# A serious new wilt disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa

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## Summary

In a recent survey of *Eucalyptus* clones in the Republic of Congo, Central Africa, a serious wilt and die-back disease of two different hybrid clones was observed. Affected trees ranged in age from approximately 6 months to 4 years. Isolations from symptomatic plant material consistently yielded a *Ceratocystis* species. On the basis of morphology and sequence data this fungus was identified as *Ceratocystis fimbriata*, a well-known wilt and canker pathogen of many economically important plants. The *Eucalyptus* isolates were compared with other *Ceratocystis* spp. based on sequence data generated from the ITS and 5.8S region of the rRNA operon. The results confirmed the identity of the *Ceratocystis* isolates from *Eucalyptus* as *C. fimbriata* and showed that they group with other *C. fimbriata* isolates from Brazil, South Africa and Europe. Inoculations on young *Eucalyptus* plants were conducted in the greenhouse and all three of the Congolese isolates tested, produced typical lesions in the bark and xylem. This study represents the first report of *C. fimbriata* as a pathogen of *Eucalyptus* in Africa. This is a serious new disease that will require considerable study in order to ensure that losses, caused by *C. fimbriata*, do not continue.

## 1 Introduction

*Eucalyptus* spp. are native to Australia and approximately 8 million hectares of plantations have been established, mostly in tropical and subtropical countries of the world. The wood from these trees is used for timber, paper and pulp, the production of rayon and viscose and for firewood (Turnbull 1991). *Eucalyptus* spp. have been grown in the Republic of the Congo since 1953 and from 1978, clonal plantations, established from vegetatively propagated trees, have been utilized (Leakey 1987). Serious disease problems have emerged on exotic *Eucalyptus* spp. in most countries where they have been planted, despite the fact that the trees have been isolated from their natural enemies. These diseases include both stem, root and leaf diseases. Diseases such as Cryphonectria canker, caused by *Cryphonectria cubensis* (Bruner) Hodges, have necessitated extensive clonal programmes to reduce losses in countries such as South Africa and Brazil (Florence et al. 1986; Hodges et al. 1986; Conradie et al. 1990). Other stem and root diseases include Eucalyptus rust caused by *Puccinia psidii* G. Winter (Ferreira 1989), Coniothyrium canker caused by *Coniothyrium zuluense* (Wingfield et al. 1997) and Pythium and Phytophthora root rot (Linde et al. 1994). Leaf diseases caused by species of *Mycosphaerella* Johanson and *Cylindrocladium* Morgan also cause serious problems, especially in tropical areas (Park and Keane 1984;

CROUS and WINGFIELD 1994; 1996). These and other diseases have led to considerable economic losses to the *Eucalyptus* industry.

Until recently, no thorough survey of the diseases affecting plantation *Eucalyptus* in the Republic of Congo had been conducted. Sporadic reports of tree deaths were made occasionally, but no detailed investigations into the causal agents were initiated. One such report was of a wilt and die-back disease, thought to be caused by an undetermined species of *Botryodiplodia* (DECLERT 1996). During 1998, a survey of diseases of Eucalypt plantations in the Pointe-Noire area of the Republic of Congo was undertaken. This led to the discovery of a wilt and die-back disease of *Eucalyptus urophylla* S. T. Blake × *Eucalyptus pellita* F. Muell. (UP) and *Eucalyptus territicornis* Sm. × *Eucalyptus grandis* Hill ex. Maid. × (TG). The affected trees ranged from 6 months to more than 4-year-old. The most common symptoms were rapid wilting and death and irregular (streaked) dark brown discoloration of the xylem.

This paper reports on the isolation, identification and pathogenicity of the causal agent of Eucalyptus wilt in the Republic of Congo. Both morphological and molecular techniques were utilized to confirm the identity of the pathogen and Koch's postulates were fulfilled in greenhouse inoculation trials. The ITS and 5.8S regions of the ribosomal RNA operon was chosen for the molecular comparisons, because these regions have previously been used to characterize species of *Ceratocystis* (HAUSNER et al. 1993; VISSER et al. 1995; WINGFIELD et al. 1996; WITTHUHN et al. 1998, 1999).

