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Original Article

Silvicultura

Chemical Control of Mycosphaerella Leaf Disease on *Eucalyptus dunnii* in Southern Brazil

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ABSTRACT

Mycosphaerella leaf disease (MLD) is a dangerous disease for eucalypt plantations; however little information is available regarding its control using fungicides in South America. In this study, we evaluated MLD control, and growth in young stands of *Eucalyptus dunnii* clones (C18 and C25). After two applications of azoxystrobin + cyproconazole (0.3 L/ha^{-1} and 0.45 L/ha^{-1}), with a spraying volume of 200 L/ha^{-1}, and trifloxystrobin + tebuconazole (0.5 L/ha^{-1} and 0.75 L/ha^{-1}), with a spraying volume of 100 L/ha^{-1}, mean severity reduction for apical branches of C18 was 74%. For middle branches, application of trifloxystrobin + tebuconazole at 0.5 L/ha^{-1} reduced severity by 27% in C18. While fungicide applications did not affect growth in height or root collar diameter, our results suggest that the application of fungicide can deter disease progression throughout the plant. Therefore, the monitoring of disease outbreaks is crucial to enable effective, early application.

Keywords: silviculture, fungicide, leaf spot.

1. INTRODUCTION

Mycosphaerella leaf disease (MLD) is one of the most harmful diseases to eucalypt species, with more than 100 species occurring on *Eucalyptus* spp. around the world (Crous et al., 2007a; Hunter et al., 2011). The disease is caused by a complex of *Mycosphaerella* spp. and *Teratosphaeria* spp., which produce leaf spots and cankers on stems and branches (Teodoro et al., 2012; Cortinas et al., 2010; Crous, 2009; Santos et al., 2001).

In South America, the disease has only been reported within the last ten years, with the first outbreak occurring in Uruguay, where *Teratosphaeria nubilosa*, one of the most severe species of the complex, was observed (Pérez et al., 2009b). The disease has since spread progressively to other regions, including Southern Brazil (Pérez et al., 2009c), where another species had been reported (Ramos & Perez, 2015; Cândido et al., 2014; Passador et al., 2012).

The leaf spots caused by MLD lead to defoliation in different portions of the crown (Crous, 2009). Together, these symptoms can affect growth and yield in young eucalypt plantations (Aguín et al., 2013; Jackson et al., 2008; Carnegie & Ades, 2002). Growth rate reductions can occur even at low levels of severity, due to reductions in photosynthetic capacity and changes in stomata control (Pinkard & Mohammed, 2006), affecting plantations in the first two years after planting (Balmelli et al., 2013).

The area of *Eucalyptus dunnii* Maiden plantations is increasing in Southern Brazil due to its adaptation to regional climatic conditions that present cold winters and the occurrence of frosts. *E. dunnii* is susceptible to species from the MLD complex (Whyte et al., 2011; Andjic et al., 2010; Pérez et al., 2009a), but little is known about the impacts of the disease on plantations or possible control mechanisms.

Due to the potential damage that MLD can cause in large plantation areas, selecting different eucalypt species or genetically resistant material may be the best option to avoid growth reductions (Passador et al., 2012). Hybridization is another option to avoid or limit damage caused by the disease, but the efficacy of hybridization has not been widely studied (Hunter et al., 2009). Furthermore, selection is usually based on inoculation tests to confirm plant resistance, but this step is very difficult because *Mycosphaerella* spp. and *Teratosphaeria* spp. have slow growth rates and little or no sporulation *in vitro* (Passador, 2011). Therefore, the inclusion of chemical control in silvicultural practices must be considered during outbreaks of the epidemic, particularly in cold regions where only a few eucalypt species can grow.

In Brazil, some fungicides are available to control foliar diseases, such those caused by *Cylindrocladium* spp., *Oidium eucalypti*, *Puccinia psidii*, and the bacterial disease caused by *Xanthomonas axonopodis*. These products include the active agents tebuconazole, trifloxystrobin, azoxystrobin and cyproconazole (Brasil, 2003) and are commonly applied to control these diseases (Santos & Auer, 2011; Ferreira et al., 2006; Bizi et al., 2005). Thus, the aim of the present study is to evaluate the effectiveness of the combined fungicides tebuconazole + trifloxystrobin and azoxystrobin + cyproconazole to reduce disease severity and the effects on growth caused by Mycosphaerella leaf disease in young *E. dunnii* plantations in Southern Brazil.

