CHAPTER 3

ENDOPHYTIC FUNGI FROM ZINGIBERACEAE

3.1 Introduction

Many reports of endophytes from monocotyledonous plants have concentrated on temperate grasses, with an emphasis on clavicipitaceous endophytes and their beneficial role to the host (Clay, 1986; Dahlman *et al*., 1991). In the tropics, comprehensive studies on endophytes in palms have been carried out by Rodrigues and Samuels (1990), Rodrigues (1994), Taylor *et al*. (1999) and Fröhlich *et al*. (2000), while Umali *et al*. (1999) and Lumyong *et al*. (2000) investigated the endophytic fungi in leaves, nodes and internodes of bamboo. These studies have shown the effects of factors such as leaf age, leaf tissue type, stage of plant growth, site, altitude, and seasonality on endophyte assemblages and colonization. Brown *et al*. (1998) investigated the endophytes in banana leaves, discussing their potential in biological control. Photita *et al.* (2001b) investigated the endophytic fungi from leaves, petioles and pseudostems of wild banana and noted that the ratio of six fungal species for every vascular plant species, a ratio used to estimate global fungal species numbers (Hawksworth, 1991), appeared to hold for *Musa* species. A wide range of the endophytic fungi isolated from wild banana also showed the potential to produce bioactive compounds (Photita, 2003).

The present study was initiated in order to establish the ecology and diversity of endophytic fungi in four wild and two cultivated Zingiberaceae in northern

Thailand. This research was initiated to establish whether the endophytes differ between zingiberaceous hosts, especially between the wild and cultivated species; whether the endophytes in ginger differ from those in other tropical hosts; and whether there is any evidence that the endophytes of Zingiberaceae are latent pathogens.

3.2 Materials and methods

3.2.1 Sample selection

Ten plants of each species were randomly selected and collected from each of two localities: *Amomum siamense* and *Etlingera littoralis* from two sites at Doi Suthep-Pui National Park (Huay Kok Ma and Medicinal Plant Garden), *Alpinia malaccensis* from two sites at Doi Suthep-Pui National Park (Huay Kok Ma and Doi Pui), *E. elatior* from one site at Queen Sirikit Botanic Garden and one site at Chiang Mai University area, *Alpinia galanga* from one site in Lampang Province (Muang) and one site in Chiang Mai Province (Hangdong), and *Zingiber officinale* from two sites (commercial and backyard) in Phayao Province. Collections (one year sampling) were made in August (wet season) and February (dry season) with the exception of *Z. officinale*, which was collected in August only (2000 and 2001). One pseudostem, two leaves (1 young, and 1 old), and one rhizome from each plant was removed, bagged and returned to the laboratory.

3.2.2 Surface sterilization and isolation of endophytes

Within 24 hour of collection samples were washed in running tap water for 15 min. Five segments (10 mm long) were then cut from each pseudostem and five

segments (10 mm^3) were cut from each rhizome. The segments were cut from scattered areas of the pseudostem and rhizome. Twenty discs (5 mm diameter) were cut from the leaves, using a sterile cork borer. Ten disks included a vein while the other ten consisted of only interveinal lamina. The surface sterilization method chosen was similar to that used for isolation of endophytes from palms (Taylor *et al*., 1999), but modified following pilot experiments, based on the thickness of the ginger tissues. Samples were triple surface sterilized by soaking in 95% ethanol for 15 s, then in a solution of sodium hypochlorite (1%) for 5 min., and finally in 95% ethanol for 15 s. Samples were dried on sterilized paper, and then transferred to 2% malt extract agar + 0.03% Rose Bengal + 0.1% streptomycin sulfate. Labeled plates were incubated at room temperature (27–30 °C) for 1 to 2 weeks (Figure 3.1). Hyphae growing out from the tissues were then transferred to tubes containing corn meal agar.

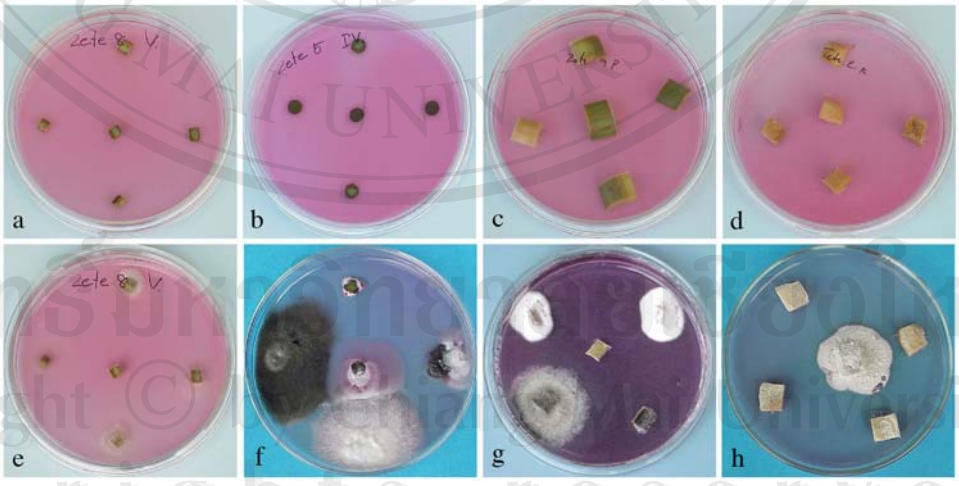

Figure 3.1 Zingiberaceous samples: vein (a), intervein (b), pseudostem (c) and rhizome (d) on malt extract agar containing Rose Bengal and streptomycin sulfate, with fungal hyphae growing out from the tissues (e–h).

3.2.3 Identification

The isolates were incubated for 4 to 8 weeks and, if sporulating, were identified to genus or species using taxonomic keys (e.g., Ellis 1971, 1976; Carmichael *et al*., 1980; Sutton, 1980; von Arx, 1981; Hyde *et al*., 2000). Isolates which failed to sporulate within 8 weeks were subcultured on corn meal agar or potato dextrose agar, each containing autoclaved strips of host leaf tissue. After 2 months, sterile isolates were identified as mycelia sterilia. These methods were similar to those used to promote sporulation in endophytic fungi of *Bambusa tuldoides* and *Livistona chinensis* (Guo *et al*., 1998; Umali *et al*., 1999).

3.2.4 Statistical analyses

The fungal isolate prevalence and intensity were calculated as follows:

$$\text{Isolate prevalence} = \frac{\text{total number of samples yielding} \geq 1 \text{ isolate}}{\text{total number of samples in that trial}} \times 100$$

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

Isolate prevalence is expressed as a percentage, as commonly used by other workers (Petrini *et al*., 1982). Intensity was calculated and used to demonstrate the degree of multiple colonization from the samples in different trials, but was not expressed as a percentage (Taylor *et al*., 1999). Relative frequency of isolation (Petrini *et al*., 1982) was used for species abundance and calculated as:

$$\text{Relative frequency} = \frac{\text{number of samples colonised by a given fungus}}{\text{total number of fungal isolates infected}} \times 100$$

A chi-squared (χ^2) goodness-of-fit test was performed to test whether the isolate prevalence for the four trials of each plant species were statistically different. Non-parametric statistical tests were used because the data in most cases did not fit the assumptions for parametric statistics, even after correction of the data by square root or logarithmic transformation (Zarr, 1999). A Mann-Whitney test was performed on isolates from old and young leaves at each site. A Kruskal-Wallis procedure was used for multisample analysis of the number of isolates recovered from vein and intervein tissues, and of the number of isolates recovered from different tissue types between wet and dry seasons. ANOVA was used to test for significant differences between the means of the species/plant for each site of collection. In all analyses, *P* values are described if $P < 0.05$. The fungal compositions of different zingiberaceous species at different sites were compared using three dimensional correspondence analysis. Species area curves were used to determine whether enough samples were taken at each site.

