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Hypovirulence detected in Brazilian isolates of *Cryphonectria cubensis*

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A single 3 kb segment of double-stranded (dsRNA) was present in three of 30 Brazilian isolates of *Cryphonectria cubensis*. These dsRNA-containing isolates showed morphological characteristics suggestive of hypovirulence and were significantly less virulent than dsRNA-free isolates. One isolate, however, with morphological characteristics suggestive of hypovirulence, showed reduced virulence, but was free from dsRNA. Conversion of virulent isolates with normal morphology to a morphology associated with hypovirulence was achieved by pairing hypovirulent and virulent isolates of the same vegetative compatibility group (VCG). This suggests that dsRNA can be transmitted to isolates of the same vegetative compatibility group by hyphal anastomosis. Converted isolates exhibited the same hypovirulence-associated traits as those of the original dsRNA-containing hypovirulent isolates. These studies suggest that a single 3 kb segment of dsRNA alters both morphology and virulence by conferring hypovirulence on the pathogen; the first such report for Brazilian isolates of *C. cubensis*.

Keywords: *Cryphonectria cubensis*, hypovirulence, dsRNA

Introduction

Extensive eucalypt plantations have been established in many countries of the world, to meet the increasing demand for pulp and paper. A canker disease caused by *Cryphonectria cubensis* (Bruner, 1916; Boerboom & Maas, 1970; Hodges, 1980; Wingfield *et al*., 1989) has severely limited the development of seedling plantations comprising susceptible species of *Eucalyptus* in areas where climatic conditions favour development of the pathogen (Alfenas *et al*., 1982). Reducing losses caused by this pathogen is thus of considerable importance to *Eucalyptus* propagation.

Currently, the only means of managing those losses is through the use of disease resistant, or at least disease tolerant, species or clones of *Eucalyptus* (Alfenas *et al*., 1983; Conradie *et al*., 1990). Another means of reducing the impact of a disease such as *Cryphonectria* canker is by reducing the virulence of the causal agent through hypovirulence, associated with the presence of double-stranded RNA (dsRNA) (Van Alfen *et al*., 1975; Jaynes & Elliston, 1980; Elliston, 1985; Van Alfen, 1988; Nuss & Koltin, 1990; Choi & Nuss, 1992a,b). Hypovirulence may have contributed greatly to the recovery of European chestnut from blight caused by *Cryphonectria parasitica* (Van Alfen *et al*., 1975; Anagnostakis, 1982a).

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This condition is associated with the presence of cytoplasmic double-stranded RNA (Day *et al*., 1977), with down-regulation of specific genes (Powell & Van Alfen, 1987), reduced sporulation and virulence, and changes in colony morphology (Anagnostakis & Jaynes, 1973; Elliston, 1985).

Much knowledge exists regarding hypovirulence in *C. parasitica* (MacDonald & Fulbright, 1991). *Cryphonectria cubensis* is a very similar fungus, but with a different host range. At this stage, very little is known about its population biology, although hypovirulence has recently been found in South African isolates (Van der Westhuizen *et al*., 1994). *Cryphonectria cubensis* is best known in Brazil and the primary aim of this study was to screen a wide array of isolates from that country for dsRNA, and associated hypovirulence-related traits.

Materials and methods

Isolates

A total of 1092 isolates of *C. cubensis* were obtained from 182 trees showing typical canker symptoms in various parts of the *Eucalyptus*-growing areas of Brazil. Bark samples were kept moist in Petri dishes containing wet filter paper and were incubated at 25–27°C for up to three weeks. Most perithecia on bark samples produced spores within seven to 14 days. Ascospore masses from

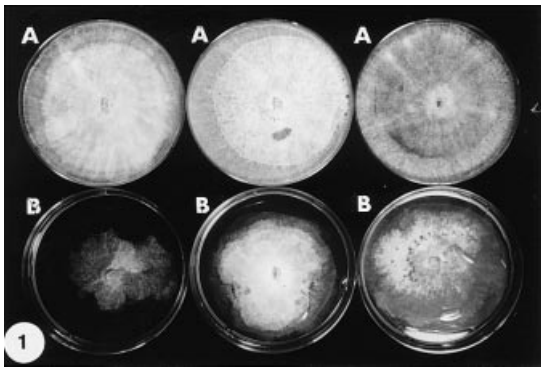

Figure 1 Comparative morphology of apparently virulent (A) and hypovirulent (B) Brazilian isolates of *Cryphonectria cubensis*.

