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Isolation, Phenotypic Characterisation and Molecular Identification of Fungal Pathogens of Sweet Pepper (*Capsicum Annuum* L.) Fruit in Sokoto State Nigeria.

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ABSTRACT

Sweet pepper (*Capsicum annum* L.) belongs to the family Solanaceae; it is an important group of vegetables extensively cultivated in almost every country of the world (Channabasavanna and Setty, 2000). A purposive sampling technique was used to select three farms in each Local Government to represent the four Agricultural zones of the state.The state has four Agricultural zones namely: Gwadabawa (Sokoto West), Isa (Sokoto East), Sokoto (Sokoto Central) and Tambuwal (Sokoto South) zones. Each zone has between 5 - 6 Local Governments' areas (Junaidu, 2005). One hundred and twenty fruits were collected for the study. Cultural, microscopic and molecular techniques were used to identify the fungi, while poisoned food techniques was used to confirm the pathogenesity of the fungi isolated. Five fungal species which include *Fusarium oxysporum, Fusarium equiseti, Fusarium culmorum, Aspergillus niger* and *Aspergillus fumigatus* were isolated, identified and were found to be pathogenic to sweet pepper fruits. *Fusarium oxysporum* had the highest frequency of occurrence of all the four Agricultural zones investigated with *Aspergillus niger* exhibiting the highest degree of virulence on sweet pepper. The findings of this study recommended that studies should be conducted on plant extracts in management of fungal diseases of sweet pepper fruit in sokoto state.

Key words: Sweet pepper, Fungal pathogens, Sokoto and Nigeria

INTRODUCTION

Sweet pepper (*Capsicum annum* L.) belongs to the family Solanaceae; it is an important group of vegetables extensively cultivated in almost every country of the world (Channabasavanna and Setty, 2000). The crop thrives best in warm climate, where frost is not a problem during the growing seasons. In general, it requires temperatures ranging from 25-35^{o}C (Olalla and Valero, 1994). The sweet pepper also known as Bell pepper, is one of the most varied and widely used foods in the world; it originated in Mexico and Central America regions and Christopher Columbus encountered it in 1493 (Kelley and Boyhan, 2009). Sweet pepper is the world's second most important vegetable after tomato (Anon, 1989). It is one of the most important vegetable grown in other parts of sub-humid and semi-arid tropics (Aliyu, 2000).

The Pepper grown worldwide consists of approximately 22 wild species and five domesticated species. The five domesticated species include *C. Annum, C. Frutescens, C.Chinenses, C. Baccatum and C. pubescens R.* (Bosland and Votava, 2000). Capsicum species can be divided into several groups based on fruit/pod characteristics ranging in pungency, colour, shape, intended use, flavor, and size. Despite their vast trait differences most cultivars of peppers commercially cultivated in the world belong to the species *C. Annum* (Lin *et al.,* 2013). There are an estimated 1,600 different varieties of pepper (*Capsicum annum*) throughout the world (Delelegn, 2011).

Diseases are major constraints to sweet pepper production in sub-Saharan Africa including Nigeria. Phytopathogenic fungi, bacteria and viruses have been reported to cause a major yield loss in pepper.The National Programme for Agriculture and Food Security (NPAFS) (2009) reported that the yield of pepper between 1999 and 2004 declined from 2939 tonnes to 2912 tonnes and 2744 tonnes to 2469 tonnes. Between 2007 and 2009 despite the increased hectrage from 22.5 hectares to 26.92 hectares of land, the yield was not commensurate (5532 tonnes to 6063 tonnes). The decline according to Jaliya and Sani (2006), and Abdulmalik *et al*., 2012) have been attributed to biotic and abiotic factors like weeds, pests, diseases and environmental stress. Information on specific diseases responsible for the decline in yield and production of sweet peppers in Sokoto State, Nigeria is very important for the development of effective control strategy for the management of diseases in farmers' fields in order to improve yields.

The study is aimed at isolation, phenotypic characterization and molecular identification of fungal pathogens of Sweet Pepper (*Capsicum annuum* L.) Fruit in Sokoto State Nigeria.

