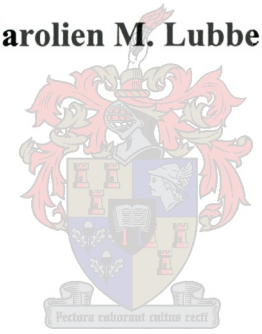
COLLETOTRICHUM DISEASES OF PROTEACEAE

Carolien M. Lubbe



Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Agriculture at the University of Stellenbosch

Supervisor: Dr. S. Denman
Co-supervisors: Prof. S.C. Lamprecht
Prof. P.W. Crous

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: Date:

COLLETOTRICHUM DISEASES OF PROTEACEAE

SUMMARY

This thesis consists of four chapters that present research findings on *Colletotrichum* diseases associated with Proteaceae worldwide. The first chapter is a review of literature regarding the taxonomy and histology of *Colletotrichum* species associated with Proteaceae. The literature is not restricted to Proteaceae hosts, as information regarding *Colletotrichum* on Proteaceae is very limited.

In chapter two, *Colletotrichum* spp. associated with proteaceous hosts growing in various parts of the world were identified based on morphology, sequence data of the internal transcribed spacer region (ITS-1, ITS-2), the 5.8S gene, and partial sequences of the ß-tubulin gene. Four species of *Colletotrichum* were associated with Proteaceae. *Colletotrichum gloeosporioides* was isolated from *Protea cynaroides* cultivated in South Africa and Zimbabwe and from a *Leucospermum* sp. in Portugal, but is known to occur worldwide on numerous hosts. A recently described species, *C. boninense,* was associated with Zimbabwean and Australian Proteaceae, but also occurred on a *Eucalyptus* sp. in South Africa. This represents a major geographical and host extension for the species, and a description of the African strains is provided. *Colletotrichum crassipes* was represented by a single isolate obtained from a *Dryandra* plant in Madeira. *Colletotrichum acutatum* was isolated from *Protea* and *Leucadendron* in South Africa as well as from other proteaceous hosts occurring elsewhere. *Colletotrichum acutatum* f. sp. *hakea* was isolated from *Hakea* in South Africa.

In chapter three, pathogenicity of these *Colletotrichum* species to certain proteas was established, relative aggressiveness of the different species tested and host response to them were compared as well as the effect that wounding had on host response. From the results obtained it is concluded that *C. acutatum* and *C. gloeosporioides* are the primary pathogens associated with *Colletotrichum* leaf necrosis, and *C. acutatum* is the main cause of anthracnose and stem necrosis of Proteaceae in South Africa.

A histological study was performed in chapter four in response to the findings from the previous chapter. The behaviour of two *C. acutatum* isolates (one originating from *Protea* and the other from *Hakea*, *C. acuataum* f.sp. *hakea*) was studied on inoculated *Protea* leaf surfaces using light and scanning electron microscopy. *Colletotrichum acutatum* from *Protea* formed melanised appressoria on the leaf surface, whereas *C. acutatum* from *Hakea* formed very low numbers of both melanised and unmelanised appressoria. Most of the appressoria formed by *C. acutatum* from *Protea* were formed on the cell junctions and on the periclinal walls of the epidermal cells. From this study it is clear that *C. acutatum* f. sp. *hakea* is not a pathogen of *Protea*. Consequently the current use of this isolate as a biological control agent of *Hakea* in South Africa poses no threat to indigenous *Protea* species. *Colletotrichum acutatum* from *Protea* (although closely related to *C. acutatum* f. sp. *hakea*), is a pathogen of *Protea*, which was confirmed by histological observations.

In conclusion, the present study has shown that several species of *Colletotrichum* are associated with diseased Proteaceae. These species differed in their pathogenicity and aggressiveness when inoculated onto certain protea cultivars. These differences could be partially explained by examining the behaviour of *C. acutatum* on the leaf surface. It is clear,

however, that the distribution of the different species, their aggressiveness on different Proteaceae and their modes of infection needs to be investigated further. This work provides a basis for future research on the long-term effective management of these pathogens in fynbos production.

COLLETOTRICHUM SIEKTES VAN PROTEACEAE

OPSOMMING

Hierdie tesis bestaan uit vier hoofstukke wat handel oor navorsing van *Colletotrichum* siektes van Proteaceae wêreldwyd. Die eerste hoofstuk is 'n oorsig van literatuur rakende die taksonomie en histologie van die *Colletotrichum* spesies wat met Proteaceae geassosieer word. Die literatuur oorsig is nie beperk tot die Proteaceae nie aangesien baie min inligting rakende *Colletotrichum* op Proteaceae bestaan.

In die tweede hoofstuk word die *Colletotrichum* spesies wat met proteas in verskeie dele van die wêreld geassosieer word, op grond van morfologie, DNS volgorde data van die interne getranskribeerde spasieerder area ("ITS-1, ITS-2"), die 5.8S geen, en gedeeltelike DNS volgordes van die ß-tubulin geen geïdentifiseer. Vier *Colletotrichum* spesies is met die Proteaceae geassosieer. *Colletotrichum gloeosporioides* is geïsoleer vanaf *Protea cynaroides* wat in Suid-Afrika en Zimbabwe gekweek is en vanaf 'n *Leucospermum* sp. in Portugal, maar is bekend op verskeie gashere wêreldwyd. 'n Spesie wat onlangs beskryf is, *C. boninense,* is met Zimbabwiese en Australiaanse Proteaceae geassosieer, maar kom ook op 'n *Eucalyptus* sp. in Suid-Afrika voor. Dit is 'n groot uitbreiding van die geografiese voorkoms en gasheerreeks van hierdie spesie en 'n beskrywing van die Afrikaanse rasse word gegee. *Colletotrichum crassipes* is verteenwoordig deur 'n enkele isolaat wat vanaf 'n *Dryandra* plant in Madeira verkry is. *Colletotrichum acutatum* is vanaf *Protea* en *Leucadendron* in Suid-Afrika asook vanaf ander proteas wat elders voorkom, geïsoleer. *Colletotrichum acutatum* f. sp. *hakea* is vanaf *Hakea* in Suid-Afrika geïsoleer.

In hoofstuk drie is die patogenisiteit van hierdie *Colletotrichum* spesies teenoor sekere proteas getoets, die relatiewe aggressiwiteit van die verskillende spesies is vergelyk, asook die gasheer se reaksie teenoor die spesies en die effek wat verwonding op die gasheer gehad het. Daar kan afgelei word vanaf die resultate dat *C. acutatum* en *C. gloeosporioides* die primêre patogene is wat met *Colletotrichum* blaarnekrose geassosieer word, en dat *C. acutatum* die hoof oorsaak is van antraknose en lootnekrose van Proteaceae in Suid-Afrika.

'n Histologiese studie is in hoofstuk vier uitgevoer in reaksie op die bevindings van die vorige hoofstuk. Die gedrag van twee *C. acutatum* isolate (een vanaf *Protea* en die ander vanaf *Hakea, C. acutatum* f.sp. *hakea*) op die oppervlaktes van geïnokuleerde *Protea* blare is bestudeer deur gebruik te maak van lig- en skandeer-elektronmikroskopie. *Colletotrichum acutatum* vanaf *Protea* vorm gemelaniseerde appressoria op die blaaroppervlak, terwyl *C. acutatum* vanaf *Hakea* klein hoeveelhede van beide gemelaniseerde en ongemelaniseerde appressoria vorm. Meeste van die appressoria wat deur *C. acutatum* vanaf *Protea* gevorm word, vorm op die aanhegtingspunte tussen selle en op die periklinale wande van die epidermale selle. Vanuit hierdie studie is dit duidelik dat *C. acutatum* f. sp. *hakea* nie 'n patogeen van *Protea* is nie. Gevolglik hou die huidige gebruik van hierdie isolaat as biologiese beheer agent van *Hakea* in Suid-Afrika geen gevaar in vir inheemse *Protea* spesies nie. *Colletotrichum acutatum* vanaf *Protea* (alhoewel dit naverwant is aan *C. acutatum* f. sp. *hakea*) is 'n patogeen van *Protea* en hierdie stelling is ook bevestig deur histologiese waarnemings.

Ter samevatting het hierdie studie getoon dat verskeie *Colletotrichum* spesies geassosieer word met siektes van Proteaceae. Hierdie spesies het van mekaar verskil rakende patogenisiteit en aggressiwiteit nadat hulle op sekere protea kultivars geïnokuleer is. Hierdie

verskille kon gedeeltelik verklaar word deur die gedrag van *C. acutatum* op die blaaroppervlaktes van verskillende protea kultivars. Dit is duidelik dat die verspreiding van die verskillende spesies, hulle aggressiwiteit op verskillende Proteaceae en hul infeksie metodes verder ondersoek moet word. Hierdie studie verskaf 'n basis vir toekomstige navorsing rakende lang-termyn effektiewe bestuur van hierdie patogene in fynbos aanplantings.

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1. A REVIEW OF THE TAXONOMY AND HISTOLOGY OF *COLLETOTRICHUM* SPECIES ASSOCIATED WITH DISEASES OF PROTEACEAE

INTRODUCTION

Members of the plant family Proteaceae form an important component of the Cape Floral Kingdom and Fynbos biome, which is internationally renowned for its exceptional floral diversity. Plant species in the Fynbos biome are unique, but many are rare or threatened with extinction due to increasing urbanisation and agricultural expansion, untimely fires and invasion from aggressive exotic plant species. Furthermore, a number of species have great commercial value, and hence the cultivation of Proteaceae as cut-flowers is a rapidly growing industry in South Africa, as well as in Australia, California, Chile, Hawaii, Israel, New Zealand, Portugal and Spain (including Madeira and Tenerife) and Zimbabwe (Forsberg, 1993; Archer, 1998; Moura & Rodriques, 2001). Commercial Proteaceae plants are propagated mostly by rooting cuttings, but seedlings and seed are also used (Wessels *et al.*, 1997).

The plant pathogen *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. causes serious diseases on propagation material and mature plants of the Proteaceae (Table 1). *Colletotrichum gloeosporioides* occurs in all the major protea growing regions of the world, but is the most severe in summer rainfall regions of South Africa (von Broembsen, 1989). Von Broembsen (1989) considered *C. gloeosporioides* the most important foliar pathogen of cultivated *Protea* spp. This pathogen commonly causes anthracnose, which is necrosis of young shoot tips and leaves. Shoots are infected a few centimetres below the growth tip. Infected tissues become necrotic and the pathogen girdles the stem. Water and nutrients are cut off from green tissues above the lesion, causing the shoot tips to wilt and a characteristic

shepherd's crook symptom to develop (Fig. 1). Other diseases caused by *Colletotrichum* on proteas include stem cankers, leaf spots and necrosis, damping-off in nurseries (Benic & Knox-Davies, 1983) and cutting dieback (von Broembsen, 1989). This pathogen is also seed-borne, causing infected seeds to become shrunken and discoloured (Benic & Knox-Davies, 1983; Benic, 1986).

Disease occurrence in fields tends to be sporadic and is dependent on climatic conditions suitable for disease development and high inoculum. Successful infection of proteas is favoured by moderate (20–25°C) temperatures and humid conditions (Forsberg, 1993). Under humid conditions, masses of orange-pink spores can be seen on the diseased tissue (Fig. 2). The stem canker phase is often associated with other fungi such as *Botryosphaeria* Ces. & De Not. (Benic & Knox-Davies, 1983), as well as with drought stress (von Broembsen, 1989; Forsberg, 1993). In nurseries, however, conditions are usually conducive to disease development, and young plant material is more susceptible to infection. *Colletotrichum* spp. also have a very wide global distribution and host range, resulting in a constantly high inoculum pressure in fynbos and nurseries (Denman pers. com.).

In spite of the general importance of *Colletotrichum* diseases of Proteaceae, relatively little is known about the identity of the species involved, the infection processes and disease epidemiology. Disease control has thus far primarily relied on the use of fungicides. Demands from the international market to produce flowers in accordance with EUREPGAP standards (Middelmann pers. com.), and the threat of pathogen resistance to fungicides, however, necessitate the use of alternative methods of disease management. Host resistance to anthracnose would therefore be of great commercial value.

Developing a technique to screen different genotypes for resistant properties, relies on a number of aspects such as the correct identification and characterisation of the pathogen, knowledge of the infection process, and conditions under which disease will develop.

THE GENERAL STATUS OF TAXONOMY AND MORPHOLOGY OF *COLLETOTRICHUM*

The taxonomy of *Colletotrichum* Corda has been beset by confusion since earliest times. The genus was erected in 1831 with *Colletotrichum dematium* Pers.: Fr. as type of the genus (Sutton, 1980; Baxter, 1981). *Colletotrichum* is the anamorph of the genus *Glomerella* Spauld. & H. Schrenk (Baxter, 1981). In the past isolates of *Colletotrichum* have frequently been misidentified as *Vermicularia* Penz. and *Gloeosporium* Desm. & Mont. (Sutton, 1992). One of the most important and widespread plant pathogenic species of *Colletotrichum,* which is also recorded as a pathogen of Proteaceae, is *C. gloeosporioides* [teleomorph *Glomerella cingulata* (Stonem.) Spauld. & H. Schrenk]. In an attempt to prevent misidentification of *C. gloeosporioides*, von Arx (1957) described various forms based on morphology and host specificity, but according to Sutton (1980) the variation in *C. gloeosporioides* was so extreme that it was meaningless to provide a standard description of the species. However, Sutton (1992) recognised five "group species", each exhibiting wide morphological and biological variation. Baxter *et al.* (1985) concluded that species identification remained problematic and even recently, Cannon (2003) mentioned that species limits remained vague. Obviously taxonomic relationships in the genus will not be resolved solely by means of morphological characteristics. The main stumbling blocks in preventing identification based on morphological features are that culture medium and light conditions influence production of conidiomata, colour of mycelia and shape and size of conidia (Nirenberg *et al.*, 2002).

Primarily the size and shape of conidia and appressoria are the features used to characterise species of *Colletotrichum* (Sutton, 1992). The range of variation in shape and dimensions of morphological structures within species groups is, however, too variable to allow differentiation between species. Furthermore, host specificity is also not a reliable character to use in species identification (Cannon *et al.*, 2000). Species like *C.*

gloeosporioides and *C. fragariae* A.N. Brooks are morphologically so similar that it is difficult to distinguish between them without considerable experience (Bonde *et al.*, 1991). Accurate, diagnostic morphological features describing *Colletotrichum* species are hampered by morphological variation induced by environmental conditions (Cannon *et al.*, 2000; Nirenberg *et al.*, 2002). Intermediate forms often occur, because considerable variation exists in size and shape of conidia, and the dimensions often overlap between species. Furthermore, shapes can easily be defined in a subjective manner (Cannon *et al.*, 2000). Another difficulty that has arisen is the loss of typical features exhibited by the reference cultures that are repeatedly subcultured, selecting "mutants" that are adapted to cultural conditions and are no longer representative of the original isolate (Cannon *et al.*, 2000).

Characterising *Colletotrichum* species using the biological species concept is difficult. Biological species refers to actually or potentially interbreeding populations reproductively isolated from other such groups, whether or not they are morphologically distinguishable (Hawksworth *et al.*, 1995). Sexual recombination of *Colletotrichum* under laboratory conditions occurs very rarely, and mating experiments are often difficult and time-consuming (Turgeon *et al.*, 1993; Cannon *et al.*, 2000). However, Guerber and Correll (2001) successfully crossed self-sterile strains of *C. acutatum* J.H. Simmonds to form the teleomorph, *Glomerella acutata* Guerber & J.C. Correll, which was the first formal description of *G. acutata* formed in culture.

Based on all the difficulties discussed above, it is therefore imperative that morphological classification be linked to molecular data sets. According to Cannon *et al.* (2000) and in retrospect of the advances in biotechnology, there is great potential for molecular analysis to supplement morphological classification systems.

A MOLECULAR APPROACH TO RESOLVING SPECIES OF *COLLETOTRICHUM*

Molecular analysis of *Colletotrichum* species

To date essentially two DNA techniques, electrophoretic banding patterns (e.g. RAPD, RFLP) and phylogenies derived from sequence data, have dominated molecular investigations of *Colletotrichum* taxonomy. Electrophoretic techniques are mostly used to compare populations and groups within species. In contrast, DNA sequence data is used to resolve genetic divergence in phylogenetic studies (Cannon *et al.*, 2000). *Colletotrichum* sequence data has been obtained only from nuclear ribosomal DNA (rDNA) (Cannon *et al.*, 2000), but other types of analysis (e.g. RAPDs) are performed on mitochondrial DNA (mtDNA) as well (Hodson *et al.*, 1993; Buddie *et al.*, 1999). ITS sequences of rDNA are generally used for differentiation of taxa at the species and sub-species level, whereas 18S and 28S sequences are more conserved than ITS, and therefore useful at the generic level. Other portions of the genome that have also been used in sequence analyses include cutinase (Soliday *et al.*, 1989), ß-tubulin (Panaccione & Hanau, 1990; Buhr & Dickman, 1993, 1994; Talhinas *et al.*, 2001), glyceraldehyde-3-phosphate dehydrogenase (Rodriguez & Redman, 1992), histone-4 (Bailey *et al.*, 1996; Talhinas *et al.*, 2001) and domain 2 (Moses *et al.*, 1996; Johnston & Jones, 1997). Sequence data obtained from these areas are often used to supplement data obtained from the ITS or 18S/28S regions, as reliable conclusions cannot be made from a single data set.

Species complexes

Colletotrichum gloeosporioides, *C. acutatum* and *C. orbiculare* (Berk. & Mont.) Arx are pivotal species around which major species aggregates are centred (Cannon *et al.*, 2000). These aggregates consist of various species that are very closely related.

The *C. gloeosporioides* aggregate has been recognised as polymorphic, but according to Sutton (1992), there is uncertainty as to how the variation should be treated. For example, *C. kahawae* J.M. Waller & P.D. Bridge (causing coffee berry disease), has been identified as distinct from other *C. gloeosporioides* strains causing other diseases (e.g. brown blight of ripe berries) of coffee trees (Waller *et al.*, 1993). This finding was based on a slower growth rate and the inability of the pathogen to use citrate or tartrate as the sole carbon source. Although Sreenivasaprasad *et al.* (1996) conceded that *C. kahawae* was different based on ITS sequence data, they stated that the differences were insufficient to justify *C. kahawae* as a separate species.

Another example that illustrates the taxonomic difficulties within the *C. gloeosporioides* complex is *C. fragariae,* one of the species within the *C. gloeosporioides* species aggregate. *Colletotrichum fragariae* is generally not morphologically distinguishable from *C. gloeosporioides*, although Gunnell and Gubler (1992) did report some distinct features. Apparently *C. fragariae* forms more abundant conidia than *C. gloeosporioides*, and does not have a teleomorph. Using ITS data from a range of strains of *C. fragariae, C. gloeosporioides* and *C. acutatum* from strawberries, Sreenivasaprasad *et al.* (1992) showed close similarity between *C. gloeosporioides* and strains of *C. fragariae.*

Buddie *et al.* (1999) also made a study of strawberry isolates using RFLPs and isoenzyme profiles. They found that *C. fragariae* and *C. gloeosporioides* strains had identical rDNA RFLP bands, but different mtDNA RFLP bands and isoenzyme profiles. They concluded tentatively that *C. fragariae* is a holomorphic species and that the mitotic strains (only able to reproduce asexually; referred to as *C. fragariae*) are only different molecularly from the meiotic (sexually reproducing strains; referred to as *C. gloeosporioiodes*) in the mtDNA and isozyme areas of the genome. Johnston and Jones (1997) had similar findings with isolates from fruit rot.

Munaut *et al.* (2001) considered *C. gloeosporioides* representative of a species aggregate. They characterised *C. gloeosporioides* isolates from Mexican *Stylosanthes* Sw. species by means of molecular and morphological analysis. The molecular analysis was conducted using PCR amplifications with the primer CgInt (synthesised from an ITS1 fragment specific to *C. gloeosporioides*) and ITS4. The endonucleases *Ava* II and *Sma* I were used to generate RFLP patterns for the entire ITS1 region. Based on these molecular analyses, they found that the Mexican isolates formed three clusters, one that grouped with reference isolates from Africa and Australia, while the other two clusters contained Mexican isolates only. Using morphological features, three groups were identified, which correlated partially with the host species they were isolated from, and to the molecular groups. Thus, in this work there appears to be a degree of overlap of some features (morphological, molecular and host specificity), but the amount of overlap is not sufficient to be used as diagnostic or to describe the species. This supports Sutton's (1980) view of the difficulties involved in characterising this fungus.

A more recent study performed by Abang *et al.* (2002) with foliar anthracnose of yam (caused by *C. gloeosporioides*) identified four different isolate groups based on growth rate and colony colour. These groups also represented three distinct virulence phenotypes. Three techniques viz. the reaction of monoconidial cultures on casein hydrolysis medium, RFLP and sequence analysis of the ITS region were used to distinguish between these groups. All the yam isolates (being *C. gloeosporioides*) were distinguished from *C. acutatum* by means of the casein medium. The ITS sequence data, as well as the RFLP data, showed that three of the groups were highly similar to *C. gloeosporioides* (correlating with morphological identification) and the fourth group did not cluster with any previously described *Colletotrichum* species, and probably represented a new taxon.

Defining taxa

Systematic concepts are not necessarily standard for all taxonomic groups due to variation in selection pressures and genetic systems (Brasier, 1997). Hodson *et al.* (1993) showed that this was the case with *Colletotrichum*. They found considerable variation within *C. gloeosporioides* strains originating from different tropical fruits regarding rDNA and mtDNA polymorphisms. RFLP bands generated from the mango isolates were almost identical (irrespective of origin), whereas isolates from avocado, banana and papaya varied considerably. Similar findings were made by Freeman *et al.* (2001), who included several Israeli isolates of *C. acutatum* and *C. gloeosporioides* from almond, avocado and strawberry. These studies demonstrate that considerable variation can occur within a species. Contrary to this fact is the little variation that can occur between species as was shown in the study of Buddie *et al.* (1999).