six perithecia on each bark sample were transferred to sterile Petri dishes, 9 cm in diameter, containing 15 mL of 2% malt extract agar (MEA), and incubated at 25–27°C for seven days. At the first transfer after isolation, some of the isolates showed symptoms described as typical for hypovirulence in *C. parasitica* (Anagnostakis & Day, 1979). These isolates (H isolates) had one or more of the following characteristics: slow growth, irregular colony margins, white or light orange colonies and infrequent pycnidial production. In contrast, most other isolates (V isolates) grew rapidly, had a dense and bright yellow to orange colour, abundant aerial mycelium and frequently produced pycnidia (Fig. 1).

Thirty isolates of *C. cubensis*, thought, on the basis of their morphology, to be dsRNA-infected, were selected from the above isolates and used in this study. In addition, 10 isolates with normal growth characteristics, two isolates of *C. parasitica* (EP 155 [ATCC 38755] and EP 713 [ATCC 52571]), and one dsRNA-containing *C. cubensis* isolate (D13–3) from South Africa were used for comparative purposes.

Extraction and purification of dsRNA

For extraction of dsRNA, cells were grown in the dark in 1 L Erlenmeyer flasks containing 250 mL of 3% malt extract broth (MEB) for two weeks at 25°C on a rotary shaker. Mycelium was harvested by centrifuging (15 min at 8000 × *g*) at 4°C. Cells were lyophilized and stored in Petri dishes at −74°C. The procedure for extraction of dsRNA from fungal mycelium was as described by Morris & Dodds (1979) and Valverde *et al.* (1990), with minor modifications. One gram of freeze dried fungal tissue was ground in 6 mL 2 × STE buffer with a mortar and pestle (1 × STE = 0·1 M NaCl, 0·05 M Tris-HCl, 0·001 M ethylenediamine tetra-acetic acid [EDTA] pH 6·8). The mixture was then transferred to 250 mL centrifuge tubes and a further 24 mL 2 × STE was added to each. To this homogenate, 3 mL of 10% sodium dodecyl sulphate (SDS) and 28 mL 1 × STE-saturated phenol were added and shaken (210 × *g*) at 25°C for 30 min. Samples were centrifuged (15 min at 10 000 × *g*) at 10°C to separate the phases, then 25 mL of the upper aqueous phase of each sample was withdrawn, adjusted to 16·5% ethanol and subjected to two cycles of cellulose (CF-11, Whatman) column chromatography. Columns were prepared as described by Valverde & Fontenot (1991), except that glass wool plugged 60 mL plastic syringes were used. Electrophoresis of dsRNA was carried out by using 1% agarose gels in 1 × TBE (0·089 M Tris, 0·089 M boric acid, 0·002 M EDTA, pH 8.0) electrophoresis buffer. Electrophoresis was performed at 40 V for two to three hours at 25°C and gels were stained with ethidium bromide (0·17 ($\mu g\,mL^{-1}$) and photographed in transmitted ultraviolet light.

Enzymatic digestions of nucleic acid samples were conducted with deoxyribonuclease I, ribonuclease-free (DNAase 1, RNAase-free) [Boehringer Mannheim] at 10 $\mu g\,mL^{-1}$ in 0·01 M MgCl2 at 30°C for 30 min and ribonuclease A (RNAase A) [Boehringer Mannheim] at 0·5 ($g\,mL^{-1}$ in 0·03 M and 0·3 M NaCl at 25°C for 30 min. Nucleic acids that hydrolyzed in the 0·03 M RNAase treatment but not in the 0·3 M treatment or in the DNAase treatment were considered to be double-stranded RNA. Thus, nucleic acids that were hydrolyzed by the RNAase in a low-salt buffer but not in a high salt buffer, and that were resistant to DNAase, were considered to be dsRNA (Hansen *et al.*, 1985).

