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SHORT COMMUNICATION

Teratosphaeria pseudoeucalypti on eucalyptus in Brazil

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ABSTRACT

A new foliar disease caused by *Teratosphaeria pseudoeucalypti* on eucalyptus (*E. globulus*, *E. urophylla* x *E. globulus* and *E. nitens* x *E. globulus*) in Brazil is described. The disease is characterized by leaf spots of variable sizes and shapes, resulting in leaf blight and premature defoliation. Based on the morphological characteristics and multilocus phylogenetic analysis of the EF-1α, β-T and ITS-2 gene regions of five isolates, the fungus was identified as *T. pseudoeucalypti*. This is the first report of this pathogen outside Australia and a method for sporulation in culture is described.

Key words: *Eucalyptus* spp., Forest Pathology, kirramyces leaf blight, phylogenetic analysis.

Global expansion of clonal plantations of *Eucalyptus* spp. has occurred in recent years to meet the growing demand for wood fiber. Concomitantly, several diseases caused mainly by fungi and bacteria have emerged in eucalypt plantations (Alfenas et al., 2009). Among the foliar diseases of eucalypts in Brazil, those caused by species of *Teratosphaeria* are of particular interest due to intense defoliation, potentially leading to a decrease in tree growth as a result of the reduced photosynthetic capacity of the diseased plants (Alfenas et al., 2009). These fungi cause a complex of diseases on *Eucalyptus* spp. known as Teratosphaeria Leaf Disease (TLD).

In Brazil, seven species have been reported associated with TLD: *T. nubilosa*, *T. suberosa*, *T. suttonii*, *T. ohnowa*, *T. perpendicularis*, *T. pseudafricana* and *T. flexuosa* (Alfenas et al., 2009; Crous et al., 2006; Pérez et al., 2009a; Teodoro et al., 2012). However, there may be other species causing TLD in eucalyptus in Brazil (Teodoro et al., 2012). In 2012, clonal trials of *Eucalyptus* spp. in southern Brazil showed severe defoliation caused by a *Teratosphaeria* sp. (Figure 1A). The aim of this study was to identify the species of *Teratosphaeria* as the causal agent of this disease.

Infected *Eucalyptus* leaves exhibiting TLD symptoms (Figure 1A-C) were collected in clonal trials of *E. globulus*, *E. urophylla* x *E. globulus* and *E. nitens* x *E. globulus* at the Horto Florestal Capão do Leão (31º 6'43" S, 52º 3'19" W), Cristal (RS), Brazil (Table 1). Leaf samples from 10-month-old plants were collected from clones that were severely affected by the fungus. The leaf samples were kept in a moist chamber for 24 h in the laboratory at room temperature (25ºC ± 3ºC). After incubation, the conidial mass from a single pycnidium was transferred aseptically to 2% malt extract agar (MEA). The isolates were grown at 20°C under a 12 h photoperiod and 40 µmol s^{-1} m^{-2} light intensity for 30 days. Representative specimens of the fungus were deposited in the local herbarium (Herbarium VIC) and pure cultures corresponding to each herbarium specimen were obtained and used in the work (but lost during storage at a later stage). The herbarium accession numbers and temporary culture codes (LPF – for Laboratório de Patologia Florestal, Departamento de Fitopatologia, UFV) were as follows: VIC 42699/LPF 521, VIC 42700/LPF 522, VIC 42701/LPF 523, VIC 42702/LPF 524, VIC 42703/DPF 606. Information for each specimen is presented in Table 1.

To induce *in vitro* sporulation, inoculum plugs of four isolates of the fungus grown on MEA (Figure 1F) were each added to a 125 mL Erlenmeyer flask containing tomato juice + calcium carbonate liquid medium (10% tomato juice and 2% $CaCO_3$) and incubated under agitation at 130 rpm (Shaker, Thermo scientific, MaxQ 4000) at 20 ± 1ºC for 10 days in the dark and 5 days in the light. After incubation, the fungal structures were mounted in lacto-glycerol (1:1:1 lactic acid:glycerol:water) and observed under a light microscope (Olympus BX53), and the images were captured with a camera (Olympus Q-Color 5TM America INC). Each isolate was assessed for conidial sizes (length and width), shape, pigmentation and septa number of 50 randomly chosen conidia. Conidia were measured using the image analysis system Image Pro Plus (Version 7.0).

