View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

South African Journal of Botany 72 (2006) 245–255

SOUTH AFRICAN JOURNAL OF BOTANY

www.elsevier.com/locate/sajb

Occurrence and possible role of endophytic fungi associated with seed pods of *Colophospermum mopane* (Fabaceae) in Botswana

A. Jordaan [b,*], J.E. Taylor [a], R. Rossenkhan [a]

[a] *Department of Biological Sciences, University of Botswana, Private Bag 0022, Gaborone, Botswana*

[b] *Electron Microscope Unit, Department of Physics, University of Botswana, Private Bag 0022, Gaborone, Botswana*

Received 18 April 2005; accepted 6 September 2005

Abstract

Endophytic fungi were isolated from different aged surface sterilised pods of *Colophospermum mopane* and succession patterns were investigated. Lignocellulolytic enzyme assays as well as histological and fine structural studies were used to investigate if succession was related to different lignocellulolytic abilities of the fungi. Representatives of the common genera (*Alternaria*, *Phoma* and *Phomopsis*) were qualitatively tested for lignocellulolytic enzyme activity. Samples of endophyte infected and uninfected pod pericarps were fixed and sectioned for light microscopy and TEM. Samples were also viewed with SEM. A fungal succession was evident as pods aged, and detached from the tree. Strains of all three genera demonstrated lignocellulolytic abilities. Microscopy studies suggested that *Phoma* was only capable of utilising moderately lignified mesophyll cells whereas *Phomopsis* and *Alternaria* could degrade heavily lignified fibres. This could explain the pattern of succession observed: *Phoma* colonised younger pods where more unlignified resources were available, whereas *Phomopsis* and *Alternaria* colonised older pods, thus making use of the remaining lignified resources. Changes in endophyte abundance and diversity are related to the age and degree of decay of the pods, and determined by the lignocellulolytic abilities of the fungi. The potential role of these endophytes is discussed.
© 2006 SAAB. Published by Elsevier B.V. All rights reserved.

Keywords: Ultrastructure; TEM; Endophyte; Fungus; Lignin

1. Introduction

One of the most important trees in Botswana is the Mopane (*Colophospermum mopane* (J.Kirk ex Benth.) J.Kirk ex J. Léonard: Fabaceae). The mopane tree provides fodder for both wild animals and livestock and is an important source of fire wood and shade. It is also the host of the 'mopane worm' or 'phane', the caterpillar form of the moth *Imbrasia belina* Westwood, which can occur on this host in high densities and forms an important part of the diet of the local people (Coates Palgrave, 2002).

Mopane is a leguminous tree that often forms monospecific stands and dominates the vegetation structure in the north east of Botswana, commonly on alluvial soils (Macala, 1996). It occurs in two forms (often inter mixed), either as a multi-stemmed shrub of about 1–2 m high, or as a small to medium-sized tree of 5–12 m which possesses an upright, narrow crown (Timberlake, 1997).

Little research has been undertaken to investigate the fungi associated with Mopane other than a study on mainly basidiomycetous pathogens (Smith and Shah-Smith, 1999) and mycorrhizae (Sinclair et al., 1998) and there are no published records of any fungi except for a *Mycosphaerella* sp. in Zimbabwe (Whiteside, 1966). Therefore, to initiate mycological studies, preliminary research was carried out to determine the diversity and occurrence of endophytic fungi associated with the leaves and pods of Mopane (Taylor et al., 2002).

The role of endophytes in non-graminaceous hosts is a much speculated topic and endophytic associations have been implicated to have a variety of effects on the host plant (Kriel et al., 2000; Wilson, 2000). Possible roles include mediation in plant–herbivore interactions (Hammon and Faeth, 1992), which maybe have a beneficial effect for the host (Miller, 1986; Carroll, 1991; Calhoun et al., 1992; Findlay et al., 1995; Azevedo et al., 2000; Miller et al., 2002), a negative effect

* Corresponding author.
E-mail address: jordaana@mopipi.ub.bw (A. Jordaan).

0254-6299/$ - see front matter © 2006 SAAB. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.sajb.2005.09.007

(promoting a positive effect on the herbivores) (Gange, 1996; Johnson et al., 2003) or are apparently neutral (Lappalainen et al., 1999; Ahlholm et al., 2002). It has recently been acknowledged that endophytes could be an important source of bioactive compounds (Miller, 1986; Petrini et al., 1992; Strobel and Long, 1998; Tan and Zou, 2001; Schulz et al., 2002). Also endophytes have been suggested as potential biocontrol agents of plants (Dorworth and Callan, 1996), or alternatively as agents in promoting host resistance to disease (Kriel et al., 2000; Tomita, 2003).

Endophytic fungi are variously defined, as discussed by Stone and Petrini (1997), and those of non-graminaceous hosts can be divided into mutualistic symbionts or latent pathogens (Sinclair and Cerkaukas, 1996), depending on whether they are parasitic and can cause disease. However, due to the difficulty of determining the pathogenicity of a fungus occurring endophytically, a more encompassing definition is often used to include all fungi which colonise living plant tissues and which remain symptomless for the whole or only part of the period of colonisation (Stone et al., 2000).

The role of endophytes in Mopane is unknown. The present study was undertaken to provide quantitative and qualitative information on the endophytic fungi isolated from mopane pods, especially in relation to the ages of pods. The association of these fungi with the pods was also investigated using various forms of microscopy to elucidate and explain the abundance patterns seen in the aforementioned isolation studies. Finally, the lignocellulolytic abilities of the endophytic fungi were tested.

2. Materials and methods

2.1. Collection site and details

The collection site was a stand of mopane trees and shrubs (*C. mopane*) adjacent to Madiela Health Post, which is situated on the A1 road, 45 km north of Mahalapye in south east Botswana. The pods were transported back to the University of Botswana in Gaborone in a cool box and were processed within 24 h of collection while being kept at 4 °C. Collections were made according to pod maturity and were taken directly from randomly chosen trees and not from the ground unless otherwise stated. Green soft immature pods (estimated to be 1 month) were collected on January 31, 2003. On April 10, 2003, yellow pods (estimated to be 2 months) and brown pods (estimated to be 2.5 months) were collected and on October 14, 2003 brown pods (estimated to be 9 months) were collected. On October 19, 2002, brown pods (estimated to be 21 months) were hand-picked from the tree. Pods that were 2.5 months old refer to those that were harvested at the beginning of the reproductive season, while 9-month-old pods were harvested at the end of the growth season. Older pods (21 months) displayed visible signs of decomposition. The 21-month-old pods were placed into wire cages in contact with the ground and left for 2 months to determine whether there was a shift in fungal abundance and diversity between pods on the tree and those on the ground. These pods were then collected (estimated to be 23 months old) (December 4, 2002) as were pods occurring on the ground which had been there for an indefinite period (estimated to be 23 months old). The last three categories of pods, i.e. brown (21 months), ground—for 2 months (23 months) and ground—indefinite period (23 months), did not contain viable seeds due to severe decomposition of the pericarp.

