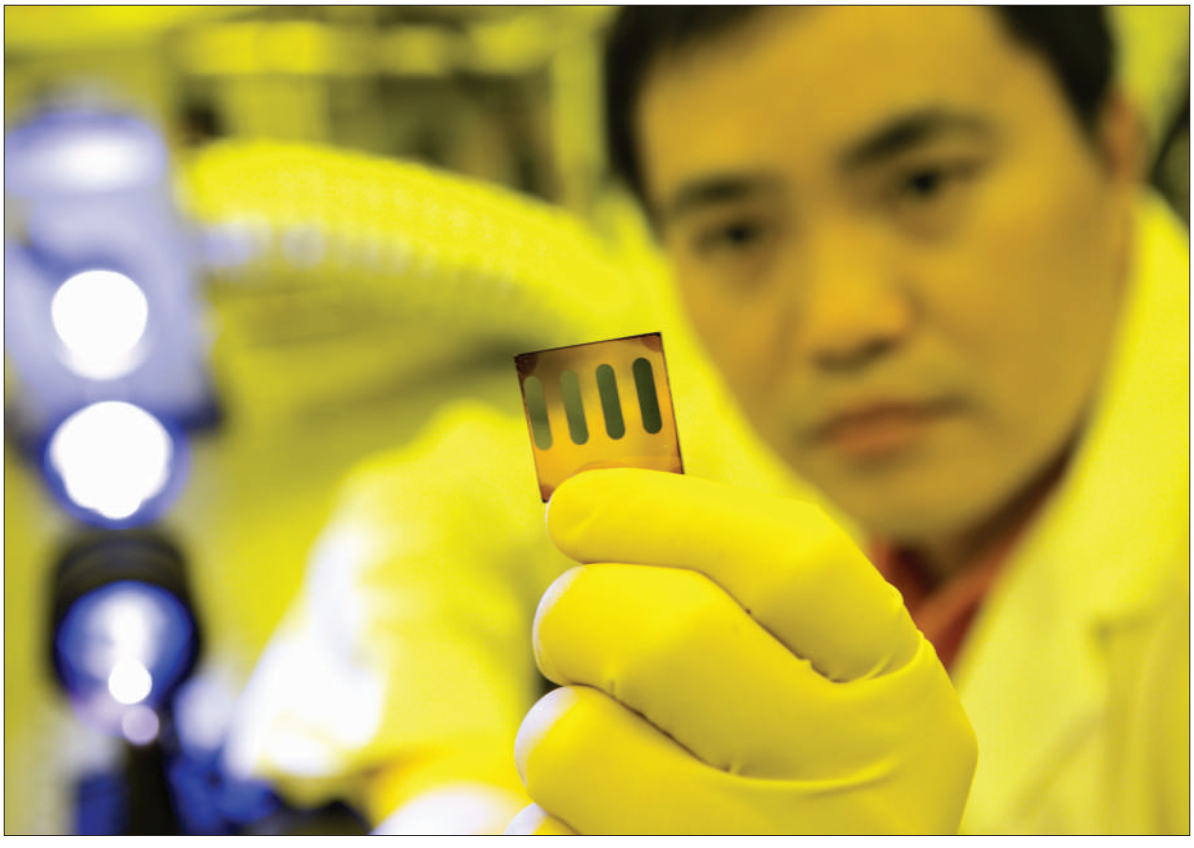

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Research Article
Morphological and Molecular Diversity of *Fusarium* Species Causing Wilt Disease in Ginger (*Zingiber officinale* Roscoe) in South Western Ethiopia

[1]Tsegaye Mekuria and [2]Tesfaye Alemu

[1]Department of Biotechnology, College of Natural and Computational Sciences, Aksum University, Axum, Ethiopia
[2]Department of Microbial Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

Abstract

Background and Objective: Ginger (*Zingiber officinale* Roscoe) is a globally popular tropical herbaceous plant with its rhizome used as raw material in the food industry, pharmaceuticals and cosmetics. The current study was conducted to isolate, identify and study cultural, morphological and molecular diversity of *Fusarium* wilt pathogen (*Fusarium* spp.) from Southwestern parts of Ethiopia. **Materials and Methods:** The infected ginger rhizome, pseudostem, leaf and soil samples were collected from ginger growing areas of Southwestern Ethiopia. Isolation of *Fusarium* wilt isolates from ginger parts were done following the standard methods, fungal pathogens were isolated from ginger parts showing vascular browning symptoms. Similarly, fungal isolates were isolated from soil using serial dilution agar plate method. **Results:** Isolation of fungal isolates revealed that four genera belonging to *Fusarium*, *Penicillium*, *Aspergillus* and *Trichoderma*. Out of 24 fungal isolates 14 isolates were *Fusarium* species. *In vitro* pathogenicity testing resulted in Pathogenicity Index (PI) values ranging from 11.0±1.6-45.4±11.6%. Cultures of *Fusarium* species on Potato Dextrose Agar (PDA) medium showed white, creamy white, dull pink and pink coloration. They imparted dull white, orange, light reddish purple, intense reddish purple and dark reddish-purple pigmentation on the reverse side. Canoe-shaped macroconidia, ovoid microconidia and short phialides were present in all of the *Fusarium* isolates. **Conclusion:** Based on their cultural and morphological characters the isolates were identified as *Fusarium oxysporum*. The isolates were confirmed to be *Fusarium oxysporum* f. sp. *zingiberi* based on their ability of causing ginger rhizome rot. Further, the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR- RFLP) profile of the Internal Transcribed Spacer1 (ITS1) rDNA region indicated genetic variation between the *Fusarium oxysporum* isolates.

Key words: *Fusarium* species, ginger wilt, genetic variation, morphology, molecular diversity

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Corresponding Author: Tesfaye Alemu, Department of Microbial Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) is a globally popular tropical herbaceous plant with its rhizome used as raw material in the food industry, pharmaceuticals and cosmetics. In Ethiopia, ginger cultivation started during the 13th century following its introduction to East Africa[1]. The major ginger growing areas in Ethiopia include wetter regions at altitude below 2000 m[2]. The producing areas in Southern Nations Nationalities and Peoples Region of the country (SNNPR) are said to be ginger belts in Ethiopia where much of the country's production and marketing activities are undertaken[3]. Large scale production of ginger in Ethiopia is limited by various problems like low quality varieties, poor pre and postharvest handling practices[3], crucial shortage of planting material[4], lack of awareness to determine the optimum rhizome seed sets[3] and postharvest deterioration due to fungal invasion[5]. Recently, ginger wilt disease has become the most threatening. The problem is more aggravated since a sudden emergence of the disease occurred in the 2012 cropping season and continues in every cropping season since then. Therefore, this study has been undertaken in consideration of being an important study examining if fungal infections are the causes for the ginger wilt disease that has largely affected ginger production in the Southwestern parts of Ethiopia.

Fusarium is one of the most ubiquitous, abundant and important genera of soil micro fungi that contains many species of environmental, agricultural and human health importance[6]. The notoriety of this genus mainly results from its pathogenicity towards a wide range of plants[6]. Many plants have at least one *Fusarium* associated disease[7]. Pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in over 100 plant species, among which there are several economically important crops[8]. Individual *F. oxysporum* isolates have narrow host ranges and can be classified depending on host range as formae speciales. The formae speciales that causes vascular wilt of ginger is known as *Fusarium oxysporum* f. sp. *zingiberi*[9,10].

Today, a wide range of molecular techniques are applied to accurately identify *F. oxysporum* isolates of which those based on detection of pathogen DNA or RNA are the most predominant[11]. Since the advent of molecular techniques, numerous studies have been conducted on molecular characterization of *Fusarium* oxysporum[12-14]. The molecular detection and identification methods of *Fusarium oxysporum* can be categorized into two; as anonymous markers (RFLP, Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR)) and sequence specific markers (ITS and Intergenic Spacer (IGS), Transposons and Translation Elongation Factor-1α (TEF-1α))[11]. The suitability of ITS regions for the detection and identification of various *Fusarium* species is reported widely[15].

