Research & Reviews: Journal of Agriculture and Allied Sciences

e-ISSN:2347-226X
p-ISSN:2319-9857

Endemic Disease of Cultivated Tomato and Microbial Screening for Infectious Agents in Ibadan, Nigeria

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Research Article

Received date: 22/02/2019
Accepted date: 11/03/2019
Published date: 20/03/2019

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Keywords: Necrosis, Blight, Diagnosis, Lesions, Mycelia, Pathogenicity

ABSTRACT

A sporadic disease outbreak was reported in a commercial farm in Ibadan, Nigeria in 2011 and investigation was carried out by a team of pathologists. Disease diagnosis and pathological screening were carried out in the field, and it turned out that the tomato plants suffered multiple infections which were instigated by diverse strains of fungi.

Standard laboratory techniques were employed in the characterization of the isolates. Pathogenicity and severity tests were also conducted (*in vitro* and *in vivo*). It was observed that *Rhizopus stolonifer*, *Aspergillus fumigatus*, and *A. sclerotirium* each caused 60% infection. Symptoms observed include leaf spots, blight of lower leaves and severe necrosis of foliages, wilting and formation of whitish mycelia mass around the root, and in extreme cases death of the entire tomato shoot. Also, fruit rots and lesions were observed on both green (unripe) and ripe fruits. A concerted disease management strategy was imminent and imperative.

INTRODUCTION

A spontaneous disease outbreak was reported in a commercial tomato field in Ibadan, Nigeria in 2011, an investigation was conducted to determine the cause of the sporadic disease outbreak. Several varieties of tomatoes cultivated in the commercial vegetable field were affected at the immature (green fruits) and matured (ripe fruits) stages. Fruit lesions, leaf necrosis and death of entire tomato plants were observed in the field. Visible mycelia growths were spotted around the root rhizosphere of some infected tomato plants suggesting the role of fungi species in the development of the disease. The infected tomato field measured about 100,000 square meters in perimeter. It was located in Apèté, a suburban community in Ibadan, Nigeria.

Tomato (*Lycopersicon esculentum Mill. Synonym Solanum Lycopersicon*) is a rich source of vitamin A (Retinol: required for good eye sight and a healthy skin), vitamin C (Ascorbic Acid- required to boost the body's metabolic functions and other enzymatic reactions), Lycopene, and potassium [1]; has come a long way, not only in distance from its point of origin, but also in terms of varietal improvement, disease resilience, enhanced storage qualities and processing techniques; today, numerous transgenic varieties and genetically modified hybrids and cybrids are cultivated in all regions of the world [2].

Such versatility coupled with a growing demand for the fresh fruits, has provided an inclination in terms of development of tomato as one of the main crops of the century [2]. The high rising demand for fresh tomato fruits is threatened majorly by field losses of tomato to pathogens [3], and inadequate storage facilities for fresh fruits, based on their highly perishable nature [4]. Majority of the diagnosed cases of disease outbreak on tomato crops results from the physiological activities of some soil borne and airborne fungi [3]; few bacteria and some tomato fruit worms like *Heliothis armigera*, which causes leaf and shoot damage in juvenile tomato plants [2].

Michael et al. [3] reported that a vast array of fungal strains can infect tomato plants and fruits on the field, culminating in postharvest losses. Alippi et al. [5] and Saygili et al. [6] also reported a sporadic and spontaneous spread of bacterial disease of cultivated tomato in Argentina known as "T*omato Pith Necrosis*" (caused by the soil borne pathogen "*Pseudomonas corrugata*") resulting in massive destruction of tomato plants and a huge financial loss to the nation's economy. Kwon and Hong [4], recorded a catastrophic outbreak of soft rot disease of tomato fruits in Jinju market, caused by the fungus "*Mucor racemosus*", which also resulted in massive economic and financial losses.

e-ISSN:2347-226X
p-ISSN:2319-9857

Therefore, this research was conducted to determine the causal agents of the spontaneous disease eruption in the commercial vegetable field, the possible mode of ingress and infection of the tomato plants, the source of overwintering of the infectious agents of the pathogens and a possible control for the disease. This will go a long way in the fight against food spoilage and food shortage in Africa and the world at large.

MATERIAL AND METHODS

Farm description

The commercial tomato farm was located in Apète village, in the suburbs of Ibadan, Ibadan, Nigeria. The vegetation of the farm is mixed cropped with economically important trees (Palm trees, mango, and Pawpaw), vegetables (Tomato, Pepper, and other leafy vegetables), and other plants like Cassava, Pineapple, and Maize plants were also cultivated in the farmland. The farm is situated in the tropical rainforest region of the Nigerian vegetation belt, and as such it enjoys optimal climatic conditions pertinent for good agricultural activities.

GPS coordinates

The commercial vegetable farm is located on the world map at Latitude: 07°26'57" N and Longitude: 03°52'20" E, respectively. The coordinates of the farm was determined using a satellite global positioning system (GPS) tracker as shown in **Figure 1**.

