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NATURAL DISEASE CONTROL BY ROOT ENDOPHYTES IN A CHANGING CLIMATE

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SUMMARY

Fungal endophytes are common symbionts in functional and apparently healthy roots and do not form any complex structures such as mycorrhizal fungi do. Root endophytes are genetically highly diverse suggesting important ecological roles. Yet, relative to above-ground endophytes, little is known about them. Ascomycete fungi of the *Phialocephala fortinii* s.l.-*Acephala applanata* cryptic species complex (PAC) that belong to the dark septate endophytes (DSE) are ubiquitous endophytic colonizers of conifer and ericaceous shrub roots, but their ecological function is largely unknown. Whilst some experimental data suggest that they are mutualists, others propose that they are antagonists. These discrepancies might be due to the fact that species assignment was not known when these studies were performed or that the effect of PAC on host plants depends on the extent of fungal colonization. On the other hand, PAC might indeed be associated with direct costs for some hosts, but might have indirect beneficial effects by conferring resistance to plants against biotic and/or abiotic stress.

Therefore, a series of *in vitro* experiments was performed to investigate the nature of interaction of PAC with Norway spruce, one of their main hosts in Europe. In addition, a quantitative real-time PCR (qPCR) approach was developed to quantify fungal DNA as a surrogate of fungal biomass in colonized roots and compared with visual assessment of fungal colonization in samples. qPCR reliably and specifically detected PAC in different types of samples and correspondence with visual assessment was very high if the fungus did not produce too many microsclerotia.

In a dual interaction experiment, Norway spruce seedlings from two different seed provenances were inoculated with isolates of the four most common PAC species to investigate possible interspecific differences in host response to PAC. Moreover, *P. subalpina* isolates of two populations from within and one from outside the natural range of Norway spruce were inoculated to study the effect of geographic origin on host response. PAC did not promote seedling growth in any case and their effects on Norway spruce were primarily isolate-dependent. Variation in virulence was much higher within than among species, but the most virulent isolates belonged all to *P. subalpina*. Disease caused by *P. subalpina* genotypes from within the native range of Norway spruce was more severe than that induced by genotypes from outside the natural distribution of Norway spruce. Moreover, virulence was not correlated with the phylogenetic relatedness of the isolates but was positively correlated with the extent of fungal colonization as measured by qPCR. Thus, these experiments suggest that PAC are neutral to pathogenic symbionts of Norway spruce in this system.

In a tripartite host-endophyte-pathogen system it was tested whether six genetically distinct isolates of *P. subalpina* varying in virulence can protect their host against the two oomycetous root pathogens *Phytophthora plurivora* (syn. *P. citricola*) and *Elongisporangium undulatum* (syn. *Pythium undulatum*). Whilst oomycete diseases are predicted to increase under climate change, the symbiotic relationship between PAC and Norway spruce might be altered in favor or to the disadvantage of either host and/or endophyte. Therefore, an elevated temperature treatment was included, to investigate whether a possible protective effect of PAC against the two pathogens might be affected. Protection of the spruce seedlings depended on the *P. subalpina* isolate, but was not affected by elevated temperature. Moreover, whilst the higher temperature significantly reduced spruce performance, it did not alter root colonization by *P. subalpina* and its influence on the host. Similarly, disease intensity of *Phytophthora* was not affected. In contrast, the influence of *Elongisporangium* on plant biomass was slightly less pronounced at elevated temperature. These results indicate that PAC can confer an indirect benefit to their host, and might therefore be tolerated in natural populations, despite negative effects on plant performance.

The results of these *in vitro* experiments corroborate findings of other studies that position PAC between neutral and pathogenic on the mutualism – antagonism continuum. Moreover, it was shown that PAC might confer an indirect benefit to their host by protecting it against harmful root pathogens. However, these findings are only the very beginning of our understanding of the ecological significance of PAC and deserve to be followed by further investigations under more natural conditions.

ZUSAMMENFASSUNG

Endophytische Pilze kommen häufig als Symbionten in funktionstüchtigen und gesunden Wurzeln vor, bilden aber im Unterschied zu den Mykorrhiza-Pilzen keine komplexen Strukturen. Im Vergleich zu Stamm- und Blattendophyten ist immer noch wenig über Wurzelendophyten bekannt, obschon angenommen wird, dass sie ökologisch sehr wichtig sind, da sie weit verbreitet und genetisch sehr vielfältig sind. Ascomyceten vom *Phialocephala fortinii* s.l.-*Acephala applanata* kryptischen Artenkomplex (PAC) gehören zu den dunklen septierten Endophyten (DSE) und sind häufige Wurzelbesiedler von Koniferen und Erikagewächsen. Die ökologische Bedeutung von PAC ist weitgehend unbekannt und Berichte verschiedener Studien sind unterschiedlich. Währendem in einigen Studien ein positiver Einfluss von PAC auf ihren Wirt festgestellt wurde, werden in anderen PAC eher als Antagonisten beschrieben. Die Diskrepanz zwischen diesen Studien könnte daran liegen, dass die Unterteilung von PAC in kryptische Arten noch nicht bekannt war, als diese Studien durchgeführt wurden. Es könnte aber auch daran liegen, dass der Einfluss von PAC auf die Pflanze von der Besiedlungsdichte des Pilzes abhängig ist oder dass PAC tatsächlich mit Kosten für die Wirtspflanze verbunden sind, ihr aber einen indirekten Vorteil verschaffen, indem sie sie vor biotischem, und/oder abiotischem Stress schützt.

Mögliche ökologische Funktionen von PAC wurden in verschiedenen *in vitro* Experimenten mit Gemeiner Fichte, einem ihrer Hauptwirten, durchgeführt. Ausserdem wurde eine quantitative Real-Time PCR (qPCR) entwickelt, um die endophytische Pilzbiomasse, basierend auf der Pilz-DNA, zu schätzen. Die Qualität der Schätzwerte wurde mit derjenigen einer visuellen mikroskopischen Messmethode verglichen. Mittels qPCR konnten PAC in unterschiedlichen Probentypen zuverlässig und spezifisch gefunden und gemessen werden. Ausserdem stimmten qPCR und visuelle Beurteilung sehr gut überein, vorausgesetzt, der Pilz bildete nicht allzu viele Mikrosklerotien.

In einer Interaktionsstudie wurden Fichtenkeimlinge von zwei verschiedenen Provenienzen mit Isolaten der vier häufigsten PAC Arten inokuliert um zu untersuchen, ob die Pflanzen unterschiedlich auf die verschiedenen Pilzarten reagieren. Zusätzlich wurden Keimlinge mit *P. subalpina* Isolaten von zwei Populationen innerhalb und einer ausserhalb des natürlichen Verbreitungsgebiets der Gemeinen Fichte inokuliert um den Effekt der geographischen Herkunft von PAC zu untersuchen. Ein wachstumsfördernder Effekt von PAC auf die Fichtenkeimlinge wurde in keinem Fall gefunden und der Effekt variierte von neutral bis hoch virulent und war in erster Linie isolatabhängig. Die Virulenz variierte innerhalb der Arten viel stärker als zwischen den Arten. Die virulentesten Isolate gehörten aber ausschliesslich zu *P. subalpina*. *P. subalpina* Isolate von innerhalb des natürlichen

Verbreitungsgebiets der Fichte waren schädlicher als Isolate von ausserhalb. Die Virulenz von PAC war nicht korreliert mit der phylogenetischen Verwandtschaft der Isolate, wogegen die Virulenz mit der Besiedlungsdichte des Pilzes positiv korreliert war. Die Resultate dieser Studie deuten an, dass PAC im hier verwendeten System neutrale bis pathogene Symbionten von Fichte sind.

In einem tripartiten Wirt-Endophyt-Pathogen System wurde getestet, ob sechs *P. subalpina* Isolate mit unterschiedlicher Virulenz die Fichte gegen die beiden Wurzelpathogene *Phytophthora plurivora* (syn. *P. citricola*) und *Elongisporangium undulatum* (syn. *Pythium undulatum*), beides Oomyzeten, schützen können. Es wird angenommen, dass durch Oomyzeten verursachte Krankheiten unter dem Klimawandel an Bedeutung gewinnen werden. Im Gegensatz dazu ist über den Einfluss steigender Temperaturen auf Endophyten nur wenig bekannt, und es ist möglich, dass die symbiontische Beziehung zum Vor- oder Nachteil von Wirt und/oder Endophyt verändert wird. Um diese Effekte zu studieren wurde eine zusätzlich Behandlung mit erhöhter Temperatur im Experiment implementiert, um deren Einfluss auf eine mögliche Schutzwirkung von PAC gegen die beiden Pathogene zu ergründen. Die Schutzwirkung hing vom *P. subalpina* Isolat ab und wurde nicht von erhöhter Temperatur beeinflusst. Währendem erhöhte Temperatur das Wachstum der Fichtenkeimlinge hemmte, wurde die Wurzelbesiedlung von PAC weder positiv noch negativ verändert. Ähnlich wurde auch die Krankheitsintensität von *Phytophthora* nicht von erhöhter Temperatur beeinflusst, wogegen der Einfluss von *Elongisporangium* auf die Pflanzenbiomasse bei erhöhter Temperatur etwas weniger stark ausgeprägt war. Die Resultate dieses Versuchs deuten an, dass PAC mit ihrer Schutzwirkung der Wirtspflanze einen indirekten Vorteil verschaffen können, was erklären würde, wieso sie trotz zum Teil schädigender Wirkung in natürlichen Populationen von ihrem Wirt toleriert werden.

Die Resultate dieser *in vitro* Studie bestätigen ältere Befunde von Untersuchungen, die PAC im neutralen bis pathogenen Bereich vom Mutualismus – Antagonismus Kontinuum ansiedeln. Es wurde jedoch gezeigt, dass PAC ihrem Wirten einen indirekten Vorteil verschaffen können, indem sie Schutz gegen Wurzelpathogene bieten. Diese Befunde sind aber erst der Anfang unseres Verständnisses der ökologischen Relevanz von PAC und müssen auch unter natürlicheren Bedingungen überprüft werden.

Chapter 1

General introduction

FROM MUTUALISM TO ANTAGONISM, A CONTINUUM OF INTERACTIONS BETWEEN FUNGI AND PLANTS

It is nowadays well believed from fossil records that when vascular plants started colonizing terrestrial habitats 350-460 million years ago, they were most likely dependent on the symbiosis of arbuscular mycorrhizal (AM) fungi (Pirozynski and Malloch, 1975; Simon et al., 1993; Remy et al., 1994; Schüssler, 2002). AM colonize plant roots internally, where they penetrate cortical cells and form coiled or branched intracellular structures. Furthermore, AM fungi also grow out into the soil, forming an extraradical mycelium, which enhances the root surface considerably and forms a mycelial network, connecting different plants (Newman et al., 1994; Robinson and Fitter, 1999). AM symbioses are mutualistic associations, which means that they are advantageous for both partners. The plant assimilates CO_2 and delivers it in form of carbohydrates to the fungus, which in turn solubilizes nutrients, in particular phosphorous and micronutrients, bound to mineral particles and provides them to the plant (Brundrett, 2004; Smith and Read, 2008). Another type of mycorrhizal symbiosis, which arose about 200 million years ago (Cairney, 2000) are ectomycorrhizae (ECM), which occur predominantly on woody perennials. ECM do not penetrate cortical cells, but form a Hartig net between epidermal and cortical cells and a dense mycelial sheath around the plant root. ECM do also form an extraradical mycelium, and they are functionally similar to AM, but it was shown that they also modify the water balance in plants by increasing the hydraulic conductance in ectomycorrhizal plants and by mobilizing water from the environment, and that they mineralize organic compounds in the soil which will then be made available to the plant (Duddridge et al., 1980; Read, 1991; Anderson et al., 1999; Muhsin and Zwiazek, 2002; Smith and Read, 2008). In contrast, the opposite of mutualism is antagonism, where the fungus profits on the expense of the plant. Whilst some antagonists impede plant growth and reproduction, or kill single individuals, others are devastating pathogens endangering entire

plant populations or even entire species. Famous examples are for instance *Phytophthora cinnamomi*, which is responsible for dieback of many plant species in the Jarrah forest in Australia, but also for oak and chestnut decline in Europe (Newhook and Podger, 1972; Brasier et al., 1993; Bergot et al., 2004), *Cryphonectria parasitica*, which almost eradicated American chestnut in the eastern United States (Anagnostakis, 1987), *Ophiostoma novo-ulmi* the cause of the Dutch elm disease (Brasier, 1991), *Phytophthora ramorum*, the causal agent of sudden oak death (Rizzo et al., 2002), and the very recently emerging pathogen *Chalara fraxinea*, which causes dieback of ash trees (Kowalski, 2006; Queloz et al., 2010).

Although the definition of the two terms 'mutualism' and 'antagonism' is very straightforward, the classification of an interaction is not as simple in nature. For instance, the effect of mycorrhizal fungi is not fixed and they can even be parasitic under certain environmental conditions (Fitter, 1990; Brundrett, 2004; Neuhauser and Fargione, 2004). Therefore, the type of plant – fungus interaction is not static but dynamic. These dynamics of interaction types especially applies to fungal endophytes which live endosymbiotically in functional plant tissues. By definition, endophytes do not form distinct structures as mycorrhizal fungi do, but they do not cause any apparent disease symptoms for a prolonged period of time or at all (Carroll, 1988; Petrini, 1991; Wilson, 1995; Saikkonen et al., 1998; Sieber, 2002; Rodriguez et al., 2009). Endophytes are ubiquitous and virtually all plants are colonized by endophytes and almost all plant tissues can harbor endophytes, as was shown from studies where endophytes were isolated from leaves and stems (e.g., Carroll, 1988; Petrini, 1991; Arnold and Lutzoni, 2007), roots (e.g., Addy et al., 2005; Sieber and Grünig, 2006) and even flowering tissues (Washington et al., 1999; Braun et al., 2003). Depending on host and fungal species, endophytes can occur locally or systemically. However, reports about their effect on host plants range from mutualistic to antagonistic (Saikkonen et al., 1998; Faeth and Sullivan, 2003; Schulz and Boyle, 2005; Sieber, 2007). Thus, in the latter case the question arises, why a plant should tolerate a pathogenic fungus within functional tissues. An answer to this apparent contradiction can be found if one sums up all direct and indirect costs and benefits that are related to this symbiosis. Thus, if the benefits outweigh the costs for both partners, mutualism occurs. In the context of natural ecosystems, a plethora of different species are interacting in diverse habitats, where environmental conditions are permanently fluctuating and changing. This can be stressful for plants with only limited physiological and/or structural adaptations to avoid environmental stress. Therefore, mutualistic endophytes might lower stress of the host plant. Indeed, endophyte mediated adaptation to stressful environments was demonstrated for *Piriformospora indica*, which enhanced tolerance to salt stress in barley (Waller et al., 2005) or *Curvularia protuberata*, which conferred thermotolerance to *Dichanthelium lanuginosum* plants from geothermal soils (Redman et al., 2002). Thermotolerance, however, depended further on the presence of an endosymbiotic

virus in the fungus (Marquez et al., 2007). Clavicipitaceous grass endophytes increased tolerance to drought and other abiotic stressors like low soil pH and aluminum toxicity (Malinowski and Belesky, 2000; Kuldau and Bacon, 2008). Furthermore, these endophytes produce alkaloids that deter a broad spectrum of herbivorous invertebrates (Siegel et al., 1987; Clay, 1988; Kuldau and Bacon, 2008). Similarly, non-clavicipitaceous endophytes might protect host plants against harmful pathogens, as was demonstrated for an assemblage of *Theobroma cacao* endophytes that reduced leaf rot by a pathogenic *Phytophthora* sp. (Arnold et al., 2003), or *Pinus monticola* endophytes that reduced white pine blister rust (*Cronartium ribicola*) in their host (Ganley et al., 2008). *Trichoderma* spp. and nonpathogenic *Fusarium oxysporum* strains, both of which often colonize roots endophytically, were shown to protect the host against harmful pathogens (Papavizas, 1985; Harman et al., 2004; Alabouvette et al., 2009). Therefore, plant – fungal endophyte mutualism occurs if the endophyte alleviates biotic and abiotic stress from the host.

PAC – WIDELY DISTRIBUTED ROOT ENDOPHYTES WITH UNKNOWN ECOLOGICAL FUNCTION

Fungal endophytes are classified into two groups, clavicipitaceous and non- clavicipitaceous endophytes (Carroll, 1988; Clay, 1988; Sieber, 2007; Rodriguez et al., 2009). Clavicipitaceous endophytes are phylogenetically related, they are restricted almost entirely to grass species, and they colonize the host plant systemically (Clay, 1988; Clay and Schardl, 2002; Sieber, 2007; Rodriguez et al., 2009). Non-clavicipitaceous endophytes in contrast form a polyphyletic group and have a broad host range (Carroll, 1988; Petrini, 1991; Schulz and Boyle, 2005; Sieber, 2007; Rodriguez et al., 2009). Moreover, there is high variability among organs colonized by non-clavicipitaceous endophytes. Therefore, it was recently proposed to group endophytes into four functional classes instead of two (Rodriguez et al., 2009). Class 1 includes the clavicipitaceous endophytes, whereas classes 2-4 distinguish all other endophytes according to their relative occurrence in the host plant (2 = systemic, 3 = stems and leaves, 4 = roots). An abundant group of class 4 endophytes are the dark septate endophytes (DSE). DSE form a polyphyletic group of ascomycetes that are characterized by melanized, septate hyphae (Read and Haselwandter, 1981; Stoyke et al., 1992; Ahlich and Sieber, 1996; Jumpponen and Trappe, 1998; Sieber, 2002; Grünig et al., 2008). They are highly abundant in arctic and alpine mycorrhizal and non-mycorrhizal plants and have been reported from a broad host range of more than 600 plant species (Stoyke and Currah, 1991; Jumpponen et al., 1998; Grünig et al., 2001; Sieber, 2002). In conifers and Ericaceae the most prevalent

members of the DSE are anamorphic helotialean ascomycetes of the *Phialocephala fortinii* s.l.-*Acephala applanata* species complex hereafter referred to as PAC (Wang and Wilcox, 1985; Ahlich and Sieber, 1996; Grünig et al., 2008). Whilst *A. applanata* is morphologically distinct (Ahlich and Sieber, 1996; Grünig and Sieber, 2005) other PAC members are cryptic species that can only be separated by molecular techniques. PAC species are widely distributed across the northern hemisphere without showing any biogeographic pattern. PAC communities are composed of up to ten species. Species abundance within these communities follow a hyperbolic distribution with a few abundant species and many rare species (Grünig et al., 2006). PAC species are little host specific (Ahlich and Sieber, 1996; Harney et al., 1997; Addy et al., 2005; Grünig et al., 2008) and their distribution seems to be mainly driven by stochastic effects rather than by environmental selection (Queloz et al., 2011). Although PAC (and DSE) are highly abundant, little is known about their influence on the host plant, and results from interaction studies are often contradictory (Jumpponen and Trappe, 1998; Grünig et al., 2008; Alberton et al., 2010; Newsham, 2011). One main reason for these discrepancies might be that cryptic species assignment was unknown when these interaction experiments were performed. Moreover, it might be possible that PAC are primarily associated with costs for their host, but that they confer an indirect benefit which compensates for these costs by conferring resistance against harmful root pathogens. On the other hand, the negative effects might be due to excessive root colonization by PAC. For instance, conifer needles hosting *Rhabdocline parkeri* senesce and die, if the endophyte exceeds a certain threshold density (Stone, 1987) or virulence of the *Bromus tectorum* seed bank pathogen *Pyrenophora semeniperda* was directly linked to mycelial growth (Meyer et al., 2010). Therefore, quantifying the extent of endophytic colonization might shed further light into the differential outcomes of plant – PAC interaction studies.

There are numerous methods that have been applied to quantify fungi in tissue and environmental samples, embracing microscopy, chemical, and biochemical techniques. Nowadays, traditional light microscopy methods (Newman, 1966; Bååth and Söderström, 1979; McGonigle et al., 1990) are still the most widely used techniques to measure hyphal length and proportional colonization of host tissue (e.g., Gamper et al., 2008; Atkin et al., 2009; Olsrud et al., 2010). However, determination of the extent of fungal colonization by microscopy is very laborious and results vary between investigators. Moreover, visual quantification is unspecific, as species designation based on hyphal morphology is almost impossible. Chemical methods measuring the amount of specific biomolecules like the fatty acid ergosterol or the carbohydrate chitin that are stored inside fungal cells or released into the environment (Wallander et al., 2001; Gessner and Newell, 2002; Olsrud et al., 2007) are much less laborious techniques than microscopy but they still lack specificity, the minimum sample size required is comparatively high (Nylund and Wallander, 1992; Gessner and Schmitt,

1996), and biomass of some fungal species in environmental samples seems wrongly measured compared with other quantification methods (Olsrud et al., 2007). Real-time quantitative PCR (qPCR) is a molecular biological technique that can be designed specifically at different taxonomic levels and allows accurate quantification of DNA copy numbers in very small samples (Heid et al., 1996; Smith and Osborn, 2009). It has been widely applied for diagnostic purposes to early and rapidly detect plant pathogens in environmental samples and in diseased tissues (Böhm et al., 1999; Ward et al., 2004; Okubara et al., 2005), but there was also some effort to estimate fungal biomass. However, there is some debate as to whether qPCR allows the measurement of fungal biomass adequately. For example, qPCR biomass estimates of *Glomeromycota* correlated only poorly with estimates obtained by visual assessment, which was most likely due to the multinucleate coenocytic mycelium (Gamper et al., 2008). On the other hand, a correlation between qPCR and mycelial dry weight was demonstrated for the Swiss needle cast causing parasite *Phaeocryptopus gaeumannii* (Winton et al., 2002) and between qPCR and hyphal length for the EM fungus *Piloderma croceum* (Raidl et al., 2005). Partial correspondence between ergosterol assays and qPCR has been demonstrated in needles infected with *P. gaeumannii* (Winton et al., 2003), and a high correlation between qPCR and ergosterol was found in tissues colonized by the conifer root pathogen *Heterobasidion annosum* (Hietala et al., 2003).

CLIMATE CHANGE AND ITS POSSIBLE CONSEQUENCES FOR SPECIES INTERACTIONS

Anthropogenic climate change and its consequences on natural ecosystems became a major concern in recent years. Expected changes over the period 1990 to 2100 are an increase in mean land surface temperature of 1.4-6°C, a rise in atmospheric concentration of CO_2 and other greenhouse gases, larger interannual variations in precipitation, and an increase of extreme events (Houghton et al., 2001). All of these factors will strongly affect species distributions, behavior, and interactions. In the past years many excellent reviews were published, describing the effect of climate change in the different fields of ecology and evolution, but the consensus of all of these publications is that there is still a need for further investigations. In general, climate change is assumed to affect the phenology of different species, to lead to range shifts of populations and communities and to lead to extinctions of vulnerable species (Walther et al., 2002; Parmesan, 2006). The effect of climate change on endophytes is poorly understood and results are inconclusive. Whilst some studies showed an increase in endophyte colonization, others suggested none. However, some endophytes might

confer tolerance to their host against rising biotic stress due to climate change (Compant et al., 2010), whereas other endophytes that occur as latent pathogens might become a threat to their host plants. For instance, *Sphaeropsis sapinea* the causal agent of shoot blight in many conifers, can occur endophytically for several years without causing any symptoms, but when the host trees suffer from water stress disease might break out (Stanosz et al., 2001). Therefore, if drought events occur more frequently under climate change, *S. sapinea* disease incidence might increase. The effects of climate change on different pathosystems are variable, but diseases in general are thought to become more devastating. Possible effects of climate change are accelerated pathogen development rates, leading to more reproductive cycles, and increased pathogen overwintering, allowing earlier disease stress. Moreover, host and pathogen ranges can be shifted, and climate change related environmental stressors might lower host resistance and induce changes in host growth and physiology (Lonsdale and Gibbs, 1996; Ayres and Lombardero, 2000; Harvell et al., 2002; Garrett et al., 2006; Lafferty, 2009; Pautasso et al., 2010; Walther, 2010).

OUTLINE OF THE THESIS

The overall goal of this thesis was to gain a deeper insight into the nature and strength of interaction of PAC with one of their main hosts Norway spruce to find possible mechanisms explaining the wide and common occurrence of this fungus in natural populations, as there is still a lack in knowledge about the interaction of PAC with their host and studies are contradictory.

Chapter 2: qPCR quantification of root endophyte biomass. A nested single-copy locus-based and a multi-copy locus-based qPCR approach were developed specifically to detect and quantify PAC DNA in colonized host tissues. Because suitability of DNA content as surrogate of fungal biomass has been disputed, these two assays were compared with each other and with two visual quantification methods. Special emphasis was also given to different fungal structures.

Chapter 3: Norway spruce-root endophyte interactions. Differences among studies examining the effect of PAC on host plants could be attributable to the fact that PAC members form a cryptic species complex rather than one single species. Therefore, this *in vitro* experiment with six isolates each of the four most abundant PAC species was performed to compare possible interspecific differences. Moreover, the geographic origin of the isolates was never considered in previous studies about PAC. Thus, six isolates each of two additional

geographic origins of *P. subalpina* (one additional from within, and one from outside the native range of Norway spruce) were also included to account for geographic origin as confounding factor.

Chapter 4: Seedling protection by *P. subalpina*. PAC might confer an indirect benefit to Norway spruce by protecting its host against harmful root pathogens. In recent years, reports about oomycetous root pathogens became an emerging problem in forests and nurseries. Moreover, modeling of the spread of infectious diseases suggested that oomycetes might particularly expand under climate change. Moreover, symbiotic associations might be shaped by climate change as well. Therefore, a tripartite interaction experiment with six *P. subalpina* isolates and two oomycetous root pathogens was performed *in vivo* under two different temperatures regimes to examine the protective potential of PAC.

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CHAPTER 2

Suitability of quantitative real-time PCR to estimate biomass of fungal root endophytes

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ABSTRACT

A nested single-copy locus-based quantitative real-time PCR (qPCR) assay and a multicopy locus-based qPCR assay were developed to estimate endophytic biomass of fungal root symbionts belonging to the *Phialocephala fortinii* sensu lato-*Acephala applanata* species complex (PAC). Both assays were suitable for estimation of endophytic biomass, but the nested assay was more sensitive and specific for PAC. For mycelia grown in liquid cultures, the correlation between dry weight and DNA amount was strong and statistically significant for all three examined strains, allowing accurate prediction of fungal biomass by qPCR. For mycelia colonizing cellophane or Norway spruce roots, correlation between biomass estimated by qPCR and microscopy was strain-dependent and was affected by the abundance of microsclerotia. Fungal biomass estimated by qPCR and microscopy correlated well for one strain with poor microsclerotia formation but not for two strains with high microsclerotia formation. The accuracy of qPCR measurement is constrained by the variability of cell volumes, the accuracy of microscopy can be hampered by overlapping fungal structures and lack of specificity for PAC. Nevertheless, qPCR is preferable because it is highly specific for PAC and less time-consuming than quantification by microscopy. There is currently no better method than qPCR-based quantification using calibration curves obtained from pure mycelia to predict PAC biomass in substrates. In this study, the DNA amount of *A. applanata* extracted from 15 mm of Norway spruce fine root segments (mean diameter, 610 µm) varied between 0.3 and 45.5 ng, which corresponds to a PAC biomass of 5.1 ± 4.5 µg (estimate and 95%-prediction interval) and 418 ± 264 µg.

