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Germination and seedling growth of groundnut seeds infected with *Macrophomina phaseolina* isolates

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Abstract

Macrophomina phaseolina is a common contaminant of groundnut (*Arachis hypogaea* L.) seeds but not much information has been documented about its adverse impact on seed health and germination. This study was carried out to determine the incidence of *M. phaseolina* on groundnut (*Arachis hypogaea*) seeds displayed for sale in local markets, characterize the isolates and determine how the fungal presence would affect the germination and seedling growth, so as to provide information on seed handling practices and planning strategies for reducing crop loss due to the fungus. The fungus was isolated using the direct plating inoculation method and then grown for a period of 7 days on a potato dextrose agar (PDA) with temperature set at 28 ^{o}C. The DNA was extracted from pure cultures and PCR was performed using primer in pair: ITS4 and ITS5. The products got from the PCR were sequenced for identification of species on the National Centre for Biotechnology Information (NCBI) database. Phylogenetic tree was generated based on the ITS1-2 gene sequences of the isolates. The 18 *M. phaseolina* isolates adopted for use in this study were all obtained from apparently healthy seeds of different varieties of groundnut sourced from local markets in Delta and Edo States, Nigeria. The experiment on germination of groundnut seeds was set up in a completely randomized design in ten replicates. The individual *M. phaseolina* isolates inhibited germination and seedling growth in varying degrees. The virulence of the isolates ranged from moderately pathogenic (50%) to extremely pathogenic (75%). Out of the 18 isolates, 14 were found to cause 100% mortality. The inoculated plants exhibited the disease symptoms (damping off, root rot, collar rot, necrosis, wilting and chlorosis) while the control remained healthy. Seedling emergence, number of leaves, root length, and stem girth of infected plants decreased when compared to the control.

Keywords: Pathogenic; ITS; Biodiversity; PCR; Phylogeny

1 Introduction

Groundnut (*Arachis hypogaea* L.), thought to have original root from South America[1], is a very useful and valuable food and cash crop used by lots of countries all over the world and it contributes immensely in solving the problem of food security and poverty reduction in some developing countries, including African nations[2]. Groundnuts are grown for their oil and protein content. Groundnut has a wide range of applications; as all parts of the plant is necessary for usage. The nut of the plant is very high in edible oil (ranging between 36 to 54%) and protein: ranging between 25 to 30% [3]. The nuts may be eaten directly in form of processed foods/snacks as good source of minerals, protein, oil and energy meals and confectioneries[4]. In addition, it provides high-quality fodder for livestock [5]. Groundnut as a plant that thrive well in the tropics needs a prolonged warm season for efficient growth.

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One major factor that impedes on profitable cultivation of groundnut is the incidence of several diseases, mainly caused by fungi, which causes huge crop losses at all stages of growth, from sowing to harvest and storage. *Aspergillus tamari, Lasiodiplodia iranensis, Macrophomina phaseolina, Penicillium citrinum, Aspergillus oryzae*, and *Aspergillus pennicillioides* were isolated from groundnut seeds by [6]. [7] and [8] reported *A. flavus, A. niger, Rhizoctonia solani. Macrophomina phaseolina, Rhizopus spp*, and *Fusarium spp*. as being responsible for reduction in plant stands due to pre-emergence and seedling rot of groundnut. A great number of plants (over 500 species) including some most significant crops in the world, such as corn, cotton and soybean, cotton are infected by the fungus *Macrophomina phaseolina* (Tassi) Goid: the cause of charcoal rot[9,10]. According to reports, charcoal rot may cause losses in yield up to 60% [11,12]. According to[13], the groundnut seed content was negatively impacted by the fungi *Aspergillus niger*, *Aspergillus flavus*, and *Macrophomina phaseolina*.[14] also reported that *M. phaseolina* infection affects the normal growth of groundnut and its content of chlorophyll, mainly in two groundnut varieties.

Seeds of groundnut deteriorate in storage due to fungal seed contaminants, which are responsible for not only decreasing oil content but also compromising germinability, whereas *M. phaseolina* broad host range suggests that it is a non-host specific fungus [12]. Increased temperatures ranging from 30-35°C and soil moisture that is low is favorable condition for *M. phaseolina*-caused diseases (e.g., seedling blight, stem rot, seedling blight and charcoal rot and root lot) [15]. This study was aimed at investigating the symptoms caused by *M. phaseolina* isolates and to see how the pathogen affected the germination of groundnut and the growth of the seedlings in Delta and Edo States in southern Nigeria, in order to contribute to information needed for planning control strategies for crop loss due to *M. phaseolina*.

2 Materials and Methods

2.1 The study area

Delta State is located between 5° 42'14.40" N, 5° 56'2.04" E. It is situated in the region of Nigeria known as the South - South geopolitical zone. Edo State is located between 6° 38' 3.12" N, 5° 55'49.44" E and situated between the Southern and Western parts of Nigeria (Figure 1 and 2). Groundnut is largely grown in large quantities in northern Nigeria. Several studies on groundnut sold in various Northern Nigerian states have been conducted. However, there are little or no studies done in the Southern part of Nigeria (In this case) Edo and Delta States.