3.3 Results

3.3.1 Isolate prevalence and intensity

A total of 4,800 samples were processed from the four wild species and 2,400 samples from the two cultivated species, *Alpinia galanga* and *Zingiber officinale.* A total of 1,195 fungal isolates were recovered from *Alpinia malaccensis*, 1,222 from *Amomum siamense*, 435 from *Etlingera elatior* and 526 from *Etlingera littoralis.* Five hundred and twenty five fungal isolates were recovered from *A. galanga*, while 933 fungal isolates were recovered from *Z. officinale*. The overall isolate prevalence (%) and intensity for the assemblages of endophytes recovered from each plant, at each

site in the wet and dry seasons are given in Table 3.1. For the four wild species there was no significant difference between isolate prevalence in each trial. However, there were significant differences between isolate prevalence in each trial with the two cultivated species, *A. galanga* (χ^2 = 12.94, df 3, *P* = 0.005) and *Z. officinale* (χ^2 = 10.23, df 3, *P* = 0.017). In all cases there were higher isolate prevalences in *A. malaccensis*, *A. siamense* and *Z. officinale* than in *E. elatior*, *E. littoralis* and *A. galanga* (Figure 3.2). Overall, there was a higher degree of multiple infections (21–38 % of samples, Table 3.1) in *A. malaccensis* and *A. siamense* than in *E. elatior*, *E. littoralis*, *A. galanga* and *Z. officinale* (4–17 % of samples, Table 3.1).

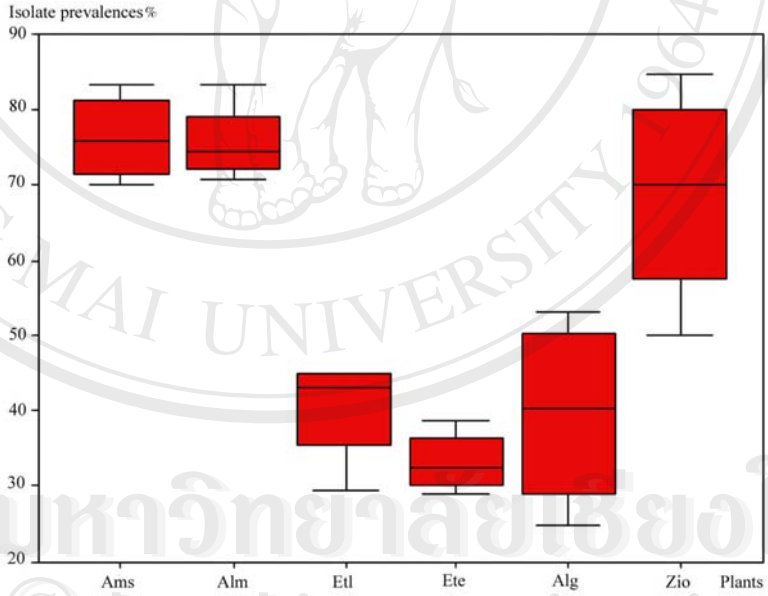

Figure 3.2 Boxplots comparing the overall isolate prevalences of endophytes recovered from wild and cultivated Zingiberaceous plant: Ams. *Amomum siamense*, Alm. *Alpinia malaccensis*, Etl. *Etlingera littoralis*, Ete. *Etlingera elatior*, Alg. *Alpinia galanga*, Zio. *Zingiber officinale*.

Table 3.1 Isolate prevalence, intensity, multiple infection and number of taxa of endophytes isolated from Zingiberaceae at each site in the wet or dry season.

Characteristic	*Alpinia malaccensis*				*Amomum siamense*			
	Wet season		**Dry season**		**Wet season**		**Dry season**	
	HKM	**DP**	**HKM**	**DP**	**HKM**	**MPG**	**HKM**	**MPG**
No. of samples	300	300	300	300	300	300	300	300
No. of isolates recovered	266	306	352	271	283	293	283	293
Total taxa recovered	20	17	18	13	19	18	23	20
Taxa/plant* (means+SD)	7±2	8±2	7±2	6±1	8±2	8±1	8±2	7±2
Taxa/plant (range)	3–8	4–10	5–10	5–8	4–12	6–11	5–11	6–11
Isolate prevalence (%)	70.7	75.0	83.7	73.7	73.0	83.3	70.0	78.7
No.(%) of samples yielding two species	41 (19%)	57 (25%)	79 (31%)	46 (21%)	54 (25%)	76 (30%)	68 (29%)	51 (21%)
No.(%) of samples yielding > two species	6 (3%)	12 (5%)	11 (4%)	2 (1%)	5 (2%)	19 (8%)	5 (2%)	3 (1%)
Intensity (no. of isolates per sample)	0.89	1.02	1.17	0.90	0.95	1.21	0.96	0.98

Characteristic	*Etlingera littoralis*				*Etlingera elatior*			
	Wet season		**Dry season**		**Wet season**		**Dry season**	
	HKM	**MPG**	**HKM**	**MPG**	**QSBG**	**CMU**	**QSBG**	**CMU**
No. of samples	300	300	300	300	300	300	300	300
No. of isolates recovered	101	140	141	144	112	94	132	97
Total taxa recovered	9	13	17	13	8	9	9	12
Taxa/plant* (means+SD)	5±1	6±1	6±1	6±1	4±1	4±1	4±1	5±1
Taxa/plant (range)	4–7	4–8	4–8	4–9	2–6	2–5	2–6	3–7
Isolate prevalence (%)	29.3	41.7	44.7	44.7	34.0	29.0	38.7	31.0
No.(%) of samples yielding two species	16 (11%)	13 (10%)	7 (5%)	8 (6%)	9 (9%)	7 (8%)	15 (13%)	4 (4%)
No.(%) of samples yielding > two species	0 (0%)	1 (1%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (1%)	0 (0%)
Intensity (no. of isolates per sample)	0.34	0.47	0.47	0.48	0.37	0.31	0.44	0.32

Table 3.1 (Continued).

