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Endophytic fungi associated with the temperate palm, *Trachycarpus fortunei*, within and outside its natural geographic range

J. E. TAYLOR[1]*, K. D. HYDE[2] AND E. B. G. JONES[3]

[1]*Department of Plant Pathology, University of Stellenbosch, Private Bag X1, Stellenbosch 7602, South Africa*
[2]*Fungal Diversity Research Project, Department of Ecology and Biodiversity, University of Hong Kong, Pokfulam Road, Hong Kong*
[3]*Department of Biology and Chemistry, The City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, N.T., Hong Kong*

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SUMMARY

Fungal endophytes associated with the palm, *Trachycarpus fortunei*, within and outside its natural geographic range were investigated. Endophytes were relatively common with colonization rates of 23–57% at the four sites sampled. The endophyte assemblages at the different sites were diverse with 75 fertile species of ascomycetes and mitosporic fungi. The assemblage composition at each site was similar and between seven and 13 species comprised 81–89% of the taxa present in relative frequencies of >1%. *Glomerella cingulata* and *Phomopsis* spp., were consistently dominant, and a large number of rare species were recorded. The diversity at each site was similar in number, but the abundance of isolates varied. The results obtained were comparable to those of previous studies of palm endophyte assemblages, but the assemblages showed more affinity with unrelated temperate hosts than with tropical palm hosts. Quantitative and qualitative differences in endophyte assemblages from old and young tissues were observed, and more isolates were recovered from old tissues independent of the age of the palm. The composition of the assemblage varied with several taxa being exclusively or more commonly isolated from old tissues (e.g. xylariaceous taxa, *Oxydothis* sp. nov.) or young tissues (e.g. *Stagonospora* spp., *Phoma multirostrata*). Some differences in the composition of the assemblage and in relative frequencies of various species were observed in trees and saplings. Significantly more isolates were recovered from the vein than intervein tissues, independent of leaf age or tree age. Tissue specificity was not exhibited by any taxa isolated from either leaf or petiole tissues, except for xylariaceous taxa in leaf tissues. Some other taxa showed a preference for leaf tissues or petioles, whereas others were equally distributed amongst all tissues. Endophyte assemblages of palms from continuous distributions were similar, but those from disjunct distributions (i.e. outside the natural geographic range of the palm, such as Australia and Switzerland), differed significantly from each other and from assemblages within continuous distributions. The relative importance values of endophyte species at the two sites in China indicates the site-specific nature of the endophyte assemblages. Most previous studies on the endophytes of palm hosts have concentrated on tropical palms. However, this study examines the endophytes of the warm temperate palm *Trachycarpus fortunei*, and investigates the relative importance of host and climate related processes.

Key words: biogeography, endophyte, fungal diversity, palm, temperate.

INTRODUCTION

Endophytic fungi have been studied for over 70 yr (Lewis, 1924). However, serious interest in endophytes has only developed in the past 20 yr (Bernstein & Carroll, 1977; Carroll *et al.*, 1977; Carroll & Carroll, 1978). Most studies have been concerned with graminaceous endophytes (Dahlman *et al.*, 1991), and have been undertaken in temperate countries. Recently, there has been interest in endophytes of tropical hosts (Dreyfuss & Petrini, 1984; Lodge *et al.*, 1996; Rodrigues & Petrini, 1996), including palms (Rodrigues & Samuels, 1990; Rodrigues, 1994; Fröhlich, 1997; Southcott & Johnson, 1997).

*Author for correspondence (tel +27 21 8084223; fax +28 21 8084956; email *jtaylor@land.sun.ac.za*).

Endophytes have been investigated at various spatial scales, ranging from small scale studies on endophyte distribution patterns in a single leaf, to those at a geographic level (Carroll, 1995). Studies at a geographic level compare endophyte assemblages of similar hosts in different geographic locations and have involved hosts in continuous distributions and those in disjunct distributions, i.e. those planted outside their natural geographic range as ornamentals. The most important variables affecting endophytic mycota and overall infection levels amongst trees growing in ecologically diverse sites is summarized by Carroll (1995), who identified areas of study that have not yet been satisfactorily investigated. For instance, very few studies have compared the endophyte assemblages associated with tropical and temperate hosts, to determine possible climate related effects which may cause differences in endophyte assemblages. Several studies have investigated endophytes of palms (Rodrigues & Samuels, 1990; Rodrigues, 1994; Fröhlich, 1997; Southcott & Johnson, 1997). Most of these have concentrated on tropical palm hosts within their natural geographic range. By contrast, this study examines the endophytes of the palm, *Trachycarpus fortunei*, which occurs in warm temperate China, to allow inferences on the more influential factor between host or climate related processes.

The aims of this study were to investigate the endophyte assemblages of disjunct and continuous populations of the host; to compare the mycota of this temperate palm host with that of tropical palms; and to identify quantitative and qualitative patterns in the distribution of the endophytes, between and within a stand of host trees and in different host tissues.

MATERIALS AND METHODS

Trachycarpus fortunei (W. J. Hooker) H. A. Wendland was selected for this study as it is a relatively small palm, with accessible palmate fronds. Although native to China, it is a popular ornamental, with a widespread distribution and is often cultivated in large stands. Collections were made in China, Australia and Switzerland during the wet season and details of each site at the time of collection are given in Table 1. Seasonal variations have been noted in endophyte assemblages in various studies (Petrini, 1991; Helander *et al.*, 1994; Rodrigues, 1994; Wilson & Carroll, 1994); therefore, collections were made at similar times during the wet season. *Trachycarpus fortunei* occurs naturally in the warm temperate areas of China, especially along the Yangtze river valley. However, it has a long history of cultivation and no truly wild specimens can be found. The site in Australia was the Royal Botanical Gardens, Sydney. The palms had been planted, but had not naturalized i.e. seeds, if produced, were not viable. At the Swiss site, a forest garden planted with many exotic species, the palms had naturalized and had grown prolifically.

The oldest and youngest (unfurled) fronds from 10 mature palm trees (above chest height), and in the 'China 1' study, five saplings or young palms (without a trunk), were removed and specimens were returned to the laboratory for processing within 1–3 d. Two healthy leaflets were removed from each leaf and eight samples were removed from both the vein and intervein (either side of the vein) areas of each leaflet. The total number of leaf discs (6 mm in diameter) extracted, in most cases, was 640. The five young trees from China were also similarly sampled giving a further 320 leaf discs. Five samples were extracted from regular intervals from each petiole, and in total 100 samples of petiole were taken per site, with an additional 50 samples of petiole for the five young trees from China.

