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Botryosphaeria dothidea endophytic in *Eucalyptus grandis* and *Eucalyptus nitens* in South Africa

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Abstract

Botryosphaeria dothidea is well recognised as a serious pathogen of various woody plants, including species of *Eucalyptus*. In South Africa, the fungus is associated with die-back and canker diseases of various *Eucalyptus* species. In the present study, *B. dothidea* was found to be the dominant taxon occurring as a latent symptomless endophyte in healthy leaves of *E. grandis* and *E. nitens* from two distinct climatic and geographical locations in South Africa. This fungus occurred consistently in trees and apparently infects all leaf parts. Disease symptoms associated with *B. dothidea* in South Africa may thus result from the manifestation of previous latent infections after the onset of stress.

Keywords: Botryosphaeria dothidea; Eucalyptus; Fungal endophytes; South Africa

1. Introduction

Eucalyptus propagation in South Africa constitutes a major component of the economically important forest industry. Limited land area restricts expansion and this is currently challenging the industry to optimisation (Denison and Kietzka, 1993). Plantations are often established in marginal areas and, in these situations, the importance of stress-related pathogens such as *Botryosphaeria dothidea* (Moug.) Ces. et de Not. is most evident. *Botryosphaeria dothidea* is pathogenic to various *Eucalyptus* species in South Africa, causing die-back and canker diseases (Smith et al., 1994). Potential pathogens of *Eucalyptus* such as *B. dothidea* have recently been shown to be present as latent endophytic infections in *Eucalyptus camaldulensis* Dehnh. (Smith et al., 1996), *Eucalyptus globulus* (Labill) (Bettucci and Saravay, 1993), *Eucalyptus grandis* Hill ex Maid. (Smith et al., 1996), *Eucalyptus nitens* (Deane et Maid.) Maid. (Fisher et al., 1993; Smith et al., 1996) and *Eucalyptus smithii* R.T. Bak. (Smith et al., 1996). These findings have raised questions regarding the role of *B. dothidea* in *Eucalyptus* diseases in South Africa.

Endophytic fungi are able to colonise healthy plant tissue without exhibiting virulence (Carroll, 1990), thus not causing obvious damage at the time of infection (McCutcheon et al., 1993). These fungi, which reside in the absence of symptoms for a more

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or less extended period of time within the plant tissues, are widespread and most probably associated with all plants (Petrini, 1986; Petrini, 1991).

The nature of endophytic relationships is variable, with some fungi such as *Rhabdocline parkeri* Shrew. surviving as discrete latent infections only to colonise Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco) needles extensively with the onset of senescence (Stone, 1987). Latent pathogens also share an endophytic relationship with their hosts, causing quiescent infections for long periods of time and symptoms only when the physiological or ecological conditions favour virulence (Tokunaga and Ohira, 1973; Nathaniels and Taylor, 1983: Kulik, 1984; Carroll, 1986; Bettucci and Saravay, 1993). Fungal endophytes can also have mutualistic relations with their hosts, often protecting plants against herbivory (Siegel et al., 1985) and insect attack (Clay, 1986).

The fact that *B. dothidea* can be endophytic in *Eucalyptus* tissue (Fisher et al., 1993; Smith et al., 1996) and the frequent observation of the presence of stromata of *B. dothidea* on dead leaf litter in *Eucalyptus* plantations (M.J. Wingfield and H. Smith, unpublished data, 1994) has led us to question whether this fungus occurs consistently as endophyte in two of the most important *Eucalyptus* species (*E. nitens* and *E. grandis*) in South Africa. The principle aim of the study was to determine whether *B. dothidea* is present in asymptomatic leaves and xylem of both *Eucalyptus* species. We also considered whether *B. dothidea* occurs consistently in trees and in various tissue types and whether the occurrence of this fungus is related to the geographical distribution of *E. grandis*.

2. Materials and methods

2.1. Sampling strategy

A first sampling constituted a preliminary study of the endophytic fungi in *E. nitens*, as this host had previously been studied by Fisher et al. (1993) and Smith et al. (1996), but also because it is one of the most widely planted cold tolerant species in South Africa. *Eucalyptus nitens* is restricted to forest areas of South Africa with cold winter conditions. The second sampling of this study concentrated on *E. grandis* which is more widely planted and could thus be considered in two distinct geographical areas. Fewer trees were sampled but more intensively than in the preliminary study of *E. nitens*.

