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Isolation and Identification of Fungi Associated with Leaf diseases of *Ficus* Tree species

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ABSTRACT

This study provides additional knowledge on the identity of fungal pathogens associated with leaf diseases of *Ficus* species found on the rocky outcrop of Amurum forest reserve with a future hope of developing effective control measures to reduce the impact of fungal pathogens and help ensure the continual survival of *Ficus* species. Disease leaves were collected and were taken to the laboratory for culture, isolation and identification of associated pathogens. A total of 9 fungi viz., *Choanephora cucurbitarum*, *Collectotrichum gloeosporiodes*, *Fusarium oxysporum*, *Fusarium solani*, *Pestalotiopsis diversiseta*, *Pestalotiopsis foedans*, *Pestalotiopsis saprophyta*, *Thermomyces lanuginosus*, and *Trichoderma harzianum* were isolated from diseased leaves. *Thermomyces lanuginosus* had the highest percentage frequency of colonization (23.33%), followed by, *Pestalotiopsis saprophyta* (20.00%), *Pestalotiopsis diversiseta* (18.33%), *Collectotrichum gloeosporiodes* (16.67%), *Choanephora cucurbitarum* (6.67%), *Pestalotiopsis foedans* (5.00%) while the lowest percentage frequency of colonization of 3.33% was recorded for *Fusarium oxysporum*, *Fusarium solani*, and *Trichoderma harzianum*. These pathogenic fungal organisms on *Ficus* species in the reserve may affect the reproductive phenology of figs which is been seen as a very important resource for frugivorous birds in Amurum forest. If *Ficus* tree species are to be properly conserve as key stone species on the rocky outcrop of Amurum Forest Reserve being an important bird area, it is necessary, to put in place mitigation strategy that will monitor and to provide adequate control measures to tackle tree diseases in the reserve so as to fully maintain ecosystem stability within the reserve.

Keywords: *Ficus* species, fungi, leaf spot, rocky outcrop, Amurum forest reserve

Introduction

Ficus, a large genus in the family Moraceae, is composed of approximately 1,000 members and is distributed in tropical and subtropical regions occupying a wide variety of ecological niches; most are evergreen, but some deciduous species are endemic to areas outside of the tropics and to higher elevations (Conservatoire, 2012; SANBI, 2016), of which some have undergone adaptive radiation in different biogeographic regions, leading to very high levels of alpha diversity (PROTA, 2016). In the tropics, it is quite common to find that *Ficus* is the most species-rich plant genus in a particular forest. In Asia as many as 70 or more species can co-exist (Harrison and Shanahan, 2005). The twig has paired stipules or a circular stipule scar if the stipules have fallen off; and the lateral veins at the base of the leaf are steep, forming a tighter angle with the midrib than the other lateral veins, a feature referred to as "tri-veined" (Frodin, 2001; Cooper and Cooper, 2004; Mabberley, 2008). *Ficus* species are characterized by their unique inflorescence and distinctive pollination syndrome, which utilizes tiny, highly specific wasps species belonging to the *Agaonidae* family for pollination (Van Noort, 2004), that enter *via*ostiole of these sub-closed inflorescences to both pollinate and lay their own eggs, has been a constant source of inspiration and wonder to biologists (Rønsted *et al.,* 2008). Their fruits distinguish them from other plants (Coates, 2002; Burrows and Burrows, 2003). The fig fruit is an enclosed inflorescence, sometimes referred to as a syconium, an urn-like structure lined on the inside with the fig's tiny flowers (Arbonnier, 2004). In the rocky outcrop of Amurum forest reserve, *Ficus* species are the most abundant tree species (Elisha and Abiem, 2015) and have been recognised as keystone species that help sustain frugivorous animals during seasons of scarcity as they display inter-specific and intra-specific asynchrony in fruiting (Abalaka, 2009). Several studies have revealed that *Ficus* contribute greatly in the study of the interaction of other flora and fauna species (Elisha and Abiem, 2015). The importance of *Ficus* tree species cannot be over emphasized because of the innumerable economic importance of the tree to humanity, their conservation and protection is of paramount importance in the reserve. However, *Ficus* tree species are attacked by one or more fungi, leading to several diseases particularly in warm and moist conditions such as heavy defoliation; trunk and branch canker (El Atta and Aref, 2013). Although these trees have evolved structural and chemical defences such as thick bark, waxy leaf coatings, root secretions, and anti-microbial toxins that prevent infection (Agrios, 2005). These “pre-formed” defences are always in place and provide general protection from all microorganisms (Agrios, 2005; Casadevall, 2007; Buckley, 2008; Blackwell *et al*., 2009). But certain fungal pathogens have developed virulence factors that enable them to overcome general plant defences (Agrios, 2005; Money, 2007; Loo, 2009) and if not controlled in one way or the other, can have devastating effects on biodiversity and forest structure that is why, there is the apparent need to conserve and protect these trees. Maintaining forest health is a worldwide challenge because the forest is in continual threat due to emerging tree diseases (pests and pathogens), shifts in climate conditions and other global change stressors despite the best biosecurity efforts (Casadevall, 2010; Fisher *et al.,* 2012; Wingfield *et al.*, 2015). Globalisation is compounding this issue, and while there are solutions- including biosecurity, biological control, breeding genetic engineering, environmentally safe chemical control and more- to protect forest, the lack of investment, capacity and coordination of global efforts in researches and proffering of solution into forest diseases are barriers (Wingfield *et al.*, 2015). The aim of this study was to identify fungal pathogens associated with leaf diseases that may lead to the decline of *Ficus* trees in the rocky outcrop habitat of Amurum forest reserve.