## 2 Materials and methods

### 2.1 Sites and isolations

Disease was observed on 2-year-old trees and 6-month-old coppice stems of *E. urophylla* × *E. pellita* (UP) hybrids from Kissoko plantation and from 4-year-old coppice stems of *E. territicornis* × *E. grandis* (TG) growing at Mengo plantation, Republic of Congo (1°00 S, 15°00 E) in 1998.

Pieces of symptomatic tissue from the leading margin of the streaked and discoloured wood were plated directly onto 2% malt extract agar (MEA) (20 g/l Biolab malt and 15 g/l Biolab agar, Biolab Diagnostics (Pty) Ltd, Midrand, South Africa). Segments of symptomatic material were also placed in Petri dishes containing moistened filter paper to induce the formation of fungal fruiting bodies. All plates were incubated at approximately 25°C to induce fungal growth. Perithecia formed in the symptomatic tissue (streaks) within a few days and single ascospore drops were transferred to separate MEA plates. Direct isolation from plant material on to MEA resulted in the abundant formation of colonies of a fungus that produced long-necked perithecia. All isolates (CMW numbers) are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

### 2.2 Greenhouse pathogenicity trials

Twenty trees, approximately 5–10 mm diameter, of an *E. grandis* × *E. camaldulensis* Denh. (GC) hybrid clone were artificially inoculated with three isolates of the suspected pathogen. The isolates were grown on MEA for 14 days before inoculation. Wounds were made into the xylem of the trees by removing the bark with a 4-mm diameter cork borer. Mycelial plugs of equal size, covered with the test fungus were placed into the wounds and the wounds sealed with parafilm to prevent desiccation of the inoculum and the wounds. Ten trees were each inoculated with sterile agar plugs as controls. Lesions on the outer bark and in the xylem were measured after 5 weeks. Pieces of symptomatic tissue were placed in Petri dishes containing moistened filter paper to confirm that the inoculated fungus was

responsible for the observed lesions. Results from the inoculation trials were statistically analysed for variances between isolates and controls using Tukey's analysis of variance procedure.

## 2.3 DNA amplification and sequencing

Isolates of *Ceratocystis fimbriata* from the Republic of Congo (CMW4769, CMW4783), Brazil (CMW4900, CMW4901) and South Africa (CMW4101) were grown on MEA plates and template DNA was obtained by scraping the mycelium with a sterile pipette tip (HARRINGTON and WINGFIELD 1995), or by picking up a single ascospore drop, and performing the polymerase chain reaction (PCR) directly on the fungal material. Primers ITS 1 (5′TCCGTAGGTGAACCTGCGG′3) and ITS 4 (5′TCCTCCGCTTATTGA-TATGC′3) were used to amplify the ITS and 5.8S regions of the ribosomal RNA operon (WHITE et al. 1990). The PCR reaction mixture included Expand® (Boehringer High Fidelity PCR, Boehringer Mannheim, Germany), 0.2 mM DNTP's, 10 × Buffer (Boehringer), 1 mM MgCl (Boehringer) and 0.75 mM of each primer. Denaturation was performed at 96°C for 1 min, followed by 35 cycles consisting of primer annealing at 55°C for 30 s, chain elongation at 72°C for 1 min and denaturation at 92°C for 1 min. Final chain elongation took place at 72°C for 5 min. The PCR products were electrophoresed in an agarose gel, stained with ethidium bromide and visualized under UV illumination.

The PCR products obtained were purified using the QIAquick PCR purification kit (QIAGEN, Germany). The PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with Amplitaq® DNA polymerase, FS (Perkin-Elmer, Warrington, UK), according to the manufacturers protocol, on a ABI PRISM™ 377 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 was used in the sequence reaction.

