Insect Associates of *Ceratocystis albifundus* and Patterns of Association in a Native Savanna Ecosystem in South Africa

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ABSTRACT Species of *Ceratocystis* Ellis and Halstead s.l. include important plant pathogens such as *C. albifundus* Morris, De Beer, and M. J. Wingfield that causes a serious wilt disease of non-native, plantation-grown *Acacia mearnsii* De Wild. trees in Africa. The aim of this study was to identify the insects associated with *C. albifundus* in South Africa and to consider the means by which the pathogen spreads. Insects were collected weekly for 77 wk in a native ecosystem using modified pitfall traps. Trapped insects were identified, and fungi were isolated using carrot baiting and by plating them onto malt extract agar. Fungi were identified using morphological characteristics and DNA sequence comparisons. Three different nitidulid (Coleoptera: Nitidulidae) beetles, *Brachypeplus depressus* Erichson, *Carpophilus bisignatus* Boheman, and *Ca. hemipterus* L, were collected, of which the most common were the *Carpophilus* spp. Two *Ceratocystis* spp., namely *C. albifundus* and *C. oblonga* R. N. Heath and Jolanda Roux, were isolated from all three insect species. Insect numbers and fungal isolates decreased significantly in the colder months of the year. Of the two *Ceratocystis* spp., *C. oblonga* was most abundant, occurring on 0.5% of the *Carpophilus* spp. *C. albifundus* was isolated from 1.1% of the *Brachypeplus* individuals and from 0.01% of the *Carpophilus* individuals. This study presents the first record of insects associated with *C. albifundus* and *C. oblonga* and provides an indication of environmental influences on fungal and insect populations, which could contribute to future disease management.

KEY WORDS insect/fungus associations, ophiostomatoid fungi, tree disease

Species of *Ceratocystis* (sensu lato) s.l. includes many plant pathogens, the majority of which infect trees (Kile 1993). This group of fungi has been known for more than a century, with the type species, *Ceratocystis fimbriata* Ellis and Halstead, described in 1890 as the causal agent of black rot of sweet potato (*Ipomoea batatas* L.) (Halsted 1890). *C. fimbriata* is widely recognized as representing a complex of cryptic species, some of which have been described recently. Examples of important pathogens in the *C. fimbriata* s.l. species complex are *C. platani* (J. M. Walter) Engelbrecht and T. C. Harrington (Engelbrecht and Harrington 2005), *C. cacaofunesta* Engelbrecht and T. C. Harrington (Engelbrecht and Harrington 2005), and *C. albifundus* Morris, De Beer, and M. J. Wingfield (Wingfield et al. 1996). Other well-known pathogens accommodated in *Ceratocystis* s.l., not related to *C. fimbriata* s.l., include the oak wilt pathogen *C. fagacearum* Bretz (Bretz 1952) and *C. paradoxa* (Dade) Moreau, which causes disease of numerous crops (Kile 1993). There are also numerous species that are best known as agents of sap stain in lumber or that have not been shown to be pathogenic (Kile 1993).

Ceratocystis spp. are well adapted to being vectored by insects. In this regard, there are two discrete groups in the genus. Those that produce fruity aromas have casual vectors such as nitidulid beetles (Coleoptera: Nitidulidae) and flies (Diptera) (Moller and DeVay 1968b, Kirisits 2004). A second suite of species, such as *C. polonica* (Siemazko) Moreau and *C. laricicola* Redfern and Minter, live in a mutualism with conifer-infesting bark beetles (Coleoptera: Scolytidae) and do not produce fruity aromas (Harrington and Wingfield 1998). Other than the production of fruity aromas, *Ceratocystis* spp. are well adapted for dispersal by insects, having long ascomatal necks that give rise to sticky masses of spores that stick easily to insect bodies. It is thought that these long necks not only reduce the competition of surrounding fungi by bearing their spore drops above the competing fungi but that they could also influence the type of insects that vector them (Malloch and Blackwell 1993). An interesting related adaptation is that some species compensate for short perithecial necks by the production of their spore masses in thread-like tendrils (Wingfield 1993).

Aside from the bark beetle associated species, the species of *Ceratocystis* that has been considered most closely in terms of its insect vectors is the oak wilt pathogen *C. fagacearum.* Numerous studies have

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shown that nitidulid beetles are the primary vectors of this fungus (Gibbs 1980, Juzwik and French 1983). Transmission of *C. fagacearum* by nitidulid beetles is significant in overland spread of the fungus and the establishment of new infection centers (Cease and Juzwik 2001). The beetles are attracted to sporulating mats on recently killed oak trees, and after feeding on these mats, they are covered in fungal propagules that they subsequently spread to other trees (Juzwik and French 1983).

Various insects that are associated with *Ceratocystis* spp. either feed on sap or on the fungi themselves. After the insects have been attracted to the fungi by the fruity odors that they produce (Lanza and Palmer 1977), the spores are ingested or they adhere to their bodies. These insects are attracted to the sweet sap associated with fresh wounds on plants, and the fungi are thus transferred to a new substrate as in the case of nitidulid beetles transmitting *C. fimbriata* (Moller and DeVay 1968b). There is also some evidence that the insects play a role in the overwintering of the fungi that do not persist on wounds for very long (Moller and DeVay 1968b). Insects are further believed to play a significant role in the spermatization of the fungi with which they are associated, as has been shown in the case of *C. fagacearum* (Thompson et al. 1955).