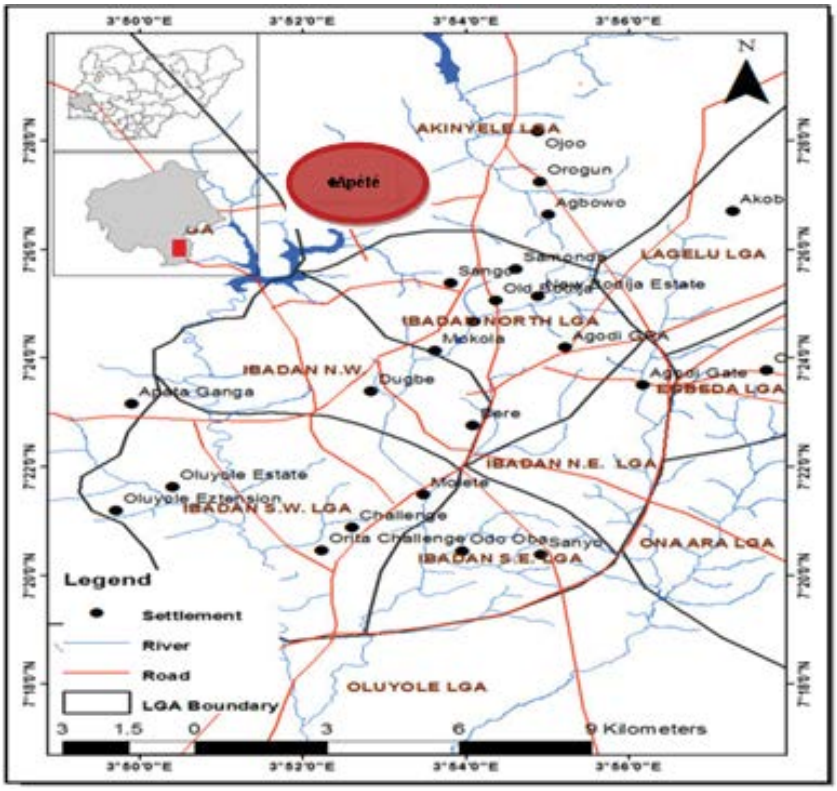

Figure 1. A space map of the farm location with GPS coordinates.

The weather conditions of the vegetable farm

The commercial vegetable farmland located in Apète, Ibadan; Oyo State, Nigeria enjoys annual ambient rainfall ranging from 788 mm to 1884 mm with mean annual average temperature of about 26.6°C and a constantly high relative humidity with annual average greater than 80%.

Field observation and disease screening

Several cultivars/varieties of tomato were screened manually on the field (using a standardized diagnostic procedure) for the presence of disease. Infected shoot, fruit and soil samples were isolated for analysis in the laboratory.

Sample collection

Infected samples numbering about Thirty-six (36) tomato shoots and fruits were randomly collected from six (6) different cultivars of tomato plants; they were carefully labeled and each placed in a sterile polyethylene bag. Soil samples were also collected from the root rhizosphere of infected tomato plants (basically from tomato shoots that showed severe disease symptoms) and wrapped using sterile filter papers; the samples were then transferred to the Pathology and Mycology research laboratory of the Department of Botany, University of Ibadan; for disease diagnosis and further analysis.

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Isolation of the causal organism(s)

The procedure employed for isolation of the causal agents was a modification of the methodology of Ijato et al. [7]. Infected leaf samples were cut into small pieces of 5 mm radial diameter using a sterile 5 mm cork borer, surface sterilized in 75% ethanol and rinsed in three separate changes of sterile distilled water, to eliminate traces of ethanol; they were then inoculated using direct plating technique into replicates of freshly prepared (full strength i.e. 39 g/L) potato dextrose agar medium (PDA).

The stems and roots of the infected tomato shoots were surfaced sterilized by swabbing with 10% sodium hypochlorite. They were then later cut longitudinally to expose the vascular bundles. The already exposed stems and roots were further cut into smaller units of 5 mm vertical length, sterilized in 75% ethanol, rinsed in three (3) successive changes of sterile distilled water and then plated on freshly prepared potato dextrose agar (for fungal growth) and nutrient agar (for Bacteria growth). The cultured samples were incubated at standard room temperature (25 ± 2°C), for a period of seven days for fungal isolates and a maximum of 48 hours for bacterial isolates, after which pure cultures were obtained from direct screening and a series of sub-culturing of the isolates.

Sample preparation for analysis

The soil samples taken from the infected plants were air dried for 24 hours at room temperature in the laboratory. Approximately, 1 g of each soil sample was suspended in 10 ml of sterile distilled water for a period of 30 mins with regular shaken by hand to enhance homogenization. One ml from each solution (stock) was pipetted into 9 ml of sterile distilled water titrated into a test tube. The process was repeated for nine (9) more test tubes, each containing 9 ml of sterile distilled water. A dilution factor of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} was obtained.

Inoculation was done using the pour plate technique/method. One ml from each dilution factor was aseptically introduced into freshly prepared media (PDA and Nutrient Agar). The medium was gently swirled and decanted to remove excess water. It was then incubated at 25 ± 2°C) for between 24 hrs - 48 hrs (for Bacterial growth) and 7 days (for Fungal growth) in an incubator.

