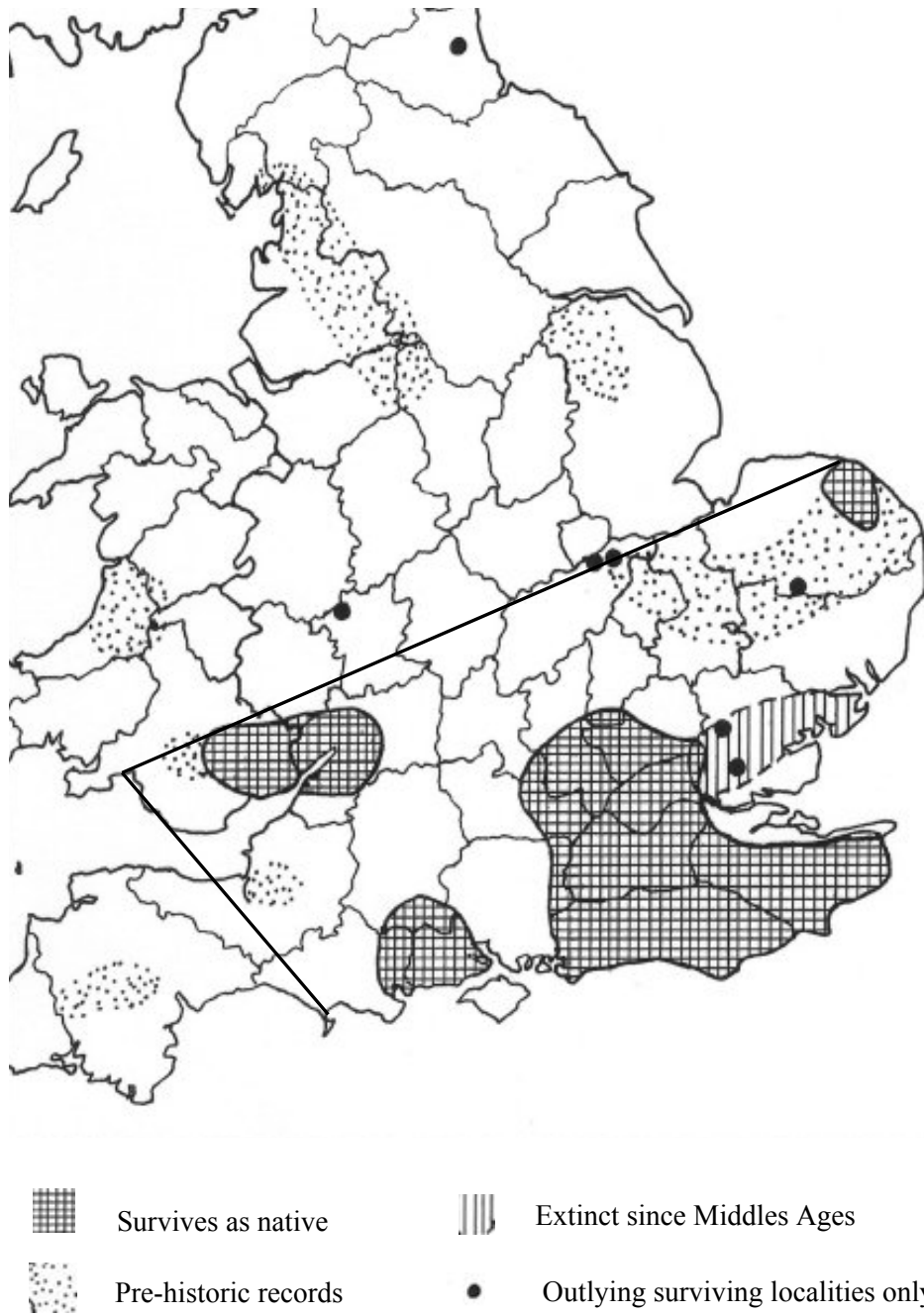
Figure 1:2: Native distribution of *Fagus sylvatica* (subsp. *sylvatica*) in the UK (Rackham, 2003) within a boundary from King's Lynn to Swansea and Weymouth.

European beech is an important forest species, which can be planted as an ornamental, amenity tree and is also very popular as garden hedging and as Bonsai specimens. It is a deciduous tree with thin, smooth, silver-grey coloured bark. Leaves are green and turn orangey brown in autumn. Flowers are unisexual and two beechnuts are formed within a four-valved prickly case. Many horticultural varieties of *F. sylvatica*, such as *F. sylvatica* var. *purpurea* (copper beech) have been developed. Its timber has a wide variety of uses including furniture and flooring. *F. sylvatica* is one of the UK's largest trees and can reach a height of 48m in its 200-year life span. Many beeches in the UK are regarded as ancient trees and are protected by the Woodland Trust as part of an initiative called the Ancient Tree Forum (http://www.ancient-tree-forum.org.uk/ancient-tree-forum).

1.2.2 *F. grandifolia* (Ehrhart), American beech

F. grandifolia is native to eastern North America. In the USA it occurs from northern Michigan to eastern Texas in the south and east toward the Atlantic Ocean. In Canada, it occurs from Nova Scotia, New Brunswick, southern Quebec and southeastern Ontario. In northeastern Mexico, it exists as the subspecies *F. grandifolia* subsp. *mexicana* (Peters, 1997). American beech is a hardwood, used as a source of timber for products such as furniture and flooring. It is also an environmentally important species in the USA as beechnuts (or "mast") offer a food source for wildlife such as black bear, grey squirrel, deer and turkey.

1.3 PESTS AND DISEASES OF *FAGUS* SPP.

Because of its thin bark, *Fagus* spp. are susceptible to a vast range of insects, fungi, bacteria, nematodes and mammals (CABI Forestry Compendium, http://www.cabi.org/fc). In the USA, more than 70 decay fungi have been recorded on

beech (Hepting, 1971). The most serious of these is beech bark disease (BBD), which is having a devastating effect on American beech. In the UK, BBD is not such a serious problem (Parker, 1974) but more recent studies have reported that *F. sylvatica* is susceptible to *Phytophthora ramorum* and *P. kernoviae,* the causal agents of sudden oak death (Brasier *et al.*, 2005).

1.4 BEECH BARK DISEASE (BBD)

BBD can affect beech (*Fagus* spp.) in both Europe and North America. It is an insect-fungus complex that results from the combined action of the felted beech scale (*Cryptococcus fagisuga* Lind.) and sequential attack by fungi belonging to the family Nectriaceae, (*Neonectria* spp.) The sap-sucking insect wounds the tree and provides an entry route for the fungus to invade and colonise the inner bark tissues. Cankers form and girdle the main stem of the tree. Many trees are broken by the wind at approximately three to five metres high (Parker, 1974), a condition known as "beech snap". Invasion by decay fungi and wood-boring insects follows and whole tree mortality usually occurs within two to five years of initial infection. The development of BBD in the forest occurs in three stages: the "advancing front", the "killing front" and the "aftermath forest". The "advancing front" describes recently invaded areas by the scale insect without indication of the *Neonectria* fungus. The "killing front" describes areas where high beech scale populations and abundant *Neonectria* infection have resulted in heavy tree mortality. The "aftermath forest" describes stands where beech mortality has occurred and remaining trees typically demonstrate lower infestation levels of BBD (Shigo, 1972).

1.4.1 *Cryptococcus fagisuga* (Lindinger), Beech scale

The felted beech scale, *Cryptococcus fagisuga* (Lindinger Hem.: Eriococcidae) is a tiny (0.5-1mm), soft-bodied, yellow insect with three stages in its life cycle: egg, crawler (or nymph, with two instars) and adult. The adults are immobile and wingless and all are female. The insect feeds on the beech sap by inserting its relatively long (2mm) stylet into the inner bark (live tissue) of the tree. The adults lay up to fifty eggs (0.3mm long), produced in strings of four to eight, which hatch approximately 25 days later to form first-instar mobile crawlers. Crawlers have well-developed legs and antennae and facilitate dispersal of the insect on the tree. Crawlers establish and overwinter and then moult to form the second instar. A second moult generates the adults, which die soon after laying their eggs. There is one generation per year. The insect has several minute glands that produce a white, woolly secretion (see Figure 1:3) that surrounds it and provides protection to the insect on the cracked surface of the bark. Often, this can cover the whole trunk of the tree and is the first indication of the presence of the insect, see Figures 1:4a and 1:4b. The insects are also dispersed from tree to tree by wind, birds and other animals including humans (Wainhouse and Gate, 1988). Scale attack alone is not fatal but heavy infestations weaken trees and facilitate entry for *Neonectria* spp. Severe damage occurs only after the subsequent invasion by *Neonectria* fungi. A phylogeographic study undertaken by Gwiazdowski *et al.* (2006) suggests that *F. sylvatica* subsp. *orientalis* (oriental beech) is the native host of *C. fagisuga* and recommend that natural enemies of the insect would be best sought in northeastern Greece, the Black Sea drainage basin, the Caucasus Mountains and northern Iran.

1mm

Figure 1:3: Beech scale "crawler" in amongst woolly secretion.

1.4.2 *Neonectria* species

The BBD fungus is a member of the Ascomycetes, order Hypocreales, family Nectriaceae, genus *Neonectria* Wollenw, Ann Mycol. 15:52. (1917). Fruiting bodies or perithecia (sexual stage or teleomorph) of the Hypocreales are brightly coloured, usually red, yellow, pink or orange. Members of the Nectriaceae have conidial states (asexual stage or anamorph) from genera such as *Cylindrocarpon, Fusarium, Lecanicillium* and *Cephalosporium.*

Neonectria is a common fungal genus occurring on stems and branches of many woody hosts. *Neonectria* spp. are able to infect the bark, cambium and sapwood and cause cankers to form beneath the bark surface. Such cankers often occur at branch bases,

wounds and scars and appear as areas of dead callus tissue that enlarge with time. In general, Nectriaceous fungi are saprophytes but can cause diseases that are of economic importance. They do not generally kill their hosts but reduce tree vigour, and cankers that girdle the main stem of the tree leaving it more vulnerable to wind breakage. Infected trees are also rendered more susceptible to wood-rotting fungi and the quality of lumber is usually affected. The first symptom of infection by *Neonectria* may be the presence of "tarry spots" that ooze red-brown exudates from the trunk of the tree (see Figure 1:4c). The red, lemon shaped ascomata are visible through cracks that develop in the surface of the bark (see Figure 1:5a) and contain asci with hyaline, septate ascospores (see Figure 1:5b). Known only on *Fagus* spp. *Neonectria coccinea* and *N. faginata* are the causal agents of BBD in Europe and North America respectively.

1.4.2.1 Neonectria coccinea *(Pers.:Fr.) Rossman & Samuels, in Rossman, Samuels, Rogerson & Lowen, Stud. Mycol. 42: 158. 1999.*

On European beech (*F. sylvatica*) the fungal component of the disease complex is *Neonectria coccinea.* It was first recognised in Europe by Hartig in 1878 and was originally named *Nectria ditissima* Tul. Other synonyms for the fungus include: *Sphaeria coccinea* Pers.:Fr., Persoon, Icon. & Descript. Fung. 2: 47, 1800, Fries, *Syst. Mycol.* 2: 412, 1823, *Nectria coccinea* (Pers.:Fr.) Fr., Summa Veg. Scand. 2: 388.1849. *Sphaerostilbe caespitose* Fuckel, Jahrb. Nassauischen Vereins Naturk. 27-28:33. 1873. and *Neonectria caespitose* (Fuckel) Wollenw., Angew. Bot. 8: 192. 1926.
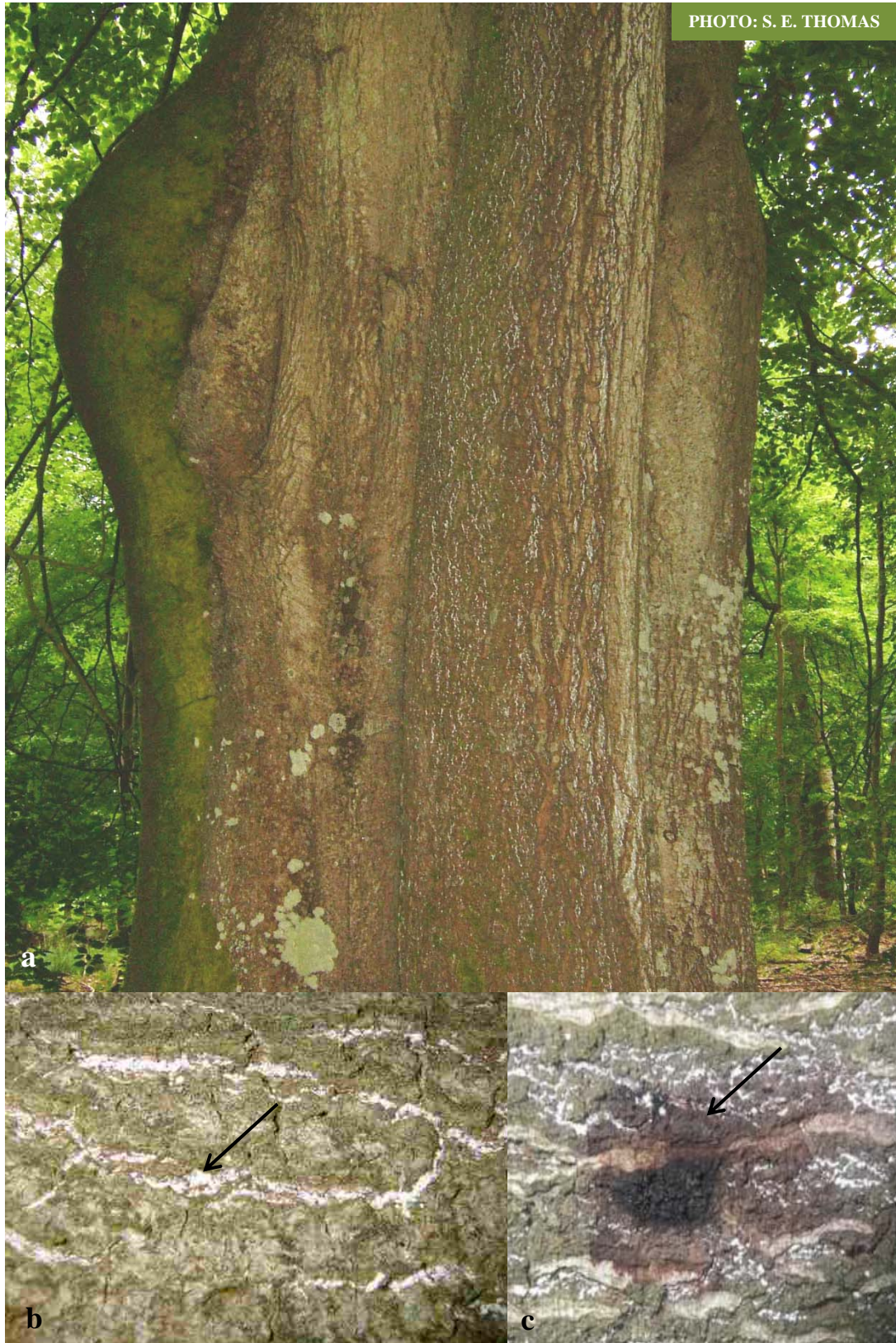
Figure 1:4: Beech bark disease; a) beech tree trunk covered in beech scale, *Cryptococcus fagisuga*; **b) close-up of the waxy covering of the scale and c) canker or "tar spot" caused by** *Neonectria*.

Perithecia typically form in groups of 5–35 and are typically 250–350μm in diameter. They are oval to sub-globose with a pointed (often darker) ostiole papilla, appearing translucent when freshly developed but become rougher and darker with age. The asci are 75–100 × 7–10μm, cylindrical with a rounded apex and contain eight ascospores. Ascospores are discharged in wet weather conditions and are disseminated by air. The spores penetrate their host via bark fissures or wounds caused by scale insects.

The asexual state of *N. coccinea* is *Cylindrocarpon candidum* (Link) Wollenw., Fus. Autogr. Del., ed. 2, no. 655. 1926. Cultures produce a floccose to fibrous yellowish-white to greyish-brown mycelium. Microconidia form after 2–3 days, initially from lateral phialides but later from well-developed conidiophores, 4–9 × 1.5–3μm, cylindrical with rounded ends, occasionally slightly curved and may develop a central septum. Macroconidia develop later from larger phialides than those producing the microconidia, Macroconidia are hyaline, cylindrical and narrowing slightly towards the apex and base, when mature with 3–7 septa, 46–80 × 6–7μm. Chlamydospores are sparsely formed in older cultures often from the cells of the macroconidia (Booth, 1977a).

1.4.2.2 Neonectria faginata *(M.L. Lohman, A.M.J. Watson & Ayres) Castl. & Rossman, in Castlebury, Rossman & Hyten, Can. J. Bot. 84: 1425. 2006.*

In North America, the fungal component of BBD is *N. faginata*. Ehrlich (1934) was the first to document this and was first described as a variety of *Nectria coccinea*: *Nectria coccinea* var. *faginata*. The fungus was subsequently renamed as *Neonectria coccinea* var. *faginata* (Rossman *et al.*, 1999) but more recent taxonomic studies by Castlebury *et al.* (2006) proposed five distinct sister species to *N. coccinea*, including

N. faginata, based on differences to *N. coccinea* in host specificity and geography. However, due to the limited number of samples available, no significant morphological differences were found and the name-change was not fully accepted. Subsequent re-evaluation of newly obtained *Neonectria* specimens by Hirooka *et al.* (2013), enabled further taxonomic studies based on morphology, phylogeny, host specificity and geography to be carried out and *N. faginata* was conclusively delimited from *N. coccinea.*

The asexual state of *N. faginata* is *Cylindrocarpon faginatum,* (Booth, 1977b) see Figure 1:6. This appears as white spore cushions that are very similar in appearance to the woolly secretions of the scale insect. Perithecia are 200–300μm in diameter and are formed in groups of 7–15 on a reddish-orange erumpent stroma. In artificial culture, aerial mycelium is white and a yellow pigmentation develops on the surface of the agar, turning reddish-brown or chocolate-brown. Chlamydospores have not been observed for *N. faginata* (Booth, 1977b).

Detailed mycological descriptions of both *N. coccinea* and *N. faginata* can be found in Booth (1977a; 1977b), Rossman *et al.* (1999), Castlebury *et al.* (2006) and Hirooka *et al.* (2013).

30 micron

Figure 1:5: *Neonectria coccinea*; **a) red ascomata bursting through the surface of beech bark (*Fagus sylvatica*) and b) asci containing ascospores.**
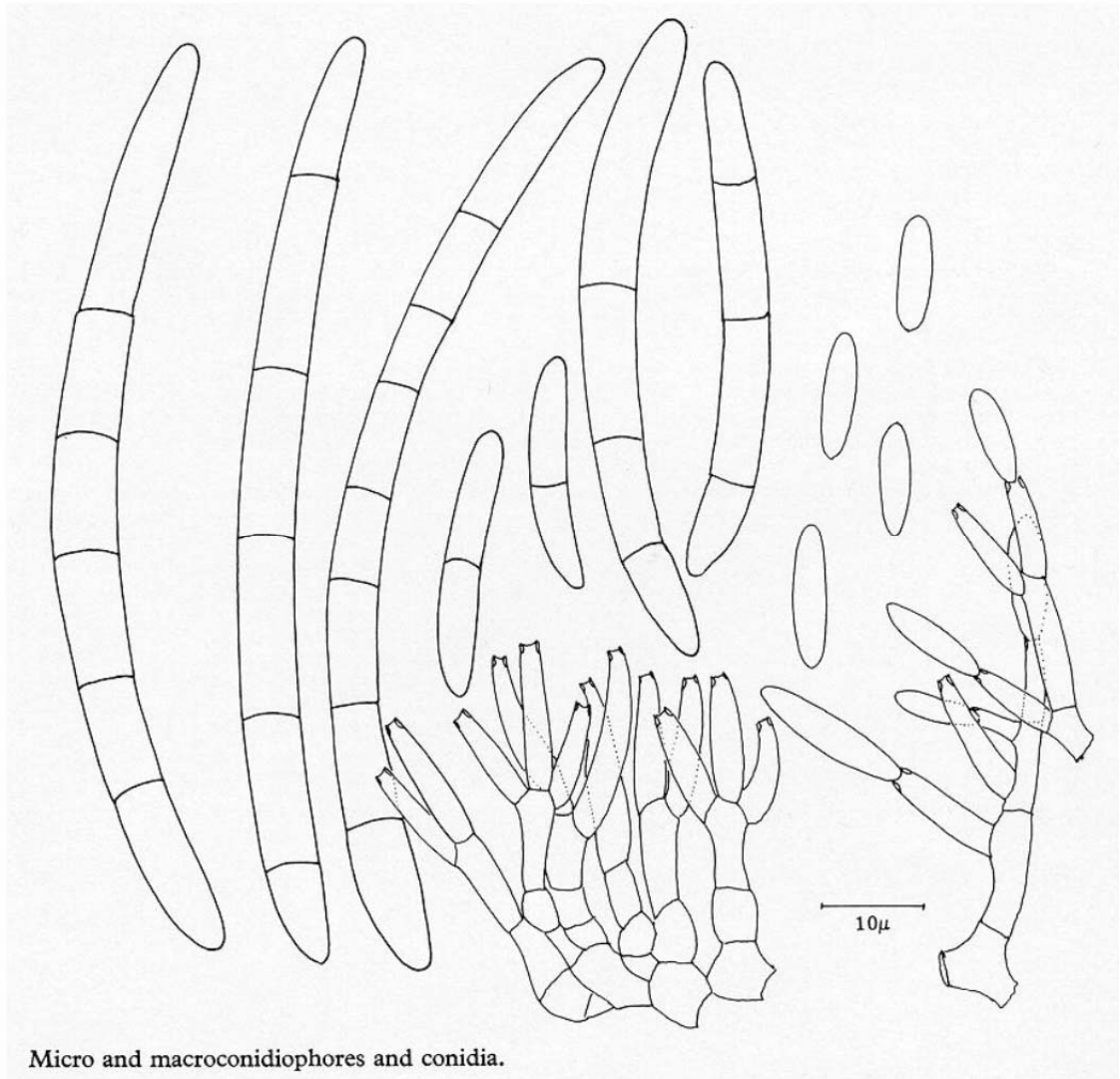
Micro and macroconidiophores and conidia.

Figure 1:6: *Cylindrocarpon faginata,* anamorphic state of *Neonectria faginata* **(Booth, 1977b).**

1.4.3 Abiotic factors affecting the development of BBD

Tree characteristics such as age, trunk (bole) diameter, bark morphology and chemical composition can also play a role in the development of the disease complex. *C. fagisuga* tends to first colonise older trees that have a rougher bark texture (Houston *et al.*, 1979; Lonsdale, 1983) and a larger bole diameter. Younger, smaller trees are generally smoother and are less favourable to the scale insects. However, less than 1% of beech trees harbour resistance to BBD (Houston and O'Brien, 1983). Latty *et al.* (2003) demonstrated that beech trees with higher nitrogen concentrations in the bark have higher beech scale populations and may also show an increased severity of BBD symptoms. The abundance of scales on a given tree can differ with the cardinal aspect of the tree bole. It is believed that greater temperature fluctuations and increased levels of sunlight place the scale insects on the south and west-facing sides of the tree bole under greater environmental pressure than those on the north and east-facing sides. Similarly, those trees situated at the forest edge will have fewer scales because of the increased sunlight (Ehrlich, 1934).

1.4.4 Incidence and distribution of BBD

In Europe, BBD occurs naturally throughout the geographical range of its principal host, *F. sylvatica* but outbreaks are restricted and relatively limited damage occurs (Wainhouse and Gate, 1988). However, in North America, where both the insect and fungus are exotic and invasive pests, the susceptibility of the native American beech (*F. grandifolia*) is far greater and the disease complex is having a severe and dramatic effect on the species. The scale insect was introduced into North America from Europe accidentally in the late 1890s on seedlings of *F. sylvatica.* It was first recorded in a botanical garden in Halifax, Nova Scotia (Houston, 1994) and by 1914 the disease was well established in stands of *F. grandifolia.* By the 1930s, the disease was present

throughout Nova Scotia and was spreading into the United States. It spread north into Quebec and south and west into New England, New York, New Jersey and Pennsylvania. By 1980, the disease was present in West Virginia and was recorded in North Carolina and Tennessee in 1993. More recent infestations have been recorded in Ohio, Ontario and Michigan (McCullough *et al.*, 2005). Figure 1:7 illustrates the distribution of BBD in North America in 2010 (US Forest Service website http://nrs.fs.fed.us/tools/afpe/maps/. BBD has the potential to continue to spread throughout the natural range of *F. grandifolia* (Koch, 2010).

1.4.5 Impact of BBD on forest composition

BBD has the potential to alter the species composition of the forests it occupies (Twery and Patterson, 1984; Griffin *et al.*, 2003). BBD can significantly reduce beech nut production on large trees (Costello, 1992) as it is trees with a diameter greater than 25cm that produce the greatest amount of seed and are also the most affected by the disease (McCullough *et al.*, 2005). Sage (1996) estimated that seed production of *F. grandifolia* has declined by 37% since the introduction of BBD. In North America, beech mast is an important autumn food source for a large number of animal species (Martin *et al.*, 1951; DeGraaf and Yamasaki, 2001). In the northern forests dominated by spruce-hardwoods, beech mast is probably the most important autumn food source for black bears (*Ursus americanus* Pallus) (Hugie, 1982). A study undertaken by Rosemier and Storer (2010) indicated that BBD has little effect on small mammals but suggest that the impact is likely to increase in the future.
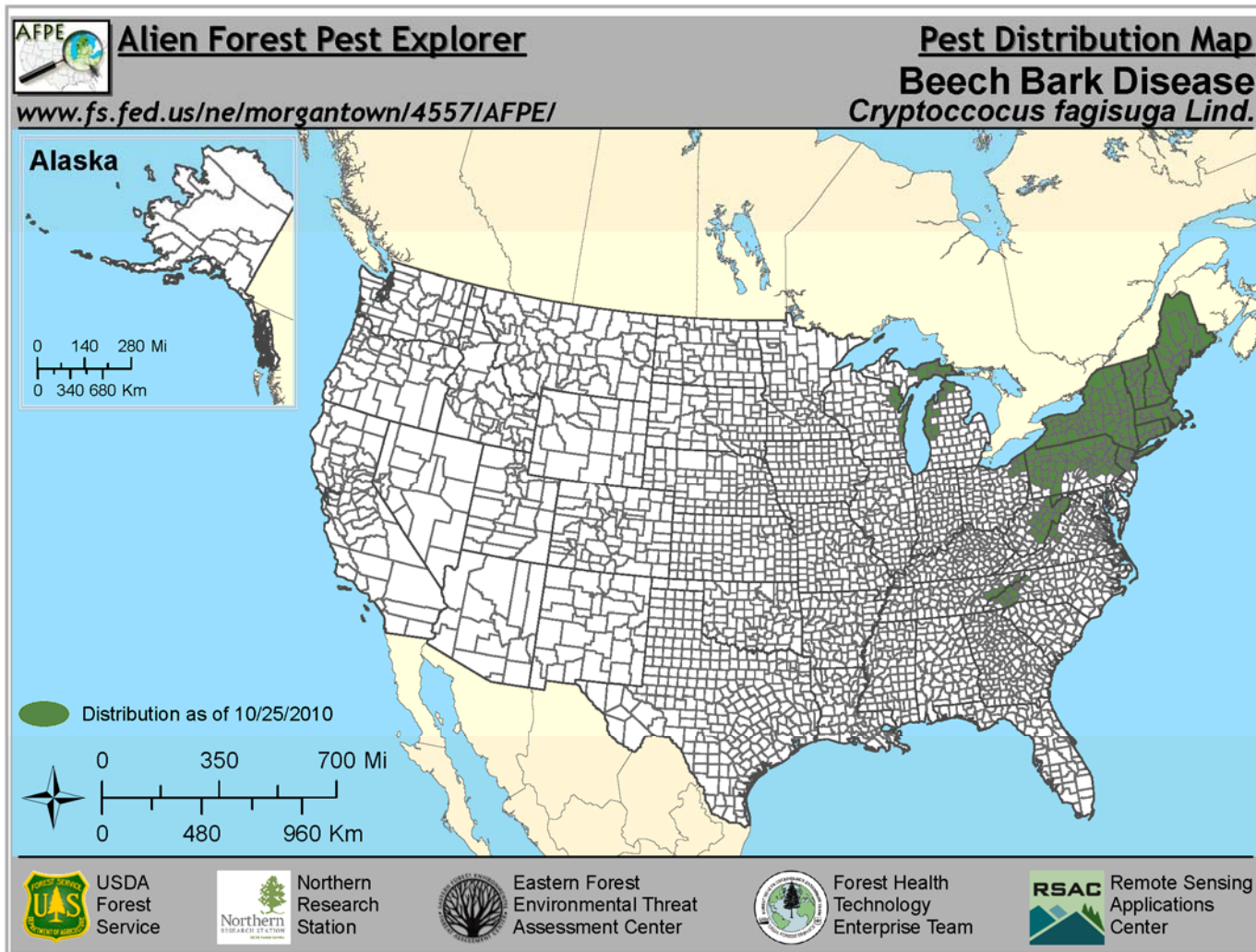
Figure 1:7: Distribution of beech bark disease in North America in 2010 (USDA Forest Service website http://nrs.fs.fed.us/tools/afpe/maps/).

1.5 CONTROL METHODS

Since *Neonectria* fungi can only infect after beech scale attack, direct control of the scale insect could be considered as the primary method of preventing the development of BBD. Contact insecticides can be used with only partial success as the insects are protected by the woolly secretion they produce. Yearly application of a tar-oil wash can have a limited effect on ornamental trees (McCullough *et al.*, 2005) but conventional control strategies are not practicable in forestry systems and removal of heavily infested trees is currently the only successful method to reduce the spread of the disease. It is estimated that between 1 and 5% of American beech trees possess natural resistance to the beech scale insect and so management practices to retain and increase these resistant trees is recommended (Koch, 2010).

1.5.1 Biological control

Biological control is a method that can be employed to control a pest organism as an alternative to conventional control methods. Many types of organisms can be used as biological control agents (BCAs): predators (Howell and Daugovish, 2013), parasitoids (Garcia and Ricalde, 2013), nematodes (Edgington and Gowen, 2010), viruses (Lacey *et al.*, 2001), bacteria (Trotel-Aziz *et al.*, 2008) and fungi (Ellison *et al.*, 2008). Within the biological control framework there are two main strategies: augmentative biological control which involves the mass production of a biological control agent or "biopesticide" which can be applied to a pest in a similar way to that of a chemical control agent, and classical biological control (CBC).

1.5.1.1 Biopesticides

A biological pesticide or biopesticide is a naturally occurring pest control product based on organisms such as microbials, nematodes, botanicals and their bioactive compounds such as antibiotics and insect pheromones (Copping and Menn, 2000). The use of microbial pesticides for control of insect pests, nematodes, weeds and plant diseases has been recognised for over 100 years and this is still an expanding area of research today (Faria and Wraight, 2007; Ravensberg, 2011). Many entomopathogenic fungi, nematophagous fungi, mycoherbicides and mycoparasites have been developed commercially (Butt *et al.*, 2001; Lacey *et al.*, 2001), however, due to a their high cost, lack of efficacy and inconsistent performance in the field, many have been unsuccessful or restricted to niche markets (Ravensberg, 2011; Glare *et al.*, 2012). Some of the most common mycoinsecticides and mycoacaricides consist of fungi belonging to the genera *Beauveria, Lecanicillium* and *Metarhizium* (Lacey *et al.*, 2001; Charnley and Collins, 2007). The development of a mycopesticide is a complex process which includes several stages: isolate selection, production and formulation, application, safety and registration (Charnley and Collins, 2007). Ravensburg (2011) recognised that the development of a new commercial product is extensive and involves many steps. For this purpose, he produced a roadmap to facilitate the successful development of microbial biopesticides. An example of a very successful product which was developed for biological control of locusts and grasshoppers in Africa, is Green Muscle (Becker Underwood, BASF, South Africa) based on *Metarhizium acridum* (Moore, 2008)*.* This product resulted from a large multi-donor programme called LUBILOSA (http://en.wikipedia.org/wiki/LUBILOSA), which took 40 scientists, 12 years and US$17 million to develop. The development of a biopesticide could be an option for either component of the BBD complex.

1.5.1.2 Classical biological control (CBC)

CBC of a pest or disease is a specific method that can be employed when the pest organism is an introduced or exotic species without its co-evolved natural enemies. It involves introduction of the pest's natural enemies, often from its centre of origin, in order to maintain the pest at a level at which it is no longer problematic. CBC has been successfully employed for the control of alien invasive weeds and exotic arthropods using both insect and fungal agents (Greathead, 1995; McFadyen, 1998). CBC is regarded as a viable option for BBD and strategies to target both the scale insect and fungal components of the complex have been considered (Houston and O'Brien, 1983; Kenis *et al.*, 2003). The twice-stabbed lady beetle, *Chilocorus stigma* Say has commonly been observed in association with the scale in North America but its inability to feed on all life stages of the beech scale has limited its success (Mayer and Allen, 1983). Another coccinellid predator (*Exochomus* sp.) and a fly (*Lestidiplosis* sp.) have been observed in association with the pest (Wainhouse and Gate, 1988). The entomopathogenic fungus *Lecanicillium muscarium* (Petch) Zare & W. Gams, comb. nov. (Zare and Gams, 2001) (formerly *Verticillium lecanii)* has also been recorded on the scale insect (Lonsdale, 1983) and preliminary *in vitro* experiments carried out by Laflamme *et al*. (2009) showed some control of beech scale with *L. muscarium*. The mycoparasite *Nematogonum ferrugineum* (Pers.) Hughes (*Gonatorrhodiella highlei* A. L. Smith) was found in association with the BBD fungus in North America in 1933 (Ayres, 1941) and in Great Britain in 1947 (Blyth, 1949). Although the fungus had been observed parasitizing large areas of *Neonectria* on infected bark, and had the ability to reduce spore production of the pathogen in laboratory tests (Shigo, 1964), it was considered ineffective in the field, as it could only colonise the *Neonectria* after severe outbreaks of the disease. Houston (1983) studied the effects of this mycoparasite on the pathogenicity of *N. faginata* and *N. galligena* in the USA and found that parasitized

isolates, inoculated into bark wounds made into the cambium, were less pathogenic than unparasitised isolates of *N. faginata*. Parasitised *N. faginata* also produced fewer and smaller cankers which produced fewer perithecia.

1.6 FUNGAL ENDOPHYTES

The term "fungal endophyte" was first used by de Bary (1866) to describe fungi that colonise the internal tissues of plants without causing disease symptoms. Different workers have proposed many variations of the term over the last 30 years. Wilson (1995) describes an endophyte as "a fungus causing apparently symptomless occupation of host plant tissues for all or part of its life-cycle". This could potentially include a gamut of fungi from latent pathogens to mutualistic symbionts. Until recently, fungal endophytes were a largely unexplored component of biodiversity.

Fungal endophytes are ubiquitous and are assumed to occur in all plants (Carroll, 1988), and with few exceptions, most endophytes belong to the *Ascomycota* and certain anamorphic genera always appear in endophyte assemblages, notably *Cryptocline, Cryptosporiopsis, Leptostroma, Phomopsis* and *Phyllosticta*. Basidiomycetes and Zygomycetes have been rarely isolated (Petrini, 1986). Exploration for fungal endophytes has led to the discovery of numerous novel taxa (Arnold *et al.*, 2000; Holmes *et al.*, 2004) and it is probable that many more undiscovered fungi exist as endophytes. Research has primarily focused on temperate plants and in particular the relationship between *Neotyphidium* spp. (*Acremonium* spp.) in the grasses (Clay, 1989). Less work has been undertaken on woody plants but several studies on trees have shown that multiple fungal endophyte species can colonise their host (Petrini and Fisher, 1990; Johnson and Whitney, 1992; Sahashi *et al.*, 2000).

In grasses, fungal endophytes are passed vertically from one generation to the next via seed. However, within trees it is believed that transmission of endophytes is horizontal via environmental inoculation and colonisation of the developing plant (Johnson and Whitney, 1992) and it is posited that in native ecosystems, endophytic assemblages differ to those in non-native ecosystems. Taylor *et al.* (1999) studied the endophytes associated with the palm *Trachycarpus fortunei* and showed that the host is relatively depauperate of fungal endophytes within its non-native range. Endophytes isolated from *Eucalyptus nitens* within its native Australian habitat differed to those isolated from the same species in England where it is non-native (Fisher and Petrini, 1990). Even within the native range of a host, endophyte assemblages can significantly differ and could be attributed to factors such as management practices (Sieber-Canavesi and Sieber, 1987). Non-biotic factors can also have an effect on the occurrence of endophytes. Altitude has been shown to affect the isolation frequency of *Quercus acuta* endophytes in Japan (Hashizume *et al.*, 2008) and a study carried out on *Fagus crenata* revealed seasonal differences in the isolation *Mycosphaerella buna* in leaves.

