CERATOCYSTIS WILT ON *EUCALYPTUS*: FIRST RECORD FROM SOUTH AFRICA

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Wilt and death of an *E. grandis* X *E. urophylla* variety was recently observed in the Zululand region of KwaZulu-Natal, South Africa. Symptoms on the dying trees included a streaking pattern of discoloration in the sapwood and cambium of stems and roots. A fungus resembling a *Ceratocystis* sp. was consistently found sporulating on diseased material and isolated from the symptomatic root and stem tissue. DNA sequence analyses of the *Ceratocystis* isolates, including multiple gene regions, identified the isolates as *C. eucalypticola*. Multiple ITS types were identified among the isolates sequenced, similar to that found in other, closely related, *Ceratocystis* species. Artificial inoculations under field conditions confirmed the pathogenicity of the isolates. This is the first report of a *Ceratocystis* sp. causing a wilt disease of *Eucalyptus* in South Africa.

Keywords

Ceratocystidaceae, canker, emerging disease, root infection, vascular wilt

Introduction

Ceratocystis wilt of *Eucalyptus* species is considered one of the most important diseases of these trees. This is particularly true in Brazil where the disease has been known for more than twenty years (Ferreira et al. 1999; Oliveira et al. 2015). It has also been found in Uruguay (Barnes et al. 2003), the Republic of Congo (Roux et al. 2000), Uganda (Roux et al. 2001), China (Li et al. 2014) and Pakistan (Alam et al. 2017). The disease results in wilting symptoms and death of susceptible trees, reduction of volumetric growth and lower cellulose yields (Mafia et al. 2013).

The causal agent of Ceratocystis wilt on *Eucalyptus* has in most cases been attributed to the aggregate species *Ceratocystis fimbriata* Ellis and Halst. (Alam et al. 2017; Barnes et al. 2003; Ferreira et al. 1999; Li et al. 2014; Roux et al. 2000). This fungus resides in the Ceratocystidaceae, as defined by de Beer et al. (2014), and the taxonomy remains open to considerable debate (Fourie et al. 2015; Harrington et al. 2014; Oliveira et al. 2015). Various other species of *Ceratocystis* have been identified from *Eucalyptus* spp., including *C. eucalypticola* (Van Wyk et al. 2012), *C. chinaeucensis* (Chen et al. 2013), *C. manginecans* (Chen et al. 2013), *C. fimbriatomima* (Van Wyk et al. 2009), *C. neglecta* (Rodas et al. 2007) and *C. piriliformis* (Barnes et al. 2003), but none of these have been associated with mortality of trees in plantations. All of these species were isolated from freshly made wounds on trees, in the absence of disease. Some species, such as *C. pirilliformis*

and *C. chinaeucensis*, can however result in lesions on artificially inoculated *Eucalyptus* plants (Chen et al. 2013; Roux et al. 2004).

Symptoms of Ceratocystis wilt include wilting of single branches and the tops of trees followed by rapid tree death as a result of vascular occlusion (Ferreira et al. 2006; Roux et al. 2000). These symptoms may also be accompanied by stem cankers (Ferreira et al. 2006). Wilting and stem cankers are typically associated with a streaked pattern of staining in the cambium and xylem (Ferreira et al. 2006; Roux et al. 2000). Infection of *Eucalyptus* trees in Brazil has been shown to take place through the roots (Ferreira et al. 2006) or via stem wounds (Ferreira et al. 1999, 2006). Spread of the pathogen has been attributed to the use of contaminated tools, especially in cutting nurseries, and the movement of clonally propagated plant material (Ferreira et al. 2011, 2013).

Wilt symptoms and death of a *E. grandis* X *E. urophylla* (GU) hybrid variety were observed in the Zululand region of the KwaZulu-Natal (KZN) Province of South Africa in 2018. The aim of this study was to identify the causal agent of the disease and obtain an indication of the geographic extent of the disease in the area.

Materials and methods

Disease symptoms and distribution

Wilt and death of young, four-month-old, *Eucalyptus* trees was first observed in March 2018 in a plantation in the Zululand region of KZN, near the city of Richards Bay. The affected trees were of a single *E. grandis* X *E. urophylla* (GU) hybrid variety, established in November 2017. Surveys of all compartments planted during

the same period, and within a 20km radius of the affected compartment, were subsequently conducted to determine the distribution of the disease in the area. Additionally, in 2019, all compartments in the area established with the affected *Eucalyptus* variety were surveyed for the presence of the disease.

