Swiss Needle Cast

cooperative

ANNUAL REPORT

2000

Gregory Filip, SNCC Director

SNCC

OREGON STATE UNIVERSITY
COLLEGE OF FORESTRY

Members of the Swiss Needle Cast Cooperative and Their 2000 Contributions

Boise Cascade Corporation	$22,500
Coos County Forestry Department	$2,500
Confederated Tribes of the Grand Ronde	$7,500
Confederated Tribes of the Siletz	$700
Hampton Resources, Inc.	$22,500
John Hancock Life Insurance	$22,500
Longview Fibre Co.	$22,500
Menasha Corporation	$22,500
Miami Corporation	$7,500
Oregon Department of Forestry	$22,500
Rosboro Lumber Co.	$7,500
Simpson Timber Co.	$22,500
Starker Forests	$22,500
Swanson Superior Forest Products, Inc.	$7,500
The Timber Company	$22,500
Weyerhaeuser Corporation	$22,500
Willamette Industries	$22,500
USDA Forest Service	In kind
USDI Bureau of Land Management	$22,500
OSU Forest Research Laboratory	$30,000 (salary)

Swiss Needle Cast Cooperative

SNCC

Annual Report

2000

Edited by Greg Filip, SNCC Director

Layout by Gretchen Bracher, FRL Publications

Second Edition

SNCC INCOME SOURCES AND EXPENDITURES 2000

Income	
Membership Dues	$303,200
Oregon State Legislature	$120,000

Expenditures (as of 9/00)	
Salaries and Wages	$134,467
OPE	44,635
Supplies and Services	83,796
Travel	17,415
Indirect Costs	27,743
Total Expenditures	$308,056

Contents

To: SNCC Members
From: Greg Filip
Date: September 2000
Subject: 2000 Annual Report

This is the fourth year for SNCC, and I thank the members for all of the support that they have given SNCC this year. Last year we were fortunate to receive $240,000 from the Oregon State Legislature to support projects this year and 2001. This year's annual report contains summaries on the progress made on our 12 projects. We had two aerial survey flights this year, one in Oregon and one in Washington, that show a continuing infestation of Swiss needle cast. Information continues to be collected on the permanent growth impact and precommercial thinning plots. Progress continues on the basic infection biology research that is summarized in this report. Projects are continuing in needle physiology, soil and foliage nutrition, and tree genetics, and progress reports are contained in this report. Foliage application of sulfur continues to look promising as a short-term management tool for SNC. Several publications concerning SNC were written this year based on results obtained through SNCC.

I would like to especially thank this year's project investigators for their fine efforts in generating new information concerning Swiss needle cast: Alan Kanaskie, Doug Maguire, Katy Kavanagh, Jeff Stone, Randy Johnson, Scott Ketchum, Cathy Rose, and Gary Chastagner. And thanks to the many graduate students who do so much of the work, Dan Manter, Lori Winton, Bryan Capitano, Pablo Rosso, Paul Reeser, Wendy Sutton, Gabe Crane, and Fatih Temel. I would also like to thank the members of the SNCC executive committee who's enthusiasm and creativity keep this cooperative moving in the right direction: Mark Gourley, Tim Tompkins, Greg Johnson, Dale Claussen, Jim Carr, Mari Kramer, Will Littke, and Alan Kanaskie. We have at least 12 projects planned for 2001; it should be another exciting and productive year.

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Highlights of 2000

This report presents the Swiss Needle Cast Cooperative activities in Swiss needle cast research. Highlights for 2000 include:

- An aerial survey was conducted over 2.9 million acres in western Oregon. A total of 283,000 acres of Douglas-fir had obvious symptoms on Swiss needle cast. In general, symptoms of Swiss needle cast were about the same in 2000 as they were in 1999. Survey maps can be obtained from Alan Kanaskie, Oregon Department of Forestry in Salem.
- An aerial survey was conducted over almost 2 million acres in western Washington. A total of 410,000 acres had symptoms. Survey maps can be obtained from Dan Omdal, Washington Department of Natural Resources in Olympia.
- Research continues on 12 different projects in 2000 including: aerial survey, growth impact studies, tree physiology, infection biology, tree genetics, alternative fungicides, precommercial thinning, nutrient trends, nutrient imbalances, sulfur/tree growth, sulfur efficacy, and fertilizer and vegetation control.
- Bryan Capitano completed his MS thesis: "The infection and colonization of Douglas-fir needles by the Swiss needle cast pathogen, *Phaeocryptopus gaeumannii* (Rhode) Petrak."
- The "Silviculture and Swiss needle cast: research and recommendations" guide was published this year by the OSU Forest Research Lab.
- Everett Hansen, Jeff Stone, and others published their research in the journal "Plant Disease" with the title "Incidence and impacts of Swiss needle cast in forest plantations of Douglas-fir in coastal Oregon."

Plans for 2001

- Continue aerial survey to monitor SNC in Oregon and Washington
- Monitor permanent plots from the growth impact study Phase III
- Determine stem and root limitations to water movement in SNC-affected trees. Determine the effects of microclimate on fungal development and physiological impact.
- Conduct infection biology studies: Factors affecting colonization rate and foliage retention; *P. gaeumannii* infection, development, and reproduction; aerobiology and epidemiology; and population biology of *P. gaeumannii*.
- Determine trends in soil and nutrient status across a gradient in SNC severity
- Characterize nutritional imbalance as a predisposing factor in SNC
- Develop early screening techniques for SNC resistance in Douglas-fir families

- Determine growth response to precommercial thinning in Douglas-fir stands with varying intensity of SNC in the Coast Range of Oregon
- Identify alternative fungicides and application timings to reduce SNC damage in stands of Douglas-fir
- Determine the effects of fertilization and vegetation control on SNC infection and growth of coastal Douglas-fir
- Determine the effects of elemental sulfur on SNC and growth of coastal Douglas-fir saplings
- Determine the efficacy of sulfur, optimal application frequency, and spreader-sticker benefits.

Background and Organization

The Swiss Needle Cast Cooperative (SNCC) was established in January 1997. Damage caused by Swiss needle cast, a native foliage disease that affects Douglas-fir, has made it imperative that new research be conducted to learn practical methods of disease detection and management to maintain the health and productivity of Douglas-fir plantations. A well-run cooperative is an efficient means of increasing and accelerating the level of forest disease research in the region. Because members participate directly in problem identification and research solutions, communications of results is speeded and results are applied more rapidly and effectively.

SNCC is located in the College of Forestry at Oregon State University. The Membership is comprised of private, county, state, and federal organizations. Membership dues vary depending on forestland ownership. One annual report, project reports, and newsletters are distributed to members each year. All projects are carried out in cooperation with specific members on their land holdings.

Purpose

The focus of SNCC will be Swiss needle cast research for forestland owners in western Oregon and Washington. The purpose of SNCC is to provide the following services:

1. Conduct research on the biology, detection, and management of Swiss needle cast in coastal Douglas-fir as related to basic infection biology and genetics, tree physiological dysfunctions, aerial and ground survey technology, disease hazard and risk rating, growth and yield impacts, and strategies for control.
2. Conduct training and workshops on research and survey results
3. Provide newsletters and reports on research and surveys, and
4. Serve as a focal point for information on Swiss needle cast.

Swiss Needle Cast Aerial Survey, 2000

Alan Kanaskie, Mike McWilliams, Keith Sprengel, Dave Overhulser

Survey Procedures

The observation plane flew at 1,500 to 2,000 feet above the terrain, following north-south lines separated by 2 miles. Observers looked for areas of Douglas-fir forest with obvious yellow to yellow-brown foliage, a symptom of Swiss needle cast. Patches of forest with these symptoms (the patches are referred to as polygons) were drawn onto 1:100,000 scale topographic maps. Each polygon was classified for degree of discoloration as either "L" (light) or "H" (heavy). Polygons classified as "H" for discoloration had very sparse crowns and brownish foliage, while those classified as "L" were predominantly yellow to yellow-brown foliage and slightly more dense crowns than those classified as "heavy".

New sketch-mapping technology was used for the first time in 2000. Instead of using paper maps, observers used computer generated topographic maps linked to a real-time Geographic Positioning System (GPS). Observers recorded damage onto computer touch screens displaying the topographic maps and the position of the aircraft. This technology allowed observers to spend less time navigating and more time mapping. Observers generally could be more precise in their mapping and could draw smaller polygons than in previous surveys.

The survey of the Coast range began on April 20 and ended on June 1, 2000. It extended from the coastline eastward until obvious symptoms were no longer visible, and from the Columbia river south to approximately the Coos/Curry county line.

Two flights were made over the west slopes of the Cascade Range; one on May 20 and one on June 2, 2000. These flights extended from the Columbia river south to near Roseburg.

Results of the Survey

Figure 1 shows the approximate size and location of areas of Coast Range Douglas-fir forest with symptoms of Swiss needle cast detected during the survey conducted in 2000. Figures1 also shows survey results for 1999-1996. The Coast Range survey covered about 2.9 million acres of forest. A total of 283,000 acres of Douglas-fir forest had obvious symptoms of Swiss needle cast; 224,500 acres north of Florence, and 58,500 acres south of Florence. This is a decrease of about 12,000 acres compared to the 1999 survey (table 1, figure 2). Most of the decrease in

the number of acres with symptoms occurred in Lincoln and Tillamook counties. South of Florence, the number of acres with symptoms increased by about 23,000 acres between 1999 and 2000. The easternmost area with obvious SNC symptoms was almost 30 miles inland from the coast, which is the same as in the 2000 survey.

A small amount of Swiss needle cast damage was mapped North of Sweet Home near Keel mountain during the Cascade range survey. These areas were visited on the ground and Swiss needle cast was confirmed. Symptoms in the Cascades were must less developed than in the Coast range, and areas with symptoms tended to occur infrequently and in small patches. By the time weather cleared enough for additional flights in the Cascades, symptoms were no longer visible because of the emergence of new foliage. Thus far the disease appears to be of concern in a few localized areas.

Although the results of the 2000 survey suggest a flattening or decrease in the total amount of area with symptoms of Swiss needle cast, it would be premature to say that the disease has peaked. The inherent variability of symptom development from year to year and the use of the new sketch mapping technology could easily explain the small change in acres mapped between 1999 and 2000. The large increase in acres

Table 1. Area of Douglas-fir forest in western Oregon with symptoms of Swiss needle cast detected during aerial surveys in 1996-2000.

Region	1996	1997	1998	1999	2000
	acres				
North of Florence	106,000	130,000	135,000	259,000	226,000
South of Florence	24,000	30,000	38,000	36,000	57,000
TOTAL	30,000	160,000	173,000	295,000	283,000

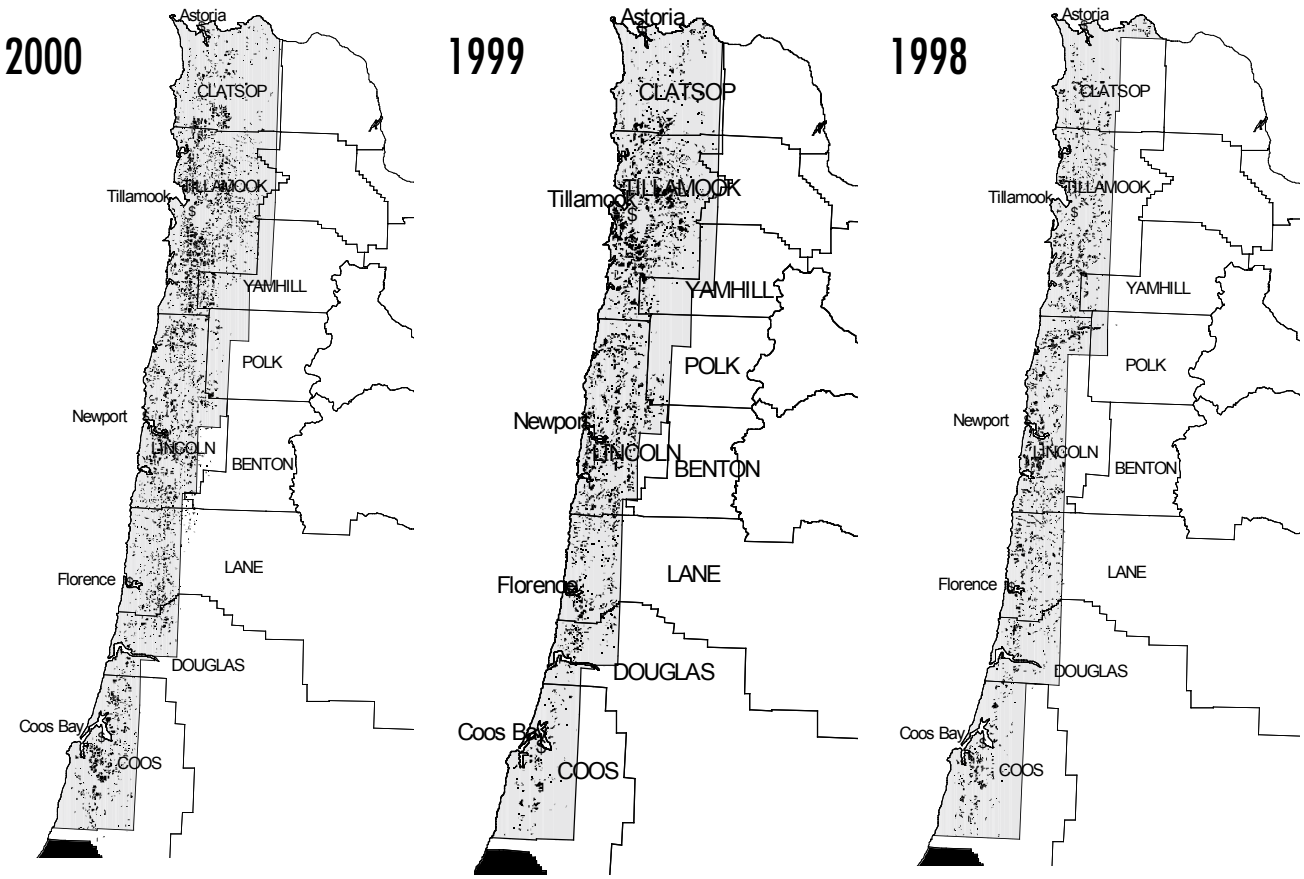

Figure 1. Approximate size and location of areas of Coast Range Douglas-fir with symptoms of Swiss needle cast detected during the aerial survey conducted in 1998, 1999, and 2000.

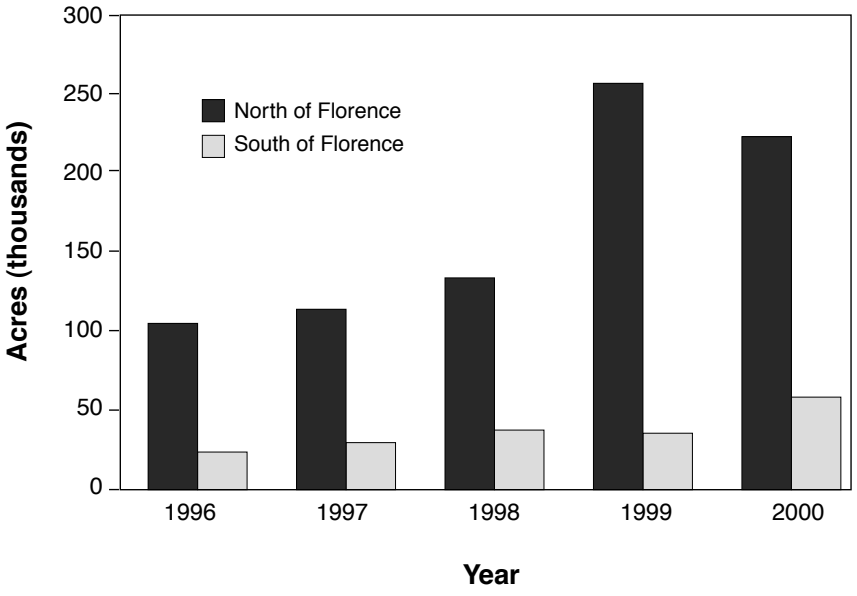

Figure 2. Area of Douglas-fir forest in western Oregon with symptoms of Swiss needle cast detected during aerial surveys from 1996 to 2000.

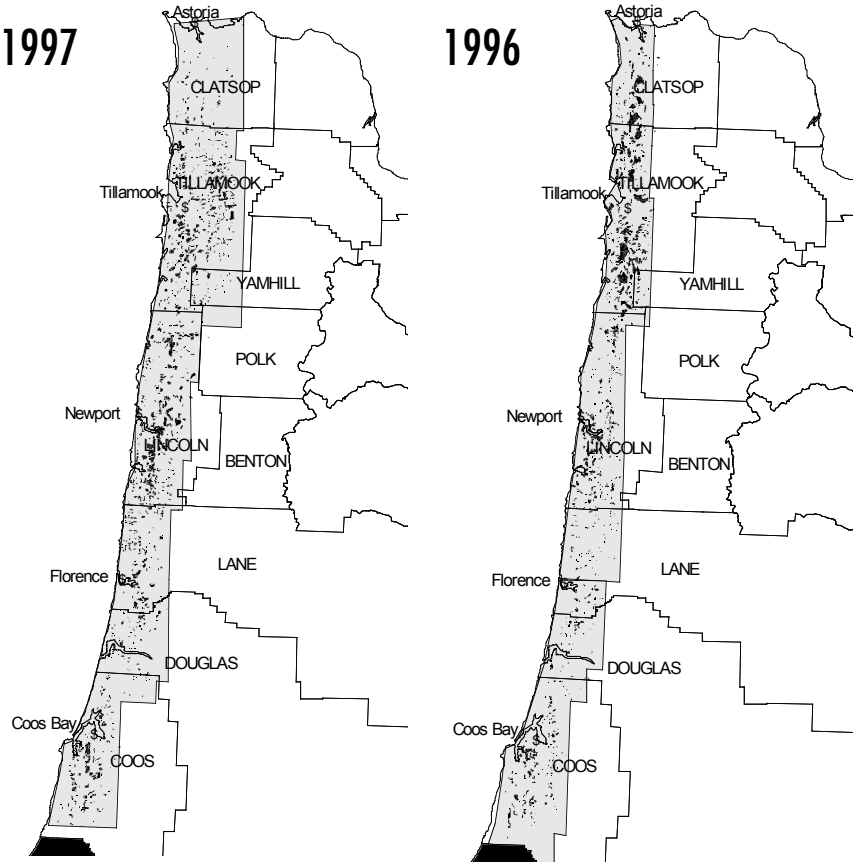

Figure 1. Approximate size and location of areas of Coast Range Douglas-fir with symptoms of Swiss needle cast detected during the aerial survey conducted in 1996, and 1997.

ɔped south of Florence suggests : disease continues to intensify hat region. Observation of areas th of Florence suggest continued ı levels of damage, with a pos- e slowing of the rate of increase : has been evident over the past years. Symptoms occurred no her inland in 2000 than in 1999. st areas with symptoms that can letected from the air are within ut 18 miles of the coast.

Some year-to-year variation in ɣey results is due to timing of the ɡıts. Because symptoms develop rapidly during April and May, later surveys detect more areas with symptoms than those conducted earlier. This was very evident in 1997 when a few extra days of surveying at the time of bud break increased the estimate of acres with symptoms from 145,000 to 393,000. This happened again to some degree in 1999, when about half of the survey was completed between April 21 and April 30, and the other half on May 21 and 22. However, the unseasonably cool spring temperatures in 1999 delayed bud-burst by about 2 to 3 weeks compared to previous years, so even though much of the survey was completed in late May, it was well within the target surveying window. Had the survey been flown later (in early June), we undoubtedly would have mapped even more acres of heavy discoloration during 1999. For the 2000 survey, about 20 percent of the flight time occurred in mid-April, 60 percent in mid-May, and 20 percent at the beginning of June. Periods of cloudy weather caused most of the delays.

Aerial survey results are conser-

vative estimates of damage because observers map only those areas where disease symptoms have developed enough to be visible from the air. Permanent monitoring plots and ground checks have shown that Swiss needle cast occurs throughout the survey area, but that symptoms often are not developed enough to enable aerial detection. Factors other than the presence of the pathogen strongly affect disease development, and these factors remain poorly understood. The shape and distribution of survey polygons and the observer's comments suggest that symptoms are most obvious on southerly aspects and on exposed ridge tops, indicating a strong environmental interaction.

Winter weather can affect symptom development. During 1999 ground checks, brown needle tips were observed commonly, and may have resulted from cold temperatures during the week before Christmas in 1998. However, this brownish discoloration was restricted to Douglas-fir, and even if it was partly due to low temperature injury, Swiss needle cast was an important factor as well. The upper crowns of many trees were barren of needles, apparently battered by the unusually frequent high-winds during the winter of 1998-1999. The winter of 1999-2000 was relatively mild and uneventful compared to the previous two years, and probably exerted little direct influence on the results of the 2000 survey.

Acknowledgments

The survey was conducted by the Oregon Department of Forestry Insect & Disease and Air Operations sections, and was funded by the USDA Forest Service Forest Health Monitoring Program and the Oregon Department of Forestry. Jack Prukop (ODF) piloted the plane. Mike McWilliams (ODF), Keith Sprengel (US Forest Service), and Dave Overhulser (ODF) were the aerial observers.

Early Testing of Douglas-fir Families for Swiss Needle Cast Resistance

Fatih Temel and Randy Johnson

The primary objective of this study is to develop techniques that will allow for early screening of Douglas-fir families for Swiss needle cast (SNC) resistance/tolerance. This is being done by inoculating seedlings from 57 families that have already been screened for SNC tolerance in older field tests. An additional 96 families are being screened so that we can better study the genetic variation of SNC tolerance over the landscape. Last year we deployed four sets of plantings in an attempt to find at least one inoculation method that would produce varying levels of infection (symptoms) in young trees. Two field sites were established on Simpson Timber Company land, one near Toledo and one near Pleasant Valley. Seedlings in two other sets were artificially inoculated and transplanted into raised beds at the Dorena Tree Improvement Center near Cottage Grove.

Artificial Inoculations

Two sets of seedlings were artificially inoculated in the mist chamber at Dorena for one week using infected branches as the inoculum source. One set of seedlings was removed from the mist chamber at the end of one week and transplanted to nursery beds, which were covered with shade cloth and well watered to maintain high humidity for an additional two weeks. The other set of seedlings remained in the chamber with high humidity an additional week without the infected branches. This set was transplanted to the nursery beds and covered with shade cloth for an additional week. The seedlings received what we thought was a high dose of spores, between 10 to 20 spores per mm^2. At present, the seedlings have the fungus in their needles based on quantitative polymerase chain reaction (PCR) by Lori Winton; however, there were no visible fruiting bodies this spring. The seedlings will be examined this coming spring to see if any symptoms develop. Based upon results to date, the artificial inoculation method is not proving effective in screening families for SNC resistance/tolerance.

Field Sites

The two-year-old seedlings in the field trials near Pleasant Valley and Toledo were visually assessed in May of this year for SNC symptoms on their 1999 foliage. Needle retention was scored on a south

facing branch from 0 (0-10% needle retention) to 9 (91-100% needle retention). Needle color was scored from 1 (yellow) to 3 (green) on the same branch. Overall foliage density (from 1=very sparse to 6=very dense foliage) and color (from 1=yellow to 3=green) were based on all branches on the seedling that had 1999 foliage. These assessments were repeated in September 2000 and include height measurements.

Branches were also sampled this spring from seedlings of 57 families in this study for detailed laboratory analyses. Samples were stored in a freezer until lab assessments were conducted throughout the summer. Needle color was assessed by comparing the needles to a color photo with four different Douglas-fir foliages, ranging from brownish yellow to dark green. Needle retention was visually estimated as in the field. We found that the correlation between the estimated foliage retention score (1-9) on the sample branch we brought back to the lab was highly correlated (r=0.94) with the actual retention that was calculated by counting all needles and needle scars on the same sample branch. Percent stomata occluded with pseudothecia was determined by observing 500 stomata from 2 needles from each sample in a family (10 needles total per family). We also measured projected needle specific area, internode length and needle length (based on 50 needles, 10 from each seedling). This fall we will also quantify amount of fungal biomass using quantitative PCR method. Preliminary data analyses from the field assessments are reported here. Results from lab assessments will be completed when DNA data is available.

Field Results

Narrow-sense heritability estimates the proportion of the total variation of individual trees controlled by the genetics of the trees; the remaining variation is that due to the environment. Likewise, family-mean heritability estimates the proportion of the variation in family means controlled by genes. The narrow-sense heritability estimates for the foliage traits were low to moderate for the both the two young trials and the two 10-year-old progeny tests measured in 1996 (Table 1). Needle retention had a higher heritability in the younger trials. Color and crown density heritabilities were similar across trial ages. Genetic correlations between the four traits scored in the two-year-old trials were high ($r>0.68$, $p<0.01$), suggesting that these traits are under the control of same set of genes. The traits we measured were also relatively stable across sites, i.e. family ranks were similar across the two sites.

In order for early testing to be successful there must be sufficient genetic correlation between the trait measured at the early age with that measured at the older age. Preliminary genetic correlation estimates among visually assessed traits at the young age with their respective counterparts assessed at age 10 were relatively strong (Table 2). The large genetic correlation estimates with age-10 retention is a function of its low estimate of heritability (the genetic correlation was calculated as the family-mean correlation divided by the geometric mean of the two family-mean heritabilities). The two color traits in these young trials were also correlated with estimated basal area growth from age 10 to 13 in the older trials.

The efficiency of early selection is calculated as

$$\text{Efficiency} = (h_{early} / h_{late})\, r_{el}$$

Where: h is the square root of the family mean heritability and r_{el} is the genetic correlation among the trait at the two different ages. Based on our preliminary estimates, the efficiency of early selection is 0.50 for color, 0.32 for crown density, and 1.42 for needle retention. This implies that selecting for an SNC trait at age 2 would be about 50% as efficient as delaying selection until age 10.

Other ongoing studies

We are also investigating the relationship between actual level of *Phaeocryptopus gaeumannii* (PG) infection and SNC symptom development. We are attempting to determine whether the more tolerant families have less PG or simply grow better for a given amount of PG. Six families (two families from each of three disease severity groups) were identified in the Acey Creek and Coal Creek progeny test plantations based on previous SNC assessments. Symptoms (color, crown density and needle retention) were scored on trees in the field and on sample branches collected for laboratory analyses. Infection level is being measured by counting occluded stomata and by estimating

fungal content with Lori Winton's PCR technique.

Field data from all 153 families in the our young progeny trials will be combined with growth and phenology data that was collected from raised beds in Corvallis to examine genecology of Douglas-fir with respect to SNC resistance.

Table 1. Heritability estimates from 2-year-old and 10-year-old progeny tests.

	Narrow-sense heritability		Family mean heritability	
Trait	2-year-old trials	10-year-old trials	2-year-old trials	10-year-old trials
Needle color	0.15	—	0.35	—
Needle retention	0.22	0.06	0.43	0.19
Foliage color	0.18	0.18	0.39	0.55
Foliage density	0.21	0.24	0.41	0.57

Table 2. Genetic correlation estimates between 2-year-old and 10-year-old progeny tests.

	Age-10 trait			
Age-2 trait	Color	Crown Density	Retention	Basal area increment
Needle color	0.56	0.30	>1.0	0.58
Needle retention	0.51	0.33	>1.0	0.09
Foliage color	0.42	0.32	>1.0	0.43
Foliage density	0.74	0.40	>1.0	0.24

Infection Biology and Epidemiology of *Phaeocryptopus gaeumannii*

1. Seedling exposure experiments

Jeffrey Stone, Loretta Winton, Paul Reeser, Dan Manter, Wendy Sutton, Everett Hansen

Abstract

Groups of potted seedlings exposed at two week intervals to *Phaeocryptopus gaeumannii* ascospores at a diseased site became infected between early May and early July. Infection occurred almost entirely in current season foliage. Only a small proportion of one year old needles became infected. Peak infection periods coincide with periods of precipitation, but once ascospore release begins, small amounts of rainfall are sufficient to maintain high infection levels. Seedlings exposed for four or eight weeks between May 27 and July 21, 1999 had higher incidences of infection and much greater numbers of pseudothecia one year after exposure than seedlings exposed for two week intervals during the same period.

Introduction

Field experiments have been conducted for the past three years to identify the peak infection period and phenological stage of the foliage most susceptible to infection. Groups of potted seedlings were exposed at the Salal site for two-week periods between mid March and late August in 1997, 1998, and 1999. In 1999 longer exposure periods were added to examine the effect of duration of exposure to *P. gaeumannii* inoculum on resultant colonization of Douglas-fir foliage. To determine whether foliage older than one-year can become infected, infection levels for two-year old foliage were compared with one-year old foliage on trees for each exposure interval.

Materials and Methods

Groups of fifteen potted seedlings were exposed to inoculum of *P. gaeumannii* at the Salal site for varying periods between April 15, 1999 and August 4, 1999. The seedlings were obtained from a container nursery and had not previously been exposed to *P. gaeumannii* inoculum. Groups of seedlings were exposed for either two-week,

four-week, or eight-week intervals so that four week exposures overlapped with two week exposures, and eight week exposures overlapped with four-week exposures (Table 1). At the end of the exposure periods, the trees were returned to OSU Botany farm for incubation. Two groups of fifteen trees were kept at the OSU Botany farm as unexposed controls. One of these groups was treated with chlorothalonil (Bravo 720) and the other was unsprayed. A third group of fifteen trees was treated with chlorothalonil (Bravo 720) and exposed at the Salal site for eight weeks (interval 5-8).

Branches were collected and analyzed for *P. gaeumannii* in May, 2000. Two 1999 (one-year old) and two 1998 (two-year old) internodes were clipped from each seedling, the needles stripped, and ten needles chosen randomly for PCR analysis. A sample of 50 needles for each internode/seedling was attached to index cards with double faced adhesive tape for visual assessment of *P. gaeumannii* colonization. Incidence of infection was determined as the proportion of fifty needles bearing pseudothecia of *P. gaeumannii*. Pseudothecial density was determined as the proportion of stomata occluded by pseudothecia for ten needles. Needles on index cards were examined under a binocular dissecting microscope with a counting grid for determination of incidence and density. Three segments of each needle were counted, the area of each segment was 0.676 mm^2. For each needle the total area counted was 2.028 mm^2, and 20.28 mm^2 for all ten needles.

Table 1. Dates of exposure to inoculum at the Salal site for groups of fifteen seedlings.

Interval	duration (weeks)	dates
1	2	4/1-4/15
1-2	4	4/1-4/29
1-4	8	4/1-5/27
2	2	4/15-4/29
2-3	4	4/15-5/13
3	2	4/29-5/13
3-4	4	4/29-5/27
3-6	8	4/29-6/24
4	2	5/13-5-27
4-5	4	5/13-6/10
5	2	5/27-6/10
5-6	4	5/27-6/24
5-8	8	5/27-7/21
6	2	6/10-6/24
6-7	4	6/10-7/8
7	2	6/24-7/8
7-8	4	6/24-7/21
8	2	7/8-7/21
9	2	7/21-8/4

Results

Previous years studies have identified a period of infection in early summer. Trees exposed at a field site with moderate Swiss needle cast become infected when exposed from early May until late June (Figure 1). In all three years infection was negligible before May 1 and after June 30, with peak infection occurring between mid May and mid June. Pseudothecia are mature and ascospores are available for release earlier. In 1999 mature ascospores were present by mid April (Figure 2), and in 1998 they were available in late March (Figure 3). Ascospores are available for infection between mid April and mid July. In both years, ascospore availability was reduced by late July. In 1999, the peak ascospore availability occurred before the peak infection period and had begun to decline as infection levels increased.

