Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation

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Rhizophora apiculata leaves of different age levels were studied for their endophyte assemblages. The number of species as well as the number of isolates of endophytes that could be recovered from the leaves increased with leaf age. The endophyte mycobiota did not remain static after leaf fall. The endophytes appeared to grow or decline depending on the environment where the leaves might fall (soil or sea water). Some endophytes such as *Cladosporium cladosporioides*, *Phyllosticta* sp. and *Sporormiella minima* declined in fallen leaves, irrespective of the environment where the leaf might fall. A few fungi that probably existed as endophytes in low frequencies in living leaves appeared in higher frequencies after leaf fall. Such growth activity of these endophytes and their capacity to produce certain enzymes are indicative of their potential role in litter degradation.

Key words: litter degradation, litter fungi, fungal endophytes, mangrove endophytes.

Introduction

A wide range of plants have been studied for the presence of symptom less fungi (endophytes) in their aerial tissues. These include grass hosts (Bacon and White, 1994), members of the *Gymnospermae* (summarized in Stone *et al.*, 2000), palms (Rodrigues, 1994; Southcott and Johnson, 1997; Taylor *et al.*, 1999; Fröhlich *et al.*, 2000) and a few tropical plants (Rodrigues and Petrini, 1997; Brown *et al.*, 1998; Suryanarayanan *et al.*, 1998, 2000, 2001; Suryanarayanan and Vijaykrishna, 2001). Endophytes isolated from plants have been screened for their potential to produce unique metabolites of pharmaceutical and agricultural importance (Petrini *et al.*, 1992). However, the role of endophytes in litter degradation in tropical plant communities, such as a mangrove community, is unknown (Hyde and Lee, 1995). Such knowledge is pertinent, as mangroves are one of the most productive natural ecosystems (Kohlmeyer and Volkmann-Kohlmeyer, 1993) and are important in soil building and global cycling of carbon dioxide and sulphur (Bandaranayake,

1998). We therefore investigated the role of foliar endophytes in mangrove litter degradation.

Materials and Methods

Collection of leaf samples

The endophyte assemblages of bright green, thin, tender, young leaves, dark green, thick mature leaves and yellow, old, senescent leaves of *Rhizophora apiculata* growing in Pichavaram mangrove forest (11°27'N, 79°47'E), which is about 200 km south of Chennai in Tamil Nadu, India, were studied. The yellow senescent leaves were collected by gently tapping the petiole. Only those senescent leaves that could be detached by this method were screened. This indicated that they were in late stage of senescence and would have soon fallen. The three types of leaves were collected and screened for endophytes at the same time. The leaves were washed thoroughly in running water. The leaf segments (0.5 cm^2) cut from the median portion of the leaves were surface sterilized and incubated on Potato Dextrose Agar medium. The leaf segments were surface sterilized following a modified method of Dobranic *et al.* (1995) as used for the mature leaves of *R. apiculata* (Suryanarayanan *et al.*, 1998). The leaf segments were dipped in 70% ethanol for 5 seconds, followed by 4% sodium hypochlorite for 90 seconds and sterile water for 10 seconds. Two hundred leaf segments from twenty leaves were screened from young, mature and senescent leaves. Further, the senescent leaves were divided into two batches, named the dry litter and the wet litter.

Dry litter

Sixty leaves were surface sterilized and were incubated in a moist chamber under sterile conditions for 21 days (26 ± 1 C). This represented a situation akin to the litter that collects in the canopy of *Rhizophora* or collects in the soil beneath the trees that is not inundated.

Wet litter

Sixty leaves were surface sterilized and submerged in diluted sterile sea water (salinity 15‰) for 21 days. The temperature during this incubation was 26 ± 1 C. The water was changed every 48 hours. This situation was similar to the leaf litter of *Rhizophora* that is present submerged in the water.

Both dry and wet litter was sampled (twenty leaves per treatment) once every seven days for 21 days. Two hundred segments of these treated leaves were surface sterilized and then screened for endophytes for each treatment, as mentioned above.