The surface sterilization methods used in this study are similar to those used in previous studies on palm endophytes (Rodrigues & Samuels, 1990; Rodrigues, 1994; Fröhlich, 1997). Surface sterilization of specimens involved dipping in 95% alcohol for 1 min, followed by 10 min in 3.25% sodium hypochlorite (Chlorox, containing 5.25% sodium

Table 1. *Climatic data at the time of collection of samples of* Trachycarpus fortunei (*following Pearce & Smith (1990)*

	Grid reference	Average rainfall (mm)	Relative humidity	Average temperature (daily min–max °C)
Shaoshan* (China 1)	27°54′N 112°24′E	180	62	26–34
Enshi* (China 2)	30°20′N 108°57′E	As above	As above	As above
Sydney (Australia)	33°52′S 151°12′E	127	63	11–19
Locarno (Switzerland)	46°9′N 18°57′E	147	59	3–11

*Data for Wuhan, 30°35′N 114°17′E.

hypochlorite diluted with distilled water at a ratio of 620:380 ml l^{-1} to give a final concentration of 3.25%) and finally, 30 s in 95% alcohol. Samples were incubated on Difco bacto malt extract agar supplemented with Sigma streptomycin sulphate (Sigma, MO, USA) (0.3 g dissolved in 1.5 ml sterile water per l of agar). To prevent fast growing fungi engulfing the plate a growth inhibitor, Rose Bengal (BDH Laboratory Supplies, Poole, Dorset, UK) (0.033 g l^{-1}), was added to the agar. Fungi growing from the leaf discs were transferred to Falcon 50 mm 1006 petri dishes (which prevent mite infestation) of malt extract agar, supplemented with Sigma streptomycin sulphate. Each dish contained a strip of autoclaved *T. fortunei* leaflet which was included to encourage sporulation (Matsushima, 1971). The isolates were sorted into species groups if sporulating, or if infertile they were placed in a black box on a cycle of 12 h cool white fluorescent light and 12 h near uv fluorescent light (Bills, 1996) to encourage sporulation. Sterile isolates were described as 'morphotypes' differentiated by cultural characteristics. Several strains of each species of sporulating and identifiable fungi were deposited in the Hong Kong University Culture Collection (HKUCC). Data were processed using Microsoft Access Version 7.0 and statistical analysis and graphical presentation was carried out with Microsoft Excel Version 7.0 and StatSoft Statistica Version 4.0. Multivariate statistics were performed using StatSoft Statistica Version 4.0 and a Multivariate Statistical Package (MVSP) (Kovach, 1995).

The rates of colonization and isolation were calculated as follows:

$$\text{Colonization rate} = \frac{\text{total number of samples yielding} \geqslant 1 \text{ isolate}}{\text{total number of samples in that trial}}$$

$$\text{Isolation rate} = \frac{\text{total number of isolates yielded in a given trial}}{\text{total number of samples in that trial}}$$

Colonization rates were expressed as percentages (Petrini *et al.*, 1982) as commonly used in the literature. Isolation rates were also calculated and used to demonstrate the degree of multiple colonization from the samples in different trials but were not expressed as percentages. A chi-squared (χ^2) goodness-of-fit test was performed to test whether the colonization rates of the five trials were statistically different.

The data in most cases did not fit the assumptions for parametric statistics, even after correction of the data by transformation (by square root or logarithmic transformation) (Zarr, 1996). Therefore, non-parametric statistical tests were used throughout. A Mann–Whitney test was performed on isolates from old and young leaves and petioles at each site. For multisample analysis, such as the investigation of the number of isolates recovered from vein and intervein tissues for all leaves (but not petioles) at each site, the Kruskal–Wallis test was used. Box plots were used to display the results graphically (Fisher & Petrini, 1987). In all analyses, P values are described as follows: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Ordination by simple correspondence analysis was performed to investigate patterns of geographic distribution on a reduced data matrix (including taxa which occurred at relative frequencies >5% in at least one type of tissue-type/site category) using MVSP (Kovach, 1995). This analysis makes no assumptions about the underlying structure of the data, but displays the data in an optimal way. The data for the correspondence analysis were transformed ($\log_e [x = 1]$) as they were highly skewed. ANOVA was used to test for significant differences between the means of the species/tree for each site. Relative importance values (RI) (Ludwig & Reynolds, 1988) were calculated to investigate site specific differences in endophyte assemblages at the two Chinese sites. These were calculated by standardizing the RI values within each site, by assigning the most common species at each site the value of 100% and computing the RI of each taxon as a percentage of it (Petrini *et al.*, 1992). The results were plotted and the RI values for individual trees were then compared.

RESULTS

Colonization and isolation rates

A total of 3256 leaf discs from *T. fortunei*, from all four sites in the three countries were processed, and 1985 isolates were recovered. The overall colonization rates (%) and isolation rates for the assemblages of endophytes recovered at each site are given in Table 2. There was a significant difference between colonization rates in each country $\chi^2 = 16.45$, df 4, $P = 0.0024$**). In all cases there were higher colonization rates in palm tissues from China than from Australia or Switzerland. Overall, there was a high degree of multiple infections (14–54% of samples, Table 2).

Tissue specificity

Leaf/petiole age analysis. Mann–Whitney tests were performed on the data at each site and with the exception of China 1 (mature) petioles ($P = 0.045$*), there was no significant difference between the number of isolates recovered from young and old petioles at any of the sites sampled ($P = <0.05$). However, at each site there were differences between the number of isolates recovered from young and old leaves (China 1 (mature), $P = 0.00015$***; China 1 (immature), $P = 0.009$**;

Table 2. *Overall colonization, overall isolation and multiple infection rates at each site*

Site	China 1 (Mature)	China 1* (Immature)	China 2	Australia	Switzerland
No. of samples	740	370	666	740	740
No. of isolates recovered	497	302	494	491	201
Overall colonization rate (%)	53.6	57.3	55.7	47.4	23.4
No. (%) of samples yielding two isolates	86 (35%)	61 (40%)	85 (35%)	60 (24%)	24 (12%)
No. (%) of samples yielding > two isolates	7 (4%)	14 (14%)	18 (11%)	34 (6%)	2 (2%)
Overall isolation rate (no. of isolates per sample)	0.67	0.82	0.74	0.66	0.27
No. of leaflets sampled	640	320	576	640	640
Overall colonization rates of leaflets only (%)	337 (53%)	179 (56%)	306 (46%)	271 (42%)	111 (17%)

*Immature palms were only collected during the pilot study, China 1.

