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A case study on fungal leaf spot diseases of orange plants

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ABSTRACT

The incidence, effect, and causal organism of fungal leaf spot diseases of orange tree (Citrus spp.) in Southeastern Nigeria were investigated. Healthy and diseased samples of orange leaves were obtained from Aba, Amagunze, Awka, Nkpor , and Onitsha all in Southeastern Nigeria. Sabouraud dextrose agar (SDA) was used for isolation of fungi. Ten trees with disease symptoms were sampled at each location using visual assessment method and their percentage frequency calculated. The occurrence of fungal leaf spot was least at Nkpor (30%) and highest at Aba (43%). The fungi identified were *Aspergillus niger*, *Fusarium solani*, *F. oxysporium*, *Botryodiplodia theobromae*, and *Geotrichium spp.* The *F. solani* occurred at highest percentage of 37.5% incidence while the lowest was *B. theobromae* at 6.25%. The pathogenicity test revealed that the major cause fungal leaf spot disease of orange was *B. theobromae*, *Fusarium solani*, and *F. oxysporium*. Although, leaf spot disease is a minor economic problem that reduces crop yield, a number of control measures need to be adopted.

Introduction

Orange tree (*Citrus sinensis* Osbek) is a tropical plant, which belongs to the family of Rutacaea. *Citrus species* are evergreen trees of small to medium stature. Some of them prick which indicate the presence of thorn on the branches while some are thornlesss especially the budded species[15] . They contain about 100 genera and nearly 150 species are known worldwide. The tree occupies the same position in the tropics as the apple in temperate America and Europe (Sing, 1960). The cultivars of orange mostly found in Nigeria include: sour lime (*Citrus aurantofolia* Christm) Swingle, bitter orange (*C. aurantium* L.), lemon (*C.* limon L.) Burm f., grape (*C. paradisi* Macf), and sweet orange (*C. sinensis* Osbek), Okigbo and Emoghene (2000).

They are grown principally for the juice of their fruits. After extraction of the juice, the pulp can then be used for feeding livestock.

However, for many years ago, the attention of many mycologists have been attracted by the microorganism associated with fungal leaf spot (Schmidt *et al.*, 1997). Many diseases such as black spot, brown spot, usually attack orange trees and shot hole caused by *Heterosporium iridis*. Moreover, it is reported that the agent of dieback and back canker was caused by *Botryodiplodia theobromae* and the gray blight leaves was caused by *Pestalotiopsis mangiferae* (Verma *et al.*, 1991). The presence of endospores (picnidia) in *Botryodiplodia theobromae* make it possible for it to persist on the surface of orange leaf and this is mostly found in the hot tropics of Africa, example, Nigeria (Asaka and Shoda, 1996). The leaf spot disease of orange, deviation from the normal growth and development of leaves are caused mostly by fungi (Chuang and Ann, 1997). Plants affected by this fungal leaf spot are estimated to number more than 25, 000; the resulting estimated annual loses to farmers are enormous worldwide (Kais, 1975). In addition, the site of infection of the host plant shows some reactions, which are visible around the areas of infection and these areas, are defined and visible as disease, which are discoloured patches or spots on the leaf.

The effective disease control measure requires a fundamental knowledge of the causes of disease (spot), symptoms on host plant, and their application to ensure successful control. These can be controlled by cultural control, like burning of all diseased leaves and irrigation of the plant early in the day so that foliage will dry before nightfall (McManus, 1999). It can also be controlled by the use of chemical control, for example, Daconil 2787. According to Ellis (200), the use of chemicals as control measure of the plant pathogens especially foliage pathogen has had only limited success in the past in Africa due to inadequate methods of application, lack of an effective chemicals and cost (Okigbo and Osuinde, 2003). The concern of the development of resistance by the pathogens and also about the chemical residue in the environment has been incorporated (Spotts and Cervantes, 1986; Osuinde *et al*., 2001).

