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**Fungal associates of the tree-killing bark beetle, *Ips typographus*, vary in virulence, ability to degrade conifer phenolics and influence bark beetle tunneling behavior**

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## Abstract

The bark beetle *Ips typographus* carries numerous fungi that could be assisting the beetle in colonizing live Norway spruce (*Picea abies*) trees. Phenolic defenses in spruce phloem are degraded by the beetle's major tree-killing fungus *Endoconidiophora polonica*, but it is unknown if other beetle associates can also catabolize these compounds. We compared the ability of five fungi commonly associated with *I. typographus* to degrade phenolic compounds in Norway spruce phloem. *Grosmannia penicillata* and *G. europhioides* were able to degrade stilbenes and flavonoids faster than *E. polonica* and grow on minimal growth medium with spruce bark constituents as the only nutrients. Furthermore, beetles avoided medium amended with phenolics but marginally preferred medium colonized by fungi. Taken together our results show that different bark beetle associated fungi have complementary roles in degrading host metabolites and thus might improve this insect's persistence in well defended host tissues.

Keywords: *Ips typographus*, fungal symbionts, *Endoconidiophora*, *Grosmannia*, *Ophiostoma*, phenolic defenses, detoxification, complementary roles

Research highlights:

- ***Ips typographus*-associated fungi have the ability to degrade conifer phenolics**
- ***Grosmannia penicillata* degrades major spruce phenolics most rapidly**
- **Efficiency of phenolic degradation varies directly with fungal virulence**
- **Bark beetles avoid phenolics but prefer fungus-colonized medium**

## 1. Introduction

Plant-insect interactions are important in shaping ecosystems worldwide and insects can affect plant fitness both positively (e.g. as pollinators) and negatively (e.g. as herbivores) (Strauss and Zangerl 2002). Plant-herbivore interactions that lower plant fitness are often not simple two-way interactions but are mediated by microorganisms and are thus complex three-way plant-herbivore-microbe interactions (Jones 1991; Scott et al. 2008). A well-studied three-way plant-herbivore-microbe interaction with large economic impacts is that between conifer trees, tree-killing bark beetles, and phytopathogenic fungi thought to assist the beetles in killing trees (Krokene 2015; Paine et al. 1997). Tree-killing bark beetles have huge negative impacts on ecosystem dynamics, forestry and carbon sequestration in conifer forests worldwide and these impacts appear to be aggravated by ongoing global climate change (Allen et al. 2010; Kurz et al. 2008; Raffa et al. 2008).

One of the best-studied tree-killing bark beetle systems is that involving the spruce bark beetle *Ips typographus*, its host tree Norway spruce (*Picea abies*), and beetle-associated ophiostomatoid fungi in the *Endoconidiophora*, *Ophiostoma* and *Grosmannia* genera (Franceschi et al. 2005; Kirisits 2004; Krokene 2015). The spruce bark beetle is a primary killer of mature Norway spruce, one of the most widespread and economically important trees in Europe. During intermittent outbreaks the beetle-fungus complex overwhelms the defensive capacity of healthy trees in mass-attacks coordinated by beetle aggregation pheromones (Christiansen and Bakke 1988; Wermelinger 2004). As the beetles tunnel into the phloem, they infect the tree with blue-stain fungi that challenge tree defenses and thus increase the virulence of the attack (e.g. Christiansen et al. 1987). Recent research has shown that beetle-associated blue-stain fungi may weaken tree defenses by metabolizing terpenes and phenolics (DiGuistini et al. 2011;

Hammerbacher et al. 2011; 2013; Wang et al. 2014). For example, *Endoconidiophora polonica*, an important fungal associate of the spruce bark beetle, depletes tree defenses by metabolizing phenolics at a higher rate than the rate at which the tree can produce them (Hammerbacher et al. 2011; 2013).

Because of its ability to invade healthy sapwood and disrupt water transport, *E. polonica* is considered to be a key fungal associate of the spruce bark beetle (Kirisits 2004; Horntvedt et al. 1983). The virulence of *E. polonica* to Norway spruce has been studied extensively since the 1980s, and healthy trees are killed when the fungus is mass-inoculated into spruce stems at densities mimicking bark beetle mass-attack densities (Christiansen 1985; Horntvedt et al. 1983; Krokene and Solheim 1998b; 2001). Consequently, the *I. typographus* – *E. polonica* interaction has emerged as a model system for conifer-bark beetle-fungus interactions (Six and Wingfield 2011). However, despite the proven ability of *E. polonica* and other ophiostomatoid fungi to challenge tree defenses, the question of whether the beetles or their fungal associates are most important in killing trees remains controversial (Krokene 2015; Six and Wingfield 2011). The paradigm for the past hundred years has been that fungi play a critical role in exhausting tree resistance (Craighead 1928; Krokene et al. 2013; Lieutier et al. 2009; Münch 1907; 1908), but inconsistencies in the patterns of association between beetle and fungal species has led some to challenge this paradigm and conclude that fungi have no role in bark beetle success (Persson et al. 2009; Six and Wingfield 2011).

