Factors influencing infection of *Acacia mearnsii* by the wilt pathogen *Ceratocystis albifundus* in South Africa

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Summary

It is well known that species of *Ceratocystis*, which cause canker and wilt diseases on trees, require wounds for infection. In this regard, a number of physical and environmental factors influence the success of wound colonization by these fungi. The aim of this study was to consider the influence of wound age, stem diameter and colonization of wounds by *Ophiostoma quercus* on the success of infection by *Ceratocystis albifundus*, an important wilt pathogen of *Acacia mearnsii* in southern and eastern Africa, under field conditions. This was performed by doing controlled inoculations on *Acacia mearnsii* trees, of different diameters, with *C. albifundus* at different time intervals after wounding and after pre-inoculation of wounds with *O. quercus* at the time of wounding. The success of infection by *C. albifundus* decreased significantly from 8 h after wounding and was higher on stems of greater diameter. Pre-infection of wounds by *O. quercus* reduced the infection success of *C. albifundus*. The overall results showed that managing wounds created during forestry operations by treating wounds with naturally occurring, non-virulent fungi, such as *O. quercus*, could reduce the prevalence of infections by *C. albifundus*.

1 Introduction

Ceratocystis albifundus De Beer, M.J. Wingf. & Morris (Ascomycetes, Microascales) is the causal agent of Ceratocystis wilt and die-back of non-native plantation-grown *Acacia mearnsii* De Wild. trees in southern and eastern Africa and is considered the most important pathogen of *A. mearnsii* trees in Africa (Morris et al. 1993; Wingfield et al. 1996; Roux and Wingfield 1997; Roux et al. 2005). The disease was first reported on *A. mearnsii* in South Africa (Morris et al. 1993), but it has subsequently also been recorded from Uganda (Roux and Wingfield 2001), Kenya and Tanzania (Roux et al. 2005). Symptoms of infection include gum exudation, wood-discoloration, stem cankers, rapid wilting and tree death (Morris et al. 1993; Roux et al. 1999). The fungus has been reported from native tree species in Africa, but has not been reported to cause disease on its native hosts (Roux et al. 2007).

Ceratocystis spp. require wounds for infection (Bretz 1952; Norris 1953; DeVay et al. 1963; Kile 1993). Such wounds can arise from wind (Roux et al. 2007), hail damage (Roux et al. 1995), growth cracks (Teviotdale and Harper 1991), insect and animal feeding (Walter 1946; Bretz 1952; DeVay et al. 1963), harvesting (Teviotdale and Harper 1991; Marin et al. 2003) and silvicultural practices such as grafting and pruning (Walter

1946; TEVIOTDALE and HARPER 1991; DUNLOP and GOODRICKE 2000). Success of infection is dependent on a number of physical and environmental factors. For example, species in the *Ceratocystis fimbriata sensu lato* (*s.l.*) species complex are able to infect their hosts when viable fungal propagules are deposited onto bark wounds (DEVAY et al. 1968). Other *Ceratocystis* spp., such as *Ceratocystis fagacearum* (Bretz) Hunt, can only infect if viable fungal propagules are deposited onto freshly exposed wood of the host (KUNTZ and DRAKE 1957). Temporal factors also affect the success of infection by *Ceratocystis* spp. For example, KUNTZ and DRAKE (1957) showed that *C. fagacearum* could not cause infection when wounds were older than 24 h. Climatic factors such as temperature and relative humidity have also been shown to influence germination of spores and infection by *Ceratocystis* spp. (COLE and FERGUS 1956). GIBBS (1980), showed that colonization of wounds by the saprophytic fungus, *Ophiostoma piceae* (Munch) H. Sydow & Sydow, prior to infection by *C. fagacearum*, prevented colonization by the pathogen.

Ceratocystis spp. have evolved several strategies to reach wounds and subsequently infect trees. The most common of these is through associations with insects including bark beetles (Coleoptera: Scolytidae) (WEBBER and GIBS 1989; KIRISITS 2004), nitidulid beetles (Coleoptera: Nitidulidae) (HIMELICK and CURL 1958; MOLLER and DEVAY 1968; HARRINGTON 1987) and flies (MOLLER and DEVAY 1968; HINDS 1972). These fungi are morphologically adapted to insect dispersal having their ascospores in sticky drops at the apices of long-necked sporocarps (DOWDING 1984). Many species of *Ceratocystis*, particularly those not associated with bark beetles (WEBBER and GIBS 1989; KIRISITS 2004), also produce fruity aromas that attract insects that subsequently visit fresh wounds on trees. In this regard, *C. albifundus* has recently been reported to be associated with nitidulid beetles (*Carpophilus* spp. and *Brachypeplus* spp.) that visit wounds on native and non-native trees in South Africa (HEATH et al. 2009).

