

# Biocontrol Potential of Forest Tree Endophytes

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## Abstract

In the natural forest environment, the spread of pathogens may have dramatic effects on ecosystem functioning. To successfully control devastating forest pathogens, application of endophytes as biocontrol agents is an emerging area of research. There are several ways by which endophytic microorganisms can protect their tree hosts against pathogens. Endophytes promote plant growth by producing beneficial secondary metabolites (e.g. phytohormones) or providing nutrients (e.g. phosphorus). Endophytes can compete with pathogens and herbivores by successfully utilizing available substrates (colonization of shared niche can restrict pathogen invasion), or endophytes can produce antagonistic metabolites. Endophytes can enhance plant resistance by triggering and priming host defensive reactions. Endophytes could provide several opportunities for application in integrated pest management (IPM) to gain sustainable forestry practices. To utilize endophytes as biocontrol agents, the mechanisms behind the possible mode of action should be determined. Novel advances in cultivation-independent techniques including next generation sequencing technology (NGS), association analyses and network inference modelling will greatly facilitate identification of endophytes and unravel potential beneficial functions of endophytic communities. A further understanding of these mechanisms could help to minimize the use of environmental harming chemicals in plant and forest tree protection. We propose simple guidelines that could facilitate the use of fungal endophytes as biocontrol agents and simultaneously study their ecological functions.

## 1. Introduction

### *1.1. Forestry and forest tree-associated microbes*

The total forest area in the world is estimated at 4 billion hectares, which covers ca. 31 % of the total land area (FRA 2010). The economic value of forest trees, apart from wood removals, could be over 70 billion Euros (€) annually (in the period 2003-2007, <http://www.fao.org/forestry/fra/fra2010/en/>). Forests currently absorb billions of tons of CO<sub>2</sub> (Canadell and Raupach 2008) globally every year and stock permanently in their biomass (289 gigatonnes) of carbon (<http://www.fao.org/forestry/fra/fra2010/en/>). This makes forests important in climate change protection (Canadell and Raupach 2008). Climate change may increase the mean temperature of Earth, and forests will likely be subject to increased frequency and intensity of stress. Temperature and moisture can affect pathogen sporulation and dispersal, and changes in climatic conditions can favour certain pathogens (La Porta et al. 2008, Lilja et al. 2010). Sustainable management of forest is not only beneficial in mitigation of harmful effects of greenhouse gases (Patosari 2007) but equally as a potential alternative bio-energy source and by conserving biological diversity. Additionally, timber and associated products from forest trees contribute substantially to the revenue generation of many countries of the world. According to estimates, the demand for wood and forest products is expected to continue to grow in the next decade (FAO 2009) However, a major threat to the numerous benefits of forestry is pests and diseases. The need to sustain timber quality, increase and safeguard the use of renewable materials and bio-energy, and demand for novel wood products provide new challenges in the area of forest biotechnology and bioeconomy, particularly in tree health protection.

From the ecological point of view, forest trees share a habitat with diverse microbes and maintain dynamic balanced relationships with them. These relationships could vary from latent commensalistic relationship to mutualism or pathogenic infections. In the last 100 years, a number of notable severe forest tree disease outbreaks have been recorded, such as chestnut blight (Milgroom and Cortesi 2004), Dutch elm tree disease (DED) (Hardwood et al. 2011), root and butt rot diseases (Asiegbu et al. 2005), poplar rust caused by the biotrophic fungal pathogen *Melampsora larici-populina* (Steenackers et al. 1996, Dowkiw and Bastien 2004, Duplessis et al. 2009, Feau et al. 2009, Bennett et al. 2011), and very recently, sudden oak death caused by the oomycete pathogen *Phytophthora ramorum* (Grünwald et al. 2012). At the other extreme are the mutualistic endophytic microbes, which play important and economically significant roles in plant nutrition, nutrient cycling, growth, and health (Sieber 2007, Parent et al. 2009, Martín and Nehls 2009, Porras-Alfaro and Bayman 2011). Many of these are increasingly being considered or exploited for integrated pest management (IPM).

Endophytes, by definition, are microorganisms that can colonize inner plant tissues without causing any harm or disease symptoms (Saikkonen et al. 1998,

Ryan et al. 2008, Porrás-Alfaro and Bayman 2011). Both fungal and bacterial endophytes are considered ubiquitous, as there are no reports of plant species devoid of endophytes (Rosenblueth and Martínez-Romero 2006). Although endophytes and their interactions with hosts have been studied for decades, many details are still not well understood (Ryan et al. 2008, Sieber and Grünig 2013). Nevertheless, there has been an overwhelming number of studies demonstrating a wide range of benefits of endophytic colonization. In this review, we will focus on microbial endophytes and their potential as biocontrol agents (BCAs) in forest trees. Bacterial endophytes have been studied to a lesser extent in comparison to endophytic fungi (Izumi 2011). Furthermore, based on extensive literature survey, utilisation of BCAs in trees and woody plants is not as common as in agricultural crops, herbaceous species and seedlings (Cazorla and Mercado-Blanco 2016).

## **2. Biological control**

### ***2.1. Concept and principles of biological control***

Throughout their lifecycle, trees accumulate considerable amounts of biomass, making them rich sources of nutrients that attract both microbial pathogens and animal pests. In certain cases, the spread of pathogens and/or pests may have dramatic effects on forest ecosystem functioning. The textbook examples of forest tree diseases with particularly deleterious consequences are chestnut blight and DED. However, there are newly emerging threats for forest trees, which constitute a significant challenge for forest pathologists today, such as sudden oak death, eucalyptus rust, ash dieback and spread of emerald ash borer, to name just a few. Spread of pests and pathogens threatens both natural forests and commercial forest tree plantations, emphasizing the need for development of integrated pest management plans for forest ecosystems, similar to ones used in agriculture. However, there are important differences between the pest management approaches in agriculture and forestry. The use of pesticides in commercial forest plantations is controversial and strongly opposed by the public (Wagner et al. 1998, Hartley 2002). Additionally, country-specific regulations on the use of chemical control substances in forestry are often stricter than in agriculture. Large-scale pesticide treatments over extensive forest areas can become prohibitively expensive. For these reasons, alternative methods to control the spread of pests and diseases of forest trees are of particular importance. Approaches that can help to reduce the use of pesticides in forestry include improved plantation management, breeding of tree varieties with an improved pathogen resistance, and biological control of pests and diseases. In particular, biological control is gaining increasing support as an alternative to the conventional chemical control, as it is generally considered more environment-friendly and having less impact on biodiversity.

The following definition of biological control was provided by Eilenberg et al. (2001): “The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be”. The concept of ‘living organisms’ adopted by the authors included viruses, but genes and gene fragments were specifically excluded. Also, the use of certain metabolites of biological origin without organisms producing them was excluded from this definition. Four strategies of biological control can be distinguished based on the origin (native or introduced), the ability to reproduce after release, and the intended duration of the control (permanent or temporal): (a) classical biological control, (b) inoculation biological control, (c) inundation biological control and (d) conservation biological control (Eilenberg et al. 2001, Roderick and Navajas 2003). The classical biological control implies the introduction of an exotic species (biological control agent) into a new environment for permanent establishment and long-term control. This control strategy is often applied to control insect pests and weeds, but it is not used for control of plant pathogens. Inoculation biological control is based on the release of an organism (the control agent) that is able to multiply after the release and to control the pest (or pathogen) for a prolonged period, but not permanently. Inundation biological control is achieved by the released organisms (control agents) themselves. In this case, the control agents are usually released in high numbers to ensure immediate effect, but their ability to multiply in the environment is limited, and they do not provide long-term control. Conservation biological control does not require an introduction or an intended release of control agents. Instead, it is based on the modification of the environment to promote the development of specific natural enemies able to target the specific pest or the pathogen.

Nowadays, there are numerous examples of successful applications of biological control in agriculture. It is widely used to control animal pests (insects and nematodes), weeds, and microbial plant pathogens. The range of approved biological control agents is very broad (more than 400 species) and includes predator and parasitoid insects, mites, entomopathogenic fungi, specialized microbial pathogens of weeds, bacteria producing insect-specific toxins, microbial antagonists of plant pathogens, viruses, etc. (Fravel 2005, van Lenteren et al. 2017). As the application areas of biological control are expanding, the market for the biological control agents grows steadily, having reached the value of approximately US\$ 1.7 billion in 2015 (van Lenteren et al. 2017).

