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Endophytic fungi from *Amomum siamense*

Boonsom Bussaban, Saisamorn Lumyong, Pipob Lumyong, Eric H.C. McKenzie, and Kevin D. Hyde

Abstract: Endophytic fungi were isolated from apparently healthy organs of the wild ginger *Amomum siamense* Criab., including leaves, pseudostems, and rhizomes, collected from two sites in Doi Suthep-Pui National Park, Thailand. Endophytes were relatively common with an isolate prevalence of 70%–83% at the two sites sampled in the wet and dry seasons. The endophyte assemblages from the two sites were diverse and comprised 7 Ascomycetes and 26 mitosporic fungi. *Colletotrichum "gloeosporioides"* (Penz.) Penz. & Sacc. in Penz., *Glomerella* spp., xylariaceous fungi, and *Phomopsis* spp. were consistently dominant as endophytes on *Amomum siamense*, but many rare species and mycelia sterilia were also recorded. There was no significant difference between the number of isolates recovered from leaves containing vein tissues and those containing intervein tissues, independent of leaf age. Most taxa showed a preference for either leaf tissue or pseudostems. Two new Ascomycetes species, *Gaeumannomyces amomi* and *Leiosphaerella amomi*, were discovered from leaves and rhizomes, respectively, and four species of *Pyricularia*, including three new species, were isolated from leaves.

Key words: Zingiberaceae endophytes, isolate prevalence, tissue specificity, seasonal effects.

Résumé : Des champignons endophytes ont été isolés d'organes apparemment sains du gingembre sauvage *Amomum siamense* Criab., soit à partir des feuilles, des pseudotiges et des rhizomes récoltés dans deux sites du Parc National Doi Suthep-Pui en Thaïlande. Ces endophytes étaient relativement communs, car leur prévalence d'isolement était de 70 % – 83 % aux deux endroits échantillonnés et ce en saisons sèches ou humides. Les populations d'endophytes de chacun des sites étaient diversifiées et comprenaient 7 Ascomycètes et 26 champignons à reproduction asexuée. Chez *l'Amomum siamense*, les endophytes régulièrement dominants étaient *Colletotrichum "gloeosporioides"* (Penz.) Penz. & Sacc. in Penz., *Glomerella* spp., des champignons du groupe des Xylariaceae et *Phomopsis* spp. mais plusieurs espèces rares ainsi que des mycéliums stériles ont aussi été observés. Il n'y avait pas de différence significative dans le nombre d'isolats provenant de feuilles contenant du tissu veineux ou intraveineux, indépendamment de l'âge de la feuille. La majorité des taxa ont montré une préférence pour le feuillage ou les pseudotiges. Deux nouvelles espèces d'Ascomycètes, *Gaeumannomyces amomi* et *Leiosphaerella amomi*, ont été découvertes respectivement dans les feuilles et les rhizomes et quatre espèces de *Pyricularia*, incluant trois nouvelles espèces, ont été isolées à partir des feuilles.

Mots clés : Zingiberaceae endophytes, prévalence des isolats, spécificité tissulaire, effets saisonniers.

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Introduction

Most reports of endophytes from monocotyledonous plants have concentrated on temperate grasses, with an emphasis on clavicipitaceous endophytes and their beneficial

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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) have 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.

Saprobic and pathogenic fungi associated with Zingiberaceae (ginger) have been reported by Rao (1963); Muthappa (1996); Doi (1977); Khurana (1980); Rathaiah (1980); Vittal (1981); Pavgi and Upadhyay (1986); Samuels (1989); and Hyde (1997). The current study was initiated to investigate the endophytic fungal assemblages of the wild ginger, *Amomum siamense* Criab., at Doi Suthep-Pui National Park, Thailand. The research was primarily motivated by the desire to establish whether the endophytes in ginger differed from those in other tropical hosts. We also wanted

to establish whether the endophytes in ginger were diverse and whether there was any evidence that the endophytes of ginger are latent pathogens.

Materials and methods

Sample selection

Ten plants of *Amomum siamense* were randomly collected from each of two localities in Doi Suthep-Pui National Park, Chiang Mai, Thailand. Collections were made at Huay Kok Ma (933 m above sea level) and Medicinal Plant Garden (600 m above sea level) in August 1999 (wet season) and February 2000 (dry season). One pseudostem, two leaves (1 young and 1 old), and one rhizome from each plant were randomly removed and returned to the laboratory.

