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Quantification of *Phaeocryptopus gaeumannii* colonization in Douglas-fir needles by ergosterol analysis

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Summary

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

1 Introduction

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

Ergosterol is a membrane sterol found in most fungi, but not in plant or other microbial cells (Gessner and Newell 1997). Therefore, it can be used as a measure of fungal biomass in the substrate under analysis. Although it is non-species-specific, ergosterol content has been successfully used to quantify total fungal biomass in various substrata and applications (e.g. Matcham et al. 1985; Johnson and McGill 1990; Desgranges et al. 1991; Schnürer 1993). Ergosterol content has been used to measure fungal biomass in soil (Davis and Lamar 1992), mycorrhizal roots (Salmanowicz and Nylund 1988; Antibus

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and SINSABAUGH 1993), leaf litter (GESSNER et al. 1991; NEWELL 1992; GESSNER and NEWELL 1997; NEWELL 2000) and foliage (NEWELL 1994; MAGAN and SMITH 1996). Total ergosterol content is better correlated with fungal biomass than free ergosterol content (NEWELL 1994); and STAHL and PARKIN (1996) and NEWELL (2000) stress that ergosterol is a better indicator of live, rather than total, fungal biomass. Ergosterol determinations of fungal biomass in this paper are therefore considered to be estimates of living fungal biomass. Furthermore, factors such as substrate nutrient availability and fungal age may alter ergosterol concentrations (JOHNSON and MCGILL 1990; BJURMAN 1994).

The potential for ergosterol content to quantify *P. gaeumannii* infection levels is unknown. Douglas-fir needles harbour a diverse assemblage of internal, endophytic fungi (CARROLL and CARROLL 1978) and epiphytic fungi (CARROLL 1979). As much as 90% of needles are infected by internal fungi by the time the needles are 3 years old (BERNSTEIN and CARROLL 1977). However, over 90% of these infections are due to only two fungal species (CARROLL 1988). One of these is *Rhabdocline parkeri*, which has a very widespread occurrence on Douglas-fir, but has restricted colonization within living needles. Infection sites of *R. parkeri* are limited to a single epidermal cell until needle senescence. Needles may be repeatedly infected, and the number of infections per needle increases as needles age. However, only a small proportion of the total needle cells is colonized, even on the most heavily infected foliage (STONE 1988). Another abundant endophytic species, *Phyllosticta abietis*, also has very limited colonization, with each infection site comprised of only a few fungal cells in healthy needles (STONE and PETRINI 1997).

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

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

2 Materials and methods

2.1 Sample storage time

All 1-year-old needles from one randomly selected lower canopy branch from each of 10 *P. gaeumannii*-infected Douglas-fir trees at Sour Grass Summit, Oregon (45°05.673′ N, 123°44.684′ W) were collected in July 1997 and pooled. The sample was split into 30 subsamples of approximately 250 mg and stored in paper coin envelopes at 0°C. At 0, 1, 2, 4, 6, and 8 months, five of the subsamples were randomly selected for ergosterol content measurements. One-half of the needles in each envelope were used for ergosterol extraction and high-performance liquid chromatography (HPLC) analysis, and one-half were used for fresh weight (FW) : dry weight (DW) ratio determinations. Ergosterol values shown in Fig. 1 are the mean and standard error of all five subsamples analysed at each sample date.

2.2 Ergosterol – fungal biomass studies

Pure cultures of the seven most common Douglas-fir foliar fungi were obtained from surface-sterilized Douglas-fir needles and grown in unshaken 250 ml Erlenmeyer flasks with 100 ml 2% potato dextrose broth (Difco, Detroit, MI, USA) at 18°C. After 3 months, mycelium was removed from each flask (one per species), rinsed with distilled H_2O, blotted dry on sterilized filter paper, macerated with a razor blade into 1 mm^2 pieces and separated into five to six samples of different biomass amounts (5–80 mg DW). One subsample was used for determination of the FW : DW ratio and the remaining portions were used for ergosterol extraction and HPLC analysis. One isolate of *Cladosporium cladosporioides*, *Hormonema dematiodes*, *Nodulisporium* sp., *Phomopsis* sp., *Rhabdocline parkeri*, *Tryblidiopsis pinastri*, and four isolates of *Phaeocryptopus gaeumannii* were analysed.