Several workers are currently doing work on the biological control of fungal leaf spot disease (Okigbo, 2001). Presently, the attention of mycologists is focused on the control measures of orange leaf spot (Okigbo and Emoghene, 2000). In addition, the use of biological control of plant pathogen could minimize some of those concerns of development of resistance by the pathogens. The control is very cheap and more durable (Osuinde *et al.*, 2001). Also, reports have been focused on the increasing number of *Bacillus subtilis* as a biological control agent (Ferreira *et al.*, 1991; Ikediugwu *et al.*, 1994; Okigbo, 2001). Another area of important in the control of fungal leaf spot disease is the ability to survey, identify and quantify diseases within a plant population, and how to set up an economic disease control measures.

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The objectives of this research include:

i. To evaluate location where fungal leaf spot disease of *Citrus sinensis* Osbek has high percentage frequency of occurrence.
ii. To study the effect of fungal leaf spot on orange leaves.
iii. Determine the extent of damage done to orange leaves by fungal isolates.
iv. Evaluate the frequency of occurrence of different fungi isolates.
v. Isolate and identify fungi that are associated with orange leaf spot.

Materials and Methods

Sources of materials

Leaves, stems, and branches of *Citrus sinensis* used in this work were obtained from orchards located in various areas like Aba, Amagunze, Awka, Nkpor, and Onitsha all in Southeastern Nigeria. The orange tree was identified using the manual of Keay (*et al.*, 1964). Samples were inspected for any visible sign of fungal leaf spot disease.

Survey and identification

The survey and identification were carried out to evaluate the frequency of fungal leaf spot diseases of orange tree. At each location, ten (10) trees were randomly sampled. Also, the frequency of occurrence of leaf spots was recorded and the number of orange trees affected by the leaf spot was expressed as percentage of the total number of orange plants at each location. However, visual assessment method was applied with which a lot of plantations can be evaluated in a very short time (Derso, 1999).

In comparing the mean occurrence of the disease at each location, analysis of variance was used. Stems, leaves, and branches of orange plant affected by fungal leaf spot diseases were collected and brought to the laboratory for identification of the pathogen that causes leaf spot disease.

The percentage frequency of occurrence of fungal leaf spot diseases was conducted at each location to find out the number of times the leaf spots occurred and the total number of plants assessed was recorded (Derso, 1999).

This was worked out as follows:

$$\text{Percentage frequency of occurrence} = \frac{C}{P} \times \frac{100}{1}$$

Where C = Total number of leaves infected by spots.

Total number of plants assessed.

Sterilization of the orange tree sample

The leaves infected with fungal spots were cut using sterilized knife through the advance margin. These were dipped into 70% ethanol solution for 1 – 2 minutes and rinsed in sterile water. Each sample was removed using sterile forceps and allowed to dry before use.

Preparation of media

Sixty-two grammes (62g) of dehydrated sabouraud dextrose agar (SDA, Biotech, Uk) were dispersed into one litre (1L) of water in McCartney bottle to form suspension. They were mixed and allowed to stay for 5 – 10 minutes. The suspension was sterilized using autoclave at 121^{o}C for 15 minutes. This was brought out and allowed to cool at 57^{o}C before antibiotics (chloramphenicle, Beecham) containing 0.5mg was added to inhibit bacteria growth. The media were then poured into sterile Petri dishes, which after few minutes gel.

Isolation and identification of fungi

One millimetre (1mm) by 1mmof the infected leaves of orange plants were cut with a sterile knife and theirs surfaces were washed by dipping them into 70% ethanol solution for 1 – 2 minutes. With sterile forceps, these leaves tissues were collected and placed onto SDA in Petri dishes. These were incubated at a room temperature of 27± 2^{o}C for 4 to 5 days to enhance fungal growth and sporulation. The cultures were under a check through out the incubation period until changes were observed on day 4 but these changes were more conspicuous on day 5. The features of each fungi isolates were carefully observed and recorded. In addition, wet mount of each isolates were prepared on a microscope slide, covered with microscopic cover and stained with lactophenol cotton blue dye. This was then mounted on the microscope and isolates were identified (Barnett and Hunter, 1972).