In PCR amplification ITS1, 5.8S and ITS2 region has been the priority of almost all researchers working with the ITS region in fungal molecular identification. The ITS1, 5.8S and ITS2 region is amplified by the universal fungal primer pairs, ITS1 and ITS4. However, a relatively lower PCR amplification success rate has been reported by this primer combination as compared to the amplification success rate attained by the primer combination ITS1/ITS2 that amplifies the ITS1 region only[16]. PCR amplification of the ITS1 region alone confers the advantage of showing interspecies and intraspecies variability among isolates. This is possible due to the fact that ITS1 is more variable region as opposed to the entire ITS1, 5.8S and ITS2 region in that it is devoid of the more conserved 5.8S ribosomal RNA gene[17]. In the present study, ITS1 region is PCR amplified with primer pairs ITS1/ITS2 to take advantage of the fast evolving nature of the ITS1 region to detect variability between isolates of the same species inferred from the morphological identification.

Isolation and identification of pathogens is a precondition to control pathogenic diseases. Investigating these pathogens and controlling them will help to increase the yield and quality of ginger product, thereby supporting the supply and finally quenching the market demand. It has been difficult to establish appropriate guidelines for the wilt disease management of ginger rhizome in Ethiopia, mainly due to the lack of knowledge about the pathogen. Therefore, this study was conducted to isolate, identify and study cultural, morphological and molecular diversity of *Fusarium* wilt pathogen (*Fusarium* sp.) from Southwestern parts of Ethiopia.

MATERIALS AND METHODS

Study area: The study was conducted during the period of December, 2013-August, 2015 at Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University Ethiopia. Sample materials were collected from the Southwestern parts of Ethiopia: Sheka Zone (Yeki district and Teppi town administration), Bench Maji Zone, (Aman district and Semen Bench district), Kaffa Zone (Gimbo district and Bonga town administration) and Jimma Zone (Shabe district) of the ginger growing areas (Fig. 1).

Sample collection: Samples of diseased ginger parts (rhizome, pseudostem and leaf) and soil were collected from the study areas and transported to the Mycology Laboratory of Department of Microbial Cellular and Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia.

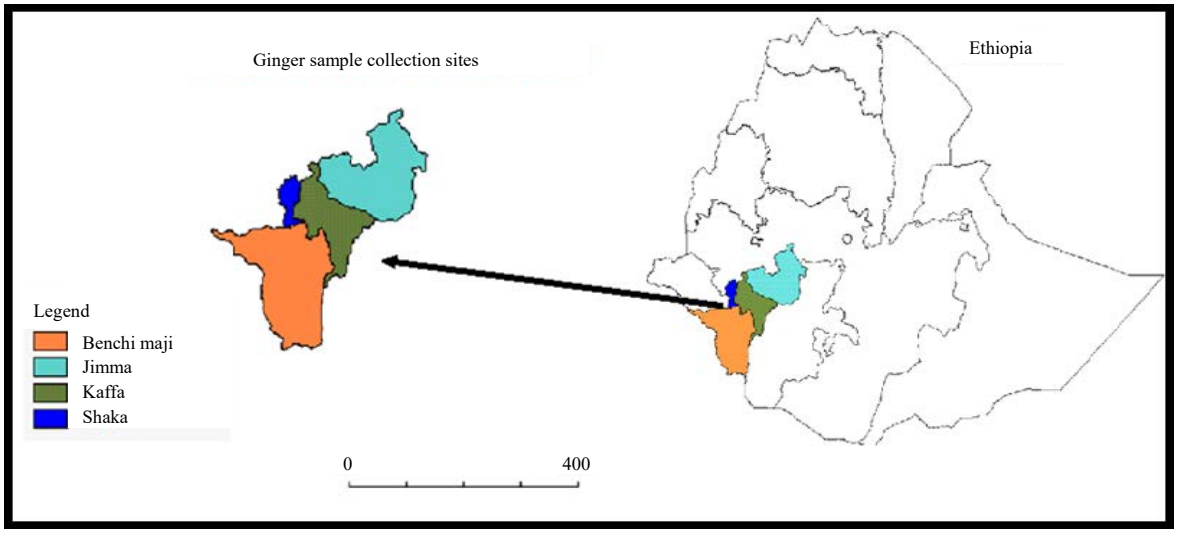

Fig. 1: Map of sample collection sites (Tsegaye Mekuria and Tesfaye Alemu, generated from GPS data using ArchGIS software)

Media preparation and sterilization: Culture media, Potato Dextrose Agar (PDA), Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA) are used to isolate and characterize the *Fusarium* isolates[7]. All glassware and culture media were autoclaved at 121°C for 15 min. Chloramphenicol was used to prevent bacterial contamination[18].

Isolation of the *Fusarium* wilt pathogen from samples: Infected ginger rhizomes, leaves and pseudostems were used for isolation of fungal pathogens using tissue transplanting technique[19]. Following surface disinfection using 1% sodium hypochlorite for 5 min, ginger tissues at the boundaries of lesion and healthy tissue were excised from the specimen tissue and placed on the surface of Potato Dextrose Agar (PDA) plates supplemented with 0.05 g L^{-1} chloramphenicol (Himedia). Fungal isolates from soil samples were obtained by serial dilution agar plating technique[20]. All the inoculated plates were incubated at 25±1°C. The cultures were periodically checked for mold growth starting from the 24th h after inoculation. A small portion of emerging mycelial hyphae were then transferred to PDA medium for further growth, purification and storage. Totally, 24 isolates were isolated of which, 14 of them were identified as *Fusarium* genus. These *Fusarium* isolates were further selected and subjected to pathogenicity, cultural, morphological and molecular studies.

Sporulation study and inoculum preparation: Three petri dishes full of mycelia from 10 days old culture grown on PDA were soaked with 20 mL sterile distilled water (per each plate), scraped with sterile scalpels and filtered through muslin cloth to remove mycelial fragments and to examine spore morphology. Spore suspension (10 µL) was placed on a hemocytometer using micropipettes. The number of spores in five square grids in the hemocytometer was counted and the concentration of spores per milliliter of the suspension was calculated[21]. The spore concentration was adjusted to 1×10^6 spores/mL and the spores were used for *in vitro* pathogenicity test[22].

***In vitro* pathogenicity test:** Fresh rhizomes were obtained from Piazza vegetable market, Addis Ababa, Ethiopia by selecting vigorous and healthy looking tubers. Rhizomes were washed in sterile distilled water and surface sterilized using 1% sodium hypochlorite for 5 min and then washed with sterile distilled water twice. Holes of approximately 2 cm deep were dug in the rhizomes using 5 mm diameter sterile cork borer. Spore suspensions were placed into the hole and the wounded areas were capped with 5 mm thick sterile agar discs to cover the openings. The treated rhizomes were incubated at 30±1°C with a periodic checkup for *Fusarial* mold growth. The pathogenicity test of the *Fusarium* isolates was then evaluated using the method[23]. Pathogenicity Index (PI) value of each isolate was assessed by evaluating the ability of the isolate to induce rot in healthy (disinfected) test rhizomes and compared with a control group inoculated with sterile distilled water.

Infected rhizomes were then placed into screw-capped air tight bottle and weighed. The weighed bottles were moistened by adding 3 mL of sterile distilled water onto filter

papers placed beneath the rhizome to keep moisture in the container and incubated for 15 days. The control experiment was done by using sterilized distilled water of equal volume placed into the holes of similarly disinfected rhizomes. At the end of the 15th day, the rhizomes were reweighed to determine the pathogenicity index of each *Fusarium* isolate. The experiment was conducted in five replicates and repeated twice:

$$\text{Pathogenicity Index (PI)} = 100 - \frac{\text{WD}}{\text{WH}} \times 100$$

where, WD is the weight of the diseased rhizome and WH is the weight of healthy rhizome.

The rhizomes were cut along the plane of inoculation using sterilized scalpel and observation was made on the type of rot developed. A portion of the mold grown over the surface was inoculated on to PDA to confirm whether the rhizome rotting was due to the inoculated pathogens.

Identification of *Fusarium* isolates: General to specific approach has been followed to identify the isolates from genus to species level beginning with cultural characters and proceeding to microscopic features and finally to PCR-RFLP for molecular diversity study. The isolates were grown on PDA plates at 30±1°C for 4-7 days. Observation was made on colony color, pigmentation, presence or absence of macroconidia, microconidia, phialides and chlamydospores. Measurements were done for the radial growth of the isolates using millimeter calibrated ruler.

