CHAPTER 3

A new species of *Cryphonectria* from South Africa and Australia pathogenic to *Eucalyptus*

ABSTRACT

Endothia gyrosa is known as the pin oak blight pathogen in North America, and also causes cankers on other trees. In South Africa and Australia the fungus has been recorded only on *Eucalyptus* spp. In a previous study, based on molecular data, Australian and South African isolates of *E. gyrosa* grouped with *C. parasitica* rather than with North American isolates of *E. gyrosa*. The aim of this study was, therefore, to compare *E. gyrosa* isolates from North America with those from South Africa and Australia and to consider whether differences observed at the molecular level can be substantiated based on morphology. Morphological features were compared from a large collection of specimens. Distinct morphological differences were observed between North American specimens and those from South Africa and Australia. In addition, growth in culture at 10 °C and 15 °C was different for those sets of isolates. Moreover, stromatal morphology in the South African and Australian specimens was more similar to that of *Cryphonectria gyrosa* than to *Endothia*. Morphological data thus confirm the differences observed at the molecular level. The fungus known as *Endothia gyrosa* from *Eucalyptus* in South Africa and Australia is consequently described as a new taxon in *Cryphonectria*.

INTRODUCTION

Endothia gyrosa (Schw.:Fr.) Fr. is a fungal pathogen best known for its association with the disease known as pin oak (*Quercus palustris* Muenchh.) blight in North America (Appel & Stipes, 1986; Roane *et al.*, 1974; Stipes & Phipps, 1971). This fungus, apparently native to North America, also causes serious cankers on exotic Formosan sweetgum (*Liquidambar formosana* Hance.) (Snow, Beland & Czabator, 1974) and other trees, including several *Quercus* spp., *Acer saccharinum* L. (Roane *et al.*, 1974), *Liquidambar styraciflua* L. (Snow *et al.*, 1974), *Castanea* spp., *Ilex opaca* Aiton (Appel & Stipes, 1986), *Fagus sylvatica* L. (Shear, Stevens & Tiller, 1917), *F. grandiflora* Ehrh., *Prunus laurocerasus* L. (Roane, 1986), *Ulmus*, *Corylus* and *Vitis* (Farr *et al.*, 1989).

In the USA, *E. gyrosa* has been reported to occur in various southeastern states (Stevens, 1917), as well as isolated areas of Kansas (Shear *et al.*, 1917; Stevens, 1917), Ohio (Appel & Stipes, 1986; Hunter & Stipes, 1978; Stevens, 1917), Michigan, Maryland, New Jersey, Connecticut, New York, California (Shear *et al.*, 1917, Stevens, 1917) and on the Pacific coast (Stevens, 1917). *Endothia gyrosa* has also been reported in China on *Quercus* (Teng, 1934) and Spaulding (1961) reported the fungus in Portugal, Spain and Italy on *Quercus* spp. and *Fagus* L. spp. Spaulding (1961) also noted that *E. gyrosa* occurred in Germany, western Europe, Ceylon, New Zealand and the Phillipines, but no hosts were recorded.

Endothia gyrosa has been reported as a pathogen of *Eucalyptus* L'Hérit from Australia (Davison & Coates, 1991; Walker *et al.*, 1985), Tasmania (Old *et al.*, 1986; Yuan & Mohammed, 1997) and South Africa (Van der Westhuizen *et al.*, 1993). There is also a single report of the fungus on *E. diversicolor* F. Muell. in Portugal (Spaulding, 1961). In southeastern and western Australia, *E. gyrosa* is found on numerous species of *Eucalyptus* (Davison & Coates, 1991; Old *et al.*, 1986; Walker *et al.*, 1985; White and Kile, 1993). The fungus was recently reported from South Africa associated with cankers on several species of *Eucalyptus*, including *E. grandis* W. Hill ex Maid., *E. nitens* (Deane & Maid.) Maid., *E. urophylla* R. T. Blake and hybrids of *E. grandis* with *E. camaldulensis* Dehnh. and *E. urophylla* (Van der Westhuizen *et al.*, 1993).

Endothia gyrosa was not originally regarded as a serious pathogen in North America (Shear *et al.*, 1917), but more recent reports describe it as relatively important (Roane *et al.*, 1974; Snow *et al.*, 1974; Stipes & Phipps, 1971). Cankers are discreet, sunken and elongated (Stipes & Phipps, 1971) with the characteristic orange stromata formed on the surface of the bark (Snow *et al.*, 1974; Stipes & Phipps, 1971). Common symptoms associated with the fungus, and more particularly with pin oak blight, are die-back, defoliation (Roane *et al.*, 1974; Stipes & Phipps, 1971), death of branches and overall tree decline (Stipes & Phipps, 1971). *Endothia gyrosa* is an opportunistic pathogen, and pruning or other wounds appear to provide infection courts (Roane *et al.*, 1974; Snow *et al.*, 1974; Stevens, 1917; Stipes & Phipps, 1971; Weir, 1925). Disease appears to be stress-related and becomes more serious when trees are

predisposed by environmental factors, such as water stress (Appel & Stipes, 1984 1986; Hunter & Stipes, 1978; Stevens, 1917).

In Australia and Tasmania, *E. gyrosa* has been found associated with elongated vertical cankers on *Eucalyptus* spp. (Walker *et al.*, 1985), cracking of the bark, kino veins (Old *et al.*, 1986), die-back of branches (Old *et al.*, 1990; Walker *et al.*, 1985), severe cankers and death of trees (Wardlaw, 1999). In artificial inoculations, the fungus was found to be pathogenic (Old *et al.*, 1986, 1990; Walker *et al.*, 1985; Yuan & Mohammed, 1999), and was especially serious in seedling inoculations (Old *et al.*, 1990). In South Africa, cankers tend to be superficial and characterized by bark cracking and slightly swollen areas on the stems (Van der Westhuizen *et al.*, 1993). The fungus on eucalypts is also an opportunist, readily infecting through wounds (Old *et al.*, 1986; White & Kile, 1993). Although water stress has not been found to predispose eucalypts to infection by *E. gyrosa*, defoliation had an aggravating effect on lesion length (Old *et al.*, 1990). Based on those results, *E. gyrosa* in Australia has been considered a mild pathogen, having no serious effect on healthy trees (Old *et al.*, 1990).

Endothia gyrosa has been known to occur in the USA from the first half of the nineteenth century (Barr, 1978; Shear *et al.*, 1917; Stevens, 1917). Its recent discovery in Australia and South Africa on a completely different host to those known in North America, is unusual. The question of the similarities and differences between the North American fungus and Southern Hemisphere *Eucalyptus* fungus was raised by Walker *et al.* (1985), who noted morphological differences between those two groups

of fungi. Those differences were mainly that the stromata of the Australian specimens were less well developed, and that the lower part or the entire perithecial body is seated in the bark and not in fungal tissue, as is observed in specimens from North America. Nonetheless, the North American and Australian specimens had similar perithecia, asci and ascospores, leading to the identification of the Australian fungus as *E. gyrosa*.

In a previous study (Chapter 2), the South African and Australian fungus was found to be different to *E. gyrosa* from North America based on PCR-based RFLPs and DNA sequence comparisons. Furthermore, the South African and Australian isolates grouped more closely with *Cryphonectria parasitica* (Murr.) Barr than with *E. gyrosa* from North America. The aim of this study was, therefore, to determine, based on morphology, whether collections of *E. gyrosa* from Australia and South Africa represent the same taxon as those from North America, and to consider their respective generic positions.

MATERIALS AND METHODS

Source of isolates

Bark samples from South Africa were collected from different localities in commercial plantations of *Eucalyptus* exhibiting typical canker symptoms (Table 1). Samples from trees in North America, used for comparative purposes, were sent by Dr. C. S. Hodges, Dept. Plant Pathology, NC State University, Raleigh, NC, USA.

Specimens of the Australian fungus, deposited by Walker *et al.* (1985) in the Plant Pathology Herbarium, Orange Agricultural Institute, were also examined (Table 1). All samples considered have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (Table 1). The type specimen of *E. gyrosa*, which had a North American origin, was found not to be useful for study (Shear *et al.*, 1917) and the co-type as listed by Shear *et al.* (1917), could not be located despite attempts to do so. Specimens of *C. gyrosa* (Berk. & Br.) Sacc., the type of *Cryphonectria*, were also examined (Table 1).

Light microscopy

Stromata together with surrounding bark tissue were removed from all bark samples using a scalpel, sectioned vertically with a razor blade, and mounted on microscope slides in lactophenol. Fifty measurements were made of all structures believed to be of taxonomic significance, including perithecia, asci, ascospores, pycnidial locules, conidiogenous cells and conidia. Due to the limited occurrence of stromata, only 30 stromata were measured for the holotype. The means and ranges of all measurements were computed. The colour designations of Rayner (1970) were assigned to standardize colours to structures.

Growth study

The growth in culture of isolates CRY 910, CRY 911, CRY 900 (South Africa) and CRY 21, CRY 906, CRY 907 (North America) were compared (Table 2). Two types

of media were used, namely 20 g/l malt extract agar (Biolab, Merck, Midrand, South Africa) and 50 g/l potato dextrose agar (Difco Laboratories, Detroit, USA). A disc 6 mm in diameter, taken from the edge of actively growing colonies, was placed in the centre of each of four 90 mm Petri dishes per isolate. Growth studies were conducted at 10 to 30 °C, with 5 °C intervals, in the dark. The colony diameters (mm) were measured daily from the third day of the experiment until the first isolates completely covered the plate on day nine. Colony diameter of each isolate was compared as an average of the four readings per isolate. Isolates are preserved in the culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Hillcrest, Pretoria, 0002 South Africa.

RESULTS

Morphological comparisons

The South African and North American fungi were found to be distinctly different from each other. The South African and Australian specimens from *Eucalyptus*, however, were morphologically indistinguishable from each other. The stromata of the North American specimens were much larger [(911-1562(-1800) μm high, (1250-)1296-1881(-2050) μm in diameter] than those of the South African specimens [(202-)389-741(-940) μm high, (186-)343-704(-868) μm in diameter]. The stromata from the North American specimens were also superficial and subclavate (Fig. 1) compared with the South African specimens where stromata were usually semi-immersed (although fully immersed and superficial stromata also occurred occasionally) (Fig.

2a, d). The ratio of the part of the stromata that are erumpent to the entire length of the stroma, is 1:2 for the South African and Australian fungus (sometimes a much as 1:4), while the ratio for the North American fungus is 1:1, since the stromata of the North American fungus are almost completely erumpent. Stromata from *Eucalyptus* were more variable in shape than those from trees in North America. Furthermore, the lower half of the stromata of the North American specimens had a typical pseudostromatic appearance with host cells distributed thoughout the stromatal tissue (Fig. 1a, d). The ectostroma and entostroma were also continuous with each other, but predominantly entostromatic. The lower parts of the stromata of the South African and Australian specimens consisted predominantly of host tissue, while the upper parts of the stromata in these specimens consisted primarily of fungal tissue (Fig. 2a, d). The South African and Australian stromata thus had typical ectostromatic discs forming the erumpent parts, and entostromatic discs containing the perithecia.

The stromatal structure of the North American *E. gyrosa* specimens were consistent with those described for *Endothia* by Micales & Stipes (1987). In contrast, the stromatal structure of the South African and Australian specimens resembled that for species of *Cryphonectria* (Micales & Stipes, 1987) rather than *Endothia*. These specimens also had stromata that were similar to the specimens of *C. gyrosa*, which is the type of *Cryphonectria*.

The pycnidial locules in the South African and Australian specimens were usually less than 10 in number, irregular and convoluted in shape (Fig. 2a, 3a). In contrast, the pycnidial stromata of the North American specimens had more than 30 locules, and

the locules were not convoluted (Fig. 1a). In most cases, the pycnidial locules in the South African specimens were large [(56-)104-368(-558) μm long, (50-)82-230(-350) μm wide] relative to the stromata, with some smaller locules also being present. The locules in the North American specimens [(47-)67-163(-279) μm long, (35-)51-107(-175) μm wide] were small relative to the stromata and were dispersed thoughout the stromata, giving them a labyrinthiform appearance (Fig. 1a). Conidia in the specimens from North America were exuded from pycnidia in large orange (15) droplets becoming sienna (15i) in colour when older. Conidia of the South African and Australian specimens were exuded either as brilliantly orange (15) or brilliantly luteous (19) spore tendrils or in orange droplets.

The conidiogenous cells in the South African specimens were longer [(4-)8-17·5(-27) μm long, 0·5-1·5(-2) μm wide; mean length/width ratio 13:1] (Fig. 2b, 3b) than those of the North American specimens [(4-)5·5-9·5(-12) μm long, 1-1·5(-2) μm wide; mean length/width ratio 7·5:1]. Furthermore, hyphae up to 71·5 μm long occurred between the conidiogenous cells in the South African specimens (Fig. 2b, 3c), but not in the North American specimens. These long hyphae often terminated in phialides, but they were never seen to produce conidia and were probably sterile. No conidiogenous cells could be seen in the Australian specimens due to the age of the specimens. The long sterile hyphae were, however, noted by Walker *et al.* (1985). Similar long hyphae have also been reported in *C. parasitica* (Shear *et al.*, 1917), although not in any other species of this group (Kobayashi, 1970; Roane, 1986; Shear *et al.*, 1917).

