A series of studies, in three western Oregon Douglas-fir plantations, was conducted to understand the physiological impacts of Swiss needle cast on Douglas-fir physiology. Four aspects of the disease complex were investigated: fungal colonization and assessment, plant-water relations, carbon assimilation and interaction with climate.

Several techniques were developed and used to assess the colonization of *Phaeocryptopus gaeumannii*, the causal fungus, in foliage (i.e., ergosterol concentration, quantitative PCR, and visual estimates of fruiting bodies). All measures of fungal colonization were significantly correlated with each other ($r \geq 0.733$) and with the amount of visible symptoms present (i.e., needle chlorosis and retention) ($r \geq 0.578$). Furthermore, removal of *P. gaeumannii* with fungicide applications reduced visible symptoms and increased tree growth.

Upon sporulation, *P. gaeumannii* produces fruiting bodies (pseudothecia) that emerge from needle stomata, significantly reducing gas exchange in Douglas-fir needles by physically impeding gaseous diffusion. Maximum rates of needle gas exchange ($\text{CO}_2$ and $\text{H}_2\text{O}$) were inversely proportional to the presence of *P. gaeumannii* in needle stomata. Anatomical and biochemical changes, such as reduced sapwood permeability...
and reduced rubisco activity, associated with prolonged disease presence further reduced the capacity and duration of needle gas exchange.

Climate was also shown to play a significant role in disease development. Differences associated with site topography (i.e., slope and aspect), influenced both fungal colonization and symptom development. For example, an increase in fungal colonization and symptom development was observed on foliage from south slopes, which typically experienced greater evaporative demands (i.e., increased temperature and/or lower relative humidity).

The cumulative effects of P. gaeumannii infection were integrated into a process-based model of photosynthesis. Modeled estimates of stomatal conductance and photosynthesis were well correlated with observed values ($R^2 = 0.777$, $R^2 = 0.820$, respectively). Yearly estimates of whole-canopy carbon assimilation, accounting for P. gaeumannii infection and site climate differences, were significantly correlated with tree height growth ($R^2 = 0.792$).
Physiological Impacts of Swiss Needle Cast on Douglas-fir.

by

Daniel K. Manter

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Dean of Graduate School

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Daniel K. Manter, Author
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Chapter 3: Dr. Rick Kelsey graciously provided the equipment and technical assistance necessary for the HPLC analysis; Dr. Jeff Stone assisted with the design, interpretation of results, and editing of the manuscript.

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Chapter 1
Introduction

Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) is the most common tree species growing in forest ecosystems of western Oregon and Washington. Due to its high quality wood and fast growth rates, it has become the preferred commercial species in industrial forestry in this region. However, since the early 1980s, unusually high levels of defoliation, growth losses, and even mortality have been observed in Coast Range Douglas-fir. A 1999 aerial survey conducted by the Oregon Department of Forestry estimated that a foliage disease, Swiss needle cast (SNC), severely affects more than 119,500 ha of forested lands in western Oregon alone (Hansen et al., 2000). Furthermore, growth studies conducted in 1996 estimated that the associated reduction in volume growth due to SNC was ca. 23 % or ca. 3.2 m³ ha⁻¹ yr⁻¹ (D.A. Maguire, unpublished data).

The causal fungus of SNC is the ascomycete Phaeocryptopus gaeumannii (Rhode) Petrak. Following SNC outbreaks in Europe and abroad in the 1920s and 30s (Boyce, 1940; Liese, 1938; Rhode, 1937; Wilson and Waldie, 1928), an extensive survey of British Columbia, Washington, Oregon and California was conducted (Meinecke, unpublished cited in Boyce, 1940). In this survey, P. gaeumannii was widely distributed throughout the natural range of Douglas-fir, although due to the lack
of symptom development in these stands, it was considered to be a relatively harmless native of the Pacific Northwest.

In light of the recent increased SNC symptom development in coastal Oregon, and estimated growth losses associated with disease, we initiated a series of studies to investigate how this once “benign” pathogen is influencing host physiology and growth. The main objectives of this research were to determine: 1) appropriate assessment techniques for fungal colonization, 2) the impact of P. gaeumannii infection on needle gas exchange (CO₂ and H₂O), 3) the influence of growing conditions on symptom development, and 4) the relationship between P. gaeumannii infection and Douglas-fir growth.

Objective 1 – In order to relate needle physiology to fungal infection, appropriate techniques must be available for assessing the presence and distribution of fungal colonization. In previous work, P. gaeumannii colonization levels have mainly been assessed by the visual observation of fungal fruiting bodies emerging from needle stomata (e.g., Hood, 1982; Michaels and Chastagner, 1984a, 1984b; Hansen et al., 2000). However, two factors may limit the usefulness of this approach. First, the emergence of fungal fruiting bodies from needle stomata occurs at least six months after the initial infection event; therefore, pseudothecial estimates of fungal colonization cannot be used to quantify P. gaeumannii presence during the initial stages of infection and colonization. Second, depending on the mechanism of pathogenesis, other measures of fungal colonization may relate better to physiological effects. For example, the number of tracheids occluded with a vascular wilt should relate to its biomass; whereas, a toxin producer may affect a disproportionately larger area as the toxin moves
systemically within plant tissues. Furthermore, the relationship between \( P. \ gaeumannii \) pseudothecia production and total biomass has not been investigated.

Objective 2 – CO\(_2\) that diffuses through needle stomata is the primary source of carbon, which is required for plant growth and metabolic function. Reductions in gas exchange and carbon assimilation caused by fungal infection have been shown for a variety of disease complexes. However, the specific impact of \( P. \ gaeumannii \) infection on needle gas exchange has not been explored, and based on observations of densely packed fungal structures in needle stomata (Stone and Carroll, 1986), an increased resistance to stomatal diffusion is a likely consequence of fungal infection.

Objective 3 — Anecdotal evidence suggests that SNC symptom development is more severe on trees growing on south slopes. If so, the mechanism(s) for this phenomenon may also relate to gas exchange and water effects. In this regard, we sought to confirm that south exposure affects symptoms, and, if so, through what mechanisms is this achieved. For example, is the greater symptom development due to increased fungal colonization or increased physiological impacts associated with site microclimate?

Objective 4 – In an effort to synthesize the findings related to the first three objectives, and to quantify the total physiological impact of \( P. \ gaeumannii \) on its host, estimates of carbon assimilation were related to Douglas-fir growth rates. To address this objective, a process-based photosynthesis model that accounts for \( P. \ gaeumannii \) infection and site microclimate was developed and used to estimate annual whole-canopy carbon assimilation rates. It was assumed, \textit{a priori}, that a strong correlation between estimated carbon budgets and growth would support the conclusion that \( P. \)
*gaeumannii* is the major factor influencing growth in the modeled stands, and that the model successfully accounts for the major physiological impacts of *P. gaeumannii* infection.
Chapter 2

Literature Review

2.1 Introduction

Parasitic fungi extract the nutrients necessary for their survival from the plant tissues that they invade, consequently reducing host growth and vigor. In addition, their metabolic activity and physical presence can have profound effects on host physiology. The discipline dedicated to the study of such effects is commonly referred to as Pathophysiology or Phytopathophysiology. This relatively young field is dedicated to quantification and elucidation of the pathogenic impacts on host physiological function.

It is not surprising that some of the most familiar and most studied foliage-inhabiting fungi (e.g., powdery mildews and some vascular wilts) cause diseases that produce obvious visible symptoms, ending in necrosis that reduces host growth and production by removing functional leaf area. However, in many cases, altered host physiology occurs prior to necrosis, and the mechanistic basis responsible for the reduced host growth and production has proven to be much more complicated.

Furthermore, in most situations, biotrophic fungi, many of which are asymptomatic, are the most common fungi associated with plant foliage. For example, even in normal, healthy Douglas-fir populations, up to 50% of the current-year needles (Manter, unpublished data) and as much as 90% of the 3-year-old needles are infected with internal, asymptomatic fungi (Bernstein and Carroll, 1977). Unlike the necrotropic fungi, the physiological impacts of these fungi have received far less attention.
However, as shown in the following discussion, their physical presence, parasitism, and metabolism may influence host physiology in a variety of ways. This is especially the case when their colonization reaches critical levels, as is the current situation regarding *P. gaeumannii*.

### 2.2 Carbon flux

In plants, atmospheric CO₂ is the ultimate source of carbon necessary for the production of energy and carbon skeletons required for growth and survival. In plants, photosynthesis is the series of reactions involved in the assimilation and reduction of atmospheric CO₂ into a usable form of carbon. Most efforts to assess a plant’s photosynthetic rates and capacity (*i.e.*, gas exchange) are derived from measurements of time-dependent changes in atmospheric CO₂ surrounding a leaf (*i.e.*, net carbon assimilation). In this discussion, we will focus on the CO₂ uptake (photosynthesis) and CO₂ evolving (respiration and photorespiration) processes determining measured rates of net carbon assimilation.

#### 2.2.1 Photosynthesis

Photosynthesis is a highly coordinated set of reactions, with the input of substrates following a convergent metabolic pathway and the end-products following a divergent metabolic pathway (Figure 2-1). The inputs are atmospheric CO₂, which must diffuse to the chloroplast where its incorporation is catalyzed by the enzyme ribulose 1,5-bisphosphate carboxylase (rubisco), and solar energy, which is captured by
pigments associated with the chloroplast photosystems and converted into usable forms, ATP and NADH. The end-products of the Calvin cycle (triose phosphates) are converted into starch within the chloroplast, or upon transport from the chloroplast converted into glucose, sucrose, and other compounds.

Steady-state photosynthesis requires that the two inputs conform to a specific stoichiometry matching the production of triose phosphates with the withdrawal of triose phosphates and conversion to starch and sucrose (Woodrow and Berry, 1988). If left unregulated, photosynthetic rates will decline due to temporarily unavailable substrates or irreversible damage. In regard to the latter, if the input of light energy exceeds energy consumption by the Calvin cycle, then reactive oxygen species may arise and damage the photosynthetic machinery (e.g., Barber, 1994).

2.2.1.1 Regulation of photosynthesis - the healthy plant

Extensive physiological investigation has shown that the rate of photosynthesis, due to the coordinated regulation of the involved branches, is typically dependent on four major factors (e.g., Farquhar et al., 1980; Sharkey, 1985; Woodrow and Berry, 1988; Stitt and Schulze, 1994; Stitt, 1996). These factors or possible "limitations" to photosynthesis include the following: (i) CO₂ – the source of all carbon to be used for growth of terrestrial plants, (ii) rubisco – the enzyme mediating the incorporation of CO₂ into the photosynthetic process, (iii) chemical energy (ATP and NADH) – the solar-derived energy necessary to reduce carbon into triose-phosphate and complete the Calvin cycle, and (iv) inorganic phosphate (P₁) – with each exported triose-phosphate
molecule generated by photosynthesis, one phosphate molecule is removed from the chloroplast, which must be replenished (Figure 2-1).

Figure 2-1. The C₃ photosynthetic system showing the convergent inputs of light and CO₂, and the divergent end-products of sucrose and starch. Abbreviations: Cₐ, CO₂ in the atmosphere; Cᵢ, intercellular CO₂; Cₙ, CO₂ in the chloroplast; PGA, 3-phosphoglyceric acid; LHC, light harvesting complex; PSII, photosystem II reaction center; PSI, photosystem I reaction center; RuBP, ribulose-1,5-bisphosphate (adapted from Woodrow and Berry, 1988).
The limitation of CO₂ on photosynthetic rates is commonly referred to as “stomatal” or “supply limitations” to photosynthesis. The availability and entry of CO₂ into a needle, and ultimately the actively photosynthesizing chloroplast, is a passive diffusion process limited mainly by stomatal (e.g., Raschke, 1975) and mesophyll (e.g., Nobel et al., 1975) resistances. CO₂ also plays an important role as a regulator of the second limitation, rubisco. The rubisco enzyme exists in both an active and inactive form, and in order to be catalytically active, rubisco must be bound to a molecule of CO₂ and a magnesium ion (Portis, 1992), the binding of which is mediated by the enzyme rubisco activase (Portis et al., 1986; Portis, 1992).

Of the 13 enzymes regulating the steps of the Calvin cycle, the carboxylation of CO₂ and ribulose 1,5-bisphosphate (RuBP) mediated by rubisco is typically the rate-limiting step. By comparison, rubisco is a remarkably poor catalyst with a turnover number of only about 3.3 s⁻¹ for each active site (Mott, 1997); therefore, its concentration and activation strongly influence photosynthetic rates.

Rubisco catalyzes both carboxylation (incorporation of CO₂ into an organic molecule) and oxidation (addition of O₂) of RuBP. Therefore, the rate of photosynthesis depends upon the relative chloroplast concentrations of CO₂ and O₂. The standard Michaelis-Menten two-substrate competitive system can be used to describe the curvilinear relationship between photosynthesis and internal CO₂ concentration (Figure A-1).

The complex set of reactions necessary for the production of ATP and NADPH are known collectively as the “light reactions” (Prézelin and Nelson 1997). The energy
and reducing power provided by these molecules is necessary to complete the reduction and regeneration phases of the Calvin cycle.

Continued functioning of the Calvin cycle requires a replenished supply of P\textsubscript{i}. During photosynthesis, triose phosphate is exported to the cytosol, taking with it one P\textsubscript{i} molecule from the chloroplast, upon conversion to sucrose the P\textsubscript{i} is liberated and returned to the chloroplast via phosphate translocator (Stitt et al., 1987). Therefore, the rate of sucrose synthesis, influenced by regulators in the cytosol (e.g., Stitt, 1990; Huber and Huber, 1991), may reduce chloroplast P\textsubscript{i} concentrations and limit rates of photosynthesis (Foyer and Spencer, 1986).

### 2.2.1.2 Regulation of photosynthesis - the diseased plant

In most disease complexes, photosynthesis declines following fungal infection (e.g., Shitenberg, 1992), although a few cases of increased photosynthesis have been reported (e.g., Ellis et al., 1981; Magyarosy et al., 1976). The mechanisms by which pathogens affect host photosynthesis are varied, and Boote et al. (1983) suggest the following categories: tissue consumers, leaf senescence accelerators, stand reducers, light stealers, photosynthetic rate reducers, assimilate sappers, and turgor reducers. All of these functional groups reduce photosynthesis by decreasing the photosynthesizing leaf area and/or its carboxylation efficiency. The former is common to most pathosystems and will not be discussed here, while the latter arises from a variety of mechanisms varying with each pathosystem, and will be discussed in more detail. In an effort to organize the various physiological impacts logically, the impacts of pathogens
on host physiology will be discussed in relation to the major photosynthetic “limitation” they influence.

2.2.1.2.1 Limitation 1 – \( \text{CO}_2 \)

Stomatal and mesophyll resistances may be increased or decreased in response to fungal infection – the majority resulting in increased resistance. As a general rule, increased stomatal resistance probably reflects a coordinated down-regulation in response to reduced biochemical activity (e.g., Wong et al., 1979). However, in some cases, changes in stomatal resistance appear to be the primary cause of disease impact, e.g., reduction of the rate of \( \text{CO}_2 \) diffusion into the leaf and the concomitant reduction in internal \( \text{CO}_2 \) concentrations, will reduce photosynthesis by limiting both the kinetics and activation of rubisco.

In *Fusarium oxysporum* f. sp. *lycoperisci*-infected tomatoes, Duniway and Slayter (1971) showed that in the late stages of disease both stomatal and mesophyll resistances increased; however, the mechanism is unknown. In those disease complexes where a mechanistic basis has been examined, several different processes may increase diffusion resistance. For example, Rubin and Artsikhovskaya (1968, as cited in Sutic and Sinclair, 1991) showed that *Plasmopora viticola* penetrates grapevine guard cells inducing stomatal closure, presumably from associated losses in turgor pressure. In other pathogen systems (e.g., sooty molds, *Altenaria* and *Apiosporium*; powdery mildew, *Erysiphe* sp.), stomatal resistance is not increased directly; however, increased resistance due to the physical presence of superficial fungal growth has been suggested. For example, Kuprevich (1947, as cited in Sutic and Sinclair, 1991) showed that
powdery mildew surface hyphae reduce CO₂ diffusion, and that the decline was related to the thickness of the surface mycelium.

The influence of toxins and/or enzymes on diffusional resistances has not been well studied. However, circumstantial evidence suggests that in some diseases the increased stomatal resistance may be mediated by increases in enzyme levels. For example, a five-fold increase in abscisic acid (ABA) levels was observed in *Verticillium albo-atrum*-infected tomatoes (Pegg and Selman, 1959), although it was not investigated whether the ABA was of host or fungal origin, and increased ABA levels have been shown to induce stomatal closure (Tardieu and Davies, 1993). More recently, McDonald and Cahill (1999) showed that stomatal closure is induced by an unknown but transmissible signal in *Phytophthora*-infected soybean.

In contrast, stimulation of photosynthesis in some diseases has been associated with a decreased stomatal resistance. For example, Farella *et al.* (1969) report that *Phytophthora infestans* induces the opening of stomatal apertures, and Turner and Graniti (1969) show that the *Fusarium* toxin, fusicoccin, inhibits stomatal closure.

### 2.2.1.2.2 Limitation 2 – rubisco

Few studies have provided evidence for a direct impact of fungal pathogens on rubisco activity, and in those cases where reduced photosynthetic efficiency is related to rubisco content (*e.g.*, Walters and Ayres, 1984), this effect was probably secondary in nature. Three possible situations will be discussed.

First, the amount of activated rubisco is dependent upon CO₂ concentrations (*Sage *et al.*, 1990; von Caemmerer and Edmondson, 1986); therefore, diseases that
increase stomatal and mesophyll resistances, limiting the diffusion and availability of internal CO$_2$, should influence the amount of active rubisco.

Second, several pathogens have been shown to enhance invertase activity (e.g., Long et al., 1975; Billet et al., 1977, Callow et al., 1980; Greenland and Lewis, 1981, 1983; Krishnan and Pueppke, 1988; Tang et al., 1992), which catalyzes the conversion of sucrose into glucose and fructose, leading to an accumulation of carbohydrates, and transcriptional activity of seven photosynthetic gene promoters, including rubisco's, is repressed by high carbohydrate concentrations (Sheen, 1990). For more detail, and evidence supporting this mechanism see Scholes (1992).

Third, pathogen consumption of nitrogen may be expected to reduce rubisco content and activity based on several studies showing a linear relationship between leaf nitrogen (N) and rubisco content and activity (e.g., Evans, 1983, 1986, 1989).

2.2.1.2.3 Limitation 3 – ATP & NADPH

The generation of ATP and NADPH is mediated by a series of reactions collectively known as the “light” reactions. Pathogenic fungi have been shown to interfere with several of these reactions. For example, a toxin from Alternaria tenuis, tentoxin, inhibits photophosphorylation or the production of ATP (Arntzen, 1972). The formation of ATP is also reduced by a toxic substance in the oat broad bean rust (Montalbini and Buchanan, 1974). Montalbini and Buchanan (1974) suggest that the toxin acts like DCMU, which inhibits noncyclic electron transport, or the transfer of an electron from water to NADP. Fungal competition for ATP can also reduce host photosynthesis (Scholes and Rolfe, 1996).
In the above systems, pathogens interrupt the transfer of electrons after light energy has been absorbed and used to initiate electron transfer; however, fungal infections have also been shown to reduce ATP and NADPH formation by limiting light interception and absorption. The causes of reduced light interception are varied and have been related to leaf rolling, epinasty and surface mycelium (e.g., Bowden and Rouse, 1991), whereas a reduction in light absorption is almost always related to a decline in the amount of chlorophyll content (i.e., chlorosis) – this being the most common symptom associated with plant diseases. There is little evidence showing direct impacts of fungal pathogens on chlorophyll content, i.e., interfering with synthesis. However, like rubisco, chlorophyll content is linearly related to N (Evans, 1989), and fungal consumption of N would be expected to reduce chlorophyll content. Instead, the major causes for reduced chlorophyll contents are probably related more to chloroplast disruption and necrosis, or down-regulation in chlorophyll synthesis in response to reduced photosynthetic rates mediated by one or more of the causal mechanisms in this discussion.

2.2.1.2.4 Limitation 4 - $P_i$

As shown by Foyer and Spencer (1986), photosynthesis is reduced by low $P_i$ concentrations in the chloroplast. In light of this, Whipps and Lewis (1981) suggested that biotrophic pathogens might decrease photosynthesis by sequestering large amounts of $P_i$ in polyphosphates within the mycelium. However, no direct evidence of a pathogen-induced photosynthetic decline associated with $P_i$ limitation has been observed. For example, when several rust-infected leaves were fed $P_i$, they exhibited no
stimulation in photosynthesis (Scholes and Farrar, 1986; Roberts and Walters, 1988; Zulu et al., 1991). Instead, the declines in photosynthesis observed by Whipps and Lewis are suggested to occur via feedback inhibition associated with high carbohydrate levels (Scholes, 1992).

2.2.2 Respiration

Respiration is essential for the production of energy and carbon skeletons required for plant metabolism and growth. Respiration, or CO$_2$ evolving reactions, is actually a collection of reactions or pathways including glycolysis, the pentose phosphate pathways, the tricarboxylic acid (TCA) cycle and the electron transport pathways (Figure 2-2). The majority of respiration begins with the anaerobic conversion of glucose (or other carbohydrates) to malate and pyruvate by glycolysis or the pentose phosphate pathways (a major source of amino acids, nucleotides, etc.) in the cytosol. These products are then imported into the mitochondria and oxidized in the TCA cycle forming ATP, NADH, and many of the precursors for biosynthesis pathways (e.g., oxaloacetate is a precursor to amino acid synthesis (Ireland, 1997)). Finally, the generated electron carriers, NADH and FADH$_2$, can be sent to either the cytochrome or alternative oxidase electron transport chains. The cytochrome pathway creates a chemiosmotic gradient to generate ATP (e.g., Mitchell, 1974), whereas energy transferred to the alternative oxidase pathway is mainly lost as heat (Lambers, 1997b; Simons et al., 1998). Readers interested in the finer details of respiration should consult other texts (e.g., Dennis et al., 1997).
Figure 2-2. The major steps of the respiratory processes and input of substrates (NADH and FADH$_2$) into the electron transport pathways. Only some of the major substrates in each of the following processes are shown (1) glycolysis, (2) oxdative pentose phosphate pathway, (3) tricarboxylic acid cycle, (4) photorespiration, and (5) cytochrome and alternative oxidase electron transport. Abbreviations: GAP, glyceraldehyde 3-P; PEP, phosphoenolpyruvate; OAA, oxaloacetate (adapted from Lambers et al., 1997a).
Finally, photorespiration (i.e., the oxygenation of rubisco and regeneration of phosphoglycerate by the PCO cycle) is also a respiratory process (i.e., CO₂ evolving), although it results in a net consumption of ATP (e.g., Canvin and Salon, 1997). The functional advantage of the photorespiratory cycle has often been questioned since it reduces plant productivity (i.e., by reducing rubisco carboxylation and consuming ATP). Furthermore, Arabidopsis mutants lacking a PCO cycle can grow normally under non-stressful conditions (Canvin and Salon, 1997). One possible role of photorespiration is protection of the photosynthetic machinery from photo-oxidative damage, i.e., photoinhibition (e.g., Osmond, 1981). Photoinhibition results when the absorption of solar energy exceeds its dissipation resulting in the formation of singlet oxygen species, which are highly reactive and may damage cell constituents (e.g., Powles, 1984). Therefore, photorespiration in stressed plants, e.g., water stress - where solar energy absorption continues but the dissipation of electrons to the carboxylation of rubisco is decreased, maintains sufficient levels of electron dissipation, avoiding the formation of singlet oxygen and photoinhibition.

2.2.2.1 Regulation of respiration - the healthy plant

Like photosynthesis the respiration reactions are highly regulated by plants and can be influenced by any of the enzymes or substrates involved. However, in general, as the “energy demand” of the plant for metabolic energy (i.e., ATP) required for the growth, maintenance, and transport processes increases, respiration increases (Day et al., 1985; Pilbeam et al., 1986; Lambers, 1997a). Typically, the bulk of the mitochondrial electron transport proceeds through the cytochrome pathway, which
operates at 50-95% of its capacity; and a minority of the electron flux passes through the alternative oxidase pathway, operating at 10-50% of capacity (Farrar and Williams, 1990). However, when substrate (e.g., glucose) levels are high, but the energy demand is low, the activity of the alternative oxidase pathway may operate closer to full capacity. Like photorespiration, the function of the alternative oxidase pathway is not well understood, but has been suggested to also play a role in excess energy dissipation (Lambers, 1997b).

2.2.2.2 Regulation of respiration - the diseased plant

In many pathosystems, infected leaves exhibit an increased rate of respiration (e.g., Scholes and Farrar, 1985, 1986). However, attributing the increased respiration to fungal or host origins has proven to be problematic. For example, rough calculations by Kneale and Farrar (1985) suggest that increased respiration rates in brown rust-infected barley pustules can be ascribed to the fungus; however, uninfected regions around pustules also exhibited an increased respiration rate (Bushnell, 1970; Scholes and Farrar, 1986). What factors then are responsible for the increased host respiration rates?

Theoretically, increases in host respiration rates may result from two sources, host and/or fungus. To a large extent, the increase in respiration is probably host-induced. First, induced defense responses to infection, such as callose deposition and aromatic defense compounds, will require an input of ATP and carbon skeletons for biosynthesis (e.g., Daly and Sayre, 1957) thereby stimulating respiration. Second, the hypersensitive response of plants enhances the production of active oxygen species (Mehdy et al., 1996), which have been suggested to up-regulate the gene encoding for
alternative oxidase (Simons et al., 1998). In other cases, the increase in respiration will be fungal-induced. First, fungal consumption of host substrates and ATP will increase the “energy demand” of the host. Second, as discussed above (section 2.2.1.2.3) fungal-induced declines in host photophosphorylation will also increase the “energy demand” of the host. Third, stimulated host growth and cell elongation associated with fungal production of growth regulators (e.g., corn smut, Wolf, 1952) will also increase host respiration.

2.3 Water Flux

This section will focus on the regulation of water movement through the soil-plant-air continuum. It is generally accepted that water loss from transpiring surfaces (i.e., leaves) creates a tension that pulls water upward from the soil through the plant xylem (Cohesion-Tension theory). Water is an essential constituent of plants accounting for more than 95% of the fresh weight of needles. From a physiological perspective, water is arguably the most important substrate in plant processes, acting as a solvent, reactant, influencing cell turgor and elongation, and much more. However, the current discussion will focus only on the relationship between water flux and its impact on plant productivity in the following context. The net balance of liquid (xylem flow) and vapor (transpirational) fluxes directly impacts stomatal conductance and, as a result, the supply of CO₂ available for photosynthesis. Thus, changes in water flux that limit stomatal conductance will limit the amount of carbon available for plant growth.
2.3.1 Regulation of water flux - the healthy plant

Fick's law states that flux rate ($F$) is equal to the water potential ($\Psi$) gradient divided by the resistance ($r$) to movement. Thus, water flux can be mathematically expressed as: $F = \Delta \Psi / r$. Water flux along a linear path in the soil-plant-air continuum can be thought of as a series of fluxes within "compartments" (e.g., soil to root, root to xylem, xylem to leaf, leaf to air) (Figure 2-3). Therefore, the flux within any compartment can be approximated using an appropriate version of Fick's law and at steady state the flux through the whole system is equal to the flux through any part. Integrating fluxes in time and/or space is much more complex, however, because the resistances and concentration gradients between the various compartments are interdependent (i.e., dotted-lines in Figure 2-3). For example, once leaf water potential falls below a critically low value, stomatal resistance increases due to stomatal closure (e.g., Saliendra et al., 1995).

