BOTRYOSPHAERIA DISEASES OF PROTEACEAE

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation 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:

SUMMARY

Fungi belonging to the genus *Botryosphaeria* are heterotrophic micromycetes that can be pathogens on woody plants. They cause serious, and in some cases devastating losses to crops through leaf necrosis, stem cankers and plant death. The Proteaceae cut-flower industry in South Africa accounts for 70% of the national cut-flower enterprise. Botryosphaeria diseases are a major impediment to production and trade of Proteaceae and there is an urgent need to investigate the etiology, epidemiology and control of these diseases. Losses of one of the most important proteas, *P. magnifica*, amount to 50% or more, locally. The main aims of this study were therefore to establish the etiology and aspects of epidemiology of Botryosphaeria stem cankers on *P. magnifica* and other Proteaceae, and to investigate methods of disease control.

Although there is a vast body of information pertaining to this fungus, which was reviewed in Chapter 1, there is relatively little information available on *Botryosphaeria* on Proteaceae. The taxonomy of *Botryosphaeria* requires thorough review, and molecular techniques need to be employed to resolve species identities.

In Chapter 2, it was found that *Phyllachora proteae*, a leaf pathogen of proteas, produced a *Fusicoccum* anamorph, which is described as *F. proteae*. A sphaeropsis-like synanamorph was associated with *F. proteae* and a new combination for *P. proteae* is proposed in *Botryosphaeria*, as *B. proteae*.

The taxonomy of *Botryosphaeria* is in disarray at both the generic and the specific level. In Chapter 3 the taxonomic history of *Botryosphaeria* is reviewed, and the genus circumscribed and distinguished from other morphologically similar genera. Although several anamorph genera have been linked to *Botryosphaeria*, based on morphological observations and phylogenetic analysis of ITS rDNA sequence data, two anamorph genera are now recognised, those with pigmented conidia (*Diplodia*), and those with hyaline conidia (*Fusicoccum*). *Botryosphaeria proteae* should thus be excluded from *Botryosphaeria*.

Several pathogenic *Botryosphaeria* spp. have an endophytic phase within their hosts. They are therefore imported unwittingly into other countries where they may pose a risk to agriculture and indigenous vegetation. The current global distribution of

Botryosphaeria spp. associated with Proteaceae is clarified and a key to these taxa associated with Proteaceae is provided in Chapter 4. Five *Botryosphaeria* spp. are associated with cut-flower Proteaceae worldwide *viz. B. lutea, B. obtusa, B. protearum, B. proteae* and *B. ribis. B. protearum* is described as a new species.

A thorough understanding of disease epidemiology is essential to effect a reduction of losses. In Chapter 5, I show that on *P. magnifica,* lesions caused by *Botryosphaeria protearum,* which lead to the formation of stem cankers, are initiated in the mid-rib vein or margin of leaves. Koch's postulates were satisfied and it was found that the number of lesions that developed from artificial inoculations correlated with starch levels present in leaves at the time of inoculation.

In Chapter 6 it is shown that *B. protearum* exists as an endophyte in leaves of *P. magnifica* in naturally occurring as well as cultivated plants. In natural stands of proteas stem cankers are rare, but in cultivated plantations the incidence is high. Nutritional analyses indicate that higher levels of nitrogen occur in leaves of cultivated plants in spring, which could enhance disease development. High levels of sodium in the leaves of wild plants may restrict disease development.

The severe economic losses caused by *B. protearum* make the search for improved methods of disease control essential. Fungicide applications form an important component of an integrated approach to disease management. In Chapter 7, *in vitro* tests demonstrate that tebuconazole, benomyl, prochloraz mc, iprodione and fenarimol reduce the mycelial growth of *B. protearum* effectively. In the field there was a 25–85% reduction in the occurrence of stem cankers by applying fungicides or sanitation pruning. The best control was achieved by using benomyl, bitertanol, fenarimol, iprodione, prochloraz manganese chloride alternated with mancozeb and tebuconazole prophylactically. If sanitation pruning is combined with regular applications of fungicides, disease can be combated.

OPSOMMING

Mikrofungi wat tot die genus *Botryosphaeria* behoort, is heterotrofiese organismes, wat patogenies op houtagtige plante kan wees. Hulle veroorsaak ernstige, en in sommige gevalle, verwoestende verliese, deur blaarnekrose, stamkankers en plantafsterwing. Die Proteaceae snyblom-industrie in Suid-Afrika maak 70% van die nasionale snyblom-industrie uit. *Botryosphaeria* siektes is 'n belangrike struikelblok in die produksie en handeldryf van Proteaceae, en daar is 'n ernstige behoefte om die etiologie, epidemiologie en beheer van siektes te ondersoek. Verliese van een van die belangrikste proteas, *P. magnifica*, beloop plaaslik 50% of meer. Die hoof doelstellings van hierdie studie was dus om die etiologie en epidemiologie van Botryosphaeria stamkankers op *P. magnifica* en ander Proteaceae vas te stel en metodes van siektebeheer te ondersoek.

Hoewel daar 'n wye hoeveelheid inligting rakende die swam bestaan, wat in Hoofstuk 1 hersien is, is daar relatief min inligting oor *Botryosphaeria* op Proteaceae beskikbaar. Die taksonomie van *Botryosphaeria* benodig deeglike hersiening, en molekulêre tegnieke word benodig om spesie-identiteite op te klaar.

In Hoofstuk 2 is gevind dat *Phyllachora proteae,* 'n blaarpatogeen van proteas, 'n *Fusicoccum* anamorf produseer, wat as *F. proteae* beskryf word. 'n Sphaeropsis-agtige synanamorf is met *F. proteae* geassosieer en 'n nuwe kombinasie vir *P. proteae* is as *B. proteae* in *Botryosphaeria* voorgestel.

Die taksonomie van *Botryosphaeria* is, beide op die genus- as die spesievlak, in wanorde. In Hoofstuk 3 word die taksonomiese geskiedenis van *Botryosphaeria* hersien, en die genus word omskryf en van ander morfologies soortgelyke genera onderskei. Hoewel verskeie anamorf genera al met *Botryosphaeria* op grond van morfologiese waarnemings en filogenetiese analise van ITS rDNA volgorde data verbind is, word twee anamorf genera nou herken, dié met gepigmenteerde konidia (*Diplodia*), en dié met deurskynende konidia (*Fusicoccum*). *Botryosphaeria proteae* moet dus van *Botryosphaeria* uitgesluit word.

Verskeie patogeniese *Botryosphaeria* spp. het 'n endofitiese fase in hul lewenssiklus. Hulle word dus onwetend in ander lande ingevoer waar hulle 'n gevaar vir landbou en inheemse plantegroei kan inhou. Die huidige wêreldverspreiding van

Botryosphaeria spp. wat met Proteaceae geassosieer word is opgeklaar, en in Hoofstuk 4 word 'n sleutel tot die taksa wat met Proteaceae geassosieer word verskaf. Vyf *Botryosphaeria* spp. word met snyblom Proteaceae wêreldwyd geassosieer, naamlik *B. lutea, B. protearum, B. proteae, B. ribis* en *B. obtusa. B. protearum* word as 'n nuwe spesie beskryf.

'n Deeglike kennis van siekte-epidemiologie is noodsaaklik ten einde verliese te verminder. In Hoofstuk 5 dui ek aan dat letsels wat lei tot stamkankers, veroorsaak deur *Botryosphaeria protearum* op *P. magnifica*, in die hoofnerf of rant van blare ontstaan. Koch se postulate is uitgevoer en daar is vasgestel dat die aantal letsels wat vanuit kunsmatige inokulasies ontwikkel het korreleer met die styselvlakke teenwoordig in die blare ten tye van die inokulasie.

In Hoofstuk 6 word getoon dat *B. protearum* as 'n endofiet in die blare van *P. magnifica*. In natuurlike standplase van proteas is stamkankers skaars, maar in verboude plantasies is die voorkoms hoog. Voedingsanalises dui aan dat hoër vlakke van stikstof in die blare van verboude plante in die lente voorkom, wat siekte-ontwikkeling moontlik kan bevorder. Hoë vlakke van natrium in die blare van natuurlike plante mag siekte-ontwikkeling beperk.

Die ernstige ekonomiese verliese wat deur *B. protearum* veroorsaak word, maak die soektog na verbeterde metodes van siektebeheer noodsaaklik. Fungisiedtoedienings maak 'n belangrike deel uit van 'n geïntegreerde benadering tot siektebeheer. In Hoofstuk 7 dui *in vitro* toetse aan dat tebuconazole, benomyl, prochloraz mc, iprodione en fenarimol die miseliumgroei van *B. protearum* effektief verminder. 'n Vermindering van 25–85% is aangetoon in die voorkoms van stamkankers in die veld, deur die toediening van fungisiedes en sanitasiesnoei. Die beste beheer is verkry deur die voorkomende toediening van benomyl, bitertanol, fenarimol, iprodione en prochloraz manganese chloride, afgewissel met mancozeb en tebuconazole, op plante in die veld. Indien sanitasiesnoei met gereelde toedienings van fungisiedes gekombineer word, kan die siekte bekamp word.

This dissertation is dedicated to my father

Evan Robert Denman

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"I believe a leaf of grass is no less than the journey-work of the stars"

Walt Whitman 1819–1892

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CHAPTER 1

Botryosphaeria: A review of its taxonomy, epidemiology, pathology and its significance in the Proteaceae cut-flower industry

Abstract

The Proteaceae cut-flower industry is an important agricultural industry in South Africa, accounting for 70% of the local cut-flower industry. Species of *Botryosphaeria* are serious pathogens of Proteaceae, severely restricting commercial production thereof. Not much is known about these pathogens on Proteaceae, and the aim of this study was to summarise essential information pertaining to the taxonomy, pathology, epidemiology and control of *Botryosphaeria* spp. associated with this crop. The taxonomy of *Botryosphaeria* requires thorough review, and molecular techniques need to be employed to resolve species identification. Thus, many of the species previously assigned to *Botryosphaeria* are arbitrary. Definitive taxonomic studies are required to establish which species of *Botryosphaeria* are associated with Proteaceae. Several pathogenic *Botryosphaeria* spp. reside endophytically within their hosts, waiting for specific stimuli to incite disease development. Aspects such as seasonal effects, stress and carbohydrate status of host tissue are associated with disease development. By implementing an integrated control programme incorporating sanitation pruning, specific cultural practices and fungicides, disease can be combated.

Introduction

The South African Proteaceae cut-flower industry has grown from humble origins where people harvested indigenous vegetation from mountainsides surrounding Cape Town, to a R220 million concern in the millennium production season (Coetzee and Littlejohn 1995; Middelmann 2000; Coetzee and Littlejohn 2001). At present it accounts for 70% of the entire South African cut-flower industry. Since the inception of the protea cut-flower industry in South Africa, products have been highly sought after, particularly in overseas markets. Today, more than 80% of the produce is

exported, mainly to Holland and Germany, but also to other European markets and to the U.S.A. (Middelmann 2000).

Cultivation of South African Proteaceae is expanding throughout South Africa and has also become established in Australia, Azores Islands, California, Canary Islands, Chile, Hawaii, Israel, Madeira, New Zealand, Portugal, Spain and Zimbabwe. Increased international competition in this trade is placing great pressure on South Africa to retain its advantage in the market place. Thus, the demand for high quality produce that is regularly supplied must be met.

One of the top selling flowers exported by South Africa is *Protea magnifica* Link. (the queen protea). Commercial production of this protea is severely restricted by leaf necrosis and stem cankers caused by *Botryosphaeria* spp. (von Broembsen 1989). Disease not only devalues aesthetic appearance by leaf blemishes and stem necrosis, but also causes losses through branch dieback and ultimate death of bushes. Locally, losses of up to 50% of a crop have been observed in some seasons (G. Jacobs, Dept. of Horticulture, University of Stellenbosch, pers. comm.), but there are records of entire plantations being destroyed by canker in the U.S.A. (Hawaii, California) (Taylor 2001; Taylor *et al.* 2001a,b).

Losses are not only experienced in the field. Occasionally flower packers do not notice small leaf blemishes when filling boxes for export. Upon arrival at the port of import, the flowers are subject to phytosanitary inspection. If any pathogens are isolated from blemishes, entire consignments can be rejected on the basis of contravention of phytosanitary regulations, and great losses ensue. Thus, significant losses attributable to *Botryosphaeria* are not only experienced in the production process, but also at various points along the marketing chain.

Since *Botryosphaeria* is such a serious pathogen of Proteaceae worldwide, studies on the etiology and epidemiology of diseases caused by this fungus are urgently required. Such studies will make it possible to develop disease management strategies and thus minimise losses. Information generated from these studies will also enhance quarantine decisions to ensure that they are justly applied to Proteaceous material being imported to and exported from various countries interested in these plants. As a first step towards these studies, information on *Botryosphaeria* needed to be reviewed. Although there is a vast body of information pertaining to this fungus there is relatively little information available on *Botryosphaeria* on Proteaceae. Thus the information on *Botryosphaeria* in general was consulted, but the scope of the

literature was confined to certain aspects. These included taxonomic issues, *Botryosphaeria* spp. associated with Proteaceae, and the pathology, epidemiology and control of diseases caused by *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & de Not. and *Botryosphaeria ribis* (Tode ex Fr.) Gross & Dugg. The latter two species were singled out because they have commonly been associated with diseases of Proteaceae (Olivier 1951; van Wyk 1973; von Broembsen 1989). The main emphasis was placed on *B. dothidea* because it is generally regarded as being the chief stem canker pathogen of proteas (von Broembsen and van der Merwe 1990).

Taxonomy and reproduction of *Botryosphaeria*

Taxonomic history of *Botryosphaeria*. The basis of any disease investigation is a thorough understanding and correct identification of disease etiology. The foundation of etiology is taxonomy. *Botryosphaeria* taxonomy is complex and many issues pertaining to the generic and species concepts are unresolved (Sutton 1980; Pennycook and Samuels 1985). The position of the genus in the higher classification of ascomycetes has not yet been resolved (von Arx and Müller 1975; Sivanesan 1984; Barr 1987; Silva-Hanlin and Hanlin 1999). Barr (1987) placed *Botryosphaeria* in the Pleosporales, and von Arx and Müller (1975) placed it in the Dothideales. Currently, however, the widely accepted classification of *Botryosphaeria* is that it is a member of the family Botryosphaeriaceae, which is accommodated in the Dothideales (Hawksworth *et al.* 1995).

At the specific level much confusion has occurred regarding the allocation of *Botryosphaeria* species to the genus. The reasons for this are many but can be attributed largely to ill-defined concepts describing morphological features and insufficient diversity of features to allow unequivocal differentiation. *Botryosphaeria* species are pleomorphic, i.e. they have more than one independent spore stage in their complete (holomorphic) life cycle (Sivanesan 1984; Barr 1987). The teleomorph (sexual form of the fungus to which the name *Botryosphaeria* applies) is represented in the subdivision Ascomycotina while the anamorph or asexual states are represented in the coelomycetes (Barr 1987). Because there is insufficient diversity amongst the taxonomic features of the teleomorphs to allow accurate differentiation at species

level (Shoemaker 1964; Laundon 1973), the identification of these fungi is largely dependent upon the taxonomy of its anamorphs (Hanlin 1990).

At least seven anamorph genera have been consistently linked to *Botryosphaeria*. Fungi from the genera *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino, *Sphaeropsis* (Sivanesan 1984) and *Fusicoccum* Corda are the most important anamorphs of *Botryosphaeria* species (Sutton 1980; Pennycook and Samuels 1985; Samuels and Singh 1986; Morgan-Jones and White 1987). However, concepts circumscribing the anamorph genera associated with *Botryosphaeria* are poorly described (Morgan-Jones and White 1987), further complicating identification. This has often resulted either in incorrect identification or the pathogen not being fully identified to species level.

***Botryosphaeria dothidea* complex.** Two species of *Botryosphaeria* that have been victim of taxonomic uncertainties are *B. dothidea* and *B. ribis*. Von Arx and Müller (1954) studied the type specimens of numerous *Botryosphaeria* spp., and synonymised many of them. Seven *Botryosphaeria* spp., *B. ribis* as well as a number of other fungi included, were synonymised with *B. dothidea*, and the latter epithet was retained since it was described first (von Arx and Müller 1954). However, there has been some debate and contrary opinion as to whether *B. ribis* is synonymous with *B. dothidea.* Witcher and Clayton (1963), Kobayashi and Oishi (1979), Maas and Uecker (1984) and Michailides (1991) supported von Arx and Müller's synonymy but Punthaligam and Holliday (1973), Rumbos (1987) and Rayachhetry *et al.* (1996) did not accept it and considered the two species separate. Recently Smith and Stanosz (2001), Zhou and Stanosz (2001), and Zhou *et al.* (2001) have also presented molecular evidence supporting the idea that they are separate species. Pennycook and Samuels (1985) first coined the term '*Botryosphaeria dothidea* complex' based on the wide range of conidial measurements attributed to this species. Clearly there are a lot of discrepancies surrounding the taxonomic correctness of the identities of fungi in this genus. This reduces the reliability of species specific literature regarding epidemiology, pathology and control, unless it is supported by in depth taxonomic studies. Nonetheless general guidelines to factors affecting the host pathogen relationships can be obtained.

With the advent of molecular biology, however, a new era in species identification has been proclaimed. Nucleic acid sequences have been used to identify

and integrate fungi at different taxonomic levels, and recent molecular studies on *Botryosphaeria* have shown that it is possible to relate anamorph and teleomorph genera through these methods (Jacobs and Rehner 1998; Zhou and Stanosz 2001). Molecular techniques must therefore be employed to differentiate species until reliable morphological features can be assigned to them.

***Botryosphaeria* spp. and diseases on Proteaceae.** A number of different *Botryosphaeria* spp. have been described from Proteaceae but in most cases these names have not been applied after detailed taxonomic studies had been carried out. Therefore, in view of the taxonomic confusion outlined above, many of the names given to these species are probably arbitrary. Consequently, their use and relevance in the various pathology studies must be considered in this context. To date the species associated with Proteaceae include *B. guabe* Petrak (Petrak 1967), *B. dothidea* (von Broembsen 1986; Taylor 2001b), *B. proteae* (Wakef.) Denman & Crous (Swart *et al.* 2000; Taylor 2001; Taylor *et al.* 2001a,b,c), *B. rhodina* (Cooke) Arx (Forsberg 1993; Taylor 2001b), and *B. ribis* (Olivier 1951; Herbert and Grech 1985; Shearer 1995).

Botryosphaeria gaube was found on dead twigs of *Grevillea* in Australia, and nothing is known about its pathogenicity or how widespread it is. *B. dothidea* on the other hand is considered a serious canker pathogen of Proteaceae wherever they occur (von Broembsen 1986; Taylor 2001b). Pruning wound rot caused by *B. rhodina* is the most serious *Botryosphaeria* disease of cultivated Proteaceae recorded in Australia, but this fungus was considered a weak, opportunistic pathogen of Proteaceae in Hawaii (Taylor 2001b). *B. ribis* caused severe die-back and death of macadamia trees (Herbert and Grech 1985), as well as cankers on proteas (Olivier 1951; van Wyk 1973) in South Africa. In view of the new developments in *Botryosphaeria* taxonomy (Jacobs and Rehner 1998; Zhou and Stanosz 2001; Chapter 3) and conflicting ideas on synonymies (see *B. dothidea* / *B. ribis* discussion above) some of these species require taxonomic reassessment and the associated disease epidemiology or biology of these pathogens needs elucidation.

Sexual reproduction of *Botryosphaeria*. Little is known about the process of sexual reproduction in *Botryosphaeria*, and whether it is homothallic or heterothallic. There are few reports where the teleomorphs have been produced in culture. Taylor (1958) reported that Fulkerson (1956) failed to produce the teleomorph of *B. ribis* in culture

after crossing isolates under a variety of conditions. Attempts by Maas and Uecker (1984) at pairing 12 isolates of *B. dothidea* in all possible combinations were also unsuccessful in inducing ascomata in culture. Similarly, Michailides and Morgan (1992) were unable to induce the teleomorph of *B. dothidea* either *in vitro* or *in vivo*. However, Taylor (1958) found ascogenous stromata and ascospores of *Botryosphaeria corticus* (Demaree & Wilcox) Arx & Müller a few times in culture. Witcher and Clayton (1963) reported perithecial production in only two of 110 pairings of *B. dothidea*, but commented that "it could not be determined whether the fruiting bodies had resulted from a cross or from a single isolate". Cesare and van Warmelo (1987) found that the teleomorph formed readily in pure cultures generated from single ascospores of only one (AC4) out of four isolates of an unnamed *Botryosphaeria* sp. They suggested that sexual reproduction was achieved through spermatisation. Thus, there is some evidence to suggest that these fungi are homothallic.

Sporulation. Obtaining spores *in vitro* is not only important for identification purposes but also for epidemiological work. There is varied success in inducing *B. dothidea* to sporulate in culture. Light seems to be an important factor in inducing conidial formation. Taylor (1958) reported that light was necessary for conidial formation in *B. corticus*. Pycnidia did not develop when *Botryosphaeria* cultures were incubated in the dark (Putterill 1919; Schreiber 1964; Smith and Fergus 1971), but were formed when cultures were placed under lights. Cultures of *B. dothidea* sporulated under continuous or 12 h exposure to fluorescent light (Luttrell *et al.* 1962; English *et al.* 1975; Shahin and Claflin 1980; Reilly and Okie 1982; Kohn and Hendrix 1983; Parker and Sutton 1993). Unfortunately no details of the light spectrum were supplied in these reports, and so it is not clear which wave lengths of light induced sporulation.

Smith and Fergus (1971) tested the effect of light intensity on sporulation using cool white fluorescent tubes and red fluorescent tubes on cultures of *B. ribis*. They found that pycnidia formed at low light intensity (20–38 ft–c), but became more abundant at high light intensity (300–470 ft–c).

Temperature appears to be of little importance in the sporulation process as long as cultures are incubated within a moderate range. Most reports indicated that conidia were formed at 20–28°C.

There are various opinions regarding the most suitable culture medium to use for inducing sporulation. Schreiber (1964), English *et al.* (1975), Shahin and Claflin (1980), Brown and Hendrix (1981) and Parker and Sutton (1993) used fresh potato dextrose agar (PDA). Oatmeal agar was recommended by Luttrell *et al.* (1962), Witcher and Clayton (1963), Milholland and Galetta (1969), Reilly and Okie (1982), Pusey *et al.* (1986) and Ramos *et al.* (1991). Cellulose agar was reported to be very effective in enhancing pycnidial formation (Morgan-Jones and White 1987). A number of researchers have induced spore formation on sterilised vegetable matter. Pycnidia developed on autoclaved corn meal (Shear and Davidson 1936; Demaree and Wilcox 1942) and sterilised *Pinus radiata* D. Don needles on malt extract agar (Chou 1976), or *Pinus resinosa* Ait. needles on PDA (Palmer *et al.* 1987). Kim *et al.* (2001) used autoclaved barley (*Hordeum vulgare* L.) grains for production of conidia of the anamorph of *B. dothidea,* and Putterill (1919) used cylinders of potato, rice grains, apple chips and Coon's solution with success. In some cases attempts to induce sporulation in culture failed (Birmingham 1924; Toole 1963; Schoeneweiss 1965; Spiers 1977; Gardner 1997).

Cultural characteristics

Pigment formation. Cultural features such as mycelium colour and production of pigments in agar medium have been used to aid identification (Grossenbacher and Duggar 1911; Salerno 1957). Witcher and Clayton (1963) tested 60 isolates of *B. dothidea* (39 from blueberry [*Vaccinium* L.] and 21 from other hosts including allspice [*Pimenta* Lindl.], apple [*Malus* Mill], grape [*Vitis* L.], and tung [*Aleurites montana* (Lour.) E. Wils.] plants) for their ability to produce pigments at a range of pH levels. Most isolates produced either a yellow or a lilac pigment at pH values between 5 and 6 up to pH 9, but the authors concluded that this was a variable characteristic and not correlated with pathogenicity as suggested by Grossenbacher and Duggar (1911). Putterill (1919) reported observing the red ("madder brown") colour that Grossenbacher and Duggar (1911) referred to in cultures of *B. dothidea*, but maintained that it was ephemeral and disappeared after a few days. Witcher and Clayton (1963) grew isolates on Czapek's solution while Grossenbacher and Duggar (1911) used starch paste and Salerno (1957) used rice medium.

Brown (1957) and Satour *et al.* (1969) demonstrated that different carbon sources affect morphological properties of *B. ribis* and *Diplodia* (anamorph of some *Botryosphaeria* spp.) respectively. Thus the results obtained in all the separate works on pigment formation are not directly comparable and in view of the confusion regarding the identification of *Botryosphaeria* spp., they may not be reliable. Since molecular techniques are now available to distinguish different species of *Botryosphaeria* (Jacobs and Rehner 1998; Zhou and Stanosz 2001) it would be interesting and might be informative to revisit the pigment forming ability of these fungi. A range of solid and liquid media with different carbon sources, pH levels and light regimes should be tested.

Growth rate. Mycelial growth of *B. dothidea* on PDA is usually rapid and initially colonies are white and floccose, but the mycelium turns grey to black after 3–5 d (Putterill 1919; Maas and Uecker 1984; Brooks and Ferrin 1994). Colonies usually cover the entire surface of a 10 cm diam Petri dish in 4 d at 25°C (Maas and Uecker 1984).

Nuclear status of mycelium. Wolf and Wolf (1939) pointed out that the mycelium of *B. ribis* was multinucleate, and Cesare and van Warmelo (1987) showed that ascospores of *Botryosphaeria* spp. (*B. proteae* among them) contained between 8–24 nuclei per spore. They postulated that the mycelium of *Botryosphaeria* could either be homokaryotic or heterokaryotic (Cesare and van Warmelo 1987). Both Wolf and Wolf (1939) and Cesare and van Warmelo (1987) noted that this was an unusual feature in Ascomycetes. If *Botryosphaeria* is heterokaryotic there are important implications for control using fungicides. However, once again the taxonomic uncertainties with species identity must be remembered, and these results must be considered in that context.

Temperature. The temperature range at which *Botryosphaeria* grows is broad (5–37°C), but most researchers reported the optimum temperature to be around 25–30°C. Witcher and Clayton (1963) noted that the minimum temperature at which growth occurred was 10°C, which is relatively high. The optimum temperature was around 25–30°C, and maximum temperature at which growth occurred was 32–35°C (Witcher and Clayton 1963). The rate or severity of disease development has been

reported to be affected by temperature with more severe disease occurring at warmer temperatures (Hwang 1983; Pusey and Bertrand 1993), but this is discussed in more detail in the pathogenicity and epidemiology section.

Pathogenicity and Epidemiology

Pathogenicity of *Botryosphaeria* spp. associated with South African Proteaceae. Substantial economic losses are presently being ascribed to *Botryosphaeria*. It is therefore ironic that *Botryosphaeria* spp. have historically been viewed as secondary opportunists associated with diseases caused by other primary pathogens (Knox Davies *et al.* 1986). Clearly the view of *Botryosphaeria* spp. and their role as pathogens has changed in recent times and a review of this topic is overdue. The following case studies involving different *Botryosphaeria* spp. demonstrate previous views that these fungi were secondary opportunists.

Olivier (1951) thought that *B. ribis* might be the cause of wilting and sudden death of *Leucadendron argenteum* (L.) R. Br., but van Wyk (1973) proved that *Phytophthora cinnamomi* Rands was the primary cause of death of *L. argenteum* trees. *P. cinnamomi* was thus the main pathogen, and *B. ribis* infections were expressed on weakened host tissue. However, van Wyk (1973) showed that *Botryosphaeria* was pathogenic to Proteaceae, but only formed small, restricted lesions, although in warm weather, bigger lesions occurred.

Benic and Knox-Davies (1983) showed that *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. was the main cause of stem die-back in *Protea compacta* R. Br. plants, but canker development was more severe when plants were inoculated with both *Colletotrichum* and *Botryosphaeria*. The latter fungus was unable to induce symptoms when inoculated into *P. compacta* plants alone, suggesting that it had an opportunistic nature.

Orffer and Knox-Davies (1989) found an association between a species of *Botryosphaeria* and *Phomopsis* (=*Phomopsis saccharata* J.-C. Kang, L. Mostert & Crous; Mostert *et al.* 2001) in cankers on *Protea repens* (L.) L. and *Protea obtusifolia* H. Buek ex Meisn. *P. saccharata* was the primary pathogen, but in about one third of the cases a *Botryosphaeria* sp. was also isolated.

In yet another example of the association of *Botryosphaeria* with primary pathogens, pycnidia of *Fusicoccum aesculi* Corda (anamorph of *B. dothidea*) were found in leaf lesions of *P. magnifica*. The waxy lesions had initially been caused by *Coleroa senniana* (Sacc.) Arx, (Serfontein and Knox-Davies 1990) but once they were colonised by *F. aesculi,* they became necrotic. In inoculation trials, leaf necrosis developed only on leaves with *C. senniana* lesions and not on healthy leaves. Serfontein and Knox-Davies (1990) concluded that the *Coleroa* thyrothecia rupture the leaf cuticle as the fruiting bodies emerge, thereby providing a port of entry for *F. aesculi*. Without the ruptured cuticle the fungus was unable to cause disease, and was thus considered an opportunistic pathogen.

On pin-cushion proteas (*Leucospermum* spp.)*, B. dothidea* was frequently isolated together with *Drechslera dematioidea* (Bubák & Wroblewski) Subram. & Jain from dark stem cankers (von Broembsen 1986). Both fungi caused lesions on stems artificially inoculated through wounds.

Botryosphaeria proteae (Wakef.) Denman & Crous was considered a leaf pathogen of *Protea* L. but could invade stem tissue weakened by insect damage or cankers caused primarily by other pathogens. Swart *et al*. (2000) concluded that *B. proteae* was mostly restricted to leaf tissue and was not important as a stem pathogen. This fungus appears to be restricted to *Protea* and *Leucospermum* R. Br. (Crous *et al*. 2001).

Latent pathogen or endophyte. Recently *Botryosphaeria* spp., particularly *B. proteae,* have been isolated as endophytes on Proteaceae (Swart *et al*. 2000; Taylor *et al*. 2001c). There are also a number of reports of *B. dothidea* having an endophytic stage or existing in plant tissues as latent pathogens on other hosts (Schoeneweiss 1965; Weaver 1974; Pennycook and Samuels 1985; Pusey *et al*. 1986; Smith *et al*. 1996). The endophytic status of *Botryosphaeria* spp. needs to be investigated since this phenomenon has serious implications for disease epidemiology and the export trade. Furthermore, since it appears that the fungus resides endophytically, its geographic distribution and host range may be much wider than originally thought.

Symptoms and diseases. *Botryosphaeria dothidea* is pathogenic to many woody plants of agricultural importance other than Proteaceae. The fungus is associated with a wide range of symptoms and diseases including shoot tip death, stem cankers,

gummosis and die-back. Leaf necrosis (Horne and Palmer 1935; Ramos *et al.* 1991), fruit rot and nut blight have also been recorded (Horne and Palmer 1935; Kobayashi and Oishi 1979; Sutton 1981; Rittenburg and Hendrix 1983; Webb 1983; Pennycook and Samuels 1985).

An unusual symptom that has been associated with some *Botryosphaeria* spp. is root disease. For example root disease was caused by *B. dothidea* on pines (Hodges 1983) in Hawaii. Another record was made by Kobayashi and Oishi (1979) where *Macrophoma castaneicola* TS Kobay. & C. Oishi, which was considered the anamorph of a *Botryosphaeria* sp., caused root disease on chestnuts (*Castanea* Mill.). Although root rot is not typically associated with *Botryosphaeria* spp., certain methods of cultivating crops may lend themselves to this form of disease.

Host range. Among the hosts affected by *B. dothidea* are almonds (*Prunus amygdalus* Batsch) (English *et al.* 1975), apples (Sutton 1981; McGlohon 1982; Latorre and Toledo 1984; Travis *et al.* 1991), blackberries (*Rubus* L.) (Maas and Uecker 1984), blueberries (De Maree and Wilcox 1942; Witcher and Clayton 1963; Milholland 1972), chestnuts (Kobayashi and Oishi 1979), mangoes (*Mangifera indica* L.) (Johnson 1992) peaches (*Prunus* L.) (Weaver 1974; Reilly and Okie 1982), Proteaceae (von Broembsen 1986), as well as many forestry and ornamental plants (Hodges 1983; McPartland and Schoeneweiss 1984).