The perithecial bases in the South African and Australian specimens were generally dark with dark necks (Fig. 2d, 3d), while the bases of the young perithecia were usually light coloured. The perithecial bases in the North American specimens were not always as dark, or only had darkened areas around the bases of the necks (Fig. 1b), similar to young perithecia in the South African and Australian samples. The perithecial bases of the North American specimens were also situated in the stromatal tissue above the level of the bark surface, while those of the South African and Australian perithecia generally occurred below the level of the bark surface, frequently surrounded with bark tissue, and not exclusively surrounded with fungal tissue. Furthermore, the North American specimens had perithecia that often occurred together with active pycnidial locules. Perithecia were seldom found together with pycnidial locules in the South African specimens. When they did occur together, the pycnidial locules were barren.

Ascus and ascospore morphology are similar for the South African and North American fungus (Fig. 1f, 2f). Asci of the South African specimens were (12-)17·5-26·5(-34) μm long and 4·5-7(-9) μm wide. Asci of the North American specimens were shorter [(9·5-)11·5-18(-20) μm long, (2·5-)3·5-6 μm wide] than the South African asci, but longer measurements up to 30 μm has been reported for asci of the North American fungus (Barr, 1978; Shear et al., 1917). Ascospore size for the South African specimens were similar to those of the North American specimens [(4·5-)6-9(-12·5) μm long, (0·5-)1-1·5(-2) μm wide for the South African specimens; (3·5-)7·5-11(-13) μm long, 0·5-1·5(-2) μm wide for the North American specimens]. The South

African specimens also had the allantoid to cyllindrical shape of the North American specimens.

When mounting *E. gyrosa* specimens from North America in lactophenol, this mounting medium always became orange-coloured. In contrast, South African and Australian specimens did not discolour lactophenol. This discolouration of lactophenol was previously noted by Roane (1986) and is due to the pigments found in *Endothia* and *Cryphonectria*. Pigments of *E. gyrosa* from North America and other species of *Endothia* and *Cryphonectria* have previously been studied (Roane & Stipes, 1978) and differences have been observed in pigment composition for different species of *Endothia* and *Cryphonectria*. Differences in discolouration of lactophenol indicates that there are probably distinct differences in pigment contents in the North American isolates and the South African and Australian fungus from *Eucalyptus* known as *E. gyrosa*.

Growth in culture

Clear differences in colony morphology were observed between the South African and North American isolates of *E. gyrosa*. Colonies of isolates from North America were fuscous black (13''''m) to buff (19''f) to cinnamon (15'') (Fig. 4*a*, *b*) and had either a uniformly fluffy or sectored appearance (Fig. 4*b*). Colonies of the South African isolates were white and fluffy (Fig. 4*a*). Similar differences were observed on both MEA and PDA. Although not produced during the growth study, South African isolates usually produce orange pycnidia on MEA and PDA, while North American

isolates seldomly produce pycnidia. The South African isolates also grew faster at 10 °C and 15 °C than the North American isolates (Fig. 5) on both MEA and PDA.

DISCUSSION

In this study we have shown conclusively that the fungus known as *E. gyrosa* from *Eucalyptus* in South Africa and Australia is morphologically different to *E. gyrosa* from North America. This is both in terms of a wide range of structures on host tissue as well as characteristics in culture. The South African and Australian fungus is also different from other species of *Endothia* in that *E. singularis* (H. & P. Syd.) Shear & Stevens has large, red stromata [2-4 mm high, 3-5 mm in diameter (Shear *et al.*, 1917)], and *E. viridistroma* Wehm. has green stromata (Barr, 1978; Roane, 1986). These morphological observations support previously presented DNA sequence and RFLP data showing that South African and Australian isolates are distinct from North American isolates (Chapter 2).

In Chapter Two of this thesis, DNA sequnces of the ITS region of the ribosomal operon, suggested that the South African and Australian fungus is more closely related to *Cryphonectria* than to *Endothia*. Our morphological observations support this view. *Endothia* species have strongly developed, widely erumpent, sub-globose, primarily entostromatic stromata consisting primarily of pseudoparenchyma, and with perithecia occurring generally above the bark surface in a diatrypoid configuration. In contrast, *Cryphonectria* species, as well as the South African and Australian *Eucalyptus* fungus, have semi-immersed stromata consisting primarily of prosenchyma and with

distinct ectostromatal and entostromatal discs. The perithecia occur underneath the bark surface and have a valsoid appearance near the periphery of the stroma when the stroma is weakly developed. This is in accordance with previous observations by Micales & Stipes (1987).

Different hosts, bark types and environments may influence the stromatal morphology of *Endothia* and *Cryphonectria*. More specifically, the number of layers of pycnidia and perithecia can be influenced by the bark of the host, while the sequence of formation of perithecia and pycnidia appears to be influenced by climatic factors (Shear *et al.*, 1917). Moreover, the degree to which perithecia are positioned to assume a diatrypoid or valsoid configuration, appears to depend on the degree of stromatal development (Cannon, 1988). The size, structure and degree to which stromata are embedded, depends on the bark, host and external factors, such as moisture (Cannon, 1988; Hodges, Alfenas & Ferreira, 1986; Kobayashi, 1970; Micales & Stipes, 1987; Roane, 1986; Shear *et al.*, 1985). This variability due to external factors and host, was the primary argument provided by Walker *et al.* (1985) to explain the differences between Australian and North American specimens. The magnitude of differences between these fungi, is, however, great and we are convinced that these are not due to host or environmental factors. Furthermore, the fact that molecular data support our morphological observations leaves us with little doubt that these fungi represent distinct taxa.

Ascospores of the South African and Australian specimens are clearly non-septate and cylindrical to allantoid (Fig. 2*f*). This is typical of the genus *Endothia* (Roane, 1986;

Shear *et al.*, 1917). However, stromatal morphology in this fungus closely resembles that of *Cryphonectria* as described by Micales & Stipes (1987). They also resemble the structure of those in *C. gyrosa*, which is the type species of *Cryphonectria*. This is consistent with the grouping of the South African and Australian isolates with *C. parasitica* and not with *E. gyrosa* in a phylogram based on DNA sequence data (Chapter 2). We, therefore, propose that the fungus from *Eucalyptus* in South Africa and Australia should reside in *Cryphonectria* and not in *Endothia* as initially suggested. The following description is thus provided for the new taxon.

PLEASE NOTE THAT THE FOLLOWING SPECIES DESCRIPTION IS PRESENTED HERE IN PRELIMINARY FORM AND SHOULD NOT BE CITED. THE DESCRIPTION WIL BE PUBLISHED FORMALLY IN SCIENTIFIC LITERATURE AT A LATER DATE.

Cryphonectria eucalypti Venter et M. J. Wingfield, sp. nov.

Etym.: refers to the occurrence of this fungus exclusively on *Eucalyptus* spp.

Coloniae albae, plumeae, margine laevi, interdum maculis stramineis (21'd), celeriter crescentes, 90 mm diametro in minime novem diebus attingentes, temperatura faustissima 25–30 °C. Stromata in areis papulosis nunc turmis gregariis nunc individuis, interdum confluentes, in cortice subimmersa, aliquando erumpentia vel omnino immersa, multilocularia, (202-)389-741(-940) μm alta, (186-)343-704(-868) μm lata; aurantiaca (15); parte superiori eustromatica, pseudoparenchymatica, parte inferiori pseudostromatica, in centro cum prosenchymate e basibus peritheciorum vel

loculis pycnidiorum in textura hospitis formato (Fig. 2a, 2d, 3a, 3d). Infra 10 loculi pycnidiorum in quoque stromate, statura variabile (56-)104-368(-558) μm longi, (50-) 82-230(-350) μm lati, forma et directione irregulari, saepe convoluti, non-ostiolati. Cellulae in parietatibus loculorum cellulas conidiogenas efficientes, hyalinae cum textura globosa, tristromaticae, (5-)6-14(-20) μm crassae. Cellulae conidiogenae phialidosae, septatae, ramosae, hyalinae, cylindricae, apicem versus plerumque subcontractae, nonnullae basibus inflatis, (4-)8-17·5(-27) μm longae, 0·5-1·5(-2) μm latae, longitudo latitudoque in ratione fere 13:1 (Fig. 2b, 3b). Hyphae steriles inter cellulis conidiogenis, interdum apicibis phialidosis, longae, septatae, cylindricae, apicem versus subcontractae, (22-)28·5-50(-71·5) μm longae, 1-1·5 μm latae (Fig. 2b, 3c). Conidia amerosporae, oblongo-cylindraceae, allantoideae, oblongae vel ovoideae, (2·5-)3-4(-5) longa, 0·5-1 μm lata, hyalinae, longitudo latitudoque in ratione fere 3·5:1 (Fig. 2c, 3b). Perithecia sub superficie corticis ad basin stromatum immersa, 1–12 in quoque stromate, globosa, subglobosa vel triangularia, parietibus fuscis, (105-)161-245(-288) μm longa, (82-)157-291(-257) μm latae (Fig. 2d, 3d), cellulis basalibus textura epidermoidea cum a superficie videntur; parietatibus (8-)13-22(-31) μm diametro. Colla fusca, gracilia, periphysata, in stromate centralia, (65-)118-385(-630) μm longa, (31-)33-53(-65) μm lata, longitudine secundum in stromate perithecii profunditatem; canale ostiolari 20-26(-28) μm lato; pariete colli (10-)11-14(-15) μm diametro, cellulis textura intricata vel porrecta cum a superficie videntur, apicem colli versus textura porrecta; colla per texturam stromatis tortuosa, ad superficiem stromatis ut ostiola, papillae vel rostra longa cum textura stromatis aurantiaca tecta erumpentia, usque ad 170 μm supra superficie stromatis extensa. Asci multi, diffluentes, in cavitate perithecii libere natantes, cylindrici vel fusiformes, (12-)17·5-26·5(-34) μm

longi, 4·5-7(-9) μm lati, longitudo latitudoque in ratione fere 4:1, dum immaturi stipitates, unitunicati, annulis apicalibus non-amyloideis refractivis, in quoque asco 8 ascosporis (Fig. 2e, 3e). Ascosporae non-septatae, hyalinae virido-tinctae, cylindricae vel fusiformes, allantoideae vel in uno extremitate sublaxatae, extremitatibus rotundatis vel subcontractis, (4·5-)6-9(-12·5) μm longae, (0·5-)1-1·5(-2) μm latae, longitudo latitudoque in ratione fere 7·5:1 (Fig. 2f, 3e).

Colonies white, fluffy with a smooth margin, sometimes with straw yellow (21'd) patches, fast growing, covering a 90 mm plate after minimum of nine days, optimum temperature 25 – 30 °C. Stromata in papulose areas either as gregarious groups or individually, sometimes confluent; semi-immersed in bark, occasionally erumpent or totally emmersed, multilocular, (202-)389-741(-940) μm high, (186-)343-704(-868) μm wide; orange (15); upper region eustromatic, pseudoparenchymatous; lower region pseudostromatic with prosenchyma in centre, comprised mainly of perithecial bases or pycnidial locules within host tissue (Fig. 2a, 2d, 3a, 3d). Less than 10 pycnidial locules per stroma, variable in size, (56-)104-368(-558) μm long, (50-)82-230(-350) μm wide, shape and orientation irregular, often convoluted, non-ostiolate. Cells in locule walls hyaline with textura globulosa, 1-3 layers thick, (5-)6-14(-20) μm. Conidiogenous cells phialidic, septate, branched, hyaline, cylindrical, usually slightly tapered towards apex, some with inflated bases, (4-)8-17·5(-27) long, 0·5-1·5(-2) μm wide, mean conidiogenous cell length/width ratio 13:1 (Fig. 2b, 3b). Sterile hyphae, occasionally with phialidic apices present amongst conidiogenous cells, long, septate, cylindrical, slightly tapered towards apex, (22-)28·5-50(-71·5) long, 1-1·5 μm wide (Fig. 2b, 3c). Conidia amerospores, oblong-cylindrical, allantoid,

or oblong to obovoid, (2·5-)3-4(-5) long, 0·5-1 μm wide, hyaline, mean length/width ratio 3·5:1 (Fig. 3*b*, 2*c*). Perithecia embedded beneath surface of bark at base of stromata, 1-12 per stroma, globose to sub-globose to triangular, dark-walled, (105-) 161-245(-288) long, (82-)157-291(-257) μm wide (Fig. 2*d*, 3*d*), basal cells with *textura epidermoidea* in surface view; perithecial walls (8-)13-22(-31) μm in diameter. Necks dark, slender, periphysate, with a central position in the stroma, (65-) 118-385(-630) μm long, (31-)33-53(-65) μm wide, length depending on depth of perithecium in stroma; ostiolar canal 20-26(-28) μm wide, neck wall (10-)11-14(-15) μm in diameter, neck cells with *textura intricata* to *textura porrecta* in surface view, with textura porrecta at apex of neck, necks wind through stromatic tissue and emerge at stromatal surface as ostioles, papillae or long beaks covered with orange stromatal tissue, extending up to 170 μm above stromatal surface. Asci numerous, evanescent, floating freely in perithecial cavity, cylindrical to fusiform, (12-)17·5-26·5(-34) μm long, 4·5-7(-9) μm wide, mean length/width ration 4:1, stipitate when immature, unitunicate with non-amyloid, refractive apical rings; asci with 8 ascospores (Fig. 2*e*, 3*e*). Ascospores aseptate, hyaline with greenish tint, cylindrical to fusiform, allantoid or slightly wider at one end, ends rounded or slightly tapered, (4·5-)6-9(-12·5) μm long, (0·5-)1-1·5(-2) μm mean ascopore length/width ratio 7·5:1 (Fig. 2*f*, 3*e*).