Growth studies

Growth rates were determined for three dsRNA-containing isolates (BSN 26·2, BSN 27·2, BSNCH 131·3), two isolates devoid of dsRNA (BSN 84·5, BSNCH 117·6) and one isolate devoid of dsRNA, but with growth characteristic of hypovirulence (BSN 18·4). Mycelial plugs of the isolates, 5 mm in diameter, were transferred to 2% potato dextrose agar (PDA), with three plates for each temperature to be tested. Plates were incubated at temperatures ranging from 10 to 30°C, at five degree intervals, in the dark, for 4 days. Growth was determined by measuring colony diameter. Mean growth rates were compared, using Tukey's procedure for comparison of means (Steel & Torrie, 1980).

Susceptibility to conversion

The three dsRNA-containing isolates of *C. cubensis* were paired with 10 virulent isolates in a modification of the technique of Anagnostakis & Day (1979). Isolates were grown on 2% PDA overlaid with dialysis membranes for seven days at 25°C, with a 16 h day provided by cool-white fluorescent light. One plug of agar and mycelium, 3 mm in diameter, was cut from the margin of each culture. One plug of each of the suspected hypovirulent isolates and one from an apparently normal isolate of the same vegetative compatibility group (VCG) were placed in pairs adjacent to each other in 9 cm diameter Petri dishes. The same

method was used to pair suspected hypovirulent isolates with apparently normal isolates of different VCGs. VCGs were determined using the method described by Anagnostakis (1977). Each pairing was repeated three times. Cultures were incubated in the dark at 25°C for five to eight days. Pairings were observed at five, seven and eight days, for indications that the apparently normal isolates had been converted to hypovirulent growth. Representative sectors from converted isolates were subcultured, and dsRNA was re-isolated from them to confirm that conversion had occurred.

Inoculations

Two-year-old trees of *E. grandis* clone ZG 14 and *E. grandis* clone TAG 5 were grown in 1·5 L containers. The former is known to be highly susceptible, while the latter is moderately tolerant to infection by *C. cubensis*, under field conditions (Authors, unpublished). Plants were maintained in a glasshouse at 23–25°C and 70–90% relative humidity.

Three of the Brazilian isolates which showed typical signs of hypovirulence (two possessing dsRNA and one from which dsRNA could not be isolated), as well as one isolate with normal morphology and devoid of dsRNA, were grown on 2% PDA at 25°C for seven days. Twenty trees of each clone (20 mm in diameter) were inoculated with each isolate, by removing a disk of bark with a 5 mm diameter cork borer. The same number of trees were used as controls. A 5 mm disc from the margin of a seven-day-old culture of *C. cubensis* was placed in each wound. A sterile PDA disk was used for the control treatments. Wounds were covered with Parafilm to prevent the inoculum from desiccating. After six weeks, the Parafilm and bark were removed and the length of discoloured cambium was measured. Mean values were tested for significance according to Tukey's procedure (Steel & Torrie, 1980). Isolations were made from the advancing edge of each cambial lesion.

Results

Presence of dsRNA

dsRNA was successfully extracted from three of the 30 isolates tested. The method used allowed the consistent detection of dsRNA elements in relatively small amounts. Results were confirmed by extractions from the two *C. parasitica* isolates: EP 713 (ATCC 52571) was known to be infected with dsRNA whereas isolate EP 155 (ATCC 38755) was not infected. The number and size of dsRNA segments isolated from the European hypovirulent strain of *C. parasitica*, EP 713, were as described by Hillman *et al.* (1992) (Fig. 2). Its virulent counterpart, strain EP 155 from Connecticut, USA., was devoid of dsRNA segments. These results were typical of those reported for the same isolates by Enebak *et al.* (1994a,b). The dsRNA from a South African hypovirulent *C. cubensis* isolate, D13-3, was characterized by

