DETECTION OF *ARMILLARIA* BASIDIOSPORE DISPERSAL

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ABSTRACT

This study used a molecular technique to identify airborne basidiospores caught using a SporeWatch volumetric spore trap in order to understand the nature of basidiospore dispersal by *Armillaria novae-zelandiae*. The spore trap was deployed during 3 successive years: year 1 within a mature podocarp-hardwood forest containing fruitbodies of *A. novae-zelandiae*, year 2 in the open at the edge of the forest, and year 3, 150 m from the forest edge. Results indicated that although a high proportion of *Armillaria* basidiospores were trapped within the forest by screening and reduced air currents, others escaped and were dispersed beyond the stand edge. Year 1 results showed the presence of *A. novae-zelandiae* on 63% of days while *A. novae-zelandiae* was recorded on 11% and 20% of days in years 2 and 3, respectively. Sensitivity analysis of the molecular technique that was used indicated that the minimum detectable basidiospore concentration was 1-10 basidiospores/ μ l, or about 1 pg/ μ l of DNA.

Keywords: *Armillaria novae-zelandiae*, spore dispersal, aerial dispersal, molecular identification, pine plantations.

INTRODUCTION

Four species of *Armillaria* are known to occur naturally in New Zealand, *A. novae-zelandiae* (G. Stev.) Herink, *A. limonea* (G. Stev.) Boesew., *A. hinnulea* Kile & Watling and a fourth species that has yet to be formally described (Hood 1989). *Armillaria novae-zelandiae* is recognised as the main cause of *Armillaria* root disease in contemporary *Pinus radiata* plantations (Hood et al. 2002b). In such stands it causes a low incidence of early mortality and more extensive, chronic, non-lethal infection leading to significant growth loss (Kimberley et al. 2002). Species of *Armillaria* spread vegetatively within the forest by means of growth of mycelium along host root systems and rhizomorphs (Dettman & van der Kamp 2001). However, it has been found that basidiospores are important in the establishment of new infection centres (Kile 1983; Hood & Sandberg 1987; Rishbeth 1988). *Armillaria novae-zelandiae* is capable of colonising freshly cut wood of *P. radiata* by means of basidiospores, and establishment of the pathogen in pine debris produced during site preparation may result in new infection centres in succeeding rotations (Hood et al. 2002a, 2008). It is likely that such infection centres may be initiated through the dispersal of basidiospores from indigenous forests in which *Armillaria* species fruit prolifically during May and June (Hood et al. 2002a), since observation indicates that fruiting is infrequent within diseased pine stands. Given the enormous number of basidiospores produced by *Armillaria* fruiting bodies, it is probable that some will travel over large distances (Stenlid & Gustafsson 2001). However, it is necessary that direct confirmation of basidiospore dispersal by *A. novae-zelandiae* be obtained.

The cryptic nature of the disease hampers efforts to follow its development in current pine forests or to predict its impact in future pine stands. Monitoring basidiospore dispersal provides a complementary approach to help resolve these questions. This study, therefore, aimed to investigate the airborne dispersal of *A. novae-zelandiae* by basidiospore trapping, followed by a polymerase chain reaction (PCR) based

identification technique to detect basidiospores of this species. Knowledge of how the pathogen spreads will help to determine the extent to which *A. novae-zelandiae* is becoming established in new stands, and if the presence of disease is increasing through successive plantations (Hood et al. 2002a; 2008).

METHODS

Airborne basidiospore collection

A volumetric spore trap was used to capture and measure air-dispersed basidiospores during the fruiting seasons over 3 years; each year at increasing distances from the assumed basidiospore source. The Burkard SporeWatch Sampler (Burkard Scientific) operates by drawing air in through an orifice at a constant rate, allowing any basidiospores present in the air to impact onto a paraffin coated tape placed on a rotating drum. The drum makes one full revolution in seven days, after which the tape is replaced. The spore trap tape was changed weekly according to the manufacturer's instructions, and start and finish times were recorded for each tape. Using these times, individual collection days (midnight to midnight) were marked on the spore trap tape.

