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Genetic diversity of *Cryphonectria cubensis* isolates in South Africa

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Cryphonectria canker caused by *Cryphonectria cubensis* is one of the most destructive diseases of *Eucalyptus* plantations in South Africa. To implement a meaningful management of plantation diseases, it is important to have an understanding of the population diversity of the pathogen. In this study, trees were surveyed to determine whether *C. cubensis* reproduces sexually in South Africa. The diversity of the South African *C. cubensis* population was assessed based on vegetative compatibility tests. Field inoculations were used to determine whether VC groups correlated with virulence. Only pycnidia were found on cankered trees, indicating that sexual reproduction does not occur. Only 23 VC groups were found amongst 100 isolates each collected from single diseased trees. A low degree of genetic diversity also indicated that sexual reproduction is absent or rare in the South African *C. cubensis* population. Inoculation studies revealed that isolates belonging to different VC groups differ significantly in their ability to cause lesions. The low level of genetic diversity enhances opportunities to capitalise on hypovirulence to reduce the impact of the pathogen in the future. It also supports the view that the fungus was recently introduced into South Africa.

INTRODUCTION

Cryphonectria canker caused by *Cryphonectria cubensis* is one of the most destructive fungal pathogens of *Eucalyptus* trees in plantations. The disease is favoured by high rainfall and temperatures above 23 °C, and has limited the establishment of eucalypts where climatic conditions favour disease development (Hodges, Geary & Cordell 1979, Alfenas, Hubbes & Couto 1982, Sharma, Mohanan & Florence 1985, Florence, Sharma & Mohanan 1986). Cryphonectria canker is, thus, considered to be a major impediment to plantation forestry in tropical and sub-tropical areas of the world (Boerboom & Maas 1970, Hodges & Reis 1974, Hodges *et al.* 1979, Hodges 1980, Gibson 1981, Florence *et al.* 1986).

Cryphonectria canker was first discovered in South Africa in 1989 (Wingfield, Swart & Abear 1989), and it has subsequently become important to identify effective management strategies for the disease. Various strategies can be implemented to manage losses due to this disease. These can include the deployment of disease tolerant clones and hybrids of *Eucalyptus*, which is practised in many countries and is considered as effective (Alfenas, Jeng & Hubbes 1983, Wingfield 1990). Chemical control is also an option but due to the low economic return of *Eucalyptus*, it is not financially viable. Another prospect for reducing the impact of Cryphonectria canker is by using hypovirulence linked to double stranded (ds) RNA (Day *et al.* 1977).

The recent discovery of dsRNA-associated hypovirulence in *C. cubensis* populations (van der Westhuizen *et al.* 1994, van Zyl *et al.* 1999) has raised interest in the potential of using mycoviruses to reduce the impact of *C. cubensis*. It is known that within natural populations of the chestnut blight pathogen *Cryphonectria parasitica*, the transfer of dsRNA particles is favoured when isolates belong to the same vegetative compatibility group (VCG) (Anagnostakis 1977, Anagnostakis & Day 1979). This is because hyphal anastomosis occurs between isolates of similar VC group, which favours the transmission of dsRNA (Choi & Nuss 1992).

The success of biological control through hypovirulence depends on the ease of spread of dsRNA, through a population with similar VC groups (Anagnostakis & Day 1979, Anagnostakis 1982, Kuhlman *et al.* 1984, Liu & Milgroom 1996). For example biological control of *C. parasitica* through hypovirulence in Europe was effective due to the low number of VC groups (Grente & Berthelay-Sauret 1978, Heiniger & Rigling 1994). This is in contrast to the situation in North America where the *C. parasitica* population is genetically diverse, and where hypovirulence has not been effective (Anagnostakis 1982, 1987).