Genomic DNA of the five isolates was extracted from mycelia grown on MEA for 30 days (Wizard Genomic DNA Purification Kit, Promega). The DNA was quantified with a Nanodrop 2000c (Thermo Scientific) and adjusted to 10 ng μL^{-1}. Target regions of the internal transcribed spacer regions 1 and 2, including the 5.8S rRNA gene (ITS), β-tubulin (β-T) and translation elongation factor 1-α (EF1-α) were amplified using the primers ITS-3 and

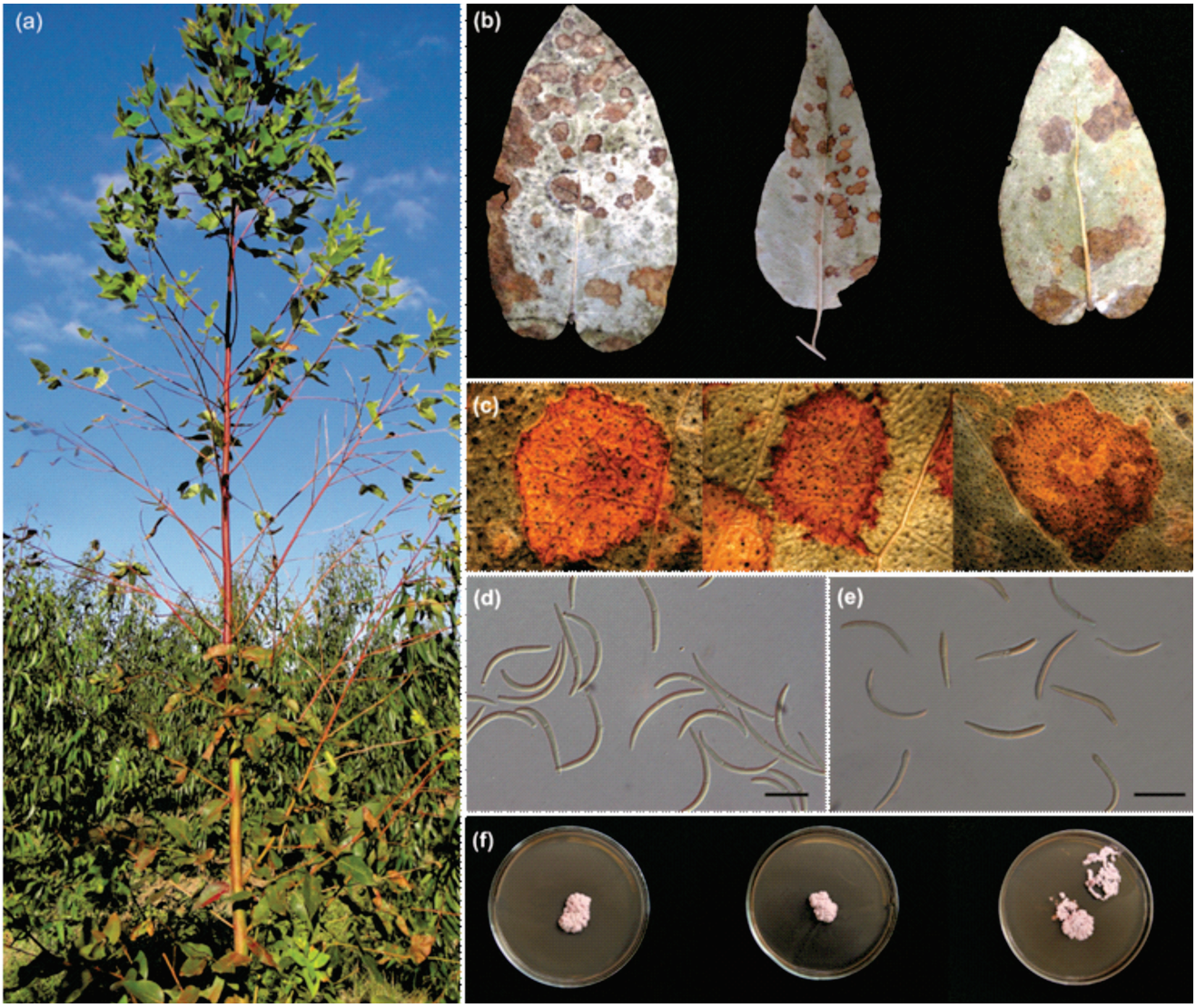

FIGURE 1 - Symptoms and signs of *Teratosphaeria pseudoeucalypti* on eucalyptus in southern Brazil: (a) Premature defoliation; (b) Leaf lesions; (c) Black conidiomata *pycnidia* formed on the lesions; (d) Conidia obtained directly from leaf lesions; (e) Conidia produced on liquid culture medium (10% tomato juice and 2% $CaCO_3$); (f) Colonies in malt-extract-agar. Scale bar = 20 µm (d) and 25 µm (e).

ITS-4 (Gardes & Bruns, 1993), Bt2a and Bt2b (Glass & Donaldson, 1995) and EF1-728F and EF1-986R (Carbone & Kohn, 1999), respectively. Polymerase chain reactions (PCR) were performed using the following ingredients for each 25 µL reaction: 12.5 µL of Dream Taq™ PCR Master Mix 2X (MBI Fermentas), 1.5 µL of 10 µM of each forward and reverse primer, 2 µL of genomic DNA, and nuclease-free water to complete the total volume. The PCR conditions were performed as described previously by Andjic et al. (2010a) and performed using a Veriti 96 (Life Technologies) thermocycler. The PCR products were purified using GFX™ DNA and a Gel Band Purification Kit (GE) following the manufacturer's instructions. Sequencing was performed by Macrogen Inc. (www.macrogen.com). The same primers that were used for PCR were used for DNA sequencing. The sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program ClustalW (Thompson et al., 1997) built in MEGA v. 5 software (Tamura et al., 2011). Alignments were checked, and manual adjustments were made when necessary. Gaps (insertions/deletions) were treated as missing data. The Bayesian Inference (BI) method was used to construct phylogenetic trees using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). The substitution model was chosen based on the Akaike information criterion (AIC) using MrModelTest v. 3.2 software (Nylander, 2004). The probability of a posterior tree distribution was calculated using an MCMC algorithm (Metropolis-coupled Markov Chain Monte Carlo) of two chains from a random tree with 10 million generations, and 25% of the first trees were discarded. The MCMC convergence and effective sample size were checked using the Tracer v. 1.4

TABLE 1 - Species and isolates of *Teratosphaeria* used in phylogenetic analyses.