2.3 Ergosterol – fungal age studies

One-millimetre-plugs obtained from a pure agar culture of *P. gaeumannii* were used to inoculate unshaken 250 ml Erlenmeyer flasks containing 100 ml 2% potato dextrose broth (Difco) at 18°C (12 flasks in all). At 1, 3, 6 and 12 months mycelia from three flasks were removed, rinsed with distilled H_2O, blotted dry on sterilized filter paper, macerated into 1 mm^2 pieces, and separated into two approximately equal samples. One sample from each flask was used for ergosterol extraction and HPLC analysis and one sample was used to determine the FW : DW weight ratio.

2.4 Detection of *P. gaeumannii* from field samples

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

2.5 Ergosterol extraction and analysis

The following extraction and analysis procedures were adapted for determination of ergosterol in green conifer needles based on previously reported methods of ergosterol determination (Johnson and McGill 1990; Gessner et al. 1991; Newell 1994). Ergosterol was extracted from approximately 125 mg FW (unless otherwise noted) of sample tissues (stored < 1 month at 0°C) by heating at 80°C for 30 min in 2 ml potassium hydroxide-methanol solutions (0.05 g KOH/ml MeOH). After cooling, samples were partitioned three times with 2 ml petroleum ether. The petroleum ether fraction was

collected and reduced to dryness. Each sample was then dissolved in 1 ml HPLC-grade methanol and filtered with a 0.2 μm nylon filter before HPLC analysis. Extraction and HPLC of pure ergosterol standards showed efficiencies were 85–93% ($n = 12$).

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

2.6 Pseudothecia density

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

2.7 Internal fungal isolations

Isolations of internal needle fungi were conducted on five needles in July 1997 and two needles in February 1998 from each pooled sample described above. Each selected needle was surface sterilized (rinsed H_2O, 1 min 95% EtOH, 5 min 3.5% NaOCl, 30 s 100% EtOH), cut into 2 mm lengths, and placed on 2% malt extract agar in Petri dishes. The Petri dishes were monitored weekly for 6 months and needle segments with hyphal growth emerging from cut ends were recorded. The percentage of segments with *P. gaeumannii* emerging, and number of segments with other fungi emerging were averaged for all needles from each branch.

2.8 Statistical analyses

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

3 Results

Ergosterol concentrations decreased linearly over time in needles stored at 0°C (Fig. 1). A significant loss in ergosterol was observed 2 months after collection, and by 8 months the ergosterol had decreased to approximately 50% of its original concentration. Based on these results all future analysis of ergosterol content was limited to needle samples stored less than 1 month.

All seven species of the most common Douglas-fir foliar fungi (*Cladosporium cladosporioides, Hormonema dematiodes, Nodulisporium* sp., *Phomopsis* sp., *Rhabdocline parkeri, Tryblidiopsis pinastri,* and *Phaeocryptopus gaeumannii*) showed a linear relationship between ergosterol content and fungal biomass when grown in culture

(Fig. 2). Furthermore, the relationship between fungal biomass and ergosterol was similar for all species tested. Tests with *P. gaeumannii* also showed that ergosterol content did not change with culture age from 1 to 12 months (Fig. 3).

Detectable amounts of ergosterol, pseudothecia, and internal fungal isolations were present in all trees sampled. Ergosterol content showed a positive linear relationship with numbers of pseudothecia (Fig. 4) and internal fungal isolations (Fig. 5). Regression parameters for these relationships are presented in Table 1. For both sample dates and all sites, ergosterol content was best correlated with pseudothecia density, followed by *P. gaeumannii* isolations, and finally all fungal isolations (Table 1).

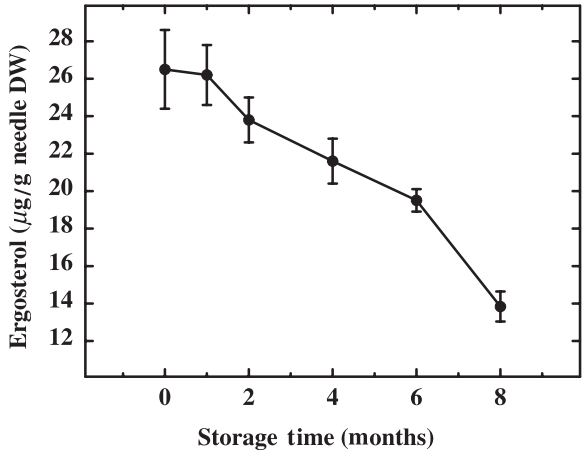

Fig. 1. Ergosterol content (mean ± SE) from Douglas-fir needles stored at 0°C over the course of 8 months, n = 5

