A new bark canker disease of the tropical hardwood tree *Cedrelinga cateniformis* in Ecuador

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Cedrelinga cateniformis is an indigenous leguminous tree that occurs naturally in the Amazonian regions of Ecuador, Peru and Colombia. This tree is economically valuable for wood production and it is also culturally important to rural communities in these countries. A canker disease has recently emerged on *C. cateniformis* in Ecuadorian plantations of this tree. The disease is characterized by severe cracks in the bark, from which kino exudes; wood discoloration is associated with these cracks; as well as die-back and stunted growth. The aim of this study was to determine the cause of the disease. Isolation from cankers consistently yielded species of *Fusarium*. These species were identified as *F. solani*, *F. oxysporum* and *F. decemcellulare* based on morphology as well as on comparisons of their partial Translation Elongation Factor-1a gene sequences. In inoculation trials, all three species resulted in cankers that were similar to those found on trees under natural conditions. The three species were also consistently re-isolated from lesions that resulted from inoculations. Results of this study suggest that the canker disease on *C. cateniformis* is caused by the three *Fusarium* spp., which have similar degrees of pathogenicity.

Key words: bark canker, *Cedrelinga cateniformis*, *Fusarium decemcellulare*, *Fusarium oxysporum*, *Fusarium solani*

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Introduction

Cedrelinga cateniformis (Ducke) Ducke (*Leguminosae*) is an indigenous tree distributed throughout the Amazonian regions of Ecuador, Brazil and Peru (Chudnoff, 1984; Valencia *et al.,* 2004). It is planted to rehabilitate deforested areas as well as for its wood and bark products such as pulp, veneer and block board (Chudnoff, 1984; Woodward, 1996). *Cedrelinga cateniformis* is also used by the indigenous people of the region for medicinal purposes (Ezaki *et al.,* 1991; Villegas, 2004). Several studies have been conducted on the ecology and distribution of *C. cateniformis* (Woodward, 1996; Valencia *et al.*, 2004; Villegas, 2004; Brienen and Zuidema, 2006). However, little is known regarding disease and pest problems that are associated with this important tree species in Ecuador.

In 2002 and 2003, a canker disease was observed for the first time on the main stems and side branches of *C. cateniformis* in plantations in Ecuador. The disease has gradually spread and increased in its severity. Currently, trees in most plantations are affected to some extent. The aim of this study was to identify the causal agent of this canker disease in Ecuador. To achieve this goal, isolations were made from symptomatic tissues, and isolates were identified based on morphology and DNA sequence comparisons. Pathogenicity tests were undertaken under field conditions.

Materials and methods

Disease incidence and symptoms

The canker disease on *C. cateniformis* trees was first observed in 2002. This was on trees growing in Rio Pitzara near Las Golon-

drinas, west of Quito in the province of Pichincha, Ecuador. Incidence of the disease has steadily increased since its first appearance, and currently large numbers of trees in plantations have been affected.

Cankers (Fig. 1A) on the bark of affected trees are characterized by long vertical cracks that exude copious amounts of resin. These cracks can extend from bases to crowns of affected trees. In some cases, similar cankers are evident on side branches (Fig. 1E). Brown discoloration is found on the inner sides of the bark and cambium in irregular shapes (Figs. 1C and 1D). These lesions can also extend into the wood (Fig. 1B). Cross-sections through cankers on stems suggest that these cankers are annual in nature and trees overgrow infected areas yearly (Fig. 1D). New infections on previously affected areas in the stems lead to death of the cambium. Severely affected trees are typically stunted and lack vigor.