Determination of inoculum size (Microbial Load)

Pure culture of each isolate were grown on a full strength (39 g/L) potato dextrose agar (PDA) slants in 14 ml McCartney bottles and used as stock cultures. A 7-day old pure culture of the isolates obtained from the serial dilution of soil samples collected from infected root rhizosphere was used to conduct this experiment. The culture medium was flooded with sterile distilled water, swirled and decanted into a sterile container; to extract the spores. 1 ml of the spore suspension was calibrated using a spore counter (Central Laboratory-University of Ibadan) and the number of spores per ml was recorded. The resulting spore values obtained was multiplied by the corresponding dilution factor so as to give a true representative of the actual spore count.

Identification of the causal agent(s)

The identification of the pathogens was done using standard laboratory techniques. The identification of the isolates were aided by information provided by Alexopoulos et al., Emmons et al., Keith Seifert, John et al., Lunn, Sarbhoy, and Schipper [8-14]. Slides were prepared for each isolates using lactophenol in cotton blue stain and examine under the Spectro-photomicroscope. Each isolates was carefully identified based on its mycelia morphology and orientation on culture plates; production of metabolites; and the presence of various fruiting bodies like the sporodochia (macrospores), phiallides, microspores, conidia and sporangiospores.

Pathogenicity test

In order to establish the fact that the isolates obtained from the diseased samples were actually the causal agents of the disease (symptoms) observed on the tomato plants and fruits; the isolates were subjected to screen-house analysis following the methods introduced by Alexander Koch [15].

Source of tomato seeds

Three (3) varieties of tomato seeds were used for this experiment. They are Cherry Tomato (*Lycopersicon esculentum var. cerasiforme*), Currant Tomato (*L. pimpinellifolium*), and *L. esculentum var. esculentum*. The seeds were obtained from the National Center for Genetic Resources and Biotechnology (NACGRAB).

Soil sterilization

The method employed for the sterilization of soil for this experiment was basically a modification of the standard technique/ methodology used by plant breeders in rural and small scale farming. Top-soil was collected from the nursery in the Department of Botany, University of Ibadan. The soil was loaded into troughs, watered and steamed to a temperature range between 100°C for one (1) hour in a controlled incinerator; in order to eliminate traces of the natural "fauna and flora" community of the soil substrate. The soil was allowed to cool before transferring to the screen house for planting.

Soil analysis

Soil analysis was conducted using standard techniques. This analysis was carried out to determine the microbial load of the

e-ISSN:2347-226X
p-ISSN:2319-9857

soil samples assayed against an uninfected soil. A total of 1 g of both sterilized and unsterilized soil sample were collected and labeled appropriately. Sterilization of soil samples were done at 100°C for six (1) hour. The collected samples were aseptically introduced into test tubes and serial dilution of the suspension were made; 1 ml dilution factor of 10^{-3} and 10^{-6} was aseptically inoculated using pour plate method, into freshly prepared potato dextrose agar (for fungal growth) and nutrient agar (for Bacteria growth).

Preparation of spore suspension

The isolates were grown to maturity (as pure cultures). At the end of the 7th day, the culture plates containing the pure isolates were then flooded with sterile distilled water, swirled and decanted into different sterile conical flasks and labeled according to the isolate. Serial dilution of collected spore samples was carried out and the inoculum size was determined using haemocytometer. The inoculum size was maintained at 3.2×10^6 spores per ml of spore sample.

Experimental design for screen house analysis

Factorial Design (FD) was used as the set up for the experiment. The test plants were laid in 3 by 3 by 13 format in the screen house. Data obtained from the experiment were represented as "Means ± Standard Deviation (SD)" and the analysis of variance was carried out using Minitab 16.0 statistical software while statistically significant means were separated using Least Significant Difference (LSD), Duncan Multiple Range Test (DMRT) and Fishers' Pairwise Comparison (FPC). Charts and graphs were employed as descriptive tools for both qualitative and quantitative data.

Field experiment

The first experiment was designed to ascertain the role of each isolates in the replication of the disease symptoms observed on the field. Approximately 50 ml of the spore suspension was aseptically decanted around the root rhizosphere of the transplanted seedlings, and it was then covered with top soil. The experiment was allowed to run for a period of one month, during which agronomic parameters of the tomato seedlings like plant height, leaf area, stem girth, number of leaves, and number of branches; were accessed weekly and the tomato plants were allowed to grow to fruiting stage under close observation in the screen house of the Department of Botany. Re-isolation of the introduced isolates was carried out from selected samples showing typical disease symptoms.

RESULTS

The result obtained so far showed that the isolates replicated the disease symptoms on the test plants at varying degrees with the exception of the control set up after the seventh (7th) day of inoculation **(Figures 2 and 3)**. The same trend was observed for day 14 **(Figures 4 and 5)**.