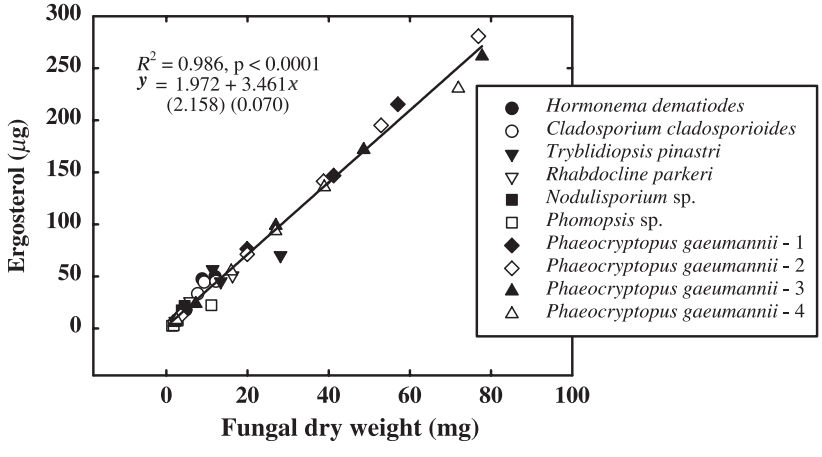

Fig. 2. Relationship between ergosterol and fungal biomass in seven common Douglas-fir foliar fungi grown in pure culture

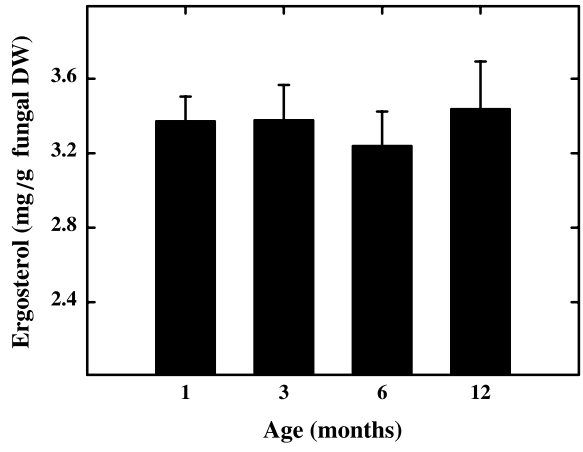

Fig. 3. Relationship between ergosterol content and *P. gaeumannii* culture age. Bars represent means ± SE, n = 3

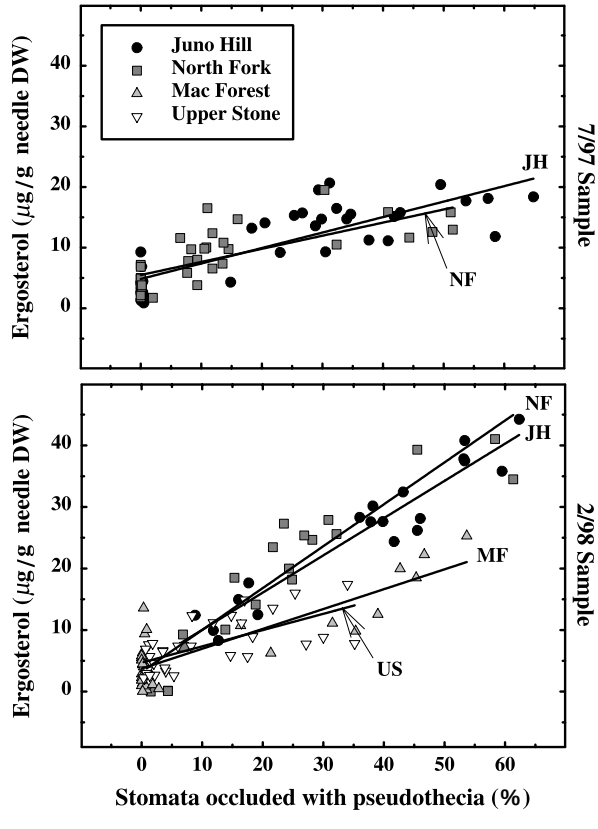

Fig. 4. Relationship between ergosterol content and *P. gaeumannii* pseudothecia density from Douglas-fir needles collected at four sites. JH, NF, MF, and US are the Juno Hill, North Fork, Mac Forest and Upper Stone sites, respectively