Despite these challenges, species and intraspecific groups are as far as possible defined in a similar way for genetically related pathogens. The sequence variation within groups and subgroups currently recognised, in contrast with the variation between the taxa studied, is examined in order to define systematic concepts on a phylogenetic basis. If this technique is applied to *Colletotrichum*, the aggregates centred around *C. gloeosporioides* and *C. acutatum* will have to be treated as species units rather than superspecific taxa (Cannon *et al.*, 2000). This means that *C. kahawae* and *C. fragariae* will rather be seen as intraspecific taxa of *C. gloeosporioides*, than as separate species (Cannon *et al.*, 2000), until further studies can provide more insight into the relationship between these species.

Genetic relationships within morphological groups

Another aspect of *Colletotrichum* phylogenetics to take into account is the complexity of the relationships among isolates from the same host. Although molecular methods are

providing very valuable information for defining groups within *Colletotrichum*, these phylogenetic groups are not necessarily equivalent in other characteristics, such as origin (Johnston, 2000).

Johnston and Jones (1997) performed a study on fruit rots and found that groups of isolates within *C. acutatum s.l.* (based on morphological and culture features), that are pathogenic to a specific host, can have independent evolutionary origins. Another example is of two genetically distinct isolate groups causing stem and leaf blights of lupin: one from New Zealand and the UK and the other from Canada and France (Lardner *et al.*, 1999). Further molecular analyses have shown that these two groups are two different varieties i.e. *Colletotrichum lupini* (Bondar) Nirenberg, Feiler & Hagedorn, comb. nov. var. *lupini* and *C. lupini* var. *setosum* Nirenberg, Feiler & Hagedorn var. nov., which were described by Nirenberg *et al.* (2002).

Freeman *et al.* (2001) characterised *C. acutatum* isolates from various hosts with different molecular techniques and compared these to morphological identifications. They found four subgroups within *C. acutatum,* and each subgroup contained isolates from various hosts, indicating genetic variation between populations of *C. acutatum*. The presence of the teleomorph in this study possibly contributed to this variation.

Correlation between symptoms and molecular profiles

Saha *et al.* (2002) identified *Colletotrichum* as the pathogen causing leaf disease of *Hevea* in India. The three disease symptoms associated with this host, namely raised spots, anthracnose, and papery lesions, were all previously attributed to *C. gloeosporioides*. These isolates were later characterised by means of RAPDs and RFLPs, and two groups were revealed by both techniques. One group caused raised spots and consisted of *C. acutatum*

isolates only, while the other group caused anthracnose and papery lesions, and consisted of *C. gloeosporioides* isolates only (Saha *et al.*, 2002).

INFECTION STRATEGIES

An understanding of conditions required for infection and disease development is essential to develop a screening protocol for Proteaceous material for resistance to *Colletotrichum*. Elucidating the infection process of the pathogen will facilitate the implementation of appropriate disease management strategies. Information about the pathogen's infection strategy would also enable the producer to be pro-active, preventing disease instead of trying to cure it.

Inoculum type

The two major types of inoculum of *Colletotrichum* are conidia (formed in acervuli) and ascospores (formed in perithecia). Both conidia and ascospores are encased in a hydrophilic mucilaginous material (spore matrix) when the fruiting bodies are still young (Bailey *et al.*, 1992). The spore matrix consists of a mixture of polysaccharides and glycoproteins (Louis *et al.*, 1988). If the fruiting bodies mature under dry conditions, the matrix forms a crust containing clusters of spores. Conidia from young acervuli are most often dispersed by water droplets while the crusty spore clusters are more often spread by wind (Nicholson & Moraes, 1980). The spore matrix prevents premature germination of conidia that are still enclosed in the acervulus, thereby ensuring distribution. The matrix also maintains spore viability under dry conditions, and protects them from the harmful effects of temperature extremes and UV light (Nicholson *et al.*, 1986).

Adhesion of inoculum on plant surfaces

Very little is known about the adhesion of *Colletotrichum* propagules to the plant surface. Young and Kauss (1984) showed that when *C. lindemuthianum* (Sacc. & Magnus) Briosi & Cavara conidia were suspended in water, 20–50% of the conidia adhered to bean hypocotyls within the hour. They also showed that this percentage could be lowered by 80% if the surface waxes were removed from the bean hypocotyls. In a study carried out on avocado, Podila *et al.* (1993) found that the surface wax induced germination and appressorium formation in spores of *C. gloeosporioides.* Waxes from non-host plants did not induce appressorium formation in *C. gloeosporioides*, and avocado wax did not induce appressorium formation in most other *Colletotrichum* species. The deposition of propagules on the plant surface can thus involve very specific physical (thigmotrophic) and/or chemical signals from the plant surface.

Spore germination

Environmental conditions play a very important role in successful spore germination. The only information on spore germination of *Colletotrichum* on Proteaceae is given by Morris (1983) who found that *C. gloeosporioides* spores did not germinate on Proteaceae if there was less than 95% RH. Temperatures between 16–32°C were optimal for germination, and spores did not survive more than 1 or 2 days on leaves of plants exposed to natural light (Morris, 1983).

In other studies on strawberries, it was discovered that in *C. acutatum*, spore germination can begin as early as 16 h after the host has been inoculated, and germ tubes can originate from one or both ends of the conidium. Germ tubes usually do not exceed the length of one plant cell (Curry *et al.,* 2002).

The presence of nutrients on the plant surface also has an effect on spore germination. Conidia of *C. acutatum* germinated poorly if they were formed on a medium containing high iron concentrations, but germinated readily if a low (10 μg/mL) iron concentration was present (Swinburne, 1986). The role that the plant surface topography played in stimulating germination of *C. acutatum* has thus far not been clarified (Bailey *et al.*, 1992).

Germ tube differentiation on the plant surface

In most *Colletotrichum* species, the formation of an appressorium is essential for host penetration (O'Connell *et al.*, 2000). One exception to the rule is *C. acutatum* infection of citrus blossoms, where the pathogen was found to penetrate the flower petals without forming appressoria (Zulfiqar *et al.*, 1996). In most hosts the conidium or ascospore germinates on the host surface to form a germ tube. During germination a septum is formed by most species to delimit the germ tube from the conidium or ascospore. The delineated tip of the germ tube adheres to the plant surface forming the appressorium. The appressorium adheres firmly to the plant surface, and ensures that the infection peg will be at a site where penetration can successfully occur (Agrios, 1997). Appressoria are often sessile and can form in the absence of a host (Lennè, 1978). Mucilagenous material often surrounds the appressorium (Bailey *et al.*, 1992), probably to protect it from adverse environmental conditions. The appressorium also synthesises proteins for example melanin (Suzuki *et al.*, 1981). Melanin protects the appressorium from harmful radiation and plays a role in the penetration process (Bailey *et al.*, 1992). *Colletotrichum acutatum* appressoria are initially unmelanised but after 24 h they are completely pigmented (Curry *et al.*, 2002). This suggests that germination should take place under conditions of very low light intensity for successful infection to take place. It has also been shown that spore germination and appressorium formation on the surfaces of both ripe and unripe avocado fruit are similar, but appressorium formation is enhanced by increasing concentrations of epicuticular wax (Prusky & Saka, 1989). This suggests that the chemical

signal from the wax can induce appressorium formation on both unripe, resistant and ripe, susceptible fruits. Curry *et al.* (2002) also found that *C. acutatum* appressoria were present on the surface of healthy and diseased plant tissue.

Penetration structures

In order for the pathogen to infect the host a penetration pore is formed in the host tissue beneath the appressorium. An infection peg develops at the base of the appressorium that will penetrate the host tissue through the pore. In some species (e.g. *C. gloeosporioides*) (Brown, 1977) the penetration pore becomes surrounded by a funnel-shaped elaboration of the inner wall layer (called the appressorial cone). This structure is continuous with the penetration peg wall and does not contain chitin or melanin. In other species, the appressorial cone is absent but a thickened ring of fungal tissue forms around the penetration pore (O'Connell *et al.*, 2000).

Penetration of the plant surface

Colletotrichum species can penetrate the host in several ways: through natural openings (stomata and lenticels), through wounds and by direct penetration. The most common means is by direct penetration (Bailey *et al.*, 1992). An interesting example of wound penetration is provided by Boher *et al.* (1983). They showed that intact cassava stems were resistant to *C. gloeosporioides*, but after insect attack, lesions formed very rapidly. The insect killed the tissue where it inserted its mouthpart and created a water-soaked area that the fungus could colonise. Another fascinating example is of *Monstera deliciosa* Liebm. plants that are infected by *C. gloeosporioides* through lesions created by the rust fungus *Puccinia paullula* Syd. & P. Syd. (Shaw, 1995).

Three methods of direct penetration are known for *Colletotrichum* spp.: mechanical force alone, secretion of cutin degrading enzymes or a combination of both (Bailey *et al.*, 1992). Dickman *et al.* (1982) showed that *C. gloeosporioides* produced cutinases when grown in liquid culture and Dickman and Patil (1986) demonstrated that cutinase-deficient mutants of *C. gloeosporioides* were not pathogenic when placed on intact papaya fruit surfaces. The secretion of cutinase by *C. gloeosporioides* has been shown by Podila *et al.* (1995) to be a directed process. Cutinase secretion was directed to the infection peg that penetrates the host tissue.

A study carried out by Prusky *et al.* (2001) found that *Colletotrichum* also alters the microenvironment to optimise conditions for degrading enzymes. The fungus secretes ammonia in order to increase the pH of the infected area to 7.5, the pH at which the cell wall degrading enzyme, pectate lyase, functions optimally. This has been proven with *C. gloeosporioides* on avocado as well as with *C. acutatum* on apples.

Infection and colonisation

Colletotrichum gloeosporioides is capable of utilising different modes of nutritional uptake, depending on the host species. On *Stylosanthes guianensis* Sw., it infects in an intracellular hemibiotrophic manner. The first phase is without symptoms, because the pathogen invades the cell wall and lumen but does not kill the cell (Ogle *et al.,* 1990). Only during the second phase does the pathogen kill the plant tissue. In the case of *C. acutatum* on strawberry, the biotrophic phase takes less than 12 h, which is such a short period that it might be considered as modified necrotrophy (Curry *et al.*, 2002).

During the second phase of infection, necrotrophic behaviour is noticed for all infection types of *Colletotrichum*. The pathogen grows extensively in the host cells, in the walls and in the intercellular spaces. The cuticles of the necrotic tissues remain intact, indicating that the

cutinase secreted during penetration is not present during colonisation (Bailey *et al.*, 1992). This is a reproductive adaptation because the formation of both acervuli and perithecia requires intact cuticles (Bailey *et al.*, 1992).

LATENT INFECTIONS

According to Agrios (1997), a latent infection is a state in which the pathogen has started the infection process but the host doesn't show any symptoms. The fungus may become latent at initiation of germination, germ-tube elongation, appressorium formation, penetration or colonisation (Prusky & Plumbley, 1992).

Host factors involved:

Germination of appressoria. A study performed by Binyamini and Schiffmann-Nadel (1972) with *C. gloeosporioides* on avocado revealed that attached appressoria remained viable on unripe fruits until the host condition was suitable for infection to take place. They showed that as soon as the fruit began to ripen, an infection peg developed and penetrated the peel and pulp. In a subsequent study (Rappel *et al.*, 1989), it was discovered that by the time the appressoria were fully developed, short infection pegs had in fact already been formed in the peel of unripe fruit, and some hyphae had developed below the skin of the fruit. The hyphae that developed from this infection peg remained dormant/inactive underneath the peel until the onset of fruit ripening. Thus the pathogen had a latent status.

Inhibition by preformed toxic compounds. Toxic compounds present in the unripe host tissue before infection, can be a possible reason for latency. An inhibitory compound that was found in unripe avocados was 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky *et al.*, 1982). This compound inhibited *in vitro* germ tube elongation at

concentrations similar to those in unripe fruit. The concentration of this compound also decreases as fruit ripening progresses. Cultivars that are more susceptible to *C. gloeosporioides* showed a more rapid decrease of this compound during ripening (Prusky *et al.*, 1991). These facts suggest a very important role for preformed toxic compounds in the latency of *C. gloeosporioides* in avocado fruit.

COLLETOTRICHUM GLOEOSPORIOIDES AS A MYCOHERBICIDE

Mycoherbicides consist of fungal inoculum that is applied to unwanted plants or weeds in order to destroy such a population (Templeton, 1985). *Colletotrichum gloeosporioides* has been employed to control the invasive weed, *Hakea sericea* Schrad. (Australian Proteaceae), in natural stands of fynbos in South Africa. The formulation of the *C. gloeosporioides* biocontrol agent is called "Hakattack" and it causes gummosis and die-back of *Hakea* (Morris, 1982b). The characteristic symptoms that develop on *Hakea* treated with "Hakattack" are stem and branch cankers that exude colourless gum, which later becomes reddish-brown (Morris, 1982a; Richardson & Manders, 1985). Growth of the pathogen is favoured by cool, moist conditions during which dark fruiting bodies form under the surface of the bark. Pink spore masses are formed which cause the bark to flake off. Subsequently exposed spores are dispersed by wind-blown rain (Morris, 1982a). Once the spores are on the host tissue they can germinate at a RH of 95% or more only, and the host parts that are infected have to be succulent, expanding leaves. Penetration occurs through stomata (Morris, 1983). Although *Hakea* is a member of the Proteaceae, no other plant species in this family is susceptible to the *C. gloeosporioides* strain used for biocontrol (Morris, 1982a; b).

CONCLUSIONS

Colletotrichum is an important plant pathogen in general and causes great losses to the protea industry at various stages in the production of flowers, from damping-off in nurseries to shoot or leaf damage in *Protea* orchards. The taxonomy of *Colletotrichum* is in a state of flux and molecular investigations, employing different techniques and using data from different areas of the genome, are needed to confirm the identity of the different species, including those associated with Proteaceae. The standardisation of systematic concepts and the amount of variation allowed in taxa are also areas that need to be addressed urgently.

Another issue related to the taxonomic uncertainty surrounding *Colletotrichum,* is the use of the *C. gloeosporioides* strain as a mycoherbicide against *Hakea* in South Africa. In view of the potential damaging effect this strain might have on natural stands of Proteaceae, it is prudent to establish the relatedness of this fungus to *Colletotrichum* species pathogenic to other, South African Proteaceae.

Colletotrichum spp. are sensitive to environmental and host effects. Infection and disease development appears to follow specific pathways that are mediated by thigmotrophic and chemotrophic responses under ideal environmental conditions. The unique surface characteristics (thick cuticle and many hairs) of the Proteaceae may play a significant role in the infection process of *Colletotrichum.* Information on infection strategies is unfortunately limited to very few *Colletotrichum* spp. and hosts. Tests need to be carried out to determine the infection processes of *Colletotrichum* on proteas, and environmental conditions required for disease development, before a screening protocol can be developed and a disease management strategy implemented.

In this thesis isolates of *Colletotrichum,* obtained from various diseased Proteaceae cultivated in different localities around the world, are identified and phylogenetically characterised. The pathogenicity and relative virulence of the isolates are compared on three

commercial hybrid cultivars of Proteaceae, and histological studies of the infection pathways of certain isolates are presented.

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Table 1. Proteaceae hosts of *C. gloeosporioides*.

Country	Host	Source
Australia	*P. compacta, P. coronata, P. cynaroides, P. longifolia, P. magnifica, P. neriifolia, P. obtusifolia, P. repens, P. stokoei*	Greenhalgh 1981
	Grevillea spp., *Leucospermum* spp., *Protea* spp.	Taylor 2001
	Serruria florida	Forsberg 1993
Portugal (Madeira Island)	*Leucadendron* spp., *L. cordifolium* "Vlam", *L. cordifolium x L. lineare* "Succession II", *L. cordifolium x L. patersonii* "High Gold", *L. glabrum* "Helderfontein", *P. cynaroides, P. magnifica x P. susannae* "Susara"	Moura and Rodrigues 2001
South Africa	*Leucospermum* spp., *Protea* spp., *P. compacta, P. cynaroides, P. eximia, P. longifolia, P. speciosa, P. stokoei*, *Serruria* spp.	Knox-Davies *et al.* 1986
	P. grandiceps, P. lacticolor, P. laurifolia, P. lepidocarpodendron, P. magnifica, P. mundii, P. nana, P. neriifolia, P. obtusifolia, P. pudens, P. repens, P. scolymocephala, P. susannae, Serruria florida	Benic 1986
	P. coronata, P. longiflora, P. punctata, P. scolymocephala	Von Broembsen 1989
USA (California)	*Protea* spp.	Taylor 2001
USA (Hawaii)	*Banksia* spp., *Leucadendron* spp., *Leucospermum* spp., *Protea* spp., *Telopea* spp.	Taylor 2001

Fig. 1. *Protea cynaroides* exhibiting the shepherd's crook symptom caused by a *Colletotrichum* sp.

Fig. 2. Masses of orange-pink spores formed by a *Colletotrichum* sp. on *Protea cynaroides* under humid conditions.

2. CHARACTERISATION OF *COLLETOTRICHUM* SPECIES ASSOCIATED WITH DISEASES OF PROTEACEAE

ABSTRACT

Colletotrichum spp. are known to occur on and cause diseases of Proteaceae, but their identities are confused and poorly understood. In this study, *Colletotrichum* spp. associated with proteaceous hosts growing in various parts of the world were identified based on morphology, sequence data of the internal transcribed spacer region (ITS-1, ITS-2), the 5.8S gene, and partial sequences of the ß-tubulin gene. Four species of *Colletotrichum* were associated with Proteaceae. *Colletotrichum gloeosporioides* was isolated from *Protea cynaroides* cultivated in South Africa and Zimbabwe and from a *Leucospermum* sp. in Portugal, but is known to occur worldwide on numerous hosts. A recently described species, *C. boninense,* was associated with Zimbabwean and Australian Proteaceae, but also occurred on a *Eucalyptus* sp. in South Africa. This represents a major geographical and host extension for the species, and a description of the African strains is provided. *Colletotrichum crassipes* was represented by a single isolate obtained from a *Dryandra* plant in Madeira. *Colletotrichum acutatum* was isolated from *Protea* and *Leucadendron* in South Africa as well as from other hosts occurring elsewhere. A *forma specialis* of *C. acutatum* was described from *Hakea* in South Africa. A pathologically distinct population of this species was found to occur on *Hakea* in South Africa. This population is characterised, and relationships with other *C. acutatum* populations are discussed.

INTRODUCTION

Members of the plant family Proteaceae are indigenous to Australia, South Africa, Central America, South America, Southeast Asia and the southwest Pacific Islands (Rebelo,

1995). Proteaceae are commercially valuable and sought after as cut-flowers on several international markets. Consequently certain species are increasingly being cultivated as active global trade in fresh cut-flower proteas and germplasm of these plants is growing. Many South African Proteaceae are cultivated in Australia, Azores Islands, Canary Islands, Chile, Israel, Madeira, New Zealand, Portugal, Spain, USA (California, Hawaii) and Zimbabwe. Likewise, some Australian Proteaceae (e.g. species of *Banksia* L.f. and *Telopea* R.Br.), are cultivated in countries other than Australia (Crous *et al.*, 2000).

One of the factors limiting commercial production of Proteaceae is damage caused by pests and diseases (Knox-Davies, 1981; Wright & Saunderson, 1995). Some pathogens cause significant losses in the field and in nurseries. Others damage the appearance of blooms, and although they are not debilitating pathogens, they are considered important for aesthetic reasons. Many pathogens associated with Proteaceae are regarded as actionable quarantine organisms and can result in rejection of consignments at the point of import, due to contravention of phytosanitary regulations (Crous *et al.,* 2000; Taylor, 2001).

Among the most devastating fungal pathogens of Proteaceae are *Colletotrichum* spp., causing seedling damping-off, shepherd's crook (anthracnose), pruning wound dieback, leaf lesions and stem dieback (Knox-Davies, 1981; Knox-Davies *et al.,* 1986; Von Broembsen, 1989). Disease occurrence in cultivated fields tends to be sporadic and is dependent on climatic conditions suitable for disease development and high inoculum levels. Successful infection of proteas is favoured by moderate (20–25°C) temperatures and humid conditions (Forsberg 1993). Only the current season's tissues are affected (Serfontein & Knox-Davies, 1990), often displaying the shepherd's crook symptom or leaf necrosis. In nurseries, conditions are often conducive to disease development, and the young plant material is especially susceptible to infection. Losses in nurseries are therefore an annual occurrence.

To date, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is the only

Colletotrichum species reported to affect Proteaceae. This pathogen has been recorded from most areas where Proteaceae are cultivated. The Proteaceae hosts include the genera *Banksia, Grevillea* R.Br. ex Knight, *Leucadendron* R.Br., *Leucospermum* R.Br., *Protea* L., *Serruria* Salisb., and *Telopea* (Greenhalgh, 1981; Benic, 1986; Knox-Davies *et al.,* 1986; Von Broembsen, 1989; Forsberg, 1993; Moura & Rodrigues, 2001; Taylor, 2001).

The morphological identification of *Colletotrichum* spp. has been beset by confusion since earliest times. The main stumbling blocks in preventing identification based on morphological features are that the culture medium and light conditions influence the production of conidiomata, the colour of the mycelia and the shape and size of the conidia (Nirenberg *et al.,* 2002). Although *C. gloeosporioides* is the only *Colletotrichum* species reported to affect Proteaceae to date, conclusions based on recent molecular data suggest that several species of *Colletotrichum* could be involved (Lubbe *et al.,* 2002).

