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Population diversity among Brazilian isolates of *Cryphonectria cubensis*

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Abstract

The devastating effect of *Cryphonectria* canker on *Eucalyptus* spp. in Brazil has necessitated the initiation of a strategy to control the disease. A potential biological control strategy for *Cryphonectria cubensis* would be to make use of dsRNA, capable of debilitating the fungus. However, success would depend on the diversity of the pathogen population. In this study, the presence of vegetative compatibility groups (VCGs) in *C. cubensis* was demonstrated by pairing isolates and observing the reaction along the line of contact between the expanding colonies. Isolates (1092) of *C. cubensis*, from 182 *Eucalyptus* trees located in five regions in Brazil, were examined using this method. On a tree, one or at most two VCGs were present. However, isolates from different but closely spaced trees, represented numerous VCGs. It is probable that the multiplicity of VCGs in the Brazilian population of *C. cubensis* will limit the natural spread of dsRNA and, hence, limit the potential for biocontrol via hypovirulence. The large number of VCGs is also indicative of a well-established, and possibly endemic population of the pathogen. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cryphonectria cubensis (Bruner) Hodges is a notorious stem canker pathogen of *Eucalyptus* trees (Bruner, 1916; Boerboom and Maas, 1970; Hodges, 1980; Wingfield et al., 1989). It is prevalent in many tropical areas of the world and, particularly, between the latitudes 30° north and south of the equator (Hodges, 1980; Old et al., 1986; Conradie et al., 1990). *Cryphonectria cubensis* has limited the establishment of seedling plantations of susceptible *Eucalyptus* spp. in areas where climatic conditions favour disease development (Hodges et al., 1979; Alfenas et al., 1982; Sharma et al., 1985). Humid conditions, high rainfall (>1200 mm year^{-1}), and temperatures greater than 23°C or higher (Hodges et al., 1976; Sharma et al., 1985) are needed for the growth and spread of the pathogen. In cooler or drier conditions, the infection rates and the extent of canker development are much lower (Hodges et al., 1979).

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C. cubensis was reported for the first time in South Africa in 1989 (Wingfield et al., 1989). Clonal forestry, which is now widely practised with *Eucalyptus* spp. in this country, gives rise to large, genetically uniform stands of *Eucalyptus* trees that will be at risk from pests and diseases, including *C. cubensis*. (Conradie et al., 1992). Current means of reducing losses due to the disease in the forestry areas of South Africa and Brazil, is through the plantings of disease-tolerant *Eucalyptus* species and clones (Conradie et al., 1990). A search for alternative methods of controlling this disease are thus being sought.

One means to reduce the impact of a tree pathogen is through the use of viruses or virus-like agents, which are capable of debilitating it. Such hypovirulence has been used successfully for limiting canker expansion and mortality of the American chestnut [*Castanea dentata* (Marsh.) Borkh.], due to infection by *C. parasitica* (Biraghi, 1950a, b; Anagnostakis and Day, 1979; Anagnostakis and Waggoner, 1981; Choi and Nuss, 1992; Chen et al., 1996). This system involves the conversion of a virulent (V) strain to hypovirulence (H) when the cytoplasmic determinants (dsRNA) of hypovirulence are transferred during hyphal anastomosis. The dsRNA is associated with the down-regulation of specific fungal genes (Powell and Van Alfen, 1987a, b), reduced sporulation and virulence, and changes in colony morphology (Anagnostakis and Day, 1979).

Transfer of dsRNA from hypovirulent to virulent isolates of *C. parasitica* is favoured when strains are of the same VCG (Anagnostakis, 1977; Glass and Kuldau, 1992; Leslie, 1993). Spread of hypovirulence could, therefore, be limited by vegetative incompatibility, although H strains that can convert V strains belonging to several already defined VCGs, termed networks (Anagnostakis, 1983), or conversion groups (Kuhlman et al., 1984) have been identified. Vegetative incompatibility is the inability of genetically different strains of a fungal species to fuse and form heterokaryons (Puhalla and Anagnostakis, 1970; Anagnostakis, 1988), and it results when different alleles are present at one or more vegetative incompatibility loci in the hyphae attempting to fuse (Anagnostakis, 1988).

The presence of a large number of VCGs in *C. parasitica*, within small geographical areas in the United States, has severely limited the natural spread of hypovirulence factors and, therefore, reduced their usefulness as biological control agents for chestnut blight (Anagnostakis et al., 1986; MacDonald and Fulbright, 1991). In a preliminary study, Van der Westhuizen et al. (1993) suggested that isolates of *C. cubensis* in South Africa represent a uniform population structure which is indicative of an introduced pathogen. This would facilitate spread of dsRNA which has also recently been discovered in the population (Van der Westhuizen et al., 1994).