Host. *Eucalyptus* species

Distribution. South Africa, Tasmania and mainland Australia.

Specimens examined

Herbarium types: Cryphonectria eucalypti: South Africa, Nyalazi, Northern Kwazulu/Natal, bark of GC747 clone of *Eucalyptus*, M. Venter, 25 February 1998, PREM 56211 (holotype); South Africa, Sabie, Mpumalanga, bark of *E. grandis*, J. Roux, August 1998, PREM 56212 (paratype); South Africa, Tzaneen, Mpumalanga, bark of *E. saligna* Smith, M. Venter, 6 February 1999, PREM 56305 (paratype); South Africa, Dukuduku, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM 56214; South Africa, Amangwe, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM56215; South Africa, Dukuduku, Northern Kwazulu/Natal, bark of *E. grandis*, M. Venter, October 1998, PREM 56216.

Previously, *Endothia* and *Cryphonectria* resided in the Valsaceae and the Gnomoniaceae, respectively, based on arrangement of perithecia (valsoid or diatrypoid), stromatic tissue type and ascospore shape and septation (Barr, 1990). Nonetheless, the stromatal morphology of these two genera is very similar (Cannon, 1988). Others were also of the opinion that these two genera are too close to reside in different families (Chen *et al.*, 1996; Walker *et al.*, 1985). The *nom. cons.* status of the Gnomoniaceae to the Valsaceae (Hawksworth *et al.*, 1996), however, placed *Endothia* and *Cryphonectria* in the same family, and supports the view that these genera are closely related. The fact that the new species, *C. eucalypti* described in this study, shares *Endothia*-like and *Cryphonectria*-like characteristics, confirms that *Endothia* and *Cryphonectria* are closely related and probably diverged recently.

In the past, emphasis has been placed on ascospore septation to differentiate *Endothia* and *Cryphonectria*. In this study, we have shown that stromatal structure is the more useful characteristic to separate the genera, and is more conserved than ascospore morphology. This is also consistent with results of molecular comparisons. Molecular studies done parallel with morphogical comparisons of available stromatal collections and herbarium specimens, should be carried out to redefine phylogenetic relationships between these two genera.

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Table 1. Specimens of *Endothia gyrosa*, *Cryphonectria eucalypti* and *C. gyrosa* used in morphological comparisons.

Identification	State	Host	Origin	Collector	Date	Herbarium allocation[1]
E. gyrosa	Anamorph, teleomorph	*Quercus phellos* L.	Raleigh, USA	L. Grand	April 1997	PREM 56218
C. eucalypti (holotype)	Teleomorph	*Eucalyptus grandis* X *camaldulensis*	Nyalazi, SA	M. Venter	February 1998	PREM 56211
C. eucalypti (paratype)	Teleomorph	*Eucalyptus grandis* W. Hill ex Maid.	Sabie, SA	J. Roux	August 1998	PREM 56212
C. eucalypti (paratype)	Teleomorph	*E. saligna* Smith	Tzaneen, SA	M. Venter	February 1999	PREM 56305
C. eucalypti	Anamorph	*E. grandis*	Dukuduku, SA	M. Venter	October 1998	PREM 56214
C. eucalypti	Anamorph	*E. grandis*	Amangwe, SA	M. Venter	October 1998	PREM 56215
C. eucalypti	Anamorph	*E. grandis*	Dukuduku, SA	M. Venter	October 1998	PREM 56216
C. eucalypti	Anamorph	*E. globulus* Labill.	Perth, Australia	M. Wingfield	August 1997	PREM 56217
C. gyrosa	Teleomorph	*Elaeocarpus dentatus* Vahl.	Omahuta Forest, New Zealand	S. J. Hughes	June 1964	DAR 14534 DAOM 93506A
C. gyrosa	Teleomorph	Dead twigs	Olinda pipe line, Maui, USA	C. L. Shear	December 1927	DAR 49895
E. gyrosa	Teleomorph	*E. saligna*	Termeil, Australia	K. Old	October 1983	DAR 49904
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	June 1984	DAR 49905
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	September 1983	DAR 49906
E. gyrosa	Teleomorph	*E. saligna*	Currowan, Clyde Mountain, Australia	K. Old	October 1983	DAR 49907
E. gyrosa	Teleomorph	*E. saligna*	Termeil, Australia	K. Old	October 1983	DAR 49909
E. gyrosa	Teleomorph	*Liquidambar* sp.	Grenada, USA	C. L. Shear & N. E. Stevens	February 1914	DAR 49897

1) PREM, National Collection of Fungi, Pretoria, South Africa; DAR, Plant Pathology Herbarium, Orange Agricultural Institute, Forest Road, Orange, N. S. W. 2800, Australia; DAOM, National Mycological Herbarium, Eastern Cereal and Oilseed Center (ECORC), Agriculture and Agri-Food Canada, Edifice Wm. Saunders Bldg. #49, Ottawa, Ontario, Canada, K1A 0C6.

Table 2. Isolates of *Endothia gyrosa* and *Cryphonectria eucalypti* used in culture comparisons.

Culture number[1]	Identification	Host	Origin	Collector	Date
CRY910	*C. eucalypti*	*Eucalyptus grandis*	Amangwe, SA	M. Venter	October 1998
CRY911	*C. eucalypti*	*E. grandis* X *camaldulensis*	Nyalazi, SA	M. Venter	February 1998
CRY900	*C. eucalypti*	*E. saligna*	Tzaneen, SA	M. Venter	February 1999
CRY906	*E. gyrosa*	*Quercus falcata*. Michx.	Raleigh, USA	C. Vernia	September 1998
CRY907	*E. gyrosa*	*Q. falcata*	Raleigh, USA	C. Vernia	September 1998
CRY21	*E. gyrosa*	*Q. borealis* Michx.	Lipman Drive, Raleigh, USA	S. Anagnostakis	-

1) Culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002.

Fig. 1. Anamorph and teleomorph states of *Endothia gyrosa*. (*a*) Anamorphic stroma (Bar = 100 μm). (*b*) Conidiogenous cells (Bar = 10 μm). (c) Conidia (Bar = 10 μm). (d) Teleomorphic stroma (Bar = 100 μm). (e) Asci (Bar = 10 μm). (f) Ascospores (Bar = 10 μm).
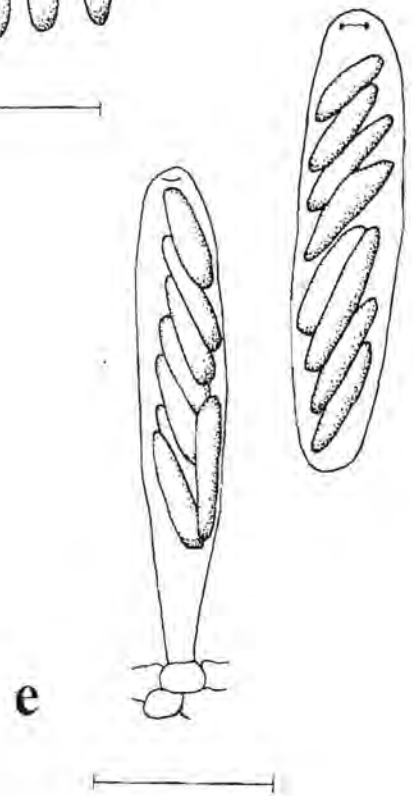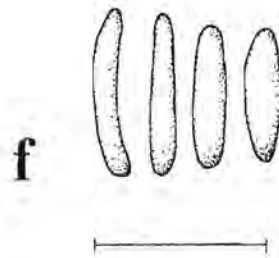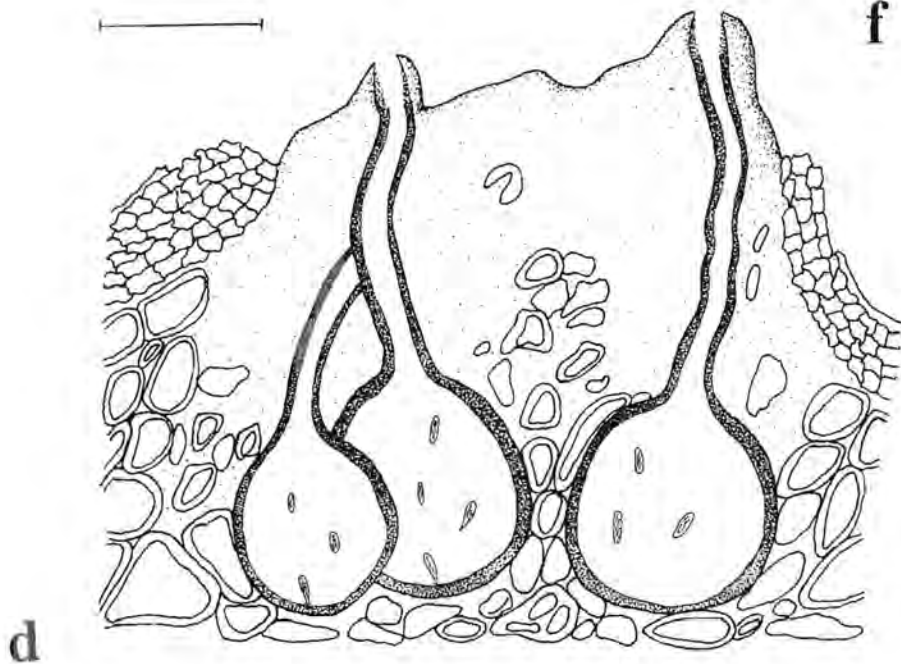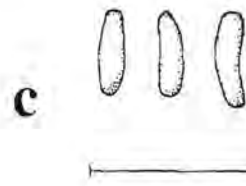
90
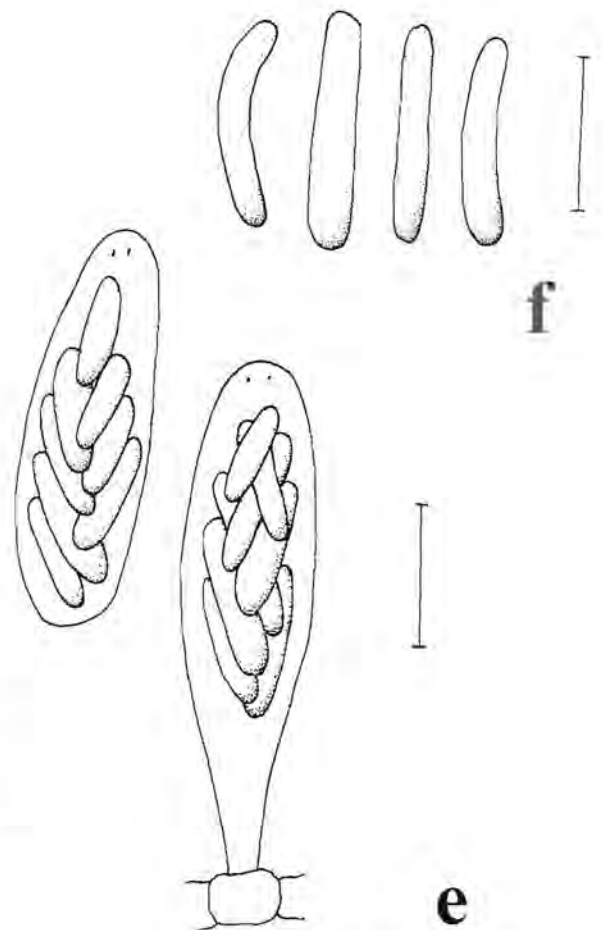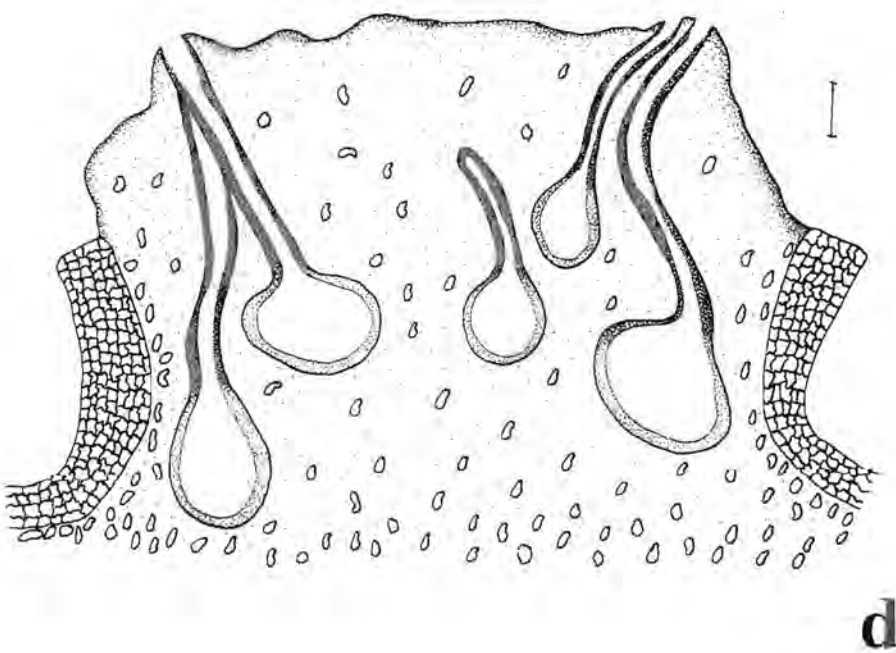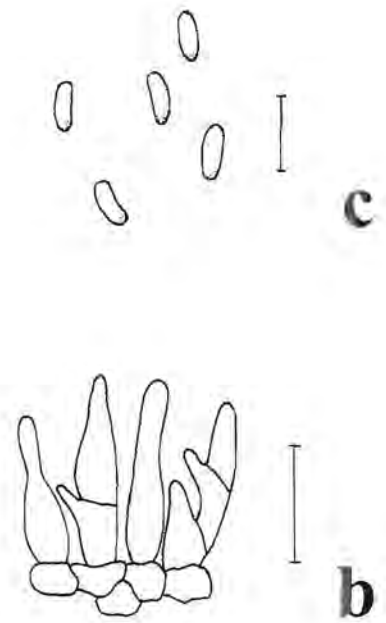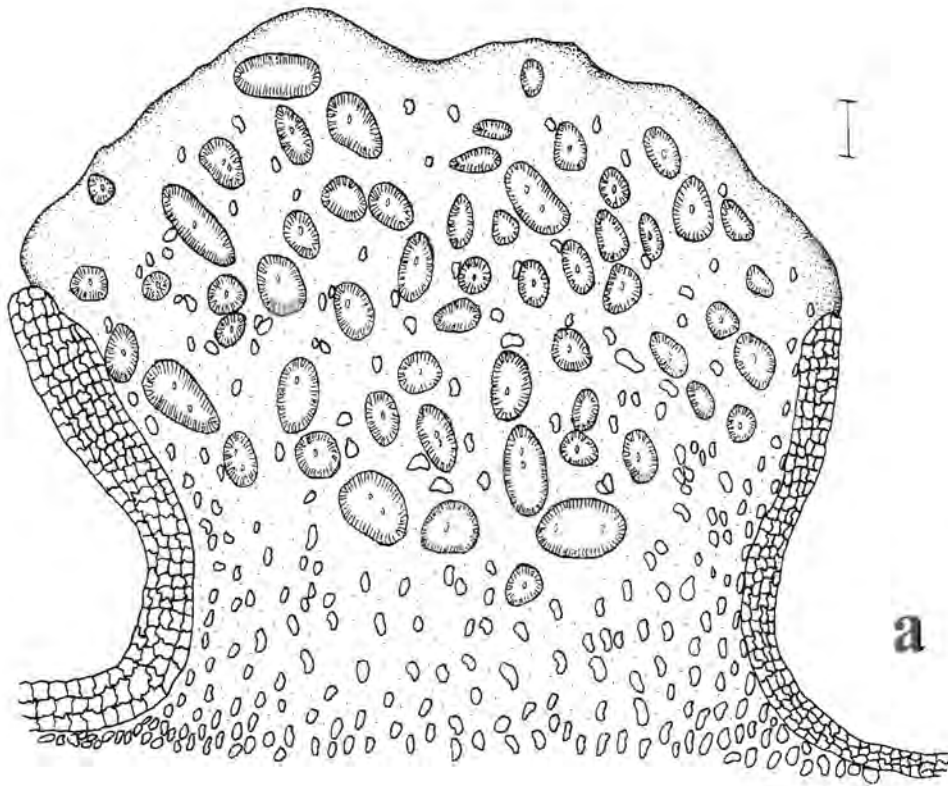
Fig. 2. Anamorph and teleomorph states of *Cryphonectria eucalypti*. (*a*) Anamorphic stroma (Bar = 100 μm). (*b*) Conidiogenous cells and sterile hyphal cell (Bar = 10 μm). (*c*) Conidia (Bar = 10 μm). (*d*) Teleomorphic stroma (Bar = 100 μm). (*e*) Asci (Bar = 10 μm). (*f*) Ascospores (Bar = 10 μm).
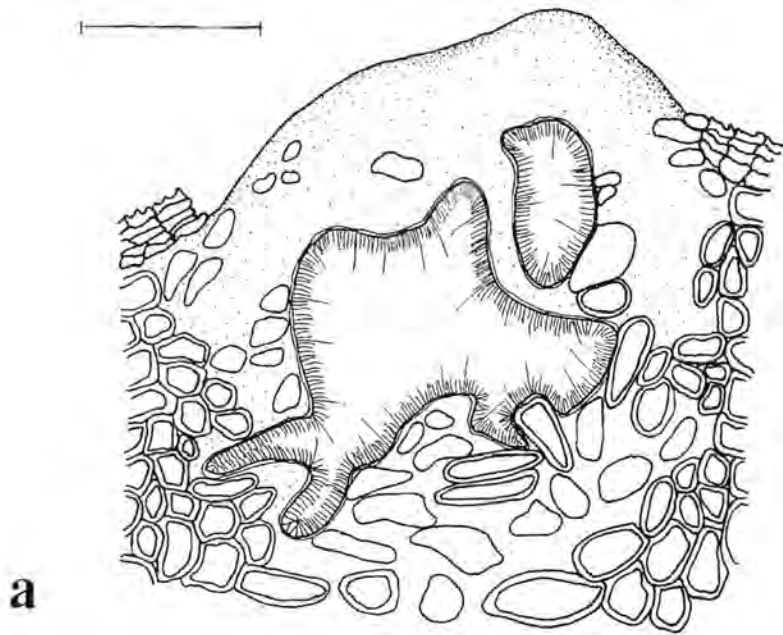
92