2. MATERIAL AND METHODS

2.1. Plant material and study area

We evaluated two *Eucalyptus dunnii* (C18 and C25) clones planted in the municipality of Otacílio Costa, in the Serrana region of Santa Catarina State, Brazil. The region has a subtropical climate with a mean temperature of 16°C, an altitude of 900 m, and regular occurrence of frosts. In the region, a natural MLD outbreak was observed in six-month-old *E. dunnii* stands in a plantation run by Klabin S. A. in 2014.

The studied plantation stands of C18 and C25 clones were selected based on similar age, site conditions and initial disease severity. At the beginning of the study, the plantations were seven months post-planting.

2.2. Experimental design

In selected areas, growth and MLD leaf spot severity were evaluated for each clone (C18 and C25) in three stands in which four plots of approximately 367 m^2 (17.5 m x 21 m; seven plantation lines x seven trees per line, with two buffer lines) were installed. In each plot, different fungicide and dosage combinations were applied. To compare the effects of fungicide application on disease severity and tree growth, plots with fungicide

application were compared with C18 and C25 plots without fungicide application (WFA). WFA plots were composed of three plots of approximately 2500 m^2 (50 m x 50 m; 20 plantation lines x 17 trees per line), in which 49 trees were randomly selected to evaluate growth and MLD severity.

Treatments were composed of areas without fungicide application (WFA) and areas with different fungicide combinations using the following active ingredient dosage and spraying volumes:

a) Trifloxystrobin + tebuconazole 0.5 L/ha^{-1}, spraying volume of 200 L/ha^{-1} (F1);
b) Trifloxystrobin + tebuconazole 0.75 L/ha^{-1}, spraying volume of 200 L/ha^{-1} (F2);
c) Azoxystrobin + cyproconazole 0.3 L/ha^{-1}, spraying volume of 100 L/ha^{-1} (F3);
d) Azoxystrobin + cyproconazole 0.45 L/ha^{-1}, spraying volume of 100 L/ha^{-1} (F4).

Fungicide composed of trifloxystrobin + tebuconazole is systemic and mesosystemic, while the fungicide composed of azoxystrobin + cyproconazole is exclusively systemic. Fungicides were applied twice using a 15-liter manually-pumped backpack sprayer. The first application occurred at the beginning of the experiment (seven months after planting) and the second application at nine months after planting. The decision to apply fungicides was based on an outbreak of the disease in the region, and the application interval was determined by climatic conditions and the company's operating procedures.

In each fungicide treatment plot, we evaluated 25 trees and in each WFA plot we evaluated 49 trees, for a total of 894 trees (300 trees each of C18 and C25 with fungicide application; and 147 trees each of C18 and C25 WFA).

2.3. Evaluation of growth and severity

Tree growth was evaluated by measuring root collar diameter (RCD) and height. Leaf spot severity was determined using a branch severity scale of 3, 6, 12.5, 25, 50, and 75% (Maxwell, 2004). Severity and defoliation were assessed for all apical, middle, and basal branches of the crown.

Measurements to assess growth in RCD and height were taken at the beginning of the study (seven months after planting) and at the end of the study (eleven months after planting and two months after the second fungicide application). MLD severity was evaluated during the first fungicide application (seven months after planting), the second fungicide application (nine months after planting), and two months after second application (eleven months after planting).

At the end of the study, we determined the Area Under the Disease Progress Curve (AUDPC) (Campbell & Madden, 1990) as follows (Equation 1):

$$AUDPC = \sum_{i=1}^{n}\left[\frac{Y_i + Y_{i+1}}{2}\right](t_{i+1} - t_i) \quad (1)$$

where n is the number of measurements, Y_i is the disease severity at each ith evaluation, and t the time at each ith evaluation.

2.4. Pathogen identification

From each study area, we collected leaves showing evidence of MLD, i.e., leaves with angular or irregular brown to pale brown colored spots with borders, that were dispersed or coalescent across the leaf surface. The following isolation methods were used: active ascospore ejection, moist chamber and collection of spore mass, and ascomata deposition on the medium. Spores were transferred to Petri dishes containing 2% malt extract agar (MEA), potato dextrose agar (PDA), 2% yeast extract agar, 2% malt extract agar, and a solution of five grams of macerated leaves in sterile water with 1 $g.L^{-1}$ of Polyvinylpyrrolidone-iodine (PVP-iodine).

As no fungi were isolated, no germination pattern or molecular techniques were performed to identify species. Genus identification was based on morphological characteristics using an optical microscope, recording size of asci and ascospores, shape, color, and number of structures.