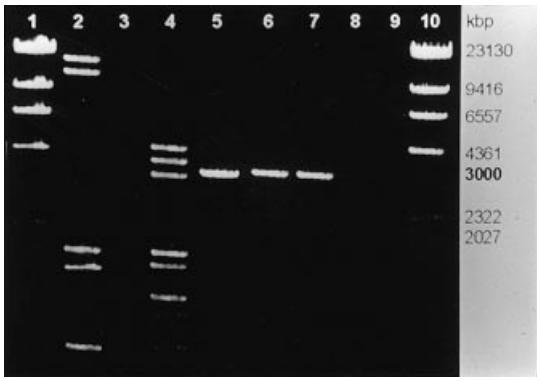

Figure 2 Agarose gel showing dsRNA extracted from hypovirulent and virulent isolates of *Cryphonectria cubensis and Cryphonectria parasitica*. Lanes: 1 and 10, DNA cut with Hind III, as molecular weight markers; 2, dsRNA recovered from EP 713; 3, absence of detectable dsRNA in virulent isolate EP 155; 4, dsRNA extracted from the South African hypovirulent isolate D13-3 – nine segments were visible on the gels but only 6 can be seen clearly in the figure; Lanes 5, 6 and 7, dsRNA recovered from apparently hypovirulent isolates BSN 26·6, BSN 27·2 and BSNCH 131·3, respectively; Lanes 8 & 9, absence of dsRNA from apparently normal (virulent) isolates BSN 84·5 and BSNCH 117·6.

nine segments (Fig. 2). The major segment of these had a molecular weight of 4·3 kb, as described by Van der Westhuizen *et al.* (1994).

The morphology of the three isolates with dsRNAs was suggestive of hypovirulence. These had originated from different geographic areas. Isolates BSN 26·6, BSN 27·2 and BSNCH 131·3 contained only a single molecular weight dsRNA with identical mobility in agarose gels (Fig. 2). By comparison with lambda DNA (Hind III digest), its molecular weight was estimated to be 3 kb. Of the 11 Brazilian isolates with normal growth, one, namely BSN 84·5, was used as control treatment (Fig. 2). This isolate showed normal colony morphology, sporulation and growth rate, and no dsRNA segments were detected in it. The nature of the dsRNA from the three isolates was also confirmed by insensitivity to both DNAase and RNAase at high ionic strength and sensitivity, to RNAase at low ionic strength.

Growth studies

All isolates failed to grow at 10°C and optimum growth was observed at 25°C. Analysis of the data showed that there was a difference ($P \leq 0{\cdot}05$) between growth rates of isolates possessing and those devoid of dsRNA (Table 1). There was also a significant difference ($P \leq 0{\cdot}05$) at 25°C, between the growth of BSN 18·4 (devoid of dsRNA, but showing characteristics of hypovirulence) and all other isolates; it grew faster ($P \leq 0{\cdot}05$) than isolates known to possess dsRNA and significantly slower than the other isolates devoid of dsRNA (Table 1).

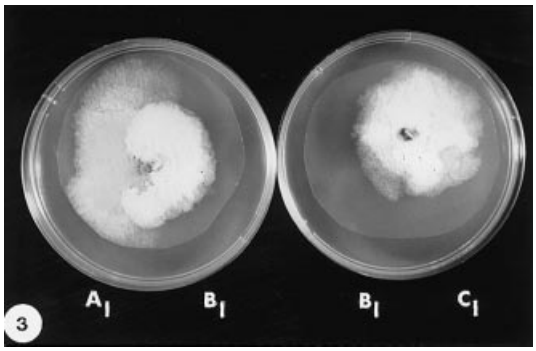

Figure 3 Pairing between hypovirulent and virulent isolates of *Cryphonectria cubensis*. No conversion of an apparently virulent isolate (A_1) paired with dsRNA-containing isolate (B_1) of a different VCG. Conversion of a virulent isolate (C_1) to a morphology typical of hypovirulence, when paired with a dsRNA-containing isolate, BSN 27·2 (B_1), of the same VCG.