Characteristic	*Alpinia galanga*				*Zingiber offinale*			
	Wet season		**Dry season**		**Year 2000**		**Year 2001**	
	CM	**LP**	**CM**	**LP**	**PC**	**PB**	**PC**	**PB**
No. of samples	300	300	300	300	300	300	300	300
No. of isolates recovered	177	77	167	104	222	254	290	167
Total taxa recovered	11	10	12	10	12	13	12	7
Taxa/plant* (means+SD)	6±1	6±1	7±1	6±2	8±1	5±1	7±2	4±1
Taxa/plant (range)	5–8	4–8	6–9	4–8	6–10	3–6	5–9	3–5
Isolate prevalence (%)	53.3	24.7	47.3	33.3	65.0	75.3	84.7	50.0
No.(%) of samples yielding two species	17 (11%)	3 (4%)	22 (15%)	4 (4%)	23 (12%)	28 (12%)	36 (14%)	17 (11%)
No.(%) of samples yielding > two species	0 (0%)	0 (0%)	2 (1%)	0 (0%)	2 (1%)	0 (0%)	0 (0%)	0 (0%)
Intensity (no. of isolates per sample)	0.59	0.26	0.56	0.35	0.74	0.85	0.97	0.56

*Means are not significantly different in each plant species according to ANOVA ($P < 0.05$) with exception of *Zingiber officinale*, means are significantly different ($P = 0.000$)

HKM = Huay Kok Ma, DP = Doi Pui, MPG = Medicinal Plant Garden, QSBG = Queen Sirikit Botanic Garden, CMU = Chiang Mai University, CM = Chiang Mai (Hangdong), LP = Lampang (Muang), PC = Phayao (Muang, commercial) , PB = Phayao (Phugamyao, backyard)

3.3.2 Tissue specificity

3.3.2.1 Leaf age analysis

Mann-Whitney tests were performed on data obtained for each plant at each site. There was no significant difference between the number of isolates recovered from young and old leaves of the two cultivated species (*A. galanga* and *Z. officinale*), at any of the sites sampled (P <0.05). However, there were significantly more isolates recovered from old leaves of *E. elatior*, than from young leaves. This applied in both the wet and dry seasons samples from Queen Sirikit Botanic Garden (wet, P = 0.007; dry P = 0.003) and Chiang Mai University area (wet, P = 0.035; dry P = 0.021). In the dry season samples of *A. malaccensis* from Huay Kok Ma (P = 0.011) and Doi Pui (P = 0.029), and of *A. siamense* from Huay Kok Ma (P = 0.005) and Medicinal Plant Garden (P = 0.000), significantly more isolates were recovered from old leaves, as compared to young leaves. In the wet season sample of *E. littoralis* from Huay Kok Ma (P = 0.023) and in the dry season sample from Medicinal Plant Garden (P = 0.019), there were differences between the number of isolates recovered from young and old leaves, with higher frequencies consistently obtained from older leaves (Appendix B).

3.3.2.2 Pseudostem, rhizome and leaf (vein and intervein) analysis

Kruskal-Wallis rank-sum test was performed on data of the number of isolates recovered from different tissue types. For both the wild and cultivated species there were significant differences in the number of isolates recovered from different tissue types. In most cases there were higher numbers of isolates recovered from leaf and pseudostem than from rhizome (Figure 3.3). However, the pattern of numbers of

isolates recovered from *E. littoralis* was pseudostem > rhizome > leaf (Figure 3.3). For vein and intervein tissues, the Kruskal-Wallis tests for the wild and cultivated species indicated that there was no significant difference in the number of isolates recovered from vein and intervein tissues irrespective of leaf age. However, there were significantly more isolates recovered from vein as compared with intervein tissues ($P = 0.000$) in both young and old leaves of *E. elatior* (Appendix B).

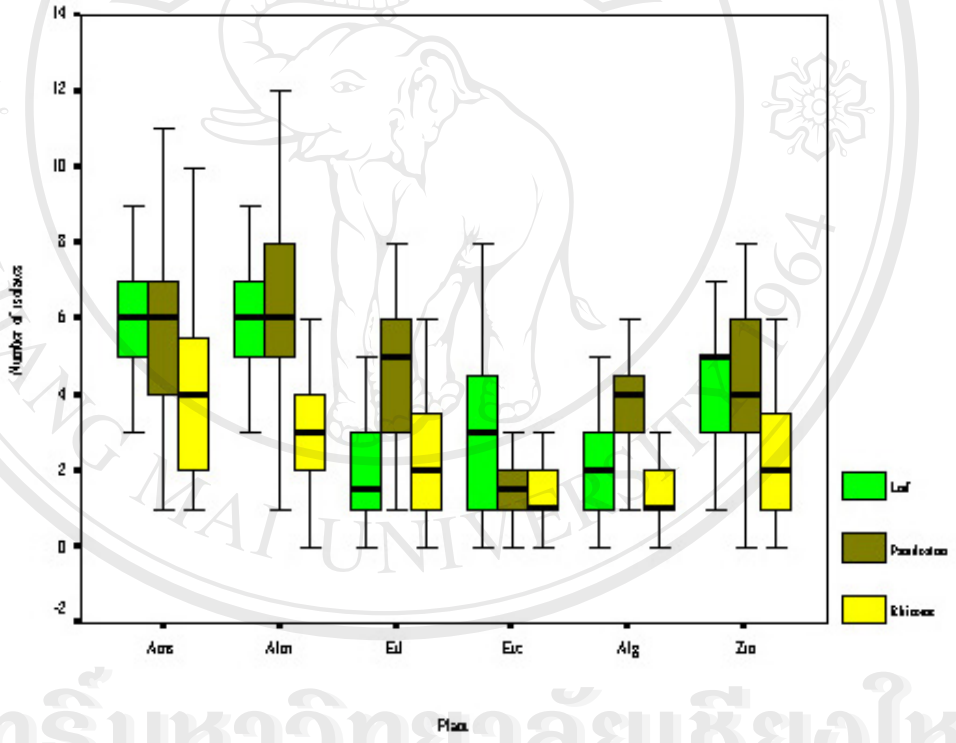

Figure 3.3 Boxplots comparing the ranked medians of the numbers of isolates, recovered from leaf, pseudostem and rhizome, from wild and cultivated Zingiberaceous plant: Ams. *Amomum siamense*, Alm. *Alpinia malaccensis*, Etl. *Etlingera littoralis*, Ete. *Etlingera elatior*, Alg. *Alpinia galanga*, Zio. *Zingiber officinale*.

3.3.3 Composition of endophytic assemblages

Of the 4,836 fungal isolates recovered, 46 taxa were observed, comprising 9 ascomycetes and 37 anamorphic fungi (6 coelomycetes and 31 hyphomycetes) (Table 3.2). In the wild and cultivated species the most dominant genus was *Colletotrichum*. The taxa occurring at relative frequencies of > 1 % at each site in different seasons are presented in Tables 3.3–3.8. Based on conidial size and colony characteristics, the *Colletotrichum* isolates could be separated into three taxa. However, these all fit the broad description of *C. gloeosporioides*. Four species of *Pyricularia* (including *P. costina* and three new species) were isolated, distinguished by conidial shape, size and septation. *Fusarium* comprised five species, which were distinguished on colony morphology and conidial size, while *Glomerella* comprised two species separated on colony morphology, spore size and spore colour. Four species of *Phomopsis* were distinguished by colony morphology, the presence/absence of α- and β-conidia and the dimensions of these conidia. Since it is difficult to identify these taxa to species level, the data of each genus has been combined for statistical comparison.

