Original Article

Susceptibility of Clonal Seedlings of *Eucalyptus* spp. to Powdery Mildew Disease

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ABSTRACT

Powdery mildew is one of the main diseases in eucalyptus clonal nursery. The present work aimed to confirm the identity of the causal agent of powdery mildew in clonal seedlings of *Eucalyptus* spp., and to evaluate the susceptibility reaction of clones to this disease. Conidia were collected in minigardens for molecular identification of the pathogen. We assessed susceptibility by testing 4 clones: Clone 1 (*Eucalyptus benthamii*), Clone 2 (*E. dunni*), Clone 3 (*E. benthamii*) and Clone 4 (*E. urophylla* x *E. globulus*). Scale of classes was used for calculations of Disease Index (DI) and Final Incidence (I). Temporal analysis was performed using the Logistic and Gompertz models. The molecular identification confirmed the identity of the isolates approximate to *Podosphaera pannosa* (teleomorphic phase of *Oidium eucalypti*). The Gompertz model obtained a better adjustment to the severity and incidence data, and clones 1 and 2 showed greater susceptibility to eucalyptus powdery mildew.

Keywords: clonal nursery, *Oidium eucalypti*, incidence.

1. INTRODUCTION

Powdery mildew diseases are caused by fungi that present powdery mycelium, and conidia that develop into chains on the surface of attacked tissues. In species of the genus *Eucalyptus*, it is considered a cosmopolitan disease reported in South Africa, Argentina, Brazil, Australia, Europe and the United States (Silva et al., 2001). It is also considered the main disease in nurseries, vegetation greenhouses and clonal mini-gardens of several species of the genus (Valeriano et al., 2015). Alfenas et al. (2009) reported that this pathogen has often been found on plants in nurseries, clonal mini-gardens with temporary flood hydroponics, or in drip irrigation tubes, where there is little or no leaf wetting.

The fungus spreads through wind, water splashes and by contact between infected and healthy plants (Furtado et al., 2000). Mucci et al. (1980) relate the disease to the etiological agent *Oidium eucalypti* Rostr. Krugner & Auer (2005) have identified *Sphaerotheca pannosa* (synonym: *Podosphaera pannosa*) by cross-inoculation, confirming the identity as *Oidium eucalypti*.

The proper identification of the pathogen species is a key step to understand all pathogen-host cycle relations. The knowledge of such interactions is essential, not only to understand the disease itself, but mainly to create control strategies aiming to interfere with its cycle (Bergamin & Amorim, 1996). The identification of phytopathogenic fungi is often based on morphological characteristics, but in many genera such characteristics are difficult to be observed in natural populations and are often affected by the environment (Faleiro et al., 2003).

According to Lima et al. (2008), the ideal classification should be based on the phylogeny of microorganisms. One of the most used regions for molecular identification of fungi is the so-called Internal Transcribed Spacer (ITS), because this region has more conserved phylogenetically sequences and, therefore, is more suitable for biodiversity studies (Reis et al., 2006). In addition, this is the most frequent region in most databases of fungal sequences.

Comparative epidemiology aims to identify similarities or differences between epidemics based on the nature or the behavior of the disease progression curve. The disease progress curve integrates the effects of the pathogen, the host and the environment into a single graph. The selection of an appropriate mathematical model to describe the disease progress curve is an important aspect of the temporal analysis, and such selection aims to estimate parameters used for a statistical analysis comparing curves (Madden et al., 2007). The interpretation of curve shapes and the determination of their components, such as the initial inoculum of the disease, progress rate, final index of the disease and area under the progress curve, are essential for the control of epidemics (Bergamin, 1995).

Within this context, knowledge of the epidemiology of *Oidium eucalypti* is key to establishing control measures at the right time, using adequate techniques to manage the disease. Thus, this study aims to confirm the identity of the causal agent of powdery mildew disease in clonal seedlings of *Eucalyptus* spp., and to evaluate the susceptibility of *Eucalyptus* spp. clones to the causal agent of the disease.