In order to maintain constant water content, the liquid flux of water into the leaf must be in equilibrium with the vapor flux out of the leaf. If we assume that the system is inelastic (i.e., no capacitance), than the previous statement described mathematically as a set of flux equations becomes:

$$g_L \cdot \text{VPD} = K_L \cdot (\Psi_{\text{soil}} - \Psi_{\text{leaf}}),$$

(1)

where $g_L$ is leaf conductance to water vapor, VPD is the leaf-to-air gradient in the concentration of water vapor, $K_L$ is leaf specific hydraulic conductance, and $\Psi_{\text{soil}}$ and $\Psi_{\text{leaf}}$ are the soil and leaf water potentials (Jones and Sutherland, 1991; Dewar, 1995; Whitehead, 1998; Bond and Kavanagh, 1999). Furthermore, the leaf conductance to
water vapor is the sum of stomatal \( (g_s) \), cuticular \( (g_c) \) and leaf boundary layer \( (g_b) \) conductances; so a rearranged version of equation 1 can be used to model the effect that changes in water flux will exert on stomatal conductance.

\[
\begin{align*}
(g_s + g_c + g_b) &= K_L \cdot (\Psi_{\text{soil}} - \Psi_{\text{leaf}})/\text{vpd}.
\end{align*}
\]

For example, equation 2 shows the proportionality between stomatal conductance and \( K_L \); and \( K_L \) is a function of root membrane permeability and fine root proliferation, sapwood permeability, the length of the vascular path, and the ratio of sapwood area to leaf area (Sperry 1995). However, as shown in Figure 2-3, these factors are not independent. As \( \Psi_{\text{soil}} \) decreases stomatal conductance decreases, stimulated by increasing abscisic acid concentrations (e.g., Zhang and Davies, 1990). Also, as \( \Psi_{\text{leaf}} \) decreases, stomatal conductance decreases (e.g., Harrington et al., 1994), and \( \Psi_{\text{leaf}} \) is related to the net balance of water flow into and out of the leaf.

Figure 2-3. An electric circuit analogy of water flux in plants. The flow of water from each compartment (boxes) is restricted by a resistance (lines), and at any point in the system, flow = \( \Delta \Psi / \Sigma r \), where \( \Psi \) is the water potential and \( r \) is the resistance. The dotted lines represent control processes with the signs indicating the effect of an increase in the first factor on the second. \( E \) denotes transpirational loss of water. (adapted from Jones 1992, figure 6.16).
2.3.2 Regulation of water flux - the diseased plant

Transpiration in diseased plants has been reported to increase (Duniway and Durbin, 1971; Fric, 1975; Hewitt and Ayres, 1975; Tissera and Ayres, 1986), decrease (Yarwood, 1947; Mignucci and Boyer, 1979), initially decrease then increase (Yarwood, 1947; Gerwitz and Durbin, 1965) initially increase then decrease (Ayres, 1976) or remain unchanged (Spotts and Ferree, 1979). In an effort to organize the causal mechanisms associated with transpirational changes, the impacts from several pathosystems will be discussed in relation to the variables defined in equation 2 above.

2.3.2.1 $g_s$

Several leaf pathogens exert a direct influence on stomatal conductance, regardless of plant water status, through both physical and biochemical means. In regard to the former, Ayres (1976) suggests that stomatal conductance, independent of stomatal openness, is reduced by the physical presence of surface hyphae on the leaf surface. Furthermore, fungal structures that obstruct conducting tissue and stomata reduce transpiration (Reed and Cooley, 1913). In regard to the latter, several pathogen-synthesized toxins appear to affect host membrane permeability and the retention of solutes within cells, which results in the loss of epidermal cell turgor and the inability of guard cells to effectively close stomatal openings. For example, fusicoccin stimulates proton fluxes across cell membranes, promoting stomatal opening (Turner and Graniti, 1976), and cercosporin has been shown to generate singlet oxygen, which damages membranes and increases solute leakage (Daub and Briggs, 1983).
2.3.2.2 $g_c$

In healthy leaves, when stomata are open, cuticular conductance is typically several orders of magnitude lower than stomatal conductance and not a significant source of water vapor loss. However, in many pathosystems, cuticular injury associated with fungal sporulation may allow significant and uncontrolled water loss resulting in leaf wilting and death. For example, upon sporulation and aecial rupturing of *Puccina lagenophorae*, open sori of *ca.* 0.2 mm in diameter increased water vapor loss significantly reducing leaf water potential (Paul and Ayres, 1984). However, cuticular injury does not invariably increase water loss. For example, Spotts and Ferree (1979) showed that the sub-cuticular fungus, *Venturia inequalis*, did not increase transpiration in McIntosh and Delicious apple leaves. In this case and others (*e.g.*, Bushnell and Gay, 1978), fungal protrusions through the cuticle are sealed by a closely fitting, hydrophobic neckband (Nusbaum, 1938) that limits water vapor loss.

2.3.2.3 $g_b$

No reports of changes in leaf boundary conductance could be located in the literature. However, it is most likely that any changes in $g_b$ were unintentionally included in estimates of stomatal and/or cuticular conductance. For example, surface mycelium or other fungal structures that increase "leaf" thickness, and the layer of still air surrounding the leaf, will decrease leaf boundary layer conductance and reduce transpiration.
2.3.2.4 $K_L$

Changes in $K_L$ may result from "long-term" changes in plant allometrics. For example, Ayres (1985) showed that foliar infections reduced the absolute size of root systems, related to reduced photosynthetic rates and transport to the roots (Whipps and Lewis, 1981). Similar changes would also be expected to occur with stem tissues. Furthermore, in woody perennials, reduced productivity and growth should result in not only reduced stem area but also a reduced permeability associated with smaller annual rings and/or "latewood" deposition. Unfortunately, despite the well-documented effects of foliar disease on biomass production (e.g., stems and to a lesser extent, roots) little attention has been focused on the relationship to water-flux.

2.3.2.6 $\Psi_{leaf}$

As discussed above (section 2.3.2.2), pathogen-induced changes in membrane permeability will influence solute concentrations in leaf cells and their ability to adequately regulate and maintain physiological active water concentrations. Furthermore, pathogenic impacts that result in abnormal stomatal function (e.g., fusicocecin) or unusually large cuticular transpiration (e.g., sori) will render the regulatory pathway between $\Psi_{leaf}$ and stomatal conductance ineffective.
Chapter 3

Quantification of *Phaeocryptopus gaeumanni* Colonization in Douglas-fir Needles by Ergosterol Analysis

Daniel K. Manter, Rick G. Kelsey, Jeffrey K. Stone

3.1 Abstract

Current assessments of infection levels of *Phaeocryptopus gaeumannii*, the incitant pathogen of Swiss needle cast disease, typically rely on surveys of abundance of fruit bodies on diseased needles. The relationship between this measure and internal fungal colonization is unknown. In this paper, we report a series of experiments to determine whether ergosterol can be used to quantify *P. gaeumannii* internal colonization within Douglas-fir needles. We found that ergosterol content in seven commonly occurring Douglas-fir foliar fungi is proportionally related to biomass, and in *P. gaeumannii* this relationship is not affected by age of the culture. Furthermore, at four sites tested, *P. gaeumannii* was the most common fungus species isolated from Douglas-fir needles, accounting for approximately 50% of the isolations. Ergosterol content in these needles was best related to *P. gaeumannii* despite the presence of other fungi. We attribute the strong relationship between ergosterol and *P. gaeumannii* to its greater contribution to total fungal biomass compared to all other fungi present within Douglas-fir needles.

3.2 Introduction

Swiss needle cast (SNC) is a foliar disease of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) associated with premature needle abscission, chlorosis and growth loss in the coastal Pacific Northwest. Since about 1990, increasing symptoms of SNC in Oregon have prompted renewed interest in *Phaeocryptopus gaeumannii* (Rhode) Petrak, the incitant pathogen, particularly methods for quantification of fungal
colonization for epidemiological studies. *Phaeocryptopus gaeumannii* is widespread on Douglas-fir in the Coast Range and western Cascade Range of Oregon, where it is believed to be endemic (Hansen et al., 2000). Disease assessments for SNC have typically relied on estimates of *P. gaeumannii* infection levels by means of counts of fruiting bodies (pseudothecia) on needles, or the development of symptoms, such as needle retention and chlorosis (e.g., Hood, 1982; Michaels and Chastagner, 1984; Hansen et al., 2000). However, the degree to which the production of pseudothecia or disease symptoms reflects the extent of hyphal colonization and live fungal biomass within Douglas-fir needles is unknown.

Ergosterol is a membrane sterol found in most fungi, but not in plant or other microbial cells (Gessner and Newell, 1997). Therefore, it can be used as a measure of fungal biomass in the substrate under analysis. Although it is non species-specific, ergosterol content has been successfully used to quantify total fungal biomass in various substrata and applications (e.g. Matcham et al., 1985; Johnson and McGill, 1990; Desgranges et al., 1991; Schnürer, 1993). Ergosterol content has been used to measure fungal biomass in soil (Davis and Lamar, 1992), mycorrhizal roots (Salmanowicz and Nylund, 1988; Antibus and Sinsabaugh, 1993), leaf litter (Gessner et al., 1991; Newell, 1992; Gessner and Newell, 1997; Newell, 2000) and foliage (Newell, 1994; Magan and Smith, 1996). Total ergosterol content is better correlated with fungal biomass than free ergosterol content Newell (1994); and Stahl and Parkin (1996) and Newell (2000) stress that ergosterol is a better indicator of live, rather than total, fungal biomass. Ergosterol determinations of fungal biomass in this paper are therefore considered estimates of living fungal biomass. Furthermore, factors such as substrate nutrient availability and
fungal age may alter ergosterol concentrations (Johnson and McGill, 1990; Bjurman, 1994).

The potential for ergosterol content, a non-species-specific measure of fungal biomass, to quantify P. gaeumannii infection levels is unknown. Douglas-fir needles harbor a diverse assemblage of internal, endophytic fungi (Carroll and Carroll, 1978) and epiphytic fungi (Carroll, 1979). As much as 90% of needles are infected by internal fungi by the time they are three-years-old (Bernstein and Carroll, 1977). However, over 90% of these infections are due to only two fungal species (Carroll, 1988). One of these is Rhabdocline parkeri, which is widely distributed on Douglas-fir, but has restricted colonization within living needles. Infection sites of R. parkeri are limited to a single epidermal cell until needle senescence. Needles may be repeatedly infected, and the number of infections per needle increases as needles age. However, only a small proportion of the total needle cells are colonized, even on the most heavily infected foliage (Stone, 1988a; 1988b). Another abundant endophytic species, Phyllosticta abietis, also has very limited colonization, with each infection site comprised of only a few fungal cells in healthy needles (Stone and Petrini, 1997).

Colonization by P. gaeumannii, however, is not limited to a few cells; it colonizes needles both internally and externally, and growth of hyphae in the host mesophyll can be extensive (Capitano, 1999). Furthermore, populations of endophytic and epiphytic fungi increase gradually with needle age and are typically negligible on 1- and 2-year-old needles (Carroll, 1979; Stone, 1988a; 1988b), which are extensively colonized by P. gaeumannii in severely diseased trees (Hansen et al., 2000). Therefore where it is present, P. gaeumannii is likely to constitute the predominant component of
fungal biomass within Douglas-fir needles, and thus any measure of total fungal biomass, such as ergosterol content, should be indicative of its internal distribution or colonization.

Due to the potential influence of the above mentioned factors on measured ergosterol concentration, we conducted the following experiments to determine whether ergosterol is a useful indicator of *P. gaeumannii* biomass and colonization within Douglas-fir needles: (i) test the effect of sample storage on ergosterol content, (ii) test the relationship between ergosterol content and fungal biomass in a number of Douglas-fir foliar fungi, (iii) evaluate the relationship between ergosterol content and age in *P. gaeumannii* cultures, and (iv) test whether ergosterol content could be used to estimate only *P. gaeumannii* infection in Douglas-fir needles obtained from the field.

3.3 Methods

3.3.1 Sample storage time

All 1-year-old needles from one randomly selected lower canopy branch from each of ten *Phaeocryptopus gaeumannii* (Rhode) Petrak-infected Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees at Sour Grass Summit, Oregon (N45°05.673' W123°44.684') were collected in July 1997 and pooled. The sample was split into 30 sub-samples of approximately 250 mg and stored in paper coin envelopes at 0 C. At 0, 1, 2, 4, 6, and 8 months, five of the sub-samples were randomly selected for ergosterol content measurements. One-half of the needles in each envelope were used
for ergosterol extraction and HPLC analysis, and one-half were used for fresh weight (FW) to dry weight (DW) ratio determinations.

3.3.2 Ergosterol - fungal biomass studies

Pure cultures of the seven most common Douglas-fir foliar fungi were obtained from surface-sterilized Douglas-fir needles and grown in unshaken 250 ml Erlenmeyer flasks with 100 ml 2% potato dextrose broth (Difco, Detroit, MI, USA) at 18°C. After three months, mycelium was removed from each flask (one per species), rinsed with distilled H₂O, blotted dry on sterilized filter paper, macerated with a razor blade into 1 mm² pieces and separated into five to six samples of different biomass amounts (5-80 mg DW). One sub-sample was used for determination of the FW to DW ratio and the remaining portions were used for ergosterol extraction and HPLC analysis. One isolate each of Cladosporium cladosporioides, Hormonema dematiodes, Nodulisporium sp., Phomopsis sp., Rhabdocline parkeri, Tryblidiopsis pinastri, and four isolates of Phaeocryptopus gaeumannii were analyzed.

3.3.3 Ergosterol - fungal age studies

One mm plugs obtained from a pure agar culture of Phaeocryptopus gaeumannii were used to inoculate unshaken 250 ml Erlenmeyer flasks containing 100 ml 2% potato dextrose broth at 18°C (12 flasks in all). At 1, 3, 6 and 12 months mycelia from three flasks were removed, rinsed with distilled H₂O, blotted dry on sterilized filter paper, macerated into 1 mm² pieces, and separated into two approximately equal
samples. One sample from each flask was used for ergosterol extraction and HPLC analysis and one sample was used to determine the FW to DW weight ratio.

3.3.4 Detection of *P. gaeumannii* from field samples

The ability to assess *P. gaeumannii* infection by means of ergosterol content from field-collected Douglas-fir needles was compared against measures of *P. gaeumannii* pseudothecial abundance and internal fungal colonization in needles from four sites showing a range of visible SNC symptoms. Three of the sites (Juno Hill [JH], North Fork [NF], and Upper Stone [US]) have been previously described in Hansen *et al.* (2000). These sites are all located in the Oregon Coast Range near Tillamook, and have high, moderate, and low levels of *P. gaeumannii* infection and SNC symptom development, respectively. The fourth site is located on the MacDonald Forest, near Corvallis, Oregon (Mac Forest [MF]). This site was chosen because it historically has shown low levels of *P. gaeumannii* infection. In July 1997, five trees from each of the Juno Hill and North Fork sites were analyzed for ergosterol content, numbers of *P. gaeumannii* pseudothecia, and internal fungal colonization. Five trees from each of the four sites were similarly sampled in February 1998. Two randomly selected lower-canopy branches were harvested from each tree sampled, and needles from each branch were removed and pooled by age class. Ergosterol concentration, *P. gaeumannii* pseudothecia density, and internal fungal colonization were determined for each needle age class on a branch from one sub-sample as outlined below.
3.3.5 **Ergosterol extraction and analysis**

The following extraction and analysis procedures were adapted for determination of ergosterol in green conifer needles based on previously reported methods of ergosterol determination (Johnson and McGill, 1990; Gessner *et al.*, 1991; Newell, 1994). Ergosterol was extracted from *ca.* 125 mg FW (unless otherwise noted) of sample tissues (stored < 1 month at 0 °C) by heating at 80 °C for 30 min in 2 ml potassium hydroxide-methanol solutions (0.05 g KOH ml⁻¹ MeOH). After cooling, samples were partitioned three times with 2 ml petroleum ether. The petroleum ether fraction was collected and reduced to dryness. Each sample was then dissolved in 1 ml HPLC-grade methanol and filtered with a 0.2 μm nylon filter before HPLC analysis.

Extraction and HPLC of pure ergosterol standards showed efficiencies were 85-93% (*n* = 12).

Quantification of ergosterol content was conducted on a Perkin Elmer HPLC system with a Lichrosphere RP-18 (Alltech, Deerfield, IL, USA) reverse-phase column. Operating conditions consisted of an isocratic HPLC-grade methanol mobile phase (flow rate 1 ml min⁻¹, 40 °C). Absorbance at 270 nm was measured for each 200 μl sample injected. The ergosterol peak was determined by comparison of the retention time (*ca.* 8 min) and UV-spectra (UV-max at 270, 280 and 295 nm) against pure ergosterol standards (Sigma-Aldrich, Co., St. Louis, MO, USA). The ergosterol concentration was determined by comparison against an ergosterol standard calibration curve (peak height vs. concentration) determined for each run. All ergosterol concentrations are reported on a per unit dry weight basis. A sub-sample from each
analyzed sample was used to derive a FW to DW ratio for determination of ergosterol sample dry weights.

3.3.6 Pseudothecia density

P. gaeumannii pseudothecia emerging from stomata were recorded from 10 needles randomly selected from each pooled sample described above. At three locations on each needle (tip, middle, and petiole thirds), the number of pseudothecia emerging from 80 consecutive stomata were determined by visually counting (at 40X magnification) stomata in the first complete row closest to the needle mid-rib. The average value for all 30 positions (3 positions/needle from 10 needles) is reported.

3.3.7 Internal fungal isolations

Isolations of internal needle fungi were conducted on five needles in July 1997 and two needles in February 1998 from each pooled sample described above. Each selected needle was surface sterilized (rinse dH2O, 1 min 95 % EtOH, 5 min 3.5 % NaOCl, 30 sec 100 % EtOH), cut into 2 mm lengths, and placed on 2 % malt extract agar in petri dishes. Petri dishes were monitored weekly for six months and needle segments with hyphal growth emerging from cut ends were recorded. The percent of segments with P. gaeumannii emerging, and number of segments with other fungi emerging were averaged for all needles from each branch.
3.3.8 Statistical analysis

Regression analyses were carried out with the Systat V. 8.0 (SPSS, Evanston, IL, USA) statistics software package.

3.4 Results

Ergosterol concentrations decreased linearly over time in freezer stored needles (Fig. 3-1). A significant loss in ergosterol was observed two months after collection, and by eight months ergosterol had decreased to approximately 50% of its original concentration. Based on these results all future analysis of ergosterol content was limited to needle samples stored less than 1 month.

Figure 3-1. Ergosterol content (mean ± SE) from Douglas-fir needles stored at 0 C over the course of 8 months, n = 5.
All seven species of Douglas-fir foliar fungi (Cladosporium cladosporioides, Hormonema dematiodes, Nodulisporium sp., Phomopsis sp., Rhabdocline parkeri, Tryblidiopsis pinastri, and Phaeocryptopus gaeumannii) showed a linear relationship between ergosterol content and fungal biomass when grown in culture (Fig. 3-2). Furthermore, the relationship between fungal biomass and ergosterol was similar for all species tested. Tests with P. gaeumannii also showed that ergosterol content did not change in cultures from 1 to 12 months old (Fig. 3-3).

Figure 3-2. Relationship between ergosterol and fungal biomass in seven common Douglas-fir foliar fungi grown in pure culture.
Detectable amounts of ergosterol, pseudothecia, and internal fungal isolations were present in all trees sampled. Ergosterol content showed a positive linear relationship with numbers of pseudothecia (Fig. 3-4) and internal fungal isolations (Fig. 3-5). Regression parameters for these relationships are presented in Table 3-1. For both sample dates and all sites, ergosterol content was best correlated with pseudothecia density, followed by *P. gaeumannii* isolations, and finally all fungal isolations (Table 3-1).

The two most common fungus species isolated from Douglas-fir needles were *Rhabdocline parkeri* and *Phaeocryptopus gaeumannii*. For both sample dates and all sites, *P. gaeumannii* accounted for ca. 50% of the fungal isolations (Table 3-2).
Incidental species recovered included *Hormonema dematioides*, *Phomopsis* sp., *Nodulisporium* sp., *Phyllosticta abietis*, *Cryptosporiopsis* sp., *Tryblidiopsis pinastri*, and non-sporulating mycelia sterilia.

Figure 3-4. Relationship between ergosterol content and *P. gaeumannii* pseudothecia density from Douglas-fir needles collected at four sites. JH, NF, MF, and US are the Juno Hill, North Fork, Mac Forest and Upper Stone sites, respectively.
Figure 3-5. Relationship between ergosterol content and *P. gaeumannii* fungal isolation or all fungal isolations from surface sterilized Douglas-fir needles collected at four sites. JH, NF, MF, and US are the Juno Hill, North Fork, Mac Forest and Upper Stone sites, respectively.
Table 3-1. Parameters for ergosterol curves shown in Figures 4 and 5.

### Ergosterol vs. *P. gaeumannii* pseudothecia density

<table>
<thead>
<tr>
<th>Site</th>
<th>R²</th>
<th>p-value</th>
<th>y-intercept</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH</td>
<td>0.6607</td>
<td>0.0001</td>
<td>4.8328</td>
<td>0.2552</td>
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<tr>
<td>NF</td>
<td>0.5298</td>
<td>0.0001</td>
<td>5.4798</td>
<td>0.2163</td>
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</table>

**February 1998**

<table>
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<tr>
<th>Site</th>
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<th>p-value</th>
<th>y-intercept</th>
<th>slope</th>
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<tr>
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<td>0.6807</td>
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<tr>
<td>MF</td>
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<td>0.0001</td>
<td>3.7330</td>
<td>0.3226</td>
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<tr>
<td>US</td>
<td>0.4336</td>
<td>0.0001</td>
<td>4.7020</td>
<td>0.2645</td>
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</table>

### Ergosterol vs. *P. gaeumannii* fungal isolations

<table>
<thead>
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<th>y-intercept</th>
<th>slope</th>
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<tbody>
<tr>
<td>JH</td>
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<td>0.0001</td>
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<td>0.1946</td>
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<tr>
<td>NF</td>
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<td>0.0001</td>
<td>5.2160</td>
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</table>

**February 1998**

<table>
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<tr>
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<th>slope</th>
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<tr>
<td>JH</td>
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<td>US</td>
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### Ergosterol vs. all fungal isolations

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<th>slope</th>
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**February 1998**

<table>
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<th>slope</th>
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<tr>
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<tr>
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<td>0.0001</td>
<td>0.9508</td>
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<tr>
<td>US</td>
<td>0.0519</td>
<td>0.1148</td>
<td>3.9656</td>
<td>0.0481</td>
</tr>
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</table>
Table 3-2. Percent of needle ends (2 mm surface sterilized fragments) from which fungal hyphae emerged. Values reported are the mean ± SE for all observations at each site. \(^1\)PG denotes *P. gaeumannii*, RP denotes *R. parkeri*, All denotes all fungal species.

<table>
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<tr>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>PG(^1)</td>
<td>RP</td>
<td>All</td>
</tr>
<tr>
<td>July 1997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH</td>
<td>0.7</td>
<td>0.0</td>
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<tr>
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<td>(0.4)</td>
<td>(0.0)</td>
<td>(0.9)</td>
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<td>NF</td>
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<tr>
<td></td>
<td>(0.8)</td>
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<td>(1.0)</td>
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<tr>
<td>February 1998</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH</td>
<td>38.3</td>
<td>2.6</td>
<td>51.1</td>
</tr>
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<td></td>
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<td>(1.1)</td>
<td>(8.8)</td>
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<td>NF</td>
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<td></td>
<td>(7.3)</td>
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<td>US</td>
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<td>57.3</td>
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<tr>
<td></td>
<td>(1.2)</td>
<td>(0.6)</td>
<td>(3.7)</td>
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</table>
3.5 Discussion

Ergosterol content appears to be a good measure of *P. gaeumanni* colonization. It correlated well with both pseudothecia density and *P. gaeumanni* isolations from surface sterilized needles. Although about 50% of fungal isolations from all sites were species other than *P. gaeumanni*, they did not appear to contribute significantly to the detectable amount of ergosterol (i.e., ergosterol content was best predicted by measures of *P. gaeumanni* only). This result is best explained by colonization of *P. gaeumanni* comprising a major proportion of the fungal biomass in needles. To our knowledge, efforts to quantify internal colonization rates of *P. gaeumanni* have not been conducted elsewhere. However, light and electron microscopy of Douglas-fir needles infected by *P. gaeumanni* has shown extensive internal and external needle colonization (Capitano, 1999), whereas many of the other foliar fungi typically have very limited colonization within the needle. *Rhabdocline parkeri*, for example, was the second most common fungus isolated. Infections by this fungus are restricted to single epidermal cells until needle senescence, and no more than about 5% of the total epidermal cells are occupied in heavily infected foliage (Stone, 1988).

In pure cultures, ergosterol content to fungal biomass (all seven species investigated) and did not vary with culture age (only *P. gaeumanni* investigated). These two results suggest that measures of ergosterol should relate directly to amounts of live fungal biomass in Douglas-fir needles. However, we did not test the effect of growing conditions (i.e., nutrient concentration) on ergosterol content in pure cultures. Medium composition has been shown to affect ergosterol content in fungal cultures (Newell, 2000). It is possible that fluctuations in nutrient levels in needles could cause
the relationship between ergosterol and fungal biomass to vary. While we cannot rule out the possibility of such an effect influencing observed ergosterol contents, we believe that it is not a significant factor. If nutrient levels caused significant changes in ergosterol content, we would expect to see a consistent change in stationary cultures over time, as nutrient levels in the media became depleted. Instead, the relationship remained linear. We also would expect to see the ratio of ergosterol content to P. gaeumannii pseudothecia count, or the ratio of ergosterol content to P. gaeumannii isolations to vary within each regression in Figs. 4 & 5. Each regression includes several needle age classes, and nutrient concentrations typically decline with needle age (Bauer et al., 1997) and increasing P. gaeumannii infection (Manter, unpublished data); however, the relationship remains linear, suggesting a constant ratio of ergosterol content to P. gaeumannii colonization level.

