Endophytic diversity of *Fraxinus excelsior* L. (European ash) and its interaction with the dieback pathogen *Hymenoscyphus fraxineus*

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by

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Research conducted under the joint supervision of

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Declaration

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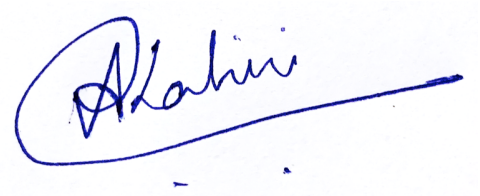

Anindita Lahiri

Abstract

European ash, *Fraxinus excelsior*, has huge economic and environmental value on the island of Ireland for forestry and ecosystem services. Given the rapid spread of *Hymenoscyphus fraxineus*, ash dieback disease, since its first Irish finding in 2012, and the extensive and severe damage caused by the pathogen in mainland Europe, methods are required to minimise the impact of this fungus. The pathogen is now past the point of eradication in Ireland, being present in all 32 counties. There is, therefore, a need to develop methods to manage the disease and minimise its impact. Manipulation of its microbiome, and its fungal endophytes in particular, is one such solution. Endophytes of ash include other pathogens that can worsen the symptoms of a dieback infected tree but also beneficial symbionts that can improve the health of the trees. This thesis explores the fungal microbiome of ash. It takes a culture based endophyte isolation approach to characterise endophytes from a European provenance trial of ash in Roosky, Co. Rosscommon, a sample from a dieback infected population in the French Alps, and a collection of different *Fraxinus* species and taxa from the National Botanic Gardens, Glasnevin. Methods were tested to optimise endophyte isolation from different tissues with contrasting health (diseased vs non-diseased) and tissue types (position on leaf, rachis, roots). The diseased material from Roosky could have been infected with *Hymenosycphus fraxineus* but we did not detect it even though it has subsequently been recorded at the site. A total of 628 endophyte isolates were cultured and these represented 214 morphotypes. Different media (MEA and MEA+*Fraxinus* leaf extract) recovered different endophyte communities.

A comprehensive sample of these isolates were further identified using DNA barcoding with the nrITS region and 119 fungal endophyte species discovered from 2 phyla and 10 classes. Fungal endophyte communities were shown to differ markedly between root and leaf samples and some evidence was found for variation between source populations. Identification of four *Hymenoscyphus* culture strains from the island of Ireland was confirmed with nrITS DNA barcoding and three distinct haplotypes discovered. These match other European strains from differing countries but phylogenetic analysis using TCS network analysis could not resolve the invasion route into Ireland. One haplotype matched UK samples but the other two matched with the samples from Latvia, Lithuania, Slovakia and Switzerland. A culture independent approach was also

undertaken in parallel to directly characterise the fungal endophytes of leaves and seeds. In this case, the nrITS region was sequenced using Illumina HiSeq (high throughput sequencing; HTS) of DNA amplicons directly from plant DNA extracted from ash (without the isolation and culture of the endophyte). The analysis also revealed a huge alpha diversity of fungi including a greater taxonomic depth of endophytes than the culture dependent method (5 phyla, 16 classes). The seed endophyte community was markedly different from the leaf communities and communities could be separated by NMDS and PCoA according to geographical location (France, Glasnevin, Roosky). There is also evidence for differing fungal communities in other ash species as the diverse sample of *Fraxinus* taxa differed from the communities found in *F. excelsior* alone. Despite the variation detected, core endophytes found in all samples of leaf, root and seed could be identified. However, there was little overlap between the community composition estimated by the culture dependent and culture independent approaches. This suggests that only a small proportion of endophytes were cultured and also that primers used to in the nrITS HTS did not amplify all the fungal taxa.

Methods are required to test the interactions of the ash dieback pathogen with endophytic fungi to assess how they influence the growth of each other. In particular, we wanted to know if any endophytes were antagonistic to *H. fraxineus*. Therefore, we undertook dual culture, *in vitro,* antagonism testing of endophytes against two strains of the pathogen. *Pyronema domesticum*, *Meyerozyma guilliermondi* and *Lecanicillium attenuatum* were found to significantly reduce the growth of the pathogen. Methods are also required to test the interaction of endophyte, pathogen and *Fraxinus* plants. This is a challenge because field-based inoculation experiments release the pathogen into the environment. Furthermore, it is also harder to control other environmental variables in field or glasshouse experiments. We therefore developed an *in vitro* tube screening system to grow ash in tissue culture, to break dormancy and to co-culture the plants with the endophytes and pathogen. We compared tissue culture of embryos with seed culture methods and found the seed culture methods to be most appropriate for establishing clonal lines of ash despite higher germination success with embryos. Survival rate of ash, post germination, was higher (100%) for seeds than embryos (43%) presumably because of their endosperm reserves. We showed that the *in vitro* method can be used for screening of dieback resistance and for endophyte interaction studies but more work is required to optimise the system. We found that the tissue culture methods can also be used to remove endophytes from ash.

The ultimate aim of the research was to provide evidence-based guidance for foresters and woodland managers to minimise the impact of the disease and to maximise the recovery of timber or minimise the damage to ash woodland ecosystems. This thesis has, therefore, made the most comprehensive assessment and characterisation of Irish ash endophytes to date and laid the foundation for further endophyte manipulation studies aiming to manage and control the disease.

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Chapter 1 Introduction

1.1 Introduction to common ash

Common ash or *Fraxinus excelsior* L. belongs to the olive family Oleaceae and grows in temperate regions of Europe except the driest Mediterranean areas due to extended dry summers and late spring frosts (Pilura and Heuertz 2003). It is a medium sized, 20 – 35 m tall, deciduous tree with large compound leaves and its crown is domed and open with ascending branches (Wardle 1961; Mitchell 1974; Boshier *et al.* 2005; Johnson and Moore 2006; Wallander 2008; Dobrowolska 2011; Praciak *et al.* 2013). Its leaves are 3 – 12 cm in size (width, length) and compound with 9 – 13 leaflets which are pinnately serrated and stalkless (Wardle 1961; Mitchell 1974; Boshier *et al.* 2005; Johnson and Moore 2006; Wallander 2008; Dobrowolska 2011; Praciak *et al.* 2013). The leaves develop in late spring and the flowers emerge before the leaf buds bursts (Thomasset *et al*. 2011). Flowers can be male, female and hermaphrodite and develop in bunches of 100 to 400 without petals and are pollinated by wind. Its fruits are known as samara they are a characteristically winged fruit, often called keys. Common ash grows well on soil where soil pH exceeds 5.5 (Beck *et al.* 2016). Common ash is mostly found as mixed stands and is rarely found as pure stands (Wardle 1961; Mitchell 1974).

Common ash is distributed throughout the European temperate zone, from the Atlantic coast of the Volga River (Figure 1.1; Pilura and Heuertz 2003). It is absent from the centre and south of the Iberian Peninsula, south of the Italian and Balkan Peninsula and northern Fennoscandia and Iceland (Meusel *et al.* 1978; Hulten *et al.* 1986; Boshier *et al.* 2005; Dobrowolska 2011).

The elasticity, hardness and pressure, shock and splintering resistance make common ash an economically highly valuable and precious plant compared to other ash species (Kerr 1995; Dobrowolska 2011; Beck *et al.* 2016). The wood is used for tool handles, walking sticks and sports equipment such as tennis racquets, hockey sticks, cricket stumps and billiard cues (Kew 2019). Hurling sticks for the traditional Irish game are mostly made from common ash. It was also used for weapon handles, agricultural implements, carriage and boat frames, before these were replaced by steel. Moreover the difference in grain between the

hardwood and sapwoood make it more valuable for veneer, furniture and flooring (Kerr 1995; Dobrowolska 2011; Beck *et al.* 2016).

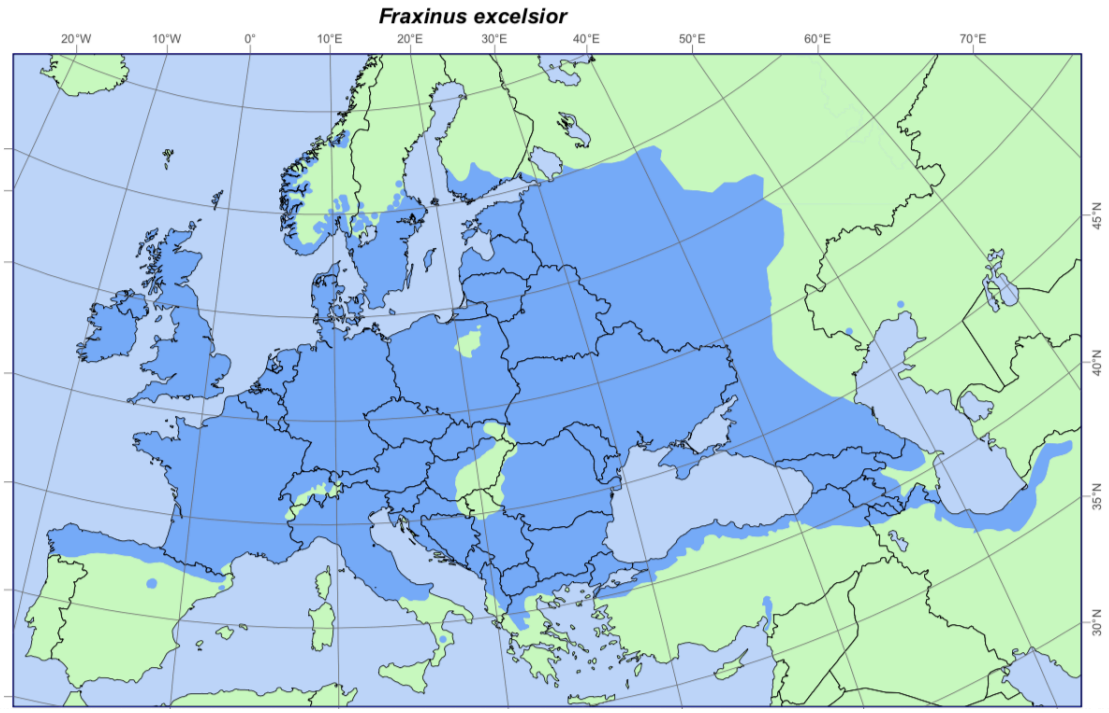


Figure 1.1 Distribution of *Fraxinus excelsior* in Europe. (Source Pilura and Heuertz 2003; http://www.euforgen.org/species/fraxinus-excelsior/).

Traditionally ash leaves were used for animal fodder and the bark used to tan calf leather (Boshier *et al.* 2005). In the 18th century England, ash leaves were used to adulterate tea leaves (Kew 2019). European ash has been used for medicine as well, for example its bark treated fever and buds were used as a slimming aid in UK (Kew 2019). Leaves were used to treat gout and leaf sap used for earache and warts (Kew 2019). In Central Europe common ash has been used mostly as an ornamental plant but in many other countries they have ethnic, cultural and mythological significance (in the 13th Century, Edda and other writers relating to Norse mythology, mentioned a mythological ash tree called Yggdrasil that supports the centre of the World) (Kew 2019). In the past it was believed that burning ash logs will drive evil spirits away. A long standing belief in Roman writing was that ash logs can be used to repel snakes or to protect against snake bite (Kerr 1995; Boshier *et al.* 2005; Dobrowolska 2011; Kew 2019).

1.2 Ash dieback disease

Ash dieback is a disease devastating *Fraxinus excelsior* populations and plantations (Stener 2013). It is caused by a fungal pathogen known as *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya (Baral *et al.* 2014). It is commonly called *Chalara* but its generic name has changed to *Hymenoscyphus* (Baral *et al.* 2014). Ash dieback disease can affect all age groups of *F. excelsior* (Kowalski *et al*. 2017). It often infects plants from fungal spores and its leaves can be easily infected. Fungal spores are present in the previous year's ash litter and are able to disperse over tens of kilometers (Forestry Commission UK 2012). Movement of diseased ash logs, following felling can also facilitate spread over large geographical areas (Forestry Commission UK 2012). The symptoms of the disease can be seen throughout the tree and are highlighted below.

1.2.1 Ash leaves

Affected ash plants often first show leaf wilt in which the pinnae (leaflets) start becoming dry and droopy because of loss of their turgor pressure (Kräutler *et al*. 2012). The leaflet petiole changes colour from green to light brown and then becomes black (Figure 1.2). The main veins are more resistant and the fungus develops within them and produces sclerotia on the rachis and can remain dormant (Douglas *et al*. 2013). Diseased leaves fall onto the ground.

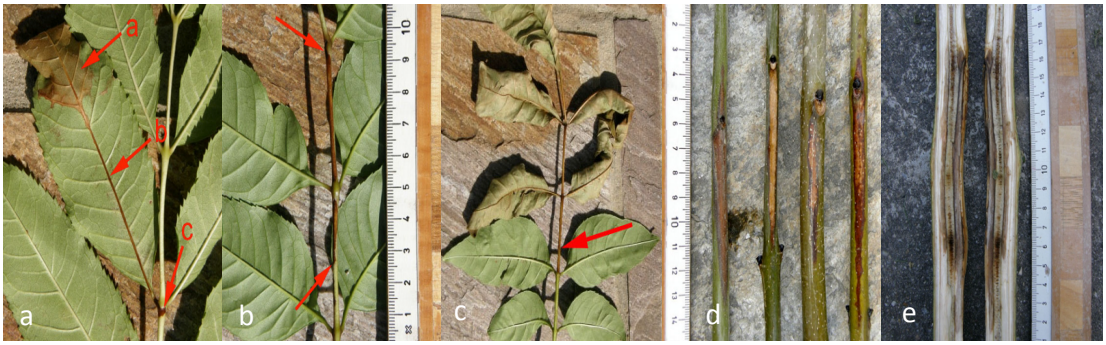


Figure 1.2 Leaf and stem symptoms of ash dieback. a-d. Spreading of the disease from leaf petiole towards stem, e. Mycelial growth.

Picture sources: Forestry Commission UK; Department of Agriculture and Teagasc, Agriculture Food Development Authority, Ireland

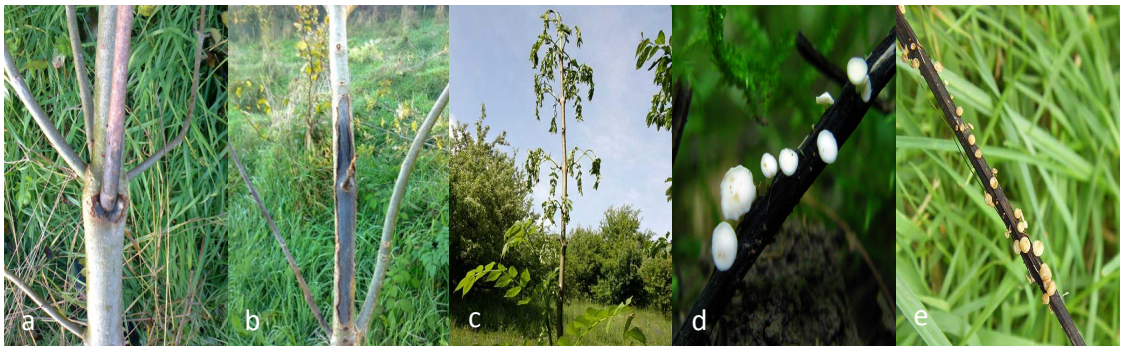


Figure 1.3 Stem symptoms of ash dieback disease. a. Epicormic branching, b. Necrotic lesion, c. Dieback of crown, d-e. Fruiting body of fungus on litter within stem.

Picture sources: Forestry Commission UK; Department of Agriculture and Teagasc, Agriculture Food Development Authority, Ireland.

1.2.2 Ash branches and stems

Ash dieback can cause a change in the colour of the stem from green to brown in young material. It is also affects the primary branches and can lead to the formation of epicormic shoots (Figure 1.3), which can become numerous (Sansford 2013; Gross *et al*. 2014). Epicormic shoots are axillary shoots that develop from each node. In mature woody parts of the tree, the mycelium can grow inside the stem and trunk and, in turn, it blocks the xylem vessels and damages the water transport system (Gross *et al.* 2014). It can be observed, when a young stem is cut longitudinally into half, as brown or black strips of necrotic tissue of damaged xylem vessels (Figure 1.2). This kind of necrotic lesion on the stem is known as canker. Cankerous growth appears on the stem and increases in length. Cankers can often look diamond or lens shaped and the colour of the stem changes to orange to red brown to black. After canker spreads, crown dieback of the tree occurs which mostly affects the upper crown. Canker of ash can also be caused by other biological organisms such as bacteria (e.g. *Pseudomonas syringae* pv. *savastanoi*) and other fungi (e.g. *Nectria galligena*) (Janse 1981; Douglas *et al.* 2013).

1.2.3 Ash roots

The fungus can be isolated from the roots of diseased ash where it weakens the root and can lead to the introduction of other fungal pathogens (Bakys 2013). One of these is the honey fungus *Armillaria borealis* that is particularly damaging (Bakys

2013).

1.3 Spread and occurrence in Europe

Ash die back disease was first observed in Poland in 1996 (Przybył 2002a; Kowalski and Łukomska 2005). Major outbreaks have occurred in several European countries and the disease has spread rapidly. Altogether, at least 28 countries (Figure 1.4) have so far reported the occurrence of ash dieback disease:

1. Austria 2005 (Chech 2006)
2. Belarus 2011 (Musolin 2017)
3. Belgium 2010 (Chandelier *et al*. 2011, 2017)
4. Croatia 2009 (Baric and Diminic 2010)
5. Czech Republic 2007 (Jankovsky and Holdenrieder 2009)
6. Denmark 2002 (Thomson 2005; Thomsen and Skovsgaard 2007)
7. Estonia 2009 (Drenkhan and Hanso 2009)
8. Finland 2007 (Rytkönen *et al*. 2010)
9. France 2008 (Ioos *et al*. 2009)
10. Germany 2002 (Schumacher *et al*. 2007; Schumacher *et al*. 2010)
11. Hungary 2008 (Szabó 2009)
12. Ireland 2012 (DAFM 2012)
13. Italy 2009 (Ogris *et al*. 2010)
14. Latvia 2007 (Rytkönen *et al*. 2010)
15. Lithuania 1996 (Gustienė and Lygis 2010)
16. Luxembourg 2013 (EPPO 2014)
17. Montenegro 2016 (Milenkovic *et al.* 2017)
18. Netherland 2010 (NPPO of the Netherlands 2013; CABI/EPPO 2013; EPPO 2014)
19. Norway 2008 (Talgø *et al*. 2009)
20. Poland 1992 (Barklund and Kowalski 1996)
21. Romania 2015 (Chira *et al.* 2016)
22. Russia 2011 (Kaliningard Oblast, R Vasaitis, pers comm.; Timmermann *et al.* 2011)
23. Serbia 2015 (Keča *et al*. 2017)
24. Slovakia 2004 (Kunca 2006)
25. Slovenia 2006 (Ogris, Hauptman and Jurc 2009)
26. Sweden 2001 (Barklund 2005)
27. Switzerland 2007 (Engesser *et al.* 2009)
28. Ukraine 2010 (Davydenko *et al.* 2013; EPPO, 2014)

Figure 1.4 Distribution of ash dieback in Europe.
Picture source https://www.cabi.org/ISC/datasheet/108083

One of the earliest European regions to report significant levels of ash dieback was Scandinavia where it was first observed in 2001. By 2004-06 the disease had spread all over the Scandinavian distribution range of ash leading to destruction of its populations (Barklund 2005; Stenlid and Barklund, pers. comm). It was first recorded in Denmark in 2003 (Thomsen 2005) and in Norway ash dieback was first documented in the southeastern parts in 2008 (Talgø *et al*. 2009).

According to Timmermann *et al.* (2011) disease free zones included Britain and Ireland, Western France, Spain, major parts of Italy and some northern, eastern and southeastern Europe (Russia except Kaliningrad Oblast, Ukraine, Moldavia and Bulgaria). However, the disease has since been reported from all of these regions.

1.4 Incidence of *Hymenoscyphus fraxineus* in Ireland and the UK

The Forest Commission UK (Kallow and Balding, online report), reported the incidence of dieback disease at the scale of 10 km grid squares in Scotland, England, Wales, Northern Ireland and Isle of Man and found that the total cases of ash dieback increased substantially between 2012 and 2018. The total numbers of infected ash plants recorded in these 6 years were 219 (Scotland), 1000 (England), 212 (Wales), 60 (Northern Ireland) and 1 (Isle of Man). The first observations in

Wales were noted in 2013. The first reported case in the Republic of Ireland was 12th of October 2012 in a forestry plantation site in County Leitrim. These plants were planted in 2009 and imported from continental Europe (DAFM, Ireland, online report).

1.5 Economic impacts of ash dieback disease in Great Britain and Ireland

For the Republic of Ireland, it is estimated that there are over 400,000 km of hedgerows and over 113,000 km in Northern Ireland. Ash is the second most important component of hedgerows after hawthorn (*Crataegus monogyna*) (McCracken *et al.* 2017). Since 1990 over 20,000 ha of ash has been planted in Republic of Ireland (McCracken *et al.* 2017). Ash timber is important as the source for the material to produce implements (hurley sticks) for the national sport of hurling. Since the first finding of ash dieback in 2012, about 306 infected sites has been detected in both the Republic of Ireland and Northern Ireland. To date over 2.1 million trees have been destroyed as part of an eradication strategy (McCracken *et al.* 2017). The eradication strategy also involved further applications of herbicides to the stumps of cut trees, excavation and burning of branch material and leaf litter, and drain clearing (McCracken *et al.* 2017). In the Republic of Ireland, about 733 ha of ash plantation land has been replanted with another ash species, costing €2.6 million (McCracken *et al.* 2017).

Ash has an important heritage and ecological value in the landscape for hedgerows and woodlands. It is a significant component of 91% of the woodland in the Republic of Ireland. Average demand for ash in Europe was 1,300,000 yr^{-1} from 1997 to 2007 (McCracken *et al.* 2017). Use of ash timber for traditional sports equipment for hurley and camogie has been traced back to the Bronze Age and ash is an important part of Gaelic heritage. Annual requirement for hurley stick manufacture is 360,000, crafted from 2,000 m^3 of ash wood that is valued €450 per m^3. Due to outbreak of ash dieback it is feared that in the long term this market will be lost and plantation logs will only be sold as firewood at prices of €60 per m^3. Ash is highly important for carbon storage in hedgerows and non-woodland patches. Black *et al.* (2014) conducted a study on Republic of Ireland hedges, hedgerows and non-woodland patches, which estimated that these habitats have a total carbon sequestration potential of 4.98 t CO2 $ha^{-1}yr^{-1}$. Ash has the potential to grow in less favourable sites or where human manipulation of the environment occurs (Dobrowolska *et al.* 2011). As a control measure, the DAFM (Department of Agriculture, Food and the Marine) in the Republic of Ireland and DARD

(Department of Agriculture and Rural Development) in Northern Ireland have strict policies of eradication and containment set out in the All-Ireland Chalara Control Strategy (Anon 2013). The National Roads Authority, Republic of Ireland, suspended the use of ash as a roadside plant from 2013 (McCracken *et al.* 2017). Moreover increasing public awareness of ash dieback has occurred through information updates on government departments websites, press releases and local and national adverts, and the development by DARD and DAFM of the Tree Check App for reporting ill-health trees. An all-Ireland conference in 2014 was organized jointly by DAFM, DARD, Agri-Food and Biosciences Institute (AfBi) of Northern Ireland and the Society of Irish Plant Pathologists which brought together presentations and discussion on the latest scientific knowledge of the disease and input from stakeholders and other interested parties (McCracken *et al.* 2017). DAFM was involved in a five year project from 2013 that aimed to identify individual ash trees that show resistance or tolerance to ash dieback and to bulk up plantings with the resistant varieties (McCracken *et al.* 2017).

In England, woodlands cover 2,634,000 ha of which broadleaved is 1,277,000 ha and ash covered woodland is 141,600 ha, which is about 5.4% of all woodland. Areas in percentage of broadleaved woodlands covered by ash trees for England, Wales and Scotland are 12.5%, 13.9% and 5.1% respectively (Smith *et al.* 2013). Apart from that, according to the countryside survey data by the Centre for Ecology and Hydrology, a further of 38,500 ha of ash in Great Britain occur in the woodlands of less than 0.5 hectares (Smith *et al*. 2013). About 17% of veteran trees recorded in the Countryside Survey of 2007 were ash (UK Centre for Ecology and Hydrology website). The total value of the social and environmental benefits of ash trees is £150 million per annum. The total social and environmental loss after the dieback disease spread is £17.3 million – £38.2 million each year (Smith *et al*. 2013). Ash is estimated to be 15% of the standing UK hardwood resource stock, equivalent to 22 million tonnes (UK Centre for Ecology and Hydrology website). According to the Forestry Commission Sawmill Survey in 2004, ash accounted for 8% of all hardwood going to UK sawmills, but in 2011 about 0.13% of the total volume sawn wood (Forestry Commission website, UK). Ash has always been recognised as the best hardwood for firewood as it is burn readily when green (UK Centre for Ecology and Hydrology website).

Other effects due to ash dieback related tree loss are a reduction in the absorption of noise pollution, a reduction in below canopy temperature control, loss of wildlife

habitat and soil erosion (Leonard and Parr 1970; Gardiner *et al.* 2006). The benefits of trees for reducing air pollution has been estimated at around £0.5m p.a. in 2012, of which ash represents £0.04m p.a (Willis *et al.* 2003). Ash also produces a high quality, friable and readily degradable litter with high rates of decomposition which are associated with the density of fungal mycelia, bacteria, protozoa and nematodes. This litter provides a nutrient source for trees which is high in nitrogen, phosphorus, potassium and sulphur. It is also rich in magnesium, manganese, calcium and low in carbon and lignin (Broome and Mitchell 2017). The fall of litter and its decomposition is one of the primary routes of nutrient and carbon cycling and also impacts soil fertility (Broome and Mitchell 2017). There are 955 biological species associated with ash plants in the UK, of which 5% are obligate associations and 6% are classified as highly associated (Broome and Mitchell 2017). These species include birds, mammals, bryophytes, fungi, vascular plants, invertebrates and lichens (Mitchell *et al.* 2014). Monitoring in eastern Europe revealed the first local extinctions of a lichen species due to loss of ash (Lohmus and Runnel 2014). Typical ash woodland communities are relatively rich in vascular plants such as *Mercurialis perennis*, *Allium ursinum*, *Hyacinthoides non-scripta*, *Oxalis acetosella*, *Dryopteris filix-mas* in upland zones, while lower altitude flora associated with ash communities are estimated to contain about 47 species (Mitchell *et al.* 2016a). Many of these species are known as ancient woodland indicator species and five of them have been listed on the Vascular Plant Red Data List in Great Britain (Cheffings and Farrell 2005).

The absence of ash trees is predicted to influence ground flora communities and in the short term may favour some bulkier light-demanding species at the expense of species which prefer damp shady conditions, such as *Athyrium filix-femina* and *Mercurialis perennis* (Mitchell *et al.* 2016a). However, seven plant species associated with ash are grasses. It is also expected that the loss of ash tree will affect some of the ruderal grass species such as *Deschampsia cespitosa* and *Rubus fruticosus agg.* (which spread rapidly and outcompete typical ash ground flora communities) (Broome and Mitchell 2017). Alternatives to ash trees which can support ash associated species the most are two species of oak (*Quercus robur* and *Q. petraea*), which can support 640 ash associated species. Hazel, hawthorn and alder may have potential to fill 15% of the gaps in broadleaved woodland areas in the first 10 years following loss of ash. After 10 years in seven of UK regions and in up to 35% of broadleaved woodland, sycamore is expected to fill the gaps (Broome and Mitchell 2017). Alexander and Green (2006) listed ash as of equal

importance to species such as oak in wood-decaying insects and greater importance than oak in terms of its leaf litter contribution. However, it is estimated that filling the gaps in broadleaved woodland due to dying ash trees will be difficult (Thomas 2016). Ash produces a large amount of seeds with good dispersal (up to 1.4 km) and creates a ready source of 'seedling bank' as the seedlings can germinate in light or heavy shade and need a small depth of well-drained but moist soil to establish (Thomas 2016).

Similar studies on economic and ecological impacts of ash dieback were carried out for other European countries including Austria (Heinze *et al.* 2017), Belgium (Sioen *et al.* 2017), Czech Republic (Rozsypálek *et al*. 2017), Denmark (Kjær *et al.* 2017), Germany (Enderle *et al.* 2017), Lithuania (Pliūra *et al.* 2017), Poland (Kowalski *et al.* 2017), Sweden (Cleary *et al.* 2017), and Switzerland (Queloz *et al.* 2017).

1.6 Endophytes of trees

There has been growing interest in understanding the microbiome, and more specifically the endophytes, of trees for various applications including forestry, horticulture, plant protection and phytoremediation (Hodkinson and Murphy 2019). The first published use of the term 'endophyte', but actually described as 'Entophytae', was in 1809 by Heinrich Friedrich Link, primarily for a group of fungi that are partly parasitic in nature (Link 1809). Furthermore, Anton de Bary used the term 'endophyte' to describe an endophyte as a parasite living inside its host's organ (de Bary 1884). The term endophyte was then also applied to bacteria and other organisms (Chanway 1996; Hallmann 1997). The term has been broadened further by others and Hodkinson and Murphy (2019) recommend a wide definition of 'any microbe that lives within plants'. Petrini (1991) defined endophytes as "..organisms inhabiting plant organs that at some time in their life cycle can colonise internal plant tissues without causing apparent harm to their host" and this is the definition followed in this thesis. According to sequence data obtained from NCBI, fungal endophytes of plants mostly belong to the five primary classes (Hardoim *et al.* 2015): Glomeromycota (40%), Ascomycota (31%), Basidiomycota (20%), Zygomycota (0.1%) and Unidentified phyla (8%). Unidentified taxa demonstrate the huge diversity of fungal endophytes remaining to be discovered and further studied (Murphy *et al*. 2015).

Endophytes can be classified in many ways but it is common to split them into Clavicipitaceous and nonclavicipitaceous types. Clavicipitaceous endophytes are endophytically associated with grasses and nonclavicipitaceous endophytes which are found in a broad range of hosts (Hodkinson and Murphy 2018). Clavicipitaceous endophytes can be split into three types: type I is transmitted vertically by maternal parents passing fungi to offspring via seeds (Saikkonen *et al*. 2002); type II endophytes exhibit stromata only in a portion of the grass tillers allowing partial seed production in the others and thus vertical transmission by seeds (White 1988); type III endophytes are transmitted vertically through seeds but many remain as epiphyllous mycelium and have the potential of horizontal transmission (Tadych *et al.* 2007). Type III endophytes of leaves, culms, and rhizomes frequently colonize the inflorescence primordia and as the inflorescence develops, the mycelium grows into the seed ovules. Within the seeds it colonizes the scutellum and embryo axis (Philipson and Christey1986) before germination.

Nonclavicipitaceous endophytes are highly diverse and can be divided into three functional groups named classes 2, 3 and 4. Class 2 endophytes colonize roots, stems, leaves and are transmitted via seed coat and/or rhizomes, and have low abundance in the rhizosphere (Rodriguez *et al.* 2009). They also colonize plants via infection structures such as appressoria or by direct penetration of plant tissues via hyphae (Ernst *et al.* 2003). They are mostly transmitted horizontally but sometimes vertically by seed coats (Redman *et al.* 2002). Class 3 endophytes occur primarily in above ground tissues by horizontal transmission through localized infection. This class of endophytes is hyperdiverse and many are associated with leaves of tropical trees (Lodge *et al.* 1996; Fröhlich and Hyde 1999; Arnold *et al*. 2000; Gamboa and Bayman 2001). Several studies indicate that the class 3 endophytes could be mutualistic but several aspects of their ecology can often be associated with pathogenic or parasitic lifestyles (Schultz *et al.* 1998).

Class 4 endophytes are grouped with dark septate endophytes (DSE) (Peyronel 1924) a paraphyletic group of fungi (Hodkinson and Murphy 2019). They form melanized structures such as inter or intracellular hyphae and microsclerotia in the roots (Jumpponen and Trappe 1998; Jumpponen 2001). DSE are present in soils and plant roots and are transmitted horizontally (Jumpponen and Trappe 1998). Colonization begins with superficial or runner hyphae which form loose networks of hyphae on the root surface. After that, hyphae grow, along the main axis of the roots and can grow between cortical cells and in the depressions between

epidermal cells (O'Dell *et al.* 1993). Colonization can be intracellular without causing any distortion to the host roots and the endophytes form closely packed thick cell clusters within the cortical cells collectively referred to as 'thick pseudoparenchymatic mass', sclerotia, microsclerotia and sclerotial bodies (Wang and Wilcox 1985; Jumpponen and Trappe 1998). Some DSE form Hartig-net or labyrinthine tissue, and in a few cases colonization of the root cortical layer results in the formation of chlamydospore-like rounded cells within cortical cells (O'Dell *et al.* 1993). Most of them are transmitted horizontally but some are transmitted vertically via the seed as with *Epichlöe* endophytes. They are extremely useful for stress resistance in metal enriched sites as they help their hosts to cope with heavy metal toxicity (Pirttilä and Frank 2011). The reason might be the adaptive metal tolerance showed in several studies (Panaccione *et al.* 2001; Colpaert *et al*. 2004; Adriaensen *et al.* 2005).

The endophytic fungi from tropical and temperate forest trees have also been extensively studied (e.g. Jumpponen 2001; Osono 2006; Arnold 2007; Sieber 2007; Saikkonen 2007; Slippers and Wingfield 2007; Albrectsen *et al.* 2010; Tejesvi *et al*. 2010). Most studies have been conducted on foliar endophytes because endophytes obtained from other parts such as bark and wood may sometimes be considered as non-endophytes and as wood inhibiting fungi instead (Boddy and Rayner 1983; Danti *et al.* 2002). Approximately 10% of trees species (from approximately 1000 tree species in the temperate region) have been investigated for foliar endophytes in the European region (Latham and Ricklefs 1993; Sieber 2007). Species richness in endophytes in the temperate region has been observed mostly in trees like *Acer* spp. (Sieber and Dorworth 1994; Pehl and Butin 1994; Vujanovic and Brisson 2002; Unterseher *et al.* 2007), *Betula* (Barengo *et al.* 2000), *Quercus* spp. (Cohen 1999; Ragazzi *et al.* 2003; Gennaro *et al*. 2003), *Abies* spp. (Carroll and Carroll 1978), *Pinus* spp. (Legault *et al.* 1989; Sieber *et al.* 1999) and *Carpinus caroliniana* (Bills and Polishook 1991). The collection of endophytes observed from these studies are mostly ubiquitous fungi and opportunistic colonisers of leaf tissues such as *Alternaria*, *Asteromella*, *Aureobasidium*, *Cladosporium* (anamorph: *Davidiella*), *Geniculosporium* (anamorph: *Hypoxylon*), *Phoma*, *Phomopsis* (anamorphic *Diaporthe*), *Ramularia* (anamorph: *Mycosphaerella*), *Sordaria* or *Xylaria* (Whitfield 2005; O'Malley 2008).

Different tissues, such as leaf tips, blades, leaf bases and petioles, can contain different endophyte species combinations (Halmschlager *et al.* 1993; Lodge *et al.*

1996; Taylor *et al.* 1999). Species richness also varies among different tree species and can vary with season, sampling site and condition of the host plant (Sieber and Hugentobler 1987). For example, endophyte species richness in *Fagus sylvatica* from north-eastern Germany and Switzerland differed (Unterseher and Schnittler 2009, 2010) and a meta-analysis of different trees from Switzerland (Sieber and Hugentobler 1987) showed variation in endophytic diversity among species.

Foliar endophytes can have a significant effect on host physiology and biochemistry (Newsham *et al.* 1998), influence multitrophic networks (Vega *et al.* 2008) and influence entire ecosystems (Selosse *et al.* 2004; Rudgers and Clay 2007). Endophytes can promote growth, inhibit the growth of the pathogens and improve stress tolerance in the host plant (Arnold *et al.* 2003; Herre *et al.* 2007; Rodriguez and Redman 2008). For example *Rhizoctonia* endophytes isolated from Norway spruce and Scots pine can improve host tree growth (Hietala *et al.* 1994; Gronberg *et al.* 2006). Phytohormone IAA (indole-3-acetic acid) production from L-tryptophan was shown in a few species of *Rhizoctonia* (Furukawa *et al.* 1996) and could be a contributing factor to growth stimulation. A yeast identified as *Rhodotorula graminis* strain WP1 isolated from the hybrid cottonwood plant (*Populus trichocarpa*) helped cottonwood growth via phytohormone production (Xin *et al.* 2009b) and *Populus* was also found to have diazotriphic nitrogen fixing bacterial endophytes (Doty *et al.* 2009; Xin *et al.* 2009a). Some endophytes can regulate the thermotolerance of their host by selective activation of heat shock protein genes (McLellan *et al.* 2007).

Many studies have been conducted on the search for novel metabolites from endophytic fungi, e.g. taxol, camptothecin, podophyllotoxin and other molecules (Sekita *et al.* 1973; Umeda *et al.* 1975; Camarda *et al.* 1976; Strobel *et al.* 1997; Li *et al.* 1998; Pelaez *et al*. 1998; Bashyal *et al.* 1999; Brady *et al.* 2000; Isaka *et al.* 2000; Wang *et al.* 2000; Yoganathan *et al.* 2004; Puri *et al.* 2005, 2006; Eyberger *et al.* 2006; Janes *et al.* 2007; Phongpaichit *et al.* 2007; Campos *et al.* 2008; Gangadevi and Muthumary 2008; Ge *et al.* 2008; Huang *et al*. 2008; Kumaran *et al.* 2008, Liu *et al.* 2008a, 2009, 2010a, 2010b; Pongcharoen *et al.* 2008; Rehman *et al.* 2008; Wu *et al.* 2008; Fernandes *et al*. 2009; Hazalin *et al.* 2009; Hussain *et al*. 2009; Kusari *et al.* 2009b; Xu *et al*. 2009a; Hu *et al*. 2010; Rosa *et al*. 2010; Mahapatra and Banerjee 2010; Scherlach *et al*. 2010; Shweta *et al.* 2010; Sutjaritvorakul *et al.* 2010; Pirttilä and Frank 2011). An interest in secondary

metabolities of endophyte was stimulated after the discovery of a taxol producing endophyte isolated from the Pacific yew in 1993 (Stierle *et al.* 1993). From the year 2002 – 2006 over 230 metabolites from 70 plant associated microbial strains were isolated and characterized by Gunatilaka (2006).

Several studies on mycorrhizal fungi of ash have been reported (Brundrett *et al.* 1990; Lang *et al.* 2011). Mycorrhiza can be considered a subgroup of endophytes (Hodkinson and Murphy 2019) but are treated separately here. Ash seems to form mostly arbuscular mycorrhizae (Brundrett *et al*. 1990; Lang *et al.* 2011).

Only a few studies have been published on the ash microbiome in recent years (Cross *et al*. 2017; Kosawang *et al.* 2018). Those studies reveal our knowledge about endophytes in healthy trees and help us to compare newly isolated endophytes with the list of organisms already revealed. Schlegel *et al.* (2016) studied ash endophytes as potential biocontrol agents. The study was conducted in *Fraxinus excelsior* and *F. ornus* L.. Firstly, the influence of exudates from isolated *F. excelsior* endophytes was tested *in vitro* (on Petri plates) against the ascospore germination of *Hymenoscyphus fraxineus*. Strong inhibitory effects on ascospore germination were recorded from the exudates of *Paraconiothyrium* sp., *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley, *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin, *Ampelomyces quisqualis* Ces. and Elsinoaceae sp.. Weak effects were observed for exudates of *Venturia* spp. and *Nemania serpens* (Pers.) Gray. Secondly, the protective effects of endophytes against the ash dieback pathogen were studied in the field (infected forest) using endophyte-free and plants pre-inoculated with endophytes. *Venturia* spp. dominated the endophytic community in the field grown trees inoculated with endophytes, but no significant effect of endophyte inoculation was seen on plants after they had been infected with *Hymenoscyphus fraxineus*. Thus, no evidence for field-based endophyte biocontrol of *H. fraxineus* was found Schlegel *et al.* (2016).

Another study by Kosawang *et al.* (2018) was conducted on fungal communities from resistant *Fraxinus* for use as biocontrol agents. Fungal isolations were undertaken from five *H. fraxineus* tolerant ash species (*Fraxinus chinensis* subsp. *rhynchophylla* (Hance) A.E. Murray*, F. lanuginosa* Koidz*., F. mandshurica* Rupr.*, F. ornus* and *F. pennsylvanica* Marshall) and endophytes identified using DNA sequencing (nuclear ribosomal ITS 1 and 2). They isolated 196 fungal taxa belonging to 15 families, 9 orders and 40 species. Most of their endophytes were

Ascomycetes except for a single Basidiomycete, namely *Peniophora* sp.. They performed antagonistic activity assays by growing the endophyte with *H. fraxineus* on half strength PDA (with ash leaf supplement). Endophyte species showing high antagonistic activity included *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley*, Epicoccum nigrum* Link, *Fusarium* sp.*, Sclerostagonospora* sp. and *Setomelanomma holmii* M. Morelet.

1.7 Aims and objectives

Little is known about the microbiome of ash, although some studies have begun to characterize its endophytic and epiphytic fungi and bacteria (Donnarumma *et al*. 2011; Hañáčková *et al.* 2017). However there is a need to further characterize and study the culturable and non-culturable endophytes of ash to understand their role in biotic and abiotic stress resistance. No studies have been reported from Ireland. The focus in this thesis is on the shoot, root and seed endophyte community of ash and its interaction with the ash dieback pathogen, *Hymenoscyphus fraxineus*. Endophytes from multiple populations and species have been sampled.

The project has established endophytic fungal cultures from ash leaflets and roots and obtained pure cultures of these fungi growing in malt extract agar media. These isolates have been used for DNA extraction and barcoding to identify the fungal endophyte species. The commonly used DNA barcoding locus known as nrITS (the nuclear ribosomal internally transcribed spacer) has been used (Begerow *et al.* 2010; Schoch *et al*. 2012) together with *tef* and the nrLSU (to a less extent). Next generation high throughput amplicon sequencing, of the same nrITS region, has also been used on seed and leaf samples of some plants to record what other fungal organisms are present inside them (the non-culturable component).

To understand ash endophyte interactions, we have used tissue culture techniques and optimized the culture systems so that the endophytic fungi could be tested *in vitro* with ash trees and the ash dieback pathogen (*Hymenoscyphus fraxineus*). We have also undertaken extensive dual culture testing (antagonism experiments) of pathogen and endophyte to assess their interactions. Such interaction testing, either with or without the host plant, was used to help determine if the endophytes can reduce disease severity or onset. It is possible that they could be used as biocontrol agents.

Therefore this thesis aimed to:

1. Isolate fungal root and shoot endophytes from populations of *Fraxinus excelsior* and also representative individuals of other ash species including *F. americana, F. angustifolia, F. dipetala, F. glabra, F. mandshurica, F. numidica, F. ornus, F. pennsylvanica*, *F. potamophila, F. pubinervis, F. texensis* and *F. xanthoxyloides*.
2. Identify the isolated fungi using DNA barcoding of the nrITS DNA region, *tef* and nrLSU and to assess the endophyte species richness of different plants and taxa and tissue types.
3. Identify the endophytes of ash using high throughput next generation sequencing (amplicon sequencing) to estimate the non-culturable components of the ash endophytic microbiome and to compare species richness estimates from different plants and tissues.
4. Compare Irish *Hymenoscyphus fraxineus* cultures with those found elsewhere, using DNA sequencing of nrITS to establish any strain variation and what it might most closely resemble.
5. To use *in-vitro* antagonism testing of endophyte against *Hymenoscyphus fraxineus.*
6. Establish an efficient plant tissue culture method for the generation of large *in-vitro* experimental populations of ash from both embryo culture and seed culture (to remove dormancy of seeds).
7. Conduct endophyte, pathogen and ash tree interaction studies to establish the role of endophytes in ash dieback disease resistance.

Chapter 2
Endophyte isolation from leaves and roots of a range of *Fraxinus* species

2.1 Introduction

The isolation process of endophytes from the host plant is often the first step for their study (Murphy *et al.* 2015). Endophytes can be tissue specific, although some are more general and can be found in both shoot and root tissues of their hosts (Paulus *et al.* 2006; Arnold 2007; Hyde *et al.* 2007; Hodkinson and Murphy, 2019). For example *Cenococcum geophilum* exists as both a mycorrhizal fungus and also a leaf endophyte (Arnold 2007). This tissue specification depends upon the many factors such as the site of initial colonisation or substances present inside tissues (Paulus *et al.* 2006; Arnold 2007; Hyde *et al.* 2007).

Because the endophytic community of a plant varies according to tissue type (White 1988; De Battista *et al.* 1990), the choice of plant tissue is an important step in the isolation process (Murphy *et al.* 2015). The time of year has also been shown to influence endophyte community composition (Haňáčková *et al.* 2017) so it is important to consider seasonal factors in endophyte isolation (Wilson and Caroll 1994; Scholtysik *et al.* 2013). Given all these factors, pilot studies are an important consideration for the study of endophytes (Fröhlich *et al.* 2000) so that the process is optimised before large scale screening is undertaken. It is very important to standardise the quantity and application time of surfactants and sterilising agents on different tissues to kill epiphytes and any other surface contaminants without killing the endophytes (Schulz *et al*. 2006; Murphy *et al.* 2015). This optimisation process is necessary because different tissues can sustain different levels of sterilisation. For example, thinner leaf tissues generally need less time for sterilisation than thicker leaves. The type and strength of surface sterilisation agents should also be examined. A common protocol involves a three step procedure with ethanol, sodium hypochlorite and ethanol again (Schulz *et al.* 1993; Bills 1996; Sieber 2002). A number of different chemicals are available for sterilisation such as 70-90% ethanol (Kosawang *et al.* 2018) 1-10% sodium hypochlorite (Gardener *et al.* 1982; Quadt-Hallmann *et al.* 1997; Reiter *et al.* 2002; Schulz *et al.* 2002; Sieber 2002; Davis *et al.* 2003; Schulz *et al.* 2015; Power *et al.* 2017), 0.1% Tween 20 (Zinniel *et al.* 2002), 0.1% mercuric chloride (Hollis 1951;

Gagné *et al.* 1987; O'Dell and Trappe 1992; Sriskandarajah *et al*. 1993; Lu *et al.* 2011) and hydrogen peroxide (Misaghi and Donndelinger 1990; McInroy and Kloepper 1994; Sieber 2002). Less commonly used sterilisation agents are propylene oxide vapour (Sardi *et al.* 1992) and formaldehyde (Schulz *et al.* 1993; Cao *et al.* 2002). Woody root tissues of alfalfa can also be sterilised by dipping in 95% ethanol for 15 seconds and flame sterlised (Gagné *et al.* 1987).

It is also important to experimentally check that surface contaminants such as epiphytes have been removed or killed. The simplest method for testing the accuracy of a sterilisation is to make imprints of the surface sterilised tissue onto a medium and to incubate the plate; if nothing grows it indicates that the sterilisation process has been successful (Pleban *et al.* 1995; Shishido *et al.* 1995; Schultz *et al.* 1998). In our studies we adopted this approach and checked the efficiency of the sterilisation procedure by making imprints of the sterilised tissues prior to their transfer and culture of endophytes on Petri dishes containing media. It is also important that tissue is rinsed well in sterile water to remove disinfectant (Schulz *et al.* 2015; Power *et al.* 2017; Kosawang *et al.* 2018). Culturing the last rinsing water onto nutrient media is another method for checking sterilisation (McInroy and Kloepper 1994) or dipping the tissues into nutrient broth (Gagné *et al.* 1987). A further check is to dip the tissues directly in a known density of bacterial or fungal suspension, slightly dry it, then surface sterilise it and then subject it to a sterility check (Petrini 1984; Coombs and Franco 2003a; Schulz *et al.* 2006).

Choice of culture media is also critical. Not all endophytes will grow on all media and some are recalcitrant to culture on any known substrates (Arnold 2007). Some endophytes can be better cultured if the media is supplemented with plant extract either specifically from the host plant or more generally from plant material (Sarhan *et al.* 2018). Therefore, it is important to culture plant material on a range of different substrates to maximise the number of fungal isolates retrieved (Murphy *et al.* 2015). Murphy *et al.* (2015) used whole plant extract with the isolation media and considered it useful to isolate the maximum number of endophytes from the hosts. Some endophytes such as *Piriformospora indica* grow on Kaefer media (Kaefer 1977) but can also be cultivated on other media such as yeast extract with peptone and glucose, potato dextrose broth, malt extract and Gamborg media supplemented with 0.2% peptone, 0.1% yeast extract and 0.1% casamino acid hydrolysate (Kumar *et al.* 2011). Meletiadis *et al.* (2001) found that filamentous fungi could be cultured effectively on five different media including antibiotic

medium 3, yeast nitrogen base medium, Sabouraud broth, RPMI 1640 and RPMI with 2% glucose. Endophytes from coffee leaves can grow on half strength PDA with 35 ppm benomyl, half strength PDA with 35 ppm rose bengal, half strength corn meal agar and Czapek Dox agar with 25 gL^{-1} $KClO_3$ (Santamaría *et al.* 2005). Endophytes from barley can grow on corn meal agar, Czapek Dox liquid agar media, malt extract with vegitone, potato dextrose agar (PDA) and Sabouraud maltose agar and apart from these two more media from whole plant extract of wall barley seemed to be useful for endophyte growth (Murphy *et al.* 2015).

Culture of endophytes from forest trees and ash in particular has also been achieved using a range of media. Endophytes from shoot, wood and bark of ash (*Fraxinus* spp.) can grow on 2% Wort agar with 2% w/v sucrose (Haňáčková *et al.* 2017). Other media also used to isolate endophytes from wood and bark from ash trees include Hagem agar (Bakys *et al.* 2009a), 1% malt extract agar (MEA) for bark, wood and twigs (Chen 2012) and 2% malt extract agar (Kowalski and Kehr 1992). To ensure the inoculation success Schlegel *et al.* (2016) incubated one disc of punched surface sterilised leaf tissue on terramycin malt agar (TMA, 20 gL^{1}, malt extract 15 gL^{-1} agar, 50 mgL^{-1} oxytetracycline). On the other hand, endophyte isolation from *Fraxinus ornus* leaflets and petioles from six sites in south and north of the Alps by Ibrahim *et al.* (2017) was carried out on terramycin malt agar. PDA and rose bengal chloramphenicol agar (RBA) were also used for isolation of endophytes from ash twigs (Kosawang *et al.* 2018).

Several studies of ash endophyte community composition have been published taking an approach that first involved isolation and culture of endophytes from the plant tissue. For example, endophyte isolation from different leaf tissues and bark in accordance with different seasonal impacts was studied by Haňáčková *et al.* (2017). They collected ash shoots from high disease pressure areas. Bark and wood fragments were surface sterilised with 96% ethanol followed by 0.47% sodium hypochlorite and then 96% ethanol again prior to culture on 2% Wort agar. Other reports for endophyte isolation and testing as biocontrol agents by antagonistic activity have also been published by Schlegel *et al.* (2016) who used MEA media with 20gL^{-1} malt extract and 15gL^{1} of agar and Kosawang *et al.* (2018) who used PDA and RBA media for antagonistic assay they used PDA with blended ash leaves (100g blended in 1L of media). Furthermore isolation of endophytic communities from healthy ash trees has been performed using different media in a number of other studies such as Hagem agar, 1% MEA and 2% MEA (Kowalski

and Kehr, 1992; Kowalski and Łukomska (2005); Bakys *et al.* 2009b; Chen 2012; Davydenko *et al.* 2013).

However, studies are still required to optimise endophyte isolation from ash to encompass a range of plant taxa and tissue types. This chapter therefore aims to:
1) Assess the recovery of endophytes from a range of ash species and their populations;
2) Compare endophyte recovery from a range of tissue types and disease symptomatic and asymptomatic material; and
3) Assess the suitability of different media for endophyte culture.

To do this we have utilized an EU provenance trial growing in Roosky, Roscommon, Ireland of *Fraxinus excelsior* material sourced from eleven European countries and also material of different species from the National Botanic Garden, Ireland including *F. americana, F. angustifolia, F. dipetala, F. glabra, F. mandshurica, F. numidica, F. ornus, F. pennsylvanica*, *F. potamophila, F. pubinervis, F. texensis* and *F. xanthoxyloides*.

2.2 Materials and Methods

2.2.1 Collection

Shoot and root endophytes from multiple healthy ash populations and species were sampled (Table 2.1, 2.2 and 2.3). Shoot endophytes were isolated from three different locations in Ireland, one is a provenance trial for forestry research in Roosky, County Rosscommon and the other the National Botanic Gardens Glasnevin (NGB), Dublin. Another collection for leaf samples was made from a single tree in Loreto Park, Dublin on 24th September 2015 and isolates from that tree named as IRE. In Roosky, all plants (all *Fraxinus excelsior*) had been grown from root-trainer pots in 2005 and 2006 before being planted out in 2007. Ash trees were planted on a grey brown podzolic soil with plant spacing at 2m x 2m. No fertiliser was used and the site was a moderately open space surrounded by farmer's fields, hedgerows and some small areas of forestry. The provenance trial (Figure 2.1) included ash from 11 European countries including Ireland. Leaflets were sampled on 23th September 2015 from trees that were 8 to 10 years old. We also collected seeds for DNA extraction for high throughput (next generation) amplicon DNA sequencing (Chapter 3).

Table 2.1 Ash leaflets collected for endophyte isolation and DNA extraction from Roosky, Roscommon. Collection date: 21/9/15; a, b, c are replicate plants within plots

No.	Name of the plots/plants	Plot number given in field	No. of leaflets collected
1.	A	13	2
2.	A1.a	5	3
3.	A1.b	5	2
4.	A1.c	5	2
5.	B	20	4
6.	C	7	2
7.	D1.a	12	2
8.	D1.b	12	2
9.	D1.c	12	4
10.	E	9	2
11.	E1.a	11	3
12.	E1.b	11	2
13.	F1.a	21	3
14.	F1.b	21	2
15.	G.a	24	2
16.	G.b	24	2
17.	G1.a	30	2
18.	G1.b	30	2
19.	H	25	2
20.	H1.a	1	2
21.	H1.b	2	3
22.	J	29	2
23.	J1	18	3
24.	K	2	4
25.	K1.b	10	2
26.	L	14	2
27.	L1	33	3
28.	M	31	3
29.	M1	15	3
30.	O1	27	3
31.	Q1	37	3
32.	R1.a	28	2
33.	R1.b	28	2
34.	S1.a	19	3
35.	S1.b	19	3
36.	T1.a	32	3
37.	T1.b	32	3
38.	T1.c	32	3
39.	U1	36	3
40.	Y	23	3

Table 2.2 Number of trees sampled from Roosky, Roscommon provenance trial for each country

Country name	Number of trees sampled
Belgium	3
Czech Republic	3
Denmark	4
France	5
Germany	4
Ireland	4
Italy	4
Lithuania	4
Netherland	3
Poland	3
UK	4

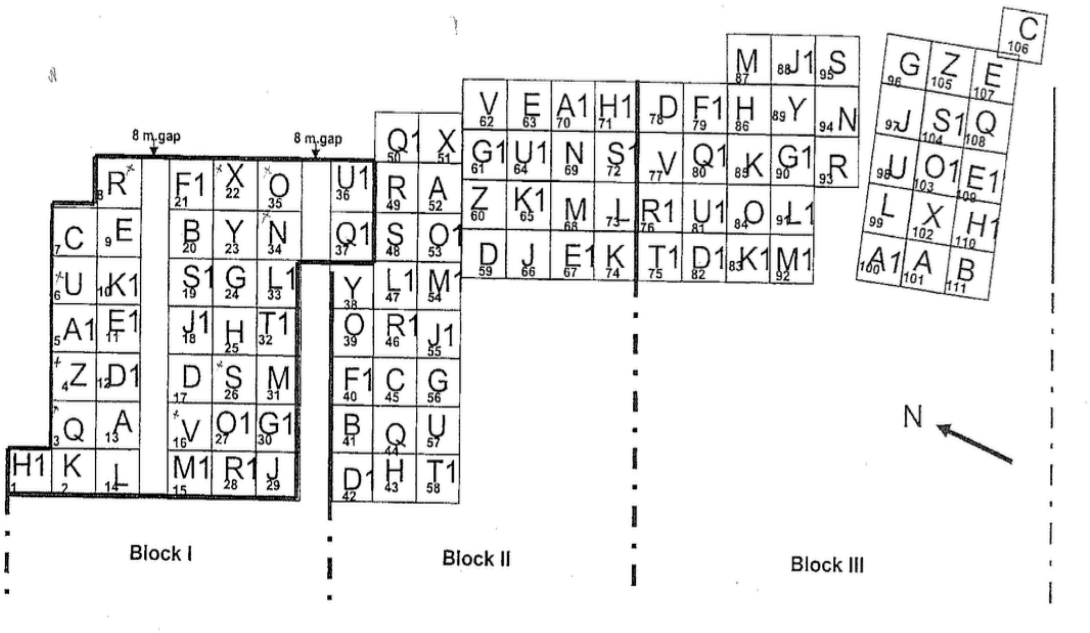


Figure 2.1 Map of ash provenance trial material at Roosky, Roscommon. Site is ca. 250 m long from Block I to Block III and 100 m wide.

Table 2.3 Ash leaflets collected form Glasnevin Botanical Garden. Plants collected on 30/9/2015

Serial No.	Name	Serial number written on them	Additional notes at the time of collection
1	*Fraxinus texensis*	XX.011046	-

2	*Fraxinus xanthoxyloides*	2003.0735	-
3	*Fraxinus glabra*	1909.011054	-
4	*Fraxinus mandshurica*	1934.011053	-
5	*Fraxinus potamophila*	1932.011031	-
6	*Fraxinus americana*	2001.1739	-
7	*Fraxinus pubinervis*	1954.011044	-
8	*Fraxinus angustifolia* 'Monophylla'	XX.011028	-
9	*Fraxinus excelsior* 'Jaspidea'	2000.3637A	-
10	*Fraxinus ornus*	1977.0039	-
11	*Fraxinus ornus*	2005.0652	-
12	*Fraxinus excelsior* ('Diversifolia Laticifolia')	XX.011040	-
13	*Fraxinus dipetala*	XX.011038	-
14	*Fraxinus excelsior* (orange bark)	XX.011029	-
15	*Fraxinus* sp..	2013.0214	-
16	*Fraxinus ornus*	1969.011033	-
17	*Fraxinus ornus*	XX.011034	Mark 83
18	*Fraxinus numidica*	1904.011032	SL/04 on Label
19	*Fraxinus angustifolia* 'Lentiscifolia'	XX.011074	-
20	*Fraxinus* sp. (no label)	2006.1641	Mark 91
21	*Fraxinus* sp. (greybud)		Mark 78
22	*Fraxinus excelsior* 'Pendula'	XX.011048	-
23	*Fraxinus pennsylvanica*	1889.011030	-

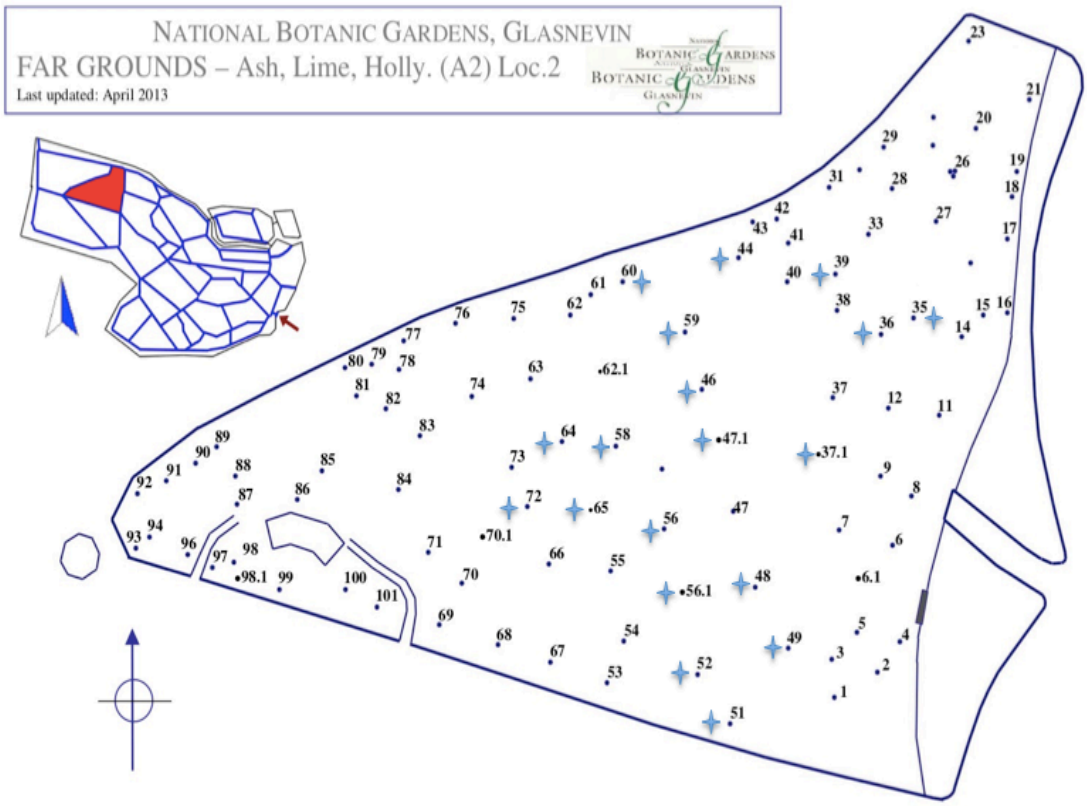


Figure 2.2 Sample location in Glasnevin Botanic Garden. The blue crosses show the selected trees used for sampling (http://botanicgardens.ie/).

The second collection was carried out at the National Botanic Garden Glasnevin (NGB) on 30th September 2015 (Figure 2.2). Leaflets were collected from mature *Fraxinus excelsior* trees and also representative individuals of other species including *F. americana, F. angustifolia, F. dipetala, F. glabra, F. mandshurica, F. numidica, F. ornus, F. pennsylvanica*, *F. potamophila, F. pubinervis, F. texensis* and *F. xanthoxyloides* trees for DNA extraction and amplicon based high throughput DNA sequencing.

Roots were also collected from a research and field bank conservation plantation in Teagasc, Kinsealy Research Centre, Malahide, County Dublin. We dug out the soil approximately two metres from the base of the tree trunks and took out the roots and collected primary, secondary and tertiary roots.

2.2.2 Isolation of endophytes

After collection, tissue samples were kept in a fridge at 4°C and cultured within 24 to 48 hours. Leaflets were surface sterilised by spraying with 70% ethanol followed by immersion for 5-7 min in 5% bleach (NaOCl) and 1 min of 70% ethanol followed by washing 5-6 times with sterile ultrapure water. Leaflets were cut into small pieces either near the edge of the leaf blade (about 4x4mm in size) or along the leaf vein (6x4mm in size) with sterile scalpels. Then they were transferred into two different media on square plates using sterile forceps (Thermo Scientific: 100mm Square Petri dish, 25 compartments: Catalogue no. 103). One set of square plates contained half strength malt extract agar with vegitone supplement (MEA) and another set contained the same media as above with additional supplementation of digested fresh ash leaves. To obtain the ash leaves for the latter medium, leaves were collected from a healthy tree and cleaned with tap water three to four times, followed by cleaning with sterile pure water two times and then digested with 50 g of ash leaves were autoclaved with the half MEA media in 1litre (by including them in the sample bottle during autoclaving (MEAF). After tissues had been sterilised and plated onto two media, they were incubated at ±18°C in the dark. After 4–10 days, fungi began to emerge from the surface sterilised tissue and started growing on the agar medium. A small section of this was then sub-cultured onto another Petri plate containing half strength malt extract agar with vegitone supplement to get a pure culture for identification.

A test for growth of endophytes on different media was conducted on a selected sample of 20 isolates obtained from leaf tissues. Four media were made up at half strength: Malt extract agar with vegitone supplement, potato dextrose media, Sabouraud agar media and corn meal agar media. A total of 5 replicates for each endophyte were included for each media. Growth observations were after 25, 43 and 64 days.

Tissues from different leaf positions were selected to see if there were differences among leaf position for endophyte recovery. Sampling of individual trees for leaf endophytes was completed for five different tissue types from healthy leaves and five different tissue regions from diseased leaves (leaves that were beginning to senesce and decay; Table 2.4). In the case of diseased leaves, the excision position or type of tissue was not fixed, as it was dependent upon the disease location on the lamina.

Table 2.4 Locations of leaf tissue isolation

	Lamina	Rachis	Middle lamina	Apex	Side vein
Healthy	H.L	H.R	H.M	H.A	H.V
Diseased	D.L	D.R	D.M	D.A	D.V

H.L = 4 cm above from the leaf base on the sides of lamina

H.M = middle lamina with midrib portion

H.R = healthy rachis

H.A = healthy apex

H.V = healthy veins it is mostly same as H.L only in 10 cm below apex

D.L = diseased leaf tissue position not fixed

D.R = diseased rachis

D.M = diseased midrib with laminar tissue

D.A = diseased apex

D.V = diseased veins

Roots were washed five times with tap water and sterilised by rinsing two times with sterile ultrapure water followed by immersion in 5% bleach (NaOCl) for 10 mins and then 70% ethanol for 1 min. They were then washed five times with sterile ultrapure water. Roots were then cut into 1 cm long pieces and cultured in a Petri dish containing half strength malt extract agar with vegitone supplement. They were kept in the dark in an incubator at ±18°C. After 10-12 days, emergent hyphae were sub-cultured onto fresh media as outlined for leaves above.

2.3 Results

2.3.1 Leaf endophyte isolation

Altogether, 545 tissues were cultured from leaves and rachises of different ash trees. 65 root tissues were cultured from three individual ash trees. Endophytes grew from most of the 545 cultured leaf tissues with a few exceptions such as trees from plot S1.b, and a few tissues of Y, G1.b and H that gave negative results for both MEA and MEAF. For further identification purposes, 13 cultures out of 25 cultures from each plate were selected on the basis of difference in morphology. 310 leaf and 100 root endophyte pure cultures were further extracted for DNA identification; detail of DNA barcoding is provided in Chapter 3.

Initial growth statistics were recorded for endophytes obtained from the square

plates (in terms of total number of isolates retrieved). The fungi were then sub-cultured onto a new Petri dish containing half strength MEA media. The number of isolates obtained on the basis of tissue type, colour and country of origin (provenance) were plotted using histograms. Morphotypes were also recorded. A culture was considered a different morphotype if it was a different colour or had different gross mycelium morphology or spores.

Figure 2.1 shows the number of fungal isolates and morphotypes retrieved and classified according to colony colour. Each bar in the histogram therefore represents the number of isolates obtained from each of the colony colour categories. White coloured isolates were by far the most common type (222 isolates) but only single isolates were obtained from types with brown, pink + black, pink + white, white with red spores, transparent orange and transparent green. Pink and green isolates were also common (52 and 69 respectively). Among other isolates orange and yellow was relatively more common than the rest (22 and 19 respectively). A range from 2 – 9 isolates were obtained for transparent brown, orange + green, green + white, transparent, green + brown, green + pink, transparent white, grey and red. The numbers inside each bar give the number of different morphotypes obtained from each colour type; for example, 44 different morphotypes were found from the white colour colony group. Numbers on the top of each bar indicate the mean number of isolates per morphotype within their respective colour category. For example, there was an average of 5.1 isolates per morphotype for the white cultures.

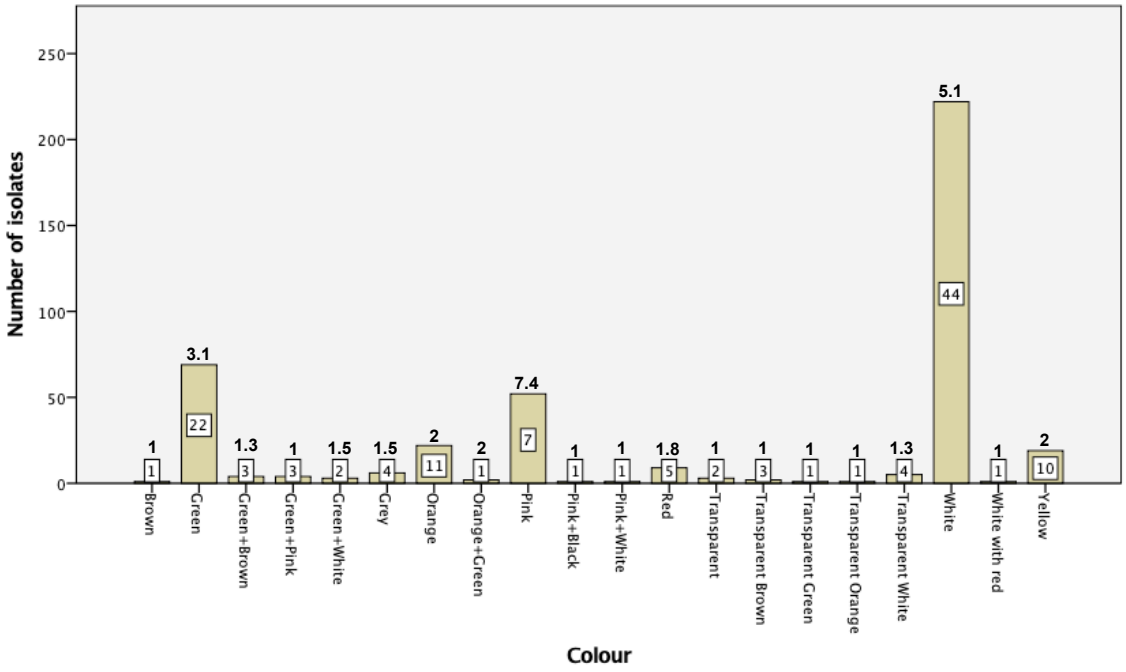


Figure 2.1 Histogram for the total number of isolates and morphotypes of endophytes according to the colour of their culture colony (for the Roosky provenance trial ash material). Number of morphotypes are shown inside the bars (indicates number of morphotypes obtained in each colour category). Numbers above each bar indicate the number of isolates per morphotype within their respective colour category.

Figure 2.2 shows the number of isolates and different morphotypes obtained from each of the tissue types. The highest numbers of isolates were retrieved from the healthy rachis (93 isolates) and the lowest number from the healthy apex and leaf margin (2). High numbers of isolates were also obtained from other tissues such as healthy leaf, healthy midrib, diseased midrib and diseased leaf isolates (isolate number ranging from 65-72). The total numbers of plant samples used for isolation from each healthy tissue type were 82 (healthy leaf = 0.9 isolates per sample), 64 (healthy midrib = 1 isolate per sample), 85 (healthy rachis = 1.1 isolates per sample), 20 (healthy side vein = 0.8 isolates per sample), 2 (healthy apex = 1 isolates per sample) and 3 (margin = 0.6 isolates per sample). For diseased leaf tissue there was 68 samples (1.1 isolates per sample), diseased rachis 10 samples (0.9 isolates per sample), diseased mid lamina 65 samples (1.1 isolates per sample), diseased apex 20 samples (1 isolate per sample) and diseased side vein

8 samples (1 isolate per sample). The maximum number of morphotypes was obtained from healthy rachis (31) and minimum number of morphotypes obtained from healthy apex and margin single morphotype for both.

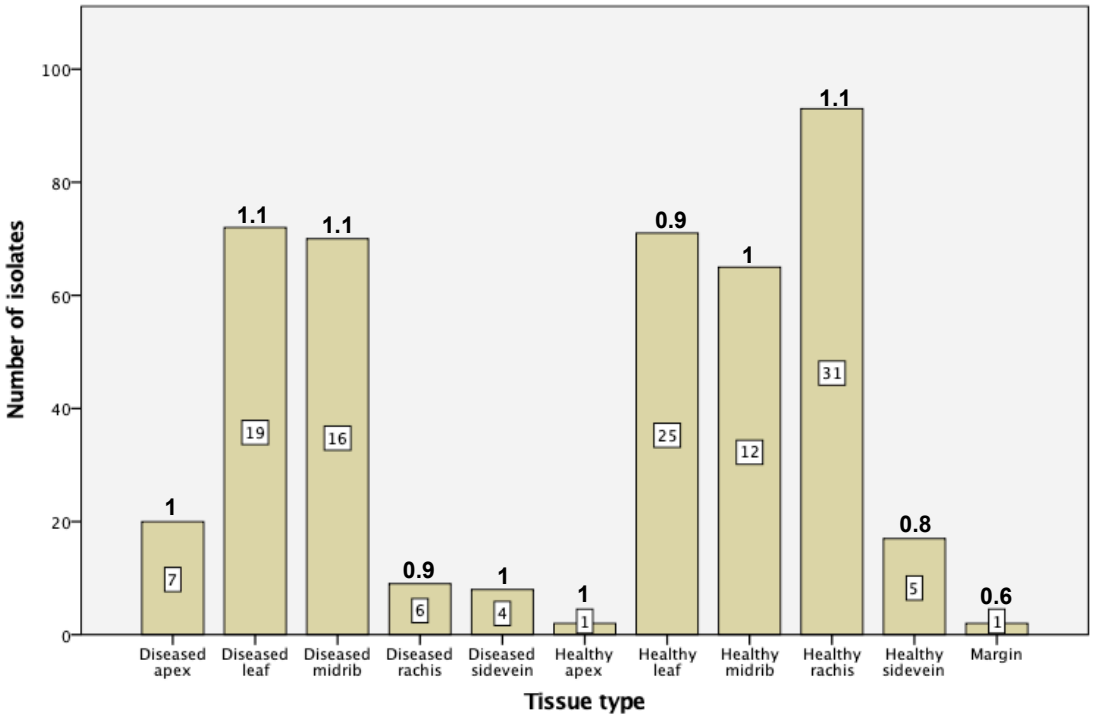


Figure 2.2 Histogram for total number of endophyte isolates and morphotypes according to tissue type (for the Roosky provenance trial ash material). Total number of morphotypes obtained in each tissue category are shown inside the bars. Numbers above each bar indicate the mean number of isolates per tissue within their respective tissue category.

A Chi square analysis was performed assessing whether endophyte recovery (number of isolates) was dependent on leaf tissue location (example - apex, leaf blade, midrib region, rachis, and vein) for both healthy and diseased samples. The Null hypothesis (H^0) for this test was that endophyte recovery was not dependent on leaf tissue location (including apex, leaf blade, midrib region, rachis, and vein) for both healthy and diseased tissue. The alternate hypothesis (H^1) for this test was that endophyte recovery was dependent of leaf tissue location (including apex, leaf blade, midrib region, rachis, and vein) for both healthy and diseased. The Chi square (X^2) value = 6.561, degrees of freedom (df) 4 and *p* value of 0.161 (details of Chi square test are given in Appendix I). Hence we do not reject the null

hypothesis and endophyte recovery is not dependent on leaf tissue location.

A Chi square analysis was performed assessing whether endophyte recovery was dependent on tissue type (healthy or diseased). The Null hypothesis (H^0) for this test was that endophyte recovery was not dependent on tissue type (healthy or diseased). The alternate hypothesis (H^1) for this test was that endophyte recovery was dependent on tissue type (healthy or diseased). The Chi square (X^2) value = 1.523, df 1 and *p* value of 0.217 which was not significant and the null hypothesis was therefore accepted: endophyte recovery is, therefore, not dependent on tissue type (details of Chi square test are given in Appendix I).

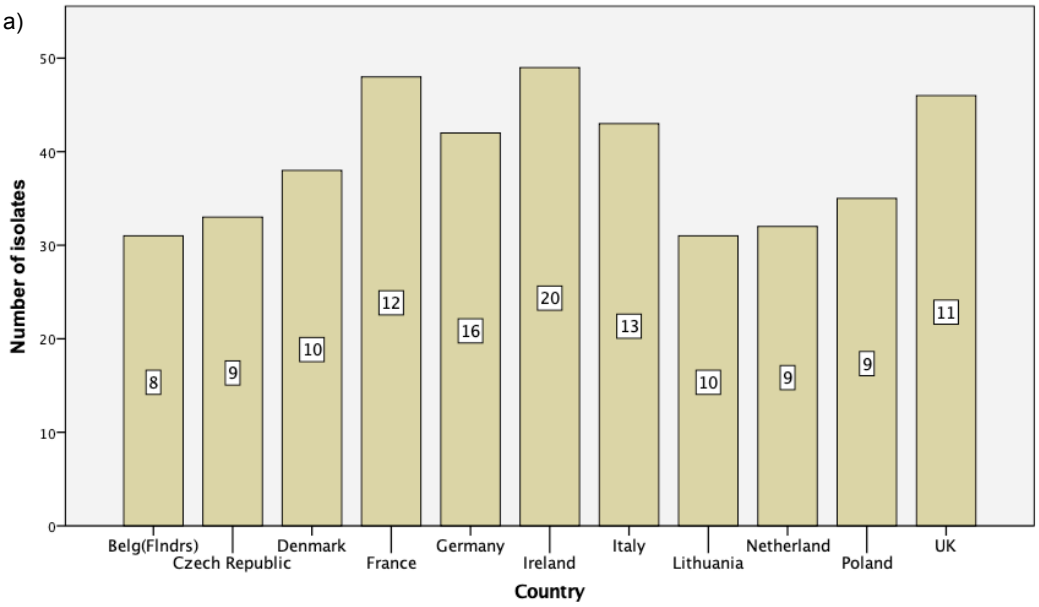
Furthermore, a Chi square analysis was performed assessing whether number of morphotypes was dependent on leaf tissue location (including apex, leaf blade, midrib region, and rachis). The Null hypothesis (H^0) for this test was that morphotype was not dependent on leaf tissue location (including apex, leaf blade, midrib region, and rachis). The alternate hypothesis (H^1) for this test was that morphotype was dependent on leaf tissue location (including apex, leaf blade, midrib region, and rachis). The Chi square (X^2) value = 327.283, df 212 and *p* value of < 0.0001 for Roosky. Therefore, the null hypothesis was rejected and endophyte recovery of morphotype is dependent on leaf tissue location for the Roosky samples (details of Chi square test are given in Appendix I).

Figure 2.3a shows the number of isolates obtained from ash material categorised according to its source (provenance). Numbers of morphotypes found in the respective country are shown inside each bar. The number of trees sampled from each country is shown in Table 2.2. The sample in Belgium was collected from 3 trees. We got 31 isolates and there was 8 distinct morphotypes among them. We can see from Figure 2.3a that a low number of isolates were obtained from the Belgium, Lithuanian and Czech Republic and Netherlands (31-33 isolates). The highest number of isolates was obtained from the Ireland provenance material (49 isolates). Denmark, France, Germany, Italy, Poland and UK provenances showed a high number of isolates range from 35-48 isolates. The highest number of morphotypes was found from the leaf tissues sampled from Ireland (20) and lowest found from Belgium (8).

Figure 2.3b shows the mean number of isolates per tree from each country. The numbers above each bar represent the mean number of morphotypes per tree. For

example in Belgium the mean number of isolates per tree was 10.33 while the mean number of morphotypes per tree was 2.7. Ireland showed the highest mean number of isolates (12.25) and Lithuania the lowest (7.8). The highest mean number of morphotypes per tree was from Ireland (5).

a)



b)

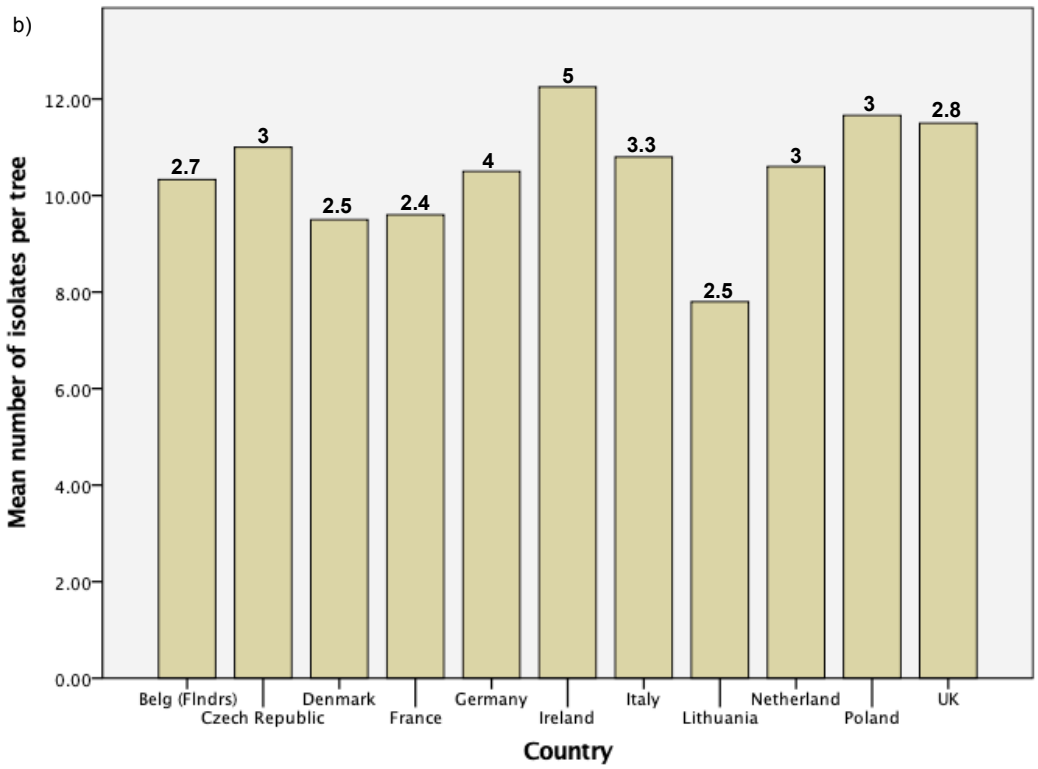


Figure 2.3a and **2.3b** Histogram of total number of endophyte isolates and morphotypes categorised according to ash tree provenance (country of origin) and, the mean number of isolates and morphotypes obtained from each tree of each country of origin. Top: numbers inside each bar indicates the number of morphotypes found from the respective country. Bottom: Mean numbers of morphotypes per tree are shown on the top of each of the bars. A Chi square analysis test was undertaken to assess whether endophyte recovery (number of isolates) was dependent on country provenance trials or not. The Null hypothesis for this Chi square test was that endophyte recovery (number of isolates) was not dependent on country provenance trials. The alternative hypothesis was that endophyte recovery (number of isolates) was dependent on country provenance trials. The Chi square (X^2) value = 56.062, df 10 and *p* value of < 0.0001. Therefore, the null hypothesis that endophyte recovery (number of isolates) was not dependent on country provenance trails was rejected and so endophyte recovery is dependent on country provenance (details of Chi square test are given in Appendix I).

2.3.1.1 Isolations from differing *Fraxinus* taxa

Summary statistics were also generated for endophytes isolated from differing *Fraxinus* taxa (including different species and some horticultural taxa) obtained from the National Botanic Gardens, Glasnevin. Statistics were generated for tissue type (Figure 2.4), colour of isolates (Figure 2.5), and a comparison made of isolate number from *F. excelsior* and other *Fraxinus* species (Figure 2.6).

Figure 2.4. shows the total number of isolates categorised according to tissue type. The maximum numbers of isolates were obtained from healthy rachis and healthy apex material (21 isolates) and the minimum number of isolates obtained from the diseased leaf and diseased midrib (8 and 9 isolates respectively). Similar numbers of samples were used for each tissue type for the healthy tissues. In case of diseased tissues, the sample number is different. Tissues sampled for each healthy leaf tissue and healthy midrib is similar in number namely 24 and 25 (0.6 isolates per sample), healthy rachis is 22 (0.90 isolates per sample) and healthy apex is 23 (0.9 isolates per sample). In the case of the diseased tissue, 12 tissues were sampled from the diseased mid lamina and 9 for tissues sampled for diseased leaf. The maximum number of morphotypes was found from the leaf tissues sampled from healthy apex material (12) and the minimum was from diseased leaves (2).

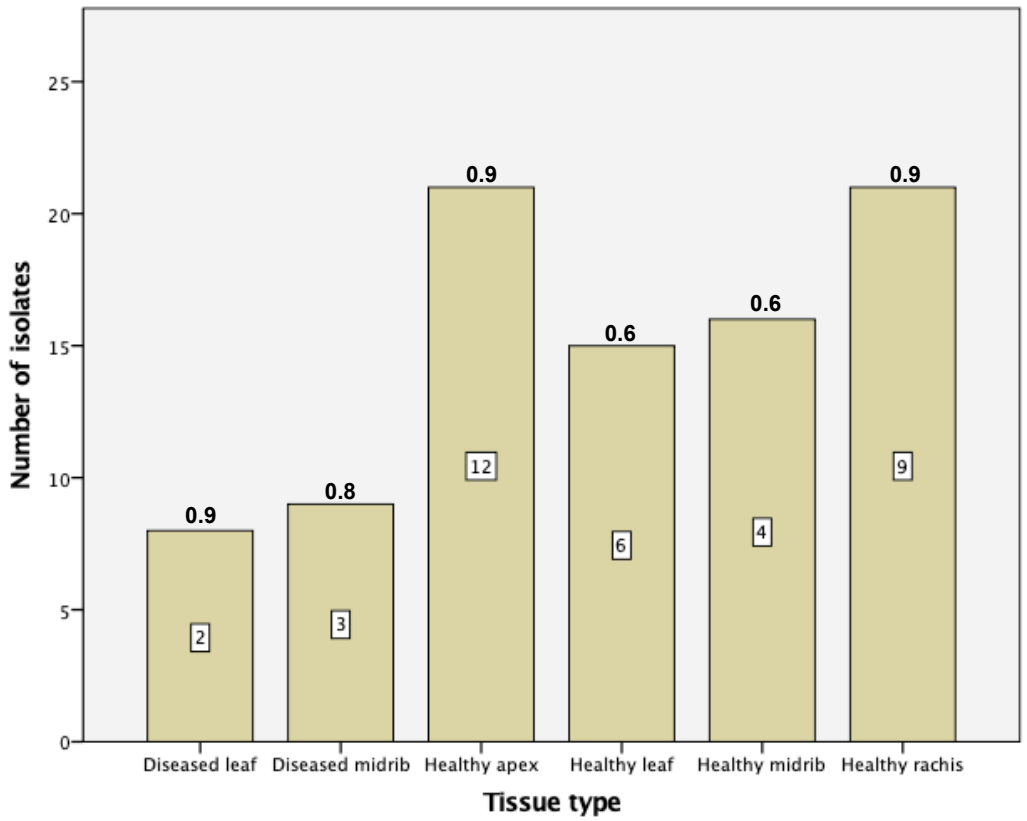


Figure 2.4 Histogram of the total number of fungal endophytes and morphotypes isolated according to source tissue type from a range of *Fraxinus* taxa at the National Botanic Garden Glasnevin. Morphotypes obtained in each tissue category are shown inside the bars. Numbers above each bar indicate the number of isolates per tissue within their respective tissue category.

A Chi square analysis was performed on the Glasnevin samples assessing whether endophyte recovery (number of isolates) was dependent on leaf tissue location (including apex, leaf blade, midrib region and rachis) for both healthy and diseased samples. The Null hypothesis for the Chi square test was that endophyte recovery (number of isolates) was not dependent on leaf tissue location (including apex, leaf blade, midrib region and rachis) for both healthy and diseased. The alternative hypothesis was that endophyte recovery (number of isolates) was dependent on leaf tissue location (including apex, leaf blade, midrib region and rachis) for both healthy and diseased samples. The Chi square (X^2) value = 10.032, df 3 and *p* value of 0.018. Therefore the null hypothesis that endophyte recovery was not dependent on tissue location was rejected and endophyte recovery was, therefore, dependant on tissue location (details of Chi square test

are given in Appendix I).

A Chi square analysis was also performed on samples from the Glasnevin site assessing whether endophyte recovery was dependent on tissue type (healthy or diseased). The Null hypothesis for the Chi square test was that endophyte recovery was not dependent on tissue type (healthy or diseased). The alternative hypothesis was that endophyte recovery was dependent on tissue type (healthy or diseased). The Chi square (X^2) value = 0.109, df 1 and *p* value of 0.741 and the null hypothesis was, therefore, not rejected: endophyte recovery is therefore not dependent on tissue type (details of Chi square test are given in Appendix I). So from type of tissue (diseased or healthy) it is difficult to assume endophyte recovery as which type will be more suitable to recover maximum endophytes.

A Chi square analysis was also performed assessing whether the number of morphotypes was dependent on leaf tissue location (including apex, leaf blade, midrib region, and rachis) samples from the Glasnevin site for both healthy and diseased samples. The Null hypothesis for this Chi square analysis was that the number of morphotypes was not dependent on leaf tissue location (including apex, leaf blade, midrib region and rachis) samples from Glasnevin for both healthy and diseased samples. The alternative hypothesis for this Chi square analysis was that the number of morphotypes was dependent on leaf tissue location (including apex, leaf blade, midrib region and rachis) samples from Glasnevin for both healthy and diseased. The Chi square (X^2) value = 70.538, df 57 and *p* value of 0.107. Therefore the null hypothesis for the Glasnevin samples cannot be rejected and the number of morphotypes is not dependent on leaf tissue location.

The hypothesis that the morphotype count across different sampling tissue regions, namely apex, leaf blade, midrib, rachis and sidevein, have the same distribution was tested using a Kruskall-Wallis test. The test gave a *p* value of 0.86, Kruskall-Wallis H = 8.152, df 4 for the Roosky site and *p* value = 0.009, Kruskall-Wallis H = 11.667, df 3 for the Glasnevin site. Therefore, we rejected the null hypothesis for the Glasnevin site but accepted the same for Roosky site. In other words, morphotype count across sampling tissue regions in the Glasnevin site do not come from the same distribution.

Figure 2.5 shows a histogram of the total number of isolates and morphotypes from

the sample of different *Fraxinus* species at Glasnevin. The fewest number of isolates were yellow + green (1 isolate) followed by red + white and brown (3 and 4 isolates, respectively). The maximum numbers of isolates were of white colour, which are 43 cultures. Green coloured endophytic fungi were also common (16 isolates). In case of the others grey and red has similar number of isolates (7) and yellow have 9 isolates. Most morphotypes (14) were also of the white category (number inside bar). Numbers on the top of each bar were indicating that single morphotype were obtained from respective number of isolates for each colour category.

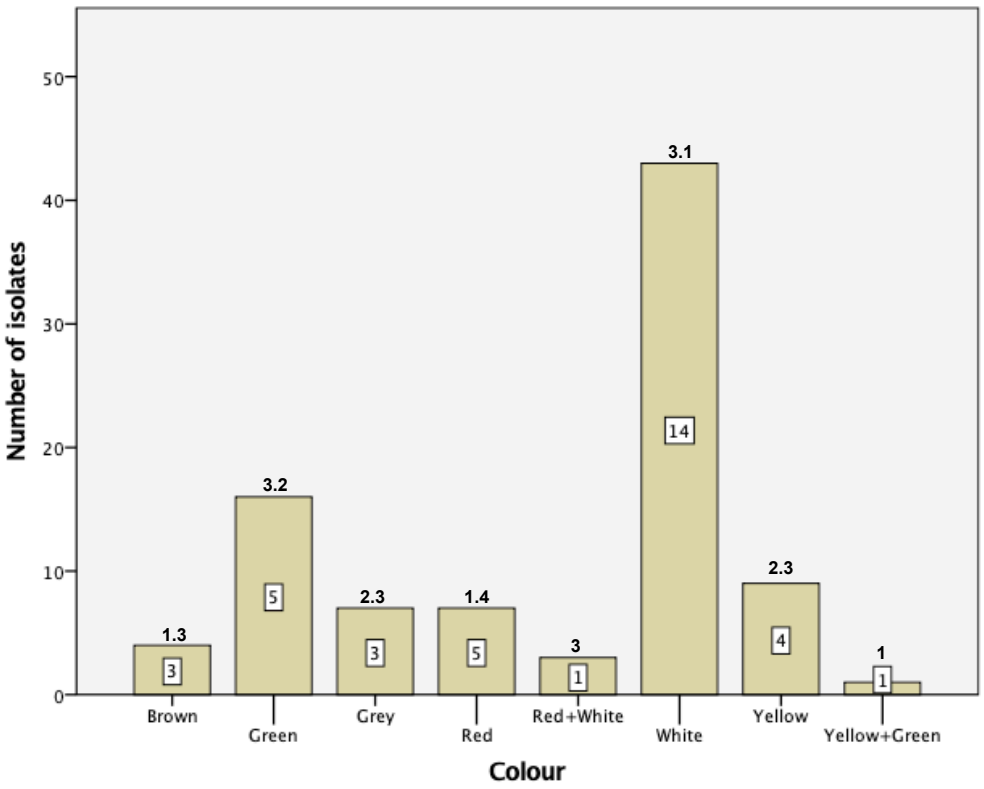


Figure 2.5 Histogram of total number of isolates and morphotypes categorised according to the colour category of the culture colony for the NBG Glasnevin data (different *Fraxinus* species). Number of isolates is the total number of outgrowths for a particular colour. Morphotype number within colour class is given inside each bar. The mean number of isolates per tree is shown above each bar.

Figure 2.6 compares the number of isolates obtained from *Fraxinus excelsior* to that obtained from the other sample of *Fraxinus* species and taxa. Here we obtained a higher number of isolates from other *Fraxinus* species than *Fraxinus*

excelsior but the number of sampled trees was higher in that category too. The average number per tree is 4.8 for *Fraxinus excelsior* and 3.7 for the other *Fraxinus* species. So they are roughly comparable once standardised to account for sample number.

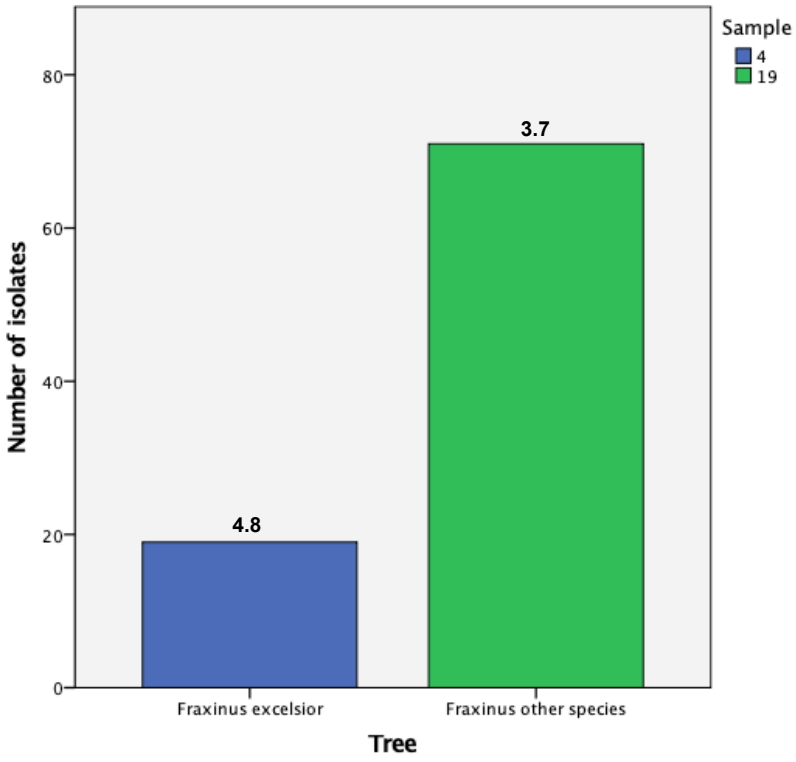


Figure 2.6 Histogram comparing the number of isolates obtained from *Fraxinus excelsior* with other *Fraxinus* taxa. The legend on the right side indicates the numbers of trees sampled per group. The mean number of isolates per tree shown above each bar.

