NOTE

Enzymatic activity of endophytic fungi of six native seedling species from Doi Suthep-Pui National Park, Thailand

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Abstract: Endophytic fungi were isolated from the stems, petioles, midribs, and leaves of seedlings of six native tree species collected from Doi Suthep-Pui National Park, Thailand. Endophytes were isolated from all tissue samples investigated, and taxa included five ascomycetes, eight anamorphic taxa, and numerous sterile mycelia. Twenty-six strains were tested for their ability to produce cellulase, mannanase, proteinase, and xylanase. The ability to produce these enzymes was distributed amongst the strains tested. Rainforest seedlings supported a diverse array of endophytes that have a wide range of enzymatic activities. The implication of enzyme production in relation to lifestyle abilities of the endophytes is discussed.

Key words: cellulase, hydrolytic enzyme, mannanase, protease, xylanase.

Résumé : Des champignons endophytes ont été isolés des tiges, des pétioles, des nervures médianes et des feuilles des plants de six espèces d'arbres indigènes recueillis dans le Parc National Doi Suthep-Pui, Thaïlande. Des endophytes ont été isolés à partir de tous les échantillons de tissus analysés et les taxons comprenaient cinq ascomycètes, huit taxons anamorphiques et de nombreux mycéliums stériles. Nous avons évalué la capacité de vingt-six souches à produire de la cellulase, de la mannanase, de la protéinase et de la xylanase. La capacité de produire ces enzymes était répartie parmi les souches analysées. Les plants issus de forêts tropicales ont soutenu le développement d'un éventail varié d'endophytes ayant une large gamme d'activités enzymatiques. L'incidence de la production des enzymes sur les habitudes de vie des endophytes est débattue.

Mots clés : cellulase, enzymes hydrolytiques, mannanase, protéase, xylanase.

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Numerous authors have reported the presence of endophytes in temperate plants (e.g., Petrini et al. 1982; Petrini and Fisher 1988), but there has been less emphasis on tropical species (Rodrigues and Petrini 1997). Recent studies on endophytes of tropical plants include bamboo (Umali et al. 1999; Lumyong et al. 2000), banana (Brown et al. 1998; Photita et al. 2001), *Cuscuta reflexa* (Suryanarayanan et al. 2000), *Parthenium hysterophorus* (Romero et al. 2001), palms (Rodrigues and Samuels 1990; Rodrigues 1994; Guo et al. 1998; Taylor et al. 1999; Fröhlich et al. 2000), and wild Zingiberaceae (Bussaban et al. 2001).

The role of endophytes in plants has been the subject of much debate. It may range from the protection of the plants against herbivory to improvement in mineral absorption and drought tolerance (Bacon and Hill 1996). Some latent pathogens live endophytically, while others have been shown to be the initial saprobes that colonize dead plant material (Guo et al. 1998). Endophytes are important producers of secondary metabolites, and there has been biotechnological interest in their potential to produce novel metabolites (Dreyfuss and Chapela 1995; Strobel et al. 1996*a*, 1996*b*; Karim et al. 1997; Peláez et al. 1998).

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Table 1. Fungal endophytes isolated from rainforest seedlings.

Endophyte	Host					
	Camellia sinensis var. *assamensis*	*Cinnamomum iners*	*Garcinia cowa*	*Litsea salicifolia*	*Manglietia garrettii*	*Trichilla connaroides*
Colletotrichum spp.	8	11	31	–	36	13
Curvularia sp.	–	–	–	–	1	–
Didymella sp.	–	–	1	–	–	–
Glomerella cingulata	3	12	28	5	12	1
Guignardia cocoicola	1	2	3	3	1	5
Helminthosporium sp.	–	–	–	–	4	–
Nigrospora sp.	1	2	1	–	2	2
Pestalotiopsis sp.	–	1	–	–	1	1
Phoma sp.	3	2	2	6	–	10
Phomopsis sp.	4	1	2	8	–	–
Sporormia sp.	1	–	–	–	–	–
Xylaria sp.	2	–	–	–	2	–
Mycelia sterilia	9	23	7	1	7	18
Unidentified sp.*	7	1	–	1	–	–

Note: Data are percent colonization rate.
*Unidentified anamorphic fungi.