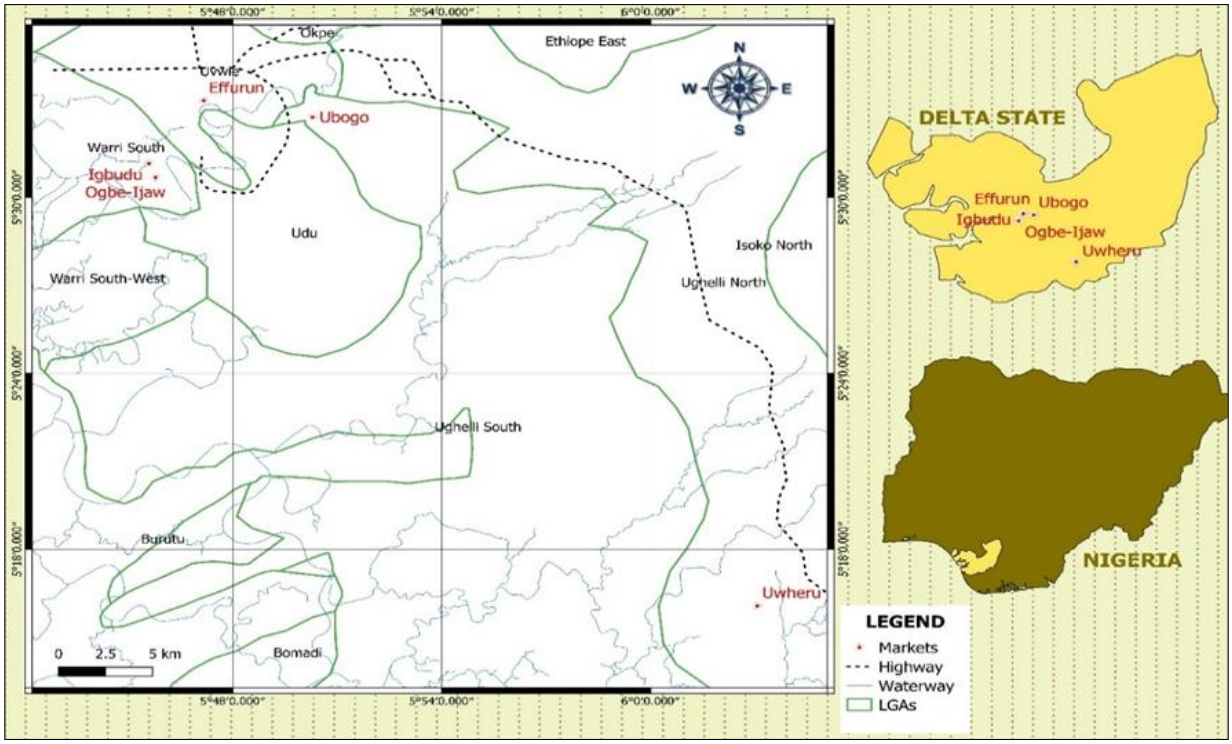


Figure 1 Sites of sample collection from Delta State

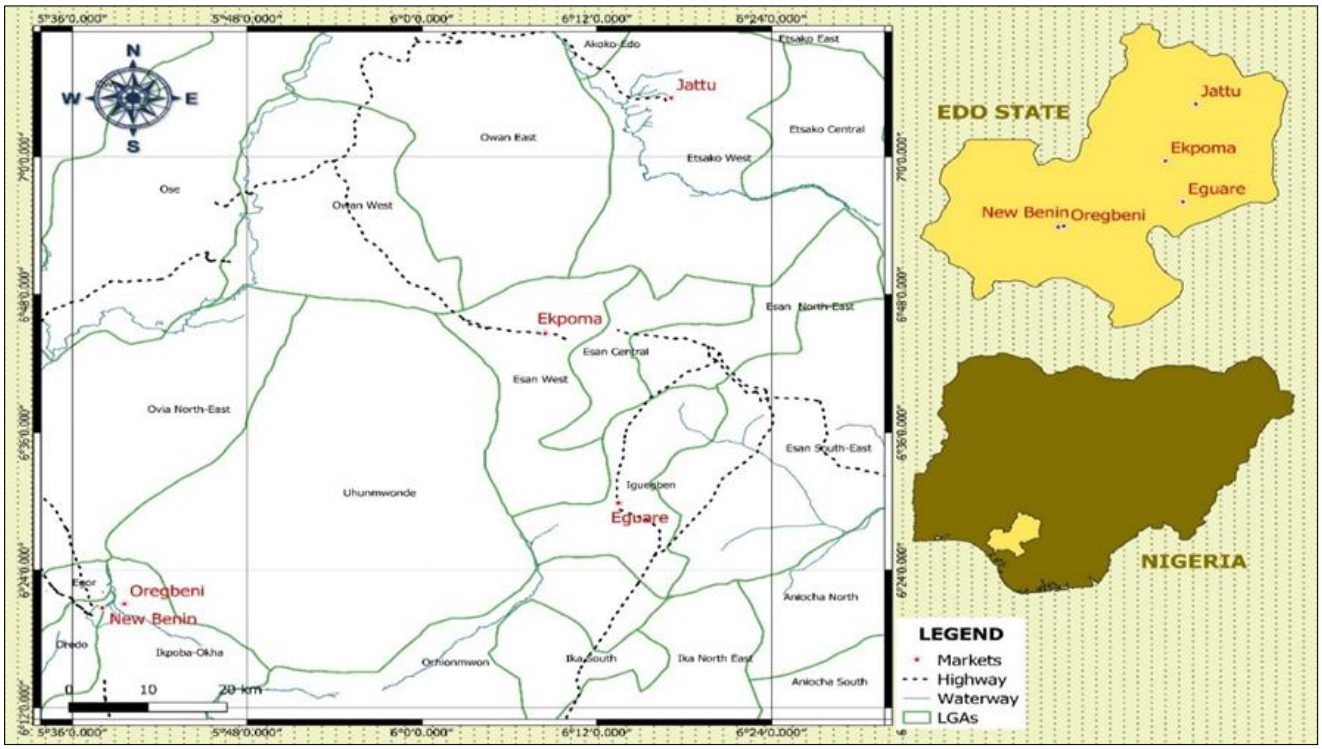


Figure 2 Sites of sample collection from Edo State

2.2 Sampling technique and sample size

Five markets each were selected from Delta and Edo States where groundnuts were sold in large quantities. The groundnut seeds were bought, labelled and stored in an airtight black polythene bag and kept in a refrigerator for adequate preservation until when needed.

The markets sampled included Igbudu, Ogbe-ijaw, Ubogo, Uwheru and Effurun markets in (Delta State) and Eguare, New Benin, Jattu, Oregbeni and Ekpoma markets (Edo State). Total number of markets sampled was ten.

2.3 Sample collection

Field trips were made to the different markets in Delta and Edo States, sample collections were made from the specified locations. Sample collection from Delta State was made in February 2017 and from Edo State, March 2017.

2.4 Isolation of the *M. phaseolina* isolates associated with groundnut seeds collected from the different markets

2.4.1 Culture media preparation

Using potato dextrose agar, the fungi associated with the various groundnut seed samples were isolated (PDA, Oxoid, England). The culture medium was autoclaved at 15psi (121 ^{o}C) for 15 minutes and preparation was based on the instruction of the manufacturer. Chloramphenicol was added to the media at the time of pouring to suppress the growth of bacteria (at a concentration of 0.02 gm per 200 ml of medium). After wiping with ethanol, inoculation and culture transfer were performed on a sterile inoculating bench.

2.4.2 Sterilization

All glassware was washed with soap, rinsed with numerous changes of tap water, distilled water and allowed to dry. They were sterilized for 24 hours at 60^{o}C in an electric oven model OVE.200.030Y.

2.4.3 Sample preparation and inoculation

The direct plating inoculation method was used for isolation of the fungi in relation with the different seeds of groundnut. This was done by carefully teasing the different groundnut samples into smaller bits using a pair of forceps

surface sterilized using cotton wool soaked with 70% ethanol and then placed in a sterilized Petri dish, after which, the teased groundnut samples were placed in an already prepared PDA in Petri dish.

2.4.4 Determination of microbial load

The microbial load of the samples were visibly determined through counting of the colony forming unit (CFU) at interval of 72hrs for fungi growth.

2.4.5 Macroscopic identification of the fungal isolates

Fungi associated with the groundnut samples were then subcultured into a freshly prepared PDA on Petri dishes, after growth it was then examined and identification was made based on their cultural and microscopic characteristics according to the methods described by [16]. The fungi were microscopically identified by examining the shape and texture of the conidia under microscope at ×40 magnification. The Commonwealth Mycological Institute Surrey received ten random isolates suspected to being *Macrophomina phaseolina* for confirmation. The identification of the isolates with the Ref: E0000375 was confirmed by CABI Identification service UK. Further characterization was done at the Regional Center for Biotechnology and Bio-resources Research Laboratory, University of Port Harcourt, Choba, Rivers State. The product of the PCR sequencing was conducted at the International Institute of Tropical Agriculture (IITA), Ibadan.

2.5 Fungal DNA Extraction

The modified version of the manufacturer's instructions for the Quick-DNA™ Fungal/Bacterial MiniPrepKit (Zymo Research Group) was adopted for the extraction of Genomic DNA.

Using a surgical blade that was heat sterilized, the mycelium of each pure fungal culture was removed from the surface of the culture medium and placed into a sterile mortar and pestle. With liquid nitrogen, the mycelia were individually frozen (-196 ^{o}C). Each mortar was filled with 750 l of the lysis solution before being blended with a pestle. Each sample was placed in an Eppendorf tube (1.5 ml), to which 200 l of distilled water was added. The samples were then centrifuged at 10,000 x g for one minute in a refrigerator. Up to 400l of supernatant was now taken to a collection tube containing a Zymo-Spin IV Spin Filter (orange top) and now centrifuged at 7,000 x g for 1 minute. The filtrate present in the collection tube was combined with 1,200 l of Genomic Lysis Buffer, and 800l of the mixture was transferred to a Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x g for 1 minute. The flow from the collection tube was made away/or discarded followed by the repetition of the preceding steps. In a new collection tube, 200l of DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column and centrifuged at 10,000 x g for 1 minute. In a new collection tube, 500 µl g-DNA Wash Buffer was added to the Zymo-Spin IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin IIC Column was transferred to a clean 1.5ml microcentrifuge tube, 100l of DNA Elution Buffer was added directly to the column matrix, and the DNA was eluted by centrifugation at 10,000 x g for 30 seconds.

2.5.1 Determination of DNA quality using nanodrop

The NanoDrop 2000 c spectrophotometer was used to test the quality and purity of DNA (Thermo fisher Scientific Inc. Wilmington, Delaware, USA). Purity was measured as a ratio of Ultraviolet (UV) light absorbance at 260 nm to that of 280 nm. The NanoDrop was connected to a computer system, and the sensor was cleaned using a cotton wool and 70 % ethanol. 1µl of Elution buffer (the solution used to re-suspend the DNA) was dispensed directly on top of the NanoDrop sensor. The Nanodrop determined the blank and the DNA samples (1 µl) were separately loaded onto the sensor. The sensor was usually wiped when a new sample was to be loaded to avoid contamination. Nanodrop measurement was taken in triplicates for each sample.

