Dothistroma Needle Blight: an emerging epidemic caused by *Dothistroma septosporum* in Colombia

C. A. Rodas[a], M. J. Wingfield[b], G. M. Granados[c], I. Barnes[b]*

[a]*Forestry Protection Programme, Smurfit Kappa Cartón de Colombia. Km 15, Carretera Cali – Yumbo, Valle, Colombia;* [b]*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (UP), South Africa, 0002 and* [c]*Department of Microbiology and Plant Pathology, FABI, UP, South Africa, 0002.*

**E-mail: Irene.barnes@fabi.up.ac.za*

Short title: Dothistroma septosporum in Colombia

Key words: *Dothistroma septosporum*, pathogenicity, DNA-based diagnostic, *Pinus* spp.

Abstract

Plantation forestry in Colombia is based mainly on non-native species of *Pinus* and *Eucalyptus*. Since 2008, a disease with symptoms similar to those of Dothistroma needle blight (DNB) have been found affecting large areas planted to *Pinus* spp. The aim of this study was to identify the causal pathogen as well as to document the levels of disease incidence and severity. Isolates from each of three forestry zones, collected from different host species, were compared based on rDNA sequence of the ITS regions. These were conclusively identified as *Dothistroma septosporum*, one of two *Dothistroma* spp. known to cause DNB. Susceptibility was greatest on low elevation *P. tecunumanii* followed by *P. kesiya* and *P. oocarpa*. *Pinus maximinoi* and high elevation *P.*

tecunumanii showed tolerance to *D. septosporum*. The disease incidence in the different zones varied significantly with the Northern zone being the most severely affected. This constitutes the first report of disease distribution and susceptibility of hosts, as well as the first consideration of the relative importance of *D. septosporum* in Colombia.

Introduction

Plantation forestry in Colombia is based mainly on non-native species of *Pinus* and *Eucalyptus*. Collectively, these species make up approximately 327 000 hectares (ha) of plantations (MADR, 2010) that provide the raw material for pulp and solid timber products (MADR, 2006). Early plantations of *Pinus* spp. were largely comprised of *P. patula* but in recent years, various other species and especially *P. tecunumanii* and *P. maximinoi,* have been planted in order to match species more appropriately to the variable sites and altitudes found in the country.

Plantations of *Pinus* spp. in Colombia have been challenged by a number of native insect defoliating pests (Vélez, 1972; Wiesner & Madrigal, 1983; Mackay & Mackay, 1986; Madrigal & Abril, 1994; Rodas, 1994) and a few diseases. In 1984, a severe infection caused by *Diplodia sapinea* (syn. *Sphaeropsis sapinea*) was recorded in *P. patula* plantations in different areas in Colombia (Hoyos, 1987; Rodas & Osorio, 2008). More recently, a diverse suite of pathogens such as *Calonectria* spp. (Lombard *et al.,* 2009), and *Fusarium circinatum* (Steenkamp *et al.,* 2012) have affected *Pinus* plantations. These problems have grown in severity and this has resulted in an increasingly large area being planted with alternative, disease-tolerant species.

Needle pathogens that have been reported in Colombia include *Lecanosticta acicola, Meloderma desmazierii* and *Dothistroma septosporum* (Gibson, 1979; Gibson, 1980;

Ivory, 1987). Gibson (1980) suggested that *M. desmazierii*, found on *P. patula* and *P. radiata,* in Colombia would not be of any concern in pine plantations. He did warn, however, that the new discovery of *L. acicola* on *P. radiata, P. patula* and *P. elliottii* in Colombia might pose a significant threat to Southern Hemisphere pine plantations. This was a valid concern considering the extensive disease epidemics that a similar needle pathogen, *Dothistroma septosporum* was causing in the Southern Hemisphere, especially in areas such as Chile and New Zealand (Alzamora *et al.*, 2004; Bulman *et al.*, 2008).

Colombia was included in the distribution list of countries where *D. septosporum* occurs (Ivory, 1987), but there is no supporting information as to where it was found or on what host it occurred. In addition, this report was made before it was accepted that two morphologically similar species, *D. septosporum* and *D. pini* cause the same needle blight symptoms, collectively referred to as Dothistroma needle blight (DNB) or red band needle blight (Barnes *et al*., 2004). The presence of *D. septosporum* in Colombia has thus never been unequivocally confirmed.

In 2008, a new and serious needle disease problem appeared in the Central zone of Colombian pine plantations. Symptoms of the disease closely resembled those of DNB. Within two years, the needle blight disease had spread throughout all three forestry zones in Colombia on the non-native *Pinus tecunumanii.* Importantly, this is a tree species relatively new to forestry outside its native range, and for which there is little knowledge of diseases and insect pests that affect it.

The aims of this study were to establish the distribution and host range of the new and serious needle disease in Colombia and to confirm the identity of the pathogen based on DNA sequence data. Furthermore, our aim was to determine the susceptibility of different *Pinus* species and provenances of *P. tecunumanii* based on disease incidence

and severity. The impact of the disease on *P. tecunumanii* in intensively managed plantations was also assessed.

Materials and Methods

Disease distribution and host range

The extent of the needle blight epidemic in Colombia, mainly on *P. tecunumanii*, was assessed from field surveys conducted throughout all the plantation areas (Fig.1) belonging to Smurfit Kappa Cartón de Colombia (SKCC). Initial observations commenced in 2008 and continued until 2012. The surveys covered three different geographic zones (North, Central and South) and 14 farms (Yanahuanca, Argentina, Campania, Tesalia, Cedral, Angela Maria, Alaska, Esmeralda, La Concha, Samaria, Volconda, Unión_B, Unión_S and D. Miguel) located in the Departments of Caldas, Risaralda, Valle del Cauca and Cauca (Fig. 1). The total area surveyed was approximately 26 730 ha and consisted of plantations of *P. patula*, *P. kesiya*, *P. maximinoi, P. oocarpa*, and two different forms of *P. tecunumanii*; a low elevation (LE) form and a high elevation (HE) form. These two forms can be distinguished by RAPD analyses and differ slightly in morphology (Dvorak *et al*., 2000). In their native environment in Central America, the LE form occurs between 450-1500 m.a.s.l. and the HE form between 1500-2900 m.a.s.l. (Dvorak *et al*., 2000). In Colombia, the optimal elevation for LE growth is between 1400 - 1900 m.a.s.l. and for HE, between 1900 – 2500 m.a.s.l.