In trees, endophytes have most commonly been isolated from leaves, twigs or branches. Specificity of endophytes for particular host and tissue types has been studied (Carroll, 1995). Petrini and Fisher (1988) isolated different endophytes from each host in a *Pinus sylvestris* and *F. sylvatica* mixed stand. Sieber and Dorworth (1994) found differences in the endophyte assemblages in leaves and twigs of *Acer macrophyllum.* Even within bark tissues, the endophytes isolated from inner bark and outer bark were found to differ. Kowalski and Kehr (1992) describe endophytes colonising inner bark as "phellophytes" and suggest that these exhibit greater host specificity than those from outer bark tissue. Endophytes have been shown to protect plants from environmental stresses such as drought and to reduce the feeding of sap-sucking insects and

herbivorous invertebrates through production of toxic metabolites and to protect against fungal pathogens (Carroll, 1988). In recent years, there has been an extensive amount of research conducted with fungal and bacterial endophytes as biological control agents of both arthropods and plant pathogens (Clay, 1989; Chanway, 1996; Evans *et al.*, 2003; Campanile *et al.*, 2007; Backman and Sikora, 2008).

1.7 MYCOBIOTA OF *FAGUS SYLVATICA*

A number of mycobiota studies have been carried out on beech trees (Chapela and Boddy, 1988b; Chapela, 1989; Hendry *et al.*, 2002) and several fungal endophyte assemblages, (Cotter and Blanchard, 1982; Sieber and Hugentobler, 1987; Kowalski and Kehr, 1992; Toti *et al.*, 1993; Viret and Petrini, 1994; Danti *et al.*, 2002), similar to those recorded for other tree hosts have been identified (Petrini and Fisher, 1988; Sieber *et al.*, 1991; Sieber *et al.*, 1999; Vujanovic and Brisson, 2002). Danti *et al.* (2002) isolated endophytic fungi from twigs of *F. sylvatica* in Italy and compared the endophytic assemblages of trees with low and high crown transparency. *Discula umbrinella* (Berk. Et Br.) Morelet is a well-documented endophyte of *F. sylvatica*. Sieber and Hugentobler (1987) reported it to be the most abundant endophyte of *F. sylvatica* twigs and leaves whilst Viret *et al.* (1993) studied the infection of beech leaves by the endophyte using electron microscopy. As with other studies of temperate trees, these have focussed on twigs, detached branches or leaves of beech. Baum *et al.* (2003) isolated *Trichoderma* spp. from *F. sylvatica* in Germany but this was only after felled beech logs had been subjected to 8-24 weeks of drying. No endophyte studies of living *F. sylvatica* stem are known.

1.8 ENTOMOPATHOGENIC FUNGI (EPF)

These are fungi that can infect and consequently kill arthropods, including insects and mites. Entomopathogens are important natural regulators of forest pests (Augustyniuk-Kram and Kram, 2012) and are of interest as biological control agents. Of particular importance are genera belonging to the order Hypocreales of the Ascomycota: *Beauveria, Metarhizium, Nomuraea, Paecilomyces, Hirsutella* and *Cordyceps* and others belonging to the order Entomophthorales of the Zygomycota. Entomopathogenic fungi such as *Beauveria bassiana* have been isolated as endophytes from several host plants including coffee (*Coffea arabica*) (Santamaría and Bayman, 2005). Elliot *et al.* (2000) consider whether plants can "use" entomopathogens as "bodyguards" by already having a mutualistic association with such fungi.

The entomopathogenic fungus *Lecanicillium muscarium* was recorded from *C. fagisuga*, (Lonsdale, 1983) but was never fully investigated as a biological control agent. *L. muscarium* has been reported infecting mites, whiteflies, aphids and scale insects worldwide. Several workers have investigated the efficacy of various strains of *L. muscarium* (Cuthbertson *et al.*, 2005a) and compatibility studies with chemicals have even been undertaken (Cuthbertson *et al.*, 2005b). The fungus was developed commercially as a biopesticide by Koppert (The Netherlands) and is available as two products: Mycotal, for biological control of whitefly and thrips, and as Vertalec, for control of aphids in glasshouses**.**

Other entomopathogenic fungal genera such as *Fusarium, Cladosporium* and *Alternaria* are also commonly associated with scale insects (Shabana and Ragab, 1997; Xie *et al.*, 2012) and could potentially be exploited as biological control agents.

Entomopathogens isolated from the cadavers of *C. fagisuga* in Armenia (its purported centre of origin) could offer a control option for beech scale and therefore BBD.

1.9 OUTLINE AND OBJECTIVES OF THE PHD THESIS

The overall objective of this research was to isolate and identify fungal endophytes and entomopathogens from the centres of origin of *F. sylvatica* and *C. fagisuga* and to investigate the potential of them as biological control agents for both components of the BBD complex, namely *Cryptococcus fagisuga* and *Neonectria* spp. The outline of the thesis is outlined in Table 1:1.

Table 1:1: Outline of research undertaken in this thesis.

Chapter 2. Surveys for fungal stem endophytes of European beech (*Fagus sylvatica*) in the UK and Armenia as potential classical biological control agents of beech bark disease.   The objective of this study was to isolate and characterise fungal endophytes of *F. sylvatica* stems in native and non-native sites. The correct site selection and timing of surveys is of great importance when surveying for BCAs. This study hypothesised that endophyte assemblages of *F. sylvatica* would differ spatially and temporally.
Chapter 3. Entomopathogenic fungi associated with beech scale (*Cryptococcus fagisuga*) in Armenia and a preliminary assessment of their potential for biological control.   The objective of this study was to isolate and characterise EPF associated with *C. fagisuga* in its centre of origin in Armenia. The minute size of *C. fagisuga* and the fact that adults cannot be cultured *in vitro*, make it a very challenging experimental target. Attempts to develop a suitable laboratory bioassay to screen the isolated EPF using *C. fagisuga* eggs and crawlers were initially made with the commercial product Mycotal.
Chapter 4. *In vitro* screening of endophytic *Trichoderma* isolates for fungal antagonism of *Neonectria coccinea* and *N. faginata*.   The objective of this study was to assess the antagonistic ability of selected *Trichoderma* endophytes isolated in Chapter 2 against *Neonectria* spp. Three modes of action: competition, mycoparasitism and antibiosis can be employed by fungal antagonists. This study hypothesised that different *Trichoderma* endophytes would possibly exhibit different modes of action and possess variable levels of antagonism against *Neonectria* spp.
Chapter 5. Evaluation of application techniques for colonisation of *Fagus sylvatica* saplings using *Trichoderma*, *Beauveria* and *Lecanicillium*.   The objective of this study was to develop spray, wound and drench application methods using isolated *Trichoderma*, *Beauveria* and *Lecanicillium*, in order to colonise *F. sylvatica* saplings for protection against BBD. In order for a BCA to be effective, it must be able to colonise and persist within the plant. This study hypothesised that the three isolates and three techniques may differ in their effectiveness at colonising different plant parts for 1, 3 and 6 month periods.

2. SURVEYS FOR FUNGAL STEM ENDOPHYTES OF EUROPEAN BEECH (*FAGUS SYLVATICA*) IN THE UK AND ARMENIA AS POTENTIAL CLASSICAL BIOLOGICAL CONTROL AGENTS OF BEECH BARK DISEASE

2.1 INTRODUCTION

Classical biological control (CBC) is a technique that can be applied for control of arthropods, weeds and fungal diseases of plants. It exploits the fact that all organisms have co-evolved natural enemies in their native habitats and is an important component of integrated pest management (IPM). Examples of biological control agents include: predators, parasitoids, herbivores, entomopathogens and fungal antagonists, including endophytes. Therefore, in CBC, the centre of origin of a pest or disease is targeted and surveyed for potential biological control agents of the organism.

C. fagisuga was introduced into North America in the 1800s, on *F. sylvatica* seedlings from Europe, where it does not pose a serious problem. Europe is therefore, the most likely location to discover natural enemies of the beech scale insect for exploitation as CBC agents.

2.1.1 Fungal endophytes

In woody plants, the most documented ecological role of fungal endophytes is insect antagonism, through the production of toxic metabolites. In elm trees, the bark endophyte *Phomopsis* sp. was shown to produce feeding deterrents to the elm bark beetle (Claydon *et al.*, 1985). Calhoun *et al.*, (1992) isolated and characterized the bioactive components of three strains of balsam fir (*Abies balsamea*) needle endophytes that cause reduced growth rate and mortality of spruce budworm larvae. Endophytes

may also protect their hosts from animal herbivores and pathogenic microbes (Carroll, 1988). As well as offering protection from pests and diseases, endophytes can also promote plant growth and provide tolerance to environmental stresses such as drought and low nutrient levels (Clay, 1989). Endophytes can be applied to seeds and propagating materials which provides them with protection from the external environmental. An example of an endophyte that has been exploited commercially is *Neotyphodium,* a fungus which enhances growth properties of tall fescue grass (*Festuca arundinacea*) without causing toxic effects to grazing cattle. It has been commercialised in the USA as the product MaxQ by Grasslanz, New Zealand (http://www.grasslanz.com).

Transmission of tree endophytes is horizontal via colonisation of the developing plant by spores (Johnson and Whitney, 1992) and are not systemically transferred to seed and seedlings as in grasses (Wilson, 2000). There are also apparent geographical and seasonal influences on endophytic assemblages of plants (Collado *et al.*, 1999) and it has been shown that the infection frequency of Douglas fir endophytes is influenced by climatic factors such as rainfall and elevation (Carroll and Carroll, 1978).

Fungal endophytes from *Fagus* spp. (and other trees) have been isolated from branches, leaves and twigs (Danti *et al.*, 2002). Other studies carried out on beech trees (Chapela and Boddy, 1988a; Chapela, 1989; Hendry *et al.*, 2002) have identified fungal endophytes belonging to Ascomycetes, Basidiomycetes, Coelomycetes and mitosporic fungi (Cotter and Blanchard, 1982; Sieber and Hugentobler, 1987; Toti *et al.*, 1993; Viret and Petrini, 1994; Kowalski and Kehr, 1997; Danti *et al.*, 2002; Unterseher *et al.*, 2013) which have been similar to those recorded for other tree hosts (Petrini and Fisher, 1990; Fisher *et al.*, 1994; Bills, 1996; Taylor *et al.*, 1999; Shamoun and Sieber, 2000;

Suryanarayanan *et al.*, 2002; Vujanovic and Brisson, 2002; Matsumura and Fukuda, 2013).

No studies of *F. sylvatica* stem endophytes are known in the UK. Mature stems may contain different or additional endophytes to those isolated from other plant organs previously studied. In particular, endophytes isolated from European beech within its native range (and especially ancient forests) may contain novel and even host specific, co-evolved endophytes that could be exploited for classical biological control of BBD in North America.

Fungal endophytes could offer a biological control option for both the insect and fungal components of the BBD complex. Within beech tissues, an applied endophyte could prevent the feeding activity of the scale insect through toxic metabolite production or could act as an entomopathogen. Similarly, the presence of an endophyte in stem tissues could exclude the *Neonectria* fungus through fungal antagonism (antibiosis or mycoparasitism) or by physically excluding it from the same ecological niche (competition) thereby preventing the spread or severity of this invasive disease complex.

Entomopathogenic fungi belonging to genera such as *Beauveria*, *Lecanicillium* and *Paecilomyces* have been isolated as endophytes from a number of plant hosts (Bills and Polishook, 1991; Gómez-Vidal *et al.*, 2006; Vega, 2008; Vega *et al.*, 2008; Vega *et al.*, 2009). Vega *et al*. (2010) studied the endophytic assemblages of coffee (*Coffea* spp.) and isolated three different species of *Beauveria* from leaf, berry, crown, peduncle and seed tissues in Colombia and Hawaii but these were not recovered in Mexico and Puerto

Rico. Giordano *et al.* (2009) also isolated *B. bassiana* from sapwood of Scots pine (*Pinus sylvestris* L.) in Italy.

The aim of this chapter is to report on the identity of endophytic fungi associated with healthy (i.e. beech scale-free), mature stems of *F. sylvatica* within its native range of the UK. Endophytes were also isolated in non-native areas of the UK and from *F. sylvatica* subsp. *orientalis,* the native host of *C. fagisuga* in Armenia for comparison. A selection of isolates with potential for biological control of BBD was made for further investigation in subsequent chapters of this thesis.

2.2 MATERIALS AND METHODS

2.2.1 Survey for endophytes in the UK

After reviewing the literature and consultation with foresters at Natural England, Crown Estate and the Forestry Commission, four ancient woodland sites were identified within the native range of *F. sylvatica* in the UK and selected for the main surveys. The four sites were: 1) Wye Valley (Little Doward), Monmouthshire (OS grid ref. SO535158); 2) Windsor Great Park (High Standinghill Woods), Berkshire (OS grid ref. SU935739); 3) Savernake Forest (Grand Avenue), Wiltshire (OS grid ref. SU240651) and 4) New Forest National Park (Beech Beds), Hampshire (OS grid ref. SU230064) (see Figures 2:1 and 2.2). At each site, only healthy trees with no presence of *C. fagisuga* were identified and marked for future reference. Each site was sampled on two occasions in June/July (visit 1) and October/November 2004 (visit 2). Three additional surveys were carried out at site 2 (Windsor Great Park), during June/July 2005 (visit 3), October 2005 (visit 4) and June/July 2006 (visit 5) in order to assess the effect of sampling over an extended period. Samples were taken from the same individual trees in order to minimise damage. Endophyte samples were also collected at three sites located outside of the UK native range of *F. sylvatica* for comparison. These were: 7) Lake District National Park (Great Hagg), Cumbria (OS grid ref. SD357860); 8) Gateshead (Chopwell Wood), Tyne and Wear (OS grid ref. NY137585) and 9) National Forest (Swandlincote), Leicestershire (OS grid ref. SK309155), see Figure 2:1. Endophyte samples were collected at these sites during August and September, 2005 (visit 1). Sites 5 and 6 were located in Armenia, see 2.2.2.
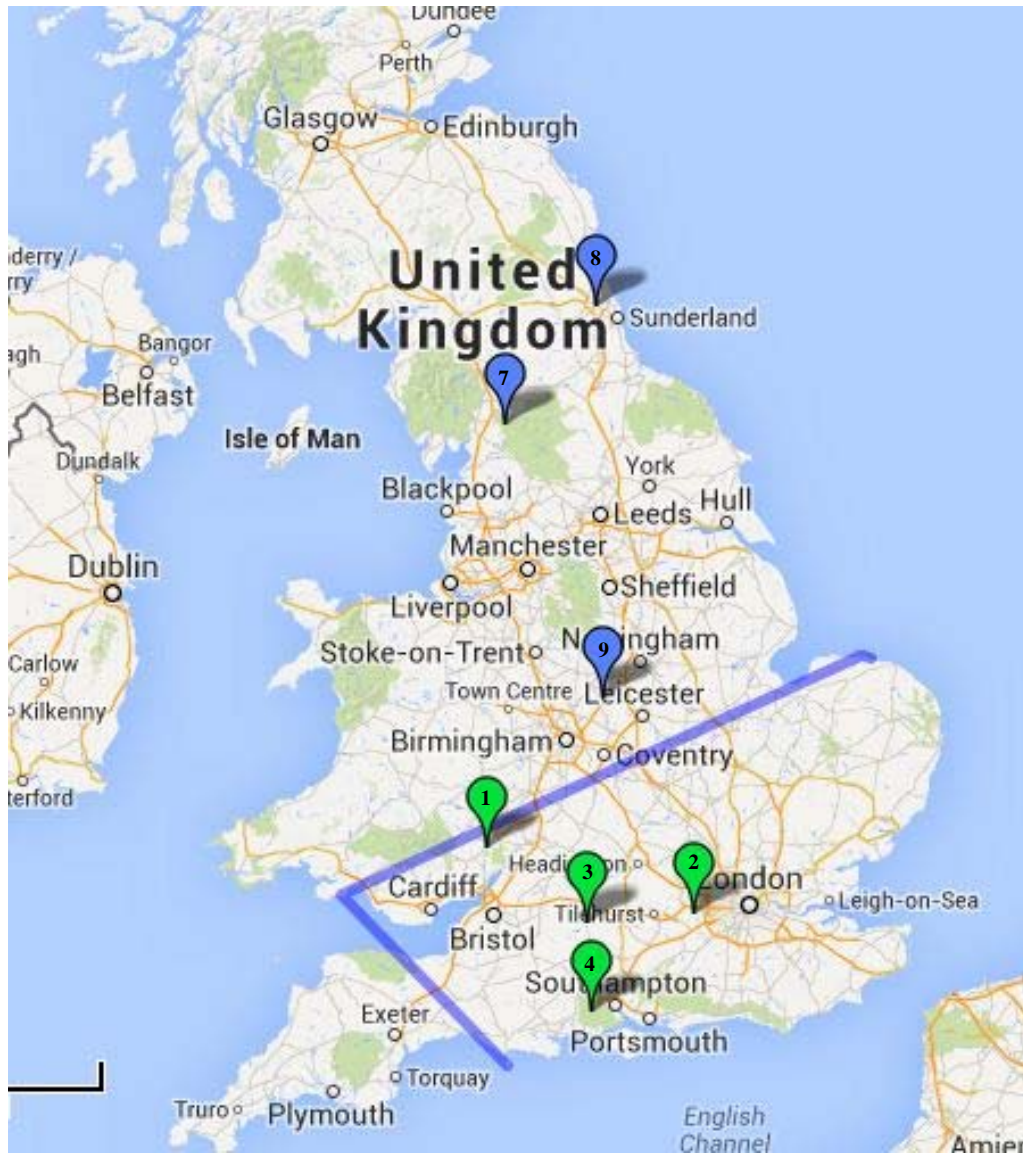
Figure 2:1: Location of *Fagus sylvatica* experimental sites in the UK, green markers represent native sites: 1) Wye Valley (Little Doward), Monmouthshire; 2) Windsor Great Park (High Standinghill Woods), Berkshire; 3) Savernake Forest (Grand Avenue), Wiltshire and 4) New Forest National Park (Beech Beds), Hampshire and blue markers indicate non-native sites: 7) Lake District National Park (Great Hagg), Cumbria; 8) Gateshead (Chopwell Wood), Tyne and Wear and 9) National Forest (Swandlincote), Leicestershire. Created from Google Maps, 2013: https://maps.google.co.uk/maps?hl=en&tab=wl.

Figure 2:2: *Fagus sylvatica* **at Beech Beds, New Forest National Park, Hampshire.**

2.2.2 Survey for endophytes in Armenia

A visit to northern Armenia was made in June 2005, to survey for endophytes of Oriental beech (*Fagus sylvatica* subsp. *orientalis*) as this is the centre of origin and host of *C. fagisuga* (Gwiazdowski *et al.*, 2006). Two suitable sites were identified for endophyte sampling: 5) Itsakar, (GPS position 40°52'60.3N, 45°16'13.8E) and 6) Dilijan National Park, Haghartsin, (GPS position 40°47'51N, 44°54'45E), see Figure 2:3.

Figure 2:3: Location of *Fagus sylvatica* subsp. *orientalis* experimental sites in Armenia: 5) Itsakar and 6) Dilijan National Park, Haghartsin. Created from Google Maps, 2013: https://maps.google.co.uk/maps?hl=en&tab=wl.

2.2.3 Isolation, storage and identification of endophytes

Endophytes were isolated directly from beech trunks in the field by taking 10 samples from 10 trees at each site. Following the method of Evans *et al.* (2003), an area of outer bark (8 x 6 cm) was removed on each tree using a machete knife and the exposed inner

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bark was cleaned with a flamed scalpel (see Figure 2:4) in order to expose the sapwood. The purpose of sampling this area was to isolate endophytes from where BBD could potentially invade. Triangular samples of tissue (0.8 x 0.8 x 0.5 cm) were carefully excised with a sterile scalpel. Five were transferred to individual 3cm diameter Petri dishes containing malt extract agar (MEA, Oxoid) and five to Potato Carrot Agar (PCA), see Appendix 8:1 for recipes. Penicillin-Streptomycin solution (Sigma Aldrich, P4333) was added to the media in order to eliminate bacterial growth. The sampled area was covered with a liquid wound sealant (Arbrex, Bio) where local forestry management practices required this.
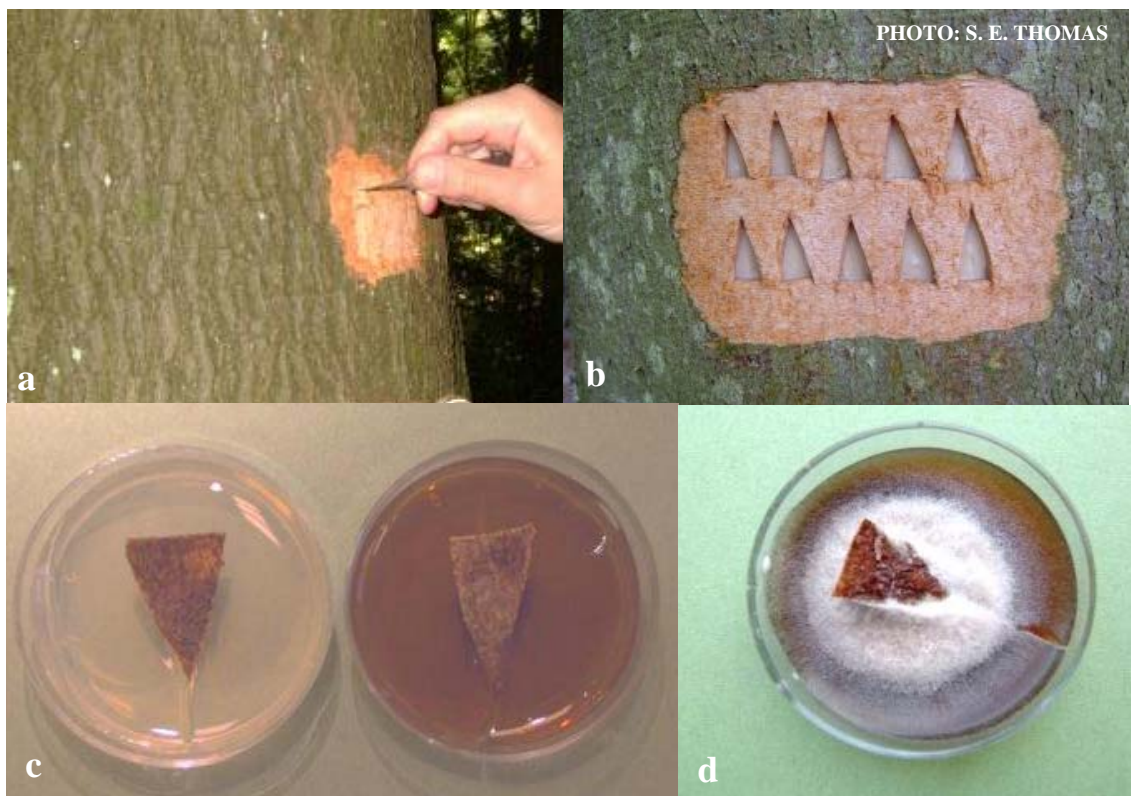
Figure 2:4: a) Removing samples from the beech trunk; b) removed bark; c) samples on selective media and d) endophyte emerging from sample.

In the laboratory, the Petri dishes were incubated at 25°C and examined regularly over an 8-week period. Emerging fungi were transferred to fresh 5cm PCA plates and incubated under black light at 25°C to induce sporulation. All isolates were maintained and stored for the duration of the study using three methods: 1) on PCA slopes in 7ml glass Bijou bottles at 10°C; 2) on PCA slopes in 7ml Bijou bottles covered with a layer of mineral oil at room temperature and 3) as 5mm inoculated agar plugs in SDW at room temperature. For each isolate, specimens were prepared by aseptically transferring small samples of mycelium to glass microscope slides. A drop of mountant (lactophenol and cotton blue) was added to the samples for staining. The mycelium was carefully teased out and a cover glass was placed on top of the specimen. The specimens were examined using a light microscope (Nikon Eclipse, E600) to determine if sporulating structures were present. With the aid of taxonomic keys, fungi were identified to species level where possible. Identifications were confirmed by taxonomy specialists Drs. Brian Sutton and Gary Samuels. Sterile or non-sporulating isolates were grouped into morphological species by gross morphology of the colonies, firstly by colour (black, brown, cream, pink or white) and then by growth pattern (1, 2, 3, 4, 5 etc.). Selected isolates were later identified molecularly using DNA sequencing (see 2.2.5).

2.2.4 Data analysis of UK endophytes

Species richness, i.e. total number of fungal species, was calculated for each of the UK sites for visit 1 and was compared using analysis of variance (ANOVA) in order to determine any differences between them. Significant differences were further examined with the use of Tukey's test to examine pairwise comparisons for species richness for each site.

Isolation frequency (IF) for each tree on visit 1 was calculated as the proportion of samples per tree yielding a fungal isolate. Data were angular transformed before performing ANOVA. For the study at Windsor Great Park (site 2), IFs were calculated for each visit and following angular transformation, differences in IF between trees and visits were examined with ANOVA with repeated measures. All statistical analyses were undertaken with GenStat, 12th Edition (VSN International Ltd.).

2.2.5 Molecular identification of selected endophytes

After morphological examination, four of the fungal endophyte isolates from the native range of *F. sylvatica* were noted as being interesting and unusual and these were selected for molecular identification. Total genomic DNA was extracted from a single loopful of biomass using a proprietary complex DNA release solution (microLYSIS-PLUS; Microzone Ltd, UK) in accordance with manufacturer's instructions. The thermal cycler lysis profile was: 15 min at 65°C, 2 min at 96°C, 4 min at 65°C, 1 min at 96°C, 1 min at 65°C, 30 s at 96°C and held at 20°C. Partial ribosomal RNA gene cluster (part of 18S small subunit RNA gene, internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, internal transcribed spacer 2 (ITS2), part of 28S large subunit ribosomal RNA gene) was amplified by PCR using primers ITS6 and ITS4. PCR products were purified and used in sequencing reactions from which excess dye was removed before subjecting to capillary electrophoresis DNA sequence analysis. Sequence trace files were checked and exported as text files before being searched against the holdings of GenBank using the BLAST algorithm. The D1/D2 of the nuclear large subunit rRNA LSU, 26S was also examined to allow identification to species level as some taxa have more reliable sets of sequences for the LSU gene. These isolates were allocated an IMI number and deposited in the CABI genetic resource collection (GRC) for future reference and safekeeping.

2.3 RESULTS

2.3.1 Total endophyte isolates

A total of 601 isolates were cultured from the 1600 samples that were taken from beech stems at all nine sites on all visits in the study (UK and Armenia). All samples were catalogued and a total of 117 different taxa were cultured. Amongst those that were identifiable according to their morphological characteristics, 78 (67%) belonged to the Ascomycetes and anamorphic fungi, 1 (<1%) to the Zygomycetes and 3 (2%) to the Basidiomycetes. The remaining non-sporulating organisms or sterile mycelia were grouped into 35 "morphospecies" (30%) according to their gross morphology (see Figures 2:5 and 2:6). Isolated genera of particular biological control interest included *Acremonium, Beauveria, Clonostachys, Paecilomyces, Trichoderma* and *Xylaria* (see Table 2:1 and Appendix 8:2 for lists of endophytes).
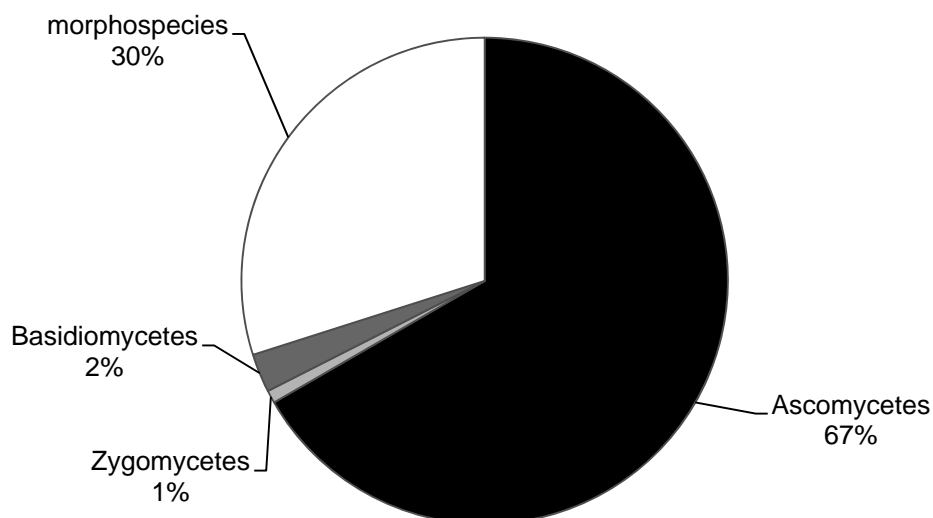
Figure 2:5: Fungal composition of isolated beech endophytes from UK and Armenia.

Table 2:1: Fungal endophytes isolated from *Fagus* stems in the UK and Armenia.

Identification		Site type/number								
		UK native				Armenia		UK non-native		
		1	2	3	4	5	6	7	8	9
Ascomycetes and anamorphic fungi										
1	*Acremonium psammosporum*	-	-	-	+	-	-	-	-	-
2	*Acremonium* sp.1	-	+	+	+	-	-	-	-	-
3	*Acremonium* sp.2	-	-	-	+	-	-	-	-	-
4	*Acremonium* sp.3	-	-	-	-	+	-	+	-	+
5	*Alternaria* sp.	-	-	-	-	-	-	+	-	-
6	*Apiognomonia errabunda*	-	+	-	-	-	-	-	-	-
7	*Aspergillus* sp.1	+	-	-	+	-	-	-	-	-
8	*Aspergillus* sp.2	+	-	-	-	-	-	-	-	-
9	*Aspergillus* sp.3	+	-	-	-	-	-	-	-	-
10	*Aspergillus* sp.4	+	-	-	-	-	-	-	-	-
11	*Aspergillus* sp.5	+	-	-	-	-	-	-	-	-
12	*Aspergillus* sp.6	+	-	-	-	-	-	-	-	-
13	*Aspergillus* sp.7	+	-	-	-	-	-	-	-	-
14	*Aspergillus* sp.8	+	-	-	-	-	-	-	-	-
15	*Aspergillus* sp.9	+	-	-	-	-	-	-	-	-
16	*Aspergillus* sp.10	+	-	-	-	-	-	-	-	-
17	*Aspergillus* sp.11	-	-	-	-	-	+	-	-	-
18	*Aspergillus* sp.12	-	-	+	-	-	-	-	-	-
19	*Asteromella* sp.	-	-	+	-	-	-	-	-	-
20	*Aureobasidium pullulans*	+	+	-	-	-	-	-	-	-
21	*Aureobasidium* sp.	-	+	-	+	-	-	-	-	-
22	*Beauveria bassiana*	-	-	+	+	-	-	-	-	-
23	*Beauveria* sp.	+	+	-	-	+	+	-	+	-
24	*Blastobotrys* sp.	+	-	-	-	-	-	-	-	-
25	*Bloxamia truncata*	-	+	-	-	-	-	-	-	-
26	*Botryotrichum piluliphorum*	-	-	+	+	-	-	-	-	-

Identification		Site type/number								
		UK native				Armenia		UK non-native		
		1	2	3	4	5	6	7	8	9
27	*Cladosporium cladosporoides*	+	-	-	-	-	-	-	-	-
28	*Cladosporium herbarum*	+	+	-	-	-	-	-	+	+
29	*Cladosporium oxysporum*	-	-	+	-	-	-	-	-	-
30	*Cladosporium* sp.	-	+	-	-	-	-	+	-	-
31	*Clonostachys rosea*	-	+	+	-	+	-	-	-	-
32	*Clonostachys* sp.	-	-	+	-	+	+	-	-	-
33	*Codineae hughesii*	-	-	-	+	-	-	-	-	-
34	*Colletotrichum* sp.	-	-	+	-	-	-	-	-	-
35	*Coniothyrium fuckelli*	+	+	+	-	-	-	-	-	-
36	*Dendrodochium aurantiacum*	-	-	+	-	-	-	-	-	-
37	*Dendrodochium* sp.	-	-	-	+	-	-	-	-	-
38	*Fusarium* sp.1	-	-	-	-	-	+	-	-	-
39	*Fusarium* sp.2	-	+	-	-	-	-	-	-	-
40	*Geniculosporium* sp.1	-	+	+	+	-	-	-	-	-
41	*Geniculosporium* sp.2	-	+	-	-	-	-	-	-	-
42	*Geotrichum candidum*	-	-	-	+	-	-	-	-	-
43	*Geotrichum* sp.	+	+	+	+	+	-	-	-	-
44	*Hormomyces* sp.	-	-	+	-	-	-	-	-	-
45	*Melanconium atrum*	+	+	+	+	-	-	-	-	-
46	*Monochaetia* sp.	-	-	-	-	+	-	-	-	-
47	*Monodictys* sp.	-	+	-	-	-	-	-	-	-
48	*Paecilomyces* sp.	-	-	-	-	-	-	-	-	+
49	*Paecilomyces variotii*	-	+	-	-	-	-	-	-	-
50	*Penicillium* sp.1	-	+	+	-	+	-	-	-	-
51	*Penicillium* sp.2	-	+	+	-	-	+	-	-	-
52	*Penicillium* sp.3	-	-	+	-	-	-	-	-	-
53	*Penicillium* sp.4	-	-	-	+	-	-	-	-	-

Identification		Site type/number								
		UK native				Armenia		UK non-native		
		1	2	3	4	5	6	7	8	9
54	*Penicillium* sp.5	-	-	+	-	-	-	-	-	-
55	*Penicillium* sp.6	-	-	-	-	+	-	-	-	-
56	*Penicillium* sp.7	-	-	-	-	-	-	-	+	-
57	*Periconiella* sp.	-	+	-	-	-	-	-	-	-
58	*Pesotum ulmi*	-	-	+	-	-	-	-	-	-
59	*Phoma dorenboschii*	-	-	+	-	-	-	-	-	-
60	*Phoma herbarum*	-	+	-	-	-	-	-	-	-
61	*Phoma* sp.	-	-	-	-	-	-	+	-	+
62	*Phoma tropica*	-	-	+	-	-	-	-	-	-
63	*Phomopsis* sp.	+	+	-	-	-	-	-	-	-
64	*Phylocephala* sp.	+	-	-	-	-	-	-	-	-
65	*Pithomyces chartarum*	-	-	+	-	-	-	-	-	-
66	*Pseudocercosporella* sp.	-	+	-	-	-	-	-	-	-
67	*Pseudopatalina conigena*	-	+	-	-	-	-	-	-	-
68	*Pseudopatalina* sp.	-	+	-	-	-	-	-	-	-
69	*Rhinocladiella* sp.	-	+	-	-	-	-	-	-	-
70	*Stagonospora* sp.	-	-	+	-	-	-	-	-	-
71	*Trichoderma harzianum*	-	-	+	+	-	-	-	-	-
72	*Trichoderma* sp.	+	+	+	-	-	-	-	-	+
73	*Trichoderma stilbohypoxyli*	-	-	+	-	-	-	-	-	-
74	*Trichoderma viride*	+	-	-	-	-	-	-	-	-
75	*Trichoderma viridescens*	+	-	+	+	-	-	-	-	-
76	*Verticillium* sp.	-	-	-	-	-	-	+	+	+
77	*Xylaria carpophila*	+	-	-	-	-	-	-	-	-
Zygomycetes										
78	*Mucor hiemalis*	-	-	+	-	-	-	-	-	-

Identification	Site type/number								
	UK native				Armenia		UK non-native		
	1	2	3	4	5	6	7	8	9
Basidiomycetes									
79	*Rhizoctonia* sp.	-	-	-	-	-	-	-	-	+
80	*Sclerotium* sp.	-	-	+	-	-	+	-	-	-
81	*Spiniger meineckellus*	+	-	-	-	-	-	-	-	-
Unidentified "morphospecies"									
83	Sterile BL01	+	+	+	+	+	+	+	+	+
84	Sterile BL02	-	-	-	+	-	+	-	-	-
85	Sterile BL03	-	+	-	-	-	-	-	-	-
86	Sterile BL04	-	-	-	+	-	-	-	-	-
87	Sterile BL05	-	+	+	-	-	-	+	-	-
88	Sterile BL06	-	-	+	-	-	-	+	-	-
89	Sterile BL07	-	+	+	+	-	+	-	-	-
90	Sterile BL08	-	-	+	-	-	-	-	-	-
91	Sterile BR01	+	+	-	-	-	+	-	-	-
92	Sterile BR02	-	+	-	-	-	-	-	-	-
93	Sterile BR03	-	-	-	+	-	-	-	-	-
94	Sterile BR04	-	+	+	-	-	-	-	-	-
95	Sterile BR05	-	-	+	-	-	+	-	-	-
96	Sterile BR06	-	+	-	-	-	-	-	-	-
97	Sterile BR07	-	+	-	-	-	-	-	-	-
98	Sterile BR08	-	+	-	+	+	+	+	-	-
99	Sterile BR09	-	+	-	-	-	-	-	-	-
100	Sterile BR10	-	+	-	-	-	-	-	-	-
101	Sterile BR11	-	-	-	-	+	-	-	-	-
102	Sterile C01	-	+	-	-	+	+	-	-	+
103	Sterile C02	-	-	+	+	+	-	-	-	-
104	Sterile C03	-	+	-	-	-	-	-	-	-

Identification		Site type/number								
		UK native				Armenia		UK non-native		
		1	2	3	4	5	6	7	8	9
105	Sterile P01	-	+	+	-	-	-	-	-	-
106	Sterile W01	+	+	+	+	+	+	-	-	-
107	Sterile W02	+	-	-	+	-	-	-	-	-
108	Sterile W03	-	+	+	+	-	-	-	-	-
109	Sterile W04	+	+	+	+	+	+	-	-	-
110	Sterile W05	-	-	-	+	-	-	-	-	-
111	Sterile W06	+	+	-	-	-	+	-	-	+
112	Sterile W07	-	+	-	+	-	-	-	-	-
113	Sterile W08	-	-	-	+	-	-	-	-	-
114	Sterile W09	+	+	+	-	-	-	-	-	-
115	Sterile W10	+	-	-	-	-	-	-	-	-
116	Sterile W11	+	-	+	-	-	-	-	-	-
117	Sterile W12	+	+	-	+	-	-	-	-	-

Where site 1= Wye Valley; site 2= Windsor Great Park; site 3= Savernake Forest; site 4=New Forest: site 5=Armenia 1; site 6= Armenia 2: site 7=Lake District; site 8=Gateshead and site 9=National Forest. BL=Black, BR=Brown, C=Cream, P=Pink, W=White. += present, - not present.