Ceratocystis albifundus resides in the *C. fimbriata* s.l. species complex, and it causes a serious wilt disease of non-native *A. mearnsii* de Wild in eastern and southern Africa (Morris et al. 1993, Roux et al. 2005). The fungus was first discovered in South Africa in the 1980s and was initially treated as *C. fimbriata* (Morris et al. 1993) until DNA sequence comparisons became available, and it was recognized as a discrete taxon (Wingfield et al. 1996). Population biology studies, its occurrence on several native African tree families, and reports only from Africa suggest that *C. albifundus* is most likely an African fungus (Roux et al. 2001, 2007, Barnes et al. 2005).

Very little is known regarding the biology of *C. albifundus*, despite the fact that it is an important pathogen. Because of its fruity aroma, it has been assumed that it is vectored by insects similar to those associated with other *Ceratocystis* spp. that produce attractive aromas. The aim of this study was to identify possible insect associates of *C. albifundus* in South Africa. Furthermore, we considered the seasonal occurrence of these vectors in a typical native savanna ecosystem.

Materials and Methods

Study Areas. Two study areas were selected for this investigation. One was located on the Leeuwfontein Collaborative Nature Reserve (Leeuwfontein) ≈60 km northeast of Pretoria, Gauteng Province, South Africa. This study area included three sites (25°23′38.3″ S, 028°37′19.5″ E; 25°22′39.1″ S, 028°37′23″ E, and 25°22′37.2″ S, 028°37′38.2″ E) and is situated in native savannah vegetation. The area was selected because *C. albifundus* has previously been isolated from several native tree species in this reserve (Roux et al. 2007) and because of its close proximity to the laboratory.

The second study area, selected for comparison of insect species, was situated ≈40 km southwest of Piet Retief, Mpumalanga Province, South Africa (26°58′68.5″ S, 030°54′28.3″ E). This study area, consisting of a single site, is within a plantation of non-native *A. mearnsii* trees, between two compartments. The one compartment was 6 yr old, and the second compartment had recently been clear felled. The two aforementioned study areas are referred to as the "native" and "non-native" study areas.

Traps and Bait. Before starting the main trials, several different trap and bait types were tested in pilot trials. The different baits tested included fermenting dough, bananas, pineapples, and a mixture of all three baits. The traps that were tested included funnel traps, panel traps, and a modified pitfall trap (Southwood and Henderson 2000). Based on results of the pilot trials, freshly cut pineapple (1-cm^2 blocks) was selected as the ideal bait, and modified pitfall traps were used for the main experiments. These traps (Fig. 1) consisted of a removable cup-shaped bottom section (115 mm diameter) in which the bait was placed beneath a sieve to prevent the insects from coming into direct contact with the bait. The top section of the trap consisted of a tube (with 3-mm-diameter holes) fitting into the cup, which allowed insects to enter the tube. The tube was sealed with a lid (Fig. 1) to prevent rainwater from entering the trap or the insects from escaping. The traps were fastened to trees at a height of ≈1.5 m with adjustable straps.

Collection of Insects from the "Native" Study Area. Trapping and collection of insects on Leeuwfontein started in mid-winter 2005 (30 June) and proceeded over a period of 77 wk. The bait was replaced, and insects were collected weekly throughout the study period. Insects were removed from the traps with an aspirator and transported to the laboratory in individual glass vials in a cool box at ≈5°C. Insect specimens were grouped based on morphological characteristics and counted, and representative samples were mounted or preserved in 70% alcohol for identification.

Collecting of Insects from "Non-Native" Study Area. Trapping of insects in the plantation of non-native *A. mearnsii* trees near Piet Retief was undertaken to compare the possible insect associations of *C. albifundus* in commercial plantations of non-native trees, with that in the native ecosystem. Sampling was performed for only a week during summer 2006 (23–26 April). For this portion of the study, the same trap design, bait, and trapping protocol as that used for the native study area was used. The bait was replaced, and insects were collected daily for 4 d. Insect numbers were not calculated for this portion of the study, because the aim here was not to monitor insect numbers or environmental conditions but merely to compare insect species to those in the native habitat.

Insects were also collected from under bark flaps on the stumps of recently felled *A. mearnsii* trees. This made it possible to compare the incidence of fungus/

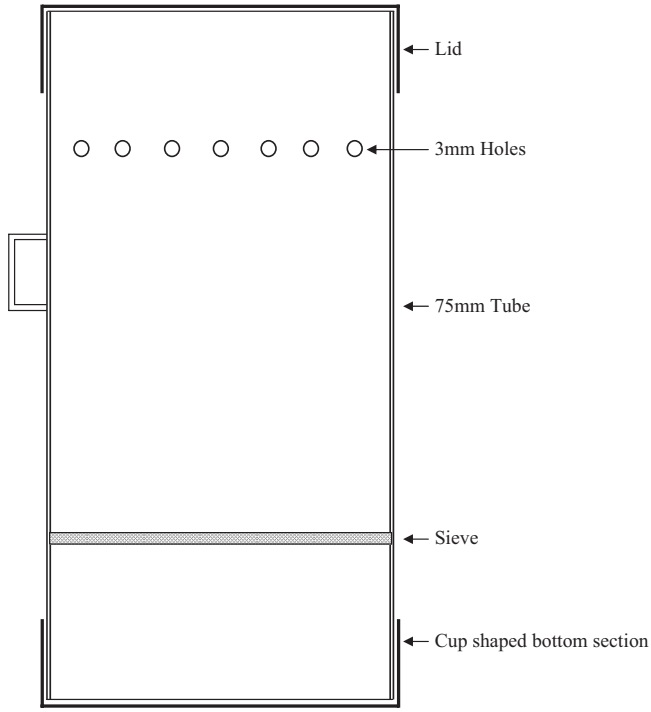

Fig. 1. Section through the trap used in this study.

insect associations for insects collected from traps and insects collected from the stumps. It was also done to confirm that the fungal isolation techniques used were effective. Sampling for this part of the study was done during January (summer) 2008.

