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Isolation of the Endophytes *Lophodermium piceae* and *Rhizosphaera kalkhoffii* from Sitka Spruce Needles in Poor and Good Growth Sites and in vitro Effects of Environmental Factors

By

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Summary

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The level of infection of symptomless green needles of Sitka spruce by *Lophodermium piceae* and *Rhizosphaera kalkhoffii* was compared in poor growth/polluted and good growth/unpolluted stands from 1989-1991. Generally isolation of these two endophytes from green needles increased with needle age. This was supported by both culturing of the endophytes on media and the total ergosterol content of needles. Statistically significant correlations were obtained between the two techniques on some sampling occasions. Over the three year experimental period there was markedly higher isolation frequency of *R.kalkhoffii* from the poor growth/polluted site than from the good growth/unpolluted site. This was less apparent for *L.piceae*. Complimentary in vitro studies showed that *R.kalkhoffii* was more tolerant of elevated sulphur dioxide, lowered water availability, and had a lower temperature optimum than *L.piceae*. The use of such endophytic fungi as possible bioindicators of tree vitality is discussed.

Introduction

In the early 1980s' the Forestry Authority in the U.K. noted markedly reduced growth and "bent top" of Sitka spruce in plantations of the coalfield sites at Afan and Tywi in industrial south Wales (COUTTS 1995) with symptoms similar to those seen on Sitka spruce plantations grown under a range of abiotic stress

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conditions. The "bent top" symptom was an indicator of forest decline, with features common to the so-called "neuartige waldsterben" or new forest decline seen in Europe (SCHUTT & COWLING 1985). Other characteristic symptoms included premature needle senescence and fall, a thinning of the crown density and reduced tree growth. A number of stresses were believed to be acting on the trees in these sites including wind, spring drought, winter water logging, historically elevated levels of air pollutants, nutrient deficiency, fungi and insects.

There has been considerable interest in the possible contributory role that needle fungi might play in bringing about the symptoms of needle discolouration and premature defoliation that are common in the affected trees in Afan, south Wales. In Europe there has been considerable debate about the role of fungal infections in the development of forest decline. For example, REHFUESS & RODENKIRCHEN 1985 and SUSKE & ACKER 1987a implicated the two endophytes *Lophodermium piceae* (Fuckel)Hhn and *Rhizosphaera kalkhoffii* Bubak in needle reddening disease whereas BUTIN & WAGNER 1985 suggested that they were early colonisers of dying or dead needles and therefore not directly involved in causing these symptoms. However, *L.piceae* has been described as both a weak parasite or saprophyte (DARKER 1967) and a pathogen (BUTIN 1986). *R.kalkhoffii* has also been found to infect trees under water stress, and spread of this fungus is facilitated by sulphur dioxide pollution (TANAKA 1980). More recently, in Scotland and north England premature loss of needles from Sitka spruce plantations was accompanied by a high level of colonization by these same two fungi (GREGORY & REDFERN 1987). However, no other information exists on the prevalence of these two fungi in needles of Sitka spruce plantations in the U.K. and whether their presence could be used as an indicator of pollution stress.

The objectives of this study were therefore to determine the patterns of colonisation of different age classes of green Sitka spruce needles by *L.piceae* and *R.kalkhoffii* in the intensively studied poor growth sites at Afan, and compare their behaviour in a good growth site at Mynydd Ddu in the Black mountains of east Powys. Complimentary in vitro studies were carried out to examine their relative activities under different abiotic factors including temperature, water availability, and sulphur dioxide.

Materials and Methods

Experimental sites

The replication within the Afan poor growth site and the good growth site in Mynydd Ddu in the Black Mountains were initially 4 trees (April, 1989) and 8 trees subsequently until October, 1991.

Sampling procedure and fungal isolation

Generally, a branch with the current, and at least two previous years needles was cut from the top whorl using tree loppers. Needles were separated into age classes and stored at 4°C in sterile Universal bottles and processed within 24-48 hrs. Up to twenty five green needles were taken from each of three year classes. Needles of each age class of needles were surface-sterilized in 20 ml of

sodium hypochlorite (7.5% available chlorine) which also contained a 1:100 solution of 95% ethanol to wet the needles (CARROLL & CARROLL 1978, PETRINI 1986). Glass Universal bottles (25 ml) containing the needles and sterilizing solution were agitated with a Hati rotomix for 30 s and left for 4.5 min. The needles were blotted dry, cut into 5 mm segments aseptically and plated onto 1% malt extract agar (MEA, Lab M Ltd, pH 4.0). Twelve segments per plate and 5 replicates plates per age class were plated out for each of four/eight replicate trees (total of 240 segments per age class). Petri plates were then incubated at 20°C and examined weekly for four weeks before identification and assessment of colonisation frequency. The fungi isolated were compared with strains of *L.piceae* and *R.kalkhoffii* obtained from the International Mycological Institute (Egham, Surrey), the Forestry Authority (south), Prof. H. BUTIN (Forest Research Institute, Braunschweig, Germany) and Dr. J. SUSKE (University of Bayreuth, Germany).