MATERIALS AND METHODS

Sources of Isolates

The fungal isolates used in this study were obtained from a study of fungal pathogens of Sweet Pepper (*Capsicum annuum* L.) Fruit in Sokoto State Nigeria Samples of healthy and physically damaged sweet pepper fruits used in this study were collected from twelve (12) farms of the four selected local government areas, from the four Agricultural zones in Sokoto State (Gwadabawa, Isa, Sokoto and Tambuwal). Both kinds of sample (physically healthy and physically damaged), were each collected in a separate sterile polythene bag and transported to the mycology laboratory of the Botany Unit of the Biological Science Department, Usman Danfodiyo University, Sokoto (within 2 hours of collection) for the isolation of fungi.

Isolation of Fungal Pathogens

Potato Dextrose Agar (PDA) Medium was used for isolation of fungi in this study. The media was prepaed according to manufacture's instructions. Portion of the sweet pepper fruits were cut aseptically with the aid of sterile scissors into small pieces (5mm). It was then placed centrally on Petri-dishes containing solidified PDA (Fawole and Oso, 1988). The plates were then incubated at room temperature ($32 \pm 2^{o}C$) in the dark for 72hours in the month of October. The fungal colony grown from the incubated plates was sub-cultured into fresh medium until pure culture was obtained.

Identification of Fungal Pathogens (Phenotype)

The Microscope used was MC30 in the mycology laboratory of Usmanu Danfodiyo University Sokoto to examinine the colony characteristics to establish identity of Fungi. A sterile needle was used in taking a little portion of the hyphae containing spores on the sterile glass slide, stained with lacto phenol cotton blue and then examined under the microscope for fungal structures.The macroscopic/culture features and the microscopic characteristics observed were then compared with fungal identification atlas for identification of the fungi (Snowdon, 1990).An isolated fungus was identified based on colony and morphological characteristics, such as color and shape observed with the microscope. The Morphological characteristics and appearance of the fungi isolated were confirmed and authenticated with the help of Mycological Atlas Book of Robert and Ellen, (1988).

Infection/Pathogenesity Study

To ascertain the Pathogenesity of the various fungi that were isolated the approach of Balogun *et al*. (2005) were employed. Fresh and apparently healthy sweet pepper fruits were surfaced-sterilized with methanol for 30 seconds and then rinsed three times in distilled water. With a 7mm diameter flame-sterilized cork borer, cylindrical cores were removed from each fruit. An equal diameter of fungal mycelium was punctured and used to inoculate the apparently healthy fruits earlier punctured. Vaseline jelly was smeared to completely seal the surface of each of the inoculated pepper fruit to prevent external infection and then incubat for 10 days in triplicate. The control was inoculated with disc of solidified potato dextrose agar medium. The symptom developed with different fungal isolates was compared to the control. The pathogen was re-isolated and identified using the same procedures described earlier.

The number of times each fungus occurs on the samples was recorded and the percentage frequency of occurrence was determied and recorded.

Molecular Identification of Fungal Pathogens

Genomic DNA was extracted from the isolated fungal pathogens using Qiagen® DNA extraction kit according to the manufacturer's instructions. The PCR was carried out using Qiagen Top Taq master mix kit. A total of 25 uL reaction mix was prepared containing: 12.5 uL Qiagen Toptaq master mix, 1uL of 10 micromolar each of ITS1 and ITS4, 1.5 uL of coral load, 1 uL of nuclease free water and 8uL of DNA template. The tubes were mixed and transferred into applied biosystems 9700 programmed thermocycler with the following cycling conditions: Initial denaturation at 94 degrees for 3 minutes, followed by 40 cycles of denaturation at 94 degrees for 30 seconds, annealing at 49 degrees for 1 minute, extension at 72 degrees for 1 minute, followed by final extension at 72 degrees for 10 minutes.The amplicons were resolved in a 1% agarose pre-stained with ethidium bromide. The gel image was viewed using a UV transilluminator in a Biorad XRS gel documentation device.