Presence of Fungal Propagules on Insect Bodies. Insect exoskeletons were inspected for the presence of fungal propagules using scanning electron microscopy (SEM). A total of 25 specimens of each of the morphological groups of insects collected from the "native" area were examined. Specimens were dried in self-indicating silica gel for 5 d. Once the critical drying point (CDP) was reached, specimens were mounted on stubs using double-sided carbon tape. One half of the specimens were mounted on their dorsal sides, and the remaining specimens were mounted on their ventral sides. Specimens were coated with gold using a Polaron E5200C sputter coater (Watford, United Kingdom) and examined using a JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

Isolation of Fungi. Three methods were used to isolate *Ceratocystis* spp. from the collected insects. Equal numbers of insects were used for each of the three isolation methods. For one third of the insects, the exoskeletons were surface disinfested by submerging the insects in 96% ethanol for 1 min, in undiluted bleach for 1 min, and in 70% ethanol for 1 min and thereafter rinsing them in sterile water. Insects were macerated onto 2% Malt Extract Agar (MEA; Biolab, Merck, Midrand, South Africa) amended with 100 mg/liter (100 ppm) streptomycin sulfate (Sigma, Steinheim, Germany) to inhibit bacterial growth and incubated at 25°C for 2–7 d. Plates were examined using a dissection microscope.

Another one third of the insects were killed with forceps and placed directly onto the surface of 2% MEA amended with streptomycin sulfate with either the dorsal or ventral sides facing the agar surface. Samples were incubated for 2–4 d at 25°C, after which plates were examined using a dissection microscope. Pure cultures were obtained by making hyphal tip transfers.

With one third of the insects, 5–10 individuals were placed between two carrot discs, ≈10 mm thick (Moller and DeVay 1968a). The carrot discs were prepared by soaking in distilled water amended with 300 mg/liter (300 ppm) streptomycin sulfate for 10 min. The carrot discs, containing the insects, were incubated at 25°C for 4–7 d. Once ascomata had formed on the carrot surfaces, single spore drops were transferred to 2% MEA amended with 100 mg/liter (100 ppm) streptomycin sulfate to obtain pure cultures. All isolates obtained in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Identification of Isolates. *Ceratocystis* isolates obtained from the insects were grouped based on culture morphology after 7 d of growth at room temperature on 2% MEA. Isolates were identified based on structural morphology following published descriptions for *Ceratocystis* spp. Fungal structures were mounted on glass slides in lactophenol and examined under a Zeiss

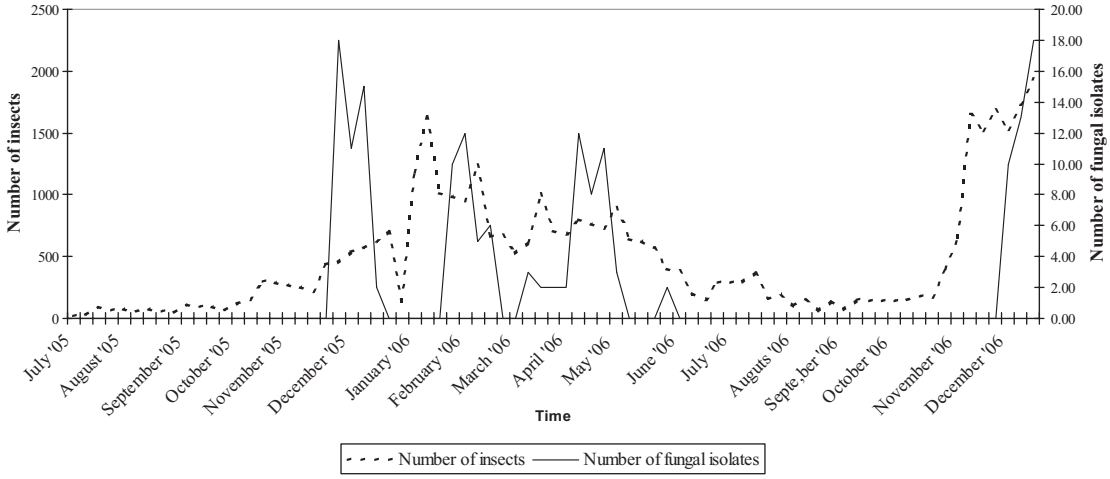

Fig. 2. Graph showing the fluctuation of the total numbers of insects and number of *Ceratocystis* isolates collected from the native study area for the duration of the study.

Axioskope (Carl Zeiss, Jena, Germany) microscope and images were captured using a HRc Axiocam digital camera and Axiovision 3.1 software (Carl Zeiss).

Identifications of the *Ceratocystis* isolates were confirmed by comparing DNA sequences for two isolates of each morphospecies with those previously published. Three gene regions were used for the DNA sequence comparisons. These were the internal transcribed spacer regions (ITS1, ITS2), including the 5.8S rDNA region, amplified using primers ITS1 and ITS4 (White et al. 1990), part of the β-tubulin gene region using primers Bt1a and Bt1b (Glass and Donaldson 1995), and the transcription elongation factor 1α (EF-1α) region using the primers EF1 F and EF2R (Jacobs et al. 2004). Polymerase chain reaction (PCR), sequencing, purifying, data processing, and data analyses protocols were the same as those used by Van Wyk et al. (2006).

Statistical Analyses of Data. Data collected in this study included the number of insects collected per week on Leeuwfontein, the number of fungal isolates obtained from these insects, and various climate variables. After insects had been collected and divided into groups based on morphological characteristics, the total number of insects residing in each morphological group was enumerated. Once fungal isolates had been obtained from the insects and identified, the total number of each species was noted.