China 2, $P = 0.00007$***; Australia, $P = 0.008$**; Switzerland, $P = 0.02114$*) with higher frequencies consistently obtained from older leaves.

Vein and intervein analysis. At the China 1 site, significantly more isolates were recovered from the vein than intervein tissues in both young and old leaves irrespective of tree age, with the numbers of isolates recovered following the pattern O/V > Y/V > O/IV > Y/IV (O, old tree; Y, young tree; V, vein; IV, intervein) (Fig. 1a). The results of the Kruskal–Wallis tests for the other three sites indicate that there were also significant differences in the number of isolates recovered from vein and intervein tissues irrespective of leaf age (China 1 (mature and immature), $P = 0.000$***; China 2, $P = 0.000$***; Australia, $P = 0.0035$*; Switzerland, $P = 0.0186$*). Therefore, it can be concluded that irrespective of tree age or leaf age, there are differences in the number of isolates in the vein and intervein tissues.

Tree age class analysis

Analysis of the influence of the age of the palm on the endophyte assemblages associated with different aged leaves and petioles is presented in Fig. 1b, c. The Kruskal–Wallis test shows that, other than with China 1 (mature) old and young petioles, there was a significant difference between the numbers of isolates obtained from young and old leaves ($P = 0.000$**), and from petioles ($P = 0.0282$*), of both age classes of the tree. Therefore, the influence of tree age is less important than leaf or petiole age.

Composition of endophytic assemblage

Of the 1985 isolates recovered from 3256 samples, 75 species in 43 genera were recorded. This total includes fertile, and therefore identifiable cultures, which represented 1728 (87%) of the total isolates recovered. It does not include *Mycelia sterilia* 'morphospecies' of which there were 209 isolates (11%). There were six main, consistently recognizable morphospecies (*Mycelia sterilia* w1, *Mycelia sterilia* w2, *Mycelia sterilia* b2, *Xylaria* sp. 1, *Xylaria* sp. 2, and *Xylaria* sp. 3) which comprised 101 isolates (5% of the total) and 112 (6%) miscellaneous *Mycelia sterilia* isolates. The remaining 43 (2%) included yeasts, actinomycetes and bacteria. Of the 77 identified species, nine were ascomycetes, 41 were coelomycetes and 25 were hyphomycetes. There were two anamorph–teleomorph connections (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. –*Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk and *Guignardia cocogena* (Cooke) Punith. –*Phyllosticta cocoicola* (Bat.) Sivan.), which were referred to using the name of the teleomorph (Hawksworth *et al.*, 1995), even though the majority of the cultures formed only the anamorph. A synopsis of the taxa occurring at relative frequencies of >1% at each site is given in Table 3.

There was a large degree of overlap in the endophyte communities, especially at the genus level. The greatest overlap occurred between the two China sites, with 14 of the genera recorded in China 1 also occurring in China 2. The overlap between the China sites (combined, 32 genera), and Australia and Switzerland was lower at 10 for each site. Eight genera recorded in Australia also occurred in Switzerland.

The effect of tissue type, tissue age and tree age on the composition of endophyte assemblages. Differences in endophyte assemblages in the different tissue types (leaf tissues vs petiole) would be reflected in tissue preferences of the individual dominating taxa. Box plots were used to compare the isolation rates of taxa (recovered from the China sites only), against tissue types (not illustrated). There was very little evidence of tissue specificity exhibited by the endophytes in this study. *Xylaria* 1 was restricted to leaf tissues, and *Guignardia cocogena*, *Xylaria* 3 and *Oxydothis*

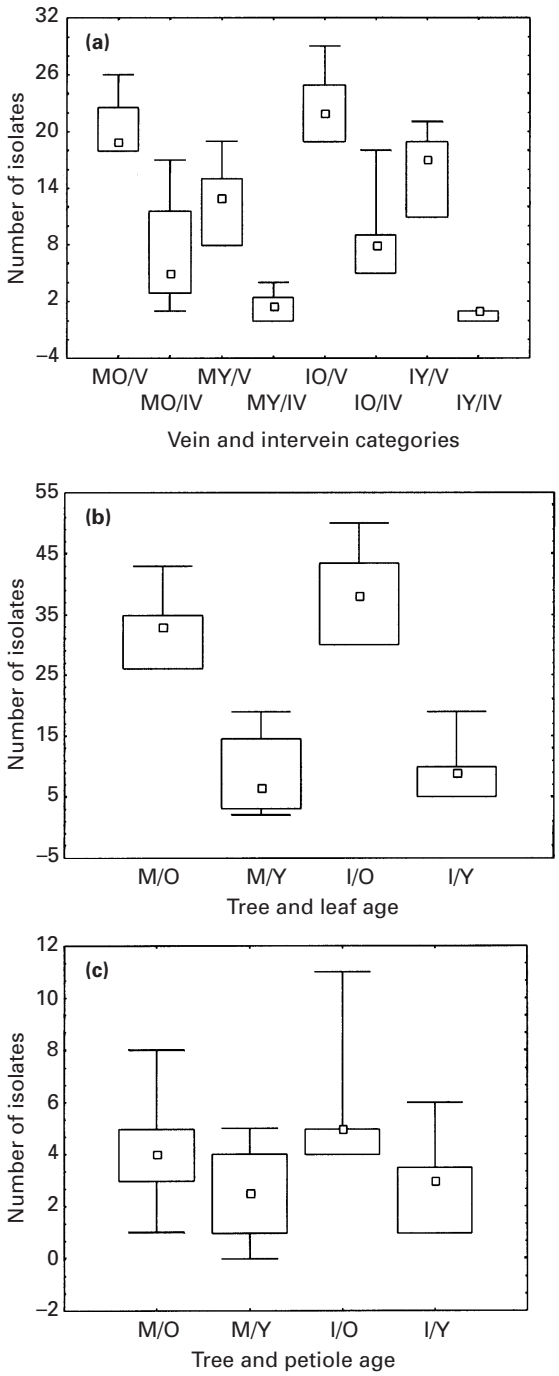

Fig. 1. Box-Whisker Plots comparing the ranked medians of the numbers of isolates, recovered from (a) vein and intervein tissue; (b) old and young leaves;(c) old and young petioles, from differently aged palms from China 1. Categories are represented by: M, mature tree; I, immature tree; O, old leaf/petiole; Y, young leaf/petiole; IV, intervein; V, vein. Minimum and maximum values are indicated by the bars above the boxes (25–75%) with the median value indicated by the smaller inner box.

sp. nov. seemed to show a preference for leaf tissues. Other taxa were more equally distributed amongst all tissues.