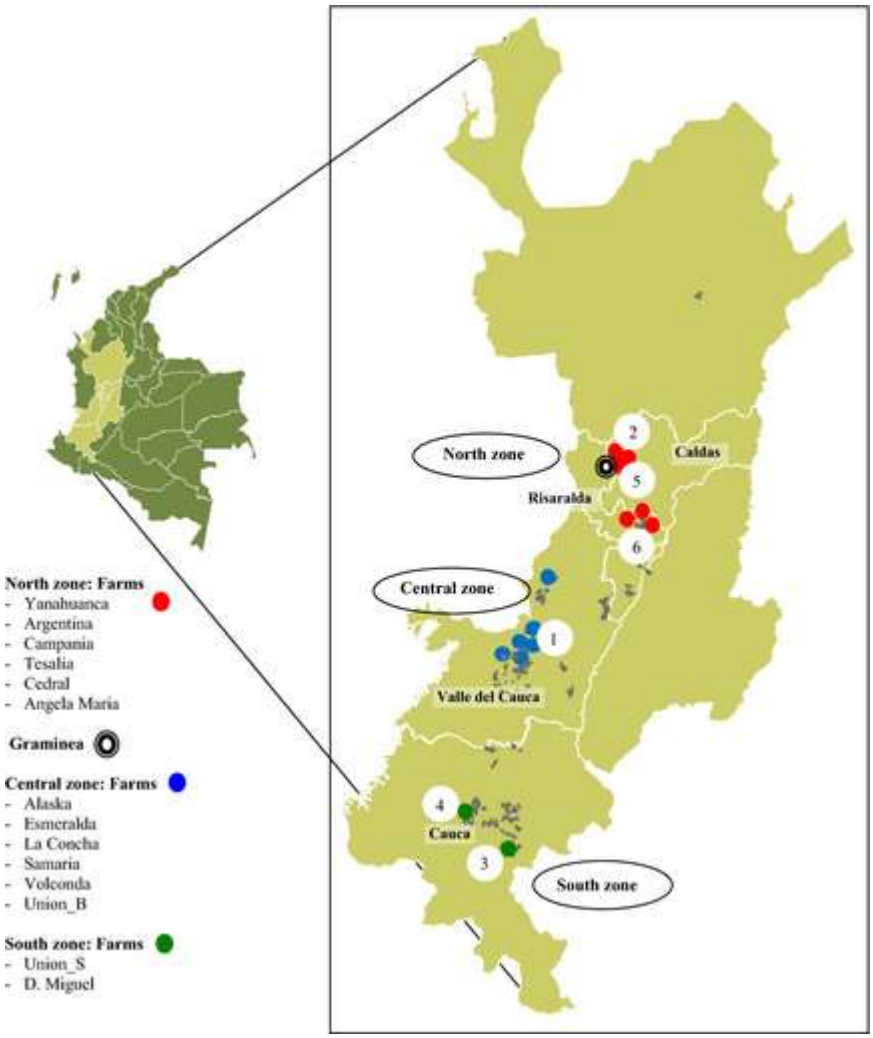


Figure 1 Geographic distribution of Dothistroma needle blight (DNB) outbreaks in Colombia in the North (red dots) Central (blue dots) and Southern Zones (green dots), on *P. tecunumanii*.The numbers represent the chronological order in which DNB was reported at the various locations. The black dot represents the location of the Graminea farm where the *P. tecunumanii* susceptibility trial was conducted.

Pathogen identification

Sample collection, isolation and morphological characterization

To verify the identity of the pathogen responsible for the disease symptoms in the surveyed areas, infected needles bearing distinct conidiomata were collected from diseased trees in each of the three forestry zones. Needles were placed in paper envelopes and stored at 4°C in preparation for subsequent laboratory studies.

Needles were prepared for isolations by first surface-disinfesting them in 0.2% sodium hypochlorite for 1 minute, rinsing with distilled water and blotting them dry with sterile paper towels. Using a Nikon SMZ645 stereoscope, fruiting structures were excised from the needles and placed on MEA 2% (w/v) malt extract (Merck, Darmstadt, Germany), 1.5% (w/v) agar (OXOID, Hampshire, United Kingdom), supplemented with 1% lactic acid, and incubated for 15 days at 24°C.

Morphological characteristics of the fungus were observed using a Nikon Eclipse E200 microscope. Microscope slides were prepared by fixing conidiomata bearing conidia, excised from diseased needles, with 1% lactic acid (Carlo Erba Reagenti, Arese, Italy).

DNA sequence based comparisons

Species identifications were made for several cultures isolated from needles collected from each of the three forestry zones and from different hosts. Mycelium was scraped from the surface of the cultures on agar, freeze-dried and crushed using the Retsch MM301 mixer mill (Haan, Germany) for 3 min at 1/30 mHz. The crushed mycelium was heated to 65°C in 800 µl DEB buffer (200 mM Tris-HCL, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) for an hour and DNA was extracted using the method described by Barnes *et al.* (2001).

The rDNA, Internal Transcribed Spacer (ITS) region was amplified for the selected isolates using primers ITS1 and ITS4 (White *et al.*, 1990). The reaction mixture consisted of ± 5 ng DNA template, 200 nM of each primer, 0.2 mM of each dNTP, 1U FastStart Taq DNA Polymerase with 10× buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM $MgCl_2$. Cycling conditions were set at 96°C for 2 min, 10 cycles

of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. An additional 30 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle with a 10 min final elongation at 72°C. PCR amplicons were visualized on 2% agarose gels and cleaned using Sephadex G-50 columns (SIGMA-Aldrich, Steinheim, Germany). Sequencing of the amplicons was carried out using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) following the manufacturer's protocols and run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Sequences were analyzed in CLC Bio (Main Workbench Version 6.6.2) and aligned using the online version of MAFFT version 6 (http://mafft.cbrc.jp/alignment/server/). Maximum parsimony phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford, 2002) using the heuristic search option with random stepwise addition and tree bisection reconnection as the tree swapping algorithm. Bootstrap analyses were conducted with 1000 randomizations. The trees were rooted with sequence data for *Lecanosticta acicola* as the outgroup taxon.

Susceptibility of ***Pinus tecunumanii*** **progenies**

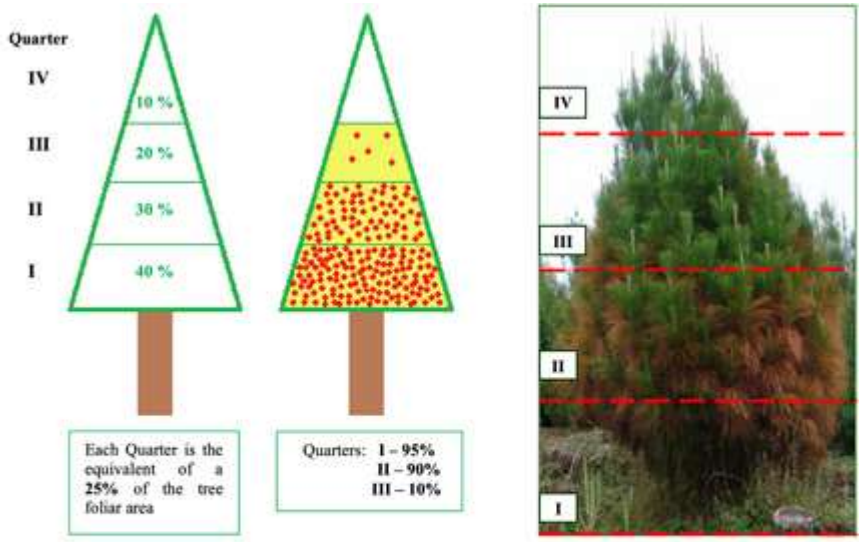
Susceptibility studies were established on the farm Graminea located at 5°25'19'' N – 75°45'56'' W, (North zone, Caldas, Fig. 1) having an altitude of 2155 m.a.s.l., annual precipitation of 2560 mm and an average temperature of 18°C. At this site, 27 different *P. tecunumanii* Low Elevation (LE) progenies (half-sib crosses where the female parent is known), had been planted approximately 2 km from an area very heavily affected by needle blight. Trees in the plot were established in November of 2008 and the first disease symptoms were recorded two years later in November 2010. A total of 1260 trees

distributed evenly in 6 blocks were used in the trial. Each block included 6 trees each of 27 different *P. tecunumanii* LE progenies. For comparative purposes, each block also included six trees each of four different *P. tecunumanii* provenances [PTEBsuH1 (LE), PTEByucu (LE), PTEAcaH1 (HE), TECASaH1 (HE)], two provenances (MAXcabH1 and MAXcabH2) of *P. maximinoi* and two provenances (PKcalvH1 and PKcalvH2) of *P. kesiya.*

The disease incidence was assessed by dividing the total number of affected trees by the total number of planted trees for each of the *P. tecunumanii* LE progenies (972 trees representing 27 progenies) as well as for the additional treatments used for comparative purposes (288 trees representing 8 provenances).

The disease severity per tree was calculated by partitioning the foliar area of the tree into equal quarters. Each quarter was then assessed individually and the relative level of disease present in that quarter of the tree was recorded as a percentage of the total crown (Fig. 2). To accommodate for the difference in conical area represented by each quarter, the data collected were then statistically re-weighted so that the bottom quarter of the tree would correspond to maximum 40% of the total area of tree infected, the second quarter represented 30%, the third 20%, and the crown 10%. The weighted percentage of each quarter was then summed up to obtain the overall severity per tree.