Slide cultures of *Fusarium* isolates were prepared[20]. Morphological species identification was done by referring to the illustrative literature[7,24,25]. Carnation Leaf Agar (CLA) was used to enhance production of macroconidia. Observation of macroconidial and microconidial shapes and septation was made using compound microscope at 400X magnification. Macroconidial and microconidial size were measured using Olympus System Microscope (OLYMPUS, BX51, BX2 series, Japan) fitted with a 12-bit quality imaging retiga camera system. Phase contrast images of the macroconidia and microconidia were captured using the Olympus System Microscope at 400X magnification.

Culture preparation and DNA extraction for molecular diversity study: The *Fusarium oxysporum* isolates were grown in liquid media in order to obtain mycelial mass for DNA extraction. Five (5 mm) agar discs were cut out from a seven day old colony with sterile cork borer and inoculated into 500 mL Erlenmeyer flasks containing 100 mL Potato Dextrose Broth (PDB). Tween 80 (10 µL) was added to each flask to help mycelia disperse evenly through the growth media.

The flasks were placed in water bath shaker incubator at 30°C temperature at 200 rpm for 3-7 days until fungal growth appeared. The mycelial mats were harvested by filtration through a sterilized thick muslin cloth, washed several times with sterile distilled water and then ground with sterilized white porcelain mortar and pestle under excess liquid nitrogen[26]. The fine powder of the mycelia obtained was weighed to 150-160 mg and kept in 2 mL capped centrifuge tubes and immediately proceeded to the DNA extraction procedure, which was managed by following the manufacturer's instructions of HIMEDIA (HiPurA Fungal DNA Purification Kit, MolBio™).

Determination of DNA concentration and purity: Concentration and purity of DNA was analyzed using NanoDroP Spectrophotometer 2000c (Thermo Scientific, UK) as well as in 1% agarose gel electrophoresis. After the test, the DNA was stored at -20°C until used for PCR[8].

Polymerase Chain Reaction (PCR) procedures: Primers ITS1: (TCCGTTGGTGAACCAGCGG) and ITS2 (GCTGCGT TCTTCATCGAT-GC) sequences were used to amplify the ITS1 region[16]. The PCR amplifications were carried out in a total reaction volume of 25 µL containing nuclease free H_2O (15.7 µL), PCR buffer (2.5 µL), $MgCl_2$ (2 µL), dNTPs (2 µL), Each primer (0.2 µL), ITS1 and ITS2, TaqDNA Polymerase (0.4 µL) and genomic DNA (2 µL). The PCR amplification was carried out using Eppendorf Master Cycler (Nexus, Germany) programmed for initial DNA denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 68°C for 1 min, extension at 72°C for 1.5 min and a final extension at 72°C for 7 min. The amplified DNA products were resolved by electrophoresis on 2% agarose gel in 1×TBE buffer; stained with ethidium bromide and photographed in gel doc system with UV-transilluminator (BioSens 750, Japan). About 100 bp DNA ladder (MBT049, Himedia) was used as a marker. The experiment was repeated three times with 3 replications. PCR products were then kept in a refrigerator at 4°C until subjected to restriction digestion experiment.

Restriction Fragment Length Polymorphism (RFLP) experiment: The PCR amplified rDNA regions were treated with 4 restriction enzymes of which 2 were hexa cutters (HindIII, PstI) and the other 2 were tetra cutters (HhaI, MspI) with the following set of conditions. For HindIII (recognition

sequence, AAGCTT) and PstI (recognition sequence, CTGCAG) digestion, reaction condition was set in a total volume of 50 µL according to the manufacturer's instructions, Himedia (34 µL of nuclease free water, 5 µL of 10X Hibuffer H2 for HindIII and 10X Hibuffer H3 for PstI, respectively, 10 µL of PCR product and 1 µL of the respective enzyme, HindIII/PstI) and incubated in water thermostat at 37°C for 1 h. A 6X loading buffer was used as a tracking dye for the agarose gel electrophoresis following restriction digestion[16].

For HhaI and MspI with recognition sequences GCGC and CCGG respectively, restriction digestion reaction was carried out in a total volume of 30 µL according to the manufacturer's instructions, Thermo Scientific (17 µL nuclease free water, 2 µL of 10X FastDigest Green Buffer, 10 µL of PCR product and 1 µL of FastDigest Enzyme, MspI/HhaI) and incubated for 5 min at 37°C in water thermostat. The 10X FastDigest Green Buffer included in the reaction was also used to track the migration of amplicons during the gel electrophoresis. The product was immediately loaded to 2% agarose gel and subjected to electrophoresis at 100 V for 1.5 h in 1X TBE buffer using a horizontal gel electrophoresis tank connected to a power pack. This experiment was conducted twice with three replicates.

Data analysis: Pathogenicity Index (PI) of isolates on ginger rhizome was analyzed using SPSS 20. Comparison of mean PI values of isolates against mean PI value of the control unit was conducted by Dunnett's test of mean comparison and mean comparison of the PI values of every isolate against one another was conducted by the Duncan's multiple mean comparison to evaluate the statistical difference in the pathogenicity of the isolates. The restriction digestion profile (the fragments produced by the restriction Enzyme MspI), that showed genotypic polymorphism between the isolates were analyzed using NTSYSpc (Numerical Taxonomy System, Applied Biostatistics, Jefferson, 2000) computer program version 2.02 h. The data (presence or absence of bands) were coded in the form of a binary matrix and a pairwise similarity matrix was constructed with the Jaccard's similarity coefficient. Unweighed Pair Group Method with Arithmetical Averages (UPGMA) dendrogram was generated with the Sequential Agglomerative Hierarchal Nested (SAHN) cluster analysis module of the NTSYSpc software[27].

RESULTS

Fungal genera isolated from samples: Upon incubation at 25±1°C for 4-7 days, hairy to cottony mold growth was observed emerging from the sample specimens, placed on PDA (leaf, pseudostem and rhizome) (Fig. 2). For the soil samples, *Fusarium* mycelia were emerged on PDA plates inoculated with the dilutions of 10^{-3}, 10^{-4} and 10^{-5} three days after inoculation at 25±1°C. The genus level identification of the isolates revealed four genera of fungi: *Fusarium*, *Trichoderma*, *Aspergillus* and *Penicillium*.

***In vitro* pathogenicity test of *Fusarium* isolates on ginger rhizomes:** The pathogenicity index data is presented in Table 1. The highest Pathogenicity Index (PI) values are recorded from isolates AAUFG6 and AAUFG7 with 45.6 and 38.6%, respectively. The PI value of all isolates exceeds that of the control group (treated with sterile distilled water), indicating that all of the test isolates have induced rhizome rot. The rots developed on all of the test rhizomes were almost

