**RESEARCH ARTICLE**

**Do colonization by dark septate endophytes and elevated temperature affect pathogenicity of oomycetes?**

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**Abstract**

*Phialocephala subalpina* is one of the most frequent dark septate root endophytes in tree roots but its function in forest ecosystems is largely unknown. A full-factorial infection experiment was performed, using six *P. subalpina* isolates, two pathogenic oomycetes (*Phytophthora plurivora* [syn. *Phytophthora citricola* s.l.] and *Elongisporangium undulatum* [syn. *Pythium undulatum*]) and two temperature regimes (17.9 and 21.6 °C) to examine the ability of *P. subalpina* to protect Norway spruce seedlings against root pathogens. Seedling survival, disease intensity and seedling growth were affected by *P. subalpina* genotype, temperature and pathogen species. Some *P. subalpina* isolates effectively reduced mortality and disease intensity caused by the two pathogens. Elevated temperature adversely affected seedling growth but did not aggravate the effect of the pathogens. *Elongisporangium undulatum* but not *P. plurivora* significantly reduced plant growth. Colonization density of *P. subalpina* measured by quantitative PCR was not affected by temperature or the presence of the pathogens. In conclusion, *P. subalpina* confers an indirect benefit to its host and might therefore be tolerated in natural ecosystems, despite negative effects on plant health and plant growth.

**Introduction**

Microorganisms are interacting in complex ways with plants and each other in natural communities. Aerial plant surfaces are colonized by epiphytic microorganisms, the rhizosphere hosts a plethora of soil microorganisms, 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, time of observation of the interaction or host and microorganism genotype. For instance, mycorrhizal fungi reduce plant growth during the early establishment of the symbiosis compared to mycorrhiza-free plants, but when the mycorrhiza is functional, these plants gain relatively more biomass and overgrow nonmycorrhized plants (Johnson *et al.*, 1997). In contrast, the nature of host–endosymbiont interactions is less clear for nonmycorrhizal endosymbionts.

Fungal endophytes are endosymbionts that colonize most functional plant tissues but do not cause disease symptoms, either for a prolonged period of time or never (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 not well understood.
Yet, the presence of fungal root endophytes in plants, in particular of dark septate endophytes (DSE), is very common. DSE are abundant root colonizers of a wide range of mycorrhizal and nonmycorrhizal plant species (Stoyke et al., 1992; Sieber, 2002; Addy et al., 2005) and form a polyphyletic group of ascomycete fungi with melanized, septate hyphae (Stoyke et al., 1992; Ahlich & Sieber, 1996; Jumpponen & Trappe, 1998; Newsham, 2011). In confers and ericaceous shrubs, the most prevalent DSE fungi belong to the *Phialocephala fortinii* s.l.– *Acephala* *applanata* cryptic species complex (PAC) (Wang & Wilcox, 1985; Ahlich & 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 & Sieber, 1996; Addy et al., 2005; Grünig et al., 2008; Walker et al., 2011) nor with climate and is assumed to be mainly driven by stochastic effects (Queloz et al., 2011). Despite the wide distribution and frequent occurrence, PAC behaves 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 provides indirect benefits to their host by protecting it against harmful root pathogens. Such protection could be vital for plant seedlings, as these are particularly susceptible to pathogens (Newhook & 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 & Campbell, 1973; Hamm & Hansen, 1982; Lilja et al., 1992). Moreover, oomycetes are globally involved in dieback of forest trees (Newhook & Podger, 1972; Brasier, 1996; Jung et al., 1996, 2005; Nechwatal & Osswald, 2001; Brasier et al., 2004; Chavarriaga et al., 2007). Two widely distributed species are *Elongisporangium undulatum* (syn. *Pythium undulatum*, Uzuhashi et al., 2010) and *Phytophthora plurivora* (syn. *Phytophthora citricola*, Jung & Burgess, 2009). *Elongisporangium 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 & Kavanagh, 2005). *Phytophthora plurivora* is primarily known as a pathogen of broadleaved tree species (Jung et al., 1996, 2005; Nechwatal & Osswald, 2001; Jung & Burgess, 2009), but can also cause significant root loss and mortality in Norway spruce seedlings (Nechwatal & Osswald, 2001). Oomycetes seem to react strongly to environmental conditions (Newhook & Podger, 1972), and might gain even more importance under climate change, as warming combined with high amounts of rainfall is predicted to create favourable conditions for their growth and development (Brasier, 1996; 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 & Lombardero, 2000; Harvell et al., 2002; Garrett et al., 2006; Walther, 2010). The effect of climate change on neutral or beneficial interactions is little understood, but is also predicted to vary considerably (Compton 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–host equilibrium might enhance this effect. Thus, the goal of this study was to examine in a tripartite host–endophyte–pathogen system whether *Phialocephala subalpina* can protect Norway spruce seedlings against *P. plurivora* and *E. undulatum* and whether this effect is 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 PAC isolates representing different multilocus microsatellite haplotypes (6_16_1, 6_2_7v, 6_37_6v, 6_53_6v, 6_70_4, 6_8_7v) (Queloz et al., 2010). These isolates belong to the globally most widely distributed PAC species, *P. subalpina* (Queloz et al., 2011), and originate from a single population (Bödmeren, Switzerland). Moreover, they have previously been shown to vary in strength of interaction with Norway spruce from neutral to highly virulent (Tellenbach et al., 2011). *Phytophthora plurivora* (syn. *P. citricola*) isolate Bu 137/7a (Nechwatal & Osswald, 2001; Jung & Burgess, 2009) and *E. undulatum* (syn. *P. undulatum*) (CBS 101728) (Uzuhashi et al., 2010) were included as soilborne root pathogens.