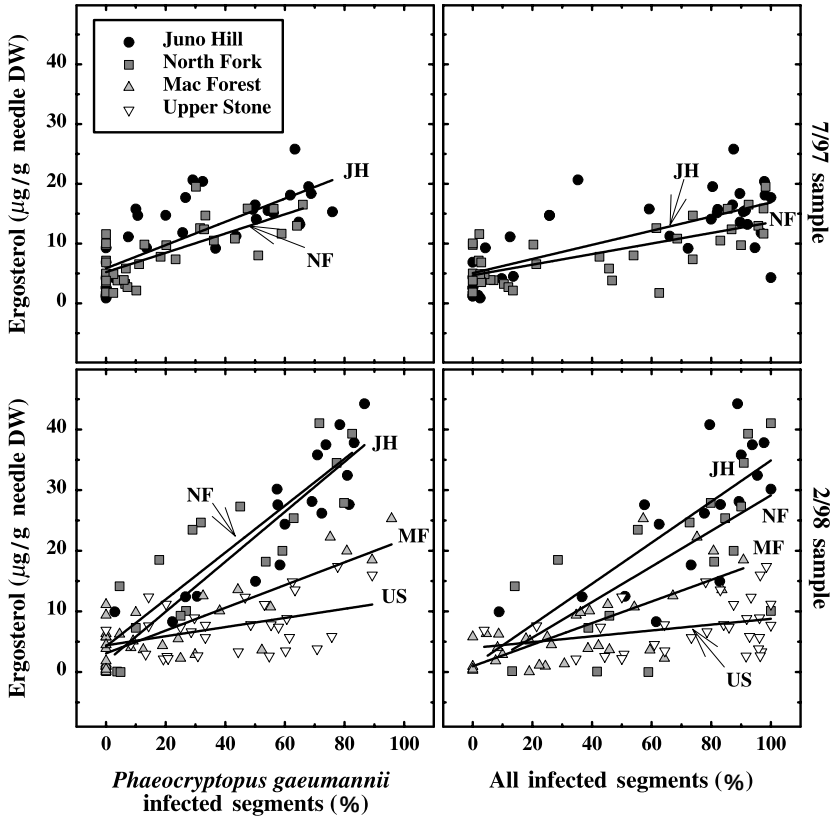

Fig. 5. Relationship between ergosterol content and *P. gaeumannii* isolation or all fungal isolations from surface-sterilized Douglas-fir needles collected at four sites. JH, NF, MF, and US are the Juno Hill, North Fork, Mac Forest and Upper Stone sites, respectively

The two most common fungus species isolated from Douglas-fir needles were *R. parkeri* and *P. gaeumannii*. For both sample dates and all sites *P. gaeumannii* accounted for about 50% of the fungal isolations (Table 2).

4 Discussion

Ergosterol content appears to be a good measure of *P. gaeumannii* colonization. It correlated well with both pseudothecia density and *P. gaeumannii* isolations from surface-sterilized needles. Although about 50% of fungal isolations from all sites were species other than *P. gaeumannii*, they did not appear to significantly contribute to the detectable amount of ergosterol (i.e. ergosterol content was best predicted by measures of *P. gaeumannii* only). This result is best explained by colonization of *P. gaeumannii* comprising a major proportion of the fungal biomass in needles. To our knowledge, efforts to quantify the internal colonization rates of *P. gaeumannii* have not been conducted elsewhere. However, light and electron microscopy of Douglas-fir needles infected by *P. gaeumannii* has shown extensive internal and external needle colonization (Capitano 1999), whereas many of the other foliar fungi typically have very limited colonization