## ***2.2. Mechanisms of biological control***

The ability of biocontrol agents to control disease and to protect plants from pathogens has attracted the attention of numerous researchers. The research field developed rapidly in the second half of the 20th century, which greatly enhanced our understanding of biocontrol mechanisms. It became clear that various biocontrol agents differ in their modes of action, and, at the same time, several dif-

ferent mechanisms can simultaneously be employed by the same biocontrol organism. The known modes of action of biocontrol agents can be classified into two principal groups: mechanisms that directly reduce growth and development of the pathogen, and mechanisms that induce host responses. The interactions between biocontrol organisms and corresponding pathogens can either be direct, where physical contact occurs between two microorganisms, or indirect, *i.e.* without direct contact (Punja and Utkhede 2003, Tjamos et al. 2010, Narayanasamy 2013). There are numerous documented cases of hyperparasitism, or mycoparasitism if both organisms are fungi, showing that a biocontrol agent can directly parasitize on the targeted plant pathogen. For example, *Ampelomyces quisqualis* parasitizes the hyphae and conidia of powdery mildew fungi (Rotem et al. 1999), *Coniothyrium minitans* targets sclerotia of *Sclerotinia* spp. (Budge and Whipps 2001, Giczey et al. 2001), and the oomycete *Pythium oligandrum* is parasitic on other *Pythium* spp. and numerous phytopathogenic fungi (Benhamou et al. 1997, Madsen and de Neergaard 1999). Mycoparasitism requires that both interacting species are in a close proximity where the hyperparasite uses the content of the pathogen's cells as a source of nutrients, often through specialized structures (*e.g.* haustoria) (Narayanasamy 2013). Another example of the hyperparasitism-based approach to control a plant pathogen is the use of hypovirulent strains of *Cryphonectria parasitica* to control chestnut blight. The hypovirulence of the chestnut pathogen is caused by dsRNA mycoviruses. The virus-carrying strains are used for inoculation of chestnut trees, where the virus can spread in the pathogen population via hyphal anastomoses, resulting in decrease in the pathogen virulence and slower canker formation. Viral infection also causes reduction in asexual spore production and in sexual reproduction of *C. parasitica* (Milgroom and Cortesi 2004).

In many cases, biocontrol agents are able to inhibit pathogen growth and development without the need for a direct contact, because the compounds or enzymes secreted by biocontrol agents may have a strong inhibitory effect on the targeted pathogen. This type of interaction is referred to as antibiosis (Narayanasamy 2013). *Trichoderma virens*, an approved biocontrol agent, is able to produce the antifungals gliotoxin and gliovirin to control root-infecting fungi (Howell 2003). The yeast *Pseudozyma flocculosa* produces antifungals that affect membrane permeability (Avis and Belanger 2001) to control powdery mildew (Cheng et al. 2003). Many species of *Trichoderma* used in biocontrol are well-known potent producers of hydrolytic enzymes: chitinases,  $\beta$ -glucanases, and proteinases. Secreted chitinases and glucanases may have an important role in the degradation of cell wall polymers of fungal plant pathogens. *Trichoderma* mutants with lower levels of secreted enzymes are less effective as biocontrol agents (Punja and Utkhede 2003). Inactivation of *Botrytis cinerea* hydrolytic enzymes by cysteine proteases secreted by *Trichoderma harzianum* has been proposed to have a role in the biocontrol of *Botrytis* infection (Kapat et al. 1998, Elad and Kapat 1999). Some *Trichoderma* species are able to produce both antibiotic compounds and secreted hydrolytic enzymes, and their synergistic action in biocontrol has been reported (Schirmbock et al. 1994, Lorito et al. 1996).

Another mechanism involved in the biocontrol of plant diseases is competition for nutrients and niche colonization. In many cases, plant pathogens are unable to penetrate intact plant surfaces, and they require specific points of entry to infect their hosts. Certain biocontrol agents are able to block the potential entry points, outgrow and outcompete the pathogen or prevent the germination of its propagules. This strategy is successfully used in the control of wound infections, where species of *Trichoderma* and *Gliocladium* are applied, but also to prevent infection of tree stumps by *Heterobasidion annosum s.l.* through application of *Phlebiopsis gigantea* immediately after cutting. More efficient nutrient utilization by biocontrol agents also contributes to the success in competition against plant pathogens (Adomas et al. 2006). Specific biocontrol yeasts compete with the fruit-rotting pathogens for availability of sugars (Filonow 1998), whereas competition for iron has a role in the control of soil-borne pathogens by pseudomonads (Kloepper et al. 1980).

All above-mentioned mechanisms refer to direct or indirect interactions between biocontrol agents and plant pathogens. However, there is an increasing number of evidence demonstrating the importance of interactions between the biocontrol agents and their host plants for efficient biocontrol (Shoresh et al. 2010). Most of these studies were made on interactions between *Trichoderma* species and their host plants (Shoresh et al. 2010), but such data have been obtained from other species, as well, e.g. *Piriformospora indica*, nonpathogenic strains of *Fusarium oxysporum* and the oomycete *Pythium oligandrum* (Punja and Utkhede 2003, Shoresh et al. 2010). The data indicate that colonization of plant tissues by biocontrol fungi is accompanied by a large-scale reprogramming of the host plant gene expression. One of the most important outcomes of these transcriptional changes is an activation of induced systemic resistance (Shoresh et al. 2010). However, biocontrol fungi are also able to induce plant responses that increase their tolerance to abiotic stressors and improve their nitrogen utilization efficiency (Shoresh et al. 2010). Induction of systemic resistance by *Trichoderma* spp. occurs via jasmonic acid and ethylene signaling pathways (Shoresh et al. 2010). It causes activation of a number of defense- and stress-related genes, including genes encoding pathogenesis-related (PR)-proteins, components of phenylpropanoid pathway, enzymes involved in cell wall reinforcement and oxidative enzymes (oxidases and peroxidases) (Shoresh et al. 2010). Additionally, biocontrol fungi are able to stimulate plant growth and increase plant vigor (Shoresh et al. 2010).

### ***2.3. Overview of biological control systems in forest tree diseases***

Biological control of pests and plant diseases is nowadays applied in agriculture and horticulture worldwide, and the number of commercially available products is growing steadily. However, the examples of biocontrol products in forestry and silviculture remain rather few (Cazorla and Mercado-Blanco 2016), despite the growing number of pathogens threatening both natural forests and commercial plantations.

Probably, the best-known example of biocontrol of a forest tree pathogen is *P. gigantea*, which controls spread of root and butt rot pathogens in the genus *Heterobasidion* in managed conifer tree plantations. It was one of the first biocontrol products that became commercially available over 50 years ago, mentioned in numerous textbooks (Campbell 1989, Van Driesche and Bellows 1996, Sun et al. 2009). *P. gigantea* is a saprotrophic white-rot fungus that can efficiently colonize freshly cut stumps of conifer trees, being able to outcompete *Heterobasidion* and prevent infection of stump surfaces. Suspension of *P. gigantea* spores (oidia) is commonly applied on the stump surface immediately after cutting. Use of *P. gigantea* as a biocontrol agent is approved in Canada, United Kingdom, Finland, Sweden, Norway, Switzerland and Poland (Pratt et al. 2000, Ravensberg 2015, van Lenteren et al. 2017). Importantly, the use of *P. gigantea*, while efficiently preventing the stump colonization by *Heterobasidion* fungi, also has a minimal impact on the structure of microbial communities in treated stumps (Sun et al. 2013, Terhonen et al. 2013a).

Another textbook example on biocontrol of trees is the use of hypovirulent strains of *C. parasitica* to control chestnut blight disease. *C. parasitica*, the causative agent of chestnut blight, is an ascomycete that was introduced to North America and Southern Europe from East Asia in the first half of 20<sup>th</sup> century. Both North-American and European chestnut species are highly susceptible to this pathogen. Its introduction had devastating consequences, as it caused nearly entire eradication of American chestnut (*Castanea dentata*) from North-American forests. The effects on European chestnut (*Castanea sativa*) were severe, but far less dramatic than in North America. In early 1950s, chestnut trees that showed symptoms of recovery from the disease were found. Cankers produced by the fungus on the trees were healing or growing very slowly. Additionally, fungal spores were produced in low numbers, and the disease progress was slower (Campbell 1989). Strains of *C. parasitica* isolated from these trees showed lower levels of virulence (hypovirulence), which was caused by the presence of double-stranded RNA (dsRNA) viruses of the family *Hypoviridae* (Milgroom and Cortesi 2004). This discovery opened a way to control the spread of chestnut blight in Europe. As these viruses are able to spread via hyphal contacts, inoculations of chestnut trees with hypovirulent strains of *C. parasitica* can be used to control the disease. However, the formation of hyphal anastomosis is possible only between compatible fungal strains, and their vegetative incompatibility can restrict the spread of virus. Large-scale inoculations with hypovirulent strains were performed in Southern Europe, and the epidemic situation there has been greatly improved. However, attempts of controlling chestnut blight using hypovirulent *C. parasitica* strains in North America had limited success, reasonable in Michigan, but a nearly complete failure was faced in eastern North America (Milgroom and Cortesi 2004). The factors determining the success of the introduction of hypovirulent strains have not been fully determined yet (Milgroom and Cortesi 2004).

A similar strategy of using hypovirulent fungal strains to control DED has been tested (Swinton and Gilligan 1999), without reaching the practical stage. Nevertheless, an alternative biocontrol-based method is currently used to prevent DED infection of elm trees. In this approach, the conidiophore suspension of *Verticillium*

*albo-atrum* strain WCS850 is injected into vascular system of elm trees. The inoculation is assumed to induce resistance of the trees and the biocontrol agent does not interact with the pathogen (Postma and Goossen-van de Geijn 2016). It was shown to be an efficient preventive treatment, but with a number of serious limitations. The treatment needs to be repeated annually for efficient protection, each tree has to be treated individually, and the treatment costs are relatively high. For these reasons, only highly valuable trees, *i.e.* old, ornamental, and characteristic landscape trees, are treated. The biocontrol product is commercialized under the trade name Dutch Trig, and its use is currently approved in The Netherlands, USA, Canada, Germany, and Sweden (Postma and Goossen-van de Geijn 2016, van Lenteren *et al.* 2017).

