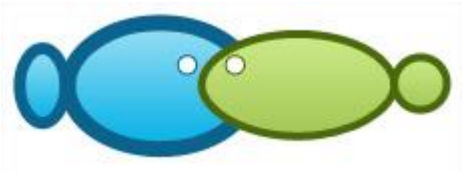

Fungal infections of mangroves in natural forests and reforestation sites from Philippines

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Abstract. The prevalence of fungal infections and the occurrence of fungi in mangrove leaves from in natural forests and reforestation sites were compared in three areas from Philippines: Central Visayas-Bais, Negros Oriental, Alcantara, Cebu and Pangangan Island, Bohol. Three diseases were identified, namely the Brown Leaf Spot disease (BRS), Black Leaf Spot disease (BLS) and White Leaf Spot disease (WLS). BRS was found in *Sonneratia alba* and *Avicennia marina* in natural forests and reforestation sites in all areas, while BLS and WLS were found only in Bais, with BLS occurring in *Ceriops decandra* and *Osbornia octodonta* in natural forests and in *Rhizophora stylosa* in reforested sites. WLS occurred only in *A. marina* in natural forests. Disease prevalence for BRS was significantly higher in reforested areas ($F=8.477$; $p=0.001$). 4 fungi genera were consistently found in infected leaves: *Aspergillus*, *Penicillium*, *Pseudocercosporella* and *Rhizopus*. The occurrence percentage was higher in natural forests than in reforestation sites for all fungi.

Key Words: Brown Leaf Spot disease, Black Leaf Spot disease, White Leaf Spot disease, disease prevalence.

Introduction. The variability of mangrove ecosystems has attracted the attention of several researchers who have contributed extensively to scientific knowledge about fungi associated with mangrove trees (Cribb & Cribb 1955; Alias et al 1999; Alias et al 2010; Sivakumar 2013). These contributions have been mostly descriptive and focused on the morphology and taxonomy of mangrove fungi (Alias et al 1999; Steinke 2000; Sarma & Hyde 2001). Only a limited number of publications reveal information on fungi causing diseases of mangroves (Teas & McEwan 1982; Wier et al 2000; Gilbert & Sousa 2002; Tattar & Scott 2004).

The first published study relating to diseases of mangroves was apparently carried out in Puerto Rico by Stevens (1920), who reported a leaf spot on *Rhizophora mangle* caused by a fungus belonging to the genus *Anthostomella* (Xylariaceae). Since then, only one other leaf disease has been described, from a mangrove species, caused by *Phyllosticta hibiscina* (Botryosphaeriaceae), resulting in the necrosis and death of leaves of *Avicennia germinans* (L.) (Black mangrove) in Florida (Olexa & Freeman 1975).

In the Philippines, a study of mangrove diseases was conducted by Sinohin (1997), in Cavite, Manila, and documented three diseases, namely Brown Leaf Spot disease, Black Leaf Spot disease and Tip Blight disease, caused by fungi. However, studies on marine fungi are limited. The occurrence and distribution of marine fungi in the Philippines, particularly in the Bais, Alcantara and Pangangan Island has been less studied, with the exception of a few studies on fungal diversity in mangrove areas in other parts of the country (Alias et al 1999; Besitulo et al 2010). Alias et al (1999) have reported 57 fungal species isolated from parts of mangroves in Boracay and Pagbilao from 2002 until now and Besitulo et al (2010) reported 66 species of fungi from Siargao Island, Mindanao.

Fungi associated with mangroves are especially well documented. A large number of studies have been conducted in East and Southeast Asia (Ho et al 1990; Nakagiri et al

2001; Sarma & Hyde 2001; Wang et al 2010), especially from areas such as China (Jones et al 1999; Tsui & Hyde 2004) and Thailand (Hyde et al 1990a; Hyde et al 1990b). Many of these fungi were found in dead plant parts, often from unidentified mangrove species (Hyde et al 1998; Kohlmeyer & Kohlmeyer 1993; Gilbert & Sousa 2002).

An increasing concern regarding mangrove health has prompted several studies to determine possible diseases of these trees caused by fungi and other bacteria (Tattar & Scott 2004; Sinohin 1997; Gilbert et al 2002). However, despite the direct benefits provided by mangroves globally, studies regarding biological factors associated with the decline of these trees remain limited (Osorio et al 2014).

The absence of records regarding diseases caused by fungi in mangroves from Philippines necessitates research. A survey of infected leaves of mangrove plants was performed at three selected sites in Bais, Alcantara and Pangangan Island. This study aims to identify diseases caused by fungi and to compare fungi disease prevalence in natural and reforested mangrove forests. The occurrence of fungi and hosts were also observed. The results of this study can serve as baseline information regarding mangrove fungi, with an emphasis on diseases caused by fungi in order to gain a more integrated understanding of the pattern of fungal infections.

Material and Method

Description of the study sites. Leaves were collected from three mangrove forests, each in natural and reforested mangrove forests located in Bais, Alcantara, and Pangangan Island. Sample collection was performed during wet season, in November and December 2016. The tropical monsoon season was characterized by relatively high temperature, oppressive humidity and plenty rainfall. There are two seasons in the Philippines based on the amount of rainfall, the wet season (June to November) and the dry season (December to May); average temperature is 28.3°C, and precipitation is 1.607 mm year^{-1} (Figure 1).

