

Factors influencing infection of eucalypts by *Cylindrocladium pteridis*

R. N. Graça^a, A. C. Alfenas^{a*}, L. A. Maffia^a, M. Titon^a, R. F. Alfenas^a, D. Lau^b and J. M. A. Rocabado^c

^aDepartment of Plant Pathology, Universidade Federal de Viçosa, Viçosa, MG, 36570-000; ^bEmbrapa Trigo, Passo Fundo, RS, 99001-970; and ^cUniversidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, 28015-620, Brazil

The pattern of *Cylindrocladium pteridis* adhesion, germination and penetration in eucalypt leaves was assessed using scanning electron microscopy. The effects of inoculum concentration, leaf wetness period, plant age and branch position of cylindrocladium leaf blight and defoliation severity were assessed in greenhouse studies using two *Eucalyptus grandis* × *E. urophylla* hybrid clones. Penetration occurred through stomata, and there was no difference in the number of penetrations between young and old leaves. Percentage leaf area with lesions and defoliation increased with the increase in inoculum concentration (1×10^2 to 10^5 conidia mL⁻¹), duration of leaf wetness period (6 to 48 h) and plant age (60 to 180 days). Branch position in plants also significantly affected the percentage leaf area with lesions and defoliation, the latter variable being significantly higher at the stem base. The highest values of lesion area were also observed on leaves at the stem base in both clones. The Pearson correlation between defoliation and leaf area with lesions was significant in all experiments ($r > 0.9$) indicating a high association between these two variables.

Keywords: *Calonectria pteridis*, cylindrocladium leaf blight, defoliation of eucalypt, *Eucalyptus grandis* × *E. urophylla* hybrids

Introduction

Cylindrocladium species infect a variety of host species and may cause significant losses, especially under tropical and subtropical conditions (Bertus, 1976; Alfenas, 1986; Dianese, 1986; Booth *et al.*, 2000; Cedeño & Carrero, 2000; Poltroniere *et al.*, 2004). Several species of this fungus infect eucalypts (*Eucalyptus* and *Corymbia*) causing diseases such as seedling damping off, root rot, stem canker and leaf blight (Mohanan & Sharma, 1985; Alfenas, 1986; Ferreira, 1989; Crous *et al.*, 1993; Booth *et al.*, 2000; Cedeño & Carrero, 2000; Alfenas *et al.*, 2004). Leaf blight and defoliation caused by *Cylindrocladium pteridis* (teleomorph: *Calonectria pteridis*) is one of the main leaf diseases found in Brazilian eucalypt plantations in warm, high rainfall regions (Ferreira, 1989; Booth *et al.*, 2000; Ferreira & Milani, 2002; Alfenas *et al.*, 2004).

Cylindrocladium pteridis was first described in 1926 as the causal agent of brown spot of the iron fern, *Polystichum adiantiforme* in Florida (Wolf, 1926). This pathogen was first reported in Brazil in 1975 (Hodges *et al.*, 1975) in Bahia and again in 1986 (Dianese, 1986) in Pará, causing

needle blight in *Pinus caribaea* var. *hondurensis*. In 1981, the fungus was reported to cause leaf spot of coconut palm (*Cocos nucifera*) in Maranhão (Silva & Souza, 1981) and in the same year it was isolated from soil samples collected from *Crotalaria* sp. in the Federal District-Brazil (Almeida & Bolkan, 1981).

The first report of *C. pteridis* in eucalypt was in the United States at the end of the 1960s when it was found in *Eucalyptus cinerea* (Sobers, 1968). In Brazil *C. pteridis* was first described on eucalypts in 1995 causing blight and defoliation on one-year-old plants of *E. grandis* (Ferreira *et al.*, 1995). The disease is currently widely distributed throughout the country and affects eucalypt plants most severely from six months to 2–3 years after planting (Alfenas *et al.*, 2004). The disease is characterized by initially small, round, light grey leaf lesions that progress to light brown coalesced spots that can occupy the entire leaf, resulting in intense defoliation in susceptible genotypes (Ferreira *et al.*, 1995). Based on the results of an artificial pruning at different levels of *E. grandis* plants (Pulrolnik, 2005), it is believed that defoliation caused by this fungus can reduce volumetric plant growth and facilitate growth of invasive plants because the increased entrance of light in the sub canopy leads to increasing competition between eucalypt and understorey plants.

*E-mail: aalfenas@ufv.br

Field observations of natural *C. pteridis* infections and controlled greenhouse inoculations indicate that resistance in eucalypts can be quantitative and there appears to be a positive correlation between increase in leaf age and susceptibility to disease (unpublished data). These factors make it difficult to separate genetic and phenotypic resistance. Similar phenomena occur in other pathosystems (Maiero, 1990; Riaz *et al.*, 1991; Stevenson, 1994; Chang & Hwang, 2003; Visker *et al.*, 2003).

Despite the fact that *Cylindrocladium* spp. are important eucalypt pathogens, few studies have evaluated the factors influencing eucalypt infection (Bolland *et al.*, 1985; Sharma & Mohanan, 1990). Knowledge of the pathogenesis of *C. pteridis* in eucalypts (from penetration to multiplication), as well as of the factors that influence these processes, are basic prerequisites for the morphological and cytochemical investigations necessary to understand mechanisms and gene expression involved in resistance. Furthermore, development of appropriate techniques for selection of resistant genotypes, including definition of the ideal inoculum concentration and leaf wetness period after inoculation, the disease distribution pattern in the plant and the developmental stage of inoculated plants with symptoms, is essential for differentiating responses of *Eucalyptus* spp. genotypes to *C. pteridis*.

Given these considerations, the objectives of this study were to determine conditions favourable for infection of *Eucalyptus grandis* × *E. urophylla* hybrids by *C. pteridis*, the effect of plant and tissue phenological stage on infection by *C. pteridis* and the mode of penetration by *C. pteridis* in the host tissue.

Materials and methods

Plant material

Sixty-day-old cuttings of two *Eucalyptus grandis* × *E. urophylla* hybrid clones (6021 and 9882), widely planted in Brazil, were grown in 8 L pots containing a mixture of soil : sand : bovine manure (3:1:1). The mixture was sterilized with methyl bromide and supplemented with 1 g L⁻¹ super phosphate (33% of P₂O₅) before use. The plants were kept in a greenhouse and fertilized twice weekly with 100 mL of a 7.5 g L⁻¹ Ouro Verde® solution (15% N, 15% P₂O₅, 20% K₂O) until reaching the suitable stage for inoculation, approximately 120 days after transplanting. Over the course of the experiments minimum temperature in the greenhouse varied from 17 to 22°C and maximum temperature from 28 to 36°C. In the mist chamber the temperature varied from 21 to 26°C.

Source and storage of *C. pteridis* isolate

A monosporic *Cylindrocladium pteridis* (PF-1) isolated from diseased plants in a commercial eucalypt plantation in Lençóis Paulista, São Paulo Brazil was used in all experiments. This isolate was identified as *C. pteridis* based on morphological features as previously described (Crous, 2002). It was maintained on colonized oat flakes at -80°C.

