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Effect of environment on the response of *Eucalyptus* clones to inoculation with *Cryphonectria cubensis*

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Summary

The ascomycete *Cryphonectria cubensis* causes severe losses in *Eucalyptus* plantations in South Africa and selection programmes for disease tolerance are necessary. The aim of this study was to use two *C. cubensis* inoculation trials, planted at different locations to assess the disease susceptibility of the clones and the effect of the environment on disease development. These two trials consisted each of 21 *Eucalyptus* clones (*E. grandis*, *E. grandis* × *camaldulensis* and *E. grandis* × *urophylla*). All trees were inoculated with a single virulent strain of *C. cubensis* and lesion widths measured 6 and 12 months after inoculation. Clones differed significantly in their tolerance to *C. cubensis*. Further, disease severity differed depending on the geographical location of the trial. A significant clone × locality (genotype × environment) interaction was observed. Therefore, screening for disease resistance should take place only in areas where clones will be commercially grown.

1 Introduction

The South African forestry industry is based on extensive plantings of exotic *Pinus* spp., *Eucalyptus* spp. and *Acacia mearnsii*. *Eucalyptus* trees are commonly infected by various fungal pathogens, including the important canker pathogen *Cryphonectria cubensis* (Bruner) Hodges. *Cryphonectria cubensis* causes severe stem cankers that result in significant losses in tropical and sub tropical areas of the world (Boerboom and Maas 1970; Hodges and Reis 1974; Hodges et al. 1979; Hodges 1980; Gibson 1981; Florence et al. 1986). Since the discovery of Cryphonectria canker in South Africa (Wingfield et al. 1989), it has become important to reduce losses due to this disease. In order to implement a management programme to achieve this goal, it has become essential to investigate the biology of *C. cubensis,* as well as to determine the tolerance of planting stock to the pathogen.

Considerable variation exists in the tolerance of *Eucalyptus* to infection by *C. cubensis* (Ferreira et al. 1977; Alfenas et al. 1983). It is, therefore, important to select eucalypt planting stock for disease tolerance, before commercial propagation is undertaken. Such an approach has already been successful in reducing the impact of Cryphonectria canker in countries such as Brazil and South Africa (Campinhos and Ikemori 1983; Wingfield 1990).

Rapid methods for evaluating differences in the susceptibility of *Eucalyptus* clones to *C. cubensis* are desirable in breeding programmes for disease tolerance. Artificial inoculation is often used to evaluate trees tolerant to *C. cubensis* and similar canker pathogens (Ferreira et al. 1977; Alfenas et al. 1983; Van der Westhuizen et al. 1992). Van der Westhuizen et al. (1992) further showed that hybrids of *E. grandis* with *E. camaldulensis, E. urophylla* or *E. tereticornis* are more tolerant to Cryphonectria canker than South African *E. grandis* clones.

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Occurrence and severity of Cryphonectria canker on *Eucalyptus* are influenced by environmental conditions, with most damage occurring in areas where the average annual temperatures are above 23°C and rainfall exceeds 2000–2400 mm per annum (Hodges et al. 1979; Alfenas et al. 1982; Sharma et al. 1985; Florence et al. 1986). The effect of the climate most likely explains the occurrence and severity of Cryphonectria canker in South Africa, in areas such as in coastal Zululand (KwaZulu Natal), where the humidity and annual average temperatures are high.

Environmental conditions and host genotype play important roles in host response to disease. Not all genotypes respond in the same way to a pathogen, for all environmental conditions. These genotype × environment (G × E) interactions for traits such as disease tolerance, complicate selection trials because tree genotypes must be tested in different environments. Very little is known on G × E interactions for disease tolerance in eucalypts. Screening different *Eucalyptus* clones for disease tolerance at different sites by artificial inoculation with *C. cubensis* should illustrate the effect of G × E interactions, for Cryphonectria canker. A significant G × E would also indicate whether selection for tolerance to Cryphonectria canker might be undertaken at a single location, or whether clones must be evaluated at different locations. When planning artificial inoculation trials, the length of time required for significant disease development to occur following inoculation is also an important factor to be considered.