2.3.2 UK endophytes

Endophyte species richness was highest at Wye Valley (site 1) during visit 1, where 24 different species of fungi were isolated and a total of 56 isolates were collected. In contrast, only five different species of fungi were isolated at the non-native site in Gateshead (site 8) from a total of 6 isolates collected. Analysis of species richness data for the seven UK sites (sites 1, 2, 3, 4, 7, 8 and 9) showed that there were significant differences between sites ($F_{6,63}$=5.5, p<0.001) see Table 2:2.

Table 2:2: Species richness and total endophytes at UK sites, visit 1.

Site Type	UK Native				UK Non-native		
Site number	1	2	3	4	7	8	9
Species richness	24^c	19^{bc}	19^{bc}	9^{ab}	10^{ab}	5^a	10^{ab}
Total isolates	56	35	42	13	22	6	15

Isolation frequencies at the four native UK sites for visit 1 and 2 were calculated and ANOVA showed that there were significant differences ($F_{3,36}$=3.65, p<0.01), using site and visit as factors. Figure 2:6 illustrates the variety of endophytes isolated from the native range of *F. sylvatica.*

2.3.2.1 Windsor Great Park temporal study

On almost all occasions, fungal endophytes were successfully isolated from the stems of beech at the Windsor site. There were only five instances when a tree did not yield a fungal isolate. Isolation frequency (44) and species richness (20) were both highest on the second sampling (October/November 2004). Isolation frequency (17) and species richness (7) were lowest on the fifth sampling (June/July 2006). ANOVA results indicated that there were significant differences between isolation frequencies over time ($F_{4,36}$ =3.59, p=0.022) at Windsor Great Park site, see Table 2:3.

Table 2:3: Isolation frequencies (IF) of endophytes for beech trees at Windsor Great Park, where IF=proportion of samples producing a fungal isolate and n=10 and total number of isolates and species richness.

	Visit				
Tree number	1	2	3	4	5
1	0.4	0.5	0	0.2	0.1
2	0.3	0.5	0.2	0	0
3	0.3	0.7	0.7	0.1	0.3
4	1.0	0.1	0.3	0	0.2
5	0.1	0.4	0.4	0.3	0
6	0.1	0.4	0.3	0.2	0.3
7	0.3	0.2	0.6	0.2	0.1
8	0.5	0.6	0.6	0.5	0.2
9	0.1	0.5	0.6	0.2	0.5
10	0.2	0.6	0.5	0.3	0.1
Total isolates	35	44	39	18	17
Species richness	19	20	13	9	7

Figure 2:6: A selection of endophyte isolates from the UK native range of *Fagus sylvatica*.

2.3.3 Armenian endophytes

A total of 24 different fungal endophytes were isolated from oriental beech (*F. sylvatica* subsp. *orientalis*) at the two sites in Armenia. A total of 30 isolates were collected at site 5 (15 different taxa) and 31 isolates at site 6 (14 different taxa). Of particular interest were *Acremonium* sp., *Beauveria* sp. and *Clonostachys* spp., as these genera are known to have entomopathogenic properties. No *Trichoderma* endophytes were isolated from *F. sylvatica* subsp. *orientalis* in Armenia.

2.3.4 Molecular identification of selected endophytes

Molecular identification of the four unusual endophytes did not unveil fungi of any biological control interest (see Table 2:4) Three of the fungi identified after sequencing of the ITS and LSU were *Phomopsis* sp., *Cosmospora* sp. and *Cylindrobasidium* sp. After ITS sequencing, the fourth isolate, IMI397378 (see Figure 2:7) was preliminary identified as 97% match to "fungal endophyte" and after Partial 26S sequence, as a 95% match to *Sporidesmium obclavatulum*. IMI397378 most likely represents a new species and may even represent a new genus as it is not currently represented in GenBank. Literature (Shenoy *et al.*, 2006) suggests RNA polymerase II second largest subunit (RPB2) primers could aid further identification.

Table 2:4: Molecular identification of four selected endophytes.

Strain number	Nearest Genbank match		Provisional Molecular ID
	ITS sequence	**Partial 26S sequence**	
IMI397374	>97% *Phomopsis columnaris*	>98% *Phomopsis* sp.	*Phomopsis* sp.
IMI397376	>98% *Cosmospora vilior*	>98% *Cosmospora coccinea*	*Cosmospora* sp.
IMI397377	>99% *Cylindrobasidium evolvens*	>99% *Cylindrobasidium leave*	*Cylindrobasidion* sp.
IMI397378	>97% to "fungal endophyte"	>95% *Sporidesmium obclavatulum*	Possible new species
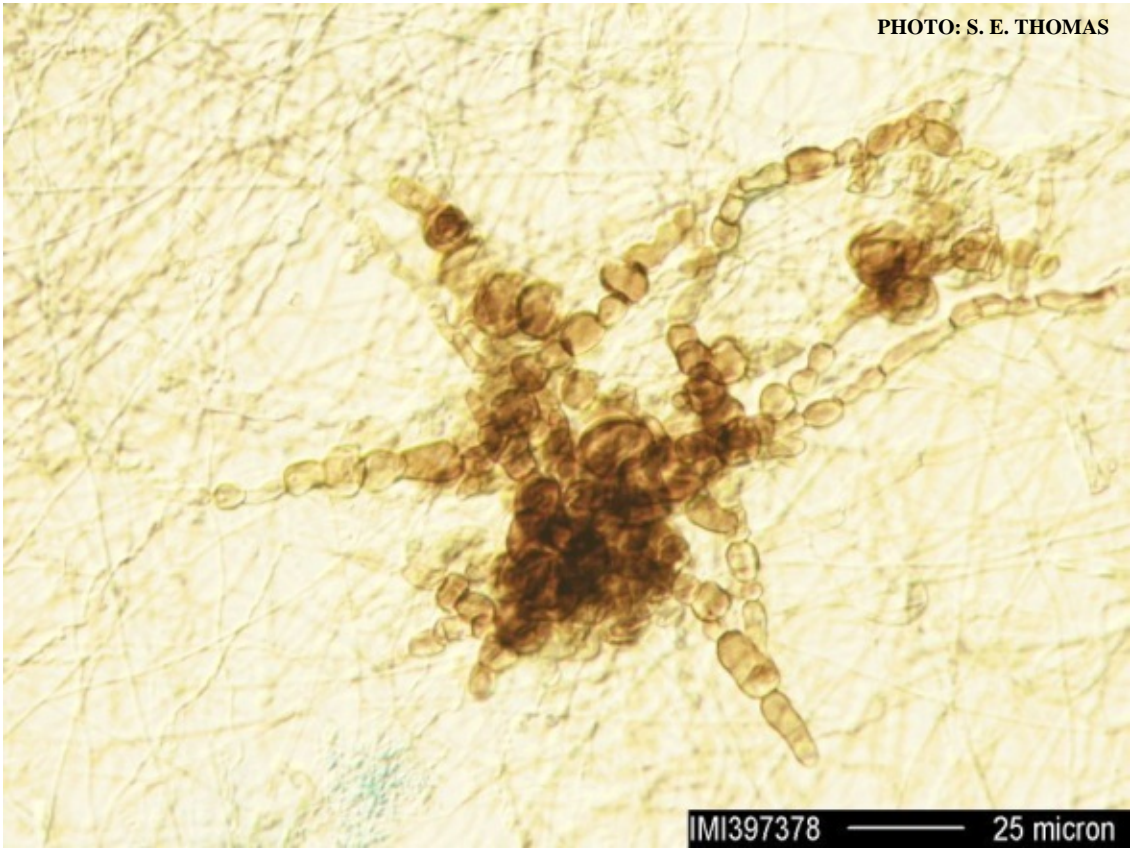
Figure 2:7: IMI397378, *Sporidesmium* **sp.**

2.4 DISCUSSION

This study is the first known effort to isolate and identify fungal endophytes of living *F. sylvatica* stems, with the intention to explore and potentially use them as biological control agents of the beech scale insect (*C. fagisuga)* and the *Neonectria* fungi that constitute the BBD complex affecting *F. grandifolia* in North America.

This study supports the findings of some previous research carried out on *F. sylvatica* in Europe. Several of the endophytic fungal genera (*Alternaria* sp., *Aureobasidium* sp., *Cladosporium* sp., *Fusarium* sp., *Melanconium* sp., *Penicillium* sp., *Phoma* sp., *Phomopsis* sp. and *Xylaria* sp.) isolated in this study were also isolated by Danti (2002) from branches of *F. sylvatica* in Italy. In addition, Griffith and Boddy (1990) isolated an analogous list of fungi from healthy bark of beech twigs which comprised of *Xylaria* sp*., Coniothyrium fuckeli, Phoma macrostoma, Phomopsis quercella, Aureobasidium pullulans, Fusarium lateritium, Penicillium* sp. and *Trichoderma* sp. as well as sterile mycela from a mixed deciduous woodland in South Wales. Chapela (1989) also identified a similar mycoflora from American beech (*F. grandifolia*) including *Fusarium* sp., *Penicillium* sp. and *Trichoderma* sp.

Many of the endophytes isolated in this study were not identifiable morphologically, as they were non-sporulating. These sterile mycelia or "morphospecies" were not studied further as they would not be useable as biological control agents. However, molecular identification of these "morphospecies" would make an interesting study as they could potentially be previously unrecorded on beech. This was the case when endophytic morphological species isolated from *Theobroma* spp. were molecularly identified (Crozier *et al.*, 2006; Thomas *et al.*, 2008).

This study has shown that the endophyte composition of beech stems varies between sites. Species richness was highest at Wye Valley, in native, ancient beech woodland. The study at the Windsor Great Park site showed that there are also temporal differences in endophyte assemblages. Therefore, it is recommended that multiple surveys for potential biocontrol agents are conducted throughout the calendar year to allow for any seasonal effect. Several studies have looked at temporal patterns of leaf and needle endophyte infection (Wilson, 2000; Suryanarayanan and Thennarasan, 2004) but very few have looked at those of other plant organs or tissues (Gazis and Chaverri, 2010).

The study also partially identified several interesting or unusual fungi. One of these was preliminarily identified as *Sporidesmium* sp. This was a most unusual fungus which warrants further investigation but falls outside the concern of this current study. Although unsuccessful in complete identification, it indicates that there are probably many new species of endophytic fungi of *F. sylvatica* which are yet to be discovered and described.

Several of the endophytic genera that have been isolated from the stems of healthy *F. sylvatica* in this study are known to possess recognised biological control activity against plant pathogens and insect pests. *Trichoderma* spp. and *Clonostachys* spp. are well-documented mycoparasites of plant diseases (Krauss and Soberanis, 2001) plus *Acremonium* spp., *Beauveria* spp. and *Lecanicillium* spp. (*Verticillium*) are known entomopathogens of insect pests (Steenberg and Humber, 1999) and have potential to be exploited as biological control agents for BBD.

Interestingly, *Beauveria* spp. were isolated as endophytes from beech stems both in the UK and Armenia. *Beauveria* spp. have been isolated as endophytes from numerous

other plant hosts and are known to produce several metabolites, including bassianin, beauvericin, bassianolide, beauveriolide, bassiacridin, oosporein, and tenellin (Vega, 2008). Members of the Hypocreales, including endophytes and EPF are able to obtain nutrients in a number of ways and function in more than one econutritional mode (Ownley *et al.*, 2010). Some isolates of *B. bassiana* and *Lecanicillium* spp. are able to exist endophytically within plant tissues (Vega *et al.*, 2008) and research has shown that EPF can in fact, be multifunctional as they can be effective suppressants of plant diseases such as *Rhizoctonia solani* and *Pythium myriotylum* in addition to insect pests (Ownley *et al.*, 2008). Biological control agents that suppress plant pathogens usually either act directly through mechanisms such as competition, mycoparasitism and antibiosis or indirectly by triggering plant responses such as induced systemic resistance that reduce or alleviate plant disease (Ownley *et al.*, 2010). As Elliot *et al.* (2000) suggested, it may be possible that *Beauveria* spp. (and possibly other EPF) that exist endophytically within plant tissues are able to switch their mode of action to become pathogenic to insects or fungi, if or when necessary. If this theory could be proved, then it could potentially be exploited for control of BBD. If an EPF could be applied to beech stem tissues and could successfully colonise saplings endophytically, it may be possible to prevent successive infection of *C. fagisuga* and/or *Neonectria faginata*. Endophytes with entomopathogenic properties have good potential to be developed for biological control of insect-pathogen disease complexes.

In summary, the use of endophytes, particularly those isolated from beech stem tissue within its centre of origin, offers a potential novel source of biological control agents for BBD. A number of interesting fungi have been isolated from *F. sylvatica* tissues as endophytes and these will be further investigated in subsequent chapters of this thesis, see Table 2:5.

Table 2:5: Endophyte isolates selected for further study in this thesis.

Fungus	ID	Chapter(s)
Beauveria bassiana	IMI502733	3 and 5
Beauveria sp.	IMI502734	3
Trichoderma viride	IMI395629	4 and 5
T. viridescens	IMI395630	4
T. viridescens	IMI395631	4
T. harzianum	IMI395632	4
T. harzianum	IMI395633	4
T. harzianum	IMI395634	4
T. stilbohypoxyli	IMI395635	4
T. harzianum	IMI395636	4 and 5
T. viridescens	IMI395637	4

3. ENTOMOPATHOGENIC FUNGI ASSOCIATED WITH BEECH SCALE (*CRYPTOCOCCUS FAGISUGA*) IN ARMENIA AND A PRELIMINARY ASSESSMENT OF THEIR POTENIAL FOR BIOLOGICAL CONTROL

3.1 INTRODUCTION

3.1.1 Entomopathogenic fungi

One possible biological control strategy for BBD is the use of entomopathogenic fungi (EPF) targeted at the beech scale, *C. fagisuga*. These are fungi that occur naturally in the environment and can be exploited for use as biological insecticides. They secrete the enzymes chitinase, protease and lipase which degrade the respective components of the insect cuticle (chitin, protein and lipid) in order to penetrate to the insect haemocoel (Khan *et al.*, 2012).

Most EPF used in biological control belong to the Clavicipitaceae: a family of fungi within the order Hypocreales and which consists of 43 genera and 321 species (Kirk *et al.*, 2008). Many commercial products based on formulations of EPF are available for control of pests such as whitefly, thrips, aphids, psyllids, mealybugs, scarab beetles, weevils and also for mites (Faria and Wraight, 2007). Some of the most common commercial mycoinsecticides and mycoacaricides consist of fungi belonging to the genera *Beauveria, Lecanicillium* and *Metarhizium* (Faria and Wraight, 2007). These fungi are good sporulating organisms that can easily be cultured, mass produced and formulated in the laboratory for use in many sectors including horticulture, agriculture and forestry. Some examples include Mycotrol, (*B. bassiana*), produced by BioWorks (USA), Mycotal (*L. muscarium)* for control of aphids and Vertalec (*L. longisporum*) for control of whitefly and thrips, both produced by Koppert Biological Systems (The

Netherlands) and GreenMuscle (*M. acridum*) produced by Becker Underwood, BASF (South Africa) for control of locusts and grasshoppers.

EPF such as *Cladosporium, Fusarium* and *Lecanicillium* are commonly associated with scale insects (Evans and Hywel-Jones, 1997). *L. muscarium* has been reported on mites, whiteflies, aphids and on scale insects including *C. fagisuga* (Lonsdale, 1983). *Beauveria, Lecanicillium* and *Paecilomyces* have also been isolated as endophytes from within living tissues of a number of plant hosts (Bills and Polishook, 1991; Gómez-Vidal *et al.*, 2006; Vega, 2008; Vega *et al.*, 2008; Vega *et al.*, 2009). EPF such as *B. bassiana* have also been established as endophytes in a number of plant hosts for biological control of arthropod pests and plant pathogens (Parsa *et al.*, 2013).

Research to investigate the efficacy of various strains of *L. muscarium* (Marshall *et al.*, 2003; Cuthbertson *et al.*, 2005a) and compatibility studies with chemical insecticides have been undertaken (Cuthbertson *et al.*, 2005b; Gurulingappa *et al.*, 2011). *L. longisporum* (Vertalec) has been evaluated for control of both aphids and powdery mildew on potted cucumber plants (Kim *et al.*, 2008). Goettel *et al.* (2008) review and conclude that *Lecanicillium* species have potential as biological control agents of plant pathogenic fungi, insects and plant parasitic nematodes. Laflamme *et al.,* (2009) undertook some preliminary laboratory assays with *L. muscarium* and *C. fagisuga* and achieved a 50% reduction in crawler population after 11 days. Treatment of eggs with the fungus was also shown to slow down hatching but did not affect mortality. EPF isolates can be selected by evaluation in the laboratory, usually by determining growth characteristics related to temperature, mass production characteristics and bioassays. For example, Yeo *et al.* (2003) studied the effect of temperature on the germination and

growth of *Beauveria, Lecanicillium, Acremonium, Paecilomyces* and *Metarhizium* species and assessed their pathogenicity to aphids in the laboratory.

The aim of this chapter is to report on EPF associated with *C. fagisuga* in its centre of origin in Armenia. A preliminary, *in vitro* evaluation of key EPF isolates (including two endophytic EPF isolates from *F. sylvatica* subsp. *orientalis* in chapter 2 of this thesis) will be made and compared to commercially available mycoinsecticides. Their suitability for development into a mycoinsecticide for biological control of *C. fagisuga* will be discussed.

3.2 MATERIALS AND METHODS

3.2.1 Survey of entomopathogenic fungi (EPF) in Armenia

In addition to the survey conducted in northern Armenia for endophytes of healthy *F. sylvatica* subsp. *orientalis* trees in June 2005, the opportunity to collect entomopathogens of *C. fagisuga* was also taken. A search for scale-infested trees was made at the two sites described in Chapter 2 (see 2.2.2 and Figure 2:3) and samples were collected.

3.2.2 Entomopathogen collection and isolation

Small sections of bark, plus scale insects (eggs, juveniles and adults) and their waxy covering were removed from the surface of the beech trees using a small chisel. Samples were placed and sealed in small plastic bags and returned to the laboratory in the UK. With the use of a dissecting microscope, mummified adults were removed from their waxy covering using fine forceps and transferred to 5cm Petri dishes containing PCA plus Penicillin-Streptomycin solution (Sigma Aldrich, P4333) to eliminate bacterial growth. The Petri dishes were incubated at 25°C and emerging fungi were transferred to fresh PCA plates.

3.2.3 Morphological identification of EPF

Specimens were prepared by aseptically transferring small samples of mycelium to glass microscope slides. A drop of mountant (acid fuchsin) was added to the samples for staining. The mycelium was carefully teased out and a cover glass was placed on top of the specimen. The specimens were examined using a light microscope (Nikon Eclipse, E600) and identified morphologically to genus and species level where possible.

3.2.4 Development of a *C. fagisuga* inoculation technique

3.2.4.1 Preparation of fungal inoculum of **L. longisporum** *(Vertalec)*

In order to establish an effective *C. fagisuga* inoculation technique, preliminary experiments with the commercially available mycoinsecticide, Vertalec (*L. longisporum*) were carried out. A sample of the product was obtained from Koppert Biological Systems, The Netherlands. 0.5g of the dried powder (5 x 10^8 spores g^{-1}) was mixed with 20ml of sterile distilled water (SDW) in a Universal tube. A drop of the suspension was transferred to each of five, 5cm PCA plates which were incubated at 25°C for 7 days to confirm the viability of the product and to produce living cultures of the fungus.

A fresh spore suspension of *L. longisporum* was produced by adding 5ml of SDW to the culture on PCA plate. The surface of the culture was gently scraped with a sterile spatula and the suspension transferred to a Universal tube using a sterile syringe. Once filtered with glass wool to remove mycelium from the suspension, the spore concentration was adjusted to 1 x 10^7 spores ml^{-1} with the use of a haemocytometer.

3.2.4.2 Collection of healthy beech scale insects

Healthy *C. fagisuga* eggs, crawlers and adults were collected from an infested beech tree (*F. sylvatica)* located in Savernake Forest in Wiltshire, UK. They were collected as described in 3.2.2, placed inside a small plastic bag and returned to the laboratory. Eggs and crawlers were separated from the adults for use in the experiments. Adult insects require attachment to xylem for sap (see 1.4.1) and were therefore not suitable for *in vitro* assay.

3.2.4.3 Inoculation of **C. fagisuga** *eggs*

The inoculation method was similar to that used by Asensio *et al.* (2005) for the

inoculation of the red scale insect (*Phoenicococcus marlatti*) with *B. bassiana,*

Lecanicillium dimorphum and *Lecanicillium* cf. *psalliotae*. *C. fagisuga* eggs were

carefully teased from their waxy covering with a fine needle. They were washed in

0.13% sodium hypochlorite for 2 minutes in a 5cm Petri dish. The eggs were rinsed in

SDW for a further 2 minutes. Eggs were inoculated by dipping into the prepared

suspension of *L. longisporum* spores (3.2.3). The eggs were carefully transferred to the

surface of 3cm Tap Water Agar (TWA) plates. A total of 100 eggs (10 per agar plate)

were transferred. Control eggs were inoculated with SDW only. All plates were

incubated at 25°C. Egg hatching was recorded daily for the first 7 days and weekly

thereafter. Inoculated eggs were observed after staining with acid fuchsin for growth of

the fungus.

3.2.4.4 Inoculation of **C. fagisuga** *juveniles/crawlers*

C. fagisuga crawlers were carefully separated from their waxy covering with the use of

a fine needle. A total of 100 crawlers was placed on filter paper contained within 5cm

Petri dishes (10 crawlers per dish). Surface sterilisation of the crawlers was not possible

due to their small size and fragility. Filter papers were inoculated with a 1ml spore

suspension (1 x 10^7 spores ml^{-1}) of *L. longisporum* and the insects were left to "self-

inoculate" by surface contact.

3.2.5 Molecular analysis of endophytic *Beauveria* isolates

Because of the potential uniqueness of the two endophytic *Beauveria* isolates

(*B. bassiana,* IMI502733 and *Beauveria* sp., IMI502734) that were isolated from

F. sylvatica subsp. *orientalis* in Armenia (chapter 2), special interest was taken in these

isolates and further identification using molecular techniques was undertaken. This was to determine any noteworthy differences in their taxonomy, compared to other *B. bassiana* isolates in the literature, including the ARSEF6444 isolate used in the commercially available product, Mycotrol.

3.2.5.1 Isolation of DNA

Approximately 2.4mg of wet weight biomass and 1.2mg of freeze dried mycelium of each organism was used for DNA extraction. The Plant DNeasy (Qiagen Ltd., Crawley, UK) method was performed following the manufacturer's instructions. As recommended by the protocol, before cell lysis visible tissue clumps were homogenised by using a sterile micropestle followed by brief vortexing. After incubation at 65°C for two hours the cell lysis solution was spun at 800 rpm for 1 min and the supernatant was transferred to a new tube.

3.2.5.2 Primers and polymerase chain reaction conditions

Conditions for Inter Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) were adapted from Grünig *et al.* (2001). Amplification reactions with ISSR-TGT 5′VHVTGTTGTTGTTGTTGT3′ primer and ISSR-UBC890 5′VHVGTGTGTGTGTGTGT3′ primer were undertaken in volumes of 20µl containing 0.5µl primer (100pmol/µl stock solution), 0.8µl of each dNTP (100mM stock, Promega Ltd., Southampton, UK), PCR buffer 10 × *Taq* (Qiagen Ltd., Crawley, UK), $0.05\mu l^{-1}$ *Taq* DNA-polymerase (Qiagen Ltd., Crawley, UK) and 1µl of stock solution of template DNA. Polymerase chain reaction conditions were as follows: an initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation step at 94°C for 1 min, an annealing step at 46°C for 1 min and an extension step at 72°C for 2 min. A final extension step (72°C for 10 min) was included. The samples were held at 10°C until

further processing occurred. At least two PCR reactions were performed per extract. Conditions for Variable Number Tandem Repeat Polymerase Chain Reaction (VNTR-PCR) were adapted from Bridge *et al.* (1997). Amplification reactions with MR 5′ GAGGGTGGCGGTTCT3′ primer, were undertaken in volumes of 25µl containing 0.5µl primer (100 pmol/µl stock solution), 2.0µl of each dNTP (100 mM stock, Promega Ltd., Southampton, UK), PCR buffer 10× *Taq* (Qiagen Ltd., Crawley, UK), 0.05 U µl^{-1} *Taq* DNA-polymerase (Qiagen Ltd., Crawley, UK) and 1µl of stock solution of template DNA. Polymerase chain reaction conditions were as follows: an initial denaturation for 6 min at 95°C was followed by 39 cycles of denaturation step at 95°C for 1 min, an annealing step at 45°C for 1 min and an extension step at 72°C for 1 min. A final extension step (72°C for 5 min) was included. The samples were held at 10°C until further processing occurred. At least two reactions were performed per extract.

3.2.5.3 Electrophoresis conditions

Aliquots (11µl) of amplification products plus 5µl loading buffer (sucrose – 40g; bromophenol blue – 0.05g; dist H$_2$O to 100ml) were mixed and separated in 1.5% (w/v) SeaKem LE agarose (Lonza Ltd., Basel, Switzerland) gels containing 5µl per 100ml Safe View Nucleic Acid Stain (NBS Biologicals Ltd., Huntington, UK). Electrophoresis was undertaken in a midi gel tank (Thermo-Hybaid, UK) in 0.5x TBE buffer (TBE stock solution 5x : 54g Tris base, 27.5g Boric acid, 20ml 0.5 M EDTA (pH 8.0)) following the protocol for electrophoresis buffers (Sambrook and Russell, 2001). Aliquots (10µl) of 100-bp ladder (25ng/µl) Invitrogen Ltd., Paisley, UK) as a molecular weight marker were added as a standard in both sides of the gel. Gels were visualised using a U:Genius gel documentation system (Syngene, UK). Images were stored as TIFF files for subsequent use.

3.2.5.4 ITS Sequencing

Partial ribosomal RNA gene clusters (part of 18S small subunit RNA gene, internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, internal transcribed spacer 2 (ITS2), part of 28S large subunit ribosomal RNA gene) were amplified by polymerase chain reaction (PCR) using primer set TW81 (fwd): 5'–GTTTCCGTAGGTGAACCTGC–3' and AB28 (rev): 5'–ATATGCTTAAGTTCAGCGGGT–3' (Sigma Genosys, UK (Curran *et al.*, 1994). PCR was undertaken in a ThermoHybaid PCR Express thermal cycler (Thermo-Hybaid, UK) using a reaction mix containing 3 pmoles of each primer, 1μl of template DNA solution and 10μl of MegaMix-Royal (Microzone Ltd, UK) containing optimised mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in $2 \times$ Reaction Buffer (6 mM $MgCl_2$) with 400 μM dNTPs made up to a final volume of 20 μl with sterilised ultrapure H_2O. Amplification conditions were: 95°C for 5 min followed by 30 cycles of 30s at 95°C, 30s at 50°C, 45s at 72°C, followed by 5 min at 72°C and held at 10°C.

Aliquots (4μl) of amplification products were assessed for quality by gel electrophoresis using 1.5 % Seakem LE agarose (BMA, UK) for 2 h at 5V cm-1 in half-strength Tris-Borate-EDTA buffer (i.e., $0.5 \times$ TBE buffer; 45 mM Tris; 45 mM Boric acid; 1.25 mM EDTA, pH 7.5 (Sambrook *et al.*, 1989) containing 5μl of SafeView Nucleic Acid Stain (NBS Biologicals Ltd, UK) per 100 ml of buffer.

Remaining unused PCR products were purified with the microCLEAN PCR Purification Kit (Microzone Ltd, UK) following the manufacturer's instructions. Purified PCR products were utilised in sequencing reactions undertaken in a Primus 96 plus thermal cycler (MWG-BIOTECH AG, Germany) by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with primer TW81 (as above).

Sequencing conditions were: 96°C for 1 min followed by 25 cycles of 20s at 96°C, 10s at 50°C, 4 min at 60°C (ramp rate: 1°C s^{-1}). Excess unincorporated BigDye was removed with DyeEx 2.0 affinity columns (Qiagen Ltd., UK) according to the manufacturer's instructions and the sequencing reaction products were suspended in HiDi Formamide (Applied Biosystems, UK). These products were separated on a capillary array 3130 Genetic Analyser (Applied Biosystems, UK). Sequence trace files were first assessed for quality using Sequencing Analysis Software v5.2 Patch 2 (Applied Biosystems, UK) and exported as text files.

3.2.6 Radial growth of *Lecanicillium, Beauveria* and *Chaunopycnis* isolates

The radial growth of EPF isolated in 3.2.2: *L. muscarium* (IMI502731), *Lecanicillium* sp. (IMI502732), *B. bassiana* (IMI502733) and *Chaunopycnis* sp. (IMI502734) was measured in order to compare to those of similar species used in the commercially available products: Mycotrol, ARSEF6444 (*B. bassiana),* Mycotal (*L. muscarium*) and Vertalec (*L. longisporum*), see Table 3:1.

Table 3:1: EPF isolates used in the experiment.

Isolate	Fungal species	Source of isolate
IMI502731	*Lecanicillium muscarium*	*C. fagisuga* entomopathogen
IMI502732	*Lecanicillium* sp.	*C. fagisuga* entomopathogen
Mycotal	*Lecanicillium muscarium*	Koppert Biological Systems
Vertalec	*Lecanicillium longisporum*	Koppert Biological Systems
IMI502733	*Beauveria bassiana*	*F. sylvatica* subsp. *orientalis*
IMI502734	*Chaunopycnis* sp.	*F. sylvatica* subsp. *orientalis*
Mycotrol (ARSEF6444)	*Beauveria bassiana*	USDA ARS collection

For each isolate, five, 9cm Sabouraud Dextrose Agar (SDA) replicate plates were prepared and the experiment was conducted at four temperatures: 10, 15, 20 and 25°C. All agar plates were inoculated centrally with a 5mm diameter agar plug of fungal inoculum. The Petri dishes were marked on the underside using a permanent marker and the cardinal diameters were measured every 2-3 days for 14 days or until cultures had reached the edge of the Petri dishes. The diameter of each colony was recorded for each isolate and temperature combination.

3.2.7 Data analysis

The mean radial growth rates (mm day^{-1}) of the fungal isolates were calculated at the four different temperatures. Growth rates were log transformed and differences in radial growth rates of the EPF isolates and differences in their growth rates at the four temperatures were examined using two-way analysis of variance (ANOVA). ANOVA was also used to examine any interaction between isolate and temperature. *Post hoc* Tukey's range test was used to compare the mean radial growth rates. All statistical analyses were undertaken with GenStat, 12th Edition (VSN International Ltd.).

3.3 RESULTS

3.3.1 Collection and culture of EPF in Armenia

The scale insect was not very widespread, with only one individual tree at the Haghartsin site being heavily infested (>100 insects per 10cm^2), see Figure 3:1. A total of thirteen different fungal isolates were cultured from cadavers of 100 *C. fagisuga* adults (see Table 3:2 and Figure 3:2). Isolated EPF of significant interest included *Lecanicillium muscarium* and *Lecanicillium* sp. as these fungi are well documented entomopathogenic fungi and have been developed and commercialised as mycoinsecticides.

Table 3:2: EPF isolated from beech scale cadavers in Armenia.

Isolate	Fungal identification	IMI number
1	*Alternaria* sp.	-
2	*Cladosporium* sp.1	-
3	*Cladosporium* sp.2	-
4	*Clonostachys* sp.	-
5	*Cylindrocarpon* sp.1	-
6	*Cylindrocarpon* sp.2	-
7	*Fusarium* sp.1	-
8	*Fusarium* sp.2	-
9	*Lecanicillium muscarium*	IMI502731
10	*Lecanicillium* sp.	IMI502732
11	*Penicillium* sp.	-
12	*Pyrenochaeta* sp.	-
13	*Verticillium* sp.	-

Figure 3:1: *Fagus sylvatica* subsp. *orientalis* trunk infested with *Cryptococcus fagisuga* in Armenia.
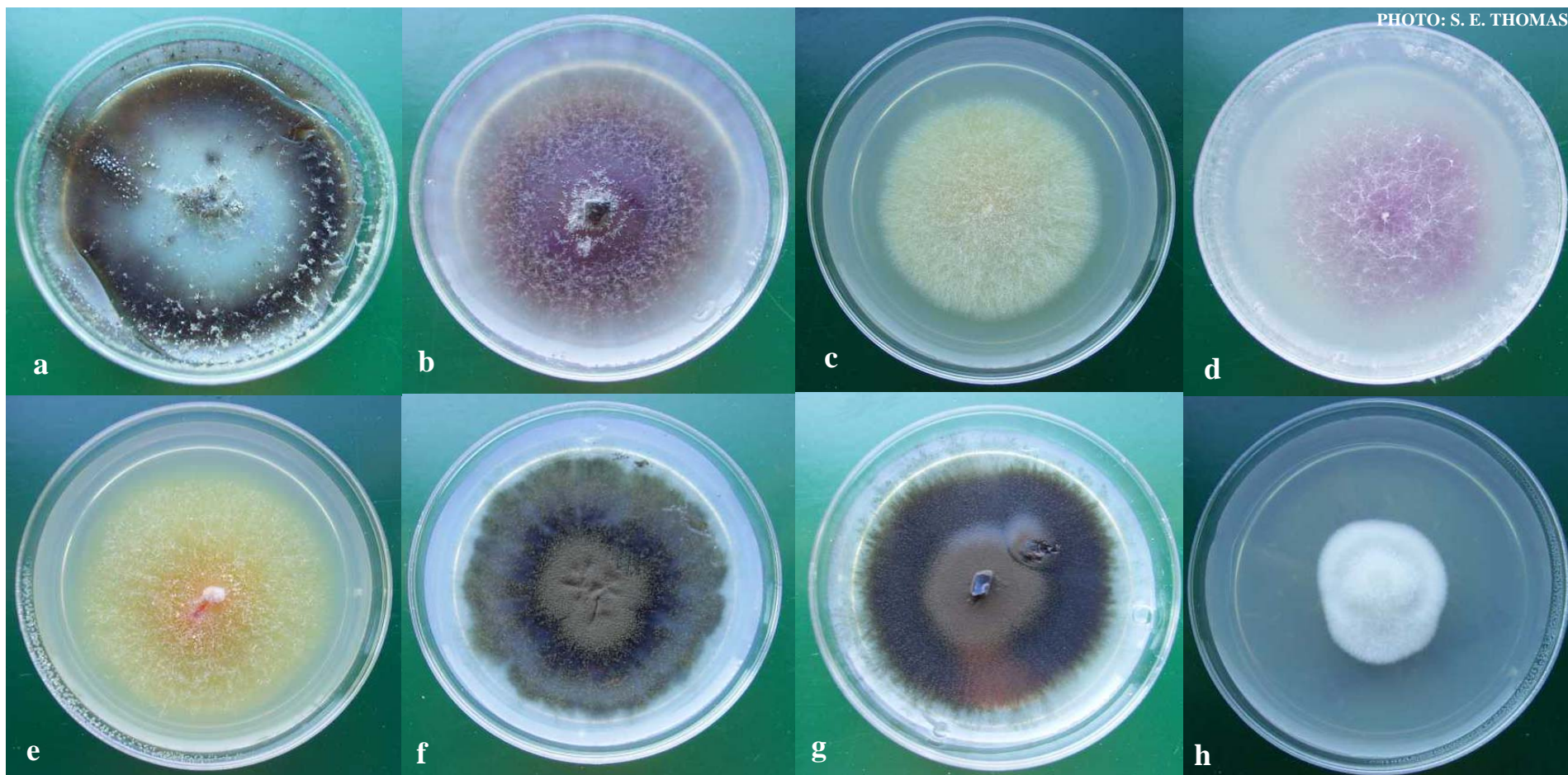
Figure 3:2: Entomopathogens isolated from the beech scale insect, *Cryptococcus fagisuga* **in culture;** *a) Verticillium* **sp.;** *b) Pyrenochaeta* **sp.;** **c)** *Fusarium* **sp.1; d)** *Fusarium* **sp.2; e)** *Cylindrocarpon* **sp.1; f)** *Cladosporium* **sp.2; g)** *Cladosporium* **sp.1 and h)** *Lecanicillium muscarium*.