2.2. Endophytes of E. nitens

Foliage and stem samples of *E. nitens* were collected from trees in the Piet Retief area, south-eastern Mpumalanga, South Africa, during March 1993. Ten trees were randomly selected in a commercial plantation block. Two branches were pruned from each tree, approximately 2 m above the ground. This material was transported to the laboratory and stored at 4°C. All samples were processed within 48 h after collection.

A random sample from each tree consisting of 10 whole, asymptomatic leaves and 20 twig portions (1 cm length), was taken. Leaves and twig portions were thoroughly washed in running tap water, after which the leaves and de-barked twigs were surface sterilised by submerging them sequentially in 70% ethanol for 1 min, undiluted bleach (3.5–5% available chlorine) for 5 min, 96% ethanol for 30 s and finally rinsed in sterile water. Subsequently, each leaf was divided into three segments and placed in groups of three in Petri dishes containing MEA (2% Biolab, malt extract agar), supplemented with 200 mg l^{-1} chloramphenicol to suppress bacterial growth. Twig portions were placed in groups of five in Petri dishes containing the same medium. All the plates were incubated at 20°C for up to 2 weeks. Emerging fungi were transferred to 2% MEA plates and incubated under a mixture of cool white and near-UV lights to induce sporulation before identification.

The relative importance (RI) values of each endophyte species isolated from the leaves (L1–L10, with 1–10 being the tree number) and xylem (X1–X10) were computed using the method of Petrini et al. (1992). Only those fungi with standardised RI values of at least 5% in either leaves or xylem were used for the ordination analyses. Simple correspondence analysis (Greenacre, 1986) was performed on the percentage frequency data of the reduced matrix, as differences in sample size for leaf and xylem samples did not allow the use of raw data. Simple correspondence analysis is particularly robust and

well suited to handle percentage data (Greenacre, 1993).

2.3. Endophytes of E. grandis

Foliage and stems were collected from two *E. grandis* trees during April 1994 (representing a post-summer sampling) in the Piet Retief area, south-eastern Mpumalanga (27°S, 30°50′E, elevation 1476 m above sea-level, 790 mm rainfall for 1993–1994 season). At the same time of the year, foliage and stems were also collected from two *E. grandis* trees in the Kwa Mbonambi area, northern KwaZulu-Natal (28°40′S, 32°10′E, elevation 74 m above sea-level, 966 mm rainfall for 1993–1994 season). Rainfall figures were in both cases below the average owing to a relatively severe drought.

At both locations, the same method of sampling, culturing, isolation and identification was used and corresponded essentially to that used in the first experiment, with minor modifications. A random sample from each tree, consisting of 50 whole,

Table 1
Incidence of fungi in leaf and xylem samples from 10 *E. nitens* trees from the south-eastern Mpumalanga

Fungal taxon	Tree number [a]										
	1	2	3	4	5	6	7	8	9	10	Total
	Sample leaf/xylem [b]										
Acremonium sp.	0/0	0/0	0/0	0/0	2/0	0/0	0/0	0/0	3/0	0/0	5/0
Alternaria alternata (Fr.) Keissler	13/0	21/0	15/0	13/1	17/0	15/0	20/1	13/1	9/0	12/0	148/3
Aureobasidium pullulans (de Barry) Arnaud	1/0	3/0	15/2	0/2	2/2	2/3	10/0	2/0	2/0	3/1	40/10
Botryosphaeria dothidea Ces et de Not.	12/0	15/0	14/0	16/0	16/8	20/0	15/4	18/0	16/0	14/0	156/12
Chaetomium globosum Kunze: Fr.	1/0	4/0	1/0	0/0	4/0	4/0	2/0	0/0	0/0	0/0	16/0
Cladosporium cladosporioides (Fresen.) de Vries	1/2	2/2	2/0	0/1	1/0	1/1	0/1	2/2	0/1	7/1	16/11
Colletrotichum gloeosporioides (Penz.) Sacc.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0	0/0	1/0	2/0
Curvularia sp.	0/0	0/0	0/0	0/0	0/0	0/0	0/1	1/0	2/0	0/0	3/1
Cytonema sp.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/4	0/4
Cytospora eucalypticola van der Westhuizen	1/0	1/5	0/0	0/7	0/0	0/0	0/0	0/3	1/1	1/14	4/30
Endomelaconium sp.	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
Epicoccum nigrum Link	1/0	0/2	2/0	8/1	1/3	6/0	4/0	6/1	5/0	2/1	35/8
Nigrospora oryzae (Berk and Br.) Petch	5/0	1/3	5/1	9/0	15/1	6/0	11/0	5/0	6/0	4/0	67/5
Nodulosporium sp.	3/0	0/0	4/0	0/0	0/0	0/0	0/0	1/0	3/0	1/0	12/0
Non-sporulating isolates	4/2	5/5	5/0	6/1	3/3	5/4	9/2	6/2	3/0	3/2	48/21
Pestalotiopsis guepinnii (Desm.) Stey.	0/3	0/0	0/0	13/0	1/0	0/0	4/0	0/0	1/0	1/0	20/3
Phoma glomerata (Cda.) Wollenw. and Hochapf.	1/0	0/1	0/0	0/1	0/0	0/0	2/0	1/0	0/0	0/0	4/2
Phoma crysanthamicola Hollos	0/0	0/0	0/0	0/0	1/0	0/0	0/0	0/0	0/0	0/0	1/0
Phoma jolyana Pirozynski and Morgan-Jones.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0	0/0	1/0
Speghazinia sp.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1
Sphaerulina sp.	2/0	0/1	1/0	0/0	3/0	3/0	1/0	0/0	0/0	0/0	10/1
Sordaria sp.	1/0	0/0	0/0	0/0	2/0	2/0	0/0	0/0	0/0	1/0	6/0
Trichoderma sp.	1/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0
Total	47/7	52/20	64/3	63/14	68/9	60/16	83/5	53/13	64/2	52/24	595/113
Segments	30/20	30/20	30/20	30/20	30/20	30/20	30/20	30/20	30/20	30/20	300/200

