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Joanna F. Dames · C.J. Straker · Mary C. Scholes

Ecological and anatomical characterization of some *Pinus patula* ectomycorrhizas from Mpumalanga, South Africa

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Abstract Ectomycorrhizal (ECM) fungi are an important component of the *Pinus patula* Schlechdt. et Cham. forest ecosystem in Mpumalanga, South Africa. ECM roots are intimately associated with accumulated litter on the forest floor and four ECM isolates where examined to determine whether they provide plant access to inorganic and organic sources of nitrogen (N) and inorganic, complexed inorganic and organic sources of phosphorus (P). In in vitro studies, all isolates were found to utilize most of the organic N compounds and organic or complexed inorganic P compounds supplied. Thus, ECM fungi could play a significant role in providing N and P to *P. patula*, especially from sources to which the host plant would not normally have access. Temperature sensitivities and pH optima of the four isolates differed. Of the ECM isolates WITS 01 and WITS 06 were collected from a high-litter site; WITS 01 mycorrhizas, identified as *Scleroderma citrinum*, were white, smooth and dichotomously branched with smooth, pale yellow, differentiated rhizomorphs. The mantle was plectenchymatous with outer and inner layers showing ring-like arrangements of hyphal bundles. The Hartig net had a palmetti shape. The WITS 02 (not identified) mycorrhizas were brown with lighter coloured root tips, with simple to dichotomous branching, smooth with no distinct mantle and sparse hyphae occurred on the root surface. The Hartig net was palmetti type with lobed haustoria. The results are discussed in relation to ECM distribution and function in nutrient cycling.

Key words Anatomy · Ectomycorrhizas · Morphology · *Pinus patula* · P and N utilization · pH and temperature

J.F. Dames (✉) · C.J. Straker · M.C. Scholes
Botany Department, University of the Witwatersrand, Private Bag 3, WITS, 2050, Johannesburg, South Africa
e-mail: Dames@rhobot.ru.ac.za, Fax: +27-46-622-5524

Present address:
Botany Department, Rhodes University, PO Box 94, Grahamstown, 6140 South Africa

Introduction

The accumulation of litter under *Pinus patula* Schlechdt. et Cham. in the Mpumalanga province of South Africa has been shown to be correlated with altitude, with thicker litter layers occurring at high-altitude sites. Thus climatic factors (cool and wet) associated with higher altitudes appear to markedly influence decomposition and nutrient cycling (Dames 1997; Schutz 1990). The accumulated litter layer under *P. patula* is very acidic, with pH 3.26 in the upper and pH 2.16 in the lower layers (Dames 1997). The acidic nature of the *Pinus* litter probably contributes to its slow decomposition as the decomposing enzymes, such as cellulases and amylases, are sensitive to changes in pH (Alexander 1977). In Mpumalanga, concern about the effects of acid rain on soil fertility and acidification has led to the application of dolomitic lime on sites with accumulated litter layers (De Ronde 1991; Olbrich 1995).

It is well established that ectomycorrhizal (ECM) fungi benefit their hosts by enhancing mineral uptake thus contributing to the cycling of nutrients in the ecosystem. ECM are a major component of the accumulated litter layers under *P. patula*. Being in intimate contact with the litter (Dames 1997), the ECM roots are well placed to optimize absorption of nutrients mineralized by saprotrophic micro-organisms. Some ECM species utilize complex, organically bound nitrogen (N) compounds by producing external acid proteases that break down protein to amino acids and short chain peptides. They are thus able to utilize proteins as their sole N source (Abuzinadah and Read 1986; Abuzinadah et al. 1986; Finlay et al. 1992; Littke et al. 1984). The major N sources in litter are likely to be proteinaceous material derived from the litter and from the microbial population itself (Abuzinadah and Read 1986; Read et al. 1989).

ECM fungi also produce surface acid phosphatases of broad specificity capable of hydrolysing phytates (Antibus et al. 1992; Vogt et al. 1991). They thus decompose organic or complexed inorganic sources of

phosphorus (P) and can increase the weathering rate of P bound in mineral form as a result of increased CO_2 and organic acid production, which lowers the rhizosphere pH (Antibus et al. 1992). ECM also produce oxalic acid, which acts as a chelating agent, releasing P from compounds complexed with iron, calcium or aluminium and increasing its availability (Gianinazzi-Pearson and Gianinazzi 1989; Nye and Tinker 1977). Up to 50% of the phosphorus in forest soils may be in an organic form and a substantial portion of this may be made available by the ECM fungi (Häussling and Marschner 1989). Both N and P are important in forest ecosystems. Nitrogen is regarded as a limiting factor and there is increasing evidence that P is immobilized in the first stages of decomposition in many forests. Decreased availability of P in forest ecosystems probably results from complexing with aluminium oxides (Attiwill and Adams 1993). Access to these compounds would contribute positively to the cycling of nutrients.

Various studies of ECM in the forestry regions of South Africa have been conducted. Marais and Kotzé (1977) examined the ECM of *P. patula* at a plantation in the Mpumalanga Province and identified *Boletus edulis* and *Amanita muscaria* as being ectotrophic, with *Tuber rapaeodorum* and *Lycoperdon umbrinum* as possible ectotrophs. Van der Westhuizen and Eicker (1987) listed 19 species of basidiomycetes, considered to be ectotrophic, which occur in the major pine-growing regions of the country, 10 of which occurred in Mpumalanga. Several studies in the Mpumalanga forestry region have classified ECM roots based on morphology alone (Carlson 1992, 1994).

This paper reports an investigation into the morphology and anatomy of ECM roots collected from a high-altitude, high-litter site of *P. patula* and into the physiology of the mycobionts. The identification method followed that of Agerer (1986, 1987–1996), which has not been applied before to ECM of *Pinus* species in South Africa. Carlson (1992) showed that the population structures of ECM associated with *P. patula* roots changed in response to artificially-applied acidic rain. Therefore, we examined the growth responses of the isolated fungi to temperature and pH. The abilities of isolated ECM fungi to utilize organic and inorganic N compounds and organic and complexed inorganic P compounds as their sole source of N or P were also investigated. The results are discussed in relation to the distribution of ECM at this site and the role of ECM in nutrient cycling.

Materials and methods

Study area

The province of Mpumalanga is a major man-made forestry region situated in the eastern part of South Africa. ECM species were collected from a 42-year-old, first-rotation *P. patula* compartment at 1350 m with an accumulated litter layer of 22 cm (Dames 1997).

Ectomycorrhizal isolates

ECM fungi were isolated by hand from 10 litter cores. Two distinct types of ECM roots were observed. Approximately 30 ECM root tips of each morphological type were sterilized by immersion in 30% H_2O_2 for 1 min, macerated and plated onto 1/2-strength modified Melin Norkans (MMN) agar medium (Erland and Söderström 1990). Plates were incubated at 25 °C for 21–30 days in the dark and examined periodically for growth and contamination. In addition to the two ECM species isolated from these ECM roots (designated WITS 01 and WITS 06), two further isolates were used in this study, one from another study site in the same area (WITS 02) and the other (WITS 04) isolated previously from *P. patula* roots by C. J. Straker (personal communication).

To confirm the mycorrhizal ability of the four isolates, *P. patula* seeds were surface sterilized in 30% H_2O_2 for 15 min and germinated on water agar. The *P. patula* seedlings were placed in 750-ml jars containing a mixture of perlite and peat moistened with MMN liquid media and inoculated with agar plugs of the isolates under sterile conditions. After 3 months in a growth chamber with a day/night cycle of 25 °C/18 °C for 12 h/8 h, seedlings were examined for ECM roots.

Growth response to temperature

The four isolates were each centrally inoculated onto MMN agar plates using 7-mm agar discs and five replicate plates incubated at 7, 18 and 30 °C. Colony diameters were measured along two intersects periodically for a period of 30 days. The results were averaged and divided by the number of days to give the overall growth rates. The growth rates for each isolate at 7 °C and 30 °C were compared to the growth rate at 18 °C. Three growth-response categories were recognized for each temperature: tolerant, growth rate >50% of that at 18 °C; semi-tolerant, growth rate 20–50% of that at 18 °C; sensitive, growth rate <20% of that at 18 °C (Hutchison 1990).

Growth response to pH

The growth response of the four ECM isolates to pH was determined on MMN agar medium. The pH of a buffer salt solution and the MMN medium were adjusted after autoclaving (Erland and Söderström 1990, 1991) to give test pH values of 2, 3, 4, 5, 6 and 7. This pH range corresponds to that in litter and soil (Dames 1997) and the higher pH values following lime application. Five replicate plates of each isolate were centrally inoculated with 7-mm agar plugs onto media at each pH (Erland and Söderström 1990). Plates were incubated in the dark at 25 °C, at which temperature all isolates grew well, with the exception of WITS 06, which grew best at 18 °C. Colony diameters were measured along two transects periodically for a period of 30 days. The results were averaged and divided by the number of days to give the overall growth rates at each pH.