Collection of isolates

Stem and root sections were collected from the wilting GU trees. Samples were placed in separate brown paper bags for each sampled tree and transported to the laboratory for processing. Symptomatic pieces of wood, from the stems and roots of trees showing signs of vascular staining, were placed in moist chambers to induce sporulation of the pathogen. Single ascospore drops produced at the apices of ascomata and/or mycelium growing on the wood and root surfaces were aseptically transferred to 2% Malt Extract Agar (MEA) in Petri dishes and incubated at room temperature (~25°C). All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Identification of isolates

Isolates were initially identified as belonging to the genus *Ceratocystis* based on morphology. Species level identification was achieved based on DNA sequence comparisons. Pure cultures were grown at 23 °C for 10 days for DNA extraction. DNA was extracted using a Zymo Research ZR Fungal/Bacterial DNA MiniPrep™ kit (Irvine California, USA). PCR amplification and sequencing of four gene regions; *bt1*, *ef1*, *ms204* and ITS, was carried out as described by Fourie et al. (2015) and *rpb2* as described by Liu et al. (2018). The resulting sequences were added to the

respective gene sequence databases for the Latin American Clade of *Ceratocystis* generated in Barnes et al. (2018) and available as TreeBASE Accession No. S22005 (http://purl.org/phylo/treebase/phylows/study/TB2:S22005). These databases contain representative type sequences (Marin-Felix et al. 2017) of all described *Ceratocystis* species for which sequence data are available.

Maximum parsimony (MP) analyses were carried out using PAUP v. 4.0b10 (Swofford 2003) with the following settings: heuristic search option, TBR branch swopping algorithm with 1 000 repeats and gaps treated as a fifth character state. Standard parameters such as tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated. Maximum likelihood analyses were performed using the GTRGAMMA setting and standard parameters in RAxML (Stamatakis 2014). For both MP and ML analyses, 1000 bootstrap replicates were conducted to determine branch support, and *C. albifundus* was used as the outgroup. Trees were visualised and edited in MEGA v. 7 (Kumar et al. 2016).

Pathogenicity trials

To fulfil the requirements of Koch's Postulates, three isolates (CMW 53160, CMW 53156, CMW 53157) obtained from diseased trees were grown on 2% MEA for 10 days at room temperature. These isolates were inoculated into the stems of healthy six-month-old GU trees under field conditions. Ten trees were inoculated for each of the test isolates and 10 trees with sterile agar to serve as controls. Inoculations were done by inserting 9 mm diameter plugs of agar overgrown with the test isolate, with the mycelium facing the cambium, into the stem wounds on each tree. Stem wounds for inoculation were made using a 9 mm diameter cork borer to remove the

bark and expose the cambium. Wounds were covered with masking tape to prevent desiccation of the inoculum and to reduce contamination.

The pathogenicity trial was assessed after six weeks by measuring the lengths of the lesions that had developed in the bark and cambium. Sections of symptomatic tissue were also collected for re-isolations to be made. Results were analysed in GenStat Version 18.2.0.18409 (VSN International 2016), using ANOVA. Significance of differences between treatments was determined with Tukey's confidence analyses at 95%.

Results

Disease symptoms and distribution

Typical symptoms of the wilt disease on the GU variety included leaf chlorosis and wilting, death of branches and in many cases entire trees (**Figure 1a**), bark lesions (**Figure 1b**), mottling of the cambium tissues under the bark (**Figure 1c**) and brown streaked discoloration of the sapwood (**Figure 1d**). Uprooting of trees revealed the presence of root lesions, with brown streaked discoloration in the root tissues. Incubation of symptomatic tissue in moist chambers resulted in the consistent and dominant growth of a fungus producing ascomata with round black bases and long necks with spore masses at their apices.