Some differences in the endophyte assemblages were recorded in differently aged tissues (China sites only were analysed). Xylariaceous taxa were found almost exclusively in old tissues (93% of total isolates), as were *Microsphaeropsis* sp. C and *Guignardia cocogena*. *Oxydothis* sp. nov. was not recorded in any young tissues. Several species showed a higher incidence in young tissues such as *Stagonospora* spp. and *Phoma multirostrata*. This observation was analysed by complete linkage (furthest neighbour) cluster analysis performed on a reduced data matrix of species occurring at $\geqslant 5\%$ frequency (isolation rates) in one type of tissue (not illustrated). This gave two main clusters separated on the basis of tissue age, regardless of tissue type or age of palm.

The main noticeable difference between the endophyte assemblages recovered from different aged trees (China 1 site only) was the absence of xylariaceous taxa from the tissues of immature palms (Table 3).

Distribution at different spatial scales

Country/site/tissue : correspondence analysis. Ordination by simple correspondence analysis was used to investigate whether variations in the fungal assemblages may be attributed to biogeographical factors (China/Australia/Switzerland), possible site related factors (indigenous/naturalized/planted) or tissue specificity.

The first three axes of Fig. 2 explain 54.6% of the inertia or variation of the data set. This is quite low indicating that the model does not correspond well with the data. Therefore, the interpretation is quite weak, and illustrative at best. When broadly interpreted, the analysis shows that the samples are separated on geographic differences. Little evidence of clustering on the basis of tissue specificity is indicated, other than in cluster C where the old and young tissues are somewhat distinct.

The relative importance of site vs geographic differences are examined in this study, but again the interpretation is quite weak due to the low inertia contribution (54.6%). In Fig. 2, the first axis of the correspondence analysis is broadly determined by the gradient Asia/Europe (clusters A & C) and Australia (B), which could either be biogeographic differences and/or indigenous/naturalized and planted stands (site differences). However, possible site differences are less influential as Axis 2 separates naturalized stands (Switzerland C) from planted (Australia B) and indigenous stands (China A).

Stand and tree level patterns

At the stand level, variations in the numbers of taxa per tree were examined, and the number of isolates and species recovered from each site were compared (Table 4) (after Bills & Polishook, 1992). With the exception of China 1 (immature), the raw data for all isolates were included; therefore endophyte taxa from a wide range and age of organs and tissues were

Table 3. *Relative frequencies* (*RF*) *and colonization rates* (*CR*) *of endophytes species from* Trachycarpus fortunei *occurring at relative frequencies of* $>1\%$

	China 1 (Mature)		China 1 (Immature)		China 2		Australia		Switzerland	
Taxa	RF (%)	CR (%)	RF (%)	CR (%)	RF (%)	CR (%)	RF (%)	CR (%)	RF (%)	CR (%)
Acremonium sp.									1.5	0.4
Alternaria alternata			1.3	0.8	3.0	2.3			5.0	1.4
Aureobasidium pullulans									1.5	0.4
Bacteria sp.									12.9	3.4
Colletotrichum sp.									1.5	0.4
Fusarium solani	1.2	0.7								
Fusarium sp. 2					1.0	0.8				
Glomerella cingulata	23.3	14.7	40.1	28.6	48.2	30.0	13.7	7.8	23.9	6.4
Guignardia cocogena	2.8	1.9	2.0	1.6	8.3	6.0	14.7	9.6		
Microsphaeropsis olivacea							1.4	0.9	2.5	0.7
Microsphaeropsis sp. C	1.4	0.9	5.0	4.1					9.0	2.3
Mycelia sterilia w 1	5.8	3.5	1.7	1.1	3.0	2.0	1.2	0.8		
Nodulosporium sp. 1							1.2	0.8		
Oxydothis sp. nov.					5.7	4.2				
Pestalotiopsis disseminata	1.0	0.5								
Pestalotiopsis caroliniana							2.7	1.6		
Phoma glomerata							1.8	1.4		
Phoma multirostrata			1.7	0.8						
Phoma nebulosa							1.4	0.9	2.0	0.4
Phoma pinodella									3.5	0.9
Phoma sp. 1							1.2	0.8		
Phomopsis sp. A							27.5	14.1		
Phomopsis sp. B							12.0	7.0		
Phomopsis sp. G							11.8	7.2	5.5	1.5
Phomopsis sp. H	42.3	25.9	37.7	25.9	10.1	6.6			7.0	1.9
Stagonospora sp. 5					3.2	2.4				
Wardomycopsis sp. nov.					1.6	1.1				
Xylaria sp. 1	3.8	2.6								
Xylaria sp. 2	1.2	0.8								
Xylaria sp. 3	1.0	0.7			1.8	1.4			5.5	1.1
Yeast sp.										
Mycelia sterilia	11.2		8.2		7.2		5.1		7.5	
Rare isolates	5.0		2.3		6.9		5.7		11.2	
Total	83.8		89.5		85.9		89.2		81.3	
Grand total	100		100		100		100		100	