The objectives of this study were: (1) to determine the effect of the genotype and the environment on tolerance to *C. cubensis* after inoculation; (2) to screen a wide selection of *Eucalyptus* clones and to identify those tolerant to disease; (3) to determine whether disease tolerance is the same for all genotypes at two locations and thus to estimate the effect of the G × E interaction on tolerance and (4) examine the effect of time on disease development by comparing results after 6 and 12 months.

2 Materials and methods

2.1 Experimental design and inoculation protocols

Cryphonectria cubensis isolate (CMW 2113) used for inoculation was chosen from a large number of isolates and represents one of the most virulent strains available in South Africa (Van heerden and Wingfield 2001). This isolate is 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, and is used routinely for annual disease tolerance screening trials.

Each of the 21 different *Eucalyptus* genotypes (see Table 2) was vegetatively propagated by taking cuttings from ramets grown as clonal hedges. Rooted cuttings of each genotype were hardened off, whereafter, 22 trees per genotype were planted in January 1996 at DukuDuku (KwaZulu-Natal) and at Sabie (Mpumalanga), in South Africa. The two sites differed in location, elevation, precipitation and temperature (Table 1). Both of these sites

Table 1. **Differences in environmental conditions between the two trials**

	(Duku-Duku) Kwazulu-Natal	(Sabie) Mpumalanga
Co-ordinates	28°22′S, 32°19′E	24°25′S, 30°31′E
Mean annual precipitation (mm)	890	1175
Mean annual temperature (°C)	23	18
Altitude above sea level (m)	20	966

are suitable for *Eucalyptus* growing in South Africa and are used for the commercial propagation of timber. The trees were planted in a randomized block design. Both the trials included the 21 *Eucalyptus* clones, planted in 22 fully randomized rows of equal length. Thus, a total of 22 trees per clone were planted with a spacing of 3 × 2.5 m. Both of the trials were surrounded by buffer rows of *E. grandis*.

The inoculum was prepared by growing the *C. cubensis* isolate in 9 cm diameter Petri dishes containing 2% malt extract agar (MEA) [20 g Biolab malt extract (Midrand, RSA), 20 g Biolab agar (Midrand, RSA), 1 l distilled water]. Before inoculation, we ensured that all trees used in the inoculation had similar circumference, by inspecting the trial visually. This was to eliminate the effect of circumference on the experiment. All trees were inoculated at the beginning of January 1997, by removing a 20 mm diameter bark disc from the main stem, approximately 140 cm from the ground, with a cork borer. All the inoculations were performed on the shadow side of the trees for the entire experiment. Similar sized discs from the edges of actively growing cultures were placed, mycelium side down, in each wound. These were sealed with masking tape to reduce desiccation. The maximum horizontal lesion diameter (lesion width) was measured 6 and 12 months after inoculation (hereafter referred to as 'time'). Previous experiments have shown that control inoculations with sterile MEA result in no disease development (M. J. Wingfield, unpublished data), thus inoculations were performed using only the pathogen.

2.2 Statistical analysis

Differences in lesion width among tree genotypes, locations, and time of recording lesion width were analysed using a two factor repeated measures ANOVA (SYSTAT version 7.0, Chicago, USA), with time as the grouping factor (within subjects, repeated measure), and location and clone as the trial factors (between subjects). A repeated measures analysis was selected because the same trees were measured at two different times, thus measurements from each time period were not independent. Lesion width data did not meet assumptions of parametric testing, as data were not normally distributed but heavily left skewed. Lesion width data were, therefore, log (10) transformed which normalized the distribution. The clone × locality interaction in the ANOVA showed statistical significance, indicating that the choice of a clone based on the mean over the localities and times would not provide an effective selection technique. The effect of this interaction on clone selection was thus determined by considering the two localities and the two measuring times as four environments and applying the Additive Main effects and Multiplicative Interaction (AMMI) model to the resulting G × E interaction (EISENBERG et al. 1996; GAUCH and ZOBEL 1996). As the interaction is analysed by principal component analysis, each clone and environment is assigned a score value. The relative magnitude of these scores represents a lesser or greater interaction effect. The following model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \sum_{n=1}^{N} \lambda_n \gamma_{in} \delta_{jn} + \rho_{in} + \text{£}_{ijk}$$