INTRODUCTION

Interactions between fungi and plants are very common in nature and range from mutualistic to pathogenic (Schulz and Boyle, 2005). The outcome of a plant-fungus interaction largely depends on the extent of colonization by the fungus, independent of whether pathogenic or mutualistic fungal species are involved (Bodles et al., 2006; Beldomenico and Begon, 2010). This is also true for endophytic species. In contrast to infections by pathogenic fungi, where disease symptoms are expressed after a comparatively short period of incubation, infection by endophytic fungi does not cause disease symptoms for prolonged periods, because once inside the tissue, endophytes assume a quiescent state either for the whole lifetime of the infected plant tissue or until the host is adversely affected by the arrival of biotic or abiotic stress (Petrini, 1991; Saikkonen et al., 1998; Stone et al., 2004; Sieber, 2007). Therefore, switching endophyte behavior from neutral to pathogenic or mutualistic can depend on the predisposition of the host tissue, environmental factors, and the extent of colonization. For instance, in conifer needles, the biomass of endophytic *Rhabdocline parkeri* thalli increase over time (Stone, 1987). It has been postulated that the needles die as soon as the endophyte's biomass exceeds a certain threshold value (Sieber, 2007). Therefore, attainment of the threshold usually coincides with natural senescence. However, the threshold can either be lowered or reached prematurely if host resistance is reduced by adverse factors. Thus, the health status of plants depends on the density of colonization by the endophyte, and vice versa.

Estimation of the extent of colonization is difficult, and there are certain conditions that must be met for techniques to determine fungal biomass. First, they should reproducibly combine target (i.e., species/genotype) specificity with accurate quantification of biomass. Traditionally, microscopy has been used to measure hyphal length or proportional colonization of host tissue (Newman, 1966; Bååth and Söderström, 1979; McGonigle et al., 1990). However, determination of fungal biomass by microscopy is very laborious, and results vary between investigators. Moreover, visual quantification is unspecific, as species designation is often difficult or impossible. Chemical methods measuring the amount of specific biomolecules stored inside fungal cells or released into the environment (e.g., the fatty acid ergosterol or the carbohydrate chitin) are also widely used (Wallander et al., 2001; Gessner and Newell, 2002). Although these methods are much less laborious than microscopy, they are nonspecific and problems can arise if used for field samples (Olsrud et al., 2007), and the minimum sample size required is comparatively high (Nylund and Wallander, 1992; Gessner and Schmitt, 1996). Quantitative real-time PCR (qPCR) (Heid et al., 1996; Smith and Osborn, 2009) combines specificity at different taxonomic levels with accurate measurement of DNA copy numbers and allows quantification of DNA in very small

samples. Different qPCR chemistries (TaqMan, SYBR green or molecular beacons) and methods are available (Heid et al., 1996; Hamelin, 2006). The choice of method used for qPCR assays largely depends on the aim of the study. While multicopy genes allow the detection of lower DNA amounts, single-copy genes give more precise measurements of DNA copy number, as the number of repeats of multicopy loci can differ between strains and even within individual strain (Birnstiel et al., 1971; Hibbett, 1992). In addition, sensitivity of qPCR can be increased by applying a nested approach, where the entire locus is initially amplified by conventional PCR, and the resulting product is then quantified with the specific primer-probe combination in a second step (de Souza et al., 2004; Schena et al., 2004). Diagnostic qPCR assays have been used for early and rapid detection of plant pathogens in the environment and in diseased tissues (Böhm et al., 1999; Ward et al., 2004; Okubara et al., 2005). However, little investigation has been done into the usefulness of qPCR to estimate fungal biomass, and considerable disagreement exists. For example, qPCR biomass estimates of multinucleate arbuscular mycorrhizal fungi (AMF) correlated only poorly with estimates obtained by visual assessment (Gamper et al., 2008). This was proposed to be due to the multinucleate nature of *Glomeromycota*. On the other hand, a correlation between qPCR and mycelial dry weight was demonstrated for the Swiss needle cast-causing parasite *Phaeocryptopus gaeumannii* (Winton et al., 2002) and between qPCR and hyphal length for the ectomycorrhizal fungus (EMF) *Piloderma croceum* (Raidl et al., 2005). Partial correspondence between ergosterol assays and qPCR has been demonstrated in needles infected with *P. gaeumannii* (Winton et al., 2003), and a high correlation between qPCR and ergosterol was found in tissues colonized by the conifer root pathogen *Heterobasidion annosum* (Hietala et al., 2003).

In the present study we tested the suitability of qPCR to estimate biomass of a common group of ascomycetous root endophytes. Members of the *Phialocephala fortinii* sensu lato-*Acephala applanata* species complex (PAC) (Stoyke and Currah, 1991; Grünig et al., 2008b) are ubiquitous root symbionts in woody plants, especially in conifers and heathland shrubs, where they are the most prominent endophytes (Ahlich and Sieber, 1996). PAC form loose networks of hyphae running mostly parallel to the root axis on the root surface but also grow inter- and intracellularly within the root cortex (Stoyke and Currah, 1993). Inside cortical cells and under certain culture conditions, PAC species can form microsclerotia, which are tight complexes of more or less isodiametric, to irregular, thick-walled cells that can endure harsh conditions and may therefore serve as resting spores and units of dispersal (Read and Haselwandter, 1981; Ahlich-Schlegel, 1997; Yu et al., 2001). The ecological role of PAC members is still controversial, despite several studies (Grünig et al., 2008a). The effects of PAC on their hosts were described as being pathogenic in some studies but beneficial in others. This variability in behavior was mainly due to the use of different, undefined isolates

and a multitude of experimental designs which either favored PAC members or the host plants (Grünig et al., 2008a). Recently, isolates characterized by specific molecular markers have become available, making PAC-host interaction studies more meaningful.

In this study we aimed (i) to develop a specific qPCR method that allows detection of all PAC members, (ii) to test the method's suitability for biomass estimation in three different experimental systems *in vitro* (liquid fungal cultures, cellophane culture and colonized roots of *Picea abies* seedlings) by using biomass estimates obtained by microscopy as reference, and (iii) to compare the reproducibility, sensitivity, and specificity of a nested single-copy qPCR assay and a multicopy qPCR assay.

MATERIALS AND METHODS

Mycelia were grown (i) in liquid cultures, (ii) in and on cellophane, and (iii) as endophytic thalli in roots of *in vitro*-grown Norway spruce (*Picea abies*) seedlings. One strain each of three PAC species was used: *A. applanata* (T1_58_1), *P. fortinii* sensu stricto (7_45_5), and *P. subalpina* (6_16_1) (Grünig et al., 2008b).

Mycelium preparation in liquid cultures. Strains were inoculated on terramycine-malt agar (TMA; 15 g liter^{-1} malt extract [Hefe Schweiz AG, Stettfurt, Switzerland], 20 g liter^{-1} agar, 50 mg liter^{-1} terramycine) and incubated at 20°C in the dark. After 1 week, one colonized agar plug (diameter, 4 mm) from the margin of the growing colony was transferred to 50 ml 2%-malt broth (20 g l^{-1} malt extract) in a 100-ml Erlenmeyer flask and incubated at 20°C on a rotary shaker at 100 rpm. After 3 weeks, the mycelium was harvested, washed twice with nanopure water, and lyophilized. Twelve weight samples per strain were taken, ranging from 1 to 40 mg.

Cellophane system. Inocula were prepared on TMA as described above. 10 days, five Petri dishes per strain containing water agar overlaid with autoclaved cellophane were inoculated with a colonized agar plug from the growing margin of the colony and incubated at 20°C for 24 days in the dark. Nine 10-mm-long and 5-mm-wide pieces of cellophane were cut from the margin of the growing colony on each Petri dish. Three pieces were pooled to represent one replicate for further analyses, resulting in a total of 15 replicates per strain. Each piece was cut in two squares of 5 by 5 mm, and one of each was randomly assigned to quantification by either microscopy or qPCR (see below).

In planta system. Mycelia were produced in malt extract broth as described above. Thalli were blotted dry on a sieve and weighed. The mycelium-to-water ratio was adjusted to 90 g l^{-1} (fresh weight) with sterile nanopure water. Thalli were homogenized with a blender for 30 s. Fifty-milliliter Falcon tubes containing a sterile 1:1 (v/v) -vermiculite-peat mixture were inoculated with 2 ml of the homogenized PAC mycelia and incubated for 3 weeks at 20°C. Two-week-old, sterile spruce seedlings were planted in the tubes. Seedlings had been produced from surface-sterilized seeds. Surface sterilization occurred by immersion in 30% H_2O_2 for 30 min, followed by rinsing with sterile nanopure water. Germination occurred on water agar at 18°C in the dark. After planting into the Falcon tubes, plants were transferred to a phytotron (16-h day [120 to 140 µE m^{-2} s^{-1}]/8-h night; temperature, 22°C/10°C; relative humidity [rH], 45%/85%). Plants were watered every other day with 3 to 4 ml deionized water and fertilized every third to fourth week with 3 to 4 ml of a 0.2% (v/v) -dilution (v/v) of a complete fertilizer (Wuxal, Maag, Switzerland). Six plants per fungal strain were harvested after 5 months. The shoot was cut off, and roots were washed under running tap water and dissected. Three 4-cm-long root pieces were excised from each root system, and lateral roots were removed. Each 4-cm-long root piece, constituting a replicate, was further subdivided into eight 0.5-cm-long segments. The outermost segments were surface sterilized (1 min in 30% H_2O_2, 10 s in 98% ethanol) and incubated on TMA to reisolate the inoculated fungal strain to accomplish Koch's postulates. From the six remaining segments, every other segment was assigned to qPCR and two of the three remaining ones to microscopic quantification. The three segments assigned to qPCR were pooled and considered one replicate. Correspondingly, the two segments assigned to microscopic quantification were considered one replicate.

Microscopic quantification of fungal biomass in cellophane and roots. Cellophane squares were stained with 0.5% trypan blue in lactophenol at 50°C overnight, mounted in 90% lactic acid on microscope slides, and analyzed with an Axiophot microscope (Zeiss, Switzerland). Pictures were taken with an AxioCam MRc5 camera at 200-fold magnification, and images were acquired with the AxioVision (4.6.3.0) software. Five pictures were taken per cellophane square (one in each corner at a distance of 0.8 mm from both abutting sides and one in the centre of the square, resulting in an analyzed area of 43,625 µm^2, i.e., 0.17% of the total square area).

The extended focus option of the AxioVision software was used to visualize the mycelia at various depths in the cellophane in the same image: starting on the uppermost layer where hyphae grew, a picture was taken every 2 µm down to the hyphae that penetrated deepest into the cellophane, resulting in 3 to 15 layers. Light intensity, exposure time, and brightness were optimized to enhance contrast. Mean hyphal width of each strain was determined and used to

categorize the width ranges for single hyphae (n=1) and hyphal strands consisting of n>1 hyphae. Lengths were measured for each category with the root analysis software WinRhizo (v2007d; Regent Instrument Inc., Canada) and multiplied by the corresponding n to obtain total hyphal length. Microsclerotia were characterized by parallel chains of short, wide cells. The width of these microsclerotial chains was several times that of single hyphae. Thus, the "length" of microsclerotia could be determined in the same way as that of hyphal strands.

For microscopy, root segments were destained in 10% KOH at 50°C overnight, and at room temperature for one more day. Then, the segments were acidified in 2% HCl for 2 min, washed twice with nanopure water, and preserved in 90%-lactic acid. The total length of the hyphae (l_{tot}) colonizing every individual root segment was estimated using a modified version of the line intersection method according to Newman (1966) (see Method S1 in the supplemental material). Per root segment, the number of intersections ($n_{int,i}$) of the hairline of the ocular lens with hyphae was counted along i = 6 transects, and the length of each transect ($l_{trans,i}$) was measured. The silhouette area (A_{tot}) was estimated based on $l_{trans,i}$. Hyphal length was calculated according to the formula $l_{tot} = \pi/2 \times A_{tot} \times \sum n_{int,i} / \sum l_{trans,i}$. In the analyses l_{tot} of both root segments was summed to represent one replicate. Moreover, the width of the microsclerotia ($l_{micro,i}$) transect line and the number of cells of the microsclerotia ($n_{micro,i}$) intersecting with the transect line were measured per root segment. These measurements were used to estimate microsclerotial biomass.

Development of qPCR. A nested single-copy qPCR assay and a one-step multicopy qPCR assay were developed, with both based on TaqMan chemistry. The single-copy, noncoding PAC-specific locus pPF-076 was chosen for the nested assay (Grünig et al., 2003; Grünig et al., 2004; Grünig et al., 2006). All available pPF-076 sequences of PAC strains were aligned and the most conserved DNA region was selected to design specific primers and a probe using the Primer Express 3.0 software (Applied Biosystems, Switzerland). In the first step, a portion (561 bp) of the locus pPF-076 was amplified with the external primers pPF-076_F1 and pPF-076_R1 (Grünig et al., 2007) in a conventional PCR. In the second step, the PCR product was subjected to the qPCR quantification step, using a specific pPF-076 qPCR primer-probe combination. Specificity of primers was tested by PCR amplification of DNA of PAC-free host plants and potential fungal contaminants in plant roots (see Table S2 in the supplemental material). The multicopy gene assay was based on the internal transcribed spacer (ITS) sequence of the rRNA locus. Primers and probe for qPCR were developed as described above. However, DNA was quantified in a single step, without the conventional PCR step.

DNA extraction and qPCR conditions. A few grains of DNA-free (dried 4 h at 180°C) silica sand were added to the cellophane and root samples to facilitate later disruption of cellulose

and plant cell walls. Then, samples were frozen at -80°C and freeze-dried for 2 days. Lyophilized mycelia, colonized cellophane squares, and colonized fine root segments were homogenized using a bead mill. DNA was extracted with the DNeasy plant mini kit (Qiagen, Basel, Switzerland) following the protocol of Grünig et al. (2003). For the cellophane and root samples the protocol was adjusted for small sample sizes: only 250 µl lysis buffer and 3 µl RNAse A were added to the ground tissue, and there was only one washing step with 500 µl chloroform-isoamyl alcohol (1:24). Extraction efficiency was estimated by extracting DNA from blank cellophane and PAC-free root samples spiked with known amounts of PAC DNA.

In step 1, PCRs were performed in a total volume of 25 µL containing 5 µL 1:25-diluted sample DNA, 2.5 µL 10× PCR buffer (GE, Switzerland), 200 µM dNTPs, 0.5 units of Taq polymerase (GE, Switzerland) and 500 nM each external primer. PCR amplification conditions were as follows: one cycle at 94°C for 2 min; 15 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 30 s; and a final cycle at 72°C for 6 min. In step 2, reactions were performed in a total volume of 25 µL containing 5 µL of the PCR product of step 1, 12.5 µL of 2× Reaction Buffer (qPCR MasterMix Plus Low ROX; Eurogentec, Belgium), 600 nM each primer, and 150 nM probe. Optimum primer and probe concentrations had been determined previously by varying primer and probe concentrations. PCR amplification conditions consisted of a denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescence was monitored during the 60°C phase. As a standard, a serial dilution of genomic DNA with known concentrations was added to each sample plate. Each sample was run in triplicate. Reaction conditions and the setup of the qPCR step of the multicopy gene assay were exactly the same as for step 2 of the single-copy gene assay.

Statistical analyses. All statistical analyses were performed with the R statistical package (R Development Core Team, 2009). A *t* test was used to test whether the slope of the regression line resulting from regression of the threshold cycle (C_T) values of the multicopy assay on the C_T values of the single-copy assay deviated significantly from 1. A full factorial ANOVA was applied to test relationships between the C_T value, strain, weight, or length of the mycelia and the area covered by microsclerotia. Only noncorrelated parameters were integrated in the models to avoid inflation of the ANOVA. Weight, length, and microsclerotial parameters were log transformed to achieve homogeneity of variances. The residuals of each model were analyzed to detect violations of the normality assumption and points with high leverages.

RESULTS

Development of qPCR. Based on the sequence data of the pPF-076 locus, forward primer pPF-076_qPCR_F (5'CGGATAGCTTCGCTGTGAATC3'), reverse primer pPF-076_qPCR_R (5'ACGCAGATCTTTCAAGGAGCTT3') and a fluorogenic probe pPF-076_qPCR_P (5'CTTTGTTGACGTACAGGATGCTCCCTCTG3') were designed, yielding PCR products of 88 bp. The probe was labeled at the 5' end with the fluorescent reporter dye Yakima Yellow and at the 3' end with the quencher Black Hole Quencher 1 (BHQ1) (Fig. 1A). Correspondingly, forward and reverse primers of the ITS locus PF-ITS_qPCR_F (5'CGTGTTTACATACTATTGTTGCTTTGG'3) and PF-ITS_qPCR_R (5'TCTCTGGCGG GCACACA'3) and the probe PF-ITS_qPCR_P (5'CCGTGGCCTCCACTGCGGG'3) yielded a product of 76 bp. The probe was labelled with the fluorescent reporter dye FAM at the 5' end and with BHQ1 at the 3' end (Fig. 1B).

The efficiency ($E = 10^{(-1/slope)}-1$) determined with a serial dilution of genomic DNA was similar for each individual qPCR assay, but differed significantly for the *A. applanata* strain in the pPF-076 assay (Fig. 2). The correlation between C_T values or DNA amounts of the single-copy and the multicopy assay was generally high but varied between different calibration methods, with DNA extracted from cellophane having the worst correlations (Table 1). The slope of the regression line of the C_T values of the multicopy assay on the C_T values of the single-copy assay did not deviate significantly from one ($t_{13} = 0.329$, $p = 0.374$), indicating that both assays are equally efficient. DNA extraction efficiency was about 70% for both cellophane and root samples. The single-copy assay was highly specific for PAC DNA. Even small amounts of PAC DNA could be detected, and no product was obtained from non-PAC DNA (see Table S2 and Fig. S3 in the supplemental material). In contrast, the multicopy assay also amplified DNA of non-PAC strains, i.e., *Acephala* sp. strain 1 and *Phaeomollisia picea*.

Table 1. Correlations between qPCR measurements obtained using the single-copy (pPF-076) and the multicopy (ITS) gene markers

	Pearson's correlation coefficient[a]			
Strain	**genomic DNA**	**liquid culture**	**cellophane cuttings**	**root segments**
A. applanata	0.996***	0.993***	0.685**	0.955***
P. fortinii s.s.	0.995***	0.994***	0.484	0.955***
P. subalpina	0.998***	0.997***	0.726**	0.932***
overall	0.988***	0.963***	0.741***	0.889***

[a] Correlations for genomic DNA were between C_T values from the two assays; otherwise, correlations were between DNA amounts determined with the two assays. Significance levels are indicated as follows: **, $p < 0.01$, ***, $p < 0.001$