The primary aim of this study was thus to determine the identity of the *Colletotrichum* spp. associated with diseases of Proteaceae being cultivated in different parts of the world.

MATERIALS AND METHODS

Isolates

Forty-eight isolates from different hosts and origins were examined in this study (Table 1). For comparison purposes, reference strains of several well-known species of *Colletotrichum* were also included in this study. Isolates were obtained from the following sources: the University of Stellenbosch culture collection (STE–U), the culture collection of the Biocontrol Unit of the Plant Protection Research Institute – Agricultural Research Council in South Africa, CABI Bioscience (IMI) in the UK, the University of Arkansas – Department of Plant Pathology, and from infected nursery material sampled at various nurseries in the Western Cape province of South Africa. The sampled nursery material was surface

disinfested in 1% sodium hypochlorite for 2 min, 70% ethanol for 1 min and rinsed in distilled water. Infected tissues were plated onto 2% potato dextrose agar (PDA, Biolab, Midrand, South Africa) amended with 1 mL/L streptomycin. Isolates were incubated at 25°C under near-ultraviolet (NUV) light with 12 h light/dark cycles. Cultures were transferred to PDA, carnation leaf agar (CLA) (Fisher *et al.*, 1982), and synthetic nutrient-poor agar (SNA) containing filter paper (Gams *et al.*, 1998) in order to stimulate sporulation and facilitate identification. Morphological observations were made from structures mounted in lactic acid. The 95% confidence intervals of conidial measurements were derived from at least 30 observations at 1000 x magnification. Slide cultures (Riddell, 1950) were made to stimulate the production of appressoria. Reference cultures were established from single conidium isolates obtained from CLA plates. Cultures of each isolate were maintained on McCartney bottles containing either PDA or malt extract agar (MEA), and sterile paraffin oil. Cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) in South Africa, at CABI Bioscience (IMI) in the UK, and the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands.

Cultural studies

One to six isolates of each species occurring on Proteaceae were selected for cultural studies. Colony colours were described from isolates incubated at 25°C under NUV light for 10 days according to the designations of Rayner (1970). Growth rates and cardinal temperature requirements for growth were determined for isolates plated onto PDA in 90 mm Petri dishes and incubated in the dark for 7 days at seven different temperatures, ranging from 5°C to 35°C at 5° intervals. Three plates were used for each isolate at each temperature. Radial mycelial growth was measured for each plate and the mean calculated at each temperature to determine the growth rates for each species.

Phylogenetic analysis

DNA was extracted from the fungal cultures according to Lee and Taylor (1990) and the ITS and ß-tubulin regions were amplified (Kang *et al.,* 2001). The ITS1 region, 5.8S rRNA gene and the ITS2 region of the nuclear-encoded ribosomal RNA gene were amplified with primers ITS1 and ITS4 (White *et al.*, 1990) and part of the ß-tubulin gene with primers T1 (O'Donnell and Cigelnik, 1997) and ßt-2b (Glass and Donaldson, 1995). The PCR products were visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) after ethidium bromide staining. Amplification products were purified following the recommended protocol of the NucleoSpin Extract 2 in 1 Purification Kit (Macherey-Nagel GmbH, Germany) and PCR primers were used to sequence both strands of the purified products using the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Ready reaction Kit (PE Biosystems, Foster City, CA, USA) according to the manufacturers instructions. Resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut).

Both the ITS and ß-tubulin sequences were assembled using Sequence Alignment Editor v2.0a11 (Rambaut, 2002), from which consensus sequences were created. These sequences together with retrievals from GenBank were aligned with Clustal W (Thompson *et al.*, 1994). Manual improvement of the final alignment based on visual inspection was made where necessary. Sequences of *Botryosphaeria ribis* Grossenb. & Duggar, *Botryosphaeria parva* Pennycook & Samuels and *Botryosphaeria dothidea* (Moug. : Fr.) Ces. & De Not were used as outgroups for both the ITS and ß-tubulin data. Neighbor joining analysis was performed with PAUP* version 4.0b10 (Swofford, 2000) on the separate and combined data sets using the Kimura-2-parameter substitution model. Alignment gaps were treated as missing character states and all characters were unordered and of equal weight. The resulting tree was evaluated with 1000 bootstrap replications to test the clade stability. Resulting trees

were printed with TreeView Version 1.6.6 (Page, 1996). A partition homogeneity test (Farris *et al.,* 1994) was conducted in PAUP (Swofford, 2000) to examine the possibility of a joint analysis of the different data sets.

RESULTS

Phylogenetic analysis

For ITS, approximately 550 bases were determined for the 48 isolates (Table 1), and added to the alignment. The manually adjusted alignment of the ITS nucleotide sequences contained 87 taxa and 542 characters including alignment gaps (data not shown). Approximately 700 bases of the β-tubulin gene were determined for the isolates and added to the alignment. The manually adjusted alignment of the β-tubulin nucleotide sequences contained 50 taxa and 455 characters including alignment gaps (data not shown). Because of the use of outgroups with β-tubulin sequences generated by a different primer combination that resulted in shorter sequence lengths, the complete sequences generated for the *Colletotrichum* isolates in this study could not be used for the phylogenetic analysis.

The result of the partition homogeneity test ($P = 0.006$; where $P = 0.05$ was taken as significantly incongruent) indicated that it was not possible to combine the different datasets, which were therefore analysed separately. New sequences were deposited in GenBank (Table 1), and the alignments in TreeBASE (SN1583).

The phylogram obtained from ITS data delimited three clades concerning *Colletotrichum* species associated with Proteaceae (Fig. 1). The first clade was supported by a 100% bootstrap value and included the ex-type strain of *Colletotrichum acutatum* J.H. Simmonds (STE-U 5292) as well as GenBank sequences of *C. lupini* (Bondar) Nirenberg, Feiler & Hagedorn (AJ301968, AJ301975). Within this clade, four well-supported groups were observed: the first group (76% bootstrap support) contained the *C. acutatum* ex-type

strain as well as three isolates from South African *Protea* (STE-U 4448, STE-U 4460, STE-U 5122) and *Pinus* (STE-U 160, STE-U 162, STE-U 164) as well as *C. acutatum* f. sp. *hakea* isolates from *Hakea* (STE-U 4461 - 4463, STE-U 4465 - 4471); the second group (96% bootstrap support) contained an isolate from apple (STE-U 5287) and a *C. acutatum* sequence from GenBank (AF207793); the third group (65% bootstrap support) contained an isolate from *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. (STE-U 5303), South African Proteaceae isolates (STE-U 4452, STE-U 4456 - 4459), as well as three *C. acutatum* sequences obtained from GenBank (AF081292, AF090853, AF411765); and the fourth group (96% bootstrap support) contained two *C. lupini* sequences from GenBank (AJ301968, AJ301975). The second clade (69% bootstrap support) was identified as *C. gloeosporioides*. The Proteaceae isolates in this clade originated from Portugal (STE-U 4450), South Africa (STE-U 4454 - 4455) and Zimbabwe (STE-U 2291). This clade also contained isolates of *C. kahawae* J.M. Waller & P.D. Bridge (STE-U 5295), *Glomerella cingulata* (Stonem.) Spauld. & H. Schrenk (AF411764, AF411769, AF411774, STE-U 5291), *C. gloeosporioides* (AJ311882, AJ311883, STE-U 5297) and a single isolate from *Vitis vinifera* L. (STE-U 4453). Two strains of *C. gloeosporioides* from the type host *Citrus* (STE-U 4297, STE-U 5295) formed a well-supported group within this clade (95% bootstrap support), as did sequences obtained from GenBank (AF411764, AF411774, AJ311882) and isolates STE-U 4453 and 5291 (83% bootstrap support). *Colletotrichum crassipes* (Speg.) Arx (STE-U 5302), a GenBank sequence of supposedly *Glomerella cingulata* (AF411775) and one isolate obtained from *Dryandra* R.Br. in Madeira (STE-U 4445) formed a well-supported (86% bootstrap support) clade sister to the second clade.

The third clade (100% bootstrap support) consisted of two groups, the first of which (98% bootstrap support) contained Proteaceae isolates from Zimbabwe (STE-U 2289 - 2290) and Australia (STE-U 2998, STE-U 3000), a South African isolate from *Eucalyptus* (STE-U

194), two GenBank sequences (AB076800, AJ301974), and sequences of *C. boninense* J. Moriwaki, Toy. Sato & T. Tsukiboshi (AB051402, AB051405). The second group (94% bootstrap support) in this clade also contained two *C. boninense* sequences (AB051400, AB051406) as well as a GenBank sequence of a *Colletotrichum* sp. (AJ301939). The third clade formed a sister clade (96% bootstrap support) to a clade containing isolates of *C. truncatum* (Schwein.) Andrus & W.D. Moore (AF451899, AF451906, AJ301945, STE-U 5294), *C. dematium* (Pers.) Grove (STE-U 5299) and *C. capsici* (Syd.) E.J. Butler & Bisby (STE-U 5304).

The phylogram obtained from the β-tubulin data (Fig. 2) showed the same three major clades as observed in the ITS phylogram. A well-supported *C. acutatum* clade emerged (Clade 1: 100% bootstrap support), but no support was obtained for groups containing the *Hakea* and *Pinus* isolates (Group 1). However, the third *C. acutatum* group observed in the ITS tree was supported (Group 2: 80% bootstrap support) in the β-tubulin tree, with the isolates from Proteaceae (STE-U 4452, STE-U 4456 - 4459) forming a subgroup with a 100% bootstrap support. The *C. gloeosporioides* clade was also well supported (Clade 2: 100% bootstrap support), and showed the same topology as the ITS clade. The two strains of *C. gloeosporioides* from *Citrus* (STE-U 5295, STE-U 4297) also formed a group within this clade (99% bootstrap support). As with the ITS tree, *C. crassipes* STE-U 5302 and an isolate from *Dryandra* (STE-U 4445) formed a clade (100% bootstrap support) sister to this one. The third well-supported clade (100% bootstrap support) contains the isolate from *Eucalyptus* from South Africa (STE-U 194) and the Proteaceae isolates (STE-U 2289 - 2290, STE-U 2998, STE-U 3000) of *C. boninense*.

Taxonomy

Colletotrichum acutatum f. sp. *hakea* Figs. 3–5

Conidiomata with masses of orange conidia. Setae developing in a dense layer around conidiomata, 60–100 µm long, 3– to 8-septate, medium brown at the base, pale brown at the bluntly rounded apex, tapering from a base 3–5 µm diam, to an apex 1.5–2 µm diam. Conidiophores branched below, at times pigmented in the lower part, or reduced to single hyaline conidiogenous cells. Conidiogenous cells subcylindrical, hyaline, smooth, tapering towards a truncate apex with visible periclinal thickening, 12–20 x 3–4 µm. Conidia hyaline, smooth, guttulate, fusoid to naviculate (widest in the upper third), with acutely rounded apex and subtruncate base with a distinct abscission scar; on SNA conidia tend to be naviculate, or to have more bluntly rounded apices, becoming clavate; (9–)11–13(–16) x (3–)4 µm (Avg. 12.5 x 4 µm). Appressoria medium brown, ovoid to clavate, 6–13 x 4–5 µm, 0(–1)-septate. Colonies on SNA with moderate, appressed, white aerial mycelium; on PDA with moderate fluffy aerial mycelium and few aerial conidia. Colonies on SNA with moderate, appressed, white aerial mycelium; on PDA with moderate fluffy aerial mycelium and few aerial conidia; rosy buff (13''f) with vinaceous buff (17'''d) centres on the surface, underneath saffron (15d) with olivaceous gray (21'''''i) centres. Cardinal temperature requirements for growth were min 5°C, opt 25°C, max 30°C. No growth was recorded at 35°C. The mean daily growth rate at 25°C was 10.2 mm/day.

Colletotrichum boninense J. Moriwaki, Toy. Sato & T. Tsukiboshi, *Mycoscience* **44**, 48, 2003. Figs. 6–7

Conidiomata with masses of orange conidia. Setae 75–140 µm long, 3– to 5-septate, medium brown at the base, pale brown at the bluntly rounded apex, tapering from a base 4–6 µm wide, to an apex 1.5–2 µm wide. Conidiophores irregularly branched, frequently with a

pigmented lower half. Conidiogenous cells subcylindrical to obovoid, to fusoid, or irregular, hyaline, smooth, generally tapering from the lower part towards a truncate apex with visible periclinal thickening, 10–25 x 3–5 µm. Conidia hyaline, smooth, guttulate, subcylindrical with bluntly rounded ends and visible abscission scar, at times tapering inconspicuously to a slightly wider apex, or appearing slightly constricted in the middle of the conidium, (14–)15–16(–18) x 5–6 µm (Avg. 15 x 6 µm). Appressoria medium brown, ovoid to irregularly lobed, 9–11 x 6–8 µm, 0–1-septate. Colonies on SNA with sparse, white aerial mycelium; on PDA with moderate gray aerial mycelium and few aerial conidia. Colonies on SNA with sparse, white aerial mycelium; on PDA with moderate gray aerial mycelium and few aerial conidia; brown vinaceous (5'''m) with rosy buff (13''f) centres on the surface, and brown vinaceous (5'''m) underneath. Cardinal temperature requirements for growth were min 10°C, opt 25°C, max 30°C. No growth was recorded at 35°C. The mean daily growth rate at 25°C was 8.1 mm/day.

CONCLUSION

In this study the *Colletotrichum* species associated with Proteaceae were characterised. Because morphological identification of *Colletotrichum* spp. is hampered by phenotypic variation (Nirenberg *et al.,* 2002), it was essential to link the morphological descriptions to molecular data. Although *C. gloeosporioides* is the only *Colletotrichum* species reported to affect Proteaceae to date, preliminary data indicated that more than one species could be involved (Lubbe *et al.*, 2002), and this was confirmed in the present study.

Four species of *Colletotrichum* (*C. acutatum, C. boninense, C. crassipes, C. gloeosporioides*) and a *forma specialis* were found to be associated with diseased Proteaceae. There does not appear to be any correlation between host specificity and symptom type among the species, with the exception of a distinct population of *C. acutatum* f. sp. *hakea*

from *Hakea*, a host to which these isolates appear to be highly specific (Morris, 1982).

Colletotrichum acutatum is known to have a wide host range and geographic distribution (Dyko & Mordue, 1998), and our data also confirm that it occurs on species of *Protea, Leucadendron* and *Leucospermum* in South Africa. This is the first report of *C. acutatum* on Proteaceae, a host family on which it appears to be a serious pathogen. Various subgroups were delineated within the *C. acutatum* clade, which correlate with previous findings (Johnston & Jones, 1997; Lardner *et al.,* 1999). The characterisation of the population from *Hakea* is of special importance to South Africa, because it is presently used as a biological control agent of *Hakea* (Morris, 1982). The latter plant originates in Australia but is considered a noxious weed in South Africa that is spreading through the indigenous fynbos vegetation. This species is still currently marketed as a specific strain of the '*C. gloeosporioides*' complex (Morris, 1982).

Colletotrichum gloeosporioides was confirmed from *Protea cynaroides* (L.) L. growing in South Africa and Zimbabwe, and from a *Leucospermum* sp. in Portugal, but has also been reported to occur on other Proteaceae elsewhere (Greenhalgh, 1981; Benic, 1986; Knox-Davies *et al.*, 1986; Von Broembsen, 1989; Forsberg, 1993; Moura & Rodrigues, 2001; Taylor, 2001). In view of the data presented here, previous reports of this species must be treated with circumspection. A relatively unknown species, *C. boninense,* was found to be associated with Zimbabwean and Australian Proteaceae, but also occurred on a *Eucalyptus* sp. in South Africa. This species was until recently treated as part of *C. gloeosporioides* complex (Moriwaki *et al.,* 2003).

Colletotrichum crassipes was represented by a single isolate obtained from a *Dryandra* plant in Madeira. A more comprehensive study of the *Colletotrichum* spp. occurring on Proteaceae in Australia, Madeira and Zimbabwe would be required to reveal the importance and distribution of *C. crassipes*, and especially *C. boninense*, which has until now only been

reported from Japan and only on non-proteaceous hosts (Moriwaki *et al.,* 2003). Once more representative global distribution data are available, a reassessment of the phytosanitary significance of *Colletotrichum* species can be made. The pathogenicity of *C. acutatum, C. acutatum* f. sp. *hakea, C. boninense* and *C. gloeosporioides* to *Protea* has subsequently been evaluated and is discussed in chapter 3 of this thesis.

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Table 1. Isolates of *Colletotrichum* spp. studied.

Anamorph/ Teleomorph	Accession no.	Other culture collection no.	GenBank no. ITS	GenBank no. B-tub	Host	Collection site [b]	Origin
C. acutatum	STE-U 5122	CBS 112994	AY376497	AY376545	*Leucospermum* sp.	C	South Africa
C. acutatum	STE-U 164	CBS 112980	AY376498	AY376546	*Pinus radiata*	B	South Africa
C. acutatum	STE-U 160	CBS 112979	AY376499	AY376547	*Pinus radiata*	B	South Africa
C. acutatum	STE-U 162	CBS 112981	AY376500	AY376548	*Pinus radiata*	B	South Africa
C. acutatum	STE-U 4448	CBS 112990	AY376501	AY376549	*Leucadendron* "Safari Sunset"	C	South Africa
C. acutatum	STE-U 4460	CBS 113006	AY376502	AY376550	*Protea cynaroides*	B	South Africa
C. acutatum	STE-U 4452	CBS 112992	AY376503	AY376551	*Protea magnifica*	C	South Africa
C. acutatum	STE-U 4456	CBS 113002	AY376504	AY376552	*Protea repens*	C	South Africa
C. acutatum	STE-U 4457	CBS 113003	AY376505	AY376553	*Protea* sp.	B	South Africa
C. acutatum	STE-U 4458	CBS 113004	AY376506	AY376554	*Protea* sp.	B	South Africa
C. acutatum	STE-U 4459	CBS 113005	AY376507	AY376555	*Protea* sp.	B	South Africa
C. acutatum	STE-U 5303	IMI 383015; CBS 112989	AY376508	AY376556	*Hevea brasiliensis*	D	India
C. acutatum	STE-U 5287	A 38; CBS 112995	AY376509	AY376557	Apple	D	USA
C. acutatum [a]	STE-U 5292	ATCC 56816; IMI 117617; CBS 111296	AY376510	AY376558	Papaya	D	Australia
C. acutatum f. sp. *hakea*	STE-U 4471	CBS 112658	AY376511	AY376559	*Hakea sericea*	A*	South Africa
C. acutatum f. sp. *hakea*	STE-U 4467	CBS 113009	AY376512	AY376560	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4466		AY376513	AY376567	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4470	CBS 112759	AY376514	AY376561	*Hakea sericea*	A	South Africa

Table 1. Continued

Anamorph/ Teleomorph	Accession no.	Other culture collection no.	GenBank no. ITS	GenBank no. B-tub	Host	Collection site [b]	Origin
C. acutatum f. sp. *hakea*	STE-U 4465		AY376515	AY376562	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4462	CBS 113007	AY376516	AY376563	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4463	CBS 113008	AY376517	AY376564	*Hakea gibbosa*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4461	CBS 112761	AY376518	AY376565	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4469	CBS 112993	AY376519	AY376566	*Hakea sericea*	A	South Africa
C. acutatum f. sp. *hakea*	STE-U 4468	CBS 112760	AY376520	AY376568	*Hakea sericea*	A	South Africa
C. boninense	STE-U 194	CBS 110779	AY376521	AY376569	*Eucalyptus* sp.	B	South Africa
C. boninense	STE-U 3000	CBS 112762	AY376522	AY376570	*Leucospermum* sp.	C	Australia
C. boninense	STE-U 2998		AY376523	AY376571	*Leucospermum* sp.	C	Australia
C. boninense	STE-U 2290		AY376524	AY376572	*Protea cynaroides*	C	Zimbabwe
C. boninense	STE-U 2289	CBS 112982	AY376525	AY376573	*Protea cynaroides*	C	Zimbabwe
C. capsici	STE-U 5304	IMI 56173; CBS 113117	AY376526	AY376574	*Arachis hypogaea*	D	Tanzania
C. caudatum	STE-U 5300	IMI 196464; CBS 113172	AY376527	AY376575	*Cymbopogon martinii*	D	India
C. coccodes	STE-U 5301	IMI 61249; CBS 112987	AY376528	AY376576	*Lycopersicon esculentum*	D	Zimbabwe
C. crassipes	STE-U 5302	IMI 359911; CBS 112988	AY376529	AY376577	*Dryas octopetala*	D	Switzerland
C. crassipes	STE-U 4445	CBS 112984	AY376530	AY376578	*Dryandra* sp.	C	Madeira
C. dematium	STE-U 5299	IMI 80025; CBS 127.57	AY376531	AY376579	*Peperomia* sp.	D	Unknown
C. gloeosporioides	STE-U 4295	IMI 356878; CBS 953.97	AY376532	AY376580	*Citrus* sp.	D	Italy

Table 1. Continued

Anamorph/ Teleomorph	Accession no.	Other culture collection no.	GenBank no. ITS	GenBank no. B-tub	Host	Collection site [b]	Origin
C. gloeosporioides	STE-U 2291	CBS 112983	AY376533	AY376581	*Protea cynaroides*	C	Zimbabwe
C. gloeosporioides	STE-U 5297	IMI 266803; CBS 112986	AY376534	AY376582	*Citrus* sp.	D	Belize
C. gloeosporioides	STE-U 4450	CBS 112991	AY376535	AY376583	*Leucospermum* "High Gold"	C	Portugal
C. gloeosporioides	STE-U 4455	CBS 113192	AY376536	AY376584	*Protea cynaroides*	B	South Africa
C. gloeosporioides	STE-U 4454	CBS 113001	AY376537	AY376585	*Protea cynaroides*	B	South Africa
C. gloeosporioides	STE-U 4453	CBS 113000	AY376538	AY376586	*Vitis vinifera*	C	South Africa
C. graminicola	STE-U 5298	IMI 84302; CBS 113173	AY376539	AY376587	*Zea mays*	D	Zimbabwe
C. kahawae	STE-U 5295	IMI 319424; CBS 112985	AY376540	AY376588	*Coffea arabica*	D	Kenya
C. orbiculare	STE-U 5296	IMI 368075; CBS 113171	AY376541	AY376589	*Xanthium spinosum*	D	Argentina
C. sublineolum	STE-U 5293	IMI 372541; CBS 112997	AY376542		*Sorghum bicolor*	D	Ethiopia
C. truncatum	STE-U 5294	IMI 217517; CBS 112998	AY376543	AY376590	*Arachis hypogaea*	D	Gambia
Glomerella cingulata	STE-U 5291	IMI 324985; CBS 113010	AY376544	AY376591	*Fragaria* sp.	D	USA

[A] STE-U 5292 is the ex-type strain of *C. acutatum.*

[B] Collection sites were as follows: A) wild stands, A*) originally from wild stands in Australia but obtained as commercial biocontrol formulation in SA, B) nurseries, C) commercial plantations, D) unknown.