where Y_{ijk} = lesion width
μ = overall mean
α_i = clone effect
β_j = time environment (locality) observations
λ_n = singular value for IPCA axis $_n$
γ_{in} = clone eigenvector value for IPCA axis $_n$
δ_{jn} = environment value for IPCA axis $_n$
ρ_{in} = residual
£_{ijk} = error.

3 Results and discussion

Most of the trees showed disease development at the time of the lesion assessment. Lesion widths differed significantly among clones ($F = 14.481$; df = 20; $p < 0.001$). Various clones displayed a high degree of disease tolerance (Table 2). This study has, therefore, shown clearly that host genotypes respond differently to infection by *C. cubensis*. It has previously been shown that clones with a high degree of susceptibility under natural field conditions are the same as those that are the most susceptible in inoculation experiments (M. J. Wingfield, unpublished data). Thus, a substantial opportunity exists to screen clones for disease tolerance through artificial inoculation.

Lesion widths differed significantly between the two different locations ($F = 209.029$; df = 1; $p < 0.001$). Mean lesion width in Mpumalanga was 43.9 mm (±2.4 SEM) after 6 months and 44.5 mm (±2.2 SEM) after 12 months. For KwaZulu-Natal, mean lesion width was 82.5 mm (±7.2 SEM) after 6 months and 109.7 (±13.9 SEM) after 12 months. In South Africa, Cryphonectria canker are known to be most damaging in the sub tropical regions (Wingfield et al. 1995). The inoculation trials in this study were thus conducted at one site that is known to have an environment that favours disease development and at another site where the disease has been never observed. Inoculation of trees at the sub tropical site (KwaZulu-Natal) resulted in significant disease development, consistent with the biology of the fungus where high humidity and temperature requirements favour disease development (Hodges et al. 1979; Alfenas et al. 1982; Sharma et al. 1985; Florence et al. 1986). In contrast, lesions in Mpumalanga

Table 2. ***Eucalyptus*** **clones selected for artificial inoculation with** ***Cryphonectria cubensis*** **with mean lesion width (mm) (±SEM), 6 and 12 months after inoculation**