MATERIALS AND METHODS

Study Site

The study was carried out in Amurum forest reserve (Fig 1), located in Laminga village, 15 km Northeast of Jos at an altitude of 1,280m above sea level and covering an area of about 300 hectares (Vickery and Jones, 2002). Amurum forest Reserve is a key biodiversity hotspot in West Africa and it is recognized internationally as an Important Bird Area (IBA) in Nigeria with at least 300 bird species such as *Lagonosticta sanguinodorsalis* (Rock Fire finch) and *Vidua maryae* (Jos Plateau Indigo bird) (Ezealor, 2001). Other fauna species include rock hyraxes, bat, rabbits and several species of reptiles (Ibrahim 2002). It comprises of three major habitats: the gallery forest, Dry savannah and rocky outcrops, all of which differ remarkably in floristic composition (Dawang *et al*., 2010). Amurum forest reserve is a vulnerable site of conservation concern because of its small size and proximity to the urban community of Jos. Though the reserve is a protected area, there are still a few sporadic cases of wood cutting and, grazing and setting of fire (Abiem, 2013).

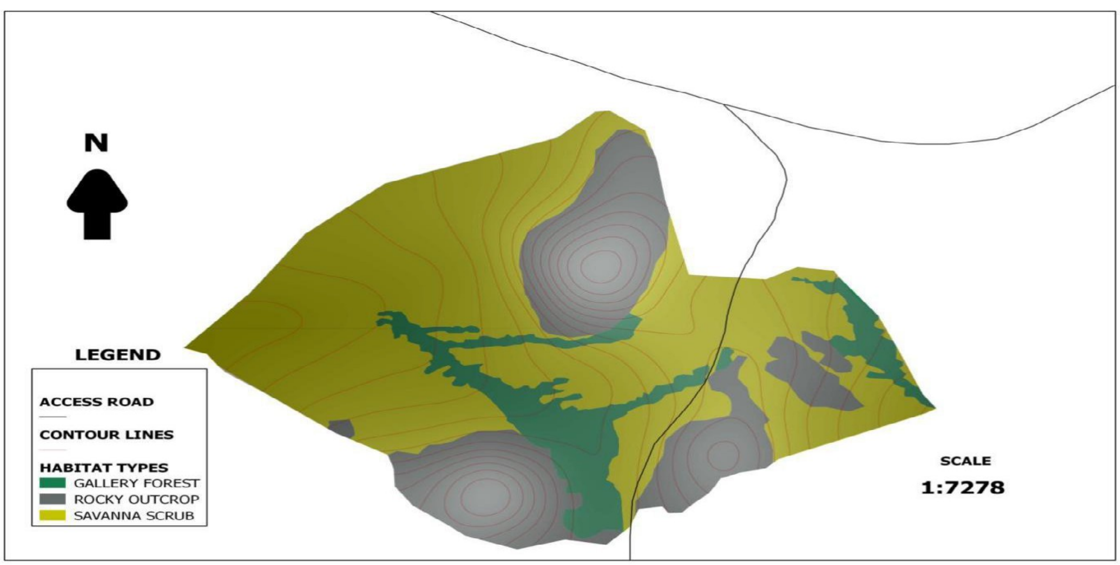

Figure 1: Map of Amurum Forest Reserve showing topography and distribution of major habitat types

Standardized quadrat plots of 20m x 20m in size were established at 30m interval randomly on the rocky outcrops. A total of 10 sample quadrat points in the study area were surveyed. The location of each quadrat point was recorded and established on the rocky outcrops using a Garmin Etrex® Global Positioning System (GPS) device. The mean annual rainfall in the areas surveyed is 1411 mm and mean annual temperatures were between 8^{o}C and 38^{o}C (Table 1).