Fig. 1. Phylogram obtained from a neighbor joining analysis of the ITS1, 5.8S rDNA and ITS2 sequence data of *Colletotrichum* isolates from Proteaceae. The tree was rooted to three *Botryosphaeria* species. Branch support is based on 1000 bootstrap replicates and is shown at the nodes. The bar represents 0.1 substitutions per site.

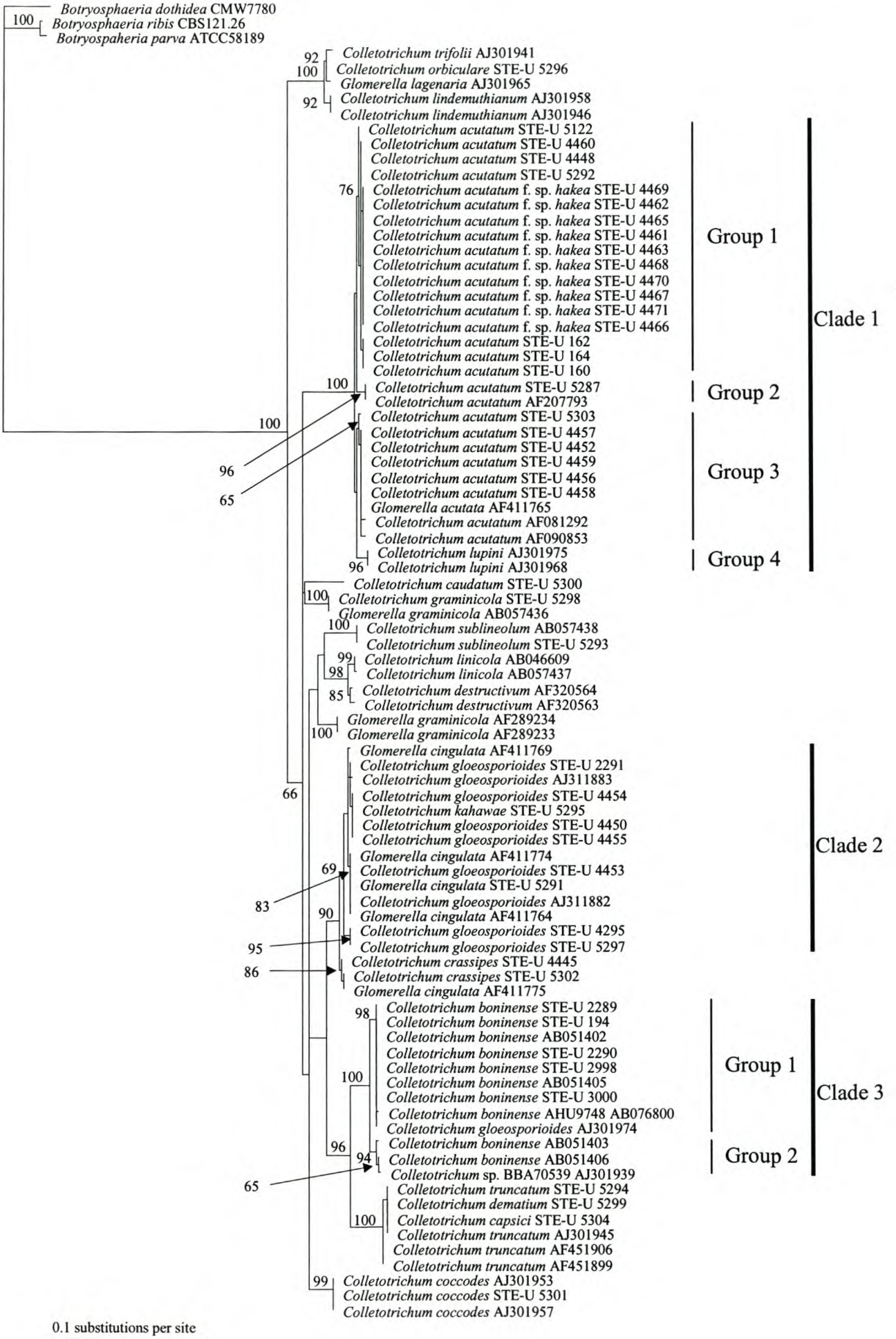
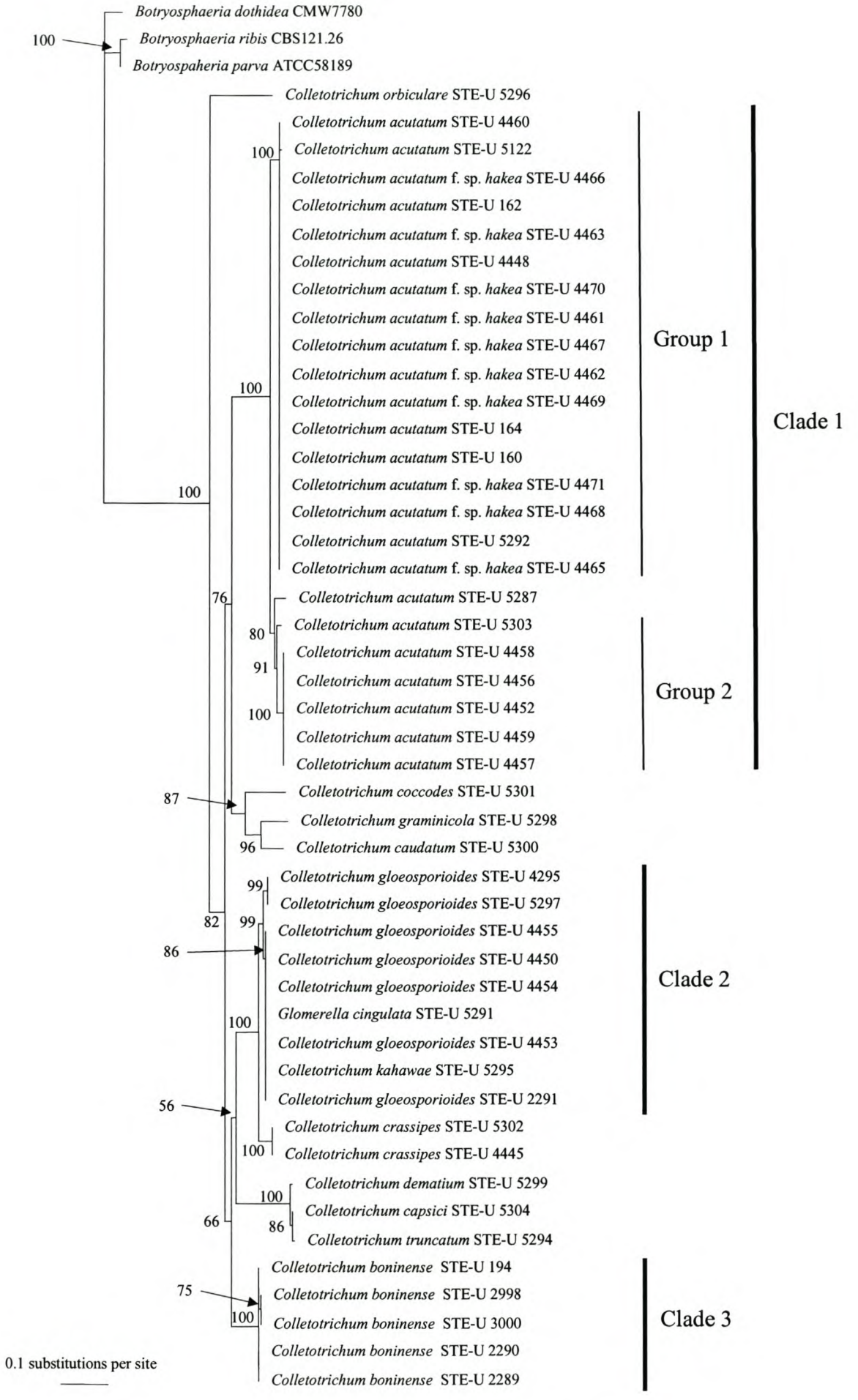


Fig. 2. Phylogram obtained from a neighbor joining analysis of the alignment of sequences from part of the ß-tubulin gene of *Colletotrichum* isolates from Proteaceae. The tree was rooted to three *Botryosphaeria* species. Branch support is based on 1000 bootstrap replicates and is shown at the nodes. The bar represents 0.1 substitutions per site.



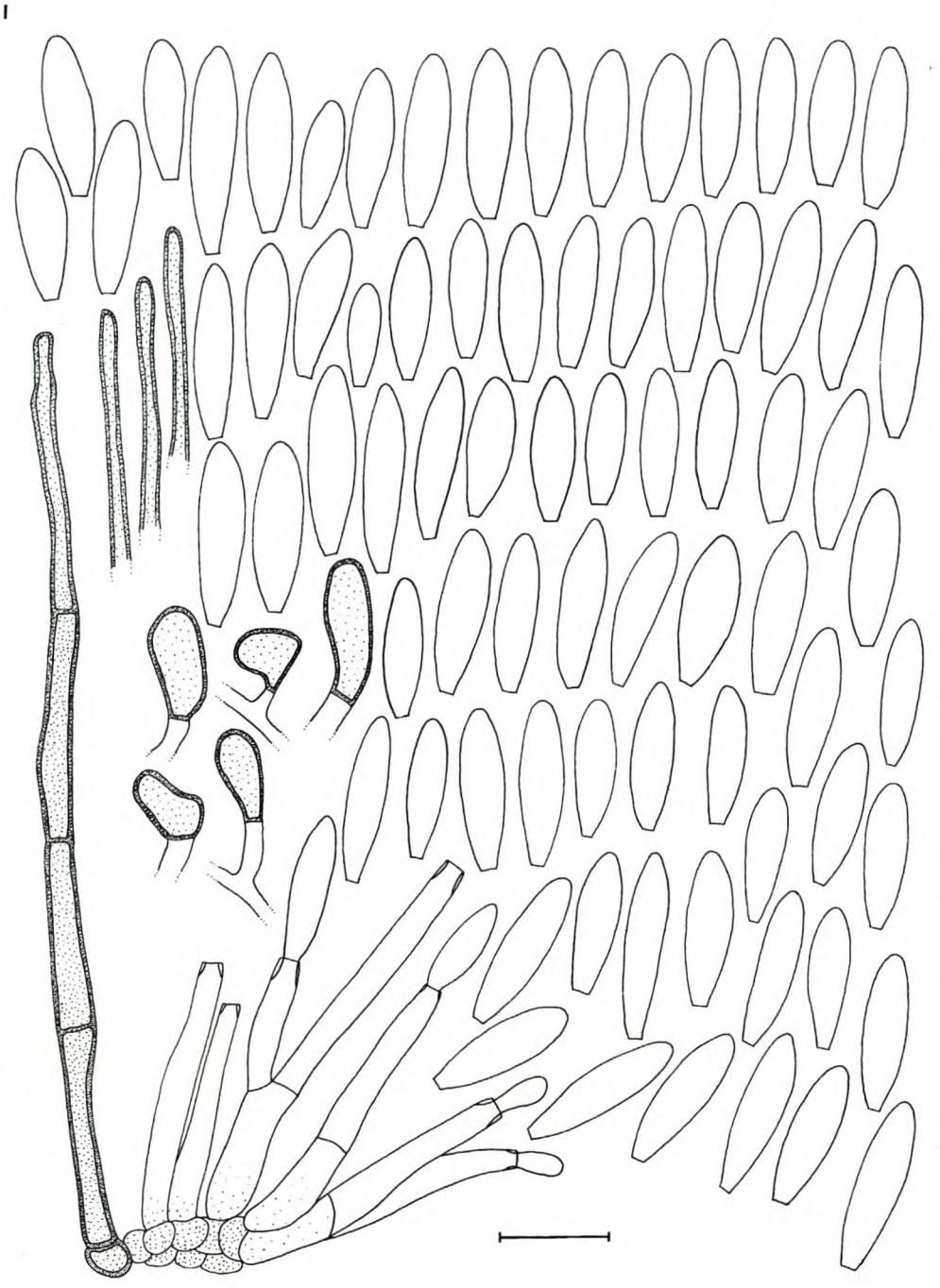


Fig. 3. *Colletotrichum acutatum* f. sp. *hakea* (STE-U 4461). Setae, conidiophores, conidia and appressoria. Bar = 10 µm.

Figs. 4-6. Conidiophores and conidia of *Colletotrichum* spp. **Figs. 4–5**. *Colletotrichum acutatum* f. sp. *hakea* (STE-U 4461). **Fig. 6**. *Colletotrichum boninense* (STE-U 194). Bar = 10 µm.

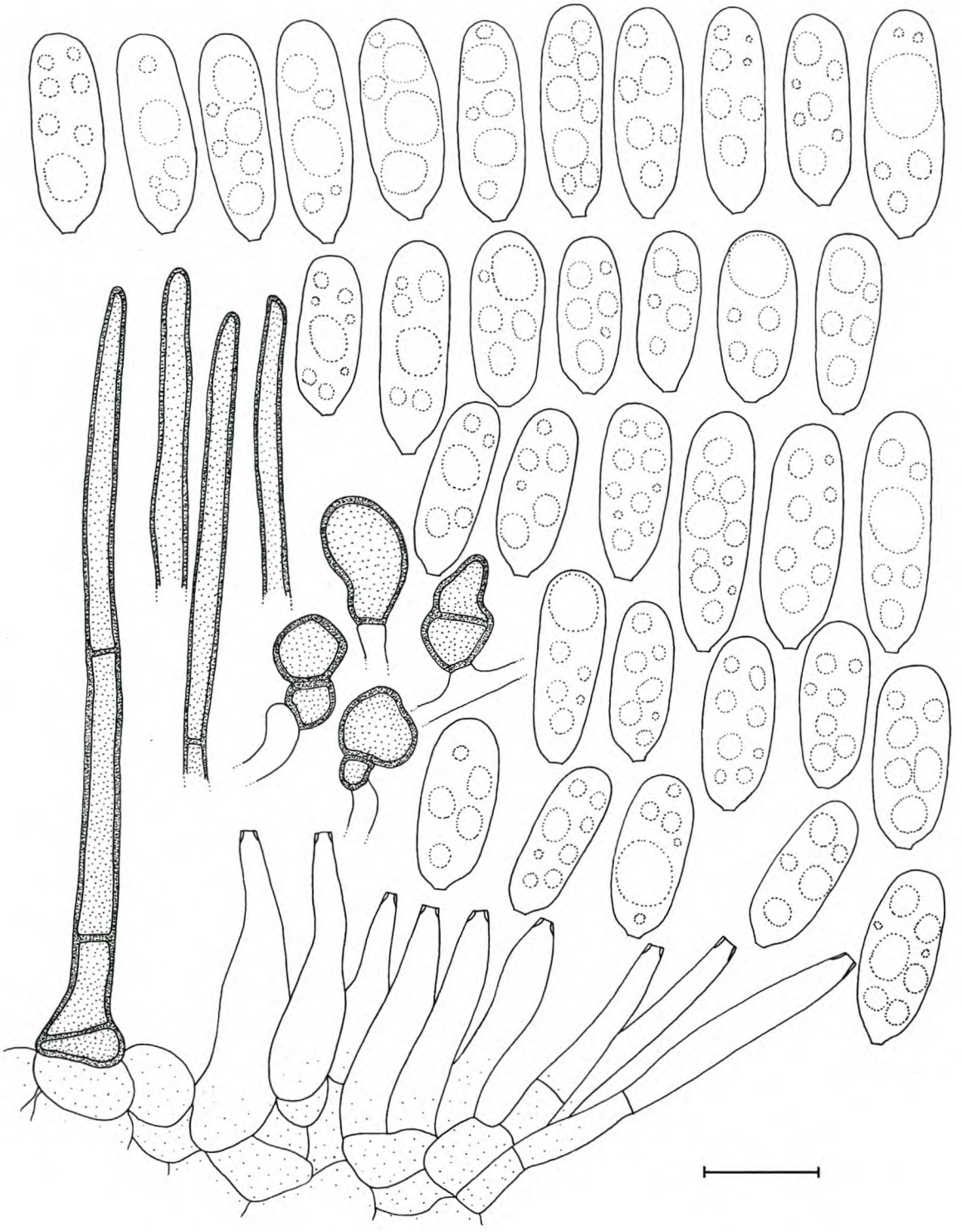


Fig. 7. *Colletotrichum boninense* (STE-U 194). Setae, conidiophores, conidia and appressoria. Bar = 10 µm.

3. PATHOGENICITY OF *COLLETOTRICHUM* SPECIES ON *PROTEA*

ABSTRACT

Colletotrichum species cause a wide range of diseases on Proteaceae. Currently four *Colletotrichum* species have been associated with diseased Proteaceae in South Africa, *viz. C. acutatum, C. acutatum,* f.sp. *hakea*, C. *boninense* and *C. gloeosporioides*. In this chapter, the pathogenicity of these taxa was evaluated on three *Protea* cultivars. The relative aggressiveness of the isolates and the effect that wounding had on the host response were compared. Results showed that *C. boninense* and *C. acutatum* f.sp. *hakea* did not cause lesions significantly different from the controls and that *C. acutatum* isolates originating from *Protea* and *C. gloeospoioides* were the primary pathogens associated with *Colletotrichum* leaf necrosis. Furthermore, *C. acutatum* also proved to be the main cause of anthracnose and stem necrosis of Proteaceae in South Africa.

INTRODUCTION

Members of the plant family Proteaceae are indigenous to Australia, South Africa, Central America, South America, Southeast Asia and the southwest Pacific Islands (Rebelo, 1995). Proteaceae are commercially valuable and sought after as cut-flowers on several international markets. Consequently certain species are increasingly being cultivated as active global trade in fresh cut-flower proteas and germplasm of these plants is growing. One of the factors limiting commercial production of Proteaceae is damage caused by pests and diseases (Knox-Davies, 1981; Wright & Saunderson, 1995). Some pathogens cause significant losses in the field and in nurseries, while others damage the appearance of blooms, and although they are not debilitating pathogens, they are considered important for aesthetic

reasons.

Among the most devastating fungal pathogens of Proteaceae is *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., which causes seedling damping-off, shepherd's crook (anthracnose), pruning wound dieback, leaf lesions and stem dieback (Knox-Davies, 1981; Knox-Davies *et al.*, 1986; Von Broembsen, 1989). Disease occurrence in cultivated fields tends to be sporadic and is dependent upon climatic conditions suitable for disease development and high inoculum levels. Moderate temperatures (20–25°C) and humid conditions favour successful infection of proteas (Forsberg, 1993). Young tissues are most affected, often displaying the shepherd's crook symptom or leaf necrosis. In nurseries losses occur annually since conditions are often conducive to disease development, and plant material is young and therefore susceptible to infection.

Until recently *Colletotrichum gloeosporioides* was the only *Colletotrichum* species recorded to affect Proteaceae. This pathogen has been reported from most areas where Proteaceae are cultivated. The Proteaceae hosts include the genera *Banksia* L.f., *Grevillea* R.Br. ex Knight, *Leucospermum* R.Br., *Leucadendron* R.Br., *Protea* L., *Serruria* Salisb., and *Telopea* R.Br. (Greenhalgh, 1981; Benic, 1986; Knox-Davies *et al.*, 1986; Von Broembsen, 1989; Forsberg, 1993; Moura & Rodrigues, 2001; Taylor, 2001). Results from Chapter 2, however, demonstrate that four species of *Colletotrichum* are associated with diseased Proteaceae. In this study, *C. boninense* and *C. gloeosporioides* as well as two groups of *C. acutatum* (one originating from *Protea* called *C. acutatum* and one group originating from *Hakea* called *C. acutatum* f. sp. *hakea*) were tested. The final taxonomic position of the two *C. acutatum* groups has not yet been resolved but they are distinct ecological groups and are therefore treated separately in this study. The aims were to establish the pathogenic status of these species on certain proteas, to compare the relative aggressiveness of the different species and the effect that wounding has on the host response. Since it was difficult to induce

disease development under conditions of artificial inoculation (pilot trials, data unpublished) a wound treatment was included to introduce the pathogen directly into plant tissues. Furthermore, leaf wounds artificially simulate insect damage as stem wounds do pruning, which are both common occurrences in commercial plantations, thereby creating conditions that occur in the field.