2.2. Isolation studies

Thirty-five pods of each age category were surface sterilised in a laminar flow cabinet as follows: The pods were first washed in sterile water for 1 min, after which they were washed in sterile water with approximately 2 ml of dishwashing detergent. The pods were then transferred to a beaker of 70% alcohol for 1 min followed by 8 min immersed in undiluted commercial bleach (3.5% sodium hypochlorite). Finally, the pods were rinsed in 70% alcohol for 30 s and were allowed to dry in the laminar flow cabinet before being dissected. Six pieces (5×5 mm) were excised from the following areas of the pod pericarp (not including the internal seed) and numbered as follows: the stalk (1), outer margin (2 and 3), inner margin (4) and pericarp wall (5 and 6). Together these pieces represented the entire pericarp. The pieces were placed around the outside of a Petri dish (labelled on the reverse with the appropriate numbers) containing malt extract agar (MEA, Biolab, MERCK, Gauteng South Africa) with a fungal growth retardant, rose bengal, at 0.033 g/l (Sigma-Aldrich Co, St Louis, USA), and supplemented with 0.2 g streptomycin sulphate (Sigma-Aldrich Co, St Louis, USA) dissolved in 2 ml sterile water. The plates were labelled 1–35, and the pod age indicated. The Petri dishes were incubated at 25 °C in the dark and were checked after 5 days for growth. All isolates that developed were subcultured onto MEA for morphological identification. The percentage colonisation of pod pieces [(No. of pod pieces which yielded ≥ 1 isolates/Total no. of pod pieces) $\times$ 100] was calculated, as were isolation frequencies (No. of isolates yielded in total/Total no. of pod pieces) to demonstrate the degree of multiple colonisation from the samples taken.

2.3. Microscopy

Material used in the microscopy studies was derived as follows. After surface sterilisation and incubation, samples where only a single endophyte had developed were taken from culture and processed for microscopy. The developing endophytes were subcultured to MEA for positive identification. Other samples were derived from pods that were unsterilised and unincubated, or pods which had been sterilised and incubated, but where no fungi developed. The latter served as controls.

Pericarps of intact pods and pods in various stages of decomposition were sputter coated with gold and examined with a Philips XL30 ESEM. To test the efficacy of the surface sterilisation method, pericarp sections before and after sterilisation were compared. Pieces of pericarp were prepared for LM and TEM as follows: Samples were pre-fixed in 2.5% Glutaraldehyde in 0.1 M phosphate buffer and post-fixed in

Table 1
A summary of colonisation and isolation rates of endophytes from Mopane pod pericarps

Pod state and age	No. of isolates	Colonisation rate (%)	Isolation rate
Green (1 month)	0	0	0
Yellow (2 months)	95	30	0.45
Brown (2.5 months)	115	37	0.55
Brown (9 months)	154	46	0.74
Brown (21 months)	143	51	0.68
Ground — for 2 months (23 months)	115	28	0.55
Ground — indefinite period (23 months)	99	32	0.47

Number of samples per pod = 6; per age category (35 × 6) = 210.
Overall colonisation rates (no. of samples yielding isolates / no. of samples).
Overall isolation rates (no. of isolates / no. of samples).

1% OsO_4. Samples were dehydrated in a graded ethanol series, embedded in LR White, sectioned and examined in a Zeiss Axioskop II light microscope or Tecnai 12 TEM. To confirm the presence of endophytes, sections of pericarp were surface sterilised and plated on DRBC medium. These cultured sections were also examined anatomically and with TEM.

2.4. Lignocellulolytic tests

Qualitative methods were used to assess the lignocellulolytic enzyme production of different strains of the fungi isolated (Table 2). The protocols are outlined by Pointing (1999) and involved a cellulolytic enzyme assay using cellulose clearance agar and a lignolytic assay using Poly-R 478 (Sigma-Aldrich Co, St Louis, USA) agar. Positive reactions for lignocellulose

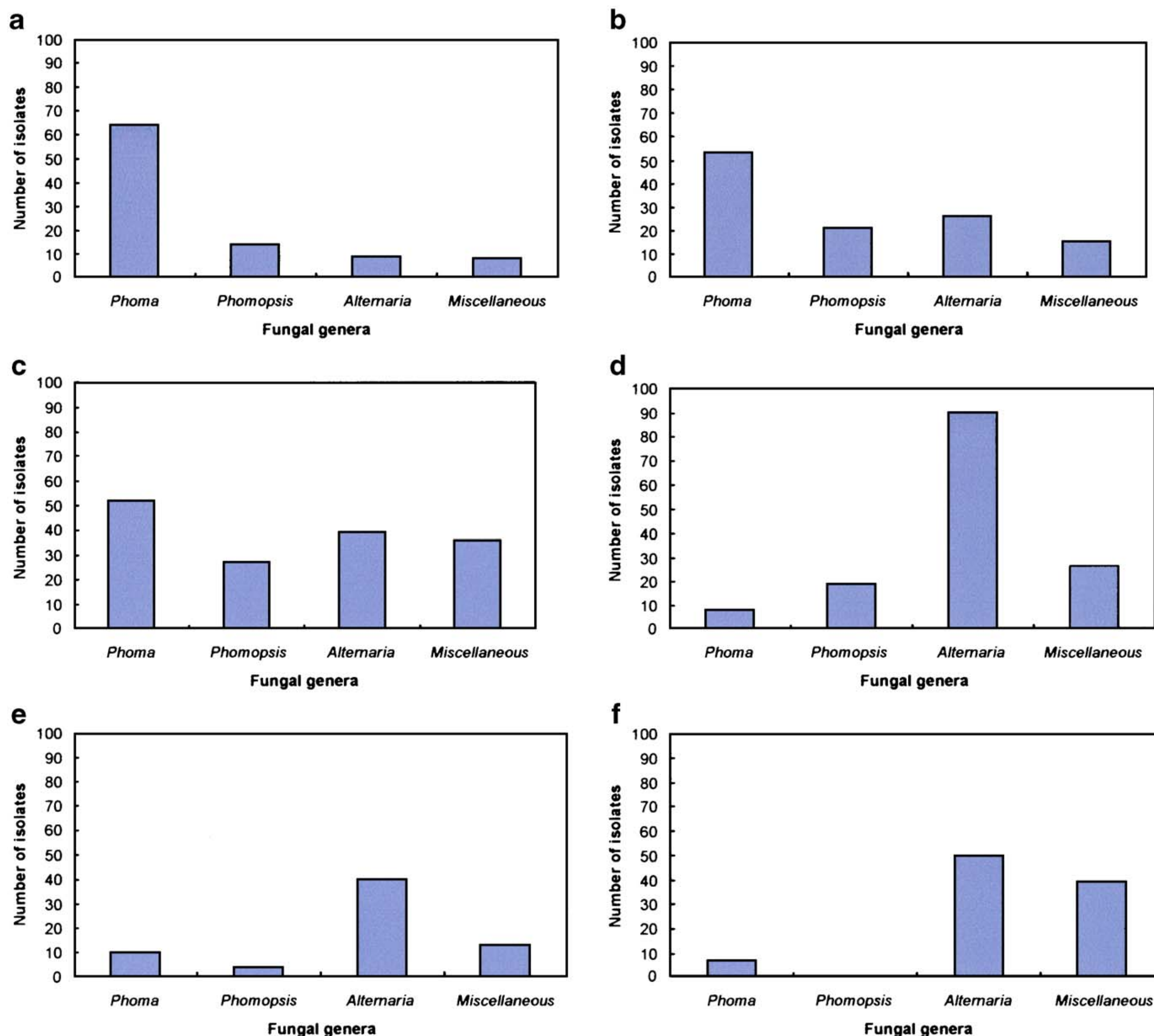

Fig. 1. Numbers of isolates of common genera for different aged pods. (a–d) Pods still attached to the tree. (a) 2 months, (b) 2.5 months, (c) 9 months, (d) 21 months. (e and f) Pods on the ground. (e) 23 months (ground for 2 months), (f) 23 months (ground for an indefinite period).

degrading enzymes involve clearance of the respective media around the growing fungal colony.