a

b

c

d

e

f
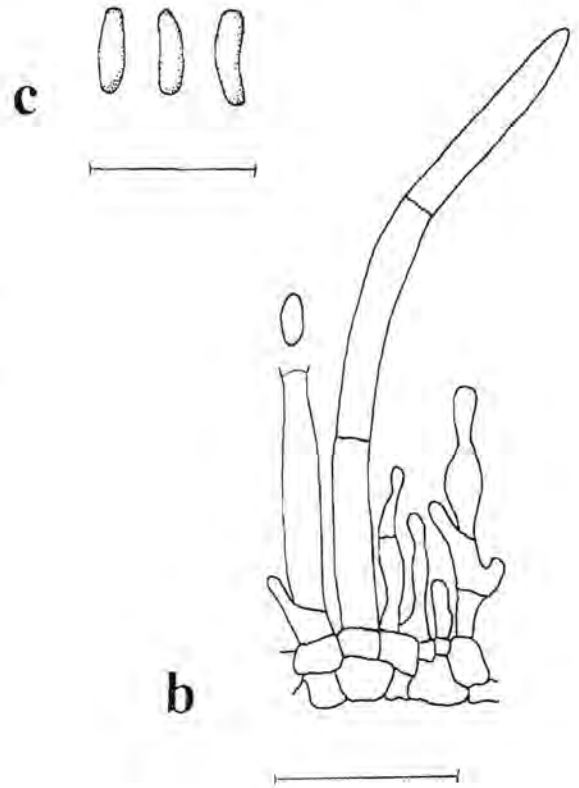
Fig. 3. Light micrographs of the anamorph and teleomorph states of *Cryphonectria eucalypti*. (*a*) Anamorph stroma containing pycnidial locules (indicated with arrow) lined with conidiogenous cells (Bar = 100 μm). (*b*) Conidiogenous cells producing conidia (Bar = 10 μm). (*c*) Sterile hyphal cell indicated with arrow (Bar = 10 μm). (*d*) Teleomorphic stroma containing perithecium (Bar = 100 μm). (*e*) Ascus containing ascospores (Bar = 10 μm).
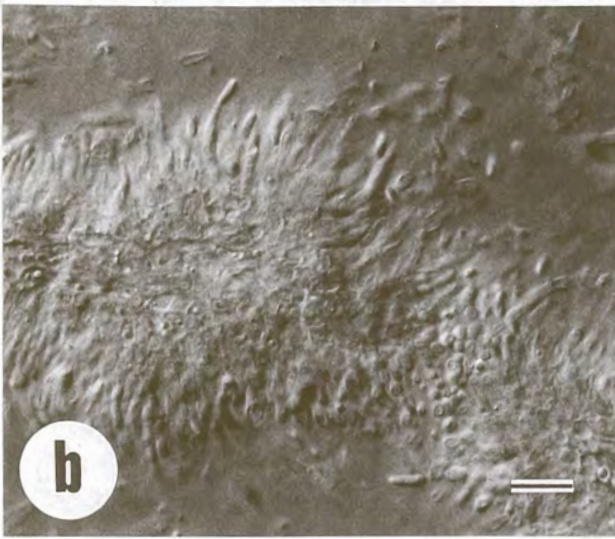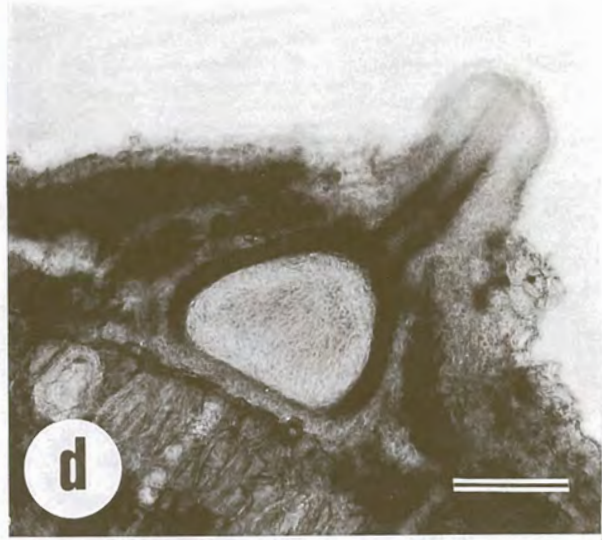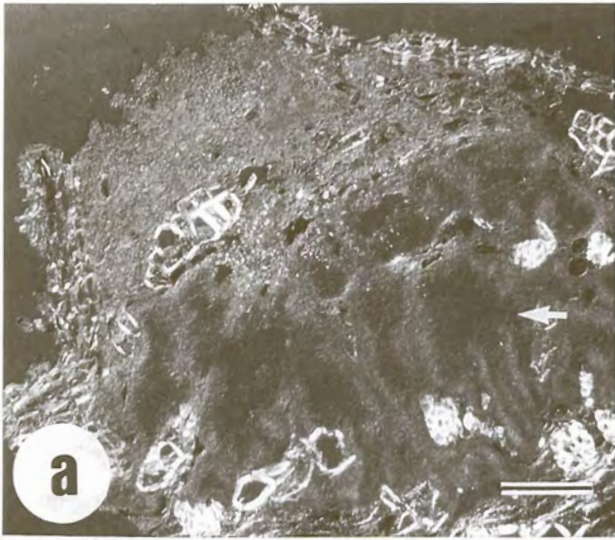
Fig. 4. (*a*) Cultures of *Endothia gyrosa* from North America and *Cryphonectria eucalypti* from South Africa on MEA and PDA. (*b*) Different growth patterns of *E. gyrosa* isolates.
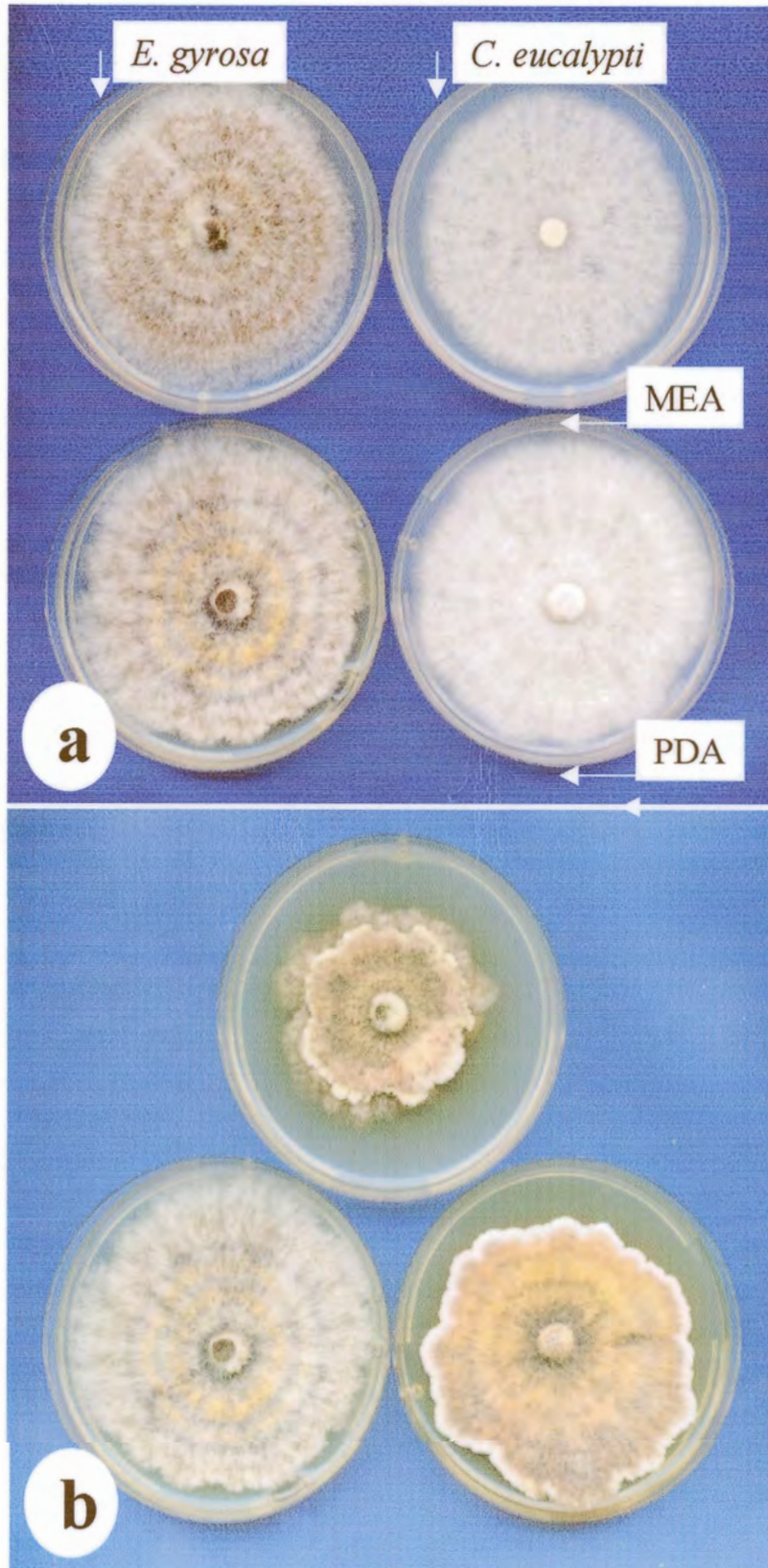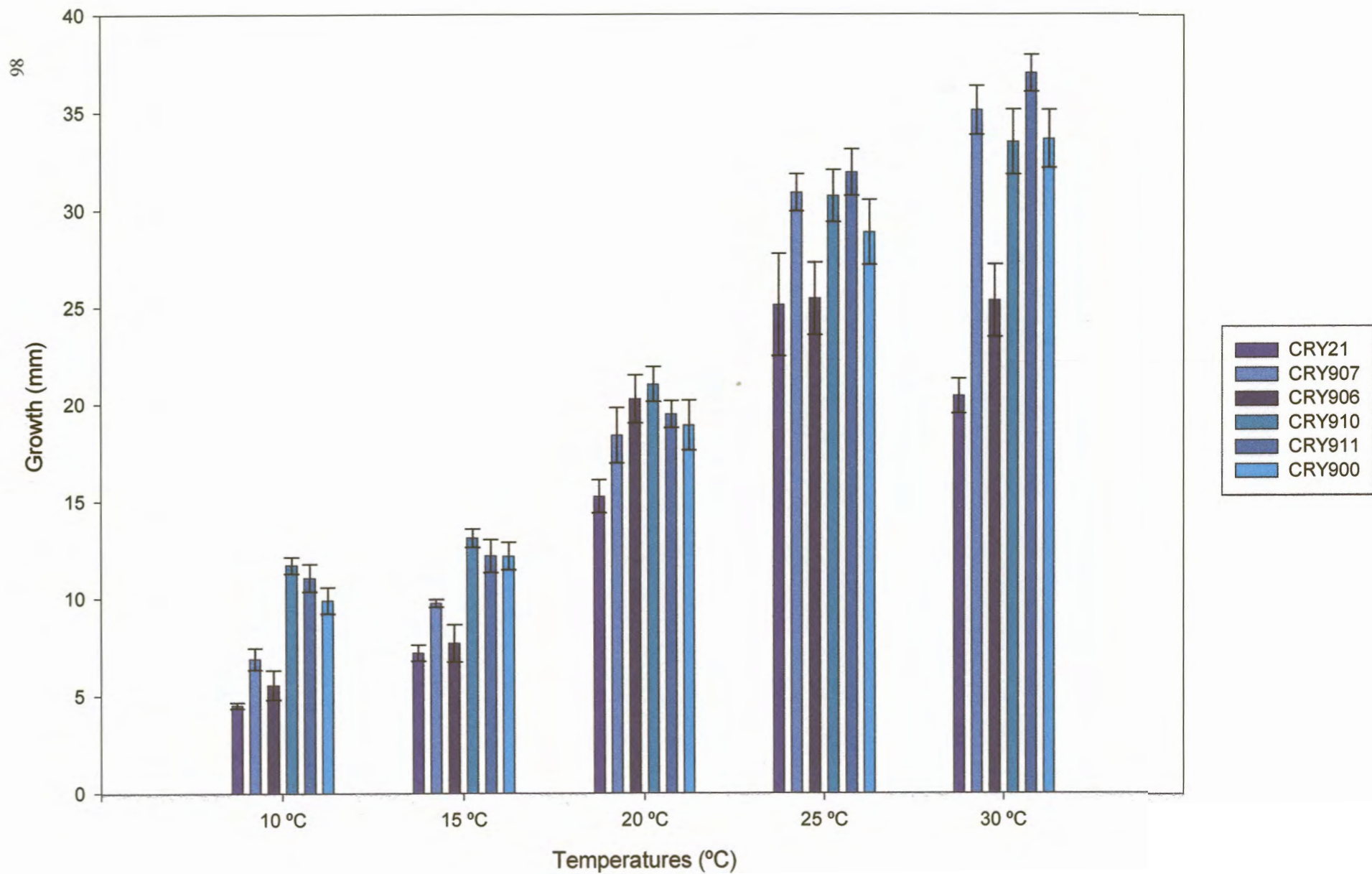
Fig. 5. Comparison of growth of isolates of *Endothia gyrosa* (CRY21, CRY 906, CRY 907) and *Cryphonectria eucalypti* (CRY 900, CRY910, CRY 911) at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C on malt extract agar (MEA). Growth is reflected as a mean of four measurements in mm after 9 days. Bars represent standard error values.