In 1998, incidence of infection in the exposed trees appeared to follow rainfall for each exposure period (Figure 4). In 1999, however, the two week period with the highest infection incidence, 6/10-6/24, had relatively little measurable precipitation (Figure 5). This suggests that once ascospore release has begun, only a small amount of precipitation is required for successful spore dissemination/infection. This can also be seen in the 1998 exposure period 6/24-7/9, which had a relatively high level of infection but the rainfall measured was only 40mm. The subsequent exposure period had a similar amount of precipitation but only half as much infection (Figure 4), but ascospore production was reduced in the 8 and 21 July in 1998 (Figure 3).

Exposing seedlings to inoculum for varying periods should have caused differences in the cumulative inoculum dose for seedlings that were exposed during peak spore release. Seedlings that were exposed for two weeks should have received a lower cumulative inoculum dose than seedlings exposed for four or eight weeks during peak spore discharge. Initial infection occurred as early as April 15 in 1999, but incidence was low. After June 10 incidence of infection greater than 50% began to be detected. For the two-, four-, and eight-week intervals ending June 24, incidence was much greater for the four- and eight-week exposures than for the two-week exposure (Figure 6). This was also true for the two- and

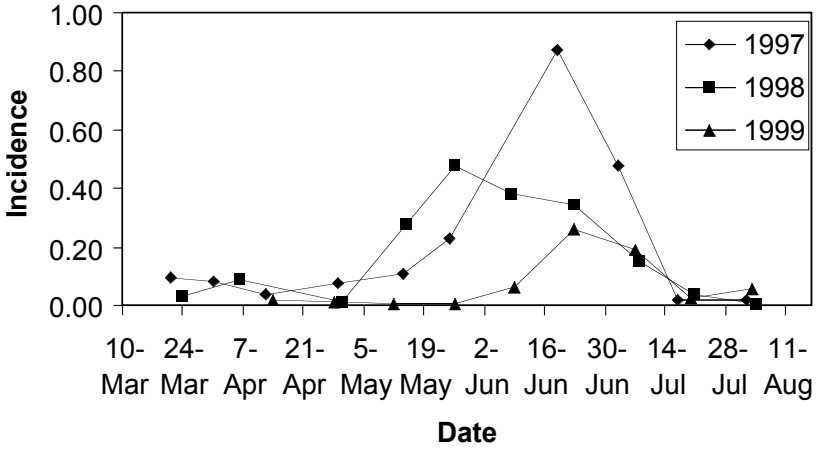

Figure 1. Infection incidence in seedlings exposed at the Salal site in 1997, 1998, and 1999.

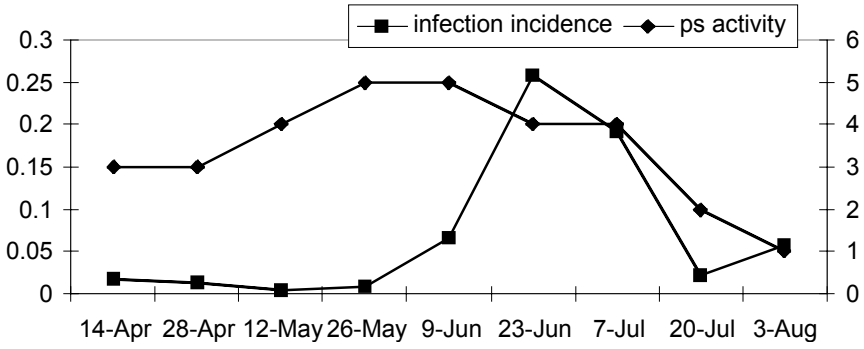

Figure 2. Incidence of infection of potted seedlings in 1999 compared with availability of mature ascospores (ps activity).

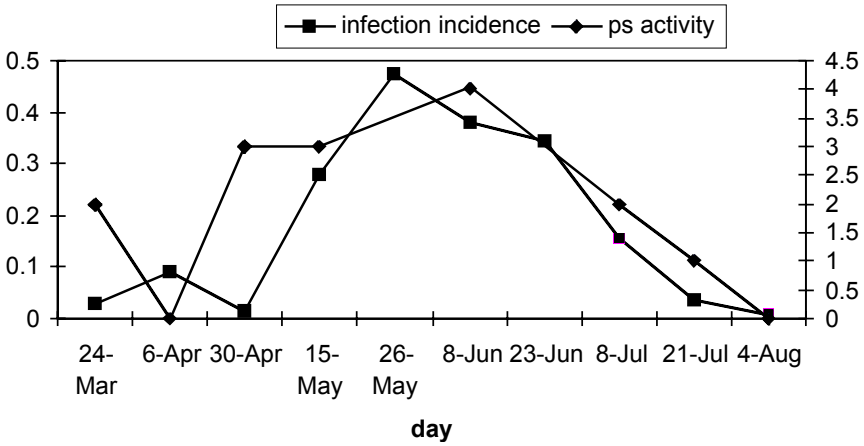

Figure 3. Incidence of infection of potted seedlings in 1998 compared with availability of mature ascospores (ps activity).

four-week exposures ending July 8, and the two-, four-, and eight-week periods ending July 21.

Infection severity, measured as normalized quantitative PCR and counts of pseudothecia on needles, also was much greater for the four- and eight-week exposure periods than for the two-week periods during the period of peak ascospore release (Figure 7). The eight week exposure period ending June 24 had quantitative PCR 7.28 times as high as sum of the separate two-week levels. Interval 5-8, which covered the entire peak spore release period (May 27- July 21), had almost twice the number of pseudothecia as the sum for the four two-week intervals over the same period, and a quantitative PCR level 5.5 times higher than for the sum of the two-week exposures. A similar trend is apparent for the four-week intervals compared to two-week intervals. Interval 6-7 (June 10-July 8) had 1.8 times as many pseudothecia and 3.9 times greater quantitative PCR value. It is clear that longer periods of exposure to inoculum lead to disproportionately higher levels of needle colonization.

Young foliage became infected when exposed to inoculum between mid April and early August. Foliage that was already one-year old at the time of exposure, however, had very slight levels of infection. Incidence of infection in 1998 needles was very low. Nine of 60 seedlings sampled had pseudothecia on needles that had been exposed to inoculum in their second growing season, and incidence of infection for these seedlings was less than 10% (Figure 8). Similarly low values were obtained with the quantitative PCR analysis of one- and two-year-old needles (Figure 9). No infection was detected in either the one- or two-year-old needles prior to the exposure period ending May 27, although most infection occurred in the period May 27 – June 24. Needles in their second growing season appear to be much less susceptible to infection than needles in their first season. Although two-year old needles can become infected, this only occurs when inoculum levels are very high. The proportion of two-year old needles that become

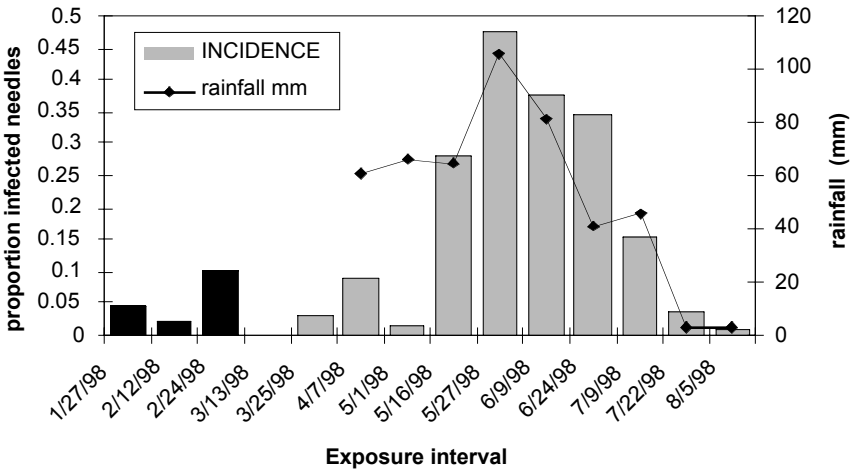

Figure 4. Incidence of infection in seedlings exposed at the Salal site in 1998 compared with rainfall for each two-week period.

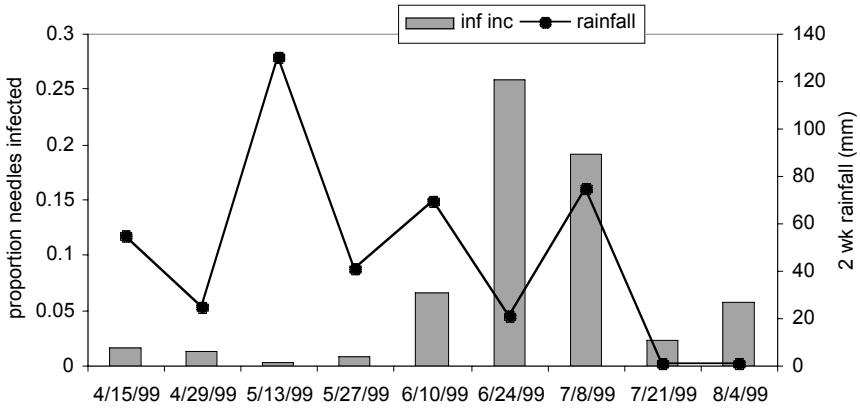

Figure 5. Incidence of infection in seedlings exposed at the Salal site in 1999 compared with rainfall for each two-week period.

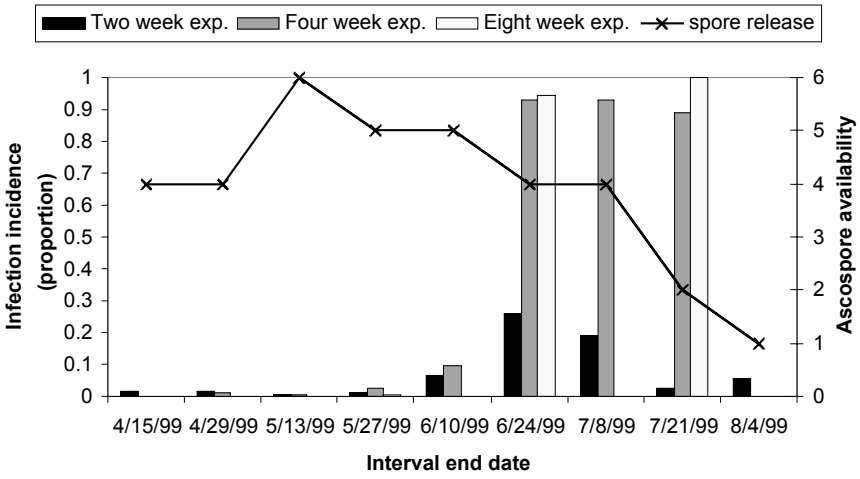

Figure 6. Incidence of infection in seedlings exposed for two-, four-, and eight-week intervals compared with ascospore production.

infected even under high inoculum levels is quite low.

Discussion

Studies with potted seedlings over the past three years confirm that infection of Douglas-fir by *Phaeocryptopus gaeumannii* occurs between late May and late July, with most infection occurring between early June to mid July. This coincides with the peak period of ascospore release at the site. Newly emerged foliage is susceptible to infection, but older foliage is much less susceptible. Very little new infection from ascospores occurs on two-year-old foliage. Since the seedlings used in these experiments were grown in a container nursery and had no exposure to ascospores of *P. gaeumannii* prior to our field exposure experiment, infections detected in two year old foliage must have resulted from new infection from ascospores in 1999, and not development of an earlier latent infection. Foliage protected from infection in its first growing season therefore should remain disease free, since older foliage is much less susceptible to infection by ascospores.

Chastagner and Byther (1983) reported an infection period for Christmas trees between late May through June. A single application of chlorothalonil in late May-early June was sufficient to provide complete control of Swiss needle cast in a Douglas-fir Christmas tree plantation in western Washington. Protectant sprays applied after June 25 were ineffective. Our results on timing of ascospore release and infection in forest plantations in western Oregon

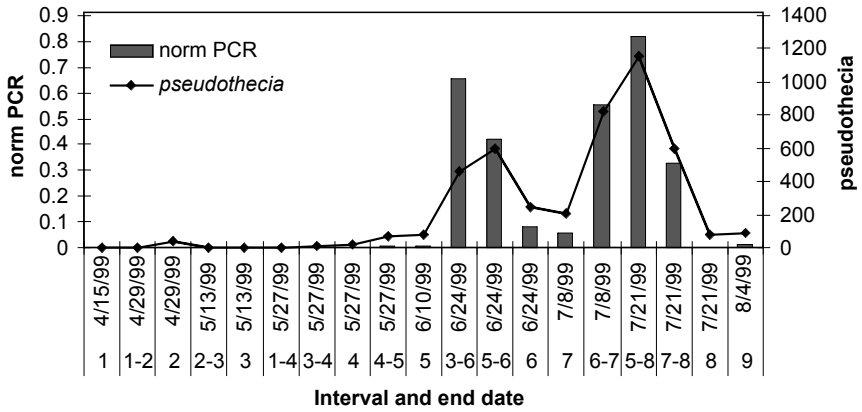

Figure 7. Infection severity as determined by quantitative PCR and direct counts of pseudothecia on needles. Values for quantitive PCR are averages of ten needles. Values for pseudothecia counts are the total for ten needles. Three sections of each needle were counted, each section was 0.676 mm^2 and had approximately 180 stomata.

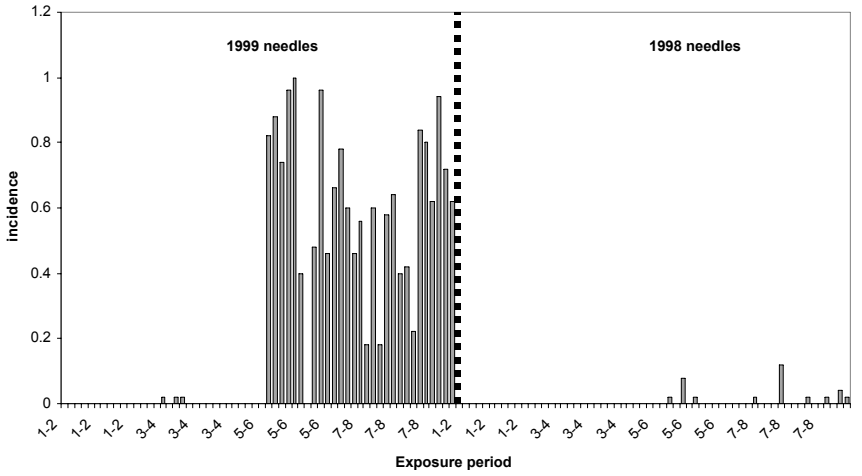

Figure 8. Incidence of infection in 1999 and 1998 needles from seedlings exposed to inoculum for four-week intervals in 1999.

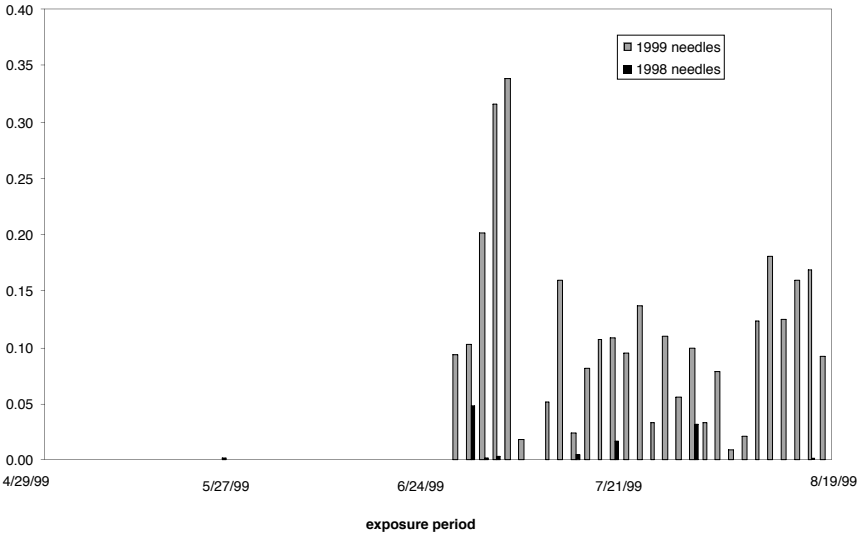

Figure 9. Quantitative PCR analysis of one-year-old and two-year-old needles for seedlings exposed at the Salal site in 1999. Both age classes were analyzed one year following exposure to inoculum.

are similar, but with a low level of infection occurring in early May and the infection period extending through mid July. The availability of ascospores also shows a sharp increase between May and June and decreases by September (Michaels and Chastagner 1984).

Much higher infection levels were found in seedlings that had been exposed to inoculum for a longer period. Exposure to higher levels of inoculum resulted in higher levels of colonization of foliage. Colonization of foliage of seedlings exposed to four or eight weeks was much higher than the sum of the colonization for seedlings exposed in the corresponding two-week intervals. One of the characteristics of the current Swiss needle cast problem is that the fungus is completely colonizing needles and producing fruit bodies earlier, i.e. after one year, than is typical of the fungus where Swiss needle cast is not severe. Where *P. gaeumannii* is typically found fruiting on three- and four-year old foliage, it follows that the foliage has been infected since its first growing season and that the fungus has remained at harmless levels for three to four years.

Our data suggest that very high levels of inoculum can lead to more rapid colonization of needles and earlier fruiting. Colonization of needles from one to a few initial infection sites is a slow process that may take several growing seasons to reach a level where needle health is sufficiently impaired to cause needle abscission. Foliage of Douglas-fir can normally tolerate low to moderate levels of colonization by *P. gaeumannii* without severe needle loss. Hood (1982) reported

widespread *P. gaeumannii* in southern British Columbia, but did not report proportions of pseudothecia for needles aged less than 3-4 years, suggesting that pseudothecia were sparse or absent from most young foliage. In severely diseased sites in western Oregon, needle loss is apparent in needles aged two years or more, and often needles age three years or more are absent. Premature abscission of needles occurs when the proportion of stomata occluded by pseudothecia exceeds 50% (Hansen et al 2000). Initial infection of a needle from many ascospores leads to more rapid colonization of the needle, earlier production of fruiting bodies in the stomata, and more adverse effects on needle physiology. It appears that above a threshold level of infection a more rapid rate of colonization occurs, possibly due to disruption of needle physiology, resultant metabolite leakage, and premature senescence, all of which could in turn lead to increased fungal growth.

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Infection Biology and Epidemiology of *Phaeocryptopus gaeumannii*

2. Field plots

Jeffrey Stone, Paul Reeser, Loretta Winton, Wendy Sutton, Dan Manter, Everett Hansen

Abstract

Disease severity varies between sites affected by Swiss needle cast in the Oregon Coast Range, but within sites measures of severity have remained unchanged over three years. Severity of disease is not increasing in coastal Oregon sites, but in sites with severe disease it is not diminishing. Disease severity is strongly influenced by site specific factors. Climatic factors that favor fungal infection and colonization are strongly correlated with disease severity measures. Three-year cumulative leaf wetness at 12-26 C is greatest for sites that have the most severe symptoms. The cumulative leaf wetness at 12-26 C between April and August is highly correlated with the amount of colonization of needles by *P. gaeumannii*. Levels of *P. gaeumannii* are negatively correlated with needle retention. Spring-summer leaf wetness at 12-26 C is a good predictor of SNC severity and could be used as a criterion for site evaluation for Douglas-fir.

Introuction

Despite periodic interest in the disease, there has been little documentation of the distribution, severity, and growth impacts of Swiss needle cast in forests in the Pacific Northwest. Most of the published research on Swiss needle cast disease comes from Christmas tree plantations or forests in Europe or New Zealand where Douglas-fir is planted as an exotic species (Boyce 1940, Chastagner 1996, Hood, 1996, Hood and Kershaw 1975). Severe defoliation and growth reduction due to Swiss needle cast disease were observed in Douglas-fir plantations in Switzerland as early as 1925 (Gaeumann 1930) and subsequently reported from other European countries (Boyce 1940), New Zealand, and Australia (Beekhuis 1978, Hood 1996). However, factors affecting the distribution and severity of the disease in the forests of the Pacific Northwest where both Douglas-fir and the pathogen are native have received little attention. In contrast to the situation in Europe and other areas where Douglas-fir is cultivated, defoliation due to Swiss needle cast has not been documented in forests of the Pacific Northwest until recently (Hansen et al 2000).

Phaeocryptopus gaeumannii, the causal agent, is widespread in the Pacific Northwest, but until recently was considered insignificant in North American forest situations (Boyce 1940, Hood 1982). Although recent concern about Swiss needle cast near Tillamook dates from 1990 or later, there were earlier observations of the disease in the vicinity, and it is almost certain that the fungus is native to the area. Meinicke (unpublished, cited in Boyce 1940) collected the fungus in 1938 near Otis Junction and Hebo near the center of the current area of severe symptoms, but stated that it was not causing defoliation or yellowing.

Hood (1982) conducted the only published survey that measured infection levels and needle retention in PNW coastal forests. Hood collected branches from young, forest-grown Douglas-fir trees in southern British Columbia and northwestern Washington and examined them for numbers *Phaeocryptopus* fruiting bodies and needle retention. The fungus was widespread, but incidence varied from greater than 80% of needles infected in wet coastal and mountain areas to less than 5% infection in the rain shadow on the east sides of Vancouver Island, the Olympic Peninsula, and the Coast Mountains of British Columbia. Hood reported a significant correlation between infection and rainfall between May and July. There was no comment on defoliation associated with high infection levels, and overall, >50% of 6-year-old needles remained on the sampled branches.

Even within the area of severe disease impact in western Oregon, there is considerable variation between sites with respect to needle retention, foliage color, and amount of fungal colonization of needles. We have been monitoring several measures of disease severity in relation to environmental and site factors to attempt to identify site characteristics that might influence fungus colonization and disease development.

Materials and Methods

Measurements of disease impact were made at nine monitoring plots first established in 1996. The nine plots are grouped in three clusters of three plots each in the vicinity of Tillamook, Oregon (Table 1). The plots in each cluster were placed in Douglas-fir plantations of the same age and, where possible, the same seed source. Plantations were selected to represent different elevations and distances from the ocean and exhibited a range of disease severity. One plot in each group has moderate to heavy symptoms of Swiss needle cast, and one site is classified as healthy.

The South Cluster of plots are all USDA Forest Service progeny test plantations. Ten trees in each of two families were selected for measurements in each plantation. Plots of the Tillamook Cluster were planted with seedlings from the same bulk seed lot, from the "Boundary" seed collection area of the Coast Ranges, at about 600 m elevation. Ten trees were randomly selected for measurements in each plantation. The North Cluster included one plot planted with the Boundary source (North Fork), and two Oregon Department of Forestry progeny test plantations. Ten trees of each of two families (different from the South plots) were selected for measurements in each of the latter plantations, and ten trees were measured at North Fork.

Each study site is equipped with weather monitoring equipment. A tipping bucket rain gauge, leaf wetness sensors, and temperature sensors are maintained at each site. Symptoms of Swiss needle cast have been measured annually at all nine sites since

Table 1. Characteristics of field plots.

SITE	Disease Severity	Elevation	MILES TO OCEAN/BAY	AGE (1996)	SEED SOURCE	Aspect
JUNO HILL	Severe	380	2.25	14	Boundary 1800FT	NE
STONE RD LOWER	Mild	430	14.75	14	Boundary 1800FT	SW
STONE RD UPPER	Healthy	1700	14.5	14	Boundary 1800FT	N
N FORK	Severe	160	4.75	10	Boundary 1800FT	SW
COAL CRK PROGENY	Moderate	220	5	10	1600FT & 1400FT	SE
ACEY CRK PROGENY	Healthy	670	8	10	1600FT & 1400FT	E
SALAL PROGENY	Moderate	370	4	9	1000FT	NW
CEDAR NORTH PROGENY	Mild	1500	7.5	9	1000FT	NW
LIMESTONE PROGENY	Healthy	890	12.25	9	1000FT	N

1999. Tree height and diameter are measured, and a visual index of crown density and transparency is made for each tree. Needle retention is determined for two lateral branches from the mid crown (fifth whorl below the terminal shoot), and foliage samples are collected for determination of infection incidence and severity for each tree. All measurements and foliage collection were done in the spring just prior to bud break.

For determination of infection incidence and severity, needles for each age class/tree were stripped from branches and combined. Fifty needles for each age class/tree were randomly selected and affixed to index cards with double-faced adhesive tape. the needles on index cards were then examined under a dissecting microscope. Incidence of infection was recorded as the proportion of 50 needles bearing pseudothecia of *P. gaeumannii*. Severity, the number of stomata occluded by pseudothecia, was determined by counting pseudothecia on ten needles. Three sections, 2.6 mm x .26 mm, of each needle were counted with the aid of a dissecting microscope. Another sample of ten randomly selected needles from each age class/tree was used for quantitative PCR analysis (Winton et al this volume).

Statistical analyses of differences in needle retention, pseudothecial density, PCR value (normalized *P. gaeumannii* DNA) between sites were carried out by ANOVA with Tukey multiple comparisons procedures. Correlations between needle retention, pseudothecial densities, PCR value, and leaf wetness were carried out with SYSTAT v. 9.0.

Results

Retention of two-year-old needles is a good indication of disease severity at the nine sites. The most severely diseased sites in each group (north, central, south) have the lowest retention of two-year-old needles. Retention of two-year-old needles varies by year, but there is no indication yet that needle retention at diseased sites is decreasing, or that SNC at healthy sites is intensifying (Table 2). This is despite an incidence of SNC on one-year-old foliage very close to 100% at all sites. Numbers of pseudothecia on one- and two-year-old needles is an indication of the degree of colonization of foliage and is inversely related to needle retention. Juno Hill, the most severely diseased site, has a average needle retention of 2.8, and pseudothecia occupying 53.4% of two-year-old needles and 22.8% of one-year-old needles. In contrast, healthy sites such as Upper Stone and Acey Creek have over 80% of second year foliage, and about 15% of stomata occluded in two-year-old needles and less than 5% for one-year-old needles (Table 2).

Pseudothecial densities on foliage are one of the best indicators of disease severity on sites, and correlate

Table 2. Three year summary of disease measurements for nine coastal study sites. Needle retention, SNC incidence, and proportion of stomata occluded by pseudothecia on one- and two-year old needles are given for spring sampling year (e.g. sp00) and needle age class (AC) year. Needle retention is estimated on a 1-9 scale, where 9 indicates 90-100% needle retention and 1 indicates 10-20% retention. Incidence is the proportion of 50 needles bearing pseudothecia of *P. gaeumannii.* Letters following numbers in rows are not statistically different at $p < 0.05$.

	North Group			Central Group			South Group		
	Acey	Coal Creek	North Fork	Upper Stone	Lower Stone	Juno	Lime-stone	Cedar	Salal
2nd yr needle retention									
sp00 AC98	8.0	5.8	1.8	8.0	8.8	1.4	7.5	7.6	6.8
sp99 AC97	7.4	6.3	4.2	8.2	6.8	2.7	6.0	6.4	5.7
sp98 AC96	8.7	6.9	6.3	8.9	8.0	4.4	8.2	7.7	6.8
3 yr avg	8.0	6.3	4.1	8.4	7.9	2.8	7.2	7.2	6.5
1 yr SNC incidence									
sp00 AC99	.989	.995	1.0	.968	.970	1.0	.980	.931	.990
sp99 AC98	.969	.912	1.0	.922	.951	.996	.995	.925	.972
2 yr avg	.979	.954	1.0	.945	.961	.998	.988	.928	.986
2nd yr pseudothecia									
sp00 AC98	.194 a	.250 a	.483 b	.211 a	.283 a	.520 b	.167 a	.178 a	.228 a
sp99 AC97	.115	.227	.341	.181	.299	.548	.134	.101	.189
2 yr avg	.155	.239	.412	.196	.291	.534	.151	.140	.209
1st yr pseudothecia									
sp00 AC99	.072 ab	.100 c	.167 d	.028 ab	.094 bc	.244 e	.044 ab	.022 a	.050 ab
sp00 AC98	.026	.057	.131	.041	.051	.212	.027	.016	.025
2 yr avg	.049	.079	.149	.035	.073	.228	.036	.019	.038

well with other measures of disease severity such as foliage retention. Pseudothecial densities on two year-old needles (1998 needles sampled in April 2000) for the two most severely diseased sites, Juno and North Fork, were statistically equivalent but higher than all other sites at $p < 0.001$. Pseudothecial densities of one-year-old needles (1999 needles sampled in April 2000) were also highest for Juno and North Fork but were significantly different from each other. In general, density of pseudothecia on one-year-old needles closely parallels the values for two year-old needles within each site (Table 2).

Pseudothecial densities on one and two year needles within sites are very highly correlated (Pearson coefficient = 0.952, $p < 0.001$), as expected. Retention of two-year-old needles is highly correlated with pseudothecial densities on two-year-old needles (Pearson coefficient = 0.912, $P < .01$) (Figure 1) and with pseudothecial densities on one-year-old needles (Pearson coefficient = 0.869, $p < 0.05$). PCR analysis of one- and two-year-old foliage agreed well with the pseudothecial density measurements. For two-year-old foliage, the Juno site had the highest colonization levels detected by quantitative PCR, followed by North Fork and Salal in one statistical group, with the remaining six sites not statistically different. Quantitative PCR values were also correlated with retention of two-year-old needles (Figure 2). Results were similar for one-year-old needles, except that Juno and North Fork were not statistically different, and Salal was intermediate between these two sites and the other sites (Table 3, Figure 3).

Because infection levels and disease severity appear to be relatively constant within sites but variable between sites, and because infection levels remain similar within sites from year to year, environmental variables that might affect fungal growth were considered. Free surface moisture is necessary for infection by most foliar plant pathogens, and in general higher infection levels are associated with prolonged periods of free moisture. The optimal temperature range for fungal growth also must be considered. Several combinations of temperature range and threshold leaf wetness values were tested for separation of sites by disease severity. A temperature range

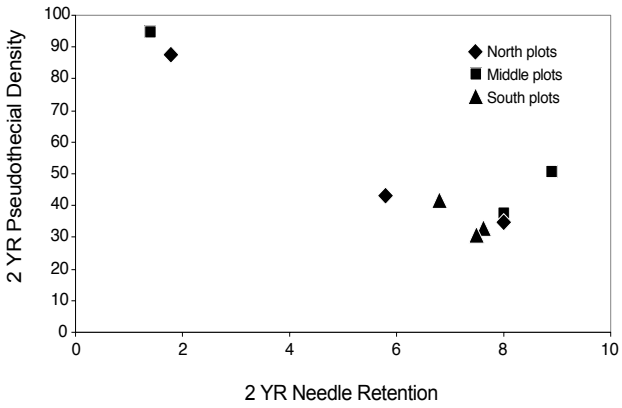

Figure 1. Relationship between pseudothecial density on two-year-old needles and retention of two-year-old needles.

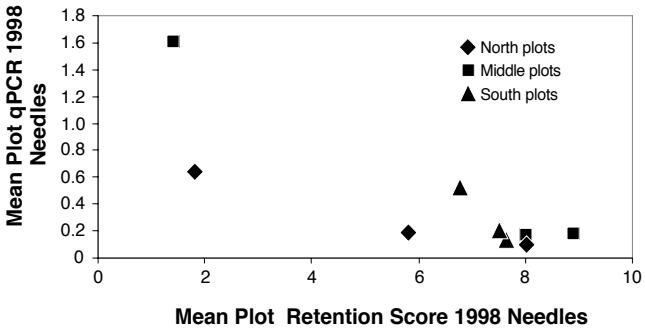

Figure 2. Relationship between PCR values for two-year-old needles and retention of two-year-old needles.

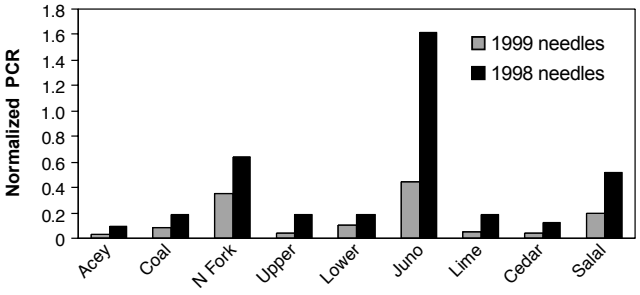

Figure 3. PCR analysis of colonization of floiage by P. gaeumannii *at nine coastal sites.*

Table 3. PCR analysis of foliage from nine coastal study sites. Letters following values in rows are not different at $p<0.01$.

