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Fungal endophytes of Proteaceae, with particular emphasis on *Botryosphaeria proteae*

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Fungal endophytes occurring in leaves and stems of three species of Proteaceae, *Protea cynaroides*, *Leucospermum cordifolium* and *Leucadendron salignum* × *laureolum* were investigated on farms in three locations in the Western Cape province of South Africa. The aims of this study were to determine if *Botryosphaeria proteae*, a fungus that has been recorded from leaf spots of *Protea* spp., was mostly restricted to leaf tissue, and whether it could occur as an endophyte in different members of Proteaceae. In this study *B. proteae* was routinely isolated in *Protea* and *Leucospermum*, although it was not a dominant taxon and did not occur in *Leucadendron*. *Botryosphaeria proteae* occurred mostly in leaves, rather than stems, suggesting that it is not important as a stem canker pathogen.

Key Words——*Botryosphaeria*; endophytes; pathogens; Proteaceae.

The Proteaceae is one of the most prominent flowering plant families in the southern hemisphere with about 300 species confined to the South-Western Cape (Rebelo, 1995). Proteas are important niche products in the world floriculture market, and are grown as cut-flower crops in Australia, California, Hawaii, Israel, New Zealand, South Africa (Forsberg, 1993) and Zimbabwe (Archer, 1998). The commercial trade in Fynbos in South Africa is predominantly a fresh-cut- and dry-flower industry (Coetzee and Middelmann, 1997). About 70% of fresh-cut-flowers and 80% of dried flowers are exported (Wessels et al., 1997).

Indigenous members of the Proteaceae are utilized in the cut-flower industry and are susceptible to many, apparently indigenous, diseases. Several species of *Botryosphaeria* Ces. & de Not. are pathogenic to proteas (van Wyk, 1973; Knox-Davies et al., 1981; Benic, 1986; von Broembsen, 1986; Denman et al., 1999). *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & de Not. is the most significant in Proteaceae (von Broembsen and van der Merwe, 1990), and is also a well-known pathogen of numerous other hosts worldwide (Müller and Arx, 1962). *Protea* diseases caused by *B. dothidea* are devastating and difficult to control (von Broembsen, 1986; von Broembsen and van der Merwe, 1990). The pathogen causes severe losses of young and mature plants of *Protea* L. (*P.*), *Leucospermum* R. Br. and *Leucadendron* Berg. in South Africa, Western Australia, Hawaii and California (Greenhalgh, 1981; Lamont et al., 1995). *Botryosphaeria proteae* (Wakef.) Denman & Crous also causes leaf spots, and has also been isolated from stems of *Protea* and *Leucospermum* species (Denman *et al.*, 1999). Other *Botryosphaeria* species have also been isolated from shoot lesions, seed heads and seeds of different proteas in South Africa (Knox-Davies et al., 1981).

Some plant diseases, particularly stem and crown cankers and die-back diseases are most severe on plants subjected to environmental stress (Schoeneweiss, 1981). Although species of *Botryosphaeria* are known to be virulent on a wide range of hosts, some cause cankers only on hosts that are weakened or stressed (Hutton, 1958; Schoeneweiss, 1965; Neely, 1968; Crist and Schoeneweiss, 1975). *Botryosphaeria dothidea* normally infects trees as an opportunistic pathogen (Crist and Schoeneweiss, 1975). Smith et al. (1996) have shown that the fungus exists as an endophyte in healthy eucalypt trees. Brown et al. (1998) have discussed the role of endophytes as latent pathogens, which become pathogenic as plants become stressed. This may be the case with *Botryosphaeria* spp. occurring on Proteaceae. In previous surveys, *B. proteae* was found to be associated with stem canker and tip blight symptoms of Proteaceae, and in a few instances also isolated as an endophyte from apparently healthy tissue (S. Denman, pers. comm.). The aims of the present study were to determine whether *B. proteae* was a dominant endophyte of Proteaceae. If it occurred in the three important members of Proteaceae chosen for this study, a further aim was to determine whether it was restricted mainly to leaf or shoot tissue.

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Materials and Methods

Collection Plant material of *Protea cynaroides*, pure and hybrid *Leucospermum cordifolium* (Salisb. ex Knight) Fourc., and a hybrid *Leucadendron salignum* P. J. Bergius × *Leucadendron laureolum* (Lam.) Fourc. cv. Safari Sunset were collected in July 1997 from farms at three localities in the Western Cape province. Samples were taken from five plants per host species at each locality, with two localities being situated near Stellenbosch (Elsenburg and Protea Heights, 15 km apart) and one in Hermanus (60 km distant). Specimens were transported in paper bags in a cooler, stored overnight at 7°C, and processed on the following day.