Several species of *Trichoderma* are well known as potent antagonists of plant-pathogenic fungi. *Trichoderma harzianum* and *T. polysporum* are approved in the USA and some EU countries as an agent against pathogens for wound treatment of shade, ornamental and forest trees (Fravel 2005, Ruocco *et al.* 2011). *Trichoderma*-based formulations are also used to control damping off caused by *Fusarium* spp. in forest nurseries (Gromovykh *et al.* 2007). Additionally, several prospective agents to control tree diseases are currently evaluated. The examples include the use of bacterial endophytic strains as potential control agents against canker-causing fungi on poplar (Ren *et al.* 2013) and against fungal and bacterial wilts (Eljounaidi *et al.* 2016) and the application of various fungal endophytes in control of cacao tree (*Theobroma cacao*) pathogens (Mejía *et al.* 2008).

Furthermore, there are a number of biocontrol products used in forestry against insect pests. Most of the commercially available formulations are based on *Bacillus thuringiensis* strains, which produce a potent insect-specific toxin. Their target species are mainly various lepidopteran larvae, *e.g.* caterpillars of gypsy moth (Ravensberg 2015, van Lenteren *et al.* 2017). Additionally, two insect viruses are approved for use against sawflies in Canada (van Lenteren *et al.* 2017).

### 3. Endophytes

#### 3.1. Endophytes in Plant Fitness

As highlighted in the introduction chapter of this book, two major groups of endophytic fungi have been recognized, clavicipitaceous endophytes (C-endophytes) and the non-clavicipitaceous endophytes (NC-endophytes) (Rodriguez *et al.* 2009). Clavicipitaceous endophytic fungi are further assigned to Class 1 and the non-clavicipitaceous endophytic fungi to Classes 2, 3 and 4 according to host range, colonized plant tissue, biodiversity, transmission, fitness benefits and *in planta* colonization (Rodriguez *et al.* 2009).



The relationship between Class 1 endophytes and grasses is usually defined as defensive mutualism. It seems that the ecological role of these endophytes is to produce bioactive alkaloids, hormones, and other metabolites (Bacon et al. 1977, Hamilton et al. 2012, Panaccione et al. 2013, Saikkonen et al. 2004, Siegel and Bush 1996, Siegel et al. 1990), which can provide protection to the host against herbivores and pathogens (Clay and Schardl 2002, Schardl et al. 2004, Saikkonen et al. 2013). Beside the metabolite production, the transcriptomes of *Epichloë festucae* and its host, *Lolium perenne*, at different developmental stages indicated that the fitness-enhancing effects of the endophyte are based both on systemic alteration of the host's hormonal responses and induction of stress response genes (Schmid et al. 2017).

Only Class 2 endophytes have been shown to have the ability to confer habitat-specific stress tolerance to host plants (Rodriguez et al. 2008). This was described as endophyte-conferred fitness benefits that is habitat-adapted if the benefits are a result of habitat-specific selective pressures such as pH, temperature and salinity (Rodriguez et al. 2009).

An important functional role of aerial non-clavicipitaceous endophytes (Class 3) in the ecosystem include possession of the ability to protect the host against pathogens/herbivores as reflected by the production of secondary metabolites (Schulz et al. 1999, Sumarah et al. 2008a), niche competition (Blumenstein et al. 2015), or induction of systemic resistance (Mejía et al. 2014). The best understood example is the impact of toxigenic foliar endophytes of white spruce against the needle herbivore *Choristoneura fumiferana* (eastern spruce budworm). The rugulosin-producing endophyte *Phialocephala scopiformis* DAOM 229536 (Helotiales, Ascomycota) delays the development of this destructive pest, exposing the larvae for an extended period to birds, parasitoids, and pathogens prior to moth formation (Miller et al. 2008). Overall, aerial endophytes are capable of modifying plant fitness partly due to metabolic production, induced resistance or competition. The mechanisms behind this are not fully understood and even some of the endophytes do not have any influence to the hosts, the extremely high biodiversity indicates that they are ecologically important.

One of the positive responses of Class 4 fungal endophyte colonization includes the modulation of plant growth via nutrient acquisition (as in mycorrhizae) (Jumpponen 2001, Mandyam and Jumpponen 2005, Newsham 2011). The other beneficial aspect is the production of plant growth-promoting phytohormones (Schulz et al. 1998, 2002, Schulz and Boyle 2005). Other notable interesting function that root fungal endophytes possess is the production of unique secondary metabolites with potential benefits to the host through promoting plant growth (Figure 3.1) or limiting pathogen spread (Figure 3.2) (Schulz et al. 1999, 2002, 2015, Mandyam and Jumpponen 2005, Tellenbach and Sieber 2012, Terhonen et al. 2016). At the other end are the bacterial root endophyte communities also known to play important roles in maintaining root health (Backman and Sikora 2008).

Functionally, the beneficial effects of bacterial endophytes can sometimes have higher impact on the host compared to rhizosphere bacteria (Pillay and Nowak 1997). Similar to rhizobacteria, bacterial endophytes like their fungal counterparts

can promote plant health and growth in many ways such as manipulation of phytohormones (Lee et al. 2004), providing necessary nutrients to the plant like solubilizing phosphate (Puente et al. 2009, Chimwamurombe et al. 2016), stimulate growth and improve survivability of host under abiotic stress conditions (Barka et al. 2006). Other essential feature of bacterial endophytes is their ability to suppress many plant pathogens. Disease suppression and growth promotion are interlinked features for the plant in such a way that both have impacts on plant health. An indirect way of disease suppression by endophytes is promotion of plant health and growth. Healthy growth conditions raise robustness of plants and reduce the host vulnerability to pathogens (Mercado-Blanco and Lugtenberg 2014).

Another beneficial feature of endophytes that has received increased attention recently is their ability to increase survivability of their host under environmental stress conditions and ultimately, to improve plant's phytoremediation capability (Taghavi et al 2005). Bacterial endophytes have also the capacity to degrade some pollutants and improve plant's overall tolerance to xenobiotic substances (Newman and Reynolds 2005). Some bacterial endophytes can produce substances like antibiotic, anti-fungal or other novel compounds (Ezra et al. 2004, Ryan et al. 2008) as well as lytic enzymes like chitinases and hydrolases (Frankowski et al. 2001, Cherrin and Chet 2002, Rashid et al. 2012) in order to reduce or inhibit pathogen growth. Additionally, bacterial endophytes can induce plant's defence response by releasing pathogen elicitors (Chimwamurombe et al. 2016).

### ***3.2. Endophytes as biocontrol agents***

#### ***3.2.1. Biocontrol potential of fungal endophytes***

There are several ways that endophytic fungi can protect their host against pathogens. The following are the possible potential mechanisms:

- i. Promoting plant growth
  - a. Production of secondary metabolites (*e.g.* phytohormones)
  - b. Providing nutrients to the host (*e.g.* translocation of phosphorus)
- ii. Competition with pathogens and herbivores
  - a. Substrate utilization and colonization of shared niche can inhibit or restrict the invasion of harmful pathogens
  - b. Production of antagonistic metabolites
- iii.
  - a. Induction of host defenses (*e.g.* induced systemic resistance, ISR)

**Aerial endophytes** Most of the studies that explore utilization of fungal endophytes in plant protection against pathogens rely on culturable endophytes. There is, however, evidence that this approach grossly underestimates the real biodiversity, as

unculturable endophytes are not accounted for. The composition of root endophytes in the same host species at different forest sites can be divided into a few major species and minor fungal lineages, which differ between sites (Menkis et al. 2004, Grünig et al. 2006, Terhonen et al. 2013b). However, among foliar endophytes (Class 3), a relatively small number of species have been reported to dominate the fungal community (Arnold et al. 2001, Gazis and Chaverri 2010, González-Teuber 2016). Inducing growth of the dominant endophytic species can have adverse negative effect on pathogens and herbivores. Preszler et al. (1996) showed that a higher infection frequency of the dominant fungal endophyte in oak trees reduced parasitism of a leaf-mining moth. Similarly, González-Teuber (2016) showed that colonization by the four dominating genera of foliar endophytes resulted in lower levels of damage by fungal pathogens and herbivores of the host plant (*Embothrium coccineum*) in natural conditions. In the tropical tree *Theobroma cacao*, a combination of the six most dominant endophytes effectively reduced *Phytophthora* sp. symptoms on the host (Arnold et al. 2003). Several other studies have also shown that endophytic fungi can help limit pathogen damage on *T. cacao* (Evans et al. 2003, Holmes et al. 2004, Rubini et al. 2005, Tondje et al. 2007, Mejía et al. 2008, Hanada et al. 2010).