2.3.2 Root endophytes isolation

Root endophytes were obtained from surface sterilised 1.5 cm long roots. Figure 2.7 shows the mean number of isolates obtained categorised into colour classes of the colony. The number of isolates ranged from 1 to 22. The maximum numbers of isolates (22 isolates) were of colours 158 and minimum number from colour 199 and N189 (1 isolate). The number of different morphotypes found from each colour are also shown inside the bars of each respective colour category.

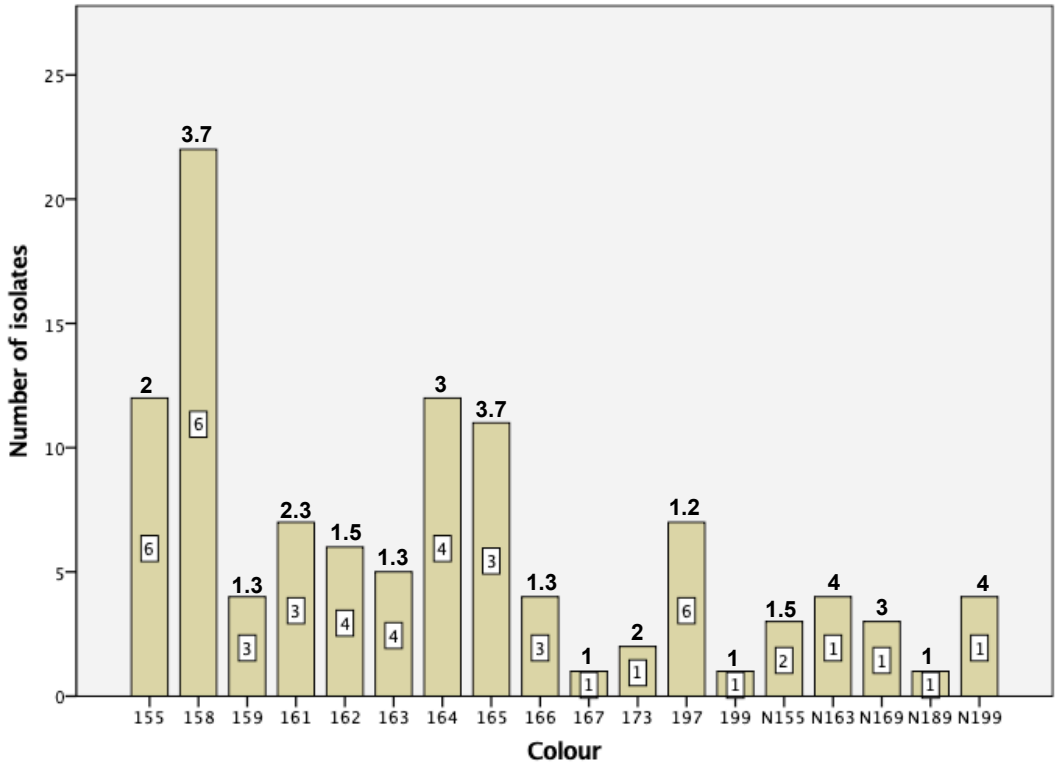


Figure 2.7 Total number of root fungal endophyte isolates categorised according to colour of colony. Morphotypes obtained in each colour category are shown inside the bars. Numbers above the bars indicate the number of isolates per morphotype within their respective colour category.

Figure 2.8 shows the mean isolate number from the different source trees. The number of isolates ranged from 20 (tree 3) to 47 (tree 2). However, when the data are standardised for number of roots sampled the number of isolates per root tree were as follows: Tree 1= 1.9, Tree 2 = 2 and Tree 3 = 1.1 (Figure 2.9).

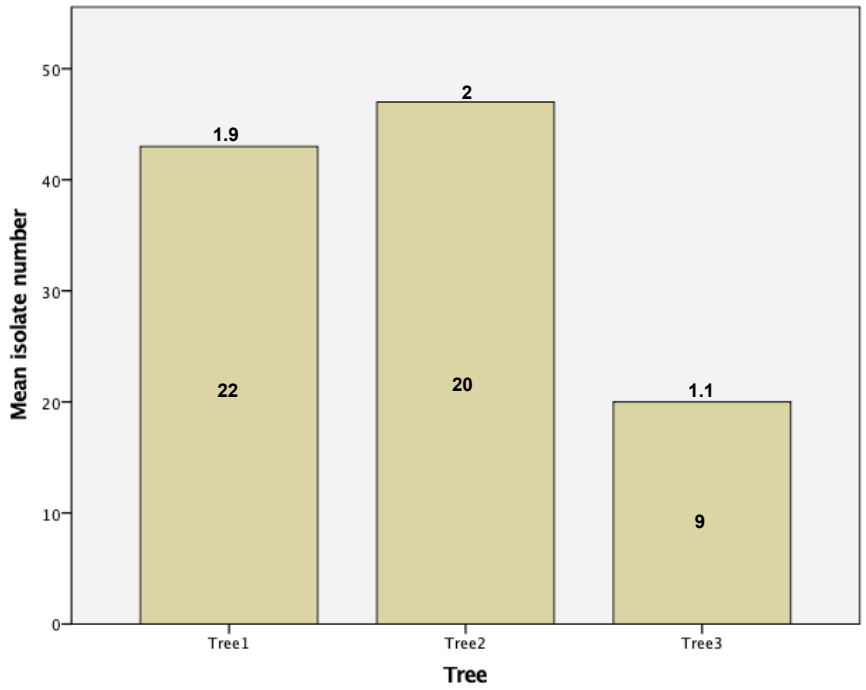


Figure 2.8 Histogram of the mean isolate number of root fungal isolates from each of three trees. Root number sampled for each tree was 23 for tree 1, 24 for tree 2 and 18 for tree 3. The numbers of isolates obtained per root are shown above each of the bars. Number of morphotypes are shown within the bars.

2.3.3 Culture media

Four different media were tested for fungal growth comparisons including ½ strength MEA, ½ strength PDA, ½ strength Sabouraud agar media and ½ strength corn meal agar media. Differences in growth were observed after 25, 43 and 64 days on 20 selected endophytes and 5 Petri dish replicates. Area of growth obtained for different media were plotted against the days on a cluster box plot graph (Figure 2.9). Once the colonies became established (days 43 and 64 data) it could be seen that the lowest median growth values in terms of colony area were recorded for MEA and the highest values for PDA and Sabouraud.

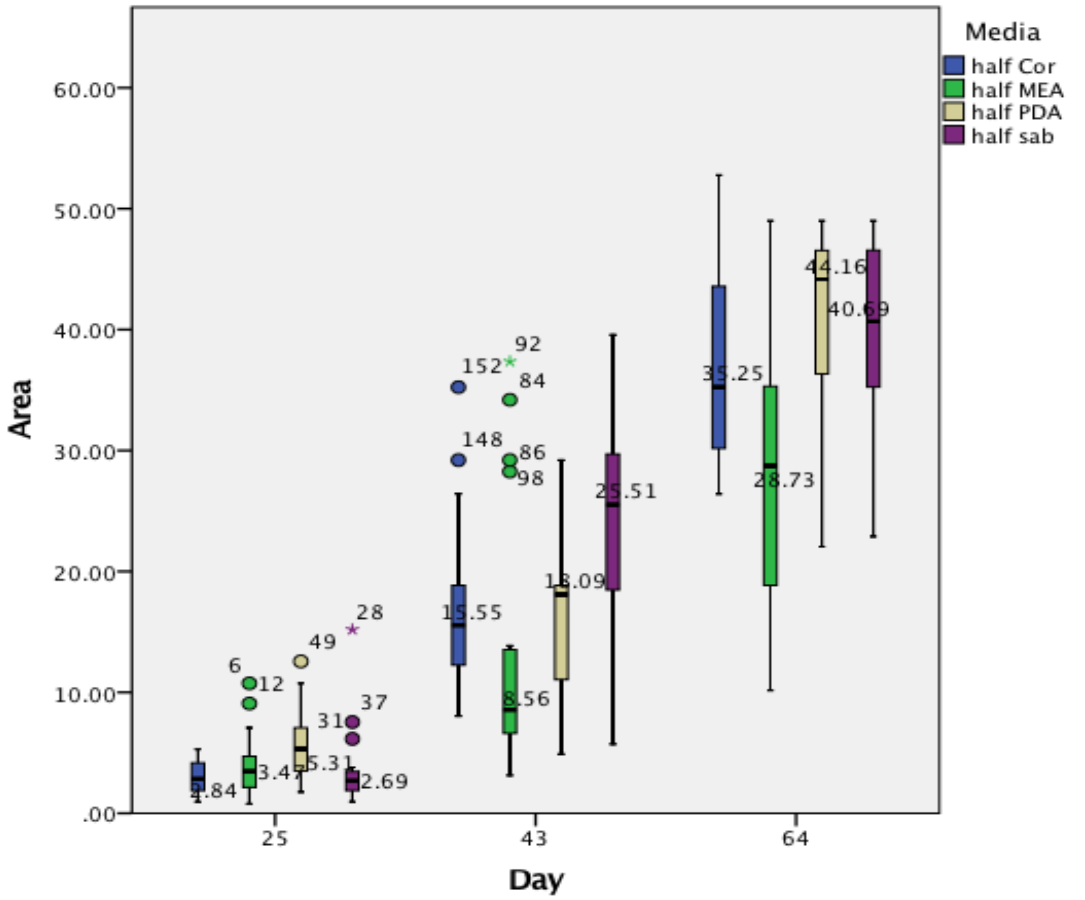


Figure 2.9 Box plots on growth of twenty selected endophytes on four different media types after 25, 43 and 64 days. Area equals growth of fungal colony in cm^2. Each boxplot shows the growth of fungal colony in each media in a particular growth interval. Maximum growth is observe for half PDA media on 64 days with a median value of 44.16.

We sampled similar sections from leaf tissues for each respective tree in each provenance country trial from Roosky and cultured on two different media MEA and MEAF. Morphologically identical and morphologically different endophyte communities were obtained from the similar tissue sections on two different media. One example of different morphotypes obtained after the same tissue was cultured on two media is shown in Figure 2.10. After healthy rachis (H.R) material from S1.a leaf tissues was cultured on each medium (MEA or MEAF) we found two morphologically distinct endophyte colonies. For further information all pictures are presented in the Appendix I (Figure 2.1 to 2.5). Only one is presented in this chapter to show the differences.

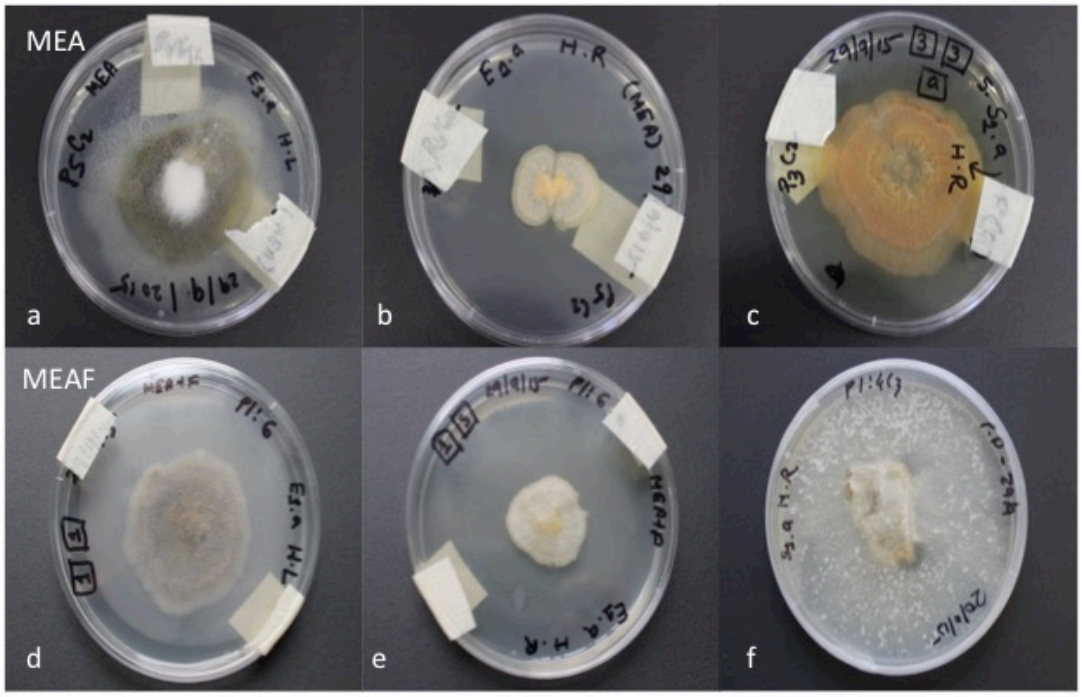


Figure 2.10 Four morphotypes of endophytic fungal cultures isolated from three leaf tissues (E1.a.H.L, E1.a.H.R and S1.a.H.R) grown on MEA (upper row) or MEAF media (lower row). The same fungal morphotypes are in each of the columns except the last column on the right side which has two different morphotypes.

We also recorded the number of endophytic fungi that were sporulating. For the 429 isolates from Roosky, 301 isolates were sporulating and 128 not sporulating. For isolates from Glasnevin Botanic Garden, we selected 90 isolates and found 48 isolates were sporulating and 42 not sporulating. From these 110 endophytic root fungi, 78 were sporulating and 32 not sporulating. We have selected 234 isolates from Roosky 77 from Glasnevin and 100 from Kinsealy for further DNA barcoding identification.

2.4 Discussion

This chapter aimed to assess endophyte isolation efficiency from a range of ash species and their populations, to compare endophyte recovery from a range of tissue types and to assess the suitability of different media for endophyte culture. To do this we used an EU provenance trial of *Fraxinus excelsior* material growing

in Ireland sourced from eleven European countries; material of different species from the NBG Ireland, including *F. americana*, *F. angustofolia, F. dipetala, F. glabra, F. mandshurica, F. numidica, F. ornus, F. pennsylvanica*, *F. potamophila, F. pubinervis, F. texensis* and *F. xanthoxyloides*; and roots from *Fraxinus excelsior* from one site (Kinsealy, Ireland).

2.4.1 Endophyte recovery success

A total of 610 tissues were sampled from leaves and roots from three sites of *Fraxinus* excelsior and other *Fraxinus* taxa and we recovered a total of 628 isolates. Only *Fraxinus excelsior* was sampled at Roosky and we obtained a 99% recovery rate from leaves (at least one endophyte isolate per tissue sampled). For the leaf material in Glasnevin we obtained 76% success with *F. excelsior*, and 79% with other *Fraxinus* species. A study conducted by Bakys *et al.* (2009b) on healthy and necrotic shoots of *Fraxinus excelsior* retrieved 204 isolates from 230 sampled tissues with 88.3% recovery. However in our study we obtained higher success rates from leaf tissues (99%) of the *Fraxinus excelsior* provenance trials compared to Bakys *et al.* (2009b). Power *et al.* (2017) isolated endophytes from 36 twig samples and 34 bud samples, cultured them in 1% MEA media and obtained 68 isolates -that is 100% recovery. It is not known why our endophyte recovery success was higher for the Roosky sample than the Glasnevin sample. The age of the trees is generally older in Glasnevin and the trees are grown further apart.

2.4.2 The influence of plant tissue type on endophyte recovery

We sampled leaf endophytes from healthy (260 from Roosky and 94 from Glasnevin) and diseased leaf tissues (170 Roosky and 21 from Glasnevin) and assessed different leaf zones (lamina, rachis, leaf apex, midribs and veins). The highest number of morphotypes and isolates (Figure 2.2) was obtained from healthy rachises (31 morphotypes, 85 isolates) and this was much higher than the number obtained from diseased rachis material (6 morphotypes, 10 isolates). Chi square analysis assessing whether endophyte recovery was dependent on tissue type (healthy or diseased) gave a p value of 0.217 was not significant which implies, endophyte recovery was not dependent on tissue type (healthy or diseased) and the null hypothesis was therefore accepted: endophyte recovery is, therefore, not dependent on tissue type (Hence despite the large differences, we do not reject the null hypothesis. However, diseased midribs (16, Figure 2.1) and diseased leaf blades (19, Figure 2.2) were a good source of endophytes with comparable numbers to the healthy material of that kind. Bakys *et al.* (2009a)

sampled healthy shoots and obtained an isolation success of 74.1% compared to dead tops (100% of samples). Fungi were isolated from 87.9 and 90.7% of samples with initial and advanced necroses, respectively. Altogether they obtained 430 isolates comprising 56 fungal taxa from healthy and diseased tissues. They found the highest number of taxa from shoots with advanced necroses (30) and lowest numbers from visually healthy shoots (20).

Endophyte isolation was carried out by Haňáčková *et al.* (2017) from shoot, bark and wood with 2% wort agar. They selected six pairs of *F. excelsior* trees in a high disease pressure area (in each pair, one was infected and one visually resistant) and isolated 884 endophytes with 54 different fungal species. They found slightly more fungal species in winter (38) than the summer (35). After DNA barcoding they found more fungal OTUs in resistant trees (32) than susceptible ones (26) even though the total number of colonies were identical (442) in both cases. They obtained 116 endophytes from shoots when combining morphological and ITS barcoding.

Ibrahim *et al.* (2017) conducted endophyte isolation from leaflet and petioles of *Fraxinus ornus* from six sites (south and north of the Alps) and recovered isolates in 97-99% of the cases from leaf material (except one site with 80%). They found 102 morphotypes based on culture morphology. However, in our current study the percent recovery was lower for *Fraxinus ornus*. We sampled 20 leaf tissues from 4 trees of *Fraxinus ornus* on one site and obtained 14 isolates with 70% recovery (Figure 2.3.a) with 3 morphotypes. We also obtained 3 morphotypes from each of *F. americana*, *F. angustifolia*, *F. excelsior*, *F. pubinervis*, F. *texensis*, *F. xanthoxyloides*, *Fraxinus* spp. and 2 morphotypes from *F. dipetala*, *F. glabra*, *F. mandshurica*, *F. pennsylvanica, F. potamophila* and *F. numidica*.

Root endophytes were only recovered from healthy tissues and a small sample (3 trees) of *Fraxinus excelsior* from the Kinsealy site. However, 110 isolates were obtained and recovery success was 100% (51 morphotypes, Figure 2.7). It is not possible to generalize about factors influencing endophyte root diversity in our study. However, endophytic root mycobiota in the forest trees is known to be affected by planting regime, selective cutting or windthrows on forest trees (Görke 1998). Schulz *et.al.* (2006) found that a maximum of 42% of root endophytes were common to both planted and naturally regenerated trees.

A number of other studies have been carried out on mycorrhizal or root endophytes on tree species such as European beech (*Fagus sylvatica*), scots pine (*Pinus sylvestris*), white cedar (*Thuja occidentalis*), paper birch (*Betula papyrifera*), douglas fir (*Pseudotsuga menziesii*), oak (*Quercus* spp.), spruce (*Picea abies*) and common ash (*Fraxinus excelsior*) (Schulz *et. al.* 2006; Lang *et al*. 2011). Ash roots are known to be colonised with arbuscular mycorrhizal (AM) fungi in mixed temperate forests (Seven and Pole 2014). A microanalysis of processed root tissues of *F. excelsior* in the Hainich National Park showed presence of *Glomus* sp. (Seven and Pole 2014). Another study of mycorrhizal species richness in Central European forest was carried out on five types of trees (two species of *Tillia* sp., *Acer* sp., *Fagus sylvatica*, *Carpinus betulus* and *Fraxinus excelsior*) on 280 soil cores (*Fraxinus excelsior* contributed 199 of the soil cores). After morphological analysis and DNA barcoding for SSU, LSU and ITS regions they found out that the relative abundance of AM fungal hyphae in roots of *Fraxinus* and *Acer* was 19±9% regardless of season and field plots and that they are strictly associated with AM fungi. Molecular analysis AM fungi in 50 samples from *Acer* and *Fraxinus* roots revealed seven different sequences for glomeromycota and two singletons in *Fraxinus excelsior* (Lang *et al.* 2011).

Another study conducted by Kowalski and Łukomska (2005) on endophyte recovery from infected *Fraxinus excelsior* seedlings assessed fragments from dead branches, living branches and dead roots and found three dominant species of endophytes by morphological identification namely *Cryptosporiopsis radicicola*, *Cylindrocarpon destructans* and *Phialocephala* sp. We have identified our root endophytes further with the nrITS DNA barcoding region and details are presented in Chapter 3.

2.4.3 The influence of media on endophyte recovery and growth

We used two different media, MEA and MEAF for foliar endophyte isolations from a range of tissue categories from healthy and diseased ash leaves. The MEAF medium used differed from MEA by the inclusion of ash leaves in medium preparation. In this way it was anticipated that the MEAF media would contain extra nutrients or metabolites that could encourage ash endophyte growth. Inclusion of host species leaves in the media preparation has proven successful with other groups of plants (Murphy *et al.* 2015).

We found that the total number of morphotypes obtained from MEA and MEAF

were 67 and 60 respectively. In many cases, the same tissue (from same region of leaf) sampled on differing media (MEA vs MEAF) gave different types of endophytes (Chapter 3, Table 3.20). Even though MEAF media has been successful during the project for the isolation of many morphologically distinct fungal colonies in comparison to MEA, we have found, after DNA barcoding, that MEA cultured the highest number of isolates and the most distinct taxa (OTUs). MEA cultured 27 distinct taxa and MEAF 17 (Chapter 3, Table 3.20). Our results contradict Murphy *et al.* (2015) who found that such whole plant extract media was more useful than other media for endophyte isolation from barley. Bakys *et al.* (2009b) isolated from healthy and diseased *Hymenoscyphus fraxineus* shoots on three different media (2% malt extract, vegetable juice agar and water agar) and found 150 strains including *H. fraxineus* and 42 other fungal taxa. However they have not evaluated the efficiency of the different media for isolation of the *H. fraxineus*. In the present study we also isolated endophytes from diseased leaf tissues but *H. fraxineus* was not cultured from the diseased parts of the leaves sampled with the method employed.

We compared the growth rate of twenty endophytes isolated from Roosky on different media (½ strength MEA, ½ strength PDA, ½ strength Sabouraud agar media and ½ strength corn meal agar media) to assess differences in growth among media types over time up to 64 days (Figure 2.9) and found out that growth on PDA and Sabouraud media was higher after 64 days. A high degree of variation was recorded among isolates for growth on MEA. However, all four media would, on our evidence, be suitable for further sub-culturing and experimental work. For later experimentation on endophytes and pathogen for antagonistic assays we chose MEA over PDA to maintain the balance of hyphal growth of endophyte and pathogen as *H. fraxineus* grows very slowly (personal observation) on artificial media such as PDA and MEA (Chapter 5).

2.5 Conclusion

In conclusion we obtained 429 different isolates (127 morphotypes) from Roosky leaf tissues, 90 different isolates (36 morphotypes) from Glasnevin leaf tissues and 110 different isolates (51 morphotypes) from Kinsealy root tissues.

We used these isolates further to determine their identity via DNA barcoding in Chapter 3 and plant-fungal interaction studies in Chapter 5. Among all these isolates, 301 leaf endophytes (from Roosky), 48 leaf endophytes (from Glasnevin) and 78 root isolates (from Kinsealy) were sporulating.

Chapter 3

Identification of culturable ash endophyte communities using DNA barcoding and a comparison with direct high throughput amplicon sequencing from differing tissues and environments

3.1 Introduction

It is important to accurately identify the fungal communities found in ash if we are to understand how they interact with the dieback pathogen *Hymenoscyphus fraxineus* and investigate the ecological role they play in ash trees in general. Fungal isolations from a diverse range of *Fraxinus excelsior* and other ash species were made in Chapter 2 using different types of isolation media. The isolates could be identified to morphotype and sometimes to a specific taxon but they required identification with DNA so that their community dynamics can be studied in this Chapter.

Plants live in association with various microorganisms in the above ground phyllosphere and below ground rhizosphere (Vorholt 2012; Bulgarelli *et al*. 2013). These can be endophytic and/or epiphytic and can act as beneficial, neutral or detrimentral associates to their host plant (Newton *et al.* 2010; Knief 2014). DNA based studies are important to know the identity of the endophyte, to understand their composition in communities and to investigate their physiological interaction with the plant. Sometimes genomic studies of individual strains are required or sometimes, as in the case of this thesis, metagenomic study of the microbial community is essential (Knief 2014).

Taxonomic identification of fungal species is also an important first step for fungal utilization for forestry, agrochemical, biofuel and pharmaceutical products (Strobel *et al*. 2004, 2008; Alho 2008; Sudhakar *et al.* 2013; Raja *et al*. 2017). Fungi are well known for their ability to produce secondary metabolites with biological activities that can be used for biocontrol or the production of new drugs (Smith *et al.* 2009; Aly *et al*. 2011; Raja *et al.* 2017). It is also important to correctly identify the pathogens relating to plant diseases such as ash dieback disease (Bialek *et al*. 2005; Rickerts *et al*. 2006). After proper identification of the causal organism, it is possible to take quarantine measures influencing international trade of the plant and its products (Wingfield *et al.* 2001; McNeil *et al*. 2004; Begerow *et al.* 2010).

Identification using only morphological characters is not adequate for most fungal species (Crous *et al*. 2007), especially in highly speciose lineages which can be controversial and problematic even for highly trained mycologists (Geiser 2004; Raja *et al*. 2017). Moreover, morphological characters can be misleading in the case of hybridisation (Olson *et al.* 2002; Hughes *et al.* 2013), cryptic speciation (Harrington *et al.* 1999; Kohn 2005; Giraud *et al.* 2008; Foltz *et al*. 2013; Lücking *et al.* 2014) and convergent evolution (Brun and Silar 2010). Some endophytic fungi and some endolichenic fungi do not produce spores in cultures and this makes it difficult to identify the species because fewer characters are available (Hyde 2008; KoKo *et al.* 2011). A further complication is that, in many cases, the anamorph and teleomorph of the same species has been given separate taxonomic names (Moore *et al.* 2011).

3.1.1 DNA barcoding of fungi

Molecular methods are straightforward to follow in comparison to some morphological, anatomical, ultrastructural or chemical procedures (Brasier 1996; Crous *et al*. 2007). PCR followed by DNA sequencing is used widely for fungal identification (White *et al.* 1990; Gardes and Burns 1993). The Consortium for the Barcode of Life (Begerow *et al*. 2010) recommends the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (nrDNA) for identification of fungi and also other regions or nuclear ribosomal DNA such as the large and small subunits of nrDNA described below (Seifert 2009; Begerow *et al*. 2010).

Fungal DNA sequencing can be undertaken with different primers for the large subunit (nrLSU-26S or 28S), small subunit (nrSSU-18S), and the entire ITS spacer region (nrITS1, 5.8S, ITS2) and has greatly improved identification in the kingdom of fungi (Bruns *et al.* 1991; Seifert *et al*. 1995; Raja *et al*. 2017). The ITS region is highly variable among morphologically distinct fungal species (White *et al.* 1990; Gardes and Burns 1993, Mohamed *et al.* 2010). These regions can be used for differing levels of taxon identification (species, genus, family, order, class and phyla) because the SSU generally evolves the slowest so has the lowest amount of variation among taxa. ITS evolves the fastest and exhibits the highest levels of variation (Bruns *et al.* 1991; Mitchell 2006; Raja *et al*. 2017). The LSU region is often combined with ITS for identification because it is slightly more conserved (Vilgalys and Hester 1990; Rehner and Samuels 1995). The ITS region is generally the most useful for identification (Schoch and Seifert 2011; Schoch *et al*. 2012;

Porras-Alfaro *et al*. 2014), as it is easy to amplify and has an appropriately large barcode gap (Schoch *et al*. 2012; Raja *et al*. 2017). Schoch *et al*. (2012) estimated that there were about 172,000 full-length fungal ITS sequences deposited in GenBank, representing approximately 2,500 genera and 15,500 species. However, there are some limitations for the ITS region as it is not suitable for some highly speciose genera that demonstrate a narrow or no barcode gap in the ITS regions (Lieckfeldt and Seifert 2000; Seifert *et al*. 2007; Schoch *et al*. 2012).

Apart from nuclear ribosomal DNA there are also a few other protein coding genes that are used for fungal identification such as the largest (*RPB1*) and second largest (*RPB2*) subunits of RNA polymerase (Stiller and Hall 1997; Matheny *et al*. 2002; Liu and Hall 2004; Reeb 2004), the translation elongation factor 1-alpha (*tef1*) (Rehner 2001), and the beta-tubulin region (*tub2/BenA*) (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997). More recently the mini-chromosome maintenance protein (*MCM7*) has also been used (Schmitt *et al*. 2009; Raja *et al*. 2011; Morgenstern *et al*. 2012; Gillot *et al*. 2015; Hustad and Miller 2015).

DNA sequencing technologies have progressed rapidly and have recently facilitated community level studies that were previously not feasible with standard Sanger sequencing (O'Brien *et al*. 2005; Nilsson *et al.* 2019). The so called next generation sequencing methods were developed in the mid 2000s and marked the beginning of high-throughput sequencing (HTS) of fungal communities. These have been improved to offer higher read length and efficient metabarcoding and community analyses (Hibbett *et al.* 2009, 2016; Nilsson *et al*. 2019). HTS platforms produce massive amounts of sequencing data (up to terabytes) in parallel (Schadt *et al*. 2010; Niedringhaus *et al*. 2011; Pareek *et al.* 2011; Liu *et al*. 2012; Knief 2014). Sequencing studies using, HTS technologies, have made it possible to sequence 3,000 complete genomes (submitted to NCBI databases) and furthermore the genetic information of 16,000 microorganisms are available as scaffold and contigs (Turnbaugh *et al.* 2007).

Several studies have been completed on the fungal communities using the HTS technologies. For example, metagenomic data for phyllosphere associated microorganism communities are available from soybean, rice, clover, tomato, *Arabidopsis* and *Tamarix* (Delmotte *et al.* 2009; Atamna-Ismaeel *et al*. 2012a; Knief *et al*. 2012; Ottesen *et al.* 2013b). Another study showed the microbial communities

of freshwater (Luo *et al*. 2012b). Extensive data are also available from the *Populus* rhizosphere (Brown *et al.* 2012).

Plant associated phyllosphere metagenomic studies have shown a certain degree of consistency of fungal communities at phylum level in terms of colonising taxa (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Vorholt 2012). The HTS methods are particularly suitable for unculturable taxa (Scholz *et al.* 2012; Knief 2014). Amplicon sequencing is used for microbial community study or for comparison between different samples. Diversity studies are often conducted with the 16S rRNA gene for bacterial identification and 18S rRNA gene or ITS for fungal identification. Functional markers are used for studies of specific functions such as the *chiA* gene for chitin degradation (Cretoiu *et al.* 2012). Other studies has been conducted to reveal the sequences of unculturable plant pathogens such as *Candidatus, Liberibacter asiaticus* which causes citrus huanglongbing (Duan *et al.* 2009). Some studies have investigated the differences of microbiome community composition among host plants, biogeographical pattern and the temporal succession of the microbiota (Redford *et al*. 2010; Rastogi *et al.* 2012; Bodenhausen *et al.* 2013; Ottesen *et al*. 2013b Bokulich *et al*. 2014; Maignien *et al.* 2014). Some studies have investigated the impact of specific treatments such as crop irrigation (Williams *et al*. 2013).

Studies of rhizosphere HTS data have addressed similar questions as the phyllosphere data but have also focused on mycorrhizal symbionts including ectomycorrhizal and endomycorrhizal fungi (Lumini *et al*. 2010; Dumbrell *et al.* 2011; Gottel *et al*. 2011; Lundberg *et al.* 2012; Yu *et al.* 2012; Navarrete *et al*. 2013; Peiffer *et al*. 2013; Zhang *et al*. 2013). Furthermore, aspects of rhizosphere interactions such as bioremediation, disease suppressiveness or possible impact of herbicide application and genetically modified plants have also been addressed (Barriuso *et al.* 2010; Rosenzweig *et al.* 2012; Dohrmann *et al*. 2013; Bell *et al.* 2014).

3.1.2 The ash microbiome

A few studies have been published on the ash microbiome in recent years. Those studies have improved our knowledge about endophytes in healthy trees and provided baseline data that can be used to compare our newly isolated endophytes from Chapter 2. Bakys *et al.* (2009a) sampled four locations in Sweden and 20-30 year old natural *Fraxinus excelsior* from mixed stands. Isolations of endophytes

were made from tissues that were visually healthy, tissues showing initial necroses, tissues showing advanced necrosis and shoots with dead tops. DNA sequencing with nrITS revealed 48 ascomycetes, 7 basidiomycetes, and 1 zygomycete. The most frequently observed genera were *Alternaria alternata*, *Aureobasidium pullulans Epicoccum nigrum* and *Gibberella avenacea*. Four common genera were found from all symptomatic categories of tissues: *Botryosphaeria stevensii*, *Valsa* sp. 118, *Lewia* sp. 924 and *Phomopsis* sp. 57. *Hymenoscyphus fraxineus* was isolated from all symptomatic tissue but not isolated from visually healthy shoots. The remaining taxa (34 taxa) were found once from any given syptomatic tissues.

Bakys *et al.* (2009b) studied symptomatic tissues of ash from two sites in Sweden and directly amplified fungal nrITS regions from 32 necrotic leaves, 32 necrotic leaf stalks and 32 discrete bark necroses on shoots. PCR products were cloned and combined with T-RFLP (terminal restriction fragment length polymorphism) analysis. *Hymenoscyphus fraxineus* was isolated from all the tissue types including 30% of the healthy tissues. Molecular data showed 25 different taxa of which 23 were found in shoot bark, 22 in leaves and 15 in leaf stalks. The most frequently observed taxa were *Cryptococcus foliicola* and *Hymenoscyphus fraxineus*, recorded in 70 and 61% of all samples, respectively, followed by *Phoma glomerata*, *P. exigua* and *Cladosporium cladosporioides* - each of them found from half of the analysed samples.

Davydenko *et al.* (2013) isolated 29 taxa by direct sequencing of nrITS from symptomatic and symptomless ash shoot segments in the Ukraine and obtained 430 fungal sequences from 376 samples. Among all samples, the most common were *Aureobasidium pullulans* (24.2%), *Venturia fraxini* (12.5%) and *Alternaria alternata* (10.6%). Other taxa including *Acremonium implicatum*, *Alternaria alternate*, *Alternaria arborescens*, *Aureobasidium pullulans*, *Cladosporium* sp. and *Epicoccum nigrum* were detected in both petiole and shoots. The most common taxa in petioles were *Aureobasidium pullulans*, *Venturia fraxini* and *Lophiostoma corticola*. *Aureobasidium pullulans* was more common in symptomatic shoots (26.5%) than in healthy shoots (11.8%). A correspondence analysis showed that *Cercospora fraxini* and *Venturia fraxini* were associated with petioles and *Alternaria alternata*, *Epicoccum nigrum*, *Cryptococcus victoriaea*, *Cytospora sp.*, *Hymenoscyphus fraxineus* and *Hysterographium fraxini* were associated with symptomatic shoots.

Scholtysik *et al.* (2013) analysed healthy leaflets from the light crown of canopy trees and shade crown of 10 year old healthy ash saplings from three different forest layers in Germany. Altogether, 213 leaflets were sampled and DNA sequenced for the ITS region. A total of 854 fungal isolates from all leaf tissues were grouped into 50 morphotypes. *Alternaria alternata* and *A. infectoria*, were isolated from 127 and 148 of the total isolates, respectively. Some species showed a higher infection rate in autumn from the understorey including *Ramularia endophylla*, *Septoria* sp., *Colletotrichum gloeosporioides*, *Phoma* sp., *Phomopsis* sp., and *Coniothyrium* sp. In addition, *Diplodina acerina*, *Discula umbrinella*, and *Fusarium lateritium* occurred exclusively from the understorey but *Alternaria* sp. was mostly found from the light crown. *Fusicladium fraxini* showed a shift of frequency with season and forest layers and *Xylaria* sp. was the most frequent in August in the shady crown sample. Overall, they found a much higher number of isolates in the understorey than the entire tree crown.

Kowalski *et al.* (2016) studied the ash microbiome from necrotic tissues in six forest sites in Poland. Identification was carried out by sequencing nrITS. About 48 taxa were identified from all fungal isolates and they were mostly Ascomycota (98.6%) with only one Basidiomycota (*Peniophora*). The frequency of *Hymenoscyphus fraxineus* varied on different sites from 43.3% to 68.3%. Six other taxa were also found in 5% of the samples, *Alternaria alternata*, *Diaporthe eres*, *Diplodia mutila*, *Fusarium avenaceum*, *F. lateritium* and *Phomopsis* spp. The endophytic fungi *Hypoxylon serpens*, *Xylaria* sp. and *Chaetomium globosum* were also found. *H. fraxineus* was isolated together with *Diaporthe eres*, *Alternaria alternata*, *Phomopsis* spp., *Diplodia mutila*, *Fusarium avenaceum*, *F. lateritium* and *Aurobasidium pullulans*. Six taxa formed fruiting bodies on the apical parts of stem and twigs of *Fraxinus excelsior* namely *Diaporthe eres*, *Diplodia mutila*, *Lophiostoma corticola*, *Phomopsis* spp., *Sirodothis* sp. and *Valsa cypri*. Other genera formed black discolouration of the dead apical parts of twigs including *Alternaria alternata*, *Cladosporium cladosporioides* and *Epicoccum nigrum*. *Melanomma pulvis-pyrius*, *Mollisia* cf. *cinerea* and *Teichospora obducens* formed ascomata on the exposed wood after degenration of the bark. Areas of local hypertrophies and canker were often found colonised with *Neonectria* sp.. The wood in the stem colonised with *Peniophora cinerea* showed symptoms of white rot.

Another study in Poland conducted by Cross *et al.* (2017), sampled diseased and healthy trees on a weekly basis. They used real time PCR quantification for *Hymenoscyphus fraxineus* and direct HTS of nrITS1 barcoding region on DNA extracts from leaflet and petiole tissues. They found that a few species such as *Phyllactinia* sp. and *Phoma* sp. were positively correlated with *H. fraxineus* whereas *Taphrina* sp., *Tilletiopsis* sp. and *Cladophialophora* sp. were negetively correlated with *H. fraxineus*. Significant changes in the ITS read percentages of fungi associated with leaf tissues were observed in August. *Hymenoscyphus fraxineus* and epiphytic yeasts (*Bullera* sp. and *Rhodotorula* sp.), biotrophs (*Exobasidium* sp. and *Phyllactinia* sp.) and endophytes with pathogenic potential (*Boeremia* sp., *Diaporthe* sp., *Epicoccum* sp., *Fusarium* sp., *Knufia* sp., *Phoma* sp. and *Pleospora* sp.) showed significant increases in ITS1 and ITS2 read percentages in one or several tissues with time. Significant declines in read percentages were observed for *Aureobasidium* sp., *Tilletiopsis* sp., *Sporobolomyces* sp. and *Taphrina* sp. towards the end of the summer. For ITS2, some genera showed positive correlation with *H. fraxineus* (*Exobasidium* sp., *Phyllactinia* sp., *Devriesia* sp., *Knufia* sp. and *Phoma* sp.). However, some other genera showed significant negative correlations (*Aureobasidium* sp., *Tilletiopsis* sp., *Sporobolomyces* sp. and *Taphrina* sp). Higher read percentages in leaflet than petiole, across the season, were found in *Naevala* sp., *Gyoerffyella* sp. and *Phyllactinia* sp.. Similarly, some genera showed higher read percentages throughout the season in petiole tissue compared to leaflet (*Exobasidium* sp., *Rhodotorula* sp., *Phallus* sp. and *Leptosphaeria* sp.). *Tilletiopsis* sp. was the most common taxon from ITS1 and ITS2 data. There was a decline in read proportion of ITS2 at the end of the season from the spore material of genera *Cladosporium* sp. and *Cladophialophora* sp. but an increase in spore material from leaf tissues for *Exobasidium* sp. and *Phyllactinia* sp..

Kosawang *et al.* (2018) studied fungal communities from resistant *Fraxinus* (*F. chinensis* subsp. *rhynchophylla* (Hance) A.E. Murray*, F. lanuginosa* Koidz.*, F. mandshurica* Rupr.*, F. ornus* and *F. pennsylvanica* Marshall) for use as potential biocontrol agents. They isolated, and DNA barcoded, 196 fungal taxa with nrITS and found 15 families, 9 orders and 40 species. Most of their endophytes were Ascomycetes (including *Boeremia exigua, Epicoccum nigrum*, *Fusarium* sp.*, Sclerostagonospora* sp. and *Setomelanomma holmii*); except a single Basidiomycete, namely *Peniophora* sp..

Cleary *et al.* (2016) analysed the microbiome of asymptomatic leaves from *Fraxinus mandshurica* collected from mixed forest strands in Far East Russia and compared the microbiome communities with *Fraxinus excelsior* from other European studies. DNA from all samples were amplified for nrITS and subjected to 454-amplicon sequencing. A total of 49 distinct fungal genera was obtained from leaflets and rachises. There were 20 dominant species (96% read sequences) that belonged to six orders of Ascomycota (Capinodiales, Pleosporales, Diaporthales, Helotiales, Hypocreales and Dothideales) and one order of Basidiomycota (Tremellales). *Hymenoscyphus fraxineus* was detected in 33% of the samples. Endophytic taxa shared between *Fraxinus excelsior* and *F. mandshurica* included *Cladosporium* sp., *Phomopsis* sp., *Phoma* sp., *Alternaria alternata*, *Cryptococcus foliicola*, *Diaporthe nobilis*, *Periconia byssoides*, *Ramularia* sp. and *Fusarium* sp., *Coniozyma leucospermi* was the dominant taxon in *Fraxinus mandshurica*.

Agostinelli (2018) studied the mycobiome of *Fraxinus excelsior* from leaf, bark and xylem collected from two seed orchards in Sweden with the Ilumina MiSeq sequencing of the ITS2 region (Ihrmark *et al.* 2012). The fungal community obtained from 272 ash samples included 59.3% Ascomycota and 38.5% Basidiomycota. They found highly diverse fungal communities across leaf, xylem and bark. Tree disease susceptibility had a contrasting effect in shaping the endophytic and epiphytic community. The dieback resistant, intermediate and susceptible classes shared about 992 of the OTUs. The dieback resistant, intermediate classes had more OTUs compared to the susceptible class. The OTU richness is different in susceptibility classes for leaves but not for xylem or bark. Fungal community composition varies for three tissue types among the susceptibility classes. Species richness was highest in bark tissues. The study also showed the seasonal variation among the fungal community species richness for leaf tissue and also for xylem samples. However no seasonal patterns were observed for bark samples. *Aureobasidium pullulans*, *Alternaria* sp., *Phomopsis* sp. and *Trichoderma* sp. were isolated and cultured and the later three species were also found from culture independent method. Other endophyte genera with pathogenic traits such as *Cladosporium* sp., *Colpoma* sp., *Phoma* sp., *Ramularia* sp. were also isolated (Kehr 1992; Thomma *et al*. 2005; Videira *et al.* 2016). *Hymenoscyphus fraxineus* was not found from their isolates.

Schlegel *et al.* (2018) studied ash and sycamore microbiomes in the Alps with ITS amplicon sequencing using culture dependant and independent methods. The

Illumina sequencing yielded 2,251 OTUs, from which 1,562 had at least 20 reads. About 1,090 OTUs were found in one single lamina or petiole sample and 55 OTUs were present in more than 10% of all samples. In contrast, 84 morphotypes were obtained for ash and sycamore from the culture dependent method and a total of 224 sequences obtained of which 141 were unique (belonging to 96 putative species). They found that the frequency of the morphotypes showed a hyperbolic distribution with a few very abundant and many rarely occurring morphotypes. *Venturia fraxini* were most abundant from *Fraxinus excelsior* and others like *Colletotrichum acutatum*, *Botryosphaeria dothidea*, and *Diaporthe* spp. were dominant for only some sample sites. They also identified (with Illumina data) *Hymenoscyphus fraxineus* from symptomless leaves of ash. Petiole samples revealed lower levels of *H. fraxineus*. On the other hand *H. albidus* was detected at very low levels from the leaf lamina from those sites where *H. fraxineus* were present.

3.1.3 Aims and objectives

This chapter aimed to identify the fungal isolates from Chapter 2 and to study the community level composition of the ash fungal microbiome in different European provenances trial material of *Fraxinus excelsior* (growing in Ireland) and different taxa of *Fraxinus* growing at the National Botanic Garden, Glasnevin. It compares the culturable fungal species with those recorded by direct high-throughput (HTS) of nrITS amplicons. It also compares fungal composition in differing ash tissues including leaves, roots and seeds. The specific objectives were to:

1) Compare the efficiency of nrITS, LSU and *tef* barcoding sequences for identification of culturable ash endophytes.
2) Sequence the nrITS region from the fungal isolates obtained in Chapter 2 and use BLAST searches and phylogenetic analyses to identify them and/or define OTUs.
3) Sequence ash material directly with a culture independent method using HTS (Illumina High Seq) of nrITS to assess the unculturable fungal community of the material.
4) To compare the endophytes found in different ash provenances and taxa.
5) To compare the endophytes found in different plant tissues including leaves, roots and seeds from both diseased and asymptomatic material.

3.2 Material and Methods

3.2.1 Collection

Collection of the leaf and root samples for endophyte isolation from European ash (*Fraxinus excelsior*) and other species of *Fraxinus* is described in Chapter 2 (Section 2.2.1).

The collection of leaf and seed samples for direct HTS of nrITS amplicons was undertaken at the same time as the collections for endophyte isolations (21st September 2015 from the provenance trial for forestry research in Roosky, County Rosscommon; and 30th September 2015 from the National Botanic Gardens, NGB, Glasnevin, Dublin). After collection, leaves were kept inside Ziplock bags filled with silica gel for drying and leaf preservation before extraction (Hodkinson *et al.* 2007). Seeds were kept in a brown envelope and placed inside the drying cabinet to remove excess moisture. Tables 3.1, 3.2 and 3.3 show the material used for direct HTS of nrITS amplicons from plant extracted DNA.

Table 3.1 Ash leaflets collected for DNA extraction and HTS from Roosky, Co. Rosscommon

No.	Name of the plots/plants	Plot number given in field	No. of leaflets collected
1.	A	13	2
2.	A1.a	5	3
3.	A1.b	5	2
4.	A1.c	5	2
5.	B	20	4
6.	C	7	2
7.	D1.a	12	2
8.	D1.b	12	2
9.	D1.c	12	4
10.	E	9	2
11.	E1.a	11	3
12.	E1.b	11	2
13.	F1.a	21	3
14.	F1.b	21	2
15.	G.a	24	2
16.	G.b	24	2
17.	G1.a	30	2
18.	G1.b	30	2
19.	H	25	2
20.	H1.a	1	2
21.	H1.b	1	3
22.	J	29	2
23.	J1	18	3
24.	K	2	4

25.	K1.b	10	2
26.	L	14	2
27.	L1	33	3
28.	M	31	3
29.	M1	15	3
30.	O1	27	3
31.	Q1	37	3
32.	R1.a	28	2
33.	R1.b	28	2
34.	S1.a	19	3
35.	S1.b	19	3
36.	T1.a	32	3
37.	T1.b	32	3
38.	T1.c	32	3
39.	U1	36	3
40.	Y	23	3

Table 3.2 Ash keys collected form Roosky, Rosscommon

No.	Plot name	Plot number	Plot name	Position of plant
1.	A	7	A	5.4
2.	B	13	B	6.6
3.	B	15	B	Not in trial gap
4.	B	17	B	1.3
5.	B (Irish)	16	B (Irish)	1.1
6.	C	12	C	2.2
7.	D	4	D	3.5
8.	D1	9	D1	4.4
9.	E1	3	E1	2.5
10.	G	2	G	5.4
11.	G1.b	14	G1.b	b
12.	H	10	H	5.5
13.	K	8	K	1.6
14.	Q	11	Q	2.3
15.	U	5	U	5.5
16.	U1	1	U1	2.4
17.	Z	6	Z	2.2

Table 3.3 Ash leaflets collected form Glasnevin Botanical Garden

No.	Name	Accession number	Additional notes at the time of

			collection
1	*Fraxinus texensis*	XX.011046	-
2	*Fraxinus xanthoxyloides*	2003.0735	-
3	*Fraxinus glabra*	1909.011054	-
4	*Fraxinus mandshurica*	1934.011053	-
5	*Fraxinus potamophila*	1932.011031	-
6	*Fraxinus americana*	2001.1739	-
7	*Fraxinus pubinervis*	1954.011044	-
8	*Fraxinus angustifolia* 'Monophylla'	XX.011028	-
9	*Fraxinus excelsior* 'Juspidea'	2000.3637A	-
10	*Fraxinus ornus*	1977.0039	-
11	*Fraxinus ornus*	2005.0652	-
12	*Fraxinus excelsior ('Diversifolia Laticifolia')*	XX.011040	-
13	*Fraxinus dipetala*	XX.011038	-
14	*Fraxinus excelsior* (orange bark)	XX.011029	-
15	*Fraxinus* sp..	2013.0214	-
16	*Fraxinus ornus*	1969.011033	-
17	*Fraxinus ornus*	XX.011034	Mark 83
18	*Fraxinus numidica*	1904.011032	SL/04 on Label
19	*Fraxinus angustofolia* 'Lentiscifolia'	XX.011074	-
20	*Fraxinus* sp. (No label)	2006.1641	Mark 91
21	*Fraxinus* sp. (greybud)		Mark 78
22	*Fraxinus excelsior* 'Pendula'	XX.011048	-
23	*Fraxinus pennsylvanica*	1889.011030	-

3.2.2 DNA extraction from fungal cultures (for Sanger sequencing) and plant material (for direct amplicon sequencing)

3.2.2.1 Fungal DNA extraction

Altogether, 625 isolates were cultured from leaf and root tissues (Chapter 2). Among them, 410 were selected for DNA barcoding identification using nrITS, nrLSU and *tef*. DNA was extracted from the pure cultures of fungal endophytes using a DNeasy Plant mini kit from Qiagen (which is also suitable for fungi). Approximately 1/8th of the fungal plate hyphal tissue was transferred, using sterile forceps and scalpels, into a 1.5 ml microcentrifuge tube with a sterile metal bead

for homogenisation and tissue disruption using a mixer mill (Retsch MM 300) for 1 min at 10 frequency / second (turning the block on the other side and running it for another minute). The rest of the DNA extraction followed the Qiagen manufacturer's protocol. The final extract volume was 50 µl and it was stored at –20°C until further use. Due to some negative results with the Qiagen kit extractions, we also used a hot CTAB extraction for 55 isolates (modified from Hodkinson *et al.* 2007). The 1/8th of the fungal plate tissue was transferred into a preheated autoclaved mortar and ground with a small amount of sterile sea sand (Macherey-Nagel 727423). The grinding step was carried out inside the laminar air flow cabinet to avoid cross contamination. The rest of the fungal extraction followed the protocol described in Box 1 for the plant material.

3.2.2.2 Plant DNA extraction and purification

DNA from leaflets and seed were extracted by a modified hot CTAB method (Hodkinson *et al.* 2007; Box 1). Cleaning of total DNA extracts after the CTAB extraction of seeds and leaves was done using the Jetquick DNA purification kit (Box 2).

Box 1 Modified DNA extraction technique (from Hodkinson *et al.* 2007)

Preheat 5ml of 2×CTAB extraction buffer at 65°C in a 12 ml chloroform resistant centrifuge tube in a water bath. Preheat mortar and pestle at 65°C in a drying oven. Weigh approximately 0.1-0.15 g of dried leaves (or 4 dried seeds at approximately 0.05-0.12 g). Grind the leaf or seed inside a preheated mortar, initially using a small amount of buffer. Then gradually add buffer as grinding continues. Finally add the remaining buffer and pour the slurry back into the 12 ml tube, screw on the lid and place it in the water bath again for 10 mins at 65°C with occasional stirring. Add 5 ml of CI. Mix it well and release the gas by opening the lid and closing the lid. Shake the tube horizontally on a shaker for 30 min. Centrifuge the tube at, 3200 relative centrifugal force (rcf), for 10 min. This equates to the maximum 4000 rpm on the centrifuge. Higher rotation speeds are also acceptable and preferable if equipment permits. Carefully remove the tube from the centrifuge being careful not to disturb the separation. Remove the aqueous phase of the solution (upper; CTAB) containing the DNA using a transfer pipette and place into a 50 ml conical based centrifuge tube. Add equal volume of

chilled isopropanol to the 50 ml tube and mix it gently (at this stage precipitation of DNA may be visible). Place into a -20°C freezer overnight, to further precipitate the DNA. Centrifuge tubes at 2000 rcf for 5 min to pellet the DNA into the base of the tube. Pour off the supernatant and add 1.5 ml of 70% ethanol, wash buffer. Mix gently. Centrifuge the sample at 2000 rcf at 3 min to pellet the DNA again. Pour off the supernatant and keep the tube, upside down on a paper towel for 5 min. Then place the open tube inside the fume hood for at least 30 min to let them dried completely. Dissolve the pellet in 0.5 ml of TE (10 mM Tris–HCl, 1.0 M EDTA) buffer by mixing with the transfer pipette and transfer the DNA solution to a labelled 1.5 ml micro centrifuge tube (with the same transfer pipette) and store at -20°C.

Box 2 Column cleaning using Jetquick purification

Binding: Initially 50µl of TE (Tris, EDTA) buffer were preheated at 65ºC. First 400 µl of binding buffer (H1,supplied) and 100 µl of uncleaned total DNA were added into the column and placed onto receiver tube. The column was centrifuged at 12,000 rcf for 1 min. Liquid was removed from the receiver tube.

Washing: Then 500 µl of wash buffer (H2, supplied) added into filter tube and centrifuged again for 12,000 rcf for 1 min. After centrifugation remove the liquid from the receiver and centrifuge the tube for second time at 12,000 rcf for 1 min. After the liquid was removed from receiver filter tube was placed into final collection tube.

Elute: Then add preheated 50 µl TE buffer into the filter tube and centrifuge the tube at 12,000 rcf for 2 mins. After cleaning the total DNA they were checked by agarose gel electrophoresis and quantified using nanodrop. The cleaned DNA samples were stored at -20°C.

3.2.2.3 High-throughput sequencing of nrITS-1

High throughput amplicon sequencing was undertaken using the ITS-1 primers for fungal DNA recommended by Novogene (estimated amplicon size = 307bp). Amplicon sequencing was carried out by Novogene on 70 samples using an Illumina High Seq 250 paired end platform and 50,000 raw tags. The ITS-1 primers were as follows:

ITS1 −5’ GGAAGTAAAAGTCGTAACAAGG 3’

ITS2-2043R −5’GCTGCGTTCTTCATCGATGC 3’

All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equidensity ratios. Then, mixed PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit before analysis by the Illumina High Seq 250 platform.

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/ FLASH/), a fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end of the same DNA fragment; and the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the Qiime (V1.7) quality controlled process. The tags were compared with the reference database (Unite database, https://unite.ut.ee/) using the UCHIME algorithm to detect chimeric sequences and chimera sequences were removed. Then the effective tags were finally obtained.

Samples used for HTS of nrITS amplicons are listed in Table 3.4.

Table 3.4 Samples selected for NGS analysis and their quantity of total DNA after column cleaning (CC)

Place	Plot	Country	Provenance	Tissue type	Name given	Name/plot name	DNA yield after CC (ng ul^{-1})
R	13	IE	ENN	Leaf	R1 *	A	93.1
R	5	BE	HOG	Leaf	R2	A1.A	90.8
R	20	IE	DON	Leaf	R5	B	81.9
R	12	IE	Control	Leaf	R8	D1.C	79.7
R	11	DE	FAR	Leaf	R10	E1.B	83.4
R	21	DE	KAR	Leaf	R13	F.O	39.2
R	24	GB	LOC	Leaf	R15	G.B	67
R	30	DK	RAV	Leaf	R17	G1.B	98.9
R	25	FR	VSP	Leaf	R18	H	63.5

R	1	DK	BRE	Leaf	R20	H1.B	91.3
R	29	FR	SAM	Leaf	R21	J	26
R	18	IT	ABE	Leaf	R22	J1	111.7
R	2	FR	ATH	Leaf	R23	K	50.6
R	10	IT	VAP	Leaf	R24	K1	73.3
R	14	FR	LAR	Leaf	R25	L	72.4
R	33	IT	CAD	Leaf	R26	L1	57.7
R	31	GB	WYW	Leaf	R27	M	69.2
R	15	IT	MON	Leaf	R28	M1	51.1
R	27	PO	WLO	Leaf	R29	O1	137
R	37	PO	WLO	Leaf	R30	Q1	66
R	28	LT	KAL	Leaf	R31	R1.A	130.1
R	19	LT	ZEI	Leaf	R34	S1.B	76.4
R	32	CZ	RAB	Leaf	R35	T1.A	139.9
R	36	PO	MIR	Leaf	R38	U1	85.4
R	23	FR	SAG	Leaf	R39	Y	55.3
G	-	a	-	Leaf	G1 **	*F. texensis*	75.9
G	-	b	-	Leaf	G2	*F. xanthoxyloides*	52.4
G	-	c	-	Leaf	G3	*F. glabra*	78.9
G	-	d	-	Leaf	G4	*F. mandshurica*	138.2
G	-	e	-	Leaf	G5	*F. potamophila*	27.7
G	-	f	-	Leaf	G6	*F. americana*	144.2
G	-	g	-	Leaf	G7	*F. pubinervis*	117.1
G	-	h	-	Leaf	G8	*F. angustifolia*	84.5
G	-	i	-	Leaf	G10	*F. ornus*	145.6
G	-	j	-	Leaf	G11	*F. ornus*	71.5
G	-	k	-	Leaf	G12	*F. excelsior* 'Diversifolia Lacinata'	53.6
G	-	l	-	Leaf	G13	*F. dipetala*	117

G	-	m	-	Leaf	G14	*F. excelsior* (Orange bark)	125.4
G	-	n	-	Leaf	G16	*F. ornus*	102.5
G	-	o	-	Leaf	G17	*F. ornus*	72.5
G	-	p	-	Leaf	G18	*F. numidica*	42.1
G	-	q	-	Leaf	G19	*F. angustifolia* 'Lentiscifolia'	63.5
G	-	r	-	Leaf	G20	*Fraxinus sp.*	56.4
G	-	s	-	Leaf	G21	*Fraxinus* (unknown)	140.1
G	-	t	-	Leaf	G22	*F. excelsior* 'Pendula'	21.5
G	-	u	-	Leaf	G23	*F. pennsylvanica*	78.6
F	-	-	ABL	Leaf	F1 °	*Fraxinus* sp.	24.2
F	-	-	ABL	Leaf	F2	*Fraxinus* sp.	27.1
F	-	-	ABL	Leaf	F3	*Fraxinus* sp.	41.2
R	3	FR	SPD	Seed	S1 w	Q (2.3)	93
R	20	IE	DON	Seed	S3	B (6.6)	102.6
R	13	IE	ENN	Seed	S5	A (5.4)	31.1
R	25	FR	VSP	Seed	S6	H (5.5)	58.2
R	20	IE	DON	Seed	S7	B (1.3)	39.2
R	2	FR	ATH	Seed	S8	K (1.6)	54.4
R	12	NL	VCS	Seed	S9	D1 (4.4)	98.8
R	9	GB	SET	Seed	S10	E (2.5)	39.4
R	4	FR	DOU	Seed	S11	Z (2.2)	48.4
R	17	IE	Control	Seed	S12	D (3.5)	247.3
R	7	IE	CUR	Seed	S13	C (2.2)	34.5
R	6	FR	SPF	Seed	S14	U (5.5)	47.9
R	36	PO	MIR	Seed	S15	U1 (2.4)	39.7
R	30	DK	RAV	Seed	S16	G1 (b)	51.8
R	24	GB	LOC	Seed	S17	G (5.4)	32.8
G	-	v	-	Seed	SG1	v	121

					□		
G	-	n	-	Seed	SG2	*F.ornus*	98.3
G	-	w	-	Seed	SG3	*F.ornus*	22.5
G	-	s	-	Seed	SG5	x	66.2
G	-	e	-	Seed	SG6	*F. potamophila*	34.2
G	-	d	-	Seed	SG7	*F. mandshurica*	11.9

*R1-R39 = Leaflets from Roosky; **G1-G23= Leaflets from Glasnevin; °F1-F3= Leaflets from France; □S1-S17= Seed extract from Roosky; □SG1-SG7=Seed extracts from Glasnevin; Belgium – BE, Czech republic – CZ, Denmark – DK, France – FR, Germany – DE, Ireland – IE, Italy – IT, Lithuania – LT, Netherland – NL, Poland – PO, UK – GB; Abeton (IT) - ABE, Athis (FR) - ATH, Au BoutduLac – ABL, Bregentved (DK)-BRE, Cadore (IT)-CAD, Currachase (IE)- CUR, Donadea (IR)-DON, Dourdan – DOU, Enniskillen (IE)-ENN, Farchau (DE)-FAR, Hoge Bos (BE)-HOG, Kalsiadorys (LT)- KAL, Karlsruhe (DE) -KAR, La Romagne (FR)–LAR, Loch Tay (GB)- LOC, Mircze (PO)- MIR, Monte Lessini (IT)– MON, Rabstejn (CZ)- RAB, Ravenholt (DK)– RAV, Saint Gatien (FR)- SAG, Saint Martin (FR) – SAM, Settrington (GB)- SET, St Paul De Salers – SPD, St. Pierre des fleurs – SPF,Vaartbos Com.seed (NL) – VCS, Val Saint Pierre (FR) – VSP, Valle Pesio (IT)– VAP, Wloszczowa (PO) – WLO, Wytham Wood (GB)– WYW, Zeimelis (LT)- ZEI; accession number XX.011046 – a, 2003.0735 – b, 1909.011054 – c, 1934.011053 – d, 1932.011031 – e, 2001.1739 – f, 1954.011044 – g, XX.011028 – h, 1977.0039 – i, 2005.0652 – j, XX.011040 – k, XX.011038 – l, XX.011029 – m, 1969.011033 – n, XX.011034 – o, 1904.011032 – p, XX.011074 – q, 2006.1641 – r, GPS -78 – s, XX.011048 – t, 1889.011030 – u. GPS-91 – v, GPS-83 – w, GPS 81- x.

3.2.2.4 DNA amplification of pure culture fungal DNA

DNA extracts of the isolated fungal DNA were used for amplification in a total volume of 25 µl using ultrapure water, dNTPs (Invitrogen) 5 x Buffer (Promega), $MgCl_2$ (Promega), *Taq* polymerase (Promega), forward and reverse primers and 1µl of DNA (approximately 100 $ngµl^{-1}$) (Table 3.5). PCR amplification for the targeted regions followed the cycles shown in Tables 3.6, 3.7, 3.8, 3.9 and 3.10.

Table 3.5 Primer information for each DNA barcoding locus used (nrITS, Tef and LSU)

Primer	Primer sequence	Reference
Forward primer		
ITS-1	5’ TCCGTAGGTGAACCTGCGG 3’	White *et al.* (1990)

ITS-1F	5’ CTTGGTCATTTAGAGGAAGTAA 3’	Gardes and Bruns (1993)
ITS-5	5’ GGAAGTAAAAGTCGTAACAAGG 3’	White *et al.* (1990)
LROR (LSU)	5’ ACCCGCTGAACTTAAGC 3’	Vilgalys and Hester (1990)
Tef-983	5’ GCYCCYGGHCAYCGTGAYTTYAT 3’	Rehner and Buckley (2005)
Reverse primer		
ITS-4	5’ TCCTCCGCTTATTGATATGC 3’	White *et al.* (1990)
LR5 (LSU)	5’ TCCTGAGGGAAACTTCG 3’	Vilgalys and Hester (1990)
Tef-1576	5’ ACHGTRCCRATACCACCRATCTT 3’	Rehner and Buckley (2005)

Table 3.6 PCR master mix reagents

Name	Microliters
DNA (100 ngµl^{-1})	1
H_2O	15.875
dNTPs (10 mM; Invitrogen)	0.5
5x Buffer (Promega)	5
F Primer (20 pmolµl^{-1})	0.25
R Primer (20 pmolµl^{-1})	0.25
$MgCl_2$ (25 mM)	2
Taq polymerase (5 units µl^{-1}; Promega)	0.125
Total	25

Table 3.7 PCR cycle conditions used for nrITS (ITS 1 & ITS 4; ITS 5 & ITS 4) primer combinations

Stages	Temperature (°C)	Time (min)	Number of cycles
Premelt	94	3	1
Denature	94	1	32
Anneal	58	1	32
Extension	72	1	32
Final extension	72	10	1
Hold	4	∞	∞

Table 3.8 PCR cycle conditions used for nrITS (ITS 1F & ITS 4) primers

Stages	Temperature (°C)	Time (min)	Number of cycles
Premelt	95	1:50	1
Denature	95	0:45	34

Anneal	56	1	34
Extension	72	1	34
Final extension	72	7	1
Hold	4	∞	∞

Table 3.9 PCR cycle conditions used for the *tef* (983F and 1576 R) primers

Stages	Temperature (°C)	Time (min)	Number of cycles
Premelt	94	1:30	1
Denature	94	0:45	32
Anneal	61	1	32
Extension	72	1	32
Final extension	72	7	1
Hold	4	∞	∞

Table 3.10 PCR cycle conditions used for the LSU (LROR and LR5) primers

Stages	Temperature (°C)	Time (min)	Number of cycles
Premelt	94	1:30	1
Denature	94	0:30	36
Anneal	53	1	36
Extension	72	1	36
Final extension	72	7	1
Hold	4	∞	∞

Successful DNA amplification was confirmed with agarose gel electrophoresis using gel red nucleic acid stain (Biotium 41003) and positive samples were selected for further sequencing. For gel electrophoresis, 1.2 g of agarose (Roche 11388991001) was added in 100 ml of TBE (Trish-borate-EDTA) buffer and heated for approximately 3 mins in a microwave and 5 µl gel red was then added into the mixture and poured into the gel caster boat with gel comb left for 30 minutes for solidification. Then the gel was placed into electrophoresis chamber and 4 µl PCR product loaded in each well with 2 µl of loading dye and run at 126 volts for 20 min. After the run was completed the gel was checked with the gel doc machine (DNR Bio-imaging system MiniBIS) and an image of the gel was recorded.

3.2.2.5 Cleaning of PCR products and cycle sequencing

Exosap was used to clean-up the PCR reactions prior to cycle sequencing and an X Terminator Kit (Applied Biosystems) used to clean up the cycle sequencing products. Exosap clean-up was done using exonuclease and alkaline phosphatase and water (Table 3.11).

Table 3.11 Exosap clean-up of PCR products (master mix)

Name	µl
Exonuclease I (20 unitsμl^{-1})	0.3
Alkaline phosphatase (1 unitsμl^{-1})	2
Ultrapure H_2O	2.7

5 µl of master mix was added to 5 µl of PCR product and then the reaction mixture was placed in the thermocycler following the cycles in Table 3.11. After the exosap clean-up, the PCR products were used for cycle sequencing process as described in Table 3.12 and 3.13 or they were sent for commercial sequencing.

Table 3.12 Cycles for exosap for PCR clean up

Temperature (°C)	Time (min)	Cycle
37	30	1
82	20	1
4	∞	∞

Table 3.13 Cycle sequencing procedure mix

Reagent	µl
H_2O	5.5
Big dye Terminator v3.1 (pink mix)	0.8
5X buffer (Applied Biosystems)	1.7
F primer or R primer (20 pmolμl^{-1})	0.5
H_2O	5.5

A total of 8.5 µl of master mix was added to 1.5 µl cleaned PCR product to give a total reaction volume of 10 µl. Two batches of cycle sequencing reactions were made, one with forward and the other with reverse primer. Both forward and reverse must be sequenced separately. The cycles required for cycle sequencing are described in the Table 3.14. The cycle sequenced products were cleaned using

X Terminator (Applied Biosystems). X Terminator reagents as shown in Table 3.15

Table 3.14 Cycle conditions for cycle sequencing

Temperature (°C)	Time (min)	Number of cycles
96	1	1
96	0:10	25
50	0:05	25
60	4	25
4	∞	∞

Table 3.15 X terminator clean-up of cycle sequencing reactions

Name	µl
SAM (Applied Biosystems)	45
Cycle sequencing reaction	10
X Terminator (Applied Biosystems)	10

55 µl (SAM and X Terminator) was added to each sample well on the plate (finished from the cycle sequencing) and the plate shaked for 30 mins before being transferred to the DNA sequencer (Applied Biosystems 3100xl Genetic Analyser) for analysis following the manufacturer's instructions.

About 32 DNA samples were sequenced using the 'in house' (Applied Biosystems 3100xl) sequencer (Botany Department TCD). The remaining samples, after being successfully amplified and cleaned, were processed for Sanger DNA sequencing commercially by Macrogen or Source Biosciences. Before sending the PCR product for sequencing, they were exosap purified, quantified using a Nanodrop lite UV spectrophotometer and diluted with pure water according to the preferred concentration requirement for the companies. Sequence traces were edited in Geneious (v.6; Biomatters), or MEGA5 (Tamura *et al*. 2011) and then used in a BLAST search to find the closest match (percent similarity) in GenBank (NCBI).

3.2.2.6 Data analysis

3.2.2.6.1 OTU cluster and species determination for amplicon sequencing

Sequences analyses were performed by Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequences for each OTU were screened for further annotation. Sequence analyses were performed by BLAST with Qiime (Version 1.7) and UNITE database for species annotation at

each taxonomic rank (kingdom, phylum, class, order, family, genus, species). The phylogenetic relationship of all OTU representative sequences was undertaken by aligning the sequences in MUSCLE (in MEGA 5) and tree produced using the neighbour joining algorithm with Kimura 3 parameter model. OTU abundance data were normalised using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalised data.

Alpha diversity was determined for 1) Observed-species and 2) Shannon calculated with QIIME (Version 1.7) and displayed with R software (Version 2.15.3); the significance of pairwise species richness and Shannon index values was assessed with Tukey and Wilcoxon tests.

Beta diversity analysis was used to evaluate differences of samples in species complexity. Beta diversity on both weighted and unweighted unifrac (Lozupone *et al.* 2011) were calculated in QIIME (v 1.7) and significance of all pairwise comparisons assessed with Tukey and Wilcoxon tests. Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software (Version 2.15.3). Principal Coordinate Analysis (PCoA) was performed to visualise the complex, multidimensional data. A distance matrix of weighted or unweighted unifrac distance among samples was obtained before it was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Non-metric multi-dimensional scaling (NMDS) analysis was also performed to comparison with PCoA. It is a ranking method applicable to ecological research. It is a non-linear model designed for a better representation of non-linear biological data structure aiming at overcoming the flaws in methods based on linear models, including PCA and PCoA (Rivas *et al.* 2013). Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted using the QIIME software (v 1.7). Diversity analysis of the results from the HTS samples were carried out by Novogene.

3.3 Results

3.3.1 Identification of cultured fungal isolates

We isolated 628 endophytes from leaf and root tissue (Chapter 2). We selected 13 morphologically distinct isolates out of 25 isolates from each of the square partitioned Petri plates, used for fungal isolation, for further identification with the DNA barcoding. Altogether, 410 isolates were selected for DNA barcoding; 310 were leaf endophytes and 100 were root endophytes. DNA samples were amplified for different barcoding loci (nrITS; nrLSU; *tef*). We successfully amplified 389 isolates. The proportion of sequences obtained for the different loci are shown in Figure 3.1. The whole ITS region (ITS1, 5.8S and ITS2; primers ITS 5 & 4) was found to be the most efficient for fungal identification, so most samples were identified with this region. The ITS 1 region alone (primers ITS 1F and 4) was also efficient in comparison to the two other regions tested (nrLSU and *tef*). The *tef* region was not reliable for our samples.

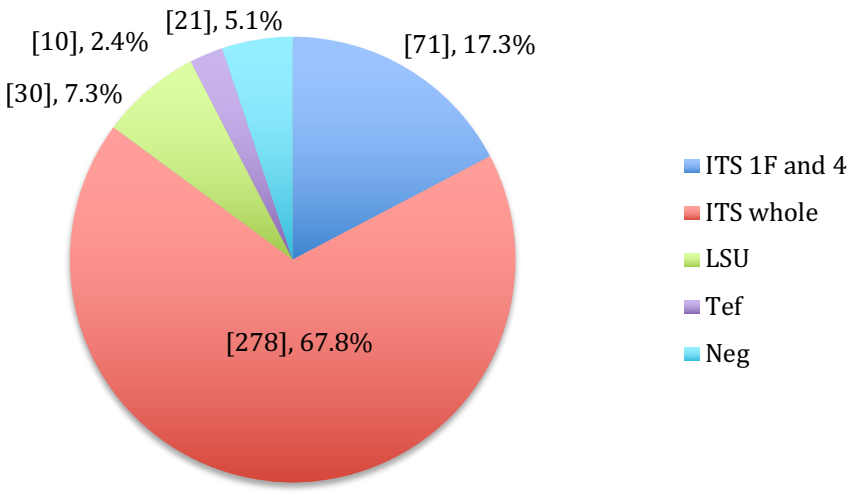


Figure 3.1 Pie chart showing the number and percentages of isolates identified using each barcoding locus. Neg=negative results (percentages for those samples which did not sequence). Numbers in parentheses indicate number of isolates identified using that locus.

Edited sequence traces were subject to BLAST searches for similarity matches (percent similarity) in NCBI GenBank for identification. We obtained 119 different OTUs out of 410 root and leaf endophyte sequences. The frequency of each of the

OTUs at each of the sites are given in Table 3.16. The detailed results for frequencies of each OTUs are shown in Appendix II (Table 1).

Table 3.16 Frequencies for each of the OTUs at each of the study sites. Roosky and Glasnevin are leaf endophytes; Kinsealy are root endophytes

No.	OTU	Roosky, leaf	Glasnevin, leaf	Kinsealy, root
		Frequencies of samples		
1.	*Acremonium alternatum*	-	-	1
2.	*Acremonium* sp.	2	2	-
3.	*Alternaria humuli*	1	-	-
4.	*Alternaria* sp.	1	-	-
5.	*Aspergillus versicolor*	-	1	-
6.	*Aureobasidium pullulans*	1	1	-
7.	*Aureobasidium* sp.	1	1	-
8.	*Bjerkandera adusta*	1	-	-
9.	*Boeremia exigua*	48	-	-
10.	*Boeremia exigua* var. exigua	7	2	-
11.	*Boeremia hedericola*	5	-	-
12.	*Boeremia* sp.	1	-	-
13.	*Boeremia strasseri*	2	-	-
14.	*Boeremia trachelospermi*	6	-	-
15.	*Botrytis cinerea*	1	-	-
16.	*Cadophora meredithiae*	-	-	1
17.	*Cadophora* sp.	1	-	5
18.	*Cladosporium allicinum*	1	-	-
19.	*Cladosporium cf. herbarum*	1	-	-
20.	*Cladosporium cladosporioides*	1	-	1
21.	*Cladosporium floccosum*	-	1	-
22.	*Cladosporium herbarum*	1	-	-
23.	*Cladosporium kenpeggii*	-	1	-
24.	*Cladosporium perangustum*	-	1	-
25.	*Cladosporium* sp.	6	6	-
26.	*Cladosporium westerdijkieae*	-	-	1
27.	*Collembolispora aristata*	-	1	-
28.	*Colletotrichum godetiae*	-	1	-
29.	*Coprinopsis* sp.	1	-	-
30.	*Cordyceps confragosa*	-	-	1
31.	*Cordyceps crassispora*	-	-	2
32.	*Cystobasidium slooffiae*	-	1	-
33.	*Dactylonectria alcacerensis*	-	-	1
34.	*Dactylonectria hordeicola*	-	-	6
35.	*Dactylonectria macrodidyma*	-	-	20

36.	*Dactylonectria torresensis*	-	-	2
37.	*Diaporthe cotoneastri*	-	2	-
38.	*Diaporthe eres*	1	1	-
39.	*Diaporthe passiflorae*	5	-	-
40.	*Diaporthe rudis*	1	2	-
41.	*Diaporthe* sp.	2	-	-
42.	*Diaporthe viticola*	2	2	-
43.	*Dictyochaeta siamensis*	1	-	-
44.	*Engyodontium album*	4	2	-
45.	*Epichloe typhina*	-	-	1
46.	*Epicoccum cf. nigrum*	1	-	-
47.	*Epicoccum nigrum*	5	-	2
48.	*Epicoccum* sp.	4	-	-
49.	*Eutypa spinosa*	-	1	-
50.	*Exophiala oligosperma*	-	2	-
51.	*Fimetariella rabenhorstii*	1	-	-
52.	*Fusarium avenaceum*	1	1	-
53.	*Fusarium culmorum*	-	1	7
54.	*Fusarium lateritium*	12	8	-
55.	*Fusarium oxysporum*	1	1	5
56.	*Fusarium oxysporum* f. sp. radicislycopersici	-	1	-
57.	*Fusarium proliferatum*	1	2	-
58.	*Fusarium* sp.	2	1	-
59.	*Fusarium tricinctum*	1	-	-
60.	*Gibberella* sp.	1	-	-
61.	*Gloniopsis calami*	-	1	-
62.	*Harzia velata*	-	-	2
63.	*Hydropisphaera* sp.	-	-	1
64.	*Ilyonectria destructans*	-	-	1
65.	*Ilyonectria radicicola*	-	-	6
66.	*Ilyonectria robusta*	-	-	2
67.	*Ilyonectria* sp.	-	-	5
68.	*Juxtiphoma eupyrena*	-	-	2
69.	*Kalmusia* sp.	1	-	-
70.	*Lecanicillium attenuatum*	-	1	-
71.	*Lecanicillium lecanii*	-	-	1
72.	*Lecanicillium muscarium*	-	1	-
73.	*Lecanicillium* sp.	-	-	1
74.	*Leptosphaerulina australis*	1	-	-
75.	*Leptosphaerulina trifolii*	1	-	-
76.	*Limonomyces roseipellis*	-	1	-
77.	*Limonomyces* sp.	-	2	-
78.	*Meyerozyma guilliermondii*	-	2	-
79.	*Mollisia* sp.	1	-	-

80.	*Mycochaetophora sp.*	-	-	1
81.	*Mycosphaerella coacervata*	28	3	-
82.	*Mycosphaerella sp.*	3	-	-
83.	*Naganishia diffluens*	4	-	-
84.	*Nectria* sp.	-	-	1
85.	*Neoceratosperma eucalypti*	1	-	-
86.	*Neonectria candida*	-	-	1
87.	*Neonectria punicea*	-	-	2
88.	*Neonectria radicicola*	-	-	1
89.	*Neonectria* sp.	-	-	9
90.	*Ophiosphaerella korrae*	2	-	-
91.	*Paraconiothyrium* sp.	1	-	-
92.	*Penicillium expansum*	-	1	-
93.	*Penicillium griseoroseum*	1	-	-
94.	*Penicillium* sp.	1	-	-
95.	*Penicillium spathulatum*	-	-	1
96.	*Phaeosphaeria gahniae*	2	-	-
97.	*Phaeosphaeria pontiformis*	1	-	-
98.	*Phlebia rufa*	1	-	-
99.	*Phlyctema vagabunda*	-	1	-
100.	*Phoma exigua*	1	-	-
101.	*Phoma exigua* var. exigua	2	-	-
102.	*Phoma multirostrata*	2	-	-
103.	*Phoma* sp.	12	2	-
104.	*Phomopsis* sp.	-	2	-
105.	*Psiloglonium* sp.	-	-	4
106.	*Pyronema domesticum*	1	1	-
107.	*Sarocladium strictum*	2	-	-
108.	*Septoria convolvuli*	1	-	-
109.	*Septoria cucubali*	5	-	-
110.	*Septoria lepidiicola*	-	1	-
111.	*Septoria protearum*	-	1	-
112.	*Sistotrema brinkmannii*	1	-	-
113.	*Trichoderma viride*	-	-	3
114.	*Ustilaginoidea virens*	1	-	-
115.	*Ustilago filiformis*	-	2	-
116.	*Vagicola dactylidis*	1	-	-
117.	*Vishniacozyma heimaeyensis*	1	-	-
118.	*Vuilleminia coryli*	-	1	-
119.	*Xylaria* sp.	5	-	-
Total no. of isolates, total no. OTUs		**219, 66**	**69, 42**	**100, 33**

The most abundant leaf OTUs were *Boeremia exigua*, *Cladosporium* sp., *Diaporthe* spp., *Epicoccum nigrum*, *Fusarium* sp., *Mycosphaerella* sp. and *Phoma* spp.. Some OTUs were only recorded in root tissues (*Ilyonectria* sp., *Nectria* sp. and *Neonectria* sp.).

Boeremia exigua (including *B. exigua* var. *exigua*) and *Mycosphaerella coacervata* were very frequently isolated from Roosky material (55 and 31 of the isolates respectively). Moderate frequencies were recorded for *Cladosporium* sp., *Dactylonectria macrodidyma*, *Fusarium laterium* and *Phoma* sp..

We isolated 374 isolates belonging to the Ascomycota and 16 to the Basidiomycota. We found a total of 37 families (Table 3.17) belonging to 23 taxonomic orders (Appendix II, Figure 1) within which Didymellaceae and Nectriaceae had highest proportion of isolates; 104 and 89 isolates respectively. Isolates belonging to Cladosporiaceae, Diaportheceae and Mycosphaerellaceae were also moderately common; 21, 20 and 45 respectively. Only one isolate was found in each of the following families; Bionectriaceae, Bulleribasidiaceae, Chaetosphaeriaceae, Clavicipitaceae, Cystobasidiaceae, Diatrypaceae, Glomerellaceae, Hydnaceae, Lasiosphaeriaceae, Psathyrellaceae and Sclerotiniaceae.

The samples belonged to 10 different taxonomic classes (Table 3.17) among which the most abundant classes were the Dothideomycetes and Sordariomycetes having 195 and 162 samples respectively. The number of isolates in other classes ranged from 1 to 7. A total of 6 isolates belonged to Leotiomycetes. The majority of the isolated endophytes belong to the subdivision Pezizomycotina (372), a moderate number to Agaricomycotina (13) and very few to Pucciniomycotina (1), Saccharomycotina (2) and Ustilaginomycotina (2).

The total number of isolates and OTUs obtained within families for all cultured leaf and root endophytes is also shown in Table 3.17 Didymellaceae, Mycosphaerellaceae and Nectriaceae are particularly common.

Table 3.17 Total number of isolates and OTUs within each class and family from all leaf and root endophytes

Class	Isolates	OTUs	Family	Isolates	OTUs
Agaricomycetes	8	7	Aspergillaceae	5	5

Dothideomycetes	195	45	Bionectriaceae	1	1
Sordariomycetes	162	50	Bulleribasidiaceae	1	1
Eurotiomycetes	7	6	Ceratostomataceae	2	1
Cystobasidiomycetes	1	1	Chaetosphaeriaceae	1	1
Tremellomycetes	5	2	Cladosporiaceae	21	9
Leotiomycetes	6	6	Clavicipitaceae	1	1
Pezizomycetes	2	1	Cordycipitaceae	13	7
Saccharomycetes	2	1	Corticiaceae	4	3
Ustilaginomycetes	2	1	Cystobasidiaceae	1	1
-	-	-	Debaryomycetaceae	2	1
-	-	-	Dermateaceae	2	2
-	-	-	Diaporthaceae	20	7
-	-	-	Diatrypaceae	1	1
-	-	-	Didymellaceae	104	14
-	-	-	Didymosphaeriaceae	2	2
-	-	-	Dothioraceae	4	2
-	-	-	Filobasidiaceae	4	1
-	-	-	Glomerellaceae	1	1
-	-	-	Helotiales	9	4
-	-	-	Herpotrichiellaceae	2	1
-	-	-	Hydnaceae	1	1
-	-	-	Hypocreaceae	3	1
-	-	-	Hypocreales	20	7
-	-	-	Hysteriaceae	5	2
-	-	-	Lasiosphaeriaceae	1	1
-	-	-	Meruliaceae	2	2
-	-	-	Mycosphaerellaceae	45	7
-	-	-	Nectriaceae	89	18
-	-	-	Phaeosphaeriaceae	6	4
-	-	-	Pleosporaceae	4	4
-	-	-	Psathyrellaceae	1	1
-	-	-	Pyronemataceae	2	1
-	-	-	Sarocladiaceae	2	1
-	-	-	Sclerotiniaceae	1	1
-	-	-	Ustilaginaceae	2	1
-	-	-	Xylariaceae	5	1

A comparison was made of leaf endophytic taxa obtained from *Fraxinus excelsior* from two sites (Roosky and Glasnevin) with other taxa of *Fraxinus* (*F. americana, F. angustifolia, F. dipetala*, *F. glabra*, *F. mandshurica*, *F. numidica, F. ornus, F. pennsylvanica, F. potamophila, F. pubinervis, F. texensis*, *F. xanthoxyloides*) from

Glasnevin (Table 3.18). The total number of tissues sampled for *Fraxinus excelsior* was 229 and for other *Fraxinus* species was 54.

A total of 69 leaf endophyte taxa was obtained from *Fraxinus excelsior* and a total of 36 leaf endophyte taxa for other *Fraxinus* taxa. Sixteen taxa were shared between the *F. excelsior* and the other *Fraxinus* taxa group (*Acremonium* sp., *Aureobasidium pullulans, Aureobasidium* sp., *Boeremia exigua* var. exigua, *Cladosporium* sp., *Diaporthe rudis*, *Diaporthe viticola*, *Engyodontium album, Fusarium avenaceum, Fusarium lateritium, Fusarium oxysporum, Fusarium proliferatum, Fusarium* sp*., Gloniopsis calami*, *Mycosphaerella coacervata* and *Phoma* sp.). The total number of tissues sampled from *F. excelsior* was 229 and the total sampled from other *Fraxinus* taxa was 54. Thus, from each tissue of *F. excelsior* we obtained 3.27 taxa and each tissue from other *Fraxinus* taxa we obtained 1.5 taxa. Some taxa were only isolated from *Fraxinus excelsior* (53 taxa) and some only from the other *Fraxinus* species (20 taxa) (highlighted in bold in Table 3.18).

Table 3.18 A comparison of taxa obtained from *Fraxinus excelsior* and other taxa of *Fraxinus*. Samples only found in *F. excelsior*, or only in the other *Fraxinus* species are highlighted in bold. Shared taxa are labelled with *. 229 tissues were sampled for *F. excelsior* and 54 tissues sampled for other *Fraxinus* taxa

No.	OTU from *Fraxinus excelsior*	OTU from other *Fraxinus* taxa
1.	*Acremonium* sp. *	*Acremonium* sp.*
2.	***Alternaria humuli***	*Aureobasidium pullulans**
3.	***Alternaria* sp.**	*Aureobasidium* sp.*
4.	***Aspergillus versicolor***	*Boeremia exigua* var. exigua *
5.	*Aureobasidium pullulans**	***Cladosporium allicinum***
6.	*Aureobasidium* sp.*	***Cladosporium floccosum***
7.	***Bjerkandera adusta***	***Cladosporium perangustum***
8.	***Boeremia exigua***	*Cladosporium* sp. *
9.	*Boeremia exigua* var. exigua *	***Collembolispora aristata***
10.	***Boeremia hedericola***	***Colletotrichum godetiae***
11.	***Boeremia* sp.**	***Cystobasidium slooffiae***
12.	***Boeremia strasseri***	***Diaporthe cotoneastri***
13.	***Boeremia trachelospermi***	*Diaporthe rudis* *
14.	***Botrytis cinerea***	*Diaporthe viticola* *
15.	***Cadophora* sp.**	*Engyodontium album* *
16.	***Cladosporium cf. herbarum***	***Exophiala oligosperma***
17.	***Cladosporium cladosporioides***	*Fusarium avenaceum* *
18.	***Cladosporium herbarum***	***Fusarium culmorum***

19.	***Cladosporium kenpeggii***	*Fusarium lateritium* *
20.	*Cladosporium* sp. *	*Fusarium oxysporum* *
21.	***Coprinopsis* sp.**	***Fusarium oxysporum* f. sp. radicislycopersici**
22.	***Diaporthe eres***	*Fusarium proliferatum* *
23.	***Diaporthe passiflorae***	*Fusarium* sp. *
24.	*Diaporthe rudis* *	*Gloniopsis calami* *
25.	***Diaporthe* sp.**	***Lecanicillium attenuatum***
26.	*Diaporthe viticola* *	***Lecanicillium muscarium***
27.	***Dictyochaeta siamensis***	***Limonomyces* sp.**
28.	*Engyodontium album* *	***Meyerozyma guilliermondii***
29.	***Epicoccum cf. nigrum***	*Mycosphaerella coacervata* *
30.	***Epicoccum nigrum***	***Penicillium expansum***
31.	***Epicoccum* sp.**	*Phoma* sp. *
32.	***Eutypa spinosa***	***Phomopsis* sp.**
33.	***Fimetariella rabenhorstii***	***Septoria lepidiicola***
34.	*Fusarium avenaceum* *	***Septoria protearum***
35.	*Fusarium lateritium* *	***Ustilago filiformis***
36.	*Fusarium oxysporum* *	***Vuilleminia coryli***
37.	*Fusarium proliferatum* *	-
38.	*Fusarium* sp. *	-
39.	***Fusarium tricinctum***	-
40.	***Gibberella* sp.**	-
41.	*Gloniopsis calami* *	-
42.	***Kalmusia* sp.**	-
43.	***Leptosphaerulina australis***	-
44.	***Leptosphaerulina trifolii***	-
45.	***Mollisia* sp.**	-
46.	*Mycosphaerella coacervata**	-
47.	***Mycosphaerella* sp.**	-
48.	***Naganishia diffluens***	-
49.	***Neoceratosperma eucalypti***	-
50.	***Ophiosphaerella korrae***	-
51.	***Paraconiothyrium* sp.**	-
52.	***Penicillium griseoroseum***	-
53.	***Penicillium* sp.**	-
54.	***Phaeosphaeria gahniae***	-
55.	***Phaeosphaeria pontiformis***	-
56.	***Phlebia rufa***	-
57.	***Phoma exigua***	-
58.	***Phoma exigua* var. exigua**	-
59.	***Phoma multirostrata***	-
60.	*Phoma* sp. *	-
61.	***Pyronema domesticum***	-
62.	***Sarocladium strictum***	-

63.	***Septoria convolvuli***	-
64.	***Septoria cucubali***	-
65.	***Sistotrema brinkmannii***	-
66.	***Ustilaginoidea virens***	-
67.	***Vagicola dactylidis***	-
68.	***Vishniacozyma heimaeyensis***	-
69.	***Xylaria* sp.**	-

*Denotes common taxa obtained from both the cases.

We collected leaf tissues from three trees in some of the Roosky plots (A1, D1, T1), from two trees (E1, F1, G, G1, H1, R1 and S1) in some other plots and a single tree in the remaining plots. Appendix II (Table 2) shows the summary data for the replicates across all plots (provenances) combined. Very few of the species were isolated in all tree replicates from the same provenance.

The frequency of OTUs retrieved from each type of healthy tissues are shown in the heat-map Figure 3.2. *Boeremia* sp., *Diaporthe* sp., *Fusarium* sp., *Mycosphaerella* sp. *and Phoma* sp. were frequently isolated from healthy rachis (H.R) material. The total number of isolates (53) and taxa (11) were also the highest for the healthy rachis material. The fewest number of isolates (2, 5) were obtained from the healthy apex (H.A) and healthy vein (H.V) material respectively. Healthy leaf tissue (H.L) also provided a high number of isolates and taxa (43, 11 respectively). *Boeremia* sp., *Cladosporium* sp., *Diaporthe* sp., *Egnyodontium* sp., *Leptosphaerilina* sp., *Mycosphaerella* sp. and *Septoria* sp. were very frequent in the H.L. Detailed numbers on frequencies for each OTU are given in Appendix II (Table 3).

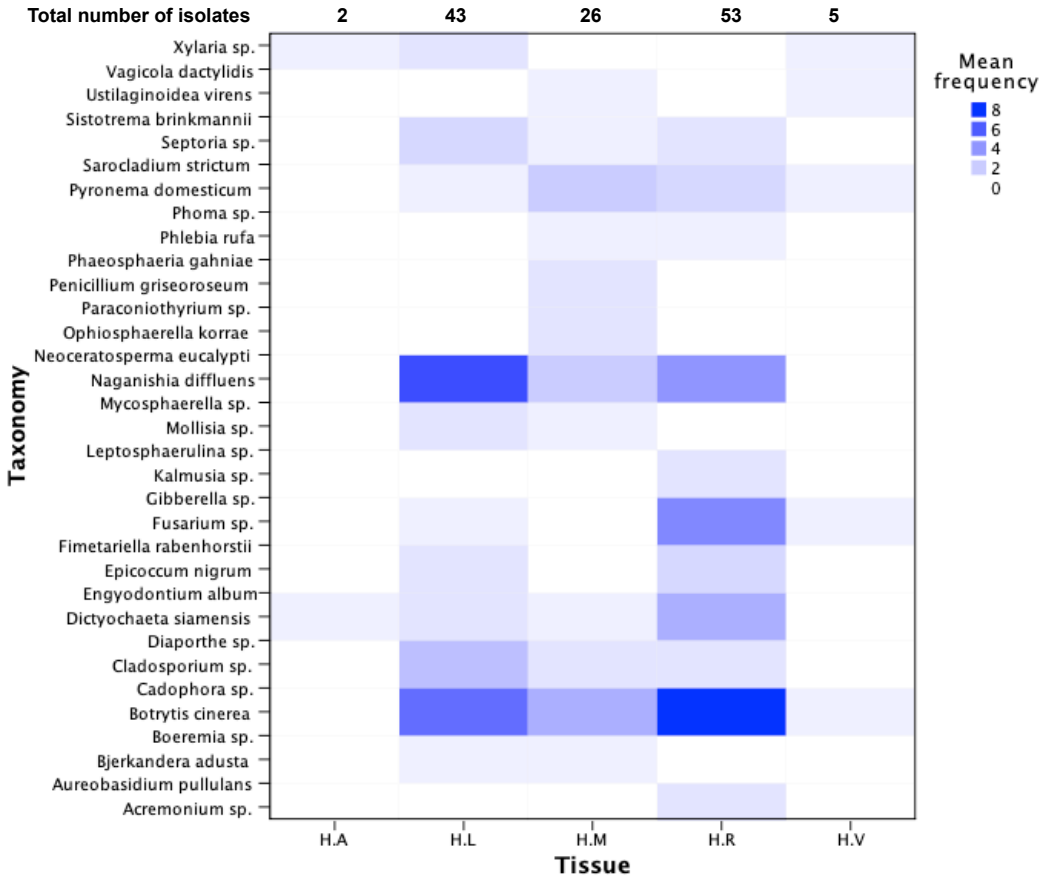


Figure 3.2 Heat-map for mean frequency of OTUs in different healthy ash leaf tissues sampled from Roosky. H.L = 4 cm above from the leaf base on the sides of lamina, H.M = middle lamina with midrib portion, H.R = healthy rachis, H.A = healthy apex, H.V = healthy veins (mostly the same as H.L only in 10 cm below apex).