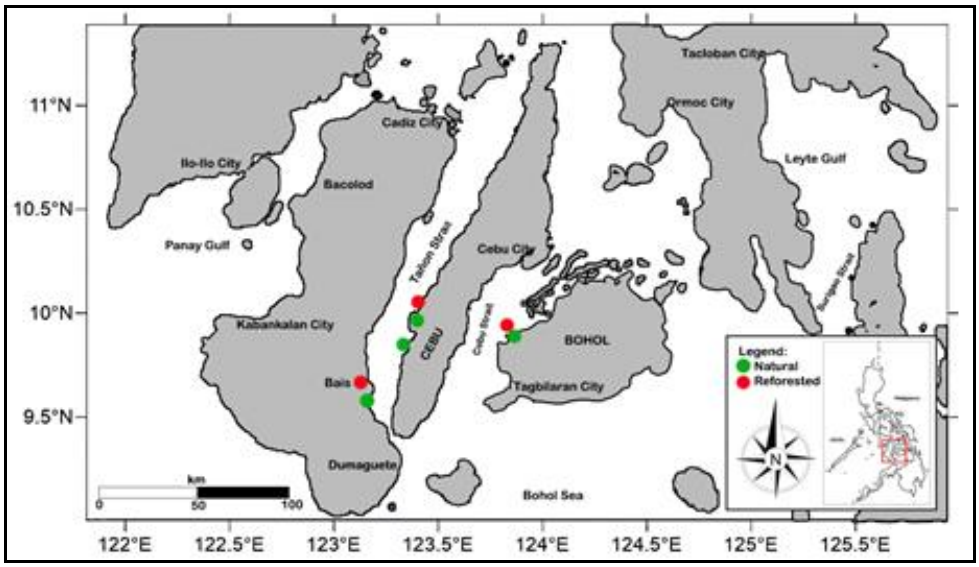

Figure 1. Study sites.

Sampling for fungal diseases. To identify diseases in natural and reforested mangrove forests, a method described by Sadaba & Barnuevo (2010) was followed. A line transect plot was established and the prevalence of mangrove species affected by diseases caused by fungi was quantified. Following the modified method of Pranasannarai & Sridhar (2003), disease prevalence was quantified as number of disease symptoms divided by total number of trees in the area ×100.

The frequency of fungi is calculated based on the percentage of occurrence of fungi. The term percentage occurrence is used to denote the number of samples on which a particular fungus was found against the total number of samples examined in the study and is calculated according to the following formula as outlined by Sarma & Hyde (2001):

Percentage Occurrence = Number of Samples with Particular Fungus Recorded, divided by the Total Number of Samples Examined × 100

Based on percentage occurrence, the following frequency groupings of fungi were performed:

1. Very frequent: occurring in more than 10% of samples.
2. Frequent: above 5% and below 10%.
3. Less frequent: above 1% and below 5%.
4. Rare: below 1%.

Collection of samples of fungal infections. About 200 infected leaves were collected from both natural and reforested mangrove forests in Bais, Alcantara, and Pangangan Island. Determination of infected leaves was performed through visual inspection. Samples of infected leaves were collected and gently washed with sterile distilled water to remove any adhering soil particles and placed in zipped plastic bags, after which all samples were processed in the laboratory (Umechuruba 2005; Capote et al 2012).

Fungal growth. Isolation of fungi was carried out according to the modified method from Choi et al (1999), Umechuruba (2005), and Capote et al (2012). After proper washing, the infected leaves were cut into 2×2 cm pieces, excluding the midrib, under aseptic conditions. Surface sterilization was done by soaking in 70% ethanol for 30 seconds, and later the segments were rinsed with sterile distilled water.

Two pieces of infected leaves of each mangrove species were placed directly on Petri dishes (size of Petri dish: 60 mm diameter and 15 mm height; Pyrex brand), which contained potato dextrose agar (PDA, 300 g/L diced potatoes, 20 g/L dextrose, and 20 g/L agar), making sure that the pieces were well separated. Three Petri dishes were used for each mangrove species. The infected leaves were then incubated at room temperature in an incubator at 27°C for 72 hours for fungal organisms to sporulate and appear (Capote et al 2012). To avoid the presence of bacteria, filter sterilized antibiotics (ampicillin and streptomycin) were added to the agar in 0.5 g/L and 0.3 g/L, respectively.

The agar plates were investigated in terms of colony morphology, including shape, margin and elevation. Morphologically dissimilar colonies were selected, and for obtaining pure colonies, a small portion of different colonies was cut and placed into new Petri dishes containing PDA.

Identification of fungi. Isolates were inoculated in the PDA media and incubated at 27°C for 72 hours for identification. Petri dishes were first examined under a dissecting microscope (a stereomicroscope) and then under a Motic stereo microscope to determine the colonial features and the morphological structures of the fungi. The microscopic examination was made from slide preparation stained with Lactophenol Cotton Blue and the covered glass was sealed with colorless nail varnish. Photomicrographs of fungal species were obtained using a Nikon Cool Pix P3 digital camera.