Infection of *A. mearnsii* by *C. albifundus* has been associated with hail wounds, as well as with pruning wounds on these trees (ROUX et al. 1995). *Acacia mearnsii* trees typically require corrective pruning to improve growth form after establishment and to correct damage caused by animal grazing (DUNLOP and GOODRICKE 2000). Wounds arising from these operations provide ideal infection sites for *C. albifundus*.

Although selection and breeding for resistance against the disease caused by *C. albifundus* has been promoted, the fungus remains one of the most important pathogens of *A. mearnsii* in South Africa. Therefore, an understanding of the factors involved in the successful infection of wounds by *C. albifundus* could contribute to improved disease management options in plantations.

In this study we aimed: (i) to consider the influence of wound age on the success of infection by *C. albifundus* under field conditions; (ii) investigate the possible influence of the colonization of wounds by the commonly occurring wound-infecting *Ophiostoma quercus* (Georgév.) Nannf. (DE BEER et al. 2003) on the success of infection by *C. albifundus* under field conditions; and (iii) to consider whether stem diameter affects the infection success of *C. albifundus*.

2 Materials and methods

2.1 Preparation of inoculum

An isolate (CMW4095) of *C. albifundus* collected in 1998 from diseased *A. mearnsii* in South Africa was selected for artificial inoculations. Only one isolate was used for the study as low variance in virulence between isolates of *C. albifundus* was proven by DE BEER (1994). An *O. quercus* isolate (CMW24164), collected from a 4-week-old wound on an *A. mearnsii* stump in 2006, also in South Africa, was selected for tests to consider the effect of pre-infection by this fungus on the infection success of *C. albifundus*. Both

isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Pure cultures of the test isolates were grown on 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with Streptomycin Sulphate (0.001 g/volume; SIGMA, Steinheim, Germany) and incubated at 25°C under natural day/night conditions for 7 days prior to inoculation. Spore suspensions were made by washing spores from the surface of 7-day old cultures, with sterile water. Concentrations of spores were adjusted to 1×10^6 spores/ml with sterile water using a Neubauer 1/100 mm haemocytometer (BEOCO, Hamburg, Germany). Suspensions were prepared no longer than ∼60 min before inoculation and were maintained at ∼5°C prior to application. Viability of the spores in the suspensions was confirmed by plating 1 ml of spore suspension onto 2% (w/v) MEA at the time of inoculation and confirming growth after 5 days.

2.2 Wounding and inoculation of trees

The study was conducted in a 2-year-old stand of *A. mearnsii* situated 35 km east of Pretoria, South Africa (S 25°23′38.3″ E 028°37′19.5″). The inoculation study was performed using a completely randomised design with regard to treatments as well as the diameter of tree stems. Trees ranging from 10 to 32 mm in diameter were wounded by cutting the stems 50 cm above the soil surface with pruning shears, thereby simulating stem reduction practices applied in silvicultural regimes for *A. mearnsii* cultivation in South Africa (Fig. 1a). This method produced a stub with a flush cut exposing the xylem cells of the stems. Inoculations commenced in March 2007 (late summer) and the study was completed in May 2007 (autumn), thus also consistent with the time at which corrective pruning often occurs.

A total of 400 trees were pruned for the experiment. Twenty different treatments were applied, with each treatment applied to 20 trees. The 20 treatments included a water control (negative control), a positive control with *C. albifundus*, a positive control with *O. quercus* and a combination of *C. albifundus* and *O. quercus* inoculated at wounding. Eight treatments included those where *C. albifundus* was inoculated onto wounds 2, 6, 8, 24, 48 (2 days), 124 (5 days), 196 (8 days) and 346 (14 days) hours after wounding. The remaining eight treatments were fresh wounds which were inoculated with *O. quercus* at the time of wounding, followed by inoculation with *C. albifundus* at 2, 6, 8, 24, 48 (2 days), 124 (5 days), 196 (8 days) and 346 (14 days) hours after wounding. Inoculations were made by dispensing 2-ml spore suspensions directly onto the wounds using disposable syringes. The entire experiment was repeated once consecutively.