The highest number of isolates and OTUs from diseased tissues (Figure 3.3) was obtained from leaf tissue (D.L) (34 isolates, 16 OTUs respectively) and midrib (D.M) material (33 isolates, 18 OTUs) with *Boeremia* sp. *Fusarium* sp., *Phaeosphaeria* sp., *Mycosphaerella* sp., *Phoma* sp., and *Septoria cucubali* being particularly common.

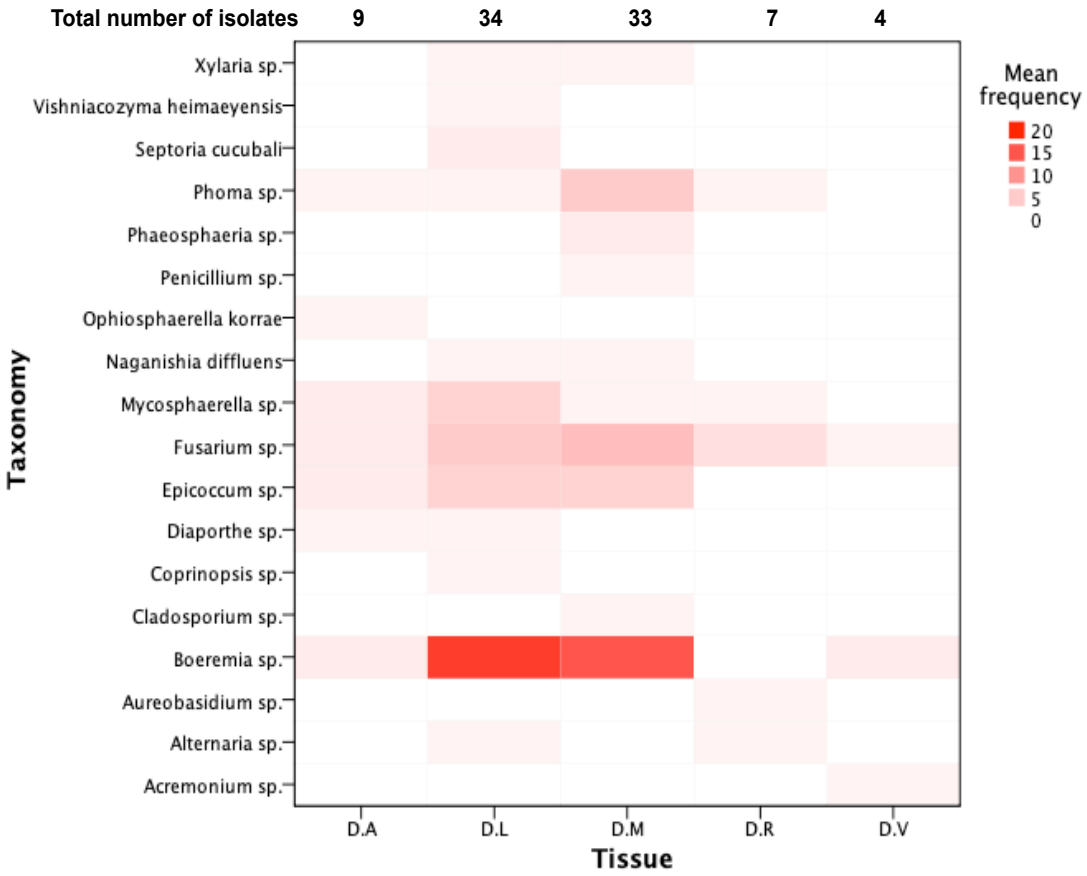


Figure 3.3 Heat-map for frequency of OTUs in different diseased ash leaf tissue sampled from Roosky. D.L = diseased leaf tissue position not fixed, D.R = diseased rachis, D.M = diseased midrib with laminar tissue, D.A = diseased apex, D.V = diseased veins.

The total number of different taxa obtained from all diseased tissues was 18 compared to 33 for healthy leaf tissue. The highest number of taxa (18) was obtained from the diseased midrib (D.M) material and the minimum number (3) obtained from diseased vein (D.V). Detailed results are found in Appendix II, Table 4 and Figure 6.

However, when the data are corrected for the number of samples, so that the results are expressed as number of OTUs obtained per tissue sample, we can see that the results are more even and that approximately one to two OTUs were obtained per sample. The diseased lamina (D.L) and healthy rachis (H.R) returned the most isolates per sample at 2.13 and 2.12 respectively (Figure 3.4).

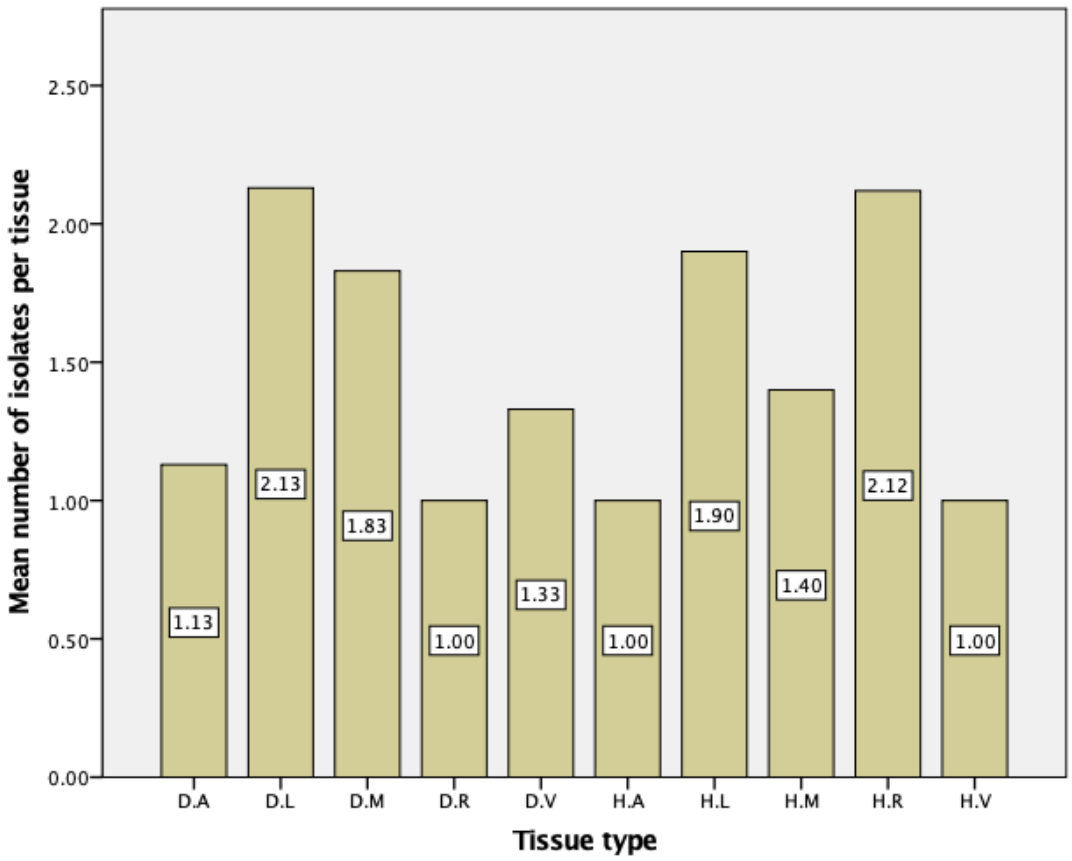


Figure 3.4 Histogram showing the number of OTUs obtained per tissue sample for all tissue types (diseased and healthy).

Endophyte isolations from leaf tissues, sampled from Roosky, were performed on two different media types: 1) malt extract agar with vegitone supplement (MEA) and 2) MEA media digested with *Fraxinus excelsior* leaves (MEA+F) prior to autoclaving. Comparisons of OTUs obtained from the two media are shown in Table 3.19.

Table 3.19 OTU frequency isolated from two media. Taxa are separated among those found on both media (MEA and MEA+F; top part of table) and those only found on one media type (bottom part of table)

	Media	
	MEA	MEA+F
OTU	Count	
Taxa found on both media		
Boeremia exigua	28	27
Boeremia hedericola	1	4

Boeremia strasseri	1	1
Boeremia trachelospermi	3	3
Cladosporium cf. herbarum	1	1
Cladosporium sp.	1	5
Diaporthe passiflorae	4	1
Engyodontium album	1	3
Epicoccum nigrum	1	5
Epicoccum sp.	3	1
Fusarium lateritium	9	3
Mycosphaerella coacervata	16	13
Mycosphaerella sp.	1	2
Naganishia diffluens	3	1
Phoma exigua	1	2
Phoma sp.	5	7
Sarocladium strictum	1	1
Septoria cucubali	3	2
Xylaria sp.	2	3
Taxa found on only one medium		
Acremonium sp.	2	0
Alternaria humuli	1	0
Alternaria sp.	1	0
Aureobasidium pullulans	0	1
Aureobasidium sp.	0	1
Bjerkandera adusta	1	0
Boeremia sp.	0	1
Botrytis cinerea	1	0
Cadophora sp.	0	1
Cladosporium cladosporioides	1	0
Coprinopsis sp.	0	1
Diaporthe eres	1	0
Diaporthe rudis	1	0
Diaporthe sp.	0	2
Diaporthe viticola	0	2
Dictyochaeta siamensis	1	0
Fimetariella rabenhorstii	0	1
Fusarium avenaceum	1	0
Fusarium oxysporum	1	0
Fusarium proliferatum	1	0
Fusarium sp.	2	0
Fusarium tricinctum	1	0
Gibberella sp.	0	1
Kalmusia sp.	1	0
Leptosphaerulina australis	1	0
Leptosphaerulina trifolii	1	0
Mollisia sp.	1	0

Neoceratosperma eucalypti	0	1
Ophiosphaerella korrae	2	0
Paraconiothyrium sp.	1	0
Penicillium griseoroseum	0	1
Penicillium sp.	1	0
Phaeosphaeria gahniae	2	0
Phaeosphaeria pontiformis	1	0
Phlebia rufa	0	1
Phoma multirostrata	0	2
Pyronema domesticum	0	1
Septoria convolvuli	0	1
Sistotrema brinkmannii	0	1
Ustilaginoidea virens	1	0
Vagicola dactylidis	1	0
Vishniacozyma heimaeyensis	1	0
Total	**115**	**104**

The total number of tissues sampled for MEA and MEA+F media were 118 and 114, respectively. Altogether 115 isolates were obtained from MEA and 104 from MEA+F media, which equates to 0.98 and 0.91 isolates per tissue, respectively. We isolated 45 different taxa on MEA media and 35 different taxa on MEA+F media; which equates to 0.38 and 0.31 taxa isolated per sample. 26 taxa were only isolated on MEA and 16 only on MEA+F.

Leaf samples were taken from the provenance trials of *Fraxinus excelsior* at Roosky, Co. Rosscommon, that included material collected from 11 countries: Belgium (BE), Czech Republic (CZ), Denmark (DK), Germany (DE), France (FR), Ireland (IE), Italy (IT), Lithuania (LT), Netherlands (NL), UK (GB) and Poland (PO). The OTU frequency from sampled leaf tissue for each country is shown in Table 3.20. Total numbers of isolates obtained per provenance ranged from 13 (BE) to 27 (FR) and the total number of taxa per provenance ranged from 8 (NL) to 20 (IE). The number of taxa isolated per sample ranged from 0.5 (CZ, FR, IT and NL) to 0.8 (IE, LT and PO). A detailed comparison between each provenance is presented in (Table 5.1, Appendix II. Total frequency counts for each provenance for all countries is shown in (Table 5.2) Appendix II.

Table 3.20 OTU frequencies obtained from all sampled leaf tissues from all provenances

	Country										
OTU	BE	CZ	DK	FR	DE	IE	IT	LT	NL	PO	GB

	Count										
Acremonium sp.	-	**1**	-	-	**1**	-	-	-	-	-	-
Alternaria humuli	-	-	-	-	-	-	-	-	-	**1**	-
Alternaria sp.	-	**1**	-	-	-	-	-	-	-	-	-
Aureobasidium pullulans	-	-	-	-	-	-	**1**	-	-	-	-
Aureobasidium sp.	-	-	**1**	-	-	-	-	-	-	-	-
Bjerkandera adusta	-	-	-	-	-	-	-	-	-	-	**1**
Boeremia exigua	-	**7**	**6**	**5**	**5**	**4**	**7**	**3**	**6**	**2**	**3**
Boeremia exigua var. exigua	**1**	**1**	**1**	**1**	**1**	**1**	-	-	-	**1**	-
Boeremia hedericola	-	**2**	-	-	**1**	-	**1**	-	-	**1**	-
Boeremia sp.	-	-	-	-	-	-	-	**1**	-	-	-
Boeremia strasseri	-	-	-	**1**	-	-	-	-	-	-	**1**
Boeremia trachelospermi	-	-	-	**3**	**1**	-	**1**	-	-	**1**	-
Botrytis cinerea	-	-	-	-	-	-	-	**1**	-	-	-
Cadophora sp.	-	-	-	-	-	**1**	-	-	-	-	-
Cladosporium cf. herbarum	-	-	**1**	-	-	-	-	-	-	-	-
Cladosporium cladosporioides	-	-	-	-	-	**1**	-	-	-	-	-
Cladosporium herbarum	-	-	-	-	-	-	-	-	-	-	**1**
Cladosporium sp.	-	-	**1**	-	-	**1**	-	**1**	**1**	-	**2**
Coprinopsis sp.	-	-	-	-	-	-	-	**1**	-	-	-
Diaporthe eres	-	-	-	-	-	-	-	-	**1**	-	-
Diaporthe passiflorae	-	-	**1**	-	**1**	**1**	-	**1**	-	**1**	-
Diaporthe rudis	-	-	-	-	**1**	-	-	-	-	-	-
Diaporthe sp.	-	-	-	-	-	**1**	-	-	-	-	**1**
Diaporthe viticola	-	-	-	-	-	**1**	**1**	-	-	-	-
Dictyochaeta siamensis	-	-	-	-	-	-	-	-	**1**	-	-
Engyodontium album	-	-	-	**2**	-	**1**	-	**1**	-	-	-
Epicoccum cf. nigrum	-	-	-	-	-	-	-	-	-	-	**1**
Epicoccum nigrum	**1**	-	-	-	-	**1**	**1**	**1**	-	-	**1**
Epicoccum sp.	-	-	-	-	-	**1**	**1**	**1**	-	-	**1**

Fimetariella rabenhorstii	-	-	-	-	-	-	-	-	**1**	-	-
Fusarium avenaceum	-	-	-	-	-	**1**	-	-	-	-	-
Fusarium lateritium	-	-	**1**	**1**	**4**	**1**	**1**	-	**2**	**1**	**1**
Fusarium oxysporum	-	-	-	-	-	-	-	-	-	-	**1**
Fusarium proliferatum	-	-	-	-	-	-	-	-	-	**1**	-
Fusarium sp.	-	-	**1**	**1**	-	-	-	-	-	-	-
Fusarium tricinctum	-	-	-	-	-	-	**1**	-	-	-	-
Gibberella sp.	-	-	-	-	-	-	-	-	-	**1**	-
Kalmusia sp.	-	-	-	**1**	-	-	-	-	-	-	-
Leptosphaerulina australis	-	-	**1**	-	-	-	-	-	-	-	-
Leptosphaerulina trifolii	-	-	-	**1**	-	-	-	-	-	-	-
Mollisia sp.	-	-	-	-	-	-	-	-	-	**1**	-
Mycosphaerella coacervata	**2**	**2**	**2**	**7**	-	**3**	**5**	**3**	**1**	**1**	**3**
Mycosphaerella sp.	**1**	-	-	-	**1**	-	-	-	-	-	**1**
Naganishia diffluens	**2**	-	-	**1**	-	-	-	-	-	-	**1**
Neoceratosperma eucalypti	-	**1**	-	-	-	-	-	-	-	-	-
Ophiosphaerella korrae	**2**	-	-	-	-	-	-	-	-	-	-
Paraconiothyrium sp.	-	-	-	**1**	-	-	-	-	-	-	-
Penicillium griseoroseum	-	-	-	-	-	-	-	-	-	**1**	-
Penicillium sp.	-	**1**	-	-	-	-	-	-	-	-	-
Phaeosphaeria gahniae	-	-	**1**	-	-	**1**	-	-	-	-	-
Phaeosphaeria pontiformis	-	-	-	-	**1**	-	-	-	-	-	-
Phlebia rufa	**1**	-	-	-	-	-	-	-	-	-	-
Phoma exigua	-	-	**1**	-	-	-	-	-	-	-	-
Phoma exigua var. exigua	-	-	**1**	-	-	-	**1**	-	-	-	-
Phoma multirostrata	-	-	**1**	-	**1**	-	-	-	-	-	-
Phoma sp.	**2**	**2**	**3**	-	**1**	**2**	-	-	**1**	-	**1**

Pyronema domesticum	-	-	-	-	-	**1**	-	-	-	-	-
Sarocladium strictum	-	-	-	-	-	-	**1**	-	-	**1**	-
Septoria convolvuli	-	-	-	-	-	-	-	**1**	-	-	-
Septoria cucubali	**1**	-	-	**1**	-	**1**	-	**1**	-	**1**	-
Sistotrema brinkmannii	-	-	-	-	-	**1**	-	-	-	-	-
Ustilaginoidea virens	-	-	-	-	-	-	-	**1**	-	-	-
Vagicola dactylidis	-	-	-	-	-	-	-	-	-	-	**1**
Vishniacozyma heimaeyensis	-	-	-	-	-	-	**1**	-	-	-	-
Xylaria sp.	-	-	-	**1**	**2**	**1**	-	-	-	-	1
Total no. of isolates	**13**	**18**	**23**	**27**	**21**	**26**	**23**	**17**	**14**	**15**	**22**
Total no. of taxa	**9**	**9**	**15**	**14**	**13**	**20**	**13**	**13**	**8**	**14**	**17**
No. of taxa per sample	**0.7**	**0.5**	**0.7**	**0.5**	**0.6**	**0.8**	**0.5**	**0.8**	**0.5**	**0.8**	**0.7**

BE-Belgium, CZ-Czech Republic, DK-Denmark, FR-France, DE-Germany, IE-Ireland, IT-Italy, LT-Lithuania, NL-Netherlands, PO-Poland, GB-UK.

Isolates were also obtained from leaf tissue sampled from different *Fraxinus* taxa at the NBG Glasnevin. The frequencies of OTUs obtained from healthy tissues are shown in the heat-map Figure 3.5. The highest number of isolates were obtained from healthy rachis (H.R) material (20 isolates; and 15 taxa) and the least from healthy middle lamina (6 isolates; 6 taxa) with midrib portion (H.M). *Cladosporium* sp., *Diaporthe* sp., and *Fusarium* sp. were particularly common in all tissue types. *Aspergillus versicolor*, *Aureobasidium pullulans*, *Aureobasidium* sp., *Boeremia exigua var. exigua*, three different species of *Cladosporium* sp., *Collembolispora aristata, Colletotrichum godetiae, Cystobasidium slooffiae, Diaporthe eres, Engyodontium album, Exophiala oligosperma,* three different species of *Fusarium* sp*.,* two different species of *Lecanicillium* sp*., Limonomyces roseipellis, Limonomyces* sp., *Phlyctema vagabunda,* two different species of *Septoria* sp*.* and *Vuilleminia coryli* were isolated from only one tissue type. Detailed information on taxa and respective frequencies are presented in Table 6 and Figure 8 in Appendix II.

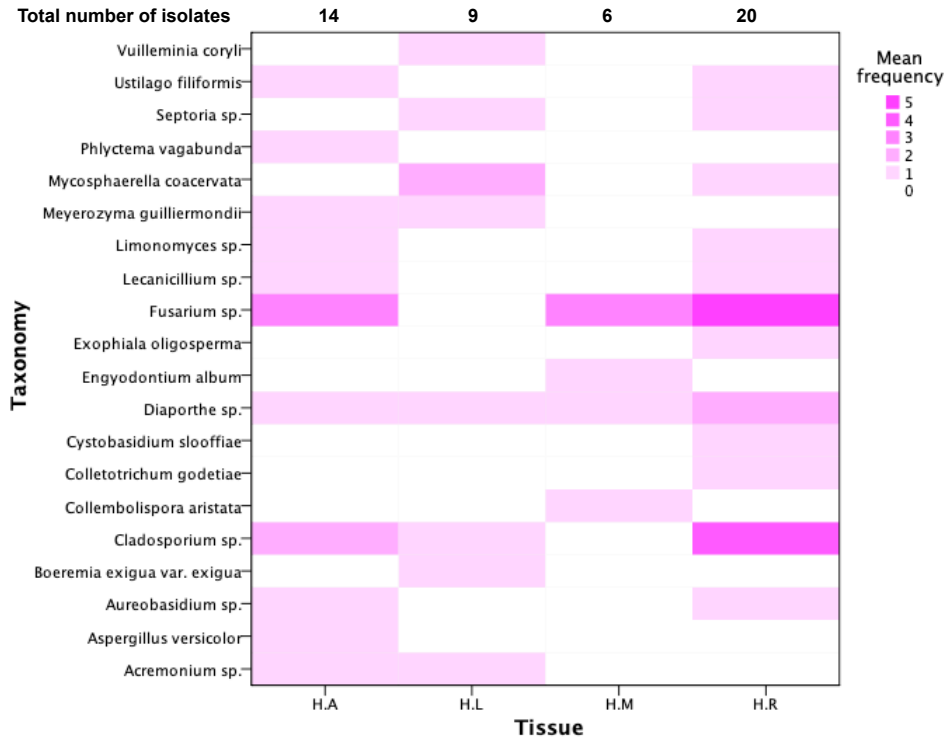


Figure 3.5 Heat-map for mean frequency of OTUs in a combined dataset of different tissues of healthy *Fraxinus* taxa from the National Botanic Gardens, Glasnevin. H.L = 4 cm above from the leaf base on the sides of lamina, H.M = middle lamina with midrib portion, H.R = healthy rachis and H.A = healthy apex.

Only six tissues were sampled for diseased leaves from the NBG Glasnevin because of availability of diseased material. Two isolates were cultured from these for diseased leaf (D.L) and three isolates cultured for diseased midrib (D.M). A total of 6 OTUs were obtained: *Gloniopsis calami*, *Phoma* sp. *Fusarium lateritium*, *Cladosporium* sp., *Fusarium oxysporum* f. sp. radicislycopersici, and *Eutypa spinosa*. *Gloniopsis calami* and *Eutypa spinosa* had not been isolated in any of our other samples.

Root endophytes obtained from Kinsealy and their frequencies per tree sampled are shown in Table 3.21.

Table 3.21 Root endophyte OTU frequencies for three trees sampled from Kinsealy. Taxa also recorded from leaf tissues are shown in bold with *.

OTU	Family	Tree

		T1	T2	T3
		Count		
Acremonium alternatum	Hypocreales	1	0	0
Cadophora meredithiae	Helotiales	0	1	0
Cadophora* sp.	Helotiales	0	5	0
Cladosporium cladosporioides*	Cladosporiaceae	0	1	0
Cladosporium westerdijkieae	Cladosporiaceae	1	0	0
Cordyceps confragosa	Cordycipitaceae	1	0	0
Cordyceps crassispora	Cordycipitaceae	2	0	0
Dactylonectria alcacerensis	Nectriaceae	0	1	0
Dactylonectria hordeicola	Nectriaceae	4	2	0
Dactylonectria macrodidyma	Nectriaceae	0	12	8
Dactylonectria torresensis	Nectriaceae	1	1	0
Epichloë typhina	Clavicipitaceae	0	1	0
Epicoccum nigrum*	Didymellaceae	0	2	0
Fusarium culmorum*	Nectriaceae	5	0	2
Fusarium oxysporum*	Nectriaceae	1	3	1
Harzia velata	Ceratostomataceae	2	0	0
Hydropisphaera sp.	Bionectriaceae	1	0	0
Ilyonectria destructans	Hypocreales	1	0	0
Ilyonectria radicicola	Hypocreales	3	3	0
Ilyonectria robusta	Hypocreales	1	0	1
Ilyonectria sp.	Hypocreales	2	2	1
Juxtiphoma eupyrena	Didymellaceae	0	1	1
Lecanicillium lecanii	Cordycipitaceae	0	1	0
Lecanicillium sp.	Cordycipitaceae	0	1	0
Mycochaetophora sp.	Helotiales	1	0	0
Nectria sp.	Nectriaceae	0	0	1
Neonectria candida	Nectriaceae	1	0	0
Neonectria punicea	Nectriaceae	2	0	0
Neonectria radicicola	Nectriaceae	0	1	0
Neonectria sp.	Nectriaceae	8	1	0
Penicillium spathulatum	Aspergillaceae	1	0	0
Psiloglonium sp.	Hysteriaceae	0	4	0
Trichoderma viride	Hypocreaceae	0	0	3
Total isolates	**100**	**39**	**43**	**18**
Total taxa (cumulative total in parentheses)	**33**	**19 (19)**	**18 (31)**	**8 (33)**

Total isolate count was highest for tree number 2 but the number of different isolate counts was more for tree number 1. A total of 19 taxa were sampled from the first tree, 31 taxa cumulatively after two trees and 33 taxa cumulatively after three trees. Thus, sampling three trees seems to have recorded most of the culturable root endophyte species richness.

Total number of tissues sampled for each tree were 39, 44 and 18. There were a few taxa also recorded from leaf tissues (*Cadophora* sp., *Cladosporium cladosporioides, Epicoccum nigrum, Fusarium culmorum, Fusarium oxysporum*; shown in bold with *) and all other genera were specific to root tissues. Detailed information of frequencies of OTUs for all three sites (Roosky, Glasnevin and Kinsealy) were shown in Appendix II (Table 7).

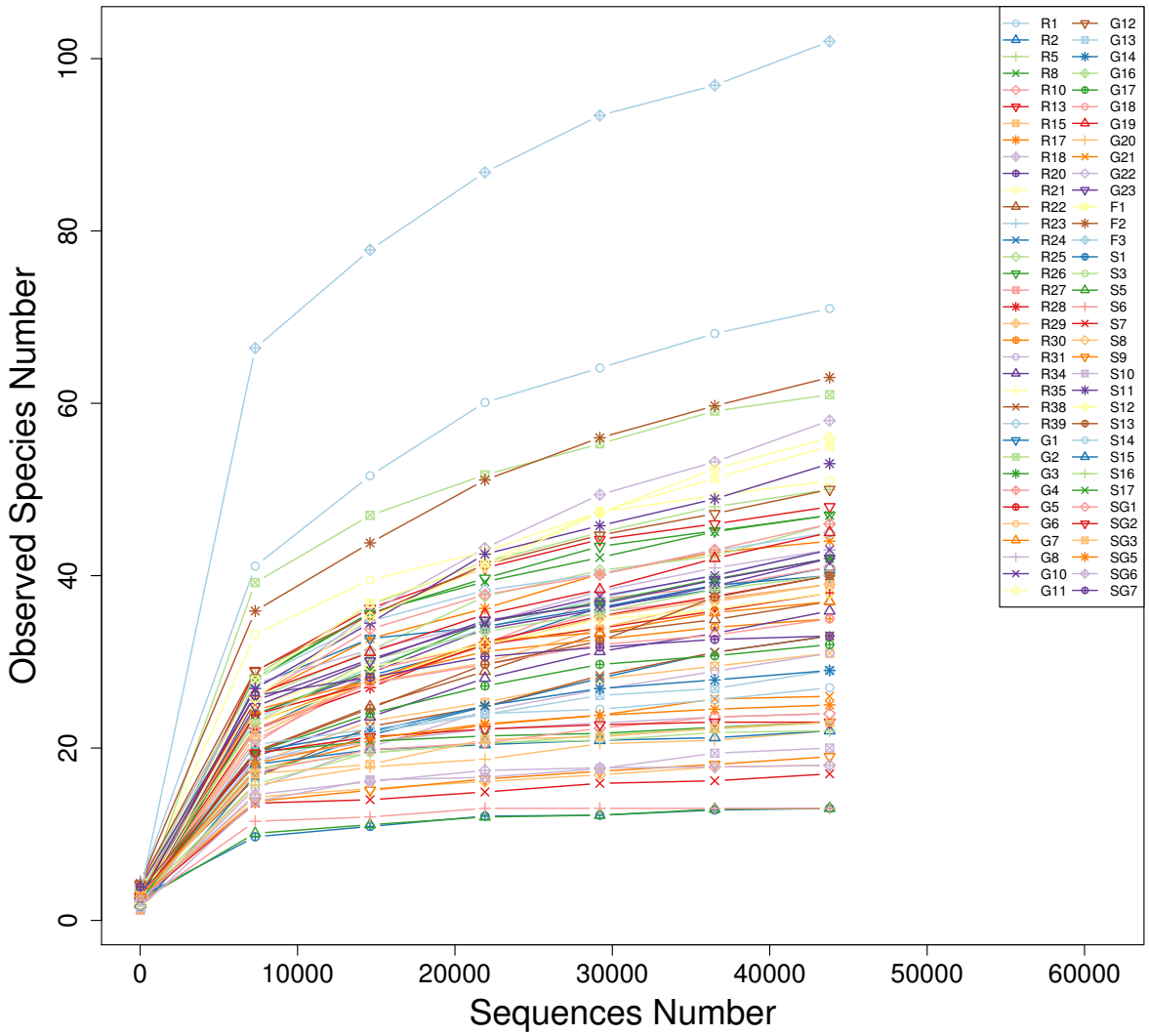
3.3.2 Community analysis of samples using direct high throughput amplicon sequencing

Samples used for high throughput amplicon sequencing (HTS) are shown in Table 3.4 (Materials and Methods). These were selected to cover a wide range of provenances, tissue types and *Fraxinus* taxa. Alpha diversity of the samples from the NGS data were represented by rarefaction curves and rank abundance curves (Figure 3.6 and 3.7), species accumulation box plots (Figure 3.8), Shannon diversity index (Figure 3.9) and Venn diagrams (Figure 3.10) OTU assigned to respective taxonomic level with total numbers obtained from seeds and leaves by HTS are shown in Table 3.22.

The rarefaction curves are created by randomly selecting a certain amount of sequencing data from the samples and then counting the number of species they represent. If the curve levels off it means that adding more sequences in the HTS will not detect many more new species. Here in our DNA samples (Figure 3.6a) most samples had started to level off as early as 7,309 reads and had levelled off reasonably well by the maximum 43,804 reads per sample. More reads would have detected some more species in most samples but our budget was limited to 50,000 raw tags (=43,804 cleaned reads). The samples from F3 (France Au BoutduLac) and R1 (Ireland, Enniskillen) showed the most OTUs and S1 seed DNA (France, St Paul De Salers) and S5 (Ireland, Enniskillen) the fewest. The species richness of F3 was strikingly higher than all other samples. The rank abundance curves (Figure 3.7a) are used to display relative species abundance in the reads. They can be used to visualise species richness and evenness. Each curve represents a single sample, plotted by OTU relative read abundance on the Y-axis and the OTU abundance rank on the X-axis. 20 species are relatively abundant in most samples but most species are rare and found only in a small number of reads.

Figure 3.6b shows the rarefaction curves and Figure 3.7b rank abundance curves for the data divided by category (France, Glasnevin, Roosky, Seed from Roosky and Seed from Glasnevin). The French sample has the highest species richness followed by Roosky and Glasnevin and the two seed groups (Roosky and Glasnevin). The two seed groups had completely levelled off by 43,804 reads and the other groups showed reasonable levelling, indicating that most species had been recorded in those samples. The rank abundance curves at group level show that roughly 20 to 40 of the species are relatively common in all groups and that all the other species (up to 100) are relatively rare and mostly found in the French group.

a)



b)

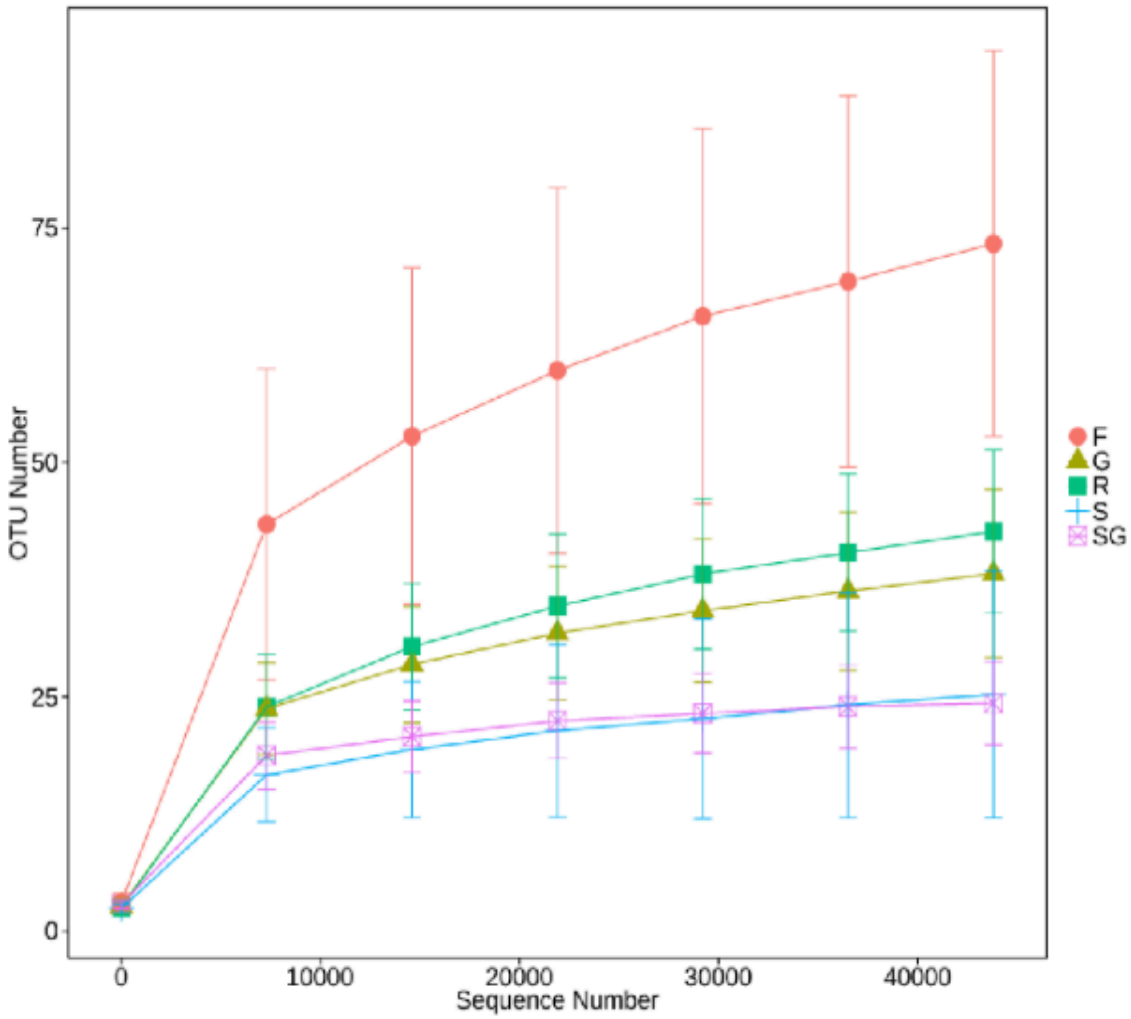
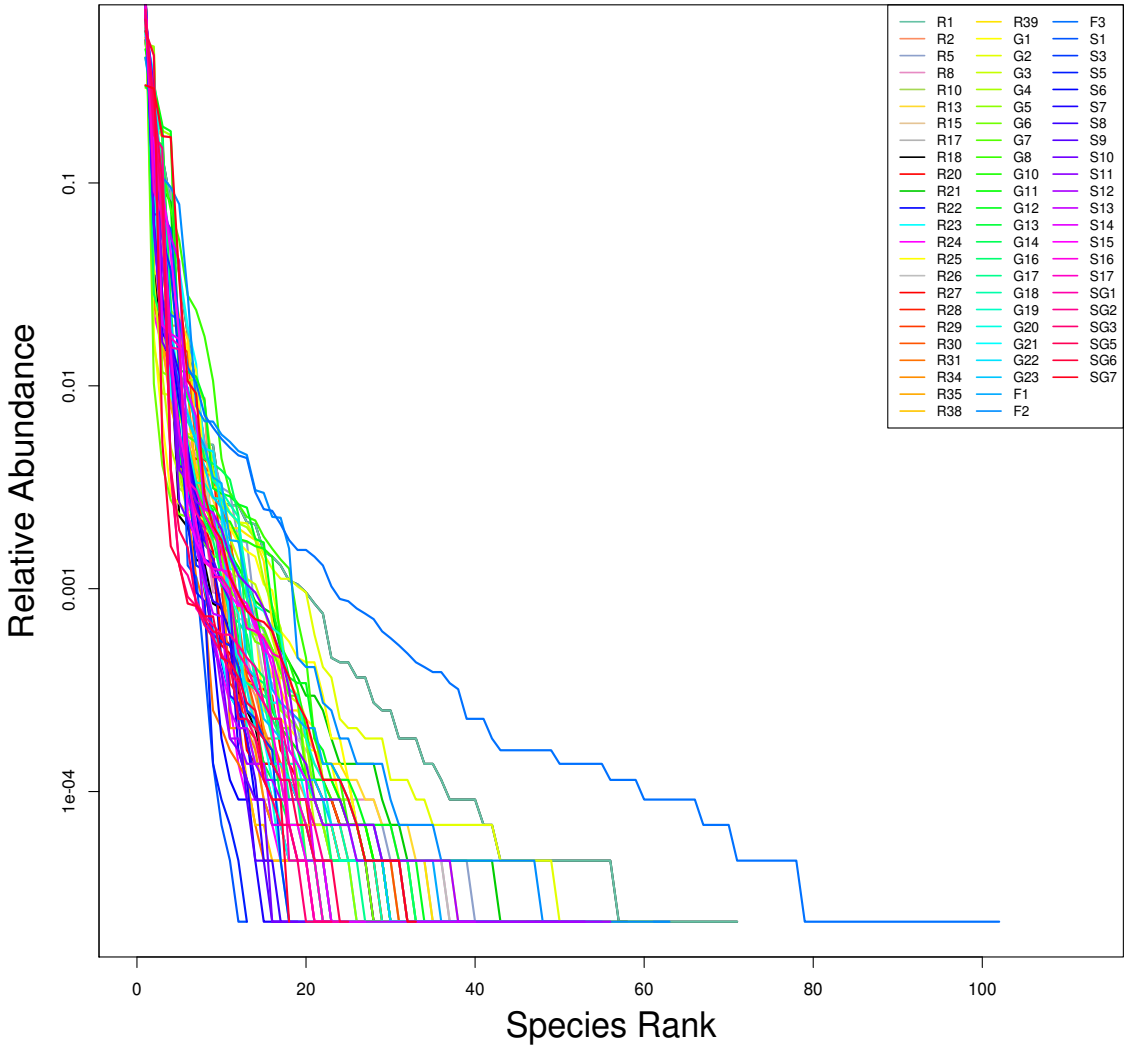


Figure 3.6 Rarefaction curves for all samples. Top: a) individual samples. Bottom: b) samples grouped by category (F=France, G=Glasnevin; S=Seed from Roosky and SG=Seed from Glasnevin). Rarefaction categories are for 10; 7,309; 14,608; 21,907; 29,206; 36,505 and 43,804 sequence reads (x axis).

a)


Relative Abundance
0.1
0.01
0.001
1e-04
0
20
40
60
80
100
Species Rank
R1
R2
R5
R8
R10
R13
R15
R17
R18
R20
R21
R22
R23
R24
R25
R26
R27
R28
R29
R30
R31
R34
R35
R38
R39
G1
G2
G3
G4
G5
G6
G7
G8
G10
G11
G12
G13
G14
G16
G17
G18
G19
G20
G21
G22
G23
F1
F2
F3
S1
S3
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
S17
SG1
SG2
SG3
SG5
SG6
SG7

b)

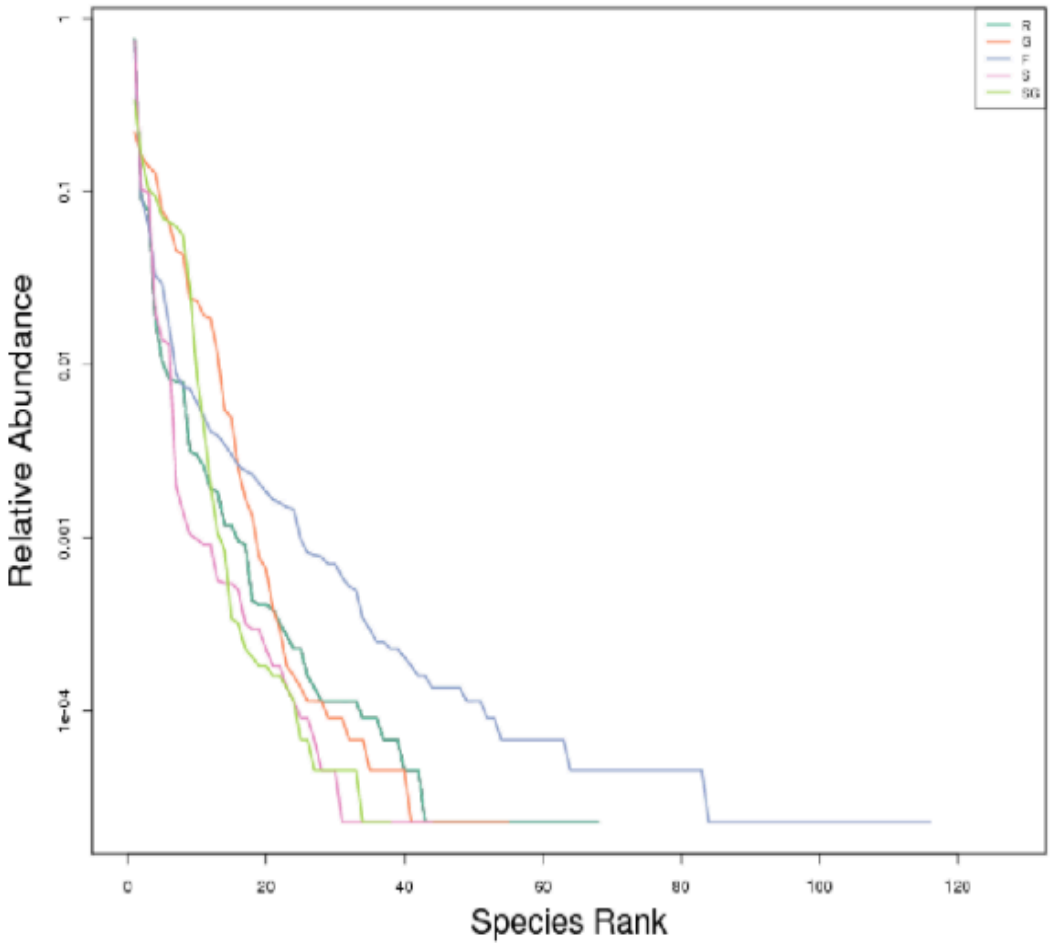


Figure 3.7a Figure 3.7b Rank abundance curves for all samples. Top: a) individual samples. Bottom: b) samples grouped by category (F=France, G=Glasnevin; S=Seed from Roosky and SG=Seed from Glasnevin).

Figure 3.8 shows the accumulation of total species richness (detected as OTUs) accumulation with increasing sample number (1 to 70 samples). It shows that a total of 60 to 70 samples adequately captures most species richness in the entire dataset (all locations and tissues) and that about two thirds of the total species number was recorded from approximately 20 samples. The data includes OTUs from several kingdoms (Chromista, Fungi and Plantae) not just fungi although most belong to the fungi.

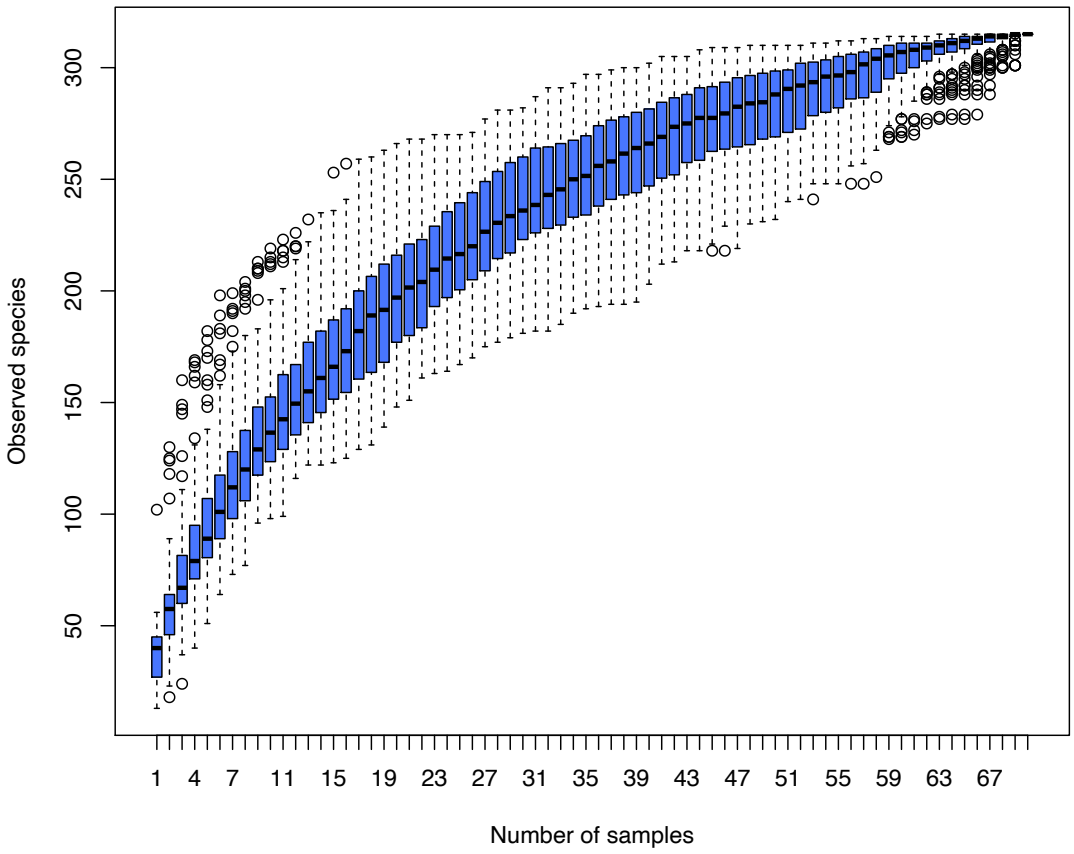


Figure 3.8 Species accumulation boxplots for all the HTS nrITS amplicon data.

Figure 3.9 shows the unique OTUs in each group and shared OTUs among groups under differing combinations. The R-G-F Venn diagram comparison (lower left) shows the leaf endophyte data for Roosky, Glasnevin and France.

Each site has a large number of unique leaf OTUs (37, 20 and 41 respectively) but also a high number of shared OTUs. 40 leaf OTUs are shared among all locations. 64 leaf OTUs are shared between Roosky and Glasnevin (upper left), 65 between Roosky and France and 51 between Glasnevin and France.

Comparisons of OTU richness in seeds are also shown in the Figure 3.9. The middle top Venn diagram shows that Roosky had a high number of unique seed endophytes (57) compared to 1 unique endophytes in the seeds from Glasnevin. However, the two groups also shared 13 core OTUs. A total of 15 endophyte OTUs are shared among Roosky leaves and Roosky seeds (lower left) but these groups have more unique (110, 54) than shared OTUs. Seeds from Roosky shared a total of 6 OTUs with leaves from Roosky and Glasnevin combined (lower right).

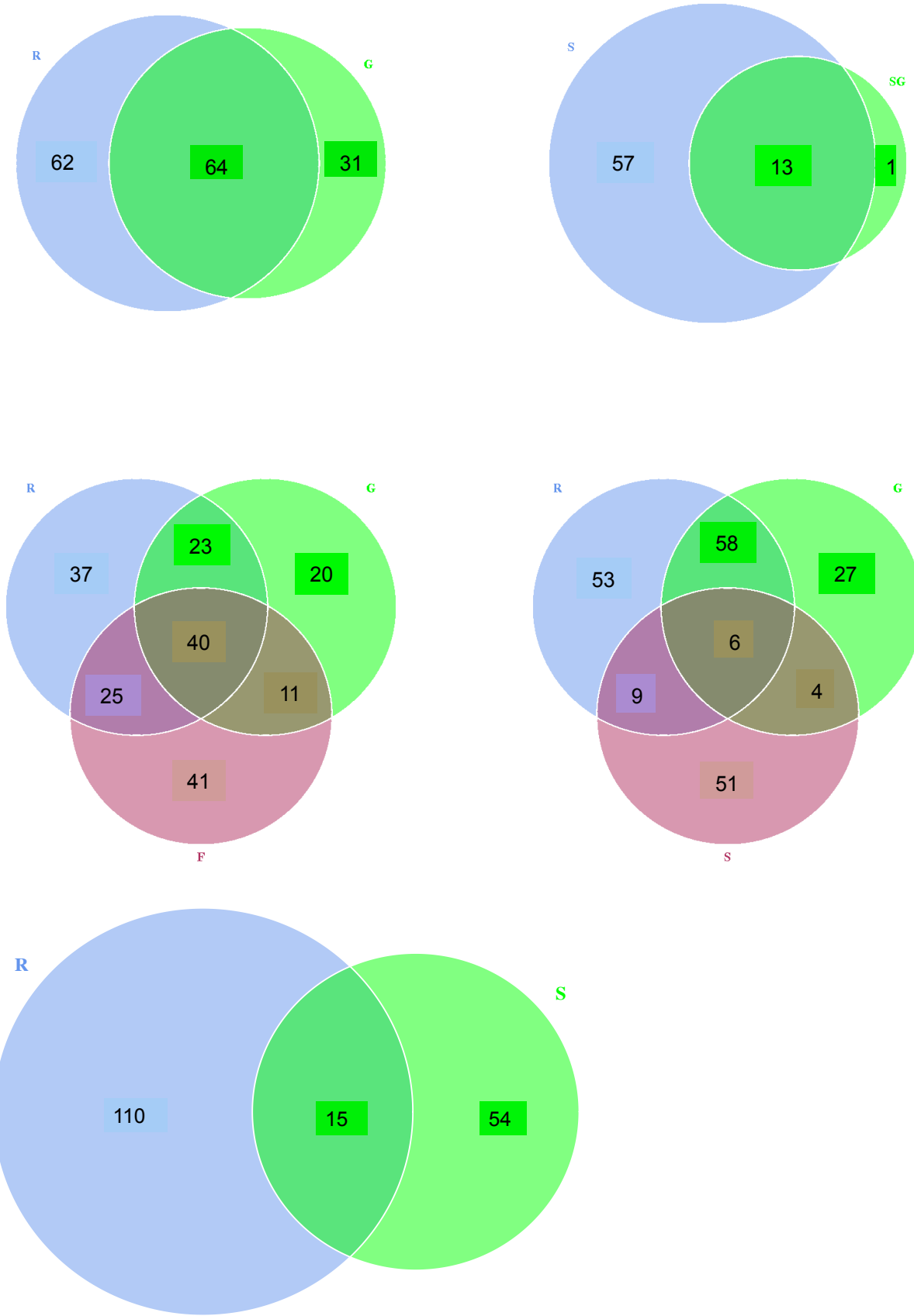
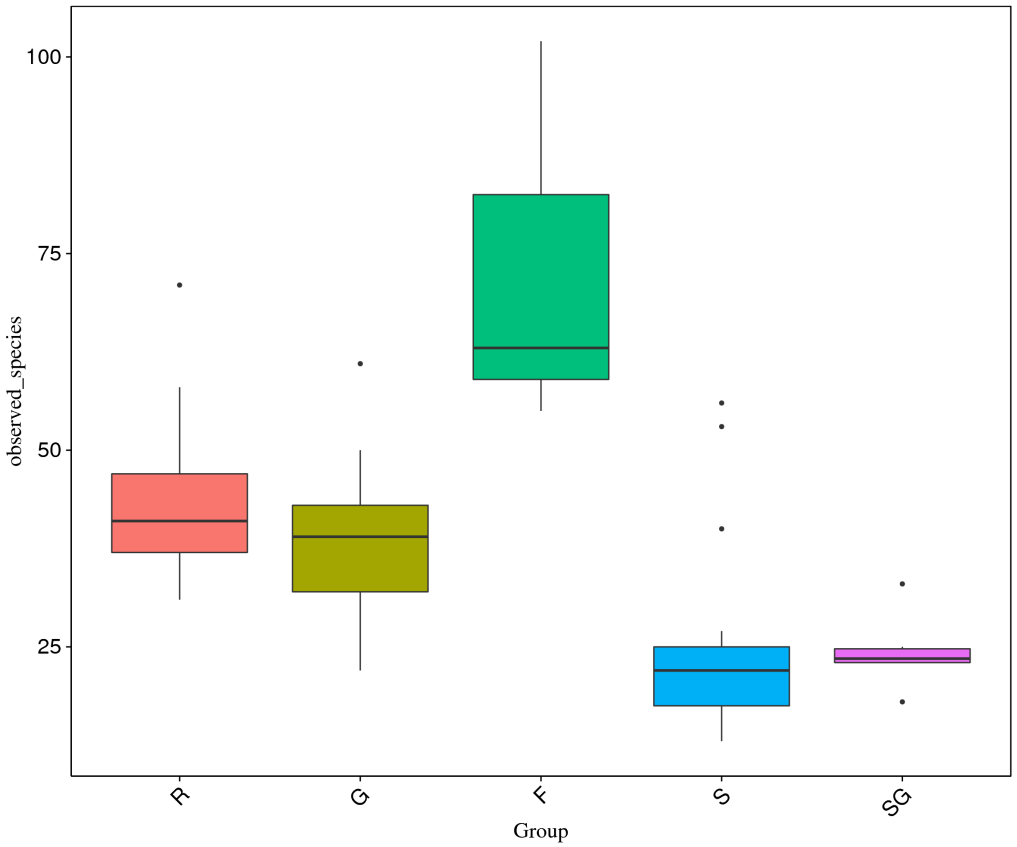


Figure 3.9 Venn diagrams of OTU distribution among groups. R=Roosky leaves, G=Glasnevin leaves, F=France leaves; S=Roosky seeds, SG=Glasnevin Seeds.

The highest diversity in terms of species richness and Shannon's index was found for the French leaf group (Figure 3.10). Relatively high species richness was also found for the Roosky and Glasnevin leaf groups. Lowest species richness was found in the two seed groups from Roosky (S) and Glasnevin (SG). However, the seed groups had relatively higher Shannon index values (comparable to leaves from Roosky and Glasnevin). For species richness values, all pairwise comparisons are significantly different ($p<0.05$) with Tukey tests except R-G, SG-G and S-SG; and for Wilcoxon test all are significantly different ($p<0.05$) except R-G and S-SG. For Shannon index values no pairwise comparisons are significantly different with either the Tukey or Wilcoxon test.



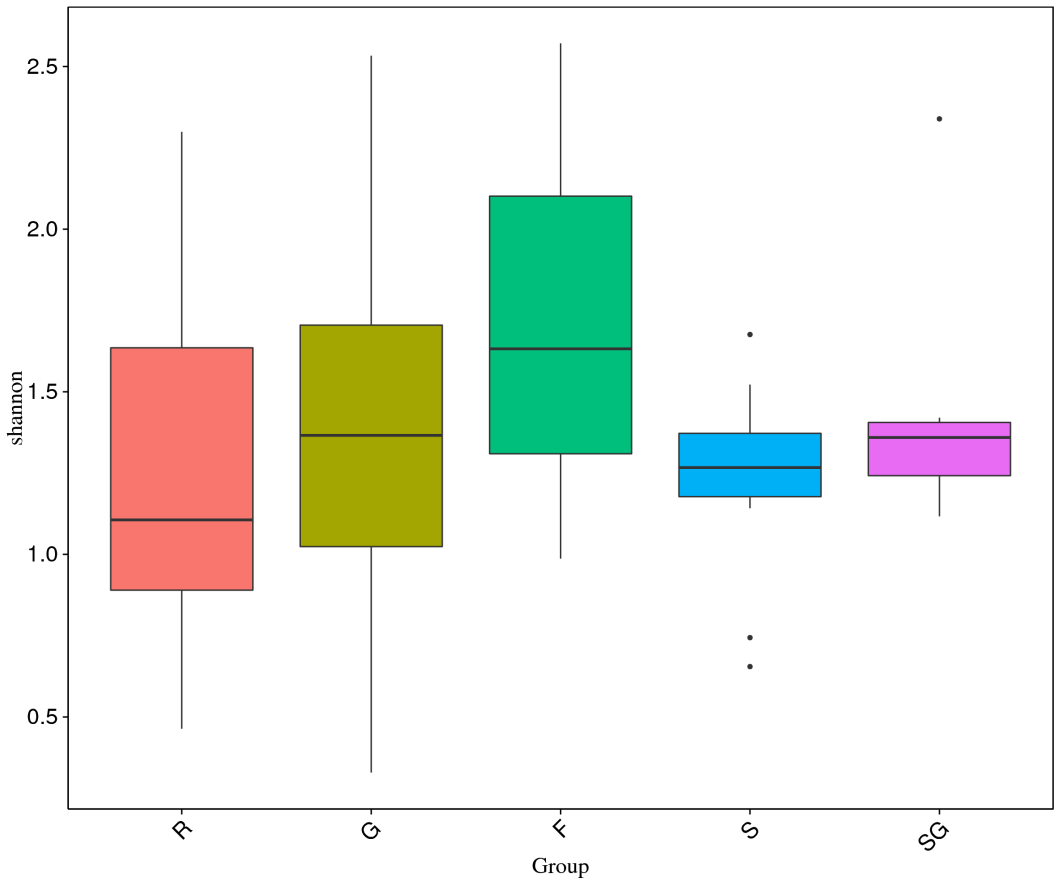


Figure 3.10 Box plots of fungal species richness (top) and Shannon indices (bottom) in the five different HTS ITS amplicon groups. R=Roosky leaves, G=Glasnevin leaves, F=France leaves; S=Roosky seeds, SG=Glasnevin seeds. All pairwise species richness comparisons are significantly different ($p<0.05$) with Tukey tests except R-G, SG-G and SG-S; and for Wilcoxon tests all are significantly different ($p<0.05$) except R-G and SG-S. For Shannon index values no pairwise comparisons are significantly different with either the Tukey or Wilcoxon test.

Beta diversity statistics/comparisons among the samples from HTS of nrITS were performed with heat-maps (Figure 3.11), UPGMA (Fig 2., Appendix II) PCoA analyses (Appendix II, Figure 3) and NMDS analysis (Figure 3.13). Figure 3.11 shows a heat-map for beta diversity (with weighted and unweighted unifrac distances among groups). The highest beta diversity levels are found between seed and leaf groups and between France and Ireland groups.

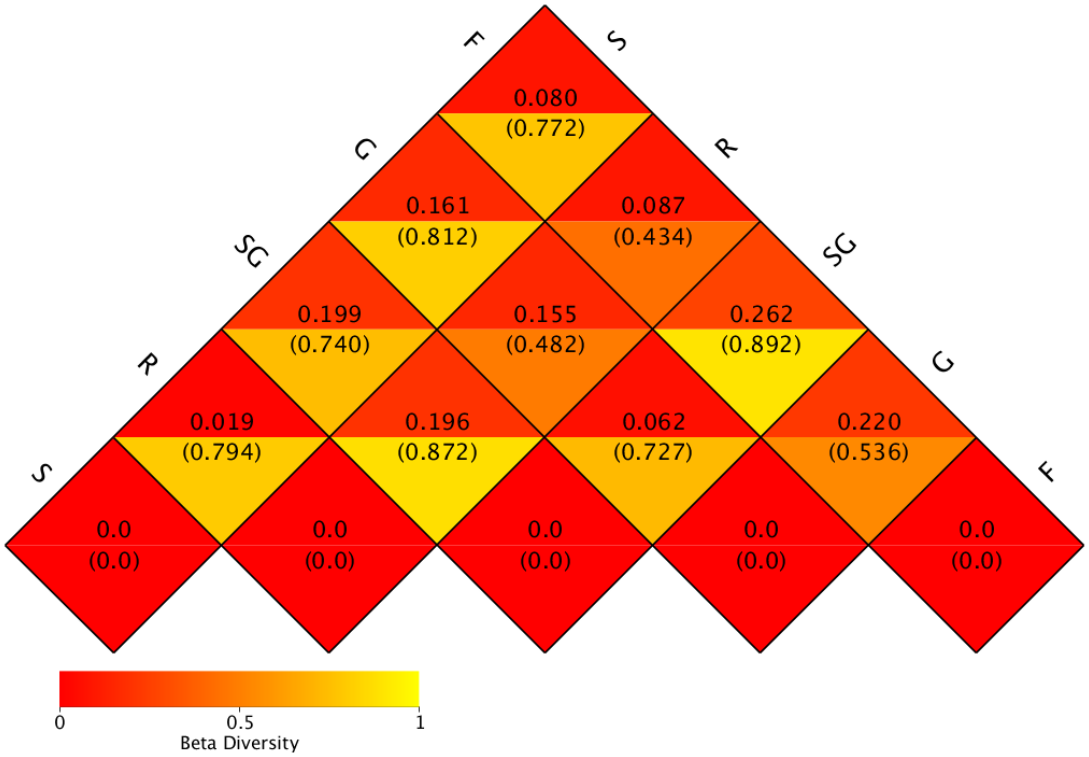


Figure 3.11 Heat-map for beta diversity for weighted unifrac distance and unweighted unifrac distances among groups. Each cell in the grid represents a pairwise dissimilarity coefficient between groups. Unweighted unfrac distances below the line; weighted unfrac distances above the line. All comparisons of beta diversity with weighted unifrac distances are significantly different with Tukey HSD at $p<0.05$ except G-F, SG-G, and S-R (in Wilcoxon tests all are significantly different except G-F, F-SG, SG-G and S-R). With unweighted unifrac distances only R-G, S-G, S-R, and SG-R are significantly different with Tukey HSD (in Wilcoxon tests R-G, S-G, S-R, and SG-R, F-R and SG-G are significantly different at $p<0.05$ level).

Appendix II, Figure 3 shows a PCoA with unweighted unifrac distances (Lozupone *et al.* 2011). It groups samples into rough clusters corresponding to either their geography (Roosky, Glasnevin, France) or tissue type (leaf or seed). A similar pattern can be seen in the NMDS plot (Figure 3.12). The Glasnevin sample is a diverse set of *Fraxinus* species in comparison to the Roosky and French samples that are only *F. excelsior*. The French samples group more closely with Roosky samples than the Glasnevin samples (despite the close geographical proximity of Roosky and Glasnevin), thus there seems to be strong influence of *Fraxinus* species on fungal community composition. F3 is the French sample with much higher species richness than the others. The *F. excelsior* samples in the mixed *Fraxinus* species group from Glasnevin also group closely to the Roosky and

French *F. excelsior* groups in the NMDS (Figure 3.12). The endophyte communities of seeds from Glasnevin and Roosky are also relatively distinct – particularly in the NMDS.

Unfortunately, the NMDS analysis here presented was undertaken by Novogene who failed to provide the original data table or details of the way the analysis was performed (e.g. number of dimensions selected for analysis, stress convergence, or number of runs). Due to the Corona virus lockdown in 2020 it has proved impossible to re-analyze these data. Until such re-analysis is undertaken all conclusions regarding the NMDS analysis must be regarded as tentative.

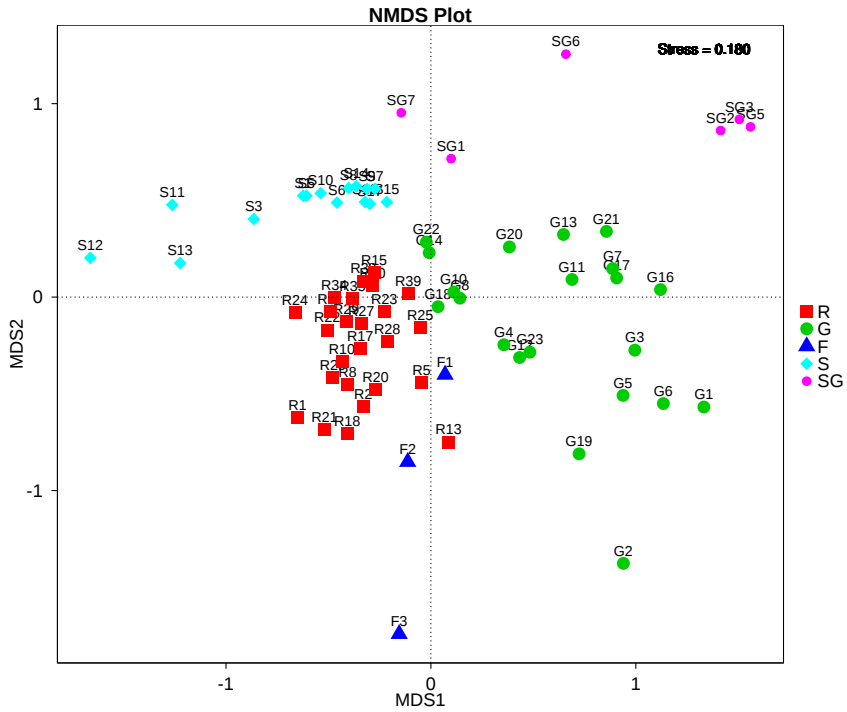


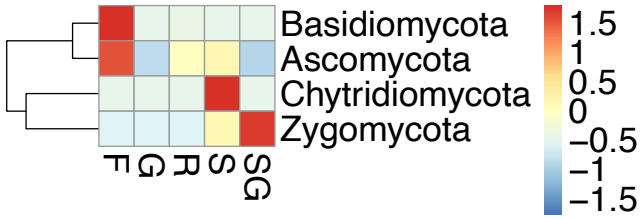
Figure 3.12 NMDS (plot of HTS samples from Roosky, Glasnevin and France. Legends on the right corresponds to R= leaves from Roosky, G= leaves from Glasnevin, F= leaves from France, S= Roosky seed and SG= Glasnevin seed. Sample number is provided. Stress=0.180.

3.3.2.1 Taxonomic composition of HTS data

The results for the 70 seed and leaf DNA samples for nrITS amplicon sequencing by HTS are shown as heat maps showing the relative abundance of different taxa among different groups. Heat maps for phyla, class, families and genera are shown in Figures 3.13 (a, b) and 3.14 (a, b).

Fungi were detected in four phyla (Ascomycota, Basidiomycota, Zygomycota and Chyridiomycota; Figure 3.13a). Ascomycota and Basidiomycota dominated the leaf samples but Zygomycota were relatively abundant in seeds from Glasnevin and Chytridiomycota relatively abundant in seeds from Roosky. The abundance of classes (Figure 3.13b) varied according to geographic source (Roosky, Glasnevin, France) or tissue type (seed or leaf). Among them Sordariomycetes and Chytridiomycetes were relatively more abundant in seed samples from Roosky and *incertae sedis* Zygomycota was high in seed samples from Glasnevin. Leotiomycetes were relatively abundant in Roosky leaf samples whereas Lecanoromycetes and Microbotryomycetes were relatively abundant for Glasnevin samples. Many taxonomic classes were relatively abundant in the French samples but rarer in the other samples such as Agaricomycetes, Taphrinomycetes, Dothideomycetes, Eurotiomycetes, Tremellomycetes and Cystobasidiomycetes (details are shown in Appendix II, Figure 9).

a)



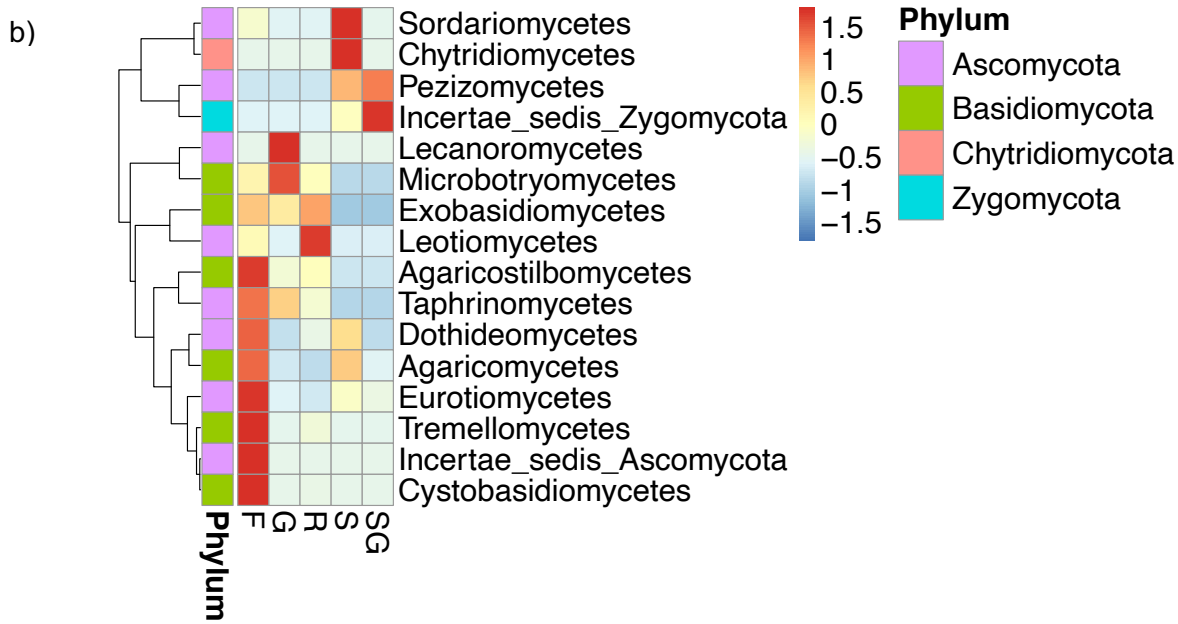


Figure 3.13 Heat map for phyla and class composition of the 70 samples screened with HTS of ITS. a) abundance heat map of phyla; b) abundance heat map of taxonomic classes. Red shows high proportion of that group in the sample group and blue a low proportion (F=France, G=Glasnevin, R=Roosky, S=Seed Roosky, SG=Seed Glasnevin). The absolute value of 'z' represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and vice versa.

Similar site specific or tissue specific patterns of abundance can be seen for orders (Appendix II, Figure 4), families (Figure 3.14a) and genera (Figure 3.14b). Samples are grouped into 35 families (Figure 3.14a). Dominant groups of families and genera differed for site and tissue type. For example 17 famlies were dominant in French leaf material but rare in other samples. *Incertae sedis* Pleoporales, Diaporthaceae, Nectriaceae and Ceratostomataceae were abundant in seed from Roosky and Mucoraceae was abundant in seed from Glasnevin. 35 genera were found in all 70 DNA samples by amplicon sequencing (Figure 3.14b). Higher abundance of 17 genera was found in leaf samples from France including *Alternaria* sp., *Ascochyta* sp., *Bensingtonia* sp., *Botryosphaeria* sp., *Bullera* sp., *Cryptococcus* sp., *Fomitopsis* sp., *Leptosphaeria* sp., *Peniophora* sp., *Phaeosphaeria* sp., *Phomopsis* sp., *Sterilitziana* sp., *Trichomerium* sp., *Trimmatostroma* sp., *Veturia* sp., *Wojnowicia* sp. and *Zymoseptoria* sp. In

Glasnevin leaves *Microstroma* sp. and *Rhodotorula* sp. were dominant and in leaves from Roosky *Acicuseptoria* sp., *Cadophora* sp., *Hannaella* sp., *Phyllactinia* sp. and *Septoriella* sp. had high scores. Seed DNA from Roosky showed 5 genera (*Diapothe* sp., *Harzia* sp., *Neofusicoccum* sp., *Paraconiothyrium* sp. and *Volutella* sp.) of relatively high abundance and two genera (*Mucor* sp. and *Talaromyces* sp.) of high abundance from Glasnevin. Heat maps showing abundance of OTUs in orders are given in Appendix II, Figure 4.

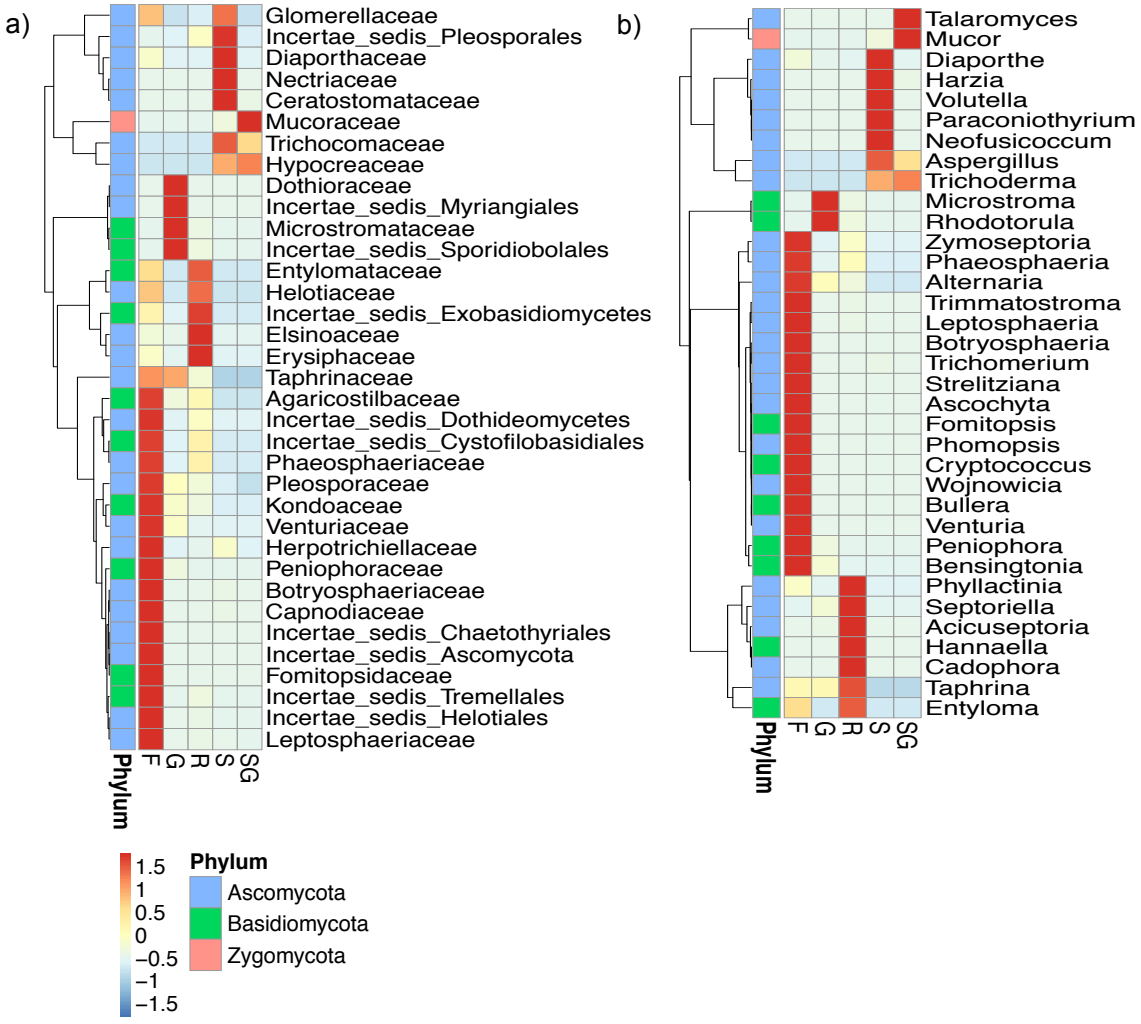


Figure 3.14 OTU abundance heat map categorised into families or genera. a) abundance heat map of families; b) abundance heat map of genera. Red shows high proportion of that group in the sample group and blue a low proportion (F=France, G=Glasnevin, R=Roosky, S=Seed Roosky, SG=Seed Glasnevin). The absolute value of 'z'

represents the distance between the raw score and the mean of the standard deviation. 'Z' is negative when the raw score is below the mean, and vice versa.

Table 3.22 OTU assigned to respective taxonomic level with total numbers obtained from seeds and leaves by HTS.

Kingdom (OTU)	Phylum (OTU)	Class (OTU)	Order (OTU)	Family (OTU)
Fungi (71)	Ascomycota (56)	Dothideomycetes (28)	Botryosphaeriales (2)	Botryosphaeriaceae (2)
			Capnodiales (3)	Capnodiaceae (2)
				Mycosphaerellaceae (1*)
			Dothideales (2)	Dothioraceae (1*)
			Myriangles (3)	Elsinoaceae (2*)
			Pleosporales (18)	Didymosphaeriaceae (1)
				Montagnulaceae (3)
				Leptosphaeriaceae (3*)
				Phaeosphaeriaceae (6*)
				Pleosporaceae (3*)
			Venturiales (2)	Venturiaceae (1*)
		Eurotiomycetes (9)	Chaetothyriales (3)	Chaetothyriaceae (1)
				Herpotrichiellaceaea (1*)
			Eurotiales (6)	Trichocomaceae (6)
			Verrucariales (1)	Verrucariaceae (1*)
		Lecanoromycetes (3)	Lecanorales (1)	Ramalinaceae (1)
			Teloschistales (2)	Teloschistaceae (2)
		Leotiomycetes (3*)	Erysiphales (1)	Erysiphaceae (1)
			Helotiales (3)	Helotiaceae (1*)
			_	Vibrisseaceae (1*)
		Pezizomycetes (1)	Pezizales (1)	Ascobolaceae (1*)
		Sordariomycetes (7*)	Diaporthales (2*)	Diaporthaceae (2*)
			Hypocreales	Clavicipitaceae

			(3*)	(1*)
				Hypocreaceae (1*)
				Nectriaceae (1)
			Glomerellales (1)	Glomerellaceae (1)
			Melanosporales (1)	Ceratostomataceae (1)
			Sordariales (2)	Sordariaceae (1*)
		Taphrinomycetes (5*)	Taphrinales (5*)	Taphrinaceae (5*)
	Basidiomyc--ota (11)		Polyporales (1)	Fomitopsidaceae (1)
		Agaricomycetes (2)	Agaricales (1*)	_
			Russulales	Peniophoraceae
		Agaricostilbomycetes (1)	Agaricostilboles (1*)	Agaricostilbaceae (1*)
			_*	Kondoaceae (*)
		Cystobasidiomycetes (1*)	_*	_*
		Exobasidiomycetes (3*)	Micostromatales (1)	Microstomataceae (1)
			Entylomatales (1)	Entylomataceae (1)
		Microbotryomycetes (1*)	Sporidiobolales (1)	Sporidiobolaceaea (1)
			_*	_*
		Tremellomycetes (4*)	Tremellales (3*)	Tremellaceae (1)
				Bulleraceae (1)
			Cystofilobasidiales (1*)	Cystofilobasidiaceae (1)
			_*	-*
	Chytridiomycota (1)	Chytridiomycetes (1)	Rhizophydiales (1)	_*
	Zygomycota (3)	Mucoromycetes (3)	Mucorales (2)	Mucoraceae (2)
			_*	Rhizopodaceae (1)
Chromista (3)	Oomycota (3)	Oomycetes (3)	Pythiales (3)	Pythiaceae (3)

* indicates unidentified reads are present in that level.

3.4 Discussion

3.4.1 Culture dependent fungal diversity and community composition

3.4.1.1 Diversity and taxonomy

A total of 410 endophyte isolates were sequenced for nrITS, nrLSU and *tef* regions (these represent the culture dependent set) and 21 remained unidentified. We found that the nrITS region was the most consistent to amplify and showed the highest taxon differentiation among the loci tested. This supports early work by White et al. (1990) and more recently the Consortium for the Barcode of Life (Begerow *et al*. 2010) that recommends nrITS for fungal identification. nrITS has the added benefit of having high taxon coverage in GenBank and UNITE databases.

We identified 141 OTU in total from the leaf and root endophyte culture samples (details are given in Table 3.16). Cultured leaf endophyte isolates from the Roosky provenance trial of *Fraxinus excelsior* and the mixed *Fraxinus* taxa sample from Glasnevin belong to two divisions (Ascomycota and Basidiomycota) and 7 and 9 classes respectively (**Basidomycota**: **Agaricomycetes**, Cystobasidiomycetes, Tremellomycetes; **Ascomycota**: **Dothideomycetes**, **Eurotiomycetes**, **Leotiomycetes**, **Pezizomycetes**, Saccharomycetes, **Sordariomycetes** and Ustilaginomycetes) bold indicates presence in both sites (details shown in Table 6.1 and 6.2 in Chapter 6). Cultured root endophytes isolates were found from one division, four class, six orders and ten families. Bionectriaceae, Ceratostomataceae and Clavicipitaceae families were unique to roots.

Two families, Psathyrellaceae and Bulleribasidiaceae (belonging to two different orders Agaricales and Tremellales) were only found in diseased leaf tissues from Roosky. Two families, Hysteriaceae and Diatrypaceae, (belonging to Hysteriales and Xylariales) were only found from diseased samples from Glasnevin (details shown in Table 6.1, 6.2 in Chapter 6).

The culture dependent method detected many taxa not detected by the culture independent method for leaves. These included:
Two sub-phyla (Sachharomycotina and Ustilaginomycotina).
Two classes (Sachharomycetes and Ustilaginomycetes).
10 orders (Agaricales, Cantharellales, Chaetosphaeriales, Corticiales, Cystobasidiales, Filobasidiales, Hysteriales, Saccharomycetales, Ustilaginales and Xylariales).
26 families (details shown Chapter 6, Table 6.1).

Other studies on the culture dependent ash mycobiome by Bakys *et al.* (2009a),

Scholtysik *et al.* (2013), Kowalski *et al.* (2016) and Kosawang *et al.* (2018) have found a total of 133 taxa, 63 families and 17 orders belonging to 3 classes (Agaricomycotina, Mucoromycotina, Pezizomycotina) and two divisions (Ascomycota and Basidiomycota). We found, from our cultured leaf and root endophytes, 119 taxa, 35 families, 23 orders and 11 classes (details shown in Table 3.16 and Chapter 6, Table 6.1 and 6.2).

Common taxa found from our cultured leaf and root endophytes with other studies on the ash mycobiome (Bakys *et al.* 2009a, Scholtysik *et al.* 2013, Kowalski *et al.* 2016 and Kosawang *et al.* 2018) are shown in Table 3.23. Two divisions, 5 classes, 15 orders, 17 families and 31 species/genera are common between our study and those listed above.

Table 3.23 Common taxa found from the ash mycobiome in our study and that of other researchers. Other studies included Bakys *et al.* (2009a), Scholtysik *et al.* (2013), Kowalski *et al.* (2016) and Kosawang *et al.* (2018).

Division	Class	Order	Family	Taxa
Ascomycota	Agaricomycetes	Agaricales	Aspergillaceae	*Alternaria* sp.
Basidiomycota	Dothideomycetes	Cantharellales	Cladosporiaceae	*Aureobasidium pullulans*
	Eurotiomycetes	Capnodiales	Dermataceae	*Alternaria* sp.
	Leotiomycetes	Chaetothyriales	Diatrypaceae	*Boeremia exigua*
	Sardariomycetes	Diaporthales	Didymellaceae	*Cadophora* sp.
		Dothideales	Didymosphaeriaceae	*Cladosporium cladosporioides*
		Eurotiales	Dothioraceae	*Cladosporium herbarum*
		Glomerellales	Glomerellaceae	*Cladosporium* sp.
		Helotiales	Hydnaceae	*Colletotrichum godetiae*
		Hypocreales	Hypocreaceae	*Diaporthe cotoneastri*
		Peziziales	Incertae sedis Helotiales	*Diaporthe eres*
		Pleosporales	Phaeosphaeriaceae	*Diaporthe* sp.
		Polyporales	Pleosporaceae	*Diaporthe viticola*
		Sordariales	Mycosphaerellaceae	*Epicoccum nigrum*
		Xylariales	Nectriaceae	*Eutypa spinosa*
			Sarocladiaceae	*Fusarium avenaceum*
			Xylariaceae	*Fusarium*

				lateritium
				Gibberella sp.
				Mollisia sp.
				Nectria sp.
				Neonectria sp.
				Paraconiothyrium sp.
				Penicillium sp.
				Phaeosphaeria pontiformis
				Phoma exigua
				Phoma multirostrata
				Phoma sp.
				Phomopsis sp.
				Sarocladium strictum
				Septoria convolvuli
				Sistotrema brinkmannii
				Xylaria sp.

Apart from above mentioned families two other families are shared with those found in Bakys *et al.* (2009a) and Kowalski *et al.* (2016) but the constituent species/genera we have found are diffierent. Those are in Sclerotiniaceae where we have isolated *Botrytis cinera* and in Cordycipitaceae, where we isolated *Engyodontium album*. Kowalski *et al.* (2016) isolated *Encoelia furfuacea* from Sclerotiniaceae and *Simplicillium lamellicola* from Cordycipitaceae. Bakys *et al.* (2009a) found *Botryotinia fuckeliana* from Sclerotiniaceae.

We found several distinct taxa from our cultured leaf and root endophytes that were not found in the above mentioned research. We found 16 unique families, 9 orders and 5 classes as listed below:
Families: Bionectriaceae, Bulleribasidiaceae, Ceratomataceae, Chaetosphaeriaceae, Clavicipitaceae, Corticiaceae, Cystobasidiaceae, Debaryomycetaceae, Filobasidiaceae, Herpotrichiellaceae, Hysteriaceae, Lasiosphaeriaceae, Meruliaceae, Psathyrellaceae, Pyronemataceae, Ustilaginaceae.

Orders: Cantharellales, Chaetosphaeriales, Corticiales, Cystobasidiales, Filobasidiales, Hysteriales, Sachharomycetales, Tremellales, Ustilaginales
Classes: Cystobasidiomycetes, Pezizomycetes, Saccharomycetes, Tremellomycetes, Ustilaginomycetes.

The culturable root fungal community was found to be highly different to the culturable leaf communities examined. Bionectriaceae, Ceratostomataceae and Clavicipitaceae families were unique to roots and 26 families were unique to leaves as listed below:

Famiilies: Bulleribasidiaceae, Chaetosphaeriaceae, Corticiaceae, Cystobasidiaceae, Debaryomycetaceae, Dermataceae, Diaporthaceae, Diatrypaceae, Didymosphaeriaceae, Dothioraceae, Filobasidiaceae, Glomerallaceae, Herpotrichiellaceae, Hydnaceae, Hysteriaceae, Lasiosphaeriaceae, Meruliaceae, Mycosphaerellaceae, Phaeosphaeriaceae, Pleosporaceae, Psathyrellaceae, Pyronemataceae, Sarocladiaceae, Sclerotiniaceae, Xylariaceae and Ustilaginaceae.

Kowalski and Lukomska (2005) studied endophytes from three types of diseased tissues, namely dying top shoots, local canker, and dead roots. They found no common genera among roots and shoots. Three genera were found from dead roots, namely *Cryptosporiopsis radicola*, *Cylindrocarpon destructans* and *Phialocephala* sp.. However, we did not find these genera in our root samples. We did find some genera in common with Kowalski and Lukomska (2005) who sampled dying top shoots and local canker and found *Cladosporium cladosporoides*, *Cystospora ambiens*, *Fusarium lateritium*, *Gloeosporidiella turgida*, *Hymenoscyphus* sp., and two species of *Phomopsis* sp. In our study, we found *Cladosporium cladosporoides* (healthy leaf and root tissue in Roosky), *Fusarium lateritium* (healthy and diseased leaf tissue in Roosky and Glasnevin) and *Phomopsis* sp. (healthy root tissue from Kinsealy) (Table 3.16, Tables 3, 4 and 6 in Appendix II). Pezizomycota were the most common in roots of barley by Murphy *et al.* (2015).

3.4.2 Community differences

Figure 3.15 shows the number of OTUs found from Roosky leaves (R), Glasnevin leaves (G) and Kinsealy roots (K) and the number of OTUs shared among these sites. The leaf samples from Roosky and Glasnevin have more unique OTUs than

shared OTUs. This difference can be explained by host species effects (as the Glasnevin sample is from a range of *Fraxinus* taxa compared to the single *Fraxinus excelsior* species at Roosky. It can also be explained by habitat and ecological differences among sites. Glasnevin is a botanic garden and Roosky a provenance trial plantation. 17 OTUs are shared among these sets and can be considered core endophytes. There are also a high proportion of unique endophytes in the roots from Kinsealy (28) with only five shared with the leaf samples in total and only 1 OTU (*Fusarium oxysporum*) is shared among Roosky (leaf), Glasnevin (leaf) and Kinsealy root (details in Figure 3.15 left one; Table 3.16). It is not known if the five OTUs (*Cadophora* sp., *Cladosporium cladosporoides*, *Epicoccum nigrum*, *Fusarium culmorum*, and *Fusarium oxysporum*) shared by leaves and roots are transmitted vertically by seed but it is a hypothesis worth investigating further.

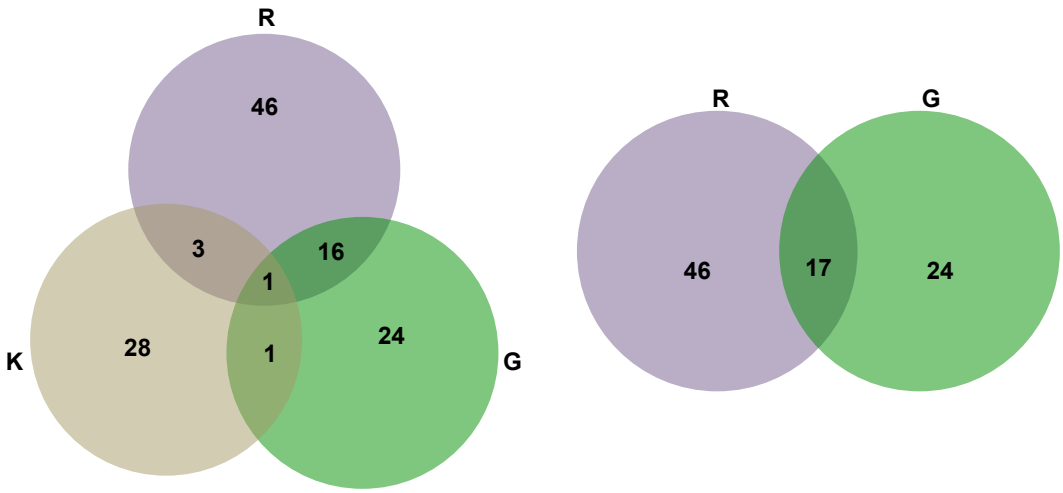


Figure 3.15 Venn diagram showing distinct and common taxa among leaf and root endophytes from all sites. Leaf endophyte Roosky=R, Glasnevin=G, and root endophyte Kinsealy=K.

Therefore the culturable endophyte communities from differing sites, tissue type (roots and shoots) and tissue health (diseased and undiseased) have been demonstrated to be highly different from each other. The culturable community are the sample that can be used for further experimentation and potential biocontrol of ash dieback disease. Our sampling has ensured we captured maximal diversity.

3.4.3 Culture independent fungal diversity and community composition

3.4.3.1 Diversity

A total 324 OTUs were obtained from 70 leaf and seed samples in nrITS amplicon high throughput sequences (using sequences with at least 97% similarity to define an OTU). From these, 212 were assigned to kingdom Fungi and 3 reads for kingdom Chromista (only found in culture independent sequences). Other OTUs were assigned to kingdom Plantae and 16 reads were for unidentified fungal isolates. The Plantae sequences were a mixture of *Fraxinus* (the host plant) and various algae. Some of these are likely to be epiphytic on the surface of the ash leaves or could be surface contaminants.

66 OTUs were detected in the leaves by the culture independent approach and these were divided into 4 phyla, 16 classes, 32 orders, and 42 families (shown in Table 3.23). The culture independent assessment detected many fungal taxa not detected by the culture dependent method. These included:

Three Phyla (Chytridiomycota, Zygomycota and Oomycota)

Eight classes (Agaricostilbomycetes, Chytridiomycetes, Exobasidiomycetes, Lecanoromycetes, Microbotryomycetes, Mucoromycetes, Oomycetes and Taphrinomycetes).

Fifteen orders (Agaricostilboles, Botryosphaeriales, Cystofilobasidiales, Erysiphales, Entylomatales, Lecanorales, Melanosporales, Micostromatales, Myriangles, Russulales, Sporidiobolales, Verrucariales, Venturiales, Taphrinales and Teloschistales).

Thirty families (Agaricostilbaceae, Ascobolaceae, Botryosphaeriaceae, Bulleraceae, Capnodiaceae, Ceratostomataceae, Chaetothyriaceae, Cystofilobasidiaceae, Elsinoaceae, Entylomataceae, Erysiphaceae, Fomitopsidaceae, Kondoaceae, Leptosphaeriaceae, Microstomataceae, Montagnulaceae, Mucoraceae, Peniophoraceae, Pythiaceae, Ramalinaceae, Rhizopodaceae, Sordariaceae, Sporidiobolaceae, Taphrinaceae, Teloschistaceae, Tremellaceae, Trichocomaceae, Venturiaceae, Verrucariaceae and Vibrisseaceae) (details are shown in Table 3.22, Chapter 6, Table 6.1 and 6.2).

