Eur. J. For. Path. 29 (1999) 161–167

ISSN 0300–1237

Wound response of *Eucalyptus* clones after inoculation with *Cryphonectria cubensis*

By L. M. VAN ZYL and M. J. WINGFIELD

Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

Summary

Twenty-five different *Eucalyptus grandis* clones were artificially wounded and inoculated with a virulent isolate of *Cryphonectria cubensis*. The capacity of wounds to close through callus production was correlated with the relative susceptibility of these clones to infection by *C. cubensis*. Clones with the greatest capacity to close wounds were those that were also most tolerant to *C. cubensis* infection. Those with a lower capacity to close wounds were most susceptible to Cryphonectria canker.

1 Introduction

The forestry industry in South Africa depends almost exclusively on exotic species of *Eucalyptus* and *Pinus*. Eucalypts and pines are planted in more or less equal proportions and, together, they cover an area of approximately 1.5 million ha (ANONYMOUS 1990). *Eucalyptus* forestry in South Africa is commonly based on a monoculture system, using a relatively small number of selected clones, and this could lead to large, genetically uniform stands that are at risk from pests and diseases (WINGFIELD et al. 1991). Strategies to ensure the planting of only disease tolerant clones of *Eucalyptus* are thus of considerable importance.

Cryphonectria cubensis (Bruner) Hodges causes a serious canker disease of *Eucalyptus* spp. planted in many tropical areas of the world (BRUNER 1916; BOERBOOM and MAAS 1970; SHARMA et al. 1984; WINGFIELD et al. 1989). *Cryphonectria cubensis* has limited the planting of susceptible *Eucalyptus* spp. in Brazil, in areas where climatic conditions favour the growth and spread of the pathogen (ALFENAS et al. 1982). The relatively recent discovery of the disease in South Africa, has prompted concern that this disease could impact negatively on the planting of *Eucalyptus* clones in the country (WINGFIELD et al. 1989).

ALFENAS et al. (1983) showed that considerable inter- and intraspecific variation in resistance to Cryphonectria canker exists within the genus *Eucalyptus*. In Brazil, cankers caused by *C. cubensis* were thus minimized by the selection of *E. grandis* clones, displaying tolerance to the pathogen (CAMPINHOS and IKEMORI 1983). In South Africa, a programme has been established to select for tolerance of *E. grandis* clones to Cryphonectria canker (VAN DER WESTHUIZEN et al. 1992). However, trials to test disease tolerance of clones are time consuming, and cannot keep pace with the demand for new and improved clones. A method that could provide an earlier assessment of disease tolerance would therefore be useful.

Received: 17.7.1997; accepted: 24.8.1998; editor: G. A. Kile

U. S. Copyright Clearance Center Code Statement: 0300–1237/99/2902–0161 $14.00/0

Cryphonectria cubensis initiates disease by infecting wounds or growth cracks in the bark, and cankers expand until the trees are girdled (Hodges et al. 1979; Boerboom and Maas 1970; Sharma et al. 1985). It is proposed that Cryphonectria canker of *Eucalyptus* can potentially be managed by incorporating host tolerance, related to the development of structural barriers to pathogen ingress. A rapid, reliable and economically efficient method to detect tolerance to pathogen ingress would expedite the exploitation of new *Eucalyptus* spp. or clones with tolerance to *C. cubensis*. An understanding of the processes through which new lateral cambial tissues (both phellogen and vascular cambium) are generated, could lead to a better understanding of disease tolerance. The objective of this study was to determine whether a relationship exists between the capacity of trees to close wounds and the relative susceptibility of different *E. grandis* clones to infection by *C. cubensis*.

2 Material and methods

2.1 Trees for inoculation

Two identical plots including 25 *E. grandis* clones ranging from highly susceptible to highly tolerant to infection by *C. cubensis* (M. J. Wingfield, unpublished data) were planted in October 1992 in Zululand, KwaZulu-Natal, South Africa. One plot was used to screen for tolerance to infection by *C. cubensis*, using artificial inoculation. The second plot was used to wound trees mechanically. Plots were designed with 40 trees in each of 25 rows, and were surrounded by two border rows of untreated trees. Clones were completely randomized in the plots. The trees were maintained using standard silvicultural procedures.