PCR	Acey	Coal Creek	North Fork	Upper Stone	Lower Stone	Juno	Lime-stone	Cedar	Salal
AC 98	.096 a	.189 a	.641 c	.185 a	.187 a	1.615 b	.188 a	.119 a	.511 c
AC 99	.032 a	.085 a	.354 c	.042 a	.101 ab	.440 c	.054 a	.038 a	.196 b

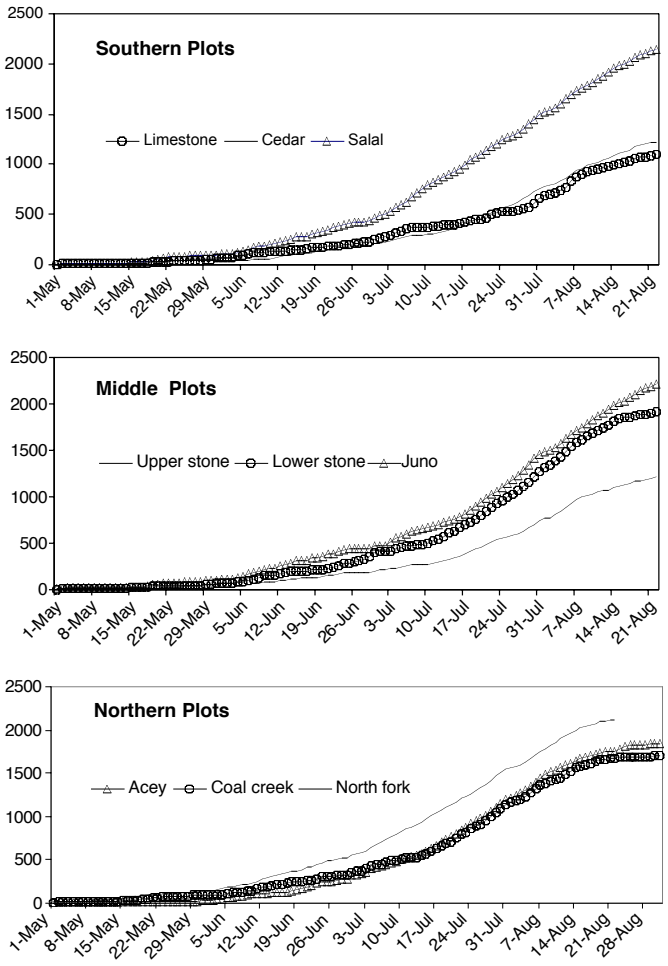

Figure 4. Cumulative leaf wetness hours at 12-26 C for the southern, middle, and northern group of study sites.

of 12-26 C and an intermediate leaf wetness threshold of 1.0 appeared to separate the three sites in each group.

For each of the three groups of study sites, the site with most severe disease has the highest cumulative leaf wetness at 12-26 C between June-August (Figures 4-6). This is the period when ascospores are released and when initial infection occurs. Growth of infection hyphae from ascospores and secondary infection from surface hyphae both should be increased under conditions of greater surface moisture. Leaf wetness hours at 12-26 C for 1999 was correlated with pseudothecial density on one-year-old needles (Pearson coefficient 0.900, $p < 0.01$). Pseudothecial densities for one-year-old needles were therefore highly correlated with the leaf wetness/temperature conditions in the year when initial infection occurred. Leaf wetness hours for 1999 were also correlated with pseudothecial densities on two-year-old needles (Pearson coefficient 0.846, $p < 0.04$). A regression model of pseudothecial density on two-year-old needles against three-year average leaf wetness (1998-2000) is significant at $p < 0.02$ with an r^2 of 0.582.

Discussion

There is a strong correlation between levels of *Phaeocryptopus gaeumannii* and defoliation and discoloration of Douglas-fir in coastal plantations in Oregon. The number of pseudothecia on one- and two-year-old foliage are highly correlated with needle retention at sites. Regardless of age, needles are abscised when more than approximately 50% of stomata are occupied by pseudothecia (Hansen et al. 2000), a consequence of impaired gas exchange (Manter et al 2000). Our evidence points to Swiss needle cast disease as the primary cause of needle loss and not a secondary colonist of foliage weakened by another agent. Consistently greater needle retention in foliage sprayed with chlorothalonil indicates the involvement of a fungal foliar pathogen in the defoliation of Douglas-fir trees on the Oregon coast. *Phaeocryptopus gaeumannii* is the only foliar pathogen present on the trees before treatment, and is the only pathogen that is abundant throughout the area of the epidemic (Hansen et al 2000).

A puzzling aspect of the current Swiss needle cast epidemic is concerns why the disease apparently intensified since about 1990. Periodic observations indicate that the fungus has long been present in the area, and periodic disease outbreaks have been noted (Russell 1981, Hansen et al 2000). Measurement of disease symptoms of Swiss needle cast over the past three years do not indicate a rapidly developing epidemic, and suggest that at coastal sites the disease is maintaining an equilibrium. Although the disease may be becoming more serious further inland from the coast, levels of infection and defoliation have remained relatively constant over three years in individual coastal study sites. Disease severity between sites is variable however, and within each group of three sites a range of disease severity can be seen that has remained constant within sites over

three years. Because the same seed source was used within each group of sites, consistent differences in disease severity between sites suggests environmental or other site related factors are involved.

Factors that may be important in the current outbreak of Swiss needle cast are increased amounts of young Douglas-fir plantations in the coastal area and subtle climatic differences between sites that favor infection and colonization by *P. gaeumannii*. In 1997 the Oregon Department of Forestry (unpublished data) investigated the history of 76,970 ha of Douglas-fir plantations 10 – 30 years old growing within 29 km of the north Oregon Coast. About 31% of these plantations had been established on sites where hemlock and spruce had dominated the previous stand. Only 20% were on sites dominated by Douglas-fir in the previous rotation. The remaining areas were mostly alder stands that had been converted to Douglas-fir. Although historical records are scant, this at least suggests that Douglas-fir is more abundant in the coastal forests than earlier this century.

Much of the land that has been converted to Douglas-fir plantations in recent decades, and where most severely affected plantations are located, lies in the *Picea sitchensis* vegetation zone, a narrow strip of coastal forest characterized by elevations generally below 150 m, proximity to the ocean, and a moderate climate. Although Douglas-fir is the natural seral dominant in the *Tsuga heterophylla* Zone, which borders the *Picea sitchensis* Zone to the east, its occurrence in the *Picea sitchensis* Zone is more sporadic, and normally it occurs in mixtures of spruce and hemlock, not as pure stands (Franklin and Dyrness 1973). An increase in the proportion of Douglas-fir in recent decades, its concentration in pure stands, and favorable climatic conditions may have enabled *P. gaeumannii* to increase above historically normal levels in coastal forests, leading to increased disease pressure. Under this increased inoculum pressure, even a naturally tolerant host population may be adversely affected.

Subtle climatic differences that are primarily responsible for the different vegetation composition of the *P. sitchensis* and *T. heterophylla* zones, also are likely important factors in disease severity. Hood (1982) found higher levels of *P. gaeumannii* in southern British Columbia and western Washington in coastal forests of Vancouver Island and the Olympic Peninsula, with lower levels in the rain shadow of eastern Vancouver Island and the interior. In our plot clusters, disease symptoms are more severe and fungal colonization greatest in sites with low elevation near the coast. At slightly higher elevations and further inland, plantations of the same age and seed source have milder symptoms of disease and needle retention of 3 to 4 years on average. The fungus is still abundant but predominantly on the older needles. Environmental differences between such nearby sites are subtle but perhaps significant. Temperatures are milder and annual rainfall is actually lower closer to the coast in the *Picea sitchensis* Zone than it is at higher elevations in the Coast Range. Hood (1982) found a significant correlation between May-July rainfall and percentage of infected needles. At our sites, measurement of leaf wetness at a temperature range where *P. gaeumannii* should be physiologically active (12-26 C) revealed differences between sites, and the cumulative leaf wetness at 12-26 C from April-August was strongly correlated with needle colonization by *P. gaeumannii*. Spring-summer leaf wetness at 12-26 C therefore appears to be a good predictor of SNC severity and could be used as a criterion for evaluating suitability of sites for Douglas-fir.

Genetic composition of the Douglas-fir plantations may also be important. In Douglas-fir progeny tests in British Columbia (Hood 1982) seedling families originating from drier areas east of the Coast Ranges were more susceptible to SNC than families collected from wet forests near the coast. In many cases in the Tillamook area, seed used in low elevation coastal plantations originated from higher elevations in the Coast Ranges. The most severe disease symptoms in our study sites are at the Juno Hill and North Fork sites. Both sites were planted with seed designated Boundary 1800 ft, but both sites are below 400 ft elevation. It might be expected that trees adapted to climates less favorable to the fungus would have less tolerance to infection than trees from areas where conditions favor colonization and evolution of genetic resistance would have a high selective benefit.

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Sulfur Fungicide Studies 1999-2000

Jeffrey Stone, Mark Gourley, Bruce Kelpsas, Gabriel Crane, Robin Rose, Scott Ketchum, and Diane Haase

Abstract

Thiolux fungicide applied to emerging foliage was effective in reducing colonization of Douglas-fir by *Phaeocryptopus gaeumannii*. Levels of infection were lowest for foliage treated with Bravo 720. Sulfur applied to the ground at the tree drip line had no effect on *P. gaeumannii* colonization. In plate studies, Bravo completely inhibited ascospore germination at concentrations as low as 0.0005%. Thiolux reduced ascospore germination by 50% at a concentration of 0.006% and also sharply inhibited hyphal growth. Plate studies support the conclusion that reduced colonization of Douglas-fir by *P. gaeumannii* in Thiolux treatments was due to contact fungicidal activity.

Field Study

A field study to test several sulfur treatments was established in a Douglas-fir plantation managed by Starker Forests. The objectives of the study were to evaluate the effect of elemental sulfur (Thiolux fungicide) in controlling infection by *P. gaeumannii* and resultant needle loss compared to chlorothalonil (Bravo fungicide). To ascertain whether sulfur fertilization may contribute to foliage retention and tree growth, a ground drench treatment was included. Ten trees were randomly selected for each of five treatments in a complete randomized design. Treatments included: 1)Untreated control, 2) Bravo fungicide @ 3.75 pts/100 gallons sprayed on the foliage, 3) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) sprayed on the foliage, 4) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) with TacTic sticker (8 oz per 100 gallons) sprayed on the foliage, and 5) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) sprayed on the ground under each tree within the drip line. The trees were approximately 12-years old at the time of treatment application, June-July 1999. Treatments were applied on June 8, June 25, and July 10, 1999 using a truck tank sprayer at 38 psi. Each tree was sprayed for 14 seconds resulting in an application rate of 2 oz Thiolux per tree.

Foliage was collected from trees in June, 2000. Two branches from each tree were collected, needles removed from the 1999 internode, and a subsample of ten needles for each tree was randomly drawn. Needle samples

were analyzed by a quantitative real-time PCR (polymerase chain reaction) assay developed specifically for quantification of *P. gaeumannii* (Winton et al unpublished). Statistical analysis was performed with Systat V. 9.0.

The results of PCR analysis are shown in Figure 1. Bravo 720 was the most effective treatment in reducing the colonization of foliage by *P. gaeumannii*. This is consistent with published results from studies on Swiss needle cast disease in Douglas-fir Christmas trees, which have shown Bravo to be effective in reducing infection by *P. gaeumannii* (Chastagner and Byther 1982, Hadfield and Douglas 1982). Ground application of Thiolux fungicide was not effective in reducing *P. gaeumannii* infection, and this treatment was not statistically different from the untreated control.

Thiolux fungicide with sticker also reduced *P. gaeumannii* infection. The amount of *P. gaeumannii* colonization in this treatment group was intermediate between the Bravo and Thiolux without sticker. Although not statistically different from the Bravo treatment, the amount of *P. gaeumannii* DNA detected was approximately ten times greater in the Thiolux with sticker treatment than for Bravo. The Thiolux without sticker treatment was intermediate between the Thiolux with sticker and untreated control and was not statistically different from either treatment (Figure 1).

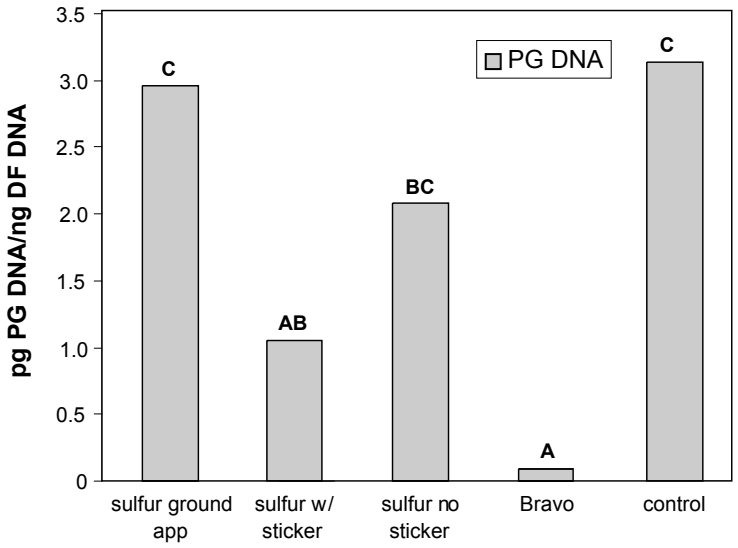

Figure 1. Effect of fungicide treatment on colonization of foliage by P. gaeumannii. Bars denoted by the same letter are not statistically different at $p < 0.05$.

Laboratory Plate Studies

Laboratory tests were also conducted on the effects of Thiolux fungicide on ascospore germination and hyphal growth of *P. gaeumannii*. Our objectives were to compare the efficacy of Thiolux fungicide in inhibiting germination and hyphal growth of *P. gaeumannii*, to compare the efficacy of sulfur fungicide to Bravo, and to ascertain the effective contact concentration required to inhibit ascospore germination.

Ascospores were germinated on culture medium containing 2% agar in water to which either test fungicides or no additions (control) were made. Thiolux was added to plates at 0.1, 0.01, 0.05, 0.001, 0.005, 0.0005 percent (w/v). Bravo was added at 0.005 and 0.0005 percent. Five needles bearing pseudothecia of *P. gaeumannii* were suspended above the agar plates for one hour to allow ascospore deposition on the plate surfaces, after which the lids were replaced and the plates incubated at 18 C in the dark. Ten plates for each fungicide concentration and control group were prepared and inoculated. Germination was assessed at 24 and 72 h, and hyphal growth was measured at 72 h following inoculation. Percent germination was determined for a group of 30 spores on each of ten replicate plates for each concentration. Spores were considered germinated if a germ hypha of least 2 µm was present. Hyphal lengths were measured as the length of the longest hypha and did not include branches. Ten germinated spores were measured for each of the ten replicate plates for each treatment. Statistical analyses for germination and length comparisons were done with Systat v 9.0.

Results for fungicide plate studies are given in Table 1. Bravo was the most effective inhibitor of ascospore germination, completely inhibiting ascospore germination at the lowest concentration tested, 0.0005% (Data for 0.005% not shown). In contrast, even the highest concentration of Thiolux did not completely inhibit ascospore germination. At 0.0005%

Table 1. Effect of fungicide type and concentration on ascospore germination and hyphal growth of Phaeocryptopus gaeumannii. Mean of ten plates for each concentration. Germination was determined from a group of 30 spores for each plate, hyphal length from ten germinated spores for each plate. Means followed by the same letter in a column are not different at $p < 0.05\%$.

Fungicide concentration (%)	Germination (%)	Hyphal length (µm)
control	97.7 e	20.38 d
Thiolux .0005	72.7 d	4.36 c
Thiolux .001	43.7 c	3.08 bc
Thiolux .005	53.0 cd	2.80 bc
Thiolux .01	35.0 bc	2.44 b
Thiolux .05	28.0 bc	1.40 b
Thiolux .1	17.7 b	0.27 a
Bravo .0005	0.0 a	0 a

Thiolux over 72% of the ascospores germinated compared to none for the same concentration of Bravo. Increasing concentrations of Thiolux further reduced ascospore germination in a concentration dependent manner. A probit analysis showed that a concentration of 0.006% Thiolux reduced germination by 50%.

The effect of Thiolux on hyphal growth is more pronounced. At 0.0005%, Thiolux reduced hyphal length to about 20% of the control. Increasing Thiolux concentration led to further reduction in hyphal length in a concentration dependent manner (Table 1).

The differences in ascospore germination and hyphal growth for some typical treatments can be readily seen in Figure 2. On water agar control plates, almost all the ascospores are germinated and have long, branched germ hyphae at both ends of the ascospores. Ascospores germinated on Thiolux at 0.005% have few germinating spores and those that are germinating have only very short, unbranched germ tubes. The Bravo treatment has completely inhibited spore germination (Figure 2).

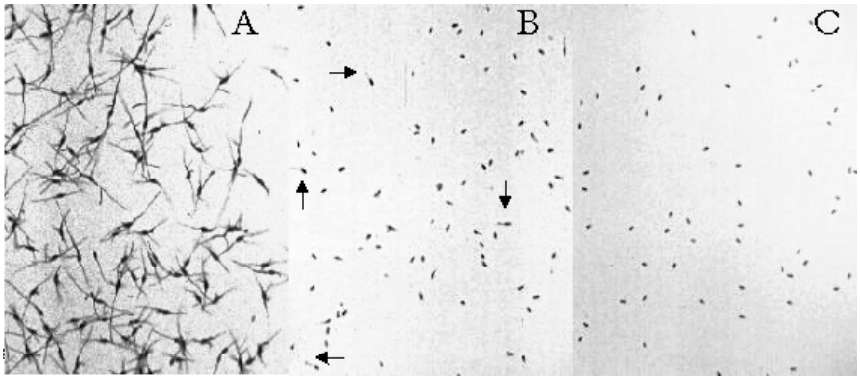

Figure 2. Typical ascospore germination and hyphal growth at 72 h after inoculation for three different treatments. A. water agar control with no fungicide addition. B. Thiolux 0.005%. Note a few germinating spores (arrows) with very short germ hyphae. C. Bravo 0.0005%. No germination.

Thiolux fungicide applied to newly emerging Douglas-fir foliage reduced colonization of the foliage by *P. gaeumannii*. Thiolux was not as effective as chlorothalonil (Bravo), however, and only the Thiolux with a sticker added reduced colonization compared to the untreated control. Thiolux without a sticker was not shown to be statistically different from the control. Sulfur applied to the ground had no effect on reducing colonization by *P. gaeumannii*. Thiolux effectively reduced ascospore germination and hyphal growth of *P. gaeumannii* in culture studies, and therefore it is likely that reduced foliage colonization was due to a contact fungicidal effect.

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Assessment of Swiss Needle Cast Disease Development

I. Comparison of biochemical, molecular, and visual methods to quantify *Phaeocryptopus gaeumannii* in Douglas-fir foliage

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Abstract

Swiss needle cast is a defoliating disease caused by the ascomycete *Phaeocryptopus gaeumannii* that has been shown to be associated with growth reductions of Douglas-fir forest plantations. A recent epidemic along the Oregon Coast has prompted efforts to quantify *P. gaeumannii* infection and colonization of foliage. Direct observation of fruiting body abundance on needle surfaces has proven to be well correlated with needle retention but it is labor intensive and difficult to achieve adequate sample sizes. Recent advances in technology have suggested biochemical and molecular methods that may provide an indirect means of estimating fungal biomass within host tissue. In this report we compare four methods to quantify infection levels of *P. gaeumannii*: fruiting body density, ergosterol content, DNA probe hybridization, and Taqman quantitative PCR. While all four techniques were significantly correlated, fruiting body density and quantitative PCR, the two methods least affected by the presence of other needle fungi, had the greatest correlation. In addition, we compared foliage colonization in nine field plots exhibiting a range of disease severity with all four methods. While all methods provided evidence that sites differed in the degree of fungal colonization, only quantitative PCR consistently separated both moderately and severely diseased sites from healthy sites as estimated by foliage color, canopy density, and growth measurements.

Introduction

Historically, the disease Swiss needle cast has resulted in economically significant defoliation only where Douglas-fir (*Pseudotsuga menziesii* (Mirb.) France) has been planted as an exotic species or Christmas tree crop (Chastagner 1996, Gaeumann 1930, Hood 1975, 1996). The causal agent of the disease, *Phaeocryptopus gaeumannii* (T. Rohde) Petr., is common and is believed to be endemic to the Pacific Northwest. Recently however, Swiss needle cast has been implicated in a serious decline of

Douglas-fir along the coastal region of Oregon (Hansen *et al* 2000). Severely diseased plantations are chlorotic, prematurely lose needles, and suffer from reduced growth. Objective, quantitative measurement of both symptoms and signs is a challenging aspect of research on this disease, which has been particularly hampered by insufficient methods for measuring the extent of colonization by the pathogen.

Accurate measurement of pathogen abundance is critical for epidemiological studies of plant pathogens. *P. gaeumannii* produces ascomata (pseudothecia) in the stomata of infected needles (Stone & Carroll 1985). Pseudothecia initials can be seen approximately nine months after ascospore infection of newly emerging needles in the spring (Capitano 1999). The proportion of stomata occupied by pseudothecia gives a good measure of colonization, but pseudothecia can only be counted the spring following ascospore infection, when needles are 9-12 months old. Incidence of needles bearing pseudothecia is a quick and objective measurement, however, in many Douglas-fir stands near the Oregon coast nearly every needle bears at least a few pseudothecia. In order to achieve an objective measure of infection, both stomata and pseudothecia can be counted to get a proportional measure of stomata occupied by pseudothecia (pseudo-thecia density). This imposes serious labor and statistical challenges, as many stomata on many needles must be counted. This becomes particularly problematic when the incidence of infected needles is low. Therefore alternative methods for quantification of colonization were investigated.

Ergosterol is a cell membrane sterol found only in higher fungi. While ergosterol cannot be used to discriminate between different fungal species, it has previously been used as a quantitative measure of both endophytic and pathogenic fungal biomass in forest foliage (Osswald et al 1986, Magan & Smith 1996).

Species-specific DNA hybridization probes have previously been designed to quantify colonization of plant pathogenic fungi in host tissue (Judelson and Messenger-Routh 1996, Goodwin et al 1990, Constabel et al 1996). In contrast to ergosterol measurements, DNA probes can be designed which do not react with organisms other than the target. Quantification is based on densitometric measurement of labeled probes hybridized to samples and compared to a standard curve of target DNA.

Real-time quantitative PCR is the most recent development in quantitative methods. The most frequently utilized application of this technique utilizes Taqman (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (Gibson et al 1996, Heid et al 1996, Livak et al 1996) in conjunction with the 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The fluorogenic Taqman probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the 5'–3' exonuclease activity of Taq DNA polymerase cleaves only annealed probe molecules. Release of the reporter dye results in a fluorescent signal which is measured by the 7700 Sequence Detection System during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (Zhang et al 1999) and roots of both crop plants and forest trees (Böhm et al 1999). Unlike end-point quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting.

The purpose of this report is to evaluate the precision and labor needs of several methods to quantify colonization levels of *P. gaeumannii* in Douglas-fir foliage. We compare pseudothecia density, ergosterol content, and two methods of measuring *P. gaeumannii* DNA in Douglas-fir needles and relate these to symptoms observed in nine field plots placed within the center of the epidemic.

Materials & Methods

Field plots, disease impacts, and sample collection. Nine disease-monitoring plots were established in Tillamook County, Oregon in 1996 (Hansen et al 2000). The plots were arranged in three clusters by latitude (Table 1). The three plots in each cluster were placed in Douglas-fir plantations exhibiting a range of symptom severity and consisted of trees of the same age and seed source where possible. Plots in the South Cluster were all USDA Forest Service progeny test plantations. Ten trees of each of two families were randomly selected for

Table 1. Estimated means of symptom and infection measurements at each Swiss needle cast field plot in Douglas-fir plantations in the vicinity of Tillamook OR.

Site	Visual Disease Severity[a]	Canopy Density (%) [b,d]	Discoloration Score[b,d]	Volume (m^3) [b,d]	Pseudothecia Density (%) [c,d]	TAQMAN2 (pg/ng) [c,d]	Ergosterol (mg/g) [b]	DNA Probe (ng) [c]
North Cluster								
Acey Crk Progeny	Healthy	46.0a	2.1a	.08a	2.6a	.89a	8.35a	41.46a
Coal Crk Progeny	Moderate	41.5a	2.3a	.06ab	5.7b	1.27a	8.18a	50.64a
North Fork	Severe	36.7a	3.0b	.05b	13.1c	3.75b	8.90a	88.38b
Tillamook Cluster								
Upper Stone	Healthy	44.5a	1.7a	.13a	4.1a	1.65a	5.38a	66.87a
Lower Stone	Mild	36.7a	1.9a	.13a	5.1a	1.80a	9.37b	57.92a
Juno Hill	Severe	17.0b	4.0b	.02b	21.2b	10.12b	21.42c	284.39b
South Cluster								
Limestone Progeny	Healthy	51.3a	1.5a	.05a	2.7a	.50a	4.65a	23.66a
Cedar Progeny	Mild	43.3ab	1.9b	.05a	1.6b	.46a	6.11b	15.59a
Salal Progeny	Moderate	42.1b	2.2b	.03b	2.5ab	.78b	6.13b	45.99b

[a] Visual disease severity was estimated in 1996 by overall impressions of needle retention, chlorosis, and growth.

[b] Comparisons between plots were performed with Fisher's 95% LSD.

[c] Comparisons between plots were performed with Welch's t-tests.

[d] Sites within clusters that were significantly different ($P<0.05$) are distinguished by different letters.

measurements in each plantation. Plots of the Tillamook Cluster and the North Fork plot in the North Cluster were planted with seedlings from the same bulk seed lot from the "Boundary" seed collection area of the Coast Ranges, at about 600 m elevation. Ten trees were randomly selected in each of the "Boundary" plantations. The North Cluster also included two plots located in Oregon Department of Forestry progeny test plantations. Ten trees of each of two families (different from the South plots) were selected in each of the latter plantations.

Plot measurement trees were monitored for growth, symptom, and pseudothecium development from 1996-2000. Tree volume was calculated from diameter and height measured in 1998 with a formula derived for young-growth trees (Bruce & DeMars 1974). Crown density and chlorotic discoloration were measured just before bud-break in spring 1999 and recorded as the average of two observers. Crown density, an estimate of needle retention, was compared to standardized diagrams and estimated in 5% increments as the percentage of sunlight being blocked by the live crown. Discoloration, an estimate of chlorosis, was described from foliage in the fifth whorl from the top of the tree in 4 classes: 1=normal green, 2=slight yellowing, 3=moderate yellowing, 4=extremely yellow or yellow-brown. For quantitative assays, secondary and tertiary branches from the fifth whorl from the apex of each tree were collected just before budburst in spring 1999. One-year-old needles were stripped from the branches, mixed, and stored at –20 °C.

Pseudothecia density. Ten one-year-old needles bearing pseudothecia were randomly selected from each tree. Needles were affixed to index cards with double-stick tape for microscope examination. Each needle was considered in three regions, basal, medial, and apical. From each region a single row of 80 stomata was randomly chosen for pseudothecium counting. The thirty regions from each tree sample were averaged and the data presented as the proportion of stomata occupied by pseudothecia.

Ergosterol extraction and quantification. Ergosterol was extracted as previously described (Manter et al 2000), from ca. 125 mg frozen foliage and quantified by HPLC (high performance liquid chromatography). All ergosterol contents are reported on a per unit dry weight basis. From each analyzed sample, a sub-sample was used to create a fresh: dry weight ratio for determination of ergosterol sample dry weights.

DNA extraction. Ten needles from each tree were placed into a 2 ml microfuge tube with two 5 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 30 s. at 4200 rpm. After pulverization, samples were incubated in 1.5 ml extraction buffer (2% CTAB (cetyltri-methylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% poly-vinylpolypyrrolidone, 0.1% 2-mercaptoethanol) at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml TE (5 mM Tris, pH 8.0, .5 mM Na_2EDTA).

DNA probe development. Probes were developed from RAPD (random amplified polymorphic DNA) PCR, a technique that amplifies arbitrary sequences throughout the genome (Welsh and McClelland 1990). Total genomic DNA was extracted from pure cultures of *P. gaeumannii* and several other fungi isolated from Douglas-fir needles (see Figure 1). Fungal cultures were maintained on potato dextrose agar (Difco). Each sample was amplified in a 20 µl volume containing 1 U Amplitherm DNA polymerase (Epicentre Technologies, Madison, WI), Amplitherm buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 750 nM RAPD primer, 2X Enhancer buffer (Epicentre Technologies), and up to 300 ng genomic DNA. Six RAPD primers were tested and subsequently used for developing hybridization probes: 709, 743, 846, 857, 886 (University of British Columbia, Vancouver, B.C.), and B02 (Operon Technologies). Reaction conditions were 40 cycles of 45 s at 92 °C denaturing, 45 s at 36 °C annealing, and 60 s at 72 °C extension. The reaction products were examined side-by-side on 2% (w/v) TBE agarose gels to identify bands that were specific for *P. gaeumannii*. One candidate amplicon from each RAPD primer was excised from the gel and purified from the agarose matrix (Qiagen, Chatsworth, CA). The purified PCR products were labeled non-radioactively with alkaline phosphatase according to the manufacturers directions (AlkPhos Direct Labeling System, Amersham-Pharmacia) and hybridized overnight to dot-blots at 60 °C.

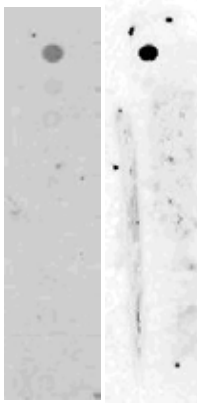

Figure 1. Replicate dot-blots demonstrating the specificity of the DNA probe. Each circle contains 1 µg of total genomic DNA. The darker the dot the more probe hybridized to the target DNA.

For dot blots, DNA samples were denatured in 10 mM Tris-HCl pH 8, 1 mM EDTA, and 200 nM NaOH and applied with mild suction to Hybond-N+ nylon membranes (Amersham-Pharmacia). After rinsing the wells with 1X SSPE, the membranes were air-dried and UV cross-linked at 12000 µJoules (UV Stratalinker 1800).

After hybridization, membranes were washed twice at 55 °C and twice at room temperature according to the AlkPhos labeling kit manufacturer's instructions. Chemiluminescent signal was generated with CDP-*Star* (Amersham-Pharmacia) and the membranes exposed for 1 hour to Kodak Biomax ML film.

For the 6 candidate probes, specificity was characterized on two replicate dot-blots prepared from 1 µg total genomic DNA extracts of each of the fungal species used to identify candidate probes as well as total genomic DNA of uninfected, newly flushed Douglas-fir needles. To ensure that candidate probes could detect *P. gaeumannii* in infected foliage, another dot-blot was prepared in which dots had increasing amounts of *P. gaeumannii* DNA as well as dots with constant amounts (about 10 µg) of the total DNA extracted from infected Douglas-fir needles (data not presented).

DNA probe hybridization assay. Assay samples consisted of 10 randomly chosen one-year-old needles from each plot tree. DNA extractions were performed as described above except that 10 µl of each sample were retained for additional quantitative PCR analysis. Dot-blotting, probe hybridization, and signal generation were performed as described above except that approximately 10 µg of each sample was applied to the membrane. In addition, a blot of duplicate, known amounts of *P. gaeumannii*

was included in each hybridization to serve as quantification standards. Signal intensities were determined by scanning films with a Molecular Dynamics Personal Densitometer model PDSI and analyzing the data with ImageQuaNT 5.0 (Molecular Dynamics). Absolute amounts of *P. gaeumannii* DNA were estimated from signal intensities by interpolation to internal standard curves.