2.5.2 Gel electrophoresis

This was done by utilizing 1.5 % agarose gel. 0.75 g of agarose powder was combined with 50 ml of TrisBoris EDTA (TBE) 1X inside a measuring flask followed by microwaving for 2 minutes to get a solution that is clear. The contents inside the conical flask were mixed with 5 µL of EZ viewing dye (Blue Light) and then emptied onto the casting tray or gel holder. The comb was put in the casting tray and left to sit at room temperature for 20 to 30 minutes to allow the gel to solidify. A set up of the electrophoretic gel unit was made, the gel holder which contains the gel was put into the gel tank and TBE 1X was emptied into the gel tank to an extent the gel becomes fully submerged. Molecular weight marker (1Kb DNA Ladder) was loaded into the first lane, and the DNA samples were loaded separately into the wells that was created by the comb on the gel. Each DNA sample (3 µL) was combined with 3 µL of 2X loading dye and then loaded in one lane on the gel. A control which has in it, the various components of the mixture of the PCR reaction with the

exception of the DNA template was also loaded. The setup was made to run for 40 minutes at 100 volts. After the experiment, the observation of the DNA fragments were made with a UV transilluminator.

2.5.3 PCR amplification and sequencing

To amplify nuclear ribosomal DNA fragments, the fungal universal primers ITS4, forward (5'-CCTCCGCTTATTGATATGS-3') and ITS5, reverse (5'-GGAAGTAAAAGTCGTAACAAGG-3') (Lane, 1991) were used (rDNA). The PCR was carried out in a 25 L, where final volume contains 3 L of genomic DNA (10ng/L), 0.1 L of Taq polymerase, 2.5 L of 10X PCR buffer, 1.0L of DMSO, 1.0 L of 2.5 mM DNTPs, 1.0L of 25 mM $MgCl_2$ (Promega), 1.0 L of each primer (concentration of 5 M), and 13.4 L of Nuclease-free water. Amplifications were carried out in a thermal cycler with an initial denaturation step of 94 °C for 5 minutes, followed by 36 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and elongation at 72 °C for 45 seconds, with a final elongation step of 72 °C for 45 seconds at 72 °C for 7 minutes. The amplicon was kept at 10 °C. The amplicon from the previous reaction was electrophoresed in 1.5 % agarose gel with TBE 1X and stained with EZ-Vision gel. Bioline's (1kb) Hyper ladder (London, United Kingdom) was used. The setup was run for 40 minutes at 100 volts, and a photograph of the gel was taken with the aid of a UV light (with Enduro Gel Documentation System, Aplegen, California, USA).

Amplified products were Sanger sequenced on an ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, United States of America). For sequencing, a DNA template, a DNA polymerase, a DNA primer, di-deoxynucleotide triphosphates (ddNTPs), and deoxynucleotide triphosphates (dNTPs) were used. Because they lack a 3'-OH group, that brings about the the formation of a phosphodiester bond between two nucleotides, each deoxynucleotide (ddNTP) that is brought and fixed at intervals terminated the chain of the DNA elongation. This halted DNA extension.

Following sequencing, a comparison of the obtained sequences were made with known sequences by making deposits of the sequenced samples of DNA to the National Centre for Biotechnology Information (NCBI) database by utilizing the Basic Local Alignment Search Tool (BLAST).

2.5.4 Phylogeny

Sequences obtained were trimmed and edited on MEGA X software. Phylogenetic tree was generated based on the ITS1-2 gene sequences of the isolates. BLAST hits aligned by Clustal X were used to construct the phylogenetic tree. The trees constructed showed the phylogenetic relationships between the fungal isolates from *Arachis hypogaea* and other fungal species on GenBank. The Neighbour Joining (NJ) approach was used to infer the evolutionary history [17]. The maximum composite likelihood approach was used to calculate the evolutionary distances [18]. MEGA X was used to do the evolutionary analyses [19].

2.6 Determination of the effect of *M. phaseolina* on the *In Vitro* germinability of groundnut seeds

The viability of groundnut seeds was determined using the floating methods described by [20]. For 30 minutes, 1000 seeds from each groundnut sample were soaked in a water bath with distilled water. The submerged seeds were used to study growth parameters, after which twenty seeds were selected and placed in a sterilized Petri dish with filter paper soaked in distilled water. The groundnut seeds in the Petri dish were then inoculated with an *M. phaseolina* spore suspension. As a control, groundnut seeds inoculated with distilled water was used. Careful observation on the emergence of seedlings in the controlled and treatments experiment were made and proper record ensured. The calculation of the percentage seedling emergence in each treatment was made based on the methods described by [21]:

$$\text{Emergence percentage (E \%)} = \frac{\text{Number of seedling that emerged} \times 100}{\text{Total number of seed sown}}$$

Seedling height and girth measurements were taken daily in each treatment for 5 weeks after planting (5WAP). The seeding girth was measured with a Vernier Caliper, and with the aid of a metre rule, the seedling height measurements were taken. The girth and height mean values were calculated and recorded. A calculation of the leaf area was made by Leaf area was calculated by making a multiplication of the length of the leaf and width of the leaf by the correlation coefficient (r) of 0.72, as suggested by [22]. The experiment was terminated at 5WAP due to plant death

2.7 Effect of culture filtrates of test fungi on germination and seedling growth of groundnut

Eighteen identified isolates of *M. phaseolina* from groundnut seeds were used. The organisms were inoculated into sterile conical flasks with ten grams of healthy surface-sterilized seeds. A 1.5cm diameter cork borer was used to pick the test fungus, which was then aseptically transferred into the sterile conical flasks and 10ml sterile distilled water. The conical flasks were vigorously shaken to obtain a homogeneous mixture before being kept in the dark for a duration

of 24 hours. The control seeds were subjected to the same procedure, but they were not inoculated. A germination test was performed. Each black polythene bag was filled with sterile sandy loam soil collected from the Edwin Clark University Staff Quarters. Each bag contained five infected groundnut seeds.

The treatments were carried out five times. The experiment required the use of 95 polythene bags. The bags were kept in the greenhouse at Edwin Clark University, Department of Biological Sciences Kiagbodo and were irrigated with water every 24 hours. A Completely Randomized Design was used for the experimental design (CRD). The appearance of the cotyledon above ground between 2 and 7 days after planting was used to record the germination of each treatment. Leaf spots, wilt, and blight were observed and recorded as disease symptoms. The growth parameters measured were stem girth, seedling emergence, shoot length, leaf area and leaf number. A weekly count of emerging leaves was conducted for a period of five weeks. Measurement of the length of root was made as well, from the root collar to the terminal bud using a meter rule. A vernier caliper was used to measure the stem girth length. In order to confirm the isolates identity, leaves from sampled were taken to the lab for re-isolation after the experiment was observed for 5 weeks.

2.8 Data Analysis

MEGA X software [19] was used to remove sequencing errors from the raw sequences of the ITS1-2 genes. The fungal ITS gene sequences were compared to sequences in GenBank. MEGA X was used to align the sequences using Clustal W, and the phylogenetic tree was constructed using BLAST hits with the highest identity threshold and query cover. Demographic data were presented as percentages.

3 Results

3.1 Gel Electrophoresis

On a 1 % agarose gel, the quality of the genomic DNA was determined. All the isolates extracted DNA was of high quality, as shown by the presence of bands on gel in Figure 3.