2. MATERIAL AND METHODS

This study was conducted at the Laboratory of Bacteriology of the Department of Phytosanitary of the Faculty of Agronomy of the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, between 2015 and 2016. We used seedlings from four distinct genetic materials, two clones of *Eucalyptus benthamii*, one clone of *E. dunnii* and one clone of *E. urophylla* x *E. globulus* obtained by donation from the company Celulose Riograndense CMPC, located in the municipality of Guaíba, RS. The material was collected in the Horto Barba Negra (30°20'41.8" S, 51°14'42.2" W), in the municipality of Barra do Ribeiro, RS.

Initially, the fungus was morphologically characterized through the evaluation of 200 conidia (Boesewinkel, 1980; Braun, 1987; Gorter, 1988) of *Eucalyptus* spp. with natural infection. The conidia collected in a same clone were considered as a single isolate. Microscopic slides of sporulated colonies were used to observe the shape, length and width of conidia in an Olympus optical microscope (400 X, MD50) containing an ocular with a micrometric ruler.

For the molecular identification of the causative agent of eucalyptus powdery mildew, the conidia of infected ministrains were sucked using a vacuum pump and transferred to Eppendorf microcentrifuge tubes

containing 70% ethanol. The isolates were identified according to the collection performed on each clone, and sent to the Biological Institute of São Paulo for DNA extraction and sequencing. Pathogen DNA extraction was performed according to the CTAB method as described by Doyle & Doyle (1991). Extracted genomic DNA samples were submitted to Polymerase Chain Reaction (PCR) for amplification of the rDNA ITS region with the primers ITS1 and ITS4 (White et al., 1990). The nucleotide sequences obtained were compared with those available on the *GenBank* for isolated pathogens. The sequences presenting the highest scores were selected and aligned, along with sequences obtained in the ClustalW algorithm sequencing. Phylogenetic analysis was conducted with 1,000 replicates by the software MEGA, version 6.0 (Tamura et al., 2013). The similarity of nucleotide sequences among isolates was calculated using the Basic Local Alignment Search Tool (BLAST) procedure from the National Center for Biotechnology Information (NCBI). A sequence of grapevine powdery mildew (*Uncinula necator*) was used as the "sister group" for the construction of the phylogenetic dendrogram.

To maintain the inoculum for the experiments, we collected leaves of the eucalyptus ministrains containing powdery mildew (source of inoculum) in the clonal mini-garden of the company that supplied the material. Then, we performed the inoculation by scraping the conidia off the leaves surface using a brush with soft bristles, and transferring the conidia of the fungus to the adaxial surfaces of the upper third of the ministrains leaves. These inoculated ministrains were kept in a growth chamber at 25 °C with a 12-h photoperiod as a permanent source of inoculum for subsequent inoculations.

The susceptibility reaction test was performed with clone seedlings (obtained by minicutting) from four clones: clone 1 (*Eucalyptus benthamii*), clone 2 (*E. dunni*), clone 3 (*E. benthamii*) and clone 4 (*E. urophylla* x *E. globulus*) at about 90 days of age. Each treatment had 40 seedlings of each clone, divided into 4 replicates of 10 seedlings. The inoculation of seedlings was performed as described in the previous paragraph for the inoculation of ministrains. The inoculated seedlings were kept in a growth chamber at 25 °C with a photoperiod of 12 h for 29 days. The irrigation of seedlings and ministrains was performed daily and manually using a graduated wash bottle, dampening only the substrate.

The experiment was completely randomized. In order to evaluate Incidence (I), the number of seedlings showing symptoms of *Oidium eucalypti* was counted weekly in each treatment.

We established a scale for severity assessment using a descriptive scale containing four classes: 0 = absence of symptoms, 1 = mild infection (presence of fungus mycelium in leaves without sporulation), 2 = intermediate infection (sporulation on the first leaf pairs), and 3 = high infection (leaf deformation, winding of the first pair of leaves and leaf fall). The scores of this descriptive scale were used to calculate the disease index (DI), in a range of 0-1, expressed by Equation 1:

$$DI = \frac{\sum(Y*X_y)}{X_t*h} \qquad (1)$$

where: Y is the scale score; X_y is the number of plants with a note Y; X_t is the total number of plants; and h is the maximum value of the scale (McKinney, 1923).