3.3.2 Inoculation of *C. fagisuga* eggs

After 5 days of incubation at 25°C, it was possible to observe growth of *L. longisporum* (Vertalec) on the inoculated beech scale eggs (see Figure 3:3b, c and d). After 28 days, none of the beech scale eggs inoculated with *L. longisporum* had hatched. Many of the inoculated eggs were no longer intact but where possible, the eggs were surface sterilised and plated onto fresh 5cm TWA. Growth of *L. longisporum* on agar confirmed Koch's postulates. After 7 days, all one hundred control eggs had hatched to produce first instar juveniles (see Figures 3:3a) and remained alive for at least 28 days on TWA plates, see Figure 3:4.
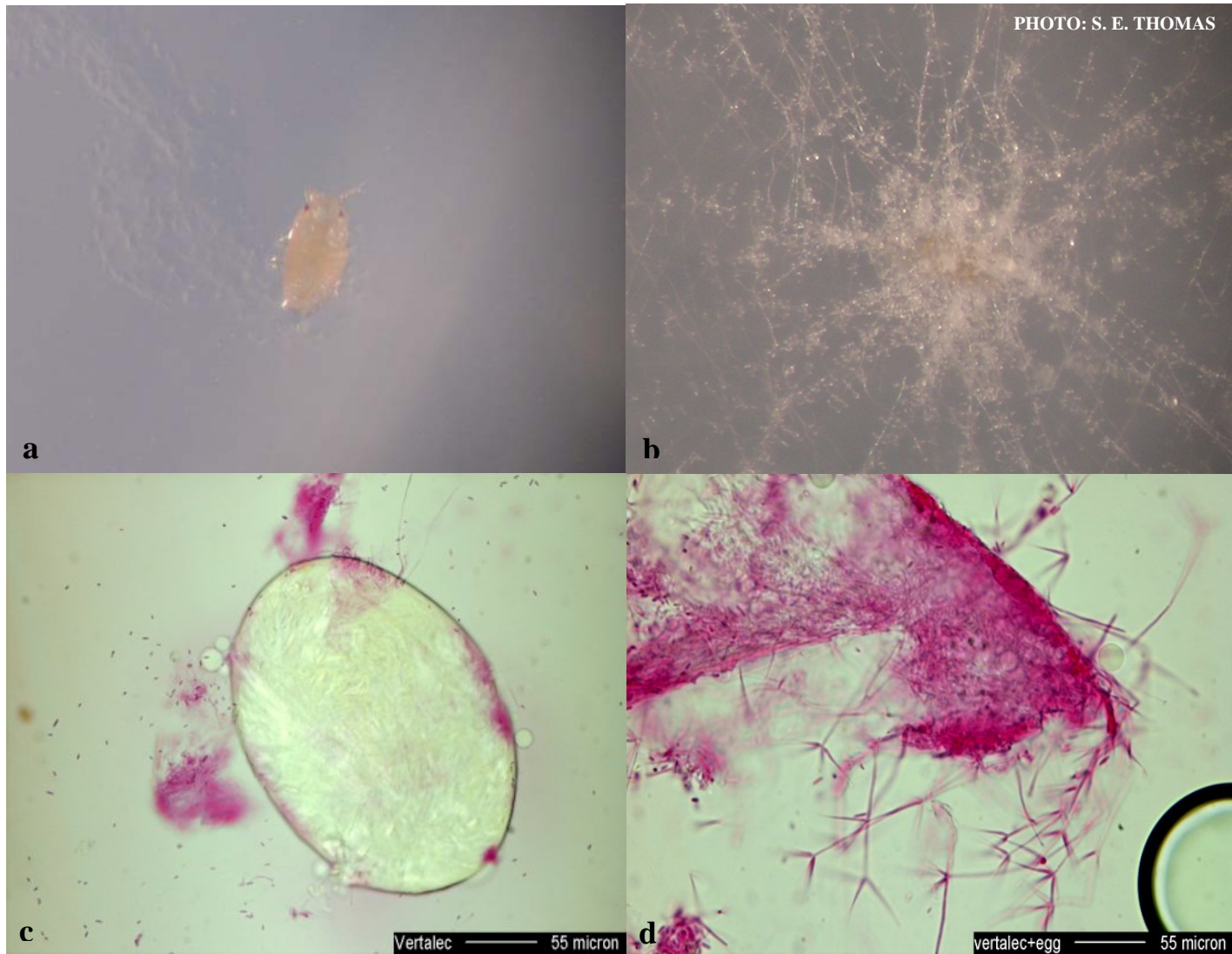
Figure 3:3: Beech scale (*Cryptococcus fagisuga*); a) control-hatchling on agar; b) egg inoculated with *Lecanicillium longisporum* (Vertalec) on agar surface; c) and d) microscopic observation of egg, 5 days after inoculation.
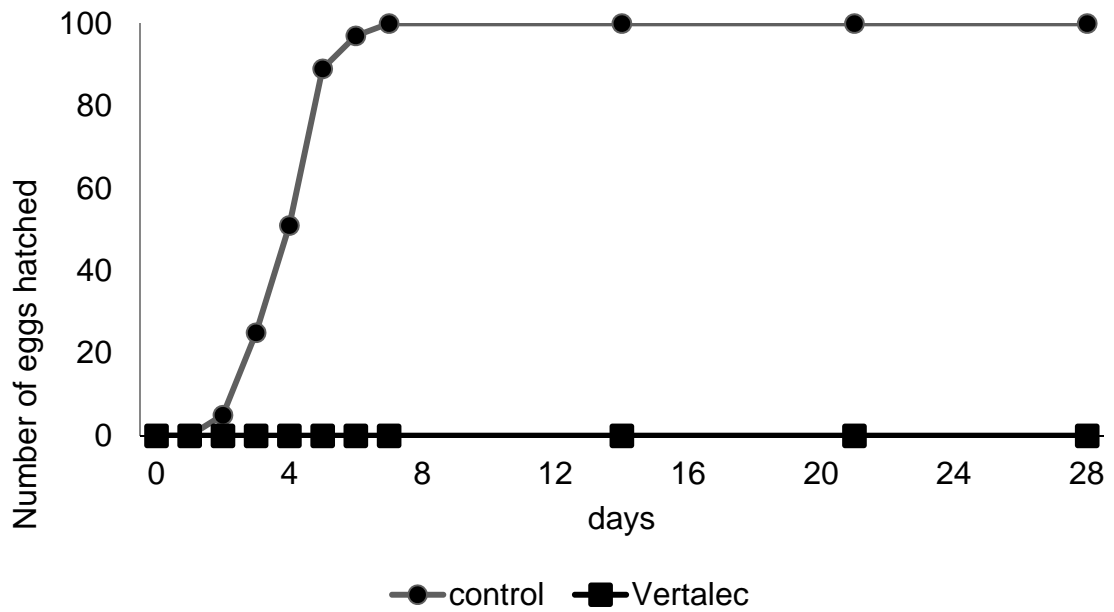
Figure 3:4: Number of *Cryptococcus fagisuga* eggs hatching to produce 1st instar juveniles on Tap Water Agar, after inoculation with *Lecanicillium longisporum* (Vertalec) over a 28 day period.

3.3.3 Inoculation of *C. fagisuga* juveniles

None of the 100 control insects placed on water-inoculated filter papers survived more than five days. Microscopic observations revealed that these crawlers had great difficulty in moving on the wet filter paper due to the surface tension. All 100 crawlers placed on *L. longisporum* inoculated filter papers also died by day 5. The cadavers that had been treated with *L. longisporum* were transferred to 5cm TWA plates and *L. longisporum* was confirmed to be present on the inoculated insects after three days incubation at 25°C (see Figure 3:5). *L. longisporum* was not re-isolated from the control insects.
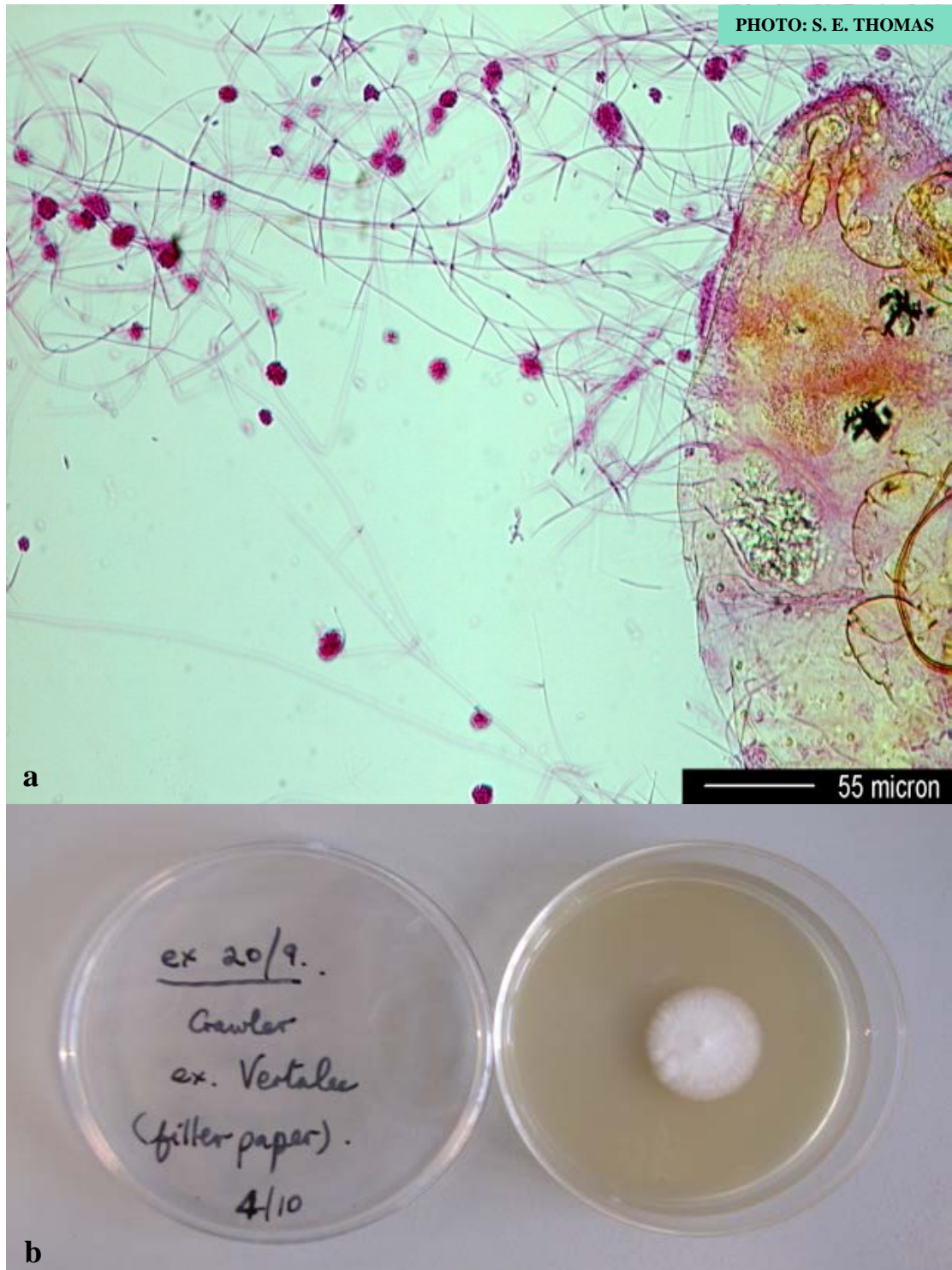
Figure 3:5: a) *Cryptococcus fagisuga* juvenile inoculated with *Lecanicillium longisporum* (Vertalec) and b) re-isolation of the fungus from inoculated crawler.

3.3.4 Molecular analysis of endophytic EPF

3.3.4.1 Sequence data and phylogenetic tree

ITS sequences for IMI502733 and IMI502734 were obtained. A Basic Local Alignment Search Tool (BLAST) search confirmed that IMI502733 is *B. bassiana.* ITS sequences of reference endophytic *B. bassiana* isolates were downloaded from GenBank for comparison (see Table 3:3). The sequences were aligned, then the evolutionary history was inferred using neighbor-joining methods described by Saitou and Nei (1987). Analysis was carried out using Mega5 software (Tamura *et al.*, 2011). The neighbor-joining tree method clustered IMI502733 with the reference isolates of *B. bassiana* including the commercial isolate Mycotrol, see Figure 3:6.

ITS sequencing revealed that isolate IMI502734 was not *B. bassiana* as identified by morphological features but was *Chaunopycnis* sp., another closely related clavicipitacious fungus. *C. alba, C. pustulata* and *Chaunopycnis* sp. were therefore added into the phylogenetic tree for further comparison.

Table 3:3: List of ITS sequences used in phylogenetic tree and their origin.

Isolate number	Species	Source	Publication	GenBank accession number
IMI502733	***Beauveria bassiana***	***Fagus sylvatica* subsp. *orientalis* stem endophyte**	**-**	**NA**
IMI389521	*Beauveria bassiana*	*Sitophilus granarius,* Coleoptera: Curculionidae	-	NA
ARSEF6444	*Beauveria bassiana*	*Diabrotica undecimpunctata* Coleoptera: Chrysomelidae	-	NA
03032	*Beauveria bassiana*	*Coffea arabica,* seed endophyte	(Vega *et al.*, 2008)	DQ287236
03042	*Beauveria bassiana*	*Coffea arabica,* epicarp endophyte	(Vega *et al.*, 2008)	DQ287232
03043	*Beauveria bassiana*	*Coffea arabica,* peduncle endophyte	(Vega *et al.*, 2008)	DQ287231
03044	*Beauveria bassiana*	*Coffea arabica,* crown endophyte	(Vega *et al.*, 2008)	DQ287233
NA	*Beauveria bassiana*		(Gurulingappa *et al.*, 2010)	GU953213
EaBb 04/01	*Beauveria bassiana*	*Timaspis papaveris,* Hymenoptera: Cinipidae	(Quesada-Moraga *et al.*, 2006)	DQ364698
IMI502734	***Chaunopycnis* sp.**	***Fagus sylvatica* subsp. *orientalis* stem endophyte**	**-**	**NA**
NA	*Chaunopycnis alba*		(Bills *et al.*, 2002)	AF389195
NA	*Chaunopycnis pustulata*		(Bills *et al.*, 2002)	AF389193
NA	*Chaunopycnis* sp.		(Slemmons *et al.*, 2013)	JX171164

Beauveria bassiana GU953213

Beauveria bassiana DQ364698

Beauveria bassiana DQ287233

Beauveria bassiana DQ287231

Beauveria bassiana DQ287232

Beauveria bassiana DQ287236

Beauveria bassiana IMI389521

Beauveria bassiana IMI502733

Beauveria bassiana ARSEF 6444

Chaunopycnis sp. IMI502734

Chaunopycnis sp. JX171164

Chaunopycnis alba AF389195

Chaunopycnis pustulata AF389193

0.02

Figure 3:6: Phylogenetic tree of EPF using ITS sequences and the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in Mega5.

3.3.4.2 DNA fingerprinting

DNA fingerprinting results showed the sequence of *B. bassiana*, IMI502733 to be unique from IMI389521 and ARSEF6444 (Mycotrol) using all three primers (MR, TGT and UBC890). This showed that there is intra-species variation and not all *B. bassiana* isolates are genetically identical. Electrophoresis gel photos are shown in Figures 3:7, 3:8 and 3:9.
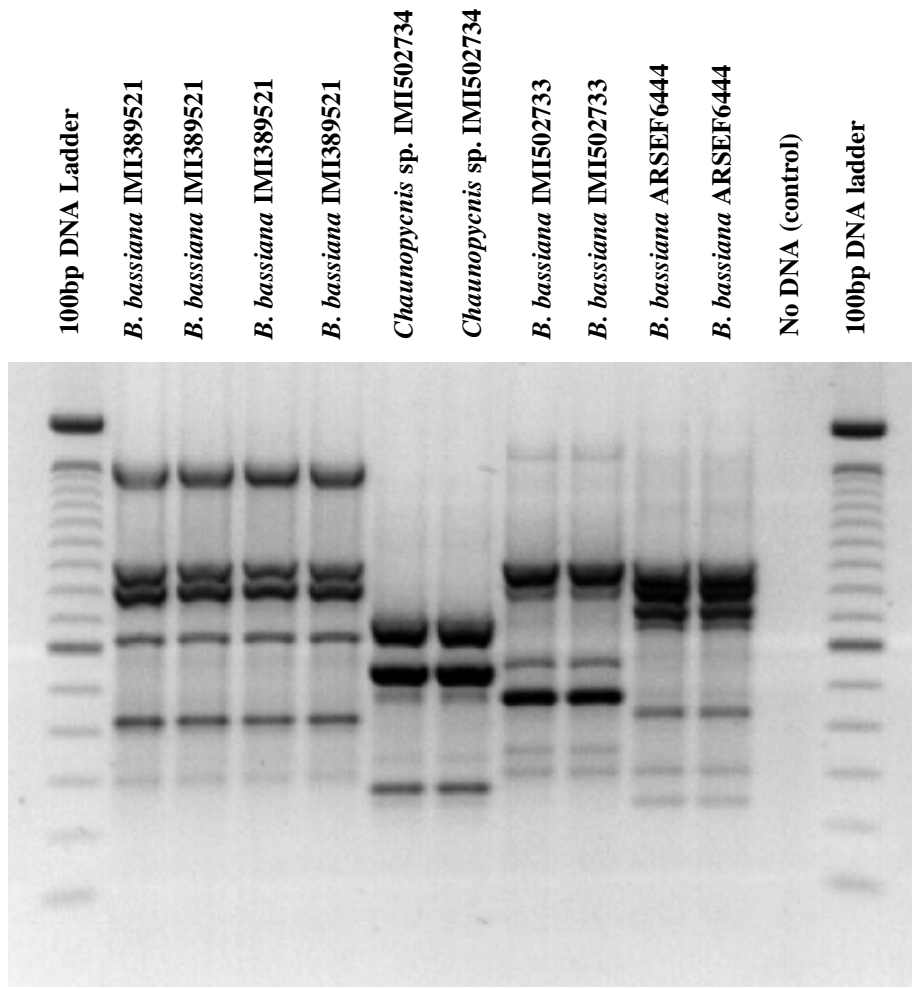
Figure 3:7: Variable Number Tandem Repeat Polymerase Chain Reaction (VNTR-PCR) fingerprints of *Beauveria bassiana* and *Chaunopycnis* isolates with MR primer.
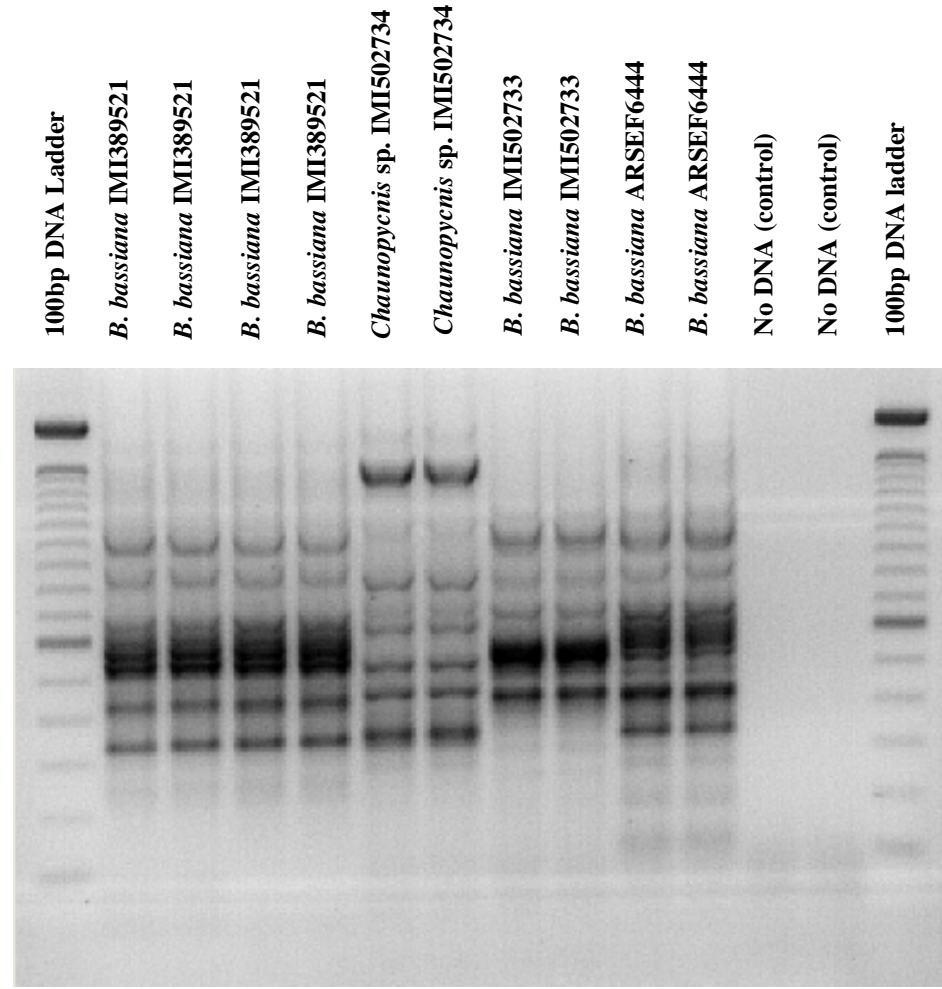
Figure 3:8: Inter-Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) fingerprints of *Beauveria bassiana* and *Chaunopycnis* isolates with TGT primer.
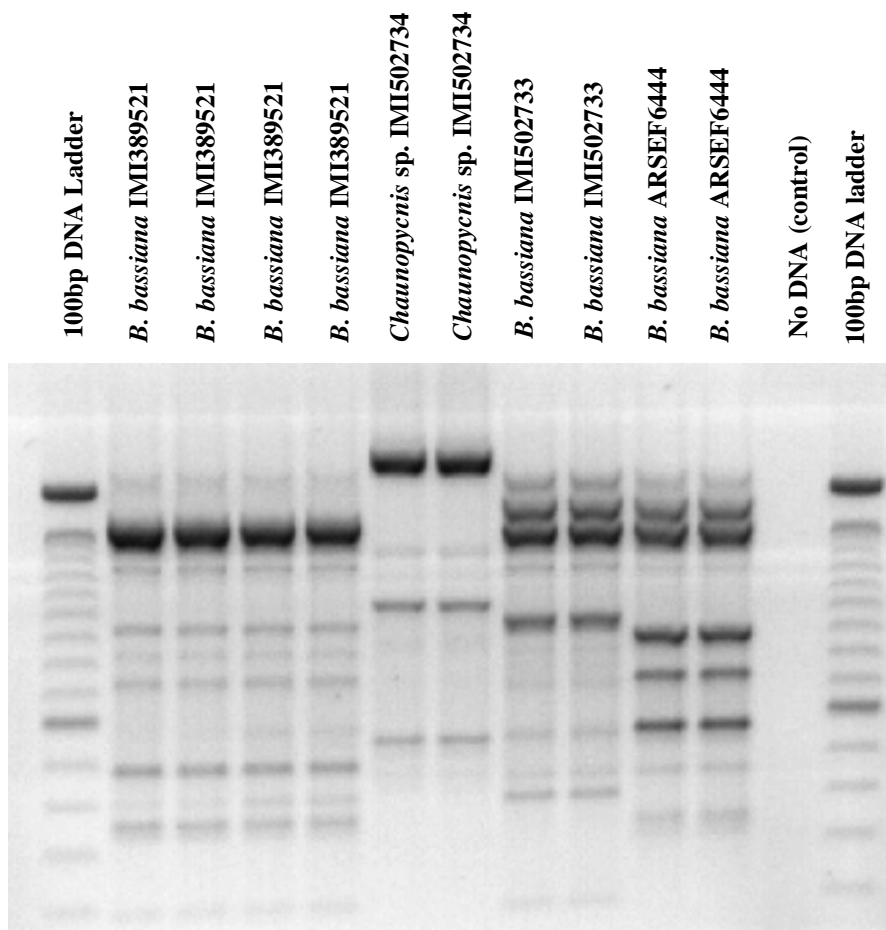
Figure 3:9: Inter-Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) fingerprints of *Beauveria bassiana* and *Chaunopycnis* isolates with UBC890 primer.

3.3.5 Radial growth of *Beauveria*, *Chaunopycnis* and *Lecanicillium* isolates

The *Beauveria* and *Chaunopycnis* isolates grew faster at 25°C. The fastest growing isolate was Mycotrol, which grew 1.81mm day^{-1} at 25°C. At 10°C, the slowest growing isolate was IMI502733, which grew at 0.36mm day^{-1}, see Figure 3:10. ANOVA showed that the three isolates grew significantly different from each other ($F_{2,48}$=107.5, p<0.001) and significantly different at different temperatures ($F_{3,48}$=429.24, p<0.001). The three isolates did not show the same interaction pattern of growth across the four different temperatures and all responses were significantly different ($F_{6,48}$=8.88, p<0.001).
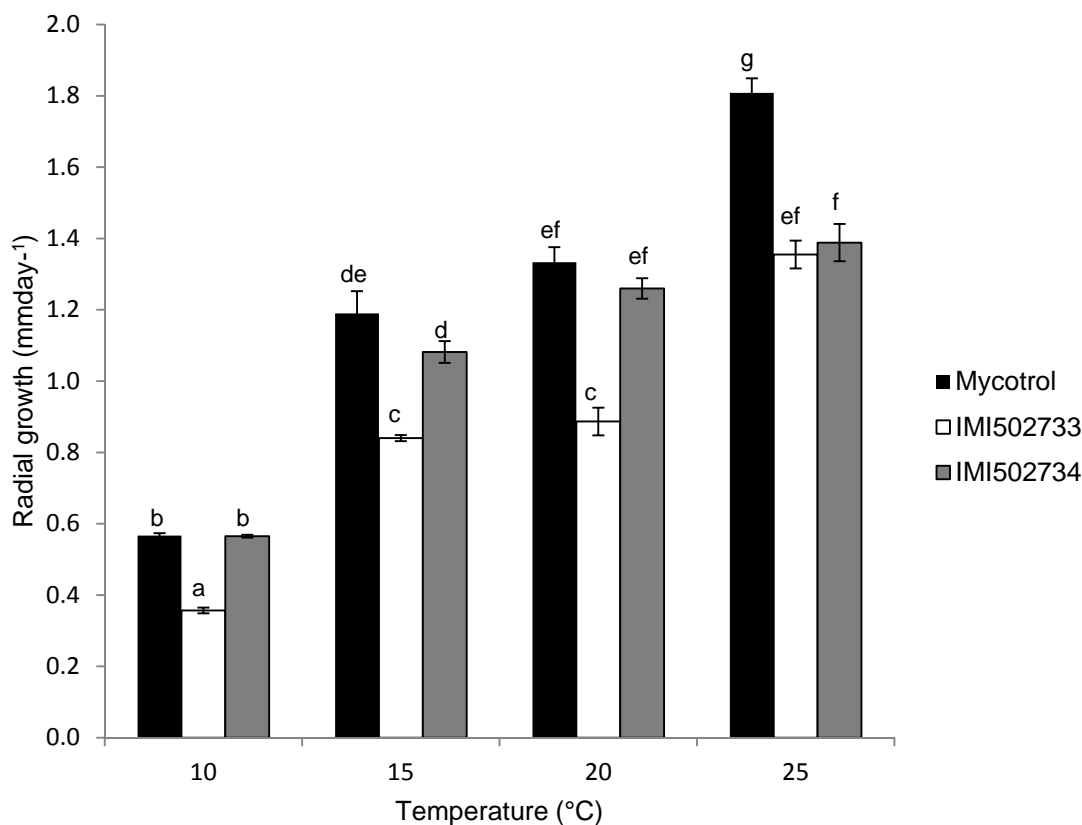
Figure 3:10: Mean radial growth (mmday^{-1}) of *Beauveria bassiana* (Mycotrol), *B. bassiana* (IMI502733) and *Chaunopycnis* sp. (IMI502734) at four temperatures. Means with different letters are significantly different (Tukey's, p<0.001).

The fastest growing *Lecanicillium* isolate was *L. muscarium* (IMI502731) at 20°C. The commercial isolates Vertalec and Mycotal grew fastest at 25°C (1.27 and 1.25mmday^{-1} respectively). The slowest growing isolate was Vertalec at 10°C, which grew at 0.33mmday^{-1}, see Figure 3:11. At 20°C, isolates IMI502731 and IMI502732 had the fastest growth rates.

ANOVA showed that the four *Lecanicillium* isolates grew significantly different from each other ($F_{3,64}$=26.31, p<0.001) and significantly different at the four temperatures ($F_{3,64}$=229.25, p<0.001). The four isolates did not show the same interaction pattern of growth across the four different temperatures and all responses were significantly different ($F_{9,64}$=28.96, p<0.001).
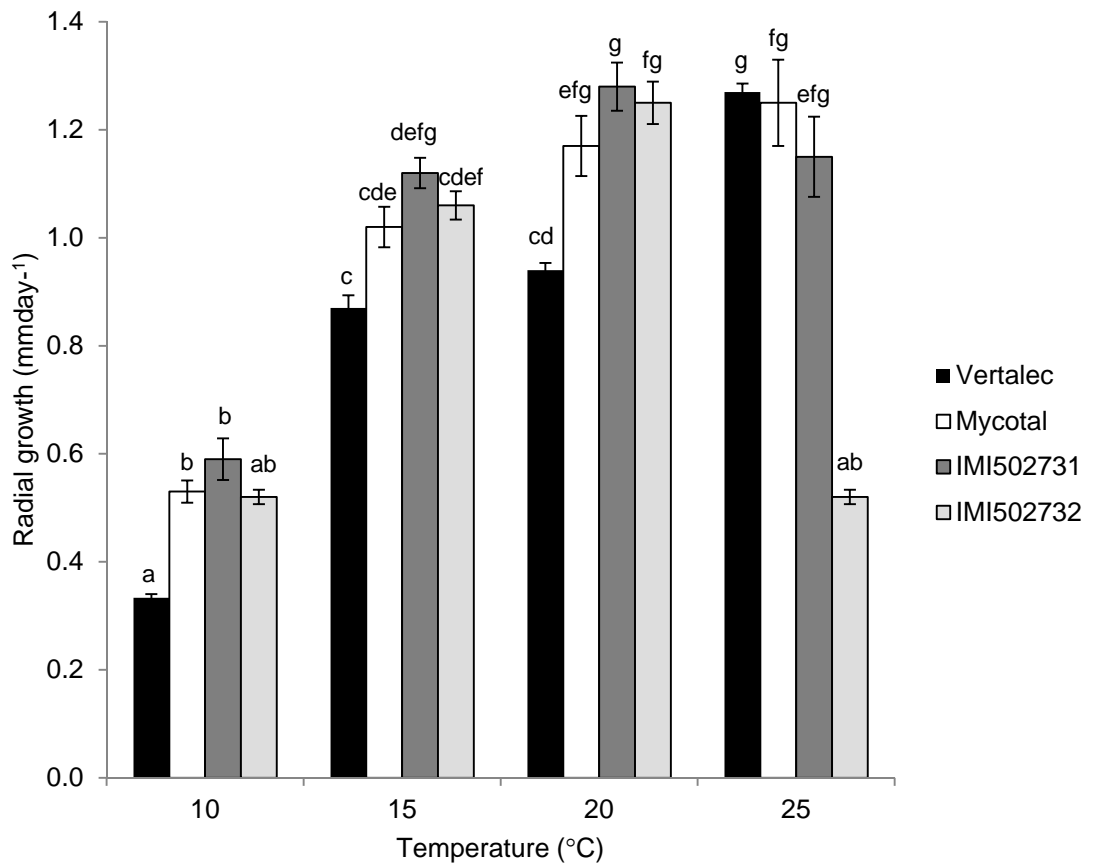
Figure 3:11: Mean radial growth (mmday^{-1}) of *Lecanicillium longisporum* (Vertalec), *L. muscarium* (Mycotal), *L. muscarium* (IMI502731) and *Lecanicillium* sp. (IMI502732) at four temperatures. Means with different letters are significantly different (Tukey's, p<0.001).

3.4 DISCUSSION

Thirteen different fungal isolates were cultured from adult *C. fagisuga* cadavers, in its centre of origin in Armenia including *L. muscarium* (IMI502731) and *Lecanicillium* sp. (IMI502732). This genus is widely used as the active ingredient of many commercial mycoinsecticides and has potential for use as a biological control agent for *C. fagisuga*. *B. bassiana* was <u>not</u> isolated from beech scale in Armenia but was previously isolated from its host, *F. sylvatica* subsp. *orientalis* as an endophyte (see chapter 2).

Development of a successful inoculation technique for *C. fagisuga* with EPFs was difficult. Preliminary *in vitro* experiments with *C. fagisuga* eggs showed that *L. longisporum* (Vertalec) was able to reduce egg hatching by 100%. However, the experiment with crawlers was unsuccessful as the control insects also died. Laflamme *et al*. (2009) also attempted a similar experiment with *L. muscarium* and *C. fagisuga* eggs but these workers only achieved a two-day delay in egg hatching compared to control eggs. This present study with *L. longisporum* achieved 100% kill of eggs. Laflamme *et al*. (2009) also infected juveniles with *L. muscarium* (Mycotal) and *Beauveria* on bark discs and both biological control agents reduced the population by 50%.

DNA sequencing of isolate IMI502733, confirmed it to be *B. bassiana* but isolate IMI502734, which had initially been identified morphologically as *Beauveria* sp. was molecularly identified as *Chaunopycnis* sp. At 25°C, there was a difference in the pattern of growth of the three isolates which corroborated its initial misidentification as *Beauveria* sp. (IMI502734). However, *Chaunopycnis* is another anamorphic genus belonging to the Clavicipitaceae family and all genera in this family are typically entomopathogenic. This fungus has previously been recorded as an endophyte of wild cocoa, *Theobroma gileri* by Thomas *et al.* (2008). Further molecular analysis of this

isolate (IMI502734) by a taxonomic expert would be worthwhile, especially as this genus is known to produce indole diterpenes (Bills *et al.*, 2002), a group of secondary metabolites associated with insect toxicity (Saikia *et al.*, 2008). Many of the fungi that synthesise these compounds are known to form symbiotic relationships with plants, insects and other fungi (Parker and Scott, 2004).

DNA fingerprinting of *B. bassiana* isolates with three primers, showed that IMI502733, an endophyte of *F. sylvatica* subsp. *orientalis*, is genetically different from two isolates originating from Coleoptera: IMI389521 (ex *Sitophilus granaries*) and ARSEF6444 (ex *Diabrotica undecimpunctata*). It may be possible that endophytic isolates of *B. bassiana* (and possibly other fungi) are molecularly different to non-endophytic ones. DNA fingerprinting of further endophytic *B. bassiana* isolates, e.g., those isolated from coffee by Vega *et al.* (2008) would make an interesting comparison.

There are some promising indicators that biological control of *C. fagisuga* with EPF should be investigated further. However, because of the impracticalities of developing *in vitro* bioassays with *C. fagisuga* (its minute size, waxy covering and dependence on mature trees for its survival) it was decided not to pursue this line of research any further. Instead, attention was re-directed towards determining further properties of the EPF in order to develop them further as biological control agent.

B. bassiana (IMI502733) had a significantly slower growth rate to the commercial isolate ARSEF6444 (Mycotrol). DNA fingerprinting of this isolate, using three different primers showed it to be genetically unique from other *B. bassiana* isolates. This information would be of great importance for its future development as a

mycoinsecticide as it for example, could be used to confirm the individual identity of the isolate if it was applied and re-isolated in the field.

Growth rates of the *Lecanicillium* isolates were significantly different, which highlights the importance of selecting the correct isolate or strain when developing a mycoinsecticide. *L. muscarium* (IMI502731) and *Lecanicillium* sp. (IMI502732) grew fastest at 20°C and these isolates of *Lecanicillium* may be better suited to the temperate beech forests of North America than an off-the-shelf product.

It would be of interest to pursue entomopathogens as a potential source of biological control agents, as it may be possible to reduce or contain beech scale populations and hence reduce the incidence of BBD. Virulence of the *L. muscarium* isolate (IMI502731) could be assessed *in vitro* with an experimental insect such as *Galleria mellonella*. Further research to assess spore production, spore viability, mass production and formulation of EPF isolates could be undertaken to gather further knowledge on their suitability as an effective mycoinsecticides for BBD.

The preliminary work carried out here gives an indication that there is potential for the use of EPF as biological control agents. The beech scale insect is exceptionally difficult to manipulate due to its small size and work with this insect *in vitro* is therefore limited. However, fungi such as *Beauveria* and *Lecanicillium* do deserve further research in the glasshouse or field. Due to the large size of mature beech trees and the vast areas of forest affected by *C. fagisuga* in North America, it would probably not be possible to treat all affected trees in a forestry situation but application to, and protection of nursery stock using an EPF could possibly be developed to provide some protection from *C. fagisuga*. Subject to successful inoculation of beech seedlings or saplings with EPF

and subsequent artificial infestation with *C. fagisuga* using the methodology developed by Houston (1982), it may be possible to assess the biological control potential of EPF *in planta*. Methods of application of EPF to beech saplings will be investigated in chapter 5 of this thesis.