A total of six evaluations for disease incidence and severity were performed in the Graminea trial in Nov 2010, Feb 2011, May 2011, Aug 2011, Nov 2011 and May 2012. Data used to calculate the mean percentage disease incidence and severity were analysed using descriptive statistics and data evaluation for assumptions of normality and homogenous provenance/progeny variances. Analysis of variance (ANOVA) for a



$$\%\ Severity = \frac{(QI * 40\%) + (QII * 30\%) + (QIII * 20\%) + (QIV * 10\%)}{100}$$

$$\%\ Severity = \frac{(95 * 40\%) + (90 * 30\%) + (10 * 20\%)}{100}$$

$$\%\ Severity = 67\%$$

Figure 2 The disease severity of DNB. This is calculated by dividing the total foliar area of a tree into quarters and then estimating the percentage (out of 100%) of infected area per each quarter. These values are then statistically analyzed using a weighted mean average model based on the conical foliar area distribution of *Pinus* in which the designated value for the major quarter (bottom of the tree) corresponds to 40%, next quarter with 30%, 20%, and the top, 10%, respectively.

completely random design (six blocks) was used to test for differences between progeny and provenances. Multiple Range test was used to compare means at the 5% level (SAS Proc Insight). Only the data collected in May 2012 were used for calculations of disease incidence while the mean over all six evaluation periods was used for the severity calculations.

Disease impact in plantations

Evaluations of the impact of the disease were made at 11 forestry farms (Yanahuanca, Argentina, Tesalia, Cedral, Alaska, Esmeralda, La Concha, Samaria, Volconda,

Table 1 Details of the forestry farms and plots in Colombia used for the evaluation and impact of Dothistroma needle blight on provenances of *P. tecunumanii*

Zone	Department	Forestry Farm	Number of plots in each farm	Coordinates		m.a.s.l.[a]	Precipitation (mm/year) 2011[b]	Planted provenance elevation	Year planted
				Latitude	Longitude				
North	**Caldas**	Yanahuanca	3	5º 26' 12'' N	75º 45' 46'' W	2350	4389	*P. tec* Suiza LE[c]	Dec-05
		Argentina	6	5º 24' 20'' N	75º 44' 55'' W	2244	3027	*P. tec* Suiza LE	Dec-05
	Risaralda	Tesalia	5	4º 48' 15'' N	75º 36' 31'' W	1986	4082	*P. tec* Suiza LE	Dec-04
			7	4º 48' 15'' N	75º 36' 31'' W	1908	4082	*P. tec* Suiza LE	Dec-05
		Cedral	6	4º 42' 57'' N	75º 38' 15'' W	1902	4346	*P. tec* Yucul LE	Oct-06
Central	**Valle del Cauca**	Alaska	4	4º 3' 24'' N	76º 25' 09'' W	1763	2276	*P. tec* Yucul LE	Dec-05
			8	4º 3' 24'' N	76º 25' 09'' W	1950	2276	*P. tec* Yucul LE	Feb-06
		Esmeralda	4	4º 3' 27'' N	76º 25' 53'' W	1763	2276	*P. tec* Yucul LE	Dec-05
		La Concha	1	4º 0' 46'' N	76º 25' 24'' W	1741	2712	*P. tec* Arcad LE	Jul-04
			6	4º 0' 46'' N	76º 25' 24'' W	1603	2712	*P. tec* Yucul LE	Dec-06
		Samaria	2	4º 1' 47'' N	76º 26' 30'' W	1680	2712	*P. tec* Suiza LE	Oct-07
			6	4º 1' 47'' N	76º 26' 30'' W	1603	2712	*P. tec* Suiza LE	Oct-07
			5	4º 1' 47'' N	76º 26' 30'' W	1745	2712	*P. tec* Suiza LE	May-08
		Volconda	5	4º 1' 47'' N	76º 26' 06'' W	1754	2712	*P. tec* Yucul LE	Jun-06
		Unión_B	5	4º 25' 13'' N	76º 15' 24'' W	1630	2902	*P. tec* Yucul LE	Aug-06
South	**Cauca**	Unión_S	8	2º 17' 04'' N	76º 33' 56'' W	2701	3102	*P. tec* Suiza LE	Dec-04
			9	2º 17' 04'' N	76º 33' 56'' W	2780	3102	*P. tec* Suiza LE	Dec-04
Total	**3**	**4**	**11**	**90**					

[a] m.a.s.l.: meters above sea level.

[b] Only the data for 2011 is presented here as an example of the amount of precipitation that can occur in one year.

[c] LE.: Low Elevation.

Unión_B, and Unión_S) distributed in the North, Central and South zones (Table 1). A total of 90 circular plots, representing 1% of the total affected area (1 plot per every three hectares of affected area) were evaluated. Each plot was 300 m^2 (9.78 m radius) and consisted of approximately 30 planted trees (plantation density of 3 x 3 m). These plots were randomly selected within severely diseased plantations that showed a wide range of altitudes and weather conditions such as precipitation (Table 1).

The impact of the disease in an area was determined by calculating the disease incidence as the total number of affected trees in each plot divided by the total number of trees in the plot. The disease severity was calculated as described above for the Graminea trial. Disease evaluations were made at least four times a year from April 2009 to January 2012 for each of the 90 plots. Abiotic factors such as precipitation, elevation and age of trees (in months) were also recorded at each farm and zone for the evaluation period. Precipitation was recorded daily throughout the evaluation period.

To determine whether the different geographic areas in Colombia influenced the presence and impact of DNB in *P. tecunumanii* plantations, data obtained from the 300 m^2 circular plots were analyzed individually per farm and per zone. Data used to calculate the mean percentage disease incidence and severity were analysed using descriptive statistics and data evaluation for assumptions of normality and homogenous variances per zone and farm. Analysis of variance (ANOVA) was used to test for differences between geographic areas (zones/farms). Duncan's Multiple Range test was used to compare means at the 5% level (SAS Proc Insight). Only the data captured in January 2012 were used to calcuate disease incidence. Severity was calculated as the mean over all evaluation periods from 2009 to 2012.

Regression analyses were used to determine wheter there was a relationship between the disease severity on *P. tecunumanii* and the variables of precipitation, elevation, age

(in months) of diseased trees and year of measurement (2009, 2010, 2011, 2012). Stepwise regression selects variables to include or exclude from a linear model according to the ratio of residual mean squares, which was set to 1.0 (Draper & Smith, 1981). Regression modeling was done using the statistical program GenStat® (Payne (Ed.), 2014).

Results

Disease distribution and host range

In June 2008, needle blight was first noticed as an important foliar disease causing significant impact on various pine species in Colombia (Fig. 3). The disease appeared in 2.5-year-old *P. tecunumanii* (Yucul provenance) plantations located at the farms Alaska (4°3´24´´N - 76°25´09´´W) and Esmeralda (4°3´27´´N - 76°25´09´´W) in the Valle del Cauca Department of the Central zone. A month later, a second report of the disease was made for 2.5 year-old *P. tecunumanii* (Suiza provenance) on the farm Argentina, (5°24´20´´N - 75°44´55´´W) in Caldas, Northern zone.

In February of 2009, a third report of needle blight emerged from the farm La Unión (Cauca, Southern zone) at 2°17´04´´N - 76°33´56´´W in a 2.5 year-old-stand of *P. tecunumanii* (Suiza provenance). A fourth report was recorded in August of the same year where 2.1-year-old *P. kesiya* began to show signs of blight in the Cauca Department (2°17´27´´N - 76°39´46´´W) on both farms located in the Southern zone. In 2010, the fifth and sixth reports corresponded to infections on *P. oocarpa* and where species emerged as being highly susceptible to needle blight in two localities, specifically the Campania (5°26´57´´N - 75°45´56´´W) and Angela Maria (4°49´18´´N - 75°36´21´´W)

farms located in the Caldas and Risaralda Departments respectively (Northern zone) (Fig. 1).