Sample collection and isolations

Wood and bark samples displaying canker symptoms were collected from seven individual trees in the vicinity of Las Golondrinas, Ecuador. Isolations were made directly from boarders between diseased and healthy tissues onto *Fusarium* Selective Medium (FSM; Nelson *et al*., 1983) and Malt Extract Agar (MEA, 2% w/v) medium. After five days of incubation at 25°C, fungal colonies were transferred on to Half-strength Potato Dextrose Agar (HPDA, Biolab, South Africa) and incubated further for seven days. For each isolate, single conidial cultures were prepared on HPDA, and the strains were grouped based on their culture morphology after seven days of growth at 25°C. Representative strains are maintained in the Mike Wingfield culture collection (CMW), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphology

Other than a small number of common contaminant fungi, all strains isolated from infected wood and bark samples were those of *Fusarium* spp. For morphological identification of isolates, single conidial cultures were prepared on HPDA and Carnation Leaf Agar (CLA; Fisher *et al*., 1982). Inoculated plates were incubated at 25ºC under continuous near-ultraviolet light and examined after 10-15 days. Gross morphological characteristics were determined based on 10-15 day-old cultures on HPDA. Microscopic features of conidia, conidiophores and chlamydospores were determined based on 10-15 day-old cultures on CLA.

DNA sequence comparisons

Two isolates were randomly selected from each of the three morphological groups (see below) for DNA sequence comparisons. These six isolates were also utilized in pathogenicity trials. Isolates were grown on HPDA for 10-15 days. Mycelium was then scraped from the surfaces of the cultures, freeze-dried, and ground to powder in liquid nitrogen using mortars and pestles. DNA was extracted from the mycelial powder as described by Möller *et al*. (1992).

A ~ 650 base pair fragment of the Translation Elongation Factor (TEF)-1α gene was amplified using the EF1 and EF2 primers, and PCR conditions described by O'Donnell *et al*. (1998). Amplified fragments were purified using High Pure PCR Product Purification Kit (Roche, USA) and sequenced in both directions. For this purpose, the BigDye terminator sequencing kit (Version 3.1, Applied Biosystems, USA) and an ABI PRISM^{TM} 3100 DNA sequencer (Applied Biosystems) were used. All PCRs and sequencing reactions were preformed on an Eppendorf Mastercycler Personal PCR Thermal Cycler (Eppendorf AG, Germany).

Gene sequences were assembled and aligned using Sequence Navigator (Version 1.0.1, Applied Biosystems) and MAFFT (Version 5.11, Katoh *et al*., 2005), respectively. The aligned sequences were checked manually and corrected where necessary. PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0b10, Swofford, 2003) was used to estimate phylogenetic relationships. For this purpose, heuristic searches based on 1000 random addition sequences and tree bisection-reconnection (TBR) were used, with the branch swapping option set on 'best trees' only. All characters were weighted equally and alignment gaps were treated as missing data. Bootstrap analysis (Hillis and Bull, 1993) was based on 1000 replications.

