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Relationships between Swiss needle cast and ectomycorrhizal fungus diversity

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Abstract: Swiss needle cast (SNC) is a disease specific to Douglas-fir (Pseudotsuga menziesii) caused by the ascomycete Phaeocryptopus gaeumannii. Here we examine characteristics of the EM fungus community that are potentially useful in predictive models that would monitor forest health. We found that mean EM density (number of colonized root tips/soil core) varied nearly 10-fold among sites of varying levels of SNC, while mean EM fungus species richness (number of species/soil core) varied by about 2.5 times. Strong relationships were found between EM and SNC parameters: EM species richness was positively correlated with both Douglas-fir needle retention ($R^2 = 0.93$) and EM density ($R^2 = 0.65$); EM density also was significantly correlated with Douglas-fir needle retention ($R^2 = 0.70$). These simple characteristics of the EM fungus community could be used to monitor forest health and generate predictive models of site suitability for Douglas-fir. Based on previous findings that normally common EM types were reduced in frequency on sites with severe SNC, we also hypothesized that some EM fungi would be stress tolerant-dominant species. Instead, we found that various fungi were able to form EM with the stressed trees, but none were consistently dominant across samples in the severely diseased areas.

Key words: anthropogenic disturbance, climate change, ecosystem health, Phaeocryptopus gaeumannii

INTRODUCTION

Swiss needle cast (SNC) disease is specific to Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) and is caused by the ascomycete Phaeocryptopus gaeumannii (T. Rohde) Petr. (Gaümann 1930 in Stone et al. 2008). SNC affects the foliage of Douglas-fir: symptoms include chlorotic needles and premature needle abscission, resulting in reduced leaf area index and vigor (Manter et al. 2000, McGuire et al. 2002, Weiskittel and McGuire 2007). Phaeocryptopus gaeumannii is native to the western United States and presumably was introduced in Europe, New Zealand and elsewhere via import of live Douglas-fir seedlings (Stone et al. 2007). It was the initial discovery of the disease in Douglas-fir plantations in Switzerland in the mid-1920s that engendered the name of the disease (Gaümann 1930). Until the mid-1980s, P. gaeumannii was considered an unimportant and minor pathogen (Boyce 1940, Hansen et al. 2000). Under most conditions, P. gaeumannii is a benign component of the tree canopy and does not contribute to premature needle shed or growth loss. Nevertheless, since the late 1980s and early 1990s SNC has caused an epidemic affecting hundreds of thousands of acres, across a wide variety of soil conditions, in the western Oregon Coast Range from Coos Bay to Astoria. Annual aerial surveys conducted by the Oregon Department of Forestry have shown a consistent increase in affected areas since the surveys began in 1996. The 2011 aerial survey mapped more than 160 000 ha with symptoms of SNC. Most diseased trees occurred within 29 km of the Pacific Ocean, but SNC symptoms extended up to 40 km inland (Kanaskie and McWilliams 2011). Stone et al. (2008) note the strong correlation of P. gaeumannii abundance and disease severity with climatic factors, particularly winter mean temperature. Growth losses in the area of epidemic infection generally are 20–50%, and annual growth losses are estimated to exceed $200 000 000 per year (Maguire et al. 2002).

It is important to consider the potential links between ectomycorrhizal (EM) fungi and SNC. Ectomycorrhizal fungi are essential for host plant nutrient uptake and play important roles in nutrient cycling in many forests (Cromack et al. 1979, Smith and Read 2008). For example, an estimated 50–70% of the net annual productivity may be translocated to roots and associated mycorrhizal fungi (Fogel and Hunt 1979, Vogt et al. 1982, Norton et al. 1990). Markkola et al. (2004) found reduced carbon allocation to roots after 100% defoliation and that fungal biomass in fine roots decreased when defoliation occurred in the year of harvest but not when the defoliation was conducted the previous year. They concluded that current photosynthates are particularly important for EM fungal symbionts.