Statistical Analyses

The density of colonization (rD%) of a single endophyte species was calculated by the method of Fisher and Petrini (1987) and was equal to the number of colonized segments divided by the total number of segments observed × 100. The Richness Index (R1) of the endophyte assemblages in the leaves of different age classes were calculated as described by Ludwig and Reynolds (1988) using the software provided by John Wiley and Sons. Mann Whitney U test was carried out to compare the endophyte assemblages of young and senescent leaves.

Detection of extracellular enzyme production

The methods described by Hankin and Anagnostakis (1975) and Rohrmann and Molitoris (1992) were used to detect the production of extracellular enzymes by the endophytes.

Amylolytic activity

GYP medium (i.e. 1 g glucose + 0.1 g yeast extract + 0.5 g peptone, 16 g agar in 1000 ml distilled water) plus 0.2% soluble starch, pH 6, was used. After 3-5 days of colony growth, the plates were flooded with iodine solution. A yellow zone around the fungal colony in an otherwise blue medium indicated amylolytic activity.

Cellulase activity

YP medium containing Na-carboxy-methylcellulose (0.5%) was used. After 3-5 days of colony growth, the plates were flooded with 0.2% aqueous congo red solution and destained with 1M NaCl (15 min each). Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity.

Laccase activity

GYP medium with 0.05 g 1-naphthol L^{-1}, pH 6, was used. As the fungus grows the colourless medium turns blue due to the oxidation of 1-naphthol by laccase.

Lipolytic activity

Tween 20 was sterilized by autoclaving for 15 min at 103 kPa pressure and 1 ml of it was added to 100 ml of sterile, cooled agar medium (Peptone 10 g, NaCl 5 g, $CaCl_2.2H_20$ 0.1 g, agar 20 g in 1000 ml of distilled water, pH 6). Clearing or precipitation around the fungal colony indicated lipolytic activity.

Pectinolytic activity

Agar medium containing 1 g yeast extract, 5 g pectin, 15 g agar (in 1000 ml distilled water), was used. This medium at pH 7 was used to detect pectate transeliminase production. The same medium at pH 5 was used to detect pectinase activity. After 3-5 days of colony growth, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. A clear zone formed around the fungal colony indicated pectinolytic activity.

Proteolytic activity

GYP medium amended with 0.4% gelatin (pH 6) was used. A solution of gelatin in water (8%) was sterilized separately and added to the GYP medium at the rate of 5 ml per 100 ml of medium (constituting 0.4% gelatin). After incubation, degradation of the gelatin was seen as a clearing in the somewhat opaque agar around the colonies. The plate was then flooded with saturated aqueous solution of ammonium sulphate which resulted in the formation of a precipitate. This made the agar opaque and enhanced the clear zones around the fungal colony.

Tyrosinase activity

Endophytes were grown in GYP medium. Formation of red brown colour around the fungal colony after addition of *p*-cresol (1.08 g /1000 ml) with 0.05% glycine to the surface of fungal colony indicated tyrosinase activity.

Results and discussion

Endophyte status in leaves of different age classes

Young, bright green, tender leaves, mature, dark green leaves and old, yellow, senescent leaves of *R. apiculata* harboured fungal endophytes. The number of endophyte isolates recovered increased with the age of the leaf (Table 1). Senescent leaves were significantly more densely colonized by endophytes when compared with young leaves (Mann-Whitney U = 134, P = 0.0015).

The number of endophytes that can be recovered from leaf tissue has been shown to increase with the age of leaves in several hosts including Douglas-fir (Stone, 1987), coastal redwood (Espinosa-Garcia and Langenheim, 1990), *Trachycarpus fortunei* (Taylor *et al.*, 1999) and *Azadirachta indica* (Rajagopal and Suryanarayanan, 2000). The increased density of colonization of older leaves is due to repeated reinfection of the leaf over time, probably from air borne inoculum (Carroll *et al.*, 1977; Bertoni and Cabral, 1988; Rodrigues *et al.*, 1993). This is especially relevant in the case of mangroves

Table 1. Density of colonization (rD%) of endophytes isolated from young, mature and senescent leaves of *R. apiculata*.