Almost nothing is known of the biology of *C. cubensis* in South Africa. Although it is known that perithecia occur rarely (Wingfield, unpubl.), this has not been verified experimentally or through systematic surveys. It is widely accepted that the pathogen was introduced into the country and that it was

absent, or at least not obvious until 1988. A reasonable hypothesis would be that the fungus is represented by a genetically uniform population. This is in contrast to the situation in Brazil, Venezuela and Indonesia where *C. cubensis* is represented by large numbers of VC groups (van Heerden *et al.* 1997, van Zyl *et al.* 1988). It is also known that perithecia of the fungus are the predominant structures on the cankers in those countries (Wingfield, unpubl.).

The objectives of this study were: (1) to determine the genetic diversity of *C. cubensis* in South Africa based on a collection of isolates from an area where the disease is prevalent; (2) to test experimentally whether sexual recombination in *C. cubensis* can be expressed in the laboratory; (3) to evaluate relative pathogenicity and thus to determine whether there are differences in the virulence of isolates representing different VC groups. The intention of this study was further to provide background knowledge that might be useful in implementing a biological control programme based on hypovirulence.

MATERIALS AND METHODS

Collection and maintenance of samples

Cryphonectria cubensis isolates were randomly collected from *Eucalyptus grandis* plantations in KwaZulu-Natal, South Africa in January 1997. One hundred bark samples showing characteristic symptoms of Cryphonectria canker were collected from individual trees in these plantations. The sampling strategy also included a survey of the diseased trees to assess whether the sexual state of *C. cubensis* was present on cankers.

To induce the production of spores, bark samples were incubated in Petri dishes lined with wet filter paper, for one week at 25–27 °C. Spore masses were removed from the tips of the fruiting bodies and transferred to sterile 9 cm diam. Petri dishes containing 2% Malt extract agar (MEA) (20 g Biolab malt extract, 20 g Biolab agar, 1 l distilled water). All the spore masses were examined under a light microscope to ascertain whether they represented ascospores or conidia. Once the cultures were pure, they were transferred to McCartney bottles containing sterile water, incubated, and after sufficient growth, stored at 4 °. All isolates are stored in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Vegetative compatibility tests

The genetic structure of the South African *C. cubensis* population was assessed in two ways. Firstly, VC groups were identified on oatmeal agar. Secondly, a medium described by Powell (1995), using a pH indicator (Bromcresol green) was used to confirm the total number of VC groups that were identified using the first technique.

VC tests on oatmeal agar

Oatmeal agar was prepared by adding 100 g oatmeal to one litre distilled water. This was steamed for two to three hours while being stirred occasionally. It was then sieved through cheesecloth, and autoclaved with the addition of 30 g l^{-1} agar (Biolab). The number of VC groups for each tree was first identified by using spore masses from six different pycnidia per bark sample. Tests to determine VC groups were made by transferring mycelial plugs from the edges of young, actively growing cultures (less than seven-days-old) onto oatmeal agar, in sterile 9 cm diam. Petri dishes. This was done only for two bark samples, because previous studies have shown that single cankers on trees are colonised by only one VC group (Van Heerden *et al.* 1997). These results influenced the sampling strategy in that only one isolate was used per bark piece collected. Thereafter, the total number of VC groups was determined by taking the isolates from all the sampling locations and pairing them against each other in all possible combinations. In a part of this study, bioassay dishes (20 cm square, Nunc Industries) were used instead of 9 cm diam. Petri dishes. This method was also used to assess the total number of VC groups present. All pairings were repeated. Plates were sealed with parafilm and incubated at 25–27° in the dark for 5–7 d.

VC groups were identified using the method described by Anagnostakis (1977). At the end of the incubation period, isolates that were vegetatively compatible had merged, forming a confluent mycelium. Incompatible genotypes had grown to a meeting point, but remained separated by a barrage reaction, formed along the line of contact.