Utilization of N compounds

To determine the abilities of the four ECM isolates to utilize inorganic and organic forms of N, a range of N-containing compounds was incorporated into MMN medium as the sole N source. The basal medium lacked malt extract and the ammonium source (Molina and Palmer 1982) and nitrogen sources were added at 60 mgN l^{-1}, BSA was added at 243 mgN l^{-1} (Abuzinadah and Read 1986). The pH of all media was adjusted to 4.5. Nitrogen sources were inorganic NH_4^+–N supplied as $(NH_4)_2SO_4$, inorganic NO_3^-–N, supplied as KNO_3, the protein bovine serum albumin (BSA), the tri-peptide glutathione, and various amino acids of microbial origin which occur naturally in litter and have been reported in forest stands (Abuzinadah and Read 1988; Fogel

1980), DL-aspartic acid, L-glutamic acid, L-arginine, DL-serine, glycine and L-alanine. BSA does not occur naturally in litter but was included as a reference to other studies (Abuzinadah and Read 1986; Finlay et al. 1992; Littke et al. 1984). Inoculation and growth measurements were as for the pH study. Isolates were qualitatively grouped into 'protein fungi' (growth on BSA exceeded that on inorganic N), 'intermediate protein users' (similar growth on BSA and inorganic N) and 'non-protein fungi' (no or poor growth on BSA) by comparing the growth curves on BSA and $(NH_4)_2SO_4$ (Abuzinadah and Read 1986).

Utilization of P compounds

To determine the abilities of the four ECM isolates to utilize organic and complexed forms of P, a range of P-containing compounds was incorporated into MMN medium as the sole P source. The basal medium lacked KH_2PO_4 and $(NH_4)_2PO_4$ (Molina and Palmer 1982), nitrogen was supplied as $(NH_4)_2SO_4$ at 60 mgN l^{-1}, and the pH was adjusted to 4.5. Inorganic P was supplied as KH_2PO_4, complexed inorganic P as $AlPO_4$, complexed P as phytic acid, organic P as RNA (which occurs naturally in the litter of plant, microbial or animal origin) and organic P as glycerophosphate (Jayachandran et al. 1992; Lapeyrie et al. 1991). All P sources were added at 15 mgP l^{-1}. Inoculation and growth measurements were as for the pH study.

Collection of ECM roots

Approximately 30–50 roots each of WITS 01 and WITS 06 were removed from 10 litter core samples and placed in sterile, distilled water for observation of fresh material and in FAA (Agerer 1987–1996). Preserved voucher specimens have been placed in the Selmar-Schonland Herbarium, Grahamstown, South Africa.

Identification of ECM roots

Morphological characters of mycorrhizal roots were examined with a dissecting microscope (Agerer 1987–1996). Chemical tests were applied to fresh whole mycorrhizas (Zak 1973) and hand-sectioned ECM roots (Agerer 1986) using cotton-blue-lactophenol, 0.1% toluidine blue, 70% ethanol, 15% KOH, lactic acid, Melzer's reagent, IKI and sulfo-vanillin (Agerer 1986; Brundrett et al. 1994; Ingleby et al. 1990). Whole mycorrhizas mounted in lactic acid were examined with a fluorescent microscope using UV (390–420 nm) and blue (450–490 nm) filters, to assess autofluorescence (Agerer 1986, 1987–1996). Anatomical details were recorded of the mantle, rhizomorphs, emanating hyphae and cross- and longitudinal sections of the ECM roots (Agerer 1987–1996).

Tracing mycorrhizal links to sporocarps

Sporocarps occurring in the study area were recorded and identified using various identification manuals (Bottomley 1948; Buczacki 1989; Levin et al. 1987; Pearson 1950; Singer 1962; Van Der Westhuizen and Eicker 1994). Sporocarps and the litter in which they were growing were removed and any connections with rhizomorphs and mycorrhizal roots were examined using a hand-held magnifying glass.

Data analysis

To determine the effects of N and P sources, overall growth rates of isolates were subjected to one-way ANOVA. Where significant differences were evident ($P \leq 0.05$), multiple comparisons using least significant differences were conducted to establish which nutrient sources contributed to the differences observed. Statistical analyses were conducted using Statgraphics version 5. The temperature, N and P growth curves for each isolate were $\log(y+1)$ transformed to produce linear growth models. The slopes of these models were tested for homogeneity and any significant differences ($P \leq 0.05$) were subjected to multiple comparisons using the Tukey test ($k \geq 3$). In cases were $k < 3$, comparisons of slopes were made using a *t*-test (Zar 1984).

Results

Culture characteristics

Culture descriptions of the four isolates: WITS 01, slow growing, pale yellow, reverse colour white, velvety culture, margins smooth, hyphae septate with clamp connections. WITS 02, fast growing, olive green/brown, reverse colour brown, flat culture, margins smooth, hyphae septate with clamp connections; WITS 04, fluffy white, reverse colour pale yellow, aerial growth, margin irregular, hyphae joining to form yellow to brown threads, hyphae septate with clamp connections; WITS 06, growth intermediate, beige, reverse colour beige, velvety culture, margin smooth, hyphae septate with clamp connections.

All isolates were inoculated individually on to germinated *P. patula*. Seedling roots showed dichotomous branching but no mantles. Roots sectioned with a freeze microtome were examined microscopically and Hartig net formation was observed in all seedlings.

Effects of temperature on growth

Growth curves of WITS 01, WITS 02, WITS 04 and WITS 06 growing at 7, 18 and 30 °C are presented in Figures 1a–d, respectively. Because unrelated isolates were used in the study and no comparisons in growth were made between isolates, the *y*-axis scale on the growth curves are different. $\text{Log}(y+1)$ transformations of the growth curves (Table 1) yielded slightly lower or similar correlation coefficients compared with those obtained from the linear regression models. Thus the cultures were mainly in the linear growth phase. WITS 01 did not grow at 7 °C, while growth at 18 °C was slower than growth at 30 °C (Fig. 1a, Table 1). WITS 02 showed slower growth at 7 °C than at 18 °C and 30 °C (Fig. 1b, Table 1). WITS 04 grew well at 7 °C, although growth was slower than at 18 °C and 30 °C (Fig. 1c, Table 1). WITS 06 grew poorly at 7 °C, with faster growth at 18 °C and no growth at 30 °C (Fig. 1d, Table 1). Based on the overall growth rates at the three temperatures (Table 2), the isolates were categorized into tolerance groupings (Hutchison 1990).

Effects of pH on growth

The growth rates of WITS 01, WITS 02, WITS 04 and WITS 06 at various pHs are presented in Figures 2a–d, respectively. Because unrelated isolates were studied and no growth comparisons were

