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Morphological, cultural and pathogenic characteristics of *Coniothyrium zuluense* isolates from different plantation regions in South Africa

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Abstract

Coniothyrium canker caused by *Coniothyrium zuluense*, is a serious stem canker disease of *Eucalyptus* species in sub-tropical regions of South Africa. This disease is typified by necrotic bark lesions that coalesce to form large kino-impregnated cankers along the stems of trees. The strategy currently used to manage Coniothyrium canker in plantations is to deploy *Eucalyptus* species or clones that are resistant to the disease. Considerable success has already been achieved in this regard, but the long-term durability of resistance is of concern. Thus, forest managers are interested in the genetic diversity of the pathogen and its potential to overcome disease resistance in planting stock. In this study, 344 isolates of *C. zuluense* from different plantation regions in South Africa were compared on the basis of colony colour, conidial morphology, growth characteristics on agar and pathogenicity to a susceptible *E. grandis* clone. Conidia of all *C. zuluense* isolates measured were similar in size and shape. The fungus is slow growing in culture, which is indicative of its apparent biotrophic habit, with optimum growth observed at 30 °C. Isolates of *C. zuluense* displayed considerable variation in colony colour and pathogenicity in inoculation trials. Variation in morphology and pathogenicity amongst isolates suggests that *C. zuluense* has been present in South Africa for an extended period of time, or that it is changing rapidly due to strong directional selection pressures.

Key words: *Eucalyptus*, stem canker

Introduction

The forestry industry in South Africa relies almost exclusively on the planting of exotic species of *Pinus* and *Eucalyptus*. These genera are planted in approximately equal proportions and about 1.5 million ha of land is currently afforested [1]. Planting of *Eucalyptus* clones is a common practice and results in large, genetically uniform stands. These plantations are at risk from damage due to pests and diseases [2, 3]. The current means of reducing losses due to disease is by planting disease resistant species and clones of *Eucalyptus* [4]. Strategies to ensure that large numbers of disease resistant clones are planted and that a high degree of genetic diversity is maintained in clonal plantations, have, therefore, been implemented [2].

Coniothyrium zuluense Wingfield, Crous & Coutinho is a serious stem canker pathogen of *Eucalyptus* trees in South Africa [5–7]. Disease symptoms were first noted on a single clone of *Eucalyptus grandis* Hill ex Maid. at Honey Farm plantation in the Zululand region of the KwaZulu-Natal province. Since its discovery in 1988, various *Eucalyptus* species, clones, and hybrids have displayed symptoms of infection. The earliest symptoms of infection by *C. zuluense* on trees are small, discrete, necrotic lesions on the young, green bark. These lesions coalesce to form large necrotic cankers on the stems that exude copius amounts of kino. Epicormic shoots are commonly produced from stems of cankered trees, indicative of partial girdling. In severely infected clones, the tops of trees die, due to the girdling effect of the cankers resulting in loss of height growth [5–7].

Coniothyrium zuluense, and the canker disease associated with it, was first observed in 1988 [7].

Currently, no information is available concerning the population characteristics of the pathogen. Knowledge regarding fungal population structures is important to programmes aimed at reducing the impact of disease, as this must affect the likely durability of disease resistant clones. The aim of this study was, therefore, to consider variability in morphological, cultural and pathogenicity characteristics, amongst a large collection of *C. zuluense* isolates.

Materials and methods

Isolates and cultures

A survey of *C. zuluense* in nine *Eucalyptus* growing regions of KwaZulu-Natal was conducted during 1995 and 1996. Pieces of bark, showing characteristic disease symptoms, were collected from each of 172 trees sampled. These bark samples were incubated at 30 °C in Petri dishes containing moist filter paper, to induce production of pycnidia. Conidial masses from 172 pycnidia were then washed onto the surface of 2% water agar (20 g agar (Biolab); 1 l distilled H_2O) in Petri dishes and spread across the medium surface. Plates were incubated for 19–24 hours at 30 °C. Single germinating conidia were then lifted from each plate with the aid of a dissecting microscope and sterile syringe needle. The germinating conidia were transferred to sterile 9-cm diameter Petri dishes containing 15 ml of 2.5% Potato Dextrose Yeast extract Agar (PDYA) [24 g Potato Dextrose extract (Merck); 1 g Yeast extract (Merck); 1 g Glucose (Merck); 40 g agar (Merck); 1 l distilled H_2O] and incubated at 30 °C. Isolates produced in this manner were stored on PDA slants in screw-capped tubes at 4 °C.