3. Results

3.1. Isolation studies

Three fungal genera namely *Phoma*, *Phomopsis* and *Alternaria* occurred prominently. For the purpose of this study, the genera have not been separated into species, but the following observations were made. The *Phoma* isolates were similar to *Phoma multirostrata* (P.N. Mathur, S.K. Menon and Thirum.) Dorenb. and Boerema, but differed in that the conidial mass was deep pink. The cultures reached a diameter of approximately 60 mm in 7 days and were very variable, ranging from almost translucent to dark brown with sparse aerial mycelium, with some isolates producing denser grey aerial mycelium. Some colonies were observed to sector. In all cases, pycnidia were produced abundantly over the surface of the colony. No attempt was made to identify the *Phomopsis* isolates, but some sporulated and all displayed the morphology typical of *Phomopsis* cultures with woolly, dense yellowish-white mycelium (55–70 mm in 7 days), and black stromatic structures produced all over the colony with some producing cream-coloured conidial masses after 28 days. The *Alternaria* isolates were considered possibly to resemble two species although there did appear to be a continuum in colony morphology from '*Alternaria* sp. 1' which produced dense woolly colonies that were brownish-grey above and blackish-brown in reverse to '*Alternaria* sp. 2' which produced dense woolly colonies that were greenish-grey above and brown below with varying amounts of pale pink. Most of the colonies grew at a rate of 60–80 mm in 7 days. The remaining fungal taxa were rarely isolated or remained sterile.

A total of 721 isolates were made from 1470 samples, and a summary of the results is given in Table 1. No isolates were obtained from 1-month green pods, but the number of isolates steadily increased as the pod matured up until the age of 9 months. The number of isolates appears to remain constant while the pods are attached to the tree, however, when the same pods are placed on the ground endophytic fungi decrease.

The colonisation rates indicated that many of the samples plated were not colonised (up to 70% of the pod age categories that yielded endophytes), and of the samples that yielded endophytes, many were colonised by more than one isolate. This was demonstrated by the higher isolation rates when compared with the colonisation rates. For the purpose of the microscopy studies, only samples that either yielded no endophytes, or only one species, were utilised. This was to ensure that anatomical and ultrastructural evidence of pod breakdown for each species could be studied separately. Of samples, which yielded only one species, the isolate was purified by isolation onto MEA and in no instances did more than one isolate develop.

Fig. 1a–f illustrates the relative proportions of different fungal genera isolated from pods of different ages. A pattern of colonisation was evident. Initially, in younger pods collected from the tree (2–9 months), *Phoma* was dominant, whereas *Alternaria* gradually increased as the pod matured and *Phomopsis* remained at a constant level. However, after the pod had remained on the tree for 21 months, *Phoma* reduced dramatically whereas *Alternaria* became dominant. When the same pods were placed on the ground (23 months), the dominance of *Alternaria* decreased and with time, the numbers of miscellaneous taxa began to increase, with *Phoma* and *Phomopsis* much reduced.

Table 2
Lignocellulolytic abilities of representative isolates of the three common genera isolated from Mopane pod pericarps

Isolate ID (UBCC#)	Pod age (months)	Lignin assay	Cellulose assay
Alternaria 550*	2	+	+
Alternaria 551*	2	−	+
Alternaria 559*	2.5	+/−	+
Alternaria 519*	9	+	+
Alternaria 520*	9	+	+
Alternaria 544	2	+	+
Alternaria 554	2.5	+	−
Alternaria 567	2.5	+	+
Alternaria 523	9	+	+
Phoma 537	2	+	−
Phoma 549	2	+	+/−
Phoma 556	2.5	−	−
Phoma 574	2.5	+	+
Phoma 524	9	+/−	+/−
Phomopsis 568	2.5	+/−	+
Phomopsis 573	2.5	+	+
Phomopsis 526	9	+	+
Phomopsis 527	9	+/−	−
Phomopsis 518	?	+/−	−

3.2. Lignocellulolytic studies

The results of the lignocellulolytic assays are given in Table 2. It should be recognised that both tests are qualitative and only give an indication of the lignocellulolytic abilities of the different isolates, but do not provide quantitative information. In addition, the cellulose assay was very difficult to interpret and the cellulolytic abilities of the different isolates were therefore based not on clearance, but rather on growth rates as has been suggested previously (Abdel-Raheem and Shearer, 2002). For *Alternaria* strains, the fungi from the older pods demonstrated greater lignocellulolytic abilities, whereas the strains of *Phoma* and *Phomopsis* from the younger pods were more consistent in their lignocellulolytic abilities.

3.3. Microscopy

SEM studies revealed that the surface sterilisation technique used was highly effective. Yellow 2 months (Fig. 2a) and brown 2.5 months unsterilised pods (Fig. 2c) showed particles over their surfaces, but more in the case of brown pods as the pods were older. Both yellow (Fig. 2b) and brown pods (Fig. 2d) were clear of these particles after surface sterilisation.

Mature 9-months brown pods often had stomata occupied by fungal growth (Fig. 2e,f). It was not always apparent whether

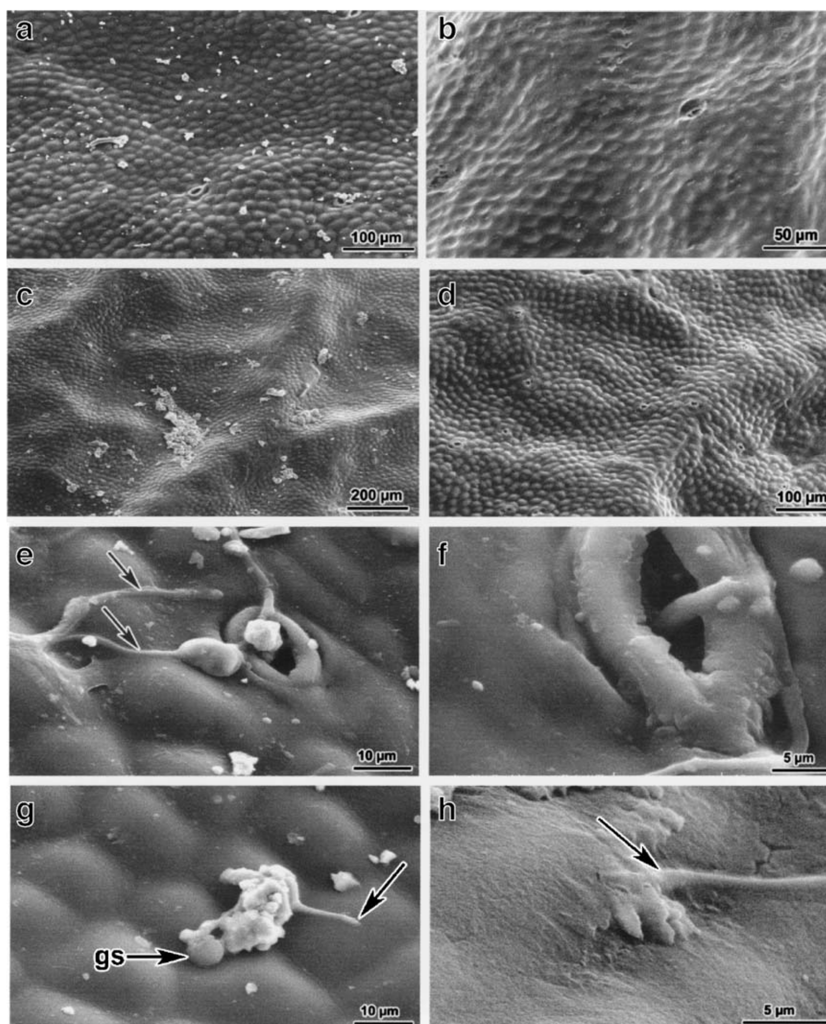

Fig. 2. SEM micrographs of the surfaces of mopane pods. (a) Yellow 2 months before surface sterilisation, (b) Yellow 2 months after surface sterilisation, (c) 2.5 months brown before surface sterilisation, (d) 2.5 months mature brown pod after surface sterilisation, (e) 9 months mature brown pod with hyphae from germinating spore penetrating stoma as well as subcuticular hyphae (arrows). (f) Hyphae penetrating stoma of 9 months mature brown pod, (g) Germinating spore and hyphae (arrow) penetrating epidermal cell, (h) Subcuticular hyphae (arrow) in mature 9 months brown pod. gs, germinating spore.

the fungi grew out of the pericarp through the substomatal chambers or whether the stomata formed a route of penetration for fungi from the outside. In most cases, it appeared that the penetrating hyphae originated from germinating spores on the pod surface that then penetrated the stomata (Fig. 2f). There were also instances of subcuticular penetration of the pericarp by hyphae originating from germinating spores in pods from all age classes (Fig. 2g). Subcuticular hyphae were also frequently observed (Fig. 2e,h).