MATERIALS AND METHODS

Isolates

Isolates, representing the *Colletotrichum* spp. described in Chapter 2 were selected for pathogenicity tests and are listed in Table 1. Only South African isolates were used to avoid quarantine implications and therefore *C. crassipes* was excluded from this study. For the same reason a *C. boninense* isolate from *Eucalyptus* was selected instead of an isolate from Proteaceae (the *C. boninense* isolates from Proteaceae originated from Australia and Zimbabwe - see Chapter 2). Isolates were obtained from the following sources: the University of Stellenbosch culture collection (STE–U 194, 4454), the culture collection of the Biocontrol Unit of the Plant Protection Research Institute – Agricultural Research Council, South Africa (STE-U 4468) and from diseased material sampled from orchards at Elsenburg in the Western Cape of South Africa (STE-U 4452, 4456).

Plant material

Rooted cuttings of the cultivars Cardinal [*P. eximia* (Salisb. ex Knight) Fourc. × *P. susannae* E. Phillips], Carnival Too (*Protea compacta* R. Br. × *P. neriifolia* R. Br.) and Rubens [*P. repens* (L.) L.] were used. Cuttings were treated with 4000 µg/ml indole buturic acid and put into a rooting bed with a surface temperature of 25°C to ensure rooting. As soon as there was sufficient root development the cuttings were transplanted into 20 cm diameter plastic pots. The growth medium consisted of a mixture of sterilised sand, peat and

polystyrene (1:1:1). The plants were 2–4 months-old and had one or two 10–15 cm shoots per plant at the time of testing.

Inoculum

The isolates were cultured on carnation leaf agar (CLA) (Fisher *et al.*, 1982) and incubated for 4 weeks at 25°C under near-UV and cool white light with a 12 h photoperiod. The contents of three 9 cm Petri dishes with one carnation leaf per dish were used per isolate. Three leaves (covered in conidial masses) were immersed in 10 mL sterile distilled water, to produce spore suspensions for each isolate. The spore suspensions were filtered through four layers of sterile muslin to remove residual mycelia and carnation leaves. The final spore concentration was adjusted to 10^6 spores/mL using a haemocytometer. A drop of Tween 20 (a wetting agent) was added to the spore suspension of each isolate.

Inoculation

Five isolates and a control (sterile water) were evaluated during the trial and each isolate was tested on 12 plants (4 per cultivar). Thus, in total 72 plants were inoculated per replicate. There were four treatments applied to each cultivar isolate combination (one plant per treatment). The treatments were plant part inoculated (leaves or stems) and wounded or non-wounded. Five insect mounting needles were attached to a cork, which was used to prick each leaf. Shoots were wounded by removing the top 1 cm of the stem growth tip. Each plant was inoculated with 10 mL spore suspension. Control plants were sprayed with sterile water only. The 10 mL spore suspension was enough to atomise plants until run-off. Thus, there were 36 plants tested for pathogenicity to leaves, 12 plants for each of the three cultivars. Two plants were used per isolate and one of these two plants was wounded while

the other was not. The same treatment structure was applied to the stems. The entire experiment was replicated.

Incubation

Plants were covered with plastic bags that were secured in place with rubber bands and incubated in the laboratory at 23–25°C for the first 48 h during which they received only low light intensities emitted by fluorescent tubes. Thereafter the bags were removed and the plants were transferred to a Conviron (controlled environment incubation room) at 25°C ± 2°C with a 12 h photoperiod for 12 days. The air was humidified using a Goldair GUH-852 home humidifier set at full capacity. The plants were watered daily.

Re-isolation

At the end of the experiment assessments were made and leaves and stems of plants were surface disinfested with 1% NaOCl for 2 min, 70% alcohol for 1 min and then rinsed in sterile filtered water. Isolations were made from every leaf (whether necrotic or not) and from each stem- or growth tip of the most recent growth flush. Only the tissue of the most recent growth flush is susceptible to *Colletotrichum* infection. Entire leaves were placed onto potato dextrose agar (PDA, Biolab, Midrand, South Africa), irrespective of necrosis, and were incubated for 14 days at room temperature (25°C ± 2°C). One piece of stem tissue from each of the stems was placed onto PDA and incubated. Where stem necrosis was evident the tissue was taken from the margin between healthy and diseased tissue. Where no necrosis was evident, the tissue was selected at random from the inoculated stem- or growth tip.

Assessments

Four parameters were measured:

Leaf lesions. The percentage of leaves with lesions was calculated as a proportion of the total number of current flush leaves per plant. The total number of current flush leaves on each plant was counted before inoculation, and after incubation the number of necrotic leaves was counted, and the percentages were calculated.

Stem lesions. The presence of stem lesions was noted and scored as being present or not. The data were then expressed as the percentage stems that developed lesions.

Leaf infections. The percentage of current flush leaves per plant that became infected with the pathogen was calculated. After surface disinfestation and isolation, the number of leaves from which the pathogen was re-isolated was recorded. The percentage of leaves infected was then calculated as a proportion of the number of leaves isolated from for each plant.

Stem infections. Counts were made of the number of necrotic stems, and after surface disinfestation and isolation the number of stems from which the pathogen was re-isolated was recorded. The percentage of infected stems was calculated as a proportion of the number of stems isolated from for each plant.

Statistical procedures

Experimental layout. The experiment was set out in a completely randomised, four-factor factorial design and was repeated once. The factors were cultivars (Cardinal, Carnival Too and Rubens), species (*C. acutatum, C. acutatum* f. sp. *hakea, C. boninense, C. gloeosporioides* plus a control), treatments (non-wounded and wounded) and plant parts (leaves and stems), totalling 72 plants per experiment. An experimental unit was a single plant.

Combined analyses of variance for the 2 experiments (repeated over time) were carried out after tests for homogeniety of variance indicated that data were of comparable type. The

analyses were carried out on the leaf lesion data, the percentage infected leaves and the infected stem data using SAS version 8.2 (SAS, 1999) statistical software. The Shapiro-Wilk test was performed to test for non-normality (Shapiro & Wilk, 1965) on all data used in the ANOVA tests. Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means in the ANOVA's. The stem lesion data were subjected to 2 × C contingency tables, with C levels for each factor, and Chi Square tests were performed.

RESULTS

Leaf lesion data

Data for the two experiments were pooled and then analysed. There was no significant four-way or three-way interaction although there was significant two-way interaction (Table 2). The significant interactions occurred for cultivar × treatment ($P = 0.0321$) data and plant part × treatment ($P = 0.0021$) data. Since there were no interactions involving the species data, only the main effects of species were considered.

The mean percentage of *Protea* leaves with lesions caused by each of the *Colletotrichum* species is presented in Table 3. Inoculation with *C. acutatum* and *C. gloeosporioides* resulted in the highest percentages of leaves with lesions (36.3% and 25.3%, respectively). Although all the *Colletotrichum* species evaluated caused leaf lesions, inoculation with *C. acutatum* f. sp. *hakea* and *C. boninense* did not cause lesions significantly different from that of the controls. The differences in leaf lesions caused by the different *Colletotrichum* species are illustrated in Figure 1.

The cultivar by treatment interaction is shown in Table 4. Generally wounding increased the percentage of leaves that developed lesions (although not always significantly at the 95% confidence level), with the exception of cultivar 'Rubens', which was unaffected by wounding (Table 4). This interaction is emphasised by the fact that the highest percentages of

leaves with lesions occurred on wounded 'Carnival Too' plants (45.6%), and more than three times as many leaf lesions occurred on wounded 'Cardinal' plants than non-wounded ones (14.2 % vs. 4.6%, respectively), although the latter was not significant at the 95% confidence level (Table 4). Figure 2 illustrates the reaction of wounded and non-wounded 'Cardinal' plants to inoculation with *C. acutatum*. Figure 3 shows the response of the three cultivars to *C. acutatum*.

Leaf susceptibility was greatly increased by wounding relative to stem susceptibility, which is reflected in significantly more wounded leaves developing lesions relative to non-wounded leaves (Table 5). Wounded leaves developed significantly more lesions than leaves that were not wounded and then inoculated (with the exception of cultivar 'Rubens', Table 4).

Stem lesion data

The Chi square tests demonstrated that there were highly significant differences ($P < 0.0001$) amongst *Colletotrichum* species with regard to the percentage stem lesions that formed (Fig. 4). *Colletotrichum acutatum* was able to cause stem lesions on 42% of the plants inoculated compared with all the other species that caused lesions on less than 10% of stems (Fig. 4).

There were also significant differences ($P = 0.0240$) amongst cultivars in the percentage of stems that developed lesions (Fig. 5). The most stem lesions were formed on 'Carnival Too' (29.2%), compared with 'Rubens' (12.8%) and 'Cardinal' (8.5%).

The treatments (wounding or non-wounding) also significantly ($P = 0.05$) affected the number of stem lesions that developed. Wounding more than doubled the number of stems that developed lesions (24%, Fig. 6), compared with the non-wounded stems (10%).

Infected leaves data

Analyses were carried out on the combined data set. There was no four-way or three-way interaction, but there was significant (P = 0.0034) two-way interaction between treatments x plant parts (Table 6). The species and cultivars were not involved in interaction and can be interpreted as main effects. There were significant differences (P = 0.0020) between the species tested and the control, but not between the cultivars (P = 0.2544).

The percentage of *Protea* leaves infected by each of the *Colletotrichum* species is presented in Table 7. All the *Colletotrichum* species tested caused significantly higher percentages of leaf infection than the control (sterile water) in which only one plant was infected. Relatively high infection levels (>30%) were obtained for all the species tested, but there were no differences between species in infection levels obtained (Table 7).

The effects of wounding and the plant part inoculated on the percentage of infected leaves are presented in Table 8. Plants with wounded leaves had significantly higher percentages of leaf infection compared to plants with no leaf or stem wounds, or plants with only stem wounds.

Stem infection data

The analysis of variance was carried out on the combined data set. There were no significant interactions so the main effects could be interpreted (Table 9). There were significant differences between the species tested (P = 0.0043) and between the treatments (wound or non-wound) (P = 0.0121).

Inoculation of the plants with any of the *Colletotrichum* species resulted in significantly more stem infections than the control plants (Table 10). However, at the 95% confidence level the differences in ability of the various species tested to infect stems, was not significant. It is noteworthy that almost 61% of stems were infected with *C. acutatum*.

Wounded plants had significantly higher percentages of infected stems (57.2%) compared to plants that were not wounded (36.7%).

DISCUSSION

The primary aim of this study was to establish the pathogenicity of four selected *Colletotrichum* species associated with diseased proteas. Although only a selection of isolates was used in the pathogenicity tests, they were representative of the species based on previous molecular tests and pilot pathogenicity tests. The results show that all four species of *Colletotrichum* could infect protea leaves and stems. *Colletotrichum acutatum* and *C. gloeosporioides* caused significantly more necrosis than the control, whereas *C. acutatum* f.sp. *hakea* and *C. boninense* did not cause lesions significantly different from the controls The most aggressive species appears to be *C. acutatum*, because it could cause the greatest lesion damage on both leaves and stems of proteas. *Colletotrichum gloeosporioides* was as aggressive on leaves of proteas as *C. acutatum*. However, it was unable to cause major stem damage, and hence should be regarded as a serious leaf pathogen of proteas, probably being less involved with anthracnose, shepherd's crook and stem canker. This is also supported by preliminary isolation data from diseased Proteaceae. An observation that was made regarding leaf infections was that infection was not always coupled with leaf necrosis, in other words, in some cases the pathogen was isolated from surface disinfested, asymptomatic material.

The *C. boninense* isolate used in this study originated from *Eucalyptus*. The lower degree of aggressiveness demonstrated might therefore be attributed to the fact that the isolate did not originate from the host onto which it was inoculated. The same explanation may apply to the *C. acutatum* f. sp. *hakea* isolate. There is very little known regarding the pathogenicity of *C. boninense,* since Moriwaki *et al.* (2003) only recently described this species from Japan. *Colletotrichum boninense* was found on diseased Proteaceae in Australia

and Zimbabwe (Chapter 2), and is therefore potentially capable of causing disease in the Proteaceae. Further testing with isolates from Proteaceae is therefore necessary to establish the aggressiveness of *C. boninense* towards proteas.

All three *Protea* cultivars tested, 'Cardinal', 'Carnival Too', and 'Rubens', were equally infected by the different *Colletotrichum* species, but demonstrated differences in susceptibility through disease expression (necrosis). Although interactions made the interpretation of results difficult, 'Carnival Too' developed a high proportion of leaf and stem necrosis, whereas 'Cardinal' had the lowest levels of leaf and stem necrosis. This suggests that 'Cardinal' is less susceptible to *Colletotrichum* than 'Carnival Too'. Results were less clear with 'Rubens', which had high levels of leaf infection but lower levels of stem infection. This probably means that 'Rubens' is more likely to develop leaf blight than anthracnose, stem necrosis and shepherd's crook. However, at this stage it is unknown whether this is the case under field conditions. It is suspected that the variation in susceptibility is not only the result of differences in internal resistance (bio-chemical resistance responses), but might also be due to the variation in the leaf surface characteristics (to be discussed in Chapter 4).

Wounding generally increased the susceptibility of the *Protea* cultivars to both infection and lesion formation. Wounding is known to predispose plants to *Colletotrichum* infection (Muimba-Kankolongo & Bergstrom, 1992; Shaw, 1995). Muimba-Kankolongo and Bergstrom (1992) attributed the higher levels of anthracnose stalk rot of wounded maize plants to the availability of sucrose to the pathogen. Sucrose is known to cause an increase in conidial germination of *C. graminicola* (Ces.) G.W. Wilson (Bergstrom, 1978). Wounding the *Protea* plants might have led to sucrose leaking from the wounded cells and therefore resulted in a higher percentage of conidial germination. The fact that leaf wounding had a more significant effect than stem wounding is possibly due to the nature of the tissue (leaf tissue being less sclerophyllous).

All leaves were isolated from, whether symptomatic or not. *C. acutatum* was yielded from leaves that had lesions on them as well as from those that did not. Because isolations were not made from the areas adjacent to necrotic leaf tissue, the necrosis evident on symptomatic leaves can not be necessarily coupled with leaf lesions when *C. acutatum* f. sp. *hakea* was inoculated onto *Protea*. A different inoculation technique using point inoculations, and exact points of back isolation will need to be conducted to prove that the lesions on the leaves are caused by *C. acutatum*. Freeman *et al.* (2002) also found that symptomless material could still yield the pathogen when they inoculated pepper, tomato and bean plants with a *C. acutatum* isolate originating from strawberry. They found that the pathogen survived and proliferated in these plants over a three month period without causing any symptoms, except on strawberry.

A factor that plays a role in the pathogenicity of *Colletotrichum* on *Protea* and which was not measured in this study, is the phenological state of the host material. Pilot trials that were conducted before the onset of this study showed that lesion development did not take place when material was inoculated during the winter months (results not shown). The *Protea* shoots of the most recent flush hardened off at the onset of winter and leaves were probably at their most resistant and no symptoms developed on plants inoculated during the winter months.

This study also acted as a precursor to developing a protocol for screening protea cultivars for resistance to *Colletotrichum*. It is apparent that in a screening protocol different species of the pathogen must be tested. If a single species only is used in tests a universal conclusion could not be made on the susceptibility of proteas to *Colletotrichum*. Furthermore, different plant parts show different susceptibilities to the different species and this must also be taken into account. The difficulties experienced in obtaining infection and disease expression under artificial conditions, also emphasise the very important effect of the

environment on the pathogen. Further studies in this regard would yield valuable information that could be used in future tests.

Finally, from the results obtained in this study, it is concluded that *C. acutatum* and *C. gloeosporioides* are the primary pathogens associated with *Colletotrichum* leaf necrosis, and *C. acutatum* is the main cause of anthracnose and stem necrosis of Proteaceae in South Africa.

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Table 1. List of *Colletotrichum* isolates used for pathogenicity testing.

Species	Isolate number	Host
C. acutatum	STE-U 4452, 4456	*Protea*
C. acutatum f. sp. *hakea*	STE-U 4468	*Hakea*
C. boninense	STE-U 194	*Eucalyptus*
C. gloeosporioides	STE-U 4454	*Protea*

Table 2. Analysis of variance for effects of species, cultivar (Cv), treatment (Tmt) and plant part (Pp) treated on leaf lesions caused by *Colletotrichum* species inoculated onto *Protea* plants.

Source of variation	Df	SS	MS	SL
Model	60	93593.5	1559.9	0.0001
Trial	1	11404.9	11404.9	0.0001
Species	4	21762.8	5440.7	0.0001
Cv	2	11840.6	5920.3	0.0001
Cv x species	8	6647.0	830.9	0.1560
Tmt	1	5822.1	5822.1	0.0015
Species x tmt	4	2317.5	579.4	0.3745
Cv x tmt	2	2929.2	1464.6	0.0321
Cv x species x tmt	8	4117.3	514.7	0.4772
Pp	1	10764.7	10764.7	0.0001
Species x pp	4	3783.3	945.8	0.1461
Cv x pp	2	65.8	32.9	0.9408
Cv x species x pp	8	1569.8	196.2	0.9365
Tmt x pp	1	5460.8	5460.8	0.0021
Species x tmt x pp	4	1454.4	363.6	0.6116
Cv x tmt x pp	2	436.1	218.0	0.6687
Cv x species x tmt x pp	8	3217.1	402.1	0.6508
Error	81	43666.4	539.1	
Corrected total	141	137259.8		

Table 3. Percentage of *Protea* leaves with lesions after inoculation with *Colletotrichum* species.

Colletotrichum species	Leaves with lesions (%) [ab]
C. acutatum	36.34 a
C. acutatum f. sp. *hakea*	11.91 bc
C. boninense	15.82 bc
C. gloeosporioides	25.32 ab
Control (sterile water)	5.98 c

[a] Means followed by the same letter are not significantly different ($P = 0.05$).

[b] Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.

Table 4. Effect of wounding on the percentage leaves with lesions after inoculating *Protea* cultivars with different *Colletotrichum* species.

	Leaves with lesions (%) [ab]	
Cultivar	Wounded	Non-wounded
Cardinal	14.20 bc	4.64 c
Carnival Too	45.62 a	19.67 bc
Rubens	21.94 b	24.32 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$).

[b] Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.

Table 5. Effect of wounding and the plant part treated on the percentage leaves with lesions after inoculating *Protea* cultivars with different *Colletotrichum* species.

	Leaves with lesions (%) [ab]	
Plant part treated	Wounded	Non-wounded
Leaf	42.40 a	16.20 b
Stem	12.11 b	16.54 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$).

[b] Leaves with lesions (%) = current flush leaves with lesions as a portion of the total number of current flush leaves.

Table 6. Analysis of variance for effects of species, cultivar (Cv), treatment (Tmt) and plant part (Pp) treated on infected leaves caused by *Colletotrichum* species inoculated onto *Protea* plants.

Source of variation	Df	SS	MS	SL
Model	60	71904.9	1198.4	0.4686
Trial	1	57.1	57.1	0.8263
Species	4	22167.7	5541.9	0.0020
Cv	2	3286.2	1643.1	0.2544
Cv x species	8	3389.0	423.6	0.9378
Tmt	1	3853.3	3853.3	0.0748
Species x tmt	4	674.5	168.6	0.9653
Cv x tmt	2	912.1	456.0	0.6802
Cv x species x tmt	8	2575.6	321.9	0.9724
Pp	1	6440.5	6440.5	0.0222
Species x pp	4	815.1	203.8	0.9514
Cv x pp	2	4600.4	2300.2	0.1494
Cv x species x pp	8	4159.2	519.9	0.8916
Tmt x pp	1	10857.9	10857.9	0.0034
Species x tmt x pp	4	2014.8	503.7	0.7878
Cv x tmt x pp	2	2765.8	1382.9	0.3149
Cv x species x tmt x pp	8	3335.5	416.9	0.9406
Error	68	80000.3	1176.5	
Corrected total	128	151905.3		

Table 7. Percentage of *Protea* leaves infected after inoculation with *Colletotrichum* species.

Colletotrichum species	Infected leaves (%) [ab]
C. acutatum	37.55 a
C. acutatum f. sp. *hakea*	46.73 a
C. boninense	31.47 a
C. gloeosporioides	46.94 a
Control (sterile water)	0.40 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$).

[b] Infected leaves (%) = current flush leaves infected as a portion of the total number of current flush leaves from which isolations were made.

Table 8. Effect of wounding and the plant part treated on the percentage leaves infected after inoculation of *Protea* cultivars with different *Colletotrichum* species.

	Infected leaves (%) [ab]	
Plant part treated	Wounded	Non-wounded
Leaf	57.66 a	28.51 b
Stem	25.38 b	33.35 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$).

[b] Infected leaves (%) = current flush leaves infected as a portion of the total number of current flush leaves from which isolations were made.

Table 9. Analysis of variance for effects of species, cultivar (Cv), treatment (Tmt) and plant part (Pp) treated on infected stems caused by *Colletotrichum* species inoculated onto *Protea* plants.

Source of variation	Df	SS	MS	SL
Model	60	108102.3	1801.7	0.6588
Trial	1	6383.2	6383.2	0.0784
Species	4	33485.1	8371.3	0.0043
Cv	2	4150.3	2075.2	0.3597
Cv x species	8	12494.4	1561.8	0.6207
Tmt	1	13269.7	13269.7	0.0121
Species x tmt	4	4606.9	1151.7	0.6810
Cv x tmt	2	1697.1	848.6	0.6559
Cv x species x tmt	8	6537.1	817.14	0.9119
Pp	1	100.6	100.6	0.8232
Species x pp	4	661.9	165.5	0.9874
Cv x pp	2	1156.4	578.2	0.7498
Cv x species x pp	8	3028.6	378.6	0.9916
Tmt x pp	1	25.1	25.1	0.9111
Species x tmt x pp	4	7211.1	1802.8	0.4680
Cv x tmt x pp	2	3172.1	1586.1	0.4564
Cv x species x tmt x pp	8	10122.6	1265.3	0.7475
Error	69	137968.8	1999.5	
Corrected total	129	246071.0		

Table 10. Percentage of *Protea* stems infected after inoculation with *Colletotrichum* species.