Ganley et al. (2008) demonstrated that fungal endophytes can mediate resistance against *Cronartium ribicola* and thereby increase host fitness in *Pinus monticola* (Western white pine). These findings were considered extremely important when searching for biocontrol agents against this aggressive invasive pathogen. *C. ribicola* was introduced to North America in the early 1900s and it has decimated native Western white pines. Ganley et al. (2008) found that pre-inoculation of *P. monticola* seedlings with fungal endophytes could increase survival against *C. ribicola*. Similarly, pre-inoculation of young needles with endophytes at approximately the same time as natural infection by *Dothistroma septosporum* occurred, the endophytes were able to modify disease severity (Ridout and Newcombe 2015).

Gazis and Chaverri (2015) showed that indigenous endophyte communities of rubber tree (*Hevea brasiliensis*) contain a high diversity of beneficial fungi (*i.e.*, *Trichoderma* and *Tolyposcladium*) that may protect the host against pathogens (*i.e.* protective mutualism). Genotypes of forest trees with beneficial endophytes could be enriched in newly established plantations. Alternatively, supporting and increasing the endophytic genera of healthy foliage could help to decrease disease incidence and pathogen populations (Christian et al. 2017). Another good example of niche competition was reported by Blumenstein et al. (2015). They showed that carbon utilization profiles of the virulent DED pathogen *Ophiostoma novo-ulmi* and endophytic isolates of four asymptomatic elm (*Ulmus* spp.) trees exhibited significant niche overlap. This suggests that some endophyte strains might protect elms against DED through competition for substrates.

### **Belowground fungal endophytes**

There are very few studies on the use of root endophytes to protect host trees against pathogens. Tellenbach and Sieber (2012) showed that some strains of *Phialocephala subalpina* could reduce disease intensity caused by the two oomycete root rot pathogens *Elongisporangium undulatum* and *Phytophthora plurivora* in *Picea abies*

(Norway spruce) seedlings. Similarly, Terhonen et al. (2016) reported that the root endophyte *Phialocephala sphaeroides* isolated from Norway spruce (Terhonen et al. 2013b) was able to prevent the infection of seedling roots by the pathogen *H. parviporum* *in vitro*. The global plant trade combined with climate change creates the risk of introducing new non-indigenous tree pathogens with risks of new disease outbreaks in native forest ecosystems (Pautasso et al. 2015). If these invasive pathogens continue to spread via the nursery pathway to forest sites during outplanting, they will potentially pose a threat to forestry globally. Consequently, protection of conifer roots in their early development by biocontrol agents against indigenous and non-indigenous root pathogens is of primary importance.

### **Protection of host trees through metabolites secreted by endophytes**

Fungal endophytes secrete a diverse range of metabolites: amides, amines, peptides, flavonoids, steroids, phenylpropanoids, lignans and terpenoids (Schulz et al. 1995, 2002, Tan and Zou 2001, Stadler and Hellwig 2005, Stadler 2011). The secreted metabolites from endophytic fungi might provide benefits to the host trees or suppress pathogen growth (Fig. 3.2) (Schulz et al. 1999, 2002, Strobel 2003, Mandyam and Jumpponen 2005, McMullin et al. 2015, Sumarah et al. 2015). The metabolites from endophytes of forest trees could be new sources of natural antimicrobials to be utilized in the fields of agriculture, pharmaceuticals and forestry.

McMullin et al. (2015) reported several antifungal metabolites from endophytes of *Picea rubens* (red spruce) and *P. mariana* (black spruce). Crude liquid culture extracts of the endophyte *Diaporthe maritima* from *P. rubens* and *P. mariana* possessed potent antifungal activity against the biotrophic pathogen *Microbotryum violaceum* (Tanney et al. 2016). They extracted and characterized three dihydropyrones, phomopsolides A, B, and C, and a stable alpha-pyrone, which demonstrated *in vitro* antimicrobial activity against *Bacillus subtilis*. Sumarah et al. (2015) extracted secondary metabolites produced by 22 strains of foliar endophytes of Western white pine, having antifungal activity. Of these, homodimeric macrolide pyrenophorol inhibited the growth of *C. ribicola*. DNA sequencing of the 22 endophytic strains revealed that the majority were *Lophodermium nitens*. These results further suggest that several antifungal metabolites produced by *L. nitens* possess the potential to increase the tolerance of the host tree to the white pine blister (Sumarah et al. 2011, Richardson et al. 2014, 2015, McMullin et al. 2015, Sumarah et al. 2015).

Endophytic metabolites can also protect the host against herbivory. The example of the anti-insect toxin (rugulosin) produced against herbivorous insect *C. fumiferana* is discussed in detail in this volume by J. D. Miller. This metabolite has been identified and characterized from phylogenetically diverse foliar endophytes of conifers (Findlay et al. 2003, Sumarah et al. 2008a, Sumarah et al. 2010). Beside rugulosin, *P. scopiformis* also produces emodin and skyrin (Calhoun et al. 1992, Miller et al. 2002). Rugulosin is toxic to the spruce budworm *C. fumiferana* at a concentration as low as 10-25  $\mu\text{M}$  (Sumarah et al. 2008b). Studies have shown that when this fungus is pre-inoculated on white spruce needles, the growth rate of *C. fumiferana* is significantly reduced (Miller et al. 2002, Miller et al. 2008, Frasz et al. 2014). It is also reported that foliar endophyte *P. scopiformis* can persist within

inoculated seedlings for at least a decade, with active rugolysin secretion in the needles (Frasz et al. 2014). Another foliar endophyte with anti-insect potential, *Diaporthe maritima*, has been reported to secrete potent secondary metabolites toxic to herbivores (Tanney et al. 2016).

Tellenbach et al. (2012) isolated four major compounds from the root fungal endophyte, *Phialocephala europaea*. Two of these compounds (sclerin and sclerotinin A) were demonstrated to significantly reduce the growth of *Phytophthora citricola sensu lato*. They concluded that two antifungal metabolites are either individually or synergistically responsible for growth inhibition of the oomycete pathogen observed *in vitro*. Similarly, Terhonen et al. (2016) observed that extracted metabolites from liquid cultures of root endophytes *P. sphaeroides* and *Cryptosporiopsis* sp. inhibited *Heterobasidion parviporum*, *Phytophthora pini* and *Botrytis cinerea* (Fig. 3.3)

### **Induction of host resistance by endophytes**

Endophytes are often confronted with host defence systems during invasive growth within plant tissues. In order to grow asymptotically in their hosts, fungal endophytes must maintain multiple balanced antagonisms – with the host and with the other microbial competitors (Schulz et al. 2015). Although most pathogenic fungi are able to overcome the defense reaction of their host, leading to disease, endophytes, on the other hand, can tolerate the host defense in order to infect and colonize the tissues (Schulz et al. 1999). By altering the hormonal balance in plants, pathogens use the defensive machinery of plants to their advantage and either induce or suppress the processes relevant for cell death and accumulation of antimicrobial compounds (Robert-Seilaniantz et al. 2011, Kovalchuk et al. 2013). The ability of endophytes to colonize host tissues asymptotically could be attributed to their ability to modulate host signaling molecules, phytohormones, e.g. endogenous concentrations of JA and SA (Navarro-Meléndez and Heil 2014). Eaton et al. (2010) observed that mutation in a mitogen-activated protein kinase of the endophytic fungus *Epichloë festucae* changed the mutualistic fungus into an invasive pathogen. The mutant produced significantly lower amounts of secondary metabolites *in vivo*, triggered a strong host defense response, caused a stunted host phenotype, reduced host anthocyanin production, and induced changes to all major host hormone signaling pathways (Eaton et al. 2010). These results underline that a complex interplay of plant and in-plant expressed fungal genes is required to maintain the mutualistic coexistence (Eaton et al. 2010).

The actual mechanism behind mediation of host disease resistance by endophytes is however unknown. Besides competition and metabolite production, endophytic colonization could activate the host genetic resistance. Inoculation of *T. cacao* leaves with the dominant foliar fungal endophyte *Colletotrichum tropicale* enhanced the expression of several host genes known to contribute to defense against pathogen and herbivore attack (Mejía et al. 2014). This indicated that foliar fungal endophytes could influence host disease resistance (Mejía et al. 2014). Raghavendra and Newcombe (2013) inoculated several genotypes of *Populus* sp. with diverse foliar endophytes (*Stachybotrys* sp., *Trichoderma atroviride*, *Ulocladium atrum* or *Truncatella angustata*), followed by challenge inoculation with

the pathogenic *Melampsora* species. The observed differences between genotypes pre-inoculated with endophytes and their controls explained 54% of the total variation in quantitative resistance (*i.e.* rust severity). They concluded that endophytes contributed significantly to quantitative resistance against *Melampsora* in leaves of poplar (Raghavendra and Newcombe 2013). Evidently, pre-inoculation with foliar endophytes can enhance resistance in host, but it is however not known whether the beneficial effects are facilitated by a single isolate or multiple endophytic isolates.