Ergosterol analysis

Samples were also taken on three occasions, in January, March and June, 1991 from the poor growth site and analysed for the total internal fungal biomass using the biochemical marker, ergosterol. Needle samples were washed in sterile distilled water (+ 0.05% Tween 80) to remove phyllopshere fungi. Between 2-4 g of needles of each age class were dried and stored at -20°C for approx. 24 hrs after which they were processed. Small sub-samples were also kept for direct plating of needles segments as described above. Needles were placed in a 60°C incubator until constant dry weight was achieved. The needles were then placed in a Waring blender and homogenised for 2 min. The ground needles were then weighed out and ergosterol extraction carried out immediately. The method used was that described previously by TOTHILL & al. 1992.

Growth media, inoculation and measurement of fungal growth in vitro

Experiments were done on a 1% malt extract agar medium (MEA, pH 4.5). In all cases molten cooled agar was poured into 9 cm Petri dishes and inoculated with 2 mm agar plugs of the test isolates and species taken from the growing margin of 14-d old colonies. In all experiments the diameter of the colonies was measured in two directions at right angles to each other, every two days for up to 27 d unless otherwise stated. The radial growth rates (mm day^{-1}) were subsequently calculated. All experiments were carried out with three replicates and each experiment was repeated three times.

The effect of temperature was examined by placing inoculated MEA agar plates at 5, 15, 20, 25 and 30°C. The effect of sulphur dioxide (SO_2) was evaluated by addition of known amounts of a filter sterilised (Millipore, 0.2µ) 50% (V/V) suplphurous acid solution to obtain a range of concentrations of SO_2 (0-100 ppm). The concentration in solution was confirmed by using a sodium thiosulphate back-titration experiment. The effect of water activity (a_w) was examined by modifying the media with glycerol in the range 0.998 (unamended) to 0.928 a_w. Plates with the same a_w were always enclosed in clean polyethylene bags and incubated at 15 and 25°C for 25-28 days. The a_w of all media were confirmed by measurement in a humidistat (Humidat ICII, Nove Sina, Switzerland). Water availability x SO_2 interactions werer investigated by adjusting the water availability to 0.998, 0.991, 0.971 and 0.928 as described previously. Filter sterilised SO_2 (0-75 ppm) was added as described previously.

In all cases analyses was carried out on the number of segments from which the two endophytes were isolated. Student t tests were used to directly compare treatment effects on individual sampling dates.

Results

Needle age and endophyte colonisation

Tab. 1 shows the relationship between needle age, colonisation frequency of fungal endophytes (predominantly *L.piceae* and *R.kalkhoffii*), and ergosterol content (μg g^{-1}) from green needles. Generally, as the needle age increased there was an increase in both isolation frequency and ergosterol content in the needles of the poor growth plots on all three sampling dates. Correlation of the isolation on media with total fungal biomass showed that there was a significant (P = 0.05) correlation in January, 1991 (r^2= 0.9957). For other treatments and dates the correlation was not significant and varied between 0.4260 and 0.5241.

Tab. 1. Comparison of mean percentage (%) needle isolation with total ergosterol (mg g-1) content of green needles in the poor growth Afan site on three sample dates in 1991.

Needle age		1991	1990	1989	1988	1987	1986	1985
January	% isolation	np*	1.8	5.1	8.2	8.3	12.4	14.9
	ergosterol	np	1.7	6.2	10.3	8.2	12.3	12.6
March	% isolation	1.6	8.7	8.3	17.2	13.8	17.1	16.3
	ergosterol	1.3	2.1	3.7	3.9	5.3	6.0	25.2
June	% isolation	np	7.4	6.1	12.9	15.8	10.0	np
	ergosterol	3.2	2.4	8.6	16.5	4.9	3.8	np

* np: needles not present

Comparison of needle colonization in the poor and good growth sites

Tab. 2 shows the changes in percentage isolation of *L.piceae* and *R.kalkhoffii* from the poor growth site at Afan with the good growth site at Mynydd Ddu. The occurrence of *L.piceae* was very low, with isolation from green needles always slightly higher on trees in the poor growth site when compared to that of vigorously growing trees.