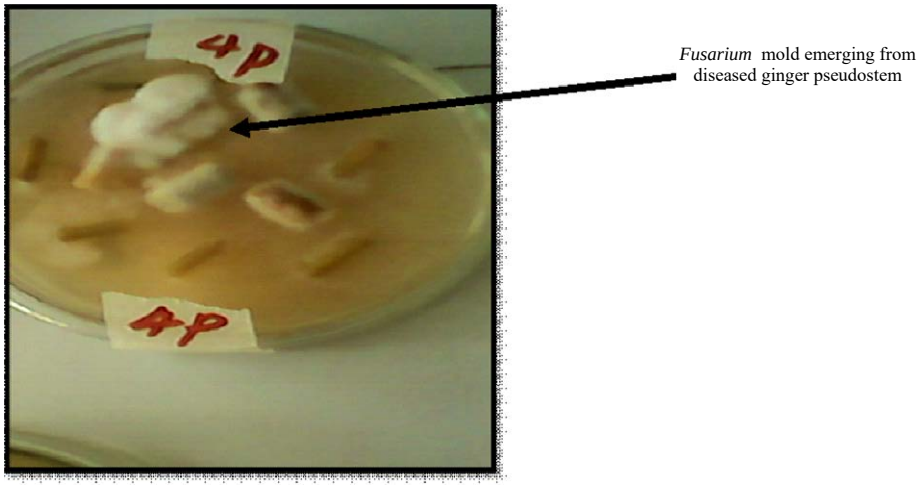

Fig. 2: *Fusarium* isolate emerging from pseudostem samples of ginger on PDA

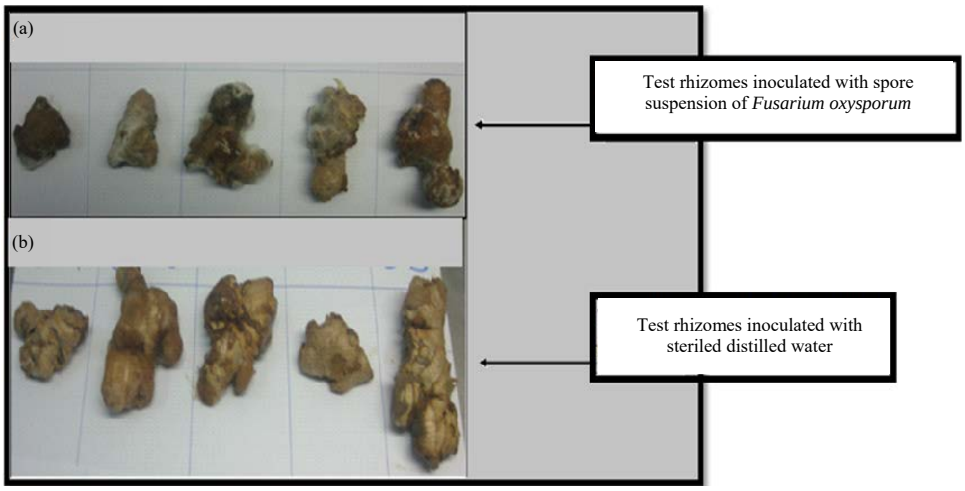

Fig. 3(a-b): A comparison of rhizomes treated with spore suspension of (a) *Fusarium* sp. and (b) A control group treated with sterile distilled water

Table 1: Pathogenicity index of *Fusarium* isolates as tested on ginger rhizomes

Isolates tested	Mean pathogenicity index±SE (%)	Mean difference in PI of isolate from control (I-J)	Significance
AAUFG1	32.46±10.66^{abc}	29.44*	0.028
AAUFG3	14.71±4.53^{bcd}	11.69	0.534
AAUFG4	19.15±11.52^{bcd}	16.13	0.331
AAUFG5	18.45±6.94^{bcd}	15.43	0.361
AAUFG6	45.35±11.57^{a}	42.33*	0.001
AAUFG7	38.69±12.28^{ab}	35.67*	0.006
AAUFG8	28.40±9.62^{abc}	25.38	0.07
AAUFG10	14.84±6.41^{bcd}	11.82	0.528
AAUFG11	16.47±6.81^{bcd}	13.45	0.451
AAUFG12	13.27±3.21^{cd}	10.25	0.602
AAUFG13	10.96±1.55^{cd}	7.94	0.704
AAUFG14	14.65±1.43^{bcd}	11.63	0.537
AAUFG15	11.19±1.71^{cd}	8.17	0.694
AAUFG16	12.96±1.21^{cd}	9.94	0.616
Control	3.02±0.20^{d}	-	-

Values are means of five replicates, I: Mean pathogenicity index of *Fusarium* isolate, J: Mean pathogenicity index of the control group, *Values have statistically significant differences with the PI value of the control group, letters indicate homogeneous subsets of Duncan's multiple range test

similar; pinkish white dense mycelia covering the external surfaces of the injured rhizomes (Fig. 3). Pigmentation with reddish pink color were observed on the rotting rhizome beneath the externally proliferated mold. When transversely split, the internal part showed pale to dark brown discoloration and the rotting usually gave off foul odor.

The one sided (>Control) Dunnett's test of the PI values of isolates AAUFG1, AAUFG6 and AAUFG7 against the PI value of the control group showed statistically significant difference ($\alpha = 0.05$) (Table 1). The mean PI values of the rest of the isolates did not show statistically significant difference ($\alpha = 0.05$) from the mean PI value of the control group (3.02). However, visual observation showed mold growth and rotting of the test rhizomes with foul odor as opposed to the control group with no rotting and mold growth on the surface or inside the test rhizomes.

Cultural and morphological identification: It based on cultural characters on PDA, the isolates showed different colony colors: dull pink, pink, creamy white and white (Fig. 4). The aerial mycelium of the isolates resulted in three different appearances that could be described as fluffy growth and adherent smooth growth with concentric circle (AAUGF5 and AAUFG10). The isolates imparted dull white, orange, light reddish purple, intense reddish purple and dark reddish purple pigmentation (Fig. 5).

Radial growth of *Fusarium oxysporum*: Mycelial growth of isolates was recorded after seven days of incubation period at 30±1°C as presented in Table 2. The radial growth data ranged from 54.20-90 mm.

Morphological characters: Microscopic observations of the morphology of *Fusarium* isolates is presented in Table 3. Macroconidia were formed on mycelial conidiophores (Fig. 6a) and were straight with 1-5 septated and of medium length (Fig. 6b). Microconidia were small to large and mostly non septated (Fig. 6c). Chlamydospores were observed in all of the isolates in single, pairs and occasionally in clumps at intercalary and terminal positions (Table 3). Phialides were short in all isolates (Fig. 6d). Microconidial size between the isolates ranged from 6.56×2.50-16.25×6.50 µm whereas macroconidial size were in the range 14.79×3.98-55.26×10.67 µm (Table 4).

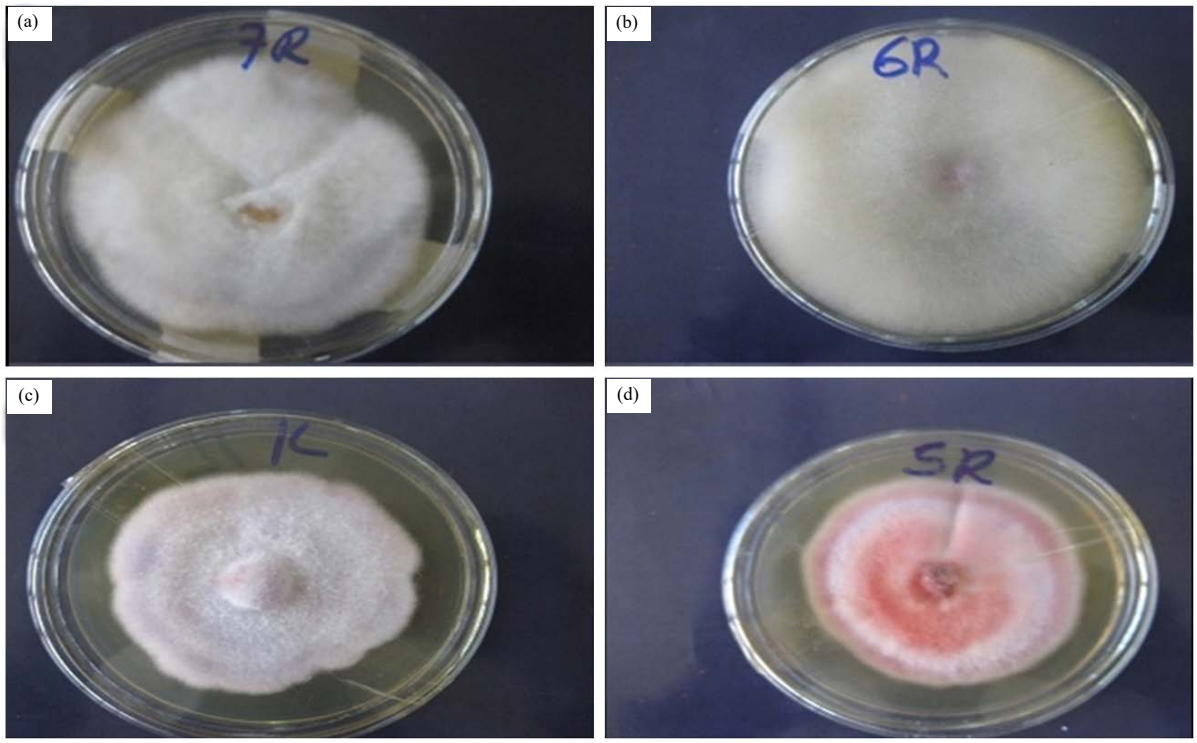

Fig. 4(a-d): Colony color of *Fusarium* isolates on PDA, (a) White, (b) Creamy white, (c) Dull pink and (d) Pink with concentric circles