RESULTS

Isolation and Identification of Fungal Pathogens of Sweet Pepper Fruit (Capsicum annuumL.) in Sokoto State, Nigeria

The fungal species and morphological characteristics are outlined in Table **1.** Five species of two genera of fungal pathogens that include *Fusarium* and *Aspergillus* were identified. The genera of *Fusarium* include: *Fusarium oxysporum, Fusarium* equiseti and *Fusarium culmorum*, the genera of *Aspergillus* include: *Aspergillus niger* and *Aspergillus fumigatus* and their relative frequency are shown in Table 2.

Percentage Occurrence of Fungal Isolates

Five fungal pathogens (*Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Fusarium equiseti*, and *Fusarium culmorum*) were found to be pathogens of Sweet pepper fruits in Sokoto State, Nigeria. *Fusarium oxysporum* had the highest frequency of occurrence of fungal pathogen in all the Sweet pepper investigated with 41.60% frequency, followed by *Fusarium equiseti* with 33.30%. *Aspergillus niger*, *Aspergillus Fumigatus* and *Fusarium culmorum* had the same frequency of occurrence of 8.30% Table 2

Table 1: Identified Fungal Isolates and their Colonial and Microscopic Characteristics

Specimen	**Appearance on petri plate**		**Description**	**Organism Identified**
	Front view	**Reverse view**		
A	White yellow and then black	White to yellow	Simple conidiophores that terminate in the globose swelling. Hyphae is septate	*Aspergillus niger*
B	Green to dark green	Colourless to yellow	Conidia heads are long, glonose to prolate. Conidiophores hyaline vesicle ovate	*Aspergillus fumigatus*
C	Light white	Creamy white	Conidia on aerial conidiophores (blastoconidia), usually borne singly on scattered denticles, fusiform to falcate, mostly three to five-septate	*Fusarium equiseti*
D	Pinkish white	Light white	Conidiophores are short, single, lateral monophialides in the aerial mycelium, later arranged in densely branched clusters. Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate.	*Fusarium oxysporum*
E	Creamy white	Creamy white	Hyphae are septate and hyaline. Phailades are cylindrical conidiophores are medium length.	*Fusarium culmorum*

Table 2: Frequency of Fungal species of Sweet Pepper, Cultivated in selected Local Government Areas. Sokoto State Nigeria, in October 2019.

Percentage frequency of occurrence

	Goronyo	**Gwadabawa**	**Bodinga**	**Kware**	Total n (%)
A. niger	0 (0.00)	9 (100.0)	0 (0.0)	0 (0.0)	9 (8.3%)
A. fumigates	0 (0.0)	0 (0.0)	9 (100.0)	0 (0.0)	9 (8.3%)
F. oxysporum	9 (20.0)	21 (46.7)	6 (13.3)	9 (20.0)	45 (41.7%)
F. equiseti	6 (16.7)	0 (0.0)	12 (33.3)	18 (50.0)	36 (33.3%)
F. culmorum	9 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (8.3%)
Total	24 (22.2%)	30 (27.8)	27 (25.0)	27 (25.0)	108 (100%)

Pathogenesity Test

Among the five isolates, *Aspergillus niger* exhibited the highest level of virulence (i.e. mycelia and/or rots covering more 82.35%, of the fruit), *Fusarium equiseti* rated as second (i.e. mycelia and/or rots covering 67.06%, of the fruit), *Fusarium culmorum* and *Aspergillus fumigates* rated as medium levels having a pathogenic effect covered 59.21%, and 50.98% of the fruit.*Fusarium oxysporum* was rated the least virulence (i.e. mycelia and/or rots covering 43.32%. of the fruit) as shown in Table 3.

Table 3: Length and Percentage of the Mycelia Growth of Fungi in Treatment Replica

Test fungus	**Original length of the fruit (mm) (A)**	**Length of mycelia growth in sweet pepper (mm)**			**Average (B)**	**%damage (B)/(A) × 100**
		Fruit 1	Fruit 2	Fruit 3		
Fusarium oxysporum	85	49	31	30.5	36.83±10.54	43.32%
Fusarium equiseti	85	79	20.5	71.5	57.00±31.83	67.06%
Aspergillus fumigates	85	49	21	60	43.33±20.11	50.98%
Aspergillus niger	85	80.5	60	69.5	70.00±10.26	82.35%
Fusarium culmorum	85	70	50.5	30.5	50.33±19.75	59.21%