Most of the dominant and economically important timber species in the Pacific Northwest are EM-dependent, including all members of the pine, oak and birch plant families (Smith and Read 2008). Douglas-fir alone is thought to have about 2000 EM fungal symbionts throughout its range (Trappe 1977) and appears unable to grow in soil without ectomycorrhizal fungi (Trappe and Strand 1969).
In a 2006 pilot study we found that Douglas-fir forests with moderate to high SNC (as measured by foliage retention) had 3–4 times fewer EM fungus species than similar Douglas-fir stands without appreciable SNC (Eberhart et al. 1996, Luoma et al. 2006) and had numbers of EM species comparable to that of sites that had experienced significant disturbance from clear-cutting and fire (Luoma and Eberhart 2006). We concluded that the degree of EM fungus diversity detected in the pilot study suggested that the belowground ecosystem was being strongly affected by SNC, alone or in combination with the previous removal of mature trees during timber harvest and postharvest silvicultural practices.

Here we present work that was undertaken to provide better understanding of relationships between EM fungus diversity and SNC in northwestern Oregon. We assumed the EM fungus community structure varied substantially across the landscapes in which SNC is found for many potential reasons, particularly those tied to variations in climate and soil conditions. We hypothesized that in comparing sites of similar age, structure and management history across a gradient of SNC disease, EM root-tip density and EM fungus species richness (at the scale of the soil core) would be inversely correlated with SNC severity (mean needle age cohorts, measured in years) due to reduced photosynthetic capacity accompanying increasing disease.

In addition, preliminary data suggested that some EM fungi were tolerated stress (sensu Grime 1979) because common Douglas-fir EM types formed by genera such as *Cenococcum* and *Rhizopogon* were less widespread in SNC-stressed stands than in other studies (Eberhart et al. 1996, Luoma et al. 2006). Because the diseased trees were mycorrhizal, albeit at low EM root-tip densities, we hypothesized that certain EM fungi (with important functional roles to keep Douglas-fir alive in the face of heavy SNC) may become more dominant on the roots that remain. Therefore we examined a second hypothesis that particular stress-tolerant EM fungi would be found as dominant members of the EM community. Specifically we hypothesized that (i) common mycorrhiza types of non-SNC affected trees, as measured by mean number of root-tips/soil core, will be less common on high SNC sites; (ii) A limited number of stress-tolerant fungi, possibly adapted to a reduced carbon supply, will be relatively more common, as measured by mean number of EM root-tips/soil core, on high SNC sites.

**Materials and Methods**

**Field sites.**—The study sites (Fig. 1) varied by degree of SNC disease symptoms (Table I), and disease severity was measured by use of a needle retention index (Hansen et al. 2000). Stands were similarly managed, un-thinned and unfertilized Douglas-fir plantations that were about 20–25 y old at the time of sampling. Additional site information can be found in Stone et al. (2005), Mainwaring et al. (2007) and Mulvey et al. (2013). Foliage retention (mean needle age cohorts, measured in years) has been found to work well for predicting tree and stand growth (Maguire et al. 2002, 2011).

Study trees had been mapped and numbered by other researchers (Stone et al. 2005, Mainwaring et al. 2007, Mulvey et al. 2013). EM roots of Douglas-fir were obtained from 350 cc soil cores, 15 cm × 5.5 cm. At each site one soil core was taken from beneath the canopy of each of 10 trees chosen randomly from the list of prenumbered trees. Soil cores were located approximately 1 m from the base of each tree and on the side of the bole closest to the nearest neighboring Douglas-fir, so as to bias for maximum Douglas-fir root density. A total of 70 soil cores were obtained (10 trees/site × 7 sites) in Feb 2007. We also sampled two sites in 2008 that included the most severely diseased site, GDH, as well as another heavily affected site (Swede Hill) near GDH (Table I).