		Lesion width (mm) ± SEM			
		Zululand inoculation		Mpumalanga inoculation	
Clone number	Clone*	6 months	12 months	6 months	12 months
SGR 036	A	41.8 ± 12.5	31.9 ± 11.9	36.7 ± 3.6	37.4 ± 3.4
SGR 071	A	54.0 ± 11.1	90.0 ± 17.3	39.7 ± 4.6	51.3 ± 7.3
SGR 112	A	125.6 ± 15.3	181.9 ± 28.7	60.6 ± 6.0	55.0 ± 8.5
SGR 127	A	138.3 ± 18.1	171.2 ± 16.1	60.3 ± 6.5	57.3 ± 7.4
SGR 137	A	63.7 ± 5.8	82.1 ± 12.2	47.7 ± 3.9	44.2 ± 8.9
SGR 170	A	108.3 ± 9.9	151.6 ± 16.6	57.8 ± 5.7	56.1 ± 7.1
SGR 171	A	83.7 ± 11.6	57.5 ± 10.5	41.2 ± 5.4	50.7 ± 5.4
SGR 198	A	84.0 ± 6.7	92.7 ± 12.4	40.3 ± 3.8	53.8 ± 8.1
SGR 202	A	87.1 ± 11.3	119.4 ± 14.9	39.5 ± 4.0	43.6 ± 7.4
SGR 223	A	113.5 ± 14.5	117.5 ± 14.9	39.1 ± 5.0	45.0 ± 4.8
SGR 380	A	88.1 ± 9.1	158.6 ± 16.9	40.7 ± 3.5	35.6 ± 4.2
SGR 417	A	75.9 ± 11.2	159.7 ± 25.8	54.2 ± 5.8	66.5 ± 14.1
SGR 470	A	45.4 ± 3.3	51.7 ± 9.3	43.0 ± 5.9	48.3 ± 8.0
SGR 474	A	70.9 ± 7.8	87.9 ± 15.0	42.6 ± 4.7	29.1 ± 2.8
SGR 479	A	115.7 ± 9.8	193.3 ± 22.4	58.9 ± 6.8	38.3 ± 6.8
SGR 516	A	151.4 ± 18.5	296.8 ± 35.6	68.6 ± 10.5	55.0 ± 11.2
SZ 5	A	38.9 ± 4.3	52.9 ± 10.8	39.0 ± 4.8	40.5 ± 3.5
SZ 12	B	66.6 ± 11.3	75.5 ± 19.2	33.5 ± 6.1	28.5 ± 3.1
SZ 11	B	75.9 ± 11.6	92.0 ± 21.4	29.5 ± 3.5	28.5 ± 3.8
SZ 9	B	52.0 ± 7.7	50.0 ± 8.6	34.1 ± 4.6	50.4 ± 10.2
SZ 15	C	33.7 ± 2.8	24.2 ± 2.9	26.4 ± 2.1	30.3 ± 2.8

*A, *Eucalyptus grandis*; B, *E. grandis* × *E. camaldulensis*; C, *E. grandis* × *E. urophylla*.

were small and did not develop significantly. Thus, environmental conditions in Mpumalanga are not conducive to disease development. It also strongly suggests that environmental factors linked to susceptibility are associated with disease development and not to the infection process.

There were significant differences in lesion width on the clones, for the two times of lesion measurement ($F = 4.228$; df = 1; $p < 0.001$). Results of this study, therefore, showed differences in the response of clones to inoculation after 6 and 12 months. It was observed that in some clones, there was a reduction in lesion size after 12 months. These clones had evidently begun to recover and such recovery might reflect a degree of disease tolerance. We assume that those clones, in which lesions continued to expand after the 6-month evaluation, have the highest degree of susceptibility.

Not all clones responded similarly at both locations as indicated by a significant clone × location (G × E) interaction ($F = 5.996$; df = 20; $p < 0.001$). AMMI analysis showed that the interaction sum of squares makes up a very large proportion of the total means of squares. The first principal component accounts for 92.5% of the interaction and thus represents virtually the entire predictable pattern of the G × E effect. This pattern is illustrated in a Bi-plot of average lesion width against the scores (Fig. 1). The clones and environments (Z1, Z2, M1 and M2) which are closest to the zero score level are least interactive and are expected to maintain the level of lesion reaction (shown on the X-axis) in all environments. As the score level increases (negatively or positively), the interaction with environment increases. As the interaction is multiplicative, high negative clone × environment scores give high positive G × E effects, as do positive scores. Negative by positive scores provide negative G × E effects and will result in reduced lesion sizes. Results indicated that some clones had similar lesion widths at both locations, whereas others had larger lesions in KwaZulu-Natal relative to Mpumalanga. If there was no G × E interaction, selection for disease tolerance would be greatly simplified because the best genotypes at one location would be suitable for planting at other locations (Matheson and Cotterill 1990; Basford and Cooper 1998). Our study shows that screening trees for tolerance to *C. cubensis* should always be conducted in the area where the clones of interest are to be propagated. However, it may be possible to focus on genotypes that respond similarly in both environments and that exhibit little G × E interaction.