2.5. Statistical analysis

The statistical design was a block design with five treatments (WFA; F1; F2; F3; F4) and three replications (plots) for each clone and treatment.

Tree growth variables and MLD severity were analyzed with analysis of variance (ANOVA), t-test for growth and MLD severity at the beginning of the study, and Tukey test for other analyses. All statistical analyses were performed with a 5% error probability, using the software StatSoft Statistica Trial 12.

3. RESULTS

3.1. Pathogen identification

Species identification was not possible despite the observation of *Mycosphaerella* spp. and *Teratosphaeria* spp. structures on *E. dunnii* leaves, including pseudothecia, bitunicate asci, and ascospores, with the identification of one sept (Figure 1).

Dark to light brown pseudothecia, globose in shape were observed on the abaxial leaf surface. Hyaline asci were cylindrical in shape, bitunicate, 33 µm long and 12 µm wide. In the asci, we observed hyaline or light brown ascospores occurring in groups of eight with one septum, with a length of 9 µm and width of 2 µm (Figure 1). We cannot confirm the species due to lack of pathogen cultures

3.2. Growth and MLD severity

With fungicide application, we observed a reduction in disease severity on some crown portions of the trees. Compared to WFA areas, MLD severity in apical branches reduced for C18 trees with all fungicide treatments. For the middle third, a reduction in MLD severity only occurred for C18 with the application of the F1 treatment. For branches of the basal third, which showed the highest levels of disease severity, no leaf spot reduction was observed (Table 1). The basal portions of the crown also showed increased severity of defoliation, due to the bottom-up defoliation pattern in the study areas (data not shown).

After the two fungicide applications, we found no differences in height or RCD for either clone between plots with and without fungicide applications.

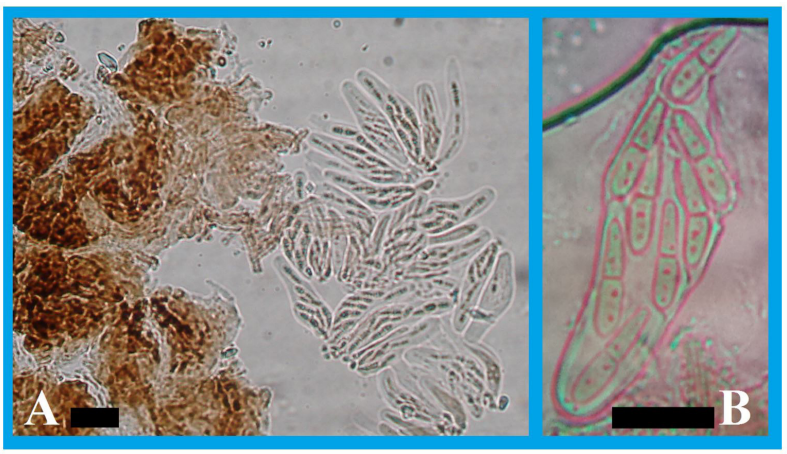

Figure 1. Asci and ascospores observed on *E. dunnii* leaves from the study areas. (A): Asci and ascospores; (B): Asci containing ascospores. Scale bars: 10 µm.

Table 1. Area Under Disease Curve Progress (AUDCP) of apical, middle, and basal branches of the crown after two fungicide applications.

Fungicide Treatment	Crown Portions					
	---Apical Branches---		--Middle Branches--		-----Basal Branches----	
	C18	C25	C18	C25	C18	C25
WFA	2.70 a	3.89 a	39.41 a	56.58 a	66.52 a	36.18 a
F1	0.68 b	3.02 a	28.87 b	55.47 a	60.18 a	33.60 a
F2	0.74 b	2.17 a	34.84 ab	54.78 a	68.91 a	33.36 a
F3	0.32 b	2.99 a	34.55 ab	53.46 a	62.93 a	35.50 a
F4	0.54 b	3.43 a	32.86 ab	55.55 a	66.97 a	34.50 a
CV (%)	21.88	20.15	8.87	7.50	12.91	15.86
***p*-value**	<0.0001	0.0805	0.0342	0.9123	0.7249	0.9602

Means followed by the same lowercase letters in columns do not differ statistically according to the Tukey test with 95% probability.
CV: coefficient of variation.

Table 2. Height increment (m), root collar diameter increment (cm), severity at the beginning of the study, and Area Under Disease Curve Progress (AUDCP) for C18 and C25 after two fungicide applications.