Host specificity. Despite the fact that *B. dothidea* is regarded by many as a non-host specific pathogen (Smith 1934; Ma *et al.* 2001a), Pusey *et al.* (1986) thought that certain biotypes may exist, which are host specific in their pathogenicity. Creswell and Milholland (1987) used a number of isolates of *B. dothidea* from a range of hosts to inoculate different cultivars of blueberry bushes. They reported that all isolates were pathogenic to blueberries, but their virulence on the different cultivars was variable, thus implying the occurrence of different pathogenic strains (biotypes). Schreiber (1964) also demonstrated that isolates of *B. ribis* from apples were less virulent than those from *Rhododendron* when wound inoculated on *Rhododendron*, thereby supporting the idea of host specificity of biotypes. Contrary to this Ma *et al.* (2001a) reported that sixty isolates of *B. dothidea* from various hosts were all capable of causing typical symptoms of panicle blight of pistachio trees (*Pistacia* spp.) in California, thus suggesting the absence of biotypes.

Distribution and cross pathogenicity of *B. dothidea*. *B. dothidea* has a global distribution on many crops, and has been reported from Australia (Johnson 1992), Chile (Latorre and Toledo 1984), Guatamala (Schieber and Zentmyer 1978), Hawaii (Hodges 1983), Japan (Kobayashi and Oishi 1979), Portugal (Phillips 1998), New Zealand (Spiers 1977; Pennycook and Samuels 1985), South Africa (von Broembsen 1986) and the U.S.A. (Weaver 1974; Maas and Uecker 1984). However, it is unknown whether the isolates from Proteaceae are able to cause disease on other crops and *vice versa* and whether a risk is involved in importing infected material. Cultivation of South African Proteaceae is rapidly expanding in many countries and material cultivated in other countries could become infected with different species of *Botryosphaeria* that have not yet been recorded on this host in South Africa. Material produced outside South Africa may then be re-imported into this country and bring with it a range of different *Botryosphaeria* pathogens that could place both the local industry and the fynbos biome at risk. It is therefore necessary to determine which species of *Botryosphaeria* occur on Proteaceae in different parts of the world. This information could then be used as a basis for Pest Risk Assessments (PRA's) and protect both the protea industry and the farmers that sustain it.

Disease initiation. Development of disease is usually determined by the intrinsic properties of the host as well as those of the pathogen, and is influenced by environmental conditions. In spite of the broad host range and wide geographic distribution of *B. dothidea*, shoot tip die-back and stem canker are the most prevalent forms of disease caused by this fungus, but disease initiation is variable. Wounds are often cited as being the main port of entry for the pathogen (Wiehe 1952; Hutton and Leigh 1958; Witcher and Clayton 1963; Schreiber 1964; English *et al.* 1975; McGlohn 1982; Fraser and Davison 1985; Forsberg 1993) and some reports maintain that they are essential for disease development (Schreiber 1964; Christ and Schoeneweiss 1975; Kohn and Hendrix 1983; Worral *et al.* 1986). On the other hand, certain authors have concluded that *B. dothidea* is both a wound and non-wound pathogen (Weaver 1974; Pusey 1989). Toole (1963) maintained that wounds facilitated canker development but were not essential for disease. Some researchers reported that *B. dothidea* is mostly a non-wound pathogen (Horne and Palmer 1935; DeMaree and Wilcox 1942; Luttrell *et al.* 1962).

Non-wound infection can begin in leaves (Luttrell *et al.* 1962; Ramos *et al.* 1991) or through lenticels or stomata either on fruit (Horne and Palmer 1935) or on young stems and branches (Brown and Hendrix 1981). Wound infections do not always occur in obvious wound sites such as insect forage wounds or hail damaged parts. Weihe (1952) discovered that the scars of perianth parts were the main infection points in tung tree (*Aleurites* J.R. Forst. & G. Forst.) tip die-back.

Disease initiation on proteas is also often associated with wounds. On *P. magnifica*, Serfontein and Knox-Davies (1990) claimed that *F. aesculi* obtained entrance to leaves through cuticles ruptured (wounded) by *Coleroa senniana*. In Australia, Forsberg (1993) maintained that Botryosphaeria stem disease of proteas manifested itself as a pruning wound rot, which was also observed in Hawaii (P.W. Crous, Dept. of Plant Pathology, University of Stellenbosch, pers. comm.).

Disease severity in wound infected material is affected by the age of the wound with fresh wounds being the most susceptible (Schreiber 1964; Wene 1979; Creswell and Milholland 1987). In fact, Wene (1979) pointed out that only fresh wounds on *Cornus stolonifera* Michx. (redtwig dogwood) were colonised by *B. dothidea* and that seven-day-old leaf scars or wounds were not colonised.

The age of plant parts also affects the success of infection. Stevens and Jenkins (1924) and Maas and Uecker (1984) indicated that disease on currants (*Ribes* L.) and blackberry stems respectively, began in the lateral buds of second year stems. Britton and Hendrix (1982) did not record any infection on inoculated old wood of peach trees, while severe gummosis developed on young wood. Weaver (1979) reported that during inoculation trials on peach trees young wood became symptomatic quickly but infections on older wood took longer to develop and express as cankers. Creswell and Milholland (1987) found that fewer infections occurred with increasing age of wounds on blueberries.

Effects of season on inoculum production, dispersal and germination. Under field conditions on various hosts, it appears that reproductive structures require between 3–24 months to form after infection has taken place. Pycnidia usually form rapidly and develop within a few weeks after infection, but pseudothecia develop slowly. Some researchers reported that pycnidia may have developed in spring, but were usually present in summer (Wiehe 1952; Weaver 1974), although Covey (1967)

maintained that fruiting structures of the fungus were never present during the summer. Wolf and Wolf (1939) reported that the pycnidial stage was present on the current season's shoots while pseudothecia typically formed in older branches. Pseudothecia only first became evident in winter (Grossenbacher and Duggar 1911; Olivier 1951; Weaver 1974).

Season also greatly influences inoculum dispersal because different moisture and temperature regimes prevail at different times of the year. Inoculum of *Botryosphaeria* spp. is usually released in association with moisture, but temperature has an effect on spore release. Weaver (1979), Sutton (1981) and Pusey (1989) reported that inoculum production and dispersal usually took place in spring, but could also occur in summer after rainfall. Windblown rain was reported to be one of the most important mechanisms in spore dispersal (Weaver 1979; Sutton 1981; Pusey 1989; Michailides and Morgan 1992).

In *Botryosphaeria* spp. there are two main components of inoculum, namely ascospores and conidia, and dispersal of the two inoculum types is not necessarily the same. Sutton (1981) and Pusey (1989) reported that ascospores of *B. dothidea* from peaches in the southeastern United States and apples in North Carolina, were airborne. Ascospore discharge occurred during or soon after rainfall or periods of wetness (dew or mist) and were mostly dispersed through the air, but could be found in run-off water as well (Sutton 1981; Pusey 1989).

Conidia on the other hand, were seldom airborne but were mostly found in run-off water dripping from branches (Sutton 1981). Conidia were dispersed throughout the year when moisture was present but never during the winter in the southeastern U.S.A. (Pusey 1989).

The most important seasonal effect on inoculum release and dispersal in the presence of sufficient moisture, is temperature. Pusey (1989) maintained that spore release did not often occur at low temperatures even in the presence of water. Neither Weaver (1979) nor Pusey (1989) detected spores of *B. dothidea* in run-off water from infected trees at the coldest times of year (January and February) in North America. However, Michailides and Morgan (1992) who were working on pistachio nuts in California, recorded conidia being spread by winter rain. In the Western Cape Province of South Africa, von Broembsen and van der Merwe (1990) stated that sporulation of *B. dothidea* on *Protea grandiceps* Tratt. occurred from spring to late

summer, and did not occur during winter. They did not, however, differentiate between ascospores and conidia.

Temperature also has a significant effect on germination of inoculum. Michailides and Morgan (1992) showed that conidia did not germinate below 12°C. Brooks and Ferrin (1994) remarked that at low temperatures there was a much lower rate of germination of conidia originating from the warm southeastern United States, than of conidia originating from California, suggesting that strains of the pathogen were adapted to environmental conditions that they existed in. Therefore warm climate strains were less able to germinate in cold temperatures.

The effects of season on infection, disease development and isolation of the pathogen. Seasonal temperatures also influence disease development. In general, warm temperatures were reported to be favourable for disease development by *B. dothidea* (Weaver 1979; Sutton and Boyne 1983; Hwang 1983; Pusey and Bertrand 1993). Optimal temperatures for panicle shoot blight caused by *B. dothidea* in California were shown to be between 27–33°C (Michailides and Morgan 1992). Weaver (1979) assessed lesion development and positively correlated mean daily air temperature at the time of inoculation with successful infections. In instances where there were *Botryosphaeria* species complexes or seasonal succession of species associated with disease, *B. dothidea* dominated during the summer months when it was warm (Britton and Hendrix 1986; Brooks and Ferrin 1994).

In contrast to the favourable effect of warm temperatures on disease development, low temperatures appear to be unfavourable for activity of the pathogen and disease development by *B. dothidea*. For example, Britton and Hendrix (1986) were unable to isolate *B. dothidea* from peach trees with gummosis at the coldest time of year, and Kohn and Hendrix (1983) could not induce infection of apple fruit below 20°C. Lesion development also ceased in winter on almond trees (English *et al.* 1975), peaches (Britton and Hendrix 1986), chapparal vegetation (Brooks and Ferrin 1994) and azaleas (Schreiber 1964).

Temperature and host physiologies as functions of season also have an effect on disease severity and expression (Pusey and Bertrand 1993). Disease severity was affected by season, with more severe disease occurring in spring and summer than in autumn (Pusey and Bertrand 1993). Disease expression in the form of cankers only became evident in mid or late summer (Demaree and Wilcox 1942; English *et al.*

1975; Shahin and Claflin 1980; Michailides and Ogawa 1986; Brooks and Ferrin 1994). White rot of apples (*B. dothidea*) only developed when fruit began to ripen in late summer (Stevens and Jenkins 1924; Sitterly and Shay 1960; McGlohn 1982; Kohn and Hendrix 1983; Maas and Uecker 1984). However, in some cases infections first became apparent in spring (Wiehe 1952).

Season also has a profound effect on the success of artificial inoculations, and in a number of cases the spring and summer inoculations were more successful than autumn or winter inoculations (Weaver 1979; Pusey and Bertrand 1993). Unwounded plants inoculated in spring or summer usually took a year to express cankers (Luttrell 1950; Weaver 1979), and Parker and Sutton (1993) suggested that *B. dothidea* underwent a long latent period before expressing disease.

Stress. Stress has been reported to increase disease severity and host susceptibility to diseases caused by *Botryosphaeria* (Funk 1964; Schoeneweiss 1965). There are a number of different types of plant stress, and these have been summarised by Schoeneweiss (1975). Stress can thus be categorised as water stress, or temperature, defoliation, transplanting and nutrient stress, with other stresses including light, toxic substances, wounding etc., which also contribute to disease development.

Water stress can be induced either by a water deficit (drought) or by a water surplus (flooding, water logging). A number of reports maintain that *Botryosphaeria* diseases are enhanced by drought stress (Shay and Sitterly 1954; Hutton and Leigh 1958; Bega *et al*. 1978; Lewis and van Arsdel 1978; Herbert and Grech 1985; Madar *et al*. 1989). However, Schoeneweiss (1975) and Wene (1979) noted that many of these reports were based on field observations and were not substantiated experimentally.

Canker length has been related proportionally to drought stress (Hutton and Leigh 1958; Rayachhetry *et al*. 1996). Some researchers have found that there was a threshhold osmotic potential of -12 to -13 bars (measured with a pressure chamber) above which *B. dothidea* did not cause cankers, but remained latent in the tissues (Crist and Schoeneweiss 1975; Wene 1979; Schoeneweiss 1981). Plants also had to be subjected to water stress for a minimum length of time before they became susceptible to canker formation, and once the stress had been relieved by irrigation, the spread of the pathogen and consequent lesion development was halted.

Contrary to the idea that drought stress enhances canker development, Old *et al*. (1990) demonstrated that it had no significant effect on the development of cankers induced by *B. ribis* in *Eucalyptus*. However, Old *et al*. (1990) did relate disease to nutritional stress induced by defoliation and low carbohydrate levels.

Carbohydrates. Diseases caused by *Botryosphaeria* spp. have been associated with carbohydrate levels of host tissues (Sitterly and Shay 1960; Hwang 1983; Kohn and Hendrix 1983; Bachi and Peterson 1985; Parker and Sutton 1993). Diseases associated with the carbohydrate status of plants can be divided into two categories, *viz*. high sugar diseases and low sugar diseases (Horsfall and Dimond 1957).

A number of reports linked disease development of *Botryosphaeria* with high levels of carbohydrates (Sitterly and Shay 1960; Kohn and Hendrix 1983). Sitterly and Shay (1960) reported that by infusing apples with sucrose or fructose, disease resistance mechanisms were overcome and disease developed. They postulated that there was some threshold carbohydrate level at which disease would be initiated. Kohn and Hendrix (1983) demonstrated that white rot of apples caused by *B. dothidea* developed only once sugar levels reached 10.5% in fruit.

The carbohydrate status of plants can be manipulated or affected by a number of factors. Drought stress was reported to affect the carbohydrate status of stressed plant tissues (Kramer 1963; Bachi and Peterson 1985), and Kramer (1963) maintained that frequently there was an increased conversion of starch to sugar in water deficient tissue. Bachi and Peterson (1985) believed that the increase in canker development by *Sphaeropsis sapinea* (Fr.) Dyko & Sutton might be attributable to an increase in the carbohydrate pool available to the pathogen.

Although most reports associated diseases caused by *Botryosphaeria* spp. with high levels of carbohydrates, there have been studies demonstrating the opposite. Old *et al*. (1990) associated the extreme susceptibility of two *Eucalyptus* spp. to *B. ribis* with reduced starch levels caused by defoliation stress. This is clearly a topic that merits further investigation, because the results could lead to significant improvement in disease control.

Control

Control. A thorough knowledge of the epidemiology of specific disease systems is vital to minimise losses and optimise the control options. The spectrum of control options available for use in an integrated control programme includes cultural practices, application of fungicides and plant resistance. Little is known about natural resistance to stem cankers in Proteaceae or other crops. However, von Broembsen and van der Merwe (1990) reported variability amongst Proteaceae in response to inoculation studies, and suggested that resistant genotypes existed for some species. Creswell and Milholland (1987) had great difficulty in selecting blueberry genotypes resistant to *Botryosphaeria* due to cultivar x isolate interaction.

Kuc *et al.* (1967) investigated the nature of resistance to white rot (*B. ribis*) of apples. Host resistance could not be attributed to inhibition of fungal pectolytic enzymes by host phenolic compounds or their oxidation products only, but other mechanisms were also involved in the resistance process. It can be concluded that little is known about host resistance to *Botryosphaeria*.

There are two aspects of cultural practices that are particularly important with respect to management of Botryosphaeria diseases on Proteaceae. These are duration of leaf or stem wetness and sanitation pruning. The period of leaf or stem wetness has been positively correlated to disease incidence if temperatures were suitable for disease development (Weaver 1979; Sutton 1981; Pusey and Bertrand 1993). Therefore, an important component of disease management is to maintain dry plant surfaces and to avoid overhead irrigation (Michailides and Morgan 1992). Wetness was not only attributable to rainfall but might also have been caused by mist or dew (Pusey 1989). Hence, choice of planting site and plant spacing must be planned to minimise free moisture on plant surfaces (Forsberg 1993).

The second aspect of cultural practices that is important for *Botryosphaeria* disease control is the implementation of sanitation pruning. In general the purpose of sanitation pruning is two-fold. Firstly it allows for the removal of diseased portions of branches from trees and thus prevents the spread of disease to uninfected material. Secondly, it reduces inoculum. Sanitation pruning is thus strongly recommended and is one of the most important disease management practices, especially on cultivated Proteaceae (Olivier 1951; Benic and Knox Davies 1983; von Broembsen and van der

Merwe 1990). Infected material that has been pruned should be burnt (Benic and Knox-Davies 1983), or buried (McLennan 1993).

The importance of sanitation pruning was also emphasised by other researchers working on crops other than proteas. Weaver (1974) and Pusey (1989) commented that a major source of *B. dothidea* inoculum affecting fruit trees (apple and peach) was found on dead wood left in the orchards, dead branches in trees and diseased bark on trees. Newly infected wood was a greater source of inoculum than old wood (Weaver 1979) and special attention should be given to removal of these branches. It was deemed crucial to destroy the wood harbouring the inoculum. Consequently all dead wood should be removed from orchards and burned (Birmingham 1924; Weaver 1979; Pusey 1989; Forsberg 1993). However, Starkey and Hendrix (1980) and Pusey (1989) suggested that inoculum on fruit tree prunings left on the orchard floor might be reduced by mowing them with a flail mower.

Burning diseased plant material was seen as an important disease management tool against *Botryosphaeria* on Proteaceae. Benic and Knox-Davies (1983) suggested burning old infested lands prior to replanting them to remove inoculum.

Prevention of unnecessary wounding and treatment of harvesting and pruning wounds was also seen as an important disease management tool (von Broembsen and van der Merwe 1990; Forsberg 1993; Brown-Rytlewski and McManus 2000). It was recommended that pruning and harvesting should be carried out in dry, cold weather where possible (Pusey 1989; Forsberg 1993).

Forsberg (1993) noted that the site layout was an important consideration in disease management and plants should be exposed to maximum sunlight and there should be good air circulation through the canopy. He suggested that growers avoid planting in warm, humid coastal environments but should rather select low rainfall areas. Indigenous populations of *P. magnifica* in South Africa can be found just below the snow line in hot, dry, montane areas of the Kouebokkeveld to Hottentots Holland mountains, the Klein Swartberge, and the Riviersonderend and central Langeberg mountain ranges (Rebelo, 1995). Thus, cultivation of these plants should occur in this type of environment. In Hawaii the climate is warm and humid and prominent Botryosphaeria stem cankers devastate protea bushes (P.W. Crous, Dept. of Plant Pathology, University of Stellenbosch, pers. comm.). Obviously the effect climate has on disease is an important aspect of disease management.

One of the most important aspects of an integrated disease management programme for the protea cut-flower industry is the judicious use of fungicides. Aesthetic beauty is the most significant quality for marketing cut-flowers, and disease devalues appearance of stems and leaves. It is therefore crucial that fungicides are applied to ensure that the aesthetic value of the product is obtained. Fungicides also perform an important role in protecting plants against infections, which could be detected during phytosanitary inspections, and place entire consignments at risk of being rejected.

There are numerous reports on the use of chemicals against *Botryosphaeria* in the Proteaceae and other industries. Fungicides previously recommended against *Botryosphaeria* on the Proteaceae included sprays with captab, captafol and mancozeb (Benic and Knox-Davies 1983), monthly applications of benomyl and captab (von Broembsen and van der Merwe 1990), and either benomyl or iprodione was recommended in Australia (McLennan 1993). None of the above mentioned chemicals are registered on Proteaceae in South Africa, although benomyl, iprodione and mancozeb are registered on ornamentals, and captafol is no longer available (Nel *et al*. 1999).

Chemical control of diseases caused by *B. dothidea* on apples, cranberries, Japanese apricots, peaches and pistatios has been reported (Starkey and Hendrix 1980; Parker and Sutton 1993; Li *et al*. 1995; Brown-Rytlewski and McManus 2000; Ma *et al*. 2001b). Schoeneweiss (1979) found that benomyl applied as a soil drench, where it was taken up by roots and transported to the stems and leaves of red-osier dogwood, effectively reduced losses caused by *B. dothidea*. Von Broembsen (1989) reported that Proteaceae were very sensitive to agricultural chemicals. The phytotoxic effects of fungicides applied under field conditions therefore need to be assessed before chemicals can be registered and recommended for use.

Conclusions

- Substantial economic losses are experienced by the protea cut-flower industry worldwide through diseases attributable to *Botryosphaeria* spp., and there is an urgent need to address the etiology, epidemiology and control of these diseases so that losses can be minimised.

- The taxonomy of *Botryosphaeria* is complex and many issues are unresolved at both the generic and the specific level. At the specific level identification of these fungi is dependent upon anamorph taxonomy, and concepts defining the anamorphs require immediate clarification.

- Molecular technology must be employed to assist with the allocation of isolates to species.

- In view of the discrepancies surrounding the taxonomy of *Botryosphaeria* spp. the taxa associated with Proteaceae require reassessment. The biology of these pathogens and the epidemiology of the diseases they cause need elucidation.

- The global distribution of *Botryosphaeria* species associated with Proteaceae needs to be documented. The potential threat that these fungi pose to other agricultural crops as well as to indigenous vegetation must be studied, and phytosanitary regulations devised where appropriate.

- The endophytic status of these pathogens prior to disease development must be investigated, and mechanisms that trigger disease need to be elucidated. A better knowledge of these factors could lead to significant improvements in formulating disease control.

- Since better disease control can be achieved with a greater understanding of the epidemiology, there is a need to reveal the epidemiology of *Botryosphaeria* diseases, and to determine factors that influence disease development.

- Improved, practical methods of disease management must be sought so that they can be implemented.

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CHAPTER 2

A taxonomic reassessment of *Phyllachora proteae*, a leaf pathogen of Proteaceae[1]

Abstract

Phyllachora proteae is a well-known leaf pathogen of *Protea* spp. In the present study this fungus was recollected from several genera and species of Proteaceae in the Western Cape Province of South Africa, and its taxonomy was reassessed. Single ascospore cultures produced a *Fusicoccum* anamorph in culture, described here as *F. proteae*. A sphaeropsis-like synanamorph with narrowly ellipsoidal, brown, thick-walled conidia, was commonly associated with *F. proteae* in culture. Based on its bitunicate asci, as well as pseudothecial and ascospore morphology, a new combination for *P. proteae* is proposed in *Botryosphaeria*, as *B. proteae*.

Introduction

The Proteaceae is one of the oldest plant families and is estimated to be more than 140 million years old. The family comprises at least 1400 species, of which 330 occur in the South African Fynbos biome, and is among the most predominant groups of flowering plants in the Southern Hemisphere (Rebelo 1995). The unique beauty and hardiness of *Protea* flowers make them highly desirable to local and international cut-flower markets. In 1996, 4.8 million kg of fresh proteas were produced in South Africa, of which 3.3 million kg were exported, earning an estimated R64.5 million (Wessels *et al.* 1997). However, strict phytosanitary regulations of importing countries frequently prevent blemished blooms from reaching potential export markets. Additionally, the marketing of low quality flowers results in consumer dissatisfaction, a loss in credibility of South African products and ultimately the forfeiting of markets to other exporting countries (Wessels *et al.* 1997).

Lesions induced by plant pathogenic organisms are a major cause of foliage and bloom spoilage. A large number of fungal pathogens is known to occur on Proteaceae in South Africa (Knox-Davies *et al.* 1987). The taxonomy of some of these has, however, changed considerably since they were first reported. The correct identification of pathogenic fungi is necessary to ensure appropriate quarantine decisions and suitable control strategies. *Phyllachora proteae* Wakef., commonly associated with leaf spots, leaf necrosis (van Wyk 1973) and stem cankers (pers. obs.) of *Protea* L., and *Leucospermum* R. Br. species, is an example of a pathogen that requires taxonomic reassessment. This fungus was described by Wakefield (1922) as having unilocular ascomata that develop under a very small epidermal clypeus, cylindrical asci, pseudoparaphyses and hyaline, aseptate, ellipsoidal ascospores, 19–22 x 8–9 μm. In a reexamination of the type material, Doidge (1942) found the ascomatal wall to be continuous with, and similar in structure to the clypeus. She noted, however, that the ascomatal stromata differed from those of other South African *Phyllachora* spp. In his study of leaf pathogens of *Protea*, *Leucadendron* and *Leucospermum* spp., van Wyk (1973) commented that the ascocarps of *P. proteae* appeared to be unilocular with pseudoparaphyses, and that the fungus should probably be transferred to *Guignardia* Viala & Ravaz or *Botryosphaeria* Ces. & De Not. The aims of this study were therefore to recollect *P. proteae*, study the type specimen, identify the anamorph, and to record new hosts and collection sites.

Materials and methods

Collection and isolation. Several farms in the Western Cape Province reporting proteas with severe leaf spots and necrosis, and stem cankers were visited. Affected plants were identified and diseased leaves and branches cut from bushes and brought back to the laboratory for study. Leaf and stem samples were incubated in Petri dishes containing moist filter paper. Single ascospore cultures were obtained from pseudothecia by squashing the contents in a drop of sterile water and spreading this onto the agar surface of dishes containing potato dextrose agar (PDA) (Biolab, Midrand, South Africa). Alternatively, pseudothecia were soaked in water for 2 h, attached to lids of Petri dishes, and ascospores ejected onto the agar surface of PDA plates. Single germinating ascospores were transferred to fresh PDA plates, and

incubated at room temperature in the dark for 5 d. Subcultures were made from five single ascospore or conidial colonies per diseased plant.

Morphological characterization. To induce sporulation, two different techniques were used. In the first, cultures were transferred to divided plates containing carnation leaf agar (Fisher *et al.* 1982) in one half of the dish and PDA in the other. In the second technique, isolates were grown on a piece of *Leucospermum* stem that had been sterilized in full strength V8 broth (Englander and Turbitt 1979), and placed on tap water agar (Biolab). All plates were incubated in the laboratory at room temperature (20–25°C) under cool white and near-ultraviolet light with a 12 h light cycle. Cultures were stored on PDA slants, with or without mineral oil, at room temperature. All fungal material was mounted in lactophenol, and at least 30 structures were measured. The range of dimensions is given with the extremes in parentheses. Reference specimens have been deposited at the National Collection of Fungi in Pretoria (PREM), and cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U).

Cultural studies. Ten isolates derived from different hosts and localities were selected for cultural growth studies on PDA. Mycelial discs 5 mm diam were cut from the periphery of actively growing cultures and placed at the center of PDA plates, with three plates per isolate at each temperature (5–40°C at 5°C intervals). Linear growth and colony color (Rayner 1970) were determined after 7 d. Two perpendicular readings were taken for each colony, using a digital caliper. Separate regression lines were fitted to data acquired for each isolate. The quadratic equation ($y=a+bx+cx^2$) described the relationship between the colony diameters after 7 d and the incubation temperature. The various coefficients (a, b, c) were compared using Student's t-LSD ($P = 0.05$). Since there were no significant differences in the coefficients of the isolates, data were combined and a single regression function was fitted to all the data (Fig. 1). The temperatures at which optimum growth, as well as where no growth occurred, were calculated from the equation.

Results

Taxonomy. In a reexamination of the type specimen of *Phyllachora proteae* (PREM 32915), it was found that this taxon had bitunicate asci, which were borne in thick-walled, brown pseudothecia. Contrary to the protologue for the species, no clypeus was observed. These observations suggest that this species would be better accommodated in *Botryosphaeria* than *Phyllachora* Nitschke ex Fuckel, and a new combination is therefore proposed. Cultures derived from single ascospores of *B. proteae* produced a *Fusicoccum* Corda anamorph with a microconidial state when cultured on PDA. As no anamorph has thus far been reported for *B. proteae*, the *Fusicoccum* state is described as new.

Botryosphaeria proteae (Wakefield) S. Denman & Crous, Mycologia 91: 510–516. 1999.   Figs.2–13

≡ *Phyllachora proteae* Wakefield, Kew Bull. 1922: 164. 1922.

Anamorph. **Fusicoccum proteae** Denman & Crous, sp. nov. Mycologia 91: 510–516. 1999.

Mycelium immersed, consisting of branched, septate, smooth, medium brown hyphae, 2.5–5 µm diam. *Pseudothecia* epiphyllous, separate, unilocular, initially solitary and discrete, becoming aggregated, immersed, substomatal, with a central, flattened ostiole, obovoid, slightly depressed, 200–300 µm wide, 200–240 µm high; wall consisting of 8–11 layers of brown pseudoparenchymatic *textura angularis*, up to 65 µm thick in upper, widest part. *Asci* fissitunicate, clavate to cylindrical, stipitate, bitunicate, 90–150 x 12–15 µm; nasse apicale visible as a notch-like indentation at the apex. *Ascospores* uni- to biseriate, hyaline, guttulate, smooth, ellipsoidal, clavate to fusiform, frequently widest in the upper third of the ascospore, tapering to obtuse ends, (15–)17–20(–21) x (5–)6–8(–9) µm. *Pseudoparaphyses* hyaline, septate, branched, frequently attached to the top and base of the pseudothecial cavity, 2–3.5 µm diam. *Conidiomata* pycnidial, eustromatic, to 450 µm diam, immersed, subepidermal, separate, dark brown, uni- to multilocular, walls consisting of dark brown *textura angularis*, ostiolate. *Fusicoccum* anamorph: *Conidiophores* hyaline, smooth, branched, subcylindrical, 1–3-septate, formed from the inner layer of the locule, 20–40 x 3–4.5 µm; intermingled with hyaline, septate paraphyses. *Conidiogenous cells* phialidic, discrete or integrated, hyaline, smooth, cylindrical,

producing the first conidium holoblastically, and subsequent conidia enteroblastically, proliferating percurrently with 1–2 indistinct proliferations, or determinate, with periclinal thickening (sensu Sutton 1980), 20–30 x 2.5–3.5 µm. *Conidia* hyaline, thin-walled, aseptate, smooth, fusiform, widest in the middle or upper third of the conidium, apex subobtuse, base truncate, (20–)22–25(–30) x (4.5–)5–6 µm. *Microconidial state*: occurrs in the same or in separate conidiomata to the *Fusicoccum* anamorph. *Microconidiophores* hyaline, smooth, branched, cylindrical, 1–3-septate, formed from the inner layers of the locule, 15–25 x 2–3 µm. *Microconidiogenous cells* phialidic, discrete or integrated, hyaline, smooth, cylindrical, determinate with prominent periclinal thickening, 6–10 x 2–3 µm. *Microconidia* medium brown, thick-walled, finely verruculose, guttulate, aseptate, subcylindrical to narrowly ellipsoid with rounded ends, (7–)8–11(–14) x 2.5–3.5 µm. *Spermatial state*: Occurs in conidiomata with the *Fusicoccum* anamorph, or in separate *spermatogonia*. *Spermatiophores* hyaline, smooth, branched, cylindrical, 1–3-septate, formed from the inner layer of the locule, 15–20 x 3–4 µm. *Spermatiogenous cells* discrete or integrated, hyaline, smooth, cylindrical, proliferating via determinate phialides with periclinal thickening, 10–12 x 2–3 µm. *Spermatia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 5–7 x 1.5–2 µm.

HOLOTYPES. SOUTH AFRICA. WESTERN CAPE: Klapmuts, on leaves of *Protea repens* (as *P. mellifera*), *P. Van Der Bijl,* No.357 (PREM 32915, teleomorph); Grabouw, Molteno Estate, on stems of *Protea grandiceps*, 5 Jun. 1997, *S. Denman* (PREM 55769, anamorph, culture ex-type STE-U 1694).

Additional specimens examined. SOUTH AFRICA. WESTERN CAPE: Elsenburg, on leaves of *Protea eximia,* 22 Jul. 1997, *S. Denman* (STE-U1695, PREM 55770, teleomorph); Stellenbosch, Devon Valley, Protea Heights Farm, on leaves of *Protea magnifica* x *Protea compacta* Hybrid "Lady Di", 20 Aug. 1997, *S. Denman* (STE-U1697, PREM 55773, teleomorph); J.S. Marais Park, on leaves of *Protea repens,* 17 Aug. 1997, *S. Denman* (STE-U 1696, PREM 55771, anamorph); Tulbach, on leaves of *Protea magnifica*, Apr. 1997, *S. Denman* (STE-U 1698, PREM 55774, anamorph); Stellenbosch, Banhoek Valley, Calenick Farm, on leaves of *Protea aristata* x *P. repens* hybrid "Venus", 18 Aug. 1997, *S. Denman* (STE-U 1699, PREM 55776, anamorph); Banhoek Valley, Calenick Farm, on leaves of *Protea cynaroides,* 18 Aug. 1997, *S. Denman* (PREM 55775, anamorph). USA. HAWAII: Maui, on leaves of *Protea* sp., Oct. 1997, *P.W. Crous* (PREM 55772).