Surveys in 2018, of compartments established during the same planting period (June to November 2017) when the affected compartment was established, showed no signs of disease. Two compartments (~ 6-month-old trees), planted to the affected GU variety in the 2018 planting season were identified in 2019 surveys. These

compartments were within a 20km radius from each other and the site at which the disease was originally observed. All affected compartments have previously been planted to various Eucalypt genotypes, with no prior reports of disease.

Identification of isolates

Cultures obtained from symptomatic plant material, stems and roots, had morphological features typical of *Ceratocystis* species *sensu* de Beer et al. (2014). These included black, round ascomatal bases with long black necks and cream-colored spore masses that exuded from the tips of the necks. Ascospores had hat-shaped sheaths and were produced in mucilaginous masses. The asexual state of the isolates had a white to grey mycelium from which tubular conidiogenous cells (phialides) gave rise to chains of hyaline cylindrical conidia. After a few days, cultures produced a fruity aroma typical of *Ceratocystis* spp.

Seven isolates were selected for DNA sequencing. Amplification of the *bt1*, *ef1*, *ms204*, *rpb2* and ITS regions resulted in amplicon sizes of approximately 610 bp, 780 bp, 900 bp, 1 200 bp and 550 bp, respectively.

For the maximum parsimony analyses, the combined dataset of the gene regions *bt1*, *ef1*, *ms204*, *rpb2,* and the ITS dataset consisted of 3257 and 546 characters, of which 9 and 7 were excluded and 231 and 121 were parsimony informative characters, respectively. The analysis resulted in 24 and 16 most parsimonious trees for the combined, and ITS gene regions, and produced tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) values of TL = 281 and 339, CI = 0.883 and 0.737, RI = 0.933 and 0.835, RC= and 0.824 and 0.616

respectively. The log likelihood of best trees obtained from maximum likelihood analyses were -6253.7 and -1637.48 for the combined gene regions and for the ITS region.

The *bt1*, *ms204* and *rpb2* sequences for all seven isolates were 100% identical to the ex-type culture of *C. eucalypticola* (CBS 124016, CMW 11536) while the *ef1* sequences had a 1bp difference from that isolate. For the purpose of this study, all isolates are considered *C. eucalypticola* based on sequences of these gene regions (**Figure 2a**). For the ITS gene region, the seven isolates contained mixed ITS sequences (**Figure 2b**) typical of many species of *Ceratocystis* (Harrington et al. 2014; Oliveira et al. 2015; Barnes et al. 2018). Three isolates (CMW 53161, CMW 53160, CMW 53158) were identified as *C. eucalypticola* (**Figure 2b**) with 100% homology to ex-type culture CBS 124016 (CMW 11536). Two isolates (CMW 53162, CMW 53157) had ITS sequences that matched those of *C. manginecans* Group 1 (Al Adawi et al. 2013), while two isolates (CMW 53156, CMW 53159) had mixed ITS signals and their sequences could not be determined in the absence of cloning (Al Adawi et al. 2013; Marin-Felix et al. 2017), which was beyond the scope of the present study.

Pathogenicity trial

Six weeks after inoculation, very small or no bark lesions were visible for the control inoculations (**Figure 3a**). In contrast, clear over-bark lesions were visible for all three *Ceratocystis* isolates tested (**Figure 3b, c**). Exposure of the cambium showed the presence of callus formation and no lesion development for the controls (**Figure 3d**), while the three *C. eucalypticola* isolates produced obvious lesions in the cambium

(**Figure 3e, f**), which extended into the xylem tissues. Statistical analyses showed significant differences between the lesion lengths associated with the three *C. eucalypticola* isolates and those of the control inoculations ($p<0.05$) (**Figure 4**). *Ceratocystis eucalypticola* was easily re-isolated from the lesions associated with the test isolates but not from the controls.

Discussion

The results of this study showed clearly that a *Ceratocystis* sp. was responsible for the wilt disease that has recently appeared in a plantation of a GU variety in KwaZulu-Natal, South Africa. This is the first report of a Ceratocystis wilt disease on *Eucalyptus* in South Africa. It is also the first report of *C. eucalypticola* causing a disease of these trees. This is particularly interesting given the fact that *C. eucalypticola* have been known from *Eucalyptus* in the country for many years (Kamgan-Nkuekam et al. 2013; Roux et al. 2004), but were not previously found on diseased trees in plantations.

