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ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH FIELD PEA (*PISUM SATIVUM* LINN) IN NORTHERN GUINEA SAVANNA OF NIGERIA

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ABSTRACT

Pisum sativum L. (field pea) is cultivated globally and valued for its protein- rich grain. The aim of the study is to identify fungi associated with field pea in Northern guinea savanna of Nigeria. Fungi were isolated through direct plating of diseased tissue. Morphological identification was done through colony texture, color, and its cultural characteristics on PDA. Size/shape of conidia and conidiophores. Molecular identification was carried out using Polymerase Chain Reaction (PCR) using universal primers ITS 1 and ITS4. The NCBI Blastx programed (http://www.ncbi.nlm.nih.gov/BLAST) V. 4 analysis was used to identify the fungi.Alignments of nucleotide sequences were done using Bio Edit sequence alignment editor software. Phylogenetic relationships of fungal isolates were analyzed using MEGA V. 7.0 software. Pathogenecity tests were carried out on the fungal isolates. A total of six different fungal diseases were recorded which include; wilt caused by *Fusarium solani*, *F. oxysporum* and *P. boydii*, leaf spot caused by *A. alternata* and *C. eragrotidis*, Leaf blight and root rot caused by *R. solani*, Pod rot caused by *T. terricola*and mold caused by *Botrytis* sp. other saprophytic fungi identified on field pea were *Penicillium singorense, Trichoderma, Chrysosporium, Cheatophoma* and *Cheatomium* spp. *Rhizoctonia solani* had the highest percentage occurrence of 55.3 % while *Cheatomium* had the least with 6.3%. To the best of our knowledge, this is the first record of causative fungi of wilt,

root rot, leaf blight, leaf spot, pod rot and gray mold diseases on field pea in Northern guinea savanna of Nigeria.

Keywords; Field pea, Fungi, Polymerase Chain Reaction (PCR)

INTRODUCTION

Pisum sativum L.(field pea) is cultivated globally and valued for its protein- rich grain. It is consumed both as fresh vegetable as well as in a dried form. In Nigeria, the plant is mainly grown as cash crop under irrigation in Kaduna and Katsina State. However, in Plateau State it is cultivated during rainy season. The inclusion of peas in crop rotation is agronomically very significant. The nutritional value of dry pea seed is similar to other grain legumes and contains 18-30 % protein, 35-50% starch and 4-7 % fiber (Shivani, 2017).

The major reason responsible for low pea productivity is fungal diseases causing significant annual losses in grain yield (Beasse *et al.,* 2000; Xue, 2000). More than twenty species infesting pea crops have been established in various parts of the world which damage the plant root, foot, aerial parts, pods and grains (Bretag *et al.,* 1995; Marcinkowska, 2008). Some important fungal diseases of field include; leaf spot, root rot and wilt among others (Laflamme, 1998). A yield loss due to fungal diseases is up to 20-80%. Fungal diseases which affect the field pea plant before flowering leads to decline of seed- set, while if infection occurs at the beginning of seed formation stage, it results in reduction in seed weight (Beasse *et al*., 2000; Ruta *et al*., 2014). Based on the literature surveyed there is no documentation of fungi associated with field pea in Northern guinea savanna of Nigeria, hence the need for this study.

MATERIALS AND METHODS Sample collection

Disease looking as described by (Hagedorn, 1991) *Pisum sativum* Were Collected From Katanga, Shika Dam and Zangon Danbarno (SabonGari Local Government Area of Kaduna State), Sabuwa Local Government Area of Katsina State, Razek Fan (BarkinLadi) and RapiyamFam (Jos South Local Government Area of Plateau State). Geographic positioning system (GPS) was used to take the coordinates of each sampling site.

Preparation of Media and Isolation of Wilt Fungi Preparation of media

Commercially formulated Potato Dextrose Agar was prepared as described by the manufacturer's instructions (Oxoid, England) and used throughout the study.

Isolation of fungi

Infected plants samples were cut with sterile surgical blades. Direct tissue plating method was used as described by (Mungo, 1996).