Treatment	Height (m)		Root Collar Diameter (cm)		Severity at beginning		AUDCP	
	C18	C25	C18	C25	C18	C25	C18	C25
WFA	1.38 a	1.08 a	2.45 a	1.64 a	22.07 a	44.08 a	108.63 a	96.64 a
F1	1.29 a	1.02 a	1.84 a	1.42 a	17.92 a	46.82 a	89.64 a	92.09 a
F2	1.29 a	1.44 a	1.89 a	2.04 a	25.65 a	42.45 a	104.40 a	90.30 a
F3	1.06 a	1.21 a	1.78 a	1.55 a	15.01 a	43.58 a	97.68 a	91.95 a
F4	1.32 a	1.42 a	1.84 a	1.98 a	29.57 a	44.44 a	100.29 a	93.48 a
CV (%)	19.7	18.0	23.0	22.6	26.2	10.67	8.5	7.7
***p*-value**	0.594	0.161	0.412	0.308	0.084	0.839	0.168	0.849

Means followed by the same letter in columns do not differ statistically according to the Tukey test with 95% probability. CV: coefficient of variation.

Similarly, MLD severity was not reduced after treatment application, thus maintaining the same severity level as observed at the beginning of the study between WFA and treatment plots (Table 2).

4. DISCUSSION

4.1. Pathogen identification

The presence of pseudothecia in leaf spots, along with bitunicate asci and ascospores with one sept, structures common to Mycosphaerella species (Alfenas et al., 2009), suggest the occurrence of Mycosphaerella and Teratosphaeria leaf disease. The disease can also be associated with cankers (Hunter et al., 2011), but this symptom was not observed in this study. Distinguishing between Mycosphaerella and Teratosphaeria species in eucalyptus is very difficult as symptoms and structures can be very similar across species and the same pathogen can have different symptoms on different hosts, causing confusion in the description of diseases caused by both species (Hunter et al., 2011).

Problems with, or lack of, *in vitro* cultures of *Mycosphaerella* spp. and *Teratosphaeria* spp. further limit identification and phylogenetic studies of these species (Crous et al., 2007b). Identification difficulties can also be attributed to the occurrence of various species in a single lesion, thus causing problems with identification based solely on morphology (Crous et al., 2006; Crous, 2009). As some species are morphologically similar and require specific molecular techniques for identification (Hunter et al., 2011; Pérez et al., 2014), the collection of cultures is necessary to identify these species using DNA analysis or ascospore germination patterns.

4.2. Growth and MLD severity

To evaluate the ability of the fungicides to control disease, the treatments used here were developed based on the lowest and highest recommended active ingredient dosages with the same spraying volume. Of the active ingredients applied in this study, trifloxystrobin + tebuconazole has recently been recommended for use in eucalypt plantations in Brazil, showing efficacy in controlling leaf diseases, such as *O. eucalypti* (Bizi et al., 2005) and *P. psidii* (Masson et al., 2011), and controlling *M. graminicola* in banana (Vidhyasekaran, 2004).

Although trifloxystrobin + tebuconazole and azoxystrobin + cyproconazole applications reduced MLD severity in the apical branches of C18, no differences were observed between active ingredients and dosages. For middle branches, only trifloxystrobin + tebuconazole with a dosage of 0.3 L/ha^{-1} reduced disease severity. Higher MLD severity levels in the lower crown are common for *E. dunnii* (Santos et al., 2001), and after repeated attacks of the disease, it can progressively affect other portions of the crown (Taole et al., 2012). Therefore, the reduction in severity in the apical third of the crown suggests that an early fungicide application, when the disease is starting to infect the lower crown, can deter disease progression. However, since the disease occurs on the abaxial leaf surface, a bottom-up oriented application is recommended (Carnegie & Ades, 2002). This application pattern was not used in this study, as it is impracticable in large plantation areas due to the difficulty in applying fungicide to the crown branches.

Possible impacts of the disease were not detected in our analysis as we found no differences in growth or severity between trees with or without fungicide application. For *E. globulus*, one of the most susceptible species to MLD, growth was affected and mortality was observed when MLD severity was greater than 40% (Balmelli et al., 2013). However, in some circumstances, the disease can reduce growth when severity levels are at least 20% (Smith et al., 2016), while another study reported that at severity levels lower than 10%, height increment was reduced by 13% and diameter by 4% (Carnegie & Ades, 2002). These variable responses indicate that a different active ingredient or dosage may be required depending on the clone, and that fungicide treatments with systemic and mesosystemic activity (treatments F1 and F2) are more effective to control the disease.