Ceratocystis eucalypticola was first described by van Wyk et al. (2012) in South Africa, based on a multi-gene phylogenetic analysis showing that isolates of a *Ceratocystis* sp. from wounds on *Eucalyptus* stems were distinct from those reported as *C. fimbriata sensu lato* on various other tree hosts. *Ceratocystis eucalypticola* resides in what has been described as the Latin American clade of *Ceratocystis* (Harrington 2000). This clade includes *C. fimbriata*, the name that has been applied to the Ceratocystis wilt pathogen of eucalypts in Brazil (Oliveira et al. 2015), *C. fimbriatomima*, *C. neglecta, C. pirilliformis* and other species also associated with eucalypts (Fourie et al. 2015).

Currently, some researchers consider *C. eucalypticola* and other species from eucalypts as different haplotypes of *C. fimbriata* (Li et al. 2016; Oliveira et al. 2015) and not distinct species. But, the taxonomy of *Ceratocystis* spp. occurring on *Eucalyptus* spp. remains to be fully resolved (Fourie et al. 2015; Harrington et al. 2014; Oliveira et al. 2015). This relates to differences in opinion regarding the identity of the type species of *Ceratocystis* i.e. *C. fimbriata*. In this regard, *C. fimbriata sesu stricto* is a pathogen of sweet potato tubers first recorded in the USA by Halsted in 1890 (De Beer et al. 2014). Isolates of that fungus represent a clonal lineage in *Ceratocystis sensu lato* (Li et al. 2016) that is very closely related to a number of other described species of *Ceratocystis*, especially *C. eucalypticola* and *C. manginecans* (Fourie et al. 2015). For the purposes of the present study, and in order to avoid confusion, we have chosen to adopt a broad taxonomic concept while recognising the relatedness of species and the existence of clonal lineages in the *C. fimbriata sensu lato* complex.

Ceratocystis eucalypticola, identified in this study, commonly infects freshly made wounds on *Eucalyptus* in the KwaZulu-Natal and Mpumalanga Provinces of South Africa (Kamgan Nkuekam et al*.* 2013; Roux et al. 2004; Van Wyk et al*.* 2012). The fungus has, however, never been associated with disease or death of *Eucalyptus* species. Results of the present study have shown that the fungus is able to kill trees under field conditions and is able to infect tree roots. This is similar to the situation with *C. fimbriata* in Brazil, where the fungus is commonly isolated from roots and soil in *Eucalyptus* plantations (Ferreira et al. 2011).

It remains to be determined how *C. eucalypticola* was able to enter trees to cause the disease described in this study. *Ceratocystis* spp. require wounds to infect trees. There were, however, no signs of stem wounds on the infected trees and, based on the presence of root lesions and progression of symptoms, the most likely and common points of infection were through the roots of trees. It is possible that insects could have damaged the roots to provide infection points but there was no obvious evidence that this was the case. Direct penetration of root hairs and roots of various plant species has been shown for *Berkeleyomyces basicola* (Nel et al. 2018), which also resides in the Ceratocystidaceae (Hood and Shew 1997; Mauk and Hine 1988) and this may be true for *C. eucalypticola* infection of the GU variety in this study.

The source of inoculum resulting in infection of the diseased trees could not be determined at the time of the disease outbreak, or from subsequent surveys of other compartments planted to *Eucalyptus*. However, the presence of root infections and development of symptoms from the roots, upwards into the root collar of affected trees, in all three compartments where the disease has been found, suggests that the inoculum originates from the soil. The origin of *C. eucalypticola*, its possible presence in the soil and its mode of infection are currently being studied so as to consider possible control strategies.