Liquid cultures. The relationships between mycelium weight ($F_{1,26}$ = 560.20, p < 0.001), strain ($F_{2,26}$ = 28.50, p < 0.001) and the DNA amount as measured by single-copy qPCR were statistically significant in the global test with all three PAC strains. The DNA amount was regressed against mycelial dry weight for every strain individually, because strain had a significant but differential effect on the relationship between DNA amount and mycelial dry weight (Fig. 3A). The 95% prediction interval was narrow, indicating that DNA amount is a good predictor of fungal biomass. For a DNA amount of 1000 ng, the width of the 95% prediction interval for mycelial dry weight was 7.8 mg for *A. applanata*, 13.0 mg for *P. fortinii* s.s. and 37.5 mg for *P. subalpina*, indicating that the accuracy of the biomass prediction based on the DNA amount was best in *A. applanata* and poorest in *P. subalpina*. Correlation between DNA amounts estimated by the single-copy assay and the multicopy assay was high (Table 1), and the biomass estimates obtained with the multicopy assay were equivalent to those obtained with the single-copy assay. The width of the 95% prediction interval for mycelial weight was 8.8 mg for *A. applanata*, 6.7 mg for *P. fortinii* s.s. and 18.6 mg for *P. subalpina*.

```
A) pPF-076:
DQ013117          CAACCGATTCAGACCAGTGAA[331 bp]CGGATAGCTTCGCTGTGAATCG
P. subalpina      ....................[331 bp].....................
P. fortinii s.s.  ....................[331 bp].....................
A. applanata      ....................[331 bp].....................

DQ013117          CTTTGTTGACGTACAGGATGCTCCCTCTGGCATCTGAGACGGAGAAGCTCC
P. subalpina      ....................................-...........
P. fortinii s.s.  ....................................-...........
A. applanata      ....................................-...........

DQ013117          TTGAAAGATCTGCGT[102 bp]TTGTTCCATTGCTGCAGATTC
P. subalpina      ...............[101 bp]...........T.........
P. fortinii s.s.  ...............[101 bp]...........T.........
A. applanata      ...............[101 bp]...........T.........

B) ITS:
AY033087          CGTGTTTACATACTATTGTTGCTTTGGCGGGCCGTGGCCTCCACTGCGGG
P. subalpina      ................................................
P. fortinii s.s.  ................................................
A. applanata      ..............................A.................

AY033087          CTCTGCTCGTGTGTGCCCGCCAGAGA
P. subalpina      ...........................
P. fortinii s.s.  ...........................
A. applanata      ..........................G
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Figure 1. Partial sequence alignments of the pPF-076 locus (A) and the ITS locus (B). Primers are underlined, the TaqMan probe is underlined and in bold, and deletions are marked as a dash.

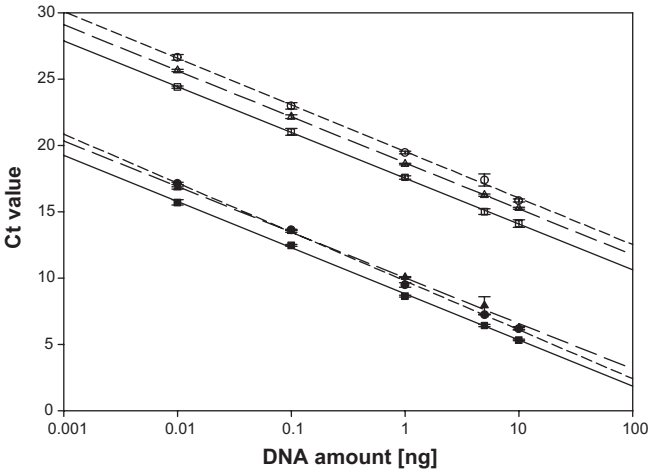

Figure 2. Regression of C_T values against the logarithm of the DNA amount of serially diluted DNA. Triangles, *A. applanata*; circles, *P. fortinii* s.s.; squares, *P. subalpina*. Filled symbols represent the single-copy assay, and open symbols show the multicopy assay.

Cellophane system. In the cellophane system, the relationships between hyphal length ($F_{1,39}$ = 12.21, p = 0.001), strain ($F_{2,39}$ = 14.77, p < 0.001) and DNA amount were significant for the single-copy assay. Due to the significant strain effect, each strain was analyzed individually. The relationship between hyphal length and DNA amount was significant for *A. applanata*, and *P. fortinii* s.s. but not for *P. subalpina* (Fig. 3B). DNA amount and hyphal lengths varied considerably among cellophane squares within and among Petri dishes (Fig. 4). The correlation between DNA amounts estimated with the single-copy and the multicopy assay was much lower compared with the other test systems, and the correlation was not

significant for *P. fortinii* s.s. (Table 1). The relationship between hyphal length and DNA amount was significant for *A. applanata* (R^2 = 0.546, $F_{1,13}$ = 15.65, p = 0.002) but not for *P. fortinii* s.s. (R^2 = 0.065, $F_{1,13}$ = 0.91, p = 0.357) and *P. subalpina* (R^2 = 0.243, $F_{1,12}$ = 3.85, p = 0.074) in the multicopy assay.

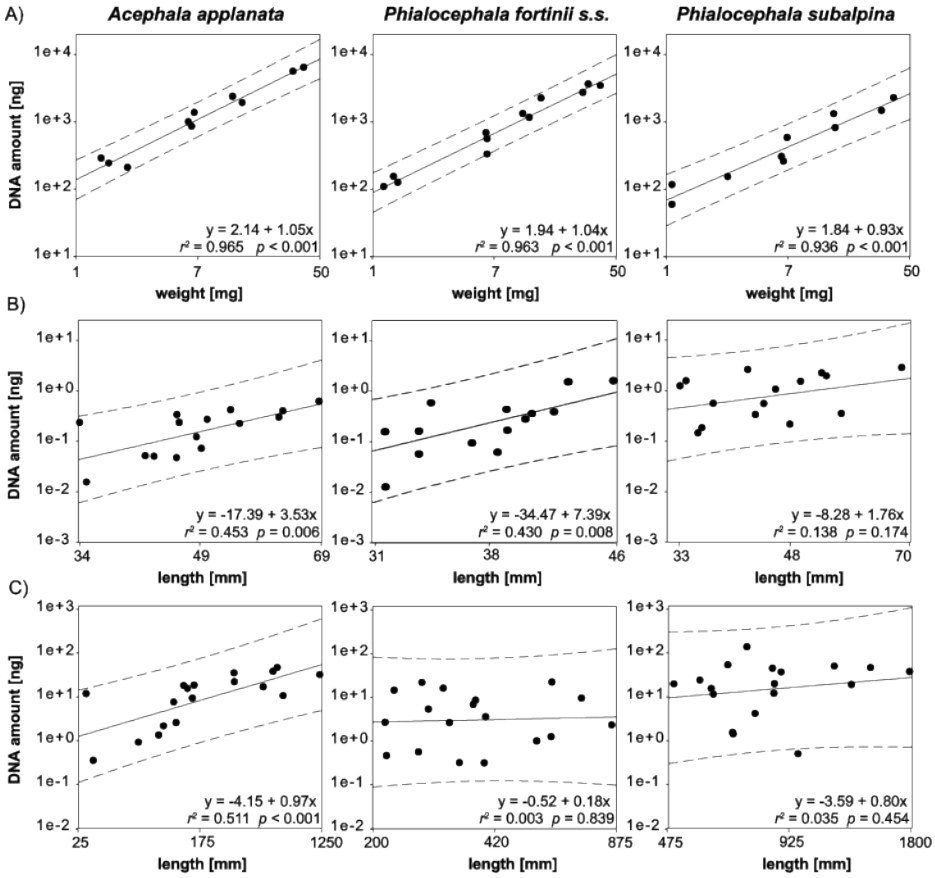

Figure 3. DNA amounts as measured by single-copy qPCR regressed on (A) dry weight of mycelia grown in liquid cultures, (B) hyphal length of mycelia grown on cellophane, and (C) hyphal length measured in *P. abies* roots (log scale axes). Dashed lines indicate the upper and lower 95% prediction intervals. In each graph the linear regression of y (the log[DNA amount]) against x (the log[weight or hyphal length]), r^2, and p-values are indicated.

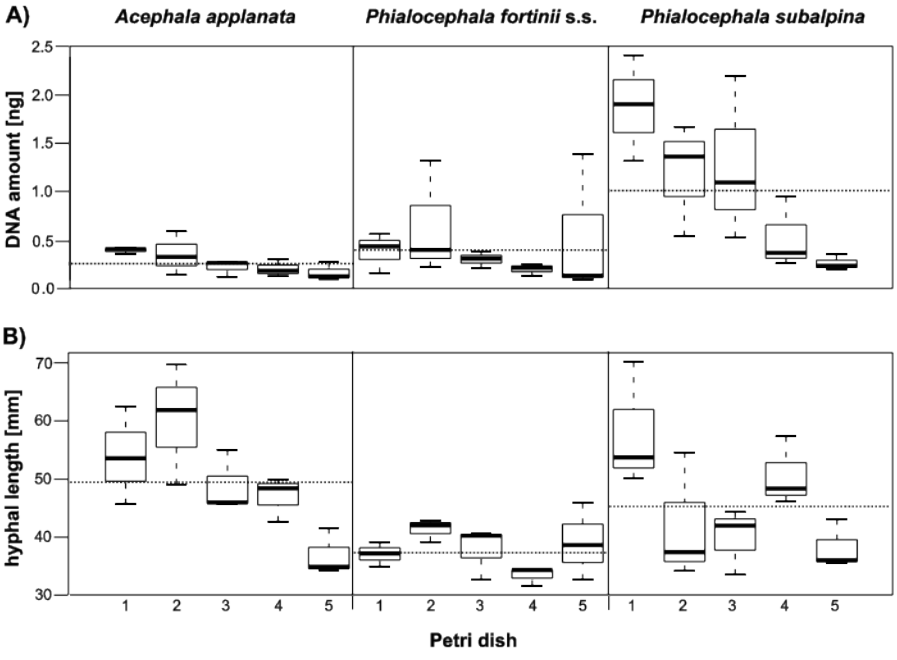

Figure 4. Fungal biomass estimates received from cellophane cultures. (A) DNA amounts as measured by single-copy qPCR; (B) hyphal length as measured with WinRhizo. Each box represents measurements from one Petri dish, and the dotted lines indicate the means for each PAC strain.

In planta system. The PAC strains could be reisolated from the roots of every plant, confirming successful colonization. *A. applanata* was reisolated from 30 of 36 root segments, *P. fortinii* s.s. from all 36 root segments, and *P. subalpina* from 32 of 36 root segments. All microsclerotial parameters correlated highly with each other ($R^2 \geq 0.956$, $p < 0.001$). Therefore, only one parameter was selected and included in the model to prevent inflation. We selected the sum of the mean silhouette area covered by microsclerotia of the two root segments originating from the same 4-cm-long root piece, because the fit of the ANOVAs was best when this parameter was used (data not shown). The area covered by microsclerotia per root segment has been defined as $s_a = 1/6 \times A_{tot} \times \sum(l_{micro,i} / l_{trans,i})$. In the global model, hyphal length ($F_{1,42} = 11.08$, $p = 0.002$) and strain ($F_{2,42} = 8.22$, $p < 0.001$) had significant influences on the DNA amount. In *A. applanata*, hyphal length ($F_{1,14} = 33.79$, $p < 0.001$), the area covered by microsclerotia ($F_{1,14} = 10.66$, $p = 0.006$) and the interaction between them ($F_{1,14} = 7.64$, $p = 0.015$) had significant influences on the DNA amount. For *P. fortinii* s.s., only the interaction between hyphal length and area covered by microsclerotia was significant ($F_{1,13} = 7.83$, $p = 0.015$). As for the mycelia grown in and on cellophane, there was no fit of the model at all for the *P. subalpina* strain ($R^2 = 0.072$, $F_{3,14} = 0.36$, $p = 0.782$), and residual analysis revealed no possibilities to improve the model. When the DNA amount was regressed against hyphal length, ignoring microsclerotia, the model of *A. applanata* was significant, whereas the models of the other two strains were not (Fig. 3C). DNA amount, hyphal length, and area covered with microsclerotia varied strongly among individual plants (Fig. 5). The correlation between DNA amounts estimated with the single-copy assay and the multicopy assay was high (Table 1), and the biomass estimates obtained with the multicopy assay were equivalent to those obtained with the single-copy assay.

DISCUSSION

Real-time qPCR was introduced in fungal ecology and pathology to detect and quantify fungi in natural samples and to investigate host-fungus interactions. The method has been used to estimate the biomass of plant pathogenic fungi in colonized plant tissues (Böhm et al., 1999; Okubara et al., 2005; Bodles et al., 2006), ectomycorrhizal fungi (EMF) growing in artificial medium and in seedlings (Landeweert et al., 2003; Schubert et al., 2003; Raidl et al., 2005), and arbuscular mycorrhizal fungi (AMF) in plant roots (Böhm et al., 1999; Gamper et al., 2008). However, the usefulness of qPCR and comparability with other methods to quantify fungal biomass has sometimes been controversial (Landeweert et al., 2003; Gamper et al., 2008). In the present study, the biomass of nonmycorrhizal fungal root symbionts was estimated by real-time qPCR and compared with estimates obtained by traditional visual

inspection methods. In addition, two qPCR assays (single-copy locus versus multicopy locus) were compared.

Comparison of single-copy and multicopy qPCR assays and determination of specificities of both assays for PAC. The efficiencies of different qPCR assays must be equal to allow comparison. This was shown to be the case for the two assays tested (Fig. 2). qPCR measurements of the two assays correlated well, except with DNA of *P. fortinii* s.s. extracted from cellophane (Table 1). Nonetheless, the C_T values of the single-copy qPCR were 6 to 10 times lower than those of the multicopy assay, indicating that the sensitivity of the single-copy qPCR was 100 to 1,000 times higher. Although nested qPCR assays are widely used (e.g., Cullen et al., 2001; Schena et al., 2004), the additional PCR run increases the risk of cross-contamination and handling errors. However, the highly significant correlation between DNA amounts estimated with the single-copy and the multicopy assay showed that this risk was low for biomass estimation of mycelium grown in liquid cultures and endophytic mycelium in plant roots. There are several reasons for the low correlation between DNA amount estimates obtained with the two assays for DNA extracted from the cellophane samples: first, the density of mycelia was lower in cellophane than in roots or pure, freeze-dried mycelium, and therefore the DNA amount was probably at the detection limit in the multicopy assay. Second, cellophane is a glucose polymer and polysaccharides are known to hamper DNA extraction and PCR amplification (Bahnweg et al., 1998; Varma et al., 2007). Therefore, qPCR measurements of the single-copy assay are probably more accurate due to dilution of PCR inhibitors after the first amplification step.

Specificity for the target microorganism(s) is one of the strengths of the qPCR method, allowing detection and quantification of microorganisms in semisterile microcosms or in the field (Schena et al., 2004; Ward et al., 2004; Okubara et al., 2005; Smith and Osborn, 2009). One aim was to develop a qPCR assay specific on the PAC level to study the nature (mutualism/antagonism) of PAC-plant interactions *in vitro*. Species specificity was not essential, as the assay should allow detecting of strains of several PAC species. Specificity was tested by amplifying DNA from the host, frequent fungal contaminants, and close relatives of PAC (Grünig et al., 2009; Münzenberger et al., 2009). While none of these control templates was amplified using the single-copy assay the multicopy qPCR resulted in the amplification of DNA of close relatives of PAC (see Table S2 in the supplemental material). As some of these taxa are also known to colonize plant roots (Grünig et al., 2009), it is possible that the multicopy assay will overestimate PAC DNA in semisterile or environmental samples. Therefore, due to the higher sensitivity and higher specificity the nested qPCR assay is superior to the multicopy approach to quantify PAC DNA in natural samples.

Suitability of qPCR to measure biomass of pure fungal mycelium. The best measurement for fungal biomass is mycelial dry weight. The strong linear relationship between mycelial dry weight and qPCR estimates of biomass demonstrates that qPCR is a reliable method for PAC biomass estimation and that the regression lines are suitable as calibration curves (Fig. 3A). Similarly, a strong correlation between mycelial dry weight and qPCR was found for the ascomycete *Phaeocryptopus gaeumannii* (Winton et al., 2002). PAC strain had a small but significant effect on measurements of DNA amount (Fig. 3A). A likely explanation for this is that the number of nuclei per unit of mycelium differs slightly among strains due to differences in cell size. Indeed, differences in growth response and cell lengths among PAC strains have been documented in previous studies (Ahlich and Sieber, 1996; Ahlich-Schlegel, 1997).

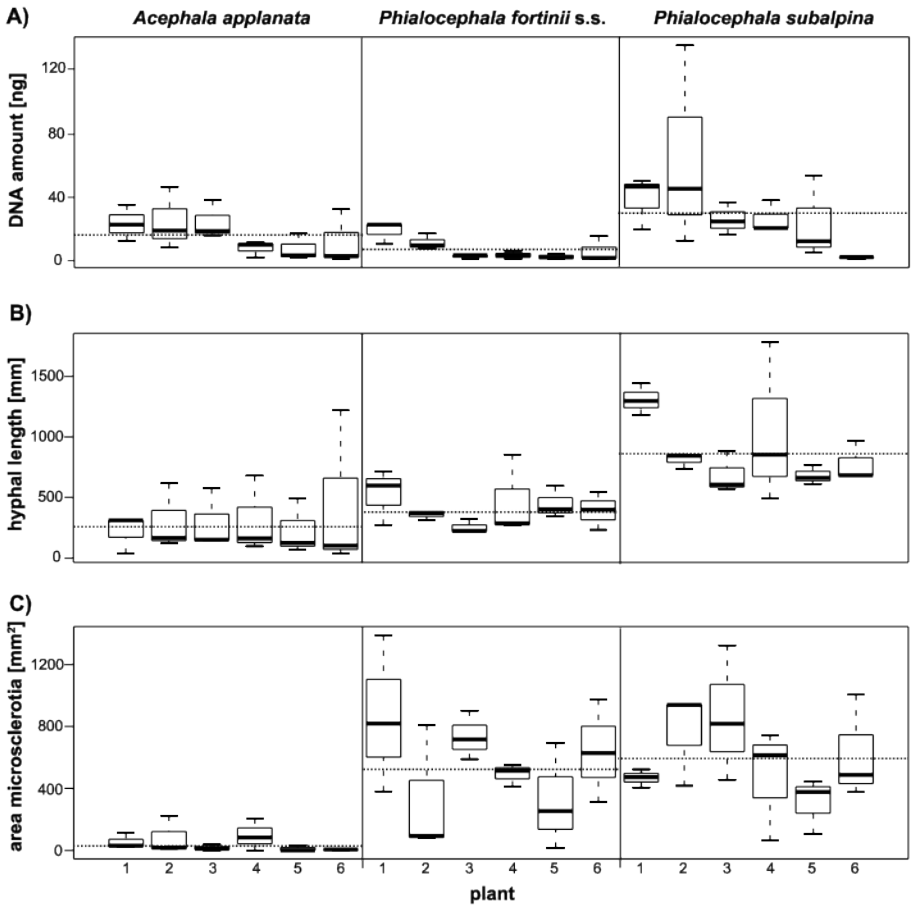

Figure 5. Fungal biomass estimates obtained from colonized roots. (A) DNA amounts as measured by single-copy qPCR; (B) hyphal length as meas–ured by the Newman method; (C) area covered by microsclerotia. Each box represents measure–ments from one plant, and the dotted lines indicate the means for each PAC strain.

Correspondence of qPCR and microscopy in determining biomass of fungal mycelia growing in and on organic substrates. The weight of mycelia growing in and/or on organic substrates cannot be measured directly, as it is impossible to properly separate substrate and mycelia. Thus, indirect methods must be used to estimate fungal biomass. Biomass was measured in cellophane and in the *in planta* system by using microscopy and qPCR. Strength of correlation between microscopic measurements and qPCR differed strongly among the

three PAC strains and between the two substrates, and correlations were lower than those obtained for mycelia grown in liquid cultures. The weak correlations between estimates obtained by qPCR and microscopy in the cellophane and the *in planta* system resulted from the weaknesses of either method. Nonetheless, a significant relationship between hyphal length and qPCR was found for *A. applanata* in both systems (Fig. 3B and C). Similarly, hyphal length correlated positively with the number of ITS loci of *Piloderma croceum* grown in agar on microscope slides (Raidl et al., 2005).

The main reason for the weak correlation of the biomass estimates obtained by qPCR and microscopy for the other two PAC strains seems to be the presence of microsclerotia. It is technically demanding to extract DNA from microsclerotia, which form tight complexes of thick-walled cells (Li et al., 1999). Therefore, proper homogenization of the sample is crucial. This was attempted by the addition of a few grains of silica sand. Ground root samples were shown to be properly homogenized, and incompletely crushed root fragments were rarely observed. Therefore, incomplete homogenization of samples was not the reason for the lack of congruence between estimates obtained by qPCR and microscopy. In addition to variable cell dimensions, the number of nuclei per cell can be a source of variation of qPCR estimates. However, hyphae and microsclerotia of PAC are consistently monokaryotic according to our observations and information from R. L. Peterson (personal communication). Thus, differing numbers of nuclei per cell can be excluded as a source of variation.

On the other hand, fungal biomass estimates using light microscopy can be hindered by (i) increases in erroneous counts of hyphae and microsclerotial cells due to increasing colonization density, (ii) underestimation of microsclerotial biomass with increasing size of microsclerotia, and (iii) limited visibility of (hyaline) fungal structures. (i) Since the pictures taken at various depths in the cellophane were merged into a single picture, WinRhizo was not able to distinguish overlapping hyphae or microsclerotia. Similarly, some overlapping hyphae and microsclerotia in roots escaped counting, although fungal structures were examined on several focal planes separately. The denser the colonization, the more often fungal structures overlapped or were overlooked. (ii) Similarly, the difficulty in accurately measuring the vertical dimension of microsclerotia (i.e., the extension of microsclerotia parallel to the optical path) increases with increasing size, density, and frequency of these structures. (iii) Hyphae of PAC are hyaline when young and thus may escape observation in both cellophane and roots, leading to an underestimation of fungal biomass. In addition, intercellular hyphae often grow parallel to host cell walls, rendering accurate counting difficult (Yu et al., 2001). Another source of error in the microscopic quantification of roots may be due to false positives (Atkins et al., 2009). Since the seedlings were grown under semisterile conditions, colonization by non-PAC species occurred, albeit rarely (as confirmed by reisolation). Some of these species produced melanized mycelia (e.g., *Cladosporium* sp.,

Virgariella sp.) and could erroneously be considered PAC. However, the potential for false positives was minimized, as the PAC strains were inoculated in the substrate under axenic conditions before the seedlings were planted and grown in the phytotron. Furthermore, colonization of roots by non-PAC fungi is expected to be more important in plants only weakly colonized by PAC, due to less competition for infection sites. This was, however, not the case in our study, as the correlation between qPCR and microscopic biomass estimates was strongest for *A. applanata*, which formed less dense endophytic hyphal and microsclerotial networks than the other two strains (Fig. 5B). Finally, line intersect sampling was used to estimate fungal biomass in roots. This method is adequate for estimation of parameters of needle-shaped objects such as hyphae but microsclerotia in roots can assume almost any shape and thus are less suitable for estimation by this method.

It is impossible to compare qPCR and visual inspection with regard to the accuracy of fungal biomass estimates, because there is no reference for the "true" amount of fungal biomass in cellophane and in roots. However, the specificity of qPCR is high and time consumption is low in contrast to visual methods. In addition, estimates of the biomass of pure mycelium obtained by qPCR were highly accurate, as indicated by narrow 95%-prediction intervals (Fig. 3A). For example, the DNA amount from samples of three pooled 5-mm-long root segments (mean diameter, 610 µm) colonized by *A. applanata* varied between 0.3 and 45.5 ng (Fig. 3C). The upper and lower limit of the 95% prediction interval of biomass for a DNA amount of 0.3 ng were 0.6 µg and 9.7 µg and for a DNA amount of 45.5 ng they were 154 µg and 683 µg based on the calibration curve obtained from pure mycelium grown in liquid culture (Fig. 3A). Thus, there is currently no better method to predict PAC biomass in and on substrates than qPCR-with the calibration curves obtained from pure mycelium grown in liquid culture. The method can also be used to detect PAC and quantify fungal biomass as DNA amount in field samples. Although significant strain differences were detected, estimated values were of the same order of magnitude, because differences in C_T values among strains were about 1 (Fig. 2). However, to gain insight into more complex interactions of PAC with their hosts in nature, primer-probe combinations would have to be developed to specifically detect single PAC strains or species.

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SUPPLEMENTAL MATERIAL

Method S1: Estimating hyphal length. To calculate total hyphal length we used a modification of the formula proposed by Newman (1966). Per root segment, the number ($n_{int,i}$) of intersecting hyphae was counted along each of six equidistant (distance = x = 0.7 mm) parallel transects perpendicular to the longitudinal axis of the root with the length of the i^{th} transect line being $l_{trans,i}$. The hairline of the eyepiece of the microscope was used to represent the transects, i.e., $n_{int,i}$ was determined for each transect by counting the hyphae intersecting with the hairline. Total hyphal length (l_{tot}) was calculated according to formula [1], where N is the total number of hyphae intersecting with all transects, A_{tot} the inspected area, i.e., the silhouette area of the root segment, and H the total length of all transects.

$$l_{tot} = \pi/2\, A_{tot}\, N/H \quad [1]$$

The silhouette area (A_{tot}) of the root segment was calculated as the sum of the area A_i of each of five adjoining equidistant (distance = x) trapezes (Fig. S1).

$$A_i = x \times \frac{(l_{trans,i} + l_{trans,i+1})}{2} \quad [2]$$

$$A_{tot} = \sum A_i \quad [3]$$

By inserting [2] in [3] A_{tot} becomes:

$$A = {}^1\!/_2\, x \times (l_{trans,1} + 2l_{trans,2} + 2l_{trans,3} + 2l_{trans,4} + 2l_{trans,5} + l_{trans,6}) \quad [4]$$

Then, N was calculated

$$N = \sum n_{int,i} \quad [5]$$

and finally H

$$H = \sum l_{trans,i} \quad [6]$$

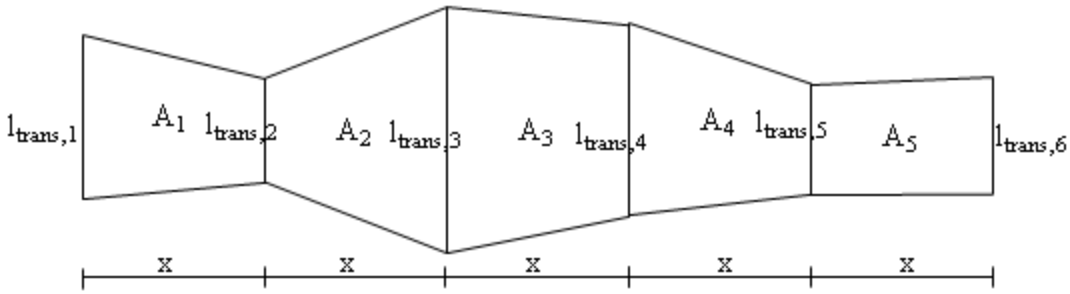

Figure S1. Schematic representation of the root silhouette area.

Table S2: C_T values received with nested single-copy qPCR (pPF-076 locus) and multi–copy qPCR (ITS locus) for *P. fortinii* s.s. DNA in two different concentrations (under–lined), close relatives of PAC (bold) and contaminants isolated from the vermiculite-peat mixture used as potting medium in the in-planta experiment.

	Species	C_T pPF-076	C_T ITS	Reference
1	*P. fortinii* s.s. (10 ng DNA)	6.2	15.3	(Grünig et al., 2006)
2	*P. fortinii* s.s. (0.01 ng DNA)	16.8	25.7	(Grünig et al., 2006)
3	***Acephala* sp. 1**	24.5	15.4	(Grünig et al., 2009)
4	***Phaeomollisia piceae***	23.9	15.0	(Grünig et al., 2009)
5	***Acephala macrosclerotiorum***	22.5	29.7	(Münzenberger et al., 2009)
6	***Phialocephala compacta***	22.8	29.2	(Grünig et al., 2009)
7	***Phialocephala glacialis***	22.7	32.2	(Grünig et al., 2009)
8	***Vibrissea truncorum***	20.4	31.9	(Grünig et al., 2009)
9	***Phialocephala sphaeroides***	22.9	32.8	(Grünig et al., 2009)
10	*Cladosporium cladosporiodes*	20.5	31.6	Present work
11	*Penicillium spinulosum*	22.2	31.7	Present work
12	*Trichoderma* sp.	23.2	32.5	Present work
13	*Virgariella* sp.	24.0	33.8	Present work
14	*Fusarium* sp.	23.6	33.6	Present work
15	*Leucosporidium scottii*	20.9	31.7	Present work
16	*Rhinocladiella atrovirens*	23.2	30.9	Present work
17	*Sporothrix* sp.	22.9	32.0	Present work
18	*Picea abies*	22.3	34.0	Present work
19	non-template control	25.3	34.7	

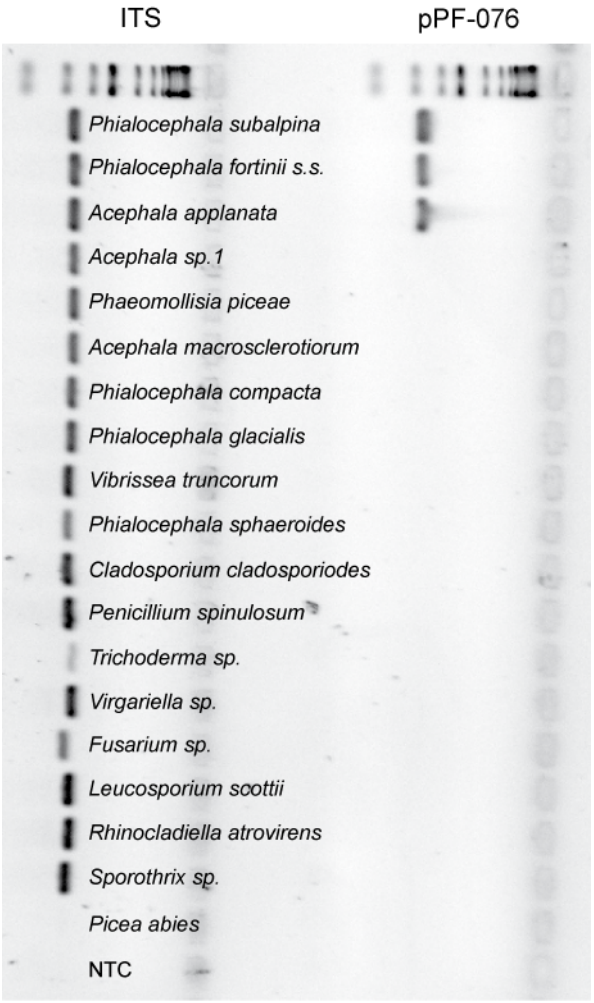

Figure S3. Amplification of the entire pPF-076 locus with the external primers pPF-076_F1 and pPF-076_R1 (Grünig et al., 2007) and the ITS locus with the primers ITS1 and ITS4 as positive control for presence/absence of fungal DNA (White et al., 1990). NTC = non-template control

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CHAPTER 3

Negative effects on survival and performance of Norway spruce seedlings colonized by dark septate root endophytes are primarily isolate-dependent

with C.R. Grünig and T.N. Sieber
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ABSTRACT