Morphological Identification of Isolated Fungi

Identification was carried out by both macroscopic and microscopic observations of pure cultures. Confirmation of the fungal identification was done using identification key (Barnett and Hunter, 1999), they were compared with already described isolates in the Department of Crop Protection diagnostic unit, Institute of Agricultural Research, ABU Zaria.

Percentage occurrence of fungi isolated was determined using the formula below (Gonzalez, 1995).

Percentage occurrence =

$$\frac{\text{Frequency of occurrence of fungi}}{\text{Total number of fungi}} \times 100$$

Molecular Identification of Fungi

Genomic DNA was isolated from mycelia in liquid medium (Phosphate Buffer Saline) using Accuprep genomic DNA extraction kits according to the manufactures recommendations through out the molecular studies. From the crude extract of DNA, two to three µl was used directly for PCR amplification.

The DNA ITS region was amplified using the universal primer pair ITS1; TCCGTAGGTAGGTGAACCTGCGG and ITS4; TCCTCCGCTTATTGATATGC. The method of Ali, (2002); Hussain *et al.* (2012) was adopted.

Gel elution;

Deoxyribo Nucleic Acid (DNA) was excised from the agarose gel and weigh in a 1.5 ml microtube. Three buffer GB (buffer GB is an agarose solubilization and DNA binding buffer used in DNA extraction from agarose gel) and incubated at 60^{o}C for 8 minutes and it was mixed by tapping the tube bottom every 2- 3 minutes till the gel melted completely. One gel of isopropanol was added to the mixture and mixed. 750µl of the mixture was transferred to a DNA mini column with a collection tube. It was then centrifuge at 11, 000 rpm for 1 minute. The flow through was discarded and the column was put back to the collection tube. The process was repeated. 750µl of DNA wash buffer containing ethanol was added to the column and centrifuge at 11,000 rpm for 1 minute. The flow through was discarded. The empty column was centrifuge at 11, 000 rpm for 1 minute to remove ethanol for optimal elution. The column was placed in a clean 1. 5ml microcentrifuge tube. Elution buffer (20µl) was added and incubated at room temperature for 1 minute. It was then centrifuge at 11, 000 rpm for 1 minute to elute the DNA. The samples were loaded on the sequencer.

Pathogenecity Test

Isolates were sub cultured onto PDA, once sporulating abundantly; they were flooded with sterilized distilled water, macerated in a blender and the suspension passed through four- folded layers of cheese cloth inside a funnel. To obtain a conidial suspension, concentration was standardized to the appropriate conidia/ml using a haemocytometer.

Seeds from three varieties of *P. sativum* were sown in sterilized pots (25cm in diammeter) containing sterilized soil. The pea plants were inoculated two weeks after emergence by spraying with appropriate conidial suspension of the different isolates. Shortly after inoculation, all seedlings were placed for 24 hours under an enclosure made of a polyethylene sheet to encourage humidity. The plants were observed daily for disease development (Mungo, 1996).

Statistical Analysis

The NCBI Blastx programed (http://www.ncbi.nlm.nih.gov/BLAST) V. 4 analysis was used to identify the fungi. Alignments of nucleotide sequences was done using Bio Edit sequence alignment editor software. Phylogenetic relationships of fungal isolates were analyzed using MEGA V. 7.0 software. Principle component analysis (PCA) was used to determine the most susceptible variety

RESULTS

Symptoms Fungal Disease using field guide by Hagedorn, (1991) of *P. sativum* Wilt disease which leads to drying of the roots which later causes foliage to loss its green color and causes death of the whole plant.

Initial symptoms of leaf blight are small circular brown spots on leaves which enlarges becoming surrounded by brown margin and light centers turning into extensive blighting on leaves and pods. Leaf spot disease causes development of circular to irregular brown spots on leaves which later turn dark brown. Lesions tan in color with well define brown margin on leaves and pods were observed to be symptoms of pod rot disease. Root rot leads to rooting of the root causing the plant to dry and die. Gray mold leads to production of powdery gray mycelia on leaf and pod as shown in Table 1.