Colletotrichum species	Infected stems (%) [ab]
C. acutatum	60.82 a
C. acutatum f. sp. *hakea*	54.17 a
C. boninense	37.68 a
C. gloeosporioides	45.83 a
Control (sterile water)	0.00 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$)

[b] Infected stems (%) = stems infected as a portion of the total number of stems from which isolations were made.

Fig. 1. Carnival Too plants exhibiting leaf necrosis after inoculation with (from left to right) sterile water (Control), *C. gloeosporioides* (STE-U 4454), *C. acutatum* (STE-U 4452), *C. acutatum* (STE-U 4456), *C. acutatum* f. sp. *hakea* (STE-U 4468) and *C. boninense* (STE-U 194).

Fig. 2. Cardinal plants with non-wounded (left) and wounded (right) leaves, inoculated with *C. acutatum*.

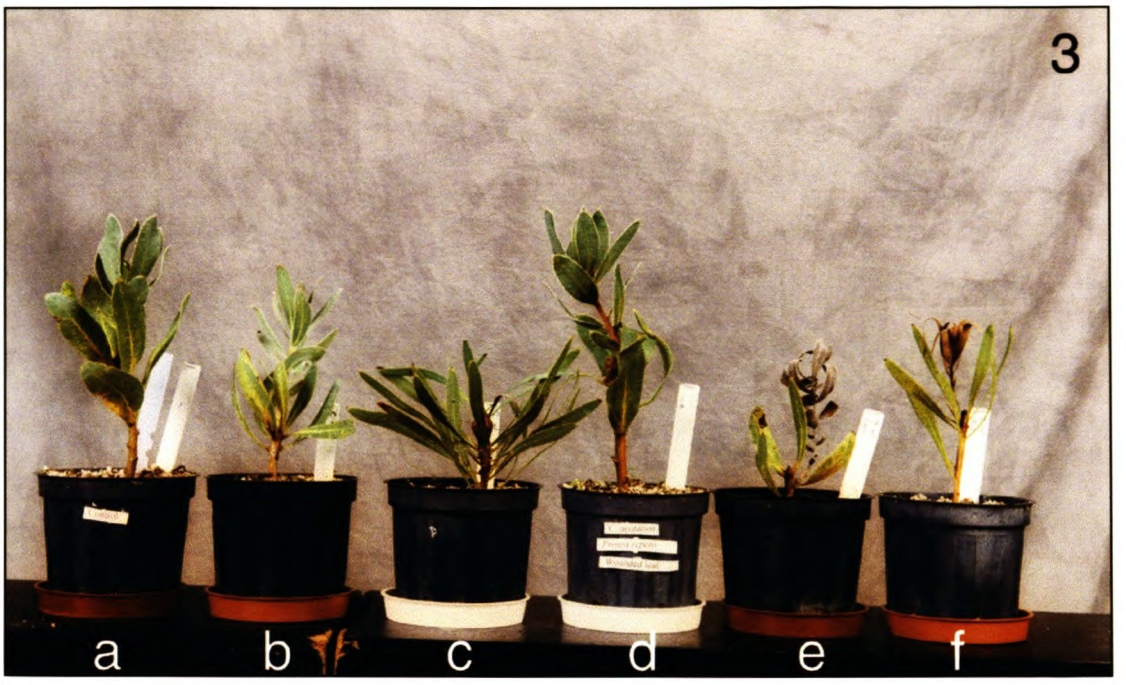

Fig. 3. Control plants (a–c) Cardinal (a), Carnival Too (b) and Rubens (c) and after inoculation with *C. acutatum* (d–f) Cardinal (d), Carnival Too (e) and Rubens (f).

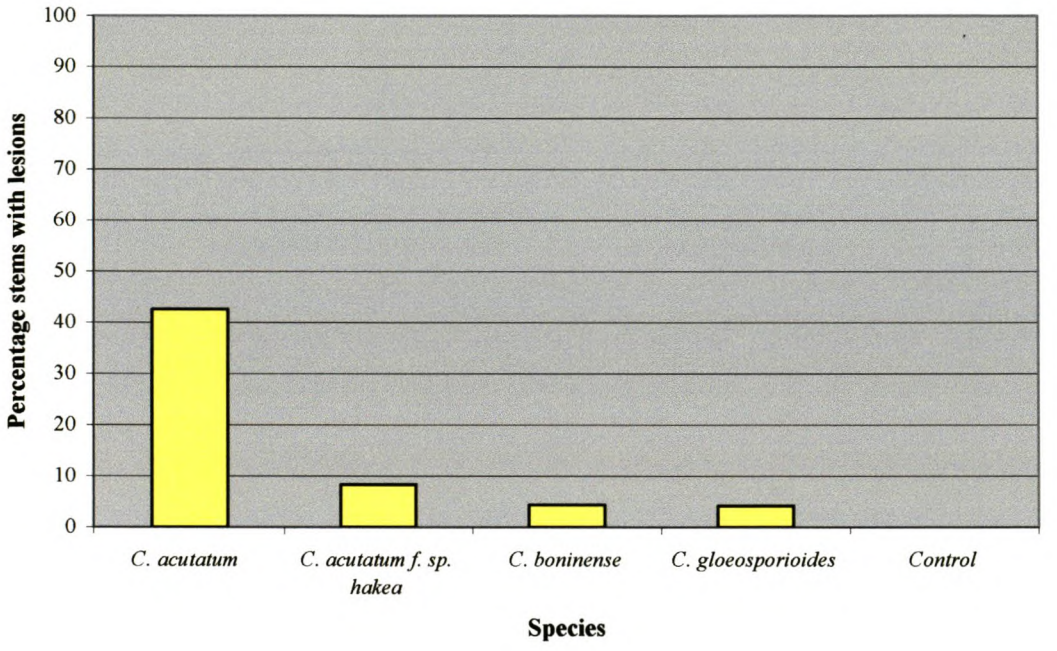

Fig. 4. Percentage of *Protea* stems with lesions for each of the *Colletotrichum* species tested for pathogenicity [Chi Square (df = 4) = 33.51; $P < 0.0001$].

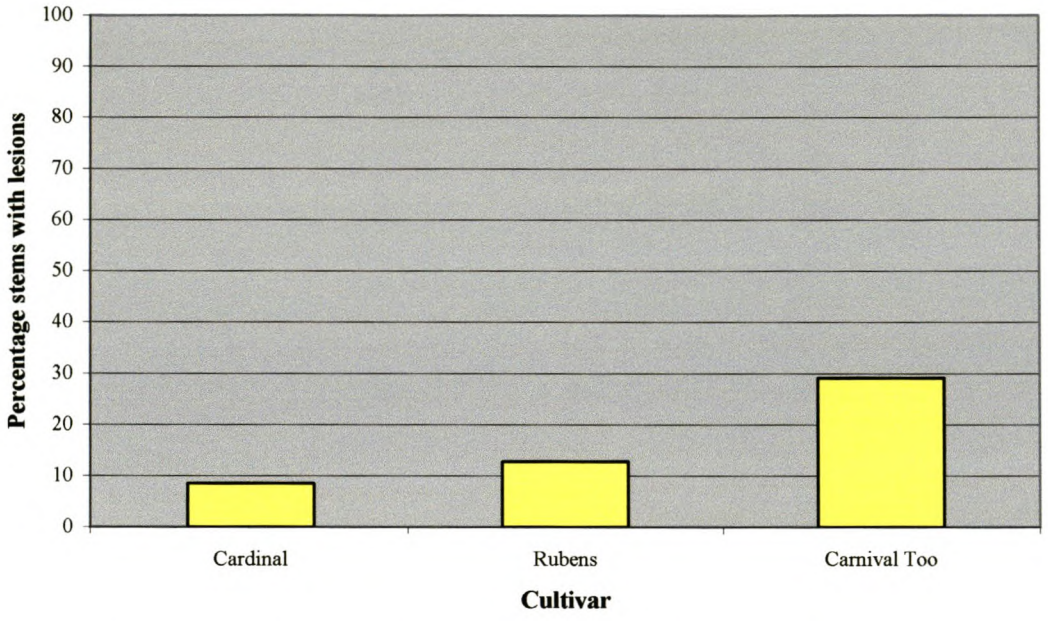

Fig. 5. Percentage of stems with lesions after inoculation of different *Protea* cultivars with *Colletotrichum* species [Chi Square (df = 2) = 8.1; $P = 0.02$].

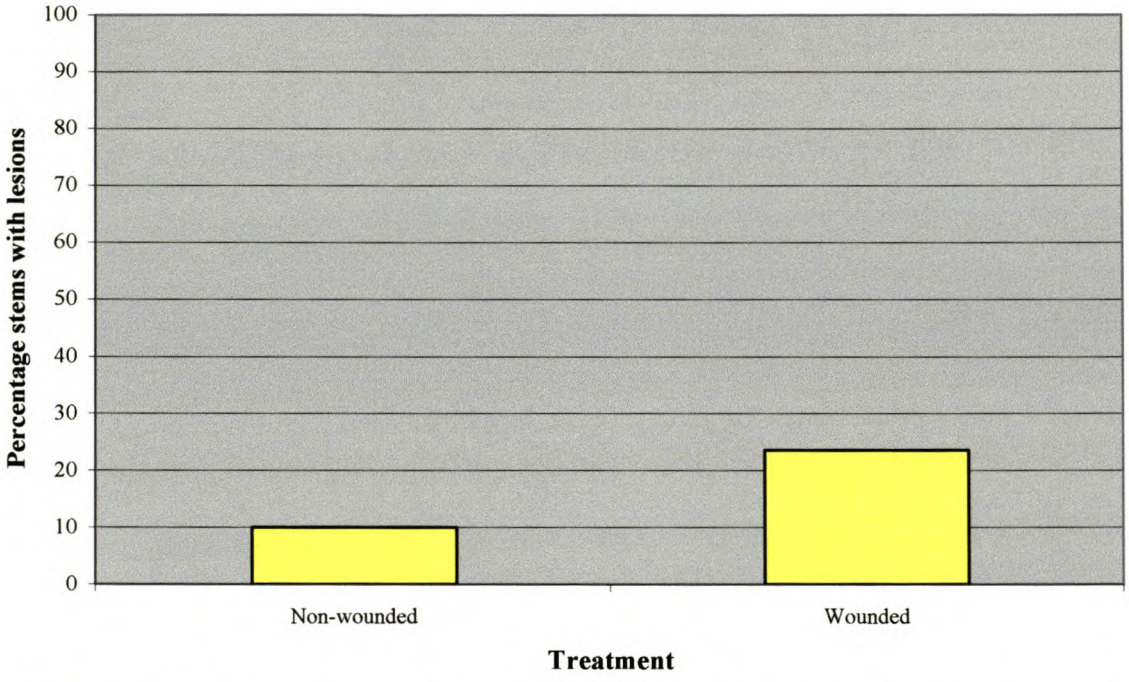

Fig. 6. Effect of wounding on the percentage stems with lesions after inoculation of *Protea* cultivars with different *Colletotrichum* species [Chi Square (df = 1) = 4.68; $P = 0.03$].

4. A HISTOLOGICAL COMPARISON OF THE INFECTION PROCESS OF *COLLETOTRICHUM ACUTATUM* ISOLATES FROM *PROTEA* AND *HAKEA* (PROTEACEAE) ON LEAF SURFACES OF *PROTEA* CULTIVARS

ABSTRACT

Colletotrichum species are known to occur on and cause severe diseases of Proteaceae. Worldwide, four *Colletotrichum* species are associated with diseased Proteaceae, *viz. C. acutatum, C. boninense, C. crassipes* and *C. gloeosporioides*. In this study, the behaviour of two *C. acutatum* isolates, originating from *Protea* and *Hakea*, respectively, was studied on inoculated *Protea* leaf surfaces using light and scanning electron microscopy. The *C. acutatum* isolate from *Protea* (*C. acutatum*) formed high numbers of melanised appressoria on leaf surfaces, whereas the *C. acutatum* isolate from *Hakea* (*C. acutatum* f.sp. *hakea*) formed relatively low numbers of both melanised and unmelanised appressoria. Most of the appressoria formed by both of the isolates were formed on the cell junctions and on the periclinal walls of the epidermal cells. From this study it is clear that *C. acutatum* f. sp. *hakea* is not a pathogen of *Protea*. Consequently the current use of this isolate as a biological control agent of *Hakea* in South Africa poses no threat to indigenous *Protea* species. *Colletotrichum acutatum* from *Protea,* although closely related to *C. acutatum* f. sp. *hakea*, is a pathogen of *Protea*, which was confirmed by histological observations.

INTRODUCTION

Colletotrichum species cause serious diseases on propagation material and mature plants of Proteaceae. Four species of *Colletotrichum* have been associated with diseased Proteaceae to date, *viz. C. acutatum* J.H. Simmonds, *C. boninense* J. Moriwaki, Toy. Sato & T. Tsukiboshi, *C. crassipes* (Speg.) Arx and *C. gloeosporioides* (Penz.) Penz. & Sacc. (Chapter 2). Within *C. acutatum* there appears to be two distinct groups, one group originating from *Protea* hosts (hereafter referred to as *C. acutatum*) and another group originating from *Hakea* (hereafter referred to as *C. acutatum* f. sp. *hakea*) (Chapter 2). Isolates previously obtained from *Hakea* were identified as *C. gloeosporioides* (Morris, 1982), but based on ITS data they have since been shown to be closely related to *C. acutatum* (Chapter 2). However, sequence data derived from the ß-tubulin gene proved unable to provide good support to justify the *Hakea* population as a distinct species (Chapter 2), regardless of the morphological, cultural and ecological differences observed between populations from *Protea* and *Hakea*. Because *C. acutatum* is accepted to be morphologically a highly variable species (Guerber *et al.*, 2003), it was decided to not yet name the *Hakea* collections as a new species, but to acknowledge the ecological and pathological differences by naming them as a *forma specialis* of *C. acutatum*. Any further information that would help clarify the taxonomic position of *C. acutatum* from Proteaceae would therefore be useful to resolve this issue.

Colletotrichum acutatum and *C. gloeosporioides* are extremely virulent pathogens of Proteaceae causing leaf necrosis and stem tip dieback (Chapter 3). *Colletotrichum acutatum* f. sp. *hakea* causes shoot and stem dieback and gummosis on *Hakea* (Morris, 1989), which can ultimately lead to the death of the shrub. Morris (1982) maintained that *C. acutatum* f. sp. *hakea* was not pathogenic to other members of the Proteaceae, and although in recent artificial inoculation tests it was able to infect proteas and cause minor leaf necrosis, isolates are

considered to have a very low virulence on *Protea* (Chapter 3). Thus the two groups in *C. acutatum* interpreted by the molecular studies, appear to represent two separate populations or ecological species.

Hakea sericea Schrad. & J.C.Wendl. is an Australian plant that was introduced into South Africa and has become an invasive weed that threatens the indigenous flora of many of the coastal mountain ranges of the southwestern, southern and eastern Cape. The Proteaceae in South Africa are taxonomically grouped into the sub-family Proteoideae whereas *Hakea* falls into the sub-family Grevilleoideae. *Colletotrichum acutatum* f. sp. *hakea* is currently being used as a commercial biological control agent against this alien invasive plant in South Africa (Lennox pers. com.). In view of the results from the molecular characterisation (Chapter 2) that indicated that the biocontrol strain was closely related to *C. acutatum*, concern about the pathogenicity of the biocontrol agent was raised, since *C. acutatum* strains are serious pathogens of Proteaceae (Chapter 3) and *C. acutatum* f.sp. *hakea* could actually infect *Proteas*. A need therefore existed to verify that the biocontrol strain was not a pathogen of *Protea*, and did not pose a disease risk to the natural fynbos vegetation.

In spite of the general importance of *Colletotrichum* diseases of Proteaceae, the infection processes and disease epidemiology remains largely unknown. Histological data would contribute to our understanding of the pathogenicity processes used by *C. acutatum* and in developing disease management strategies. Furthermore, it might assist in unravelling the species status of this pathogen.

The two major types of inocula of *Colletotrichum* species are conidia (formed in acervuli) and ascospores (formed in perithecia). The adhesion of these propagules to the plant surface is an area of research that has not received much attention. Hamer *et al.* (1988) suggested that a physical signal was necessary for attachment of *Colletotrichum graminicola* (Ces.) G.W. Wilson

(thigmotrophic response). Podila *et al.* (1993) found that the surface wax on avocadoes induced germination and appressorium formation in the spores of *C. gloeosporioides*. The leaf surfaces of most Proteaceae are covered with a very thick cuticle, and often many trichomes are present (Rebelo, 2001). These features may have a significant influence on the infection process of *Colletotrichum* on members of the Proteaceae.

In a study on strawberries it was discovered that with *C. acutatum* and *C. fragariae* A.N. Brooks spore germination begins as early as 16 hours post inoculation (hpi), and that germ tubes can originate from one or both ends of the conidium. These germ tubes usually did not exceed the length of one plant cell (Curry *et al.,* 2002). In contrast with the findings of Curry *et al.* (2002), Leandro *et al.* (2001) witnessed conidial germination of *C. acutatum* on strawberry leaves within 3 hpi. This difference might be attributed to Leandro *et al.* (2001) using excised leaves for inoculation, whereas Curry *et al.* (2002) inoculated attached leaves.

The formation of an appressorium by most *Colletotrichum* species is essential for host penetration (O'Connell *et al.*, 2000). One exception is *C. acutatum* infection of citrus blossoms, where the pathogen was found to penetrate the flower petals without forming appressoria (Zulfiqar *et al.*, 1996). In most hosts the conidium germinates on the host surface to form a germ tube. During germination a septum is formed to delimit the germ tube from the conidium (Agrios, 1997). On strawberry leaves *Colletotrichum acutatum* and *C. fragariae* formed one septum in the conidium upon germination, and formed another septum between the conidium and the germ tube (Curry *et al.,* 2002). The delineated tip of the germ tube adheres to the plant surface, subsequently forming the appressorium. The appressorium adheres firmly to the plant surface and ensures that the infection peg will be at a site where penetration can successfully take place (Agrios, 1997). In some *Colletotrichum* spp. the appressorium can be separated by a septum from the germ tube and in such cases the germ tube and spore are frequently devoid of

cytoplasm (Dean, 1997). Appressoria are often sessile and can form in the absence of a host (Lennè, 1978). The appressorium can also be surrounded by mucilagenous material (Bailey *et al.*, 1992), probably to protect it from adverse environmental conditions.

The appressorium of *Colletotrichum* species has a thick two or three-layered wall, one of which is melanised (Bell & Wheeler, 1986). The appressorium synthesises melanin (Suzuki *et al.*, 1981) to protect itself against harmful radiation and to aid the penetration process (Bailey *et al.*, 1992). *Colletotrichum acutatum* appressoria on strawberries are initially unmelanised, but after 24 h they are completely pigmented (Curry *et al.*, 2002). This suggests that germination should take place under conditions of very low light intensity for successful infection to take place.

Colletotrichum species can penetrate the host in several ways: through natural openings (stomata, lenticels and the apical cells of trichomes), through wounds, and by direct penetration (Bailey *et al.*, 1992).

Clearly there are many aspects of the infection processes that can be examined in histological investigations. In this study scanning electron microscopy was used to study the leaf surfaces of the three different *Protea* cultivars used in pathogenicity tests. The aim of this approach was to search for differences between the cultivars that might help explain the observed differences in disease susceptibility. Further experiments examined the behaviour of *C. acutatum* and *C. acutatum* f. sp. *hakea* conidia on cleared and stained leaf surfaces of *Protea* using light microscopy. Since it was difficult to induce disease development under conditions of artificial inoculation a wound treatment was included to introduce the pathogen directly into plant tissues. Furthermore, leaf wounds artificially simulate insect damage as stem wounds do pruning, which are both common occurrences in commercial plantations, thereby creating conditions that occur in the field.

MATERIALS AND METHODS

Plant material

Potted rooted cuttings of the following cultivars were used: 'Cardinal' [*P. eximia* (Salisb. ex Knight) Fourc. × *P. susannae* E. Phillips], 'Carnival Too' (*Protea compacta* R. Br. × *P. neriifolia* R. Br.) and 'Rubens' [*P. repens* (L.) L.]. Cuttings were treated with 4000 µg/ml indole buturic acid and placed into a rooting bed with a surface temperature of 25°C to ensure rooting. As soon as sufficient root development had occurred the cuttings were transplanted into plastic pots. The growth medium was a mixture of sterilised sand, peat and polystyrene (1:1:1). All the trial plants had one or two 10–15 cm young shoots at the time of inoculation.

Colletotrichum acutatum isolates and inoculation

Two *C. acutatum* isolates were used for inoculation: STE-U 4452 (isolated from *Protea magnifica* Link. in South Africa; referred to as *C. acutatum*) and STE-U 4461 (isolated from *Hakea sericea* in South Africa; referred to as *C. acutatum* f. sp. *hakea*). Spore pellets of both isolates were produced following the protocol of Morris (1983). The spores were resuspended in sterile water and a spore suspension with a concentration of 1×10^6 spores/mL was made. Detached or attached leaves from the three cultivars were either wounded (by means of needle prick) or not wounded. The detached leaves or attached leaves (on potted plants) were atomised with the spore suspension until run-off. Detached, inoculated leaves were placed in Petri dishes with moist filter paper linings and the dishes were sealed with Parafilm. These dishes were incubated at 25°C in an incubation room with near-ultraviolet and white lights set at 12 h cycles. Plants with attached leaves were covered with plastic bags and incubated at 25°C in the laboratory at 22°C with 10 h fluorescent cool white light.