Site selection

The site selected for the first year of collection (2005) was Hinehopu Scenic Reserve, located on Highway 30 between Lakes Rotoiti and Rotoehu, Bay of Plenty, New Zealand (38°02'19.58" S, 176°29'48.53" E). The stand at this site is a mature podocarp-hardwood forest (Type D3; Nicholls 1965). The spore trap was placed in the forest within a few metres of a fallen tree bearing many fruitbodies of *A. novae-zelandiae*. In 2006, the spore trap was placed in the open on private property at the edge of the same forest, and in 2007 it was positioned on a golf course ca 370 m from the 2006 site and ca 150 m distant from the nearest edge of the same forest. Basidiospore collection in the first year was carried out between 26 May and 17 August (80 days), in the second year between 12 May and 18 August (99 days), and in the third year between 2 May and 30 August (121 days). Deployment of the trap was timed to coincide approximately with the onset of fruiting of *A. novae-zelandiae* based on past records from indigenous forests, and to run well beyond the period when fruiting had finished.

PCR reactions

PCR reactions were carried out by scraping a sterile pipette tip along a single line over one marked day on the tape. The scraped material was then dipped into an aliquot of PCR cocktail and mixed to act as template DNA for the reaction. All scrapings were tested with the *A. novae-zelandiae* specific AnzF (5'-GCT CCC TTT GCC TAG G-3') / AnzR2 (5'-CCG ATC CTT GAC TTG TAT TCT G-3') primer pair that was developed by S. Dodd (Landcare Research). PCR amplification was carried out in a 15 µl reaction containing 0.5 µM of both forward and reverse primers, 0.45 U Taq DNA polymerase (Roche), 1 x reaction buffer (Roche), 1.5 mM MgCl₂ and 0.2 mM each of dATP, dGTP, dCTP and dTTP (Roche). The PCR conditions were: one cycle at 94°C for 2 min, 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, one cycle of 72°C for 7 min, and a 4°C hold cycle. PCR products were separated by electrophoresis on 1% agarose gels, stained using ethidium bromide and visualised and photographed under UV light.

Sensitivity analysis

A dilution series of *A. novae-zelandiae* basidiospores was prepared in order to determine the minimum number of basidiospores detected by the PCR method. A small aliquot of basidiospores collected from *A. novae-zelandiae* fruiting bodies was added to 0.5 ml of sterile distilled water and the concentration determined using a haemocytometer. After adjusting to 10⁷ basidiospores/ml the suspension was serially diluted to range between 10⁷ and 2 × 10² basidiospores/ml. The sensitivity of the PCR reaction was assessed by adding 5 µl of each basidiospore suspension to each PCR reaction, representing a dilution series of 5 × 10⁴ to 1 basidiospores per PCR reaction.

The minimum concentration of DNA that was detectable by the PCR protocol was established using a serial dilution series of purified *A. novae-zelandiae* DNA ranging in concentration between 10 ng/µl and 10 fg/µl.

Through comparison of the intensity of bands generated by the PCR reaction with known numbers of basidiospores and known concentrations of DNA, it was possible to estimate the minimum number of basidiospores detected by the assay.

RESULTS

Sensitivity analysis

The PCR reaction was sensitive enough to detect 10⁴ basidiospores/ml (or 10 basidiospores/μl; equivalent to 50 basidiospores in the PCR reaction) but lower concentrations of basidiospores were not detected. The limit of sensitivity of DNA detection was 1 pg/μl; there were no PCR products for the 100 fg/μl or 10 fg/μl concentrations. The PCR product generated from the 1 pg/μl dilution of DNA was less intense than the PCR product from the 10 basidiospore/μl sample. There was no PCR product in the 1 basidiospore/μl sample (equivalent to 5 basidiospores in the PCR reaction). Therefore, an assay sensitivity of 1 pg/μl for purified DNA was achieved. Based on C-values this equates to between 8 and 17 basidiospores/μl (Kullman et al. 2008).

PCR reactions

Positive results for the 2005 spore traps were obtained through to the 11th week of trapping (from 26 May to 4 August). The majority of the PCR products on positive collection days produced bright bands, indicating a large amount of DNA and therefore representing many basidiospores. Results for the 2006 tapes produced less intense bands at a lower frequency and were recorded until the 5th week of trapping (from 12 May to 15 June). In 2007, positive results were observed until the 9th week of trapping (from 12 May to 3 July) and the majority of positive bands were more intense than in 2006, indicating a greater concentration of basidiospores. Figure 1 graphically represents basidiospore trapping results for each year. When the first 84 days of spore trapping for each year were compared, basidiospores of *A. novae-zelandiae* were detected on 63% of days in 2005, compared with 11% in 2006 and 20% in 2007 (Table 1). Data were not recorded for technical reasons on 4 days in 2005 and 6 days in 2007.