Inoculum production

Oat flakes colonized with mycelia of *C. pteridis* were transferred to Petri dishes containing oat-dextrose agar to produce inoculum. After transfer, the dishes were kept at 25 ± 1°C, under a 12 h photoperiod and 20 μmols photons s⁻¹m⁻² of light intensity until the mycelium covered the surface of the dishes. After this period, the fungal cultures were submitted to physical stress in order to stimulate conidial production as previously used for *A. solani* (Rodriguez, 2003; Scheuermann, 2006). For this, 20 mL of sterilized distilled water were added to the culture surface which was then scraped with a sterilized paint brush to remove all aerial mycelia present. Thereafter, an additional 10 mL of sterile distilled water were added to each dish. The culture remained submerged for two days, after which the water was discarded and the culture was blotted dry using sterile gauze. The culture was maintained under laboratory conditions (mean temperature 25°C) for approximately two days until sporulation occurred on the culture medium surface. Inoculum suspensions were prepared by adding 20 mL distilled water + 0.05% Tween 20 to the culture surface followed by scraping with a sterilized, soft-bristled paint brush to release conidia. Suspensions were filtered through a double layer of sterile gauze and adjusted to the desired conidial concentration, which varied with the assay performed.

Penetration of *C. pteridis* in eucalypts

In order to characterize the pattern of adhesion, germination and penetration of *C. pteridis* in eucalypt leaves, two old leaves (the second leaf pair near the main stem located on the second branch, counted from the base to apex), and two young leaves (the first leaf pair counted from the youngest shoot of the penultimate branch, from the base to apex) of each of three plants of clone 6021 were randomly collected. Leaf petioles were wrapped in moist cotton and placed in a moisture chamber (Gerbox + moist foam + nylon screen) at 25°C. The leaves were inoculated by dropping 10 μL of a 1 × 10⁴ conidia mL⁻¹ suspension on the adaxial or abaxial surface. Five fragments (4 mm²) of each inoculated leaf were collected 6 h after inoculation (one fragment = one experimental unit). The fragments were set in glutaraldehyde (4%, buffered with 0.1 M sodium cacodylate, pH 7) for 2 h at room temperature and washed three times for 20 min with sodium cacodylate buffer. The fragments were then post-fixed in osmium tetroxide (OsO₄ 2%) for 1 h, at 5°C. After fixing, fragments were washed three times at 15 min intervals with sodium cacodylate, dehydrated in an alcohol series (30, 50, 70, 80, 95 and 100%), and dried using a critical point dryer (Balzers, model CPD020, BAL-TEC AG). The fragments were mounted on a metal support and then covered with gold using a cathodic sprayer in a sputter coater coupled to a freeze-drying unit (Balzers, FDU010 BAL-TEC AG). The sample was examined using a scanning electron microscope (LEO, model 1430VP, Carl Zeiss) operated at

variable acceleration ranging from 10 to 20 kv. The number of stomata, conidia, germ tubes, penetrations per stoma and direct penetrations in the central area (0.09 mm^2) of each of the five fragments were counted. The data are presented as a number of events per mm^2 of leaf area.

Severity of leaf blight and defoliation using different *C. pteridis* inoculum concentrations

The effect of inoculum concentration on disease development was studied by inoculating eucalypts (clones 6021 and 9882) with suspensions of 1×10^2 , 1×10^3 , 1×10^4 and 1×10^5 conidia mL^{-1} . Plants at 120 days after transplanting were atomized with a 5 L sprayer equipped with a fan type nozzle with pressure regulator to evenly cover the abaxial and adaxial surfaces of all leaves. After inoculation the plants were kept in a mist chamber during 48 h under 40% ambient light and then transferred to the greenhouse. A completely randomized experimental design was used with five plants (replicates) per treatment. Five uninoculated plants of each clone were held under the same conditions already described for use as controls.

The percentage of leaves dropped (defoliation) was evaluated in all branches present in the basal, median and apical thirds of the plants at 0, 10, 20, 30, 40 and 50 days after inoculation. Only main branches were considered and secondary shoots were not evaluated. Leaf-lesioned area was quantified 7 days after inoculation by means of digital imaging of the second (old) and penultimate (young) leaf pairs from the base of branches located in the middle of the stem base (lower third of the plant). The images obtained were analyzed using the software Quant (Vale *et al.*, 2003) to estimate percentage leaf area with lesions. The area under the disease progress curve (AUDPC) was estimated by plotting percentage defoliation over time.

Severity of leaf blight and defoliation caused by *C. pteridis* under different leaf wetness periods

To evaluate the effect of leaf wetness period on infection by *C. pteridis*, plants of clone 6021 at 120 days after transplanting were inoculated with suspensions of 1×10^4 conidia mL^{-1} . After inoculation the plants were maintained in the mist chamber for 0, 6, 12, 24 and 48 h, at 40% ambient light and then transferred to the greenhouse. A completely randomized experimental design was used with five plants (replicates) per treatment. The pathogen inoculation process and evaluation of disease severity were performed according to the methods used in the study of the effect of inoculum concentration.

Severity of leaf blight and defoliation in plants of different ages inoculated with *C. pteridis*

In order to evaluate the effect of eucalypt plant age on infection by *C. pteridis*, 60-day-old cuttings of clones 6021 and 9882 were transplanted 60, 120 and 180 days before inoculation. The plants were inoculated with a

suspension of 1×10^4 conidia mL^{-1} and held in a mist chamber for 48 h under 40% ambient light and then transferred to the greenhouse. A completely randomized experimental design with five replicates (plants) per treatment was used. As control, five non-inoculated plants of each clone (transplanted at the same intervals and held under the same conditions as inoculated plants), were used. Pathogen inoculation and evaluation of disease severity were performed according to the methods used in the study of the effect of inoculum concentration.

Distribution of leaf blight and defoliation caused by *C. pteridis* in the plant

To characterize the distribution pattern of leaf blight and defoliation induced by *C. pteridis* in eucalypts, five plants of each clone 6021 and 9882 at 90 days after transplanting were inoculated with a suspension of 1×10^4 conidia mL^{-1} , held in a mist chamber for 48 h under 40% ambient light, and then transferred to the greenhouse. The main stem was divided by height into intervals of 0–20, 20–40, 40–60, 60–80, 80–100 and above 100 cm and defoliation was examined on all branches in each height interval at 0, 10, 20, 30, 40 and 50 days after inoculation. Only main branches were considered and secondary shoots were not evaluated. Leaf area with lesions was quantified 7 days after inoculation using a digital image analysis of all leaves on branches situated in the middle section of each plant third (basal, median and apical). The images obtained were analyzed using the Software Quant (Vale *et al.*, 2003) to estimate the percentage leaf area with lesions. The AUDPC was estimated using the percentage defoliation. The average incubation period in each plant third was calculated as the amount of time between inoculation and appearance of disease symptoms in at least one leaf in 50% of the branches in each plant third. Evaluations were carried out 6 and 12 h after inoculation and at 12 h intervals thereafter. The latent period was determined in leaves on branches in each plant third by observing vegetative or reproductive structures of the pathogen using a pocket magnifying glass (20× magnification). A completely randomized experimental design was used with five replicates (one plant = one experimental unit).