Ficus trees with symptoms were identified (as seen in Plate 1) and samples of infected leaves were collected in separate sterilized polythene bags and taken to the laboratory for the isolations of the associated pathogens.

Table 1: Description of location surveyed to collect samples used for the identification of fungal pathogen on the Rocky outcrop

Sampling site	Average rainfall (mm/year)	Altitude (m)	GPS Location	
			Latitude (^{o})	Longitude (^{o})
Q001	1411	1350	**N**09.52522	**E**008.58847
Q002	1411	1346	**N**09.52496	**E**008.58831
Q003	1411	1353	**N**09.52422	**E**008.58851
Q004	1411	1350	**N**09.52374	**E**008.58848
Q005	1411	1341	**N**09.52299	**E**008.58160
Q006	1411	1340	**N**09.52334	**E**008.58618
Q007	1411	1322	**N**09.52359	**E**008.58640
Q008	1411	1333	**N**09.52397	**E**008.58544
Q009	1411	1351	**N**09.52617	**E**008.58658
Q010	1411	1341	**N**09.52640	**E**008.58741

Incidence and Severity

Data was collected for disease incidence and severity following formulas illustrated below:

Incidence of disease attack was calculated according to the formula of Mardji (2001) as follows:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plant assessed}} \times 100\%$$

$$\text{Disease severity (\%)} = \frac{\text{Sum of all disease rating}}{\text{Total no.of Rating} \times \text{Maximum Disease Grade}} \times 100\%$$

Assessment of the infection on individual tree species encountered in the rocky outcrop of Amurum forest reserved was based on the symptoms observed. The scoring system of the symptoms (Table 2) was modified from Mardji (2001).

Table 2: Scoring system used to assess disease severity on the individual tree in the forest reserve site

Disease symptom	Severity	Score
Nil (no infection or symptom present)	0	0
Low (number of infected leaves and number of lesions on each leaf are few or little defoliation or chlorosis has occurred or plant looks sound	>1 – 25	1
Medium (number of infected leaves and number of lesions on each leaf are many or much defoliation and chlorosis has occurred	>25 – 50	2
Severe (number of infected leaves and number of lesions on each leaf are abundant or abundant defoliation or chlorosis has occurred	>50 – 75	3
Highly severe (number of infected leaves and number of lesions on each leaf are very extensive or extensive defoliation or chlorosis has occurred	>75 - 100	4
Dead (all leaves are wilted or defoliated)	>100 & Above	5

Source: Mardji (2001)

Isolation and identification of fungal species from diseased leaves

Using a scalpel, a section of unhealthy and healthy tissues was excised from the diseased leaves. The cut portions were surface sterilized by soaking in ethanol (75%, v/v) for 40 seconds, followed by 4 min in hypochlorite (1%, v/v) and subsequently soaking in ethanol (75%, v/v) for 30 seconds again to remove residual hypochlorite, finally rinsed in sterile distilled water three times and blot-dried on clean, sterile paper to remove sterilant. The pieces were transferred with a sterile tweezer, shaking off excess water onto a plate of PDA containing 2% potato dextrose agar and supplemented with streptomycin sulphate at 50 mg/l to prevent bacterial growth. Six pieces of leaf tissues were placed on two Petri plates and incubated in an inverted position at 25°C. After incubation for 3-5 days, the fungal hyphae growing from the diseased leaf tissues were cut off from the edge of the colonies and subcultured to obtain pure culture on a plate of PDA for purification. For storage, the fungal isolates were maintained on PDA slant (Aneja, 2010).