For the temporal analysis of the behavior of the disease for each clone, the variables I and DI were submitted to a non-linear adjustment in relation to time for each clone. For this, the following recommended models for polycyclic diseases were used: Logistic and Gompertz. These models are composed of three parameters: y_0, the initial disease index; y_{max}, the maximum (asymptotic) disease index; and r, the progress rate of the epidemic. The models were adjusted using the non-linear method and the PROC NLIN routine of the statistical package SAS 9.4®. The most appropriate model was chosen taking into account the highest value of the coefficient of determination (R^2). The estimated parameters of the models were compared by t test between clones in pairs taking into account their errors (Madden et al., 2007). A correlation analysis of the residues was also used to relate the DI to I data (Spolti et al., 2015).

In addition, I and DI data were analyzed by mixed models using the repeated measures method. The covariance structure that presented the best fit was selected according to Akaike and Bayesian information criteria (Silva et al., 2015). The means of the final evaluation, at 29 days, were compared by Tukey test at 5% probability.

3. RESULTS AND DISCUSSION

The causal agent of eucalyptus powdery mildew disease in the clones evaluated in this study was confirmed as *Oidium eucalypti,* by molecular identification (sequencing of

the ITS region). The four sequenced isolates were molecularly identical, presenting a 516 bp fragment size. Therefore, we opted to present only one sequence. It aligned to sequences in the *GenBank* corresponding to the species *Podosphaera pannosa* (Figure 1). Silva & Alfenas (1994) reported that eucalyptus powdery mildew is similar to that of roses, considering that the disease, for the second crop, has as its causal agent *Sphaerotheca pannosa* (Vallr. ex. Fr.) Lev.

The result of the identification are similar to the obtained by other authors, such as by Silva et al. (2003), who observed, during the nursery phase in the Minas Gerais region, powdery mildew in *Eucalyptus citriodora* and other species of the same genus caused by the fungus *Sphaerotheca pannosa* (synonym: *Podosphaera pannosa*), which is described as the sexual phase of *O. eucalypti*. In Brazil, in eucalyptus, only the anamorphic phase of the etiological agent has been found and identified as *Oidium eucalypti* Rostrup. (Ferreira, 1989). In 2014, in Korea, the first occurrence of powdery mildew caused by *Podosphaera pannosa* was identified in *Corymbia citriodora* (Cho et al., 2016).

The manifestation of powdery mildew in eucalyptus seedlings may be attributed to its high susceptibility to the disease, favorable environmental conditions and availability of inoculum (Silva et al., 2001). As shown in the phylogenetic dendrogram (Figure 1), we observe that there are previous records of *Podosphaera pannosa* occurring in other *Eucalyptus* and *Rosa* spp. species.

The symptoms and the structure of powdery mildew in clonal eucalyptus seedlings can be seen in Figure 2. The shape of the conidia ranged from ellipsoid to ovoid (Figure 2B). The conidial chain formation was observed in the four isolates studied, and the mean values of the 200 conidia were 32.5×20.7 μm (length x width), with a maximum value of 40×22.5 μm and a minimum of 27.5×17.5 μm.