Methods for the potential use of endophytes for biological control of the *Neonectria* component of BBD are also explored in this thesis (chapter 4). Development of a biopesticide by mass production and application of a beech scale-specific strain of an entomopathogen, such as *B. bassiana* or *L. muscarium* from Europe, could offer an effective biological control agent for the beech scale, which could consequently reduce infection by the *Neonectria* component of the BBD complex.

4. *IN VITRO* SCREENING OF ENDOPHYTIC *TRICHODERMA* ISOLATES FOR FUNGAL ANTAGONISM OF *NEONECTRIA COCCINEA* AND *N. FAGINATA*

4.1 INTRODUCTION

A second biological control strategy for BBD is to target the pathogen, *Neonectria coccinea* or *N. faginata* with an antagonistic fungus. As *Trichoderma* spp. are known to be effective antagonists of plant pathogens, endophytic *Trichoderma* isolates from the study in chapter 2 were selected for further investigation. The antagonistic ability of nine endophytic *Trichoderma* isolates was assessed *in vitro* against two isolates of the fungal (and most acute) component of the BBD complex, *N. coccinea* and *N. faginata*, in order to determine their suitability as biological control agents.

4.1.1 Morphology and taxonomy of the genus *Trichoderma*

Trichoderma is a genus of asexual fungi that belongs to the ascomycete order Hypocreales and the sexual stage associated with it is the genus *Hypocrea*. *Trichoderma* appears as green, yellow or white colonies on agar media, producing abundant quantities of spores (or conidia). Conidiophores are hyaline with many branches. Phalides can be singular or can occur in groups. Conidia are ovoid, one-celled and occur in small terminal clusters (Barnett and Hunter, 1998), see Figure 4:1. *Trichoderma* was first described by Persoon in 1794 and originally included just four species (Samuels, 1996). Rifai (1969) used morphological and colony characteristics to review and describe nine "aggregate species" of *Trichoderma*: *T. auroviride* Rifai, *T. hamatum* (Bonord.) Bain., *T. harzianum* Rifai, *T. koningii* Oudem., *T. longibrachiatum* Rifai, *T. piluliferum* Rifai, *T. polysporum* (Link: Fr.) Rifai, *T. pseudokoningii* Rifai and *T. viride* Pers.. Since the introduction of molecular techniques, the identification of new

Trichoderma species has rapidly accelerated and by 2006, over one hundred species had

been identified (Druzhinina *et al.*, 2006).
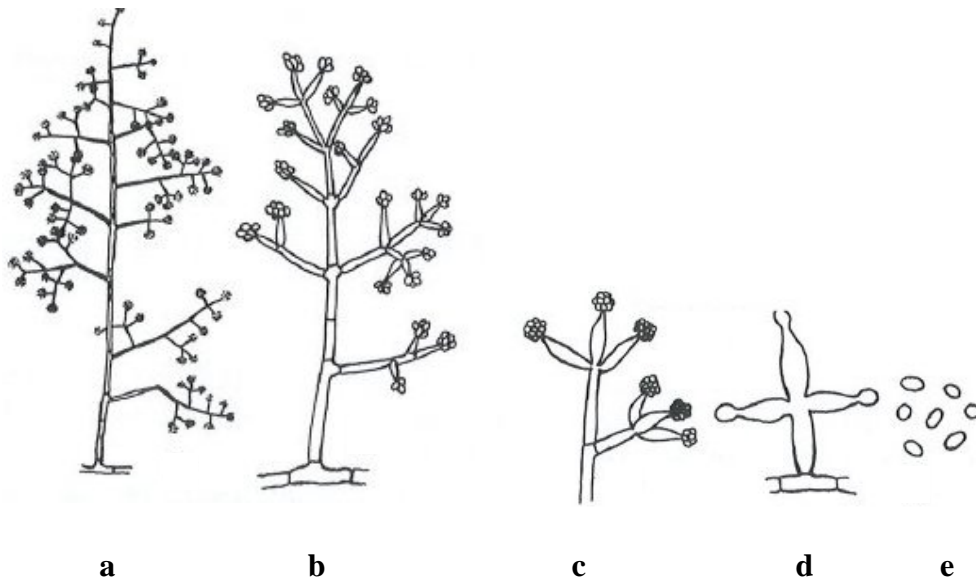
Figure 4:1: Typical morphology of *Trichoderma*; a) and b) conidiophores with extensive branching; c) and d) phialides and e) conidia (Barnett and Hunter, 1998).

4.1.2 Ecology of *Trichoderma* spp.

Trichoderma is a ubiquitous genus that generally occurs as a plant epiphyte or

saprophyte on decaying plant material or soil (Samuels, 1996). The genus can be found

at all latitudes but some species are limited in their geographical distribution (Samuels,

2006). *Trichoderma* has also been isolated from temperate trees such as aspen, oak and

beech (Cotter and Blanchard, 1982; Chapela, 1989; Ragazzi *et al.*, 1999). More

recently, species of *Trichoderma* have been isolated as endophytes from mature

Theobroma cacao trees (Holmes *et al.*, 2004; Samuels *et al.*, 2006; Bailey and Melnick,

2013) however, *Trichoderma* spp. have only been isolated from felled stems of healthy

F. sylvatica trees (Baum *et al.*, 2003).

4.1.3 *Trichoderma* spp. as biological control agents

Fungi belonging to the genus *Trichoderma* have been widely investigated as biological control agents for plant diseases (Bell *et al.*, 1982; Papavizas, 1985; Harman *et al.*, 1989; de Melo and Faull, 2000). Several species of *Trichoderma* are now available as commercial biocontrol agents in the agricultural industry. Some examples of such products on the market are: Trichomic (Trichodex, Spain, http://www.amcchemical.com) which contains a cocktail of five *Trichoderma* spp. and is antagonistic to a variety of soil pathogenic fungi. T-22 HC (Bioworks, USA, http://www.bioworksbiocontrol.com) is a strain of *T. harzianum* that provides protection against plant root pathogens such as *Pythium, Rhizoctonia, Fusarium, Cylindrocladium* and *Thielaviopsis.* Some more examples include Vinevax (Key Industries, New Zealand, http://www.keyindustries.co.nz), a *T. harzianum* strain produced for the protection of vines and trees against vascular trunk diseases such as *Eutypa lata* and TRICHOgold (Myagri, Malaysia, http://myagrigroup.com), a plant defence booster employing two *Trichoderma* species, *T. harzianum* and *T. viride*, to protect vegetables, ornamental crops, trees, shrubs, turf grasses and fruits crops, tobacco and plantation crops against soil-borne pathogens.

Trichoderma species have been successfully used as biological control agents against soil-borne and seed-borne pathogens, as well as diseases of the phyllosphere for economically important crops such as cotton, onion, pea and apple (Papavizas, 1985; Tronsmo, 1986; Chet, 1987) and also wood decay fungi (Bruce and Highley, 1991; Schubert *et al.*, 2008b). *Trichoderma* species have many advantageous characteristics that make them good biocontrol candidates. They are fast growing, which enables them to establish themselves quickly once applied and are good sporulators to ensure regeneration of the biocontrol agent. They are also tolerant of a wide range of

environmental factors (Tronsmo and Hjeljord, 1997), which means that it is possible to match a species or even specific isolate to the appropriate environmental conditions. Some workers have applied mixtures of *Trichoderma* spp. or isolates to maximise their effect (Harman *et al.*, 1989; Krauss and Soberanis, 2001).

4.1.4 Mechanisms of fungal antagonism

There are three main, direct mechanisms demonstrated by biological control fungi such as *Trichoderma* that enable them to function against plant pathogens (Tronsmo and Hjeljord, 1997; Harman *et al.*, 2004). These mechanisms are competition, mycoparasitism and antibiosis (Howell, 2003). Another indirect mechanism employed by *Trichoderma* spp. is induced systemic resistance, whereby the fungus triggers the defence mechanisms within the host plant. De Meyer *et al.* (1998) showed that treating soil with *T. harzianum* (T-39) made leaves of bean plants resistant to diseases caused by the fungal pathogens *Botrytis cinerea* and *Colletotrichum lindemuthianum*.

4.1.4.1 Competition

The first mechanism is competition, which was first described by Wicklow (1992) as the simultaneous demand by two individuals for space and resources. *Trichoderma* species are able to compete with other fungi for carbon and nitrogen resources as well as for physical space. For example, *T. harzianum* is able to control *B. cinerea* (grey mould) on grapes by colonizing the blossom tissue and excluding the pathogen from the infection site (Gullino, 1992). Sivan and Chet (1986) demonstrated that competition for nutrients is the major mechanism used by *T. harzianum* to control *Fusarium oxysporum* f. sp. *melonis*.

4.1.4.2 Mycoparasitism

Mycoparasitism is described as the direct parasitism of one fungus by another through the production of lytic enzymes (Dennis and Webster, 1971a; Dennis and Webster, 1971b; Dennis and Webster, 1971c). Four stages can be recognised in the process. The first is chemotrophic growth whereby the antagonist (*Trichoderma*) is attracted to a chemical stimulus produced by the pathogen. The second stage is a recognition process in which lectins are thought to play a vital role (Elad *et al.*, 1983). The third stage is attachment of hyphae. Hyphal interaction studies undertaken by Dennis and Webster (1971c) and Tronsmo (1986) showed that *Trichoderma* hyphae exhibited coiling of the pathogen hyphae. The final stage in the process is degradation of the host's cell wall through the production of lytic enzymes such as chitinases and 1, 3-β-glucosidases (Elad *et al.*, 1983).

4.1.4.3 Antibiosis

Antibiosis is the process by which toxic metabolites or antibiotics produced by one organism have a direct effect on another organism. Interest in metabolite production by *Trichoderma* species has increased ever since Weindling (1934) reported the toxic effects that culture filtrates of *T. lignorum* had on *Rhizoctonia solani* and prompted interest in this genus for biological control of plant diseases. *Trichoderma* spp. are known to produce soluble secondary metabolites and lytic enzymes such as cellulases, chitinases and glucanases which inhibit growth of pathogenic fungi. *T. virens* produces the anitibiotics viridian, viridol, gliovirin and gliotoxin. Studies have since been conducted with species in this genus and have focussed on this concept.

In this chapter, nine endophytic *Trichoderma* isolates were screened *in vitro* against the BBD pathogens *N. coccinea* and *N. faginata* by assessing three direct mechanisms of antagonism: competition, mycoparasitism and antibiosis. The potential of *Trichoderma* spp. as biological control agents for BBD will be discussed.

4.2 MATERIALS AND METHODS

4.2.1 Fungal cultures and their maintenance

4.2.1.1 Neonectria inoculum

An isolate of *N. coccinea* (IMI113898 ex *F. sylvatica,* UK) and of *N. faginata* (IMI268223 ex *F. grandifolia,* Canada) were obtained from the Genetic Resource Collection (GRC) at CABI (formerly the International Mycological Institute, IMI). Both isolates were maintained on two media types: Potato Carrot Agar (PCA) and 3% Malt Extract Agar (MEA) at 25°C (see Figure 4:2).
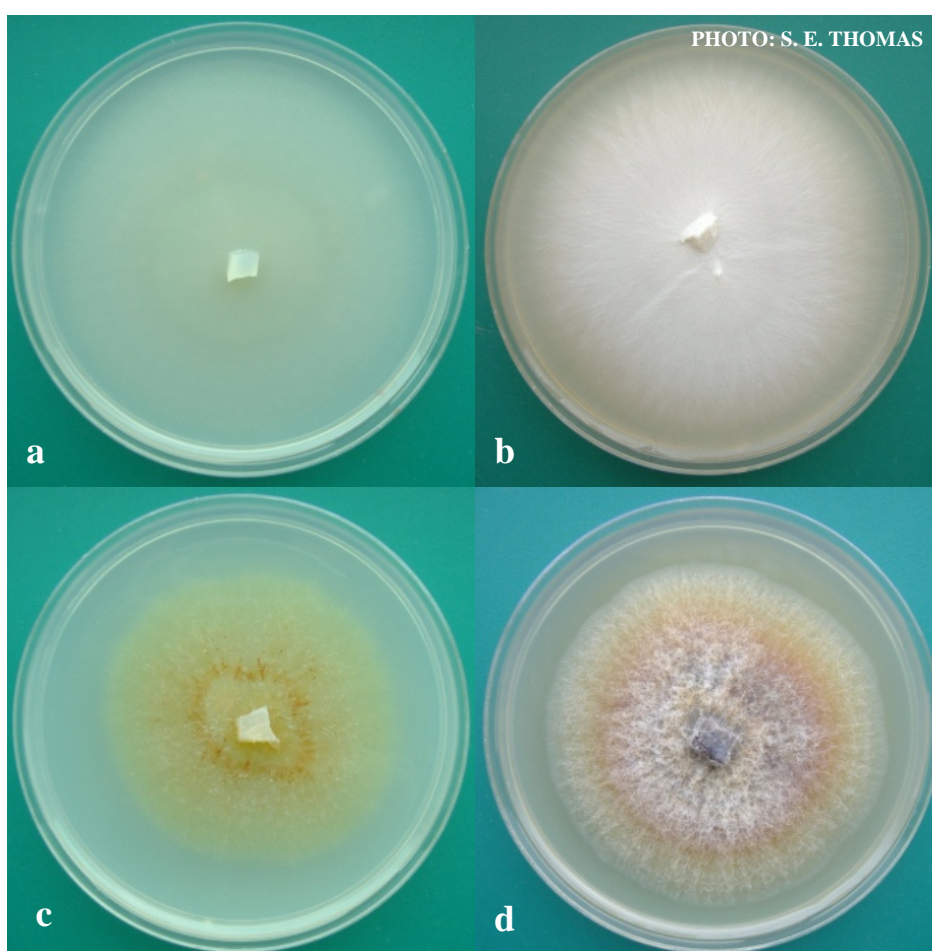
Figure 4:2: *Neonectria* **isolates maintained on agar in the laboratory. a) IMI113898 on PCA, b) IMI113898 on MEA, c) IMI268223 on PCA and d) IMI268223 on MEA.**

4.2.1.2 Trichoderma inoculum

Nine *Trichoderma* stem endophyte isolates were selected from those isolated from the native range of *F. sylvatica* in the UK, identified to species level by Gary Samuels (formerly of USDA) and deposited in the CABI-GRC (see Table 4:1). All isolates were grown on PCA for 10 days at 25°C with near-UV light for the induction of sporulation.

Table 4:1: *Trichoderma* isolates used in the study.

Isolate number	IMI number	Identification
1	IMI395629	*Trichoderma viride*
2	IMI395630	*T. viridescens*
3	IMI395631	*T. viridescens*
4	IMI395632	*T. harzianum*
5	IMI395633	*T. harzianum*
6	IMI395634	*T. harzianum*
7	IMI395635	*T. stilbohypoxyli*
8	IMI395636	*T. harzianum*
9	IMI395637	*T. viridescens*

4.2.2 Growth rates of *Neonectria* isolates

The growth rates of *N. coccinea* (IMI113898) and *N. faginata* (IMI268223) were established by measuring colony diameter on two different media (PCA and 3% MEA) at 15, 20, 25 and 30°C. 9cm agar plates were inoculated centrally with a 5mm agar plug of the pathogen. Both *Neonectria* isolates were replicated five times on both MEA and PCA. Within each incubator, agar plates were arranged randomly and incubated in the dark. Colony diameter was measured daily for 10 days and the radial growth rates (mmday^{-1}) of the isolates were calculated on the two media types at the four

temperatures. Radial growth rates were log transformed and analysis of variance (ANOVA) was used to determine any differences between growth rates of the two pathogens, the two media types and growth rates of both pathogens at the four temperatures. Interactions between pathogen isolate, media type and temperature were also analysed using GenStat, 12th edition (VSN International Ltd.).

4.2.3 Measurement of competition through dual plate interactions between *Neonectria* and *Trichoderma* isolates

Dual culture interaction plates were set up following the method of Badalyan *et al.* (2004). 5mm inoculum plugs of *Neonectria* and *Trichoderma* were taken from the growing edge of 3-day old cultures and placed on opposite sides of a 9cm agar plate (see Figure 4:3).
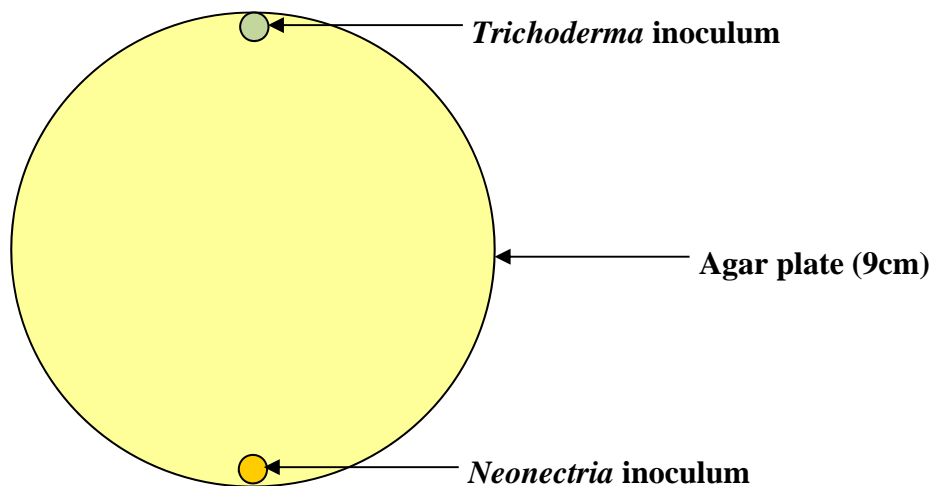
Figure 4:3: Dual plate set-up. Agar plate inoculated with agar plugs of *Trichoderma* and *Neonectria*.

Each *Neonectria*/*Trichoderma* combination was replicated five times on both MEA and PCA. Plates were sealed and placed in the dark at 25°C. Negative controls were set up by inoculating agar plates with *Neonectria* plugs and un-inoculated agar plugs. Antagonism towards the *Neonectria* isolates was scored using the method of Badalyan (2004) according to 3 types (A, B, and C) and 4 sub-types (C_{A1}, C_{A2}, C_{B1} and C_{B2}), see Table 4:2 for descriptions of the interaction types. Each interaction type and sub-type was assigned a score from 1-5, to calculate an antagonism index (AI).

$$AI = \Sigma N \times I$$

where N= number (frequency) of each type or sub-type and I = score.

Table 4:2: Fungal interaction types and descriptions with corresponding score (Badalyan *et al.*, 2004).

Type	description	score
A	deadlock with mycelial contact	1
B	deadlock at a distance	2
C	replacement, overgrowth without deadlock	3
C_{A1}	partial replacement after initial deadlock with mycelial contact	3.5
C_{B1}	partial replacement after initial deadlock at a distance	4
C_{A2}	complete replacement after initial deadlock with mycelial contact	4.5
C_{B2}	complete replacement after initial deadlock at a distance	5

Therefore, a maximum, cumulative AI score of 20 could be recorded for each *Trichoderma* isolate against both *Neonectria* pathogens on both media types. *Trichoderma* isolates with a cumulative AI ≥18 were considered to be highly antagonistic as these isolates could completely replace the *Neonectria* pathogens on both media types.

4.2.4 Measurement of the mycoparasitic ability of *Trichoderma* endophytes vs. *N. coccinea* and *N. faginata* using the pre-colonised plate (PCP) method

The nine endophytic *Trichoderma* isolates (see Table 4:1) were screened for their mycoparasitic ability using a modification of a pre-colonised plate (PCP) method (Foley and Deacon, 1985; Krauss *et al.*, 1998; Evans *et al.*, 2003). Inoculum strips (2.5 × 0.5 cm) from 7-day old sporulating cultures of the potential antagonists were placed on top of 9cm agar plates (MEA and PCA), pre-colonised with one of the beech bark disease pathogens (*N. coccinea;* IMI113898 and *N. faginata*; IMI268223, see Figure 4:4). Five replicate plates were prepared for each treatment. All plates were incubated at 25°C in the dark and sampled at one-week intervals either until plates were 100% colonised by the *Trichoderma* isolate or for a maximum of five weeks. Fifteen agar-plug samples were removed from the PCP with a sterilised 5mm cork borer and placed onto 9cm PCA plates (see Figures 4:4 and 4:7). Agar plugs were observed for the presence of the mycoparasite (*Trichoderma*) and the percentage colonisation of the *Neonectria* isolate was determined. For this study, *Trichoderma* isolates scoring over 50% colonisation were considered to be effective mycoparasites of *Neonectria* spp. Angular transformation of the percentage colonisation data was performed before determining any differences between *Trichoderma* isolates using ANOVA. ANOVA was also used to determine any differences between pathogen isolate and media type as well as any interactions between *Trichoderma* isolate, pathogen isolate and media type.
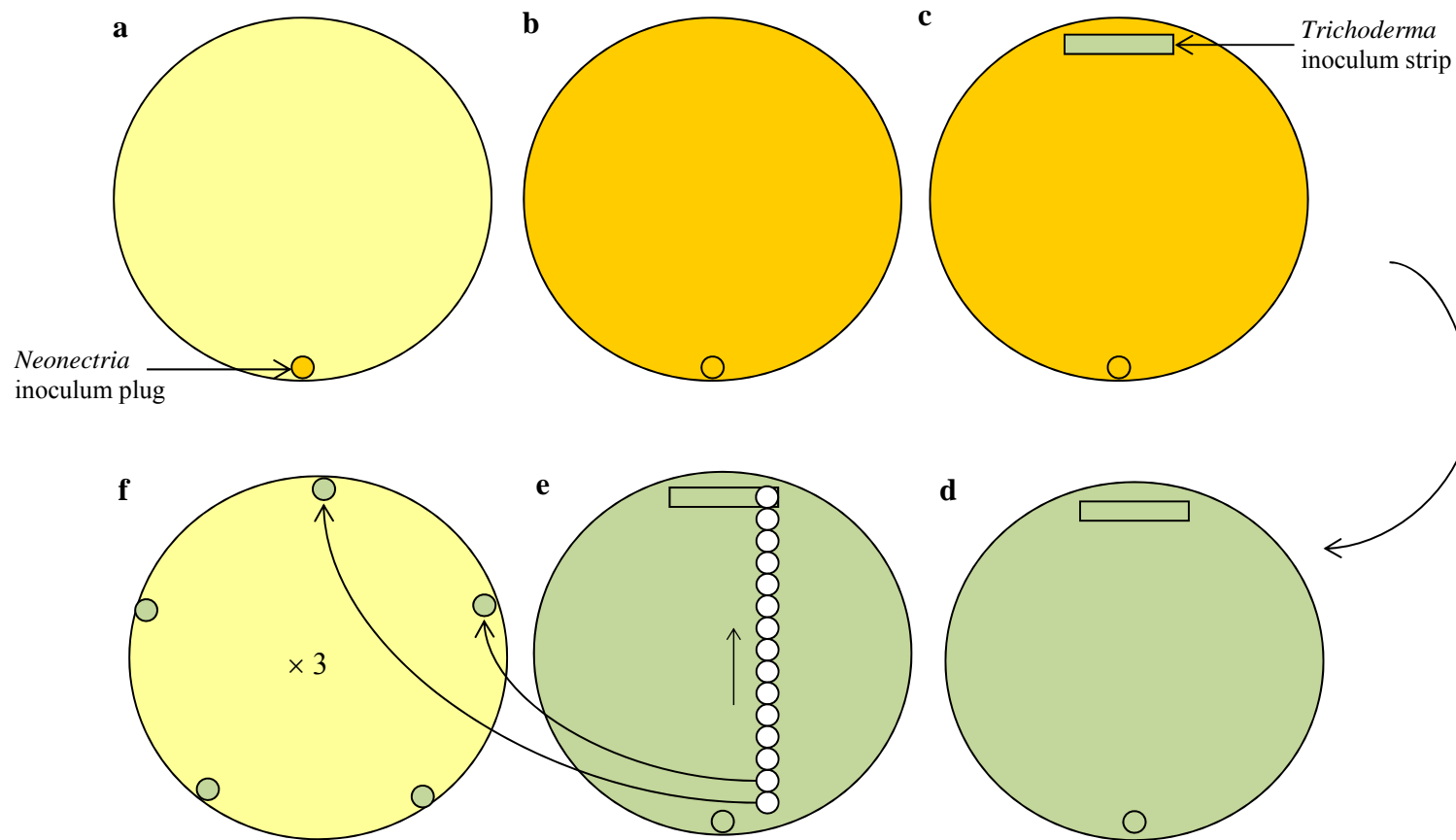
Figure 4:4: Pre-colonised Plate (PCP) method.
a) inoculate agar plate with a 5mm plug of *Neonectria* isolate; b) once fully colonised; c) place strip of *Trichoderma* inoculum (0.5 × 2.5cm) at opposite edge; d) incubate for 24h at 25°C, then invert and incubate; e) every 7 days, remove x15, 5mm plugs from the distal edge towards the *Neonectria* inoculum and f) transfer agar plugs to fresh agar plates and observe growth of *Trichoderma* and/or *Neonectria*.

4.2.5 Measurement of antibiosis by *Trichoderma* isolates through production of soluble inhibitory metabolites

To assess and measure any antibiosis effect produced by *Trichoderma*, the growth rates of the *Neonectria* isolates were measured both with and without *Trichoderma* soluble metabolites incorporated into two types of agar media using methods developed by Srinivasan *et al.* (1992).

4.2.5.1 Preparation of spore suspensions

Spore suspensions of the nine *Trichoderma* isolates (see Table 4:1) were prepared by adding 5ml of 0.05%, Tween 80 (see Appendix 8:1 for recipe) to the surface of two-week-old cultures grown on 5cm PCA plates. After gently scraping the surface of the culture with a sterilised spatula, the spore suspension was transferred to a sterile Universal bottle. The suspensions were subsequently filtered through glass wool to remove mycelia and the spore concentration adjusted to 1×10^6 spores ml^{-1}.

4.2.5.2 Inoculation of **Trichoderma** *isolates and extraction of metabolites*

Three 250ml conical flasks containing 150ml of 3% malt extract broth (MEB) and three flasks containing 150ml of Potato Carrot Broth (PCB) were inoculated with 1ml of the prepared spore suspension for each *Trichoderma* isolate. One uninoculated flask of PCB and one of MEB served as controls. All flasks were placed inside a temperature controlled orbital incubator (Gallenkamp) at 25°C, 110rpm for 7 days in the dark.

After 7 days growth, the mycelium was removed by vacuum filtration and the filtrate sterilised by passing through a disposable 0.22µm-membrane filter unit (Stericup, Millipore). The sterile culture filtrates were stored at -20°C until use. Before being incorporated into the agar medium, the sterile filtrates were defrosted and then placed in a 90°C water bath for 2h to destroy enzyme activity (Srinivasan *et al.*, 1992). The sterile

filtrates were added to an equal volume of corresponding double strength agar, (PCA and 3% MEA) and poured into 5cm Petri dishes and allowed to solidify. Control agar plates were prepared by incorporation of the uninoculated filtrates. Five agar plates were prepared per flask/filtrate.

4.2.5.3 Effect of **Trichoderma** *soluble metabolites on radial growth of the* **Neonectria** *pathogens*

Agar plates were inoculated centrally with a 5mm plug of the pathogen (*N. coccinea* IMI113898 and *N. faginata* IMI268223), from the growing edge of a 7-day-old colony. Five replicate plates were prepared per filtrate for each *Trichoderma/Neonectria*/Agar combination and all plates were incubated at 25°C. Colony diameter of the *Neonectria* isolates was measured after 10 days, once controls had reached the edge of the agar plate. Inhibition of *Neonectria* mycelial growth was measured as the difference between mean radial growth in the presence and absence of the fungal filtrate. The percentage inhibition data was inverse logit transformed and differences in inhibition by the nine *Trichoderma* isolates, the two pathogens and the two media types were analysed using ANOVA. Any interactions between *Trichoderma* isolate, pathogen isolate and media type were also determined by ANOVA.

4.3. RESULTS

4.3.1 Growth rates of *Neonectria* isolates

Both *Neonectria* isolates grew at all four temperatures (see Figure 4:5). At 15, 20 and 25°C, IMI113898 grew faster than IMI268223 and at 30°C, both pathogens grew best on PCA. Growth of IMI113898 was fastest on PCA at 20°C (3.98mmday^{-1}). Growth of IMI268223 was fastest on MEA at 25°C (3.26mmday^{-1}). For all isolates, growth was slowest at 30°C. There were significant differences in growth rates of the two pathogens ($F_{1,77}$=253.82, $p<0.001$) and significant differences between the two media types ($F_{1,77}$=15.45, $p<0.001$). There were also significant differences in growth rates of the pathogens at the four different temperatures ($F_{3,77}$=3151.11, $p<0.001$) and significant interactions between media type, pathogen isolate and temperature ($F_{3,77}$=32.92, $p<0.001$).
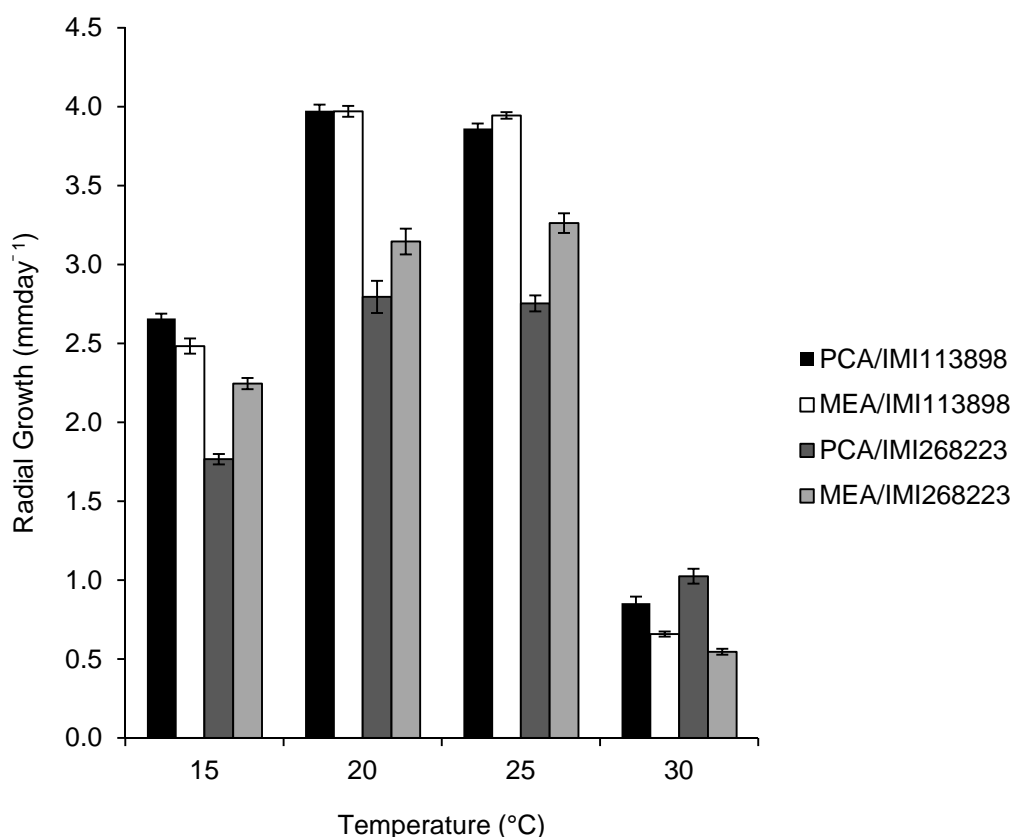
Figure 4:5: Mean radial growth rate (mmday^{-1}) of *Neonectria* isolates IMI113898 and IMI268223 on Potato Carrot Agar (PCA) and Malt Extract Agar (MEA) at different temperatures.

4.3.2 Dual plate results

Results from the dual plate studies show that antagonism interactions between the *Trichoderma* and *Neonectria* isolates varied according to media type and isolates in the interactions. Tables 4:3 and 4:4 show the types of interaction and the corresponding scores.

Table 4:3: Interaction types of the dual plate studies between *Neonectria* and *Trichoderma* isolates and their corresponding Antagonism Index (AI) score on Malt Extract Agar (MEA).

| *Trichoderma* isolate | *Neonectria coccinea* IMI113898 | | *Neonectria faginata* IMI268223 | | AI |
	Interaction	Score	Interaction	Score	
IMI395629	C_{A2}	4.5	C_{A1}	3.5	8
IMI395630	A	1	C_{A1}	3.5	4.5
IMI395631	A	1	A	1	2
IMI395632	C_{A2}	4.5	C_{A2}	4.5	9
IMI395633	C_{A2}	4.5	C_{A2}	4.5	9
IMI395634	C_{A2}	4.5	C_{A2}	4.5	9
IMI395635	C_{A2}	4.5	C_{A2}	4.5	9
IMI395636	C_{A2}	4.5	C_{A2}	4.5	9
IMI395637	C_{A2}	4.5	C_{A2}	4.5	9

Table 4:4: Interaction types of the dual plate studies between *Neonectria* and *Trichoderma* isolates and their corresponding Antagonism Index (AI) score on Potato Carrot Agar (PCA).

| *Trichoderma* isolate | *Neonectria coccinea* IMI113898 | | *Neonectria faginata* IMI268223 | | AI |
	Interaction	Score	Interaction	Score	
IMI395629	A	1	C_{A1}	3.5	4.5
IMI395630	A	1	C_{A2}	4.5	5.5
IMI395631	A	1	C_{A1}	3.5	4.5
IMI395632	A	3.5	C_{A2}	4.5	8
IMI395633	C_{A2}	4.5	C_{A2}	4.5	9
IMI395634	A	1	C_{A2}	4.5	5.5
IMI395635	C_{A1}	3.5	C_{A1}	3.5	7
IMI395636	C_{A2}	4.5	C_{A2}	4.5	9
IMI395637	A	1	A	1	2

All interactions resulted in antagonism of the *Neonectria* isolate by *Trichoderma*. On MEA, interactions A (deadlock with mycelial contact) and C_{A2} (complete replacement after initial deadlock with mycelial contact) were observed between *Neonectria* and *Trichoderma*. On PCA, interactions A (deadlock with mycelial contact), C_{A1} (partial replacement after initial deadlock with mycelial contact) and C_{A2} (complete replacement after initial deadlock with mycelial contact) were observed between *Neonectria* and *Trichoderma*. IMI395633 and IMI395636, two isolates of *T. harzianum* were the most antagonistic of the nine *Trichoderma* isolates screened. They were able to completely replace *Neonectria coccinea* (IMI113898) and *N. faginata* (IMI268223) after initial deadlock with mycelial contact and scored 9 on the antagonistic index on both media types (cumulative AI score of 18). Figure 4:6 shows the three different interaction types (see Appendix 8:3 for complete set of dual plate interactions).
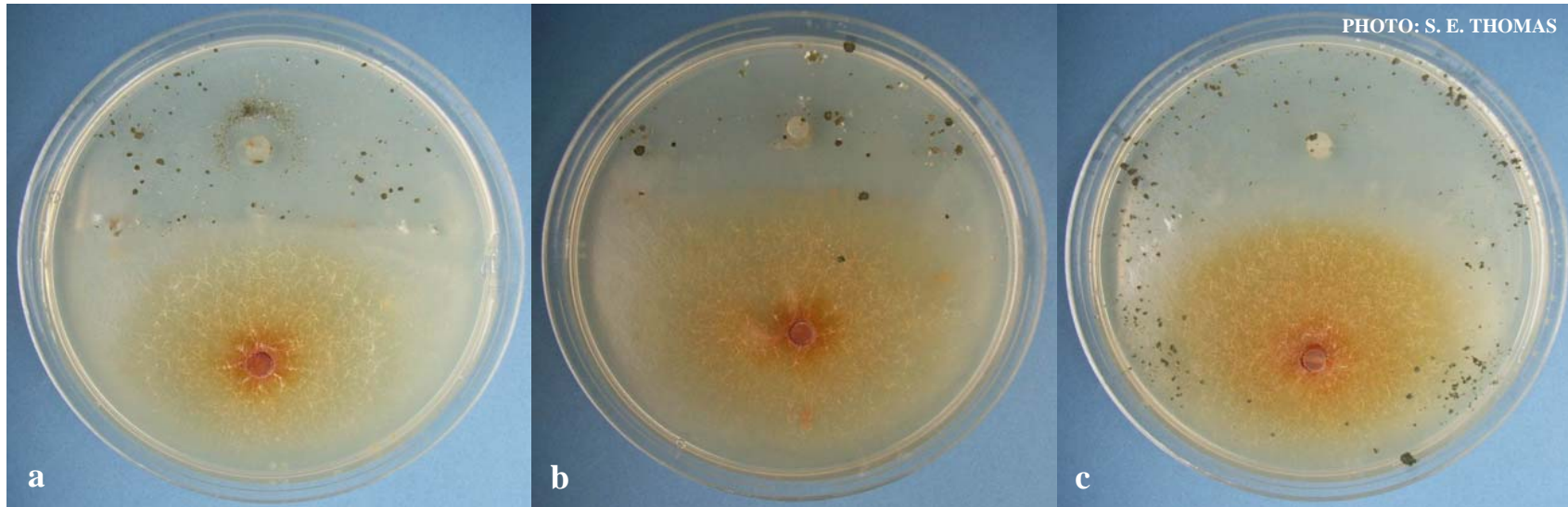
Figure 4:6: Examples of the dual plate interactions observed between *Neonectria faginata* and *Trichoderma* isolates on Potato Carrot Agar; a) IMI395631 interaction C_{A1}; b) IMI395636 interaction C_{A2} and c) IMI395637 interaction A.