The highest alpha diversity in terms of species richness and Shannon's index was found for the French leaf group (Figure 3.10). Relatively high species richness was also found for the Roosky and Glasnevin leaf groups. Lowest species richness was found in the two seed groups from Roosky (S) and Glasnevin (SG). No seed was sampled from France. However, the seed groups had relatively higher Shannon index values (comparable to leaves from Roosky and Glasnevin). Thus they are

much more species poor but have comparable evenness. It is not known why the French samples had the highest richness despite being the smallest sample, however, it could be due to the isolated island status of Ireland which hosts lower animal and plant diversity (Parnell and Curtis 2012) might also be expected to host lower fungal diversity. The French population was also from a natural forest in the Alps compared to a plantation in Roosky and botanic garden in Glasnevin. Seed diversity is discussed below.

3.4.4 Community differences on seeds

The fungal communities of each geographical location (Roosky, Glasnevin, France) and plant tissue type (leaf vs seed) differed considerably. The highest beta diversity levels are found between seed and leaf groups and between France and Ireland groups. The NMDS (Figure 3.12) groups samples into rough clusters corresponding to either their geography (Roosky, Glasnevin, France) or tissue type (leaf or seed). A similar pattern can be seen in the PCoA plot (Appendix II, Figure 3). The Glasnevin sample is a diverse set of *Fraxinus* species in comparison to the Roosky and French samples that are only *F. excelsior* which might partly explain their difference. The French leaf samples group more closely with Roosky samples than the Glasnevin samples (despite the close geographical proximity of Roosky and Glasnevin), thus there seems to be strong influence of *Fraxinus* species on fungal community composition. The *F. excelsior* samples in the mixed *Fraxinus* species group from Glasnevin also group closely to the Roosky and French *F. excelsior* groups in the NMDS. The heat-maps (Figures 3.13 to 3.14 also show considerable differences in community composition of leaves among groups at different taxonomic levels. For example, the dominant families and genera vary closely with group (site or seed vs leaf).

The heatmaps and NMDS show that the endophyte communities of seeds are distinct from those of leaves. In addition, the seed endophyte community from Glasnevin and Roosky are also relatively distinct. From 15 seed DNA samples collected from Roosky we obtained a total of 12 taxa (Table 3.24) among which *Aspergillus niger* and *Harzia acremonioides* were the most abundant reads with 247 and 154 reads respectively. Less abundant species in terms of read number were *Mucor abundans*, *Aspergillus penicillioides* and *Cladophialophora chaetospira* with read numbers of 1, 2 and 2 respectively (data not shown). 9 OTUs were found from 6 samples from Glasnevin including seeds from *Fraxinus excelsior*, *F. mandshurica*, *F. ornus* and *F. potamophila* and among these high read abundance

was found for *Aspergillus niger* (82 reads) details are shown in Appendix II, Figures 10 and 11.

Table 3.24 Endophytes identified from seed taken from two sites, Roosky and Glasnevin. OTUs highlighted in bold are shared among sites and considered core.

Seed endophytes	Roosky R	Glasnevin G
Aspergillus niger	1	1
Aspergillus nominus	1	0
Aspergillus penicilloides	1	1
Botryosphaeria stevensii	1	0
Cladophialophora chaetospira	1	0
Harzia acremoniodes	1	1
Mucor abundans	1	1
Paraconiothyrium brassilliense	1	0
Paraconiothyrium hawaiiense	1	1
Penicillium brevicompactum	1	1
Rhizopus arrhizus	1	0
Sordaria fimicola	0	1
Talaromyces minioluteus	0	1
Volutella ciliata	1	1

Aspergillus niger, A. penicilloides, Harzia acremonoides, Mucor abundans, Paraconiothyrium hawaiiense, Penicillium brevicompactum and *Volutella ciliata* were present in seeds from both sites and can be considered core endophytes of ash seeds. *Botryosphaeria stevensii* is commonly present in leaf samples from Glasnevin and Roosky and seed from Roosky. Likewise *Sordaria fimicola* is commonly present in leaf samples from Roosky and seed samples from Glasnevin (details in Table 3.24, Appendix II, Figures 10 and 11).

Among above mentioned list of 14 endophytes found from seeds of *Fraxinus* spp. some of them are also reported in other plants including ash. *Harzia acremonoides* reported from syptomatic ash petiole in north western Spain (Trapiello *et al.* 2017); *Botryosphaeria stevensii* has been reported from twigs of *Vitis vinifera* (González V and Tello ML 2011); *Volutella ciliata* has been reported as a culturable endophyte from roots of *Pinguicula vulgaris* (Quilliam and Jones 2012); *Paraconiothyrium brasiliense* was reported for first time from chinese maple leaves (Paul and Lee 2014); *Talaromyces minioluteus* was isolated from *Silybum marianum* and

produces biological active compounds (Kaur *et al.* 2016); *Aspergillus* sp. and *Penicillum* sp. Were reported as endophytes from *Taxus globosa* (Soca-Chafre *et al.* 2011).

Hayatgheibi H (2013) isolated *Lophodermium pinastri* as an endophyte from ash seeds and *Pyrenochaeta corni* was noted as an endophyte of ash seeds by Cleary *et al.* (2013). In the present study we did not isolate these endophytes from seeds.

3.4.5 Comparison of OTUs obtained from high throughput amplicon sequencing (HTS) and Sanger sequencing

Very large differences were found in OTU diversity and community composition recorded by the culture dependent (Sanger sequencing) and culture independent approach (direct HTS sequencing of plant material) despite the fact the same nrITS DNA barcoding region was used in each.

A total of 108 OTUs was detected from leaves using the culture dependent method compared to 88 for the culture independent method (Appendix II, Figures 5 to 7). The taxonomic breadth of fungal OTUs was much higher in the culture independent approach. Two subdivisions were obtained from the culture dependent sequencing approach (Agaricomycotina and Pezizomycotina) but fungi detected from five subdivisions using the culture independent HTS sequencing approach (Agaricomycotina, Pezizomycotina, Pucciniomycotina, Taphrinomycotina and Ustilaginomycotina). In addition, a total of 11 classes were detected with direct amplicon sequencing compared to 9 in the culture dependent sample (Tables 3.25 and 3.26). It was particularly noteworthy that only two OTUs were shared between the two approaches (*Alternaria* sp. and *Phaeosphaeria* sp.). Core OTUs found in both Irish sites (Roosky and Glasnevin) are listed in Table 3.27. None of these are shared between the culture dependent and culture independent methods.

The nrITS region for fungal identification was the same so the results cannot be explained by database coverage. However, the primers differed to amplify the nrITS region. Furthermore, it is known that many fungi such as those in the Glomerales are not directly culturable. However, the culturable fungi would be expected to be detected by the direct HTS of plant material as they are present. Perhaps, they are rarer than expected and are not detected because of competitive template processes in the PCR.

Other studies on the ash mycobiome have been conducted using the HTS method but only a small subset of taxa are shared with our analysis. *Phyllactinia* sp. was found by Cross *et al.* (2017) positively correlated with *Hymenoscyphus fraxineus* and *Taphrina* sp.,*Tilletiopsis* sp. endophytes were negatively correlated. We have also found *Phyllactinia fraxini, Tilletiopsis washingtonensis* and 5 species of *Taphrina sp.* from our healthy leaf samples. However we did not detect *Hymenoscyphus fraxineus* from those leaves as they were from healthy leaf samples. A high number of reads (14,901) of *Phyllactinia fraxini* suggest that it is a core mycobiome component of ash leaves (Figure 5, Appendix II). Cleary *et al.* (2016) found *Mycosphaerella* sp., *Cladosporium* sp. and *Phoma* sp. commonly present in asymptomatic leaves of *Fraxinus mandshurica*. Our study support this finding because we also found *Mycosphaerella* sp., *Cladosporium* sp. and *Phoma* sp. as commonly present in healthy tissues. These three taxa were cultured but not detected with the HTS method. In the culture dependent method we isolated *Cladosporium* spp. (8 and 7 isolates) and *Mycosphaerella* spp. (23 and 3 isolates) from healthy leaf tissue sampled from Roosky and Glasnevin respectively (details in Table 3 and 6, Appendix II) and only 1 isolate was obtained from diseased tissue for *Cladosporium* spp.. No isolates obtained from diseased leaf tissue for *Mycosphaerella* spp. in Glasnevin (details in Table 4, Appendix II). This suggests that *Mycosphaerella* sp. and *Cladosporium* sp are present in high numbers in healthy leaves. Higher numbers of isolates were also found for *Phoma* sp. (8 and 9 isolates) in healthy and diseased tissue sampled from the Roosky site. Since the Roosky site was only a *Fraxinus excelsior* provenance trial, it suggests that *Phoma* sp. Are also a common endophyte for *Fraxinus excelsior*.

Schlegel *et al.* (2018) compared culture dependent and independent methods in ash and sycamore. They found that the most abundant OTUs for the diseased area (HTS Illumina data) were *Mycosphaerella* sp., two *Cladosporium* spp., *Preussia minima* and one *Venturia fraxini* genotype. For the symptomless area, the most abundant OTUs were *Paraconiothyrium* sp., *Colletotrichum godetiae* and another *Mycosphaerella* sp. We have only sampled healthy leaf tissues and seeds for the culture independent method and also found two species of *Paraconiothyrium* spp. but not from leaf samples from Roosky and Glasnevin (details in Table 3.24). Cleary *et al.* (2016) have also found *Paraconiothyrium sp.* from leaves of *Fraxinus mandshurica* in East Russia. We have isolated *Paraconiothyrium* sp. by the cultured dependent method from leaf samples of *Fraxinus excelsior* at the Roosky site (Table

3.16). According to Schlegel *et al.* (2016), *Paraconiothyrium* sp. has strong antibiotic compounds which can inhibit ascospore germination and mycelial growth (Kosawang *et al*. 2018) of *H. fraxineus*.

Agostinelli (2018) studied the mycobiome of *Fraxinus excelsior* from leaf, bark and xylem using a culture independent HTS method and found *Aureobasidium pullulans*, *Alternaria* sp., *Phomopsis* sp. and *Trichoderma* sp. as common species. However in the present study, we found *Alternaria* sp., using both methods but *Aureobasidium pullulans* (from leaf samples from Roosky and Glasnevin), *Phomopsis* sp. (only from leaf sample at Glasnevin) and *Trichoderma* sp. (root sample, Kinsealy) detected only by the culture dependent method (Table 3.16).

Table 3.25 Comparison for classes and families found in NGS (HTS) and Sanger sequencing for leaf samples from Roosky (R)

Sanger sequencing		NGS	
Class	Family	Class	Family
Agaricomycetes	Aspergillaceae	Agaricomycetes	Botryosphaeriaceae
Dothideomycetes	Bulleribasidiaceae	Dothideomycetes	Chaetothyriaceae
Eurotiomycetes	Chaetosphaeriaceae	Eurotiomycetes	Cladosporiaceae
Leotiomycetes	Cladosporiaceae	Exobasidiomycetes	Cystofilobasidiales
Sordariomycetes	Cordycipitaceae	Lecanoromycetes	Didymosphaeriaceae
Tremellomycetes	Diaporthaceae	Leotiomycetes	Dothideales
	Didymellaceae	Microbotryomycetes	Entylomataceae
	Didymosphaeriaceae	Sordariomycetes	Erysiphaceae
	Dothioraceae	Taphrinomycetes	Helotiaceae
	Filobasidiaceae	Tremellomycetes	Helotiales
	Hydnaceae		Leptosphaeriaceae
	Hypocreales		Melanommataceae
	Lasiosphaeriaceae		Microstromataceae
	Meruliaceae		Peniophoraceae
	Mycosphaerellaceae		Phaeosphaeriaceae
	Nectriaceae		Pleosporaceae
	Phaeosphaeriaceae		Pleosporales
	Pleosporaceae		Ramalinaceae
	Psathyrellaceae		Sordariaceae
	Sarocladiaceae		Sporidiobolaceae
	Sclerotiniaceae		Strelitzianaceae
	Xylariaceae		Taphrinaceae
			Tremellales
			Venturiaceae

Table 3.26 Subdivisions and families for the isolates from NGS (HTS) and Sanger sequence data for G samples from Glasnevin (G) Botanic Garden

Sanger sequencing		NGS	
Class	Family	Class	Family
Agaricomycetes	Aspergillaceae	Agaricomycetes	Botryosphaeriaceae
Cystobasidiomycetes	Cladosporiaceae	Agaricostilbomycetes	Erysiphaceae
Dothideomycetes	Cordycipitaceae	Dothideomycetes	Helotiaceae
Eurotiomycetes	Corticiaceae	Eurotiomycetes	Helotiales
Sachharomycetes	Cystobasidiaceae	Exobasidiomycetes	Kondoaceae
Sordariomycetes	Debaryomycetaceae	Lecanoromycetes	Leptosphaeriaceae
Ustilaginomycetes	Diaporthaceae	Leotiomycetes	Melanommataceae
	Diatrypaceae	Microbotryomycetes	Microstromataceae
	Didymellaceae	Taphrinomycetes	Peniophoraceae
	Dothioraceae		Phaeosphaeriaceae
	Glomerellaceae		Ramalinaceae
	Herpotrichiellaceae		Sporidiobolaceae
	Hypocreales		Strelitzianaceae
	Mycosphaerellaceae		Taphrinaceae
	Nectriaceae		Trichomeriaceae
	Ustilaginaceae		Xanthorioideae

Table 3.27 Common and shared genera between Roosky and Glasnevin obtained from NGS (HTS) and Sanger sequencing data for leaf samples.

Sanger	NGS
Acremonium sp.	*Acicuseptoria rumicis*
*Alternaria humuli**	*Alternaria infectoria**
Aureobasidium pullulans	*Bacidina flavoleprosa*
Aureobasidium sp.	*Botryosphaeria stevensii*
Boeremia exigua var. exigua	*Cadophora orchidicola*
Cladosporium sp.	*Herpotrichia parasitica*
Diaporthe eres	*Microstroma juglandis*
Diaporthe rudis	*Peniophora cinerea*
Diaporthe viticola	*Phaeosphaeria vagans*
Engyodontium album	*Phyllactinia fraxini*
Fusarium lateritium	*Rhodotorula bacarum*
Fusarium oxysporum	*Sclerostagonospora opuntiae*
Fusarium proliferatum	*Septoriella phragmitis*
Fusarium sp.	*Strelitziana eucalypti*
Mycosphaerella coacervata	*Taphrina antarctica*
*Phaeosphaeria gahniae**	*Phaeosphaeria carcicola**
*Phaeosphaeria pontiformis**	*Phaeosphaeria vagans**

Phoma sp.	*Trimmatostroma cordae*
Pyronema domesticum	*Xanthoria parietina*

* indicates common genera from Roosky site with NGS and Sanger sequencing that are not present in the Glasnevin site.

3.5 Conclusions

This chapter has revealed extensive endophyte diversity in *Fraxinus excelsior* and other *Fraxinus* taxa. Only a few taxa were shared between the two approaches of sampling endophytic diversity and the two lists need to be combined to fully represent the diversity present. The ash dieback pathogen, *Hymenoscyphus fraxineus*, was not detected by either approach in any of the samples even though was known to exist in the French samples (and possibly exist in Roosky). It is also clear that different sampling localities, different tissues (especially roots, shoots and seeds) support largely different communities. The culturable endophytes are of most interest for practical application as they represent the taxa that are now immediately available for testing biotic or abiotic stress resistance in ash. We have tested a small sample of this diversity in Chapter 5.

Chapter 4

Plant tissue culture of *Fraxinus excelsior* for *in vitro* biocontrol experimentation

4.1 Introduction

Fraxinus excelsior L. is an important forest tree for timber, furniture, veneer, flooring, traditional medicine and sports equipment including the hurley stick (Kew 2019; Lahiri *et al.* 2019). Tissue culture methods are required to improve ash micropropagation from seed and plantlets. This is important because ash germplasm needs *ex-situ* conservation and because ash is under serious threat from dieback disease (*Hymenoscyphus fraxineus*) that is destroying the forest ash stands across Europe (Kowalski 2006; Kowalski and Holdenrieder 2009a,b). Ash dieback spread in Europe from Central and Northern parts and was first reported from the Baltic state of Poland in the 1990s followed by many other countries including Germany, Austria, the Czech Republic, Switzerland, France, Hungary and recent reports from Slovenia, UK and Ireland (Juodvalkis and Vasiliauskas 2002; Przybyl 2002b; Barklund 2005; Heydeck *et al*. 2005; Thomsen and Skovsgaard 2006; Cech 2007; Kowalski and Holdenrieder 2008; Szabo 2008; Jankovsky and Holdenrieder 2009; Ogris *et al.* 2009). Trees of all ages are affected by the disease.

The seed dormancy period is relatively long and seeds can remain dormant in the soil for six years (Puchner 1922). The delayed germination is due to its relatively small embryo that requires a period of enlargement before the enveloping coats can be broken (Lakes States Forest Experiment Station 1935). European ash seed dormancy is often referred to as morpho-physiological (Baskin and Baskin 1998; Drâghici and Abrudan 2010). To break the dormancy for commercial purposes the seeds need a warm: cold stratification to remove phenolic inhibitors (Suszka *et al.* 1996). Dormancy can also be broken through tissue culture (Raquin *et al.* 2002).

Tissue culture also offers methods for controlled experiments on ash in sterile conditions and has the potential to isolate disease free material from pathogen-infected material (Lahiri *et al*. 2019). However, it is also known that even after tissue culture establishment of plants, endophytic bacteria and fungi may be present (Donnarumma *et al.* 2009). The endophytic community may also help

control disease infection and severity in natural stands of ash. Therefore, experimental systems are required for testing of endophytic fungi on *in-vitro* grown plants and to test them for bio-control applications Raquin *et al.* (2002) conducted some work on ash seed culture for *Fraxinus excelsior* and *F. angustifolia* but did not focus on endophytes or biocontrol. Here we expand that work with Irish *F. excelsior*. We optimized the media and growth conditions necessary for the germination and growth of explants. We also checked for endophytes on seeds and embryos of ash by culturing them on half strength malt extract agar media. We wanted to compare the media used by Raquin *et al.* (2002) with an alternative half strength MS media. We used two alternative *in-vitro* methods for the co-culture experimentation of fungal pathogens, endophytes and *in-vitro* grown ash. One of these is based on agar for shoot fungal endophytes and the other on perlite for root fungal endophytes; the latter being better aerated and hence theoretically more suitable for fungal mycelium growth.

Therefore, this chapter aimed to:

1. Find an optimal and rapid method to culture seed and embryos of ash to break dormancy.
2. Compare seed and embryo germination rates and establish the best way to obtain tissue culture explants/plantlets rapidly for further experimentation.
3. Establish an optimal method to establish large numbers of *in-vitro* cultured plantlets.
4. Develop an *in-vitro* testing system for plant, pathogen and endophyte interaction studies.

4.2 Materials and Methods

4.2.1 Collection and initial testing

Ash seeds were collected from a single mother tree to maintain uniform maternal stock. The tree was located at Loreto Park, Churchtown, Dublin 14 (N53º17'19.08", W6º16'8.63") and seeds were collected on 29th September 2016. The samaras (keys; fruits) were collected from the lower branches of the tree from random points and stored at 4°C until used. Five seeds and embryos were cultured from freshly collected seed to test a potential protocol before undertaking a full *in-vitro* experiment and to help refine the full experimental set up. The procedures were modified from the tissue culture study of *Fraxinus* by Raquin *et al.* (2002). A second tissue culture experiment was conducted on a large scale and using 6 months old cold-stored samaras. A third experiment was conducted the following

year on freshly collected seeds. The seeds for the third experiment were collected on the 1st September 2018 from a tree at Marlay Park, Rathfarnham, Dublin 16 (N53º26'92.70", W6º26'83.41"). In this experiment, a series of seeds were collected from the lower branches of a single tree and an *in-vitro* experiment for germination was conducted with half strength Murashiige and Skoog (MS) media, with either Agar or Phytagel as a gelling agent, to see if there is any effect on germination with different gelling agent.

4.2.2 Sterilisation process and media preparation

In the initial experiment, the first step was to select healthy undamaged fruits without holes in them that might indicate insect contamination by weevils; *Lignyodes bisclwffi* (Blatchley), *L. helvolus* (LeConte) and *L. horridulus* (Casey) (https://www.srs.fs.usda.gov/pubs/gtr/gtr_so096.pdf). This material was collected fresh without cold storage. After sorting, the pericarp was removed (seed depericarped) and the seed soaked in 7% calcium hypochlorite for at least 20 mins with occasional stirring (3-4 times) to clean the seed coat throughout its surface. After each round of stirring, the old solution was replaced with a fresh one. After 20-22 minutes, all the solution was discarded.

Seeds that had been kept in cold storage were used for the full experiment (experiment 2). Seeds were depericarped and washed once with sterile distilled water. Then they were soaked in 0.3 M NaOH for 20 mins. The liquid was discarded and the seeds were kept in 0.2 % calcium hypochlorite overnight at 4°C (to rehydrate the seeds which had become dry during storage). After discarding the liquid, they were transferred to 2% calcium hypochlorite for 2 hours for sterilisation. Finally, seeds were thoroughly washed with sterile distilled water (five to six times). They were transferred to half strength MMS media with 0.6% agar and without any additional sugars and kept in the dark for two weeks at ± 18°C. After two weeks, seeds were kept in continuous light for 1600 $\mu mol.m^{-2}$ s^{-1} PAR light intensity at ± 18°C.

For the third experiment, seeds were collected fresh (note only mature seeds should be used; personal observation/experimentation) on 1st of September 2018, soaked overnight and treated with the same protocol for dormancy breaking as followed for the cold stored seeds.

The media was modified from that reported in Raquin *et al.* (2002). We chose a half strength commercially available MS media (Sigma M5524). The modified MS media with no added sugar showed good results and is outlined in Table 4.1. We chose half strength MS media without any added sugar because seeds already have a lot of storage food as carbohydrate (including starch) and protein. Therefore, they did not need the full strength media for the initial germination experiment.

Table 4.1 The MMS media per litre (Sigma M5524) components

	Name	Concentration (mg or %)
Macronutrients	NH_4NO_3	825
	KNO_3	950
	$MgSO_4$	90.35
	$CaCl_2$	166.1
	KH_2PO_4	85
	K_2HPO_4	-
	Fe EDTA	-
Micronutrients	H_3BO_3	3.1
	$CoCl_2.6H_2O$	0.0125
	$CuSO_4.5H_2O$	0.0125
	$FeSO_4.7H_2O$	13.9
	$MnSO_4.H_2O$	8.45
	KI	0.415
	$Na_2MoO_4.2H_2O$	-
	$ZnSO_4.7H_2O$	4.3
	NaFe-EDTA of 5ml/l ($FeSO_4.7H_2O$;Na_2-EDTA)	13.9; 18.63
Gelling agent	Phytagel*	-
	Agar**	0.6%
Sugar	None	
Vitamins	None	
Hormones	None	

*Phytagel (Sigma P8169); **Agar (Sigma A-5054)

In our third experiment, after the seeds were germinated, the plantlets were transferred into full strength MS media without sucrose. Due to unsatisfactory growth of the plantlets after being transferred, we changed our media to MS media (Sigma 5519; Table 4.2); the components are different from the first one and we added 1% sucrose with the full strength MS media.

Table 4.2 The MS media per litre (Sigma M5519) components

	Name	Concentration (mg or %)
Macronutrients	NH_4NO_3	1650
	KNO_3	1900
	$MgSO_4$	180.70
	$CaCl_2$	332.20
	KH_2PO_4	170
	K_2HPO_4	-
	Fe EDTA	-
Micronutrients	H_3BO_3	6.20
	$CoCl_2.6H_2O$	0.0250
	$CuSO_4.5H_2O$	0.0250
	$FeSO_4.7H_2O$	27.80
	$MnSO_4.H_2O$	16.90
	KI	0.830
	$Na_2MoO_4.2H_2O$	0.250
	$ZnSO_4.7H_2O$	8.60
	NaFe-EDTA of 5ml/l ($FeSO_4.7H_2O$;Na_2-EDTA)	27.80; 37.260
Gelling agent	Phytagel*	0.8%
	Agar**	0.8%
Sugar	Sucrose	1%
	Glycine	2
Vitamins	Myo-inositol	100
	Nicotinic acid	0.50
	Pyridoxine hydrochloride	0.50
	Thiamine hydrochloride	0.10
Hormones	NAA****	0.020; 0.010; 0.007

*Phytagel (Sigma P8169); **Agar (Sigma A-5054); Sucrose (AnalaR product 102744B); ****NAA (Sigma 317918).

4.2.3 Tissue culture

4.2.3.1 Initial experiment

Surface sterilised seeds were transferred onto the modified MS media (MMS; Table 4.1). A total of 10 Petri plates were included for initial testing with either 5 seeds per plate or 5 embryos per plate. Embryos were dissected from the seed coat under a microscope (40x magnification) with scalpels and forceps. Ease of removal depends on dehydration status of the seed (fresher the easier). Sterile seeds were handled on clean Petri dishes viewed under the dissecting microscope and given a cut along their middle. The half seed containing the embryo was carefully removed from the seeds without damaging any tissues and placed on to a media containing Petri dish and sealed with parafilm on the edges. For the seed

treatments, the ends and sides of the seeds were removed and the remaining edgeless seed transferred to the growth media.

4.2.3.2 Full, second, experiment

Seed material was cultured in two ways. Half of them were dissected and the embryos cultured on half strength MMS media with 0.6 % agar and no additional sugars. Five embryos were cultured in each Petri dish. The remaining half of the Petri dishes was used for seed culture as described above for their seed coat removal (5 seeds per Petri dish). All the Petri dishes were sealed with parafilm and kept in the dark at ± 18°C.

After four weeks in continuous light, the seedlings were transferred into full strength MS media supplemented with 10g sucrose per litre. They were transferred into test tubes filled with media with the help of sterile forceps and scalpel and kept in continuous light at +/- 18°C for further growth.

4.2.3.3 Third experiment

Seeds collected from Marlay Park were cultured on Petri dishes containing ½ MS with Agar or Phytagel. After culturing they were kept in dark at ± 18°C. The seeds were also cut along the edges as in the above experiment to facilitate the movement of media inside the seeds and to prevent any physical barrier to germination. Each Petri dish contained 5 cut seeds or embryos.

4.2.4 Growth conditions

Petri plates were kept at room temperature and dark conditions for 14 days for initiation of germination. From the third week onwards they were given a continuous light exposure 1600 $\mu mol.m^{-2}\ s^{-1}$ PAR for 48 hours followed by dark for the rest of the hours in that week. From the fourth week and fifth week the light exposure was increased by exposing them for 72 hours of light followed by 48 hours of dark. From the sixth week, the Petri plates were kept under constant light exposure 1600 $\mu mol.m^{-2}\ s^{-1}$ PAR. Temperature was maintained at +/- 18 °C throughout.

During the second experiment, the Petri dishes were kept in the dark for one and half weeks and then place under continuous light 1600µmol.$m^{-2}s^{-1}$ PAR at +/- 18° C. Furthermore, 100 seeds were grown on MEA to test for endophyte presence.

For the third experiment, Petri dishes were kept in the dark for three weeks to initiate germination, then from the fourth week Petri dishes were kept under a light exposure of 1600 µmol.m^{-2} s^{-1} PAR. Temperature was maintained at +/- 18 °C throughout.

4.2.4.1 Methods for endophyte screening on Perlite

For Perlite experiments, well rooted ash plantlets were transferred into test tubes containing 1.5g of Perlite and 10ml of full strength MS media with 1 % of sucrose supplement. 52 plants were transferred into Perlite to test their growth. For the third Perlite experiment germinated explants were transferred onto full strength MS with 0.6% Agar without any sucrose to maintain the continuity for further experiment with the endophytes. After a few days it was observed that the growth of the plants was not satisfactory and that they did not produce roots. Therefore we changed the MS media for these plantlets and used a different MS media (Sigma M5519). This is Murashige and Skoog Basal medium; the previous one was the Murashige and Skoog Basal salt mixture (Sigma M5524). The components of this MS media (Sigma M5519) are slightly different and are given in Table 4.2. For rooting, we have used different concentrations (7, 10, 20 mgL^{-1}) of auxin NAA (1-Napthaleneacetic acid) (Sigma 317918) dissolved in 1N NaOH prior to pH adjustment.

4.3 Results

4.3.1 Initial experiment

Among the 10 Petri dishes each containing single explants, 4 Petri dishes with embryo showed positive germination results. However, the 4 Petri dishes with cut seeds showed no germination. Two Petri dishes were contaminated. The first germination responses were observed after 21 days; plumule and radicle formation started, and the seedlings showed distinct growth from 35 to 46 days. After 46 days, green leaf formation had taken place. The shoot and roots were clearly observed by the end of this period. Most of the seeds from the seed cutting cultures did not germinate. However one did germinate after 40 days of culture. The rest of the seeds had no response in the MMS media.

4.3.2 Full experiment results

In the full experiment, we performed seed cut culture and embryo culture experiments. Five seeds or embryos were cultured on each Petri dish. Table 4.3 and Table 4.4 and Figure 4.3 showed the results for initiation of germination.

Table 4.3 Germination results for embryo culture

Plate number	Germinated embryos	Negative response	Survival after continued culture
1	5	0	2
2	5	0	3
3	2	3	1
4	4	1	3
5	1	4	0
6	5	0	1
7	5	1	2
8	5	2	1
9	5	0	2
10	6	0	4
11	5	0	2
12	6	0	2
13	5	1	3
14	5	0	1
15	3	2	2
16	5	0	2
Total	*72*	*14*	*31*

Percentage germination rate 83.7%

Table 4.4 Germination results for seed cut culture

Plate number	Germinated seeds	Germination initiated	Negative response	Survival after continued culture
1	1	2	2	3
2	1	4	0	5
3	2	1	1	3
4	1	2	2	3
5	1	0	4	1
6	3	0	2	3
7	1	0	4	1
8	2	0	3	2
9	2	1	3	3
10	4	1	0	5
11	4	0	2	4
12	1	2	2	3
13	1	2	2	3
14	1	0	4	1

15	4	1	0	5
16	1	4	2	5
17	1	4	2	5
18	2	3	2	5
19	1	3	2	4
20	4	0	2	4
21	1	1	3	2
22	1	2	2	3
23	3	1	1	4
Total	*43*	*35*	*48*	*77*

Percentage germination rate 62%

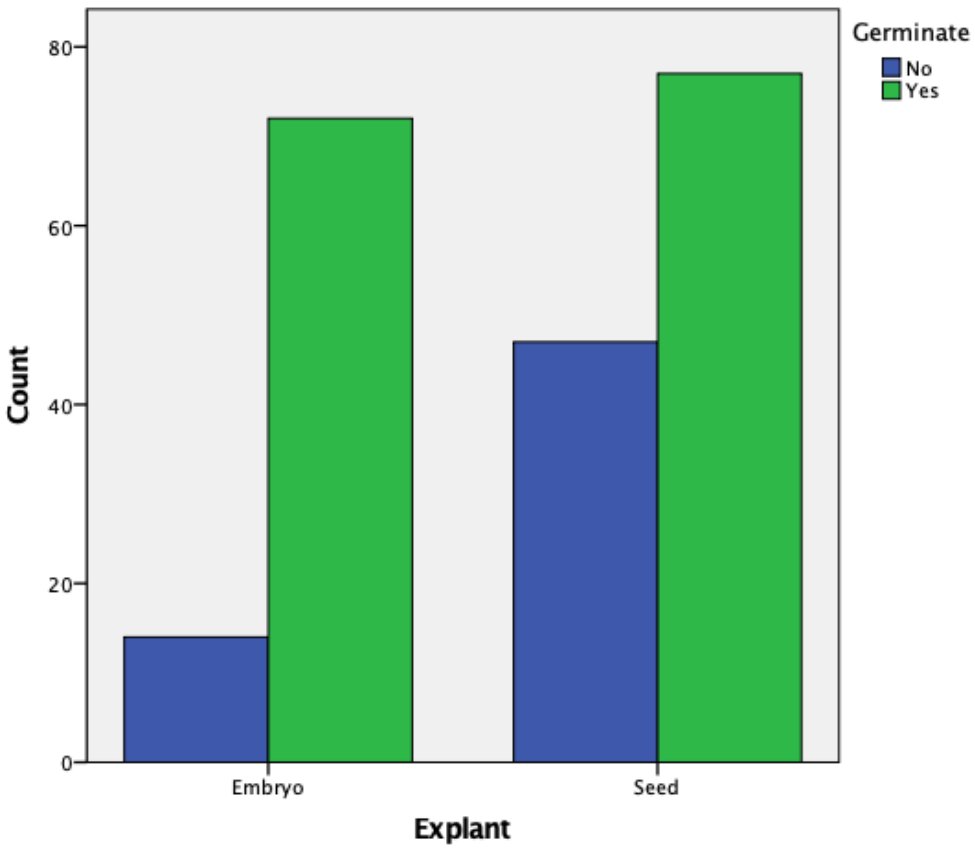


Figure 4.1 Bar chart for germination success and explant type. Germination success was 83.7 for the embryos and 62% for the seed cutting treatment.

A Chi square analysis was undertaken assessing whether germination rate was dependent on explant type or not. The null hypothesis for this Chi square test was that germination rate was not dependent on explant type (seed or embryo). The alternate hypothesis was that germination rate was dependent on explant type. The Chi square (X^2) value = 11.521 and degrees of freedom (df) 1 and the *p* value is 0.001 (details are given in Appendix III). Therefore the null hypothesis that the

germination rate is not dependent on explant type was rejected. Germination success was higher in embryo culture but survival higher for seed cut culture (Table 4.3, 4.4; Figure 4.2).

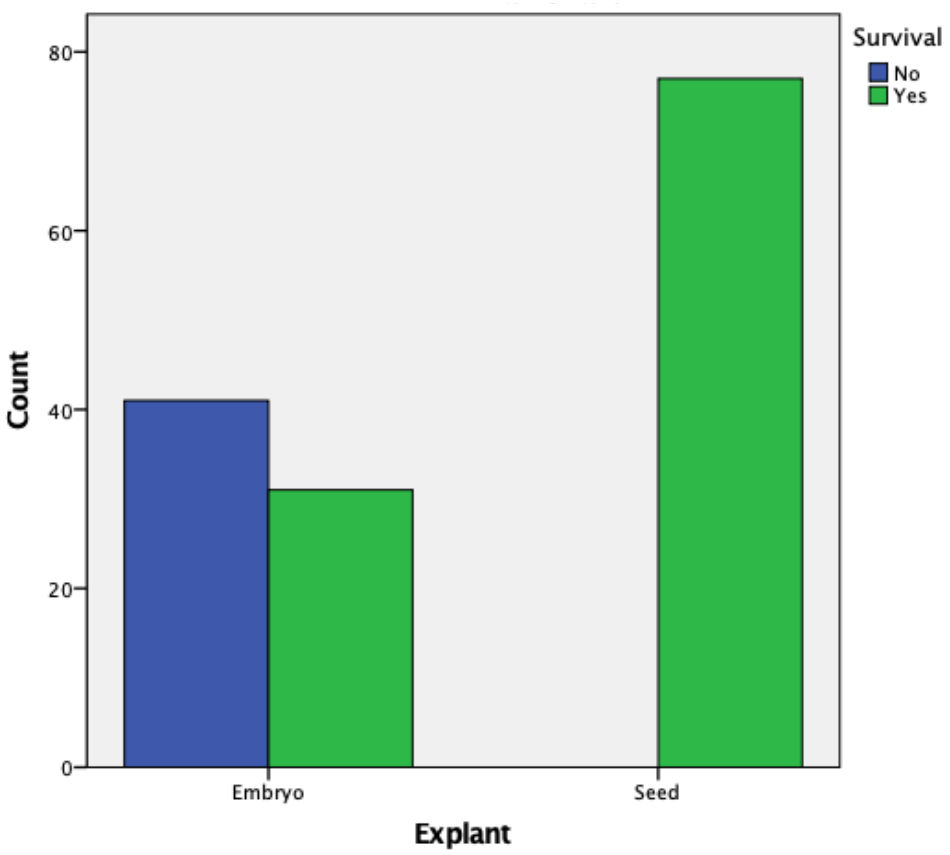


Figure 4.2 Bar chart for the survival rate of the explants as germinated embryos or seeds. Survival rate was 43% for the embryos and 100% for the cut seeds.

A Chi square test was performed to test whether survival of germinated plants was dependent on explant type or not. The null hypothesis for this test was survival of germinated plants was independent on explant type. The alternative hypothesis was whether survival of germinated plants was dependent on explant type. The Chi square (X^2) value was 60.493. The df was 1 and the *p* value was 1.326×10^{-13} (details are given in Appendix III). The null hypothesis that the survival post germination is independent of explant type was therefore also rejected. In the Figure 4.2 there was no bar for no survival for the seed explant (as all survived) so it clear that the cut seed plants had highest survival.

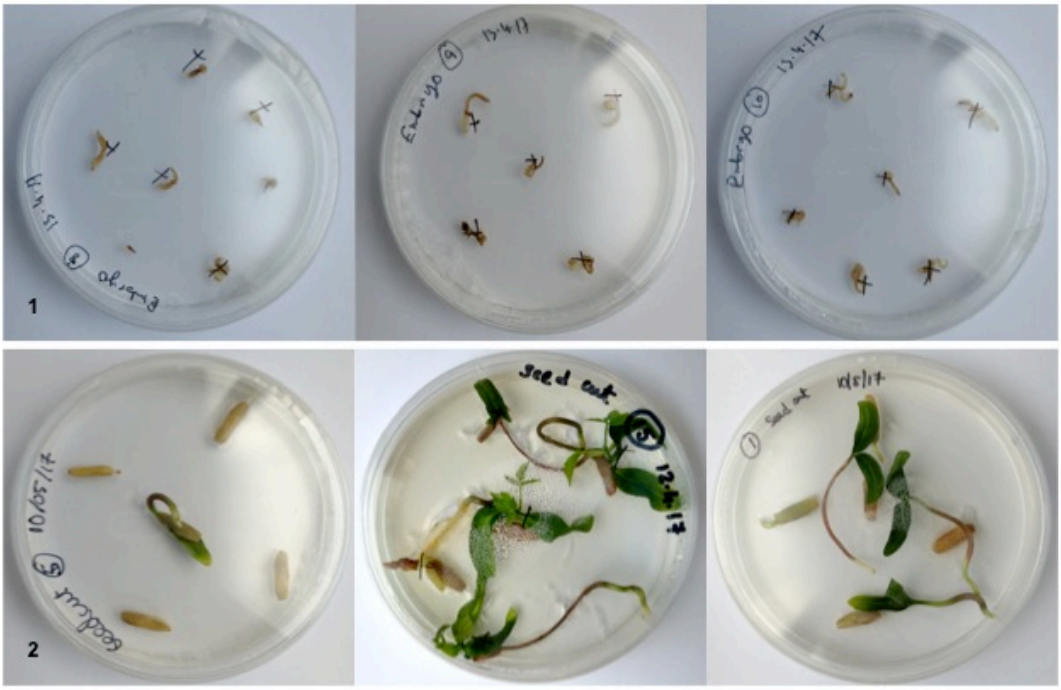


Figure 4.3 Tissue culture of ash seeds and embryos. 1) Germinated embryos after embryo culture (upper three plates), 2) Germinated seeds after seed cutting treatment (lower three plates).

The results for the third experiment of germination percentages of seeds are shown in Figures 4.4 and Table 4.5 and 4.6. Figure 4.5 shows the growth of the rooted plantlets after being transferred into test tubes with MS solid media (agar) and MS liquid media with Perlite.

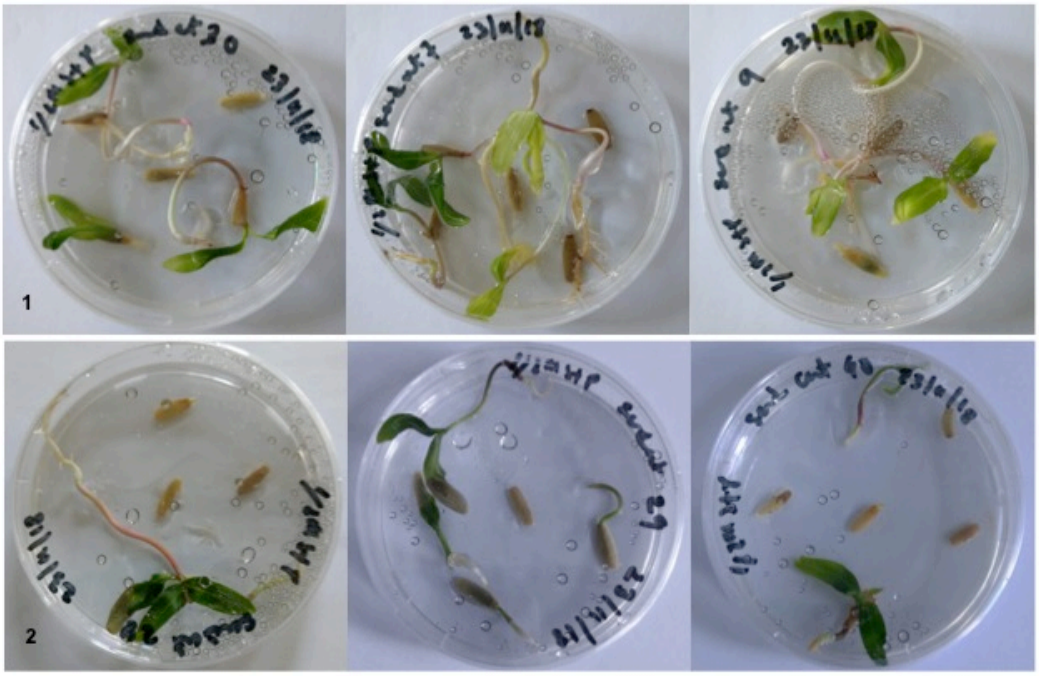


Figure 4.4 Tissue culture of ash seeds in third experiment. 1) and 2) Germinated seeds after seed cutting treatment.

Figure 4.5 Tissue culture of ash plantlets from the first and second experiment. 1) and 2) showing well rooted plantlets in MS+1% sucrose media. 3) more rooting observed in the same media, 4) Rooted plantlets after being transferred to perlite liquid MS+1% sucrose media.

Seed and embryo germination results for the third experiments.
Germination was marginally better on Phytagel than agar (Table 4.5 and Table 4.6). However, the overall germination was markedly higher for the embryos than the cut seed treatment (Table 4.6)

Table 4.5 Germination of seeds and embryos for MS with Agar/Phytagel

No.	Media	Germinated seeds or embryos	Total number of seeds or embryos (n)	(%) germination
1	½ MS+P°	147	365	40.27
2	½ MS+A°°	106	370	28.64
3	½ MS+P	18	205	8.80
4	½ MS+A	30	205	15.60
5	½ MS+P	59	270	21.80
6	½ MS+A	83	265	31.30
7	½ MS+P	78	225	32.00
8	½ MS+A	20	220	9.00
9	½ MS+P	25	95	26.30
10	½ MS+A	2	40	5.00
11	½ MS+P	79	220	36.00
12	½ MS+A	63	195	32.30
13	½ MS+P+e•	25	65	38.50
14	½ MS+A+e••	56	80	70.00
15	½ MS+P+e	45	50	90.00
16	½ MS+A+e	44	50	88.00

°half strength MS media with Phytagel for seeds, °° half strength MS media with Agar for seeds, • half strength MS media with Phytagel for embryo, •• half strength MS media with Agar for embryo.

Table 4.6 Total number of germinated seeds and embryos (combined from Table 4.5 above).

No.	Media	Total germinated seeds or embryos	Total number of seeds or embryos (n)	(%) germination
1	½ MS+P°	406	1380	29.42
2	½ MS+A°°	306	1295	23.62

3	½ MS+P+e•	100	130	77.00
4	½ MS+A+e••	70	115	61.00

° half strength MS media with Phytagel for seeds, °° half strength MS media with Agar seeds, • half strength MS media with Phytagel for embryo, •• half strength MS media with Agar for embryo.

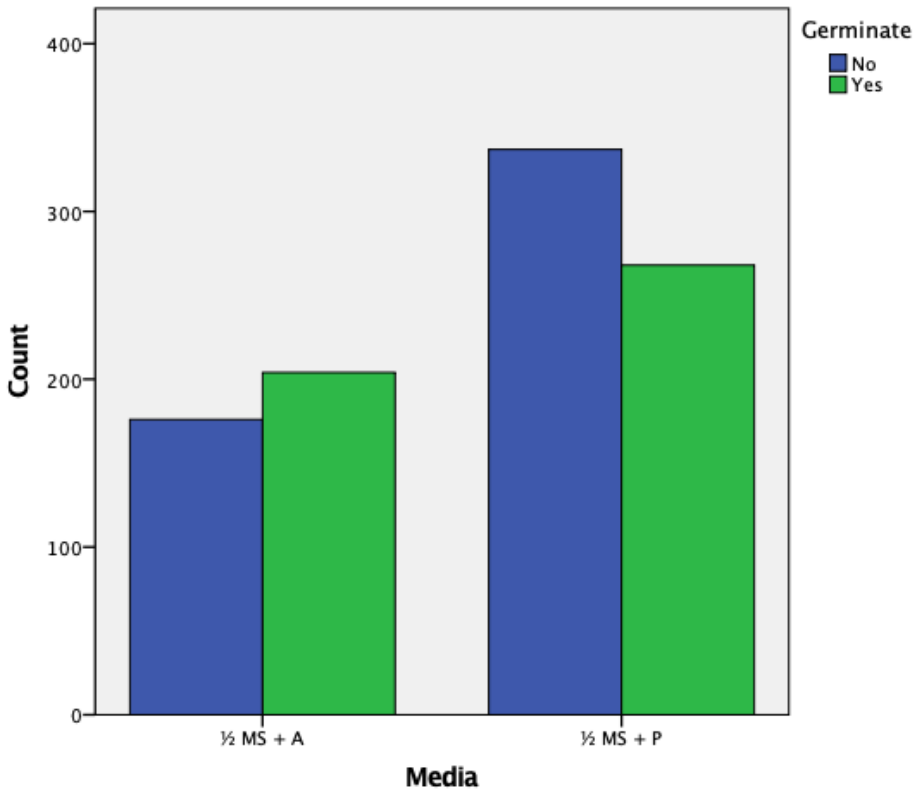


Figure 4.6 Germination rates of seeds for two media (different gelling agents: A=Agar and P=Phytagel).

A Chi square test analysis was performed to see if germination of seeds was dependent on the gelling agent. The null hypothesis for this test was germination of seeds were independent of gelling agent. The alternate hypothesis was germination of seeds were dependent of gelling agent. The Chi square (X^2) value = 8.240, df was 1 and the *p* value was 0.004 (details are given in Appendix III). Therefore the null hypothesis that germination rate was not dependent on gelling agent was rejected. The results can also be observed from Figure 4.6 that there were dependency on the gelling agent for germination.

4.3.2.1 Rooting

In the first and second experiments, the rooting of the germinated plantlets was observed on 10 ml full strength MS (Sigma 5524) with 1% sucrose and 1.5 g of Perlite. After four weeks the roots had grown 1 - 2 cm in length. In the third experiment, plantlets mostly did not show rooting except a few. The only difference between the two experimental setups was absence of sucrose in the media. Therefore, we subsequently tried five different treatments to establish the best rooting media. Those media were full strength MS (Sigma 5519) with no sucrose, full strength MS (Sigma 5519) with 1% sucrose and full strength MS (Sigma 5519) with 1% sucrose and 7, 10, 20 mgL^{-1}NAA. Observations are presented in the Table 4.7.

The rooting results (Table 4.7) demonstrate that sucrose was essential for rooting of the ash plantlets. The highest rooting (68.5%) was observed for the 1% sucrose medium. We also found that 20 mgL^{-1} auxin (NAA) was better in combination with 1% sucrose as it showed 51.2% of rooting in the plantlets compared to 10% rooting with 7 mgL^{-1}. However, none of the NAA treatments were as good as sucrose alone.

Table 4.7 Rooting of plantlets on five different media.

No	Media	Total plantlet/embryo	Rooting	Not rooting	% rooting
1	MS+no sucrose+p*	626	49	577	8.5
2	MS+no sucrose+e**	95	0	95	0
3	MS+1% sucorse	278	113	165	68.5
4	MS+1% sucrose+7mg NAA	54	5	49	10.2
5	MS+1% sucrose+10mg NAA	17	4	13	31.0
6	MS+1% sucrose+20mg NAA	62	21	41	51.2

* full strength MS without sucrose for plantlets, **full strength MS without sucrose for embryo

4.3.3 Endophyte culture

Twenty Petri dishes (with 5 explants per plate) were tested for endophyte culture of sterilized cut seed and 20 Petri dishes (with 5 explants per plate) for endophyte culture from isolated embryos. No endophytes emerged from these cultures. Thus it is assumed that they are endophyte free (or 'culturable endophyte free').

4.4 Discussion

4.4.1 Dormancy breaking

Embryo culture showed higher success in germination rate than seed cutting treatment. Our result is in agreement with Raquin *et al.* (2002) who found the embryo germination rates were high for *Fraxinus excelsior* and that the embryos could be successfully grown on to become plantlets and established trees. However in the case of our embryo culture experiments, it was difficult to grow the germinating embryos on further as most of them turned brown fairly quickly, presumably due to suboptimal nutrition or incomplete development. They did not recover and grow back in more than 60% of the cases after they were transferred to fresh full strength MS media. The germination rates of cut seeds were relatively low compared to embryo culture (62 vs. 83%; Chi square p = 0.0011) but when they did germinate, growth was rapid, presumably because they have access to the nutrients and other essential components from the endosperm. Therefore, despite the lower germination rate success with seeds, the plantlet establishment was higher from the seed cut cultures than embryo culture, (survival 43 vs 100%; Chi square $p = 1.326 \times 10^{-13}$). The seed cutting technique was therefore an effective method to break the dormancy as described by Sambeek *et al.* (2007) and ensure subsequent survival.

Seed cutting and embryo culture allowed rapid germination in a sterile system with no dormancy because the seed coat is not restricting the growth of the relatively small embryo. The seed coats are made up of five to eight layers of cells (Steinbauer 1937; Finch-Savage and Clay 1997; Chmielarz 2009). The endosperm is separated from the innermost layer of cells by a suberized membrane. The exact composition of the suberized membrane is unknown but some chemical tests show presence of cutin and suberin (Steinbauer 1937). This membrane is the resistant layer outside the embryo. It might prevent the embryo from rapidly expanding. To break the dormancy in the wild, a 5°C temperature is ideal to enlarge the embryo and help the various digestion of stored starch carbohydrate and protein in the seed coat (Steinbauer 1937; Nikolaeva *et al.* 1985; Chmielarz 2009).

4.4.2 *In-vitro* plantlet establishment and micropropagation

Half strength MMS media was shown to be sufficient for initial germination. After germination of the plantlets they were cultured onto full strength MS media with 1% of sucrose as a sugar supplement. The plantlets started growing roots without any hormone supplement for the first and second experiments. In case of third experiment, initially germinated plantlets were transferred into full strength MS without sucrose supplement for further growth and rooting. However, we later found that sucrose is essential for rooting by comparing five different rooting media. Four media contained full strength MS with 1% sucrose with either 0, 7, 10, or 20 mg of NAA L^{-1}; one of which lacked sucrose. After a month, the highest degree of rooting was observed in the media supplemented with 1% sucrose without hormone (68.5%) and the media with the highest (20 mgL^{-1}) of NAA (51.2%). Other studies have found good rooting in 7mg L^{-1} NAA or even lower concentrations such as 0.5mgL^{-1} (Lebedev and Shestibratov 2016). A few studies have used a combination of NAA and IBA for successful ash rooting (Preece *et al*. 1987; Kim *et al*. 1998; Sambeek *et al.* 2007). It is not clear why full strength MS with 1% sucrose performed better than the same treatment with NAA (20mgL^{-1}). However we have shown how to efficiently break the dormancy of *Fraxinus excelsior* seed (with cut seeds) and get aseptic plantlets for future experiments. It is also possible to establish a large number of disease/endophyte free plantlets (below).

4.4.3 Tissue culture for the removal or reduction of endophytes

We checked for endophytes in seed and embryo by culturing them in half strength Malt extract agar and did not detect any in our samples. In comparison, we have isolated 518 endophyte cultures from ash leaf material and 110 endophyte cultures from roots using the same sterilisation, media and culture technique (Chapter 2). Therefore we can conclude that the tissue culture methods are suitable for endophyte removal in *Fraxinus excelsior*. We only tested for fungal endophytes and it is possible that bacterial endophytes may remain. However, no detectable bacterial endophytes were found from the surface sterilised seeds or embryos. It is important for *in-vitro* performed ecophysiological studies involving endophytes to remove any endogenous endophytes so that the effect of the target endophyte(s) can be established. We have shown through our endophyte isolation experiments that embryo culture and seed cutting *in-vitro* offers a way to achieve this goal.

Some other studies have shown the presence of endophytes after micropropagation in several other species such as banana (Dubois *et al.* 2004) and

mint (Reed *et al*. 1995). Our studies are the first, to date, on *Fraxinus* endophytes and *in-vitro* micropropagation. Some other studies have also demonstrated the efficiency of tissue culture to obtain disease free plantlets. In many cases tissue culture has been conducted to obtain disease free plantlets in a small amount of time such as in sandalwood (Rao *et al.* 1978), apple (Lane 1978), vanilla (Philip 1986), horticultural pot plants (Rout *et al.* 2006) and forest trees (Karnosky 1981).

4.4.4 Tissue culture for endophyte, pathogen and plant interaction studies

Culture systems are needed for many studies involving microbe and ash interactions. For example, systems are required to test genotypic variation in ash resistance to diseases such as dieback disease. Furthermore, studies are required to study the interaction of ash with its microbiome and its endophytes. Here we provide a description of two alternative culture systems for the co-culture of endophytes, plant and pathogens. Agar with MS media with sucrose supplement is suitable as a general *in-vitro* culture system and perlite most suitable for root endophytes (including mycorrhizal fungi) and pathogenic fungi that prefer a well-aerated media (See Lahiri *et al.* 2019, Supplement; and Chapter 5). Perlite has been found to be suitable for growing endophytes including mycorrhizal fungi in several other studies but never in ash. These include studies on wheat with *Glomus fasciculatum* (Sreenivasa *et al.* 1988); *G. mosseae* (Hawkins *et al*. 2000) and spruce with *Amanita* (Schrey *et al.* 2005).

4.5 Conclusions

Ash dieback is a potential threat to the ash population throughout Europe and is spreading rapidly by its spores that are able to travel over long distances. As common ash has high economic value and covers huge land areas in plantations and woodlands, it is vital to find ways of improving its disease resistance. The *in vitro* culture methods we have developed will aid in the study of disease resistance in ash. The *in vitro* antagonistic and *in vitro* biocontrol assays including endophyte and pathogen are performed in an enclosed and otherwise sterile environment so that the pathogen will not escape into the environment and so that the direct interaction of the microbes can be assessed without the influence of other organisms

4.6 Supplement

In vitro Methods for Plant-Microbe interaction and Biocontrol Studies in European ash (*Fraxinus excelsior* L.)

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Abstract

In vitro tissue culture systems are required for plant-microbe interaction studies on European ash, *Fraxinus excelsior*. Methods are needed for plant micropropagation and for physiological experimentation including pathogen/resistance testing and biocontrol studies. For example, systems are required for experiments on ash dieback disease, caused by the ascomycete fungus *Hymenoscyphus fraxineus,* that is killing ash plantations and natural populations across its native range. Methods are also needed to optimise the number of endophytes cultured from ash tissue and to taxonomically identify them. We present endophyte isolation protocols

and media for ash, provide an optimised DNA barcoding procedure for endophyte identification and describe *in vitro* tissue culture methods suitable for ash-microbe interaction studies in both roots and shoots. Methods for both embryo culture and seed culture (with precutting) and for the bulking up of genotypes via single node culture are outlined. We also discuss the potential of tissue culture for establishing microbe/endophyte free cultures.

Keywords Ash dieback disease, DNA barcoding, *Fraxinus excelsior*, fungal endophytes, *Hymenoscyphus fraxineus*, *in vitro* culture, microbiome, tissue culture

15.1 Introduction

There has been growing interest in understanding the microbiome, and more specifically the endophytes, of trees for various applications including forestry, horticulture, plant protection and phytoremediation. The first published use of the term 'endophyte', but actually described as 'Entophytae', was in 1809 by Heinrich Friedrich Link, primarily for a group of fungi that are partly parasitic in nature (Link, 1809). The term endophyte was then also applied for bacteria and other organisms (Chanway, 1996; Hallmann, 1997). Petrini (1991) defined endophytes as "organisms inhabiting plant organs that at some time in their life cycle can colonise internal plant tissues without causing apparent harm to their host". We follow that definition in this paper. According to sequence data obtained from National Centre for Biological Information (NCBI), fungal endophytes of plants mostly belong to the five primary classes (Hardoim *et al.,* 2015): Glomeromycota (40%), Ascomycota (31%), Basidiomycota (20%), Zygomycota (0.1%) and unidentified phyla (8%). Unidentified taxa demonstrate the huge diversity of fungal endophytes remaining to be discovered and further studied (Murphy *et al*., 2015 Hodkinson, 2018).

Little is known about the endophytic community of European ash, *Fraxinus excelsior* L., but research has recently focused on this species because of the threat of ash dieback disease that is destroying ash populations and plantations across Europe (Kowalski, 2006; Kowalski and Holdenreider, 2009a, b). This chapter describes what is known about the microbiome and endophytic community of *F. excelsior* and outlines *in vitro* methods for the experimental study of endophyte, pathogen and ash tree interactions. Such methods are needed to reduce the risk of spreading the disease in open field sites of greenhouse environments. *In vitro* culture also allows precision so that experiments can include only the target organisms, in this case the dieback disease pathogen (*Hymenoscyphus fraxineus*), the endophyte species (or combinations of species) and the ash tree.

15.2 Ash Dieback Disease

Fraxinus excelsior is an important forest tree for timber, furniture, veneer, flooring, traditional medicine and sports equipment, including the Hurley stick used in the Irish sport hurling. Ash dieback disease is caused by a fungal pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya (Baral *et al.,* 2014). It was originally, and commonly, called *Chalara* but its generic name was changed to *Hymenoscyphus* after the full characterisation of its sexual and asexual stages, teleomorph and anamorph, respectively (Baral *et al.,* 2014). Ash dieback disease can affect all age groups of *F. excelsior* (Kowalski *et al*., 2017). It often infects plants from fungal spores and leaves can be easily infected. Fungal spores are present in the previous year's ash litter and are able to disperse over tens of kilometers (Forestry Commission UK, 2015). Movement of diseased ash logs, following felling can also facilitate spread over large geographic areas (Forestry Commission UK, 2012).

The symptoms of the disease can be seen throughout the tree. Affected ash often first show leaf wilt in which the pinnae (leaflets) become dry and droopy because of loss of their turgor pressure (Kräutler *et al*., 2012). The leaflet petiole changes colour from green to light brown and then becomes black. The main veins are more resistant than minor ones and the fungus develops within them and can produce sclerotia on the rachis that can remain dormant (Douglas *et al*., 2013). The disease causes a change in colour of the stem from green to brown in young material and then to black. It also affects the primary branches, and can lead to the formation of epicormic shoots, which can become numerous (Sansford, 2013; Gross *et al*., 2014). In mature woody parts of the tree, the mycelium can grow inside the stem and trunk and, in turn, it blocks all the xylem and damages the water transport system (Gross *et al.,* 2014). It can be observed when a young stem is cut longitudinally to reveal brown or black strips of necrotic damaged xylem tissue. The necrotic lesion on the stem is known as canker and can often look diamond or lens shaped. Once the disease develops, crown die back occurs which mostly affects the upper crown. Canker of ash can also be caused by other biological organisms such as bacteria *Pseudomonas syringae pv. savastanoi* (Smith, 1908) Young *et al.* 1978, and other fungi such as *Nectria galligena* Bres. (Janse, 1981; Douglas *et al.,* 2013). The fungus can also be isolated from the roots of diseased ash where it weakens the root and can lead to the introduction of other fungal pathogens (Bakys, 2013). One of these is the honey fungus *Armillaria borealis* Marxm. & Korhonen, 1982, that is particularly damaging (Bakys, 2013).

15.3 Geography of Ash Dieback Disease

Ash die back disease was first observed in Poland in 1996 (Przybył, 2002; Kowalski and Łukomska, 2005). Major outbreaks have occurred in several European countries and the disease has spread rapidly. Altogether, at least 23 countries have so far reported the occurrence of ash dieback disease

(Timmermann *et al.,* 2011). One of the earliest European regions to report significant levels of ash dieback was Scandinavia, where it was first observed in 2001. By 2004−06 the disease had spread throughout the Scandinavian ash distribution range leading to destruction of its populations (Barklund, 2005; Stenlid and Barklund, pers. comm). It was first recorded in Denmark in 2003 and in Norway ash dieback was first documented in the south−eastern parts in 2008 (Thomsen, 2005). According to the report of Forest Commission Survey of UK (Broome and Mitchell, 2017), the total number of cases of ash dieback has significantly increased between 2012 to 2017 in Scotland, England, Wales and Northern Ireland. The first reported case in the Republic of Ireland was in October 2012 in a forestry plantation site in County Leitrim and it has spread throughout the country (DAFM, n.d.). Thus, the disease has now spread throughout most of the natural distribution range of *F. excelsior*.

15.3.1 The Ash Microbiome

A few studies have been published on the ash microbiome in recent years. Those studies reveal our knowledge about endophytes in healthy trees and help us to compare our newly isolated endophytes with the list of organisms already revealed. Schlegel *et al.* (2016) studied ash endophytes as potential biocontrol agents. The study was conducted in *F. excelsior* and *F. ornus* L.. First, the influence of exudates from isolated *F. excelsior* endophytes was tested *in vitro* (on Petri dishes) against the ascospore germination of *H. fraxineus*. Strong inhibitory effects on ascospore germination were recorded from the exudates of *Paraconiothyrium* sp., *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley, *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin, *Ampelomyces quisqualis* Ces. and Elsinoaceae sp.. Weak effects were observed for exudates of *Venturia* spp. and *Nemania serpens* (Pers.) Gray. Second, the protective effects of endophytes against the ash dieback pathogen were studied in the field (infected forest) using endophyte−free and

plants pre−inoculated with endophytes. *Venturia* spp. dominated the endophytic community in the field grown trees inoculated with endophytes, but no significant effect of endophyte inoculation was seen on plants after they had been infected with *H. fraxineus*. Thus, no evidence for field−based endophyte biocontrol of *H. fraxineus* was found.

Another study by Kosawang *et al.* (2018) was conducted on fungal communities from resistant *Fraxinus* for use as biocontrol agents. Fungal isolations were undertaken from five *H. fraxineus*, tolerant ash species (*F. chinensis* subsp. *rhynchophylla* (Hance) A.E. Murray*, F. lanuginosa* Koidz.*, F. mandshurica* Rupr.*, F. ornus* and *F. pennsylvanica* Marshall) and endophytes identified using DNA sequencing (internal transcribed spacer (ITS) 1 and 2). They isolated 196 fungal taxa belonging to 15 families, 9 orders and 40 species. Most of their endophytes were ascomycetes except a single basidiomycete, namely *Peniophora* sp.. They performed antagonistic activity assays by growing the endophyte with *H. fraxineus* on half−strength PDA (with ash leaf supplement). Endophyte species showing high antagonistic activity included *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley*, Epicoccum nigrum* Link, *Fusarium* sp.*, Sclerostagonospora* sp. and *Setomelanomma holmii* M. Morelet. In our endophyte isolations from ash (Table 15.1 and 15.2) we also found three of these species from leaf tissues, namely *Boeremia exigua, Epicoccum nigrum,* and *Fusarium* sp..

15.4 *In vitro* culture of ash for plant-microbe interaction studies

Tissue culture methods are required to improve ash micropropagation from seed and plantlets. They provide an enclosed system for the co−culture of pathogen, endophyte and plant and hence prevent unintentional release of the microorganisms into the environment. The *in vitro* systems allow precise control of the target endophytes and pathogen and exclusion of other microbes. Hence, they represent ideal phenotyping systems in ash. Tissue culture methods are also

needed for ash because germplasm needs conserving *ex situ,* as field collections are threatened by dieback disease. We therefore describe the methodology we have successfully used for the collection, isolation and culture of ash endophytes and describe *in vitro* systems for plant−microbe interaction studies in ash.

Table 15.1. Predominant endophytes from leaf and rachis tissue

Sample name	**Place**	**Provenance trial**	**Organism**	**Accession number GenBank**	**Identifi-cation Score %**
H1.a.H.M	Denmark	Bregentved	*Phoma sp.*	JX160059	99
K.H.M	France	Athis	*Diaporthe viticola*	KC145904	99
H1.aD.M	Denmark	Bregentved	*Phoma exigua var. exigua*	EU343168	96
T1.a.H.L	Czech Republic	Rabstejn	*Boeremia exigua*	KT004579	100
H1.b.H.R	Denmark	Bregentved	*Mycosphaerella coacervata*	EU167596	99
H1.b.D.M	Denmark	Bregentved	*Boeremia exigua*	KX618484	99
H1.a.H.L	Denmark	Bregentved	*Mycosphaerella coacervata*	EU167596	99
S1.a.H.R	Lithuania	Zeimelis	*Epicoccum nigrum*	MF509753	99
L1.H.M	Italy	Cadore	*Aureobasidium pullulans*	HG532077	98

K.H.R	France	Athis	*Diaporthe viticola*	KC145904	95

15.4.1 Seed Collection and Culture

Endophytes can be isolated from different tissues of host plant such as leaf, leaf rachis and roots. Some endophytes have also been isolated from seed (Marshall *et al.*,1999; O'Hanlon *et al.,* 2012). Isolation should be made within 24-48 hours of collection of the plant material before the decay of host tissue. For ash, we collected plant material and surface sterilised it with 5% sodium hypochlorite and 70% ethanol prior to culture to kill epiphytic and contaminating microorganisms. After the surface sterilisation process, the tissue was rinsed in sterile pure water to remove all surfactants. Surface−sterilised tissues were then dissected to prepare the explant that might, for example, be a leaf section, rachis section or root section. This was done immediately prior to plating. If the plant material is sterilised after dissection, the surfactants can more readily enter the plant cells and remain in the cut/damaged plant material and hence reduce the efficiency of endophyte isolation. Surface−sterilised host tissues from leaf, rachis and roots were then cultured onto Petri dishes containing a suitable growth medium such as malt extract agar modified with Vegitone (MEA) (Sigma 38954). We have also successfully used half−strength of MEA and half−strength MEA with additional leaves from healthy ash trees (MEA+F). For this MEA+F medium, ash leaves were collected from a healthy tree and cleaned with tap water three to four times, followed by cleaning with sterile pure water two times and then digested and autoclaved with the half MEA media (by including them in the sample bottle during autoclaving). We assume that MEA+F media will help to promote the endophyte growth differently than the MEA because it will contain extra metabolites from the ash leaf. In addition to MEA, we have found other commercial media to be good for fungal culture including corn meal agar, potato dextrose agar, Sabouraud maltose agar and Czapek dox liquid medium (Murphy *et al.,* 2015). The separate use of a range of

different media maximises the species richness of endophytes recovered because some endophytes do not grow on some media (Murphy *et al.,* 2015).

After plating out, the Petri dishes were sealed with Parafilm or masking tape and kept inside the incubator in dark condition at ±18°C for at least 4−21 days. We found that most endophytes start growing out within 5−7 days of culture. After appearance of mycelial growth, small subcultures were transferred onto a fresh medium in a Petri dish containing half−strength MEA media to maintain single isolates. Where more than one endophyte emerged from the same host tissue, each had to be separately subcultured. Pure cultures can be obtained from single spore sub−culture and can then be preserved in long−term storage. Two principal methods are used to preserve fungal isolates; preparing spore suspensions in ultra pure water at 0–4ºC and cryogenic storage at –80ºC. Cryogenic preservation is preferred for longer−term storage, and is accomplished by freezing the fungal cultures in a solution of glycerol (typically 10 or 20% glycerol). Multiple storage tubes for each pure fungal isolate are kept because each cryotube cannot be refrozen after thawing without significant damage to the sample.

Table 15.2. List of predominant endophytes from root tissue

Sample Name	**Place**	**Tree No.**	**Organism**	**Accession number GenBank**	**Identification score %**
SITI-14-1b	*KRC	1	*Fusarium oxysporum*	MH055398	99
SITI-15-1b	KRC	1	*Cordyceps crassispora*	AB067714	100
SITI-15	KRC	1	*Neonectria candida*	LT821512	99

S1T1-11	KRC	1	*Acremonium alternatum*	NR144913	93
S4T2-6b	KRC	2	*Lecanicillium lecani*	FJ515768	100
S4T2-9-2b	KRC	2	*Dactylonectria macrodidyma*	MH06373	99
S4T2-10-b	KRC	2	*Dactylonectria macrodidyma*	MH06373	98
S5T3-2c	KRC	3	*Dactylonectria macrodidyma*	MF567498	99
S5T3-A-6a	KRC	3	*Fusarium culmorum*	MG274304	99
S6T3-D-4	KRC	3	*Ilyonectria robusta*	LT821483	92

*Kinsealy Research Centre: KRC

15.4.2 Identification of fungal isolates

Fungi can be identified using a combination of morphological examination and DNA barcoding (Hawksworth, 2001; Nilsson *et al.,* 2008; Moore *et al.,* 2011). We extracted DNA from pure cultures of ash fungal endophytes using DNeasy Plant mini kits from Qiagen. Using sterile forceps and scalpels, approximately one−eighth of the fungal plate tissue was transferred into a 1.5 ml microcentrifuge tube with a sterile metal bead for homogenisation and tissue disruption. Disruption of the fungal tissue was done with bead homogenisation using a mixer mill (Retsch MM 300) for 1 min at 10 Hz. The mill block was then inverted and bead homogenisation run for a further 1 min. The rest of the DNA extraction followed the Qiagen manufacturer protocol. The final volume was 50 µL. it was stored at –20°C until

further use. We have also found that the CTAB method described in Hodkinson *et al.* (2007) is an efficient general method for fungal endophyte DNA extraction (Beekwilder *et al*., 2019, Chapter 9).

DNA extracts were then used for PCR amplification of the nuclear ribosomal ITS using the ITS 5 and ITS 4 primers of White *et al.* (1990). The ITS region includes a small part of the 18S, all of the 5.8S and some of the 26S nrDNA. ITS 1F (Nilsson *et al.,* 2008; Begerow *et al.,* 2010) and ITS 4 also work well. After successful amplification, PCR products were cleaned using Exosap and processed for Sanger DNA sequencing (commercially by Macrogen or Source Biosciences; or using an in-house Applied Biosystems 3100xl Genetic Analyser). Sequence traces were edited in Geneious (v.6; Biomatters), or MEGA5 (Tamura *et al.*, 2011) and then used in a BLAST search to find the closest match (similarity) in GenBank (NCBI).

We isolated 310 leaf endophytes on half−strength MEA and half−strength MEA+F media from leaf and leaf rachis tissues of healthy *F. excelsior* and its close relatives. Furthermore, we have isolated over 100 root endophytes on half−strength MEA media from three trees of *F. excelsior*. *Boeremia exigua pseudolilacis*, *Phoma* sp., *Mycosphaerella coecervata*, *Aureobasidium pullulans*, *and Diaporthe viticola* were predominantly isolated from leaf tissues and *Acremonium alternatum, Dactylonectria sp., Fusarium oxysporium, Fusarium culmorum, Neonectria* sp., *Cordyceps crassispora* and *Lecanicillium lecani* from root tissues. Predominant fungi are listed in Table 15.1 and 15.2.

The choice of potential endophyte for biocontrol experimentation, from the large list of potential isolates, can be based on a number of criteria. Information can be obtained about the isolate's antagonistic activity against *H. fraxineus in vitro* (as described in Kosawang *et al.,* 2018). Cultures with high spore formation may also be preferred (Murphy *et al.,* 2015). In addition, some basic information about the identified endophytes were also obtained from the online database (Mycobank,

UNITE) and used as selection criteria. For example, known pathogens were avoided and known endophytes selected.

15.5 Methods for *In Vitro* Plant−Microbe Interaction Studies

In vitro co−culturing of fungal microbes with host plants for plant/endophyte and plant/endophyte/pathogen testing requires a source of plant material that can come from seed or from the micropropagation of existing stocks. For this process we grew *in vitro* sterile (endophyte free) plantlets from seeds and embryos of *F. excelsior*, which were then used for direct experimentation or propagated further by micropropagation. Although, several studies have found that endophytes can be vertically transmitted via seed, we found that no endophytes could be retrieved in endophyte isolation experiments from a total of 100 surface−sterilised ash seeds divided equally between half−strength PDA and half−strength MEA media and incubated at 18°C (Lahiri, pers. obs.).

15.5.1 Tissue Culture of Ash

Healthy undamaged fruits (samara) without holes in them that might indicate insect contamination by weevils such as *Lignyodes bisclwffi* (Blatchley), *L. helvolus* (LeConte) and *L. horridulus* (Casey) were selected (Thomasset *et al*., 2014). Fruits were depericarped and seeds surface sterilised with 7% calcium hypochlorite and rinsed with ultra pure sterile water. Seeds were then directly transferred to Petri dishes containing half−strength Murashige and Skoog (MS) media (Sigma M5524) with 0.6% agar and pH 5.7 (Mettler Toledo FE 20) and then kept in dark at ±18°C.

Old seeds, that are not from the same year, need to be kept in dry cold storage at 4–6°C. Those seeds need stratification with the same surface−sterilisation process. For this purpose, depericarped seeds were soaked in 0.3 M NaOH for 20 mins. The liquid was discarded and the seeds soaked in 0.2% calcium hypochlorite overnight at 4°C (to rehydrate the seeds which had become dry during storage). After discarding the liquid, the seeds were transferred to 2% calcium hypochlorite

for 2 hours for sterilisation, and then thoroughly washed, five to six times, with sterile distilled water. They were then transferred to half−strength MS media with 0.6% agar and without any additional sugars and kept in the dark for two weeks at ±18°C.

In vitro plantlets were also grown from germinated embryos cultured from surface−sterilised and stratified seeds. Dissection of embryos from the seed coats was carried out under a dissecting microscope (10X magnification; Jenco USA; ZM-500) with sterile forceps and scalpels. Sterile seeds were placed on a clean Petri dish under the dissecting microscope and cut along their middle. The half seeds containing the embryos were then carefully removed without damaging any tissues and placed on a Petri dish containing half−strength MS media, sealed with Parafilm on the edges and kept in ±18°C.

Germinated embryos and seedlings were transferred into individual test tubes of 25 x 150mm (Fisherbrand 14-961-34) with polypropylene closure of 25mm (Kimble 73660-25) containing full strength MS media with 1% of sucrose (BDH AnalaR: lot 236 K 18057286), pH 5.7, kept in light at ± 18°C. Plantlets began to root after a few weeks of transferring to test tubes without any hormone supplement.

Our studies (unpublished) have found embryo culture is the most effective for seedling initiation (83% germination) but seed cutting (62% germination) is the most successful for large scale *in vitro* propagation because the plantlets generated by seed cutting are most robust and hence show the highest post establishment survival (survival recorded from embryo cultured plantlets was 43% compared to 100% for seed established plantlets).

In vitro plantlets can be used directly or propagated further using single node culture. For micropropagation, single node explants were dissected from the plantlets and rooted on full strength MS media with 1% sucrose. They were then grown under 1600 µmol $m^{-2}s^{-1}$ PAR to fill the culture tube once more before being propagated once more. In this way large stocks of identical genotype (ramets) were

produced. Using such micropropagated stocks in plant−microbe interaction studies reduces variability caused by genotypic variation in a seed batch.

15.5.2 *In Vitro* Biocontrol and Plant−Microbe Interaction Testing

Some endophytes reside in the shoot and some in the roots of ash. Therefore, culture methods are required for both these types. We have found that agar culture system work well for shoot endophytes and perlite works well for root endophytes as outlined below.

To test the effects of the application of endophytes to ash plant tissue, we used *in vitro* plantlets growing on MS media with 0.6% agar (Sigma 9002-18-0). An endophyte inoculant solution was first obtained by diluting (9:1) with ultrapure water and 1ml of the spore suspension was streaked out onto the agar surface to obtain single spore colonies, which were then subcultured individually. The surface of the sporulating subcultures were each washed with 5 ml of pure water and diluted to a spore concentration of 10^6 spores per millilitre. We used one of two methods to introduce the endophytes into the nodes of the *in vitro* plantlets: by either making a nick with scalpel at the node and then dusting spores from the pure culture plates onto leaf surface or by spraying the spore suspension onto the leaf. For non−sporulating cultures, a 'fluffed out' agar core (5mm diameter) was added to the base of the plantlet upon establishment.

Root endophytes were tested on seedlings growing on MS and perlite (Vitax: Perlite) without any agar. The perlite allows aeration required by the root fungi for growth through the medium. Plantlets were inoculated with either a spore solution (as outline above) or by fluffed out agar plug (as outline above). In this case the potential candidate root endophyte was introduced near the root tips (meristems) of *in vitro* plantlets.

An alternative approach for *in vitro* endophyte inoculation is to establish the experiment from seed and to coat the seeds with endophyte spore solution prior to germination. In this way the endophyte establishes with the seed as it germinates.

Biocontrol Experimentation

For plant/endophyte/pathogen testing, the culture system outlined above can be simply extended for a second microbe. This can be undertaken for both root and shoot endophytes and a pathogen. We have optimised the biocontrol experiments for *H. fraxineus* but the method is applicable to a wide range of pathogens so is not restricted to ash dieback disease studies.

We separately inoculated ten replicates of *in vitro* grown ash plantlets with *H. fraxineus* for each of root endophytes. Growth and disease development of the ash plantlets were recorded at several time intervals. Comparisons were made among plantlets with endophytes and plantlets without endophytes (for controls) in a fully randomised experimental design under 1600 $\mu mol.m^{-2}\ S^{-1}$ PAR light.

15.6 Conclusion

Ash dieback is a potential threat to the ash population throughout Europe and is spreading rapidly by its spores that are able to travel over long distances. As common ash has high economic value and covers huge land areas in plantations and woodlands, it is vital to find ways or improving its disease resistance. The *in vitro* culture methods we have developed will aid in the study of disease resistance in ash. The *in vitro* antagonistic and *in vitro* biocontrol assays including endophyte and pathogen are performed in an enclosed and otherwise sterile environment so that the pathogen will not escape into the environment and so that the direct interaction of the microbes can be assessed without the influence of other organisms.

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15.7 References

Bakys, R. (2013). Dieback of *Fraxinus* in Baltic sea region. *Swedish University of Agricultural Sciences*, *Uppsala*, 1–48.

Baral, H-O., Queloz, V. and Hosoya, T. (2014). *Hymenoscyphus fraxineus*, the correct scientific name for the fungus causing ash dieback in Europe. *IMA Fungus*, **5**, 79–80.

Barklund, P. (2005). Ash dieback sweeping over south and central Sweden. *SkogsEko,* **3**,11–13. (In Swedish).

Beekwilder, J., Murphy, B. R. , Mathuna, E. M. , Barry , A. and Hodkinson , T. R. (2019). Isolation, diversity and potential use of endophytes in the biomass and bioenergy crop *Miscanthus*. In *Endophytes for a Growing World*, ed. T R. Hodkinson, F. M. Doohan, M. J. Saunders and B. R. Murphy. Cambridge: Cambridge University Press, Chapter 9.

Begerow, D., Nilsson, H., Unterseher, M. and Maier, W. (2010). Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied Microbiology and Biotechnology*, **87**, 99–108.

Brome A. and Mitchell, R. J. (2017). *Ecological impacts of ash dieback and mitigation methods*. Research Note. London: Forestry Commission UK, 1– 16.

Chanway, C. P. (1996). Endophytes: they're not just fungi! *Canadian Journal Botany,* **74**, 321–322.

DAFM (n.d.). Ash Dieback (*Chalara*). https:// www.agriculture.gov.ie/forestservice/

treediseases/ ashdiebackchalara/

Douglas, G. C., Pliura, A., Dufour, J. *et al*. (2013). Common Ash (*Fraxinus excelsior* L.). In *Forest tree breeding in Europe: Current State of the Art and perspectives, vol. 2,* ed. L. E. Pâques, *Springer, Managing Forest Ecosystems,* **25**, 403–462.

Forestry Commission UK (2015). *Chalara* dieback: managing ash trees and woodlands. www.forestry.gov.uk/ forestry/ infd-92pjkx.

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V. and Sieber, T. N. (2014). *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. *Molecular Plant Pathology,* **15**, 5–21.

Hawksworth, D. L. (2001). The magnitude of fungal diversity: the 1.5 million species estimated revisited. *Mycological Research*, **105**, 1422–1432.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F. and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, **43**, 895–914.

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

Hodkinson, T. R. (2018). Evolution and taxonomy of the grasses (Poaceae): a model family for the study of species rich groups. *Annual Plant Reviews Online*, doi:10.1002/ 9781119312994. apr0622.

Hodkinson, T. R., Waldren, S., Parnell, J. A. N. *et al.* (2007). DNA banking for plant breeding, biotechnology and biodiversity evaluation. *Journal of Plant Research*, **120**,17–29.

Janse, J. D. (1981). The bacterial disease of ash (*Fraxinus excelsior*), caused by *Pseudomonas syringae subsp.savastanoi pv. fraxini*. *European Journal of Forest Pathololgy,* **11**,306–315.

Kosawang, C., Amby, B. D., Bussaban, B. *et al.* (2018). Fungal communities associated with species of *Fraxinus* tolerant to ash dieback, and their potential for biological control. *Fungal Biology*, **122**, 110–120.

Kowalski, T. (2006). *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology*, **36**, 264–270.

Kowalski, T. and Holdenrieder, O. (2009a). Pathogenicity of *Chalara fraxinea*. *Forest Pathology*, **39**, 1–7.

Kowalski, T. and Holdenrieder, O. (2009b). The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology*, **39**, 304–308.

Kowalski, T. and Łukomska, A. (2005). Studies on *Fraxinus excelsior* L. dieback in Włoszczowa Forest Unit stands. *Acta Agrobotanica*, **59**, 429–440.

Kowalski, T., Bilański, P. and Kraj, W. (2017). Pathogenicity of fungi associated with ash dieback towards *Fraxinus excelsior*. *Plant Pathology,* **66**, 1228–1238.

Kräutler, K. and Kirisits, T. (2012). The ash dieback pathogen *Hymenoscyphus pseudoalbidus* is associated with leaf symptoms on ash species (*Fraxinus* spp.). *Journal of Agricultural Extension and Rural Development,* **4**, 261–265.

Link, H. F. (1809). Observationes in ordines plantarum naturales, disserta- tio prima, complectens anandrarum ordines Epiphytas, Mucedines, Gas- tromycos et Fungos, Der Gesellschaft Naturforschender Freunde zu Berlin, Berlin, Germany.

Marshall, D., Tunali, B. and Nelson, L.R. (1999). Occurrence of fungal endophytes in species of wild *Triticum*. *Crop Science*, **39**, 1507–1512.

Moore D., Robson, G. D. and Trinci, A. P. J. (2011). *21st Century Guidebook to Fungi*. Cambridge: Cambridge University Press, pp. 41–81.

Murphy, B. R., Batke, S. P., Doohan, F. M. and Hodkinson, T.R. (2015). Media manipulations and the culture of beneficial fungal root endophytes. *International Journal of Biology*, **7**, 94–102.

Nilsson, R. H., Kristiansson, E., Ryberg, M., Hallenberg, N. and Larsson, K-H. (2008). Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*, **4**, 193–200.

O'Hanlon, K. A., Knorr, K., Jørgensen, L. N., Nicolaisen, M. and Boelt, B. (2012). Exploring the potential of symbiotic fungal endophytes in cereal disease suppression. *Biological Control*, **63**, 69–78.

Petrini, O. (1991). Fungal endophytes of tree leaves. In *Microbial Ecology of Leaves*. eds Andrews and S. S. Hirano. New York, NY: Springer-Verlag, pp. 179–197.

Przybył, K. (2002). Fungi associated with necrotic apical parts of *Fraxinus excelsior* shoots. *Forest Pathology*, **32**, 387–394.

Sansford, C. E. (2013). Pest Risk Analysis for *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) for the UK and the Republic of Ireland. *Forestry Commission,* 1–128.

Schlegel, M., Dubach, V., Buol, L. and Sieber, T. N. (2016). Effects of endophytic fungi on the ash dieback pathogen. *FEMS Microbiology Ecology*, **92**, 1–18.

Tamura K., Peterson D., Peterson N. *et al.* (2011). MEGA5: Molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28**, 2731–2739.

Thomasset, M., Hodkinson, T. R., Restoux, G. *et al.* (2014). Thank you for not flowering: conservation genetics and gene flow analysis of native and non-native populations of *Fraxinus* (Oleaceae) in Ireland. *Heredity,* **112**, 596–606.

Thomsen, I. M. (2005). Frost damage of ash. *Videnblade Skovbrug*, **8**, 2–1. (In Danish).

Timmermann, V., Borja, I., Hietala, A.M., Kirisits, T. and Solheim, H. (2011). Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *OEPP/EOPP Bulletin*, **41**,14–20.

White, T. J., Bruns, T. D., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*, ed. M.A. Innis, D.H. Gelfand, J.J., Sninsky, and T.J. White. New York: Academic Press, pp. 315–322.

Chapter 5

Ash dieback (*Hymenoscyphus fraxineus*) strain variation and endophyte/pathogen antagonism and biocontrol studies

5.1 Introduction

European forests are characterised by low tree species diversity (Svenning and Skov 2007) and trees of economic importance are even less diverse and often planted in monocultures, so a new disease that arrives or develops in Europe can potentially have a tremendous effect on the ecosystem, forestry and landscape development (Gross *et al.* 2012). The increase in global interconnectedness also enhances the chances of introduction of exotic pathogens into the forest ecosystem (Moslonka-Lefebvre *et al*. 2011). In the last decades alarming numbers of diseased trees were reported, for example Dutch elm disease caused by the ascomycete *Ophiostoma novo-ulmi* (Brasier 1991), chestnut blight, caused by the fungus *Cryphonectria parasitica* (Murrill) M.E. Barr (Robin and Heiniger 2001), Phytophthora disease of alder caused by *Phytophthora alni* (Brasier *et al*. 2004b) and now ash dieback caused by invasive ascomycete fungi *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya (Baral *et al.* 2014).

Ash dieback of *Fraxinus excelsior* was first reported from Poland in the mid-1990s, after which the disease spread rapidly and covered large parts of Europe (Chandelier *et al.* 2011; Kowalski and Holdenrieder 2008; Leonhard *et al*. 2009; McKinney *et al.* 2011). The pathogen causing ash dieback was first identified as the novel Ascomycota fungus *Chalara fraxinea* T. Kowalski (Kowalski 2006; Kowalski and Holdenrieder 2009a). Later the teleomorph was studied and described as *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips (Kowalski and Holdenrieder 2009b). However, a study of molecular markers on healthy and disease ash stands revealed that *H. albidus s.l*. is composed of two species and the new species was described as *Hymenoscyphus pseudoalbidus* (Queloz *et al.* 2011). This latter species was recognised as the causal organism of disease, whereas *H. albidus* is non-pathogenic and the only species, of the two, found in healthy ash stands (Husson *et al.* 2011; Queloz *et al.* 2011). Baral *et al.* 2014 renamed *Hymenoscyphus pseudoalbidus* as *Hymenoscyphus fraxineus* as the correct scientific name for the causal agent of the ash dieback pathogen. A phylogenetic study of *H. fraxineus* and *H. albidus,* with three other species, showed that *H. fraxineus* and *H. albidus* are phylogenetically closely related (Gross and

Han 2015). Also, morphological studies showed that *H. albidus* and *H. fraxineus* differ from each other; *H. fraxineus* has croziers at the ascus base (Zheng and Zhuang 2013; Gross and Han 2015). It is grouped in the Ascomycota class Leotiomyctes and family Helotiaceae (Kowalski and Holdenrieder 2009b).

Hymenoscyphus fraxineus probably infects the leaves by ascospores (Kraj *et al*. 2012; Rytkönen *et al.* 2010) which produce hyphae that spread along the rachis into the stem and form extensive bark canker and then the distal end of the shoots dies (Schumacher 2011). Shoot infections are thought to be at the end of the infection cycle as fruiting bodies of the pathogen have very rarely been seen on the shoots (T. Kowalski, pers comm). Pseudosclereotia develop in the petiole of the fallen ash leaf litter during the winter and conidia are produced. However, germination of conidiospores has rarely been observed as they may behave as spermatia (Kirisits *et al.* 2009). However, Fones *et al.* (2016) demonstrated germination of conidia *in planta* and in leaf litter. During the following summer, apothecia are formed and a new infection cycle starts after they release spores infected that infect new leaves (Engesser *et al*. 2009; Kirisits *et al*. 2010; Kowalski and Holdenrieder 2009b; Schumacher *et al*. 2007, 2010; Gross *et al.* 2012). Ascospores are dispersed by wind and the disease front can move up to 75 km per year (Gross *et al.* 2014a; Haňáčkova *et al.* 2015).

An investigation on herbarium samples (1990) and fresh samples from Japan suggested that the likely origin is from East Asia (Hosoya *et al.* 1993; Zhao *et al.* 2012) and it was subsequently also reported in East China (Zheng and Zhuang 2013), East Russia (Marčiulynienė *et al.* 2013) and Korea (Han *et al.* 2014). However, *H. fraxineus* has been reported as a pathogen from its native range (Gross and Han 2015). The European common ash (*Fraxinus excelsior*) is highly susceptible to *H. fraxineus* infection, except for ~5% which showed partial resistance (McMullan *et al.* 2018). A genome sequencing study of 44, *H. fraxineus* isolates from Europe and 9 from Japan was carried out by McMullan *et al.* (2018) using SNPs (single nucleotide polymorphisms) and found that, at the genome level, Japanese and European strains are divergent. More diversity was present in the Japanese strains than in European ones (McMullan *et al.* 2018). Estimates from *H. fraxineus* microsatelite allelic richness suggests that two haplotypes may have invaded Europe (Gross *et al.* 2014a). Genomic studies revealed proteins involved in pathogenicity such as glucosyl hydrolase which are associated with cell wall degrading enzymes (cellulase, pectinoesterases and cutinases), oxidoreductase

and cytochrome P450 (monooxygenase reaction for destruction of ash tissue-derived aromatic compounds with antifungal activity) (Collemare *et al.* 2008; McMullan *et al.* 2018). *Hymenoscyphus fraxineus* also showed a low complexity of transcribed domains that help effectors to be more flexible and diverse, driven by adaptive evolution (Mesarich *et al.* 2015; McMullan *et al.* 2018).

Considering the complexity of the *Hymenoscyphus fraxineus* genome and its potential to disperse spores over a vast range of area, it is important to take precautionary measures to save ash trees from extinction or major decline. Endophytes may have the potential to play a major role as biocontrol agents to outcompete plant-pathogens (Terhonen *et al.* 2019). Endophytes may also play a role in the production of bioactive substances that enhance the host-endophyte relationship (Strobel 2003) as well as increase plant defences against pathogens (Zabalgogeazcoa 2008). Endophyte interaction with the plant can range from antagonism to mutualism, depending on the species (Saikkonen *et al*. 1998; Schulz and Boyle 2005). To control diseases in the host, some endophytes may induce a plant defence mechanism which affects pathogen growth, whilst other endophytes may affect pathogen growth by producing antibiotic/or antifungal metabolites (Zabalgogeazcoa 2008). Results from a study on the application of liquid extracts of endophyte cultures to pathogens showed that the growth of several species of pathogens was retarded (Liu *et al.* 2001; Park *et al*. 2005; Inácio *et al*. 2006; Kim *et al*. 2007). Much research has been carried out on the bioactive compounds found in endophytes (Strobel *et al.* 1997). Some researchers have shown that taxol can be produced from several fungi isolated from trees, such as *Pestalotiopsis guepinii*, an endophyte of the Wollemi pine (*Wollemia nobilis*, Araucariaceae) (Strobel *et al.* 1997), *Periconia* sp. from the evergreen tree *Torreya grandifolia* (Taxaceae; Li *et al.* 1998), *Seimatoantlerium nepalense*, a coelomycete from Himalayan yew (*Taxus wallachiana*; Bashyal *et al.* 1999), *Tubercularia* sp. strain TF5 from *Taxus mairei* (Wang *et al.* 2000), *Bartalinia robillardoides* isolated from the medicinal tree Bael (*Aegle marmelos*, Rutaceae; Gangadevi and Muthumary, 2008), *Phyllosticta spinarum* from *Cupressus* sp. (Cupressaceae; Kumaran *et al.* 2008), *Xylaria* sp., *Sordaria* sp., *Metarhizium anisopliae* and *Coniothyrium diplodiella*, isolated from *Taxus chinensis* (Liu *et al.* 2009), and *Pestalotiopsis* species isolated from *Taxus cuspidata* (Kumaran *et al.* 2010). Another anticancer drug, Camptothecin, can be isolated from several endophytic fungi such as *Entrophospora infrequens* and *Neurospora* (from *Nothapodytes foetida*) (Puri *et al.* 2005; Rehman *et al.* 2008). Liu *et al.* (2010b) isolated *Xylaria* M20 and Kusari *et al.* (2009b) isolated *Fusarium*

solani from *C. acuminata*. An analog of Campothecin is also produced by *Fusarium solani* but the host is *Apodytes dimidiata* (Shweta *et al.* 2010). Podophyllotoxin, another important metabolite for medical purposes can be isolated from *Phialocephala fortini* (Eyberger *et al.* 2006) and *Trametes hirsuta* (Puri *et al.* 2006) isolated from *Podophyllum*.

Endophytes from ash that may have potential as biocontrol agents have been studied by many researchers. Bakys *et al*. (2009a) isolated endophytes from healthy and diseased shoots of ash from four locations in Sweden and studied their potential as biocontrol agents. They selected 24 taxa, including *H. fraxineus,* and tested for pathogenicity. Pathogenicity experiments were setup by artificial inoculation of selected taxa on one year old ash trees cultured on agriculturally rich soil in a bare root nursery. Among 24 taxa only four taxa, *A. alternata*, *E. nigrum*, *H. fraxineus* and *Phomopsis* sp. 57, caused visual necrosis on bark and cambium. *Hymenoscyphus fraxineus* was the most pathogenic and caused necrosis on 3 trees out of 6, of which one died. *Phomopsis* sp. 57 caused necrosis on 2 trees out of 12, of which one died. Re-isolation of the fungi from the necrotic tissues showed identical morphology with the respective inoculated isolates. Another study on pathogenicity using a similar method (Bakys *et al.* 2009b) was carried out for 8 isolates of *Hymenoscyphus fraxineus* on 86 treated trees and 10 (1 year old) control trees. Results from pathogenicity tests showed all 8 isolates caused necroses on bark and cambium with mortality rates ranging from 36% to 18%.