In all cases there was a large degree of overlap in the endophyte communities at the genus level (Tables 3.3–3.8). In the wet season, five to nine of the taxa were recorded from two sites of each plant species, whereas in the dry season six to eight taxa occurred at both sites. Six to eleven taxa recorded in the wet season also occurred in the dry season. Twenty six taxa were recorded only from the wild species, while one taxa were recorded only from the cultivated species (Tables 3.2). Almost taxa recovered from the cultivated species were also recovered from the wild species.

Table 3.2 A comparison of the total fungal taxa recovered from zingiberaceous species.

Taxa	*Alpinia malaccensis*	*Amomum siamense*	*Etlingera littoralis*	*Etlingera elatior*	*Alpinia galanga*	*Zingiber offinale*
Acremonium polychroma	+	+				
Acremonium sp.			+	+		
Alternaria alternata	+					
Aspergillus niger	+	+	+			
Byssochlamus nivea			+			
Chaetomium globosum	+		+			
Cladosporium cladosporioides		+				
Colletotrichum gloeosporioides	+	+	+	+	+	+
Cordana sp.				+		
Curvularia brachyspora			+	+	+	
Cylindrocarpon sp.		+				
Cylindrocladium sp. 1	+	+	+		+	
Cylindrocladium sp. 2		+				
Dactylaria sp.	+					
Drechslera australiensis		+				
Eupenicillium crustaceum	+	+	+	+	+	+
Fusarium spp.	+	+	+	+	+	+
Fusicoccum sp.	+	+				
*Gaeumannomyces amomi**	+	+				+
Gelasinospora sp.		+	+		+	+
Geniculosporium sp. 1	+	+	+	+	+	+
Geniculosporium sp. 2		+				
Gilmaniella humicola	+	+				
Glomerella spp.	+	+	+	+	+	+
Humicola fuscoatra	+	+	+	+	+	
Idriella lunata						+
*Leiosphaerella amomi**	+	+				
Nigrospora oryzae	+	+				

Table 3.2 (Continued).

Taxa	*Alpinia malaccensis*	*Amomum siamense*	*Etlingera littoralis*	*Etlingera elatior*	*Alpinia galanga*	*Zingiber offinale*
Nodulisporium sp.			+			+
Paecilomyces sp.			+		+	
Papulaspora sp.		+				
Penicillium sp.	+	+	+			+
Periconia sp.	+					
Pestalotiopsis sp.	+	+		+		
Phoma sp.	+	+				
Phomopsis spp.	+	+	+	+	+	+
Phyllosticta capitalensis	+	+	+	+	+	+
Pyricularia costina	+	+	+			+
*Pyricularia kookicola**		+				
*Pyricularia longispora**	+	+				
*Pyricularia variabilis**		+				
Stachybotrys sp.	+	+				
Talaromyces flavus	+	+	+	+	+	+
Thermomyces sp.		+				
Trichoderma sp.	+	+			+	+
Xylariaceous taxa	+	+	+	+	+	+
Ascomycetes (9 taxa)	7	7	7	4	5	6
Anamorphic fungi (37 taxa)	22	28	14	10	10	10
Total taxa (46 taxa)	**29**	**35**	**21**	**14**	**15**	**16**

*Described during the present study

Table 3.3 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Alpinia malaccensis* occurring at isolate prevalences of >1 %.

Taxa	Wet season				Dry season				Plant part		
	Huay Kok Ma		Doi Pui		Huay Kok Ma		Doi Pui				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Colletotrichum gloeosporioides	35.3	31.3	18.9	19.3	30.9	36.3	7.4	6.7	26.9	30.5	2
Eupenicillium crustaceum	3	2.7	3.3	3.3	2.5	3	0	0	0.5	0.5	11
Fusarium spp.	6	5.3	10.1	10.3	0.8	1	0.7	0.7	1.9	16	2.5
Glomerella spp.	15	13.3	16.7	17	23.6	27.7	37.3	33.7	29.4	19.5	0
Phoma sp.	0.7	0.6	1.9	2	0	0	0	0	0.7	1	0
Phomopsis spp.	9.4	8.3	16.7	17	5.4	6.3	14.7	13.3	15.5	5	0.5
Phyllosticta capitalensis	1.9	1.7	2.6	2.7	0.3	0.3	0.4	0.3	1.9	0	0
Pyricularia spp.	7.9	7	2.6	2.7	0.3	0.3	2.6	2.3	4.6	0	0
Talaromyces flavus	0	0	0	0	4.2	5	9.2	8.3	0.4	1	17.5
Xylariaceous taxa	1.1	1	8.8	9	20.7	24.3	11.4	10.3	11.6	17	3.5
Mycelia sterilia	16.1		15.3		8.8		14.7				
Rare isolates*	3.6		3.1		2.5		1.6				
Total	80.3		81.6		88.7		83.7				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Wet season

Huay Kok Ma: *Chaetomium globosum*, *Gaeumannomyces amomi*, *Geniculosporium* sp. 1, *Gilmaniella humicola*, *Humicola fuscoatra*, *Leiosphaerella amomi*, *Periconia* sp., *Phoma* sp., *Stachybotrys* sp., *Trichoderma* sp.

Doi Pui: *Aspergillus niger*, *Dactylaria* sp., *Fusicoccum* sp., *Gaeumannomyces amomi*, *Geniculosporium* sp. 1, *Stachybotrys* sp.

Dry season

Huay Kok Ma: *Alternaria alternata*, *Aspergillus niger*, *Cylindrocladium* sp. 1, *Fusarium* spp., *Acremonium polychroma*, *Leiosphaerella amomi*, *Penicillium* sp., *Phyllosticta capitalensis*, *Pyricularia costina*

Doi Pui: *Alternaria alternata*, *Fusarium* spp., *Leiosphaerella amomi*, *Nigrospora oryzae*, *Pestalotiopsis* sp., *Phyllosticta capitalensis*

Table 3.4 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Amomum siamense* occurring at isolate prevalences of >1 %.

Taxa	Wet season				Dry season				Plant part		
	Huay Kok Ma		Medicinal Plant Garden		Huay Kok Ma		Medicinal Plant Garden				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Colletotrichum gloeosporioides	33.1	32.7	21.8	27	20.7	20	26.8	26.3	28.8	37.5	6
Eupenicillium crustaceum	1.4	1.3	6.2	7.7	0	0	0	0	1	0	10.5
Fusarium spp.	5.4	5.3	4.6	5.7	5.9	5.7	1.7	1.7	1.4	13.5	8.5
Glomerella spp.	13.2	13	11	13.7	20.4	19.7	16.3	16	17	22.5	3
Phomopsis spp.	9.8	9.7	8.3	10.3	7.6	7.3	4.7	4.7	9.5	9	0.5
Phyllosticta capitalensis	7.1	7	4.6	5.7	0	0	0	0	4.9	0.5	1
Pyricularia spp.	5.1	5	4.6	5.7	3.5	3.3	8.8	8.7	8.5	0	0
Talaromyces flavus	0	0	0	0	2.8	2.7	1.4	1.3	0	0	6
Xylariaceous taxa	2.4	2.3	26.9	33.3	12.8	12.3	16.9	16.7	18.8	11	11
Mycelia sterilia	15.9		5.6		9		8.5				
Rare isolates*	6.6		6.4		17.3		14.9				
Total	77.5		88		73.7		76.6				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Wet season

Huay Kok Ma: *Gaeumannomyces amomi*, *Gilmaniella humicola*, *Humicola fuscoatra*, *Leiosphaerella amomi*, *Papulaspora* sp., *Pestalotiopsis* sp., *Phoma* sp., *Thermomyces* sp., *Trichoderma* sp.