2.2 Pathogenicity tests

One-year-old trees were inoculated during October of 1993, when trees are most susceptible to infection. Trees were inoculated 20 cm above the soil line, using a cork borer (10 mm in diam.). This was done to remove the bark and expose the cambium. Care was taken not to injure the xylem deeper than the cambium. Inoculum of *C. cubensis* was prepared by culturing the fungus in Petri dishes on 2% w/v Potato Dextrose Agar (PDA) at 25°C for 7 days. A 10 mm disc of PDA, overgrown with an isolate of *C. cubensis* (D21–13 from Kwambonambi, KwaZulu-Natal, South Africa) known to be virulent in previous tests, was used to inoculate 20 trees for each clone. A sterile 10 mm diameter disc of PDA was used to inoculate the remaining 20 trees for each clone that represented the controls. All wounds were covered with masking-tape to limit contamination and the inoculum from drying out. Six weeks after inoculation, the masking-tape was removed and the canker widths on each tree was measured. Data, were then expressed as mean lesion width.

2.3 Wounding study

This experiment was conducted at the same time as the pathogenicity study. All the trees were mechanically wounded, 20 cm above the soil line, using a sharpened, oval-shaped, 25 mm × 45 mm wounding instrument (Fig. 1). This wounding instrument was specifically designed to provide a relatively large wound that would not be rapidly closed by callus. Bark was removed to expose the cambium, with care being taken to avoid injury to the

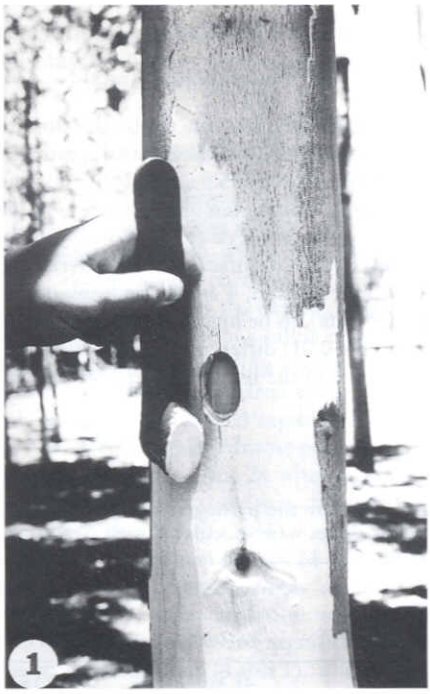

Fig. 1. Wounding tool used to remove an oval-shaped bark disc from trees

vascular cambium. *Cryphonectria cubensis* cultures were prepared by growing a virulent isolate of the fungus in Petri dishes on PDA. A 25 mm × 45 mm disc of colonized agar (similar in shape to the wounding tool), was used to inoculate 20 trees of each clone. Sterile PDA discs were used to inoculate the remaining 20 trees per clone, that served as controls. All wounds were covered with masking-tape to limit contamination and the inoculum from drying out. Six weeks after inoculation, the masking-tape covering the wounds was removed and the width of the exposed wound area between the callus tissue was measured. The circumference of each tree was measured at the time of inoculation/wounding and 6 weeks thereafter. This was done to indicate the average increase in tree diameter over a 6-week period.

2.4 Statistical analysis

The data obtained from this study were statistically analysed using a two factorial analysis of variance to assess differences in susceptibility towards Cryphonectria infection and the ability of *E. grandis* clones to close wounds. Means were tested for significance according to Tukey's procedure (Steel and Torrie 1980). Virulence of the pathogen on the various clones was correlated using Spearman's rank correlation coefficient (r_s) with the ability of these clones to close wounds (Steel and Torrie 1960). Spearman's rank correlation coefficient was also used to show the correlation between relative increase in diameter of trees and their ability to close wounds (Steel and Torrie 1960).

3 Results

3.1 Pathogenicity tests

Inoculation of clones with *C. cubensis* gave rise to the development of cankers of variable size. No Cryphonectria cankers occurred in the control treatment. Mean lesion width was greatest on clones 25 (226.7 mm) and 24 (185.5 mm) (Table 1). This confirmed the results of a previous study, using the same clones inoculated with *C. cubensis*, showing that these clones are highly susceptible to the pathogen (M. J. WINGFIELD unpublished data). Clones 22 and 23 were also highly susceptible to infection, but differed significantly ($p = 0.01$) from clones 25 and 24 (Table 1). Clones 1, 2, 3, 4 and 5 are known, from past field inoculations (M. J. WINGFIELD unpublished data), to be highly tolerant to infection by *C. cubensis*. These clones showed no significant ($p = 0.01$) differences in susceptibility amongst each other, but differed significantly ($p = 0.01$) from the highly susceptible clones (Table 1). The remaining