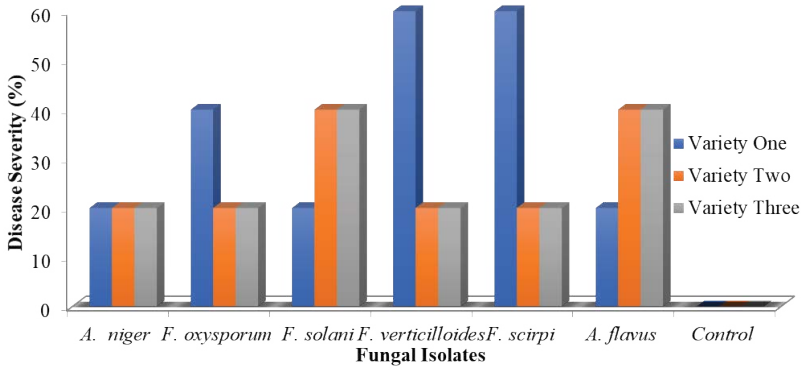

Figure 2. The percentage disease severity of each fungal isolates after the 7th day of inoculation into disease free tomato seedlings.

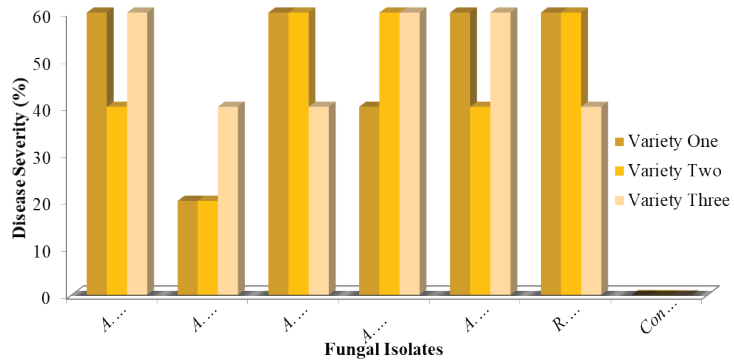

Figure 3. The percentage disease severity for the other fungal isolates after the 7th day of inoculation into disease free tomato seedlings.

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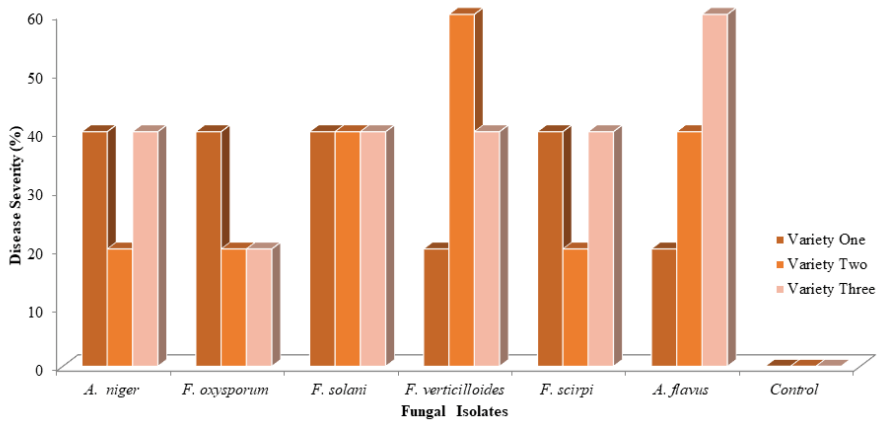

Figure 4. The percentage disease severity of each fungal isolates after the 14th day of inoculation into disease free tomato seedlings.

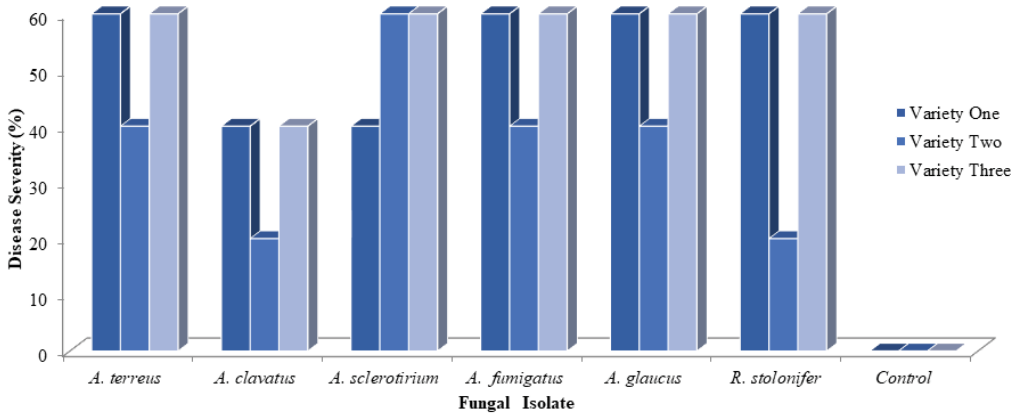

Figure 5. The percentage disease severity of the other isolates after the 14th day of inoculation into disease free tomato seedlings.