Climate data were obtained from the Range and Forage Department, Roodeplaat Research Station of the Agricultural Research Council (ARC; 25°60′41″ S, 028°35′42″ E), ≈30 km from Leeuwfontein. The weather data collected included rainfall (mm/d), daily maximum temperature (°C), daily minimum temperature (°C), wind speed (M/s), maximum daily relative humidity (%), and minimum daily relative humidity (%).

For the purpose of statistical analyses, the data were divided into two sets. One dataset was used to determine which variables influenced the number of fungal isolates obtained during the study period. Data for the number of fungal isolates were transformed into binary values where values below zero were converted to zero and those greater than zero to one. Data were subjected to the stepwise logistic procedure determining the predictive variables using SAS (SAS Institute 2001).

The second data set was used to determine which climatic variables had an influence on the number of insects collected during the study period. Data were subjected to a stepwise regression on the predictive variables using SAS (SAS Institute 2001). The significance level for entry of variables into the model was set at 0.05, and the significance level for variables remaining in the model was set at 0.1.

Results

Collection of Insects from the "Native" Study Area. Insects were found in the traps at Leeuwfontein throughout the study period, with 38,262 insects collected over the 77 wk study period. A clear seasonal trend was observed in the number of insects collected, with the lowest numbers of insects (4,752) collected in the winter and spring (June 2005–October 2005 and June 2006–October 2006) seasons, respectively (Fig. 2).

At the start of the 2005/2006 summer (mid-October), the number of insects increased gradually, and these fluctuated over the summer months, gradually decreasing from early May 2006 to stabilize at relatively low numbers in winter 2006 (June–September). A similar trend was observed for the 2006 summer, with the number of insects increasing from the end of October. A total of 33,510 insects were collected during the summer and autumn months during the study period. In contrast to the summer of 2005/2006, the number of insects collected in the summer of 2006 increased more rapidly at the beginning (October) of the 2006 summer season (Fig. 2).

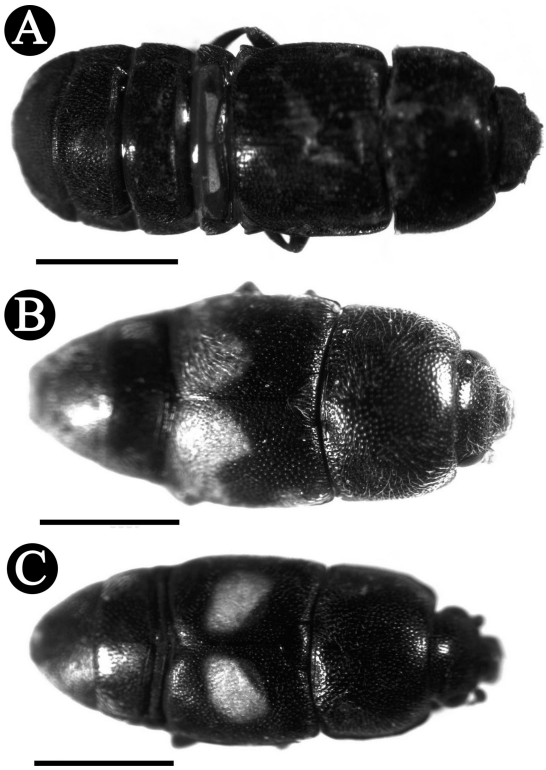

Fig. 3. Insects collected from the non-native study site: (A) *B. depressus*, (B) *C. hemipterus*, and (C) *C. bisignatus*. Scale bar = 1,000 μm.

Three main groups of insects were identified based on morphological characteristics. These represented two *Carpophilus* spp. (28,252 insects) and one *Brachypeplus* sp. (2,041 insects). The two *Carpophilus* spp. were identified as *Ca. bisignatus* Boheman and *Ca. hemipterus* L. (Fig. 3). The *Brachypeplus* sp. was identified as *B. depressus* Erichson (Fig. 3). Other insects collected in the traps included Coleoptera, Diptera, and Hymenoptera (7,969). The numbers of insects collected for each of the two *Carpophilus* spp. differed significantly, with *Ca. bisignatus* being more abundant (21,188) than *Ca. hemipterus* (7,064).

Collection of Insects from "Non-Native" Study Area. The same three species of nitidulid beetles (*Ca. bisignatus*, *Ca. hemipterus*, and *B. depressus*) collected in the native study area were found in the *A. mearnsii* plantations. Other insects collected in the traps included Coleoptera and Diptera. Mainly *Ca. hemipterus* (6) and *B. depressus* (23) were collected in 2008 from under bark flaps from the stumps of recently felled trees.

Presence of Fungal Propagules on Insect Bodies. SEM showed hat-shaped ascospores on the dorsal sides of some insects (Fig. 4). These spores had similar morphology to those produced by *Ceratocystis* spp. isolated from the insects, varying from 4 to 6 μm in size. Ascospores were observed on 2 of the 25 *B. depressus* beetles scanned. Of the 50 *Carpophilus* beetles examined, ascospores were observed on only 2 *Ca. hemipterus* and 3 *Ca. bisignatus* individuals. The spores occurred singly on the insects and were not abundant. No spores were observed on the ventral sides of the beetles.