Isolation A stem piece (5 cm long) with two leaves from the previous growing season, was removed from shoots cut from each plant in the field. Each leaf of *Lsp. cordifolium* and *Leucadendron* cv. Safari Sunset was cut into 18 parts. Leaf laminas of *P. cynaroides* were cut into 33 parts, and their petioles cut transversely into five equal parts. Stem pieces of the three genera were cut transversely into five equal parts that were again split longitudinally. A pilot study was conducted to determine the optimal surface-sterilization regime, which was subsequently followed here. After dissection, plant material was surface-sterilized in 70% ethanol for 1 min, 3% NaOCl for 3 min, and finally in 70% ethanol for 30 s. Surface-sterilized samples were plated on potato dextrose agar plates (PDA, Biolab, Randburg, South Africa) supplemented with cyclosporin (Sandimmun®, Sandoz, Randburg, Johannesburg, 50 mg/ml) (5 mg/l PDA), to suppress the growth of fast growing isolates (Dreyfuss, 1987). Depending on the size of the leaf and stem pieces, 4 to 7 pieces were plated on a 90-mm Petri dish. All dishes were incubated at room temperature. Petri dishes were checked every second day for signs of fungal development. Hyphal tip isolations were made from each colony and transferred to PDA slants.

Fungal colonies were transferred from PDA slants onto divided plates, containing PDA in one half of the dish and carnation leaf agar (CLA; Fisher et al., 1982) in the other half to enhance sporulation. Plates were incubated at room temperature on the laboratory bench, and examined weekly for the presence of reproductive structures to facilitate identification. Sterile isolates were checked every week for up to one month, after which they were incubated under near-ultraviolet light for a period of up to three months. If they remained sterile after this time, they were assigned to "sterile morphotypes" depending on the characteristics of each culture.

Statistical analysis A regression analysis was carried out to verify whether the raw colonization rates (non-transformed data of total number of isolates) and raw infection rates (non-transformed data of number of infected tissue pieces) were correlated. In addition, confirmatory statistical analysis was carried out when appropriate on selected frequency data using Chi-square tests ($P=0.05$). These statistical analyses were carried out using Microsoft Excel Ver. 8.0. For the statistical evaluation, the infection frequency by a fungal species was defined as the total number of pieces of a given tissue colonized by a given taxon. Community ordination was performed on the complete matrix, containing all identified taxa, as well as on a reduced matrix of the raw data of the colonization frequencies that contained only those taxa with a relative importance (dominance) index (Ludwig and Reynolds, 1988) of at least 5%. The resulting matrices were then analyzed by simple correspondence analysis using the package SimCA 2.1 (Greenacre, 1990).

Results

Identification A total of 2700 samples from *Leucospermum*, *Leucadendron* and *Protea* from three sites were processed and 1279 fungal isolates were recovered. Twenty-five genera consisting of 31 species were recorded from the 1279 isolates. This total includes fertile and therefore identifiable cultures, which represented 954 (74.6%) of the total isolates recovered. It does not include sterile mycelia of which there were 325 (25.4%) isolates, consisting of eight recognizable morphospecies. The total number of isolates from the Hermanus samples was at least twice (678) that recovered from the Elsenburg samples (265) and the Protea Height samples (336) (Table 1). Seventeen species were recorded from Elsenburg, and 20 and 26 species respectively from Protea Heights and Hermanus.

In Fig. 1 infection and colonization rates were highly correlated and the dependence of both variables was linear, and therefore the raw infection data were used for further analyses. Of the 39 taxa isolated in this study, including sterile mycelia, 14 were present at RI values of more than 5%. Correspondence analysis carried out on this reduced matrix showed that some samples were characterized by rather homogeneous fungal assemblages (Fig. 2). The percentage of inertia explained by the first four axes was approximately 75%, which

Table 1. Number of isolates recovered from *Protea*, *Leucospermum* and *Leucadendron* at three different locations in the Western Cape province.

Locality	Elsenburg				Protea Heights				Hermanus			
Genus	*Protea*	*Lsp*[a]	*Lcd*[b]	Total	*Protea*	*Lsp*[a]	*Lcd*[b]	Total	*Protea*	*Lsp*[a]	*Lcd*[b]	Total
No of samples	230	230	440	900	230	230	440	900	230	230	400	900
No of isolates	158	83	24	265	162	103	162	427	322	180	176	678

a) *Leucospermum*, b) *Leucadendron*.