Furthermore, meteorological variables and pathogen resistance to fungicides should also be considered (Cools et al., 2010; Ngando et al., 2015). Different eucalypt species present varying degrees of tolerance to MLD, as can be observed with *E. globulus* and *E. maidenii*: *E. globulus* tolerates greater leaf spot severity and defoliation caused by the disease, while growth increments of *E. maidenii* are affected at lower levels of severity and defoliation than *E. globulus* (Balmelli et al., 2016).

Given that the fungicide applications used here did not reduce disease severity, no growth response was observed during the evaluation period. Two fungicide applications can be considered a cost-effective strategy to control MLD progression at disease outbreak (Carnegie & Ades, 2002); however, in this study, the disease was already found on *E. dunnii* leaves. When fungicide applications are repeated, control of the disease can be observed, as well as differences in growth rates. For *E. globulus*, Smith et al. (2016) observed a volume that was 17% higher and greater growth in height and diameter in areas with MLD control. However, these results occurred only with several fungicide applications over a period of two years. This practice is, therefore, considered economically impracticable, as well as presenting environmental and operational issues (Balmelli et al., 2014), particularly in forest stands with a five or seven-year rotation. Thus, this practice can only be implemented during disease outbreaks with informed planning, and fungicide application at the beginning of infection.

Mean severity reduction after two applications for C18 in apical branches was 75, 73, 88, and 80% for F1, F2, F3 and F4, respectively. For the middle branches of the crown, the severity reduction was 27% for treatment F1. According to Carnegie & Ades (2002), after six consecutive applications of protectants and systemic fungicides, the reduction of MLD severity can be even greater, up to 66% with defoliation presenting a reduction of 50%, leading to beneficial effects for height and diameter growth. Although chemical control of MLD is related to reductions in defoliation for *E. globulus* (Smith et al., 2016), in this study, even with a reduction in severity in the upper-crown, defoliation was similar in areas with and without fungicide application, with a bottom-up pattern (data not shown).

Considering our results, selection of resistant genetic material (Pérez et al., 2009b) is an economical option that can avoid the negative impacts on growth and production caused by MLD. As stated by Carnegie & Ades (2002), even though chemical control reduces impacts of MLD, genetically resistant material is a better mechanism to mitigate MLD. Forest companies must implement selection and management of resistant material as a key mechanism to avoid damage caused by Mycosphaerella leaf disease in eucalypt plantations. In so doing, when the disease occurs at low severity levels, control using fungicides can promote the full growth potential of the selected material.

Although resistance management is common in forestry, the propagation and planting of resistant material can be limited. For example, *E. urophylla* and *E. pellita* hybrids in Australia were attacked by MLD after *E. grandis* hybrids with high susceptibility to the disease were substituted in plantations (Andjic et al., 2010). In Brazil, this scenario is concerning, as the genetic foundation of *Eucalyptus* spp. clones is limited, which can cause significant impacts on forest production in the case of low resistance (Cândido et al., 2014), particularly considering that the number and dispersion of *Mycosphaerella* and *Teratosphaeria* species are increasing in South America.

Although changing species is an option in critical areas (Smith et al., 2016), it can require significant modifications to the production chain and industrial processes, thus limiting the choice of species. As such, *E. dunnii* plantations must be monitored and the

viability of new plantations in areas where the disease occurs should be considered.

In conclusion, forest managers must develop resistant material to avoid the effects of Mycosphaerella leaf disease (Maxwell, 2004). Until resistant material is developed, managers must also consider adapting silvicultural practices such as fertilization with nitrogen and phosphorus to promote tree vigor, leaf phase change, and recovery after MLD attack (Pinkard et al., 2007; Wardlaw, 2007; Carnegie & Ades, 2001) as alternative control measures to ensure desirable growth and fiber characteristics. In the present study, we analyzed stands under current attack by MLD. Two fungicide applications showed a reduction in severity in the apical and middle third of the crown. *E. dunnii* growth, on the other hand, did not differ between areas with and without fungicide application. Therefore, the applied chemical control is suitable to limit disease progression throughout the tree crown, but care should be taken to ensure that application occurs at disease outbreak. This practice can demand a greater number of applications over shorter intervals. As such, it can be economically unviable particularly in large plantation areas and given that MLD outbreaks usually occur during adverse climatic conditions (Smith et al., 2016).

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