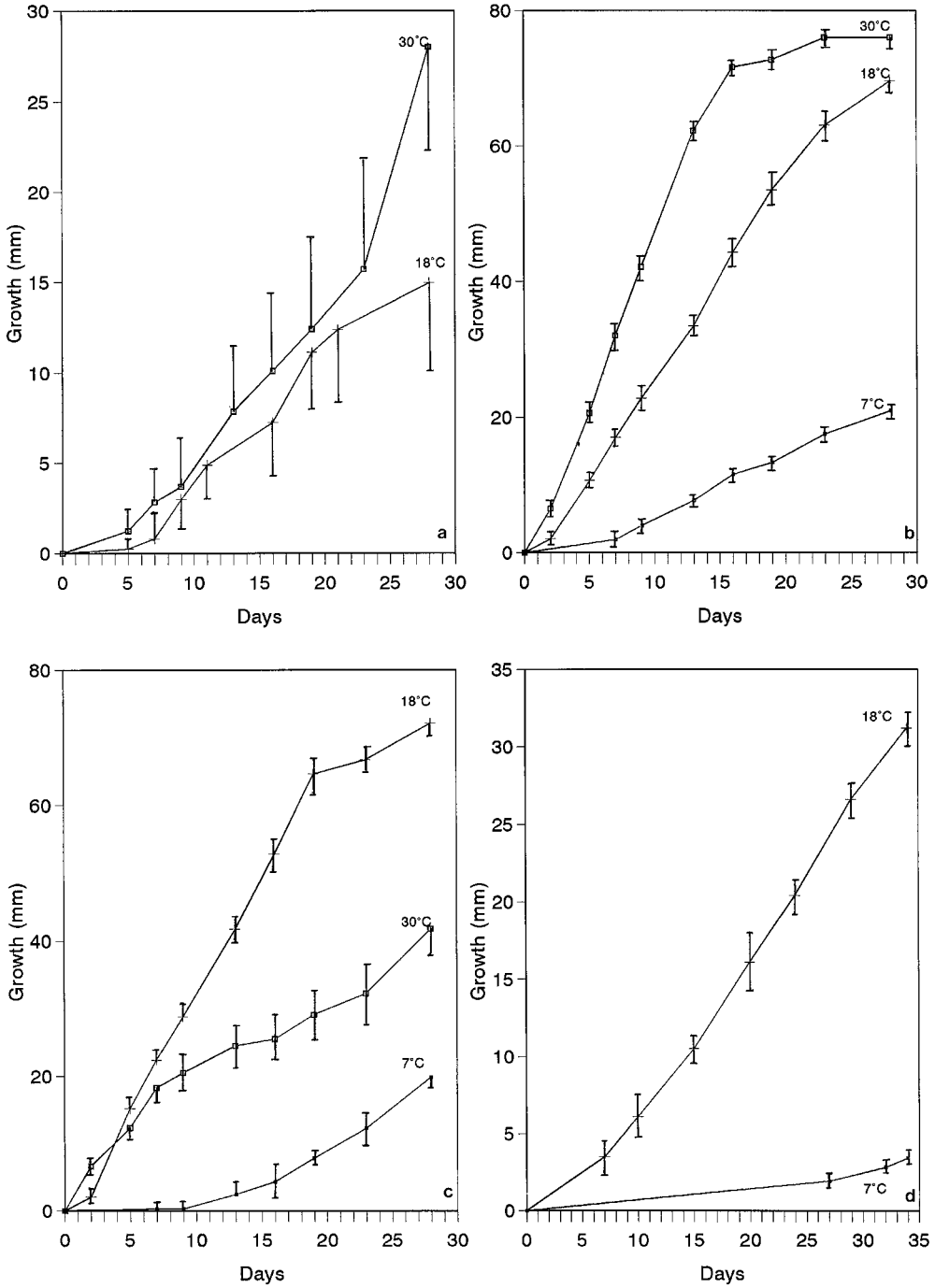

Fig. 1a–d Growth curves of ectomycorrhizal (ECM) isolates at 7 °C, 18 °C and 30 °C; **a** WITS 01 (no growth at 7 °C); **b** WITS 02; **c** WITS 04; **d** WITS 06 (no growth at 30 °C). Data points are the means of 5 replicates ± SD

Table 1 Linear growth models obtained from $\log(y+1)$ transformation of growth curves for ectomycorrhizal (ECM) isolates growing on Melin Norkans (MMN) medium at various temperatures. Growth models within rows followed by same letters are not significantly different ($P<0.05$)

Isolate	Temperature			
	7 °C	18 °C	30 °C	Significance
WITS 01	No growth	$y=0.22+0.04x$ r 1.00[a]	$y=0.13+0.05x$ r 0.94[b]	$t_{0.05,(1),14}=-2.5$
WITS 02	$y=0.15+0.05x$ r 0.97[a]	$y=0.57+0.06x$ r 0.89[b]	$y=0.69+0.07x$ r 0.82[bc]	$F_{0.05,(1),2.21}=6.5$
WITS 04	$y=-0.11+0.05x$ r 1.00[a]	$y=0.61+0.05x$ r 0.84[b]	$y=0.72+0.04x$ r 0.84[bc]	$F_{0.05,(1),2.22}=5.89$
WITS 06	$y=0.04+0.02x$ r 1.00[a]	$y=0.31+0.04x$ r 0.95[b]	no growth	$t_{0.05,(1),8}=-7.5$

Table 2 Tolerance groupings of ECM fungal isolates based on temperature responses

Isolate	7 °C	30 °C
WITS 01	Sensitive	Tolerant
WITS 02	Semi-tolerant	Tolerant
WITS 04	Semi-tolerant	Tolerant
WITS 06	Sensitive	Sensitive

made between isolates, the *y*-axis scale for each isolate is different. WITS 01 did not grow at pH 2 but showed maximal growth at pH 4–5 (Fig. 2a). WITS 02 showed maximal growth at pH 3–5 (Fig. 2b). WITS 04 did not grow at pH 2 and only poorly at pH 3. Maximal growth occurred at pH 5–7 (Fig. 2c). WITS 06 grew well at pH 2, with maximal growth pH 3–5 (Fig. 2d).

Growth on N sources

All isolates grew on the amended media, with the exception of WITS 01, which did not grow on BSA, and WITS 04, which grew extremely poorly on ammonium.

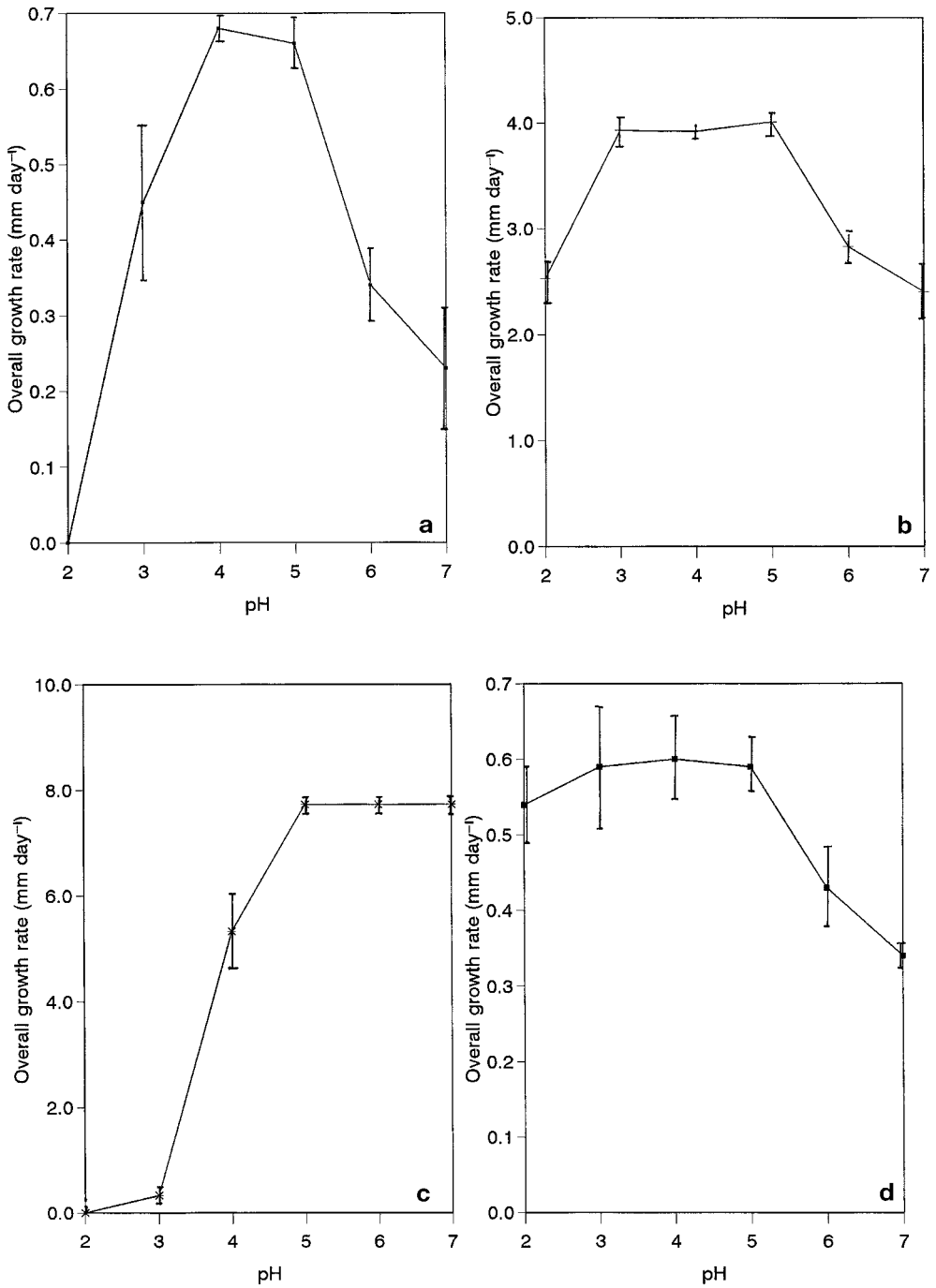

Fig. 2a–d Growth response of ECM isolates on media at different pH values; **a** WITS 01; **b** WITS 02; **c** WITS 04; **d** WITS 06. Data points are the means of 5 replicates ±SD