Cultures. Cultures were characterised morphologically after growing for 1 mo in the dark at 25°C. The colony margins were crenate to irregular and moderate to sparse, gray aerial mycelium, occasionally sectored, with black conidiomata, which occurred over the entire colony surface, but aggregated in dense masses along the outer colony margins. In several plates ascomata were also observed to develop on PDA. Colony colour (underneath) ranged from buff (21″f) to olivaceous gray (23‴′′I) or iron gray (23‴′′k), and smoke gray (19‴′′i) on the surface.

Temperature requirements for growth. Min 5°C, opt 25°C, max 35°C. No growth was recorded at 40°C. The mean daily growth rate at 25°C was 7 mm/d (Fig. 1).

Hosts. *Protea cynaroides* L.; *P. eximia* (Salisb. ex Knight) Fourc.; *P. grandiceps* Tratt.; *P. magnifica* Link.; *P. repens* (L.) L. and hybrids with cultivar names, *P. aristata* (E. Phillips) x *P. repens* cultivar "Venus", *P. magnifica* x *P. compacta* (R. Br.) cultivar "Lady Di" and a *Leucospermum* sp.

Known distribution. South Africa (Western Cape Province), and U.S.A. (Hawaii).

Discussion

In the present study *P. proteae* was reexamined and it was found that it was a species of *Botryosphaeria*, for which the name *B. proteae* is proposed. This is consistent with previous suggestions (Doidge 1942, van Wyk 1973) that *Phyllachora* was not an appropriate genus for this fungus. Furthermore, it has been shown that the anamorph of *B. proteae* is a species of *Fusicoccum*, now known as *F. proteae*. In culture as well as on host material, a microconidial state with thick-walled brown conidia is also frequently observed, accompanied by a spermatial state with spermatia that are sterile in culture.

A number of *Botryosphaeria* spp. have been associated with Proteaceae. These include *B. dothidea* (Moug.) Ces & De Not. [=*Botryosphaeria ribis* (Tode ex Fr.) Gross & Dugg.; von Arx and Müller 1954] on *Protea, Leucospermum* and *Leucadendron, B. banksiae* Hansf. on *Banksia* L.f. (Hansford 1954), and *B. gaubae* Petr. on *Grevillea* R. Br. ex Knight (Petrak 1968). *Botryosphaeria proteae* differs from *B. banksiae* in that it does not have periphyses in the ostiolar region. Furthermore, ascospores of *B. banksiae* (13–15 μm), and *B. gaubae* (10–13 μm), are much wider than those of *B. proteae* (5–9 μm), and none are widest in the upper third

of the ascospore as in the case of *B. proteae* (Hansford 1954, Petrak 1968). Ascospores of *B. dothidea* are similar in size (18–23 x 7–9 µm; Arx and Müller 1954), but differ in shape, and in the anamorph produced in culture.

Botryosphaeria proteae is unusual in that the obovoid pseudothecia have a wider wall layer in the apical part, which was incorrectly referred to as a clypeus by Wakefield (1922). Furthermore, the presence of abundant pseudoparaphyses, the frequent occurrence of cylindrical asci with uniseriate ascospores, its distinct cultural characteristics, as well as the microconidial form suggest that this species may not be a typical species of *Botryosphaeria*.

Botryosphaeria is commonly ascribed to collections of bitunicate ascomycetes that have multi- or uniloculate, black ascomata occurring separately, or grouped to aggregated on a common basal stroma (Sivanesan 1984). Pseudothecia are ostiolate and may be embedded in the host tissue or erumpent. The centrum contains numerous filamentous pseudoparaphyses (Hanlin 1990), and although Sivanesan (1984) reports that interthecial tissues usually disintegrate, it is frequently not the case as observed in *B. proteae*, as well as in other species of *Botryosphaeria* (Pennycook and Samuels 1985). Ascospores are hyaline, one celled, often inequilateral, and may become brown and 1–2 septate with age. Some discrepancy still exists, however, regarding the presence/absence of mucilaginous caps on ascospores of *Botryosphaeria* and related genera. Barr (1987), in her key to the genera of the Botryosphaeriaceae, mentioned that ascospores usually lack a gel coating or appendages, thereby implying that some species may well have these features. Hanlin (1990) also stated that ascospores may have a thin gelatinous coat. However, the gelatinous sheath should be distinguished from the mucilaginous caps found in *Guignardia* Viala & Ravaz.

The genus *Botryosphaeria* seems to be beset with unresolved taxonomic issues. A number of authorities have thus stated that the whole complex is in urgent need of revision (Sutton 1980, Pennycook and Samuels 1985). Sivanesan (1984) treated 12 species of *Botryosphaeria,* and subsequent to his treatment several additional species have been described (Pennycook and Samuels 1985, Sivanesan and Sutton 1985, Bisset 1986, Shang 1987, Gardner and Hodges 1988, Ramesh 1991, Subileau *et al.* 1994, Yuan 1996, Gardner 1997). A number of these have possibly been incorrectly assigned to *Botryosphaeria*, and there may be many more that have been incorrectly allocated to morphologically similar genera. The bulk of recent literature suggests that *Guignardia*, which has been confused with *Botryosphaeria* in

the past, is clearly segregated and always associated with *Phyllosticta* Pers. anamorphs (Sivanesan 1984, Hanlin 1990).

The genus *Botryosphaeria*, on the other hand, has been associated with several anamorph form genera. Sivanesan (1984) listed several anamorph states of *Botryosphaeria*. These included *Botryodiplodia* (Sacc.) Sacc., *Dothiorella* Sacc., *Diplodia* Fr., *Macrophoma* (Sacc.) Berl. & Vogl. and *Sphaeropsis* Sacc. Sutton (1980) placed *Macrophoma* in synonymy with *Sphaeropsis*, and stated that there are several genera available for other species originally described in *Macrophoma*. The similarities between *Dothiorella* and *Fusicoccum* were extensively discussed by Sutton (1977; 1980), and are be dealt with elsewhere (Crous and Palm 1999). Pennycook and Samuels (1985) and Phillips and Lucas (1997) broadened the concept of *Fusicoccum* to include taxa with conidiomata ranging from unilocular pycnidia to complex multilocular eustromatic structures. Simple or branched conidiophores also produced conidia via phialides, while conidia were thin-walled, hyaline, aseptate, fusiform, and had a distinct truncate base (Pennycook and Samuels 1985). The genus *Macrophomopsis* Petrak was distinguished from *Fusicoccum* by having conidiogenous cells with percurrent proliferations (annellides sensu Sutton 1980). However, Pennycook and Samuels (1985) found the same mode of conidiogenesis in specimens of *Fusicoccum*, and subsequently reduced *Macrophomopsis* to synonymy with it.

Fusicoccum proteae, the anamorph of *B. proteae,* is similar to other species in the genus that have branched conidiophores, and hyaline, thin-walled, fusiform conidia. The mode of conidiogenesis by producing conidia via determinate or percurrently proliferating phialides is also more common in *Fusicoccum* than is reported in literature (Pennycook and Samuels 1985). *Botryosphaeria proteae* is an unusual species of the genus, however, in having a microconidial state with brown, thick-walled conidia.

Notwithstanding this morphological variation, it is interesting to speculate whether *B. protea* will cluster with those taxa with typical *Fusicoccum* or typical *Sphaeropsis* or *Diplodia* anamorphs. Molecular studies aimed at elucidating its phylogenetic position in *Botryosphaeria* and the Dothideales are currently in progress (Chapters 3 and 7).

The occurrence of *B. proteae* on species of *Protea* and *Leucospermum* in South Africa and Hawaii, leads suggests that this taxon may have a much wider

distribution than previously thought. Presently very little is known about the distribution, host range, and pathogenicity of *B. proteae*. Further collections and inoculation trials are presently underway to characterise its importance as a pathogen of Proteaceae.

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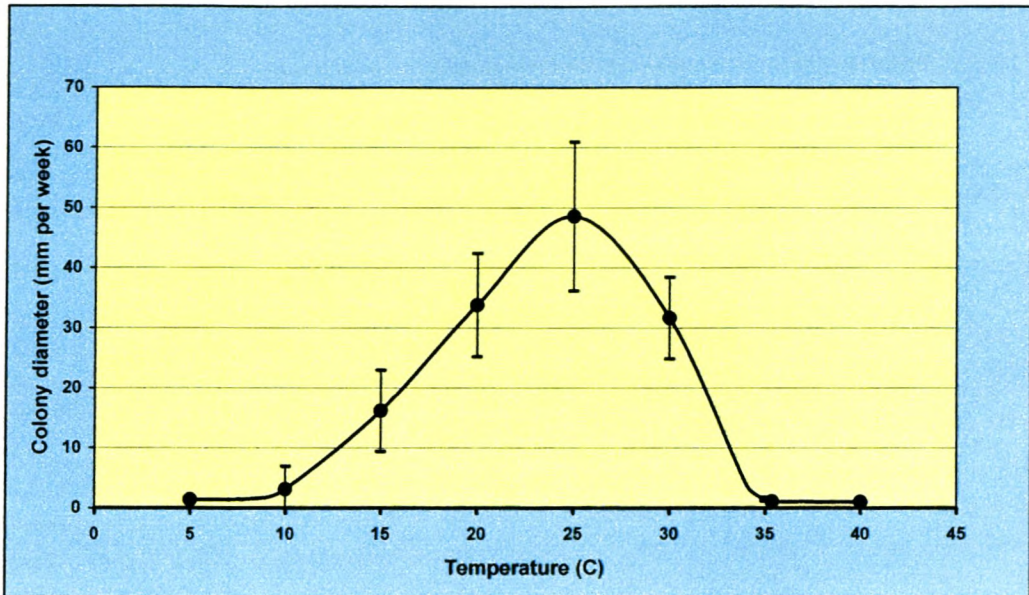
Fig. 1. Temperature growth relationship of *B. proteae*.
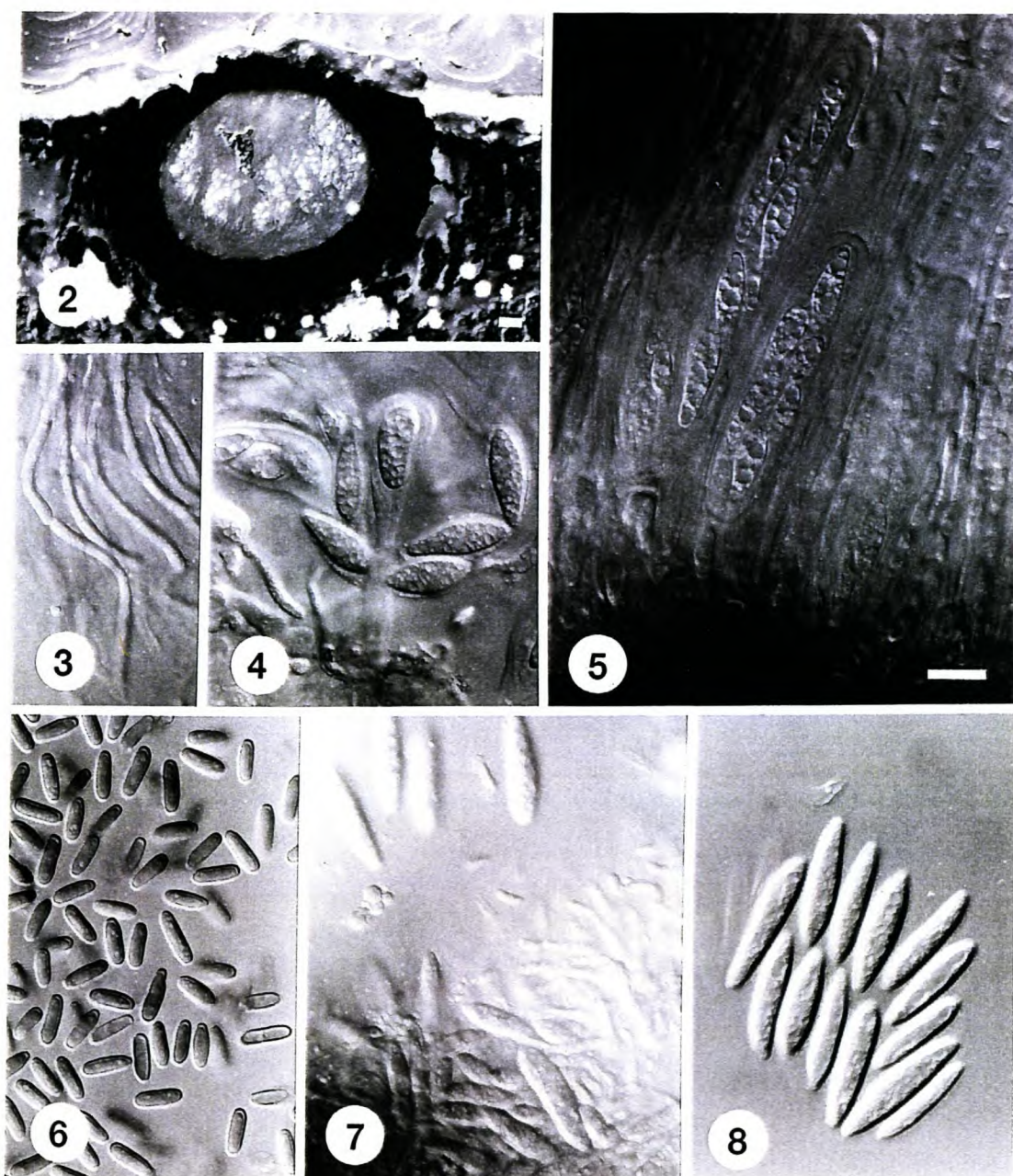
Figs. 2–8. *Botryosphaeria proteae* and its anamorphs. 2. Transverse off-centre section through a pseudothecium (type). 3. Pseudoparaphyses (PREM 55773). 4. Ascospores and broken asci (PREM 55773). 5. Asci and ascospores (type). 6. Brown conidia of the microconidial state (PREM 55773). 7. Spermatia and conidia of *Fusicoccum proteae* (PREM 55773). 8. Conidia of *Fusicoccum proteae*. Bars = 10 µm.
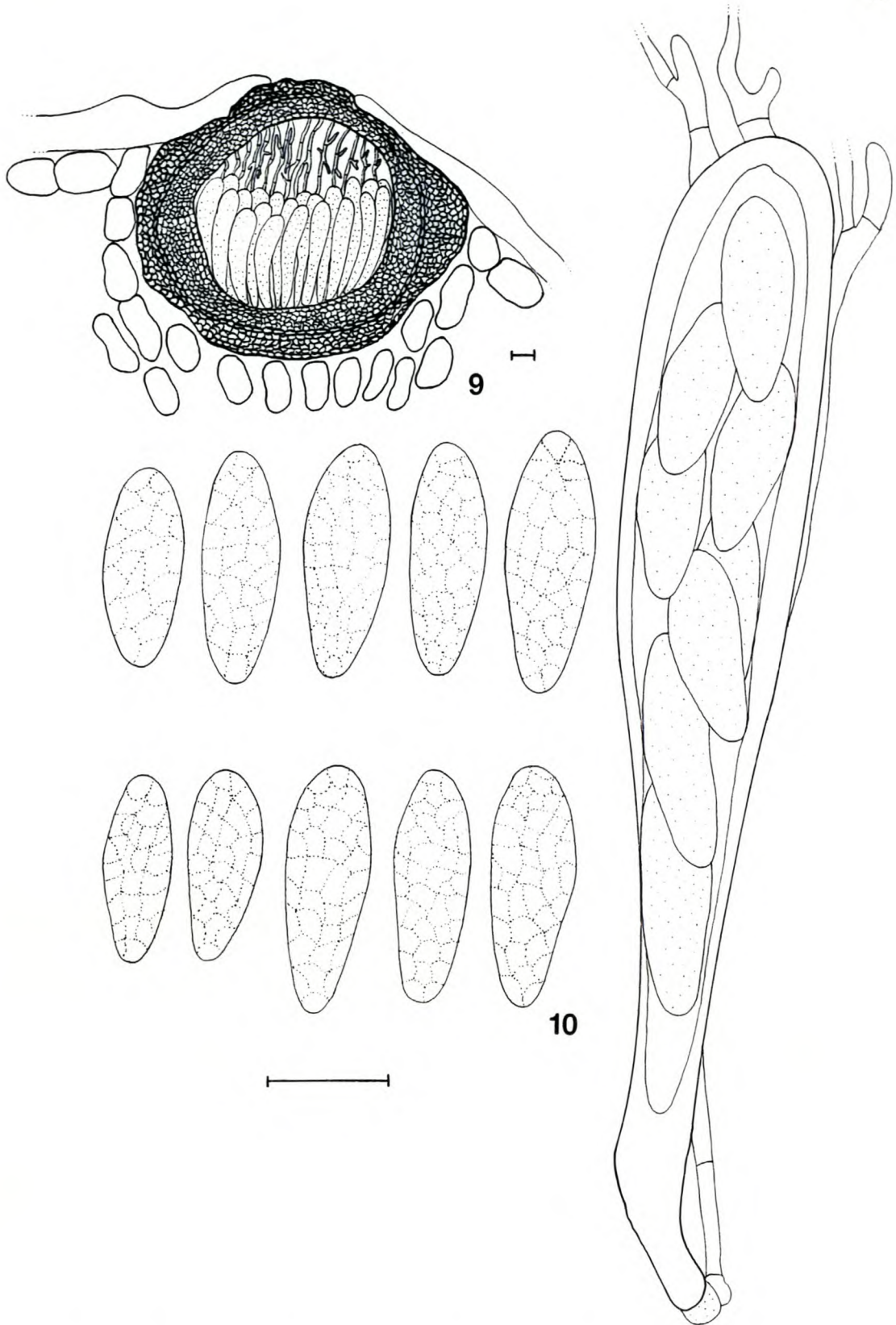
Figs. 9–10. *Botryosphaeria proteae.* 9. Transverse off-centre section through a pseudothecium, and ascospores (type). 10. Ascospores, ascus and pseudoparaphyses (PREM 55773). Bars = 10 μm.
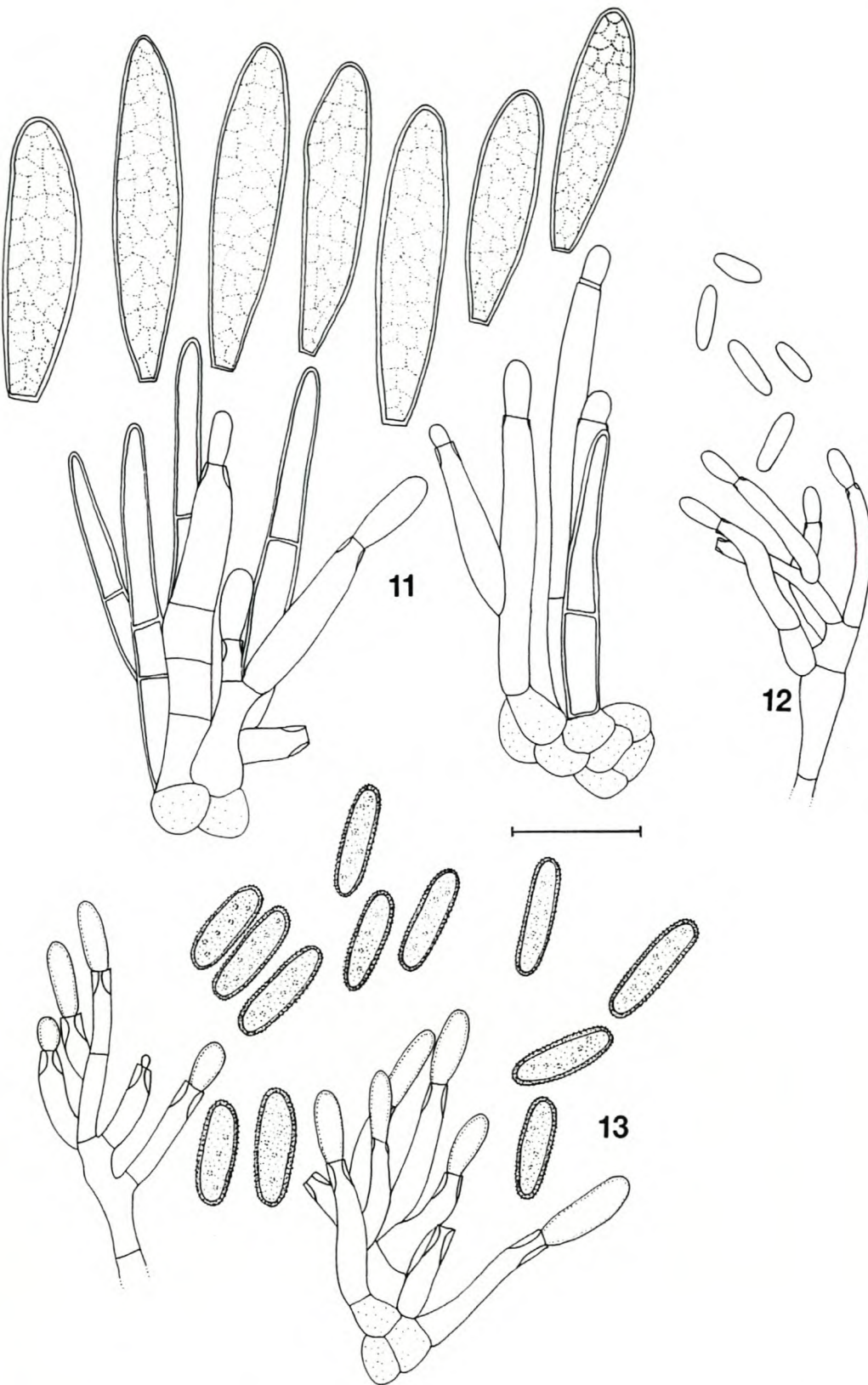
Figs. 11–13. Anamorphs and spermatia of *Botryosphaeria proteae* (type). 11. Fusiform conidia, conidiophores and paraphyses of *Fusicoccum proteae*. 12. Spermatiophores and spermatia. 13. Conidiophores and conidia of microconidial state (PREM 55773). Bar = 10 µm.

CHAPTER 3

An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny[1]

Abstract

The taxonomic history of *Botryosphaeria* is reviewed and the genus is circumscribed and distinguished from other morphologically similar genera. Several anamorph genera have been linked to *Botryosphaeria*. Based on morphological observations and phylogenetic analysis of ITS rDNA sequence data, two groups of anamorphs are recognised. Anamorphs with conidia that are pigmented when mature are placed in *Diplodia*, while those with hyaline conidia are accommodated in *Fusicoccum*. *Botryosphaeria proteae*, a species with both conidial types, should be excluded from *Botryosphaeria* based on its ascomatal wall anatomy, anamorph morphology and ITS rDNA phylogeny.

Introduction

Botryosphaeria Ces. & De Not. is a species-rich genus with a cosmopolitan distribution (Barr 1987). Species are saprophytic, occasionally parasitic and endophytic (Smith *et al.* 1996; Chapter 5), and can cause die-back and canker diseases of woody hosts (von Arx 1987). They occur on a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts, on woody branches, herbaceous leaves, stems and haulms of grasses, on twigs and in the thalli of lichens (Barr 1987).

The taxonomy of *Botryosphaeria* is problematic at several levels of classification. The position of the genus in the higher classification of ascomycetes has

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not yet been resolved (von Arx and Müller 1975; Sivanesan 1984; Barr 1987; Silva-Hanlin and Hanlin 1999). Secondly, in *Botryosphaeria*, much confusion has occurred regarding the allocation of species to the genus. The reasons for this are many. Teleomorphs are uncommonly encountered in nature (Shoemaker 1964; Laundon 1973; Jacobs and Rehner 1998), or are difficult to induce in culture (Laundon 1973), and there is insufficient diversity of teleomorph features to allow unequivocal differentiation at the species level (Shoemaker 1964; Laundon 1973). Furthermore, concepts defining morphological features of the teleomorph (eg. perithecial or pseudoperithecial ascomata, uni- or bitunicate asci and hamathecium) have been historically slow to develop. Ontogenic studies on the majority of species allocated to *Botryosphaeria* have not been conducted (Sivanesan 1984), which has also hindered the correct placement of many species. Therefore, the assignment of many species to this genus is questionable (see synonymies in von Arx and Müller 1954). This has also resulted in problems with the differentiation of species in superficially similar genera such as *Physalospora* Niessl. A number of species of *Botryosphaeria* were reduced to synonymy (von Arx and Müller 1954), only later to be recognised as distinct (Shoemaker 1964; Laundon 1973).

The taxonomy of *Botryosphaeria* is thus largely dependent upon the taxonomy of its anamorphs, which are the more commonly encountered morphs (Hanlin 1990). Morgan-Jones and White (1987) maintained that the identification of *Botryosphaeria* species is by no means a simple task because the characters used to circumscribe anamorph genera associated with *Botryosphaeria* are poorly described and inconsistently applied. Morphological changes in conidia as they age also make identification difficult (Laundon 1973).

The recent advent of molecular techniques and application of sequence data of ribosomal RNA genes to fungal phylogenetic studies have contributed greatly to the phylogenetic reconstruction of fungi (Bruns *et al.* 1991; Lee and Taylor 1991; Berbee and Taylor 1992; O'Donnell and Gray 1995; O'Donnell *et al.* 1997). Among the variable regions of rDNA, the internal transcribed spacers (ITS), which often vary between and within species (Lee and Taylor 1991; Peterson and Kurtzman 1991), have been successfully used to investigate phylogenies of *Pezizales* (Momol and Kimbrough 1994), *Leptosphaeria* Ces. & De Not. (Morales *et al.* 1993; 1995), *Alternaria* Nees (Jasalavich *et al.* 1995) and *Mycosphaerella* Johanson (Stewart *et al.* 1999). Jacobs and Rehner (1998) used ITS sequence data to link several anamorphs to *Botryosphaeria*, which in turn helped to clarify the taxonomy of the genus.

In this chapter, an overview of the taxonomy of *Botryosphaeria* is given and molecular data that support two morphological groups within the genus is presented. Special emphasis is placed on characters of the anamorphs, which are regarded as important in defining the genus.

Historical review

Taxonomic history of higher classification of *Botryosphaeria*. The genus *Botryosphaeria* was introduced in 1863 by Cesati and De Notaris, who designated *Botryosphaeria dothidea* (Moug.) Ces. & De Not. as the type species (Johnson 1992). At this time, the taxon was placed under the group name *Sphaeria* (Munk 1953) and anamorph genera were not explicitly linked to teleomorphs. In the late nineteenth century, two systems of classification emerged. In *Sylloge Fungorum,* Saccardo grouped species based on spore-shape, septation and colour, an artificial system that was very practical to use (Munk 1953), but did not reveal phylogenetic relationships. Another system, proposed by Lindau (1897), attempted to place fungi in natural (phylogenetic) groups. This system, considered by some (Luttrell 1951; Wehmeyer 1975) as the earliest, significant classification, is the system upon which higher taxonomy of the Ascomycetes is currently based. *Botryosphaeria* was allocated to the Melogrammataceae in the Sphaeriales (Lindau 1897). At that time, the Sphaeriales included fungi with clearly differentiated carbonaceous ascomata with or without a stroma, while the Dothideales were characterised by the formation of asci in locules embedded in stromata, and not in distinct peridia. A single family, the Dothideaceae, which was restricted to compound (multiloculate) forms, was placed in this order. Von Höhnel (1907) established the family Pseudosphaeriaceae to accommodate taxa with single-locule, multiascal ascostromata, and placed *Botryosphaeria* in this family, which was later allocated to the order Dothideales (von Höhnel 1909).

In the period from 1909–1928, the classification of *Botryosphaeria* was subjected to much rearrangement. Theissen and Sydow (1915) created a subfamily Botryosphaerieae and put *Botryosphaeria* into this subfamily in the Pseudosphaeriaceae. This family was treated as an 'anhang' and was not placed in any order. A year later, Theissen (1916) allocated the Pseudosphaeriaceae to the Myriangiales. However, by 1917 Theissen and Sydow thought that the Pseudosphaeriaceae should be united with the

Dothideaceae (Luttrell, 1951). A year later, Theissen and Sydow (1918) created a subclass the Dothidiineae into which the order Pseudosphaeriales, family Botryosphaeriaceae, and genus *Botryosphaeria* were assigned. Petrak (1923) rejected Theissen and Sydow's classification and placed *Botryosphaeria* in the subfamily Pseudosphaerieae, which he put in the Pleosporaceae (Sphaeriales). One of the main reasons for this reshuffling of the classification of *Botryosphaeria* was confusion regarding ontogeny and morphology of true perithecia, ascostromata and interthecial tissues.

Miller (1928) showed that there was a fundamental difference between the tissues forming the perithecium and those forming the boundary of the locule. He also showed how these different tissue types were correlated with features of the centrum. Taxa allocated to the Sphaeriales had true perithecia and paraphyses (or in some cases periphyses) while those assigned to the Dothideales had ascostromatic ascomata and lacked paraphyses. Thus, *Botryosphaeria* species (Pseudosphaeriaceae) were allocated to the Dothideales because they lacked true perithecial walls (Miller 1928).

Nannfeldt (1932) re-grouped the Euascomycetes into three orders. The ascostromatic forms, where asci formed in cavities in pre-formed stromata, were accommodated in the Ascoloculares. The true Sphaeriales, i.e. species in which the asci developed in a hymenium, were accommodated in the Ascohymeniales. Although these groups were not accepted by many at the time, they were consistent with the bitunicate and unitunicate groups later proposed by Luttrell (1955).

Concepts based on morphological features resulting from the ontogeny of the perithecial wall and the development of centrum tissues, were further developed by Miller (1938) and three orders were recognised. The Sphaeriales had perithecia and paraphyses, the Dothideales encompassed ascostromatic forms without paraphyses and the Pseudosphaeriales included ascostromatic forms with interthecial threads that appeared in the ascomatal cavity before the asci arose. Although details of the development of hamathecial tissues were beginning to take form, conflicting opinions regarding the taxonomic value of these structures predominated. Miller (1938) established a new order, the Pseudosphaeriales, and retained the position of *Botryosphaeria* in the family Pseudosphaeriaceae. Thus, *Botryosphaeria* was placed in the Pseudosphaeriales and not the Dothideales, where Miller had classified the genus in 1928.

Luttrell (1951) recognised two major morphological groups in the pyrenomycetous fungi. He also emphasised the significance of ontogenetic characters of ascomata in classification. The two major morphological groups were those with single-walled asci, or unitunicate ascomycetes, and those with double-walled asci, the Loculoascomycetes, commonly referred to as bitunicate ascomycetes (Luttrell 1955). Luttrell also identified eight types of centrum development and highlighted the taxonomic value of sterile, interthecial tissues. He provided an explanation why the original name of the order Pseudosphaeriales was no longer tenable. The type of the family Pseudosphaeriaceae, and the type of the genus *Pseudosphaeria*, had been transferred to the Dothideales. Therefore, Luttrell (1955) replaced the name Pseudosphaeriales with Pleosporales, based on the most important genus in the group with that type of centrum development, and assigned *Botryosphaeria* to the Pleosporales.

Luttrell's views were promoted by Barr (1972; 1976; 1979; 1983; 1987). In Barr's earlier work (1972; 1976), she had not studied specimens of *B. dothidea* in which the interthecial tissues were clearly visible and, despite the clear demonstration by Parguey-Leduc (1966) that *B. dothidea* exhibited a *Pleospora* centrum-type, she classified *Botryosphaeria* in the *Dothideales*. However, later Barr (1979) acknowledged that *Botryosphaeria* species had a centrum typical of the Pleosporales, and concluded that the genus should reside in this order. This view was retained in later publications (Barr 1983; 1987).