Taxa occurring at $<1\%$ RF in the each country:
China 1 (Mature): *Alternaria alternata*, *Aureobasidium pullulans*, *Coniochaeta nepalica*, *Curvularia lunata*, *Fusicoccum parvum*, *Geniculosporium serpens*, *Nigrospora oryzae*, *Oxydothis* sp. nov., *Microsphaeropsis* sp. B, Mycelia sterilia w 2, *Nodulisporium fuscum*, *Phoma multirostrata*, *Phoma nebulosa*, *Phoma pomorum*, *Stagonospora* sp. 2, Unidentified coelomycete sp. 1, Unidentified coelomycete sp. 2, Unidentified coelomycete sp. 3.
China 1 (Immature): *Microsphaeropsis* sp. A, *Nigrospora oryzae*, *Oxydothis* sp. nov., *Periconia* sp. nov., *Phoma pomorum*, *Wardomycopsis* sp. nov.
China 2: *Cladosporium cladosporioides*, *Diaporthe* sp., *Fusarium* sp. 1, *Fusarium graminearum*, *Fusarium semitectum*, *Fusarium solani*, *Geniculosporium serpens*, *Glomerella*-like sp., *Idriella licualae*, *Microsphaeropsis* sp. C, *Nodulisporium* sp. 1, *Nigrospora oryzae*, *Pestalotiopsis* sp. 2, *Phaeosphaeria* sp., *Phoma nebulosa*, *Stagonospora* sp. 6, Unidentified ascomycete sp. 2, Unidentified coelomycete sp. 4, Unidentified hyphomycete sp. 4, Unidentified hyphomycete sp. 7, *Xylaria* sp. 1.
Australia: *Alternaria* sp. 1, *Asochyta palmicola*, *Coleophoma crateriformis*, *Colletotrichum acutatum*, *Fusarium solani*, *Fusicoccum parvum*, *Nodulisporium fuscum*, *Pestalotiopsis palmarum*, *Phomopsis* sp. D, *Phomopsis* sp. F, *Phomopsis* sp. J, *Phomopsis* sp. I, *Septoria* sp. 1, *Sporormiella minimoides*, *Pithomyces sacchari*, Unidentified coelomycete sp. 6.
Switzerland: *Actinomycete*, *Colletrotrichum acutatum*, *Fusicoccum aesculi*, *Epicoccum nigrum*, *Fusarium semitectum*, *Fusarium tricinctum*, *Geniculosporium serpens*, Mycelia sterilia b 2, Mycelia sterilia w 1, *Penicillium* sp., *Pestalotiopsis caroliniana*, *Phoma* sp. 2, *Phoma glomerata*, *Phoma multirostrata*, Unidentified hyphomycete sp. 8, Yeast sp. 1, Yeast sp. 2, Yeast sp. 3, Yeast sp. 4.

considered. Miscellaneous categories such as *Mycelia sterilia* were included in the analysis as a 'species'. The four sites were relatively homogenous in terms of the number of species recovered, with no significant difference between the means of species per tree at each site. The range of species per tree varied between 9 (9–18) for China 2 and 10 (4–14) for Switzerland and China 1.

The RI values (Ludwig & Reynolds, 1988) of the endophytes species isolated from palm material at

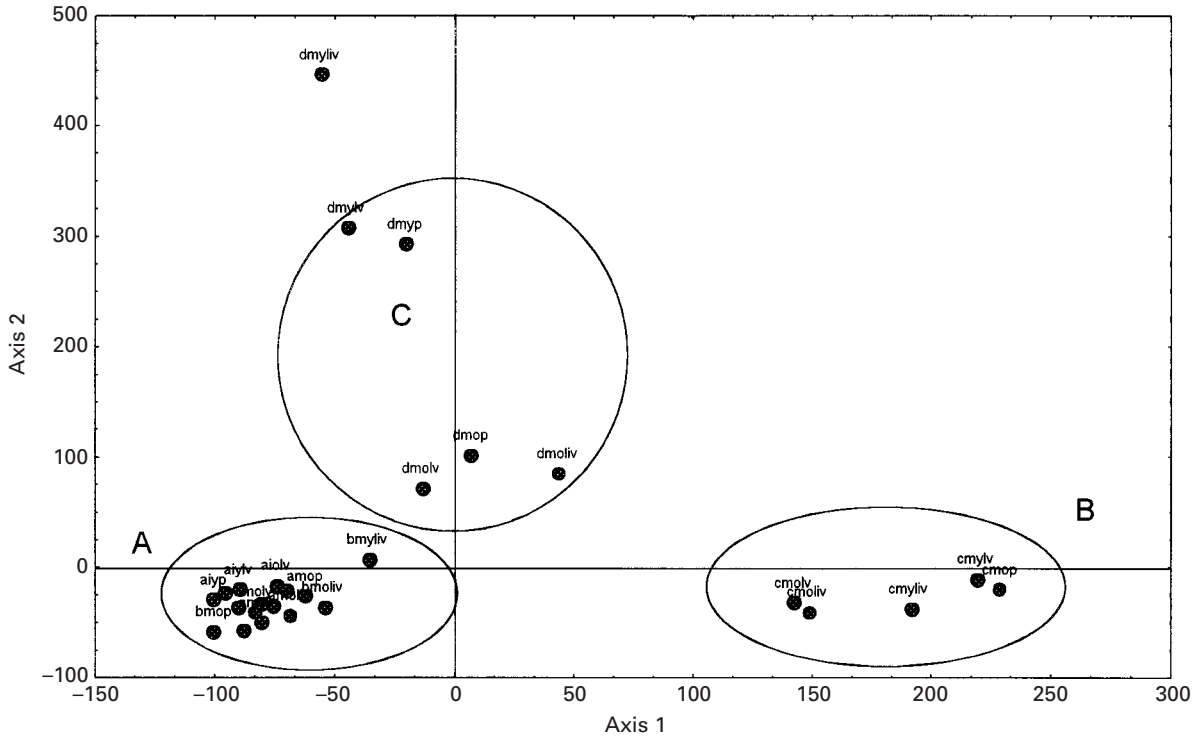

Fig. 2. Results of ordination by simple correspondence analysis (data log transformed ($\log_e [x = 1]$)). The combinations of letters on the scatterplots encode the provenance of the samples. Sites are shown as: a, China 1; b, China 2; c, Australia; d, Switzerland 1. Tissue-types are indicated by: m, mature palm; i, immature palm; o, old leaf/petiole; y, young leaf/petiole; v, vein; iv, intervein, p, petiole. The inertia of the model explained by the first three axes of the plot is 54.6%.