Pathogenicity test

The infected leaves of orange tree used for this test were obtained from different locations such as Aba, Amagunze, Awka, Nkpor, and Onitsha all in Southeastern Nigeria. Using a sterile knife, 1mm slices of infected leaf tissues were cut and dipped (washed) into 70% ethanol solution for 1 to 2 mniutes. The slices were removed with sterile forceps and rinsed in sterile water. Thereafter, the slices were then placed on the Petri dishes containing SDA and were incubated at room temperature (27± 2^{o}C) for 4 to 5 days to enhance fungal growth and sporulation. Isolated fungi were identified with the manuals of Barnett and Hunter (1972).

Determination of percentage frequency of isolates

This was done by thorough observation of each fungal isolates. The frequency of occurrence of each isolates was kept on periodic basis. The identification of the isolates was done using the method of Barnett and Hunter (1972). However, the number of times each fungus was isolated in each week was expressed as a percentage of the total period (Derso, 1999; Okigbo, 2001). This was calculated as follows:

$$\text{Percentage frequency of occurrence} = \frac{A}{N} \times \frac{100}{1}$$

Where A = Number of times of occurrence of each isolates.

N = Total number of week in a month

Design

The experimental design used was completely randomised design, with three (3) treatments and five (5) replicates. Analysis of variance was used to determine the work (Cyprain, 199).

Results

Sources of materials

The leaves, stems, and branches of *Citrus spp* were obtained from orchards located in various areas like Aba, Amagunze, Awka, Nkpor, and Onitsha all in Southeastern Nigeria. They all have visible signs of fungal leaf spot diseases. Those with visible signs of dead necrotic tissues were isolated and identified.

Survey and identification

There was leaf spot disease in all the locations studied. The survey and identification showed that the incidence of fungal leaf spot diseases were lowest at Nkpor (9 leaves infected with spots) and highest at Aba (13 leaves infected with spots) in ascending order.

However, both younger plants and older ones were surveyed and identified with a branch brought to the laboratory for critical identification and isolation. In each location, the number of plants assessed were properly recorded together with the number of leaves infected by fungal leaf spot and their percentage frequency of occurrence were determined (Table1). The occurrence of the fungal leaf spot diseases was lowest at

Nkpor (30%) and more abundant at Aba (50%) in ascending order.

The mean leaf spot diseases of orange trees assessed in different locations (Aba, Amagunze, Awka, Nkpor, and Onitsha) in Southeastern Nigeria were equally determined (Table 2). The sampling time was conducted for four (4) weeks. In each week, the number of leaves infected by leaf spot diseases at each location was recorded. In the first week, the number of leaves infected with spot diseases occurred highest at Aba with 5 leaves infected. In the second week of experiment, Aba and Awka had 4 leaves infected by spots; Amagunze had 3 leaves infected while Nkpor and Onitsha recorded 2 leaves each infected with spots. In the third week, Onitsha had the number of leaf spots with 4 leaves infected, Amagunze had 3 leaves infected, Aba and Nkpor also had 2 leaves each infected whereas at Awka one leaf was infected by fungal leaf spot disease. By the fourth week, the incidence of fungal leaf spot diseases occurred highest at Awka with 4 leaves infected by spots, Onitsha had 3 leaves infected, both Aba and Amagunze had 2 leaves each infected while the lowest of the spots was found at Nkpor with one (1) leaf infected by spots.

Isolation and identification of fungi

The fungi isolated from diseased leaves of *Citrus sinensis* were identified. These included: *Aspergillu niger, Fusarium solani, Fusarium oxysporium, Botryodiplodia theobromae*, and *Geotrichium spp*. *Fusarium spp* occurred more abundant than the fungi isolates in the study. The least ones were *B. theobromae* (Table 3). *Fusarium solani* occurred at 31.25% and *Geotrichium spp* occurred at 25%.

Also, tests were conducted to determine the fungi occurring at each plant location and the frequency of occurrence (%) (Table 4). Three (3) fungi *Geotrichium spp, B. theobromae*, and *Fusarium spp* were isolated at Onitsha. Two (2) fungi each were isolated at Aba, Amagunze, and Nkpor. The *B. theobromae* isolated at Awka were able to produce picnidium after 10 days of continues incubation of the Petri dish under florescent light.