TaqMan quantitative PCR development. TaqMan probe/primer sets were designed as described in Winton et al (in prep). Briefly, the *P. gaeumannii* probe/primer set was based upon sequence information of the low copy number B-tubulin gene of 6 isolates of *P. gaeumannii*, and 2-3 isolates each of the Douglas-fir needle fungi listed in Figure 1. The specificity and sensitivity of the *P. gaeumannii* probe/primer set was tested on genomic DNA extracted from the isolates described above, as well as uninfected Douglas-fir needles and infected needles collected both before and after pseudothecial development. The Douglas-fir probe/primer set was based upon a LEAFY/FLORICAULA-like gene involved in floral development (Weigel *et al* 1992, Bradley *et al* 1996) and serves as an endogenous control.

TaqMan assay PCR conditions and analysis. Reaction volumes were 25 µl (5 µl of DNA template, 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems), 150 nM of each TaqMan probe, and 60 nM of each primer) performed in MicroAmp optical 96-well plates with caps (PE Biosystems). Two no template controls were included in each assay to confirm that chemical stock solutions were not contaminated with template DNA. Real-time quantitative PCR was performed on an ABI Prism 7700 sequence detector (PE Biosystems) programmed according to the universal thermal cycler protocol (2 min. at 50 °C, 10 min. at 95 °C, and 40 cycles of 95 °C for 15 s. and 1 min. at 60 °C).

Assay standards were obtained from uninfected, greenhouse grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described above except that ribonuclease A treatment was included. After quantitation by OD_{260}, both DNA samples were mixed and ten-fold serial dilutions prepared to serve as one-tube quantification standards for each probe/primer set and included in each assay. *P. gaeumannii* standards ranged from 1 pg to 10 ng and Douglas-fir standards ranged from 100 pg to 1000 ng. Standards were run in duplicate for each assay and used to calculate regression equations by which to quantitate unknown samples by interpolation. *P. gaeumannii* quantities were normalized to the amount of Douglas-fir host DNA present in individual samples to compensate for pipetting errors and both DNA extraction and PCR amplification efficiencies.

To determine whether *P. gaeumannii* could be detected before appearance of fruiting bodies, samples of current year needles were collected between July 1999 and February 2000 at the Juno Hill and Upper Stone sites, the two plots exhibiting high and low symptom extremes, respectively, within the Tillamook cluster. Needles were stored at –20 °C and DNA was extracted as described above.

Comparisons of infection levels among plots were estimated from two separate plot tree DNA extractions. The first set (TAQMAN1), consisted of the same set of randomly chosen needles used for the DNA probe hybridization assay. The second set (TAQMAN2), were randomly selected from a pool of pseudothecia-bearing needles and used for pseudothecia density estimates prior to DNA extraction. DNA from both sets were extracted as described above except that needles used for TAQMAN2 were periodically removed from the freezer to count pseudothecia and were finally air dried for approximately 3 weeks prior to extraction.

Statistical analyses. Data were analyzed with the statistical package Statgraphics Plus 4.0 (Statistical Graphics Corp.). All comparisons except discoloration scores were made after log transformation of the data. Plots were compared with ANOVA using Fisher's protected least significant differences (LSD) for measurements of ergosterol, canopy density, discoloration, and wood volume. Because variances between some plots were unequal, pseudothecia density, quantitative PCR, and the DNA probe comparisons between plots were made with Welch's t-tools. Correlations between disease impact measurements and quantitative methods were tested on individual tree data using Pearson correlation with Dunn-Sidak probabilities.

Results

Disease impacts. There were measurable differences in disease impacts between sites (Table 1).

In each cluster, trees at the more severely diseased sites were more chlorotic, had thinner crowns, and reduced growth when compared to the healthy sites within clusters (P<0.05 from Fisher's 95% LSD). For example, the average crown density at North Fork was about 20% less than at Acey Creek (95% confidence interval from 2% to 36%). Average crowns at Juno Hill, the most severely diseased site by all measures, were 63% less dense than at Upper Stone (52%-71%), and tree crowns at Salal were 21% (6%-34%) less dense than at Limestone. Average wood volumes at North Fork, Juno Hill, and Salal were about 50% (22%-68%), 87% (78%-92%), and 57% (40%-70%) less then their respective healthy sites. Increased chlorosis was significantly correlated (P<0.0001) with reductions in both canopy density (r=-0.70) and wood volume (r=-0.47). In addition, canopy density was significantly correlated with wood volume (r=0.56; P<0.0001).

Pseudothecia density. North Fork and Juno Hill, the two most severely diseased plots, both differed significantly from their respective healthy plots within clusters (P<0.0005 from Welch's t-tests; Table 1). Median pseudothecia density at North Fork was estimated to be 6.5 times higher than at Acey Creek (95% confidence interval from 4.1 to 10.2) and Juno Hill was 8.5 times higher than Upper Stone (3.0-24.5). Of the moderately diseased sites, there was suggestive evidence (P=0.019) that Coal Creek had about twice the amount of pseudothecia than Acey Creek (1.1-3.4). While neither of the mildly diseased sites had significantly more pseudothecia than their corresponding healthy plots, the Cedar Progeny site had a median density about half that of the healthy plot (0.37-0.92; P=0.01, Welch's t-test).

Ergosterol content. Only in the Tillamook cluster could the high disease site be distinguished from its healthy counterpart (P<0.05; protected 95% LSD; Table 1). The median ergosterol content at Juno Hill was estimated to be 4 times higher than at Upper Stone (95% confidence interval from 2.8-5.6 times). In addition, the median at the mildly diseased Lower Stone was about 1.7 (1.2-2.4) times higher than the healthy site in that cluster. In the south cluster there were no significant differences between the healthy and most highly diseased site in the cluster. However, the mildly diseased Cedar site was estimated to have a median slightly higher than its healthy counterpart Limestone (Table 1).

DNA Probe hybridization. Of 6 candidates, a 900-base fragment generated from primer B02 was the most specific for *P. gaeumannii* while still retaining enough sensitivity to detect the fungus in lightly infected needles. When tested against a variety of purified DNA from fungi commonly isolated from or found on Douglas-fir, the 900-base probe hybridized strongly to *P. gaeumannii* (Figure 1) but on all three blots also hybridized weakly to the fungus *Rasutoria pseudotsugae*, a close relative of *P. gaeumannii* (L.M. Winton, unpublished data). The other needle fungi tested were not detected, and the probe did not bind to purified Douglas-fir DNA, which of course would be present in any foliage sample.

In each plot cluster, there were significant differences in estimated *P. gaeumannii* DNA between the most and least severely diseased sites (P<0.05; Welch's t-tests; Table 1). North Fork, Juno Hill, and Salal were estimated to be 2.4 (95% confidence interval: 1.4-4.3; P=0.0002), 4.3 (2.9-6.6; P<0.0001), and 2.2 (1.0-4.8; P=0.024) times higher, respectively, in *P. gaeumannii* DNA than the healthy plots within their clusters.

TaqMan quantitative PCR. Both sets of needles yielded similar results, were well correlated (r=0.86), and revealed significant differences between healthy and moderately to severely diseased plots within clusters (P <0.05; Welch's t-tests). However neither of the mildly diseased sites could be distinguished from their healthy counterparts (Table 1). For TAQMAN2, median *P. gaeumannii* DNA at North Fork, Juno Hill, and Salal were estimated to be about 4.5 (95% confidence interval: 3.1-6.4; P<0.0001), 9.7 (3.7-25.6; P<0.0002), and 1.7 (1.0-2.9; P=0.026) times higher, respectively, than at the respective healthy plots.

Differences in *P. gaeumannii* DNA among plots could be estimated well before pseudothecium formation (Figure 2). For example, *P. gaeumannii* DNA could be detected within 2 weeks of infection at Juno Hill and within 4 weeks at Lower Stone.

Comparison of methods. All four of the methods used to estimate infection levels were significantly correlated (P<0.0001; Table 2). Correlations were high, but somewhat different, between pseudothecia density and quantitative PCR on the two

sets of needles. Pseudothecia density had a higher correlation with DNA extracted from the same set of needles (TAQMAN2) than with a random sampling of needles (TAQMAN1). Ergosterol and the DNA probe, the two less specific assays, both had similar and lower correlations than did quantitative PCR when compared to pseudothecia density. The DNA probe, which is more specific for *P. gaeumannii* than ergosterol, had a slightly higher correlation with pseudothecia density than did ergosterol. The DNA Probe was better correlated with the quantitative PCR assay performed on the same DNA extraction (TAQMAN1) than it was with the second quantitative PCR experiment (TAQMAN2). Correlations were significant ($P<0.0001$) and similar ($0.46 \leq r \leq 0.49$) between chlorosis and pseudothecia density, quantitative PCR, and DNA probe hybridization. However, increased yellowness appeared to be slightly better correlated with ergosterol content ($r=0.52$). A similar situation was seen with correlations between canopy density and ergosterol ($r=-0.53$), while canopy density correlated less well with the three more specific methods ($-0.36 \leq r \leq -0.48$).

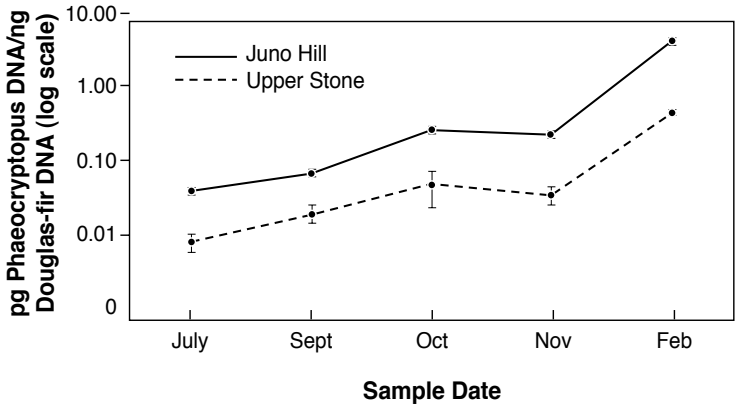

Figure 2. TaqMan estimates of P. gaeumannii *DNA in current year needles collected periodically from the two most extreme sites in the Tillamook cluster. Verticle bars represent standard error of the means. Bud-break, and ascospore infection, began in late May 1999, about 6 weeks before the first sample collection. Bud-break at Upper Stone began about 4 weeks before the first collection date. Recognizable pseudothecia initials were first observed in February 2000 at Juno Hill and in April 2000 at Upper Stone.*

Table 2. Pearson product moment correlation coefficients for comparisons of quantitative methods.

	Pseudothecia	TAQMAN1[a]	TAQMAN2[b]	DNA Probe
Pseudothecia				
TAQMAN1	.79			
TAQMAN2	.85	.86		
DNA Probe	.53	.67	.58	
Ergosterol	.47	.56	.42	.65

[a] TAQMAN1 was a random sampling of needles on which the DNA probe assay was also performed.

[b] TAQMAN2 were the same needles from which pseudothecia density was estimated.

Discussion

Improved methods to detect and quantify infection by *P. gaeumannii* will facilitate many studies investigating the effects of foliage infection and colonization by *P. gaeumannii*. This will aide in evaluating disease resistance in trees and enable investigation of the progress of needle colonization over time and in relation to various nutritional, environmental, and chemical factors.

Although pseudothecia density is well correlated with symptoms such as chlorosis and needle retention, it has severe limitations. No expensive materials other than a microscope are needed, but it is very time-consuming, tedious, and can be subject to misidentification errors. The common Douglas-fir needle fungus *Rasutoria pseudotsugae*, as well as species of *Rhizosphaera* and *Stomiopeltis* are also frequently found on Douglas-fir needles and can be mistaken for *P. gaeumannii* unless workers are well-trained and observant. In addition, because it is impossible to count every stomate on each needle in a sample, it is necessary to consider only a small fraction of the available stomata. This has important statistical considerations when pseudothecia are unevenly dispersed over the needle surface. In this case, a randomly assigned stomatal row might easily give either erroneously high or low estimates, depending upon the pattern of pseudothecial clustering. Another

problem with counting pseudothecia is that it is unavailable in early phases of infection before pseudothecia have formed.

Measurement of ergosterol is less cumbersome than counting pseudothecia on individual needles, and is sensitive enough to quantify fungal biomass in needles not yet producing pseudothecia. One limitation of ergosterol, however, is that it is nonspecific. Because it is a component of fungal cell membranes, any fungal species on or within needles will contribute to the total ergosterol and may lead to erroneously high estimates of *P. gaeumannii* colonization. In trees and sites with moderate to heavy colonization by *P. gaeumannii*, the relative contribution of other fungi within and on needle surfaces is probably minimal and ergosterol might provide a good approximation of *P. gaeumannii* biomass. A second problem with the use of ergosterol is variation in ergosterol content of cells over time in response to temperature, availability of nutrients, and age of cells. This effect has the potential to confound field experiments and also demands that samples be processed quickly. However, ergosterol may still prove to be a useful technique to estimate fungal biomass in needles, as it is relatively inexpensive, rapid, and can be applied to a large number of samples. It would be most useful in combination with pseudothecia counts or quantitative PCR as a separate indication of total fungal biomass present.

Hybridization of labeled DNA probes to dot-blotted sample DNA has the potential to be very specific. However in this study, all 9 of the probes tested also cross-reacted with fungi closely related to *P. gaeumannii*. The lack of specificity most likely was a consequence of the length of the probe necessary to generate a signal with enough sensitivity to detect DNA in low amounts. We tested probes ranging from approximately 300 to 900 bases in length, and all hybridized in varying degrees to at least one of the other fungi tested. In an attempt to increase the specificity, we also tested a radiolabeled oligonucleotide that was homologous to an 18-base region in the inter-transcribed spacer of nuclear ribosomal genes (data not presented). Although this probe was highly specific, it did not detect *P. gaeumannii* in any but the most heavily infected needles.

Real-time quantitative PCR is a relatively new technology that derives specificity from three oligonucleotides, two serving as PCR primers and one fluorescently labeled internal probe. The method capitalizes upon the sensitivity of PCR and in some systems has been reported to have a lower detection limit of 1 DNA molecule (citation). Another benefit of quantitative PCR is that it has a large range, in this study our standards displayed a linear range of 5 orders of magnitude. This enables samples with widely differing amounts of infection to be directly compared without the added complications of handling samples multiple times to dilute them to an appropriate concentration. Real-time quantitative PCR has the advantage of speed, technical simplicity, very low detection limits, and unparalled specificity.

Quantitative PCR is the only method to date that can detect and quantify *P. gaeumannii* early in the disease cycle. Although sample collection did not begin early enough to capture the start of the infection cycle, there were significant differences in *P. gaeumannii* DNA at the two extreme sites in the Tillamook cluster by July 1999. At this time, needles were less than 6 weeks old. Ascospore release, and subsequent infection of trees at the nearby Salal site, occurred between mid-June and early-July in 1999 (unpublished data). Because the rate of increase was not different between the two sites, it is possible that the higher amount of *P. gaeumannii* DNA at Juno Hill is the result of greater initial inoculum load, rather than faster growth rate. All methods displayed large differences in variance among plots, this may be a consequence of host variability.

The quantitative methods presented here illustrate that it is possible to quickly detect the presence of *P. gaeumannii* and to assess the total amount of *P. gaeumannii* colonization of Douglas-fir of needles at any time of year, regardless of the presence of pseudothecia. This should provide a very sensitive, standardized method for comparing total *P. gaeumannii* DNA within infected foliage that can be used in a number of planned or already in progress studies.

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Assessment of Swiss Needle Cast Disease Development

II. Simultaneous one-tube quantification of host and pathogen DNA using TaqMan real-time PCR

L.M. Winton, J.K. Stone, L. Watrud, and E.M. Hansen

Abstract

Phaeocryptopus gaeumannii is a widespread foliar parasite of Douglas-fir. Although normally innocuous, the fungus also causes the defoliating disease Swiss needle cast in heavily infected needles. The extent of *P. gaeumannii* colonization in Douglas-fir foliage was estimated with real-time quantitative PCR using TaqMan chemistry. In order to derive a normalized expression of colonization, both pathogen and host DNA were simultaneously amplified but individually detected by using species-specific primers and TaqMan probes labeled with different fluorescent dyes. Detection of host DNA additionally provided an endogenous reference, which served as both an internal positive control and adjusted for errors introduced by sample-to-sample differences in DNA extraction and PCR efficiencies. The genes employed for designing the TaqMan probes and primers were B-tubulin for the pathogen and a LEAFY/FLORICAULA-like gene involved in floral development for the tree host. Both probe/primer sets exhibited high precision and reproducibility over a linear range of four orders of magnitude. This eliminated the need to analyze samples in multiple dilutions when comparing lightly to heavily infected needles. Quantification of the fungus within needles was successful as early as one month after initial infection. Real-time PCR is the only method currently available to quantify *P. gaeumannii* colonization early in the first year of the colonization process.

Introduction

Swiss needle cast has recently been implicated in a serious decline of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) France) along the coastal region of Oregon (Hansen et al 2000). The disease is caused by internal needle colonization and subsequent stomatal blockage by ascomata of the fungus *Phaeocryptopus gaeumannii* (T. Rohde) Petr. Normally extensive colonization is found only in older (3-4 year) foliage, but severe disease symptoms are associated with extensive colonization of foliage less than one year old (Hansen et al 2000). Objective, quantitative measurement

of the extent of foliage colonization by the pathogen in order to understand factors affecting its growth in young needles is a challenging aspect of research on this disease. Although direct observation of ascomata (pseudothecia) abundance on needle surfaces has proven to be well correlated with symptoms, it is labor intensive and unavailable during the first year of the disease cycle before fruiting bodies have developed.

Real-time PCR is the most recent development in quantitative diagnostic methods and promises to be useful at very low levels of infection. The most frequently utilized application of this technique utilizes Taqman (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (Gibson et al 1996, Heid *et al* 1996, Livak et al 1996) in conjunction with the 7700 Sequence Detection System (PE Applied Biosystems). The fluorogenic Taqman probe, labeled on opposite ends with a reporter dye and a quencher dye, anneals between the PCR primers. During the extension phase of the PCR, the 5'→3' exonuclease activity of Taq DNA polymerase cleaves only annealed probe molecules. Release of the reporter dye results in an intense fluorescent signal which is measured by the 7700 Sequence Detection System during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (Zhang et al 1999) and roots of both crop plants and forest trees (Böhm et al 1999). Unlike conventional end-point quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting. In addition, because different reporter dyes can be attached to separate species-specific TaqMan probes, it is possible to simultaneously quantify both the host and pathogen DNA in infected tissues.

This report describes the development of a multiplexed TaqMan assay to simultaneously quantify both *P. gaeumannii* and Douglas-fir DNA in infected foliage and derive a normalized measurement of pathogen infection.

Materials & Methods

Fungal cultures and DNA extraction. The fungal isolates used in this study are listed in Table 1. *P. gaeumannii* isolates were obtained by suspending needles bearing only *P. gaeumannii* pseudothecia over water agar and incubating in a moist chamber at 17 °C. Identical methods were used to obtain isolates of the epiphytic fungi *Rasutoria pseudo-tsugae* and an undescribed *Stomi-opeltis* species, both of which commonly occur with *P. gaeumannii* on Douglas-fir foliage. Endophytic fungal cultures were isolated from surface sterilized needles. All fungal cultures were maintained on potato dextrose agar (Difco). Fungal cultures were prepared for extraction by scraping about 30 mg mycelium from the agar surface. Collected mycelium was placed into 2 ml microfuge tubes with 1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) and 1 ml CTAB extraction buffer (2% CTAB (cetyltrimethylammonium bromide), 100 mM Tris, pH 8.0, 20 mM Na_2EDTA pH 8, 1.4 M NaCl, 1% polyvinylpolypyrrolidone, 0.1% 2-mercaptoethanol) and shaken in a Mini-Beadbeater (Biospec Products) for 30 s at 5000 rpm. After mixing, samples were incubated at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol and further purified to reduce PCR

Table 1. Fungal isolates obtained from Douglas-fir foliage used to design the P. gaeumannii-specific TaqMan probe/primer set and to test its specificity.

Species	GenBank accession number
Phaeocryptopus gaeumannii	
Phaeocryptopus gaeumannii	
Phaeocryptopus gaeumannii	
Phaeocryptopus gaeumannii	
Phaeocryptopus gaeumannii	
Rasutoria pseudotsugae	
Stomiopeltis sp.	(data not available)
Cladosporium cladosporioides	
Cladosporium herbarum	
Rhizosphaera oudemansii	
Hormonema dematioides.	
Alternaria alternata	
Allantophomopsis lycopodina	
Thysanophora penicilliodes	

inhibitors by passing the extract over QiaAmp Spin Columns (Qiagen Inc., Valencia, CA) according to the manufacturers instructions.

Douglas-fir needles (ten needles per sample) were placed into 2 ml microfuge tubes with two 5 mm glass beads, frozen in liquid nitrogen, and pulverized in a Mini-Beadbeater for 30 s at 4200 rpm. After pulverization, samples were incubated in 1.5 ml extraction buffer at 65 °C for 2 hours. The DNA was purified in 24:1 chloroform:isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml TE (5 mM Tris, pH 8.0, 0.5 mM Na_2EDTA).

Standards. Assay standards were prepared from genomic DNA extracted from uninfected, greenhouse grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described above except that ribonuclease A treatment was included to facilitate accurate spectrophotometric quantitation. After quantitation by OD_{260}, both DNA samples were mixed so that the DNA standards used were comparable to infected foliage. Five ten-fold serial dilutions were prepared for use in calibration experiments and to serve as dual-species, one-tube quantification standards to be included in each assay. *P. gaeumannii* standards ranged from 1 pg to 10 ng and Douglas-fir standards ranged from 100 pg to 1000 ng.

Probe and Primer Design. The *P. gaeumannii* probe/primer set was based upon B-tubulin sequences obtained from PCR amplicons of genomic DNA extracted from the isolates listed in Table 1. PCR was performed in 50 µl reactions (1X enzyme buffer, 200 µM dNTP, 0.4 µM T1 and T22, 2.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO), and 1 µl template DNA). Reaction conditions were 35 cycles of 60 s at 94 °C denaturing, 60 s at 55 °C annealing, and 60 s at 72 °C extension. After amplification, PCR products were prepared for direct sequencing by isopropanol precipitation. Cycle sequencing in both 5' to 3' and 3' to 5' directions with primers T1 and RT1 were performed using dye-terminator chemistry on an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, CA). Contigs were assembled and the overlapping sequences edited using the Staden package (Staden 1996). Because species-specific priming sites must be conserved within species to be useful, five *P. gaeumannii* isolates from different locations were sequenced. Sequence alignments were generated with ClustalX (Thompson et al 1997) and compared for regions unique to *P. gaeumannii* but invariant within the species.

The Douglas-fir probe/primer set was designed to be included in multiplex PCR as an endogenous reference and served as both an internal positive control (IPC) and as a normalizer. The IPC was used to distinguish uninfected needles from PCR inhibition. Normalization served to adjust *P. gaeumannii* estimates of colonization for differently sized needles, pipetting errors, and sample-to-sample variation in both DNA extraction and PCR amplification efficiencies. The Douglas-fir probe/primer set was based upon homologs of the LEAFY/FLORICAULA genes, which control the transition from vegetative to floral development in *Arabidopsis* (Weigel et al 1992) and *Antirrhinum* (Bradley et al 1996). Sequences were obtained from three Douglas-fir cDNA clones (Steve Strauss, unpublished data.) and *Pinus radiata* (GenBank accession #U76757), aligned with ClustalX, and compared for regions common to all sequences.

After identification of target regions, final selection of TaqMan primer and probe sequences were performed using the ABI Primer Express program (PE Applied Biosystems) according to the manufacturer's instructions. Both TaqMan probes used in this study were obtained from the Oligo Factory (PE Applied Biosystems) and contained a TAMRA quencher dye conjugated to the 3'-terminal nucleotide. The *P. gaeumannii* probe contained the reporter dye FAM linked to the 5'terminal nucleotide, while the Douglas-fir probe employed VIC as the reporter.

TaqMan PCR conditions and analysis. Reactions were performed in 15 µl aliquots with 1X TaqMan Universal Master Mix (PE Applied Biosystems), 150 nM *P. gaeumannii* FAM-labeled probe, 150 nM Douglas-fir VIC-labeled probe, 60 nM forward and reverse primers for both organisms, and 5 µl DNA template (various concentrations). Real-time quantitative PCR was performed by an automated ABI Prism 7700 SDS (Sequence Detection System, PE Biosystems) in MicroAmp optical 96-well plates or single tubes (PE Biosystems). Thermal cycling was

completed in less than 2 h and conditions consisted of 10 min at 50 C and 5 min at 95 °C and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The 7700 SDS software collected data for both reporter dyes every 7 s from each well, generating a fluorescence profile for each amplification. The threshold cycle (CT) was recorded for each dye as the cycle at which fluorescent signal, associated with an exponential growth of PCR product, exceeded background fluorescence.

PCR controls in every assay included no template (negative) controls and the genomic DNA standards (positive) for both *P. gaeumannii* and Douglas-fir. Combined standards in ten-fold dilutions (described above) were run in duplicate for each assay. Standard curves for both *P. gaeumannii* and Douglas-fir were generated by plotting the known DNA amounts against the CT calculated by the SDS software and calculating a regression equation. Unknown samples were quantitated from measured CT-values by interpolation using the regression equation. Estimates of *P. gaeumannii* colonization of Douglas-fir host foliage were obtained by dividing *P. gaeumannii* DNA estimates by Douglas-fir DNA estimates for individual samples.

Validation Experiments. The specificity and sensitivity of the probe/primer sets were tested using genomic DNA from five isolates of *P. gaeumannii*, 2-3 isolates each of nine other Douglas-fir needle fungi (Table 1), uninfected, greenhouse grown Douglas-fir foliage, and infected foliage both with and without visual sign of *P. gaeumannii*. Infected foliage was collected one month, 13 months, and 25 months after initial infection from a highly diseased site on Juno Hill near Tillamook, Oregon (Hansen et al 2000). According to the instruction manual for the 7700, the instrument is more sensitive to differences in the lower range of initial template amounts. Therefore, calibration experiments with spectrophotometrically measured amounts of Douglas-fir and *P. gaeumannii* DNA were performed on each probe/primer set, both separately and together, to determine their sensitivity and linear dynamic range. In addition, RNase treated genomic DNA extracted from infected needles was compared to untreated DNA for differences in quantification and possible interference by RNA transcripts.

Because the TaqMan assay was intended to provide an indirect measure of *P. gaeumannii* biomass in foliage, the relationship between mycelium dry weight and estimated DNA content was examined for a single *P. gaeumannii* isolate. The isolate was grown for three months in 2% potato dextrose broth at 18 °C. Harvested mycelium was then rinsed and dried overnight at 40 °C. Prior to DNA extraction, dried mycelium of variously sized pieces were weighed.

Precision, or reproducibility, of the TaqMan assay on field samples was evaluated on Douglas-fir foliage collected from Juno Hill. One-year-old needles were stripped from branches, pooled, and separated into twelve sub-samples of 10 needles randomly chosen from the pool. Prior to DNA extraction, all samples were stored at –20 °C. Intra-assay precision, which could be affected by reaction-to-reaction differences in pipetting volumes and PCR efficiency and measurement among wells, was evaluated on 12 replicates of one of the DNA sub-samples analyzed in a single assay. Inter-assay variability, which could additionally be affected by slight differences in reaction components, was evaluated on the same DNA sub-sample amplified over five separate assays. Inter-sample reproducibility, which could be affected by sample-to-sample differences in PCR efficiency, sample selection, and DNA extraction, was evaluated on separate extractions of 12 sub-samples amplified in a single assay.

Results

The sequences, locations, and amplicon sizes of the TaqMan probe/primer sets constructed for the quantification of *P. gaeumannii* and Douglas-fir in this study are listed in Table 2. Neither of the TaqMan probe/primer sets significantly amplified DNA extracted from any of the fungi cultured from Douglas-fir needles other than *P. gaeumannii*. The *P. gaeumannii* set did not amplify DNA extracted from uninfected needles and the Douglas-fir set did not amplify DNA extracted from *P. gaeumannii* isolates. These results indicate no undesired cross-reactivity with non-target genomic DNA present in the sample preparations. The *P. gaeumannii* set successfully amplified DNA extracted from all five *P. gaeumannii* isolates and infected needles, whether there were obvious signs of the fungus or not.

The Douglas-fir probe/primer set was successful in detecting

Douglas-fir DNA in both infected and uninfected needles. Therefore, this gene proved useful as both an internal positive control (IPC) and as a normalizing gene. Samples were recorded as uninfected when the *P. gaeumannii* gene did not amplify and the Douglas-fir IPC gene was positive. Samples that resulted in negative reactions for the Douglas-fir gene were recorded as failed PCR reactions. The importance of the normalizing effect is evident in Figure 1, where both host and pathogen DNA content were estimated from total genomic DNA extracted from infected needle samples. *P. gaeumannii* DNA varied among samples and was directly proportional to the length of time following initial infection (Figure 1A). However, the amount of Douglas-fir DNA was similar, but not identical, for all six samples, and probably reflects differences in needle sizes and DNA extraction efficiencies (Figure 1B). Additional evidence for normalizing performance was provided by the DNA standards of known concentrations. Despite being diluted over four orders of magnitude, the average ratio of CT-values (*P. gaeumannii* CT: Douglas-fir CT) for all 10 multiplex reactions was 1.025 (±0.005 SE).

When run either separately or multiplexed, both probe/primer sets displayed high precision over a linear range of at least four orders of magnitude (Figure 1). The correlations between CT and known DNA quantities were high for both *P. gaeumannii* (R=0.998) and Douglas-fir (R=0.997). *P. gaeumannii* was quantifiable between 100 fg and 10 ng (Figure 1A) while Douglas-fir was quantifiable from 1 ng to 1 µg

Table 2. TaqMan probe/primer sets.

Probe and primer	Sequence (5′→3′)	Target	Position	Fragment length (bp)
PGBT308F[a]	GGTACAATGGCACGTCTGATCTC	P. gaeumannii	308	
PGBT429R[b]	GGACGCCTATATCGCAAGTCA	P. gaeumannii	429	122
PG336BTUBP-FAM[c]	CGAGCGCATGAACGTCTACTTCAACG	P. gaeumannii	336	
LFY989F[a]	GGTCACAAACCAAGTATTTCGACA	Douglas-fir	989	
LFY1102R[b]	TGTTCAACATCCAGGCAATGA	Douglas-fir	1102	114
LFY1015P-VIC[c]	TAACCGGCGCCTGAATGCTTCG	Douglas-fir	1015	

[a] Forward primer.
[b] Reverse Primer.
[c] TaqMan probes: The P. gaeumannii probe is labeled with the reporter dye FAM (6-carboxy-fluorescein; emission 518 nm) on the 5′ end, the reporter dye for the Douglas-fir probe is VIC, both probes are labeled with TAMRA (6-carboxy-tetramethyl-rhodamine; 582 nm) on the 3′ end as a quencher.