Table 4. *Total numbers of isolates and taxa isolated from all palm parts sampled from* Trachycarpus fortunei *at the four different sites*

	China 1	China 2*	Australia	Switzerland 1
Total isolates	497	409	491	201
Total species	32	33	30	33
Species/tree† (means + SD)	11 ± 3	12 ± 3	12 ± 3	10 ± 4
Species/tree (range)	4–14	9–18	7–16	4–14

(Number of samples = 740, except China 2 = 666).
*Only eight trees were considered owing to loss of some samples.
†Means are not significantly different according to ANOVA ($P < 0.05$).

several sites were examined for the China 1 (mature only) and China 2 sites, and included the endophyte assemblages from young and old, leaves and petioles from mature plants. At both sites the two most frequent endophytes were the same, although in different proportions (Table 3). However, there were few similarities with the less frequent endophytes (occurring at $< 0.6\%$ RI). The differences in the assemblages of the less frequent and rare species therefore indicated the site specific nature of the endophyte assemblages. By comparing the RI values of randomly chosen individual trees with the total RI values for each site, it was apparent that the endophyte assemblage for individual trees can vary markedly.

Sample size analysis. In order to determine whether enough samples were taken at each site, it was necessary to examine the relationship between increasing sample size and the recovery of endophytes. By comparing the number of species recovered, plotted against the number of trees sampled (selected at random), it was possible to estimate whether the sampling was thorough (Fig. 3).

The number of species at the China 1 site reaches an asymptote, whereas the number at the other sites do not level off (Fig. 3). Therefore it is possible that the Australia and Switzerland sites and possibly China 2, were undersampled. Further sampling would have recovered more species, but other than a lower estimate of diversity, the significance of this

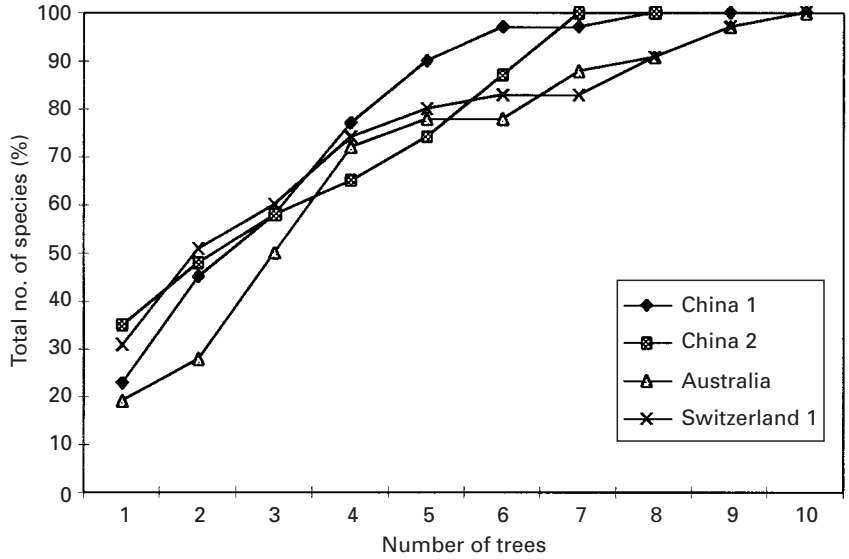

Fig. 3. Incremental increase in the number of species isolated plotted against the number of trees sampled.

result on the interpretation of the data is minimal, as in most analyses in this study, the rare taxa were excluded.

DISCUSSION

Colonization and isolation rates

As noted previously, colonization rates are defined as the total number of samples yielding one or more isolate, divided by the total number of samples in the trial (Eqn 1), and isolation rates as the total number of isolates yielded in a given trial divided by the total number of samples in that trial (Eqn 2). A great variation in colonization rates has been reported in other studies on palm endophytes from as low as 12.5% (Rodrigues & Samuels, 1990) to 80.8–89.2% (Fröhlich, 1997). In several studies, leaflets only (no petioles) were examined and the colonization rates were 21–30% (Rodrigues, 1994) and 20.3% (Southcott & Johnson, 1997).

In general, the colonization rates of this study appear to be closer to those of Rodrigues (1994). Potential reasons for differences in colonization rates obtained in this study, and those obtained previously from palms, could be genuine, or because of minor variations in the methodology. The protocols used here were very similar to those of Fröhlich (1997) but differed slightly from those of Rodrigues (1994).

The palms sampled outside their geographic range had lower colonization rates. This agrees with the results of similar studies on other hosts from disjunct distributions (Fisher *et al.*, 1993; McCutcheon *et al.*, 1993; Fisher *et al.*, 1994). By contrast, Southcott & Johnson (1997) found no significant difference between the colonization rates of native and non-native palms in Bermuda.

The reason for the particularly low colonization rates in Switzerland, compared with the Australian site, is unknown. The palms were the healthiest sampled and the habitat was very favourable for their growth and reproduction. There were distinct differences, however, in the adjacent vegetation at the sites. *Trachycarpus fortunei* in the Royal Botanical Gardens, Sydney was surrounded by native and non-native palms, whereas it was the only palm species in the forest garden at Villa la Palma, Switzerland. If native palms act as a source of endophyte inoculum, then the lack of other palms in Switzerland could explain the low colonization rates.

Composition of endophytic assemblage

Several taxa such as *Glomerella cingulata*, *Phomopsis* spp. and *Guignardia cocogena* dominated the assemblages at each site. The assemblages comprised ubiquitous taxa often recovered from many other hosts (e.g. *Glomerella cingulata*, *Alternaria alternata*, *Phoma* spp., *Fusarium solani*) and in the China sites only, host specific taxa (*Oxydothis* sp. nov., *Wardomycopsis* sp. nov., *Periconia* sp. nov.). Of these host specific taxa, *Oxydothis* spp. have been previously reported as endophytes of palms (Rodrigues, 1994; Fröhlich, 1997), but not in such high proportions (<1%). In addition, species with affinities to *Wardomycopsis* (Minter, 1985) such as *Wardomyces* sp. (Rodrigues, 1994; Southcott & Johnson, 1997) and *Mammaria* sp. (Fröhlich, 1997), have also been isolated from palms.