Visual observations of the needle blight disease on different species of pines distributed in all three zones showed that the most susceptible species were *P. tecunumanii* LE, followed by *P. oocarpa*, and *P. kesiya* (Fig. 3). The most tolerant species were *P. patula*, followed by *P. tecunumanii* HE and *P. maximinoi* (Fig. 3).

Pathogen identification

Sample collection, isolation and morphological characterization

The early symptoms of needle blight in Colombia included yellow bands on the green needles, developing into dark-red to brown bands. In the advanced stages of the disease, infection proceeded upwards from the bases of trees and irregular-shaped acervuli emerged from the necrotic needle tissues. Hyaline, 2-5 septate, cylindrical spores, typical of *Dothistroma* spp. were observed in the excised conidiomata from infected needles.

Isolations from infected needles yielded typical callus-like *Dothistroma* cultures of various colours ranging from grey to pink. Some of the cultures produced a red exudate in the isolations medium. Six cultures were retained for further study and these included two from the Northern zone (farms Argentina and Tesalia), two from the Central zone (farms Alaska and Esmeralda), and two from the Southern zone (farms Don Miguel and

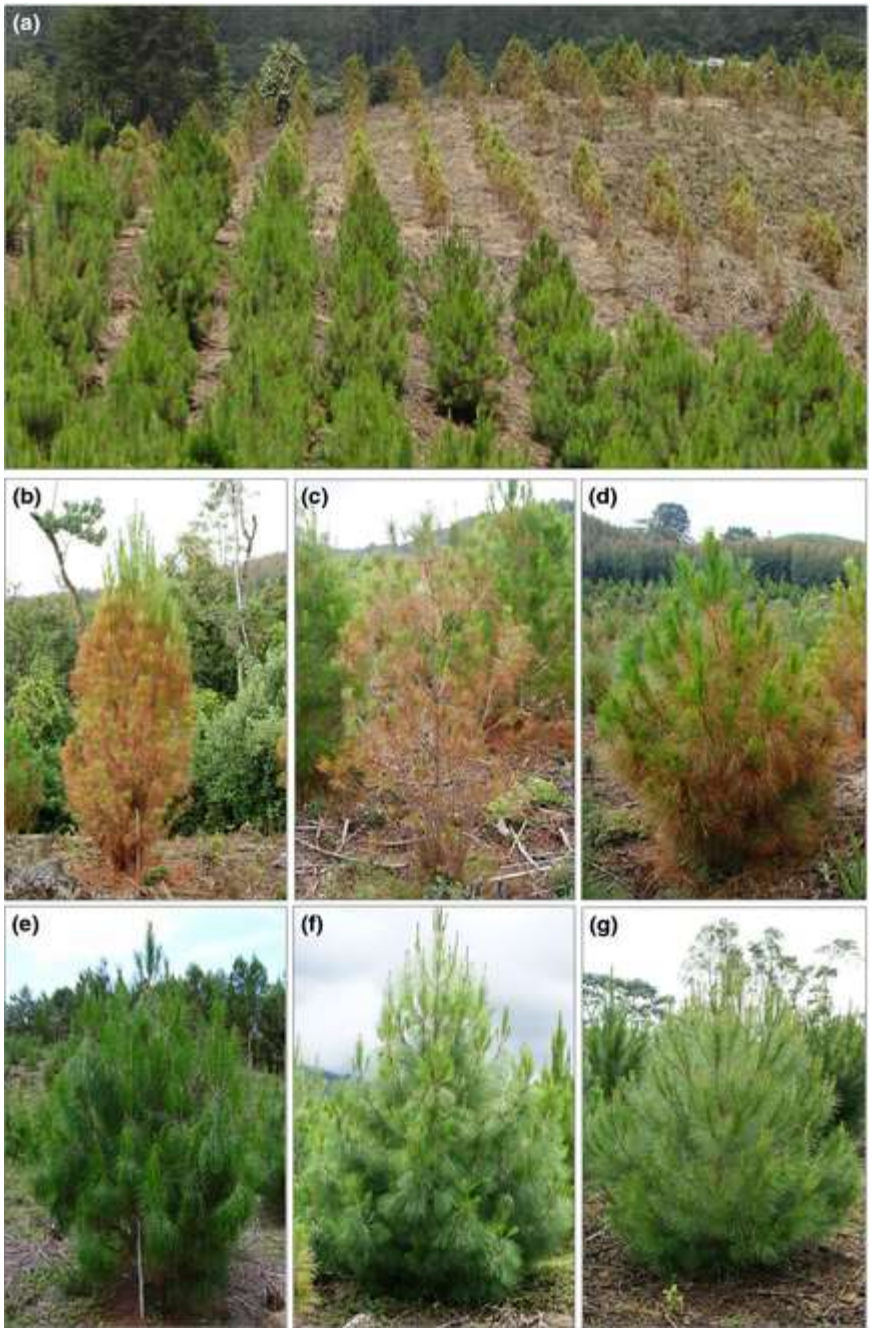


Figure 3 Dothistroma needle blight (DNB) symptoms on different pine species in Colombia. (a) Tolerant and susceptible *Pinus* species to DNB. Pine species susceptible to DNB include: (b) *P. tecunumanii* from low elevation (LE), (c) *P. oocarpa* and (d) *P. kesiya.* Tolerant species to DNB include: (e) *P. patula,* (f) *P. tecunumanii* high elevation (HE) and (g) *P. maximinoi.*

Unión). These isolates and representative needle samples are maintained in the Mycological Herbarium at SKCC, Colombia. The cultures are also maintained in the

culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA sequence-based comparisons

The six isolates amplified using ITS primers produced amplicons of approximately 500bp. All ITS sequences were identical and gave Blast search results of 100% similarity to the ITS regions of *D. septosporum* available on GenBank (e.g. AY808288 from Chile and AY808289 from Ecuador). The alignment of the ITS sequences for these isolates with those of known identity and closely related species generated a dataset of 455 characters. Of these, 35 characters were parsimony-informative. Phylogenetic analyses generated eight trees with a length of 161 and a consistency and retention index of 0.870 and 0.611 respectively. One representative tree is presented in Fig. S1.

Susceptibility of *Pinus tecunumanii* progenies

Disease incidence

All 972 trees representing *P. tecunumanii* Low Elevation (LE) (PTLE) progenies in the Graminea trial became naturally infected with *D. septosporum* and the disease incidence ranged from 99.2% to 100% (mean 99.9%). In stands used for comparative purposes, provenances having disease incidences of 100% included *P. tecunumanii* LE (PTEBsuH1 and PTEByucu), and *P. kesiya* (PKcalvH1 and PKcalvH2). Low disease incidence was recorded on *P. maximinoi* (MAXcabH1 (8.33%) and MAXcabH2 (6.66%). High levels of tolerance to infection were recorded in *P. tecunumanii* high elevation (HE)

PTEAcaH1 and TECASaH1, where no symptoms of needle blight were observed (Fig. 4).