It was observed that the level of disease severity varied immensely from species to species after each week of inoculation **(Figures 2-5)**. As indicated in **Table 1**, it was observed that *Rhizopus stolonifer, Aspergillus glaucus, A. sclerotirium, A. terreus, Fusarium scirpi* and *F. verticilloides* each had 60% disease expression on variety one of the test plants used for this experiment (+++); whereas, *A. clavatus, A. flavus, F. solani* and *A. niger* had mildly significant levels of about 20% disease symptom expression on the experimental tomato plants (+). *F. oxysporum* and *A. fumigatus* each had two-fifth of the test plants infected (++) i.e. 40% symptoms expression based on their microbial activities within the tomato plants ($P<0.05$) [16].

Table 1. Disease severity scoring adopted for the in-situ experiment conducted in the screen house using disease free tomato plants.

S/N	OBSERVATIONS	SIGN INDICATION
1	Zero plant part infected	-
2	One-fifth ”	+
3	Two-fifth ”	++
4	Three-fifth ”	+++
5	Four-fifth ”	++++
6	All ”	+++++

The same effect was noticed on variety two of the test plants at day 7 **(Tables 2 and 3)**. It was observed that *R. stolonifer, Aspergillus fumigatus,* and *A. sclerotirium* exhibited high levels of disease symptom expressed physiologically on the observed tomato plants (+++) which was calculated to fall within 60% of the body area of the test plants; followed by *A. glaucus, A. terreus, A. flavus* and *F. solani* (40%); the other isolates had little significant effect on the tomato plants (20%). A similar trend was also described for variety three of the tomato plants with organisms like *A. glaucus, A. fumigatus and A. terreus* showing 60% (+++) disease expression on the inoculated tomato plants, seconded by *R. stolonifer, A. sclerotirium, A. clavatus, A. flavus* and *F. solani* each having 40% disease effect; while *F. scirpi, F. verticilloides, F. oxysporum* and *A. niger* had 20% level of disease expression on the inoculated plants after the 7th day of inoculation **(Table 4)**.

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Table 2. Disease severity measurement and the level of aggressiveness of each isolates on disease free Tomato seedlings at day 7.

Fungal Isolate	Variety One		Variety Two		Variety Three	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
A. *niger*	Yes	+	Yes	+	Yes	+
F. oxysporum	Yes	++	Yes	+	Yes	+
F. solani	Yes	+	Yes	++	Yes	++
F. verticilloides	Yes	+++	Yes	+	Yes	+
F. Scirpi	Yes	+++	Yes	+	Yes	+
A. flavus	Yes	+	Yes	++	Yes	++
A. terreus	Yes	+++	Yes	++	Yes	+++
A. clavatus	Yes	+	Yes	+	Yes	++
A. sclerotirium	Yes	+++	Yes	+++	Yes	++
A. fumigatus	Yes	++	Yes	+++	Yes	+++
A. glaucus	Yes	+++	Yes	++	Yes	+++
R. stolonifer	Yes	+++	Yes	+++	Yes	++
Control	No	-	No	-	No	-

Key:
- Healthy
+ Tolerable Disease State
++ Mildly Severe Disease State
+++ Severe Disease State
++++ Extremely Severe Disease State
+++++ Death of all plants.

Table 3. Disease severity measurement and the level of aggressiveness of each isolates on disease free tomato seedlings at day 14.

Fungal Isolates	Variety one		Variety two		Variety three	
	incidence	severity	incidence	severity	Incidence	severity
A. niger	Yes	++	Yes	+	Yes	++
F. oxysporum	Yes	++	Yes	+	Yes	+
F. solani	Yes	++	Yes	++	Yes	++
F. verticilloides	Yes	+	Yes	+++	Yes	++
F. Scirpi	Yes	++	Yes	+	Yes	++
A. flavus	Yes	+	Yes	++	Yes	+++
A. terreus	Yes	+++	Yes	++	Yes	+++
A. clavatus	Yes	++	Yes	+	Yes	++
A. sclerotirium	Yes	++	Yes	+++	Yes	+++
A. fumigatus	Yes	+++	Yes	++	Yes	+++
A. glaucus	Yes	+++	Yes	++	Yes	+++
R. stolonifer	Yes	+++	Yes	+	Yes	+++
Control	No	-	No	-	No	-

Key:
- Healthy
+ Tolerable Disease State
++ Mildly Severe Disease State
+++ Severe Disease State
++++ Extremely Severe Disease State
+++++ Death of all plants.

Table 4. Percentage disease severity of each fungal isolates after the 7th day of inoculation into disease free tomato seedlings.

Fungal Isolates	Percentage Disease Severity (%)		
	Variety One	Variety Two	Variety Three
A. niger	20	20	20
F. oxysporum	40	20	20
F. solani	20	40	40
F. verticilloides	60	20	20
F. Scirpi	60	20	20

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p-ISSN:2319-9857

A. flavus	20	40	40
A. terreus	60	40	60
A. clavatus	20	20	40
A. sclerotirium	60	60	40
A. fumigatus	40	60	60
A. glaucus	60	40	60
R. stolonifer	60	60	40
Control	0	0	0

Data were represented as Means ± Standard Deviation (SD) and N=3.