VC tests with Bromcresol green

In these tests, the medium of Powell (1995) was used where the Potato dextrose agar (PDA) was replaced with MEA. This medium thus contained 24 g malt extract, 2 g yeast extract, 200 mg tannic acid, 100 mg methionine, 2 mg biotin, 2 mg thiamine and 20 g l^{-1} agar and the pH indicator bromcresol green (50 mg l^{-1}) was added to enhance the visualisation of the incompatible reactions. To confirm the total number of VC groups identified on the oatmeal agar, VC tests were done by transferring cubes (4 mm^3) from the edges of young cultures (less than 7 d old) to 9 cm diam. Petri dishes containing 15 ml of this medium. Two cubes were placed in close contact (1 mm apart), 5 mm from the edge of the Petri dish, with the mycelium side down. Six pairs of cubes were placed on one Petri dish, sealed with parafilm and incubated in the dark at 25° for 7 d. Compatible reactions had a confluent lawn of mycelium with no reaction at the contact zone. Incompatible reactions were characterised by a dark coloured reaction when viewed from the bottom of the Petri dish, at a slight angle.

Population analysis

Each VC group was assigned a number (SA1–SA23). The most common VC groups in the *C. cubensis* population were determined by identifying the number of representatives for each VC group. The genotypic diversity (G) of the population was determined as proposed by Stoddart & Taylor (1988). Genotypic diversity (G), was estimated as: $G = 1/\Sigma pi^2$ where pi is the observed frequency of the genotype. The maximum percentage of genotypic diversity, for the total set of isolates, was also determined by dividing the genotypic diversity by the total sample size (G/N).