Susceptibility to conversion

Pairing of a hypovirulent, dsRNA-containing isolate of *C. cubensis* with a normal, non-dsRNA-containing, morphologically stable isolate of the same VCG, resulted in conversion of the normal isolate to a morphology typical of the hypovirulent isolate (Fig. 3). Subcultured sectors of converted mycelium maintained the hypovirulence morphology and dsRNA was extracted from these converted isolates. Pairing of a hypovirulent isolate with an apparently normal isolate of a different VCG did not result in conversion (Fig. 3) and dsRNA could not be extracted from the recipient strain.

Inoculations

Isolate BSN 84·5 contained no detectable dsRNA and was highly virulent in pathogenicity tests (Table 1). This isolate had normal cultural characteristics on PDA and sporulated readily. Isolate BSN 18·4, with morphological characteristics typical of hypovirulence, but found to be devoid of dsRNA, was significantly ($P \leq 0{\cdot}05$) less virulent than BSN 84·5. These dsRNA-free isolates were more virulent ($P \leq 0{\cdot}05$) than the dsRNA-containing isolates (BSN 27·2 and BSNCH 131·3) (Table 2). There were also significant ($P \leq 0{\cdot}05$) differences in the susceptibility of the two clones used. Clone ZG 14 was significantly more susceptible to *C. cubensis* than clone TAG 5 for all isolates tested. These results are consistent with those of Van der Westhuizen *et al.* (1992). *C. cubensis* was re-isolated from all lesions.

Discussion

Results of this study showed that dsRNA-mediated hypovirulence occurs in Brazilian isolates of *C. cubensis*. It was, however, interesting that only 30 of the 1092 randomly collected isolates studied exhibited a morphology typical of hypovirulence, and only three of these were found to contain dsRNA. The small number of isolates showing symptoms characteristic of hypovirulence could be attributed to a high number of vegetative compatibility groups (VCGs) in the population (Van Zyl *et al.*, 1998). Mycelial incompatibility is considered to act as a cellular defense mechanism by preventing the spread of agents such as viruses and suppressive cytoplasmic determinants among the population (Anagnostakis, 1977; MacDonald & Fulbright, 1991; Glass & Kuldau, 1992; Leslie, 1993).

Virulent strains of *C. cubensis* are disseminated by ascospores and conidia that are transported mechanically. Sporulation, particularly ascospore production, is decreased in hypovirulent strains (Anagnostakis & Jaynes, 1973; Hillman *et al.*, 1990). Until now, agents conferring hypovirulence have not been shown to be transmitted via ascospores of *C. parasitica* (Anagnostakis, 1982a,b). They are transmitted through conidia, with a frequency that depends largely on the hypovirulent agent, rather than the fungal genotype (Russin & Shain, 1985).

Most of the 30 apparently hypovirulent isolates used in this study were free from dsRNA segments. Some hypovirulent isolates in *C. parasitica* have also been found not to contain dsRNA (Griffin *et al.*, 1983; Jaynes & Elliston, 1982). Possible explanations for the present results could be a low concentration of dsRNA in the fungus, latency of the dsRNA, or an absence of dsRNA in the part of the thallus that was subcultured for assay.

In three of the 30 Brazilian *C. cubensis* isolates showing morphology typical of hypovirulence, a single

Table 1 Average colony diameter of dsRNA-containing and dsRNA-free isolates of *Cryphonectria cubensis* after incubation for four days at different temperatures

		Mean colony diameter (mm)[a]				
Isolates	Description	10°C	15°C	20°C	25°C	30°C
BSN 26·2	dsRNA-containing	0	5·2 a	11·7 a	25·6 a	17·3 a
BSN 27·2	dsRNA-containing	0	4·8 a	10·2 a	23·6 a	15·8 a
BSNCH 131·3	dsRNA-containing	0	5·6 a	11·9 a	25·1 a	18·1 a
BSN 18·4	dsRNA-free	0	7·5 a	18·3 b	36·8 b	27·2 b
BSN 84·5	dsRNA-free	0	16·3 b	34·8 c	60·3 c	42·8 c
BSNCH 117·6	dsRNA-free	0	15·6 b	32·5 c	58·7 c	39·9 c

[a] Mean of three replicates. Values in columns followed by different letters differ significantly ($P \leq 0{\cdot}05$), according to Tukey's procedure for comparison of means.