Statistical analysis

Statistical analyses were carried out using the SAS statistical package (The SAS Institute Inc., Version 6.01). Values of leaf area with lesions, defoliation and AUDPC were submitted to analysis of variance.

The effects of leaf age (old and new leaves) and leaf surface (abaxial and adaxial) were based on means and standard deviations of adhesion, germination and penetration events and the number of stomata observed in the scanning electron microscopy analyses.

Regression analyses were performed on leaf area with lesions and defoliation from the experiments on branch position on the stem, inoculum concentration and leaf wetness period after inoculation. Regression models were

selected based on coefficients of determination and mean square, normality and homogeneity of residuals. Since in all experiments young leaves presented low leaf area with lesions, average disease severity in the old leaf pair of the branches evaluated was used in statistical analyses. Similarly, leaf fall was not observed in the median and apical thirds of the plants and therefore only the values of defoliation measured in the basal third of the plant were used in statistical analyses.

Means of leaf area with lesions and defoliation were compared using Fisher's LSD ($P \leq 0.5$) in the experiment to evaluate plant age.

Pearson correlation analyses were performed on the average values of leaf area with lesions and defoliation in all experiments.

For each study (penetration, inoculum concentration, leaf wetness period, plant age, and symptom distribution in the plant), two independent experiments were conducted. Three and five replications per treatment were used in the first and second experiments, respectively. Since the data showed the same trend, only data from the second experiment for each study is presented.

Results

Penetration of *C. pteridis* in eucalypts

Six hours after inoculation all conidia observed possessed at least one germ tube, regardless of the treatment. Each conidium produced on average two germ tubes which frequently branched and developed along the epidermis with no visibly defined orientation (Fig. 1a,b), until finding a stoma and penetrating it. On average, 10 adhered, germinated conidia and three stomatal penetration events were observed on the abaxial leaf surface of young leaves inoculated with *C. pteridis*. On the adaxial leaf surface of leaves of the same age, approximately five adhered germinated conidia were observed, but no penetration events were found. The same pattern occurred in old leaves, with 15 adhered conidia and three stomatal penetration events observed on average on the abaxial surface and approximately four adhered, germinated conidia but no penetration events on the adaxial surface. Although not quantified, it was observed that young and old leaves differed in size, and usually the old were larger. All penetrations observed occurred through stomata (Fig. 2). Neither appressorial development nor direct penetration was observed. In the central area of the leaf fragments evaluated, stomata were observed only on the abaxial leaf surface, independent of leaf age (Fig. 2). However, a few stomata were observed in the adaxial leaf surface outside the area used to assess germination and penetration events. Swelling of the germ tube (Fig. 1g,i) and formation of appressorium-like structures over stomata that were closed at the time of penetration (Fig. 1f) were observed. Penetration occurred in open stomata without altering germ tube morphology (Fig. 1d,e,k). A germ tube growing over an open stoma without penetrating it was observed (Fig. 1h).

Table 1 Analysis of variance of area under the disease progress curve (AUDPC), defoliation and leaf area with lesions for experiments with *Cylindrocladium pteridis* on eucalypts: (1) inoculum concentration, (2) leaf wetness period, (3) plant age and (4) distribution pattern of the disease symptoms in the plant

Source	df	F value	P
1. Inoculum concentration			
1.1. AUDPC			
Clone	1	30.48	< 0.0001
Conc.	4	81.15	< 0.0001
Clone * Conc.	4	4.04	0.0078
1.2. Defoliation			
Clone	1	33.11	< 0.0001
Conc.	4	81.43	< 0.0001
Clone * Conc.	4	4.45	0.0047
1.3. Leaf area with lesions			
Clone	1	0.02	0.8967
Conc.	4	61.91	< 0.0001
Clone * Conc.	4	0.04	0.9967
2. Leaf wetness			
2.1. AUDPC			
Wet. period	4	13.29	< 0.0001
2.2. Defoliation			
Wet. period	4	24.35	< 0.0001
2.3. Leaf area with lesions			
Wet. period	4	23.54	< 0.0001
3. Plant age			
3.1. AUDPC			
Clone	1	2.32	0.1411
Age	2	122.55	< 0.0001
Clone * Age	2	0.98	0.3915
3.2. Defoliation			
Clone	1	5.67	0.0256
Age	2	185.71	< 0.0001
Clone * Age	2	1.93	0.1671
3.3. Leaf area with lesions			
Clone	1	10.64	0.0033
Age	2	43.48	< 0.0001
Clone * Age	2	2.51	0.1021
4. Symptoms distribution			
4.1. AUDPC			
Clone	1	0.93	0.3389
Height	5	25.34	< 0.0001
Clone * Height	5	0.72	0.6109
4.2. Defoliation			
Clone	1	2.78	0.1023
Height	5	38.38	< 0.0001
Clone * Height	5	0.91	0.4808
4.3. Leaf area with lesions			
Clone	1	3.08	0.0922
Height	2	38.55	< 0.0001
Clone * Height	2	5.28	0.0125

Severity of leaf blight and defoliation with different *C. pteridis* inoculum concentrations

Analysis of variance showed a significant effect ($P \leq 0.01$) of clone, inoculum concentration and clone × concentration on defoliation and AUDPC (Table 1), indicating different responses of genotypes to concentrations of inoculum