Root endophytes are common and genetically highly diverse suggesting important ecological roles. Yet, relative to above-ground endophytes, little is known about them. Dark septate endophytic fungi of the *Phialocephala fortinii* s.l.–*Acephala applanata* species complex (PAC) are ubiquitous root colonizers of conifers and Ericaceae, but their ecological function is largely unknown. Responses of Norway spruce seedlings of two seed provenances to inoculations with isolates of four PAC species were studied *in vitro*. In addition, isolates of *Phialocephala subalpina* from two populations within and one outside the natural range of Norway spruce were also included to study the effect of the geographic origin of *P. subalpina* on host response. The interaction of PAC with Norway spruce ranged from neutral to highly virulent and was primarily isolate-dependent. Variation in virulence was much higher within than among species, nonetheless only isolates of *P. subalpina* were highly virulent. Disease caused by *P. subalpina* genotypes from the native range of Norway spruce was more severe than that induced by genotypes from outside the natural distribution of Norway spruce. Virulence was not correlated with the phylogenetic relatedness of the isolates but was positively correlated with the extent of fungal colonization as measured by quantitative real-time PCR.

INTRODUCTION

Symbiotic fungi of plants are widespread and highly diverse contributing to adaptation and selection in plant communities (Dobson and Crawley, 1994; Clay, 2001; Gilbert, 2002; Selosse et al., 2004). While research has been focused largely on the two extremes of these symbioses (i.e., mycorrhizae and pathogens), seemingly neutral associations such as endophytes, which live inside the host tissue, are of increasing interest (Carroll, 1988; Saikkonen et al., 1998; Sieber, 2002; Rodriguez et al., 2009). Endophytic fungi colonize functional plant tissues of aerial plant parts (Carroll, 1988; Petrini, 1991; Rodriguez et al., 2009) as well as roots (Stoyke et al., 1992; Varma et al., 1999; Sieber, 2002; Addy et al., 2005; Mandyam and Jumpponen, 2005; Schulz and Boyle, 2005; Summerbell, 2005), but apparent disease symptoms do not develop at all or for prolonged periods of time (Carroll, 1988; Saikkonen et al., 1998; Sieber, 2007; Rodriguez et al., 2009). Endophytes can have beneficial effects on their host plant by promoting plant growth (Varma et al., 1999; Mandyam and Jumpponen, 2005; Schulz, 2006; Rodriguez et al., 2009), by increasing tolerance to abiotic stress (Varma et al., 1999; Clay, 2001; Mandyam and Jumpponen, 2005; Rodriguez et al., 2009), by the production of compounds that are toxic for herbivores (Carroll, 1988; Clay, 2001; Arnold et al., 2003; Selosse et al., 2004; Schulz, 2006; Sieber, 2007; Miller et al., 2008), and by protecting the plant either directly by occupation of infection sites, by hyper-parasitism and antibiosis, or indirectly by inducing the plant's defence system (i.e., acquired resistance) against harmful pathogens (Arnold et al., 2003; Selosse et al., 2004; Schulz, 2006; Sieber, 2007; Oelmüller et al., 2009). In contrast, weak pathogens might slow down plant growth by allocating resources that are taken up or produced by the plant, or penetrate living host cells and kill them. They might remain latent in the host until the host's physical status or environmental conditions change and trigger pathogenicity in the endophyte (Sinclair and Cerkauskas, 1997; Saikkonen et al., 1998; Sieber, 2007; Barrett et al., 2009). However, the true nature of interaction with their host and the ecological significance of endophytic fungi is still not fully understood and it is assumed that they stretch a continuum from mutualism to antagonism, which was also shown for mycorrhizal fungi (Carroll, 1988; Johnson et al., 1997; Jumpponen, 2001; Schardl et al., 2004; Schulz and Boyle, 2005; Rodriguez et al., 2009).

Dark septate endophytes (DSE) are abundant fungal root colonizers of a wide range of mycorrhizal and nonmycorrhizal plant species, particularly of woody plants, forming a polyphyletic group of fungi that are characterized by melanized, septate hyphae (Stoyke et al., 1992; Ahlich and Sieber, 1996; Jumpponen and Trappe, 1998b; Summerbell, 2005). In conifers and ericaceous shrubs the most prevalent members of the DSE are anamorphic Helotialean ascomycetes of the *Phialocephala fortinii* s.l.–*Acephala applanata* species

complex hereafter referred to as PAC (Wang and Wilcox, 1985; Ahlich and Sieber, 1996; Grünig et al., 2008). While *A. applanata* is morphologically distinct (Ahlich and Sieber, 1996; Grünig and Sieber, 2005), other PAC members are cryptic species that can only be identified using molecular techniques. PAC species are widely distributed across the northern hemisphere without showing any biogeographic pattern. PAC communities are composed of up to ten species, which seem to show little host-specificity apart from *A. applanata* and do not correlate with climate (Ahlich and Sieber, 1996; Harney et al., 1997; Addy et al., 2005; Grünig et al., 2006; 2008). Consequently, species composition of PAC communities is considered as purely stochastic (Queloz et al., 2011).

The frequency of PAC, their high genetic diversity and their versatile effects on plants suggest that they are important components of many ecosystems. Reports about the effects of PAC on host fitness are contradictory, with effects ranging from beneficial to pathogenic for different hosts, growing conditions and fungal isolates (literature summarized in Grünig et al., 2008). However, the test systems varied greatly among studies (Jumpponen, 2001; Grünig et al., 2008; Newsham, 2011), and all experiments were performed without PAC-species assignment and little or no attention was paid to variability among isolates, population and geographic origin of PAC. Therefore, we have performed this study to improve our understanding of the host–PAC interaction. We included 36 genotypically well-defined PAC isolates and studied their effects on disease intensity and performance of Norway spruce seedlings from two seed provenances under controlled environmental conditions *in vitro*. Specifically, we have studied (i) interactions of six isolates each of four PAC species to examine isolate and species effects on host response, and (ii) those of six isolates each of two European and one North-American *Phialocephala subalpina* population to investigate the effect of the geographic origin of *P. subalpina* from within and outside the native range of Norway spruce. In addition, we have examined (iii) whether disease intensity is correlated with the genetic relationship of the isolates or the extent of PAC colonization.

MATERIALS AND METHODS

Host plant and fungal isolates. Norway spruce (*Picea abies* Karst.), a European native species, is the main host of PAC in Europe. Seedlings from two different seed provenances [Central Alps and Swiss Plateau (890–970 and 520 m asl), Switzerland] were used to account for genetic variability of the host in the native range. To study differences among PAC species, six isolates each of the four globally most prominent species were included (*Phialocephala fortinii* sensu stricto (s.s.), *P. subalpina, P. europaea* and *Acephala applanata*). All 24 isolates originated from the same PAC community (Bödmeren, BOD)

(Table S1). In addition, six isolates each of two *P. subalpina* populations from Finland (Kevo, KEV) and Oregon (Noonday, NOD) were included to study the influence of geographic origin (Table S1).

Experimental set-up. Fungal isolates were grown on terramycine-malt agar [TMA; 15 g l^{-1} malt extract (Hefe Schweiz AG, Stettfurt, Switzerland), 20 g l-1 agar, 50 mg l-1 terramycine] at 20°C in the dark. After 1 week, one colonized agar plug (diameter 4 mm) from the margin of the growing colony was transferred to 50 ml of 2% malt broth (20 g l^{-1} malt extract) in 100 ml Erlenmeyer flasks and incubated at 20°C on a rotary shaker at 100 rpm. After 18 days, the mycelium was harvested, and washed twice with sterile water. Thalli were blotted dry on a sieve and weighed. The thallus to water ratio was adjusted to 186 g l^{-1} (fresh weight) with sterile nanopure water. Thalli were homogenized with a blender for 30 s. Fifty-millilitre Falcon tubes containing a sterile 1:1 (v/v) vermiculite : peat mixture were inoculated with 1 ml of the homogenized PAC mycelium and incubated for 17 days at 20°C in the dark. Twelve-day-old, sterile spruce seedlings were planted in the tubes. Seeds had been surface-sterilized by immersion in 30% H_2O_2 for 30 min, followed by rinsing with sterile nanopure water. Germination occurred on water agar at 18°C in the dark. After planting into the Falcon tubes, plants were transferred to a phytotron [16 h day (120–140 µE m^{-2} s^{-1})/8 h night, temperature (22°C/10°C) and relative humidity (rH 45%/rH 85%)]. Tubes were randomly distributed and rearranged regularly to exclude block effects. Plants were watered every other day with 3–4 ml of deionized water and fertilized every third to fourth week with 3–4 ml of a 0.2% dilution (v/v) of a complete fertilizer (Wuxal, Maag, Switzerland). Each treatment was replicated 10 times.

Data collection. Seven months after inoculation, the experiment was terminated and virulence and seedling performance were measured. Virulence, i.e., the degree of damage caused to the seedlings, was expressed as disease intensity and reduction of seedling performance. Disease intensity which includes the two components disease incidence, i.e., the number of affected seedlings (e.g., mortality rate), and disease severity, i.e., the percentage of necrotic or chlorotic surface area of the needles, was scored: (0) seedlings without any apparent disease symptoms, (1) seedlings with less than 50% of the needle surface necrotic, (2) seedlings with more than 50% of the needle surface necrotic or chlorotic, and (3) seedlings dead. Thereafter, the seedlings were harvested. The shoot was cut off, and roots were washed under running tap water, scanned on a standard flatbed scanner to measure root length, using the root analysis software WinRhizo (v2007d, Regent Instruments Inc., Canada), and dissected. Three 5-mm-long root segments were excised from the root system of three randomly selected seedlings per treatment: one from the growing region of the root, one from the middle of the root, and

one close to the hypocotyl. The segments were surface-sterilized (1 min in 30% H_2O_2, 10 s in 98%-EtOH) and incubated on TMA to re-isolate PAC isolates to verify Koch's postulates. Fungal re-isolations on TMA were genotyped with microsatellites (Queloz et al., 2010) to verify identity. To estimate the extent of fungal colonization, PAC DNA content per mg root weight was measured by means of nested qPCR. Similar to above, four 5-mm-long root segments from the same root regions (an additional segment from the middle of the root) were processed as described previously (Tellenbach et al., 2010). The seedlings were dried at 50°C for two consecutive days and performance of the plants was measured using the following parameters: total biomass, needle, shoot, root mass, R/S ratio and SRL.

Statistical analyses. All statistical analyses were performed with the R statistical package (R Development Core Team, 2009). Disease intensity was analysed with a proportional odds model using the *lrm* function in the *Design* package with seedling provenance, and either PAC isolate, PAC species or geographic origin, and the respective interactions as explanatory variables. Effects of PAC inoculations on performance of spruce seedlings compared with uninoculated control plants were analysed by linear regression. Two linear mixed-effects models, using the *lme* function in the *nlme* package, were performed (i) to analyse the effect of different PAC species and (ii) to analyse the effect of different geographic origins of *P. subalpina*. In both models we set PAC isolates as random variables and as fixed effects seedling provenance, and either (i) PAC species or (ii) geographic origin, and the respective interaction with seedling provenance. Uninoculated control plants were not included in this analysis. Selection of random model terms was done using a Wald test, whereas for the fixed effects the *F*-values were calculated. Correlation between genetic distance and difference in virulence among the 18 *P. subalpina* isolates from the three different geographic regions was tested with a Mantel test. Pairwise genetic distances among isolates were calculated with the D_c criterion using microsatellite data, whereas dissimilarity in virulence was calculated accordingly with the $(\delta\mu)^2$ in Populations 1.2.30 (Langella, 1999) treating disease severity as a locus with the four disease categories as alleles. The influence of the extent of colonization and disease incidence was investigated using a Pearson's correlation of the logarithm of DNA content and number of healthy seedlings within individual treatments.

RESULTS

All PAC isolates were successfully re-isolated from inoculated roots (on average from 94.1% of the root segments; range: 33–100%), whereas there were no PAC in the uninoculated control seedlings. Genotyping of recovered isolates confirmed the presence of the inoculated isolate in every case. In addition, no cross-contaminations were observed.

Effects of isolates of different PAC species on disease intensity and performance of spruce seedlings. Disease intensity ranged from 0 to 80% of dead or heavily diseased seedlings depending on the isolate (Fig. 1D). Compared with the control, the overall isolate effect on disease intensity was significant (χ^2 = 150.16, d.f. = 48, $P < 0.001$), but significance of this effect was due to only a few isolates. For example, mortality rates were highest for *P. subalpina* isolates 6_16_1 and 6_2_7v, amounting to up to 70% (Fig. 1D). Variation of disease intensity was higher within than among PAC species, but PAC species differed significantly in regards to overall disease intensity (χ^2 = 31.65, d.f. = 8, $P < 0.001$). Disease intensity of plants inoculated with *A. applanata* was higher on average but mostly only moderate compared with seedlings inoculated with one of the other three PAC species. Disease intensity was least severe when seedlings had been inoculated with *P. fortinii* sensu stricto (s.s.) or *P. europaea*. Furthermore, disease intensity did not differ between the two seed provenances, neither in the analysis of different isolates nor in the analysis of different PAC species (χ^2 = 12.31, d.f. = 25, $P = 0.984$; χ^2 = 5.49, d.f. = 5, $P = 0.359$).

Regarding plant performance, there was a strong among-isolate effect on all parameters (Fig. 1A–C, Fig. S1, Table S2). Seedlings inoculated with isolates 6_16_1 and 6_2_7v differed significantly from the uninoculated controls with respect to all plant performance parameters, in contrast to seedlings inoculated with isolates 6_37_6v, 6_53_6v and T1_55_1 tthat did not differ from the controls. Although some isolates had no significant adverse effect on biomass gain, infections were associated with costs and growth promotion was never observed. Effects on biomass parameters varied considerably among fungal isolates ($\chi^2 > 75$, d.f. = 1, $P < 0.001$) according to the mixed effects models (Table S3). However, different PAC species had a differential effect only on specific root length (SRL) ($F_{3,20}$ = 3.518, $P < 0.05$). Seedlings from the two seed provenances inoculated with PAC differed only in the root-associated parameters root mass ($F_{1,450}$ = 9.932, $P < 0.01$) and root-to-shoot ratio (R/S ratio) ($F_{1,450}$ = 54.154, $P < 0.001$) (Table S3), with the provenance from the Swiss Plateau generally exhibiting reduced root mass and a lower R/S ratio (Fig. S1C, Fig. 1B). Furthermore, there was no significant interaction between species and provenance, indicating that PAC species affected seedlings from both provenances equally.

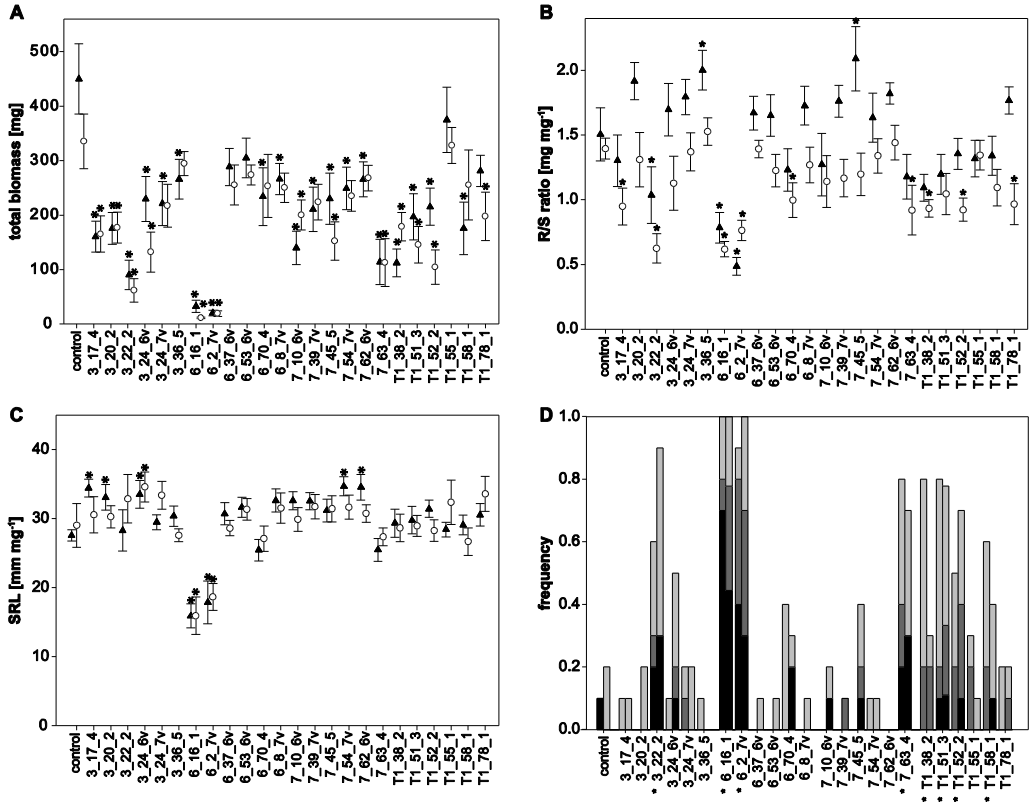

Figure 1. Effects of inoculations with various isolates of four PAC species on A) total biomass, B) root-to-shoot (R/S) ratio, C) specific root length (SRL), and D) the disease intensity of Norway spruce seedlings. Asterisks indicate significant differences to the control. A-C) Triangles indicate the seed provenance from the Central Alps (VS) and open circles the provenance from the Swiss Plateau (TG). D) Black indicates the proportion of dead seedlings, dark gray the proportion of seedlings with more than 50% of the needle surface necrotic or chlorotic, and gray the proportion of seedlings with less than 50% of the needle surface necrotic. The first number/letter of the isolate labels indicates the species: 3 = *Phialocephala europaea*, 6 = *P. subalpina*, 7 = *P. fortinii* s.s., T1 = *Acephala applanata*.

Effects of *P. subalpina* isolates originating from three geographic regions on disease intensity and performance of spruce seedlings. Disease intensity of *P. subalpina* isolates from KEV and NOD origin varied within the same range as the isolates from BOD origin (see above), and depended on both the isolate (χ^2 = 124.45, d.f. = 36, $P < 0.001$) and geographic origin (χ^2 = 19.70, d.f. = 6, $P < 0.01$). In addition to the two highly virulent BOD isolates, the KEV isolate 6_K93_394 significantly harmed seedlings. In contrast, plants inoculated with isolates from NOD were equally vigorous as the controls (Fig. 2D). Overall, disease intensity was lowest for isolates from NOD and highest for those from BOD. The two seed provenances were not differentially diseased, because neither provenance nor the interaction term was significant when comparing isolates (χ^2 = 10.28, d.f. = 19, P = 0.946) or geographic origin (χ^2 = 2.06, d.f. = 3, P = 0.725).

There were high among-isolate effects on performance (Fig. 2A–C, Fig. S2, Table S4), but unlike isolates from BOD no isolate from KEV or NOD had an adverse effect on all performance parameters of either seed provenance compared with the control (Fig. 2A–C, Fig. S2A–C). Biomass of seedlings inoculated with *P. subalpina* isolates from different geographic origins varied within a much narrower range compared with biomass of seedlings inoculated with isolates belonging to different species from the same geographic origin. There was also considerable variation in plant performance among *P. subalpina* isolates ($\chi^2 > 85$, d.f. = 1, $P < 0.001$) in the mixed effects models (Table S5), but there were no significant differences among isolates from different geographic origin. Similar to the comparison among different PAC species, seedlings from different seed provenances differed significantly in root mass ($F_{1,338} = 7.323$, $P < 0.01$) and R/S ratio ($F_{1,338} = 36{,}679$, $P < 0.001$). In addition, seedlings from the two provenances differed significantly in SRL ($F_{1,338} = 4.954$, $P < 0.05$) (Table S5). The interaction between seed provenance and geographic origin was only

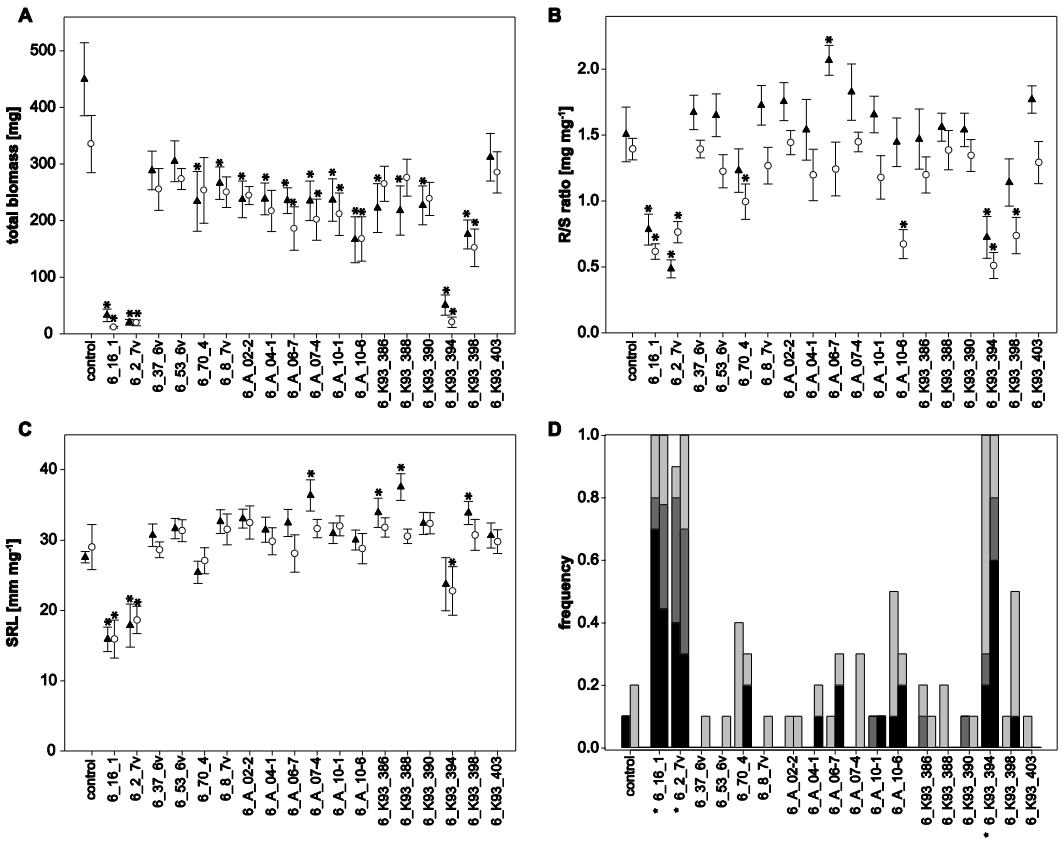

Figure 2. Effects of inoculations with various *P. subalpina* isolates from different geographic origin on A) total biomass, B) root-to-shoot (R/S) ratio, C) specific root length (SRL), and D) the disease intensity of Norway spruce seedlings. Asterisks indicate significant differences to the control. A-C) Triangles indicate the seed provenance from the Central Alps (VS) and open circles the provenance from the Swiss Plateau (TG). D) Black color indicates the proportion of dead seedlings, dark gray the proportion of seedlings with more than 50% of the needle surface necrotic or chlorotic, and gray the proportion of seedlings with less than 50% of the needle surface necrotic. The first six isolates to the right of the control are from Switzerland (BOD), the next six isolates from the USA (NOD) and the last six isolates from Finland (KEV).

significant for R/S ratio ($F_{2,338}$ = 3.061, p < 0.05). This significant interaction indicates that seedlings from the two provenances allocate resources to roots and shoots differently, depending on the geographic origin of the isolate.

Genetic distance of PAC isolates, DNA quantity and disease intensity. Disease intensity and genetic distance of *P. subalpina* were not correlated (Mantel test: r = -0.22, P = 0.98). There was, however, a negative correlation (r = -0.565, P < 0.001) between the endophyte's extent of colonization, represented by the mean DNA amount, and the number of healthy seedlings per treatment (Fig. 3). Extent of colonization of isolates 6_A_10-6 and T1_55_1 was much lower than that of all other isolates. However, these measurements cannot be considered as outliers because both isolates were successfully reisolated, and measurements were of the same order of magnitude for all replicates and seedlings from both provenances. Seedlings infected by isolate T1_55_1 performed equally well as the controls, and there was no difference in disease intensity (Fig. 1A–D). In contrast, plants infected by isolate 6_A_10-6 performed equally or worse than plants infected with other PAC isolates and disease intensity was more pronounced (Fig. 2A–D). The correlation between extent of colonization and the number of healthy plants increased substantially, when data of these two isolates were omitted (r^2 = -0.724, P < 0.001).

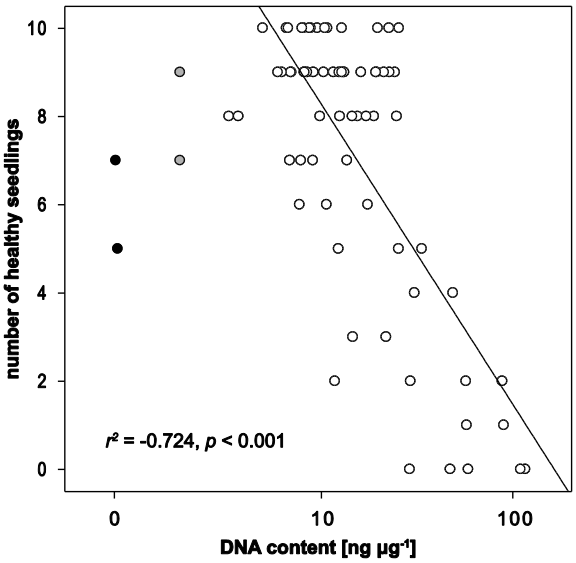

Figure 3. Correlation of PAC DNA content per root weight (ng μg^{-1}) with the number of healthy plants (data from both seed provenances pooled). Black filled dots represent isolate 6_A_10-6 and gray filled dots isolate T1_55_1. These two isolates were excluded from regression analysis due to abnormal behavior (details see main text).

DISCUSSION

Interactions between *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (PAC) members and Norway spruce ranged from neutral to highly virulent, but a mutualistic interaction was not observed for any isolate in our experiment. The type of interaction of PAC with Norway spruce was primarily isolate-dependent and variation in virulence was much higher within than among species; nonetheless only isolates of *P. subalpina* were highly virulent. Surprisingly, *P. subalpina* originating from the same region as the Norway spruce seedlings had a more adverse effect on their host than isolates from outside the natural range of Norway spruce.