Results from agricultural crops clearly indicate that fungal endophytes can help plants resist pathogens. Fungal endophytes could provide several opportunities for utilizing them in integrated pest management to gain sustainable forestry practices. To utilize endophytes as biocontrol agents, the mechanisms behind the possible inhibition of the pathogen should be determined. We propose simple guidelines that could facilitate evaluation of the potential use of fungal endophytes as biocontrol agents and simultaneously study their other ecological functions:

- i. The fungal endophytes of healthy host trees should be isolated, correctly identified and stored in culture collections
- ii. Screening of potential biocontrol endophytes against a specific pathogen should be done *in vitro* by the dual-culture method
- iii. Bioassay of liquid culture extracts against the host pathogen should be performed to test if the antagonistic effect is due to extractable metabolites
- iv. Inoculation studies of the most promising endophytes on seedlings of host trees should be performed to assess host reaction
- v. The ability of fungal endophytes to protect the host from the pathogen should be determined
- vi. The persistence of inoculated endophytes in the host tree should be determined in the host's natural environment

#### **Use of endophytic bacteria in biocontrol**

Despite the fact that the majority of research of BCAs is based on agricultural crops, there have been few successful studies on the application of bacterial endophytes on forest trees. To apply endophytic microbes as a commercial product for biocontrol or plant growth promotion, the product must be officially registered as a plant protection product (PPP). Until 2014, there have been no reports of bacterial endophytes as plant protection products (Mercado-Blanco and Lugtenberg 2014). Endospore-forming bacteria can potentially be a superior alternative to fungal endophytes (Melnick et al. 2008). Many factors can influence the ability of an endophyte as BCAs. Some of the factors include population dynamics and host colonization patterns, motility of the endophyte within host tissues, the ability to induce systemic resistance, and host specificity (Backman et al. 1997, Melnick et al. 2008).

It is likely that the most efficient BCAs are not isolates of one species, but rather a community of different microbes that contain desirable genes, which can further improve a plant's health when colonized. An earlier study showed that single inoculation of an endophytic strain led to depression of growth in red clover, whereas inoculation with mixture of endophytes resulted in growth promotion (Sturz et al. 1997). A consortium of some endophytic fungi along with endophytic

bacteria reduced 90% of the damage caused by root pathogenic nematode (*Radopholus similis*) on banana plants (Glick 2015). A significant proportion of the indigenous endophytic bacterial community in plant roots can possess the capacity to produce antibiotics against fungal pathogens (Mercado-Blanco and Lugtenberg 2014). Application of indigenous bacterial root endophytes (*Pseudomonas* spp.) was shown to have inhibitory effect *in vitro* against *Verticillium* wilt in Olive trees (Prieto et al. 2011). *Verticillium* wilt is caused by the soil-borne pathogenic fungus *Verticillium dahliae*. In the last two decades, *Verticillium* wilt of olive (VWO) has become a major problem in olive plantations, as it is difficult to manage (López-Escudero and Mercado-Blanco 2011). Melnick et al. (2011) reported that about 23% of the 69 endospore-forming bacterial isolates of *T. cacao* demonstrated *in vitro* chitinase production. Additionally, a considerable proportion of the endophytes showed antagonism against one of the three known plant pathogens: 49% inhibited *Phytophthora capsici*, 33% inhibited *Moniliophthora perniciosa* and 42% inhibited *Moniliophthora roreri*. Furthermore, 22% of the isolates had the capacity to inhibit all three pathogens (Melnick et al. 2011).

Despite the promising results, it does not seem that a single application of a BCA would ensure long-term protection of plants. This is partly because the inoculation of bacteria had no permanent effect on the native bacterial communities of cacao trees (Melnick et al. 2011). Some endophytes appear to be more aggressive than the others and can out-compete and displace others (Verma et al. 2004). Perhaps, in order to have biocontrol agents that can cause a long-term shift in the native endophyte community and provide long-term protection of plants, we may need to look into the genetic traits of more aggressive endophytes and focus on genetic modification of inoculated endophytes. Downing et al. (2000) managed to use a genetically modified strain of endophytic *Herbaspirillum seropedicae* as a biocontrol against larvae of African sugar cane borer. The gene encoding insecticidal protein was extracted from *B. thuringiensis* strain. Melnick et al. (2008) evaluated the potential of four *Bacillus* isolates to act as BCA on cacao trees (*T. cacao*). The results revealed increased resistance of foliage of inoculated cacao plants against a pathogenic oomycete, black pod rot (*P. capsici*). They suggested that *Bacillus* spp. suppressed the pathogen by increasing systemic resistance of the host. The disease suppression continued for >68–70 days. Re-inoculation may be required because of perennial non-deciduous nature of cacao tree leaves (Melnick et al. 2008). In another study, Brooks et al. (1994) evaluated the use of bacterial endophytes as BCAs. The endophytes were extracted from surviving oak trees in an area where oak wilt caused by *Ceratocystis fagacearum* was epidemic. Around 21% of bacterial isolates were tested *in vitro* for inhibitory effect against oak wilt. Interestingly, oak samples that were pre-inoculated with *Pseudomonas denitrificans* prior to introduction of *C. fagacearum* displayed 50% reduction in oak wilt occurrence and 17% reduction in crown loss (Brooks et al. 1994).

A potential disadvantage in the use of endophytic bacteria as BCAs is that some of the prominent bacterial endophytes are also known as latent plant pathogens and a few are closely related to human pathogens. Under certain conditions or in different plant genotypes, these bacterial species may become pathogenic against plants (Kobayashi and Palumbo 2000, Misaghi and Donndelinger 1990, Ulrich et

al. 2008). There is also the theoretical risk of BCAs being pathogenic to humans. In the past, there have been recorded outbreaks of salmonella that were suggested to originate from *Salmonella* endophytes in alfalfa sprouts (Ponkä et al. 1995, Rosenblueth and Martínez-Romero 2006). Therefore, prior to application of any endophyte as a BCA, all aspects of potential hazard to ecosystem and human health must be evaluated and considered.

Another disadvantage of bacterial endophytes as BCAs is lack of field results. Most of the studies of antagonisms of endophytes against host pathogens have been conducted *in vitro* (Rosenblueth and Martínez-Romero 2006). These experiments may have a different outcome when tested in natural habitats with much larger plant-microbe interactions and versatile competition between microbes.

## 4. Methods for isolation, identification and bioassay of endophytes

### 4.1. Isolation methods

Conventional isolation methods on artificial media require sufficient surface sterilization of the plant samples (*e.g.* leaves, stems and roots) (Schulz et al. 1998). The objective is to remove and kill the epiphytic microbial growth on the plant surfaces. This is achieved by applying strong oxidant or general disinfectant, followed by rinsing with sterile water to remove the sterilant (Stone et al. 2004). A combination of the sterilant with a wetting agent (ethanol) can also improve the efficacy of sterilization (Stone et al. 2004). Sometimes surfactants (*e.g.* Tween 20) are combined with the sterilant to lower the surface tension (Stone et al. 2004). The sterilization protocol usually consists of several steps: optional washing of the plant sample (*e.g.* root samples to remove soil particles) under tap water, sterilization in ethanol, followed by sodium hypochlorite (or hydrogen peroxide) treatment. An additional sterilization step with ethanol can be included, after which the plant material is rinsed 3 to 5 times with sterile water (Petrini and Dreyfuss 1981). The shorter version with sufficient success is soaking the plant tissues in ethanol, then in sodium hypochlorite and finally in ethanol (Luginbuhl and Muller 1980, Petrini et al. 1992, Schulz et al. 1993, Sieber et al. 1999).

The incubation times and concentrations of ethanol and sterilant vary between studies. Below is listed the most common method (see also Stone et al. 2004, Fröhlich et al. 2000, Arnold 2002, Arnold et al. 2003, Terhonen et al. 2013b):

- |      |                                     |                 |
|------|-------------------------------------|-----------------|
| i.   | Ethanol 70%-99%                     | 5 s to 1 min    |
| ii.  | NaOCl 0.5 %-10%                     | 2 min to 10 min |
| iii. | Or H <sub>2</sub> O <sub>2</sub> 3% | 5 min           |
| iv.  | Ethanol 70%-99%                     | 30 s to 2 min   |
| v.   | Sterile water (3-5 x)               | 30 s            |



The time required for each step can vary depending on the plant tissue, *e.g.* needles with thick wax layer need longer sterilization time than deciduous leaves (Schulz et al. 1998, Schulz and Boyle 2005, Hyde and Soyong 2008). The sterilization must be sufficient to remove microbes from the plant surface but not excessive to destroy the tissue (Schulz and Boyle 2005). A pilot study may be necessary to determine suitable sterilization method before the actual experiment. It is crucial to confirm that the surface sterilization is successful, as all bacteria and fungi that are subsequently isolated from the plant samples are presumed to be endophytic. This can be verified by pressing the sterilized plant tissue on to a suitable agar plate as described by Schulz et al. (1998), or by incubating the last rinsing water on an agar plate. However, the review by Lodewyckx et al. (2002) highlighted that no protocol of surface sterilization can kill 100% of surface bacteria, unless disinfection is able to penetrate interior tissues and thereby killing endophytic bacteria as well.