As shown in Table 3-1, slopes of the ergosterol regressions vary over time and between sites. We attribute this to the inaccuracy of either pseudothecia density or fungal isolations for estimation of internal colonization rates. A greater slope suggests to us that the amount of internal colonization per unit pseudothecium (or fungal isolation) is increasing. Such a relationship is supported by Capitano’s (1999) work showing greater internal hyphal growth of P. gaeumannii at the Juno Hill site, which consistently had the highest observed slopes compared to other sites studied. The increase in slope over time is also consistent with increasing amounts of internal hyphal growth and the number of external surface hyphae radiating from pseudothecia (Capitano, 1999).
In summary, ergosterol content appears to be a good measure of fungal biomass in Douglas-fir needles mainly related to *P. gaeumannii* colonization. Unlike pseudothecia density and fungal isolation measures of *P. gaeumannii*, it is non-species specific and measures colonization levels of other foliar fungi present in Douglas-fir needles. However, the relative contribution of other fungi appears to be negligible at all sites, regardless of *P. gaeumannii* infection levels. Changes in the slope of the linear relationship between ergosterol and *P. gaeumannii* infection levels suggest that the amount of internal hyphae varies over time and with site. The cause, effect, and relative importance of differences in internal hyphae growth, and their relationship to Swiss needle cast disease are unknown and deserve further attention.

### 3.6 Acknowledgements

This research was funded through the Swiss Needle Cast Cooperative at Oregon State University - a consortium of industrial, federal, and state landowners in Oregon and Washington. The comments of an anonymous reviewer helped improve the paper. The use of trade names is for information and convenience of the reader and does not constitute official endorsement or approval by the USDA.
3.7 Literature cited


Chapter 4

Assessment of Swiss Needle Cast Disease Development. Temporal and Spatial Investigations of Fungal Colonization and Symptom Development

Daniel K. Manter, Loretta M. Winton, Gregory M. Filip, Jeffrey K. Stone
4.1 Abstract

Swiss needle cast (SNC) is a foliar disease of Douglas-fir caused by the fungus *Phaeocryptopus gaeumannii*. SNC occurs throughout the natural range of Douglas-fir, and its increasing severity has become a concern throughout coastal Oregon and Washington. This study monitored the development of fungal colonization and symptoms from three Douglas-fir plantations bi-annually in 1998 and 1999. Quantification of fungal colonization was determined by several methods: ergosterol content, pseudothecia density, and quantitative PCR. All measures of fungal colonization were significantly correlated with each other and with disease symptoms. We also show that fungicide applications reduced both *P. gaeumannii* colonization and symptom development. Variation in fungal colonization was detected within sites and within individual tree canopies. Trees on south slopes at our coastal sites had higher fungal colonization and more severe symptoms compared to north slopes. However, for interior sites, north slopes had higher fungal colonization and symptoms. Within individual trees, fungal colonization was consistently higher in the upper portions of the canopy and in south facing foliage. Spatial patterns of *P. gaeumannii* development varied at the macro- and micro-scales and are consistent with observed patterns of symptom development.

4.2 Introduction

Swiss needle cast (SNC) is a foliar disease of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) caused by the fungus *Phaeocryptopus gaeumannii* (Rhode)
Petrak. SNC occurs throughout the range of Douglas-fir; and since the mid-1980s, forest managers and researchers have noticed an increase in its frequency and severity. Aerial surveys conducted by the Oregon Forestry Department in 1999 detected ca. 119,500 hectares of plantations with visible chlorosis and premature needle loss (Hansen et al., 2000), and extensive growth losses have been observed in these stands. For example, based on a 1996 survey, Maguire (unpublished data) estimated that on 52,611 hectares of forested lands in western Oregon, SNC caused a ca. 23% reduction in volume growth or an implied growth loss of about 3.2 m³ ha⁻¹ yr⁻¹. In addition to observations of a correlation between P. gaeumannii and disease symptoms over the landscape (Hansen et al., 2000), recent work has suggested a mechanistic basis of impact in which P. gaeumannii reduces Douglas-fir photosynthetic capacity by occluding needle stomata (Manter et al., 2000). Despite the apparent relationship between colonization and symptoms, few published reports have detailed the relationship between P. gaeumannii colonization and symptom development in Douglas-fir plantations or forests, although they have reported extensive fruiting of P. gaeumannii throughout the range of Douglas-fir. Furthermore, evidence for the pathogenicity of P. gaeumannii has been demonstrated primarily in forest plantations where Douglas-fir is planted as an exotic (Gaeumann, 1930; Hood and Kershaw, 1975) and Christmas tree plantations (Michaels and Chastagner, 1984a) but not in forest plantations in western North America.

The distribution, colonization and pathogenicity of P. gaeumannii in the northwestern United States have received intermittent attention in forest plantations. One of the earliest surveys for P. gaeumannii, conducted in 1938, (Meinicke,
unpublished data, cited in Boyce, 1940) found that P. gaeumannii was present in many stands but was not cited as the cause of defoliation. More recently, Hood (1982) surveyed branches from Douglas-fir in British Columbia and northwestern Washington and found an ubiquitous distribution of P. gaeumannii fruiting bodies in 3- to 5-year-old needles, with incidence appearing to vary with rainfall. For example, in wet coastal sites more than 80% of the needles surveyed were infected, but in drier sites less than 5% of the surveyed needles were infected. These observations are supported by studies showing that ascospore production (Michaels and Chastagner, 1984b) and germination (Capitano, 1999) require free moisture.

Within the Oregon fog-belt, where the current SNC outbreak is at its highest levels, considerable heterogeneity in symptom development has been noted between stands (Hansen et al., 2000), despite the high rainfall and frequent fog-events common throughout the coastal Pacific Northwest. In fact, anecdotal evidence suggests that symptom development is greater on south compared to north slopes. Although it is known that slope and aspect can greatly influence environmental conditions in forest ecosystems, especially those that are typified by limited moisture regimes, such environmental limitations are often overlooked in the wet and cool regions of the Pacific Northwest. If such spatial variations in environment do exist over the landscape, how, and to what extent, they may be influencing symptom development is unknown. For example, does environment limit the colonization and development of P. gaeumannii, or does the environment mediate the effective impact of the fungus on its host?
In the case of *P. gaeumannii*, the interaction between slope-aspect and fungal development is largely unknown; however, previous research has typically shown that foliar fungi are more abundant on wet, humid sites (e.g., north slopes), and have higher colonization rates in the lower portion of tree canopies (Diem, 1971; Sinclair, 1997). Many fungal pathogens require high relative humidities and/or free water for germination and growth (Diem, 1971); thus, higher colonization of *P. gaeumannii* on south-aspect slopes would suggest a pattern of colonization opposite to the norm, if moisture was the main factor limiting colonization. Michaels and Chastagner (1984a) showed that ascospore production decreases with darkness, and has a clear temperature optimum at *ca.* 20 C, and Capitano (1999) showed that spore germination was optimized at *ca.* 22 C. Thus, increased temperatures and/or light levels associated with south slopes may lead to greater symptom development due to increased fungal colonization. However, the interactions between environment, fungal colonization (hyphal growth and/or pseudothecia), and symptom development have not been studied, nor have these relationships been examined in forest plantations.

In addition to greater fungal colonization on south slopes there is also the potential for greater physiological impacts. Research conducted by our laboratory has shown that stomatal occlusion caused by *P. gaeumannii* pseudothecia reduces host photosynthesis through both stomatal and non-stomatal limitations (Manter *et al.*, 2000), both of which may be potentially exacerbated due to the increased temperature and high light conditions on south slopes. For example, stomatal limitations may limit photosynthesis due to the increased stomatal closure in response to the high evaporative demand; and non-stomatal limitations may be increased due to the presence of
photoinhibition, which is more likely under high temperature and light conditions (Demmig-Adams and Adams, 1992).

Historically, *P. gaeumannii* infection has been quantified by visual assessments of numbers of pseudothecia emerging from stomata; however, it is unknown if pseudothecial estimates of colonization are the best predictors of disease impact. Manter *et al.* (2000) showed that at least 75% of the impact on photosynthetic capacity was related to pseudothecia density. Any remaining impacts have yet to be determined. Therefore, the total impact of SNC on Douglas-fir growth, physiology and symptom development may be best predicted by pseudothecia and/or some other estimate of fungal colonization.

Due to our lack of knowledge regarding the observed spatial heterogeneity of symptom development and fungal colonization, the main objectives of this study were to evaluate the relationship between colonization by *P. gaeumannii* and symptom severity at several scales: (1) among three stands with different levels of needle retention and chlorosis, (2) among trees on north- and south-facing plots within a stand, and (3) among various locations within individual tree canopies.

4.3 Methods

4.3.1 Sample sites

Three 12- to 15-year-old Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) plantations with varying levels of Swiss needle cast were chosen for study. Paired permanent plots were created on north- and south-facing slopes (10 - 30%) at each site
(Appendix I). Each permanent plot consisted of a group of six infected trees and six uninfected controls. Control trees were kept disease free by spraying foliage with chlorothalonil (Bravo 720, rate = 66 ml / 3.78 L, applied until run-off) by means of a backpack sprayer. Fungicide applications were conducted in 1998 and 1999 at bud break (90% trees had broken bud) and one month following bud break. The high disease site is located on the Siuslaw National Forest, near Beaver, OR (Beaver north (BN) plot - N45° 17.55' W123° 46.46'; Beaver south (BS) plot N45° 17.43' W123° 46.35'), the medium disease site is located on the Siuslaw National Forest near Hebo, OR (Hebo north (HN) plot – N45° 13.87' W123° 48.51'; Hebo south (HS) N45° 13.85' W123° 48.19'), and the low disease site is located on the MacDonald-Dunn Forest near Corvallis, OR (Mac north (MN) plot – N44° 37.68' W123° 19.45'; Mac south (MS) plot N44° 37.48' W123° 19.57').

4.3.2 Tree sampling

At each sample date two primary lateral branches from each tree were removed, placed in plastic bags, and transported back to the laboratory on ice. Once in the laboratory, needles from each sampled tree were pooled by age class and stored at 0°C. All measures of fungal and symptom development were determined on sub-samples from the pooled samples within one month of collection. All analyses from these trees were limited to branches from the south side of the lower half of the tree canopy. Ergosterol content, pseudothecia density, needle retention and chlorosis measurements were conducted on branches harvested in July 1998, December 1998, May 1999, and
November 1999. Quantitative PCR measurements were conducted on branches harvested in May 1999 and November 1999.

4.3.3 Canopy position sampling

Three infected trees from each of five SNC affected sites were sampled in February 2000. Sites were the BN, BS, HN, HS stands described above, and a stand at Sour-grass Summit (N45° 05.673' W123° 44.684'). One branch from each of the north-top, north-bottom, south-top, and south-bottom quadrants of each tree was sampled and analyzed for fungal colonization and symptom development.

4.3.4 Fungal and symptom quantification

Ergosterol content, *Phaeocryptopus gaeumannii* (Rhode) Petrak DNA content (*i.e.*, quantitative PCR) and pseudothecia counts were determined according to the methods outlined in Manter *et al.* (2001) and Winton *et al.* (2001). Ergosterol content (µg ergosterol g⁻¹ needle dry weight), quantitative PCR (pg *P. gaeumannii* DNA ng⁻¹ *P. menziesii* DNA), and pseudothecia density (% of stomata with visible pseudothecia) were measured on randomly selected sub-samples of ca. 25, 10, and 10 needles, respectively, from the needle collections described above. Needle retention was visually estimated to the nearest 10 % for each needle-age class, and needle chlorosis was scored on a scale of 0 - 3, with 0 being green, 1 slight, 2 moderate and 3 severe yellowing.
4.3.5 **Weather data**

At each site air temperature and relative humidity were recorded hourly with temperature / RH dataloggers (Spectrum Technologies, Inc., Plainfield, IL). Dataloggers were protected from direct radiation by a radiation shield (Spectrum Technologies, Inc.) and placed in an open area (ca. 5 m radius) at approximately 1.5 m above the ground. The maximum, minimum and mean values were calculated for each day.

4.3.6 **Analysis**

All reported values of fungal colonization and symptoms are the mean and standard error for all trees sampled at each site (e.g., BN, BS, etc.). Paired t-tests and Pearson correlations were calculated with Systat 8.0 (SPSS Inc., Chicago, IL, USA), and linear regressions were calculated with Plot 4.0 (SPSS Inc.). Canopy position data were analyzed with PROC MIXED (SAS Institute Inc., Cary, NC, USA), where canopy position was a fixed effect and tree and site attributes were random effects.

4.4 **Results**

*Phaeocryptopus gaeumannii* was present in all plot trees sampled. All measures of fungal abundance detected similar levels of fungal colonization and development (Figures 4-1 to 4-3) and were highly correlated ($r \geq 0.685$) with each other (Table 4-1). Furthermore, all measures of fungal colonization (i.e., pseudothecia density, ergosterol content and quantitative PCR) were significantly correlated with symptom development.
(i.e., needle retention \[r \leq -0.423\] and chlorosis \[r \geq 0.552\]) (Table 4-1). A marginal improvement in the correlations was observed when the mean values for all six trees were used (i.e., needle retention \[r \leq -0.451\] and chlorosis \[r \geq 0.628\]) (Table 4-2).

The Beaver site consistently had the highest and fastest rates of fungal colonization, for all assays, followed by the Hebo and Mac sites, respectively (Figures 4-1 to 4-3). Pseudothecia density was ca. 50, 20 and 5 % (Figure 4-1), and ergosterol content was ca. 28, 12 and 8 µg mg\(^{-1}\) needle dry weight (Figure 4-2), for 18-month-old needles at the Beaver, Hebo and Mac sites, respectively. In general, fungal colonization increased over time, with the largest increases during the late summer / early fall (Figures 4-1 & 4-2), except during the spring sample (May 1999) when abscission of the most heavily-infected needles occurred (Figure 4-4). Like fungal colonization, symptom development (i.e., needle retention and chlorosis) also increased over time; however, the largest increases occurred during the late winter / early spring (Figures 4-4 & 4-5). To account for this time lag between fungal colonization and symptom development, the correlation between fungal colonization (December 1998 sample date) and subsequent symptom development (May 1999 sample date) was determined. In this case, the correlation between fungal colonization and symptom development was greatly improved (i.e., needle retention \[r \leq -0.816\] and needle chlorosis \[r \geq 0.756\]) (Table 4-3). Furthermore, the apparent decline in fungal colonization during the spring months (Figures 4-1 to 4-3), coinciding with needle abscission (Figure 4-4), suggests that a successful correlation between fungal colonization and symptoms will be dependent upon the sample date when fungal colonization was measured (i.e., measurements in the spring or later will underestimate fungal colonization).
Figure 4-1. Mean pseudothecia density of *P. gaeumannii* over time from six untreated Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error. Missing observations were due to needle abscission.
Figure 4-2. Mean ergosterol content over time from six untreated Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error. Missing observations were due to needle abscission.
Figure 4-3. Mean quantitative PCR (P. gaeumannii DNA per unit Douglas-fir DNA, pg ng\(^{-1}\)) over time from six untreated Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error. Analysis was not conducted for the June 1998 and December 1998 sample dates. Missing observations for the May 1999 and November 1999 sample dates were due to needle abscission.
Table 4-1. Pairwise Pearson correlation coefficient for all fungal colonization and symptom development measures (n > 204). Correlations were conducted using all observations. All correlations are significant at p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Pseudothecia Density</th>
<th>Ergosterol Content</th>
<th>Quantitative PCR</th>
<th>Needle Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudothecia Density</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ergosterol Content</td>
<td>0.818</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>0.765</td>
<td>0.685</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>-0.542</td>
<td>-0.485</td>
<td>-0.423</td>
<td>-</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>0.665</td>
<td>0.552</td>
<td>0.603</td>
<td>-0.721</td>
</tr>
</tbody>
</table>

Table 4-2. Pairwise Pearson correlation coefficient for all fungal colonization and symptom development measures (n > 34). Correlations were conducted using the mean value for each date-site-slope-treatment-age combination (i.e., mean value for six trees). All correlations are significant at p ≤ 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Pseudothecia Density</th>
<th>Ergosterol Content</th>
<th>Quantitative PCR</th>
<th>Needle Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudothecia Density</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ergosterol Content</td>
<td>0.885</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>0.814</td>
<td>0.733</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>-0.644</td>
<td>-0.578</td>
<td>-0.451</td>
<td>-</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>0.725</td>
<td>0.628</td>
<td>0.677</td>
<td>-0.750</td>
</tr>
<tr>
<td>Chlorosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4-4 shows that the correlations between fungal colonization (June 1998 sample date) and needle symptoms (May 1999 sample date) were significantly lower compared to when the winter (December 1998) fungal colonization and spring (May 1999) symptom measurements were used (Table 4-3).

Consistent differences in pseudothecia density between the north and south plots were detected at each site, especially for the winter samples (i.e., December 1998 and November 1999) (Figures 4-1 to 4-3). Pseudothecia density was significantly higher for the south plot than the north plot at the coastal sites (i.e., Beaver and Hebo), but the opposite trend was seen for the interior site (i.e., Mac). The difference in pseudothecia density between plots varied depending upon the needle cohort and age and ranged from ca. 0 - 40 % (Figure 4-1). North and south plots also differed in the amount of needles retained for any given level of fungal colonization. At all three sites, the south plots had lower needle retention at a given level of pseudothecia density than to the north plots (Figure 4-6).

Application of chlorothalonil successfully limited colonization in all needle cohorts assayed. The most effective control was achieved on the newly expanding needles (i.e., 1998 cohort), and at the end of this study no pseudothecia were observed in these needles (Figure 4-7). In addition, fungal colonization (i.e., pseudothecia density) was reduced in the fully expanded, older needles (i.e., 1997 and 1996 cohorts) that were infected prior to application (Figure 4-7). Application of chlorothalonil not only limited fungal colonization, but also decreased needle abscission and chlorosis. Table (4-5) shows the average difference in fungal colonization and symptom development between the sprayed and unsprayed branches.
Figure 4-4. Mean needle retention over time from six untreated Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error.
Figure 4-5. Mean needle chlorosis over time from six untreated Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error. Missing observations were due to needle abscission.
Figure 4-6. Relationship between the mean needle retention (May 1999 sample) and pseudothecia density (December 1998 sample) from Douglas-fir trees in north and south slope plots at three sites.

North Plots
$R^2 = 0.962, p < 0.001$
$Y = 103.92 - 1.06 * X$
$(1.57) (0.05)$

South Plots
$R^2 = 0.970, p < 0.001$
$Y = 99.91 - 1.47 * X$
$(2.12) (0.08)$
Figure 4-7. Mean *P. gaeumannii* pseudothecia density over time from six sprayed (chlorothalonil) Douglas-fir trees in a south and north slope plot at three sites. Error bars are one standard error. Missing observations were due to needle abscission.
Table 4-3. Pairwise Pearson correlation coefficient between fungal colonization (December 1998 sample) and symptom development (May 1999 sample) (n = 12). Correlations were conducted using the mean value for each site-slope-treatment-age combinations (i.e., mean value for six trees). All correlations are significant at p ≤ 0.05. Quantitative PCR was not measured for the December 1998 sample.

<table>
<thead>
<tr>
<th></th>
<th>Pseudothecia Density</th>
<th>Ergosterol Content</th>
<th>Quantitative PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Needle Retention</td>
<td>-0.902</td>
<td>-0.816</td>
<td>NA</td>
</tr>
<tr>
<td>Needle Chlorosis</td>
<td>0.810</td>
<td>0.756</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 4-4. Pairwise Pearson correlation coefficient between fungal colonization (June 1998 sample) and symptom development (May 1999 sample) (n = 12). Correlations were conducted using the mean value for each site-slope-treatment-age combinations (i.e., mean value for six trees). All correlations are significant at p ≤ 0.05. Quantitative PCR was not measured for the December 1998 sample.

<table>
<thead>
<tr>
<th></th>
<th>Pseudothecia Density</th>
<th>Ergosterol Content</th>
<th>Quantitative PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Needle Retention</td>
<td>-0.679</td>
<td>-0.661</td>
<td>NA</td>
</tr>
<tr>
<td>Needle Chlorosis</td>
<td>0.712</td>
<td>0.731</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 4-5. Difference in fungal colonization and symptom development between untreated and treated (chlorothalonil) needles, average for all age classes. The mean difference and paired t-tests were pooled over all dates, ages and plots at each site. Negative values are reductions due to spraying. Pseudo is pseudothecia density, Erg is ergosterol content, PCR is quantitative PCR, Reten is needle retention, and Chior is needle chlorosis. Diff is the difference in observed means, t is the t-value, df are the degrees of freedom, p is the p-value.

| Measure | Beaver | | | Hebo | | | | Mac | |
|---------|--------|--------|-----|--------|--------|-----|--------|--------|
| Pseud   | -15.64 | -4.17  | 19  | 0.001  | -7.58  | 25  | 0.000  | -3.44  | 25  | 0.004 |
| Erg     | -9.47  | -4.91  | 17  | 0.000  | -5.95  | 23  | 0.000  | -4.68  | 23  | 0.000 |
| PCR     | -2.19  | -3.20  | 13  | 0.007  | -1.07  | 19  | 0.101  | -0.36  | 20  | 0.546 |
| Reten   | 9.22   | 4.49   | 25  | 0.000  | 10.55  | 4.64 | 25  | 0.000  | 4.30   | 25  | 0.010 |
| Chior   | -0.48  | -3.12  | 19  | 0.006  | -0.36  | 25  | 0.000  | -0.03  | 25  | 0.014 |

Significant differences in both fungal colonization and symptom development were detected within tree canopies (Table 4-6). For example, pseudothecia density was ca. 10% higher in the upper portion of the canopy, and ca. 4% greater on the south side of the tree. Symptom development showed a similar pattern to fungal colonization, also being highest in the upper canopy and on the south side of the tree. Needle retention was reduced by ca. 15% in the upper canopy, and reduced by ca. 10% on the south side of the tree.

Ambient daily temperature and relative humidity varied substantially between sites, and at each plot within a site (data not shown). The coastal sites consistently had higher average humidity and lower average temperature than the interior site. Within a site, the south-facing plots typically experienced a lower average relative humidity than the north plots, with the largest differences occurring during the summer of 1999. However, differences between the average temperatures at each plot varied depending upon the site location. For example, at the coastal sites the south plots had...
the higher average daily temperatures, whereas at the interior site the north plot had the higher average temperature. The different pattern in average daily temperature, at the interior site is consistent with the observed daily maximum and minimum temperatures. For example, the interior south slope consistently reached a higher maximum daily temperature compared to the north slope, like its coastal counterparts. However, its minimum daily temperature was consistently lower than the north slope, unlike its coastal counterparts resulting in a lower average daily temperature.

Table 4-6. Fungal colonization and symptom development at four canopy positions. Means with different letters are significantly different at p ≤ 0.05. Standard errors are in parentheses.

<table>
<thead>
<tr>
<th>Measure</th>
<th>South</th>
<th>North</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top</td>
<td>Bottom</td>
</tr>
<tr>
<td>Pseudothecia Density</td>
<td>17.5(4.5)a</td>
<td>12.6(3.5)b</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>0.8(0.2)c</td>
<td>0.8(0.2)d</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>76.7(3.4)d</td>
<td>86.7(4.1)e</td>
</tr>
<tr>
<td>Needle chlorosis</td>
<td>1.5(0.2)f</td>
<td>0.9(0.2)g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudothecia Density</td>
<td>6.9(1.5)h</td>
<td>4.0(0.9)i</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>0.6(0.1)j</td>
<td>0.3(0.1)k</td>
</tr>
<tr>
<td>Needle Retention</td>
<td>91.7(3.1)l</td>
<td>98.3(1.7)m</td>
</tr>
<tr>
<td>Needle chlorosis</td>
<td>1.2(0.2)m</td>
<td>0.5(0.1)n</td>
</tr>
</tbody>
</table>
4.5 Discussion

The pathogenicity of *P. gaeumannii* as the causal agent of Swiss needle cast is supported by the findings in this study. In all of the sites studied here, which exhibit typical Swiss needle cast symptoms, the amount of symptom development was strongly correlated with the amount of *P. gaeumannii* colonization, particularly pseudothecia density. A strong correlation between fungal colonization and symptom development was also detected at a variety of scales (*e.g.*, stand, tree and branch), and removal of the fungus through fungicide applications consistently resulted in a reduction in symptom severity. Our observed correlations between symptom development and fungal colonization (Table 3) are higher than those reported in other studies (Hansen et al., 2000; Winton et al., 2001), and may be attributed to the following: (i) sample branches were limited to the bottom quadrant of each tree – minimizing the potential effect of an interaction with environmental differences present within the tree canopy, (ii) correlations were conducted using mean values from six trees at each site – minimizing tree to tree variation arising from host genotype, microclimate differences and/or errors due to our limited sampling within each tree, and (iii) symptom development was assessed five months after fungal colonization – accounting for the substantial time for symptoms to appear. Furthermore, fungal colonization should be measured during the winter months before needle abscission removes the most heavily infected needles resulting in an underestimation of fungal colonization.

Our observed pattern of fungal colonization (Beaver > Hebo > Mac) is consistent with previous observations that high moisture is required for both ascospore production (Michaels and Chastagner, 1984b) and germination (Capitano, 1999),
because rainfall and RH were consistently highest at the Beaver site, followed by the Hebo and Mac sites, respectively (data not shown). Furthermore, the “abnormally” high colonization rates in the Mac 1997 cohort of needles occurred during an abnormally wet spring at that site (data not shown); because spore deposition was not measured, however, it was not possible to determine whether ascospore production or germination was the major factor responsible for the higher fungal colonization rates.

The frequently observed higher symptom development on south slopes may be related to both an increase in fungal colonization and increased physiological impact. First, south slopes have greater needle loss for a given level of pseudothecia presence than their north counterparts. Second, at the coastal sites studied, fungal colonization was significantly higher on south slopes.

The exact cause of higher levels of needle loss on south-facing slopes was not studied here. However, physiological impacts related to non-stomatal and stomatal limitations to photosynthesis may interact with the south slope microclimate to cause more physiological damage and needle loss.

In regard to the former, energy consumption by the Calvin cycle is reduced in *P. gaeumannii*-infected needles (Manter et al., 2000), potentially resulting in excess energy, which if not dissipated thermally can lead to singlet oxygen formation, photo-inhibition, and damage (Demmig-Adams and Adams, 1992). The presence of photodamage in *P. gaeumannii*-infected needles has not been investigated; however, preliminary data suggest that thermal dissipation (*i.e.*, non-photochemical quenching) increases with *P. gaeumannii* infection (Will Littke, personal communication). Thus, if the amount of thermal dissipation cannot adequately shunt the excess energy, then
photo-oxidation may result; and such a scenario is most likely under high light and
temperature conditions where photo-inhibition through singlet oxidation is exacerbated
by inadequate thermal dissipation (Demmig-Adams and Adams, 1992).

Alternatively, due to the greater evaporative demand on south slopes, greater
stomatal limitations to gas exchange may be limiting host production, reducing vigor
and increasing needle abscission.

Fungal colonization of needles was observed to occur faster on south coastal
sites and the north interior site, especially during the cooler winter months. Of the two
climatic variables measured here, temperature is the most likely factor influencing
fungal colonization. *Phaeocryptopus gaeumannii* ascospore production (Michaels and
Chastagner, 1984a), hyphal growth and germination (Capitano, 1999) have been
observed to increase with temperature until maximized at *ca.* 20 C. At our sites, during
the winter months the observed air temperatures were consistently below 20 C, so at the
sites with warmer average temperatures (*i.e.*, south coastal sites and north interior site),
faster growth and higher colonization, as observed, is consistent with these
observations.