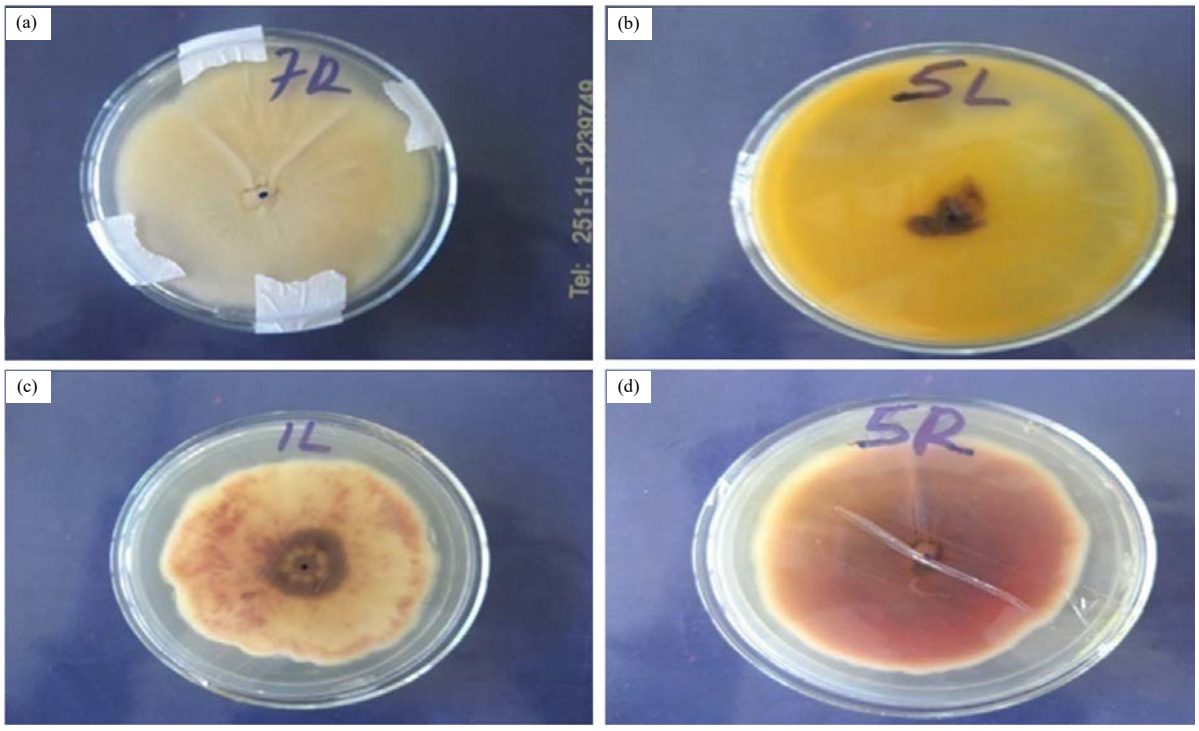

Fig. 5(a-d): Colony pigmentations of *Fusarium* isolates on PDA, (a) Dull white, (b) Yellow/orange, (c) Light reddish purple with dark purple at center and (d) Intense reddish purple

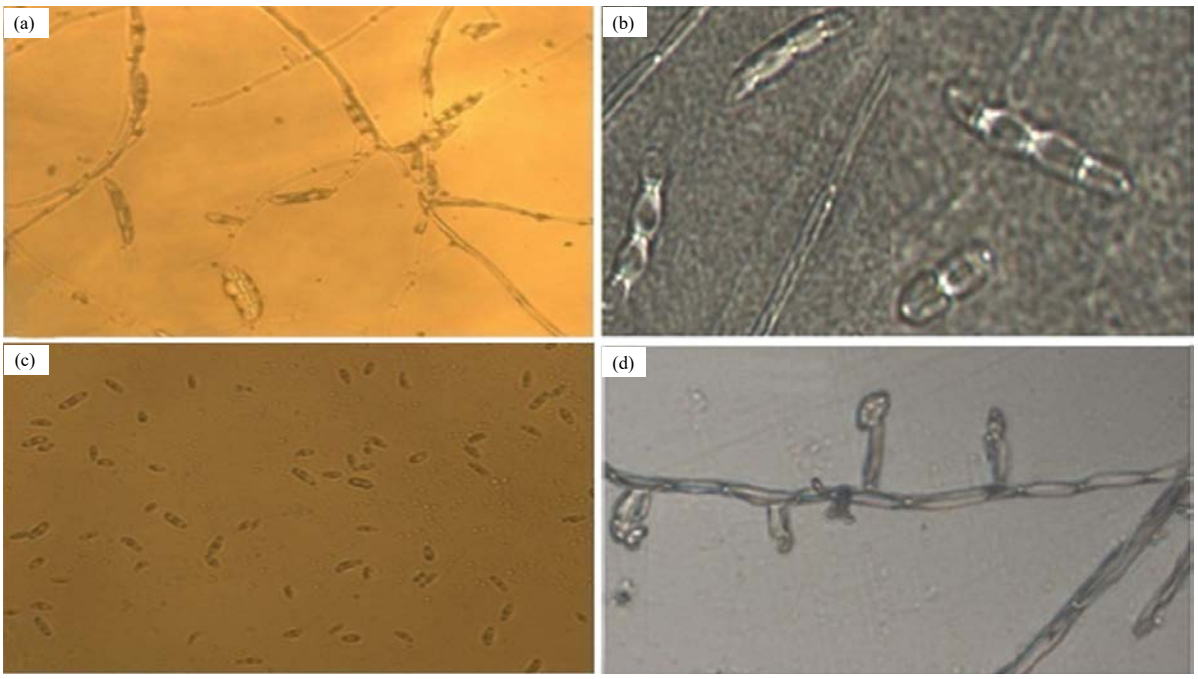

Fig. 6(a-d): Morphological features of *Fusarium oxysporum*, (a) Macroconidia on mycelial conidiophores, (b) Macroconidial shape and septation, (c) Shape and septation of Microconidia and (d) Short phialides branching from mycelium

Table 2: Cultural characteristics of *Fusarium oxysporum* (radial growth, colony color and colony reverse) grown at 30±1°C on PDA

Isolates	Mean radial growth 7 DAI (mm)	Colony color	Colony reverse
AAUFG1	66.80^{cd}	Pink	Dark reddish purple in the center with the rest red tinged
AAUFG 3	54.20^{e}	Pink	Reddish purple tinge
AAUFG 4	55.80^{de}	Dull pink	Orange
AAUFG 5	90.00^{a}	Pink with concentric circles	Intense reddish purple
AAUFG 6	90.00^{a}	Creamy white	Orange with dark purple at the center
AAUFG7	90.00^{a}	Creamy white	Dull yellow with purple at the center
AAUFG8	90.00^{a}	White cottony	Dull white
AAUFG10	67.40^{cd}	Dull pink with concentric circles	Orange
AAUFG11	75.53^{bc}	Pink	Light reddish purple
AAUFG12	81.83^{ab}	Dull pink	Light reddish purple
AAUFG13	81.17^{ab}	Dull pink	Light reddish purple
AAUFG14	80.83^{ab}	Dull pink	Light reddish purple
AAUFG15	75.00^{bc}	Dull pink	Light reddish purple
AAUFG16	78.67^{abc}	Dull pink	Light reddish purple

Values are means of 3 replicates, DAI: Days after Inoculation, letters indicate Duncan's homogeneous subsets for multiple mean comparison

Table 3: Macro and microconidial shapes, septation, phialides, chlamydospores presence

Isolates	Macroconidial general shape	Septa	Microconidial shape	Septa	Phialide	Chlamydospores present/absent
AAUFG1	Straight	3	Ovoid	0	Short	+single, in pair, at intercalary and terminal
AAUFG3	Straight with bent basal cells	1-3	Ovoid to elliptical	0	Short	+pairs, clumps at terminal and intercalary
AAUFG4	Straight	3-5	Ovoid to elliptical	0	Short	+pairs
AAUFG5	Straight	2-5	Ovoid to elliptical	0	Short	+intercalary terminal
AAUFG6	Straight with hooked basal and apical cells	3	Ovoid to cylinder	0	Short	+pairs
AAUFG7	Straight with hooked basal and apical cells	3	Ovoid to cylinderic	0	Short	+pairs
AAUFG8	Straight with pedicellate basal cells	3	Cylinderic	0	Short	+terminal
AAUFG10	Straight, broad at the middle	3	Elliptical to fusiform	0-1	Short	+in pairs
AAUFG11	Straight with blunt apical and basal cells	3	Ovoid to elliptical	0-1	Short	+in pairs
AAUFG12	Straight with bent apical and basal cells	1-3	Elliptical	0	Short	+single
AAUFG13	Straight	1-3	Elliptical	0	Short	+single
AAUFG14	Straight	1-3	Elliptical	0	Short	+ in clumps
AAUFG15	Straight	1-3	Elliptical	0	Short	+ in pairs
AAUFG16	Straight	1-3	Elliptical	0	Short	+ in pairs