Fungi were indentified to genus level according to various mycological references: Choi et al (1999), Quimio & Hanlin (1999), and Lu et al (2012).

Results and Discussion

Fungal diseases. Three foliar diseases were identified in the present study (Figure 2, 3 and 4), namely Brown Leaf Spot disease (BRS), Black Leaf Spot disease (BLS) and White Leaf Spot disease (WLS). The leaf symptoms are presented in Figure 2, 3 and 4, and

were identified from different hosts: *Avicennia marina, Ceriops decandra*, *Osbornia octodonta*, *Rhizophora stylosa*, and *Sonneratia alba.*

Brown Leaf Spot disease is characterized by the presence of light to dark brown areas with irregular shapes margins in various sizes, with or without necrotic lesions. This was found in *Sonneratia alba* and *Avicennia marina* (Figure 2A to 2D).

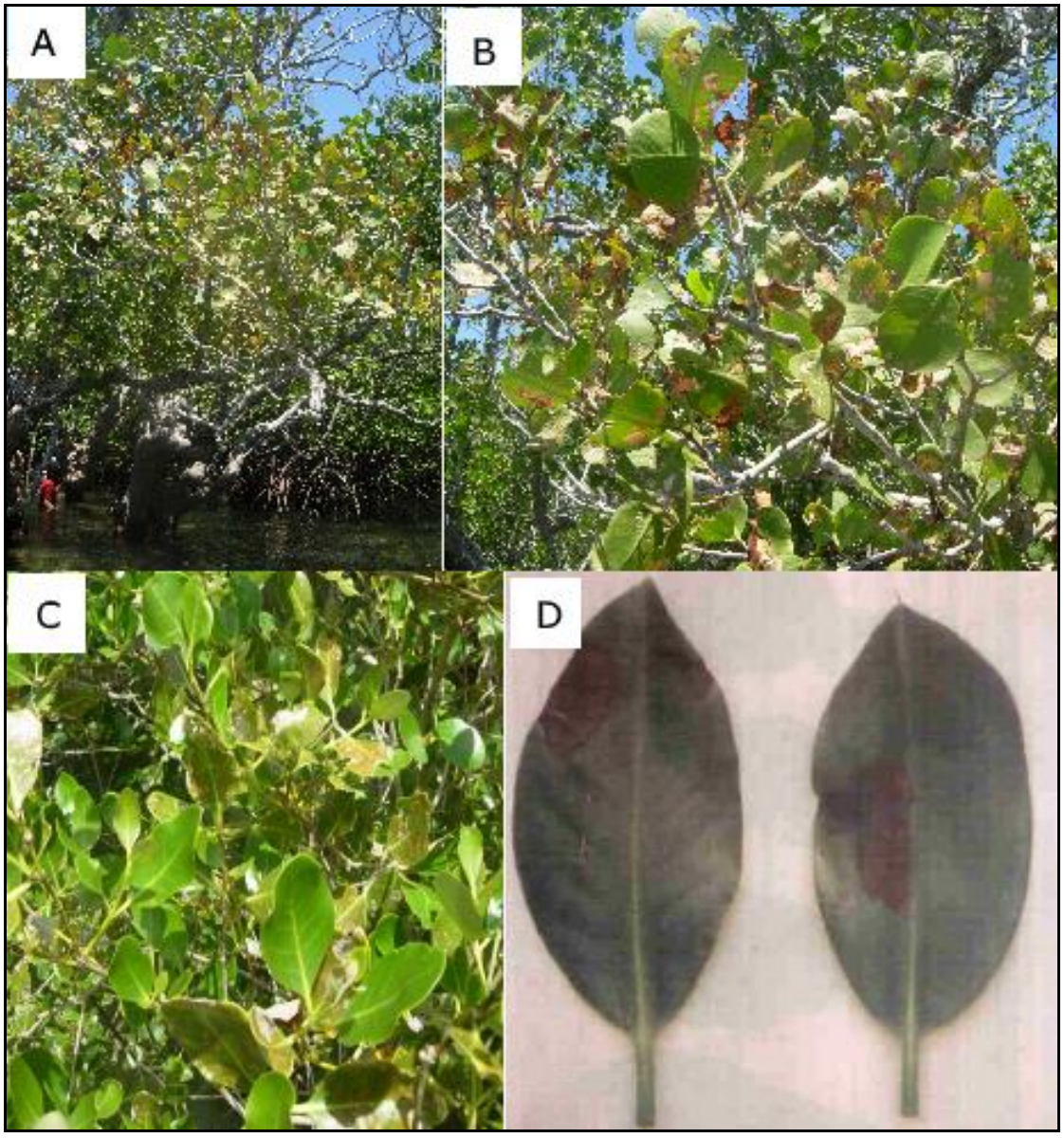

Figure 2. A - *Sonneratia alba* tree in Panganan Island. B - Infected leaves with Brown Leaf Spot disease in *Sonneratia alba* tree, Pangangan Island. C - Infected leaves with Brown Leaf Spot disease in *Avicennia marina* tree, Alcantara. D - Close up of Brown Leaf Spot disease in *Rhizophora stylosa* from Cavite, Manila, identified by Sinohin (1997).