Most symptomless surface sterilised pericarps (i.e. those not displaying any outward signs of fungal colonisation) displayed profuse fungal growth after incubation and there was considerable histological and ultrastructural evidence for the breakdown of lignified walls. A few surface sterilised pods had no endophytes growing out of them and these served as controls. When these 2.5-months pods were examined anatomically it was clear that the surface sterilisation process did not damage the pods, as the overall structure was intact (Fig. 3a). Therefore, it was safe to conclude that fungi caused the breakdown of not only lignified, but also unlignified cell walls.

In the 9-months mature brown pods, the mesophyll was largely intact although some cells showed signs of degradation (Fig. 3b). Most mesophyll cells had thick, lignified walls. The fibres were heavily lignified.

In sections of unsterilised pods that had been on the ground for 23 months, the mesophyll and epidermal cells were almost totally decomposed (Fig. 3c). The only remaining part of the epidermal layer was the thick, cutinized wall. Fungal growth was abundant in mesophyll and epidermal regions (Fig. 3c). The upper layer of the heavily lignified fibres was also degraded and lignin has completely disappeared from the walls, while the inner cell layers also showed loss of lignin from the walls (Fig. 3c).

In 9-months pericarps that yielded *Alternaria* the mesophyll was completely broken down, including most of the lignified parenchyma cells (Fig. 3d). Most of the epidermal cells were also destroyed. Light micrographs showed that the lumen of fibres was heavily infested by *Alternaria* hyphae and that cell walls were digested by the enzymatic action of the fungus as was evident by the extensive white areas in the walls (Fig. 3e).

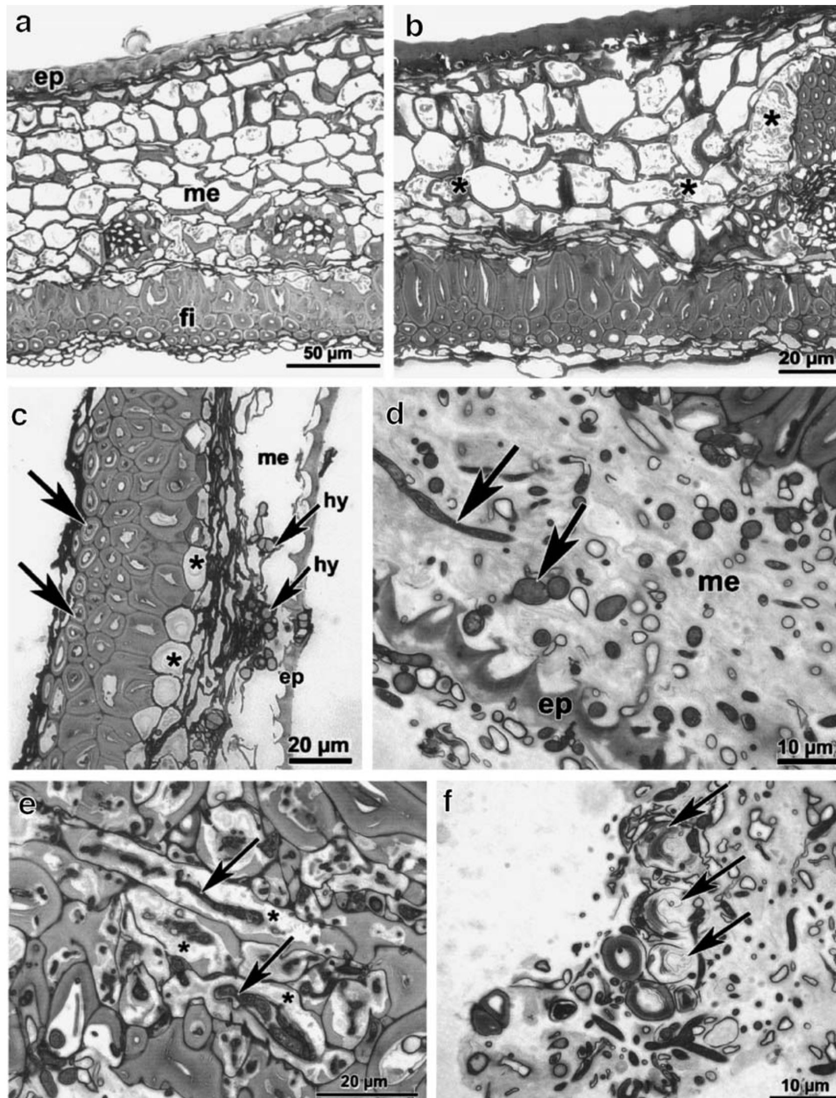

Fig. 3. Light micrographs of pericarp sections of *C. mopane* in various stages of decomposition. (a) Incubated pericarp section of 2.5 months mature brown pods with no endophytes emerging (control), (b) Unincubated 9 months pericarp section showing initial stages of decomposition in mesophyll (*), (c) 23 months unincubated pericarp section showing extensive decomposition of the mesophyll as well as the outer layer of fibres (*) and the inner layers (arrows). An abundance of fungal hyphae is present in the mesophyll region, beneath the epidermis, (d) Complete decomposition of the mesophyll (me) by *Alternaria* hyphae (arrows) with remaining epidermal walls, (e) Incubated 9 months pericarp section showing proliferous colonisation of fibres by *Alternaria* (arrows) and decomposition of lignified walls (*). (f) Lignified mesophyll cells decomposed by *Alternaria*, showing swollen state of remaining cell components (arrows). ep, epidermis; hy, hyphae; me, mesophyll.

Mesophyll cell walls that had been broken down by *Alternaria* often appeared swollen (Fig. 3f).

Fine structural studies supported the evidence that *Alternaria* sp. had the ability to degrade the lignified walls of fibres in 9-month pod pericarps. Cell wall areas that were in close contact with hyphae displayed evidence of enzymatic degradation by the fungus (Fig. 4a–d). Some areas appeared to be etched by the action of lignocellulolytic enzymes secreted by the fungus (Fig. 4a), while other areas were completely broken down or in various stages of decomposition. Some cell walls only had a granular component remaining due to the action of lignocellulolytic enzymes (Fig. 4b). Hyphae of *Alternaria* were able to penetrate and grow right through the lignified walls as was evidenced by the numerous holes in the cell walls (Fig. 4c,d). Lignified mesophyll cell walls were almost completely degraded by *Alternaria*. The fine structure of the lignified walls showed that they were degraded to a much larger extent than the lignified walls of fibres. Only granular and fibrillar substances remained where the lignified component used to be and the cell walls were freely traversed by hyphae (Fig. 6a).