[a] Ten leaves and 10 xylem segments were randomly collected from each of 10 trees.
[b] Number of isolates from 10 leaves (30 leaf segments)/number of isolates from 20 xylem segments. Thus: for Tree 5, two *Acremonium* isolates were found in the leaves and none in the xylem segments.

healthy leaves and 20 twig portions (10 cm length), was taken. Leaves and twig portions were surface sterilised. Each leaf was then cut into five segments which were placed in groups of two and three respectively on the surface of MEA in Petri dishes. Each twig portion was de-barked before sterilisation, divided into 10 segments (1 cm each) and placed in groups of five on the surface of MEA in Petri dishes. Thus, a total of 250 leaf and 200 xylem segments per tree at each location were examined in this experiment. A χ^2 test was conducted to test whether *B. dothidea* was evenly distributed in leaves.

3. Results

3.1. Endophytes of E. nitens

A total of 23 fungal taxa and several non-sporulating isolates were collected and identified from leaves and xylem of *E. nitens* trees (Table 1). Of these, only seven had an RI of greater than 5%. Results of the ordination by simple correspondence analysis are presented in Fig. 1. The total inertia explained by the first three components was 88%, thus indicating that the model fitted the data appropriately.

The leaf samples formed a compact cluster, typified by species such as *Alternaria alternata* (Fr.) Keissler, *B. dothidea*, *Epicoccum nigrum* Link, *Aureobasidium pullulans* (de Barry) Arnaud and *Nigrospora oryzae* (Berk. and Br.) Petch. These fungi were thus prevalent in leaves. In contrast, the xylem samples formed a less defined group and *Cytospora eucalypticola* van der Westhuizen was the dominant species in xylem segments. The fungal assemblages of the xylem samples can thus be considered less homogeneous than those of the leaf samples.

Fungi were also more frequent in the leaves than in the xylem. This is an additional reason for the rather diffuse cluster formed by the xylem samples in the correspondence analysis. The compact cluster of all leaf samples indicates that *B. dothidea* occurred consistently in all leaves of all 10 trees. In contrast, it occurred in the xylem of only two trees.