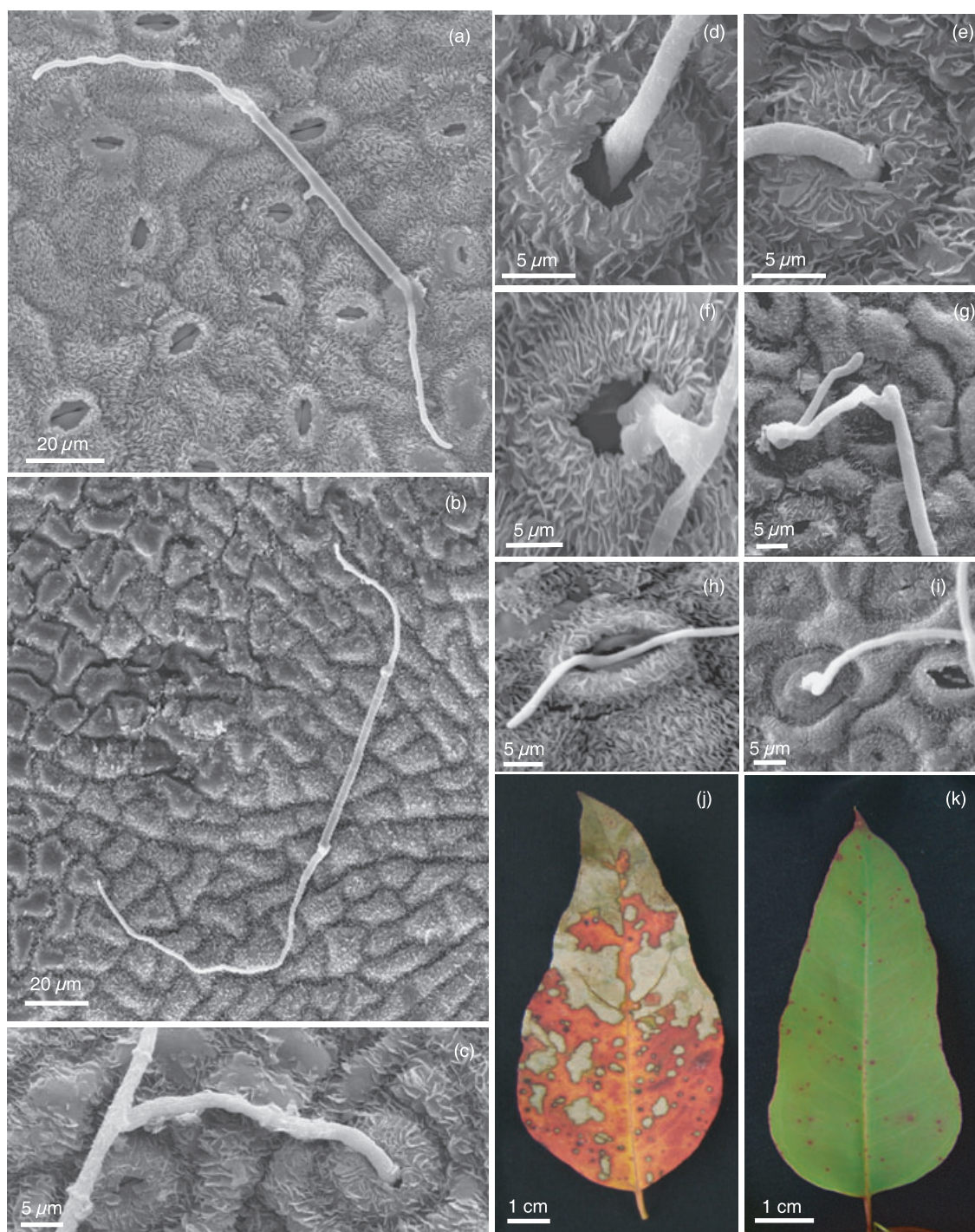


Figure 1 Germination and penetration of *Cylindrocladium pteridis* in eucalypt leaves shown by scanning electron microscope: (a) conidial germination on abaxial and (b) adaxial surface; (c, d, e) penetration through an open stoma with no morphological alteration of the germ tube; (f) appressorium-like structure over a closed stoma; (g) tip of a germinated conidium and penetration through stoma with swelling and emission of a new germ tube; (h) germ tube growing over an open stoma; (i) penetration in immature stoma with swelling of the tip of the germ tube. (Young leaf – a, b, c, g, h, i; old leaf – d, e, f). Eucalypt leaves inoculated with *C. pteridis*: (j) expanded leaf exhibiting large area with lesions; (k) young leaf showing minute spots.

used. A significant effect ($P \leq 0.01$) on leaf area with lesions was only observed for inoculum concentration.

Defoliation at 50 days after inoculation increased with the increase in inoculum concentration (Fig. 3a). A linear

relationship between inoculum concentration and percentage defoliation was observed for the two clones tested. However, at all concentrations the average percentage defoliation in clone 6021 was lower than in

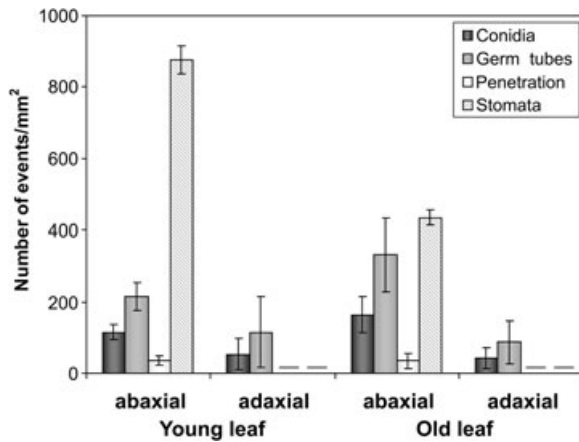


Figure 2 Number of conidia, germ tubes and stomatal penetrations by *Cylindrocladium pteridis* and number of stomata per mm² of leaf area in the abaxial and adaxial surfaces of young and old leaves of *Eucalyptus grandis* × *E. urophylla* hybrid clone (6021), at 6 h after inoculation, observed with a scanning electron microscope. Values correspond to mean of five replicates. Error bars correspond to ± standard deviation of the mean.

clone 9882. At the highest inoculation concentration used, average defoliation on plants of clones 6021 and 9882 was about 35 and 54%, respectively. The lowest percent defoliation, 4.9% for clone 6021 and 12.6% for clone 9882, occurred at the lowest inoculum concentration tested (Fig. 3a).

No significant effect of clone on leaf area with lesions was found and therefore mean values for the two clones were used for statistical analyses (Table 1). Lesioned leaf area increased with the increase in inoculum concentration (Fig. 3b). Old leaves from plants inoculated with suspensions of 1×10^2 and 1×10^3 conidia mL⁻¹ had 1.5 and 10% leaf area with lesions, respectively, with the percentage increasing to 29.5 and 56% when the plants were inoculated with suspensions of 1×10^4 and 1×10^5 conidia mL⁻¹, respectively.

The Pearson correlation coefficient ($r = 0.83$, $n = 5$) between percentage leaf area with lesions and defoliation indicated a positive association between the two variables used to assess disease severity. The AUDPC increased with the increase in inoculum concentration for both clones. Clone 6021 showed lower AUDPC values, regardless of the concentration used. The control plants, inoculated with water and Tween 20, showed no disease symptoms.

Severity of leaf blight and defoliation caused by *C. pteridis* under different leaf wetness periods

Defoliation, leaf area with lesions and AUDPC were significantly affected ($P \leq 0.01$) by wetness period after inoculation (Table 1). Defoliation was not observed in the median and apical thirds of the plants in any treatment and defoliation and leaf area with lesions values were therefore obtained only from the basal third.