Specimen preparation

Scanning electron microscopy (SEM). In the first experiment SEM was used to study the leaf surfaces of three different *Protea* cultivars as well as the behaviour of *C. acutatum* and *C. acutatum* f. sp. *hakea* on these leaf surfaces. Detached leaves of the three *Protea* cultivars were inoculated and sampled as described above. Leaf tissue was fixed overnight in 3% gluteraldehyde buffered in 0.2 M sodium cacodylate (pH 7). Tissue was rinsed with 0.05 M cacodylate buffer and post-fixed for 2 h in the same solution. The tissue was dehydrated with an ethanol series (30, 50, 70, 80, 90 and 100%) and kept in 100% ethanol. The material was then critical point dried in carbon dioxide and the leaf pieces (5mm × 5 mm) were mounted on stubs. All specimens were gold/palladium-coated in a Polaron Sputter coater and examined using a Leo F440 scanning electron microscope operating at 5 or 10 kV.

Lightmicroscopy. In a pilot study for the second and third experiments, pieces of leaf tissue (5 mm × 5 mm) were excised from the detached leaves in the Petri dishes or from attached leaves on plants at various time intervals (6, 24, 48 or 72 h) after inoculation, and cleared overnight in glacial acetic acid : ethanol (1 : 3). The leaf pieces were stained by gentle heating over an alcohol burner for 1–2 minutes in 0.05% trypan blue in lactophenol. The stained leaf tissue pieces were mounted in clear lactophenol and examined with 40× magnification using a Nikon light microscope. The number of structures considered in each parameter measured (described below) were counted. There were no differences in results between attached or detached leaves so all further work was carried out on detached leaves only. The behaviour *of C. acutatum* and *C. acutatum* f.sp. *hakea* on *Protea* were determined as described above, but on detached leaves only. A Nikon microscope and Zeiss M35W camera was used to photograph infection structures at 40× and 100× magnification.

Experiment layout

Behaviour of *C.acutatum.* The experimental design was a randomised block design. Two pivotal parameters were assessed, *viz*. germination and appressoria. The criteria considered in the germination parameter were (a) not germinated, (b) germinated without the formation of appressoria and (c) germinated with the formation of appressoria. To determine the effect of cultivar, time intervals and wound treatment on germination type, a 4-factor factorial treatment design was used. The factors were: three cultivars (Cardinal, Carnival Too and Rubens), three time intervals (24, 48 and 72 hrs after inoculation), two wound treatments (not wounded and wounded) and three germination types (ungerminated, germinated without formation of appressoria and germinated with the formation of appressoria). With regard to the appressorial parameter, to determine the effect of cultivar, time intervals, wound treatments and cell locations on appressorium type, a 5-factor factorial design was used. The factors were: three cultivars (Cardinal, Carnival Too and Rubens), three time intervals (24, 48 and 72 h after inoculation), two wound treatments (not wounded and wounded), four appressoria types (globose, lobed, sessile and globose, sessile and lobed) and four locations (cell junction, epidermal cell, stoma, trichome). There were two replicates of each treatment combination.

Behaviour of *C. acutatum* f.sp. *hakea.* The experimental structure and design was the same as that for the previous experiment, except for the time intervals. To determine the effect of cultivar, time intervals and wound treatment on germination type, a 4-factor factorial treatment design was used with the following time intervals as part of the time factor: 6, 24 and 48 h after inoculation. To determine the effect of cultivar, time intervals, wound treatments and locations on appressorium type, a 5-factor factorial design was used which included the following time intervals: 6, 24 and 48 h after inoculation. An experimental unit was three leaves and only detached leaves were used.

Parameters measured

In both the light microscopy experiments counts of infection structures were made from the cleared and stained material. Numbers of ungerminated and germinated conidia that did not form appressoria were recorded, as well as the number of conidia that formed appressoria. The number of globose and lobed appressoria were determined, as well as the numbers of sessile globose appressoria and sessile lobed appressoria. Furthermore, the number of globose or lobed appressoria that formed at different locations (the stoma, periclinal wall of the epidermal cell, cell junction and trichome) were determined. The percentages of ungerminated and germinated (no appressoria) conidia, as well as appressoria were calculated as the proportion of the total number of conidia counted for each factor combination. The percentages of appressorium types formed were calculated as the proportion of the total number of conidia that formed appressoria.

Statistical treatment of data

Counts were transformed to percentages and working logit transformation before subjected to analysis of variance using SAS version 8.2 (SAS, 1999) statistical software. Shapiro-Wilk test was performed to test for non-normality (Shapiro & Wilk, 1965). Student's t-least significant difference was calculated at the 5% confidence level to compare treatment means. Pearson's product moment correlation test was performed to determine the correlation between the germination data of *C. acutatum* and *C. acutatum* f.sp. *hakea* (Ott, 1998).

RESULTS

Statistical results

Evidence of non-normality was found in some cases due to high kurtosis and not skewness. A high occurrence of zero values was responsible for kurtosis. The results of the analysis of variance were regarded as valid (Glass *et al.*, 1972).

Description of the cultivar surfaces

The following descriptions of the surface characteristics of the three *Protea* cultivars were made from observation by means of SEM. The entire leaf surface (adaxial and abaxial) of Carnival Too is covered with trichomes (Fig. 1). The surface of Cardinal is covered with an extensive wax layer (Fig. 2) and trichomes are also found but only on the leaf edges (Fig. 3). The surface of Rubens is also covered with wax (Fig. 4), but with a visibly shallower layer than Cardinal. Rubens does not have trichomes on the leaf surface.

Behaviour of *C. acutatum*

Germination. The conidia of this isolate germinated within 24 hpi and were able to form germ tubes from one or both ends of the conidium. In some cases a septum bisected the conidium shortly after germination. Another septum delimited the germ tube from the conidium.

A significant interaction ($P < 0.0001$) occurred for cultivar × germination type (Table 1). In general about 40% of the conidia were ungerminated. No significant difference ($P = 0.05$) was observed between the three cultivars regarding the percentages of ungerminated conidia (Table 2). However, Carnival Too had a significantly higher percentage of germinated conidia (lacking appressoria) in comparison with the other cultivars, and significantly higher percentages of germinated conidia with appressoria were formed on Cardinal and Rubens than on Carnival Too

leaves. Another significant interaction ($P = 0.0460$) occurred for treatment × germination type. A significantly higher percentage of germinated conidia with appressoria formed on non-wounded leaves than on wounded leaves (Table 3). The percentages of ungerminated and germinated (without appressoria) conidia did not differ significantly with regard to wounded and non-wounded leaves.

Appressoria. Four different types of appressoria formed, *viz.* globose appressoria, lobed appressoria and sessile forms of both of these. Appressoria developed on the end of well-defined germ tubes or in a sessile fashion, i.e. directly from the conidium. Instances occurred where more than one appressorium originated from one conidium. Septa were noticed between the appressoria and the germ tubes and coupled with this were conidia that were devoid of cytoplasm. The typical appressorium was globose (Figs. 5–6) and 100% of the appressoria that formed were melanised (Figs. 7–8). Appressoria formed on all three *Protea* cultivars (Fig. 9). Secondary conidiation took place on the leaf surface when *C. acutatum* (*Protea*) was inoculated onto *Protea* (Fig. 10). No penetration of the host was observed by means of the light microscopy or SEM.

A significant interaction ($P = 0.0136$) was found for appressorium type × treatment × location (Table 4). The data presented in Table 5 shows that globose and sessile, globose appressoria were most commonly formed and the highest mean percentage of conidia that formed these appressoria were found on the cell junctions. The second most common location for appressorium formation was the periclinal wall of the epidermal cell. Very low numbers of lobed appressoria or sessile lobed appressoria were formed. Wounding appeared to significantly ($P = 0.05$) increase the percentage of appressoria that formed on the cell junction (Table 5).

Another significant interaction ($P < 0.0001$) occurred for appressorium type × time interval × location. Globose appressoria on the cell junctions significantly increased from 24 to 72 hpi,

whereas the percentage globose appressoria on the periclinal walls of the epidermal reached a maximum after 48 hpi (Table 6). Low percentages of globose appressoria occurred on the trichomes and only after 48 hpi. Sessile, globose appressoria formed within 24 hpi and formed in significantly higher percentages on the cell junctions than on the periclinal walls of the epidermal cells (Table 6).

A significant (P = 0.0003) appressorium type × cultivar × location interaction was recorded. The incidences of different appressoria types at different locations on the leaves of the three Protea cultivars are given in Table 7. The highest percentage of appressoria that formed was globose appressoria on the cell junctions of leaves of Rubens (Table 7). The second highest percentages were globose appressoria on the cell junctions of the other two cultivars. It was noted that a significantly higher percentage of globose appressoria formed on the periclinal walls of the epidermal cells of Cardinal and Carnival Too compared to Rubens. Carnival Too also exhibited significantly higher numbers of globose appressoria on trichomes than the other cultivars. No significant differences were observed between the cultivars regarding the formation of sessile globose appressoria.

Behaviour of *C. acutatum* f. sp. *hakea*

The infection process of this isolate was essentially similar to that observed for *C. acutatum*. However, very few appressoria formed and those that formed, were reduced in size (Figs. 11–12), compared to *C. acutatum* (*Protea* isolate), and not all were melanised (Fig. 13). No direct penetration of the host was observed by means of the light microscopy or SEM.

Germination. A significant interaction (P = 0.0389) occurred for cultivar × germination type Table 8). The data presented in Table 9 indicate that the highest percentage of ungerminated conidia occurred on Cardinal. Carnival Too had significantly higher percentages of germinated

conidia (appressoria absent) than Cardinal. No significant difference was noted for the percentages of conidia that germinated and formed appressoria on the three cultivars.

Another significant interaction (P = 0.0417) took place between time interval and germination type. A significantly higher number of ungerminated conidia were present at 48 hpi than at 6 and 24 hpi (Table 10). No significant increase or decrease in numbers of germinated conidia (without appressoria) or appressoria formed occurred from 6 hpi to 48 hpi. Very low percentages of appressoria formed after inoculation with this isolate.

Appressoria. A significant appressorium type × location interaction ($P < 0.0001$) occurred (Table 11). Incidences of appressoria types recorded at the four locations on Protea leaves are given in Table 12. Most conidia that germinated to form appressoria, formed globose appressoria on the cell junction followed by globose appressoria that formed on the periclinal walls of the epidermal cells (Table 12).

A significant interaction (P = 0.0291) occurred for treatment × time interval × location (Table 13). There was a trend towards no significant difference between the percentage appressoria on wounded and non-wounded plants at the same appressorium location and time interval after inoculation. However, the incidence of appressoria on the trichomes of wounded plants increased significantly from 6 to 48 hpi.

Results on appressorium location of *C. acutatum* f. sp. *hakea* after inoculation of leaves of the three *Protea* cultivars are given in Table 14. A significant interaction (P = 0.007) occurred for cultivar × time interval × location. The highest percentage of appressoria was noted on the trichomes of Carnival Too at 48 hpi and a significant increase of appressoria occurred from 6 to 48 hpi. The appressoria on Cardinal, Carnival Too and Rubens formed mostly on the cell junction and the periclinal wall of the epidermal cell. Cardinal showed no significant increase in the percentage appressoria formed from 6 to 24 hpi and neither did Rubens from 6 to 48 hpi.

A comparison between *C. acutatum* and *C. acutatum* f. sp. *hakea*

No significant correlation was found between the *C. acutatum* and *C. acutatum* f. sp. *hakea* isolates regarding the germination data (i.e. ungerminated, germinated conidia and appressoria formed) ($P = 0.4300$). A significant positive correlation was found between the two isolates for the appressorium type and location data ($P < 0.0001$; $r = 0.51885$).

DISCUSSION

Analyses of the behaviour of *C. acutatum* on various *Protea* cultivars indicated that both cultivar and wounding had an effect on the percentages of conidial germination and appressorial formation. The fact that higher percentages of appressoria formed on non-wounded leaves than on wounded leaves, might be because the pathogen can easily penetrate the host through wounds (as was observed), thus eliminating the necessity for appressorium formation. *Colletotrichum* species can penetrate the host in several ways – through natural openings (stomata and lenticels), through wounds and by direct penetration (Bailey *et al.*, 1992). The most common means is by direct penetration (Bailey *et al.*, 1992). Morris (1983) discovered that "*C. gloeosporioides*" (= *C. acutatum* f. sp. *hakea*) penetrated *Hakea* leaf tissue through the stomata and the apical cells of trichomes. The *C. acutatum* isolate used in this study most often formed appressoria on the cell junctions and on the periclinal wall of the epidermal cell of the *Protea* cultivars, which suggests that natural openings or wounds on the host are not required for penetration by this isolate. The trichomes of Carnival Too leaves might also have served as point of entry as a number of germinated conidia was observed on these structures. Wounding and cultivar had an effect on the type and location of the appressoria. It seems that wounds provide nutrients to the pathogen, and it can therefore stay in an epiphytic phase (forming appressoria on the cell junctions and growing between cells) for longer. Without the nutrients leaking from wounds the pathogen is forced to

go into a necrotrophic phase, forming appressoria on the periclinal wall of the epidermal cell, and penetrating the host cell in order to survive.

The high percentages of globose appressoria formed on the cell junctions of Rubens indicate that this cultivar is very susceptible to infection. The pathogenicity tests performed in Chapter 3 also found Rubens to be a susceptible cultivar. Carnival Too exhibited lower numbers of appressoria than Rubens, which is difficult to reconcile with the high susceptibility this cultivar showed in pathogenicity tests performed in Chapter 3. However, it is possible that the pathogen could have penetrated the trichomes (the entire leaf surface of Carnival Too is covered with trichomes), without the formation of appressoria. Furthermore, *C. acutatum* has already been reported to infect citrus flower petals without forming appressoria (Zulfiqar *et al.*, 1996). The high level of resistance exhibited by Cardinal in the pathogenicity tests is possibly due to the extensive wax layer on the leaf surface that was observed by means of the SEM.

This study found globose appressoria to form at higher incidences than lobed appressoria, which appears to be a characteristic of *C. acutatum*. Curry *et al.* (2002) also reported the formation of globose appressoria by *C. acutatum* on strawberry. All the appressoria of the protea isolate which was pathogenic to *Protea* spp. were melanised, and this feature was also noted for *C. acutatum* on strawberries (Curry *et al.*, 2002; Leandro *et al.*, 2002).

All the features developed after the inoculation of the *Protea* cultivars with the *C. acutatum* f. sp. *hakea* isolate indicate a non-host interaction (Heath 1981) and it is therefore concluded that *C. acutatum* f. sp. *hakea* is not pathogenic to *Protea*. The features referred to are: the high numbers of conidia that remained ungerminated, the lack of significant differences between cultivars with regard to the percentage of conidia that germinated to form appressoria, the low percentages of appressoria that formed and the formation of unmelanised appressoria, as well as the trend towards no significant differences between the percentage of appressoria formed on

wounded and non-wounded plants. Although Heath (1981) stated that inoculated non-host plants commonly do not develop visible symptoms of infection, the pathogenicity data from Chapter 3 however, showed that *Protea* leaves did develop lesions after inoculation with *C. acutatum* f. sp. *hakea.* However, the percentage of leaves with lesions did not differ significantly from the control plants. In other words, no significant symptoms of disease were noticed, which also confirms the non-host status of *C. acutatum* f. sp. *hakea.*

In most *Colletotrichum* species, the formation of an appressorium is essential for host penetration (O'Connell *et al.*, 2000) and melanisation of the appressorium is known to aid the penetration process (Bailey *et al.*, 1992). Because these processes so rarely took place with *C. acutatum* f. sp. *hakea*, it further supports the previous data indicating a non-compatible host-pathogen interaction. Horowitz *et al.* (2002) studied pathogenic and non-pathogenic lifestyles of *C. acutatum* and found that in non-pathogenic interactions the conidia germinated but produced thin, straight germ tubes. They furthermore found that appressoria were formed but without penetration pegs, and that the fungus grew epiphytically on the host tissue after several days of incubation. These findings corroborate observations of appressorial behaviour in this study, as well as in Chapter 3, and offer an explanation why the *Hakea* isolate was re-isolated from surface sterilised *Protea* tissue.

Light and scanning electron microscopy of the two *C. acutatum* isolates isolated from *Protea* and *Hakea* spp., have shown that marked differences occur in the pre-penetration infection process of these two isolates on *Protea* spp. From this study it is clear that *C. acutatum* f. sp. *hakea* is not a pathogen of *Protea.* Consequently the use of this isolate as a biological control agent of *Hakea* in South Africa poses little threat to *Protea* species in this country. *Colletotrichum acutatum*, although closely related to *C. acutatum* f. sp. *hakea* (Chapter 2), is a

pathogen of *Protea*. This was proved by pathogenicity testing (Chapter 3) and supported by histological observations.

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Table 1. Analysis of variance for the effects of cultivar (Cv), time interval (Ti), treatment (Tmt), and germination types (Gt) on the germination status of *C. acutatum* on *Protea* leaves.

Source of variation	Df	SS	MS	SL
Model	54	37854.4	701.0	0.1395
Experiment	1	0.0	0.0	1.0000
Cv	2	0.0	0.0	1.0000
Ti	2	0.0	0.0	1.0000
Cv x ti	4	0.0	0.0	1.0000
Tmt	1	0.0	0.0	1.0000
Cv x tmt	2	0.0	0.0	1.0000
Ti x tmt	2	0.0	0.0	1.0000
Cv x ti x tmt	4	0.0	0.0	1.0000
Gt	2	4858.6	2429.3	0.0133
Cv x gt	4	18896.1	4724.0	0.0001
Ti x gt	4	1138.9	284.7	0.6833
Cv x ti x gt	8	620.3	77.5	0.9952
Tmt x gt	2	3341.3	1670.6	0.0460
Cv x tmt x gt	4	5177.9	1294.5	0.0521
Ti x tmt x gt	4	1408.9	352.2	0.5906
Cv x ti x tmt x gt	8	2412.6	301.6	0.7649
Error	35	17361.7	496.0	
Corrected total	89	55216.1		

Table 2. Comparison of light microscopy counts (%) [a] of ungerminated and germinated conidia as well as appressoria formed by *Colletotrichum acutatum* on inoculated leaves of three *Protea* cultivars.

Germination status	Cardinal [b]	Carnival Too [b]	Rubens [b]
Ungerminated	40.6 a	33.6 ab	41.2 a
Germinated (no appressoria)	8.7 c	50.1 a	10.0 c
Germinated + appressorium	50.6 a	16.3 bc	48.9 a

[a] Percentage calculated as a proportion of the total number of conidia counted on each cultivar.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 3. Comparison of light microscopy counts (%)[a] of ungerminated and germinated conidia as well as appressoria formed by *Colletotrichum acutatum* on wounded and non-wounded, inoculated leaves of *Protea* cultivars.

	Treatment [b]	
Germination status	Non-wounded	Wounded
Ungerminated	35.1 ab	41.8 ab
Germinated (no appressoria)	17.7 c	28.2 bc
Germinated + appressorium	47.2 a	30.1 bc

[a] Percentage calculated as a proportion of the total number of conidia counted within each treatment (wounded or non-wounded).

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 4. Analysis of variance for the effects of cultivar (Cv), time interval (Ti), treatment (Tmt), appressorium types (At) and location on the appressoria formed by *C. acutatum* on *Protea* leaves.

Source of variation	Df	SS	MS	SL
Model	170	100432.8	590.8	0.0001
Experiment	1	0.0	0.0	1.0000
Cv	2	0.0	0.0	1.0000
Ti	2	0.0	0.0	1.0000
Cv x ti	4	0.0	0.0	1.0000
Tmt	1	0.0	0.0	1.0000
Cv x tmt	2	0.0	0.0	1.0000
Ti x tmt	2	0.0	0.0	1.0000
Cv x ti x tmt	3	0.0	0.0	1.0000
At	3	24656.7	8218.9	0.0001
Cv x at	6	229.0	38.2	0.4495
Ti x at	6	13084.3	2180.7	0.0001
Cv x ti x at	12	346.8	28.9	0.7144
Tmt x at	3	38.6	12.9	0.8059
Cv x tmt x at	6	14.6	2.4	0.9990
Ti x tmt x at	6	185.8	30.9	0.5818
Cv x ti x tmt x at	9	19.7	2.2	1.0000
Location	3	32429.9	10809.9	0.0001
Cv x location	6	1674.9	279.2	0.0001
Ti x location	6	1541.2	256.9	0.0001
Cv x ti x location	12	771.4	64.3	0.0942
Tmt x location	3	240.1	80.0	0.1140
Cv x tmt x location	6	870.4	145.1	0.0024

Table 4 Continue

Source of variation	Df	SS	MS	SL
Ti x tmt x location	6	116.6	19.4	0.8114
Cv x ti x tmt x location	9	305.8	33.9	0.5601
At x location	3	12586.6	4195.5	0.0001
Cv x at x location	6	1102.0	183.7	0.0003
Ti x at x location	6	8519.8	1419.9	0.0001
Cv x ti x at x location	12	376.3	31.4	0.6523
Tmt x at x location	3	441.2	147.1	0.0136
Cv x tmt x at x location	6	356.9	59.5	0.1820
Ti x tmt x at x location	6	343.5	57.3	0.2014
Cv x ti x tmt x at x location	9	180.4	20.0	0.8646
Error	99	3894.6	39.3	
Corrected total	269	104327.3		

Table 5. Light microscopy counts (%)[a] of appressorium type formed by germinated *Colletotrichum acutatum* conidia and location of these appressoria on inoculated leaves of three wounded or non-wounded *Protea* cultivars.

