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# Inhibition of phytopathogens by fungal root endophytes of Norway spruce

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## HIGHLIGHTS

- Root endophytes produce diverse kinds of metabolites with potential antifungal property.
- Root endophytes were able to inhibit the growth of plant pathogens *in vitro*.
- Inoculation with root endophyte increased Norway spruce root shoot ratio.
- Inoculation with root endophyte prevented the root rot pathogen infection *in vitro*.
- Root endophytes might serve as the first root protectors of young seedlings.

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## ABSTRACT

Fungal endophytes have been reported to inhibit growth of phytopathogenic microbes and some also are known to promote root growth. If endophytes of conifers can protect their host roots against invaders the screening of endophytes for their biocontrol abilities is relevant. The aim of this research was to screen for the potential inhibitory effects of selected Norway spruce root endophytes during interaction, *in vitro*, with well-known genera of phytopathogens (*Heterobasidion parviporum*, *Phytophtora pini*, *Botrytis cinerea*) and test the endophytes ability to protect Norway spruce seedlings against *H. parviporum* infection. The root endophytes and their metabolites were able to form inhibition zones in paired cultures with the pathogens. Higher numbers of unique metabolites were observed in culture extracts of *Cryptosporiopsis* sp. further suggesting that stronger inhibitory effect observed could be due to acquisition of more diverse metabolite pool. However, this endophyte decreased and retarded the root growth of Norway spruce seedlings. The endophyte *Phialocephala sphareoides* was able to inhibit all phytopathogens, promoted the root shoot ratio of Norway spruce seedlings and was able to prevent under *in vitro* conditions the infections of seedling roots by the pathogen *H. parviporum*.



## 1. Introduction

Fungal endophytes colonize plant tissues without any visible disease symptoms with a continuum of unknown relationship with the host (Saikkonen et al., 1998). The host–endophyte relationship in the plant roots is thought to differ from mycorrhizal symbioses as the cellular interface where specialized structures for nutrient transfer (e.g. arbuscules) are lacking (Brundrett, 2006). Meta-analysis of root endophytes on plant performance have revealed positive effects on total, shoot and root biomass (Newsham, 2011). The two most prominent explanations for the observed positive responses of the host to root endophyte colonisation include the modulation of plant growth via nutrient acquisition (as in mycorrhizae) (Jumpponen, 2001; Mandyam and Jumpponen, 2005; Newsham, 2011; Reininger and Sieber, 2013) and the production of plant growth promoting phytohormones (Schulz and Boyle, 2005; Schulz et al., 2002, 1998). Endophytic fungi might also suppress the growth of pathogenic fungi via production of secondary metabolite or competing within the ecological niche (Mandyam and Jumpponen, 2005). These observations and hypotheses could partly explain the existence of these ubiquitous fungi in their host plants but the ecological roles and functions of these endophytes, especially their general effects on the colonized hosts are difficult to define, and despite their apparent great abundance, have not been fully resolved (Sieber and Grünig, 2013). The most frequently isolated endophytic fungi from the roots of Norway spruce (*Picea abies* (L.) H. Karst) are the so-called dark septate endophytes (DSEs)

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(Ahlich and Sieber, 1996; Grünig et al., 2002; Queloz et al., 2005; Terhonen et al., 2014) that belong to Class 4 of non-clavicipitaceous endophytes (Rodriguez et al., 2009). DSE hyphae are both septate and melanised and they can form specialized structures in the host roots, referred to as microsclerotia (Jumpponen and Trappe, 1998a; Mandyam and Jumpponen, 2005). Most of these DSE fungi belong to the *Phialocephala fortinii* s.l.–*Acephala applanata* cryptic species complex (PAC) in roots of Norway spruce (Ahlich and Sieber, 1996; Grünig et al., 2002, 2004; Terhonen et al., 2014). According to previous studies (Miller et al., 2002; Sumarah et al., 2010, 2011; Tellenbach et al., 2013) it can be hypothesised that fungal endophytes can produce antifungal substances in addition to host metabolites (Schulz et al., 1999). PAC member have also been noted to suppress pathogens (Tellenbach et al., 2013) and reduce mortality and disease intensity caused by the pathogens in Norway spruce (Tellenbach and Sieber, 2012). All these facts validate the possible role that fungal endophytes could play as biocontrol agents against root pathogens.