Table 1. **Parameters for ergosterol regressions shown in Figs 4 and 5**

	Site	R^2	p-value	*y*-intercept	Slope
Ergosterol versus *P. gaeumannii* pseudothecia density					
July 1997					
	JH	0.66	0.0001	4.83	0.255
	NF	0.53	0.0001	5.48	0.216
February 1998					
	JH	0.91	0.0001	3.98	0.605
	NF	0.85	0.0001	3.20	0.681
	MF	0.72	0.0001	3.73	0.323
	US	0.43	0.0001	4.70	0.264
Ergosterol versus *P. gaeumannii* fungal isolations					
July 1997					
	JH	0.59	0.0001	5.85	0.195
	NF	0.53	0.0001	5.22	0.161
February 1998					
	JH	0.77	0.0001	1.69	0.412
	NF	0.75	0.0001	4.22	0.387
	MF	0.68	0.0001	3.080	0.188
	US	0.14	0.0001	4.40	0.0754
Ergosterol versus all fungal isolations					
July 1997					
	JH	0.50	0.0001	5.05	0.118
	NF	0.50	0.0001	4.69	0.0885
February 1998					
	JH	0.50	0.0007	0.999	0.339
	NF	0.41	0.0027	−0.210	0.294
	MF	0.50	0.0001	0.951	0.178
	US	0.05	0.11	3.97	0.0481

JH, Juno Hill; NF, North Fork; MF, Mac Forest; US, Upper Stone sites.

within the needle. *Rhabdocline parkeri*, for example, was the second most common fungus isolated. Infections by this fungus are restricted to single epidermal cells until needle senescence, and even in heavily infected 4-year-old needles no more than about 5% of the total epidermal cells are occupied (Stone 1988).

In pure cultures, ergosterol content was proportional to fungal biomass in all seven species investigated and did not vary with culture age (only *P. gaeumannii* investigated). These two results suggest that measures of ergosterol should relate directly to amounts of live fungal biomass in Douglas-fir needles. However, we did not test the effect of growing conditions (i.e. nutrient type and concentration) on ergosterol content in pure cultures. Medium composition has been shown to affect ergosterol content in fungal cultures Newell (2000). It is possible that fluctuations in nutrient levels in needles could cause the relationship between ergosterol and fungal biomass to vary. Although we cannot rule out the possibility of such an effect influencing observed ergosterol contents, we believe that it is not a significant factor. If nutrient levels caused significant variation in ergosterol content, we would expect to see a consistent change in stationary cultures over time, as nutrient levels in the media became depleted. Instead, the relationship remained linear. We also would expect to see the ratio of ergosterol content to *P. gaeumannii* pseudothecia count, or the ratio of ergosterol content to *P. gaeumannii* isolations to vary within each regression in Figs 4 and 5. For instance, each regression includes several needle-age classes,

Table 2. **Percentage of needle ends (2 mm surface-sterilized fragments) from which fungal hyphae emerged. Values reported are the mean (± SE) for all observations at each site**

	Current year needles (1997 cohort)			One year-old needles (1996 cohort)			Two year-old needles (1995 cohort)		
Site	PG[1]	RP	All	PG	RP	All	PG	RP	All
July 1997									
JH	0.7 (0.4)	0.0 (0.0)	2.5 (0.9)	42.1 (6.0)	4.2 (1.7)	70.1 (7.0)	44.9 (6.0)	5.7 (3.2)	90.5 (3.1)
NF	2.1 (0.8)	0.0 (0.0)	4.0 (1.0)	21.2 (4.4)	13.2 (3.5)	49.6 (7.1)	41.2 (6.0)	17.9 (4.0)	83.3 (5.1)
February 1998									
JH	38.3 (7.4)	2.6 (1.1)	51.1 (8.8)	39.2 (5.6)	13.3 (5.0)	75.7 (7.7)	65.8 (15.1)	8.1 (3.6)	94.2 (1.3)
NF	30.6 (7.3)	0.1 (0.1)	50.7 (7.9)	31.8 (7.1)	1.0 (0.7)	55.4 (8.0)	40.0 (7.3)	13.7 (4.8)	83.2 (5.8)
US	30.3 (6.8)	4.6 (1.8)	57.3 (8.7)	46.8 (6.5)	9.8 (4.3)	83.4 (5.9)	41.1 (7.2)	14.4 (7.2)	77.3 (6.3)
MF	1.7 (1.2)	0.9 (0.6)	11.2 (3.7)	3.1 (2.2)	8.5 (3.2)	20.7 (5.3)	15.5 (8.0)	11.9 (5.2)	40.1 (7.6)

[1]PG denotes *P. gaeumannii*; RP denotes *R. parkeri*; All denotes all fungal species.
JH, Juno Hill; NF, North Fork; MF, Mac Forest; US, Upper Stone sites.

and nutrient content in needles typically declines with needle age (BAUER et al. 1997) and increasing *P. gaeumannii* infection (MANTER, unpublished data); however, the relationship remains linear suggesting a constant ratio of ergosterol content to *P. gaeumannii* colonization level.