Interaction of PAC species along the mutualism–antagonism continuum. High among-isolate variation was observed in host response to inoculation with 24 PAC isolates of four different species. Similarly, high among-isolate variation was regularly observed in other studies for mutualists (Munkvold et al., 2004; Koch et al., 2006) and pathogens (e.g., Robin and Desprez-Loustau, 1998; Barrett et al., 2009; Rowe and Kliebenstein, 2010) indicating that it is essential to study many isolates to fully appreciate diversity of host–fungus interactions. Our findings corroborate findings of other studies showing predominantly a neutral to pathogenic effect of PAC isolates (for a detailed review, see Grünig et al., 2008; but see also Jumpponen and Trappe, 1998a; Mandyam and Jumpponen, 2005; Schulz, 2006; Newsham, 2011). Thus, the highly isolate-specific response of the PAC seedlings in our experiment explains some of the conflicting results observed in previous studies where only few isolates were studied.

Small, yet significant differences were observed among PAC species for disease intensity and SRL of Norway spruce seedlings. Among-species differences were mainly due to the two highly virulent *P. subalpina* isolates which caused more disease and greater reduction of plant biomass than any other isolate. However, phylogenetic relatedness and virulence of PAC were correlated neither within nor between PAC species. Based on these results, pathogenicity can be excluded as a main driver of PAC speciation which contrasts with data from other species complexes, where higher virulence and adaptation to a particular host led to speciation (Thomsen and Jensen, 2002; Marin et al., 2005).

The ecological role of PAC species. The negative correlation of PAC DNA content and disease intensity demonstrates that the extent of PAC colonization is tightly linked to virulence. A similar relationship was also reported for the conifer needle endophyte *Rhabdocline parkeri* or the *Bromus tectorum* seed bank pathogen *Pyrenophora semeniperda* (Stone, 1987; Meyer et al., 2010). Colonization by PAC was associated with net costs for

Norway spruce seedlings in our system. In nature, the whole root system of apparently healthy adult spruce trees is colonized by PAC (i.e., from root tips to coarse roots, Menkis et al., 2004; Grünig et al., 2008). However, it can only be speculated about the costs of PAC in the field because it is impossible to determine them, but PAC might have adverse effects on adult trees as well.

Three factors or combinations of them could explain the apparent discrepancy between the results of our study with the observations from natural ecosystems, where PAC are very common (Ahlich and Sieber, 1996; Addy et al., 2005; Grünig et al., 2008; Queloz et al., 2011; Walker et al., 2011). First, some effects of PAC might be beneficial for adult trees whereas they are associated with net costs for seedlings. For instance, PAC could accelerate root turnover or decompose dead organic matter in the rhizosphere, releasing nutrients similar to mycorrhizal fungi (Mandyam and Jumpponen, 2005; Schulz, 2006; Finlay, 2008). The released nutrients could then be readily utilized by PAC colonized plants, which might therefore exploit a broader area in the rhizosphere compared with those not hosting PAC. For example, under high soil N content, colonization by PAC led to an increase of biomass and foliar N and P levels of *Pinus contorta*, in particular when organic matter was added, indicating decomposer activity and nutrient uptake accelerating function of PAC (Jumpponen et al., 1998). However, an accelerated root turnover is only effective if fungal virulence is low, so that the root can acquire nutrients from the soil over a sufficiently long period of time (Fitter, 2002; Yanai and Eissenstat, 2002). In addition, the plant must possess enough resources to compensate for root loss. Therefore, the process of resource exploitation, root death and production of fresh fine roots must be well balanced in a healthy plant, which probably holds true for adult trees but not for seedlings, since adult trees are better in compensating photosynthate loss due to symbionts. Furthermore, adult trees might harbour weak pathogens that have more severe effects on conspecific seedlings to reduce intraspecific competition pressure from offspring germinating in the immediate vicinity. This hypothesis, known as the Janzen-Connell hypothesis, was first proposed as a mechanism to maintain biodiversity in tropical forests, but has since been shown to apply to temperate forests as well (Packer and Clay, 2000; Gilbert, 2002; Reinhart and Clay, 2009; Bever et al., 2010). Second, presence of a competing microbial soil community in forest ecosystems reduces the probability of successful PAC infections, thus, leading to a reduction of adverse effects caused by PAC. The infection pressure was high in our system because sterile, 12-day-old Norway spruce seedlings were planted into soil substrate solely colonized by PAC. In nature, density of colonization of the rhizosphere by single PAC genotypes is assumed to be much lower as they are under control of other root endophytes, mycorrhizal fungi and other members of the rhizosphere community. This was demonstrated for ectomycorrhizal fungi that suppressed deleterious effects of PAC on *Betula platyphylla* var. *japonica* seedlings

(Hashimoto and Hyakumachi, 2001). Third, PAC species confer an indirect benefit on their hosts by direct suppression of other, more severe root pathogens, enhance stress tolerance of the plants or by spillover on more susceptible plants. A protective effect of symbiotic fungi has been demonstrated for ecto- and endomycorrhizal fungi (Newsham et al., 1995; Azcón-Aguilar and Barea, 1997; Whipps, 2004), for leaf endophytes (Carroll, 1988; Clay, 2001; Arnold et al., 2003) and also for PAC (Narisawa et al., 2004). An indirect effect might also occur if plants host weak pathogens to suppress competing vegetation by spillover, provided that these pathogens are generalists and more virulent for the competitors (Holt, 1977; Begon et al., 1992; Power and Mitchell, 2004).

Coevolution of *Phialocephala subalpina* and Norway spruce. *P. subalpina* isolates from outside the natural range of Norway spruce were less virulent and had a lower variability in virulence than isolates from within the range. This observation contrasts with the rapid epidemic spread of exotic pathogens into native host populations (e.g., chestnut blight or Dutch elm disease, Anagnostakis, 1987; Brasier, 1991). However, exotic pathogens usually are completely absent before introduction and have never been in contact with the new host before. In contrast, host and PAC species used in our experiments are coevolving for a long time. The geographic mosaic theory (Thompson, 2005) postulates that interactions evolve locally leading to a geographic mosaic of interactions. For instance, populations of the ectomycorrhizal species *Rhizopogon occidentalis* performed better with tree provenances collected from the same site than with provenances from geographically distant sites (Hoeksema and Thompson, 2007). Evidence for local adaptation has also been reported for plant pathogens (Sacristán and García-Arenal, 2008). For example, isolates of the rust *Melampsora lini* from Western Australia were more virulent on host lines (*Linum marginale*) from Western Australia than they were on a broad range of host lines collected throughout eastern Australia (Burdon et al., 2002). Restricted gene flow in both pathogen and host resulting in genetic differentiation of their populations is a prerequisite for local adaptation. Although it is not known how PAC fungi disperse, long-distance gene flow is considered to be rather restricted (Grünig et al., 2008; Queloz et al., 2011). Norway spruce is native to Europe and does not occur naturally in North America. Consequently, the prerequisites for local adaptation are met. Therefore, the higher virulence of *P. subalpina* from within the range of the host suggests that PAC are symbionts that have locally adapted to Norway spruce to maximize exploitation of the host.

Conclusions. Our results corroborate findings from other studies that demonstrated that PAC vary in virulence from neutral to pathogenic and differentially affect plant performance. Growth performance and health conditions mainly depend on the PAC isolate, but some

effects can be attributed to species and geographic origin of these isolates. However, virulence does not strictly follow the species limits, and pathogenic and non-pathogenic isolates occur in all species. Nonetheless, highly virulent isolates were only found among the *P. subalpina* isolates. Moreover, *P. subalpina* isolates from within the native range of Norway spruce were more virulent than isolates from outside, indicating that they have adapted to a symbiotic lifestyle and maximized host exploitation. Thus, PAC might play a key role in the establishment of plant communities in forest and heathland ecosystems given their high abundance and wide distribution in the Northern hemisphere. Nonetheless, it is premature to draw any definitive conclusions about the role of PAC, and there remain many open questions about more complex interactions of PAC with different hosts and members of the rhizosphere community.

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SUPPLEMENTAL MATERIAL

Table S1. Detailed list of PAC isolates used in the experiments

Isolate	Species	Experiment[1]	Origin	Host species[2]	Reference
3_17_4	*P. europaea*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
3_20_2	*P. europaea*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
3_22_2	*P. europaea*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
3_24_6v	*P. europaea*	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
3_24_7v	*P. europaea*	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
3_36_5	*P. europaea*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
6_16_1	*P. subalpina*	*a,b*	BOD	*Picea abies*	(Grünig et al., 2006)
6_2_7v	*P. subalpina*	*a,b*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
6_37_6v	*P. subalpina*	*a,b*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
6_53_6v	*P. subalpina*	*a,b*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
6_70_4	*P. subalpina*	*a,b*	BOD	*Picea abies*	(Grünig et al., 2006)
6_8_7v	*P. subalpina*	*a,b*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
6_A_02-2	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_A_04-1	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_A_06-7	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_A_07-4	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_A_10-1	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_A_10-6	*P. subalpina*	*b*	NOD	N.A.	(Queloz et al., 2011)
6_K93_386	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
6_K93_388	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
6_K93_390	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
6_K93_394	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
6_K93_398	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
6_K93_403	*P. subalpina*	*b*	KEV	*Pinus sylvestris*	(Ahlich and Sieber, 1996)
7_10_6v	*P. fortinii* s.s.	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
7_39_7v	*P. fortinii* s.s.	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
7_45_5	*P. fortinii* s.s.	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
7_54_7v	*P. fortinii* s.s.	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
7_62_6v	*P. fortinii* s.s.	*a*	BOD	*Vaccinium myrtillus*	(Grünig et al., 2006)
7_63_4	*P. fortinii* s.s.	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_38_2	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_51_3	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_52_2	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_55_1	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_58_1	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)
T1_78_1	*A. applanata*	*a*	BOD	*Picea abies*	(Grünig et al., 2006)

[1]Plant-PAC interactions using isolates of different species (*a*) and isolates from different geographic origins (*b*).
[2]N.A.= random sample of roots, details about host not available

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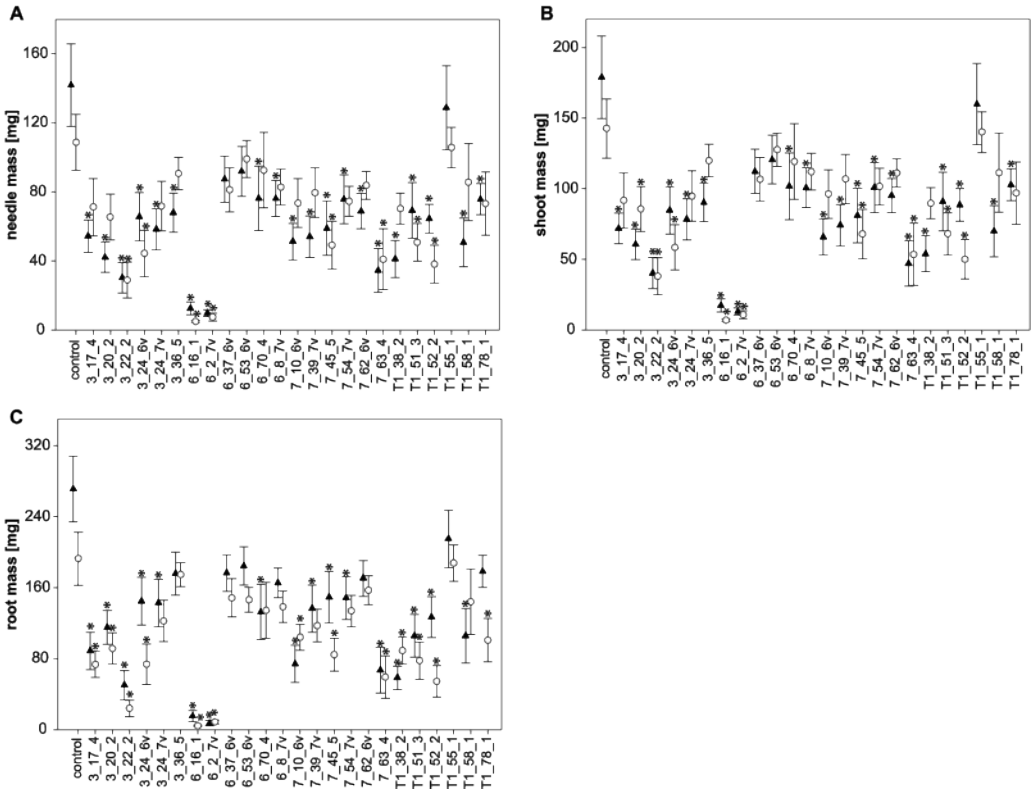

Figure S1. Effects of inoculations with various isolates of four PAC species on A) needle, B) shoot, and C) root mass. Asterisks indicate significant differences to the control. Triangles indicate the seed provenance from the Central Alps (VS) and open circles the provenance from the Swiss Plateau (TG). The first number/letter of the isolate labels indicates the species: 3 = *Phialocephala europaea*, 6 = *P. subalpina*, 7 = *P. fortinii* s.s., T1 = *Acephala applanata*.

Table S2. Mean plant performance and DNA content (± SD) of seedlings from the Central Alps (VS) and the Swiss Plateau (TG), inoculated with different PAC species

measure	Control		*P. europaea*		*P. subalpina*		*P. fortinii* s.s.		*A. applanata*	
	VS	TG	VS	TG	VS	TG	VS	TG	VS	TG
needle mass (mg)	141.9 (±75.9)	108.8 (±51.5)	53.1 (±35.5)	62.1 (±44.5)	59.0 (±50.2)	62.3 (±53.9)	57.3 (±40.7)	67.0 (±43.2)	71.7 (±53.1)	71.0 (±49.6)
shoot mass (mg)	178.8 (±92.9)	142.7 (±66.4)	71.0 (±43.1)	81.4 (±54.9)	77.6 (±63.5)	81.7 (±67.7)	77.3 (±50.5)	89.5 (±54.7)	94.3 (±64.7)	93.1 (±62.7)
root mass (mg)	271.0 (±116.8)	192.6 (±94.9)	119.5 (±80.3)	93.2 (±71.2)	113.5 (±94.5)	98.2 (±84.7)	124.2 (±84.2)	109.1 (±64.4)	131.6 (±90.5)	109.4 (±84.1)
R/S ratio (mg mg^{-1})	1.5 (±0.7)	1.4 (±0.3)	1.6 (±0.6)	1.2 (±0.6)	1.3 (±0.6)	1.1 (±0.4)	1.6 (±0.6)	1.2 (±0.5)	1.3 (±0.4)	1.1 (±0.4)
SRL (mm mg^{-1})	27.6 (±2.5)	29.0 (±10.1)	31.5 (±6.2)	31.5 (±7.3)	25.7 (±8.9)	25.7 (±8.4)	31.8 (±5.6)	30.5 (±5.2)	29.8 (±5.0)	29.8 (±7.2)
DNA (ng ug^{-1})	-	-	24.5 (±26.1)	29.8 (±21.1)	25.4 (±46.5)	34.9 (±48.4)	17.8 (±14.6)	10.9 (±5.2)	19.6 (±26.3)	24.4 (±40.4)

Table S3. Significance levels of the parameters in the interaction with different PAC species

factor	total biomass	needle mass	shoot mass	root mass	R/S ratio	SRL
	F	*F*	*F*	*F*	*F*	*F*
species (SP)	$0.253_{(3,20)}$	$0.346_{(3,20)}$	$0.343_{(3,20)}$	$0.226_{(3,20)}$	$1.029_{(3,20)}$	$3.518_{(3,20)}$*
provenance (PRO)	$2.608_{(1,450)}$	$0.593_{(1,450)}$	$0.436_{(1,450)}$	$9.932_{(1,450)}$**	$54.154_{(1,450)}$***	$0.458_{(1,450)}$
SP × PRO	$0.225_{(3,450)}$	$0.472_{(3,450)}$	$0.469_{(3,450)}$	$0.260_{(3,450)}$	$1.791_{(3,450)}$	$0.367_{(3,450)}$

Type III *F*-statistics from linear mixed effect models, followed by the degrees of freedom in parentheses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

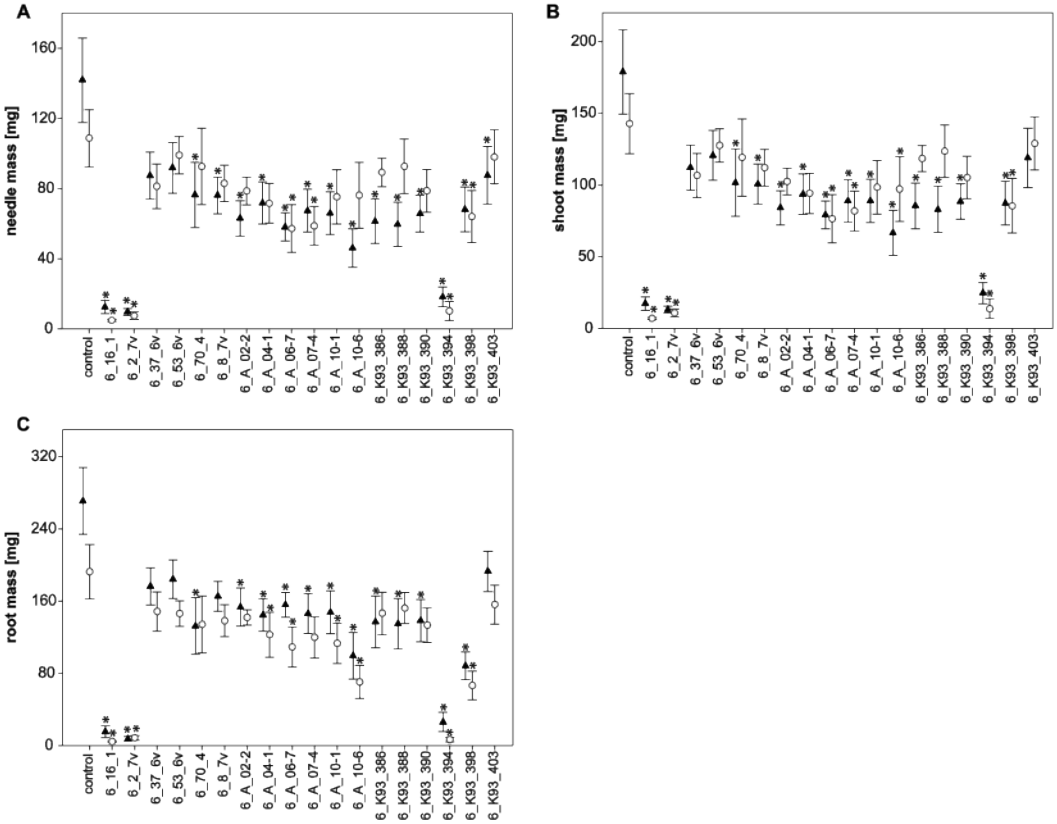

Figure S2. Effects of inoculations with various *P. subalpina* isolates from different geographic origin on (A) needle, (B) shoot and (C) root mass. Asterisks indicate significant differences to the control. Triangles indicate the seed provenance from the Central Alps (VS) and open circles the provenance from the Swiss Plateau (TG). The first six isolates to the right of the control are from Switzerland (BOD), the next six isolates from the USA (NOD) and the last six isolates from Finland (KEV).

Table S4. Mean plant performance and DNA content (± SD) of seedlings from the Central Alps (VS) and the Swiss Plateau (TG), inoculated with *P. subalpina* isolates from different geographic origin

measure	Control		BOD		KEV		NON	
	VS	TG	VS	TG	VS	TG	VS	TG
total biomass (mg)	449.8 (±203.4)	335.3 (±159.5)	191.0 (±154.3)	180.0 (±148.8)	200.9 (±134.3)	206.1 (±131.3)	224.7 (±104.1)	204.6 (±108.7)
needle mass (mg)	141.9 (±75.9)	108.8 (±51.5)	59.0 (±50.2)	62.3 (±53.9)	60.1 (±42.5)	72.1 (±48.4)	62.1 (±34.4)	69.6 (±41.6)
shoot mass (mg)	178.8 (±92.9)	142.7 (±66.4)	77.6 (±63.5)	81.7 (±67.7)	81.3 (±53.9)	95.8 (±60.8)	83.5 (±42.5)	91.7 (±50.7)
root mass (mg)	271.0 (±116.8)	192.6 (±94.9)	113.5 (±94.5)	98.2 (±84.7)	119.6 (±85.4)	110.2 (±78.0)	141.2 (±66.5)	112.9 (±65.8)
R/S ratio (mg mg^{-1})	1.5 (±0.7)	1.4 (±0.3)	1.3 (±0.6)	1.1 (±0.4)	1.4 (±0.6)	1.1 (±0.5)	1.7 (±0.6)	1.2 (±0.5)
SRL (mm mg^{-1})	27.6 (±2.5)	29.0 (±10.1)	25.7 (±8.9)	25.7 (±8.4)	32.0 (±8.1)	29.7 (±6.9)	32.4 (±5.6)	30.5 (±6.3)
DNA (ng μg^{-1})	-	-	25.4 (±46.5)	34.9 (±48.4)	19.4 (±18.4)	28.3 (±49.1)	7.0 (±5.3)	9.5 (±10.7)

Table S5. Significance levels of the parameters in the geographic origin analysis

factor	total biomass	needle mass	shoot mass	root mass	R/S ratio	SRL
	F	***F***	***F***	***F***	***F***	***F***
geographic origin (GO)	$0.394_{(2,15)}$	$0.254_{(2,15)}$	$0.305_{(2,15)}$	$0.456_{(2,15)}$	$1.181_{(2,15)}$	$3.291_{(2,15)}$
provenance (PRO)	$1.523_{(1,338)}$	$1.038_{(1,338)}$	$0.901_{(1,338)}$	$7.323_{(1,338)}$**	$36.679_{(1,338)}$***	$4.954_{(1,338)}$*
GO × PRO	$0.510_{(2,338)}$	$0.499_{(2,338)}$	$0.475_{(2,338)}$	$0.720_{(2,338)}$	$3.061_{(2,338)}$*	$1.032_{(2,338)}$

Type III *F*-statistics from linear mixed effect models, followed by the degrees of freedom in parentheses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

CHAPTER 4

Is a tripartite host-endophyte-pathogen interaction affected by elevated temperature?

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FEMS Microbiology Ecology (submitted)

ABSTRACT

Plants harbor a multitude of microbial endosymbionts, some of which have adopted an apparently parasitic lifestyle. Ascomycetes of the *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (PAC) are ubiquitous root endophytes of conifers and ericaceous shrubs, differing in their effect on Norway spruce (*Picea abies*) seedlings from nearly neutral to highly virulent. Here we tested in a tripartite host-endophyte-pathogen system whether six genetically distinct isolates of the common PAC species *P. subalpina* varying in virulence can protect their host against the oomycete root pathogens *Phytophthora plurivora* (syn. *P. citricola*) and *Elongisporangium undulatum* (syn. *Pythium undulatum*) at different temperatures. Protection of the spruce seedlings depended on the *P. subalpina* isolate, but was not affected by elevated temperature. Moreover, whilst temperature significantly reduced spruce performance, it did not alter root colonization by *P. subalpina* and the endophyte's influence on the host. Similarly, disease intensity of *Phytophthora* was not affected. In contrast, the influence of *Elongisporangium* on plant biomass was slightly less pronounced at elevated temperature. These results indicate that PAC confer an indirect benefit to their host, and might therefore be tolerated in natural populations, despite negative effects on plant performance.

INTRODUCTION

In natural communities a multitude of microbes are interacting in complex ways with plants and each other. Aerial plant surfaces are colonized by epiphytic microbes, the rhizosphere hosts a plethora of soil microbes and most functional plant tissues are colonized by endosymbionts. Microbial endosymbionts are ubiquitous, and, depending on their effects on the host, they are considered as mutualistic, pathogenic, or neutral. However, the nature of host-endosymbiont interactions is usually not fixed and depends on a multitude of factors, such as environmental conditions, host and microbe genotype, time of observation of the interaction. For instance, mycorrhizal associations with plants could be considered pathogenic in the early stage of the symbiosis, since plants infected with the fungus accumulate initially less biomass than uninfected plants. Later on, these initial expenses are compensated by gain in biomass of mycorrhizal plants compared to mycorrhiza-free plants (Johnson et al., 1997). However, for non-mycorrhizal and presumably non-pathogenic endosymbionts the nature of this interaction is much less clear.

Fungal endophytes colonize most functional plant tissues without causing visible disease symptoms at all or for prolonged periods of time, and no distinct mycorrhiza-like structures are formed (Saikkonen et al., 1998; Brundrett, 2004). Some endophytes can have beneficial effects on their plant hosts such as increased tolerance to drought, heat, or high levels of metal concentrations (Clay, 2001; Rodriguez et al., 2009), the production of compounds that are toxic for herbivores or make the infected tissues unpalatable for them, or the deterrence of harmful pathogens (Carroll, 1988; Clay, 2001; Arnold et al., 2003; Selosse et al., 2004; Sieber, 2007; Miller et al., 2008). Whilst these effects have been relatively well studied for common leaf and needle endophytes, the ecological significance of naturally occurring root endophytes in protecting plants against pathogens is much less understood.

Yet, in most plants, fungal root endophytes are very common, in particular dark septate endophytes (DSE) that are abundant fungal root colonizers of a wide range of mycorrhizal and non-mycorrhizal plant species (Stoyke et al., 1992; Sieber, 2002; Addy et al., 2005; Summerbell, 2005). DSE form a polyphyletic group of mainly ascomycetous fungi with melanized, septate hyphae (Stoyke et al., 1992; Ahlich and Sieber, 1996; Jumpponen and Trappe, 1998; Summerbell, 2005). In conifers and Ericaceae the most prevalent DSE fungi known belong to the *Phialocephala fortinii* s.l.-*Acephala applanata* cryptic species complex (PAC) (Wang and Wilcox, 1985; Ahlich and Sieber, 1996; Grünig et al., 2008). All known PAC species are widely distributed across the northern hemisphere without showing any biogeographic pattern (Queloz et al., 2011). PAC communities are composed of up to ten species, but species composition neither correlates with host species (Ahlich and Sieber,