The most efficient management strategy to reduce the impact of diseases in plantation forestry, based on vegetative propagation, is through the breeding and selection of disease tolerant genotypes, and this is universally true for *Eucalyptus* spp. (Wingfield et al. 2013). In this respect, considerable variation in susceptibility to *Ceratocystis* has been shown for different *Eucalyptus* genotypes in Brazil

(Guimarães et al. 2010; Zauza et al. 2004). Identification of relative susceptibility in *Eucalyptus* varieties can emerge from natural infection as well as by using artificial inoculation tests. However, because of variation in aggressiveness between pathogen strains (Oliveira et al. 2016), it will be important to have a comprehensive understanding of the strains used in such studies. This variation in aggressiveness of strains was also observed in the present study, and it needs to be considered in future work to select disease tolerant planting material as well as studies to obtain an understanding of the genetic variation in *C. eucalypticola.*

A previous population genetic study of *C. eucalypticola* isolates in South Africa showed that the fungus represented a clonal lineage and was most likely introduced into the country (Van Wyk et al. 2006). Assuming that isolates of the pathogen that have emerged from the present study represent the same genetic entity as the fungus previously reported in South Africa, the likelihood of an accidental introduction would be supported. What remains curious is that the fungus, while present in the country, has not previously emerged as a pathogen causing tree disease. This question and others raised in this study form the basis of ongoing investigations regarding a new and potentially threatening disease problem in South Africa.