Surface sterilization and isolation of endophytes

Samples were washed in running tap water for 15 min. Five segments (10 mm long) were then cut from each pseudostem, and five rhizome segments from each plant were cut into 10-mm^3 cubes. Twenty discs (5 mm diam.) were cut from the leaves, using a sterile cork borer. The discs either included a vein (10 samples) or consisted of only interveinal lamina (10 samples). The surface sterilization method chosen was similar to that used for the isolation of endophytes from palms (Taylor et al. 1999), but modified based on the thickness of the ginger tissues, following pilot experiments. 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 filter paper and were then transferred to 2% malt extract agar + 0.03% Rose Bengal + 0.1% streptomycin sulphate. Labelled plates were incubated at room temperature (27°C–30°C) for 1–2 weeks, and hyphae growing out from the tissues were transferred to tubes containing corn meal agar.

Identification

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

Statistical analyses

Isolate prevalence and intensity were calculated as follows:

[1]   Isolate prevelence =

$$\frac{\text{Total number of samples yielding} \geq 1 \text{ isolate}}{\text{Total number of samples in that trial}} \times 100$$

[2]   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 percent, as commonly used by other workers (Petrini et al. 1982). Intensity was also calculated and used to demonstrate the degree of multiple colonization from the samples in different trials, but was not expressed as a percentage. A Chi-squared (χ^2) goodness-of-fit test was performed to test whether the isolate prevalence for the four trials was statistically different. Nonparametric statistical tests were used because the data in most cases did not fit the assumptions for parametric statistics. A Mann–Whitney test was performed on isolates from old and young leaves and pseudostems at each site (Taylor et al. 1999). A Kruskal–Wallis rank-sum test was used for multisample analysis of the number of isolates recovered from vein and intervein tissues for all leaves at each site and for the number of isolates recovered from different tissue types at each site between wet and dry seasons (Petrini et al. 1982). In all analyses, P values are described as follows: $P < 0.05$, $P < 0.01$, and $P < 0.001$, for Chi-squared, Mann–Whitney, and Kruskal–Wallis, respectively.

Results

Isolate prevalence and intensity

A total of 1200 samples were processed from two sites in the wet and dry seasons, and 1222 fungal isolates were recovered. The overall isolate prevalence (%) and intensity for the assemblages of endophytes recovered at each site in wet and dry seasons are given in Table 1. There was no significant difference between isolate prevalence in each trial. In all cases, there was a rather high isolate prevalence, and overall there was a high degree of multiple infections (22%–38% of samples, Table 1).

Tissue specificity

Leaf and pseudostem age analysis

Mann–Whitney tests were performed on data at each site, and there was no significant difference between the number of isolates recovered from young and old leaves at any of the sites sampled ($P < 0.05$). However, with the exception of the dry season samples from the Medicinal Plant Garden, there were significantly more isolates recovered from old leaves, as compared with young leaves ($P = 0.000$), according to the Kruskal–Wallis rank-sum test. There was no significant difference in the number of isolates recovered from vein and intervein tissues, irrespective of leaf age.

Composition of endophytic assemblages

Of the 1222 fungal isolates recovered, 33 taxa were identified, comprising 7 Ascomycetes and 26 mitosporic fungi (6 Coelomycetes and 20 Hyphomycetes). Dominant genera were *Colletotrichum*, *Glomerella*, and *Phomopsis*. The taxa occurring at relative frequencies of >1% at each site in different seasons are presented in Table 2. Based on conidial size and colony characteristics, the *Colletotrichum* isolates could be separated into three taxa. However, these all fit the broad description of *Colletotrichum* "*gloeosporioides*" (Penz.) Penz. & Sacc. in Penz (Sutton 1980). Four species of *Pyricularia*, including *Pyricularia costina* and three new species, were isolated and distinguished by conidial shape, size, and septation. *Fusarium* comprised five species, which were distinguished on colonial morphology and spore size, while *Glomerella* and *Phyllosticta* each comprised two species separated on colonial morphology, spore size, and spore color. *Phomopsis* comprised four species, which were distinguished by colonial morphology, the presence and (or) absence of α- and β-conidia, and the dimensions of these conidia. These species were the dominant fungal endophytes in this study. Since it is difficult to identify these taxa to spe-

Table 1. Isolate prevalence, intensity, multiple infection, and number of taxa of endophytes isolated from *Amomum siamense* at each site in the wet and dry season.

Characteristic	Wet season		Dry season	
	Huay Kok Ma	Medicinal Plant Garden	Huay Kok Ma	Medicinal Plant Garden
No. of samples	300	300	300	300
No. of isolates recovered	284	362	283	293
Total taxa recovered	19	18	23	20
Taxa per plant* (means + SD)	8±2	8±1	8±2	7±2
Taxa per plant (range)	4–12	6–11	5–11	6–11
Isolate prevalence (%)	73	83.3	70	78.7
No. (%) of samples yielding two species	54(25%)	76(30%)	68(29%)	51(21%)
No. (%) of samples yielding > two species	5(2%)	19(8%)	5(2%)	3(1%)
Intensity (no. of isolates per sample)	0.99	1.21	0.96	0.98

*Means are not significantly different according to ANOVA ($P < 0.05$).

cies level, we have combined the data of each genus for statistical comparison.