Sequences for the Congolese, Brazilian and South African isolates were aligned with sequences for *Ceratocystis* spp. obtained from Genbank and WINGFIELD et al. (1996) (Table 1). Nucleotide sequences were manually aligned by inserting gaps and analysed in PAUP* 4.0 [Phylogenetic Analysis Using Parsimony (*and other methods)] (SWOFFORD 1998). Missing data were treated as a 'fifth character' (NEWSTATE). Analysis was carried out using parsimony with trees generated by heuristic searches with tree bisection reconnection (TBR) branch swopping and MULPAR effective. For the heuristic search, Maxtrees was set to increase automatically by 100 and taxa were added by simple stepwise addition with polytomic collapse. Bootstrap values of the branching points were determined using bootstrap analysis with a 1000 replicates (FELSENSTEIN 1988). *Petriella setifera* (J.C. Schmidt) Curzi, *Microascales*, which is related to the genus *Ceratocystis*, was used as outgroup taxon and was treated as a monophyletic sister group to the ingroup.

# 3 Results

## 3.1 Symptoms and isolations

Approximately 50% of the trees in the investigated stands were dead or dying. Hybrid UP clones at Kissoko plantation showed symptoms of wilt, followed by death. The xylem was extensively discoloured with a distinct streaky appearance (Fig. 1). The streaking was more intense and concentrated towards the base of the trees. At Mengo plantation, trees showed signs of wilt and die-back in a first rotation coppice of a 4-year-old TG clone. More than half of the trees in this stand were dead or dying. Many of the affected trees exuded kino and the stems of many trees were cracked. Extensive kino pockets were observed in the xylem. Epicormic shoots were also common on many of the trees. Some trees showed a streaked discoloration of the xylem. Isolations made from the diseased 2-year-old UP and the 6-month-old coppice at Kissoko consistently yielded a *Ceratocystis* sp., both from

*Table 1.* **List of isolates used in DNA sequence comparisons**

| Species | Isolate numbers[1] | Origin | Host | Genebank number[2] |
|---|---|---|---|---|
| *Ceratocystis adiposa* | CMW1622 | Japan | Unknown | AF043606 |
| *C. albofundus* | CMW2475 | South Africa | *Acacia mearnsii* | F043605 |
| *C. albofundus* | CMW2148 | South Africa | *A. mearnsii* | AF264910 |
| *C. coerulescens* | CMW3263 | Norway | *Picea* sp. | U756618 |
| *C. eucalypti* | CMW3254 | Australia | *Eucalyptus sieberi* | U75627 |
| *C. fagacearum* | CMW2651 | USA | *Quercus* sp. | AFO43598 |
| *C. fimbriata* | CMW4769 | Republic of Congo | *Eucalyptus* hybrid | AF264905 |
| *C. fimbriata* | CMW4783 | Republic of Congo | *Eucalyptus* hybrid | AF264906 |
| *C. fimbriata* | CMW4101 | South Africa | *Acacia mearnsii* | AF264909 |
| *C. fimbriata* | CMW4900 | Brazil | *Eucalyptus grandis* | AF264907 |
| *C. fimbriata* | CMW4901 | Brazil | *E. grandis* | AF264908 |
| *C. fimbriata* | CMW2220 | Europe | *Platanus* sp. | AFO43604 |
| *C. fimbriata* | CMW2228 | Europe | *Platanus* sp. | AF264902 |
| *C. fimbriata* | CMW2242 | Europe | *Platanus* sp. | AF264903 |
| *C. fimbriata* | CMW1547 | Papua New Guinea | *Ipomoea batatas* | AF264904 |
| *C. fimbriata* | C854 | USA | *I. batatas* | AFO07749 |
| *C. paradoxa* | CMW1546 | New Zealand | *Musa* sp. | AFO43607 |
| *C. virescens* | CMW0460 | USA | *Quercus* sp. | U75625 |
| *Petriella setifera* | | | Unknown | AFO43596 |