The fungal isolates were identified based on their macro and micro morphological characteristics using keys of Domsh and Grams (1980), Barnett and Hunter (1998) and Sajeewa *et al*. (2012). The following morphological characteristics: colony growth, presence or absence of aerial mycelium, colony colour, presence of wrinkles and furrows and pigment productions were observed. Microscopic examination of fungal isolates was carried out. A clean slide with a drop of lacto phenol cotton blue in its centre was used. A portion of the mycelium at the edge of the colony at the desired stage was picked and dropped in lacto phenol cotton blue. This was teased with the aid of a mounting needle and covered carefully with a cover slip by applying little pressure to eliminate air bubbles. The slide was mounted on the light microscope, focused and observed with x10, x40 and x100 objective lenses respectively.

Pathogenicity of the isolates

To confirm that the pathogens were responsible for the disease, detached healthy leaves removed from each tree sample were inoculated with the fungal culture *in vitro* as described by El-Gali and Zahra (2014). Before inoculation, leaves were surface sterilized by immersion in 0.5% hypochlorite for 2 min, and subsequently soaking in ethanol (75%, v/v) for 15 seconds, then rinsed in sterile distilled water (SDW), and then air-dried in a laminar flow hood and maintained in Petri dish containing sterile distilled water. For each isolate, three fully expanded leaves were inoculated by placing a PDA plug (10 cm^2) of the fungal mycelia on upper surfaces of the leaves, and each leaf was slightly wounded on both sides with a sterile needle prior to inoculation. The Petri dishes were covered and leaves were monitored for the development of disease symptoms and compared with the original symptoms. The pathogen was re-isolated from the leaf after seven days and compared with original culture isolate.

Percentage frequency of colonization

The frequency of fungi from foliar part was estimated using the following formula:

$$\textbf{PFC (\%)} = \frac{\text{Number of leave pieces colonized by a pathogen}}{\text{Total number of leave piece}} \times 100\%$$

RESULTS

Study sites and sampling

In total, observed data from 39 *Ficus* tree species were recorded and five *Ficus* tree species were encountered in the 10 quadrate sampled plots in the rocky outcrop during the survey. They are: *Ficus abutilifolia* (Miq.) Miq., *Ficus cordata* Thunb, *Ficus ingens* (Miq.) Miq, *Ficus mamornata* Bojer ex Baker, and *Ficus umbellata*Vahl.

In this study, symptoms of several diseases were observed on *Ficus* spp. (Plate 1) in the reserve viz.: branch decay, branch splits, chlorosis, stem canker, spots on leaves, and

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leaves wrinkle (Table 3) and the percentage frequency of occurrence of the disease symptoms observed in *Ficus* tree species (Figure 2). Leaf spot has the highest frequency of occurrence with 87.18%, followed by chlorosis (41.03%), branch split (33.33%), stem canker (10.26%) and lowest percentage frequency was observed with branch decay and leave wrinkle with 5.13%.

a). *F. abutilifolia* showing leaf spot

b). *F. abutilifolia* showing canker on trunk

c). *F. abutilifolia* showing leaf spots

d). *F. cordata* showing leaf spot

e). *F. umbellata* showing leaf spot

f). *F. ingens* showingleaf blotch

Plate 1: *Ficus* species showing disease symptoms

Table 3: Summary of the number of Diseased trees base on each type of disease symptoms encountered on *Ficus* species in the rocky outcrop of Amurum Forest Reserve.

Habitat	Species	Total	BD	BS	CH	C	LS	LW
Rocky Outcrop	*Ficus abutilifolia*	15	0	3	9	1	14	0
	Ficus cordata	14	1	8	6	0	11	2
	Ficus ingens	5	0	1	0	1	5	0
	Ficus mamornata	3	0	1	1	2	3	0
	Ficus umbellata	1	1	0	0	0	1	0
	Total	39	2	13	16	4	34	2

Key: **BD** = Branch decay, **BS** = Branch split, **CH** = Chlorosis, **C** = Canker, **LS** = Leaf spot, **LW** = Leaf wrinkle