1996; Harney et al., 1997; Addy et al., 2005; Grünig et al., 2008), nor climate and is assumed to be mainly driven by stochastic effects (Queloz et al., 2011). Despite the wide distribution and frequent occurrence, PAC behave in a range from nearly neutral to highly virulent on Norway spruce seedlings, showing only small differences among species (Grünig et al., 2008; Tellenbach et al., 2011). One possible explanation for this apparent contradiction might be that PAC provide indirect benefits to their host by protecting it against harmful root pathogens. This might be particularly important for plants in the seedling stage, as these are particularly susceptible to pathogens (Newhook and Podger, 1972). In nurseries, damping-off of conifer seedlings occurs frequently and is often due to oomycete root pathogens of the genera *Pythium* and *Phytophthora* (Hendrix and Campbell, 1973; Hamm and Hansen, 1982; Lilja et al., 1992; Linde et al., 1994). Moreover, oomycetes are globally involved in dieback of forest trees (Jung and Blaschke, 1996; Chavarriaga et al., 2007). In temperate forests, these pathogens are associated with dieback of *Alnus* spp. (Brasier et al., 2004), *Quercus* spp. (Brasier, 1996; Jung et al., 1996; Jung et al., 1999; Balci and Halmschlager, 2003), *Fagus sylvatica* (Nechwatal and Osswald, 2001; Jung et al., 2003; Jung et al., 2005), and conifers (Newhook and Podger, 1972; Pratt et al., 1976; Hamm et al., 1988; Chavarriaga et al., 2007). Two widely distributed species are *Elongisporangium undulatum* (syn. *Pythium undulatum*, Petersen, 1910; Uzuhashi et al., 2010) and *Phytophthora plurivora* (syn. *P. citricola*, Jung and Burgess, 2009). *E. undulatum* is often found in nurseries causing remarkable loss to pine and spruce seedlings (Lilja et al., 1992), and it was shown to cause disease and mortality in conifer seedlings in infection trials (Lilja, 1994; Shafizadeh and Kavanagh, 2005). *P. plurivora* is primarily known as a pathogen of broadleaved tree species (Jung et al., 1996; Nechwatal and Osswald, 2001; Jung et al., 2005; Jung and Burgess, 2009), but can also cause significant root loss and mortality in Norway spruce seedlings (Nechwatal and Osswald, 2001). Oomycetes seem to react strongly to environmental conditions (Newhook and Podger, 1972), and might gain even more importance under climate change, as warming combined with heavy rainfall are predicted to create favourable conditions for them (Brasier, 1996; Bergot et al., 2004; Desprez-Loustau et al., 2007). The effects of climate change on different pathosystems are variable, but diseases in general are expected to become more damaging (Ayres and Lombardero, 2000; Harvell et al., 2002; Garrett et al., 2006; Lafferty, 2009; Walther, 2010). The effect of climate change on neutral or beneficial interactions is little understood, but is predicted to vary considerably, too (Compant et al., 2010; Van der Putten et al., 2010).

Norway spruce forests are considered to be highly vulnerable to climate change (Ohlemüller et al., 2006), and the question arises whether disturbance of the endophyte-pathogen equilibrium might enhance this effect. Thus, goal of this study was to examine in a tripartite host-endophyte-pathogen system, whether *P. subalpina* can protect Norway spruce

seedlings against *P. plurivora* and *E. undulatum* and whether this effect and the *P. subalpina* relationship are altered by elevated temperature.

MATERIALS AND METHODS

Host plant and fungal isolates. The experiment was performed with Norway spruce (*Picea abies*) seedlings from a central alpine provenance (Fully, Switzerland) and six genetically distinct isolates (based on microsatellite multilocus haplotyes) of the globally most widely distributed PAC species, *Phialocephala subalpina* (Queloz et al., 2011), originating from Bödmeren, Switzerland, with known virulence (Tellenbach et al., 2011) were chosen as endophytic symbionts. Furthermore, *Phytophthora plurivora* (syn. *Phytophthora citricola*) isolate Bu 137/7a (Nechwatal and Osswald, 2001; Jung and Burgess, 2009) and *Elongisporangium undulatum* (syn. *Pythium undulatum*) (CBS 101728) (Petersen, 1910; Uzuhashi et al., 2010) were used as soilborne root pathogens.

Experimental procedures. *P. subalpina* isolates were grown on terramycine-malt agar (TMA; 15 g l^{-1} malt extract [Hefe Schweiz AG, Stettfurt, Switzerland], 20 g l^{-1} agar, 50 mg l^{-1} terramycine) in Petri dishes at 20°C in the dark. After one week, one colonized agar plug (diameter = 4 mm) from the margin of the growing colony was transferred to 50 ml 2%-malt broth (20 g l^{-1} malt extract) in 100-ml Erlenmeyer flasks and incubated at 20°C on a rotary shaker at 100 rpm. After 23 days, the mycelium was harvested, and washed with sterile nanopure water. Thalli were blotted dry on a sieve and weighed. Thalli were homogenized with a blender for 30 s and the thallus to water ratio was adjusted to 55 g l^{-1} (fresh weight) with sterile nanopure water. 50-ml Falcon tubes containing a sterile 1:1:1 (v/v/v)-silica sand:vermiculite:sphagnum peat mixture and a 1-ml pipette tip as spacer for the addition of *P. plurivora* or *E. undulatum* later on were inoculated with 1 ml of the homogenized *P. subalpina* mycelium and rinsed with 3 ml sterile nanopure water to distribute the inoculum more evenly in the substrate, whereas 4 ml sterile nanopure water was added to uninoculated control tubes. Then, tubes were incubated for 14 days at 20°C in the dark. Thirteen-day-old, sterile spruce seedlings were planted in the tubes. Seedlings had been produced from surface-sterilized seeds. Surface-sterilization occurred by immersion in 30% H_2O_2 for 30 min, followed by rinsing with sterile nanopure water. Germination occurred within twelve days on water agar at 18°C in the dark. After planting the seedlings into the Falcon tubes, plants were transferred to a phytotron [16h day (120-140 µE m^{-2} s^{-1}) / 8h night, temperature (24°C / 15°C), and relative humidity (rH 45% / rH 85%)]. Tubes were randomly distributed in non-heated or heated water baths to expose them to two different temperature regimes. Thus, temperature in

the tubes fluctuated between 21.0 and 14.7°C (day/night; mean 17.9°C) for the low temperature treatment, corresponding to average June temperatures recorded at the climate measuring station in Sion (http://www.meteoschweiz.admin.ch/), which lies about 20 km apart from the seed origin in Fully, and between 26.2 and 16.5°C (day/night; mean 21.6°C) for the elevated temperature regime. After planting, all seedlings were fertilized with 5 ml of a 0.2%-dilution (v/v) of complete fertilizer (Wuxal, Maag, Switzerland). Thereafter, plants exposed to low temperature were watered every other day with 3-4 ml deionized water. Plants exposed to elevated temperature were watered equally but once a week they were given an additional 1 ml deionized water to compensate for higher evaporation. In the first month, plants of both treatments were given 4 ml 0.2%-complete fertilizer weekly and thereafter once every three weeks.

The inoculum of *P. plurivora* and *E. undulatum* was prepared as follows. Two colonized plugs were punched out with a cork borer (diameter = 4 mm) from the margin of three-day old cultures growing on 10%-carrot juice agar (CA; 100 ml l^{-1} carrot juice, 20 g l^{-1} agar, pH = 7.0) plates and used to inoculate sterile vermiculite-millet-carrot juice (VMC, vermiculite:millet:carrot juice = 50:4:35 [v:v:v]) inoculation medium in 50-ml Falcon tubes. The cultures were incubated at room temperature. After two months, the VMC medium was rinsed with nanopure water to remove excess nutrients and used for inoculation. Inoculation of the seedlings with *P. plurivora* and *E. undulatum* occurred 49 days after planting. The pipette tip was pulled out of the substrate, and replaced by 1 ml inoculated VMC medium, controls received sterile VMC medium. Then, tubes were flooded with deionized water for 72 h to induce sporangia-formation of *P. plurivora* and *E. undulatum*. Thereafter, watering and fertilizing occurred as described above. We applied a full-factorial experimental design, consisting of two different temperature regimes with six different *P. subalpina* isolates and an uninoculated control treatment, and with two pathogens and a sterile inoculate. Each treatment was replicated 10 times, resulting in 420 experimental units.

Data collection. Twenty two days after pathogen inoculation, virulence, i.e., the degree of damage caused to the seedlings, was expressed as disease intensity and reduction of seedling performance. Disease intensity, which includes the two components disease incidence, i.e., the number of affected seedlings (e.g., mortality rate), and disease severity, i.e., the percentage of necrotic or chlorotic surface area of the needles, was scored: (0) seedlings without any apparent disease symptoms, (1) seedlings with less than 50% of the needle surface necrotic, (2) seedlings with more than 50% of the needle surface necrotic or chlorotic, (3) seedlings dead. Dead seedlings were removed and processed as described below. Scoring of the seedlings and removal of dead plants was repeated weekly. The experiment was

terminated 70 days after pathogen inoculation, disease intensity was scored again, and plants were harvested.

The shoot was cut off, and roots were washed under running tap water, scanned on a standard flatbed scanner to measure root lengths, using the root analysis software WinRhizo (Pro 2009c, Regent Instrument Inc., Canada). Then, three 5-mm long root segments were excised from the root system of all dead seedlings that had been removed during the experiment and from two randomly chosen seedlings at harvest: one from the periphery of the root system, one from the middle of the root system, and one close to the hypocotyl. Two segments were surface-disinfected (1 min in 30%-H_2O_2, 10 s in 98%-EtOH) and incubated on TMA to re-isolate *P. subalpina* and the third one without surface disinfection was laid on PARP medium (Jeffers and Martin, 1986) to re-isolate *P. plurivora* and *E. undulatum* to verify Koch's postulates. Similarly, from the same two seedlings and an additional seedling per treatment, four 5-mm long root segments from the same root regions (an extra segment was excised from the middle of the root system) were pooled, freeze-dried, weighed and analyzed, using a nested qPCR to estimate the colonization density by *P. subalpina* (Tellenbach et al., 2010). Afterwards, the seedlings were dried in an oven at 50°C for two consecutive days, and the following plant growth-related parameters were measured: Total biomass, needle mass, shoot mass, root mass, the root-to-shoot ratio (R/S ratio, i.e., root mass divided by shoot mass), and specific root length (SRL, i.e., root length divided by root mass).

Statistical analyses. All statistical analyses were performed with the R statistical package (R Development Core Team, 2009). Seedling survival in relationship to temperature, *P. subalpina* isolate and pathogens was analyzed with a Cox proportional hazard model using the *coxph* function in the *Survival* library. An individual plant was censored in the analysis when it survived until the end of the experiment. Similarly, ordinal health categories were analyzed using a proportional odds model using the *lrm* function in the *Design* library. Plant growth-related parameters were analyzed using an ANOVA. Prior to the analysis all plant growth-related variables were transformed in order to reduce skewness and achieve homogeneity in variance. R/S ratio, SRL and DNA content were transformed with the logarithm and the remaining variables with the square-root. For the ANOVAs, the best model was selected according to AIC (Akaike Information Criterion) comparisons, in the survival analysis and the proportional odds model the best model was selected using likelihood ratio tests that are asymptotically χ^2 distributed. Significant differences in the ANOVAs were further investigated using Tukey's HSD post-hoc test.

RESULTS

Koch's postulates were fulfilled for both *P. subalpina* and the two pathogens. On average, *P. subalpina* was successfully reisolated from 65% of all seedlings at low and 70% at elevated temperature, *P. plurivora* from 63% and 39%, and *E. undulatum* from 90% and 77%.

Seedling health and survival. Seventy days after pathogen inoculation (i.e., 119 days after planting), seedling health and mortality varied considerably among the two temperature treatments, different *P. subalpina* isolates, and the presence of *P. plurivora* or *E. undulatum* (Fig. 1). No seedling mortality was observed at either temperature when *P. subalpina*, *P. plurivora*, and *E. undulatum* were absent. Mortality of *P. subalpina* inoculated plants ranged from 0% to 50% at low and 10% to 60% at elevated temperature, when no pathogen was inoculated. In the absence of *P. subalpina,* mortality of seedlings inoculated with *P. plurivora* was 30% and 60%, whereas mortality of seedlings inoculated with *E. undulatum* was 90% and 80% (Fig. 1). Significance of the parameters in the survival analysis and in the Cox proportional hazard regression was the same (Table 1). Seedling survival and health depended on temperature, *P. subalpina* isolate, and pathogen. Moreover, the significant *P. subalpina* isolate by pathogen interaction indicates that *P. subalpina* and the pathogens influenced each other and that some *P. subalpina* isolates suppressed the pathogens more effectively than others. For example, isolate 6_2_7v reduced mortality caused by *P. plurivora* and 6_70_4, 6_8_7v, 6_16_1 and 6_2_7v that caused by *E. undulatum* particularly at lower temperature (Fig. 1a). Elevated temperature led to a general increase in disease intensity but did not have any differential effect on the ability of *P. subalpina* isolates to prevent disease nor to cause more severe disease symptoms. Likewise, elevated temperature did not alter the ability of the pathogens to cause disease, since there was neither a significant temperature by *P. subalpina* nor a significant temperature by pathogen interaction, nor a significant three-way interaction (Table 1).

Table 1. Effects of isolate, temperature and presence of a pathogen on survival and health status of the seedlings

factor		Survival analysis	Odds model
	df	Wald χ^2	Wald χ^2
isolate (I)	6	22.526**	46.004***
temperature (T)	1	8.114**	9.458**
pathogen (P)	2	93.114***	19.782***
I × P	12	40.695***	28.771**

**, $P < 0.01$; ***, $P < 0.001$.

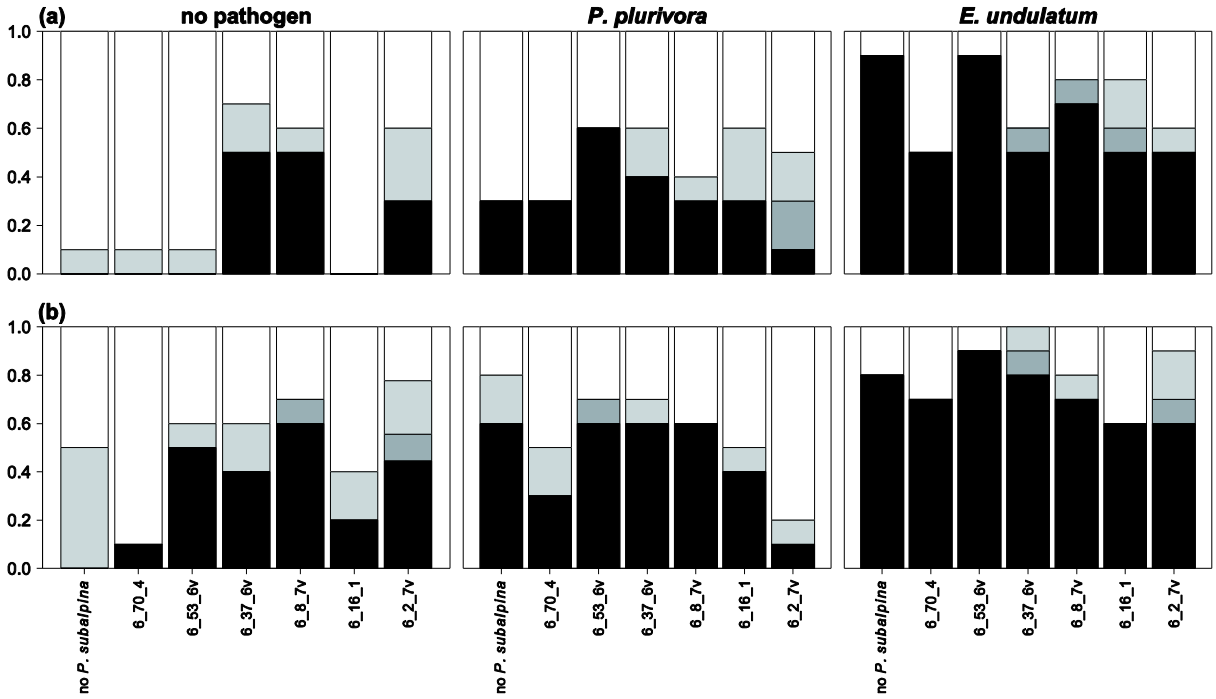

Figure 1. Proportional distribution of health status of the seedlings at a) low and b) elevated temperatures. Black indicates dead, dark grey heavily diseased, grey mildly diseased and white healthy seedlings.

Plant growth-related parameters. Plant growth-related parameters were highly variable among seedlings inoculated with different *P. subalpina* isolates, the two pathogens and in both temperature regimes (Table S1). *E. undulatum* was highly virulent on *P. subalpina*-free controls and seedlings inoculated with isolates 6_37_6v and 6_53_6v (Fig. 2), resulting in survival of fewer than three seedlings. ANOVAs were performed for each pathogen separately, one with all *P. subalpina* isolates for *P. plurivora* (analysis 1, Table 2a) and one with a reduced dataset for *E. undulatum* comprising only those *P. subalpina* isolates that led to survival of at least three seedlings at either temperature (analysis 2, Table 2b).

Non-inoculated seedlings (control) had the highest biomass (i.e., total biomass, needle, shoot, and root mass) at both temperatures. Over all treatments, total biomass ranged from 4.96 to 368.9 mg, needle mass from 2.45 to 154.98 mg, shoot mass from 4.71 to 186.60 mg, and root mass from 0.25 to 191.54 mg per plant. Analysis 1 showed that *P. plurivora* had no effect on biomass except for a slightly significant reduction in shoot weight, whereas elevated temperature and *P. subalpina* significantly decreased all biomass parameters (Table 2a). Tukey's HSD test revealed that seedlings inoculated with *P. subalpina* isolate 6_2_7v had significantly lower biomass than the controls or seedlings inoculated with any other isolate. Moreover, biomass of seedlings inoculated with isolate 6_16_1 differed significantly from the controls. Significant differences among other isolates or the control were less consistent and depended on the biomass parameter. Analysis 2 revealed that inoculation with *P. subalpina* and *E. undulatum* led to a significant reduction in biomass of all tissues and that temperature significantly decreased total, needle and shoot, but not root mass (Table 2b). Whereas

temperature alone had no effect on root biomass, the interaction between temperature and *E. undulatum* had an effect on any biomass measure as indicated by the significant temperature by *E. undulatum* interaction. In fact, root mass of *E. undulatum*-infected seedlings at low temperature was significantly more reduced than root mass of pathogen-free seedlings, whereas there were no differences at elevated temperature, as shown by Tukey's HSD test. As in analysis 1, *P. subalpina* isolate 6_2_7v infected seedlings had significantly less biomass than the other seedlings in analysis 2. Moreover, there was a distinct protective effect of isolates 6_70_4, 6_8_7v, 6_16_1 and 6_2_7v against *E. undulatum* (Fig. 2). For any of these isolates, at least 30% of *E. undulatum*-infected seedlings survived, whereas almost all *P. subalpina*-free seedlings were killed by *E. undulatum.* Moreover, root biomass of surviving seedlings colonized by some *P. subalpina* isolates was high (Fig. 2).

The root-shoot (R/S) ratio ranged from 0.05 to 2.62 and differed significantly only among different *P. subalpina* isolates in both ANOVAs (Table 2a&b). This significant effect was due to a R/S ratio reduction by isolate 6_2_7v, indicating that seedlings infected with this particular isolate had proportionally less root than shoot mass compared to plants inoculated with the other isolates and the controls.

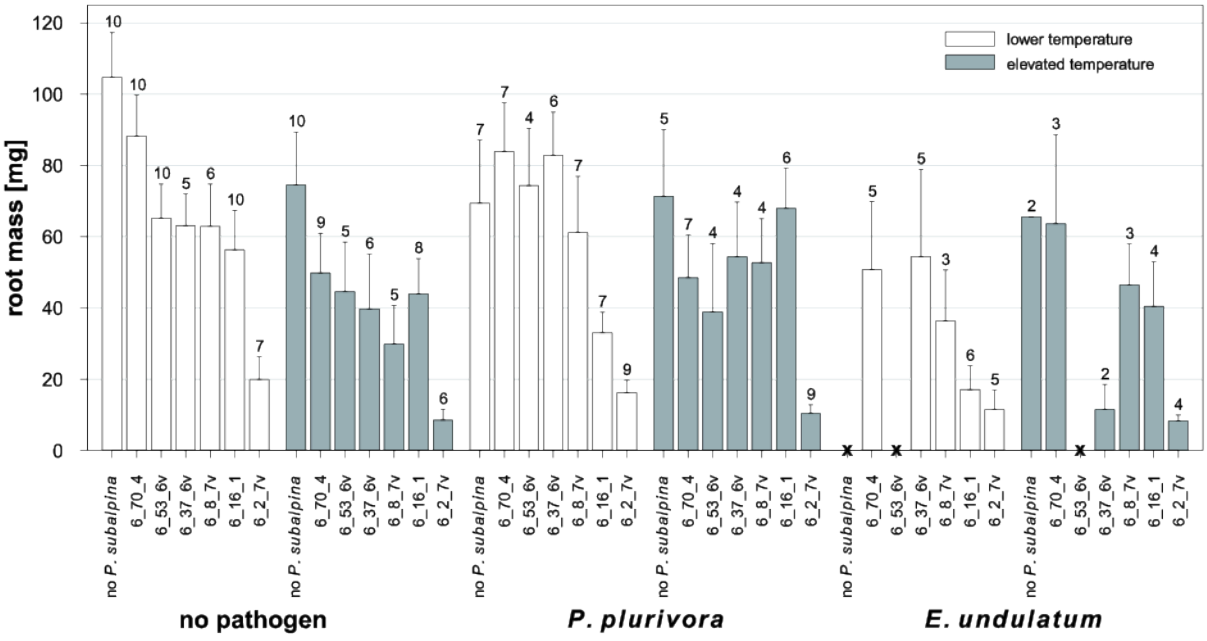

Figure 2. Mean root mass (± SEM) of seedlings without pathogen, with *P. plurivora*, and with *E. undulatum.* Numbers above the bars indicate the number of surviving seedlings, "X" indicates treatments with too few replicates for ANOVA (details see main text).

Specific root length (SRL) ranged from 13.04 to 101.22 mm mg^{-1} and was significantly increased by elevated temperature for both pathogens (Table 2a&b). However, there was a significant temperature by *E. undulatum* interaction, which was due to significant differences of SRL at low and elevated temperature in the absence of *E. undulatum* inoculation.

Table 2. Effects of isolate, temperature and presence of a pathogen on plant growth related parameters[a]

a) Analysis 1:	total biomass	needle mass	shoot mass	root mass	R/S ratio	SRL
P. plurivora	***F***	***F***	***F***	***F***	***F***	***F***
temperature (T)	$33.226_{1,177}$***	$38.663_{1,183}$***	$42.009_{1,177}$***	$17.234_{1,183}$***	–	$21.897_{1,190}$***
P. subalpina (*sub*)	$21.895_{6,177}$***	$16.825_{6,183}$***	$20.212_{6,177}$***	$16.654_{6,183}$***	$6.511_{6,186}$***	–
P. plurivora (*pluri*)	$3.202_{1,177}$.	$3.392_{1,183}$.	$3.997_{1,177}$*	$1.815_{1,183}$	–	$2.032_{1,190}$
T × *sub*	$1.970_{6,177}$.	–	$2.042_{6,177}$	–	–	–
T × *pluri*	$3.289_{1,177}$.	$2.421_{1,183}$	$2.603_{1,177}$.	$3.123_{1,183}$.	–	–
b) Analysis 2[b]: ***E. undulatum***						
temperature (T)	$9.168_{1,87}$**	$15.415_{1,87}$***	$15.028_{1,87}$***	$3.037_{1,87}$.	$1.412_{1,87}$	$8.836_{1,90}$**
P. subalpina (*sub*)	$19.448_{3,87}$***	$14.444_{3,87}$***	$16.793_{3,87}$***	$16.590_{3,87}$***	$7.037_{3,87}$***	–
E. undulatum (*und*)	$8.136_{1,87}$**	$5.188_{1,87}$*	$5.982_{1,87}$*	$8.707_{1,87}$**	$3.101_{1,87}$.	$0.640_{1,90}$
T × *und*	$10.014_{1,87}$**	$8.299_{1,87}$**	$8.266_{1,87}$**	$9.905_{1,87}$**	$2.351_{1,87}$	$4.855_{1,90}$*

Type III F–statistics from a linear model, followed by the degrees of freedom. ·, P <0.1; *, P < 0.05; **, P < 0.01; ***, P < 0.001. – parameters excluded by model reduction

[a]interactions that were excluded by the AIC criterion for all plant growth-related parameters are not shown
[b]excluding the control treatment and isolates 6_37_6v and 6_53_6v (<3 surviving seedlings)

Fungal colonization density. Colonization density of *P. subalpina*, represented by DNA content per root dry weight, varied among isolates (Table 3), but was neither significantly affected by elevated temperature, nor by the presence of a pathogen. After model reduction according to the AIC criterion, both, *P. subalpina* isolate and pathogen were retained as factors, but only *P. subalpina* isolate was significant ($F_{5,100} = 8.99$, $P < 0.001$). Moreover, the absence of a significant pathogen by isolate interaction indicated that pathogen presence did not significantly alter endophyte colonization density. As revealed by Tukey's HSD test, isolate 6_2_7v had a significantly higher colonization density than all other isolates except isolate 6_8_7v (Table 3). Moreover, isolates 6_70_4 and 6_8_7v differed significantly from each other.

Table 3. Mean *P. subalpina* colonization density ± SD (ng DNA per mg root dry weight)

isolate	Without pathogen		*P. plurivora*		*E. undulatum*	
	low	elevated	low	elevated	low	elevated
6_70_4	4.9 (±2.7)	3.7 (±1.8)	10.1 (±7.5)	10.2 (±6)	6.9 (±2.3)	6.9 (±4.1)
6_53_6v	7.7 (±2.6)	13.1 (±6.7)	11.9 (±5.8)	10.4 (±7.2)	7.8 (±4.1)	15.9 (±5.4)
6_37_6v	9.4 (±2.1)	9.2 (±5.5)	8.3 (±9.8)	6.9 (±4.1)	11.8 (±3.5)	15.1 (±4.7)
6_8_7v	17.4 (±8.5)	14.7 (±4.5)	15.6 (±9.9)	13.8 (±6.2)	19.7 (±4.5)	16.1 (±5.8)
6_16_1	15.2 (±11.1)	19.9 (±10.1)	21.0 (±28.8)	6.0 (±4.2)	22.7 (±15.7)	10.7 (±5.9)
6_2_7v	28.9 (±16)	17.9 (±8.9)	18.6 (±13.6)	40.3 (±27.5)	43.9 (±9.8)	25.5 (±19.6)

DISCUSSION

Protection of Norway spruce by *P. subalpina* against oomycete root pathogens. The ability of six genetically distinct *Phialocephala subalpina* isolates to control two oomycete root pathogens of Norway spruce was tested *in vitro*. The effect of these six isolates on Norway spruce ranged from neutral to highly virulent *in vitro* in a previous study (Tellenbach et al., 2011). *P. subalpina* isolates varying in virulence were selected intentionally, since plant response to isolates differing in virulence might differ (Niks and Marcel, 2009), which then in turn might affect the interaction with pathogens. The effect of these isolates on host seedlings was similar in the current study and the experiment of Tellenbach et al. (2011) with some being slightly more and others slightly less aggressive. However, comparability of these two studies might be limited because experimental conditions were not exactly the same (i.e., substrate composition, temperature regime, duration). Nonetheless, the most virulent isolate was highly virulent in both studies.