Differences in lesion width after 6 and 12 months were different at the two test locations, which is indicated by the significant time × location interaction observed ($F = 5.048$; df = 1; $p < 0.001$). This significant time × location interaction shows that the effect of time of assessment for disease tolerance is different in Mpumalanga and KwaZulu-Natal. Thus, it may be necessary to retain trials for longer periods at some places. In Mpumalanga, the lesion sizes were generally smaller than those for Zululand. This could be due to the fact that the two environments differ in such a way that the Zululand climate is more conductive for disease development.

There was a significant time × location × clone interaction showing that the effect of time of recording results was not the same at both locations, for all the clones ($F = 2.992$; df = 20; $p < 0.001$). A significant time × location × clone interaction implies that the effect of time at which assessments are made would not be the same for all the clones at both locations. In contrast, there was no significant time × clone interaction indicating that all the clones respond similarly in terms of lesion development after 6 and 12 months ($F = 1.159$; df = 20; $p > 0.25$). This indicates that individual clones react similarly over the two time periods, within a single location.

Inoculation of *Eucalyptus* clones provides a reasonably simple strategy to select planting stock that is tolerant to Cryphonectria canker, prior to commercial planting of these clones. In this study, we have confirmed that clones differ in their susceptibility to the pathogen and the potential utility of this technique. However, these inoculation