Conclusions

This study presents a first report of Ceratocystis wilt of *Eucalyptus* in South Africa. While the disease has emerged in a single *Eucalyptus* variety, its occurrence in three compartments, and the reported importance of Ceratocystis wilt of *Eucalyptus* in Brazil is of considerable concern to South Africa. The fungus causing the disease

was identified as *C. eucalypticola*, related to the sweet potato pathogen *C. fimbriata,* and other closely related species of *Ceratocystis sensu* de Beer et al. (2014). Most of these species represent clonal lineages that might be considered host-adapted varieties, although a robust system to define such differences has yet to be considered. The origin and the mode of infection of *C. eucalypticola* infecting the single *Eucalyptus* variety in this study remains to be determined. Likewise, studies to select disease-tolerant planting stock will be important for the management of the disease. The fact that it has been found affecting only a single GU variety suggests that will be a viable strategy.

Acknowledgements

We thank the members of the Tree Protection Co-operative Programme (TPCP) and the University of Pretoria for funding and facilities to undertake the identification of the pathogen in this study. We thank the National Research Foundation (Grant 95875) for financial support.

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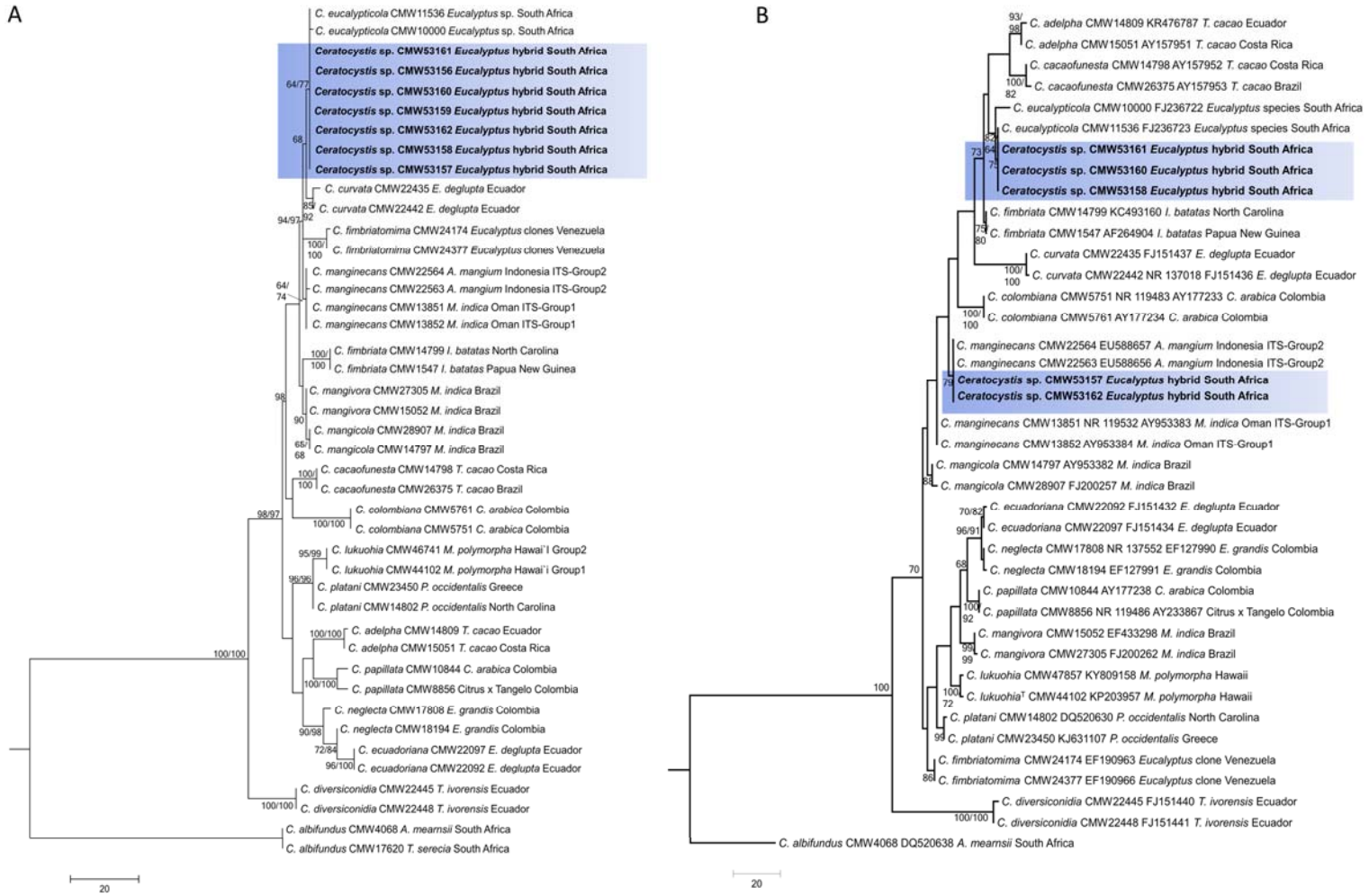
List of figures