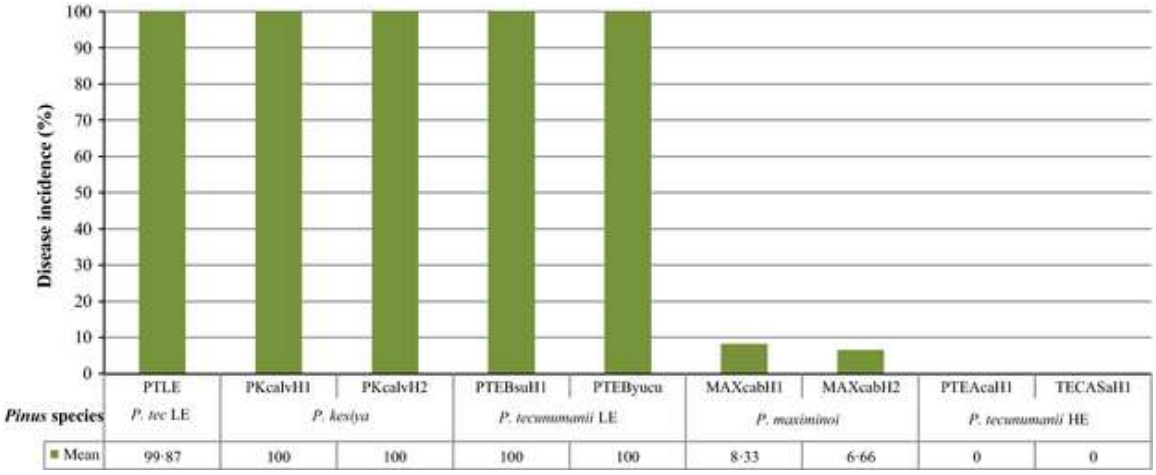


Figure 4 Disease incidence of *Dothistroma septosporum* on different progenies of *P. tecunumanii* LE and other pine species in the Graminea susceptibility trial in the North zone.

Disease severity

Pinus tecunumanii LE (PTLE) progenies differed in the range of severity of damage (Table 2) from 0% to 85% (mean 39.7%). The provenances used for comparison that had similarly high levels of severity included *P. tecunumanii* LE PTEBsuH1 (39.1%), followed by *P. kesiya* PKcalvH2 (38.6%), *P. tecunumanii* LE PTEByucu (37.4%), and *P. kesiya* PKcalvH1 (28.2%). The lowest recorded disease severity was found in the provenances of *P. maximinoi* MAXcabH2 (0.76%) and MAXcabH1 (0.66%). *Pinus tecunumanii* HE provenance TECASaH1 and PTEAcaH1 showed no signs of infection (Table 2). There were significant differences between the mean disease severity observed in *P. tecunumanii* LE (PTLE) progenies and the provenances (MAXcabH1, MAXcabH2, PKcalvH1, PKcalvH2, PTEAcaH1, PTEBsuH1, PTEByucu, TECASaH1) used for comparative purposes ($p<0.05$) (Table 2).

Table 2 Severity of disease caused by *D. septosporum* expressed as a Mean % on 27 different progenies of *P. tecunumanii* LE (PTLE), and provenances of other pine species, in the Graminea susceptibility trial

Pine species	Progeny / Provenance	Disease severity (Mean %[a])	SD[b]	CV[c]	n[d]
P. tecunumanii LE (27 progenies)	PTLE	39.72a	20.45	51.48	633
P. kesiya	PKcalvH1	28.15b	27.20	96.63	33
	PKcalvH2	38.55ab	26.23	68.04	29
P. tecunumanii LE	PTEBsuH1	39.10ab	19.57	50.05	29
	PTEByucu	37.37ab	17.54	46.49	19
P. maximinoi	MAXcabH2	0.76c	2.33	303.89	26
	MAXcabH1	0.66c	2.77	416.02	27
P. tecunumanii HE	PTEAcaH1	0c	0	0	23
	TECASaH1	0c	0	0	29

[a]Duncans multiple range test provided significance levels for the difference between pairs of means calculated between the different progenies of *P. tecunumanii* LE and provenances of other pine species.
[b]Standard Deviation
[c]Coefficient of Variation
[d]Sample number

Diseases impact in different forestry zones

Disease incidence

The differences observed in the mean disease incidence between zones were statistically significant ($p < 0.05$). The highest average disease incidence for 27 plots (98.1%, $n = 27$) was recorded in the Northern zone. This was followed by the Central zone with 96.8% ($n = 46$) infection. The Southern zone had the lowest disease incidence, with an average of 50.6% ($n = 17$; Table 3).

Table 3 Disease incidence of *D. septosporum* on *P. tecunumanii* in three forestry zones

Forestry Area		Disease incidence (Mean %[a])	n
Zone	North	98.09a	27
	Central	96.77a	46
	South	50.64b	17

[a]Duncans multiple range test provides significance levels ($p \leq 0.05$) for the difference between pairs of means. Means per zone followed by different letters were significantly different.

Disease severity

Differences in disease severity between zones and farms were statistically significant ($p<0.05$; Table 4). *P. tecunumanii* in the Northern zone was the most severely affected by DNB with an average disease severity of 42.4% (n = 27) followed by the Central zone with at 33.7% (n = 46). The lowest disease severity (15.7%; n = 17) was recorded in the Southern zone, which had approximately half the disease severity found in the Central zone (Table 4).

For the individual farms, the highest disease severity was found in Argentina (Caldas) and Cedral (Risaralda), both in the North zone with 51.3% (n = 6) and 50.81% (n = 6) respectively. The lowest disease severity levels, 23.3% (n = 13) and 15.7% (n = 17), were recorded for Samaria (Valle del Cauca in the Central zone) and Unión_S (Cauca, in the Southern zone) respectively (Table 4).

Table 4 Disease severity of *D. septosporum* on *P. tecunumanii* calculated in plots of 300 m^2 in 11 farms and three forestry zones

Forestry Area		Severity (Mean %[a])	Number of plots
a) Zone	North	42.38a	27
	Central	33.65b	46
	South	15.68c	17
b) Farms	Yanahuanca	37.97b	3
	Argentina	51.33a	6
	Tesalia	34.80c	12
	Cedral	50.81a	6
	Alaska	37.86c	12
	Esmeralda	34.29c	4
	Volconda	35.05c	5
	La Concha	35.98c	7
	Samaria	23.34d	13
	Unión_B	45.16b	5
	Unión_S	15.68d	17

[a] Severity expressed as mean% with significance levels calculated for the differences in pairs of means using Duncan's mulitple range test.

In the North and Central zones, all four variables (age, elevation, precipitation and year) appeared to have an influence on disease severity (Table 5). The impact of the variables on disease severity, however, differed between zones and were not consistent. In the North, these variables contributed to 25% of the observed disesae severity while in the Central zone, they accounted for as much as 53.2%. Age of trees was related to severity in both the North and Central zones. The South zone included only one farm (Union S) and there was no indication of a relationship between disease severity and age (Table 5).

Table 5 Effect of elevation, precipitation and age of trees on the disease severity in different zones and at different farms

Forestry Area		Variable in model	Adjusted R^2	P[a]	SER[b]
a) Zone	North	Year	15,4	<0.001	15,10
		Elevation	19,7	<0.001	14,70
		Age	21,0	0,016	14,60
		Age.Precipitation	22,6	0,009	14,50
		Precipitation.Elevation	24,0	0,013	14,30
		Age.Elevation	25,1	0,028	14,20
	Central	Age.Elevation	22,4	<0.001	12,00
		Year	25,9	<0.001	11,80
		Elevation	32,0	<0.001	11,30
		Age.Year	44,5	<0.001	10,20
		Age	51,6	<0.001	9,52
		Age.Precipitation	52,8	<0.001	9,40
		Year.Elevation	53,2	0,016	9,36
	South	Age	1,0	0,138	7,93
b) Farm	Yanahuanca	Age	50,2	<0.001	10,50
		Age.Year	51,3	0,187	10,30
		Year	54,5	0,079	10,00
	Argentina	Age.Precipitation	32,0	<0.001	11,40
		Precipitation.Year	38,4	0,005	10,80
	Tesalia	Age.Year	26,0	<0.001	12,00
		Age	28,6	0,023	11,80
		Age.Precipitation.Year	31,9	0,013	11,50
	Cedral	Age	52,2	<0.001	10,80
		Age.Year	55,5	0,02	10,40
		Year	57,8	0,038	10,10
	Alaska	Year	31,9	<0.001	11,00
		Age	49,7	<0.001	9,48
		Age.Precipitation	56,5	<0.001	8,81
		Precipitation.Year	59,5	0,001	8,50
	Esmeralda	Age	25,2	<0.001	6,39
		Age.Year	28,7	0,08	6,25
		Year	52,0	<0.001	5,12
	La Concha	Age.Year	75,8	<0.001	7,93
		Year	83,2	<0.001	6,61
	Samaria	Year	4,5	0,004	7,40
		Precipitation	6,2	0,057	7,33
	Volconda	Age	62,4	<0.001	9,24
	Unión_B	no relationship	n/a	n/a	n/a
	Unión_S	Age	1,0	0,138	7,93

[a] P is the probability of including a variable in the model.
[b] Standard Error of the Regression model.