Fungus	Reference isolates[1]	Host	Location	Haplotypes[2]	GenBank accession number		
					EF-1α	β-T	ITS-2
T. eucalypti	CMW 19453	*Eucalyptus nitens*	Settlement Rd, New Zealand	KE1	EU101585	EU101529	FJ793234
T. eucalypti	MUCC 635	*E. nitens*	Roses Tier, TAS, Australia	KE2	EU101614	EU101557	FJ793250
T. eucalypti	MUCC 632	*E. nitens*	Kinglake, VIC, Australia	KE3	DQ632726	DQ632631	DQ632661
T. eucalypti	CMW 19455	*E. nitens*	Coxs, New Zealand	KE4	EU101628	EU101571	FJ793260
T. eucalypti	MUCC 626	*E. grandis* x *E. tereticornis*	Kyogle, N-NSW, Australia	KE5	EU101602	EU101546	FJ793241
T. eucalypti	MUCC 630	*E. grandis* x *E. tereticornis*	Kyogle, N-NSW, Australia	KE6	EU101606	EU101550	FJ793245
T. eucalypti	MUCC 631	*E. grandis* x *E. tereticornis*	Kyogle, N-NSW, Australia	KE7	EU101626	EU101569	FJ793258
T. pseudoeucalypti	MUCC 598	*E. grandis* x *E. camaldulensis*	Harrisville, S-QLD, Australia	KE8	EU101592	EU101536	FJ793215
T. pseudoeucalypti	MUCC 610	*E. grandis* x *E. camaldulensis*	Mirian Vale, C -QLD, Australia	KE9	EU101599	EU101543	FJ793221
T. pseudoeucalypti	MUCC 704	*Eucalyptus spp.*	FNQ, Australia	KE10	FJ793205	FJ793209	FJ793213
T. pseudoeucalypti	MUCC 705	*Eucalyptus spp.*	FNQ, Australia	KE11	FJ793206	FJ793210	FJ793214
T. pseudoeucalypti	MUCC 615	*Eucalyptus spp.*	Davies Creek, FNQ, Australia	KE12	EU101613	EU101556	FJ793231
T. pseudoeucalypti	LPF0521	*E. urophylla* x *E. globulus*	Cristal, RS, Brazil		KF986726	KF986721	KF986716
T. pseudoeucalypti	LPF0522	*E. nitens* x *E. globulus*	Cristal, RS, Brazil		KF986727	KF986722	KF986717
T. pseudoeucalypti	LPF0523	*E. nitens* x *E. globulus*	Cristal, RS, Brazil		KF986728	KF986723	KF986718
T. pseudoeucalypti	LPF0524	*E. urophylla* x *E. globulus*	Cristal, RS, Brazil		KF986729	KF986724	KF986719
T. pseudoeucalypti	LPF606	*E. globulus*	Cristal, RS, Brazil		KF986730	KF986725	KF986720
T. cryptica	CBS110975	*E. globulus*	Australia		DQ235119	DQ658234	AF309623
T. destructans	CMW19832	*E. grandis*	Sumatra, Indonesia		DQ235113	DQ632623	DQ632665
T. molleriana	CBS117924	*Eucalyptus* sp.	Portugal		DQ235104	DQ240115	DQ239968
T. nubilosa	CMW11560	*E. globulus*	Tasmania		DQ240176	DQ658236	DQ658232
T. suttonii	CMW22484	*E. urophylla*	China		DQ632714	DQ632616	DQ632705
T. viscida	MUCC455	*E. grandis*	Mareeba, Australia		EF031498	EF031485.1	EF031474
T. zuluensis	CBS117835	*E. grandis*	Mexico		DQ240161	DQ240108	DQ239987
Dothistroma septospora	CMW13122	*Pinus mugo*			AY808260	AY808195	AY808295

[1]Designation of isolates and culture collections: CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; MUCC = Murdoch University Culture Collection, Perth, Western Australia; LPF = Laboratory of Forest Pathology, Universidade Federal de Viçosa, Minas Gerais, Brazil; CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands. [2]Haplotypes according to work performed by Andjic et al. (2010a).

(Rambaut & Drummond, 2007). Phylogenetic trees were viewed and edited in the FigTree v. 1.3.1. (http://tree.bio.ac.uk/software). The sequences obtained in this study were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank), from which sequences of seven isolates of *T. eucalypti*, five isolates of *T. pseudoeucalypti* and a representative of each of the species *T. cryptica, T. suttonii, T. zuluensis, T. molleriana, T. nubilosa, T. destructans* and *T. viscila* were retrieved for comparison (Table 1). The isolates of *T. eucalypti* and *T. pseudoeucalypti* used in phylogenetic analysis were considered distinct haplotypes as described elsewhere (Andijc et al., 2010a). The sequences of *Dothistroma septospora* were used as an outgroup (Table 1).

The conidia of the studied isolates showed morphological characteristics similar to those of *T. pseudoeucalypti* Andjic, T.I. Burgess previously described and found in Australia (Andjic et al., 2010a). The fungus sporulated profusely in culture. The conidia produced *in vitro* were hyaline to light brown and straight to variously curved with narrowing ends and 0-3 septa (three septa were rare), while conidia obtained directly from the leaves were larger and more curved (Figure 1D-E). In addition, conidia examined directly from the leaves were longer (37 to 66 µm - average 53.3 µm) and wider (3 to 5 µm - average 3.7 µm) in relation to those produced *in vitro*, 18.5 to 59 µm (average 37.4 µm) and 2.5 to 5 µm (average 3.5 µm), respectively.