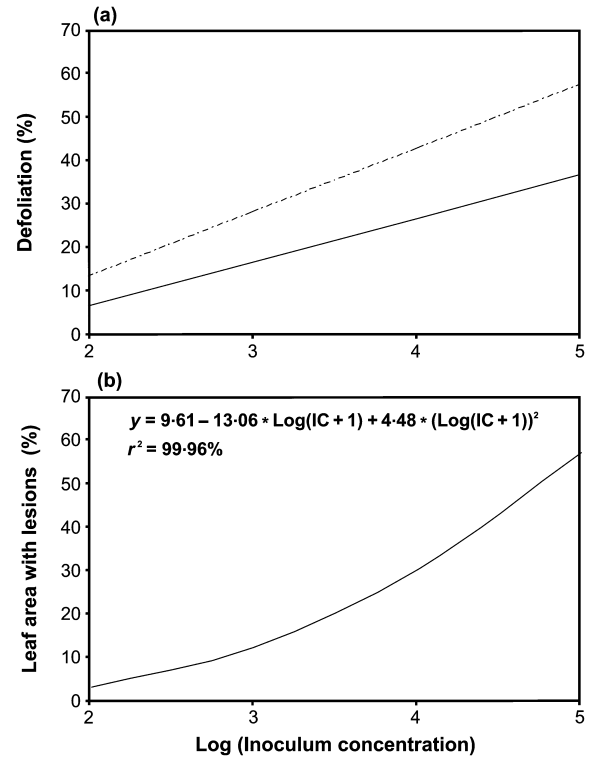


Figure 3 (a) Percentage defoliation in branches from the basal third of plants of two hybrid clones of *Eucalyptus grandis* × *E. urophylla* (9882, dashed line [$y = -16.17 + 14.71 \cdot \text{Log}(IC + 1)$; $r^2 = 94.45\%$] and 6021, solid line [$y = -13.64 + 10.08 \cdot \text{Log}(IC + 1)$; $r^2 = 98.32\%$]) inoculated with different inoculum concentrations (IC) of *Cylindrocladium pteridis*, at 50 days after inoculation (d.a.i.). (b) Mean leaf area with lesions as a function of inoculum concentration measured in the second pair of completely expanded leaves (old leaves), situated on the middle branch of the basal third of 6021 and 9882 plants, at 7 d.a.i. with *C. pteridis*.

There was an increase in disease severity (defoliation and lesioned leaf area) with an increase in wetness period (Fig. 4a,b). At 50 days after inoculation, defoliation reached 29.9 and 44.5% in plants kept in the mist chamber for 24 and 48 h, respectively, whereas average percentage defoliation was only 5.1 and 3.7% in plants kept in the mist chamber for 6 and 12 h, respectively.

Old leaves, on branches of the lower third of plants kept in the mist chamber for 6 and 12 h, presented the same percentage leaf area with lesions (5.1% on average). When the wetness period increased to 24 and 48 h, leaves of the same phenological stage had 20 and 26% leaf area with lesions, respectively.

The Pearson correlation coefficient ($r = 0.99$, $n = 5$) between leaf area with lesions and defoliation indicated a high association between the two variables used to assess disease severity. The AUDPC increased with increase in leaf wetness period. Control plants inoculated and kept under greenhouse conditions without mist did not develop disease symptoms.

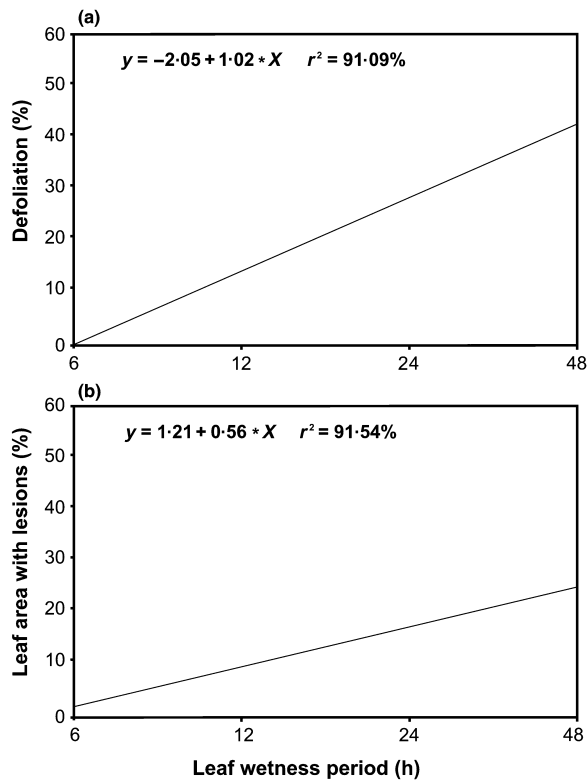


Figure 4 (a) Percentage defoliation on branches of the basal third of plants of hybrid clone 6021 of *Eucalyptus grandis* × *E. urophylla* exposed to different leaf wetness periods, measured 50 days after inoculation (d.a.i.) with *Cylindrocladium pteridis*. (b) Percentage leaf area with lesions in the second pair of fully expanded leaves (old leaves) situated on the middle branch of the basal third of 6021 plants, measured 7 d.a.i. with *C. pteridis*.

Progress of leaf blight and defoliation in plants of different ages inoculated with *C. pteridis*

The clone × age interaction did not significantly affect leaf area with lesions, defoliation or the AUDPC. However all the variables evaluated were significantly affected ($P < 0.01$) by time of transplant (age) before inoculation (Table 1). Genotype had a significant effect on percentage leaf area with lesions and defoliation but not on AUDPC values. Since defoliation was not observed in the median and apical thirds in any treatment, disease severity was assessed only in branches in the basal third of the plants.

Defoliation varied significantly according to the age of the plants of both clones and was higher in older plants (Fig. 5a). Sixty-day-old plants showed no defoliation.

Leaf area with lesions of both clones was similar for 120 and 180-day-old plants (Fig. 5b). Old leaves from 180-day-old plants presented 18.5 and 28.3% leaf area with lesions, in clones 6021 and 9882, respectively. The average percentage leaf area with lesions in 120-day-old plants in clones 6021 and 9882 were 15.5 and 26.4%, respectively. Sixty-day-old plants presented the lowest leaf area with lesions values, 0.97 and 1.2%, respectively, for clones 6021 and 9882.

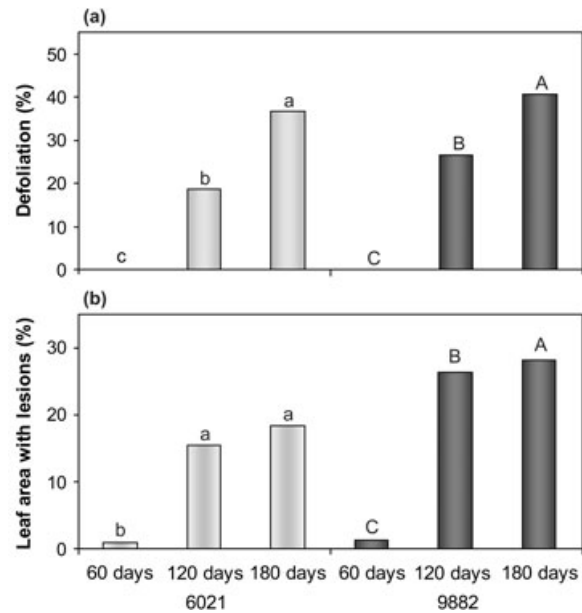


Figure 5 (a) Percentage defoliation on branches of the basal third of different aged plants of two *Eucalyptus grandis* × *E. urophylla* hybrid clones (6021 and 9882), measured 50 days after inoculation (d.a.i.) with *Cylindrocladium pteridis*. (b) Percentage of leaf area with lesions measured in the second pair of totally expanded leaves (old leaves) situated in the middle branch of the basal third of plants of different ages of 6021 and 9882 clones, measured 7 d.a.i. with *C. pteridis*. Means followed by the same letter do not differ by the Fisher test (LSD; $P \leq 0.05$).