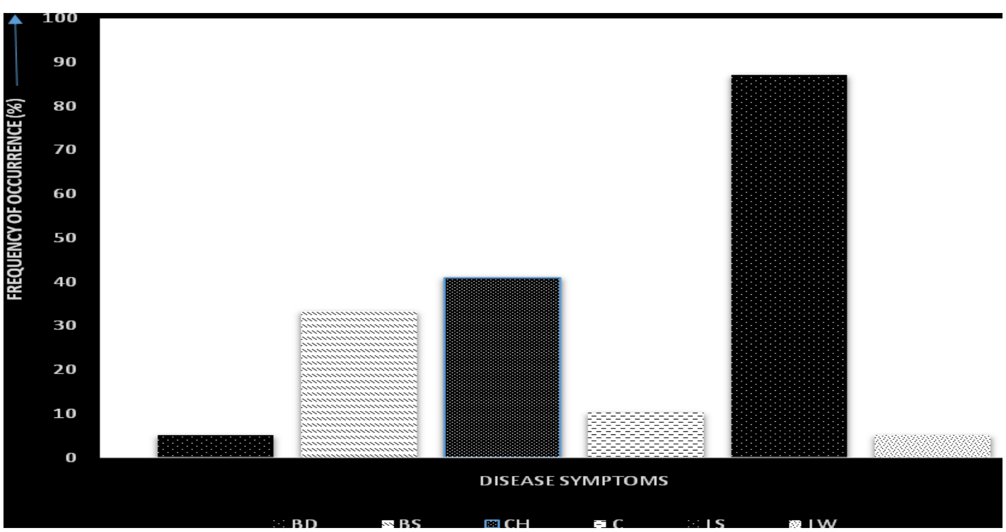

Figure 2: Percentage Frequency of disease symptoms in Amurum forest reserve

Key: BD = Branch decay, **BS** = Branch split, **CH** = Chlorosis, **C** = Canker, **LS** = Leaf spot, **LW**=Leaf wrinkle

Figure 3 shows the disease incidence rates in each of the *Ficus* tree species present in the Rocky habitat types in Amurum Forest Reserve.*Ficus abutilifolia*had the highest rate of disease incidence with 38.46%, followed by *Ficus cordata*with 35.90%, *Ficus ingens* with 12.82%, *Ficus mamornata* with 7.69%, while*Ficus umbellata* had the lowest rate of disease incidence with 2.56%.

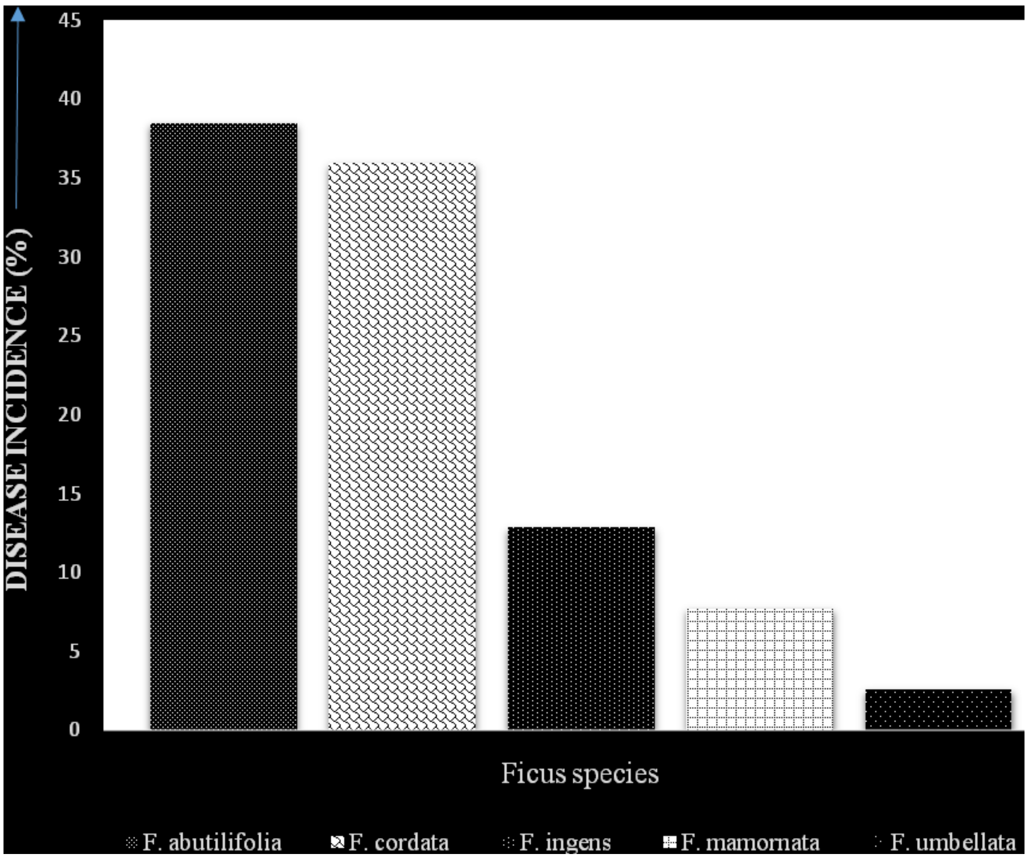

Figure 3: Incidence of disease in the different *Ficus* species encountered in the rocky outcrop of Amurum forest Reserve