The orders proposed by Luttrell (1955; 1973) and Barr (1979; 1987) were not accepted by von Arx and Müller (1975) and von Arx (1987). They felt the ordinal boundaries did not enable the correct groupings of related genera. The orders comprised a mixture of unrelated genera (von Arx 1987) and there was overlap of some features amongst the orders (von Arx and Müller 1975). Furthermore, von Arx and Müller (1975) did not support the placement of what they considered closely related genera, such as *Guignardia* Viala & Ravaz and *Botryosphaeria*, in different orders (Dothideales and Pleosporales, respectively) (Luttrell 1973). They delimited a single order, the Dothideales, which comprised two sub-orders and 24 families. They felt that this was a more appropriate means of dealing with the taxonomy of this very large, heterogeneous group, at least until a more natural method of classification could be developed. Thus, *Botryosphaeria* was maintained in the Botryosphaeriaceae, but was placed once again in the Dothideales. Hence the two major systems of classification that prevailed at the end of 1975, and which remain in common use, are those of Barr (1987), in which

Botryosphaeria was placed in the Pleosporales, and von Arx and Müller (1975), who placed the genus in the Dothideales.

Eriksson (1981) emphasised that *Botryosphaeria* species have a centrum typical of the Pleosporales with pseudoparaphyses and pseudothecia. Currently, however, the widely accepted classification of *Botryosphaeria* is that it is a member of the family Botryosphaeriaceae accommodated in the Dothideales (Hawksworth *et al.* 1995).

***Botryosphaeria* species described to date**. The Index to Saccardo's Sylloge Fungorum lists 105 *Botryosphaeria* species recorded up to 1920. A further ten species are listed in The Index of Fungi (Petrak's Lists number 1–8, 1920–1939). Von Arx and Müller (1954) studied the type specimens of *Botryosphaeria* species and synonomised 183 species, reducing them to 11 taxa. It is fair to assume that von Arx and Müller (1954) treated all *Botryosphaeria* species recorded up to 1954. The Index of Fungi (vols. 1–6) lists 81 additional species of *Botryosphaeria* published from 1954–1999. During this 45-year period, 32 species were relocated to *Botryosphaeria* from other genera, 11 species have been synonymised and at least 34 species have been described as new.

Taxonomy

Botryosphaeria Ces. & De Not., Comm. Soc. Crittog. Ital. 1: 211. 1863; emend. Sacc., Michelia 1: 42. 1877.

= *Melanops* Nitschke ex Fuckel, Symb. Mycol.: 225. 1870.

= *Thuemenia* Rehm, Flora 62: 123. 1879.

= *Coutinia* Alm. & Cam., Rev. Agr. Lisboa 1: 392. 1903 (non Vellozo, 1799).

= *Phaeobotryosphaeria* Speg., Ann. Mus. Nac. 17: 120. 1908.

= *Cytosporina* (Henn.) Höhn., Kais. Akad. Wiss. Wien Naturw. 120: 437. 1911.

= *Amerodothis* Theiss. & Syd., Ann. mycol. 13: 295. 1915.

= *Phaeobotryon* Theiss. & Syd., Ann. mycol. 13: 664. 1915.

= *Epiphyma* Theiss., Verh. Zool.-Bot. Ges. Wien 66: 306. 1916.

= *Pyreniella* Theiss., *ibid* : 371. 1916.

= *Desmotascus* F. Stevens, Bot. Gaz. 68: 476. 1919.

= *Rostrosphaeria* Tehon & Daniels, Mycologia 19: 112. 1927.

= *Neodeightonia* C. Booth, Mycol. Pap. 119: 19. 1969.

= *Caumodothis* Petr., Sydowia 24: 276. 1971.

The features that characterise *Botryosphaeria* include the production of ascostromatic pseudothecia, described as uniloculate by Sivanesan (1984) but generally considered multiloculate (Hanlin 1990). Pseudothecia are ostiolate, solitary or botryose, on a common basal stroma and may be embedded in the host tissue or erumpent. Cellular pseudoparaphyses are prevalent in the centrum (Dennis 1981; Hanlin 1990). The asci are bitunicate, stalked or sessile, clavate and contain eight hyaline ascospores (Dennis 1981; Sivanesan 1984; Hanlin 1990). Ascospores have a uni- to bi-seriate arrangement, are aseptate and they vary from ovoid to fusoid to ellipsoid in shape. They are often inequilateral with the widest part in the middle. Ascospores may become brown and 1–2-septate with age, are smooth and thin-walled, but can occasionally be slightly verruculose after discharge (Sivanesan 1984; Hanlin 1990). They may possess evanescent hyaline appendages (Sivanesan 1984; Pennycook and Samuels 1985) or a thin gelatinous coat (Hanlin 1990).

Closely related teleomorph genera. Barr (1987) differentiated *Botryosphaeria* from *Auerswaldiella* Theiss. & Syd., *Discochora* Höhn., *Dothidotthia* Höhn., *Neodeightonia* C. Booth and the lichenicolous genus *Homostegia* Fuckel based on a number of morphological features. For instance, the ascomata of *Auerswaldiella* species (with amerosporous ascospores) and *Homostegia* species (phragmosporous) are borne in pulvinate stroma. *Dothidotthia* species are separated from *Botryosphaeria* species by their 1-septate, yellow-brown to dark-brown ascospores.

Another genus that has been confused with *Botryosphaeria* is *Discochora*. Barr (1987) placed *Guignardia* in *Discochora*. Although the name *Discochora* pre-dates *Guignardia* (Bissett 1986), the name *Guignardia* has subsequently been conserved. Therefore, Barr's reference to *Discochora* being closely related to *Botryosphaeria* actually pertains to *Guignardia*. *Guignardia* species are separated from *Botryosphaeria* species by having smaller ascospores, distinct mucilaginous caps on the apices of the ascospores, and *Phyllosticta* Pers. anamorphs (van der Aa 1973; Punithalingam 1974; Hanlin 1990). Barr *et al.* (1986) used *Neodeightonia* for *Dothidotthia*, but *Neodeightonia* had been reduced to synonymy with *Botryosphaeria* by von Arx and Müller (1975). Barr (1987) supported the latter synonymy.

Physalospora is a long-standing name that was misapplied to *Botryosphaeria* species, probably because the concept of unitunicate and bitunicate asci was only developed in 1951 by Luttrell (Luttrell 1951; 1955). *Physalospora* differs from *Botryosphaeria* in that the species have unitunicate asci with non-septate ascospores and a hamathecium composed of paraphyses (Hanlin 1990). Many *Physalospora* species were placed in *Botryosphaeria* by von Arx and Müller (1954). Since then, another 32 *Physalospora* species have been relocated to *Botryosphaeria* (Index of Fungi, vols. 1–6).

Species of *Otthia* Nitsche have short-stalked, cylindrical, bitunicate asci (Denis 1981; Sivanesan 1984). Ascospores are hyaline when young but become brown and 1–septate when mature, and are slightly constricted at the septum (Dennis 1981; Sivanesan 1984). Booth (1958) reviewed the history of the genus and designated *Otthia spiraeae* (Fuckel) Fuckel as the lectotype species. Booth (1958) identified *Diplodia sarmentorum* (Fr.) Fr. as the anamorph of *O. spiraeae*. Laundon (1973) expressed doubt over the tenability of *Otthia*, because the anamorphs were clearly related to those of *Botryosphaeria*. Von Arx (1974) listed *Otthia* as the teleomorph of *Aplosporella* Speg. However, von Arx's concept for *Aplosporella* is indistinguishable from the anamorph of *B. obtusa* (Schw.) Shoemaker, i.e. *Sphaeropsis* Lév. sp. Hawksworth *et al.* (1995) described *Aplosporella* as being stromatic and having 1-celled, brown, holoblastically produced conidia. The description of *Aplosporella* given by Sutton (1980) is also very similar to that of *Diplodia sarmentorum*. Furthermore, Booth (1958) studied the type material of *Otthia quercus* Fuckel, and its measurements are identical to those of *Botryosphaeria quercuum* (Schw.) Sacc. It seems, therefore, that *Aplosporella* is indistinguishable from *Sphaeropsis* Sacc. It is thus likely that *Otthia* should be synonymised with *Botryosphaeria*, but further morphological and molecular studies need to be conducted to confirm this.

Anamorph genera associated with *Botryosphaeria*. *Botryosphaeria* species are pleomorphic ascomycetes with coelomycetous anamorphs (Barr 1987), which traditionally include the genera *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino and *Sphaeropsis* (Sivanesan 1984). More recently, *Fusicoccum* Corda species have been recorded as anamorphs of some *Botryosphaeria* species (Sutton 1980; Pennycook and Samuels 1985; Samuels and Singh 1986; Morgan-Jones and White 1987; Chapter 2 this dissertation). Other anamorph genera have also been associated occasionally with

Botryosphaeria. These include *Chaetodiplodia* P. Karst., *Colletotrichella* Höhn., *Diplodiella* Petr., *Kabatia* Bubák, *Pellionella* (Sacc.) Petch., *Placosphaeria* (De Not.) Sacc., *Rhynchodiplodia* Briosi & Farneti, *Selenophoma* Maire, *Striodiplodia* Zambett., and *Strionemadiplodia* Zambett. (Barr 1987). *Phyllosticta* was linked to *Botryosphaeria* by von Arx (1987) and Jacobs and Rehner (1998), but this genus should be reserved for anamorphs of *Guignardia* (van der Aa 1973; Punithalingam 1974; Sivanesan 1984; Hanlin 1990).

Anamorph genera with hyaline conidia.

Fusicoccum Corda in Sturm, Deutschlands Flora 2: 111. 1829.

= *Macrophomopsis* Petr., Ann. Mycol. 22: 108. 1924.

A revised description of *Fusicoccum* is provided by Crous and Palm (1999).

According to Sutton (1980), *Fusicoccum* includes coelomycetes with fusiform, hyaline, non-septate conidia produced holoblastically in stromatic conidiomata. He regarded *Fusicoccum* as the genus best suited to accommodate the anamorphs of the *Botryosphaeria ribis* (Tode ex Fr.) Gross & Dugg. / *B. dothidea* complex, an opinion later also shared by Maas and Uecker (1984). Pennycook and Samuels (1985) accepted this concept but commented that the specimen examined by Sutton (1980) (Saccardo in PAD, now the neotype designated by Crous and Palm 1999), is immature, with most conidiogenous loci appearing to produce only one holoblastic conidium. Based on observations of cultures and older material, Pennycook and Samuels (1985) expanded the generic circumscription of *Fusicoccum* to include species with pycnidial conidiomata with enteroblastic conidiogenesis (Pennycook and Samuels 1985), with proliferation occurring at the same level, resulting in periclinal thickening, or percurrently resulting in annellations (Crous and Palm 1999). Pennycook and Samuels (1985) examined the type specimen of *Macrophomopsis* and found this genus indistinguishable from the earlier described *Fusicoccum*. Consequently they recommended that *Macrophomopsis* be placed in synonymy under *Fusicoccum*.

Sutton (1980) noted that Petrak (1922) first made the link between *Botryosphaeria* (*B. berengeriana* De Not.) and *Fusicoccum*, but at that time Petrak referred to *Fusicoccum* as *Dothiorella*. This probably marks the beginning of an extended confusion regarding the application of the name *Dothiorella* to specimens that have hyaline spores (Petrak 1922; von Arx and Müller 1954; Luttrell *et al.* 1962; Bezuidenhout and Marasas 1978; Johnson 1992), which should probably have been

referred to as *Fusicoccum*. Recently, Crous and Palm (1999) re-examined the type of *Dothiorella*, and considered it synonymous with the earlier described genus *Diplodia* (see below). Thus, taxa with hyaline conidia previously referred to as *Dothiorella* and associated with *Botryosphaeria* teleomorphs will need to be carefully re-examined to confirm their correct taxonomic placement.

Anamorph genera with pigmented conidia.

Diplodia Fr., Ann. Sci. Nat. Bot. Sér. 2, 1: 302. 1834.

= *Sphaeropsis* Sacc., *nom. cons.*, Michelia 2: 105. 1880.

= *Dothiorella* Sacc., Michelia 2: 5. 1880.

= *Macrophoma* (Sacc.) Berl & Vogl., Atti Soc. Venet.-Trent. Sci. Nat. 10: 4.

  1886, and Sacc., Syll. Fung. Addit. 1-4: 306. 1886.

  ≡ *Phoma* Westend. subgen. *Macrophoma* Sacc., Syll. Fung. 3: 65. 1884.

= *Lasiodiplodia* Ell. & Everh., Bot. Gaz. 21: 92. 1896. (additional synonyms listed in Sutton, 1980) Type: *D. mutila* Fr., Ann. Sci. Nat. Bot., Sér. 2, 1: 302. 1834.

Mycelium immersed or superficial, branched, septate, heavily melanized, dark brown. *Conidiomata* pycnidial, ostiolate, formed in uni- or multi-loculate stromata, comprising single, thin-walled pycnidia to large erumpent pustules containing up to 20 pycnidial locules, each with a prominent ostiole, immersed or erumpent, separate or aggregated. *Paraphyses* present or lacking. *Conidiophores* (where present) hyaline, simple, occasionally septate, rarely branched, cylindrical, arising from the inner layers of the pycnidial cavity. *Conidiogenous cells* holoblastic, hyaline, cylindrical, determinate or proliferating percurrently, borne on flattened, pale brown cells lining the inside of the pycnidial cavity. *Conidia* variable in colour, ornamentation and septation; initially hyaline, thick-walled, smooth or granular, aseptate with a central guttule, becoming 1-euseptate in some cases; mature conidia light to dark brown with melanin often being deposited on the inner surface of the outer wall (i.e irregularly verruculose), in some species longitudinal striations evident. Both young and mature conidia can occur concurrently in the same pycnidium, resulting in a mixture of hyaline and dark conidia.

The above description of *Diplodia* is an amended version of several descriptions, and represents a new generic concept proposed in the present paper. According to Sutton (1980) the original generic description of *Diplodia* was compiled by Fries based on a sample collected by Montagne in 1834, and identified as *Diplodia*

mutila Fr. The teleomorph of *D. mutila* was discovered by Stevens (1936) who cited it as *Physalospora mutila* (Fries) N.E. Stevens. However, von Arx and Müller (1954) reduced *P. mutila* to synonymy under *Botryosphaeria quercuum* (Schw.) Sacc. Shoemaker (1964) renamed *P. mutila* as *Botryosphaeria stevensii* Shoem., and separated it from *B. quercuum* based on conidial characteristics. He did not, however, name the anamorphs "because this would have raised the problem of taxonomic distinctions between *Diplodia* Fr., *Sphaeropsis* Lév., and *Dothiorella* Sacc." (Shoemaker 1964). Sivanesan (1984) accepted *D. mutila* as the anamorph of *B. stevensii* and because *Diplodia* predates *Sphaeropsis* and *Lasiodiplodia* (Sutton 1980), this genus should be adopted to accommodate the dark-spored anamorphs of *Botryosphaeria*.

The following anamorph genera are here considered synonyms of *Diplodia*:

Macrophoma (Sacc.) Berl & Vogl. has been commonly applied to *Botryosphaeria* anamorphs with hyaline conidia (Tehon and Daniels 1927; Funk 1964; Smerlis 1970). Sutton (1980) re-examined the type specimen and found it to be a later name for *Sphaeropsis*. Phillips and Lucas (1997) examined the causal agents of excoriose on grapevines in Portugal, namely *Macrophoma flaccida* (Viala & Ravaz) Cav. and *Macrophoma reniformis* Viala & Ravaz, and reported that these species represented later names for *Fusicoccum aesculi* Corda, the anamorph of *B. dothidea*.

Because many *Botryosphaeria* species with *Macrophoma* anamorphs have conidia described as hyaline to pale brown (Funk 1964; Bezuidenhout and Marasas 1978), it is unclear whether they would be better accommodated in *Fusicoccum* or in *Diplodia*. They all need to be re-examined to determine their correct generic placement.

Dothiorella Sacc. A great deal of confusion has surrounded the type specimen and generic concept of *Dothiorella*, which was discussed in detail by Sutton (1977) and Crous and Palm (1999). *Dothiorella pyrenophora* Sacc., the type species, is typified by Berkeley's English material of *Dothiora pyrenophora* Fr. (Berk. Exs. No. 282, K 54913). Crous and Palm (1999) re-examined this specimen, and found conidiomata to be variable (unilocular to multilocular, eustromatic). Conidiophores were branched, septate, holoblastic, and gave rise to smooth to finely verruculose, brown, 1-euseptate conidia, indistinguishable from those of *Diplodia*. *Dothiorella* should therefore be considered a synonym of *Diplodia*, and all anamorphs of *Botryosphaeria* that were placed in *Dothiorella* need to be re-examined.

Lasiodiplodia Ell. & Everh. Laundon (1973) stated that anamorphs of *Botryosphaeria* have to be studied at two stages, namely at conidial dehiscence from conidiogenous cells, when conidia are regarded as 'mature', and after discharge from the pycnidium, when conidia are regarded as 'aged'. Conidia vary greatly in septation, ornamentation and colour at these two stages. Young conidia are hyaline, and become pigmented with age. Conidial septation and ornamentation also develop with age. In the case of the type species, *L. theobromae* (Pat.) Griffon & Maubl., Uduebo (1975) clearly illustrated that the conidial wall ornamentation is made up of deposits of melanin on the inside of the wall, creating the illusion of striations on surfaces of conidia. The paraphyses observed in conidiomata of *L. theobromae* are characteristic of this species, but not unique, as they also occur in other anamorphs of *Botryosphaeria* (Zambettakis, 1954). Thus on the basis of these observations, there is justification for including *Lasiodiplodia* as a synonym of *Diplodia* until it can be proven otherwise.

Sphaeropsis Sacc. The distinction between *Diplodia* and *Sphaeropsis* has never been clear (Hesler 1913; Shoemaker 1964). Percurrent proliferation seen in conidiogenous cells has been regarded more typical of *Sphaeropsis* as defined by Sutton (1980) than of *Diplodia*. However, isolates of *Diplodia* also produce percurrent proliferation in conidiogenous cells. Conidial septation is another feature that has been used to separate these genera. In *Sphaeropsis* conidia are aseptate but become euseptate prior to germination (Sutton 1980), but in *Diplodia* conidia become 1-euseptate as they mature (Sutton 1980). There are a number of reports of 'ageing' conidia of *Botryosphaeria* anamorphs developing septa (Witcher and Clayton 1963; Shoemaker 1964; Maas and Uecker 1984; Pennycook and Samuels 1985; Samuels and Singh 1986; Rayachhetry *et al*. 1996). Thus the concept pertaining to maturity of conidia is vague, and one must question the value of using septation to distinguish the two genera.

Materials and Methods

Isolate collection and examination. Fresh plant material with typical disease symptoms caused by *Botryosphaeria* spp. was obtained from species of the *Proteaceae* sampled in the Western Cape Province, South Africa. Material was treated as described in Chapter 2. Slide preparations were made by squashing fruiting structures in a drop of water or lactophenol, and examining them under a Zeiss Axioskop light microscope.

When necessary, ascomata and conidiomata were rehydrated, and sections made using a Leica CM 1100 cryostat freezing microtome.

DNA sequencing and phylogenetic analysis. The methods employed in DNA isolation, PCR amplification, sequencing and phylogenetic analysis are identical to those used by Crous *et al*. (2000a). Data were compared with those generated by Jacobs and Rehner (1998), in which a *Dothidea* sp. was used as outgroup.

Results

Phylogenetic analysis. DNA sequences were determined for approximately 470 bp of ITS1, 5.8S and ITS2 regions of the rRNA gene of *Botryosphaeria* isolates included in this study (Table 1). These sequences were algorithmically aligned with those generated by Jacobs and Rehner (1998) (Table 1), and manually adjusted for improvement. The alignment (data not shown, available from corresponding author) contains 369 constant characters and 242 variable characters, of which 215 were parsimony informative. A single most parsimonious tree (Fig. 1) was generated using PAUP 4* (Swofford 1999) with the branch and bound option and 1000 bootstrap replicates using the sequences of *Dothidea insculpta* Wallr. and *D. hippophaes* (Pass.) Fuckel as outgroups. The neighbour-joining method included in PAUP* 4 (Swofford 1999) was also used for the analysis, and produced an identical tree topology. In the phylogenetic tree (Fig. 1), isolates of *B. proteae* formed a clade paraphyletic to other *Botryosphaeria* isolates with 100% bootstrap support. The major *Botryosphaeria* clade is composed of dark- and hyaline-conidial groups, and has 100% bootstrap support. All the pigmented (dark) conidial isolates (Jacobs and Rehner 1998) formed a clade with 99% bootstrap support. Three subclades were found in the dark-conidial clade. The hyaline-conidial group was only supported by a bootstrap value of 54%. Jacobs and Rehner (1998) demonstrated 83% bootstrap support for the hyaline clade, so the decline in support with addition of more *B. dothidea* isolates suggests that the hyaline strains may not represent a true division in *Botryosphaeria*. Two subclades were formed in the hyaline group, the *B. dothidea* isolates (Group 3) from Jacobs and Rehner (1998) with a 96% bootstrap support, and a second subclade (99% bootstrap support) comprising three groups of strains. The *B. dothidea* strains from *Protea*

formed one of the subclades with 99% bootstrap support. *Fusicoccum luteum* Pennycook and Samuels (Group 2 in the Jacobs and Rehner 1998) and two *B. ribis* and two *B. dothidea* strains (Group 1 in the Jacobs and Rehner 1998) formed the other subclades. The significance of the phylogenetic differences between the hyaline-conidial clades remains uncertain, but they might represent species.

Jacobs and Rehner (1998) discussed the phylogenetic information obtained from ITS sequence data for *Botryosphaeria*. Doubts were raised where morphological characters and ITS phylogeny were contradictory. In the alignment presented here, however, it was observed that sufficient informative characters supported the phylogenetic tree topology (Fig. 1), which correlated with morphological characters used to distinguish species in *Botryosphaeria*. More sequence data and other gene trees will be required, however, to support the observations made in these studies.

Discussion

Anamorphs of *Botryosphaeria* can essentially be differentiated into two groups based on conidium colour, namely a hyaline group typified by *Fusicoccum*, and a dark-conidial group represented by *Diplodia*. Anamorphs of *Botryosphaeria* should either be placed in *Fusicoccum* or *Diplodia*. Species of *Fusicoccum* are those with hyaline conidia that can become translucent brown and septate prior to germination. *Diplodia* anamorphs of *Botryosphaeria* have 0–1-euseptate conidia that are opaque brown when mature and they can have prominent melanin deposits on the insides of the conidial walls, which give the impression of striations, or the conidial walls can be smooth. *Fusicoccum* spp. are always less than 10 μm wide, while those of *Diplodia* are wider than this.

Results from this study suggest, however, that *Botryosphaeria* is monophyletic. The clade representing the hyaline *Fusicoccum* anamorphs received only 54% bootstrap support, and this may reduce even further as more taxa are added. The question then arises if two separate anamorph genera should be retained for *Botryosphaeria*. In culture, hyaline conidia of several *Fusicoccum* species are known to turn brown with age, making them indistinguishable from those of *Diplodia* species. Given the present impetus to merging anamorph and teleomorph genera in

accordance with the true phylogeny, the future may see us moving to a system where only one anamorph name is available for asexual *Botryosphaeria* species. If the monophyly of *Botryosphaeria* also holds with other data sets, the older, valid name for anamorphs of *Botryosphaeria* would be *Fusicoccum* (1829), and not *Diplodia* (1834).

Of special interest in this study was the clustering of isolates of the recently described *Botryosphaeria proteae* (Chapter 2). This species is rather unusual because it has a *Fusicoccum* anamorph, as well as a dark-spored synanamorph (or microconidial state) and spermatia. Isolates commonly form both anamorphs in culture. Both synanamorphs also occur on diseased host material. If the argument presented in this chapter, where there are two anamorph groups associated with *Botryosphaeria* is correct, the placement of *B. proteae* raises serious problems. The molecular data (Fig. 1) show, however, that isolates of *B. proteae* reside in a clade outside *Botryosphaeria*. The correct generic placement of *B. proteae* is thus uncertain, and it will have to be compared to other, similar genera to try and resolve its correct generic affinity.

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Table 1. Isolates used in phylogenetic studies

Species	Reference	GenBank	Strain No.
Diplodia quercina	Jacobs and Rehner 1998	AF027753	K.J. 93.29[1]
Sphaeropsis sp. (*B. obtusa*)	Jacobs and Rehner 1998	AF027759	K.J. 93.56
Sphaeropsis sapinea	Jacobs and Rehner 1998	AF027758	K.J. 94.07
		AF027757	K.J. 94.05
		AF027756	K.J. 93.31
		AF027753	K.J. 93.29[1]
Lasiodiplodia theobromae (*B. rhodina*)	Jacobs and Rehner 1998	AF027761	K.J. 93.27
		AF027760	K.J. 93.40
		AF027762	K.J. 93.41
Fusicoccum aesculi (*B. dothidea*)	Jacobs and Rehner 1998	AF027741	K.J. 93.42
		AF027743	K.J. 94.09
		AF027742	K.J. 93.03
		AF027744	K.J. 94.11
Fusicoccum luteum	Jacobs and Rehner 1998	AF027745	K.J. 93.52
Fusicoccum sp. (*B. dothidea* complex)	Present study	AF195774	STE-U4360
		AF196294	STE-U4356
		AF196295	STE-U4361
		AF196296	STE-U4357
		AF196297	STE-U4362
		AF196298	STE-U1802
Fusicoccum aesculi (*B. dothidea*)	Jacobs and Rehner 1998	AF027746	K.J. 93.12
		AF027751	K.J. 93.23
		AF027750	K.J. 93.54
		AF027749	K.J. 94.26
		AF027748	K.J. 94.27
		AF027747	K.J. 94.23

Table 1. Continued

Species	Reference	GenBank	Strain No.
Fusicoccum proteae (*B. proteae*)	Present study	AF1962996	STE-U4358
		AF1963007	STE-U4354
		AF1963018	STE-U4355
		AF1963029	STE-U4359

[1] K.J. = cultures of K. Jacobs (Jacobs and Rehner 1998)

[2] STE-U cultures maintained at the Department of Plant Pathology, University of Stellenbosch.
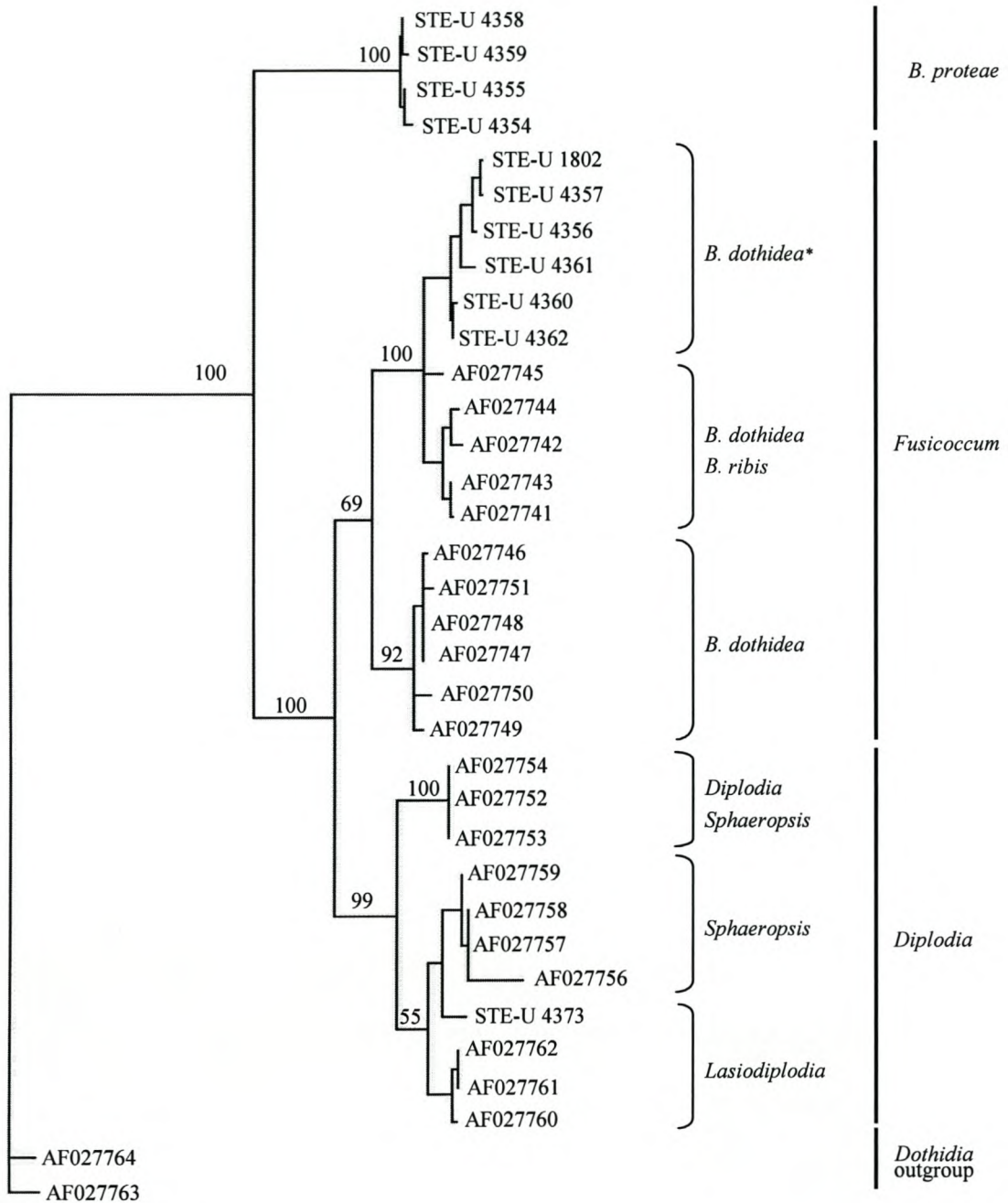
*Host: *Protea*

Fig. 1. The most parsimonious tree with a tree length of 400 steps (CI = 0.777, RI = 0.915, RC = 0.712) derived from a branch and bound search in PAUP 4 (Swofford 1999) with 1000 randomisations of sequence input orders and 1000 bootstrap replications using ITS1, 5.8S and ITS2 data. The tree is rooted with outgroups *Dothidea insculpta* and *D. hippophaes*. The bootstrap values and branch lengths are indicated above and below the branches.

CHAPTER 4

Botryosphaeria species associated with Proteaceae

Abstract

In this study the identity of *Botryosphaeria* spp. associated with Proteaceous hosts growing in various localities around the world, was established based on morphology and sequence data. Five *Botryosphaeria* spp. were associated with Proteaceae. *B. lutea* was isolated from *Banksia* and *Buckinghamia* spp. from Australia, and a single isolate was obtained from *P. cynaroides* in South Africa. *B. proteae* was associated with South African Proteaceae only. However, it was isolated from these hosts cultivated in Australia, Hawaii, Madeira, Portugal and South Africa. Another *Botryosphaeria* sp., that was exclusive to South African Proteaceae, represents a new taxon that is described as *B. protearum*. This pathogen was also found on South African Proteaceae cultivated in Australia, Hawaii, Madeira, Portugal and South Africa. *B. ribis* was associated with both South African and Australian Proteaceae and was isolated from material collected in Australia, Hawaii and Zimbabwe. A single occurrence of *B. obtusa* as an endophyte was recorded from *P. magnifica* in South Africa. The present study has, for the first time, clarified the current global distribution of *Botryosphaeria* spp. associated with Proteaceae. A key to these taxa associated with Proteaceae is also provided.

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). Because Proteaceae are commercially valuable and sought after as cut-flowers on several international markets, worldwide cultivation and an increase in global trade of certain species has emerged. Many of the South African Proteaceae are currently cultivated in Australia, Azores Islands, Canary Islands, Chile, Israel, Madeira, New Zealand, Portugal, Spain, U.S.A. (California, Hawaii) and Zimbabwe. Similarly some Australian Proteaceae (e.g. *Banksia* and *Telopea* spp.), are cultivated

in countries other than Australia. Consequently, there is an increasing movement of fresh proteas as well as germplasm around the world.

One of the factors limiting commercial production of Proteaceae is damage caused by pests and diseases. Some pathogens cause significant losses in the field and in nurseries through disease and plant death. Others damage the aesthetic appearance of blooms, and although they are not severe pathogens, they are considered important. Many pathogens associated with Proteaceae are regarded as actionable quarantine organisms and can be the cause of rejection of consignments at point of import based on contravention of phytosanitary regulations (Crous *et al.* 2000b; Taylor 2001).