Some of the controversies around the importance of fungi in tree-killing by bark beetles can probably be traced back to the strong emphasis on *E. polonica* as the most virulent fungus in the spruce bark beetle-Norway spruce system. Geographical variation in the degree of association between *E. polonica* and *I. typographus* has been taken as evidence that factors other

than fungi are critical for sustained bark beetle outbreaks, since these beetles are also extremely successful in areas where *E. polonica* is rare or absent (Persson et al. 2009; Six and Wingfield 2011). Yet it is possible that other fungi may contribute to sustaining bark beetle outbreaks since the spruce bark beetle can vector a diverse assembly of ophiostomatoid fungi in addition to *E. polonica* (Krokene and Solheim 1996; Solheim 1992b; Kirisits 2004). For example, early inoculation studies showed that the beetle associate *Grosmannia penicillata* may induce extensive necrotic lesions in Norway spruce phloem that may be even longer than those induced by *E. polonica* (Horntvedt et al. 1983; Solheim 1988). Also, our recent discovery that *Grosmannia* fungi can synthesize spruce bark beetle pheromone components (Zhao et al. 2015) suggests that these fungi have a long co-evolutionary history with the beetle and may play a role in tree colonization.

In this article we revisit *G. penicillata* and other common fungal associates of the spruce bark beetle that have been largely neglected in the scientific literature over the last 30 years in favor of *E. polonica*. We test the hypothesis that beetle-associated fungi other than *E. polonica* can help overcome tree defenses by assessing their virulence, ability to metabolize spruce phenolics and growth rate on medium containing phenolics. We also investigated if spruce bark beetle behavior is affected by phenolics and if they have a selective preference towards specific fungal species based on fungal ability to detoxify phenolic defense compounds.

## 2. Materials and methods

### 2.1. Inoculation of Norway spruce with bark beetle-associated fungi

Ten healthy Norway spruce trees (DBH =  $12.6 \pm 0.75$  cm (mean  $\pm$  SD)) each from a different family, were selected from a stand planted in 1990 at the Hogsmark Experimental Farm in Ås, SE Norway (for details about the stand see (Gebauer et al. 2011)). On June 17, 2014 the trees were prepared for inoculation by removing dead branches from the lower 2 m of the stem. The following day all trees were inoculated with fungus growing on standard malt agar (2% malt and 1.5% agar) or with malt agar as a control into bark wounds made using a 5 mm cork borer (Krokene et al. 2010). Each tree was inoculated with 11 different fungal strains from five different species (Table S1), plus the agar control. Inoculations were made in three rings encircling the stem at 0.9, 1.3 and 1.7 m above ground, with four evenly spaced inoculations per ring. The position of individual strains was randomized between trees to avoid systematic placement or neighborhood effects.

### 2.2. Harvesting and processing of plant material for chemical analysis

Phloem samples were harvested on August 15, 2014, about 8 weeks after inoculation. We carefully removed the outer cork bark over the inoculation sites to expose the necrotic lesions in the inner bark and measured the full length of the lesions. Phloem plugs for chemical analyses were collected using a 5 mm cork borer. Plugs were collected immediately above and below the inoculation wound (two plugs in total per inoculation site). The two plugs were pooled, wrapped in aluminum foil, immediately placed in liquid nitrogen, and stored at  $-80$  °C until analysis. For chemical analyses we selected one strain per fungal species, except for the most virulent species *G. penicillata* for which we included two strains (Table S1).

Samples were ground under liquid nitrogen to a fine powder and lyophilized at 0.34 mbar pressure using an Alpha 1-4 LD plus freeze dryer (Martin Christ GmbH, Osterode, Germany). Approximately 20 mg dried tissue was extracted with 1 mL analytical grade methanol containing 100  $\mu\text{g mL}^{-1}$  chlorogenic acid (Sigma) as an internal standard for 4 hours at 4 °C. The extract was centrifuged at 3200 g and 0.8 mL supernatant was recovered. Insoluble material was re-extracted with 0.6 mL methanol for 16 hours. Supernatants were combined and stored at -20 °C for LC-ESI-MS analysis.

### 2.3. *Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)*

Chromatography was performed on an Agilent 1200 HPLC system. Separation was achieved on a DXB-C18 (4.6  $\times$  50 mm, 1.8  $\mu\text{m}$  particle size) column (Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-1 min, 100% A; 1-7 min, 0-65% B in A; 7-8 min 65-100% B in A; 8-9 min 100% B and 9-10 min 100% A. The total mobile phase flow rate was 1.1 ml  $\text{min}^{-1}$ . The column temperature was maintained at 25 °C.