Table 1. **Lesion and wound widths for the pathogenicity and wounding study, 6 weeks after 25 *Eucalyptus grandis* clones were inoculated with *Cryphonectria cubensis*[1]**

Clone No.	Stem diameter increase after 6 weeks (mm)	Pathogenicity study[2] Lesion width (mm)	Wounding study[2] Width inoculated (mm)	Wounding study[2] Width control (mm)
1	42.54	41.3a	10.1a	6.9a
2	43.87	48.8ab	11.7ab	9.0abc
3	68.00	50.5abc	11.9ab	7.1ab
4	25.56	51.2abc	12.2ab	10.4abcd
5	53.76	51.4abc	12.8ab	10.9abcde
6	32.01	52.3abc	13.5abc	11.7abcde
7	59.34	52.3abc	13.3abc	11.4abcde
8	76.31	52.6abc	13.9abc	12.0abcde
9	68.90	53.9abc	14.4abc	13.7abcdef
10	42.89	56.7bcd	14.6abc	13.4abcdef
11	39.23	57.1bcd	15.0abc	13.9abcdef
12	46.57	57.4bcd	15.8abcd	14.5bcdef
13	57.82	57.7bcd	15.6abcd	14.6bcdef
14	66.00	57.9bcd	16.2abcde	14.9cdef
15	51.26	59.5bcde	16.9abcde	16.0cdef
16	35.76	62.5cde	16.8abcde	15.8cdef
17	35.08	65.2cdef	16.7abcde	15.7cdef
18	76.93	66.1cdef	17.2abcde	15.9cdef
19	23.56	70.8cdef	17.6abcde	16.1cdef
20	65.34	78.5defg	17.9bcde	16.8def
21	19.35	84.7efgh	18.3bcde	17.0def
22	36.00	95.6fgh	19.1bcde	17.5def
23	49.03	101.3gh	20.8ede	18.3ef
24	64.46	185.5h	23.7de	20.6f
25	68.94	226.7i	22.9e	20.1f

[1] Each value represents the average of 20 replications. Values in each column followed by different letters, differ significantly at $p = 0.01$ according to Tukey's procedure for comparison of means.
[2] Two identical plots of 25 *E. grandis* clones were used for the pathogenicity and wounding study. For the pathogenicity study, trees were inoculated by inserting inoculum into a wound made with a 10 mm cork borer. For the wounding study, a 25 mm wide ellipsoidal wound was made on stems, and half of these wounds were filled with inoculum. Results show widths of exposed wounds at the termination of the experiment.

clones showed no significant difference in susceptibility amongst each other, and were thus considered to be moderately susceptible (or moderately tolerant) to infection (Table 1).

3.2 Wounding study

Clones exhibited significant ($p = 0.01$) differences in their ability to close wounds, through callus production for both the inoculated and control wounding treatments. Ranking of susceptibility based on the exposed wound width between callus tissue, 6 weeks after inoculation with *C. cubensis*, was positively correlated ($r_s = 0.93$; $P = 0.01$) with the ability of clones to produce callus. Thus, the smaller the exposed wound width on clones in both the control and inoculation treatments, the more callus tissue had accumulated. This was true for both the inoculated and control wounding treatments. However, inoculated trees showed a reduced ability to close wounds compared with the uninoculated, control treatments (Table 1). In clone 1, the callus developed rapidly. The accumulation of callus was somewhat less in clones 2, 3, and 4 (Table 1). Almost no callus tissue was produced in the wounded control or inoculated susceptible clone 24 when compared with the more tolerant clones 1,2, and 3. Slightly better wound closure was displayed by clones 25, 23, and 22, respectively, when compared with clone 24 (Table 1).

The lack of substantial callus formation in clones 24 and 25, which were shown to be highly susceptible to cankering by *C. cubensis*, was significantly correlated with susceptibility in field inoculation studies, based on lesion width ($r_s = 0.94$; $p = 0.01$) (Table 1). Most of the remaining 17 clones did not show any significant differences in their ability to form callus tissue and thus to close wounds (Table 1). These clones are considered either moderately susceptible or moderately tolerant to *C. cubensis* infection. No significant correlation was observed between the wound closing ability of clones and growth of trees ($r_s = 0.051$) (Table 1).