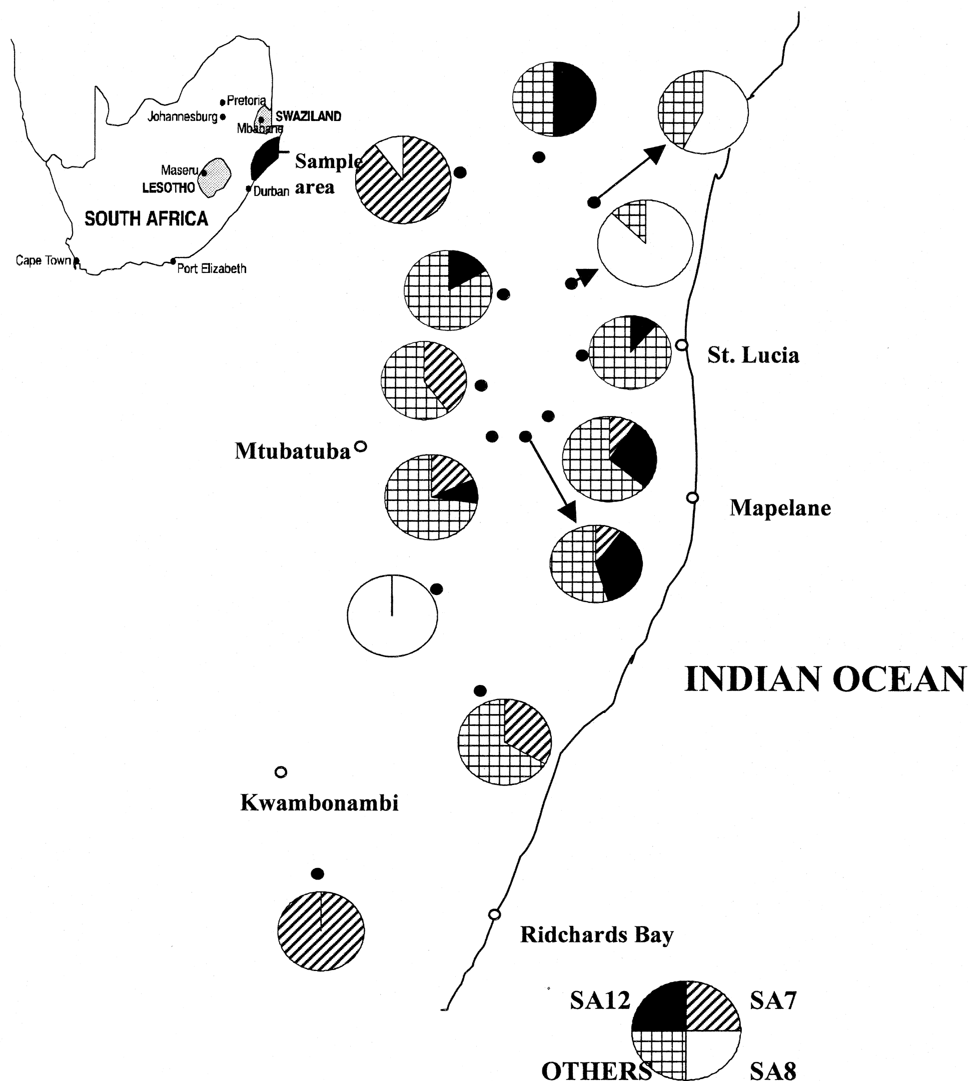

Fig. 1. Distribution of the three most common VC groups in the South African *Cryphonectria cubensis* population sampling area. Each circle indicates the different sampling areas from which *C. cubensis* isolates were obtained. □, % isolates of VC group SA8; ■, % isolates of VC group SA12; ▨, % isolates of VC group SA7; ▦, % isolates not belonging to the three most common VC groups.

Assessment of isolate sexuality

One *C. cubensis* isolate representing each VC group known in South Africa was selected for this study. In addition, five Colombian *C. cubensis* isolates obtained from a mass of ascospores from a single perithecium, as well as five single ascospore cultures were included. One freshly cut stem section (1 cm diam and 15 cm in length) for each isolate, from the *E. grandis* clone (TAG5), known to be moderately tolerant to *C. cubensis* infection, was used for this test. Prior to inoculation, the stem pieces were surface sterilised by wiping the surfaces with 70% ethanol. The ends were then coated with melted paraffin wax to reduce desiccation. The bark was removed with a 5 mm diam. cork borer, and the wounds were inoculated under laboratory conditions with discs of agar taken from the actively growing margin of 7 d old MEA cultures. The wounds were sealed with parafilm to prevent drying and contamination. Five stem sections were inoculated with a sterile MEA disc as a control treatment. The stem pieces were placed in a plastic container lined with wet filter paper, incubated at 25–28° and regularly inspected for the presence of fruiting structures. The spores produced were examined microscopically to ascertain whether they represented ascospores or conidia.

Field inoculations

A field inoculation trial was conducted in January 1998 to test for differences in pathogenicity amongst *C. cubensis* isolates represented by the VC groups. Three year old coppice shoots of the *E. grandis* clone ZG14, which is known to be highly susceptible to *C. cubensis* infection, were used for the inoculation study. The trees were grown in KwaZulu-Natal, South Africa, and maintained using standard silvicultural procedures. *Cryphonectria cubensis* isolates representing a wide range of different VC groups in South Africa were used as inoculum. These included the 23 different South African VC groups as well as, five isolates that belong to the same VC group which originated from the same location. Isolates for inoculation were grown on 2% MEA plates. Trees were inoculated approximately 140 cm from the ground, by

Table 1. Genotypic diversity in the South African population of *Cryphonectria cubensis*.

Genotype	Frequency
11	1
2	2
2	3
1	4
2	6
2	7
1	14
1	15
1	20

Number of genotypes: 23.
Total sample size (N): 100.
Genotypic diversity (G): 9.578
Maximum percentage of genotypic diversity: (G/N): 0.095%.

removing a cambial disc from the main stem with a 15 mm diam. cork borer. Similar sized discs from actively growing MEA cultures of each isolate were placed in each wound, and the wounds were wrapped with masking tape to reduce desiccation. Sterile MEA discs were used in the control treatment. Ten trees were inoculated for each isolate and the control. After 11 weeks the masking tape was removed from the point of inoculation and lesion lengths were measured.