Although *C. cubensis* has been known in Brazil for a considerable time, almost no information is available concerning the diversity of its population in that country. It has, however, previously been suggested that the fungus is native to Indonesia (Bruner, 1916; Hodges et al., 1986), thus implying that it was introduced into Brazil. The aim of this study was to determine the genetic variability amongst isolates of *C. cubensis* from Brazil. This would then provide clues as to the origin of the fungus in Brazil, and also whether dsRNA-mediated hypovirulence might be a means to reduce the impact of the disease in that country.

2. Materials and methods

2.1. Isolates and cultures

A survey of *C. cubensis* in *Eucalyptus* growing areas of Brazil (Table 1) was conducted during April 1993. One piece of bark per tree, showing characteristic disease symptoms, was collected. All bark pieces were surface-disinfested with 90% ethanol. These

Table 1
Origin of Brazilian isolates of *Cryphonectria cubensis* used for vegetative compatibility tests

Region	Total number	
	Trees	Isolates
Aracruz	12	72
Teixeira de Freitas Nursery	113	678
Teixeira de Freitas Plantation	32	192
Mogi Guacu Nursery	22	132
Mogi Guacu Plantation	3	18

bark samples were kept moist in petri dishes containing wet filter paper and incubated at 25–27°C for 3 weeks. This was done to induce production of pycnidia and perithecia. Most of the perithecia on bark samples began to produce ascospores within 7–14 days after incubation. Ascospore masses from six perithecia per bark sample were transferred to sterile 9 cm diameter petri dishes containing 15 ml of 2% malt extract agar (MEA) (20 g malt extract, Biolab; 20 g agar, Biolab; 1 l distilled water) and incubated at 25–27°C. Isolates were stored on MEA slants in screw-capped tubes at 4°C.

2.2. VCGs per perithecium

Ascospore masses from 10 perithecia per piece of bark from each tree in the five regions were selected. Ascospores were washed onto petri dishes containing 2% water agar (20 g agar, Biolab; 1 l distilled water) and spread across the medium surface. Plates were incubated for 19–24 h at 25°C. Twenty single-germinated ascospores were then isolated from each plate with the aid of a dissecting microscope and sterile syringe needle, and incubated at 25°C in petri dishes containing 2% MEA.

Tests for determining VCGs were made by transferring small pieces of agar containing mycelium (<3 mm in diameter) from the edges of rapidly growing single ascospore cultures, less than 7 days old, onto oatmeal agar in square, sterile assay plates (30 cm^2). Oatmeal agar was prepared by adding 100 g oatmeal to 1 l distilled water, steaming for 2 h and then sieving through cheese cloth before sterilizing at 120°C for 20 min. Each isolate was paired with itself and with each of the other isolates. Mycelial plugs were positioned, not greater than 5 mm apart on the surface of plates, which were then sealed with Parafilm and incubated at 25–27°C in the dark for 5–7 days. Two replicates of each plate were made.

The presence of VCGs was determined using the method described by Anagnostakis (1977). At the end of the incubation period, mycelia that were vegetatively compatible had merged, forming a confluent mycelium. Incompatible mycelia had grown to a meeting point on the agar, but remained separated by a 'barrage-like' reaction formed along the line of contact between paired colonies (Fig. 1).

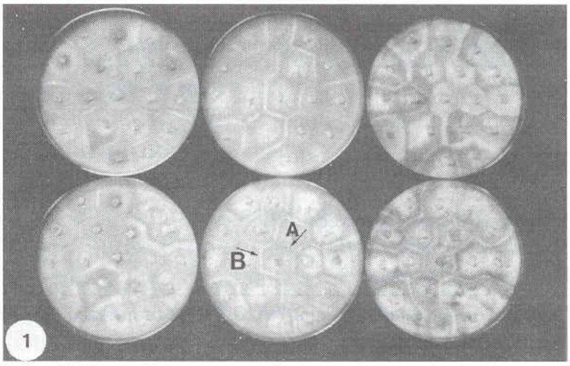

Fig. 1. *Cryphonectria cubensis* isolates, from six randomly selected trees, were paired on a petri dish. (A) Compatible strains merged along the line of contact; and (B) 'barrage' reaction zone formation along the line of contact of incompatible strains.

2.3. VCGs per tree

The number of VCGs present on each tree was determined using ascospore masses from six perithecia per bark sample. The technique of Anagnostakis (1977) was used as described previously. In this study, however, 9 cm diameter, sterile petri dishes were used rather than the assay plates utilized previously.

2.4. VCGs per region

In regions, where more than 20 trees were sampled, isolates from six randomly selected trees were paired against each other. Initial screening of isolates was done, and those with the same VCGs were excluded in order to reduce the number of isolates used. Twenty isolates were then paired against each other using the 30 cm^2 assay plates, in order to determine the total number of VCGs for each region.

3. Results

3.1. VCGs per perithecia

At the end of the incubation period, mycelia from cultures originating from ascospores from single perithecia had merged, forming a confluent lawn of mycelium. This indicates the existence of only one VCG amongst all ascospores in a perithecium. None of the 20 single ascospore cultures from each of the