The increased fungal colonization and symptom development in the upper and
south side of tree canopies is also consistent with a temperature effect. The validity of
such a temperature effect needs to be verified experimentally. Furthermore, future
studies regarding the role of water stress and/or photo-oxidation damage in determining
symptom development need to be conducted.

The strong correlation between fungal colonization measures indicates that any
of these measures may be successfully used in future assays of Swiss needle cast for
both research and management activities. However, we recommend that future studies may benefit from quantifying Swiss needle cast through pseudothecia density counts for the following reasons. First, pseudothecia counts had the best correlation with symptom development. Second, a mechanistic pathway relating pseudothecia density and physiological impact exists (Manter et al., 2000). And third, pseudothecia counts appeared to show the greatest sensitivity in detecting colonization differences at a variety of scales. However, under some conditions (e.g., routine monitoring or less detailed work, especially prior to the formation of pseudothecia) quantitative PCR may be the most suitable measure of fungal colonization. The benefits of quantitative PCR are: (i) its ability to detect P. gaeumannii colonization prior to pseudothecia formation, (ii) it is well correlated with pseudothecia density, and (iii) is relatively inexpensive and non-labor intensive.

Finally, sampling schemes should account for several factors affecting colonization heterogeneity and its relationship to symptom development. These factors include: (i) within and between tree variation, (ii) method errors, (iii) canopy position, (iv) the substantial time-lag between fungal colonization and symptom development, and (v) appropriate sample dates for fungal colonization and symptom measurements.

4.6 Acknowledgements

The authors thank Wendy Sutton for help with the ergosterol and quantitative PCR assays. This research was funded through the Swiss Needle Cast Cooperative at Oregon State University - a consortium of industrial, federal, and state landowners in Oregon and Washington.
4.7 Literature cited


Chapter 5

Pseudothecia of Swiss Needle Cast Fungus, Phaeocryptopus gaeumannii, Physically Block Stomata of Douglas-fir, Reducing CO₂ Assimilation

Daniel K. Manter, Barbara J. Bond, Kathleen L. Kavanagh, Pablo H. Rosso, and Gregory M. Filip

5.1 Abstract

The following studies represent an effort to investigate the timing and mechanism of impact of Swiss needle cast (SNC) on Douglas-fir (Pseudotsuga menziesii) needle physiology (i.e., gas exchange). SNC is a foliar disease caused by the fungus Phaeocryptopus gaeumannii, which occurs throughout the range of Douglas-fir, and until recently has been considered unimportant. However, recent surveys show the SNC currently affects more than 52,611 hectares of forested lands in western Oregon causing a reduction in growth of ca. 23% or an implied growth loss of about 3.2 m³ ha⁻¹ yr⁻¹ for 1996 alone.

Gas exchange of artificially inoculated 2-year-old Douglas-fir seedlings was monitored on a monthly basis using A/Ci curve analysis. No impact of fungal presence on gas exchange was noted until the emergence of fungal fruiting structures (pseudothecia) from needle stomata. Once present, however, maximum stomatal conductance and CO₂ assimilation rates were inversely proportional to the presence of pseudothecia. A/Ci curve analysis showed that declines in CO₂ assimilation appeared to be due to both stomatal and non-stomatal limitations. Stomatal limitations to CO₂ assimilation were the direct result of reduced CO₂ diffusion through blocked stomata. Non-stomatal limitations arose, in part, from an indirect effect of pseudothecia development on rubisco activation. For example, in both SNC-affected foliage and foliage with artificially blocked stomata (i.e., external application of petroleum jelly), the amount of rubisco activation showed a strong, positive relationship with daily
maximum stomatal conductance. A mechanism is proposed that outlines the impact of pseudothecia development on stomatal conductance and CO₂ assimilation rates.

5.2 Introduction

Swiss needle cast (SNC) is a foliar disease caused by the fungus Phaeocryptopus gaeumannii (Rhode) Petrak. SNC occurs throughout the range of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) and until recently has been considered unimportant. However, recent surveys show that SNC currently affects more than 52,611 hectares of forested lands in western Oregon (Hansen et al., 2000) causing a reduction in volume growth of ca. 23 % or an implied growth loss of ca. 3.2 m³ ha⁻¹ yr⁻¹ for 1996 alone (D.A. Maguire, unpublished data).

Parasitic fungi extract the nutrients necessary for their growth and survival from the plant tissues that they invade, consequently reducing host growth and vigor. In addition to direct absorption of nutrients, fungi may also reduce host photosynthate production (e.g., Sutic and Sinclair, 1991; Scholes, 1992) through a variety of biochemical and/or structural means. Biochemical processes include changes in host processes (e.g., electron transfer chain, Montalbini et al., 1981) and enzymes (e.g., rubisco, Gordon and Duniway, 1982; Walters and Ayres, 1984) or the introduction of fungal enzymes, which regulate host physiology abnormally (e.g., invertase, Tang et al., 1992). Structural means include the loss of functional host tissue (e.g., necrosis) and physical blocking of intercellular spaces or stomata (Ayres 1976, 1981; Sutic and Sinclair, 1991). The latter has been suggested to be the major initial impact of P.
gaeumannii due to the presence of fungal fruiting bodies (pseudothecia) that emerge from needle stomata.

The hypothesis that P. gaeumannii affects Douglas-fir needle gas exchange mainly through blockage of stomata is largely based on circumstantial evidence. For example, microscopic work has shown that internal colonization by P. gaeumannii is limited to intercellular spaces with no obvious development of haustoria, penetration or necrosis of needle tissue (Capitano, 1999), and pseudothecial initials can be observed densely packed into needle stomata (Stone and Carroll, 1986). Based on these observations and preliminary field data (Manter, unpublished data) showing reduced gas exchange in Douglas-fir stands infected with P. gaeumannii, we conducted the following studies in order to quantify the impacts of P. gaeumannii infection on Douglas-fir needle physiology, especially the factors controlling CO2 assimilation rates, and to determine the mechanism of impact.

5.3 Methods

5.3.1 Plant material and inoculations

All measurements were conducted on potted 2-year-old Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) seedlings (Georgia-Pacific Corp., Cottage Grove, OR, USA). In May 1998, 50 seedlings were inoculated by placing seedlings in a chamber with an overhead misting system. Mist was applied for 15 sec every 60 min from 8 am to 8 pm, and 15 sec every 120 min from 8 pm to 8 am. An inoculum source was provided by branches infected with Phaeocryptopus gaeumannii (Rhode) Petrak,
collected from Sour Grass Summit, OR, USA, suspended over the target seedlings. Inoculum levels were monitored by weekly spore counts on glass slides suspended over the target seedlings (ca. 1 spore / mm²). After a two-week inoculation period, seedlings were incubated for two weeks in a greenhouse maintained at 21 °C under ambient light and humidity conditions. Immediately following the incubation period, a second round of inoculation (with newly-collected inoculum-source branches) and incubation treatments was applied. Following inoculation, seedlings were maintained in an outdoor cold frame at Oregon State University, Corvallis until future measurements. For each step of the inoculation procedure (i.e., inoculation, incubation, and storage), seedling position was varied haphazardly. To create non-infected control branches, two branches on each seedling were covered with bags ("D"-bag w/ polypropylene window, Northwest Mycological Consultants, Corvallis, OR, USA) during the inoculation and incubation periods. The bagging of branches was successful in reducing overall infection; however, some infection was eventually detected.

5.3.2 Fungal infection

The presence of *P. gaeumannii* was determined by visual estimates of fungal fruiting bodies (pseudothecia) emerging from stomata. Estimates of the percentage of needle stomata that were occluded with pseudothecia (i.e., "pseudothecia counts") for each branch were calculated by averaging pseudothecia counts from three positions on each needle present. At each position, one from each longitudinal third of the needle, pseudothecia counts were conducted by visually counting the number of pseudothecia
emerging from 100 consecutive stomata from the first complete row closest to the needle mid-rib.

5.3.3 Gas exchange measurements

A LiCor 6400 portable infrared gas exchange system (LiCor, Lincoln, NE, USA) was used to determine response curves of CO₂ assimilation (A/Cᵢ response curve, i.e., CO₂ assimilation rate versus calculated internal CO₂ concentration). During measurements, cuvette conditions were maintained at PAR 2000 μmol m⁻² s⁻¹, temperature 25 °C, [H₂O vapor] ≥ 18 mmol mol⁻¹ air, [CO₂] 40 Pa, and flow rate 100 μmol m⁻² s⁻¹, unless otherwise noted. A/Cᵢ response curves were measured by varying the cuvette CO₂ concentration, allowing equilibration to a steady state (cuvette [CO₂] coefficient of variation < 2%), and logging measurements every 10 sec for 1 min. CO₂ was varied in the following order: 40, 30, 20, 60, 80, 100, 120, 160, and 200 Pa.

Temporal variation in gas exchange was measured each month from October 1998 to June 1999 using one bagged (i.e., control) and one unbagged (i.e., infected) 1-year-old branch from at least two seedlings with exception of March (six seedlings) and April and May (five seedlings) – sample sizes were increased in March due to increased variation associated with the development of *P. gaeumannii*-pseudothecia. Beginning in March, low levels of infection (pseudothecia) were observed in most infected branches and some control branches. As a result, control branch needles were preferentially selected based on the absence of pseudothecia, and infected branch needles were selected based on the presence of pseudothecia.
A/C\textsubscript{i} curves were used to estimate some of the major limitations to net uptake of carbon into a plant following methods described by Farquhar \textit{et al.} (1980), Sharkey (1985), Harley and Sharkey (1991) and Harley \textit{et al.} (1992). All A/C\textsubscript{i} curve calculations are in Appendix I, and a list of abbreviations and parameters can be found in Appendix II.

5.3.4 Imaging chlorophyll fluorescence

In April 1999, needles (1998 cohort) from the six seedlings selected for the April A/C\textsubscript{i} curve analysis were also analyzed for chlorophyll fluorescence. Following gas exchange, sample branches (one infected and one control branch per seedling) were removed, re-cut under water, and then dark-adapted for 1 h. After the dark treatment, four or five needles were removed from each branch, cut transversely in half, and placed side-by-side on index cards creating two samples per branch (\textit{i.e.}, one sample with four or five needle tip-halves, and one with four or five needle petiole-halves). For each card, a 1-cm\textsuperscript{2} sample was measured for chlorophyll fluorescence. For each 1-cm\textsuperscript{2} sample, a two-dimensional image of fluorescence was created by means of an imaging fluorometer, a device that measures time-dependent fluorescence from an array of 31,680 positions per sample. A description of the imaging fluorometer used can found in Ning \textit{et al.} (1995).

For each position, an estimate of quantum yield (Y') was calculated from measurements of F\textsubscript{m} (the maximum fluorescence signal), F\textsubscript{s} (the low, steady-state level of fluorescence 105 sec after illumination), and F\textsubscript{dark} (the background level of
fluorescence); where \( Y = (F_m - F_s)/(F_m - F_{dark}) \). A more detailed explanation of the parameters and equations can be found in Ning et al. (1995) and Bowyer et al. (1998).

5.3.5 Rubisco activation

In order to confirm \( A/C_i \) estimates of rubisco activation (i.e., \( V_{cmax} \), see Appendix I), spectrophotometric assays of initial and total rubisco activity (\( R_I \) and \( R_T \), respectively) were measured from a random sample of ten seedlings. From each seedling, two samples (one infected and one control branch) of six needles (1998 cohort) were analyzed and the percent of activated rubisco (i.e., rubisco activation, \( R_{ACT} \)) was calculated as \( R_I / R_T \times 100 \). Needles were homogenized in 3 ml of extraction buffer (100 mM Bicine, 5 mM EDTA, 0.75 % (w/w) polyethylene glycol, 14 mM \( \beta \)-mercaptoethanol, and 1 % (v/v) Tween80; pH adjusted to 7.8 using 2N KOH). The extract was centrifuged at 13,000 g for 40 sec, and 50 \( \mu \)l of the supernatant was added to each of two samples of 900 \( \mu \)l of analysis buffer (100 mM Bicine (pH 8.0 at 25 C), 25 mM KHCO\(_3\), 20 mM MgCl\(_2\), 3.5 mM ATP, 5 mM phosphocreatine, 80 nkat glyderaldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, 80 nkat creatine phosphokinase, and 0.25 mM NADH). For initial rubisco activity 50 \( \mu \)l RuBP was added to one sample of the analysis buffer immediately (total preparation time was \( ca. < 2 \) min.) and changes in \( A_{340} \) were measured 15 sec later when a steady slope was observed. For total (fully activated) rubisco activity, RuBP was added after 15 min of activation and changes in \( A_{340} \) were measured.
In order to test the hypothesis that reduced stomatal conductance causes a decline in rubisco activation, we also measured rubisco activation on needles that were artificially induced to have lower stomatal conductance. To achieve this we covered the abaxial surface of selected needles (1999 needles with no visible pseudothecia only) with one of three different amounts of petroleum jelly (0, 50 and 100 % of projected leaf area). Each treatment level was applied to one secondary lateral branch on each of six randomly selected seedlings. Pre (< 30 min) and post (ca. 1 hr) treatment stomatal conductance was measured using a LiCor 6200 under natural conditions. One day after treatment, initial and total rubisco activity were measured on needles exposed to full sunlight.

5.3.6 Statistical analysis

All reported values are the mean for each measured branch. Within each sample date, differences in gas exchange parameters and fungal infection (pseudothecia density) between infected and non-infected branches were tested using a paired t-test (n = # of seedlings, see Gas Exchange Measurements). All linear regressions were calculated using Sigma Plot 4.0 (Jandel Scientific, San Rafael, CA, USA).

5.4 Results

5.4.1 Seasonal variation

The rate of net CO₂ assimilation differed seasonally in both infected and control branches (Figure 5-1a). Net assimilation declined by ca. 40 % between December and
January, associated with a reduction in stomatal conductance of ca. 60 % (Figure 5-1b). Assimilation rates remained constant at these reduced levels until March and the onset of budbreak. In March, assimilation declined another ca. 20 % for both infected and control branches. At this time, respiration rates also increased ca. 50 % (e.g., $R_{\text{day}}$ ca. 4 [March] and $R_{\text{day}}$ ca. 2 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ [October-February]) (Figure 5-1c). By June, assimilation rates of control branches recovered to 87 % of pre-winter rates and conductance ca. 80 %. In infected branches, however, no recovery in assimilation rates and conductance was observed; in fact, assimilation and conductance were at their lowest observed values, i.e. 26 and 15 % of the pre-winter values, respectively.

Seasonal changes in rubisco activation ($V_{\text{cmax}}$) mirrored changes in stomatal conductance (Figures 5-2a & 5-1b). $V_{\text{cmax}}$ declined ca. 22 % between December and January (Figure 5-2a) as compared to the 60 % decline in stomatal conductance (Figure 5-1b). $V_{\text{cmax}}$ remained depressed during the winter until recovery in the spring, which is also when stomatal conductance recovered. By May, $V_{\text{cmax}}$ recovered to pre-winter values (e.g., $V_{\text{cmax}}$ = 37.55 [May] and $V_{\text{cmax}}$ = 37.53 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ [October-December]). Similar to rubisco activation, RuBP regeneration ($J_{\text{max}}$) showed some seasonal changes, declining in the winter and recovering during the spring months (Figure 5-2b).

5.4.2 The impact of fungal infection

$P. gaeumannii$ internal and external hyphal colonization and biomass increases gradually following inoculation (Capitano, 1999, Manter, unpublished data); however,
the first sign of physiological impact due to infection occurred 10 months after inoculation, or only after pseudothecia emerged from > 5% of the needle stomata, i.e., March sample date (Figures 5-1 to 5-3). Reductions in net assimilation were associated with development of pseudothecia. For example, in April, when 18.9% of the stomata

Figure 5-1. Seasonal patterns of needle physiology (gas exchange) in 2-year-old Douglas-fir seedlings infected with *P. gaeumannii*. For each sample date, treatment differences were tested using a paired t-test, * denotes p < 0.05. Net assimilation rate was measured at PAR 2000 μmol m⁻² s⁻¹, temperature 25 °C, [H₂O vapor] ≥ 18 mmol mol air⁻¹, [CO₂] 40 Pa, and flow rate 100 μmol m⁻² s⁻¹. Stomatal conductance is the average rate of conductance measured during the entire A/C₁ curve. Calculations of Day Respiration can be found in Appendix I.
Figure 5-2. Seasonal patterns of biochemical limitations to gas exchange in 2-year-old Douglas-fir seedlings infected with *P. gaeumannii*. For each sample date treatment differences were tested using a paired t-test, * denotes $p < 0.05$. Calculation of $V_{cmax}$ and $J_{max}$ can be found in Appendix I.

![Graph showing seasonal patterns of biochemical limitations to gas exchange](image)

contained *P. gaeumannii* pseudothecia, net assimilation rates in ambient conditions (*i.e.*, PAR *ca.* 2000 μmol m$^{-2}$ s$^{-1}$, [CO$_2$] 35.5 Pa, [H$_2$O vapor] *ca.* 18 mmol mol$^{-1}$ air) declined by 50 %, compared to control branches (Figures 5-1a & 5-3). Other physiological changes associated with infection development include stomatal conductance reduced by 37 %, rubisco activation reduced by 40 %, and RuBP regeneration reduced by 31 % compared with control branches (Table 5-1).
Figure 5-3. Seasonal patterns of *P. gaeumannii* infection in inoculated 2-year-old Douglas-fir seedlings. For each sample date treatment differences were tested using a paired t-test, * denotes $p \leq 0.05$.

No true control seedlings were used in our study, because branches adjacent to inoculated branches were covered with bags to obtain non-infected branches. It is possible that fungal growth on the infected branches influenced the physiology of the entire seedling via water stress, growth regulator, or source/sink relations. However, as discussed below, none of these factors appear to be associated with *P. gaeumannii* infection, and no "compensatory" effect (e.g., an increase in net CO$_2$ assimilation rate) was observed in the control branches; all physiological parameters remained relatively constant throughout the study, except during the winter depression (Figures 5-1 to 5-3).
Table 5-1. Infection level and A/C\textsubscript{i} curve parameters from six 2-year-old Douglas-fir seedlings infected with \textit{P. gaeumannii}, April 1999.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>p-value \textsuperscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Infected</td>
</tr>
<tr>
<td>Pseudothecia Count</td>
<td>0.23(0.14)</td>
<td>18.91(2.83)</td>
</tr>
<tr>
<td>Stomatal Conductance</td>
<td>52.70(9.32)</td>
<td>33.18(5.02)</td>
</tr>
<tr>
<td>CO\textsubscript{2} Assimilation Rate</td>
<td>4.40(0.36)</td>
<td>2.20(0.24)</td>
</tr>
<tr>
<td>V\textsubscript{cmax}</td>
<td>32.71(3.55)</td>
<td>19.43(1.52)</td>
</tr>
<tr>
<td>J\textsubscript{max}</td>
<td>99.69(12.13)</td>
<td>68.70(11.78)</td>
</tr>
<tr>
<td>Day Respiration</td>
<td>3.94(0.76)</td>
<td>4.29(0.30)</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Pseudothecia Count is the percent of stomata occluded with pseudothecia (see “Methods” for further explanation). Stomatal conductance is the average value from the entire A/C\textsubscript{i} curve measurement. CO\textsubscript{2} assimilation rates are single point measurements of assimilation at [CO\textsubscript{2}] ca. 35.5 Pa, PAR ca. 2000 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}, [H\textsubscript{2}O vapor] > 18 mmol mol\textsuperscript{-1} air. \textit{V\textsubscript{cmax}}, \textit{J\textsubscript{max}}, and day respiration are derived from A/C\textsubscript{i} curves as described in the text.

\textsuperscript{2}One control and one infected branch from each of six 2-year-old Douglas-fir seedlings were measured. Values reported are the mean and standard errors of the mean.

\textsuperscript{3}p-values were calculated using paired t-tests between control and infected branches for the six seedlings sampled.

The relationship between fungal infection and stomatal conductance was determined by a regression between the percent decline in stomatal conductance (\textit{g\textsubscript{sw.control}} - \textit{g\textsubscript{sw.infected}} / \textit{g\textsubscript{sw.control}} \times 100) and pseudothecia presence (infected - control) based on the observations from each seedling in April - June (Figure 5-4). A strong positive-linear relationship between pseudothecia presence and the percent decline in stomatal conductance was detected (adjusted \textit{R\textsuperscript{2}} = 0.927, \textit{p} < 0.0001).
Figure 5-4. Relationship between stomatal conductance and *P. gaeumannii* pseudothecia in 2-year-old Douglas-fir seedlings. Each observation represents differences between sample branches from each of the seedlings measured during the April – June sample dates. Percent Decline in Stomatal Conductance = \((g_{sw\_control} - g_{sw\_infected}) / g_{sw\_control}\) * 100. Percent Difference in Pseudothecia Count = infected – control.

Figure 5-5 depicts the average A/C\(_i\) or “CO\(_2\) demand” curve (calculated by inputting the mean values of \(V_{cmax}, J_{max}\) and \(R_{day}\) [from Table 5-1] in equation 2) and “CO\(_2\) supply” curve (calculated by inputting the mean values of \(g_{sc}\) [from Table 5-1] in equation 4 when \(C_a = 35\) Pa). The “CO\(_2\) demand” curve represents the response of photosynthesis to internal CO\(_2\) concentration, demonstrating for the infected branches, photosynthesis is lower at all internal CO\(_2\) concentrations. The “CO\(_2\) supply” curve depicts the amount of CO\(_2\) entering the needle for a given stomatal conductance and atmospheric CO\(_2\) concentration. The intersection of these two curves, or the point
where the supply of CO₂ entering the needle through the stomata and the biochemical demand for CO₂ are equal, has been termed the "operating point" and should approximate the realized rate of CO₂ assimilation at the designated C_a concentration (Jones, 1985). When this analysis is applied to our April measurements, predicted net assimilation rates (i.e., the "operating point") at ambient CO₂ (35 Pa) are ca. 4.7 and 1.9 μmol CO₂ m⁻² s⁻¹ for control and infected branches, respectively. These rates are similar to the measured rates of 4.4 and 2.2 μmol CO₂ m⁻² s⁻¹, respectively (Table 5-1).

Figure 5-5. Average A/C_i curves for control and P. gaeumannii-infected branches in 2-year-old Douglas-fir seedlings sampled in April 1999. Supply and demand curves were determined from the average values of V_{cmax}, J_{max}, R_{day} and conductance (Table 1) for each treatment (control [solid] and infected [dashed]) using the equations found in Appendix I.
Changes in net CO₂ assimilation rates may occur through changes in either the CO₂ demand curve and/or the CO₂ supply curve. As shown in Figure 5-5 both curves are affected by *P. gaeumannii* infection. If we assume stomatal conductance and CO₂ supply affect infected needles first (see discussion below), followed by changes in rubisco activation and CO₂ demand, then it appears that net CO₂ assimilation in infected needles is limited by approximately equal stomatal and biochemical limitations. For example, as stomatal conductance declines, the operating point shifts from $A_{\text{control}}$ to $A_2$, resulting in a ca. 26% decline in net CO₂ assimilation. Next, as the demand curve changes, the operating point shifts from $A_2$ to $A_{\text{infected}}$, reducing net CO₂ assimilation by ca. 34% or slightly more than half of the total decline in net ambient assimilation rate due to infection.

5.4.3 Rubisco activation

Similar to the $A/C_i$ curve analysis, spectrophotometric assays of rubisco activation also showed that branches infected with *P. gaeumannii* had a reduced amount of activated rubisco compared to control branches (78.7 ± 3.2 and 48.0 ± 23.7% for the control and infected branches, respectively). However, no difference in the total rubisco activity was detected (10.1 ± 1.0 and 10.5 ± 0.9 µmol m⁻² s⁻¹ for the control and infected branches, respectively).

Spectrophotometric assays of rubisco activation in needles treated with petroleum jelly showed that rubisco activation is lower in treated branches (i.e., lower stomatal conductance). In these seedlings, pre-treatment stomatal conductance did not significantly differ between branch samples (stomatal conductance was 142.7 ± 9.07,
143.7 ± 12.7, and 139.5 ± 7.2 mmol m² s⁻¹ for 0, 50 and 100 % treatments, respectively); however, stomatal conductance was significantly reduced following treatment with petroleum jelly (stomatal conductance was 69.8 ± 8.67, 48.86 ± 7.4 and 27.92 ± 4.5 mmol m² s⁻¹ for 0, 50 and 100 % treatments, respectively). The overall decline in stomatal conductance (e.g., ca. 50 % in controls) was associated with an increased vapor pressure deficit during the post-treatment measurements. A strong linear relationship (adjusted $R^2 = 0.736, p < 0.0001$) exists between the percent decline in stomatal conductance following treatment ($gsw_{\text{treatment}_0} - gsw_{\text{treatment}_i} / gsw_{\text{treatment}_0} \times 100$, where $i$ is either the 50 or 100 % treatment level) versus the percent decline in rubisco activation ($R_{\text{ACT}_{\text{treatment}_0}} - R_{\text{ACT}_{\text{treatment}_i}}$, where $i$ is either the 50 or 100 % treatment level) (Figure 5-6a). A similar relationship between the percent decline in stomatal conductance due to $P. gaeumannii$ infection ($gsw_{\text{control}} - gsw_{\text{infected}} / gsw_{\text{control}} \times 100$) versus the percent decline in rubisco activation ($V_{\text{cmax}_{\text{control}}} - V_{\text{cmax}_{\text{infected}}} / V_{\text{cmax}_{\text{control}}} \times 100$) was obtained using the A/Ci curve analysis of diseased seedlings ($R^2 = 0.777, p < 0.0001$) (Fig. 5-6b).

5.4.4 Chlorophyll fluorescence

Chlorophyll fluorescence was measured to assess both quantum efficiency (e.g., Ning et al., 1995; Bowyer et al., 1998) and the presence of “patchy” stomatal closure (e.g., Buckley et al., 1997). Figure 5-7 shows typical images of quantum efficiency ($Y'$) from one control and one infected sample of needles from the same seedling. As can be seen in this figure no spatial variation in quantum efficiency can be observed within measured needles. In other words, each set of needles measured had a quantum
Figure 5-6. Relationship between changes in stomatal conductance and rubisco activation. Panel a was determined from spectrophotometric analysis of rubisco activation from petroleum jelly treated needles. Each observation represents differences between treated needles from each of six seedlings measured in July 1999. Percent decline in rubisco activation = $R_{ACT \_treatment \_0} - R_{ACT \_treatment \_i}$, where i is either the 50 or 100 % treatment level. Percent decline in stomatal conductance = $g_{sw \_treatment \_i} / g_{sw \_treatment \_0} \times 100$, where i is either the 50 or 100 % treatment level. Stomatal conductance was measured 1 hr after treatment under ambient conditions. Panel b was determined from $A/C_{i}$ curve analysis of P. gaeumannii infected seedlings. Each observation represents differences between control and infected branches. Percent decline in rubisco activation = $V_{cmax \_control} - V_{cmax \_infected} / V_{cmax \_control} \times 100$. Percent decline in stomatal conductance = $g_{sw \_control} - g_{sw \_infected} / g_{sw \_control} \times 100$. Stomatal conductance is the average stomatal conductance measured during $A/C_{i}$ curve analysis at optimal conditions.
efficiency that showed a unimodal distribution of $Y'$ values (data not shown).