To establish the functional role of endophytes, it would be useful to determine their patterns of substrate ultilization and enzyme production (Carroll and Petrini 1981). We report on endophytes isolated from seedlings of six native rainforest trees in northern Thailand, one of the few reports of endophytes from seedlings of native tropical plants (Rodrigues 1994). We tested the ability of selected isolates to produce cellulase, mannanase, protease, and xylanase to elucidate their possible roles.

Six seedling species (Table 1), all large, native rainforest tree species, were selected for this study in an area of Doi Suthep-Pui National Park at 1000 m above sea level. The seedlings were grown in a nursery that was situated within deciduous forest. Five healthy seedlings of each species, approx. 50 cm high, were randomly selected, returned to the laboratory, and processed within 24 h.

From each plant, five old and five young leaves were selected, and all stems were cut from the seedlings and treated (6 seedlings × (*i*) 5 specimens (5 old leaves, 5 young leaves), (*ii*) 5 petioles, and (*iii*) 1 stem = 336 samples) for isolation of endophytic fungi following the method of Photita et al. (2001). Colonization rate is expressed as a percentage.

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

Twenty-six fungal isolates were tested for cellulase, mannanase, and xylanase production using 1% carboxymethyl cellulose, 1% locust bean gum (Difco Laboratories, Detroit, Mich.), and 1% xylan as carbon source, respectively (Downie et al. 1994; Pointing 1999). A modified agar diffusion method incorporating Congo red dye (Downie et al. 1994) was used as a qualitative assay. Protease activity was assayed using casein hydrolysis medium, which contained 1% skimmed milk. After incubation at 30°C, the diameter of the clear zone was measured.

The 336 tissue samples yielded five ascomycetes, eight anamorphic taxa, and numerous sterile mycelia (Table 1). Endophytes were isolated from all types of tissue. The sterile mycelia were not separated into morphospecies, and no basidiomycetes were isolated.

Most of the endophytes were isolated from several plant species, and all plant species hosted at least six endophytic taxa (Table 1). *Colletotrichum* spp., *Glomerella cingulata*, and sterile mycelia were the most common taxa isolated from most hosts. Some fungi may be specific to certain host seedlings. *Helminthosporium* sp. and *Curvularia* sp. were isolated only from *Manglietia garrettii* Craib, *Didymella* sp. only from *Garcinia cowa* Roxb., and *Sporormia* sp. only from *Camellia sinensis* (L.) var. *assamica* (Mast.) Kitamura. *Glomerella cingulata*, *Guignardia cocoicola*, and sterile mycelia occurred in all hosts. The colonization rate from each host and each plant tissue type is presented in Table 2. Most taxa were isolated from the stem, while the number of taxa isolated from other plant tissues varied within a low range. Fewer taxa were isolated from the young leaves.

The endophytes found were similar to those found in mature plants of other species at Doi Suthep-Pui National Park (Bussaban et al. 2001; Photita et al. 2001) and in other tropical plants (e.g., Suryanarayanan et al. 2000). Mature plants, however, tend to support a larger fungal assemblage (e.g., Taylor et al. 1999). The current data supports the hypothesis that endophytic fungi colonize plant tissues via inoculum from the surrounding environment (Fröhlich et al. 2000). As seedling leaves age, more spores will fall onto them, and thus, older leaves can be expected to have a larger and more diverse endophyte assemblage.

There was considerable variation in enzyme production between the endophytes tested (Table 3). The knowledge of enzyme production by fungi provides some idea of their biotechnological potential and may give insight into the lifestyles of endophytes. There were some interesting trends in the results that hint at possible roles for some of the endophytes isolated from the various seedlings. Most strains of

Table 2. Comparison of colonization rate in different seedling species and tissue types.

	Colonization rate of each taxa per tissue type													
	Old tissue					Young tissue								
Seedling species	Stem	Petiole	Top midrib	Between vein	Bottom midrib	Top intervein	Bottom intervein	Top vein	Petiole	Top midrib	Between vein	Bottom midrib	Top intervein	Bottom intervein	Top vein
Camellia sinensis var. *assamica*	9	5.6	2	2.3	4	1	2	1.6	0	1.3	1	1.5	1	1	0
Cinnamomum iners	6	6	6.6	4.6	3.6	3	2.6	4.6	3.3	5.3	6	4.6	1.3	2.3	2.6
Garcinia cowa	9.8	4.4	6.8	7.4	5.2	6.8	6.4	6.2	1	5.2	4.8	5.2	4.6	5.6	4
Listea salicifolia	12.6	4.4	1.6	5	2.8	2.8	2.2	1	0	0	0	1	1	1	1.4
Manglietia garrettii	6.6	4.6	6.4	4.8	5.4	3.8	4	4.8	5.4	5.6	3.6	3.6	3.8	3.2	5.5
Trichilla connaroides	8.8	10	6.6	3.8	1.6	4.8	4.6	7	0	2	6.6	2.2	1.6	0	1.6

Table 3. Production of enzymes by endophytes.