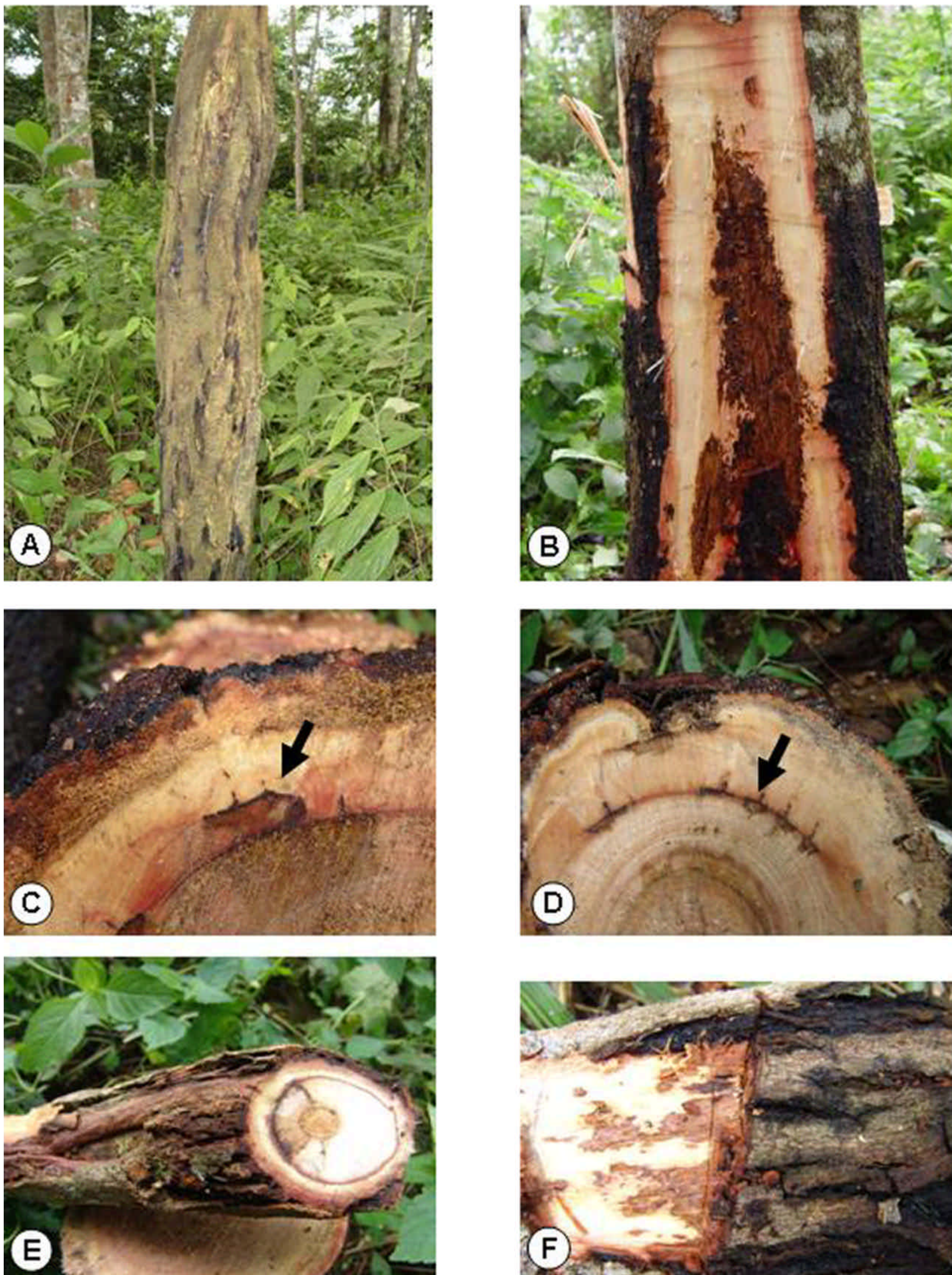

Fig. 1. Disease symp toms on *Cedrelinga cateniformis*. **A.** External cracking of the bark with kino exudation. **B.** Internal symp toms of wood discolouration. **C, D.** Annual cankers (arrows) visible in cross-section of main stem. **E.** Side branch with symptoms. **F.** Wood discolouration in side branch.

Each of the TEF-1α sequences for the isolates studied were analyzed using the Basic Local Alignment Search Tool for Nucleotide sequences (BLASTN, Altschul *et al.*, 1997) and the *Fusarium* ID database (http://fusarium.cbio.psu.edu; Geiser *et al.*, 2004). The phylogenetic analysis included 63 TEF-1α partial gene sequences representing 51 *Fusarium* spp. (Fig. 2), which were obtained from GenBank for the purpose of comparison. Three, four and 13 isolates, respectively, from the *F. oxysporum*, *F. lateritium* and *F. solani* species complexes, were included in the analysis. This was to ensure that the three clades in each of *F. oxysporum* (O'Donnell *et al.*, 1998) and *F. lateritium*, and all the mitotic and meiotic species in the *F. solani* (Aoki *et al.*, 2003; O'Donnell, 2000) complexes were represented. *Cylindrocladium pacificum* J.C. Kang, Crous & C.L. Schoch was used as the outgroup taxon.