Identification of *T. pseudoeucalypti* is difficult and inconclusive based solely on morphology, as many *Teratosphaeria* species are morphologically indistinguishable (Andjic et al., 2010b). Examples include *T. eucalypti* and *T. pseudoeucalypti*, which are considered morphologically similar (Andjic et al., 2010a), making it difficult to identify these species based on this trait. Currently, the distinction between species is primarily based on DNA sequence comparisons (Crous, 2009). Considering the three gene regions (β-T, EF-1α and ITS-2), there were no differences among the Brazilian isolates. Bayesian inference of concatenated genes grouped the isolates from Brazil with isolates of *T. pseudoeucalypti* (Figure 2),

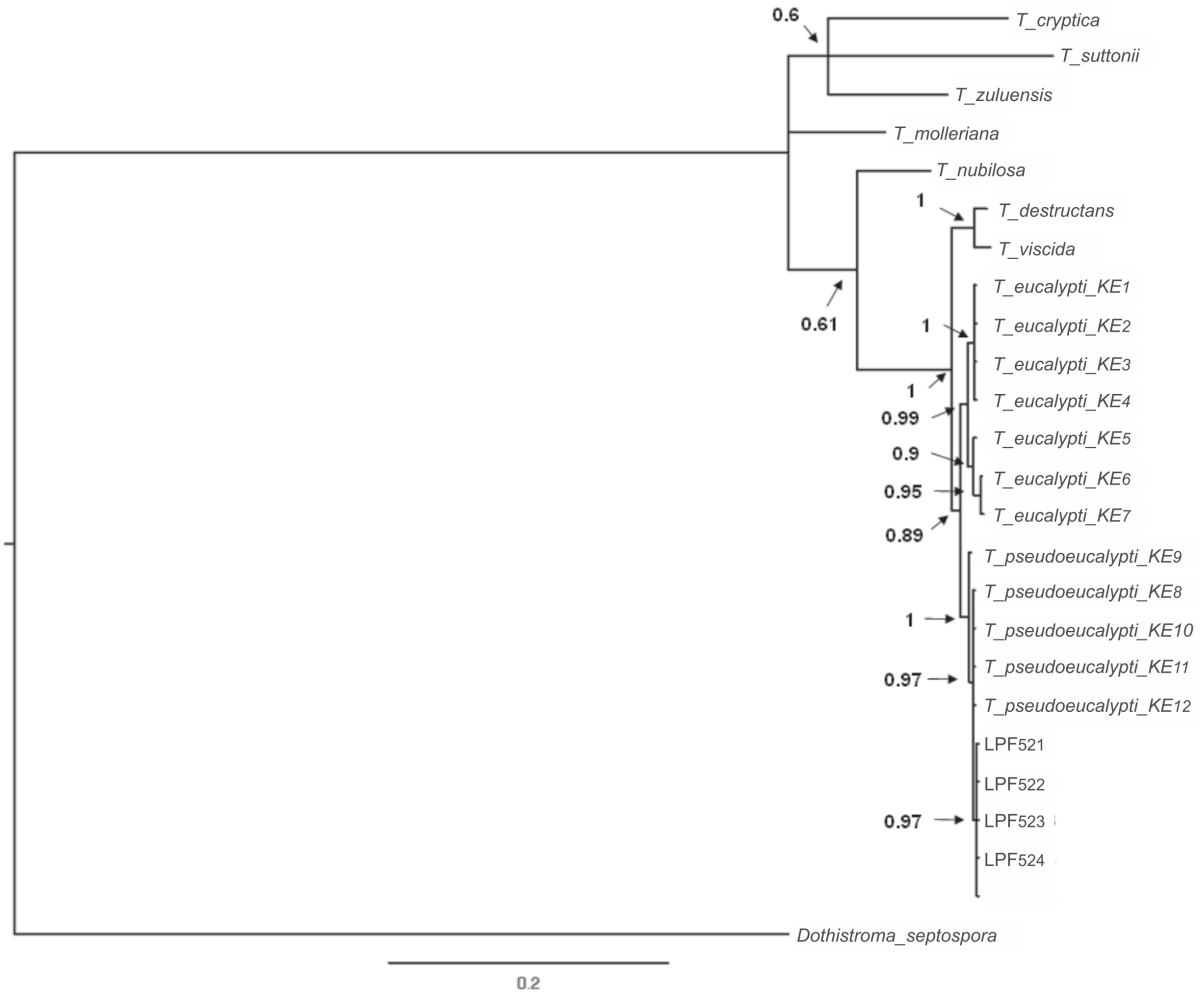

FIGURE 2 - Multilocus phylogenetic tree for partial sequences of the EF-1α, β-tubulin and ITS-2 genes by Bayesian analysis for isolates obtained in this study (LPF). Probability values are indicated on the branches. *Dothistroma septospora* was used as an outgroup. The identification of isolated *Teratosphaeria eucalypti* and *T. pseudoeucalypti* are according to the identification of a haplotype (KE) by Andjic et al. (2010a) as indicated in the Table 1. The scale bar indicates the fraction of substitution per site.

though the sequences of the Brazilian isolates differed slightly from those previously described in Australia by Andjic et al. (2010a).

This is the first report of *T. pseudoeucalypti* outside Australia. The fungus was found in four clones (*E. globulus*, *E. urophylla* x *E. globulus* and *E. nitens* x *E. globulus*) severely affected by the disease. In each case, one of the parents was *E. globulus*, considered one of the most susceptible species to *Teratosphaeria* spp. (Passador et al., 2012). According to Hunter et al. (2011), *T. destructans*, *T. pseudoeucalypti* and *T. viscidus* are most destructive on *E. grandis* and its hybrids in tropical and subtropical regions. In our study, the clones were highly susceptible to *T. pseudoeucalypti* with diseased leaves covered by pycnidia and cirri of conidia, which may constitute an important source of inoculum.