An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards of all analytes except for isorhapontin, rhapontigenin and taxifolin glucoside for which partially purified plant extracts were used for optimization. The ion spray voltage was maintained at -4500 V. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 10 psi. Multiple reaction monitoring (MRM) was used to monitor analyte precursor ion  $\rightarrow$  product ion fragmentation (See supplemental table S2 for details). Both Q1 and

Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity of compound detection for quantification was verified by external calibration curves (Hammerbacher et al. 2014). The phytohormones jasmonic acid and jasmonate-isoleucine were analyzed using the method described in Ohse et al. (2017).

#### *2.4. Fungal growth on spruce bark constituents and minimal medium amended with selected polyphenols*

To determine fungal growth on spruce constituents, a growth medium was prepared by steam sterilizing water agar (2% w vol<sup>-1</sup>) amended with 5% spruce bark extract prepared by boiling 200 g fresh bark with 1 L (20% stock solution) of water for 20 minutes (for exact chemical profile see Supplemental table S3). Growth medium was dispensed in Petri dishes (Ø=5.2 cm). After the medium solidified, an agar plug (Ø=4 mm) from a 14-day-old fungal culture was placed in the middle of each Petri dish, which was sealed with Parafilm and incubated at room temperature in the dark (n = 5). Fungal growth was measured every 24 hours until growth reached the margins of the Petri dish.

Minimal medium (6 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 4 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub>\*7H<sub>2</sub>O, 1 mg L<sup>-1</sup> CuSO<sub>4</sub>\*5H<sub>2</sub>O, 1 mg L<sup>-1</sup> MnCl<sub>2</sub>, 20 µg L<sup>-1</sup> biotin, 200 µg L<sup>-1</sup> pyridoxine, 200 µg L<sup>-1</sup> thiamine, 2mg L<sup>-1</sup> inositol, 2 mg L<sup>-1</sup> L-arginine and 2% w vol<sup>-1</sup> agar) was amended with 1 mg mL<sup>-1</sup> glucose, astringin, coniferyl alcohol or catechin as organic carbon sources and dispensed in Petri dishes as above. Fungal growth on the different carbon sources was measured as described above (n = 5).



## 2.5. *In vitro* bark beetle choice assays

To investigate the behavior of bark beetles in response to high concentrations of phenolics in the medium a choice test arena was made by mixing 7% spruce inner bark ground to a fine powder with 4% agar and dispensing it in square Petri dishes (120 mm × 120 mm) after heat sterilization (121 °C for 20 min). Five Petri dishes were filled with medium amended with 1 mg/mL catechin dissolved in DMSO before dispensing and five Petri dishes were filled with non-amended medium. Medium in plates were allowed to set and then the medium was cut into two equal pieces. One piece of each plate was removed and replaced with either a catechin-amended piece or a non-amended piece. Each resulting test arena, therefore, contained 50% non-amended medium and 50% catechin-amended medium in two adjoining rectangles. The surface of the medium was scratched gently using a sterile pipette tip to create a rough surface to facilitate beetle movement over the plate. Fungus amended test arenas were made by placing a 4 mm *E. polonica* mycelium plug on the section with catechin and a sterile agar plug on the non-amended section. The plates were then incubated for 3 days before the onset of the experiment. Four bark beetles (equal sex ratios) were introduced into each of four plates with and without fungus. The tunneling preference in each section was recorded after 12 hours and represented as a percentage choice of beetles tunneling in each section.

To study the behavior of beetles in the presence of different fungal species, a single batch of 7% spruce inner bark medium was dispensed into square Petri dishes. Each test arena (Petri dish) was divided into four equal sections. Three sections were inoculated with a 4 mm agar plug colonized by one strain of five different fungal species and on the last section a sterile agar plug was placed as an uninoculated control. Fungi were allowed to colonize the medium for 3 days before bark beetles were introduced. The choice assay was performed in two different

combinations of fungi where the first combination presented a choice between *E. polonica*, *G. europhioides*, *O. bicolor* and uninoculated control (n = 5 replicates with four beetles in each replicate) or the second combination between *E. polonica*, *G. penicillata*, *O. piceae* and uninoculated control (n = 4 replicates with four beetles in each replicate) to adult beetles. The tunneling preferences in each zone were represented as percentage choice of beetles that tunneled in each zone recorded after 12 hours.

Beetles were reared in the laboratory in small spruce logs (Ø 30 cm, length 40 cm) (Schlyter et al. 1987) and newly emerged adult beetles were used for the choice experiments. Prior to introduction into the test arenas, adult beetles were surface sterilized in a disinfectant solution containing 10% bleach and 2% ethanol for 30 seconds and then rinsed three times with clean water. Among the four beetles introduced into each test arena were at least one male and female.

## 2.6. Statistical analysis

All statistical analyses were conducted using the program R ([www.r-project.org](http://www.r-project.org)). Differences between inoculation treatments in necrotic lesion lengths and analyte levels were tested using ANOVA followed by Tukey's post-hoc test using the package *laercio*. Fungal growth rates were determined by using the slope of the linear growth curves in an ANOVA followed by Tukey's post-hoc test. Percentage tunneling behavior of bark beetles in individual test arenas was analyzed using the Kruskal-Wallis non-parametric test.