There was a large degree of overlap in the endophyte communities at the genus level. In the wet season, eight of the taxa recorded from Huay Kok Ma also occurred at the Medicinal Plant Garden, whereas in the dry season seven taxa occurred at both sites. Six taxa recorded in the wet season also occurred in the dry season.

The effect of tissue type on the composition of endophytic assemblages

Differences in endophyte assemblages in the different tissue types (leaf tissues versus pseudostem versus rhizome) would reflect the tissue preferences of the individual dominant taxa. There was little evidence of tissue specificity exhibited by the endophytes isolated in this study. However, *Talaromyces flavus* (Klöcker) A.C. Stolk & R.A. Samson and *Eupenicillium crustaceum* Ludwig were restricted to rhizome tissues, and *Phyllosticta* spp. and *Pyricularia* spp. were restricted to leaf tissues. *Phomopsis* spp. appeared to have a higher occurrence on leaf and pseudostem tissues than on the rhizomes, whereas *Colletotrichum "gloeosporioides"* and *Glomerella* spp. appeared to have a higher occurrence on pseudostem tissues than on the leaves and rhizomes, respectively. *Fusarium* spp., however, seemed to occur recurrently on the pseudostems and rhizomes. The rare species *Gaeumannomyces amomi* and *Leiosphaerella amomi* B. Bussaban, recently described by Bussaban et al. (2001), were recovered from both the rhizomes and the leaves (Table 2).

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 was compared (Table 1). Both sites in the wet and dry seasons were relatively homogeneous in terms of the number of taxa recovered, with no significant difference between the means of taxa per plant at each site. The range of taxa per plant varied (Table 1). In the wet season, the three most frequent endophytes at both sites were the same, although they occurred in different proportions (Table 2). However, there were differences in the three most frequent endophytes at both sites during the dry season. The differences in the assemblages of the most fre-

quent endophytes in the different seasons and sites, therefore, indicated the seasonality and site-specific nature of the endophyte assemblages.

Discussion

Composition of endophytic assemblages

As no previous studies have been reported on the endophytic fungi associated with members of the Zingiberaceae, there is no data available for comparison. Of the taxa identified here from *Amomum siamense*, *Colletotrichum "gloeosporioides"*, *Glomerella* spp., *Phomopsis* spp., *Fusarium* spp., 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, 1995; Rodrigues 1994; Menendez et al. 1995; Taylor et al. 1999; Lumyong et al. 2000). In the present study, *Colletotrichum "gloeosporioides"* and *Glomerella* spp. were the most frequent species isolated. A survey of fungal endophytes associated with *Trachycarpus fortunei* (Hook.) H. Wendl. (palm) also recorded *Glomerella* (teleomorph of *Colletotrichum*) as the most dominant genus (Taylor et al. 1999).

Xylariaceous taxa were a frequent isolate in this study and are commonly isolated from many hosts in tropical regions (Rodrigues and Samuels 1990; Whalley 1993; Rodrigues 1994). Xylariaceous taxa were the second most dominant species isolated from leaves of *Licuala ramsayi* (Mueler) Domin. in the Australian tropics (Rodrigues and Samuels 1990).

In this study, *Talaromyces flavus* and *Eupenicillium crustaceum* (teleomorphs of *Penicillium* sp.) were commonly isolated from rhizomes of *Amomum siamense*. *Penicillium* spp. have been commonly recovered as endophytes from leaves and roots of various hosts, such as *Alnus glutinosa* (L.) Gaertn., *Cuscuta reflexa* Roxb., *Picea abies* (L.) Karst., *Picea marina* (Mill.), and *Sorbus* spp. (Cappellano et al. 1987; Summerbell 1989; Valla et al. 1989; Holdenrieder and Sieber 1992; Suryanarayanan et al. 2000).

Isolate prevalence and intensity

Isolate prevalence is an indication of the number of endophytic fungi. In this study, the isolate prevalence was

Table 2. Relative frequencies (RF) (%) and colonization frequencies (CF) (%) of endophytes from *Amomum siamense* occurring at relative frequencies of >1%.

| | Wet season | | | | Dry season | | | | Plant part | | |
| | Huay Kok Ma | | Medicinal Plant Garden | | Huay Kok Ma | | Medicinal Plant Garden | | | | |
Taxa	RF	CF	RF	CF	RF	CF	RF	CF	Leaf (CF)	Pseudostem (CF)	Rhizome (CF)
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 spp.	7.1	7	4.6	5.7	0	0	0	0	4.9	0.5	1
Pyricularia spp. (including *P. costina*)	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	76.8		87.4		73.7		76.6				
Grand total	100		100		100		100				