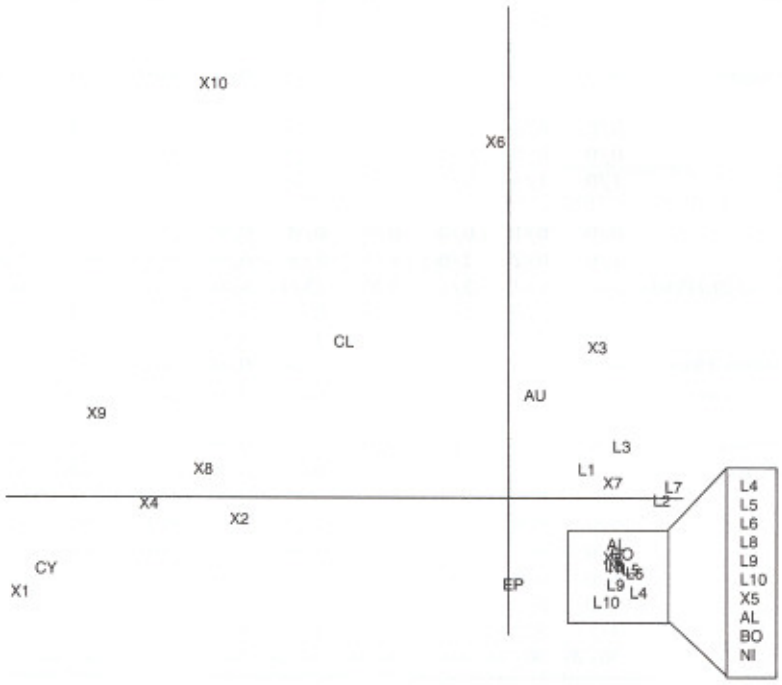

Fig. 1. Correspondence analysis map of the incidence of the most common endophytic fungi from leaf and xylem tissue of 10 *E. nitens* trees. L1–L10, leaves for trees numbered 1–10; X1–X10, xylem for trees numbered 1–10; AL, *A. alternata*; AU, *A. pullulans*; BO, *B. dothidea*; CY, *C. eucalypticola*; CL, *C. cladosporioides*; EP, *E. nigrum*; NI, *N. oryzae*.

The 10 trees were sampled in a random way throughout a commercial compartment of *E. nitens*. Thus, the distribution of this fungus in the leaves seems to be evenly poised over a fairly large area, implying a high inoculum pressure.

3.2. Endophytes of E. grandis

Having established that *B. dothidea* occurs consistently in all *E. nitens* trees sampled and that the fungus is present in *E. grandis* leaves (Smith et al., 1996), we undertook an intensive sampling of a smaller number of *E. grandis* trees from different areas to establish whether this fungus occurs widespread. The analysis by χ^2 showed that *B. dothidea* is distributed homogeneously in the leaves ($\chi^2 = 0.877$, $P > 0.01$), not being more prevalent near the leaf tip or base. The result was consistent at both locations (Table 2).

The total number of isolates obtained from the post-summer sampling of *E. grandis* was larger in the Piet Retief area than the Kwa Mbonambi area (Table 3). This was due to the higher frequency at which a *Cochliobolus* sp. and *C. eucalypticola* were isolated, as well as to the frequent presence of a *Mycosphaerella* species, which was absent from the sample taken from the Kwa Mbonambi area. Sterile Mycelium 1 and 2 were found to be absent from the sample taken from Piet Retief area. *Botryosphaeria dothidea* was the most prominent taxon isolated from *E. grandis* from both geographical locations and was approximately equally frequent in both samples taken from the two locations (Table 3).

Table 2
Incidence of *B. dothidea* from leaves of *E. grandis* from two geographic locations

Species and sample location [a]	Tree number [b]									
	Tree 1/leaf segments					Tree 2/leaf segments				
	1	2	3	4	5	1	2	3	4	5
GNN	36	35	47	46	44	38	30	50	40	38
GSET	41	44	44	43	44	38	37	34	39	39

[a] G, *E. grandis*; NN, northern KwaZulu-Natal; SET, south-eastern Mpumalanga.
[b] Two trees sampled with five segments from each of 50 randomly collected leaves.

Table 3
The incidence of endophytic fungi from *E. grandis* from two distinct geographical areas