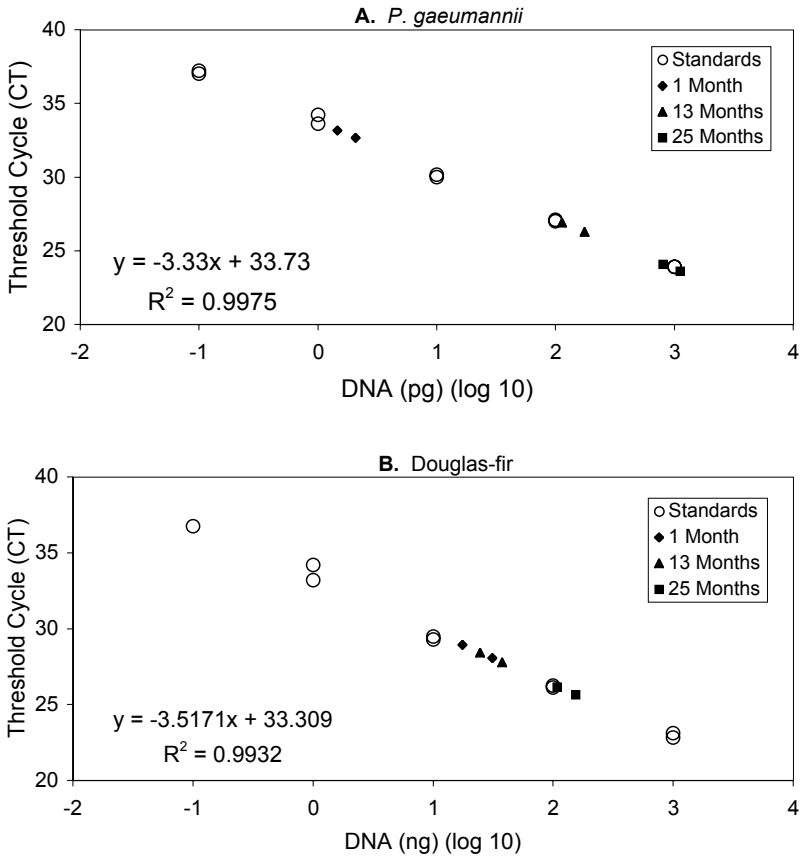

Figure 1. Standard curves and unknown samples demonstrating the simultaneous quantification of P. gaeumannii *(A) and Douglas-fir (B) DNA present in foliage samples using TaqMan real-time PCR. Cycle thresholds (CT) were plotted against the log of genomic DNA standards of known concentration and linear regression equations were calculated for the quantification of unknown samples by interpolation. Unknowns consisted of foliage samples collected from the most recent three needle cohorts at the highly diseased Juno Hill site near Tillamook, OR. Individual cohorts had been initially infected one month, 13 months, and 25 months prior to sample collection.*

(Fig. 1B). These data were used to determine the appropriate dilution for DNA extracted from samples consisting of 10 needles and ensured that uninfected, lightly infected, and heavily infected foliage could all be quantified in a single assay. There was a linear relationship (R=0.97) between *P. gaeumannii* DNA and biomass (Figure 2). Differences in estimated DNA quantities between RNase treated and untreated infected needle extracts were minor, therefore to save costs and the potential for error introduced by additional steps, RNase treatment was not routinely performed in subsequent DNA extractions.

P. gaeumannii colonization estimates among the three experiments designed to assess the precision of the TaqMan system were not significantly different (P=0.35 ANOVA F-test; Figure 3). The standard error for the intra-assay experiment (± 0.36 SE), in which 10 replications of a single DNA sample were amplified concurrently, was slightly less than that for the same sample amplified over 5 separate assays (± 0.48 SE). The greatest error was evident when different sub-samples from a pool of needles were processed individually (± 0.81 SE).

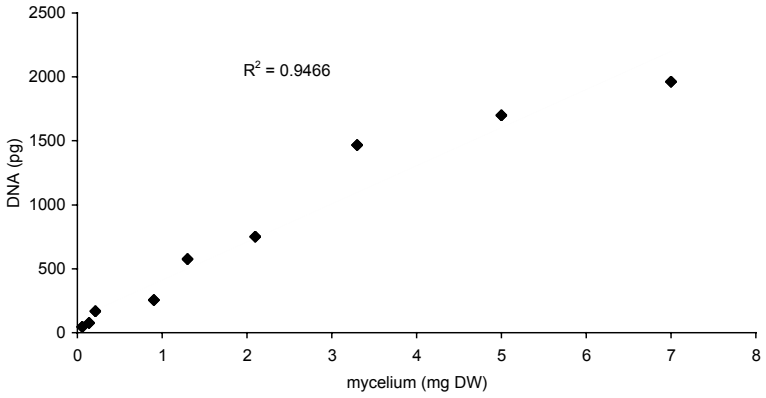

Figure 2. Relationship between biomass estimates of dried P. gaeumannii *mycelium using real-time PCR (DNA) and dry weight (DW).*

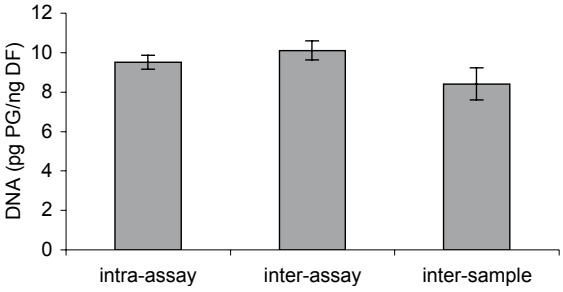

Figure 3. Reproducibility of TaqMan estimates of P. gaeumannii *(PG) colonization of Douglas-fir (DF) foliage. Intra- and inter-assay reproducibility was evaluated on replicate reactions of DNA extracted from one foliage sub-sample amplified in single and multiple assays respectively. Inter-sample reproducibility was evaluated in a single assay of ten separate DNA extractions of different sub-samples. Vertical bars represent standard errors.*

Discussion

Accurate detection and quantification of pathogen colonization in host tissue is an important step in research on many plant diseases. These data are necessary for research on the ecology and epidemiology of plant pathogens, and aid in evaluating potential resistant germplasm. Traditionally, quantitative methodologies have relied either upon culturing the pathogen from infected tissues or visual identification and enumeration of pathogen propagules. While feasible for some pathogenic fungi at some stages of the disease cycle, many pathogens either lack distinctive characters at critical stages of the infection process, or produce so many propagules that enumeration becomes unwieldy. To date, Swiss needle cast research has depended upon estimating the proportion of stomata occluded with fruiting bodies, nearly one year following initial infection. While this method has been useful for comparing severely diseased with infected, but apparently healthy plantations, real-time PCR is the only method suitable for quantification early in the disease cycle, before fruiting bodies have developed.

This is the first report on the application of real-time PCR technology to simultaneously quantify both pathogen and host DNA to derive a relative measurement of pathogen colonization of host tissue. Used in conjunction with the automated ABI Prism 770 sequence detector, species-specific PCR primers and fluorogenic TaqMan probes enabled accumulating amplicons to be detected in real-time during the extension phase of the PCR reaction. In the early stages of developing this technique, we determined optimal probe and primer concentrations to prevent one species from being out competed in the multiplex PCR reaction (data not

presented). This provided confidence that we scored uninfected needles accurately and enabled comparisons between needles of different sizes and developmental stages. In addition, multiplexing resulted in doubled assay output compared to separate tube amplifications.

Calibration experiments with known amounts of target DNA demonstrated that starting quantities that differed over 4 orders of magnitude were detected within the linear dynamic range of the TaqMan system. This enabled accurate quantification of *P. gaeumannii* colonization in severely diseased foliage and in apparently healthy foliage without the need to examine multiple dilutions of each sample. Precision and intra-assay reproducibility among replications were high for both the *P. gaeumannii* and Douglas-fir DNA standards as well as naturally infected foliage. Reproducibility decreased somewhat when replicates were compared between assays. Variance was greatest when sub-samples from a single tree were examined. But because the average colonization estimate for several sub-samples was not significantly different from that of a single sample, one unreplicated sample per tree appears to be sufficient for routine stand-level quantification of *P. gaeumannii* colonization. A normal variance estimate for the entire sub population or treatment group should be sufficient for comparative analyses.

We are currently modifying the technique to detect and quantify *P. gaeumannii* ascospores deposited on grease-coated tape by an air-sampling spore trap. Initial tests are promising and have successfully detected ascospores on both laboratory and field inoculated tapes. Previous attempts to directly enumerate ascospores using microscopy have been confounded by the abundance of similar spores found in diseased forests. The application of real-time PCR will be critical for both observational and planned studies that address environmental and management parameters affecting inoculum potential in forest settings.

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Identification of Alternative Fungicides and Application Timings to Reduce Swiss Needle Cast Damage in Stands of Douglas-fir Timber

Gary Chastagner

Abstract

Protectant Studies - Although applications of Compass, Daconil Weather Stik, Heritage and Benlate plus Dithane significantly reduced disease at the site with the lowest disease pressure, only chlorothalonil types of fungicides were effective in controlling Swiss needle cast (SNC) at the sites with higher disease pressure. Applications of Daconil Weather Stik using a high pressure sprayer provided excellent control of SNC. Sprayed trees also had needles that had a darker green color than the unsprayed trees and also lost fewer 1999 needles. Laboratory tests also showed that Thiolux and Golden Dew sulfur fungicides were equally effective in inhibiting the germination and subsequent germtube growth of ascospores on fungicide amended media. A series of additional protectant trials were established during this past spring to further study the potential use of sulfur to control SNC.

Inoculum Disruption Studies - During fall 1999, a series of four inoculum disruption plots were established. Overall, treatments had no effect on disease incidence this past spring. Treatments containing Sylitt caused a small, but significant reduction in disease severity and the disease index. Treatments containing Sylitt also resulted in a small, but significant reduction in pseudothecia maturation. Unlike previous years, treatments had no effect on inoculum production at any of the test sites. Given the lack of effectiveness of any treatment in reducing inoculum production, it does not appear feasible to use fall applications of fungicides to manage SNC in timber stands, particularly along the coast.

Introduction

This project has two objectives: 1) to identify fungicides that are effective in controlling Swiss needle cast (SNC) when applied as protectants in the spring and 2) determine the effectiveness of fall applications of selected fungicides in disrupting inoculum production and reducing subsequent disease development. In addition, we are examining the role of SNC in the discoloration and needle loss problems in timber stands along the Washington coast.

Protectant studies - Fungicides have played an important role in the management of SNC in Christmas tree plantations, and there is increasing interest in using them as a short-term approach to mitigate damage to trees in selected stands of timber.

During 1999/2000, three replicated field trials were conducted in timber stands along the Oregon and Washington coasts to identify alternatives to chlorothalonil types of fungicides that are effective in protecting newly developing Douglas-fir needles from infection by *Phaeocryptopus gaeumannii* (Table 1). Applications of various rates and combinations of the fungicides were applied to newly developing needles on trees at each site. A single application of each treatment was applied to a single branch (Yankee/Beaver and Grays Harbor) or tree (Starker) in each of five blocks at each test site. The length of a new growth was measured at the time of application. An additional application of two of the treatments was made approximately 28 days later. The following spring the effect of these treatments on disease development, needle color, and needle loss was assessed using the following rating scales.

Table 1. Products included in 1999/2000 fungicide protectant trials.

Trade name and formulation	Active ingredient
Beam 75W	tricyclazole
Benlate 50W	benomyl
Compass 50W	trifloxystrobin
Daconil Weather Stik 720	chlorothalonil
Daconil Ultrex 82.5WDG	chlorothalonil
Dithane 80DF	mancozeb
Golden Dew Sulfur 92%	sulfur
Heritage 50WP	azoxystrobin
KTU 3616 30SC	carpropamid
Ultrafine oil	paraffinic oil

Assessment Methods and Scales

Various combinations of the following assessment methods were used to evaluate the effects various treatments have on disease development. Pseudothecia assessments are typically done by examining the lower surfaces of needles with a dissecting microscope.

Disease Incidence - This is the number of needles out of ten that have one or more SNC pseudothecia on them.

Disease Severity – Disease severity is rated on a 0 to 6 scale where 0 = none, 1 = <1%, 2 = 1 – 10%, 3 = 11-25%, 4 = 26-50%, 5 = 51-75%, and 6 = >75% of stomates on the needles are plugged with pseudothecia. These ratings are made with the aid of a card that illustrates sections of needles with 180 stomates that have 1, 10, 25, 50, and 75% of the stomates plugged with pseudothecia.

Disease Index - A disease index is calculated by multiplying the disease incidence times disease severity. Thus our disease index ranges from 0 to 60.

Needle Loss – Needle loss is rated on a scale of 0 to 10, where 0 = none, 1 = 1-10%, 2 = 11-20%, 3 = 21-30%,…., and 10 = 91-100% loss.

Needle Color – We rate needle color on a 1 to 6 scale, where 1 = healthy appearing dark green needles, 2 = healthy appearing green needles, 3 = needles with a slight yellow mottling on a green background that may also have brown spots or tips on the needles, 4 = dull green needles with moderate chlorosis that may also have brown spots or tips on the needles, 5 = extensive yellowing/browning, and 6 = uniformly yellow needles that may have some brown spots or tips.

Results from 1999/2000 trials

Disease levels varied among the three sites (Tables 2, 3 and 4). Trees at the Yankee/Beaver site had the highest disease index while trees at the Grays Harbor site had the lowest. At the Grays Harbor site, single applications of Compass, Daconil Weather Stik and Benlate plus Dithane along with two applications of Heritage significantly reduced the disease index compared to the unsprayed checks (Table 2). Needles on some of the fungicide treated branches also had a darker green color than the unsprayed check. Loss of 1999 needles was very low (0.4 to 1.8) at this site and none of the treatments had any effect on this parameter.

The disease level at the Starker site was intermediate, and fewer of the fungicides were effective in reducing disease development (Table 3). The highest rate of Daconil Weather Stik was the only treatment that significantly reduced disease incidence and the disease index compared to the unsprayed check. None of the treatment had any effect on needle color (ratings ranged from 1.2 –2.2) and needle loss (ratings ranged from 0.0 – 0.6).

At the Yankee/Beaver test site, the only treatments that were effective in reducing disease incidence and the

Table 2. Effect of protectant fungicide sprays on the development of Swiss needle cast at the Grays Harbor test site.

Treatment[1]	Product/ 100 gal	Disease[2] Incidence	Index	Needle color[2]
Check	-	10.0 a	16.0 a	2.2 ab
Golden Dew Sulfur*	20 lb	10.0 a	16.0 a	2.0 abc
Ultrafine oil	0.5%	10.0 a	16.0 a	2.6 a
KTU 3616 30SC	4 oz	9.4 a	15.4 a	1.8 bc
Heritage 50WG	8 oz	10.0 a	14.0 ab	2.2 ab
Golden Dew Sulfur*	10 lb + 2X	10.0 a	14.0 ab	1.8 bc
Benlate 50W*	1 lb	4.6 bcd	10.6 abc	1.6 bc
Benlate 50W plus Ultrafine oil	1 lb 0.5%	6.0 bcd	8.0 abc	2.6 a
Daconil Ultrex 82.5WDG	5 lb	5.2 abcd	7.8 abc	1.6 bc
Beam 75WP	14 oz	7.6 abc	7.6 abc	1.4 c
Benlate 50W*	2 lb	7.6 abc	7.6 abc	1.8 bc
Dithane 80DF	4 lb	4.6 bcd	6.6 abc	1.8 bc
Daconil Ultrex 82.5WDG	2.5 lb	6.0 abcd	6.0 bc	1.4 c
Compass 50W*	1 oz	4.2 bcd	4.8 bc	2.0 abc
Compass 50W*	2 oz	4.8 abcd	4.2 c	2.0 abc
Heritage 50WG*	8 oz + 2X	3.6 cd	3.6 c	1.8 bc
Daconil Weather Stik 720	2.75 pt	3.2 cd	3.2 c	1.4 c
Benlate 50W plus Dithane 80DF*	1 lb 4 lb	2.6 cd	2.6 cd	1.8 bc
Daconil Weather Stik 720	5.5 pt	1.8 d	1.8 d	1.6 bc

[1] Treatments were applied with a hand sprayer on June 3, 1999 when the new growth was 1.8 inches long. Treatments that were applied twice (2X) were reapplied on June 28th. (*) = Triton B-1956 @ 1 pt/100 gallons added with the fungicide.

[2] Disease assessments and needle color was evaluated on April 13, 2000. Numbers in columns followed by the same letter are not significantly different, P=0.05, DMRT.

Table 3. Effect of protectant fungicide sprays on the development of Swiss needle cast the following spring at the Starker test site.

Treatment[1]	Product/100 gal	Disease[2] Incidence	Index
Compass 50W*	1 oz	10.0 a	30.0 a
Dithane 80DF	4 lb	10.0 a	30.0 a
Ultrafine oil	0.5%	10.0 a	26.0 ab
Beam 75WP	14 oz	10.0 a	26.0 ab
Golden Dew Sulfur*	20 lb	10.0 a	24.0 abc
KTU 3616 30SC	4 oz	10.0 a	24.0 abc
Golden Dew Sulfur*	10 lb + 2X	10.0 a	24.0 abc
Benlate 50W*	2 lb	10.0 a	24.0 abc
Daconil Ultrex 82.5WDG	2.5 lb	10.0 a	24.0 abc
Check	-	10.0 a	22.0 abc
Heritage 50WG	8 oz	10.0 a	22.0 abc
Benlate 50W*	1 lb	10.0 a	20.0 abcd
Benlate 50W plus Dithane 80DF*	1 lb /4 lb	10.0 a	18.0 abcd
Heritage 50WG*	8 oz + 2X	8.4 a	16.4 abcd
Benlate 50W plus Ultrafine oil	1 lb /0.5%	9.8 a	15.8 abcd
Compass 50W*	2 oz	7.8 a	15.8 abcd
Daconil Ultrex 82.5WDG	5 lb	9.0 a	15.0 bcd
Daconil Weather Stik 720	2.75 pt	8.0 a	10.0 cd
Daconil Weather Stik 720	5.5 pt	4.2 b	6.2 d

[1] Treatments were applied with a hand sprayer on June 3, 1999 when the new growth was 1.8 inches long. Treatments that were applied twice (2X) were reapplied on June 28th. (*) = Triton B-1956 @ 1 pt/100 gallons added with the fungicide.

[2] Disease assessments and needle color was evaluated on April 13, 2000. Numbers in columns followed by the same letter are not significantly different, P=0.05, DMRT.

disease index were the highest rates of Daconil Weather Stik and Daconil Ultrex (Table 4). Although treatments had no effect on the loss of 1999 needles (ratings ranged from 0.2 to 2.2), the needles on branches treatments with various chloro-thalonil products had a darker green color than the needles on the unsprayed branches.

Applications of the two fungicides that inhibit melanin biosynthesis (Beam and KTU 3616), Golden Dew sulfur and the addition of spray oils to Benlate had no effect on disease development. Given the apparent effectiveness of Thiolux sulfur applications in controlling SNC in other trials in Oregon, two laboratory spore germination tests were conducted to obtain a better understanding of why the applications of Golden Dew sulfur failed to provide any disease control in our protectant trials.

Moistened SNC infected needles were suspended over plates of water agar that had been amended with various concentrations of thiolux and Golden Dew Sulfur. After 48 hrs incubation at 20°C, the percentage of ascospores that had been discharged onto the agar surface and germinated was determined. The length of the germtubes on the germinated spores was also measured.

About 85% of the ascospores on water agar had germinated after 48 hours incubation (Table 5). The addition of Thiolux or Golden Dew sulfur to the agar caused a significant reduction in germination. The extent of reduction was dependent on the concentration of sulfur in the media. Both sources of sulfur were equally effective in inhibiting germination.

Table 4. Effect of protectant fungicide sprays on the development of Swiss needle cast and needle color the following spring at the Yankee/Beaver test site.

Treatment[1]	Product/ 100 gal	Disease[2] Incidence	Disease[2] Index	Needle color[2]
Heritage 50WG*	8 oz + 2X	10.0 a	40.0 a	3.2 ab
Check	-	10.0 a	38.0 ab	3.2 ab
Heritage 50WG	8 oz	10.0 a	36.0 ab	3.2 ab
Golden Dew Sulfur*	20 lb	10.0 a	34.0 ab	2.8 abcd
KTU 3616 30SC	4 oz	10.0 a	34.0 ab	3.0 abc
Golden Dew Sulfur*	10 lb + 2X	10.0 a	34.0 ab	3.0 abc
Compass 50W*	2 oz	10.0 a	34.0 ab	2.8 abcd
Compass 50W*	1 oz	10.0 a	32.0 abc	2.8 abcd
Ultrafine oil	0.5%	10.0 a	32.0 abc	3.6 c
Beam 75WP	14 oz	10.0 a	32.0 abc	2.8 abcd
Benlate 50W*	1 lb	10.0 a	32.0 abc	2.8 abcd
Benlate 50W plus Dithane 80DF*	1 lb 4 lb	9.2 ab	31.6 abc	2.6 abcde
Dithane 80DF	4 lb	9.6 a	30.6 abcd	2.2 bcde
Benlate 50W plus Ultrafine oil	1 lb 0.5%	10.0 a	30.0 abcd	3.2 ab
Benlate 50W*	2 lb	9.2 ab	25.8 bcde	2.0 cde
Daconil Ultrex 82.5WDG	2.5 lb	6.8 bc	19.8 cde	1.6 e
Daconil Weather Stik 720	2.75 pt	7.4 abc	18.6 def	1.8 de
Daconil Ultrex 82.5WDG	5 lb	5.0 cd	16.6 ef	2.0 cde
Daconil Weather Stik 720	5.5 pt	4.2 d	7.4 f	1.8 de

[1] Treatments were applied with a hand sprayer on June 2, 1999 when the new growth was 2.5 inches long. Treatments that were applied twice (2X) were reapplied on June 29th. (*) = Triton B-1956 @ 1 pt/100 gallons added with the fungicide.

[2] Disease assessments and needle color evaluated on April 12, 2000. Numbers in columns followed by the same letter are not significantly different, P=0.05, DMRT.

Table 5. Germination of Phaeocryptopus gaeumannii ascospores on water agar amended with two formulations of sulfur.

Sulfur concentration µg ai/ml	Percent germination[1] (48 hr @ 20ºC) Thiolux 80W	Golden Dew 92%
0	85.5±2.5	85.5±2.5
4	49.7±5.1	41.0±5.1
8	21.3±3.7	21.4±3.4
40	4.8±1.1	8.6±1.7
80	0.0±0.0	2.7±1.0
400	1.7±1.3	3.1±1.6
800	0.0±0.0	0.0±0.0

[1] Averages ± standard errors of two experiments.

In addition to inhibiting germination, both the Thiolux and Golden Dew sulfur inhibited the growth of germtubes (Table 6).

Based on these data, the lack of control we observed with Golden Dew sulfur is likely the result of the fact that we used lower rates of product then were used in the Oregon trials with Thiolux or due to different coverage and/or weathering properties between these two formulations of sulfur.

2000/2001 Trials - A series of five protectant trials were established during this past spring in a timber stand along the Washington coast. The first trial involves eight treatments with two reduced risk fungicides – cyprodinil and triflumizole. The second and third trials represent extensions of the Oregon State University sulfur studies that were established in Oregon this past spring. One of these trials involves early and late hand sprayer applications of Thiolux at 15 lbs per 100 gallons of water. The other trial involves two applications of Thiolux at 15, 30 and 60 lbs per acre. In the fourth trial, we are looking at the effect of spray coverage on the control of SNC with a single application of Daconil Ultrex and Daconil Weather Stik. Two rates of each product are being tested and the applications were applied to wet the foliage or on a per acre basis. The fifth trial is designed to examine the effect of sulfur product (Thiolux and Golden Dew), rate (15, 30, 60 and 90 lbs), spray coverage (spray to wet the foliage or on a per acre basis) and timing (early, late, or early and late) on the control of SNC. The effectiveness of the treatments in these trials in protecting needles

Table 6. Length of germtubes on germinated Phaeocryptopus gaeumannii ascospores on water agar amended with two formulations of sulfur.

Sulfur concentration µg ai/ml	Germtube length after 48 hr @ 20ºC[1] (µ) Thiolux 80W	Golden Dew 92%
0	17.5±1.1	17.5±1.1
4	5.5±0.5	4.8±0.4
8	3.8±0.4	4.0±0.3
40	2.8±0.1	4.0±0.8
80	-	2.8±0.1
400	-	3.0±0.1
800	-	-

[1] Averages ± standard errors of two experiments.

from SNC will be assessed during spring 2001.

Inoculum Disruption Studies - Another approach to controlling SNC with fungicides is to use fungicides to disrupt inoculum production. Disrupting the development of fruiting bodies and subsequent production of inoculum is a form of sanitation and sanitation is a very effective way of reducing the development of single cycle diseases such as SNC.

We have been examining the potential of using applications of systemic fungicides during the fall to disrupt the development of pseudothecia and reduce the production of ascospore inoculum the following spring for the past three years. These studies have involved making applications of fungicides to trees during the fall and then assessing the effects of the treatments on the development of pseudothecia and production of ascospore inoculum the following spring. Samples of needles are collected from the treated branches and pseudothecia maturation and inoculum production are assessed as follows:

Pseudothecia Maturity - The development or maturity of pseudothecia on the ten needles that are used to assess inoculum production are rated on a 0 to 4 scale where 0 = none, 1 = <10%, 2 = 10-25%, 3 = 25-50%, and 4 = >50% of the pseudothecia are fully erupted out of the stomates.

Inoculum production – We determine the ability of pseudothecia on needles to release ascospores by mounting ten needles across a 1 cm wide window in a piece of filter paper that is mounted in the top of a petri plate lid. The exposed lower surfaces of the needles are then sprayed with water and the lid is positioned above a plate of water agar. Plates are sealed and incubated at 20C. After 24 hours, the surface of the agar is then sprayed with rose bengal to stop the germination of spores and the number of spores released are counted using a compound microscope. This provides us with the number of spores released from ten 1-cm-long sections of needle.

In a 1997/98 trial, we found that a single application of Syllit 50W, Cleary's 3336 50W, and especially Benlate 50W in November significantly disrupted the development of pseudothecia and production of inoculum the following spring. Three test sites were used in our 1998/99 studies. Applications of fungicides were applied to whole trees or branches on trees at two sites along the Oregon coast (Starker and Yankee/Beaver) and a Manke Timber site near Cinnebar, WA. Although a single fall application of Sylitt resulted in a significant reduction in inoculum production at the Manke site, none of the treatments at the Starker and Yankee/Beaver sites were effective in disrupting inoculum production. Application timing (September through late November) at the Manke site had no effect on the efficacy of the treatments.

During fall 1999, a series of four additional inoculum disruption plots were established at the three sites used in 1998/99 and a site near Grays Harbor, WA. Treatments included a single application of Benlate 50W (2lbs/100 gallons), Sylitt (3lbs/100 gallons), and Benlate plus Sylitt. These same materials were also mixed with Ultrafine spray oil (0.5%) in an effort to increase their effectiveness. Checks consisted of unsprayed branches and branches sprayed with only the Ultrafine oil. A single application was made to trees at each test site during late November, 1999. All the plots were a RCB design with ten blocks.

The effects of these treatments in disrupting inoculum production was determined by assessing pseudothecia incidence, severity, maturity and ascospore production on samples collected on April 24-26, May 22-24, and June 19-21, 2000. Overall, treatments had no effect on disease incidence, but treatments containing Sylitt caused a small, but significant reduction in disease severity and the disease index (data not shown). Treatments containing Sylitt also resulted in a small, but significant reduction in pseudothecia maturation. There were significant differences in the level of disease among the four test sites (Table 7).

Overall, pseudothecia maturity and inoculum production differed among the test sites (Table 8 and 9).

Table 7. Differences among test sites relating to overall disease levels[1].

	Disease		
Test site	Incidence	Severity	Index
Yankee/Beaver	9.98 a	4.7 a	46.8 a
Starker	9.97 a	2.8 b	27.9 b
Manke	9.96 a	2.6 c	25.4 c
Grays Harbor	9.40 b	2.4 c	23.6 d

[1] Overall data for samples collected during April, May and June, 2000. Numbers in columns followed by the same letter are not significantly different, P=0.05, DMRT.

A higher percentage of the pseudothecia on the needles from the trees at the Yankee/Beaver test site were fully erupted out of the stomates and they produced the highest levels of inoculum on all three sample dates. Unlike previous years, treatments had no effect on inoculum production at any of the test sites (data not shown).

Given the lack of effectiveness of any of the treatments in reducing inoculum production, it does not appear that it will be feasible to use fall applications of fungicides in an effort to manage SNC in timber stands, particularly along the coast.

Table 8. Difference in overall pseudothecia maturity ratings by test site and sample date.

	Pseudothecia maturity[1]		
Test site	April 24-26	May 22-24	June 19-21
Yankee/Beaver	4.1 a	3.1 a	3.7 a
Grays Harbor	2.2 b	2.2 b	2.3 b
Manke	1.9 bc	1.9 bc	2.1 b
Starker	1.7 c	2.1 bc	2.2 b

[1] Numbers in columns followed by the same letter are not significant, P=0.05, DMRT.

Table 9. Differences in overall levels of inoculum production among test sites[1].

	No. of ascospores[2]		
Test site	April 24-26	May 22-24	June 19-21
Yankee/Beaver	465.7 a	2,066.7 a	362.0 a
Grays Harbor	33.0 b	568.3 b	135.6 b
Starker	17.7 b	459.7 b	117.8 b
Manke	5.4 b	335.0 b	76.3 b

[1] Overall data for all treatments by sample date.

[2] Numbers of ascospores released from pseudothecia on 10 cm of needle. Numbers in columns followed by the same letter are not significantly different, P=0.05, DMRT.

Additional Studies

During spring 1999, stands of Douglas-fir timber along the central Washington coast exhibited extensive discoloration and trees had very sparse crowns because of the premature loss of needles. Trees that were examined in these stands hand high levels of SNC sporulation on their needles.

In an effort to obtain some information on the role of SNC on the condition of the trees in these stands, a series of paired plots were installed at two sites. Applications of Daconil Weather Stik 720 (5.5 pts/100 gallons) was applied to half of the trees at each site using a high pressure sprayer. At a Grays Harbor County timber site, there are three replications of ten treated and check trees. At a Rayonier timber site, all of the trees within one of five 60' X 100' paired plots were sprayed. Data were collected on shoot development on all of the trees in the plots at the time of spraying.

Samples of 1999 growth were collected from each tree in these plots during April 2000 and examined for the incidence and severity of pseudothecia. Samples were also rated for needle color and the loss of 1999 needles. Although disease incidence was fairly high at both sites, the overall disease index ratings were relatively low because of the low numbers of stomates plugged with pseudothecia on infected needles (Tables 10 and 11). No pseudothecia were found on any of the needles examined from the trees that had been sprayed with Daconil Weather Stik at the Grays Harbor site and very few infected needles were found on the sprayed trees at the Rayonier site. The sprayed trees at both sites also had needles that had a darker green color than the needles on the unsprayed trees and these trees had also lost fewer 1999 needles.

In an effort to better understand the role of SNC on the condition of the trees in these stands, all the trees that were sprayed in 1999 were sprayed again during June 2000 and additional disease and foliage

Table 10. Effect of high pressure ground based applications of Daconil Weather Stik 720 on the development of Swiss needle cast at Grays Harbor test site[1].

		Disease		Needle	
Treatment	Prod./ 100 gal	Inc.	Index	Color	Loss
Check	-	9.9 a[2]	16.2 a	3.0 a	2.0 a
Daconil Weather Stik 720	5.5 pt	0.0 b	0.0 b	1.8 b	1.5 b

[1] Trees were sprayed to drip on June 10, 1999 when the new growth was 1.1 inches long. Disease and needle color/loss data were taken on April 6, 2000.

[2] Numbers in columns followed by the same letter are not significantly different, P=0.05, t-test.

Table 11. Effect of high pressure ground based applications of Daconil Weather Stik 720 on the development of Swiss needle cast at the Rayonier test site[1].

		Disease		Needle	
Treatment	Prod./ 100 gal	Inc.	Index	Color	Loss
Check	-	9.2 a[2]	15.2 a	3.5 a	0.3 a
Daconil Weather Stik 720	5.5 pt	0.1 b	0.1 b	2.1 b	0.1 b

[1] Trees were sprayed to drip on June 11, 1999. Disease and needle color/loss data were taken on April 6, 2000.

[2] Numbers in columns followed by the same letter are not significantly different, P=0.05, t-test.

assessments will be made during spring 2001.