**Laboratory.**—Soil cores were kept cool in the field and stored at −20°C immediately after returning from the field.
When soil cores were thawed they were examined within 48 h. Roots were extracted from the soil cores by wet sieve-washing over a 1 mm grate. The contents of the sieve were spread evenly, with sufficient water to cover, in the bottom of a 38 × 17 × 2 cm tray that was divided into 36 compartments by an inserted Plexiglas partition (Eberhart et al. 1996). Roots were examined with a stereomicroscope at 15–30× magnification. Each EM type encountered was classified by morphological characteristics similar to those described in Ingleby et al. (1990) and Goodman et al. (1996) including color, texture, presence/absence of rhizomorphs and emanating hyphae. Morphotype identities were determined by comparison to the EM character database maintained by J. Eberhart (unpubl), some of which had been determined to genus or species by DNA sequencing (Luoma and Eberhart 2006). The total number of EM types per soil core and total number of mycorrhizal root tips in each core were recorded for 10 soil cores from seven sites in 2007 and two sites in 2008. Representative samples (a minimum of two tips each) of the predominant mycorrhiza types were saved in CTAB buffer for molecular analysis of the fungal DNA.

**Molecular analysis.**—For the most common EM morphotypes in the 2008 samples, we extracted DNA following the method described by Avis et al. (2003) with a plant DNA extraction kit (REDExtract-N-Amp Plant PCR Kit, Sigma, St Louis, Missouri). The ITS region (ITS1, 5′-8S and ITS2) of the rRNA operon was amplified with primer set ITS1-F/ITS4 (White et al. 1990, Gardes and Bruns 1993). PCR was performed with the REDExtract-N-Amp Plant PCR Kit with PCR cycling conditions modified from Gardes and Bruns (1993). Cycling conditions included an initial denaturation at 94°C for 3 min followed by 35 PCR cycles (93°C, 35 s; 55°C, 53 s; 72°C, 30+0.5 s per cycle) and 72°C for 10 min. Amplified ITS products were visualized in 2% agarose gels stained with ethidium bromide under UV illumination. For sequencing, samples producing a single PCR product were purified before sequencing with ExoSAP-IT (USB, Cleveland, Ohio). Sequencing was performed by the Center for Genome Research and Biocomputing Core Laboratory at Oregon State University with an ABI Prism 3730 genetic analyser (Applied Biosystems, Foster City, California) with the forward primer ITS1F. Sequences were identified by querying the GenBank database with the nucleotide-nucleotide (BLASTN) query option on the National Center for Biotechnology Information website (Altschul et al. 1997). We set a functional species match criterion at 98% sequence similarity over at least 500 bp with corresponding sporocarp sequences, although not all matches at that level of similarity had corresponding species determination. Taxon assignment of EM fungi to our morphotypes was made with the BLASTN results. These were added to the EM morphotype identities in the EM character database maintained by J. Eberhart. Our manually trimmed sequences were submitted to GenBank (TABLE II).

**Statistical analysis.**—Number of EM types per soil core (species richness) and total number of EM tips per soil core (feeder root density) were used as response variables. The data were used to test for gradient responses to SNC disease.
ANOVA was used for comparisons across the sites. When appropriate, Fisher’s protected least significant difference test was used to determine whether study site means differed from one another. Linear regression was used to examine gradient responses in EM density and EM fungus species richness to among-block variation in SNC severity (foliage retention). Linear regression also was used to measure the association between EM density and EM fungus type (species) richness. To address the second hypothesis, we examined the mean number of EM root-tips/soil core and constancy of EM types across soil cores on high SNC sites from the 2008 dataset. When necessary to meet the assumptions of normality and constant variance (Sabin and Stafford 1990), we transformed the dependent variables; EM density was square-root transformed, EM fungus richness was log-transformed. Analyses were carried out with StatView 5.1 software (SAS Institute 1999).