A number of fungal pathogens that occur on Proteaceae have already been introduced into other countries as endophytes or latent pathogens in cuttings or on seed (Crous *et al.* 2000b). This raised concern regarding the risk that these fungi pose to agriculture and indigenous vegetation.

Petrini (1991) defined endophytes as "organisms inhabiting plant organs that at some stage in their life can colonise plant tissues without causing apparent harm to their host". Therefore, latent pathogens are endophytes until they initiate disease.

Among the most important fungal pathogens of Proteaceae are *Botryosphaeria* spp., causing leaf necrosis, shoot die-back, stem cankers and plant death. Recently it has been demonstrated that some *Botryosphaeria* spp. have an endophytic or possibly latent phase in their life cycles (Smith *et al.* 1996; Swart *et al.* 2000). This contributes to exporting fungal pathogens unwittingly to countries importing plant material.

Botryosphaeria diseases have been recorded in most areas where Proteaceae are cultivated (Olivier 1951; van Wyk 1973; Benic and Knox-Davies 1983; von Broembsen 1986; Orffer and Knox-Davies 1989; Serfontein and Knox-Davies 1990; Forsberg 1993; Moura and Rodrigues 2001; Taylor *et al.* 2001a,b), but the species involved remain uncertain. This is largely due to the taxonomic difficulties encountered in identifying members of this genus (Shoemaker 1964; Laundon 1973; Morgan-Jones and White 1987; Jacobs and Rehner 1998; Chapter 3). However, with recent advances in molecular taxonomy many of the problems previously encountered can now be resolved (Jacobs and Rehner 1998; Zhou *et al.* 2001; Chapter 3).

The correct identification of *Botryosphaeria* spp. associated with Proteaceae that are cultivated in both the Northern and Southern Hemispheres, will enable the global movement of these pathogens to be monitored. It will also contribute to appropriate application of quarantine decisions. Accurate species identities are also

required for developing disease management strategies since different species do not react in the same way to different hosts and environmental conditions (Britton and Hendrix 1982, 1986).

The aims of this study were to establish the identity of *Botryosphaeria* spp. isolated from different Proteaceae growing in parts of the world. A table listing the different *Botryosphaeria* spp. and their proteaceous hosts at various localities (Table 1), and a key to *Botryosphaeria* spp. associated with Proteaceae are also provided.

Materials and methods

Isolates. Isolates (Table 2) were obtained by making single spore isolations from mature fruiting bodies present in diseased material or by isolating the pathogen from the margin between symptomatic and healthy plant material. Isolations were also made from completely asymptomatic, visually healthy tissue. The plant material used for isolations represented a wide range of genera and species of Proteaceae collected in many countries. Plant tissue was surface disinfested by placing it in 70% ethanol for 30 sec, 1% NaOCl for 1 min, 30 sec in ethanol and rinsed in sterile water for 1 min. Pieces of tissue were excised from the margin between necrotic and apparently healthy tissue lesion and plated on 2% potato dextrose agar (PDA, Biolab, Midrand, South Africa). Hyphae growing out of the pieces of tissue were sub-cultured onto divided plates containing PDA in one half of the dish and carnation leaf agar (Fisher *et al.* 1982) in the other. Divided plates were incubated for 3–6 wk at 25°C under near-ultraviolet and cool white fluorescent light with a 12 h light cycle. *Botryosphaeria* cultures were subsequently identified based on growth morphology and if cultures had sporulated, on conidial or ascospore morphological features.

In addition to isolations, single conidial or ascospore isolates were obtained using the method described in Chapter 2. Cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) (Table 2).

DNA extraction. Isolates (Table 2) were cultured on PDA and incubated at 25°C under continuous fluorescent light for 7–10 d. Methods for DNA extraction were adapted from those described by Crous *et al.* (2000a). Mycelium was scraped from

the surface of actively growing cultures and placed in eppendorf tubes containing 1 mL of SDS extraction buffer (2% SDS, 50 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA; pH 8). The contents were boiled for 5 min after which the tubes were placed in a mortar. Liquid nitrogen was poured over the tubes, to freeze their contents. The tubes were then left on the bench top to thaw. TE buffered phenol was added to the tubes at a ratio of 2:3 (phenol to sample). The sample was then agitated for 15 min and centrifuged for 15 min at 14 000 rpm. The aqueous phase was transferred to a clean eppendorf tube and 1 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the extraction procedure repeated. RNase A was then added to the solution, which was incubated overnight at 37°C. Proteinase K was then added at a concentration of 300 µg/µL, and further incubated for another 4 h at 37°C. Thereafter, the phenol extraction procedure was repeated. Nucleic acids were precipitated by adding NaAc pH 5.5 at a final concentration of 50 mM, and adding an equal volume of absolute ethanol. The suspension was left on the laboratory bench for 30 min, then centrifuged at 14 000 rpm for 15 min. The resulting pellet was washed with 70% ethanol, dried at 60°C for an hour and then resuspended in 100 µL of sterile Sabax water. All genomic DNA was stored at 4°C until amplification.

DNA amplification. The primers ITS1 (5'TTTCCGTAGGTGAACCTGC3') and ITS4 (5'TCCTCCGCTTATTGATATGC') (White *et al.* 1990) were used to amplify part of the nuclear rRNA operon using PCR. The amplified region included the 3'end of the 16S (small subunit) rDNA gene, the first internal transcribed spacer (ITS1), the 5.8S rDNA gene, the second ITS (ITS2) region and the 5'end of the 26S (large subunit) of the rDNA gene. The reaction mixture contained 5 µL of diluted sample, 1x buffer, 8 mM $MgCl_2$, 500 µM of each of the dNTPs, 2.5U (Bioline) Taq and 10 pM of each primer and made up to a total volume of 25 µL with sterile water. The cycling conditions comprised denaturing at 96°C for 5 min followed by 30 cycles of denaturation at 96°C (30 sec), annealing 55°C (30 sec) and elongation at 72°C (90 sec). A final elongation step at 72°C for 7 min was included. PCR products were run on ethidium bromide stained, 1% agarose gels, using electrophoresis and were visualised using UV illumination

DNA sequencing and analysis. PCR products were purified according to the manufacturer's instructions using a commercial kit (Nucleospin Extract 2 in 1 Purification Kit, Machery-Nagel GmbH & Co., Germany). Sequencing reactions were carried out using ABI PRISM Big Dye Terminator Cycle v3.0 Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, U.S.A.) according to the maunfacturer's recommendations. The reaction was done on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). Raw sequence data were analysed using EditView 1.0.1 (http://www.appliedbiosystems.com) and manually aligned by inserting gaps. Phylogenetic analyses were undertaken using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 2000). Gaps were treated as a fifth character and all characters were unordered and of equal weight. The data matrix consisted of two outgroup taxa and 61 ingroup taxa, each sequence containing 528 characters (including gaps). Heuristic searches were carried out using stepwise simple addition and tree bisection and reconstruction (TBR) as the branch swapping algorithm to find maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Given the large number of taxa used in the study, branch support was determined using 100 bootstrap replicates (Felsenstein 1985). Representative *Botryosphaeria* sequences from the preliminary clades were used to obtain sequences from GenBank using a standard nucleotide-nucleotide BLAST search (Altschul *et al*. 1997). The representative sequences from GenBank were included in the final analyses. Trees were rooted to *Mycosphaerella africana* Crous & Wingf. and *Guignardia bidwellii* (Ellis) Viala & Ravaz.

Results

PCR and phylogenetic analyses. PCR products of ca. 580 base pairs (bp) were obtained for all isolates tested in this study. All sequences consisted of 528 characters after manual alignment by inserting gaps. Of these, 51 variable characters were parsimony-uninformative and 208 were parsimony-informative, and used to obtain 159 most parsimonious trees, of 470 steps using heuristic searches (CI = 0.821, RI = 0.952, RC = 0.782, HI = 0.179) (Fig. 1).

Five distinct clades emerged and sequences obtained from GenBank enabled identification of four of the clades. The GenBank sequence of *Botryosphaeria ribis*

(Tode ex Fr.) Gross & Dugg (AF27741, Jacobs and Rehner 1998) grouped with a clade of 10 isolates from Proteaceae (100% bootstrap support). Sequence data for eight isolates corresponded with the *Fusicoccum luteum* Pennycook & Samuels sequence in GenBank (AF27745, Jacobs and Rehner 1998; Zhou and Stanosz 2001) (94% bootstrap support). A single isolate grouped with the *Botryosphaeria obtusa* (Schwein.) Shoemaker (100% bootstrap support) and 7 isolates grouped with *B. proteae* (Wakefield) S. Denman & Crous (AF196299, AF196300, AF196301 AF196302, Chapter 3) (100% bootstrap support).

The majority of the isolates (20) formed a separate, strongly supported clade (100% bootstrap support). This clade was clearly distinct from any other *Botryosphaeria* sequences. Previously, representative sequences from this clade had been reported to form part of the *B. dothidea* complex (Chapter 3). However, results of this work show that the group is closer to *B. ribis* than to *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & de Not., but is clearly separate (72% bootstrap support) from *B. ribis*. It is thus considered a new taxon.

No isolates of *B. ribis* were found in the material from South Africa or Maderia. All but one of the *F. luteum* isolates were isolated from Australian Proteaceae (*Banksia* and *Buckinghamia*), growing in Australia (Table 2). The single *F. luteum* isolate (STE-U4393) that was not obtained from Australia, occurred as an endophyte on *Protea cynaroides* L. in South Africa (Table 2). The isolates comprising the unidentified *Botryosphaeria* sp. all occurred on South African Proteaceae, but were obtained from many localities worldwide, including Australia, Madeira, Portugal and South Africa (Table 1). Similarly, in this study *B. proteae* was restricted to South African Proteaceae, but was present in many countries including Australia, Hawaii (U.S.A.), Madeira and Portugal. The single isolate of *B. obtusa* was obtained from a wild protea growing in a nature reserve in South Africa.

Botryosphaeria protearum S. Denman & Crous, sp. nov.   Figs. 2–14

Anamorph. **Fusicoccum protearum** S. Denman & Crous, sp. nov.

> *Latin description to be inserted when this chapter is submitted for publication. The present description is not to be cited.*

Ascomata pseudothecial, embedded in host tissue, up to 600 µm diam., becoming erumpent, solitary or botryose, stromatic, dark brown to black, with central, black

ostioles; pseudothecial wall 6–15 cell layers thick, composed of brown *textura angularis*. *Asci* clavate to subcylindrical, 110–200 x 15–21 μm, 8-spored, bitunicate with a well-developed apical chamber, which becomes inconspicuous at maturity, interspersed amongst filiform, branched, septate pseudoparaphyses, 3–5 μm wide. *Ascospores* irregularly biseriate, hyaline, nonseptate, granular, becoming light brown with age, (25–)26–33(–37) x (9–)10–12(–13) μm, sometimes inequilateral, fusiform, widest in the middle with obtuse ends. *Pycnidia* embedded in host tissue, solitary or botryose, stromatic, globose, up to 500 μm diam; pycnidial wall, 4–8 cell layers thick, composed of brown *textura angularis*, becoming hyaline towards the inner region. *Conidiophores* 0–1-septate, hyaline, subcylindrical, rarely branched, 7–20 (–30) x 3–5 μm. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 7–12 x 3–5 μm, rarely proliferating percurrently with 1–2 proliferations, predominantly proliferating at the same level with minute periclinal thickening, which becomes more prominent in older conidiogenous cells. *Conidia* hyaline, granular, ovoid to clavate when young, becoming irregularly fusoid when mature, widest in the middle with an obtuse apex and bluntly rounded or minutely flattened base (inconspicuous in older, permanent mounts), (20–)25–30(–40) x 7–8(–10) μm *in vivo*. *Spermatial state* occurring in conidiomata with the *Fusicoccum* anamorph, or in separate spermatogonia. *Spermatiophores* hyaline, smooth, branched, cylindrical, 0–2-septate, straight, unbranched or branched above, 12–17 x 2–3 μm. *Spermatiogenous cells* discrete or integrated, hyaline, smooth, cylindrical, proliferating via determinate phialides with periclinal thickening, 5–12 x 1.5–2.5 μm. *Spermatia* hyaline, smooth, aseptate, rod-shaped with rounded ends, 3–6 x 1–1.5 μm.

HOLOTYPES. SOUTH AFRICA. WESTERN CAPE: Baanbreek Farm, Porterville, on stems of *Protea magnifica* Link. 27 Jul. 1997, *S. Denman* (PREM XXXX [Number still to be obtained], teleomorph, culture ex-type STE-U 4361); Devon Valley, Protea Heights Farm, Stellenbosch, on stems of *Leucadendron salignum* P.G. Bergius x *L. laureolum* (Lam.) Fourc. cv Silvan Red, 31 Oct. 1997, *S. Denman & J. Taylor* (PREM XXXX [Number still to be obtained], anamorph, culture ex-type STE-U 1775).

Additional specimens examined. SOUTH AFRICA. WESTERN CAPE: Elsenburg, Stellenbosch, on leaves of *Protea eximia* (Salisb. ex Knight) Fourc., 22 Jul. 1997, *S. Denman* (STE-U 4360, teleomorph); Osdam Farm, Porterville, on stems of *P. magnifica* 16 Jun. 1997, *S. Denman* (B2088.2, teleomorph), Baanbreek Farm, Porterville, on leaves of *P. magnifica,* 29 Jul. 1997, *S. Denman* (STE-U 1803,

anamorph); Baanbreek Farm, Porterville, on leaves of *P. magnifica,* 29 July 1997, *S. Denman* (STE-U 4362, anamorph); Florialis Estate, Madeira on leaves of *Protea compacta* R. Br. x *P. susannae* E. Phillips cv "Pink Ice"*,* Apr. 2000, *S. Denman* and *J. Taylor* (STE-U 4397, anamorph).

Cultures. Colonies initially translucent to white, gradually darkening from the centre, olive green to grey (4–7 d), ultimately becoming charcoal black (14–21 d). Initially a moderate amount of aerial mycelium was formed but this collapsed as hyphae reached the edges of Petri dishes, resulting in flat colonies with rims of loose aerial mycelium at the edge of the dish. Black conidiomatal initials were sometimes formed, beginning in the centre of colonies and spreading over the entire colony surface. Colony colour, based on the colour charts of Rayner (1970) was greenish black (33""'k) underneath and olivaceous grey (23""'i) to iron grey (25""'k), on the surface. The aerial mycelium around the edge of the dish ranged from smoke grey (21""'f) to pale olivaceous grey (21""'f).

Temperature requirements for growth. Min 5°C, opt 25°C, max 35°C. No growth was recorded at 40°C. The mean daily growth rate at 25°C was 25.5 mm/d (Fig. 15).

Hosts. *Protea cynaroides* L., *P. eximia* , *P. magnifica, P. neriifolia* R. Br., *P. repens* (L.) L., *Leucadendron salignum* P.G. Bergius x *L. laureolum* (Lam.) Fourc. cv Slivan Red, *L. tinctum* I. Williams and other *Leucadendron* spp.

Known distribution. Australia (Queensland), Madeira, Portugal, South Africa (Western Cape Province).

Discussion

This study represents the first attempt to comprehensively characterise the species of *Botryosphaeria* associated with Proteaceae. Material was collected extensively from a wide range of hosts and the results will provide a foundation for future pathology and biogeographical studies. A large number of new host and distribution records are made and a new species of *Botryosphaeria* from Proteaceae is described.

Fusicoccum luteum is newly reported on Australian Proteaceae (*Buckinghamia*) in Australia, and on *P. cynaroides* in South Africa. Zhou and Stanosz (2001) demonstrated that an isolate obtained from *Banksia* in Australia and previously mis-identified as *B. ribis* represented *F. luteum*. Results of this study

confirm the presence of *F. luteum* on *Banksia* and extend the host range to *Buckinghamia*. Shearer *et al.* (1995) described a devastating disease of *Banksia coccinea* R. Brown caused by *B. ribis* along the southwestern coast of Australia. In light of the findings by Zhou and Stanosz (2001) and results of the present work, it is possible that the pathogen described by Shearer *et al.* (1995), is *F. luteum*. It therefore seems that *F. luteum* is fairly widely distributed on Australian Proteaceae.

The isolated occurrence of *F. luteum* on Proteaceae in South Africa is probably not representative of the relative occurrence of this fungus on Proteaceae. This view is based on the fact that the fungus appears to be common on Proteaceae in Australia and on kiwi fruit in New Zealand (Pennycook and Samuels 1985). *F. luteum* has been found commonly on grapevines by Phillips *et al.* (2002) who recently described the teleomorph as *Botryosphaeria lutea* A.J.L. Phillips. In the Western Cape Province it has also been commonly associated with grapevines as well as stone and pome fruit trees, which are cultivated alongside Proteaceae orchards (P.W. Crous, Dept. of Plant Pathology, University of Stellenbosch, pers. comm.). *F. luteum* was isolated as an endophyte of *P. cynaroides* and thus cannot be described as a pathogen of South African Proteaceae at this stage. This isolate was particularly interesting as it formed its teleomorph in culture, a feature not previously recorded for this fungus.

The new species, *Botryosphaeria protearum* is reported for the first time from Australia, Maderia, Portugal and South Africa. This pathogen was found on South African Proteaceae in their native habitat as well as on cultivated plants. The fact that it was isolated only from South African Proteaceae suggests that this *Botryosphaeria* sp. is indigenous to South Africa. Its occurrence in other countries where South African Proteaceae are cultivated suggests that it was introduced with South African protea germplasm.

Botryosphaeria protearum was not isolated from any Australian Proteaceae, indicating that it may be specific at the sub-family level, and might only occur on South African Proteoideae. The family Proteaceae is comprised of two sub-families, namely the Proteoideae and the Grevilleoideae. Members of the former occur mainly in southern Africa while members of the latter occur primarily in Australia (Rebelo 1995). Crous *et al.* (2000b) and Taylor *et al.* (2001a,b) suggested that many of the fungal pathogens of Proteaceae are host specific, and current evidence suggests that this is true for *B. protearum*.

Present results, as well as those from previous studies (Crous *et al.* 2000b; Taylor *et al.* 2001a,b), confirm that *B. proteae* is associated with only South African Proteaceae. This evidence supports the view that some *Botryosphaeria* spp are host specific on Proteaceae. However, this study included a relatively small sample of Australian Proteaceae from a limited area and more extensive collections are needed to confirm the hypothesis regarding host specificity.

In contrast to the apparent host specificity of *B. proteae* and *B. protearum*, *B. ribis* has a wide host range. Results of the present study demonstrate for the first time that *B. ribis* occurs on both South African and Australian Proteaceae, cultivated in Hawaii, and on *P. cynaroides* in Zimbabwe. This pathogen has been isolated from apples (*Malus* Mill.) (Stevens and Jenkins 1924), currants (*Ribes* L.) (Grossenbacher and Duggar 1911), *Eucalyptus* (Fraser and Davidson 1985), mangoes (*Mangifera indica* L.)(Ramos *et al.* 1991), *Melaleuca* (Rayachhetry *et al.* 1996), Proteaceae (Olivier 1951) and walnuts (*Juglans* L.) (Rumbos 1987) amongst others. However, there has been some confusion about the identity of *B. ribis* (Chapter 1), and therefore earlier reports in the literature must be interpreted with some circumspection.

Botryosphaeria ribis has been reported on various Proteaceae. Schieber and Zentmyer (1978) considered *B. dothidea* synonymous with *B. ribis* and described a serious trunk canker disease of *Grevillea robusta* Cunn. in Guatemala. In South Africa, Olivier (1951) reported *B. ribis* on *Leucadendron* and Herbert and Grech (1985) isolated a fungus of this name from *Macadamia* trees. In the present study it was found on South African Proteaceae cultivated in Hawaii and Zimbabwe, but not in South Africa.

The South African samples were collected from Proteaceae growing in the cool, winter rainfall region in the Western Cape Province. In the previous records of *B. ribis* on Proteaceae, the hosts were growing in warm, humid climates (Schieber and Zentmyer 1978; Herbert and Grech 1985). Results presented here demonstrate that *B. ribis* was isolated only from areas with high temperatures and humidity [Australia (Queensland), U.S.A. (Hawaii) and Zimbabwe]. These climatic conditions may be a prerequisite for infection by *B. ribis*. In South Africa the cultivation of Proteaceae is currently expanding in the warm, humid, summer rainfall regions, and this might lead to the appearance of *B. ribis* in South Africa. There is a good chance that this pathogen is already present on native Proteaceae in these areas, and that it might pose a threat to Proteaceae cultivation.

The isolated incidence of *B. obtusa* on *P. magnifica* is difficult to explain, but it has commonly been associated with apples (Stevens and Jenkins 1924). Apple orchards in the valley the Groot Winterhoek Nature Reserve, from where this sample was from proteas, and this might explain its origin on protea. Wider sampling may reveal broader distribution of this fungus. This is, however, also the first report of *B. obtusa* on *P. magnifica*.

The present study has clarified the current global distribution of *Botryosphaeria* spp. associated with Proteaceae. A key to identify the taxa associated with Proteaceae is provided and this will help alleviate taxonomic confusion in the future. Very little is known about the pathogenicity of these species. Further work is clearly justified and should elucidate the importance of the various species of *Botryosphaeria* associated with Proteaceae worldwide.

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Table 1. Host and localities of *Botryosphaeria* spp. associated with Proteaceae

Species	Host	Locality	Reference
B. gaube	*Grevillea victoriae*	Australia	Petrak 1967
B. dothidea	*Grevillea wilsonii*	Hawaii	Taylor *et al.* 2001b
	Leucadendron spp.	Hawaii	Taylor *et al.* 2001b
	Leucospermum spp.	Hawaii	Taylor *et al.* 2001b
	P. compacta	Hawaii	Taylor *et al.* 2001b
	P. magnifica x *P. compacta* cv Lady Di	Hawaii	Taylor *et al.* 2001b
	P. compacta x *P. susannae* cv Pink Ice	Hawaii	Taylor *et al.* 2001b
	P. cynaroides	Hawaii	Taylor *et al.* 2001b
	Teleopea sp.	Hawaii	Taylor *et al.* 2001b
	Leucospermum spp.	South Africa	von Broembsen 1986
B. lutea	*Banksia* sp.	Australia	Present study
	Buckinghamia sp.	Australia	Present study
	P. cynaroides	South Africa	Present study
B. obtusa	*P. magnifica*	South Africa	Present study
B. proteae	*P. eximia*	Australia	Crous *et al.* 2000
	P. magnifica	Australia	Crous *et al.* 2000
	P. obtusifolia	Australia	Crous *et al.* 2000
	Protea spp.	California	Taylor *et al.* 2001a
	P. compacta	Hawaii	Taylor *et al.* 2001b
	P. compacta x *P. susannae* cv Pink Ice	Hawaii	Taylor *et al.* 2001b
	P. cynaroides	Hawaii	Taylor *et al.* 2001b
	P. magnifica x *P. compacta* cv Lady Di	Hawaii	Taylor *et al.* 2001b
	P. neriifolia cv mink	Hawaii	Taylor *et al.* 2001b
	P. laurifolia	Hawaii	Taylor *et al.* 2001b
	P. neriifolia	Hawaii	Taylor *et al.* 2001b

Table 1. Continued

Species	Host	Locality	Reference
	P. aristata x *P.repens* cv Venus	South Africa	Chapter 2
	P. cynaroides	South Africa	Swart *et al.* 2000
	P. eximia	South Africa	Chapter 2
	P. grandiceps	South Africa	Chapter 2
	P. magnifica	South Africa	Chapter 2
	P. magnifica x *P. compacta* cv Lady Di	South Africa	Chapter 2
	L. cordifolium	South Africa	Swart *et al.* 2000
B. protearum	*Leucadendron* sp.	Australia	Present study
	P. magnifica	Australia	Present study
	P. cynaroides	Hawaii	Present study
	Leucadendron tinctum	Madeira	Present study
	Leucadendron sp.	Portugal	Present study
	Leucadendron cv Silvan Red	South Africa	Present study
	P. cynaroides	South Africa	Present study
	P. magnifica	South Africa	Present study
	P. neriifolia	South Africa	Present study
	P. repens	South Africa	Present study
B. rhodina	*Protea* spp.	Australia	Forsberg 1993
	Banksia dryandroides	Hawaii	Taylor *et al.* 2001b
	Leucospermum sp.	Hawaii	Taylor *et al.* 2001b
	P. cynaroides	Hawaii	Taylor *et al.* 2001b
	Teleopea sp.	Hawaii	Taylor *et al.* 2001b
B. ribis	*Buckinghamia* sp.	Australia	Present study
	Banksia coccinea	Australia	Shearer *et al.* 1995
	Grevillea sp.	Hawaii	Taylor 2001

Table 1. Continued

Species	Host	Locality	Reference
	Leucadendron cv Safari sunset	Hawaii	Present study
	Leucospermum sp.	Hawaii	Present study
	P. cynaroides	Hawaii	Present study
	Teleopia sp.	Hawaii	Present study
	L. argenteum	South Africa	Olivier 1951
	P. cynaroides	South Africa	Olivier 1951
	P. cynaroides	Zimbabwe	Present study
	Macadamia integrifolia	South Africa	Herbert and Grech 1985
	Macadamia tetraphylla	South Africa	Herbert and Grech 1985
Diplodia grevilleae	*Grevillea robusta*	Pakistan	Ahmad 1972
Dothiorella banksiae	*Banksia integrifolia*	Australia	Hansford 1954
***Fusicoccum* spp.**	*Leucospermum* spp.	Hawaii	Taylor *et al.* 2001b
	P. compacta	Hawaii	Taylor *et al.* 2001b
	P. magnifica x *P. compacta* cv Lady Di	Hawaii	Taylor *et al.* 2001b
	P. compacta x *P. susannae* cv Pink Ice	Hawaii	Taylor *et al.* 2001b
	P. cynaroides	Hawaii	Taylor *et al.* 2001b
	P. laurifolia	Hawaii	Taylor *et al.* 2001b
	P. neriifolia	Hawaii	Taylor *et al.* 2001b
	Teleopea sp.	Hawaii	Taylor *et al.* 2001b

Table 2. Collection data of *Botryosphaeria* isolates from Proteaceae studied

Accession No.	GenBank No.	Species	Host[a]	Locality	Symptom	Collector	Reference
STE-U 2051	AF452526	*B. ribis*	*Leucospermum* sp.	Hawaii, U.S.A.	Canker[b]	P.W. Crous	Present study
STE-U 2055	AF452523	*B. ribis*	*Ldn.* cv Safari Sunset	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 2057	AF452524	*B. ribis*	*P. cynaroides*	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 4379	AF452525	*B. ribis*	*P. cynaroides*	Zimbabwe	Canker	C. Saywood	Present study
STE-U 4381	AF452522	*B. ribis*	*P. cynaroides*	Zimbabwe	Canker	C. Saywood	Present study
STE-U 4371	AF452518	*B. ribis*	*P. cynaroides*	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 4376	AF452527	*B. ribis*	*P. cynaroides*	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 2100	AF452521	*B. ribis*	*Telopia* sp.	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 3036	AF452519	*B. ribis*	*Buckinghamia* sp.	Australia	Canker	P.W. Crous	Present study
STE-U 4382	AF452520	*B. ribis*	*P. cynaroides*	Zimbabwe	Canker	C. Saywood	Present study
KJ94.09	AF027741	*B. ribis*	*M. quinquenervia*	Florida, U.S.A.	-	M. Rayachhetry	Jacobs and Rehner 1998
STE-U 4384	AF452535	*B. protearum*	*P. cynaroides*	South Africa	Die-back	S. Denman	Present study
STE-U 1775	AF452539	*B. protearum*	*Ldn.* cv Silvan Red	South Africa	Die-back	S. Denman	Present study
STE-U 1776	AF452538	*B. protearum*	*Ldn* cv Silvan Red	South Africa	Canker	S. Denman	Present study
STE-U 2930	AF452528	*B. protearum*	*Leucadendron* sp.	Australia	Leaf necrosis	P.W. Crous	Present study
STE-U 4363	AF452540	*B. protearum*	*P. magnifica*	South Africa	Canker	S. Denman	Present study
STE-U 4368	AF452542	*B. protearum*	*P. repens*	South Africa	Canker	S. Denman	Present study
STE-U 4387	AF452546	*B. protearum*	*P. magnifica*	South Africa	Endophyte[c]	S. Denman	Present study
STE-U 4388	AF452532	*B. protearum*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
STE-U 4389	AF452529	*B. protearum*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
STE-U 2147	AF452534	*B. protearum*	*P. cynaroides*	Hawaii, U.S.A.	Leaf necrosis	P.W. Crous	Present study
STE-U 4390	AF452533	*B. protearum*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
STE-U 4392	AF452545	*B. protearum*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
STE-U 4369	AF452536	*B. protearum*	*P. repens*	South Africa	Canker	S. Denman	Present study
STE-U 4391	AF452541	*B. protearum*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
STE-U 2988	AF452537	*B. protearum*	*P. magnifica*	Australia	Leaf necrosis	P.W. Crous	Present study

Table 2. Continued

Accession No.	GenBank No.	Species	Host[a]	Locality	Symptom	Collector	Reference
STE-U 4397	AF452530	*B. protearum*	*Ldn. tinctum*	Madeira	Die-back	S. Denman	Present study
STE-U 4398	AF452531	*B. protearum*	*Leucadendron* sp.	Portugal	Die-back	S. Denman	Present study
STE-U 4366	AF452543	*B. protearum*	*P. neriifolia*	South Africa	Canker	S. Denman	Present study
STE-U 4367	AF452544	*B. protearum*	*P. neriifolia*	South Africa	Canker	S. Denman	Present study
STE-U 4365	AF452547	*B. protearum*	*P. magnifica*	South Africa	Canker	S. Denman	Present study
STE-U 4360	AF195774	*B. protearum*	*P. eximia*	South Africa	Canker	S. Denman	Chapter 3
STE-U 4356	AF196294	*B. protearum*	*P. magnifica*	South Africa	Leaf necrosis	S. Denman	Chapter 3
STE-U 4361	AF196295	*B. protearum*	*P. magnifica*	South Africa	Canker	S. Denman	Chapter 3
STE-U 4362	AF196297	*B. protearum*	*P. magnifica*	South Africa	Leaf necrosis	S. Denman	Chapter 3
STE-U 4357	AF196296	*B. protearum*	*P. magnifica*	South Africa	Canker	S. Denman	Chapter 3
STE-U 1802	AF196298	*B. protearum*	*P. magnifica*	South Africa	Leaf necrosis	S. Denman	Chapter 3
STE-U 3085	AF452554	*B. lutea*	*Buckinghamia* sp.	Australia	Leaf necrosis	P.W. Crous	Present study
STE-U 3088	AF452555	*B. lutea*	*Buckinghamia* sp.	Australia	Leaf necrosis	P.W. Crous	Present study
STE-U 3091	AF452549	*B. lutea*	*Buckinghamia* sp.	Australia	Leaf Necrosis	P.W. Crous	Present study
STE-U 2953	AF452550	*B. lutea*	*Buckinghamia* sp.	Australia	Leaf necrosis	P.W. Crous	Present study
STE-U 4393	AF452548	*B. lutea*	*P. cynaroides*	South Africa	Endophyte	L. Swart	Present study
STE-U 4402	AF452551	*B. lutea*	*Banksia* sp.	Australia	Canker	M.J. Wingfield	Present study
STE-U 4401	AF452552	*B. lutea*	*Banksia* sp.	Australia	Canker	M.J. Wingfield	Present study
STE-U 4403	AF452553	*B. lutea*	*Banksia* sp.	Australia	Canker	M.J. Wingfield	Present study
ATCC 56125	AF293480	*B. lutea*	*Eucalyptus* sp.	Australia	Canker	E.M. Davidson	Smith and Stanosz 2001
KJ93.52	AF027745	*B. lutea*	*A. deliciosa*	New Zealand	-	G. J. Samuels	Zhou and Stanosz 2001
STE-U 2271	AF452562	*B. proteae*	*P. magnifica* cv Lady Di	Hawaii, U.S.A.	Leaf spot	P.W. Crous	Present study
STE-U 4378	AF452560	*B. proteae*	*Protea* sp.	Australia (USDA interception)	Leaf spot	M.E. Palm	Present study

Table 2. Continued

Accession No.	GenBank No.	Species	Host[a]	Locality	Symptom	Collector	Reference
STE-U 4385	AF452561	*B. proteae*	*Protea* sp.	Australia (USDA interception)	Leaf spot	M.E. Palm	Present study
STE-U 4386	AF452558	*B. proteae*	*Protea* sp.	Australia (USDA interception)	Leaf spot	M.E. Palm	Present study
STE-U 4399	AF452557	*B. proteae*	*P. cynaroides*	Madeira	Leaf spot	S. Denman	Present study
STE-U 4400	AF452559	*B. proteae*	*P. repens*	Portugal	Leaf necrosis	S. Denman	Present study
STE-U 2269	AF452563	*B. proteae*	*P. laurifolia*	Hawaii, U.S.A.	Canker	P.W. Crous	Present study
STE-U 4358	AF196299	*B. proteae*	*P. cynariodes*	South Africa	Endophyte	L. Swart	Chapter 3
STE-U 4354	AF196300	*B. proteae*	*P. cynariodes*	South Africa	Endophyte	L. Swart	Chapter 3
STE-U 4359	AF196302	*B. proteae*	*P. repens*	South Africa	Leaf necrosis	S. Denman	Chapter 3
STE-U 4355	AF196301	*B. proteae*	*P. repens*	South Africa	Leaf necrosis	S. Denman	Chapter 3
STE-U 4373	AF452556	*B. obtusa*	*P. magnifica*	South Africa	Endophyte	S. Denman	Present study
KJ93.56	AF027759	*B. obtusa*	Hardwood shrub	New York, U.S.A.	-	G.J. Samuels	Jacobs and Rehner 1998
97–94	AF241175	*B. dothidea*	*Populus nigra*	New Zealand	-	G.J. Samuels	Smith and Stanosz 2001
–	AF216533	*Guignardia bidwellii*	*Vitis sp.*	New York, U.S.A.	-	A.B. Baudoin	Crous *et al.* 2001
STE-U 794	AF283690	*Mycosphaerella africana*	*E. viminalis*	South Africa	-	P.W. Crous	Crous *et al.* 2001

[a] *Ldn. = Leucadendron; P.= Protea; M = Melaleuca; E = Eucalyptus.*

[b] Cankers were all stem cankers.