Pathogenicity tests

The six isolates used in the pathogenicity study were randomly selected from each of the three *Fusarium* morphological groups. These were the same strains used in the DNA sequence comparisons. The isolates were grown on HPDA for seven days and then inoculated onto stems of *C. cateniformis* trees in two separate field stands referred to as Stand 394 and Stand 480.

A random selection of test and control trees was used in the pathogenicity tests. Each of the six isolates was inoculated into stems of 20 test trees in each stand. Twenty other trees in each stand were used for control inoculations. A five mm-diameter wound was made on the main stems of test and control trees by removing the bark with a cork borer and exposing the cambium. Test trees were inoculated with test strains on HPDA plugs (five mm in diameter) that were cut from the margins of seven-day-old cultures, and placed in these wounds with the mycelial surface facing the cambium. Control trees were treated likewise, but using sterile HPDA plugs. All inoculation wounds were covered with insulation tape to prevent desiccation of the inoculum and wounds.

Four weeks after inoculation, lesion lengths were measured. The results were subsequently analyzed using SAS Analytical Programmes 2002. Re-isolations were made from lesions on the test and control trees after lesions had been measured.

Results

Sample collection and isolation

A total of 139 fungal strains were isolated from cankers on *C. cateniformis*. Based on their morphology, these isolates were separated into three groups, which represented *Fusarium oxysporum* Schltdl. (emend. W.C. Snyder & H.N. Hansen), *Fusarium solani* (Mart.) Sacc. (emend. W.C. Snyder & H.N. Hansen) and *Fusarium decemcellulare* Brick. *Fusarium solani* (89 isolates) was encountered more frequently than *F. oxysporum* (27 isolates) and *F. decemcellulare* (23 isolates).

DNA sequence comparisons

BLASTN analyses showed that TEF-1α sequences from the isolates had the highest similarity with sequences from isolates of *F. solani*, *F. oxysporum* and *F. decemcellulare* (data not shown). Phylogenetic analyses based on the aligned TEF-1α sequence data contained 69 in-group taxa. Each sequence in the aligned data consisted of a total of 687 characters including gaps. Of these, 279 were constant. Among the variable characters, 323 were parsimony-informative.

Analyses of these sequence data resulted in 1373 most parsimonious trees, one of which is presented (Fig. 2). In this tree, the in-group taxa separated into two clades with bootstrap support values (BP) of 99% and 83%. One of these clades (83% BP) consisted of the *F. solani-Nectria haematococca* species complex and its close relative, the *F. decemcellulare-N. rigidiuscula* species complex. Four of the isolates from *C. cateniformis* grouped in this clade. Of these, two isolates (CMW18783 and CMW18785) grouped closely (100% BP) with the *F. decemcellulare* strain, and the other two isolates (CMW18782 and CMW18786) grouped with mating populations III, V, VI and VII of *F. solani.*

The second clade (99% BP) consisted of all the remaining *Fusarium* spp. including our isolates CMW18777 and CMW18778. Within this clade, isolates CMW18777 and CMW18778 grouped (98% BP) with those representing the *F. oxysporum* species complex.