The other two isolated species; *Phoma* and *Phomopsis* also appeared to have lignin-degrading abilities, but to a lesser extent. Breakdown of lignified mesophyll cell walls were conspicuous in pericarp sections from pericarps of all 2–9-month pods that only had *Phoma* associated with the tissues (Fig. 5a). As with *Phomopsis*, the cell walls had a typical “ghost” appearance in light micrographs, indicating the breakdown of some components of the walls (Fig. 5b). Unlike *Alternaria* and *Phomopsis* however, the distribution of *Phoma* was confined to the mesophyll layer (Fig. 5c). The fibre layer

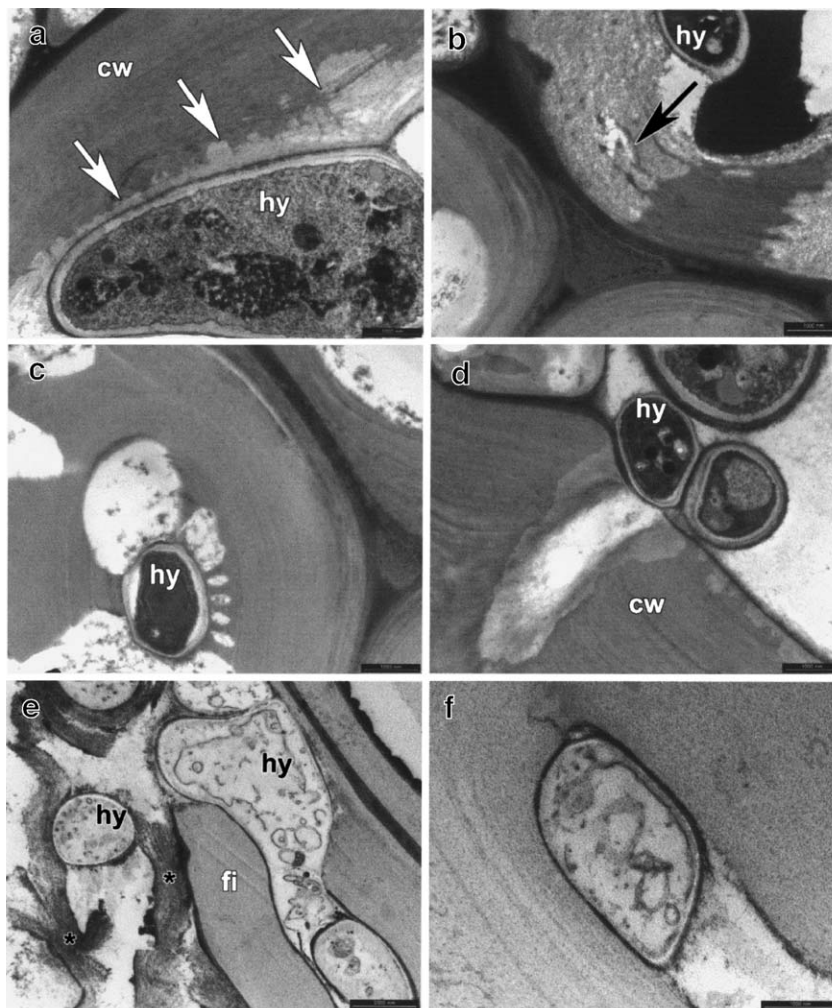

Fig. 4. Transmission electron micrographs of *Alternaria* and *Phomopsis* in association with the lignified walls of fibres in 9 months brown incubated pods. (a) Hyphae of *Alternaria* appressed to lignified wall showing etching of wall due to probable enzymatic action (arrows). (b) Granular fibrillar component remaining (arrow) after enzymatic action of *Alternaria* hyphae. (c) Digestion of cell walls resulting in holes due to action of *Alternaria* hyphae (hy). (d) Penetration of *Alternaria* hyphae into lignified cell wall of fibre. (e) *Phomopsis* hyphae occupying lumen of fibre. Degradation of adjacent lignified mesophyll cells (*) by *Phomopsis*. (f) *Phomopsis* hyphae in lumen of fibre but no ultrastructural evidence of breakdown of wall. cw, cell wall; hy, hyphae.

was intact and no evidence was found that this fungus had the ability to use this layer as a carbon source or to colonise the tissue (Fig. 5c). The fine structure of the lignified cell walls associated with *Phoma* also showed some evidence of enzymatic breakdown by the fungus. Areas in close contact with fungal hyphae appeared more electron-translucent, indicating dissolution of cell components due to enzymatic breakdown (Fig. 6b).

In the case of *Phomopsis*, isolated from the 9-month brown pods, this fungus also attacked lignified mesophyll cell walls (Fig. 5d) but to a lesser extent than *Alternaria*. In light micrographs, only a faint outline of where the lignified mesophyll cell wall used to be was often visible (Fig. 5e). This also resulted in "ghost" cell walls. Unlike *Phoma*, *Phomopsis* was able to colonise fibres, but in light micrographs no clear evidence of the breakdown of fibres was noted. There was also no clear ultrastructural evidence that this fungus had the ability to degrade the highly lignified walls of fibres even though hyphae were closely associated with them (Fig. 4e,f). However, there was a dramatic distinction at the boundary between the fibres and mesophyll cells in that the fungus clearly broke down the walls of the latter (Fig. 4e).

Fine structural studies showed that during the initial stages of the breakdown of mesophyll cell walls, the inner layers first became granular and fibrillar, confirming some enzymatic action by *Phomopsis* (Fig. 6c). Later, the entire fine structure of the "ghost" cell walls seen in light micrographs appeared swollen, granular and traversed by fine electron dense lamellae (Fig. 6d). The lamellae probably represent cellulose microfibrils that have become more visible after the breakdown of the lignin component. These cell walls were always associated with conspicuous hyphae that were closely appressed to the cell wall (Fig. 6d).

4. Discussion

In this study, the definition of the fungi being isolated has been a problem. This is because the pod pericarp is not strictly a

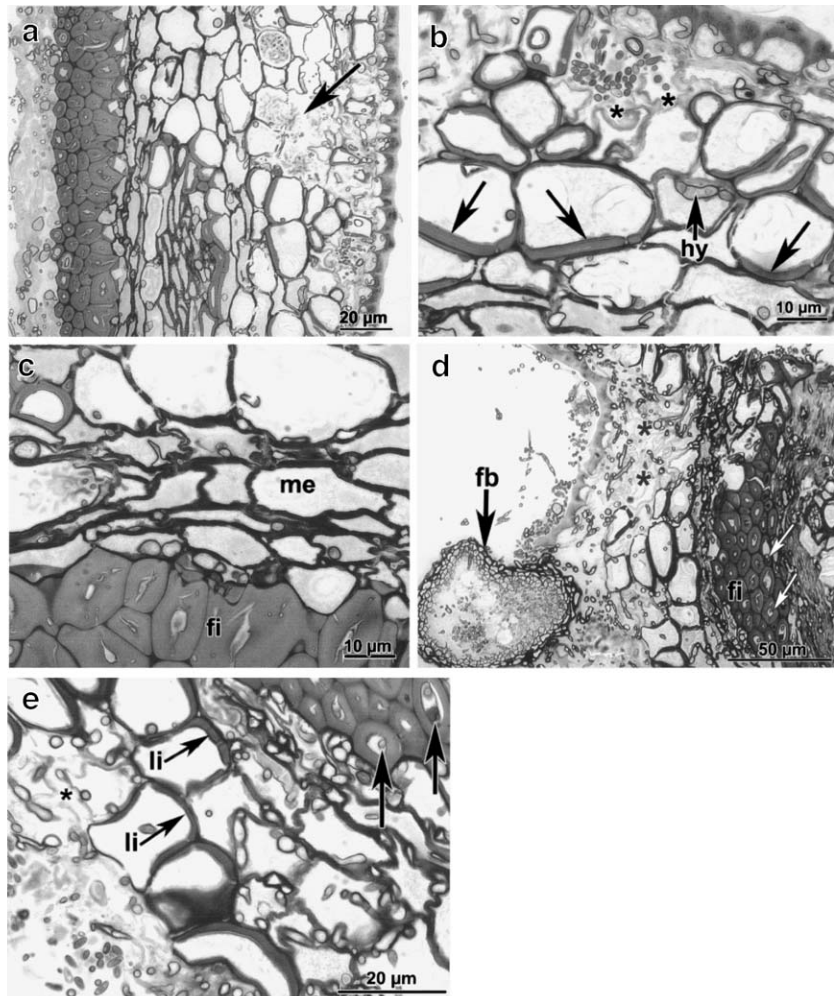

Fig. 5. Incubated pericarp sections of 9 months brown pods yielding either *Phoma* or *Phomopsis*. (a) *Phoma* occupying mesophyll and causing breakdown of lignified walls of fibres (arrow). (b) Hyphae (hy) of *Phoma* within mesophyll cells (*). Arrows indicate lignified walls that are still intact. (c) Hyphae of *Phoma* colonising the mesophyll (arrows) but absent from fibres. (d) *Phomopsis* with fruiting body causing breakdown of mesophyll (*) as well as colonising fibres (arrows). (e) *Phomopsis* hyphae in lignified mesophyll cells as well as in lumen of fibres (arrows). (*) indicates "ghost" cell wall being broken down. fb, fruiting body; fi, fibres; hy, hyphae; li, lignin; me, mesophyll.