CHAPTER 4

Pathogenicity of *Cryphonectria eucalypti* to *Eucalyptus* clones in South Africa

ABSTRACT

Eucalyptus spp. are planted in many parts of the world for the production of timber and pulp, and in South Africa they form a major component of the forestry industry. The canker pathogen, *Cryphonectria eucalypti*, is pathogenic to *Eucalyptus* spp. in Australia and Tasmania and occurs in all of the major *Eucalyptus* growing areas of South Africa. This study was undertaken to consider the pathogenicity of *C. eucalypti* to *Eucalyptus* clones in South Africa. Fifteen isolates of *C. eucalypti* were initially screened for their virulence on a susceptible *E. grandis* clone (ZG14) in the field. A plot consisting of 42 different clones of *Eucalyptus* was subsequently challenged with a selected virulent isolate of *C. eucalypti* to determine whether clones differ in their tolerance to the pathogen. Lesions of varying size were produced by *C. eucalypti* in all trials, seven weeks after inoculation. Results showed that *C. eucalypti* is capable of causing significant lesions on *Eucalyptus* in South Africa and that disease development is strongly dependant on environmental factors. All of the clones tested were susceptible to *C. eucalypti*, but varying levels of tolerance to the pathogen existed. This should enable the establishment of a breeding programme aimed at reducing the impact of *C. eucalypti* in South Africa.

INTRODUCTION

Recent studies have shown that a fungus reported as *E. gyrosa* on *Eucalyptus* L'Hérit from Australia and South Africa, is not the same species as *E. gyrosa* from North America (Chapters 2, 3). Isolates from Australia and South Africa had identical RFLP (Restriction Fragment Length Polymorphisms) patterns for the ITS1, ITS2 and 5.8S regions of the Internal Transcribed Spacer region of the ribosomal RNA operon, but differed from those of North American isolates. In a phylogenetic analysis based on sequences of the ITS regions and 5.8S rRNA gene, Australian and South African isolates grouped separately from North American isolates. Moreover, the South African and Australian isolates grouped with *Cryphonectria parasitica* (Murr.) Barr in a different clade to *E. gyrosa* from North America. The molecular differences were substantiated when morphological and physiological characteristics of the fungi were considered. The South African and Australian fungus from *Eucalyptus* was consequently described as a new species of *Cryphonectria*, namely *C. eucalypti* Venter & M. J. Wingfield (Chapter 3). *Endothia gyrosa* from *Eucalyptus* will, therefore, be referred to as *C. eucalypti* for the remainder of this chapter.

Typical symptoms of *C. eucalypti* infection on *Eucalyptus* in Australia included bark cracks and cankers with associated exudation of kino, die-back of coppice shoots, branches and stems, and in severe cases, tree death (Old *et al.*, 1986; Walker *et al.*, 1985). Based on pathogenicity trials, *C. eucalypti* was viewed as a mild pathogen in the absence of stress, although it may cause girdling and death of seedlings (Old *et al.*, 1986, 1990). *Endothia gyrosa* has, however, been associated with severe cankers on *E. nitens* (Deane & Maid.) Maid. in Tasmania (Wardlaw, 1999). In that situation, annual cankers developed in the bark,

while more severe cankers also extended into the cambium. The annual cankers were shed from trees with the bark, thereby causing little damage. Infections reaching the cambial area, however, caused die-back and tree death. Furthermore, in pathogenicity trials in Australia, *C. eucalypti* was amongst the most pathogenic species in a test that included a *Phoma* sp. and *Seridium eucalypti* Nag Raj (Yuan & Mohammed, 1999).

In South Africa, cankers caused by *C. eucalypti* are usually superficial on the bark (Fig. 1) (Van der Westhuizen *et al.*, 1993). These cankers rarely cause kino exudation or damage to the cambium and have no apparent influence on timber quality or yield. *Cryphonectria eucalypti* has, however, recently been found associated with serious cankers extending into the cambium of *E. smithii* Denn. ex Smith in the Mooirivier area near Pietermaritzburg (Dr. J. Roux, personal communication), and it was closely associated with stunted *Eucalyptus* seedlings near Tzaneen.

Eucalyptus spp. make up approximately 40 % of forestry plantations in South Africa (Anonymous, 1998). Vegetative propagation of *Eucalyptus* clones to improve timber quality and yield, is widely practiced (Denison & Kietzka, 1993; Leakey, 1987). Clonal propagation, however, increases the threat of disease outbreaks and a clear understanding of such problems is needed in order to reduce risks (Chou, 1981; Wingfield *et al.*, 1991). The conflicting reports regarding the potential for *C. eucalypti* to cause disease and death of *Eucalyptus* spp. prompted us to investigate its pathogenicity to *Eucalyptus* clones in South Africa. The aim of this study was, therefore, to test the virulence of several isolates of *C. eucalypti* on a clone of *Eucalyptus* in field inoculations. A virulent isolate of the fungus was

then selected and used to screen a larger number of clones to determine whether they display differences in tolerance.

MATERIALS AND METHODS

Source of isolates

Stromata associated with typical cankers on *Eucalyptus* spp. from commercial plantations in various regions of South Africa, were collected. Isolations were made by placing the stromata on malt extract agar (Biolab, Merck, Midrand, South Africa). Fifteen isolates were randomly chosen for the initial inoculations (Table 1). Isolates were maintained on 2% malt extract agar at 4 °C. All isolates are preserved in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Screening of isolates

Inoculations were done on the tree trunk using the technique described by Van der Westhuizen *et al.* (1993). A negative control consisting of a sterile MEA plug was also included. Lesions were measured by removing the bark and exposing the cambial discolouration, since no external discolouration of the bark around the lesions was visible on the particular clone used. Reisolations were conducted at the time when lesion lengths were recorded.

Inoculations with fifteen isolates of *C. eucalypti* were done in a two-year-old coppice stand of a *Eucalyptus grandis* W. Hill ex Maid. clone (ZG14) at the Flatcrown farm near KwaMbonambi, northern Kwazulu/Natal. Twenty trees per isolate were inoculated in January 1998 and lesion lengths were measured seven weeks later. A second inoculation consisting of two blocks of trees of the same clone at different locations in the same compartment, was done in March 1998 with the same isolates used in the first inoculation, and results were recorded after seven weeks. Trees in two different blocks were inoculated in order to determine whether the variation in the data obtained for isolates in the first inoculation, was linked to the isolates or the environment. Combined analysis of variance were performed on all data. Combining experiments, with identical treatments, in an appropriate ANOVA, is a valid analysis (Freeman, 1973). Differences between the means of isolates were evaluated using appropriate multiple comparison methods (Tukey's Multiple Range and the t-test) (SAS, 1989).