Figure 1

Figure 2


A
C. eucalypticola CMW11536 Eucalyptus sp. South Africa
C. eucalypticola CMW10000 Eucalyptus sp. South Africa
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C. curvata CMW22435 E. deglupta Ecuador
C. curvata CMW22442 E. deglupta Ecuador
C. fimbriatomima CMW24174 Eucalyptus clones Venezuela
C. fimbriatomima CMW24377 Eucalyptus clones Venezuela
C. manginecans CMW22564 A. mangium Indonesia ITS-Group2
C. manginecans CMW22563 A. mangium Indonesia ITS-Group2
C. manginecans CMW13851 M. indica Oman ITS-Group1
C. manginecans CMW13852 M. indica Oman ITS-Group1
C. fimbriata CMW14799 I. batatas North Carolina
C. fimbriata CMW1547 I. batatas Papua New Guinea
C. mangivora CMW27305 M. indica Brazil
C. mangivora CMW15052 M. indica Brazil
C. mangicola CMW28907 M. indica Brazil
C. mangicola CMW14797 M. indica Brazil
C. cacaofunesta CMW14798 T. cacao Costa Rica
C. cacaofunesta CMW26375 T. cacao Brazil
C. colombiana CMW5761 C. arabica Colombia
C. colombiana CMW5751 C. arabica Colombia
C. lukuohia CMW46741 M. polymorpha Hawai`i Group2
C. lukuohia CMW44102 M. polymorpha Hawai`i Group1
C. platani CMW23450 P. occidentalis Greece
C. platani CMW14802 P. occidentalis North Carolina
C. adelpha CMW14809 T. cacao Ecuador
C. adelpha CMW15051 T. cacao Costa Rica
C. papillata CMW10844 C. arabica Colombia
C. papillata CMW8856 Citrus x Tangelo Colombia
C. neglecta CMW17808 E. grandis Colombia
C. neglecta CMW18194 E. grandis Colombia
C. ecuadoriana CMW22097 E. deglupta Ecuador
C. ecuadoriana CMW22092 E. deglupta Ecuador
C. diversiconidia CMW22445 T. ivorensis Ecuador
C. diversiconidia CMW22448 T. ivorensis Ecuador
C. albifundus CMW4068 A. mearnsii South Africa
C. albifundus CMW17620 T. serecia South Africa
20
B
C. adelpha CMW14809 KR476787 T. cacao Ecuador
C. adelpha CMW15051 AY157951 T. cacao Costa Rica
C. cacaofunesta CMW14798 AY157952 T. cacao Costa Rica
C. cacaofunesta CMW26375 AY157953 T. cacao Brazil
C. eucalypticola CMW10000 FJ236722 Eucalyptus species South Africa
C. eucalypticola CMW11536 FJ236723 Eucalyptus species South Africa
Ceratocystis sp. CMW53161 Eucalyptus hybrid South Africa
Ceratocystis sp. CMW53160 Eucalyptus hybrid South Africa
Ceratocystis sp. CMW53158 Eucalyptus hybrid South Africa
C. fimbriata CMW14799 KC493160 I. batatas North Carolina
C. fimbriata CMW1547 AF264904 I. batatas Papua New Guinea
C. curvata CMW22435 FJ151437 E. deglupta Ecuador
C. curvata CMW22442 NR 137018 FJ151436 E. deglupta Ecuador
C. colombiana CMW5751 NR 119483 AY177233 C. arabica Colombia
C. colombiana CMW5761 AY177234 C. arabica Colombia
C. manginecans CMW22564 EU588657 A. mangium Indonesia ITS-Group2
C. manginecans CMW22563 EU588656 A. mangium Indonesia ITS-Group2
Ceratocystis sp. CMW53157 Eucalyptus hybrid South Africa
Ceratocystis sp. CMW53162 Eucalyptus hybrid South Africa
C. manginecans CMW13851 NR 119532 AY953383 M. indica Oman ITS-Group1
C. manginecans CMW13852 AY953384 M. indica Oman ITS-Group1
C. mangicola CMW14797 AY953382 M. indica Brazil
C. mangicola CMW28907 FJ200257 M. indica Brazil
C. ecuadoriana CMW22092 FJ151432 E. deglupta Ecuador
C. ecuadoriana CMW22097 FJ151434 E. deglupta Ecuador
C. neglecta CMW17808 NR 137552 EF127990 E. grandis Colombia
C. neglecta CMW18194 EF127991 E. grandis Colombia
C. papillata CMW10844 AY177238 C. arabica Colombia
C. papillata CMW8856 NR 119486 AY233867 Citrus x Tangelo Colombia
C. mangivora CMW15052 EF433298 M. indica Brazil
C. mangivora CMW27305 FJ200262 M. indica Brazil
C. lukuohia CMW47857 KY809158 M. polymorpha Hawaii
C. lukuohia^T CMW44102 KP203957 M. polymorpha Hawaii
C. platani CMW14802 DQ520630 P. occidentalis North Carolina
C. platani CMW23450 KJ631107 P. occidentalis Greece
C. fimbriatomima CMW24174 EF190963 Eucalyptus clone Venezuela
C. fimbriatomima CMW24377 EF190966 Eucalyptus clone Venezuela
C. diversiconidia CMW22445 FJ151440 T. ivorensis Ecuador
C. diversiconidia CMW22448 FJ151441 T. ivorensis Ecuador
C. albifundus CMW4068 DQ520638 A. mearnsii South Africa
20