Schlegel *et al.* (2016) studied ash endophytes as potential biocontrol agents. The study was conducted using *Fraxinus excelsior* and *F. ornus* L.. Firstly, the influence of exudates from isolated *F. excelsior* endophytes was tested *in vitro* (on Petri plates) against the ascospore germination of *Hymenoscyphus fraxineus*. Strong inhibitory effects on ascospore germination were recorded from the exudates of *Paraconiothyrium* sp., *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley, *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin, *Ampelomyces quisqualis* Ces. and *Elsinoaceae* sp.. Weak effects were observed for exudates of *Venturia* spp. and *Nemania serpens* (Pers.) Gray. Secondly, the protective effects of endophytes against the ash dieback pathogen were studied in the field (infected forest) using endophyte free and plants pre-inoculated with endophytes. *Venturia* spp. dominated the endophytic community in the field grown trees inoculated with endophytes, but no significant effect of endophyte inoculation was seen on plants

after they had been infected with *H fraxineus*. Thus, no evidence for field-based endophyte biocontrol of *H. fraxineus* was found.

Kosawang *et al.* (2018) studied fungal communities from resistant *Fraxinus* (*F. chinensis* subsp. *rhynchophylla* (Hance) A.E. Murray*, F. lanuginosa* Koidz.*, F. mandshurica* Rupr.*, F. ornus* and *F. pennsylvanica* Marshall) for use as potential biocontrol agents. They performed antagonistic activity assays by growing the endophyte with *H. fraxineus.* Endophyte species showing high antagonistic activity included *Boeremia exigua, Epicoccum nigrum* and *Fusarium* sp.; we also recovered these three species from leaf tissues in our own work (Chapter3).

This chapter aimed to select potential biocontrol root and shoot endophytes from our identified collection of endophytes (Chapter 3) on the basis of online data available in several databases (MycoBank, CBS-KNAW and GenBank) and from research articles. Any potential plant pathogens were removed from the list. After this selection the remaining endophytes were tested for antagonistic activity against two strains (Irish and Northern Irish) of *Hymenoscyphus fraxineus*. A sample of the endophytes which showed good antagonism activity against *H. fraxineus* were further tested for co-culture experiments with *in vitro* grown *Fraxinus excelsior* plantlets. The physiological changes among plants, with a) endophyte or pathogen (in separate replicate sets) or b) with plant endophyte and pathogen, was recorded against control plantlets. We also aimed to identify putative strains of *H. fraxineus* from Irish and Northern Irish sources and to analyse the molecular DNA variation of these in a European context.
The specific objectives of this chapter were to:

1) Confirm identity and assess strain variation, using nrITS sequences, of *Hymenoscyphus fraxineus* obtained from Irish and Northern Irish sources and to compare these with European strains.
2) Select potential endophytes as biocontrol agents from our studies guided also by previously reported studies.
3) Assess *in vitro* antagonistic activity of selected root and shoot endophytes against two strains (Irish and Northern Irish) of *H. fraxineus* pathogens.
4) Co-culture *in vitro* grown, endophyte-free, plantlets with potential biocontrol endophytes (from the antagonism study) and the pathogen (Irish strain) to assess biocontrol potential.

5.2 Material and Methods

5.2.1 Strain variation in *Hymenoscyphus fraxineus*

Irish and Northern Irish *Hymenoscyphus* isolates were obtained from AFBI, Belfast (Richard O'Hanlon) and Teagasc, Ashtown, Dublin (Brian McGuinness). These were supplied as putative *H. fraxineus* but needed to be confirmed using DNA barcoding of nrITS before inclusion in subsequent endophyte/pathogen interaction studies. Some samples were rejected as *H. fraxineus* on the basis of their morphology and DNA sequences thus the barcoding was an essential step. The DNA analysis also allowed a comparison to be made between the material from Ireland and sequences available for samples from several other European and global countries (Austria, Canada, China, Croatia, Czech Republic, Estonia, Finland, France, Germany, Ireland, Italy, Japan, Latvia, Lithuania, Poland, Serbia, Slovakia, Switzerland, UK, Ukraine).

Four isolates were tested to check their identity as *H. fraxineus*. These were: H1 from Teagasc; and H3 – RANT 2, H5 – 4795D and H8 – 80/13 from AFBI. Total genomic DNA of the putative *H. fraxineus* isolates was extracted and purified using the hot CTAB method as outlined in Chapter 3 (Section 3.2). The nrITS1 and 2 regions were amplified and sequenced using ITS 4 and ITS 5 primers (as listed in Chapter 3 Section 3.2). Sequences were compared in Geneious v. 11.0.5.

Four isolates were confirmed as *Hymenoscyphus fraxineus* by BLAST searching (NCBI GenBank) and these were used for comparative DNA alignment with other strain sequences available in GenBank. A Blast search was undertaken against H1 and all sequences with up to 98% similarity and 100% coverage were downloaded (excluding duplicate batch sequences). Haplotypes were identified in PopArt (Population Analysis with Reticulate Trees; Leigh and Bryant 2015) and then coded by country of origin as listed in their GenBank entry under 'Source'.

Two types of haplotype network analyses were run in PopArt, namely TCS (Clement *et al.* 2000) and median-joining networks (Bandelt *et al.* 1999). Patterns found in the median-joining network did not differ from the TCS network analysis so only the latter is described/shown. TCS networks are constructed using an agglomerative approach where clusters are progressively combined with one or more connecting edges (see Clement *et al.* (2000). The number of mutations

represented by the network's edges are shown as hatch marks along edges (branches).

5.2.2 Selection of potential endophytes for antagonistic tests

After searching in the online databases and literature available, we selected 10 potential leaf endophytes and 6 potential root endophytes for testing against two strains (Irish and Northern Irish) of *Hymenoscyphus fraxineus* pathogens (Table 5.1). Images of these selections are shown in Appendix IV (Figure 5.1 and 5.2).

Table 5.1 Selected root and shoot endophytes for testing as potential biocontrol agents; and two selected strains of the dieback pathogen *Hymenoscyphus fraxineus*.

Name given	Sources (plots/tree name)	Location (country, provenance)	Source tissue	Name
Leaf endophytes				
E1	H	Roosky (France, VSP)	healthy leaf lamina	*Naganishia diffluens*
E2	S1	Roosky (Lithuania, ZEI)	healthy leaf rachis	*Epicoccum nigrum*
E3	J	Roosky (France, SM)	healthy leaf lamina	*Engyodontium album*
E4	G	Roosky (UK, LOC)	healthy leaf lamina	*Bjerkandera adusta*
E5	O1	Roosky (Poland, SZC)	healthy midrib	*Mollisia* sp.
E6	O1	Roosky (Poland, SZC)	healthy leaf rachis	*Gibberella* sp.
E7	Ire (Loreto park)	Loreto park, Dublin	healthy midrib	*Pyronema domestic um*
E8	*Fraxinus texensis*	NGB, Glasnevin	healthy midrib	*Collemboliospora aristata*
E9	*Fraxinus americana*	NGB, Glasnevin	healthy leaf rachis	*Exophiala oligosperma*
E10	*Fraxinus americana*	NGB, Glasnevin	healthy apex	*Meyerozyma guillier mondi*
Root endophytes				
ER1	tree 2	Kinsealy	root	*Lecanicillium attenu atum*
ER2	tree 1	Kinsealy	root	*Cordyceps perangustam*
ER3	tree 1	Kinsealy	root	*Penicillium*

				spathulatum
ER4	tree 1	Kinsealy	root	*Harzia velata*
ER5	tree 1	Kinsealy	root	*Hydropisphaera* sp.
ER6	tree 2	Kinsealy	root	*Psilloglonium* sp.
Pathogens				
H1	-	Teagasc	-	*Hymenoscyphus fraxineus*
H8	80/13	AFBI	-	*Hymenoscyphus fraxineus*

5.2.2.1 Experimental setup for antagonism activity test for root and shoot endophytes with two strains of pathogens

Petri dishes containing autoclaved half strength malt extract agar (MEA) with vegitone supplement (Sigma: 38954) were set up with a 1 × 1 cm plug of fungal mycelium tissue, from a pure endophyte single culture plate, on one corner of the Petri dishes and a 1 × 1 cm plug of fungal tissue, from a pure single culture plate of pathogen (H8), on the opposite corner of the same Petri dish and sealed with Parafilm. Five replicates for each shoot (10 isolates) and root (6 isolates) endophyte were included. All Petri dishes were incubated in the dark at ± 20ºC and growth was measured at 7 days intervals for 8 weeks.

After the above experiment, endophytes were further selected from the Petri dishes where pathogen growth was very slow or did not grow at all (E1, E2, E3, E4, E6, E7 and E9). The experiments was then repeated using the selected endophytes (five replicates each) but this time the pathogen was introduced three weeks earlier than the endophytes. This was because from the first experiment it was observed that the pathogen growth was very slow in several cases and that the endophyte outcompeted the pathogen. After three weeks, the endophytes were added to the *Hymenoscyphus* culture Petri dishes, which were then sealed and incubated as described above. Growth measurements were taken at 7 day intervals for 5 weeks.

Another experiment for the second strain of *Hymenoscyphus* pathogen (H1) was carried out following the above method, and for this experiment the pathogen was plated out three weeks earlier than the endophyte (as in the repeated experiment above). All Petri dishes were incubated at ± 20ºC and growth measurements taken at 7 days intervals for 5 weeks.

5.2.3 Establishment of *in vitro* plantlets for co-culture experiments

Ash seeds were collected in Marley Park, Dublin, Ireland from a single mother tree (details in Chapter 4 section 4.2.1). Germination was carried out using surface-sterilised endophyte-free seeds (cut seeds) on half strength Murashige and Skoog (MS) media with 0.6% agar (details in Chapter 4 section 4.2.2). Seedlings were transferred onto full strength MS media with 0.6% agar and 1% sucrose for further growth (details in Chapter 4 section 4.2.2.3) and then placed onto a rooting media of full strength MS with 0.6% agar, 1% sucrose and 20mgL^{-1} NAA (details in Chapter 4 section 4.2.3.1). Well rooted plantlets were transferred onto 10ml of full strength MS with 0.6% agar for shoot endophytes and 10ml of full strength MS with 1.5g of Perlite for root endophytes for three weeks. Co-culturing experiments were carried out on the previously described media where the plantlets had been maintained before the experiment.

5.2.3.1 Preparation of inocula for co-culture experiments

Plant-pathogen interaction studies were carried out using two leaf endophytes (E7 and E10), 1 root endophyte (ER1) and 1 strain of pathogen (H1). Endophyte and pathogen strains were checked under the microscope and for those that were sporulating we calculated the spore count and for those that were not sporulating a hyphal count was carried out to prepare the required concentration of inocula. Spore counting was performed for the leaf endophyte (E7) by producing a spore suspension solution. A Petri dish with a pure single culture was first flooded with 1ml of ultrapure water. The spore suspension solution from the Petri dish was then collected in a 1.5ml microcentrifuge tube. One microlitre of TWEEN 20 was added and it was vortexed gently for 10 sec. This spore suspension solution was used to make serial dilutions (9:1) with ultrapure water and 1ml of the spore suspension obtained. Spore suspensions were shaken for 30 min on a horizontal shaker before counting. Then 10µl of spore solution was placed on a haemocytometer slide and spores counted at 10x and 40x magnifications. No further dilutions were needed in the preparation of the inocula because the first dilution spore count was approximately 10^6 the desired concentration for the inocula.

The other two endophytes (E10 and ER1), were prepared by CFU count because it was too difficult to separate hyphal fragments into single fragments for counting (even after gentle crushing and shaking with the addition of 1µl TWEEN 20). For CFU counting, the hyphal inoculant was obtained by the same method described above for the spore suspension. 1ml of hyphal suspension was serially diluted (9:1;

for a CFU count of 10^5) with ultrapure water and vortexed gently for 10 sec. Finally, 10µl of hyphal suspension was pipetted out onto Petri dishes with water agar and streaked out onto the surface of the Petri dishes with a sterile loop to obtain single colonies. Petri dishes were incubated at 20ºC after being sealed with Parafilm. CFU value was calculated for each dilution from 5 replicate of Petri dishes.

Inocula preparation of the pathogen was by hyphal count. Petri dishes with a pure *Hymenoscyphus* culture were flooded with 1ml of ultra-pure water and the hyphal suspension was gently ground with an autoclaved mortar with 1µl of TWEEN 20. The 1ml hyphal suspension was collected in a 1.5ml microcentrifuge tube and shaken for 30 mins on a horizontal shaker before counting. Hyphal fragments were counted under the microscope at 10X and 40X magnification. No dilutions were needed as the hyphal count was as required.

The concentration of the inocula used for co-culture experiment are shown in Table 5.2. The concentrations of spore suspension were obtained by following the protocol for haemocytometer. Colony forming unit (CFU) counts were obtained by dividing the concentration (initial suspension) and dilution factor with the mean value. For the hyphal counts, first the field-of-view (FOV) of the microscope was calculated (41.13), then that value multiplied with the mean value of hyphal count (324) to give us a hyphal count (1.33×10^4) for the 10µl suspension (as initially that amount was taken onto the slide). To get a value for 1ml that value was multiplied by 100 (1.33×10^6).

Table 5.2 The concentrations of inocula used for co-culture biocontrol experiment

Name given	Type	Spore/CFU/hyphal count in 1ml suspension
E7	Shoot	1.5×10^6
E10	Shoot	2.4×10^5
ER1	Root	1.35×10^5
H1	Pathogen	1.33×10^6

5.2.3.2 Plant-endophyte-pathogen interaction experiments

We used 10 replicates of plantlets for the shoot endophyte experiment and 8 replicates for the root endophyte study. Four treatments were prepared for individual endophyte assays, 1 – control *in vitro* plantlets without any pathogen or

endophyte; 2 – pathogen treated plantlets; 3 – endophyte treated plantlets; and 4 – endophyte and pathogen treated plantlets. All test tubes for shoot endophyte interaction assays contained 10ml MS (full strength) with 0.6% agar; and all test tubes for root endophyte interaction assays used 10 ml MS with 1.5g of Perlite. Endophytes were introduced to the *in vitro* plantlets a week earlier than the pathogen. Shoot endophytes were introduced by painting the spore/hyphal suspensions onto each lamina of individual plantlets with a paint brush before being transferred into the test tube and closed with a polypropylene closure and sealed with parafilm. Leaves from the control set were painted with ultrapure water and sealed in the same way as above. One week later, the pathogen was introduced for the plant/pathogen and plant/pathogen/endophyte treatments by spraying the hyphal suspension onto the plantlets. Before spraying, and to make sure the hyphal fragments were coming out from the spray nozzle, a test spraying on a microscope slide was performed and checked under the microscope for hyphal fragments presence.

Root endophytes were introduced one week earlier than the pathogen, as for the shoot endophyte experiments. Root endophytes were introduced to the *in vitro* plantlets by dipping the roots for 2 min in the hyphal suspension and placing them into the test tube; then they were closed with polyprepene closure and sealed with parafilm. Control *in vitro* plantlets were dipped in ultrapure water and sealed in the same way as above. One week later, the pathogen was introduced for the plant/pathogen and plant/pathogen/endophyte sets by spraying the hyphal suspension onto the plantlets. Plantlets were kept at ± 18°C with 16h of light and 8h dark. All experiments were performed in sterile and fully enclosed environments to avoid exposure of spores into the environment. After five weeks the plantlets were harvested and recorded for fresh weight, number of leaves, number of roots, leaf size, and shoot/root ratios. The plant tissues were kept in a drying oven at 65°C for three days and the dry weight was measured. Fresh leaves from three individual trees from each set were collected and further processed for tissue sections for endophyte penetration under the microscope. For root endophytes, three roots for each treatment set were selected.

5.2.3.3 Processing of leaf and root tissues for checking endophyte penetration into the plant cells

Plant tissues (root/shoot) were first rinsed with deionised water, 2 times and kept in a 50ml Falcon tube. Then they were submerged in 50% (v/v) ethanol for 24h. The

following day, the plant tissue was rinsed 3 times in deionised water and treated with 5% potassium hydroxide (KOH) at 90°C in a water bath. After 2h, the 50ml tubes were taken out from the water, rinsed, and, solution carefully discarded without losing any tissues and then they were rinsed 4 times with deionised water. A treatment of 2% lactic acid (wt/vol) was applied to the rinsed tissue for 2 min. Staining of the tissue was done with 0.05% (wt/vol) trypan blue in 50°C in a water bath for 5h. The trypan blue was discarded through a strainer and tissues were placed in 50% glycerin (vol/vol) for 24h. Slide preparations carried out in 50% glycerol were examined under a microscope at 10x and 20x magnification and photographs taken. For root slide preparation, a squash technique was applied to spread the cells in a single plane for better visualisation.

5.3 Results

5.3.1 *Hymenoscyphus* strain variation in a European context

The final *Hymenoscyphus* matrix contained 243 sequences from GenBank and 4 from this project. It was 781 bp in length and contained 21 segregating sites. The analysis revealed 9 unique haplotypes in total and three unique haplotypes in the Irish material. Variable regions in the Northern Irish and Irish strain are shown in Figure 5.1. It included 2 insertion deletions (INDELS) of adenine at 857bp and at 847bp (Figure 5.1). This generated three unique Irish/Northern Irish haplotypes. One of the mutations in the Irish material was excluded from the TCS analysis (Figure 5.2) because it was only found in one sample (and was removed automatically by the PopArt programme).

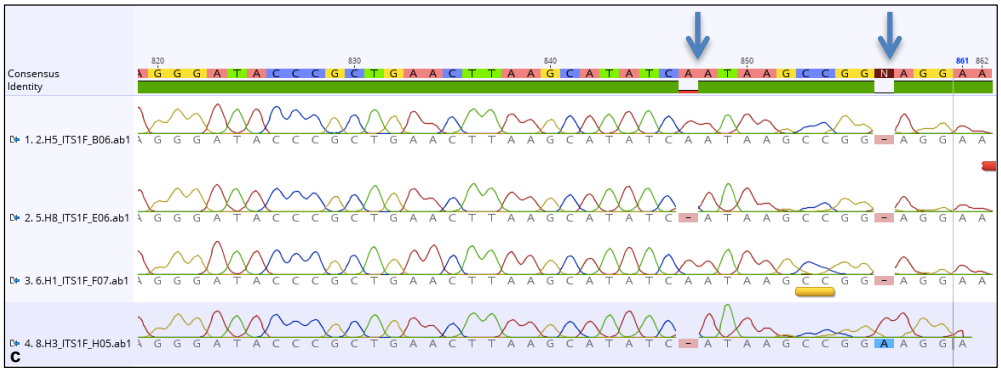

Figure 5.1 Trace file showing the nucleotide variations at two regions of the nrITS sequences for the Irish *Hymenoscyphus* isolates. H5 = 4795D, H8 = 80/13, H1 = Teagasc and H3 = RANT2). INDELS include: extra adenine at 847bp for H5 and H1 and

extra adenine at 857bp for H3. These variants generated three different haplotypes (1 = AA, A; 2 = A, A; and 3 = A, AA).

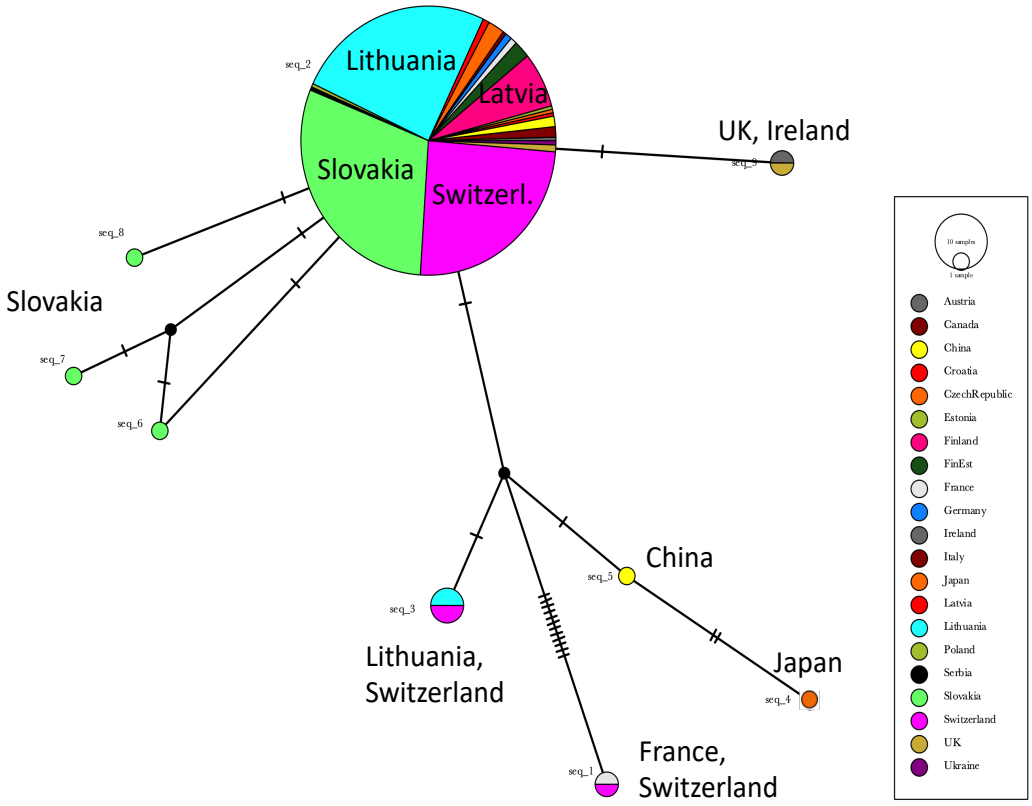

Figure 5.2 TCS network for *Hymenoscyphus fraxineus* strain variation in a European context. Size of pie is proportional to number of samples (smallest pie = 1 sample). The Irish and UK samples are found in the large pie and a satellite pie differing by one bp.

The TCS analyses revealed that most *Hymenoscyphus* sequences in GenBank, with high sequence similarity to the samples sequenced here, shared an identical haplotype (the large circle/pie in the analysis). The majority of samples were from Latvia, Lithuania, Slovakia and Switzerland. Two of the Irish samples (H3, H8)

belonged to this haplotype group. The other two Irish samples (H1, H5) belonged to Haplotype 9 and grouped with two samples from the UK. Some samples from France and Switzerland (bottom of network) were the most different and separated by 13 inferred mutations from their closest haplotypes. These were accession numbers HM193455 and MH864151 and are *H. albidus* (the non/less pathogenic sister taxon of *H. fraxineus*).

5.3.2 Antagonistic activity assay of ten shoot endophytes and six root endophytes with two strains of *Hymenoscyphus fraxineus* pathogen (H1 – Teagasc and H8 – 80/13).

5.3.2.1 Endophyte antagonism tests with *Hymenoscyphus* strain H8

The results for the antagonism experiments with *Hymenoscyphus* strain H8 are shown in Figure 5.3. A wide range of growth rates and interactions can be seen. For example, Endophyte E10 (*Meyerozyma guilliermondi*) showed maximum growth after 21 days (24.67 cm mean growth) whereas the pathogen grew much less (0.48 cm mean growth). However, in the case of the E5 endophyte (*Mollisia* sp.), the pathogen and endophyte grew in a similar way. On the other hand, the E9 endophyte (*Exophiala oligosperma*) showed poorer growth compared to the pathogen after 42 and 49 days. The details can be found in Appendix IV (Table 5.1). In six instances the pathogen showed very slow growth or did not grown at all, for example E1, E2, E3, E4, E6 and E7; those samples were repeated again and data shown in Figure 5.4

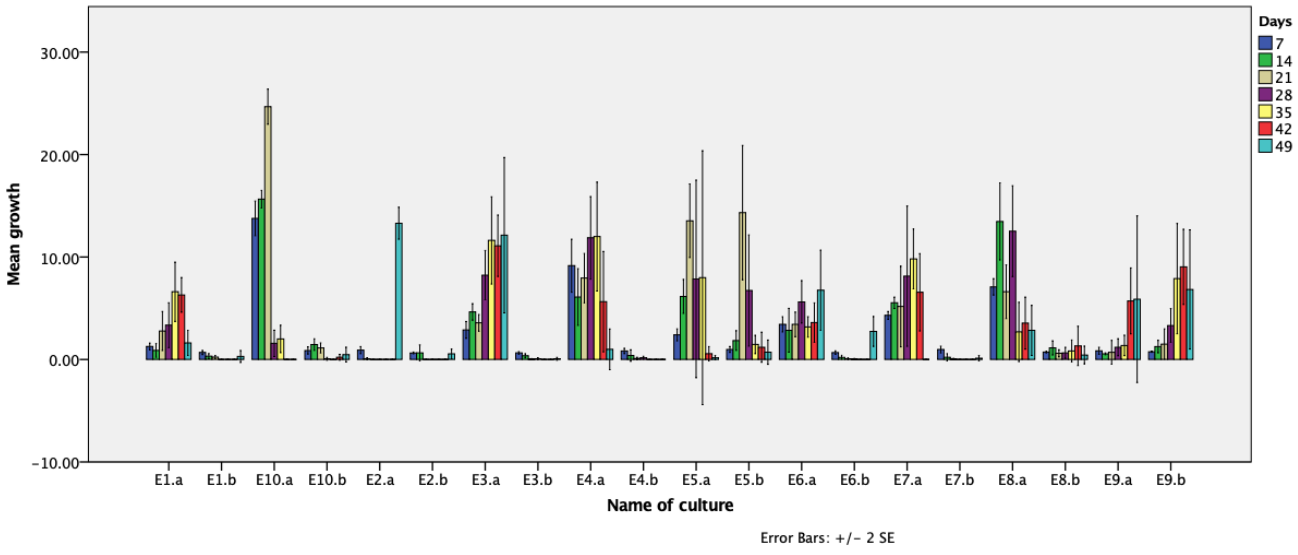

Figure 5.3 A mean growth at seven day intervals for 10 leaf endophytes co-grown with *Hymenoscyphus* strain H8 – 80/13. Growth (y axis) is in cm^2. Legend shows the day intervals. Endophytes are labelled as E1.a – E10.a and pathogen as E1.b – E10.b. Error bars are +/- 2 SE (=95% confidence limits).

Figure 5.4 shows the repeated samples. Again, a wide range of growth rates and interactions were observed. For example, the maximum growth of the endophytes was recorded for the E7 endophyte (*Pyronema domesticum*) after 28 days and the minimum for the E1 endophyte (*Naganishia diffluens*) after 14 days. In the case of endophytes E2 (*Epiccocum* sp.) and E9 (*Exophiala oligosperma*), the pathogens grew more than the endophytes but 95% confidence bars are large for some of the means. Details of the values are shown in Appendix IV (Table 5.2).

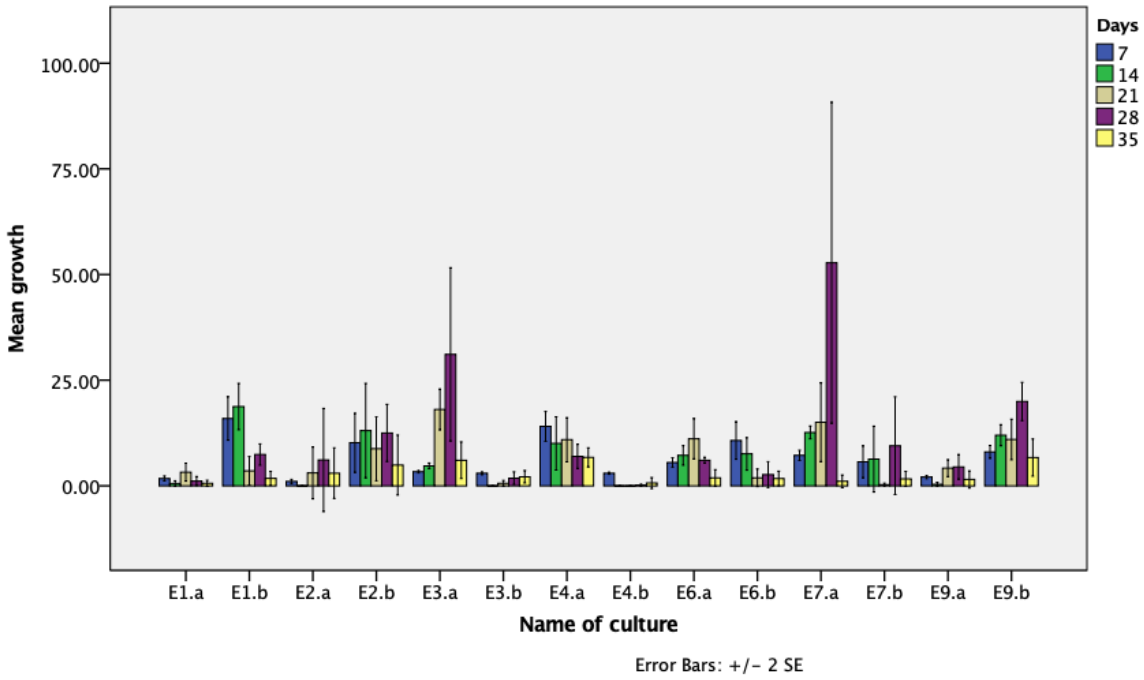

Figure 5.4 Mean growth at seven day intervals for seven leaf endophytes and the *Hymenoscyphus* pathogen strain H8 – 80/13. Growth (y axis) is in cm^2. Legend shows the day intervals. Endophytes are labelled as E1.a – E9.a and pathogen as E1.b – E9.b. Since the sample number in each bar is 5 so Error bars are +/- 2.776 SE (=95% confidence limits).

5.3.2.2 Endophyte antagonism tests with *Hymenoscyphus* strain H1

The antagonistic assay for the leaf endophytes was also carried out for another strain of *Hymenoscyphus* (Irish strain H1). Comparisons of growth among all endophytes and pathogen are shown in Figure 5.5. Among all leaf endophytes, E7 (*Pyronema domesticum*) showed the maximum mean growth against the pathogen. Endophyte E10 (*Meyerozyma guilliermondi*) also showed higher growth compared to the others. E5 showed a considerable amount of growth compared with the *Hymenoscyphus* strain (details of the values are given in Appendix IV (Table 5.3).

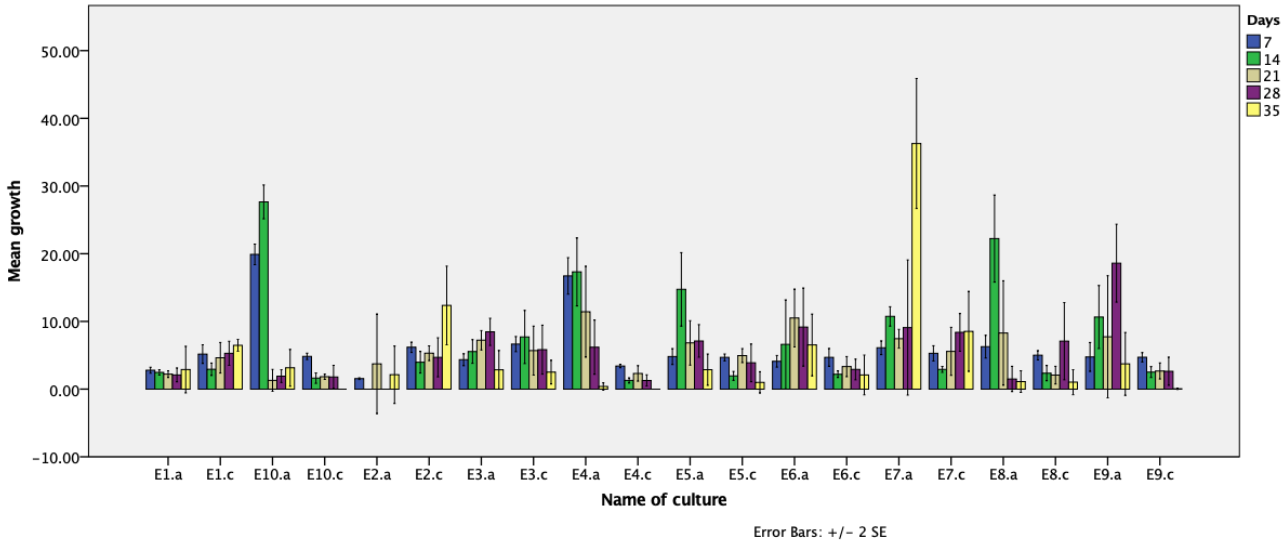

Figure 5.5 Mean growth for seven day intervals for 10 leaf endophytes and one strain of *Hymenoscyphus* pathogen (H1). Growth (y axis) is in cm^2. Legend shows the day intervals. Endophytes are labelled as E1.a – E9.a and pathogen as E1.c – E10.c. Since the sample number in each bar is 5 so Error bars are +/- 2.776 SE (=95% confidence limits).

5.3.2.3 Root endophyte and *Hymenoscyphus fraxineus* antagonism tests

A selected set of six root endophytes were also tested for antagonistic activity with the two *Hymenoscyphus* strains (Figure 5.6 and 5.7). All root endophytes showed considerably greater growth than *Hymenoscyphus* strain H8 (Figure 5.6). Among the six tested root endophytes, ER1 (*Lecanicillium attenuatum*), ER2 (*Cordyceps perangustam*) and ER5 (*Hydroposphaera* sp.) had the greatest effect against the pathogen. Maximum growth was observed on day 35 for ER1 and ER2 and at day 49 for ER5. On the other hand, endophyte, ER3 (*Penicillium spathulatum*) and ER4

(*Harzia velata*) showed a similar range of mean growth at day 42 and 49. Details of the values are given in Appendix IV (Table 5.4).

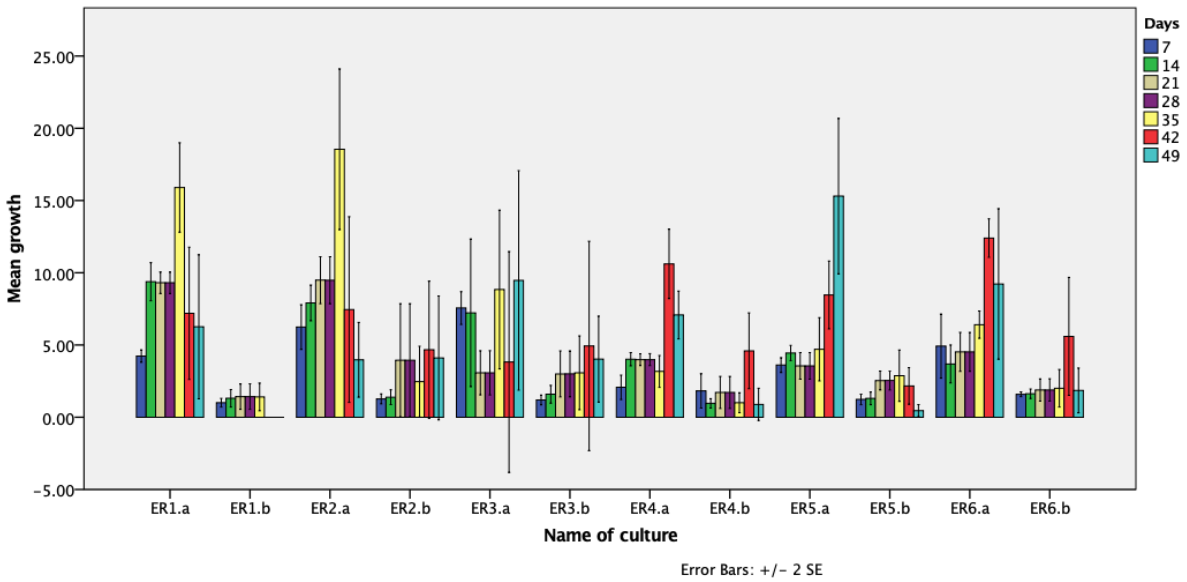

Figure 5.6 Mean growth at seven days intervals for six root endophytes and *Hymenoscyphus* pathogen strain H8. Growth (y axis) is in cm^2. Legend shows the day intervals. Endophytes are labelled as ER1.a – ER6.a and pathogen as ER1.b – ER6.b. Since the sample number in each bar is 5 so Error bars are +/- 2.776 SE (=95% confidence limits).

Results for the root endophyte antagonism assay for six endophytes co-cultured with *Hymenoscyphus fraxineus* strain H1 are shown in Figure 5.7. The greatest growth of endophytes against pathogen was observed for endophyte ER2 (*Cordyceps perangustam*) and ER3 (*Penicillium spathulatum*) at 35 days. However, in case of endophyte ER5 (*Hydroposphaera* sp.), the pathogen had a greater growth than the endophyte at the 28 days interval (but there was some overlap in the confidence limits). The growth of endophyte ER4 (*Harzia velata*) increased considerably after 35 days (18.76cm mean growth) and pathogen growth almost stopped (0.06cm mean growth). Similar observations were found for endophytes ER1 (*Lecanicillium attenuatum*) and ER6 (*Psilloglonium* sp.); after 35 days pathogen growth had almost stopped whereas the endophytes were still growing. Detailed values are given in Appendix IV (Table 5.5).

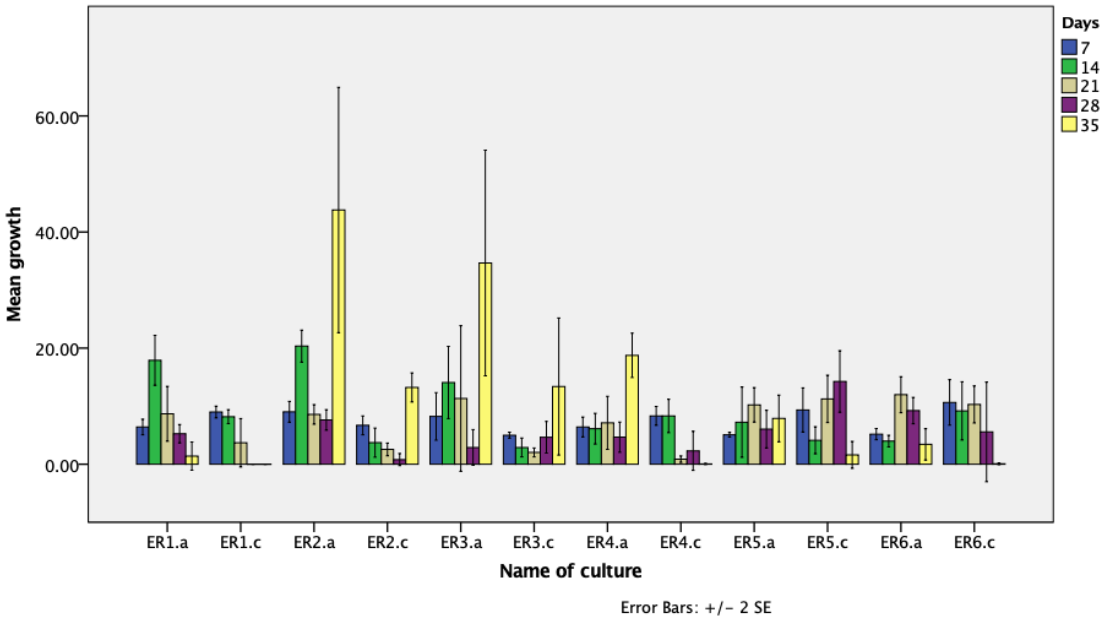

Figure 5.7 Mean growth at seven days intervals for six root endophytes and *Hymenoscyphus* pathogen strain H1. Growth (y axis) is in cm^2. Legend shows the day intervals. Endophytes are labelled as ER1.a – ER6.a and pathogen as ER1.c – ER6.c. Since the sample number in each bar is 5 so Error bars are +/- 2.776 SE (=95% confidence limits).

Photographs of the maximum growth achieved by the endophytes are shown in Figures 5.8 (a,b,c and d) and 5.9 (a,b,c,d,e and f).

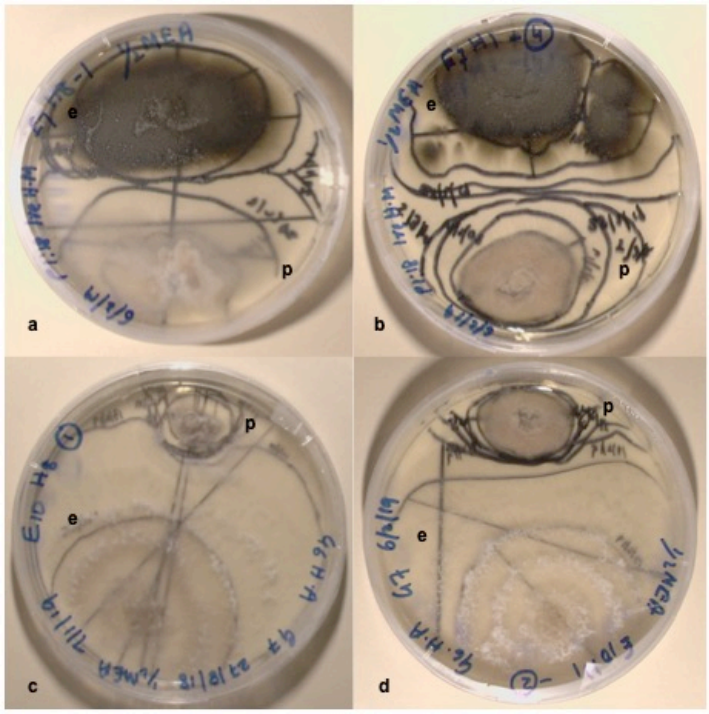

Figure 5.8 Images of antagonistic activity of two leaf endophytes (E7 – *Pyronema domesticum* and E10 – *Meyerozyma guilliermondi*) with two pathogens (H8 and H1). Upper row (a and b) represent the growth of endophytes E7 (labelled as e) and pathogen H8 and H1 (labelled as p) from left to right. Lower row (c and d) shows endophyte E10 (labelled as e) and pathogen H8 and H1 (labelled as p) from left to right.

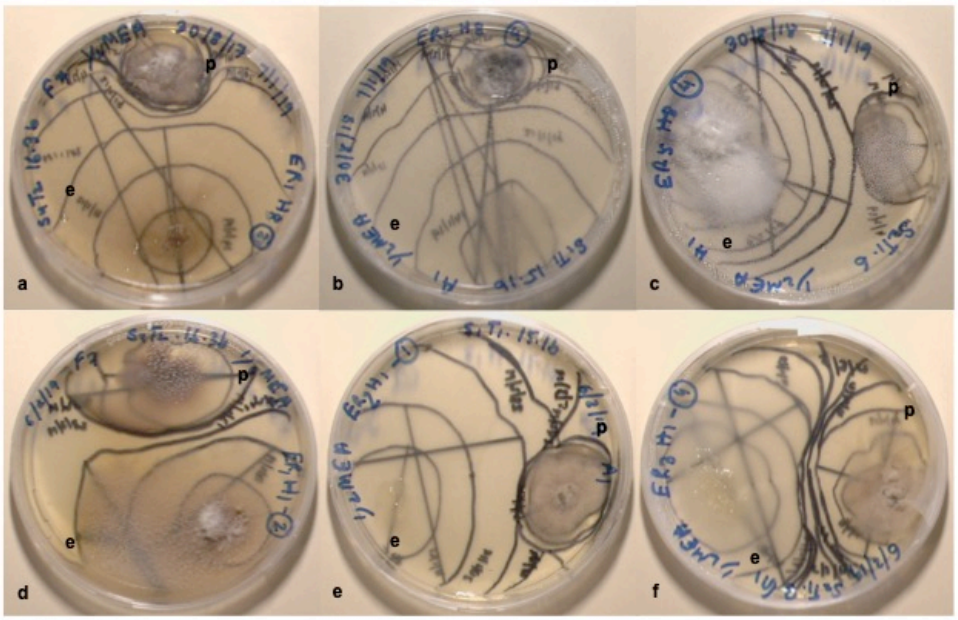

Figure 5.9 Images of antagonistic activity of root endophytes with *Hymenoscyphus* pathogen. Upper row left to right: ER1 – *Lecanicillium attenuatum*, ER2 – *Cordyceps perangustam* and ER5 *Hydroposphaera* sp.) with *Hymenoscyphus* pathogen H8. Lower row left to right: root endophytes (ER1 – *Lecanicillium attenuatum* and ER2 – *Cordyceps perangustam* and ER3 – *Penicillium spathulatum*) with *Hymenoscyphus* pathogen H1. Endophytes are labelled as 'e' and pathogen as 'p'.

Images of the other leaf and root endophytes which showed antagonism against pathogens are shown in Appendix IV (Figure 5.3 and 5.4).

5.3.3 Co-culture experiment

5.3.3.1 Shoot endophyte experiments

At five weeks from the start of the experiment plantlets were harvested and physiological parameters including shoot and root length, number of leaves/roots, leaf sizes, fresh and dry weights were compared (Figures 5.10, 5.5 in Appendix IV, 5.11 and 5.12). Plantlets were also scored 0 – 5, according to the severity of the visible disease infection. Plantlets were scored 0 for no visual infections, 1 for 1-20% infection (slight infection), 2 for 21 – 40% infection (plant ½ infected), 3 for 41 – 60% (plant ¾ infected), 4 for 61% - 80% infection (plantlets >¾ infected but not dead), and 5 for >81% infection (plant dying up or dead). A comparison of disease score is shown in Figure 5.13.

Figure 5.10 shows the growth of shoots and roots and Figure 5.5 (Appendix IV) shows the number of leaves in the biocontrol experiments. Growth of shoots and roots among treatments varied considerably and there is no recorded negative effect of the pathogen on the plants. Furthermore, the differences among treatment means are not large and the variance around the mean is high.

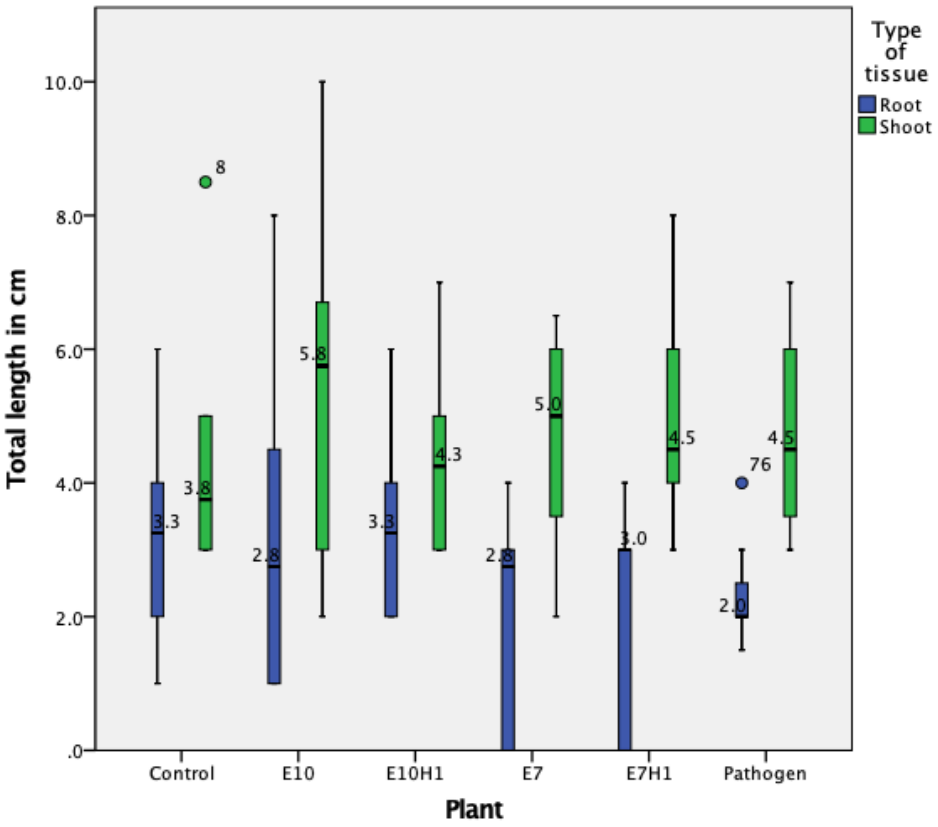

Figure 5.10 Each boxplot shows the comparison of total length of shoots and roots for 10 replicates in the leaf endophyte co-culture biocontrol experiment. Pathogen treatment = H1 on its own. Control = plants without endophyte or pathogen. Total length of shoots are higher for E10 treated plantlets and lower for control plantlets. Higher root lengths were observed for E10 treated plantlets and lower values for pathogen treated plantlets.

The same degree of variation was found for the number of leaves and roots in the biocontrol experiments (Appendix IV, Figure 5.5) and again there is little noticeable effect of the pathogen compared to the control. Furthermore, the dry and fresh weights follow a similar pattern (Figure 5.12).

Figure 5.11 shows that the mean leaf sizes for control plantlets were higher than the pathogen but with large overlapping confidence bars (95% confidence limits). The mean leaf area (for all three leaves measured in 10 replicates) were highest for endophyte plus pathogen treated plantlets (E10H1) and greater than for the endophyte E10 alone treated plantlets. The lowest leaf area was recorded for endophyte (E7H1) plus pathogen but this difference is within the confidence limits of the pathogen alone.

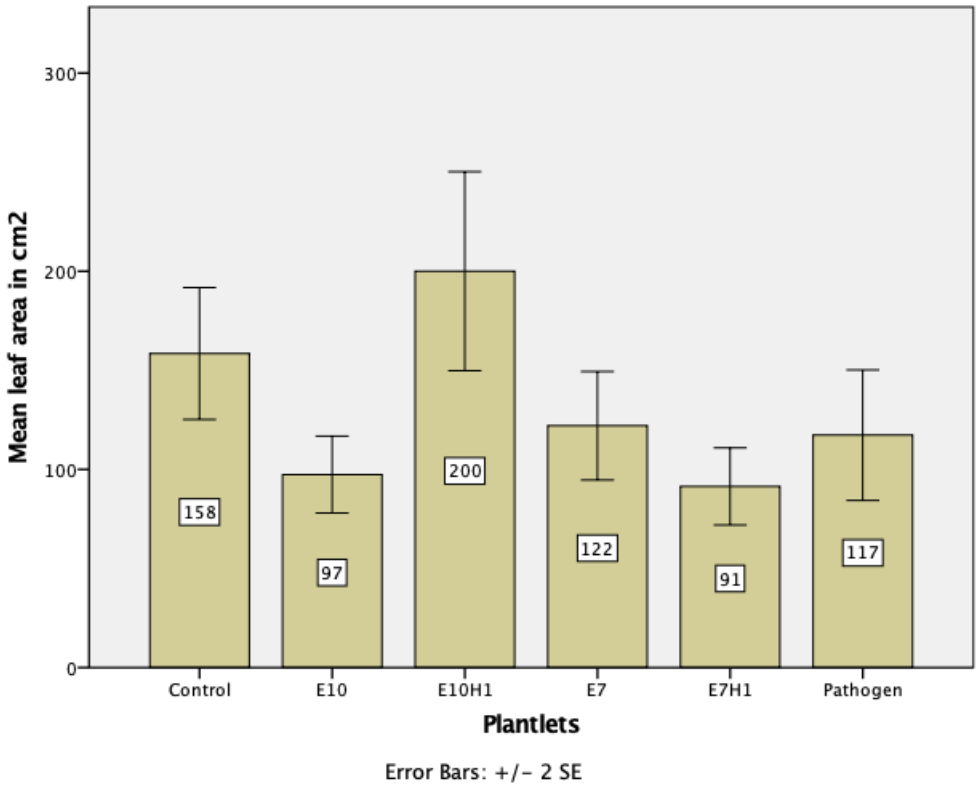

Figure 5.11 A comparison of mean leaf area across all categories of plantlets for shoot endophytes. Three leaf lamina were measured for each replicate plantlet and the mean value for total leaf for all replicates are given inside the bars. Since the sample number in each bar is 10 so Error bars are +/- 2.262 (=95% confidence limits). Pathogen = H1.

Figure 5.12 shows a comparison of total fresh weight (upper) and dry weight (lower) across all treatments. Total fresh weight showed less variation compared to total dry weights. The highest fresh weight was observed for E10 endophyte treatment (0.3g mean). Total dry weight was highest for control plantlets (0.03g mean) .

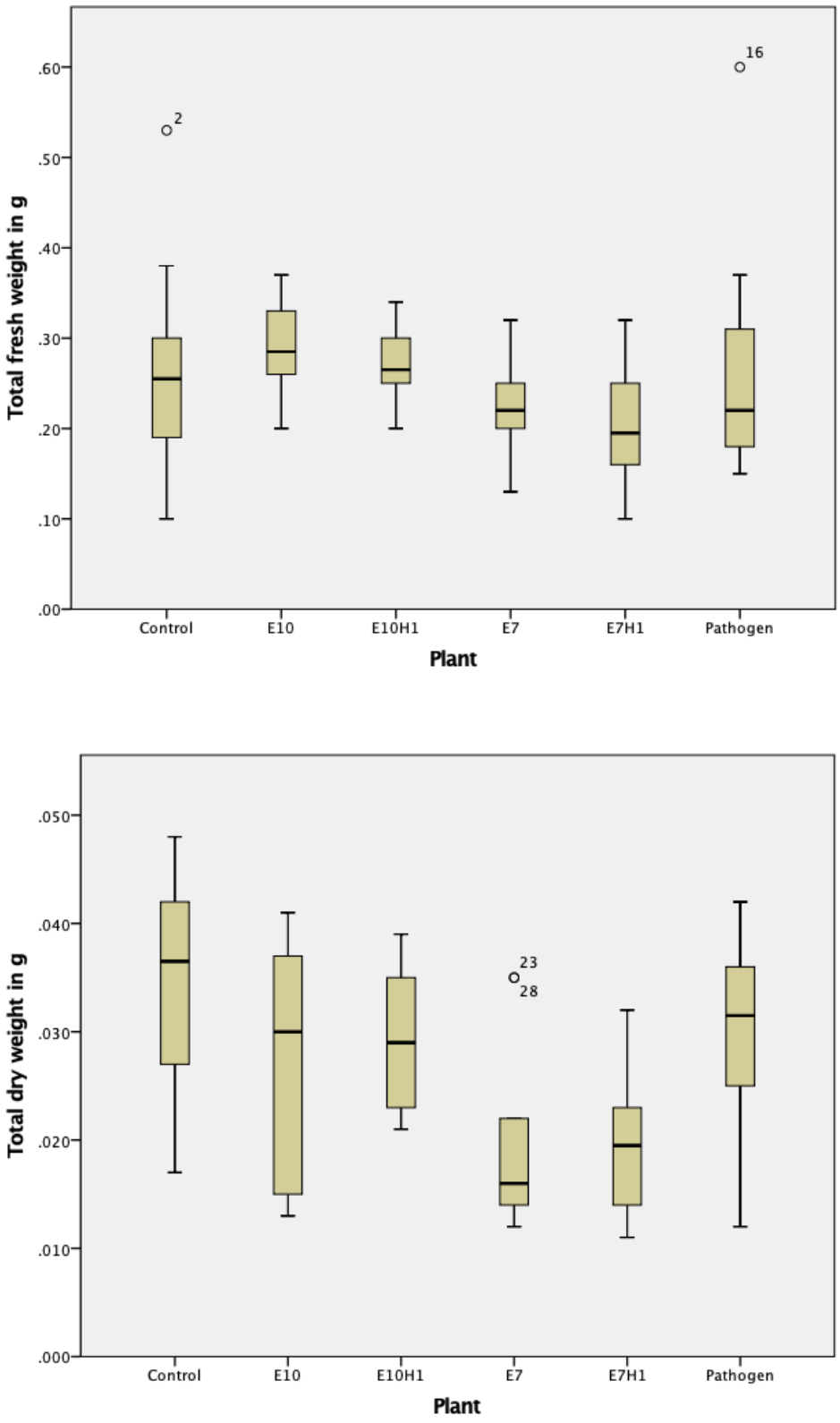

Figure 5.12 A comparison of total fresh (upper) and dry weight (lower) of plantlets for 10 replicates for all treatments. Pathogen = H1. Bars in each box plot represent the ranges of fresh weight and dry weight values in the plantlets on that particular treatment. Higher fresh weight was observed for E10 and lower for E7H1 (E7

endophyte and pathogen treated) plantlets. Higher dry weight was observed for the control treated plantlets and lower are dry weight was found in E7 treated plantlets.

The boxplot for disease score in Figure 5.13 shows that the pathogen, endophyte E7 and endophyte E7 plus pathogen (E7H1) treatments were in a similar range, whereas endophyte E10 treated plantlets had a consistently higher score (between 3 and 4) compared with the others. Whereas the others had a more variable range from 1 to 5. Endophyte E10 plus pathogen treated plantlets were scored 4 which means that no plants died due to infection; this also applies for E10 treated plantlets. No box plots are shown for the control as they were free from infection and hence scored as 0.

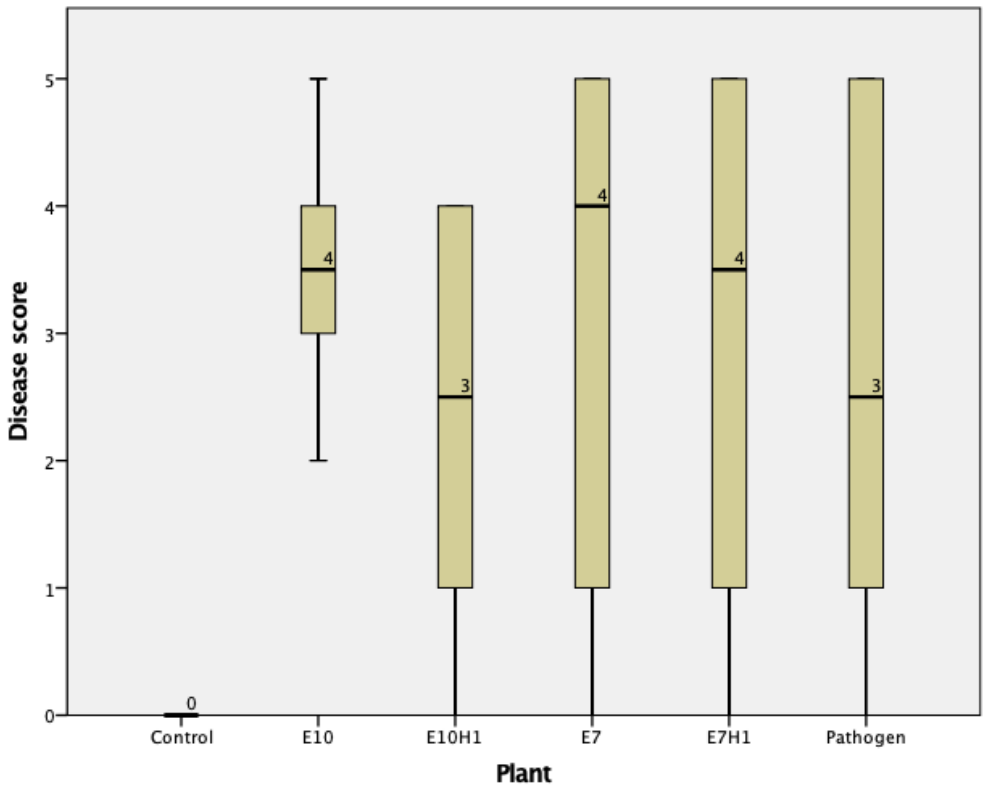

Figure 5.13 A comparison of disease scores (0 – 5) between different treatments among plant replicates for shoot endophytes. Median values are shown in the boxplots. Pathogen = H1. The bars in the box plot represents the range of values present. Maximum disease score was observed in E10 treated plantlets (3.6) and minimum disease score was observed in E10H1 treated plantlets (2.4) (for details see Appendix IV.

5.3.3.2 Root endophyte experiments

The co-culture experiment on root endophytes for 8 replicates were similarly harvested after five weeks, as for the shoot endophytes. Growth parameters such as shoot and root length, number of leaves/roots, leaf sizes, fresh and dry weights were compared (Figures 5.14, 5.15, 5.16, and 5.17). Disease scores are presented in the boxplot in Figure 5.18.

Figure 5.14 shows the comparison of the shoot and root length among the replicates of the root endophyte co-culture experiment. Overall variation in the median value among the treatments for shoot length varies greatly (4.3 – 6.5 cm) compared to root length which is between 1 – 2 cm. The highest shoot growth is observed for the pathogen treated plantlets.

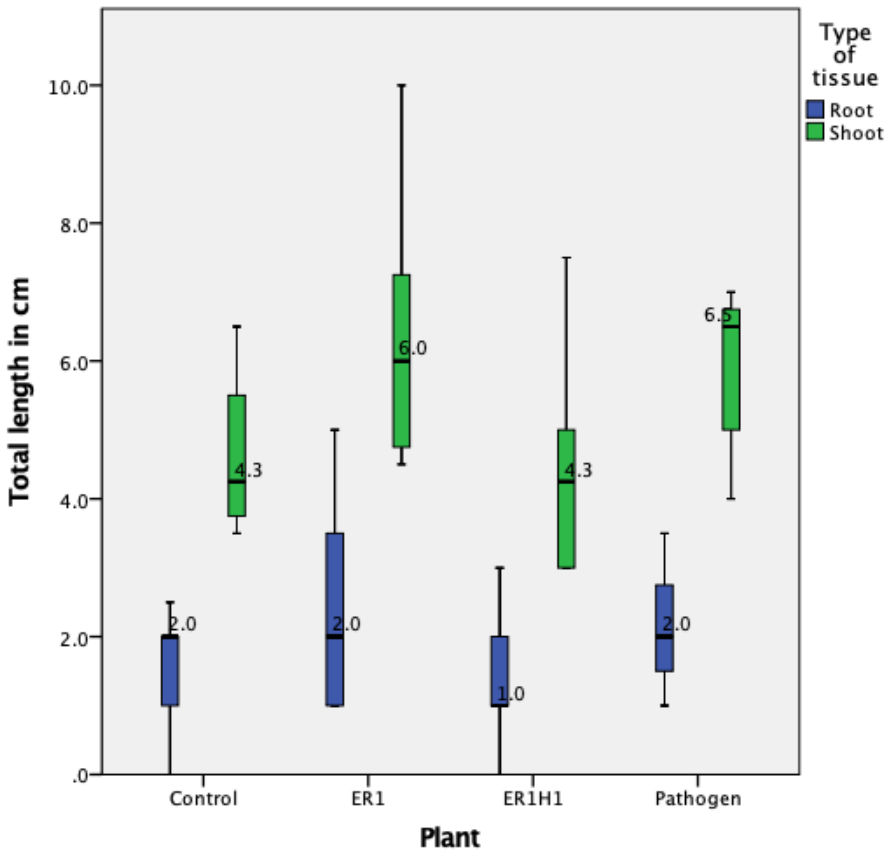

Figure 5.14 A comparison of total shoot and root length among all treatments, median values are shown in the boxplots for the root endophyte biocontrol experiment. Pathogen = H1.

The total number of leaves produced for the varying treatments were similar. However, the control plants and endophyte treated plants produced less than, or equal to, the number of leaves in the pathogen treatment (mean 8.1 leaves with 7 median value). Mean values for the number of roots ranged from 3.8 to 4.6 and did not vary much among treatments.

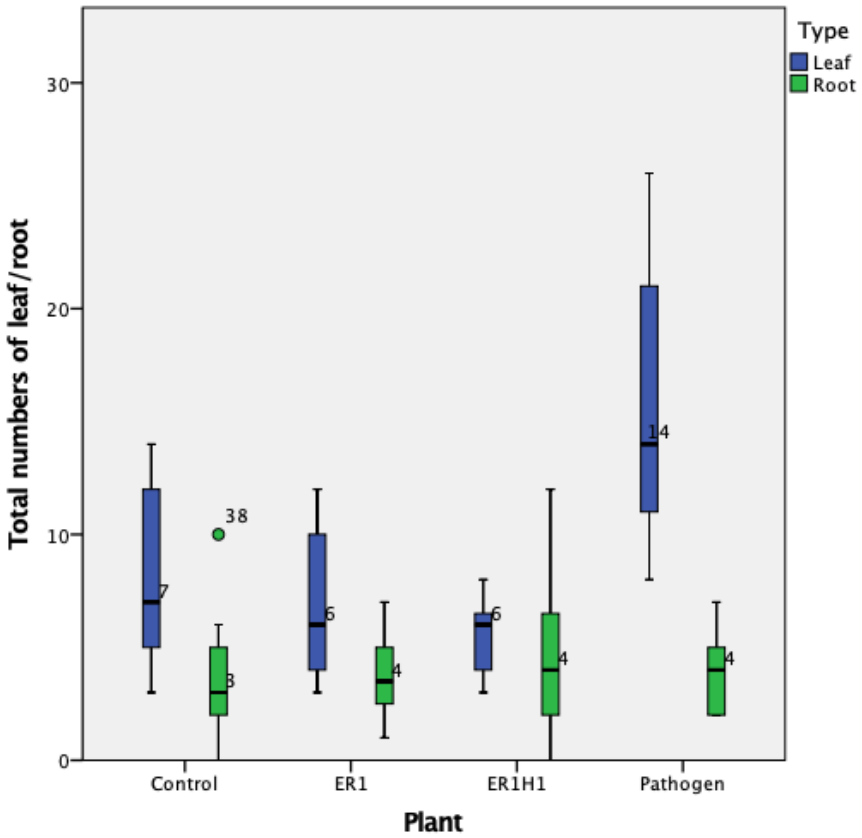

Figure 5.15 Total number of leaves and roots among all treatments for the root endophyte biocontrol experiment; individual lines in each box denotes median values. Pathogen = H1.

Mean leaf areas for three leaves for all replicate plantlets were higher for pathogen treated (191, 182 and 174 cm^2 mean values for leaf 3, 2 and 1) and lower in control plantlets (109, 88 and 119 cm^2 mean values for leaf 3, 2 and 1). Control plantlets had smaller leaf lamina than the pathogen treated ones; however, among endophyte treated (ER1) and endophyte plus pathogen treated plantlets produced slightly higher mean values (136, 149 and 184 cm^2 mean values for leaf 3, 2 and 1) for the size of leaflets.

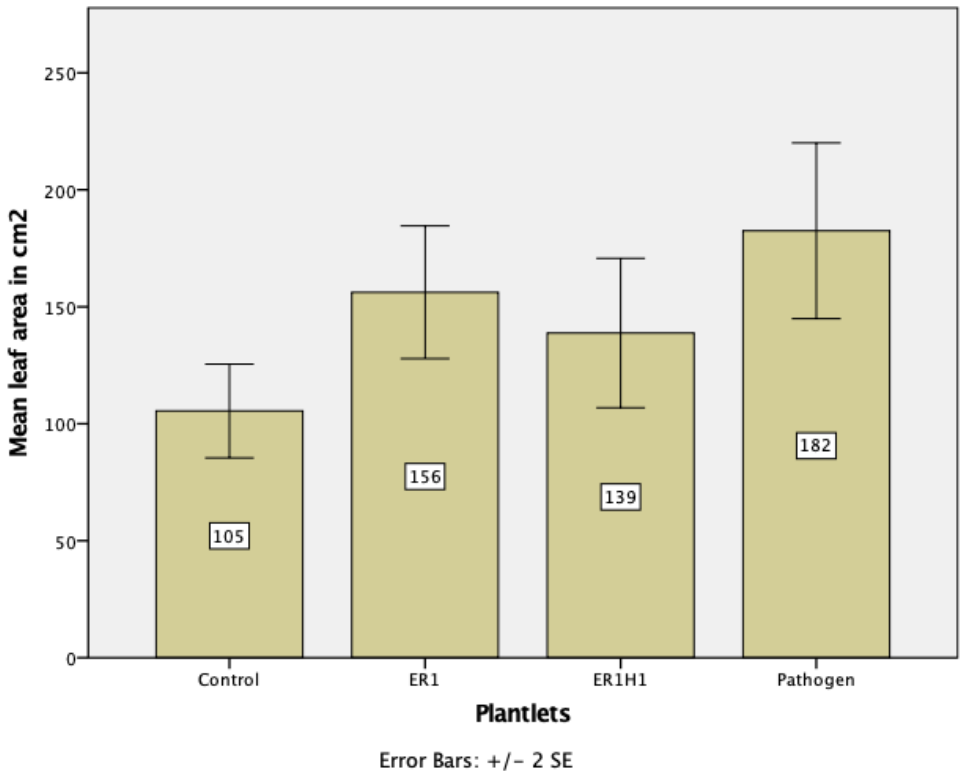

Figure 5.16 A comparison of mean leaf area in (cm^2) across all categories of plantlets for root endophytes. Three leaf lamina were measured for each replicate and the mean value for all leaves (24) for all replicates are given inside the bars. Since the sample number in each bar is 5 so Error bars are +/- 2.365 SE (=95% confidence limits). Pathogen = H1.

Figure 5.17 shows the total fresh weight (upper) and dry weight (lower) in all replicates for the root endophyte biocontrol experiments. Plants showed a range variation from 0.02 – 0.3g fresh weight. The highest fresh weight was obtained from pathogen treated plantlets with a 0.21g median value (0.2 mean) and the lowest in endophyte treated plantlets, with a 0.05g median value (0.1 mean). Endophyte plus pathogen (ER1H1), pathogen treated, and control treatments were in a similar range 0.28g, 0.33g and 0.26g mean values for 8 replicates. Dry weight values showed a less variable range, mostly from 0.02 – 0.03g median values. Pathogen and pathogen plus endophyte had slightly higher dry weight than endophyte treated and control plantlets.

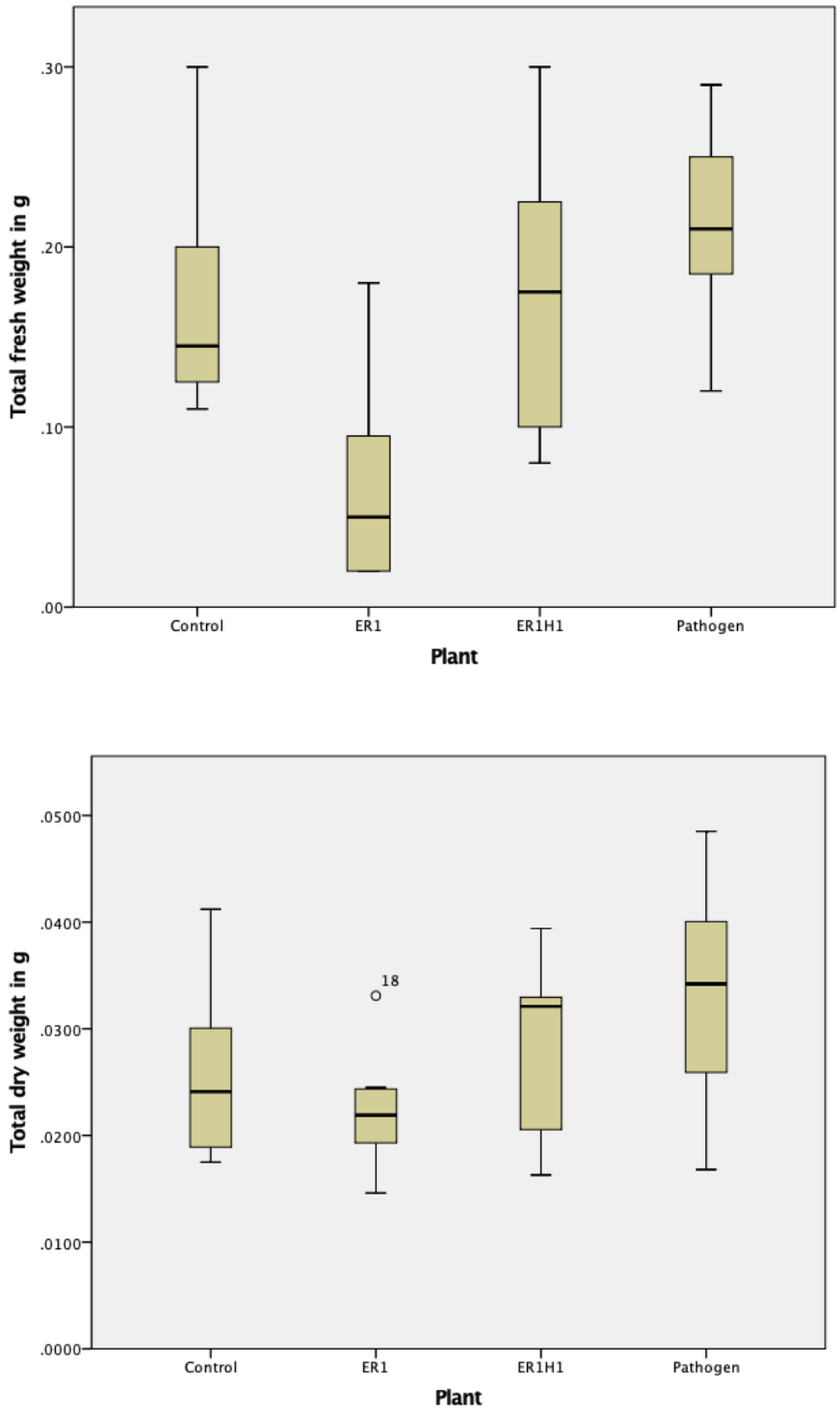

Figure 5.17 A comparison of total fresh (upper) and dry weight (lower) of plantlets for 8 replicates in all root endophyte biocontrol experiment treatments. Pathogen = H1.

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From the disease score boxplot graph it was evident that the pathogen plus endophyte treatment had higher disease scores (median disease score of 5) than only pathogen treated plantlets (median disease score of 2). Surprisingly, the endophyte treatment had double the disease score compared with the pathogen treatment. This might be because the endophyte is pathogenic or that the strain of pathogen we have used is not as infectious for an *in vitro* environment. As we have seen earlier, *Hymenoscyphus* strains have high variation. Furthermore, the experiment was only run for a short period of time as it was designed for rapid screening purposes.

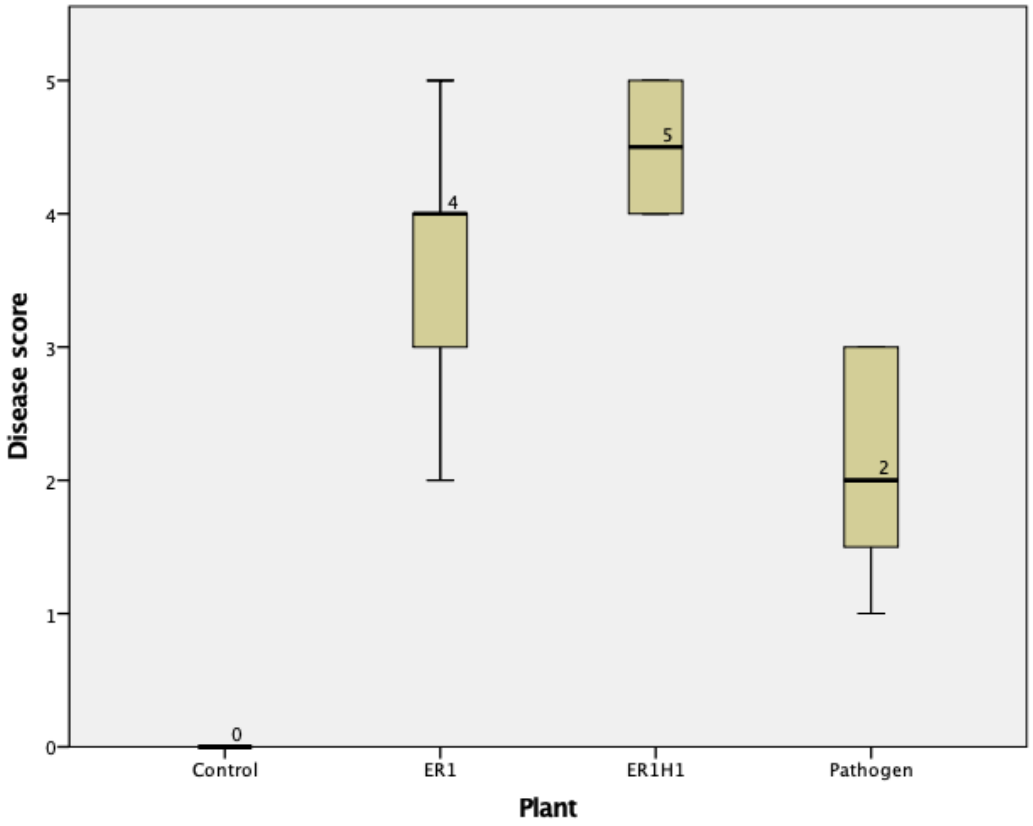


Figure 5.18 A comparison of disease scores (0 – 5) between different treatments among plant replicates for the root endophyte biocontrol experiment. Median values are shown in the boxplots. Pathogen = H1.

Images from the shoot endophyte experiment of plantlets for control and pathogen are shown in Figure 5.19. a and b; endophyte treated (E7 and E10) plantlets shown in Figure 5.20. c and d; and endophyte plus pathogen treated plantlets (E7H1 and

E10H1) shown in Figure 5.21. e and f. Images for root endophyte treatments are shown in Appendix IV – Figure 5.6a, b, c and d.

Figure 5.19 Shoot endophyte biocontrol experiment images 1. a) Control plantlets after five weeks, b) Pathogen H1 plantlets after five weeks.

Figure 5.20 Shoot endophyte biocontrol experiment images 2. c) Endophyte (E7) treated plantlets after five weeks, d) Endophyte treated (E10) plantlets after five weeks.

Figure 5.21 Shoot endophyte biocontrol experiment images 3. e) Plantlets treated with endophyte and pathogen (E7H1) after five weeks, f) Plantlets treated with endophyte and pathogen (E10H1) after five weeks.

Differences in tissue characteristics were found with the stained and processed leaf tissue sections of control, pathogen treated, endophyte treated (E7 and E10) and endophyte plus pathogen treated (E7H1 and E10H1) plantlets under 10x and 20x magnification (Figure 5.22 a – f).

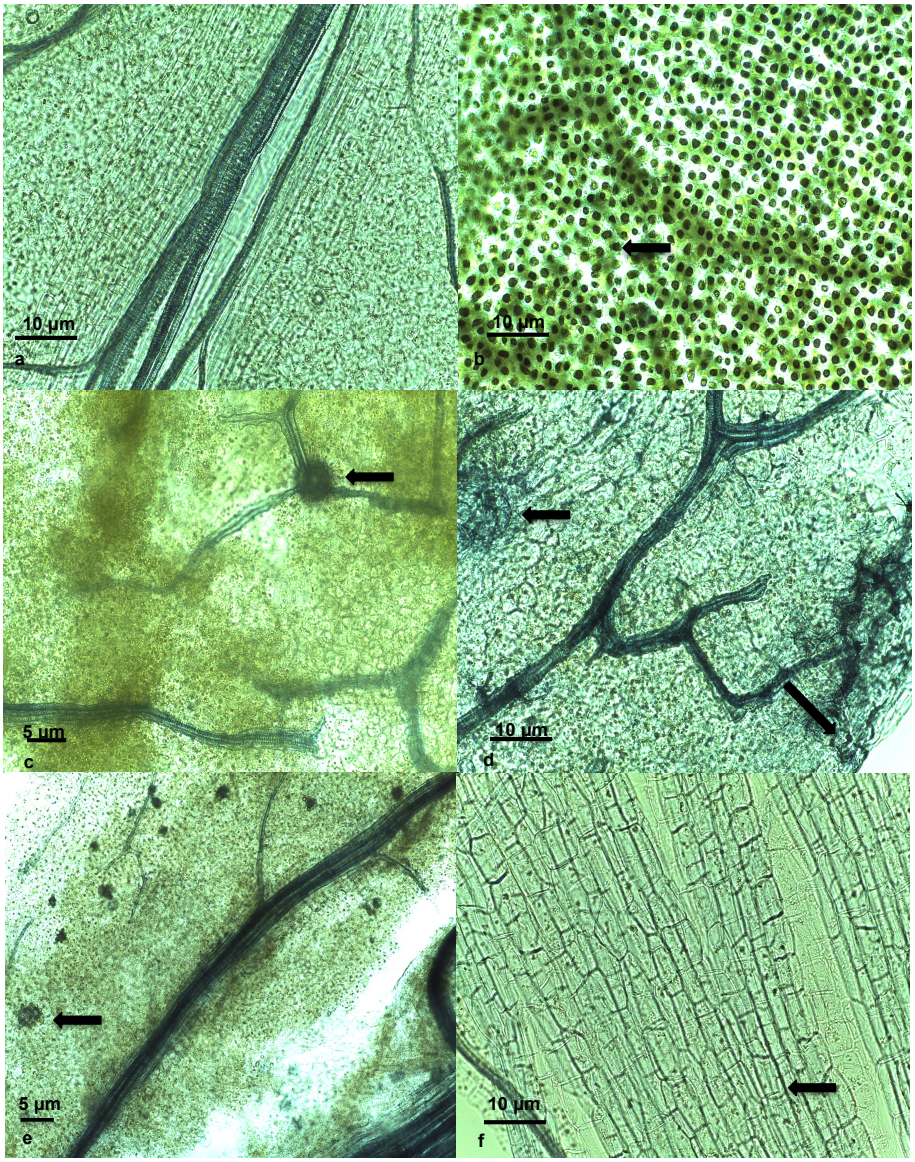


Figure 5.22 Leaf tissue microscopy from the biocontrol experiment. a) stained leaf tissues from control plantlets under 20x magnification, b) leaf tissues treated with pathogen with depositions of spores inside the cells (arrow) under 20x magnification, c) leaf tissues from endophyte E7 treated plantlets with sporangia in between leaf veins under 10 x magnification, d) leaf tissues treated with endophyte E10 with mycelial fragments which are stained darker than the leaf cells in 20x magnification, e) leaf tissues from endophyte and pathogen treated plantlets (E7H1) under 10x magnification; sporangia are stained dark, f) leaf tissue from endophyte and pathogen treated (E10H1) plantlets with mycelia inside the cells.

We also tested for endophytes within the roots for the co-culture experiments with root endophytes. The stained roots for control, pathogen treated, endophyte treated (ER1) and endophyte plus pathogen treated (ER1H1) plantlets are shown in Figure 5.23 a, b, c and d.

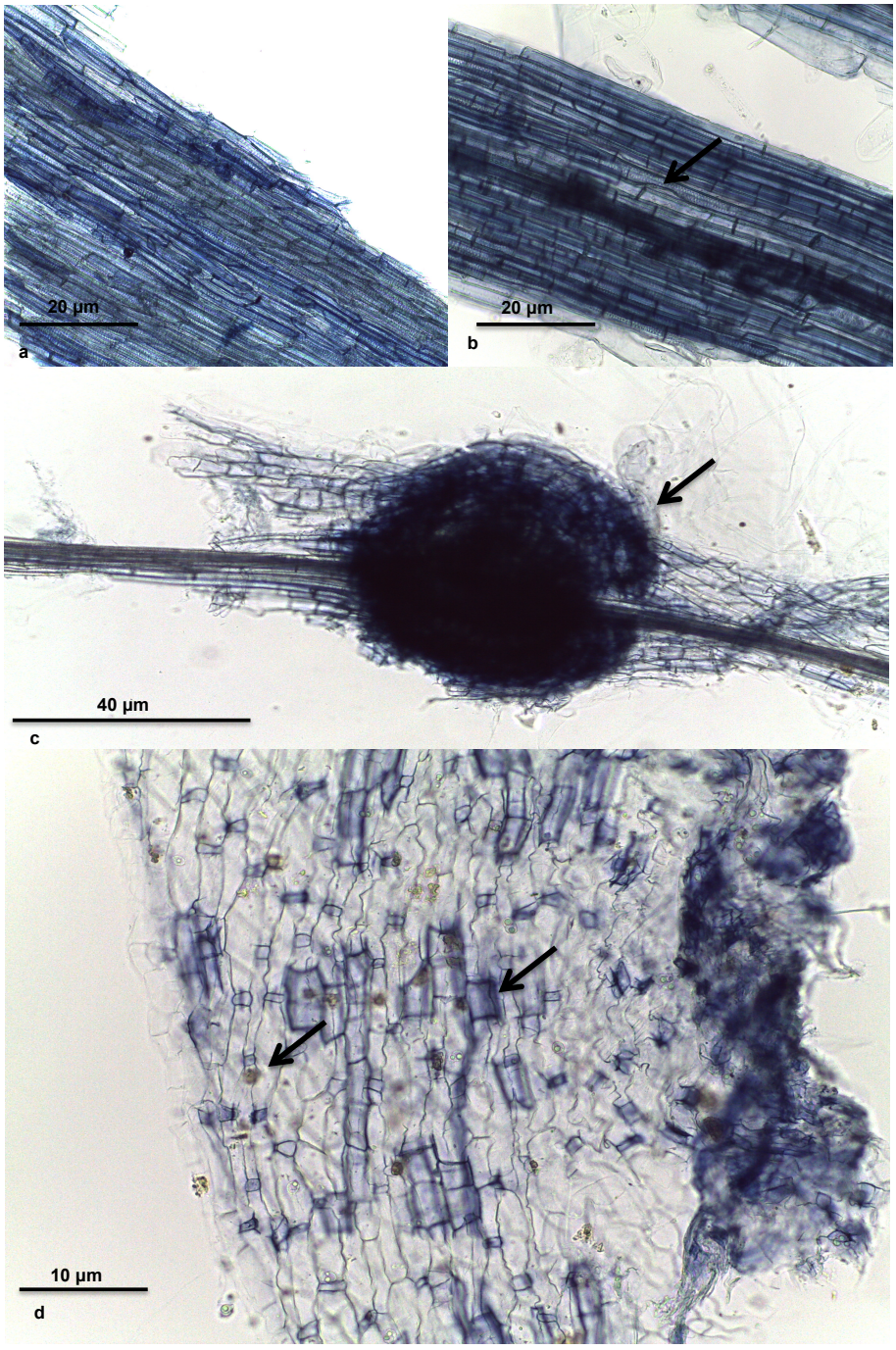


Figure 5.23 Root sections from the root endophyte biocontrol experiment. a) stained root cells of control plants under 10x magnification showing absence of

fungal mycelia, b) root cells treated with pathogen with mycelia growing inside the root cell walls, c) root cells for plantlets treated with endophyte (ER1) under 10x magnification with large sporangia stained dark blue in the junctions of the root cells, d) root cells under 20x magnification from the plantlets treated with endophyte and pathogen (ER1H1). Cells with mycelial growth are visible as dark blue patches with a brown mass of spores in between cells.

5.4 Discussion

5.4.1 Assessment of *Hymenoscyphus fraxineus* strain variation

We detected some variation in the nrITS sequences of *Hymenoscyphus fraxineus* obtained from Irish and Northern Irish material (Figure 5.1). The four strains of *H. fraxineus* (three Northern Irish and one Irish) have three different haplotypes. This is the first report of strain variation in Ireland. The TCS analysis showed that the Irish and Northern Irish strains shared an identical haplotype with most of the GenBank sequences of previously sequenced *H. fraxineus* from other European sites. The two Northern Irish strains (H3 and H8) shared a haplotype with samples from Latvia, Lithuania, Slovakia and Switzerland and one Irish strain (H1) and Northern Irish (H5) were the same as haplotype 9 and grouped with UK samples. It is not possible to infer the invasion route of *Hymenoscyphus* into Ireland from these data but is noteworthy that strain variation is present. Gross *et al.* (2014) estimated (using DNA barcoding for nrITS, EF1-α, calmodulin and actin gene) that two different and major lineages of *Hymenoscyphus fraxineus* may have invaded Europe. It is not clear which of these our samples group with.

5.4.2 Antagonistic response of *Hymenoscyphus fraxineus* to the selected endophytes

5.4.2.1 Shoot endophytes

We tested 10 leaf endophytes and 6 root endophytes for antagonism activity against one Irish strain (H1) and one northern Irish (H8) strain separately and found out that among leaf endophytes, *Pyronema domesticum* (E7) and *Meyerozyma guilliermondi* (E10) showed considerable amounts of antagonistic activity against both strains of *H. fraxineus*. A study conducted by Kosawang *et al.* (2018) tested antagonistic activity against *H. fraxineus* ascospore germination and found strong inhibitory *Hymenoscyphus* growth effects for the leaf endophytes *Boeremia exigua, Epicoccum nigrum,* and *Fusarium* sp. We isolated *Boeremia exigua, Epicoccum nigrum,* and *Fusarium* sp. as endophytes from leaf tissues of ash but did not use *Boeremia exigua* and *Fusarium* sp. in our biocontrol

experiments because they have been reported as potential pathogens. We tested antagonistic activity of *Epicoccum nigrum* for both strains of *H. fraxineus* but these failed to reduce the growth of the pathogen; in fact the pathogen mostly outcompetes *Epicoccum nigrum.*

Schlegel *et al.* (2016) tested the inhibitory effect of endophytes against *H. fraxineus* ascospore germination and found a strong inhibition on germination for *Paraconiothyrium* sp., *Boeremia exigua*, *Kretzschmaria deusta*, *Ampelomyces quisqualis* Ces. and Elsinoaceae sp.. and weak effects for *Venturia* spp. and *Nemania serpens.* We isolated *Boeremia exigua* and *Paraconiothyrium* sp. from leaf tissues of *Fraxinus excelsior* but did not assay inhibitory effects on ascospore germination.

5.4.2.2 Root endophytes

We tested six root endophytes isolates for antagonism against both the pathogen strains (H1 and H8) and found that *Cordyceps perangustum* (ER2) and *Penicillium spathulatum* (ER3) showed strong antagonistic effects against the Irish *H. fraxineus* strain (H1). The endophytes *Lecanicillium attenuatum* (ER1), *Cordyceps perangustum* (ER2) and *Hydroposphaera* sp. (ER5) showed antagonistic activity against Northern Irish *H. fraxineus* strain (H8). No other research has been reported yet on root endophyte antagonistic assay against *H. fraxineus*; thus this is the first report of such interactions.

5.4.3 Biocontrol potential: growth differences among control, endophyte treated, pathogen treated and endophyte plus pathogen treated plantlets

Co-culturing of *in vitro* grown (endophyte-free) plantlets with two leaf endophytes *Pyronema domesticum* (E7) and *Meyerozyma guilliermondi* (E10) and one root endophyte *Lecanicillium attenuatum* (ER1) with Irish *H. fraxineus* strain (H1) showed no biocontrol effect against the pathogen. We were successful in establishing the endophytes inside the leaf and root cells (Figure 5.22 and 5.23) but unfortunately endophyte treated plantlets showed higher disease scores than the pathogen treatment (mean value of 4 and 3 for leaf and root endophytes; whereas pathogen treated plantlets scored 3 and 2 respectively). The microscopy (Figure 5.22) showed that leaf cell structure in control, endophyte-treated and endophyte-pathogen treated plantlets was different from the only pathogen treated plantlets (which showed higher amounts of deposition in laminar cells). Mean dry weights were higher (0.04g) in control than the other treatments.

Meyerozyma guilliermondi (E10) treated plantlets, E10 plus pathogen treated, and only pathogen treated had similar dry weights (0.03g) but endophyte *Pyronema domesticum* (E7) treated and E7 plus pathogen treated plants had lower dry weights (0.02g).

The root endophyte biocontrol experiments with *Lecanicillium attenuatum* (ER1) showed similar mean value. The endophyte (ER1) treatment had the lowest dry weight accumulation (0.02g). Endophyte isolates may sometimes, under certain conditions, show pathogenic effects on the host plant as they can exist in many states along a symbiotic-pathogenic continuum (Hodkinson and Murphy, 2019). This might explain the high disease score for the endophyte treated plantlets in our studies. It could be that they are pathogenic in plant establishment but not after that. A biocontrol study on *ex vitro* grown trees of *Fraxinus excelsior* for 24 isolated endophyte taxa was performed by Bakys *et al.* (2009a) and they found that 4 taxa *A. alternata*, *E. nigrum*, *Chalara fraxinea* and *Phomopsis* sp. 57 had high scores of visual necrosis on bark and cambium of *Fraxinus excelsior*. Another *ex vitro* biocontrol assay was performed by Schlegel *et al.* (2016) to investigate potential protective effects of endophytes against the ash dieback pathogen. In the field (infected forest) using endophyte free and plants pre-inoculated with endophytes, no significant effect of endophyte *Venturia* spp. was recorded after trees were infected with *Hymenoscyphus fraxineus*. However, our study related to *in vitro* conditions and no biocontrol assay on *in vitro* ash plantlets has previously been reported.

Another reason for our inconclusive biocontrol results is that the pathogen may need more time to produce a higher infection rate; our plantlets were only grown with pathogen for four weeks which may not be enough time for *Hymenoscyphus fraxineus* to show sufficient symptoms of dieback. Treatment with endophyte, on the other hand, showed more infection in the *in vitro* plantlets. During the *in vitro* establishment of plants it was seen that ash plantlets need an external carbohydrate source in the form of sucrose. Furthermore sucrose, has also been shown to be essential for the growth and rooting of ash plants *in vitro* by Lebedev and Shestibratov (2016). We did not use any sucrose for our co-culture experiment, and the plantlets were weak in the beginning and also after the endophyte treatment, so the endophytes were taking up all the nutrients available from the ash plantlets, leaving the plantlets with a lack of nutrients to establish themselves and support endophyte growth at the same time. Consequently, a positive establishment of the relationship between plant and endophytes was not

possible. Detailed information of the results of the co-culture experiment are presented in Appendix IV (Table 5.6 and 5.7).

5.5 Conclusions

Our antagonistic assays and biocontrol experiments have provided preliminary data for future biocontrol experimental trials. More biocontrol experiments need to be undertaken to find endophytes with inhibitory affects against *Hymenoscyphus fraxineus*. The culture system also needs to be modified to improve endophyte establishment without reducing the growth of the plantlets. Many internal factors (such as host physiology, interaction of host with the endophytes, media used) add up during the co-culture experiments and more trails are required to standardise the experiments and encourage positive beneficial relationship with the endophyte which in turn will be helpful for both (plant and endophytes) to increase the defense against the pathogens. It is also important to test biocontrol against different *Hymenoscyphus fraxineus* strains and our sequencing of nrITS has identified differing strains of the pathogen for future use.

Chapter 6
Final Discussion

This thesis set out to:

1. Isolate root and shoot fungal endophytes from populations of *Fraxinus excelsior* and also representative individuals of other ash species including *F. angustifolia*, *F. dipetala*, *F. glabra, F. mandshurica*, *F. ornus*, *F. texensis*, *F. pennsylvanica* and *F. potamophila*.
2. Identify the culture dependent isolated fungi using DNA barcoding of the nrITS DNA region (compared to the LSU and *tef* regions), to assess the endophyte species richness and community composition of different plants, taxa and tissues.
3. Directly identify the endophytes of ash using HTS (Illumina High Seq amplicon sequencing) to estimate the culture independent components of the ash endophytic mycobiome and to compare species richness and community composition estimates from different plants and tissues (leaves and seeds) and geographical regions.
4. Compare Irish *Hymenoscyphus fraxineus* cultures with those found elsewhere, using DNA sequencing of nrITS, to detect any strain variation and to investigate its invasion route in to Ireland.
5. To use *in vitro* antagonism testing of endophytes against *H. fraxineus* to screen potential endophytes for biocontrol activity.
6. Establish an efficient plant tissue culture method for the generation of large *in vitro* experimental populations of ash from both embryo culture and seed culture (to remove dormancy of seeds) and develop an *in vitro* pathogen testing process.
7. Conduct endophyte, pathogen and ash tree interaction studies to establish the role of endophytes in ash dieback disease resistance.