Protection of Norway spruce against disease was observed, depending on *P. subalpina* isolate and pathogen. Presence and viability of both pathogens were also demonstrated by a high reisolation frequency. To estimate the pathogen-control efficacy of *P. subalpina*, disease was quantified by estimating incidence and severity, and by measuring different plant growth parameters. All these components must be assessed to fully appreciate the ability of an endophyte to control disease. For instance, the number of surviving plants alone is a poor predictor for the fitness of a plant population as demonstrated for isolate 6_2_7v. This isolate conferred the best protection against the two pathogens in terms of mortality. However, biomass of the surviving seedlings was very poor. Under field conditions, these seedlings would certainly be outcompeted. In contrast, the non-aggressive isolate 6_70_4 also provided very good protection against both pathogens combined with good plant growth. Thus, there is no linear relationship between *P. subalpina* virulence and protection against root pathogens. This is also supported by the absence of a relationship between protective effects and *P. subalpina* colonization density, which was previously shown to be positively correlated with seedling mortality (Tellenbach et al., 2011).

In general, the protection against pathogens by endophytes and nonpathogenic isolates of fungal pathogens occurs either directly by antagonism or indirectly by the induction of host resistance. Antagonism occurs when the fungus attacks and penetrates the hyphae of a pathogenic fungus (i.e., by mycoparasitism), when it sequesters antibiotics and/or toxins that limit pathogen growth, or when it competes for nutrients or space in the host tissue. These different mechanisms of protection against pathogens have been well documented in agricultural systems (Whipps, 2001; Tripathi et al., 2008), in particular for *Trichoderma* spp. (Papavizas, 1985; Harman et al., 2004) and non-pathogenic *Fusarium oxysporum*

(Alabouvette et al., 2009). In our study, competition between *P. subalpina* and root pathogens for infection sites on the roots might not be the sole protection mechanism since there was no relationship between colonization density and protective capability. In contrast, protective effects of *Neotyphodium coenophialum* against *Rhizoctonia zeae* in tall fescue (*Festuca arundinacea*) seemed primarily to be linked to endophyte colonization density (Gwinn and Gavin, 1992). Systemic induced resistance in conifers is well known and could be another possible protection mechanism conferred to Norway spruce seedlings by *P. subalpina* (Bonello and Blodgett, 2003). Formation of thicker cell walls is one mechanism of induced resistance. For example, thickening of the exodermis, as expressed by a reduction of specific root length (SRL), prevented the invasion of root pathogens in experiments of (Eissenstat and Achor, 1999). Acquired resistance due to cell wall thickening seems however to be unlikely according to our results, as there were no differences in SRL among different *P. subalpina* isolates and the control.

Studies demonstrating protective effects of endophytes are scarce and have primarily been performed with endophytes in herbaceous plant species in agricultural systems. The protection of different grass species against leaf pathogens by systemic *Neotyphodium* spp. endophytes is indistinct, since some studies demonstrated reduction of disease incidence and lesion size of pathogens (Gwinn and Gavin, 1992; Tian et al., 2008), whilst others demonstrated none (Welty et al., 1991) or even increased susceptibility of endophyte infected plants (Wäli et al., 2006). A protective effect was also demonstrated for non-systemic wheat leaf endophytes (Dingle and McGee, 2003). Root endophytes (including DSE and PAC) protected diverse crop plants against a variety of pathogens (reviewed in Kageyama et al., 2008). There are some reports for woody species, where leaf endophytes prevented rot of *Theobroma cacao* leaves caused by *Phytophthora* sp. (Arnold et al., 2003), or needle endophytes that increased survival of western white pine (*Pinus monticola*) infected with white pine blister rust (*Cronartium ribicola*) (Ganley et al., 2008). Apart from mycorrhizal fungi that are known to protect plant seedlings against pathogens (Newsham et al., 1995; Azcón-Aguilar and Barea, 1997; Whipps, 2004), little is known about protective effects of other tree root endophytes, and nearly all studies performed on woody species were concerned with the protection of stems and leaves.

The effect of elevated temperature. Control of either pathogen by any *P. subalpina* isolate was not influenced by elevated temperature. Moreover, the among-isolate differences in the reduction of plant growth remained the same at both temperatures although plant biomass was reduced at higher temperature. Studies that examined the influence of temperature on tripartite interactions are scarce, and many of them dealt in fact with bipartite systems and extrapolated the results to tripartite systems (Köhl et al., 1999; Singh et al., 2009; Thomson et al., 2010).

Nonetheless, there are some studies on true tripartite systems demonstrating dependency of plant protection from the type of interaction between temperature, other environmental parameters, pathogen isolate and antagonist species as shown for the protection of Douglas fir seedlings against *F. oxysporum* or bean plants against *Botrytis cinerea* (Strobel and Sinclair, 1991; Hannusch and Boland, 1996).

Elevated temperature did not significantly affect *P. subalpina* colonization and its influence on Norway spruce. The effect of elevated temperature on endophytes seems to be ruled by a complex set of additional factors as shown for *N. coenophialum*, where biomass in *F. arundinacea* was temperature-dependent in one study (Ju et al., 2006), but not in another (Brosi et al., 2011). Inconsistency of temperature effects on fungal isolates was also observed in arbuscular mycorrhiza (AM), where elevated temperature did not affect fungal colonization and plant biomass in one study (Antunes et al., 2011), whilst it varied in another (Staddon et al., 2004). Seasonal and site factors can modify the effect of temperature on fungal endophytes and mycorrhiza, as demonstrated for AM fungi that peaked during summer months, whilst non-AM fungi peaked in fall (Heinemeyer et al., 2004), or for differences in ECM abundance at different sites (Clemmensen et al., 2006). Furthermore, whether temperature affects endophyte colonization and behavior depends on the host species. Whilst DSE colonization in grass roots significantly increased under elevated temperature, it was not affected in roots of ericaceous shrubs (Olsrud et al., 2010). There was no apparent effect of elevated temperature on overall colonization by AM fungi in an artificially warmed plot supporting a species-rich plant community but colonization of the most frequent species, *Avena barbata*, was significantly higher (Rillig et al., 2002).

Neither *P. plurivora* nor *E. undulatum* disease intensity increased on Norway spruce at elevated temperature, although both pathogens show increased vegetative growth in culture at higher temperature. The growth optimum of *P. plurivora* lies at approximately 25°C (Jung and Burgess, 2009), whereas the growth optimum of *E. undulatum* lies at 30°C (Robertson, 1980). Sporangium production by *E. undulatum*, however, was shown to occur only at temperatures between 18 and 20°C (Goldie-Smith, 1952), whilst nothing is known of the optimum temperature for sporangium production in *P. plurivora*. In oomycetes the critical step for pathogenesis and rapid colonization of uninfected host tissues is the proliferation of asexual zoospores, which are considered the major infectious units (Erwin and Ribeiro, 1996). Therefore, reduced sporangium production might explain the better performance of surviving seedlings infected with *E. undulatum* at elevated temperature. Nonetheless, the mortality caused by *E. undulatum* at elevated temperature was still high. In contrast, there was no response of *P. plurivora* with respect to temperature. Therefore, it is likely that *P. plurivora* does not pose a major threat to Norway spruce seedlings in temperate forests, when temperature increases.

In our experiment, Norway spruce seedling biomass was reduced by approximately 30% at elevated temperature, indicating that these seedlings were more stressed under this condition. Similarly, mean biomass of black spruce (*Picea mariana*) seedlings grown at 30°C/24°C was reduced by about 60% compared to biomass of seedlings grown at 22°C/16°C (Way and Sage, 2008). Moreover, soil heating experiments showed bell-shaped growth curves for conifer trees with a maximum at around 20°C soil temperature (Lopushinsky and Max, 1990). Therefore, seedlings outside this temperature range might be slightly stressed and develop some mechanisms to evade heat stress, which in turn might also induce disease resistance against pathogens (Sandermann, 2004; Wang et al., 2006).

Conclusions. In this study we have demonstrated that PAC, represented by *P. subalpina* can prevent disease induced by the two oomycete root rot pathogens *E. undulatum* and *P. plurivora* in Norway spruce seedlings. Furthermore, the symbiotic relationship between *P. subalpina* and spruce remains constant at elevated temperature. Therefore, protection of plants against more harmful pathogens might have led to the tolerance of PAC, and could explain, why this symbiosis is widely distributed globally (Ahlich and Sieber, 1996; Queloz et al., 2011). PAC might also play a stabilizing role in protecting Norway spruce against oomycete root pathogens under climate change. However, since this study was performed *in vitro*, the findings must be verified in field trials as well. Moreover, it is not clear whether this protective effect also occurs in adult trees, which are densely colonized by PAC (Ahlich and Sieber, 1996; Sieber and Grünig, 2006; Grünig et al., 2008). Our study also gives valuable insights into the interaction of plants and endophytes, since data on the effects of elevated temperature are still scarce and inconclusive (Compant et al., 2010; Van der Putten et al., 2010). However, there may be other confounding factors like host and endophyte genotype or species, or other biotic and abiotic factors that need further consideration, before any conclusions about the wide distribution of PAC and their role in natural ecosystems can be drawn. Consequently, the protection of plants against pathogens by fungal endophytes under changing environmental conditions is complex. In both cases, basic knowledge of the components leading to this complexity can only be gathered with experiments performed under strictly controlled conditions, and it will be challenging, setting-up field experiments to study the PAC-host interaction in a more natural environment.

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Table S1. Mean values and standard deviations of different plant growth related parameters.

temperature	pathogen	*P. subalpina* isolate	n[a]	total biomass [mg]		needle [mg]		shoot [mg]		root [mg]		RS ratio[b]		SRL [mm mg^{-1}]	
				mean	sd[c]	mean	sd[c]	mean	sd[c]	mean	sd[c]	mean	sd[c]	mean	sd[c]
low	none	no *P. subalpina*	10	225.505	84.13	93.1	42.36	120.83	50.69	104.68	39.88	0.982	0.41	26.541	4.679
		6_16_1	10	126.392	69.97	54.39	31.05	70.036	38.2	56.356	34.81	0.798	0.32	24.162	3.971
		6_2_7v	7	56.979	43.08	29.09	22.44	36.943	26.72	20.036	16.61	0.476	0.24	32.847	30.39
		6_37_6v	5	131.17	33.84	49.56	16.65	68.078	19.12	63.092	19.82	0.951	0.24	30.016	2.222
		6_53_6v	10	157.813	55	69.88	28.7	92.625	35.68	65.188	30.34	0.743	0.35	29.488	3.704
		6_70_4	10	183.51	61.26	71.84	23.57	95.247	28.36	88.263	36.05	0.935	0.25	26.888	2.951
		6_8_7v	5	170.652	50.78	73.95	31.13	97.732	34.79	72.92	17.29	0.794	0.17	26.206	7.153
	P. plurivora	no *P. subalpina*	7	180.719	68.09	86.78	35.52	111.29	38.68	69.429	46.82	0.662	0.44	31.413	12.12
		6_16_1	7	70.994	21.86	28.52	9.841	37.873	11.3	33.121	15.25	0.901	0.35	26.747	3.556
		6_2_7v	9	44.687	23.03	20.81	10.84	28.406	13.29	16.281	10.53	0.555	0.18	28.67	11.98
		6_37_6v	6	178.69	57.94	73.78	29.36	95.818	34.35	82.872	29.65	0.905	0.3	30.136	4.099
		6_53_6v	4	163.947	64.71	67.58	37.73	89.615	40.78	74.332	32.24	0.915	0.47	28.259	3.382
		6_70_4	7	170.316	58.43	64.52	21.57	86.371	28.78	83.944	35.95	1.005	0.36	28.272	4.184
		6_8_7v	7	141.331	96.44	60.72	45.48	80.186	55.32	61.146	41.83	0.718	0.2	30.026	4.374
	E. undulatum	no *P. subalpina*	1	184.96	–	88.23	–	114.37	–	70.59	–	0.617	–	22.134	–
		6_16_1	5	73.932	49.92	40.34	37.27	53.756	45.63	20.176	16.26	0.468	0.37	34.659	6.055
		6_2_7v	5	32.212	21.76	15.42	9.066	20.65	10.74	11.562	12.12	0.514	0.27	23.003	5.632
		6_37_6v	5	110.176	88.69	42.96	31.39	55.734	36.55	54.442	54.72	0.896	0.47	32.01	11.02
		6_53_6v	1	167.35	–	58.99	–	77.06	–	90.29	–	1.172	–	31.357	–
		6_70_4	5	120.288	68.47	51.44	23.51	69.548	32	50.74	42.6	0.662	0.41	30.005	3.608
		6_8_7v	3	93.853	27.66	45.32	6.297	57.443	7.23	36.41	24.67	0.632	0.43	34.297	6.12
elevated	none	no *P. subalpina*	10	151.567	82.64	57.92	31.16	76.961	37.23	74.606	46.87	0.912	0.27	28.007	5.915
		6_16_1	8	95.146	46.99	38.59	14.14	51.141	19.4	44.005	27.99	0.789	0.29	33.395	4.226
		6_2_7v	5	28.886	15.51	13.9	6.198	19.626	8.24	9.26	8.212	0.41	0.27	38.178	23.26
		6_37_6v	6	100.137	59.97	44.65	20.11	60.358	24.78	39.778	37.66	0.595	0.38	27.788	5.839
		6_53_6v	5	102.946	54.78	44.85	21.94	58.352	26.05	44.594	31.1	0.707	0.3	35.167	9.361
		6_70_4	9	92.552	51.04	29.32	15.78	42.697	20.94	49.856	33.41	1.185	0.71	33.375	4.963
		6_8_7v	4	79.773	52	34.32	20.98	47.167	25.18	32.605	27.07	0.607	0.28	35.726	7.223
	P. plurivora	no *P. subalpina*	4	170.347	26.62	61.35	7.113	83.797	6.202	86.55	28.37	1.044	0.38	31.073	5.028
		6_16_1	6	128.873	47.18	45.28	15.66	60.91	21.27	67.963	27.42	1.116	0.3	28.56	3.273
		6_2_7v	9	30.131	15.48	14.27	7.565	19.672	9.984	10.459	7.345	0.531	0.28	40.518	15.67
		6_37_6v	4	117.3	55.88	44.61	21.15	62.928	27.2	54.373	30.78	0.866	0.21	32.724	2.723
		6_53_6v	4	90.433	66.74	38.55	25.41	51.528	32.42	38.905	38.48	0.599	0.48	33.439	7.568
		6_70_4	7	97.979	47.07	36.12	14.2	49.47	18.16	48.509	31.76	0.924	0.41	35.974	8.332
		6_8_7v	4	104.948	39.86	39.09	12.28	52.22	16.02	52.727	24.83	0.977	0.22	30.897	4.263
	E. undulatum	no *P. subalpina*	2	124.815	3.444	43.75	1.874	59.215	3.373	65.6	0.071	1.11	0.06	27.123	0.962
		6_16_1	4	82.692	41.86	30.95	13.97	42.165	17.87	40.528	25	0.909	0.41	30.758	7.464
		6_2_7v	4	28.585	8.966	14.16	6.034	20.203	6.747	8.383	3.234	0.426	0.17	33.671	18.86
		6_37_6v	2	44.695	43.27	26.62	28.84	33.105	33.4	11.59	9.871	0.407	0.11	30.068	9.936
		6_53_6v	1	153.96	–	62.39	–	86.66	–	67.3	–	0.777	–	27.433	–
		6_70_4	3	129.207	54.06	51.88	6.152	65.52	11.06	63.687	43.26	0.915	0.52	33.841	5.244
		6_8_7v	3	101.34	24.22	40.17	4.49	54.793	6.118	46.547	19.82	0.841	0.3	28.515	2.163

[a]number of surviving seedlings
[b]unit-free (mg mg^{-1})
[c]dash '–' symbolizes treatments, where no standard deviation could be calculated due to n = 1

CHAPTER 5

General discussion and outlook

ARE PAC MUTUALISTIC SYMBIONTS?

It was demonstrated in both interaction experiments (Chapters 3&4) that the effect of *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (PAC) members in a dual interaction system ranges from nearly neutral to highly pathogenic. Thus, the term "endophyte" does not seem applicable for some PAC isolates, as endophyte infections are defined as being asymptomatic over a long period in time or the entire life-span of the plant (Carroll, 1988; Petrini, 1991; Wilson, 1995; Saikkonen et al., 1998; Sieber, 2002; Rodriguez et al., 2009). On the other hand, any symbiotic microorganism infecting intact and healthy plant tissues is associated with costs for its host. This is also true for mycorrhizal fungi during the initial phase of the symbiosis establishment (Johnson et al., 1997). However, the results obtained in this work are based on *in vitro* experiments and care should be taken about extrapolations to the situation in nature. Nonetheless, the results in this thesis give an impression about the strength of the Norway spruce – PAC symbiosis and indicate that PAC can be pathogenic under the given conditions. The value of microcosms and *in vitro* systems is often debated since they do not represent natural systems. However, in the simplification of these systems lies their strength, as they give indications of the basic underlying mechanisms of interactions (Srivastava et al., 2004; Benton et al., 2007). The complexity of these systems can then be sequentially increased to uncover all net costs and benefits. Thus, I have chosen such a bottom-up approach to gain a deeper insight into the Norway spruce – PAC symbiosis, starting with a dual host – fungus system to examine differences among cryptic species (CSP) and different geographic origins from within and outside the range of Norway spruce. Thereafter, I have integrated two different environmental stressors – pathogens and temperature – as it was known from other plant – endophyte systems that some endophytic fungi can provide protection against either of these factors (Redman et al., 2002; Marquez et al., 2007). However, Norway spruce seedlings were indifferent against temperature stress, regardless of whether they were colonized or not by PAC. On the other hand, temperature did

not seem to modify this symbiosis, since neither PAC colonization nor disease incidence and severity were altered. This contrasts with PAC growth rates which were shown to increase linearly up to a temperature of 28°C on MEA plates (V. Reininger, pers. comm.). Thus, there seems to be some cross-talk between Norway spruce and PAC, regulating fungal growth within the host. Whilst PAC did not seem to confer adaptation to elevated temperature in Norway spruce, I could show that some PAC strains provided protection of Norway spruce seedlings against oomycete root pathogens. Similarly, PAC-colonized conifers might be protected against other common root pathogens. Consequently, they might have an advantage compared with PAC-free plants, which in turn might ultimately have led to the ubiquity of this mutualism.

FACTORS AFFECTING THE PAC – NORWAY SPRUCE INTERACTION

There is still a debate whether PAC have a promoting effect on plant growth. Two recent meta-analyses reported that dark septate endophytes (DSE) are neutral to slightly beneficial (Alberton et al., 2010), by increasing host root and shoot biomass (Newsham, 2011). However, these results have to be interpreted carefully since in the first study only 11 articles were analyzed, whereas in the second 18 out of 56 possible research articles met the criteria for meta-analysis, and many studies reporting antagonistic effects of DSE were therefore omitted. There are many confounding factors that reduce the comparability among different studies. First of all, DSE are often treated as an entity (e.g., Jumpponen, 2001; Alberton et al., 2010; Newsham, 2011) although DSE is a form taxon which comprises many different, sometimes completely unrelated species (Jumpponen and Trappe, 1998a; Sieber and Grünig, 2006; Grünig et al., 2008). For instance, when focusing only on the effect of *Phialocephala fortinii* s.l. and excluding other DSE species, variability of cumulativ effects on different plant biomass parameters was much larger and there was no longer a significant effect on total biomass due to the fungus (Newsham, 2011). Diversity is not only high between species but also within them, as clearly demonstrated for four PAC species in Chapter 3, where within-species variability was higher than between-species variability. Therefore, variation is expected to be even higher at the taxonomically still higher DSE-level. Moreover, discrepancies among the studies arise from different experimental set-ups.

Size, quantity, and type of inoculum can have a considerable influence on the outcome of an experiment (Vanachter et al., 1988). In my experiments, PAC mycelium grown in liquid cultures was fragmented in a blender and used as inoculum which has the advantage that the fungus can be more evenly distributed in the entire substrate. A more evenly distributed PAC inoculum in the substrate probably corresponds better to the natural situation than selective

application of colonized agar blocks to single positions in the root system. Growth trials of the liquid inoculum on agar plates and subsequent inspection of growing hyphae did not reveal any abnormalities, indicating that using this inoculum type was justified. The influence of inoculum quantity and quality on fungal growth and behavior has been demonstrated for instance for *Rhizoctonia solani* infections in cabbage, where disease incidence and severity increased significantly with higher propagule density (Keinath, 1995), or for the protection of tomatoes against *Botrytis cinerea*, which depended on antagonist spore density (Nicot et al., 2002). Moreover, arbuscular mycorrhizal (AM) fungal colonization of leek (Klironomos and Hart, 2002) or biocontrol efficacy of *Coniothyrium minitans* against *Sclerotinia sclerotiorum* in lettuce depended highly on inoculum type (Jones et al., 2004). Furthermore, a bigger inoculum size increased the competitive success of different wood decaying fungi on wood blocks (Holmer and Stenlid, 1993).

The outcome of an experiment may also depend on when the fungal inoculum was applied relative to planting the seedlings. Therefore, the experiment described in Chapter 3 was complemented with another experiment in which the effect of the point in time of inoculation on biomass gain was studied. Addition of the fungus earlier than or together with the spruce seedlings to the soil substrate was more harmful than addition several days after the spruce seedlings (Appendix A Fig. 1), indicating that developing fine roots are particularly susceptible to pathogenic PAC isolates and/or that wounds caused by planting allow the fungus to circumvent the plant defenses. This conclusion is also supported by the fact that root colonization was considerably higher in seedlings where *P. subalpina* was pre- or co-inoculated (Appendix A Fig. 2). Consequently, when conclusions about different DSE studies are drawn, the point in time of inoculation should be taken into consideration as an important confounding factor. In my experiments the substrates were inoculated with PAC several days before addition of the seedlings, giving the fungus an advantage compared to the seedling, which is justified as germinating seedlings in natural forests are readily exposed to a PAC community (Ahlich et al., 1998) that might persist over years (Queloz et al., 2005).