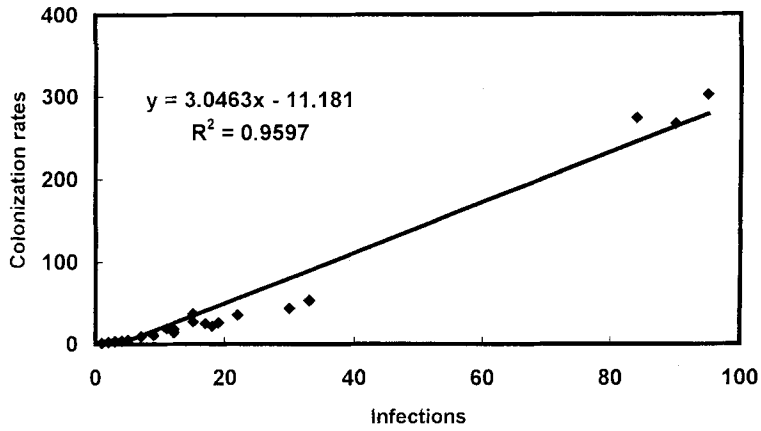

Fig. 1. Regression analysis of raw colonization rates (non-transformed data of total number of isolates) and raw infection rates (non-transformed data of number of infected tissue pieces) of fungal endophytes isolated from Proteaceae.