Differences in lesion length among different VC groups of *C. cubensis*, and differences in lesion lengths among isolates from the same VC group, were analysed using separate one-way ANOVAs. Since it was irrelevant to determine differences amongst isolates, post-hoc pairwise comparisons were not performed. Data were log transformed to meet the assumptions of ANOVA.

RESULTS

Vegetative compatibility tests and population analysis

The only fruiting bodies observed on the bark samples from cankers were pycnidia, which represent the asexual state of *C. cubensis*. Thus, the isolates used for the VC testing were all obtained from conidia. Results indicated that, for the bark samples investigated, each was colonised by a single VC group of *C. cubensis*. Based on the premise that trees were infected with a single VC group, a single isolate from each tree was then used to determine the number of VC groups in the Zululand plantation area. The total number of VC groups for the area was 23. Both of the VC testing methods gave reliable results with a good resolution. The method described by Powell (1995) confirmed the identification of the VC groups determined using the oatmeal agar method.

Three VC groups (SA7, SA8 and SA12) made up 49% of the total sample. Vegetative compatibility group SA7 occurred most commonly in all trees and 20 of the 100 isolates represented this VC group. SA8 was represented by 14 isolates and SA12 by 15 isolates (Fig. 1). The genotypic diversity (G) for the total number of genotypes (VC groups) was estimated to be 9.6. The maximum percentage of genotypic diversity (G/N) was 0.095% (Table 1).