Table 3 Overall growth rates (mm day^{-1}) of ECM fungal isolates on media amended with various N sources. Growth rates within columns followed by the same letters are not significantly different

N source	Isolates			
	WITS 01	WITS 02	WITS 04	WITS 06
$(NH_4)_2SO_4$	0.44^{ab}	2.34^{c}	0.42^{c}	1.33^{a}
KNO_3	0.47^{a}	1.81^{d}	7.64^{a}	0.68^{h}
BSA	0.00^{d}	2.29^{c}	7.64^{a}	1.04^{de}
Glutathione	0.37^{bc}	2.98^{a}	7.54^{b}	1.22^{bc}
DL-aspartic	0.31^{c}	2.37^{bc}	7.38^{c}	0.85^{gh}
L-glutamic	0.36^{bc}	2.13^{bc}	7.40^{bc}	0.89^{fg}
L-arginine	0.40^{ab}	2.60^{ab}	7.45^{bc}	1.34^{a}
DL-serine	0.37^{bc}	2.36^{c}	7.11^{d}	1.08^{cd}
Glycine	0.43^{ab}	1.91^{d}	7.45^{bc}	1.00^{ef}
L-alanine	0.29^{c}	2.38^{bc}	7.40^{bc}	1.17^{b}
Significance tests	$F_{9\,40}=21.66$ $P\leq 0.0001$	$F_{9\,39}=10.34$ $P\leq 0.0001$	$F_{9\,40}=999.99$ $P\leq 0.0001$	$F_{9\,35}=26.99$ $P\leq 0.0001$

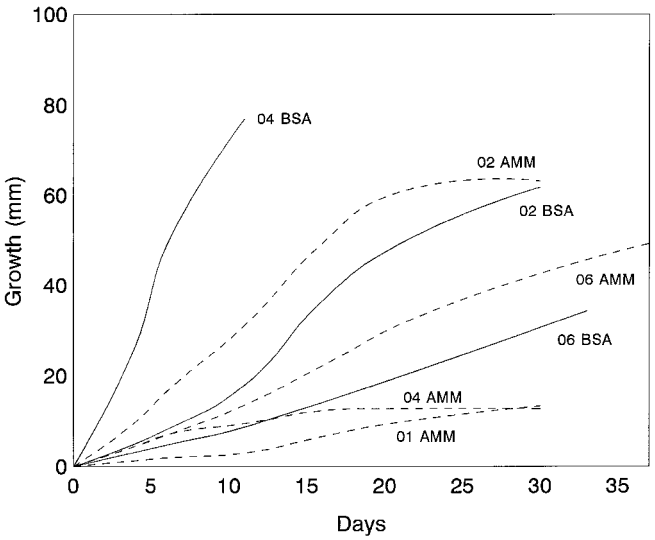

Fig. 3 Growth of ECM fungal isolates (numbered on graph) on media with $(NH_4)_2SO_4$ (*AMM*) and bovine serum albumin (*BSA*) as sole N source

Significant differences between growth rates on the various N media were evident between the isolates (Table 3). WITS 04 can be categorized as a 'protein user', as growth on BSA exceeded that on $(NH_4)_2SO_4$ (Fig. 3). WITS 02 and WITS 06 are both 'intermediate protein users', as these isolates showed similar growth on BSA and $(NH_4)_2SO_4$. WITS 01 was a 'non-protein user', being unable to grow on BSA.

Following $\log(y+1)$ transformation of the growth curves, WITS 01, WITS 02 and WITS 06 showed no significant effects of N source on their growth rates. WITS 04 showed significant differences attributable to slower growth on $(NH_4)_2SO_4$.

Growth on P sources

All isolates grew on all P-amended media (Table 4). Significant differences between growth rates on the various media were evident between the isolates. $\text{Log}(y+1)$ transformations of the growth curves showed no significant effects of P source on the growth of any isolate.

Morphology, anatomy and chemical characters of WITS 01 and WITS 06

Morphological, anatomical and chemical characters of WITS 01 and WITS 06 are presented in Table 5 and Fig. 4 and 7, respectively. Anatomical characters are presented in Fig. 5 and 6 for WITS 01 and Fig. 8 for WITS 06. Two main types of sporocarps were collected from the study site and identified as *Scleroderma citrinum* Pers. and *Boletus pinicola* Vitt.. The identifications were confirmed by G. C. A. Van Der Westhuizen. Rhizomorphs of *S. citrinum* were similar to rhizomorphs observed on WITS 01 ECM roots and connections between roots and sporocarps were confirmed. *B. pinicola*

Table 4 Overall growth rates (mm day^{-1}) of ECM fungal isolates on media amended with various P sources

P source	Isolates			
	WITS 01	WITS 02	WITS 04	WITS 06
KH_2PO_4	0.37^{a}	1.36^{a}	0.44^{bc}	0.97^{a}
$AlPO_4$	0.41^{a}	1.11^{b}	0.52^{b}	0.90^{a}
RNA	0.30^{b}	0.76^{c}	0.11^{d}	0.18^{c}
Glycerophosphate	0.20^{c}	1.20^{ab}	0.80^{a}	0.76^{b}
Phytic acid	0.33^{b}	1.17^{b}	0.38^{c}	0.87^{ab}
Significance tests	$F_{4\,20}=5.86$ $P\leq 0.003$	$F_{4\,20}=13.35$ $P\leq 0.0001$	$F_{4\,20}=31.75$ $P\leq 0.0001$	$F_{4\,20}=68.89$ $P\leq 0.001$

Table 5 Characteristics of ECM *Pinus patula* roots from Mpumalanga, South Africa

Ectomycorrhizal characteristics	Isolate WITS 01	Isolate WITS 06
Morphology – whole mycorrhiza		
Type of ramification	Dichotomous	Simple to dichotomous
Length of ramification	2294.8 ± 98.8 μm	Simple: 886.5 ± 305.9 μm Dichotomous: 1660.3 ± 622.2 μm
Length of unramified ends	814.3 ± 218.2 μm	614.0 ± 188.3 μm
Diameter of unramified ends	385.6 ± 13.0 μm	438.6 ± 36.2 μm
Shape of unramified ends	Straight	Straight, older occasionally beaded
Morphology – mantle		
Mantle description	Shiny, smooth	No distinct mantle, mycorrhiza Smooth
Mantle colour	White	
Chemical tests		
Cotton blue – lactophenol	Blue	Blue
Toluidine blue 0.1%	Mantle – no change	Blue
Ethanol 70%	No change	No change
KOH 15%	No change	No change
Lactic acid	No change	No change
Melzer's reagent	Orange	No change
IKI	No change	No change
Sulfo-vanillin	Red	Red
Autofluorescence		
390–420 nm	Entire fluoresces green	Tip fluoresces green
450–490 nm	Entire fluoresces yellow	Tip fluoresces green
Siderophilous granulations	None	None
Anatomic characteristics		
Dolipore/Woronin bodies	None	None
Lactifers	None	None
Anatomy – outer mantle		
Hyphal arrangement	Plectenchymatous, loose, evidence of ring-like arranged hyphal bundles	None
Shape of hyphal cells	Elongated, rectangular with clamps	
Thickness of walls	0.3–0.4 μm	
Length and diameter of hyphal cells	37 × 3–4 μm	
Anatomy – inner mantle		
Hyphal arrangement	Plectenchymatous, hyphae more compact, evidence of ring-like arranged hyphal bundles	
Shape of hyphal cells	Elongated, rectangular	
Thickness of walls	0.4–0.4 μm	
Length and diameter of hyphal cells	4–5 × 3–4 μm	
Rhizomorphs	Present	None
Colour	Pale yellow	
Connection with mantle	Restricted	
Margin	Smooth	
Mode of ramification	Undifferentiated young, Older differentiated, thick central hyphae, hyphae compactly woven	
Mode of hyphal connections	Amastomoses between middle part of hyphal cell and a clamp and between inverted clamps	
Arrangement of hyphae	Parallel, interwoven, branching	
Diameter of rhizomorphs	Young: 22 ± 8 μm Older: 90 ± 29 μm	
Enamating hyphae		
Occurrence	Occasional, hyaline	Sparse
Septa	Present with clamps	
Diameter	5 μm	
Thickness of wall	0.3–0.5 μm	
Cystidia	None	None

Table 5 (continued)