*Taxa occurring at <1% RF in different seasons at each site. **Wet season**—Huay Kok Ma: *Gaeumannomyces amomi, Gilmaniella humicola, Humicola fuscoatra, Leiosphaerella amomi, Papulospora* sp., *Pestalotiopsis* sp., *Phoma* sp., *Thermomyces* sp., and *Trichoderma* sp. Medicinal Plant Garden: *Drechslera australiensis, Fusicoccum* sp., *Gelasinospora* sp., *Geniculosporium* sp. 2, *Humicola fuscoatra, Nigrospora oryzae, Phoma* sp., and *Stachybotrys* sp. **Dry season**—Huay Kok Ma: *Aspergillus niger, Cylindrocarpon* sp., *Cylindrocladium* sp. 1, *Cylindrocladium* sp. 2, *Eupenicillium crustaceum, Geniculosporium* sp. 2, *Gilmaniella humicola, Nigrospora oryzae, Penicillium* sp., *Pestalotiopsis* sp., *Phyllosticta* spp., and *Trichoderma* sp. Medicinal Plant Garden: *Cladosporium cladosporioides, Cylindrocladium* sp. 1, *Eupenicillium crustaceum, Gaeumannomyces amomi, Geniculosporium* sp. 1, *Geniculosporium* sp. 2, *Gilmaniella humicola, Gliomastix murorum* var. *polychroma, Humicola fuscoatra, Nigrospora oryzae,* and *Phyllosticta* spp.

comparatively high in both sites in both wet and dry seasons (70%–83%). The isolate prevalence by endophytes on *Amomum siamense* are high when compared with the isolate prevalence of endophytes in other hosts, e.g., *Licuala ramsayi, Euterpe oleraceae* Mart., *Juncus bufonius* (L.), *Juncus imbricatus* (L.) var. *chamnissonis,* and *Trachycarpus fortunei,* with 12.5%, 21%–30%, 34%, 45.8%, and 23%–57% isolate prevalence, respectively (Rodrigues and Samuels 1990; Rodrigues 1994; Menendez et al. 1995; Taylor et al. 1999). 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 *Trachycarpus 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 *Amomum siamense* at the two sites during the wet and dry season may result from climatic conditions, including higher humidity, temperature, and rainfall (mean relative humidity 70%–80%, mean temperature 20°C–23°C, mean yearly rainfall 1350–2500 mm). *Amomum siamense* is also a plant that occurs on the forest floor under other trees and plants. Endophyte innocula from these plants and high humidity on the forest floor may account for the high colonization rates of endophytes in *Amomum siamense.*

Tissue specificity

Leaf and pseudostem 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 (Rodrigues and Samuels 1990; Rodrigues 1994). Frequency of colonization and species diversity has also been found to increase with the age of organs or tissues in several hosts (Carroll et al. 1977; Petrini and Carroll 1981; Rodrigues 1994; Bernstein and Carroll 1977; Brown et al. 1998; Taylor et al. 1999; Umali et al. 1999). The factors that may contribute to 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; Epinosa-Gracia and Longenheim 1990). *Amomum siamense* occurs beneath trees, and transmission of endophyte to *Amomum siamense* may be from spore innocula from the trees.

Vein and intervein analysis

The results of this study indicated that there was no significant difference in the number of isolates recovered from

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vein and intervein tissues. This differs from results reported from palms (Rodrigues and Samuels 1990) and other monocotyledons (Brown et al. 1998).

The role of endophytes in *Amomum siamense*

The endophytes isolated from *Amomum siamense* included commonly isolated genera, e.g., *Colletotrichum* "*gloeosporioides*", *Glomerella* spp., and *Phomopsis* spp. (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, by providing protection from insect attack and by possibly inhibiting the development of plant pathogens (Thomson et al. 1986; Latch 1993; Stone et al. 2000). Endophytic fungi may develop as pathogens or saprobes (Latch 1993; Brown et al. 1998), and some of the endophytes isolated from *Amomum siamense* are possible latent pathogens. *Colletotrichum capsici* (Syd.) Butl. & Bisby, *Colletotrichum* "*gloeospoioides*", *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *zingiberi* E.E. Trujillo, *Fusarium solani* (Mart.) Sacc., *Phomopsis* sp., *Phyllosticta zingiberi* F. Stevens & Ryan, and *Pyricularia curcumae* Y. Rathaiah are known to be pathogens of Zingiberaceae (Pavgi and Upadhyay 1986; Rathaiah 1980; Sontirat et al. 1994; Farr et al. 1995). Two species (*Colletotrichum* "*gloeosporioides*" and *Phomopsis* sp.) were associated with leaf disease of *Amomum siamense* 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). This is interesting because 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 also needs 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 compounds (Dreyfuss and Petrini 1984; Hyde 2000).

Acknowledgements

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