Table 1: Fungal Diseases of *Pisum sativum*

FUNGAL DISEASE	SYMPTOMS	NAME OF FUNGI (Watanabe, 2002)
Wilt	Drying of the root which later cause foliage to loss its green color and wilting/death of the whole plant	*Fusarium solani, F. oxysporum* and *Pseudallescheria boydii*.
Leaf Blight	Initial symptoms on leaves are small circular brown spot which enlarges becoming surrounded by brown margin light centers turning into extensive brightening on leaves and pods.	*Rhizoctonia solani*.
Leaf Spot	Circular to irregular brown spots on leaves which later turns dark brown	*Alternaria alternata*.
Pod Rot	Lesions tan in color with well define brown margin on leaves and pod.	*Thielavia terricola*.
Root Rot	The root rots causing the plants to dry and die.	*Rhizoctonia solani*.
Grey mold	Plants become grey with powdery grey mycelia on both leaf and pod.	*Botrytis* sp.

Morphological Features of Isolated Fungi from Disease *P. sativum*

Microscopic observation showed macro and micro conida are present, macro conidia is curved and of average length with size range of 31.5 – 59.4 µm in length and width of 4.6 – 6.2 µm, micro conidia is 1- celled with size range of 7.2 – 15 µm length and width of 2.4 -3.9 µm, the organism was identified as *Fusarium solani*. *Pseudallecheria boydii* had conidia are curved size ranges from 8 – 40 x 5 – 10 µm length/width ratios. Microscopic examination reveals only macro conidia which has 2 -3 cells long curved at the edge with size range of 29.1 – 45 µm length and width of 2.9 – 4.8 µm it was identified as *Fuusarium oxysporum*. *Rhizoctonia solani* have microscopic character of light brown mycelium long septate with branches usually setting off from main hyphae. *Alternaria alternata* has conidia which are dark with both cross and longitudinal septa, it is variously shaped.*Curvularia eragrotidis* shows ovoid conidia two celled, with dark conidiophores *Thielavia terricola* conidia is small 1- celled hyaline ovoid to elongate, pycnidia is round black. *Botrytis* sp. have conidiophore tall, slender hyaline branched irregularly in upper portion bearing clusters of conidia. *Penicillium singorense* have Conidiophores arising from the mycelium single in synnema. *Trichoderma* sp. has 1-celled conidia hyaline borne in small terminal

clusters. *Chaetophoma* sp. has pycnidia dark small globose to irregular without ostiole, clusters of conidia hyaline 1- celled ovoid. *Chrysosporium* sp. with conidiophore.*Chaetomium* sp. has black pycnidia subglobose covered sparsely with bristles at the apex ar straight, ascospores are brown circular as indicated in Plate 1 to 4.

Molecular Identification of Isolated Fungi from *P. sativum* in Northern guinea savanna of Nigeria From the studies of wilted field pea plant, three fungal pathogens were isolated. Polymerase Chain Reaction (PCR) carried out on the samples shows bands with size of 600 base pairs. Lane 1 was identified after sequencing as *F. solani* which shows 100 % query cover and > 99 % percentage identity to *Fusarium solani* with accession number of MK271238.1. Lane 2 shows similarity to *Pseudallescheria boydii* showing similarity of 100 % query cover and 90 % percentage identity to *P. boydii* with accession number of KX788160.1. Lane 3 was found similar to *Fusarium oxysporum* with sequence query cover of 60 % and percentage identity of 90 % to *F. oxysporum* accession number MF576369.1 NCBI. Lane 4 is the control shows no bands as shown in Fig 1A.