The Pearson correlation coefficient ($r = 0.92$, $n = 5$) between leaf area with lesions and defoliation indicated a high degree of association between the two variables used to assess disease severity.

The highest AUDPC values were observed for 180-day-old plants. Sixty-day-old plants did not defoliate and therefore had null AUDPC values. Control plants for each time after transplanting did not exhibit any disease symptoms.

Distribution of leaf blight and defoliation caused by *C. pteridis* in the plant

A significant interaction was found between clone and branch position (clone × height) for leaf area with lesions, but not for defoliation and AUDPC (Table 1). Branch position along the main stem significantly affected all variables evaluated.

Defoliation was significantly higher in branches located near the base of the plants tending to zero toward the apex, regardless of the clone inoculated (Fig. 6a). Branches located 0 to 20 cm above the base of the plant of clones 6021 and 9882 presented average defoliation of 20.8 and 25.7%, respectively. No defoliation was observed above a height of 80 cm in either clone (Fig. 6a).

As for defoliation, the greatest values of leaf area with lesions were found in leaves from branches on the lowest

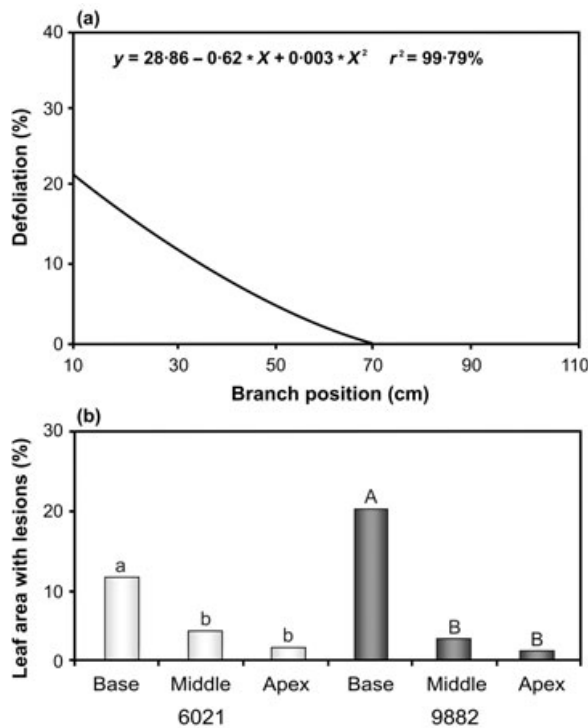


Figure 6 (a) Defoliation as a function of branch position along the stem in plants of two hybrid clones (6021 and 988) of *Eucalyptus grandis* × *E. urophylla*, measured 50 days after inoculation (d.a.i.) with *Cylindrocladium pteridis*. Branch position: 10 = 0 to 20, 30 = 20 to 40, 50 = 40 to 60, 70 = 60 to 80, 90 = 80 to 100 and 110 = above 100 cm. (b) Mean of leaf area with lesions in branches at different positions (base, middle and apex) of 6021 and 9882 plants, at 7 d.a.i. with *C. pteridis*. Means followed by the same letter do not differ by the Fisher test (LSD; $P \leq 0.05$).

third of the plant, independently of the clone evaluated. The leaf area with lesions in leaves from the median and apical regions was significantly lower than for the basal region (Fig. 6b).

A high Pearson correlation coefficient ($r = 0.97$, $n = 5$) between leaf area with lesions and defoliation indicated a high association between the two variables used to assess disease severity.

The greatest values of AUDPC were observed in branches near the base of the plants. Branches above 80 cm height exhibited no defoliation and thus presented zero AUDPC values.

The average incubation period was 36 h, independent of height and clone evaluated (Fig. 7a,b). At 48 h after inoculation, all plant branches had at least one infected leaf, regardless of the clone. No pathogen structures were observed at the end of the evaluation and it was therefore not possible to determine the latent period of the disease.

Discussion

Scanning electron microscope analysis showed a tendency of no difference in number of penetrations by germ tubes

of *C. pteridis* between young and old leaves, although more stomata were found in young leaves. Therefore the resistance in young leaves is probably related to mechanisms that act after pathogen penetration. The difference observed between number of penetrations on the abaxial and adaxial surfaces of young and old leaves may be explained by the fact that penetration of *C. pteridis* occurs exclusively through stomata on the abaxial surface, regardless of leaf age. No stomata were observed on the central area of the fragments from the adaxial leaf surface of the hybrid clone studied (*E. grandis* × *E. urophylla*). However, a few stomata were observed outside the area assessed. This pattern of stomatal distribution was also found in *Eucalyptus microcorys* (Sharma & Mohanan, 1990).

The same type of leaf penetration was observed in a *Cylindrocladium quinqueseptatum* – *E. microcorys* pathosystem, in which penetration occurred only via stomata (Bolland *et al.*, 1985). In contrast, Sharma & Mohanan (1990) concluded that leaf penetration by *C. quinqueseptatum* in *E. grandis* was mainly through epidermal cells, although a pressorium formation and penetration through stomata were infrequently observed.

Leaf blight and defoliation of eucalypts caused by *C. pteridis* can be influenced by host genotype, inoculum concentration, leaf wetness period after inoculation, plant age and phenological leaf stage. The present study has demonstrated that all these variables significantly affect disease severity.

Increasing inoculum concentration linearly increased defoliation values in both *E. grandis* × *E. urophylla* hybrid (6021 and 9882) clones. However, average percentage defoliation in clone 6021 was lower than in clone 9882 at all concentrations tested. Leaf area with lesions also increased with increasing inoculum concentration, but no significant differences were observed between the clones. It is believed that at high inoculum concentrations a greater number of penetrations occur, resulting in greater disease severity. Similar results to those found in this study were reported for other pathosystems (Dillard, 1989; Chakraborty, 1990; Chang & Hwang, 2003).

Plants of both clones inoculated with 1×10^4 conidia mL⁻¹ or higher displayed similar symptoms to those observed in the field. However, at the highest inoculum concentration several lesions were observed on the main stem and branches which are not commonly found in natural conditions, except in high levels of infection. These stem and branch lesions eventually formed mini-cankers that culminated in broken branches.

The severity of leaf blight and defoliation in eucalypts caused by *C. pteridis* was directly proportional to leaf wetness period. Inoculated plants incubated for 6 and 12 h in a mist chamber presented very low leaf area with lesions and defoliation; at least 24 h of leaf wetness was necessary to produce satisfactory disease symptoms. Leaf wetness mainly affects spore germination, during which water absorption and hydrolytic enzyme activation occurs.