The artificial medium used for isolation of endophytic fungi usually contains 2% malt extract (MEA) or potato dextrose agar (PDA) together with additional antibiotics to prevent any bacterial growth (*e.g.* penicillin, streptomycin sulphate, tetracyclin) (Petrini et al. 1992, Schulz et al. 1998). Several different types of medium can be used for isolation of bacterial endophytes, *e.g.* Yeast Peptone Dextrose Agar (YPDA), Brain Heart infusion medium (BHI), Luria agar (LA), King's B agar (KBA) (Cankar et al. 2005, Long et al. 2010, Rashid et al. 2012). However, Tryptic soy agar (TSA) seems to be the most common medium used (Sturz et al. 1998, Surette et al. 2003, Rashid et al. 2012). Bills and Polishook (1992) showed that more isolates and species can be recovered using several types of media. To reveal the true diversity of endophytic species can be difficult by using only culture-based methods (Arnold et al. 2007). The problem with culture-based methods is that many fast-growing fungi will be isolated at the expense of unculturable or slow-growing fungi (Hyde and Soyong 2007, Hyde and Soyong 2008, Unterseher and Schnittler 2009). Many unculturable fungi may escape detection (Guo et al. 2001, Duong et al. 2006, Hyde and Soyong 2007, Tao et al. 2008). Molecular methods including pyrosequencing of PCR amplicons could be used to overcome such limitations (Nilsson et al. 2009). But if the aim is to screen biologically active endophytes, then direct culturing is the method of choice. Molecular methods could be applied to understand the distribution of certain individual (possibly biocontrol) species in community dynamics.

After sterilization and plating, fungal endophytes are grown in the dark and usually at room temperature (~21 °C). For bacterial endophytes, they are commonly incubated at 30°C and for long term storage, they are suspended in 20% glycerol solution at -80°C (Long et al. 2010, Rashid et al. 2012). For the root endophytes, the temperature can be adjusted lower than air temperature to mimic conditions in the boreal forests, where the annual soil temperature can be lower than 10 °C (Jungqvist et al. 2014). The growth of fungal endophytes is slow, and they are normally allowed to grow for at least three weeks. The growth rate of endophytic bacteria is much higher and the incubation time is limited to 2-10 days (Long et al.

2010, Rashid et al. 2012). To make sure that no cross-contamination happens between endophytes, the plates should be observed weekly and subculture the emerging fungal hyphae or bacteria into new plates. Solis et al. (2016) have described the use of dilution-to-extinction method for cultivation of foliar endophytic fungi, based on a modified method of Unterseher and Schnittler (2009). According to the method described by Solis et al. (2016), samples are homogenized (*e.g.* blender) and filtered through sieves to obtain hyphal particles of desired sizes. Particles are then washed and strongly diluted before plating onto a malt extract agar in 48 well plates. This type of cultivation method could be used together with conventional ones to increase the number of detected species (Unterseher and Schnittler 2009).

#### ***4.2. Identification and storage of cultures***

Two of the most important requirements for endophytic fungi as biocontrol agents are: 1) Identity of the species verified, and 2) The isolate being stored in culture collection for easy access for other researchers. Isolated bacterial endophytes can be identified genotypically by PCR-amplification of 16S rDNA and BOX-PCR profiling of genomic DNA (Moore et al. 2006). To support genotypic characterization, phenotypic identification methods can be used (tolerance to target pollutants, antibiotic and heavy metals) (Moore et al. 2006). The identification of fungal endophytes can be quite challenging, especially as most of the root endophytes are sterile ascomycetes. In these cases, the identification will rely on amplification of certain gene region, sequencing, and analysis. A crucial step in the identification of fungal endophytes is the availability of accurate reference database, as the majority of newly detected species of fungi have never been described. Also public sequence databases have errors and also incomplete taxonomic sampling (Hibbett and Taylor 2013). For example, about 10% of fungal internal transcribed sequence (ITS) sequences in the international database are insufficiently identified (INSD: GenBank, EMBL, and DDBJ) (Benson et al. 2006, Nilsson et al. 2006), and more than 50% of the fungal ITS sequences are deplete of crucial information such as country of origin (Ryberg et al. 2009). To have proper identification, it is recommended to use two to three different databases: UNITE (Abarenkov et al. 2010), SAF (spruce-associated fungi) (Ovaskainen et al. 2010) and BLAST search against GenBank (NCBI) (Altschul et al. 1997). The reliability of identification by sequence analysis could be improved by increasing the number of regions sequenced and by extending the coverage of the reference database (Ryberg et al. 2009, Ovaskainen et al. 2010). The target regions for species identifications can include multiple DNA loci of fungi (*e.g.* the ITS region, partial SSU and LSU regions, beta-tubulin gene, elongation factor), and the taxonomic resolution among closely related endophytic species can be improved by choosing at least three different DNA regions. Identification to species level of the members of *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (PAC) requires methods employing multi-locus molecular markers, such as single-copy restriction fragment length polymorphism (RFLP), microsatellite analysis, sequencing of loci, or a combination of them (Grünig et al. 2008, Queloz

et al. 2008, 2010). Storing of the fungal cultures in public collection centers is highly recommended. This allows easy access to other researchers. There are several centers where fungal strains can be deposited, for example the Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherlands, the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, American Type Culture Collection (ATCC), Centre for Agriculture and Bioscience International (CABI), UK, and Royal Botanic Gardens, Kew, UK, the VTT Culture collection, VTT Technical Research Centre of Finland, Espoo, Finland, and MycoBank (Crous et al. 2004). For bacteria the German Collection of Microorganisms and Cell Cultures (DSMZ) and The ATCC Bacteriology Collection are good examples of culture collections.

### ***4.3. Bioassays for biocontrol properties of endophytes***

Initial primary screening for antagonistic activities can be accomplished with the dual-culture method (Fig. 4A). In the dual-culture method, agar pieces of equal sizes containing hyphae of the pathogen and the endophyte are placed on an artificial medium and growth of the both fungi is monitored. The endophyte usually grows slower and therefore it may be necessary to place the endophyte on the plate a few days before the pathogen. Based on the results, the inhibitory effect of culture extracts against the pathogen can be evaluated by several different approaches. One approach is to use disc diffuse assays (Simplified method of Oxford Discs Method) (Vincent and Vincent 1944, De Beer and Sherwood 1945). The agar plates are inoculated with the test pathogen (bacteria or fungi). The filter discs containing the crude metabolic extracts are placed over the inoculated pathogen, and potential formation of the inhibition zone is monitored. Other agar-plate-filter systems can be designed as in Figure 4B. Such a system is particularly suitable for screening of fungal or oomycetes pathogens. The agar plug including hyphae of pathogen is placed in the middle of the plate, and filter paper discs with and without metabolic extract are placed at equal distances from the pathogen (Fig. 4A). The extracted individual metabolites with varying concentrations can be tested using similar approach as mentioned above in microtiter plates. The aim is to find the minimal inhibitory concentration against the pathogen. The extraction methods are well described in several articles (Sumarah et al. 2010, 2011, Zhao et al. 2012, Tellenbach et al. 2012). Usually, the endophytes are grown in suitable liquid cultures (Zhao et al. 2012, Tellenbach et al. 2012) and the metabolites are extracted three times with equal amount of ethyl acetate (EtOAc), or acetonitrile, and the EtOAc filtrate is dried (evaporated to dryness with a rotary evaporator). Then the extract can be fractionated with silica gel column (*e.g.* Zhao et al. 2012) or screened by LC-MS using electrospray ionization in both positive and negative ion mode (Sumarah et al. 2011). Using LC-MS, the observed major peaks can be isolated by HPLC and analyzed by MS and NMR to characterize the structure of the metabolite. After this, the metabolite can solely be tested for antagonistic ability. Sometimes the extracted

major compounds are not antifungal and there might be several smaller metabolites responsible for the inhibition. After the major compounds have been separated and collected with silica gel columns, they can be tested for antagonistic activity before further purification. Zhao et al. (2012) used CC over SephadexLH-20 using a mixture of methanol and chloroform in a ratio of 1 to 1 as eluent and crystallization in petroleum ether: ethyl acetate (2:1, v/v) to yield the desired compounds. Isolation of active compound will facilitate *in vitro* testing for antagonistic activity as well as additional functional identification.

## **5. Application of cultivation-independent techniques to unravel the functional relevance of endophytes**

Our understanding of composition and functioning of plant microbiota was greatly enhanced with the advent of ‘omics’ technologies and the use of next generation sequencing (NGS). Available techniques allow large-scale surveys of the entire microbial community of a given plant. In addition, they make possible the detection of unculturable, slow-growing, or rare species, which are often overlooked when traditional methods are used. Furthermore, the application of cultivation-independent techniques boosted the studies on plant microbiome function and its role in plant health and stress tolerance (Guttman et al. 2014). One of the emerging research directions are metagenome-wide association studies (MWAS). In this approach, a relative abundance of a certain gene in the metagenome is used to establish an association with an occurrence of a disease of interest (Wang and Jia 2016). There are numerous examples of application of MWAS to study associations between human microbiome and diseases, such as type 2 diabetes, obesity, and rheumatoid arthritis (Wang and Jia 2016). The success of MWAS in human and animal models suggests that its applications can be extended to analyze associations between forest tree microbiome and diseases. Network models represent an alternative approach to establish a link between microbiome composition and function. They can provide new opportunities for plant disease management as they are used to identify keystone species crucial for plant health and functioning (Desprez-Loustau et al. 2016, Poudel et al. 2016, van der Heijden and Hartmann 2016).