#### Experimental procedures

*Phialocephala 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 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 100-mL Erlenmeyer flasks and incubated at 20 °C on a rotary shaker at 100 r.p.m. After 23 days, the...
mycelium was harvested and washed with sterile nanopure water. Mycelia were blotted dry on a sieve and weighed. Then, they 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. Fifty millilitre 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 to exclude seedborne fungi and bacteria. Surface sterilization occurred by immersion in 30% \(H_2O_2\) for 30 min, followed by rinsing with sterile nanopure water. Germination occurred within 12 days on water agar at 18 °C in the dark. After planting the seedlings into the Falcon tubes, plants were transferred to a phytopotron \([16\text{-h day (120–140} \mu E \text{ m}^{-2} \text{s}^{-1})/8\text{-h night, temperature (24 °C/15 °C})\) and relative humidity (rH 45%/rH 85%)]. Tubes were randomly distributed in nonheated 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.meteo schweiz.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 3 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 3-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 2 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 removed and replaced by 1 mL inoculated VMC medium, and 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, that is, the degree of damage caused to the seedlings, was assessed as disease intensity and reduction of seedling performance. Disease intensity, which includes the two components disease incidence, that is, the number of affected seedlings (e.g. mortality rate), and disease severity, that is, the percentage of necrotic or chlorotic surface area of the needles, was scored: (0) seedlings without any apparent disease symptoms, (1) seedlings with < 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 and 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 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 & 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 analysed, using a nested quantitative PCR (qPCR) to estimate the colonization density of *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).

**DNA extraction and qPCR conditions**

Conditions for DNA extraction and qPCR were the same as in the study by Tellenbach et al. (2010): samples were frozen at −80 °C and freeze-dried for 2 days. A few grains of DNA-free silica sand (dried for 4 h at 180 °C) were added to the lyophilized root samples to facilitate the disruption of plant cell walls. Then, samples were frozen again at −80 °C and homogenized using a bead mill. DNA was extracted using a CTAB/DNasey plant mini kit (Qiagen, Basel, Switzerland) extraction, following the protocol of Grünig (2003), with adjustments 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).

A nested qPCR was performed using the external primers pPF-076_F1 and pPF-076_R1 (Grünig et al., 2007) in step 1 and the qPCR primers and probe pPF-076_qPCR_F, pPF-076_R, pPF-076_P (Tellenbach et al., 2010) in step 2. The PCR in step 1 was 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. 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 serially diluted genomic DNA with known concentrations was added to each sample plate, and each sample was run in triplicate.