White Leaf Spot disease is characterized by the presence of white areas with irregular shape margins in various sizes, sometimes with necrotic lesions. This was found in *Avicennia marina* (Figure 3A and 3B).

Figure 3. A - *Avicennia marina* tree in Bais City, infected with White Leaf Spot disease. B - Close up of infected leaves of *Avicennia marina* tree in Bais City with White Leaf Spot disease.

Black Leaf Spot disease is characterized by the presence of irregular spots with smooth borders, with a prominent halo around the spots, which later turns brown to orange, as the disease progress. This was found in *Ceriops decandra* and *Osbornia octodonta* (Figure 4).

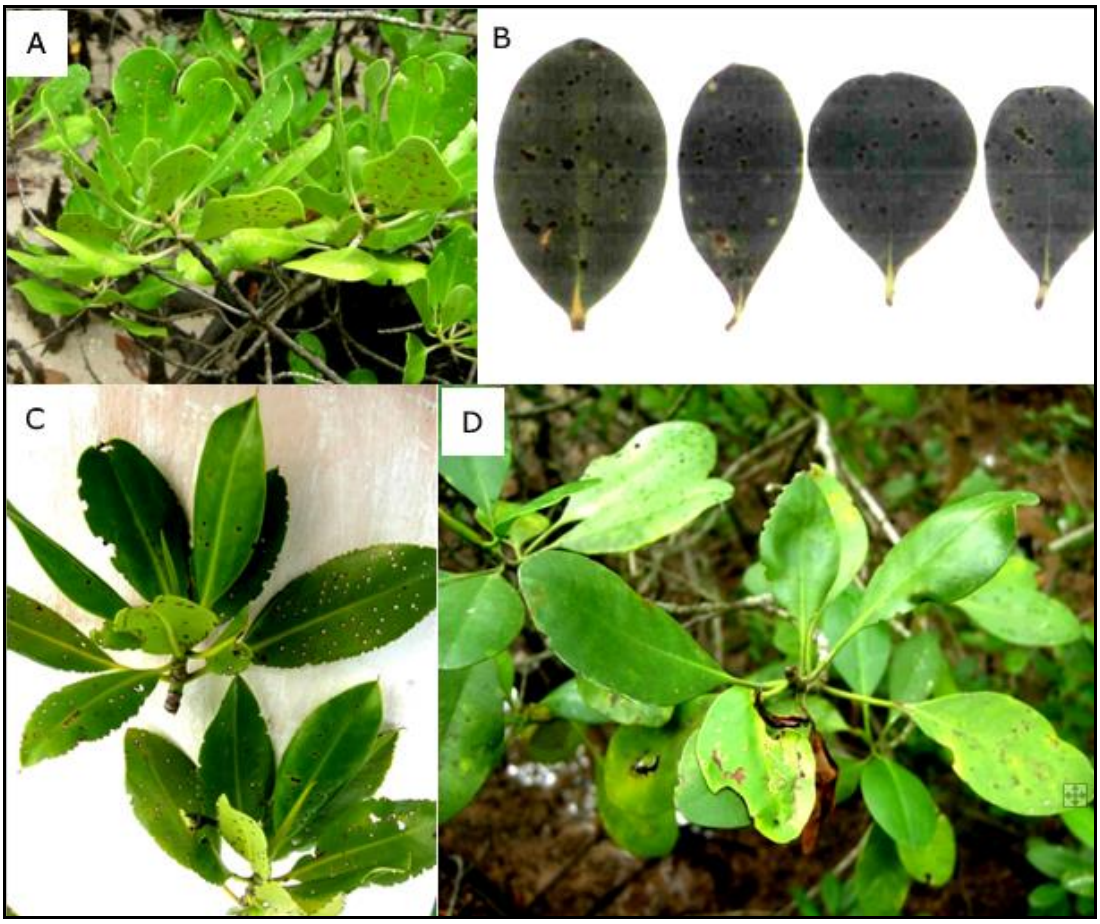

Figure 4. A - Black Leaf Spot disease in *Osbornia octodonta*, Bais City. B - Close up of Black Leaf Spot disease in *Sonneratia caseolaris* from Cavite-Manila, identified by Sinohin (1997). C - Black Leaf Spot disease found in *Rhizophora stylosa* in Bais City. D - Black Leaf Spot disease found in *Ceriops decandra* in Bais City.

Disease prevalence. The disease prevalence of BRS disease in Bais reforested areas was higher (31.15%±9.03%) than in natural forests (15.51%±3.71%). Comparing the two forests using Independent *T*-test, the results showed a significant difference (*t*=-3.967, p=0.002, n=14, with a confidence level of 95%). In Alcantara and Pangangan Island, the disease prevalence was significantly higher in natural forests, with the values of 54.91%±9.07% and 31.61%±4.95%, respectively, compared with the reforested sites with the values of 17.73%±7.81% and 16.00%±14.07%, respectively. Table 1 shows the results of the independent T-test of BRS disease prevalence in the three sites.