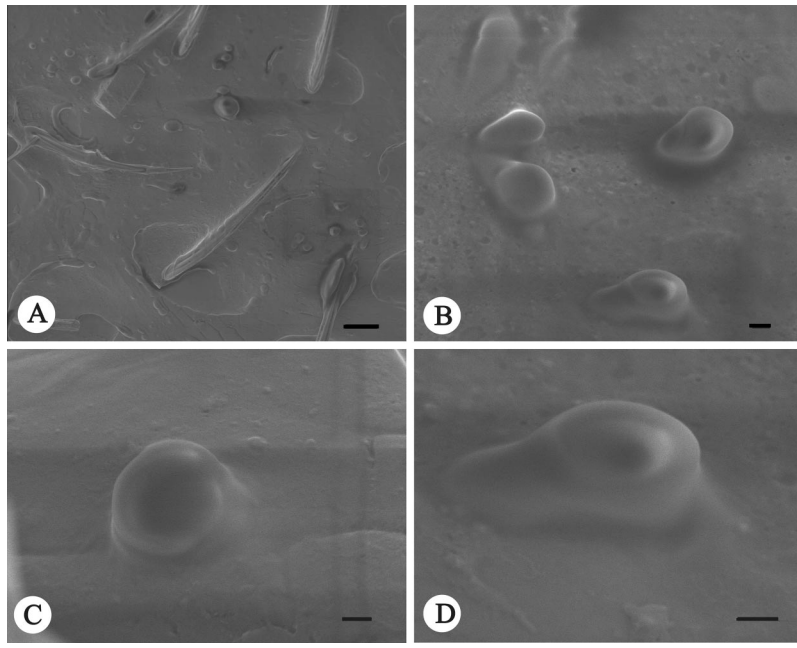

Fig. 4. SEM images showing (A) fungal propagules on the insect body and hat-shaped ascospores characteristic of *Ceratocystis* spp. observed on (B) *B. depressus*, (C) *Ca. bisignatus*, and (D) *Ca. hemipterus*.

Table 1. Association percentages for *Ceratocystis* spp. isolated from three nitidulid species collected from traps and from under bark flaps in South Africa

	Ceratocystis albifundus		*Ceratocystis oblonga*	
	Traps	Bark flaps	Traps	Bark flaps
Brachypeplus depressus	3.2%	35%	0.44%	91%
Carpophilus hemipterus	0.08%	50%	1.2%	100%
Carpophilus bisignatus	0.02%	—	1.5%	—

Identification of Isolates. The *Ceratocystis* spp. obtained in this study represented two species. Some isolates were easily identified as *C. albifundus* based on morphology because this species is the only *Ceratocystis* sp. known to produce ascomata with light-colored bases. This is in contrast to the second species obtained that had white, fast growing colonies that turned brown with time, with dark-colored ascomatal bases and granular hyphae. It was not possible to accurately distinguish these isolates from closely related species such as *C. savannae* Kamgan-Nkuekam and Jolanda Roux (Kamgan-Nkuekam et al. 2008), necessitating DNA sequencing to confirm their identity.

Analyses of DNA sequences showed that isolates with light colored ascomatal bases grouped within the *C. albifundus* clade (Bootstrap support of 100%). Isolates that could not be identified based on morphology alone grouped in a clade (Bootstrap support, 98%) with the recently described species, *C. oblonga* R. N. Heath and Jolanda Roux (Heath et al. 2008), distinct from all other species in the *C. moniliformis* s.l. Hedgcock group.

Association of Fungi with Insects. Of the three methods used to isolate *Ceratocystis* spp. from the insects, only two yielded positive results. When the insects were surface disinfected, they yielded only bacteria, and therefore, these insect numbers were excluded from the levels of association calculations. *C. oblonga* was isolated from the insects that were placed directly on agar and those placed between the carrots, whereas *C. albifundus* was obtained only using the carrot baiting technique. For the calculation of association levels for *C. albifundus*, only the number of insects placed between carrots was included.

In total, 22 of the 680 (3.2%) *B. depressus* individuals used in the carrot baiting yielded isolates of *C. albifundus.* Two isolates were obtained from 2,354 *Ca. hemipterus* (0.08%) and one isolate was obtained from 7,062 (0.02%) *Ca. bisignatus* individuals. In contrast, 137 of the 18,832 (0.73%) *Carpophilus* spp. yielded *C. oblonga* and only 3 (0.44%) of the *B. depressus* individuals yielded this fungus. Of the *C. oblonga* isolates obtained from *Carpophilus* spp., 29 isolates were from *Ca. hemipterus* and 108 isolates were from *Ca. bisignatus* (Table 1).

Both the insect numbers and success of fungal isolation showed considerable seasonal fluctuation (Fig. 2). *Ceratocystis* spp. were obtained from the insects only during the summer months, with the first isolates obtained in the 22nd week (24–30 November 2005) of sampling. Successful isolation of *Ceratocystis* spp. ceased in week 48 (1–8 June 2006) of sampling. *Ceratocystis* spp. began to reappear in isolations at the beginning of summer in week 75 (30 November 2006) of the sampling period (Fig. 2). A total of 165 *Ceratocystis* isolates were obtained from 30,293 insects during the entire study period. However, because one third of the insects (10,098) had been surface sterilized, and no fungal isolates were expected from them, the effective number of insects from which *Ceratocystis* isolates could be expected was 20,195.

Isolation of *Ceratocystis* spp. from the insects collected from traps in the non-native study area gave similar results to those obtained from the native study site. In this area, *C. oblonga* (11 isolates) was isolated only from *Carpophilus* spp., and all but one of the *C. albifundus* isolates (7) originated from the *Brachypeplus* sp., with one isolate obtained from *Ca. bisignatus.*

The six *Ca. hemipterus* and 23 *B. depressus* collected from under bark flaps of stumps from recently felled *A. mearnsii* trees in 2008 yielded a total of 38 *Ceratocystis* isolates. Six of the *Ca. hemipterus* individuals yielded six *C. oblonga* isolates and three of the same insects gave rise to three *C. albifundus* isolates. Twenty-one *C. oblonga* isolates and 8 *C. albifundus* isolates were collected from the 23 *B. depressus* insects collected from under the bark flaps. No *Ca. bisignatus* were obtained from the stumps (Fig. 3).