2.3 Assessment of infection

The experiments were terminated 6 weeks after each treatment commenced as this time period has been shown to be sufficient for disease development on trees of similar size under field conditions in South Africa (Roux et al. 1999). Inoculated stubs were collected by cutting the stems at the soil surface and transporting the samples in separate plastic bags to the laboratory. In the laboratory, the diameter of each stem was measured and the bark removed. Every inoculated wound was assessed visually for the presence or absence of vascular discoloration. Re-isolations were performed to correlate with visual assessment of *C. albifundus* infection.

2.4 Statistical analyses

Statistical analyses were performed using SAS version 8.2 (SAS Statistical Software 2001). The infection data were converted to binary format with the presence of infection
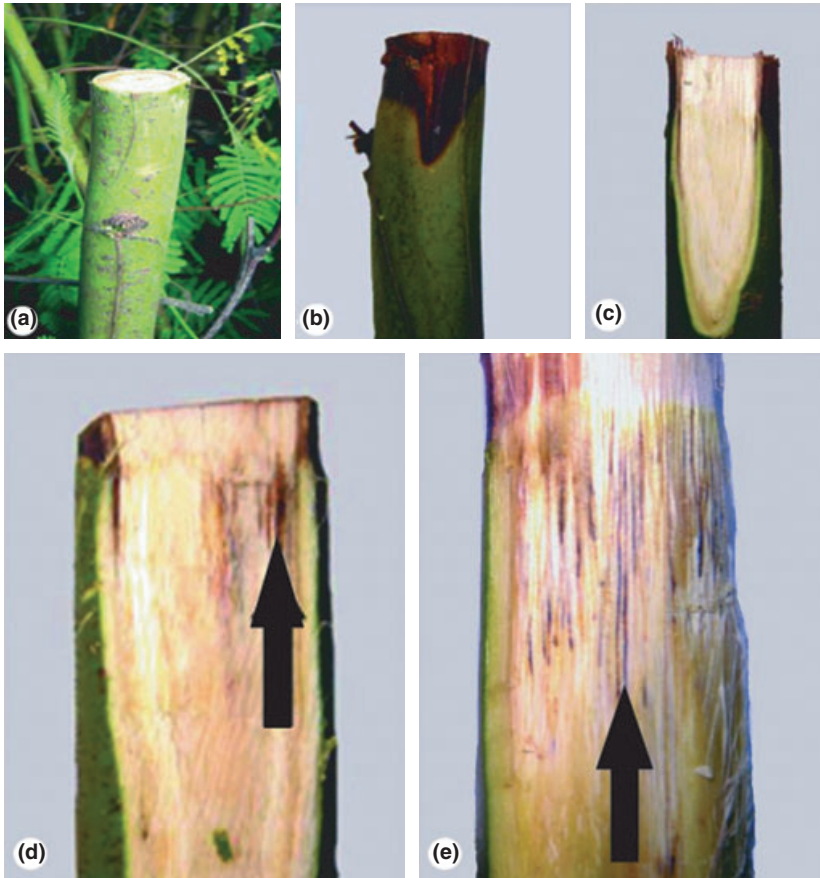
Fig. 1. Wound morphology and symptoms of infection on *Acacia mearnsii*. (a) typical wound made for the inoculation; (b) external symptoms including dying of the tips of the stems; (c) water control showing no signs of infection; (d) vascular streaking caused by *Ceratocystis albifundus* (indicated by arrow); (e) vascular streaking caused by *Ophiostoma quercus* (indicated by arrow) on positive treatment with *O. quercus*.

represented by a '1' and the absence of infection represented by '0'. Because this response variable was binary, a logistic model could be applied to reproduce the logit as a linear model of the predictor variables. The predictor variables were treatment (a categorical variable with 20 levels), replicate (a categorical variable with two levels) and tree diameter (a continuous variable). The logistic analysis models the variable $\lin\{p/(1 - p)\}$, the logit, as a linear function of the predictor variables, where p is the probability of a specific combination of predictor values to yield an infection.

The significance of each predictor to predict the logit was calculated. The concordance percentage was calculated to serve as a measure of the 'goodness of fit' of the applied model. Confidence intervals for the true model parameters were calculated using the estimated parameters and their standard errors. Furthermore, to provide for specific chosen combinations of predictor values (treatment, replicates and tree diameter), not present in the data, such combinations were added to the dataset whereas the response variable was absent. This was performed to exclude the 'spiked' observations when the model was fitted,

and to include them when estimated p-values were calculated for the specific added combinations. Finally, 95% confidence intervals were calculated for the p-values for the 'spiked' observations.