Fig 1B shows PCR reaction using the primers ITS 1 and ITS 4. Size of the bands is 600bp. Lane 1 and lane 2 after sequencing were identified as *Curvularia eragrotidis* with 100 % query cover and >99 % identity to *C. eragrostidis* accession number MK182359.1. Lane 3 showed results of *Alternaria alternata* after sequencing with Query cover of 100 % and similarity of > 99 % to *A. alternata* accession number of MK518414.1. Lane 4 was identified as *Thielavia terricola*with sequence query cover of 100 % and percentage identity > 95 % to *T. terricola* accession no. MH443360.1. Lane 5 was identified as *Penicillium singorense* with query cover of 100 % and percentage identity of > 99 % to *P. singorense* with accession number of MH512975.1. Lane 6 shows no band it is control

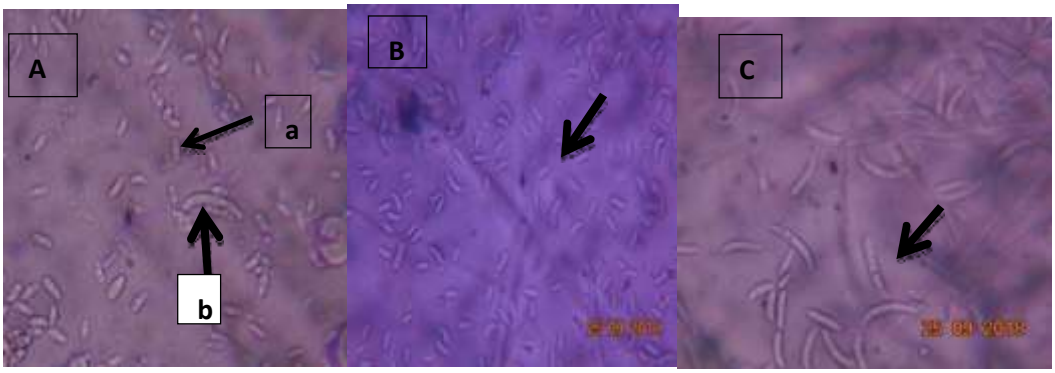


Plate 1: A= (a= micro conidia. b= Macro conidia).B= Conidia C= Macroconidia. Stain= Lactophenolcottonblue. Mg x 40.



Plate 2: A: Branched septate hyphae B= Conidia with both cross and longitudinal septa C= Conidia curved 2 or more cells. Stain= Lactophenal cotton blue. Mg x 40.

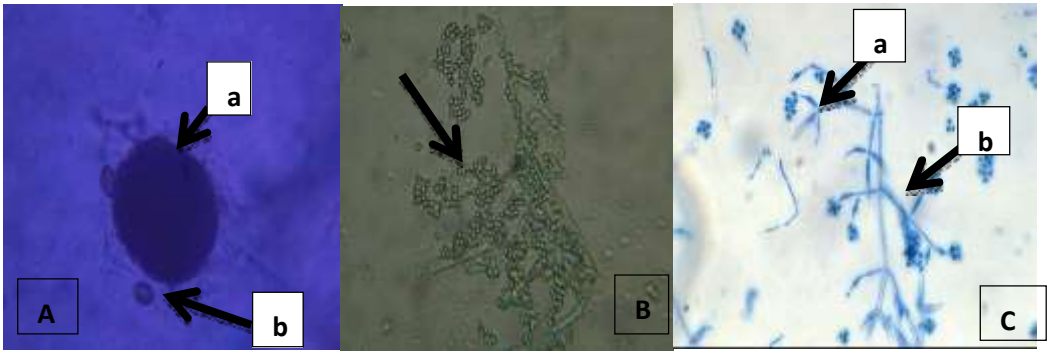


Plate 3: A= *T. terricola* Aa= Cleistotheciablak, Ab= Ascospore B= *Botrytis* sp. Conidia hyaline one cell. C= *Trichoderma*sp. Ca= Conidia hyaline borne in small clusters. Cb= Conidiophore hyaline. Stain Lactophenol cotton blue. Mg x40.