The level of damage of diseased trees was generally medium (Table 4) on the disease severity scale. *Ficus abutilifolia* recorded highly severe on the disease severity scale while *Ficus cordata* recorded severe on the disease severity scale. *Ficus ingens* and *Ficus umbellate* recorded medium on the disease severity scale. *Ficus mamornata* recorded low on the disease severity scale.

The severity of disease in *Ficus mamornata* was lowest with 20%, while the highest rate of disease severity was recorded for *Ficus abutilifolia* (98.67%), followed by *Ficus cordata* (57.14%), *Ficus umbellata* (40%) and *Ficus ingens* (28%) as seen in Table 4

Table 4: Severity of disease on *Ficus* species in the rocky outcrop habitat of Amurum forest Reserve

Habitat	Tree species	Healthy trees	Diseased Trees					Severity (%)	Level of Damage
			Low	Medium	Severe	Highly severe	Dead		
Rocky Outcrop	*Ficus abutilifolia*	0	0	0	1	14	0	98.67	Highly Severe
	Ficus cordata	0	1	7	5	1	0	57.14	Severe
	Ficus ingens	0	3	2	0	0	0	28.00	Medium
	Ficus mamornata	1	3	0	0	0	0	20.00	Low
	Ficus umbellata	0	0	1	0	0	0	40.00	Medium

The details of fungi isolated from diseased leaves of each tree species and their percentage frequency of colonization, are indicated on Tables 5 and 6. A total of nine (9) fungi species were isolated from the five *Ficus* tree species encountered during the survey. The fungal isolates are: *Choanephora cucurbitarum* (Berk. & Ravenel) Thaxt., *Collectotrichum gloeosporiodes* (Penz.) Penz. & Sacc., *Fusarium oxysporum* Schlecht, *Fusarium solani* (Mart.) Sacc., *Pestalotiopsis diversiseta* Maharachch. & K.D. Hyde, *Pestalotiopsis foedans* (Sacc. & Ellis) Steyaert, *Pestalotiopsis saprophyta* Maharachch.& K.D. Hyde, *Thermomyces lanuginosus* Tsikl., and *Trichoderma harzianum* Rifai

They were made up of the following fungal classes; Ascomycetes (*Collectotrichum gloeosporiodes* (Penz.) Penz. & Sacc., *Fusarium oxysporum* Schlecht, *Fusarium solani* (Mart.) Sacc., *Thermomyces lanuginosus* Tsikl, *Trichoderma harzianum* Rifai), Deuteromycetes (*Pestalotiopsis diversiseta* Maharachch.& K.D. Hyde, *Pestalotiopsis foedans* (Sacc. & Ellis) Steyaert, *Pestalotiopsis saprophyta* Maharachch.& K.D. Hyde) and Zygomycetes (*Choanephora cucurbitarum* (Berk. & Ravenel) Thaxt).

Thermomyces lanuginosus had the highest percentage frequency of colonization (23.33%), followed by, *Pestalotiopsis saprophyta* (20.00%), *Pestalotiopsis diversiseta* (18.33%), *Collectotrichum gloeosporiodes* (16.67%), *Choanephora cucurbitarum*(6.67%), *Pestalotiopsis foedans* (5.00%) while the lowest percentage frequency of colonization of 3.33% was recorded for *Fusarium oxysporum*, *Fusarium solani* and *Trichoderma harzianum*.