### 3. Results

#### 3.1. Inoculation of Norway spruce with bark beetle-associated fungi

To determine the virulence of common blue-stain fungi associated with the spruce bark beetle, we inoculated three strains of *E. polonica* and two strains each of *G. penicillata*, *G. europhioides*, *O. bicolor* and *O. piceae* into the inner bark of ~25-year-old Norway spruce trees. The necrotic phloem lesions induced by the different fungi when measured eight weeks after inoculation ranged from 154 mm for *G. penicillata* to 18 mm for *O. bicolor* (Fig. 1). Both *G. penicillata* isolates induced significantly longer lesions than all other species, except for one strain of *G. europhioides*. The lesion formed by the agar control treatment (lesion length: 11 mm;  $p < 0.05$ ) was significantly less than that induced by all other fungi except *O. bicolor* and one isolate of *O. piceae*. Lesion lengths induced by *E. polonica* were not significantly different from those of the two *G. europhioides* isolates or one of the *O. piceae* isolates, but were significantly longer than those induced by *O. bicolor* (Fig. 1).

#### 3.2. Hormonal responses of Norway spruce to different blue-stain fungi

To quantify the defense activation induced in Norway spruce by the different blue-stain fungi, we measured the levels of jasmonic acid and its bioactive form jasmonate-isoleucine in infected phloem tissue using liquid chromatography – tandem mass spectrometry. Combined levels of jasmonic acid and the jasmonate-isoleucine conjugate were on average higher in fungus-infected phloem than in agar-inoculated controls, with the exception of phloem infected by *O. piceae* (Fig. 2). However, jasmonate levels were significantly higher than in the control only in phloem infected by *E. polonica*, *G. europhioides*, and one of the *G. penicillata* strains (p

< 0.05). Jasmonate responses to the second *G. penicillata* strain and *O. bicolor* were highly variable between replicates and did not differ significantly from any of the other treatments.

### 3.3. Phenolic levels in Norway spruce infected by different blue-stain fungi

To determine the impact of different blue-stain fungi on Norway spruce phloem phenolic chemistry, we extracted and quantified phenolics from lesions induced by these fungi. On average, levels of stilbene glucosides, flavonoids and lignans were lower inside the necrotic lesions induced by the different fungal species than in those induced by inoculation of uncolonized agar as a control (Fig. 3). Phenolic levels were also negatively correlated with the virulence measured for the different fungal isolates (Supplemental Fig. 1), as more virulent fungi showed significantly higher phenolic degradation rates than less virulent fungi ( $y = 78.3 e^{(-0.017x)}$ ;  $R^2 = 0.29$ ;  $p < 0.001$ ).

The most striking decline in phenolics in fungal lesions was recorded for the stilbene glucosides astringin, isorhapontin and piceid. These metabolites occurred in significantly lower amounts in lesions induced by the *Grosmannia* species included in this study ( $p < 0.0001$ ). There were no significant differences in stilbene levels in lesions induced by *E. polonica* or the two *Ophiostoma* species relative to the agar control.

The flavonoids taxifolin, catechin and its dimer, proanthocyanidin B1, also occurred at lower concentrations in fungal lesions than in the agar-inoculated controls. But, similar to the patterns observed for the stilbenes, these declines were only significant for the two *Grosmannia* species ( $p < 0.0001$ ).

The phenolic metabolites that were least susceptible to fungal degradation were the lignans, which are coniferyl alcohol dimers. These metabolites declined significantly only in

lesions induced by one of the *G. penicillata* isolates ( $p < 0.0001$ ). Lignans also declined in lesions induced by *G. europhioides* and *E. polonica*, but the reductions were not significant ( $p > 0.05$ ). Phloem inoculated with the two *Ophiostoma* species had similar lignan levels as the control treatment.

### 3.4. Phenolic degradation products in Norway spruce infected by different blue-stain fungi

In order to determine if fungal biotransformation products accumulated in lesions induced by the different fungal stains included in this study, we quantified the stilbene biotransformation products described by Hammerbacher et al. (2013). Astringin lactones and stilbene dimers were found to be too unstable for reliable analyses (data not shown), but the stilbene aglucones resveratrol, piceatannol and rhapontigenin could be detected in all the lesions as well as in the agar-inoculated control treatment (Fig. 4). Quantities of aglucones were higher in fungal lesions than in the control treatments and levels were proportional to fungal virulence ( $y = 49.1 e^{(0.135x)}$ ;  $R^2 = 0.1$ ;  $p < 0.002$ ; see supplemental Fig. 2). However, aglucone levels were significantly higher than the control only in lesions induced by the most virulent *G. penicillata* isolate included in this study ( $p < 0.05$ ; Fig. 4).