RESULTS

Fewer than 1 in 10,000 root tips were non-EM. Mean EM root density varied by nearly 10-fold among sites (TABLE I), while mean EM type (species) richness varied by about 2.5 times (TABLE I) in 2007. In the 2008 sample, these response variables were nearly identical for richness and root density (TABLE I) at the GDH and GDS sites; therefore the GDS data were not included in the regressions. Treating mean logEM richness as a dependent variable of mean EM density produced a regression model of: 

$$Y = 0.417 + 0.001 * X; R^2 = 0.65, P = 0.03$$

(FIG. 2). Mean square-root EM density treated as dependent on mean years needle retention produced a regression model of 

$$Y = -1.583 + 5.932 * X; R^2 = 0.70, P = 0.02$$

(Fig. 3). For the main 2007 sample, mean log EM richness treated as dependent on mean years needle retention produced a regression model of 

$$Y = 0.002 + 0.232 * X; R^2 = 0.90, P = 0.001$$

(Fig. 4). When data from the 2008 sample of the GDH site were added to that model, the EM richness vs. years needle retention model improved to 

$$Y = 0.023 + 0.226 * X; R^2 = 0.92, P = 0.0001$$

(Fig. 4).

From the 20 soil cores examined in the 2008 Green Diamond sample, a total of 31 EM types were identified by morphotyping and molecular methods. Fifteen of the 31 types were assigned to taxa with ITS sequencing (TABLE II). Twenty-four of the 31 EM types found at the Green Diamond site were recorded from one soil core each. An additional four EM types were recorded from only two soil cores each. Only one EM type (Cenococcum geophilum) was regularly represented in the Green Diamond soil cores with 40% constancy (8 of 20 cores). The Lactarius luculentus morphotype was found in four soil cores and was confirmed by ITS sequencing of samples from two soil cores. The L. luculentus type matched...
(100% similarity) within each core and between the cores. Two EM types that were tentatively assigned to the *L. luculentus* type based on morphology later were found to be different taxa when sequenced (*Tylospora* sp. and *Hydnotrya* sp.). Among EM morphotypes found in two different cores each, matches to their own types in the other core were at least 98% similar for four of the types. Two distinct single-core EM morphotypes matched at the family level (83–88%) between two soil cores. The eight

![Regression plot of mean EM type (species) richness (log transformed) against mean root density](image1)

*Fig. 2.* Regression plot of mean EM type (species) richness (log transformed) against mean root density ($R^2 = 0.65$, $P = 0.03$, per site $n = 10$).

![Regression plot of mean EM root density (square-root transformed) against mean years needle retention](image2)

*Fig. 3.* Regression plot of mean EM root density (square-root transformed) against mean years needle retention ($R^2 = 0.70$, $P = 0.02$, per site $n = 10$).
other single-occurrence EM morphotypes were distinct and did not match the other types, even at the family level based on the ITS sequences. Those results (13/15) produced a success rate of 87% at correctly discerning species-level morphotypes.

DISCUSSION

The hypothesis that EM density and species richness are correlated with SNC severity was supported. Across a gradient of SNC disease (as measured by mean years of needle retention), EM root-tip density and EM fungus species richness varied by factors of nearly 10 and 2.5 respectively. That EM fungus species richness was highly correlated with EM root-tip density is not surprising in that the root tips represent available habitat for EM fungi. The reductions in EM characteristics that we measured did not reveal threshold responses (Figs. 2–4) to the amount of needle loss. Maguire et al. (2011) studied growth reductions in Douglas-fir stands across a gradient of SNC disease and similarly did not find threshold responses to needle loss. Their model predicts a growth loss of approximately 35% on our most severely diseased site (Green Diamond).

The 2007 data demonstrated a strong correlation between EM species richness and Douglas-fir needle retention ($R^2 = 0.90$). Addition of the 2008 GDH data was made to test the scope of inference of the model over time, especially at severely diseased sites. This allowed for the possibility that the model would show more variance. However, the results strengthened that correlation (Fig. 4) and the $R^2$ value increased to 0.93, which increases our confidence in the robustness of our model. In severely diseased SNC areas, the low values of EM root density that we observed were less strongly correlated with needle retention than was EM species richness. Even though 2008 root density was twice that observed in 2007 on the Green Diamond Hemlock site (but still low), species richness in 2008 was slightly lower. The 31 EM types (species) obtained from 20 soil cores contrasts with an expected number of 60–65 species found in 20 soil cores (of the same volume) from mature Douglas-fir stands in the Cascade Range not affected by SNC (Luoma et al. 2006 and unpubl data).