Over the same period the isolation of *R.kalkhoffii* was much higher than that of *L.piceae*, particularly in the poor growth site at Afan. There was also a more marked decrease in isolation from the good growth site than that of *L.piceae*. Indeed, isolation of *R.kalkhoffii* was much higher on all dates, and for all age classes in the Afan than the Mynydd Ddu site. On some dates there were significantly higher isolation frequencies from the poor than the good growth site.

In vitro comparison of effects of environmental factors on growth of *L.piceae* and *R.kalkhoffii*

Tab. 3 compares the effect of temperature, SO_2 and water activity on growth of the an isolate of *L.piceae* and *R.kalkhoffii*. This shows that there are significant differences between temperature optima, SO_2 tolerance and a_w optima for these two species. *L.piceae* had a higher temperature optimum, was more sensitive to SO_2 and less tolerant of a_w than *R.kalkhoffii*.

Tab. 2. Comparison of the percentage isolation of Lophodermium piceae and Rhizosphaera kalkhoffii from green symtomless Sitka spruce needles in the poor growth Afan 2 site and at the good growth Mynydd Ddu sites.

	Percentage occurrence on needle segments							
L.piceae								
Needle age class	1987		1988		1989		1990	
Site sampled	Afan 2	M.Ddu	Afan 2	M.Ddu	Afan 2	M.Ddu	Afan 2	M.Ddu
Date								
April 1989	0.4	1.3	1.3	0.4	-	-	nn	nn
September 1989	0	0	2.1	0	-	0	nn	nn
December 1989	1.7	5.0	2.1	1.7	0.8	3.3	nn	nn
March 1990	0.8	0	0.8	0	0.2	0	nn	nn
June 1990	0.4	0	0.2	0	0	0	0	0
September 1990	np	np	1.7	0.8	0.9	0	0	0
December 1990	2.1*	0.4	0.8	0.4	0	0	0	0
March 1991	nd	nd	1.87	0	0.6	0.8	0.2	0
June 1991	nd	nd	1.3	1.3	0.6	0.8	0.4	0
October 1991	nd	nd	2.7	2.1	1.67	0.8	1.1	0
R.kalkhoffii								
Needle age class	1987		1988		1989		1990	
Site sampled	Afan 2	M.Ddu	Afan 2	M.Ddu	Afan 2	M.Ddu	Afan 2	M.Ddu
Date								
April 1989	10.8*	0.4	1.7	0	np	np	nn	nn
September 1989	0.4	0	1.3	1.7	0	0	nn	nn
December 1989	13.3*	1.7	0.8	1.7	0.4	0.8	nn	nn
March 1990	4.6	0.8	2.9	0	0.2	0	nn	nn
June 1990	np	np	2.8	0	0.2	0	0	0
September 1990	np	np	6.9*	0.4	3.5	1.7	0.4	0
December 1990	np	np	6.9*	0.8	2.5	0.4	1.5	0
March 1991	nd	nd	5	0.4	4.6	3.3	1.3	0
Jume 1991	nd	nd	2.9	0.8	1	0.8	0.2	0
October 1991	nd	nd	3.2	1.3	1.7	2.5	1.3	0.8

np: needles not present; nn: no needles present; nd: not determined
*, significant difference (P = 0.05)

Discussion

Throughout the study period there was a relatively consistent relationship between needle age and isolation frequency of *L.piceae* and *R.kalkhoffii* from symptomless green needles of Sitka spruce. Both direct isolation from needle segments and ergosterol measurements reflected the trend. However, the lack of specificity of ergosterol means that it cannot be used to look at individual component species and endophytic successions in needles. OSSWALD & al. 1986 found it was a useful method for determining the effect of different environmental conditions on infection but also found that the ergosterol content of *L.piceae* and

R.kalkhoffii differed considerably. Thus direct isolation and ergosterol measurements should be seen as complimentary methods. Previously, LEGAULT & DESSUIEAULT 1989 showed that the microflora of *Pinus* spp. increased with needle age while CARROLL & al. 1977 and PETRINI & CARROLL 1981 working with needle endophytes of some European conifers showed that although frequency of infection was low in very young needles, there was a increase in infection with needle age. The only exception to this was in the work of SUSKE & ACKER 1987b who found no increase in infection with needle age by *L.piceae* and *R.kalkoffii* on Norway spruce plantations.