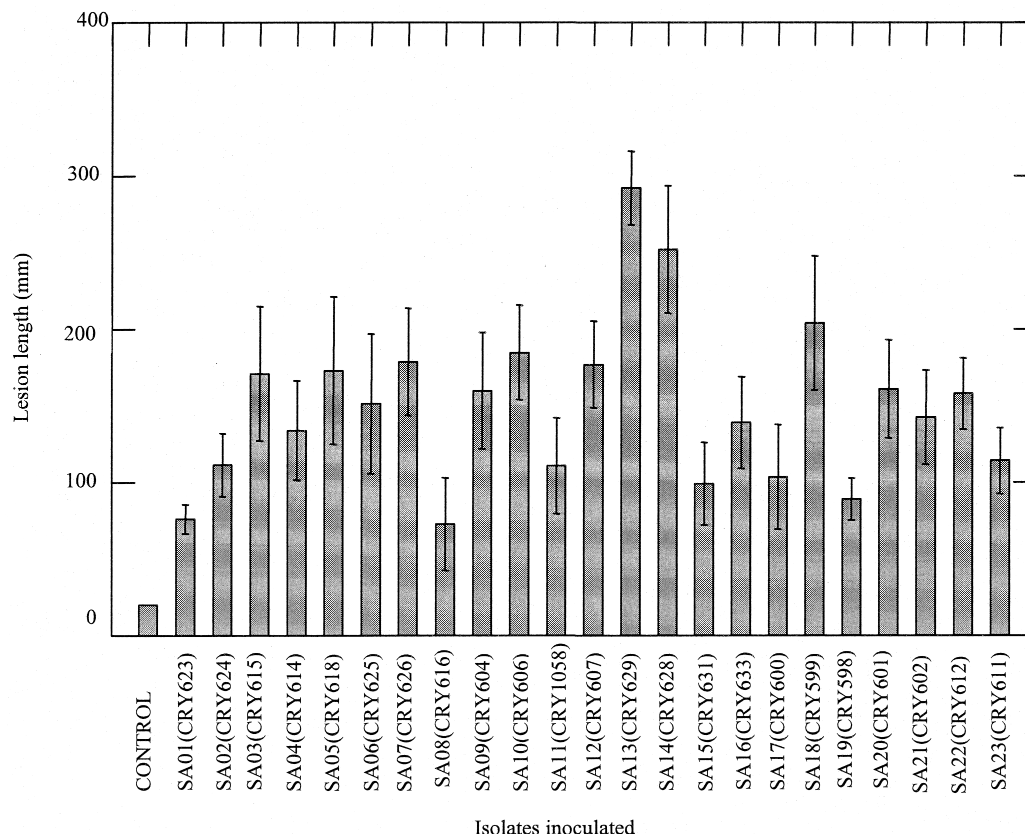

Fig. 2. Mean lesion length after inoculation with 23 isolates representing the 23 VC groups of *Cryphonectria cubensis* in South Africa on the *Eucalyptus grandis* clone (ZG14). Bars represent means (±SEM) lesion lengths for each isolate. Lesion lengths differ significantly from each other ($F = 3.99$; D.F. = 23; $P < 0.001$).

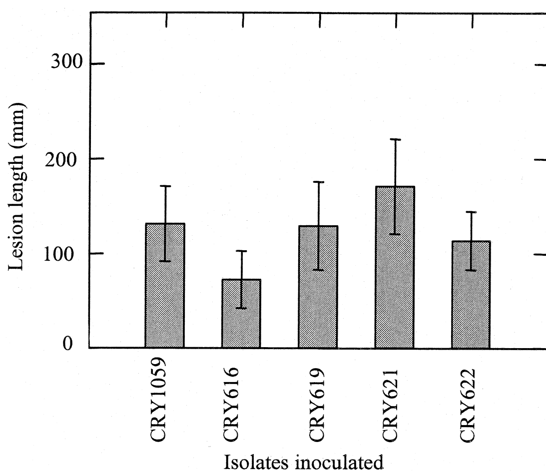

Fig. 3. Mean lesion length after inoculation with four different isolates representing the same VC group (SA 8) of *Cryphonectria cubensis* on the *Eucalyptus grandis* clone (ZG14). Bars represent mean ($\pm$SEM) lesion lengths for each isolate. Lesion lengths did not differ significantly from each other ($F = 0.88$; D.F. $= 4$; $P > 0.6$).

Assessment of isolate sexuality

Six weeks after inoculation of the *Eucalyptus* branch pieces, fruiting bodies of the inoculated fungus were evident on the surface of the bark. At this time, *C. cubensis* had colonised all inoculated stem pieces completely. All the stem pieces inoculated with South African genotypes of the fungus resulted in the formation of pycnidia only. Inoculation with the five Colombian isolates obtained from ascospore masses, however, resulted in the formation of perithecia only. The five single ascospore cultures from Colombia also resulted in the formation of perithecia. The control inoculations showed no lesions or fruiting bodies. Examination of the fruiting structures using light microscopy confirmed the presence of conidia from the South African isolates and ascospores from the Colombian isolates.

Field inoculations

All trees inoculated with the 23 isolates representing different VC groups from the South African *C. cubensis* population developed distinct cambial lesions 11 weeks after inoculation. Control inoculations resulted in no lesion development. Lesion lengths differed significantly amongst the *C. cubensis* isolates of different VC groups ($F = 3.99$; D.F. $= 23$; $P < 0.001$) (Fig. 2). Lesion lengths for the five isolates of the same VC group did not differ significantly from each other ($F = 0.88$; D.F. $= 4$; $P > 0.6$) (Fig. 3).

DISCUSSION

Results of this study clearly indicate that the South African *C. cubensis* population is represented by a small number of genetic entities. Thus, 23 VC groups were identified from a sample of 100 randomly collected isolates. This is in contrast to the situation in South America and Indonesia where a large number of VC groups represent the population (van Heerden *et al.* 1997, van Zyl *et al.* 1998). We must therefore conclude that the fungus has been present in the latter countries for extended periods of time, but that it was recently introduced into South Africa. This would be consistent with results of surveys in the late 1970's (Wingfield, unpubl.) that failed to show any evidence of Cryphonectria canker in the country at that time.