+ represents chlamydospores are present

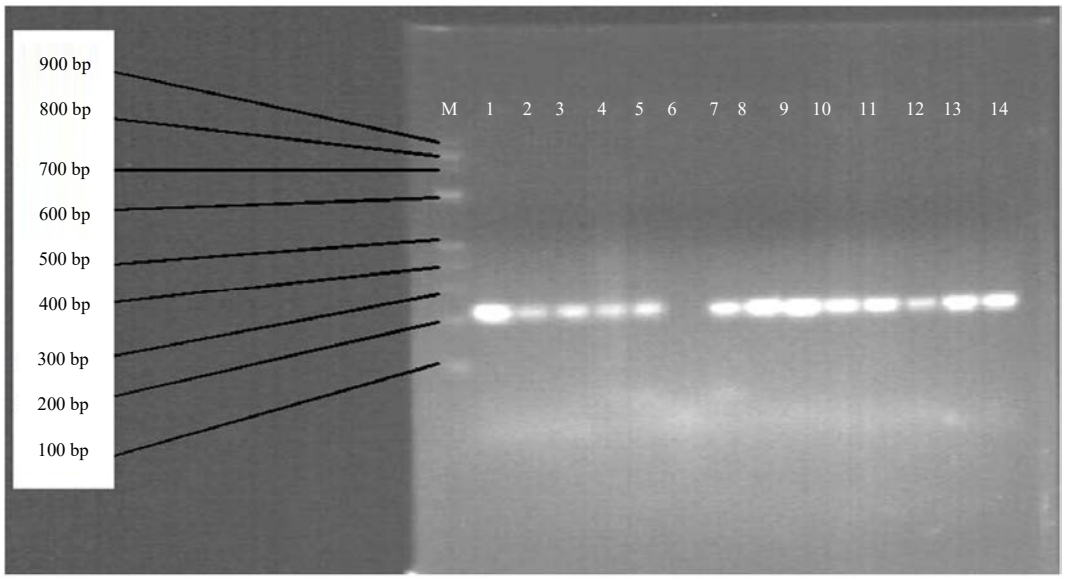

Fig. 7: PCR amplification products of the ITS1 region with primers ITS1 and ITS2
Lane M: 100 bp molecular marker, Lanes 1-14: PCR products of ITS1 regions of *Fusarium oxysporum*

Table 4: Microscopic measurements of macroconidial and microconidial size dimensions

	Spore count	Macroconidial spore size (length × breadth µm)		Microconidial spore size (length × breadth µm)	
Isolates	Number of spores (mL)	Minimum	Maximum	Minimum	Maximum
AAUFG1	2.12×10^6	25.45×6.81	38.32×8.47	9.87×4.45	13.16×5.60
AAUFG3	1.16×10^6	23.94×5.46	39.78×6.32	7.55×3.36	14.26×5.60
AAUFG4	1.42×10^6	32.24×6.23	56.44×5.79	10.23×4.88	16.08×5.62
AAUFG5	1.32×10^6	23.85×4.71	45.99×3.88	7.85×2.65	14.50×5.55
AAUFG6	2.20×10^6	17.54×3.59	32.32×5.12	10.34×4.0	16.25×6.50
AAUFG7	1.09×10^6	29.50×7.12	47.43×6.45	10.55×4.53	15.50×6.56
AAUFG8	2.01×10^6	33.50×12.19	55.26×10.67	8.42×3.57	13.31×5.36
AAUFG10	1.35×10^6	27.69×4.46	35.58×6.99	6.56×2.50	12.6×4.41
AAUFG11	1.04×10^6	26.44×4.61	36.2×5.25	7.55×2.81	11.54×6.12
AAUFG12	1.71×10^6	17.17×3.37	30.46×4.82	9.53×4.70	12.89×5.73
AAUFG13	1.92×10^6	27.69×2.96	35.98×5.56	6.56×3.25	8.94×6.51
AAUFG14	1.95×10^6	14.79×3.98	25.48×5.96	9.55×3.88	12.12×5.23
AAUFG15	2.70×10^6	19.97×3.91	29.25×4.52	8.45×3.82	12.52×5.60
AAUFG16	2.12×10^6	15.33×5.03	30.55×6.14	8.92×3.88	13.23×5.81

As of the collated data, (colony color, pigmentation, radial growth) and more importantly the presence of microconidia and the canoe-shaped macroconidia, all of the isolates are identified as *Fusarium* species. Further, the species was confirmed to be *Fusarium oxysporum* based on the possession of short phialides[7], the size and number of septa of macroconidia and microconidia with reference to Leslie and Summerell[7] and Gerlach *et al.*[24].

Molecular characterization: The DNA concentration ranging from 23.7-1086 ng μL^{-1} was obtained by the employed DNA extraction kit (HiPurA™ Fungal DNA Purification Kit, MOLBIO™).

PCR amplification of internal transcribed spacer1 (ITS1) region: Approximately, 220 bp sized single band amplicons of the ITS1 rDNA region were obtained from the PCR amplification, performed with the universal primer pairs ITS1 and ITS2 in all of the isolates. Lane 6, which resulted with no band, has shown a band of similar size, 220 bp in another experiment of this study (Fig. 7).

Restriction Fragment Length Polymorphism (RFLP) analysis: The restriction digestion experiment resulted in a band of equal size with the PCR product (220 bp) in all except one of the restriction enzymes used. The restriction enzymes, HindIII, PstI and HhaI left the PCR product undigested leaving the

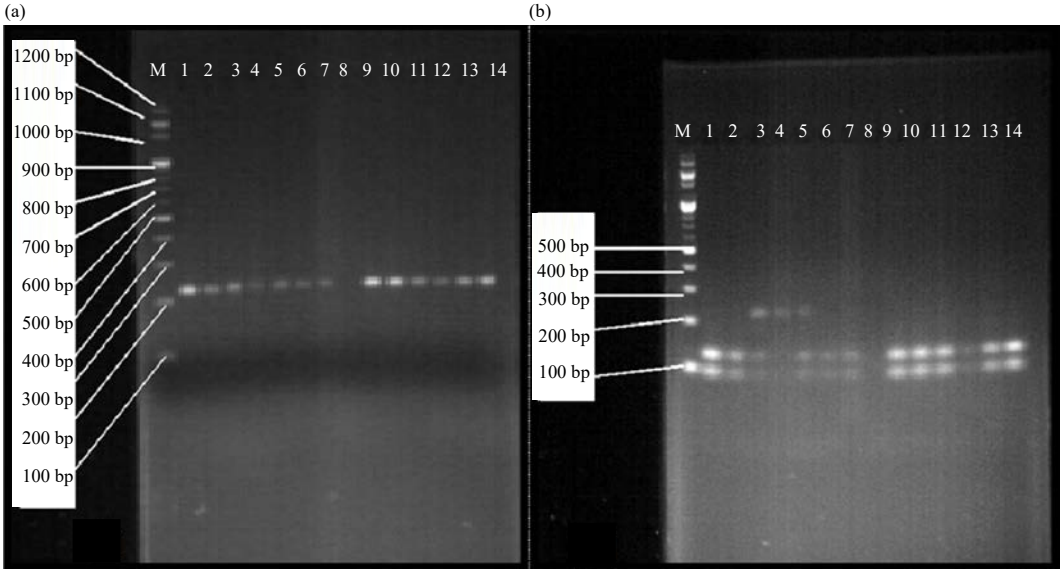

Fig. 8(a-b): Restriction pattern of PCR amplified ITS1 region of *Fusarium oxysporum* digested with (a) PstI and (b) MspI

Table 5: PCR-RFLP band patterns generated by restriction digestion of ITS1 region of *Fusarium oxysporum* with the restriction enzymes (HhaI, HindIII, PstI and MSPI)