Fungal taxon	Tree, sampling area [a]		
	GP	GK	Total
	Leaf samples/xylem samples [b]		
Acremonium sp.	1/0	0/0	1/0
Alternaria alternata	26/7	75/23	101/30
Aspergillus sp.	0/0	1/0	1/0
Aureobasidium pullulans	0/11	12/3	12/14
Botryosphaeria dothidea	403/3	405/20	808/23
Chaetomium globosum	0/0	4/0	4/0
Cladosporium cladosporioides	6/6	2/31	8/37
Cladobotruim sp.	2/0	0/0	2/0
Cochliobolus sp.	67/0	9/0	75/0
Cytospora eucalypticola	150/25	51/28	201/53
Drechslera sp.	1/0	0/0	1/0
Epicoccum nigrum	4/3	0/0	4/3
Fusarium sp.	0/0	4/0	4/0
Guignardia sp.	9/0	87/0	96/0
Mycosphaerella sp.	371/0	0/0	371/0
Nigrospora oryzae	4/0	22/5	24/5
Nodulosporium sp.	0/19	0/0	0/19
Non-sporulating isolates 1	0/0	149/0	149/0
Non-sporulating isolates 2	0/0	52/3	52/3
Pestalotiopsis guepinii	2/5	3/6	5/11
Phoma glomerata	0/0	3/0	3/0
Phomopsis sp.	2/0	0/0	2/0
Rosulomyces sp.	6/0	0/0	6/0
Sordaria sp.	2/0	0/0	2/0
Stachybotrys sp.	0/0	0/9	0/9
Tricoderma sp.	0/3	0/0	0/3
Ulocladium sp.	0/1	0/0	0/1
Valsa sp.	1/0	0/0	1/0
Total	1057/83	879/128	1936/211
Segments	500/400	500/400	1000/800

[a] G, *E. grandis*; P, Piet Retief area, south-eastern Mpumalanga; K, Kwa Mbonambi area, northern KwaZulu-Natal.
[b] A total of 250 leaf and 200 xylem segments were taken from each two trees at each location.

4. Discussion

A large number of fungal taxa were isolated from asymptomatic and apparently healthy *E. grandis* and *E. nitens* trees in this study. Symptomless infections

were found to occur in both the leaves and the xylem of healthy trees. *Botryosphaeria dothidea* was the most prominent taxon. It was evenly distributed in leaves and was also present in the xylem of *E. grandis* from two distinct geographical locations.

Botryosphaeria dothidea is considered to be one of the most important pathogens of *Eucalyptus* in South Africa. It is responsible for substantial losses owing to mortality of young transplants, die-back and canker diseases (Smith et al., 1994). *Botryosphaeria dothidea* is often particularly pathogenic in stressed trees (Wene and Schoeneweiss, 1980; Pusey, 1989; Old et al., 1990), thus making it of particular importance in a country such as South Africa with a relatively low rainfall and restricted land area suitable for establishing *Eucalyptus* plantations. Based on the terminology of Petrini (1991), we suggest that *B. dothidea* in leaves and xylem of *Eucalyptus* in South Africa be considered as symptomless endophytic infections by a latent pathogen. Additional research concerning the influence of stress on onset of virulence may contribute significantly to our knowledge of the biology and significance of this fungus.

The endophytic assemblages observed for both *E. grandis* and *E. nitens* in this study are similar to those found by Fisher et al. (1993). They reported that *C. eucalypticola* had a low incidence in England whereas this was the dominant taxon isolated from Australia, and suggested that plants outside their natural habitat become infected with less host-specific fungi and also by more cosmopolitan fungi, as already reported by Espinosa-Garcia and Langenheim (1990) for *Sequoia sempervirens* (D. Don) Endl. *Botryosphaeria dothidea* was also isolated at higher frequencies from *E. grandis* and *E. nitens* in South Africa than was *C. eucalypticola*. The difference in frequency was, however, less obvious than that reported by Fisher et al. (1993). This might be explained by the similarity of climatic conditions in South Africa and Australia and also the nearness of the two countries in comparison. *Botryosphaeria dothidea* is probably native to South Africa as this fungus is widespread on *Eucalyptus* in the country (Smith et al., 1994). It has also been found associated with diseases of indigenous plants such as *Leucospermum cordifolium* (Salisb. ex Knight) Fourcade (Von Broembsen, 1986), *Protea magnifica* Andr. (Serfontein and Knox-Davies, 1990) and *Leucodendron argenteum* (L.) R. Br. (Wager, 1970).

The higher number of isolates obtained during the post-summer sampling of *E. grandis* from the Piet Retief area may be due to the abrupt rise in temperature and humidity during summer, which could boost infections. In contrast, a lower number of isolates were obtained during the post-summer sampling of *E. grandis* from the Kwa Mbonambi area. This may be due to the less dramatic climatic changes, with summer temperatures sometimes above ideal for germination of fungal spores. The set-up of this experiment, on the other hand, does not allow us to exclude the possibility of the influence of geographic factors, which has been found in many cases to be of major importance in determining the endophytic assemblages of a given host plant (Kowalski, 1993; Fisher et al., 1993; Fisher et al., 1994). Further and more intensive samplings are necessary to clarify the influence of geographic location on endophytes in South Africa.

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