The highest disease severity was found with a 48 h leaf wetness period. As found in the scanning electron

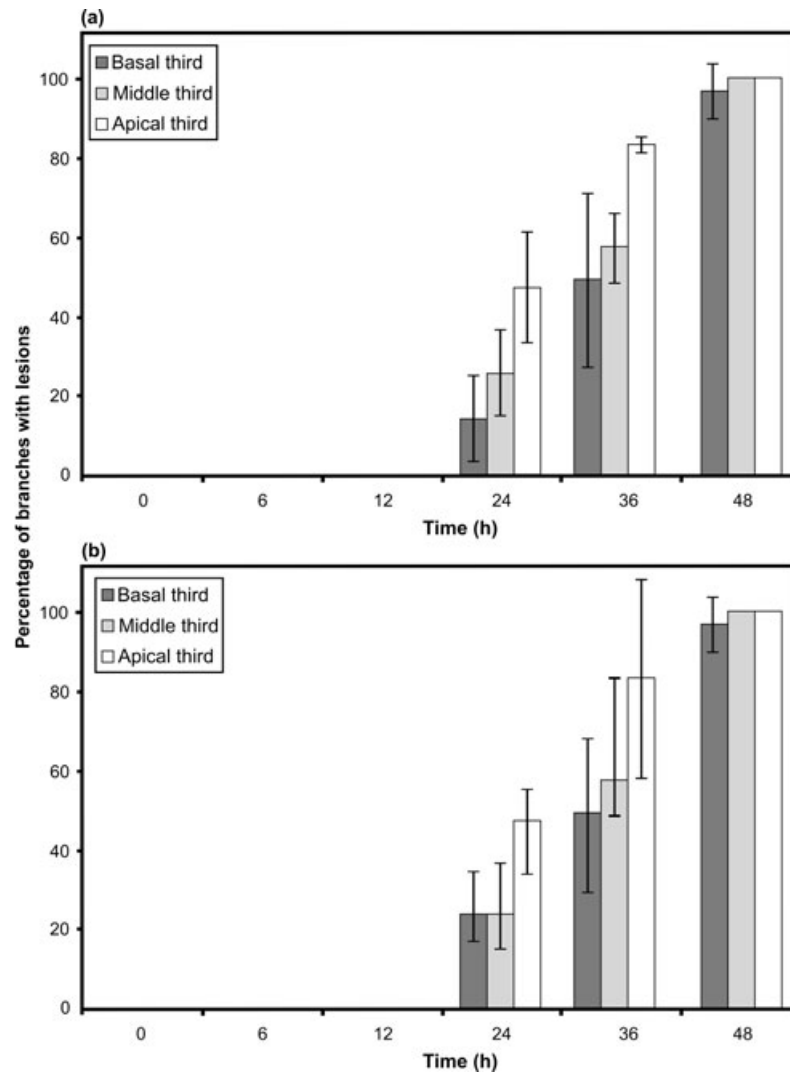


Figure 7 Percentage of branches with symptoms in the basal, middle and apical parts of plants of two hybrid clones, a) 6021 and b) 9882, of *Eucalyptus grandis* \times *E. urophylla* after 0–48 h inoculation with *Cylindrocladium pteridis*. Error bars correspond to \pm standard deviation of the mean.

microscopy studies, germ tubes of *C. pteridis* grow on the leaf surface and then penetrate through stomata. Thus, under long leaf wetness periods a higher number of germ tubes probably reach the penetration sites, resulting in increased disease severity. Free water was also essential for conidial germination of *C. quinqueseptatum* and disease progression on *E. microcorys* leaves (Bolland *et al.*, 1985). This fungus was also severely pathogenic on clove seedlings under conditions of high humidity, but in a dry atmosphere, development of disease was abruptly decreased (Reitsma & Sloof, 1950).

In this study, plant age significantly affected *C. pteridis* infection in both clones. Older plants were more susceptible than younger plants. While 180-day-old plants had the highest levels of defoliation, independent of the clone, 60-day-old plants showed no defoliation and lower leaf area with lesions in both clones. Plants of clone 6021 presented lower disease severity, regardless of age.

The effect of age on disease progression has been the focus of many studies (Pfender, 2004; Whalen, 2005).

Very young potato (*Solanum tuberosum*) plants are susceptible to *Phytophthora infestans*, while potato plants of intermediate age are resistant and old plants (more than 70 days after planting) become susceptible again (Mooi, 1965; Fry & Apple, 1986; Pfender, 2004). Young adlay plants (*Coix lacryma*) inoculated with *Bipolaris coicis* presented less severe disease than older plants (Chang & Hwang, 2003). Although there are still no conclusive explanations for the influence of age on eucalypts infection by *C. pteridis*, it is believed that relatively older plants that possess a greater number of old leaves will be more predisposed to infection. Therefore, plant age at the time of inoculation should be considered in studies of this pathosystem.

In the present study, it was found that branch position along the main stem significantly affected values of leaf area with lesions and defoliation, which were both significantly greater in branches at the base of the stem in both clones. The leaf area with lesions was also higher in leaves at the base of the plants, regardless of the clone

evaluated. Defoliation was not observed above 80 cm height in either clone. A similar pattern of disease distribution was previously reported on *E. microcorys* plants infected by *C. quinqueseptatum* (Bolland et al., 1985).

Older leaves have been found to be more susceptible to disease than younger leaves in other pathosystems, in agreement with the results of the present study. It was believed that basal leaves of potato plants in the field would be more severely infected by *P. infestans* because of the more favourable microclimate in the lower portions of the plant (Harrison, 1992). However, inoculation of this oomycete in potato detached leaves showed that old leaves are more susceptible to blight than young leaves, regardless of the plant architecture or environmental conditions (Visker et al., 2003). Young potato (Rodriguez, 2003) and tomato (*S. lycopersicum*) (Scheuermann, 2006) plant leaves are more resistant to *Alternaria solani* due to a higher number of cells per unit area and probably a greater quantity of transcripts related to the plant defence response.

Percentage leaf area with lesions and defoliation were significantly greater in the basal third of the plants, in both clones and in all experiments. No defoliation was observed and percentage leaf area with lesions was very low in the median and apical plant parts. Based on these results, basal leaves of branches and plants at the same age should be evaluated in studies on the *C. pteridis* – *Eucalyptus* spp. pathosystem.

Leaf area with lesions correlated positively to defoliation in all assays. Therefore, evaluation of either of these variables will lead to similar and consistent results. However, defoliation is considered the best variable for estimating disease severity in eucalypts since it is more easily and rapidly assessed.

In this study, a screening technique was developed to select resistant genotypes of eucalypts to cylindrocladium leaf blight, caused by *C. pteridis* and other studies involving inoculation of the pathogen. These findings may also be applied to other *Cylindrocladium* species that infect eucalypts.

Acknowledgements

We are grateful to Dr Charles S. Hodges for review of this manuscript. We thank the Federal University of Norte Fluminense and the Aracruz Celulose S/A for permitting access to laboratories and equipment necessary to carry out scanning electron microscopy. This work was supported by CNPq (National Counsel of Technological and Scientific Development).

References

Alfenas AC, 1986. Fungos do gênero *Cylindrocladium* como patógenos florestais no Brasil. *Fitopatologia Brasileira* **11**, 275–7.

Alfenas AC, Zauza EA, Mafia RG, Assis TF, 2004. *Clonagem e Doenças do Eucalipto*. Viçosa, MG, Brazil: Editora UFV.