Colony and conidial morphology

A total of 344 single conidial isolates of *C. zuluense* representing two from each tree sampled were transferred to 4% w/v Potato Dextrose Agar (Difco) plates in order to observe colony colour and growth characteristics in culture. Colony colour was rated using mycological colour charts of Rayner [8]. Thirty conidia from 10 randomly selected pycnidia (each from different trees) were measured for each of the nine plantation regions sampled. Spore morphology was determined by measuring the length and width of thirty conidia per pycnidium.

Growth studies

Growth rates and temperature requirements were determined for each of the 344 *C. zuluense* isolates collected. Isolates were transferred to PDA (5 mm diameter mycelial plugs) with three plates for each temperature and isolate to be tested. Plates were incubated in the dark at temperatures ranging from 10–35 °C, at 5°C intervals for 30 days. Growth of isolates was determined by measuring colony diameter.

Pathogenicity tests

Pathogenicity tests were conducted on 6-month-old trees of an *E. grandis* clone (ZG 14) that is known to be highly susceptible to Coniothyrium canker under natural conditions in KwaZulu-Natal. In the first trial, twenty trees were inoculated for each of the 344 different single conidial isolates. Inoculations were done by removing a 10-mm diameter disc of bark from the trees at breast height, and replacing this with a PDA disc of agar bearing the fungus, or an uninoculated disc in the case of the 20 controls. Inoculation wounds were covered with masking tape to prevent desiccation of the inoculum. Lesion lengths were measured 6 weeks after inoculation. This experiment was repeated in the second trial using all pathogenic isolates selected from the first trial and 20 randomly selected non-pathogenic isolates. Assessment of relative pathogenicity of isolates was achieved by grouping the lesion lengths produced by the isolates into six classes (10 mm; 11–20 mm; 21–30 mm; 31–40 mm; 41–50 mm; 51+ mm). An isolate causing a lesion length of 10 mm was non-pathogenic based on the fact that the cork borer had a diameter of 10 mm and any further increase in lesion length (above 10 mm) was attributed to a pathogenic response. All results were analysed by means of a two factorial analysis of variance, and for significance of differences using Tukey's procedure for the comparison of means at a 5% confidence level.

Results

Colony and conidial morphology

Isolates of *C. zuluense* varied considerably in colony colour (Figure 1). Surface colony colour of all 344 isolates considered in this study, varied from olive grey (V23″″b), isabella (19″i) greenish glaucous (33″″f) to a greyish olive (21″″) colour. Colonies viewed from

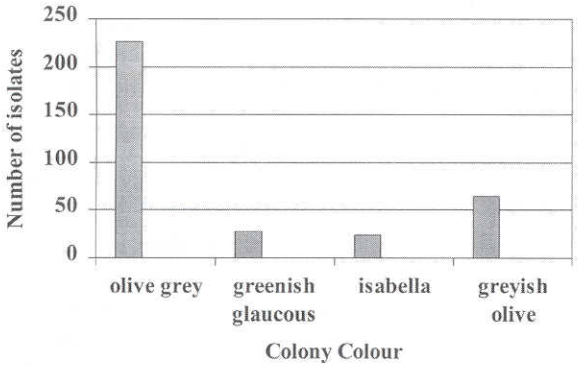

Figure 1. Differences in surface colony colour of 344 *Coniothyrium zuluense* isolates grown on PDA in Petri dishes for 30 days at 30 °C. Bars represent the number of *C. zuluense* isolates in each colour class, and colours are those of Rayner [8].