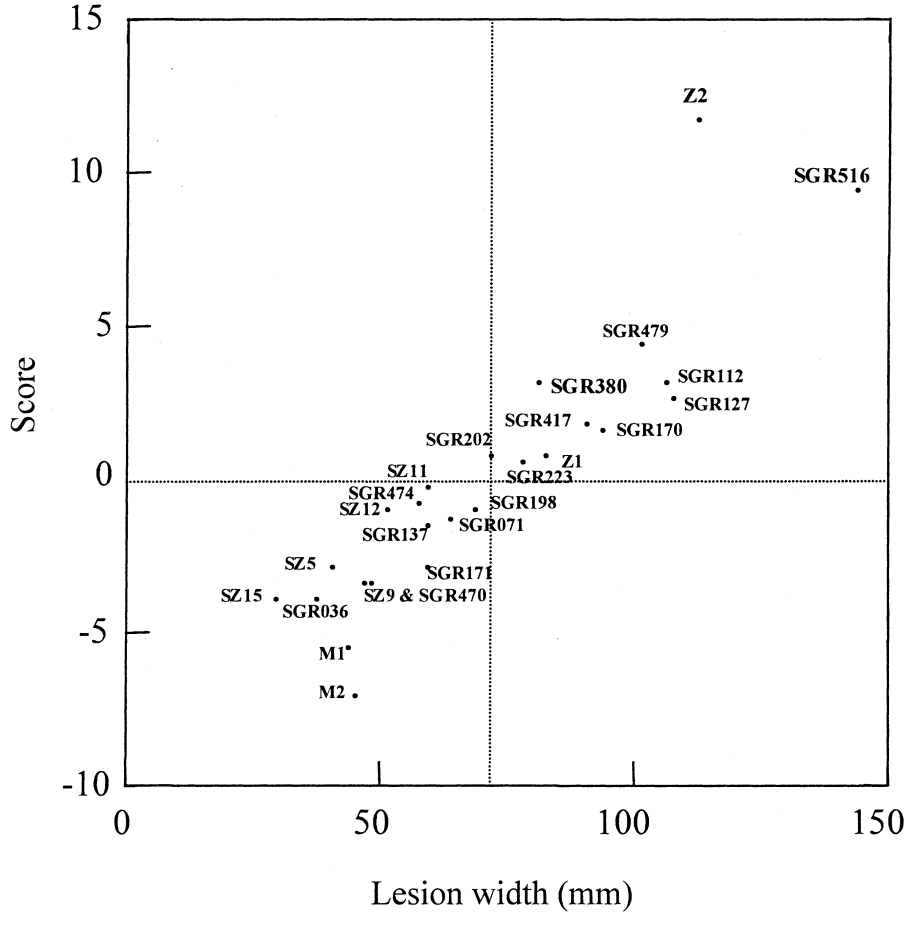

Fig. 1. Bi-plot of IPCA (Principal component analysis) scores (Y-axis) and average lesion width (mm) (X-axis) for all the clones and the four environments. Z1, average lesion width for all the clones, 6 months after inoculation in Zululand; Z2, average lesion width for all the clones 12 months after inoculation in Zululand; M1, average lesion width for all the clones, 6 months after inoculation in Mpumalanga; M2, average lesion width for all the clones 12 months after inoculation in Mpumalanga

experiments require established trees which is time consuming and costly. Alternative approaches to select disease tolerant planting stock, such as inoculation of small plants in greenhouse trials has yielded valuable and similar results to field trials (Conradie et al. 1992). Furthermore, Van Zyl and Wingfield (1999) also showed that the capacity of clones to recover from wounds caused by mechanical damage is directly correlated with their susceptibility to *C. cubensis*. In the future it might, therefore, be possible to include the capacity to recover from physical wounds as a selection criterion in breeding programmes. In addition, molecular markers linked to disease tolerance would provide an ideal method to select planting stock. We are, therefore, working towards producing such markers.

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Résumé

Effet de l'environnement sur la réponse de clones d'Eucalyptus a l'inoculation par Cryphonectria cubensis

L'ascomycète *Cryphonectria cubensis* provoque des pertes graves aux plantations d'Eucalyptus en Afrique du Sud et des programmes de sélection pour la tolérance à la maladie sont nécessaires. Le but de cette étude a été d'évaluer la sensibilitié de clones et l'effet de l'environnement sur le développement de la maladie, dans des essais d'inoculation de *C. cubensis*, plantés dans deux sites différents. Chaque essait comprenait 21 clones d'Eucalyptus (*Eucalyptus grandis, E. grandis* x *camaldulensis,* et *E. grandis* x *urophylla*). Tous les arbres ont été inoculés avec la même souche virulente de *C. cubensis* et la taille des lésions a été mesurée 6 et 12 mois après l'inoculation. Les clones differaient significativement pour leur tolerance a *C. cubensis*. Par ailleurs, la gravité de la maladie variait selon la localisation géographique de l'essai. Une interaction significative clone x localisation (génotype x environnement) a été observée. Ainsi, le tri du matériel végétal pour sa résistance à la maladie ne devrait avoir lieu que dans les zones où les clones seront utilisés.

Zusammenfassung

Der Einfluss von Umweltbedingungen auf die Reaktion von Eucalyptus-Klonen nach einer Inokulation mit Cryphonectria cubensis

Der Ascomycet *Cryphonectria cubensis* verursacht in Eucalyptusplantagen in Südafrika schwere Schäden und macht Selektionsprogramme auf Krankheitstoleranz not wending. In der vorliegenden Untersuchung wurden auf zwei Versuchsflächen mit *C. cubensis* Inokulationen an unterschiedlichen Standorten die Anfälligkeit der Klone und der Einfluss der Umwelt auf die Krankheitsentwicklung registriert. Jede Versuchsfläche enthielt 21 Eucalyptus-Klone (*Eucalyptus grandis, E. grandis* x *camaldulensis* und *E. grandis* x *urophylla*); alle Bäume wurden mit einem virulenten Isolat von *C. cubensis* inokuliert, die Grösse der Nekrosen wurde nach 6 und 12 Monaten gemessen. Die einzelnen Klone unterschieden sich bezüglich ihrer Toleranz gegenüber *C. cubensis* signifikant. Ausserden differierte die Krankheitsintensität in Abhängigkeit vom Standort. Es wurde eine signifikante Klon x Standort (Genotyp x Umwelt) – Interaktion nachgewiesen. Die Krankheitsresistenz sollte deshalb nur in Gebieten erfasst werden, wo die betreffenden Klone auch kommerziell angebaut werden.

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