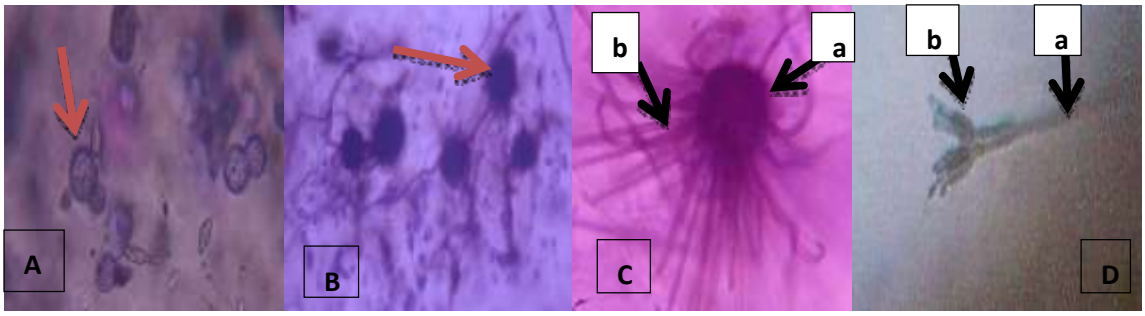


Plate 4: A=*Chrysosporium*sp. Conidia one cell globose. B= *Chaetophoma* sp. Ba= Pycnidia dark small globose. C= *Cheatomium*sp. Ca= Pycnidia black subgloboseCb= Appendeges on pycnidia. D= *P. singorense.* Ba= Conidiophore. Bb= hyphae. Stain= Lactophenol cotton blue. Mg x 40.

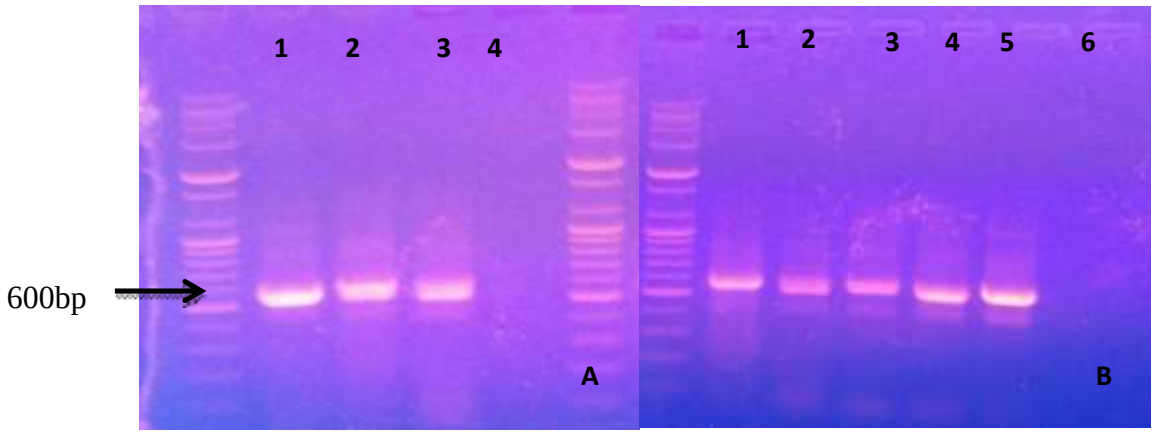


Fig 1= Polymerase Chain Reaction (PCR) using ITS1 and ITS4 Primers A= Wilt Fungi (Lane 1= *F. solani,* Lane 2= *P. boydii,* Lane 3= *F. oxysporum* and Lane 4= Control). B= (Lane 1 and 2 = *C. eragrotidis,* Lane 3= *A. alternata.* Lane 4= *T. terricola,* 5= *P. singorense,* Lane 6= Control)

Molecular Phylogeny of Isolated Fungi from *P. sativum* in Northern Guinea Savanna of Nigeria