Statistical Analyses of Data. Analyses of one climate data set, to determine which variables influenced the number of fungal isolates obtained, indicated that there were linear relationships present. Analyses indicated that the number of *C. oblonga* isolates obtained was influenced by minimum daily relative humidity ($\chi^2 = 10.81$, $P = 0.001$, df = 1). The association of predicted probabilities and observed responses for this interaction produced a concordance percentage of 78.4%. Analyses further indicated that the number of *C. albifundus* isolates obtained was influenced by an interaction between wind speed and the number of *B. depressus* collected ($\chi^2 = 9.31$, $P = 0.0023$, df = 1). The association of predicted probabilities and observed responses for this interaction produced a concordance percentage of 95.2%.

Analyses of the second data set, to determine which climatic variables influenced the number of insects collected, showed the presence of linear relationships. With regard to the number of *B. depressus* collected, a number of variables had a linear relation ($F = 13.64$, $R^2 = 0.5426$). These included daily maximum temperature, maximum daily relative humidity, daily minimum temperature, minimum daily relative humidity, the interaction between daily maximum temperature and maximum daily relative humidity, and the interaction between daily minimum temperature and minimum daily relative humidity. However, only daily maximum temperature (t-value = 2.81, $P = 0.0064$, df = 1) and maximum daily relative humidity had significant probability values (t-value = 2.73, $P = 0.0080$, df = 1).

With regard to the number of *Carpophilus* spp. collected, only two of the tested variables had a linear relationship ($F = 13.43, R^2 = 0.4896$). These were daily maximum temperature and maximum daily relative humidity. The linear relation between the climatic variable, daily maximum temperature, and the number of insects collected was significant (t-value = 4.55, $P > 0.0001$, df = 1). The linear relationship with maximum daily relative humidity identified was, however, not supported by the probability value (t-value = −0.29, $P = 0.7762$, df = 1).

Discussion

Before this study, nothing was known regarding the mode of spread of the wilt pathogen *C. albifundus.* We have thus confirmed that, similar to other *Ceratocystis* spp., *C. albifundus* has associations with nitidulid beetles. Virtually nothing is known of the biology of nitidulid beetles in South Africa, and this study was, therefore, also the first to consider the influence of climate on the population numbers of these insects in the country and to identify some of the fungi associated with them. Results also provide valuable information that might be used to limit spread of the disease of *A. mearnsii* caused by *C. albifundus* in South Africa.

Three insect species were identified as associates of *C. albifundus* and *C. oblonga* collected in this study. These were *B. depressus*, *Ca. hemipterus*, and *Ca. bisignatus.* Very little is known regarding these insects in Africa, and none has been reported to be associated with fungi in South Africa. *Brachypeplus depressus* has, however, been reported to be the most common *Brachypeplus* sp. in Africa (Kirejtshuk and Barclay 2007).

Carpophilus spp. have previously been reported as vectors of *Ceratocystis* spp. (Moller and DeVay 1968b, Juzwik and French 1983). *Carpophilus hemipterus* in particular is a known agricultural pest and has been reported as an associate of *C. fimbriata* (Moller and DeVay 1968b). No evidence could, however, be found of prior associations between fungi and *Ca. bisignatus* and *B. depressus.*

It was interesting that only two species of *Ceratocystis* were collected in this study. Various other *Ceratocystis* spp. are known to occur on wounds of trees in the areas studied. These include *C. savannae* in the native study area (Kamgan et al. 2008), and *C. obpyriformis* R. N. Heath and Jolanda Roux, and *C. polyconidia* R. N. Heath and Jolanda Roux in the nonnative study area (Heath et al. 2008). All of these fungi occur on wounds and are presumably vectored by insects similar to those encountered in this study. The results may suggest that there could be some host specialization in terms of the species of *Ceratocystis* vectored or that some level of competition exists between these fungi. Alternatively, these *Ceratocystis* spp. may occur at much lower levels than *C. albifundus* and *C. oblonga.* Our data could also have been skewed by the low number of *Ceratocystis* spp. recovered from the insects collected in traps.

Ceratocystis oblonga was more commonly isolated from nitidulid beetles than was *C. albifundus. C. oblonga* is a species recently described from cut stumps of non-native *A. mearnsii* in South Africa (Heath et al. 2008). The relatively large numbers of isolates of *C. oblonga* compared with those of *C. albifundus* could be caused by differences in the odors produced by the two fungi, with *C. oblonga* being more attractive to the insects than *C. albifundus.* Alternatively, competition between the fungi on the substrate or isolation technique could have influenced these results. *C. oblonga* grows fast (83 mm in 7 d) (Heath et al. 2008) compared with the relatively slow-growing *C. albifundus* (20 mm in 8 d) (Wingfield et al. 1996) on 2% MEA. This could account for the fact that *C. albifundus* was more frequently isolated from insects subjected to carrot baiting than from insects placed on 2% MEA plates.