4 Discussion

The results of this study show that an association exists between postwounding tissue changes in different *Eucalyptus* clones and their respective tolerance to infection by *C. cubensis*. Clones known to be highly tolerant to *C. cubensis*, closed wounds significantly faster (in the presence or absence of the pathogen), than those known to be highly susceptible to the pathogen. However, it was not possible to show significant differences between the clones that displayed an intermediate response to infection. Results from this study are similar to those of Wensley (1966) who examined the rate of wound closure (callus formation) in peach branches, and its relation to disease resistance. This author also found that the wound closure rate varied with date of wounding, age and diameter of the branch, the age of the tree, and the cultivar. Thus, most rapid wound closure occurred on the most canker-resistant cultivar. Wensley (1966) also concluded that more rapid wound closure was a characteristic of cultivars that were relatively resistant to infection by *Leucostoma* spp.

Most trees produce the same sequence of tissue changes in response to wounding, although timing and degree of response varies with clone and time of the year (Biggs 1984, 1985, 1986). Plant resistance is also not solely due to rates of callus production after wounding (Kolattukudy 1980; Biggs 1984, 1989; Biggs and Miles 1988). Rather, suberin accumulation is more important than the actual number of new phellem cells, or the thickness of the new suberized layers. Thus, a positive correlation exists between the rate of suberin accumulation in wounds and their ability to resist infection. It is therefore possible that the more resistant *Eucalyptus* clones, used in this study, could resist pathogen colonization, due to the deposition of thick suberin layers.

The results of this study showed that when large wounds are inoculated (wounding study), resulting cankers are less well developed, than when smaller wounds are inoculated. One explanation for this result might be that large disks of inoculum dry out rapidly and thus represent an ineffective inoculation technique. Alternatively, large wounds could result in heightened levels of response in trees, and thus less effective inoculations. This would be consistent with the findings of SCHMELZER et al. (1989), who suggested that after wounding, several signals are generated that result in high levels of expression in genes only in cells adjacent to the wound site. Such expression levels continue to decline gradually, the more distant the cells are from the injured area. From the rapid activation of numerous defence mechanisms a large variety of compounds are produced, including pigments, phytoalexins and structural compounds, such as lignin, suberin and other cell wall constituents (SCHMELZER et al. 1989; FREYTAG et al. 1994). Production and deposition of these substances in cells adjacent to the wound site, could be responsible for a restriction in pathogen ingress.

Data from this study showed clones of *E. grandis* that form callus tissue more rapidly after wounding, are less susceptible to *C. cubensis*, than those that respond less rapidly. These results have the potential for future practical application for management of canker disease in *Eucalyptus* clones. Knowledge of the duration of wound susceptibility, as influenced by host genotype and local environmental conditions, could be useful for future breeding and selection for clones of *Eucalyptus* tolerant to *C. cubensis*. Determination of the rate of wound healing in a clone could be used as an indication of its tolerance to *C. cubensis*.

Acknowledgements

We are grateful to the South African Forestry Industry and to the Foundation of Research Development for financial support. Mr G.H.J. KEMP also provided assistance.

Résumé

Réponse à la blessure de clones d'Eucalyptus après inoculation par Cryphonectria cubensis

Vingt-cinq clones d'*Eucalyptus grandis* ont été blessés artificiellement et inoculés avec un isolat agressif de *Cryphonectria cubensis*. L'aptitude des blessures à se refermer par production de cals a été corrélée avec la sensibilité relative des clones à l'infection par *C. cubensis*. Les clones les plus aptes à refermer les blessures étaient ceux qui étaient aussi les plus tolérants à *C. cubensis*, les moins aptes étaient les plus sensibles au chancre à *Cryphonectria*.

Zusammenfassung

Wundreaktion von Eukalyptus-Klonen nach Inokulation mit Cryphonectria cubensis

Es wurden 25 verschiedene Klone von *Eucalyptus grandis* künstlich verletzt und mit einem virulenten Stamm von *Cryphonectria cubensis* inokuliert. Die Fähigkeit dieser Klone zum Wundverschluss durch Kallusbildung war mit ihrer relativen Anfälligkeit gegenüber einer Infektion durch *C. cubensis* korreliert. Die Klone mit der grössten Fähigkeit zum Wundverschluss waren auch gegenüber einer *C. cubensis*-Infektion am tolerantesten. Diejenigen mit der geringsten Fähigkeit zur Überwallung waren gegenüber dem *Cryphonectria*-Krebs sehr anfällig.