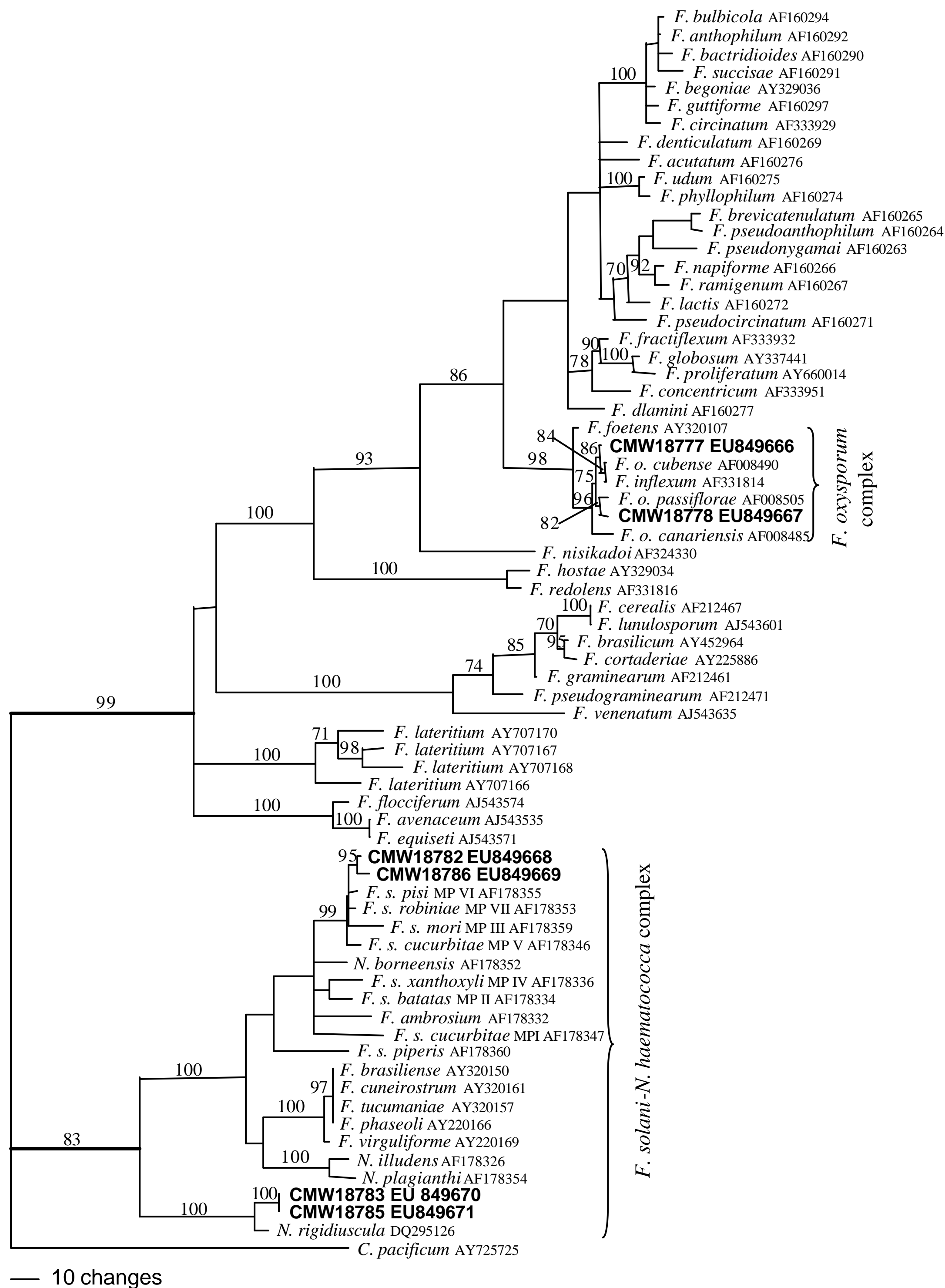

Fig. 2. One of 1373 equally most parsimonious trees obtained using TEF-1a partial gene sequences for 69 in-group taxa (Length, 1373; Consistency index = 0.479; Retention index, = 0.857, Rescaled consistency index = 0.410). Bootstrap values of 70% and higher are indicated above nodes. Accession numbers are shown next to each sequence obtained from GenBank. Thick lines lead to clades.