In a study conducted by Silva et al. (2001), isolates from powdery mildew in *Eucalyptus urophylla* and *Rosa* sp. presented ellipsoid-ovoid conidia with mean sizes of 26.2×14.2 μm and 27.2×14.2 μm, respectively. For *Dhalia* sp., the same authors found ovoid-cylindrical conidia with a mean size of 32×16.8 μm. Our study shows similar results for the morphology of conidia of powdery mildew. We observed a similarity in length and width to the powdery mildew of *Dhalia* sp., and the same format as the powdery mildew of *E. urophylla* and *Rosa* sp. Mucci et al. (1980) found *Oidium* conidia
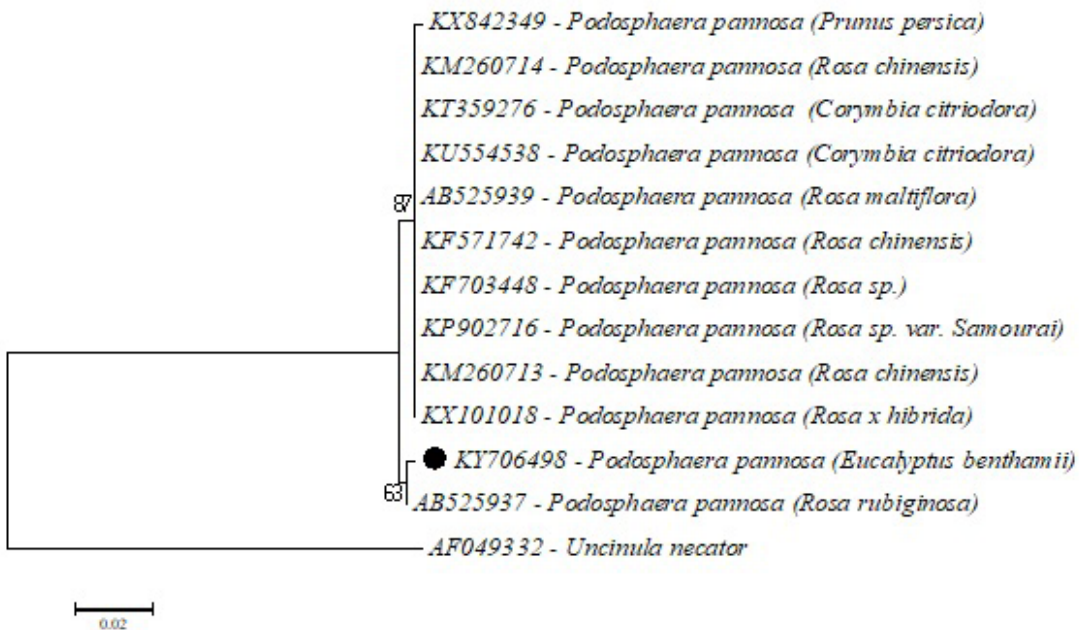
Figure 1. Phylogenetic dendrogram constructed for the isolate of powdery mildew from clonal seedlings of *Eucalyptus* spp. based on the Neighbor-joining statistical method, derived from the sequences of the ITSr DNA regions and aligned by the Tamura-Nei Model.
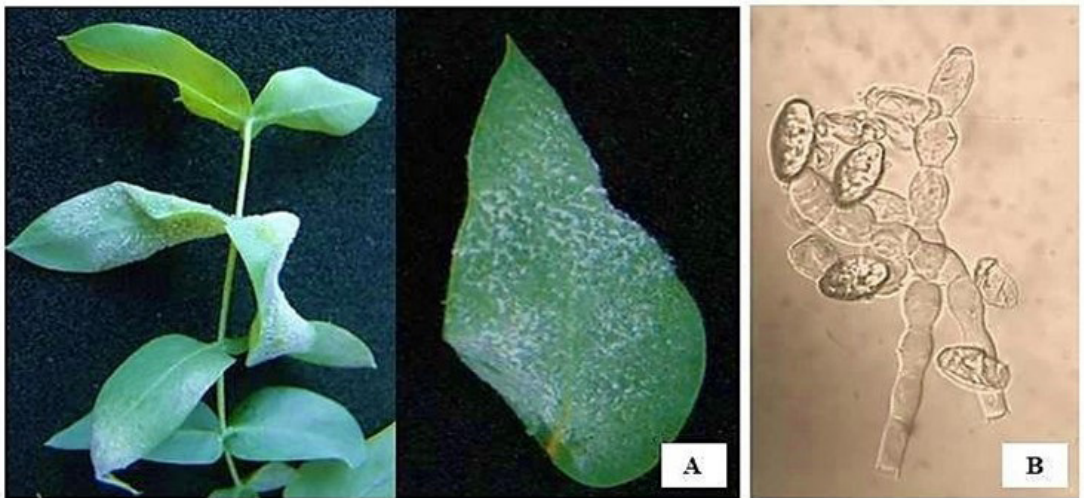
Figure 2. Symptoms of powdery mildew in clonal seedlings of *Eucalyptus* spp. and detail of the sporulation in the leaf (A); conidiophores and conidia of *Oidium eucalypti* (B).

originating from eucalyptus, with dimensions ranging between 21-35.75 × 13-20 μm. This demonstrates that the morphology of *O. eucalypti* conidia obtained in this study is similar both in shape and size to fungus conidia.