The main findings are summarised below and their limitations and recommendations for further study outlined.

6.1 Isolation of foliar and root endophytes from *Fraxinus excelsior* and other ash taxa (Chapter 2)

Considering the current scenario of constantly declining numbers of ash trees throughout Europe due to the dieback pathogen *Hymenoscyphus fraxineus* and to the fact that the disease currently beyond the hope of eradication, we have tried to find an alternative solution to the problem by finding potential endophytes that can

be either encouraged or applied as biocontrol agents. We have studied the mycobiome of different ash populations (leaf and seed) in a European provenance trial situated in Roosky, Co. Roscommon. This trial has subsequently been found to have ash dieback diseased plants (Douglas, personal communication). We also sampled ash trees from Kinsealy, Co. Dublin for root endophyte isolations and a collection of different *Fraxinus* taxa (*F. angustifolia, F. dipetala, F. glabra, F. mandshurica, F. ornus, F. texensis, F. pennsylvanica* and *F. potamophila*) for leaf and seed endophytes from the National Botanic Garden, Glasnevin. We recovered endophytes from different categories of healthy and diseased tissues of leaves and roots (Chapter 2) to isolate fungal cultures and characterise them for potential biocontrol agents against *H. fraxineus*.

We sampled a total of 610 tissues for foliar and root endophytes in all three Irish sites (Roosky, Glasnevin and Kinsealy) and recovered a total of 628 isolates (taxonomic details in Table 6.1 and 6.2). Hence the recovery success for the three sites were 0.99 (leaves, Roosky), 0.78 (leaves, Glasnevin) and 1.69 (roots, Kinsealy) isolates per single leaf/root tissue sample (Chapter2). In another study, by Bakys *et al.* (2009a) obtained 150 endophyte strains from 220 sampled tissues from leaf stalks (necrotic and healthy) and bark (wounds and canker), which is an average of 0.7 isolates per tissue sampled. Furthermore, Bakys *et al.* (2009b) made isolations from four different categories of shoots (healthy, necrotic, advance necrotic and dead tops) and found an 88.3% success rate of obtaining at least one endophyte. In our study, we found fungal growth from 492 of 545 of leaf samples which equates to 90.3%. Bakys *et al.* (2009b) also found a success rate of 87.9% from tissue within initial necrosis and 90% with tissue showing advanced necrosis. We compared diseased and healthy material and found similar levels of endophyte recovery success although the species and morphotypes varied. Another study on shoots, bark and wood from resistant and susceptible ash trees by Haňáčková *et al.* (2017) obtained 884 different isolates and found differences among tissue types.

We studied the influence of media for isolation of foliar ash endophyte for the first time with half strength MEA and MEAF media (the MEAF contained leaves of ash in the media preparation) and retrieved endophytes from 92% and 95% of tissues sampled. Bakys *et al.* (2009a) compared three types of agar media (2% malt extract, vegetable juice agar and water agar) but did not evaluate the percentage

recovery according to the media used. However, they mentioned a 0.7 endophyte average recovery per attempted isolation.

We sampled foliar endophytes from different ash tissue (healthy and diseased) and populations and found 19 (4 trees sampled) and 71 (19 tree sampled) different isolates from *Fraxinus excelsior* and the sample of other *Fraxinus* taxa from second site, Glasnevin. Ibrahim *et al.* (2017) recovered endophytes from *Fraxinus ornus* leaflets and petioles and recorded 97 – 99% presence of endophytes. We sampled *Fraxinus ornus* from Glasnevin and retrieved 14 endophyte isolates from 20 leaf tissues with a 70% recovery success (Chapter 2, Figure 2.7).

We isolated foliar endophytes from the EU provenance trial, representing 11 countries, and obtained a range of isolate numbers from 31 to 49. The highest number of endophytes was found from the Irish samples and lowest from Lithuania. The highest number of morphotypes was found from endophytes obtained from UK sourced samples (46 isolates and 4.2 morphotype per sample). We could not find on other studies on endophyte isolation of *Fraxinus excelsior* from different provenance trails (Chapter 2, Figure 2.3 and 2.4).

Aerial colony growth of twenty selected isolates from the first site (Roosky) were monitored on half strength of MEA, PDA, corn meal agar and Sabouraud media for 25, 43 and 64 days. We found that PDA and Sabouraud had the highest growth rates on average across the endophytes studied (Chapter 2, Figure 2.10). Other studies such as Bakys *et al.* (2009a) (2% malt extract, vegetable juice agar and water agar) and Haňáčková *et al.* (2017) (2% wort agar) compared different media but did not compare colony growth rates among isolates.

We isolated root endophytes from 65 root tissues from three healthy *Fraxinus excelsior* trees (from Kinsealy, Ireland) and obtained 110 endophytes and 100% presence of endophytes from all root tissues (Chapter 2, Figure 2.9). Kowalski and Łukomska (2005) carried out a study on root endophytes from infected roots of *F. excelsior* and found three dominant species colonising the roots. Among all endophytes obtained from three sites, Roosky (429 isolates), Glasnevin (90 isolates) and Kinsealy (110 isolates), 301, 48 and 78 were sporulating respectively.

Table 6.1 Detailed taxonomic information on endophytes obtained from the Roosky provenance trial.

Division	Sub-division	Class	Order	Families
Ascomycota	**Pezizomycotina**	**Sordariomycetes**	**Hypocreales**	**Hypocreaceae**
				Cordycipitaceae
				Nectriaceae
				Sarocladiaceae
			Diaporthales	**Diaporthaceae**
			Chaetosphaeriales	Chaetosphaeriaceae
			Sordariales	Lasiosphaeriaceae
			Xylariales	**Xylariaceae**
		Dothideomycetes	**Dothideales**	**Dothioraceae**
			Pleosporales	**Didymellaceae**
				Didymosphaeriaceae
				Pleosporaceae
				Phaeosphaeriaceae
			Capnodiales	**Cladosporiaceae**
				Mycosphaerellaceae
		Leotiomycetes	Helotiales	Sclerotiniaceae
				Dermateaceae
		Eurotiomycetes	**Eurotiales**	**Aspergillaceae**
		Pezizomycetes	Peziziales	Pyronemataceae
Basiodiomycota	**Agaricomycotina**	**Agaricomycetes**	Polyporales	Meruliaceae
			Cantharellales	Hydnaceae
			Agaricales	Psathyrellaceae
		Tremellomycetes	Filobasidiales	Filobasidiaceae
			Tremellales	Bulleribasidiaceae

Bold indicates common isolates from healthy and diseased tissues.

Table 6.2 Detailed information on endophytes obtained from NGB, Glasnevin

Division	Subdivision	Class	Order	Families
Ascomycota	**Pezizomycotina**	**Sordariomycetes**	**Hypocreales**	**Hypocreaceae**
				Nectriaceae
			Glomerellales	Glomerellaceae
			Diaporthales	Diaporthaceae

			*Xylariales	*Diatrypaceae
		Eurotiomycetes	**Eurotiales**	**Aspergillaceae**
			Chaetothyriales	Herpotrichiellaceae
		Dothideomycetes	Dothideales	Dothioraceae
			Pleosporales	Didymellaceae
			Capnodiales	**Cladosporiaceae**
				Mycosphaerellaceae
			*Hysteriales	*Hysteriaceae
		Leotiomycetes	Helotiales	Dermateaceae
		*Pezizomycetes	*Peziziales	*Pyronemataceae
	Saccharomycotina	Saccharomycetes	Saccharomycetales	Debaryomycetaceae
Basidiomycota	Pucciniomycotina	Cystobasidiomycetes	Cystobasidiales	Cystobasidiaceae
	Agaricomycotina	Agaricomycetes	Corticiales	Corticiaceae
	Ustilaginomycotina	Ustilaginomycetes	Ustilaginales	Ustilaginaceae

*Isolates present only in *Fraxinus excelsior*. Bold indicates common isolates from both *Fraxinus excelsior* and other *Fraxinus* taxa.

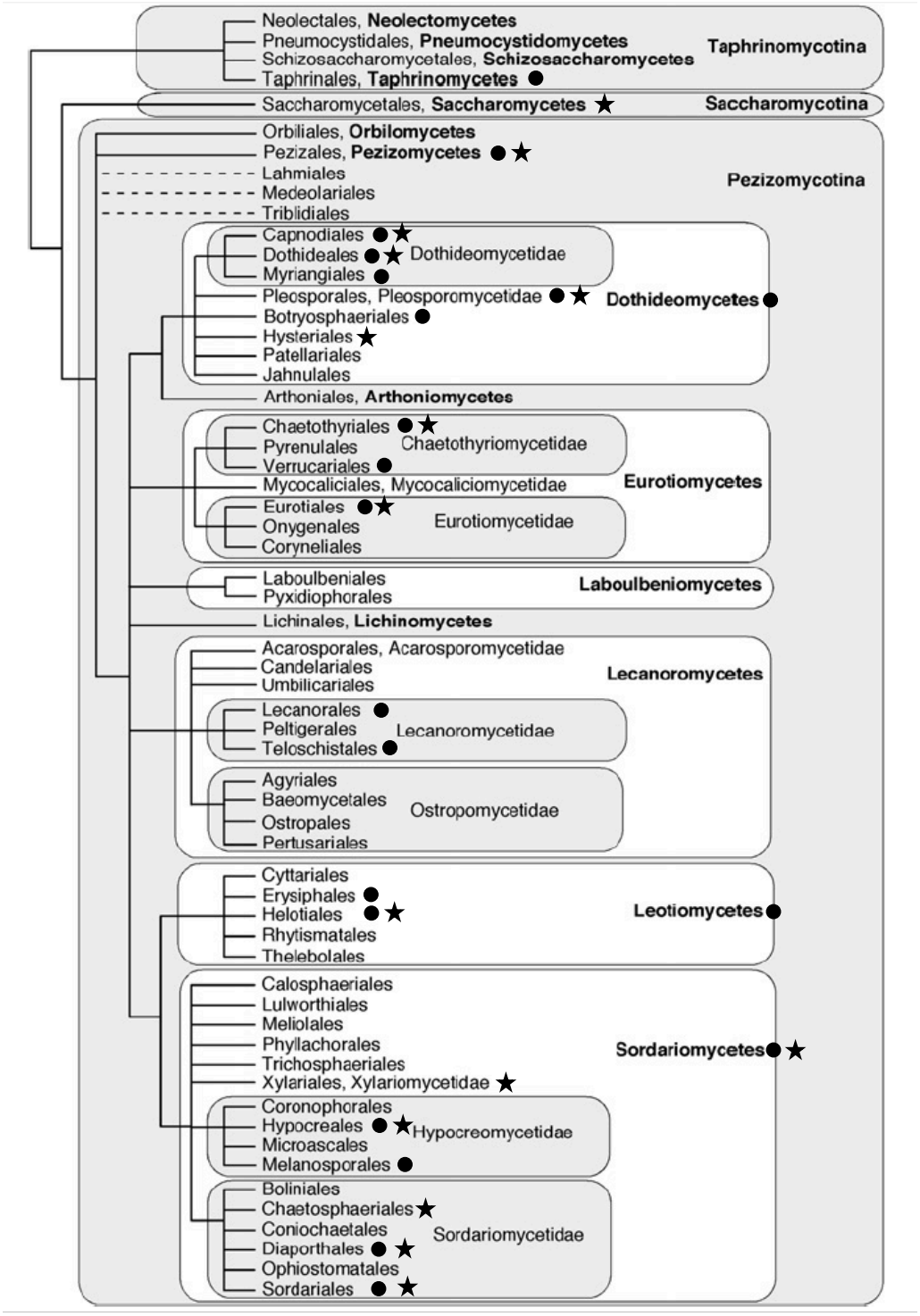
6.2 Molecular DNA barcoding characterisation of culture dependent fungal isolates and a HTS DNA study of culture independent fungal endophyte diversity (Chapter 3)

We further characterised a total of 410 different root and leaf endophyte isolates with DNA sequences for nrITS, nrLSU and *tef* barcoding regions. nrITS was found to be the most reliable and discriminating locus for DNA barcoding of the fungal isolates obtained in this study. This is in agreement with the Consortium for the Barcode of Life (Begerow *et al*. 2010) and other studies on fungal endophytes (Brasier 1996; Crous *et al*. 2007). A huge alpha diversity was detected in terms of species richness of all sample sites and tissue types (e.g. Figure 3.14, and 3.10) for both studies (culture dependent and culture independent). Cultured roots and shoots of ash varied greatly in their species composition and only a few taxa were shared (*Cadophora* sp., *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Fusarium culmorum* and *Fusarium oxysporum*). Community composition also varied greatly across groups sampled and is most striking between seed and leaf material and material collected from different ash taxa in comparison to *Fraxinus excelsior* (for the HTS data). *Fraxinus excelsior* fungal communities from France grouped more closely with *Fraxinus excelsior* communities from Ireland than the *Fraxinus excelsior* in Ireland (Roosky) and the mixed sample of *Fraxinus*

species/taxa from Ireland (NBG Glasnevin) (see the NMDS analysis, Figure 3.12). By looking at taxon sharing it is possible to identify a core group of fungi present in all sites. This core group is a candidate group for the endophyte species most commonly found in ash and which can be considered the core mycobiome. A comparison of fungal species lists for seed and leaf also revealed very few species in common (only *Aspergillus niger*, *Aspergillus penicilloides*, *Harzia acremoniodes*, *Mucor abundans*, *Paraconiothyrium hawaiiense*, *Penicillium brevicompactum* and *Volutella ciliata* were found in common). The ones in common could be candidates for endophytes that are vertically transmitted but further work will be required to test such assumptions.

The community taxonomic composition recorded using the culture dependent and independent approaches was highly different as summarised in Figure 6.a and 6.b. This creates a problem for data interpretation as it is not clear which taxa are the most abundant in the samples. The lists can be combined to represent the overall mycobiome estimation for the plants but it is unknown why the community estimations vary so much. This is, however, a common result found in other studies too (e.g. Høyer *et al.* 2019 for barley; Beekwilder *et al.* 2019 for grasses).

a)


Neolectales, Neolectomycetes
Pneumocystidales, Pneumocystidomycetes
Schizosaccharomycetales, Schizosaccharomycetes
Taphrinales, Taphrinomycetes
Taphrinomycotina
Saccharomycetales, Saccharomycetes
Saccharomycotina
Orbiliales, Orbilomycetes
Pezizales, Pezizomycetes
Lahmiales
Medeolariales
Triblidiales
Pezizomycotina
Capnodiales
Dothideales
Myriangiales
Dothideomycetidae
Pleosporales, Pleosporomycetidae
Botryosphaeriales
Hysteriales
Patellariales
Jahnulales
Dothideomycetes
Arthoniales, Arthoniomycetes
Chaetothyriales
Pyrenulales
Verrucariales
Chaetothyriomycetidae
Mycocaliciales, Mycocaliciomycetidae
Eurotiales
Onygenales
Coryneliales
Eurotiomycetidae
Eurotiomycetes
Laboulbeniales
Pyxidiophorales
Laboulbeniomycetes
Lichinales, Lichinomycetes
Acarosporales, Acarosporomycetidae
Candelariales
Umbilicariales
Lecanoromycetes
Lecanorales
Peltigerales
Teloschistales
Lecanoromycetidae
Agyriales
Baeomycetales
Ostropales
Pertusariales
Ostropomycetidae
Cyttariales
Erysiphales
Helotiales
Rhytismatales
Thelebolales
Leotiomycetes
Calosphaeriales
Lulworthiales
Meliolales
Phyllachorales
Trichosphaeriales
Xylariales, Xylariomycetidae
Sordariomycetes
Coronophorales
Hypocreales
Microascales
Melanosporales
Hypocreomycetidae
Boliniales
Chaetosphaeriales
Coniochaetales
Diaporthales
Ophiostomatales
Sordariales
Sordariomycetidae

b)

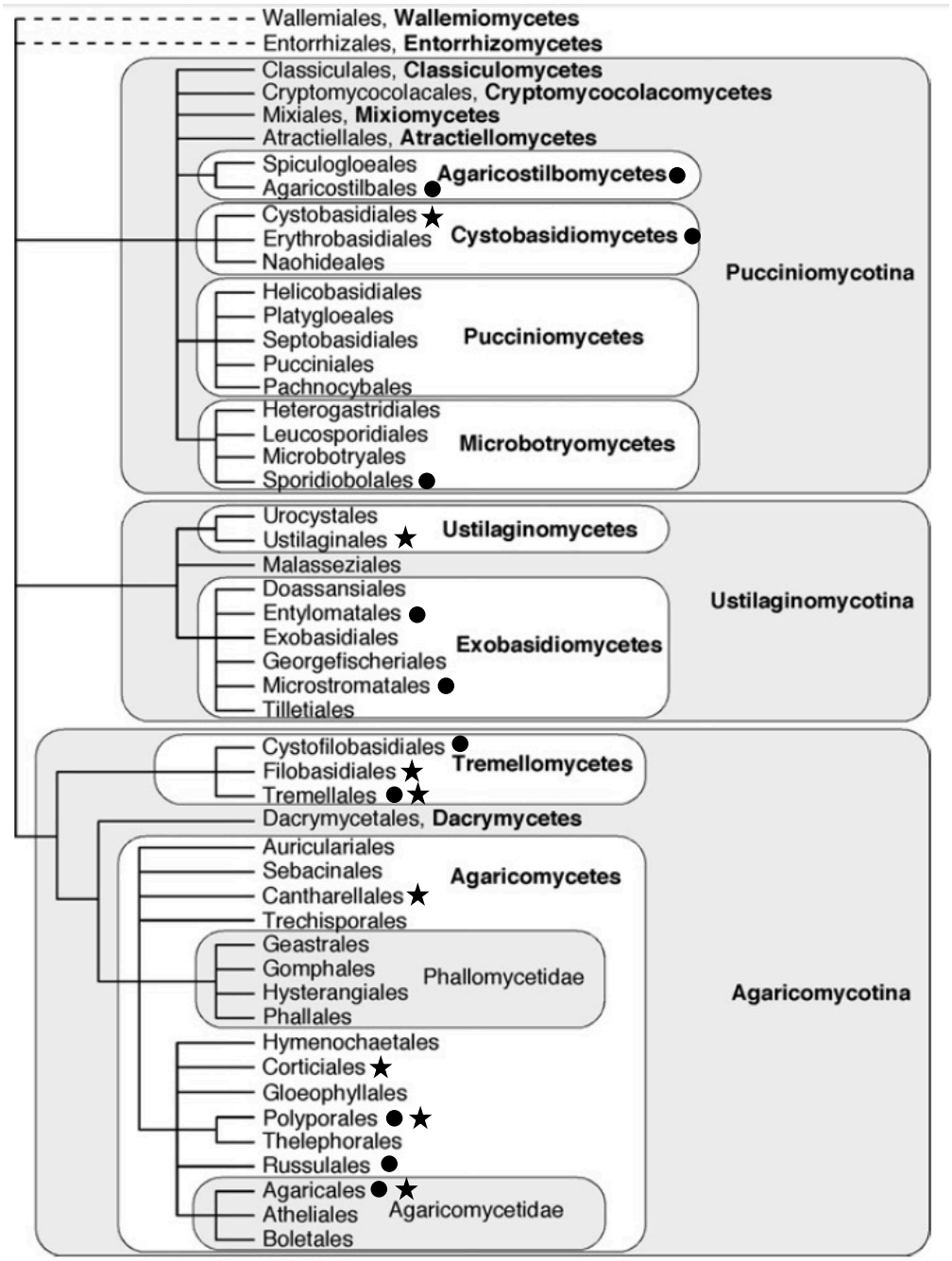


Figure 6.a and 6.b Taxonomic distribution of endophytes recorded in this thesis. 6.a are Ascomycotina and 6.b are Basidiomycotina. Endophytes recorded using the culture dependent method are shown using a ⋆ and endophytes found with the culture independent approach shown as a ● symbol. Source of background phylogenetic tree graphics: Hibbett *et al.* (2007). The British Mycological Society and Elsevier.

6.3 *In vitro* growth systems for plant fungal interaction studies (Chapter 4)

To test our endophytic isolates as suitable potential biocontrol agents against dieback of ash we established *in vitro* culturable endophyte free ash plantlets grown by tissue culture techniques. This allowed material to be tested in a sealed environment that prevents disease escape. It also allows for the careful control of environmental variables which is harder to achieve with growth cabinets, glasshouse or field experiments. It allows plant material to be cloned so that ramets (clones) can be tested rather than a mixed sample of plant genotypes that would increase inter-replicate experimental variation. Establishment of *in vitro* endophyte free plantlets from healthy ash trees, was undertaken via a rapid seed cutting culture and a slower embryo culture approach to generate a large number of plantlets.

In vitro methods remove the dormancy problem of seed germination in ash (Raquin *et al*. 2002; Lahiri *et al.* 2019). Germination of seeds (de-pericarped cut seeds) was lower at 62% (Chapter 4, Table 4.3) than from cultured embryos at 83% (Chapter 4, Table 4.4) respectively. Raquin *et al.* (2002) also found higher germination rates for embryo germination than seeds. However, we found the survival rate of seed germinated samples was higher than embryos (100% compared to 43%) (Chapter 4, Figure 4.2). This contradicts the results to Raquin *et al.* (2002) as they found 90% of the plants developed normally from the embryo germination treatments after 10 weeks. In our experiment, the seedlings from embryo culture did not survive well after being transferred onto new media (MS) with additional 1% sucrose. Hence the seed cutting technique was, overall, more efficient to break dormancy and establish ramet material than embryo culturing. This is also in agreement with Sambeek *et al.* (2007). The seed cutting technique is likely to be more successful because of the additional supply of storage carbohydrate and other nutrients from the seed endosperm which was absent from the embryo cultures. As ash seed coats are known to be made up of five to eight layers of cells (Steinbauer 1937; Finch-Savage and Clay 1997; Chmielarz 2009). The endosperm layer is separated from most layers by a suberised membrane and this membrane is the resistant layer outside the embryo (Steinbauer 1937). In wild conditions dormancy of seeds is known to break after a period of 5°C. This temperature is ideal to enlarge the embryo and also help the digestion of various storage starch carbohydrate and proteins in the seed coat (Steinbauer 1937; Nikolaeva *et al.* 1985; Chmielarz 2009). An experiment on dormancy breaking from ash fruits and

seeds was carried out by Villiers and Wareing (1964) and it showed enlargement of ash embryos on imbibed and naked seeds. We found that stratified de-pericarped seeds (with partial seed cuts) have been effective explants for the establishment of rapid regeneration of ash plantlets (Lahiri *et al.* 2019).

Rooting of the plantlets was most successful (68.5%) with MS media with 1% additional sucrose supplement (Chapter 4, Table 4.7). Addition of auxins (NAA) improved rooting (Chapter 4, Table 4.7) which is in agreement with a study carried out by Lebedev and Shestibratov (2016). However, they used lower concentrations of NAA for rooting (7mg L^{-1} NAA compared to the highest of 20mg L that we tested). However, we also found high rooting without NAA in some cases. Abbott (2000) showed that the *in vitro* rooting of *Fraxinus excelsior* was also dependent on the physiological state of the explant which may be a reason, why we have obtained higher rates of *in vitro* rooting without any hormones.

The tissue culture process successfully removed all detectable culturable endogenous endophytes from our seeds and embryos. Therefore, these methods can be used as alternatives to obtain infection free ash seedlings for future experimentation where sterile plants are required. It can also be used for restoration of ash stands to fill the gaps created by the invasive pathogen. However, it is clear that some endophytes are present as they were detected in the HTS of seed material. We applied the culture techniques developed in Chapter 4 for endophyte/pathogen/host interaction studies in Chapter 5.

6.4 *Hymenoscyphus fraxineus* strain variation (Chapter 5)

An assessment of Irish strain variations of *Hymenoscyphus fraxineus* in European context was carried out. Four strains of *Hymenoscyphus fraxineus* (three Northern Irish and one Irish) were sequenced for nrITS and assessed for haplotypes. An alignment of nucleotides for four isolates showed single variations in each (H3, H8 and H1); except H5 (4795D) with two variations. Gross *et al.* 2014 estimated a total of two haplotype of pathogens to invade Europe. In our alignment we have found more than two haplotypes (Chapter 5, Figure 5.1). A TCS mapping from the available data of *Hymenoscyphus fraxineus* from GenBank with our identified strains showed that two of the strains (H3 and H8) shared haplotypes with samples from Latvia, Lithuania, Slovakia and Switzerland and other two strains (H5 and H1) were placed in the Haplotype 9 group with samples from UK (Chapter 5, Figure 5.2).

6.5 Endophyte and pathogen interaction studies (Chapter 5)

We tested 10 leaf endophytes and 6 root endophytes for potential biocontrol activity and evaluated the antagonistic activity of these endophytes against *H. fraxineus* (Irish strain H1 and Northern Irish strain H8). From the antagonism assay we have discovered that *Pyronema domesticum* (E7, *Fraxinus excelsior*, isolated from healthy midrib) and *Meyerozyma guilliermondi* (E10, *Fraxinus americana,* isolated from healthy apex) were most effective against both strains of pathogen (H1 and H8). We do not know the antagonism mechanism but it is likely to be antibiosis by fungal metabolites (Kosawang *et al.* 2018).

Kosawang *et al.* (2018) tested antagonistic activity of 10 endophyte isolates (from twigs) from other *Fraxinus* taxa (*F. chinensis* subsp. *rhynchophylla*, *F. lanuginose*, *F. mandshurica*, *F. pennsylvanica* and *F. ornus*) and found strong inhibition by *Boeremia exigua, Epicoccum nigrum* and *Fusarium* sp.. We also tested *Epicoccum nigrum* (E2, *Fraxinus excelsior*, isolated from healthy rachis) and found it was not antagonistic against the strains of pathogen we have used (Chapter 5, Figure 5.3, 5.4 and 5.5). The other isolates *Boeremia exigua* and *Fusarium* sp. are also known as potential pathogens (Bottalico *et al.* 1994; Rai *et al.* 2009; Michielse *et al.* 2009). Another study conducted by Schlegel *et al.* (2016) tested the inhibitory effect of ascospore germination of *H. fraxineus* for 41 endophyte exudates and found a strong inhibitory effect for *Paraconiothyrium* sp., *Boeremia exigua*, *Kretzschmaria deusta*, *Ampelomyces quisqualis* Ces. and Elsinoaceae sp.. We did not assess the germination of ascospores in the presence of endophytes but we did retrieve *Paraconiothyrium* sp. and *Boeremia exigua* from our cultured leaf samples from *F. excelsior*.

Moreover, we also tested six root endophytes for antagonistic activity against the same two strains of pathogen and found that the most efficient was *Cordyceps perangustum* (ER2). However, *Lecanicillium attenuatum* (ER1), *Penicillium spathulatum* (ER3) and *Hydroposphaera* sp. (ER5) were found to be effective for pathogen strain H1 (Irish) and H8 (Northern Irish) respectively (Chapter 5, Figure 5.6 and 5.7). No other studies were found in the literature that have tested the antagonistic activity of root endophyte isolates against *H. fraxineus*.

Endophtye, pathogen and *Fraxinus excelsior* co-culturing (Chapter 5)

Co-culturing of *in vitro* grown plantlets with endophyte and pathogen was undertaken with two leaf endophytes *Pyronema domesticum* (E7, *Fraxinus*

excelsior, healthy midrib) and *Meyerozyma guilliermondi* (E10, *Fraxinus americana,* healthy apex) and one root endophyte *Lecanicillium attenuatum* (ER1) on the basis of the results of the antagonism studies. The results were not conclusive/effective as the endophyte treated plantlets showed higher average disease scores than the pathogen treated set (Chapter 5, Figure 5.13). Thus, the pathogen was not shown to reduce growth or develop sufficiently in the system. Total fresh weight was approximately the same in the E10 endophyte treated replicates as the pathogen treated replicates and E7 treated replicates showed a lower growth than pathogen treated plantlets (Chapter 5 Figure 5.12). In the case of the combined endophyte and pathogen treated replicates (E7H1 and E10H1), E10H1 showed similar average growth as the pathogen mentioned above but E7H1 showed a lower degree of growth than the pathogen treated plantlets. The root endophyte ER1 (*Lecanicillium attenuatum*) reduced growth *in vitro* (Chapter 5, Figure 5.17). No other *in vitro* co-culture studies were found in the literature for *Fraxinus excelsior* on these endophytes. An *ex vitro* study, conducted by Bakys *et al*. (2009a) on pathogenicity of isolated endophytes on one year-old *Fraxinus excelsior,* found that *Alternaria alternata*, *Epicoccum nigrum*, *H. fraxineus* and *Phomopsis* sp. 57 had high visual necrosis on bark and cambium. Another *ex vitro* study by Schlegel *et al.* (2016) using endophyte free and plants pre-inoculated with the endophyte *Venturia* sp. (which they frequently isolated from ash shoots) showed no significant effects of the endophyte on plants infected with *Hymenoscyphus fraxineus*. Thus, the search for potential biocontrol agents needs to be substantially expanded.

6.6 Priorities for future research

More research is needed on isolates from other plant tissue types such as wood, seed, bark and branches to get a fuller picture of the ash mycobiome. Studies on the seasonal impacts on endophyte isolation are also needed in the Irish environment to see how the mycobiome varies over the year and between years. Endophyte studies on ash are also required from a high disease pressure area to better compare the relationship between *Hymenoscyphus fraxineus* and the endophyte communities of diseased and healthy trees. The leaf litter is believed to be major source of disease spread, so studies on its mycobiome are urgently needed. Further optimisation of the *in vitro* method for endophyte interaction studies is also required. The tissue culture approach developed here could be used more widely as a method for the rapid screening of genetic resistance in ash.

6.7 Conclusions

This thesis aimed to provide information on the mycobiome of ash that can ultimately be used to develop future management tools for foresters and woodland managers to control ash dieback disease. Endophytes could provide an alternative, or complementary, approach to other dieback control measures such as the introduction of genetically resistant ash populations or the application of silviculture management methods including the removal of ash litter. For that purpose, we undertook a comprehensive assessment on the methods to isolate foliar and root endophytes from Irish ash. We characterised the mycobiome using both a culture dependent and culture independent approach to understand the core mycobiome species present and how they are assembled in communities of different geographical regions and tissue types. There was little overlap observed between the two characterisation approaches. We also found endophytes that are antagonistic to *Hymenoscyphus fraxineus* and developed a rapid *in vitro* method for plant/endophyte/pathogen interaction studies. The results presented in this thesis have provided the most comprehensive study, to date, of ash endophytes on the island of Ireland. The data collected in this thesis on the microbiome of ash is also of relevance to other trees in Ireland that are threatened by new diseases. For example, the horsechestnut, *Aesculus hippocastanum*, is threatened by the bleeding canker disease (McEvoy *et al.* 2016) (*Pseudomonas syringae* pv. *aesculi*) and a similar approach could be taken to find biocontrol agents for this species. Ash itself could also be under threat from other pathogens such as *Nectria* and, likewise, a microbiome approach for biocontrol might have some value.

7 References

Abbott JIE. 2000. Micropropagation of ash (*Fraxinus excelsior* L.). Digitised thesis from Trinity College Library Dublin, The University of Dublin, pp 51 – 185.

Adriaensen K, Vralstad T, Noben JP, Vangronsveld J and Colpaert JV. 2005. Copper-adapted *Suillus luteus*, a symbiotic solution for pines colonizing Cu mine spoils. *Applied and Environmental Microbiology* **71**: 7279 – 7284.

Agostinelli M. 2018. Fungal assemblages in forest trees – influence of external and internal conditions. *Acta Universitatis agriculturae Sueciae* **2018** (77): 1 – 68.

Albrectsen B, Björkén L, Varad A, Hagner A, Wedin M, Karlsson J and Jansson S. 2010. Endophytic fungi in European aspen (*Populus tremula*) leaves – diversity, detection, and a suggested correlation with herbivory resistance. *Fungal Diversity* **41**:17 – 28.

Alexander KBJ and Green T. 2006. The value of different tree and shrub species to wildlife. *British Wildlife* **18**: 18 – 28.

Alho CJR. 2008. The value of biodiversity. *Brazilian Journal of Biology* **68**: 1115 – 1118.

Aly AH, Debbab A and Proksch P. 2011. Fifty years of drug discovery from fungi. *Fungal Diversity* **50**: 3 – 19.

Anon. 2013. All-Ireland *Chalara* Control Strategy. https://www.dardni.gov.uk/publications/all-ireland-chalara-control-strategy (accessed 23rd November 2015).

Arnold AE, Maynard Z, Gilbert GS, Coley PD and Kursar TA. 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters* **3**: 267 – 274.

Arnold AE, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N and Herre EA. 2003. Fungal endophytes limit pathogen damage in a tropical tree.

Proceedings of the National Academy of Science USA **100**: 15649 – 15654.

Arnold AE. 2007. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews* **21**: 51 – 66.

Atamna-Ismaeel N, Finkel O, Glaser F, Von Mering C, Vorholt JA, Koblizek M, Belkin S and Béjà O. 2012a. Bacterial anoxygenic photosynthesis on plant leaf surfaces. *Environmental Microbiology Reports* **4**: 209 – 216.

Bakys R, Vasaitis R, Barklund P, Ihrmark K and Stenlid J. 2009a. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology* **58**: 284 – 292.

Bakys R, Vasaitis R, Barklund P, Thomsen IM and Stenlid J. 2009b. Occurrence and pathogenicity of fungi in necrotic and nonsymptomatic shoots of declining common ash (*Fraxinus excelsior*) in Sweden. *European Journal of Forest Research* **128**: 51 – 60.

Bakys R. 2013. Dieback of *Fraxinus* in Baltic sea region. Swedish University of Agricultural Sciences, Uppsala. 1 – 48.

Bandelt H, Forster P and Röhl A. 1999 Median‐joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37 – 48.

Baral H-O, Queloz V and Hosoya T. 2014. *Hymenoscyphus fraxineus*, the correct scientific name for the fungus causing ash dieback in Europe. *International Mycological Association* **5** (1): 79 – 80.

Barengo N, Sieber TN and Holdenrieder O. 2000. Diversity of endophytic mycobiota in leaves and twigs of pubescent birch (*Betula pubsescens*). *Sydowia* **52**: 305 – 320.

Baric´ L and Diminic´ D. 2010. First report of the pathogenic fungus *Chalara fraxinea* Kowalski on common ash (*Fraxinus excelsior* L.) in Gorski Kotar. *Glasilo biljne zas˘tite* **10**(1–2), Supplement, 33 (In Croatian).

Barklund P and Kowalski T. 1996. Endophytic fungi in branches of Norway spruce with particular reference to *Tryblidiopsis pinastri*. *Canadian Journal of Botany* **74**: 673 – 8.

Barklund P. 2005. Ash dieback sweeping over South and Central Sweden. *SkogsEko* **3**:11 – 13. (In Swedish).

Barriuso J, Marin S and Mellado RP. 2010. Effect of the herbicide glyphosate on glyphosate-tolerant maize rhizobacterial communities: a comparison with pre-emergency applied herbicide consisting of a combination of acetochlor and terbuthylazine. *Environmental Microbiology* **12**: 1021 – 1030.

Bashyal B, Li JY and Strobel G, Hess WM and Sidhu R. 1999. *Seimatoantlerium nepalense*, an endophytic taxol producing coelomycete from Himalayan yew (*Taxus wallachiana*). *Mycotaxon* **72**: 33 – 42.

Baskin CC and Baskin JM. 1998. Seeds. Ecology, biogeography, and evolution of dormancy and germination. *Academic Press*, *USA*. 37.

Beck P, Caudullo G, Tinner W and de Rigo D. 2016. *Fraxinus excelsior* in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz, J., de Rigo D, Caudullo G, Houston Durrant T, Mauri, A. (Eds.), European Atlas of Forest Tree Species.

Beekwilder J, Murphy BR, Mathuna EM, Barry A and Hodkinson TR. 2019. Isolation, diversity and potential use of endophytes in the biomass and bioenergy crop *Miscanthus*. In *Endophytes for a Growing World*, ed. Hodkinson TR, Doohan FM, Saunders MJ and Murphy BR, Cambridge: Cambridge University Press, Chapter 9.

Begerow D, Nilsson H, Unterseher M and Maier W. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied Microbiology and Biotechnology* **87**: 99 – 108.

Bell TH, Hassan SE, Lauron-Moreau A, Al-Otaibi F, Hijri M, Yergeau E and St-Arnaud M. 2014. Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-contaminated soils is related to plant

phylogeny. *International Society for Microbial Ecology Journal* **8**: 331 – 343.

Bialek R, González GM, Begerow D and Zelck UE. 2005. *Coccidioido mycosis* and blastomycosis: advances in molecular diagnosis. *FEMS Immunology and Medical Microbiology* **45**: 355 – 360.

Bills GF and Polishook JD. 1991. Microfungi from *Carpinus caroliniana*. *Canadian Journal of Botany* **69**: 1477 – 1482.

Bills GF. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants: systematics, ecology and evolution. *American Phytopathological Society Press*, Saint Paul, Minnesota, pp. 31 – 65.

Black K, Green S, Mullooly G. and Povida A, 2014. Towards a national hedgerow biomass inventory for the LULUCF sector using LiDAR remote sensing (2010-CCRP-DS-1.1): Final report, EPA, Dublin. https://www.epa.ie/pubs/reports/research/climate/ccrp-32-for-webFINAL.pdf.

Boddy L and Rayner ADM. 1983. Origins of decay in living deciduous trees: the role of moisture content and a re-appraisal of the expanded concept of tree decay. *New Phytologist* **94** :623 – 641.

Bodenhausen N, Horton MW and Bergelson J. 2013. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *Public Library of Sciences ONE* **8**(2): e56329. DOI: 10.1371/journal.pone.0056329.

Bokulich NA, Thorngate JH, Richardson PM and Mills DA. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of Sciences of the United States of America* **111**(1): E139–E148. DOI: 10.1073/pnas.1317377110.

Boshier D, Cordero J, Harris S, Parnell J, Rendell S, Savill P, Stewart J, Cundall N,

Hubert J, Samule S, Eriksen B, Wallander E, Martinsson O, Bellido JA, Fayos PG, López RM, Roldán M, Verdú M, Mateu I, Montilla AI, Spanos K, Kyriakidis D, Papi R, Blada I, Palada M, Popescu F, Postolache D, Canova I, Durkovic J, Gömöry D, Krajmerová D, Paule L and Slobodník B. 2005. *Ash species in Europe: biological characteristics and practical guidelines for sustainable use* (Oxford Forestry Institute, University of Oxford, United Kingdom, 2005). pp.128.

Bottalico A, Capasso R, Evidente A and Vurro M. 1994. Process for the production and purification of cytochalasin B from *Phoma exigua* var. heteromorpha. *Applied Biochemistry and Biotechnology* **48**: 33 – 36.

Brady SF, Wagenaar MM, Singh MP, Janso JE and Clardy J. 2000. The cytosporones, new octaketide antibiotics isolated from an endophytic fungus. *Organic Letters* **2**: 4043 – 4046.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T and Man In't Veld W A. 2004b. *Phytophthora alni* sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on *Alnus* trees. *Mycological Research* **108**: 1172 – 1184.

Brasier CM. 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151 – 161.

Brasier CM. 1996. Low genetic diversity of the *Ophiostoma novo-ulmi* population in North America. *Mycologia* **88**: 951 – 964.

Broome A and Mitchell RJ. 2017. Ecological impacts of ash dieback and mitigation methods. Forestry Commission Research, UK. pp 1- 16.

Brown SD, Utturkar SM, Klingeman DM, Johnson CM, Martin SL, Land ML, Lu T-YS, Schadt CW, Doktycz MJ and Pelletier DA. 2012. Twenty-one genome sequences from *Pseudomonas* species and 19 genome sequences from diverse bacteria isolated from the rhizosphere and endosphere of *Populus deltoides*. *Journal of Bacteriology* **194**: 5991 – 5993.

Brun S and Silar P. 2010. *In Evolutionary Biology − Concepts, Molecular and Morphological Evolution*, Pontarotti P. Ed. Springer, Berlin Heidelberg, pp 317 – 328.

Brundrett M, Murase G and Kendrick B. 1990. Comparative anatomy of roots and mycorrhizae of common Ontario trees. *Canadian Journal of Botany* **68:** 551 – 578.

Bruns TD, White TJ and Taylor JW. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**: 525 − 564.

Bulgarelli D, Rott M, Schlaeppi K, Ver Loren Van Themaat E, Ahmadinejad N, Assenza F, Rauf P, Huettel B, Reinhardt R, Schmeizer E, Peplies J, Gloeckner EO, Amann R, Eickhorst T and Schulze-Lefert P. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**: 91 – 95.

Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren Van Themaat E and Schulze-Lefert P. 2013. Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology* **64**: 807 – 838.

CABI/EPPO, 2013. *Hymenoscyphus pseudoalbidus*. [Distribution map]. Distribution Maps of Plant Diseases, No.October. Wallingford, UK: CABI, Map 1060 (Edition 2) (/isc/abstract/20133421493).

Camarda L, Merlini L and Nasini G. 1976. Metabolites of Cercospora. Taiwapyrone, an [alpha] pyrone of unusual structure from *Cercospora taiwanensis*. *Phytochemistry* **15**: 537 – 539.

Campos FF, Rosa LH, Cota BB, Caligiorne RB, Rabello ALT, Alves TMA, Rosa CA and Zani CL. 2008 Leishmanicidal metabolites from *Cochliobolus* sp., an endophytic fungus isolated from *Piptadenia adiantoides* (Fabaceae). *PLoS Neglected Tropical Diseases*. doi:101371/annotation/49748d99-fe4d-4c28-b77a-306d0cf7062e.

Cao LX, You JL and Zhou SN. 2002. Endophytic fungi from *Musa acuminata* leaves and roots in South China. *World Journal of Microbiology and Biotechnology* **18**: 169 – 171.

Carroll GC and Carroll F. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific northwest. *Caadian Journal of Botany* **56:** 3034 – 3043.

Cech TL and Hoyer-Tomiczek U. 2007. Aktuelle Situation des Zurücksterbens der Esche in Österreich [Actual situation of dieback of ash in Austria]. *Forstschutz Ak- tuell* **40**: 8 – 10, (in German).

Cech TL. 2006. Ash dieback and premature leaf shedding in Austria. *Forstschutz Aktuell* **37**: 18 – 20 (In German).

Chandelier A, Delhaye L, Claessens H and Lassois L. 2017. Ash dieback in Wallonia, southern Belgium: research on disease development, resistance and management options. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 53 – 60.

Chandelier A, Delhaye N and Helson M. 2011. First report of the ash dieback pathogen *Hymenoscyphus pseudoalbidus* (anamorph: *Chalara fraxinea*) on *Fraxinus excelsior* in Belgium. *Plant Disease* **95**(2): 220.

Chanway CP.1996. Endophytes: they're not just fungi! *Canadian Journal Botany*, **74**: 321 – 322.

Cheffings CM and Farrell L. (eds), Dines TD, Jones RA, Leach SJ, Mckean DR, Pearman DA, Preston CD, Rumsey FJ and Taylor I. 2005. The Vascular Plant Red Data List for Great Britain. Species Status No. 7. Joint Nature Conservation Committee, Peterborough pp. 1–116.

Chen J. 2012. Fungal community Survey of *Fraxinus excelsior* in New Zealand. Master thesis. Swedish University of Agricultural Sciences, Uppsala.

Chira D, Chira F, Taut I, Popovici O, Blada I, Donita N, Bandiu C, Gancz V, Biris IA, Popescu F, Tanasie S and Dinu C. 2016. Evolution of ash dieback in Romania. In: R. Vasaitis, R. Enderle (Editors), Dieback of European

Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management. *Uppsala, Swedish University of Agricultural Sciences*, p. 185 – 194.

Chmielarz P. 2009. Cryopreservation of dormant European ash (*Fraxinus excelsior*) orthodox seeds. *Tree Physiology* **29**: 1275 – 1285.

Cleary M, Nguyen D, Stener LG, Stenlid J and Skovsgaard JP. 2017. Ash and ash dieback in Sweden: a review of disease history, current status, pathogen and host dynamics, host tolerance and management options in forests and landscapes. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 195 – 208.

Cleary M, Nguyen D, Marčiulynienė D, Berlin A, Vasaitis R and Stenlid J. 2016. Friend or foe? Biological and ecological traits of the European ash dieback pathogen *Hymenoscyphus fraxineus* in its native environment. *Nature Scientific Reports* **6**: 21895. DOI: 10.1038/srep21895.

Cleary MR, Arhipova N, Gaitnieks T, Stenlid J and Vasaitis R. 2013. Natural infection of *Fraxinus excelsior* seeds by *Chalara fraxinea*. *Forest Pathology* **43**(1): 83 – 85.

Clement M, Posada D and Crandall KA. 2000 TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657 – 1659.

Cohen SD. 1999. Technique for large scale isolation of *Discula umbrinella* and other foliar endophytic fungi from *Quercus* species. *Mycologia* **91**: 917 – 922.

Collemare J, Pianfetti M, Houlle AE, Morin D, Camborde L, Gagey MJ, Barbisan C, Fudal I, Lebrun MH and Böhnert HU. 2008. *Magnaporthe grisea* avirulence gene ACE1 belongs to an infection-specific gene cluster involved in secondary metabolism. *New Phytologist* **179**: 196 – 208.

Colpaert JV, Muller LAH, Labaerts M, Adriaensen K and Vangronsveld J. 2004. Evolutionary adaptation to Zn toxicity in populations of Suilloid fungi. *New Phytologist* **162**: 549 – 559.

Coombs JT and Franco CMM. 2003a. Isolation and identification of actino bacteria from surface sterilised wheat roots. *Applied Environment Microbiology* **69**: 5603 – 5608.

Cretoiu MS, Kielak AM, Abu Al-Soud W, Sørensen SJ and Van Elsas JD. 2012. Mining of unexplored habitats for novel chitinases - *chiA* as a helper gene proxy in metagenomics. *Applied Microbiology and Biotechnology* **94**: 1347 – 1358.

Cross H, Sonstebo JH, Nagy NE, Timmermann V, Solheim H, Borja I, Kauserud H, Carlsen T, Rzepka B, Wasak K, Vivian-Smith A and Hietala AM. 2017. Fungal diversity and seasonal succession in ash leaves infected by the invasive ascomycete *Hymenoscyphus fraxineus*. *New Phytologist* **213**: 1405 – 1417.

Crous PW, Braun U, Schubert K and Groenewald JZ. 2007. Delimiting *Cladosporium* from morphologically similar genera. *Studies of Mycology* **58**: 33 – 56.

Danti R, Sieber T and Sanguinetti G. 2002. Endophytic mycobiota in bark of European beech (*Fagus sylvatica*) in the Apennines. *Mycological Research* **106**: 1343 – 1348.

Davis EC, Franklin JB, Shaw AJ and Vilgalys R. 2003. Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. *American Journal of Botany* **90**: 1661 – 1667.

Davydenko K, Vasaitis R, Stenlid J and Menkis A. 2013. Fungi in foliage shoots of *Fraxinus excelsior* in eastern Ukraine: a first report on *Hymenoschyphus pseudoalbidus*. *Forest Pathology* **43**(6): 462 – 467.

De Battista JP, Bacon CW, Severson R, Plattner RD and Bouton JH. 1990. Indole

acetic acid production by the fungal endophyte of tall fescue. *Agronomy Journal* **82**: 878 – 880.

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, Mering CV and Vorholt JA. 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 16428 – 16433.

Dobrowolska D, Hein S, Oosterbaan A, Wagner S, Clark J and Skovsgaard JP. 2011. A review of European ash (*Fraxinus excelsior* L.): implications for silviculture, *Forestry: An International Journal of Forest Research*, **84**(2): 133 – 148.

Dohrmann AB, Küting M, Jünemann S, Jaenicke S, Schlüter A and Tebbe CC. 2013. Importance of rare taxa for bacterial diversity in the rhizosphere of Bt- and conventional maize varieties. *International Society of Microbial Ecology Journal* **7**: 37 – 49.

Donnarumma F, Capuana M, Vettori C, Petrin G, Giannini R, Indorato C and Mastromei G. 2011. Isolation and characterisation of bacterial colonies from seeds and *in vitro* cultures of *Fraxinus* spp. from Italian sites. *Plant Biology* **13**: 169 – 176.

Doty SL, Oakely B, Xin G, Kang JW, Singleton G, Khan Z, Vajzovic A and Staley JT. 2009. Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis* **47**: 23 – 33.

Douglas GC, Pliura A, Dufour J, Mertens P, Jacques D, Fernandez-Manjares J, Buiteveld J, Parnuta G, Tudoroiu M, Curnel Y, Thomasset M, Jensen V, Knudsen MA, Foffová E, Chandelier A and Steenackers M. 2013. Common Ash (*Fraxinus excelsior* L.). *Forest tree breeding in Europe: Current State of the Art and perspectives, Managing Forest Ecosystems* **25**: 403 – 462.

Drâghici C and Abrudan IV. 2010. Dormancy breaking of *Acer* and *Fraxinus* seeds – brief review. *Bulletin of the Transilivania University of Bra*sov **3**:29 – 32.

Drenkhan R and Hanso M. 2009 [Common ash dieback in Estonia and in Europe]. *Eesti Loodus* **60** (3): 14 – 19. (In Estonian).

Duan Y, Zhou L, Hall DG, Li W, Doddapaneni H, Lin H, Liu L, Vahling CM, Gabriel DW, Williams KP, Dickerman A, Sun Y and Gottwald T. 2009. Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus Liberibacter* asiaticus' obtained through metagenomics. *Molecular Plant-Microbe Interactions* **22**: 1011 – 1020.

Dubois T, Gold CS, Coyne D, Paparu P, Mukwaba E, Athman S, Kapinduand S and Adipala E. 2004. Merging biotechnology with biological control: Banana *Musa* tissue culture plants enhanced by endophytic fungi. *Uganda Journal of Agricultural Sciences* **9**: 445 – 451.

Dumbrell AJ, Ashton PD, Aziz N, Feng G, Nelson M, Dytham C, Fitter AH and Helgason T. 2011. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist* **190**: 794 – 804.

Enderle R, Fussi B, Lenz HD, Langer G, Nagel R and Metzler B. 2017. Ash dieback in Germany: research on disease development, resistance and management options. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 89 – 105.

Engesser R, Queloz V, Meier R, Kowalski T and Holdenrieder O. 2009. Ash dieback in Switzerland. *Wald und Holz* **6**: 24 – 27. (In German).

Ernst M, Mendgen KW and Wirsel SGR. 2003. Endophytic fungal mutualists: seed-borne *Stagonospora* spp. Enhance reed biomass production in axenic microcosms. *Molecular Plant–Microbe Interactions* **16**: 580 – 587.

Eyberger AL, Dondapati R and Porter JR. 2006. Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *Journal of Natural Products* **69**: 1121 –1124.

Fernandes MDRV, Silva TAC, Pfenning LH, Costa-Neto CM, Heinrich TA, Alencar SM, Lima MA and Ikegaki M. 2009. Biological activities of the fermentation extract of the endophytic fungus *Alternaria alternata* isolated from *Coffea arabica* L. *Brazilian Journal of Pharmaceutical Sciences* **45**: 677 – 685.

Finch-Savage WE and Clay HA. 1997. The influence of embryo restraint during dormancy loss and germination of *Fraxinus excelsior* seeds. In Basic and Applied Aspects of Seed Biology. Eds. R.H. Ellis, M. Black and A.J. Hong. Kluwer Academic Publishers, Dordrecht, pp 245–253.

Foltz MJ, Perez KE and Volk TJ. 2013. Molecular phyologeny and morphology reveal three new species of *Cantharellus* within 20 m of one another in western Wisconsin, USA. *Mycologia* **105**(2): 447 – 461.

Fones HN, Mardon C and Gurr SJ. 2016. A role for the asexual spores in infection of *Fraxinus excelsior* by the ash-dieback fungus *Hymenoscyphus fraxineus*. Nature Sceintific Reports **6** (34638): 1 – 10.

Fröhlich J and Hyde KD. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodiversity and Conservation* **8**: 977 –1004.

Fröhlich J, Hyde KD and Petrini O. 2000. Endophytic fungi associated with palms. *Mycological Research* **104**: 1202 – 1212.

Furukawa T, Koga J, Adachi T, Kishi K and Syono K. 1996. Efficient conversion of 1-tryptophan to indole-3-acetic acid and/or tryptophol by some species of *Rhizoctonia*. *Plant Cell Physiology* **37**: 899 – 905.

Gagné S, Richard C, Rousseau H and Antoun H. 1987. Xylem-residing bacteria in alfalfa roots. *Canadian Journal of Microbiology* **33**: 996 – 1005.

Gamboa MA and Bayman P. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: *Meliaceae*). *Biotropica* **33**: 352 – 360.

Gangadevi V and Muthumary J. 2008. Taxol, an anticancer drug produced by an endophytic fungus *Bartalinia robillardoides* Tassi, isolated from a medicinal plant, *Aegle marmelos* Correa ex Roxb. *World Journal Microbiology Biotechnology* **24**: 717 – 724.

Gardes M and Bruns TD. 1993. ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. Molecular Ecology **2**: 113 – 118.

Gardiner B, Palmer H and Hislop M. 2006. The principles of using woods for Shelter, Available at: www.forestry.gov.uk/pdf/fcin081.pdf/$FILE/fcin081.pdf.

Gardner JM, Feldman AW and Zablotowicz RM. 1982. Identity and behavior of xylem-residing bacteria in rough lemon roots of Florida citrus trees. *Applied Environment Microbiology* **43**: 1335 – 1342.

Ge HM, Shen Y, Zhu CH, Tan SH, Ding H, Song YC and Tan RX. 2008. Penicidones A-C, three cytotoxic alkaloidal metabolites of an endophytic *Penicillium* sp. *Phytochemistry* **69**: 571 – 576.

Geiser DM. 2004. *In Advances in Fungal Biotechnology for Industry*, Agriculture and Medicine, Springer, pp 3 − 14.

Gennaro M, Gonthier P and Nicolotti G. 2003. Fungal endophytic communities in healthy and declining *Quercus robur* L. and *Q. cerris* L. trees in Northern Italy. *Journal of Phytopathology* **151**: 529 – 534.

Gillot G, Jany JL, Coton M, Le Floch G, Debaets S, Ropars J, López-Villavicencio M, Dupont J, Branca A, Giraud T and Coton E. 2015. Insights into Penicillium roqueforti Morphological and Genetic Diversity. *Public Library of Science One*, **10**(6): e0129849. DOI: 10.1371/journal.pone.0129849.

Giraud T, Refrégier G, Le Gac M, de Vienne DM and Hood ME. 2008. Speciation in fungi. *Fungal Genetics and Biology* **45**(6): 791 − 802.

Glass NL and Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes *Applied and Environmental Microbiology* **61**(4): 1323 − 1330.

González V and Tello ML. 2011. The endophytic mycota associated with *Vitis vinifera* in central Spain. *Fungal Diversity* **47**: 29 – 42.

Görke C. 1998. Mykozönosen von Wurzeln und Stamm von Jungbäumen unterschiedlicher Bestandsbegründungen. *The Bibliography of Systematic Mycology* **173**: 1 – 462.

Gottel NR, Castro HF, Kerley M, Yang ZM, Pelletier DA, Podar M, Karpinets T, Uberbacher E, Tuskan GA, Vilgalys R, Dotycz MJ and Schadt CW. 2011. Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* **77**: 5934 – 5944. DOI: 10.1128/AEM.05255-11.

Gronberg H, Kaparakis G and Sen R. 2006. Binucleate *Rhizoctonia* (*Ceratorhiza* spp.) as non mycorrhizal endophytes alter *Pinus sylvestris* L. seedling root architecture and affect growth of rooted cuttings. *Scandinavian Journal of Forest Research* **21**: 450 – 457.

Gross A and Han JG. 2015. *Hymenoscyphus fraxineus* and two new *Hymenoscyphus* species identified in Korea. *Mycological Progress* **14**: 19.

Gross A, Grünig CR, Queloz V and Holdenrieder O. 2012. A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Forest Pathology* **42**: 252 – 264.

Gross A, Holdenrieder O, Pautasso M, Queloz V and Sieber TN. 2014. *Hymenoscyphus pseudoalbidus*, the causal agent of European ash

dieback. *Molecular Plant Pathology* **15**(1): 5 – 21.

Gross A, Hosoya T and Queloz V. 2014a. Population structure of the invasive forest pathogen *Hymenoscyphus pseudoalbidus*. *Molecular Ecology* **23**(12): 2943 – 2960.

Gunatilaka AAL. 2006. Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of Natural Product* **69**: 509 – 526.

Gustienė A and Lygis V. 2010. *Chalara fraxinea* in Lithuanian ash stands: a pilot study. Poster presentation at the EPPO workshop on *Chalara fraxinea*, 30 June – 2 July 2010, Oslo, Norway.

Hallmann J, Quadt-Hallmann A, Mahaffee WF and Kloepper JW. 1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, **43**: 895 – 914.

Halmschlager E, Butin H and Donaubauer E. 1993. Endophytic fungi in leaves and twigs of *Quercus petraea*. *European Journal of Plant Pathology* **23**: 51 – 63.

Han J-G, Shrestha B, Hosoya T, Lee H-K, Sung G-H and Shin H-D. 2014. First report of the ash dieback pathogen *Hymenoscyphus fraxineus* in Korea. *Microbiology* **42**(4): 391 – 396.

Haňáčková Z, Havrdova L, Cerny K, Zahradnik D and Koukal O. 2017. Fungal Endophytes in Ash shoots- diversity and inhibition of *Hymenoscyphus fraxineus*. *Baltic forestry* **23**(1): 89 – 106.

Haňáčkova Z, Koukol O, Havrdov L and Gross A. 2015. Local population structure of *Hymenoscyphus fraxineus* surveyed by an enlarged set of microsatellite markers. *Forest Pathology* **45**: 400 – 407.

Hardoim PR, Overbeek LS, Berg G, Prittilä AM, Compant S, Campisano A, Döring M and Sessitsch A. 2015. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews*, **79**: 293 –

320.

Harrington TC and Rizzo DM. 1999. *In Structure and Dynamics of Fungal Populations*, Worrall JJ, Ed. Kluwer Press, Dordrecht, The Netherlands, pp 43 − 71.

Hawkins H-J, Johansen A and George E. 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil* **226**: 275 – 285.

Hayatgheibi H. 2013. Studies on the microflora associated with the seeds of European ash (*Fraxinus excelsior*) and the infection biology of the pathogen *Hymenoscyphus pseudoalbidus* causing ash dieback. Master thesis. Swedish University of Agricultural Sciences, Uppsala, pp 1 - 32.

Hazalin NAMN, Ramasamy K, Lim SSM, Wahab IA, Cole ALJ and Majeed ABA. 2009. Cytotoxic and antibacterial activities of endophytic fungi isolated from plants at the National Park, Pahang, Malaysia. *BMC Complementary and Alternative Medicine*. doi:10.1186/1472-6882-9-46.

Heinze B, Tiefenbacher H, Litschauer R and Kirisits T. 2017. Ash dieback in Austria – history, current situation and outlook. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 33 – 52.

Herre EA, Meíja LC, Kyllo DA, Rojas E, Maynard Z, Butler A and Van Bael SA. 2007. Ecological implications of anti pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology* **88**: 550 – 558.

Heydeck P, Bemmann M and Kontzog H-G. 2005. Triebsterben an Gemeiner Esche (*Fraxinus excelsior*) im nordostdeutschen Tiefland [Widespread dying of common ash (*Fraxinus excelsior*) in the lowlands of Northeastern Germany]. *Forst und Holz* **60**: 505 – 506 (in German).

Hibbett D, Abarenkov K, Kõljalg U, Öpik M, Chai B, Cole J, Wang Q, Crous P, Helgason T, Herr JR, Kirk P, Lueschow S, O`Donnell K, Nilsson RH,

Oono R, Schoch C, Smyth C, Walker DM, Porras-Alfaro A, Taylor JW and Geiser DM. 2016. Sequence-based classification and identification of fungi. *Mycologia* **108**: 1049 – 1068.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueiden C, Hawsworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichwardt R, Longcore J, Miądlikowka J, Miller A, Moncalvo JM, Standridge SM, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüβler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J and Zhang N. 2007. A higher-level phylogenetic classification of the Fungi. *Mycological Research*, **111**: 509 – 547.

Hibbett DS, Ohman A and Kirk PM. 2009. Fungal ecology catches fire. *New Phytologist* **184**: 279 – 282.

Hietala AM, Sen R and Lilja A. 1994. Anamorphic and teleomorphic characteristics of a uninucleate *Rhizoctonia* sp. isolated from the roots of nursery grown conifer seedlings. *Mycological Research* **98**: 1044 – 1050.

Hodkinson TR and Murphy BR. 2019. Endophytes for a growing world. In *Endophytes for a Growing World*, ed. T R. Hodkinson, F. M. Doohan, M. J. Saunders and B. R. Murphy. Cambridge: Cambridge University Press, Chapter 1.

Hodkinson TR, Waldren S, Parnell JAN, Kelleher CT, Salamin K and Salamin N. 2007. DNA banking for plant breeding, biotechnology and biodiversity evaluation. *Journal of Plant Research* **120**: 17 – 29.

Hodkinson TR. 2018. Evolution and taxonomy of the grasses (*Poaceae*): a model family for the study of species rich groups. *Annual Plant Reviews*

Online, doi: 10.1002/ 9781119312994. apr0622.

Hollis JP. 1951. Bacteria in healthy potato tissue. *Phytopathology* **41**: 350–367.

Hosoya T, Otani Y and Furuya K. 1993. Materials for the fungus flora of Japan (46). *Transaction of the Mycological Society of Japan* **34**(4): 429 – 432.

Høyer AK, Jørgensen HJL, Jensen B, Murphy BR and Hodkinson TR. 2019. Emerging methods for biological control of Barley diseases including the role of endophytes. In *Endophytes for a Growing World*, ed. Hodkinson TR, Doohan FM, Saunders MJ and Murphy BR, Cambridge: Cambridge University Press, Chapter 5.

Hu Z-Y, Li Y-Y, Lu C-H, Lin T, Hu P and Shen Y-M. 2010. Seven novel linear polyketides from *Xylaria* sp. NCY2. *Helvetica Chemica Acta* **93**: 925 – 933.

Huang Z, Cai X, Shao C, She Z, Xia X, Chen Y, Yang J, Zhou S and Lin Y. 2008. Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. *Phytochemistry* **69**: 1604 – 1608.

Hughes KW, Petersen RH, Lodge DJ, Bergemann SE, Baumgartner K, Tulloss RE, Lickey E and Cifuentes J. 2013. Evolutionary consequences of putative intra-and interspecific hybridization in agaric fungi. *Mycologia* **105**(6): 1577 − 1594.

Hultén E and Fries M. 1986. *Atlas of North European vascular plant*s (North of the Tropic of Cancer), Vols. I-III. (Koeltz scientific books, 1986).

Hussain H, Akhtar N, Draeger S, Schulz B, Pescitelli G, Salvadori P, Antus S, Kurtán T and Krohn K. 2009. New bioactive 2,3-epoxycyclohexenes and iso-coumarins from the endophytic fungus *Phomopsis* sp. from *Laurus azorica*. *European Journal of Organic Chemistry* **2009**: 749 – 756.

Husson C, Scala B, Caël O, Frey P, Feau N, Ioos R and Marçais B. 2011 *Chalara fraxinea* is an invasive pathogen in France. *European Journal of Plant Pathology* **130**(3): 311 – 324.

Hustad VP and Miller AN. 2015. Studies in the genus *Glutinoglossum*. *Mycologia* **107**(3): 647 − 657.

Hyde KD and Soytong K. 2007. Understanding microfungal diversity – a critique. *Cryptogamie Mycologie* **28**: 281 – 289.

Hyde KD, Bussaban B, Paulus B, Crous PW, Lee S, Mckenzie EHC, Photita W and Lumyong S. 2007. Biodiversity of saprobic fungi. *Biodiversity and Conservation* **16**: 17 – 35.

Hyde KD and Soytong K. 2008. The fungal endophyte dilemma. *Fungal Diversity* **33**: 163 − 173.

Ibrahim M, Sieber TN and Schlegel M. 2017. Communities of fungal endophytes in leaves of Fraxinus ornus are highly diverse. *Fungal Ecology* **29**: 10 – 19.

Ihrmark K, Bödeker IT, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE and Lindahl BD. 2012. New primers to amplify the fungal ITS2 region evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology* **82**(3): 666 − 677.

Inácio ML, Silva GH, Teles HL, Trevisan HC, Cavalheiro AJ, Bolzani VS, Young MCM, Pfenning LH and Araujo AR. 2006. Antifungal metabolites from *Colletotrichum gloeosporioides*, an endophytic fungus in *Cryptocarya mandioccana* Nees (Lauraceae). *Biochemical Systematics of Ecology* **34**: 822 – 824.

Ioos R, Kowalski T, Husson C and Holdenrieder O. 2009. Rapid in planta detection of *Chalara fraxinea* by a real-time PCR assay using a dual-labelled probe. *European Journal of Plant Pathology* **125**: 329 – 335.

Isaka M, Jaturapat A, Kladwang W, Punya J, Lertwerawat Y, Tanticharoen M and Thebtaranonth Y. 2000. Antiplasmodial compounds from the wood decayed fungus *Xylaria* sp. BCC 1067. *Planta Medica* **66**: 473 – 475.

Janěs D, Kreft S, Jurc M, Seme K and Štrukelj B. 2007 Antibacterial activity in higher fungi (mushrooms) and endophytic fungi from Slovenia. *Pharmaceutical Biology* **45**: 700 –706.

Jankovsky L and Holdenrieder O. 2009. *Chalara fraxinea* – ash dieback in the Czech Republic. *Plant Protection Science* **45**:74 – 78.

Janse JD 1981. The bacterial disease of ash (*Fraxinus excelsior*), caused by *Pseudomonas syringae subsp. savastanoi pv. fraxini*. *European Journal For Pathology* **11**: 306 – 315.

Johnson O and More D. 2006. Collins tree guide. ed. UK edition. Harper Collins Publishers, pp 464.

Jumpponen A and Trappe JM. 1998. Dark septate endophytes: a review of facultative biotrophic root colonizing fungi. *New Phytologist* **140**: 295 – 310.

Jumpponen A. 2001. Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* **11**: 207 – 211.

Juodvalkis A and Vasiliauskas A. 2002. Lietuvos uosynø dþiûvimo apimtys ir jas lemiantys veiksniai [The extent and possible causes of dieback of ash stands in Lithuania]. *LÞÛU Mokslo Darbai. Biomedicinos Mokslai* **56**: 17 – 22 (in Lithuanian with English summary).

Kaefer E. 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Advances in Genetics* **19**: 33 – 131.

Karnosky DF. 1981. Potential for forest tree improvement via tissue culture. *Biosciences*. **31**(2): 114 – 120.

Kaur A, Raja HA, Swenson DC, Agarwal R, Deep G, Falkinham III JO and Oberlies NH. 2016. Talarolutins A–D: Meroterpenoids from an endophytic fungal Isolate of *Talaromyces minioluteus*. *Phytochemistry* **126**: 4 – 10.

Keča N, Kirisits T and Menkis A. 2017. First report of the invasive ash dieback pathogen *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *F. angustifolia* in Serbia. *Baltic Forestry* **23**(1): 56 – 59.

Kehr RD. 1992. Pezicula canker of *Quercus rubra* L. caused by *Pezicula cinnamomea* (DC.) Sacc. *European Journal of Forest Pathology* **22**(1): 29 – 40.

Kerr G. 1995. Silviculture of ash in southern England, *Forestry: An International Journal of Forest Research* **68**(1): 63 – 70.

Kim HY, Choi GJ, Lee HB, Lee SW, Kim HK, Jang KS, Son SW, Lee SO, Cho KY, Sung ND and Kim JC. 2007. Some fungal endophytes from vegetable crops and their anti-oomycete activities against tomato late blight. *Letters in Applied Microbiology* **44**: 332 – 337.

Kim M-S, Klopfenstein NB and Cregg BM. 1998. *In vitro* and *ex vitro* rooting of micropropagated shoots using three green ash (*Fraxinus pennsylvanica*) clones. *New Forests* **16**: 43 **–** 57.

Kirisits T, Matlakova M, Mottinger-Kroupa S, Cech T and Halmschlager E. 2009. The current situation of ash dieback caused by *Chalara fraxinea* in Austria. In: Proceedings of the Conference of IUFRO Working Party 7.02.02, Eğirdir, Turkey, 11–16 May 2009. Ed. by Doğmuş - Lehtijärvi T, SDU *Faculty of Forestry Journal*, ISSN: 1302-7085, Serial: A, Special Issue, pp. 97 – 119.

Kirisits T, Matlakova M, Mottinger-Kroupa S, Halmschlager E and Lakatos F. 2010. *Chalara fraxinea* associated with dieback of narrow-leafed ash (*Fraxinus angustifolia*). *Plant Pathology* **59**: 411.

Kjær ED, Mckinney LV, Hansen LN, Olrik DC, Lobo A, Thomsen IM, Hansen JK and Nielsen LR. 2017. Genetics of ash dieback resistance in a

restoration context – experiences from Denmark. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 106 – 114.

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, Mering CV and Vorholt JA. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *The ISME Journal: Multidisciplinary Journal of Microbial Ecology* **6**: 1378 – 1390.

Knief C. 2014. Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Frontiers in Plant Science* **5**(216): 1 – 23.

Kohn LM. 2005. Mechanism of fungal speciation. *Annual Review of Phytopathology* **43**: 279 – 308.

KoKo TW, Stephenson SL, Bahkali AH and Hyde KD. 2011. From morphology to molecular biology: can we use sequence data to identify fungal endophytes? *Fungal Diversity* **50**: 113 − 120.

Kosawang C, Amby BD, Bussaban B, McKinney LV, Xu J, Kjær ED, CollingeDB and Nielsen LR. 2018. Fungal communities associated with species of *Fraxinus* tolerant to ash dieback, and their potential for biological control. *Fungal Biology*, **122**: 110 – 120.

Kowalski T and Holdenrieder O. 2008. A new fungal disease of ash in Europe (in German). *Schweizerische Zeitschrift für Forstwesen* **159**: 45 – 50.

Kowalski T and Holdenrieder O. 2009a. Pathogenicity of *Chalara fraxinea*. *Forest Pathology* **39**: 1 – 7.

Kowalski T and Holdenrieder O. 2009b. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology* **39**: 304 – 308.

Kowalski T and Kehr RD. 1992. Endophytic fungal colonization of branch bases in several forest tree species. *Sydowia* **44**(2): 137 – 168.

Kowalski T, Bilański P and Kraj W. 2017. Pathogenicity of fungi associated with ash dieback towards *Fraxinus excelsior. Plant Pathology* DOI: 10.1111/ppa.12667.

Kowalski T, Kraj W and Bednarz B. 2016. Fungi on stems and twigs in initial and advanced stages of dieback of European ash (*Fraxinus excelsior*) in Poland. *European Journal of Forest Research* **135**: 565 – 579.

Kowalski T and Łukomska A. 2005. Studies on *Fraxinus excelsior* L. dieback in Włoszczowa Forest Unit stands. *Acta Agrobotanica* **59**: 429 – 440.

Kowalski T. 2006. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology* **36**(4): 264 – 270.

Kraj W, Zarek M and Kowalski T. 2012. Genetic variability of *Chalara fraxinea*, dieback cause of European ash (*Fraxinus excelsior* L.). *Mycological Progress* **11**(1): 37 – 45.

Kräutler K and Kirisits T. 2012. The ash dieback pathogen *Hymenoscyphus pseudoalbidus* is associated with leaf symptoms on ash species (*Fraxinus* spp.). *Journal of Agricultural Extension and Rural Development* **4**(9): 261 –265.

Kumar V, Sahai V and Bisaria VS. 2011. High-density spore production of *Piriformospora indica*, a plant growth-promoting endophyte, by optimization of nutritional and cultural parameters. *Bioresource Technology* **102**: 3169 – 3175.

Kumaran RS, Muthumary J and Hur BK. 2008. Production of taxol from *Phyllosticta spinarum*, an endophytic fungus of *Cupressus* sp. *Engineering in Life Sciences* **8**: 438 – 446.

Kumaran RS, Kim HJ and Hur B-K. 2010. Taxol promising fungal endophyte, *Pestalotiopsis* species isolated from *Taxus cuspidata*. *Journal of Biosciences and Bioengineering* **110**: 541 – 546.

Kunca A. (ed.) 2006. *Occurrence of Pest Agents in Slovak Forests in 2005 and Prognosis for 2006*. *Forest Research Institute, Zvolen* 89 pp. (In Slovakian).

Kusari S, Zühlke S and Spiteller M. 2009b. An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *Journal of Natural Product* **72**: 2 – 7.

Lahiri AL, Douglas GC, Murphy BR and Hodkinson TR. 2019. The in vitro methods for plant-microbe interaction and biocontrol studies in European Ash (*Fraxinus excelsior* L.). In *Endophytes for a Growing World*, ed. Hodkinson TR, Doohan FM, Saunders MJ and Murphy BR, Cambridge: Cambridge University Press, Chapter 15.

Lakes States Forest Experiment Station. 1935. Recent results from seed dormancy. *Forest Research Digest* 1 – 2. May.

Lane WD. 1978. Regeneration of apple plants from shoot meristem-tips. *Plant Science Letters* **13** (3): 281 – 285.

Lang C, Seven J and Polle A. 2011. Host preferences and differential contributions of deciduous tree species shape mycorrhizal species richness in a mixed Central Europen forest. *Mycorrhiza* **21**(4): 297 – 308.

Latham R and Ricklefs R. 1993. Global patterns of tree species richness in moist forests – energy- diversity theory does not account for variation in species richness. *Oikos* **67**: 325 – 333.

Lebedev V and Shestibratov K. 2016. Large-scale micropropagation of common ash. *Biotechnology* **15**(1 **–** 2): 1 **–** 9.

Legault D, Dessureault M and Laflamme G. 1989. Mycoflora of the needles of *Pinus banksiana* and *Pinus resinosus*. 1. Endophytic fungi. *Canadian Journal of Botany* **67**: 2052 – 2060.

Leigh JW and Bryant D. 2015. Popart: full‐feature software for haplotype network construction. *Methods Ecology and Evolution* **6**: 1110 – 1116.

Leonard RE and Parr SB. 1970. Woodland trees as a sound barrier. *Journal of Forestry* **68**: 282 – 283.

Leonhard S, Strasser S, Nannig A, Blaschke M, Schumacher J and Immler T. 2009. A new disease of ash. First record of ash dieback in Bavaria (in German). *LWFaktuell* **71**: 60 – 63.

Li JY, Sidhu RS, Ford EJ, Long DM, Hess WM and Strobel GA. 1998. The induction of taxol production in the endophytic fungus *Periconia* sp. from *Torreya grandifolia*. *Journal of Industrial Microbiology and Biotechnology* **20**: 259 – 264.

Lieckfeldt E and Seifert KA. 2000. An evaluation of the use of ITS sequences in the taxonomy of the hypocreales. *Studies in Mycology* **45**: 35 – 44.

Link HF. 1809. Observationes in ordines plantarum naturales, dissertatio prima, complectens anandrarum ordines Epiphytas, Mucedines, Gastromycos et Fungos. Berlin, Germany: Der Gesellschaft Naturforschender Freunde zu Berlin.

Liu CH, Zou WX, Lu H and Tan RX. 2001. Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi. *Journal of Biotechnology* **88**: 277 – 282.

Liu F, Cai X-L, Yang H, Xia XK, Guo ZY, Yuan J, Li MF, She ZG and Lin YC. 2010a. The bioactive metabolites of the mangrove endophytic fungus *Talaromyces* sp. ZH-154 isolated from *Kandelia candel* (L) Druce. Planta Medica **76**:185 – 189.

Liu K, Ding X, Deng B and Chen W. 2009. Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis*. *Journal of Industrial Microbiology and Biotechnology* **36**: 1171 – 1177.

Liu K, Ding X, Deng B and Chen W. 2010b. 10-Hydroxycamptothecin produced by a new endophytic *Xylaria* sp., M20, from *Camptotheca acuminata*. *Biotechnology Letters* **32**: 689 – 693.

Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L and Law M. 2012. Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology* **2012**(251364): 1 – 11. DOI: 10.1155/2012/251364.

Liu X, Dong M, Chen X, Jiang M, Lv X and Zhou J. 2008a. Antimicrobial activity of an endophytic *Xylaria* sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin. *Applied Microbiology and Biotechnology* **78**: 241 – 247.

Liu YJ and Hall BD. 2004. Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny. *Proceedings of the National Academy of Sciences of the United States of America* **101**(13): 4507 − 4512.

Lodge DJ, Fisher PJ and Sutton BC. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* **88**: 733 – 738.

Lodge DJ, Hawksworth DL and Ritchie BJ. 1996. Microbial diversity and tropical forest functioning. *Ecological Studies* **122**: 69 – 100.

Lohmus A. and Runnel K. 2014. Ash dieback can rapidly eradicate isolated epiphyte populations in production forests: A case study. *Biological Conservation* **169**: 185 – 8.

Lozupone C, Lladser ME, Knights D, Stombaugh J and Knight R. 2011. UniFrac: an effective distance metric for microbial community comparison. *The International Society of Microbial Ecology Journal* **5**: 169 – 172.

Lu Y, Chen C, Chen H, Zhang J. and Chen W. 2012. Isolation and identification of endophytic fungi from *Actinidia macrosperma* and investigation of their bioactivities. *Evidence based Complementary and Alternative Medicine*. DOI: 10.1155/2012/382742.

Lücking R, Dal-Forno M, Sikaroodi M, Gillevet PM, Bungartz F, Moncada B, Yánez-Ayabaca A, Chaves JL, Coca LF and Lawrey JD. 2014. A single macrolichen constitutes hundreds of unrecognized species. *Proceedings of the National Academy of Sciences of the United States of America* **111** (30): 11091 − 11096.

Lumini E, Orgiazzi A, Borriello R, Bonfante P and Bianciotto V. 2010. Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. *Environmental Microbiology* **12**: 2165 – 2179.

Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J, Engelbrektson A, Kunin V, Rio TG, Edgar RC, Eickhorst T, Ley RE, Hugenholtz P, Tringe SG and Dangl JL. 2012. Defining the core *Arabidopsis thaliana* root microbiome. *Nature* **488**: 86 – 90.

Luo CW, Tsementzi D, Kyrpides N, Read T and Konstantinidis KT. 2012b. Direct comparisons of Illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. *Public Library of Science ONE* **7**:e30087. DOI: 10.1371/journal.pone.0030087.

Mahapatra S and Banerjee D. 2010. Diversity and screening for antimicrobial activity of endophytic fungi from *Alstonia scholaris*. *Acta Microbiologica et Immunologica Hungarica* **57**: 215 – 23.

Maignien L, Deforce EA, Chafee ME, Eren AM and Simmons SL. 2014. Ecological succession and stochastic variation in the assembly of *Arabidopsis thaliana* phyllosphere communities. *American Society for Microbiology* **5**: e00682–13. DOI: 10.1128/mBio.00682-13.

Marčiulynienė D, Cleary MR, Shabunin D, Stenlid J and Vasaitis R. 2013. Detection of *Hymenoscyphus pseudoalbidus* in Primorye region, far east Russia. COST Action FP1103 Fraxback, 4th MC meeting & workshop, 2013 Sep 4–6, Malmö.

Matheny PB, Liu YJJ, Ammirati JF and Hall BD. 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *American Journal of Botany* **89**: 688 – 698.

McCracken AR, Douglas GC, Ryan C, Destefanis M and Cooke LR. 2017. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European

Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 125 –139.

McEvoy A, O'Regan F, Fleming CC, Moreland BP, Pollock JA, McGuinness BW and Hodkinson TR. 2016. Bleeding canker of horse chestnut (*Aesculus hippocastanum*) in Ireland: incidence, severity and characterization using DNA sequences and real‐time PCR. *Plant Pathology* **65**(9): 1419 – 1429.

McInroy JA and Kloepper JW. 1994. Studies on indigenous endophytic bacteria of sweet corn and cotton. In: O'Gara F, DowlingDN, Boesten B (eds) Molecular ecology of rhizosphere microorganisms. *VCH*, Weinheim, Germany, pp 19 – 28.

McKinney LV, Nielsen LR, Hansen JK and Kjær ED. 2011. Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity* **106**: 788 – 797.

McLellan CA, Turbyville TJ, Wijeratne EM, Kerschen A, Vierling E, Queitsch C, Whitesell L, Gunatilaka AAL. 2007. A rhizosphere fungus enhances *Arabidopsis* thermotolerance through production of an HSP90 inhibitor. *Plant Physiology* **145**: 174 – 182.

McMullan M, Rafiqi M, Kaithakottil G, Calvijo BJ, Bilham L, Orton E, Percival-Alwyn L, Ward BJ, Edwards A, Saunders DGO, Accinelli GG, Wright J, Verweij W, Koutsovoulos G, Yoshida K, Hosoya T, Williamson L, Jennings P, Ioos R, Husson C, Hietala AM, Vivian-Smith A, Solheim H, MaClean D, Fosker C, Hall N, Brown JKM, Swarbreck D, Blaxter M, Downie JA and Clark MD. 2018. The ash dieback invasion of Europe was founded by two genetically divergent individuals. *Nature Ecology and Evolution*. https://doi.org/10.1038/s41559-018-0548-9.

McNeil M, Roberts AMI, Cockerell V and Mulholland V. 2004. Real-time PCR assay for quantification of *Tilletia caries* contamination of UK wheat seed. *Plant Pathology* **53**: 741 – 750.

Meletiadis J, Meis JFGM, Mouton JW and Verweij PE. 2001. Analysis of growth

characteristics of filamentous fungi in different nutrient media. *Journal of Clinical Microbiology* **39**(2): 478 – 484.

Mesarich CH, Bowen JK, Hamiaux C and Templeton MD. 2015. Repeat containing protein effectors of plant-associated organisms. *Frontiers in Plant Sciences* **6**: 872.

Meusel H, Jager E, Rauschert S and Weinert E. 1978. *Vergleichende Chorologie der Zentraleurop.ischen Flora* (Gustav Fischer Verlag Jena, 1978).

Michielse CB and Rep M. 2009. Pathogen profile update: *Fusarium oxysporum*. *Molecular Plant Pathology* **10**(3): 311 – 324.

Milenkovic I, Jung T, Stanivukovic Z and Karadžic D. 2017. First report of *Hymenoscyphus fraxineus* on *Fraxinus excelsior* in Montenegro. *Forest Pathology* **47**(5): e12359.

Misaghi IJ and Donndelinger CR. 1990. Endophytic bacteria in symptom-free cotton plants. *Phytopathology* **80**: 808 – 811.

Mitchell AF. 1974. *A field guide to the trees of Britain and northern Europe* (Harper Collins, 1974) pp. 1 – 416.

Mitchell JI and Zuccaro A. 2006. Sequences, the environment and fungi. *Mycologist* **20**: 62 – 74.

Mitchell RJ, Bailey S, Beaton JK, Bellamy PE, Brooker RW, Broome A, Chetcuti J, Eaton S, Ellis CJ, Farren J, Gimona A, Goldberg E, Hall J, Harmer R, Hester AJ, Hewison RL, Hodgetts NG, Hooper RJ, Howe L, Iason GR, Kerr G, Littlewood NA, Morgan V, Newey S, Potts JM, Pozsgai G, Ray D, Sim DA, Stockan JA, Taylor AFS and Woodward S. 2014. The potential ecological impact of ash dieback in the UK. Joint Nature Conservation Committee, Report No. 483. Peterborough 2014.

Mitchell RJ, Hewison R, Hester A, Broome A and Kirby K. 2016. Potential impacts of loss of *Fraxinus excelsior* due to ash dieback on woodland vegetation in Great Britain. *New Journal of Botany* **6**(1): 2 – 15.

Mohamed R, Jong PL and Zali MS. 2010. Fungal diversity in wounded stems of *Aquilaria malaccensis*. *Fungal Diversity* **43**: 67 – 74.

Moore D, Robson GD and Trinci APJ. 2011. *21st Century Guidebook to Fungi*. Cambridge: Cambridge University Press, pp. 41 – 81.

Morgenstern I, Powlowski J, Ishmael N, Darmond C, Marqueteau S, Moisan MC, Quenneville G and Tsang A. 2012. A molecular phylogeny of thermophilic fungi. *Fungal Biology* **116**(4): 489 – 502.

Moslonka-Lefebvre M, Finley A, Dorigatti I, Dehnen-Schmutz K, Harwood T, Jeger MJ, Xu X, Holdenrieder O and Pautasso M. 2011. Networks in plant epidemiology: from genes to landscapes, countries, and continents. *Phytopathology* **101**: 392 – 403.

Murphy BR, Batke SP, Doohan FM and Hodkinson TR. 2015. Media manipulations and the culture of beneficial fungal root endophytes. *International Journal of Biology*, **7**: 94 – 102.

Murphy BR, Nieto LM, Doohan FM and Hodkinson TR. 2015 Profundae diversitas: the uncharted genetic diversity in a newly studied group of fungal root endophytes. *Mycology*, **6**(3-4): 139 – 150.

Musolin DL, Selinkhovkin AV, Shabunin DA, Viacheslav BZ and Baranchikov YN. 2017. Between Ash Dieback and Emerald Ash Borer: Two Asian Invaders in Russia and the Future of Ash in Europe. *Baltic Forestry* **23**(1): 316 – 333.

Navarrete AA, Kuramae EE, De Hollander M, Pijl AS, Van Veen JA and Tsai SM. 2013. Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiology Ecology* **83**: 607 – 621.

Newsham KK, Lewis GC, Greenslade PD and McLeod AR. 1998. *Neotyphodium lolii*, a fungal leaf endophyte, reduces fertility of *Lolium perenne* exposed to elevated UV-b radiation. *Annals of Botany* **81**: 397 – 403.

Newton AC, Fitt BDL, Atkins SD, Walters DR and Daniell TJ. 2010. Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends in Microbiology* **18**: 365 – 373.

Niedringhaus TP, Milanova D, Kerby MB, Snyder MP and Barron AE. 2011. Landscape of next-generation sequencing technologies. *Analytical Chemistry* 83: 4327 – 4341.

Nikolaeva MG, Razumova MV and Gladkova VN. 1985. Reference Book on Dormant Seed Germination. 'Nauka' Publishers Leningrad Branch, 346 p (in Russian).

Nilsson RH, Anslan S, Bahram M, Wurzbacher C, Baldrian P and Tedersoo L. 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. *Nature Reviews Microbiology* **17**: 95 – 109.

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM and Vilgalys R. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied and Environmental Microbiology* **71**: 5544 – 5550.

O'Dell TE, Massicotte HB and Trappe JM. 1993. Root colonization of *Lupinus latifolius* Agardh. and *Pinus contorta* Dougl. by *Phialocephala fortinii* Wang & Wilcox. *New Phytologist* **124**: 93 – 100.

O'Donnell K and Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103 − 116.

O'Malley MA. 2008. 'Everything is everywhere: but the environment selects': ubiquitous distribution and ecological determinism in microbial biogeography. *Studies in History and Philosophy of Biological and Biomedical Sciences* **39**: 314 – 325.

Ogris N, Hauptman T and Jurc D. 2009. *Chalara fraxinea* causing common ash dieback newly reported in Slovenia. *Plant Pathology* **95**: 1173.

Ogris N, Hauptman T, Jurc D, Floreancig V, Marsich F and Montecchio L. 2010. First report of *Chalara fraxinea* on common ash in Italy. *Plant Disease* **94**:133.

Olson, Å and Stenlid, J. 2002. Pathogenic fungal species hybrids infecting plants. *Microbes and Infection* **4**(13): 1353 – 1359.

Osono T. 2006. Role of phyllosphere fungi of forest trees in the development of decomposer fungal communities and decomposition processes of leaf litter. *Canadian Journal of Microbiology* **52**: 701 – 716.

Ottesen AR, Peña AG, White JR, Pettengill JB, Li C, Allard S, Rideout S, Allard M, Hill T, Evans P, Strain E, Musser S, Knight R and Brown E. 2013b. Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BioMed Central Microbiology* 13: 114. doi: 10.1186/1471-2180-13-114.

Panaccione DG, Sheets NL, Miller SP and Cumming JR. 2001 Diversity of *Cenococcum geophilum* isolates from serpentine and non-serpentine soils. *Mycologia* **93**: 645 – 652.

Pareek CS, Smoczynski R and Tretyn A. 2011. Sequencing technologies and genome sequencing. *Journal of Applied Genetics* **52**: 413 – 435.

Park JH, Choi GJ, Lee HB, Kim KM, Jung HS, Lee SW, Jang KS and Cho KY. 2005. Griseofulvin from *Xylaria* sp. strain F0010, and endophytic fungus of *Abies holophylla* and its antifungal activity against plant pathogenic fungi. *Journal of Microbiology and Biotechnology* **15**: 112 – 117.

Parnell J and Curtis T. 2012. Webb's An Irish Flora (8th Edition). Illustrations by Elaine Cullen, Cork Univerity Press, pp 1 – 560. ISBN 978-185918-478-3.

Paul NC and Lee HB. 2014. First record of endophytic *Paraconiothyrium brasiliense* isolated from Chinese maple leaves in Korea. *The Korean Journal of Mycology* **42**(4): 349 – 352.

Paulus B, Kanowski J, Gadek P and Hyde KD. 2006. Diversity and distribution of

saprobic microfungi in leaf litter of an Australian tropical rainforest. *Mycological Research* **110**: 1441 – 1454.

Pehl L and Butin H. 1994. Endophytische Pilze in Blättern von Laubbäumen und ihre Beziehung zu Blattgallen (Zoocecidien). *Mitt aus der Biologischen Bundesanstalt f¨ur Land und Forstwirtschaft Berlin-Dahlem* **297**: 1 – 56.

Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, Buckler ES and Ley RE. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 6548 – 6553. DOI: 10.1073/pnas.1302837110.

Peláez F, Collado J, Arenal F, Basilio A, Cabello A, Díes Matas MT, García JB, Del Val AG, González V, Gorrochategui J, Hernández P, Martín I, Platas G and Vicente F. 1998. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycological Research* **102**: 755 – 761.

Petrini O. 1984. Endophytic fungi in British Ericaceae. *Transaction of British Mycological Society* **83**: 510 – 512.

Petrini O. 1991. Fungal endophytes of tree leaves. In Microbial Ecology of Leaves . ed J. H. Andrews and S. S. Hirano. New York, NY: Springer Verlag, pp. 179 – 197.

Peyronel B. 1924. Prime ricerche sulla micorizae endotrofiche e sulla microflora radicola normalle della fanerograme. *Revista de Biologia Tropical* **6**: 17 – 53.

Philip VJ and Nainar SAZ. 1986. Clonal Propagation of *Vanilla planifolia* (Salisb.) Ames using Tissue Culture. *Journal of Plant Physiology* **122**(3): 211 – 215.

Philipson MN and Christey MC. 1986. The relationship of host and endophyte during flowering, seed formation, and germination of *Lolium perenne*. *New Zealand Journal of Botany* **24**: 125 – 134.

Phongpaichit S, Nikom J and Rungjindamai N, Skayaroj J, Hutadilok-Towatan N, Rukachaisirikul V and Kirtikara K. 2007. Biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. *FEMS Immunology and Medical Microbiology* **51**: 517 – 525.

Pilûra A and Heuertz M. 2003. EUFORGEN technical guidelines for genetic conservation and use for common ash (*Fraxinus excelsior*). *Technical Reports on Bioversity International*.

Pilūra A, Bakys R, Suchockas V, Marčiulynienė D, Gustienė A, Verbyla V and Lygis V. 2017. Ash dieback in Lithuania: disease history, research on impact and genetic variation in disease resistance, tree breeding and options for forest management. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 150 – 165.

Pirttilä AM and Frank AC. 2011. Endophytes of forest trees - Biology and applications, ed Pirttilä AM and Frank AC, series editors Shri Mohan Jain, Hely Häggman, Alvin D. Yanchuk, Springer International Publication. pp 151 – 311.

Pleban S, Ingel F and Chet I. 1995. Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in the greenhouse using endophytic *Bacillus* spp. *European Journal for Plant Pathology* **101**: 665 – 672.

Pongcharoen W, Rukachaisirikul V, Phongpaichit S, Kühn T, Pelzing M, Sakayaroj J and Taylor WC. 2008. Metabolites from the endophytic fungus *Xylaria* sp. PSU-D14. *Phytochemistry* **69**: 1900 – 1902.

Porras-Alfaro A, Liu KL, Kuske CR and Xiec G. 2014. From Genus to Phylum: Large-Subunit and Internal Transcribed Spacer rRNA Operon Regions Show Similar Classification Accuracies Influenced by Database Composition. *Applied and Environmental Microbiology* **80**(3): 829 − 840.

Power MWP, Hopkins AJM, Chen J, Bengtsson SBK, Vasaitis R and Cleary MR.

2017. European *Fraxinus* species introduced into New Zealand retain many of their native endophytic fungi. *Baltic Forestry* **23**(1): 74 – 81.

Praciak A, Dixon C, Fyson GF, Pasiecznik N, Rushforth K, Sassen M, Sheil D, Correia CS and Van Heist TC. 2013. The CABI encyclopedia of forest trees. CABI, Oxfordshire, UK.

Preece JE, Christ PH, Ensenberger L and Zhao J. (1987). Micropropagation of ash (*Fraxinus*). Comb. Proc. *International Plant Propagate Society* **37**: 366 – 372.

Przybył K. 2002a. Fungi associated with necrotic apical parts of *Fraxinus excelsior* shoots. *Forest Pathology* **32**: 387 – 394.

Przybyl K. 2002b. Causes of decline of young ash trees and stands. *Central Board of State Forests, Warsaw, Poland* (in Polish).

Puchner H. 1922. Die verzogerte Keimung von Baumsamereien Forstwiss. *Centralbl* **44**: 445 – 455.

Puri SC, Nazir A, Chawla R, Arora R, Riyaz-Ul-Hasan S, Amna T, Ahmed B, Verma V, Singh S, Sagar R, Sharma A, Kumar R, Sharma RK and Qazi GN. 2006. The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *Journal of Biotechnology* **122**: 494 – 510.