Pathogenicity

All test trees in both stands displayed symptoms of dark-brown to black discoloration of the vascular tissue and wood, four weeks after inoculation (Fig. 3). Lesions on the control trees were either non-existent or small and light brown, which probably resulted from wood oxidation. In both stands, test trees showed significantly ($p < 0.0001$) larger lesions than control trees (Fig. 4). There was no significant difference ($p < 0.155$) in mean lesion length for any of the isolates tested in either of the test

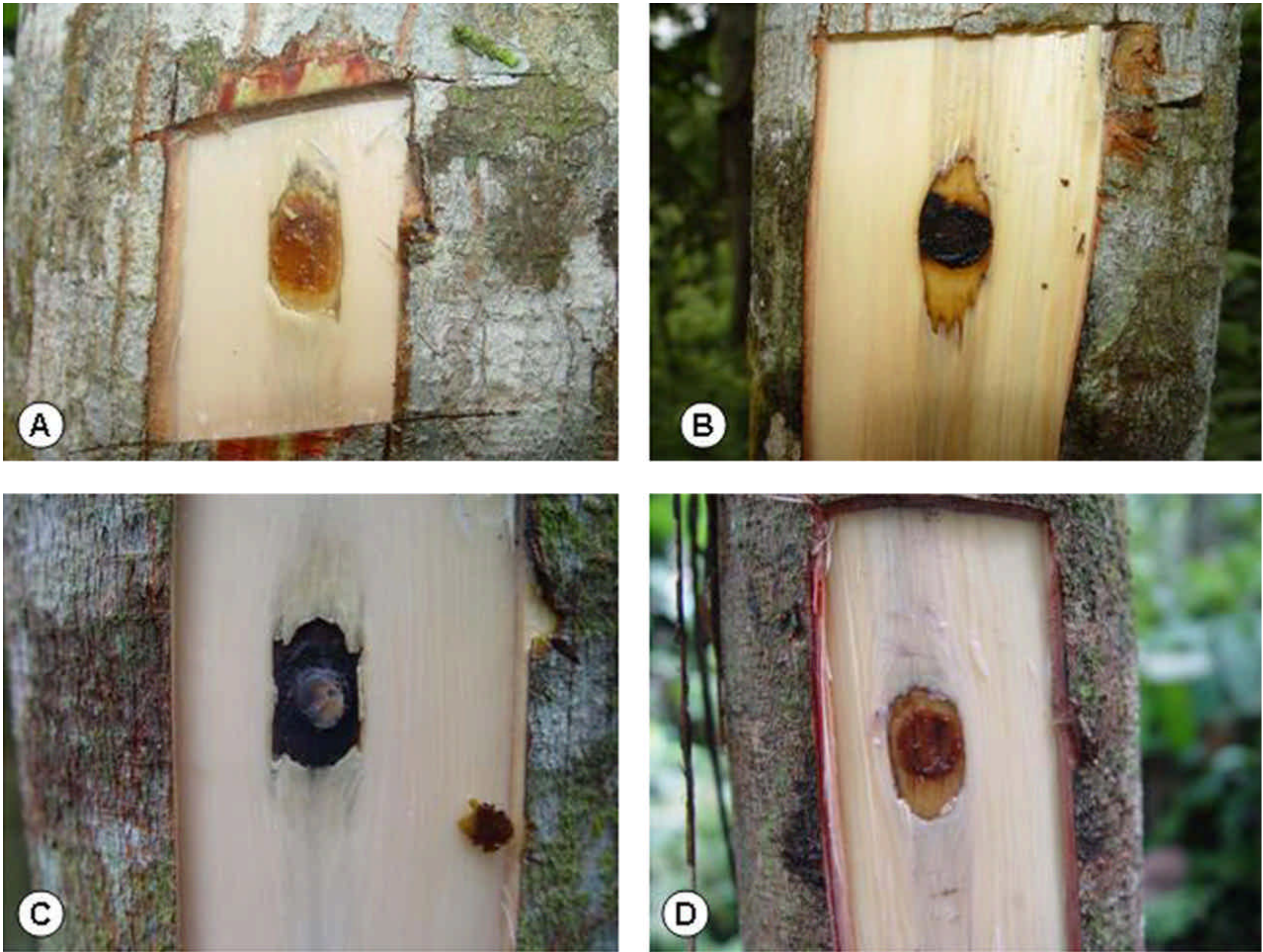

Fig. 3. Symptoms associated with inoculations on *Cedrelinga cateniformis*. **A.** Control. **B.** *Fusarium decemcellulare* (CMW18783). **C.** *Fusarium oxysporum* (CMW18777). **D.** *Fusarium solani* (CMW18782).