Tab. 3. Comparison of the effect of (i) temperature, (ii) sulphur dioxide (S02, pH 3.5) and (iii) water activity (aw, pH 5.5) on mean in vitro growth rates of an isolate of L.piceae and R.kalkhoffii on a 1% malt extract medium.

	Radial growth rates (mm day^{-1})				
(i) Temperature (°C)	5	15	20	25	30
L.piceae	0.2	0.48	1.10	1.05	0
R.kalkhoffii	0.42	1.39	0.91	0.18	0

(ii) Sulphur dioxide	Radial growth rate (mm day^{-1})							
Temperature (°C)	15				25			
SO_2 (ppm)	0	25	50	75	0	25	50	75
L.piceae	0.24	0.25	0.2	0	0.56	0.18	0.14	0
R.kalkhoffii	0.91	1.06	1.13	1.1	0.08	0.19	0.22	0

(iii) Water activity	Radial growth rate (mm day^{-1})									
Temperature (°C)	15					25				
A_w	0.997	0.99	0.98	0.97	0.95	0.997	0.99	0.98	0.97	0.95
L.piceae	0.36	0.46	0.39	0.31	0.2	1.1	1.02	0.83	0.56	0.31
R.kalkhoffii	1.33	1.42	1.0	0.52	0.32	0.13	0.32	0.78	0.91	0.69

The literature available on isolation of *L.piceae* from green needles of spruce (predominantly Norway spruce) has shown the frequency to be very variable. For example, BARKLUND 1987 in Sweden found that *L.piceae* dominanted the endophytic flora in unthinned stands of Norway spruce. Almost 90% of 2, 3 and 4 year old needles were found to be infected, with the highest frequency of isolation occurring in needles approx. 2.5 years old. After this time infection appeared to decrease with other unidentified endophytic fungi invading the needles. SUSKE & ACKER 1987b demonstrated that *L.piceae* was consistently the major endophytic coloniser of healthy needles during their study in the latter half of 1985. They found a mean isolation frequency of only 3.3% in second year needles. Later studies (SUSKE & ACKER 1989) using cultural, ultrastructural and immunocytochemical methods showed that endophytic fungi (particularly *L.piceae*) in green symptomless needles increased from about 0.8% in age class 1 to 2.8% in older second year needles, to 5.8% in third year needles and rising to 33.4% in five year old needles.

In our study higher levels of isolation of *R.kalkhoffii* were obtained than that of *L.piceae* in the poor growth plots when compared to the good growth site. There is only one other record of these two fungi in Sitka spruce plantations. GREGORY & REDFERN 1987 found that in Sitka spruce plantations showing symptoms of premature needle loss there was a high level of isolation of both *R.kalkhoffii* and *L.piceae* in northen Britain, although they suggested that neither fungus had a clear status as pathogens in Europe. However, there has not previously been a detailed study of the patterns of colonisation of Sitka spruce needles by these endophytes, particularly in stands under environmental stress in the U.K. In Germany, BUTIN & WAGNER 1985 stated that *R.kalkhoffii* has been associated with defoliation of Norway spruce but only in a secondary capacity. By contrast, DIAMANDIS 1978 and COWLING & al. 1988 showed that *R.kalkhoffii* could be an extremely fast colonist of stressed needles. Physiological changes in needles due to atmospheric pollutants may also result in them becoming predisposed to colonisation by *R.kalkhoffii* rather than *L.piceae*. It is possible that the colonisation frequency by these two fungi could be indicative of pollution or other abiotic stress directly or indirectly due to effects on physiuology of the plant.

In vitro studies with an isolate of *R.kalkhoffii* and *L.piceae* from these plantations also suggest that they may respond in different ways to environmental stress. This study has shown for the first time that the lower temperature optimum, the higher tolerance of dissolved SO_2 alone, and to SO_2 at reduced water availability (SMITH 1993) may contribute to the ecological fitness of *R.kalkhoffii* in colonising green needles, when compared to *L.piceae*. SMITH 1993 has also demonstrated that in interaction studies on media isolates of *R.kalkhoffii* were more dominant that those of *L.piceae* in the presence of dissolved SO_2 (25-50 ppm). The complex nature of these interactions warrants more detailed studies to determine whether endophytes can be used as bioindicators of pollution.

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