### 3.5. In vitro fungal growth on spruce bark extract

Bark beetle-associated blue-stain fungi were grown on agar amended with 5% spruce bark extract to determine their *in vitro* growth rate on a medium containing the typical composition of host phenolics (Table S3). The growth rate of all five fungal species differed significantly from each other ( $p < 0.05$ ) (Fig. 5). *Endoconidiophora polonica* grew fastest, closely followed by the two *Grosmannia* species. The two *Ophiostoma* species included in this

study grew much slower on this medium, but the slightly more virulent *O. piceae* grew faster than *O. bicolor* ( $p < 0.05$ ).

### 3.6. *In vitro* fungal growth on spruce phenolics as the only carbon source

To determine the impact of different phenolics on fungal growth and to test if the fungi could utilize phenolics as a carbon source, a representative compound from each major class of phenolics was offered to different fungal species in the solid minimal medium. The stilbene glucoside astringin and the flavan-3-ol catechin were chosen due to their abundance in spruce phloem, and the phenylpropanoid coniferyl alcohol was included because it is the monomeric subunit of all lignans identified in spruce. The fungal growth rate was measured and normalized to growth rate on glucose as a carbon source.

Fungal growth on glucose differed significantly among all fungi included in this study (Fig. 5;  $p < 0.05$ ). *Ophiostoma piceae* grew fastest on glucose followed by *E. polonica* and *O. bicolor*, while the growth rate of the two *Grosmannia* species was extremely low. Most of the tested fungi grew as well or better on phenolics than on glucose and for some species growth on phenolics differed significantly from that on glucose (Table 1). *Grosmannia penicillata* grew well on astringin and also grew significantly faster on coniferyl alcohol and catechin than on glucose ( $p < 0.05$ ), while the growth rate of *G. europhioides* on phenolics was similar to that measured on glucose. *Endoconidiophora polonica*, on the other hand, grew faster on media containing coniferyl alcohol, which is mainly found in wood, than on glucose but this result was statistically non-significant ( $p = 0.10$ ). The two *Ophiostoma* species showed opposing growth trends on phenolics, where *O. bicolor* grew faster on catechin and astringin ( $p < 0.05$ ), whereas *O. piceae* had similar growth rates on all phenolics and glucose ( $p = 0.16$ ).

### 3.7. Influence of phenolic content and fungal identity on bark beetle tunneling behavior

In order to determine if higher phenolic content influences the behavior of *I. typographus*, we conducted a choice assay, where beetles were released onto an arena with similar-sized squares of catechin-amended and non-amended bark agar. Catechin was chosen, because it is abundant in spruce phloem, easy to access and is highly induced in spruce trees after simulated bark beetle attack (Hammerbacher et al., 2014). Catechin was also shown to be detoxified by *E. polonica* previously (Wadke et al., 2016). Beetles chose to tunnel into the non-amended agar over the catechin-amended agar ( $p = 0.04$ ), while approximately 20% of the beetles made no choice in these assays (Fig. 6A). However, the catechin-amended agar became highly attractive to bark beetles with a fungal associate, *E. polonica*, growing on it ( $p = 0.002$ ), while no beetles chose the non-amended sterile agar in this assay (Fig. 6B; Supplemental Table S4).

In a second experiment, assays were conducted to study if *I. typographus* prefers to tunnel into medium colonized by specific species of blue-stain fungi. A choice assay was conducted where we released beetles onto bark agar colonized by different fungi and recorded the beetles' tunneling behavior. Most beetles preferred to tunnel into medium colonized by a fungus over the sterile control, but only *E. polonica* was statistically more attractive than other treatments in one of the two assays conducted (Fig 7C,  $p < 0.046$ ). The beetles also tunneled into medium colonized by *O. bicolor*, *G. penicillata* and *G. europhioides*, but this occurred more rarely than for *E. polonica* (Fig. 7; Supplemental table S5). Beetles never tunneled into bark medium colonized by *O. piceae* or bark medium without fungus.

#### 4. Discussion

Most bark beetles colonizing conifers are accompanied by specialized fungi that the beetles inoculate into the host tree. The spruce bark beetle, *I. typographus*, carries numerous fungal associates, including species from the genera *Endoconidiophora*, *Ophiostoma* and *Grosmannia* that infect the phloem and sapwood of their common host Norway spruce, *Picea abies* (Jankowiak et al. 2009; Kirisits 2010; Persson et al. 2009; Repe et al. 2013; Viiri and Lieutier 2004; Yamaoka et al. 1997). Depending on the geographical region and timing of attack, the spruce bark beetle is associated with different assemblages of these fungi (Kirisits 2004). Recent studies showed that *E. polonica*, the best-known fungal associate of the spruce bark beetle, degrades phenolic compounds produced in Norway spruce as a defense against beetle and fungal invasion, and even utilizes phenolics as a carbon source (Wadke et al. 2016). However, it was not known if phenolics are deterrent to bark beetles and if other bark beetle-associated fungi also can degrade spruce phenolic defenses. In this study we inoculated Norway spruce trees with different strains of the most consistent fungal associates of the spruce bark beetle, analyzed the phenolic contents in the lesions induced by these fungi and measured fungal virulence. To determine if tree metabolites may be used as carbon sources by the fungi we performed *in vitro* feeding assays on different media. Finally, to study if fungal metabolism of spruce defense compounds elicits a positive or negative response in bark beetles, we conducted behavioral assays under controlled conditions. In addition to *E. polonica* which was shown previously to degrade these compounds, our results showed that the two *Grosmannia* species but not the *Ophiostoma* species, had significant abilities to degrade spruce phenolics. In fact, the *Grosmannia* species degraded phenolics more efficiently than *E. polonica* and were more virulent towards Norway spruce. Finally, we showed that bark beetles are deterred by catechin, a