Endophyte	Young leaf	Mature leaf	Senescent leaf
Ascomycetes			
Yeast	0.5	-	-
Glomerella sp. MG 108	-	-	5.5
Sporormiella minima	4.5	2	10
Xylariaceous form MG 284	-	-	1
Mitosporic taxa			
Acremonium sp. MG 1	-	-	1.5
Alternaria alternata	-	1	0.5
Alternaria sp. MG 92	-	1	4
Alternaria sp. MG 94	-	0.5	1.5
Aureobasidium sp. MG 268	0.5	1	5.5
Cladosporium cladosporioides	-	0.5	4.5
Curvularia lunata	1	-	0.5
Curvularia pallescens	-	-	0.5
Drechslera sp. MG 76	-	-	0.5
Fusarium sp. MG 57	0.5	0.5	-
Nodulisporium sp. MG 351	1	-	1
Pestalotiopsis sp. MG 98	-	1	1
Phialophora sp. MG 3	-	0.5	1
Phoma sp. MG 97	1.5	1	2.5
Phoma sp. MG 167	-	0.5	-
Phomopsis sp. MG 11*	-	1.5	1
Phyllosticta sp. MG 90*	5.5	30.5	38
Phyllosticta sp. MG 123	-	-	1
Pithomyces sp. MG 262	0.5	-	-
Sporothrix sp. MG 347	-	0.5	1
Sterile forms	4.5(3)**	13 (9)	15(8)
Total rD%	20	55	98
Species Richness (R1)	2.98	4.68	5.13

* Initially reported as sterile form by Suryanarayanan *et al.* (1998).
**No. of morphotypes of sterile forms within brackets

because leaves persist on mangroves for several months (Tomlinson, 1986). In addition, the endophyte assemblage of senescent leaves of *R. apiculata* was more diverse as indicated by species richness (R1) (Table 1) suggesting that the susceptibility of leaves to endophytes and possibly saprobes increased with leaf age.

Endophytes in detached leaves

Our results show that the endophyte population present in the mangrove leaf may not be static after the leaf falls. We found that *Cladosporium cladosporioides*, *Phyllosticta* sp. (MG 90) and *Sporormiella minima* whose