Puri SC, Verma V, Amna T, Qazi GN and Spiteller M. 2005. An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *Journal of Natural Products* **68**: 1717 – 1719.

Quadt-Hallmann A, Hallmann J and Kloepper JW. 1997. Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. *Canadian Journal of Microbiology* **43**: 254 – 259.

Queloz V, Grunig CR, Berndt R, Kowalski T, Sieber TN and Holdenrieder O. 2011. Cryptic speciation in *Hymenoscyphus albidus*. *Forest Pathology* **41**: 133 – 142.

Queloz V, Hopf S, Schoebel CN, Rigling D and Gross A. 2017. Ash dieback in Switzerland: history and scientific achievements. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 68 – 78.

Quilluam RS and Jones DL. 2012. Evidence for host-specificity of culturable fungal root endophytes from the carnivorous plant *Pinguicula vulgaris* (Common Butterwort). *Mycological Progress* 11: 583 – 585.

Ragazzi A, Moricca S, Capretti P, Dellavalle I and Turco E. 2003. Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining *Quercus* species in Italy. *Forest Pathology* **33**: 31 – 38.

Rai M, Deshmukh P, Gade A, Ingle A, Kövics GJ and Irinyi L. 2009. *Phoma Saccardo*: distribution, secondary metabolite production and biotechnological applications. *Critical Reviews in Microbiology* **35**:182 – 96.

Raja H, Schoch CL, Hustad V, Shearer C and Miller A. 2011. Testing the phylogenetic utility of MCM7 in the Ascomycota. *MycoKeys* **1**: 63 – 94.

Raja HA, Miller AN, Pearce CJ and Oberlies NH. 2017. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products* **80**(3): 756 – 770.

Rao PS and Bapat VA. 1978. Vegetative propagation of sandalwood plants through tissue culture. *Canadian Journal of Botany* **56**(9): 1153 – 1156.

Raquin C, Jung-Mullera B, Dufourb J and Frascaria-Lacoste N. 2002. Rapid seedling obtaining from European ash species *Fraxinus excelsior* (L.) and *Fraxinus angustifolia* (Vahl.). *Annals of Forest Science* **59**: 219 – 224.

Rastogi G, Sbodio A, Tech JJ, Suslow TV, Coaker GI and Leveau JHJ. 2012. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial

community composition on field-grown lettuce. *The ISME Journal: Multidisciplinary Journal of Microbial Ecology* **6**: 1812 – 1822.

Redford AJ, Bowers RM, Knight R, Linhart Y and Fierer N. 2010. The ecology of the phyllosphere: Geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environmental Microbiology* **12**: 2885 – 2893.

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ and Henson JM. 2002. Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science* **298**: 1581.

Reeb V, Lutzoni F and Roux C. 2004. Contribution of RPB2 to multilocus phylogenetic studies of the Pezizomycotina (euascomycetes, Fungi) with special emphasis on the lichen-forming Acarosporaceae and evolution of polyspory. *Molecular Phylogenetics and Evolution* **32**: 1036 – 1060.

Reed BM, Buckley PM and DeWilde TN. 1995. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro Cellular & Developmental Biology* **31**(1): 53 – 57.

Rehman S, Shawl AS, Kour A, Andrabi R, Sudan P, Sultan P, Verma V and Qazi GN. 2008. An endophytic *Neurospora* sp. from *Nothapodytes foetida* producing camptothecin. *Prikladnaia Biokhimiia i Mikrobiologiia* **44**: 225 – 231.

Rehner S and Samuels GJ. 1995. Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73**(Supplement 1): S816 – S823.

Rehner S. 2001. Primers for elongation factor 1-a (EF1-a). Available from http://ocid.nacse.org/research/deephyphae/ EF1primer.pdf

Rehner SA and Buckley E. 2005. A Beauveria phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* **97**(1): 84 – 98.

Reiter B, Pfeifer U, Schwab H and Sessitsch A. 2002. Response of endophytic bacterial communities in Potato plants to infection with *Erwinia carotovora* subsp. *atroseptica*. *Applied and Environmental Microbiology* **68**(5): 2261 – 2268.

Rickerts V, Just-Nubling G, Konrad F, Kern J, Lambrecht E, Bohme A, Jacobi V and Bialek R. 2006. Diagnosis of invasive aspergillosis and mucormycosis in immune compromised patients by semi-nested PCR assay of tissue samples. *European Journal of Clinical Microbiology and Infectious Diseases* **25**: 8 – 13.

Rivas-Ubach A, Pérez-Trujillo M, Sardans J, Albert Gargallo-Garriga A, Parella T and Peñuelas J. 2013. Ecometabolomics: optimized NMR-based method Albert. *Methods in Ecology and Evolution* **4**: 464 – 473.

Robin C and Heiniger U. 2001. Chestnut blight in Europe: diversity of *Cryphonectria parasitica*, hypovirulence and biocontrol. *Forest Snow and Landscape Research* **76**: 361 – 367.

Rodriguez RJ and Redman R. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of Experimental Botany* **59**: 1109 – 1114.

Rodriguez RJ, White JF, Arnold AE and Redman RS. 2009. Fungal endophytes: diversity and functional roles. *New Phytologist* **182**(2) : 314 – 330.

Rosa LH, Gonçalves VN, Caligiorne RB, Alves TMA, Rabello A, Sales PA, Romanha AJ, Sobral MEG, Rosa CA and Zani CL. 2010. Leishmanicidal, trypanocidal, and cytotoxic activities of endophytic fungi associated with bioactive plants in Brazil. *Brazilian Journal of Microbiology* **41**: 420 – 430.

Rosenzweig N, Tiedje JM, Quensen JF, Meng QX and Hao JJJ. 2012. Microbial communities associated with potato common scab-suppressive soil

determined by pyrosequencing analyses. *Plant Disease* **96**: 718 – 725. DOI: 10.1094/PDIS-07-11-0571.

Rout GR, Mohapatra A and Jain SM. 2006. Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. *Biotechnology advances* **24**(6): 531 – 560.

Rozsypálek J, Dvořák M, Longauer R, Botella L, Prouza M, Palovčíková D and Jankovský L. 2017. Ash and ash dieback in the Czech Republic. Ash dieback on the island of Ireland. (eds) Vasaitis R and Enderle R, Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management, The Report on European Cooperation in Science & Technology (COST) Action FP1103 FRAXBACK, pp 79 – 88.

Rudgers JA and Clay K. 2007. Endophyte symbiosis with tall fescue: how strong are the impacts on communities and ecosystems? *Fungal Biology Reviews* **21**: 107 – 124.

Rytkönen A, Lilja A, Drenkhan R, Gaitnieks T and Hantula J. 2010. First record of *Chalara fraxinea* in Finland and genetic variation among isolates sampled from Åland, mainland Finland, Estonia and Latvia. *Forest Pathology* **41**: 169 –174.

Saikkonen K, Faeth SH, Helander M and Sullivan TJ. 1998. Fungal endophytes: A continuum of interactions with host plants. *Annual Review of Ecology and Systematics* **29** : 319 – 343.

Saikkonen K, Ion D and Gyllenberg M. 2002. The persistence of vertically transmitted fungi in grass metapopulations. *Proceedings of the Royal Society B: Biological Sciences* **269**: 1397 – 1403.

Saikkonen K. 2007. Forest structure and fungal endophytes. *Fungal Biology Reviews* **21**: 67 – 74.

Sambeek JWV and Preece JE. 2007. *In vitro* propagation of Fraxinus species. *Propagation for Micropropagation of Woody Trees and Fruits*. Published by Springer pg179 – 192.

Sansford CE. 2013. Pest Risk Analysis for *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) for the UK and the Republic of Ireland. *Forestry Commission* 1 – 128.

Santamaria J and Bayman P. 2005. Fungal epiphytes and endophytes of Coffee leaves (*Coffea arabica*). *FEMS Microbiology Ecology* **50**: 1 – 8.

Sardi P, Sarachhi M, Quaroni S, Petrolini B, Borgonovi GE and Merli S. 1992. Isolation of endophytic Streptomyces strains from surface-sterilised roots. *Applied Environment Microbiology* **58**: 2691 – 2693.

Sarhan MS, Patz S, Hamza MA, Youssef HH, Mourad EF, Fayez M, Murphy B, Ruppel S and Hegazi NA. 2018. G3 PhyloChip analysis confirms the promise of plant-based culture media for unlocking the composition and diversity of the Maize root microbiome and for recovering unculturable candidate divisions/phyla. *Microbes and Environments* **33**(3): 317 – 325.

Schadt EE, Turner S and Kasarskis A. 2010. A window into third-generation sequencing. *Human Molecular Genetics* **19**(R2): 227 – 240.

Scherlach K, Boettger D, Remme N and Hertweck C. 2010. The chemistry and biology of cytochalasans. *Natural Product Reports* **27**: 869 – 886.

Schlegel M, Dubach V, Buol L and Sieber TN. 2016. Effects of endophytic fungi on the ash dieback pathogen. *FEMS Microbiology Ecology* **92**(9): 1 – 8.

Schlegel M, Queloz V and Sieber TN. 2018. The Endophytic Mycobiome of European Ash and Sycamore Maple Leaves – Geographic Patterns, Host Specificity and Influence of Ash Dieback. *Frontiers in Microbiology* **9**(2345): 1 – 20.

Schmitt I, Crespo A, Divakar PK, Fankhauser JD, Herman-Sackett E, Kalb K, Nelsen MP, Nelsen NA, Rivas-Plata E, Shimp AD, Widhelm T and Lumbsch HT. 2009. New primers for promising single-copy genes in fungal phylogenetics and systematics. *Persoonia* **23**: 35 – 40.

Schoch CL and Seifert KA. 2011. DNA barcoding in fungi. In *Access Science*, McGraw-Hill. DOI: 10.1036/1097-8542.YB110060.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W and fungal Barcoding Consortia. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of United States of America* **109** (16): 6241 – 6246.

Scholtysik A, Unterseher M, Otto P and Wirth C. 2013. Spatio-temporal dynamics of endophyte diversity in the canopy of European ash (*Fraxinus excelsior*). *Mycological Progress* **12**: 291 – 304.

Scholz MB, Lo CC and Chain PSG. 2012. Next generation sequencing and bioinformatic bottlenecks: the current state of metagenomic data analysis. *Current Opinion in Biotechnology* **23**: 9 – 15.

Schrey SD, Schellhammer M, Ecke M, Hampp R and Tarkka MT. 2005. Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. *New Phytologist* **168**: 205 – 216.

Schultz B, Guske S, Damann U and Boyle E. 1998. Endophyte-host-interaction II Defining symbiosis of the endophyte-host interaction. *Symbiosis* **25**(1): 213 –227.

Schulz B and Boyle C. 2005. The endophytic continuum. *Mycological Research* 109: 661 – 686.

Schulz B, Boyle C and Sieber T. 2006. Microbial root endophytes, ed. Schulz B, Boyle C and Sieber T, series ed. Varma A. *Springer* - International Publisher Science, Technology, Medicine, pp 1 – 350.

Schulz B, Boyle C, Draeger S, Römmert A-K and Krohn K. 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* **106**(9): 996 – 1004.

Schulz B, Haas S, Junker C, Andrée N and Schobert M. 2015. Fungal endophytes are involved in multiple balanced antagonisms. *Current Science* **109**(1): 39 – 45.

Schulz B, Wanke U, Draeger S and Aust H-J. 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilisation methods. *Mycology Research* **97**:1447 – 1450.

Schumacher J, Kehr R and Leonhard S. 2010. Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. *Forest Pathology* **40**: 419 – 429.

Schumacher J, Wulf A and Leonhard S. 2007. First record of *Chalara fraxinea* T. Kowalski sp. nov. in Germany – a new agent of ash decline. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **59**: 121 – 123 (In German).

Schumacher J. 2011. The general situation regarding ash dieback in Germany and investigations concerning the invasion and distribution strategies of *Chalara fraxinea* in woody tissue. *European and Mediterranean Plant Protection Organization Bulletin* **41**: 7 – 10.

Seifert KA, Samson RA, Dewaard JR, Houbraken J, Lévesque CA, Moncalvo JM, Louis-Seize G and Hebert PD. 2007. Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *Proceedings of the National Academy of Sciences*, *United States of America* **104**: 3901 – 3906.

Seifert KA, Wingfield BD and Wingfield MJ. 1995. A critique of DNA sequence analysis in the taxonomy of filamentous Ascomycetes and ascomycetous anamorphs. *Canadian Journal of Botany* **73**(S1): 760−767.

Seifert KA. 2009. Progress towards DNA barcoding of fungi. *Molecular Ecology Resources* **9** (Supplement 1): 83 – 89.

Sekita S, Yoshihira K and Natori S. 1973. Structures of chaetoglobosin A and B, cytotoxic metabolites of *Chaetomium globosum*. *Tetrahedron Letters* **23**: 2109 – 2112.

Selosse M-A, Baudoin E and Vandenkoornhuyse P. 2004. Symbiotic microorganisms, a key for ecological success and protection of plants. *Comptes Rendus Biologies* **327**: 639 – 648.

Seven J and Polle A. 2014. Subcellular nutrient element localization and enrichment in ecto and arbuscular mycorrhizas of field-grown beech and ash trees indicate functional differences. *Public Library of Science ONE*, **9**(12): e114672.

Shishido M, Loeb BM and Chanway CP. 1995. External and internal root colonisation of lodge pole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. *Canadian Journal of Microbiology* **41**: 707 – 713.

Shweta S, Zuehlke S, Ramesha BT, Priti V, Kumar MP, Ravikanth G, Spiteller M, Vasudeva R and Shaanker UR. 2010. Endophytic fungal strains of Fusarium solani, from *Apodytes dimidiata* E Mey ex Arn (Icacinaceae) produce camptothecin, 10- hydroxycamptothecin and 9-methoxycamptothecin. *Phytochemistry* **71**: 117 – 122.

Sieber T and Hugentobler C. 1987. Endophytic fungi in leaves and twigs of healthy and diseased beech trees (*Fagus sylvatica* L.). *European Journal of Plant Pathology* **17**: 411 – 425.

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

Sieber TN and Dorworth CE. 1994. An ecological study about assemblages of endophytic fungi in *Acer macrophyllum* in British-Columbia – in search

of candidate mycoherbicides. *Canadian Journal of Botany* **72:** 1397 – 1402.

Sieber TN, Rys J and Holdenrieder O. 1999. Mycobiota in symptomless needles of *Pinus mugo* ssp. uncinata. *Mycological Research* **103**: 306 – 310.

Sieber TN. 2002. Fungal root endophytes. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots: the hidden half. *Dekker*, New York, pp 887 – 917.

Sioen G, Roskams P, Cuyper B and Steenackers M. 2017. Ash dieback in Flanders (Belgium): research on disease development, resistance and management options. Dieback of European Ash (*Fraxinus* spp.): Consequences and Guidelines for Sustainable Management. Uppsala, Swedish University of Agricultural Sciences, p. 61 – 67.

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

Smith C, Johnson A, Fernall D, Mitchell M, Harper G. and Cotterill A. 2013. (rev.) Fraser R, Hanley N, Petts J and Potter C. *Chalara* in Ash Trees: A framework for assessing ecosystem impacts and appraising options. Department for Environment Food & Rural Affairs. The National Archives, Kew, London. pp, 3 – 45.

Smith D and Ryan MJ. 2009. Fungal sources for new drug discovery. *In Access Science*, McGraw-Hill Companies. DOI: 10.1036/1097-8542.YB090090.

Soca-Chafre G, Rivera-Orduña FN, Hidalgo-Lara ME, Hernandez-Rodriguez C, Marsch R and Flores-Cotera LB. 2011. Molecular phylogeny and paclitaxel screening of fungal endophytes from *Taxus globosa*. *Fungal Biology* **115**: 143 – 156.

Sreenivasa MN and Bagyaraj DJ. 1988. Selection of suitable substrate for mass multiplication of *Glomus fasciculatum*. *Plant and soil* **109**(1): 125 – 127.

Sriskandarajah S, Kennedy IR, Yu DG and Tchan YT. 1993. Effects of plant growth regulators on acetylene reducing associations between *Azospirillum*

brasilense and wheat. *Plant Soil* **153**: 165 – 178.

Steinbauer GP. 1937. Dormancy and germination of *Fraxinus* seeds. *American Society of Plant Biologists* **12** (3): 813 – 824.

Stener LG. 2013. Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. *Scandinavian Journal of Forest Research* **28** (3): 205 – 216.

Stierle A, Strobel G and Stierle D. 1993. Taxol and taxane production by *Taxomyces andreanae*,an endophytic fungus of Pacific yew. *Science* **260**: 214 – 216.

Stiller JW and Hall BD. 1997. The origin of red algae: implications for plastid evolution. *Proceedings of the National Academy of Sciences of the United States of America 94*: 4520 – 4525.

Strobel G, Hess W, Li J-Y, Ford E, Sears J, Sidhu RS and Summerell B. 1997. *Pestalotiopsis guepinii*, a taxol-producing endophyte of the Wollemi pine, *Wollemia nobilis*. *Australian Journal of Botany* **45**: 1073 –1082.

Strobel GA, Daisy B, Castillo U and Harper J. 2004. Natural products from endophytic microorganisms. *Journal of Natural Products* **67**: 257 – 268.

Strobel GA, Knighton B, Kluck K, Ren Y, Livinghouse T, Griffin M, Spakowicz D and Sears J. 2008. The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL50072). *Microbiology* **154**: 3319 – 3328.

Strobel GA. 2003. Endophytes as sources of bioactive products. *Microbes and Infection* **5**: 535 – 544.

Sudhakar T, Dash S, Rao R, Srinivasan R, Zacharia S, Atmanand M, Subramaniam B and Nayak S. 2013. Do endophytic fungi possess pathway genes for plant secondary metabolites? *Current Science* **104**(2): 178 – 182.

Suszka B, Muller C and Bonnet-Masimbert M. 1996. Seeds of forest broadleaves from harvest to sowing. Translated by A.G. Gordon. INRA, France.

Sutjaritvorakul T, Whalley AJS, Sihanonth P and Roengsumran S. 2010. Antimicrobial activity from endophytic fungi isolated from plant leaves in Dipterocarpous forest at Viengsa district Nan province, Thailand. *Journal of Agricultural Science and Technology* **6**: 309 – 315.

Svenning J-C and Skov F. 2007. Ice age legacies in the geographical distribution of tree species richness in Europe. *Global Ecology and Biogeography* **16**: 234 – 245.

Szabo I. 2008 First report of *Chalara fraxinea* affecting common ash in Hungary. *New Diseases Reports* **18**: 30.

Tadych M, Bergen M, Dugan FM and White JF. 2007. Evaluation of the potential role of water in spread of conidia of the *Neotyphodium* endophyte of *Poa ampla*. *Mycological Research* **111**: 466 – 472.

Talgø V, Sletten A, Brurberg MB, Solheim H and Stensvand A. 2009. *Chalara fraxinea* isolated from diseased ash in Norway. *Plant Disease* **95**: 548.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: Molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731 – 2739.

Taylor JE, Hyde KD and Jones EBG. 1999. Endophytic fungi associated with the temperate palm, *Trachycarpus fortunei*, within and outside its natural geographic range. *New Phytologist* **142**: 335 – 346.

Tejesvi M, Ruotsalainen A, Markkola A and Pirttilä AM. 2010. Root endophytes along a primary succession gradient in Northern Finland. *Fungal Diversity Research Series* **41**: 125 –134.

Terhonen E, Blumenstein K, Kovalchuk A and Fred OA. 2019. Forest Tree Microbiomes and Associated Fungal Endophytes: Functional Roles and Impact on Forest Health. *Forests* **10**(42): 1 – 33.

Thomas PA. 2016. Biological Flora of the British Isles: *Fraxinus excelsior*. *Journal of Ecology* **104**(4): 1158 – 209.

Thomasset M, Fernández-Manjarres JF, Douglas GC, Frascaria-Lacoste N, Raquin C and Hodkinson TR. 2011. Molecular and morphological characterization of reciprocal F1 hybrid ash (*Fraxinus excelsior* × *F. angustifolia*, Oleaceae) and parental species reveals asymmetric character inheritance. *International Journal of Plant Sciences* **172**: 423 – 433.

Thomma BPHJ, Van Esse HP, Crous PW and De Wit PJGM. 2005. *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology* **6**: 379 – 393.

Thomsen IM and Skovsgaard JP. 2006. Toptørre i ask: klimaskade eller svampeangreb [Crown dieback of ash: climatic damage or fungal attack]. *Skoven* **38**: 408 – 411 (In Danish).

Thomsen IM, Skovsgaard JP, Barklund P and Vasaitis R. 2007. A fungal disease is the cause of shoot dieback of ash. *Skoven* **05**: 234 – 236. (In Danish).

Thomsen IM. 2005. Frost damage of ash. *Videnblade Skovbrug* **8**: 2 – 1. (In Danish).

Timmermann V, Borja I, Hietala AM, Kirisits T and Solheim H. 2011. Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *European and Mediterranean Plant Protection Organization Bulletin* **41**: 14 – 20.

Trapiello E, Schoebel CN and Rigling D. 2017. Fungal community in symptomatic ash leaves in Spain. *Baltic Forestry* **23**(1): 68 – 73.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R and Gordon JI, 2007. The human microbiome project. *Nature* **449**: 804 – 810.

Umeda M, Ohtsubo K, Saito M, Sekita S and Yoshihira K. 1975. Cytotoxicity of new cytochalasans from *Chaetomium globosum*. *Experientia* **31**: 435 – 438.

Unterseher M, Reiher A, Finstermeier K, Otto P and Morawetz W. 2007. Species richness and distribution patterns of leaf-inhabiting endophytic fungi in a temperate forest canopy. *Mycological Progress* **6**: 201 – 212.

Unterseher M and Schnittler M. 2009. Dilution-to-extinction cultivation of leaf-inhabiting endophytic fungi in beech (*Fagus sylvatica* L.) – different cultivation techniques influence fungal biodiversity assessment. *Mycological Research* **113**: 645 – 654.

Unterseher M and Schnittler M. 2010. Species richness analysis and its rDNA phylogeny revealed the majority of cultivable foliar endophytes from beech (*Fagus sylvatica*). *Fungal Ecology* **3**: 366 – 378.

Vega FE, Posada F, Aime MC, Pava-Ripoll M, Infante F and Rehner SA. 2008. Entomopathogenic fungal endophytes. *Biological Control* **46**: 72 – 82.

Videira SIR, Groenewald JZ, Braun U, Shin HD and Crous PW. 2016. All that glitters is not *Ramularia*. *Studies in Mycology* **83**: 49 – 163.

Vilgalys R and Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**(8): 4238 – 4246.

Villiers TA and Wareing PF. 1964. Dormancy in fruits of *Fraxinus excelsior* L. *Journal of Experimental Botany* **15**(2): 359 – 367.

Vorholt JA. 2012. Microbial life in the phyllosphere. *Nature Reviews Microbiology* **10**: 828 – 840. DOI: 10.1038/nrmicro2910.

Vujanovic V and Brisson J. 2002. A comparative study of endophytic mycobiota in leaves of *Acer saccharum* in Eastern North America. *Mycological Progress* **1**: 147 – 154.

Wallander E. 2008. Plant Systematics and Evolution, **273**: 25 – 49.

Wang CJK and Wilcox HE. 1985. New species of ectendomycorrhizal and psuedomycorrhizal fungi: *Phialophora findlandia*, *Chloridium paucisporum* and *Phialocephala fortinii*. *Mycologia* **77**: 951 – 958.

Wang J, Li G, Lu H, Zheng Z, Huang Y and Su W. 2000. Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. *FEMS Microbiology Letters* **193**: 249 – 253.

Wardle P. (1961) Biological Flora of the British Isles: *Fraxinus excelsior* L. *Journal of Ecology*, **49**: 739 – 751.

White JFJ. 1988. Endophyte-host associations in forage grasses. I. A proposal concerning origin and evolution. *Mycologia* **80**: 442 – 446.

White TJ, Bruns T, Lee S and Taylor J. 1990. *Amplification and direct sequencing of fungal ribosomial RNA genes for phyologenetics*, in: Innis MA, Gelfand DH, Sninsky JJ and White TJ. (Ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, USA: pp. 315 – 322.

Whitfield J. 2005. Biogeography: is everything everywhere? *Science* **310**: 960 – 961.

Williams TR, Moyne AL, Harris LJ and Marco ML. 2013. Season, irrigation, leaf age, and *Escherichia coli* inoculation influence the bacterial diversity in the lettuce phyllosphere. *Public Library of Science ONE* **8**: e68642. DOI: 10.1371/journal.pone.0068642.

Willis KG, Garrod G, Scarpa R, Powe N, Lovett A, Bateman IJ, Hanley N and Macmillan DC. 2003. The Social and Environmental Benefits of Forests in Great Britain. Report to Forestry Commission. Centre for Environmental Appraisal and Management, University of Newcastle upon Tyne.

Wilson D and Carroll GC (1994) Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*. *Mycologia* **86**: 635 – 647.

Wingfield MJ, Slippers B, Roux J and Wingfield BD. 2001. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. *Bioscience* **51**: 134 – 140.

Wu S-H, Chen Y-W, Shao S-C, Wang LD, Li ZY, Yang LY, Li SL and Huang R. 2008. Ten-membered lactones from *Phomopsis* sp., an endophytic fungus of *Azadirachta indica*. *Journal of Natural Products* **71**: 731 – 734.

Xin G, Glawe D and Doty SL. 2009a. Characterization of three endophytic, indole-3-acetic acid- producing yeasts occurring in *Populus* trees. *Mycological Research* **113**: 973 – 980.

Xin G, Zhang G, Kang JW, Staley JT and Doty SL. 2009b. A diazotrophic, indole-3-acetic acid- producing endophyte from wild cottonwood. *Biology and Fertility of Soils* **45**: 669 – 674.

Xu S, Ge HM, Song YC, Shen Y, Ding H and Tan RX. 2009a. Cytotoxic cytochalasin metabolites of endophytic *Endothia gyrosa*. *Chemistry Biodiversity* **6**: 739 – 745.

Yoganathan K, Yang L-K, Rossant C, Huang Y, Ng S, Butler MS and Buss AD. 2004. Cochlioquinones and epi-cochlioquinones: antagonists of the human chemokine receptor CCR5 from Bipolaris brizae and *Stachybotrys chartarum*. *The Journal of Antibiotics* **57**: 59 – 63.

Yu L, Nicolaisen M, Larsen J and Ravnskov S. 2012. Succession of root-associated fungi in *Pisum sativum* during a plant growth cycle as examined by 454 pyrosequencing. *Plant and Soil* **358**: 216 – 224. DOI: 10.1007/s11104-012-1188-5.

Zabalgogeazcoa. 2008. Review. Fungal endophytes and their interaction with plant pathogens. *Spanish Journal of Agricultural Research* **6** (Special issue): 138 – 146.

Zhang W, Wu X, Liu G, Chen T, Zhang G, Dong Z, Yang X and Hu P. 2013. Pyrosequencing reveals bacterial diversity in the rhizosphere of three *Phragmites australis* ecotypes. *Geomicrobiology Journal* **30**: 593 – 599. DOI: 10.1080/01490451.2012. 740145.

Zhao Y-J, Hosoya T, Baral H-O, Hosaka K and Kakishima M. 2012. *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. *Mycotaxon* **122** : 25 – 41.

Zheng H and Zhuang W. 2013. Four new species of the genus *Hymenoscyphus* (fungi) based on morphology and molecular data. *Science China Life Sciences* **56**: 90 – 100.

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG and Vidaver AK. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology* **68**(5): 2198 – 2208.

Web pages references

Forestry research UK
http://www.forestry.gov.uk/website/searchall.nsf/GoogleResults?open=&cx=001774383426470524382%3Aa5mnshoy9zo&cof=FORID%3A9&ie=UTF-8&q=symptoms

Forestry Comission UK
https://webarchive.nationalarchives.gov.uk/20180705114710/https://www.forestry.gov.uk/chalara

Department of Agriculture Food and the Marine Ireland
https://www.agriculture.gov.ie/forestservice/treediseases/ashdiebackchalara/

Kew
Kew 2019 http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:609009-1

Irish times
https://www.irishtimes.com/sport/ash-crisis-runs-straight-to-roots-of-gaa-1.554945

European Commission
http://forest.jrc.ec.europa.eu/european-atlas-of-forest-tree-species/atlas-download-page/

European and Mediterranean Plant Protection Organization
EPPO, 2014. PQR database. Paris, France: European and Mediterranean Plant Protection Organization. (http://www.eppo.int/DATABASES/pqr/pqr.htm)

European forest genetic resources programme
http://www.euforgen.org/species/fraxinus-excelsior/

Centre for Agriculture and Bioscience International
https://www.cabi.org/ISC/datasheet/108083

UK center for Ecology and Hydrology
http://www.cs2007.ceh.ac.uk/sites/default/files/pdfs/Distribution%20of%20Ash%20trees%20in%20CS_9thJan2013.pdf

Forestry research UK
http://www.forestry.gov.uk/forestry/infd-94pgy5

European and Mediterranean Plant Protection Organization
EPPO, 2014. PQR database. Paris, France: European and Mediterranean Plant Protection Organization. http://www.eppo.int/DATABASES/pqr/pqr.htm

CAB International (CABI) and EPPO are international organization
CABI/EPPO, 2013. *Hymenoscyphus pseudoalbidus*. [Distribution map]. Distribution Maps of Plant Diseases, No.October. Wallingford, UK: CABI, Map 1060 (Edition 2

FLASH (V1.2.7, http://ccb.jhu.edu/software/ FLASH/)

Unite database, https://unite.ut.ee/

http://www.drive5.com/usearch/manual/chimera_formation.html

Uparse v7.0.1001 http://drive5.com/uparse/

https://www.uniprot.org/

http://www.mycobank.org/quicksearch.aspx

Kew, http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:609009-1

Department of Marine and Natural Resources
https://www.agriculture.gov.ie/media/migration/forestry/publications/Ash_low.pdf

United States Department of Agriculture
https://www.srs.fs.usda.gov/pubs/gtr/gtr_so096.pdf

Appendix I – Chapter 2

Chi square analysis assessing whether endophyte recovery (number of isolates) was dependent on leaf tissue location (example - apex, leaf blade, midrib region, rachis, and vein) for both healthy and diseased (Figure 2.2).

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	6.561[a]	4	.161
Likelihood Ratio	6.630	4	.157
N of Valid Cases	435		

a. 2 cells (20.0%) have expected count less than 5. The minimum expected count is 1.21.

Chi square analysis assessing whether endophyte recovery was dependent on tissue type (healthy or diseased) (Figure 2.2).

Chi-Square Tests					
	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.523[a]	1	.217		
Continuity Correction[b]	1.047	1	.306		
Likelihood Ratio	1.589	1	.207		
Fisher's Exact Test				.293	.153
N of Valid Cases	438				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 9.93.
b. Computed only for a 2x2 table.

Chi square analyses assessing whether number of morphotype was dependent on leaf tissue location (example - apex, leaf blade, midrib region, and rachis) (Figure 2.2).

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	327.283[a]	212	.000
Likelihood Ratio	303.345	212	.000
N of Valid Cases	280		

a. 263 cells (97.4%) have expected count less than 5. The minimum expected count is .06.

Chi square analysis test for assessing whether endophyte recovery (number of isolates) was dependent on country provenance trials or not (Figure 2.3b).

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	56.062[a]	10	.000
Likelihood Ratio	54.228	10	.000
N of Valid Cases	430		

a. 11 cells (50.0%) have expected count less than 5. The minimum expected count is 1.80

Chi square analysis assessing whether endophyte recovery (number of isolates) was dependent on leaf tissue location (example - apex, leaf blade, midrib region and rachis) for both healthy and diseased Figure 2.4).

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	10.032[a]	3	.018
Likelihood Ratio	11.587	3	.009
N of Valid Cases	115		

a. 1 cells (12.5%) have expected count less than 5. The minimum expected count is 4.78.

Chi square analysis assessing whether endophyte recovery was dependent on tissue type (healthy or diseased) (Figure 2.4).

Chi-Square Tests					
	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.109[a]	1	.741		
Continuity Correction[b]	.001	1	.970		
Likelihood Ratio	.112	1	.738		
Fisher's Exact Test				1.000	.499
N of Valid Cases	115				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.57.
b. Computed only for a 2x2 table

Images 2.1 to 2.5 show morphotype variation observed in Roosky site after common leaf region of a single leaflet were sampled on two media. Details of plot information are shown in Table 2.1.

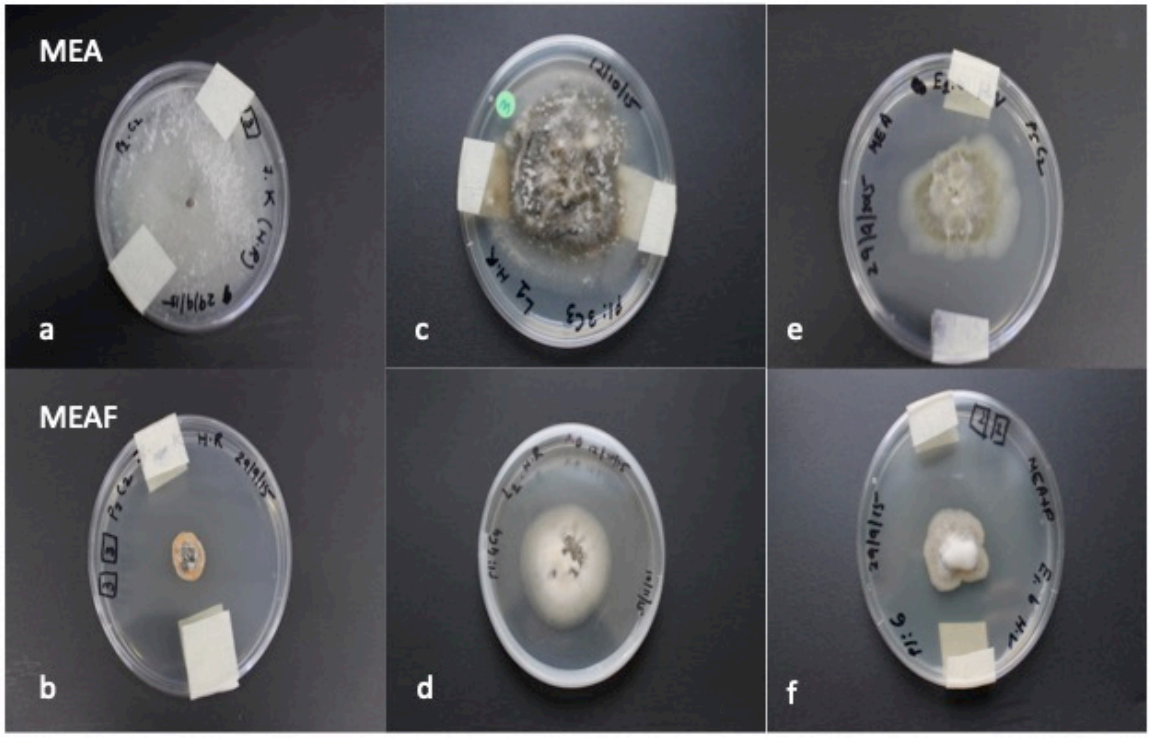


Figure 2.1 Morphotype variation observed in endophytes from Roosky from a common leaf region of a single leaflet isolated on two media (plate 1). Upper row shows healthy rachis (a and c) and healthy vein (e) sampled on MEA; lower row shows endophytes from the same leaf regions as above, healthy rachis, (b and d), and healthy vein (f) sampled on MEAF. The same single leaflets were used for MEA and MEAF. Plot information: K (a and b), L1 (c and d) and E1.b (e and f).

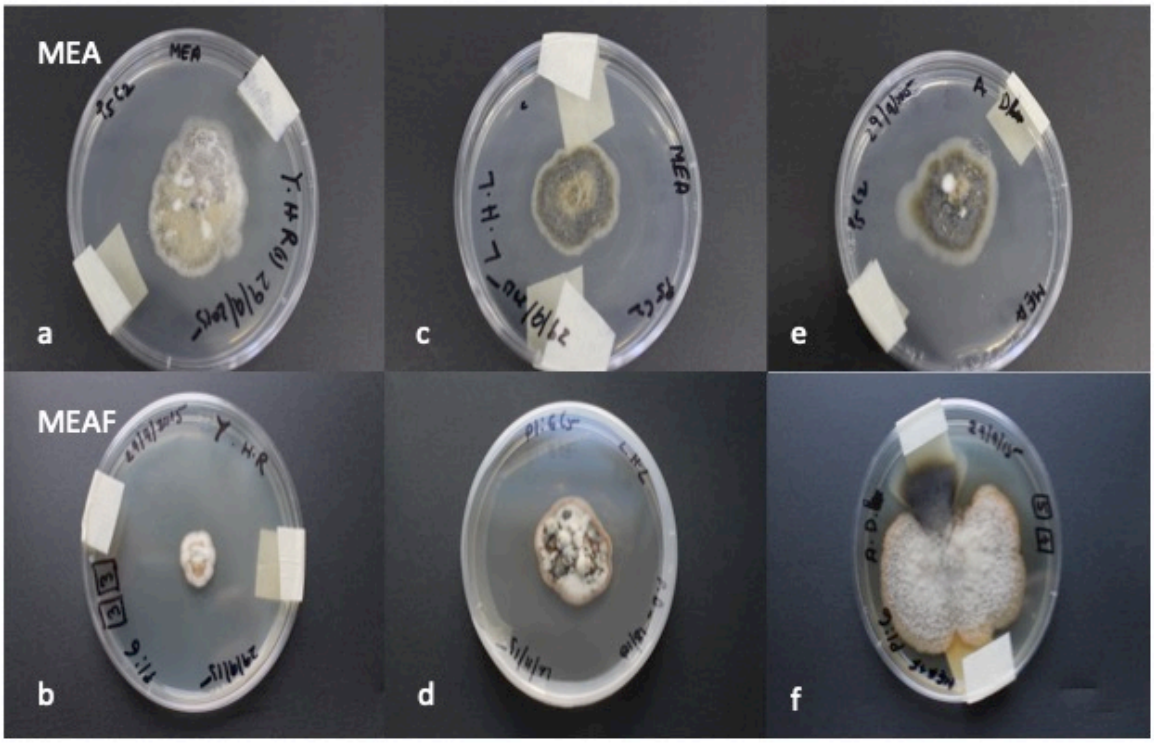


Figure 2.2 Morphotype variation observed in endophytes from Roosky from a common leaf region of a single leaflet isolated on two media (plate 2). Upper row shows endophytes from healthy rachis (a), healthy leafblade (c), and diseased leafblade (e) sampled on MEA; lower row shows endophytes from the same leaf regions as above, healthy rachis (b), and healthy leafblade (d), diseased leafblade sampled on MEAF (f). The same single leaflets were used for MEA and MEAF. Plot information: Y (a and b), L (c and d) and A (e and f).

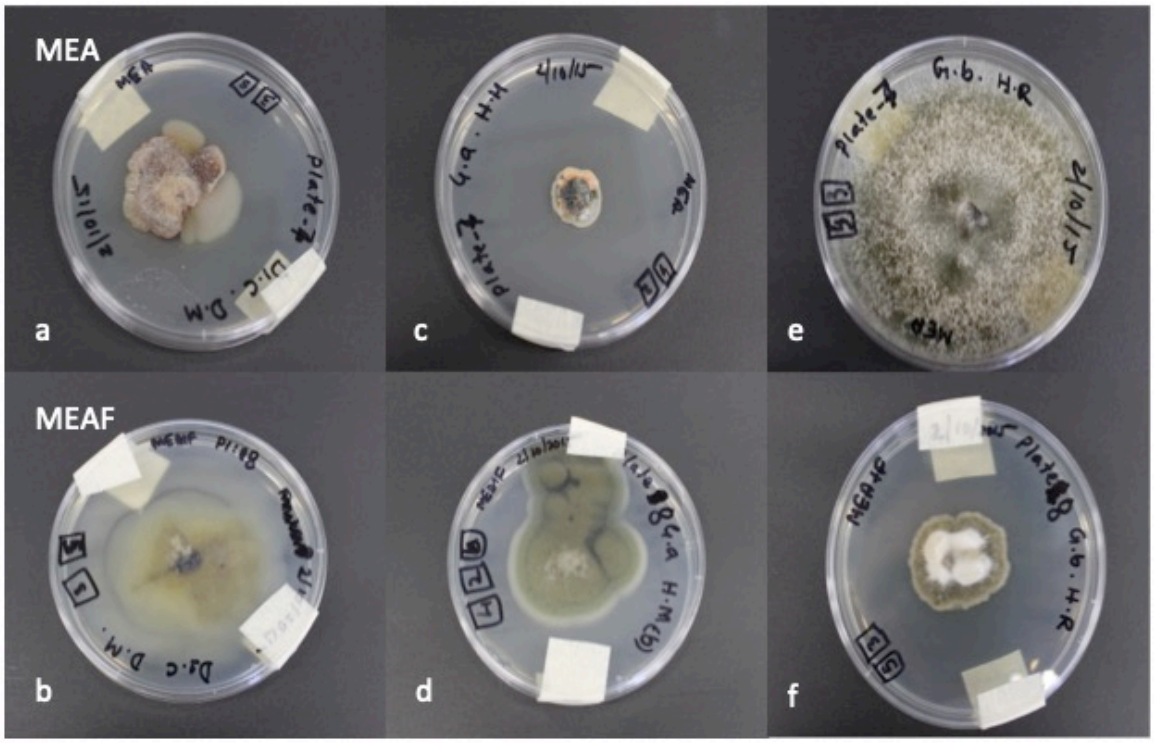


Figure 2.3 Morphotype variation observed in endophytes from Roosky from a common leaf region of a single leaflet isolated on two media (plate 3). Upper row shows endophytes from diseased midrib (a), healthy midrib (c), and healthy rachis (e) sampled on MEA; lower row shows endophytes from the same leaf regions as above, diseased midrib (b), and healthy midrib (d), healthy rachis sampled on MEAF (f). The same single leaflets were used for MEA and MEAF. Plot information: D1.c (a and b), G.a (c and d) and G.b (e and f).

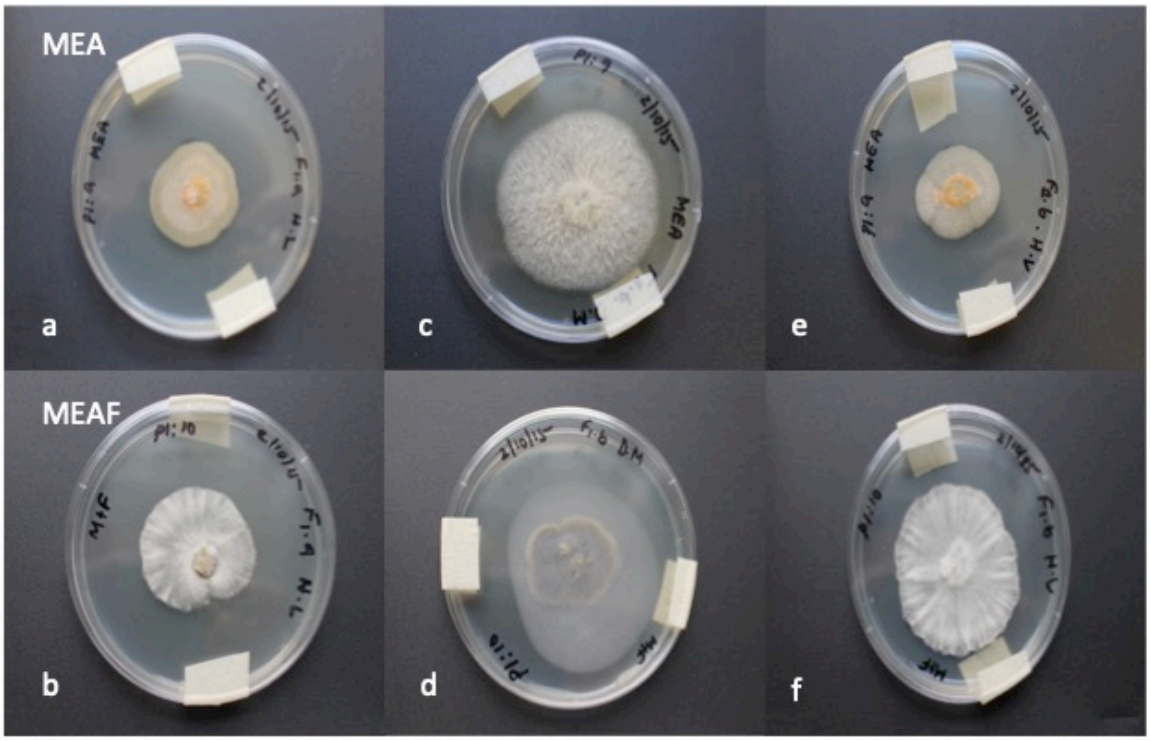


Figure 2.4 Morphotype variation observed in endophytes from Roosky from a common leaf region of a single leaflet isolated on two media (plate 4). Upper row shows endophytes from healthy leaf (a), diseased midrib (c), and healthy vein (e) sampled on MEA; lower row shows endophytes from the same leaf regions as above, healthy leaf (b), and diseased midrib (d), healthy vein.

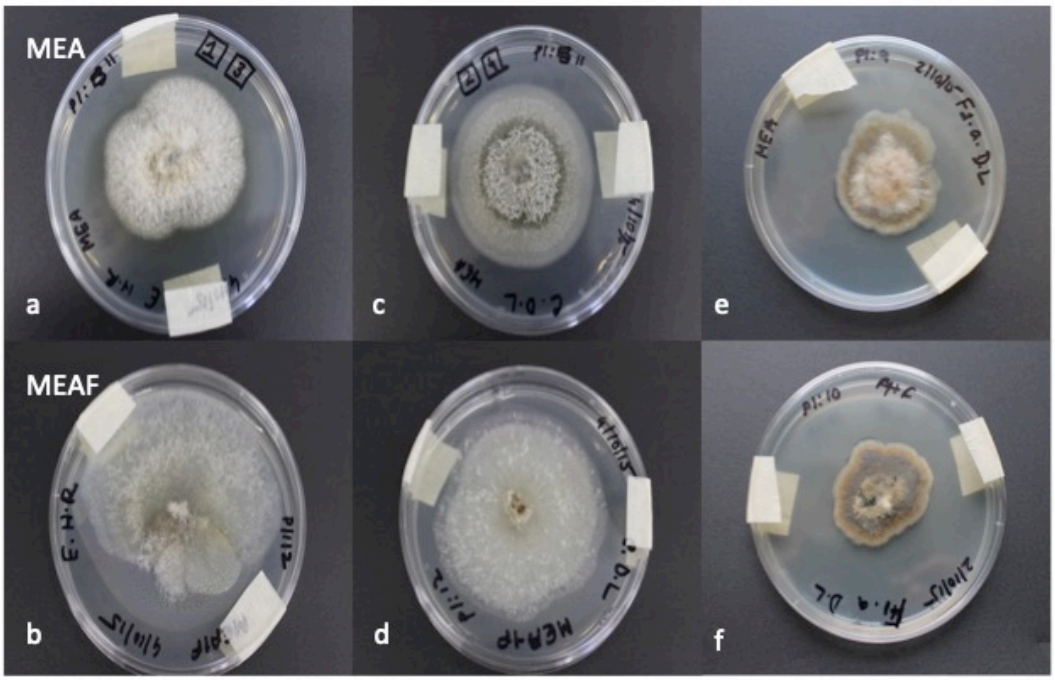


Figure 2.5 Morphotype variation observed in endophytes from Roosky from a common leaf region of a single leaflet isolated on two media (plate 5). Upper row shows endophytes from healthy rachis (a), diseased leafblade (c), and diseased leafblade (e) sampled on MEA; lower row shows endophytes from the same leaf regions as above, healthy leaf (b), and diseased midrib (d), healthy vein sampled on MEAF (f). The same single leaflets were used for MEA and MEAF. Plot information: E (a and b), C (c and d) and F1.a (e and f).

Appendix II – Chapter 3

Table 1 Detailed information for all OTUs from the culture dependent isolates obtained from three sites.

No	Plot name	Plant name	◆TT	Place	Provenance trials from	Media	Targeted region	°F/R /F+R	BLAST result	Family	Accession no.	IV	QC
1	H1	a	H.L	DK	Bregentved (DK)	ME	ITS 1F & 4	F	*Leptosphaerulina australis*	Pleosporacea e,	MH862995.1	87	98
2	H1	a	H.M	DK	Bregentved	ME	ITS whole	F+R	*Phoma*	Didymellacea e	JX160059	99	82
3	H1	a	D.M	DK	Bregentved	ME	ITS whole	F+R	*Phoma exigua var. exigua*	Didymellacea e	EU343168.1	96	83
4	H1	b	H.R	DK	Bregentved	ME	ITS whole	R	*Mycosphaerella coacervata*	Mycosphaerell aceae	EU167596.1	99	99
5	H1	b	D.M	DK	Bregentved	ME	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KT004579.1	87	94
6	K	o	H.L	FR	Athis	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerell aceae	EU167596.1	99	100
7	K	o	H.R	FR	Athis	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	99
8	K	o	D.L	FR	Athis	ME	ITS whole	F+R	*Septoria cucubali*	Mycosphaerell aceae	GU214698.1	90	95
9	A1	a	H.M	BE	Hoge Bos	ME	ITS whole	F	*Phoma sp*	Didymellacea e	JX160059.1	96	95
10	A1	a	D.L	BE	Hoge Bos	ME	ITS whole	F+R	*Mycosphaerella coacervata*	Mycosphaerell aceae	KP942874.1	98	97
11	A1	b	H.R	BE	Hoge Bos	ME	LSU	R	*Naganishia diffluens*	Filobasidiacea e	NG_058351. 1	91	97
12	A1	b	D.M	BE	Hoge Bos	ME	LSU	R	*Naganishia diffluens*	Filobasidiacea e	NG_058351. 1	85	62
13	H1	a	H.L	DK	Bregentved	MF	ITS	F	*Phoma sp.*	Didymellacea	JX160059.1	99	99

							whole			e			
14	H1	a	H.R	DK	Bregentved	MF	ITS whole	F	*Phoma sp.*	Didymellaceae	JX160059.1	99	98
15	H1	a	D.M	DK	Bregentved	MF	ITS 1F & 4	F	*Cladosporium*	Cladosporiaceae	KT826668.1	96	93
16	H1	b	H.M	DK	Bregentved	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
17	H1	b	D.M	DK	Bregentved	MF	ITS whole	F	*Phoma multirostrata*	Didymellaceae	EF585392.1	99	100
18	K	o	H.M	FR	Athis	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX826469.1	99	99
19	K	o	H.R	FR	Athis	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
20	K	o	D.L	FR	Athis	MF	ITS whole	F+R	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
21	A1	a	D.L	BE	Hoge Bos	MF	LSU	R	*Septoria cucubali*	Mycosphaerellaceae	GU214698.1	99	97
22	A1	b	H.L	BE	Hoge Bos	MF	ITS whole	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	99	100
23	A1	b	H.R	BE	Hoge Bos	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
24	A1	b	D.A	BE	Hoge Bos	MF	ITS whole	F+R	*Phoma sp.*	Didymellaceae	JX160059.1	99	99
25	L1	o	H.L	IT	Cadore	ME	ITS whole	F+R	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	98	96
26	L1	o	H.R	IT	Cadore	ME	ITS whole	F+R	*Boeremia exigua*	Didymellaceae	KT004579.1	100	99
27	L1	o	D.M	IT	Cadore	ME	ITS whole	F+R	*Epicoccum sp.*	Didymellaceae	AJ279452.1	85	99
28	S1	a	H.L	LT	Zeimelis	ME	ITS 1F &	F	*Botrytis cinerea*	Sclerotiniacea	MH329278.1	99	100

							4			e			
29	S1	a	H.R	LT	Zeimelis	ME	ITS whole	F+R	*Epicoccum nigrum*	Didymellaceae	MF509753.1	99	99
30	S1	a	D.M a	LT	Zeimelis	ME	ITS whole	F+R	*Boeremia exigua*	Didymellaceae	KX826469.1	99	100
31	S1	a	D.M b	LT	Zeimelis	ME	ITS whole	F+R	*Epicoccum sp.*	Didymellaceae	MF788189.1	95	91
32	T1	a	H.R	CZ	Rabstejn	ME	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171690.1	99	99
33	T1	a	D.M	CZ	Rabstejn	ME	ITS whole	F+R	*Boeremia exigua var. exigua*	Didymellaceae	MF435055	98	98
34	T1	b	H.M 1	CZ	Rabstejn	ME	ITS whole	F+R	*Boeremia exigua isolate*	Didymellaceae	KT004579.1	100	99
35	T1	b	H.M 2	CZ	Rabstejn	ME	ITS whole	F+R	*Phoma sp.*	Didymellacea e	JX160059.1	100	99
36	T1	b	D.L	CZ	Rabstejn	ME	ITS whole	F+R	*Boeremia exigua strain*	Didymellaceae	KX826469.1	100	99
37	L1	o	H.M	IT	Cadore	MF	ITS whole	F+R	*Aureobasidium pullulans isolate*	Dothioraceae,	KX067792.1	99	99
38	L1	o	H.R	IT	Cadore	MF	ITS whole	F+R	*Mycosphaerella coacervata*	Mycosphaerell aceae	EU167596.1	99	99
39	L1	o	D.M	IT	Cadore	MF	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	99	100
40	S1	a	H.M	LT	Zeimelis	MF	LSU	R	*Septoria cucubali*	Mycosphaerell aceae	GU214698.1	96	99
41	S1	a	H.R	LT	Zeimelis	MF	ITS whole	R	*Engyodontium album*	Cordycipitacea e	LN808868.1	97	97
42	S1	a	D.L	LT	Zeimelis	MF	ITS 1F & 4	F	*Mycosphaerella coacervata*	Mycosphaerell aceae	EU167596.1	98	100
43	T1	a	H.L	CZ	Rabstejn	MF	ITS	F+R	*Boeremia exigua*	Didymellaceae	KT004579.1	100	100

							whole						
44	T1	a	H.M	CZ	Rabstejn	MF	ITS whole	F+R	*Boeremia hedericola*	Didymellaceae	MK432727	100	97
45	T1	a	D.L	CZ	Rabstejn	MF	ITS whole	F+R	*Boeremia hedericola*	Didymellaceae	MK432727	99	98
46	T1	b	H.L	CZ	Rabstejn	MF	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171690.1	100	99
47	T1	b	H.R	CZ	Rabstejn	MF	ITS whole	F	*Phoma sp.*	Didymellaceae	AY514900.1	99	99
48	T1	b	D.M	CZ	Rabstejn	MF	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KX618484.1	100	100
49	E1	a	H.L	DE	Farchau	ME	ITS whole	F+R	*Boeremia hedericola*	Didymellaceae	MK432727	99	100
50	E1	a	H.R	DE	Farchau	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	100	100
51	E1	a	D.V 1	DE	Farchau	ME	ITS 1F & 4	F	*Uncultured Acremonium*	Hypocreaceae	HG936339.1	99	100
52	E1	b	H.V	DE	Farchau	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	100	99
53	E1	b	D.V	DE	Farchau	ME	LSU	F	*Boeremia trachelospermi*	Didymellaceae	KY064032.1	96	98
54	Y	o	H.R 1	FR	Saint Gatien	ME	ITS whole	F	*Boeremia exigua strain*	Didymellaceae	KX826470.1	100	99
55	Y	o	D.A	FR	Saint Gatien	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
56	Y	o	D.V	FR	Saint Gatien	ME	LSU	F	*Boeremia trachelospermi strain*	Didymellaceae	KY064032.1	97	99
57	L	o	H.L	FR	La Romagne	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX826470.1	100	99

58	L	o	H.R	FR	La Romagne	ME	ITS whole	F	*Kalmusia* sp.	Dothideomycetes	MG065740.1	99	100
59	L	o	D.L 2	FR	La Romagne	ME	ITS whole	F	*Xylaria sp.*	Xylariaceae	MF135147.1	100	99
60	A	o	H.L	IE	Enniskillen	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
61	A	o	D.L	IE	Enniskillen	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX826469.1	99	99
62	E1	a	H.L	DE	Farchau	MF	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KT004579.1	100	99
63	E1	a	H.R	DE	Farchau	MF	ITS whole	F	*Fusarium lateritium*		JQ693397.1	99	100
64	E1	a	D.L	DE	Farchau	MF	ITS whole	R	*Mycosphaerella sp.*	Mycosphaerellaceae	KY367494.2	99	98
65	E1	b	H.V	DE	Farchau	MF	ITS whole	F	*Phoma sp.*	Didymellaceae	AY514900.1	99	100
66	E1	b	D.L	DE	Farchau	MF	ITS 1F & 4	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	99	99
67	Y	o	H.R	FR	Saint Gatien	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	96
68	Y	o	D.V	FR	Saint Gatien	MF	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	99	100
69	L	o	H.L	FR	La Romagne	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
70	L	o	D.A	FR	La Romagne	MF	ITS whole	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	100	100
71	A	o	H.V	IE	Enniskillen	MF	ITS 1F & 4	F	*Sistotrema brinkmannii*	Hydnaceae,	KM232477.1	99	100
72	A	o	D.L 1	IE	Enniskillen	MF	ITS whole	F	*Epicoccum nigrum*	Didymellaceae	GU934519.1	100	99

73	D1	a	H.L	NL	Vaartbos Com.seed	ME	LSU	F	*Diaporthe eres*	Diaporthaceae	MF190081.1	91	92
74	D1	a	H.R	NL	Vaartbos Com.seed	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	KT004553.1	99	100
75	D1	a	D.M	NL	Vaartbos Com.seed	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	100
76	D1	b	D.L	NL	Vaartbos Com.seed	ME	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171690.1	100	100
77	D1	c	H.L	NL	Vaartbos Com.seed	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1		
78	D1	c	H.R	NL	Vaartbos Com.seed	ME	ITS whole	F	*Dictyochaeta siamensis*	Chaetosphaeriaceae	KX609952.1	99	99
79	D1	c	D.Ma	NL	Vaartbos Com.seed	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	KT004553.1	99	99
80	D1	c	D.Mb	NL	Vaartbos Com.seed	ME	ITS whole	F	*Phoma sp.*	Didymellaceae	JX160059.1	99	99
81	G	a	H.L	GB	Loch Tay	ME	ITS whole	F	*Bjerkandera adusta*	Meruliaceae	MF120203.1	100	99
82	G	a	H.M	GB	Loch Tay	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
83	G	a	D.A	GB	Loch Tay	ME	ITS whole	F	*Epicoccum sp*	Didymellaceae	MF788189.1	100	99
84	G	b	H.L	GB	Loch Tay	ME	ITS 1F & 4	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	98	100
85	G	b	H.R	GB	Loch Tay	ME	LSU/ITS 1F& 4	F+R	*Not worked*				
86	G	b	D.M	GB	Loch Tay	ME	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KT004579.1	100	99
87	D1	a	H.L	NL	Vaartbos Com.seed	MF	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	99	99

88	D1	a	H.M	NL	Vaartbos Com.seed	MF	ITS whole	F	*Boeremia exigua strain*	Didymellaceae	KY949620.1	99	100
89	D1	a	D.L	NL	Vaartbos Com.seed	MF	LSU/ITS 1F& 4	F	*Not worked*				
90	D1	b	D.L	NL	Vaartbos Com.seed	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	98
91	D1	c	H.L	NL	Vaartbos Com.seed	MF	ITS whole/1F &4	F+R	*Not worked*				
92	D1	c	H.R	NL	Vaartbos Com.seed	MF	ITS whole	F	*Fimetariella rabenhorstii*	Lasiosphaeriaceae	KP050669.1	99	88
93	D1	c	D.M	NL	Vaartbos Com.seed	MF	ITS whole	F	*Boeremia exigua strain*	Didymellaceae	KX826469.1	100	92
94	G	a	H.M a	GB	Loch Tay	MF	ITS whole	F	*Cladosporium herbarum*	Cladosporiaceae	MH047193.1	99	99
95	G	a	H.M b	GB	Loch Tay	MF	ITS whole	R	*Cladosporium sp.*	Cladosporiaceae	KT826671.1	97	97
96	G	a	D.A	GB	Loch Tay	MF	ITS whole	F	*Epicoccum cf. nigrum*	Didymellaceae	JQ676202.1	99	97
97	G	b	H.L	GB	Loch Tay	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	86
98	G	b	H.R	GB	Loch Tay	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	97
99	G	b	D.M	GB	Loch Tay	MF	ITS 1F & 4	F	*Xylaria sp.*	Xylariaceae	MF135147.1	89	86
100	J1	o	H.L	IT	Abeton	ME	ITS whole/1F &4	F	*Not worked*				
101	J1	o	H.R	IT	Abeton	ME	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KX618484.1	99	96

102	J1	o	D.L	IT	Abeton	ME	ITS whole	R	*Vishniacozyma heimaeyensis*	Bulleribasidiaceae	KY105824.1	99	100
103	F1	b	H.V	DE	Karlsruhe	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	AF310980.1	99	95
104	F1	b	D.M	DE	Karlsruhe	ME	ITS whole	F	*Phaeosphaeria pontiformis*	Phaeosphaeriaceae,	LC171716.1	99	93
105	F1	a	H.L	DE	Karlsruhe	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	AF310980.1	97	90
106	F1	a	H.R	DE	Karlsruhe	ME	ITS whole	F	*Diaporthe rudis*	Diaporthacea e	KR909216.1	99	95
107	F1	a	D.L	DE	Karlsruhe	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KY419536.1	99	95
108	G1	b	H.R .b	DK	Ravenholt	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	97	94
109	G1	b	D.R .1	DK	Ravenholt	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	AF310980.1	99	93
110	G1	a	H.L	DK	Ravenholt	ME	ITS whole	R	*Cladosporium cf. herbarum*	Cladosporiaceae	MH399503.1	82	95
111	G1	a	H.R	DK	Ravenholt	ME	ITS whole	F	*Diaporthe passiflorae*	Diaporthacea e	KR534744.1	98	93
112	G1	a	D.L	DK	Ravenholt	MEA	ITS whole	R	*Boeremia exigua var. exigua*	Didymellaceae	KP174710.1	88	98
113	J1	o	H.L	IT	Abeton	MF	ITS whole	F	*Diaporthe viticola*	Diaporthacea e	FJ228188.1	99	98
114	J1	o	H.R	IT	Abeton	MF	ITS whole	F	*Boeremia exigua*	Diaporthacea e	KX618484.1	98	98
115	J1	o	D.L	IT	Abeton	MF	ITS whole	F+R	*Boeremia hedericola*	Didymellacea e	MK432727	100	97
116	F1	b	H.V	DE	Karlsruhe	MF	ITS whole	F	*Xylaria*	Xylariaceae	MF135147.1	100	97

117	F1	b	D.M	DE	Karlsruhe	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX826470.1	99	95
118	F1	a	H.L	DE	Karlsruhe	MF	ITS whole	F	*Xylaria sp.*	Xylariaceae	MF135147.1	98	98
119	F1	a	H.R.1	DE	Karlsruhe	MF	ITS whole	F	*Diaporthe passiflorae*	Diaporthaceae	KC143196.1	92	80
120	F1	a	H.R.2	DE	Karlsruhe	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	LC171667.1	89	95
121	F1	a	D.L	DE	Karlsruhe	MF	ITS whole	F	*Phoma multirostrata*	Didymellaceae	EF585391.1	99	84
122	G1	b	H.R	DK	Ravenholt	MF	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171702.1	99	93
123	G1	b	D.M	DK	Ravenholt	MF	ITS whole	F	*Phoma exigua*	Didymellaceae	FJ228178.1	90	92
124	G1	a	H.L	DK	Ravenholt	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX826470.1	99	94
125	G1	a	H.R	DK	Ravenholt	MF	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171702.1	99	96
126	G1	a	D.L	DK	Ravenholt	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	100	98
127	E	o	H.L	GB	Settrington	ME	ITS whole	F	*Vagicola dactylidis*	Phaeosphaeriaceae,	NR_154507.1	98	90
128	E	o	H.R	GB	Settrington	ME	ITS whole	F	*Mycosphaerella sp.*	Mycosphaerellaceae	KF128849.1	95	99
129	E	o	D.La	GB	Settrington	ME	LSU	R	*Naganishia diffluens*	Filobasidiaceae	NG_058351.1	96	98
130	C	o	H.M	IE	Currachase	ME	ITS whole	F	*Diaporthe passiflorae*	Diaporthaceae	KC143196.1	100	96
131	C	o	D.L	IE	Currachase	ME	ITS whole	F	*Boeremia exigua isolate*	Didymellaceae	KY419536.1	99	99

132	Q1	o	H.L	PO	Wloszczowa	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KY949620.1	99	99
133	Q1	o	H.R.a	PO	Wloszczowa	ME	ITS 1F & 4	F	*Sarocladium strictum*	Sarocladiaceae	KU214512.1	95	97
134	Q1	o	H.R.b	PO	Wloszczowa	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	100	99
135	Q1	o	D.A	PO	Wloszczowa	ME	ITS whole	F	*Diaporthe passiflorae*	Diaporthaceae	NR_120155.1	98	100
136	A1	c	H.M	BE	Hoge Bos	ME	ITS whole	F	*Ophiosphaerella korrae*	Phaeosphaeriaceae,	KP690985.1	99	99
137	A1	c	D.A	BE	Hoge Bos	ME	ITS whole	F	*Ophiosphaerella korrae*	Phaeosphaeriaceae,	KP690985.1	99	100
138	E	o	H.L.a.1	GB	Settrington	MF	ITS whole/1F &4	F+R *	*Not worked*		KX872911.1		
139	E	o	H.R	GB	Settrington	MF	ITS whole	R	*Diaporthe sp.*	Diaporthaceae	JX624276.1	99	98
140	E	o	D.A	GB	Settrington	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	99
141	E	o	D.M.1	GB	Settrington	MF	LSU	F	*Epicoccum nigrum*	Didymellaceae	KT207732.1	99	99
142	C	o	H.M	IE	Currachase	MF	ITS whole	R	*Mycosphaerella coacervata*		KX096658.1	99	100
143	C	o	H.R.1	IE	Currachase	MF	ITS whole	R	*Phoma sp.*	Didymellaceae	MG098328.1	99	100
144	C	o	H.R.2	IE	Currachase	MF	ITS whole	R	*Diaporthe sp.*	Diaporthaceae	KU712217.1	99	100
145	C	o	D.L	IE	Currachase	MF	ITS whole	F	*Diaporthe viticola*	Diaporthaceae	KC145833.1	99	97
14	A1	c	H.M	BE	Hoge Bos	MF	ITS 1F &	F	*Phlebia rufa*	Meruliaceae,	LN611092.1	98	100

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147	A1	c	D.M	BE	Hoge Bos	MF	ITS 1F & 4	F	*Epicoccum nigrum*	Didymellaceae	LC168776.1	98	99
148	B	o	H.L	IE	Donadea	MF	LSU	F	*Cladosporium*	Cladosporiaceae	MH047202.1	99	100
149	B	o	H.R	IE	Donadea	MF	LSU	R	*Boeremia exigua*	Didymellaceae	MG816487.1	81	99
150	B	o	D.L	IE	Donadea	MF	ITS whole	F+R	*Epicoccum sp.*	Didymellaceae	MK460786	99	98
151	M1	o	H.L	IT	Monte Lessini	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	83
152	M1	o	H.R	IT	Monte Lessini	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	97
153	M1	o	D.L	IT	Monte Lessini	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	93
154	H	o	H.L	FR	Val Saint Pierre	ME	LSU	F	*Boeremia trachelospermi*	Didymellaceae	KY064032.1	92	98
155	H	o	H.L	FR	Val Saint Pierre	ME	ITS 1F & 4	F	*Leptosphaerulina trifolii*	Pleosporaceae	MF169500.1	86	58
156	H	o	D.M	FR	Val Saint Pierre	ME	ITS whole	F	*Boeremia strasseri*	Didymellaceae	MF113458.1	88	99
157	R1	b	H.L	LT	Kalsiadorys	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	100	100
158	R1	b	H.A	LT	Kalsiadorys	ME	LSU	R	*Diaporthe passiflorae*	Diaporthaceae	NG_042673.1	99	100
159	R1	b	D.A	LT	Kalsiadorys	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	100	100
160	R1	a	H.M	LT	Kalsiadorys	ME	ITS 1F & 4	F	*Ustilaginoidea virens*	Hypocreales	JF271122.1	100	99
16	R1	a	D.M	LT	Kalsiadorys	ME	ITS 1F &	F	*Boeremia exigua*	Didymellacea	KX618484.1	99	99

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162	B	o	H.L	IE	Donadea	ME	ITS 1F & 4	F	*Cladosporium cladosporioides*	Cladosporiaceae	MF475944.1	89	94
163	B	o	H.L.ii	IE	Donadea	ME	ITS 1F & 4	F	*Engyodontium album*	Cordycipitaceae	LT549076.1	99	100
164	B	o	H.R	IE	Donadea	ME	LSU	F	*Septoria cucubali*	Mycosphaerellaceae	GU214698.1	100	100
165	B	o	D.M	IE	Donadea	ME	LSU	F	*Phaeosphaeria gahniae*	Phaeosphaeriaceae	MG386127.1	96	100
166	T1	c	H.L	CZ	Rabstejn	MF	ITS 1F & 4	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
167	T1	c	H.R	CZ	Rabstejn	MF	LSU/ITS 1F& 4	F+R	*Not worked*				
168	T1	c	H.M	CZ	Rabstejn	MF	LSU	R	*Neoceratosperma eucalypti*	Mycosphaerellaceae	NG_058084.1	84	60
169	T1	c	D.L	CZ	Rabstejn	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	99
170	M1	o	H.M	IT	Monte Lessini	MF	LSU	F	*Boeremia trachelospermi*	Didymellaceae	KY064032.1	99	99
171	M1	o	D.M	IT	Monte Lessini	MF	LSU	R	*Phoma exigua var. exigua*	Didymellaceae	EU343386.1	99	99
172	H	o	H.L	FR	Val Saint Pierre	MF	LSU	R	*Naganishia diffluens*	Filobasidiaceae,	NG_058351.1	99	98
173	H	o	H.R	FR	Val Saint Pierre	MF	LSU	F	*Boeremia trachelospermi*	Didymellaceae	KY064032.1	92	100
174	H	o	D.M	FR	Val Saint Pierre	MF	LSU/ITS 1F& 4	F+R	*Not worked*				
175	H	o	D.L	FR	Val Saint Pierre	MF	LSU	R	*Boeremia exigua*	Didymellaceae	MG816487.1	87	92
17	R1	b	H.M	LT	Kalsiadorys	MF	ITS 1F &	F	*Boeremia sp*	Didymellaceae	MH931265.1	99	99

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177	R1	b	D.M	LT	Kalsiadorys	MF	ITS 1F & 4	F	*Boeremia exigua*	Didymellaceae	KX618484.1	99	100
178	R1	a	H.L	LT	Kalsiadorys	MF	LSU	R	*Septoria convolvuli*	Mycosphaerellaceae	MF540356.1	91	40
179	R1	a	H.R	LT	Kalsiadorys	MF	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	99	100
180	R1	a	D.L	LT	Kalsiadorys	MF	ITS 1F & 4	F	*Uncultured Coprinopsis*	Psathyrellaceae,	JX135081.1	94	100
181	T1	c	H.L	CZ	Rabstejn	ME	ITS 1F & 4	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	96	100
182	T1	c	H.R.a	CZ	Rabstejn	ME	ITS 1F & 4	F	*Uncultured Acremonium*	Hypocreales	HG936339.1	99	100
183	T1	c	D.M	CZ	Rabstejn	ME	ITS 1F & 4	F	*Penicillium sp.*	Aspergillaceae	MH931281.1	97	100
184	O1	o	H.M	PO	Szczecinek	ME	ITS whole	F	*Ascomycota sp.*	Division	LT821522.1	93	98
185	O1	o	D.L	PO	Szczecinek	ME	ITS 1F & 4	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	99	100
186	M	o	H.L	GB	Wytham Wood	ME	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	100	100
187	M	o	H.R	GB	Wytham Wood	ME	ITS whole	F	*Fusarium oxysporum*	Nectriaceae	EU520062.1	99	99
188	M	o	D.L1	GB	Wytham Wood	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	AF310980.1	100	100
189	J	o	H.Ma	FR	Saint Martin	ME	ITS whole	R	*Paraconiothyrium sp.*	Didymosphaeriaceae	KY367491.2	99	99
190	J	o	D.M	FR	Saint Martin	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	100	99
19	U1	o	H.L	PO	Mircze	ME	LSU	F+R	*Septoria cucubali*	Mycosphaerell	GU214698	98	100

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192	U1	o	H.R.a	PO	Mircze	ME	LSU/ITS 1F& 4	F+R*	*Not worked*				
193	U1	o	H.R.b	PO	Mircze	ME	Tef	R	*Fusarium proliferatum*	Nectriaceae	MH087479.1	84	53
194	U1	o	D.L	PO	Mircze	ME	Tef	R	*Alternaria humuli*	Pleosporaceae	JQ672434.1	98	85
195	O1	o	H.L	PO	Szczecinek	MF	ITS 1F & 4	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
196	O1	o	H.R	PO	Szczecinek	MF	Tef	R	*Gibberella sp*	Nectriaceae	EU552097.1	98	100
197	O1	o	D.A	PO	Szczecinek	MF	LSU/ITS 1F& 4		*Not worked*				
198	M	o	H.M	GB	Wytham Wood	MF	LSU/ITS 1F& 4	F+R	*Not worked*				
199	M	o	D.M	GB	Wytham Wood	MF	ITS whole	F	*Boeremia strasseri*	Didymellaceae	MF113474.1	98	98
200	J	o	H.L	FR	Saint Martin	MF	ITS whole	R	*Engyodontium album*	Cordycipitaceae	LN808868.1	99	96
201	J	o	H.R	FR	Saint Martin	MF	ITS whole	F	*Engyodontium album*	Cordycipitaceae	LT549076.1	96	98
202	J	o	D.A	FR	Saint Martin	MF	LSU/ITS 1F& 4	F+R	*Not worked*				
203	U1	o	H.M	PO	Mircze	MF	ITS 1F & 4	F	*Penicillium griseoroseum*	Aspergillaceae	KY218671.1	99	100
204	U1	o	D.M.i	PO	Mircze	MF	LSU	R	*Boeremia exigua*	Didymellaceae	MG816487.1	79	63
205	U1	o	D.M.ii	PO	Mircze	MF	LSU	F	*Boeremia trachelospermi*	Didymellaceae	KY064032.1	95	100
20	K1	b	H.L	IT	Valle Pesio	MF	LSU	F	*Sarocladium*	Hypocreales	KM249100.1	90	69

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207	K1	b	H.R	IT	Valle Pesio	MF	ITS whole	F	*Boeremia exigua*	Didymellaceae	KX618484.1	100	99
208	K1	b	D.M	IT	Valle Pesio	MF	ITS 1F & 4	F+R	*Not worked*				
209	K1	b	D.L	IT	Valle Pesio	MF	ITS whole	F	*Epicoccum nigrum*	Didymellaceae	MF509753.1	99	99
210	Ireland	o	Margin	IE		ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	100	100
211	Ireland	o	H.R	IE		ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	93	99
212	Ireland	o	D.R	IE		ME	ITS whole	F	*Fusarium avenaceum*	Nectriaceae	KP170730.1	100	100
213	Ireland	o	D.M	IE		ME	ITS whole	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	100	100
214	K1	b	H.L	IT	Valle Pesio	ME	ITS whole	F	*Boeremia exigua*	Didymellaceae	KY949620.1	99	100
215	K1	b	D.R	IT	Valle Pesio	ME	ITS 1F & 4	F	*Fusarium tricinctum*	Nectriaceae	MH931273.1	100	100
216	H+	o	Ascospores	FR	Val Saint Pierre	ME	ITS whole	F	*Fusarium sp.*	Nectriaceae	KY949613.1	99	99
217	M1	o	H.M	IT	Monte Lessini	MF	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
218	A1	c	D.R	BE	Hoge Bos	MF	ITS whole	F	*Mycosphaerella sp.*	Mycosphaerellaceae	KF128849.1	100	100
219	Q1	o	D.L.a	PO	Wloszczowa	MF	ITS whole/1F &4	F	*Not worked*				

220	Q1	o	D.L.b	PO	Wloszczowa	MF	ITS whole	F+R	*Boeremia hedericola*	Didymellaceae	MK432727	100	97
221	G1	b	D.R	DK	Ravenholt	MF	ITS whole	F	*Aureobasidium sp.*	Dothioraceae	KX611011.1	99	99
222	Ireland	o	H.M	IE		MF	ITS whole	F	*Pyronema domesticum*	Pyronemataceae	NG_027655.1	99	99
223	Ireland	o	H.R.a	IE		MF	ITS whole	F	*Cadophora sp*	Helotiales	JN941367.1	100	97
224	Ireland	o	H.R.b	IE		MF	ITS whole	R	*Boeremia exigua*	Didymellaceae	MG872327.1	98	100
225	Ireland	o	D.H.R	IE		MF	ITS whole	R	*Phoma sp.*	Didymellaceae	MG098328.1	99	100
226	B	o	H.A	IE	Donadea	ME	ITS whole	R	*Xylaria*	Xylariaceae	MF135147.1	99	100
227	H1	a	H.R.a	DK	Bregentved	ME	ITS whole	R	*Phaeosphaeria gahniae*	Phaeosphaeriaceae	NR_156675.1	93	86
228	D1	a	D.L	NL	Vaartbos Com.seed	ME	ITS whole	R	*Boeremia exigua*	Didymellaceae	LC171690.1	99	99
229	T1	a	D.R	CZ	Rabstejn	ME	ITS whole	F	*Alternaria sp.*	Pleosporaceae	MG065787.1	98	99
230	G	a	D.R	GB	Loch Tay	ME	ITS whole	F	*Phoma sp.*	Didymellaceae	JX160059.1	100	100
231	H1	a	H.R.b	DK	Bregentved	ME	ITS whole	F	*Fusarium sp.*	Nectriaceae	KU712220.1	100	99
232	K1	b	H.R	IT	Valle Pesio	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	99
233	G1	1.1.1.1	H.A	NGB	XX.011046	ME	ITS whole	F	*Diaporthe cotoneastri*	Diaporthaceae	KC843328.1	98	99

234	G1	1.2 1	H.M	NGB	XX.011046	ME	ITS whole	F	*Collembolispora aristata*	Mitosporic Helotiales	NG_042760.1	99	99
235	G1	1.3 1	H.R	NGB	XX.011046	ME	ITS whole	R	*Cladosporium sp.*	Cladosporiaceae	KY436103.1	94	99
236	G2	2	H.L	NGB	2003.0735	ME	ITS whole	R	*Mycosphaerella coacervata*	Mycosphaerellaceae	KX096658.1	99	100
237	G2	2	H.R	NGB	2003.0735	ME	ITS whole	R	*Fusarium lateritium*	Nectriaceae	LC171689.1	99	99
238	G2	2	H.A	NGB	2003.0735	ME	ITS whole	R	*Aureobasidium sp.*	Dothioraceae	KY436129.1	99	100
239	G3	3	H.M	NGB	1909.011054	ME	ITS whole	R	*Diaporthe cotoneastri*	Diaporthaceae	KY977583.1	99	99
240	G3	3	D.M	NGB	1909.011054	ME	ITS whole	R	*Fusarium lateritium*	Nectriaceae	LC171689.1	99	99
241	G4	4	H.M	NGB	1934.011053	ME	ITS whole	F	*Engyodontium album*	Cordycipitaceae	LT549076.1	100	100
242	G4	4	H.R	NGB	1934.011053	ME	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	84	94
243	G5	5	H.R	NGB	1932.011031	ME	ITS 1F & 4	F	*Cystobasidium slooffiae*	Cystobasidiaceae,	AF444589.1	99	100
244	G5	5	D.M	NGB	1932.011031	ME	ITS 1F & 4	F	*Cladosporium sp*	Cladosporiaceae	MF475925.1	84	93
245	G6	6	H.L	NGB	2001.1739	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
246	G6	6	H.R	NGB	2001.1739	ME	ITS whole	F	*Exophiala oligosperma*	Herpotrichiellaceae,	KY034051.1	94	98
247	G6	6	H.A	NGB	2001.1739	ME	ITS whole	F	*Meyerozyma guilliermondii*	Debaryomycetaceae	MG846137.1	94	99
248	G7	7	H.M	NGB	1954.011044	ME	ITS 1F & 4	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	100	100

249	G7	7	H.R	NGB	1954.011044	ME	ITS whole/1F &4	F+R	*Not worked*				
250	G7	1.4 7	H.A	NGB	1954.011044	ME	ITS 1F & 4	F	*Fusarium culmorum*	Nectriaceae	MH864973.1	99	100
251	G8	8	H.L	NGB	XX.011028	ME	ITS 1F & 4	F	*Vuilleminia coryli*	Corticiaceae,	NR_160186.1	86	100
252	G8	8	H.R	NGB	XX.011028	ME	ITS 1F & 4	F	*Lecanicillium muscarium*	Cordycipitaceae	DQ007050.1	92	98
253	G8	8	H.A	NGB	XX.011028	ME	ITS 1F & 4	F	*Ustilago filiformis*	Ustilaginaceae	MH855347.1	82	98
254	G9	9	H.M	NGB	2000.3637A	ME	LSU	F	*Fusarium avenaceum*	Nectriaceae	MG274300.1	95	100
255	G9	9	D.L	NGB	2000.3637A	ME	LSU	R	*Gloniopsis calami*	Hysteriaceae	NG_059715.1	90	40
256	G10	1.5 10	H.L	NGB	1977.0039	ME	ITS 1F & 4	F	*Meyerozyma guilliermondii*	Debaryomycetaceae	MG846137.1	99	100
257	G10	10	H.R	NGB	1977.0039	ME	ITS whole	R	*Fusarium lateritium*	Nectriaceae	MH424128.1	88	92
258	G10	1.6 10	H.A	NGB	1977.0039	ME	ITS whole/1F &4	F	*Not worked*				
259	G10	10	D.M	NGB	1977.0039	ME	ITS 1F & 4	F	*Fusarium oxysporum f. sp. radicislycopersici*	Nectriaceae	MH865886.1	96	97
260	G11	11	H.L	NGB	2005.0652	ME	ITS whole	F	*Cladosporium floccosum*	Cladosporiaceae	MF472979.1	99	99
261	G11	11	H.R	NGB	2005.0652	ME	ITS whole	F	*Aureobasidium pullulans*	Dothioraceae	KY294714.1	99	100

262	G12	12	H.R	NGB	XX.011040	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	99	100
263	G12	12	H.A	NGB	XX.011040	ME	ITS whole	R	*Aspergillus versicolor*	Aspergillaceae	MF476081.1	95	98
264	G13	1.7 13	H.R	NGB	XX.011038	ME	ITS whole	R	*Diaporthe rudis*	Diaporthaceae	KT323191.1	99	98
265	G13	13	H.A	NGB	XX.011038	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	99	100
266	G14	14	H.R	NGB	XX.011029	ME	ITS whole	F	*Fusarium lateritium*	Nectriaceae	JQ693397.1	99	100
267	G14	14	H.A	NGB	XX.011029	ME	ITS whole	R	*Fusarium lateritium*	Nectriaceae	LC171689.1	96	100
268	G14	14	D.M	NGB	XX.011029	ME	ITS whole	R	*Eutypa spinosa*	Diatrypaceae	EF155486.1	99	100
269	G15	1.7.1.1.	D.L	NGB	2013.0214	ME	ITS whole	F	*Phoma sp.*	Incertae sedis	JX160059.1	100	99
270	G15	15	H.L	NGB	2013.0214	ME	ITS 1F & 4	F	*Diaporthe eres*	Diaporthaceae	MH003631.1	92	95
271	G15	15	H.R	NGB	2013.0214	ME	ITS 1F & 4	F	*Ustilago filiformis*	Ustilaginaceae,	MH855350.1	83	93
272	G15	15	H.A	NGB	2013.0214	ME	ITS whole	R	*Phlyctema vagabunda*	Dermateaceae	KT923788.1	96	100
273	G16	1.8 16	H.L	NGB	1969.011033	ME	ITS 1F & 4	F	*Not worked*				
274	G16	16	H.R	NGB	1969.011033	ME	ITS 1F & 4	F	*Cladosporium allicinum*	Cladosporiaceae	MH857286.1	99	99
275	G17	1.9 1	H.M	NGB	XX.011034	ME	ITS 1F & 4	F	*Fusarium oxysporum*	Nectriaceae	MH575293.1	96	98

		7											
276	G17	17	H.R	NGB	XX.011034	ME	ITS 1F & 4	F	*Limonomyces sp*	Corticiaceae	MH347299.1	90	86
277	G18	1.10 18	H.L	NGB	1904.011032	ME	ITS 1F & 4	F	*Uncultured Acremonium*	Hypocreales	HG936339.1	99	100
278	G18	18	H.R	NGB	1904.011032	ME	ITS 1F & 4	F	*Septoria protearum*	Mycosphaerellaceae	MH863068.1	89	96
279	G19	19	H.L	NGB	XX.011074	ME	ITS 1F & 4	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	92	99
280	G19	19	H.R	NGB	XX.011074	ME	ITS whole	R	*Fusarium sp.*	Nectriaceae	KU712220.1	79	86
281	G19	19	H.A	NGB	XX.011074	ME	TEF	R	*Lecanicillium attenuatum*	Cordycipitaceae	EF468782.1	97	95
282	G20	20	H.L	NGB	2006.1641	ME	ITSwhole / 1F&4	F+R	*Not worked*				
283	G20	20	H.R	NGB	2006.1641	ME	ITS whole	F	*Diaporthe rudis*	Diaporthaceae	KR909216.1	99	99
284	G20	20	H.A	NGB	2006.1641	ME	ITS 1F & 4	F	*Limonomyces roseipellis*	Corticiaceae	EU622846.1	88	96
285	G21	21	H.L	NGB	Mark 78	ME	ITS 1F & 4	F	*Septoria lepidiicola*	Mycosphaerellaceae	MH865062.1		
286	G21	21	H.R	NGB	Mark 78	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	90	100
287	G21	21	H.A	NGB	Mark 78	ME	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	99	100
288	G22	22	H.R	NGB	XX.011048	ME	ITS 1F & 4	F	*Cladosporium kenpeggii*	Cladosporiaceae	KY646222.1	94	100
289	G22	22	H.A	NGB	XX.011048	ME	ITS 1F & 4	F	*Uncultured Acremonium*	Hypocreales	HG936339.1	99	100

290	G23	1.11 23	H.R	NGB	1889.01103	ME	ITS whole	F	*Colletotrichum godetiae*	Glomerellaceae	LT717068.1	100	99
291	G23	23	H.A	NGB	1889.01103	ME	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MG548567.1	84	91
292	3A	24		NGB	1969.011033	ME	ITS whole/1F &4	F+R	*Not worked*				
293	3C	24		NGB	1969.011033	ME	ITS whole/1F &4	F+R	*Not worked*				
294	2D	25		NGB	XX.011074	ME	ITS whole	F	*Diaporthe viticola*	Diaporthaceae	KC145833.1	100	100
295	2B	25		NGB	XX.011074	ME	ITS whole	F	*Fusarium proliferatum*	Nectriaceae	MH055399.1	99	100
296	2C	25		NGB	XX.011074	ME	ITS whole	F	*Exophiala oligosperma*	Herpotrichiellaceae	KY034051.1	99	100
297	4A	1.12 26		NGB	1904.011032	ME	ITS whole	F	*Diaporthe viticola*	Diaporthaceae	KC145833.1	99	100
298	2VE	25		NGB	XX.011074	ME	ITS whole	F	*Fusarium proliferatum*	Nectriaceae	MH055399.1	100	100
299	4VB	26		NGB	1904.011032	ME	ITS whole	F	*Penicillium expansum*	Trichocomaceae	KX243327.1	99	100
300	4VC	26		NGB	1904.011032	ME	ITS whole	F	*Engyodontium album*	Cordycipitaceae	LT549076.1	99	100
301	3EE	24		NGB	1969.011033	ME	ITS whole	F	*Boeremia exigua var. exigua*	Didymellaceae	KX618486.1	100	100
302	1VA	27		NGB	XX.011048	ME	ITS 1F & 4	F	*Pyronema domesticum*	Pyronemataceae	HQ115722.1	99	99

303	3VB	24		NGB	1969.011033	ME	ITS 1F & 4	F	*Cladosporium sp.*	Cladosporiaceae	MK111508.1	99	100
304	3ED	24		NGB	1969.011033	ME	LSU	F	*Phomopsis*	Diaporthaceae	AF439633.1	95	98
305	5ED	28		NGB	1889.01103	ME	ITSwhole /1F&4	F	*Not worked*				
306	4EE	26		NGB	1904.011032	ME	ITS whole	F	*Phoma sp*	Didymellaceae	JX160059.1	100	99
307	5VA	28		NGB	1889.01103	ME	ITS whole	F	*Mycosphaerella coacervata*	Mycosphaerellaceae	EU167596.1	99	100
308	2VA	25		NGB	XX.011074	ME	LSU	F	*Phomopsis sp.*	Diaporthaceae	AF439633.1	91	99
309	5VB	28		NGB	1889.01103	ME	ITS whole	F	*Cladosporium perangustum*	Cladosporiaceae	MF473185.1	99	99

◆TT= tissue taken, °F/R/F+R= Forward/Reverse/Forward+ Reverse primers, *1= *Fraxinus texensis*, 2= *Fraxinus xanthoxyloides*, 3= *Fraxinus glabra*, 4= *Fraxinus mandshurica*, 5= *Fraxinus potamophila*, 6= *Fraxinus americana*, 7= *Fraxinus pubinervis*, 8= *Fraxinus angustifolia* Monophylla, 9= *Fraxinus excelsior* Juspidea, 10= *Fraxinus ornus*, 11= *Fraxinus ornus*, 12= *Fraxinus excelsior* Diversifolia Laticifolia, 13= *Fraxinus dipetala*, 14= *Fraxinus excelsior* (orange bark), 15= *Fraxinus,* 16= *Fraxinus ornus*, 17= *Fraxinus ornus*, 18= *Fraxinus numidica*, 19= *Fraxinus angustofolia* Lentiscifolia, 20= *Fraxinus* sp. (No label), 21= *Fraxinus* unknown (greybud), 22= *Fraxinus excelsior* Pendula, 23= *Fraxinus pennsylvanica*, 24= *Fraxinus ornus*, 25= *Fraxinus angustofolia* Lentiscifolia, 26= *Fraxinus numidica*, 27= *Fraxinus excelsior* Pendula, 28= *Fraxinus pennsylvanica*.

Table 2 Comparisons of leaf endophyte OTU frequencies from individual trees.

	a	b	c	o
OTUs	Count			
Acremonium sp.	1	0	1	0
Alternaria humuli	0	0	0	1
Alternaria sp.	1	0	0	0
Aureobasidium pullulans	0	0	0	1
Aureobasidium sp.	0	1	0	0
Bjerkandera adusta	1	0	0	0
Boeremia exigua	13	16	2	17
Boeremia exigua var. exigua	2	2	0	3
Boeremia hedericola	3	0	0	2
Boeremia sp.	0	1	0	0
Boeremia strasseri	0	0	0	2
Boeremia trachelospermi	0	1	0	5
Botrytis cinerea	1	0	0	0
Cadophora sp.	0	0	0	1
Cladosporium cf. herbarum	1	0	0	0
Cladosporium cladosporioides	0	0	0	1
Cladosporium herbarum	1	0	0	0
Cladosporium sp.	4	0	0	2
Coprinopsis sp.	1	0	0	0
Diaporthe eres	1	0	0	0
Diaporthe passiflorae	2	1	0	2
Diaporthe rudis	1	0	0	0
Diaporthe sp.	0	0	0	2
Diaporthe viticola	0	0	0	2
Dictyochaeta siamensis	0	0	1	0
Engyodontium album	1	0	0	3
Epicoccum cf. nigrum	1	0	0	0
Epicoccum nigrum	1	1	1	2
Epicoccum sp.	2	0	0	2
Fimetariella rabenhorstii	0	0	1	0
Fusarium avenaceum	0	0	0	1
Fusarium lateritium	4	2	1	5
Fusarium oxysporum	0	0	0	1
Fusarium proliferatum	0	0	0	1
Fusarium sp.	1	0	0	1
Fusarium tricinctum	0	1	0	0
Gibberella sp.	0	0	0	1
Kalmusia sp.	0	0	0	1
Leptosphaerulina australis	1	0	0	0
Leptosphaerulina trifolii	0	0	0	1
Mollisia sp.	0	0	0	1

Mycosphaerella coacervata	3	8	3	15
Mycosphaerella sp.	1	0	1	1
Naganishia diffluens	0	2	0	2
Neoceratosperma eucalypti	0	0	1	0
Ophiosphaerella korrae	0	0	2	0
Paraconiothyrium sp.	0	0	0	1
Penicillium griseoroseum	0	0	0	1
Penicillium sp.	0	0	1	0
Phaeosphaeria gahniae	1	0	0	1
Phaeosphaeria pontiformis	0	1	0	0
Phlebia rufa	0	0	1	0
Phoma exigua	0	1	0	0
Phoma exigua var. exigua	1	0	0	1
Phoma multirostrata	1	1	0	0
Phoma sp.	5	4	1	2
Pyronema domesticum	0	0	0	1
Sarocladium strictum	0	1	0	1
Septoria convolvuli	1	0	0	0
Septoria cucubali	2	0	0	3
Sistotrema brinkmannii	0	0	0	1
Ustilaginoidea virens	1	0	0	0
Vagicola dactylidis	0	0	0	1
Vishniacozyma heimaeyensis	0	0	0	1
Xylaria sp.	1	2	0	2
Total	*61*	*46*	*17*	*95*

Table 3 Leaf endophyte OTUs retrieved and their frequencies for each healthy tissue sampled from Roosky.