common spruce phenolic, but that in the presence of beetle-associated fungi, medium with high phenolic content became attractive. These data demonstrate that multiple fungal associates of the spruce bark beetle can metabolize spruce defenses and thereby elicit positive responses from beetles.

#### 4.1. *Virulent fungi degrade spruce defense metabolites more rapidly*

The necrotic lesions induced in spruce phloem and sapwood by fungal infection mirror the active tree defense response, as the necrotic tissues are impregnated with chemical defenses such as terpenoid oleoresins and phenolic compounds (Franceschi et al. 2005). It is generally agreed that longer lesions develop in response to infection by more virulent fungi, which induce a stronger defense reaction in the host (Krokene and Solheim 1998a; Solheim 1992b). Studies on fungus-tree-beetle interactions over the last 30 years have focused strongly on *E. polonica*, as it has been considered to be the most virulent and therefore most important fungal associate of the spruce bark beetle (Krokene 2015). However, our comparisons of two or more strains of multiple fungal species revealed that *G. penicillata* strains can induce significantly longer phloem lesions than strains from other common beetle associates, including *E. polonica*. It is, therefore, possible that other beetle-associated fungi, which have been largely neglected for the last three decades, fulfill similar roles as *E. polonica* in metabolizing tree defenses during bark beetle host colonization and brood production. The different fungi associated with the spruce bark beetle may thus form a redundant and interchangeable community where different species may be important to the beetle in different regions or during different population phases.

Inoculation with *E. polonica* elicits many defense responses in Norway spruce, including the biosynthesis of terpenoid oleoresins and phenolic compounds (Franceschi et al. 2005; Zhao et

al. 2010; 2011). These defense responses are triggered by an accumulation of the defense hormone jasmonic acid and its derivatives (Wasternack et al. 2006). Direct treatment of spruce trees with methyl jasmonate has also been shown to trigger the accumulation of defense metabolites and the formation of additional resin ducts and phloem parenchyma cells necessary for storage of these *de novo* synthesized compounds (Erbilgin et al. 2006; Franceschi et al. 2005; Krokene 2015). We quantified jasmonic acid and jasmonic acid-isoleucine levels in fungal lesions to determine whether inoculation with different beetle-associated fungi triggered the accumulation of these defense hormones. Compared with the control, we found significantly higher jasmonate levels in phloem inoculated with *G. penicillata*, *G. europhioides* and *E. polonica*, the three most virulent fungal species used in this study.

Due to the up-regulation of defense hormones, we also expected to find increased concentrations of phenolics and other defense metabolites in phloem colonized by these fungi. However, despite extensive necrotic lesion formation and elevated jasmonate levels in fungus-colonized phloem, we observed a marked decrease in stilbenes, flavonoids and lignans compared to phloem treated with agar as a control. In fact, we found a significant negative correlation between lesion length and the concentration of total phenolics (Supplemental Fig. 1), with lower phenolic levels being found in longer lesions. An explanation for this pattern could be that virulent fungi metabolize phenolic compounds faster and more efficiently than non-virulent fungi. The hypothesis that virulent fungi are more proficient degraders of tree defense metabolites is further strengthened by the positive correlation we observed between lesion length and the amount of stilbene aglucones, which are known products of fungal degradation of spruce stilbene glucosides (Hammerbacher et al. 2013). This hypothesis is also supported by previous research demonstrating that *E. polonica* metabolizes phenolics in Norway spruce phloem at a

higher rate than the trees can produce them (Hammerbacher et al. 2013). In our study, strains of *G. penicillata* were the most virulent of those tested, and also metabolized spruce phenolics more rapidly than strains of the non-virulent species *O. piceae* or *O. bicolor*. Our results further suggest that *G. penicillata* is even more effective than *E. polonica* in degrading spruce-derived phenolic compounds.