Neighbor-joining phylogenetic tree showing the diversity of fungi isolated from Nigerian field pea (bold font) relative to global sequences (GenBank accession numbers shown). The tree is based on analysis of nucleotide sequences derived from ITS gene of the fungi is shown on Fig 4.8. The isolate **Tt** was related to previously identified *Thielavia* spp. and closely related to *Thielavia terricola* with bootstrap of 99 %. The isolate **Fs** closely related to *Fusarium solani* with bootstrap of 100 %. The isolate **Aa** was related to different species of *Alternaria* spp. and closely related to *Alternaria alternata.* The isolate **Ce** was related to previously identified *Curvularia*spp. and closely related to *Curvularia eragrosticola* with bootstrap of 99 %. The isolate **Pb** was closely related to other species of *Pseudallescheria*spp. with bootstrap of 100 %. The isolate **Ps** was

.

related to previously identified *Penicillium*spp.and closely related to *Penecilliumsingorense*with bootstrap of 98 % as indicated in Fig 2.

Percentage Occurrence of Fungal Isolates from *P. sativum* based on Locations Zangon danbarno has the highest percentage occurrence of *Rhizoctonia solani*. (55.6 %), *Fusarium* sp. (44. 5 %). Katanga has percentage occurrence of *Fusarium* sp. (35.7 %), *Rhizoctonia* sp. (28.6 %), *Alternaria* sp. (7.1 %), *Thielavia* sp. (14.3 %), *Botrytis* sp. (7.1 %) and *Chrysosporium*sp. (7.1 %). Shika dam has percentage occurrence of *Fusarium* sp. (33.3 %), *Curvularia* sp. (5.6 %).*Rhizoctonia* sp. (25 %), *Penecillium* sp. (5.6 %) and *Thielavia terricola* (41. 7 %). Sabuwa has the least percentage occurrence in all isolated fungi with 0 %. Rapiyan fan has percentage occurrence of *Fusarium* sp. (44.5 %), *Rhizoctonia solani* (16.7 %),

Curvularia sp

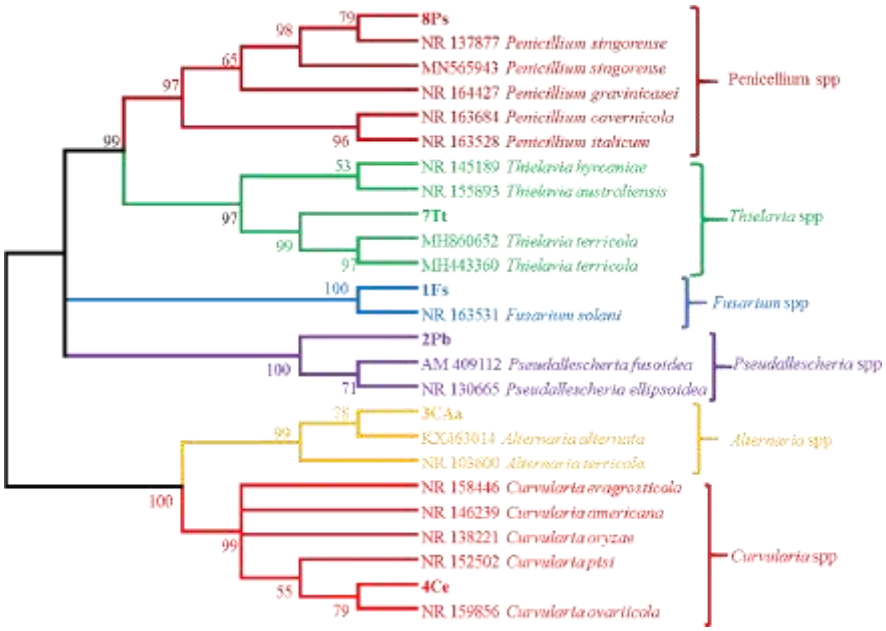


Fig 2: Neighbor-joining phylogenetic tree showing the diversity of fungi isolated from Nigerian field pea (bold font) relative to global sequences (GenBank accession numbers shown). The tree is based on analysis of nucleotide sequences derived from ITS gene of the fungi.