	Tissue				
	H.A	H.L	H.M	H.R	H.V
OTU	Count				
Acremonium sp.	0	0	0	1	0
Aureobasidium pullulans	0	0	1	0	0
Bjerkandera adusta	0	1	0	0	0
Boeremia exigua	0	7	3	15	1
Boeremia exigua var. exigua	0	1	0	0	0
Boeremia hedericola	0	1	1	0	0
Boeremia sp.	0	0	1	0	0
Boeremia trachelospermi	0	1	1	1	0
Botrytis cinerea	0	1	0	0	0
Cadophora sp.	0	0	0	1	0
Cladosporium cf. herbarum	0	1	0	0	0
Cladosporium cladosporioides	0	1	0	0	0
Cladosporium herbarum	0	0	1	0	0
Cladosporium sp.	0	3	1	1	0
Diaporthe eres	0	1	0	0	0
Diaporthe passiflorae	1	0	1	2	0
Diaporthe rudis	0	0	0	1	0
Diaporthe sp.	0	0	0	2	0
Diaporthe viticola	0	1	0	0	0
Dictyochaeta siamensis	0	0	0	1	0
Engyodontium album	0	2	0	2	0
Epicoccum nigrum	0	0	0	1	0
Fimetariella rabenhorstii	0	0	0	1	0
Fusarium lateritium	0	1	0	5	1
Fusarium oxysporum	0	0	0	1	0
Fusarium proliferatum	0	0	0	1	0
Fusarium sp.	0	0	0	1	0
Gibberella sp.	0	0	0	1	0
Kalmusia sp.	0	0	0	1	0
Leptosphaerulina australis	0	1	0	0	0
Leptosphaerulina trifolii	0	1	0	0	0
Mollisia sp.	0	0	1	0	0
Mycosphaerella coacervata	0	12	4	6	0
Mycosphaerella sp.	0	0	0	1	0
Naganishia diffluens	0	1	0	1	0
Neoceratosperma eucalypti	0	0	1	0	0
Ophiosphaerella korrae	0	0	1	0	0
Paraconiothyrium sp.	0	0	1	0	0
Penicillium griseoroseum	0	0	1	0	0

Phaeosphaeria gahniae	0	0	0	1	0
Phlebia rufa	0	0	1	0	0
Phoma sp.	0	1	3	3	1
Pyronema domesticum	0	0	1	0	0
Sarocladium strictum	0	1	0	1	0
Septoria convolvuli	0	1	0	0	0
Septoria cucubali	0	1	1	1	0
Sistotrema brinkmannii	0	0	0	0	1
Ustilaginoidea virens	0	0	1	0	0
Vagicola dactylidis	0	1	0	0	0
Xylaria sp.	1	1	0	0	1
Total	2	43	26	53	5

Table 4 Comparison of OTU frequencies for diseased leaf tissue sampled from Roosky.

	Tissue				
	D.A	D.L	D.M	D.R	D.V
OTU	Count				
Acremonium sp.	0	0	0	0	1
Alternaria humuli	0	1	0	0	0
Alternaria sp.	0	0	0	1	0
Aureobasidium sp.	0	0	0	1	0
Boeremia exigua	1	11	10	0	0
Boeremia exigua var. exigua	1	3	2	0	0
Boeremia hedericola	0	3	0	0	0
Boeremia strasseri	0	0	2	0	0
Boeremia trachelospermi	0	0	1	0	2
Cladosporium sp.	0	0	1	0	0
Coprinopsis sp.	0	1	0	0	0
Diaporthe passiflorae	1	0	0	0	0
Diaporthe viticola	0	1	0	0	0
Epicoccum cf. nigrum	1	0	0	0	0
Epicoccum nigrum	0	2	2	0	0
Epicoccum sp.	1	1	2	0	0
Fusarium avenaceum	0	0	0	1	0
Fusarium lateritium	0	1	2	1	1
Fusarium tricinctum	0	0	0	1	0
Mycosphaerella coacervata	2	3	1	0	0
Mycosphaerella sp.	0	1	0	1	0
Naganishia diffluens	0	1	1	0	0
Ophiosphaerella korrae	1	0	0	0	0
Penicillium sp.	0	0	1	0	0
Phaeosphaeria gahniae	0	0	1	0	0
Phaeosphaeria pontiformis	0	0	1	0	0

Phoma exigua	0	0	1	0	0
Phoma exigua var. exigua	0	0	2	0	0
Phoma multirostrata	0	1	1	0	0
Phoma sp.	1	0	1	1	0
Septoria cucubali	0	2	0	0	0
Vishniacozyma heimaeyensis	0	1	0	0	0
Xylaria sp.	0	1	1	0	0
Total	9	34	33	7	4

Table 5.1 Leaf endophyte OTU frequencies for each provenance from the EU provenance trial in Roosky.

	Provenance													
OTU	ABE	ATH	BRE	CAD	CUR	DON	ENN	FAR	HOG	KAL	KAR	LAR	LOC	LOR
	Count													
Acremonium sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Alternaria humuli	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alternaria sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aureobasidium pullulans	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Aureobasidium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bjerkandera adusta	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Boeremia exigua	2	2	1	1	1	1	1	2	0	2	3	1	2	1
Boeremia exigua var. exigua	0	0	0	0	0	0	0	1	1	0	0	1	0	1
Boeremia hedericola	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Boeremia sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Boeremia strasseri	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Boeremia trachelospermi	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Botrytis cinerea	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cadophora sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Cladosporium cf. herbarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Cladosporium herbarum	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Cladosporium sp.	0	0	1	0	0	1	0	0	0	1	0	0	1	0
Coprinopsis sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Diaporthe eres	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diaporthe passiflorae	0	0	0	0	1	0	0	0	0	1	1	0	0	0
Diaporthe rudis	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Diaporthe sp.	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Diaporthe viticola	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Dictyochaeta siamensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Engyodontium album	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Epicoccum cf. nigrum	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Epicoccum nigrum	0	0	0	0	0	0	1	0	1	0	0	0	0	0

Epicoccum sp.	0	0	0	1	0	1	0	0	0	0	0	0	1	0
Fimetariella rabenhorstii	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium avenaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Fusarium lateritium	0	0	0	1	0	0	0	2	0	0	2	0	0	1
Fusarium oxysporum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium proliferatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Fusarium tricinctum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gibberella sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kalmusia sp.	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Leptosphaerulina australis	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Leptosphaerulina trifolii	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mollisia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mycosphaerella coacervata	0	3	2	2	1	0	1	0	2	2	0	1	3	1
Mycosphaerella sp.	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Naganishia diffluens	0	0	0	0	0	0	0	0	2	0	0	0	0	0

Neoceratosperma eucalypti	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ophiosphaerella korrae	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Paraconiothyrium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium griseoroseum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phaeosphaeria gahniae	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Phaeosphaeria pontiformis	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Phlebia rufa	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Phoma exigua	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma exigua var. exigua	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Phoma multirostrata	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Phoma sp.	0	0	3	0	1	0	0	1	2	0	0	0	1	1
Pyronema domesticum	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Sarocladium strictum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Septoria convolvuli	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Septoria cucubali	0	1	0	0	0	1	0	0	1	0	0	0	0	0

Sistotrema brinkmannii	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Ustilaginoidea virens	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Vagicola dactylidis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vishniacozyma heimaeyensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Xylaria sp.	0	0	0	0	0	1	0	0	0	0	2	1	1	0
Total	5	6	12	6	6	8	4	10	13	10	11	5	12	8

Abeton (IT) - ABE, Athis (FR) - ATH, Bregentved (DK)-BRE, Cadore (IT)-CAD, Currachase (IE)- CUR, Donadea (IR)-DON, Enniskillen (IE)-ENN, Farchau (DE)-FAR, Hoge Bos (BE)-HOG, Kalsiadorys (LT)- KAL, Karlsruhe (DE) -KAR, La Romagne (FR)–LAR, Loch Tay (GB)- LOC, Loreto park (IE)-LOR.

Continuation of Table 5.1 (**for rest of the provenances)** Leaf endophyte OTU frequencies for each provenance from the EU provenance trial in Roosky

	Provenance													
OTU	MIR	MON	RAB	RAV	SAG	SAM	SET	SZC	VCS	VSP	VAP	WLO	WYW	ZEI
	Count													
Acremonium sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Alternaria humuli	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Alternaria sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Aureobasidium pullulans	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Aureobasidium sp.	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Bjerkandera adusta	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Boeremia exigua	1	2	7	5	1	0	1	0	6	1	2	1	0	1
Boeremia exigua var. exigua	0	0	1	1	0	0	0	1	0	0	0	0	0	0
Boeremia hedericola	0	0	2	0	0	0	0	0	0	0	0	1	0	0
Boeremia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Boeremia strasseri	0	0	0	0	0	0	0	0	0	1	0	0	1	0
Boeremia trachelospermi	1	1	0	0	1	0	0	0	0	2	0	0	0	0
Botrytis cinerea	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Cadophora sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium cf. herbarum	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Cladosporium cladosporioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium herbarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cladosporium sp.	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Coprinopsis sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diaporthe eres	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Diaporthe passiflorae	0	0	0	1	0	0	0	0	0	0	0	1	0	0
Diaporthe rudis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diaporthe sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Diaporthe viticola	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Dictyochaeta siamensis	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Engyodontium album	0	0	0	0	0	2	0	0	0	0	0	0	0	1
Epicoccum cf. nigrum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Epicoccum nigrum	0	0	0	0	0	0	1	0	0	0	1	0	0	1
Epicoccum sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Fimetariella rabenhorstii	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Fusarium avenaceum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium lateritium	0	0	0	1	1	0	0	0	2	0	0	1	1	0
Fusarium oxysporum	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Fusarium proliferatum	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Fusarium tricinctum	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Gibberella sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Kalmusia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leptosphaerulina australis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leptosphaerulina trifolii	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Mollisia sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Mycosphaerella coacervata	0	2	2	0	2	1	0	1	1	0	1	0	0	1
Mycosphaerella sp.	0	0	0	0	0	0	1	0	0	0	0	0	0	0

Naganishia diffluens	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Neoceratosperma eucalypti	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Ophiosphaerella korrae	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paraconiothyrium sp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Penicillium griseoroseum	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Phaeosphaeria gahniae	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phaeosphaeria pontiformis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phlebia rufa	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma exigua	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Phoma exigua var. exigua	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Phoma multirostrata	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma sp.	0	0	2	0	0	0	0	0	1	0	0	0	0	0
Pyronema domesticum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sarocladium strictum	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Septoria convolvuli	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Septoria cucubali	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Sistotrema brinkmannii	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ustilaginoidea virens	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Vagicola dactylidis	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Vishniacozyma heimaeyensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xylaria sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	6	6	18	11	5	4	6	4	14	7	6	5	4	7

Mircze (PO)- MIR, Monte Lessini (IT)– MON, Rabstejn (CZ)- RAB, Ravenholt (DK)– RAV, Saint Gatien (FR)- SAG, Saint Martin (FR) – SAM, Settrington (GB)- SET, Szczezecinek (PO)- SZC, Vaartbos Com.seed (NL) – VCS, Val Saint Pierre (FR) – VSP, Valle Pesio (IT)– VAP, Wloszczowa (PO) – WLO, Wytham Wood (GB)– WYW, Zeimelis (LT)- ZEI.

Table 5.2 Total number of isolates obtained from each provenance from Roosky

No.	Country	Provenance	Total number of isolates
1.	Belgium	Hoge Boss	13
2.	Czech Republic	Rabstejn	18
3.	Denmark	Bregentved	12
		Ravenholt	11
4.	France	Athis	6
		La Romagne	5
		Saint Gatien	5
		Saint Martin	4
		Val Saint Pierre	7
5.	Germany	Farchau	10
		Karlsruhe	11
6.	Ireland	Currachase	6
		Donadea	8
		Enniskillen	4
		Loreto park	8
7.	Italy	Abeton	5
		Cadore	6
		Monte Lessini	6
		Valle Pesio	6
8.	Lithuania	Kalsiadorys	10
		Zeimelis	7
9.	Netherland	Vaartbos Com.seed	14
10.	Poland	Mircze	6
		Szczezecinek	4
		Wloszczowa	5
11.	UK	Loch Tay	12
		Settrington	6
		Wytham Wood	4

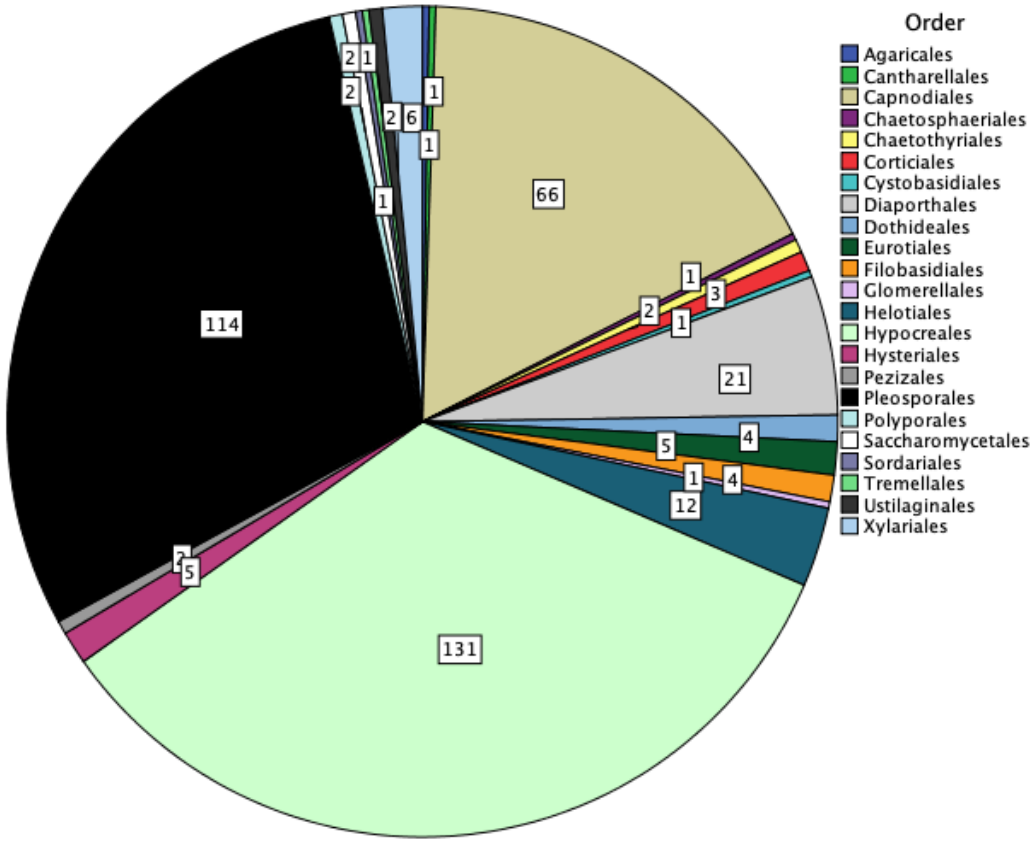

Figure 1 Number of isolates in each of the taxonomic orders obtained.

Table 6 Comparison of isolate frequencies from healthy tissues sampled from Glasnevin.

	Tissue			
	H.A	H.L	H.M	H.R
OTU	Count			
Acremonium sp.	1	1	0	0
Aspergillus versicolor	1	0	0	0
Aureobasidium pullulans	0	0	0	1
Aureobasidium sp.	1	0	0	0
Boeremia exigua var. exigua	0	1	0	0
Cladosporium allicinum	0	0	0	1
Cladosporium floccosum	0	1	0	0
Cladosporium kenpeggii	0	0	0	1
Cladosporium sp.	2	0	0	2
Collembolispora aristata	0	0	1	0
Colletotrichum godetiae	0	0	0	1
Cystobasidium slooffiae	0	0	0	1
Diaporthe cotoneastri	1	0	1	0
Diaporthe eres	0	1	0	0
Diaporthe rudis	0	0	0	2
Engyodontium album	0	0	1	0
Exophiala oligosperma	0	0	0	1
Fusarium avenaceum	0	0	1	0
Fusarium culmorum	1	0	0	0
Fusarium lateritium	2	0	1	4
Fusarium oxysporum	0	0	1	0
Fusarium sp.	0	0	0	1
Lecanicillium attenuatum	1	0	0	0
Lecanicillium muscarium	0	0	0	1
Limonomyces roseipellis	1	0	0	0
Limonomyces sp.	0	0	0	1
Meyerozyma guilliermondii	1	1	0	0
Mycosphaerella coacervata	0	2	0	1
Phlyctema vagabunda	1	0	0	0
Septoria lepidiicola	0	1	0	0
Septoria protearum	0	0	0	1
Ustilago filiformis	1	0	0	1
Vuilleminia coryli	0	1	0	0
Total	*14*	*9*	*6*	*20*

Table 7 Detailed information of frequencies of OTUs for all three sites (Roosky, Glasnevin and Kinsealy).

No.	OTU	Roosky	Glasnevin	Kinsealy
		Frequency		
1.	*Acremonium alternatum*	-	-	1
2.	*Acremonium* sp.	2	2	-
3.	*Alternaria humuli*	1	-	-
4.	*Alternaria* sp.	1	-	-
5.	*Aspergillus versicolor*	-	1	-
6.	*Aureobasidium pullulans*	1	1	-
7.	*Aureobasidium* sp.	1	1	-
8.	*Bjerkandera adusta*	1	-	-
9.	*Boeremia exigua*	48	-	-
10.	*Boeremia exigua* var. exigua	7	2	-
11.	*Boeremia hedericola*	5	-	-
12.	*Boeremia* sp.	1	-	-
13.	*Boeremia strasseri*	2	-	-
14.	*Boeremia trachelospermi*	6	-	-
15.	*Botrytis cinerea*	1	-	-
16.	*Cadophora meredithiae*	-	-	1
17.	*Cadophora* sp.	1	-	5
18.	*Cladosporium allicinum*	1	-	-
19.	*Cladosporium cf. herbarum*	1	-	-
20.	*Cladosporium cladosporioides*	1	-	1
21.	*Cladosporium floccosum*	-	1	-
22.	*Cladosporium herbarum*	1	-	-
23.	*Cladosporium kenpeggii*	-	1	-
24.	*Cladosporium perangustum*	-	1	-
25.	*Cladosporium* sp.	6	6	-
26.	*Cladosporium westerdijkieae*	-	-	1
27.	*Collembolispora aristata*	-	1	-
28.	*Colletotrichum godetiae*	-	1	-
29.	*Coprinopsis* sp.	1	-	-
30.	*Cordyceps confragosa*	-	-	1
31.	*Cordyceps crassispora*	-	-	2
32.	*Cystobasidium slooffiae*	-	1	-
33.	*Dactylonectria alcacerensis*	-	-	1
34.	*Dactylonectria hordeicola*	-	-	6
35.	*Dactylonectria macrodidyma*	-	-	20
36.	*Dactylonectria torresensis*	-	-	2
37.	*Diaporthe cotoneastri*	-	2	-
38.	*Diaporthe eres*	1	1	

39.	*Diaporthe passiflorae*	5	-	-
40.	*Diaporthe rudis*	1	2	-
41.	*Diaporthe* sp.	2	-	-
42.	*Diaporthe viticola*	2	2	-
43.	*Dictyochaeta siamensis*	1	-	-
44.	*Engyodontium album*	4	2	-
45.	*Epichloe typhina*	-	-	1
46.	*Epicoccum cf. nigrum*	1	-	-
47.	*Epicoccum nigrum*	5	-	2
48.	*Epicoccum* sp.	4	-	-
49.	*Eutypa spinosa*	-	1	-
50.	*Exophiala oligosperma*	-	2	-
51.	*Fimetariella rabenhorstii*	1	-	-
52.	*Fusarium avenaceum*	1	1	-
53.	*Fusarium culmorum*	-	1	7
54.	*Fusarium lateritium*	12	8	-
55.	*Fusarium oxysporum*	1	1	5
56.	*Fusarium oxysporum* f. sp. radicislycopersici	-	1	-
57.	*Fusarium proliferatum*	1	2	-
58.	*Fusarium* sp.	2	1	-
59.	*Fusarium tricinctum*	1	-	-
60.	*Gibberella* sp.	1	-	-
61.	*Gloniopsis calami*	-	1	-
62.	*Harzia velata*	-	-	2
63.	*Hydropisphaera* sp.	-	-	1
64.	*Ilyonectria destructans*	-	-	1
65.	*Ilyonectria radicicola*	-	-	6
66.	*Ilyonectria robusta*	-	-	2
67.	*Ilyonectria* sp.	-	-	5
68.	*Juxtiphoma eupyrena*	-	-	2
69.	*Kalmusia* sp.	1	-	-
70.	*Lecanicillium attenuatum*	-	1	-
71.	*Lecanicillium lecanii*	-	-	1
72.	*Lecanicillium muscarium*	-	1	-
73.	*Lecanicillium* sp.	-	-	1
74.	*Leptosphaerulina australis*	1	-	-
75.	*Leptosphaerulina trifolii*	1	-	-
76.	*Limonomyces roseipellis*	-	1	-
77.	*Limonomyces* sp.	-	2	-
78.	*Meyerozyma guilliermondii*	-	2	-
79.	*Mollisia* sp.	1	-	-
80.	*Mycochaetophora sp.*			
81.	*Mycosphaerella coacervata*	29	4	-
82.	*Mycosphaerella sp.*	3	-	

83.	*Naganishia diffluens*	4	-	-
84.	*Nectria* sp.	-	-	1
85.	*Neoceratosperma eucalypti*	1	-	-
86.	*Neonectria candida*	-	-	1
87.	*Neonectria punicea*	-	-	2
88.	*Neonectria radicicola*	-	-	1
89.	*Neonectria* sp.	-	-	9
90.	*Ophiosphaerella korrae*	2	-	-
91.	*Paraconiothyrium* sp.	1	-	-
92.	*Penicillium expansum*	-	1	-
93.	*Penicillium griseoroseum*	1	-	-
94.	*Penicillium* sp.	1	-	-
95.	*Penicillium spathulatum*	-	-	1
96.	*Phaeosphaeria gahniae*	2	-	-
97.	*Phaeosphaeria pontiformis*	1	-	-
98.	*Phlebia rufa*	1	-	
99.	*Phlyctema vagabunda*	-	1	-
100.	*Phoma exigua*	1	-	-
101.	*Phoma exigua* var. exigua	2	-	-
102.	*Phoma multirostrata*	2	-	-
103.	*Phoma* sp.	12	2	-
104.	*Phomopsis* sp.	-	2	-
105.	*Psiloglonium* sp.	-	-	4
106.	*Pyronema domesticum*	1	1	-
107.	*Sarocladium strictum*	2	-	-
108.	*Septoria convolvuli*	1	-	-
109.	*Septoria cucubali*	5	-	-
110.	*Septoria lepidiicola*	-	1	-
111.	*Septoria protearum*	-	1	-
112.	*Sistotrema brinkmannii*	1	-	-
113.	*Trichoderma viride*	-	-	3
114.	*Ustilaginoidea virens*	1	-	-
115.	*Ustilago filiformis*	-	2	-
116.	*Vagicola dactylidis*	1	-	-
117.	*Vishniacozyma heimaeyensis*	1	-	-
118.	*Vuilleminia coryli*	-	1	-
119.	*Xylaria* sp.	5	-	-

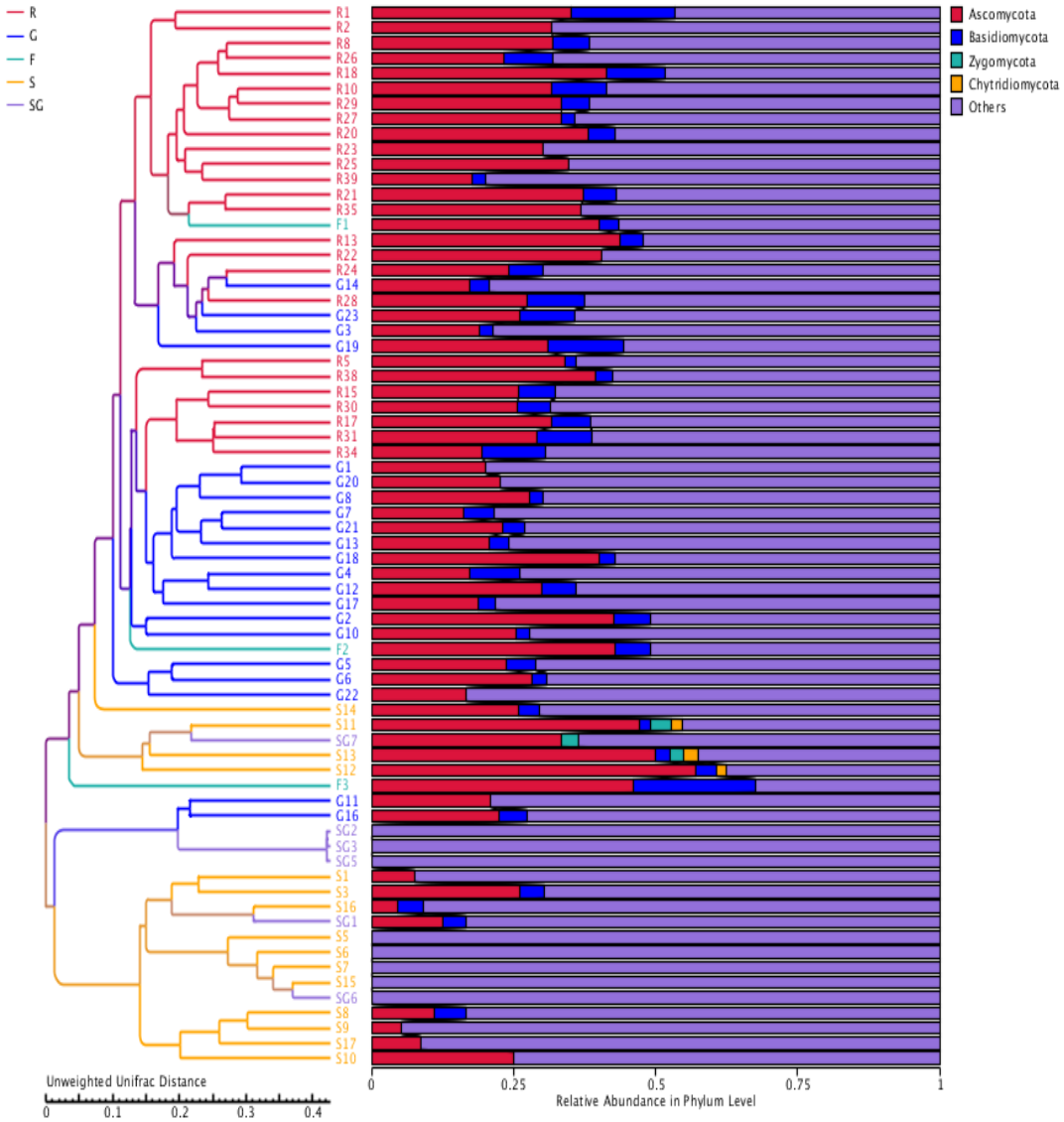

Figure 2 UPGMA cluster tree based on unweighted unifrac distance.

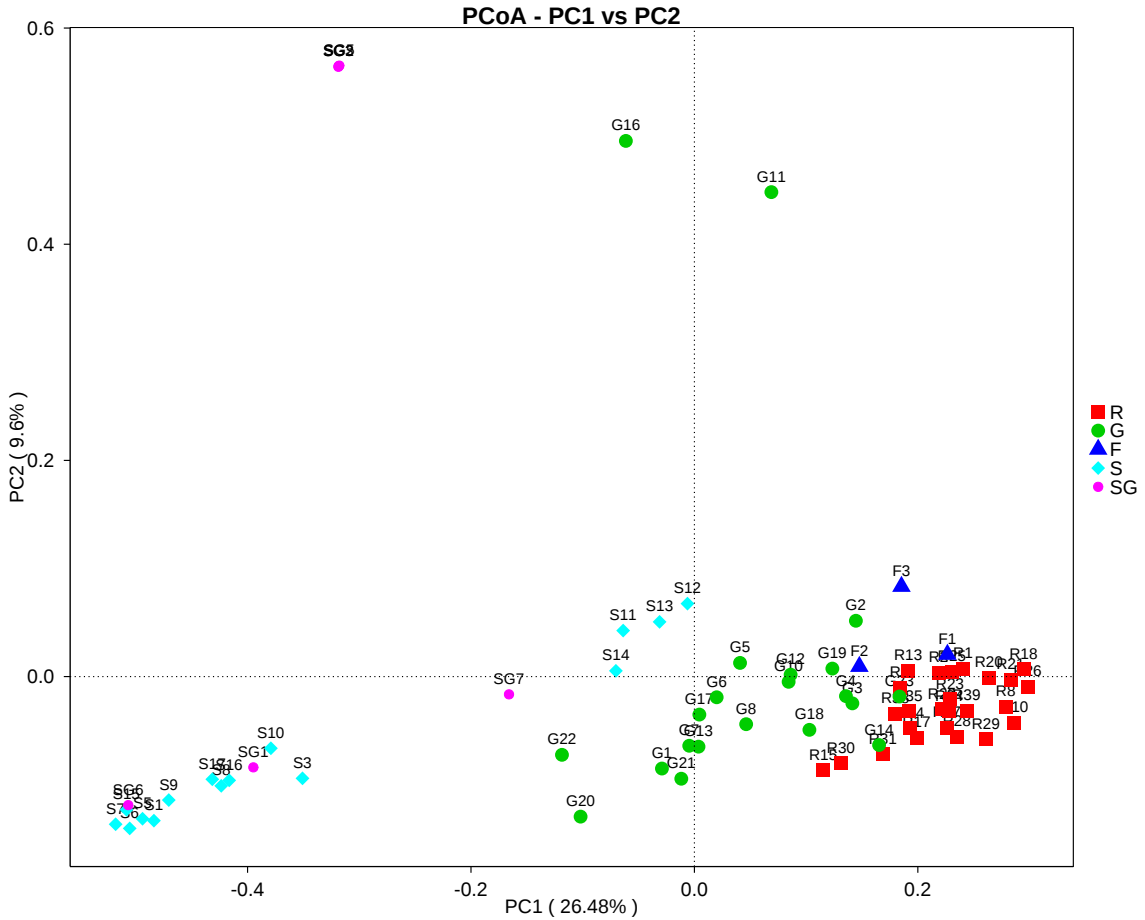

Figure 3 PCoA with unweighted unifrac distances.
R= Roosky samples, G= Glasnevin samples, F= France sample, S= seed from Roosky samples, SG= seeds from Glasnevin samples

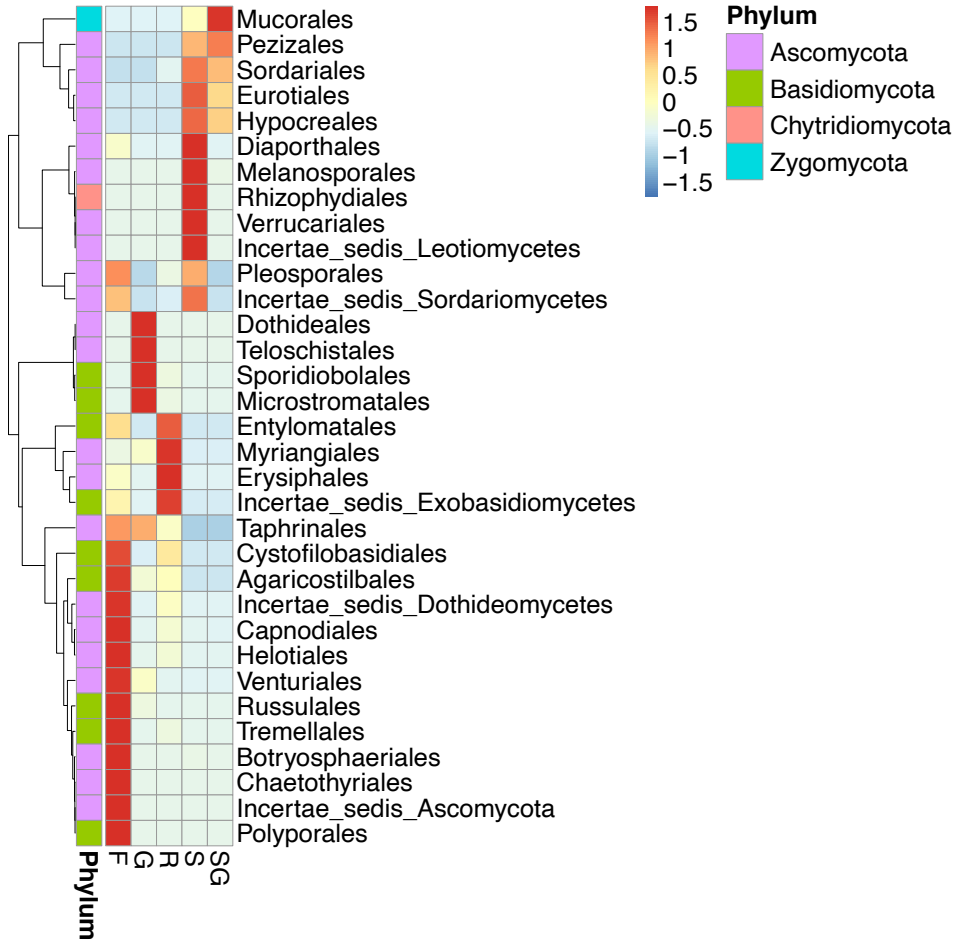

Figure 4 Heat map showing abundance of OTUs in orders.

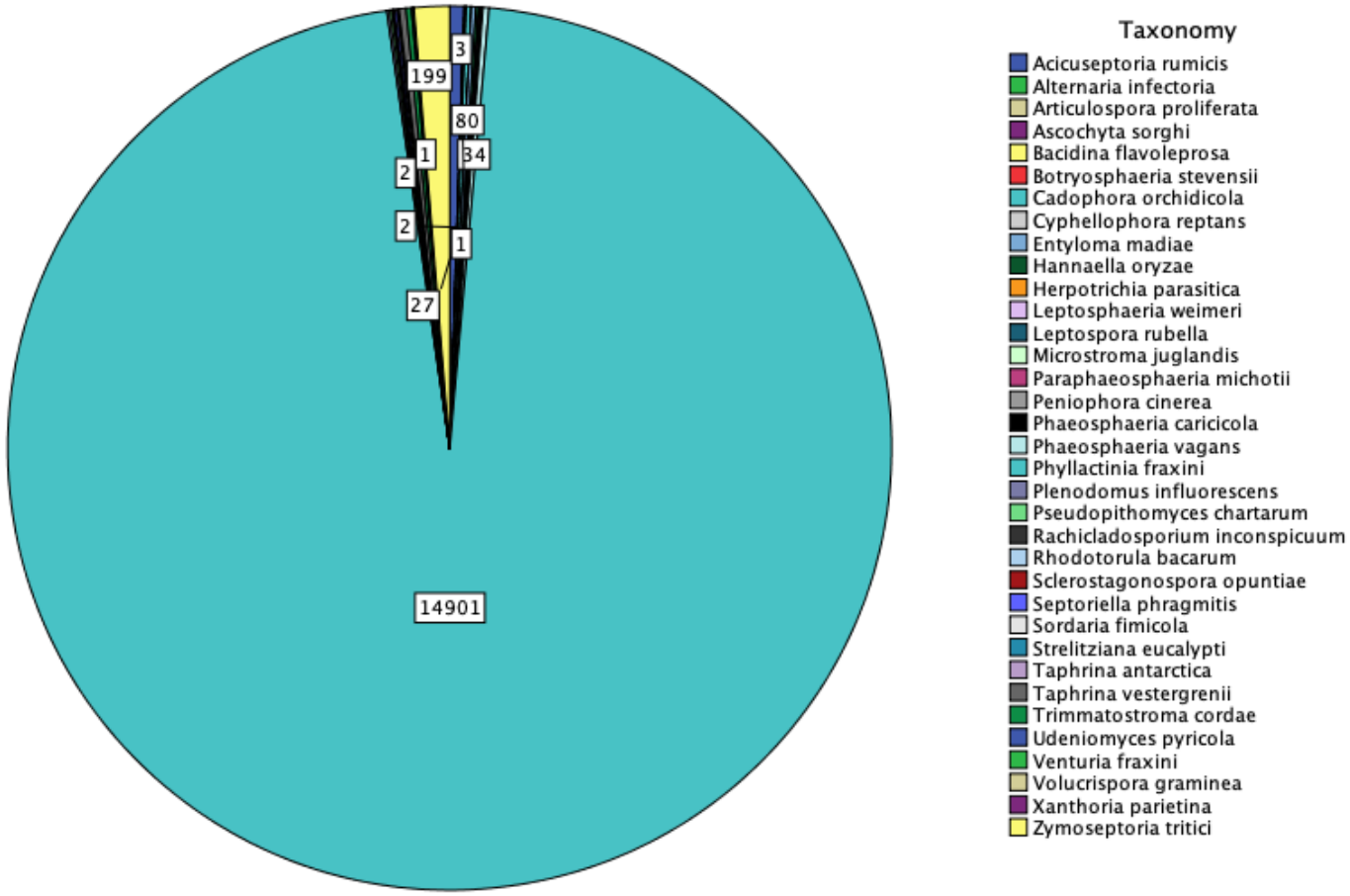

Figure 5 Pie chart showed taxa obtained from NGS for 28 tDNAs from leaf tissues of R samples from Roosky.

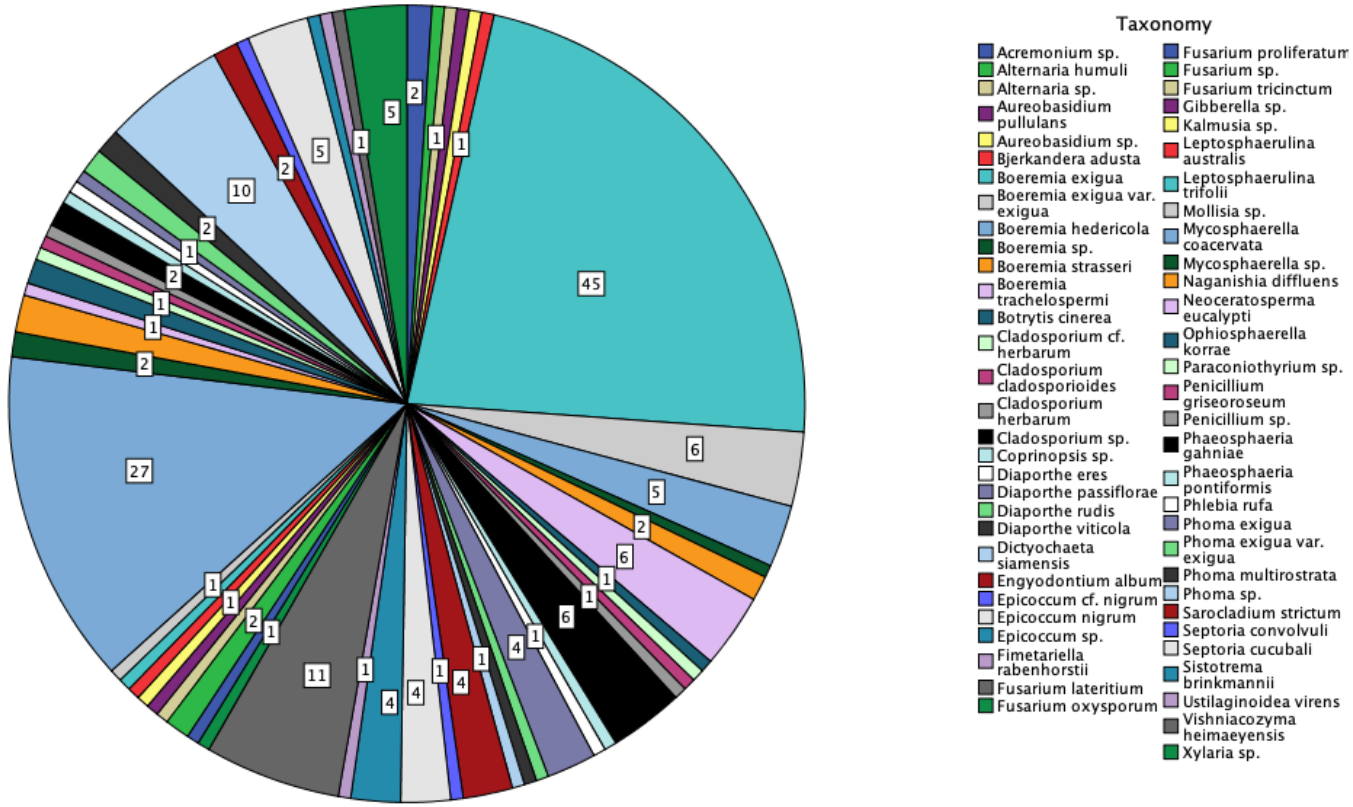

Figure 6 Pie chart for taxa obtained from 200 cultured leaf endophytes for R samples from Roosky by Sanger sequencing.

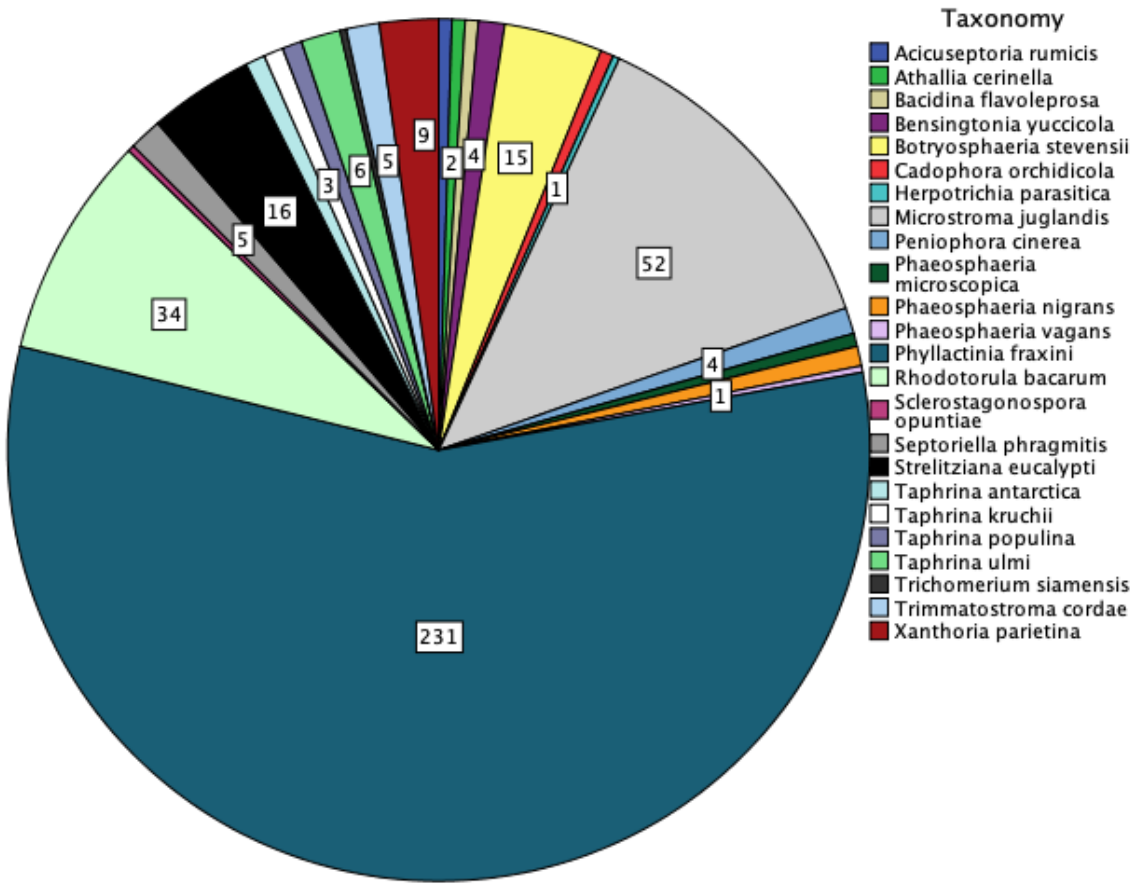

Figure 7 HTS read number data for taxa obtained for 21 leaf tDNAs for samples from Glasnevin.

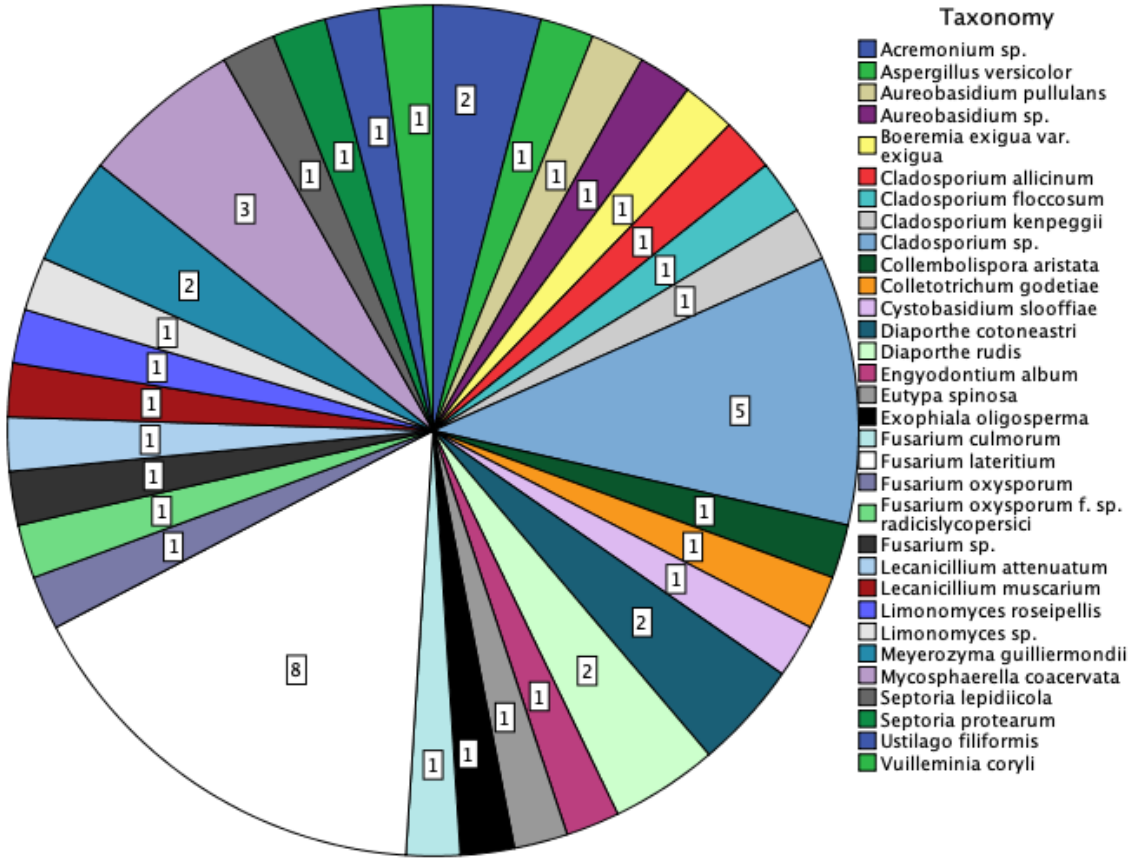

Figure 8 Pie chart for taxa obtained from cultured 49 endophytes for G samples done by Sanger sequencing for Glasnevin Botanic Garden.

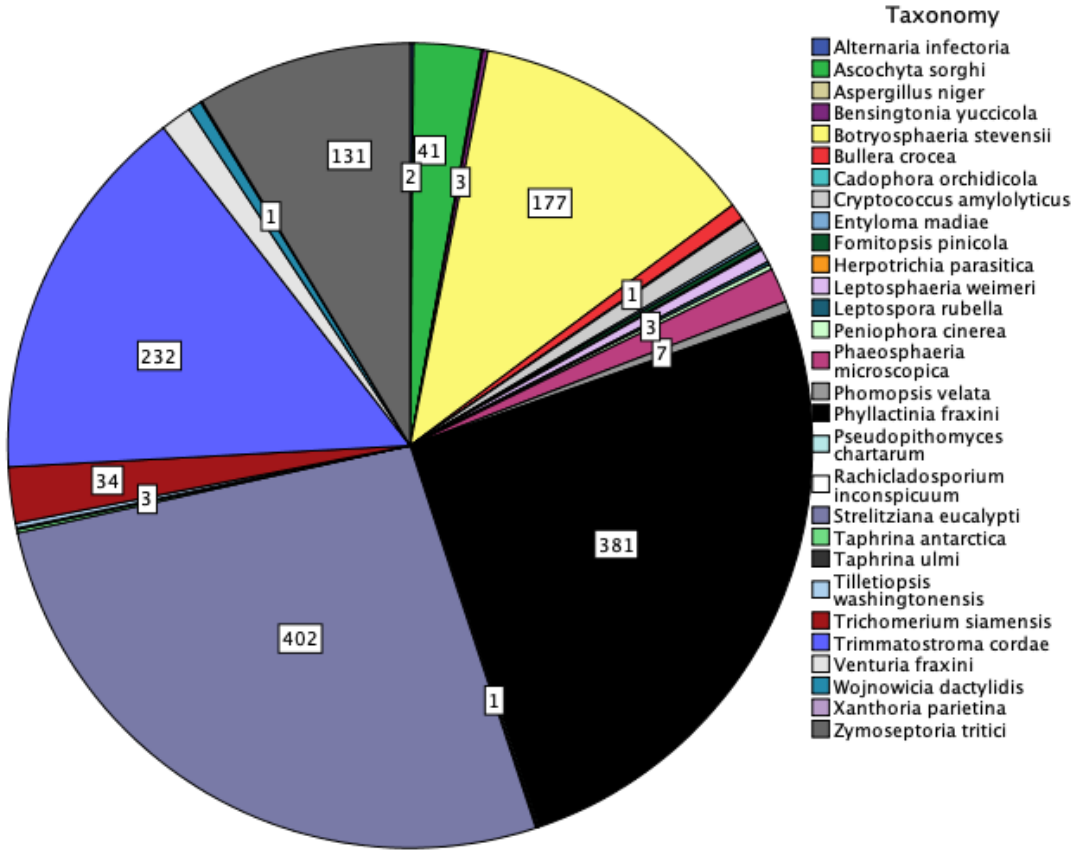

Figure 9 Pie chart showing the read abundance identified to species level in the French *Fraxinus excelsior* leaves

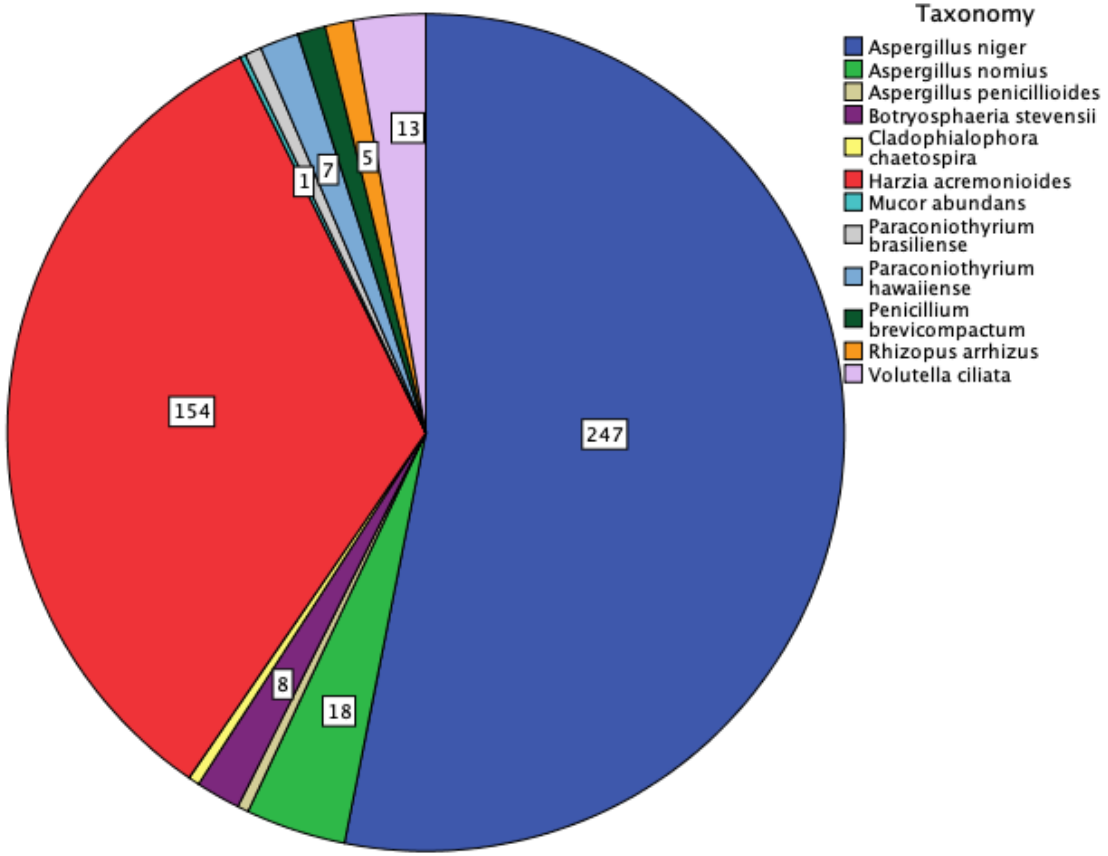

Figure 10 Taxa obtained for seed DNA collected from Roosky, provenance trials by the HTS method.

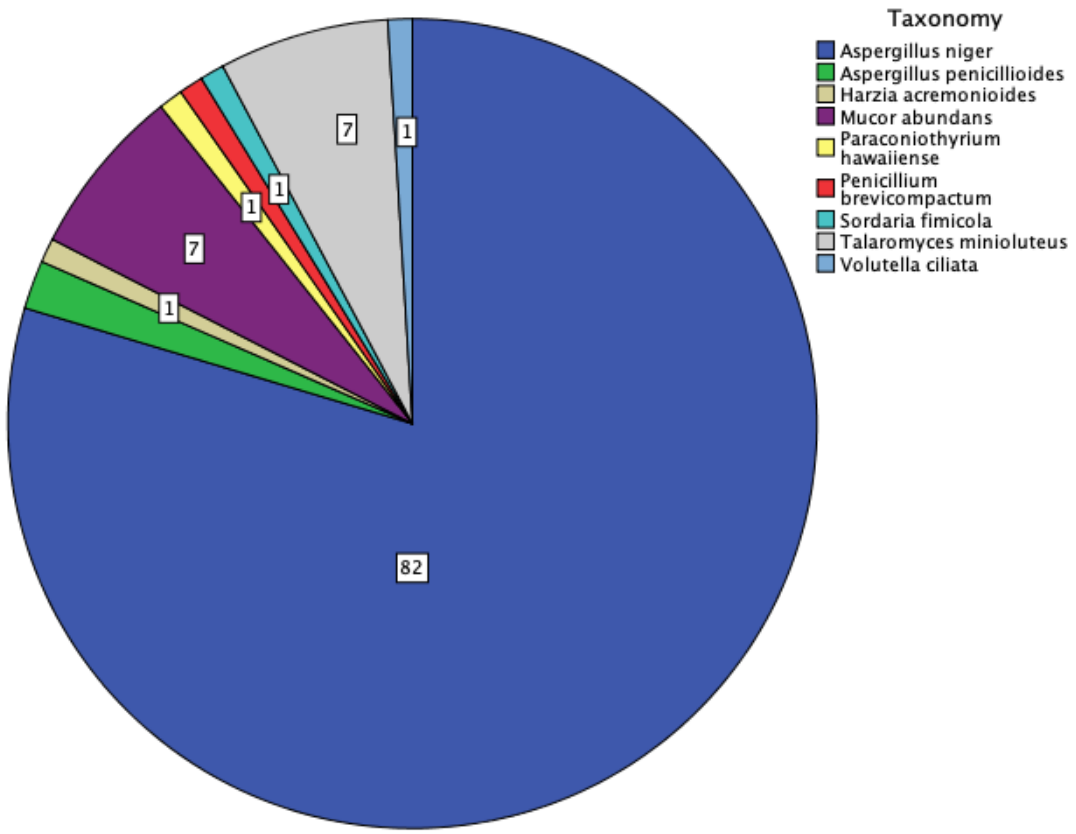

Figure 11 Taxa obtained from seed DNA collected from National Botanic Garden, Glasnevin (SG samples).

Appendix III – Chapter 4

Chi square analysis assessing whether germination rate was dependent on explant type (Figure 4.1).

Chi-Square Tests					
	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	11.521[a]	1	.001		
Continuity Correction[b]	10.496	1	.001		
Likelihood Ratio	12.098	1	.001		
Fisher's Exact Test				.001	.000
N of Valid Cases	210				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 24.98.
b. Computed only for a 2x2 table.

Chi square analysis assessing whether survival of germinated plants was dependent on explat type or not (Figure 4.2).

Chi-Square Tests					
	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	60.493[a]	1	.000		
Continuity Correction[b]	57.672	1	.000		
Likelihood Ratio	76.903	1	.000		
Fisher's Exact Test				.000	.000
N of Valid Cases	149				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 19.81.
b. Computed only for a 2x2 table

A Chi square test analysis was performed to see if germination of seeds was dependent on the gelling agent (Figure 4.6).

Chi-Square Tests					
	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8.240[a]	1	.004		
Continuity Correction[b]	7.868	1	.005		
Likelihood Ratio	8.245	1	.004		
Fisher's Exact Test				.005	.003
N of Valid Cases	985				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 182.09.
b. Computed only for a 2x2 table.

Appendix IV – Chapter 5

Selected images of Petri dishes with leaf and root endophytes and pathogen for the antagonism testing, and biocontrol experiment, are shown in Figure 5.1.1, 5.1.2, 5.1.3 and 5.2 respectively.

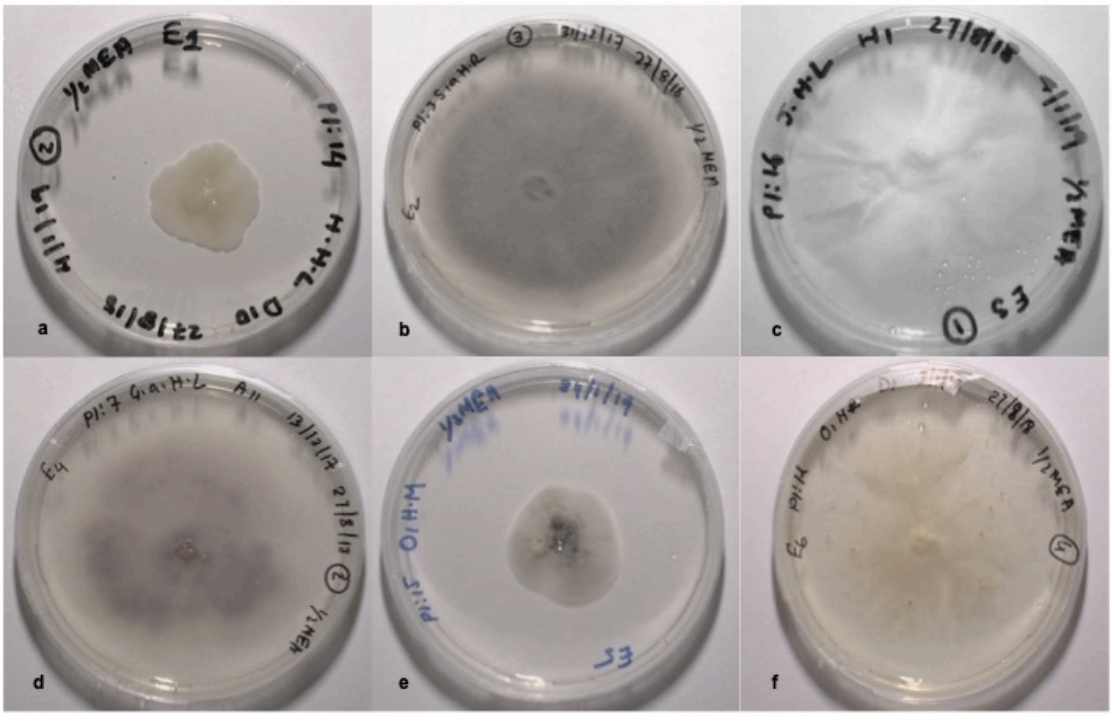

Figure 5.1.1 Endophytes used in the antagonism testing (plate 1). a) leaf endophyte E1 - *Naganishia diffluens*, b) leaf endophyte E2 - *Epiccocum* sp., c) leaf endophyte E3 - *Engyodontium album*, d) leaf endophyte E4 - *Bjerkandera adusta*, e) leaf endophyte E5 - *Mollisia* sp., f) leaf endophyte E6 - *Gibberella* sp.

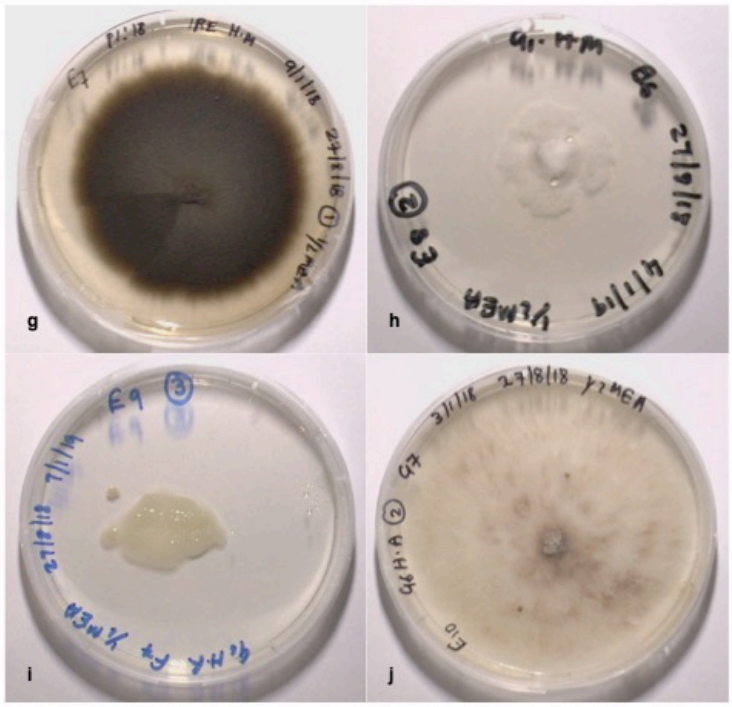

Figure 5.1.2 Endophytes used in the antagonism testing (plate 2). g) leaf endophyte E7 - *Pyronema domesticum*, h) leaf endophyte E8 - *Collemboliospora aristata*, i) leaf endophyte E9 - *Exophiala oligosperma,* j) leaf endophyte E10 - *Meyerozyma guilliermondi*.

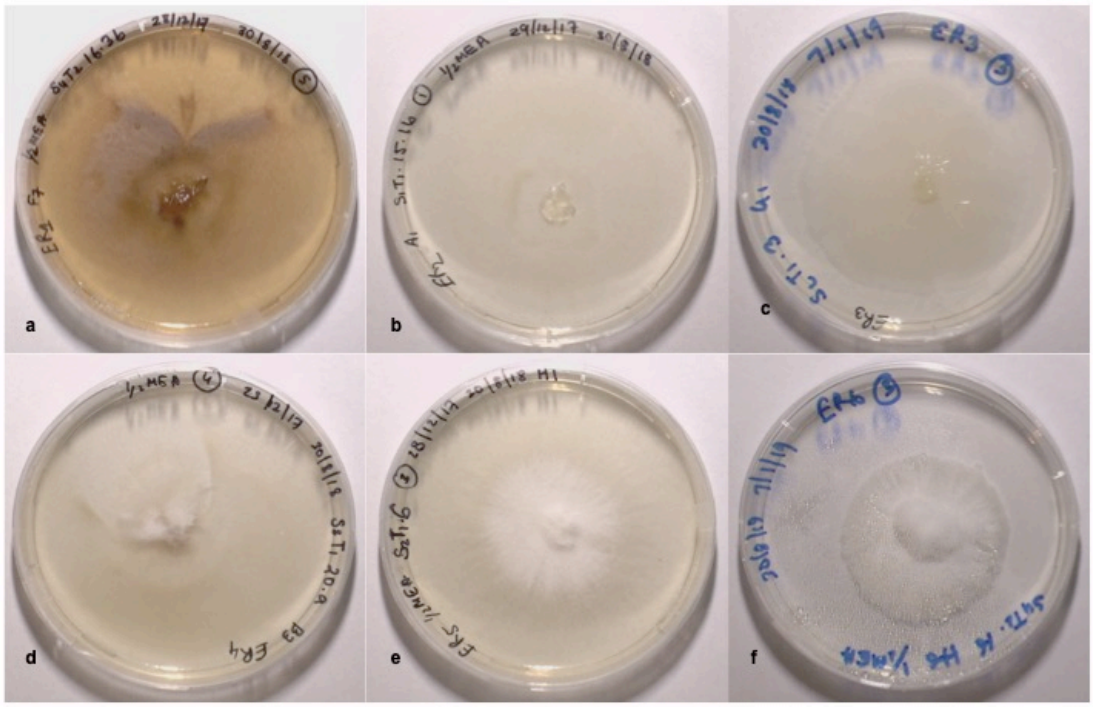

Figure 5.1.3 Endophytes used in the antagonism testing. a) root endophyte ER1 - *Lecanicillium attenuatum*, b) root endophyte ER2 - *Cordyceps perangustam, c) root endophyte ER3 - Penicillium spathulatum,* d) root endophyte ER4 - *Harzia velata*, e) root endophyte ER5 - *Hydropisphaera* sp., f) root endophyte ER6 - *Psilloglonium* sp..

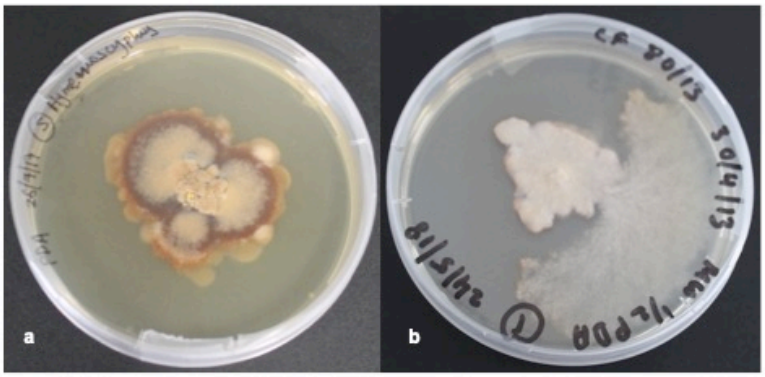

Figure 5.2 Pathogen used in the antagonism testing (plate 4). a) pathogen Irish strain H1 (Teagasc) – *Hymenoscyphus fraxineus*, b) pathogen Northern Irish strain H8 (80/13)- *Hymenoscyphus fraxineus.*

Images of Petri dishes for antagonistic assays with two pathogen strains H8 and H1 for leaf and root endophytes were shown in Figure 5.3.1, 5.3.2 and 5.4 respectively.

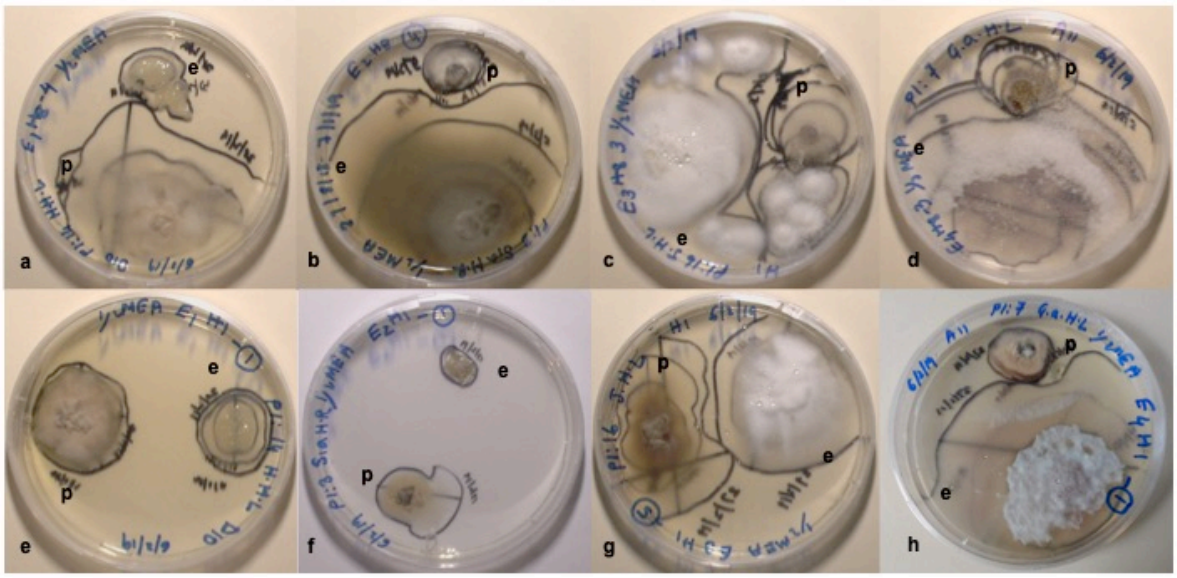

Figure 5.3.1 Antagonism assays with pathogen strains H8 and H1 (plate 1). Upper row shows Petri dishes for leaf endophyte E1, E2, E3 and E4 with Northern Irish strain H8 showed in a, b, c and d respectively. Lower row shows leaf endophyte E1, E2, E3 and E4 with Irish strain H1, in e, f, g and h respectively. Endophytes were denoted as e and pathogen as p in all photos.

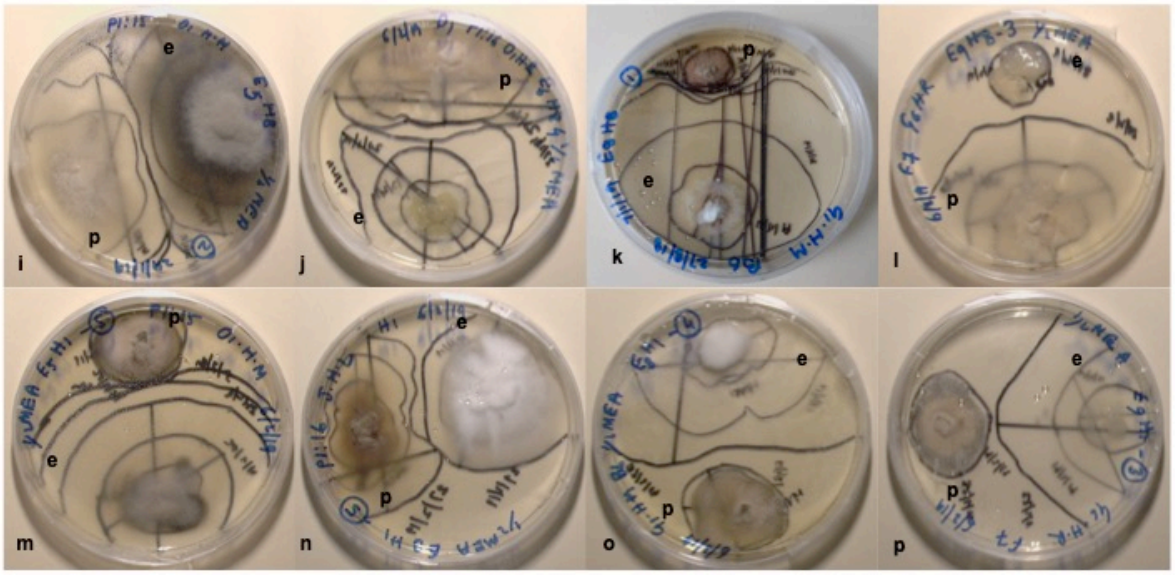

Figure 5.3.2 Antagonism assays with pathogen strains H8 and H1 (plate 2). Upper row shows Petri dishes for leaf endophyte E5, E6, E8 and E9 with Northern Irish strain H8, i, j, k and l respectively. Lower row shows leaf endophyte E5, E6, E8 and

E9 with Irish strain H1, m, n, o and p respectively. Endophytes were denoted as e and pathogen as p in all photos.

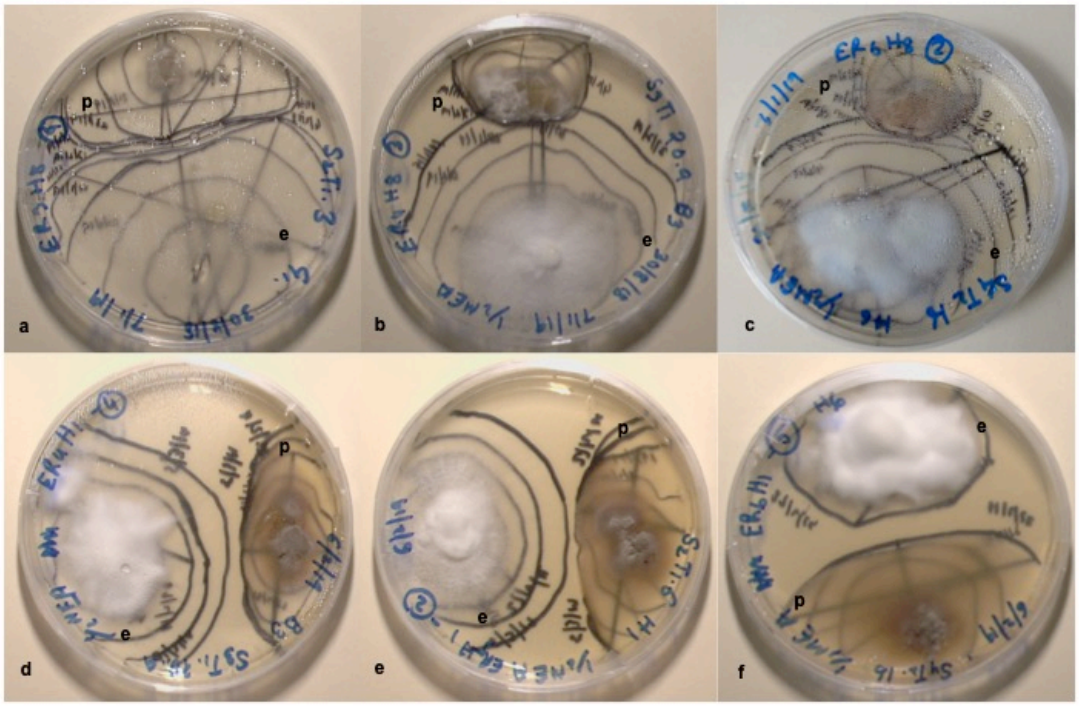

Figure 5.4 Antagonism assays with pathogen strains H8 and H1 (plate 3). Upper row shows Petri dishes for root endophyte ER3, ER4 and ER6 with Northern Irish strain H8, a, b and c respectively. Lower row shows root endophyte ER4, ER5 and ER6 with Irish strain H1, d, e and f respectively. Endophytes were denoted as e and pathogen as p in all photos.

Table 5.1 Mean growth of the endophytes from leaf and pathogen (H8 -80/13) in cm^2 for day interval

Name of the culture	Days of interval mean growth in cm^2						
	7	14	21	28	35	42	49
E1.a	1.27	0.89	2.78	3.35	6.61	6.30	1.62
E1.b	0.68	0.32	0.24	0.00	0.00	0.00	0.30
E2.a	0.91	0.07	0.00	0.00	0.00	0.00	13.29
E2.b	0.64	0.65	0.00	0.00	0.00	0.00	0.55
E3.a	2.88	4.64	3.57	8.23	11.62	11.10	12.13
E3.b	0.64	0.36	0.03	0.08	0.00	0.00	0.07
E4.a	9.16	6.10	7.95	11.88	12.00	5.64	0.99
E4.b	0.84	0.39	0.10	0.17	0.00	0.00	0.00
E5.a	2.41	6.15	13.53	7.86	7.98	0.56	0.17

E5.b	0.97	1.85	14.33	6.73	1.47	1.20	0.71
E6.a	3.43	2.85	3.43	5.62	3.18	3.60	6.76
E6.b	0.68	0.21	0.07	0.03	0.00	0.00	2.74
E7.a	4.31	5.54	5.17	8.14	9.82	6.56	0.00
E7.b	0.97	0.21	0.04	0.00	0.00	0.00	0.13
E8.a	7.09	13.47	6.61	12.52	2.69	3.55	2.84
E8.b	0.73	1.14	0.60	0.65	0.83	1.33	0.44
E9.a	0.85	0.55	0.70	1.19	1.37	5.71	5.89
E9.b	0.76	1.26	1.50	3.32	7.90	9.04	6.83
E10.a	13.77	15.65	24.67	1.58	2.01	0.00	0.00
E10.b	0.88	1.46	1.13	0.07	0.00	0.22	0.48

Table 5.2 Mean growth of the endophytes from leaf and pathogen (H8 -80/13) in cm^2 repeated for day interval

Name of the culture	Days interval of mean growth in cm^2				
	7	14	21	28	35
E1.a	1.73	0.56	3.23	1.12	0.59
E1.b	15.94	18.76	3.55	7.41	1.75
E2.a	1.03	0.00	3.07	6.10	2.99
E2.b	10.19	13.06	8.77	12.50	4.90
E3.a	3.34	4.72	18.08	31.10	6.05
E3.b	2.97	0.00	0.61	1.80	2.14
E4.a	14.06	10.03	10.91	6.98	6.73
E4.b	2.96	0.00	0.00	0.13	0.65
E6.a	5.53	7.20	11.13	6.05	1.86
E6.b	10.74	7.55	1.90	2.64	1.73
E7.a	7.21	12.63	15.03	52.79	1.06
E7.b	5.67	6.32	0.27	9.48	1.63
E9.a	2.08	0.37	4.18	4.48	1.51
E9.b	8.01	11.98	10.96	19.97	6.69

Table 5.3 Mean growth of the endophytes from leaf and pathogen (H1 -Teagasc) in cm^2 for day interval

Name of culture	Days of interval of mean growth in cm^2				
	7	14	21	28	35
E1.a	2.79	2.46	2.21	2.1	2.88
E1.c	5.17	2.93	4.63	5.28	6.47
E2.a	1.54	0	3.74	0	2.12
E2.c	6.18	3.96	5.31	4.69	12.36
E3.a	4.34	5.56	7.22	8.46	2.86
E3.c	6.66	7.71	5.7	5.83	2.52

E4.a	16.72	17.32	11.43	6.2	0.4
E4.c	3.4	1.28	2.32	1.28	0
E5.a	4.81	14.73	6.82	7.12	2.87
E5.c	4.69	1.95	4.96	3.88	0.99
E6.a	4.1	6.61	10.51	9.17	6.52
E6.c	4.68	2.2	3.32	2.9	2.09
E7.a	6.11	10.74	7.44	9.09	36.28
E7.c	5.28	2.91	5.58	8.38	8.53
E8.a	6.26	22.24	8.28	1.5	1.11
E8.c	5.01	2.36	2.06	7.08	1.03
E9.a	4.75	10.65	7.74	18.61	3.72
E9.c	4.7	2.52	2.68	2.64	0.05
E10.a	19.89	27.65	1.3	1.91	3.14
E10.c	4.84	1.62	1.82	1.78	0

Table 5.4 Mean growth of the endophytes from root and pathogen (H8 -80/13) in cm^2 for day interval

Name of culture	Days of interval of mean growth in cm^2						
	7	14	21	28	35	42	49
ER1.a	4.24	9.38	9.31	9.31	15.9	7.19	6.26
ER1.b	1	1.31	1.43	1.43	1.41	0	0
ER2.a	6.24	7.91	9.49	9.48	18.54	7.45	3.97
ER2.b	1.27	1.39	3.94	3.94	2.47	4.67	4.11
ER3.a	7.56	7.23	3.08	3.08	8.84	3.82	9.47
ER3.b	1.19	1.59	3	3	3.07	4.93	4.02
ER4.a	2.07	4.01	3.99	3.99	3.17	10.61	7.08
ER4.b	1.83	0.96	1.72	1.72	1.01	4.6	0.89
ER5.a	3.61	4.45	3.56	3.55	4.71	8.46	15.3
ER5.b	1.23	1.3	2.54	2.54	2.87	2.17	0.46
ER6.a	4.93	3.68	4.53	4.52	6.41	12.4	9.22
ER6.b	1.59	1.62	1.89	1.89	2.01	5.6	1.85

Table 5.5 Mean growth of the endophytes from root and pathogen (H1 -Teagasc) in cm^2 for day interval

Name of culture	Days of interval of mean growth in cm^2				
	7	14	21	28	35
ER1.a	6.41	17.9	8.68	5.25	1.39
ER1.c	9	8.19	3.7	0	0
ER2.a	9.01	20.35	8.57	7.63	43.79
ER2.c	6.69	3.72	2.56	0.79	13.23
ER3.a	8.24	14.07	11.32	2.88	34.66

ER3.c	5	2.88	2.03	4.64	13.38
ER4.a	6.41	6.11	7.13	4.66	18.76
ER4.c	8.32	8.31	0.86	2.3	0.06
ER5.a	5.07	7.24	10.23	6.03	7.89
ER5.c	9.35	4.12	11.25	14.25	1.6
ER6.a	5.18	3.98	11.97	9.24	3.43
ER6.c	10.65	9.19	10.29	5.57	0.07

Measurements for growth parameters for co-culture experiment for control, pathogen treatment, endophyte treatment and endophyte-pathogen treatment for two leaf endophytes (E7 and E10) and one root endophyte (ER1) are shown below in Tables 5.6 (1, 2, 3, 4, 5 and 6) and 5.7 (1, 2, 3 and 4) respectively.

Table 5.6.1 Measurements for control plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	12/2/19	20/3/19	0	0.25	0.035	4:3.5	6	2	78	67	89
2	12/2/19	20/3/19	0	0.53	0.042	5:5.5	20	2	107	369	60
3	12/2/19	20/3/19	0	0.18	0.017	3:3.5	6	2	196	167	120
4	12/2/19	20/3/19	0	0.26	0.039	5:6	6	1	201	202	211
5	12/2/19	20/3/19	0	0.23	0.037	3:2	14	4	97	102	123
6	12/2/19	20/3/19	0	0.19	0.027	3:2	16	1	200	164	155
7	12/2/19	20/3/19	0	0.38	0.048	3.5:1.5	4	2	105	126	45
8	12/2/19	20/3/19	0	0.30	0.043	8.5:3	17	6	125	98	65
9	12/2/19	20/3/19	0	0.30	0.036	5:4	8	3	324	355	367
10	12/2/19	20/3/19	0	0.10	0.027	3:1	12	1	129	107	201
Average			0	0.27	0.04	4.3:3.2	10.9	2.4	156	176	144

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.6.2 Measurements for pathogen treated plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	12/2/19	20/3/19	5	0.37	0.025	3.5:2	4	6	24	39	56
2	12/2/19	20/3/19	2	0.18	0.033	5.5:2	12	1	314	25	72

3	12/2/19	20/3/19	5	0.15	0.015	4:2.5	6	1	132	75	67
4	12/2/19	20/3/19	3	0.23	0.032	6:2	11	1	18	107	51
5	12/2/19	20/3/19	4	0.21	0.036	5:1.5	8	3	109	76	35
6	12/2/19	20/3/19	5	0.60	0.042	3:4	8	3	123	98	34
7	12/2/19	20/3/19	1	0.31	0.036	7:2	22	4	335	236	305
8	12/2/19	20/3/19	1	0.21	0.028	7:2	14	5	156	167	178
9	12/2/19	20/3/19	2	0.29	0.031	4:3	6	4	154	179	208
10	12/2/19	20/3/19	0	0.15	0.012	3.5:1.5	4	10	58	65	23
Average			2.8	0.27	0.03	4.9:2.3	9.5	3.8	142	107	103

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.6.3 Measurements for endophyte (E7) treated plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	12/2/19	20/3/19	4	0.23	0.016	5:3	3	4	228	206	179
2	12/2/19	20/3/19	5	0.20	0.012	6:3	3	1	201	187	233
3	12/2/19	20/3/19	1	0.26	0.035	6.5:2.5	16	3	35	47	51
4	12/2/19	20/3/19	5	0.20	0.013	2:1	3	1	118	127	145
5	12/2/19	20/3/19	0	0.21	0.014	6:0	11	0	245	14	71
6	12/2/19	20/3/19	4	0.23	0.016	3:0	3	0	156	115	126
7	12/2/19	20/3/19	5	0.13	0.015	3.5:0	8	0	169	233	128
8	12/2/19	20/3/19	1	0.32	0.035	4:3	11	2	136	177	24
9	12/2/19	20/3/19	4	0.25	0.017	5:4	4	4	47	71	26
10	12/2/19	20/3/19	1	0.20	0.022	5:3	14	1	141	21	4
Average			3	0.22	0.02	4.6:2	7.6	1.6	148	120	99

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.6.4 Measurements for endophyte (E10) treated plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	12/2/19	20/3/19	4	0.28	0.041	6.3:7	8	3	54	42	31
2	12/2/19	20/3/19	3	0.26	0.037	6.7:1.5	16	2	61	59	79
3	12/2/19	20/3/19	4	0.35	0.014	3:1	6	8	87	109	132
4	12/2/19	20/3/19	4	0.28	0.039	6:8	8	1	69	133	118
5	12/2/19	20/3/19	5	0.37	0.013	3:1	6	1	265	206	135

6	12/2/19	20/3/19	3	0.30	0.031	5:4	4	4	89	104	120
7	12/2/19	20/3/19	5	0.33	0.015	2:1	4	2	87	65	30
8	12/2/19	20/3/19	3	0.29	0.033	5.5:4.5	9	3	109	90	73
9	12/2/19	20/3/19	3	0.20	0.029	7:1	6	4	147	71	126
10	12/2/19	20/3/19	2	0.23	0.027	10:4	10	2	165	25	41
Average			3.6	0.29	0.03	5.5:3.3	7.7	3	113	90	89

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.6.5 Measurements for endophyte and pathogen (E7H1) treated plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	19/2/19	20/3/19	3	0.18	0.023	8:3	6	2	176	163	152
2	19/2/19	20/3/19	4	0.23	0.020	4:3	6	5	21	43	87
3	19/2/19	20/3/19	2	0.28	0.019	7.5:4	9	5	35	24	33
4	19/2/19	20/3/19	5	0.16	0.016	6:3	4	3	29	10	39
5	19/2/19	20/3/19	5	0.19	0.021	4:2	3	3	42	65	25
6	19/2/19	20/3/19	4	0.25	0.011	3:3	6	2	89	97	73
7	19/2/19	20/3/19	1	0.32	0.032	3.5:0	18	0	115	79	126
8	19/2/19	20/3/19	5	0.10	0.011	5:4	4	1	189	154	119
9	19/2/19	20/3/19	0	0.20	0.023	4:0	8	0	172	124	103
10	19/2/19	20/3/19	0	0.14	0.014	5:0	6	0	107	120	131
Average			3	0.21	0.02	5:2.2	7	2.1	98	88	89

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.6.6 Measurements for endophyte and pathogen (E10H1) treated plantlets for the leaf endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	19/2/19	20/3/19	3	0.25	0.035	7:6	10	3	123	101	84
2	19/2/19	20/3/19	4	0.25	0.023	3:2	4	2	45	65	24
3	19/2/19	20/3/19	1	0.20	0.024	3.5:2	3	3	292	242	287
4	19/2/19	20/3/19	3	0.26	0.039	4.5:3	6	4	142	197	119
5	19/2/19	20/3/19	2	0.34	0.037	5:4	16	2	178	189	122
6	19/2/19	20/3/19	4	0.30	0.027	4:3	8	5	32	20	31
7	19/2/19	20/3/19	2	0.28	0.021	3:3.5	14	4	278	290	303

8	19/2/19	20/3/19	0	0.27	0.031	5:4	15	2	335	546	507
9	19/2/19	20/3/19	1	0.33	0.032	5:4.5	16	4	318	225	412
10	19/2/19	20/3/19	4	0.20	0.022	3:2	4	2	156	132	205
Average			2.4	0.27	0.03	4.3:3.4	9.6	3.1	190	201	209

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.7.1 Measurements for control plantlets for the root endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	15/2/19	25/3/19	0	0.12	0.022	3.5:1	6	2	145	102	198
2	15/2/19	25/3/19	0	0.30	0.041	6.5:2	14	2	172	65	72
3	15/2/19	25/3/19	0	0.15	0.019	5:1	10	6	159	118	33
4	15/2/19	25/3/19	0	0.14	0.019	4:2	6	2	106	121	138
5	15/2/19	25/3/19	0	0.17	0.026	4:0	8	0	147	32	120
6	15/2/19	25/3/19	0	0.23	0.029	4.5:2	4	10	27	89	109
7	15/2/19	25/3/19	0	0.13	0.032	3.5:2	3	4	146	126	159
8	15/2/19	25/3/19	0	0.11	0.018	6:2.5	14	4	51	53	43
Average			0	0.17	0.03	4.6:1.6	8.12	3.75	120	88	109

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.7.2 Measurements for pathogen treated plantlets for the root endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	15/2/19	25/3/19	1	0.22	0.034	7:3	14	4	37	68	82
2	15/2/19	25/3/19	1	0.26	0.039	6.5:3.5	20	6	95	98	203
3	15/2/19	25/3/19	3	0.19	0.026	4.5:2.5	10	4	163	121	117
4	15/2/19	25/3/19	2	0.29	0.049	5.5:2	22	2	349	372	356
5	15/2/19	25/3/19	3	0.12	0.017	6.5:2	14	4	259	218	238

6	15/2/19	25/3/19	2	0.24	0.035	6.5:1	12	7	127	189	109
7	15/2/19	25/3/19	2	0.20	0.026	7:2	26	2	211	215	282
8	15/2/19	25/3/19	3	0.18	0.041	4:1	8	2	151	176	143
Average			2.1	0.21	0.03	5.9:2.1	15.75	3.87	174	182	191

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.7.3 Measurements for endophyte (ER1) treated plantlets for the root endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	15/2/19	25/3/19	4	0.02	0.020	4.5:1	6	7	182	129	94
2	15/2/19	25/3/19	4	0.18	0.033	10:5	4	4	204	135	162
3	15/2/19	25/3/19	5	0.08	0.019	7.5:2	6	4	219	222	245
4	15/2/19	25/3/19	4	0.02	0.024	7:1	12	2	314	234	118
5	15/2/19	25/3/19	4	0.11	0.025	5:4	8	6	152	69	154
6	15/2/19	25/3/19	3	0.02	0.020	6:2	3	1	93	164	201
7	15/2/19	25/3/19	2	0.04	0.024	4.5:1	12	3	195	43	24
8	15/2/19	25/3/19	3	0.06	0.015	6:3	4	3	110	198	87
Average			4	0.07	0.02	6.3:2.4	6.87	3.75	184	149	136

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

Table 5.7.4 Measurements for endophyte and pathogen (ER1H1) treated plantlets for root endophyte co-culture experiment

No.	D.O.I	D.O.O	DS	Fresh weight	Dry weight	S: R Ratio	No. of leaf	No. of roots	Leaf area		
1	15/2/19	25/3/19	4	0.19	0.033	4.5:1	7	12	10	15	27
2	15/2/19	25/3/19	4	0.23	0.039	3:1	6	5	287	72	40
3	15/2/19	25/3/19	5	0.16	0.033	3:0	3	0	123	145	45
4	15/2/19	25/3/19	4	0.22	0.033	7.5:3	6	2	110	230	133
5	15/2/19	25/3/19	5	0.11	0.018	4:1	4	2	120	147	144
6	15/2/19	25/3/19	5	0.30	0.032	5:1	4	8	169	162	154
7	15/2/19	25/3/19	4	0.09	0.023	5:3	6	4	310	192	208

8	15/2/19	25/3/19	5	0.08	0.016	3:1	8	4	137	168	182
Average			5	0.17	0.03	4.4:1.4	5.50	4.63	158	141	117

D.O.I = date of inoculation; D.O.O = date of observation; DS = Disease score = S: R = shoot (S) and root (R) ratio.

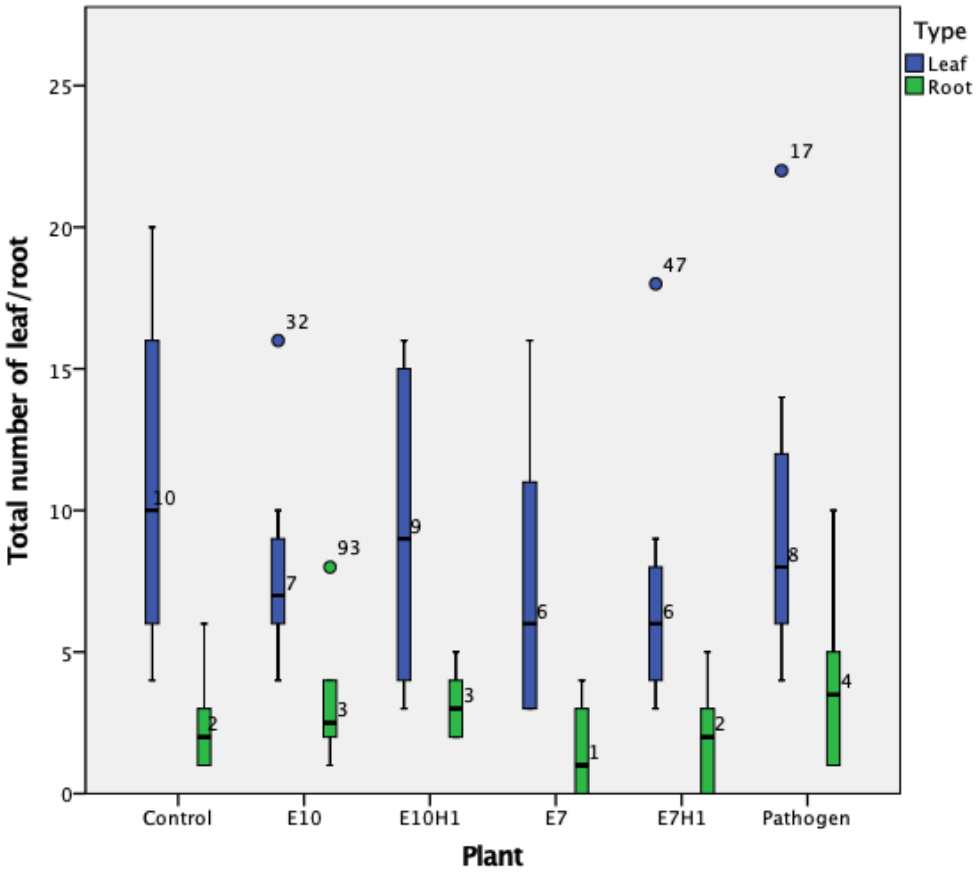

Figure 5.5 Number of leaves and roots for 10 replicates for all plantlets in the biocontrol experiment. Pathogen treatment = H1 on its own. Control = plants without endophyte or pathogen.

Images of the root endophyte biocontrol experiment are given below. Plantlets for control and pathogen are shown in Figure 5.6a and b; plantlets for endophyte (ER1) treatment are shown in Figure 5.6c and plantlets for endophyte plus pathogen treatment (ER1H1) are shown in Figure 5.6d.

Figure 5.6a and 6b Root endophyte biocontrol experiment (plate 1). a) Control plantlets treated with ultrapure water for co-culture experiments after five weeks, b) Plantlets treated with pathogens after five weeks.

Figure 5.6c and d Root endophyte biocontrol experiment (plate 2). c) Plantlets treated with endophyte (ER1) for co-culture experiments after five weeks, d) Plantlets treated with endophyte and pathogen (ER1H1) for co-culture experiments after five weeks.