Despite much reduced EM fungus species richness on severely diseased SNC sites, we found that the proportion of the Douglas-fir root tips colonized by EM fungi remained at almost 100%. These results agree with the findings of Saikkonen et al. (1999), Cullings et al. (2005), and Kuikka et al. (2003), all of whom noted little response of EM colonization (proportion of root tips colonized) to defoliation. Our results also support the theoretical model proposed by Gange (2007) in which he predicted that mycorrhizal species richness would decline in the face of moderate to severe defoliation.
Our second hypothesis was that some EM fungus species could be more tolerant of a reduced carbon supply and therefore exhibit increased relative abundance in severely diseased SNC sites. However, our results did not support this conclusion. Instead, various fungi were able to form EM with the stressed trees, with none consistently dominant across samples in the most severely diseased areas: the majority of distinct EM types (24 of 31) were found in only one core, and an additional four EM types were found in only two cores each. Thus no single EM type was dominant; only the *Cenococcum geophyllum* type was regularly represented in the soil cores (40% constancy, 8/20 cores). *Cenococcum geophyllum* (likely a species complex [Douhan et al. 2007]) is ubiquitous in PNW forests dominated by ectomycorrhizal trees. The next most common EM type was identified from its ITS sequence as *Lactarius luctulentus* and was found with only 20% constancy (4/20 soil cores).

Neither of these EM types is considered a candidate for our concept of a stress-tolerant species (sensu Grime 1979) that would dominate a SNC site (i.e. a combination of relatively high constancy across cores and mean root-tip abundance within cores). The *Cenococcum* type has been reported as occurring with 97–100% constancy in soil cores from our region (Kolaczkowski 2005, Luoma et al. 2006) but its presence on severely diseased trees in this study is greatly reduced from that found in healthy Douglas-fir forests.

An alternative to the second hypothesis is that these results are consistent with a “survival of the survivors” scenario. We propose that, while the diversity of EM fungi decreases as SNC disease progresses, particular survivors (albeit not dominant) may reflect patterns of reverse establishment (i.e. community disassembly). With this hypothesis, the remaining EM species supporting Douglas-fir growth on these sites are able to persist simply due to variation in tolerance to SNC-caused decline (e.g. reduced carbohydrates) and the ability of EM fungi to (re-)colonize roots of trees affected by SNC. When compared to stands with little or no SNC, aggregate species richness (approximately a 50% reduction; data not shown) was not affected as severely as local, soil-core species richness (70–80% reduction, Table I and Luoma et al. 2006). Avis et al. (2008) note that variables such as species richness may demonstrate different responses to treatments in a soil core when contrasted with responses at a stand. The highly patchy soil environment may provide opportunities for particular EM species to persist due to each species’ unique adaptive advantages in a given location. For instance, *Cenococcum* was able to maintain a well-distributed presence across soil cores in the severely diseased stands but it was not dominant, in terms of constancy or abundance, to the extent that would be expected in healthy stands.

Although particular EM fungi did not become dominant, we did find some distinctive responses of EM fungi to SNC. Defoliation, presumably due to SNC, was strongly associated with a reduction in mycorrhizae of *Cenococcum geophyllum* (8/20 cores), *Rhizopogon villosulus* (two cores), *Lactarius rubrilacteus* (zero cores), *Rhizopogon vinicolor* (zero cores), and *R. vesiculosis* (zero cores). These species have been found to be common EM associates of Douglas-fir in other studies (Luoma et al. 2006, Beiler et al. 2010). It is of interest to note that these species are thick-mantled EM types and their reduction in defoliated systems is similar to findings of Markkola et al. (2004) who noted that the most marked decline in thick-mantled mycorrhizae occurred with repeatedly defoliated seedlings. They concluded that carbon limitation strongly influenced EM fungus composition but not percentage of root tips colonized, which was maintained at about 97% (Markkola et al. 2004), similar to our study. Cullings et al. (2005) also noted that a *Cenococcum* sp. was significantly reduced (in sequence-based assessments of soil samples) by artificial defoliation of pine.