Furthermore, it was observed after the 14th day of inoculation that *A. glaucus, A. terreus, A. fumigatus, A. sclerotirium* and *Rhizopus stolonifer* remained highly virulent on at least two (2) out of the three (3) varieties of tomato plants used for this experiment, producing similar symptoms observed in the field **(Figures 6-8)**, with about 60% of the tomato shoots infected **(Table 5)**. While isolates like *F. verticilloides, A. niger, A. clavatus, A. flavus, F. solani, F. scirpi* and *F. oxysporum* declined drastically in their capacity to induce disease situation on the inoculated tomato plants ($P<0.05$) with an average level of disease expression of ≤ 40% on the three (3) varieties of tomatoes used for this experiment **(Figures 9-13)**.

(a) Formation of Fungal mycelia around infected tomato root (field) (b) Formation of Fungal mycelia around inoculated tomato root

Figure 6. Application of Koch's postulate for the re-establishment of disease symptoms.

(a) Necrotic Lesions on infected plants in the field (Original Symptom) (b) Necrotic Lesions on inoculated plants

Figure 7. Application of Koch's postulate for the re-establishment of disease symptoms.

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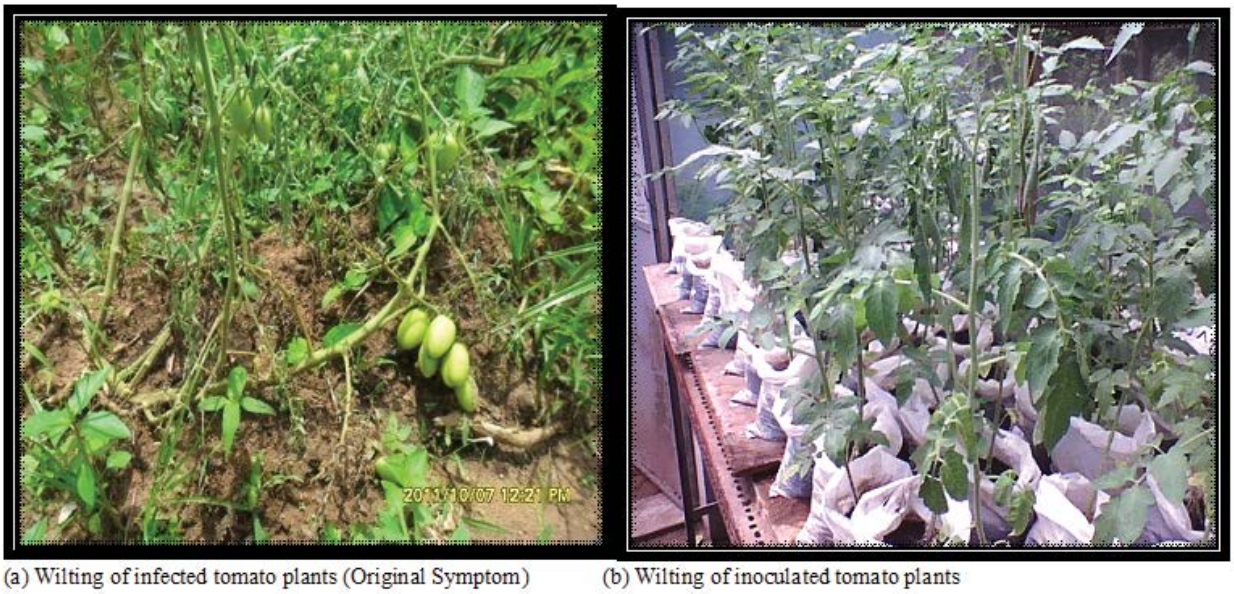

Figure 8. Application of Koch's postulate for the re-establishment of disease symptoms.

Table 5. Percentage disease severity of each fungal isolates after the 14th day of inoculation into disease free tomato seedlings.

Fungal Isolates	Percentage Disease Severity (%)		
	Variety One	Variety Two	Variety Three
A. niger	40	20	40
F. oxysporum	40	20	20
F. solani	40	40	40
F. verticilloides	20	60	40
F. Scirpi	40	20	40
A. flavus	20	40	60
A. terreus	60	40	60
A. clavatus	40	20	40
A. sclerotirium	40	60	60
A. fumigatus	60	40	60
A. glaucus	60	40	60
R. stolonifer	60	20	60
Control	0	0	0

Data were represented as Means ± Standard Deviation (SD) and N=3.

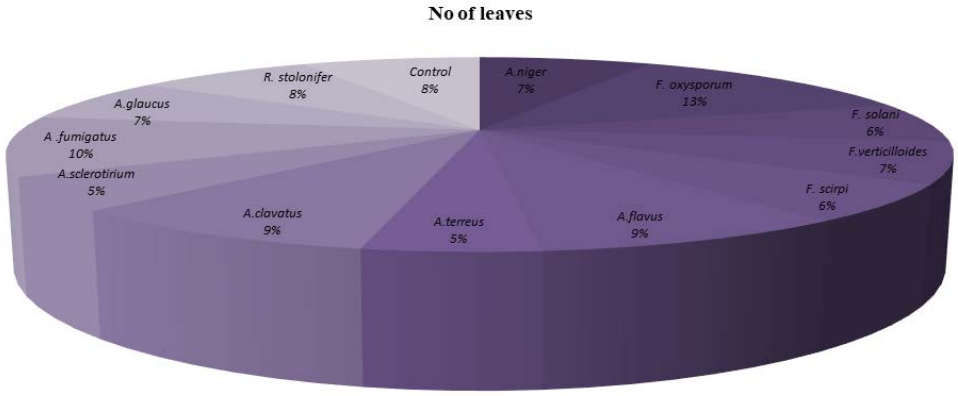

Figure 9. The leaflet count for tomato plants re-inoculated with fungal pathogens.