Acknowledgements

Personnel who worked on this project include John Staley, Joe Hudak, Kathy Riley, Jenny Glass, and Jan Sittnick. Portions of this research were also supported by the Pacific Northwest Christmas Tree Association and the Washington State Commission on Pesticide Registration. The assistance of Starker Forest, Rayonier, and Grays Harbor County Forestry Department is greatly appreciated.

Tree Physiology Studies

Dan Manter and Katy Kavanagh.
Cooperators: Greg Filip, Jeff Stone, Wendy Sutton and Lori Winton.

Abstract

Results from two studies are contained in this report. Study 1 investigated the role of *P. gaeumannii*-produced toxins in the development of Swiss needle cast symptoms. Extracts from three *P. gaeumannii* isolates had no apparent pathogenic effect on Douglas-fir needles. In study 2 we report the results of two years of disease surveys from our field plots. Across all sites we found a significant correlation between fungal colonization and symptom development, irrespective of the quantification technique. Several of the factors influencing the relationship between fungal colonization and symptom development are discussed, and some recommendations for future SNC assessments are proposed.

Study 1
No apparent toxins involved in the development of Swiss needle cast symptoms.

Background

In several other fungal disease complexes (e.g., *Dothistroma pini* on Radiata pine, *Helminthosporium sacchari* on sugarcane), fungal produced toxins play a central role in the development and impact of the fungus on host tissue. A *P. gaeumannii*-produced toxin may help to explain the impact of SNC on Douglas-fir physiology and growth; as well as, some of the variation in SNC symptom development – particularly in needles showing little sign of fungal hyphae or pseudothecia development.

Objective

In this study we tested for the possible role of *Phaeocryptopus gaeumannii* produced toxins in the development of Swiss needle cast symptoms.

Methods

Culture Extractions.

Three isolates (coastal OR, interior OR and coastal WA) of *Phaeocryptopus gaeumannii* were grown in 2 % potato dextrose broth at 20 C. After six months mycelium was harvested, blotted-dry on sterilized filter paper, and 1 g of each isolate was homogenized in acetone, ethyl acetate and ethanol. After homogenization, samples were heated at 80 C for 30 min, evaporated to dryness and re-dissolved in 1 ml of dH_20.

Needle Injections.

On March 29, 2000, six current-year needles on one branch of a three-year-old Douglas-fir seedling were injected with one of the following treatments using a hypodermic needle: 100 ml fungal extract (repeated for each of the nine possible isolate-solvent combinations), a dH_2O control, and a negative control (i.e., needle puncture only). All treatments were repeated on three seedlings. After a six-month incubation period, under ambient conditions in the greenhouse, all treated needles were harvested and scored for chlorosis and lesions surrounding the injection point.

Results

No visible chlorosis was evident on any of the injected needles (data not shown). Figure 1 shows the average lesion size for each treatment. No significant differences in any of the treatments were detected ($p = 0.640$).

Conclusion

This study was by no means a comprehensive test for presence of a toxin in *P. gaeumannii*; however, if a toxin is present, and an important aspect in the development of SNC symptoms than we believe this study should have detected one. First, the inoculation levels used in this study were several times greater than levels showing strong symptom development in the field. Second, these techniques have been successful in detecting toxins in other disease systems (e.g., *Dothistroma pini*). The lack of a toxin in the development of Swiss needle cast lends support to our previous studies showing that the first, and major, impact of *P. gaeumannii* on Douglas-fir needles is through the occlusion of stomata by fungal structures.

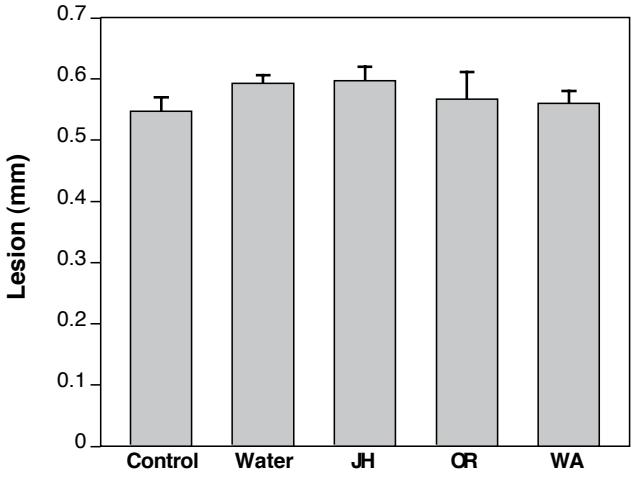

Figure 1. Lesion length in needles injected with Phaeocryptopus gaeumannii *hyphal extracts. No significant differences in solvents were detected ($p > 0.1$); therefore, each bar for the fungal culture extracts (JH = coastal Oregon isolate, OR = interior Oregon isolate, WA = coastal Washington isolate) is the pooled mean and standard error for all three solvent systems (JH, OR and WA). The control treatment consisted of a needle puncture only, and the water treatment was an injection of distilled H_20 only.*

Study 2

Factors affecting SNC symptom development. Lessons learned from two years of disease surveys.

Background

In previous studies, the impact of Swiss needle cast on Douglas-fir needle retention (Hansen et al. 2000) and physiology (Manter et al. 2000) has been shown to be proportional to the number of stomata occluded by *Phaeocryptopus gaeumannii* pseudothecia. However, in another study, Winton et al. (*in preparation*) note considerable variation in the relationship between fungal colonization and symptom development, reporting that only about 60 % of symptom development can be accounted for by estimating fungal colonization. Based on our bi-annual 1998 and 1999 surveys of fungal colonization and symptom development in our field plots, we report on some of the factors responsible for the variation in the relationship fungal colonization and symptom development. Consideration of these factors in the design of future studies should greatly improve disease impact predictions

from assessments of *P. gaeumannii* colonization.

Objectives

The objectives of these surveys were: (i) to provide disease assessments for our on-going physiology studies, (ii) identify some of the factors affecting symptom development, and (iii) improve future sampling protocols for SNC assessments.

Methods

Sample Sites.

Three 12-15 year-old Douglas-fir plantations with varying levels of Swiss needle cast were chosen for study. Paired permanent plots were created on north- and south-aspect slopes (ca. 30 %) at each site. 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, the medium disease site is located on the Siuslaw National Forest near Hebo, OR, and the low disease site is located on the MacDonald-Dunn Forest near Corvallis, OR.

Tree Sampling.

At each sample date two 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 southern-aspect branches from 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.

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.

Fungal and Symptom Quantification.

Ergosterol content (mg ergosterol g^{-1} needle dry weight), quantitative PCR (pg *Phaeocryptopus gaeumannii* DNA ng^{-1} *Pseudotsuga menziessii* 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.

Results

All measures of fungal abundance detected similar levels of fungal colonization and development and were highly correlated with each other. Furthermore, all measures of fungal colonization were significantly correlated with needle retention and chlorosis (Table 1). A marginal

Table 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 $p < 0.01$.

	Pseudothecia Density	Ergosterol Content	Quantitative PCR	Needle Retention
Pseudothecia Density	-			
Ergosterol Content	0.818	-		
Quantitative PCR	0.765	0.685	-	
Needle Retention	-0.542	-0.485	-0.423	-
Needle Chlorosis	0.665	0.552	0.603	-0.721

Table 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 $p < 0.01$.

	Pseudothecia Density	Ergosterol Content	Quantitative PCR	Needle Retention
Pseudothecia Density	-			
Ergosterol Content	0.885	-		
Quantitative PCR	0.814	0.733	-	
Needle Retention	-0.644	-0.578	-0.451	-
Needle Chlorosis	0.725	0.628	0.677	-0.750

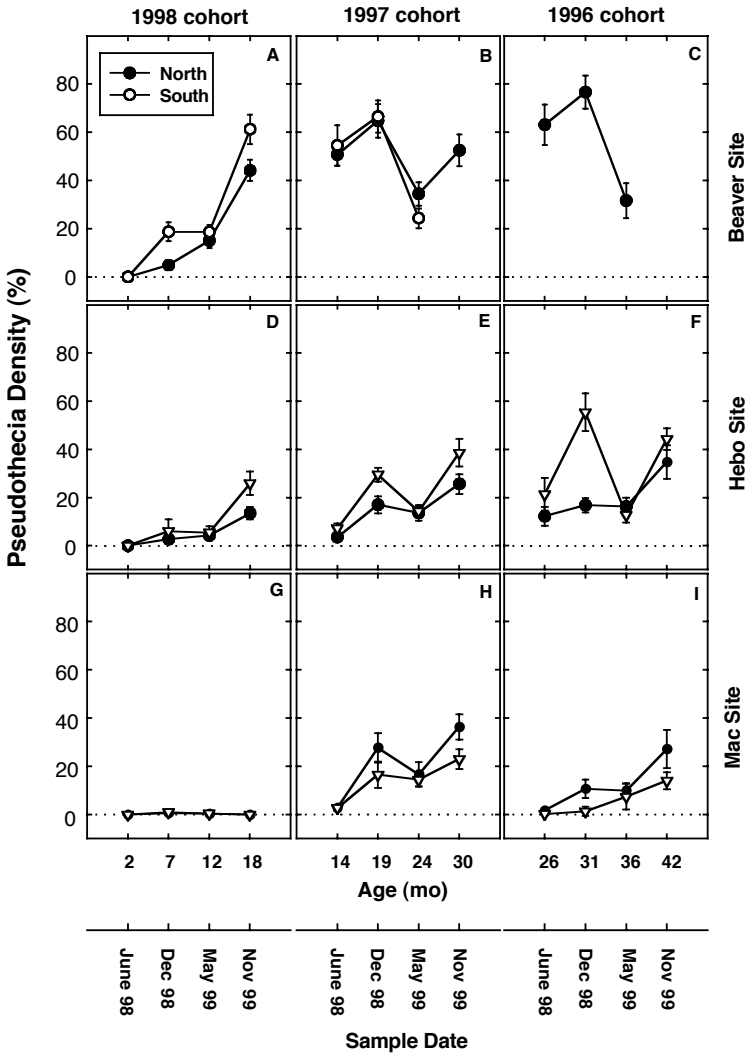

Figure 2. Mean pseudothecia density of Phaeocryptopus gaeumannii *over time from six untreated Douglas-fir trees in a southern- and northern-aspect plot at three sites. Error bars are one standard error. Missing observations were due to needle abscission.*

improvement in the correlations was observed when the mean values for all six trees were used (Table 2).

In general, fungal colonization increased over time, with the largest increases during the fall (Fig. 2), except during the spring sample (May 1999) when abscission of the most heavily infected needles occurred (Fig. 3). Like fungal colonization, symptom development (i.e., needle retention and chlorosis) also increased over time; however, the largest increases occurred during the spring (Fig. 3).

To account for this time lag between fungal colonization and symptom development, the correlation between fungal colonization (December 1998 sample date) and symptom development (May 1999 sample date) was conducted. In this case, the correlation between fungal colonization and symptom development was greatly improved (Table 3). Furthermore, the decline in fungal colonization during the spring months (Fig.2), coinciding with needle abscission (Fig. 3), suggests that a successful correlation between fungal colonization and symptoms will be dependent upon the sample date when fungal colonization was measured (i.e., fungal colonization measurements in the spring or later will underestimate fungal colonization). Table 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 3).

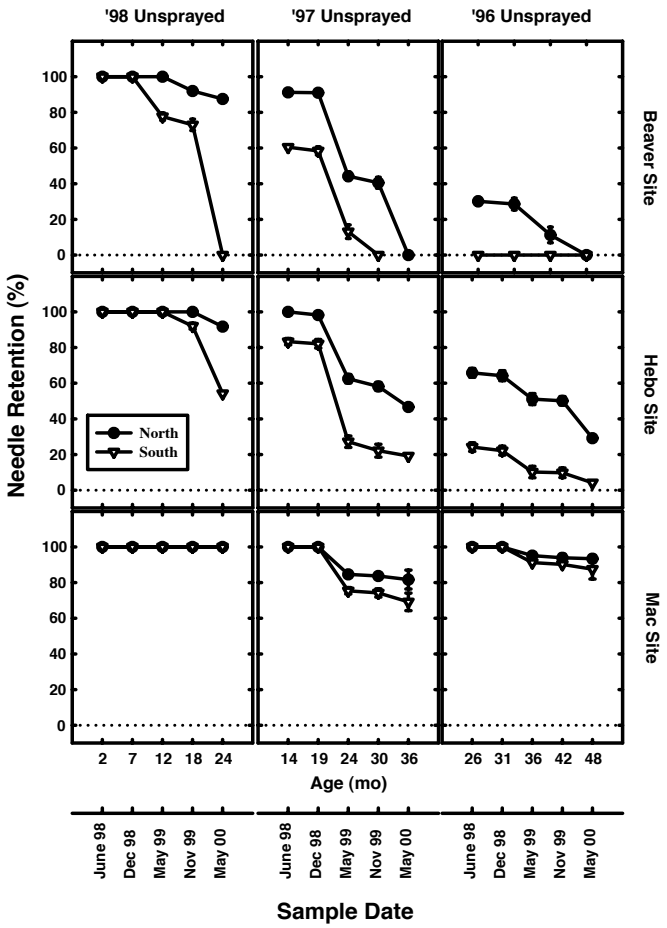

Figure 3. Mean needle retention over time from six untreated Douglas-fir trees in a southern- and northern-aspect plot at three sites. Error bars are one standard error.

Table 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 $p < 0.01$. Quantitative PCR was not measured for the December 1998 sample.

	Pseudothecia Density	Ergosterol Content	Quantitative PCR
Needle Retention	-0.902	-0.816	NA
Needle Chlorosis	0.810	0.756	NA

Table 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 $p < 0.01$. Quantitative PCR was not measured for the December 1998 sample.

	Pseudothecia Density	Ergosterol Content	Quantitative PCR
Needle Retention	-0.679	-0.661	NA
Needle Chlorosis	0.712	0.731	NA

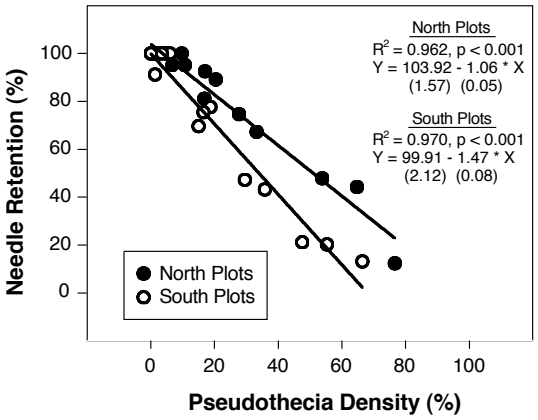

Figure 4. Relationship between the mean needle retention (May 1999 sample) and pseudothecia density (December 1998 sample) from Douglas-fir trees on northern- and southern-aspect plots from three sites.

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). 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 % (Fig. 2). 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 (Fig. 4).

Significant differences in both fungal colonization and symptom development were detected within tree canopies (Table 5). For example, pseudothecia density was ca. 10 % higher in the upper portion of the canopy, and ca. 4 % greater on the southern-side of the tree. Symptom development showed a similar pattern to fungal colonization, also being highest in the upper canopy and on the southern-side of the tree. Similar to the slope effect found at our sites, infected needles growing in the higher light and temperature environments (i.e., upper canopy and southern quadrants) exhibited greater symptom development for any given level of fungal colonization. For example, the amount of needle loss per unit pseu-

Table 5. 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.

Measure	South	North	
Pseudothecia density	17.5(4.5)[a]	12.6(3.5)[b]	Top
Quantitative PCR	0.8(0.2)[a]	0.8(0.2)[a]	Top
Needle retention	76.7(3.4)[a]	86.7(4.1)[b]	Top
Needle chlorosis	1.5(0.2)[a]	0.9(0.2)[b]	Top
Pseudothecia density	6.9(1.5)[b]	4.0(0.9)[c]	Bottom
Quantitative PCR	0.6(0.1)[a]	0.3(0.1)[b]	Bottom
Needle retention	91.7(3.1)[a]	98.7(1.7)[c]	Bottom
Needle chlorosis	1.2(0.2)[b]	0.5(0.1)[c]	Bottom

dothecia density (e.g., (100 – needle retention) / pseudothecia density) was 1.33, 1.2, 1.06 and 0.43 for the upper-south, lower-south, upper-north and lower-north canopy positions.

Conclusions

The pathogenicity of *Phaeocryptopus 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.

Based on our observed correlations between symptom development and fungal colonization, we suggest that the following factors be considered in future SNC assessments: (i) sample branches from a consistent canopy position – minimizing the effect of environmental differences within the tree canopy, (ii) pool values from several trees at each site – minimizing tree to tree variation arising from host genotype, microclimate differences and/or errors due to limited sampling within each tree, (iii) account for the substantial time for symptoms to appear, and (iv) 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.

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Effects of Fertilization and Vegetation Control on Swiss Needle Cast Infection and Growth of Coastal Douglas-fir Saplings

Gabriel Crane, Robin Rose, Scott Ketchum, and Diane Haase

Introduction

There are currently thousands of acres of Swiss needle cast infected stands in Oregon and Washington with poor prognosis for growth. The potential to increase vigor and growth within these stands through silvicultural manipulation is unknown. This study was initiated in May 1999 to determine if silvicultural treatments (fertilization and weed control) can alleviate SNC infection levels and enhance growth of Douglas-fir saplings.

The major objective of this study is to determine if fertilization and vegetation control treatments influence the height and diameter growth of SNC infected saplings. The influence of treatments on SNC infection severity will also be measured. A sub-objective of this study is to determine if chlorophyll fluorescence can be used as a viable tool to measure the severity of SNC infection.

Methods

Site selection

Three replicated studies were established at three different sites along an east/west transect of the Oregon Coast Range. Study replicates installed across this gradient were chosen because the greatest level of growth loss associated with SNC has been found in the coastal regions and tends to decrease eastward toward the Willamette Valley.

The site nearest the coast is located between Toledo and Siletz (South Drake) on The Timber Company's ownership. The second site is midway between the coast and the Willamette Valley near Eddyville (Bushy Peterson) on Starker Forest lands. The third site is on the western valley fringe close to Summit (Charlie Olson), also on Starker Forest lands. Each site was established in existing 9 to 10-year-old plantations of Douglas-fir saplings.

Experimental Design

On each site, the study is a randomized complete block design with five replications (blocks) of each treatment. An exception to this is the "Bushy Peterson" site where there are only four replications due to limited space. The six treatments consist of a 2x3 factorial with two levels of weed control and three levels of fertilization. Each treatment plot is 70 ft. x 70 ft. and encompasses 25-35 operationally planted trees of which 15 are identified for evaluation in the study. Trees with forked stems or which originated from natural regeneration were not used for measurement trees. Each tree is clearly identified with an aluminum tag, marking paint, and flagging. Individual plots were marked in each corner with white PVC stakes approximately one meter in height. Flagging was strung between stakes completing the plot layout.

Treatments

There are two vegetation control treatments:

1) No control
2) Control of all competition for three consecutive years

All treatment plots by block were assigned at random. On September 7-9, 1999, all woody vegetation on the South Drake and Bushy Peterson sites was manually cut to ground level. The Charlie Olson site did not have a significant amount of woody vegetation and therefore did not necessitate manual control of woody vegetation. In addition, weed control plots received a herbicide application broadcast with a backpack sprayer. Fall application of Sulfometuron (Oust) at 3 oz/a and Accord at 1.5 qt/a were used to eliminate all woody and herbaceous competitors. Additional spring and fall applications of herbicide will be used to maintain weed-free conditions.

The three fertilizer treatments are:

1) Unfertilized control
2) 200 grams of 9-17-17 per tree / application
3) 200 grams of 17-17-17 per tree / application

The controlled-release fertilizer formulations with minor elements included are from Simplot Company and are identical with the exception of nitrogen content (Table 1). Applications were initially made on September 8-10, 1999 to each of the 15 trees in the plots designated for fertilization. Fertilizer blends were surface applied around the base of the tree. Fertilizer was tossed by hand into the middle of the tree at approximately two feet off of the ground and allowed to scatter as it fell through the branches. Fertilizer applications were made again in both April and October 2000, and will be repeated each year of the study using the same rates and methods.

Data Collection

Morphology

Initial diameter at breast height (DBH) and height were measured on the 15 study trees in each plot at all sites in late May 1999. Second measurements were taken mid-October 1999 on all 1260 trees. The results from these measurements establish a baseline for both height and DBH growth over all treatments and sites.

Future measurements will take place at the end of each growing season. Seasonal growth is calculated by subtracting height values and DBH values. Stem volume is calculated using the formula for a cone: $1/3(\pi/4\ d^2) * h$, where d is the DBH and h is the height. Basal area is calculated as the area of a circle with diameter equivalent to DBH. Tree morphology and nutrient manipulation will not effectively be assessed until the end of the 2000-growing season at the earliest.

Swiss Needle cast infection

An initial SNC assessment was made in mid-July 1999 on a branch at breast height. This same branch will be used for continuing assessments of SNC infection on a yearly basis during

Table 1. Simplot Controlled-Release Fertilizer Formulations

					17-17-17					
	N	P	K	S	Mg	Zn	Fe	Cu	Mn	B
%	17.41	17.18	16.79	0	.06	.06	.06	.02	.06	.01
					9-17-17					
	N	P	K	S	Mg	Zn	Fe	Cu	Mn	B
%	9.49	17.18	16.79	0	.06	.06	.06	.02	.06	.01

the spring. The assessment consists of estimating the percentage of needle retention for each year of needles on the main stem of the branch and also on a lateral marked for future assessments (Figure 1).

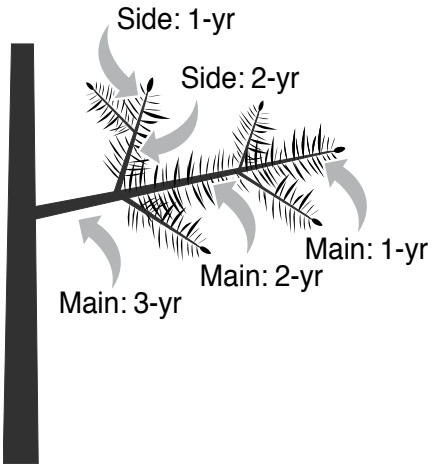

Figure 1. Needle retention assessment diagram

Nutrient analysis

Initial foliar (Table 2) and soil nutrient (Tables 3 and 4) analyses were performed on samples from each site in late October 1998. One and two-year-old foliage was collected from mid-canopy on 10 trees at each site and a composite sample from each site was sent for analyses.

During November of 2000, a sample of current year's foliage will be collected from 3-5 trees from the top one-third of the crown from each treatment replication for foliar nutrient analyses. Foliar samples will be dried for 48 hours at 68° C. Needles will be removed from the stems and the weight of 100 needles will be determined. Samples then will be pooled by block and treatment and assessed for nutrient concentration using standard laboratory

Table 2. Initial foliar nutrient levels, October 1998.

Site	Needle Wt/200	N %	P %	K %	Ca %	Mg %	B ppm	Fe ppm	Mn ppm	Cu ppm	Zn ppm	S %
Charlie Olson	1.40	1.85	0.21	0.92	0.32	0.09	18	94	518	4	20	0.12
Bushy Peterson	1.25	1.82	0.20	1.02	0.33	0.08	19	61	371	4	18	0.13
South Drake	1.22	1.81	0.16	0.79	0.21	0.10	20	50	25	3	15	0.16

Table 3. Initial soil exchangeable nutrient levels, October 1998.

	pH	P ppm	K ppm	Ca meq/100g	Mg meq/100g	Na meq/100g	N %	NH_4-N ppm	NO_3-N ppm
Light Infection—Charlie Olson									
A-Horizon	4.80	28.48	448.5	5.0	2.30	0.08	0.38	8.10	1.24
B-Horizon	5.40	2.81	390.0	3.2	2.10	0.06	0.09	5.59	0.89
Moderate Infection—Bushy Peterson									
A-Horizon	5.00	4.73	241.8	2.2	1.40	0.10	0.19	4.96	0.98
B-Horizon	5.10	2.59	265.2	1.2	0.91	0.08	0.12	3.95	0.67
Heavy Infection—South Drake									
A-Horizon	4.80	1.65	429.0	0.8	1.20	0.20	0.40	10.00	3.31
B-Horizon	4.60	1.46	237.9	0.8	1.20	0.19	0.35	7.51	2.87

	Fe ppm	Mn ppm	Cu ppm	Zn ppm	SO_4-S ppm	CEC meq/100g	Incub. N ppm	C %	S %	B ppm
Light Infection—Charlie Olson										
A-Horizon	58.0	32.80	0.18	0.70	9.00	48.04	59.58	10.69	<0.01	0.51
B-Horizon	1.4	3.88	0.02	0.02	51.50	25.20	5.96	1.25	<0.01	0.17
Moderate Infection—Bushy Peterson										
A-Horizon	12.4	10.66	0.10	0.16	9.60	26.07	20.85	2.96	<0.01	0.26
B-Horizon	2.0	4.28	0.06	0.06	43.40	25.40	5.35	1.60	<0.01	0.20
Heavy Infection—South Drake										
A-Horizon	5.8	10.28	0.04	0.24	15.50	53.32	32.16	6.55	<0.01	0.47
B-Horizon	7.4	9.62	0.04	0.34	18.10	52.82	16.37	5.97	<0.01	0.39

Table 4. Initial soil total nutrient levels, October 1998.

	P ppm	K ppm	Ca ppm	Mg ppm	Mn ppm	Fe ppm	Cu ppm	Zn ppm	Na ppm
Light Infection—Charlie Olson									
A-Horizon	1477.4	1754.5	2032.1	3311.8	1569.4	35754	23.8	87.0	89.6
B-Horizon	851.4	1906.2	1035.6	4054.4	1168.2	41285	27.8	97.1	87.2
Moderate Infection—Bushy Peterson									
A-Horizon	542.6	1410.6	739.1	4609.4	667.9	31305	19.2	84.5	99.0
B-Horizon	485.7	1245.3	475.6	4371.6	553.0	37954	23.1	85.9	88.2
Heavy Infection—South Drake									
A-Horizon	729.1	1468.6	452.9	2736.4	463.8	42345	26.3	85.3	109.6
B-Horizon	757.0	1262.1	415.5	2759.2	506.1	48199	26.5	79.1	102.3

procedures. Nutrient content will be calculated by taking the product of nutrient concentration and average dry weight of 100 needles. Relative nutrient concentration, content, and dry weight will be calculated (relative to the control treatments) and vector diagrams will be constructed (Haase and Rose 1995) to facilitate a thorough examination of nutrient responses to the fertilizer treatments.

Results and Discussion

Vegetation control

A vegetation survey was conducted in July 2000 in order to ensure that the vegetation control treatments had good efficacy over all three sites. Vegetation cover over all three sites was reduced by approximately 85% in the vegetation control plots (Figure 2a & 2b). In addition, vegetation height was reduced by approximately 75% (Figure 3a & 3b). Virtually all vegetation that remains consists of herbaceous species. Shrubs and woody competitors were successfully eliminated from all vegetation control plots. Herbicide treatments will continue for the duration of the study.

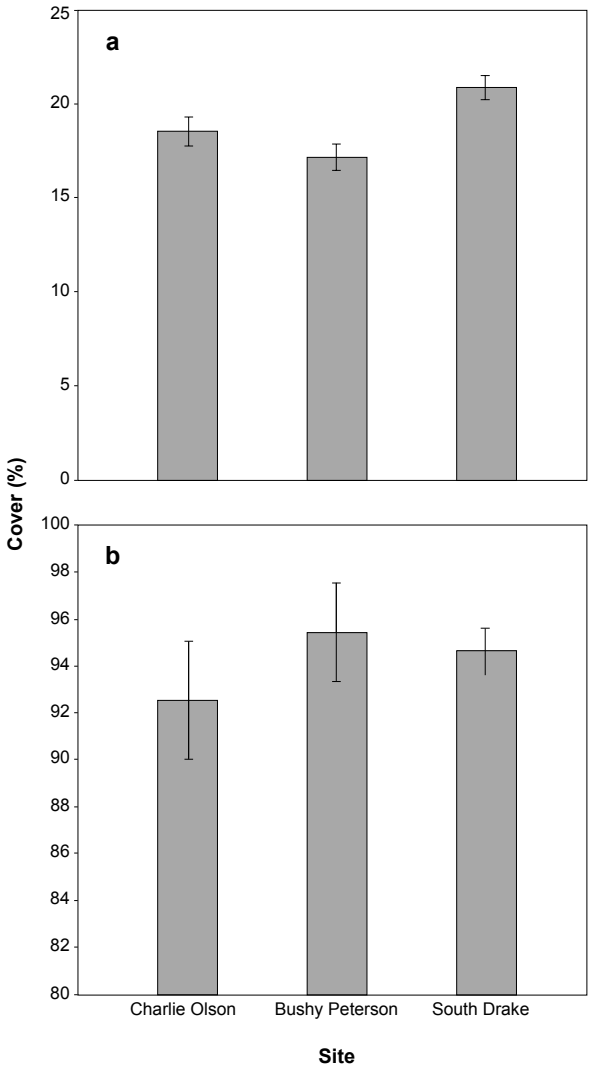

Figure 2. Site differences in cover – (a) vegetation control and (b) no vegetation control. Each bar represents the mean of 60 samples; error bars are the standard error of the mean.

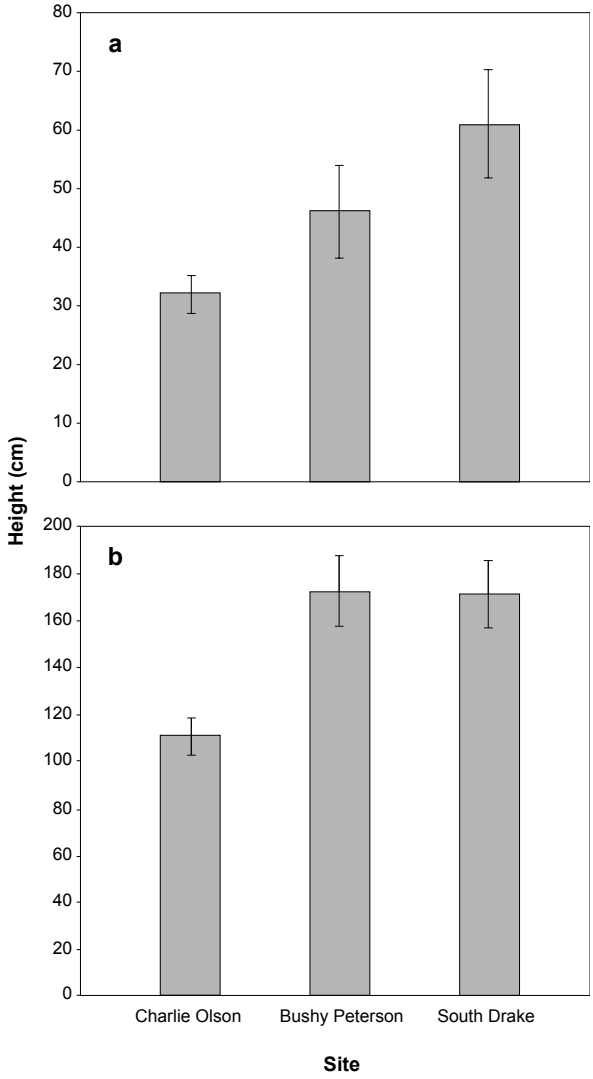

Figure 3. Site differences in height – (a)vegetation control and (b) no vegetation control. Each bar represents the mean of 60 samples; error bars are the standard error of the mean.

Morphology

The most heavily infected site (South Drake) had 12% less height growth than the moderately infected site (Bushy Peterson). The least infected site (Charlie Olson) had 6% less height growth than Bushy Peterson (Figure 4). DBH growth decreased on an eastward gradient (Figure 5). Bushy Peterson had 7.5% less DBH growth than South Drake, while Charlie Olson had 20% less DBH growth than South Drake.