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Table 5: Fungal species associated with each *Ficus* tree species on the rocky outcrop of Amurum forest reserve

TREE SPECIES	FUNGAL SPECIES								
	Choanephora cucurbitarum	***Collectotrichum gloeosporiodes***	***Fusarium oxysporum***	***Fusarium solani***	***Pestalotiopsis diversiseta***	***Pestalotiopsis foedans***	***Pestalotiopsis saprophyta***	***Thermomyces lanuginosus***	***Trichoderma harzianum***
Ficus abutilifolia	-	+	+	+	+	-	-	+	+
Ficus cordata	-	+	-	-	+	-	-	-	+
Ficus ingens	+	-	+	+	-	+	-	+	-
Ficus mamornata	-	-	-	-	-	-	+	+	-
Ficus umbellata	-	+	-	-	-	-	+	+	-

Key -= Absent
+= Present

Table 6: Frequencies (%) of fungi isolated from disease leaves of *Ficus* species

Fungal	Frequency of Colonization (%)
Choanephora cucurbitarum	6.67
Collectotrichum gloeosporiodes	16.67
Fusarium oxysporum	3.33
Fusarium solani	3.33
Pestalotiopsis diversiseta	18.33
Pestalotiopsis foedans	5.00
Pestalotiopsis saprophyta	20.00
Thermomyces lanuginosus	23.33
Trichoderma harzianum	3.33

DISCUSSION

This study provides additional knowledge on the identity of fungal pathogens associated with leaf disease of *Ficus* species in the Rocky outcrops habitat of Amurum forest reserve with a futurehope of developing management strategies to reduce the impact of disease and help ensure the continual survival of *Ficus* species as key stone species in the reserve.

The majority of pathogens identified in this study represent first records for Amurum forest reserve. During our investigation, Leaf spot disease was the most prominent and common disease found occurring on *Ficus* tree species encountered during the survey in the rocky outcrop habitat of the reserve. These spots developed as small, scattered, circular to oval dead areas in the leaves.The disease was wide spread because of free water on leaf surface of the tree. This agrees with Agrios (2005), who stated many pathogens, especially foliar pathogens, need a film of water on the plant to begin growth, penetrate the host, and establish infection. This is why wet weather is so important in the development of many fungal diseases. Stovold (2004) similarly stated that wet conditions favour most diseases especially leave diseases. Morphologicalidentification of the fungal pathogens isolated from foliar part of *Ficus* tree species revealed the causal agents of leaf spot to be*Choanephora cucurbitarum* (Berk. & Ravenel) Thaxt., *Collectotrichum gloeosporiodes* (Penz.) Penz. & Sacc., *Fusarium oxysporum* Schlecht, *Fusarium solani* (Mart.) Sacc., *Pestalotiopsis diversiseta* Maharachch. & K.D. Hyde, *Pestalotiopsis foedans* (Sacc. & Ellis) Steyaert, and *Pestalotiopsis* saprophyta. These pathogens have also been recorded by Rajput *et al*., (2010), Sajeewa *et al*. (2012) and Mane (2012) as fungal pathogens affecting foresttree species, which points to the fact that, it is important to keep surveillance of tree disease as these fungal pathogens can easily cause diseases or death in similar or other tree species.

Most of the fungi found associated with the leave disease of *Ficus* trees belong to the fungi group Ascomycetes. Ascomycetes have been recorded to cause epidemics to forest trees of which the Chestnut blight was the first of such epidemics, which virtually eliminated the most common tree species in the eastern U.S., the American chestnut, from its natural range in less than 40 years (Loo, 2009), so also the impact of Dutch elm disease which extended well beyond the death of 100 million mature elm trees in the middle of the 20th century (Money, 2007), followed by other tree canker diseases incited by the group Ascomycetes.

A majority of the genera isolated are pathogens of agricultural crops: *Choanephora*, *Collectotrichum*, *Fusarium*, *Pestalotiopsis*. The genera *Trichoderma* is a saprophytic fungi and also an opportunistic, avirulent plant symbionts as well as being parasites of other fungi as stated by Gary *et al*. (2004). *Thermomyces lanuginosus* have been identified as a major phylloplane fungus (Hudson, 1978).

CONCLUSION AND RECOMMENDATION

These pathogenic fungal organismson *Ficus* species in the reserve can affect the reproductive phenology of figs which is been seen as a very important resource for frugivorous birds in Amurum forest. If *Ficus* tree species are to be properly conserve as key stone species on the rocky outcrop of Amurum Forest Reserve being an Important Bird Area, it is necessary, to put in place mitigation strategy that will monitor and provide adequate control measures to tackle treediseases in the reserve so as to fully maintain ecosystem stability within the reserve.It is therefore recommended that Eco-friendly control measures should be employed within the reserve to reduce the inoculum level of these pathogenic fungalorganism.

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