(18.0 %), *Alternaria* sp. (16.7 %), *Thielavia* sp. (11.1 %), *Pseudallascheria* sp. (5.6 %) and *Trichoderma* sp. (5.6 %). Razek fan has percentage occurrence of *Fusarium* sp. (37. 5 %), *Rhizoctonia* sp. (18.8 %), *Culvularia* sp. (17 %), *Alternaria* sp. (18.8 %), *Thielavia* sp. (12.5 %), *Cheatophoma* sp. (6.3 %), and*Chaetomium* sp.(6.3 %) as shown in Fig 3.

Pathogenicity Tests for Fungal Isolates on Two Varieties of *P. sativum*

Principal component analysis on two varieties of field pea is shown on Fig 4. However, the green seeded short variety is more susceptible to *Alternaria* leaf spot, *Rhizoctonia* blight and *Rhizoctonia* root rot, *Phoma* pod rot, *Botrytis* gray mold and *Fusarium* wilt than the gray seed tall variety.

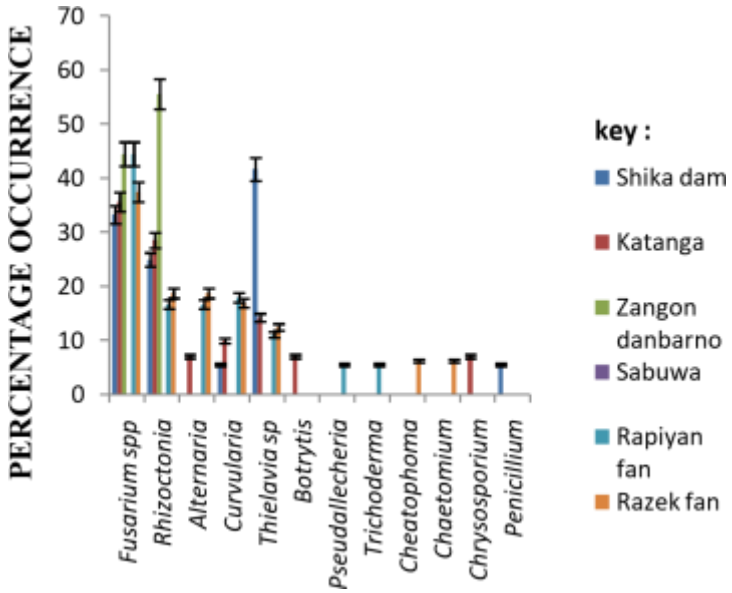
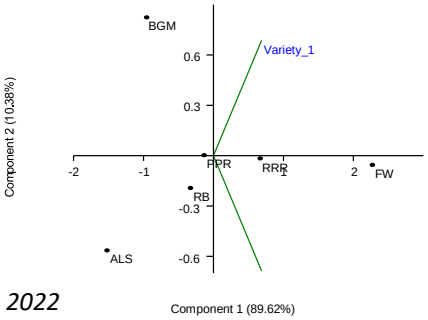


Fig 3: Percentage Occurrence of Fungal Isolates from *P. sativum* in Northern Guinea Savanna Nigeria



Key: BGM = *Botrytis* gray mold, FW = *Fusarium* wilt, PPR= *Thielavia* pod rot,
ALS = *Alternaria* leaf spot, RRR = *Rhizoctonia* root rot, RB = *Rhizoctonia* blight, Variety 1 = Gray seeded tall variety