#### 4.2. Bark beetle-associated fungi can utilize host phenolics as carbon sources

In a previous study *E. polonica* was found not only to degrade phenolic host defense compounds but also to use them as a carbon and energy source for growth and other cellular processes (Wadke et al. 2016). In order to investigate if other bark beetle associated fungi also utilize phenolics as carbon sources, we compared their growth on glucose to growth on spruce bark extract. *E. polonica* and the two *Grosmannia* species included in this study grew much better on spruce extract than on glucose, suggesting that these species require host metabolites for optimal performance. The two studied *Ophiostoma* species, while also growing faster on spruce extract than on glucose, outperformed the *Grosmannia* species on glucose, suggesting that spruce metabolites are less important for their growth and development. Interestingly, *E. polonica* outperformed the *Grosmannia* strains on both types of medium, but grew more poorly in living host phloem. This suggests that *E. polonica* may be less adapted to face the induced chemical defenses in Norway spruce phloem than the *Grosmannia* strains included in this study. Solheim (1991) also showed that induced resin chemicals caused almost complete inhibition of *E. polonica* growth in an *in vitro* assay.

We then determined the ability of the different fungal associates of the spruce bark beetle to grow on the three major phenolic metabolites in spruce (astringin, coniferyl alcohol and

catechin) when offered to fungi in artificial medium. Both *G. penicillata* and *O. bicolor* grew significantly better on astringin, the major phenolic compound present in spruce phloem than on coniferyl alcohol, the major subunit of lignin in conifer wood. This difference is in agreement with the typical location of fungal infection for these species. *Grosmannia* and *Ophiostoma* species are usually observed in the phloem and cambial layer around the beetle gallery. On the other hand, *E. polonica* grew similarly on all three compounds and lives in both the phloem and the wood, colonizing the wood of infected trees after an initial growth phase in the phloem (Solheim 1992a; Solheim 1992b). Within the phloem, *E. polonica* tends to be outcompeted by other phloem-colonizing blue-stain fungi (Solheim 1992a; Solheim 1992b). Thus overall, the competitive advantage of different blue-stain fungi in the phloem might be due to underlying differences in how the species utilize host defense metabolites.

#### *4.3. Bark beetles are deterred by phenolics, and choose fungus-colonized medium more frequently than uncolonized medium*

In a choice assay, bark beetles avoided an artificial diet amended with higher concentrations of catechin, a common phenolic occurring in high concentrations in Norway spruce phloem. Similar results were also observed by Faccoli and Schlyter (2007) with other Norway spruce phenolics. This evidence suggests that phenolic compounds could be anti-feedants for *I. typographus*. Interestingly, the bark beetles preferred the high-phenolics diet when it was colonized by *E. polonica*, providing evidence that fungal degradation of these compounds has a positive effect on bark beetle tunneling behavior. When beetles were allowed to select among bark agar with natural concentrations of spruce phenolics, but colonized by different blue-stain fungi, they also clearly avoided fungus-free bark agar and a medium that was colonized by the saprophytic *O. piceae*. The fact that beetles actively chose diets colonized by

fungi demonstrates that fungal colonization is not perceived by adult beetles as antagonistic, except for species like *O. piceae* (Six and Wingfield 2011). Our data also show that bark beetles did not prefer medium colonized by the fungi that were most efficient in catabolizing host defense compounds (Hammerbacher et al. 2013), suggesting that an intermediary level of host compound degradation might be sufficient. Instead, other fungal traits might be more important for the beetles, such as fungal production of volatiles (Kandasamy et al. 2016; Zhao et al. 2015) or essential nutrients (Bentz and Six 2006; Bracewell and Six 2014; Hofstetter et al. 2006). However, it has never been shown that bark beetles can actively choose their fungal associates in nature. Thus the full relevance of the laboratory assay is not known.

In addition to degrading tree defenses and providing nutrients, associated fungi may benefit bark beetle larvae by hastening tree death. *Grosmannia* species, which are very effective colonizers of spruce phloem and cambium, cause long necrotic lesions that will eventually lead to tree mortality. On the other hand, tree death may be more rapid with *E. polonica*, which invades the sapwood and disrupts water transport. From the beetle's perspective, the importance of rapid tree killing is not clear, and it may be most critical to block trees from producing induced resins that can kill eggs and larvae (Krokene et al. 2013).

#### 4.4. Conclusion

The assemblage of fungal species associated with the spruce bark beetle may be highly variable in time and space (Solheim 1992a; Krokene and Solheim 1996; Jankowiak et al. 2009; Kirisits 2010; Persson et al. 2009; Repe et al. 2013; Viiri and Lieutier 2004; Yamaoka et al. 1997). The fact that this beetle seems to have no obligate associations with specific fungal species has been taken as evidence that fungi are not important for the successful completion of

the beetle life cycle (Six and Wingfield 2011). In this study we have shown that several common fungal associates of the spruce bark beetle can all degrade host phenolic compounds and use them as carbon sources, and that the efficacy of degradation depends on fungal virulence. This result suggests that different assemblages of fungal species may play similar roles in promoting successful bark beetle reproduction and that the relationship between the beetle and individual fungal associates may be loose. Results from *in vitro* bioassays showed that bark beetles avoided food sources containing high levels of unmodified phenolics, but chose these food sources in the presence of *E. polonica*. Interestingly, *I. typographus* did not show a particular preference for virulent fungi with higher potential for degrading host defense metabolites. Taken together our results suggest several ways in which associated fungi may be important for bark beetle survival and persistence in the host. Future studies are needed to elucidate the most important ways and to determine if assemblages of different fungal species are more beneficial than a single species.