As shown in Table 1, slopes of the ergosterol regressions vary over time and with site. We attribute this to the inaccuracy of either pseudothecia density or fungal isolations for estimation of internal colonization rates. A greater slope suggests to us that the amount of internal colonization per unit pseudothecium (or fungal isolation) is increasing. Such a relationship is supported by CAPITANO's (1999) work showing greater internal hyphal growth of *P. gaeumannii* at the Juno Hill site, which consistently had the highest observed slopes compared to other sites studied. The increase in slope over time is also consistent with increasing amounts of internal hyphal growth and the number of external surface hyphae radiating from pseudothecia (CAPITANO 1999).

In summary, ergosterol content appears to be a good measure of fungal biomass in Douglas-fir needles mainly related to *P. gaeumannii* colonization. Unlike pseudothecia density and fungal isolation measures of *P. gaeumannii*, it is non-species-specific and therefore will be influenced by colonization levels of other foliar fungi present in Douglas-fir needles. However, the relative contribution of other fungi appears to be negligible at our western Oregon sites, regardless of *P. gaeumannii* infection levels. Changes in the slope of the linear relationship between ergosterol and *P. gaeumannii* infection levels suggest that the amount of internal hyphae varies over time and with site. The cause, effect, and relative importance of differences in internal hyphae growth, and their relationship to SNC disease are unknown and deserve further attention.

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Résumé

Quantification de la colonisation des aiguilles de Douglas par Phaeocryptopus gaeumannii, par analyse de l'ergostérol

L'évaluation du niveau d'infection de *Phaeocryptopus gaeumannii*, agent de la rouille suisse des aiguilles de *Pseudotsuga menziesii*, est basée habituellement sur l'abondance des fructifications chez les aiguilles malades. La relation entre cette mesure et la colonisation interne n'est pas connue. Dans le présent article, une série d'expériences est décrite visant à savoir si l'ergostérol peut être utilisé pour quantifier la colonisation interne des aiguilles par *P. gaeumannii*. Nous avons trouvé que le contenu en ergostérol chez sept champignons foliaires fréquents chez le Douglas, est proportionnel à la biomasse; dans le cas du *P. gaeumannii* cette relation n'est pas affectée par l'âge de la culture. Dans quatre sites testés, *P. gaeumannii* était le champignon le plus fréquemment isolé des aiguilles (environ 50% des isolements). La teneur en ergostérol de ces aiguilles était très liée au *P. gaeumannii* malgré la présence d'autres champignons. Nous attribuons cette relation forte entre l'ergostérol et le *P. gaeumannii* à la plus grande contribution de celui-ci à la biomasse fongique totale, comparée à celle de tous les autres champignons présents dans les aiguilles de Douglas.

Zusammenfassung

Quantifizierung des Myzels von Phaeocryptopus gaeumannii in Douglasiennadeln durch den Nachweis von Ergosterol

Der Infektionsgrad von *Phaeocryptopus gaeumannii*, dem Erreger der Rostigen Douglasienschütte, wird meist aufgrund der Anzahl der Fruchtkörper auf den erkrankten Nadeln bestimmt. Die

Beziehung zwischen diesem Parameter und der Besiedlung der Nadelgewebe durch das Pilzmyzel ist nicht bekannt. Es wurde deshalb experimentell geprüft, ob der Ergosterolnachweis zur Quantifizierung des Pilzmyzels in der Nadel geeignet ist. Bei sieben häufig vorkommenden Nadelpilzen der Douglasie wurde ein Zusammenhang zwischen dem Ergosterolgehalt und der Pilzbiomasse festgestellt. Dieser wurde bei *P. gaeumannii* nicht vom Alter der Kultur beeinflusst. An vier untersuchten Standorten war *P. gaeumannii* mit ≈ 50% der häufigste Pilz, der von Douglasiennadeln isoliert werden konnte. Der Ergosterolgehalt in diesen Nadeln war, unabhängig von anderen Pilzarten, am besten mit der Präsenz von *P. gaeumannii* korreliert. Dieser starke Zusammenhang zwischen dem Ergosterolgehalt und dem Vorkommen von *P. gaeumannii* wird auf den im Vergleich zu den anderen Pilzen grösseren Anteil dieser Art an der Pilzbiomasse in den Nadeln zurückgeführt.

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