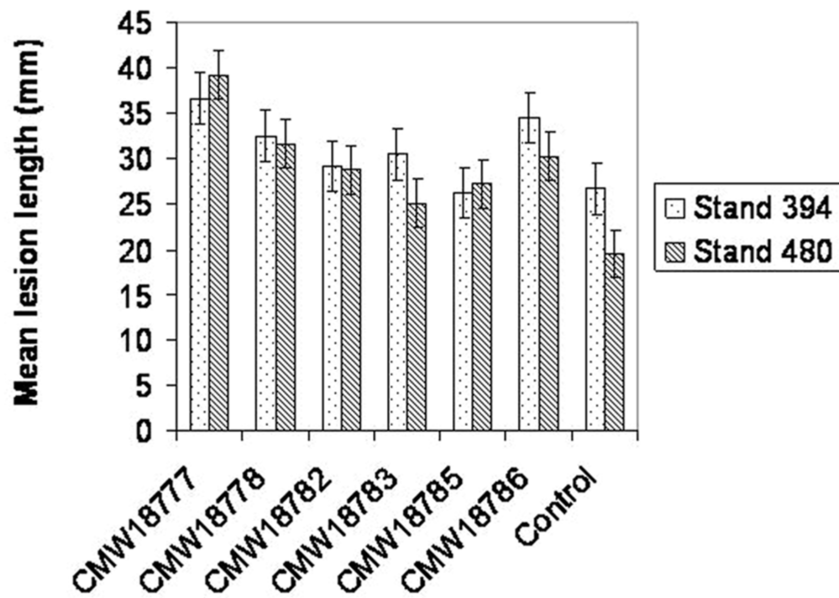

Fig. 4. Histogram showings mean lengths of lesions induced by each isolate in Stand 394 and Stand 480.

stands (Fig. 4). Consequently, data from the two stands were combined and compared to those for the control trees.

There was no significant difference ($p < 0.0007$) in lesion length produced by the three *Fusarium* species inoculated in this study. However, comparison of individual isolates showed that *F. oxysporum* isolate CMW18777 resulted in the longest lesions ($\bar{x}$ = 37.9; Table 1). This was thus the strain most pathogenic to *C. cateniformis*. *Fusarium decemcelullare* isolate CMW18785 resulted in the smallest lesions ($\bar{x}$ = 27.0; Table 1). All isolates produced significantly ($p < 0.0001$) longer lesions than those observed on control trees (Fig. 4). Re-isolation from lesions on test trees consistently yielded the inoculated fungi. No *Fusarium* species were isolated from the small lesions found on the control trees.

Table 1. Mean length of lesions on *C. cateniformis* stems four weeks after inoculation with test isolates.

***Fusarium* spp.**	**Strain No.**	**Mean lesion lengths (mm)**		
		Stand 394[a]	**Stand 480[b]**	**Combined[c]**
F. decemcellulare	CMW18783	30.5	25.2	28.0
	CMW18785	26.3	27.2	27.0
F. oxysporum	CMW18777	36.7	39.2	37.9
	CMW18778	32.5	31.6	32.0
F. solani	CMW18782	29.2	28.8	28.6
	CMW18786	34.5	30.3	32.4
Control		26.8	19.5	23.1

[a]$R^2 = 0.282$; F – value = 6.13; $p < 0.0001$; [b]$R^2 = 0.566$; F – value = 18.58; $p < 0.0001$; [c]$R^2 = 0.436$; F – value = 13.49; $p < 0.0001$

Discussion

This study provides the first description of a new and serious disease of *Cedrelinga cateniformis* in the Amazonian basin of Ecuador. Results have also shown that three species of *Fusarium* are closely associated with this annual canker disease. The emergence of this disease on *C. cateniformis* is of considerable concern as the tree is valuable for re-afforestation purposes in Ecuador and it also has economic and medicinal values to people residing in the Amazonian regions of Ecuador, Colombia and Peru (Woodward, 1996; Valencia *et al*., 2004). There have been no previous reports of diseases on this tree species and the canker disease appears to have emerged locally.