4.3.3 Pre-colonised plate (PCP) study

The nine *Trichoderma* isolates were able to colonise the two *Neonectria* pathogens. After five weeks, all *Trichoderma* isolates showed 100% colonisation of both isolates (IMI113898 and IMI268223) on MEA (see Table 4:5 and Figure 4:7. On PCA, colonisation ranged from 13-65% for *N. coccinea* (IMI113898) and from 25-58% for *N. faginata* (IMI1268223). Of the nine isolates, IMI395636 had the highest percentage colonisation of both pathogens on the two media types. There were significant differences in colonisation by the *Trichoderma* isolates ($F_{8,144}$=3.17, p=0.002), significant differences in colonisation of the two pathogen isolates ($F_{1,144}$=7.51, p=0.007) and colonisation of the pathogens was significantly different on the two media types ($F_{1,144}$=779.16, p<0.001). However, there were no significant interactions ($F_{8,144}$=1.37, p=0.214) between *Trichoderma* isolate, pathogen isolate and media type.

Table 4:5: Percentage colonisation of *Neonectria* pathogens (IMI113898 and IMI268223 by *Trichoderma* isolates on Potato Carrot Agar (PCA) and Malt Extract Agar (MEA) after 5 weeks.

Trichoderma isolate	*Neonectria coccinea* IMI113898		*Neonectria faginata* IMI268223	
	PCA	MEA	PCA	MEA
IMI395629	16	100	43.94	100
IMI395630	18.7	100	25.36	100
IMI395631	13.3	100	53.34	100
IMI395632	15.98	100	46.68	100
IMI395633	15.98	100	33.32	100
IMI395634	17.30	100	26.60	100
IMI395635	59.94	100	50.68	100
IMI395636	65.34	100	58.66	100
IMI395637	36.02	100	50.60	100
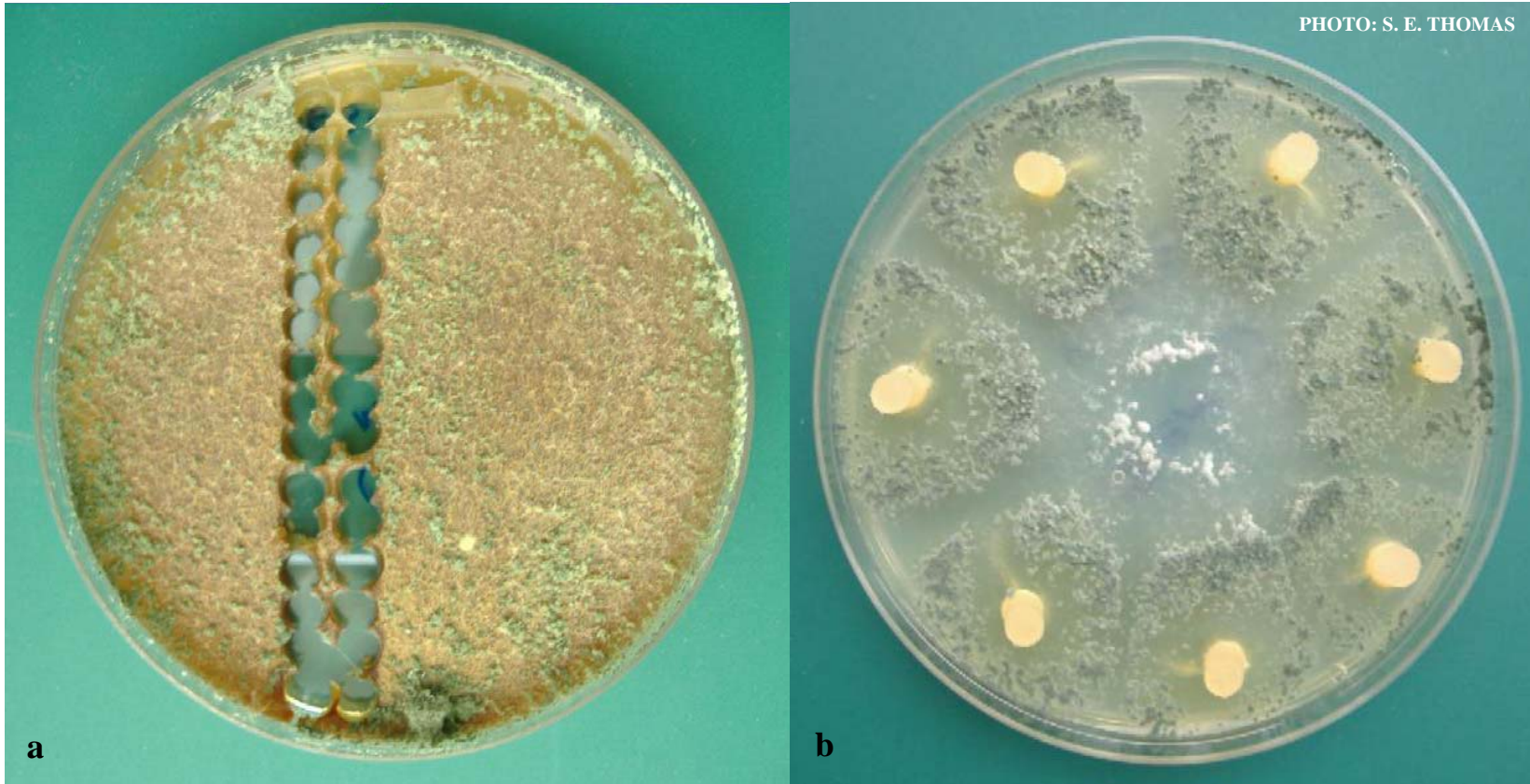
Figure 4:7: Pre-colonised Plate (PCP) method; a) after 2 weeks sampling and b) *Trichoderma* emerging from agar plugs.

4.3.4 Effect of *Trichoderma* soluble metabolites on radial growth of the *Neonectria* pathogens

The metabolites extracted from all nine of the *Trichoderma* isolates had some inhibitory effect on the growth of at least one the *Neonectria* pathogens *in vitro* (see Table 4:6). The greatest inhibition was observed with *T. viride* (IMI395629) which inhibited radial growth of both *Neonectria* pathogens when incorporated into both types of agar media used. On MEA, this isolate inhibited growth of *N. faginata* by 18%. A number of isolates actually promoted growth of the pathogens on agar, for example, IMI395634 promoted growth of IMI113898 by 5.49% on MEA

Table 4:6: Percentage inhibition of growth of *Neonectria* with nine *Trichoderma* isolates with standard error in parentheses. Positive figures indicate inhibition of growth and negative figures indicate promotion of growth.

Trichoderma isolate	*Neonectria coccinea* IMI113898		*Neonectria faginata* IMI268223	
	PCA	**MEA**	**PCA**	**MEA**
IMI395629	1.92 (1.11)	18.37 (0.59)	-2.17 (0)	7.45 (0.61)
IMI395630	2.38 (0.30)	2.61 (0.65)	2.64 (0.33)	2.12 (0.61)
IMI395631	4.98 (0.31)	0 (0)	0.71 (0.35)	3.19 (0.61)
IMI395632	3.98 (0.31)	-0.33 (0.66)	-0.72 (0.36)	0.35 (0.70)
IMI395633	5.28 (0.33)	-0.34 (0.69)	4.76 (1.37)	0.00 (0.69)
IMI395634	2.00 (0.58)	-5.49 (1.10)	0 (0.69)	3.14 (1.04)
IMI395635	1.00 (1.00)	-3.26 (1.09)	3.88 (1.55)	1.16 (1.16)
IMI395636	2.40 (0.79)	-2.36 (0.34)	1.68 (0.67)	0.31 (0.31)
IMI395637	0.30 (0.30)	1.33 (0.88)	-1.59 (0.32)	-1.59 (0.32)

There were significant differences in inhibition of *Neonectria* growth between the nine *Trichoderma* isolates ($F_{8,504}=8.97$, $p<0.001$), and on the two media types ($F_{1,504}=11.60$, $p<0.001$) but there were no significant differences in inhibition of the two pathogen isolates ($F_{1,504}=1.52$, $p=0.219$). However, there were significant interactions ($F_{8,504}=6.96$, $p<0.001$) between *Trichoderma* isolate, pathogen isolate and media type.

4.4 DISCUSSION

This study is the first to investigate the potential use of *Trichoderma* endophytes for biological control of the fungal component of the BBD complex. It has assessed the antagonistic mechanisms of nine endophytic *Trichoderma* isolates from healthy *F. sylvatica* stems against *N. coccinea* and *N. faginata.*

It was important to conduct thorough preliminary experiments with both *Neonectria* spp. at different temperatures with different media types to ensure that the pathogen could growth sufficiently in artificial culture before subsequent *in vitro* experiments were conducted.

Results indicated that *Trichoderma* endophytes have potential as biological control agents of *Neonectria,* as all nine isolates exhibited notable levels of competition, mycoparasitism and antibiosis in *in vitro* experiments. All nine *Trichoderma* isolates were good competitors for nutrients in dual plate interactions and the methodology used by Badalyan *et al*. (2004) provided an effective means by which to quantify the dual plate interactions between the *Trichoderma* and *Neonectria* isolates. Two isolates of *T. harzianum* (IMI395633 and IMI395636), exhibited the highest level of antagonism towards the pathogen isolates (AI=9 out of a possible 10) on both media types. All of the *Trichoderma* isolates were mycoparasitic to *N. coccinea* (IMI113898) and *N. faginata* (IMI268223) but IMI395636 had the highest percentage colonisation against both varieties of the pathogen in the mycoparasitism study. Some of the *Trichoderma* isolates produced soluble metabolites that were inhibitory to the growth of the pathogens when incorporated into growth media. The greatest inhibition of *Neonectria* growth was observed with *T. viride* (IMI395629) which inhibited radial growth of both pathogen isolates when incorporated into both PCA and MEA. De Melo and Faull

(2000) screened fourteen isolates of *Trichoderma* against *Rhizoctonia solani* in dual culture on potato dextrose agar and all inhibited growth of the soil-borne pathogen. Three isolates of *T. koningii* inhibited mycelial growth by 79% and *T. harzianum* reduced viability of *R. solani* by 81.8%. Some of the *Trichoderma* isolates in this study produced metabolites that actually promoted growth of the *Neonectria* pathogens. *T. harzianum* (IMI395634) promoted growth of *N. coccinea* (IMI113898) by 5.49% on MEA. With hindsight and more resources, it would have been interesting to have identified and quantified the *Trichoderma* metabolites. It should also be considered that although some of the *Trichoderma* isolates did not inhibit growth of the pathogen, it could be possible that inhibitory metabolites are only produced by *Trichoderma* when it is in physical contact with the *Neonectria,* and are not produced when simply grown in liquid broth (in the absence of the pathogen). In addition, it could be possible that inhibitory metabolites produced by *Trichoderma* could be effective in deterring feeding of *C. fagisuga*, as many endophytes (including *Trichoderma* spp.), have been shown to protect plants from insect herbivory (Wilson, 2000; Faeth, 2002; Gange *et al.*, 2012).

Due to the challenging nature of this disease complex, i.e. the pathogen requires *C. fagisuga* to be present for infection and the fact that the disease only affects mature beech trees, *in vitro* screening is the only real practical method for assessing the antagonistic ability of the *Trichoderma* endophytes. However, *in vitro* study does not allow the most realistic assessment of *Trichoderma*'s full potential. It is suggested that bioassays performed on wood (beech) blocks would provide a more realistic assessment of how the *Trichoderma* endophytes might perform against *Neonectria* spp. in nature and should be used for future work.

There are many examples in the literature which demonstrate the ability of *Trichoderma* species to also induce plant resistance (Harman *et al.*, 2004). For example, in experiments conducted by De Meyer *et al.* (1998), *T. harzianum* reduced the incidence of grey mould (*Botrytis cinerea*) symptoms on tomato, pepper, tobacco, lettuce and bean by 25-100%. It may therefore be possible that endophytic *Trichoderma* isolates from *F. sylvatica* could also induce plant resistance to BBD.

As it is the *Neonectria* component of the BBD duo that causes the major damage to *F. grandifolia* in North America, focussing on control of the pathogen could be an effective biological control strategy. Application of a *Trichoderma*-based inoculum could be used to either protect young healthy seedlings or saplings from infection by *Neonectria* or to prevent beech scale-infested mature trees from subsequent attack by the pathogen.

Trichoderma species possess many advantageous characteristics which make them preferable for use as biological control agents for plant pests and diseases. In particular, newly described endophytic isolates of *Trichoderma* have received an increasing amount of consideration in recent years (Samuels *et al.*, 2006; Bailey *et al.*, 2008; Chaverri *et al.*, 2011; Bailey and Melnick, 2013). The results of this study indicate that *Trichoderma* endophytes isolated from the native range of *F. sylvatica* would make good candidates for development into biological control agents for BBD. However, further assessment of <u>all</u> *Trichoderma* isolates obtained in this study could be worthwhile in order to select the most effective isolate. Additional experiments to assess the competitive ability of the isolates could be carried out on wood blocks (Schubert *et al.*, 2008b), mycoparasitism could be further examined by undertaking hyphal interaction studies (Inbar *et al.*, 1996) and the soluble metabolites produced by

Trichoderma isolates could be identified using High-Performance Liquid Chromatography (HPLC) techniques. Further research and development into mass production and formulation of endophytic *Trichoderma* isolates would be also be required before any *Trichoderma* product could be produced for control of BBD.

T. harzianum (IMI395636) scored highest in both the mycoparasitism and antagonism plate tests (≥59% and AI=18 respectively) and was therefore selected for further investigation in an *in planta* study (chapter 5).

5. EVALUATION OF APPLICATION TECHNIQUES FOR COLONISATION OF *FAGUS SYLVATICA* SAPLINGS USING *TRICHODERMA*, *BEAUVERIA* AND *LECANICILLIUM*

5.1 INTRODUCTION

Fungal endophytes of trees are horizontally transmitted, i.e. they are passed from one tree to another via spores, in contrast to endophytes of grasses which are transmitted vertically via seed, i.e. the seed is already infected with the endophyte before germination (Wilson, 2000). It is likely that deciduous trees become infected with spores of endophytic fungi that are present in leaf litter. These spores are dispersed by insects, rain and wind to the trunk, branches, leaves and roots where they germinate and colonise these plant tissues (Malloch and Blackwell, 1992).

Stone (1987) undertook a study with Douglas-fir (*Pseudotsuga menziesii)* and the endophyte *Rhabdocline parkeri* and verified the spore attachment, germination, penetration and colonisation of Douglas-fir needles. Studies have shown that endophyte-free tree tissues can become infected with the application of spore suspensions to stems and leaves. Posada and Vega (2005) were able to successfully infect seedlings of cocoa (*Theobroma cacao*) with *Beauveria bassiana* by application of a spore suspension to germinating seeds. The same workers (Posada and Vega, 2006; Posada *et al.*, 2007) demonstrated successful inoculation and colonisation of coffee (*Coffea arabica*) seedlings with *B. bassiana* in a pest management strategy for biological control of coffee berry borer (*Hypothenemus hampei*). Brownbridge *et al.* (2012) developed two methods to establish *B. bassiana* as an endophyte of *Pinus radiata* seedlings. The fungus was applied as a seed coating and as a root dip and was subsequently recovered from surface sterilised samples after 2, 4 and 9 months, at low

levels. Regliński *et al.* (2012) established that *Trichoderma atroviride* was able to promote stem and root growth of *Pinus radiata* seedlings and enhance resistance to conifer blight (*Diplodia pinea* (Desm.) Kickx.). Gurulingappa *et al.* (2010) demonstrated that it was possible to colonise six different crop plants (cotton, wheat, bean, corn, tomato and pumpkin) with endophytic entomopathogenic isolates of *B. bassiana*, *Lecanicillium lecanii* and *Aspergillus parasiticus,* when conidial suspensions were applied to leaves. The endophytic fungi also reduced aphid reproduction although they did not cause direct mortality.

More than fifteen products based on *Lecanicillium* spp. have been developed commercially against a variety of pests worldwide (Faria and Wraight, 2007). Goettel *et al*. (2008) reviewed a number of studies that assessed three species of *Lecanicillium* for control of plant diseases, pest insects and plant parasitic nematodes and concluded that this genus has potential for development as a biological control agent for a number of pest types due to its antagonistic, parasitic and disease resistance inducing characteristics.

There are also several biopesticides formulated with *Trichoderma* sp. on the market for the protection of vines and trees from decay fungi. Key Industries (New Zealand, http://www.keyindustries.co.nz) produce formulations of the bio-inoculant *T. harzianum* under the brand name of Vinevax. It is a dressing which creates a living barrier for pruning wounds. It is used to protect orchard trees against many wood decay fungi, including silver leaf and grapevines against dieback, dead arm and vine decline diseases. It is available as a wettable powder spray and brush-on paste and also as an inoculated dowel implant that can be inserted into the trunk of grapevines, as well as a high activity liquid bio-injection plant defence booster which can protect them against

Armillaria root rot and protect orchard and ornamental trees against wood and root decay fungi. Another novel product on the market for the prevention of Dutch elm disease is Dutch Trig (http://www.dutchtrig.com), a "biovaccine" developed to protect elm trees from infection from *Ophiostoma novo-ulmi*. The product is a pure suspension of *Verticillium albo-atrum* conidia, originally isolated from a potato field in the Netherlands. Usually, *V. albo-atrum* would infect the xylem tissue of a tree and cause wilting symptoms, however, this specific strain of *Verticillium* is only mildly pathogenic which is sufficient to induce the tree's natural defence mechanisms in the healthy elm without causing disease symptoms. The manufacturer claims that Dutch Trig will protect the injected tree against Dutch elm disease during one growing season and is effective as a preventative treatment for American elm (*Ulmus americana*) and European elm (*Ulmus laevis*) although no supporting data supporting this has been published.

In this study, three fungal isolates *T. harzianum* (IMI395636), *B. bassiana* (IMI502733) and *L. muscarium* (IMI502732) were evaluated for their ability to colonise saplings of *F. sylvatica* using three different application methods as a step towards developing them as biological control agents for BBD.

5.2 MATERIALS AND METHODS

5.2.1 Plant source and maintenance

Twenty-50cm cell-grown *F. sylvatica* saplings were obtained from Hedge Nursery Telford, Shropshire, UK (http://www.hedgenursey.co.uk/). They were planted in a sterilised (121°C for 20 mins) soil mix (50% multi-purpose compost and 50% sand) in square pots (10cm x 10cm x 14cm). The beech saplings were maintained in a glasshouse at approximately 18°C by day and 5°C at night on a 12h light:12h dark cycle and were watered freely twice weekly.

5.2.2 Inoculum preparation

Inoculum of three fungal isolates obtained in chapters 2 and 3 were prepared for the sapling inoculation experiments: *Trichoderma harzianum* (IMI395636)*, Lecanicillium* sp. (IMI502732) and *Beauveria bassiana* (IMI502733), see Table 5:1 for details.

Table 5:1: Fungal isolates used for sapling colonisation experiments.

Isolate number	Fungal species	Source
IMI395636	*Trichoderma harzianum*	*Fagus sylvatica* endophyte, UK
IMI502732	*Lecanicillium* sp.	*Cryptococcus fagisuga*, Armenia
IMI502733	*Beauveria bassiana*	*Fagus sylvatica* subsp. *orientalis* endophyte, Armenia

Spore suspensions were prepared by adding 5ml of sterile distilled water (SDW) plus 0.05% Tween 80 to the surface of a fungal culture growing on a 9cm Potato Carrot Agar (PCA) plate. Spores were gently scraped with a sterilised spatula and the spore suspension was transferred to a sterile Universal bottle using a sterile syringe. The suspensions were filtered through glass wool to remove any agar lumps. The concentration of the spore suspensions were measured with the use of a

haemocytometer and adjusted to 1×10^7 spores ml^{-1}. Spore viability was ensured by checking growth on PCA after 24h, see Figure 5:1.

Figure 5:1: Fungal spore suspensions in bijou bottles and spore viability checks on PCA.

5.2.3 Inoculation techniques

The fungi were applied to the beech plants using three techniques in order to target different plant parts and potential entry points: foliar spray, stem wound/application and soil drench, similar to those used by Posada *et al.* (2007) for the inoculation of coffee plants with *B. bassiana*.

5.2.3.1 Foliar spray

The spore suspensions were applied with a 125ml handheld aerosol spray bottle (Azlon), see Figure 5:2a. Aluminium foil was used to cover the top of the plant pot to avoid soil inoculation. Ten ml of spore suspension was applied per plant to the surface

of the leaves. Small cardboard tags were tied to the leaves in order to distinguish them from new leaves that would inevitably grow post inoculation. Each plant was covered with a plastic bag for 24h to maintain humidity and promote germination of the fungi.

5.2.3.2 Stem wounding/application

A section of bark, approximately 20mm x 4mm, was removed from the beech stem using a sterile scalpel, see Figure 5:2b. Approximately 5ml of the spore suspensions were applied to the wound using a sterilised, artist's paintbrush. Once dried, the wound was sealed using Parafilm M (Pechiney Plastic Packaging Company, Illinois, USA). Previous attempts to inject the spore suspension into the stem of the beech saplings using a small hypodermic needle and syringe failed due to the small diameter of the sapling stems.

5.2.3.3 Soil drench

Twenty ml of the spore suspensions were applied to the surface of the soil in each pot. Pots were placed in plastic bags for 24h to maintain humidity.

For all treatments, four replicate plants were prepared plus four control plants were also prepared for each application technique. Controls received SDW plus 0.05% Tween 80 only. The plants were labelled with fungus/application technique/month/replicate and placed in a randomized design in the glasshouse chamber where they were maintained (as in 5.2.1) until isolation.
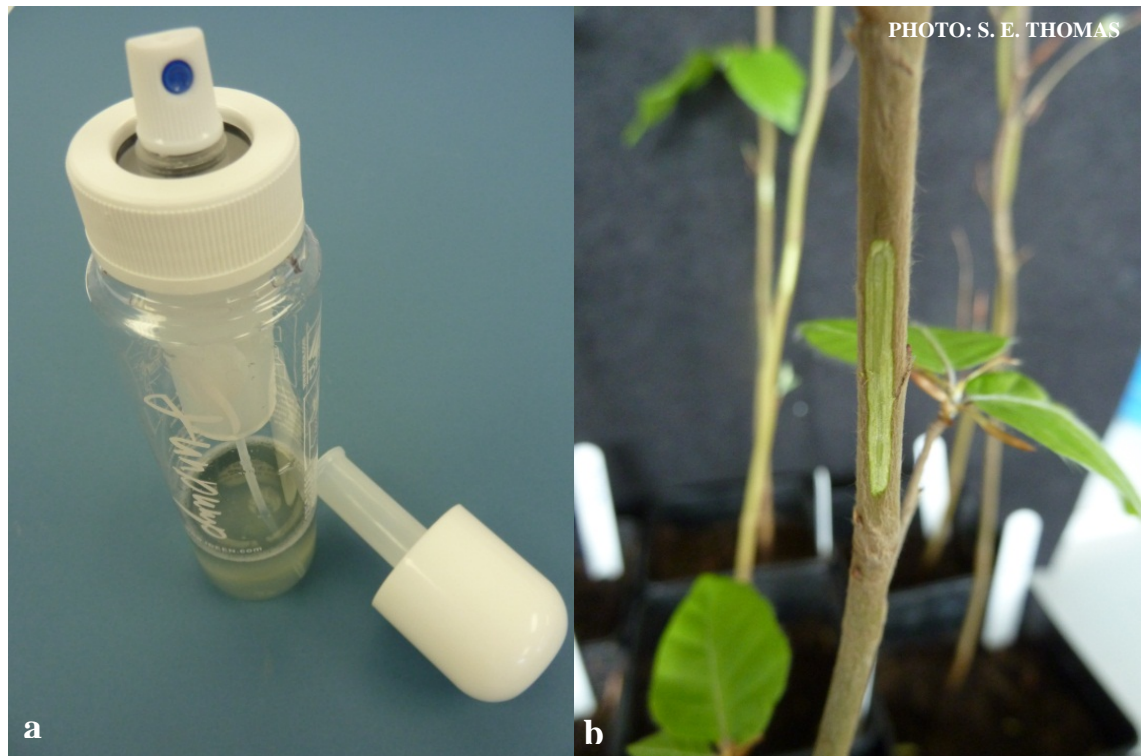
Figure 5:2: a) Spray applicator bottle for the foliar spray application and b) beech sapling with removed section of stem bark for the wound/application treatment.

5.2.4 Isolation techniques

After 1, 3 and 6 months, selected plants were removed from their pots and their roots were washed with SDW to remove the soil. 48 plants were prepared for each time interval (4 plants for each isolate/application technique combination (36) plus 12 control plants), totalling 144 plants for the whole experiment. Whole plants were surface sterilised by washing in 2% sodium hypochlorite (NaOCl) for 5 minutes followed by two sequential rinses in SDW. Sterilised saplings were placed onto sterile Kimwipes (Kimberley Clark) and patted dry before being dissected with a sterilised scalpel.

5.2.4.1 Roots and Stems

Six, 1cm root and six, 1cm stem segments (1-6) were cut from each sapling and transferred to 9cm PCA plates (six per plate, see Figures 5:3 and 5:5). Plates were sealed with Parafilm M sealing film and placed in an incubator at 20°C in the dark. Root and stem segments were assessed for emerging fungal growth for 7-10 days.

5.2.4.2 Leaves

Six leaves were selected (see Figures 5:3 and 5:4) and sterilised by washing in 0.525% sodium hypochlorite (NaOCl) for two minutes. Leaves were rinsed twice in sterile distilled water and dried on sterilised tissue paper. A single $1cm^2$ section of the lamina including the midrib was cut from six leaves and placed onto 9cm PCA plates and labelled 1-6. Leaf sections were assessed for emerging fungal growth for 7-10 days.

The proportion of saplings scoring positively for the re-isolated endophyte was recorded for each fungal isolate for root, stem and leaf tissues. Standard error was recorded as SE = √[p (1 - p)/n, where n= number of saplings and p=proportion of saplings scoring positively. A sapling/agar plate was recorded as positive even if only one sample was recorded as having the fungal endophyte growing out of it.
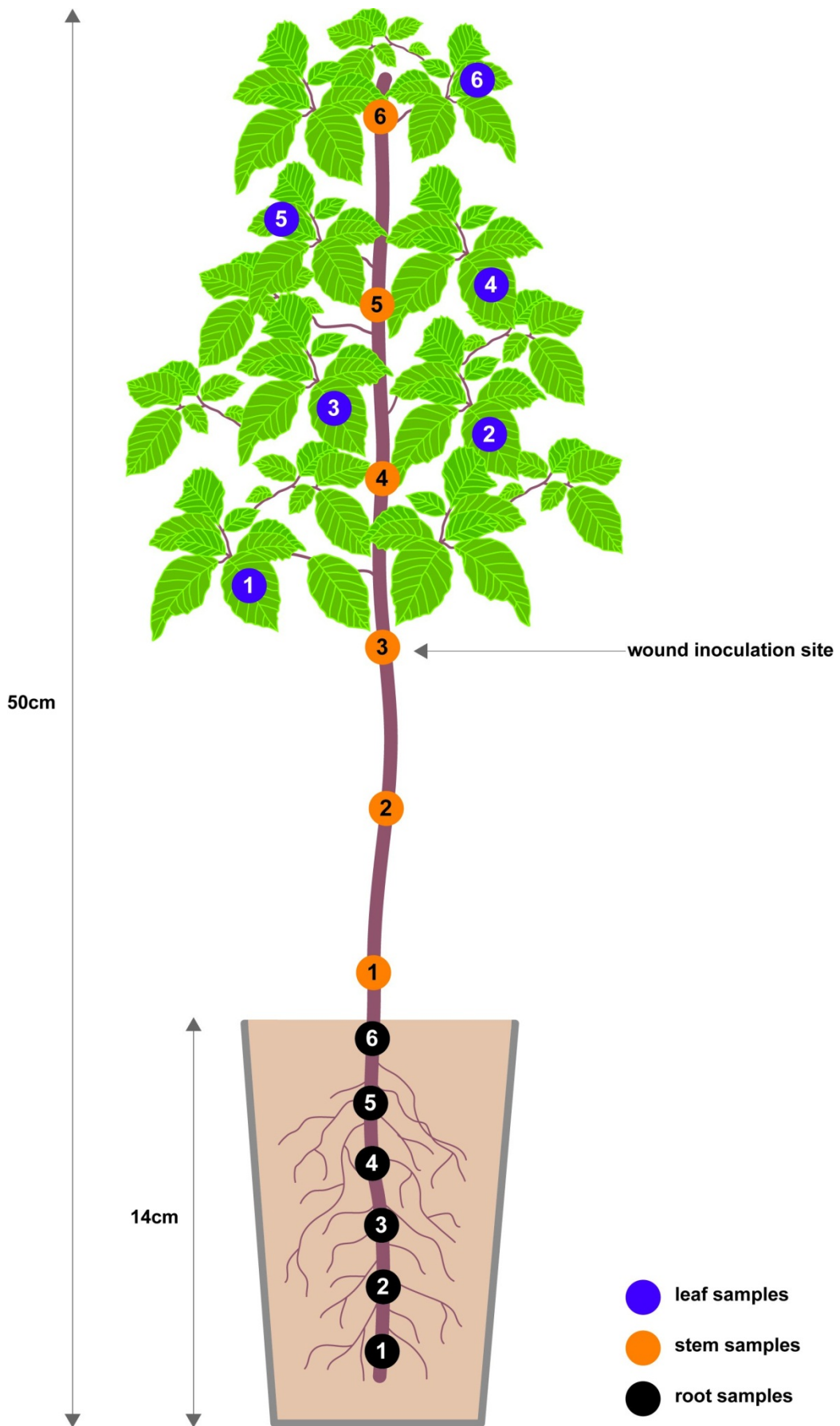
Figure 5:3: Diagrammatical representation of a beech sapling showing the location of the areas of root, stem and leaves that were dissected.
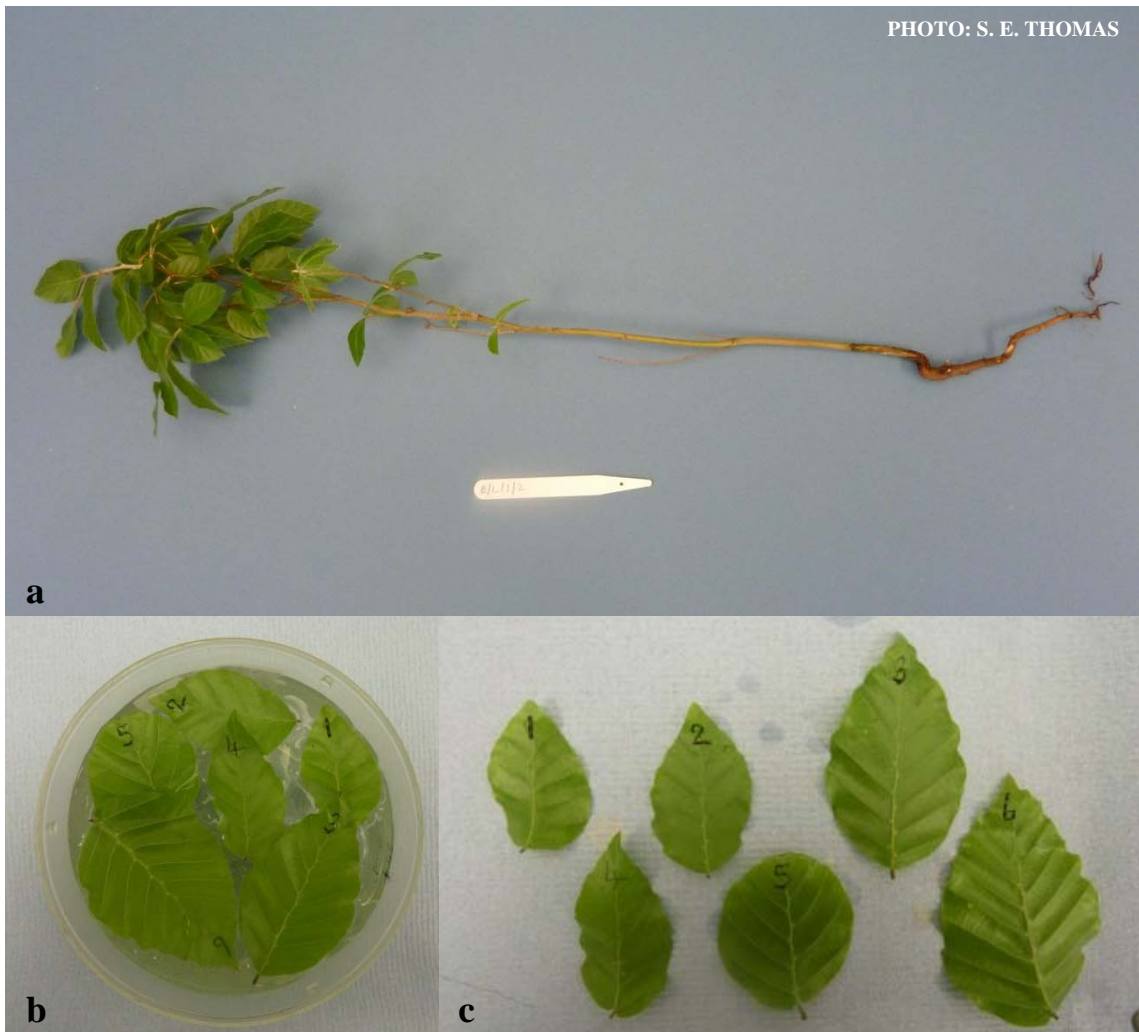
Figure 5:4: a) Beech sapling, with washed roots before sterilisation and dissection; b) beech leaves being rinsed in SDW after sterilisation and c) beech leaves prior to dissection.
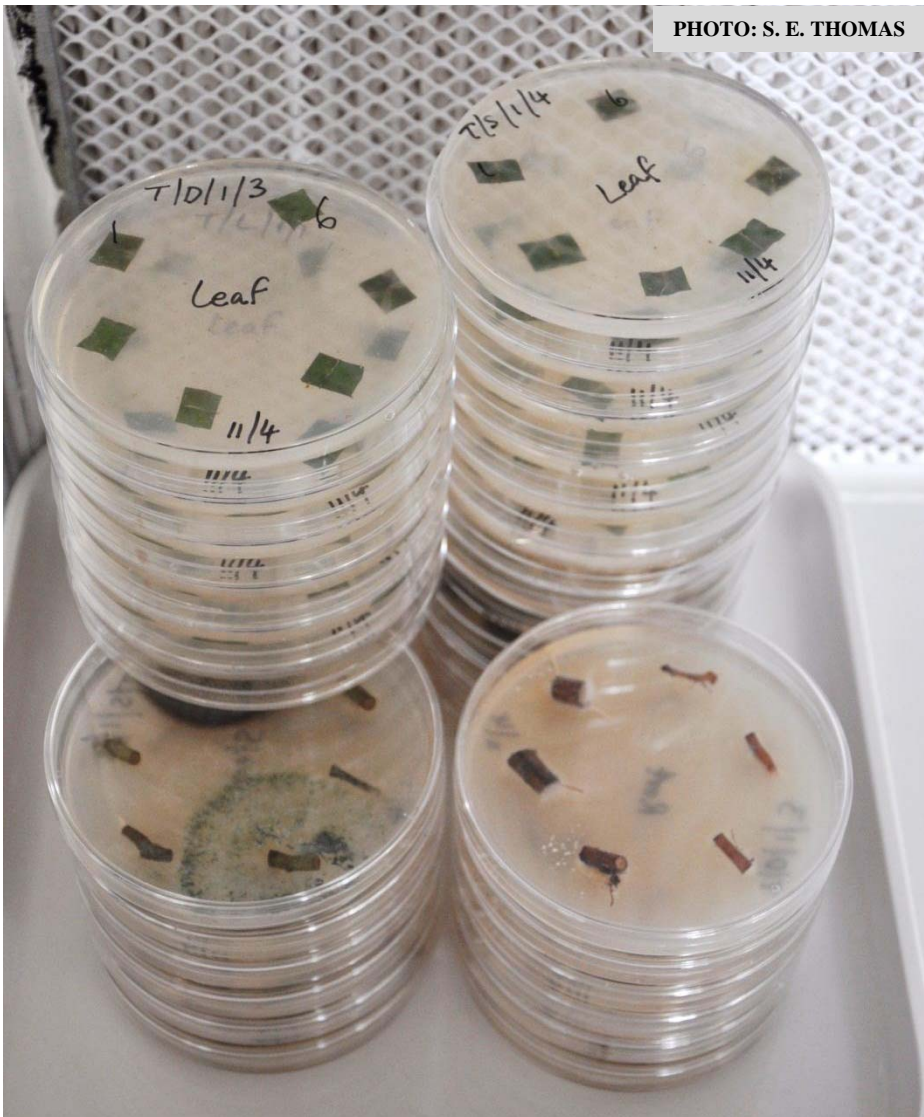
Figure 5:5: Sections of beech root, stem and leaf tissue on agar plates.