	Restriction enzymes used					
Isolates	HhaI	HindIII	PstI	MSPI digestion	Cut frequency	Band pattern (bp)
AAUFG1	X	X	X	✓	1.0	120, 100
AAUFG3	X	X	X	✓	1.0	120, 100
AAUFG4	X	X	X	✓	0.1	220, 120 and 100
AAUFG5	X	X	X	X	0.0	220 only
AAUFG6	X	X	X	✓	0.1	220, 120 and 100
AAUFG7	X	X	X	✓	1.0	120, 100
AAUFG8	X	X	X	✓	1.0	120, 100
AAUFG10	X	X	X	X	0.0	220 only
AAUFG11	X	X	X	✓	1.0	120, 100
AAUFG12	X	X	X	✓	1.0	120, 100
AAUFG13	X	X	X	✓	1.0	120, 100
AAUFG14	X	X	X	✓	1.0	120, 100
AAUFG15	X	X	X	✓	1.0	120, 100
AAUFG16	X	X	X	✓	1.0	120, 100

✓: There is digestion, X: There is no digestion

isolates undifferentiated whereas the restriction Enzyme, MSPI produced band fragments of size different from the PCR products in most of the isolates (Table 5). Digestion with the restriction enzyme, MSPI resulted in band patterns with approximate band lengths of 220, 120 and 100 bp in reference to 100 DNA ladder (Fig. 8a-b). Ten of the isolates (71%) yielded the 100 and 120 bp fragments (Table 6). Two isolates (AAUFG5 and AAUFG10) yielded 220 bp fragments only; where the PCR product is left undigested indicating that the enzyme MSPI has no restriction site for these isolates, lane 4 (Fig. 8b). The remaining two isolates showed a special band pattern of 220, 120 and 100 bp, lane 3 and lane 5 (Fig. 8b). The speciality of this band pattern is that the MSPI restriction digested product of these isolates is composed of undigested PCR amplicon of 220 bp and cut down fragments of 120 and 100 bp.

Clustering of *Fusarium oxysporum* isolates using PCR-RFLP data: The UPGMA dendrogram resolved the isolates into two major clusters, cluster-I and cluster-II (Fig. 9). The overall similarity existing among the isolates ranged between 0-100%. The similarity between cluster-I and cluster-II was 6%. The similarity between sub cluster-I and sub cluster-II was in the range 53-76% (Fig. 9).

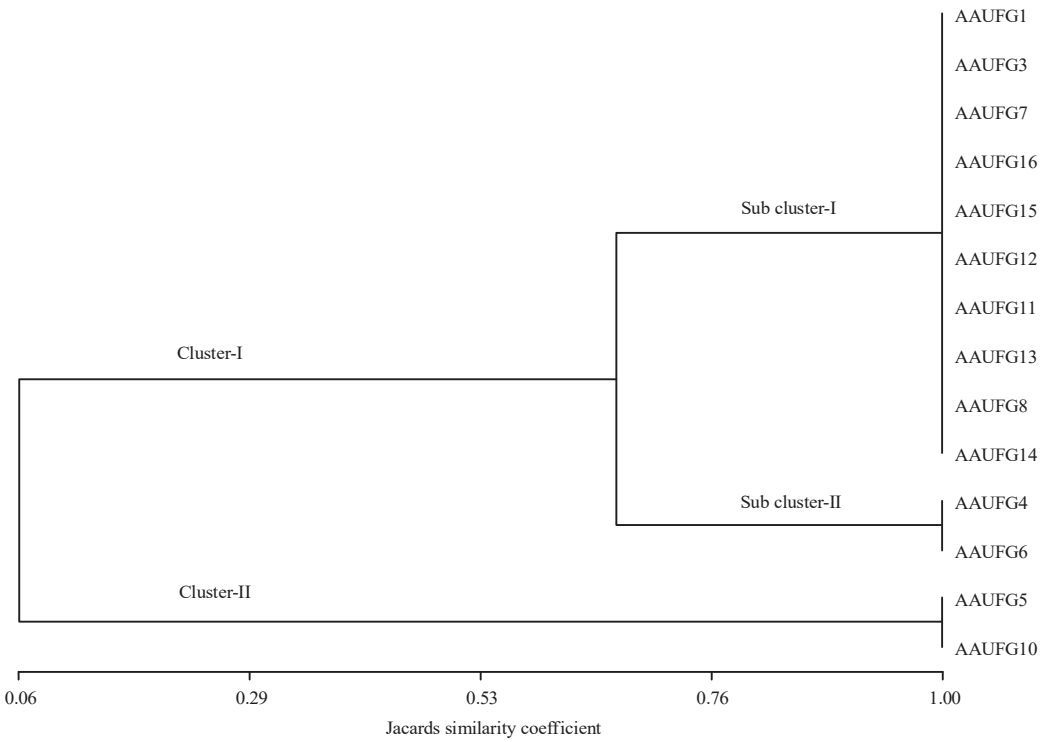

Fig. 9: UPGMA dendrogram of genetic relationships among *F. oxysporum* isolates based on Jaccard's coefficient of similarity generated from ITS1-RFLP data

Table 6: Summary table grouping isolates according to similarity in band patterns

Band pattern (bp)	Isolates that attained the band pattern	No. of isolates	Percentage
220 only	AAUFG5 and AAUFG10	2	14.29
120-100	AUFG1, AAUFG3, AAUFG7, AAUFG8, AAUFG11, AAUFG12, AAUFG13, AAUFG14, AAUFG15, AAUFG16	10	71.43
220, 120 and 100	AAUFG4 and AAUFG6	2	14.29
	Total	14	100.00

Cluster-I bearing 85.71% of the isolates diverged into two sub clusters; sub cluster-I aggregates 71.43% of the total isolates with 100% similarity between each other whereas sub cluster-II held two isolates (AAUFG4 and AAUFG6) with 100 similarity. Cluster-II bears only two isolates (AAUFG5 and AAUFG 10) with 100% similarity between the two. The cladogram resulted in two groupings that agreed with the cultural characters on PDA (colony color, adherent growth with and without concentric rings) and one grouping contrary to the grouping with cultural characters (Fig. 4). Briefly, two isolates that appeared pink in color and imparted concentric circles were placed alone separately in cluster-II (AAUFG5 and AAUFG10) (Fig. 4).

Isolates from soil which were similar in colony color, all of them appearing in dull pink color were placed in the same sub-cluster in the UPGMA cladogram (AAUFG12, AAUFG13, AAUFG14, AAUFG15 and AAUFG16). The contrary clustering of the dendrogram was that isolates AAUFG6 and AAUFG7, which were consistently showing similar colony color and pigmentation are grouped in separate sub clusters, AAUFG7 under sub cluster-I and AAUFG6 under sub cluster-II (Fig. 4).

DISCUSSION

It is clearly indicated from this study that the genus level identification of the fungal isolates revealed four fungal genera: *Fusarium*, *Trichoderma*, *Aspergillus* and *Penicillium*. Similarly, different reports[5] have also identified *Aspergillus*, *Eurotium*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* on ginger rhizome from Southern parts of Ethiopia. In the present study out of 24 fungal isolates identified, 14 fungal isolates were identified as *Fusarium oxysporum*. Four fungal pathogen isolates were resulted in higher PI values (Table 1), than a previous report by Berza *et al*.[5] which was 25.78% for *Fusarium* species.

Qualitative observation of the pathogenicity test of the *Fusarium* isolates on ginger rhizome showed white and pink mycelial growth on the surface of rhizome with red pigmentation imparting underneath the moldy surface. It has been also observed that brown discoloration of the tissues near the site of inoculation[28].

Visual inspection of the cultural characters of isolates on Potato Dextrose Agar (PDA) resulted in white, creamy white, dull pink and pink colony colors. Similarly, Gupta *et al.*[29] reported white and dull white mycelial colors of *Fusarium oxysporum* f. sp. *zingiberi*. Two creamy white isolates in the present study have fluffier aerial mycelium than that of Moreira *et al.*[30] who identified *Fusarium oxysporum* with cream colored culture on PDA from infected ginger. Even though Seifert[25] and Chaithra *et al.*[31] reported that colony color is dramatic and it could not be used as an identification criterion, a consistent colony color between subsequent batch cultures was noted in the present study.