No significant differences between needle segments were detected (i.e., tip and petiole halves, data not shown), therefore, the average value of the tip and petiole halves for each branch-treatment combination was used for subsequent analyses. No difference in quantum efficiency was detected between control and infected branches from the same seedling (0.693 ± 0.014 and 0.686 ± 0.014 for the control and infected branches, respectively). In addition, no differences were detected in $F_m$, $F_s$ or $F_{dark}$ (data not shown).

Figure 5-7. Imaging chlorophyll fluorescence ($Y'$) of 2-year-old Douglas-fir seedlings infected with *P. gaeumannii*. Panels a and c are images of chlorophyll fluorescence ($Y'$). Panels b and d are digital images showing the presence of pseudothecia from the region outlined in the adjacent panel.
5.5 Discussion

5.5.1 Seasonal variation

Seasonal changes in needle physiology (i.e., gas exchange) were observed for both infected and control branches of 2-year-old Douglas-fir seedlings. However, no difference between infected and non-infected branches was noted until there were obvious pseudothecia present in infected needles. Depressions of needle physiology during the winter are a typical phenomenon in conifers, particularly at higher elevations (e.g., Havranek and Tranquillini, 1995). In our work, winter depressions of Douglas-fir needle physiology have been detected in seedlings (present study) and field trees, ca. 15-years-old (K.L. Kavanagh, unpublished data). Although the cause(s) of these depressions were not directly investigated, other work with conifers suggests that increased levels of abscisic acid (Qamaruddin et al., 1993) may be one of the factors responsible for the increased stomatal closure and decline in conductance. Similar to the disease mechanism presented below, the reduced supply of CO₂ from the low stomatal conductance may cause a down-regulation in the activation of rubisco. However, “normal seasonal changes in cell physiology” (Havranek and Tranquillini, 1995, and references therein), e.g., membrane permeability, should not be overlooked.

5.5.2 Fungal impacts and mechanism

Following pseudothecia emergence, stomatal conductance and carbon assimilation are both reduced in infected branches.
Based on our studies here we propose the following mechanism through which *P. gaeumannii* affects Douglas-fir needle physiology and potential productivity (Figure 5-8). The greatest fungal impact results from the formation of pseudothecia, a resultant decline in stomatal conductance and the development of stomatal and non-stomatal limitations to CO₂ assimilation (steps 1-5). Additional non-stomatal limitations to CO₂ assimilation may also arise from an as yet undetermined fungal effect (step 3b). Once assimilation rates have been reduced, needles will be less productive and over time give rise to the common Swiss needle cast disease symptoms of chlorosis, needle abscission, and growth loss (step 6).

In our model, the major impact of disease occurs with the emergence of fungal pseudothecia. At this point, stomatal conductance and net CO₂ assimilation are reduced. Understanding the mechanisms responsible for the changes in either one of these parameters is complicated by the fact that both have been shown to influence the other. It is our contention, however, that a decrease in stomatal conductance (and increased stomatal limitations to net CO₂ assimilation) occurs first due to the formation of pseudothecia, followed by an increase in biochemical limitations to net CO₂ assimilation due to reduced rubisco activation.

A direct effect of pseudothecia on stomatal conductance is expected considering the physical presence of pseudothecia in needle stomata. Furthermore, the strong relationship between pseudothecia presence and decline in stomatal conductance suggests that pseudothecia presence is the causal factor reducing stomatal conductance in SNC-affected Douglas-fir foliage. If the relationship presented in figure 5-4 is extrapolated to zero stomatal conductance (*i.e.*, 100% decline in stomatal conductance),
then only ca. 58% of the stomata need be occupied by pseudothecia. This may be due to the presence of other fungal structures that also block gas exchange through needle stomata. One structure, pseudothecia initials (i.e., generative hyphae), may prove to be the best indicator of fungal impact on stomatal conductance, because these structures can be found densely packed into needle stomata with or without attached mature pseudothecia (Stone and Carroll, 1986). Secondly, surface hyphae in *P. gaeumannii*-infected foliage can at times form relatively dense mats of hyphae on the needle surface.
(Capitano, 1999), and these structures may also physically block needle gas exchange, as has previously been shown with powdery mildew (Ayres 1976; 1981).

Concurrent with the decline in stomatal conductance, net CO₂ assimilation is reduced through both stomatal and biochemical means. We suggest that the biochemical limitations, due to rubisco deactivation, result from the reduced stomatal conductance and internal CO₂ concentration. In other studies, the amount of activated rubisco was affected by other means such as water stress (Kanechi et al., 1996; Tezara et al., 1999), feedback inhibition (e.g., Scholes, 1992), nitrogen concentration (Farquhar et al., 1980) and fungal toxins (Scholes, 1992). However, if any of these other factors were limiting rubisco activation in P. gaeumannii-infected needles, then we would have expected them to be present during the long infection period prior to pseudothecia formation. Instead our research shows that reduced rubisco activation, due to fungal presence, was only present after pseudothecia formation. Furthermore, any fungal consumption of key nutrients such as nitrogen does not appear to be related to the decline in rubisco activation as no change in total rubisco activity (R_T) was detected in infected branches.

Two possible mechanisms are likely to explain the relationship between stomatal conductance and rubisco activation. The first potential mechanism involves what is commonly referred to as “patchy” stomatal closure (e.g., Terashima et al., 1988; Terashima, 1992; Mott, 1995). In this scenario, stomata are closed in groups, resulting in a needle-wide conductance distribution that has distinct modes; typically, it is bimodal with one region possessing a normal conductance and the other little to no conductance (e.g., Buckley et al., 1997). If such a distribution is present, A/C_i curve
analysis may erroneously show non-stomatal limitations due to an over-estimate of \( C_i \) (Terrashima et al., 1988).

In the case of *P. gaeumannii*-infected foliage, however, patchy stomatal closure does not appear to be responsible for our observed biochemical limitations to assimilation. First, spectrophotometric assays of rubisco activation, which do not rely on estimates of \( C_i \), confirmed that rubisco activation is reduced in infected needles. Second, chlorophyll fluorescence images show that quantum efficiency, in both infected and control needles, conform to a unimodal distribution.

The second possible mechanism is that the reduced CO\(_2\) supply due to lower stomatal conductance results in a reduction in the amount of activated rubisco. In order for rubisco to act as a carboxylase and fix carbon, it must first be activated. One of the steps in activation involves carbamylation of the active site with a CO\(_2\) molecule (Lorimer, 1981). Therefore, following the decline in stomatal conductance, and a reduction in the supply of internal CO\(_2\), it is possible that the amount of activated rubisco declines resulting in our observed reductions in \( V_{c\text{max}} \). In order to test the sensitivity of rubisco activation in Douglas-fir to internal CO\(_2\) concentration, we measured rubisco activation from healthy needles that had an artificially reduced stomatal conductance. Based on these studies, rubisco activation in Douglas-fir needles is influenced by decreasing stomatal conductance and the resulting decline in internal CO\(_2\) concentration. In fact, rubisco activation showed a linear relationship with maximal stomatal conductance, decreasing as stomatal conductance decreased. To our knowledge, a linear relationship between maximum stomatal conductance (and the associated decline in internal CO\(_2\) concentration) and rubisco activation has not
previously been shown. However, rubisco activation has been shown to decline below some threshold level of internal CO₂, which varies between species (e.g., Sage et al., 1990; von Caemmerer and Edmondson, 1986).

Reductions in rubisco activation in *P. gaeumannii*-infected foliage also appear to arise directly from the decline in internal CO₂ concentration for the following reasons. First, biochemical limitations to net assimilation were consistently associated with changes in stomatal conductance (*i.e.*, *P. gaeumannii*-infected seedlings, petroleum jelly-treated seedlings, and winter-associated physiological depressions). Second, no changes in any of the chlorophyll fluorescence parameters were detected following fungal infection. From these data we can infer that *P. gaeumannii* infection appears to have no direct impact on the level and function of energy capture and electron transfer, or any of the other physiological processes typically associated with changes in chlorophyll fluorescence (e.g., Kraus and Weis, 1991). Third, physical reductions in stomatal conductance (*i.e.*, petroleum jelly treated needles) of healthy needles resulted in reduced rubisco activation. And fourth, no differences in total rubisco activity were detected.

Reduced stomatal conductance and CO₂ supply may not fully account for the changes in rubisco activation of *P. gaeumannii* infected needles (Figure 5-6a & b). For example, in the petroleum jelly-treated trees, when stomatal conductance declined by 50 %, rubisco activation was reduced by ca. 21 %; however, in SNC-affected seedlings, when fungal presence caused a 50 % reduction in stomatal conductance, rubisco activation was reduced by ca. 43 %. Assuming that the observed differences are not related to the differing methodologies (spectrophotometric vs. A/Cᵢ curve determination
of rubisco activation), then only ca. 50% of the non-stomatal limitations in *P. gaeumannii*-infected foliage can be attributed to the stomatal conductance mechanism explained above. The increased sensitivity to stomatal conductance and internal CO₂ supply in *P. gaeumannii*-infected needles, if it is truly present, deserves further attention.

Finally, only after the decline in net assimilation occurs do we reach the final stage of disease development, or symptoms expression. During this stage, the commonly observed patterns of chlorosis, needle abscission and growth loss develop in *P. gaeumannii* infected foliage.

### 5.6 Acknowledgements

This research was funded through the Swiss Needle Cast Cooperative at Oregon State University - a consortium of industrial, federal, and state landowners in Oregon and Washington. The authors wish to thank Dr. Larry S. Daley and Li Ping for kindly providing us with instruction and use of their imaging chlorophyll fluorometer; and Dr. Lailiang Cheng and Guillaume Gruere for assistance with the spectrophotometric rubisco assays. We are also grateful to Dr. Jeffrey Stone for review and helpful discussions.
5.7 Literature cited


Chapter 6

Regulation of Stomatal Conductance in Douglas-fir Needles Infected with *Phaeocryptopus gaeumannii*

Daniel K. Manter and Kathleen L. Kavanagh
6.1 Abstract

*Phaeocryptopus gaeumannii*, a foliar pathogen of Douglas-fir, is the causal agent of Swiss needle cast. The presence of its pseudothecia in needle stomata has been shown to reduce maximum stomatal conductance ($g_s$) and photosynthesis. However, over the course of a typical day, trees do not maintain maximum rates of gas exchange. Instead, they regulate stomatal openings in response to a variety of climatic and host cues, and this daily-integrated pattern of gas exchange should be a good indicator of potential carbon assimilation and growth. We monitored daily patterns of $g_s$, and the response to vapor pressure deficit (VPD) in *P. gaeumannii*-infected trees.

Maximum $g_s$ was inversely proportional to *P. gaeumannii* fruiting bodies (pseudothecia) emerging from stomatal chambers, and proportional to branch estimates of leaf specific conductance. In all plants a negative linear relationship between $g_s$ and VPD was observed; however, the slope varied with *P. gaeumannii* infection levels and site microclimate. We attribute the change in slope to a shift in the equilibrium between the liquid and vapor fluxes in the plant that directly impact $\Psi_{\text{leaf}}$ and, as a result, stomatal regulation. Stomatal regulation consistently maintained $\Psi_{\text{leaf}}$ above a critical threshold; however, the time it took to reach this value varied. In plants with a reduced water vapor flux only (*i.e.*, $g_s$) critical $\Psi_{\text{leaf}}$ values were reached later in the day, however, this delay was offset by reductions in the liquid flux capacity (*i.e.*, $K_L$) of the xylem.

The decline in daily-integrated stomatal conductance varied with *P. gaeumannii* infection and site surveyed. It was observed that the greatest reductions occurred on
trees growing on south-facing slopes where total-plant leaf specific conductivity was most strongly affected by *P. gaeumannii* infection. Finally, because carbon assimilation relies on the passive diffusion of CO\textsubscript{2} into needles, we suggest that the observed patterns of daily-integrated stomatal conductance, and CO\textsubscript{2} diffusion, will directly influence the potential growth and survival of *P. gaeumannii*-infected Douglas-fir trees.

6.2 Introduction

Infection of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needles by the foliar pathogen, *Phaeocryptopus gaeumannii* (Rhode) Petrak, reduces gas exchange (H\textsubscript{2}O and CO\textsubscript{2}) by apparent blockage of needle stomata (Manter *et al.*, 2000). The physical obstruction of gas exchange is not surprising given the growth pattern of *P. gaeumannii*, which produces dense mats of hyphae in stomatal antechambers and fruiting bodies (pseudothecia) at stomatal openings (Stone and Carroll, 1986). In artificially inoculated seedlings, Manter *et al.* (2000) report that maximal stomatal conductance and net assimilation rates are inversely proportional to the number of stomata occluded with pseudothecia. The study reported here investigates stomatal regulation in Douglas-fir trees with a range of maximum stomatal conductances due to the presence of *P. gaeumannii*-infection.

Plants regulate stomatal openings in order to minimize water loss and maximize carbon gain (Cowan, 1977; Williams *et al*. 1996), typically resulting in a decline in stomatal conductance over the course of a day. Various factors such as water vapor pressure of the air at the leaf surface (Ball *et al.*, 1987; Leuning, 1995), soil water availability (Tardieu and Davies, 1993; Van Wijk *et al.*, 2000), xylem conductivity
(Sperry, 1986), and plant water potential (e.g., Wullschleger et al. 1998) have all been shown to affect stomatal conductance. Furthermore, species differ in their relative response to these variables, for example, *P. menziesii* responds more strongly to VPD (e.g., Van Wijk et al., 2000) than other species, particularly angiosperms (Bond and Kavanagh, 1999). While all of the above factors can affect stomatal regulation, it is becoming increasingly evident that many plants regulate stomatal apertures, in an effort to maintain leaf water potentials (\(\Psi_{\text{leaf}}\)) above a critical threshold (Jones 1990; Tardieu, 1993; Saliendra et al., 1995; Bond and Kavanagh, 1999) avoiding injury such as xylem cavitation (Tyree and Sperry, 1988).

Several authors (e.g., Jones and Sutherland, 1991; Dewar, 1995; Whitehead, 1998; Bond and Kavanagh, 1999) have proposed that the interactive effects of the above factors on stomatal regulation can be modeled mathematically by:

\[
g_s \cdot VPD = K_L \cdot (\Psi_{\text{soil}} - \Psi_{\text{leaf}}),
\]

where \(g_s\) is stomatal conductance, VPD is the leaf-to-air vapor pressure gradient, \(K_L\) is total plant leaf-specific conductance, and \(\Psi_{\text{soil}}\) and \(\Psi_{\text{leaf}}\) are water potential of the soil and leaf, respectively. This equation assumes that the transpirational flux of water vapor (\(g_s \cdot VPD\), water loss from the leaf) is in equilibrium with the liquid flux of water through the plant (\(K_L \cdot [\Psi_{\text{soil}} - \Psi_{\text{leaf}}]\), water supplied to the leaf). Due to the elasticity of plant cells and the contribution of stored water to the total water flux in plants, these two fluxes are not always in equilibrium (e.g., Phillips et al., 1997); however, this model does provide a useful formulation for investigating which factors influence stomatal regulation and how. Furthermore, the contribution of stored water and
capacitance to water flux in young Douglas-fir (20- years-old) has been estimated at only ca. 7 % of the total daily water flux (N. Phillips, personal communication).

The two plant water flux components contained in equation 1, are both diffusional equations based on Fick’s Law, e.g., the observed flux rate is equal to a conductance \( (g_s, K_L) \) times a driving force \( (VPD, \Psi_{soil} - \Psi_{leaf}) \). The two conductance parameters are related to the permeability of that feature. For example, \( g_s \) is determined by the openness of the stomata, and \( K_L \), the permeability of the whole-plant from the soil to the leaf. Thus \( K_L \) is comprised of a root, stem and branch component that varies among species (Tyree and Ewers, 1991) and with plant size an age (Hubbard et al., 1999). The variations in \( K_L \) have been attributed to above and belowground pathlength (Waring and Sylvester, 1994; Sperry et al., 1998), root membrane permeability, xylem structure \( (e.g., \) tracheid diameter and length), branch junctions and the ratio of leaf area to sapwood area (Sperry, 1995). Since water movement through the roots, stem and branches occurs through dead, cylindrical xylem cells, the influence of many of these factors on a theoretical maximum \( K_L \) can be described using Poiseulle’s Law:

\[
K_L = \frac{(K_S \cdot SA)}{(l \cdot \eta \cdot LA)} \tag{2}
\]

\[
k_s = \frac{(\pi / 128 (\Sigma d^3))}{SA} \tag{3}
\]

where \( k_s \) is average sapwood permeability (specific conductivity), \( SA \) is sapwood cross sectional area, \( \eta \) is the viscosity of water at a given temperature, \( LA \) is the supplied leaf area and \( d \) is the xylem cell diameter \( (e.g., \) Whitehead, 1998; Bond and Kavanagh, 1999). Therefore, much of the variation in \( K_L \) can be attributed to changes in the xylem architecture \( (\) total area and cell diameter) relative to the supplied leaf area (Whitehead, 1998).
Equation 4 is a reorganized version of equation 1,

$$\Psi_{\text{leaf}} = \Psi_{\text{soil}} - (g_s \cdot \text{VPD}) / K_L$$  \hspace{1cm} (4)

and based on this equation the variety of factors ($\Psi_{\text{soil}}$, $g_s$, VPD, and $K_L$) influencing $\Psi_{\text{leaf}}$ are evident. For example, if the leaf water loss increases (↑ VPD), $\Psi_{\text{leaf}}$ will decline; or if the water supplied to the leaf decreases (↓ $\Psi_{\text{soil}}$ and / or ↓ $K_L$) decline, $\Psi_{\text{leaf}}$ will decline. In other words, the net balance of water loss and supply determines $\Psi_{\text{leaf}}$.

In a previous study, the above equations have been successfully employed to predict diurnal changes in stomatal conductance in four species, including Douglas-fir (Bond and Kavanagh, 1999). As VPD increased over the course of the day, water loss eventually exceeded the water supply capacity, reducing $\Psi_{\text{leaf}}$, and inducing stomatal closure.

In *P. gaeumannii*-infected trees, we expect a similar pattern of stomatal regulation to occur. However, the timing and degree of stomatal closure in response to water loss (VPD) may be altered. If the supply capacity in *P. gaeumannii*-infected trees is not altered, then due to their lower maximum $g_s$, infected needles should be losing less water early in the day (low VPDs) maintaining higher $\Psi_{\text{leaf}}$ and $g_s$ over the course of the day (high VPDs).

Previous observation has suggested that climate may influence *P. gaeumannii* colonization and symptom development. For example, trees growing on south slopes tend to have greater symptom development (*e.g.*, needle abscission). Some of the greater symptom development has been related to increased fungal colonization during the fall and winter; however, even at similar infection levels south slope trees exhibit
greater symptom development (Manter et al. 2001). We suspect that the influence of climate on stomatal regulation may be a contributing factor. Since stomata are the entry points of CO₂ into the leaf, the time course of stomatal conductance will influence the carbon assimilation of the leaf (Williams et al. 1996). Thus the higher incident solar radiation associated with south slopes, which increases air and soil temperatures, VPD and lowers Ψ̂_soil (Grace et al., 1981; Jones, 1992), may cause earlier stomatal closure, reduced daily carbon assimilation rates, and greater symptom development.

The objectives of this study were to quantify patterns of stomatal regulation (diurnal and VPD-response) in _P. gaeumannii_ -infected seedlings and field trees accounting for the interactive effects of Ψ̂_leaf, Ψ̂_soil, and K_L. Diurnal patterns of stomatal conductance in field trees growing under different climates (north and south slopes) were also quantified.

### 6.3 Methods

#### 6.3.1 Seedling studies

##### 6.3.1.1 Plant material

Two-year-old Douglas-fir seedlings (_Pseudotsuga menziesii_ (Mirb.) Franco, Starker Forests Inc., Corvallis, OR, USA) with different levels of _Phaeocryptopus gaeumannii_ (Rhode) Petrak infection were selected from an ongoing study. Seedlings were incubated for 2, 4, or 8 weeks in the understory of _P. gaeumannii_ -infected trees (Salal plot, see Hansen et al., 2000) from May to July 1999. Peak spore release at this
site occurred in June 1999; and in general, seedlings exposed in May had no \( P. \) 
gaeumannii infection, and those incubated in June had increasing infection with 
increasing incubation times (J.K. Stone, unpublished data).

6.3.1.2 Gas exchange measurements

In April 2000, 5 seedlings with varying levels of \( P. \) gaeumannii were measured 
for the response of stomatal conductance (\( g_s \)) to vapor pressure deficit (VPD). A LiCor 
6400 was used to manipulate VPD by changing air temperature and allowing RH to co-
vary. Air temperatures were set at 15, 18, 20, 25, 28, and 30 C, and following a 45 min 
equilibration period, gas exchange was logged every 20 sec for 1 min. During 
measurements PAR was maintained at 2000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) and \( CO_2 \) concentration at ca. 
35 Pa.

6.3.2 Field studies

6.3.2.1 Site selection

Three 12- to 15-year-old Douglas-fir plantations (i.e., Beaver, Hebo, and Mac 
sites) with varying levels of Swiss needle cast as described in Manter et al. (2001) were 
chosen for study. Paired permanent plots were created on north- and south-aspect 
slopes (10 - 30 %) at each site. Each permanent plot consisted of a group of six infected 
trees and six artificially created controls. Artificial “control” trees were sprayed with a 
fungicide to prevent new \( P. \) gaeumannii infections (Chlorothalonil, rate = 66 ml / 3.78
L, applied until run-off). Fungicide applications were conducted in 1998, 1999, and 2000 at bud break (90% trees had broken bud) and one month following bud break.

6.3.2.2 Gas exchange measurements

In the summer months (June – September 1999), diurnal measurements of gas exchange and water potential were measured on all twelve trees at each site. Concurrent measurements at both plots within a site (i.e., north- and south-facing slopes) were conducted using two sets of equipment. Diurnal measurements on one sun-exposed branch per tree included pre-dawn water potentials, bi-hourly gas exchange, all needle cohorts present, (Li-1600, LiCor, Lincoln, NE) and leaf water potential (Scholander-Hammel pressure chamber, PMS Instruments, Corvallis, OR) measurements commencing at dawn or immediately after foliage dried-off. At the end of the day, all measured foliage was removed and measured for one-sided projected leaf area (Agimage, Decagon Devices, Pullman, WA).

In July 2000 the response of stomatal conductance to vapor pressure deficit was measured by means of a LiCor 6400 (as described above) on 1-year-old needles from seven branches of the Beaver-south trees, selected for varying levels of \( P. gaeumannii \) pseudothecia density (0-56.1 %).
6.3.2.3 Disease assessment

Surveys for the presence of *P. gaeumannii* were conducted on all needles measured for gas exchange, and the average pseudothecia density for each set of needles measured is reported. Pseudothecia density is the percent of stomata with visible fungal fruiting bodies determined by the methods outlined in Manter *et al.* (2000).

6.3.2.4 Hydraulic conductance

In September 2000, one 4-year-old branch from each of three spray and three nospray trees at each site was harvested, sealed in a plastic bag, and stored at 0°C. Within 48 hrs stem water flux was measured on a 3-5 cm segment from the base of the main stem from the current-, 1-, and 2-year-old nodes as outlined in Kavanagh *et al.* (1999). Four consecutive 60-sec flux measurements were made on each stem segment by connecting one end of the stem segment to tubing containing a solution of degassed dH₂O pressured to 12.5 kPa by gravity and measuring the weight of water efflux from the opposite end of the stem segment. Total leaf area (LAᵢ) from each age class on a sample branch was determined by multiplying the leaf area to dry weight ratio determined from a sub-sample of *ca.* 50 needles by the total needle dry weight present on the branch. From this data a branch-leaf specific conductance (K_L_B, μmol m⁻² s⁻¹ MPa⁻¹) was calculated.

\[ K_{L_B} = \frac{F}{p} / LA_i, \]  (5)
Where $F$ is the average flux ($\mu$mol s$^{-1}$), $p$ is the pressure gradient (1 MPa), and $LA$ is the total leaf area downstream of the stem segment ($m^2$).

### 6.3.2.5 Meteorological data

All climatic data required for model parameterization (RH, T, and PAR) were collected continuously in 1999 and 2000 at 15-min intervals using mini-dataloggers from Spectrum Technologies (Plainfield, IL, USA). One set of dataloggers was installed at a height of 1.5 m in an opening (radius > 5 m) at each study site.

### 6.4 Results

Fungicide applications were successful in preventing infections of $P. gaeumannii$ in foliage produced after the initial treatment (1998). A detailed 2-year survey of $P. gaeumannii$ infection in these plots has been presented elsewhere (Manter et al. 2001). $Phaeocryptopus gaeumannii$ infection levels in all needles used for gas exchange analysis in this study are presented below.

Branch-level stomatal response curves measured with the controlled-environment cuvette of the LiCor 6400 showed that $g_s$ declined with increasing VPD (Figure 6-1). All response curves showed a significant negative linear relationship between $g_s$ and VPD that varied with $P. gaeumannii$ infection level and tree age (seedling vs. sapling) (Table 6-1). Similar to Manter et al. (2000), maximum $g_s$, estimated as the y-intercept of the curve, was inversely proportional to the amount of pseudothecia present in needle stomata (Figure 6-2). The slope of the response curve
also declined as a linear function of pseudothecia present (Figure 6-2). Interestingly, the parameters describing the stomatal response curves varied between the seedling and field trees, *i.e.*, *P. gaeumannii* had a greater impact on both the y-intercept and slope in the seedlings (Figure 6-2). The response to increasing VPD, or slope of the curve, decreased with increasing disease levels (Figure 6-1). Furthermore, at high VPDs, $g_s$ was higher in the infected seedlings versus the control; whereas in the field trees, $g_s$ was always lower than the control, regardless of VPD.

**Figure 6-1.** Stomatal conductance response to controlled VPD (LiCor 6400) in 1-year-old *P. gaeumannii*-infected needles from Douglas-fir seedlings and field trees. Pseudo is the percent of stomata occluded with pseudothecia (± 4 %). Seedling measurements were conducted in April 2000, and field trees were conducted in July 2000.
Table 6-1. Controlled cuvette (LiCor 6400) VPD response curve parameters (y-intercept and slope) for *P. gaeumannii*-infected seedlings and field trees. Pseudo is the percent of stomata occupied with pseudothecia for all needles in the cuvette.

<table>
<thead>
<tr>
<th>Seedlings</th>
<th>Field Trees</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R²</strong></td>
<td><strong>R²</strong></td>
</tr>
<tr>
<td>0.995</td>
<td>0.977</td>
</tr>
<tr>
<td>0.966</td>
<td>0.984</td>
</tr>
<tr>
<td>0.997</td>
<td>0.966</td>
</tr>
<tr>
<td>0.956</td>
<td>0.966</td>
</tr>
<tr>
<td>0.880</td>
<td>0.958</td>
</tr>
</tbody>
</table>

Figure 6-2. Relationship between the controlled cuvette (LiCor 6400) VPD response curve parameters (y-intercept and slope) and *P. gaeumannii* infection levels.
Figure 6-3 shows a typical diurnal pattern of $g_s$ from branches in the field trees. In all branches, $g_s$ declined as VPD increased over the course of a day. As seen in the VPD response curves, maximum $g_s$ (i.e., early in the day) declined with increasing $P. gaeumannii$ pseudothecia. Over the course of the day, $P. gaeumannii$-infected needles exhibited less of a change in $g_s$ (e.g., declining from ca. 125 to 60, and 160 to 80 mmol m$^{-2}$ s$^{-1}$ for the Beaver-north 1-year-old nospray and spray needles, respectively. Pseudothecia densities from measured trees are presented in Table 6-2, and a more detailed 2-year assessment of $P. gaeumannii$ colonization is reported in Manter et al. (2001). In general, no detectable $P. gaeumannii$ colonization was present in the sprayed trees, and for the nospray trees $P. gaeumannii$ colonization from highest to lowest was at the Beaver, Hebo, and Mac sites, respectively.