Table 1. Differences in conidial size of isolates of *Coniothyrium zuluense*, collected from nine different plantation regions in KwaZulu-Natal

Plantation*	No. of isolates	Width and length of conidia (μm)**
Aboyni	11	2–3 (2.5) × 3.5–5 (4.5)
Fairbreeze	16	2–3.5 (2.5) × 3.5–5 (4)
Futululu	70	2.5–3.5 (3) × 3.5–4.5 (4)
Honey Farm	50	2.5–3 (2.5) × 3–5 (4)
Palm Ridge	55	2.5–3 (3) × 4–5.5 (5)
Shire	10	2.5–3 (2.5) × 3.5–6 (4.5)
Teranera	28	2.5–3.5 (3) × 4–4.5 (4)
Teza	81	2.5–3.5 (3) × 4.5
Trust	23	2.5–3 (2.5) × 4–4.5 (4)

*Plantations in Zululand, KwaZulu-Natal Province, South Africa.

**Each size measurement represents the range of conidial widths (averages in parentheses) and lengths, computed from an average of 3 conidia derived from 1 randomly collected pycnidium. A total of 30 conidia from 10 pycnidia were examined for each isolate.

below were either black or rust coloured with white margins. There was no predominant colony colour for isolates from any specific region. Conidia of all isolates measured were similar in size and shape. Conidia, from lesions on trees from all nine regions sampled, were pale brown in colour, thick-walled, smooth to verruculose and broadly ellipsoidal. The apices of these conidia were obtuse and the bases sub-truncate to bluntly rounded (Table 1).

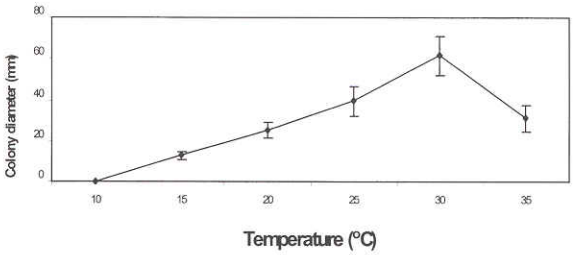

Figure 2. Ranges of average colony diameters for 344 single conidial isolates of *Coniothyrium zuluense* after incubation for 30 days at different temperatures between 10–35 °C. Vertical bars represent the standard error.

Growth studies

Coniothyrium zuluense is slow growing in culture, which is indicative of its apparent biotrophic habit [7]. The fungus failed to grow at 10 °C (Figure 2). At 15 °C the mean colony diameter for all isolates was 13 mm. Best growth was observed at 30 °C (61 mm diameter) followed by 25 °C (39 mm diameter), 35 °C (31 mm diameter) and 20 °C (25 mm diameter), respectively (Figure 2). All isolates displayed the same temperature optima, minima and maxima and grew at the same rate at each temperature.

Pathogenicity tests

Isolates of *C. zuluense* differed markedly in their relative pathogenicity and virulence (Figure 3). Lesion lengths obtained from pathogenicity trials were divided into 6 classes. Of the 344 isolates tested, 251 (73%) were non-pathogenic. The remaining 93 (27%) isolates produced lesions varying between 15–61 mm long. In the second trial, pathogenic isolates produced lesions comparable to those produced in the first trial while non-pathogenic isolates still produced no lesions.

Control inoculations developed no symptoms and inoculation points were covered with callus tissue. The most pathogenic isolates of *C. zuluense* gave rise to a distinct swelling of the stem tissue around the inoculation site after 6 weeks. Tissue surrounding the inoculation points was necrotic. These symptoms were similar to those associated with natural infections.

Discussion

Morphological and cultural characteristics of *C. zuluense* presented in this study were similar to those

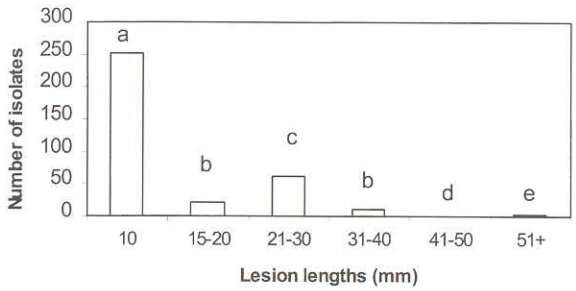

Figure 3. Ranges of lesion lengths associated with inoculation of 344 *Coniothyrium zuluense* isolates. Bars represent the number of *C. zuluense* isolates within each of six different lesion ranges (10 mm; 15–20 mm; 21–30 mm; 31–40 mm; 41–50 mm; 51+ mm) produced after inoculation of a susceptible *Eucalyptus grandis* clone (ZG 14). Each of the 344 isolates was inoculated onto the stems of 20 trees and means computed. Different letters above the bars represent significant differences ($P = 0.05$) according to Tukey's procedure for comparison of means (CV = 24.6%).