Fig 4: Principal component analysis on two varieties of *P. sativum*

DISCUSSION

Fusarium solani was isolated from wilt disease samples from Zangon danbarno, Katanga and Shika dam. Sequencing results shows >99 % similarity to *F. solani.* This report is in line with the report of (Porter *et al.,* 2015). Babu*et al.,* (2014) reported *Psuedallescheria boydii* on crop field soil in Korea. The fungus is soil borne and is strongly promoted by agricultural and industrial pollution (Guarro*et al.,* 2006; Mark *et al.,* 2008). In this study, the fungi was isolated from the roots of wilted field pea in plateau state of Nigeria, since the fungi have been reported to be soil borne this suggest possible transmission of the pathogen from the soil to the root of field pea. The agricultural and industrial effluents in the state might have favored the survival of *P. boydii*.*Fusarium oxysporum* was isolated and identified from wilted field pea from Katanga, Shika dam and Zangon danbarno irrigated fields and Rafiyan fan and Razek fan rain- fed fields in Plateau state. This report is in line with the reports of Kapoor*et al.,* 2010; Kuldhar*et al.,* 2013; Jeetendra, 2014).*Rhizoctonia solani* was isolated from both leaf blight and root rot disease of field pea in these studies. *Rhizoctonia* root rot was only recorded in Zangon danbarno while *Rhizoctonia* leaf blight was recorded in Zangon danbarno, Katanga and Shika dam. Hwang *et al.,* (2006) reported *Rhizoctonia* seedling blight on field pea in Canada. The identification of *Rhizoctonia solani* to cause root rot disease in field pea agrees with the report of (Abdulaziz and Rashad, 2010; Mathew *et al.,* 2012; Laxmirawat *et al.,* 2014; Sharma *et al.,* 2015).

Alternaria alternata size and shape of conidia is similar to the report of Schwartz, 1980; Watanabe, 2002). The isolation and identification of *A. alternata* from leaf spot disease of field pea is in line with the report of (Laflamme, 1986). *Curvularia eragrotidi* swas found to induce leaf spot on field pea in northern guinea savannah of Nigeria, however, Ferreira *et al.,* (2014) have reported *Curvularia eragrotidis*to cause leaf spot on other host plants *Thielavia terricola* was isolated from pod rot on field pea in this study. The morphology was similar to the report of Raj kumar and Aneja, 2007; Mahesh *et al.,* (2016). *Botrytis* gray mold was observed on field pea plant in this study from the samples in Katanga. The symptom was similar to the report of Pande *et al.,* (2001).

The occurrence of all these pathogenic fungi discussed above have been reported on other host plants especially cereals, tomato and potato Emechebe *et al.,* (1980); Bagwari *et al.,* (2014); Modibbo *et al.,* (2016). Tobih *et al.,* 2016;Ajayi- ojetunde and Bradley, (2017); Sarika *et al.,* 2017; *Botrytis* blight, 2018; Donald, 2019; Kiersten *et al.,* 2019; Su-Han *et al.,* 2019). This suggest possible transmission of the fungus from rice to files pea as field pea was found to be in rotation or adjacent to field pea farms. Phylogenetic relationship shows that the fungal isolates were similar to isolates from China, Argentina, Canada, America, Tajistan among others, this suggest possible importation of the isolates along with the seeds since *P. sativum* is an exotic plant in Nigeria.

Penicillium singorense, *Trichoderma*, *Chrysosporium*, *Cheatophoma* and *Cheatomium* spp. are saprophytic fungi. The fungi might have invaded the infected plant tissues after invasion of the pathogenic fungi. These fungi have been reported as saprophytes by many researchers (Hunter and Barnet, 1999; Alemu, 2014; Rasime *et al.,* 2013; Cobus*et al.,* 2015). To the best of our knowledge, this is the first record of causative fungi of wilt, root rot, leaf blight, leaf spot, pod rot and gray mold diseases on field pea in Northern guinea savanna of Nigeria.

The high percentage occurrence of

Fusarium spp. and *Rhizoctonia solani* which are soil borne fungi observed in Zangon danbarno may be attributed to the practice of crop rotation with susceptible hosts. While *Alternaria* and *Curvularia* spp. were found to have high percentage occurrence in Rapiyan fan and Razek fan which may be attributed to high relative humidity in the sampling locations which favored the development of the fungi.

CONCLUSION

A total of six fungal diseases were recorded on field pea in Northern guinea Savanna of Nigeria which include; wilt caused by *Fusarium solani, F. oxysporum* and *P. boydii*, leaf spot caused by *A. alternata* and *C. eragrotidis*, Leaf blight and root rot caused by *R. solani*, Pod rot caused by *T. terricola*and mold caused by *Botrytis* sp.

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