Table 1
Independent Sample T-Test of Brown Leaf Spot disease in Bais, Alcantara and Pangangan Island

Sites	*Forest type*	*Disease Prevalence*	*T-Test*	*p*
Bais	Natural Reforested	19.44±22.85 78.53±35.66	-3.533	0.004*
Alcantara	Natural Reforested	54.91±9.07 17.73±7.81	6.948	0.000*
Pangangan Island	Natural Reforested	31.61±4.95 16.00±14.07	2.563	0.028*

Comparing the BRS disease prevalence in the three sites using Two-Way ANOVA, the results show no significant differences between forests (F=1.393; p=0.246), and sites (F=0.329; p=0.722).

Black Leaf Spot disease was only found in Bais, both in natural and reforested sites, and the results of the T-Test showed no significant difference between the forests (t=0.561; p=0.585; n=14). WLS disease was observed only in Bais natural forests with symptoms being observed in *A. marina.*

The BRS disease prevalence was higher in reforested forests compared with natural forests in Bais, whereas in Alcantara and Pangangana Island the disease prevalence was higher in natural forests compared with reforestation sites. There are very few other studies of mangrove diseases in the natural forests and reforested sites in the Philippines, but the available data are similar to those presented here. Sinohin (1997) showed that in Cavite, Manila, mangrove plants suffers from foliar diseases. Leaf Spot disease and Brown Leaf Spot disease infected *Rhizophora apiculata* and *Rhizophora stylosa*, respectively. These results indicated that foliar diseases appear to vary between hosts.

The results of the present study indicate fungal infections, as the symptoms of diseases were observed on five different of mangrove species in both natural forests and reforested sites. Gilbert et al (2002) observed the mangrove forests from the Caribbean coast of the Republic of Panama, and the results showed that leaves of *A. germinans* (L.) and *Languncularia racemosa* (L.) were disease free, due to a high level of phenolic compounds, especially tannins. The research also suggests that salt excretion in leaves serve as an important defense against fungal attacks or colonization.

Frequency of occurrence. Based on the frequency of occurrence, natural forests present higher values compared with reforested areas in the three study sites. In natural sites, the higher frequency of occurrence was observed for *Pseudocercosporella* (13.39%) and *Rhizopus* (9.92%). These two genera were categorized as Very Frequent. In reforested areas, the higher frequency of occurrence was found for *Aspergillus* (4.29%) and *Rhizopus* (3.31%), and categorized as Less frequent.

Table 2

List of fungi identified from infected leaves of mangroves in natural and reforestation sites in Bais, Alcantara and Pangangan Island

Forest type	*Fungi*	*Frequency of Occurrence*	*Frequency Grouping*
Natural	*Aspergillus*	11.11	Very Frequent
	Penicillium	10.71	Very Frequent
	Pseudocersosporella	9.92	Very Frequent
	Rhizopus	13.49	Very Frequent
Reforestation	*Aspergillus*	4.29	Less frequent
	Penicillium	3.47	Less frequent
	Pseudocersosporella	3.31	Less frequent
	Rhizopus	3.47	Less frequent

The frequency of occurrence can help establish the most pathogenic common fungi present in an area. In fungal communities that are extremely rich in species, most species are likely to be sparsely distributed and only a few fungi will dominate a given area (Sarma & Hyde 2001). The number of fungi identified in the present study was lower than that reported by other studies. Alias et al (1999) reported 57 species from Luzon and Visayas regions, and Besitulo et al (2010) reported 66 species of fungi from Siargao Island, Mindanao. The difference in the frequently collected species between this study and previous fungal studies in other parts of the Philippines could be attributed to the different location and the mangrove tree species present in the study area, sample collection and different substrata. Most of the fungi reported in the mangroves from Philippines were isolated from woods (Gacutan & Uyenco 1983; Jones et al 1988; Alias et al 1999; Besitulo et al 2010).

The present study shows that the four genera, *Aspergillus*, *Penicillium*, *Pseudocercosporella* and *Rhizopus*, were found to be very frequent fungi in natural forests and less frequent in reforested sites. According to Hyde et al (1998), relatively few fungi have been reported as pathogens of mangrove plants, as compared with the number of saprophytic fungi identified on decaying mangrove wood and leaves. For instance, the intertidal fungus *Cytospora rhizophorae* is thought to be parasitic on the prop root of *Rhizophora* spp. (Kohlmeyer & Kohlmeyer 1979). Hyde (1996) reported that *Phomopsis mangrovei*, which is probably pathogenic, was described from dying prop roots of *Rhizophora apiculata* in Thailand.