The research on the impact of microbial endophytes on plant disease resistance is still in its infancy. The effects of pathogens on endophytic community and *vice versa* observed in a few available studies differ between experimental models (Hardoim et al. 2015). Therefore, it is currently not possible to draw any general conclusions. However, some pioneering studies indicate that there might be a correlation between the structure of endophyte communities and host plant resistance or susceptibility to pathogens (Ardanov et al. 2012, Martín et al. 2013).

## **6. Prospects of beneficial endophytic microbiomes in forest management: Implication for disease resistance research and tree breeding**

Advances in cultivation-independent techniques including next generation sequencing technology, association analyses and network inference modelling greatly facilitate the identification of potentially beneficial components of endophytic communities. However, even if the identified microorganisms show highly promising results *in vitro*, the transition to field application often presents a major challenge (Martín et al. 2015). The reproducibility of field trials might be influenced by climatic conditions, but other crucial factors for the success of endophytes as biocontrol agents are interactions with other members of the endophytic community, and the host tree genotype. Studies on several tree species clearly demonstrate that the host tree genotype influences the structure of endophytic community (Ahlholm et al. 2002, Balint et al. 2013, Pautasso et al. 2015). Thus, attempts of using endophytic microorganisms to control plant pathogens must take into consideration their interactions with particular plant (tree) genotypes (Newton et al. 2010, Chakraborty and Newton 2011, Desprez-Loustau et al. 2016). At the same time, future tree breeding programs should take into account the interactions of trees with beneficial microbiota and aim at the development of tree varieties with improved capabilities to interact with microbial inoculants. An important challenge is the identification of genetic determinants influencing interactions between host plants and their microbiota (Schlaeppli and Bulgarelli 2015). The biocontrol prospects of endophytes are an emerging research field with huge potential to transform disease management practices in agriculture and forestry.

## **7. Concluding remarks**

There are still many gaps in our knowledge of endophytes and their interaction with host plants, as well as their true beneficial effects. However, with recent advances in biotechnology, we are now able to perform much more comprehensive analyses on the whole communities rather than few culturable strains. The recent increased interest on the study of endophytes can also improve our understanding of plant pathogens and their mechanisms of infection. Endophytes possess many similar traits as pathogenic microbes, yet they are able to colonize plants without triggering any visible defence response. Biological control of plant pests and pathogens is increasingly becoming an integral part of integrated pest management (IPM) strategies for many agricultural crops (Paulitz and Belanger 2001, Punja and Utkhedde 2003, van Lenteren et al. 2017). There are examples of complete replacement of chemical pesticides by biological control agents, such as the use of predator mites to control thrips and whiteflies on sweet pepper. In particular, tree endophytes are considered as promising potential biocontrol agents due to their adaptations to a

lifestyle within woody tissues (Cazorla and Mercado-Blanco 2016). The identification of candidate biocontrol agents is greatly accelerated by current advances in the fields of cultivation-independent techniques and bioinformatics. However, several issues need to be addressed to enable commercial applications of endophytes as biocontrol agents in forest trees:

- Development of cultivation methods to produce inoculum of the species in question on industrial scale, which is not a trivial task for many of the endophytes.
- Development of application techniques. This is particularly challenging for mature trees, as inoculations of individual trees on a large scale will be prohibitively expensive, thus, biocontrol agents ideally should be able to spread efficiently in the targeted tree population. Inoculation of seedlings before out-planting is technically more feasible, but it does not solve the problem of controlling disease in existing forests or plantations.
- Interactions of potential biocontrol agents with existing tree microbiota. Introduced biocontrol agents will need to establish themselves in ecological niches pre-occupied by resident microorganisms.
- Role of host tree genotype in the biocontrol efficiency. The breeding strategies aimed at the improvement of interactions between trees and beneficial microorganisms are particularly important to address this issue.
- Influence of climatic factors on the biocontrol efficiency.

These issues illustrate that development of novel biocontrol strategies for forest trees will be challenging. However, as our understanding of interactions between trees and their respective microbiota increases, new approaches to improve forest tree health and to combat diseases in economically feasible and environmentally friendly ways will definitely emerge.

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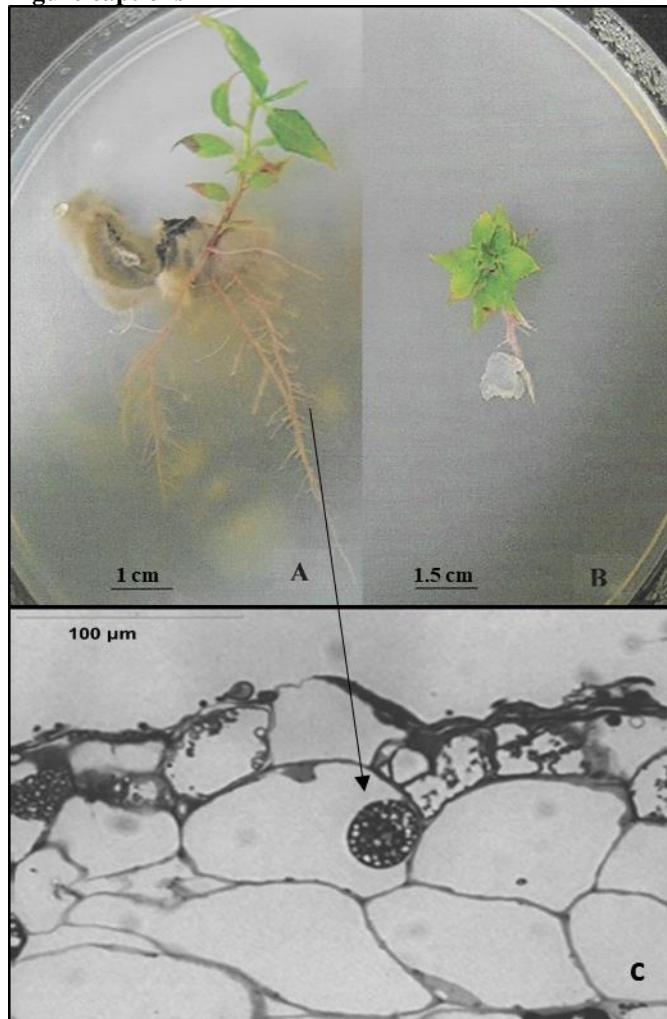
**Figure captions**

Figure 3.1 A) Hybrid aspen (*Populus tremula* × *tremuloides*) cuttings inoculated with root endophyte *Cadophora* sp.; B) Hybrid aspen cuttings growing without root endophyte; C) after inoculation with root endophyte, microscerotia could be observed in the roots. Modified from Terhonen 2008.