The diurnal pattern of $\Psi_{\text{leaf}}$ for the same days presented in Figure 6-3 is shown in Figure 6-4. In all trees, $\Psi_{\text{leaf}}$ rapidly declined until a threshold value of ca. -2.1 MPa, where stomatal regulation maintained $\Psi_{\text{leaf}}$ at or above this value. Significant differences in treatments were observed early in the day. In contrast to the predicted vapor fluxes (i.e., stomatal conductance), $\Psi_{\text{leaf}}$ declined to the threshold values faster in south-facing trees as compared to north trees, and nospray trees declined faster than spray trees.
Figure 6-3. Diurnal patterns of stomatal conductance in Douglas-fir needles from field plots with varying levels of *P. gaeumannii* infection. Data collected August 1999. Each observation is the mean and standard error of six trees. 99 are the current-year-needles and 98 are the 1-year-old needles.
Figure 6-4. Diurnal patterns of $\Psi_{\text{leaf}}$ in Douglas-fir needles from field plots with varying levels of $P. \text{gaeumannii}$ infection. Data collected August 1999. Each observation is the mean and standard error of six trees.
Table 6-2. Mean values of $\Psi_{\text{leaf}}$, $\Psi_{\text{soil}}$, $\Sigma g_s$, and VPD from diurnal gas exchange measurements of 1-year-old needles from field trees with varying levels of $P$. gaeumannii infection. $\Psi_{\text{leaf}}$ and $\Psi_{\text{soil}}$ are the leaf and soil water potential, respectively. $\Sigma g_s$ is the integrated daily stomatal conductance or the area under the diurnal stomatal conductance curve (mmol m$^{-2}$) calculated using the trapezoidal method. VPD is the leaf-to-air vapor pressure gradient. Pseudo is the percent of stomata occupied with pseudothecia measured in June 2000. Means (standard error) with different letters from each site are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>Site</th>
<th>Slope</th>
<th>Trt</th>
<th>$\Psi_{\text{leaf}}$</th>
<th>$\Psi_{\text{soil}}$</th>
<th>$\Sigma g_s$</th>
<th>VPD</th>
<th>Pseudo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beaver North Spray</td>
<td>10.5 (0.5)$^a$</td>
<td>3.2 (0.2)$^a$</td>
<td>689 (67)$^a$</td>
<td>876 (99)$^a$</td>
<td>0.0 (0.0)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaver North Nospray</td>
<td>11.1 (0.4)$^b$</td>
<td>3.2 (0.2)$^b$</td>
<td>644 (52)$^b$</td>
<td>876 (99)$^b$</td>
<td>12.8 (1.8)$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaver South Spray</td>
<td>12.8 (0.5)$^c$</td>
<td>3.3 (0.2)$^c$</td>
<td>569 (52)$^c$</td>
<td>1098 (103)$^c$</td>
<td>0.0 (0.0)$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaver South Nospray</td>
<td>14.3 (0.5)$^d$</td>
<td>3.2 (0.2)$^d$</td>
<td>496 (41)$^d$</td>
<td>1098 (103)$^d$</td>
<td>19.1 (1.8)$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebo North Spray</td>
<td>12.0 (0.8)$^a$</td>
<td>3.5 (0.2)$^a$</td>
<td>522 (73)$^a$</td>
<td>1064 (107)$^a$</td>
<td>0.0 (0.0)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebo North Nospray</td>
<td>12.5 (0.8)$^b$</td>
<td>3.4 (0.3)$^b$</td>
<td>506 (62)$^b$</td>
<td>1064 (107)$^b$</td>
<td>2.1 (0.5)$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebo South Spray</td>
<td>13.3 (0.5)$^c$</td>
<td>3.5 (0.2)$^c$</td>
<td>452 (47)$^c$</td>
<td>1293 (148)$^c$</td>
<td>0.0 (0.0)$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hebo South Nospray</td>
<td>13.9 (0.8)$^d$</td>
<td>3.5 (0.2)$^d$</td>
<td>403 (46)$^d$</td>
<td>1293 (148)$^d$</td>
<td>3.3 (0.8)$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac North Spray</td>
<td>18.4 (0.8)$^a$</td>
<td>8.7 (0.8)$^a$</td>
<td>448 (103)$^a$</td>
<td>1342 (186)$^a$</td>
<td>0.0 (0.0)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac North Nospray</td>
<td>18.3 (0.8)$^a$</td>
<td>8.9 (0.8)$^a$</td>
<td>442 (105)$^a$</td>
<td>1342 (186)$^a$</td>
<td>0.1 (0.1)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac South Spray</td>
<td>19.1 (0.9)$^a$</td>
<td>10.0 (1.9)$^a$</td>
<td>335 (87)$^a$</td>
<td>1702 (214)$^a$</td>
<td>0.0 (0.0)$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac South Nospray</td>
<td>19.4 (0.6)$^a$</td>
<td>10.1 (2.1)$^a$</td>
<td>336 (87)$^a$</td>
<td>1702 (214)$^a$</td>
<td>0.0 (0.0)$^a$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The overall reduction in diurnal stomatal conductance was estimated by integrating the area under the diurnal $g_s$ curves ($\Sigma g_s$), 1-year-old needles only. Table 6-2 reports the average value from all diurnal curves conducted in 1999. A significant reduction in $\Sigma g_s$ was associated with $P$. gaeumannii infection (spray vs. nospray) at each site. The reduction was greatest at the high disease site (Beaver) and lowest at the low disease site (Mac). Plot aspect also had a significant impact on $\Sigma g_s$. $\Sigma g_s$ was significantly lower on the south-aspect plots, which also had a higher average VPD (i.e., $\Sigma g_s$ was reduced on the south-aspect plot sprayed trees by 17.5, 13.5, and 25.2 %, and VPD was 20.2, 17.7, and 21.1 % higher for the Beaver, Hebo, and Mac, respectively).
Differences in $\Sigma g_s$ did not appear to be related to $\Psi_{\text{soil}}$, which was not significantly different for any of the treatments at the Beaver and Hebo sites. $\Psi_{\text{soil}}$ at the Mac site, was lower than the Beaver and Hebo sites due to soil drying during the late summer months (i.e., August-September, data not shown); however, the average $\Psi_{\text{soil}}$ for the treatments at the Mac site over the course of the entire study was not significantly different. Despite the reduced stomatal conductance and water vapor loss, $\Psi_{\text{leaf}}$ values were significantly lower in the nospray trees at the Beaver and Hebo sites.

A $g_s$ response curve to VPD was generated from all of the ambient porometric measurements collected in 1999 (Figure 6-5). Significant negative linear relationships were observed for all treatments. Like the controlled environment measurements (i.e., LiCor 6400), the y-intercepts and slopes (Table 6-3) were inversely proportional to $P$. gaeumannii pseudothecia density (Table 6-2). However, the relationships varied with plot-aspect. For example, the y-intercepts for the Beaver-south trees were 112.5 and 91.5 (spray and nospray trees, respectively); whereas, the Beaver-north trees were 138.8 and 118.4, respectively. As a result of the slope changes associated with fungal infection (i.e., pseudothecia density) and microclimate (i.e., plot-aspect), a significantly higher $g_s$ was observed in the diseased trees at the higher VPD values. The intersection of the spray and nospray curves occurred at ca. 1500 Pa for the north and at ca. 2500 Pa for the south plots at both the Beaver and Hebo sites. No significant differences between any of the treatments at the low disease Mac site were observed.

Estimates of the whole-plant leaf specific conductance ($K_{L}$) were determined from branch level estimates of maximum hydraulic conductance, normalized by either supplied leaf area ($K_{L,B}$) or cross-sectional sapwood area ($K_{S,B}$). Significant
Figure 6-5. Stomatal response to ambient VPD in 1-year-old needles from field plots with varying levels of *P. gaeumannii* infection. Data collected June - September 1999. Each observation is the mean and standard error of six trees.
Table 6-3. Ambient VPD response curve parameters (y-intercept and slope) in 1-year-old needles from field plots with varying levels of *P. gaeumannii* infection.

<table>
<thead>
<tr>
<th>Site</th>
<th>Slope</th>
<th>Trt</th>
<th>$R^2$</th>
<th>y-intercept</th>
<th>slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beaver</td>
<td>North</td>
<td>Spray</td>
<td>0.665</td>
<td>138.8 (4.3)</td>
<td>-0.044 (0.003)</td>
</tr>
<tr>
<td>Beaver</td>
<td>North</td>
<td>Nospray</td>
<td>0.798</td>
<td>118.4 (3.0)</td>
<td>-0.029 (0.003)</td>
</tr>
<tr>
<td>Beaver</td>
<td>South</td>
<td>Spray</td>
<td>0.406</td>
<td>112.5 (4.7)</td>
<td>-0.033 (0.003)</td>
</tr>
<tr>
<td>Beaver</td>
<td>South</td>
<td>Nospray</td>
<td>0.666</td>
<td>91.5 (3.7)</td>
<td>-0.023 (0.002)</td>
</tr>
<tr>
<td>Hebo</td>
<td>North</td>
<td>Spray</td>
<td>0.648</td>
<td>139.5 (6.5)</td>
<td>-0.038 (0.005)</td>
</tr>
<tr>
<td>Hebo</td>
<td>North</td>
<td>Nospray</td>
<td>0.586</td>
<td>127.0 (6.0)</td>
<td>-0.031 (0.004)</td>
</tr>
<tr>
<td>Hebo</td>
<td>South</td>
<td>Spray</td>
<td>0.643</td>
<td>130.5 (6.7)</td>
<td>-0.036 (0.004)</td>
</tr>
<tr>
<td>Hebo</td>
<td>South</td>
<td>Nospray</td>
<td>0.696</td>
<td>118.3 (5.2)</td>
<td>-0.031 (0.003)</td>
</tr>
<tr>
<td>Mac</td>
<td>North</td>
<td>Spray</td>
<td>0.625</td>
<td>173.9 (13.1)</td>
<td>-0.059 (0.008)</td>
</tr>
<tr>
<td>Mac</td>
<td>North</td>
<td>Nospray</td>
<td>0.626</td>
<td>172.3 (12.9)</td>
<td>-0.059 (0.008)</td>
</tr>
<tr>
<td>Mac</td>
<td>South</td>
<td>Spray</td>
<td>0.731</td>
<td>181.4 (12.6)</td>
<td>-0.060 (0.006)</td>
</tr>
<tr>
<td>Mac</td>
<td>South</td>
<td>Nospray</td>
<td>0.739</td>
<td>181.8 (12.3)</td>
<td>-0.060 (0.006)</td>
</tr>
</tbody>
</table>

differences in $K_{LB}$ (Figure 6-6) and $K_{SB}$ (data not shown) were observed in both the disease and plot-aspect treatments. Although a significant reduction in both leaf and sapwood areas was associated with fungal infection (data not shown) the ratio did not change (Figure 6-7). In other words, defoliated trees produced less sapwood and maintained a constant balance between leaf area and sapwood area for all treatments.

Instead, changes in $K_{LB}$ and appear to be associated with changes in stem permeability. Visual estimates of the percentage of latewood present in each annual ring, from main stem cores taken at breast height, showed a significant correlation with stem permeability (Figure 6-8).
Figure 6-6. Branch estimates of leaf specific conductance in field trees with varying levels of *P. gaeumannii* infection. Bars are the mean and standard error of three trees.
Figure 6-7. Total leaf area (all age classes) versus cross-sectional stem area (1997 node) for 3-year-old branches from field trees with varying levels of P. gaeumannii infection. Each observation is the mean of three trees.

\[ R^2 = 0.783 \\
\hat{y} = 20.775 - 0.197x \]

Figure 6-8. Branch-stem and -leaf specific conductance (\(K_{S,B}\) and \(K_{L,B}\), respectively) versus the percent of latewood present in sapwood cores taken at breast height from field trees with varying levels of P. gaeumannii infection. Each observation is based on one tree, conductance values for a given branch node (e.g., 1998) are plotted against the percent latewood for the corresponding annual increment (e.g., 1998).

\[ R^2 = 0.948 \\
\hat{y} = -0.0124 + 0.4311x \]

\[ R^2 = 0.566 \\
\hat{y} = 1.735 - 0.0124x \]
6.5 Discussion

In a previous study, Manter et al. (2000) found that the presence of pseudothecia in needle stomata reduced maximal needle conductance. However, if the regulation of stomata, at least in the short-term, is dependent upon $\Psi_{\text{leaf}}$ (Jones, 1990; Tardieu, 1993; Saliendra et al., 1995), we hypothesized that the reduced water loss under favorable conditions (i.e., low VPD) would allow needles to maintain stomata open longer during the day (i.e., high VPD).

In this study, temporal changes in stomatal conductance were assessed using a variety of time scales and experimental methods. In all cases, we found that maximum stomatal conductance was lower in *P. gaeumannii*-infected seedlings, and that the slope of the VPD response curve decreased with increasing infection. However, the intercept and slope of the VPD response curves also varied with plant age (2-year-old seedling vs. 15-year-old field trees) and climate (north vs. south slopes). As discussed below, our observations are consistent with the hypothesis that stomata regulate $\Psi_{\text{leaf}}$ above a critical threshold, and that $\Psi_{\text{leaf}}$ is influenced by the net balance of water loss and supply (e.g., Saliendra et al., 1995).

In the controlled environment studies (LiCor 6400), the reduced water loss associated with *P. gaeumannii* pseudothecia and reduced stomatal conductance at the low VPDs, appeared to influence stomatal conductance at the higher VPDs. For example, as maximal stomatal conductance declined the slope of the VPD response curve declined (Figure 6-1, Table 6-1). We interpret this observation to imply that the reduced water loss resulted in higher $\Psi_{\text{leaf}}$ and a reduced induction of stomatal closure.
Unfortunately, this cannot be verified since $\Psi_{\text{leaf}}$ was not determined in the LiCor 6400 studies. We also observed in the controlled environment studies that the $g_{\text{c}}$-VPD response curves differed between the seedlings and field trees. The factor(s) responsible for the differing response is unknown but may be related to differences in stem transport and water supply, which influenced the diurnal response curves as discussed below.

In all field trees at ambient conditions, $\Psi_{\text{leaf}}$ was regulated in order to maintain $\Psi_{\text{leaf}}$ above a critical threshold as previously reported (Jones, 1990; Tardieu, 1993; Saliendra et al., 1995). The average $\Psi_{\text{leaf}}$ for diseased trees was lower than healthy foliage (Table 6-2), but in all cases the values never fell below $-2.3$ MPa, typically hovering around $-2$ MPa late in the afternoon. The reason for the lower $\Psi_{\text{leaf}}$ in the diseased trees appears to be the related a faster decline in $\Psi_{\text{leaf}}$, despite the reduced water loss early in the day. Equation 4 shows that $\Psi_{\text{leaf}}$ is the net balance of the water loss and supply. Therefore, the observed declines in the diseased trees, which have a reduced water loss, suggest that the water supply component is severely diminished. In this study we measured branch-level estimates of $K_L$, which is a major determinant of water supply (e.g., Tyree, 1988; Waring and Silvester, 1994). As expected from equation 1, $K_{L-B}$ was significantly reduced in $P. gaeumannii$-infected trees. Equations 2 & 3 show that cross-sectional sapwood area and cell diameter will influence $K_{L-B}$. We did not directly measure cell diameters; however, $K_{L-B}$ was inversely related to the amount of latewood present in measured branches. Since latewood cells have a smaller diameter compared to earlywood cells (Megraw, 1986) this estimate should be related to the average cell diameter of the conducting tissue. Changes in $K_{L-B}$ could not be
attributed to LA / SA ratios as shown by Whitehead et al. (1984). Although P. gaeumannii infection did cause a significant reduction in the amount of leaf area present, constant LA / SA ratios were maintained due to reduced sapwood growth.

Presently, we have focused on the relationship between pseudothecia presence and gs; however, other fungal structures should not be overlooked, e.g., hyphae in stomatal chambers and on the leaf surface. Previous studies have reported that P. gaeumannii hyphae are densely packed in needle stomata (Stone and Carroll, 1986), and one would expect that these structures can physically block water loss much like that reported for pseudothecia. Furthermore, Ayres (1976; 1981) showed that surface hyphae can increase leaf resistance, and significant development of surface hyphae has been observed with P. gaeumannii (Capitano, 1999). Thus, the contribution of such fungal structures warrants further investigation. However, in Manter et al. (2000) no impact on gs was present prior to the formation of P. gaeumannii pseudothecia, but when surface and stomatal hyphae should have been present. Unfortunately, no effort was made to quantify the presence of either of these structures, and due to the monthly sample dates it is not clear whether they were present in samples with “normal” gs or if they developed between sample dates.

The interaction of P. gaeumannii with the balance between water loss and supply leads to differing stomatal regulation patterns and a potential compensation at high VPD. For example, as shown in Figure 6-5, when VPD is greater than ca. 1500 or ca. 2500 Pa for the north and south plots, respectively, stomatal conductance in diseased foliage is higher than undiseased foliage, whereas below these VPD levels stomatal conductance is lower. Thus, the relative productivity of foliage will be dependent upon
temporal changes in VPD and the amount of *P. gaeumannii* infection. In the current study, because productivity (*i.e.*, photosynthesis) is dependent upon CO$_2$ diffusion (*i.e.*, stomatal conductance), we estimated relative productivity as the total area under the diurnal stomatal conductance curves ($\Sigma g_s$). It was observed that despite the potential for compensation at high VPD, $\Sigma g_s$ was significantly reduced in all foliage infected with *P. gaeumannii*. The decline ranged from ca. 3.1% when *P. gaeumannii* pseudothecia density was 2.1% to 12.8% when *P. gaeumnani* pseudothecia was 19.1%. Also apparent was a greater limitation to water loss on south sites as compared to north sites. For example, $\Sigma g_s$ was ca. 18.6% lower and VPD was ca. 19.6% higher on south vs. north sites.

As previously reported, symptom development of *P. gaeumannii*-infected foliage is greater on trees growing on south slopes (Manter *et al.*, 2001). The trends in $\Sigma g_s$ observed here suggest that these differences are related to photosynthetic production capacities in these environments. For example, we observed that not only was $\Sigma g_s$ lower in trees from south-facing plots but also that the reductions associated with fungal infection were greater on south-facing plots. We attribute this to the greater reductions in sapwood growth and stem transport capacity (*i.e.*, $K_{l-B}$) observed on the south-facing plots.

The work presented here supports our previous hypothesis that the impact of *P. gaeumanni* on Douglas-fir trees arises from a general decline in host vigor due to reductions in stomatal conductance and photosynthetic activity (Manter *et al.* 2000, 2001). Furthermore, in previous studies, it was observed that symptom development (*e.g.*, needle abscission and chlorosis) was higher in *P. gaeumannii*-infected trees
growing on south slopes in the Oregon Coast Range (Manter et al. 2001). This is consistent with the greater limitations on total integrated-daily stomatal conductance reported here in trees from south slopes in the Oregon Coast Range.

In summary, several anatomical and physiological responses to *P. gaeumannii* infection contribute to the observed patterns of stomatal conductance – potentially reducing the total daily and seasonal patterns of CO₂ and H₂O diffusion. These include: (i) reduced water loss due to an increased stomatal resistance from the physical barrier to flow imposed by the physical presence of fungal structures, *i.e.*, pseudothecia, and (ii) reduced water supply due to an increased hydraulic resistance associated with reduced growth and greater latewood production. Furthermore, increased evaporative demands (*i.e.*, VPD) on trees growing on south slopes impose a further reduction on the total integrated-daily conductance in both healthy and diseased trees.

### 6.6 Acknowledgements

We are grateful to Dr. Barbara Bond for the loan of several LiCor gas exchange analyzers. This research was funded through the Swiss Needle Cast Cooperative at Oregon State University - a consortium of industrial, federal, and state landowners in Oregon and Washington.
6.7 Literature cited


Chapter 7

A Process-Based Photosynthesis Model for Douglas-fir Accounting for the Foliar Pathogen, *Phaeocryptopus gaeumannii*. I. Model Development

Daniel K. Manter, Barbara J. Bond, Kathleen L. Kavanagh, and Gregory M. Filip
7.1 Abstract

A processed-based photosynthesis model that accounts for physiological changes induced by the foliar pathogen, *Phaeocryptopus gaeumannii* is proposed. This model consists of two sub-models. The first estimates stomatal conductance assuming that the liquid (supply) and vapor (loss) fluxes of water in the soil-plant-atmosphere are in equilibrium, and that the fluxes are controlled by stomata maintaining plant water potential above a critical threshold level. The second predicts photosynthetic rates based on Farquhar estimates of photobiochemistry. For the modeled population of coastal Douglas-fir, field observations of photosynthesis were predicted to a high degree of accuracy ($R^2 = 0.777$) based solely on stomatal conductance and fungal impacts on photobiochemistry, regardless of leaf nitrogen concentration and age.

7.2 Introduction

*Phaeocryptopus gaeumannii* (Rhode) Petrak is a foliar pathogen of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and the causal agent of Swiss needle cast disease (SNC). Recent surveys indicate that SNC is currently reaching unprecedented epidemic levels in the Pacific Northwest (Hansen *et al.*, 2000). Furthermore, volume growth of Douglas-fir in severely diseased coastal Oregon plantations is currently reduced by ca. 23 % (D.A. Maguire, *personal communication*). The observed growth decline is not surprising considering that SNC reduces carbon assimilation by reducing both leaf area (Hansen *et al.* 2000) and photosynthetic capacity (Manter *et al.*, 2000). Therefore, the development of a process-based photosynthesis model is intended to
serve two objectives: first, to test if observed growth rates in SNC-affected stands are correlated with total carbon assimilation rates, and second, to provide a predictive tool that can be used to estimate SNC impacts over larger time and spatial scales.

*Phaeocryptopus gaeumannii* is one of many inconspicuous (until recently) fungi residing in Douglas-fir foliage (*e.g.*, Carroll and Carroll, 1978; Manter *et al.* 2001a). Douglas-fir needles, like other long-lived conifer needles, harbors asymptomatic fungal colonists (Carroll and Carroll, 1978; Stone, 1988a; 1988b). These infections increase rapidly both within (*e.g.*, *Rhabdocline parkeri* infections approximately doubled annually, Stone, 1988b) and between conifer needles (*e.g.*, ca. 10, 50, and 90 % of 1-, 2-, and 3-year-old needles, respectively, harbored internal foliar fungi, Bernstein and Carroll, 1977; Manter, *unpublished data*). Unfortunately, however, the physiological impacts of these ubiquitous fungi typically receive little, if any, consideration in age-related physiological changes. For example, age related declines in photosynthetic capacity have often been observed in conifer needles (*e.g.*, Wang *et al.*, 1995; Jach and Ceulemans, 2000); however, in all cases, the role of fungal infection has been unintentionally ignored. Furthermore, the common factors associated with the decline in photosynthetic capacity (*i.e.*, a decline in nitrogen concentration and rubisco activity, Jach and Ceulemans, 2000) are typical consequences of fungal parasitism (*e.g.*, Scholes, 1992; Bauer *et al.*, 2000; Manter *et al.*, 2000).

Another impetus for investigating the role of foliar fungi in regulating needle biochemistry arises from the increasing reliance upon physiological models to address both predictive and global-scale objectives (*i.e.*, global warming and climate change).
7.3 Methods

7.3.1 Plant material

The plant material necessary for estimation of background parameters consisted of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings and field trees from two separate studies. The field trees consisted of 12- to 15-year-old field trees from three coastal Oregon plantations. Two of the sites are located in the Coastal Range (*i.e.*, Beaver and Hebo), heavy and moderate levels of *Phaeocryptopus gaeumannii* (Rhode) Petrak infection, respectively. The third site is from the Willamette Valley with low levels of *P. gaeumannii* infection. A more detailed description of the sites and *P. gaeumannii* infection levels is reported in Manter *et al.* (2001b). Two-year-old Douglas-fir seedlings (Starker Forests Inc., Corvallis, OR, USA) with different levels of *P. gaeumannii* infection were also selected. To vary rates of infection, seedlings were incubated for 2, 4, or 8 weeks in the understory of *P. gaeumannii*-infected trees (Salal plot, see Hansen *et al.*, 2000) from May to July 1999.

7.3.2 A/C\textsubscript{i} curves

All A/C\textsubscript{i} curves (*i.e.*, carbon assimilation over a range of calculated internal CO\textsubscript{2} concentrations) were conducted using a LiCor 6400 portable infrared gas exchange system (LiCor, Lincoln, NE, USA). Estimates of photobiochemical parameters (*e.g.*, \(V_{\text{max}}\), rubisco activity; \(J_{\text{max}}\), electron transport capacity; and \(R_d\), respiration) were calculated according to the model proposed by Farquhar *et al.* (1980). During measurements, cuvette conditions were maintained at PAR 2000 \(\mu\text{mol m}^{-2}\text{s}^{-1}\),
temperature 25°C, [H₂O vapor] ≥ 18 mmol mol⁻¹, [CO₂] 40 Pa, and flow rate 100 μmol m⁻² s⁻¹, unless otherwise noted. A/Cᵢ response curves were measured by varying the cuvette CO₂ concentration, allowing equilibration to a steady state (cuvette [CO₂] coefficient of variation < 2%), and logging measurements every 10 sec for 1 min. CO₂ was varied in the following order: 40, 30, 20, 60, 80, 100, 120, 160, and 200 Pa.

Temperature dependencies of photobiochemical parameters were determined from A/Cᵢ curves conducted at air temperatures of 10, 18, 23, 25, 28, 30, and 35°C following a 45 min equilibration period.

### 7.3.3 P. gaeumannii infection levels

All estimates of *P. gaeumannii* infection levels were estimated by the presence of pseudothecia (*i.e.*, fruiting bodies) emerging from needle stomata. All reported values, percent of stomata occupied with pseudothecia (*i.e.*, pseudothecia density), are the mean value of for each sampled tree based on the sampling procedures outlined in Manter *et al.* (2000).

### 7.3.4 Nitrogen assessment

Following A/Cᵢ analysis of the field trees (12 per site) in June 1998, 2 g of 1-year-old needles adjacent to the cuvette were collected and used to determine the percent concentration of total combustible N on a dry weight basis. Analysis was performed by the Central Analytical Laboratory (Crop and Soil Science Department, Oregon State University, Corvallis) using a Leco CNS 2000.
7.4 Model development

This model consists of two separate, albeit related, processes. First, stomatal conductance is calculated assuming that stomatal regulation operates in such a manner as to maintain plant water potential above a threshold level (Bond and Kavanagh, 1999). Second, photo-biochemistry is estimated based on measured responses to $C_i$.