Medicinal Plant Garden: *Drechslera australiensis*, *Fusicoccum* sp., *Gelasinospora* sp., *Geniculosporium* sp. 2, *Humicola fuscoatra*, *Nigrospora oryzae*, *Phoma* sp., *Stachybotrys* sp.

Dry season

Huay Kok Ma: *Aspergillus niger*, *Cylindrocarpon* sp., *Cylindrocladium* sp. 1, *Cylindrocladium* sp. 2, *Geniculosporium* sp. 2, *Gilmaniella humicola*, *Nigrospora oryzae*, *Penicillium* sp., *Pestalotiopsis* sp., *Trichoderma* sp.

Medicinal Plant Garden: *Cladosporium cladosporioides*, *Cylindrocladium* sp. 1, *Eupenicillium crustaceum*, *Gaeumannomyces amomi*, *Geniculosporium* sp. 1, *Geniculosporium* sp. 2, *Gilmaniella humicola*, *Acremonium polychroma*, *Humicola fuscoatra*, *Nigrospora oryzae*

Table 3.5 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Etlingera littoralis* occurring at isolate prevalences of >1 %.

Taxa	Wet season				Dry season				Plant part		
	Huay Kok Ma		Medicinal Plant Garden		Huay Kok Ma		Medicinal Plant Garden				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Colletotrichum gloeosporioides	37.6	12.7	15	7	22	10.3	36.8	17.7	9	31	4.5
Cylindrocladium sp. 1	0	0	6.4	3	0	0	0.7	0.3	0.8	1.5	0.5
Eupenicillium crustaceum	5.9	2	20	9.3	0.7	0.3	6.3	3	0.4	4	16.5
Fusarium spp.	14.9	5	0	0	3.5	1.7	4.2	2	0.4	8.5	3
Gelasinospora sp.	0	0	4.3	2	0	0	0.7	0.3	0.6	0	1
Geniculosporium sp. 1	0	0	3.6	1.7	0	0	1.4	0.7	0.1	1.5	1.5
Glomerella spp.	7.9	2.7	11.4	5.3	36.2	17	3.5	1.7	6	15	1
Nodulisporium sp.	0	0	2.9	1.3	0	0	0	0	0.3	1	0
Paecilomyces sp.	0	0	0	0	2.8	1.3	0	0	0.1	0	1.5
Phomopsis spp.	7.9	2.7	12.1	5.7	5.7	2.7	8.3	4	3.3	8.5	1
Phyllosticta capitalensis	5.9	2	0	0	3.5	1.7	0	0	1	1.5	0
Talaromyces flavus	5	1.7	0.7	0.3	0.7	0.3	2.8	1.3	0.3	1.5	3
Xylariaceous taxa	11.9	4	15	7	4.3	2	29.2	14	6.4	8	7
Mycelia sterilia	3		0		14.2		4.9				
Rare isolates*	0		8.6		6.4		1.2				
Total	100		91.4		93.6		98.8				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Wet season

Medicinal Plant Garden: *Acremonium* sp., *Byssochlamus nivea*, *Chaetomium globosum*, *Humicola fuscoatra*, *Talaromyces flavus*

Dry season

Huay Kok Ma: *Acremonium* sp., *Curvularia brachyspora*, *Eupenicillium crustaceum*, *Humicola fuscoatra*, *Nodulisporium* sp., *Penicillium* sp., *Pyricularia costina*, *Talaromyces flavus*

Medicinal Plant Garden: *Aspergillus niger*, *Chaetomium globosum*, *Cylindrocladium* sp. 1, *Gelasinospora* sp., *Geniculosporium* sp. 1

Table 3.6 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Eltlingera elatior* occurring at isolate prevalences of >1 %.

Taxa	Wet season				Dry season				Plant part		
	Queen Sirikit Botanic Garden		Chiang Mai University		Queen Sirikit Botanic Garden		Chiang Mai University				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Acremonium sp.	0	0	0	0	2.3	1.0	0	0	0	0	1.5
Colletotrichum gloeosporioides	21.4	8	35.1	11	37.9	16.7	39.2	12.7	4.3	10.5	0.5
Cordana sp.	0	0	0	0	0	0	3.1	1	0.1	0.5	0.5
Eupenicillium crustaceum	0	0	0	0	0	0	6.2	2	0.3	0	2
Fusarium spp.	9.8	3.7	7.5	2.3	8.3	3.7	4.1	1.3	0.1	6	7
Geniculosporium sp. 1	0	0	6.4	2	0	0	19.6	6.3	2	2	1
Glomerella spp.	0	0	8.5	2.7	12.9	5.7	5.2	1.7	0.6	0	0
Phomopsis spp.	13.4	5	11.7	3.7	3.8	1.7	6.2	2	0.8	4.5	0
Phyllosticta capitalensis	3.6	1.3	0	0	2.3	1	1	0.3	0.1	0.5	0
Talaromyces flavus	7.1	2.7	2.1	0.7	3	1.3	1	0.3	0	2	4
Xylariaceous taxa	39.3	14.7	17	5.3	12.1	5.3	5.2	1.7	0.6	4	5.5
Mycelia sterilia	4.5		9.6		17.4		8.2				
Rare isolates*	0.9		2.1		0		1				
Total	100		97.9		100		99				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Wet season

Queen Sirikit Botanic Garden: *Curvularia brachyspora*

Chiang Mai University: *Pestalotiopsis* sp., *Talaromyces flavus*

Dry season

Chiang Mai University: *Humicola fuscoatra*, *Phyllosticta capitalensis*, *Talaromyces flavus*

Table 3.7 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Alpinia galanga* occurring at isolate prevalences of >1 %.

Taxa	Wet season				Dry season				Plant part		
	Chiang Mai		Lampang		Chiang Mai		Lampang				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Colletotrichum gloeosporioides	47.5	28	18.2	4.7	29.3	16.3	35.6	12.3	18	18	2
Cylindrocladium sp. 1	6.8	4	0	0	1.2	0.7	0	0	0	5	2
Eupenicillium crustaceum	3.4	2	10.4	2.7	3.6	2	0	0	1.5	2.5	1.5
Fusarium spp.	5.1	3	18.2	4.7	14.4	8	21.2	7.3	1.5	23.5	5
Gelasinospora sp.	0	0	3.9	1	0	0	0	0	0.1	0.5	0.5
Geniculosporium sp. 1	0	0	11.7	3	7.8	4.3	4.8	1.7	2.8	2	0.5
Glomerella spp.	2.8	1.7	10.4	2.7	6.6	3.7	2.9	1	2.1	3.5	1.5
Humicola fuscoatra	4	2.3	6.5	1.7	0	0	0	0	0.6	3	0.5
Paecilomyces sp.	0	0	0	0	2.4	1.3	0	0	0.5	0	0
Phomopsis spp.	16.4	9.7	3.9	1	13.8	7.7	10.6	3.7	5.9	8	1.5
Phyllosticta capitalensis	0	0	0	0	1.8	1	14.4	5	2.3	0	0
Talaromyces flavus	3.4	2	5.2	1.3	0	0	4.8	1.7	0.6	0	5
Trichoderma sp.	4	2.3	0	0	0	0	0	0	0.6	1	0
Xylariaceous taxa	4.5	2.7	11.7	3	11.4	6.3	3.8	1.3	3.3	1	6
Mycelia sterilia	2.3		0		7.2		1				
Rare isolates*	0		0		0.5		0.9				
Total	100		100		100		100				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Dry season

Chiang Mai: *Curvularia brachyspora*, *Cylindrocladium* sp. 1

Lampang: *Curvularia brachyspora*

Table 3.8 Relative frequencies (RF) and isolate prevalences (IP) of endophytes from *Zingiber officinale* occurring at isolate prevalences of >1 %.