(A) Healthy Sweet Pepper fruit, just after inoculation. (B) Damaged fruits of after pathogenesity testing of the fungal species isolated in the study

Molecular Identification of Fungal Pathogens

Gel-image below (Figure 1) showed that isolate 2 (band size 599 base pair) is *Aspergillus niger,* isolate 3 (band size 544 base pair) is *Fusarium oxysporum,* isolate 4 (band size 596 base pair) is *Aspergillus fumigate* and isolate 5 with band size 585 is *Fusarium culmorum* (Ferrer, *et al*. 2001)

The PCR products were sequenced, following Basic local alignment search tool (BLAST) analysis in GenBank (NCBI) revealed that bands 2, 3, 4 and 5 were *Aspergillus niger*, *Fusarium sterilihyphosum*, *Fusarium sterilihyphosum* and *Fusarium proliferatum* Appendix 7.

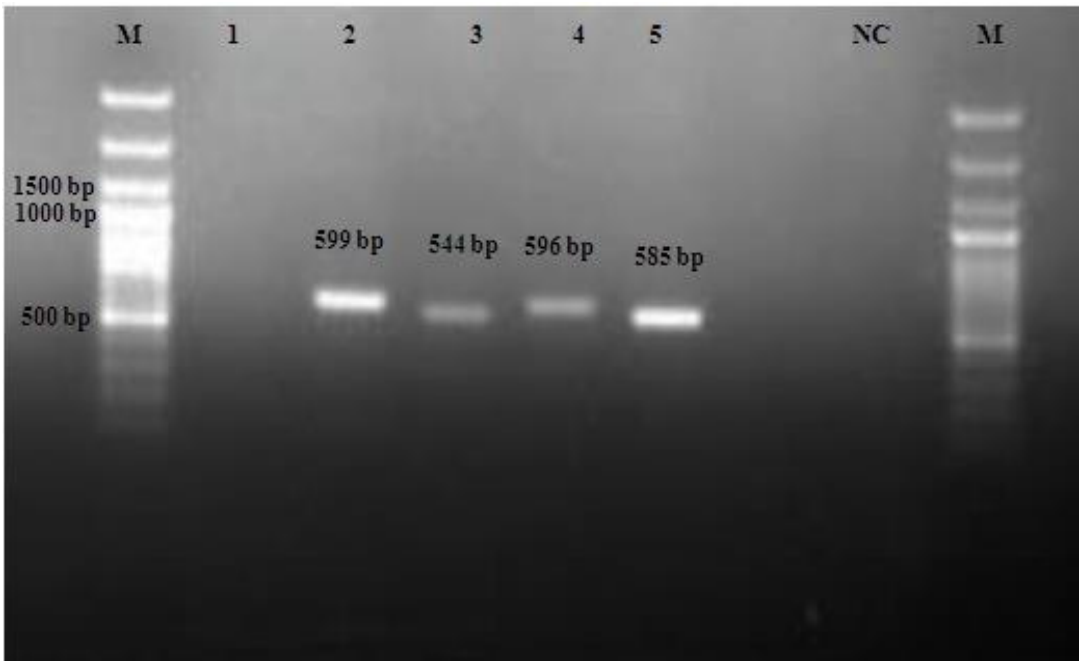

Figure 1: Gel electrophoresis image showing bands indicative of positive amplification of the internal transcribed spacer region of fungi isolated in this study. M =molecular ladder, 1-5 are samples amplified, NC = Negative control. Note that no band for sample number 1.

Sequenced PCR Products

Aspergillus niger

AGGTATCCTACTGATCCGAGGTCACCTGGAAAAATGGTTGGAAAAYGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGC
CCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCCGGAGAGGGGGACGGCGACCCAACACACAAGC
CGGGYTTGAGGGCAGCAATGACSCTCGGACMGGSATGCCCCCCGGAATACCAGGGGGCGCAATGKGCGTTCAAAGACTCGATGATTCA
CTGAATTCTGCAATTCACATTASTTATCGCATTTCSCTGCGTTCTTCATCGATGCCGGAACCAARARATCCATTGTTGAAAGTTTTAACTG
WTTKCKATYCATCCAACTTWAACTTCCCTTWAACWAAAGGGYTTCTGGGGGKCTCCCGCGGGGGCGGGCCCGGGGSTGAAAGCCCCC
CCCGCCCAAGAATGGSGGGCCCGCCGAAACAACTAAGGWACARWAAACACGGGKGGGAGGTGGGGCTCTSTGACAACCCTACCCTCC
CTAATGATCCTTCCCTACGGAACCTACSGAA