**Statistical analyses**

All statistical analyses were performed using the R statistical package (R Development Core Team, 2009). Seedling survival in relationship to temperature, *P. subalpina* isolate and pathogens was analysed in a survival analysis, 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 categories of disease intensity were analysed with a proportional odds model using the *lrm* function in the *Design* library. Plant growth–related parameters were analysed using an *ANOVA*. Prior to the analysis, all plant growth–related variables were transformed with the logarithm, and the remaining variables with the square-root. For the *ANOVAS*, the best model was selected according to Akaika Information Criterion (AIC) comparisons. In the survival analysis and the proportional odds model, the best model was selected using likelihood ratio tests that are asymptotically chi-squared distributed. Significant differences in the *ANOVAS* were further investigated using Tukey’s HSD *post hoc* test.

**Results**

Koch’s postulates were fulfilled for *P. subalpina* and the two pathogens. On average, *P. subalpina* was successfully re-isolated 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 survival and disease intensity**

Survival and disease intensity varied considerably among the two temperature treatments, *P. subalpina* isolates, and the presence or absence of the two pathogens (Fig. 1). Mortality of plants inoculated with *P. subalpina* 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% at low and 60% at high temperature, whereas mortality of seedlings inoculated with *E. undulatum* was 90% and 80% at the respective temperature (Fig. 1). Significance of the parameters was the same in the survival analysis and the proportional odds model (Table 1): elevated temperature, inoculation with *P. subalpina* and/or the two pathogens significantly increased mortality and disease intensity of Norway spruce seedlings. Moreover, the significant interaction between *P. subalpina* and pathogens (1 × P) indicates that some *P. subalpina* isolates suppressed *P. plurivora* and/or *E. undulatum* disease more effectively than others. For example, inoculation of seedlings with the isolate 6_2_7v reduced mortality caused by *P. plurivora*, whereas inoculation with isolates 6_70_4, 6_8_7v, 6_16_1 and

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6.2.7v reduced mortality caused by *E. undulatum* (Fig. 1). *Elongisporangium undulatum* was highly virulent on *P. subalpina*-free controls and seedlings inoculated with isolate 6.53.6v, resulting in survival of fewer than three seedlings at both temperatures (Figs 1 and 2). Elevated temperature led to a general increase in disease intensity, but this increase was the same for all *P. subalpina* isolates as indicated by the absence of a significant interaction between temperature and *P. subalpina*. Likewise, temperature did not alter the expression of disease symptoms caused by the two pathogens.

**Plant growth-related parameters**

Noninoculated seedlings (control) had the highest biomass (i.e. total biomass, needle, shoot, and root mass) at both temperatures. Biomass varied strongly between the two temperature regimes and among seedlings inoculated with different *P. subalpina* isolates and the two pathogens (Supporting Information, Table S1). 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. ANOVAs were performed for each pathogen separately. One analysis was performed with all seedlings inoculated with *P. plurivora* and any *P. subalpina* isolate (including the *P. subalpina*-free control inoculates) (analysis 1, Table 2a) and another one with a reduced data set containing the data of seedlings inoculated with *E. undulatum* comprising only those *P. subalpina* isolates (i.e. 6.16.1, 6.2.7v, 6.70.4, 6.8.7v) that led to survival of at least three seedlings at either temperature (analysis 2, Table 2b).

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* isolate 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 at lower temperature. Significant
differences among other isolates or the control were less consistent and depended on the biomass parameter.

In the reduced data set of analysis 2, inoculation with different *P. subalpina* isolates distinctly affected seedling biomass parameters. Likewise, all seedling biomass parameters were significantly decreased by inoculation with *E. undulatum*. In contrast, temperature significantly decreased total biomass, needle and shoot mass, but not root mass (Table 2b). However, the interaction between temperature and *E. undulatum* was significant for all four biomass parameters, indicating that temperature differently affected *E. undulatum*-free and infected seedlings at either temperature. *Post hoc* tests with Tukey’s HSD revealed for each biomass parameter that this significant

![Fig. 2. Bar charts depicting the effect of temperature and inoculation with the root pathogens *Phytophthora plurivora* or *Elongisporangium undulatum* in combination with *Phialocephala subalpina* on mean root mass (± standard error) of Norway spruce seedlings. Root mass is grouped by pathogen treatment (no pathogen, *P. plurivora* and *E. undulatum*) and temperature regime. In total, there were n = 10 seedlings per treatment. Numbers above the bars indicate the number of surviving seedlings, and ‘X’ indicates treatments with less than two surviving seedlings.](image)