e-ISSN:2347-226X
p-ISSN:2319-9857

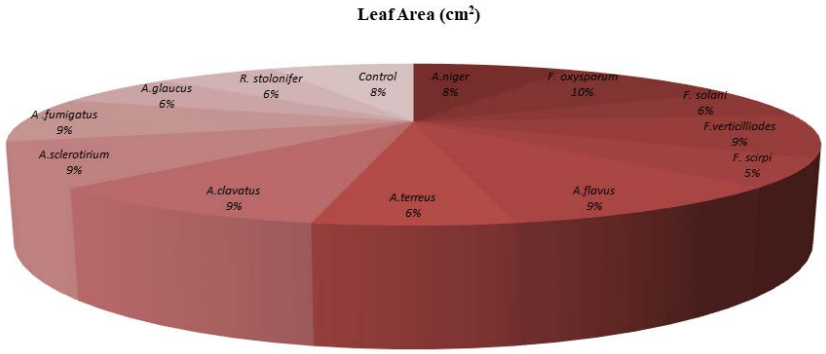

Figure 10. The percentage leaflet area for tomato plants re-inoculated with fungal pathogens.

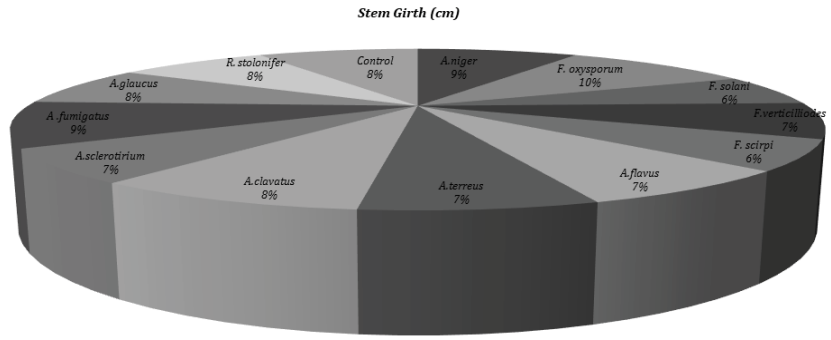

Figure 11. The percentage stem girth for tomato plants re-inoculated with fungal pathogens.

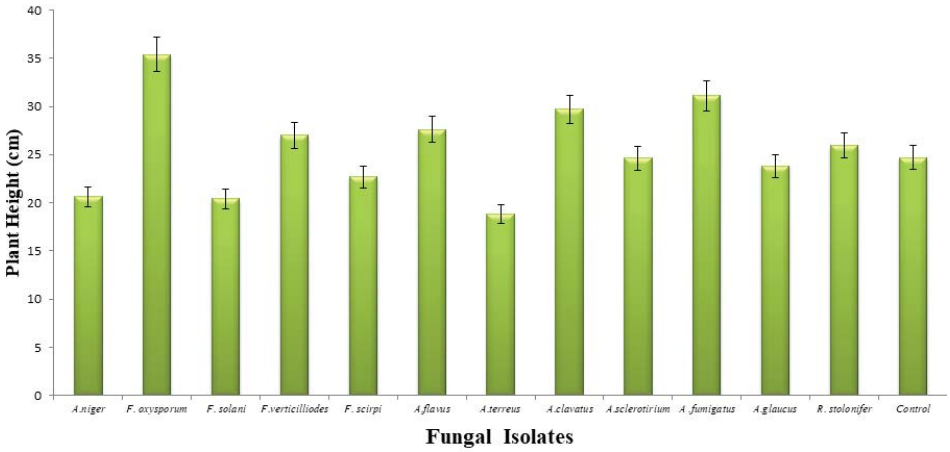

Figure 12. The plant height for tomato plants re-inoculated with fungal pathogens.

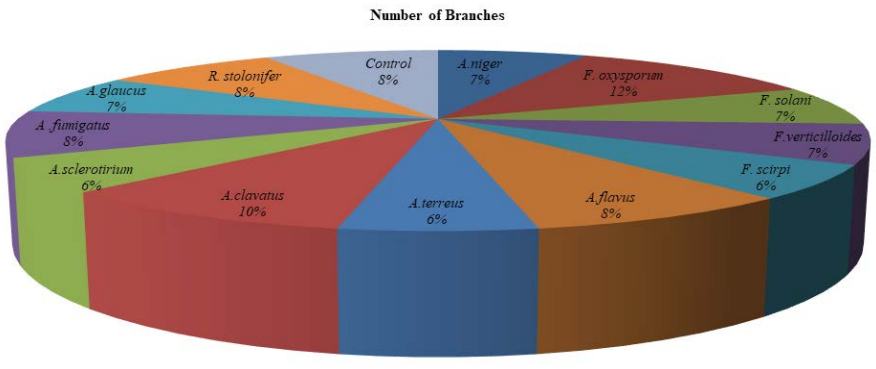

Figure 13. A comparison of the number of branches on the re-infected tomato seedlings at day 7.