3 Results

3.1 Assessment of infection

The only exterior symptoms observed on inoculated stems, 6 weeks after inoculation, were death of the tips of the stems (Fig. 1b), yellowing of the bark and staining of the wood. *Ceratocystis* infection was distinguished from infection by *O. quercus* based on the fact that *C. albifundus* causes light to dark brown streaking (Fig. 1d) whereas *O. quercus* resulted in stain, when present, having a distinct blue colour (Fig. 1e). The majority (95%) of the 40 negative water controls (Fig. 1c) showed no symptoms of *C. albifundus* or *O. quercus* infection. Those control treatments that did show infection (5%) were considered to be due to natural infection since *C. albifundus* naturally occur in the Gauteng Province (ROUX et al. 2007). Re-isolation of *C. albifundus* from infected stems followed a similar trend to infection assessed visually (Table 1). The chi-square probability value showed significant treatment differences within the total dataset (p < 0.0001). The predictor variable, replicate, had a significant effect on the analyses (p = 0.0008). This could be due to the trees used in the repeat trial entering the dormancy period due to the change in season (ROBINSON et al. 2004). However, no interaction existed between the predictor variables treatment and replicates (p = 0.4745), therefore treatment means could be calculated across replicates.

3.2.1 Time after wounding

The time after wounding had a significant influence on the infection success of *C. albifundus*. The greatest number of stems with symptoms of *C. albifundus* infection

Table 1. **Comparison of *Ceratocystis albifundus* isolated from vascular streaking to visual infection assessed.**

| Treatment	Number of visual symptoms observed	Number of *Ceratocystis albifundus* isolates obtained
Water control	2	0
Ceratocystis albifundus, 0 h	30	10
Ophiostoma quercus, 0 h	15	3
C. albifundus and *O. quercus*, 0 h	28	5
C. albifundus, 2 h	28	9
O. quercus, 2 h	15	0
C. albifundus, 6 h	31	9
O. quercus, 6 h	16	1
C. albifundus, 8 h	29	5
O. quercus, 8 h	11	0
C. albifundus, 24 h	18	2
O. quercus, 24 h	9	0
C. albifundus, 48 h	9	0
O. quercus, 48 h	8	0
C. albifundus, 124 h	5	0
O. quercus, 124 h	10	0
C. albifundus, 196 h	6	0
O. quercus, 196 h	5	0
C. albifundus, 346 h	3	0
O. quercus, 346 h	7	0
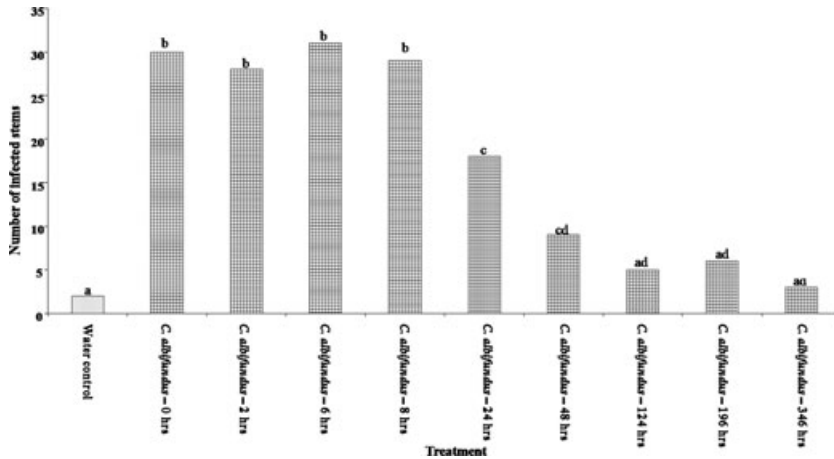
Fig. 2. Number of horizontal stem cuts (wounds) infected with *Ceratocystis albifundus* assessed visually for each treatment. Different letters above bars indicate treatment results significantly different from each other at a 5% significance level. Figure shows combined data for both experiments (2 × 20 trees).

was observed in the 6-h treatment where *C. albifundus* was inoculated alone, with 31 of the 40 trees (77.5%) showing infection. Infection of stems inoculated with *C. albifundus* at the time of wounding, 3, 6 and 8 h after wounding was significantly higher than that of all other treatments ($p < 0.0001$ to $p = 0.0344$). The levels of infection of stems that were inoculated with *C. albifundus* at 124, 196 and 346 h after wounding were not significantly different from the control treatments for this experiment, nor did they differ significantly from each other (Fig. 2).