In addition to reduced carbon allocation to roots after 100% defoliation, Markkola et al. (2004) found that fungal biomass in the fine roots decreased when 100% defoliation occurred in the year of harvest but not when the defoliation was conducted the previous year. They concluded that current photosynthates are particularly important for EM fungal symbionts. Although not examined in our study, the initial response of EM fungi to reduced photosynthetic might be a reduction in reproductive capacity. In an experiment to simulate pine sawfly (*Neodiprion sertifer*) herbivory, Kuikka et al. (2003) removed all 1 y old needles of *Pinus sylvestris* in each of two successive years and found that EM fungi reduced investment in reproduction (sporocarp production) in response to the short-term defoliation. Sporocarp biomass and species richness also were negatively affected by the defoliation (−50% and −100% respectively). They concluded that EM fungus symbionts were able to allocate proportionally less carbon to sexual reproduction due to resource limitation in the host. As with our findings, average mycorrhizal colonization percentage was high (93.5%) and did not differ between the defoliated and control treatments (Kuikka et al. 2003). A finding of a meta-analysis by Barto and Rillig (2010) was that defoliation had little or no effect on EM root colonization percentage (although root density was not examined).

**Carbon limitation and forest succession.**—*Picea sitchensis* (Sitka spruce) is a long-lived but seral dominant
that generally outperforms Douglas-fir in the *Picea sitchensis* zone (Franklin and Dyrness 1973). The zone is typified by a wet and mild climate with minimal summer drought that is further ameliorated by frequent fog and low clouds. *Tsuga heterophylla* and *Thuja plicata* also are important tree species within this zone (Franklin and Dyrness 1973). The SNC epidemic area has substantial overlaps with the area covered by the *Picea sitchensis* zone (Stone et al. 2008, Fig. 1). Growth analyses of competing tree species by Kunstler et al. (2012) have identified leaf mass per unit area as an important functional trait related to species’ niche similarity and competitive ability. The chronic infection of Douglas-fir needles with high *P. gaeumannii* has reduced growth of young Douglas-fir trees in the Sitka spruce zone, thereby providing a competitive advantage to spruce and hemlock during forest succession (Stone et al. 2008). The distribution of Douglas-fir in lower elevations of the western Oregon Coast Range was sporadic before initiation of commercial logging and conversion of large areas to plantation forestry, This led Stone et al. (2008) to conclude that the Sitka spruce zone exists in part as a result of Douglas-fir growing poorly due to Swiss needle cast disease. Stone et al. (2008) also note that winter temperature increases predicted for the Pacific Northwest correlate with an increased severity and distribution of SNC as a result of climate change. It appears that anthropogenic disturbance has interacted with regional climate change to perturb the health of forest ecosystems in northern coastal Oregon.

The hypothesis that net primary productivity is an important driver of competitive interactions between Sitka spruce and Douglas-fir can be applied to the belowground ecosystem as well. Direct measurements of carbohydrates in the EM of SNC affected Douglas-fir have not been made but should be, especially considering that Saffell (2013) found that as SNC symptoms increased, stem carbohydrate storage decreased linearly. To us, her results suggest that such a reduction would have a downstream effect on carbohydrates available to the roots. This could identify the specific mechanism involved in the decline of EM fungus diversity.

Our results and those of others suggest that reduced availability of carbohydrates to the roots of Douglas-fir could alter the species richness and abundance of the EM fungus community and reduce the presence of Douglas-fir host-specific EM fungus species. Molina et al. (1992) point out the ecological importance of EM host-specificity to plants’ abilities to adapt or migrate in response to rapid climate change. We suggest that significant consequences for SNC affected forests include a serious potential for continuing decline in EM fungus species richness, even outside the Sitka spruce zone.

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**Literature Cited**