Taxa	Wet season 2000				Wet season 2001				Plant part		
	Phayao (commercial)		Phayao (backyard)		Phayao (commercial)		Phayao (backyard)				
	RF%	IP%	RF%	IP%	RF%	IP%	RF%	IP%	Leaf (IP%)	Pseudostem (IP%)	Rhizome (IP%)
Colletotrichum gloeosporioides	31.5	23.3	45.7	38.7	51.4	49.7	38.3	21.3	39.8	29	11.5
Eupenicillium crustaceum	5.9	4.3	0.4	0.3	1.4	1.3	1.2	0.7	0.8	1	6
Fusarium spp.	9.9	7.3	5.1	4.3	6.2	6	0	0	1.1	16	6
Geniculosporium sp.	2.7	2	1.2	1	0	0	3.6	2	0.9	2.5	1.5
Glomerella spp.	11.7	8.7	21.3	18	10.3	10	13.8	7.7	13	11	3.5
Phomopsis spp.	18.9	14	8.7	7.3	9.7	9.3	12.6	7	11.6	8.5	1.5
Phyllosticta capitalensis	0.9	0.7	2.8	2.3	0.7	0.7	15	8.3	4.4	0.5	0
Talaromyces flavus	1.8	1.3	0.8	0.7	0.7	0.7	0	0	0.6	0.5	1
Trichoderma sp.	1.4	1	0	0	0	0	0	0	0	1	0.5
Xylariaceous taxa	4.1	3	5.5	4.7	7.9	7.7	0	0	3.3	4.5	5.5
Mycelia sterilia	10.4		7.5		10		15.6		7	11	9.5
Rare isolates*	0.8		1		1.7		0				
Total	100		100		100		100				
Grand total	**100**		**100**		**100**		**100**				

*Taxa occurring at < 1 % IP in different seasons at each site:

Wet season 2000

Phayao (commercial): *Gelasinospora* sp., *Phyllosticta capitalensis*

Phayao (backyard): *Eupenicillium crustaceum*, *Idriella lunata*, *Nodulisporium* sp., *Penicillium* sp., *Talaromyces flavus*

Wet season 2001

Phayao (commercial): *Gaeumannomyces amomi*, *Gelasinospora* sp., *Phyllosticta capitalensis*, *Pyricularia costina*, *Talaromyces flavus*

Phayao (backyard): *Eupenicillium crustaceum*

Correspondence analysis also indicated that the fungal communities on wild and cultivated Zingiberaceae were similar as expressed by the close cluster formed by leaves, pseudostems and rhizomes of four wild and two cultivated zingiberaceous species collected from various sites (Appendix B).

3.3.4 Effect of tissue type on the endophytic assemblages

Differences in endophyte assemblages in the different tissue types (leaf tissues vs pseudostem vs rhizome) may reflect the tissue preferences of the individual dominant taxa. There was little evidence of tissue specificity exhibited by the endophytes isolated in this study (Tables 3.3–3.8). However, *Talaromyces flavus* and *Eupenicillium crustaceum* were restricted to rhizome tissues, while *Phyllosticta capitalensis* and *Pyricularia* spp. (including *P. costina*, *P. kookicola*, *P.longispora* and *P. variabilis*) were restricted to leaf tissues. *Colletotrichum gloeosporioides*, *Glomerella* spp. and *Phomopsis* spp. appeared to have a higher occurrence on leaf and pseudostem tissues than on the rhizomes, whereas *Fusarium* spp. appeared to have a higher occurrence on pseudostem and rhizomes tissues than on the leaves. The rare species *Gaeumannomyces amomi* and *Leiosphaerella amomi* described during this study were recovered from both the rhizomes and the leaves.

3.3.5 Host species and site level patterns

At the site level, variations in the numbers of taxa per plant were examined, and the number of isolates recovered from each site in wet and dry seasons were compared (Table 3.1). In each species collection, both sites in the wet and dry seasons were relatively homogenous in terms of the number of taxa recovered, with no significant difference between the means of taxa per plant at each site. However, the number of taxa recovered from *Z. officinale* in a commercial field was significantly higher than in a backyard (P = 0.000). The range of taxa per plant varied (Table 3.1). In all plants, the most frequent endophyte at each site was *C. gloeosporioides*, although it occurred in different proportions (Tables 3.3–3.8). However, there were differences in the other most frequent and less frequent endophytes at each site during the wet and dry season among the zingiberaceous species.

3.3.6 Determination of adequacy of sampling size

Species area curves for each collection of zingiberaceous plants showed the slopes of the curves were declining with the increase of sample size. At about 10 samples, the slopes were close to zero (Figure 3.4). Although some of the curves did not completely level off, the number of samples was large enough to obtain a highly representative result.