published by Wingfield et al. [7]. Data regarding spore morphology and growth characteristics were also consistent with those previously published [5, 7]. In a study by Taylor and Crous [9], conidial colour and dimensions of *Coniothyrium leucospermi* were found to vary *in vivo* and *in vitro* and under different light regimes. In the current study, conidia were only examined from pycnidia occurring on host tissue (i.e., *in vivo*). Results of colony colour and pathogenicity studies showed evidence of considerable variation in isolates of *C. zuluense*.

There was a wide diversity in colony colour for *C. zuluense* isolates. The majority of isolates (66%) were olive grey (V23''''b) which is consistent with results of Wingfield et al. [7]. The remaining (34%) isolates had colony colours varying between isabella (19''i), greenish glaucous (33'''f) and greyish olive (21''''). It is of interest that there were no consistent patterns of colony colour linked to the origin of isolates or their ability to cause disease.

A considerable degree of variation was observed in the virulence of the isolates tested in this study. It was particularly interesting that 73% of all *C. zuluense* isolates were not able to cause any obvious disease on the susceptible *E. grandis* clone. A relatively small number (27%) of isolates were able to cause necrotic lesions. This lack of pathogenicity is not due to a loss of virulence because all isolates were the same age when inoculations were made. In addition, isolates used in the original description [7] were included in this study and remained pathogenic despite being stored at 4 °C for 5 years. In a previous study, non-pathogenic isolates, isolates of intermediate virulence and isolates with high levels of virulence were subjected to DNA sequencing [10]. Sequence data from the ITS 1, ITS 2 and the 5.8S RNA gene of these isolates clearly showed all represent *C. zuluense*.

No sexual state has been found for *C. zuluense*, despite the fact that considerable effort has been made to find a teleomorph [5, 7]. The assumption is, therefore, made that the fungus exists predominantly in an asexual form and would thus have a limited capacity to adapt. Sexual reproduction in a population leads to recombinations that could subsequently result in a rapid increase in virulence. The presence of spermatogonia in some cultures, however, suggests that a sexual state may occur, but has yet to be discovered [5, 7]. If it is present, it seems unlikely to occur on *Eucalyptus*, which have been very carefully examined, but it could be present on a native South African plant species. The sudden appearance of the disease in South Africa, as well as diversity in colony morphology and virulence, favours the hypothesis that *C. zuluense* originated from native plants in South Africa.

Eucalyptus clones that are highly susceptible to *C. zuluense* fail to grow effectively, which leads to significant losses to the South African forestry industry. A large number of clones that are currently available for planting, are susceptible [5, 6, 7]. There is also evidence to suggest that clones previously known to be resistant, are beginning to show signs of infection [5, 7]. This indicates that the pathogenicity of *C. zuluense* is changing. In plantation programmes, regular deployment of new resistant clones and hybrids, imposes strong directional selection on pathogen populations. This is especially true for asexually reproducing fungi because they must constantly adapt to changes in their environment to survive [4]. Such pressure might have led to the pathogenic variation in *C. zuluense*.

Eucalyptus species are being propagated extensively outside Australia, where most of these species are native, with about 8 million hectares currently grown in plantations [7]. *C. zuluense* is a potential threat to these plantations, particularly in areas with a tropical or sub-tropical climate. Dedicated efforts are, therefore, needed to avoid the spread of this fungus to other countries. In this regard, it is important to note that control strategies can only be successful if populations rather than individuals are targeted [10]. Future research will, therefore, focus on understanding the population structure of *C. zuluense* in South Africa. Such information will be valuable in understanding the evolution of the population in response to the deployment of new disease resistant clones.

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