		Treatment[b]	
Appressorium type	Location of appressorium	Non-wounded	Wounded
Globose	Cell junction	46.8 b	54.5 a
	Periclinal wall of epidermal cell	33.8 c	26.5 d
	Stoma	0.2 gh	1.7 gh
	Trichome	3.5 f-h	5.5 fg
Lobed	Cell junction	0.1 h	0.0 h
	Periclinal wall of epidermal cell	0.1 h	0.0 h
	Stoma	0.0 h	0.0 h
	Trichome	0.0 h	0.0 h
Sessile globose	Cell junction	10.6 e	6.7 ef
	Periclinal wall of epidermal cell	5.0 fg	4.8 fg
	Stoma	0.0 h	0.1 h
	Trichome	0.0 h	0.0 h
Sessile lobed	Cell junction	0.1 h	0.1 h
	Periclinal wall of epidermal cell	0.0 h	0.0 h
	Stoma	0.0 h	0.0 h
	Trichome	0.0 h	0.0 h

[a] Percentage calculated as a proportion of the total number of appressoria counted within each treatment (wounded or non-wounded).

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 6. Light microscopy counts (%)[a] of appressorium type formed by germinated *Colletotrichum acutatum* conidia and location of these appressoria on inoculated leaves of three *Protea* cultivars over time.

Appressorium type	Location of appressorium	Hours post-inoculation (hpi) [b]		
		24	48	72
Globose	Cell junction	17.4 e	54.1 b	61.3 a
	Periclinal wall of epidermal cell	7.7 f	41.1 c	30.4 d
	Stoma	0.0 h	1.1 gh	0.9 gh
	Trichome	0.0 h	3.7 f-h	7.1 fg
Lobed	Cell junction	0.0 h	0.0 h	0.1 h
	Periclinal wall of epidermal cell	0.0 h	0.0 h	0.2 h
	Stoma	0.0 h	0.0 h	0.0 h
	Trichome	0.0 h	0.0 h	0.0 h
Sessile globose	Cell junction	47.8 b	0.0 h	0.0 h
	Periclinal wall of epidermal cell	26.5 d	0.0 h	0.0 h
	Stoma	0.2 g	0.0 h	0.0 h
	Trichome	0.0 h	0.0 h	0.0 h
Sessile lobed	Cell junction	0.4 h	0.0 h	0.0 h
	Periclinal wall of epidermal cell	0.0 h	0.0 h	0.0 h
	Stoma	0.0 h	0.0 h	0.0 h
	Trichome	0.0 h	0.0 h	0.0 h

[a] Percentage calculated as a proportion of the total number of appressoria counted at each time interval.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 7. Light microscopy counts (%)[a] of appressorium type formed by germinated *Colletotrichum acutatum* conidia and location of appressoria on inoculated leaves of three *Protea* cultivars.

		Cultivar[b]		
Appressorium type	Location of appressorium	Cardinal	Carnival Too	Rubens
Globose	Cell junction	45.8 b	43.9 b	59.1 a
	Periclinal wall of epidermal cell	34.5 c	32.6 c	25.1 d
	Stoma	0.9 g-i	0.8 hi	0.8 hi
	Trichome	1.4 g-i	15.0 e	0.0 i
Lobed	Cell junction	0.0 i	0.0 i	0.1 hi
	Periclinal wall of epidermal cell	0.0 i	0.0 i	0.2 hi
	Stoma	0.0 i	0.0 i	0.0 i
	Trichome	0.0 i	0.0 i	0.0 i
Sessile globose	Cell junction	10.3 ef	6.0 f-h	9.4 ef
	Periclinal wall of epidermal cell	6.8 fg	1.6 g-i	5.3 f-i
	Stoma	0.1 hi	0.0 i	0.0 i
	Trichome	0.0 i	0.0 i	0.0 i
Sessile lobed	Cell junction	0.2 hi	0.0 i	0.0 i
	Periclinal wall of epidermal cell	0.0 i	0.0 i	0.0 i
	Stoma	0.0 i	0.0 i	0.0 i
	Trichome	0.0 i	0.0 i	0.0 i

[a] Percentage calculated as a proportion of the total number of appressoria counted for each cultivar.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 8. Analysis of variance for the effects of cultivar (Cv), time interval (Ti), treatment (Tmt), and germination types (Gt) on the germination status of *C. acutatum* f.sp. *hakea* on *Protea* leaves.

Source of variation	Df	SS	MS	SL
Model	54	34937.3	646.9	0.0274
Experiment	1	0.0	0.0	1.0000
Cv	2	0.0	0.0	1.0000
Ti	2	0.0	0.0	1.0000
Cv x ti	4	0.0	0.0	1.0000
Tmt	1	0.0	0.0	1.0000
Cv x tmt	2	0.0	0.0	1.0000
Ti x tmt	2	0.0	0.0	1.0000
Cv x ti x tmt	4	0.0	0.0	1.0000
Gt	2	25686.9	12843.5	0.0001
Cv x gt	4	3537.9	884.5	0.0389
Ti x gt	4	3461.7	865.4	0.0417
Cv x ti x gt	8	389.6	48.7	0.9912
Tmt x gt	2	454.0	227.0	0.4552
Cv x tmt x gt	4	267.9	66.9	0.9098
Ti x tmt x gt	4	513.4	128.3	0.7597
Cv x ti x tmt x gt	8	625.9	78.2	0.9621
Error	17	4679.4	275.3	
Corrected total	71	39616.8		

Table 9. Comparison of light microscopy counts (%)[a] of ungerminated and germinated conidia as well as appressoria formed by *Colletotrichum acutatum* f. sp. *hakea* on inoculated leaves of three *Protea* cultivars.

	Cultivar [b]		
Germination Types	Cardinal	Carnival Too	Rubens
Ungerminated	59.4 a	37.2 bc	49.8 a-c
Germinated (no appressoria)	34.8 c	54.3 ab	44.4 a-c
Germinated +appressorium	5.8 d	8.6 d	5.8 d

[a] Percentage calculated as a proportion of the total number of conidia counted on each cultivar.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 10. Comparison of light microscopy counts (%)[a] of ungerminated and germinated conidia as well as appressoria formed by *Colletotrichum acutatum* f. sp. *hakea* on inoculated leaves of *Protea* cultivars over time.

	Hours post inoculation (hpi) [b]		
Germination Types	6 hpi [B]	24 hpi	48 hpi
Ungerminated	40.8 b	46.0 b	64.8 a
Germinated (no appressoria)	50.8 ab	45.2 b	34.0 b
Germinated + appressorium	8.3 c	8.8 c	1.2 c

[a] Percentage calculated as a proportion of the total number of conidia counted at each time interval.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 11. Analysis of variance for the effects of cultivar (Cv), time interval (Ti), treatment (Tmt), appressorium type (At) and location on the appressoria formed by *C. acutatum* f.sp. *hakea* on *Protea* leaves.

Source of variation	Df	SS	MS	SL
Model	105	69604.7	662.9	0.0001
Experiment	1	0.0	0.0	1.0000
Cv	2	0.0	0.0	1.0000
Ti	2	0.0	0.0	1.0000
Cv x ti	3	0.0	0.0	1.0000
Tmt	1	0.0	0.0	1.0000
Cv x tmt	2	0.0	0.0	1.0000
Ti x tmt	2	0.0	0.0	1.0000
Cv x ti x tmt	2	0.0	0.0	1.0000
At	1	15928.2	15928.2	0.0001
Cv x at	2	263.6	131.8	0.4735
Ti x at	2	534.3	267.1	0.2272
Cv x ti x at	3	582.9	194.3	0.3521
Tmt x at	1	9.2	9.2	0.8190
Cv x tmt x at	2	34.4	17.2	0.9054
Ti x tmt x at	2	0.4	0.2	0.9989
Cv x ti x tmt x at	2	15.4	7.7	0.9565
Location	3	16852.8	5617.6	0.0001
Cv x location	6	5798.1	966.4	0.0004
Ti x location	6	2611.2	435.2	0.0397
Cv x ti x location	9	4889.1	543.2	0.0072
Tmt x location	3	1009.9	336.7	0.1399
Cv x tmt x location	6	1225.7	204.3	0.3379

Table 11. Continue

Source of variation	Df	SS	MS	SL
Ti x tmt x location	6	2809.3	468.2	0.0291
Cv x ti x tmt x location	6	1222.3	203.7	0.3396
At x location	2	10247.5	5123.8	0.0001
Cv x at x location	4	588.6	147.1	0.5018
Ti x at x location	4	477.0	119.3	0.6031
Cv x ti x at x location	6	2194.9	365.8	0.0763
Tmt x at x location	2	105.2	52.6	0.7393
Cv x tmt x at x location	4	529.9	132.5	0.5535
Ti x tmt x at x location	4	1560.6	390.1	0.0829
Cv x ti x tmt x at x location	4	114.1	28.5	0.9545
Error	34	5865.0	172.5	
Corrected total	139	75469.7		

Table 12. Light microscopy counts (%)[a] of appressorium type and location of *Colletotrichum acutatum* f. sp. *hakea* conidia after inoculation of *Protea* leaves.

	Location of appressorium[b]			
Appressorium type	Cell junction	Periclinal wall of epidermal cell	Stoma	Trichome
Globose	48.9 a	31.8 b	0.7 d	12.8 c
Lobed	3.6 d	2.4 d	0.0 d	0.0 d

[a] Percentage calculated as a proportion of the total number of appressoria counted.

[b] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 13. Light microscopy counts (%)[a] of appressorium location of *Colletotrichum acutatum* f. sp. *hakea* conidia after inoculation of wounded and non-wounded *Protea* leaves over time.

		Location of appressorium[c]			
Treatment	Hpi[b]	Cell junction	Periclinal wall of epidermal cell	Stoma	Trichome
Non-wounded	6	27.9 bc	19.9 b-e	0.0 f	4.4 ef
Wounded		21.0 b-e	22.5 b-d	3.3 ef	6.3 d-f
Non-wounded	24	33.1 ab	13.9 c-f	0.0 f	5.9 d-f
Wounded		22.5 b-d	19.2 b-e	0.0 f	16.7 b-f
Non-wounded	48	15.6 b-f	25.0 bc	0.0 f	18.8 b-e
Wounded		25.0 bc	0.0 f	0.0 f	50.0 a

[a] Percentage calculated as a proportion of the total number of appressoria counted at each time interval.

[b] Hpi = hours post-inoculation.

[c] Means followed by the same letter are not significantly different ($P = 0.05$).

Table 14. Light microscopy counts (%)[a] of appressorium location of *Colletotrichum acutatum* f. sp. *hakea* conidia after inoculation of leaves of three *Protea* cultivars over time.

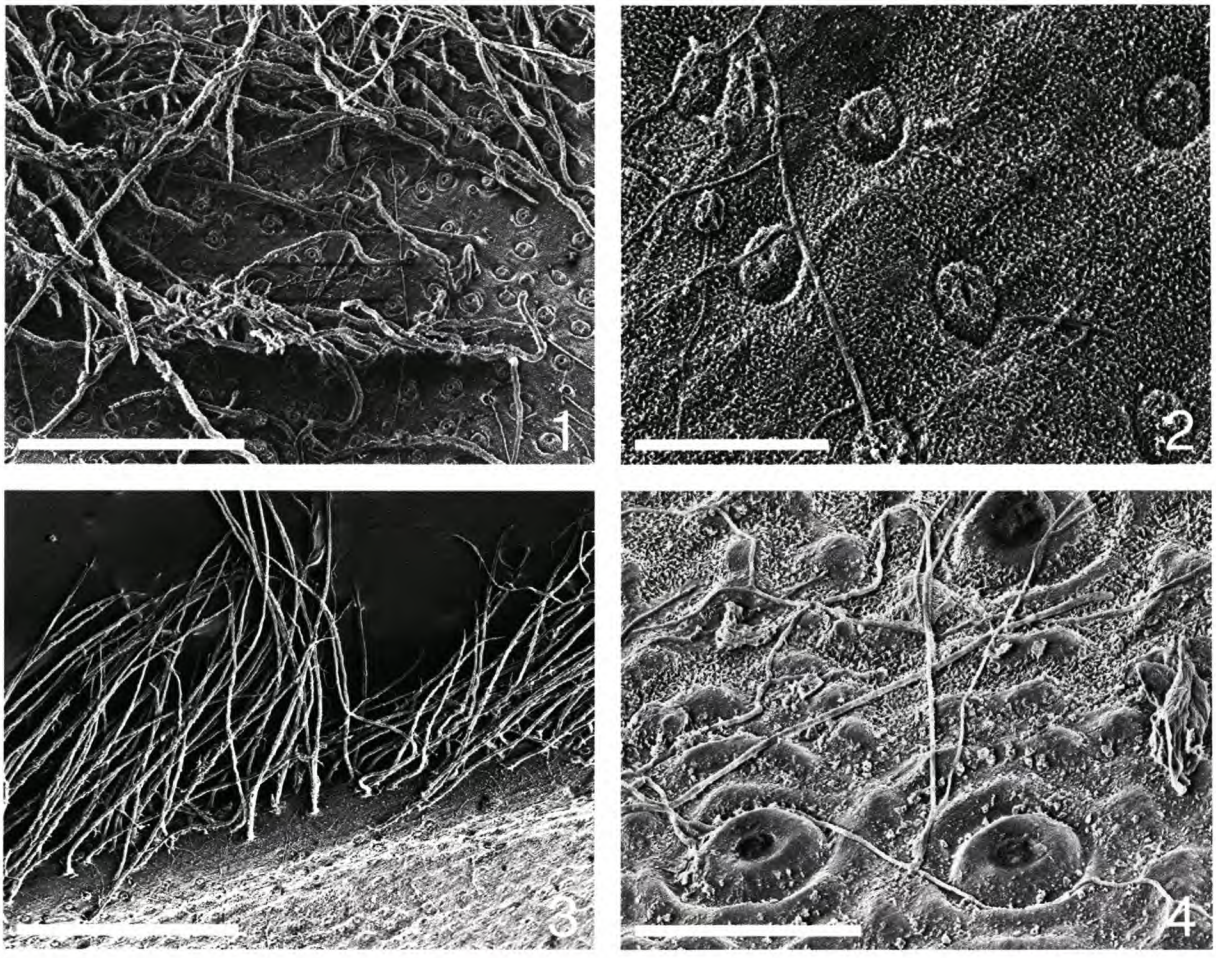
		Location of appressorium [c]			
Cultivar	Hpi [b]	Cell junction	Periclinal wall of epidermal cell	Stoma	Trichome
Cardinal	6	24.2 b-e	23.8 b-e	0.0 h	4.2 f-h
	24	32.5 bc	17.5 c-h	0.0 h	0.0 h
	48	- [d]	-	-	-
Carnival Too	6	23.4 b-f	20.0 b-g	2.2 gh	8.7 e-h
	24	26.4 b-e	10.4 e-h	0.0 h	26.5 b-e
	48	3.1 gh	12.5 d-h	0.0 h	68.8 a
Rubens	6	33.5 bc	16.5 c-h	0.0 h	0.0 h
	24	29.7 b-d	20.3 b-g	0.0 h	0.0 h
	48	37.5 b	12.5 d-h	0.0 h	0.0 h

[a] Percentage calculated as a proportion of the total number of appressoria counted at each time interval.

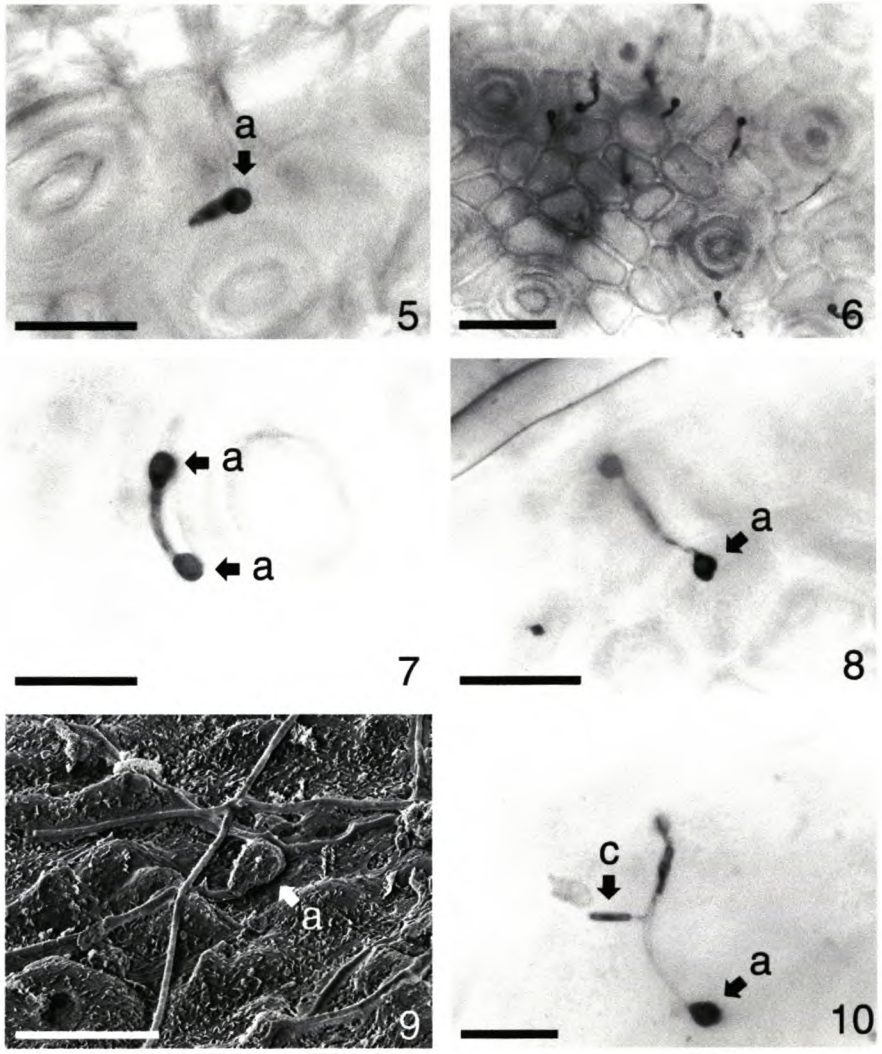
[b] Hpi = hours post-inoculation.

[c] Means followed by the same letter are not significantly different ($P = 0.05$).

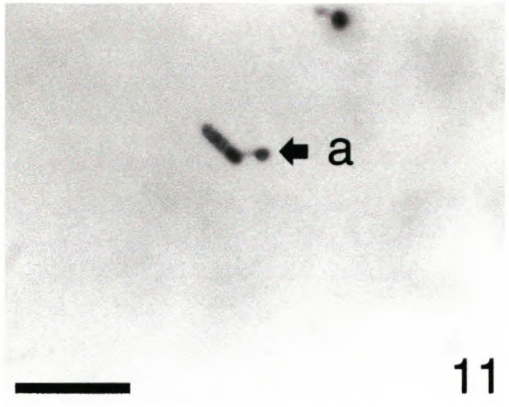
[d] Not evaluated.

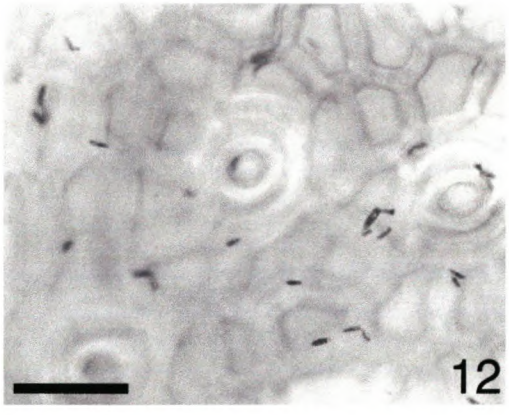


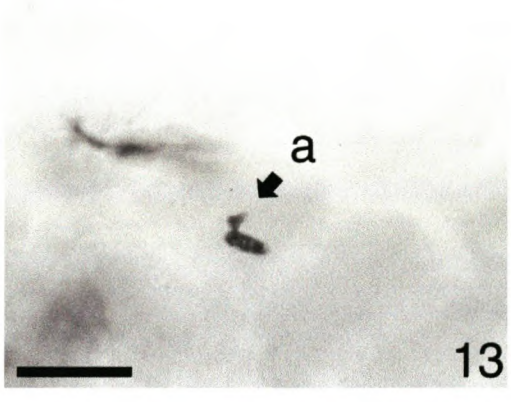
Figs. 1–4. The leaf surface characteristics of the *Protea* cultivars inoculated with *C. acutatum* species as observed by means of SEM. **Fig. 1**. The leaf surface of Carnival Too is covered with trichomes. Scale bar = 500 μm. **Fig. 2**. The surface of Cardinal is covered with an extensive wax layer. Scale bar = 100 μm. **Fig. 3**. Trichomes are found on the leaf edges of Cardinal. Scale bar = 1 mm. **Fig. 4**. The surface of Rubens is uneven and covered with a wax layer. Scale bar = 100 μm.



Figs. 5–10. Infection structures formed by *C. acutatum* on the leaf surfaces of the inoculated *Protea* cultivars. **Fig. 5**. A sessile, melanised appressorium visualised by means of light microscopy, a = appressorium. Scale bar = 25 µm. **Fig. 6**. Sessile and non-sessile, melanised appressoria. Scale bar = 100 µm. **Fig. 7**. Globose, melanised appressoria. The appressoria originate from separate spores, not visible in this field, a = appressorium. Scale bar = 25 µm. **Fig. 8**. A lobed, melanised appressorium, a = appressorium. Scale bar = 25 µm. **Fig. 9**. An appressorium on the surface of Rubens observed by means of SEM, a = appressorium. Scale bar = 50 µm. **Fig. 10**. Secondary conidiation, a = appressorium, c = secondary conidium. Scale bar = 25 µm.







Figs. 11–13. Infection structures formed by *C. acutatum* f. sp. *hakea* on the leaf surfaces of the inoculated *Protea* cultivars (observed by means of light microscopy). **Fig. 11**. A conidium that germinated to form a germtube and melanised appressorium, a = appressorium. Scale bar = 25 µm. **Fig. 12**. Ungerminated and germinated conidia. Scale bar = 50 µm. **Fig. 13**. An unmelanised appressorium, a = appressorium. Scale bar = 25 µm.