Fröhlich (1997) noted that the endophyte assemblages of tropical palms showed more affinities to those of non-angiospermous and dicotyledonous hosts in tropical regions than to monocotyledonous temperate hosts. Temperate and tropical palms have many endophyte genera in common. Fröhlich (1997) reported *Phomopsis* spp. and *Colletotrichum* spp. as the second and fourth highest recorded genera,

respectively, isolated from *Licuala* spp. in Australia and Brunei. In other studies of palms there are also considerable overlaps of species and genera (Rodrigues & Samuels, 1990; Rodrigues, 1994). The relative proportions of taxa comprising the assemblages, however, were often quite different. For instance, *Phomopsis* is the most common genus shared by this study and that of Rodrigues (1994), but it is recovered at only 3% relative frequency in the latter investigation. The endophyte assemblages of all previous studies on palms have been dominated by xylariaceous taxa (Rodrigues & Samuels, 1990; Rodrigues, 1994; Fröhlich, 1997), whereas the proportion of xylariaceous taxa in this study was 3% of the total isolates. A trend towards higher proportions of xylariaceous fungi in endophyte assemblages from tropical compared with temperate hosts has previously been noted (Pereira *et al*., 1993; Petrini *et al*., 1995; Lodge *et al*., 1996; Fröhlich, 1997). This difference in the composition of the endophyte assemblages appears to be a climatic affect, as when the host *Gynoxis oleifolia* from Ecuador, was sampled within tropical latitudes, but in temperate habitats (i.e. at high altitudes), the endophyte assemblage recorded was analogous to those of hosts in temperate habitats (Fisher *et al*., 1995).

The most common genera recorded from monocotyledonous, temperate *Juncus* species (Juncaceae) (Menendez *et al*., 1995) and *Quercus ilex*, a temperate, evergreen, dicotyledonous angiosperm (Fagaceae) (Fisher *et al*., 1994), were also commonly recorded from *T. fortunei*. Xylariaceous genera were absent from the endophyte assemblage of the *Juncus* spp. (Menendez *et al*., 1995) and in relatively low proportions compared with non-xylariaceous taxa in the assemblages from *Q. ilex* (see Fisher *et al*., 1994). Therefore, the high relative proportions of non-xylariaceous taxa reported in temperate studies are reflected in the endophyte assemblages recorded from *T. fortunei*.

Several of the species encountered here have previously been reported as pathogens of palms and other tropical and temperate plants (Rodrigues, 1994; Brown *et al*., 1998). These include *Colletotrichum gleosporioides-Glomerella cingulata* (Arx, 1987; Carroll, 1990), *Colletotrichum acutatum* (Sutton, 1980), *Alternaria alternata* (Arx, 1987), *Curvularia lunata* (Arx, 1987), *Pestalotiopsis palmarum* (Mordue & Holliday, 1971) and *Fusarium solani* (Arx, 1987).

Tissue specificity

Leaf/petiole age analysis. In several studies, differences in the species composition and frequencies of endophytes recovered for different tissue types of a given host have been recorded (Rodrigues, 1994, 1996). Petrini *et al*. (1992) concluded that different plant tissues and organs may in fact resemble distinct microhabitats. Espinosa-Garcia & Langenheim (1990) expanded this concept by demonstrating that each individual of a host species may function as a distinct ecosystem.

The results of this study, showing a general increase in the number of endophytes recovered with increasing tissue age, are in agreement with those obtained for tropical palms (Rodrigues & Samuels, 1990; Rodrigues, 1994; Fröhlich, 1997). Rodrigues (1994), however, found no significant difference between young and old leaves of immature palms. Frequency of colonization and/or species diversity has also been found to increase with the age of the organs or tissues in several hosts in temperate countries (Bernstein & Carroll, 1977; Carroll *et al*., 1977; Petrini & Carroll, 1981; Cabral, 1985; Sieber & Hugentobler, 1987; Bertoni & Cabral, 1988; Espinosa-Garcia & Langenheim, 1990; Carroll, 1991). This trend of increasing infection with increasing tissue age is thought to be due to repeated re-infection of host tissue over time from airborne endophytes, as opposed to outgrowth from a few initial infection sites (Carroll *et al*., 1977; Stone, 1987; Bertoni & Cabral, 1988). This hypothesis has been supported by isozyme studies (Rodrigues *et al*., 1993) and other studies investigating the genetic diversity of endophyte isolates (Hämmerli *et al*., 1992; McCutcheon *et al*., 1993). Changes in infection rates are probably due to physical alterations of the plant tissue or degradation of the leaf cuticle which may make plant hosts more susceptible to invasion with age (Stone, 1987; Espinosa-Garcia & Langenheim, 1990).

High rates of multiple infection have been previously recorded in palms (43.2–50.7% of all samples) (Fröhlich, 1997), and temperate deciduous trees (2–27%) (Fisher & Petrini, 1990). Possible reasons for these results, and for the similarly high multiple infection rates of this study, could be the relatively large size of the sampling unit, i.e. discs of 6 mm diameter. As Carroll (1995) points out, the size of the sampling unit is many times larger than the limited area occupied by the endophyte which may be restricted to single cell lumina (Stone, 1987; Suske & Acker, 1987, 1989). Therefore, multiple infections can be expected. A higher number of smaller samples could reduce this problem. However, in this study disc size was felt to be the optimum compromise for the sampling unit size: quantity sampled ratio (Petrini *et al*., 1992). Also, it is likely that if consistent between tissue types, the multiple infection frequencies represent real differences in the colonization of the tissues.

Tissue age had little influence on the composition of the endophyte assemblages in a previous study on palms (Fröhlich, 1997). In this study, differences in endophyte assemblages in different aged tissue were recorded, but to determine the causes behind these

trends is beyond the scope of this study. Similar results have been reported in other studies, for the differential distribution of some species in young and old tissues (Sieber & Hugentobler, 1987; Sieber-Canavesi & Sieber, 1987). Endophytic assemblages have been reported to change both qualitatively and quantitatively as the plant tissue ages (Espinosa-Garcia & Langenheim, 1990; Barklund & Kowalski, 1996). Although changes in the diversity, abundance and species richness of the endophyte assemblages were evident, no clear sequence of species replacement has ever been demonstrated.

Tree age analysis. During this study, tree age was not found to be an influencing factor, either quantitatively (Fig. 1a–c) or qualitatively (Table 3), the only obvious difference being the absence of xylariaceous taxa from immature palm hosts. Some evidence for partiality by endophyte taxa based on the age of the host was reported by Rodrigues (1994), and this was attributed to ecological factors such as the position of the tree in the canopy and the subsequent affects of the differing microenvironments on individual endophyte taxa.