Pathogenicity test

The following fungi were isolated during the pathogenicity test from the diseased leaves of orange tree: *Botryodiplodia theobromae, Fusarium oxysporium* and *F. solani. B. theobromae* occurred at diameter of 4.5 while *F. solani* and *F. oxysporium* occurred at diameter of 1 cm (Table 5)

Discussion

The visible symptoms of leaf spot diseases observed in this work were attributed to be the major cause of diseases that affect photosynthesis in many economic plants such as orange, mango, and strawberry (Okigbo and Osuinde, 2003; Ellis, 2006). The fungal leaf spot diseases have been found to occur as first unfold at the early rain and this continues until the leaves become old. The symptoms observed could be compared with that of McManus (1999) in a study on leaf spot diseases of cranberry in which he observed that the spots scattered around the leaf showing different colours. Also, Erik (2000) in the study of gray leaf spot disease of corn observed similar symptoms to the one in this study. These confirm our work on the symptoms of fungal leaf diseases. Ellis (2006) observed that the fungus *Mycosphaerella fragariae* caused the fungal leaf spot disease of strawberry. He observed that the symptoms of leaf spot first appear on the upper surface as circular, deep purple spots on the leaf surface. These spots enlarge and the centres turn grayish to white on older leaves and light brown on young leaves. These were inline with our observation on orange spots. In this our work, it was observed that definite reddish purple to rusty brown border surrounds the spots. McManus (1999) noted that leaf spots first appear as dark to purple blotches on the upper surface of the current year leaf and by the following year, the spots enlarge and fade to yellow.

This study also shows that there are leaf spot diseases in all the locations. All these locations are in Southeastern Nigeria. According to Okigbo and Osuinde (2003), they observed the presence of fungal leaf diseases of mango in different locations in Nigeria. Also, another worker has found the leaf spot diseases in Virginia (Hansen, 2000). There is variation in occurrence of this leaf spot disease. The least occurrence of this leaf spot disease at Nkpor might be probably due to urban nature of Nkpor and the awareness created in that area among orchard owners. Similarly, the highest frequency occurrence of leaf spot disease observed at Aba might be due to the area being the orange cultivating centre in Southeaster Nigeria and also the presence of one of the largest markets (Ariaria) in Nigeria which makes it that oranges are brought from different locations into Aba town.

This work also showed that younger plants had fewer symptoms of fungal leaf spot disease than the older plants. This was in line with Derso (1999) on *Phaeoramularia* leaf and fruit spot disease of citrus inwhcih he observed that the older plants were more affected by fungal leaf spot diseases. Also, Okigbo (2001) observed it in leaf spot disease of mango caused by *Macrophoma mangiferae*. Ellis (2006) also reported that there were differentiations between older and young leaves infected by leaf spot while the leaf spot disease on the younger leaves were dark brown, the older leaves were yellow. This colour differentiation was also observed in this our work, whereas the leaf spot disease of younger leaves were dark brown, the older leaves were yellowish. This showed that the leaf spot disease affects many other plants but have different casual organisms. Ebo and Okoh (1980) equally observed 40% of occurrence of fungi encountered on the study of fungi associated with some vegetable leaves.

The percentage of *A. niger* recorded in all the orange leaf with spot diseases sampled in this work was 31.25%. This high percentage observed in this work might have been due to ubiquitous nature of *A. niger* and also its presence as a contaminant in the laboratory. In the study of Ebo and Okoh (1980), they also observed high occurrence of *A. niger* in some of vegetable leaves in Nigeria. The presence of *B. theobromae* and *A. niger* were among the fungi disease isolated by Okigbo and Osuinde (2003) as a cause of fungal leaf spot disease of mango in Southeastern Nigeria. However, in Alfalfa, the prominent fungi that cause fungal leaf spot disease did not include *A. niger* and *B. theobromae*, rather, they were *Peronospora trifoliorum, Phoma medicaginis*, and *Leptosphaerulina briosiana* (Schwartz et al., 2006). The result indicated that the cause of fungal leaf disease was specific for a particular plant. The presence of *R. stolonifer* and *Mucor spp* as laboratory contaminants and also in the leaf was an indication that these were ordinary saprophytes. They were virulent in the pathogenicity test carried out. The orange tree being exposed in the fields were conducive for the deposit of spores, both the ones that were pathogenic and ones that were saprophytic.