This study represents the first report of *C. oblonga* from a host other than *A. mearnsii* and it expands the geographic range of this fungus. It was predominantly isolated from the two *Carpophilus* spp. The study performed in the *A. mearnsii* plantations showed that *C. oblonga* has a percentage association with *Carpophilus* spp. of 100%. A large number of isolates of *C. oblonga* (21) also originated from *B. depressus* (23) collected from *A. mearnsii.*

Differences in isolation success were observed between insects collected in traps compared with those collected from stumps of recently felled *A. mearnsii* trees. Isolations from insects collected from bark flaps resulted in an association of 100% for *C. oblonga* with *Ca. hemipterus* and 91% with *B. depressus. C. albifundus* had a 50% association with *Ca. hemipterus* and 35% association with *B. depressus.* These levels of association were noticeably higher than those obtained for *C. albifundus* with *B. depressus* (3.2%) and *Carpophilus* spp. (0.03%) collected in traps in the native study sites. Similar results have been reported in previous studies with *C. fagacearum* where low associations (0.7%) were found between *C. fagacearum* and *Ca. truncatus* Muttay collected from traps in contrast to high association levels (79.8%) obtained from insects collected from fungal mats (Norris 1956, Gibbs 1980, Juzwik and French 1983). The lower recovery rates of *Ceratocystis* spp. from insects collected in traps could be because of the fact that the insects and spores are exposed to harsh environmental conditions during flight possibly reducing the viability and number of spores. In contrast, insects collected from fungal mats would not have been exposed to the same severe environmental conditions. The fact that we isolated the *Ceratocystis* spp. from free-flying insects collected in traps and from insects obtained from fungal mats clearly shows that these insects play a role in the spread of these fungi.

Climatic factors had a significant influence on the population fluctuation of the nitidulid beetles. Not surprisingly, these insects were much more abundant in the spring and summer months (87%) than in the colder winter months (12%). These observations are similar to those for studies on the transmission of *C.*

fagacearum by free-flying and fungus-mat-inhabiting nitidulids in Minnesota (Yount et al. 1955, Juzwik and French 1983). In those studies, it was shown that nitidulid beetle numbers are strongly influenced by temperature, with the insects overwintering under the bark of trees, in debris, or in rotting fruit on the soil surface during the winter (James et al. 1995, Hossain and Williams 2003). Although we did not consider where these insects might occur during the winter, they most likely occupy similar niches in South Africa to those of the better-studied Northern Hemisphere species.

Climatic factors had an influence on the number of fungal isolates obtained. For *C. oblonga*, minimum relative humidity was the only climatic variable with a significant influence on recovery. Interestingly, the number of *C. albifundus* isolates obtained was influenced by wind speed. This result was unusual as the only instance where winds have been reported to play a role is in the dissemination of fungal spores in the frass of insects (Iton 1960), and it might be incidental and an aberration.

Ceratocystis albifundus infects wounds on trees, and results of this study suggest strongly that nitidulid beetles are the primary vectors that move the pathogen from one tree to another. *Acacia mearnsii* trees require corrective pruning to improve growth form after establishment and to correct damage caused by animal grazing (Dunlop and Goodricke 2000). We assume that these wounds are visited by nitidulid beetles that transmit the pathogen. Given that the insects do not seem to be active in the winter months, pruning should be undertaken only during this period to restrict the spread and infection by the fungus.

In this study, we showed that *C. albifundus* and *C. oblonga* are involved in a relationship with three nitidulid beetles that most likely act as their vectors. It seems that these relationships are not specific because both fungal species were isolated from more than one insect species. This is not surprising because both the fungi produce fruity odors and are expected to have a loose association with the insect vectors (Himelick and Curl 1958). There are at least 11 *Ceratocystis* spp. known from Africa; however, no research regarding the possible insect associations of these species or their role in the biology and possible control strategies of the fungal pathogens have been performed. Further research is needed for the possible insect associations of these species on the continent because this could provide valuable information to facilitate management and control strategies.

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References Cited

Barnes, I., G. Nakabonge, J. Roux, B. D. Wingfield, and M. J. Wingfield. 2005. Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda. Plant Pathol. 54: 189–195.

Bretz, T. W. 1952. The ascigerous stage of the oak wilt fungus. Phytopathology 42: 435–437.

Cease, K. R., and J. Juzwik. 2001. Predominant Nitidulid species (Coleoptera: Nitidulidae) associated with spring oak wilt mats in Minnesota. Can. J. For. Res. 31: 635–643.

Dunlop, R. W., and T. G. Goodricke. 2000. Black wattle (*Acacia mearnsii*), pp. 124–127. *In* D. L. Owen (ed.), South African Forestry Handbook 2000. The South African Institute for Forestry. Menlopark, South Africa.

Engelbrecht, C.J.B., and T. C. Harrington. 2005. Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. Mycologia 97: 57–69.

Gibbs, J. N. 1980. Role of *Ceratocystis piceae* in preventing infection by *Ceratocystis fagacearum* in Minnesota. Transactions of the British Mycological Society 74: 171–174.

Glass, N. L., and G. C. Donaldson. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61: 1323–1330.

Halsted, B. D. 1890. Some fungous disease of the sweet potato. Agric. Coll. Exp. Stan. Bull. 76: 1–32.

Harrington, T. C., and M. J. Wingfield. 1998. The *Ceratocystis* species on conifers. Can. J. Bot. 76: 1446–1457.

Heath, R. N., G. Meke, A. Mbaga, B. D. Wingfield, M. J. Wingfield, and J. Roux. 2009. *Ceratocystis* species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species. Fung. Div. 34: 41–68.

Himelick, E. B., and E. A. Curl. 1958. Transmission of *Ceratocystis fagacearum* by insects and mites. Plant Dis. Rep. 42: 538–545.

Hossain, M. S., and D. G. Williams. 2003. Phenology of *Carpophilus* beetle populations (Coleoptera: Nitidulidae, *Carpophilus* spp.) in fruit dump in Northan Victoria. Aust. J. Exp. Agric. 43: 1275–1279.