Ectomycorrhizal characteristics	Isolate WITS 01	Isolate WITS 06
Mantle thickness	22.7 ± 7.8 μm	
Cross section		
Measurement of mantle hyphae	Tangential: 2.8 μm Radial: 2.8–5.6 μm Wall thickness: 0.2–0.5 μm	
Root cells with Hartig net		
Tannin cells	Number of rows: 1 Shape: oval/rectangular Measurements: 11 × 12 μm	Number of rows: 1–5 Shape: rectangular Measurements: 30 × 9 μm
Cortical cells	Number of rows: 1–2 Shape: oval Measurements: 59 × 35 μm	Number of rows: 1–3 Shape: oval/circular Measurements: 35 × 40 μm
Hartig net		
Thickness of Hartig net around tannin cells	0.5–1.0 μm	0.5 μm
Thickness of Hartig net around cortical cells	2.7–5.4 μm	0.5 μm
Number of hyphal rows around tannin cells	1	1
Number of hyphal rows around cortical cells	1–2	1
Shape of Hartig net around tannin cells	Common type palmetti	Common type palmetti
Shape of Hartig net around cortical cells	Common type palmetti	Common type palmetti with lobed haustoria
Longitudinal sections		
Measurement of mantle hyphae	Tangential: 5.6 μm Radial: 5.6 μm Wall thickness: 0.3–0.5 μm	
Root cells with Hartig net		
Tannin cells	Number or rows: 1 Shape: rectangular Measurements: 25 × 12 μm	Number of rows: 1–5 Shape: oval/rectangular Measurements: 33 × 10 μm
Cortical cells	Number of rows: 1–2 Shape: oval to rectangular Measurements: 32 × 43 μm	Number of rows: 1–3 Shape: rectangular Measurements: 36 × 28 μm
Hartig net		
Thickness of Hartig net around tannin cells	1–2 μm	0.1–0.5 μm
Thickness of Hartig net around cortical cells	2–4 μm	0.28–0.56 μm
Number of hyphal rows around tannin cells	1	1
Number of hyphal rows around cortical cells	1	1
Shape of Hartig net around tannin cells	Common type palmetti	Common type palmetti
Shape of Hartig net around cortical cells	Common type palmetti	Common type palmetti with lobed haustoria

sporocarps had no rhizomorphs and connections with roots were not possible.

Discussion

The method described by Erland and Söderström (1990) was effective in isolating ECM fungi from colonized root tips. Roots of *P. patula* seedlings inoculated with the isolates showed some dichotomous branching and Hartig net formation. The formation of only a few mycorrhizal roots suggests that, under the experimental conditions used, the fungal isolates were less vigorous in the colonization of roots (Pera and Alvarez 1995).

Only two distinct morphological types of ECM root were observed in the litter and soil at the study site, not only at the time of sampling but throughout the study period. This poor mycorrhizal diversity may be caused by the lack of inoculum of other mycorrhizal types. Most mycorrhizal species presently associated with *Pinus* spp. in South Africa were introduced in litter and humus after repeated failure to establish *Pinus* spp. (Poynton 1980). Baar and De Vries (1995) in The Netherlands concluded that the removal of litter and humus from stands of *P. sylvestris* resulted in an increase in ECM types, with inoculum presumably being supplied from adjacent areas. This immigration was linked to removal of large amounts of N and phenolics with the litter and humus, which implies that the thick litter layers of the study site suppressed mycorrhizal diversity. Whether this holds true for *P. patula* and whether low-altitude, thin litter layer sites have a greater mycorrhizal diversity requires further investigation.

Conclusive identification of the ECM from the observed sporocarps and rhizomorph connections was difficult. WITS 01 was identifed as *Scleroderma citrinum* because of the direct rhizomorph connections observed. In Agerer's (1987–1996) atlas, an ECM similar to WITS 01 was described for *S. citrinum* forming a mycorrhizal association with *Betula pendula*. However, several differences were noted. The ECM roots of *Betula* showed monopodial-pyramidal branching as op-

Fig. 4a,b WITS 01 – White dichotomously branched ECM roots showing pale yellow rhizomorphs (*dbr* dichotomous branching, *m* mantle, *r* rhizomorphs) *bar* 150 μm

posed to the dichotomous branching observed on *P. patula*. Dichotomous branching of ECM roots has only been observed in *Pinus* spp. (Marks and Kozlowski 1973). The rhizomorphs from the *Betula* ECM roots were white as opposed to pale yellow; however, colour is variable, especially with age, and photographically may be dependent on the light source (Agerer 1986).

Several *Scleroderma* species are mycorrhizal with a broad host range (Miller 1982; Trappe 1962). *S. citrinum* is acidophilous and mainly distributed in raw humus and peat (Agerer 1987–1996). In South Africa, the species is widely distributed throughout the wetter parts, occurring under *Pinus* spp. (Van der Westhuizen and Eicker 1994). Although sporocarps of *Betula pinicola* were prevalent at the study site, positive connections with WITS 06 roots could not be made. The species has not been previously recorded in Mpumalanga (Levin et al. 1987; Van der Westhuizen and Eicker 1994). Pearson (1950) reported *B. pinicola* under *Pinus* in the Western Cape, the only known record of the species. Species similar to *B. pinicola* recorded in Mpumalanga include *Suillus granulatus*, *S. bovinus* and *B. edulis* (Lundquist 1986; Van der Westhuizen and Eicker 1987, 1994). *B. pinicola* has been reported under conif-

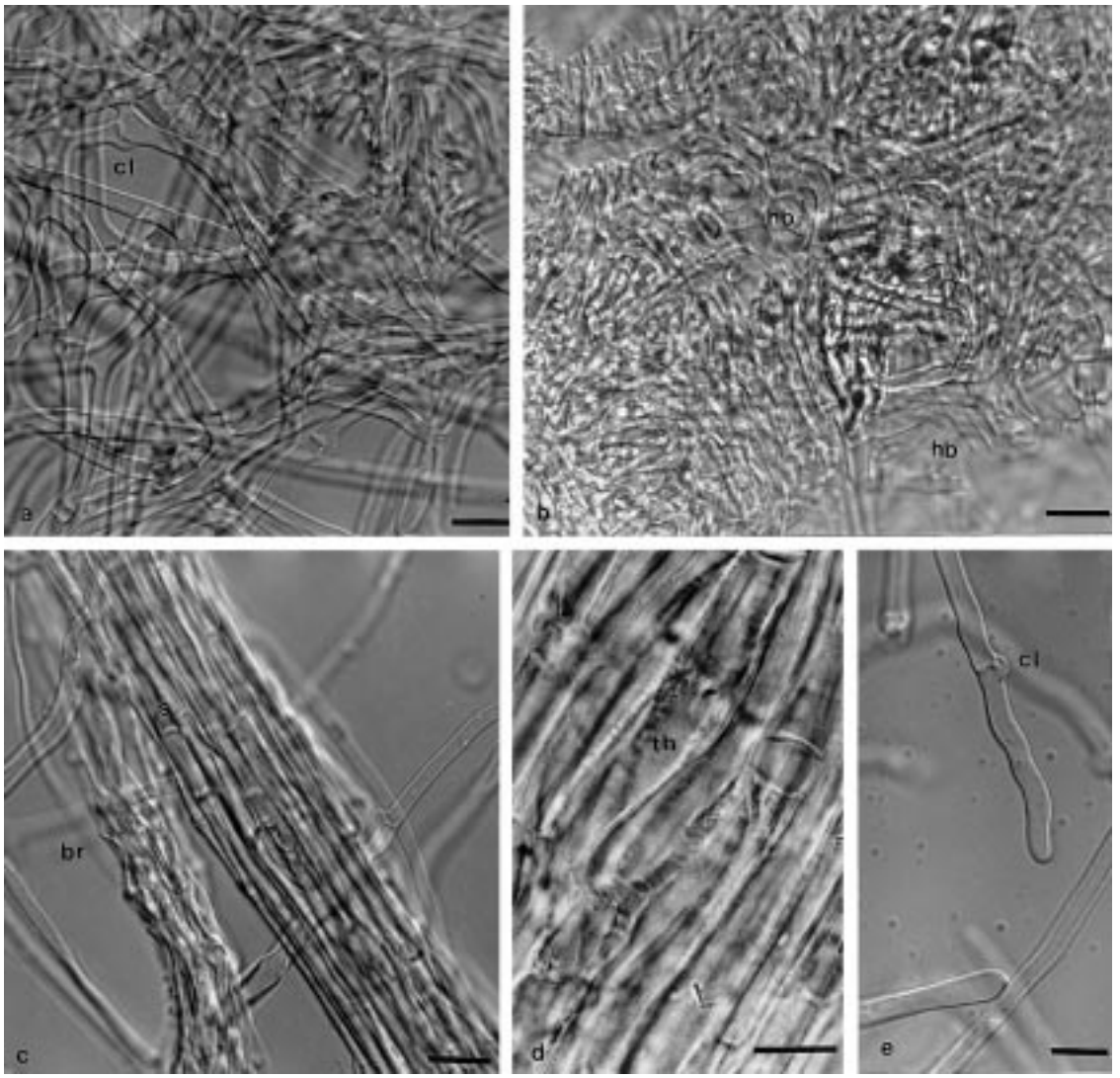

Fig. 5a–e WITS 01 plectenchymatous mantle; **a** loose outer mantle, ring-like hyphal bundles; **b** inner mantle, hyphae more compact; **c/d** rhizomorphs, hyphae compactly woven; **e** emanating hyphae showing clamp connections (*hb* ring-like arranged hyphal bundles, *cl* clamp connections, *br* branching, *ica* inverted clamp anastomoses, *s* septum, *th* thick central hyphae) *bar* 10 μm

ers and some deciduous species (Dickinson and Lucas 1979).