According to the susceptibility reaction test, all clones studied were susceptible. We observed symptoms caused by the pathogen. Among the mathematical models tested, the Gompertz model was the best fit for the progress curves for both incidence (I) and disease (DI) data for the four clones (R^2 between 0.78 and 0.97). The tested models explain the progress rate because the disease caused by *Oidium eucalypti* is polycyclic, in which the inoculum potential of the pathogen during the crop cycle is one of the main factors that determine the degree of incidence and the severity of the disease, being even more important at the beginning of plant development (Bergamin, 1995).

By the Gompertz model, the growth rate is accentuated at the onset of the disease, and rapidly changes to a slower growth (Madden et al., 2007). The clones studied showed a similar behavior. Chelal & Hau (2015) used this same model to analyze the behavior of tomato plants infected with *Oidium neolycopersici*, obtaining high values (higher than 0.98).

Incidence (I) and disease index (DI) curves, produced by the Gompertz model, presented a different behavior for the four clones evaluated (Figure 3).

We observed that the DI of the clone 1 (*E. benthamii*) showed a higher rate of disease progression in the first
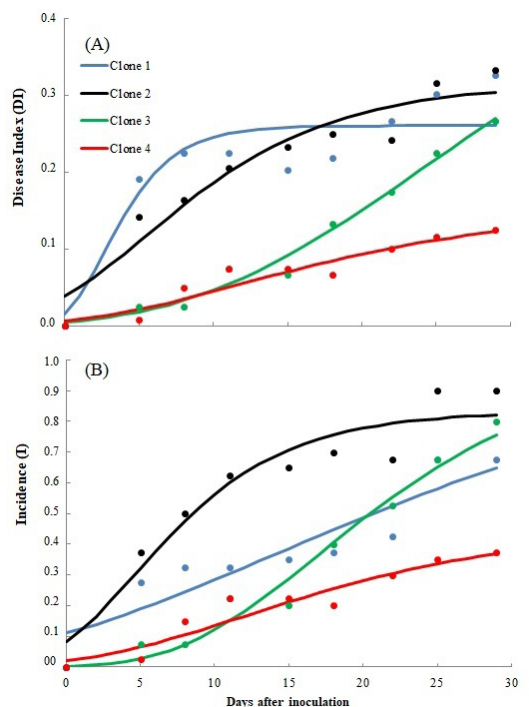
Figure 3. Temporal progression of disease index – DI (A) and incidence – I (B) of epidemics of powdery mildew (*Oidium eucalypti*) in four eucalyptus clones according to the Gompertz Model. Clone 1 (*Eucalyptus benthamii*); Clone 2 (*E. dunni*); Clone 3 (*E. benthamii*); Clone 4 (*E. urophylla* x *E. globulus*).

days after inoculation of powdery mildew (Figure 3A). With the passage of days, the disease tendency was towards a stabilization, which indicates that the pathogen could

colonize the tissue easier at the beginning, proving to be more damaging to the infected plants of that clone. Subsequently, the DI values stabilized ($y$max = 0.261), as shown in Table 1. Observing the curve of the clone 2 (*E. dunnii*), the disease also increased its intensity with the passage of time, standing out because it presented the highest disease index. The curves of the clones 3 (*E. benthamii*) and 4 (*E. urophylla* x *E. globulus*) initially presented low values of disease incidence and severity. However, from 12 days after inoculation, we observed that for the clone 3, the DI tended to increase the severity of the disease, while for the clone 4 there was a tendency to stabilize the disease (Figure 3A).

In contrast, the I curves presented a different behavior when compared to DI curves. The incidence of the causal agent of powdery mildew in clone 2 (*E. dunnii*) was the highest compared to the other clones, in addition to reaching higher incidence rates in a shorter time. We also observed a propensity to increase the incidence of the disease by the behavior of the curves of the clones 1 and 3, both clones of *E. benthamii*.

In Figure 3, the clone 4 (*E. urophylla* x *E. globulus*) had the lowest DI and I of the disease. Thus, it was considered the clone least susceptible to powdery mildew disease compared to the others as evaluated in this study. Silva et al. (2014), in a study evaluating the partial resistance of eucalyptus rust (*Puccinia psidii*) in different species of *Eucalyptus*, observed that *Eucalyptus urograndis* (*E. grandis* x *E. urophylla*) presented a low average number of pustules per leaflet, low severity, long average latent period and low values

of area below the disease progression curve, thus having a higher partial resistance to rust. This demonstrates that a eucalyptus hybrid may present a greater resistance and low incidence to fungal diseases.