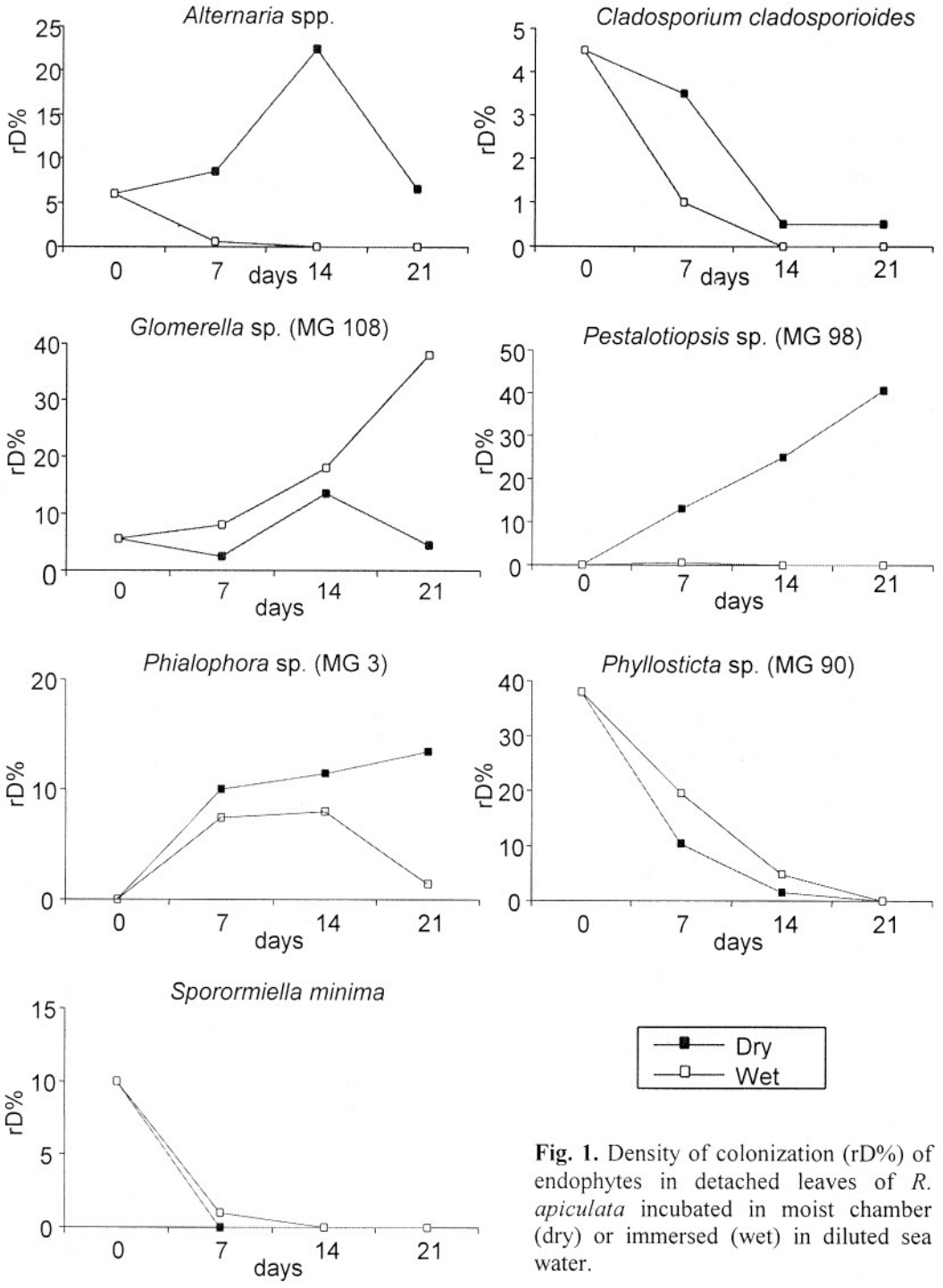

Fig. 1. Density of colonization (rD%) of endophytes in detached leaves of *R. apiculata* incubated in moist chamber (dry) or immersed (wet) in diluted sea water.

density of colonization increased with the age of the mangrove leaf (Table 1), declined in the detached leaves irrespective of whether the leaves were immersed or not (Fig. 1). This suggested that the ubiquitous endophytes such as *Phyllosticta* and *Sporormiella* (Petrini and Carroll, 1981; Carroll, 1990; Suryanarayanan *et al*., 1999; Suryanarayanan *et al*., 2001) had little role to play in mangrove litter degradation. It is possible that these fungi are more adapted to an endophytic mode of life. This is not surprising as different endophyte species could have different roles or functions (Wilson, 2000).

Role of environment in endophyte status of detached leaves

The status of a few other endophytes in detached leaves of *R. apiculata* depended on the environment in which the leaves were maintained. The density of colonization of *Alternaria* spp., *Pestalotiopsis* sp. (MG 98) and *Phialophora* sp. (MG 3) increased in detached leaves that were not immersed in sea water (Fig. 1). The density of colonization of *Pestalotiopsis* sp. and *Phialophora* sp. increased up to 21 days of incubation. These two endophytes were recovered in very low frequencies from leaves that remained attached (Table 1). Thus, some of the endophytes that were relatively less dense in the leaves before abscission became more common after the leaf fall. It is also possible that depending on the environment where the leaf falls (submerged in seawater or terrestrial), different endophytes that were dormant in intact leaves could become active. This was substantiated by the fact that several fungi which were not isolated as endophytes from senescent leaves that were screened immediately after collection (Table 1) were present in such leaves that were incubated in dry or wet conditions (Table 2). Occurrence of these fungi in detached leaves was not due to fresh infections since the leaves were incubated in sterile conditions which preclude such infections. Therefore, it is likely that these fungi were present initially in rather low frequencies and were not recovered when the attached leaves were screened for endophyte presence; they could have become active after leaf fall.

We found that while the density of colonization of most of the endophytes declined in detached leaves incubated under water, the density of colonization of *Glomerella* sp. increased (Fig. 1). The increased density of colonization of the endophytes in detached leaves was due only to their growth since fresh infections from the air were ruled out. Thus, endophytes of different ecological categories occur in the leaves of *R. apiculata*; some become active after the leaf falls on the ground or become lodged in the canopy, others start growing if the leaf is immersed. Some endophytes such as *Phyllosticta* and *Sporormiella* may stop their growth after leaf fall. The growth of endophytes in detached leaf tissue can be taken as a sign of fungal degradation of litter. Apart

Table 2. Sporulating fungi isolated only from detached yellow senescent leaves incubated in dry and wet conditions.