[c] Endophytes were all isolated from healthy leaves.

Key to *Botryosphaeria* spp. associated with Proteaceae

1. Conidia pigmented at maturity, *Diplodia* or diplodia-like   2

1. Conidia hyaline at maturity, *Fusicoccum*   3

2. Conidia uniform, ovoid to subcylindric with truncate base and

    obtuse apex, walls 0.5–1 µm thick, warty or finely roughened,

    sienna brown at maturity, 0(–1) septate, 20–26 x 9–12 µm; ascospores

    broadly fusiform, widest in the middle, hyaline, smooth, 25–33 x 7–12 µm   ***B. obtusa***

2. Conidia medium brown, subcylindrical, finely verruculose,

    7–14 x 2.5–3.5 µm; synanamorph: conidia hyaline, fusiform,

    20–30 x 4.5–6 µm; ascospores hyaline, guttulate, smooth, ellipsoidal,

    clavate – fusiform, frequently widest in the upper one third of the

    ascospore, tapering to obtuse ends, 15–21 x 5–9 µm; colony slow growing

    (<40 mm/wk), colony margins crenate to irregular, mycelium moderate,

    occasionally sectored, buff to iron grey   ***B. proteae***

3. Colony growth slow (<40 mm/wk on PDA at 25°C)   4

3. Colony growth fast (>90 mm/wk on PDA at 25°C)   5

4. Conidia hyaline, fusiform, 20–30 x 4.5–6 µm; synanamorph:

    Conidia medium brown, subcylindrical, finely verruculose,

    7–14 x 2.5–3.5 µm; ascospores hyaline, guttulate, smooth, ellipsoidal,

    clavate – fusiform, frequently widest in the upper one third of the

    ascospore, tapering to obtuse ends, 15–21 x 5–9 µm; colony slow growing

    (<40 mm/wk), colony margins crenate to irregular, mycelium moderate,

    occasionally sectored, buff to iron grey   ***B. proteae***

5. Colonies producing yellow pigment in culture

    initially; conidia fusiform – ellipsoidal, base truncate or bluntly

    rounded, 14–32 x 4.5–9 µm; ascospores hyaline, guttulate, smooth, oval

    to broadly fusiform, widest in the upper one third of the

    ascospore, tapering to obtuse base and apex, 18–28.5 x 7.5–12 µm;

    colony growth rapid (>90 mm/wk), colony margins smooth,

    mycelium moderate, grey to dark grey   ***B. lutea***

5. Colonies not producing yellow pigment in culture   6

6. Conidia on average < 25 µm in length, ovoid, apex rounded,

    base tapered, 17–24 x 7–11 µm; ascospores ovoid, hyaline, widest in the

upper one third of the ascospore 17–28 x 7–12 µm; mycelium thick,

woolly, grey (14–21 d on PDA at 25°C)   ***B. ribis***

6.   *Fusicoccum* anamorph with conidia on average > 25 µm in length

irregularly fusoid, apex obtuse, base bluntly rounded 20–40 x 9–13 µm;

ascospores fusiform, widest in the middle with obtuse ends, sometimes

inequilateral, hyaline becoming light brown with age, 25–37 x 9–13 µm;

mycelium flattened in the centre, with a rim of loose aerial mycelium at the

edge of the dish (14–21 d on PDA at 25°C)   ***B. protearum***
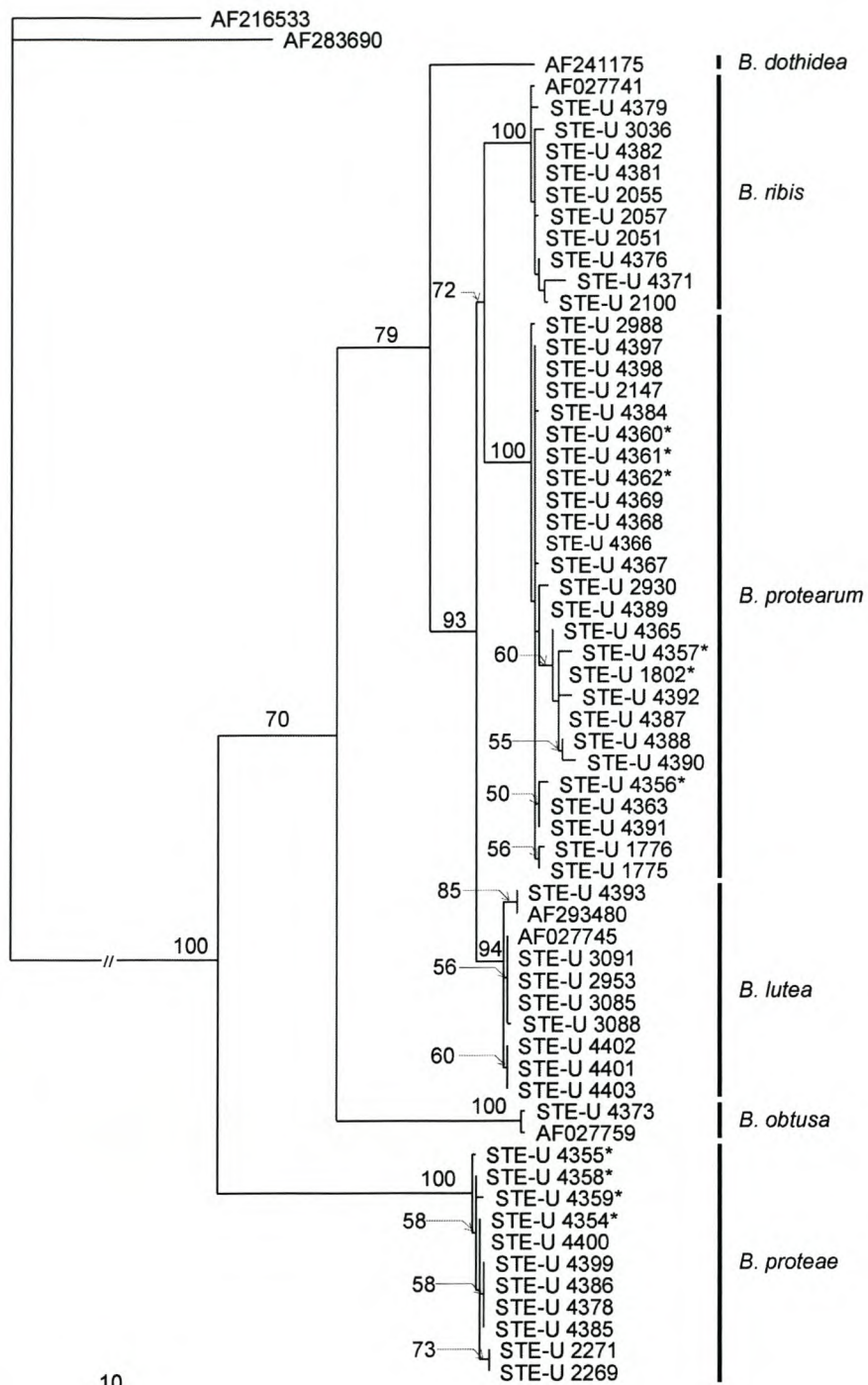
Fig. 1. The phylogram of one of the 159 most parsimonious trees derived from the alignment of ITS1 5.8S rDNA and ITS2 sequence data of *Botryosphaeria* isolates from Proteaceae. The tree is rooted to *Mycosphaerella africana* Crous & Wingf. and *Guignardia bidwellii* (Ellis) Viala & Ravaz outgroups. Branch support is given above the branches for 100 bootstrap replicates. The bar represents 10 changes.
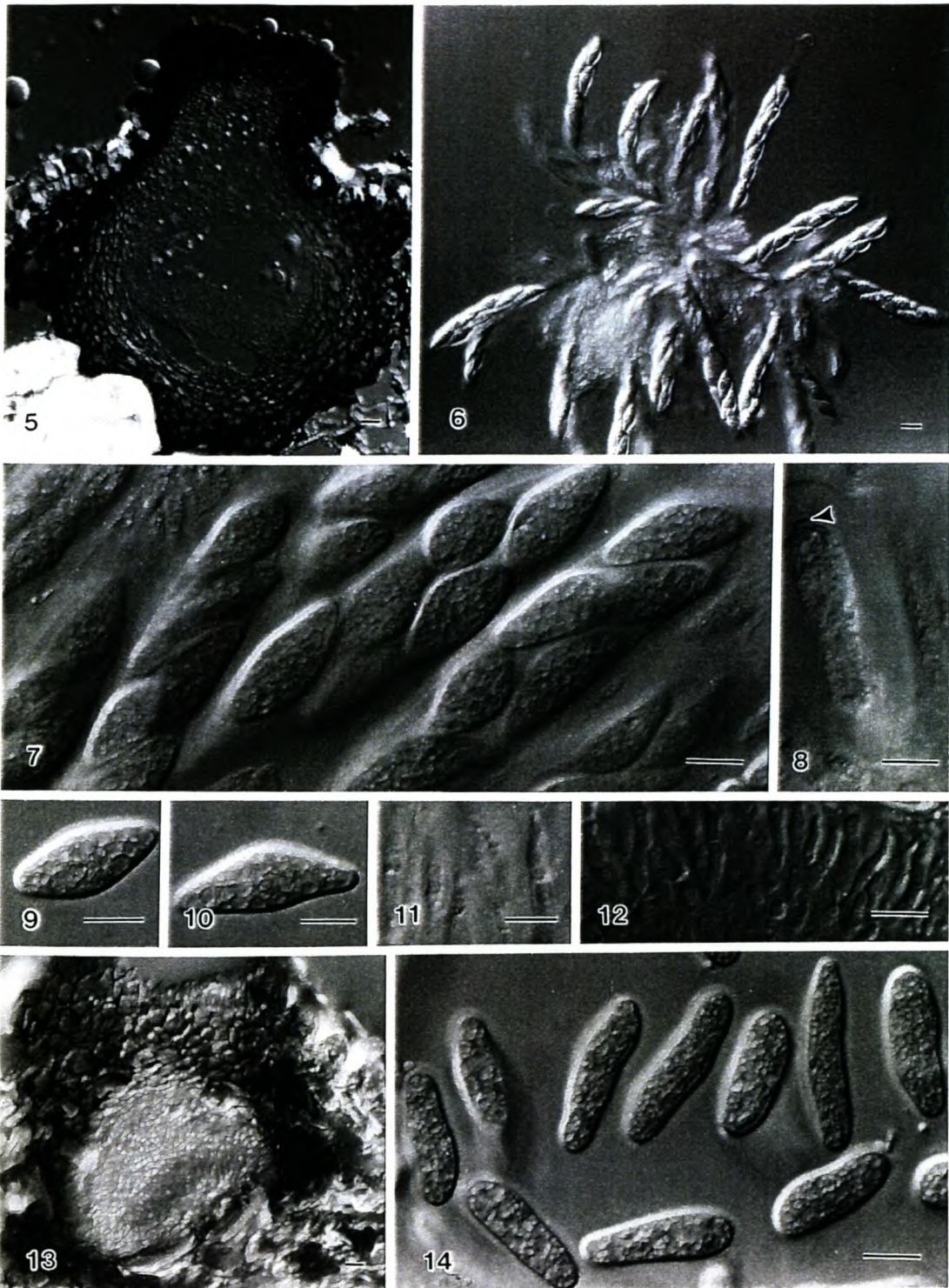
Fig. 2. Asci, ascospores and pseudoparaphyses of *Botryosphaeria protearum.*
Bar = 10 μm.
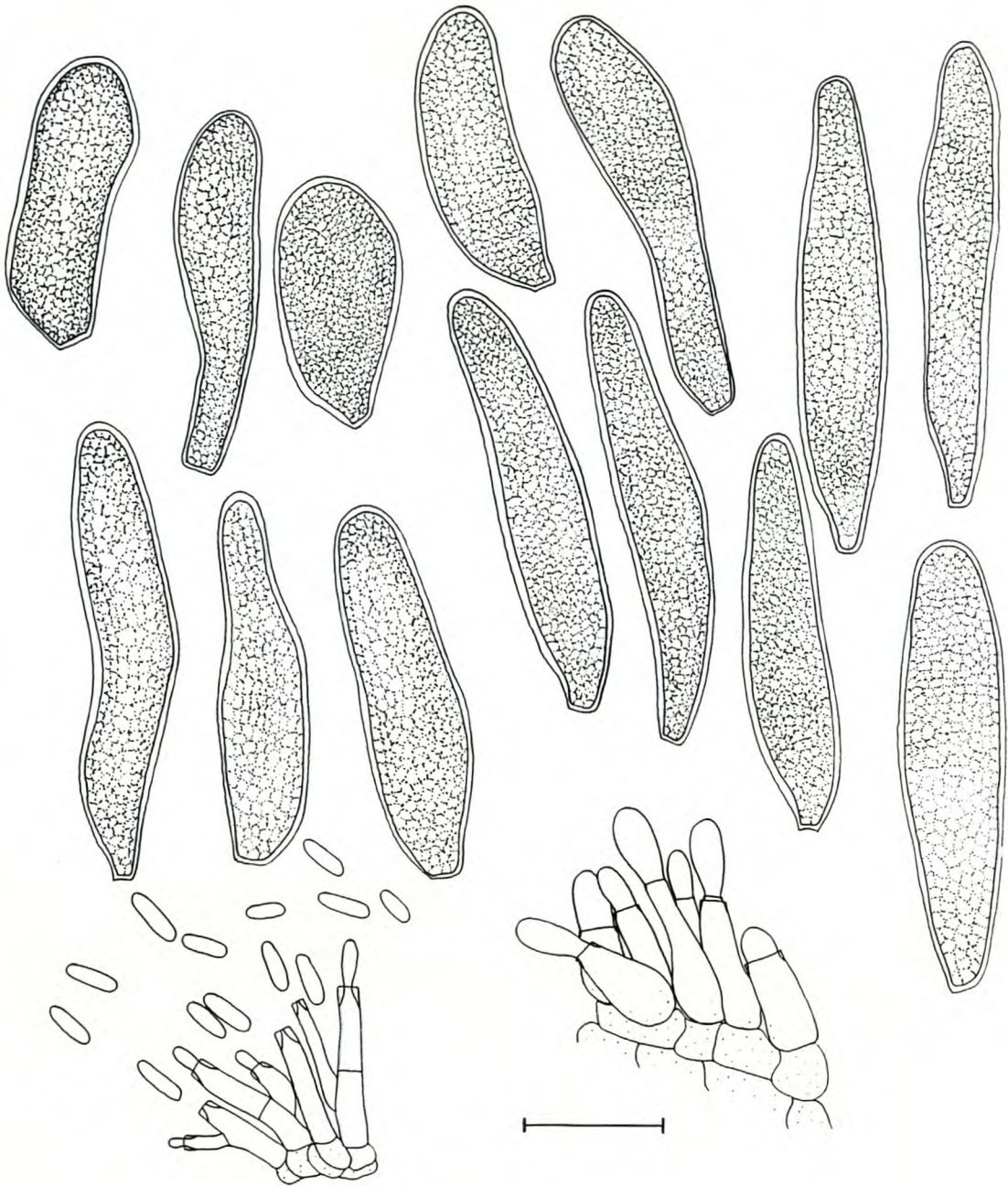
Figs. 3–4. *Fusicoccum protearum.* 3. Conidia and conidiophores. 4. Spermatia and spermatiophores. Bar = 10 μm.

Figs. 5–14. *Botryosphaeria protearum* and its anamorph *Fusicoccum protearum*. 5. Vertical section through a pseudothecium. 6, 7. Asci and ascospores. 8. Ascus with apical chamber (arrowed). 9, 10. Ascospores. 11. Pseudoparaphyses. 12. Conidiophores. 13. Vertical section through a pycnidium. 14. Conidia. Bars = 10 μm.
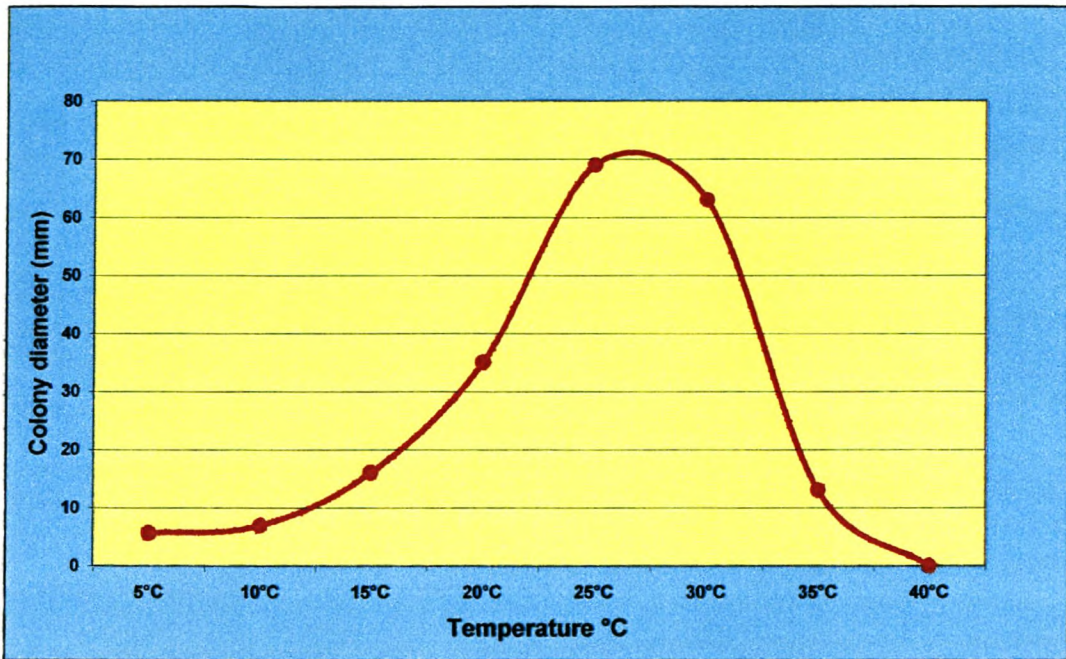
Fig. 15. Temperature growth relationship of *B. protearum.*

CHAPTER 5

Symptom development, pathogenicity and factors associated with stem cankers caused by *Botryosphaeria protearum* on *Protea magnifica* in the Western Cape Province of South Africa

Abstract

Several aspects of disease development were investigated. Disease symptoms were monitored over time and described in naturally infected plants. Furthermore, the extent of leaf and stem tissue colonisation was determined at different times during the year, corresponding with the various phenological stages of the host. To determine whether *B. protearum* was a pathogen, Koch's postulates were carried out on healthy *P. magnifica* plants growing in commercial plantations. Inoculations were done at different times during the year, again corresponding with the phenological stages of the host. Finally, the carbohydrate status of *P. magnifica* plants adjacent to the inoculated plants was monitored at the time of inoculations. Results of this study revealed that disease symptoms were first evident in the leaves. Lesions usually began forming in the mid-rib vein or at the leaf margin. The necrosis spread down the mid-rib vein of the leaf into the stem, where cankers were formed. The same phenomenon was also observed during artificial inoculations. When monitoring lesion development it was found that *B. protearum* was always isolated 20–80 mm ahead of the visible lesion in October. In January, May and July it was only sporadically isolated beyond the visible lesion, and it was not isolated ahead of the visible lesion during the March sampling date. A significant positive correlation was also observed between starch levels of leaves at the time of inoculation, and the number of lesions that ultimately developed.

Introduction

Commercial production of Proteaceae cut-flowers is severely restricted by diseases caused by *Botryosphaeria* spp. (Greenhalgh 1981; von Broembsen 1986; Forsberg 1993; Taylor 2001). Typical disease symptoms include leaf spot and necrosis (van

Wyk 1973; Taylor 2001; Chapter 2), pruning wound die-back (von Broembsen 1989; Forsberg 1993), stem cankers and die-back (Benic and Knox-Davies 1983; Benic 1986; von Broembsen 1986), and basal and tip die-back which is common in nursery mist beds (Benic 1986).

Manifestations of diseases caused by *Botryosphaeria* spp. vary according to host and locality. In Australia, wound rot and pruning wound die-back were noted as the most significant *Botryosphaeria* diseases of cut-flower proteas (Forsberg 1993; Taylor 2001). Serious losses caused by *Botryosphaeria*-associated pruning wound die-back have also been observed in Hawaii (P.W. Crous, Dept. of Plant Pathology, University of Stellenbosch, pers. comm.). In South Africa, leaf spot and necrosis, often initiated from a wound, was the most common form of disease caused by *Botryosphaeria proteae* (Wakef.) S. Denman & Crous (van Wyk 1973; Swart *et al.* 2000; Chapter 2). However, under conditions of extreme stress, this pathogen has also been associated with stem tip die-back and cankers (Swart *et al.* 2000; Taylor 2001). Of all the *Botryosphaeria* diseases of Proteaceae, stem cankers have been observed to be the most serious and economically significant form of disease.

Losses attributable to cankers caused by *Botryosphaeria protearum* S. Denman & Crous exceed 50% of a *Protea magnifica* Link. crop in some seasons in the Porterville district of the Western Cape Province (G. Jacobs, Dept. of Horticulture, University of Stellenbosch, pers. comm.), rendering this pathogen the most serious stem canker pathogen of *P. magnifica* in that area. Stem cankers caused by *Botryosphaeria* spp. were also reported to be devastating on older *Protea* L. and *Leucadendron* R. Br. plants in regions of California and Hawaii, where entire plantations have been lost as a result of this disease (Taylor 2001; Taylor *et al.* 2001a, b).

Disease development is determined by the intrinsic properties of the host as well as those of the pathogen and is influenced by environmental conditions. In general stress, temperature and the time of year (season) have been implicated as factors affecting Botryosphaeria disease development (Wiehe 1952; Schoeneweiss 1965, 1975, 1981; English *et al.* 1975; Weaver 1979; Sutton and Boyne 1983; Hwang 1983; Pusey and Bertrand 1993).

There are many different types of plant stresses and these have been discussed by Schoeneweiss (1975, 1981). Drought stress is the most important stress factor associated with diseases caused by *Botryosphaeria* spp. (Schoeneweiss 1965, 1975, 1981). Reportedly, drought stress could initiate disease, and greatly enhanced lesion

development (Shay and Sitterly 1954; Hutton and Leigh 1958; Bega *et al.* 1978; Lewis and van Arsdel 1978; Herbert and Grech 1985; Madar *et al.* 1989).

Drought stress has been reported to affect the carbohydrate status of plant tissues (Kramer 1963; Bachi and Peterson 1985) and some *Botryosphaeria* diseases have been linked to the carbohydrate status of plant tissue. Carbohydrate levels usually fluctuate seasonally and coincide with changes in host tissue physiology. However, under conditions of extreme stress these natural fluctuations might be affected by changes imposed on plants by the environment (Kramer 1963).

Carbohydrate levels in host tissues affect the development of diseases caused by *Botryosphaeria* spp. For example, Bachi and Peterson (1985) believed that the increase in canker development by *Sphaeropsis sapinea* (Fr.) Dyko & Sutton might be attributable to an increase in the carbohydrate pool available to the pathogen. Kohn and Hendrix (1983) demonstrated that white rot of apples caused by *B. dothidea* (Moug. ex Fr.) Ces. & de Not. developed only once sugar levels reached 10.5% in fruit. Contrary to these reports, Old *et al.* (1990) associated the extreme susceptibility of two *Eucalyptus* spp. to *B. ribis* (Tode ex Fr.) Gross & Dugg.with reduced starch levels caused by defoliation stress.

Disease expression and disease development also appear to be affected by season. For instance, disease was first observed in spring or summer when the host plant was actively growing and reproducing (Demaree and Wilcox 1942; English *et al.* 1975; Michailides and Ogawa 1986; Brooks and Ferrin 1994). Lesion development was more severe in spring and summer than in autumn (Pusey and Bertrand 1993), but ceased in winter on some crops such as almond trees (English *et al.* 1975), peaches (Britton and Hendrix 1986), chapparal vegetation (Brooks and Ferrin 1994) and azaleas (Schreiber 1964).

Botryosphaeria cankers are a major impediment to commercial production of *P. magnifica*, but little is known regarding the initiation and development of stem cankers on Proteaceae, or the factors that influence disease development. Although *B. protearum* has recently been isolated from Proteaceae (Chapter 4), its pathogenicity remains to be proven. A four-part study was therefore initiated to investigate aspects of disease development and to fulfil Koch's postulates.

Development of disease symptoms on *P. magnifica* plants naturally infected with *B. protearum* in commercial plantations was monitored over a two year period, and the symptoms were described. Furthermore, the extent of tissue colonisation of

naturally infected plants with leaf necrosis and stem cankers was determined five times during the year, corresponding with the different phenological stages of the host. The time of year that these growth stages were evident, were also used for inoculate healthy *P. magnifica* plants to confirm Koch's postulates. Finally, in an attempt to understand the onset of disease, the carbohydrate status of *P. magnifica* plants was monitored at each inoculation date.

Materials and methods

Symptom development on naturally infected plants. Fifteen *P. magnifica* plants were selected at random in a commercial orchard (Osdam Farm) that had a history of Botryosphaeria stem canker disease. The farm was situated at 32°56.60'S, 19°02.80'E in the Porterville district, Western Cape Province. At the onset of the experiment, the plants were tagged and any visually unhealthy tissue was removed by pruning. Symptoms of disease were monitored monthly from July 1998 until September 2000, and described.

Extent of host tissue colonisation. Five branches bearing typical symptoms were selected randomly in the orchard five times a year, namely July and October 2000, and January, March and May 2001. The sampling dates corresponded with the different phenological stages of the host. Samples were transported to the laboratory in a cooler box and kept in a cold room (5°C). Isolations were made within 24 h of sampling. Plant tissue was surface disinfested by placing it in 70% ethanol for 30 sec, 1% sodium hypochlorite (NaOCl) for 1 min, 30 sec in ethanol and rinsed in sterile water for 1 min.

The positions of necrotic parts of the leaves and branches were documented. Host tissue was excised from the margin between necrotic and apparently healthy tissue, as well as from visually healthy tissue beyond the necrotic areas on stems (up to 140 mm beyond the lesion margin). The tissue was cut out of the plant material at 20 mm intervals down the length and breadth of both the leaf and stem lesions and plated on 2% potato dextrose agar (PDA, Biolab, Midrand, South Africa). Fungi growing out of the pieces of tissue were sub-cultured onto divided plates containing PDA in one half of the dish and carnation leaf agar (Fisher *et al.* 1982) in the other.

Divided plates were incubated for 3–6 wk at 25°C under near-ultraviolet and cool white fluorescent light with a 12 h light cycle. *Botryosphaeria* cultures were subsequently identified using the key presented in Chapter 4.

Artificial inoculations. The inoculation trials were conducted in a commercial plantation of *P. magnifica* plants at Osdam Farm. The experiment began in October 1998 (experiment 1) and was repeated in October 1999 (experiment 2). Inoculations were carried out in January, March, May, July and October each year, corresponding with the different phenological stages of plant development. Six isolates of *B. protearum* (STE-U 1802, 4356, 4357, 4360, 4361 and 4526) originating from *P. magnifica* (Chapter 4) were randomly selected for inoculations, and the control plants were inoculated with PDA plugs only. Isolates were grown on PDA for 7 d prior to inoculation. For inoculations, six visually healthy plants were selected per isolate. Three plants were inoculated on the leaves of the most recent flush and the other three plants were inoculated on the previous flush. On each inoculation date, a single leaf per isolate x plant x flush combination, was inoculated. Leaf inoculations were made by placing a plug of inoculum, 5 mm in diam. on the mid-rib vein of the leaf and securing it by binding it onto the leaf with Parafilm. A polythene bag was placed over the entire leaf, and a thin brown paper packet was placed over the plastic bag to protect the leaf from sun damage. After 5 d the bags and discs were removed from the leaves. Lesion length was measured every 4 wk. The experiment was set out in a completely randomised design with three replications per isolate x flush combination. The entire experiment was repeated the following year.

Disease incidence was recorded by counting the number of inoculated plants per inoculation date on which lesions developed. Disease severity was gauged by assessing the relative patterns of lesion development. Five categories were defined (Fig. 1 a–e). In the least severe case, no lesion development took place (Fig. 1a). In the next category, a very mild form of disease occurred. A leaf lesion formed, but it did not kill the leaf (Fig. 1b). In the middle category the leaf was killed but no stem cankers were formed (Fig. 1c). In the next category, the leaf was killed and a small stem canker developed (Fig. 1d). The most severe disease occurrence resulted in a canker forming and spreading rapidly, killing the entire branch (Fig. 1e). In each experiment the outcome of the inoculations was categorised and the number of

infections in each category were counted. Readings were taken for 16 mo after each respective set of inoculations. At the end of each experiment isolations were made as described previously from the margin between healthy and necrotic tissues.

Carbohydrate readings. In each experiment, six plants were selected at random amongst the inoculated proteas, and tagged. On each inoculation date leaves and branches of the old and new flushes were collected from these six plants for carbohydrate analyses. The material was transported to the laboratory in a cooler box and stored for 24 h in a cold room. The fresh weight of leaves and stems of each flush were noted for each plant. Material was then lyophilised and the dry weight recorded. The dried leaves and stems were milled in a coffee grinder, and the pulverised material was sent to the Dept. of Horticulture, University of Stellenbosch, for carbohydrate analyses. The levels of non-reducing and reducing sugars as well as starch were measured for all samples. Carbohydrate analyses were carried out according to the methods described by Theron and Jacobs (1996). The analyses were carried out using a Sanplus Segmented Flow Analysis System (Skalar, DE Breda, The Netherlands) using the manufacturer's recommendations.

Statistical analyses. Data obtained from the field trials were subjected to statistical analyses. Chi-square tests were performed on the incidence and severity of lesions that formed on plants inoculated at different dates. Since isolates were regarded as representative of this species it was not the aim of this study to compare isolates. More than 20% of the counts were below 5, thus the data were combined for these analyses (Snedecor and Cochran 1989). The Pearson's correlation coefficient was calculated for starch levels at the time of inoculation and the number of lesions that developed in the trial period.

Results

Symptom development on naturally infected plants. Leaf necrosis that lead to stem cankers developed on only eight of the 15 plants monitored in the test period (Table 1). Two of the plants died from causes other than *Botryosphaeria*, two plants

remained asymptomatic and in three plants, leaf necrosis that did not develop into stem cankers was recorded.