The genotypic diversity for the South African *C. cubensis* population was estimated at 9.6. In a study conducted by Liu *et al.* (1996), it was observed that a *C. parasitica* population from Europe that comprised 50 isolates, grouped into three VC groups with a genotypic diversity of 7.2. In contrast, isolates of *C. parasitica* collected from Maryland represented 31 VC groups in a sample size of 53 and a genotypic diversity of 49.3. This indicates that genotypic diversity is a reliable measure that can be used to compare data from different pathosystems with different sample size, and that the South Africa population of *C. cubensis* is genetically uniform.

Both of the VC testing methods used in this study gave reliable results with good resolution. This indicates that the method used by Cortesi, Milgroom & Bisiach (1996) and Cortesi, Rigling & Heiniger (1998) for *C. parasitica* on the medium described by Powell (1995), is sufficiently sensitive to study populations of *C. cubensis*. Likewise the oatmeal agar technique was equally effective and its utility was previously shown by van Zyl *et al.* (1998) who used it to study the population diversity amongst Brazilian isolates of *C. cubensis*.

An interesting observation in this study was that some VC groups were extremely common. Vegetative compatibility groups (SA7, SA8, and SA12) comprised 49% of the total sample (Fig. 1). Cortesi *et al.* (1996, 1998) made similar observations for *C. parasitica* when identifying the VC groups from Italy and Switzerland. These most common VC groups would most likely be the first to be targeted for transformation with mycoviruses.

It is known that hypovirus transmission occurs between isolates of similar or related VC groups (Anagnostakis 1977). The frequency at which virus dissemination takes place is also dependent on the number of *vic* genes that differ between the isolates (Liu & Milgroom 1996). No studies have thus far been conducted on the genetics of VC types in *C. cubensis* and such research would be desirable. The low number of genetic entities of *C. cubensis* in South Africa, suggests that strategies linked to the reduction in pathogen virulence such as dsRNA mediated-hypovirulence, could be successful in reducing the impact of the pathogen in the future. It also implies that sexual recombination is limited in the South African *C. cubensis* population.

In this study, we were able to gain significant insight into the variability amongst isolates of *C. cubensis* from South Africa. It was possible to show that the fungus apparently lacks sexual recombination in the field. It was also possible to show evidence of lack of sexual recombination in laboratory tests with South African isolates, although sexual structures were readily formed by isolates from South America. Given that there is good evidence that Cryphonectria canker was not present in South Africa prior to 1979, it would be reasonable to expect that, in the absence of sexual reproduction, the *C. cubensis* population will remain genetically uniform in South Africa in the foreseeable future.

In contrast to the South African situation, collections from Colombia indicated that perithecia are the predominant fruiting structures associated with *C. cubensis* (Wingfield, unpubl.). These observations were further confirmed in the *Eucalyptus* stem inoculations where the Colombian isolates of *C. cubensis* produced perithecia readily. Results of stem inoculations with the single ascospore cultures resulted in the formation of perithecia and thus confirm the results of Hodges *et al*. (1979) who showed that *C. cubensis* is homothallic. Although the majority of perithecia on cankers in South America and Indonesia appear to result from homothallism (Hodges *et al*. 1979, van Heerden *et al*. 1997, van Zyl *et al*. 1998), the high level of genetic diversity in these countries suggest that sexual recombination occurs there.

Different South African isolates of *C. cubensis* representing the same VC group had levels of pathogenicity that were not significantly different from each other ($P > 0.6$). In contrast, inoculations with isolates representing different VC groups had distinctly different levels of pathogenicity ($P < 0.001$). This indicates that isolates of different VC groups do not only differ at their *vic* loci, but also in genes linked to pathogenicity. These results show that it will be important to select isolates carefully, when initiating studies to compare the tolerance of different *Eucalyptus* clones to Cryphonectria canker.

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