Possible interactions between isolates and the environment were investigated by means of an AMMI analysis (Additive Main Effects and Multiplicative Interaction Model) (Gauch & Zobel, 1996). This analysis makes it possible to analyze the interaction between isolates and environment and employs a principal component analysis of the interaction sums of squares. In this way the interaction is separated into a predictable portion (usually the first principal component), the rest being relegated to unpredictable noise (Eisenberg *et al.*, 1996). The first principal component provides score values for isolates and trials and these are plotted against the overall means of the isolates and trials. The score values are represented on the Y-axis and their deviation from the zero line (positive or negative), reflects the degree of interaction which is exhibited by an isolate or a trial. All control treatments resulted in zero values for

lesion development. These were not included in the AMMI analyses because they would not contribute to the evaluation of the interaction.

Screening of Eucalyptus *clones*

One isolate with a high level of virulence (CRY920), was selected from the isolate screening trial for inoculation of 42, eighteen-month-old clones located on the Amangwe estate near KwaMbonambi, northern Kwazulu/Natal. The 42 clones were planted in a randomized block design consisting of ten blocks, in which each clone was planted once. Each block consisted of two identical rows. Four extra rows, identical to the design of the first block, were planted at the side of the trial. One of these extra rows was used to inoculate a negative control using sterile agar plugs, and all other rows (thus maximum 23 trees per clone) were inoculated with the test fungus. The trial was surrounded by two border rows of trees.

Inoculations were made in October 1998 and lesions measured in December 1998. Both external bark lesions as well as lesions at the cambium surface developed in all the inoculated trees unlike the ZG14 clones where no external bark lesions were visible. Lengths and widths of external lesions were measured for all trees, since reading of internal cambial lesions resulted in too much damage to the tree. The experiment could not be repeated due to the cost and complexity of establishing such a large clonal trial. The same trees could not be used for inoculation since *E. gyrosa* is an endophyte of *Eucalyptus* (personal communication, Dr. Dennis Wilson) and would persist in the host tissue long after inoculation. This may influence the data to be more variable.

Measurements were analyzed with one and two way analysis of variance. Differences between the clones were evaluated using multiple comparison methods (Tukey's Multiple Range and the t-test) (SAS, 1989).

RESULTS

Screening of isolates

Two weeks after inoculation, localized cracking and kino exudation were visible around the inoculation points (Fig. 2*a*). No symptoms were visible in the control trees (Fig. 2*b*). After seven weeks, *C. eucalypti* was found sporulating on the surface of most lesions that developed after inoculation. No external discolouration of the epidermis was produced and lesions consisted only of kino veins in the cambium (Fig. 2*c*) that resulted in a swelling on the bark surface. Reisolations consistently resulted in the recovery of the inoculated fungus.

The lengths of cambial lesions were measured and used in statistical analyses since the lesions remained reasonably constant in width (30 mm) throughout the trial. Significant differences (Pr > 0·0001) between lesion lengths were observed for the two inoculation times and for the two separate blocks used in the second inoculation (Table 2). The first inoculation had the greatest mean lesion length for all trees (136·9 mm), while mean lesion length in the second block was lower (82·6 mm) than the first block (103·8 mm) of the second inoculation.

Significant differences existed between isolates in all inoculations (Pr > 0·0001) (Table 2, Fig. 3-6). Cambial lesions varied from 15 mm (CRY924) to 285 mm (CRY920) in length for

all three inoculations. All lesions differed significantly in size from the control (Pr>0·0001), which produced no or only small lesions. Means per isolate for all inoculations combined were of the same size order and represented a continuum of values between 132 mm and 100 mm when ranked from the highest to the lowest overall mean for the three data sets combined. In this ranking, lesion lengths for isolates did not differ significantly from those directly above or below them in the ranking. Isolates that ranked low, however, did differ significantly from those with a high ranking. Due to the continuous change between values, it was not possible to establish distinct groupings based on the virulence of the isolates.

Lesion lengths were, however, grouped in arbitrary clusters based on size. These groupings were done to define the virulence of each isolate since the isolates did not form natural groupings. In these clusters, isolates fell in three relative virulence groups, namely a highly virulent group (120-132 mm), a medium virulent group (110-119 mm) and a low virulent group (100-109 mm). Based on this ranking, isolates CRY916, CRY923, CRY920 and CRY924 were the most virulent. Three groups and close size order of lesion lengths were also observed for each of the two inoculation studies separately, including the two separate blocks inoculated on the same date.

Ranking positions of isolates based on mean lesion length differed for each of the three inoculation events. For example, isolate CRY917 ranked highest in the first inoculation (Fig. 3), but was fifteenth in the first block of the second inoculation (Fig. 4), sixth in the second block of the second inoculation (Fig. 5), and eighth in the ranking based on overall mean for all three inoculations (Fig. 6). Some lesion lengths were consistently above the average lesion size for all isolates (e.g. CRY923, CRY916), while others were either below or above

average size (e.g. CRY921, CRY912, CRY902) (Fig. 3-5). Some isolates (e.g. CRY923 in the high pathogenicity group and CRY925 in the medium pathogenicity group) held constant positions in the groups, but others, such as CRY917, was different in its position in groups, for each inoculation. This shift in ranking made selection of the most pathogenic isolates difficult, and ultimately this choice needed to be relatively arbitrary.

Combined analysis of variance for the results of the three isolate inoculations showed a significant interaction of experiment with isolates (Pr>0·0001) (Table 2). This was confirmed when the different mean lesion lengths for each inoculation were plotted graphically (Fig. 7). The slopes of each graph representing an isolate, differed from those of the graphs for the other isolates because the pathogenicity of isolates did not vary uniformly between inoculations. Most graphs for isolates showed a decrease in mean, except for those representing the control, which showed an increase for block one of the second inoculation trial (Fig. 7a), and CRY916 (Fig. 7b) and CRY917, which showed a decrease for block one of the second inoculation trial. The means for each isolate in the first inoculation trial differed significantly in most cases from the means of each of the blocks of the second inoculation trial. The majority of means for the two blocks of the second inoculation trial, differed significantly from each other, even though the two blocks of the second inoculation were established at the same time. Only the control inoculation showed no significant difference between the three inoculation trials. The differences in slopes in the graphs (Fig. 7) likely indicates interaction with the environment. This finding is further supported by the difference in ranking of isolates for each inoculation.

Analysis of data using the AMMI model (Fig. 8) showed that some isolates exhibited greater interaction with external factors, while others were more stable. All isolates, having the same size order of lesions, could be divided into three groups: a group with little interaction (score values 0±1), a group with moderate interaction (±1 to ±2), and a group showing high levels of interaction (±2 to ±4). Isolates CRY916, CRY917 and CRY923 showed a high degree of interaction, while isolates CRY922, CRY913 and CRY925 showed little interaction. Each of the three inoculation trials also had high interaction with external factors. The first block of the second inoculation trial had a negative value (-5·8), while the first inoculation and the second block of the second inoculation had positive values (1·9 and 3·9 respectively) (Fig. 8).

Screening of Eucalyptus *clones*

Cambial lesions, as well as external bark discolouration (Fig. 2*d*), were produced on all of the clones. Significant differences in lesion length were observed between blocks within the trial (Pr>0·0321), as well as between the different clones (Pr>0·0001) (Table 3, Fig. 9). Based on length, width and area (length x width), means for each clone formed a continuum of values in the mean rankings. Thus, no clone differed significantly from the clone directly below or above it in ranking. Levels of tolerance to *C. eucalypti* were, however, visible because clones with a low ranking differed significantly from those with a high ranking. Four tolerance groups were identified in the rankings based on the length, width and area of lesions. For example, the four groups based on the mean lesion area were: highly susceptible (4039·5 - 4900·3 mm), moderately susceptible (3032·2 - 3776·0 mm), moderately tolerant

(2011·5 - 2951·0 mm) and highly tolerant (1216·3 - 1931·1 mm) (Fig. 9). The controls formed no lesions in any of the inoculations conducted.

Rankings of the clones based on lesion width and lesion area, were similar, especially in the highly susceptible and highly tolerant groups, but differed from the rankings of the measurements based on length. In a correlation analysis, rankings based on width and area of lesions were more similar to each other (r = 0·91) than the rankings between length and area (r = 0·71), while those between length and width were poorly correlated (r = 0·39). Lesion area appeared to be the best measure for tolerance in this case, and was thus chosen for assessing the tolerance of the clones due to the high correlation with lesion width. Based on the rankings of lesion area, clones GC 575, GU115 and GC962 were the most tolerant, while clones GC747 and GC796 were the least tolerant to infection by *E. gyrosa* (Fig. 9).

DISCUSSION

Results of this study show that *C. eucalypti* is capable of causing distinct lesions on *Eucalyptus* spp. in South Africa. High levels of interaction between isolates, host and environment also exist. In South Africa, symptoms associated with *C. eucalypti* (reported as *E. gyrosa*) in the past have been of superficial cracks on the surface of tree bark (Van der Westhuizen *et al.*, 1993), with no cambial cankers. The interaction that exists for *C. eucalypti* isolates with the environment could explain the enigma surrounding the fact that *C. eucalypti* is generally not a serious pathogen in field situations, but causes distinct disease in inoculation studies. In Australia and Tasmania, *C. eucalypti* is reported to be capable of causing tree death (Walker *et al.*, 1985; Wardlaw, 1999), although it most commonly causes

annual cankers (Yuan & Mohammed, 1997). Results of this pathogenicity study, as well as recent reports of *C. eucalypti* associated with severe stem cankers in the Tzaneen and Mooirivier areas, suggest that *C. eucalypti* is more important in South Africa than previously recognized.

In South Africa, *C. eucalypti* occurs in all the major *Eucalyptus* growing areas and has also been reported from Swaziland (Dr. J. Roux, personal communication). The fungus has also been found on numerous species of *Eucalyptus* in the country (Van der Westhuizen *et al.*, 1993). In the current inoculation trials, lesions were produced on all 42 clones tested. *Cryphonectria eucalypti* has also been found sporulating on ZG36, TAG524, GC748, GC747 and TAG555 prior to inoculation. This wide host range is consistent with the situation in Australia, where *C. eucalypti* is reported on 20 *Eucalyptus* spp. (Davison, 1982; Davison & Coates, 1991; Old *et al.*, 1986; Walker *et al.*, 1985; Wardlaw, 1999; White & Kile, 1993).

Cryphonectria eucalypti has been found to persist for one year in lesions associated with field inoculations in Australia (Old *et al.*, 1986). This is probably explained by the fact that the fungus is an endophyte in the bark and twigs of some *Eucalyptus* spp. (Dr. Dennis Wilson, personal communication). *Botryosphaeria dothidea* Ces. & De Not., which causes serious disease after the onset of stress conditions such as drought, hot winds and frost, is also an endophyte of *Eucalyptus* spp. (Smith *et al.*, 1996a; Smith, Wingfield & Wingfield, 1996b). The isolate-environment interaction exhibited by the *C. eucalypti* isolates, and its endophytic status makes this pathogen similar to *B. dothidea*.

Variation in results and low levels of reproducibility for pathogenicity tests using *E. gyrosa*, have been encountered before (Old *et al.*, 1986). The differences in lesion length for the isolate trials could be explained by interactions between isolates and external factors, including the host. This was confirmed by the AMMI analysis. Based on the production of significantly large lesions in the artificial inoculations, this fungus can thus be aggressive in conditions conducive to disease. This is typical of facultative pathogens of woody plants (Colhoun, 1973; Schoeneweiss, 1975, 1981).

The environment, for instance different temperatures and the availability of water, can interact and influence the resistance of a host to disease development by facultative pathogens (Colhoun, 1973; Schoeneweiss, 1975, 1981). *Cryphonectria eucalypti* was found on virtually every tree in a very dry compartment in the Tzaneen region of South Africa, and was also very common in one of the coldest compartments in that same area. In Australia, trees weakened by defoliation were found to be more susceptible to *C. eucalypti* (Old *et al.*, 1990). In the same study, water stress did not predispose the trees to *C. eucalypti*, although it was thought that the *Eucalyptus* trees used in that study might have had a high degree of tolerance to water stress. It is, therefore, difficult to name a single factor that may have been responsible for the interaction shown by the statistical analyses of the data in this study. It is more likely that a combination of factors, unique for each environment, may predispose a tree to disease (Colhoun, 1973).

Determining the level of interaction of an isolate with external factors is crucial when selecting an appropriate isolate for disease screening. Selection of an isolate producing large lesions may not be the best choice if that isolate shows a high level of interaction, since it

will give more variable results. Selecting an isolate with low interaction would in all likelihood yield more stable results (Eisenberg *et al.*, 1996; Gauch & Zobel, 1996). This, however, needs to be determined experimentally for each pathogen.