From this work, it has shown that fungi cause leaf spot diseases and this leaf spot causes leaf to fall prematurely. And these according to Schwartz *et al*., (2006) were green factories that produce food for the plants. The repeated defoliation of leaves makes them weaker. It was also observed that most of the

fungi that cause the diseases require a well surface for excellent time.

The leaf spot disease was worldwide as far as plants were concerned but they were usually insignificant (McManus, 1999). In this study, 43% frequency of occurrence of fungal leaf spot was obtained as the highest. Ellis (2006) noted that these leaf spot diseases did not cause economic damage. The primary damage of leaf disease was a loss of plant vigour through reduced area. If the average of this becomes significant, the plant will become weaken resulting in increase susceptibility to disease. Also, *Nameth et al.*, (2002) while working leaf spot diseases on ornamental trees and shrubs also believed that the leaf spot disease was a minor problem. This inline with the 43 incidence observed in this work.

Leaf spot diseases are not significant enough to justify the use of fungicide spray. However, McManus (1999) observed that if the disease cannot be brought under control by modified cultural practices, then, judicious fungicide use might prevent economic loss.

Conclusion/Recommendation

Although leaf spot disease is a minor problem that reduces crop yield, a number of control measures need to be adopted. These include:

(1) Modifying cultural practices is a key to managing leaf spot disease.

(a) Removing and burying of diseased leaves in the fall will greatly reduce the fungal inoculums available for infection and will frequently provide sufficient disease control.

(b) Manage the fertility levels. This is very important because excessive nitrogen promotes lush, succulent tissue that is prone to leaf spot disease.

(c) Water the nursery early in the day so that the leaf will dry before the nightfall.

Chemical control

Under severe disease condition, a fungicide spray programme is recommended Hansen (2000). Usually 4 to 6 spray of fungicide containing chlorothanlonil (e.g. Daconil 2787), mancozeb (e.g. Fore, Dithane) will control the disease (Hansen, 2000).

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Table 1: The number of plants and leaves affected by fungal leaf spot diseases and their frequency of occurrence (%) in Southeastern Nigeria

Location	No. of plants assessed	% of frequency of occurrence
Nkpor	30	30.00
Amagunze	30	33.33
Awka	30	36.00
Onitsha	30	40.00
Aba	30	43.00

Table 2: The mean leaf spots of orange tree assessed in different locations in Southeastern Nigeria

Sampling Time	Locations and number of leaf affected by leaf spots				
Weeks	Aba	Amagunze	Awka	Nkpor	Onitsha
1	5	2	2	4	3
2	4	3	4	2	2
3	2	3	1	2	4
4	2	2	4	1	3

Table 3: Frequency of occurrence (%) of fungal isolates

Fungi	Frequency of occurrence (%)
Aspergillus niger	31.25
Fusarium solani	37.50
Fusarium oxysporium	9.38
Botryodiplodia theobromae	6.25
Geotrichium spp	15.63

Table 4: Fungi occurring at each plant location and the frequency of occurrence (%)

Plant location	Fungi isolated	% of frequency of occurrence
Aba	*Aspergillus niger*	40.00
	Geotrichium spp	40.00
Amagunze	*Aspergillus niger*	60.00
	Fusarium solani	33.33
Awka	*Btryodiplodia theobromae*	50.00
Nkpor	*Fusarium solani*	66.67
	Fusarium oxysporium	83.33
Onitsha	*Geotrichium spp*	60.00
	Botryodiplodia theobromae	50.00
	Fusarium oxysporium	16.67

Table 5: Pathogenicity test for some the isolated fungi from *Citrus spp*.

Fungi isolated	Diameter growth in culture
Botryodiplodia theobromae	4.5cm
Fusarium solani	1cm
Fusarium oxysporium	1cm