7.4.1 Stomatal conductance sub-model

Several authors (e.g., Jones and Sutherland, 1991; Dewar, 1995; Whitehead, 1998; Bond and Kavanagh, 1999) have suggested that the vapor and liquid fluxes of water are in equilibrium through the soil-plant-atmosphere continuum, and can be described mathematically:

$$g_s \cdot \text{VPD} = K_L \cdot (\Psi_{soil} - \Psi_{leaf}),$$

(1)

where $g_s$ is stomatal conductance, VPD is the leaf-to-air vapor pressure gradient, $K_L$ is leaf-specific hydraulic conductance, and $\Psi_{soil}$ and $\Psi_{leaf}$ are water potential of the soil and leaf, respectively. Estimation of $g_s$ using equation 1 was similar to that proposed by Bond and Kavanagh (1999). Calculations were as follows:

(1). A maximum stomatal conductance ($g_{s\text{max}}$) based on the presence of P. gaeumnnii was calculated using equation 2 (adapted from figure 2, Manter and Kavanagh, 2001):

$$g_{s\text{max}} = 150 \cdot ((100 - (-0.325 + 1.026 \cdot \text{pseudo}))/100),$$

(2)

where pseudo is the percent of stomata occupied with P. gaeumnii pseudothecia.

(2). A preliminary value of $\Psi_{leaf}$ was estimated using a reorganized equation 1.

$$\Psi_{leaf} = \Psi_{soil} - (g_{s\text{max}} \cdot \text{VPD} / K_L)$$

(3)
Based on the results of Manter and Kavanagh (2001), which showed that *P. gaeumannii* infections reduced branch-level estimates of leaf specific conductance, a *P. gaeumannii*-adjusted whole-tree $K_L$ was used. The branch-level data of Manter and Kavanagh (2001) was converted to a whole-tree level, accounting for fungal-induced changes in the percentage of latewood ($\%$ loss in $K_L = -29.492 + 0.9935 \cdot LW$, see Figure 8, Manter and Kavanagh, 2001) using equation 4,

$$K_L = 1.1 \cdot (100 - ((-29.492 + 0.9935 \cdot LW)/100)),$$

(4)

where 1.1 is a typical whole-tree $K_L$ calculated using healthy, current-year Douglas-fir needles (Bond and Kavanagh, 1999) and LW is the percent of latewood in each sapwood annual ring.

(3). If $\Psi_{\text{leaf}} < \Psi_{\text{threshold}}$

(a). $\Psi_{\text{leaf}} = \Psi_{\text{threshold}}$

(b). An adjusted $K_{L\text{adj}}$, based upon the relationship between $K_L$ and $\Psi_{\text{xylem}}$ (Kavanagh et al., 1998), was calculated from equation 5.

$$K_{L\text{adj}} = \left(\frac{K_L}{100}\right) \cdot \left(118.06 \cdot \exp\left(\frac{-\Psi_{\text{leaf}}}{4.83}\right)^{0.9}\right)$$

(5)

(c). $g_s = K_L \cdot (\Psi_{\text{soil}} - \Psi_{\text{leaf}}) / \text{vpd}$

(4). If $\Psi_{\text{leaf}} > \Psi_{\text{threshold}}$

(a). $g_s = g_{s\text{max}}$

(5). A light-limited $g_s$, based on data from Leverenz (1981), was calculated from equation 6:

$$g_{s\text{par}} = g_{s\text{max}} \cdot (1 - \exp(-0.017 \cdot \text{PAR})),$$

(6)
where PAR is photosynthetically active radiation (μmol m\(^{-2}\) s\(^{-1}\)).

(6). Finally, the modeled stomatal conductance was set equal to:

\[ g_{\text{model}} = \min(g_{\text{max}}, g_{\text{par}}) \]  

(7)

### 7.4.2 Photosynthesis sub-model

#### 7.4.2.1 Farquhar biochemistry

The model of photosynthesis is based on the biochemistry model outlined in Farquhar et al. (1980), with some modifications due to the impacts of *P. gaeumannii* infection. As suggested by Farquhar et al. (1980), net CO\(_2\) assimilation can be expressed mathematically as:

\[ A = V_c - 0.5 \cdot V_o - R_d = V_c \cdot \left(1 - \frac{0.5 \cdot O}{\tau \cdot C_i}\right) - R_d, \]  

(8)

where \(V_c\) and \(V_o\) are rubisco carboxylation and oxygenation rates, respectively. \(C_i\) and \(O\) are the intercellular partial pressures of CO\(_2\) and O\(_2\), \(R_d\) is the respiration rate in the presence of light, and \(\tau\) is the specificity factor for rubisco (Jordan and Ogren, 1984). The rate of \(V_c\) is limited by either the rubisco activation \((W_c)\) or RuBP regeneration \((W_j)\), therefore equation 8 becomes,

\[ A = \left(1 - \frac{0.5 \cdot O}{\tau \cdot C_i}\right) \min(W_c, W_j) - R_d \]  

(9)

\[ W_c = \left(\frac{V_{\text{max}} \cdot C_i}{C_i + K_c \cdot (1 + O/K_o)}\right), \]  

(10)

where \(V_{\text{max}}\) is the maximum rate of carboxylation, and \(K_c\) and \(K_o\) are Michaelis-Menten constants for carboxylation and oxygenation, respectively. The rate of
carboxylation limited by RuBP regeneration is based upon the rate of electron transport (J) and can be expressed mathematically as:

\[ w_j = \left( \frac{J \cdot C_i}{4(C_i + O/r)} \right), \]  

(11)

and

\[ J = \frac{\alpha \cdot I}{\left(1 + \frac{\alpha^2 \cdot I^2}{J_{\text{max}}^2}\right)^0.5}, \]  

(12)

where \( \alpha \) is the efficiency of light energy conversion on an incident light basis and \( J_{\text{max}} \) is the light-saturated rate of electron transport.

Before proceeding with the photosynthesis estimation procedure several unknown parameters must first be defined (i.e., \( K_c, K_o, \tau, R_d, V_{c_{\text{max}}}, \text{and } J_{\text{max}} \)). \( K_c, K_o, \) and \( \tau \) are intrinsic properties of rubisco. Estimates of \( K_c \) and \( K_o \) have proven variable among \( C_3 \) species (Evans and Seeman, 1984; Keyes, 1986), whereas \( \tau \) is remarkably constant (Jordan and Ogren, 1984; Woodrow and Berry, 1988). As in Harley et al. (1992) we chose to model \( K_c, K_o \) and \( \tau \) based on the temperature dependencies outlined in Jordan and Ogren (1984) using equation 13:

\[ \text{Parameter} = \exp(c - \Delta H_a / (R \cdot T_k)), \]  

(13)

where \( c \) is a scaling constant, \( \Delta H_a \) is an activation energy, \( R \) is the universal gas constant and \( T_k \) is leaf temperature (K) (see Table 1, Harley et al., 1992). Whereas the temperature dependencies of \( V_{c_{\text{max}}} \) and \( J_{\text{max}} \) can be mathematically expressed as:

\[ \text{Parameter} = \frac{\exp(c - \Delta H_a / (R \cdot T_k))}{1 + \exp((\Delta S \cdot T_k - \Delta H_d) / (R \cdot T_k))}, \]  

(14)

where \( \Delta H_d \) is the energy of deactivation and \( \Delta S \) is an entropy term. All parameter estimates used in equation 14 are shown in figure 7-1.
Figure 7-1. Temperature dependency of $V_{\text{cmax}}$, $J_{\text{max}}$ and $R_d$ for six 2-year-old Douglas-fir seedlings infected with varying amounts of *P. gaeumannii* (e.g., pseudothecia density was ca. 0, 5, 10, 20, 40 and 50 %). Parameters were estimated from A/C$_i$ curves at each air temperature following a 45-min equilibration time using a LiCor 6400. To account for fungal-induced changes, all parameters were relativized by dividing by parameter estimates at 25°C. Measurements were conducted in April 2000.
For $V_{cmax}$, $J_{max}$, and $R_d$, a Douglas-fir specific temperature dependency was estimated using 2-year-old seedlings with a range of $P. gaeumannii$ infection levels (Figure 7-1). Due to $P. gaeumannii$ impacts on $V_{cmax}$ and $J_{max}$ (see equations 15 and 16 below), all values for a given seedling (Figure 7-1) were relativized by parameter estimates at 25 C. In previous studies (e.g., Farquhar et al., 1980, Harley et al., 1992), $V_{cmax}$ has been estimated from leaf nitrogen concentrations. However, based on an assessment of this relationship in 1-year-old needles from 36 coastal Oregon Douglas-fir trees (12 from each of the high, medium and low disease plantations), we found no relationship between leaf nitrogen concentration and $V_{cmax}$ (Figure 7-2).

However, using the same trees, a strong correlation between $V_{cmax}$ and $P. gaeumannii$ infection levels was observed (Figure 7-3a). Therefore, $V_{cmax}$ (25 C) was estimated using equation 15.

$$V_{cmax_{25}} = 30.641 \cdot \exp(-0.041 \cdot \text{pseudo})$$  \hspace{1cm} (15)

Previous studies (e.g., Harley et al., 1992; Wullschleger, 1993) report a strong correlation between $V_{cmax}$ (25 C) and $J_{max}$ (25 C) exists. In our sample population, a significant relationship between $J_{max}$ (25 C) and $V_{cmax}$ (25 C) was also observed (Figure 7-3b), and is defined by equation 16.

$$J_{max_{25}} = 23.914 + 1.955 \cdot V_{cmax_{25}}$$  \hspace{1cm} (16)

Finally, because neither the effect of temperature nor $P. gaeumannii$ infection level had a significant effect on $R_d$ (Figures 1c & 3c), $R_d$ was set to a constant of 1.64 $\mu$mol m$^{-2}$ s$^{-1}$. 
Figure 7-2. Relationship between leaf nitrogen concentration and $V_{cmax}$ (25 C) in 1-year-old needles from three coastal Oregon Douglas-fir plantations. Six healthy and six diseased (pseudothecia density = 0 – 30 %) trees were sampled from western Oregon Douglas-fir plantations (n = 36). Measurements were conducted in June 1998.
Figure 7-3. Relationships used to estimate Farquhar-biochemistry parameters, $V_{c\text{max}}$, $J_{\text{max}}$, and $R_d$, in 1-year-old needles from three coastal Oregon Douglas-fir plantations. Six healthy and six diseased (pseudothecia density = 0 – 30 %) trees were sampled from western Oregon Douglas-fir plantations (n = 36). Measurements were conducted in June 1998.
7.4.2.2 Photosynthesis estimation procedure

All climatic data required for model parameterization (RH, T, and PAR) were collected using HOBO dataloggers from Spectrum Technologies, Inc. (Plainfield, IL). One set of dataloggers was installed at a height of 1.5 m in an opening (radius > 5 m) at each study site. *Phaeocryptopus gaeumannii* infection levels were adapted from disease assessments conducted at the same study sites and reported in Manter et al. (2001b). The remaining unknown, and driving variable for the above biochemical equations, is $C_i$, which results from the interaction of $A$ and $g_s$ based on the flux equation:

$$C_i = C_a - \frac{A}{g_s},$$

where $C_a$ is the atmospheric CO₂ concentration (assumed to be 35.5 Pa) outside the leaf boundary layer. Due to the interactive effects of $A$ and $g_s$ on $C_i$, we cannot estimate $C_i$ independent of $A$ and $g_s$ estimates. This problem was overcome by first estimating $g_s$ (see stomatal conductance sub-model), then simultaneously solving for the two unknowns ($A$ and $C_i$) using equations 9 and 17. For all observations, unique values of $A$ and $C_i$ were estimated using the model procedure in SAS Vers. 6.12 (SAS Institute Inc., Cary, NC, USA).

7.5 Model validation

Model estimates were tested against diurnal gas exchange measurements from at least three infected and three non-infected trees (*i.e.*, sprayed with the fungicide chlorothalonil, see Manter et al., 2001b) at each site. On each sample date, gas exchange was measured on the three youngest needle cohorts from one sun-exposed,
lower-canopy branch. 1998 measurements of CO₂ and water fluxes were recorded using a LiCor 6200 portable infrared gas exchange analyzer, and 1999 measurements consisted of water flux using a LiCor 1600.

Figure 7-4 shows a plot of the predicted vs. measured stomatal conductance values. Overall, modeled values were highly correlated with observed values ($R^2 = 0.777$); however, the model tended to slightly underestimate stomatal conductance (slope = 0.818). Regression analysis showed that none of the treatments (i.e., site, $P$).

Figure 7-4. Measured vs. predicted stomatal conductance for diseased and healthy needles (pseudothecia density = 0 – 61 %) for current-, 1-, and 2-year-old needles from three western Oregon Douglas-fir plantations. Each point is the mean of six trees ($n = 289$) measured in 1998 and 1999.
*gaeumannii* infection level, needle age) significantly affected the relationship between observed and predicted stomatal conductance values (data not shown). The major limitation of our stomatal conductance model arises from an overestimation of stomatal conductance during the mid-morning hours. For example, figure 7-5 depicts a typical diurnal curve for both observed and predicted stomatal conductance at the low disease site. Clearly, observed values of stomatal conductance decline earlier than the modeled values. Two factors may be responsible. First, our estimates of $\Psi_{\text{leaf}}$, are not time-dependent, and may overestimate $\Psi_{\text{leaf}}$ due to unaccounted for loss of water prior to the current time-step. Second, our model assumes that stomatal closure occurs only at the critical value of $\Psi_{\text{leaf}}$ (-2.1 MPa); however, stomatal conductance begins to decline at higher $\Psi_{\text{leaf}}$ (Webb, 1991).

Figure 7-5. Diurnal pattern of measured and predicted stomatal conductance for healthy current-, 1- and 2-year-old needles (mean pseudothecia density = 0, 0 and 4 %, respectively) from the low disease site on July 9, 1998. Each observation is the mean and standard error for three trees.
Some models (e.g., Williams et al., 1996) have overcome this limitation by including an algorithm for determining a time dependent $\Psi_{\text{leaf}}$. However, for the purpose of modeling $P. \text{gaemannii}$ impacts on needle physiology this is not necessary. Overall, our model was well correlated with observed values of stomatal conductance, and clearly shows the impact of $P. \text{gaemannii}$ infection on stomatal conductance. For example, figure 7-6 compares the diurnal patterns of stomatal conductance on a typical day from a healthy and diseased tree at our heavy disease site. Also evident in figure 7-6 is the limitation imposed on maximum (i.e., early morning) stomatal conductance by $P. \text{gaemannii}$ infection. Also, approximately equal effects resulting from the lack of a capacitance factor in our model are present in both the diseased and healthy needles.

Figure 7-6. Diurnal pattern of measured and predicted stomatal conductance for healthy (mean pseudothecia density = 0 %) and diseased (mean pseudothecia density = 15 %) 1-year-old needles from the high disease site on July 7, 1998. Each observation is the mean and standard error for three trees.
Figure 7-7. Measured vs. predicted net assimilation for diseased and healthy needles (pseudothecia density = 0 – 61 %) for current-, 1-, and 2-year-old needles from three western Oregon Douglas-fir plantations. Each observation is the mean of six trees (n = 89) measured in 1999.

Similar to stomatal conductance, our estimates of photosynthesis were highly correlated with observed values ($R^2 = 0.792$), again with a slight underestimation (slope = 0.820) (Figure 7-7). The observed time-lag and over-estimation of early morning values were not as apparent for photosynthesis estimates (Figure 7-8) as compared to that for stomatal conductance. Figure 7-8 shows a typical diurnal curve for three age classes of needles with no $P. gaeumannii$ infection from the low disease site. For this, and all days measured, no significant differences in net photosynthesis between needle age classes were detected for needles from the low disease site (Table 7-1). In contrast, several other studies (e.g., Jach and Ceulemans, 2000) have shown that photosynthesis declines with needle age, concurrent with a decline in N concentration and $V_{cmax}$. Two
factors may be responsible for the lack of an age-related decline in $V_{\text{cmax}}$ from our sample population. First, several researchers have shown a curvilinear relationship between leaf N and photosynthesis (Evans 1983, 1989; DeJong and Doyle, 1985; Sinclair and Horie, 1989); therefore, it is possible that the concentrations of leaf N are at or near the asymptote of the curve. Second, the number of $P. \gaeumannii$ (and other fungal) infections, which strongly impacts photosynthetic capacity, are too low to have a significant impact on photosynthetic capacity.

However, at the other two sites sampled, as the amount of infection increases with needle age the photosynthetic capacity declines (Table 7-1). Declines in photosynthesis due to $P. \gaeumannii$ were also present on a tree-level basis with a good fit to the diurnal patterns of measured and observed photosynthesis (Figure 7-9).

Figure 7-8. Diurnal pattern of measured and predicted net assimilation for healthy current-, 1- and 2-year-old needles (mean pseudothecia density = 0, 0 and 4 %, respectively) from the low disease site on August 4, 1998. Each observation is the mean and standard error for three trees.
Table 7-1. Observed maximum net assimilation and P. gaeumannii infection levels from three western Oregon Douglas-fir plantations. $A_{\text{max}}$ is the average maximum rate of photosynthesis, and PD is the average pseudothecia density, pooled for six trees from four days of diurnal measurements at each site. Standard errors are in parentheses. For each parameter, means for the 1- and 2-year-old age classes were compared with the current-year age class using a paired t-test. Means with different letters are significantly different $p \leq 0.05$.

<table>
<thead>
<tr>
<th>age-class</th>
<th>Low Disease Site</th>
<th>Moderate Disease Site</th>
<th>High Disease Site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{\text{max}}$</td>
<td>PD</td>
<td>$A_{\text{max}}$</td>
</tr>
<tr>
<td>current-yr</td>
<td>11.0 (1.5)$^a$</td>
<td>0.0 (0.0)$^x$</td>
<td>10.3 (0.9)$^a$</td>
</tr>
<tr>
<td>1-yr</td>
<td>9.2 (1.3)$^a$</td>
<td>3.1 (2.3)$^y$</td>
<td>7.6 (1.2)$^b$</td>
</tr>
<tr>
<td>2-yr</td>
<td>10.3 (1.6)$^a$</td>
<td>0.2 (0.1)$^y$</td>
<td>3.8 (0.8)$^c$</td>
</tr>
</tbody>
</table>

Figure 7-9. Diurnal pattern of measured and predicted net assimilation for healthy (mean pseudothecia density = 0 %) and diseased (mean pseudothecia density = 15 %) 1-year-old needles from the high disease site on August 7, 1998. Each observation is the mean and standard error for three trees.
7.6 Conclusions

The model of Douglas-fir photosynthesis proposed here achieves a high degree of accuracy for needles from various sites and age classes in coastal Oregon. One weakness of the model is an over-estimation of early morning stomatal conductance that results from the underlying assumption that liquid and water vapor fluxes within a plant are in equilibrium. Our model also shows that, in some Douglas-fir populations, rubisco activity ($V_{\text{cm}}$) is not related to leaf N concentration. In the current situation, this arises from high N concentrations and the strong impact of fungal infection on photosynthetic activity.

Furthermore, results of this modeling exercise strongly suggest that physiological investigations – particularly those concerned with needle development – consider the role of fungal infections. Such details may be onerous, but not impossible, considering the characteristics of foliar fungi such as P. gaeumannii. For example, especially at low to moderate infection levels, visible signs of the fungus are barely perceptible to the naked eye. Also, the reliance of “disease assessments” on the more obvious symptoms such as needle chlorosis is unreliable due to the long time-lag between fungal infection and symptom development (Manter et al. 2001). The benefits of increased awareness of the physiological impacts of asymptomatic fungi on canopy carbon assimilation are far-reaching – ranging from a simple causal factor for anomalous physiological behavior in “healthy” trees, to increased accuracy in regional and global assessments of carbon sequestration considering the global distribution and frequency of these fungi.
7.7 Acknowledgements

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7.8 Literature cited


CHAPTER 8

A Process-Based Photosynthesis Model for Douglas-fir
Accounting for the Foliar Pathogen, Phaeocryptopus gaeumannii.
II. Cumulative Effects on Net Canopy Carbon Assimilation,
Needle Abscission and Growth

Daniel K. Manter, Barbara J. Bond, Kathleen L. Kavanagh, and Gregory M. Filip
8.1 Abstract

A process-based photosynthesis model was used to scale-up estimates of carbon assimilation for several Douglas-fir needle age classes in terms of both time and space. Seasonal estimates varied with the greatest production during the summer months and ca. 20% of carbon assimilation between October - April. The model employed accounts for the impacts of a foliar pathogen on carbon assimilation, and needle carbon budgets became negative after 25% of needle stomata harbored visible fungal fruiting bodies. However, on a whole-tree level all modeled trees maintained a positive canopy carbon budget due to the large contribution of carbon assimilation from current-year needles. The influence of carbon budgets on observed patterns of needle retention and growth in the modeled sites is also discussed.

8.2 Introduction

Models have become an integral part of forest ecophysiology – allowing for the integration and simulation of complex processes at various temporal and spatial scales (McMurtrie, 1993; Williams et al., 1996). Various incarnations of photosynthesis process models have been developed (Jarvis et al., 1985; Running and Coughlan, 1988; McMurtrie, 1993; Williams et al., 1996; Manter et al., 2001a). These models provide a convenient tool to explore ecosystem processes and functions at scales impossible to adequately capture through direct measurement. The current simulation of net carbon assimilation employs a Farquhar-based photosynthesis model (Manter et al., 2001a) to investigate several aspects of carbon assimilation and allocation in Douglas-fir
(Pseudotsuga menziesii (Mirb.) Franco) with varying levels of Swiss needle cast disease (SNC). The two main objectives of this study were to assess the relative impacts of Phaeocryptopus gaeumannii (Rhode) Petrak, the causal pathogen, on canopy carbon assimilation, and to test if observed growth rates in SNC-affected trees are correlated with total carbon assimilation rates.

Phaeocryptopus gaeumannii is a foliar pathogen of Douglas-fir and the causal agent of Swiss needle cast disease (SNC). Until recently it was considered insignificant in North American forest situations despite its widespread distribution and incidence (Boyce, 1940; Hood, 1982). Recent surveys indicate that SNC is currently reaching unprecedented disease levels in the Pacific Northwest, affecting more than 52,611 hectares of forested lands in western Oregon alone (Hansen et al., 2000). Furthermore, volume growth in severely diseased coastal Oregon stands is currently reduced by ca. 23% (D.A. Maguire, personal communication). The observed growth decline is not surprising considering that SNC reduces carbon assimilation by reducing both leaf area (Hansen et al., 2000) and gas exchange (Manter et al., 2000). For example, P. gaeumannii reduces photosynthetic capacity through both stomatal and non-stomatal limitations (Manter et al., 2000) and the diurnal pattern of stomatal conductance is further limited by a reduction in the liquid flux through xylem tissues (Manter and Kavanagh, 2001).

Assessments of the fungal impacts on canopy carbon assimilation have implications regarding the physiology of Douglas-fir that are important from both an applied and a basic perspective. While it has been shown that carbon assimilation rates may be reduced in diseased foliage (Shitenberg, 1992; Manter et al., 2000), a
quantitative assessment of the cumulative effects of seasonal and canopy carbon budgets have not been attempted.

Optimality theory has often been applied to explain short-term regulation in the relationship between water loss and carbon gain (Cowan, 1977; Williams et al., 1996). Therefore, one would expect that over the long-term, plants would also act to maximize carbon gain. One possible means by which plants can maximize carbon gain is to shed their less productive parts. For example, Witowski (1996) examined branch-level carbon budgets in Pinus sylvestris and found that once carbon budgets became negative, branches were shed. If the resources can then be redistributed to more productive tissues (both new and existing) then this should result in an increased canopy carbon budget. Extending this to a smaller scale, one can envision a similar process influencing needle retention. For example, once a needle or group of needles (i.e., branch node) becomes a carbon sink, optimality theory would predict that these needles should be shed. The simulation reported here is used to estimate a yearly budget of canopy carbon assimilation for a range of P. gaeumannii-infected needles from field trees undergoing various rates of needle abscission.

The discussed model simulation also provides the opportunity to explore the relationship between canopy carbon assimilation and growth in both healthy and diseased trees. Ledig (1969) showed that growth could be reliably predicted by four factors – photosynthesis, respiration, leaf area and duration of function. Hourly estimates of photosynthesis and respiration per unit leaf area, accounting for climatic and disease differences have been scaled-up to a yearly estimate of canopy carbon assimilation. It is assumed a priori that that a strong correlation between estimated
carbon assimilation and growth would support the conclusion that *P. gaeumannii* is a major factor influencing growth in the modeled stands, and that the model successfully accounts for the major physiological impacts of *P. gaeumannii* infection.

The ability to scale-up pathogen impacts on canopy carbon assimilation is an important step in understanding (i) needle abscission, (ii) global carbon budgets, and (iii) growth rates.

### 8.3 Methods

#### 8.3.1 Study sites

Growth and model parameters were obtained from measurements of 12- to 15-year-old field Douglas-fir trees (*Pseudotsuga menziesii* (Mirb.) Franco) from three coastal Oregon plantations with varying levels of Swiss needle cast. The sites have been previously described in Manter *et al.* (2001b). Briefly, the two sites with severe and moderate levels of SNC are located in the Coastal mountain range (*i.e.*, Beaver and Hebo, respectively), and the third site is from the Willamette valley with relatively low levels of *Phaeocryptopus gaeumannii* (Rhode) Petrak infection. At each site two permanent plots consisting of six healthy and six diseased trees were studied. At the onset of this study, disease severity was uniform within the stands; therefore, the healthy trees were created by annual applications of the fungicide chlorothalonil. In an effort to assess the interaction between climate and *P. gaeumannii*-impacts on physiology, plots from a north- and south-facing slope were created within each plantation (Manter *et al.*, 2001b).
8.3.2 Leaf area estimates

Various techniques have been proposed to estimate whole-tree leaf areas (McDowell et al., 2001). However in this case, the best method should be easily parameterized, account for temporal changes in leaf area caused by *P. gaeumannii-*induced needle abscission, and be able to predict the relative leaf areas of all needle age classes present. Therefore, an allometric equation relating cross-sectional sapwood area (cm$^2$) and leaf area (m$^2$), for each of the youngest four needle age classes, was determined using healthy trees with full needle complements (i.e., 100 % needle retention), and modifying these estimates in modeled trees (i.e., varying levels of *P. gaeumannii*-induced needle abscission) using visual estimates of needle retention (0 – 1) as follows.