Lesions were always initiated on the leaves, usually in the mid-rib vein (Fig. 2) or at the leaf margin (Fig. 3). The pathogen always migrated down the mid-rib vein of the leaf (Figs. 4, 5) killing the vein tissue in its path, which often resulted in differentially necrotic tissues in the leaf (Fig. 6). Typically, a single leaf (and occasionally two leaves) on a branch displayed symptoms (Figs. 7, 8). Symptoms of new infections usually appeared on leaves from October to January. Occasionally disease development ceased at this point, with no canker being formed on the stem. Alternatively the pathogen entered the stem and a small canker became visible (Fig. 9). If a canker did develop, it sometimes spread both upwards and downwards in the stem (Fig. 10), but more commonly in the downward direction only. Stem cankers usually became visible 8–26 wk after leaf necrosis was first noted, and cankers could become severe (Fig. 11). Disease often resulted in branch or bush death (Fig. 12). Disease seldom developed on the newest flush, but frequently developed on the flush that had formed two seasons previously. Anamorph fruiting structures of *Botryosphaeria* were observed in the same year that symptoms appeared, and occurred on most of the leaves examined in May and July (Table 2).

Extent of host tissue colonisation. *Botryosphaeria protearum* was isolated from all except one branch of the symptomatic material sampled (Table 2). It was always isolated from the margins of the lesions between diseased and healthy tissue. In October 5/5 of the samples tested yielded *B. protearum* a distance ranging from 20–80 mm ahead of the visible lesion. In January, May and July it was only sporadically isolated beyond the visible lesion, and it was not isolated ahead of the visible lesion at all in March.

Artificial inoculations. There was a low overall incidence of lesion development (29% and 22% of the inoculated material in experiments 1 and 2 respectively) (Table 3). No disease developed in the control plants, and the pathogen was re-isolated from the margin between healthy and necrotic tissue of inoculated plants. Symptoms displayed by artificially inoculated plants were similar to those shown by naturally infected plants.

With regard to the null hypothesis that there was no association between the experiments and the number of lesions that developed from inoculations carried out at different times of the year, the Chi-square test was significant ($\chi^2 = 20.2$, $P = 0.001$). In the first trial the incidence of disease was lower than expected (under the null hypothesis that there is no association) in January, March and May, but was much higher than expected in October (Table 3). In the second experiment the reverse trend occurred where disease incidence was higher than expected in January, March and May, but much lower in October.

The second null hypothesis proposed that there was no association between experiments and disease severity. Results of the Chi-square test indicated that there was a significant association ($\chi^2 = 11.52$, $P = 0.021$) between the experiments and the incidence of disease severity. In the first experiment there were significantly more small cankers formed than expected (Table 4). In the second trial the trends were reversed, and there were fewer small cankers than expected (Table 4).

Carbohydrates. In both experiments the starch levels fluctuated throughout the year. There was a significant positive correlation ($r = 0.6$, $P = 0.01$, $n = 20$) between the starch levels of the leaves at the time of inoculation and the number of lesions that developed in the test period (Fig. 13). Although the starch levels were high in July 2000 (19 mg/g dry leaf weight) there was not a corresponding increase in infections. However, if the July 2000 data were excluded from the correlation analysis, the correlation strengthened considerably ($r = 0.79$, $P < 0.001$, $n = 18$). There was no correlation between the levels of reducing sugars at the time of inoculation and the number of lesions that developed ($r = 0.0147$, $P = 0.9508$, $n = 20$). Neither was there any correlation between the non-reducing sugars at the time of inoculation and the number of lesions that developed ($r = 0.0305$, $P = 0.8982$, $n = 20$).

Discussion

Observations on naturally infected *P. magnifica* plants in the Porterville district revealed that disease symptoms usually followed a specific path beginning with leaf necrosis that frequently ended in severe stem cankers, branch die-back and death of *P. magnifica* bushes. In separate studies on Botryosphaeria twig blight and tip die-back

Luttrell *et al.* (1962) and Ramos *et al.* (1991) respectively, also noticed that lesions which led to the formation of stem cankers were initiated in leaves. The fact that the disease is initiated in the leaves makes the use of fungicides in disease control, promising.

Wounds are often cited as being the primary site of entry for *Botryosphaeria* pathogens (Wiehe 1952; Hutton and Leigh 1958; Witcher and Clayton 1963; Schreiber 1964; English *et al.* 1975; McGlohn 1982; Fraser and Davidson 1985). However, some researchers have maintained that *Botryosphaeria* spp. are mostly non-wound pathogens, able to infect healthy tissue (Horne and Palmer 1935; DeMaree and Wilcox 1942; Luttrell *et al* 1962). In this study, while monitoring symptoms associated with natural infections, no evidence of wounds being a pre-requisite for stem canker development caused by *B. protearum* on *P. magnifica* was observed. Nonetheless, on other Proteaceous hosts, insect or pruning wounds have been frequently associated with *Botryosphaeria* diseases (von Broembsen 1986, 1989; von Broembsen and van der Merwe 1990; Forsberg 1993; Chapter 2).

Disease expression in naturally infected plants was often first visible in October and continued until the end of February. Von Broembsen and van der Merwe (1990) stated that sporulation and inoculum dispersal of *B. dothidea* on *Protea grandiceps* Tratt. took place during spring and summer only, and not during winter. If this is also true for *B. protearum,* infection must have already taken place during the previous spring or summer, to allow disease expression in spring.

If disease expression takes place one or more seasons after inoculum dispersal and infection, *B. protearum* must undergo a period where it resides endophytically in the leaf until conditions are favourable for disease development. The endophytic (latent) nature of *Botryosphaeria* pathogens prior to disease initiation in other hosts, has been implied by Schoeneweiss (1965), Weaver (1974), Pennycook and Samuels (1985), Pusey *et al.* (1986) and Smith *et al.* (1996). This aspect of the relationship between *B. protearum* and *P. magnifica* merits further investigation.

Although the present studies on the extent of tissues colonisation were only conducted over one year and need to be repeated over a longer time, the results suggest that the pathogen is very active at certain times of the year (spring) and less active at others. Isolating the pathogen beyond the visible margin of the lesion emphasises the importance of pruning well below (10–15 cm) the visible lesion to ensure eradication of the pathogen during sanitation pruning.

The results of the correlation between starch levels and the number of lesions that formed in this study appear to be contrary to the findings of Old *et al.* (1990). The latter showed that reduced starch levels were accountable for the extreme susceptibility of two *Eucalyptus* spp. to infection by *B. ribis* (Old *et al.* 1990). The positive correlation between the number of lesions that developed and high leaf starch levels at inoculation obtained in the present investigation may be a false correlation, and does not necessarily indicate a role for starch in infection. Further studies on this matter might provide some interesting and useful insight into interactions between the *B. protearum* and *P. magnifica*.

This study has yielded important information regarding symptoms associated with the initiation and development of disease caused by *B. protearum* on *P. magnifica*. This knowledge should help farmers, extension officers and researchers to identify the disease in its early stages and will form a vital part of an integrated control strategy against this disease. Koch's postulates have also been satisfied thus confirming that *B. protearum* is indeed a serious leaf necrosis and stem canker pathogen of *P. magnifica*.

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Table 1. **Disease status of *P. magnifica* plants naturally infected with *B. protearum*[1]**

No symptoms of Botryosphaeria disease	Leaf symptoms only	Stem cankers	Plant dead
2	3	8	2

[1] Observations made on 15 plants observed from July 1998 to September 2000.

Table 2. **Extent of *P. magnifica* leaf and stem colonisation by *B. protearum* and presence of fruiting structures at different times of year**

Date sampled	Samples yielding *B. p*[1]	*B.p* from lesion margins[2]	*B. p* beyond lesion margins[3]	Fruitbodies[4]
July 2000	5/5	5/5	3/5	4/5
October 2000	5/5	5/5	5/5	1/5
January 2001	5/5	5/5	2/5	1/5
March 2001	5/5	5/5	0/5	0/5
May 2001	4/5	4/4	2/4	3/4

1 *B. p = Botryosphaeria protearum.*

2 Number of samples yielding *B. protearum* from the margin between necrotic and healthy tissue.

3 Number of samples yielding *B. protearum* beyond the lesion.

4 Number of samples containing *B. protearum* pycnidia.

Table 3. **Disease incidence according to inoculation date compared with expected incidence as calculated by χ^2 test**

Month of inoculation	No. of lesions experiment 1		No. of lesions experiment 2	
	Actual	Expected [1]	Actual	Expected
January	6^2	8	9	7
March	4	8	10	6
May	1	4	6	3
July	9	9	7	7
October	32	23	8	17
Total infection[3]	29%		22%	

[1] Values calculated by the χ^2 test. $\chi^2 = 20.2$, $P = 0.001$.

[2] Based on 36 inoculations per date. No lesions developed in controls therefore they were excluded.

[3] Calculation based on 180 plants inoculated per year (controls excluded).

Table 4. **Severity of disease in inoculated tissue compared with the expected incidence of disease severity as calculated by χ^2 test**

Severity	Experiment 1	Experiment 2	Expected[1]
	Actual[2]	Actual	
No lesion	128	140	134
Leaf lesion	23	24	23.5
Leaf dead	9	11	10
Small canker	16	3	9.5
Large spreading canker	4	2	3

[1] Values calculated by the χ^2 test. $\chi^2 = 10.3$, $P = 0.0354$.

[2] Counts based on 180 plants inoculated per year. No lesions developed in controls therefore they were excluded.
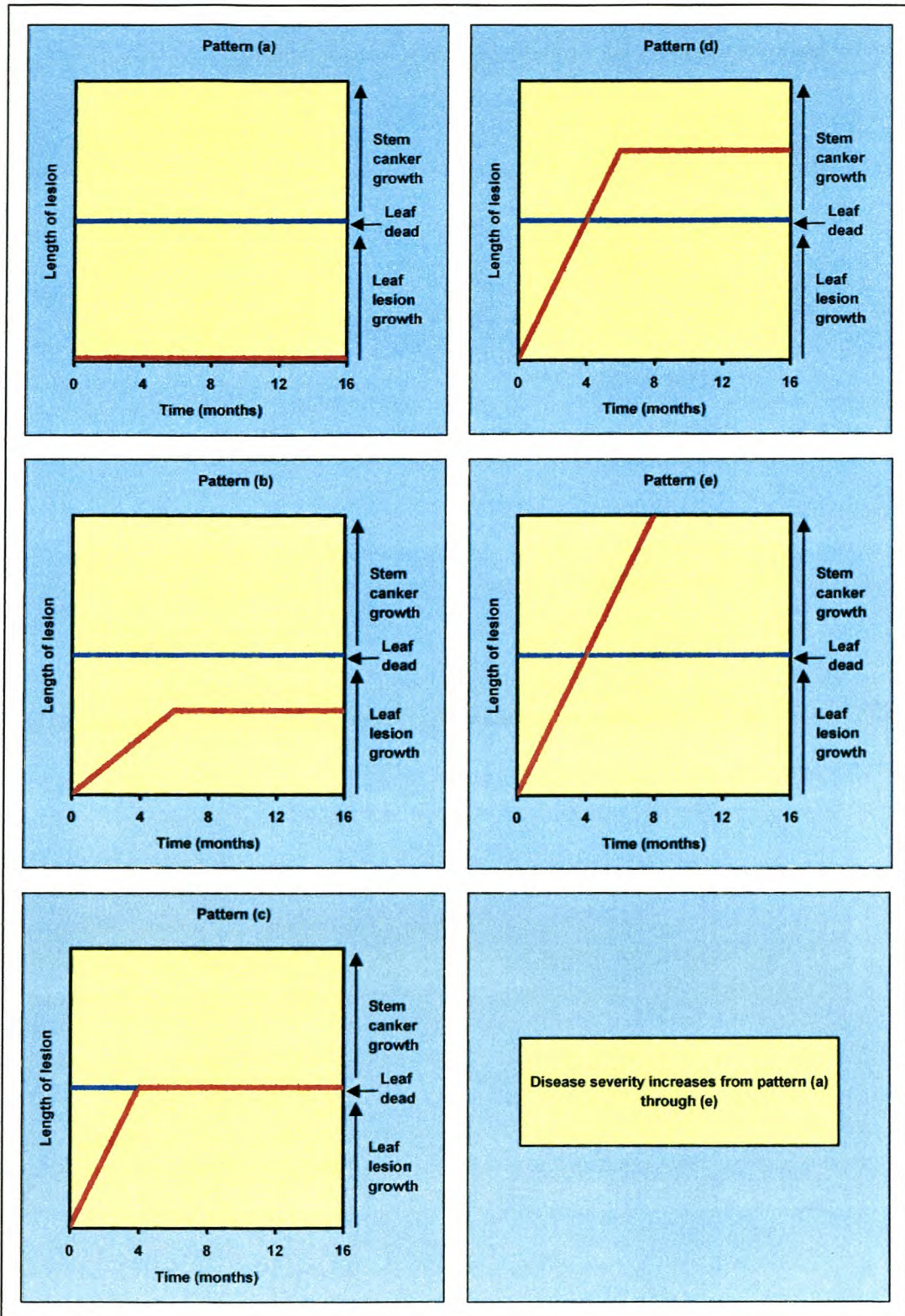
Fig. 1. Disease severity patterns on inoculated *P. magnifica* plants.

Figs. 2–5. Symptoms of disease caused by natural *B. protearum* infection on *Protea magnifica*. 2. Lesion beginning in the mid-rib vein. 3. Lesion beginning on the leaf margin. 4, 5. Lesions usually spread to stem via the mid-rib vein of the leaf.

Figs. 6–12. Stem cankers of *P. magnifica* caused by natural *B. protearum* infection. 6. Differentially necrotic leaf tissue as a result of the pathogen killing vein tissue. 7. Two leaves on a branch displaying disease symptoms. Note that necrosis was initiated from the leaf margin (right hand leaf). 8. A single leaf on a branch displaying disease symptoms. 9. Typical stem canker symptoms with a single dead leaf on a branch and a small stem canker. 10. Stem canker spreading both upwards and downwards in the stem. 11. Severe stem canker on *P. magnifica* caused by *B. protearum*. 12. *P. magnifica* branches dying as a result of stem cankers caused by *B. protearum*.
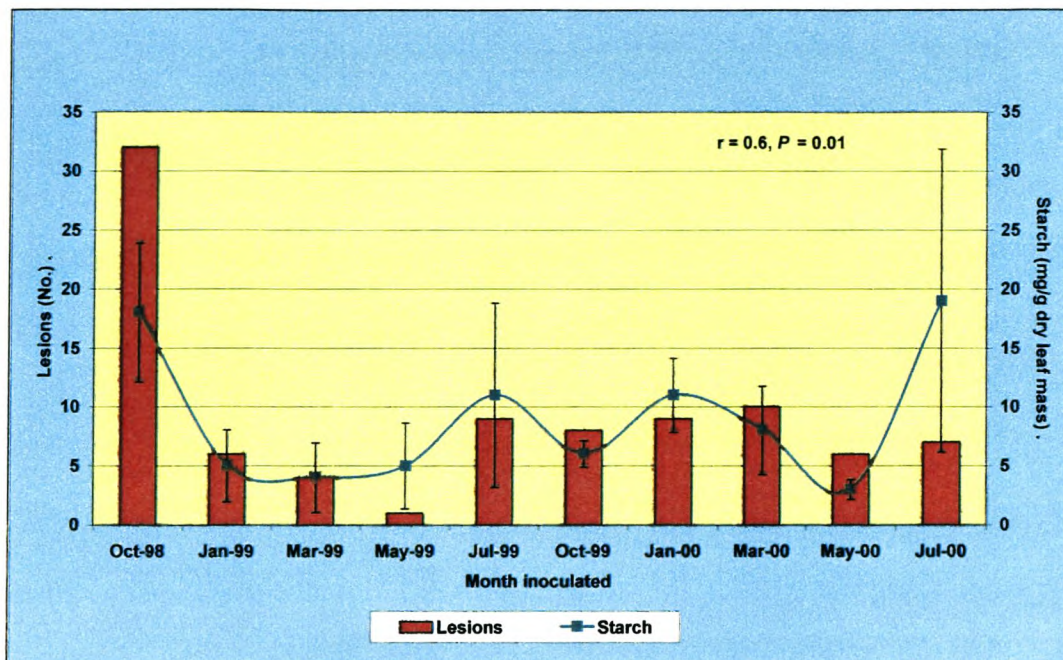
Fig 13. The number of lesions formed by *B. protearum* on artificially inoculated *P. magnifica* over a two year period compared with leaf starch levels present at the time of inoculation.

CHAPTER 6

A spatial and temporal analysis of endophytic *Botryosphaeria* spp. on leaves of *Protea magnifica* in the Western Cape Province of South Africa, and related nutrient status of leaf tissues

Abstract

Stem cankers caused by *Botryosphaeria protearum* are notably rare and in most cases absent in natural stands of *Protea magnifica,* but in cultivated plantations the incidence is high. Healthy leaves were examined to determine whether *B. protearum* occurred in both habitat types. The spatial and temporal occurrence of endophytic *Botryosphaeria* spp. in leaf tissues of *P. magnifica* from the two different situations was also investigated. Furthermore, the nutritional status of healthy leaves from plants growing in natural sites was compared with that of unblemished leaves from cultivated sites. *B. protearum* was isolated from leaves originating from both natural and cultivated stands of *P. magnifica.* Lack of disease in the natural site could therefore not be attributed to absence of the pathogen. Two species of *Botryosphaeria,* namely *B. proteae* and *B. protearum* were found to reside as endophytes in *P. magnifica* in the present study. However, *B. proteae* was not isolated from cultivated plants. Nutritional analyses indicated that there were higher levels of nitrogen in the cultivated plants in spring than in plants from the natural site. Additionally, there were much higher levels of sodium in the plants from the natural site compared with cultivated plants. These results suggest that agricultural practices could be affecting the nutritional status of cultivated plants, and that manipulation of these practices might lead to a reduction of disease.

Introduction

Botryosphaeria spp. cause leaf necrosis, stem cankers and branch die-back of Proteaceae wherever they are cultivated. These diseases can lead to plant death and result in devastating losses to the cut-flower industry (Olivier 1951; van Wyk 1973;

Greenhalgh 1981; Benic 1986; Knox-Davies *et al.* 1986; 1987; von Broembsen 1986; Serfontein and Knox-Davies 1990; von Broembsen and van der Merwe 1990; Lamont *et al.* 1995; Taylor 2001; Chapter 2). *Botryosphaeria protearum* S. Denman & Crous causes serious stem cankers on one of the most commercially important cut-flowers, *Protea magnifica* Link (Chapter 4). Effective control of this pathogen on proteas is urgently needed, and would add substantial value to this important cut-flower industry in South Africa.

Little is known about the relationship between *B. protearum* and *P. magnifica*. Recent work (Chapter 5) has revealed that lesions caused by *B. protearum* are initiated in the leaves of *P. magnifica*, from where they spread to the stem. Therefore, in this study certain aspects of the relationship between the fungus and *P. magnifica* were investigated in the leaves of the host.

Previous work on the epidemiology of diseases caused by *Botryosphaeria* spp. on hosts other than Proteaceae has suggested that these pathogens can reside in healthy, asymptomatic plant tissue as latent pathogens prior to the onset of disease (Schoeneweiss 1965; Weaver 1974; Pennycook and Samuels 1985; Pusey *et al.* 1986; Smith *et al.* 1996). However, many researchers believed that *Botryosphaeria* spp. were wound-infecting, opportunistic pathogens (Witcher and Clayton 1963; Schreiber 1964; Christ and Schoeneweiss 1975; Kohn and Hendrix 1983). This was particularly true for *Botryosphaeria* spp. on Proteaceae (Benic and Knox-Davies 1983; Knox Davies *et al.* 1986). Recently *Botryosphaeria proteae* (Wakef.) S. Denman & Crous has been shown to reside endophytically in leaves of *Protea* spp. other than *P. magnifica*, and also *Leucospermum* spp. (Swart *et al.* 2000; Taylor *et al.* 2001). This prompted the idea that *B. protearum* might also have endophytic status for a period of its life cycle.

Many definitions have been offered for the term endophyte, which are summarised by Wilson (1995). Petrini (1991) defined endophytes as "organisms inhabiting plant organs that at some stage in their life can colonise plant tissues without causing apparent harm to their host". Latent pathogens are included as endophytes by both Petrini (1991) and Wilson (1995). Essentially the definitions given by Petrini (1991) and Wilson (1995) are concordant and this concept will be followed in the present chapter.

Disease in the form of stem cankers has been observed to be lower, and in most cases absent, in natural stands of *P. magnifica*. This is in contrast to cultivated plantations where the incidence is high. The apparent difference in disease incidence could be attributable to the absence of the pathogen, different environmental effects, or farming practices that give rise to conditions that are conducive to disease development.

In this study, asymptomatic leaves were examined to determine whether *B. protearum* occurred in both habitat types. The spatial and temporal occurrence of endophytic *Botryosphaeria* spp. in leaf tissues of *P. magnifica* from the two different situations was also investigated. The nutritional status of healthy leaves from plants growing in natural sites was also compared with that of unblemished leaves from cultivated sites.

Materials and Methods

Study sites. Three sites in the Porterville district of the Western Cape Province were selected for this study. Two of the sites were commercial farms, namely Baanbreek and Osdam. The farms were situated alongside each other at 32°56.60'S, 19°02.80'E and at an altitude of 850 m above sea level. *Botryosphaeria* disease problems had been reported at both of these farms. A similar fungicide spray programme was carried out at both sites. Mancozeb (Sancozeb, 800 g a.i./kg, WP, Sanachem) was applied fortnightly throughout the year. After rain, or very high relative humidity for a 48 h period an application of prochloraz/prochloraz zinc complex (Chronos, 500 g a.i./kg, WP, UAP) was made. The Chronos spray was followed 7 d later with an application of flusilazole (Capitan, 250 g a.i./kg, EC, UAP). A single application of benomyl (Benlate, 500 g a.i./kg, WP, Du Pont) was made after the first flush had completed elongation (October – November). Plants on both farms were irrigated during summer.

The third site was a natural stand of *P. magnifica* plants in a conservation area, the Grootwinterhoek Nature Reserve (33°02.54'S, 19°09.00'E, elevation 1400 m above sea level), where stem cankers were noticeably absent. The site in the conservation area was approximately 8 km from the commercial farms.

Occurrence and distribution of *Botryosphaeria* spp. within leaves. Five healthy plants were randomly selected at each of the three sites and tagged. The plants at Baanbreek and Osdam were 3–5-years-old and those at Groot Winterhoek were estimated to be 5–7-years-old, at the onset of the study.

Branches (one from each bush) were cut five times a year, namely January, March, May, July and October, corresponding with the different phenological stages of the host. The two branches selected from each site for isolations usually had three years of growth (three flushes). A single leaf was removed for isolations from each of the flushes of these two branches from each site (Baanbreek, Osdam and Groot Winterhoek), on each sampling date. There were ten sampling dates beginning July 1998 and ending in May 2000.

To facilitate mapping the intra-leaf distribution of *Botryosphaeria* isolates grid diagrams depicting the leaves were made (Fig. 1). Prior to dissection leaves were triple disinfested by dipping in 70% ethanol for 30 sec, 3.5% sodium hypochlorite solution (NaOCl) (undiluted commercial bleach) for 10 min, 70% ethanol for 1 min, and rinsed in sterile water for 1 min. Each leaf was dissected into 5 mm squares as determined by the grid lines. Ten leaf pieces were plated per Petri dish containing 2% potato dextrose agar (PDA, Biolab, Midrand, South Africa). Plates were incubated on a laboratory bench at room temperature (approx. 22–25°C) for 3–6 wk. Fungi growing from the leaf pieces, and having the overall characteristics of *Botryosphaeria* spp., were sub-cultured onto divided plates containing PDA in one half of the dish and carnation leaf agar (Fisher *et al.* 1982) in the other. Divided plates were incubated for 3–6 wk at 25°C under near-ultraviolet light and fluorescent cool white light with a 12 h light photo-period. Isolates were identified by microscopic examination and using the key to *Botryosphaeria* spp. on Proteaceae (Chapter 4).

Occurrence of *B. protearum* within branches. To determine the number of leaves that were infected with *B. protearum* per branch, a single branch with three flushes was cut from each of six completely asymptomatic 5-year-old plants. Plants were selected randomly at one of the commercial sites, Osdam, and isolations were carried out in November (2000), January and March (2001). The branches were cut up according to the flushes, and the number of leaves per flush was recorded for each branch. Leaves were dissected into five parts (apex, base, sides and a middle region),

surface disinfested as described previously, and the parts plated separately on Petri dishes containing PDA. Plates were incubated for 2–3 wk and emerging cultures were treated as described above.

Nutrient analyses. The leaves and stems of five branches from each site were sent to commercial laboratories for carbohydrate and macro- and micro-element analyses. The carbohydrate analyses were carried out by the Deptartment of Horticulture, University of Stellenbosch (Chapter 5). The element analyses were conducted by the Analytical Services of Technology Management Laboratory, Agricultural Research Council, Infruitec-Nietvoorbij. The protocol applied by the latter laboratory was in accordance with the official methods of analysis supplied by the association of analytical chemists (Helrich 1990). Readings were made with ICP-AES, Liberty II atomic emission spectrometer (Varian Instruments PTY Ltd., 1993). Although samples were collected, data were lost for the July sampling dates at the Groot Winterhoek Nature Reserve.

Statistical analyses. The occurrence data were submitted to χ^2 tests to elucidate significant associations between occurrence and site, species, flush, position on leaf and time of year. In the absence of any significant associations, χ^2-goodness of fit tests for uniform distribution, were performed on the row and/or column totals.

The data resulting from the carbohydrate and macro- and micro-element analyses were tested for normality and log-transformed if not normal. All variables were then subjected to analysis of variance, and Student t-LSD tests ($P = 0.05$) when one or more effects were found to be significant ($P \leq 0.05$). The years and flushes were treated as blocking factors, and site and month as treatment factors. In order to compare trends between the three sites a regression function (linear or quadratic) was fitted to the data of each site and the intercepts and slopes compared by means of pair-wise Student t-tests. Data were pooled when not significantly different ($P > 0.05$) (Fig. 2).

Results

Occurrence and distribution of *Botryosphaeria* spp. within leaves. Two species of *Botryosphaeria* were found to exist endophytically in *P. magnifica* plants sampled in the study area, namely *B. proteae* and *B. protearum*. There was a low occurrence of both *Botryosphaeria* spp. at all three sites (Table 1). The occurrence of *Botryosphaeria* as an endophyte was not influenced by the month in which sampling occurred (χ^2 = 1.3, P< 0.26). Overall, only 17% (27/158) of all the dissected leaves gave rise to isolates of *Botryosphaeria* spp. (Table 1). Usually only one to three isolates were obtained from a leaf.

A significant association (χ^2 = 26.7, P < 0.001) with regard to the number of isolates was evident between site and species of *Botryosphaeria* isolated (Table 1). *Botryosphaeria proteae* was isolated only from the Groot Winterhoek Nature Reserve. However, the occurrence of *B. protearum* was similar at all three sites (χ^2 = 1.68, P = 0.917).

There was no association between the species and the position on the leaf where they resided (χ^2 = 4.56, P = 0.102). The association of the total number of isolates with position on the leaf was not significant (χ^2 = 3.9, P = 0.142). Although the null hypothesis was not rejected by the χ^2 test of goodness of fit, the data did show a systematic pattern with a tendency towards more isolates occurring in the margin pieces and fewer in the laminar pieces (Table 2). The margin data contributed 2.45 units, and the laminar data 1.25 units to the χ^2 value of 3.9.

Occurrence of *B. protearum* within branches. On Osdam farm more than one leaf on a flush could be infected with *B. protearum* (Table 3). There was a higher incidence of *B. protearum* on the older flush (1998 flush) than on the younger flushes (1999 and 2000 flushes).

Nutrient analyses. Differences in macro- and micro-elements in plant tissue from the commercial sites and the natural site were evident for boron, copper, nitrogen, phosphorous and sodium (Fig. 2). In general, the regression lines demonstrated that an almost constant concentration of the above-mentioned elements was maintained

over time at the commercial sites, with the exception of nitrogen, where there was an increase in the concentration at the end of July and in mid October (Fig. 2). A degree of cyclicity in the concentration of boron and sodium was evident in plants from the natural site, and peak concentrations were recorded in October and January, while lowest levels were reached in May (Fig. 2). Significantly higher overall levels of magnesium, phosphorous and sodium were also found in plants from the natural site compared with those from the commercial sites (Table 4).

There were no site by month interactions for any of the three carbohydrates (non-reducing sugars, reducing sugars or starch). There were significant differences between the sites with regard to starch (P = 0.01). The mean starch levels at the Baanbreek commercial site were significantly higher than those at Osdam and the Groot Winterhoek Nature Reserve (Table 5). Although this difference in the mean starch levels was present between the two commercial sites, the fluctuation of starch levels throughout the year followed the same trends described in Chapter 5.

Discussion

This study has confirmed that *Botryosphaeria* spp. reside endophytically in *P. magnifica*. *B. proteae* has previously been recorded as an endophyte of *Protea neriifolia* R. Br., *P. nitida* Mill., *P. repens* (L.) L. (Taylor *et al*. 2001), and also of *P. cynaroides* and *Leucospermum cordifolium* (Salisb. Ex. Knight) Fourc. (Swart *et al*. 2000), but this is the first record of both *B. protearum* and *B. proteae* as endophytes of *P. magnifica*.

There were site differences with regard to presence of *B. proteae*. These differences can probably be attributed to the effects of cultural practices. The plants at the two commercial farms are sprayed with fungicides and this is probably the reason why *B. proteae* was not detected at these sites.

The presence of *B. protearum* at all three localities was in contrast to the restricted distribution of *B. proteae*. However, the incidence of *B. protearum* was relatively low at all three sites. The low occurrence of the *Botryosphaeria* spp. might be attributable to low availability of inoculum. In the nature reserve, only a small, relatively dispersed population of *P. magnifica* is present, which suggests that inoculum levels are low due to the restricted size of the host population. At the

commercial sites, although the plant populations are large the use of fungicides and sanitation pruning keep levels of inoculum down.

Although not statistically significant, isolates of both *B. proteae* and *B. protearum* tended to reside in tissues near the leaf margin and the areas surrounding the mid-rib vein. Leaf lesions that lead to the formation of stem cankers most often begin near the leaf tip, margin or in the mid-rib vein (Chapter 5). Furthermore, it was found that up to eight leaves of the older flushes per branch could be infected with *B. protearum* as an endophyte. Typically, *B. protearum* stem cankers are first noticed in the field by death of a single leaf on a flush, and a stem canker developing below the dead leaf (Chapter 5). Occasionally, two leaves on a flush become symptomatic simultaneously (Chapter 5). However, in this study the pathogen was isolated as an endophyte from more than one leaf of the older flushes of some branches. This raises interesting questions as to why symptoms develop in this manner.

The fact that the occurrence of *B. protearum* was similar for all three sites eliminated the possibility that the lack of disease in the nature reserve might be attributed to the absence of the pathogen. Therefore the status of the host and environment must be considered.

Development of disease is due to the successful interaction of the host, the pathogen and the environment. Thus, an important component of plant diseases is the effect that the environment has on disease development. Although the natural stand of *P. magnifica* was only a few km away from the commercial sites, it was situated at a much higher altitude (1440 m) than the farms (850 m), and was positioned above the snow line. Thus, temperatures will be slightly lower at the natural site all year round. The negative effects of low temperatures on growth of various *Botryosphaeria* spp. have been reported (Kohn and Hendrix 1983; Britton and Hendrix 1986; Brooks and Ferrin 1994). Thus lower temperatures may be one of the factors affecting disease occurrence at the two different habitats.

Differences between cultivated *P. magnifica* plants and those growing in the wild emerged from the nutrient composition analyses. Cultivation practices clearly had an effect on the macro- and micro-element composition of the plants. For example the dramatic increase in nitrogen levels at the end of July and in mid October on the commercial sites can be attributed to fertilisation. Experiments now need to be

carried out to test the effect that fertilisation with nitrogen has on canker development of plants.