[1] CMW numbers represent cultures maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. C – Culture collection of T.C. Harrington, Department of Plant Pathology, Iowa State University.
[2] Genebank numbers are those for sequences already published.

incubated tissue and from isolations made on agar. The same fungus was isolated from the TG coppice at Mengo. The *Ceratocystis* sp. sporulated abundantly in the brown streaks found in symptomatic tissue. The fungus was identified as *C. fimbriata* on the basis of perithecial morphology and size, hat-shaped ascospores, chlamydospore morphology and the presence of a characteristic *Chalara* anamorph. Cultures of the fungus have been deposited in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), South Africa.

### 3.2 Greenhouse pathogenicity trials

All isolates tested produced lesions on the outer bark as well as in the xylem of the inoculated trees (Table 2). The typical streaking associated with *C. fimbriata* infection on naturally infected trees was also evident in many of the inoculated trees. No symptoms developed on trees inoculated as controls and in all cases these inoculation wounds were covered with callus (Table 2). Significant differences in variance (p = 0.05) were found between control inoculations and trees inoculated with the test organism. The inoculated pathogen was consistently re-isolated from lesions and never from control trees.

### 3.3 DNA amplification and sequencing

Sequences were manually aligned by the insertion of gaps, resulting in a total of 530 characters. A heuristic search of the sequence data generated two most parsimonious trees

*Fig. 1.* Wood discoloration caused by *Ceratocystis fimbriata* on an approximately 2-year-old *E. urophylla* × *E. pellita* (UP) clone (approximately 15 cm diameter)

*Table 2.* **Lesions produced on *E. grandis* × *E. camaldulensis* clones in glasshouse inoculation trials**

| Isolate | Lesion length (mm)[1] |
|---------|-----------------------|
| CMW 4786 | 50.7a |
| CMW 4769 | 47.2a |
| CMW 4781 | 40.2a |
| Control | 9.0b |

[1] All values for test isolates represent an average of 20 measurements and those for the control an average of 10 measurements. Values followed by a different letter differ significantly from each other (p = 0.05). CV = 37.5%.