Pigmentation varied from dull white to orange, light reddish purple, intense reddish purple and dark reddish purple falling in between the wide range of possible pigmentation described by Gerlach and Nirenberg[24]. It is also similar to the findings of Chaithra *et al.*[31] who identified *Fusarium oxysporum* f. sp. *lycopersici*. Pigmentation is a prominent secondary character that can be used in the identification of *Fusarium* species[7].

Basically, similar pigmentations but with noticeable variations in intensity of the pigments in subsequent batch cultures of the isolates were observed in the present study. The variation in pigmentation intensity between batches may most probably be accounted to the sensitivity of the organisms towards light and pH of the growth medium as explained by Leslie and Summerell[7].

Radial growth of mycelium incubated for 4 days at 30±1°C on PDA varied from 33.40-90 mm diameters on 90 mm diameter petri plates. It has been reported that a radial growth of 3.2-4.5 cm at 4 days of incubation[32] and on PDA where the upper value was by far lower than the present study; this might be probably due to three reasons: (1) the growing temperature was 26±1°C in case of Bayraktar and Dolar[12] whereas the growing temperature in the present study was 30±1°C, (2) the two extraordinarily fast growing isolates (AAUFG 6 and AAUFG 7) in the present study raised up the upper value of the range (90 mm); (3) the other possible reason is that the isolates of the former study were from guava wilt being a different formae specials.

Microconidial size between the isolates ranged from 6.56×2.50-16.25×6.50 µm whereas macroconidial size was in the range 14.79×3.98-55.26×10.67 µm. Both microconidial and macroconidial size in the present study are quite broader covering a wider range of size when compared to a report from India that identified *Fusarium oxysporum* f.sp. *zingiberi* with microconidial size ranging from 5.20×4.00-12.30×5.70 µm and macroconidial[10] size ranging from 16.20×4.70-32.0×5.7 µm, whereas it becomes narrower in terms of microconidial size but broader in terms of macroconidial size when compared with a recent report by Chebte[33] measured microconidial length ranging from 1.02-30.47 µm and macroconidial length of 47.51-79.63 µm for *Fusarium oxysporum* isolated from onion basal rot.

Comparison of these pathological, cultural and morphological records with previous works by Gupta *et al.*[29] and Moreira *et al.*[30] and standard literature of Leslie and Summerell[7] and Gerlach and Nirenberg[24], these features of the isolates complied with the descriptions of *Fusarium oxysporum* f. sp. *zingiberi.* Accordingly, the fungal isolates are identified as *Fusarium oxysporum* f. sp. *zingiberi*.

To support the cultural and morphological identification, a molecular identification study PCR-RFLP of ITS1 region was conducted. The PCR amplified product subjected to gel electrophoresis showed a single band of amplicon size 220 bp for all the *Fusarium oxysporum* isolates. This is in line with Moricca *et al.*[28] who reported a 220 bp PCR amplicons of *Fusarium oxysporum* f. sp. *zingiberi*. The length of the amplified ITS1 region was in the range reported by Santamaria *et al.*[17] who conducted an analysis of Fungal ITS1 sequences deposited in the ITS1 database, from a few nucleotides to 1400 nucleotides for Fungal ITS1 regions. In an exploration managed during the present study in the ITSoneDB, collections of 711 ITS1 sequences all from *Fusarium oxysporum*, ITS1 sequences as short as 69 bp nucleotides (accession, GU361934) and as long as 1644 bp nucleotides (accession, JX967529) have been observed. The majority of the sequences deposited in the database laid in the range 135-183 bp in length. In this regard, the isolates in current study seem to be monomorphic to this marker (ITS1) that resulted in similar band length in all isolates as opposed to the sequences deposited in the ITSoneDB.

Digestion of the amplified ITS1 region of all the *Fusarium oxysporum* isolates with restriction enzyme (MspI) showed three different band types. It gave ITS1/ITS2 PCR products of ten of the isolates broken into two fragments of 120 and 100 bp length; Two isolates left undigested giving the 220 bp PCR product itself and two other isolates resulted in both cut and uncut fragments giving the band pattern, 220, 120 and 100 bp on the gel. As explained that Burgess *et al.*[18] the organisms with amplicons yielding such band patterns are said to be heterozygous for the employed molecular marker

and only co-dominant markers are endowed with the ability to show this sort of polymorphism.

Genetic variability analysis of *Fusarium oxysporum* f. sp. *zingiberi* resulted in three haplotypes similar to Mishra *et al*.[34] who studied *Fusarium oxysporum* f. sp. *zingiberi* isolates using DNA Amplification Finger printing (DAF) technique.

Another molecular study conducted on *Fusarium oxysporum* f. sp. *zingiberi* using RAPD technique and UPGMA dendrogram phylogenetic analysis clustered 19 isolates into two major groups with 20% similarity Senapati and Ghose[10] whereas the UPGMA dendrogram generated in the present study also tended a similar bifurcating node with 6% Jaccard's similarity coefficient. Higher level of genetic polymorphism has been detected between *Fusarium oxysporum* f. sp. *zingiberi* identified in the present study. The finding that *Fusarium oxysporum* has three haplotypes is also in line with hitherto study in Ethiopia[35] that worked on PCR amplification of Translation Elongation Factor 1 alpha (TEF-1α) gene and restriction digestion of the amplicon using restriction enzymes, AluI and MseI resulted in 3 clades of *Fusarium oxysporum* which inturn also becomes similar to the finding of O'Donnell *et al*.[36].

Restriction digestion with HindIII in the present study also gave a similar result with the finding of Hussain *et al*.[37] who experimented digestion of PCR amplified ITS region of *Fusarium oxysporum* from onion basal rot with Hind III; left the PCR products undigested. This enables the deduction that this restriction enzyme (Hind III) does not have restriction site in the ITS1 region of *Fusarium oxysporum*. On the contrary, the restriction enzyme PstI did not show digestion on any of the *Fusarium oxysporum* isolates in the present study by Hussain *et al*.[37].

Generally, the *Fusarium oxysporum* strains identified in this study were diverse in their pathological, cultural, morphological and molecular characters considered in the present study. Accordingly, this finding strengthens the idea that *Fusarium oxysporum* is one of the most variable members of the genus *Fusarium*, regarding macroscopic and microscopic characteristics[24]. The molecular diversity finding also confirms the explanation that *F. oxysporum* and *F. solani* from the tropics are polyphyletic[7].

CONCLUSION

The fourteen *Fusarium* isolates were morphologically identified as *Fusarium oxysporum*. *In vitro* pathogenicity test of the *Fusarium* isolates showed that they were different in virulence where AAUFG6 was the most pathogenic followed by AAUFG7, AAUFG1 and AAUFG8, respectively. The virulence level of the *Fusarium oxysporum* isolates was directly proportional to their respective radial growth measurement; the fast growing the isolate is the highly pathogenic.

Based on cultural characters (colony color and reverse color) the *Fusarium* isolates are categorized as white, creamy white and pink on the adverse side and dull white, light reddish purple, intense reddish purple and dark reddish purple on the reverse side. Regarding radial growth, they were rated as fast growing, moderate growing and slow growing. Microscopically, macroconidia of the isolates were mostly straight with 1-5 septa and oval to elliptical microconidia with 0-1 septate. Phialides were short and chlamydospores were in pairs and in clumps at intercalary and terminal positions.

Restriction digestion of the PCR amplified ITS1 region with MspI showed intra-species genotypic variation between the isolates. The UPGMA dendrogram generated from the similarity matrix data of ITS1/ITS2 PCR-RFLP band profiles indicated that the genetic polymorphism between the species of *Fusarium oxysporum* in the study areas.

SIGNIFICANCE STATEMENT

Ginger (*Zingiber officinale*) is attacked by different pathogens, particularly fungi, bacteria and nematodes in the field. This study discovered that *Fusarium* species is responsible for the ginger wilt disease observed in the study area, causing reduction in the productivity of the crop. The study described the level of pathogenicity as well as the cultural, morphological and molecular features of the isolated pathogens. Since knowledge on the nature of the pathogenic agent is a precondition to take actions on disease prevention and control, the finding in this study could be beneficial for agricultural experts to design a reasonable disease management strategy against ginger *Fusarium* wilt disease. The finding of the study also indicated the power of the DNA barcode ITS1 marker to detect intra species variation among the *F. oxysporum* isolates that was not explored before. It will serve as stepping stone for researchers who wish to work on intra species variation among other fungal species.

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