Colombia has a tropical climate and although the plantations of *P. tecunumanii* were situated at different altitudes, only a minimal level of variation in annual temperature was recorded for each zone (North = 17°C, Central = 20°C and South = 19.2°C). Temperature was considered a constant environmental factor and was, therefore, not included in the stepwise regression analyses.

In the 11 farms analysed, there was no consistent effect of age, precipitation or year on disease severity (Table 5). At farm La Concha the highest adj. R^2 of 83.2% was found

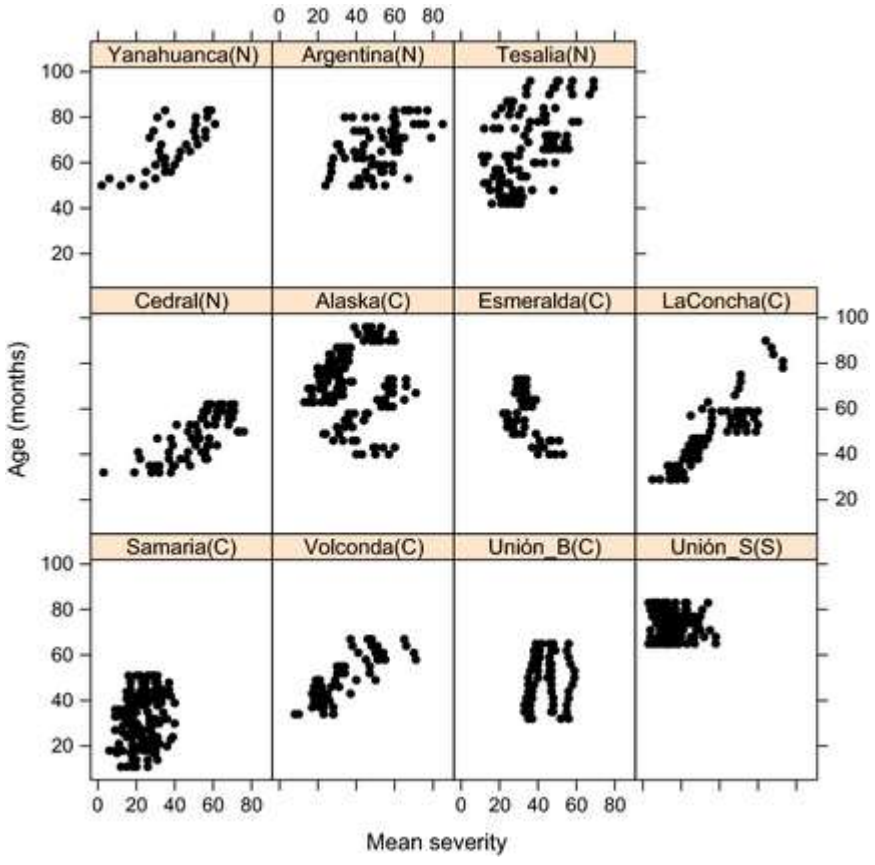


Figure 5 Comparisons between the mean severity (as a %) of *D. septosporum* infection at each of 11 farms in the North (N), Central (C) and South (S) zones with the age of tees at the respective farms. There was no clear correlation between disease severity or tree age at any of the farms.

for a model between severity and age and year. At farm Volconda, age alone accounted for 62.4% of the variation in severity. Age of trees was related to severity at most farms with the exceptions being Samaria, Union B and Union S (Table 5). When the age of trees in months was plotted against mean severity for each farm, no clear pattern or relationship could be determined (Fig. 5)

Discussion

Needle blight first appeared as a serious disease on pines in Colombia in 2008 where it resulted in very severe damage to various *Pinus* spp. DNA sequence comparisons for isolates from all three forestry zones and based on the rDNA ITS regions confirmed the identity of the pathogen as *D. septosporum*. Infection by *D. septosporum* in Colombia appears to occur throughout the year, which is different to the situation in New Zealand, for example, where the defoliation is distinctly seasonal (Bulman *et al.,* 2008). The occurrence of DNB throughout the year and without seasonal patterns in this study is consistent with the fact that high levels of precipitation are favourable for needle infection (Brown *et al.,* 2003).

The climate in large areas of Colombia where pine forestry is practiced, such as those considered in this study, is conducive to DNB outbreaks. For example, the average daily temperature throughout the year for the South, Central and Northern zones was approximately 19°C, 20°C and 17°C respectively. A wide range of temperatures have been reported for *D. septosporum* infection (Gadgil, 1974), but generally long periods (48 hours after inoculation) of needle wetness (Gadgil, 1974; 1977) and warmer temperatures contribute to higher disease severity. A minimum daily average temperature

of 10°C and a long period of high air humidity is necessary for spore production (Dvorak *et al.,* 2012).

Precipitation was exceptionally high throughout the evaluation period and could explain why it was not shown to be a significant variable for disease severity. It is important to record that the La Niña phenomenon occurred during two consecutive years from 2009 and 2011 in Colombia and would have significantly saturated the environment with high moisture and humidity conditions. In 2011 alone, precipitation values ranged from 2712 – 4389 mm at the 11 farms evaluated. Without the effects of temperature and precipitation, the strongest variable contributing to disease severity in this study was age. This is not unexpected when we consider that all the evaluated trees were under the age of eight. Young trees are most susceptible to DNB infection (Gibson 1972) and this is most probably why the year was also considered as an important variable at some farms. Thus, both environmental conditions and biotic factors were all highly conducive to disease development and infection by *D. septosporum* in the Colombian areas considered in this study.

An important element of this study was to confirm the identity of the pathogen associated with DNB in Colombia. Although the disease had been reported in the country previously (Gibson, 1979; 1980), this was based on incidental reports and molecular techniques were not available to confirm the identity of the pathogen. In recent years, there has been some considerable progress in refining the identity of the pathogen (Barnes *et al.,* 2004; Ioos *et al.,* 2010; McDougal *et al.,* 2011) and confirming the differences between *D. septosporum* and *D. pini*. To date, only *D. septosporum* has been found in the Southern Hemisphere and Central America (Groenewald *et al.,* 2007; Barnes *et al*., 2014) and it seemed likely that this would be the species present in Colombia.

Confirmation of the identity of *D. septosporum* in Colombia will now allow for comparisons to be drawn from studies on the pathogen elsewhere.

During the last approximately half century, DNB has emerged as one of the most important constraints to pine plantation forestry in the Southern Hemisphere. This has almost exclusively been associated with the wide-scale plantings of *P. radiata*, which is highly susceptible to the disease (Gibson *et al.,* 1964; Peterson, 1966; Gibson, 1972; Bulman *et al.,* 2008). The disease has lead to the cessation of planting this tree species in many countries, notably in Africa and South America. Many new tree species have been tested and established for plantation development and it is worrying that very little is known relating to their susceptibly to pests and pathogens. The infections leading to DNB that have emerged in Colombia since 2008 and reported in this study keenly reflects this situation. While DNB had been documented on *P. tecunumanii, P. maximinoi* and *P. kesiya* in Central America (Evans, 1984), this is the first situation were large areas of *P. tecunumanii* have been severely damaged by this, or any other serious tree pathogen (Lombard *et al.,* 2009; Steenkamp *et al.,* 2012). This is of concern because the tree is being increasingly planted outside its native range in the tropics and Southern Hemisphere.