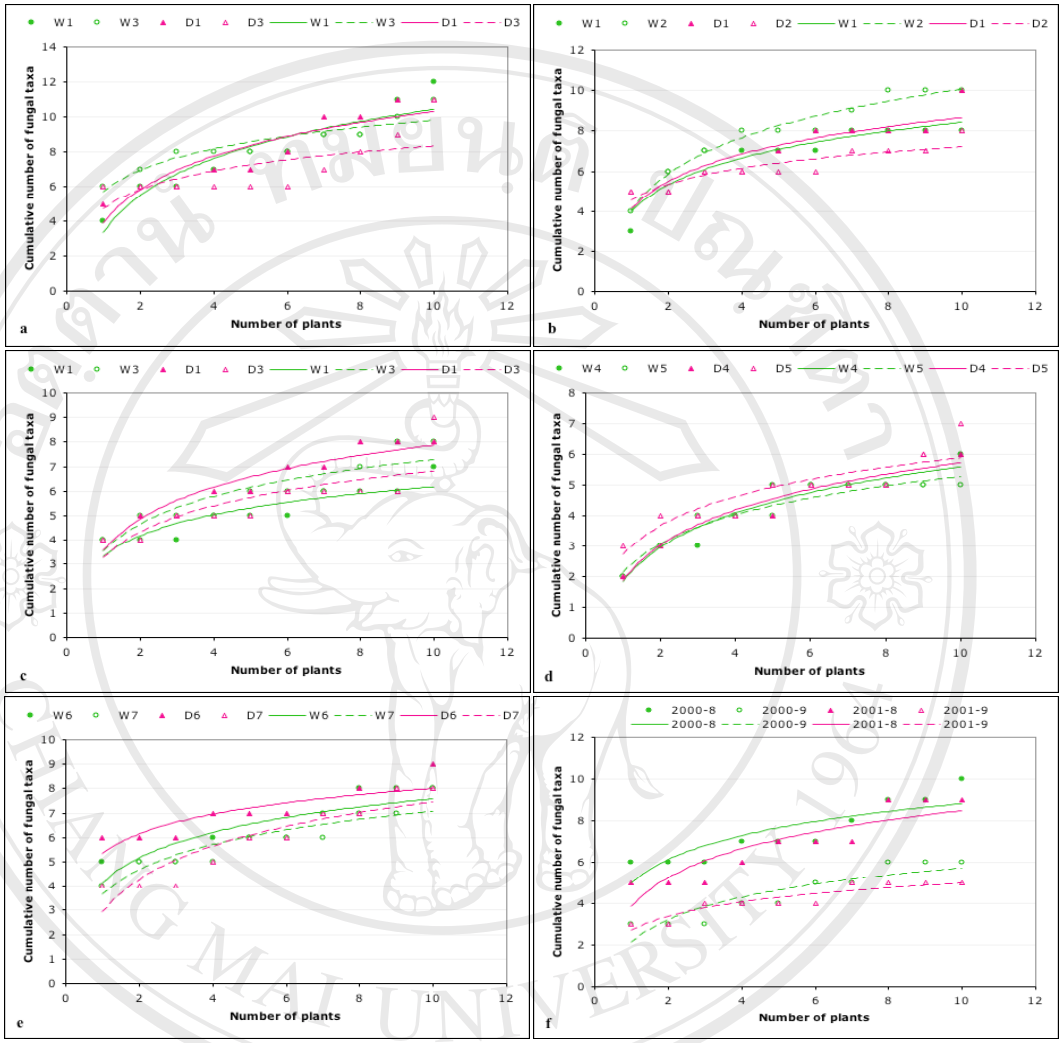

Figure 3.4 Species area curves for endophytic fungi collected from Zingiberaceae: a. *Amomum siamense*, b. *Alpinia malaccensis*, c. *Etlingera littoralis*, d. *E. elatior*, e. *Alpinia galanga*, f. *Zingiber officinale*. W. wet season, D. dry season, 1. Huay Kok Ma, 2. Doi Pui, 3. Medicinal Plant Garden, 4. Queen Sirikit Botanic Garden, 5. Chiang Mai University area, 6. Chiang Mai Province (Hangdong), 7. Lampang Province (Muang), 8. Phayao Province (commercial), 9. Phayao Province (backyard), 2000 and 2001 = year.

3.4 Discussion

3.4.1 Isolate prevalence and intensity

Isolate prevalence is an indication of the number of endophytic fungi. In the present study, the isolate prevalence of the wild and cultivated species varied from 25–85%. A great variation in isolate prevalence has been reported for palm endophytes, from as low as 12.5 % (Rodrigues and Samuels, 1990) to as high as 80.8–89.2 % (Fröhlich *et al*., 2000). The isolate prevalence of *Amomum siamense*, *Alpinia malaccensis* and *Zingiber officinale* was comparatively high in both sites in all collections (50–85%). The isolate prevalence by endophytes in these three species is high when compared to the isolate prevalence of endophytes in *Etlingera littoralis*, *E. elatior* and *Alpinia galanga* (29–54%), and in other hosts, e.g., *Licuala ramsayi*, *Euterpe oleraceae*, *Juncus bufonius*, *J. imbricatus* var. *chamnissonis*, *Musa acuminata* and *Trachycarpus fortunei* with 12.5%, 21–30%, 34%, 45.8%, 42–56% and 23–57% isolate prevalence, respectively (Rodrigues and Samuels 1990; Rodrigues 1994; Menendez *et al*. 1995; Taylor *et al*. 1999; Photita *et al*., 2001b). The climatic conditions were different, with higher humidity and rainfall in this study than in that of Taylor *et al*. (1999). Taylor *et al*. (1999) showed that the endophyte isolate prevalence in *T. fortunei* declined with decreasing relative humidity and rainfall. Carroll and Carroll (1978) demonstrated that Douglas-fir was more heavily infected in moist sites than in dry sites and suggested that differences in elevation, humidity, density of canopy cover, and innate host susceptibility were likely to cause the observed differences in endophyte infection among sites. Therefore, the high isolate prevalence of zingiberaceous species (*A. siamense* and *A. malaccensis*) collected at the

sites in Doi Suthep-Pui National Park during the wet and dry season may result from climatic conditions, including high humidity, temperature, and rainfall (mean relative humidity 70–80 %, mean temperature 20–23 °C, mean yearly rainfall 1350–2500 mm). Zingiberaceae occur on the forest floor, growing under other trees and plants. Endophyte inocula from these plants, and high humidity on the forest floor, may account for the high colonization rates of endophytes in *A. siamense* and *A. malaccensis*. In the collection of *E. littoralis*, however, the low isolate prevalence (especially in leaves) by endophytes may result from the plant having a thick and waxy leaf that restricts fungal penetration.

3.4.2 Tissue specificity

3.4.2.1 Leaf age analysis

The results of this study show a general increase in the number of endophytes recovered with increasing tissue age, especially from the leaf tissues. This is in agreement with data obtained for tropical palms and banana (Rodrigues and Samuels, 1990; Brown *et al*., 1998; Photita *et al*., 2001b). Frequency of colonization and species diversity has also been found to increase with the age of organs or tissues in several other hosts (Carroll *et al*., 1977; Petrini and Carroll, 1981; Bernstein and Carroll, 1977; Bertoni and Cabral, 1988; Umali *et al*., 1999; Hata and Futai, 1993; Kumaresan and Suryanarayanan, 2002; Toofanee and Dulymamode, 2002). The factors that may contribute to a change in the endophytic community with leaf age include weathering of the leaf cuticle, the presence of wounds, increased exposure to propagules with time, and changes in leaf

physiology and chemistry (Petrini and Carroll, 1981; Stone, 1987; Espinosa-Gracia and Longenheim, 1990).

3.4.2.2 Pseudostem, rhizome and leaf (vein and intervein) analysis

The results of this study indicated that there was a significant difference in the number of fungal isolates recovered from different tissue types of zingiberaceous species. This is in agreement with the data obtained from various studies (Petrini *et al*., 1992a; Fisher *et al*., 1993; Sieber and Dorworth, 1994; Blodgett *et al*., 2000; Photita *et al*., 2001b).

The results of vein and intervein analysis indicated that there was no significant difference in the number of fungal isolates recovered from vein and intervein tissues of Zingiberaceae studied. This is similar to results reported from *Musa acuminata* (Photita *et al*., 2001b) and *Cordemoya integrifolia* (Toofanee and Dulymamode, 2002). However, more fungal isolates recovered from vein tissues of *Etlingera elatior* were found. This result is in agreement with previous studies from palms (Rodrigues and Samuels, 1990; Rodrigues, 1994; Fröhlich *et al*., 2000) and other plants (Wilson and Carroll, 1994; Brown *et al*., 1998). Rodrigues and Samuels (1990) demonstrated endophytic fungi from leaf blade of *Licuala ramsayi*, was more concentrate in the veins. Rodrigues (1994) found higher number of fungal isolates from vein tissues of *Euterpe oleracea* than intervein. Frö hlich *et al*. (2000) reported more fungi were recovered from vein than from intervein tissues of palms, *Licuala ramsayi* and *Licuala* sp. 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 and Carroll, 1994), all of which in this case favour the vein and petiole tissues.