with similar topologies and minor differences in branch lengths. The search resulted in 246 parsimony informative characters and 75 uninformative characters. The consistency index (CI) and retention index (RI) for these trees were 0.692 and 0.778, respectively. Isolates from the Republic of Congo grouped with *C. fimbriata*, separately from other *Ceratocystis* spp. with which they were compared, with a bootstrap value of 100% (Fig. 2). The Congolese isolates (CMW4769, CMW4783) grouped with *C. fimbriata* isolates from *Eucalyptus* in Brazil (CMW4900, CMW4901), *Acacia mearnsii* de Wild. in South Africa (CMW4101) and sweet potato isolates from the USA (C854) and Papua New Guinea (CMW1547). This clade
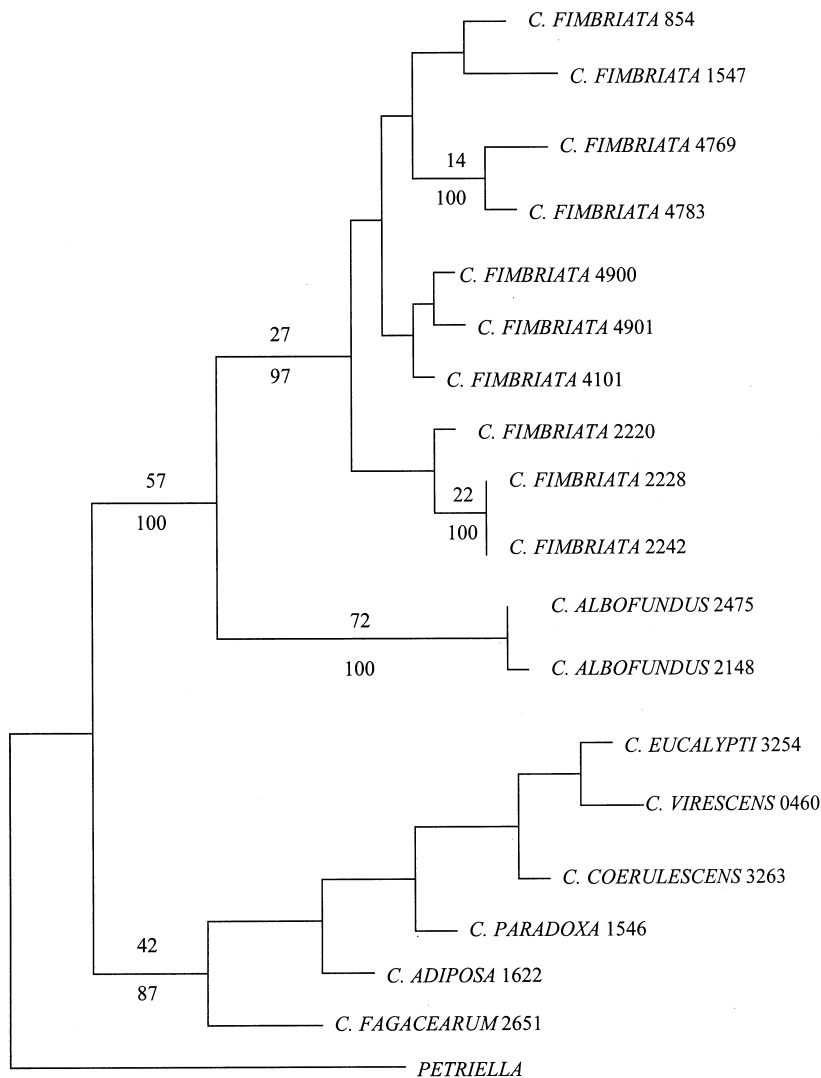


*Fig. 2.* Phylogram generated using the heuristic search option. Bootstrap confidence intervals are indicated below, and branch lengths above the branches of the tree. The origin and host of all isolates are presented in Table 1

was separate from the clade containing *C. fimbriata* isolates from *Platanus* spp. in the Northern Hemisphere (CMW2220, CMW2228, CMW2242).

## 4 Discussion

As far as we are aware, this report represents the first record of a *Ceratocystis* sp. as a pathogen of *Eucalyptus*. We are, however, aware of the recent discovery of a similar disease by colleagues in Brazil (LAIA et al. 1999). This study is also the first example of a serious vascular wilt disease of *Eucalyptus* caused by a fungus. The appearance of the disease, at a time when the clonal propagation of these trees is increasing greatly, is of concern and deserves further study.

    *Ceratocystis* spp. are well-known causal agents of wilt diseases and are amongst the most serious pathogens of woody plants in the world (KILE 1993; WINGFIELD et al. 1993). *Ceratocystis fimbriata* is the best known of these species and has a wide host range including sweet potato (HALSTED and FAIRCHILD 1891), coffee (PONTIS 1951), cocoa (KILE 1993), gmelina (MUCHOVEJ et al. 1978), fruit trees such as peach and almond (DE VAY et al. 1963; TEVIOTDALE and HARPER 1991), poplar (WOOD and FRENCH 1963; GREMMEN and DE KAM 1976), *Acacia decurrens* (RIBEIRO et al. 1988) and many others. The fungus produces slimy droplets of spores from perithecia and also produces sweet-smelling aromatics, which are thought to play a role in insect dispersal (HANSSEN 1993; CHRISTEN et al. 1997). Trees usually require wounds for the initiation of infection (DE VAY et al. 1963; TEVIOTDALE and HARPER 1991) and these wounds are usually visited by insects that transmit spores to them (CRONE and BACHELDER 1961; HINDS 1972). At this stage, we know very little concerning the factors associated with disease development on *Eucalyptus* in the Congo, but we suspect that it will be similar to the situation on other trees, elsewhere in the world.