Xylem water potential

On August 8-10, 2000, diurnal xylem water potentials were measured on each site. Figure 6 exhibits the patterns of diurnal xylem water potential for the Charlie Olson, Bushy Peterson, and South Drake sites, respectively. No trends between different treatments and xylem water content were found. It was observed that the most heavily infected site, South Drake, had the greatest amount

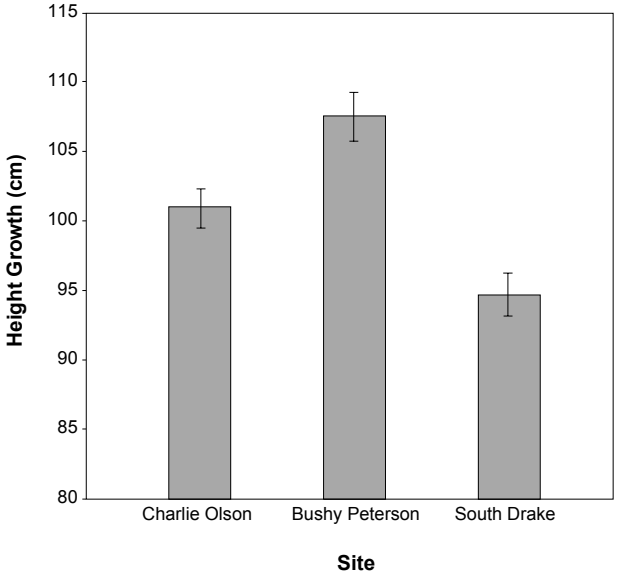

Figure 4. 1999 height growth by site (cm). Each bar represents the height growth mean of all measured trees at each site; error bars are the standard error of the mean.

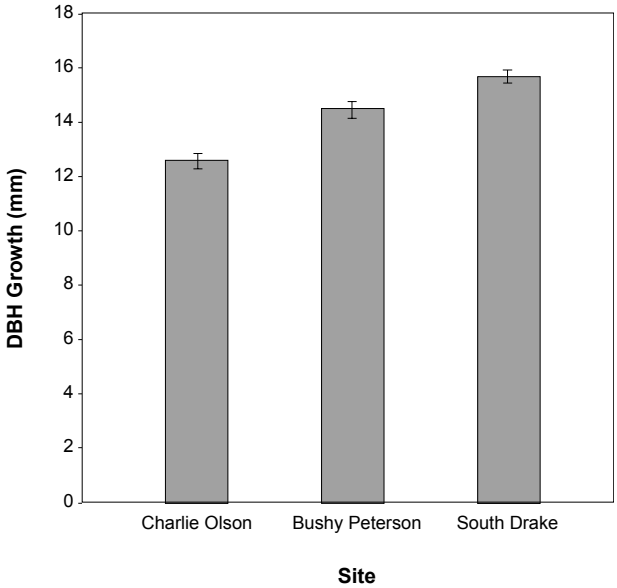

Figure 5. 1999 DBH growth by site (mm). Each bar represents the DBH growth mean of all measured trees at each site; error bars are the standard error of the mean.

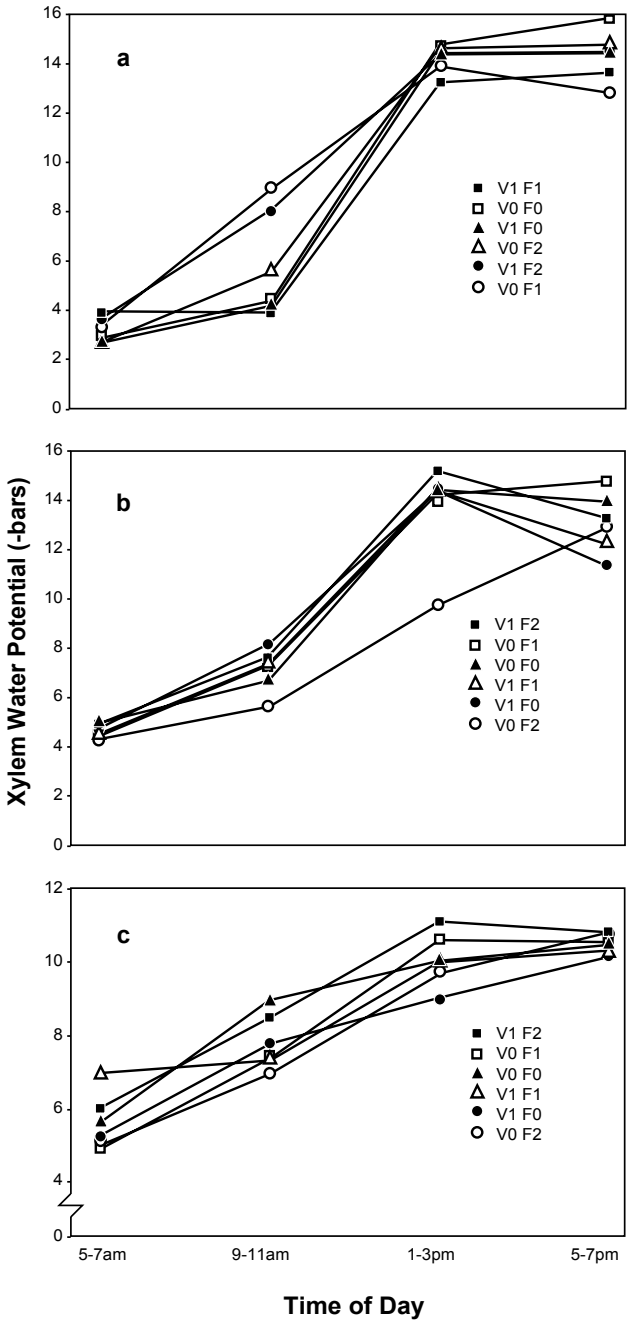

Figure 6. Diurnal xylem water potential, (a) Charlie Olson, sampled on August 8, 2000, (b) Bushy Peterson, sampled on August 10, 2000, and (c) South Drake, sampled on August 9, 2000. Each symbol represents the mean of eight trees.

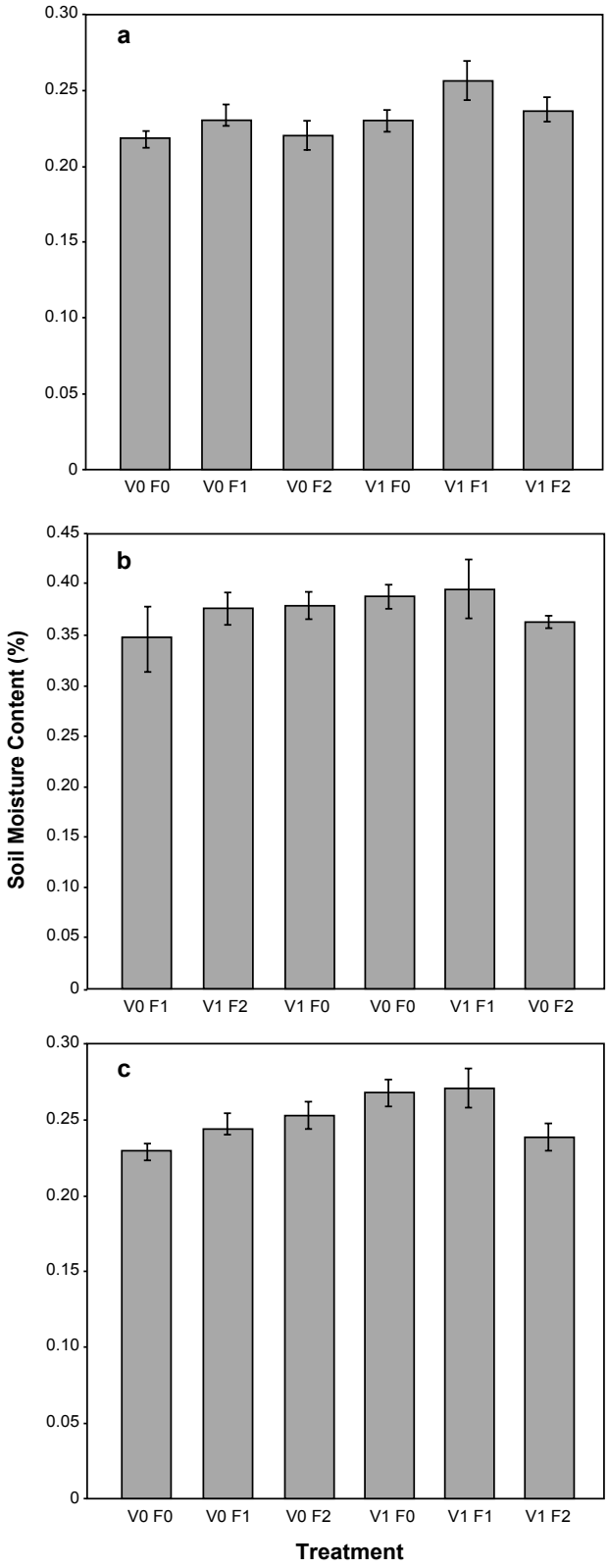

Figure 7. Soil moisture content (%), (a) Charlie Olson, sampled on August 8, 2000; (b) Bushy Peterson, sampled on August 10, 2000; and (c) South Drake, sampled on August 9, 2000. Each bar represents the mean of eight soil samples; error bars are the standard error of the mean.

of moisture stress during pre-dawn measurements. However, this same site showed the least amount of moisture stress during the highest stress period (1-3 pm). Increased soil moisture may in part be responsible for the lower overall xylem water potentials at this site.

Soil moisture content

Soil samples from all sites were taken August 8-10, 2000 in order to determine the soil moisture content (Figure 7). Total soil moisture content (%) decreased on an east to west gradient (38%, 25%, and 23%, respectively). There were no trends found between individual treatments and soil moisture (%).

Objective 2: Evaluate the usefulness of chlorophyll fluorescence as a tool to detect SNC infection.

Rationale

One problem with SNC research is the lack of a consistent, objective, quantification of infection. Chlorophyll fluorescence may be an ideal test for this purpose. Under optimum conditions of photosynthesis, the dissipation of absorbed light energy via chlorophyll fluorescence is minimal. However, when plant conditions change, chlorophyll fluorescence emissions can also change. Hence, any factor that affects the photosynthetic rate (as would be expected from SNC infection) will produce corresponding changes in fluorescence emission. Thus, fluorescence can provide information about the overall photosynthetic activity of the plant and its responses to disturbances. The following null hypothesis is being tested:

Chlorophyll fluorescence is not significantly correlated with SNC infection level.

Methods

Fluorescence tests dealing with yield (a measure of photosynthetic efficiency (PSII)) and dark - adapted non-photosynthetic quenching (occurs during photoinhibition or photodamage) are being explored. All tests are done

on trees that have been tested for the amount of SNC infection using the PCR procedure.

Results

Initial data from both tests are not significantly correlated with SNC infection level. However, trends in maximum and steady state fluorescence have been observed that warrant further investigation.

Upcoming Year

Fall 2000

- Second year growth measurements
- 3rd fertilizer application
- Herbicide application to vegetation control plots

Winter 2001

- PCR analysis on current year foliage
- Foliar nutrient analyses

Spring 2001

- Needle retention assessment
- Herbicide application to vegetation control plots
- 4th fertilizer application

Fall 2001

- 3rd year growth measurements complete
- PCR analysis on current year foliage

Effect of Elemental Sulfur on Swiss Needle Cast Infection and Growth of Coastal Douglas-fir Saplings

Gabriel Crane, Robin Rose, Scott Ketchum, and Diane Haase

Introduction

In 1997 a few individual members of the Swiss Needle Cast Cooperative ran an unreplicated study to examine if elemental sulfur would have an impact on SNC infection as well as improve tree growth. The observational study indicated that Douglas-fir saplings had an 18% increase in growth when treated with an unknown amount of sulfur suspended in water and sprayed on the trees.

These results were encouraging enough to warrant further investigation to better understand the potential for sulfur applications to lessen SNC impacts on young Douglas-fir saplings.

Research Highlights

- SNC infection levels are significantly lowered using foliage applications of Bravo fungicide (3.75 pts/100 gallons)
- SNC infection levels are significantly lowered using foliage applications of sulfur (Thiolux) diluted with water (25 lbs per 100 gallons) with TacTic sticker (8 oz per 100 gallons).
- Overall, treatment applications have had little or no effect on foliar nutrients. However, the following trends have been recognized:
    - Treatment applications of both sulfur with sticker and sulfur with no sticker led to significantly increased levels of foliage sulfur.
    - Trees that received the Bravo fungicide treatment had significantly greater foliage nitrogen levels than the control treatment

Objective

The objective of this study is to determine if applications of elemental sulfur (Thiolux) influence SNC infection level and subsequent growth of coastal Douglas-fir saplings. The following null hypotheses are tested:

1) There is no significant difference in SNC infection level between Thiolux ground, Thiolux foliage, Thiolux foliage w/sticker, or Bravo fungicide applications, and control treatments.

2) There is no significant difference on sapling diameter and height growth between Thiolux ground, Thiolux foliage, Thiolux foliage w/sticker, or Bravo fungicide applications, and control treatments.

3) There is no significant difference in foliage nutrient concentration in saplings between Thiolux ground, Thiolux foliage, Thiolux foliage w/sticker, or Bravo fungicide applications, and control treatments.

Materials and Methods

Site

The sulfur study site is installed near Toledo, Oregon in an existing nine-year-old Douglas-fir plantation.

Experimental design

This experiment utilizes a complete randomized design with 10 replications (trees) for each of the five treatments for a total of 50 trees in the study. One of five treatments was assigned randomly to each of these 50 trees. Selected trees were at least 6 m apart in order to avoid treatment drift from nearby applications. Trees with forked stems or which originated from natural regeneration were not used for measurement trees. Each tree is clearly identified with an aluminum tag, marking paint, and flagging.

Treatments

There are five treatments in the study:

1) Untreated control
2) Bravo fungicide @ 3.75 pts/100 gallons sprayed on the foliage
3) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) sprayed on the foliage
4) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) with TacTic sticker (8 oz per 100 gallons) sprayed on the foliage
5) Sulfur (Thiolux) diluted with water (25 lb per 100 gallons) sprayed on the ground under each tree within the drip line

Treatments were applied on June 8, June 25, and July 10, 1999 using a truck tank sprayer at 38 psi. Each tree was sprayed for 14 seconds resulting in an application rate of 2 oz Thiolux per tree.

Morphology

Initial diameter at breast height (DBH) and height were measured at the beginning (May) and end (October) of the 1999 growing season. Seasonal growth is calculated by subtracting height values and DBH values. Stem volume is calculated using the formula for a cone: $1/3(\pi/4\ d^2) * h$, where d is the DBH and h is the height. Basal area will be calculated as the area of a circle with diameter equivalent to DBH.

Swiss Needle cast infection

An initial SNC assessment was made in mid-July 1999 on a branch at breast height. This same branch is used for continuing assessments of SNC infection. An assessment will be made on a yearly basis during the spring from the same branch where the previous assessment was taken. The assessment consists of ocularly estimating the percentage of needle retention for each year of needles on the main stem of the branch and also on a lateral marked for future assessments (Figure 1).

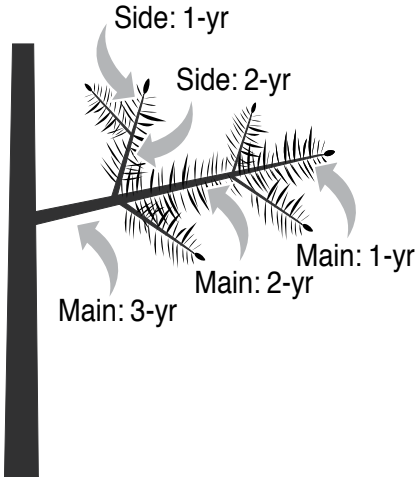

Figure 1. Needle retention assessment diagram

In April 2000, current-year foliage was sampled on all 50 trees. Using the Polymerase Chain Reaction (PCR) procedure, the relative amount of *Phaeocryptopus gaumanii* fungus within needles was identified. PCR results in a ratio of picograms of *Phaeocryptopus* DNA to nanograms of Douglas-fir DNA. PCR is being used because it has the advantage of speed, technical simplicity, low detection limits, and specificity over other procedures (SNCC Annual Report 1999).

Nutrient analysis

Foliar and soil nutrient analyses were performed on samples from this site in December 1999. Current-year foliage was collected from mid-canopy on four random trees from each treatment and analyzed. Soil was collected from the A-horizon around the base of the same four trees

at three different locations within the drip line.

During November of 2000, a sample of current year's foliage will be collected from four trees from each treatment. Foliar samples will be dried for 48 hours at 68 C. Needles will be removed from the stems and the weight of 100 needles will be determined. Nutrient content will be calculated by taking the product of nutrient concentration and average dry weight of 100 needles. Relative nutrient concentration, content, and dry weight will be calculated (relative to the control treatments) and vector diagrams will be constructed (Haase and Rose 1995) to facilitate a thorough examination of nutrient responses to the sulfur treatments.

Results and Discussion

Morphology

There were no significant differences between treatments in DBH (Figure 2) or height growth (Figure 3). These results were expected, as the respective treatments have not yet had time to integrate treatment effects into significant growth impacts.

Swiss needle cast infection

During May of 2000, needle retention was assessed on all 50 trees (Figure 4). This data will be used to analyze possible correlations between treatments and needle retention once the next year's assessment is completed in May 2001. It is anticipated that needle retention will be greatest in those treatments that have the least amount of infection.

The PCR procedure showed significant differences in infection levels between treatments. Initially a log transformation was used to normalize data due to unequal variances among samples. The resulting data from the transformation was back-transformed to get a relative scale (Figure 5). The Bravo treatment had a significantly decreased infection level when compared to all other treatments. The sulfur w/sticker was also significantly effective at lowering SNC infection levels when compared with the control treatment, but less effective than the Bravo treatment. All other treatments were not significant in lowering SNC infection levels when compared to the control treatment.

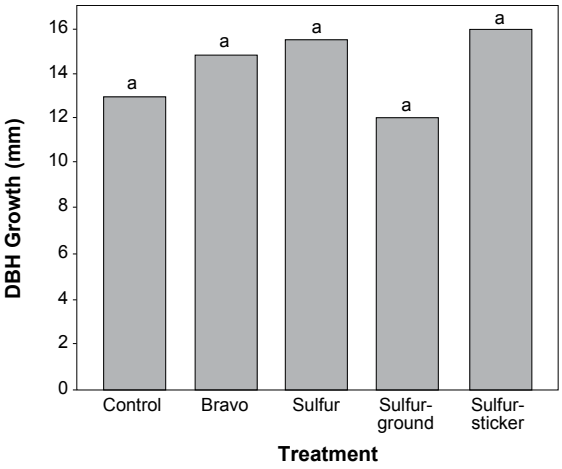

Figure 2. 1999 DBH growth by treatment (mm). Bars associated with the same letter (a) are not significantly different at the p≤ 0.05 level using Waller-Duncan's mean comparison.

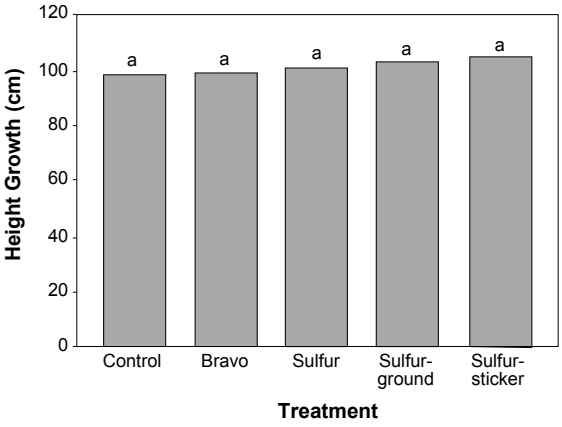

Figure 3. 1999 height growth by treatment (cm). Bars associated with the same letter (a) are not significantly different at the p≤ 0.05 level using Waller-Duncan's mean comparison.

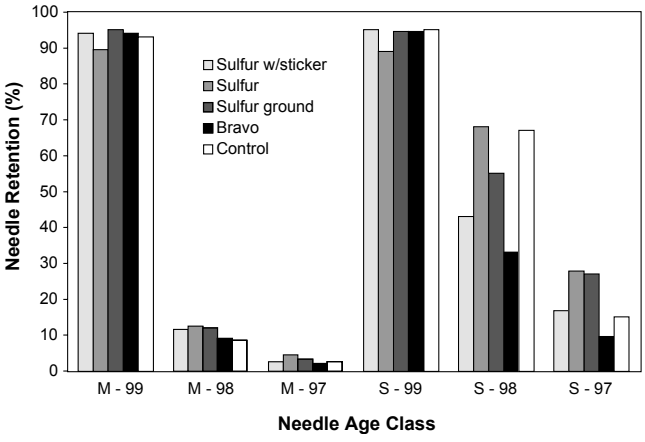

Figure 4. Needle retention assessment by treatment, May 2000.

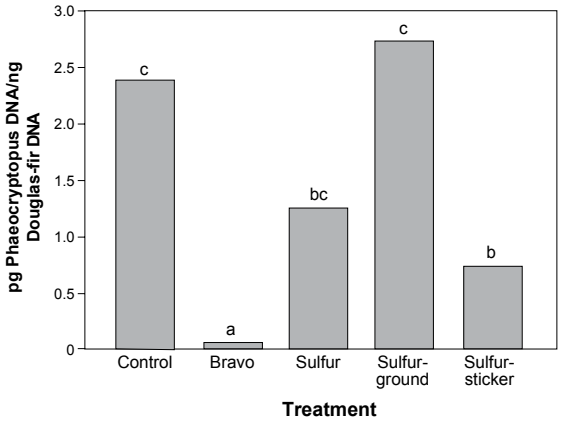

Figure 5. Polymerase Chain Reaction (PCR) results (pg Phaeocryptopus DNA / ng Douglas-fir DNA), 1999 needles, sampled April 25, 2000. Bars associated with the same letter (a, b, or c) are not significantly different at the p≤ 0.05 level using Waller – Duncan's mean comparison.

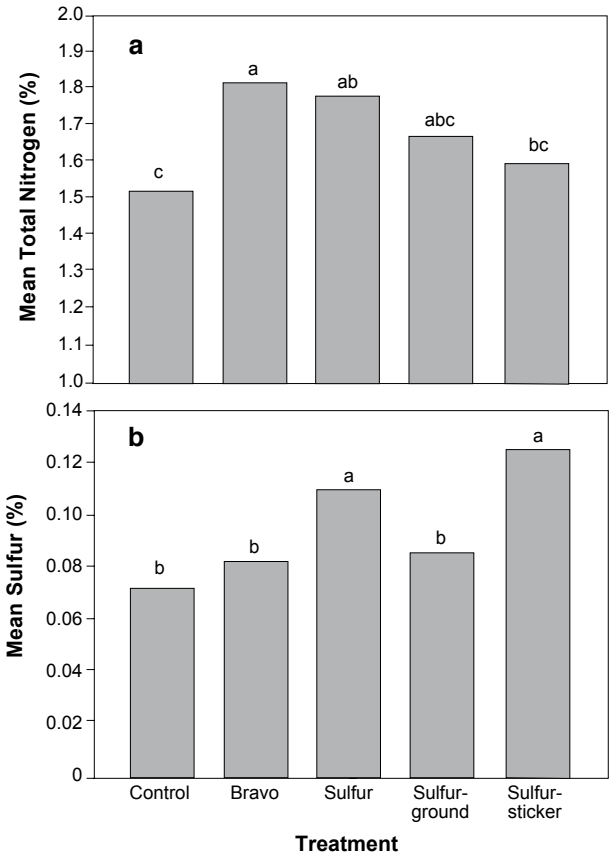

Figure 6. Foliage (a) nitrogen levels (%)and (b) sulfur levels (%), 1999 needles, sampled December 1999. Bars associated with the same letter (a, b, or c) are not significantly different at the p≤ 0.05 level using Fischer's LSD.

Nutrient analysis

Trees that received the Bravo fungicide treatment had significantly greater foliar nitrogen levels than the control treatment (Figure 6a). The sulfur foliage application also had moderately significant increases in foliar nitrogen when compared to the control treatment. This data suggests that the foliage with the least amount of infection tend to have significantly higher amounts of nitrogen compared to trees with increased infection levels.

Foliar treatment applications of both sulfur with sticker and without sticker led to significantly increased levels of foliage sulfur (Figure 6b). Future exploration is needed to determine how much sulfur needles absorb and how much residual sulfur is left on the needle surface as a residue. All other treatments showed no significant increases. However, more time may be needed in order to allow sulfur from the ground application to be translocated to the foliage. Further investigation is needed to determine if the sulfur is acting as a fungicide on the surface of the needle or if there are positive physiological effects resulting from sulfur absorption.

Upcoming Year

Fall 2000

- Second year growth measurements complete

Winter 2001

- PCR analysis on current year foliage
- Foliar and soil nutrient analyses

Spring 2001

- Needle retention assessment

Fall 2001

- Third year growth measurements complete

Reference

Annual Report. 1999. Swiss Needle Cast Cooperative. FRL Publishing. Corvallis, Oregon. 76p.

Nutritional Imbalance as a Predisposing Factor in Swiss Needle Cast Disease

Part I. The Role of Foliar Free Amino Acids in Douglas-fir Susceptibility to an Endemic Fungus

Cathy Rose, Kathleen Kavanagh, Richard Waring

Cooperators: Dan Manter, Dave Roche, Gabe Crane, Scott Ketchum

Introduction

The nutritional mechanism

Swiss needle cast (SNC) continues to intensify in Douglas-fir forests, though a mechanism is lacking to explain increased tree vulnerability to this normally endemic fungus. Clues to the problem may be found in the altered age-class structure and nutritional status of managed forests compared to historical conditions. Climate and soil conditions in coastal Oregon and Washington are conducive to nutritional imbalances in Douglas-fir forests, including moderate to high supplies of N and low supplies of S and base cations. Forest management practices (i.e. harvest, burn, weed control, fertilization) may exacerbate the nutritional imbalance to trees by increasing soil N availability and concurrently decreasing the availability of other more readily leached nutrients. When N supply is high and/or S supply is low to conifer needles, N accumulates as free amino acids (FAA), an excellent nutrient source for many fungal endophytes. High foliar concentrations of FAA may cause infection rates of *P. gaeumannii* to intensify, thereby accelerating needle loss and inducing even higher N concentrations in emerging needles. Over time, reduced carbon uptake and root growth may compromise the ability of infected trees to re-balance the foliar N:S:C ratio.

Our nutritional mechanism postulates that development of *P. gaeumannii* is favored by high levels of foliar FAA in young Douglas-fir forests (1-150 y) with high N availability. Low S availability may accelerate progression of the disease. Furthermore, once established in the foliage, the fungus may exacerbate imbalanced nutrition in the trees (via reduced carbon uptake) and improve nutrition to the fungus.

Physiological responses of trees and fungi to nutritional imbalances are well documented (Kelly and Lambert 1972, Turner and Lambert 1979). First, high N and low S availability cause foliar FAA to accumulate in

conifer needles and diffuse onto the leaf surface and into intracellular spaces, providing a favorable medium for fungal nutrition and growth (Robinson and Hodges 1981, Nasholm and Ericsson 1990, Ericsson et al. 1993, Marschner 1995). Although the nutritional physiology of *P. gaeumannii* is poorly understood, a variety of closely related ascomycetes achieve rapid growth via direct assimilation of foliar FAA (Thorne 1950, Barton-Wright 1952, Pelletier and Keitt, 1954, Cochrane 1963, Hancock and Huisman 1981). In addition, the concentration of amino acids in foliage of *Pinus radiata* has been shown to be a strong indicator of a tree's susceptibility to the needle cast fungus *Dothistroma* (Turner and Lambert 1986). These studies provide a foundation for our hypothesis that nutritional imbalances in intensively managed Douglas-fir forests have resulted in chronic high leaf levels of foliar FAA (from high soil N and/or low S), favorable to the nutrition of *P. gaeumannii*.

Recent observations indicate that sulfur applied to Douglas-fir foliage reduces SNC disease symptoms and improves tree growth (unpublished data, Maguire). These findings are consistent with the nutritional imbalance hypothesis. A variety of studies indicate that many soils in western Oregon and Washington are deficient in S (Gessel 1969, Gessel et al. 1965, 1973, Heilman et al. 1983). Sulfur is required for the synthesis of proteins from FAA, thus sulfur deficiency causes foliar FAA to accumulate in conifer needles (Kelly and Lambert 1972 Turner and Lambert 1978). As with excess N, the net effect of S deficiency is improved foliar nutrition for *P. gaeumannii*. It is also possible that S has a fungicidal effect. The lack of widespread symptoms of S deficiency in Douglas-fir forests of Oregon and Washington supports a fungicidal rather than a nutritional effect of S on tree nutrition.

Hypothesis

Elevated concentrations of foliar FAA (an indicator of nutritional imbalance) are associated with Douglas-fir susceptibility to SNC disease in Oregon and Washington.

Objectives

1. Determine if infected trees have abnormally high concentrations of total foliar N and foliar FAA.
2. Document whether FAA concentrations in fully-expanded needles remain elevated as needles age, or decline with needle age, as expected in healthy stands.

Methods

Sample collection

Four 12-20 year-old Douglas-fir plantations with varying treatments and/or levels of Swiss needle cast infection were chosen for study. At each site, three trees per treatment or disease level were selected to monitor FAA. Thirty to fifty needles were collected from each needle age class on 3 well-illuminated branches per tree. All age classes present were sampled. The samples were placed in a cooler and shipped to the University of Idaho for foliar FAA analysis. The sites and treatments sampled are outlined in Table 1. Foliar samples were collected at 2 dates prior to budbreak, soon after bud break, and at least one sample following the completion of needle expansion.

FAA analysis

Frozen plant tissue was weighed into a test tube, and homogenized with a Polytron in 80% ethanol. Insoluble polyvinlypyrrolidone (PVPP) was added to the extract to reduce interference problems with phenolics (Sanderson and Perera 1966). The ethanol solution was then evaporated to precipitate proteins, pigments and lipids, and produce an aqueous solution of amino acids (Stuart 1935, Loomis and Shull 1937, Draper 1976, Harborne 1984). Quantitative determination of FAA was made colorimetrically with ninhydrin, (1,2,3 triketohydrindene hydrate), a commercially available reagent that reacts with intact amine groups to produce a red-purple color product with maximum absorption at 570 nm (Moore and Stein 1954).

Progress to date

- Four sets of samples were collected at 4 sites in Oregon (at least 2 sets each pre- and post-budbreak)
- Laboratory analysis is complete on approximately 500 samples; analysis of final set of samples is still underway. This report contains 80% of the data. Still missing are Coal Cr. and Beaver Cr. late April and Coal Cr, Beaver Cr and South Drake late June.
- Analysis is underway for total N, S, and Ca on a subset of samples.

Table 1. Description of foliar treatments and sampling protocol for each site used in documenting foliar amino acid concentrations in coastal Douglas-fir saplings infected with Phaeocryptopus gaeumannii.

Site	Location	Foliar samples taken from:
Juno Hill	Juno Hill, near Tillamook, OR	1) 3 unsprayed control branches from 3 trees. 2) 3 branches from the same 3 trees that had surface applications of Bravo. Applications by Alan Kanaskie. Not sure of the rate. All branches were sprayed at bud break in 1995-1998.
South Drake	South Drake, near Toledo, OR	1) 3 unsprayed control trees. 2) 3 trees with surface application of sulfur (Thiolux) at 2 ounces per tree. Applications were made on June 8, June 25 and July 10th 1999.
Beaver Cr.	Beaver Cr., Siuslaw NF, OR	1) 3 unsprayed control trees and 2) 3 trees with surface application of chlorothalonil (Bravo 720, rate = 66 ml / 3.78 L, applied until run-off. Applications were made 1998, 1999 and 2000 at budbreak and one month later.
Coal Cr.	Coal Cr., near Nehalem, OR, ODF	1) 3 trees with low SNC disease symptoms (retain 3 years of foliage) and 2) 3 trees with high SNC disease (retain only 1.2 years of foliage). No foliar applications have been made to these trees.