3.2.2 Pre-inoculation with Ophiostoma quercus

Pre-inoculation of stems with *O. quercus* had a significant influence on the infection success of *C. albifundus*. Stems inoculated with *O. quercus* and *C. albifundus* concurrently at the time of wounding exhibited the highest levels of infection and differed significantly from all other treatments. All other treatments inoculated with *O. quercus* at the time of wounding, and *C. albifundus* at different time intervals thereafter, were significantly different from the control treatment (Fig. 3). Pre-inoculation with *O. quercus* at time of wounding and with *C. albifundus* at 196 h was not significantly different from the control treatments. All other treatments differed significantly from the control treatments.

3.2.3 Influence of stem diameter on infection

The diameter of the stems, and thus surface area of the wound exposed to infection, had a significant influence on the infection success of *C. albifundus* ($p = 0.0002$). The analysis of maximum likelihood estimates produced an estimate value of 0.0617 for tree diameter as a predictor variable with 1 degree of freedom (Fig. 4). This indicated that with every 10 mm increase in stem diameter, the probability of infection by *C. albifundus* increased by 0.62%.
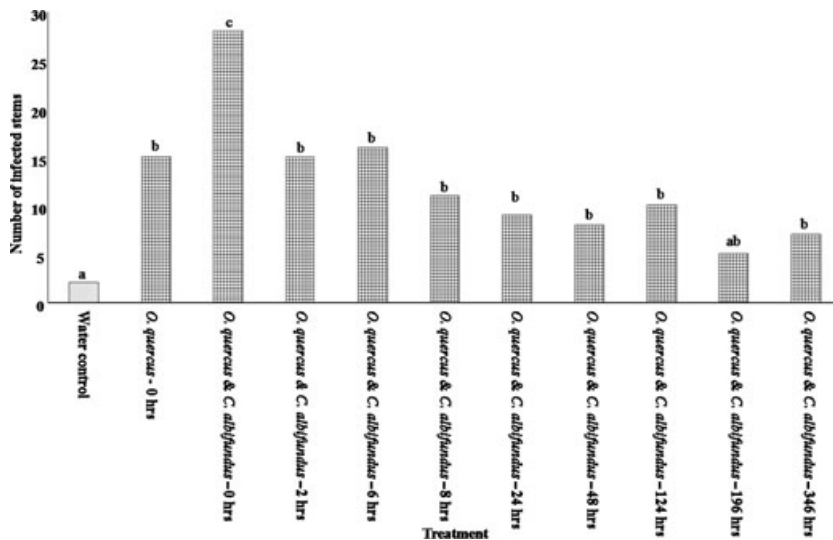
Fig. 3. Number of horizontal stem cuts (wounds) infected with *Ceratocystis albifundus* assessed visually for each treatment. Different letters above bars indicate treatment results significantly different from each other at a 5% significance level. Figure shows combined data for both experiments (2 × 20 trees).
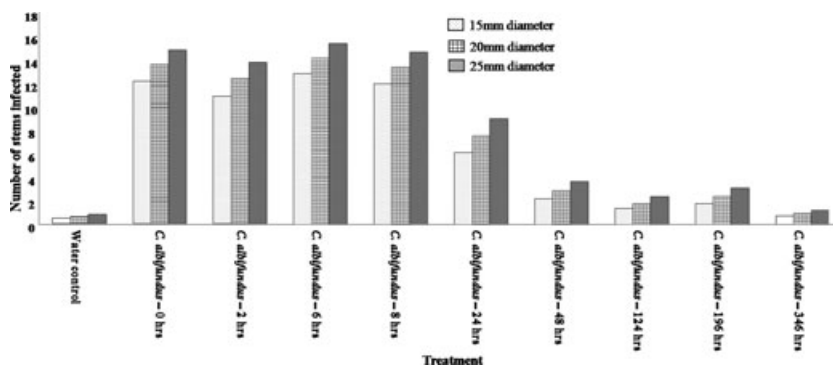

Fig. 4. The effect of diameter on infection success of *Ceratocystis albifundus* on horizontal stem cuts (wounds) obtained from analysis for specific chosen combinations of the predictor values (treatment, replicates and tree diameter) at a 5% significance level obtaining a percentage concordance of 80.5% and Wald chi-squared value of 13.67 and p = 0.0002. Figure shows combined data for both experiments (2 × 20 trees).