The genetic diversity of the pathogen allows *D. septosporum* to adapt in new and changing environments (Dale *et al.,* 2011; Drenkhan *et al*., 2013). Breeding programs can be successful as long as *D. septosporum* is not able to reproduce sexually and generate genetic diversity (Hirst *et al.,* 1999). In this regard, nothing is known about the mating structure of the *D. septosporum* population in Colombia and this is a topic that requires attention. It will also be important to identify species and progenies (such as *P. tecunumanii* HE and *P. maximinoi* in this study) that have natural tolerance to *D. septosporum* in order to avoid high disease incidence in the future. This is especially

important due to the increasing dispersal and impact of *D. septosporum* in native pine environments in the Northern Hemisphere (Drenkhan *et al*., 2013), as well as other continents (Watt *et al.*, 2009).

Dothistroma needle blight poses a serious threat to the future of pine forestry in Colombia. This is because several provenances of *P. tecunumanii* LE, considered to be important for the future of this industry in the country, are clearly highly susceptible. In addition, other species of importance such as *P. oocarpa* and *P. kesiya* also display varying levels of susceptibility to infection. This is of concern, especially considering that planting of the previously important species *P. patula* has been substantially reduced in some areas due to the negative impact of another pathogen, *Sphaeropsis sapinea* (Rodas & Osorio, 2008). However, there are also good prospects to resolve the DNB problem through breeding and selection. For example, in this study we showed that some provenances of *P. tecunumanii* HE showed tolerance to DNB and could be used as a commercial species in areas that are affected by *D. septosporum* but that have optimal elevation ranges for the growth of the tree. Indeed, hybrids between tolerant genotypes of these provenances and *P. patula* already hold promise for the future (M.J. Wingfield, unpublished).

In Colombia, disease incidence and severity was highest in the North and Central zones. During the 3-year evaluation period, and afterwards, trees in the South displayed substantial recovery of foliage and this contributed to low records of mean disease incidence and severity. It was not clear from this study why the disease severity in the South was lower, especially since the environmental conditions and host plant species were the same. One option to resolve this question would be to investigate the population genetics of the pathogen and to determine whether there could be a genetic explanation for the phenomenon. The durability of selected disease-tolerant planting stock would be

negatively affected if both mating types and a high genetic diversity of the pathogen were present in Colombia.

Clearly a great deal of work is required to resolve the DNB problem in Colombia. In addition to breeding and selection for disease tolerance, it will be necessary to better understand the genetics of the pathogen in the country. Such studies and others including those aimed at better understanding the biology of the pathogen in Colombia will be important goals for the future.

Acknowledgements

The authors gratefully acknowledge L. Perafan, M. Zapata and Marie F. Smith (stats4science, Pretoria) for providing statistical support. We thank Smurfit Kappa Cartón de Colombia, the Department of Science and Technology (DST), the National Research Foundation (NRF) and the THRIP initiative of the Department of Trade and Industry, South Africa for financial support. We also thank the Claude Leon Foundation for supporting a Postdoctoral Fellowship for Irene Barnes. This study would also not have been possible without the support of the Tree Protection Co-operative Programme (TPCP), based at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Authors have declared no conflict of interest.

References

Alzamora RM, Hauer P, Peredo H, 2004. Evaluación de pérdidas de volumen comercial de *Pinus radiata* por efecto de *Dothistroma septospora* en distintos escenarios de manejo y control químico, en la provincia de Valdivia [Evaluation of commercial volume losses to *Pinus radiata* caused by *Dothistroma septospora* under varying forest management and chemical control conditions in the province of Valdivia]. *Bosques* **25**, 15-27.

Barnes I, Roux J, Wingfield MJ, Coetzee MPA, Wingfield BD, 2001. Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. *Plant disease* **85**, 317-321.

Barnes I, Crous PW, Wingfield BD, Wingfield MJ, 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma, D. septosporum* and *D. pini*. *Studies in Mycology* **50**, 551-565.

Barnes I, Wingfield MJ, Carbone I, Kirisits T, Wingfield BD, 2014. Population structure and diversity of an invasive pine needle pathogen reflects anthropogenic activity. *Ecology and Evolution* **18,** 3642-3661.

Brown A, Rose D, Webber J, 2003. Red Band Needle Blight of Pine. *Forestry Commission Information Note (FCIN)* **49**, 1-6.

Bulman L, Ganley RJ, Dick M, 2008. Needle diseases of radiata pine in New Zealand. Scion, New Zealand Forest Research Institute Ltd.

Dale AL, Lewis KJ, Murray BW, 2011. Sexual reproduction and gene flow in the pine pathogen *Dothistroma septosporum* in British Columbia. *Phytopathology* **101**, 68-76.

Devey ME, Groom KA, Nolan MF, Bell JC, Dudzinski MJ, Old KM, Matheson AC, Moran GF, 2004. Detection and verification of quantitative trait loci for resistance to Dothistroma needle blight in *Pinus radiata. Theoretical and Applied Genetics* **108**, 1056-1063.

Draper N, Smith H, 1981. Applied Regression Analysis (2nd Ed.). John Wiley & Sons, New York, pp. 709.

Drenkhan R, Hantula J, Vuorinen M, Jankovský L, Müller MM, 2013. Genetic diversity of *Dothistroma septosporum* in Estonia, Finland and Czech Republic. *European Journal of Plant Pathology* **136,** 71-85.

Dvorak M, Drapela K, Jankovsky L, 2012. *Dothistroma septosporum*: spore production and weather conditions. *Forest Systems* **2**, 323-328.

Dvorak WS, Hodge GR, Gutiérrez EA, Osorio LF, Malan FS, Sanger TK. 2000. *Pinus tecunumanii*. In: Conservation and Testing of Tropical and Subtropical Forest Species by the

CAMCORE Cooperative. College of Natural Resources, NCSU. Raleigh, NC. USA. pp: 188-209.

Evans HC, 1984. The genus *Mycosphaerella* and its anamorphs *Cercoseptoria Dothistroma and Lecanosticta* on pines. Surrey U.K.: Commonwealth Agricultural Bureaux. *CMI Mycological Paper* **153**, 1-102.

Gadgil PD, 1974. Effect of temperature and leaf wetness period on infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand Journal of Forestry Science* **4**, 495-501.

Gadgil PD, 1977. Duration of leaf wetness periods and infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand Journal of Forestry Science* **7**, 83-90.

Gibson IAS, 1972. Dothistroma blight of *Pinus radiata. Annual Review of Phytopathology* **10**, 51-72.

Gibson IAS, 1979. Pests and diseases of pines in the tropics. *European Journal of Forest Pathology* **9**, 126-127.

Gibson IAS, 1980. Two pine needle fungi new to Colombia. *Tropical Pest Management* **26**, 38-40.

Gibson IAS, Christensen PS, Munga FM, 1964. First observations in Kenya of a foliage disease of Pines caused by *Dothistroma pini* Hulbary. *Commonwealth Forestry Review* **43**, 31-48.

Groenewald M, Barnes I, Bradshaw RE, Brown AV, Dale A, Groenewald JZ, Lewis KJ, Wingfield BD, Wingfield MJ, Crous PW, 2007. Characterization and distribution of mating type genes in the Dothistroma needle blight pathogens. *Phytopathology* **7**, 825-834.

Hirst P, Richardson TE, Carson SD, Bradshaw RE, 1999. *Dothistroma pini* genetic diversity is low in New Zealand. *New Zealand Journal of Forestry Science* **29**, 459-472.

Hoyos C, 1987. Determinación del agente causal de la muerte descendente del *Pinus patula* en el Valle del Cauca [Determination of the causal agent of dieback of *Pinus patula* in the Cauca Valley]. Medellín, Colombia: Universidad Nacional de Colombia, Tesis pregrado.

Ioos R, Fabre B, Saurat C, Fourrier C, Frey P, Marçais B, 2010. Development, comparison, and validation of real-time and conventional PCR tools for the detection of the fungal pathogens causing brown spot and red band needle blights of Pine. *Phytopathology* **100**, 105-114.