An updated list of ECM fungi associated with *P. patula* in South Africa includes *S. citrinum* as shown in this study, *Boletus edulis*, *Amanita muscaria*, *Tuber rapaeodorum*, *Lycoperdon umbrinum* (Marais and Kotzé 1977) and *Thelephora terrestris* (Van Greuning and Van Der Westhuizen 1990). The majority of the species listed were observed under *P. patula*, with the exception of *Tuber rapaeodorum* and *Thelephara terrestris*. Other fungal species reported in *P. patula* plantations and known to be ECM are *Tricholoma* sp., *Russula* sp., *Laccaria laccata*, *Rhizopogon luteolus*, *Suillus brevipes*, *S. subluteus* and *Lycoperdon perlatum* (Lundquist 1986; Miller 1982; Natarajan et al. 1992; Trappe 1962). Of these species, only *L. laccata* was observed in the Mpumalanga plantations. Comparisons were made between the ECM roots from this study and other reported *P. patula* ECM from India (Mohan et al. 1993a, b). The WITS 01 roots slightly resembled those produced by *Amanita muscaria* but lacked the distinctive cystidium-like emanating hyphae reported by Mohan et al. (1993b) and this association was discounted. The WITS 06 roots resembled the mycorrhizas produced by *L. laccata* in that both lack rhizomorphs and hyphal strands. However, *L. laccata* has a distinct prosenchymatous mantle which was not evident on the WITS 06 roots. It was concluded that these present *P. patula* ECM are different from those reported by Mohan et al..

Variation between the isolates in growth rate at different temperatures was evident (Table 1) and has been observed both between and within species (Marks and Kozlowski 1973). Hutchison (1990) studied the temperature responses of a large selection of northern hemisphere ECM fungal isolates. *Boletus edulis*, *Amanita muscaria* and *A. rubescens* were sensitive to both 7 °C

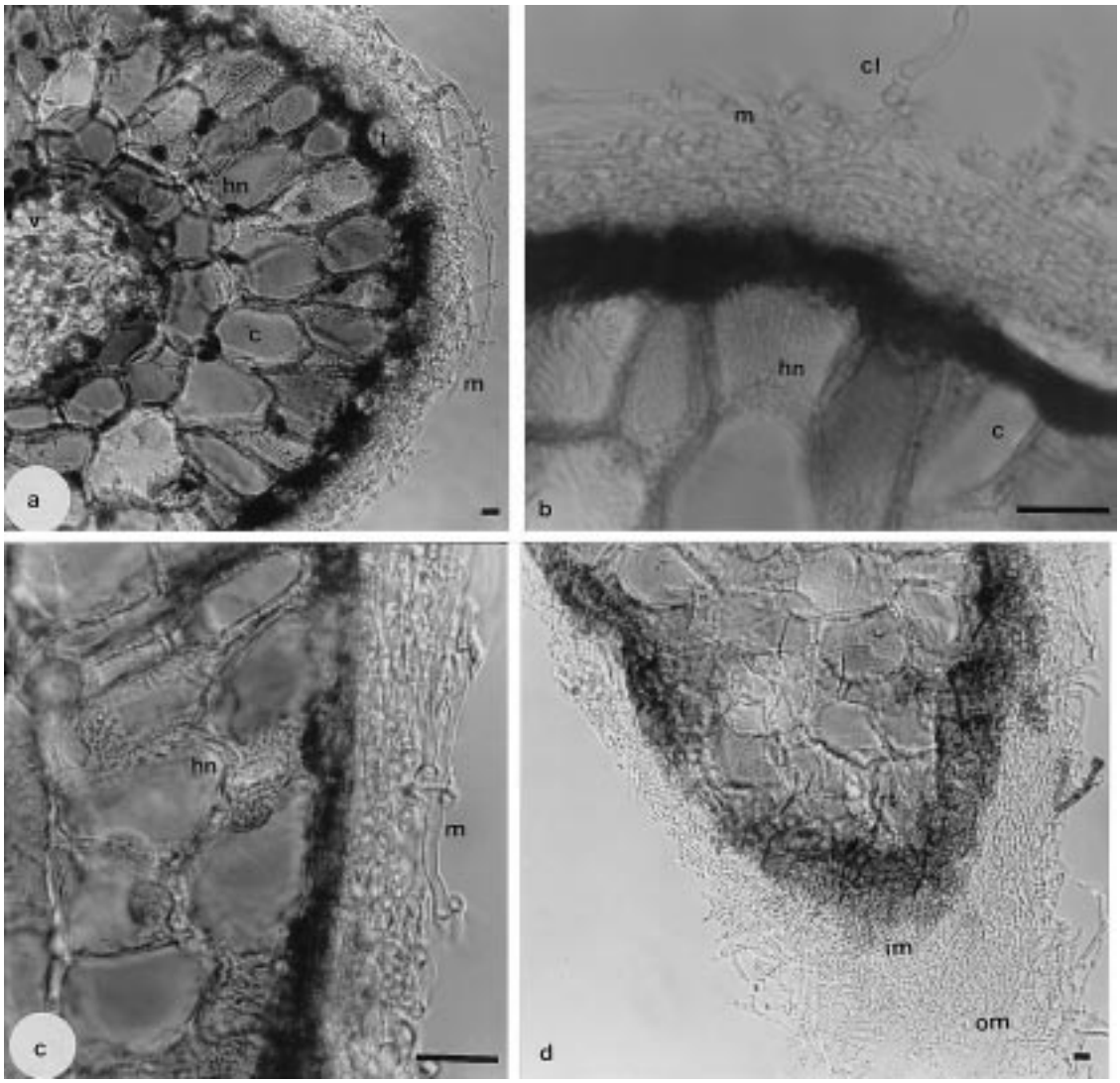

Fig. 6 WITS 01 **a/b** cross section of ECM root, **c/d** longitudinal section of ECM root, (*m* mantle; *hn* common palmetti Hartig net; *t* tannin cells; *c* cortical cells; *v* vascular tissue; *cl* clamp connection; *om* loose outer mantle; *im* compact inner mantle) *bar* 10 μm

and 30 °C, with some *A. muscaria* isolates semi-tolerant to 30 °C. *Suillus granulatus* and *Scleroderma citrinum* were sensitive to 7 °C and tolerant to 30 °C. The temperature response of WITS 01, identified as *S. citrinum*, was comparable to isolates recorded by Hutchison (1990). The semi-tolerant response to 7 °C of WITS 02 and WITS 04 suggests that these isolates belong to either the *Hebeloma* or *Laccaria* genera (Hutchison 1990). WITS 04 was initially thought to be *Amanita* sp., because of the presence of these fruiting bodies at the time of root collection (C. J. Straker personal communication). However, the indentification was not verified by the temperature response. Species of *Boletus*, *Amanita*, *Scleroderma* and *Laccaria* have been recorded in the Mpumalanga plantation (Van der Westhuizen and Eicker 1994), but the latter genus was noted only infrequently by the present authors.