4 Discussion

Results of this study showed clearly that infection of wounds on *A. mearnsii* by *C. albifundus* is strongly influenced by the condition of the wounds. Wounds became less susceptible to infection over time and after 24 h they had a very low level of susceptibility. Likewise, pre-inoculation of wounds with *O. quercus* significantly decreased the ability of *C. albifundus* to infect wounds. Potential of infection also increased as the size of the stems increased.

Loss of susceptibility to infection by *C. albifundus* with increasing wound age was not surprising. Similar results have been found in many studies of pathogens, including *C. fimbriata*, *Leucostoma cincta* and *Leucostoma persoonii*, which infect woody plants (BOSTOCK and MIDDLETON 1987; BIGGS 1989; TEVIOTDALE and HARPER 1991). Specifically in the case of *Ceratocystis*, the results of this study are similar to those on *C. fagacearum* where wounds on oak trees are typically found not to be susceptible to infection after 24 h (MORRIS et al. 1955; KUNTZ and DRAKE 1957; GIBBS 1980).

The present study did not investigate the possible reasons why wound susceptibility decreases with time. Previous studies attributed loss of susceptibility of wounds to *C. fimbriata* on Almond trees to the fact that the thin film of moisture required on the wound for the germination of spores disappears with time. Other studies have attributed the increase in resistance of wounds to infection with time to the accumulation of an impervious lingo-suberized layer (BIGGS 1984, 1985a,b, 1989; OVEN et al. 1999; STOBBE et al. 2002; ROBINSON et al. 2004). Numerous authors have stated that this layer is responsible for preventing moisture loss from the wound as well as preventing fungal infection (BIGGS 1992; WOODWARD 1992). The formation of a necrophylactic periderm has also been reported as a wound response that could prevent fungal infection (BIGGS 1985a). It is, however, unlikely that the formation of the necrophylactic periderm is the primary defence against fungal infection as this layer only forms after the impervious lingo-suberized layer has formed (WOODWARD 1992; OVEN and TORELLI 1994; OVEN et al. 1999).

Pre-inoculation of wounds with *O. quercus* had a significant effect on infection with *C. albifundus*. Thus, colonization of fresh wounds by *O. quercus* approximately 2 h prior to infection by *C. albifundus* could reduce the chance of infection by the pathogen significantly. In a similar study, GIBBS (1980) showed that where *O. piceae* was inoculated onto fresh wounds approximately 24 h prior to *C. fagacearum*, the chance of infection by the pathogen was greatly reduced. *Ophiostoma quercus* was selected for the present study because it is commonly found on wounds on *A. mearnsii* trees (R. N. Heath, unpublished data) and has not been reported to decrease the value or structural integrity of the timber. Damage caused by sapstain fungi such as *O. quercus* have been reported to be cosmetic (SEIFERT 1993). Another consideration was that *Ophiostoma* spp. have a biology similar to that of *Ceratocystis* spp. and are also carried to wounds by insects (BRASIER 1990; KIRISITS 2004). GIBBS (1980) suggested that colonization of wounds by *O. piceae* may be common in nature, reducing the natural occurrence of oak wilt. The same situation seems to be possible in South Africa in the case of *C. albifundus* infection and the colonization of wounds with *O. quercus*.

The diameter of the stems inoculated in this study had a significant effect on the infection success of *C. albifundus*. As the stem diameter increased, and thus the size of the exposed wound (horizontal cut) surface area, the probability of infection increased. This could be due to a more rapid drying of the smaller stems compared with the larger stems. The fact that stem diameter has an influence on the infection success of *C. albifundus* makes the results of this study applicable to the prescribed pruning protocols for *A. mearnsii* trees in South Africa. Current silvicultural prescriptions for the management of *A. mearnsii* suggest that pruning should be undertaken on stems 2–4 m tall (DUNLOP and GOODRICKE 2000). If the age at which pruning conducted is reduced, the diameter of the stems would be smaller and infection be less likely.

Successful management of plantation forest diseases relies on a combination of selection and breeding and sound silvicultural practices. The results obtained in this study could assist forestry companies in southern and eastern Africa in formulating silvicultural regimes that could reduce the impact of *C. albifundus* infection and mortality after pruning. The results obtained in this study could also be applied to better management of numerous tree growing industries. Furthermore, this study also raises the possibility of using *O. quercus* to reduce the infection of recently cut stems by *C. albifundus*. Although this study supports findings obtained in previous studies using other *Ceratocystis* spp., this is the first

study of its kind on the African continent and the first to provide possible management prescriptions for the fully regulated forestry management system followed in numerous countries world-wide.

Acknowledgements

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