One 5-year-old branch from each of the 12 healthy Mac forest trees was harvested in September 2000 for leaf area and branch diameter determinations. The leaf area for each age class ($L_{Ax}$, where x denotes age class) was determined by multiplying the total leaf dry weight present on the branch by a projected leaf area to dry weight ratio determined from a random sub-sample of 100 needles from each branch. These branch-level, leaf area determinations were used to create an allometric equation relating branch cross-sectional area (SA, at *ca*. 5 cm from junction of main stem) and leaf area. The relationship between leaf area ($L_{A0}$ = current-year, $L_{A1}$ = 1-year-old, etc.) and branch cross-sectional area for the four age-classes are as follows.

\[
\begin{align*}
L_{A0} &= 0.1965 \times SA, \quad R^2 = 0.8614 \\
L_{A1} &= 0.1497 \times SA, \quad R^2 = 0.9039 \\
L_{A2} &= 0.0528 \times SA, \quad R^2 = 0.8257 \\
L_{A3} &= 0.0055 \times SA, \quad R^2 = 0.2186
\end{align*}
\]
A whole-tree estimate of leaf area for each age class \( (TLA_x) \) was then estimated by measuring diameters of all living branches on each tree and summing the estimates of \( LA_x \). The whole-tree leaf area (TLA) is the sum of \( TLA_x \) for all needle age classes. Similar to other studies (McDowell et al., 2001), a consistent linear relationship between sapwood cross-sectional area at breast height (SABH) and estimates of TLA was observed:

\[
TLA = 0.4045 \times SABH, \quad R^2 = 0.8983. \tag{5}
\]

Finally, the relative distribution of the needle age classes for the whole-tree \( (RLA_x = TLA_x / TLA) \) was determined to be 47.1 ± 1.5, 36.4 ± 1.4, 14.2 ± 0.9, and 2.3 ± 0.4 for the current-, 1-, 2-, and 3-year-old needles, respectively.

From the above calculations, whole-tree \( TLA_x \) for all modeled trees was estimated by equations 5 & 6:

\[
TLA_x = TLA \times RLA_x \times NR_x, \tag{6}
\]

which could be estimated at any point in time from only DBH (no heartwood was present in sampled trees) and needle retention (NR) measurements. Estimates of leaf areas derived from equation 1 were verified by determining estimates of TLA and \( TLA_x \), using the same methods outlined for the Mac trees above, for three diseased trees from the Beaver site. All estimates of TLA and \( TLA_x \) computed by the two methods were within 5% of each other (data not shown).

### 8.3.3 Weather data

At each site, weather data used to parameterize the model (PAR, Temp, and RH) were recorded hourly with dataloggers (Spectrum Technologies, Inc., Plainfield, IL)
placed in an open area (ca. 5 m radius) at approximately 1.5 m above the ground. Continuous measurements (24-hour) beginning on June 16, 1998 and ending on May 31, 1999 were recorded.

8.3.4 Disease assessments

The parameter quantifying *P. gaeumannii* colonization required for model estimates is the proportion of needle stomata with visible pseudothecia present, or pseudothecia density. Model inputs (Table 8-1) were adapted from the biannual estimates reported in Manter et al. (2001b). Additionally, modeled estimates of stomatal conductance are dependent upon the percent of latewood present in sapwood. Estimates used in the current simulation were adapted from Manter and Kavanagh (2001) and are shown in Table 8-2. Needle retention values necessary for the estimation of whole-tree leaf areas shown in Table 8-1 were also adapted from Manter et al. (2001b).

8.3.5 Growth data

In May 2000, estimates of height growth in all modeled trees were calculated by measuring the 1995-1999 internode lengths, on the main stem. Diameter growth was also calculated for the same years by calculating the area of the sapwood rind, whose average radius of the rind was determined from two increment cores (north and west aspect) and DBH.
Table 8-1. Pseudothecia density and needle retention values input into model. The first letter corresponds to the site (B = Beaver, H = Hebo, M = Mac), the second letter is the slope-aspect (N = north, S = south), and the third and fourth letters are the spray treatment (NS = nospray, S = spray). Date is the month of the year modeled using the listed values; 6-11 = June 1998 - November 1998, 12-5 = December 1998 - May 1999. Year is the needle cohort.

<table>
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</tbody>
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Table 8-2. Values of the percent of latewood present in sapwood input into model.
The first letter corresponds to the site (B = Beaver, H = Hebo, M = Mac), the second
letter is the slope-aspect (N = north, S = south), and the third and fourth letters are the
spray treatment (NS = nospray, S = spray). Reported values for each site code were
used for all needle cohorts and months modeled.

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<tr>
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<td>MSS</td>
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8.3.6 Gas exchange model estimates

At each hour over the entire sample period (June 16, 1998 – May 31, 1999), an
estimate of stomatal conductance ($g_s$, mmol m$^{-2}$ s$^{-1}$) and net assimilation ($A_{net}$, μmol m$^{-2}$
s$^{-1}$) was calculated for needles from each age class at the sample sites by using the
model described in Manter et al. (2001a). Estimates were dependent upon the site- and
age class-specific values of climate data (temperature, RH, PAR, VPD), $P. gaeumannii$
colonization (pseudothecia density), and leaf specific hydraulic conductance (mean
tracheid diameter, approximated by the percent of latewood in the sapwood). Net
assimilation estimates were scale-up over time by multiplying the sum of each hourly
measurement for the time period by 3600 s hr$^{-1}$. Estimates were also scaled-up to a
canopy estimate by multiplying by the whole-tree leaf area (m$^2$).
8.4 Results

Over the entire study period, climate datasets from each site had less than 20 days of missing data; where data were missing, values from the corresponding paired site (north or south plot) were used. Average winter and summer values for PAR and temperature are shown in Table 8-3. It is not surprising that light levels and temperature where highly correlated and exhibited similar patterns at the various sites. For example, winter PAR and temperature values, from high to low, were recorded at the Beaver, Mac, and Hebo sites; whereas in the summer, sites were ranked as Mac > Beaver > Hebo. For all three sites, climates also varied with plot-aspect. In the winter and summer seasons, south plots had a higher average PAR and temperature regime.

Table 8-3. Total net assimilation per unit leaf area (1998 cohort from sprayed trees only), average PAR, and air temperatures by season. ¹The first letter corresponds to the site (B = Beaver, H = Hebo, M = Mac), the second letter is the slope-aspect (N = north, S = south). ²Winter is the average value from October 1998 – April 1999, Summer is the average value from June 1998 – September 1998 and May 1999.

<table>
<thead>
<tr>
<th>Site ¹</th>
<th>Winter²</th>
<th>Summer</th>
<th>Winter</th>
<th>Summer</th>
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<tr>
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<td>217.6</td>
<td>455.5</td>
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The pattern of gas exchange for current-year needles (1998 cohort) over time is shown in Figure 8-1. Net assimilation was highest in the summer and declined in the winter as climate limited gas exchange. Prior to *P. gaeumannii* pseudothecia development (November) small differences in $A_{net}$ were associated with the six plots - July and August values were all ca. 175 g m$^{-2}$. After November, however, $A_{net}$ at the various sites diverged and the site with the highest pseudothecia density had the lowest $A_{net}$ (*i.e.*, Beaver-south).

Seasonal patterns of gas exchange were summed for each season. Similar to the daily-integrated stomatal conductance patterns reported in Manter and Kavanagh (2001), $A_{net}$ was lower at the south slopes compared to their north counterparts in the summer (Table 8-3). However, in the winter, south slopes were able to photosynthesize at greater levels because PAR and temperature were not as limiting (Table 8-3). The contribution of winter (October – April) net assimilation varied, ranging from 7.6 – 23.5 % of the total yearly carbon assimilation (Table 8-3).

Estimates of the total yearly carbon budgets in needles with varying levels of *P. gaeumannii* show that once infection levels are high enough, needles will become a sink for carbon. Figure 8-2 shows these estimates for each age class of needles from all six plots versus the amount of pseudothecia present in those needles. Despite differences in climate, the relationship is highly significant when examined on a yearly basis, and once pseudothecia density increases above ca. 25 %, carbon budgets become negative.

When scaled-up to the canopy level all modeled trees were estimated to have positive carbon budgets (Table 8-4). Current-year needles were the largest contributors to the carbon gain of all trees, and at the Beaver site they were the only needle-age class
Figure 8-1. Total monthly estimates of net assimilation per unit leaf area for the current-year needles at three Douglas-fir plantations (June 16, 1998 – May 31, 1999).
with a positive carbon gain. Differences in the rates of net assimilation per unit leaf area (Figure 8-1 & Table 8-3) and leaf areas (Figure 8-3) combined to produce very different patterns in total canopy carbon budgets for the model year at the various north and south plots within each site. At two of the three sites (Beaver and Mac) yearly estimates of carbon gain were higher on the south slopes, whereas, at the Hebo site the south plot was lower.

Figure 8-2. Total net assimilation per unit leaf area for the year (June 16, 1998 – May 31, 1999) versus *P. gaeumannii* pseudothecia density (%). Net assimilation is the modeled value for each age class present within a plot, PD is the average percentage of stomata with visible pseudothecia from six trees.

Height (Figure 8-4) and diameter (Figure 8-5) growth showed similar patterns for the five-year period at all sites. Prior to the fungicide treatment in 1998, the two groups of trees at each site had similar growth rates; however, after treatment, their
growth rates quickly diverged for the high and moderate disease sites. For example, the sprayed trees had higher growth rates compared to the unsprayed trees for 1998 and 1999 at the north plots, whereas, the response at the south plots was delayed until 1999.

Figure 8-3. Total whole-tree leaf area by needle age class for all modeled trees.
The relationship between the estimate of canopy carbon assimilation ($A_{\text{net}}$ in 1998) and growth rates was assessed by regression analysis (Figure 8-6). Across all plots, a significant linear relationship was found for height growth ($R^2 = 0.792$) but not diameter growth ($R^2 = 0.124$). Within each site, as carbon assimilation increased due to fungicide spraying and the prevention of new $P. gaeumannii$ infections (Table 8-4), diameter growth increased (Figure 8-5).

Table 8-4. Total net assimilation (kg) for each needle cohort for the model-year. 1Age is the needle cohort. 2The first letter corresponds to the site (B = Beaver, H = Hebo, M = Mac), the second letter is the slope-aspect (N = north, S = south), and the third and fourth letters are the spray treatment (NS = nospray, S = spray). Missing values are due to needle abscission. Corresponding $P. gaeumannii$ infection levels are in Table 8-1.

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Figure 8-4. Height growth in six diseased (nospray) and six healthy (spray) Douglas-fir trees on north and south slope plots from three plantations.
Figure 8-5. Sapwood growth in six diseased (nospray) and six healthy (spray) Douglas-fir trees on north and south slope plots from three plantations.
Figure 8-6. Total net assimilation versus height and sapwood growth. Net assimilation is the model-year estimate and growth parameters are the mean of six spray or six nospray trees from site.

8.5 Discussion

The photosynthesis model employed in this simulation has previously been verified using leaf-level gas exchange measurements during the summer months (Manter et al., 2001a). The specific aims of this paper were to scale-up these measurements over time and space to determine the effect of *P. gaeumannii* on its host's carbon assimilation and growth.

8.5.1 *P. gaeumannii* and the carbon budget

Modeled changes in carbon assimilation over time are dependent upon both *P. gaeumannii* colonization and climatic changes. The colonization of Douglas-fir needles by *P. gaeumannii* begins early in the summer when newly emerging needles are
infected by deposited ascospores (Hansen and Lewis, 1997). Colonization of needles increases over time punctuated by the appearance of pseudothecia in needle stomata (Capitano, 1999). At this time, gaseous diffusion of \( \text{CO}_2 \) and \( \text{H}_2\text{O} \) appears to be physically impaired by these fungal structures (Manter et al., 2000). In the current simulation, the emergence of pseudothecia in November 1998 for the current-year needles, and the decline in stomatal diffusion, results in a decline in carbon assimilation. As needles age, and pseudothecia density increases the carbon budget continues to be reduced. The canopy carbon budgets remained positive although at the high and moderate disease sites needles older than one year often had negative carbon budgets. The yearly estimates of canopy carbon assimilation were significantly related to the presence of \( P. \text{gaeumannii} \) pseudothecia present, regardless of the climatic conditions.

It has been hypothesized that needle abscission begins when needles become carbon sinks (McMurtrie et al., 1986; Cannell and Morgan, 1990). In a healthy plant, the carbon budget of needles is largely attributed to a reduction in photosynthesis, since mature needles import little photosynthate (Sprugel et al. 1991). Several factors have been hypothesized to account for the decline in photosynthesis of older needles, particularly, light (Schoettle and Smith, 1991) and nutrient (Balster and Marshall, 2000) availability.

In this paper, we have attempted to determine carbon budgets in needles that are photosynthesizing at differing rates due to the presence of a foliar pathogen. Estimated carbon budgets are such that once approximately 25% of needle stomata contain visible \( P. \text{gaeumannii} \) pseudothecia needles become carbon sinks. In previous reports, using the same population of needles, this corresponds to the \( P. \text{gaeumannii} \) colonization level
where needle abscission begins (Manter et al. 2001b) suggesting that needle longevity is related to carbon budgets. However, in many cases, heavily infected needles (up to 60% of stomata with *P. gaeumannii* pseudothecia) may be retained on trees (Hansen et al., 2000; Manter et al., 2001b), even though their carbon budgets are predicted here to be negative. If needle retention is solely related to carbon budgets, then the factors contributing to the retention of such needles are unknown and deserve further attention. Possible factors could be related to inaccurate carbon budget estimates in the current simulation, *i.e.*, model error or translocation of photosynthate from neighboring needles; or variation in the time required for needle abscission to be achieved.

In regard to the latter, it is possible that needle abscission may be triggered by the negative carbon budget but not be realized for a significant and variable amount of time. For example, needle abscission induced by water stress may be delayed until the relief of the stress (Jones, 1992). Therefore, the apparent time lag before needle drop may be due to a “programmed sequence” leading up to abscission, potentially requiring the accumulation of chemical cues (ethylene or ABA), translocation of nutrients from the sink, or accumulation of carbon (translocation or synthesis) necessary to carry out the final active steps of needle abscission.

**8.5.2 Climate and the carbon budget**

Seasonal changes in climate also had a significant impact on modeled estimates of carbon. For the healthy trees, a significantly greater contribution of summer carbon assimilation to the total carbon budgets (76.5 – 92.4%) is not surprising due to the lower light levels and temperatures during the winter months. However, a previous
study predicted that winter photosynthesis may be much higher (30 - 40% of the total) in Douglas-fir growing in the coastal Pacific Northwest (Emmingham and Waring, 1976). Some of the discrepancy may be due to the photosynthesis model employed here. In the model, carbon assimilation is influenced by a temperature effect on rubisco activity (equation 14, Manter et al., 2001a), and this equation was used to model rubisco activity throughout the entire simulation, regardless of season. However, Strain et al. (1976) report that seasonal shifts in the temperature response of photosynthesis are possible, and the temperature optima for coastal Oregon Douglas-fir was closer to 10°C in 1994 (Coulombe et al., 2001) as opposed to the ca. 28°C optima used here. Therefore, the carbon assimilation rates reported here during the winter months may be underestimated. The impact may affect the sites differently because of the differing average winter temperatures; however, the treatments within each plot should be similar.

It was suggested above that the canopy carbon budgets were influenced mainly by pseudothecia density and not climate. The lack of a strong effect of the climates modeled here on the total carbon budget can be related in part to differences between winter and summer carbon assimilation. At the two coastal sites, the south slopes had lower summer photosynthesis but higher winter photosynthesis. The greater summer limitations on the south plots are consistent with Manter and Kavanagh’s (2001) report that daily-integrated stomatal conductance for these south plots were lower compared to the north. However, in the winter, evaporative demand is greatly reduced and stomatal regulation of water flux is not a major limitation of photosynthesis; therefore, the warmer and dryer climate of the south slopes permits higher rates of winter
photosynthesis. When scaled-up to a yearly basis the differences between the slopes may become minimal because the winter photosynthesis provides some compensation for the reduced summer rates.

8.5.3 Whole-tree scaling

In a previous study, Manter and Kavanagh (2001) report that on a branch level a consistent relationship between leaf area and sapwood area was found regardless of disease level or needle abscission rates. In this paper, however, a change in this ratio at the whole-tree level is inherent in the methods employed (i.e., the sapwood based leaf area determination was reduced by visual estimates of needle retention), and was supported by direct observation. Although this appears to be contradictory, it could be due to the different levels of organization under investigation. First, as suggested in Manter and Kavanagh (2001) the loss of leaf area is balanced by a decreased stem growth (as the current simulation predicts), resulting in a statistically insignificant change in the leaf area to stem area ratio on a branch level. However, when the changes in leaf and stem area are scaled-up to the whole tree level the changes become significant. A second possibility is that because the main stem is comprised of ca. 15 years of diameter growth, it contains a "history" of growth under various disease levels (i.e., greater stem growth associated with previously low disease levels); whereas, the branch "history" reflects only the past 5 years.

All of the carbon required for plant growth is acquired through photosynthesis; therefore, if net assimilation and its allocation can be determined, so can growth (Ledig, 1969). In this paper, the relationship between growth and estimates of canopy carbon
budgets for an entire year (June 1998 – May 1999) was assessed. When *P. gaeumannii* colonization was limited by fungicide applications, carbon budgets and growth (height and diameter) were increased. At all plots, a single positive linear relationship between total carbon assimilation and height growth was found. Diameter growth also increased as carbon budgets increased, although the relationship had much more variation and was not significant across all plots.

It is unclear if the more variable relationship between carbon budgets and diameter growth is due to experimental error or differences in carbon allocation to diameter growth. However, we suspect that the latter may be a major factor. If the priority of allocation proceeds from height to root and finally to stem diameter growth, then some of the variation in diameter growth may be due to differences in allocation to root biomass. Although, the allocation to root biomass was not quantified, it was precisely those sites where the soil-root interface may be most limiting (*i.e.*, south slopes) that showed a delay in diameter growth.

8.6 Acknowledgements

This research was funded through the Swiss Needle Cast Cooperative at Oregon State University - a consortium of industrial, federal, and state landowners in Oregon and Washington.
8.7 Literature Cited


Chapter 9

Dissertation Summary

This research was conducted to understand the physiological impacts of Swiss needle cast on Douglas-fir physiology. Four aspects of the disease complex were investigated: fungal colonization and assessment, plant-water relations, carbon assimilation and interaction with climate.

Chapters 3 & 4 describe studies related to the assessment of *P. gaeumannii* colonization using ergosterol analysis, and observed patterns of fungal colonization at three western Oregon Douglas-fir plantations. Ergosterol is a non-species specific technique; however, it was a good measure of total living *P. gaeumannii* biomass in Douglas-fir needles, due to its high amount of fungal biomass compared to all other foliar fungi present. Two other techniques were also used to assess the colonization of *P. gaeumannii* in foliage (quantitative PCR and visual estimates of fruiting bodies). All measures of fungal colonization were significantly correlated with each other (*r* ≥ 0.733) and the amount of visible symptoms present (*i.e.*, needle chlorosis and retention) (*r* ≥ 0.578). Furthermore, removal of *P. gaeumannii* through fungicide applications reduced visible symptoms and increased growth. Climate was also shown to play a significant role in disease development. Differences associated with site topography (*i.e.*, slope and aspect), influenced both fungal colonization and symptom development. For example, an increase in fungal colonization and symptom development was observed on south-facing foliage, which typically experienced greater evaporative demands (*i.e.*, increased temperature and/or lower relative humidity).
In an effort to understand the timing and mechanism of *P. gaeumannii*'s impact on host physiology, Chapter 5 investigated gas exchange in artificially inoculated seedlings over time. Upon sporulation, *P. gaeumannii* produces fruiting bodies or pseudothecia that emerge from needle stomata significantly reducing gas exchange in Douglas-fir needles by physically impeding gaseous diffusion. Maximum rates of needle gas exchange (CO₂ and H₂O) were inversely proportional to the presence of *P. gaeumannii* in needle stomata.

Maximum rates of gas exchange are not maintained indefinitely, however, since stomatal apertures are regulated in order to maintain plant water potentials above a critical threshold. Chapter 6 explored patterns of stomatal regulation in *P. gaeumannii*-infected field trees. In theory, the reduced maximum vapor flux (i.e., stomatal conductance) should allow diseased needles to keep needle stomata open longer during the day, *i.e.*, before low water potentials induce stomatal closure. However, anatomical changes (reduced sapwood permeability) limit the liquid flux of water through xylem tissues and thus the recharge of transpired water in needles. Therefore, even though diseased needles are losing less water per unit time, their water potentials are declining at rates similar to those in healthy needles were transpirational loses are greater.

In Chapters 7 & 8 the cumulative effects of *P. gaeumannii* infection were integrated into a process-based model of photosynthesis. The model predicts H₂O and CO₂ gas exchange in two steps. The first estimates stomatal conductance assuming that the liquid and vapor fluxes of water in the soil-plant-atmosphere are in equilibrium, and that the fluxes are controlled by stomata maintaining plant water potential above a critical threshold level. The second predicts photosynthetic rates based on Farquhar
estimates of photobiochemistry. Modeled estimates of stomatal conductance and photosynthesis were well correlated with observed values ($R^2 = 0.777$, $R^2 = 0.820$, respectively).

Model estimates of gas exchange were scaled-up in terms of both time and space. Seasonal estimates varied, with the greatest production during the summer months, and ca. 20% of carbon assimilation in the winter (Oct – April). The model successfully accounted for the impacts of \textit{P. gaeumannii} on carbon assimilation, and once 25% of needle stomata harbored visible fungal fruiting bodies needle carbon budgets became negative. However, on a whole-tree level all modeled trees maintained positive canopy carbon budgets, due to the large contribution of carbon assimilation from current-year needles, which were significantly correlated with observed height growth ($R^2 = 0.792$).

Finally, the effects of \textit{P. gaeumannii} on host physiology presented here, and the mechanisms through which infection alters host processes - giving rise to the symptoms associated with Swiss needle cast, supports the hypothesis that \textit{P. gaeumannii} is the causal agent of Swiss needle cast.
Bibliography


McDonald, K.L. and D.M. Cahill. 1999. Evidence for a transmissible factor that causes rapid stomatal closure in soybean at sites adjacent to and remote from hypersensitive cell death induced by *Phytophthora sojae*. *Physiological and Molecular Plant Pathology* 55:197-203.


Appendices
Appendix I. Field Sites

Figure A-3. Field site locations.
Figure A-2. Plot design and layout. Paired-plots created at three Douglas-fir plantations (Beaver, Hebo and Mac) with varying levels of Swiss needle cast.
Appendix II. A/C\textsubscript{i} Curve Analysis and Calculations

A/C\textsubscript{i} curves can be used to estimate some of the major underlying biochemical processes influencing gas exchange and the net uptake of carbon into a plant (assimilation) (Farquhar \textit{et al.}, 1980; Sharkey, 1985; Harley and Sharkey, 1991; Harley \textit{et al.}, 1992). According to their models, CO\textsubscript{2} assimilation (\textmu mol m\textsuperscript{-2} s\textsuperscript{-1}) can be modeled by equations 1 and 2. See Appendix II for variable definitions.

\begin{equation}
A = V_c - 0.5V_o - R_{day}
\end{equation}

\begin{equation}
A = (1 - 0.5\frac{V_o}{\tau \cdot C_i}) \cdot \text{min}(W_s, W_f, W_p) - R_{day}
\end{equation}

\begin{equation}
\tau = \exp(-3.9489 + \frac{28.9}{0.00831 \cdot T_k})
\end{equation}

Implicit in equation 1 is that for each carboxylation event one molecule of CO\textsubscript{2} is assimilated, and for every two oxygenations one CO\textsubscript{2} molecule is released. C\textsubscript{i} is the calculated internal CO\textsubscript{2} concentration (Pa) based on equations 4-6.

\begin{equation}
A = g_{sc}(C_o - C_i)
\end{equation}

\begin{equation}
E = g_{sw}(W_o - W_i)
\end{equation}

\begin{equation}
g_{sc} = \frac{g_{sw}}{160}
\end{equation}

Furthermore, according to Farquhar \textit{et al.} (1980) if rubisco assumes Michaelis-Menten enzyme kinetics based on a competitive two-substrate (O\textsubscript{2} and CO\textsubscript{2}) system, then

\begin{equation}
W_c = \frac{V_{c \text{max}} \cdot C_i}{C_i + K_c(1 + \frac{O}{K_o})}
\end{equation}

\begin{equation}
K_c = \exp(35.79 - 80.47 / 0.00831 \cdot T_k)
\end{equation}

\begin{equation}
K_o = \exp(9.59 - 14.51 / 0.00831 \cdot T_k)
\end{equation}
assuming that for every four electrons produced enough ATP and NADPH are generated for completion of the Calvin cycle and regeneration of RuBP. And the potential rate of electron transport is dependent upon the following:

$$J = \frac{\alpha \cdot I}{(1 + \frac{\alpha^2 I^2}{J_{\text{max}}^2})^{0.5}}$$  \hspace{1cm} (11)$$

Quantum-use efficiency, or $\alpha$, was assumed to be 0.18 (mol e\textsuperscript{-} mol\textsuperscript{-1} absorbed photons) for both control and inoculated branches; Ehrlinger and Pearcy (1983) showed that quantum-use efficiency and light-absorption is relatively constant among several C\textsubscript{3} plants.

$$W_p = 3 \cdot TP\text{U} + \frac{V_o}{2} = 3 \cdot TP\text{U} + \frac{V_e \cdot 0.5 \cdot \dot{Q}}{C_i \cdot \tau}$$  \hspace{1cm} (12)$$

Finally, equation (2) was solved iteratively for $V_{\text{max}}$ and $R_{\text{day}}$ by assuming that $W_c$ occurs at low $C_i$ values. Wullschleger (1993) suggests using the portion of the curve where $C_i < 30$ Pa, however when $V_{\text{cmax}}$ values are low, the best fit may be obtained using larger portions of the curve (e.g., $C_i < 50$ Pa). Therefore, for each curve the largest range of $C_i$ values that produced the best fit to the $W_c$ form of equation 2 was used to determine $V_{\text{cmax}}$ and $R_{\text{day}}$. After determining $V_{\text{max}}$ and $R_{\text{day}}$, $J_{\text{max}}$ and TPU were determined by solving the entire A/C\textsubscript{i} response curve for the full-version of equation 2 (see Figure A-1).
Figure A-3. The components of an A/C\textsubscript{i} curve. The closed circles comprise a typical A/C\textsubscript{i} curve consisting of three underlying biochemical processes. \( W_c \) is the rate of carboxylation limited by rubisco activation, \( W_j \) is the rate of carboxylation limited by RuBP regeneration, and \( W_p \) is the rate of carboxylation limited by inorganic phosphate.
## Appendix III. Abbreviations and Units in Chapter 5

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<thead>
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<th>Abbreviation</th>
<th>Parameter</th>
<th>Units</th>
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<tr>
<td>A</td>
<td>Assimilation rate</td>
<td>$\mu$mol m$^{-2}$ s$^{-1}$</td>
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<tr>
<td>$C_a$</td>
<td>Atmospheric CO$_2$ concentration</td>
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<tr>
<td>$C_i$</td>
<td>Internal CO$_2$ Concentration</td>
<td>Pa</td>
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<tr>
<td>E</td>
<td>Transpiration rate</td>
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<td>$F_m$</td>
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</tr>
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<td>$F_s$</td>
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<td>$F_{dark}$</td>
<td>Machine background fluorescence</td>
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<td>I</td>
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<td>J</td>
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<td>$O$</td>
<td>Internal oxygen concentration</td>
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<td>Evolution of non-photorespiratory CO$_2$ in light</td>
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