- Data analysis continues. Estimated completion date for analysis and draft manuscript is February 1, 2000.

Results

Are the foliar FAA concentrations higher than expected?

Using the values published by van den Driessche and Webber (1977) for coastal Douglas-fir saplings on Vancouver Island as a baseline, we evaluated the foliar FAA concentrations found in coastal Douglas-fir saplings with high to moderate levels of SNC disease.

Fully expanded foliage:

Current year foliage

With the exception of Coal Cr, all current year needles contained higher concentrations of foliar FAA compared to Vancouver Island (Table 2, Figure 2). Depending on the Coast Range site evaluated, foliar FAA concentrations were approx 30-100% higher than expected (Figures 1 and 2). However the foliar FAA concentrations in current year foliage were below levels found in fertilized stands on Vancouver Island (Figure 2).

One year old foliage

On all sites, foliar FAA concentrations were elevated compared to both unfertilized and fertilized Vancouver Island stands (Table 2). Depending on the Coast Range site evaluated, foliar FAA concentrations were approximately 75-125% higher than expected (Figures 1 and 2).

New expanding foliage:

Current year foliage

With the exception of South Drake, all expanding needles had high concentrations of foliar FAA compared to the Vancouver Island saplings (Table 1). Depending on the Coast Range site evaluated, foliar FAA concentrations were approximately 50-100% higher than expected. None of the Coast Range sites have FAA values comparable to fertilized stands.

Do foliar FAA concentrations decline with needle age?

The seasonal patterns of foliar FAA concentrations were consistent across all sites. The highest concentrations were found in new expanding needles (May) with significantly lower levels once the needles had fully matured (Figure 1). With the exception of a few samples, the concentration of foliar FAA did not drop with needle age (Figure 1).

Do foliar FAA concentrations vary with treatment?

Foliar free amino concentrations varied with treatment at Beaver Cr. In all cases the control (non-sprayed heavily infected saplings) had reduced levels of FAA. The fungicide sprayed saplings had foliar FAA levels 20-75% higher compared to the controls (Figure 1C). A similar trend was seen at the South Drake site (sulfur spray) with the control (non-sprayed) saplings having 10-20% lower foliar FAA concentrations compared to sulfur treated seedlings (Figure 1B). At the Juno Hill site, which also had sprayed and unsprayed samples, only a few samples had elevated foliar FAA in

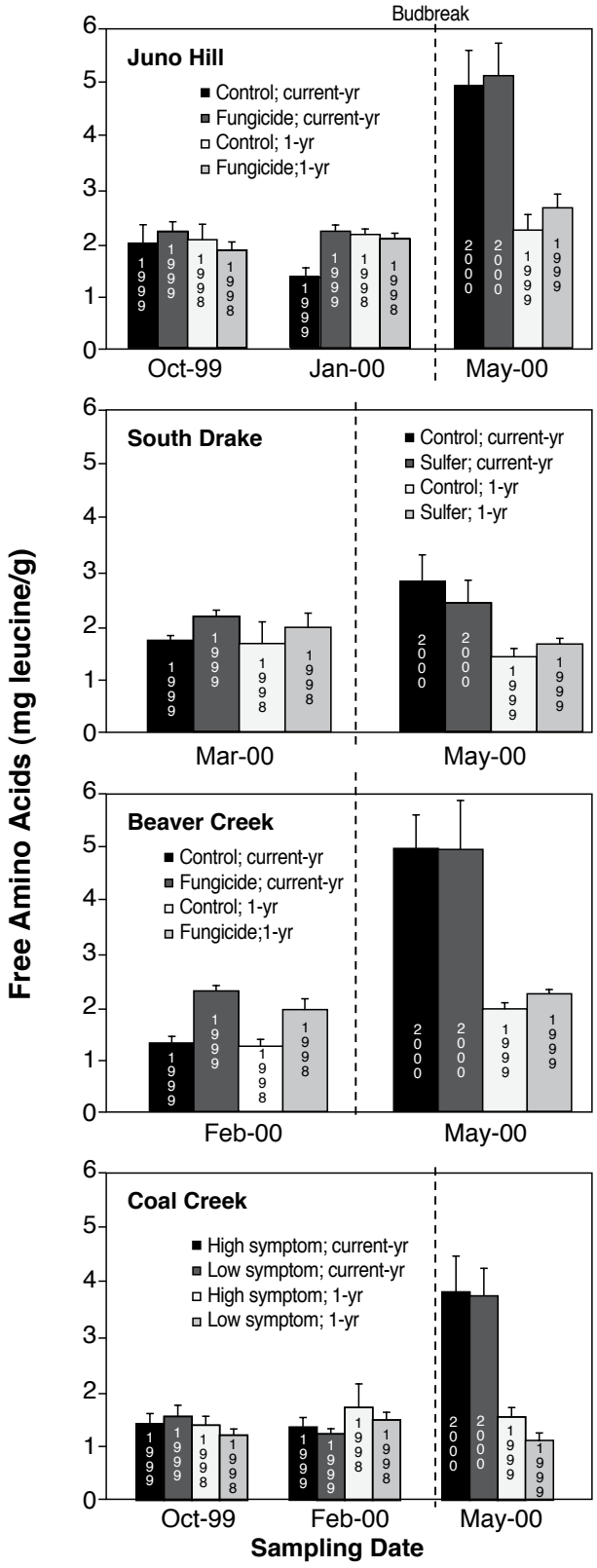

Figure 1. Seasonal values of foliar free amino acid concentrations in coastal Douglas-fir infected with Phaeocryptopus gaeumannii *by site. Dates superimposed on bars indicate year the needle flushed. For a description of the sites and treatments see Table1. Bars represent standard errors. N=3.*

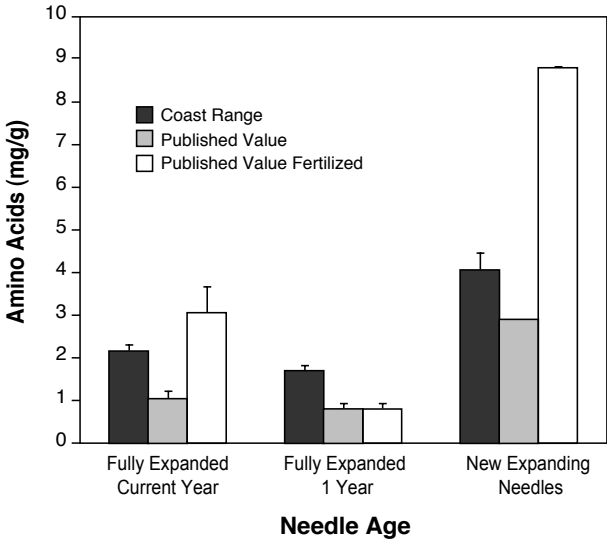

Figure 2. Mean values of foliar free amino acid concentrations in coastal Douglas-fir infected with Phaeocryptopus gaeumannii *across all Coast Range sites compared to mean values published for similar needle ages in unfertilized and fertilized sapling Douglas-fir on Vancouver Island (van den Driessche and Weber 1977). Bars represent standard errors. n is variable between 3-9. A value without standard error bars are not means across needle ages and indicate only one concentration was reported for that age class.*

the sprayed compared to control samples (Figure 1A). At Coal Cr, where trees were not treated with a fungicide, there are no differences in FAA concentrations relative to disease symptoms (Figure 1D).

Conclusions

- Relative to reported values, foliar FAA concentrations in Douglas-fir foliage from Coast Range forests are high overall (most sites, and needle age classes). In some cases these values are higher than published values for fertilized saplings. In particular, current-year needles have high peak concentrations of foliar FAA in the post-budbreak period compared to published values for needles of non-fertilized Douglas-fir. Foliar FAA levels do not decline with needle age.

- High overall concentrations of foliar FAA indicate that Douglas-fir in the Coast Range areas we sampled have high N availability. As a result, these forests may provide superior nutrition to *Phaeocryptopus gaeumannii*. Our finding that foliar FAA levels decline in trees with high levels of infection compared to adjacent trees with low levels of infection is consistent with the hypothesis that this fungus is metabolizing FAA.

Table 2. Foliar amino acid concentrations in coastal Douglas-fir saplings infected with Phaeocryptopus gaeumannii compared to values for unfertilized and fertilized Douglas-fir saplings on Vancouver Island (van den Driessche and Weber 1977). ** indicates statement is true.

	Fully expanded foliage						New expanding foliage		
	Current year foliage			1 year old foliage			Current year foliage		
Site	Similar to published values for unfertilized DF trees	Higher than published values for unfertilized DF trees	Similar to values published for fertilized DF trees	Similar to published values for unfertilized DF trees	Higher than published values for unfertilized DF trees	Similar or higher to values for fertilized DF trees	Similar to published values for unfertilized DF trees	Higher than published values for unfertilized DF trees	Similar or higher to values for fertilized DF trees
Juno Hill		**			**	**		**	
Beaver Cr.		**			**	**		**	
South Drake		**			**	**	**		
Coal Cr.	**				**	**		**	

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Growth Impact Study

Growth Trends in the First 2-yr Growth Period Following Establishment of Phase III Permanent Plots

Doug Maguire, Alan Kanaskie, Bill Voelker, Greg Johnson, and Randy Johnson

Preliminary analyses from Phases I and II of the SNCC Growth Impact Study indicated some loss in height growth prior to 1997 and substantial reductions in basal area growth. These growth losses in any given year increased with increasing severity of SNC symptoms. Because the growth analyses in Phases I and II were retrospective, indices of Swiss needle cast intensity were available only for 1997, and the initial conditions in stand variables were rough reconstructions at best. The uncertainty about historical development of Swiss needle cast on these sites make the correlations between disease intensity and past growth patterns difficult to interpret. This weakness has implications both for identifying causal mechanisms of past growth fluctuations (and disease development) and for making justifiable predictions of future growth losses.

The objectives of Phase III of the growth impact study were: 1) to monitor SNC symptoms and the growth of individual trees on permanent monitoring plots; 2) to provide an improved estimate of growth losses associated with a given level of SNC; 3) to assess the annual fluctuations and longer-term trends in SNC severity; and 4) to monitor SNC development and tree growth through the course of the current SNC intensification.

Methods

In the later winter/early spring of 1998, a network of 77 permanent plots was established across locations previously sampled in Phases I and II. The plots were square, 0.08-ha (1/5-ac) plots, measuring 31.8 x 31.8 m. Each plot was centered on the 5th point of the ODF transect established in Spring 1997 (Phase I plots were centered on the 3rd point). The sampling strategy provided several advantages over selection of entirely new locations: 1) some previous information on the status of Swiss needle cast at each plot center was available; 2) a significant cost saving was gained by not having to locate, map, and document a new set

of plot locations; and 3) the sampled locations represented a standard list sample (random sample with probability proportional to acreage) from a well-defined target population of Douglas-fir plantations (5-30 years of age, within 18 miles of the coast), so inferences can be extended to the population with statistical validity.

On each measurement plot, all trees were tagged at breast height and measured for total height, height to crown base, and dbh. All trees were remeasured in late winter/early spring of 2000. Ten dominant or codominant trees on each plot were scored for SNC at time of plot establishment in 1998, and during annual visits in the spring of both 1999 and 2000.

In the initial analysis of growth for the first 2-yr period, variation in needle retention in 1998 was assumed to represent the variation in SNC intensity. Needle retention was determined as the number of years of needle retention in each third of the crown, and was averaged to arrive at an individual tree value, and averaged again for the 10 sample trees to arrive at a plot value (ret98). A simple growth model was then fitted to the data, with foliage retention as one possible predictor variable:

$$[1]\ \ln[\text{bagdf}] = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + \ldots + b_k \cdot X_j + b_{k+1} \cdot \text{ret98}$$

where bagdf=plot-level basal area growth of Douglas-fir, X_i=plot- level predictor variable, and ret98=average foliage retention in 1998 for the plot.

Table 1. Attributes of plots in the SNCC Growth Impact strdy (n=76).

Plot attribute	Mean	Min	Ma
Douglas-fir			
RET98 (yrs)	2.32	1.07	3.07
basal area growth (m^2/ha/2yrs)	3.2	0.3	6.7
tph	607.1	86.5	1630.2
Dq (cm)	19.6	3.8	34.7
basal area (m^2/ha)	17.6	0.6	38.7
SDI	375.9	25.9	711.1
Western hemlock			
basal area growth (m^2/ha/2yrs)	1.1	0.0	5.6
tph	232.4	12.4	1519.1
Dq (cm)	15.9	6.1	56.4
basal area (m^2/ha)	4.6	0.0	20.6
SDI	102.2	1.3	392.2
Other conifers			
Dq (cm)	12.1	6.1	24.7
basal area (m^2/ha)	1.5	0.0	21.1
SDI	34.0	1.3	426.8
Red alder			
basal area growth (m^2/ha/2yrs)	0.3	0.0	1.6
tph	101.5	12.4	741.0
Dq (cm)	12.6	6.1	35.3
basal area (m^2/ha)	1.4	0.0	10.5
SDI	34.2	1.3	267.5
Other hardwoods			
Dq (cm)	8.5	6.1	12.1
basal area (m^2/ha)	0.7	0.0	5.0
SDI	19.9	1.3	146.4
All species			
basal area growth (m^2/ha/2yrs)	4.0	0.3	7.4
tph	848.1	259.4	2025.4
Dq (cm)	18.6	3.8	33.9
basal area (m^2/ha)	21.4	0.7	45.2
SDI	470.0	27.4	1035.6

Results

Initial SDI for the plots ranged from 27.4 to 1035.6 (Table 1). The database consists of 3,736 Douglas-fir trees distributed across 76 plots (one plot was lost to precommercial thinning during the first growth period). The most common species after Douglas-fir is western hemlock with 715 trees, although it occurs on only 38 plots.

The following model was one of the better 3-variable models examined:

[1] $\ln[bagdf] = -0.761 + 0.343 \cdot RD_{DF} - 0.0443 \cdot BA_{TOT} + 0.589 \cdot ret98$

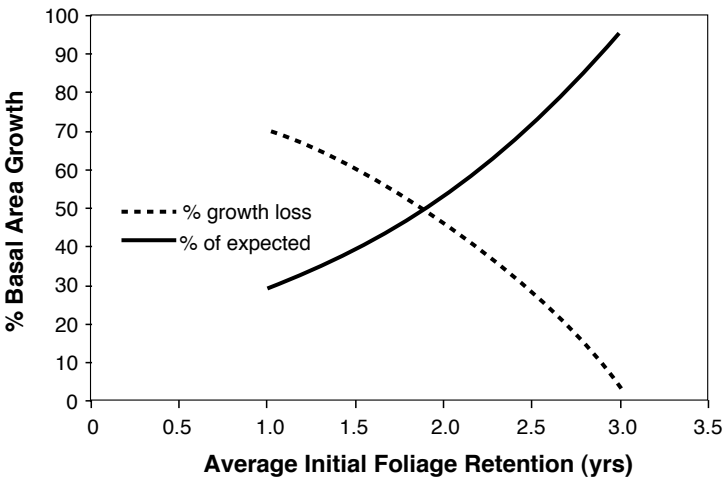
where bagdf = plot-level basal area growth of Douglas-fir

RD_{DF} = initial Douglas-fir relative density (Curtis 1982)

BA_{TOT} = initial total plot basal area (all species)

ret98 = initial average foliage retention (1998)

All variables were significant (all $p<0.001$), and this simple model accounted for approximately 58% of the variation in Douglas-fir basal area growth. As expected, basal area growth of the plots increased as foliage retention increased (Fig. 1)



Analyses of the plot data are ongoing and include: 1) height growth responses; 2) cubic volume growth responses; 3) growth relative to change in SNC rating over the 2-yr growth period; 4) test of the efficacy of live crown length:sapwood area ratio for estimating SNC and associated growth losses; and 5) direct relationship between growth and soil/foliar chemistry (link to project on soil and foliar nutrients; see later in this volume). The dynamic relationship between Douglas-fir growth and the growth of other species on site will also be examined, particularly with reference to Douglas-fir condition.

Trends in Soil and Foliar Nutrients across a Range in Swiss Needle Cast Severity

Doug Maguire, Dick Waring, Kermit Cromack, and Jim Boyle

Introduction

Preliminary data collected by various cooperators over the last several years suggest that tree nutrition may play a role in the intensification of Swiss needle cast (SNC). This intensification has developed over the past decade with an apparent epicenter in north-coastal Oregon. Neither nitrogen fertilization alone nor blended fertilizers seem to have reduced disease symptoms or associated growth losses. However, the ratio of calcium to nitrogen was observed to be low on many sites with severe SNC, prompting experimental application of lime. This treatment appears to have improved the condition of severely impacted trees, and also seems to have allowed a subsequent response to nitrogen fertilization.

The literature on atmospheric deposition ("acid rain") prompts some speculation on possible mechanisms leading to the current SNC epidemic. Acid deposition may accelerate leaching of calcium from soil profiles as Ca++ ions are displaced by H+ ions (Reuss and Johnson 1986, Lawrence et al. 1995, McLaughlin and Percy 1999; but also see Yanai et al. 1999) . And, acid deposition from industrial pollution is typically accompanied by nitrogen deposition (McLaughlin and Percy 1999), causing nitrogen enrichment of sites (Fenn et al. 1998). The net potential effect can be a serious imbalance between nitrogen and calcium, as well as between nitrogen and other nutrients (Ericsson et al. 1995). Research on the effects of excess nitrogen deposition on forested sites indicates that excess nitrogen in plant tissues is stored primarily as arginine, a relatively simple amino acid that contains no sulfur (Ericsson et al. 1995, Kätzel and Löffler 1997, Fenn et al. 1998). Additional lines of evidence suggest that fungi such as *Phaeocryptopus gaeumannii* derive nutrition from free amino acids, particularly arginine, in the intercellular spaces of needles. Hence, a case can be made for a possible mechanism by which relatively abundant available soil nitrogen leads to an excess of free arginine in Douglas-fir needles, which in turns provides a stimulating substrate for *Phaeocryptopus gaeumannii* (see report by Kavanagh and Rose in this volume). The other effect of excess nitrogen in plant tissues is a relative scarcity of calcium and associated weakening of the cell wall and cell membrane, possibly causing the cell to leak amino acids. This weakening of cell walls due to calcium shortage has been hypothesized to lead to red spruce decline in

the northeastern U.S.A. by leaving needles more susceptible to frost damage (DeHayes et al. 1999). In the case of coastal Oregon Douglas-fir forests, previous occupation of sites by red alder may have been a source of abundant nitrogen, and/or may have induced a depletion of calcium through increased leaching.

Although these speculated mechanisms have considerable appeal and although the limited data do suggest a significant role of nutrient dynamics, no comprehensive analysis of soil or foliage nutrient content has previously been conducted in the Oregon Coast Range. A conclusive study of mechanisms by which soil nutrients might influence the decline associated with Swiss needle cast would be a monumental undertaking. As a more modest first effort, a preliminary analysis of soil nutrients across a subset of the 75 existing Growth Impact Study plots and 23 Precommercial Thinning Study control plots was undertaken to gain some insight as to whether there may be a link between SNC severity and current soil and tree nutrient status. Other studies have demonstrated that imbalances in plant nutrients resulting from differential availability in the soil solution can dramatically alter the health and productivity of trees.

The original objectives of the study were: 1) to identify any apparent imbalances of tree nutrients in soil samples collected from 50 SNCC Growth Impact Study plots; and 2) to test the hypothesis that SNC severity is correlated with the degree of one or more of these imbalances. After considerable discussion of possible mechanisms, we decided that foliage sampling would provide some key, and perhaps greater insight into mechanisms driving the SNC outbreak. As a results, the modified objectives **were: 1) to identify any apparent imbalances of nutrients in both soil and foliage samples from 25 SNCC Growth Impact Study plots, half at the severe end of the SNC gradient, and half at the healthy end; and 2) to test the hypothesis that SNC severity is correlated with the degree of one or more nutrient imbalances in the soil and/or foliage.**

Methods

A subset of 25 SNCC permanent plots, either from the Growth Impact Study or PCT Study (control plots), were selected to represent the two disease extremes, specifically, severe SNC and virtually no SNC. The 1997 foliage retention ratings served as the basis of plot selection, with the 1999 ratings confirming that these plots represented the extremes of SNC (Table 1). A composite soil sample from each plot was collected during tree remeasurements in late winter/early spring of 2000. Selection of soil and foliage samples was linked to five randomly-selected SNC-rating trees per plot. Exact location of soil samples was determined by selecting a random azimuth from the tree and a random distance between 0 and 5 m from the tree. The duff and loose organic matter were lightly scraped away from the sample point, and a 2-cm-diameter cylindrical core of mineral soil was extracted from the top 10 cm with a standard soil probe. A second core was removed at the same distance from the tree but on the opposite side. The ten soil cores were placed together in the same plastic bag to produce a composite sample that was placed in a cooler and transported to the lab. Foliage samples were collected from the same five randomly-selected SNC-rating trees. The target foliage sample was the south-most branch in the fifth whorl from the top of the tree. All 1999 shoots arising from lateral buds on the terminal shoot were removed. Shoots from all trees were placed in a

Table 1. SNCC permanent plots form which soil and foliage samples were collected for nutrient analysis. Numbered polts are from the Growth Impact Study, named plots are from the Precommercial Thinning Study. Plots from which only soil was sampled are indicated by "s".

Plot	1999 Foliage retention (yrs)
Cloverdale	0.80
119	0.88
Juno Hill	1.06
83	1.36 s
51	1.45
94	1.67 s
16	1.69
20	1.69 s
Chopping Block	1.73
East Beaver	1.75
101	1.76
39	1.79
Powerline	1.80
85	1.85
Smith Creek	1.87
108	2.90
Steinburger	2.94
5	2.96
25	3.00 s
77	3.01
58	3.06
60	3.22
Music Rd	3.28
Jensen	3.37
7	3.52
Hoag Pass	3.61

Table 2. Macro- and micro-nutrients analyzed in soil and foliage samples.

Foliage	Soil
C (%)	C (%)
P (%)	P (ppm)
K (%)	K (ppm)
N (%)	N (%)
	NH_4-N (ppm)
	NO_3-N (ppm)
S (%)	S (%)
Ca (%)	Ca (ppm)
Fe (ppm)	
Mg (%)	Mg (ppm)
Mn (ppm)	
Cu (ppm)	
B (ppm)	
Zn (ppm)	

single plastic bag, and the composite sample was transported to the lab in a cooler. Due to the height of some of the sample trees, foliage was sampled from lower in the crown than the fifth whorl, and on four of the plots no foliage was sampled at all (Table 1).

In addition to the 25 permanent plots, foliage and soil samples were collected from a randomly selected cluster of 5 trees in one additional plantation with SNC as severe as any other known location in north-coastal Oregon (Cloverdale in Table 1).

Soil samples were analyzed for all macronutrients except iron, and foliage samples were analyzed for all macronutrients and some micronutrients (Table 2). A fresh subsample was also reserved for assessment of fungal mass by PCR (polymerase chain reaction) techniques in Jeff Stone's lab at OSU.

Site variables were also collected during plot establishment in the Growth Impact Study. A set of these variables was included in the analysis to test for the trends across such conditions as elevation and distance from coast (Table 3). Some information was also available on species composition of the previous stand, and on composition of the plantation as a whole from walk-through surveys (Table 3).

Table 3. Site variables included in the analysis for predicting trends in average foliage retention (n=70)

Variable	Range
plantation age	12-28
aspect	8-360
slope, %	10-85
elevation, ft	75-1121
miles from coast	2.8-19.8
landowner site index	100-130
PCT indicator	0 or 1
burn indicator	0 or 1
alder currently present	0 or 1
hemlock currently present	0 or 1
pure Douglas-fir indicator	0 or 1
alder previously present	0 or 1
alder previously dominant	0 or 1
Douglas-fir previously dominant	0 or 1

Regression analysis was applied to explore the potential relationship between SNC severity and soil and foliage nutrient status. SNC severity was indexed by either foliage retention or PCR index. Because the relative abundance or relative availability among nutrients was fundamental to the hypothesis of a nutrient imbalance, and because some of the relationships could be nonlinear, an implied multiplicative equation served as the basic exploratory model. Regression fits were accomplished by transforming the multiplicative nonlinear model into a linear model by logarithmic transformation:

$$[1]\ \ln(retx) = b_0 + b_1X + b_2X_2 + \ldots + b_kX_k$$

where retx = average foliage retention (years)

X_i = *i*th nutrient or ln of *i*th nutrient

Multivariate Euclidean distances (EDs) were computed to gain some insight into the degree of departure of actual nutrient content from "optimal" nutrient content. Only five macronutrients were considered: N, P, K, Ca, and Mg. Optima were specified on a percentage dry weight basis and were taken from research literature on balanced nutrition (Ericsson 1994). Distances were also computed for each nutrient as a relative or percentage departure from the optima. These Euclidean distances were then introduced as potential predictor variables in the regression models. Finally, a principal components analysis of soil and foliar nutrients was performed to gain some insight into the gradients in soil and foliar conditions.

Table 4. Results of chemial analysis of composite soil samples (n=26).

Soil	Range
pH	4.3-6.1
C (%)	3.8-19.3
P (ppm)	3-34
K (ppm)	125-386
N (%)	0.15-1.05
NH_4-N (ppm)	3.8-13.4
NO_3-N (ppm)	0.5-11.5
S (%)	0.00-0.11
Ca (ppm)	0.4-10.4
Mg (ppm)	0.4-10.0

Results and Discussion

The 26 composite soil samples covered a wide range in soil condition (Table 4). A similarly wide range in foliar nutrient content was observed; in particular, foliar nitrogen ranged from 0.85 to 1.74 % (Table 5).

In the foliar chemistry analysis, nitrogen had the strongest correlation with mean foliage retention, followed by carbon and sulfur (Table 6). Foliar sulfur also played a dominant role in the two-variable models, with the additional variable being either foliar carbon or foliar calcium. Variance from optimal chemical composition (ED) also emerged as a strong predictor, although both calcium and potassium contributed additional explanatory power despite the fact that they are represented in ED (Table 6).

In contrast to the foliar analysis, results from soil chemistry were more limited. Several simple regression relationships were found, the strongest of which involved sulfur, nitrogen, and pH (Table 7). However, none of the two-variables models contained predictors that each exerted a significant effect individually. Several statistically significant models appeared when all soil and foliar variables were screened together (Table 8). The strongest two-variable model contained foliar sulfur and soil sulfur, both of which displayed a negative relationship with foliage retention. In the three-variable models, soil sulfur appears consistently, as does either foliar nitrogen or foliar sulfur, and all are negatively correlated with foliage retention. However, the third variable is either foliar calcium or soil ammonium; these latter two variables are both positively related to foliage retention. The several statistically significant four-variable models were dominated by these same variables, but foliar carbon also enters with a negative relationship to foliar retention. The strongest model explains 92% of the variation in foliage retention, and contains foliar calcium, soil nitrogen, soil ammonium, and variance from optimum. As with all other regression models, the effects are consistently negative for soil nitrogen and variance from optimum nutrient concentrations, but positive for foliar calcium and soil ammonium (Table 8).

The analysis for site history is not yet complete, primarily because more information still needs to be collected for the PCT plots. However, two analyses have been completed to date: 1) relationship between foliage retention and site variables for the 13 GIS plots in the soil/foliar chemistry study; and 2) relationship between foliage retention and site variables for the 70 GIS plots with complete information. Although the same variables generally emerge as significant in both analyses, as one would expect the amount of variation explained is much higher for the 13 plots representing extreme SNC conditions (R^2>0.60 vs.R^2<0.35). Plots that have received precommercial thinning have significantly greater needle retention than those that have not, and those stands that currently have red alder exhibit poorer needle retention than those that do not. When distance from coast is added, it also has a positive relationship with needle retention. In the reduced dataset, both fire and red alder presence in the previous stand are positively related to foliage retention, but fire only marginally so. Clearly, more work is needed to sort out the influ-

Table 5. Results from chemical analysis of composite foliage samples (n=23).

Foliage	Range
C (%)	51.0-53.7
P (%)	0.11-0.23
K (%)	0.41-0.77
N (%)	0.85-1.74
S (%)	0.08-0.12
Ca (%)	0.16-0.62
Mg (%)	0.10-0.21
Fe (ppm)	41-428
Mn (ppm)	118-906
Cu (ppm)	3-15
B (ppm)	7-25
Zn (ppm)	11-22

Table 6. Significant regression relationships between foliage retention and foliar chemistry (n=23). Direction of effect is indicated by sign on each variable.

Variables	R^2
-N%	0.64
-C%	0.57
-S%	0.56
-C%,-S%	0.74
+Ca%,-S%	0.70
+Ca%,-ED	0.79
+K%,+Ca%,-ED	0.87

Table 7. Significant regression relationships between foliage retention and soil variables (n=26). Direction of effect is indicated by sign on each variable.

Variables	R^2
-S%	0.65
-N%	0.57
+pH	0.52

ence, if any, of red alder in both the previous stand and current stand. Although thinning very consistently displays a significantly positive correlation with needle retention, the causal mechanism is anything but clear. A genuine positive effect of thinning on the growth and vigor of the tree is certainly a possibility, but others include immobilization of excess soil nitrogen during microbial decomposition of thinning slash, or even perhaps a tendency to thin stands with light SNC and to avoid stands with severe SNC.

The results obtained for PCR assay as a response variable were generally consistent with the results above treating foliage retention as the response. The same variables generally appeared, and their effects were the same: precommercial thinning, higher foliar calcium and higher pH were associated with lower fungal biomass, and higher foliar nitrogen, foliar carbon, soil nitrogen and soil sulfur were all associated with higher *Phaeocryptopus* biomass. Greater variance from optimum nutrient concentrations was also associated with higher fungal biomass. The one major difference between the analyses of foliage retention and PCR assay was the repeated appearance of a negative relationship between foliar zinc and *Phaeocryptopus* biomass.

Principal components analyses on the foliage data only, on the soils data only, and on the combined soils and foliage data generally suggest gradients typically associated with pH. At the high pH end of the gradient are high calcium and magnesium, and at the low end high sulfur and nitrogen. Sites with the poorest foliage retention are those with high relative content of both foliar and soil nitrogen and sulfur. Sites with relatively high soil and foliar calcium are those with the longest foliage retention. High foliage retention is associated with low fungal mass as assessed by the PCR assay, so the major trends in nutrients are consistent. However, the reduced correlation of foliar calcium and enhanced correlation of zinc in the PCR analyses warrants much further consideration of the relationships among foliage retention, total foliage mass, fungal biomass, and total photosynthetic capacity.

Table 8. Significant regression relationships between foliage retention and combined soil and foliar nutrients (n=22). Direction of effect is indicated by sign on each variable.

Variables	R^2
-soilS%	0.70
-soilN%	0.63
-folN%	0.63
-soilS%,-folS%	0.79
-folN%,-soilS%	0.76
-folS%,-soilN%	0.75
-folN%,+soilNH_4,-soilS%	0.82
+folK%,+folCa%,-ED	0.87
+folCa%,-soilS%,-ED	0.83
-folC%,-folS%,+soilNH_4,-soilN%	0.87
-folC%,-folS%,+soilNH_4,-soilS%	0.86
+folCa%,+soilNH_4,-soilN%,-ED	0.92

In summary, the working hypothesis that abundant available nitrogen in soils may at least contribute to the Swiss needle cast epidemic in north coastal Oregon appears supported by the data collected to date. Further analysis of these data will include evaluation of the assumption that there is excess available nitrogen on these sites vs. a shortage of calcium or other nutrient. The work proposed for the year 2001 involves collection of samples from plots that have been treated with either various types of nutrient amendments or thinning. The influence that these treatments have on soil and foliar nutrient status will provide key insights into nutrient dynamics on the sites, into possible mechanisms contributing to the intensification of Swiss needle cast, and into any promising ameliorating treatments.

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