living tissue once the green pods turn yellow. The tissue may therefore be in the initial stages of senescence, which is part of the normal maturation process of pod development. After pod fill, while the pericarp tissues are still green and beginning to turn yellow, lignification of various tissues such as the epidermis and mesophyll occurs. This is associated with loss of cytoplasm from some of these cells, which may indicate that some of the pericarp tissues may be in the process of loosing their viability, but overall the pod is still alive/functional (Jordaan and Krüger, 2005). Only pericarp tissues of pods that have turned brown have lost their viability.

The pericarp is first colonised while the pods are becoming yellow (i.e. still alive/functional) (Jordaan, unpublished observations), therefore according to the definition of endophytes stated previously, the fungi are thus endophytes. Consequently it seems correct to refer to these fungi as endophytes, even though most of the study is concerned with the colonisation of essentially dead pericarp tissue. Furthermore, it must be taken into account that the green pods used in this study were still very young as they were sampled before pod fill. They, therefore, may not have been colonised via horizontal infection. It is only during the latter stages of pod fill that the green pods become lignified. Then the pod is most susceptible to colonisation because lignification of the epidermis causes the guard cells to lose their ability to regulate opening and closing of stomata, resulting in stomata staying permanently open (Jordaan and Krüger, 2005). As was demonstrated in the SEM micrographs, the open stomata appear to be an easy route of penetration of fungi into the pericarp. A study undertaken of endophytes from different aged leaves (young, mature senescent and dead) and pods (Taylor et al., 2002) revealed that the same fungi were found to colonise living leaf tissues as were isolated from the various aged pods in the present study.

Endophytes have been shown to produce the enzymes necessary to infect and colonise host plants (Carroll and Petrini, 1983; Sieber-Canavesi and Sieber, 1993; Urairuj et al., 2003), and to become established to varying degrees. In the above ground tissues of some hosts, endophyte infections are restricted (Stone, 1987, 1988; Stone et al., 1994; Stone and Petrini, 1997), whereas other studies have demonstrated more

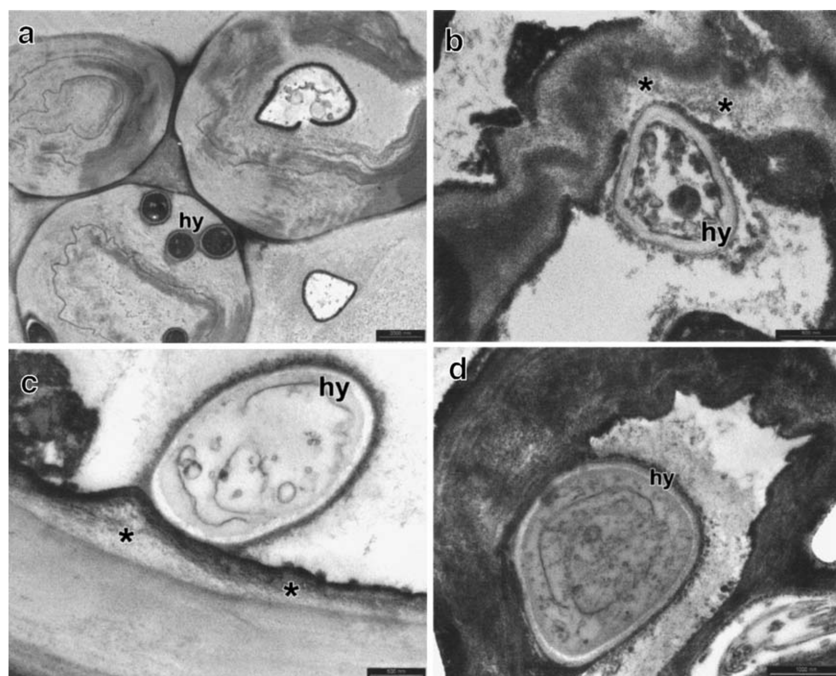

Fig. 6. Transmission electron micrographs of *Alternaria*, *Phoma* and *Phomopsis* causing breakdown of lignified mesophyll cell walls in incubated 9 mo pericarp sections. (a) *Alternaria* with hyphae growing through partially digested and swollen walls with lignin component probably removed. (b) *Phoma* associated with electron lucent region (*) due to enzymatic action of the fungus. (c) *Phomopsis* in the initial stages of causing breakdown of inner layer of lignified mesophyll cell wall (*). (d) More advanced breakdown of lignified mesophyll cell wall by *Phomopsis* hyphae. hy, hyphae.

extensive colonisation (Deckert et al., 2001). In plant pathology literature, there are similar accounts of pathogens invading plant tissues, and then remaining latent (Sinclair and Cerkaukas, 1996), but when these pathogens become active they develop symptoms of disease, thus distinguishing them as latent pathogens and not purely mutualistic endophytes (Sinclair and Cerkaukas, 1996).

There are a number of reports of endophytes being capable of litter degradation of their hosts (Carroll and Petrini, 1983), that is of endophytes becoming active after senescence and continuing even after leaf fall (Wildman and Parkinson, 1979; Cabral, 1985; Sieber-Canavesi and Sieber, 1993; Hata et al., 1998; Kumaresan and Suryanarayanan, 2002; Osono, 2002). Recently, several studies have directly investigated the decomposing abilities of the endophytes at different stages of the succession using enzyme assays and decomposition tests (Osono and Takeda, 1999; Muller et al., 2001; Urairuj et al., 2003).

The aforementioned studies bear some relevance to the research carried out in the present study. In terms of diversity and abundance, over time the major Mopane fungal taxa seem to show a succession demonstrated by a relative change in abundance. Also, the numbers of isolates increase until the 9-month brown-pod stage and they decreases, probably due to changes in fungal abundance possibly resulting from diminishing resources. Similar studies have directly investigated succession trends of endophytes in relation to the relative abilities of the different fungi to decompose plant tissues, and their ultimate role in litter degradation. Litter degradation may be a very important, but overlooked, ecological role of endophytes.

The differing abilities of endophytes to utilise various host substrate components and the associated changes in fungal succession and colonisation of tissues have been clearly demonstrated in previous studies (Osono, 2002; Osono and Takeda, 2001, 2002; Kumaresan and Suryanarayanan, 2002; Sieber-Canavesi and Sieber, 1993). For instance Osono and Takeda (2002) demonstrated that changes in fungal succession were related to available resources in the leaves (lignin and carbohydrates) and fungal succession and colonisation were a result of the different abilities of the fungi to utilise the various substrates.

The above-mentioned studies (Osono, 2002; Osono and Takeda, 2001, 2002; Kumaresan and Suryanarayanan, 2002; Sieber-Canavesi and Sieber, 1993) suggested that certain endophytic fungi may have a greater role in litter degradation than previously acknowledged. It was speculated that the lignocellulolytic abilities of the endophytic fungi would be necessary to enable them to colonise the interior of the leaf and that the fungi would maintain a presence in senescent and dead leaves, and be in a position to exploit the various carbon compounds, including recalcitrant lignocellulose resources, and thus play a part in litter decomposition (Osono and Takeda, 1999). In other substrate utilisation studies it has been demonstrated that endophytes which colonise different tissue types have varying lignocellulolytic abilities and that the patterns of substrate utilisation may enable partitioning of the substrate resources (Carroll and Petrini, 1983).