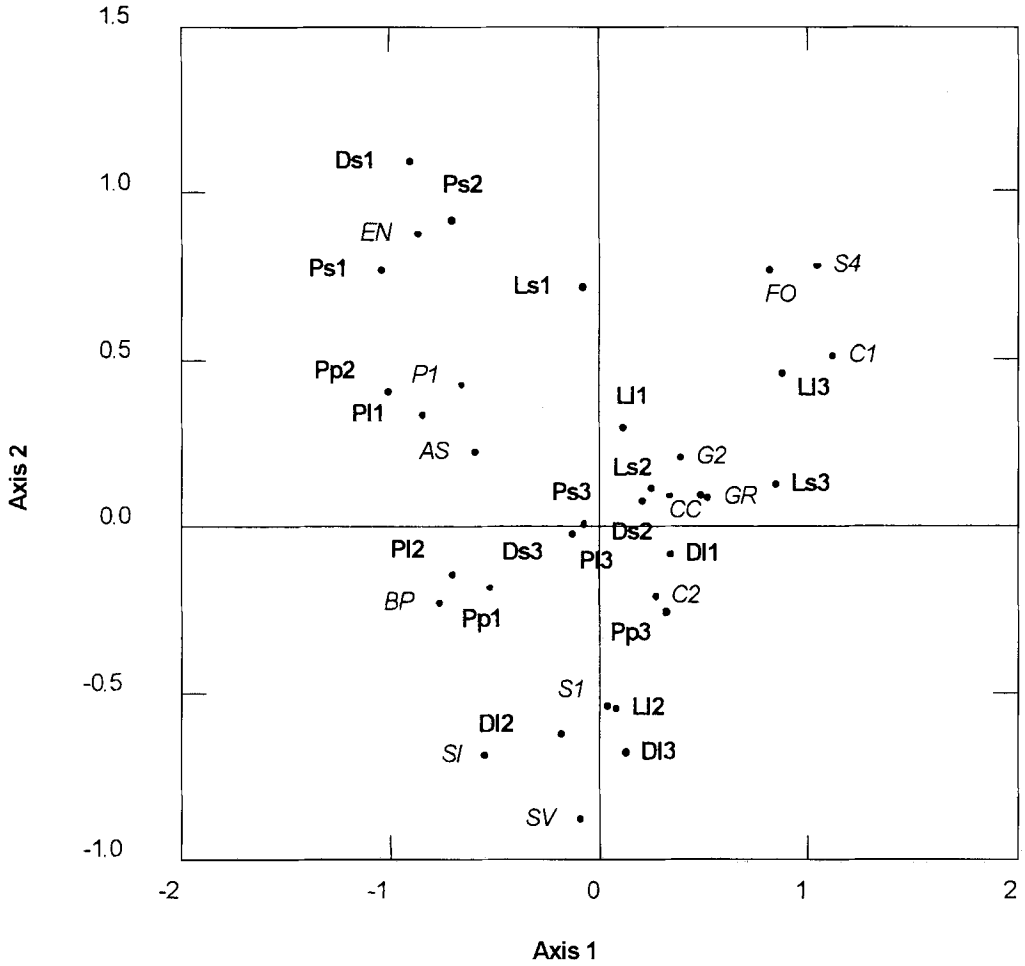

Fig. 2. Correspondence analysis of colonization frequencies of taxa with a relative importance index of at least 5%. The percentage of inertia explained by the first four axes was approximately 75%. Codes used in the analysis: P=*Protea*; L=*Leucospermum*; D=*Leucadendron*; l=Leaves; p=Petiole; s=Stem; 1=Elsenburg; 2=Protea Heights; 3=Hermanus; AS=*Alternaria* Nees sp.; BP=*Botryosphaeria proteae*; CC=*Cladosporium cladosporioides* (Fresen.) de Vries; C1=*Coniothyrium* Corda sp. 1; C2=*Coniothyrium* sp. 2; EN=*Epicoccum purpurascens* Ehrenb. ex Schltdl.; FO=*Fusarium oxysporum* Schltdl.: Fr.; GR=*Gliocladium roseum* Bainier; G2=*Gliocladium* Corda sp. 2; P1=*Phoma* Sacc. sp. 1; Sl=*Sporormiella isomera* S. I. Ahmed & Cain; SV=*Stemphylium vesicarium* (Wallr.) Simmons; S1=Sterile sp. 1; S4=Sterile sp. 4.

indicates a rather good fit of the model to the data. The distribution of the fungal taxa is shown in Fig. 3. A Chi-square analysis of the distribution revealed that the three hosts were colonized by different fungal assemblages ($P<0.005$).

All *Botryosphaeria* isolates were identified as *B. proteae* (Denman et al., 1999). *Botryosphaeria proteae* was not one of the most dominant endophytes (Figs. 3, 4), being the 7th most frequent endophyte of *Protea* and only the 11th most frequent in *Leucospermum*. It was not isolated from *Leucadendron*. It was also predominantly isolated from the leaves of *Leucospermum* and *Protea* collected at Protea Heights and Hermanus, and from the petioles of *Protea* collected at Protea Heights.

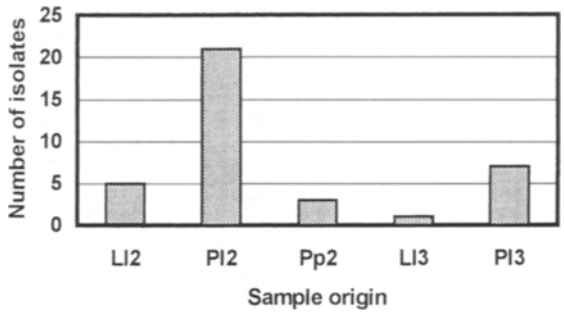

Fig. 4. The distribution of *Botryosphaeria proteae* in the different plant parts and sites. P=*Protea*; L=*Leucospermum*; l=leaves; p=petiole; 2=Protea Heights; 3=Hermanus.

Discussion

Although *B. proteae* was not a dominant endophyte in the material studied, it did occur in leaves and petioles of two of the members of Proteaceae sampled. The reason for the low colonization of plant material at the Elsenburg location is unknown. The higher endophyte colonization at the Hermanus location might be due to the high moisture level of this site near the coast. Moisture levels in the form of rain, dew, and fog interception affect endophyte assemblages by favouring high infection rates (Carroll and Carroll, 1978; Carroll, 1995).

The endophyte communities at the three locations were remarkably similar. This may result from a continuous distribution within the native habitat of the proteas in the South Western Cape. Most dominant species, e.g *Alternaria* sp., *Cladosporium cladosporioides*, *Epicoccum purpurascens*, *Gliocladium roseum*, *Gliocladium* sp. 2 and sterile sp. 1, occurred at all three locations, but there were variations in less abundant and rare species (Fig. 3). Some evidence of host specificity is indicated in the ordination of the data by correspondence analysis (Fig. 2). *Ascochyta* spp., *B. proteae*, *Fusarium oxysporum* and *Sporormiella isomera* only occurred in Protea Heights and Hermanus. *Coniothyrium* sp. and *Phomopsis* spp. did not occur in Protea Heights, while sterile sp. 5 and the *Pleospora* sp. only occurred in Hermanus. *Botryosphaeria proteae* showed a degree of host specificity being recorded only from *Protea* and *Leucospermum*. *Fusarium oxysporum* was mainly associated with leaves of *Leucospermum* in Hermanus. *Stemphylium vesicarium* and *Sporormiella isomera* occurred only in leaves and petioles, and *Coniothyrium* sp. 2, *Pestalotiopsis* sp. and *Pleospora* sp. occurred only in leaves. *Chaetomium* sp. was associated only with stems.