Norway spruce together with Scots pine (*Pinus sylvestris* L.) forms the basis for raw materials of the forest sector in Finland contributing several billion EUR net income yearly (Ministry of Agriculture and Forestry, 2015). One fourth of the forest area is covered by Norway spruce (Peltola, 2008) and 105 million Norway spruce seedlings are produced in nurseries annually in Finland (Finnish Food Safety Authority, 2014). Thus, interest in the biology and ecology of fungal endophytes in forest ecosystems with special emphasis on sustainable management strategies for forestry is high. The constant need to sustain timber quality gives new challenges in the area of forest biotechnology, particularly in tree health protection. In native forest ecosystems trees and fungi share overlapping habitats whereas the balance depends on a diverse scope of factors ranging from host type to ecological and environmental disturbances. Efficient forest management has changed the environment into favouring pathogen *Heterobasidion annosum sensu lato* in the stands where it originally has been rare. Nowadays in Finland, this pathogen is the main cause of wood rot in Norway spruce, decreasing the commercial value of these trees for 50 million EUR annually (Asiegbu et al., 2005; Mattila and Nuutinen, 2007; Peltola and Ihalainen, 2011). Fine roots of conifers are equally susceptible under *in vitro* conditions (Adomas et al., 2007; Asiegbu et al., 1993, 1994) and in non-suberized lateral roots (Heneen et al., 1994). These results suggest that this pathogen can infect roots of all ages (Asiegbu et al., 2005; Li and Asiegbu, 2004). The disease control for *H. annosum s.l.* is challenging because this pathogen is able to spread from diseased stumps to neighbouring trees through root contacts (Oliva et al., 2011) and it can stay viable for decades in stumps of different tree species (Gunulf et al., 2012; Piri, 1996) creating a long-lasting threat to newly planted seedlings. This pathogen can stay infective also in root residuals for years after stump removal and spread vegetatively to nearby Norway spruce seedlings (Piri and Hamberg, 2015). The proportion of diseased forest stands and associated production losses are expected to increase in the foreseeable future due to year-round logging. Furthermore, pathogens of the genera *Phytophthora* are often causative agents for damping-off disease of conifer tree seedlings in nurseries (Lilja et al., 2010). Additionally 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). Recently, *Phytophthora plurivora* Jung & Burgess and *Phytophthora pini* L.H. Leonian have been isolated from Finnish forestry nurseries (Lilja et al., 2011; Rytkönen et al., 2012, 2013) and Norway spruce seedlings were found to be susceptible to Finnish isolates of *P. plurivora* and *P. pini* when wounded tissue was inoculated with live mycelia (Rytkönen et al., 2012, 2013). If these oomycetes continue to spread via the nursery pathway to forest out plantings sites, they will potentially pose a threat to forestry not only in Finland but globally. Consequently, protecting conifer roots at a very early stage during their development with possible new biocontrol agents against *H. annosum s.l.* and other non-indigenous root pathogens deserves to be explored. If fungal endophytes can promote host root growth and/or protect host roots against invaders the screening of endophytes for their biocontrol abilities is of biotechnological relevance.

The aim of this research was to screen for the potential inhibitory effects of selected root endophytes during interaction *in vitro* with two well-known genera of pathogens of forest trees (*Heterobasidion parviporum* Niemelä & Korhonen and *P. pini*). The study was also repeated with Ascomycetes plant pathogen *Botrytis cinerea* Pers. Additional objective was the chemical characterization and bioassay of the metabolites secreted by these Norway spruce root endophytes and *H. parviporum*. To utilize these root endophytes as biocontrols, the mechanisms behind the possible inhibition of the root pathogen should be determined. Characterizing the secreted metabolites produced by root endophytes, followed by tests of biological activities will facilitate their potential use as biocontrol agents, as well as provide further insight on the ecological relevance of endophytes. To further examine the biocontrol potential of these endophytes; their ability to protect Norway spruce seedlings against *H. parviporum* was tested in a tripartite host–endophyte–pathogen system in order to understand the mechanism and ecological consequences of the existence of these ubiquitous endophytes in various host roots.

## 2. Material and methods

### 2.1. Endophytes, pathogens and host material

The pathogens used in this study were *Heterobasidion parviporum* (courtesy of Kari Korhonen: isolate 03014, collected from Kuhmoinen, Central Finland, from *Abies sibirica* and isolate 04009 collected from Norway spruce, Liljendal, South Finland by K. Lipponen), *Phytophtora pini* (Ph443 2007 collected from Rhododendron, Rytkönen et al., 2012). *Botrytis cinerea* (isolate B05.10, isolated from *Vitis vinifera*, kindly provided by Mehmet Ali Keçeli, Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki). Based on initial screening (Terhonen et al., 2014) two root endophytes (strain 513 and 222), isolated from roots of Norway spruce, were selected for the inhibitory study. A non-inhibitory endophyte strain 5992 (Terhonen et al., 2014) was used to characterize the secondary metabolites. These isolates were identified based on DNA sequence comparisons for the internal transcribed space ITS1 and ITS2 regions of the ribosomal DNA. The specimens of these endophytic isolates are stored in culture collections of Forest Pathology Group at Department of Forest Sciences, University of Helsinki. The inoculation experiments were performed with Norway spruce seeds collected from a single tree from South Finland; Haapastensyrjä, Tree Breeding Station (60°37′ N, 24°26′ E).

### 2.2. DNA extraction and PCR protocol

The endophytic isolates were transferred to Petri plates containing 2% malt extract agar (MEA), pre-covered with cellophane membrane and allowed to grow for use in DNA isolation. Pieces of hyphae harvested from the cellophane, was ground in 1.5 ml Eppendorf tube with sand and a micropestle in 50 µl of TE buffer (1 ml 1 M TRIS-HCl, 0.2 ml 0.5 M EDTA pH 8). The samples were microwaved full power for 30 s and incubated at 90 °C for 5 min, centrifuged maximum speed (17,000×*g*) for 1 min and 10 µl of