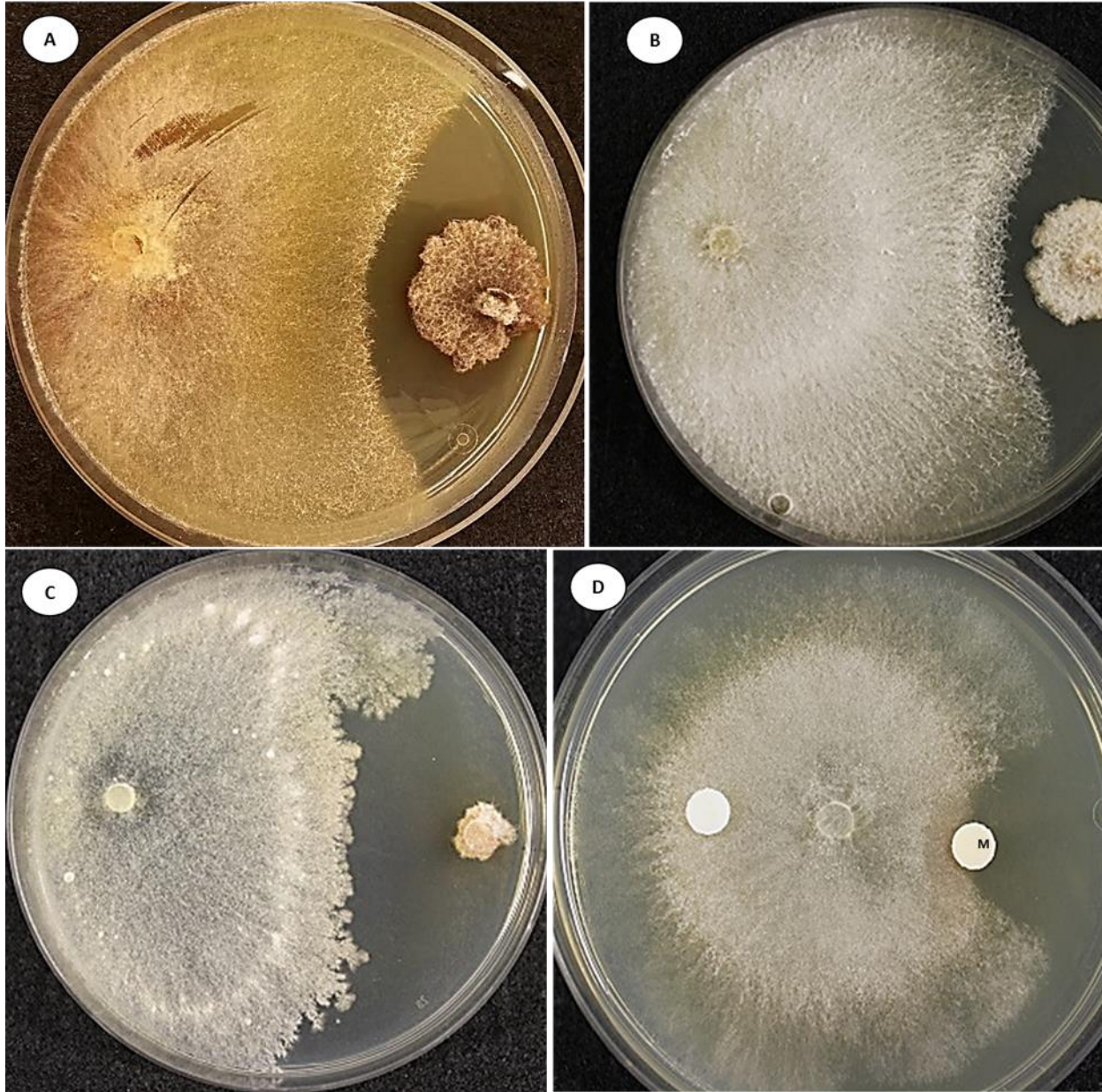


Figure 3.2 A) Growth of *Heterobasidion parviporum* after 11 days in co-culture with the root endophyte *Cryptosporiopsis* sp. 513. B) Growth of *Cryphonectria parasitica* after 11 days in co-culture with the root endophyte *Cryptosporiopsis* sp. 513. C) Growth of *Botrytis cinerea* after 8 days in co-culture with *Cryptosporiopsis* sp. 513 (Terhonen et al. 2016, modified from Fig 3). D) Growth of *B. cinerea* after 5 days. The root endophytic strain *Cryptosporiopsis* sp. 513 was

previously isolated by Terhonen et al. (2013b, 2016) and Fig. 3.2C is from Terhonen et al. (2016).

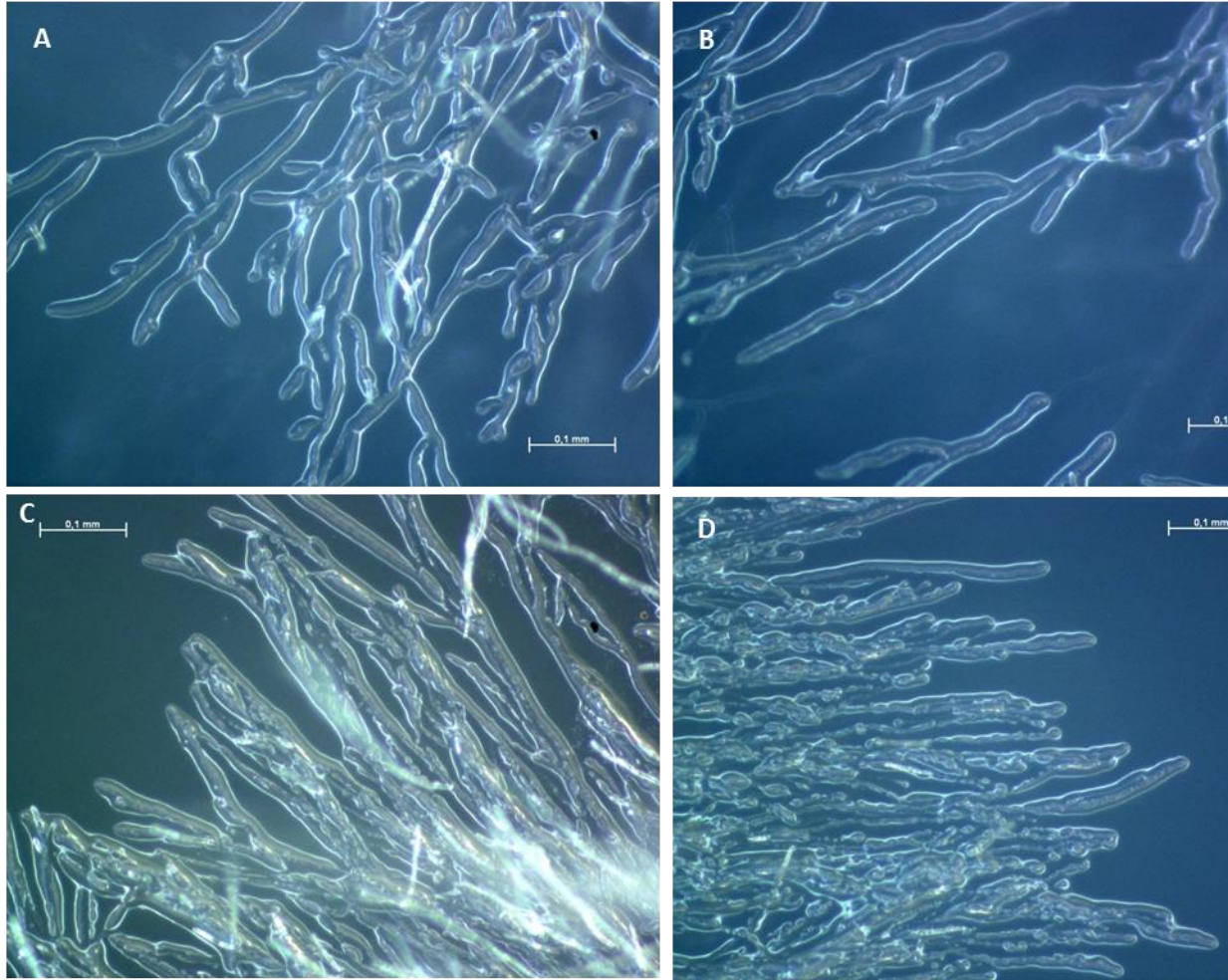


Figure 3.3. A) The hyphae of *B. cinerea* growing towards metabolites extracted from *Phialocephala* sp. 222; B) Control hyphae from the same plate; C) The hyphae of *C. parasitica* in the presence of metabolites extracted from *Phialocephala* sp. 222 D) Control hyphae from the same plate. All pictures were taken 6 days post inoculation. The root endophyte *Phialocephala* sp. 222 was previously isolated from by Terhonen et al. (2013b).

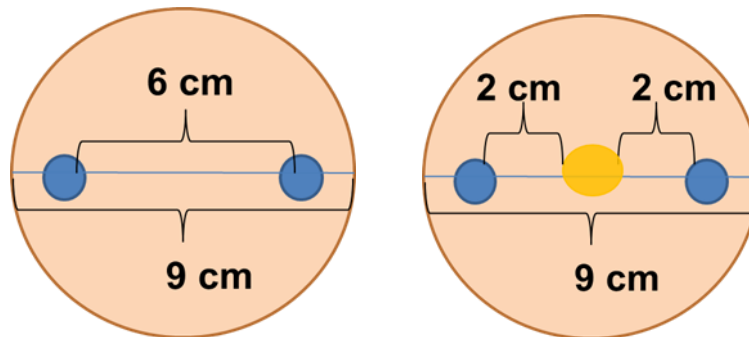


Figure 4A) The set up of the inhibition screening between pathogenic fungi and endophyte; agar plugs ( $\approx 5$  mm) containing hyphae of endophytic and pathogenic fungi are placed on 2% MEA plate at a distance of 6 cm from each other. B) Hyphae of fungal pathogen ( $\approx 5$  mm) was placed in the middle of 2% MEA plate and two filter papers exposed and evaporated from ethyl acetate ( $\approx 6$  mm) with and without broth extracts of endophytes. Modified from earlier published figure by Terhonen et al. (2016).

**Abbreviations:**

ACC	1-aminocyclopropane-1-carboxylic acid
AHL	N-acyl-L-homoserine lactone
BCAs	Biocontrol agents
BHI	Brain Heart infusion medium
C-endophytes	Clavicipitaceous endophytes
DED	Dutch elm tree disease
DSEs	Dark septate endophytes

dsRNA	Double-stranded RNA
EtOAc	Ethyl acetate
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IPM	Integrated pest management
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
K	Potassium
KBA	King's B agar
LA	Luria agar
LC-MS	Liquid chromatography–mass spectrometry
MEA	Malt extract agar
MS	Mass spectrometry
MWAS	Metagenome-wide association studies
N	Nitrogen
NC-endophytes	Non-clavicipitaceous endophytes
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance spectroscopy
P	Phosphorus
PAC	<i>Phialocephala fortinii</i> s.l.- <i>Acephala applanata</i> species complex
PDA	Potato dextrose agar
PGPR	Plant growth-promoting rhizobacteria
PPP	Plant protection product
PR proteins	Pathogenesis-related proteins
RFLP	Restriction fragment length polymorphism
SA	Salicylic acid
SAR	Systemic acquired resistance
TSA	Tryptic soy agar
VWO	<i>Verticillium</i> wilt of olive trees
YPDA	Yeast Peptone Dextrose Agar