Several of the endophytes reported here have previ-

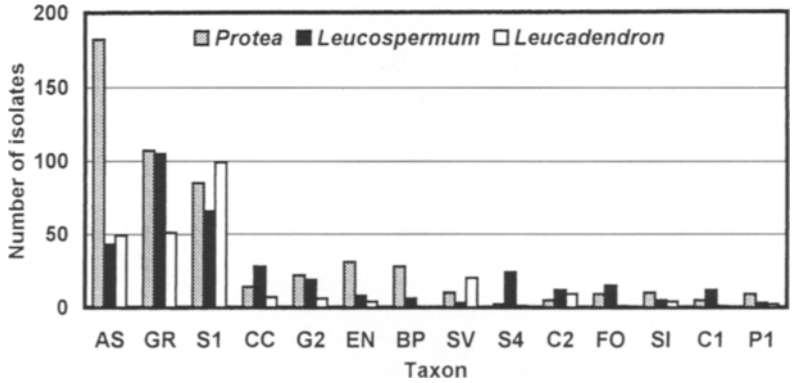

Fig. 3. Distribution of the most important fungal endophytes in *Protea*, *Leucospermum* and *Leucadendron*. Codes used in the analysis: AS=*Alternaria* sp.; BP=*Botryosphaeria proteae*; CC=*Cladosporium cladosporioides*; C1=*Coniothyrium* sp. 1; C2=*Coniothyrium* sp. 2; EN=*Epicoccum purpurascens*; FO=*Fusarium oxysporum*; GR=*Gliocladium roseum*; G2=*Gliocladium* sp. 2; P1=*Phoma* sp. 1; SI=*Sporormiella isomera*; SV=*Stemphylium vesicarium*; S1=Sterile sp. 1; S4=Sterile sp. 4. Taxa of less than 5% relative importance: *Protea*: *Ascochyta* Lib. sp. 1.; *Ascochyta* sp. 2; *Bipolaris australiensis* (M. B. Ellis) *Tsuda* & Ueyama; *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.; *Pestalotiopsis* Steyaert sp.; *Phomopsis* (Sacc.) Bubák sp. 1; *Phomopsis* sp. 2; *Pleospora* Rabenh. ex Ces. & De Not. sp.; *Sordaria* Ces. & De Not. sp.; Sterile sp. 2; Sterile sp. 3; Sterile sp. 5; Sterile sp. 6; Sterile sp. 7; *Thielavia hyrcaniae* Nicot ex Nicot & Longis; unidentified hyphomycete; *Xylaria* Hill ex Schrank sp. *Leucospermum*: *Ascochyta* sp. 2; *Chaetomium* Kunze sp.; *Gilmaniella subornata* Morinaga; *Lophotrichus* R. K. Benj. sp.; *Pestalotiopsis* sp; *Phomopsis* sp. 1; *Pleospora* sp.; *Sordaria* sp.; Sterile sp. 2; Sterile sp. 7; unidentified hyphomycete; *Xylaria* sp. *Leucadendron*: *Arthrinium* Kunze sp.; *Ascochyta* sp. 1; *Ascochyta* sp. 3; *Chaetomium* sp.; *Penicillium* Link sp. 1; *Periconia* Tode sp.; *Phomopsis* sp. 2; Sterile sp. 2; Sterile sp. 5; Sterile sp. 7; Sterile sp. 8; *Thielavia hyrcaniae*; unidentified hyphomycete.

ously been reported as plant pathogens of ornamental plants. These include *B. proteae* (van Wyk, 1973; van Wyk et al., 1975; Denman et al., 1999), *C. cladosporioides* (von Arx, 1987), *Colletotrichum gloeosporioides* (Benic and Knox-Davies, 1983; von Arx, 1987) and *F. oxysporum* (von Arx, 1987; Swart et al., 1999).

In other studies (unpublished data), pycnidia of *B. proteae* have been observed on symptomatic stems of *Lsp. lineare* R. Br. × *Lsp. cordifolium* cv. Succession, *P. cynaroides*, *P. grandiceps* Tratt., *P. repens* (L.) L. and *P. magnifica* Link. The canker infection was secondary, resulting from colonized insect wounds. *Botryosphaeria proteae* is a leaf pathogen (van Wyk, 1973; van Wyk et al., 1975; Denman et al., 1999), and in this study was primarily associated with leaf tissue. It is therefore probable that *B. proteae* is a secondary pathogen of Proteaceae stem cankers. Stem inoculations on *Protea*, *Leucospermum* and *Leucadendron* spp. (data not shown) have not produced disease. These studies are, however, only preliminary, and further research is required to elucidate the role of *B. proteae* as a leaf and stem pathogen of Proteaceae.

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