Large differences in the leaf sodium levels were evident. The sodium content of leaves of cultivated plants was much lower than those of plants from the natural site, except at the May sampling date. It has been shown that high concentrations of sodium are unfavourable for growth of some *Botryosphaeria* spp. Wene (1979) amended culture media either with KCl or NaCl to determine the effect of water potential on growth of *B. dothidea*. Mycelial growth of this fungus was negatively impacted at a much lower concentration of NaCl than KCl, indicating that the pathogen was sensitive to sodium. Brown (1957) also found that sodium in the form $NaNO_2$ inhibited mycelium growth of *B. ribis* (Tode ex Fr.) Gross & Dugg. Both of these examples support the view that sodium might have an inhibitory effect on disease development. Further studies need to be conducted to explore this phenomenon.

From these results it appears that agricultural practices affected the nutritional status of the plants at the commercial sites. Differences in the nutritional status of commercially cultivated plants may affect disease development, and this needs to be investigated further. If disease development is affected by cultivation practices, these practices can be manipulated to minimise their impact on the nutritional status of plant tissues, and thereby reduce disease.

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Table 1. Endophytic occurrence of *Botryosphaeria* spp. in *P. magnifica* leaves obtained from different sites in Porterville

Site	No. of leaves		No. of isolates	
	Dissected	Yielding *Bot*[1] spp.	*Bpm*[2]	*Bp*[3]
Baanbreek Farm[4]	48	7	15	0
Osdam Farm[4]	54	8	18	0
Grootwinterhoek Nature Reserve[5]	56	12	11	16
Total	**158**	**27**	**44**	**16**

[1] *Bot = Botryosphaeria* spp.

[2] *Bpm = Botryosphaeria protearum.*

[3] *Bp = Botryosphaeria proteae.*

[4] Commercial sites.

[5] Natural site.

Table 2. Occurrence and position of *Botryosphaeria* isolates in asymptomatic *P. magnifica* leaves obtained from different study sites in Porterville (May 1998 – 2000)

Species	No. of isolates per plant part[1]		
	Margin	Midrib	Laminar tissue
B. protearum	17	13	14
B. proteae	10	5	1
Total	**27**	**18**	**15**

[1] Occurrence is expressed as the number of isolates that were obtained from 158 leaves that were dissected.

Table 3. Incidence[1] of endophytic *Botryosphaeria protearum* on *P. magnifica* leaves of different flushes of branches at Osdam Farm

1998 flush	1999 flush	2000 flush
0–8	0	0–1

[1]Incidence is expressed as the number of infected leaves per flush per branch out of 18 branches.

Table 4. Overall mean concentration of macro- and micro-elements in leaves from different sites in Porterville (May 1998 – 2000)

Site	Concentration of elements [1]		
	Mg (%)	P(%)	Na (mg/kg)
Baanbreek[2]	0.109 b	0.027 c	1516.4 b
Osdam[2]	0.109 b	0.037 b	1284.3 c
Groot Winterhoek[3]	0.156 a	0.044 a	2034.8 a

[1] Concentration expressed as the mean of 40 readings.

[2] Natural site.

[3] Numbers followed by different letters in each column differ significantly ($P \leq 0.05$).

Table 5. Mean concentration of starch in leaves from different sites in Porterville (May 1998 – 2000)

Site	Concentration of starch (mg/g dry mass)[1]
Baanbreek[2]	10.01 b[4]
Osdam[2]	5.489 a
Groot Winterhoek[3]	6.293 a

[1] Concentration expressed as the mean of 54 readings.

[2] Commercial sites.

[3] Natural site.

[4] Numbers followed by the same letter do not differ significantly ($P = 0.01$).

Table 6. Starch levels in leaves sampled at different dates in Porterville (May 1998 – 2000)

Sampling date	Concentration of starch (mg/g dry mass) [1]
July	10.80 **a**
October	9.97 **a**
January	6.55 **ab**
March	6.23 **ab**
May	4.85 **b**

[1] Concentration expressed as the mean of 36 readings per sampling date.

[2] Numbers followed by the same letter do not differ significantly ($P < 0.05$).

Fig. 1. Diagram of a *P. magnifica* leaf mounted on the right hand side of a page with cell references on which to indicate the location of endophytic *Botryosphaeria* spp., and the intact leaf to be dissected on the left hand side.
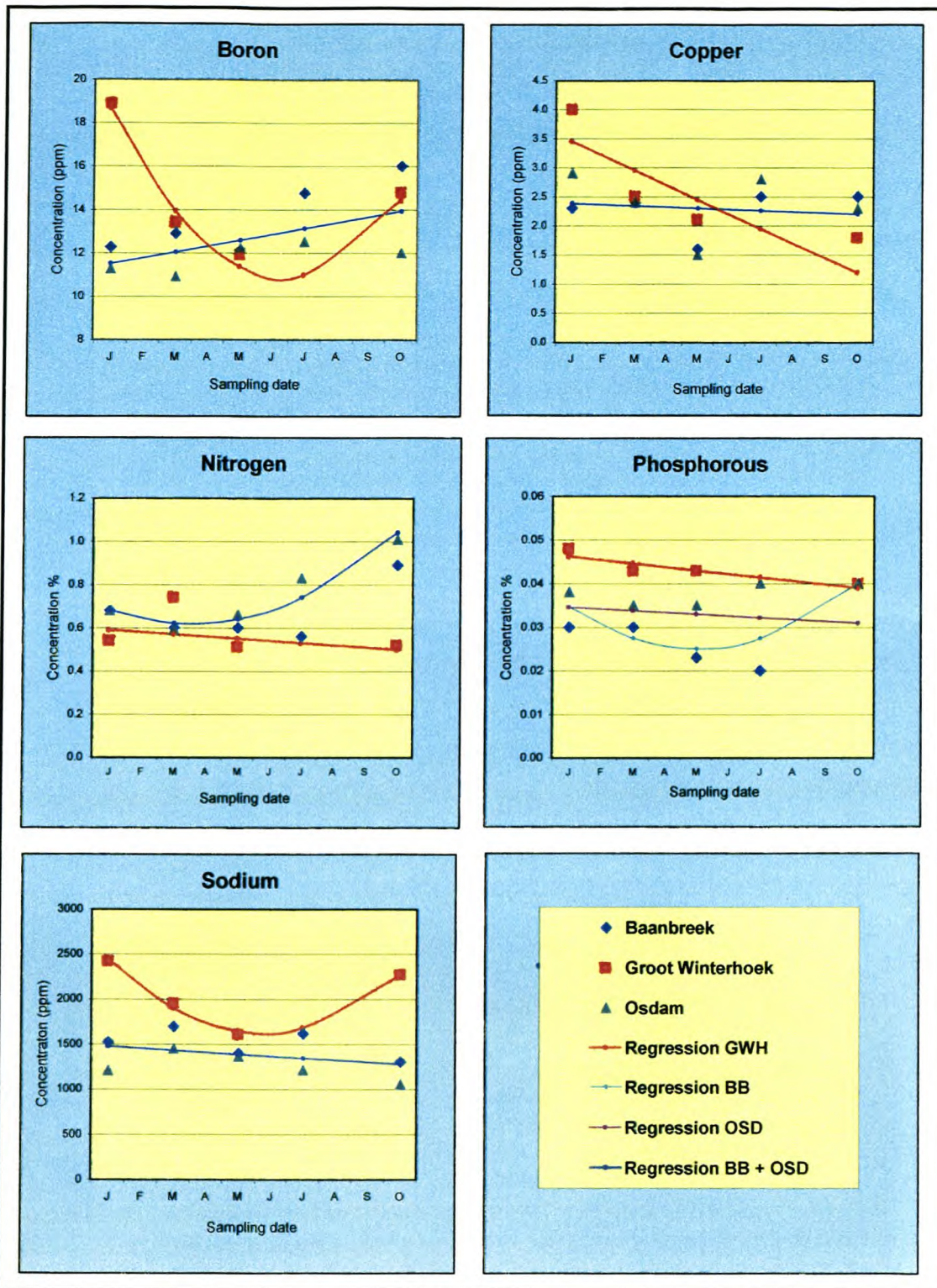
Fig. 2. Mean concentration of different macro- and micro-elements in *P. magnifica* leaves sampled at various dates between May 1998–2000 in Porterville.

CHAPTER 7

Evaluation of fungicides for the control of *Botryosphaeria protearum* on *Protea magnifica* in the Western Cape Province of South Africa

Abstract

A range of fungicides was tested *in vitro* for their effect on mycelial inhibition. Selected products showing potential for disease control were then further tested under field conditions. The most effective fungicides in the *in vitro* tests were tebuconazole, benomyl, prochloraz mc, iprodione and fenarimol. In field trials a 25–85% reduction in the occurrence of stem cankers caused by *Botryosphaeria protearum* was achieved if fungicides were applied or sanitation pruning was implemented. The best control was obtained with prophylactic treatments of benomyl, bitertanol, prochloraz mc / mancozeb or tebuconazole. Sanitation pruning reduced the occurrence of cankers by 30% compared with the controls.

Introduction

Proteaceae cut-flowers are an important agricultural crop in South Africa comprising 70% of the entire national cut-flower industry. Most of the produce is exported (80%), particularly to the Netherlands and Germany, but also to other European markets and to the U.S.A. (Wessels *et al*. 1997; Middelmann 2000). The value of these flowers lies in their aesthetic beauty and it is essential to produce perfect blooms with unblemished leaves and stems.

Production of one of the most important commercial proteas, the queen protea (*Protea magnifica* Link) is severely restricted by leaf necrosis and stem cankers caused by *Botryosphaeria* spp., with *B. protearum* S. Denman & Crous being the major stem canker pathogen in the Porterville district of the Western Cape Province (Chapter 5). Disease devalues the appearance of cut-flower stems and leaves and also causes losses through branch die-back and ultimate death of bushes. Furthermore, since these proteas are mostly exported they are subject to phytosanitary inspections and the presence of infected material places entire consignments at risk of being

rejected. It is therefore imperative that disease caused by these pathogens is controlled.

Integrated control using a variety of strategies is generally advocated to reduce the impact of *Botryosphaeria* infection on *Protea* spp. (von Broembsen and van der Merwe 1990; Forsberg 1993). The judicious use of fungicides is an important component of this integrated strategy (von Broembsen and van der Merwe 1990). Lesions caused by *B. protearum* that lead to the development of stem cankers on *P. magnifica* are initiated through leaf infection (Chapter 5). Thus, chemicals applied to foliage could reduce disease by curbing the pathogen in leaves before cankers develop. Fungicides previously recommended against *Botryosphaeria* on Proteaceae in South Africa included sprays with captab, captafol and mancozeb (Benic and Knox Davies 1983), and monthly applications of benomyl and captab (von Broembsen and van der Merwe 1990). In Australia, Mc Lennan (1993) suggested spraying with either benomyl or iprodione. None of the above mentioned chemicals are registered on Proteaceae in South Africa, although benomyl, iprodione and mancozeb are registered on other ornamentals (Nel *et al.* 1999).

Fungicides can be applied as soil drenches. Schoeneweiss (1979) showed effective control of *B. dothidea* (Moug. ex Fr.) Ces. & de Not. on red-osier dogwood (*Cornus sericea* L.), where benomyl was applied as a soil drench. This was because the chemical was taken up by roots and transported to the stems and leaves. Although this might be an attractive option, soil drenches, as a general practice in production of queen proteas, would be uneconomical. Moreover, certain chemicals such as benomyl are considered environmentally unfriendly, especially when applied to the soil (Fry 1982).

Chemical control of diseases caused by *B. dothidea* on apples (*Malus* Mill.), cranberries, apricots (*Prunus* L.), peaches (*Prunus persica* [L.] Batsch) and pistatio (*Pistacia* L.) has been reported as effective (Starkey and Hendrix 1980; Parker and Sutton 1993; Li *et al.* 1995; Ma *et al.* 2001). This evidence implied that opportunities exist for using chemicals to reduce the impact of stem cankers on *P. magnifica*. However, von Broembsen (1989) maintained that Proteaceae are very sensitive to agricultural chemicals. She suggested that the phytotoxic effects of fungicides applied under field conditions need to be assessed, before registration can be recommended.

Very little work has been conducted in South Africa on the control of *Botryosphaeria* on Proteaceae. In addition, subsequent to the last studies on chemical control of *Botryosphaeria* on this host (von Broembsen and van der Merwe 1990) new and promising fungicides have become available for this purpose. The aims of this study were thus to test the efficacy of a range of fungicides on *in vitro* mycelial inhibition of *B. protearum*. Selected products showing potential for disease reduction were further tested under field conditions. Phytotoxic responses to the chemicals were also monitored and evaluated.

Materials and methods

Selection of fungicides for *in vitro* tests. Both contact and systemic fungicides were selected for *in vitro* screening tests. This is because most fungicide application programmes that prevent pathogen populations from developing fungicide resistance combine and / or alternate the two types. Mancozeb (Sancozeb, 800 g a.i./kg, WP, Sanachem) a dithiocarbamate contact fungicide that is currently used as a general fungicide in the protea industry and is registered for use on ornamentals in South Africa was included. A representative from the dicarboximide group of fungicides, iprodione (Rovral Flo, 255 g a.i./L, SC, Rhône Poulenc) was also selected. Other contact fungicides such as chlorothalonil, (Bravo, 500 g a.i./L, SC, Efekto) which is registered in South Africa for use on ornamentals but not used by protea growers, quintozene (pentachloronitrobenzene (PCNB), 750 g a.i./kg, WP, Plaaskem) and thiram (Thiram, 750 g a.i./kg, WP, Sanachem) were also tested.

The systemic fungicides selected for *in vitro* testing included: prochloraz manganese chloride (prochloraz mc) (Octave, 500 g a.i./kg, WP, AgrEvo) which is an imidazole fungicide that is commonly used by industry and registered on ornamentals; tebuconazole (Folicur, 250 g a.i./L, EW, Bayer) a triazole fungicide not registered for use either on Proteaceae or ornamentals in South Africa; fenarimol (Rubigan, 120 g a.i./L, EC, Dow AgroSciences) a pyrimidine fungicide registered for use on roses; benomyl (Benlate, 500 g a.i./kg, WP, Du Pont) a benzimidazole fungicide registered on ornamentals in South Africa; kresoxim-methyl (Stroby, 500 g a.i./kg, WG, BASF) one of the new strobilurine fungicides (not registered for use on proteas or ornamentals) and fosetyl-Al (Aliette, 800 g a.i./kg, WP, Rhône Poulenc) which

induces host defence mechanism(s) and is registered for use on Proteaceae in South Africa.

***In vitro* tests on mycelial inhibition.** The fungicides were dissolved in sterile water and added to molten 2% potato dextrose agar (PDA) (Biolab, Midrand, South Africa) at a range of concentrations from 0–100 μg a.i./mL. Benomyl was first dissolved in chloroform (Schoeneweiss 1979) due to its insolubility in water. For the controls, PDA without the addition of chemicals was used. All the chemicals were tested at the following concentrations 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 μg a.i./mL. Additionally, chlorothalonil, mancozeb and thiram were tested at 10, 25 and 100 μg a.i./mL (Li *et al.* 1995).

Four isolates of *B. protearum* were used for each chemical tested (STE-U 1799, 1800, 1801, 1802). The isolates originated from stem cankers on *P. magnifica* and are maintained in the Deptartment of Plant Pathology culture collection (STE-U), at the University of Stellenbosch.

Fungicide-amended medium was poured into Petri dishes (90 mm diam.) to a depth of approximately 20 mm. Each plate was inoculated with a 5 mm diam. mycelial disc cut from the actively growing margins of *B. protearum* on PDA. Mycelial growth was recorded by marking the periphery of the fungal colonies along two perpendicular lines on the back of the Petri dishes after 4 d incubation at 22°C in the dark. Three replicate plates per isolate-fungicide-concentration combination were tested in the experiment and the entire experiment was repeated once.

The mean colony diameters for each isolate and fungicide-concentration were calculated. Percentage inhibition was then calculated by subtracting mean colony diameters from those of the controls. Percentage inhibition data were plotted against the chemical concentrations tested. EC_{50} values were computed from straight lines rather than from the more complicated functions of the original response curves, because it was easier and this method should be seen as no more than a convenient mathematical device to obtain the best possible estimates of the required parameters (Finney, 1952). Transformations suggested for quantitative response curves include the log-, inverse- (or reciprocal), square-root and square transformation (Armitage, 1971). All EC_{50} values were calculated on the transformed scale and converted back to the original scale to enable comparison of the fungicides on the same scale. Due to

a lack of normality the EC_{50} values were log transformed and subjected to an analysis of variance (ANOVA) and subsequent pair-wise Student's t-tests.

Field trials. Two hundred and twenty five *P. magnifica* plants (five rows each containing 45 plants) were selected in a commercial orchard on Osdam Farm, which had a history of Botryosphaeria stem canker disease. The farm was situated at 32°56.60'S, 19°02.80'E in the Porterville district of the Western Cape Province. At the onset of the experiment, the plants were tagged and any visually unhealthy tissue was removed by pruning.

Four of the fungicides that gave the best inhibition of *B. protearum* mycelial growth *in vitro* were selected for field trials. These were benomyl, fenarimol, iprodione and tebuconazole. In addition, two commercially available mixtures of fungicides that were tested *in* vitro were included in the trial. The one mixture comprised systemic fungicides and the other mixture comprised contact fungicides. The mixture of systemic fungicides was Toreador (a combination of carbendazim (133 g a.i./L) and tebuconazole (167 g a.i./L), SC, Bayer). The mixture of contact fungicides was Dirac Express (a combination of iprodione (78 g a.i./kg) and thiram (532 g a.i./kg), WG, Rhône Poulenc). The mixtures were applied alternately to simulate an anti-fungicide-resistance strategy, and were designated t/d. Bitertanol (Baycor, 300 g a.i./L, EC, Bayer) was also used even though it had not been included in the *in vitro* tests. There were thirty plants per treatment for the above treatments.

Another two fungicides that were tested *in vitro* and were included in the field trial were the contact fungicide mancozeb, and the systemic fungicide prochloraz mc. The protea growers apply these two chemcials as a standard practice, by alternating them. Therefore this treatment was included in the trial to evaluate the performance of other treatments relative to the currently employed practice. There were fifteen plants that received this treatment. Thus, in total, there were seven chemical treatments applied in the field trials.

The rates of fungicide application were followed according to Nel *et al.* (1999) for ornamental plants, and are as follows: benomyl (0.5 g a.i./L); bitertanol (0.24 g a.i./L); fenarimol (0.042 g a.i./L); iprodione (0.51 g a.i./L); mancozeb (1.6 g a.i./L); prochloraz mc (0.75 g a.i./L) and tebuconazole (0.375 g a.i./L). Approximately 0.33 L per plant was dispensed on each application date. Fungicides were applied every 14 d at bud break (August until November) (eight applications per year during these

months) after which they were applied at monthly intervals (eight applications for the rest of the year). Chemical applications began in August 1998 and ended in July 2000.

Apart from the chemical treatments, a non-fungicide treatment was included. This was pruning stem cankers once every month. The treatment was included because sanitation pruning is an essential component of integrated control of Botryosphaeria stem canker. It was necessary to determine whether by employing sanitation pruning only as a control measure high quality blooms could be produced. This treatment was referred to as the "cut only" treatment. Fifteen plants received the "cut only" treatment.

In the controls neither pruning nor fungicides were applied. Control plants were sprayed with water on each fungicide application date. Fifteen plants served as controls.

The experiment was laid out as a randomised block design. There were nine treatments and 10 replicates (except for the control, the cut only treatment, and the standard treatment of alternating prochloraz mc with mancozeb, where there were only five replicates each). Three plants made up an experimental unit in each replicate. The experiment was conducted once.

At the onset of the experiment the 1996 and 1997 flushes were present as main branches on which canker formation could be noted (refer to Chapter 5 for a description of a "flush"). Within the first two months of initiation of the experiment the 1998 flush began to emerge and by the end of the experiment the 2000 flush was present as a very young developing flush.

Plants were inspected every month and notes and sketches were made regarding disease development on every plant. The flush and position of cankers on each plant were recorded. Once cankers had clearly developed, they were cut out (except on the control plants) and brought back to the laboratory where isolations were made as stated previously (Chapter 5).

In each treatment the total number of cankers yielding isolates of *Botryosphaeria* was recorded. Plant mortality was determined at the end of the experiment. The number of cankers was expressed as a percentage of the total number of surviving plants in each treatment. The percentage data were submitted to

χ^2 tests to elucidate significant associations between fungicide and annual growth (flush).

Results

In vitro trials. EC_{50} values could not be calculated for fosetyl-Al and kresoxim-methyl because both these fungicides were ineffective in inhibiting the mycelial growth at the concentrations tested. Data for these two treatments were therefore not included in the analysis of variance.

The analysis of variance revealed similar results ($P = 0.1427$) for the two *in vitro* experiments, and the data were thus pooled (Table 1). The two main effects analysed were fungicide treatments and isolates. There was no significant isolate x treatment interaction ($P = 0.0741$), so the main effects (isolates and treatments) could be interpreted. There were significant differences among the isolates ($P = 0.0063$) and among the treatments ($P < 0.001$) (Table 1).

The effects of the fungicide treatments enabled grouping of these chemicals based on the efficacy with which they inhibited mycelial growth. Three clusters were formed indicating the efficacy of the different treatments. The most effective fungicides inhibited growth at low EC_{50} values and included tebuconazole, benomyl, prochloraz mc, iprodione and fenarimol. A single fungicide, quintozene, fell into the moderately effective group where 50% inhibition occurred at relatively high concentrations. Most of the contact fungicides (chlorothalonil, mancozeb and thiram) formed a group where inhibition only occurred at very high concentrations (Table 2). With the exception of iprodione, the fungicides in the first group were all systemic. The two most effective fungicides were tebuconazole and benomyl with EC_{50} values of 0.38 and 0.69 μL a.i./mL respectively (Table 2). Fenarimol had a significantly higher EC_{50} value than tebuconazole and benomyl, but still fell in the most effective group.

Mancozeb was the least effective fungicide *in vitro* and high concentrations were required to inhibit mycelial growth (18.72 μL a.i./mL). The other contact fungicides also required high concentrations to inhibit mycelial growth (13.46 and 15.79 μL a.i./mL for chlorothalonil and thiram respectively).

In spite of differences in growth rates amongst the isolates, the lack of treatment x isolate interaction indicated that all four isolates showed a similar reaction to the different fungicides. Therefore the fungicide effects apply to the pathogen as a whole. However, isolate STE-U 1799 appeared to be more sensitive to the fungicides than the other three isolates tested (Table 3), which indicates slight variation between individuals.

Field trials. There was significant association between the treatments and the annual growth (flushes) ($\chi^2 = 73.37$, $P < 0.001$). In general a higher percentage of cankers caused by *B. protearum* was recorded on the older growth (1996 and 1997 flushes) than on the new growth (1998–2000), except in the control treatment where an equal number of cankers were formed on both flushes (Fig. 1). In the older flushes, there was also a higher percentage of cankers than expected as calculated by the Chi-square test, for the benomyl, bitertanol, fenarimol, tebuconazole and prochloraz mc alternated with mancozeb treatments. In contrast, there was a lower percentage of cankers formed than expected in the control, t/d and cut treatments. The reverse trend occurred in the younger flushes (1998 – 2000).

Decreases ranging from 25 – 85% in the occurrence of stem cankers were recorded if fungicides or sanitation pruning was applied. The best control was achieved if benomyl, bitertanol, fenarimol, iprodione, tebuconazole and prochloraz mc alternated with mancozeb treatments were used prophylactically (Fig. 1). None of the fungicides elicited any phytotoxic response under the field conditions of the experiment.

Discussion

The results of this study have demonstrated that fungicides are very effective in reducing the impact of *B. protearum* on *P. magnifica* in the field. The most effective fungicides were bitertanol, tebuconazole and benomyl as well as the alternation of prochloraz mc with mancozeb. Bitertanol, tebuconazole and prochloraz are systemic, demethylation inhibiting fungicides more commonly know as sterol biosynthesis inhibitors (SBI's). These fungicides are able to penetrate the plant cuticle and are thus easily taken up by plant tissues (Kuck *et al.* 1995). Both bitertanol and prochloraz

have excellent activity against ascomycetous fungi, but have limited translaminar mobility (Kuck *et al.* 1995), and therefore good coverage of leaf surfaces is essential for effective control. Tebuconazole has excellent broad-spectrum fungicidal properties and exhibits acropetal distribution throughout leaf tissue (Kuck *et al.* 1995). This chemical should therefore provide protection throughout the leaf even if fungicide leaf coverage is not good. Benomyl is a benzimidazole fungicide with excellent broad-spectrum activity. This fungicide binds tightly to plant surfaces and degrades very slowly, thereby offering excellent protectant activity (Delp 1995). A significant reduction in the occurrence of cankers was also achieved with the contact fungicide iprodione.

Results presented here showed that the curative effect of benomyl, bitertanol, fenarimol, tebuconazole and as well as the alternation of prochloraz mc with mancozeb was not as good as the prophylactic effects, evident by the higher than expected occurrence of cankers in the older flushes. Because *B. protearum* has an endophytic status for a period in its life cycle, it is probable that some of the leaves of the 1996 and 1997 flushes were already infected with the pathogen, which then resided endophytically in the leaves prior to the onset of the experiment. Therefore, if disease developed in the 1996 and 1997 flushes, it was considered that the pathogen had been in the leaves before the trial began, and the fungicides had been unable to eradicate *Botryosphaeria* once infection had taken place. Other researchers working on the control of *B. dothidea* white rot of apples, reported poor curative ability of tebuconazole (Parker and Sutton 1993), which is consistent with the results obtained in the present study.

However, a very effective reduction in the percentage of cankers was obtained if the fungicides were applied preventatively. This was demonstrated by the large reduction in the formation of cankers on the 1998–2000 flushes relative to the controls and to the 1996–1997 flushes, and for the lower than expected occurrence in some of the treatments. At the onset of the trial the 1998 flush had not yet developed therefore this flush, as well as subsequent flushes, were protected by the fungicides. The decrease in disease incidence can therefore be attributed to the protectant effects of the chemicals.

Results presented here have confirmed the importance of using fungicides as part of an integrated disease management strategy, because in the absence of fungicide sprays the incidence of Botryosphaeria disease is very high. Furthermore,

without chemical control leaf blemishes caused by other fungi completely deface the foliage, rendering the flowers unmarketable.

The reduction in the occurrence of cankers achieved by pruning diseased material out of the bushes was much lower than that obtained with the chemicals. In this treatment the leaves of the plants were also badly spoiled by leaf spots caused by other fungal pathogens. None-the-less, by employing sanitation pruning only, a 30% reduction in disease was obtained, and this is highly significant in economic terms. Therefore by combining sanitation pruning with chemical applications the highest level of disease control should be achieved and high quality blooms will be produced.

Although none of the chemicals tested in the trial conditions elicited any phytotoxic response, it has been reported that tebuconazole applied under extremely hot conditions (+30°C) and low humidity levels can burn leaf tips of some proteas (G. Nieuwoud, SAFCOL, pers. comm.). Farmers are thus advised to apply chemicals under moderate weather conditions and preferably early in the morning or late in the afternoon.

In this study it has been shown that fungicides are very effective at reducing Botryosphaeria disease incidence. The best fungicides were benomyl, bitertanol, tebuconazole and the standard practice of alternating prochloraz mc with mancozeb. Regular use of these fungicides will result in appreciable decreases in disease incidence especially if combined with sanitation pruning. Additional studies need to be carried out to optimise the application intervals, and to devise a spray programme that utilises both contact and systemic fungicides to prevent the development of fungicide resistance.

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Table 1. Analysis of variance on the *in vitro* fungicide trial data to test for significant effects

Source	DF	SS	MS	F value	*P* value
Experiment	1	0.71	0.71	2.25	0.1427
Treatment	9	77.96	8.66	27.35	0.0001
Isolates	3	4.59	1.53	4.83	0.0063
Treatment x isolate	26	13.84	0.53	1.68	0.0741
Error	36	11.04	0.32		

Table 2. Mean[1] EC$_{50}$ value of fungicides on mycelial growth of *B. protearum* in *in vitro* fungicide trials

Fungicide (a.i.)	Log transformed EC$_{50}$ value	Back transformed EC$_{50}$ value (μg./mL)
benomyl (S)[2]	0.45 a[3]	1.57
fenarimol (S)	1.05 **b**	2.86
fosetyl-Al (S)	Could not be calculated[4]	Could not be calculated
kresoxim-methyl (S)	Could not be calculated	Could not be calculated
prochloraz mc (S)	0.55 **ab**	1.73
tebuconazole (S)	0.28 a	1.32
chlorothalonil (C)	2.60 **de**	13.46
iprodione (C)	0.60 **ab**	1.82
mancozeb (C)	2.93 **e**	18.72
quintozene (C)	1.91 **c**	6.75
thiram (C)	2.76 **de**	15.79

[1] Mean over all isolates.

[2] S = systemic fungicide, C = contact fungicide.

[3] Numbers followed by the same letter do not differ significantly from each other ($P > 0.05$).

[4] Mycelial growth was not reduced to 50% at the highest concentration of fungicide tested, thus EC$_{50}$ values could not be calculated.

Table 3. **Mean**[1] **EC$_{50}$ value of isolates of *B. protearum* from *in vitro* fungicide trials**

Isolate number[2]	Log transformed EC$_{50}$ value	Back transformed EC$_{50}$ value (μg./mL)
STE-U 1799	1.17 **a**[3]	3.22
STE-U 1800	1.58 **b**	4.85
STE-U 1801	1.68 **b**	5.37
STE-U 1802	1.57 **b**	4.81

[1] Mean over all the fungicides.

[2] All cultures stored at the Dept. of Plant Pathology, University of Stellenbosch, (STE-U).

[3] Numbers followed by the same letter do not differ significantly from each other ($P > 0.05$).
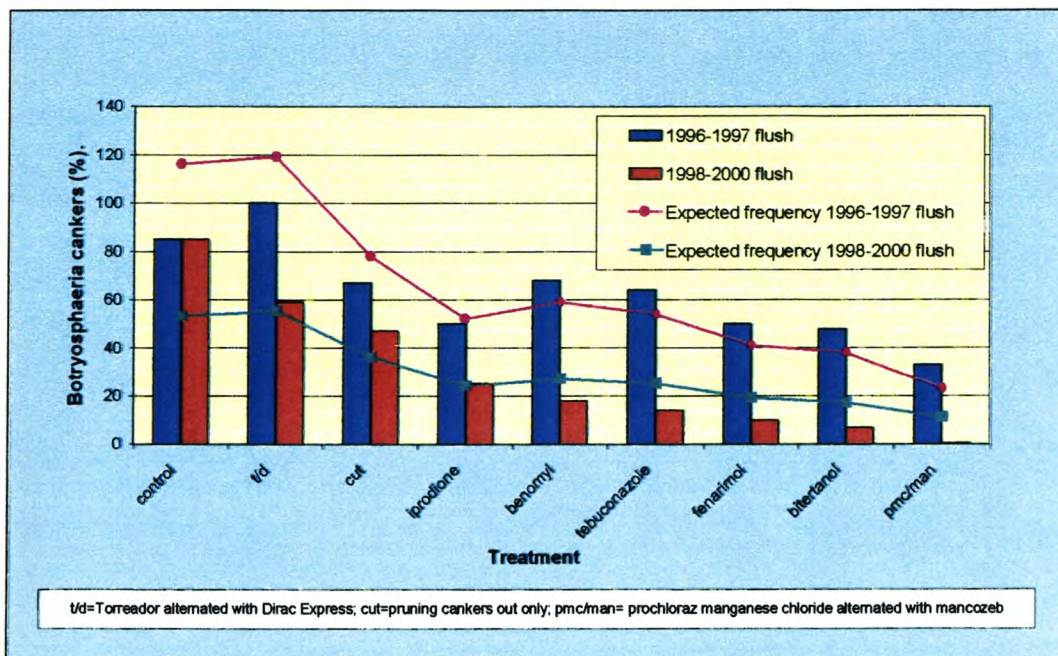
Fig. 1. The percentage of cankers caused by *Botryosphaeria protearum* in various flushes of *Protea magnifica* plants treated with different fungicides.

* Expected frequencies were calculated under the null hypothesis that there is no association between flush and treatment.