Incubation Condition	*Fungi*
Dry	*Acremonium* sp., *Ascochyta* sp., *Curvularia leonensis*, *Corynespora* sp., *Fusarium* sp.1, *Fusarium* sp2., *Penicillium* sp, *Fusicoccum* sp., *Zygosporium masonii*
Wet	*Acremonium* sp., *Massarina* sp., *Penicillium* sp.

Table 3. Extracellular enzyme production by mangrove endophytes.

Endophyte	*Pestalotiopsis* sp. MG 98	*Glomerella* sp. MG 108
Amylase	-	-
Cellulase	+	+
Laccase	+	+
Lipolytic activity	+	+
Pectate transeliminase	Not tested	+
Pectinase	Not tested	+
Proteolytic activity	-	+
Tyrosinase	-	-

from this, it is evident that endophytes can survive in abscised mangrove leaves. Such leaves could be an important source of endophyte inoculum (Wilson, 2000).

Endophytes and litter degradation

Litter degrading organisms are expected to produce extracellular enzymes that degrade the plant litter. This is obvious from several studies on aquatic hyphomycetes that colonize submerged leaves in fresh water ecosystems (Chamier, 1985). However, the role of fungal endophytes in litter degradation is not clearly understood. Substrate utilization studies on fungal endophytes of various plants have conclusively demonstrated that most endophytes are able to utilize most substrates present in the cell walls of the hosts (Carroll and Petrini, 1983; Sieber-Canavesi *et al.*, 1991; White *et al.*, 1991). In the present study, *Pestalotiopsis* sp. and *Glomerella* sp., the two endophytes that grew in detached leaves under dry and immersed conditions respectively, were screened for their capacity to produce some extracellular enzymes. Both taxa produced cellulase, laccase and lipolytic enzymes (Table 3). *Glomerella* sp. also produced pectate transeliminase, pectinase and proteolytic enzymes. This suggested that the endophytes could degrade cuticular waxes on the leaf surface as well as some of the cell wall constituents (Carroll and Petrini, 1983). Laccase is a lignin-modifying enzyme (Archibald and Roy, 1992) and hence the endophyte assemblage of *R. apiculata* leaves as a whole, appear to possess the complete enzyme array to degrade leaf litter.

The role of endophytic fungi in plant decomposition has been postulated over several years, since endophytes are already present in senescent tissues of plants and as such they may initiate tissue decomposition before being overwhelmed by ubiquitous saprobic fungi (Petrini *et al.*, 1992; Wilson, 2000). Although litter fungi are known to be associated with decaying mangrove leaves (Fell and Master, 1980; Newell, 1986; Hyde, 1990), the involvement of endophytic fungi in litter degradation is not clearly understood (Wilson, 2000; Suryanarayanan *et al.*, 2001). Wilson (1993) provided evidence for the role of endophytes in leaf tissue degradation. He showed that endophyte-free Oregon white oak leaf discs remained green and intact for eighteen months when plated out on PDA medium but endophyte-infected leaf discs turned brown and were rapidly degraded. Our study revealed that some of the foliar endophytes of the mangrove grew in fallen leaves (as reflected by their density of colonization) and that at least some of them had a degradative potential (as revealed by substrate degradation tests). Therefore, a syllogistic conclusion is that foliar endophytes are involved in litter decomposition.

We used surface sterilized mangrove leaves to suppress the regular saprobes and to discern the growth of endophytes in senescent tissues. This situation is not akin to that obtained in nature where fallen leaves are rapidly colonized by litter fungi which outgrow other fungi including terrestrial ones (Bärlocher and Kendrick, 1974). This may be the reason why endophytes are generally not encountered when dead mangrove leaves are sampled for litter fungi. However, failure to recover terrestrial (or endophytic fungi) fungi from submerged leaves does not necessarily exclude them from participating in litter degradation (Bärlocher, 1982; Godfrey, 1983). Thus, it would be worthwhile for future studies to address the role of endophytes in litter degradation.

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