The evaluation of incidence and severity of leaf spot caused by *Cylindrocladium candelabrum* on juvenile crops of *E. benthamii* showed a disease incidence in trees of 2.6-43.8% and mean severity data between 1.2 and 2.9, evidencing a susceptibility of this species to the pathogen (Schultz et al., 2015).

The parameters y_0, r and y_{max}, estimated by the Gompertz model for *O. eucalypti* epidemics on the different clones evaluated, presented a significant difference only for incidence data (Table 1). The clone 1 had the highest initial disease index ($y_0 = 0.110$), differing significantly from the clone 3 ($y_0 = 0.002$). The progress rates of the epidemic were higher for the clones 2 ($r = 0.177$) and 3 ($r = 0.106$). By analyzing the parameter y_{max}, we noted that the clones 1 and 3 showed 100% of incidence. Such values agree with the behavior of the curves represented by the model, in which the clones of *E. benthamii* and *E. dunnii* showed a high incidence of powdery mildew. The expression of symptoms of powdery mildew is directly related to the genetic differences of each eucalyptus clone. Grigoletti et al. (2005) reported *E. benthamii* as a species highly susceptible to *Oidium* sp. under nursery and greenhouse conditions. With respect to *E. dunnii*, we detected a high genetic variability of resistance to rust caused by *Puccinia psidii*, presenting a coefficient of genetic variation of 36.07 and 70% for evaluated progenies; they are thus rust-immune (Pinto et al., 2014).

Table 1. Parameters[1] estimated by the Gompertz model for temporal analysis of the disease index and the incidence of *Oidium eucalypti* in seedlings of four *Eucalyptus* spp.

Analysis	Clone	Parameters estimated by Gompertz model									
		R^2	y_0	± error		r	± error		y_{max}	± error	
Disease Index (DI)	1[2]	0.85****	0.015	0.048		0.390	0.240		0.261	0.022	
	2	0.96****	0.038	0.018	ns	0.138	0.038	ns	0.317	0.027	ns
	3	0.92****	0.005	0.000		0.062	0.034		0.597	0.446	
	4	0.78****	0.007	0.010		0.094	0.071		0.151	0.070	
Incidence (I)	1	0.93****	0.110	0.032	A*	0.056	0.077	B*	1.000	0.000	A*
	2	0.97****	0.084	0.044	AB	0.177	0.038	A	0.832	0.044	B
	3	0.92****	0.002	0.004	B	0.106	0.014	A	1.000	0.000	A
	4	0.78****	0.021	0.021	AB	0.094	0.071	AB	0.452	0.210	B

[1] Coefficient of variation (R^2), initial disease index (y_0) and asymptotic (y_{max}), and epidemic rate of progress (r) estimated by Gompertz model; [2]Clone 1 (*Eucalyptus benthamii*); clone 2 (*E. dunnii*); clone 3 (*E.benthamii*); clone 4 (*E.urophylla* X *E.globulus*); **** p<0.0001; ns: Not significant; * Means followed by the same letter do not differ from one another by the test *t* at 5% probability. Being: clone 1 (*Eucalyptus benthamii*), clone 2 (*E. dunni*), clone 3 (*E. benthamii*) and clone 4 (*E. urophylla* x *E. globulus*).

Sánchez Márquez et al. (2011), studying the occurrence of fungal species in juvenile and adult leaves of plantations of *E. globulus*, observed that, in general, juvenile leaves are more prone to infection by fungi, pathogens and endophytes than adult leaves. According to James & Bell (2001), adult leaves of *E. globulus* and other species have thicker cuticles and a lower stomatal density than juvenile leaves. In comparative studies with juvenile leaves of susceptible and resistant genotypes of *E. globulus*, anatomical characteristics such as densities of palisade mesophylls, cuticle thickness and stoma wax cover were associated with resistance (Smith et al., 2007). Such characteristics may explain the low susceptibility to severe and incidence of powdery mildew by the hybrid clone (*E.urophylla* x *E. globulus*).