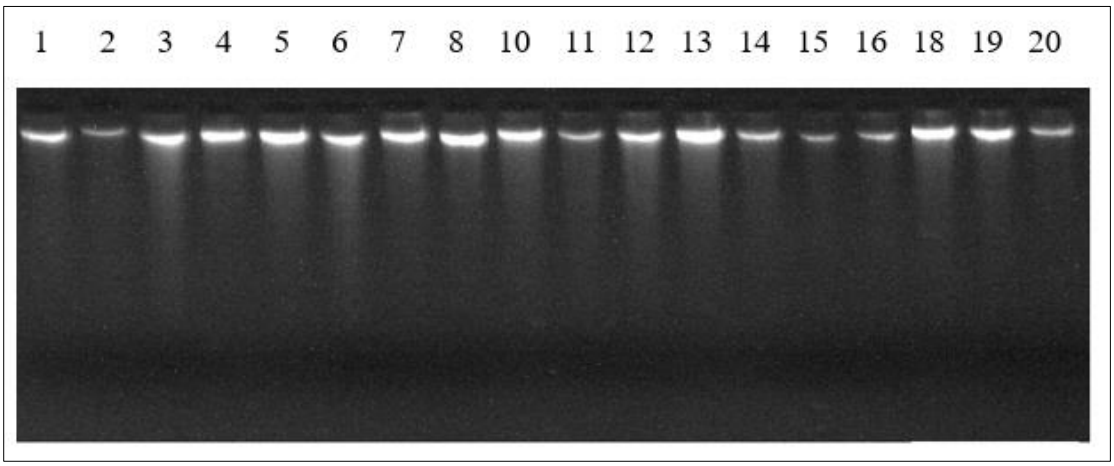


Figure 3 Gel electrophoresis of genomic DNA extracted from *Macrophomina phaseolina* isolated from *Arachis hypogeae*

3.2 Polymerase Chain Reaction

ITS4, forward (5'-TCCTCCGCTTATTGATATGS-3') and ITS5, reverse (5'-GGAAGTAAAAGTCGTAACAAGG-3') universal primers were used to successfully amplify extracted genomic DNA. Figure 4 shows the banding pattern produced by each species on agarose gel.

3.3 Amplified Products

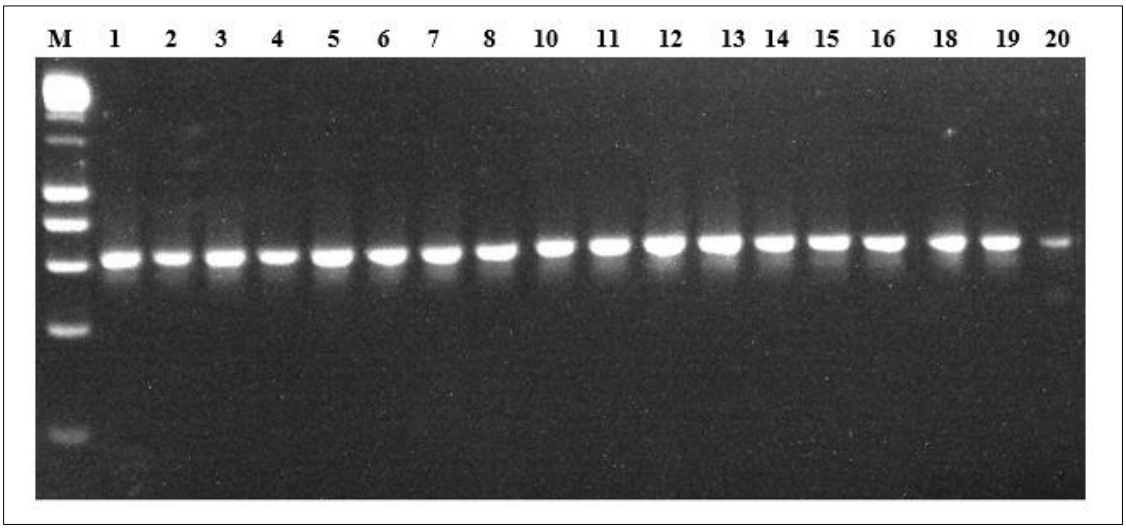


Figure 4 Gel electrophoresis showing the results of the amplification of PCR gotten from *M. phaseolina* DNA samples isolated from *Arachis hypogeae* using fungal Universal Primers; ITS4 and ITS5. M- DNA Marker (1Kb Ladder from Bioline); Bands are based on the alignment of their sequenced ITS region with published sequences in the NCBI database

3.4 DNA Sequences and blasting

The BLAST sequenced results showed and proved that all of the unknown fungal samples were *Macrophomina phaseolina* Tassi Goid. In order to allot accession number to each sequence, the fungal isolates sequences were deposited at the GeneBank: Table 1, while the nucleotide sequence obtained from the different *M. phaseolina* strain is presented in Table 2

Table 1 Putative taxonomic affinities of sequence types inferred from BLAST Searches of ITS sequences obtained from fungal isolates of *Arachis hypogaea* (L.)

Sample Origin		**Host (Groundnut)**	**Putative taxonomy affinity (Gene bank number)**	**% Similarity**	**Nucleotide length (bp)**	**Accension number**	**Strain**
ID	**(State/Market)**						
A1	Edo/Jattu	Ogoja	*M. phaseolina* (MK454909.1)	99.82	573	MN603095	RCBBR_AEAOL1
A2	Edo/Jattu	Ogoja	*M. phaseolina* (MK454909.1)	99.63	576	MN603095	RCBBR_AEAOL2
A3	Edo/Jattu	Gyada	*M. phaseolina* (MN603095.1)	99.81	574	MN689697	RCBBR_AEAOL3
A4	Edo/Jattu	PeelyPeely	*M. phaseolina* (MN603095.1)	99.81	575	MN689698	RCBBR_AEAOL4
A5	Edo/Egwuare	Red groundnut	*M. phaseolina* (MH864182.1)	99.62	577	MN689699	RCBBR_AEAOL5
A6	Edo/Egwuare	Gyada	*M. phaseolina* (MH864182.1)	99.62	576	MN689700	RCBBR_AEAOL6
A7	Edo/Ekpoma	PeelyPeely	*M. phaseolina* (MH864182.1)	99.62	577	MN689701	RCBBR_AEAOL7

A8	Edo/Ekpoma	PeelyPeely	*M. phaseolina* (MH864182.1)	99.62	575	MN689702	RCBBR_AEAOL8
A10	Edo/Ekpoma	Gyada	*M. phaseolina* (MN603095.1)	100	575	MN689703	RCBBR_AEAOL10
A11	Delta/Effurun	PeelyPeely	*M. phaseolina* (MH864180.1)	99.45	576	MN689704	RCBBR_AEAOL11
A12	Delta/Effurun	Khaki	*M. phaseolina* (KF951678.1)	99.44	583	MN689705	RCBBR_AEAOL12
A13	Delta/Effurun	Ogoja	*M. phaseolina* (MH864184.1)	99.26	575	MN689706	RCBBR_AEAOL13
A14	Delta/Effurun	Gyada	*M. phaseolina* (HQ649832.1)	99.63	577	MN689707	RCBBR_AEAOL14
A15	Delta/Ubogo	Gyada	*M. phaseolina* (MK454909.1)	99.45	578	MN689708	RCBBR_AEAOL15
A16	Delta/Ubogo	Gyada	*M. phaseolina* (MK454909.1)	99.82	575	MN689709	RCBBR_AEAOL16
A18	Delta/Igbudu	Red groundnut	*M. phaseolina* (MK454909.1)	99.63	574	MN689710	RCBBR_AEAOL18
A19	Delta/Igbudu	Ogoja	*M. phaseolina* (MH864181.1)	99.45	576	MN689711	RCBBR_AEAOL19
A20	Delta/Ogbijaw	Gyada	*M. phaseolina* (HQ649832.1)	99.63	576	MN689712	RCBBR_AEAOL20

Table 2 Nucleotide sequence obtained from different *Macrophomina phaseolina* strain

Strains	Sequences
RCBBR_AEAOL	GGTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGACG
RCBBR_AEAOL	GGTCACCTTGTAGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTTCTACTACGCTTGAGGCAAGAC
RCBBR_AEAOL	TCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGACG
RCBBR_AEAOL	GTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGACG
RCBBR_AEAOL	G-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGAC
RCBBR_AEAOL	GGTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGAC
RCBBR_AEAOL	GGTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGAC
RCBBR_AEAOL	GGTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGAC
RCBBR_AEAOL	GGTCACCTTG-AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTATGAGGCAAGAC

RCBBR_AEAOL	TCTGATCGAGGTCACCTTG- AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTA TGAGGCAAGAC
RCBBR_AEAOL	GAGGTCACCTTG- AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTA TGAGGCAAGACG
RCBBR_AEAOL	GAGGTCACCTTG- AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTA TGAGGCAAGACG
RCBBR_AEAOL	TCGAGGTCACCTTG- AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTA TGAGGCAAGACG
RCBBR_AEAOL	GGTCACCTTG- GAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTAC GAGGCAAGACGC
RCBBR_AEAOL	TCGAGGTCACCTTG-- GAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTAC GAGGCAAGACG
RCBBR_AEAOL	GGTCACCTTG- AGAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTA TGAGGCAAGACG
RCBBR_AEAOL	GAGGTCACCTTG- GAAAGTTCAGAAGGTTCGTCCGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTAC GAGGCAAGACGC

3.4 Phylogenetic analysis

The results are shown in figure 5a and 5b

3.4.1 Original tree

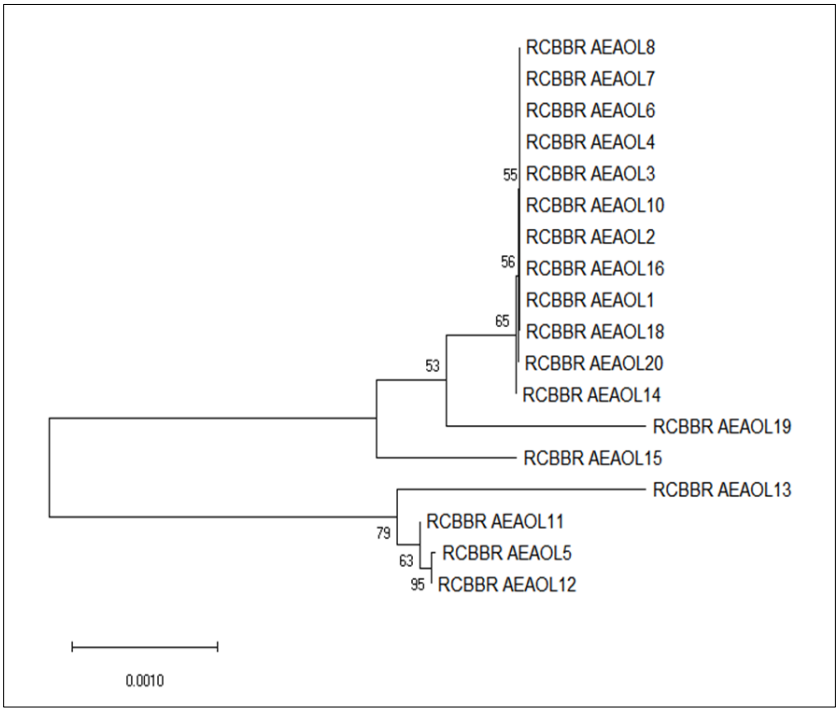


Figure 5a Molecular Phylogenetic analysis of *M. phaseolina* isolates of *Arachis hypogaea* using maximum composite likelihood method from Edo and Delta States, Nigeria

3.4.2 Bootstrap tree

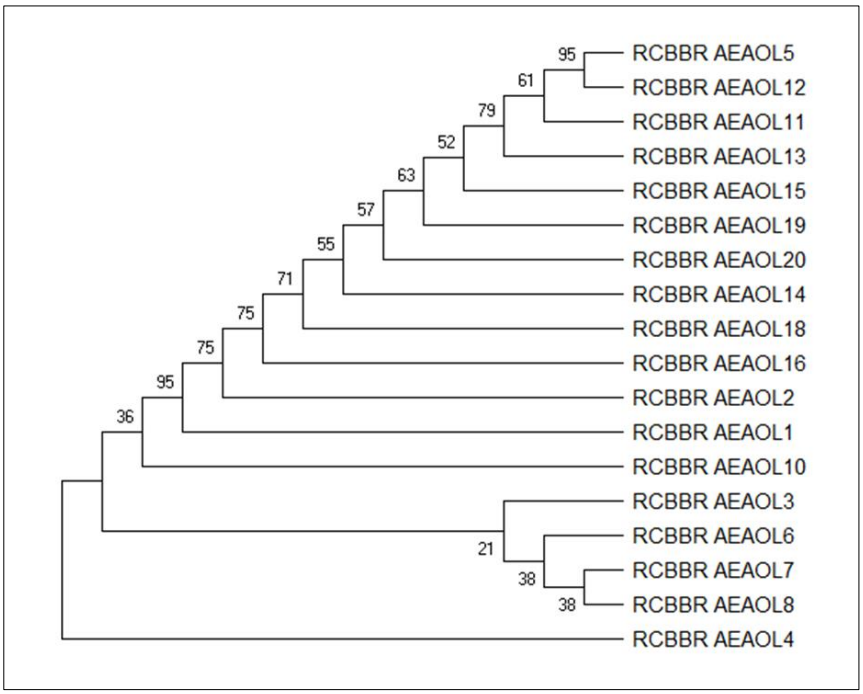


Figure 5b Molecular Phylogenetic analysis of *M. phaseolina* isolates of *Arachis hypogaea* using maximum composite likelihood method from Edo and Delta States, Nigeria

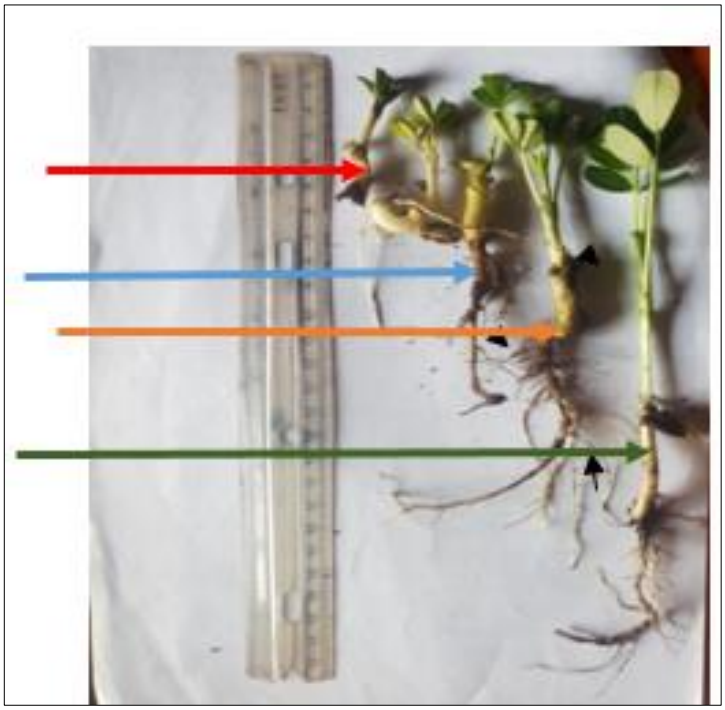
This is a phylogenetic tree for the 18 strains of *Macrophomina phaseolina*. They were compared to see the strains that are mostly related to each other. The shorter the vertical lines between the samples, the closer the strains are in evolution (i.e, the more closely related the strains are to each other). The longer the vertical lines, the farther apart the strains are in evolution.

3.6 Effect of culture filtrates of test fungi on germination and seedling growth of groundnut

Six types of disease symptoms were observed to be associated with the groundnut seedlings. These were Damping off (S_1), root rot (S_2), collar rot (S_3), wilting (S_4), chlorosis (S_5) and necrotic lesions (S_6). The percentage of disease symptoms of each isolates are presented in Table 3.

Table 3 Disease symptoms associated with groundnut seedlings

Isolates	Type of symptoms	Total (%) of seedlings in which present
AEAOL 1	Damping off	12
	Chlorosis	37
	Necrotic lesions	50
AEAOL8	Root rot	13
	Wilting	31
	Chlorosis	43
	Necrotic lesions	56
AEAOL 13	Collar rot	19
	Wilting	37
	Necrotic lesions	69
AEAOL 19	Wilting	43
	Necrotic lesions	43
Control	Symptomless	



Green arrow = Control, Orange arrow= Damping off, Blue arrow= Root rot, Red arrow= Collar rot.