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total biomass</th>
<th>Needle mass</th>
<th>Shoot mass</th>
<th>Root mass</th>
<th>R/S ratio</th>
<th>SRL</th>
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<tr>
<td></td>
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</tr>
<tr>
<td><strong>(a) Analysis 1:</strong> <em>P. plurivora</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (T)</td>
<td>33.226,177***</td>
<td>38.663,183***</td>
<td>42.009,177***</td>
<td>17.234,183***</td>
<td>–</td>
<td>21.897,1,190***</td>
</tr>
<tr>
<td><em>P. plurivora</em> (plur)</td>
<td>3.022,177***</td>
<td>3.392,183***</td>
<td>3.997,177***</td>
<td>1.815,183***</td>
<td>–</td>
<td>2.032,190***</td>
</tr>
<tr>
<td>T × sub</td>
<td>1.970,177†</td>
<td>–</td>
<td>2.042,177†</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>T × plur</td>
<td>3.289,177†</td>
<td>2.421,183†</td>
<td>2.603,177†</td>
<td>3.123,183†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>(b) Analysis 2:</strong> <em>E. undulatum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (T)</td>
<td>9.168,1,87**</td>
<td>15.415,1,87***</td>
<td>15.028,1,87***</td>
<td>3.037,1,87†</td>
<td>1.412,1,87</td>
<td>8.836,1,00**</td>
</tr>
<tr>
<td><em>P. subalpina</em> (sub)</td>
<td>19.448,1,87***</td>
<td>14.444,1,87***</td>
<td>16.793,1,87***</td>
<td>16.590,1,87***</td>
<td>7.037,3,87***</td>
<td>–</td>
</tr>
<tr>
<td><em>E. undulatum</em> (und)</td>
<td>8.136,1,87**</td>
<td>5.188,1,87†</td>
<td>5.982,1,87†</td>
<td>8.707,1,87†</td>
<td>3.101,1,87†</td>
<td>0.640,1,00</td>
</tr>
<tr>
<td>T × und</td>
<td>10.014,1,87***</td>
<td>8.299,1,87***</td>
<td>8.266,1,87***</td>
<td>9.905,1,87***</td>
<td>2.351,1,87</td>
<td>4.855,1,00†</td>
</tr>
</tbody>
</table>

Type III F–statistics from a linear model, followed by the effect and residual degrees of freedom (lowercase numbers).

†P < 0.1; *P < 0.05; **P < 0.01; ***P < 0.001 – parameters excluded by model reduction according to the AIC criterion.

†Interactions that were excluded by the AIC criterion for all plant growth–related parameters are not shown.

Excluding *Phialocephala subalpina*-free seedlings and seedlings inoculated with *P. subalpina* isolates 6_37_6v and 6_53_6v (< 3 surviving seedlings).
interaction was due to a significant biomass reduction of E. undulatum-infected seedlings at low temperature, whereas there were no differences at elevated temperature between E. undulatum-free and infected seedlings. 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 (Figs 1 and 2). Moreover, root mass of surviving E. undulatum-infected seedlings colonized by P. subalpina isolates 6_70_4 or 6_8_7v was almost as high or higher (at elevated temperature) as root mass of E. undulatum-free seedlings inoculated with the same P. subalpina isolate (Fig. 2).

The root-to-shoot (R/S) ratio ranged from 0.05 to 2.62 and differed significantly only among seedlings inoculated with different P. subalpina isolates in both ANOVAs (Table 2). 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.

SRL ranged from 13.04 to 101.22 mm mg⁻¹ and was significantly increased by elevated temperature for both pathogens (Table 2). However, there was a significant interaction between temperature and E. undulatum, which was due to differences in SRL at low and elevated temperature. These differences were significant for seedlings inoculated with E. undulatum, but not for E. undulatum-free seedlings.

**Fungal colonization density**

The amount of DNA of P. subalpina in the root samples was determined using a nested qPCR (Tellenbach et al., 2010) and used as a measure for colonization density of P. subalpina. Colonization density varied among different P. subalpina isolates, but was within the same order of magnitude (Table 3). It 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 (F₅,₁₀₀ = 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.