Isolates showing a high degree of interaction may not be pathogenic in a particular environment, but may become serious elsewhere. This is important in determining the potential damage that a pathogen might cause. Genotype x environment interaction is well recognized in forestry (Matheson & Cotterill, 1990; Basford & Cooper, 1998) and is supported by the AMMI model. The pathogenicity of an isolate with a positive score value is augmented in environments with positive score values, for instance CRY 916 in the first inoculation and second block of the second inoculation (Fig. 8). This might have been the situation in the *E. nitens* stand in Tasmania where severe cankers led to tree death (Wardlaw, 1999). Environments with negative score values will have a diminishing effect on pathogenicity for isolates with positive score values (Eisenberg *et al.*, 1996).

Different levels of susceptibility to *C. eucalypti* have previously been observed for five species of *Eucalyptus* (Old *et al.*, 1986; Yuan & Mohammed, 1999). The varying levels of tolerance observed for different clones in the present study indicates that a selection and breeding program to develop trees tolerant to *C. eucalypti* could be established. Such programmes have already been established for *Cryphonectria cubensis* (Bruner) Hodges in South Africa (Denison & Kietzka, 1993) and Brazil (Alfenas, Jeng & Hubbes, 1983) as well as for Eucalyptus rust in Brazil (Dianese, Moraes & Silva, 1984). The endophytic nature of *E. gyrosa* also requires further study.

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Table 1. Isolates of *Cryphonectria eucalypti* used in the inoculation trials.

Isolate number[a]	Host	Origin[b]	Collector	Date
CRY902	*Eucalyptus* sp.	Amangwe	J. Roux	July 1997
CRY912	*Eucalyptus* sp.	Amangwe	J. Roux	July 1997
CRY913	*E. grandis*	Dukuduku	J. Roux	July 1997
CRY914	*E. grandis*	Dukuduku	J. Roux	July 1997
CRY915	*E. grandis*	Sabie	M. Venter	December 1997
CRY916	*Eucalyptus* sp.	Futululu	J. Roux	July 1997
CRY917	*E. grandis*	Graskop	M. Venter	December 1997
CRY918	*Eucalyptus* sp.	KwaMbonambi area	J. Roux	July 1997
CRY919	*Eucalyptus* sp.	KwaMbonambi town	J. Roux	July 1997
CRY920	*Eucalyptus* sp.	Nseleni	J. Roux	July 1997
CRY921	*Eucalyptus* sp.	Nyalazi	J. Roux	July 1997
CRY922	*Eucalyptus* sp.	Nyalazi	J. Roux	July 1997
CRY923	*E. grandis*	Piet Retief	M. Venter	December 1997
CRY924	*E. saligna* Smith	Tzaneen	J. Roux	October 1997
CRY925	*E. saligna*	Tzaneen	J. Roux	October 1997

a) Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

b) All areas or estates other than Tzaneen, Piet Retief, Graskop and Sabie, are in KwaZulu/Natal, while the former regions are in the Mpumalanga provence.

Table 2. Combined ANOVA for lesion length measurements of the three inoculation sets of the ZG14 trial with *Cryphonectria eucalypti*.

Source	SS	df	MS	F	p
Isolate	604184·8	15	40279·0	30·6	0·0001
Inoculation	353943·9	2	176972·0	130·4	0·0001
Isolate x inoculation	117328·6	30	3911·0	2·97	0·0001

SEM for inoculations = 9·2

SEM for isolates = 21·0

SEM for isolates x inoculation interaction = 36.3

Table 3. Two way ANOVA for lesion length, width and area (l x b) associated with *Cryphonectria eucalypti* inoculations of 42 *Eucalyptus* clones.

Source	SS	df	MS	F	P
Block (length)	4198·7	10	419·9	2·0	0·0321
Clone (length)	40121·4	41	978·6	4·7	0·0001
Block (width)	3849·1	10	384·9	2·3	0·0137
Clone (width)	40607·3	41	990·4	5·8	0·0001
Block (area)	44396136·0	10	4439613·6	2·6	0·0055
Clone (area)	319068267·8	41	7782152·9	4·5	0·0001

Length: SEM (Clones) = 4·4

Width: SEM (Clones) = 3·9

Area: SEM (Clones) = 398·2

Fig. 1. Disease symptoms associated with *Cryphonectria eucalypti* on *Eucalyptus* in South Africa. (*a*), (*b*) Longitudinal cracking of the bark associated with cankers caused by *C. eucalypti*. (*c*), (*d*) Orange stromata of *C. eucalypti* sporulating around cankers.

Fig. 2. Lesion types associated with inoculation by *Cryphonectria eucalypti* into *Eucalyptus grandis* clone ZG14. (*a*) Cracking and kino exudation around inoculation point. (*b*) Healthy control inoculation. (*c*) Cambial lesions produced in the cambium. (*d*) Discolouration of the bark.
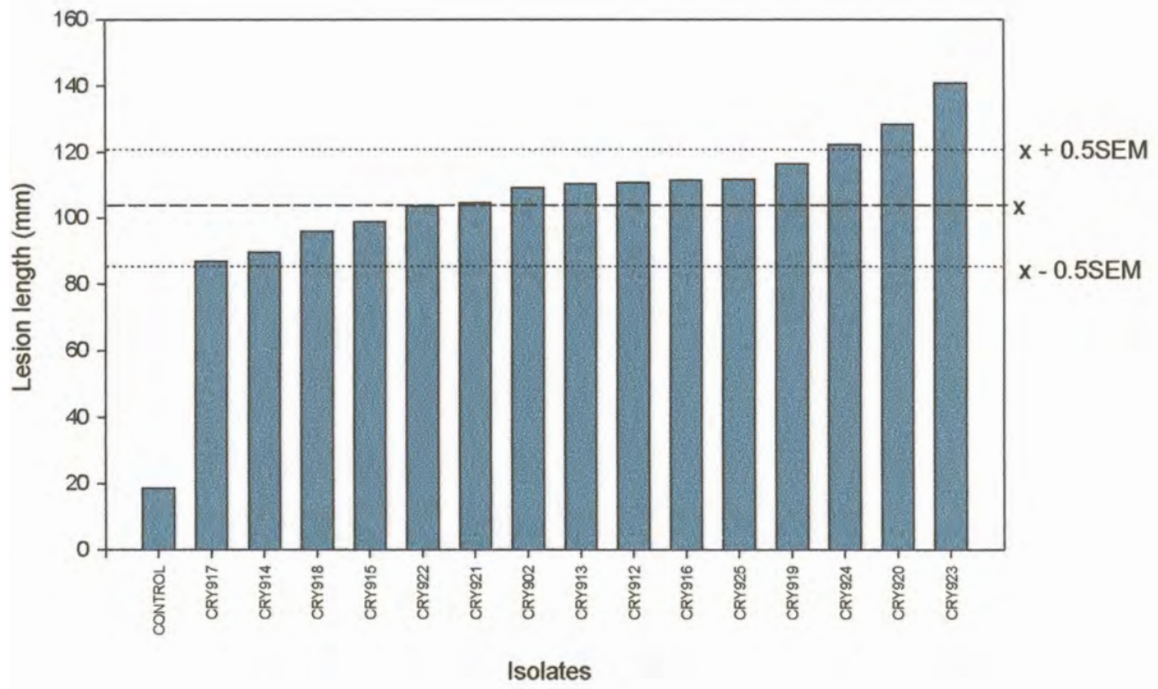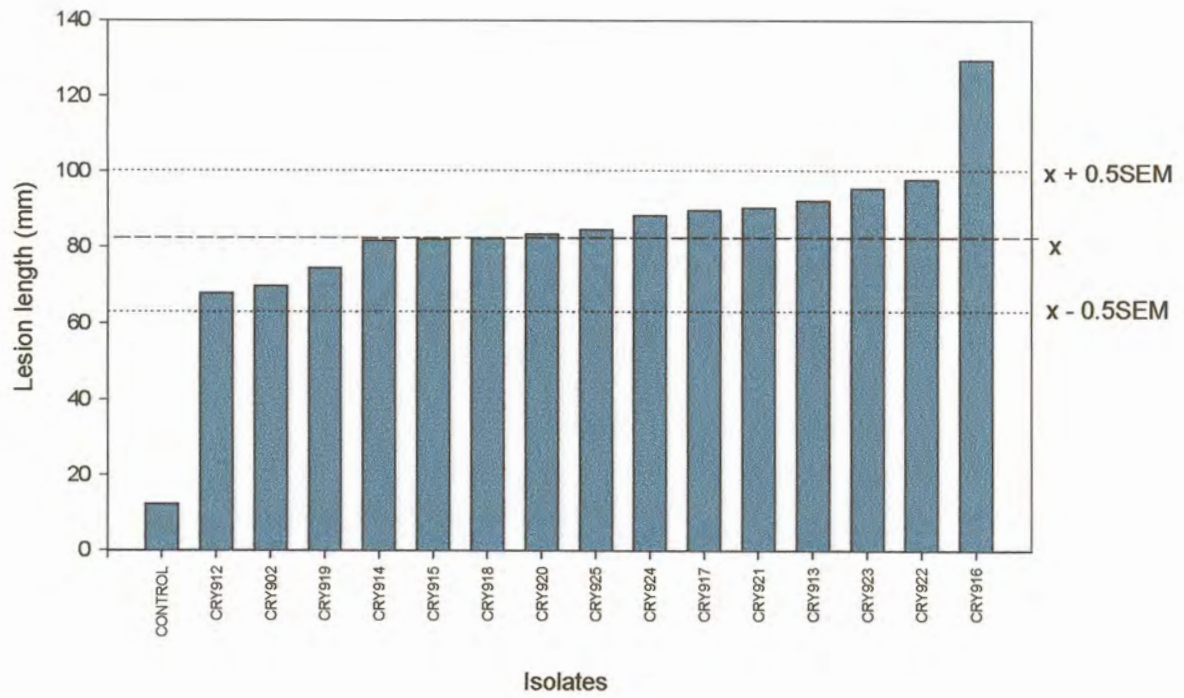
Fig. 3. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the first inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.
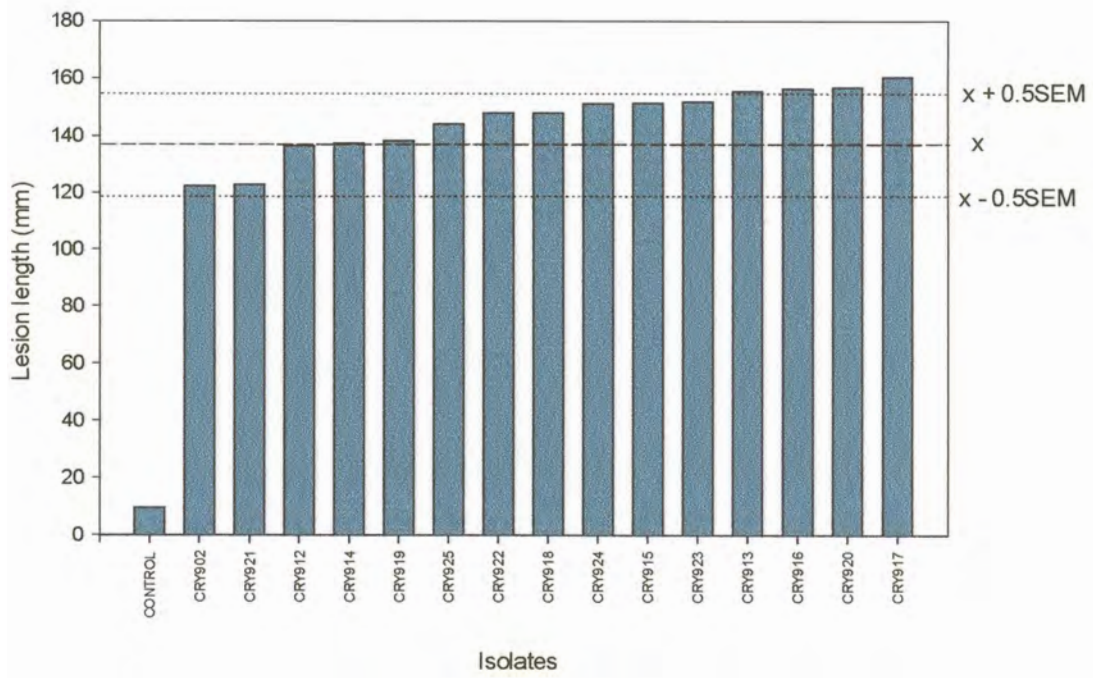
Fig. 4. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the first block of the second inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

Fig. 5. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control for the second block of the second inoculation of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.