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## Tables:

Table 1. Percentage growth of bark beetle-associated blue-stain fungi on minimal growth media amended with different spruce metabolites relative to growth on minimal media with glucose as the sole carbon source (n = 3; <sup>b,c</sup> p < 0.05 between different substrates (ANOVA)).

	Astringin	Coniferyl Alcohol	Catechin
<i>Grosmannia penicillata</i> (1)	480 <sup>a</sup>	222 <sup>b</sup>	247 <sup>b</sup>
<i>G. europhioides</i>	112 <sup>a</sup>	116 <sup>a</sup>	89 <sup>a</sup>
<i>Endoconidiophora polonica</i>	98 <sup>a</sup>	122 <sup>a</sup>	91 <sup>a</sup>
<i>Ophiostoma piceae</i>	108 <sup>a</sup>	104 <sup>a</sup>	103 <sup>a</sup>
<i>O. bicolor</i>	102 <sup>a</sup>	86 <sup>b</sup>	114 <sup>c</sup>

## Figure legends:

Fig. 1. Lesion lengths induced by fungal associates of the European spruce bark beetle *Ips typographus* in Norway spruce phloem eight weeks after inoculation (n=10; ANOVA, Tukey's post-hoc test: p < 0.05). Control treatments were wounded and inoculated with agar without fungus. Each bar represents data from one fungal isolate. Blue bars represent isolates that were included in the chemical analyses. *Ep* = *Endoconidiophora polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata*, *Ge* = *G. europhioides*, *Op* = *O. piceae*. See Supplementary Table S1 for details on the different fungal isolates.

Fig. 2. Jasmonic acid and jasmonic acid isoleucine content of Norway spruce phloem eight weeks after inoculation with bark beetle-associated blue-stain fungi (n=10; ANOVA, Tukey's post-hoc test: p < 0.05). *Ep* = *Endoconidiophora polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata*, *Ge* = *G. europhioides*, *Op* = *O. piceae*.

Fig. 3. Total stilbene glucoside, flavonoid and lignan content in Norway spruce phloem 8 weeks after inoculation with bark beetle-associated blue-stain fungi (n=10; ANOVA, Tukey's post-hoc test: p < 0.05). *Ep* = *Endoconidiophora polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata*, *Ge* = *G. europhioides*, *Op* = *O. piceae*.

Fig. 4. Accumulation of stilbene aglucones (degradation products of stilbene glucosides) in Norway spruce phloem 8 weeks after inoculation with bark beetle-associated blue-stain fungi (n=10; ANOVA, Tukey's post-hoc test:  $p < 0.05$ ). *Ep* = *Endoconidiophora polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata*, *Ge* = *G. europhioides*, *Op* = *O. piceae*.

Fig. 5. Growth rate of bark beetle-associated blue-stain fungi on spruce extract or on minimal medium with glucose as a carbon source (n=5; ANOVA, Tukey's post-hoc test:  $p < 0.05$ ). *Ep* = *Endoconidiophora polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata* (GP1), *Ge* = *G. europhioides*, *Op* = *O. piceae*.

Fig. 6: Choice assay using adult bark beetles and spruce inner bark agar supplemented with catechin (1 mg/ml). (A) Adult beetles made feeding tunnels only in bark agar where no catechin was added (Kruskal-Wallis chi-squared = 6.39,  $df = 2$ ,  $P=0.040$ ). (B) The feeding inhibition in the presence of catechin was completely abolished when catechin-supplemented bark agar was colonized by the blue stain fungus *Endoconidiophora polonica* (Kruskal-Wallis chi-squared = 11.76,  $df = 2$ ,  $P=0.002$ ; *Ep* = *E. polonica*, w/o = without, No choice = percentage of beetles that did not tunnel into any sections).

Fig. 7. (A) Four-choice assay setup showing adult beetles tunneling actively in *Endoconidiophora polonica* colonized agar as evident from the frass. (B) Box plot (thick line: median, boxes: first quartile, error bars: third quartile) showing bark beetle tunneling into uncolonized bark agar (Control) or bark agar colonized by different bark beetle-associated blue-stain fungi in 4-choice assays (Kruskal-Wallis chi-squared = 13.043,  $df = 6$ ,  $p = 0.042$ ). *Ep* = *E. polonica*, *Ob* = *Ophiostoma bicolor*, *Gp* = *Grosmannia penicillata* (GP1), *Ge* = *G. europhioides*, *Op* = *O. piceae*.