5.2.5 Identification of recovered fungi

All emerging fungi were examined and any that initially appeared to be the original cultures (i.e. *Trichoderma, Beauveria* and *Lecanicillium*) were sub-cultured onto fresh PCA plates. Any other emerging fungi were ignored. The sub-cultures were morphologically identified and microscopic observations were made and compared to the original corresponding isolates (see Figures 5:8 and 5:9). All recovered *Trichoderma, Beauveria* and *Lecanicillium* were further identified using ITS sequencing as described in 3.2.5 and DNA fingerprinting was carried out in order to confirm that the original isolates of *B. bassiana* (IMI502733) and *T. harzianum* (IMI395636) were re-isolated using three different primers: TGT, MR and UBC889.

5.3 RESULTS

5.3.1 Re-isolation of endophytes

Two of the three fungal isolates applied to beech saplings were recovered in low proportions from root, stem and leaf tissues after one, three and six months (see Table 5:1). *T. harzianum* (IMI395636) was re-isolated after one, three and six months (see Figure 5:8) and *B. bassiana* (IMI502733) was re-isolated after one and three months but not after six month (see Figure 5:9). *L. muscarium* (IMI502732) was not recovered at any point during the experiment.

5.3.1.1 Trichoderma harzianum *(IMI395636)*

One month after application as a soil drench, *T. harzianum* (IMI395636) was successfully re-isolated from root sections of all four plants (p=1) (see Figure 5:6) and from stem sections of three plants (p=0.75). Three months post-application, *Trichoderma* was re-isolated from root sections of two plants (p=0.5) and from leaf

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sections of one plant (p=0.25). *Trichoderma* was not re-isolated from any plant tissues, six months after it had been applied to the soil.

After stem wounding/application, *Trichoderma* was successfully re-isolated from stem tissues after one, three and six months (p=0.75, 1, 0.75 respectively). Three months after application to the stem, it was re-isolated from leaf sections of two plants (p=0.5).

One month after application as a foliar spray, *Trichoderma* was re-isolated from root, stem and leaf tissues (p=0.5, 0.5 and 0.75 respectively) and only at a low incidence from leaves (p=0.25) after six months.

Figure 5:6: Re-isolation of *Trichoderma harzianum* IMI395636 from beech root tissue, 1 month after soil drench application.

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5.3.1.2 Beauveria bassiana *(IMI502733)*

B. bassiana (IMI502733) was not recovered from beech roots, stems or leaves after application as a soil drench.

However, when applied as a stem inoculum it was successfully recovered from leaves, 1 (p=0.25) and 3 months (p=0.25) after application and from root tissues after three months (p=0.25).

When applied as a foliar spray, *Beauveria* was re-isolated from leaves after 1 and 3 months (p=0.25), see Figure 5:7. *Beauveria* was not isolated from any plant tissues from any application technique after six months.
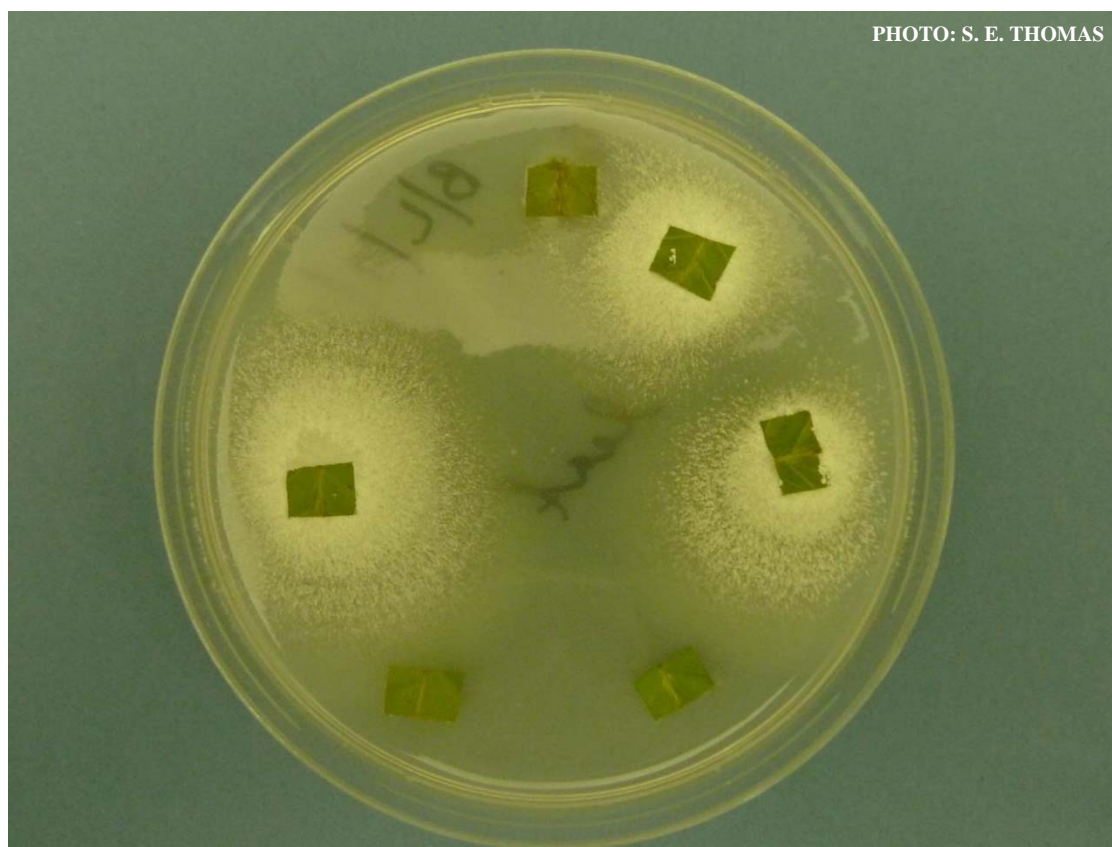
Figure 5:7: *Beauveria bassiana* **(IMI502733) growing out of beech leaf sections, on Potato Carrot Agar, 1 month after application to saplings as a foliar spray.**

5.3.1.3 Lecanicillium muscarium (IMI502732)

L. muscarium (IMI502732) was not re-isolated from any of the beech tissues (p=0) sampled during the experiment.

Table 5:2: Proportion (p) of *F. sylvatica* sapling tissues that were positive for the applied fungi (n = 4) after 1, 3 and 6 months.

Treatment		Tissue re-isolated	1 Month	3 Months	6 Months
Fungus	Application method				
Trichoderma harzianum IMI395636	soil drench	root	1 (0)	0.5 (0.25)	0
		stem	0.75 (0.22)	0	0
		leaf	0	0.25 (0.22)	0
	stem wound	root	0	0	0
		stem	0.75 (0.22)	1 (0)	0.75 (0.22)
		leaf	0	0.5 (0.25)	0
	foliar spray	root	0.5 (0.25)	0	0
		stem	0.5 (0.25)	0	0
		leaf	0.75 (0.22)	0	0.25 (0.22)
Beauveria bassiana IMI502733	soil drench	root	0	0	0
		stem	0	0	0
		leaf	0	0	0
	stem wound	root	0	0.25 (0.22)	0
		stem	0	0	0
		leaf	0.25 (0.22)	0.25 (0.22)	0
	foliar spray	root	0	0	0
		stem	0	0	0
		leaf	0.25 (0.22)	0.25 (0.22)	0
Lecanicillium muscarium IMI502732	soil drench	root	0	0	0
		stem	0	0	0
		leaf	0	0	0
	stem wound	root	0	0	0
		stem	0	0	0
		leaf	0	0	0
	foliar spray	root	0	0	0
		stem	0	0	0
		leaf	0	0	0

Multiple root, stem or leaf pieces were placed on each plate for each sapling. A plate was recorded as positive even if the isolate was only recovered from one sample. Standard Error = $\sqrt{[p (1 - p)/n]}$
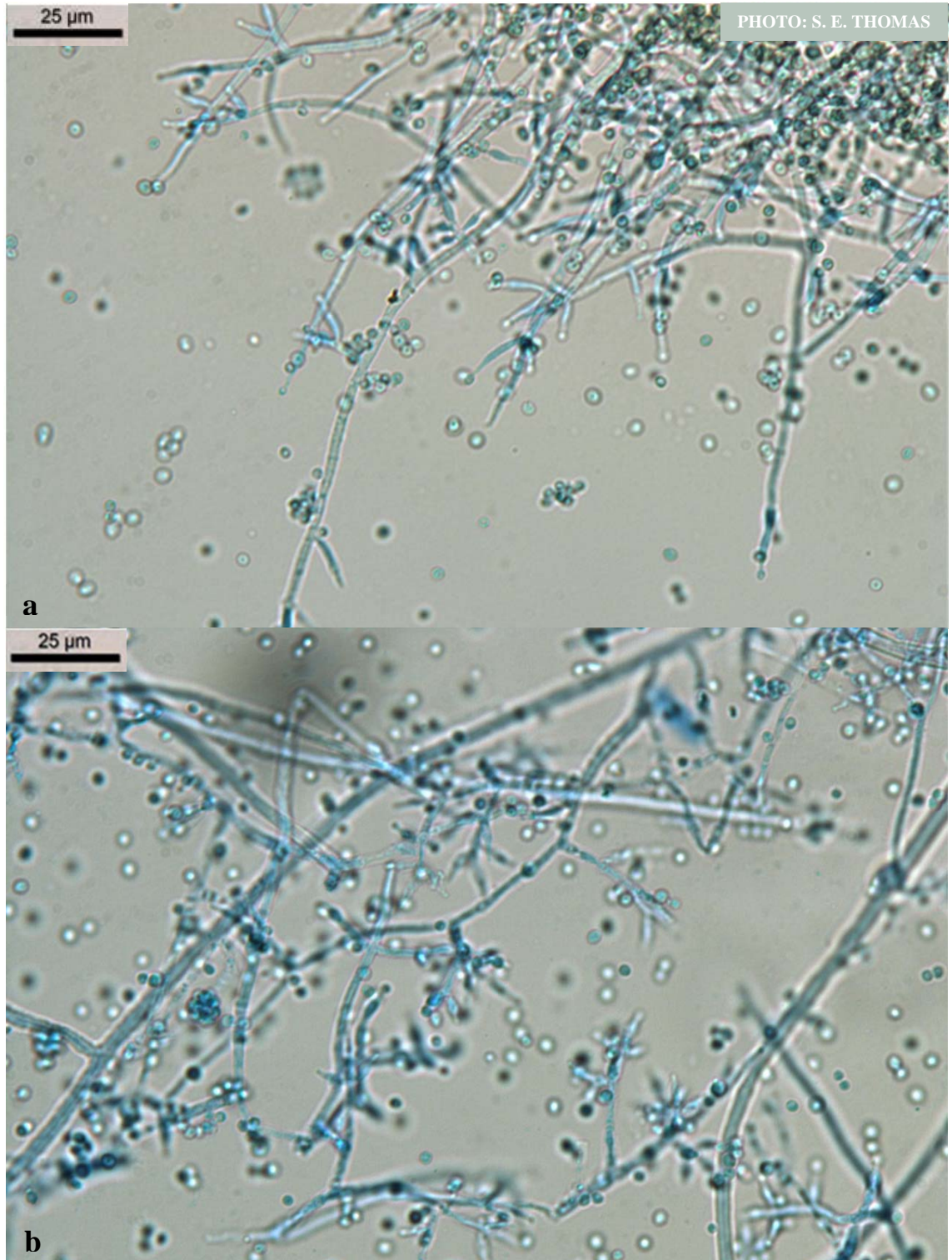
Figure 5:8: *Trichoderma harzianum* (IMI395636); a) original culture and b) re-isolated culture.

a

b
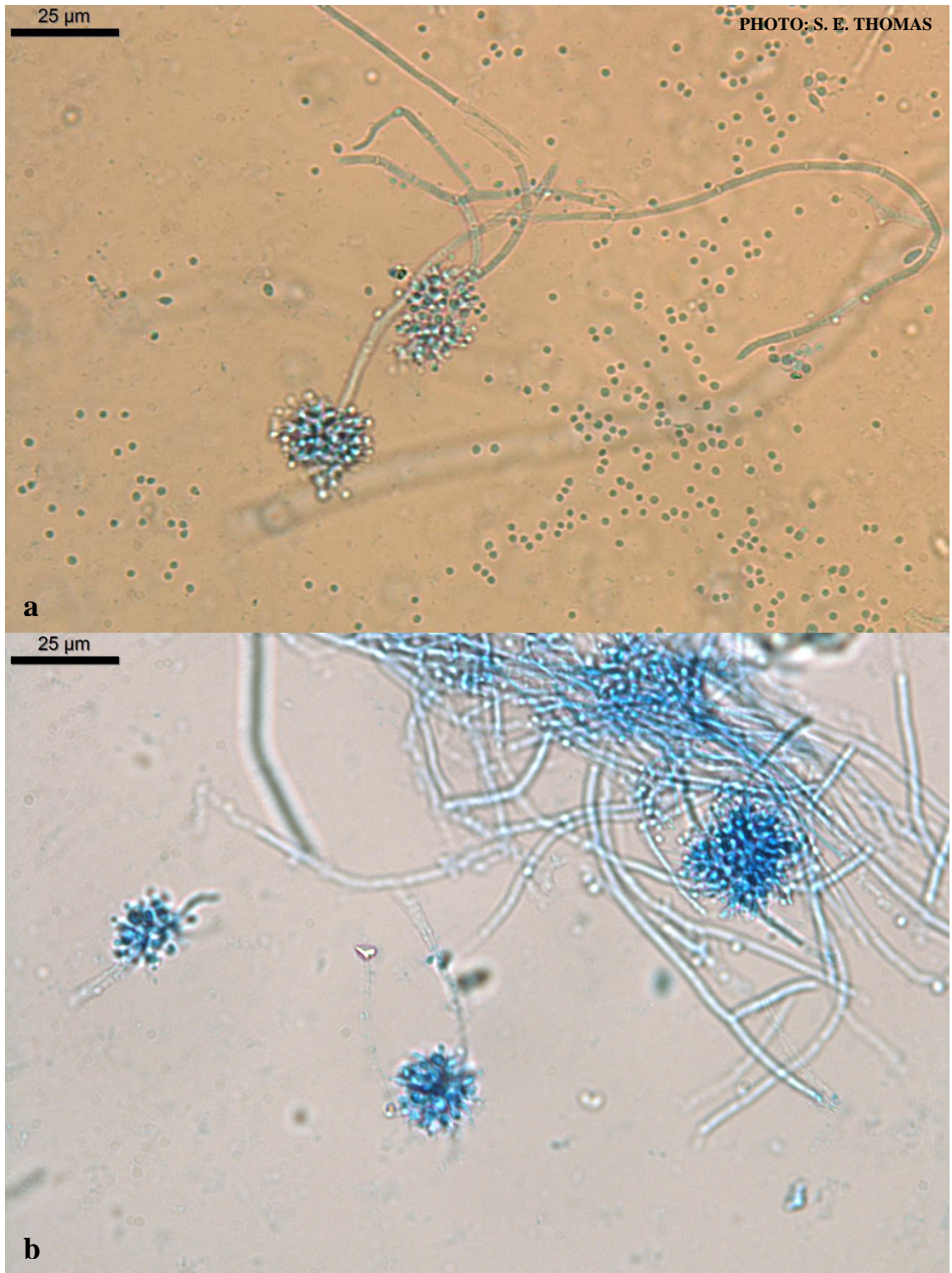
Figure 5:9: *Beauveria bassiana* **(IMI502733); a) original culture and b) re-isolated culture.**

5.3.1.4 Molecular identification

ITS sequences for the recovered fungi obtained after 1 month, 3 months and 6 months showed that they were identical to the applied fungi. A BLAST search of the recovered *Beauveria* sequences returned a 100% match corresponding to *Beauveria bassiana* strain ATCC MYA 4866 (JQ906772.1) and the recovered *Trichoderma* sequences returned a 100% match to *T. harzianum* (*Hypocrea lixii*) strain P49P11 (JQ278697.1).

5.3.1.5 DNA Fingerprinting

DNA fingerprinting results with three different primers (TGT, MR and UBC889) confirmed that the recovered *B. bassiana* was identical to the originally applied isolate *B. bassiana* (IMI502733) and unique from a reference isolate (IMI389521) obtained from CABI's GRC. Results also showed that the recovered *T. harzianum* was identical to the originally applied isolate *T. harzianum* (IMI395636) and unique from a reference isolate (IMI204016) obtained from CABI's GRC. Results of the gel using primer UBC889 are shown in Figure 5:10.
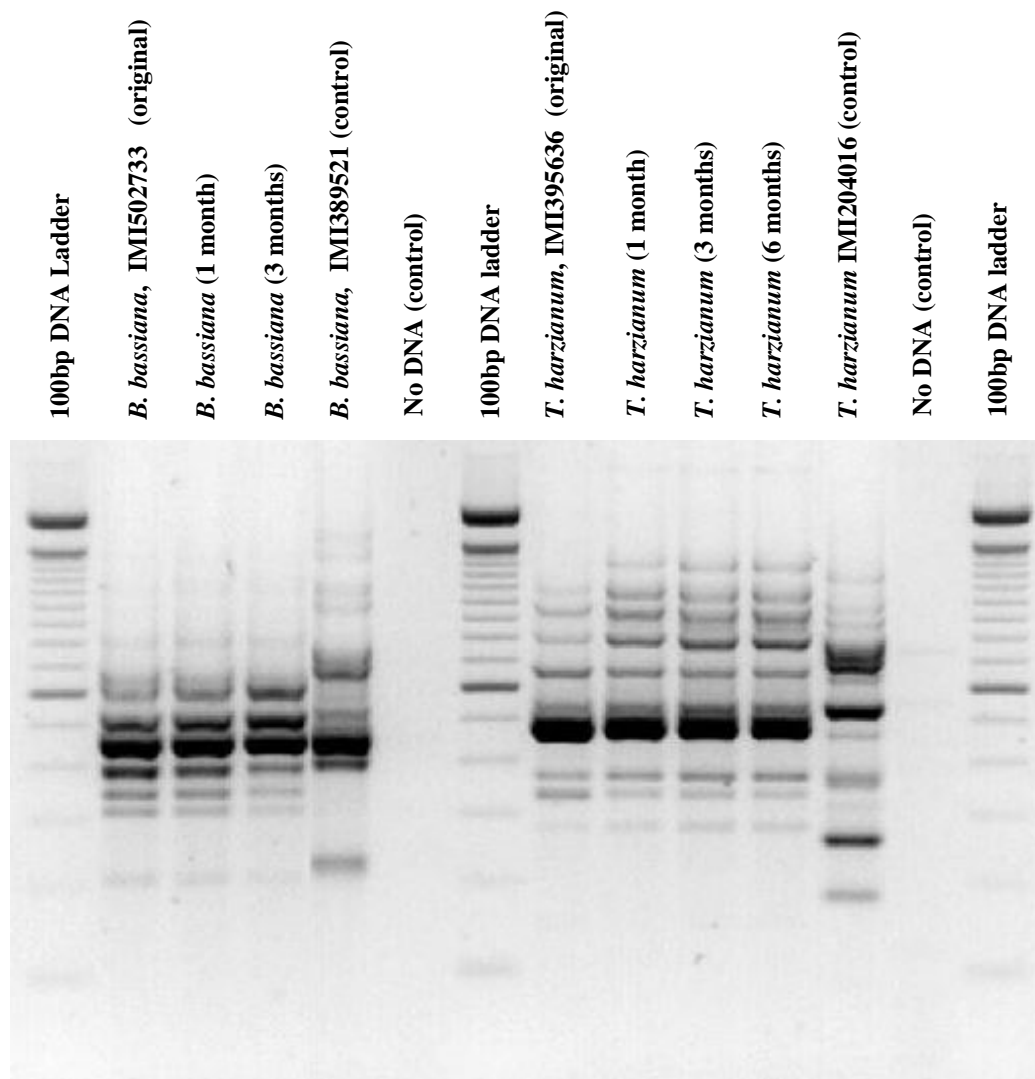
Figure 5:10: Gel showing identical fingerprints for the original, applied isolates of *Beauveria bassiana* (IMI502733) and *Trichoderma harzianum* (IMI395636) and those recovered from beech saplings after 1, 3 and 6 months with primer UBC889.

5.4 DISCUSSION

This study has demonstrated that it is possible to successfully inoculate *F. sylvatica* saplings with *T. harzianum* (IMI395636) using three different application techniques and *B. bassiana* (IMI502733) with foliar spray and stem wounding application techniques. Recovery of these two fungi from beech saplings was achieved up to six months post application, albeit at low incidence. *L. muscarium* (IMI502732) was not re-isolated from beech saplings during this experiment.

Although infection of the beech saplings was successful, colonisation of the whole saplings was not achieved, as there was very limited movement of the applied fungus within the saplings i.e. between plant parts/organs. When applied as a soil drench, both *Beauveria* and *Trichoderma* were recovered from roots. When applied as a foliar spray, successful re-isolation of these fungi from leaves was achieved and after stem/wound application, re-isolation of these fungi from stem tissues was good. When assessing individual stems, it was noted that *Trichoderma* was almost always recovered from the original inoculation site/wound at one, three and six-month assessments. The only exception to this general trend was with *Trichoderma* which was recovered from stem tissue when applied to leaves and soil as well as when applied to the stem. This result could possibly be explained as accidental inoculation e.g. splashing or dripping of spores, however, there was no evidence to support this.

Bailey *et al.* (2008) assessed fifteen isolates of *Trichoderma*, for their ability to colonise *Theobroma cacao* seeds/seedlings using five inoculation methods. They found that most of the isolates were able to establish an endophytic relationship, however, the study only lasted a number of weeks, rather than months so the longevity of these endophytic relationships was not fully assessed.

The establishment of *B. bassiana* as an endophyte has previously been demonstrated for *Zea mays* (Bing and Lewis, 1991), *Theobroma cacao* (Posada and Vega, 2005), *Papaver somniferum* (Quesada-Moraga *et al.*, 2006) and *Pinus radiata* (Brownbridge *et al.*, 2012) following artificial inoculation in the laboratory. Posada *et al.* (2007) used soil drench, foliar spray and injection methods to inoculate coffee plants with *B. bassiana* and successfully recovered it six months after injection from one individual plant. A study by Gurulingappa *et al.* (2010) showed that, when inoculated into soil, *B. bassiana* could establish as an endophyte in the leaves, stems and roots of wheat but not cotton. Posada and Vega (2005) found that while *B. bassiana* could infect cocoa plants, it did not persist beyond two months. If time had allowed, it would have been interesting to have extended this experiment beyond the six-month period to investigate the longer term persistence of these fungi.

L. muscarium (IMI502732) was not re-isolated at any stage during this experiment but it is not known whether inoculation of the saplings was unsuccessful or whether this isolate was unable to establish an endophytic relationship with the beech saplings. Similarly, Gurulingappa *et al.* (2010) could not re-isolate *L. lecanii* from any parts of wheat or cotton when grown in inoculated soil. The *Lecanicillium* isolate in this study originated from a dead scale insect, whereas the *Trichoderma* and *Beauveria* isolates were originally isolated as beech endophytes from stem tissue. An alternative explanation for the unsuccessful infection could be that insects are required to vector this fungus and artificial application is not effective. Anderson *et al.* (2007) experimented with an isolate of *L. lecanii*, cotton (*Gossypium hirsutum*) and the greenhouse aphid (*Aphis gossypii*) and found that it was possible to transfer the entomopathogenic endophyte between both hosts. They successfully transferred the

fungus from *L. lecanii*-infected aphids to cotton leaves and from cotton-infected leaves to aphids. The levels of colonisation were variable but *L. lecanii* killed the aphids without causing obvious disease symptoms in leaves of cotton. Results of this study indicate that *L. muscarium* (IMI502732) would not be suitable for use as an endophytic biological control agent for BBD. Without successful colonisation of beech saplings it would not be possible to assess its biological control potential against BBD. However, other *Lecanicillium* spp. isolates (including Mycotal and Vertalec) should not be discounted for use as a biological control agent for BBD and deserves further research.

Due to a previous failed attempt to germinate sufficient beech seed for the experiment, young saplings that had been grown in soil plugs were purchased for the study. A consequence of this was that it was possible that other fungi could already be present in the system. Indeed, some "contaminating endophytic fungi" were recovered from both the treated and untreated saplings but this was unavoidable and a more realistic representation of the natural environment. No cross contamination of the applied fungi occurred between treatments and none of the three fungi applied were re-isolated from the control plants which were treated with SDW plus 0.05% Tween 80 only.

This study has indicated that selection of an appropriate fungal isolate and the inoculation method used are both important factors when attempting to establish a plant-fungal endophyte relationship in the glasshouse. The development of a successful BCA requires the selection of an effective isolate (antagonist and/or entomopathogen). However, it is also necessary to be able to effectively apply the agent to the target where it must persist. In this case for BBD, the target area for a BCA is the trunk of the beech tree. In conclusion, the *Trichoderma* and *Beauveria* isolates show potential for endophytic colonisation of beech saplings. it would be recommended to complete

further colonisation studies with these isolates using adjuvanats which could improve application and survival of the fungi. Other recommendations would be to undertake repeat applications of the fungal inocula to all plant parts (i.e., leaves, stem *and* roots) to achieve increased colonisation of beech saplings by endophytes. A higher spore concentration, formulated with a chemical adjuvant could also enhance application and colonisation of the sapling by the fungi. Progression to assess *B. bassiana* (IMI502733) and *T. harzianum* (IMI395636) for their effectiveness against BBD in the glasshouse or field would be the next step towards development of a BCA.

6. GENERAL DISCUSSION

6.1 INTRODUCTION

Among the thousands of invasive species that have invaded North America, insect pests and fungal pathogens pose the greatest threat to forest ecosystems (Lovett *et al.*, 2006). A non-indigenous, fungal pathogen is often highly destructive to closely related species of the pathogen's host species, yet in its native range, the host is relatively unaffected by the pathogen, presumably due to host–pathogen co-adaptation (Parker and Gilbert, 2004). According to Loo (2009), beech bark disease (BBD) and six other new fungal disease introductions are having serious impacts on important native tree species in North American forests.

With concern surrounding the detrimental effect of chemical pesticides on the environment, as well as the impracticality of their use in forest systems, there has been an increased amount of interest in the use of biological methods to control forest pests and diseases. Rishbeth (1961) was one of the first to begin research in this area and showed that inoculation of pine logs (*Pinus* spp.) with *Phlebiopsis gigantea* could be used as an alternative to chemicals to successfully prevent entry of the basidiomycete pathogen *Heterobasidion annosum,* the causal agent of root rot.

There is potential for fungi to be used in this way to prevent further spread of the invasive BBD complex in North America. Any such biological agent to be introduced would probably need to be sought from the pest's centre of origin and would need to become established and persist in the environment long-term, in order to be successful for control. This thesis has reported on fungal endophytes isolated from healthy stems of *F. sylvatica* and entomopathogenic fungi (EPF) of *C. fagisuga* cadavers and explored the option of using them as biological control agents for both the insect and pathogen

components of the invasive BBD complex. A number of isolates considered to have the highest potential for biological control were selected and preliminary *in vitro* and *in vivo* assessments versus *C. fagisuga* and *N. coccinea* were carried out. Three application techniques were also used to evaluate three selected fungal isolates *in planta* for their capability to colonise and persist in beech saplings over a six-month period. Undertaking laboratory research with an insect-fungus, invasive disease complex was very challenging. The work in this thesis has begun to further knowledge of the use of antagonistic fungi (endophytes and EPF) and verified that isolates of *B. bassiana, L. muscarium* and *Trichoderma* spp. would make good candidates for biological control of BBD and justify further research and development.

6.2 *FAGUS* ENDOPHYTES

Over six hundred fungal endophyte isolates were obtained from trunks of *F. sylvatica* and *F. sylvatica* subsp. *orientalis* in this study, many of which were previously unreported in the literature. These endophytes were occupying the living cambium tissue of healthy trunks-the tissue vulnerable to attack by *C. fagisuga* and *N. coccinea*. A small number of the endophytes with recognized antagonistic properties towards plant pathogens and insect pests were isolated, including *Beauveria* spp., *Paecilomyces* sp. and *Trichoderma* spp. Species of *Beauveria* and *Lecanicillium* have been reported as entomopathogenic endophytes and can be antagonistic to both insects and plant pathogens (Ownley *et al.*, 2010). Endophytic *Beauveria* spp. were isolated from beech stems, both in the UK and Armenia. Despite *B. bassiana* having been isolated as an endophyte of numerous plant hosts, including trees *Pinus radiata* (Reay *et al.*, 2012) and *Theobroma cacao* (Evans *et al.*, 2003), no previous record of *Beauveria* spp. has been reported on *F. sylvatica*. Five different species of *Trichoderma* were isolated in this study, including *T. stilbohypoxyli*, a species common in the tropics (Lu and Samuels, 2003) and originally reported as a mycoparasite of *Stilbohypoxylon* in Puerto

Rico. *Trichoderma* species*,* including *T. viride* have previously been recorded as endophytes of *F. grandifolia* in USA. Cotter and Blanchard (1982) found that the genera most commonly isolated from healthy *F. grandifolia* in USA were *Alternaria, Aureobasidium, Cladosporium, Coniothyrium Epicoccum, Fusarium, Geotrichum, Nodulosporium, Penicillium, Phoma, Rhinocladiella* and *Trichoderma* as well as many unidentifiable fungi and bacteria. These workers also isolated endophytic fungi from cankered trees but found that healthy beech trees did not contain any unique fungal endophytes that could be attributed to their apparent absence of BBD. It would be of interest to sample and record the endophytes present in the cambium tissue of *F. grandifolia* in North America using the same technique described earlier in the thesis (chapter 2) in order to draw comparisons with the fungal endophytes isolated from *F. sylvatica* in Europe. This may further the understanding of the mutualistic relationship presented between *Fagus* spp. and fungal endophytes, in order to develop any potential role in biological control of pests and diseases such as BBD.

With the exception of the temporal study at Windsor, the endophyte surveys were carried out in summer/autumn and merely provided a snapshot of the endophytic flora present at that time. At Windsor, the same trees were sampled on five separate occasions and significantly different endophytes were isolated at these times. This is not only biologically interesting but it also highlights the importance of the correct timing of surveys, or multiple surveys, when biological control agents are being sought. This is another piece of valuable research to be done by subsequent workers.

The key purpose of this study was to identify potential biological control agents for BBD and so only trunks of healthy trees were sampled for endophytes. Not all endophytes isolated in chapter 2 of this thesis were investigated for their biological

control potential and the majority of them were not studied further. A small, manageable number of priority isolates were chosen for subsequent study but it is possible that beneficial isolates could have been overlooked, including *Clonostachys* spp.*, Paecilomyces* sp. and alternative isolates of *Trichoderma* spp. Likewise, many isolates which may have been taxonomically interesting were unidentifiable, due to a lack of sporulation. Molecular identification was limited to a small number of the most unusual isolates but if additional resources had been available, then more in-depth molecular identification of *Beauveria* spp., *Chaunopycnis* sp., *Sporidesmium* sp., *Trichoderma* spp. and the non-sporulating fungi would have been worthwhile. It could also have been of interest to isolate and identify endophytes of other *F. sylvatica* tissues such as leaves, seeds and roots for comparison and to evaluate the organ specificity of endophytes within *F. sylvatica* trees.

6.3 ENTOMOPATHOGENIC FUNGI

The field survey undertaken for entomopathogens of *C. fagisuga* in Armenia was limited by time and hence was only qualitative. However, this study did identify thirteen EPF, including two isolates of *Lecanicillium* (IMI503731 and IMI502732), a commonly used genus in commercial biopesticides. The use of EPF for biological control of *C. fagisuga* has not been previously considered, therefore, more thorough surveys for further EPF isolates could be implemented within *C. fagisuga*'s centre of origin to enable more extensive screening and selection of host-specific strains.

Due to their minute size and dependence on beech sap, it was very challenging to experiment with *C. fagisuga* juveniles and impossible to experiment with adult insects *in vitro*. However, it <u>was</u> possible to maintain and experiment with *C. fagisuga* eggs in the laboratory. Successful inoculation and colonisation of the egg coating was achieved

with *L. longisporum* (Vertalec) spores, which subsequently prevented the eggs from hatching. This could be one practicable methodology that could be used to interrupt the life-cycle of *C. fagisuga* and hence reduce populations. It was possible to inoculate, infect and re-isolate *L. longisporum* from juvenile insects, however, it was not possible to maintain untreated *C. fagisuga* juveniles *in vitro* for more than 5 days. Improved artificial rearing methods of juveniles might progress this line of research. Development of successful techniques to rear *C. fagisuga* juveniles *in vitro* are essential in order to further evaluate EPF as potential biocontrol agents for BBD. Alternatively, *in vivo* assays using wax moth larvae (*Galleria mellonella*) as a model laboratory insect, may prove useful to assess the virulence of the EPF isolates from the centre of origin of *C. fagisuga*, (*B. bassiana,* IMI502733 and *Lecanicillium* sp., IMI502732) and compare with commercial strains (Hussein *et al.*, 2012).

B. bassiana (IMI502733) had a significantly slower mean radial growth rate than the active ingredient of the commercial mycoinsecticide, Mycotrol but this slower growth rate might not be a disadvantage for the fungus and could possibly be attributed to the fact that this endophytic isolate was somehow "protected" within the *F. sylvatica* stem and therefore does not require such a fast growth rate for its survival. Similar to experiments conducted by Leland *et al.* (2005) on *B. bassiana,* conidia production, tolerance to solar radiation and production of beauvericin could be evaluated for isolate IMI502733 to further assess its suitability as a BCA. However, it should be noted that whilst laboratory screening can provide valuable information (virulence, dose, germination etc.) about an isolate, these results do not necessarily coincide with its future performance in the field.

6.4 *TRICHODERMA* SPP. AS ANTAGONISTS OF *NEONECTRIA COCCINEA* AND *N. FAGINATA*

Trichoderma is an interesting genus because it is known to possess several biological control mechanisms, exhibit different modes of action and many new endophytic species have recently been described (Samuels, 2006; Samuels *et al.*, 2006; Samuels and Ismaiel, 2009; Chaverri *et al.*, 2011). *Trichoderma* is widely used as a biological control agent and extensive research has been carried out with it against plant pathogenic fungi (Howell, 2003). Examples of their use against important pathogens of forest trees include *Armillaria* spp. and *Heterobasidion annosum* (Holdenrieder, 1984; Nicolotti *et al.*, 1999). Schubert *et al.* (2008a) found that *T. atroviride* could be used as an effective wound treatment for biological control of wood decay fungi in urban trees.

In this thesis, nine *Trichoderma* endophytic isolates from the native range of *F. sylvatica* were selected for *in vitro* assessment with two isolates of *Neonectria* spp. It was demonstrated that *T. harzianum* (IMI395636) has the highest potential for biological control of *N. faginata* as it scored highly in assessments examining the three mechanisms of fungal antagonism: competition, mycoparasitism and antibiosis. However, *in vitro* assessment of these mechanisms is only indicative of how they may perform in variable environmental conditions. For example and with regard to the production of toxic metabolites; these may only be produced by the antagonist (i.e. *Trichoderma*) in the presence of the pathogen (i.e. *Neonectria*). Future work should further evaluate the antagonistic properties of the nine *Trichoderma* isolates against *Neonectria coccinea* and *N. faginata* on *F. sylvatica* wood blocks, as Schubert *et al.* (2008a; 2008b) did with *Trichoderma* spp. and *Ganoderma* spp. on *F. sylvatica*. Hyphal interactions between *Trichoderma* and *Neonectria* isolates could be studied with the use

of scanning electron microscopy (SEM) (Chet *et al.*, 1981; Inbar *et al.*, 1996; de Melo and Faull, 2000) to observe in detail the mechanisms of antagonism.

This is the first known effort to target *Neonectria* spp. with *Trichoderma* spp. for the prospective control of BBD. The work carried out in this thesis has demonstrated that *Trichoderma* spp. have good potential for biological control of the plant pathogen and further advances the approaches of Ayres (1941) and Blyth (1949) with *Nematogonium ferrugineum*. Application of a highly competitive *Trichoderma* isolate to trees already infested with *C. fagisuga*, could prevent subsequent infection by *N. faginata,* the most detrimental half of the BBD complex. It is possible that this concept to target the fungal component of the disease complex could be an effective method to slow or halt the spread of BBD in the field, saving significant numbers of beech trees in North America. Due to the difficulties mentioned in rearing *C. fagisuga in vitro*, it is recommended that future research for biological control of BBD using beech endophytes is directed toward the *Neonectria* component of the disease complex.