Figure 3

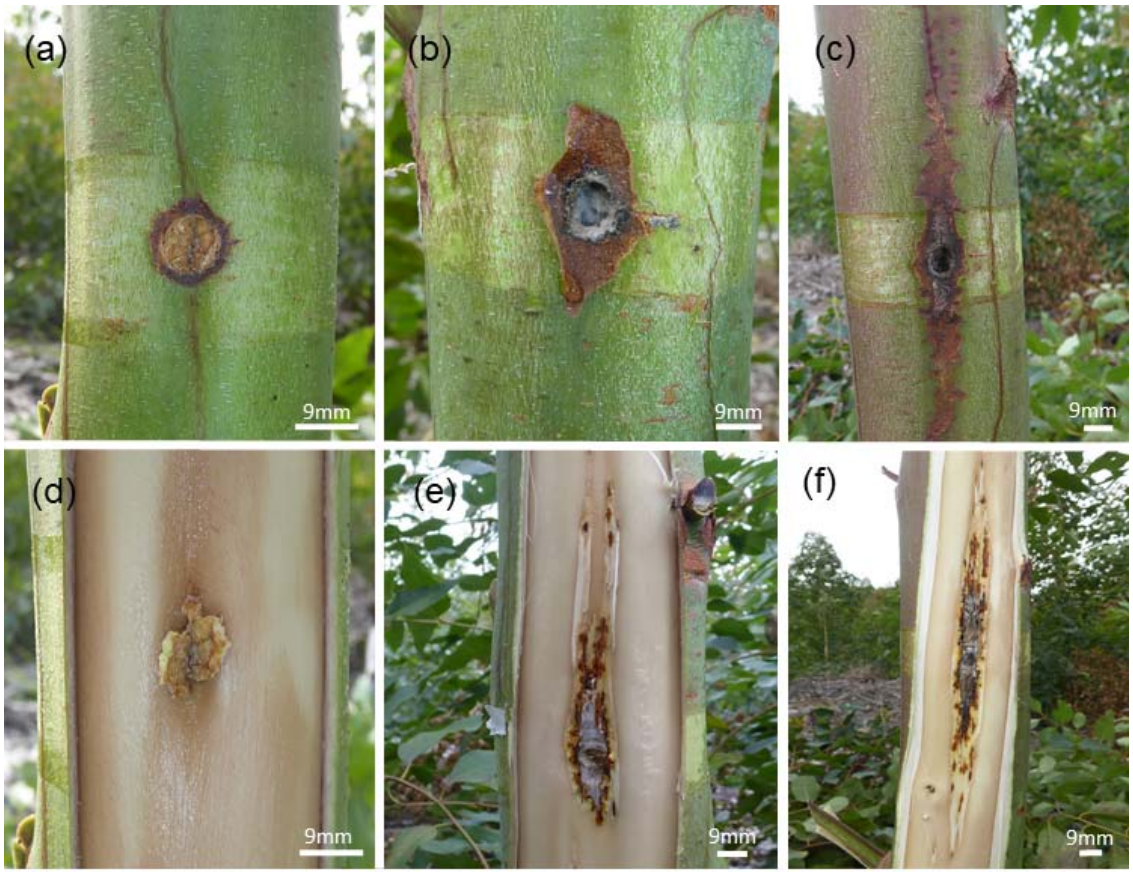
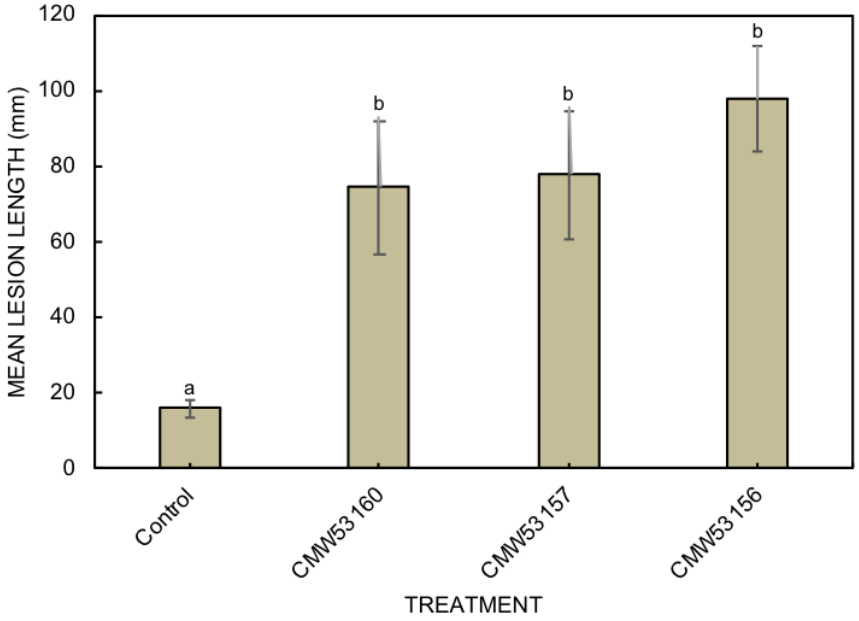


Figure 4


120
100
80
60
40
20
0
MEAN LESION LENGTH (mm)
a
b
b
b
Control
CMW53160
CMW53157
CMW53156
TREATMENT