The *Fusarium* spp. isolated from diseased samples of *C. cateniformis* were identified as *F. solani, F. oxysporum* and *F. decemcellulare.* These identifications emerged from both morphological and DNA sequence comparisons. Isolations from diseased bark and wood samples consistently yielded the three *Fusarium* spp.; among which, *F. solani* was the most commonly isolated species. In contrast, *F. decemcellulare* and *F. oxysporum* were encountered less frequently. This might suggest that *F. solani* is the most important factor resulting in the canker disease.

Pathogenicity tests with isolates of *F. solani*, *F. decemcellulare* and *F. oxysporum* from *C. cateniformis* showed that all three fungi are pathogenic to this tree species. Inoculations also showed that the three *Fusarium* spp. have comparable degrees of pathogenicity on *C. cateniformis*. This result was somewhat unusual as it was expected that a single species might emerge as more important than the other two. For the present, a reasonable interpretation would be that a complex of three *Fusarium* spp. is responsible for the cankers on *C. cateniformis.* Future studies will need to consider the infection process and whether one of the three *Fusarium* spp. plays a dominant role in disease development.

It was not surprising that *F. solani* emerged as one of the fungi associated with the canker disease on *C. cateniformis*. This fungus or at least members of the complex to which it belongs (O'Donnell, 2000) is regularly associated with cankers and die-back of legumes and trees in tropical regions (Nelson *et al*., 1981; Leslie and Summerell, 2006) and is a pathogen of many crops, for example it has been reported to cause cankers on *Populus deltoides* Bartram ex Marshall (cottonwood), *Liriodendron tulipifera* L. (yellow-popular), and *Juglans nigra* L. (black walnut) (Toole, 1963; Tisserat, 1987); and annual cankers on *Acer saccharum* Marshall (sweet maple) and several other hardwood tree species (Pawuk and Wood, 1972).

Fusarium decemcellulare is also commonly found in the tropics associated with canker and die-back of various tree species (Leslie and Summerell, 2006). The host range of this pathogen includes fruit trees such as *Persea*

americana Mill. (avocado) (Dravas and Kotze, 1987) and *Mangifera indica* L. (mango) (Ploetz *et al*., 1996). Other host tree species include *Cordia alliodora* (Ruiz & Pav.) Cham. (Spanish Elm) (Albuquerque and Bastos, 1990), *Annona squamosa* L. × *A. cherimola* Mill. (atemoya) (Togawa and Nomura, 1998) and *Ziziphus mauritiana* Lam. (Indian jujube) (Wadia and Monaharachary, 1980). To the best of our knowledge, it has not previously been associated with a canker disease on a leguminous tree but its wide host-range cannot lead us to discount its involvement in the canker disease on *C. cateniformis*.

Fusarium oxysporum is generally associated with vascular wilt and root diseases on a wide range of plants (Nelson *et al*., 1981; Leslie and Summerell, 2006). Although the fungus belongs to a complex of species (Snyder and Hansen, 1940), it has not been associated with canker diseases.

The emergence of a serious new disease on a tree that has grown rapidly and free of problems in Ecuador for many years is of concern. It is impossible to predict the origin of the disease or why it might have appeared so suddenly. The fact that three *Fusarium* spp. are associated with the disease is also unusual, although one of these, *F. solani* appears to be the most likely primary agent. Future studies will consider the relative importance of the three fungi and will focus on selecting trees with tolerance to infection.

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