The pH optima for most ECM fungi are between pH 3 and pH 5, fungi being generally considered to be acidophilic (Marks and Kozlowski 1973). WITS 01 (Fig. 2a) and WITS 02 (Fig. 2b) had pH optima within this range. WITS 04 (Fig. 2c) exhibited optimal growth above pH 5, being less adapted to acidic conditions. WITS 06 (Fig. 2d) grew well under very acidic conditions (pH 2) but was tolerant of a wide range of pH values up to pH 5. It must be noted that the optimal pH for fungal growth may differ from that for actual infection of host roots (Erland and Söderström 1990). The decline in pH of forest soils in Mpumalanga as a result of acid deposition is a major concern for the industry. Studies by Carlson (1992) showed no significant differences in the degree of mycorrhizal colonization between root cores acidified with artificial rain of different pH, but differences in mycorrhizal species composition were noted. Such changes could have important consequences for the nutrient status of the trees. The use of lime to increase soil pH has been suggested (Ol-

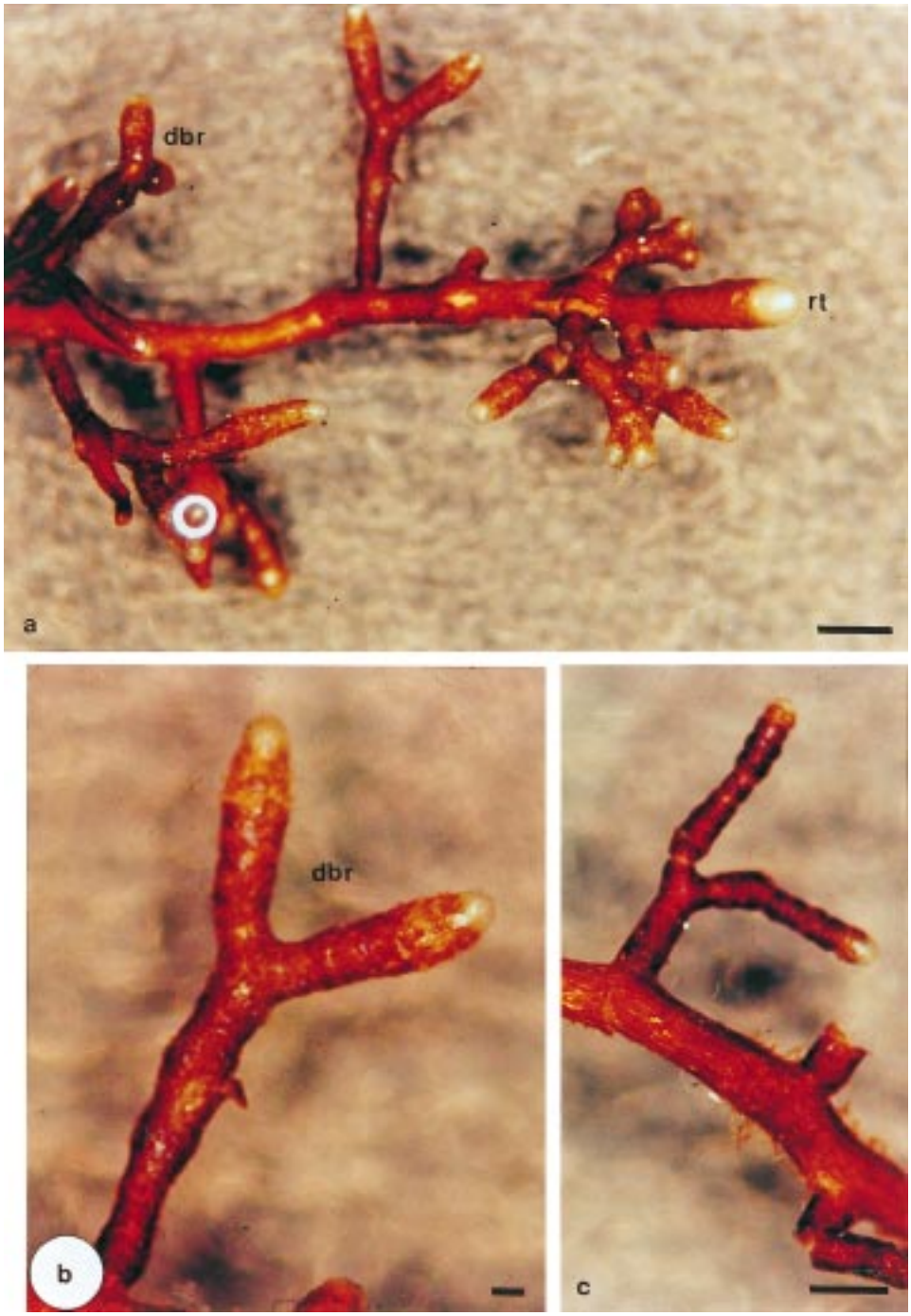

Fig. 7 WITS 06 **a/b** brown dichotomously branched ectomycorrhizal roots showing lighter coloured root tip; **c** beaded ECM roots (*dbr* dichotomous branching; *rt* root tip) *bar* 150 μm

brich 1995). Liming would not only reduce acidity but also reduce the levels of toxic metals and the leaching of cations, and would improve nutrient availability (Kreutzer 1995). All these factors could affect mycorrhizal composition, hyphal growth and colonization.

All isolates utilized the various N sources, with the exception of WITS 01, which did not grow on BSA, and differences between the isolates in overall growth rates were apparent (Table 3). However, the isolates utilized the N sources with equal efficiency, with the exception of WITS 04, which grew poorly on $(NH_4)SO_4$. In accordance with other studies, all isolates grow to some extent on $(NH_4)_2SO_4$-amended media (Abuzinadah and Read 1986; Finlay et al. 1992; Read et al. 1989). This result was expected in so far as the ECM fungi are growing in an acidic environment with an accumulation of organic material, and under these conditions the main form of inorganic nitrogen would be NH_4^+ (Marschner 1995; Read et al. 1989). Growth on nitrate was slightly lower than on ammonium, with the exception of WITS 04, which grew better on NO^{3-}. These results support previous conclusions that, in general, ECM fungi can utilize both nitrate and ammonium, although some differences between ECM fungal species

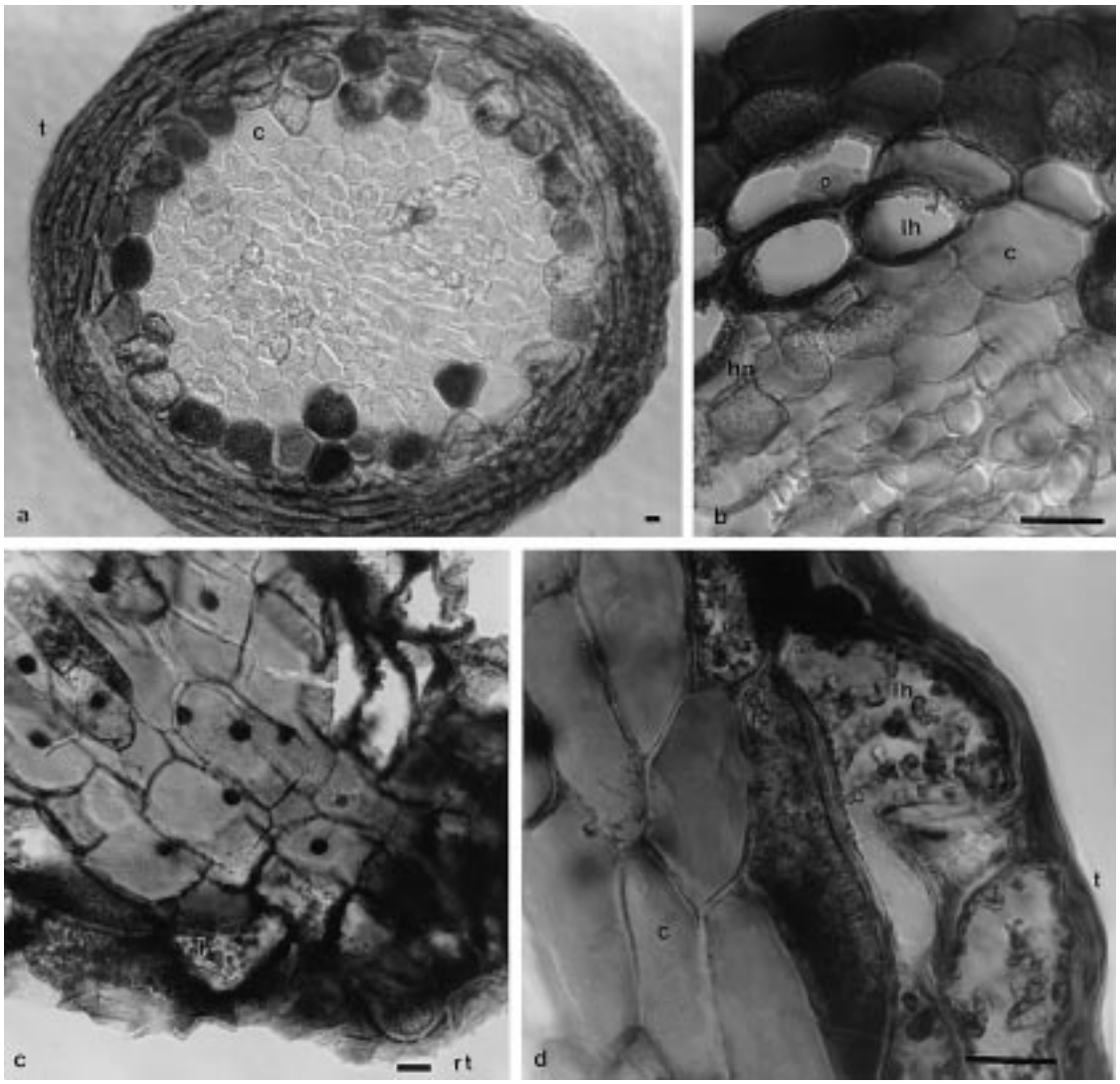

Fig. 8 WITS 06 **a/b** cross section of ECM root; **c/d** longitudinal section of ECM root; (*hn* common palmetti Hartig net; *lh* lobed haustoria; *c* cortical cells; *t* tannin cells; *rt* root tip) *bar* 10 μm