In the present study, tissue specialisation was recorded and substrate utilisation patterns appear to be dependent on the availability of the constituents of the tissues over time. The ability of the endophytes to penetrate the Mopane pod pericarp and infect tissues was directly demonstrated by microscopy studies and indirectly by lignocellulolytic assays. The

endophytes are considered to result from horizontal infection, as none was isolated from very young green pods. Thus, during the earliest stages of pod development they are entirely absent. However, these fungi have been isolated from mature green pods (Jordaan, unpublished observations) and in the present study many were isolated from yellow (2 months) and brown pods (2.5 months) and they continue to increase in abundance until the pods mature. However while they remain on the tree, the relative abundance of the different fungi changes over time.

The extent of breakdown and colonisation of lignified tissues were distinct to each of the three dominant fungal species isolated. The presence of *Alternaria* sp. in incubated pericarp sections was always associated with the breakdown of both lignified mesophyll cell walls as well as heavily lignified fibres, suggesting an ability to secrete lignocellulolytic enzymes and to degrade lignin. The data are supported by the lignocellulolytic tests. Lignified mesophyll cells appeared to be less resistant to enzymatic breakdown as they were in a far more advanced state of degradation than the walls of fibres.

The TEM results in this study are significant in terms of demonstrating apparent ultrastructural degradation of lignin cell walls associated with hyphae, of all three dominant isolates. Lignin is a strong cell wall polymer that is generally considered as being resistant to most fungi (Brett and Waldron, 1990). In addition, the lignified mesophyll cell walls appear to be less resistant to breakdown than the lignified walls of the fibres as only *Alternaria* had the ability to break down the latter. This may be due to differences in composition, structural linkages and whether the lignin is of the condensed or the uncondensed type (Lewis and Paice, 1989). Lignin is water impermeable and insoluble in water (Fry, 2004). Mesophyll cell walls that had been broken down by *Alternaria* often appeared swollen, indicating that the lignin component had been removed. Therefore, the remaining wall components that often appear are sponge-like and swollen due to the uptake of water.

The major route whereby endophytes gain entry into Mopane pods may be through the stomata or cuticle. In the latter case, subcuticular hyphae were sometimes seen. *Rhabdocline parkeri* Sherwood, J.K. Stone and G.C. Carroll, an endophyte of Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) also enters the host directly through the cuticle and cell wall (Stone, 1987). Subcuticular penetration was also seen during the infection of *Fagus sylvatica* L. leaves by the endophyte *Discula umbrinella* (Berk. and Broome) M. Morelet (Viret et al., 1993). Unlike young green mopane pods, the epidermis, including the guard cells of mature brown pods are highly lignified (Jordaan and Krüger, 2005), resulting in the stomata being permanently open, thereby serving as a route for fungal infection.

The changes in the abundance and diversity of the endophytes are therefore apparently related to the age and degree of decay of the pods, and are determined by the lignocellulolytic abilities of the fungi. It would appear that all three endophytes show lignocellulolytic activities. *Alternaria* demonstrates the greatest ability as it breaks down more heavily lignified tissue than *Phoma* and *Phomopsis*, although the lignocellulolytic assays were inconclusive being qualitative, but at least positively demonstrated the lignocellulolytic abilities of the fungi. A possible scenario for the action of the fungi is as follows: *Phoma* and *Phomopsis* initially dominate but these fungi possibly decrease because the substrate that they are able to degrade is already fully utilised and they do not have the abilities to degrade the more heavily lignified tissues. *Alternaria* on the other hand can utilise heavily lignified tissues, thus it persists and increases over time. Miscellaneous soil fungi may eventually replace all the 'endophytes'.

One possible advantageous role of endophytes in mopane is that *Phoma* and *Phomopsis* may act as early colonisers that degrade the pericarp, thus allowing the pericarp to become water permeable. This allows effective germination of seeds in an arid environment when conditions are favourable. As colonisation continues (i.e. by *Alternaria*), the protective pericarp becomes much more deteriorated and the seed loses viability. This is reflected by the short-term reproductive strategy of Mopane, in that it produces large quantities of viable but not particularly robust seeds with large cotyledons. Rapid recycling of seeds that do not germinate would add to nutrients available for the following year's growth. In contrast to this observation, Wilson (1993) suggested that endophytes may have actually caused the evolution of certain plant defences such as hard-shelled seeds, which may have evolved as a response to, among other things, attack by fungi.

C. mopane produces many large, one-seeded pods but there is no permanent seed bank. Therefore, effective decomposition of pods and non-germinated seeds and a resultant recycling of nutrients may be of prime importance to this species and other species that benefit from the resources released. As the pericarps of pods consist of highly lignified tissue, endophytes that display lignocellulolytic abilities may significantly contribute to the decay of pods.

References

Abdel-Raheem, A., Shearer, C.A., 2002. Extracellular enzyme production by freshwater ascomycetes. Fungal Diversity 11, 1–19.

Ahlholm, J., Helander, M., Elamo, P., Saloniemi, I., Neuvonen, S., Hanhimaki, S., Saikkonen, K., 2002. Micro-fungi and invertebrate herbivores on birch trees: fungal mediated plant–herbivore interactions or responses to host quality? Ecology Letters 5, 648–655.

Azevedo, J.L., Maccheroni Jr., W, Pereira, J.O., de Araújo, W.L. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electronic Journal of Biotechnology 3. http://www.ejbiotechnology.info/content/vol3/issue1/full/4/. 1 April 15 2000 [cited 8 June 2004].

Brett, C., Waldron, K., 1990. Physiology and Biochemistry of Plant Cell Walls. Unwin Hyman, London.

Cabral, D., 1985. Phyllosphere of *Eucalyptus viminalis*: dynamics of fungal populations. Transactions of the British Mycological Society 83, 501–511.

Calhoun, L.A., Findlay, J.A., Miller, J.D., Whitney, N.J., 1992. Metabolites toxic to spruce budworm from balsam fir needle endophytes. Mycological Research 96, 281–286.

Carroll, G.C., 1991. Fungal associates of woody plants as insect antagonists in leaves and stems. In: Barbosa, P., Krischik, V.A., Jones, C.G. (Eds.), Microbial Mediation of Plant–Herbivore Interactions. John Wiley, New York, pp. 253–271.

Carroll, G.C., Petrini, O., 1983. Patterns of substrate utilisation by some fungal endophytes from coniferous foliage. Mycologia 75, 53–63.

Coates Palgrave, M., 2002. Keith Coates Palgrave Trees of Southern Africa. Struik Publishers, South Africa.

Deckert, R.J., Melville, L.H., Peterson, R.L., 2001. Structural features of a *Lophodermium endophyte* during the cryptic life-cycle phase in the foliage of *Pinus strobus*. Mycological Research 105, 991–997.

Dorworth, C.E., Callan, B.E., 1996. Manipulation of endophytic fungi to promote their utility as vegetation biocontrol agents. In: Redlin, S.C., Carris, L.M. (Eds.), Endophytic Fungi in Grasses and Woody Plants. APS Press, Minnesota, pp. 209–216.

Findlay, J.A., Li, G., Penner, P., Miller, J.D., 1995. Novel diterpenoid insect toxins from a conifer endophyte. Journal of Natural Products 58, 197–200.

Fry, S.C., 2004. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytologists 161, 641–675.

Gange, A.C., 1996. Positive effects of endophyte infection on sycamore aphids. Oikos 75, 500–510.