Almeida OC, Bolkan HA, 1981. Ocorrência e distribuição o gênero *Cylindrocladium* no Distrito Federal. *Fitopatologia Brasileira* **6**, 223–8.

Bertus AL, 1976. *Cylindrocladium scoparium* Morgan on Australia native plants in cultivation. *Journal of Phytopathology* **85**, 15–25.

Bolland L, Tierney JW, Tierney BJ, 1985. Studies on leaf spot and shoot blight of eucalypts caused by *Cylindrocladium quinqueseptatum*. *European Journal of Forest Pathology* **15**, 385–97.

Booth TH, Jovanovic T, Old KM, Dudzinski MJ, 2000. Climatic mapping to identify high-risk areas for *Cylindrocladium quinqueseptatum* leaf blight on eucalypts in mainland South East Asia and around the world. *Environmental Pollution* **108**, 365–72.

Cedeño L, Carrero C, 2000. *Cylindrocladium pteridis* causando manchas foliares en eucaliptos de Portuguesa-Venezuela. *Revista Florestal Venezolana* **44**, 101–6.

Chakraborty S, 1990. Expression of resistance to *Colletotrichum gloeosporioides* and *Stylosanthes scabra* at different inoculum concentrations and day-night temperatures. *Australasian Journal of Agricultural Research* **41**, 89–100.

Chang SW, Hwang BK, 2003. Effects of plant age, leaf position, inoculum density, and wetness period on *Bipolaris coicis* infection in Adlays of differing resistance. *Plant Disease* **84**, 821–6.

Crous PW, 2002. *Taxonomy and Pathology of Cylindrocladium (Calonectria) and Allied Genera*. St Paul, MN, USA: APS Press.

Crous PW, Phillips AJL, Wingfield MJ, 1993. New records of *Cylindrocladium* and *Cylindrocladiella* spp. in South Africa. *Plant Pathology* **42**, 302–5.

Dianese JC, 1986. Problemas patológicos das florestas plantadas do Vale do Rio Jarí. *Fitopatologia Brasileira* **11**, 274–5.

Dillard HR, 1989. Effect of temperature, wetness duration, and inoculum density on infection and lesion development of *Colletotrichum coccodes* on tomato fruit. *Phytopathology* **79**, 1063–6.

Ferreira FA, 1989. *Patologia Florestal: Principais Doenças Florestais no Brasil*. Viçosa, MG, Brazil: Sociedade de Investigações Florestais.

Ferreira FA, Alfenas AC, Moreira AM, Demuner NL, 1995. Mancha-de-pteridis doença foliar de eucalipto em áreas tropicais brasileiras. *Fitopatologia Brasileira* **20**, 107–10.

Ferreira FA, Milani D, 2002. *Diagnose Visual e Controle das Doenças Abióticas and Bióticas do Eucalipto no Brasil*. Viçosa, MG, Brazil: Universidade Federal de Viçosa.

Fry WE, Apple AE, 1986. Disease management implications of age-related changes in susceptibility of potato foliage to *Phytophthora infestans*. *American Journal of Potato Research* **63**, 47–56.

Harrison JG, 1992. Effects of the aerial environment on late blight of potato foliage, a review. *Plant Pathology* **41**, 384–416.

Hodges CS, Reis MS, Ferreira FA, 1975. Uma nova enfermidade de acículas de pinus no Brasil causada por *Cylindrocladium pteridis*. *Brasil Florestal* **6**, 8–11.

Maiero M, 1990. Inheritance of collar rot resistance in the tomato breeding lines c1943 and NC EBR-2. *Phytopathology* **80**, 1365–8.

- Mohan C, Sharma JK, 1985. *Cylindrocladium* causing diseases of *Eucalyptus* in Kerala, India. *Transactions of the British Mycological Society* **84**, 538–9.
- Mooi JC, 1965. Experiments on testing field resistance to *Phytophthora infestans* by inoculating cut leaves of potato varieties. *European Potato Journal* **8**, 182–3.
- Pfender WF, 2004. Role of phenology in host susceptibility and within-plant spread of stem rust during reproductive development of perennial ryegrass. *Phytopathology* **94**, 308–16.
- Poltroniere LS, Alfenas AC, Zauza EA, Trindade DR, Poltroniere MC, 2004. *Cylindrocladium pteridis* leaf spot of apricot tree in the state of Pará, Brazil. *Fitopatologia Brasileira* **29**, 104.
- Pulrolnik K, Reis GG, Reis MGF, Monte MA, Fontan ICI, 2005. Crescimento de plantas de clone de *Eucalyptus grandis* (Hill ex Maiden) submetidas a diferentes tratamentos de desrama artificial, na região do cerrado. *Revista Árvore* **29**, 495–505.
- Reitsma J, Sloof WC, 1950. Leaf diseases of clove seedlings caused by *Gloeosporium piperatum* E. & E. and *Cylindrocladium quinqueseptatum* Boedijn & Reitsma. *Contributions of General Agriculture Research Station, Bogor* **109**, 50–9.
- Riaz M, Bockus WW, Davis A, 1991. Effects of wheat genotype, time after inoculation, and leaf age on conidia production by *Drechslera tritici-repentis*. *Phytopathology* **81**, 1298–301.
- Rodriguez MAD, 2003. *Componentes da Resistência de Batata a Alternaria solani*. Viçosa, MG, Brazil: Universidade Federal de Viçosa, PhD thesis.
- Scheuermann KK, 2006. *Identificação de Genes Diferencialmente Expressos na Interação Tomateiro – Alternaria solani*. Viçosa, MG, Brazil: Universidade Federal de Viçosa, PhD thesis.
- Sharma JK, Mohan C, 1990. Studies on conidial germination of *Cylindrocladium quinqueseptatum*, causing leaf blight of eucalypts in India. *European Journal of Forest Pathology* **20**, 15–23.
- Silva GS, Souza EAP, 1981. Mancha foliar do coqueiro causada por *Cylindrocladium pteridis* Wolf. *Fitopatologia Brasileira* **6**, 515–7.
- Sobers EK, 1968. Morphology and host range of *Cylindrocladium pteridis*. *Phytopathology* **58**, 1265–70.
- Stevenson WR, 1994. The potential impact of field resistance to early blight on fungicide inputs. *American Potato Journal* **71**, 317–24.
- Vale FXR, Fernandes Filho EI, Liberato JR, 2003. QUANT. A software plant disease severity assessment. In: *Proceedings of the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2003*. 105.
- Visker MHPW, Keizer LCP, Budding DJ, Van Loon, LC, Colon LT, Struik PC, 2003. Leaf position prevails over plant age and leaf age in reflecting resistance to late blight in potato. *Phytopathology* **93**, 666–74.
- Whalen MC, 2005. Host defense in a developmental context. *Molecular Plant Pathology* **6**, 347–60.
- Wolf FA, 1926. Brown leaf spot of leather leaf fern. *Journal of the Elisha Mitchell Scientific Society* **42**, 55–62.