    There has been only one other report of a *Ceratocystis* sp. from *Eucalyptus*. This is *Ceratocystis eucalypti* Yuan and Kile that was collected from wounds on the stems of *Eucalyptus sieberi* L. Johnson and *Eucalyptus globoidea* Blakely in Australia (KILE et al. 1996). There is, however, little chance of mistaking *C. fimbriata* and *C. eucalypti*. The latter species has very large elongated, fusiform ascospores, whereas *C. fimbriata* has characteristic hat-shaped ascospores. *Ceratocystis eucalypti* is not known as pathogenic to *Eucalyptus* (KILE et al. 1996).

    Morphological characteristics of *C. fimbriata* associated with Eucalyptus wilt in the Congo are typical of those described for the fungus. Recently, considerable data pertaining to the phylogenetic relationships between *Ceratocystis* spp. have become available (HAUSNER et al. 1993; VISSER et al. 1995; WITTHUHN et al. 1998, 1999). A number of species in *Ceratocystis sensu stricto* have been shown to represent species complexes (VISSER et al. 1995; HARRINGTON et al. 1996; WINGFIELD et al. 1996) and based on restriction fragment length polymorphism (RFLP) analysis, isolates of *C. fimbriata* can also be distinguished from each other (WITTHUHN et al. 1999). For the present, *C. fimbriata* remains a discrete species. However, WEBSTER and BUTLER (1967) have also previously presented data that might suggest that this fungus represents a number of closely related, but different species. They, however, concluded, on the basis of hybridization studies, that *C. fimbriata* represents one species including several strains that differ in morphology and cultural characteristics. As additional molecular data become available, this situation is likely to be revised.

    The recent discovery of a *Ceratocystis* sp. causing a serious wilt disease of black wattle (*Acacia mearnsii*) in South Africa aptly illustrates the difficulty with morphological identification of species in the *C. fimbriata* group (MORRIS et al. 1994). The pathogen was first reported as *C. fimbriata* but, later, based largely on sequence data, was described as a new species that is now known as *Ceratocystis albofundus* Wingfield, De Beer and Morris (WINGFIELD et al. 1996). Recent RFLP analysis have confirmed this subdivision of *C. albofundus* and *C. fimbriata* (WITTHUHN et al. 1999). It was also shown that there are

differences in the RFLP patterns for *C. fimbriata* isolates from *Platanus* spp. and those of *Prunus* and *Populus* spp. (WITTHUHN et al. 1999).

Pathogenicity tests on young trees in the greenhouse confirmed the likely role of *C. fimbriata* as the causal agent of the *Eucalyptus* disease in the Congo. Inoculations onto identical clones as those from which *C. fimbriata* was isolated was not possible. This was due to South African quarantine regulations that preclude the importation of clones. We have, however, shown that *C. fimbriata* from dying *E. grandis* hybrids in the Republic of Congo is capable of causing disease on a South African *E. grandis* hybrid. The symptoms are similar to those usually associated with *C. fimbriata* infection on other species of woody crops (LEATHER 1966; MUCHOVEJ et al. 1978; KILE and WALKER 1987; RIBEIRO et al. 1988). In the future, we would hope to conduct pathogenicity tests on established trees in the Republic of Congo. Such tests will expand our understanding of disease development, and perhaps more importantly, will allow us to compare the susceptibility of different species and hybrids.

Currently the Republic of Congo relies entirely on clonal plantations with 43 000 ha being planted. The threat of a serious disease, such as that caused by *C. fimbriata*, is enhanced by the planting of identical clones over a wide area. To avoid large-scale losses, careful management is required to ensure a diversity of clones to reduce the impact of losses. Ultimately, the most effective means of reducing the impact of diseases can only be successfully achieved through selection of disease-tolerant planting stock.