PAC-host interactions also depend on the substrate (i.e., pH, organic and inorganic compounds). Although Newsham (2011) mentions the different substrates used in the studies included in his meta-analysis, it was not tested whether these substrates had a differential influence on the interaction of DSE with their host. In the experiments described in Chapter 3 vermiculite-peat substrate (pH = 4.5) was used, whereas in Chapter 4 vermiculite-peat-sand substrate (pH = 3.8) had to be used because preliminary tests showed that the infection success of *Phytophthora plurivora* was higher in this substrate type (data not shown). Differences in substrate type might also explain why some *P. subalpina* isolates were more aggressive in the first study than in the second, whereas others were less (Chapters 3&4). The importance of soil pH for the presence of DSE was demonstrated in field studies, where DSE

occurred more frequently in roots growing in more acidic soils (Kattner and Schönhaar, 1990; Ahlich et al., 1998; Postma et al., 2007). Similarly, mycorrhization of containerized *Pinus pinea* seedlings by four ectomycorrhizal (ECM) fungi was higher in acidic peat-vermiculite substrate than in neutral peat-composted pine bark substrate (Rincón et al., 2005), pointing out the importance of the pH level.

Third, the availability of nutrients in the substrate might influence the effects of PAC (and DSE) on their plant hosts. For this purpose, I have performed an experiment where seedlings inoculated with nine different PAC isolates were exposed to different complete fertilizer treatments: none, three-weekly, and weekly amendment. Disease severity was highest when no fertilizer was applied (Appendix B Fig. 1a), which was probably due to a combined effect of fungal infection and nutrient depletion. In contrast, disease severity in the other two treatments was comparable (Appendix B Fig. 1b&c), but interestingly, the highly virulent isolate 6_2_7v was even more aggressive when fertilizer was applied weekly. Furthermore, fertilization significantly affected biomass (Appendix B Table 1), but there was no significant growth promotion by PAC inoculation in any treatment (Appendix B Fig. 2). This finding contrasts with another study, where PAC promoted *Pinus contorta* growth (Jumpponen et al., 1998). However, growth promotion seems to occur predominantly if organic nitrogen is applied (Newsham, 2011). One mechanism that was proposed explaining the different outcomes depending on nitrogen form is that DSE might mineralize organic compounds such as peptides or amino acids (Newsham, 1999; Addy et al., 2005; Mandyam and Jumpponen, 2005). In contrast, inorganic nitrogen in the commercial fertilizer applied in the present work is readily available for the plants and does not need to be broken down by PAC. Another purpose of this experiment was to test whether PAC might be opportunistic pathogens that harm stressed plants. This hypothesis could clearly be rejected, as even under nutritional beneficial conditions the effect (neutral to pathogenic) of any single isolate on spruce seedlings was the same for all three treatments (Appendix B Fig. 2).

POSSIBLE MECHANISMS EXPLAINING THE PROTECTION OF NORWAY SPRUCE BY PAC

In Chapter 4 it was shown that some PAC isolates protect Norway spruce against both oomycete pathogens. The protective ability of PAC may be one reason for the evolution of PAC – tree symbioses. However, selection pressure must have been high for the establishment of such a costly host-endophyte association, requiring a constant challenge by soilborne pathogens, which is indeed the case in forests (Hansen and Goheen, 2000). It can

only be speculated about the mechanisms of PAC-pathogen antagonism. As concluded in Chapter 4, competition for space on roots does not seem to be the main mechanism, as there was no relationship between *P. subalpina* colonization density and presence of either pathogen. Antagonistic fungi can directly attack other fungi by so-called mycoparasitism. There are different types of mycoparasitism where the antagonistic fungus is either biotrophic or necrotrophic. In biotrophic interactions the mycoparasite penetrates the host hyphae without killing them. Often, haustorium-like structures are formed within the host which serve to support growth of the external mycelium of the parasite, and the colonized host cells are only killed after the parasite has sporulated (Van Den Boogert and Deacon, 1994; Jeffries, 1995). Complete internal colonization of *Phytophthora cinnamomi* hyphae was demonstrated for a PAC isolate *in vitro* (O. Holdenrieder, pers. comm.). It is possible that PAC might sequester secondary metabolites toxic to *E. undulatum* and *P. plurivora* (i.e., antibiosis). Antibiosis is well-known from other ascomycetes, e.g., *Trichoderma* spp. (Harman et al., 2004; Alabouvette et al., 2009), and some effects against *P. plurivora* were observed for the culture filtrate of one PAC isolate (Appendix C Fig. 1). A third mechanism by which PAC could protect plants against harmful pathogens is the induction of resistance. This can occur on different levels, on the anatomical level by structural modifications of the cell wall that impede the intrusion of pathogens and on the metabolic level by triggering the chemical plant defense. Structural modifications of the cell wall include the formation of papillae or lignitubers and deposition of lignin and suberin (Fellows, 1928; Bonello et al., 1991; Woodward, 1992; Asiegbu et al., 1994; Børja et al., 1995). Deposition of electron-dense material and suberin-lamellae-like thickenings was observed in *Asparagus officinalis* roots colonized by PAC (Yu et al., 2001). The chemical plant defense includes the synthesis of secondary metabolites that are toxic for pathogens or the production of enzymes like hydrolases that degrade the fungal cell wall (Mauch et al., 1988; Woodward and Pearce, 1988; Pearce, 1996; Witzell and Martín, 2008). Therefore, the induction of these defense mechanisms by PAC might block root invading pathogens. Localized and systemic induced resistance has well been documented for different tree pathogens and might be an important mechanism of defense (Faeth and Wilson, 1997; Krokene et al., 1999; Bonello and Blodgett, 2003; Eyles et al., 2010). However, the results of Chapter 4 are inconclusive, whether induced resistance against *P. plurivora* and *E. undulatum* occurred or not and concluding the mechanisms involved. As there were no differences in specific root length (SRL, Chapter 4 Table 2) between *P. subalpina* inoculated seedlings and the uninoculated control seedlings, structural reinforcement of the cell wall due to PAC seems not to occur, and thus, defense against the two pathogens is probably not based on an altered root anatomy (Eissenstat and Achor, 1999). In contrast, strong lignituber-formation upon colonization with a *Phialocephala fortinii* s.s. isolate was observed in *Salix purpurea* (Grünig et al., 2008). However, to explore

the hypothesis of induced resistance, more detailed microscopic examinations are necessary, combined with genetic and metabolic studies that investigate the expression of host defense-related genes and others that examine the molecular profile of different PAC isolates to detect virulence factors. A comparison of the transcriptome of *Pinus sylvestris* seedlings infected with root-colonizing mutualistic, saprotrophic or pathogenic fungi, for instance, revealed different expression patterns for each fungus (Adomas et al., 2008). Thus, such studies might answer the question of why certain PAC isolates become pathogenic whilst others cause little or no harm to their host, and they might give information about cross-talk between plant host and PAC. Ultimately, these results might give valuable insight into the evolution of endophytism in general.

DOES CLIMATE CHANGE AFFECT PAC AND OOMYCETE DISEASES?

The results of the temperature experiment described in Chapter 4 suggest that climate change will have little influence on the Norway spruce – PAC symbiosis, and virulence of *P. plurivora* will not increase, whereas virulence of *E. undulatum* might decrease. However, as noted above, care should be taken when generalizing from *in vitro* experiments to natural conditions. Moreover, in my experiments I have focused exclusively on temperature as the changing factor, but there are other factors like atmospheric CO_2 and ozone concentration or shifts of frequency and amount of precipitations (i.e., water stress and heavy rainfall) that are predicted to change also. Apart from temperature, increase in atmospheric CO_2 is the most intensely studied factor. However, since CO_2 levels are already 10-50 times higher in the soil than in the atmosphere (Lamborg et al., 1983) the effect of elevated atmospheric CO_2 on microbial processes in the rhizosphere might be minor and only indirect by direct effects on the shoot (Sadowsky and Schortemeyer, 1997). As for elevated temperature, studies on the effect of other climate change factors on endophytes are scarce (Compant et al., 2010). Results from an *in vitro* study on the influence of elevated CO_2 on the effect of DSE colonization on host seedlings are ambiguous because some isolates significantly increased host biomass, whereas others (including two PAC isolates) did not promote plant growth (Alberton et al., 2010). However, as there were only one or two isolates used per fungal species, it is not clear whether this effect is isolate- or species-specific. In a field experiment DSE colonization was affected by elevated temperature, but not by elevated CO_2 (Olsrud et al., 2010). Increased DSE colonization did not seem to affect plant biomass. Interestingly, DSE root colonization was not affected in the combined treatment of temperature and elevated CO_2. Thus, it might be important to study the combination of different climate change factors with regards to PAC and other DSE as well. In contrast, elevated CO_2 levels

were shown to promote growth of extraradical mycelium of AM and ECM, colonization of the host plant, and/or to reinforce positive mycorrhizal effects on host plants (Rillig et al., 2002; Alberton et al., 2005; Smith and Read, 2008; Compant et al., 2010; Pritchard, 2011). Moreover, in the recent years free air concentration enrichment (FACE) facilities have been established to study plant pathogen responses to changing atmospheric conditions in the field, but most of these studies were done on stem and leaf pathogens and studies on root pathogens are virtually absent (Chakraborty et al., 2008; Eastburn et al., 2011; Pritchard, 2011). There are few container and greenhouse studies on the effect of elevated CO_2 levels on soilborne pathogens of crop and ornamental plants. However, the results of these studies range from pathogen inhibition to strong enhancement of disease symptoms (reviewed in Manning and v. Tiedemann, 1995). In a recent study, beech (*Fagus sylvatica*) seedlings infected with *P. plurivora* displayed more severe disease symptoms and had a higher mortality at elevated CO_2 levels, compared with *P. plurivora*-infected seedlings at ambient CO_2 levels. Furthermore, seedling mortality was drastically reduced by nitrogen fertilization (Fleischmann et al., 2010).

OUTLOOK

The results of the experiments described in my thesis give some ideas about the basic nature of the Norway spruce – PAC interaction *in vitro*, indicating that PAC might act primarily as opportunistic parasites. However, there is a need to further investigate these results in a more natural context, as artificial systems do not cover the entire complexity of the interactions in nature. For instance, discrepancies between axenic and open pot cultures were shown for PAC and *P. contorta* seedlings. In the axenic cultures, there was an evident growth-promoting effect of PAC, whereas there was none in the open pot cultures (Jumpponen and Trappe, 1998b). Therefore, both microcosm experiments used in this work (i.e., the dual culture experiment in Chapter 3 as well as the tripartite interaction experiment performed under different temperature regimes in Chapter 4) need to be expanded to mesocosm studies outdoors. These findings will then need to be tested in the field in different locations, where PAC are known to occur naturally. Ideally, experiments would be performed in a FACE facility where the soil can be heated. At all three experimental stages, PAC infection should be controlled by specifically inoculating seedlings. Furthermore, in this thesis the model host was Norway spruce, but it is known that PAC have a broad host range, particularly many conifer tree and ericaceous shrub species (Wang and Wilcox, 1985; Ahlich and Sieber, 1996; Addy et al., 2000; Grünig et al., 2008). Before any generalizations can be drawn it is therefore necessary to understand whether the interaction patterns found in this work are a general

phenomenon also applicable to other hosts. Furthermore, these two experiments were performed in favor of PAC because they were inoculated under axenic conditions and were allowed to colonize the whole substrate before the plants were added. In natural systems, however, PAC have to deal with a broad bacterial and fungal rhizosphere community, and little is known about the dynamics and possible "steady states" of these interactions. Despite the fact that PAC colonize mycorrhizal and nonmycorrhizal roots and were occasionally isolated from ECM root tips (e.g., Ahlich and Sieber, 1996; Jonsson et al., 1999; Kaldorf et al., 2004; Grünig et al., 2008) little is known about interactions of PAC and ECM fungi. Thus, studying this interaction *in vivo* might reveal that ECM suppress PAC on host roots. Similarly, an excessive growth of virulent PAC in their hosts in natural populations might be controlled by non-virulent PAC isolates or other root-endophytic fungi, or by soil microbes.

Despite controlled infection experiments, there is also a need for more profound field investigations. Field studies are also needed to gain insight into the interaction of adult trees with PAC as controlled infection studies with adult trees are impossible. Moreover, these studies will give valuable information about the effect of environmental factors that could have been omitted in simplified *in vitro* systems. An ideal field study would comprise a spatio-temporal approach, in which all plants and their endosymbiotic communities (i.e., endophytes, mycorrhizae), other soil microbes, and the soil chemistry of various ecosystems with known PAC occurrence were assessed. The reason, why a temporal approach would be necessary is that it was shown for ECM that the community is not constant in the course of time and changes strongly between seasons (van der Heijden and Vosatka, 2000; Koide et al., 2007; Walker et al., 2008). In contrast, little is known about temporal fluctuations of PAC communities, although the same genotypes can persist over several years in the population (Queloz et al., 2005). However, permanent occurrence of PAC does not exclude the possibility that there are seasonal differences in abundance and, hence, the relative importance of PAC might fluctuate seasonally. Next-generation sequencing technologies allow massive parallel sequencing and were recently successfully used to determine molecular OTU composition in ECM and AM (Jumpponen et al., 2010; Dumbrell et al., 2011). Therefore, this method could be used to characterize the seasonal community composition of the PAC community. Moreover, massive parallel sequencing would also help to resolve the fine-scale distribution of PAC and would allow the relative abundance of different PAC OTUs to be quantified without the need of culturing methods. Then, the relative importance of PAC might also fluctuate on a vertical gradient in the soil similar to the differences observed for the community composition of ECM and non-defined endophytes in different soil horizons (Rosling et al., 2003; Baier et al., 2006; Lindahl et al., 2007). Finally, such a large-scale study would shed light on many cryptic processes in the rhizosphere that are still poorly understood.

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APPENDIX A

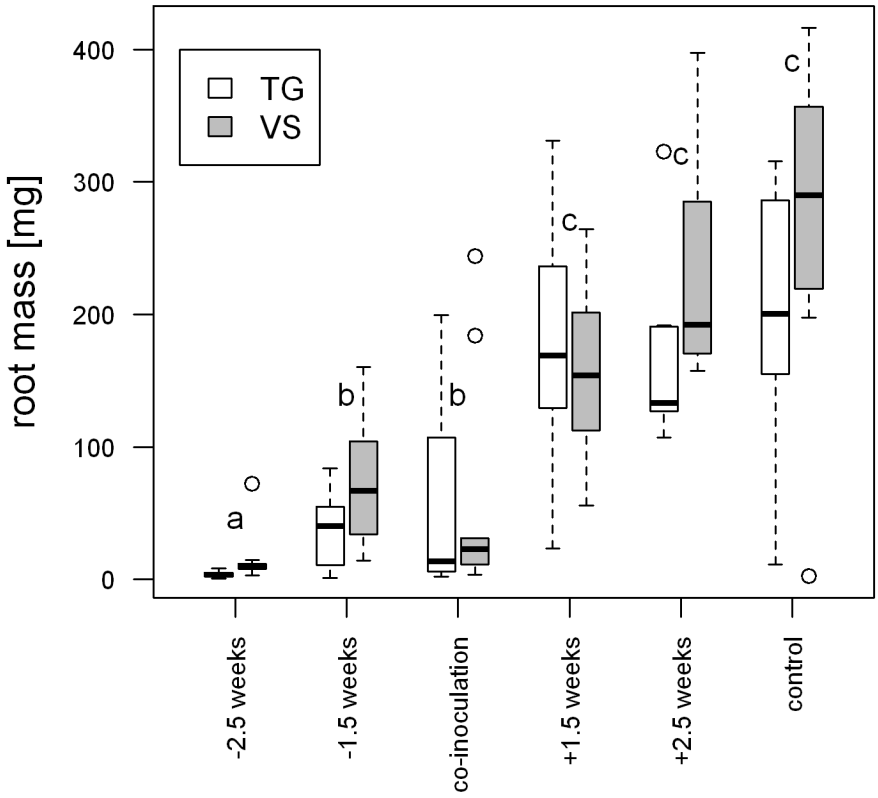

Figure 1. Root mass of Norway spruce seedlings of two provenances (TG = Swiss Plateau, VS = Central Alps) inoculated with *P. subalpina* isolate 6_16_1 at different points in time relative to the time when the seedlings were planted. Negative signs indicate that the fungus was inoculated before the seedlings were planted and positive signs indicate that the fungus was inoculated after the seedlings were planted. There were significant differences among the different points in time of inoculation ($F_{5,111}$ = 39.0, p < 0.001) and the two seedling provenances ($F_{1,111}$ = 4.9, p < 0.05). Different letters indicate significant differences among points in time of inoculation according to a TukeyHSD post-hoc test. Apart from the points in time of inoculation number of replicates and experimental conditions were exactly the same as in Chapter 3.

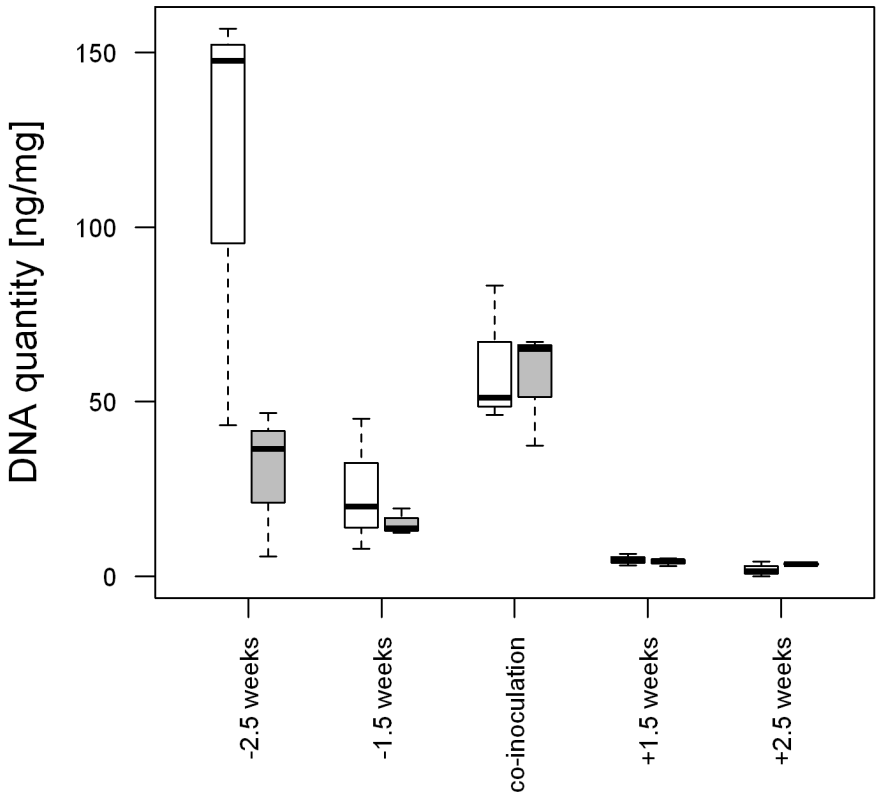

Figure 2. PAC DNA quantity ng per mg root weight of Norway spruce seedlings inoculated with *P. subalpina* isolate 6_16_1 at different points in time relative to the time when the seedlings were planted. Negative signs indicate that the fungus was inoculated before the seedlings were planted and positive signs indicate that the fungus was inoculated after the seedlings were planted. For incubation conditions see caption of Fig. 1.

APPENDIX B

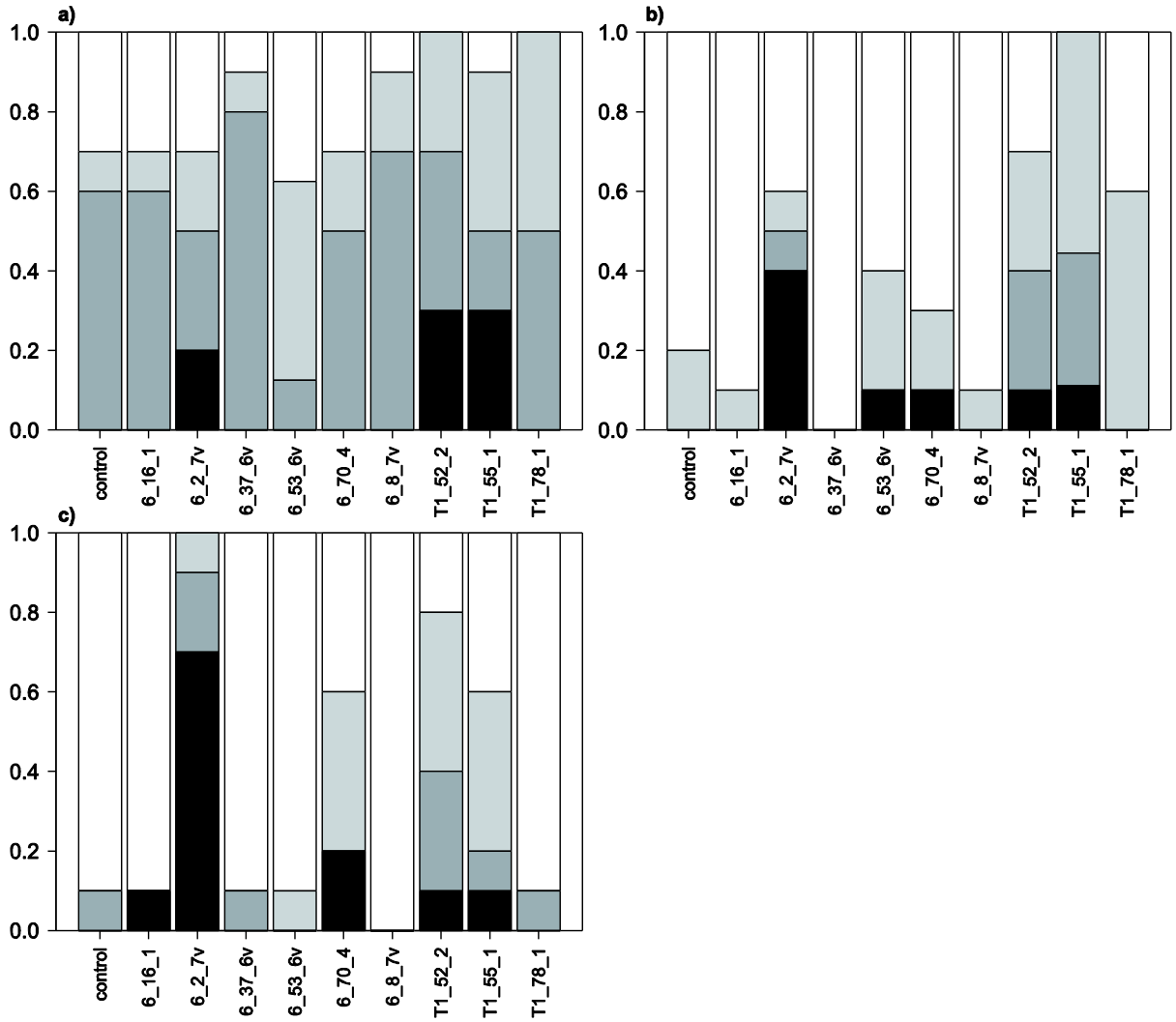

Figure 1. Proportional distribution of disease intensity of Norway spruce seedlings under three different fertilization regimes after 4 months of growth in a phytotron. Growing conditions were the same as in Chapter 3. a) no fertilization, b) 3-weekly fertilization, c) weekly fertilization with 3-4 ml of a 0.2%-dilution (v/v) of a complete fertilizer (Wuxal, Maag, Switzerland). Preparation and experimental conditions were the same as in Chapter 3. Each column represents a different PAC isolate. Black indicates dead, dark grey heavily diseased, grey mildly diseased and white healthy seedlings.

Table 1. Effects of fertilization, CSP and their interaction on plant growth parameters in a linear mixed effects model with isolate as random factor. For details see caption of Appendix B Fig. 1.

	needle mass	shoot mass	root mass	R/S ratio	SRL
	F	***F***	***F***	***F***	***F***
fertilization (f)	$152.608_{2,254}$***	$162.254_{2,254}$***	$134.106_{2,254}$***	$10.88_{2,254}$***	$3.455_{2,254}$*
CSP	$0.007_{1,7}$	$0.007_{1,7}$	$0.109_{1,7}$	$0.698_{1,7}$	$0.394_{1,7}$
f×CSP	$0.211_{2,254}$	$0.142_{2,254}$	$0.335_{2,254}$	$8.743_{2,254}$***	$6.535_{2,254}$***

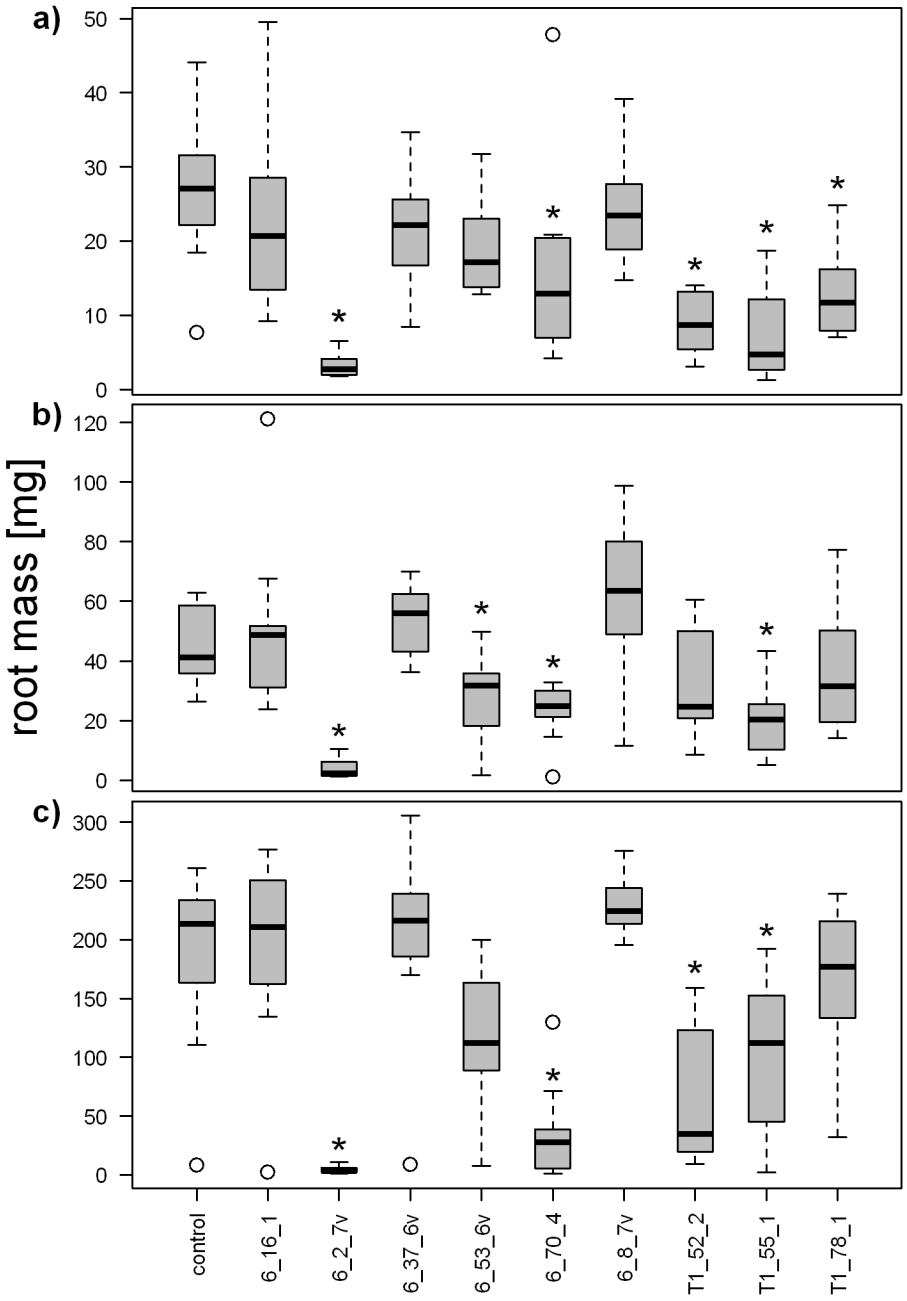

Figure 2. Root mass of Norway spruce seedlings inoculated with different *P. subalpina* (codes starting with "6") and *A. applanata* isolates (codes starting with "T1") under three different fertilization regimes: a) no fertilization, b) 3-weekly fertilization, c) weekly fertilization. Asterisks indicate significant differences from the respective control, determined with a linear regression model (see chapter 3 for details). For details see caption of Appendix B Fig. 1. See also Appendix B Table 1 for significances of the different plant performance parameters.

APPENDIX C

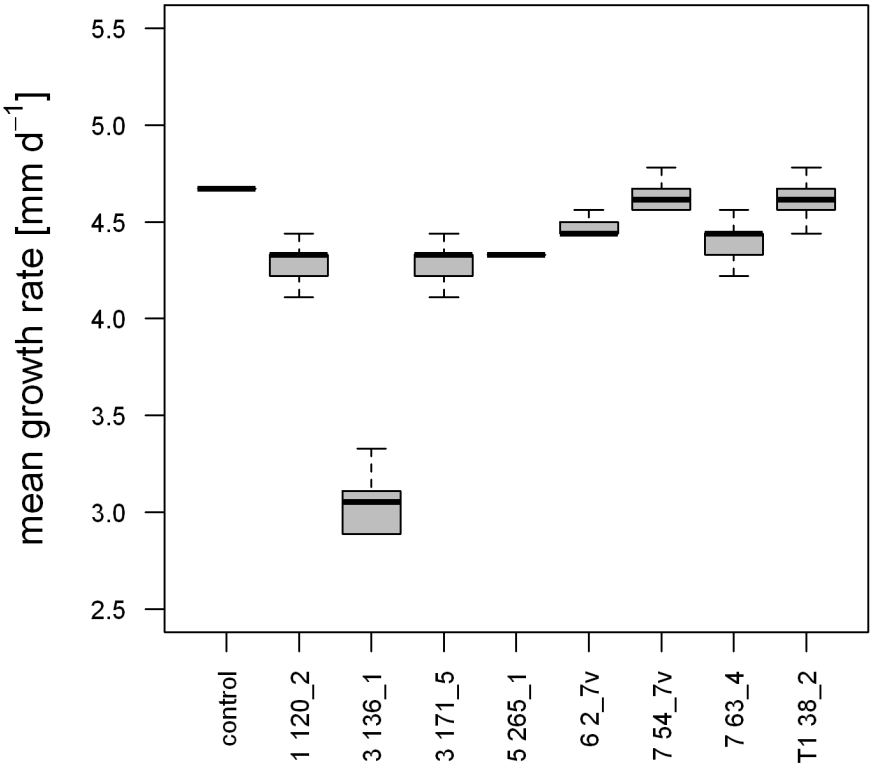

Figure 1. Growth rate of *Phytophthora plurivora* isolate 137/7a growing on an (1:1)-carrot – PAC carrot broth culture filtrate (v:v). On the x-axis the codes for the PAC isolates are shown, with the first digit indicating the cryptic species number. The control consisted of uninoculated carrot broth that was treated the same way as the PAC inoculated broths.

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Publications:

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	Born, J., **Tellenbach, C.**, Pluess, A.R., Nilus, R., Burslem, D., and Ghazoul, J. Differential performance of dipterocarp seedlings in response to soil moisture and microtopography (in preparation)
2010	**Tellenbach, C.**, Grünig, C.R., and Sieber, T.N. Suitability of quantitative real-time PCR to estimate biomass of fungal root endophytes. Applied and Environmental Microbiology (doi:10.1128/AEM.00907-10)
	Schoebel, C.N., **Tellenbach, C.**, Spaak, P., and Wolinska, J. Temperature effects on parasite prevalence in a natural hybrid complex. Biology Letters (doi:10.1098/rsbl.2010.0616)
2007	**Tellenbach, C.**, J. Wolinska, and P. Spaak. Epidemiology of a *Daphnia* brood parasite and its implications on host life-history traits. Oecologia (doi: 10.1007/s00442-007-0826-8)
	Keller, B., J. Wolinska, **C. Tellenbach**, and P. Spaak. Reproductive isolation keeps hybridizing *Daphnia* species distinct. Limnology and Oceanography 52:984-991.

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2010	Talk: "*Daphnia* parasites in Greifensee". **Tellenbach, C.**, P. Spaak. Swiss-Russian *Daphnia* Seminar 2010. Fribourg, Switzerland.
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	Talk: "Dunkelseptierte Wurzelendophyten von Nadelbäumen – Freunde oder Feinde?". **Tellenbach, C**., C. Grünig, T. Sieber, O. Holdenrieder. Annual Meeting of the Mycological Society of Switzerland. Luzern, Switzerland.
	Poster: „ PAC – Friend or Foe?". **Tellenbach, C.**, C. Grünig, T. Sieber, O. Holdenrieder. Biology09. Bern, Switzerland.
	Talk: „ PAC – Friend or Foe?". **Tellenbach, C.**, C. Grünig, T. Sieber, O. Holdenrieder. Mycology Symposium 2009. Zürich, Switzerland.
2008	Talk: „ PAC – Friend or Foe?". **Tellenbach, C.**, C. Grünig, T. Sieber, O. Holdenrieder. GEDIHAP Kickoff Meeting. Zürich, Switzerland.
2005	Talk: "The Influence of a Fungal Brood Parasite on Life-History Traits of its *Daphnia* Host". **Tellenbach, C.**, J. Wolinska, P. Spaak. 7th International Symposium on Cladocera. Herzberg, Switzerland.
	Poster: "Starving with full gut? The influence of suspended sediments on the fitness of *Daphnia hyalina* in turbid and ultra-oligotrophic Lake Brienz, Switzerland". Rellstab, C., **C. Tellenbach**, P. Spaak. 7th International Symposium on Cladocera. Herzberg, Switzerland.
	Poster: "A *Daphnia* brood parasite – its seasonal pattern and vertical distribution"; **Tellenbach, C.**, J. Wolinska, C. Rellstab, P. Spaak. 4rd International Symposium of Ecological Genetics, Antwerp, Belgium.

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