**Discussion**

**Protection of Norway spruce by P. subalpina against oomycete pathogen treatments**

The ability of six genetically distinct P. subalpina isolates to control two oomycete root pathogens of Norway spruce was tested in vitro. The degree of protection depended on P. subalpina isolate and pathogen, and the protective effect was not an artefact due to the failure of the pathogen to establish in the plants, because presence and viability of both pathogens were demonstrated by high re-isolation rates. To estimate the efficacy of P. subalpina to control the pathogens, 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 nonaggressive isolate 6_70_4 also provided very good protection against both pathogens combined with good plant growth. Thus, there is no linear relationship between pathogen presence and pathogen density.

**Table 3.** Mean Phialocephala subalpina colonization density ± standard deviation (ng DNA per mg root dry weight) of each pathogen treatment at low and elevated temperature

<table>
<thead>
<tr>
<th>Isolate</th>
<th>No pathogen</th>
<th>P. plurivora</th>
<th>E. undulatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>Elevated</td>
<td>Low</td>
</tr>
<tr>
<td>6_70_4</td>
<td>4.9 ± 2.7</td>
<td>3.7 ± 1.8</td>
<td>10.1 ± 7.5</td>
</tr>
<tr>
<td>6_53_6v</td>
<td>7.7 ± 2.6</td>
<td>13.1 ± 6.7</td>
<td>11.9 ± 5.8</td>
</tr>
<tr>
<td>6_37_6v</td>
<td>9.4 ± 2.1</td>
<td>9.2 ± 5.5</td>
<td>8.3 ± 9.8</td>
</tr>
<tr>
<td>6_8_7v</td>
<td>17.4 ± 8.5</td>
<td>14.7 ± 4.5</td>
<td>15.6 ± 9.9</td>
</tr>
<tr>
<td>6_16_1</td>
<td>15.2 ± 11.1</td>
<td>19.9 ± 10.1</td>
<td>21.0 ± 28.8</td>
</tr>
<tr>
<td>6_2_7v</td>
<td>28.9 ± 16</td>
<td>17.9 ± 8.9</td>
<td>18.6 ± 13.6</td>
</tr>
</tbody>
</table>

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between interaction strength of *P. subalpina* 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).

The six *P. subalpina* isolates used in this study were selected based on their virulence exhibited on Norway spruce seedlings in a previous experiment (Tellenbach et al., 2011). The virulence of the selected isolates ranged from neutral to highly virulent, as this corresponds to a more natural situation where isolates differing in strength of interaction with their host co-occur within the same plot (Quéloz et al., 2011; Tellenbach et al., 2011). Moreover, plant response to isolates differing in virulence is distinct (Niks & Marcel, 2009), which in turn might affect the interaction with pathogens. The effect of the *P. subalpina* isolates was similar in the current study compared to the study of Tellenbach et al. (2011) with some isolates 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).

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; Harman et al., 2004; Tripathi et al., 2008; Alabouvette et al., 2009). In our experiment, we cannot determine the exact mechanisms of antagonism, but the data suggest that competition between *P. subalpina* and the two root pathogens for infection sites is not the sole protection mechanism as there was no relationship between *P. subalpina* colonization density and protective capability. Moreover, mechanical- and chemical-induced resistance is well known in conifers and could be another protection mechanism conferred to Norway spruce seedlings by *P. subalpina* (Bonello & Blodgett, 2003). But as there were no significant differences in SRL, indicating the formation of thicker cell walls (e.g. by thickening of the exodermis; Eissenstat & Achor, 1999), mechanical-induced resistance seems to be less likely. In contrast, the production of toxins with antifungal activities against *P. plurivora* and *E. undulatum* as shown for *Pinus strobus* needle endophytes in disc diffusion assays against *Saccharomyces cerevisiae* and *Microbotryum violaceum* (Sumarah et al., 2011) is likely and needs further consideration.

In contrast to mycorrhizal fungi, PAC can colonize roots of all age classes and can thus also be detected in the bark of coarse roots close to the stem base of mature trees (Grüning et al., 2008). PAC frequently colonizes root tips (Menkis et al., 2004; Grüning et al., 2008; Wagg et al., 2008) where they have to compete with ectomycorrhizal fungi. Interestingly, the primary roots of fungus-free conifer seedlings planted into forest soil as bait are usually much faster colonized by PAC than by ectomycorrhizal fungi (Ahlich et al., 1998). However, colonization of established ectomycorrhizae (ECM) by PAC is significantly less frequent than that of nonmycorrhizal root tips (Grüning et al., 2008). Mycorrhizal fungi are known to protect plant seedlings against pathogens (Newsham et al., 1995; Azcón-Aguilar & Barea, 1997; Whipps, 2004), and Richard et al. (1971) showed that ECM formed by *Suillus granulatus* prevented DSE (probably PAC) from colonizing and adversely affecting *Picea mariana* seedlings. Similarly, PAC colonization of ECM of *P. abies* and *Pseudotsuga menziesii* was less frequent and less dense than that of nonmycorrhizal roots (V. Reininger, unpublished). ECM certainly form a physical barrier against colonization by other fungi including PAC. However, as PAC seems to be the faster colonizers, they might play an important protective role against pathogens early in the life of a spruce seedling, but complete exploitation of the root might be controlled by delayed colonization by ECM.