Table 2 Lesion development on two clones of *Eucalyptus grandis* after inoculation with dsRNA-containing and dsRNA-free isolates of *Cryphonectria cubensis*

Isolate	Description[a]	Canker length[b] (mm)	
		ZG 14	TAG 5
BSN 27·2	Apparent H, dsRNA-containing	0·4 a	0·2 a
BSNCH 131·3	Apparent H, dsRNA-containing	0·7 a	0·3 a
BSN 18·4	Apparent H, dsRNA-free	89·5 b	16·0 b
BSN 84·5	Apparent V, dsRNA-free	138·0 c	30·0 c

[a]H refers to a morphology suggestive of hypovirulence. V refers to a normal morphology suggestive of virulence.
[b]Mean of 20 trees. Values in each column followed by different letters differ significantly ($P \leq 0{\cdot}05$), according to Tukey's procedure for comparison of means (coefficient of variance = 27·4).

dsRNA segment of 3 kb was found. This is similar to findings of Rigling *et al*. (1989) and Hillman *et al*. (1992), where single dsRNA segments were associated with hypovirulence in *C. parasitica*. However, the results are in contrast to those of most other fungi studied, including well-studied strains of *C. parasitica*, where the dsRNA represents a complex banding pattern (Anagnostakis & Day, 1979; Chung *et al*., 1994; Lakshman & Tavantzis, 1994; Romaine *et al*., 1994).

Van der Westhuizen *et al*. (1994) showed that a hypovirulent South African isolate of *C. cubensis* had a complex dsRNA banding pattern. The South African dsRNA was characterized by nine different segments, with the largest segment 4·3 kb in size. This is in contrast with the Brazilian dsRNA banding pattern, represented by a single 3 kb segment. In *C. parasitica*, Choi & Nuss (1992a,b) and Shapira *et al*. (1991) showed that all genetic information of the hypovirulence-associated virus resides within the single largest dsRNA segment. Hybridization studies on the South African and Brazilian dsRNA segments from *C. cubensis* are still required, in order to determine whether there is homology between these different dsRNAs.

Hypovirulent isolates of *C. cubensis* were able to convert compatible, virulent, virus-free isolates of the same VCG to hypovirulence after anastomosis. The converted isolates exhibited the same composition of hypovirulence-associated traits as displayed by the original dsRNA-containing hypovirulent isolates. These results indicate that dsRNA confers hypovirulence in the Brazilian population of *C. cubensis*. The characteristics of this hypovirulence are also similar to those found in *C. parasitica* (Anagnostakis & Day, 1979; Anagnostakis, 1982a; Nuss, 1992). Conversion of dsRNA-free isolates to the hypovirulence phenotype is coincident with transmission of dsRNAs during anastomosis with compatible hypovirulent isolates, providing the basis for biological disease control in *C. cubensis*.

Results of this study have shown, for the first time, that dsRNAs are found in the Brazilian isolates of *C. cubensis*, and that these agents can reduce growth and virulence of the pathogen. Many questions must, however, be resolved before hypovirulence can be considered a useful tool for disease management. Maintenance and spread of hypovirulence is a major concern, because hypovirulent isolates grow and sporulate at a slower rate than do virulent isolates. They are therefore at a competitive disadvantage. However, studies recently conducted by Chen *et al*. (1996), on a synthetic viral transcript, showed that an engineered hypovirulent strain (CHV1–713) had the capacity to transmit hypoviruses via nuclear inheritance to ascospore progeny. This implies that the transmission of the virus is not hindered by vegetative incompatibility. To evaluate the potential for biological or molecular control of *Cryphonectria* canker of *Eucalyptus*, additional characterization of dsRNA, hypovirulence, and mycelial compatibility among the Brazilian isolates of *C. cubensis* is required.

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