Figure 6 Symptomatic *Arachis hypogaea* seedlings

Figure 7*A. hypogaea* seedling with necrosis (arrow)

Results of the percentage germination of seeds (*In vitro*) infected with isolates from Delta State (Variety A) and Edo State (Variety B) are shown in Figure 9. The reduction in all the seeds that was inoculated with *M. phaseolina* was very significant except isolate 7 for Variety A. However, no growth was observed in 5 of the 18 isolates for both groundnut varieties. After five weeks, the percentage of seedlings emerged was lower in all the treatments that was inoculated for only seeds inoculated with *M. phaseolina* (Figure 10). The control had 75 % seedling emergence, 50 % in isolates AEAOL 8, 25 % in isolates AEAOL 1 and AEAOL 13 (Delta State), and 0% in the other fourteen treatments, demonstrating the fungus high virulence and ability to penetrate and kill the seed. No significant decrease between the number of leaves of groundnut seed (*Arachis hypogaea*) inoculated with *M. phaseolina* for isolate AEAOL 1, AEAOL 8, AEAOL 13 and AEAOL 19 in comparison to the control. The number of leaves increased across treatments; the mean values for the number of leaves were: control (14.8), isolate AEAOL 1 (6), isolate AEAOL8 (7.4), isolate AEAOL19 (5.8) whereas the least was recorded in isolate AEAOL 13 (5.2). It was observed that the remaining infected groundnut seeds recorded no growth (Figure 11). Five weeks after infecting the seeds, there was no form of observable significant difference between the leaf area of groundnut inoculated with *M. phaseolina* for isolate AEAOL 1, AEAOL 8, AEAOL 13 and AEAOL 19 in contrast to the control. On the other hand, it was observed that the remaining *M. phaseolina* isolate-infected groundnut seeds recorded no growth (Figure 12). In this study, the stem girth also increased with weeks after planting in all the treatments when compared to the control until when the experiment was terminated. The control treatment, had the highest stem girth of 0.7 cm at (5 WAP) and the least was recorded in isolate AEAOL 13 having 0.35 cm which showed significant difference. However, it was observed that the remaining *M. phaseolina* isolate- infected groundnut seeds recorded no growth (Figure 13). Shoot length decreased in all treatments within 5 weeks of planting when comparison was made with the control, with the highest shoot length observed/recorded in the control treatments at 14.9 cm. The least shoot length was recorded in isolate AEAOL 19 treatment, with 2.9 cm. On the other hand, the remaining *M. phaseolina* infected groundnut seeds recorded no growth (Figure 14).

A B C

Figure 8 A: Healthy groundnut plant (control). B: Symptomatic isolate AEAOL 1 (yellow arrow = wilting, red arrow= leaf chlorosis). C: Symptomatic Isolate AEAOL 8 (black arrow = necrosis, blue arrow = wilting)

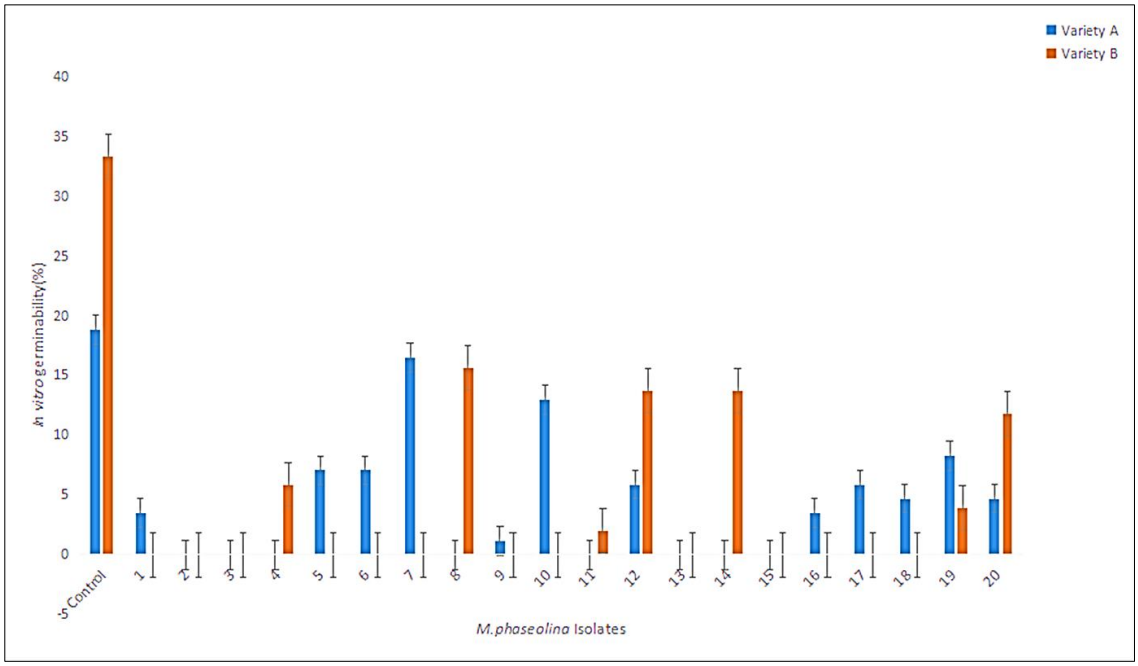


Figure 9 Percentage (%) germination of groundnut (Arachis hypogaea) seeds *In vitro*

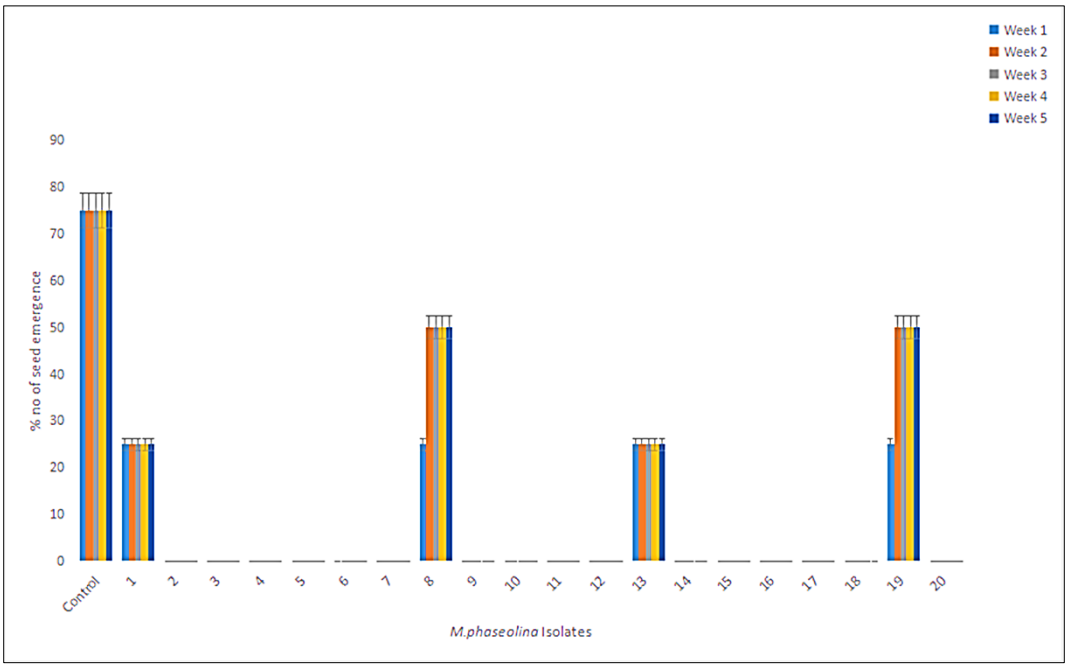


Figure 10 Effect of *M. phaseolina* isolates on percentage number of seedling emergence of groundnut seed (*Arachis hypogaea)*