6.5 COLONISATION OF BEECH SAPLINGS

It was possible to inoculate and re-isolate two of the three fungal isolates that were applied to beech saplings in an experimental glasshouse set-up. However, the levels of recovery of *T. harzianum* (IMI395636) and *B. bassiana* (IMI502733) were very low and localised to the inoculated plant parts. No recovery of *Lecanicillium* sp. (IMI502732) from *F. sylvatica* plant parts was achieved. Reasons for this are unknown but for example, it may be possible that light or humidity levels in the glasshouse were not favourable for the growth and establishment of this isolate. In order to achieve systemic, endophytic infection of a plant, it may be necessary to treat the seeds with the endophyte inoculum to first infect the cotyledon, the emerging radicle and hypocotyl

and consequently the seedling. Bailey *et al.* (2008) and Ownley *et al.* (2008) achieved good levels of seedling colonisation using this method. Alternatively, application of a more concentrated spore suspension, could possibly improve colonisation levels of *F. sylvatica* saplings. Ownley *et al.* (2008) inoculated tomato, cotton and bean seeds with different spore concentrations of *B. bassiana* and found that as the concentration of spores increased, the proportion of plant tissues from which *B. bassiana* was recovered also increased. Brownbridge *et al.* (2012), achieved colonisation of *P. radiata* seedlings with two endophytic *B. bassiana* isolates obtained from mature pine trees originally, but these could not be detected after nine months. Posada and Vega (2005) achieved successful colonisation of *T. cacao* with *B. bassiana* but they were not able to re-isolate it beyond two months. Anderson *et al.* (2007) and Gurulingappa *et al.* (2010) both successfully colonised crop plants with a *Lecanicillium lecanii* isolate that had originated as an endophyte of *Gossypium hirsutum*. The *Lecanicillium* sp. isolate (IMI502732) used in this study, was <u>not</u> initially recovered as an endophyte of *F. sylvatica* (but rather as an EPF of *C. fagisuga*) which could explain its inability to colonise the beech saplings in this study.

All spore suspensions in this study were prepared with Tween 80, a non-ionic surfactant and emulsifier. However, advanced formulation of *Trichoderma* and *Beauveria* spores in a suitable oil or water-based carrier could potentially increase viability and promote growth and establishment of these fungi during the initial stage of sapling colonisation. Due to the apparent lack of systemic growth of these isolates, longer-term endophytic establishment of these fungi in beech saplings may require multiple application methods (i.e. to soil, stem and leaves) or repeated applications as they grow and develop.

If improved levels of colonisation could be achieved, then it may be possible to artificially infest beech saplings with *C. fagisuga* (Houston, 1982) to directly assess the potential of these fungi for biological control of the insect pest.

6.6 SUMMARY

Thousands of potential biological control agents have been researched over the last ninety years, yet only a very small number have proved successful as commercially viable products (Slininger *et al.*, 2003). The development of a fungal biological control agent requires several stages and can take many years (Butt *et al.*, 2001; Taylor *et al.*, 2011). Firstly, fungi must be isolated from the environment and accurately identified. Basic information such as their ecological properties must be determined and then laboratory screening and field testing can help to determine and prioritise the most antagonistic or virulent isolates.

Work in this thesis isolated and identified suitable fungal endophytes and entomopathogenic fungi as candidates for biological control of *C. fagisuga* and *N. coccinea* and preliminary *in vitro, in vivo* and *in planta* screening of priority isolates was carried out. However, in order to progress these potential biological control agents further towards the development of a marketable mycopesticide, further work would need to be undertaken. Mass production of fungal inoculum either on a solid substrate or in liquid media is crucial for successful commercialisation (Wraight *et al.*, 2001) and formulation of fungal agents can enhance their efficacy and provide protection from the environment (Jones and Burges, 1998). Use of the most appropriate application technology should also be considered in order to optimise delivery of the biopesticide to its target pest (Bateman and Chapple, 2001). Before registration of a new product, risk assessments must be undertaken to consider its fate in the environment and its toxicity

to humans. Once a new product makes it to the shelf, it must then be possible to incorporate the biological control agent into an effective integrated pest management (IPM) package.

As BBD continues to invade beech forests in North America, methods of control are urgently needed. Whilst complete control of BBD is almost certainly impossible, strategies to slow and reduce the spread of the insect and/or pathogen components are possible with further research and development. Studies within this thesis have demonstrated that fungi, i.e., endophytes and entomopathogens <u>do</u> have potential for further development as biological control agents of BBD and strategies to control both *C. fagisuga* and *Neonectria* spp. have been explored. It may in fact, be possible to use a collection of biological control agents to capitalise on the potential benefits of different isolates or even different genera of suitable fungi. The use of biological control methods, the development of disease resistant genotypes, good forest management practices and stringent biosecurity measures are required as components of an IPM strategy to prevent further spread of this invasive disease complex.

6.7 CONCLUSIONS

1. Many fungal endophytes isolated from of *F. sylvatica* stems, including genera with potential for biological control of Beech Bark Disease

2. Endophyte species richness was highest in Wye Valley, a native ancient woodland site

3. Five different species of *Trichoderma* were isolated as stem endophytes of beech

4. Two species of *Beauveria* were isolated as stem endophytes of beech in the UK and Armenia

5. A potentially new endophytic species was identified (*Sporidesmium* sp.)

6. Spatial differences in the endophyte assemblages of beech were observed at different sites in the UK

7. Temporal differences in the endophyte assemblages of beech were observed at Windsor Great Park site

8. *Lecanicillium muscarium* was isolated from *C. fagisuga* cadavers in its centre of origin in Armenia

9. *C. fagisuga* proved very difficult to work with *in vitro,* however it was possible to work with the egg stage

10. Mycotal (*L. muscarium*) prevented hatching of *C. fagisuga* eggs by 100%

11. *Trichoderma* (IMI395636) proved to be a good mycoparasite of *Neonectria* spp. *in vitro*

12. Colonisation of *F. sylvatica* saplings was achieved at low levels with *Trichoderma* and *Beauveria*

13. DNA fingerprinting was used to confirm that *Trichoderma* and *Beauveria* isolates successfully colonised beech saplings at low levels

14. *Lecanicillium* did not colonise beech saplings

6.8 RECOMMENDED FOLLOW-ON WORK

1. Further assess antagonistic properties of *Trichoderma* against *Neonectria* spp. on *F. sylvatica* wood blocks

2. Undertake hyphal interaction studies with *Trichoderma* spp. and *Neonectria* spp.

3. Identify *Trichoderma* soluble metabolites using HPLC

4. Develop an effective inoculation technique for *C. fagisuga* crawlers using Mycotal and Vertalec, possibly on *F. sylvatica* wood discs

5. Assess virulence of *B. bassiana* (IMI502733) and *Lecanicillium* sp. (IMI502732) on *C. fagisuga* eggs or crawlers (see point 4 above) or alternatively, by using *Galleria mellonella* larvae

6. Undertake further genetic analysis of endophytic *B. bassiana* (IMI502733) and compare to other *B. bassiana* endophytic isolates

7. Develop formulation and application techniques for endophytes and entomopathogens to improve colonisation of beech saplings

8. Challenge endophyte or entomopathogen-inoculated beech saplings with *C. fagisuga* insects.

9. Undertake further identification of endophytes and entomopathogens, including *Trichoderma* spp., *Sporidesmium* sp., *Beauveria* spp., *Chaunopycnis* sp. and non-sporulating fungi using molecular techniques.

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8. APPENDICES

Appendix 8:1: Recipes

Potato Carrot Agar (PCA)

20g washed, peeled, grated organic potatoes
20g washed, peeled, grated organic carrots
20g Oxoid Technical Agar No 3 (LP0013)
1 litre tap water

Boil for 1 hour, autoclave at 15psi and 121°C for 15 minutes

Malt Extract Agar (MEA)

50g Malt Extract Agar (Oxoid-CM0059)
1 litre distilled water

Autoclave at 15psi and 115°C for 10 minutes

Sabouraud Dextrose Agar (SDA)

65g Sabouraud Dextrose Agar (Oxoid-CM0041)
1 litre distilled water

Autoclave at 15psi and 121°C for 15 minutes

Tween

10 drops Tween 80 (Polyoxyethylene Sorbitan Monooleate Sigma-P8074)
1 litre distilled water

Shake and autoclave at 15psi and 121°C for 15 minutes

Appendix 8:2: Endophytes isolated from *F. sylvatica*

Isolate number	Identification	Site number	Visit number
B001-01A	*Aspergillus* sp.3	1	1
B001-02A	*Aspergillus* sp.8	1	1
B001-02B	Sterile W12	1	1
B001-03A	*Aspergillus* sp.3	1	1
B001-04A	*Aspergillus* sp.7	1	1
B001-06A	*Trichoderma* sp. vb	1	1
B001-07A	Sterile BL1	1	1
B001-08A	*Aspergillus* sp.3	1	1
B001-09A	*Aspergillus* sp.3	1	1
B001-09B	*Aspergillus* sp.2	1	1
B001-09C	*Aspergillus* sp.2	1	1
B001-10A	*Aspergillus* sp.5	1	1
B002-01A	*Aspergillus* sp.3	1	1
B002-02A	*Coniothyrium fuckelli*	1	1
B002-02B	*Aspergillus* sp.3	1	1
B002-03A	*Aureobasidium pullulans*	1	1
B002-06A	*Aspergillus* sp.6	1	1
B002-08A	*Aspergillus* sp.6	1	1
B002-09A	*Phomopsis* sp.	1	1
B003-01A	*Aspergillus* sp.5	1	1
B003-03A	*Aspergillus* sp.3	1	1
B003-03B	*Aureobasidium pullulans*	1	1
B003-03C	Sterile W9	1	1
B003-05A	Sterile BL1	1	1
B003-05B	Sterile BL1	1	1
B003-06A	*Aspergillus* sp.3	1	1
B003-08A	*Cladosporium cladosporoides*	1	1
B004-01A	Sterile W10	1	1
B004-04A	*Cladosporium herbarum*	1	1
B004-05A	*Aureobasidium pullulans*	1	1
B004-09A	Sterile W10	1	1
B005-01A	Sterile BL1	1	1
B005-02A	*Aspergillus* sp.7	1	1
B005-03A	*Aspergillus* sp.3	1	1
B005-03B	*Aspergillus* sp.7	1	1
B005-03C	Sterile BL1	1	1
B005-04A	*Aureobasidium pullulans*	1	1
B005-04B	Sterile BL1	1	1
B005-04C	*Aureobasidium pullulans*	1	1
B005-05A	*Aspergillus* sp.2	1	1
B005-05B	*Melanconium atrum*	1	1
B005-06A	*Aureobasidium pullulans*	1	1
B005-08A	Sterile W1	1	1

Isolate number	Identification	Site number	Visit number
B005-09A	*Melanconium atrum*	1	1
B006-02A	Sterile W1	1	1
B006-05A	Sterile W1	1	1
B006-08A	*Aspergillus* sp.1	1	1
B007-01A	*Aspergillus* sp.9	1	1
B007-03A	*Xylaria carpophila*	1	1
B007-07A	Sterile BL1	1	1
B007-09A	Sterile W11	1	1
B008-02A	Sterile BL1	1	1
B008-03A	*Aspergillus* sp.1	1	1
B009-02A	*Aspergillus* sp.4	1	1
B009-03A	*Aspergillus* sp.10	1	1
B010-02A	Sterile W1	1	1
B011-01A	Sterile BL1	2	1
B011-03A	*Cladosporium herbarum*	2	1
B011-05A	Sterile W1	2	1
B011-07A	Bacteria	2	1
B012-04A	Sterile BL7	2	1
B012-05A	Sterile BL1	2	1
B012-09A	*Pseudopatalina conigena*	2	1
B013-02A	Sterile W1	2	1
B013-05A	*Aureobasidium pullulans*	2	1
B013-08A	Sterile BR2	2	1
B014-01A	Sterile BL1	2	1
B014-02A	Sterile W1	2	1
B014-02B	Sterile BL1	2	1
B014-03A	Sterile BL1	2	1
B014-04A	*Phoma herbarum*	2	1
B014-04B	Sterile W9	2	1
B014-05A	*Pseudocercosporella* sp.	2	1
B014-06A	*Aureobasidium pullulans*	2	1
B014-07A	Sterile BL5	2	1
B014-08A	Sterile BL1	2	1
B014-09A	*Pseudopatalina* sp.	2	1
B014-10A	Sterile BL2	2	1
B015-08A	*Apiognomonia errabunda*	2	1
B016-06A	*Aureobasidium pullulans*	2	1
B017-06A	*Pseudopatalina conigena*	2	1
B017-07A	*Bloxamia truncata*	2	1
B017-09A	*Aureobasidium pullulans*	2	1
B018-02A	*Rhinocladiella* sp.	2	1
B018-03A	Sterile BL1	2	1
B018-04A	*Bloxamia truncata*	2	1
B018-09A	*Paecilomyces variotii*	2	1

Isolate number	Identification	Site number	Visit number
B018-10A	Sterile BL1	2	1
B019-09A	Sterile BL1	2	1
B020-04A	*Aureobasidium* sp.	2	1
B020-08A	Sterile BL1	2	1
B021-07A	*Cladosporium oxysporum*	3	1
B021-07B	Sterile W9	3	1
B022-05A	*Geotrichum* sp.	3	1
B022-09A	Sterile BL1	3	1
B023-01A	Sterile BL7	3	1
B023-02A	*Aspergillus* sp.12	3	1
B023-03A	*Aspergillus* sp.12	3	1
B023-05A	*Aspergillus* sp.12	3	1
B023-06A	Sterile BL7	3	1
B024-01A	*Pithomyces chartarum*	3	1
B024-07A	Sterile BL1	3	1
B025-02A	*Geotrichum* sp.	3	1
B025-03A	Sterile BR4	3	1
B025-06A	Sterile BL1	3	1
B025-07A	Sterile BL1	3	1
B025-08A	Sterile BL1	3	1
B025-09A	*Coniothyrium fuckelli*	3	1
B025-10A	*Geniculosporium* sp.1	3	1
B026-05A	Sterile BL1	3	1
B026-05B	*Melanconium atrum*	3	1
B026-07A	Sterile BL1	3	1
B026-08A	*Melanconium atrum*	3	1
B026-09A	Sterile BL1	3	1
B026-10A	Sterile BL1	3	1
B027-01A	Sterile W1	3	1
B027-02A	*Botryotrichum piluliphorum*	3	1
B027-08A	Sterile BL7	3	1
B027-09A	Sterile W1	3	1
B027-09B	*Coniothyrium fuckelli*	3	1
B027-10A	*Cladosporium oxysporum*	3	1
B027-10B	Sterile BL1	3	1
B028-06A	*Cladosporium oxysporum*	3	1
B029-01A	Sterile BL1	3	1
B029-02A	*Trichoderma* sp. vd	3	1
B029-04A	*Penicillium* sp.3	3	1
B029-06A	*Cladosporium oxysporum*	3	1
B030-01A	Sterile BL5	3	1
B030-02A	*Cladosporium oxysporum*	3	1
B030-03A	*Penicillium* sp.5	3	1
B030-04A	Sterile W1	3	1

Isolate number	Identification	Site number	Visit number
B030-05A	Sterile BL8	3	1
B030-06A	Sterile W11	3	1
B030-09A	Sterile BL8	3	1
B032-01A	*Botryotrichum piluliphorum*	4	1
B032-01B	Sterile BR8	4	1
B033-02A	*Melanconium atrum*	4	1
B034-08A	Sterile BL4	4	1
B035-02A	Sterile BL4	4	1
B036-01A	*Beauveria bassiana*	4	1
B036-04A	Sterile BL4	4	1
B036-05A	Sterile BL4	4	1
B036-07A	Sterile BL1	4	1
B037-01A	Sterile BL1	4	1
B037-06A	*Acremonium psammosporum*	4	1
B037-08A	Sterile W4	4	1
B040-03A	*Acremonium* sp.2	4	1
B041-03A	Contaminated	1	2
B041-04A	Sterile W1	1	2
B041-05A	No growth	1	2
B041-07A	Sterile W1	1	2
B041-09A	Sterile BL1	1	2
B042-01A	*Blastobotrys* sp.	1	2
B042-06A	Sterile BL1	1	2
B042-07A	*Blastobotrys* sp.	1	2
B042-08A	Sterile W2	1	2
B042-09A	Sterile W2	1	2
B042-10A	*Aureobasidium pullulans*	1	2
B043-01A	Sterile W3	1	2
B043-02A	No growth	1	2
B043-04A	Sterile W6	1	2
B043-06A	Sterile W3	1	2
B043-07A	Sterile BL1	1	2
B043-08A	Sterile W4	1	2
B043-10A	*Xylaria carpophila*	1	2
B044-02A	Sterile BL1	1	2
B044-03A	Sterile BL1	1	2
B044-05A	No growth	1	2
B044-05B	Sterile BL1	1	2
B044-06A	Sterile W4	1	2
B044-10A	*Beauveria* sp.	1	2
B045-02A	Sterile W1	1	2
B045-06A	*Beauveria* sp.	1	2
B045-08A	Sterile BL1	1	2
B045-10A	Sterile W2	1	2

Isolate number	Identification	Site number	Visit number
B046-01A	Sterile W9	1	2
B046-02A	Sterile W2	1	2
B046-03A	Sterile W2	1	2
B046-04A	No growth	1	2
B046-05A	Yeast	1	2
B046-06A	*Spinager meineckellus*	1	2
B046-07A	*Geotrichum* sp.	1	2
B046-08A	No growth	1	2
B046-09A	Sterile BR1	1	2
B046-10A	Sterile W9	1	2
B047-02A	Sterile BL1	1	2
B047-03A	*Phylocephala* sp.	1	2
B047-03B	Sterile W11	1	2
B047-04A	Sterile BL1	1	2
B047-05A	Sterile W2	1	2
B047-06A	Sterile W1	1	2
B047-07A	Sterile BL1	1	2
B047-09A	Sterile W4	1	2
B047-09B	*Geotrichum* sp.	1	2
B047-10A	*Spinager meineckellus*	1	2
B048-01A	No growth	1	2
B048-03A	Yeast	1	2
B048-05A	No growth	1	2
B048-06A	Sterile W1	1	2
B048-07A	Sterile W1	1	2
B049-03A	Sterile W9	1	2
B049-04A	Sterile W9	1	2
B049-06A	Sterile W9	1	2
B049-07A	*Trichoderma* sp.	1	2
B049-08A	*Trichoderma* sp. vd	1	2
B049-09A	Penicillium sp.2	1	2
B049-10A	*Geotrichum* sp.	1	2
B050-02A	*Geotrichum* sp.	1	2
B050-07A	Sterile W1	1	2
B050-08A	Yeast	1	2
B050-09A	Yeast	1	2
B050-10A	*Spinager meineckellus*	1	2
B051-02A	Sterile W1	2	2
B051-03A	Sterile W1	2	2
B051-05A	Sterile BL1	2	2
B051-07A	Sterile BR8	2	2
B051-09A	*Geotrichum* sp.	2	2
B052-01A	Contaminated	2	2
B052-05A	Sterile BL1	2	2

Isolate number	Identification	Site number	Visit number
B052-08A	*Acremonium* sp.1	2	2
B052-09A	*Acremonium* sp.1	2	2
B052-10A	Sterile BL1	2	2
B053-02A	Sterile BR9	2	2
B053-03A	No growth	2	2
B053-05A	Sterile BL3	2	2
B053-06A	Sterile W3	2	2
B053-07A	Sterile BR1	2	2
B053-08A	*Geniculosporium* sp.1	2	2
B053-09A	*Geniculosporium* sp.2	2	2
B054-04A	*Aureobasidium pullulans*	2	2
B055-01A	Sterile BR6	2	2
B055-02A	*Penicillium* sp.1	2	2
B055-05A	Sterile BL1	2	2
B055-09A	Sterile BL1	2	2
B056-03A	*Melanconium atrum*	2	2
B056-03B	*Melanconium atrum*	2	2
B056-04A	Sterile W6	2	2
B056-05A	Sterile W6	2	2
B056-06A	*Melanconium atrum*	2	2
B057-04A	Sterile W3	2	2
B057-09A	Sterile W3	2	2
B058-02A	Sterile W3	2	2
B058-05A	*Geniculosporium* sp.1	2	2
B058-06A	*Clonostachys rosea*	2	2
B058-07A	Sterile W1	2	2
B058-08A	Sterile W12	2	2
B058-09A	Sterile W3	2	2
B059-02A	Sterile BR9	2	2
B059-03A	*Monodictys* sp.	2	2
B059-04A	Sterile BL1	2	2
B059-05A	Sterile W6	2	2
B059-07A	Sterile BL7	2	2
B060-01A	Sterile BL1	2	2
B060-02A	*Geniculosporium* sp.1	2	2
B060-03A	Sterile W6	2	2
B060-05A	Sterile BL1	2	2
B060-06A	Sterile W1	2	2
B060-10A	*Periconiella* sp.	2	2
B061-01A	*Dendrodochium aurantiacum*	3	2
B061-01B	*Hormomyces* sp.	3	2
B061-02A	Sterile C2	3	2
B061-03A	Sterile W4	3	2
B061-04A	*Dendrodochium aurantiacum*	3	2

Isolate number	Identification	Site number	Visit number
B061-06A	*Penicillium* sp.2	3	2
B061-08A	Sterile BL1	3	2
B061-09A	Contaminated	3	2
B061-10A	*Dendrodochium aurantiacum*	3	2
B062-01A	*Trichoderma harzianum*	3	2
B062-02A	Acremonium sp.1	3	2
B062-03A	*Trichoderma harzianum*	3	2
B062-04A	*Aspergillus* sp.12	3	2
B062-05A	No growth	3	2
B062-05B	No growth	3	2
B062-06A	*Colletotrichum* sp.	3	2
B062-07A	*Trichoderma harzianum*	3	2
B062-08A	*Geotrichum* sp.	3	2
B062-09A	No growth	3	2
B062-10A	*Penicillium* sp.1	3	2
B063-02A	*Pesotum ulmi*	3	2
B063-03A	Sterile W1	3	2
B063-03B	*Penicillium* sp.1	3	2
B063-04A	*Sclerotium* sp.	3	2
B063-05A	*Trichoderma harzianum*	3	2
B063-08A	*Dendrodochium aurantiacum*	3	2
B063-09A	*Trichoderma harzianum*	3	2
B064-01A	Sterile W1	3	2
B064-02A	*Penicillium* sp.1	3	2
B064-03A	*Clonostachys* sp.	3	2
B064-03B	*Clonostachys* sp.	3	2
B064-04A	*Coniothyrium fuckelli*	3	2
B064-05A	Sterile BL1	3	2
B064-06A	*Dendrodochium aurantiacum*	3	2
B064-06B	*Acremonium* sp.1	3	2
B064-07A	Sterile W9	3	2
B064-08A	*Dendrodochium aurantiacum*	3	2
B064-09A	Contaminated	3	2
B064-10A	Sterile BL7	3	2
B065-01A	Sterile W1	3	2
B065-01B	*Penicillium* sp.1	3	2
B065-02A	Sterile W1	3	2
B065-03A	*Penicillium* sp.1	3	2
B065-03B	Contaminated	3	2
B065-04A	*Trichoderma* sp.	3	2
B065-06A	*Coniothyrium fuckelli*	3	2
B065-07A	*Trichoderma harzianum*	3	2
B065-08A	*Acremonium* sp.2	3	2
B065-09A	*Phoma dorenboschii*	3	2

Isolate number	Identification	Site number	Visit number
B065-10A	*Penicillium* sp.1	3	2
B066-01A	*Asteromella* sp.	3	2
B066-02A	*Asteromella* sp.	3	2
B066-02B	*Asteromella* sp.	3	2
B066-03A	*Acremonium* sp.1	3	2
B066-04A	*Acremonium* sp.1	3	2
B066-05A	*Mucor hiemalis*	3	2
B066-06A	Sterile W4	3	2
B067-01A	*Penicillium* sp.2	3	2
B067-04A	*Acremonium* sp.1	3	2
B067-05A	*Penicillium* sp.2	3	2
B067-05B	*Penicillium* sp.2	3	2
B067-07A	Sterile W3	3	2
B067-09A	Bacteria	3	2
B067-09B	*Acremonium* sp.1	3	2
B067-10A	*Mucor hiemalis*	3	2
B068-01A	*Clonostachys rosea*	3	2
B068-02A	*Clonostachys rosea*	3	2
B068-05A	*Clonostachys rosea*	3	2
B068-06A	Acremonium sp.1	3	2
B068-07A	*Clonostachys rosea*	3	2
B068-08A	*Clonostachys rosea*	3	2
B068-09A	*Stagonospora* sp.	3	2
B068-09B	Sterile BR5	3	2
B068-10A	*Clonostachys rosea*	3	2
B069-01A	*Trichoderma stilbohypoxyli*	3	2
B069-02A	*Acremonium* sp.1	3	2
B069-04A	*Geotrichum* sp.	3	2
B069-06A	*Trichoderma stilbohypoxyli*	3	2
B069-07A	Sterile P1	3	2
B069-08A	Sterile BL6	3	2
B069-09A	Sterile BL1	3	2
B069-10A	*Phoma tropica*	3	2
B070-04A	*Clonostachys rosea*	3	2
B070-05A	Sterile W9	3	2
B070-05B	Sterile BL5	3	2
B070-08A	*Beauveria bassiana*	3	2
B070-09A	*Clonostachys rosea*	3	2
B071-01A	Sterile W12	4	2
B071-02A	Sterile W12	4	2
B071-03A	Sterile C2	4	2
B071-05A	Sterile BL2	4	2
B071-05B	Sterile BL1	4	2
B071-06A	*Aureobasidium* sp.	4	2

Isolate number	Identification	Site number	Visit number
B071-07A	Sterile W12	4	2
B072-03A	Sterile W2	4	2
B072-04A	Sterile W5	4	2
B072-05A	Sterile W2	4	2
B072-06A	Sterile W2	4	2
B072-07A	Sterile W2	4	2
B072-08A	*Aspergillus* sp.1	4	2
B072-09A	Sterile W1	4	2
B073-02A	Sterile W7	4	2
B073-03A	Sterile W7	4	2
B073-05A	*Geniculosporium* sp.1	4	2
B073-09A	Sterile W2	4	2
B073-10A	*Aspergillus* sp.1	4	2
B074-01A	Sterile BR3	4	2
B074-03A	*Acremonium* sp.1	4	2
B074-04A	*Acremonium* sp.1	4	2
B074-09A	Sterile BL1	4	2
B075-01A	*Trichoderma* sp. vd	4	2
B075-02A	Sterile BL1	4	2
B075-04A	Sterile W1	4	2
B075-05A	*Geotrichum* sp.	4	2
B075-05B	No growth	4	2
B075-06A	Sterile W7	4	2
B075-07A	Sterile W1	4	2
B075-08A	Sterile W1	4	2
B075-09A	*Penicillium* sp.4	4	2
B076-02A	Sterile W2	4	2
B076-03A	No growth	4	2
B076-05A	No growth	4	2
B076-06A	Sterile W2	4	2
B076-09A	Sterile BL7	4	2
B077-03A	Sterile W3	4	2
B077-04A	*Penicillium* sp.4	4	2
B077-06A	*Beauveria bassiana*	4	2
B077-08A	*Trichoderma harzianum*	4	2
B077-09A	Sterile W3	4	2
B077-10A	*Beauveria bassiana*	4	2
B078-01A	Sterile W4	4	2
B078-02A	Sterile W4	4	2
B078-04A	*Dendrodochium* sp.	4	2
B078-05A	*Aureobasidium* sp.	4	2
B078-06A	*Beauveria bassiana*	4	2
B078-08A	*Geotrichum* sp.	4	2
B079-01A	No growth	4	2

Isolate number	Identification	Site number	Visit number
B079-02A	*Geotrichum candidum*	4	2
B079-04A	*Trichoderma* sp. vd	4	2
B079-05A	Sterile W1	4	2
B079-06A	*Codineae hughesii*	4	2
B079-09A	*Acremonium* sp.1	4	2
B079-10A	No growth	4	2
B080-01A	Sterile W8	4	2
B080-04A	*Dendrodochium* sp.	4	2
B080-05A	Sterile W7	4	2
B080-07A	Sterile W7	4	2
B080-10A	Sterile BL1	4	2
B082-04A	*Penicillium* sp.1	2	3
B082-07A	Sterile BL1	2	3
B083-02A	Sterile BL1	2	3
B083-03A	Sterile C1	2	3
B083-05A	Sterile BR4	2	3
B083-06A	*Clonostachys rosea*	2	3
B083-07A	*Clonostachys rosea*	2	3
B083-08A	Sterile C1	2	3
B083-08B	Sterile BL1	2	3
B083-10A	*Cladosporium* sp.	2	3
B084-03A	Sterile BL1	2	3
B084-04A	Sterile BL1	2	3
B084-10A	No growth	2	3
B085-03A	No growth	2	3
B085-04A	Sterile BL1	2	3
B085-06A	No growth	2	3
B085-07A	Sterile BL1	2	3
B086-02A	Sterile C1	2	3
B086-03A	*Trichoderma* sp.	2	3
B086-10A	Sterile BL1	2	3
B087-03A	Sterile BL1	2	3
B087-04A	Sterile BL1	2	3
B087-05A	Sterile BL1	2	3
B087-06A	Sterile BL1	2	3
B087-08A	*Clonostachys rosea*	2	3
B087-10A	Sterile BL1	2	3
B088-02A	*Beauveria* sp.	2	3
B088-03A	Sterile BR4	2	3
B088-06A	*Beauveria* sp.	2	3
B088-07A	Sterile BL1	2	3
B088-08A	Sterile P1	2	3
B088-10A	*Beauveria* sp.	2	3
B089-02A	Sterile BL1	2	3

Isolate number	Identification	Site number	Visit number
B089-05A	Sterile C1	2	3
B089-06A	Sterile BR1	2	3
B089-08A	Sterile W7	2	3
B089-09A	*Coniothyrium fuckelli*	2	3
B089-10A	Sterile BL1	2	3
B090-01A	*Penicillium* sp.1	2	3
B090-04A	*Clonostachys rosea*	2	3
B090-05A	No growth	2	3
B090-06A	*Penicillium* sp.1	2	3
B090-07A	*Phomopsis* sp.	2	3
B090-07B	No growth	2	3
B091-01A	No growth	5	1
B091-02A	Sterile W4	5	1
B091-03A	*Geotrichum* sp.	5	1
B091-04A	*Clonostachys rosea*	5	1
B091-06A	*Monochaetia* sp.	5	1
B091-08A	*Clonostachys rosea*	5	1
B092-01A	*Beauveria* sp.	5	1
B092-06A	No growth	5	1
B093-01A	Sterile BR8	5	1
B093-06A	Sterile C2	5	1
B093-07A	Sterile W4	5	1
B094-01A	Sterile BR11	5	1
B094-03A	*Penicillium* sp.1	5	1
B094-06A	*Acremonium* sp.3	5	1
B095-06A	Contaminated	5	1
B096-01A	Sterile BL1	5	1
B096-02A	Sterile BL1	5	1
B097-01A	Sterile BL1	5	1
B097-02A	Sterile C1	5	1
B097-06A	*Beauveria* sp.	5	1
B097-08A	*Penicillium* sp.6	5	1
B098-01A	Sterile BL1	5	1
B098-02A	Sterile BL1	5	1
B098-06A	*Penicillium* sp.6	5	1
B098-07A	Sterile BL1	5	1
B098-09A	Sterile BL1	5	1
B098-10A	Sterile W4	5	1
B099-06A	*Clonostachys* sp.	5	1
B099-07A	*Penicillium* sp.6	5	1
B100-01A	Sterile BL1	5	1
B100-02A	Sterile BL1	5	1
B100-03A	Sterile W1	5	1
B100-06A	Sterile BL1	5	1

Isolate number	Identification	Site number	Visit number
B101-01A	Sterile C1	6	1
B101-02A	Sterile BL1	6	1
B101-03A	*Clonostachys* sp.	6	1
B102-01A	*Penicillium* sp.2	6	1
B103-01A	No growth	6	1
B103-06A	Sterile BL1	6	1
B104-01A	*Clonostachys* sp.	6	1
B104-03A	*Clonostachys* sp.	6	1
B104-04A	*Clonostachys* sp.	6	1
B104-06A	*Clonostachys* sp.	6	1
B104-08A	*Clonostachys* sp.	6	1
B105-01A	Sterile BR1	6	1
B105-02A	No growth	6	1
B105-03A	Sterile W1	6	1
B105-06A	Sterile BL7	6	1
B105-08A	Sterile BR5	6	1
B105-09A	Sterile BL7	6	1
B106-01A	No growth	6	1
B106-01B	Sterile BL1	6	1
B106-02A	*Sclerotium* sp.	6	1
B106-06A	Sterile C1	6	1
B107-01A	*Aspergillus* sp.11	6	1
B107-02A	Sterile BL1	6	1
B107-06A	Sterile BL1	6	1
B107-07A	Sterile W4	6	1
B108-01A	*Beauveria* sp.	6	1
B108-02A	Sterile BL1	6	1
B108-03A	Sterile W6	6	1
B108-06A	Sterile W6	6	1
B109-06A	Sterile BR1	6	1
B110-01A	*Fusarium* sp.1	6	1
B110-02A	Sterile BR1	6	1
B110-03A	No growth	6	1
B110-04A	Sterile BL2	6	1
B110-06A	Sterile BL7	6	1
B110-07A	Sterile BL1	6	1
B110-08A	Sterile BR8	6	1
B112-03A	*Phoma* sp.	7	1
B112-04A	*Verticillium* sp.	7	1
B112-05A	Sterile BL6	7	1
B112-06A	Phoma sp.	7	1
B112-07A	Phoma sp.	7	1
B112-08A	*Verticillium* sp.	7	1
B112-08B	Sterile BL5	7	1

Isolate number	Identification	Site number	Visit number
B113-07A	*Phoma* sp.	7	1
B113-08A	Sterile BL1	7	1
B113-09A	*Cladosporium* sp.	7	1
B113-10A	*Phoma* sp.	7	1
B114-04A	Sterile BL6	7	1
B114-06A	*Verticillium* sp.	7	1
B115-10A	Sterile BL1	7	1
B116-04A	Sterile BR8	7	1
B116-04B	*Acremonium* sp.3	7	1
B116-10A	Sterile BL1	7	1
B117-05A	Sterile BR8	7	1
B117-10A	*Alternaria* sp.	7	1
B118-08A	Sterile BR8	7	1
B121-05A	Sterile BL1	8	1
B121-10A	Sterile BL1	8	1
B123-06A	*Beauveria* sp.	8	1
B126-03A	*Cladosporium herbarum*	8	1
B126-09A	*Verticillium* sp.	8	1
B126-10A	*Penicillium* sp.7	8	1
B131-02A	*Trichoderma* sp.	9	1
B131-06A	Sterile C1	9	1
B131-06A	*Verticillium* sp.	9	1
B132-01A	Sterile W6	9	1
B133-06A	*Rhizoctonia* sp.	9	1
B134-02A	*Verticillium* sp.	9	1
B134-03A	Phoma sp.	9	1
B134-05A	*Verticillium* sp.	9	1
B135-06A	Contaminated	9	1
B136-01A	*Cladosporium herbarum*	9	1
B136-06A	*Cladosporium herbarum*	9	1
B136-07A	*Paecilomyces* sp.	9	1
B137-01A	*Trichoderma* sp.	9	1
B137-02A	Sterile BL1	9	1
B139-01A	*Acremonium* sp.3	9	1
B139-01B	*Cladosporium herbarum*	9	1
B141-07A	*Fusarium* sp.2	2	4
B141-09A	*Cladosporium herbarum*	2	4
B143-02A	*Trichoderma* sp.	2	4
B145-01A	*Penicillium* sp.2	2	4
B145-03A	No growth	2	4
B145-06A	Sterile BL1	2	4
B146-01A	*Beauveria* sp.	2	4
B146-08A	*Beauveria* sp.	2	4
B147-01A	Sterile BL1	2	4

Isolate number	Identification	Site number	Visit number
B147-02A	Sterile BR10	2	4
B148-02A	Sterile W1	2	4
B148-06A	Sterile W1	2	4
B148-08A	Sterile W1	2	4
B148-09A	Sterile BL1	2	4
B148-10A	Sterile W1	2	4
B149-04A	Sterile BL1	2	4
B149-05A	Sterile W1	2	4
B150-06A	Sterile W4	2	4
B150-07A	*Beauveria* sp.	2	4
B150-08A	No growth	2	4
B151-05A	Sterile C3	2	5
B153-01A	Sterile BR2	2	5
B153-04A	*Cladosporium* sp.	2	5
B153-05A	Sterile BR7	2	5
B154-02A	Sterile C1	2	5
B154-02B	*Cladosporium* sp.	2	5
B154-04A	Sterile BL1	2	5
B156-05A	*Penicillium* sp.1	2	5
B156-08A	No growth	2	5
B156-09A	*Penicillium* sp.1	2	5
B157-08A	Sterile BL1	2	5
B158-01A	Sterile BL1	2	5
B158-02A	No growth	2	5
B159-01A	Sterile C1	2	5
B159-02A	Sterile BL1	2	5
B159-06A	Sterile C1	2	5
B159-07A	Sterile BL1	2	5
B159-09A	Sterile C1	2	5
B160-08A	Sterile BL1	2	5

Appendix 8:3: Dual Plate interactions between *Trichoderma* isolates (upper) and *Neonectria* isolates (lower) on Malt Extract Agar (MEA) and Potato Carrot Agar (PCA).

	IMI113898		IMI268223	
	MEA	PCA	MEA	PCA

	IMI113898		IMI268223	
	MEA	PCA	MEA	PCA

IMI395632

IMI395633

IMI395634

	IMI113898		IMI268223	
	MEA	PCA	MEA	PCA
IMI395635				
IMI395636				
IMI395637				