A study by Sarma & Hyde (2001) described that some fungi occur more frequently on certain tissues or organs than others. Hence, the frequency of occurrence differs depending of the number of samples collected belonging to individual substrata (organs), for instance wood, pneumatophores, prop roots, leaves or roots. Several fungi were encountered only in roots and stems of living *Avicennia* or *Rhizophora* and appear to be host specific. Hyde et al (1990a) investigated the distribution of fungi on *Sonneratia griffithii* and showed that some were more common on pneumatophores, like *Aigialus grandis* and *Massarina velatospora*, while others were common on twigs, *Saccardoela mangrovei* and *Savoryella longispora*.

Fungi identification. Four genera of marine fungi were isolated and identified during the course of the study, both from natural and reforested areas in all sites. All four genera belong to division Ascomycota. These are *Aspergillus*, *Penicillium*, *Pseudocercosporella* and *Rhizopus*. The colony, hyphal and spore characteristics are summarized in Table 3, with images in Figure 5, 6, 7, 8 and 9.

In Bais natural forest, all four genera were isolated from leaves of *A. marina*, *R. stylosa*, *S. alba, C. decandra,* excepting *O. octodonta*, which does not have *Penicillium*. In reforested areas*,* all fungi genera were isolated from leaves of *A. marina* and *R. stylosa*.

In Alcantara, all four genera were isolated from the leaves of *A. marina* and *S. alba* in both natural forests and reforested areas. Meanwhile, in Pangangan Island, all

four genera were isolated from infected leaves of *S. alba* in natural forest, and leaves of *A. marina* in reforested areas.

Table 3

The morphological characteristics of fungi

Fungus	*Colony*	*Colony characteristics*	*Hyphal characteristics*	*Spore characteristics*
Aspergillus	Pale to light green	Circular with smooth margins, elevated	Hyphae are septate	Conidiophores bearing metulae and or phialide with conidia
Penicillium	Light grey	Circular form with raised elevation and smooth surface	Hyphae are septate	Conidiophores with phialides and conidia
Pseudocercosporella	White	Irregular form, with raised elevation and rough surface	Hyphae are septate	Conidia and conidiophores
Rhizophus	Dark brown to black	Circular form with raised elevation and smooth surface	Hyphae are septate	Long necked perithecium with asci and ascospores

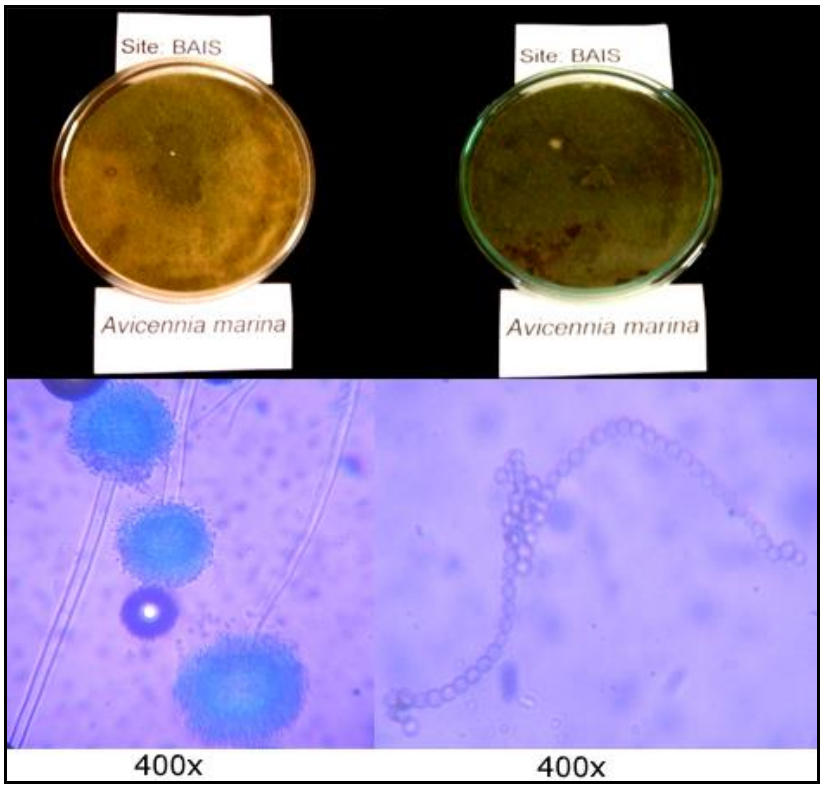

Figure 5. Colonial appearance of *Aspergillus* identified in *Avicennia marina* (upper left and upper right). Conidiophores bearing metulae and/or phialides with conidia (lower left), conidia (lower right).