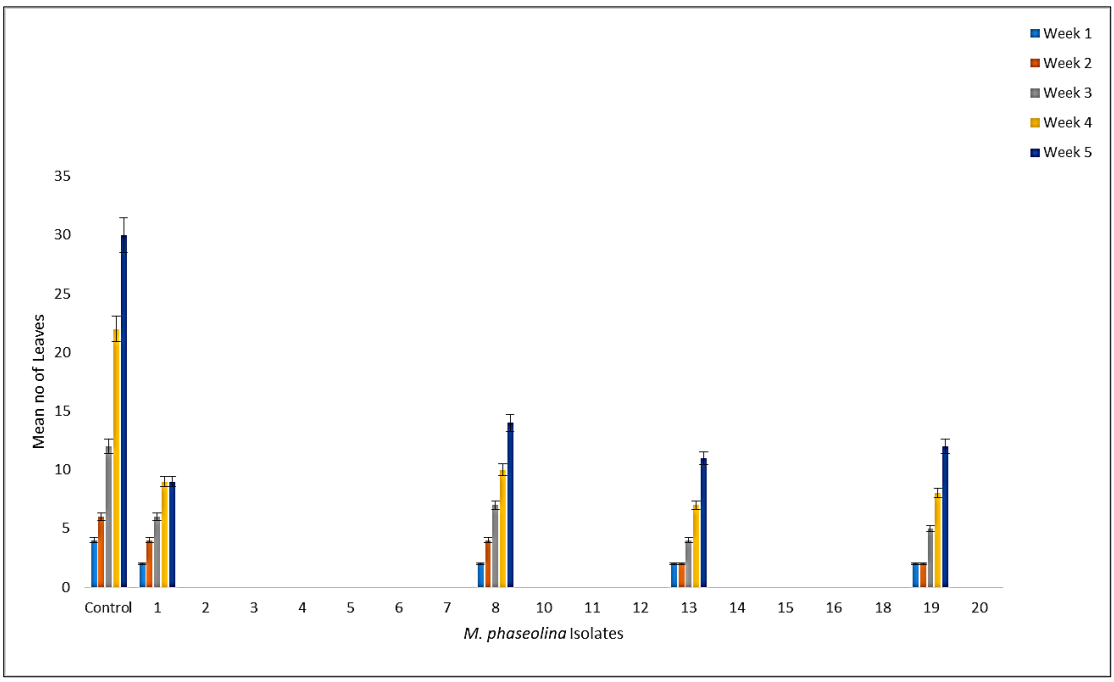


Figure 11 Effect of *Macrophomina phaseolina* isolates on leaf number of groundnut (*Arachis hypogaea*) five weeks after planting

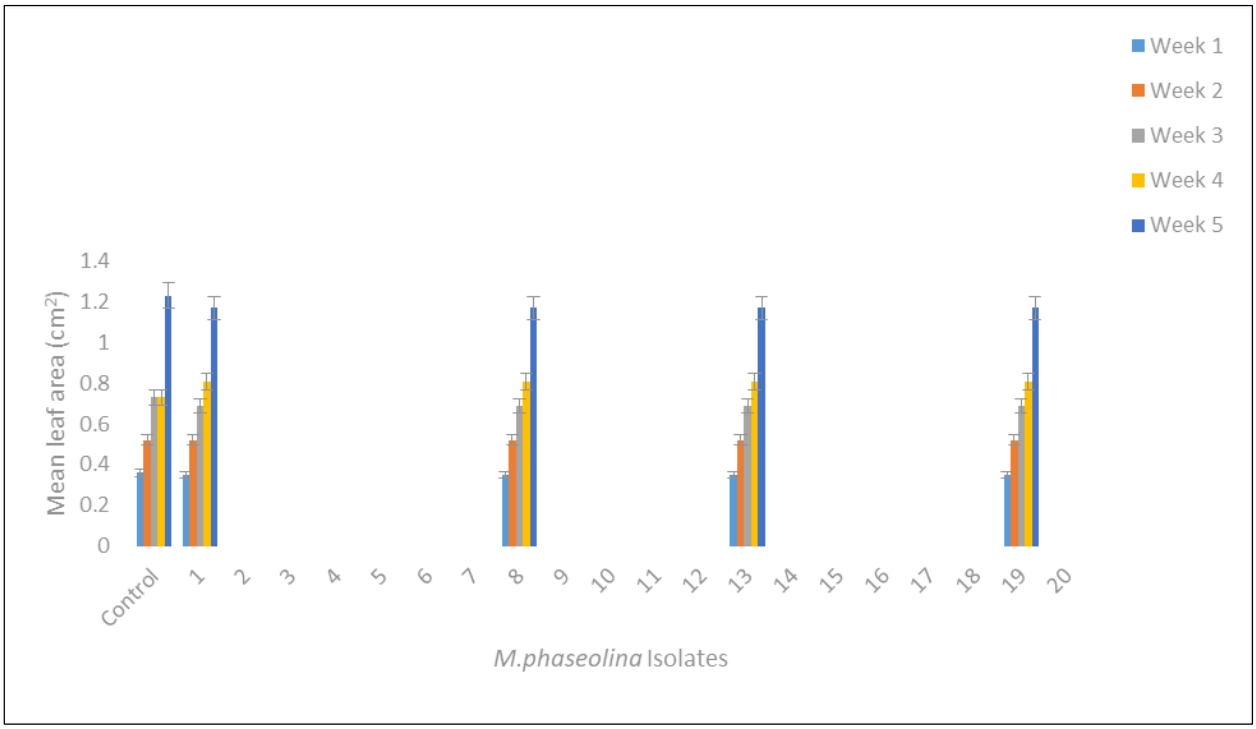


Figure 12 Effect of *Macrophomina phaseolina* isolates on leaf area of groundnut (*Arachis hypogaea*) five weeks after planting

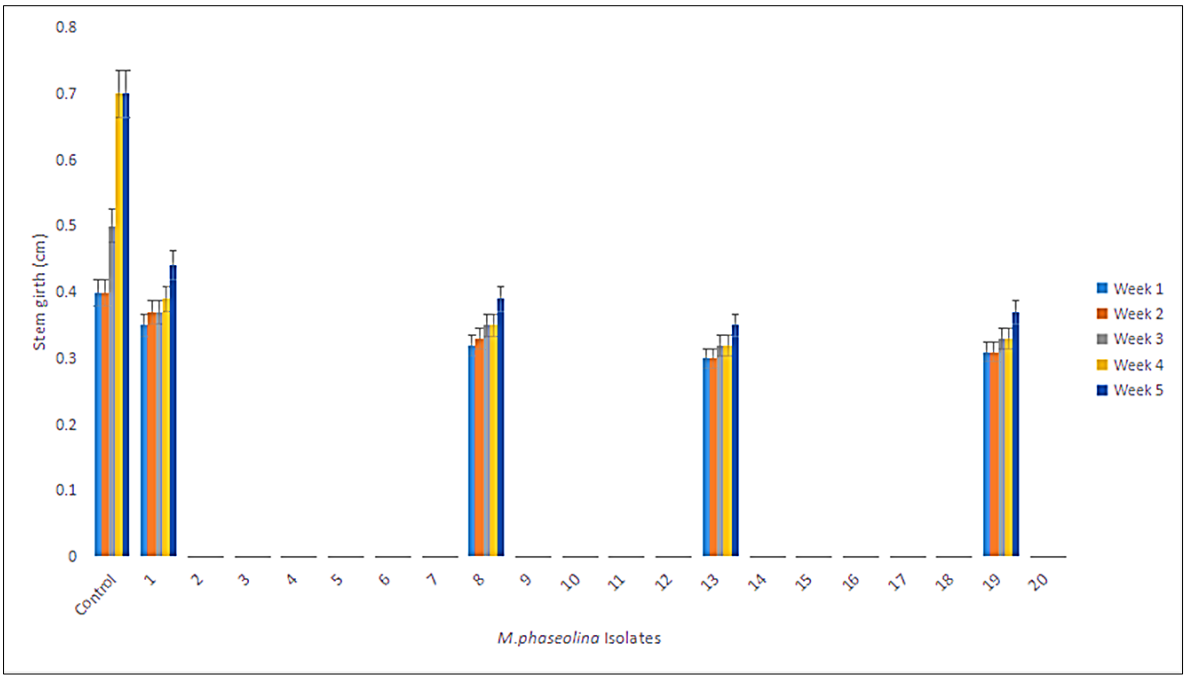


Figure 13 Effect of *Macrophomina phaseolina* isolates on stem girth of groundnut (*Arachis hypogaea*) five weeks after planting