Iton, E. F. 1960. Studies on a wilt disease of cacao at River Estate. II. Some aspects of wind transmission, pp. 47–58. *In* Annual Report on Cacao Research 1959–1960: Imperial College of Tropical Agriculture, University of the West Indies, St. Augustine, Trinidad.

Jacobs, K., D. R. Bergdahl, M. J. Wingfield, S. Halik, K. A. Seifert, D. E. Bright, and B. D. Wingfield. 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycol. Res. 108: 411–418.

James, D. G., B, Vogele, and R. J. Faulder. 1995. Seasonal abundance of *Carpophilus* spp. (Coleoptera: Nitudulidae) in fallen citrus fruit in the Murrumbidgee Irrigation Area of Southern New South Wales. Plant Prot. Qtly. 10: 103–106.

Juzwik, J., and D. W. French. 1983. *Ceratocystis fagacearum* and *C. piceae* on the surface of free-flying and fungus-mat-inhabiting nitidulids. Phytopathy 73: 1164–1168.

Kamgan Nkuekam, G., K. Jacobs, Z. W. De Beer, M. J. Wingfield, and J. Roux. 2008. *Ceratocystis* and *Ophiostoma*

species, including three new taxa, associated with wounds on native South African trees. Fung. Div. 29: 37–59.

Kile, G. A. 1993. Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*, pp. 173–183. *In* M. J. Wingfield, K. A. Seifert, and J. F. Webber (eds.), *Ceratocystis* and *Ophiostoma:* taxonomy, ecology, and pathogenicity. APS Press, St. Paul, MN.

Kirejtshuk, A. G., and M.V.L. Barclay. 2007. On *Brachypeplus* (*Brachypeplus*) *parallelus* and *B.* (*B.*) *euparallelus* sp. n. from Africa with notes on synonymy in the subgenus *Brachypeplus* (Coleoptera: Nitidulidae: Cillaeinae). Studies and reports of District Museum Prague-East. Taxonom. Ser. 3: 115–128.

Kirisits, T. 2004. Taxonomy and systematics of bark and ambrosia beetles, pp. 181–235. *In* F. Lieutier, K. R. Day, A. Batisttisti, J.-C. Grégoire, and H. F. Evans (eds.), Bark and woodboring insects in living trees in Europe, a synthesis. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lanza, E., and J. K. Palmer. 1977. Biosynthesis of monoterpenes by *Ceratocystis moniliformis.* Phytochemistry 16: 1555–1560.

Malloch, D., and M. Blackwell. 1993. Dispersal biology of the Ophiostomatoid fungi, pp. 195–206. In M. J. Wingfield, K. A. Seifert, and J. F. Webber (eds.), *Ceratocystis* and *Ophiostoma:* taxonomy, ecology, and pathogenicity. APS Press, St. Paul, MN.

Moller, W. J., and J. E. DeVay. 1968a. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata.* Phytopathy 58: 123–126.

Moller, W. J., and J. E. DeVay. 1968b. Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. Phytopathy 58: 1499–1507.

Morris, M. J., M. J. Wingfield, and C. De Beer. 1993. Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata.* Plant Pathol. 42: 814–817.

Norris, D. M. 1956. Association of insects with the oak tree and *Endoconidiophora fagacearum* Bretz. PhD thesis, Iowa State College, Ames, IA.

Roux, J., T. A. Harrington, J. P. Steimel, and M. J. Wingfield. 2001. Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus.* Mycoscience 42: 327–332.

Roux, J., G. Meke, B. Kanyi, L. Mwangi, A. Mbaga, G. C. Hunter, G. Nakabonge, R. N. Heath, and M. J. Wingfield. 2005. Diseases of plantation forestry tree species in Eastern and Southern Africa. S. Afr. J. Sci. 101: 409–413.

Roux, J., R. N. Heath, L. Labuschagne, G. Kamgan Nkuekam, and M. J. Wingfield. 2007. Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus*, on native South African trees. For. Pathol. 37: 292–302.

SAS Institute. 2001. SAS statistical software, SAS/STAT user's guide, version 8.2. SAS Institute, Cary, NC.

Southwood, T.R.E., and P. A. Henderson. 2000. Ecological methods, 3rd ed. Blackwell, Oxford, England.

Thompson, N. E., B. L. Hadley, Jr., and A. R. Jeffrey. 1955. Transmission of *Endoconidiophora fagacearum* by spore-infested nitidulids caged on wounded healthy oaks in Pennsylvania. Plant Dis. Rep. 39: 58–60.

Van Wyk, M., N. A. Van der Merwe, J. Roux, B. D. Wingfield, G. N. Kamgan, and M. J. Wingfield. 2006. Population genetic analyses suggest that the *Eucalyptus* fungal pathogen *Ceratocystis fimbriata* has been introduced into South Africa. S. Afr. J. Sci. 102: 259–263.

White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), PCR protocols: a sequencing guide to methods and applications. Academic, San Diego, CA.

Wingfield, M. J. 1993. Problems in delineating the genus *Ceratocystiopsis*, pp. 21–25. *In* M. J. Wingfield, K. A. Seifert, and J. F. Webber (eds.), *Ceratocystis* and *Ophiostoma:* taxonomy, ecology, and pathogenicity. APS Press, St. Paul, MN.

Wingfield, M. J., C. De Beer, C. Visser, and B. D. Wingfield. 1996. A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. Syst. Appl. Microbiol. 19: 191–202.

Yount, W. L., A. R. Jeffery, and H. E. Thompson. 1955. Spores of *Endoconidiophora fagacearum* on the external surface of the body of nitidulids. Plant Dis. Rep. 39: 54–57.

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