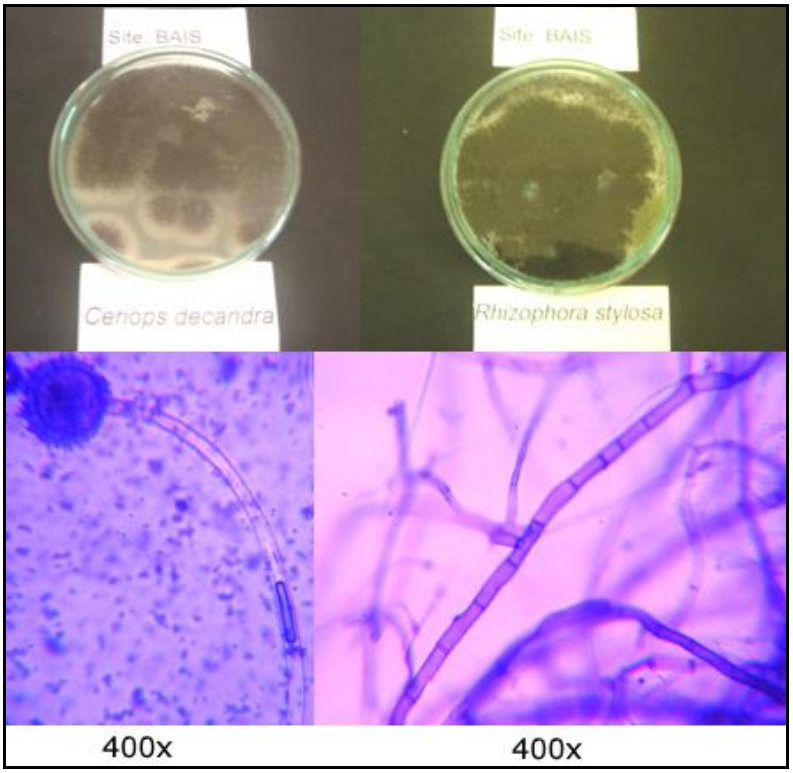

Figure 6. Colonial appearance of *Ceratocystis* identified in *Ceriops decandra* (upper left) and *Rhizophora stylosa* (upper right). Subglobose ascomatal base (lower left), Flask-shaped primary phialide producing cylindrical conidia (lower right).

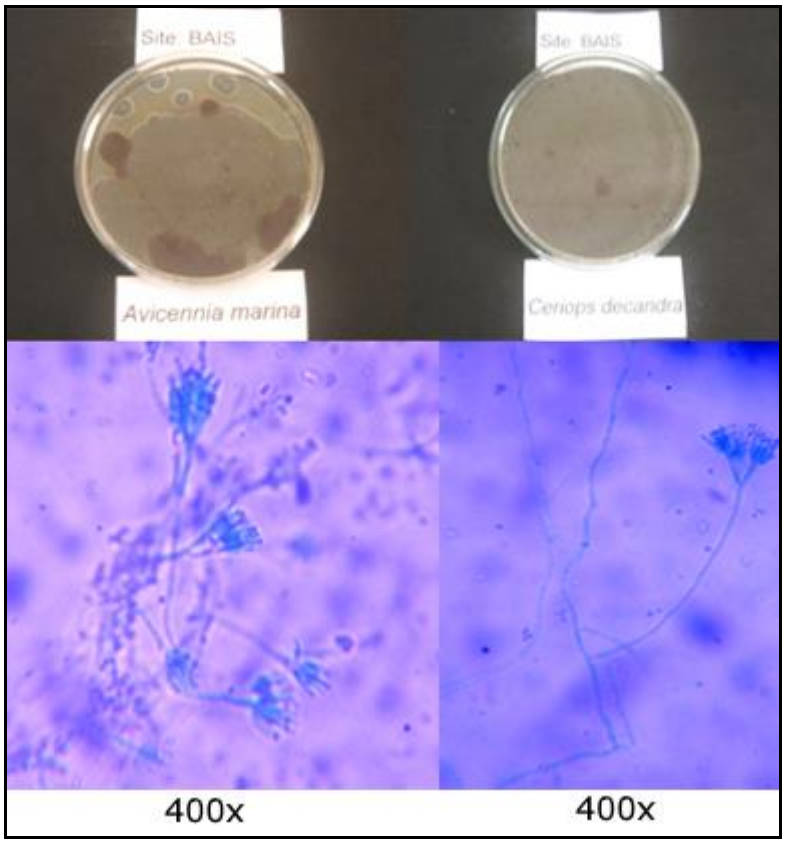

Figure 7. Colonial appearance of *Penicillium* identified from *Avicennia marina* (upper left) and *Ceriops decandra* (upper right). Conidiophores with phialides and conidia (lower left and right).