**The effect of elevated temperature**

Control of either pathogen by any *P. subalpina* isolate was not influenced by elevated temperature. Moreover, the relative among-isolate differences in the reduction in 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 (Köhl et al., 1999; Singh et al., 2009; Thomson et al., 2010). Nonetheless, some studies were carried out using true tripartite systems demonstrating dependency of plant protection from the interaction between temperature, other environmental parameters, pathogen isolate and antagonist species as shown for the protection of Douglas fir seedlings against *Fusarium oxysporum* or bean plants against *Botrytis cinerea* (Strobel & Sinclair, 1991; Hannusch & Boland, 1996).

Elevated temperature did not significantly affect *P. subalpina* colonization and its influence on Norway spruce. However, the effect of elevated temperature on endophytes and mycorrhiza seems to be ruled by a complex set of additional factors as contradictory effects were found in different studies (e.g. Staddon et al., 2004; Ju
et al., 2006; Compant et al., 2010; Antunes et al., 2011; Brosi et al., 2011), and whether temperature affects endophyte as well as mycorrhiza colonization and behaviour further depends on host species (Rillig et al., 2002; Compant et al., 2010; Olssrud et al., 2010).

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 & 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 & 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, elevated temperature did not aggravate P. plurivora disease symptoms of the seedlings, and it is therefore 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 (P. 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 & Sage, 2008). Moreover, soil heating experiments showed bell-shaped growth curves for conifer trees with a maximum at around 20 °C soil temperature (Lopushinsky & 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 (Sandermann, 2004; Wang et al., 2006).

Conclusions

In this study, we have demonstrated that P. subalpina, the most abundant and widespread PAC species, can reduce disease intensity caused by the two oomycete root rot pathogens E. undulatum and P. plurivora in Norway spruce seedlings. However, suppression of pathogens is strongly P. subalpina strain dependent, and only two of the six strains constantly reduced mortality (6.2_7v, which, however, had a strong negative impact on host biomass) or both mortality and adverse effects on plant growth (6.70.4). Therefore, protection provided by PAC against more harmful pathogens might prompt plants to tolerate PAC despite negative effects of some strains on plant growth and health, and could explain why this symbiosis is globally widely distributed (Ahlich & Sieber, 1996; Queloz et al., 2011). Moreover, PAC might also play a stabilizing role in protecting Norway spruce against oomycete root pathogens under climate change. However, because this study was performed in vitro, the findings must be verified in field trials as well, and it is further not clear whether this protective effect also occurs in adult trees, which are densely colonized by PAC (Ahlich & Sieber, 1996; Sieber & Grünig, 2006; Grünig et al., 2008). Our study also gives valuable insights into the interaction between plants and endophytes, because 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.

Acknowledgements

We would like to thank Veronika Netzer, Christine Rupflin and Angelo Duò for technical assistance and Pascal Zaffarano, Lioba Paul, Ottmar Holdenrieder and Steve Woodward for their helpful comments. We also thank Thomas Jung for providing the P. plurivora isolate, Aria Minder and Tania Torossi and the Genetic Diversity Center (GDC), ETH, Zürich, Switzerland, where qPCR was performed. This work was supported by SNF grant 3100A0-113977 from the Swiss National Science Foundation, Bern, Switzerland. This work was also partly supported by the GEDIHAP project of the Competence Center Environment and Sustainability (CCES) of the ETH Domain, Zürich, Switzerland.

References


Jung T & Burgess TI (2009) Re-evaluation of *Phytophthora citricola* isolates from multiple woody hosts in Europe and...


Supporting Information

Additional Supporting Information may be found in the online version of this article:

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

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