References

ALFENAS, A. C.; HUBBES, M.; COUTO, L., 1982: Effect of phenolic compounds from *Eucalyptus* on the mycelial growth and conidial germination of *Cryphonectria cubensis*. Can. J. Bot. **60**, 2535–2541.

–; JENG, R.; HUBBES, M., 1983: Virulence of *Cryphonectria cubensis* or *Eucalyptus* species differing in resistance. Eur. J. For. Path. **13**, 197–205.

ANONYMOUS, 1990: The South African forestry and forestry products industry. Dept. of Environmental Affairs, Forestry and Environmental Conservation Branch, Pretoria, South Africa. Leaflet: Facts 88/89.

BIGGS, A. R., 1984: Boundary-zone formation in peach bark in response to wounds and *Cytospora leucostoma* infection. Can. J. Bot. **62,** 2814–2821.

–, 1985: Suberized boundary zones and the chronology of wound response in tree bark. Phytopathology **75,** 1191–1195.

–, 1986: Phellogen regeneration in injured peach tree bark. Ann. Bot. **57,** 463–470.

–, 1989: Effect of pruning technique on *Leucostoma* infection and callus formation over wounds in peach trees. Plant Dis. **73,** 771–773.

–; MILES, N. W., 1988: Association of suberin formation in uninoculated wounds with susceptibility to *Leucostoma cincta* and *L. persoonii* in various peach cultivars. Phytopathology **78,** 1070–1074.

BOERBOOM, J. H. A.; MAAS, P. W. T., 1970: Canker of *Eucalyptus grandis* and *E. saligna* in Surinam caused by *Endothia havanensis*. Turrialba **20,** 94–99.

BRUNER, R. F., 1916: A new species of *Endothia*. Mycologia **8,** 239–242.

CAMPINHOS, E.; IKEMORI, Y. K., 1983: Mass production of *Eucalyptus* spp. by rooting cuttings. Silviculture **8,** 770–775.

FREYTAG, S.; ARABATZIS, N.; HAHLBROCK, K.; SCHMELZER, E., 1994: Reversible cytoplasmic rearrangements precede wall apposition, hypersensitive cell death and defense-related gene activation in potato/*Phytophthora infestans* interactions. Planta **194,** 123–135.

HODGES, C. S., 1980: The taxonomy of *Diaporthe cubensis*. Mycologia **72,** 542–548.

–; GEARY, T. F.; CORDELL, C. E., 1979: The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. Plant Dis. Rep. **63,** 216–220.

KOLATTUKUDY, P. E., 1980: Cutin, suberin, and waxes. In: The Biochemistry of Plants. Ed. by STUMPF, P. K.; CONN. E. E. New York: Academic Press. pp. 571–645.

SCHMELZER, E.; KRÜGER-LEBUS, S.; HAHLBROCK, K., 1989: Temporal and spacial patterns of gene expression around sites of attempted fungal infection in parsley leaves. The Plant Cell **1,** 1191–1200.

SHARMA, J. K.; MOHANAN, C.; FLORENCE, E. J. M., 1984: Nursery diseases of *Eucalyptus* in Kerala. Eur. J. For. Path. **14,** 77–89.

STEEL, R. G. D.; TORRIE, J. H., 1960: Principles and Procedures of Statistics. 1st edn. New York: McGraw-Hill Book Co., pp. 481.

–; –, 1980: Principles and Procedures of Statistics. 2nd edn. New York: McGraw-Hill Book Co., pp. 631.

VAN DER WESTHUIZEN, I. P.; WINGFIELD, M. J.; KEMP, G. H. J.; SWART, W. J., 1992: Comparative susceptibility of *Eucalyptus grandis* clones and hybrids to *Cryphonectria cubensis*. 30th Congr. South African Soc. for Plant Pathology, Cintsa, East London, Eastern Cape Province, South Africa. 23–26 January, Phytophylactica **24,** 98.

WENSLEY, R. D., 1966: Rate of healing and its relation to canker of peach. Can. J. Plant Sci. **46,** 257–264.

WINGFIELD, M. J.; SWART, W. J.; ABEAR, B. J., 1989: First record of *Cryphonectria* canker of *Eucalyptus* in South Africa. Phytophylactica **21,** 311–313.

–; –; KEMP, G. H. J., 1991: Pathology consideration in clonal propagation of *Eucalyptus* with special reference to the South African situation. In: Proc. 1991 IUFRO Symp. Intensive Forestry, The Role of *Eucalyptus*. Durban, September 1991. pp. 811–820.