e-ISSN:2347-226X
p-ISSN:2319-9857

DISCUSSION

The long term exposure of the extrapolated fungal pathogens to healthy tomato seedlings resulted in the production of infections (similar to those observed on the field) on the tomato plants after a period of intense observation in a microcosm. Majority of the isolates were able to replicate the disease symptoms observed on the field, and symptoms observed (leaf spots, blight of lower leaves, general necrosis, wilting and death of some of the tomato plants) was observed basically on the older leaves, stem and roots of the affected plants closest to the soil, while younger leaves and those newly formed were not significantly affected.

This possibly suggest the mode of entry and spread of the disease (from bottom to top of the plants); and the origin of the pathogens (soil borne in nature) as stated by Michael et al. [3]. Therefore, it is possible that the disease spread can be facilitated by rain splash or spilled water from the infected soil. A similar pattern of infection was also described by FAO (Food and Agricultural Research Organization) of the United States of America [2].

Pathogens like *A. terreus, A. sclerotirium, A. fumigatus, A. glaucus, and Rhizopus stolonifer* showed high levels of aggressiveness on all the cultivars of tomato plants investigated. Disease symptoms produced tend to increase and become more noticeable as it spread across the whole shoot with respect to time, age, and developmental stages of the tomato plants. The disease trend was synonymous to those reported by Alippi et al. [17] and Saygili et al. [6]. This result also corresponds with the documentations/report of the Food and Agricultural Research Organization of the United States of America [2].

Majority of the tomato plants inoculated with the extrapolated pathogens showed reduction in plant height, stem girth, leaflet area, folia branches and number of foliages during the pathogenicity test conducted in the serene seclusion of the microcosm. This observation might be partly due to the fact that the effects of the highly complicated pathogenic activities of the fungal pathogens (within the host plants) had adverse effects on the developmental stages and probably the growth rate of the infected tomato seedlings, resulting in loss of vigor (wilting), depreciation in stem girth, reduction of leaf area; suggesting an uncompensated loss of photosynthetic activities due to reduced plant size and decrease in yield of the tomato plants. These findings correspond with the report from the Food and Agricultural Research Organization of the United States of America [2], stating that most of the damages inflicted on tomato plants by pathogens significantly affects the quality and yield of the tomato plants at various developmental stages.

Tomato plants inoculated with *Fusarium oxysporum, A. clavatus* and *A. niger* showed positive increase in some of the agronomic parameters of their host plants, like plant height, stem girth and leaf area; although, symptoms like leaf spots, foliar necrosis and blight of the infected tomato plants still persist. A possible explanation for the increase in agronomic parameters observed might be due to the fact that the activities of these fungal isolates might trigger off the responses of some growth hormones inherent in the host plant; or these pathogens might produce a substantial amount of growth hormone themselves, resulting in an increase in growth rate or an accelerated developmental stage in the affected tomato plants; this situation is synonymous to the foolish rice seedlings disease caused by the fungus *"Gibberella fujikuroi" (*syn- *Fusarium moniliforme and Fusarium verticilloides)* reported in Japan [15] where the infected rice plants have good vegetative growth.

This report was in contrast with the report given by Mohamed, who categorically stated that *Fusarium oxysporum* caused a significant decrease in the percentage germination and reduction in some of the agronomic parameters such as plant height among other morphological data stated for the infected tomato plants.

CONCLUSION

The pathogens isolated from the diseased tomato samples produced the same symptoms as those initially observed on the field. Disease symptoms like general leaf blight, localized and general necrosis, damping-off of tomato seedlings, wilting and death of matured plants with both ripe and unripe berries were most common. Healthy tomato berries that came in contact with the soil developed the disease too, suggesting that the pathogens responsible for the infectious disease were mostly soil borne.

Currently in Nigeria, reports of spontaneous outbreak of diseases on field crops and vegetables, most especially tomato have been a major source of concern both to farmers and consumers of tomato and tomato products; culminating in a short fall in income to the government in form of decline in payable tax on goods. Therefore, great concern should be shown and solutions proffered to combat these microbial insurgents and the menace they carry out on tomato plants in other to alleviate scarcity of food materials and to strengthen the food security of the nation and the world at large.

ACKNOWLEDGMENT

I will like to acknowledge the efforts of Chief John O. and Mrs. Esther Etaware for their moral and financial support towards the completion of this research. Your benevolence and benignity will never go unrewarded, God bless you both.

e-ISSN:2347-226X
p-ISSN:2319-9857

ETHICAL STATEMENT

This is to confirm that Prof. O. J. Oyetunji declares that he has no conflict of interest and that he actively participated in the research both in the field and in the procurement of materials for disease analysis in the laboratory.

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