It is intriguing to consider the possible origin of *C. fimbriata* on *Eucalyptus* in the Republic of Congo. At the present time, there are no reports of this fungus causing disease in this or any other African countries. This might be due to the fact that intensive surveys for the pathogen, which can be inconspicuous and difficult to isolate, have not been undertaken. However, our sequencing data show that the fungus from *Eucalyptus* in the Republic of Congo is most similar to *C. fimbriata* isolates from South America and *C. fimbriata* from *Acacia mearnsii* in South Africa. These data might imply that the fungus originated in South America where *C. fimbriata* is a well-known pathogen of a wide range of crops. Further phylogenetic and biogeographic studies are planned to consider this question more completely.

## Acknowledgements

## Résumé

*Une grave maladie nouvelle de l'Eucalyptus en Afrique centrale, le flétrissement à Ceratocystis fimbriata*

Au cours d'une prospection récente portant sur des clones d'Eucalyptus en République du Congo, Afrique centrale, une grave maladie de flétrissement et de dépérissement a été observée sur deux clones hybrides. Les arbres touchés étaient âgés d'environ 6 mois à 4 ans. Les isolements à partir du matériel qui présentait des symptômes ont fourni de façon constante une espèce de *Ceratocystis*. D'après la morphologie et le séquençage, ce champignon a été identifié comme le *C. fimbriata*, un agent de flétrissement et de chancre bien connu sur de nombreuses plantes d'intérêt économique. Les isolats provenant d'Eucalyptus ont été comparés à d'autres espèces de *Ceratocystis* pour leur séquence de l'ITS et de la région 5.8S de l'ARN ribosomique. Les résultats ont confirmé que les isolats de l'Eucalyptus étaient le *C. fimbriata* et ont montré qu'ils se regroupent avec d'autres isolats du Brésil, d'Afrique du sud et d'Europe. Les trois isolats congolais ont été inoculés à de jeunes plants d'Eucalyptus en serre ; ils ont produit des lésions typiques dans l'écorce et le bois. Cette étude est la première mention du *C.*

*fimbriata* comme parasite de l'Eucalyptus en Afrique et l'un des deux seuls rapport récents au niveau international. C'est une maladie nouvelle sérieuse qui devra faire l'objet d'études importantes en vue de s'assurer que les pertes induites par ce pathogène ne sont pas en expansion.

## Zusammenfassung

*Eine bedrohliche neue Welkekrankheit bei Eucalyptus in Zentralafrika, verursacht durch Ceratocystis fimbriata*

Im Rahmen einer Inventur von *Eucalyptus*-Klone in der Republik Kongo, Zentralafrika, wurde eine schwere Welkekrankheit, die mit Zweigsterben einhergeht, an zwei verschiedenen Hybridklonen entdeckt. Die befallenen Bäume hatten ein Alter von sechs Monaten bis zu vier Jahren. Isolierungen ergaben regelmässig eine *Ceratocystis*-Art. Diese wurde aufgrund von morphologischen Merkmalen und Sequenzvergleichen als *Ceratocystis fimbriata* identifiziert. Dieser Pilz ist ein bekannter Welke- und Rindennekroseerreger an vielen Nutzpflanzen. Die Isolate von *Eucalyptus* wurden anhand der ITS und 5,8S Sequenzen des rRNA-Operons mit anderen *Ceratocystis* spp. verglichen. Dies bestätigte die Identität der Isolate von *Eucalyptus* als *C. fimbriata* und zeigte, dass sie mit anderen Isolaten dieser Art aus Brasilien, Südafrika und Europa eine Gruppe bilden. In Infektionsversuchen an jungen Eukalyptuspflanzen im Gewächshaus verursachten alle drei Isolate aus dem Kongo die typischen Läsionen in Rinde und Xylem. Diese Untersuchung ist der Erstnachweis für *C. fimbriata* als Pathogen an *Eucalyptus* in Afrika und weltweit einer von nur zwei derartigen Nachweisen.

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