Ivory MH, 1987. Diseases and disorders of Pines in the tropics. A field and laboratory manual. Overseas development administration by the Oxford Forestry Institute.

Lombard L, Rodas CA, Crous PW, Wingfield BD, Wingfield MJ, 2009. *Calonectria* (*Cylindriocladium*) species associated with dying *Pinus* cuttings. *Persoonia* **23**, 41-47.

Mackay W, Mackay E, 1986. Las hormigas de Colombia: Arrieras del género *Atta* (Hymenoptera: Formicidae) [Leaf-cutting ants of Colombia: *Atta* genus (Hymenoptera: Formicidae)]. *Revista Colombiana de Entomología* **12**, 23-30.

Madrigal A, Abril G, 1994. Biología y hábitos del insecto-palo (*Libethroidea inusitata* Hebard) defoliador del *Pinus patula* en Antioquia [Biology and habits of the defoliator stick-insect (*Libethroidea inusitata* Hebard) of *Pinus patula* in Antioquia]. *Crónica Forestal y del Medio Ambiente* **19**, 25-36.

MADR (Ministry of Agriculture and Rural Development), 2006. Colombia: A land of opportunity for investment in forestry. Colombia, 1-17.

MADR (Ministry of Agriculture and Rural Development), 2010. Balance of Government, achievements and challenges of the Agricultural sector. 2002-2010. Dynamic balance of the Colombian agriculture in the last eight years. Colombia, 1-40.

McDougal RL, Schwelm A, Bradshaw RE, 2011. Dothistromin biosynthesis genes allow inter- and intraspecific differentiation between Dothistroma pine needle blight fungi. *Forest Pathology* **41**, 407-416.

Payne RW (Ed.), 2014. Introduction to GenStat® *for Windows*™ (17th Edition), VSN International, Hemel Hempstead, Hertfordshire, UK. Http://www.genstat.co.uk/.

Peterson GW, 1966. Penetration and infection of Austrian and Ponderosa pine by *Dothistroma pini*. *Phytopathology* **56**, 894-895.

Rodas CA, 1994. *Chrysomima semilutearia* (Felder & Rogenhofer) Nuevo defoliador de plantaciones forestales en Colombia [*Chrysomima semilutearia* (Felder & Rogenhofer) New defoliator of forest plantations in Colombia]. Smurfit Carton Colombia **164**, 1-7.

Rodas CA, Osorio E, 2008. Segundo informe: Afección de *Diplodia* en plantaciones de *Pinus patula* [Infection of *Diplodia* in *Pinus patula* plantations]. Smurfit Kappa Carton Colombia 1-9.

Steenkamp E, Rodas CA, Kvas M, Wingfield MJ, 2012. *Fusarium circinatum* and pitch canker of *Pinus* in Colombia. *Australasian Plant Pathology* **41**, 483-491.

Swofford DL, 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Vélez RA, 1972. El defoliador del ciprés *Glena bisulca* Ringde [The cypress defoliator, *Glena bisulca* Ringde]. *Revista Facultad Nacional de Agronomía* **29**, 7-8.

Watt MS, Kriticos DJ, Alcaraz S, Brown AV, Leriche A, 2009. The hosts and potential geographic range of Dothistroma needle blight. *Forest Ecology and Management* **257**, 1505-1519.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, with TJ (eds.). PCR Protocols: A sequencing guide to methods and applications. San Diego: Academic Press.

Wiesner RL, Madrigal CA, 1983. Primary insect pests of Cypress, *Pinus patula* and Eucalyptus in Colombia. In: First international seminar of forestry insect pest management. SOCOLEN-FUNDEF. Medellín, Colombia, 1-33.

Supporting Information

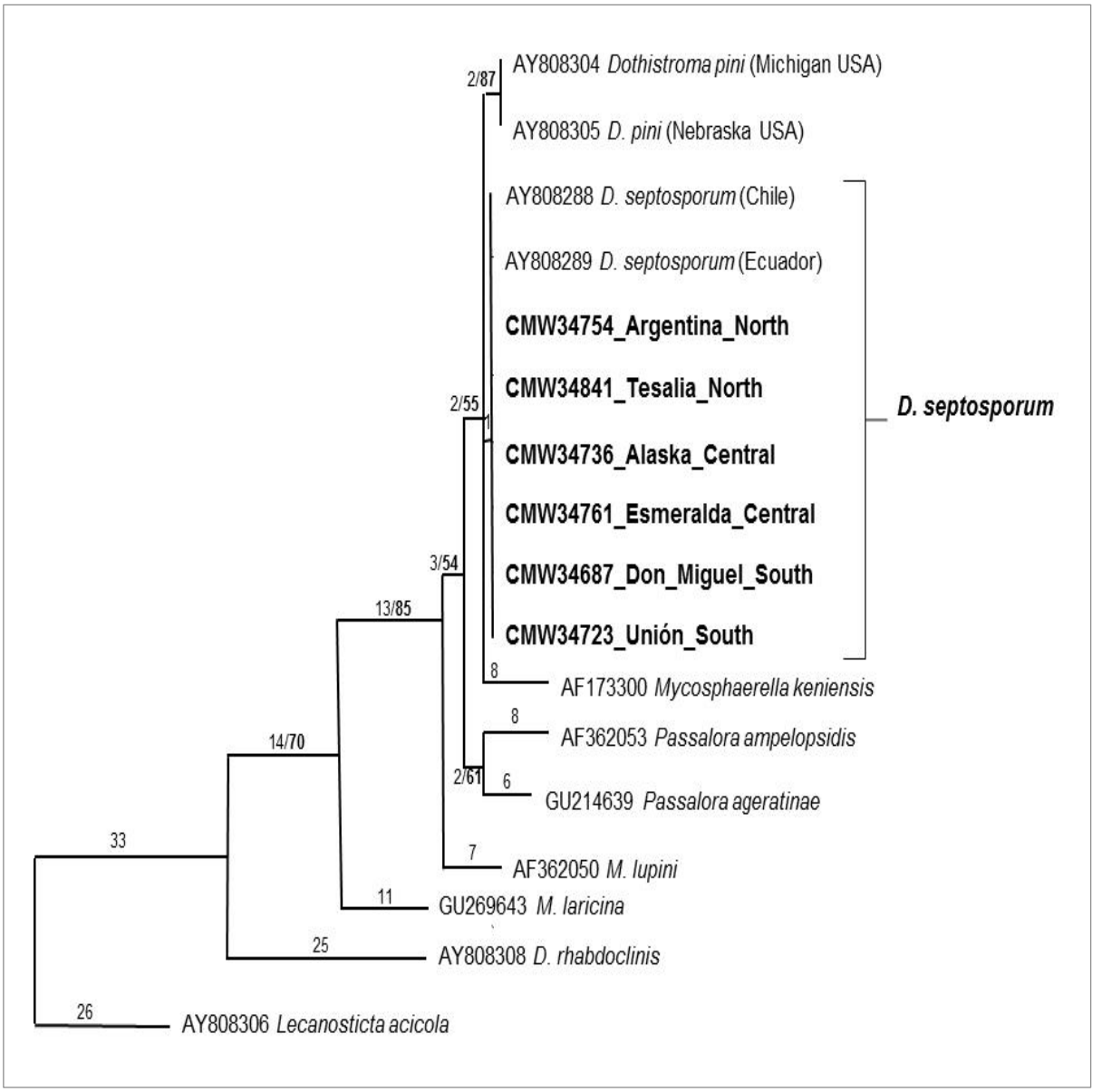


Figure S1 Phylogenetic tree showing the placement of the isolates from Colombia in the *Dothistroma septosporum* clade. From a data set of 455 aligned characters of the ITS region, 35 were parsimony informative and phylogenetic analysis generated 8 trees with length of 161 and a consistency and retention index of 0.870 and 0.611, respectively.