Hammon, K.E., Faeth, S.H., 1992. Ecology of plant–herbivore communities: a fungal component. Natural Toxins 1, 197–208.

Hata, K., Futai, K., Tsuda, M., 1998. Seasonal and needle age-dependent changes of the endophytic mycobiota in *Pinus thunbergii* and *Pinus densiflora* needles. Canadian Journal of Botany 76, 245–250.

Johnson, S.N., Douglas, A.E., Woodward, S., Hartley, S.E., 2003. Microbial impacts on plant–herbivore interactions: the indirect effects of a birch pathogen on a birch aphid. Oecologia 134, 388–396.

Jordaan, A., Krüger, H., 2005. Development and functional anatomy of pods of *Colophospermum mopane*. Australian Journal of Botany 53, 55–67.

Kriel, W.M., Swart, W.J., Crous, P.W., 2000. Foliar endophytes and their interactions with host plants, with specific reference to the Gymnospermae. In: Callow, J.A. (Ed.), Advances in Botanical Research, vol. 33. Academic Press, London, pp. 1–34.

Kumaresan, V., Suryanarayanan, T.S., 2002. Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Diversity 9, 81–91.

Lappalainen, J.H., Koricheva, J., Helander, M.L., Haukioja, E., 1999. Densities of endophytic fungi and performance of leafminers (Lepidoptera: Eriocraniidae) on birch along a pollution gradient. Environmental Pollution 104, 99–105.

Lewis, N.G., Paice, M.G., 1989. Plant Cell Wall Polymers. American Chemical Society, Washington.

Macala, J.P., 1996. *Colophospermum mopane*: a fodder tree for feeding livestock. PHANE, Proceedings of the First Multidisciplinary Symposium on Phane. University of Botswana, Gaborone, pp. 34–39.

Miller, J.D., 1986. Toxic metabolites of epiphytic and endophytic fungi of conifer needles. In: Fokkema, N.J., Heuvel, J.V.D. (Eds.), Microbiology of the Phyllosphere. Cambridge University Press, Cambridge, pp. 223–231.

Miller, J.D., Mackenzie, S., Foto, M., Adams, G.W., Findlay, J.A., 2002. Needles of white spruce inoculated with rugulosin-producing endophytes contain rugulosin reducing spruce budworm growth rate. Mycological Research 106, 471–479.

Muller, M.M., Valjakka, R., Suokko, A., Hantula, J., 2001. Diversity of endophytic fungi of single Norway spruce needles and their role as pioneer decomposers. Molecular Ecology 10, 1801–1810.

Osono, T., 2002. Phyllosphere fungi on leaf litter of *Fagus crenata*: occurrence, colonization, and succession. Canadian Journal of Botany 80, 460–469.

Osono, T., Takeda, H., 1999. Decomposing ability of interior and surface fungal colonizers of beech leaves with reference to lignin decomposition. European Journal of Soil Biology 35, 51–56.

Osono, T., Takeda, H., 2001. Organic chemical and nutrient dynamics in decomposing beech leaf litter in relation to fungal ingrowths and succession during three year decomposition processes in a cool temperate forest in Japan. Ecological Research 16, 649–670.

Osono, T., Takeda, H., 2002. Comparison of litter decomposing ability among diverse fungi in a cool temperate deciduous forest in Japan. Mycologia 94, 421–427.

Petrini, O., Sieber, T.N., Toti, L., Vivet, O., 1992. Ecology, metabolite production and substrate utilisation in endophytic fungi. Natural Toxins 1, 185–196.

Pointing, S.B., 1999. Qualitative methods for determining lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2, 17–33.

Schulz, B., Boyle, C., Draeger, S., Rommert, A.K., Krohn, K., 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research 106, 996–1004.

Sieber-Canavesi, F., Sieber, T.N., 1993. Successional patterns of fungal communities in needles of European silver fur (*Abies alba* Mill.). New Phytologist 125, 149–161.

Sinclair, J.B., Cerkaukas, R.F., 1996. Latent infection vs. endophytic colonisation by fungi. In: Redlin, S.C., Carris, L.M. (Eds.), Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution. A. P.S. Press, Minnesota, pp. 3–30.

Sinclair, R.C., Jefwa, J., Maghembe, J., Eicker, A., 1998. Arbuscular mycorrhizal fungi of Africa: taxonomy and distribution with comments on ecologyICOM II. Second International Conference on Mycorrhiza. Uppsala, Sweden, 5–10 July.

Smith, P.P., Shah-Smith, D.A., 1999. An investigation into the relationship between physical damage and fungal infection in *Colophospermum mopane*. African Journal of Ecology 37, 27–37.

Stone, J.K., 1987. Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas fir. Canadian Journal of Botany 65, 2614–2621.

Stone, J.K., 1988. Fine structure of latent infections by *Rhabdocline parkeri* on Douglas-fir, with observations on uninfected epidermal cells. Canadian Journal of Botany 66, 45–54.

Stone, J.K., Petrini, O., 1997. Endophytes of forest trees: a model for fungus–plant interactions. In: Carroll, G.C., Tudzynski, P. (Eds.), The Mycota V Part B Plant Relationships. Springer-Verlag, Berlin, pp. 129–140.

Stone, J.K., Viret, O., Petrini, O., Chapela, I.H., 1994. Histological studies on host penetration and colonisation by endophytic fungi. In: Petrini, O., Ouellette, G.B. (Eds.), Alteration of Host Walls by Fungi. Proceedings of the Symposium held at the 6th International Congress of Plant Pathology. Montreal, 1993. St. Paul, U.S.A., pp. 115–126.

Stone, J.K., Bacon, C.W., White Jr., J.F., 2000. An overview of endophytic microbes: endophytism defined. In: Bacon, C.W., White Jr., J.F. (Eds.), Microbial Endophytes. Marcel Dekker, New York, pp. 3–29.

Strobel, G.A., Long, D.M., 1998. Endophytic microbes embody pharmaceutical potential. ASM News 64, 263–268.

Tan, R.X., Zou, W.X., 2001. Endophytes: a rich source of functional metabolites. Natural Product Reports 18, 448–459.

Taylor, J.E., Bojosi, B.W., Jordaan, A., 2002. A preliminary study of the prevalence of endophytes in leaves and seed pods of *Colophospermum mopane* (Leguminosae) in Botswana. Seventh International Mycological Congress, Oslo, Norway.

Timberlake, J., 1997. A review of the ecology and management of *Colophospermum mopane*. Proceedings of a Workshop on the Management of Mopane in Southern Africa. Ogongo Agricultural College, Namibia, pp. 106–110.

Tomita, F., 2003. Endophytes in Southeast Asia and Japan: their taxonomic diversity and potential applications. Fungal Diversity 14, 187–204.

Urairuj, C., Khanongnuch, C., Lumyong, S., 2003. Ligninolytic enzymes from tropical endophytic Xylariaceae. Fungal Diversity 13, 209–219.

Viret, O., Scheidegger, C., Petrini, O., 1993. Infection of beech leaves (*Fagus sylvatica*) by the endophyte *Discula umbrinella* (teleomorph, *Apiognomonia errabunda*)-low temperature scanning electron microscopy studies. Canadian Journal of Botany 71, 1520–1527.

Whiteside, J.O., 1966. A revised list of plant diseases in Rhodesia. Kirkia 5, 87–196.

Wildman, H.G., Parkinson, D., 1979. Microfungal succession on living leaves of *Populus tremuloides*. Canadian Journal of Botany 57, 2800–2811.

Wilson, D., 1993. Fungal endophytes: out of sight but should not be out of mind. Oikos 68, 379–384.

Wilson, D., 2000. Ecology of woody plant endophytes. In: Bacon, C.W., White Jr., J.F. (Eds.), Microbial Endophytes. Marcel Dekker, New York, pp. 389–420.