Fig. 6. Mean lesion length values, sorted from the lowest to the highest values, of the fifteen isolates of *Cryphonectria eucalypti* and a negative control over all three inoculation sets of the ZG14 clone of *Eucalyptus grandis* in KwaZulu/Natal. Lesions were measured seven weeks after inoculation.
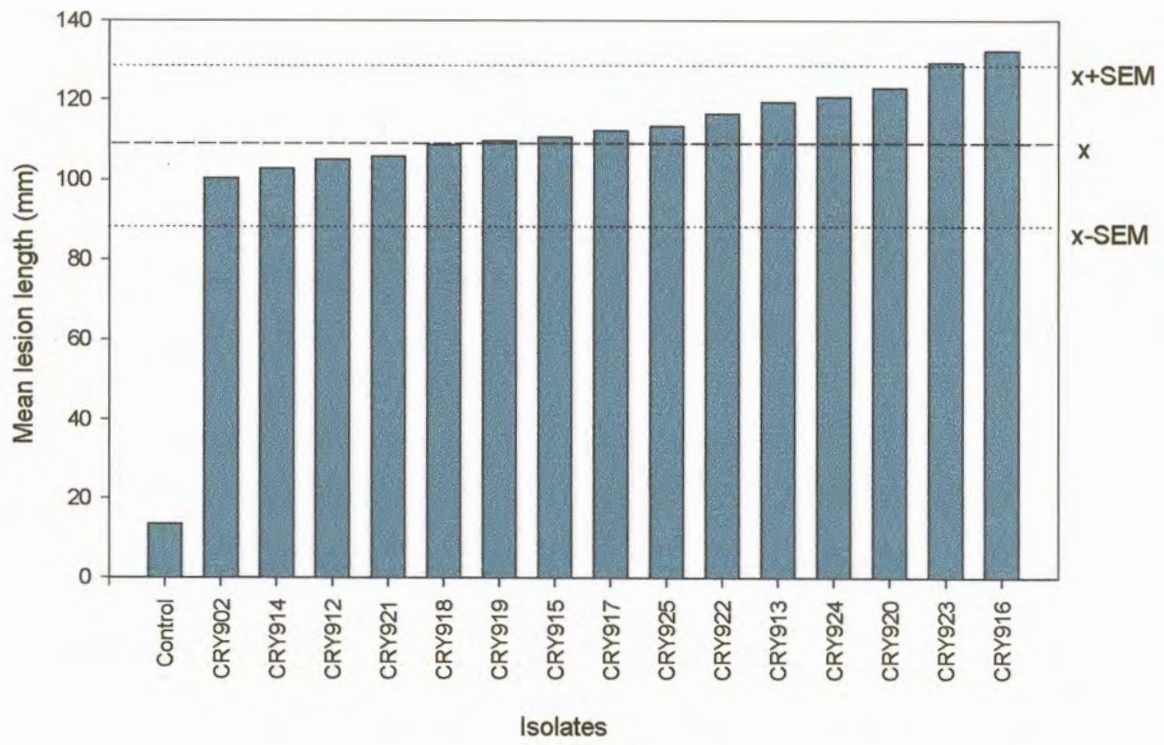
Fig. 7a-d. Graphs representing least square means for the three sets of inoculations of *Cryphonectria eucalypti* isolates on a clone of *Eucalyptus grandis* (ZG14). Results represent three isolates of *E. gyrosa* (CRY916, CRY925, CRY923) and a negative control inoculation. These three isolates were chosen for presentation because they represent characteristic trends for the larger group of isolates.
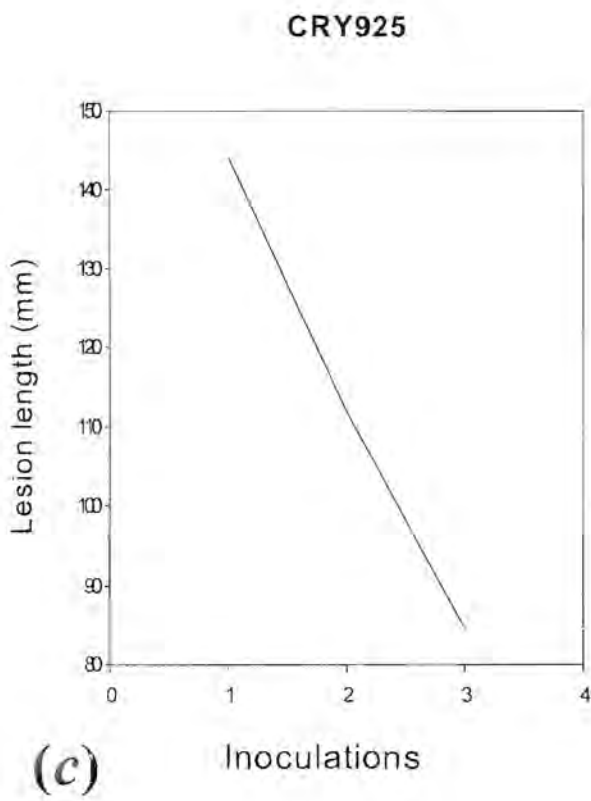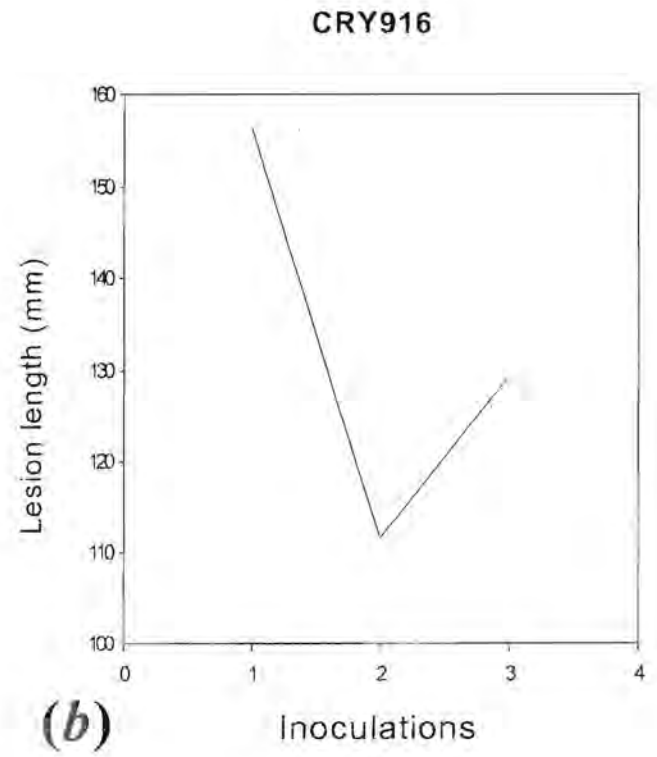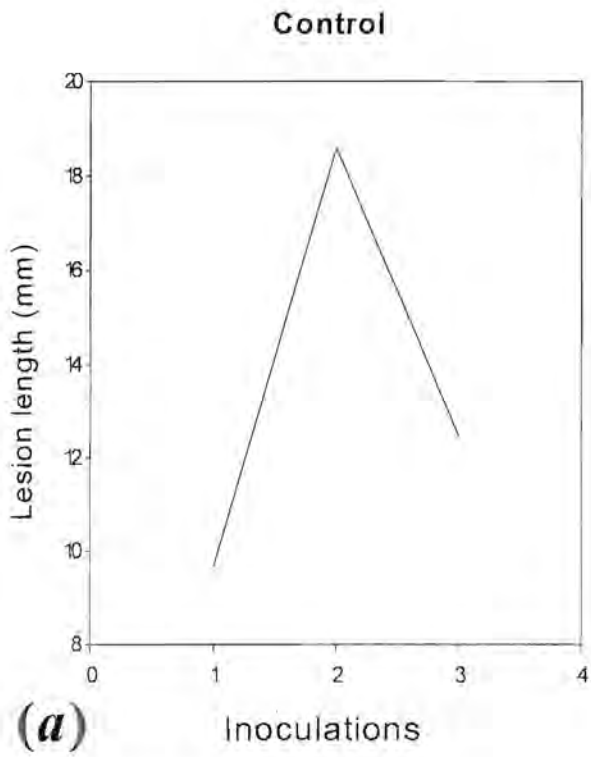
Control

CRY916

(a)

(b)

CRY925

CRY923

(c)

(d)
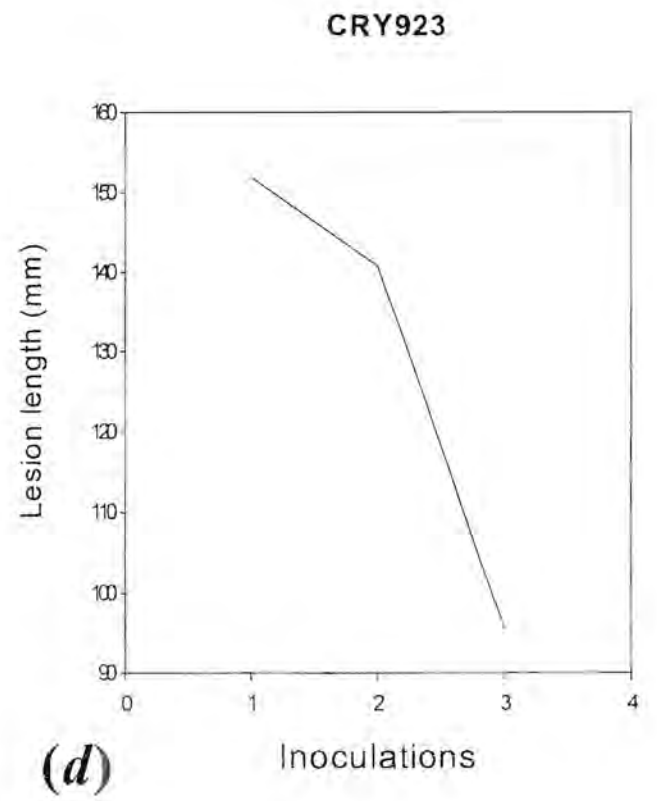
Fig. 8. Score values obtained from an AMMI analysis (Additive Main Effects and Multiplicative Interaction Model) plotted against the overall lesion length for each isolate of *Cryphonectria eucalypti* in the ZG14 inoculation trial. Score values for the different inoculations of the ZG14 trial are also included: ZG1 represents the first inoculation, ZG2.1 represents the first block of the second inoculation and ZG2.2 represents the second block of the second inoculation.
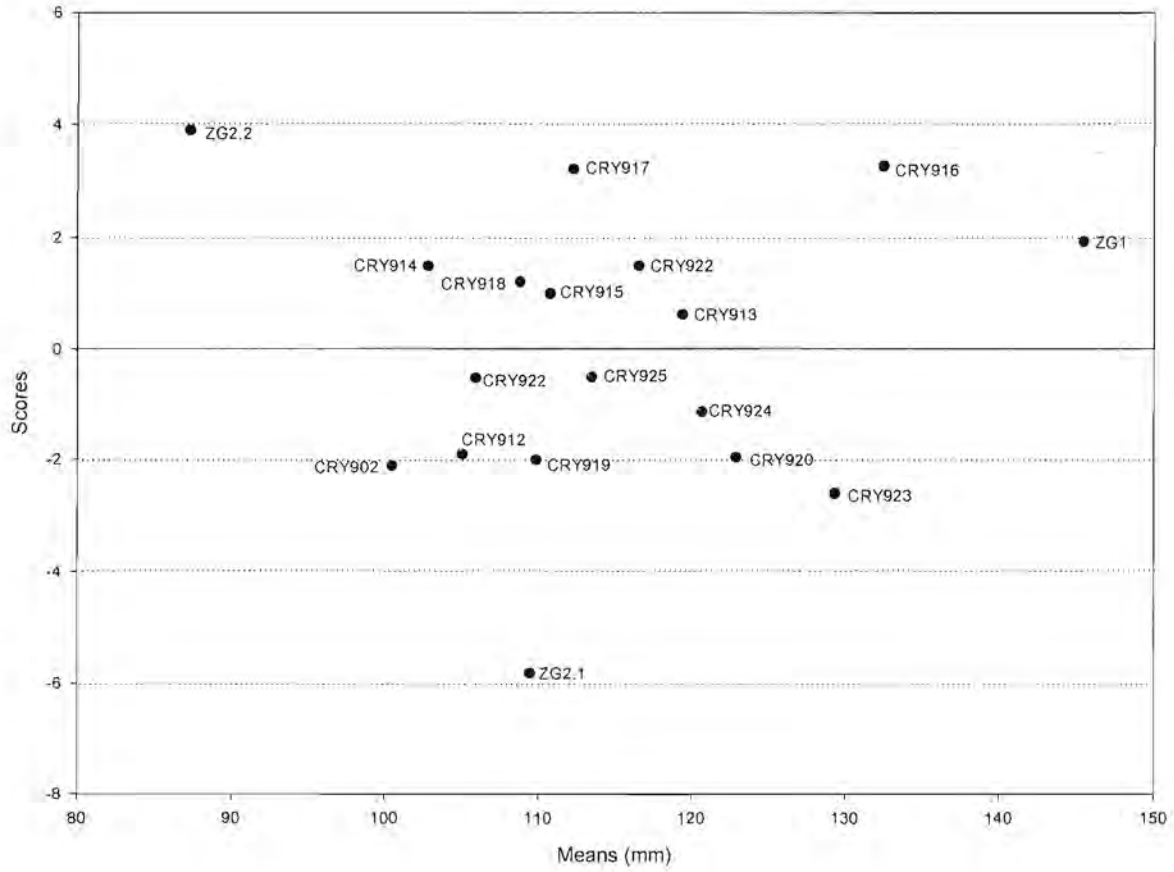
Fig. 9. Mean lesion area (length x width) values of 42 clones of *Eucalyptus grandis* after inoculation with a highly pathogenic isolate of *Cryphonectria eucalypti*, namely CRY920. Lesions were measured six weeks after inoculation.