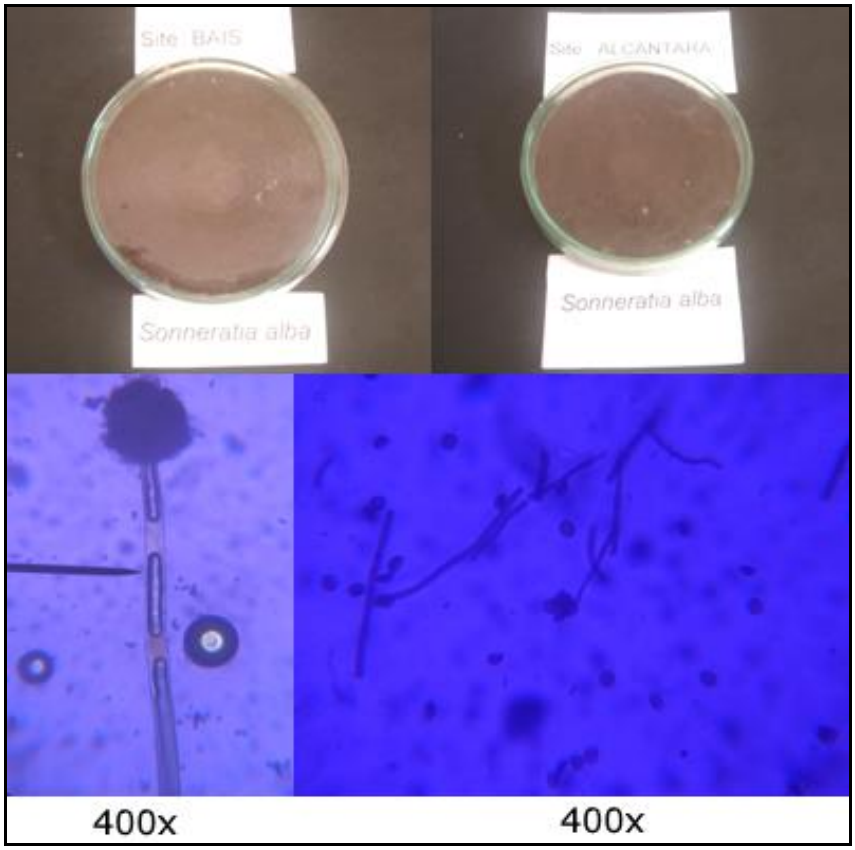

Figure 8. Colonial appearance of *Penicillium* identified from *Sonneratia alba* (upper left) and *Ceriops decandra* (upper right). Conidiophores with phialides and conidia (lower left and right).

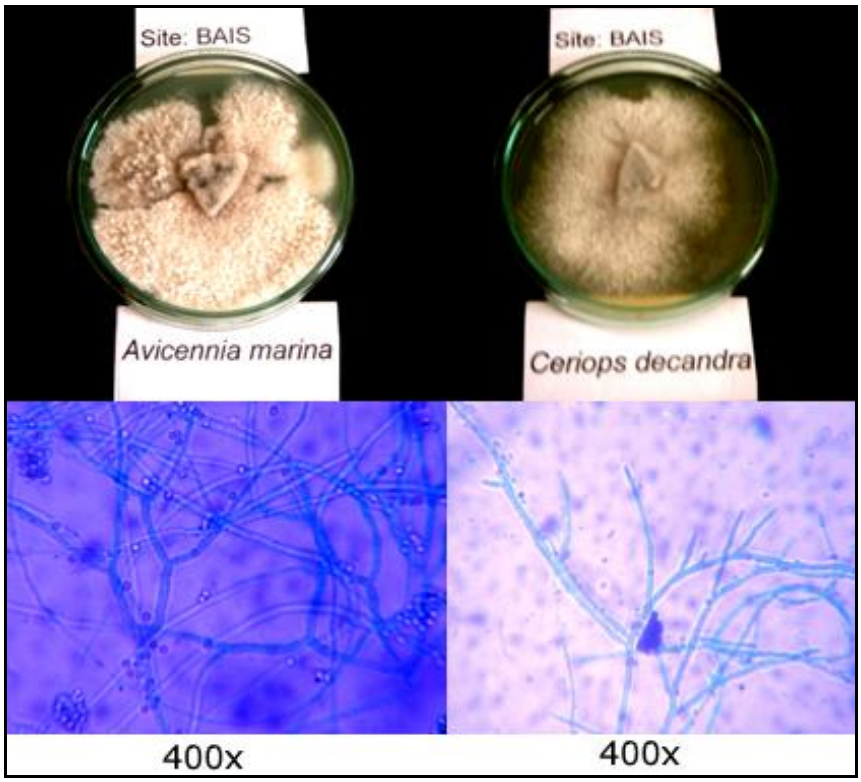

Figure 9. Colonial appearance of *Pseudocercosporella* identified from *Avicennia marina* (upper left) and *Ceriops decandra* (upper right). Conidia and conidiophores (lower left and right).

Overall, all four fungi were identified in five different hosts in this study. This result is consistent with previous studies on fungal host plants. Kohlmeyer & Kohlmeyer (1979) described that individual fungal species are often found on a wide variety of host plants and the host trees present affect the fungal species composition (Hyde & Jones 1988). Some fungi are often more common on some hosts than others when the hosts are near one another (Leong et al 1991; Alias & Jones 2000). In addition, fungal diversity may also vary from one mangrove to the next (Alias & Jones 2000; Alias & Jones 2009).

Conclusions. Based on the findings of the study, there are three foliar diseases identified in both natural mangrove forests and reforested mangrove areas, namely Brown Leaf Spot disease, Black Leaf Spot disease and White Leaf Spot disease. The Brown Leaf Spot disease prevalence was higher in reforested sites compared with natural forests in Bais, whereas in Alcantara and Pangangan Island diseases prevalence was higher in natural forests compared with reforestation sites. Fungi belonging to four genera, *Aspergillus*, *Penicillium*, *Pseudocercosporella* and *Rhizopus* were isolated from infected leaves in both natural forests and reforested areas in the three study sites.

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