3.4.3 Composition of endophytic assemblages

No previous studies have reported on the endophytic fungi associated with members of the Zingiberaceae. However, of the taxa identified here from the six zingiberaceous species, *Colletotrichum gloeosporioides*, *Glomerella* spp., *Phomopsis* spp., *Fusarium* spp., *Phyllosticta capitalensis* and xylariaceous taxa have been previously recorded as endophytes in various herbaceous and woody tree host plants (Petrini *et al*., 1982; Bettucci and Saravey, 1993; Fisher *et al*., 1993, 1995a, b; Rodrigues, 1994; Menendez *et al*., 1995; Okane *et al*., 1998, 2001, 2003; Taylor *et al*., 1999; Guo *et al*., 2000; Lumyong *et al*., 2000; Suryanarayanan *et al*., 2000; Photita *et al*., 2001b; Baayen *et al*., 2002; Hata *et al*., 2002; Rodrigues *et al*., 2004). In the present study, *Colletotrichum gloeosporioides* was the most frequent species isolated. A survey of fungal endophytes associated with *Trachycarpus fortunei* (palm) also recorded *Glomerella* (teleomorph of *Colletotrichum*) as the most dominant genus (Taylor *et al*., 1999). Xylariaceous taxa and/or sterile mycelia were also frequent, and are commonly isolated as endophytes from many hosts in tropical regions (Rodrigues and Samuels, 1990; Whalley, 1993; Rodrigues, 1994; Lodge *et al*., 1996; Fröhlich *et al*., 2000; Photita *et al*., 2001b; Kumaresan and Suryanarayanan, 2002; Sangtong, 2002).

Xylariaceous taxa were the most dominant species isolated from leaves of *Licuala ramsayi* in the Australian tropics (Rodrigues and Samuels, 1990) and Amazonian palm

Euterpe oleracea (Rodrigues, 1994). Xylariaceous anamorph was the most dominant taxa isolated from fronds of *L. ramsayi* and *Licuala* sp. in Australia and Brunei, respectively (Fröhlich *et al*., 2000).

The percentage of colonies that did not sporulate (sterile mycelia) identified in the present study is typical of the numbers in other studies in the tropics (Brown *et al.*, 1998; Umali *et al*., 1999; Lumyong *et al*., 2000; Photita *et al*., 2001b; Kumaresan and Suryanarayanan, 2002; Sangtong, 2002). Guo *et al.* (1998) inoculated the mycelia obtained from natural palm petioles onto a palm petiole in a flask and obtained better sporulation. They were able to identify two species that were saprobes of *Livistona chinensis* in this way. The evidence of Guo *et al*. (1998) suggests that some of the mycelia sterilia isolated in endophyte studies may in fact be specific to that host or host family. Altough in the current study the same incubation method (Guo *et al*., 1998) were used, some endophytic species cannot be identified to species level and often are only labeled as sterile mycelia.

In this study *Talaromyces flavus* and *Eupenicillium crustaceum* (teleomorphs of *Penicillium* spp.) were commonly isolated from rhizomes of the six zingiberaceous species. *Penicillium* spp. have been commonly recovered as endophytes from leaves and roots of various hosts, such as *Alnus glutinosa*, *Cuscuta reflexa*, *Ficus benghalensis*, *Picea abies*, *P. marina* and *Sorbus* spp. (Cappellano *et al*., 1987; Summerbell, 1989; Valla *et al*., 1989; Holdenrieder and Sieber, 1992; Suryanarayanan *et al*., 2000; Suryanarayanan and Vijaykrishna, 2001).

Several of the species encountered in the present study have previously been reported as pathogens of Zingiberaceae and other plants (van der Aa, 1973; Rodrigues, 1994; Brown *et al*., 1998; Farr *et al*., 1989). These include *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Phomopsis* sp. and *Pyricularia costina*. *Phyllosticta capitalensis* isolated as endophyte, from zingiberaceous plants in the present study is known to be parasitic on Orchidaceae (van der Aa, 1973). This fungus was often detected from healthy leaves of several dicotyledonous and monocotyledonous plants, e.g., *Arundina chinensis*, *Enkianthus perulatus*, *Musa acuminata*, *Rhododendron* spp., *Smilax china* (Okane *et al*., 1998, 2001, 2003; Kumaresan and Suryanarayanan, 2001; Baayen *et al*., 2002; Rodrigues *et al*., 2004).

3.4.4 The role of endophytes in Zingiberaceae

The endophytes isolated from the six zingiberaceous species included commonly isolated genera, e.g. *Colletotrichum, Glomerella* and *Phomopsis* (Petrini *et al*., 1982; Bettucci and Saravey, 1993; Fisher *et al*., 1993, 1995; Rodrigues, 1994; Menendez *et al*., 1995; Taylor *et al*., 1999), as well as taxa that had not been previously isolated as endophytes, e.g. *Gaeumannomyces amomi*, and *Leiosphaerella amomi*. Endophytes in tropical plants are thought to benefit the host plant by enhancing absorption of soil nutrients such as phosphorus, providing protection from insect attack, and may also inhibit the development of plant pathogens (Thomson *et al*., 1986; Latch, 1993; Stone *et al*., 2000; Arnold *et al*., 2003). Endophytic fungi may develop as pathogens or saprobes (Latch, 1993; Brown *et al*., 1998; Photita *et al*., 2004), and some of the endophytes

isolated from Zingiberaceae are possible latent pathogens. For example, *Colletotrichum capsici*, *C. gloeospoioides*, *Fusarium oxysporum* f. sp. *zingiberi*, *F. solani*, *Phomopsis* sp. and *Pyricularia curcumae* are known to be pathogens of Zingiberaceae (Pavgi and Upadhyay, 1986; Sontirat *et al*., 1994; Farr *et al*., 1989). Five species (*Alternaria alternata*, *Colletotrichum gloeosporioides*, *Phomopsis* sp. *Phyllosticta capitalensis* and *Pyricularia costina*) were associated with leaf disease of Zingiberaceae on the plants sampled in this study.

This study has shown that zingiberaceous species contain similar endophytic fungal communities as those of other monocotyledons (Rodrigues, 1994; Menendez *et al*., 1995; Taylor *et al*., 1999; Photita *et al*., 2001b). This is interesting as it places doubt on the well-used phrase that endophytes are beneficial to the host. It is not clear that any of the fungi isolated here as endophytes may benefit the host, and certainly further work should be conducted on this aspect. Of the fungi isolated, *Gaeumannomyces amomi*, *Leiosphaerella amomi* and *Pyricularia* spp. are probably unique to the Zingiberaceae and their role needs further investigation. Although the fungi isolated in this study are not generally unique to Zingiberaceae it is believed that they may be an excellent source of new bioactive compounds (Dreyfuss and Petrini, 1984; Hyde, 2001; Strobel *et al*., 2004).