are apparent which may reflect their distribution (Keller 1996; Read et al. 1989). Growth on the glutathione-amended medium was variable but high. Abuzinadah and Read (1986) found that glutathione was a poor N source for *Laccaria laccata* and *Pisolithus tinctorius* and attributed this to the decline in pH of the medium as glutathione was utilized. All fungi tested by Abuzinadah and Read (1986) utilized alanine and the isolates in this present study utilized alanine and a range of other amino acids. Abuzinadah and Read (1986) showed that a broad-spectrum acid protease brings about proteolysis and that the N released is absorbed by ECM fungi as amino acids or small peptides rather than ammonium. A GS-GOGAT pathway is present in ECM fungi, indicating that glutamate can readily enter the cell, where it forms the major component of the N assimilation pathway (Rudawska et al. 1994). This suggests a tight cycling of organic molecules by ECM as the process involves depolymerization rather than mineralization; thus the normal saprotrophic decomposition pathway is short circuited (Vogt et al. 1991).

The demonstration that ECM fungi can utilize a range of amino acids and a protein as their sole source of N (Table 3) supports growing evidence that ECM and ericoid mycorrhizal fungi are important in providing host plant access to organic sources of N (Abuzinadah and Read 1986; Andersson et al. 1997; Baar et al. 1997; Finlay et al. 1992; Keller 1996; Read et al. 1989). Uptake of N from a protein source has been demonstrated in axenically grown mycorrhizal plants of *Betula pendula*, *Picea mariana*, *Pinus contorta*, *P. sylvestris*, *Eucalyptus grandis* and *E. maculata* (Andersson et al. 1997). The roots of *P. patula* (approximately 50% of which are mycorrhizal) were shown to be distributed mainly in the litter layers (Dames 1997). Results from litter decomposition and nutrient cycling studies within sites in which litter had accumulated indicate that N was immobilized within the litter during decomposition (Dames 1997). These findings highlight the importance

of ECM, which are intimately associated with the litter layers, for providing access to both inorganic and organic N compounds. Abuzinadah and Read (1986), using the pure protein BSA as a model compound, showed that different ECM fungal species had a wide range of degrading capabilities; this is also shown by the isolates from the present study (Fig. 3). Thus acid rain (Carlson 1992) or management strategies such as fertilization (Carlson 1994) and burning or clearing of the accumulated litter layers (Baar and De Vries 1995) in *P. patula* plantations changes the ECM fungal composition and may also alter access to organically bound nitrogen. Andersson et al. (1997) indicated that the proteolytic activity of *Paxillus involutus* was strongly influenced by pH, resulting in reduced uptake of ^{15}N by mycorrhizal plants from an organic source. This was ascribed to the altered protonation state of the amino acids.

Proteolytic ability and the ability to break down lignin, holocellulose and lignocellulose (Maijala et al. 1991; Trojanowski et al. 1984) make some ECM fungi effective competitors of saprotrophs. Although the cellulolytic ability of the isolates in this study was not examined, ECM roots and rhizomorphs were observed growing within decaying wood stumps, suggesting some cellulase activity. Growth of ECM roots within woody debris may be important in maintaining mycorrhizal diversity after disturbances, such as clear felling, and may help sustain mycorrhizas during drought conditions. Woody debris retains moisture longer than litter layers, as well as providing nutrients (Harvey et al. 1976; Parke et al. 1983; Vogt et al. 1995).

All isolates utilized the various P sources, although differences between isolates in overall growth rates were apparent (Table 4). However, each isolate utilized each P source with equal efficiency. Differences in utilization of various P sources, both between and within ECM fungi species, have been observed (Antibus et al. 1992). Organic phosphates such as glycerophosphate and RNA are probably broken down by fungal phosphatases and the resulting phosphate is absorbed by the roots (Häussling and Marschner 1989; Lapeyrie et al. 1991). Antibus et al. (1992) noted that pH had a significant effect on phosphatase activity, with maximal activity occurring at pH 4.5–5.0 for *Scleroderma citrinum*. Recent studies by Leake and Miles (1996) showed that the ericoid mycorrhizal fungus *Hymenoscyphus ericae* can hydrolyse DNA and use it as a sole source of P. The phosphodiesterase was extracellular and wall bound, with a pH range of 3.0–6.5 and an optimum of 4.5–5.5. A similar system may be operative in ECM.

Complexed compounds such as phytic acid and $AlPO_4$ are probably solubilized by the action of ECM fungi or by a combination of enzyme activity and solubilization. The N source is important in the solubilization process. NH_4^+–N, which is found mainly in acidic soils, stimulates proton extrusion and results in acidification; this increases solubilization (Lapeyrie et al. 1991). Phosphatase activity is also affected by the N source, with increased activity occurring when N is supplied as NH_4^+ (Kieliszewska-Rokicka 1989). Where NO_3^-–N is the main N source, mainly in calcareous soils, oxalic acid excretion is stimulated and the ECM fungi act as a solubilizing agent by acidification and as a chelating agent by chelating iron, calcium and aluminium (Lapeyrie et al. 1991). Rock phosphates are not solubilized by ECM fungi, probably due to their crystalline structure, although ECM fungi in association with other micro-organisms may be important in this process (Antibus et al. 1992; Lapeyrie et al. 1991).

The utilization by ECM, arbuscular and ericoid mycorrhizal seedlings of organic N and P sources has been demonstrated in several studies (Abuzinadah et al. 1986; Jayachandran et al. 1992; Myers and Leake 1996). The contribution made by ECM fungi to the cycling of N and P in *P. patula* plantations is of significance for the long-term sustainability of sites with accumulated litter layers. Trees growing at these sites acquire a significant proportion of nutrients from the litter layers, as evidenced by the distribution of roots mainly within the litter layer and the large nutrient reserves within the litter pool. The litter N pool is 2.5 times larger than the soil pool, while the litter P pool is similar to that of the soil. The P-fixing capacity and the Al^{3+} toxicity of the soil decreases the ability of roots to exploit soil nutrient reserves, and it was suggested that a plant-litter-plant nutrient cycling process is operational in *P. patula* sites with accumulated litter layers (Dames 1997). The extent to which mycorrhizas can utilize these organic sources under field conditions, in competition with the microbial population, is not known and requires further investigation (Vogt et al. 1991).

Proteolytic and solubilization capabilities of ECM fungi are important factors determining distribution. Mineral soils are progressively transformed into organic soils through the input of organic matter and other soil processes (Abuzinadah and Read 1988). 'Non-protein fungi' may be successful in newly planted first-rotation sites, whereas 'protein fungi' would become more important as the stands mature and in successive rotations. This succession of ECM fungi has been reported in a number of ecosystems (Dighton et al. 1986; Mason et al. 1983; Natarajan et al. 1992) but has not been studied in the Mpumalanga plantations. However, observations during the present study suggest some succession, with *Amanita* spp. and *Lycoperdon* spp. occurring under younger trees (up to 15 years) and *Boletus* spp. and *Suillus* spp. occurring in older stands. *Scleroderma* spp. occurred mainly from 10 years onward. This area merits further research. Because of the different capacities of ECM fungi for utilizing organic and inorganic compounds, an effort should be made to assess the ECM fungal species occurring in particular forestry areas. Combined with information on succession, ECM fungi suited to specific areas could then be encouraged.

The understanding of forest ecosystems has become crucial to long-term timber production. These ecosys-

tems cannot be fully understood if the role of ECM is ignored. ECM formed by different fungi are structurally distinct and differ, for example, in nutrition and growth-promoting efficiency (Agerer 1994). Thus, correct identification of ECM is essential for interpretation of interactions observed. In this respect, DNA fingerprinting of cultures, sporocarps and ECM root tips (Rygiewicz and Armstrong, 1994) should provide invaluable data, especially when linking physiological characteristics to specific mycorrhizas, in future work.

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