3 ***Fusarium sterilihyphosum***

TTCCTACCTGATCCGAGGTCACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGC
TATGGAAGCTCGACGTGACCGCCAATCAATTTGGGGAACGCGATTTAACTCGCGAGTCCCAACACCAAGCTGGGCTTGAGGGTTGAAA
TGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACA
TTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGA
AGTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGT
ATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTYTMCTTCCA

4 ***Fusarium sterilihyphosum***

AYCTACCTGATCGAGGTCAACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGCT
ATGGAAGCTCGACGTGACCGCCAATCAATTTGGGGAACGCGATTTAACTCGCGAGTCCCAACACCAAGCTGGGCTTGAGGGTTGAAAT
GACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT
TACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAA
GTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGTA
TGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTYTMCTTCCA

5 ***Fusarium proliferatum***

TCCTACCTGATCGAGGTCACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGCTA
TGGAAGCTCGACGTGACCGCCAATCAATTTGGGGAACGCGATTTAACTCGCGAGTCCCAACACCAAGCTGGGCTTGAGGGTTGAAATG
ACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAA

GTTACATATAGAAACAGAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCGCCGAGGCAACAATTGGTA
TGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGACTTYTMCTTCCA

DISCUSSION AND CONCLUSION

Isolation and identification of fungal pathogens associated with sweet pepper fruit in this research revealed five fungal species from two genera of *Aspergillus* and *Fusarium*. Out of the fungal species isolated, *Fusarium oxysporum* was found to have the highest rate of occurrence in all the four Agricultural zones studied. The fungus *Fusarium oxysporum* was frequently incriminated in diseases of sweet paper as reported by Abada and Ahmad, (2014) that isolated *Alternaria spp., F. oxysporum, Pythium spp., Rhizontonia solani, Sclerotium rolfsii* and *Trichoderma spp*. from the roots of wilted sweet pepper plants in Egypt. In line with this, Jamiolkowska, (2009) Recovered *Alternaria alternata, Fusarium oxysporum, F. equiseti, F. solani* and *Colletotrichum coccodes* from stem base and root rot of hot pepper. Similarly, Sanogo and Caepenter, 2006, Alegbejo *et al.,* (2006) Isolated from wilted chile pepper (*Capsicum annuum*) in New Mexico, *Verticillium dahliae, Pythium capsici, R.solani*, and species of *Fusarium, Penicillium*, and *Aspergillus.* Alegbejo *et al*. (2006) Identified from root (*P. capsici*) and stem (*P. capsici* and *F. oxysporum*) of stem rot and wilt diseases of pepper plant in Northern Nigeria.

Determination of the Pathogenisity of fungal species isolated in this research on Sweet Pepper fruit revealed that the isolated fungi are pathogenic to sweet pepper fruit with *Aspergillus niger* exhibiting the highest degree of virulence. This is similar to Balogun *et al*., 2005 who's, reported the same result with *Aspergillus niger* having the highest level of virulence in Ilorin, North central Nigeria.

Five Fungal species, namely *Aspergillus niger, Fusarium equiseti, Fusarium culmorum, Aspergillus Fumigatus* and *Fusarium oxysporum* were identified and were found to be pathogenic to Sweet Pepper fruit in the State. *Fusarium oxysporum* was found to have the highest rate of occurrence in all the four Agricultural zones studied.

However, *Aspergillus niger* exhibited the highest level of virulence with Mycelia and/or rots covering about 82.35% of the Sweet Pepper fruit.

RECOMMENDATIONS

From the findings of this study it can be recommended that studies should be conducted on the use of environmentally friedly plant extracts in management of fungal diseases of sweet pepper fruit in sokoto state.

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