Vein and intervein analysis. The recovery of significantly higher rates of endophytes from vein compared with intervein tissues is consistent with other studies on palms (Rodrigues & Samuels, 1990; Fröhlich, 1997) and other monocotyledons (Brown *et al*., 1998) and also with non-palm hosts (Wilson & Caroll, 1994). The physical properties of the leaf might affect spore retention and spore deposition, such as the behaviour of water reaching the leaf and the pattern of runoff and evaporation (Wilson & Caroll, 1994), all of which in this case favour the vein and petiole tissues.

The consistently greater number of endophytes obtained from the veins in this study could be because the leaflets have induplicate leaves which are V-shaped in cross section (Uhl & Dransfield, 1987). Rainwater falling on the leaf surface would naturally collect in the troughs formed by the veins, thus affecting evaporation rates and subsequently waterborne spore deposition. Variable results, however, have been obtained for palms with reduplicate ($\wedge$-shaped in cross section) leaflets (Rodrigues, 1994; Fröhlich, 1997; Southcott & Johnson, 1977).

Fröhlich (1997) reported that hyphomycetes, and in particular xylariaceous anamorphs, were more common in the blades of fronds (leaves), whereas the coelomycetes dominate the petioles. This is also the case in the tropical host *Manilkara bidenata* (Sapotaceae) (Lodge *et al*., 1996) where, 90% of all of the xylariaceous taxa were recorded in leaf tissues. Other studies in temperate habitats which report xylariaceous taxa in low proportions such as in *Q. ilex* (Fisher *et al*., 1994) and *Gynoxis oleifolia* (Fisher *et al*., 1995), also indicate that they occur predominantly in leaf tissues. However, this is not always the case (Sieber *et al*., 1991). In general in temperate countries, endophyte assemblages, dominated by non-xylariaceous taxa, have revealed no clear trends in distribution of taxa which could be related to tissue specificity.

Distribution at different spatial scales

Country/site/tissue : correspondence analysis. The site separations were produced by variations in types and relative importance of the dominant endophyte species. The gradient on the first axis is produced by the high relative importance of *Glomerella cingulata* and also *Alternaria alternata*, *Phomopsis* sp. H, and *Microsphaeropsis* sp. C in both the Chinese and Swiss sites. The high incidence of taxa such as *Oxydothis* sp. nov., *Stagonospora* sp. 5, *Wardomycopsis* sp. nov. and the xylariaceous taxa, are important in clustering the China 1 and 2 sites away from the sites of the other two countries. The gradient of the second axis is greatly influenced by the high incidence of *Guignardia cocogena* at the Chinese and Australian sites. The site-specific nature of some of the dominant Australian taxa such as *Phomopsis* A, *Phomopsis* sp. B, are responsible for the clustering at this site.

Several studies have shown that site-specific factors may influence the level of infection (Carroll, 1995). However, efforts were made to reduce this variable in this study and only the China 2 site differed slightly from the other sites in being more open and exposed.

The palms sampled in this study were collected from continuous and disjunct distributions, and clear patterns in the endophyte assemblages are reflected (Fig. 2, Table 3). In Fig. 2, the two China sites clustered together. Similarities in endophyte assemblages, for a particular host, when compared over several widely separated sites are attributed to geographical continuity (Carroll, 1995). Rodrigues (1994) reported site related differences between the numbers of endophyte isolates recovered, but similar assemblage compositions, from two differing ecological zones (100 m apart) within the natural range of the palm *Euterpes olercaea*. The quantitative differences were related to the higher diversity of plant species at one site contributing to an increased source of inoculum. Several other studies on the distribution of endophytes within the host's natural geographic range (Espinosa-Garcia & Langenheim, 1990; Fisher & Petrini, 1990; Rollinger & Langenheim, 1993; Rodrigues, 1994) illustrate that endophytes show a remarkable degree of similarity when compared over several dispersed sites, providing there is a geographic continuity of the hosts between the sites.

The endophytic mycota of plant hosts occurring outside their natural range, in disjunct distributions,

have been reported to show both quantitative and qualitative differences when compared with the mycota of the host within its natural range (Carroll *et al*., 1977; Espinosa-Garcia & Langenheim, 1990; Fisher *et al*., 1993, 1994). In general, these studies indicate that endophyte assemblages on plant hosts occurring outside the natural range are depauperate and contain different species than those in native habitats. In this study, diversity and abundance measures can be estimated using direct counts as the sample sizes are the same in all sites (Ludwig & Reynolds, 1988). A comparison of the total numbers of taxa and isolates for each site where 740 samples were taken indicates that the numbers of taxa at each site are similar (30–34), but the abundance of isolates is variable (201–497). Although the species richness is constant and the abundance variable, the endophyte assemblages of geographically disjunct sites are depauperate. For example, what are considered host specific taxa, e.g. *Oxydothis* sp.nov. *Wardomycopsis* sp.nov., were only recovered from the two Chinese sites.

Stand and tree level patterns

Petrini *et al*. (1992) state that rare taxa are recovered sporadically due to environmental influence on the spatial distribution of the endophytes, competitive interactions, and because of sampling and isolation techniques. The optimum number of individuals and sampling units has been estimated to be up to 40 individuals with 30–40 sampling units per individual (Petrini *et al*., 1992). In this study 10 individuals were sampled with 74 sampling units per individual, because of the large size of the leaves. Australia and Switzerland and possibly China 2 were undersampled. The sampling, however, was carried out on the results of the pilot study which suggested that this combination of sampling was adequate. The China 2 results may be affected by the accidental loss of two of the young leaves, which meant that only eight trees could be represented. Sampling, especially outside the natural range of the host, must therefore take into account site differences with accommodation made for hosts in non-native habitats.

This study has provided important information regarding the effect of climate on the endophyte assemblage recovered. Of particular interest is the similarity of the endophyte assemblages of this temperate palm with those of unrelated hosts from temperate habitats. The most obvious difference is the paucity of xylariaceous taxa in the endophyte assemblage compared with tropical palms, and other tropical hosts, previously sampled. This study also reinforced earlier findings on plant hosts sampled in continuous and disjunct distributions, and in the endophyte assemblages recovered from different tissue types, tissue age and host age classes.

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