		Enzyme production ratio			
Endophyte	Host	Cellulase	Mannanase	Protease	Xylanase
Colletotrichum sp.	Mg	1	1	+	0
	Cs	3	4	+	2
	Ci	3	3	+	4
Pestalotiopsis sp.	Mg	3	4	–	0
	Gc	0	0	+	0
	Cs	0	4	+	0
	Ls	0	3	–	2
Phoma sp.	Mg	2	2	+	0
	Gc	4	4	+	0
	Tc	2	0	+	2
	Cs	4	0	–	0
	Ls	4	0	–	0
Phomopsis sp.	Mg	2	0	+	3
	Gc	4	4	–	4
	Tc	4	4	–	4
	Ls	3	2	+	1
	Ci	4	4	–	4
Xylaria sp.	Mg	4	3	+	1
	Gc	3	0	+	0
	Tc	4	4	+	4
	Cs	4	4	–	3
	Ls	4	0	–	4
Mycelia sterilia	Mg	2	0	–	1
	Gc	2	3	–	1
	Tc	3	4	–	4
	Cs	2	2	–	2
	Ci	0	4	+	0

Note: Enzyme production ratio = the ratio of clear zone diameter to that of colony diameter. The extracellular enzymatic reactions were classified into the following four types: (*i*) strong reaction, the extracellular enzyme ratio was higher than or equal to 2; (*ii*) medium reaction, the extracellular enzyme ratio was less than 2 but more than 1; (*iii*) weak reaction, the extracellular enzyme ratio was equal to or less than 1; and (*iv*) no reaction, there is no reaction at all. In the case of protease, the reaction was small and could only be measured as + or –. Ci, *Cinnamomum iners* Reinw. ex Bl.; Cs, *Camellia sinensis* var. *assamica*; Gc, *Garcinia cowa*; Ls, *Litsea salicifolia* Nees ex Roxb.; Mg, *Manglietia garrettii*; and Tc, *Trichilla connaroides* (Wighta & Arn.) Bentv.

Pestalotiopsis did not produce any cellulases or xylanases but showed some evidence of mannanase production. *Pestalotiopsis* species are mostly weak pathogens and often develop on leaf spots produced by other pathogens (Guba 1961). Most *Phoma* strains tested did not produce any mannanases or xylanases but did produce cellulases. These fungi are similar to the second group of Carroll and Petrini (1981), which are probably incapable of penetrating living cells. Such endophytes may require simple carbon sources for active growth, a nutritional feature that is common in symbiotic fungi (Carroll and Petrini 1981).

The production of cellulases, mannanases, and xylanases by *Phomopsis*, *Xylaria*, and sterile mycelia strains is interesting. Carroll and Petrini (1981) suggested that such fungi may be latent pathogens or vigorous decomposers after plant death. These taxa may be dormant in the leaves until plant death, and the evidence here indicates that they may then adopt a saprobic lifestyle and degrade dead leaves and possibly wood. *Diaporthe* species (anamorphs of *Phomopsis*) are commonly found on leaves and on wood (Wehmeyer 1933).

Xylaria species are also commonly found on degrading wood and many species have the unusual ability amongst ascomycetes to degrade lignins (Urairuj et al. 2003).

The ability of the sterile mycelia to produce cellulases, mannanases, and xylanases may also reflect their role in nature in degrading dead leaves or stems. Guo et al. (1998, 2000) have shown that many endophytic sterile mycelia are also saprobic ascomycetes (e.g., *Astrosphaeriella bakeriana* and *Myelosperma tumidum*) that can be found on decaying plant material. This study indicates that the endophytes within the rainforest seedlings may also have the ability to become saprobes following plant death.

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