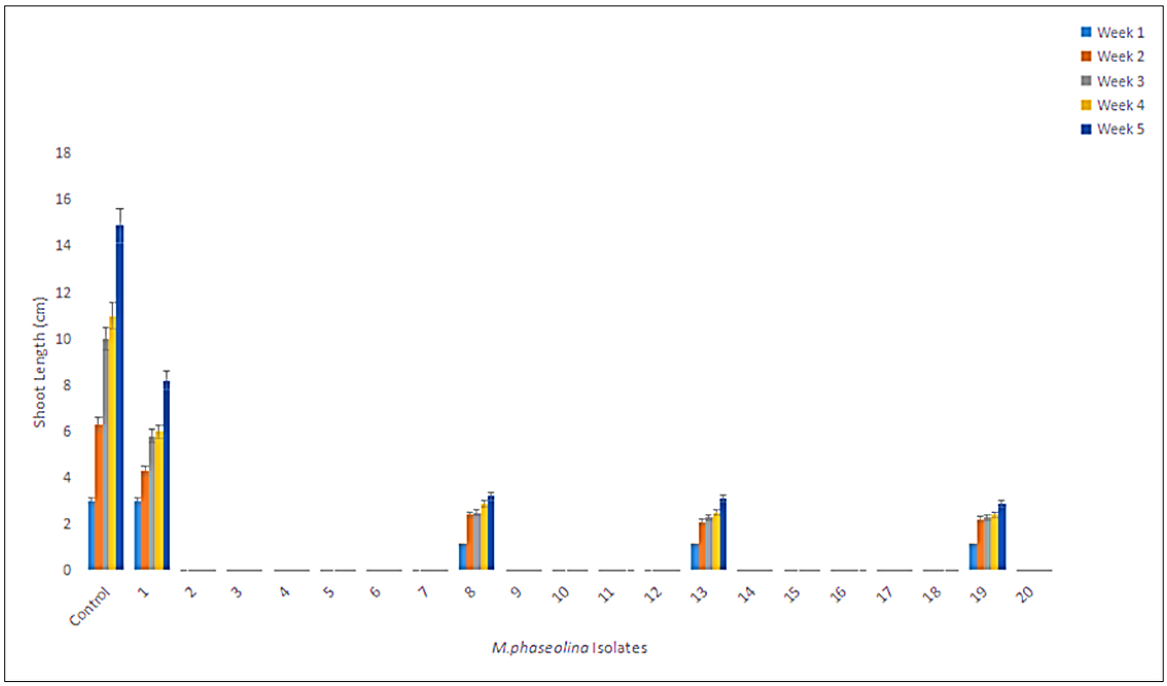


Figure 14 Effect of *Macrophomina phaseolina* isolates on shoot length of groundnut (*Arachis hypogaea*) five weeks after planting

4 Discussion

Macrophomina phaseolina is a fungus that is soil-borne and have effects on more than 500 species of plants from over 100 families globally. It can creates diseases like charcoal rot, seedling blight, stem rot and root rot [23]. According to [24], when there is a rise in temperatures (between 30-35^{o}C) and when the soil moisture is lower than 60%, this fungus has the potential to create significant losses in the production of crops like sorghum and soybean. Groundnut cultivars lost 100 percent of their output in the worst-case scenario, when disease developed during the pre-emergence stage[25]. Seeds are essential in agriculture for growing healthy crops. Around 90% of all crops on the globe are grown from seeds. Since seeds carry a range of pathogens that could be associated in the field or during post-harvest storage, they have also been connected to disease transmission.

The molecular identification procedure used ITS region sequencing and DNA barcoding. Comparison were made between ITS rDNA sequences with the ones in the database by utilizing NCBI-BLAST. Nine strains out of the 18 *M. phaseolina* that was isolated were recognized by DNA barcoding, with a 97 to 99 percent accuracy rate. According to [26], one of the most crucial tools for identifying fungal species isolated from environmental sources is the ITS rDNA region sequence. As a result, it is frequently used to identify the fungal population in soil and to improve on conventional identifications. As a result of their wide distribution, functional stability, adequate conservation, and sufficient length to provide a thorough understanding of evolutionary relationships, ITS rRNA genes are good and useful markers for phylogenetic research[27].

The biodiversity of *M. phaseolina* isolates was also clearly detected between Delta and Edo States in this investigation. Isolates distribution and quantity differ from one isolation location to another. Nine strains of *M. phaseolina* isolates were found in both Delta and Edo States, out of the eighteen *M. phaseolina* isolates. Three strains of *M. phaseolina*, MK454909.1, MN603095.1 and MH864182.1 were identified from Edo State isolates, whereas six strains of *M. phaseolina* were identified from Delta State isolates: MH864180.1, KF951678.1, MH864184.1, HQ649832.1, MK454909.1 and MH864181.1. Numerous variations of *M. phaseolina* pathogenicity, physiology, morphology, and genotyping have been discovered [28, 29]. It has proven challenging to distinguish *M. phaseolina* that is isolated from particular hosts or regions despite the fact that many isolates have been investigated for genetic and pathogenic diversity in the strains of the *M. phaseolina*. According to [30], the lack of a strong correlation between geographic origin and genotype points to the vast diversity of *M. phaseolina* strains, which is consistent with the results of our work. The 18 pure fungal cultures was compared to identify which strains were most closely related. Isolates AEAOL 5 and AEAOL 12 from Eguare Market in Edo State and Effurun Market in Delta State were found to be the most closely related, while isolates AEAOL 7 and AEAOL 8 from Ekpoma Market (both in Edo State) were also shown to be closely related. Genetic

techniques like random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) were used by researchers [31, 9, 32, 33] in order to gain more information on the genetic make-up and variation in *M. phaseolina*.

The findings of this study indicate that *M. phaseolina* infected groundnut seeds after an inoculum of the pathogen was sprayed on the seed surface. *Macrophomina phaseolina* was shown to be the cause of the symptoms observed. The findings for the *M. phaseolina* isolates used in this study were consistent with previous findings [34, 31,9, 35, 36] which revealed that *M. phaseolina* has no host-specificity and highly shows difference in terms of virulence or aggressiveness, even among isolates gotten from the same plant [37]. The *M. phaseolina* isolates induced various symptoms in groundnut seedlings. Inoculating healthy groundnut seeds with *M. phaseolina* isolates lowered seedling emergence, leaf number, root length, and stem girth decreased in isolates compared to controls. This finding conforms with the discovery of [38], who observed that the soil-borne fungus *Rhizoctonia solani* lowers wheat root yield as well as their soft and dry weight. It is known that pathogens may secrete a set of enzymes that cause seedling death. . Extracellular enzymes, in addition to these intracellular enzymes, have been reported in *R. solani* and *Fusarium solani*, both of which produce extracellular enzymes like amylase, protease, pectinase, catalase, pectinase, cellulose laccase and lipase [39, 40]. Aqueous hydrolytic enzymes produced by *Macrophomina phaseolina* degrade plant cell wall components like pectin, lignin, hemicellulose and cellulose [41]. The ability of the *M. phaseolina* (used in this study) to produce these hydrolytic enzymes could be related to the symptoms observed, including seedling death. These effects can lower yield and quality of groundnut.

5 Conclusion

The findings revealed that the groundnut samples were infected with *M. phaseolina*. Since farmers purchase seeds for their crops from the market, seed banks, or use old seeds that was previously saved from the fruits of a previous crop, to reduce seed contamination farmers must receive health education as well as training in food safety and hygienic handling. Appropriate storage methods would reduce the rate of fungal growth and survival. Selection and treatment of seeds before planting would reduce crop loss and should be enforced; the attendant negative impact on food security will be reduced. This research finding is unique as it is presently the only (first) documented report found on effect of *M. phaseolina* on seed germination and seedling growth of groundnut.

Compliance with ethical standards

Disclosure of conflict of interest

Authors have declared that no competing interests exist.

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