TABLE 1: Percentage of days on which basidiospores were detected each year between 26 May and 17 August (84 days). Days with no data available are excluded.

Year	No. days detected	No. days not detected	% of days
2005	50	29	63
2006	9	75	11
2007	16	64	20

DISCUSSION

To assess basidiospore dispersal by *Armillaria novae-zelandiae*, basidiospores were trapped from the air using a volumetric spore trap that was placed within a native forest in the first year and then at increasing distances from the forest over the following two years. In 2005 spore trapping produced positive results on a far greater number of days than occurred in 2006 and 2007. It appears that the vegetation may have interfered with basidiospore movement within the forest and that the comparatively still air may also have assisted in preventing basidiospores from spreading beyond the forest edge. Reduced wind speed within forest canopies results in steeper spore dispersal gradients than in open areas (Stenlid & Gustafsson 2001). These results also concur with previous spore dispersal studies, which indicate that the vast majority of spores fall within a few metres of the fruiting body (Stenlid & Gustafsson 2001). Once spores escape from the forest, their concentration decreases rapidly with distance due to wind shear and atmospheric turbulence (Aylor 2003).

FIGURE 1: Basidiospore trapping results for (a) 2005, (b) 2006 and (c) 2007. Relative abundance of basidiospores estimated by PCR product intensity: 0=no PCR product, 1=faint, 2=moderate, 3=bright. X indicates days on which data were not collected. Note starting dates vary for each year and X-axis records consecutive days of spore trap operation.

In 2006 the spore trap was placed at the forest edge, while in 2007 the spore trap was sited in the open beyond the forest edge further from the assumed basidiospore source. In both 2006 and 2007 there was a reduction in the number of *A. novae-zealandiae* positive days, compared to 2005, as well as in the quantities of basidiospores that were collected. Results suggest that there was little dilution of airborne basidiospore between the edge of the forest and up to 150 m from the forest edge. The detection of airborne basidiospores at distances of up to 150 m supports earlier results using partly buried pine stem segments as basidiospore traps (Hood et al. 2002a).

The production and release of basidiospores depends on a number of conditions, such as the season (Stenlid & Gustafsson 2001), inherent diurnal rhythm and weather. Wind direction and strength, atmospheric turbulence and rainfall are all factors known to influence the dispersal and hence capture of basidiospores (Aylor 1990). Variations in spore release and dispersal during and between the different years of the present study may explain the variable intensities of PCR product bands observed between 2006 and 2007, and also account for several intervals when basidiospores were not detected, for example during the 33-40 day period in 2005 (Fig. 1a).

During part of this study tests were made to determine the minimum basidiospore concentration required to produce a positive PCR result. The minimum detectable number of basidiospores was 10/μl, which corresponded well with the C-value and suggested that there was minimal difference between purified DNA and basidiospores as template. Detection of *A. novae-zealandiae* DNA did not seem to be affected by non-specific DNA present on the tape, nor by the use of whole basidiospores. Direct counting of basidiospores on the tape will be undertaken in the future, in order to validate the efficiency of the molecular identification technique and the procedure for taking samples for PCR analysis. Destructive sampling of a greater area of the spore trap tape may provide more accurate results.

The results of this work confirm that, despite a significant level of entrapment within the indigenous forest, many *Armillaria* basidiospores escape, thus providing potential inoculum for spread to pine plantations and establishment of new infection centres. The small fraction of spores that is dispersed longer distances is crucial for the establishment of new populations in distant localities and to achieve genetic exchange between populations (Stenlid & Gustafsson 2001). Future work in this project will investigate dispersal over greater distances in order to assess this directly. There is strong indirect evidence that establishment of *A. novae-zealandiae* is occurring in pine plantations as a result of airborne basidiospores. There is a need to ascertain the extent to which this long distance dispersal is occurring in order to understand the likely impact of this disease in the future.

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