By observing the proportion of the disease for both the DI_f and the I_f values, we found that the clone 4 (hybrid) showed a significant difference from the other clones, with a DI_f and an I_f ratio of 0.1250 and 0.3750, respectively. Thus, this clone stands out as the least susceptible to the disease. On the other hand, the clones 1 and 3 of *E. benthamii* and the clone 2 of *E. dunnii* were the most susceptible, presenting high proportions and exceeding the value of 0.6000 when assessed for final incidence of the disease (Table 2).

The correlation between DI and I data throughout the experiment showed that the clone 1 reached, over the evaluations, higher ratios of IDI (greater than 0.4) and a maximum incidence ratio of 0.8. This clone developed higher and lower DIs during the first half of the evaluated period, which justifies the greater slope of the line (Figure 4). In contrast, clone 2 presented low DI values. However, it presented high I values throughout the evaluations, reaching the ratio 1. Clone 3 also had high I values, reaching a ratio greater than 0.8. Clone 4 presented values lower than 0.3 for DI and lower than 0.8 for I (Figure 4). This comparison between disease proportions among clones once again reinforces the susceptibility of the clones 1 and 2 to the disease.

Mafia et al. (2012) analyzed the Pearson correlation coefficients obtained between eucalyptus rust severity values (*Puccinia psidii*), evaluated *ex vitro* at 20 days, and values of incidence of disease with the pathogen inoculated in explants *in vitro*. The values were 0.93, 0.98 and 0.98% at 7, 11 and 14 days after inoculation, respectively. The authors observed a

Table 2. Final disease index (DI_f) and final incidence (I_f) obtained at the 29 days of evaluations of the disease caused by *Oidium eucalypti* in the different eucalyptus clones.

Clone	DI_f	I_f
Eucalyptus benthamii (clone 1)	0.3267 A*	0.6750 A
Eucalyptus dunnii (clone 2)	0.3333 A	0.9000 A
Eucalyptus benthamii (clone 3)	0.2667 AB	0.8000 A
E.urophylla x *E.globulus* (clone 4)	0.1250 B	0.3750 B
Variation causes	**Probability (F test)**	
Clone	0.0018	<0.0001
Period	<0.0001	<0.0001
Clone × Period	0.0529	0.0454

* Means followed by the same letter do not differ from one another by the test Tukey at 5% probability.
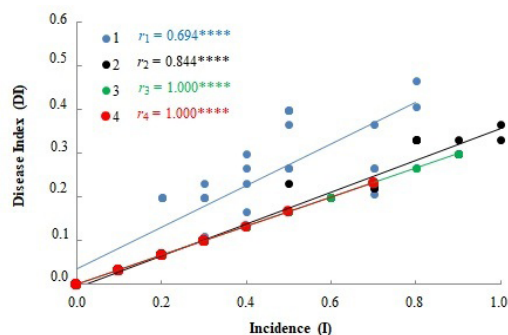
Figure 4. Correlation of residues between disease index (DI) and incidence (I) of *Oidium eucalypti* in four eucalyptus clones. Clone 1 (*Eucalyptus benthamii*); Clone 2 (*E. dunnii*); Clone 3 (*E.benthamii*); Clone 4 (*E. urophylla* X *E. globulus*); p <0.0001; R, Pearson correlation coefficients. Porto Alegre, RS, 2015.

different behavior among eucalyptus clones and reported that the most resistant clones had the same behavior between evaluation intervals.

Valeriano et al. (2015) obtained a significant difference in five different clones of *E. urograndis* from the first evaluation of incidence of powdery mildew at 7 days, with a lower incidence of the pathogen in two clones, and at the last evaluation 35 days after the onset of the disease.

With the data obtained in this study, we verified that the use of resistant clones might avoid the incidence of diseases, contributing to the success in the management of diseases in seedlings of this forest species.

4. CONCLUSIONS

Oidium eucalypti is the causal agent of powdery mildew disease in clonal seedlings of *Eucalyptus*, as confirmed by molecular analysis.

Clone 1 (*Eucalyptus benthamii*), clone 2 (*E. dunnii*) and clone 3 (*E. benthamii*) are considered more susceptible to powdery mildew, and clone 4 (*E. urophylla* x *E. globulus*) is the least susceptible. The latter had the lowest disease index at the end of the evaluation.

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