Teratosphaeria spp. were first reported on eucalyptus in Australia and likely dispersed from Australia to other countries, including South Africa and Spain (Crous et al., 2004), Brazil (Pérez et al., 2009a), Portugal (Hunter et al., 2008), Chile (Crous et al., 2009), and Uruguay (Pérez et al., 2009b). Recently, plantings of hybrid *E. grandis* x *E. camaldulensis* in Queensland, Australia suffered severe outbreaks and damage by *T. pseudoeucalyti*. The impact of this disease in Australia has increased annually (Andjic et al., 2010a). The leaf symptoms caused by *T. pseudoeucalypti* are variable and similar to those caused by *T. eucalypti* and *T. destructans,* depending on the host and number of mature leaves at the time of infection. In Queensland, *T. pseudoeucalypti* is considered the most aggressive species of *Teratosphaeria* established in the region and has been identified in nurseries and in the field (Andjic et al., 2010a).

The identification of *T. pseudoeucalypti* and the consequent damage to the eucalyptus plants in Brazil has led to substantial concern regarding the dissemination of this pathogen into other countries in South America where *E. globulus* is planted extensively, such as Uruguay, Chile, Colombia, Ecuador and Peru (Potts et al., 2004). Another problem is that eucalyptus plantations in South America, and especially in Brazil, have large planting areas of the same genetic material; thus, potential losses caused by the disease can increase considerably.

Currently, *Teratosphaeria nubilosa* is the most important *Teratosphaeria* species causing TLD in eucalyptus in Brazil (Teodoro et al., 2012). With the identification of *T. pseudoeucalypti* in south Brazil and the damage in plantations in Australia, this species of *Teratosphaeria* could become one of the most aggressive species to eucalyptus in Brazil. The most appropriate strategy for the control of TLD would be the development of resistant or tolerant clones; however, further research is needed to select clones that are resistant to this new species of *Teratosphaeria* sp. In addition, knowledge of the life cycle of the pathogen is essential for setting strategies for disease control and reducing economic losses.

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