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ABSTRACT

Morphological and Molecular Characterizations of wilt disease of Ginger (*Zingiber officinale* Roscoe) Caused by *Fusarium sp*

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Ginger rhizome, pseudostem, leaf and soil samples were collected from ginger growing areas of southwestern Ethiopia. Fungal pathogens were isolated from ginger parts showing vascular browning symptoms and soil following standard methods. Isolation revealed fungal isolates belonging to four genera: *Fusarium*, *Penicillium*, *Aspergillus* and *Trichoderma*. Out of 24 fungal isolates 14 isolates were *Fusarium* species. In vitro pathogenicity test resulted in pathogenicity index (PI) values ranging from 10.96% +1.55 (AAUFG13) to 45.35% +11.57 (AAUFG6). Cultures of *Fusarium* species on 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 pigmentations on the riverse side. Canoe shaped macroconidia, ovoid microconidia and short phialides were pertinent to all of the *Fusarium* isolates. Based on their cultural and morphological characters the isolates were identified as *Fusarium oxysporum*. The ability of the isolates to induce rhizome rot confirmed the forma specials rank of the isolates as *F. oxysporum* f. sp. *zingiberi* (Foz). PCR- RFLP profile of the ITS1 rDNA region indicated genetic variation between the *Fusarium* isolates. *In silco* RFLP digestion of ITS1 rDNA sequences of *F. oxysporum* retrieved from the fungal ITSoneDB was performed using online restriction mapper software and restriction enzymes used in the actual experiment. The result of the *in silco* restriction digestion simulation was similar with the actual PCR_RFLP study supporting that the isolates are *Fusarium oxysporum*.

Key words: *Fusarium wilt*, ITS1, Pathogenicity, PCR-RFLP, Rizhome rot

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List of Acronyms

AAU:	Addis Ababa University
ACP:	All Agricultural Commodities Program
AFLP:	Amplified Fragment Length Polymorphism
APS:	American Phytopathological Society
CSA:	Central Statistics Agency
EIA	Ethiopian Investment Agency
ETS	External Trade Statistic
ITS:	Internal Transcribed Spacer
ITS1:	Internal Transcribed Spacer 1
ITS2:	Internal Transcribed Spacer 2
ITSoneDB:	Fungal ITS1 region sequence Data Base
RAPD:	Randomly Amplified Polymorphic DNA
rDNA:	ribosomal Deoxy Ribose Nucleic Acid
RFLP:	Restriction Fragment Length Polymorphism
RNA:	Ribose Nucleic Acid
rRNA:	ribosomal Ribose Nucleic Acid
SNNPRS IEP:	SNNPRS Investment Expansion Process
SSR:	Simple Sequence Repeat

INTRODUCTION

Ginger (*Zingiber officinae Roscoe*), a member of the plant family Zingiberaceae, is a globally popular tropical herbaceous perennial, with its rhizome merited for its culinary and medicinal properties. Ginger is thought to be originated in South East Asia where India and China are frequently mentioned to be homelands (Jansen, 1981b; Vasala, 2001). Ginger entered to Africa through two gates; East Africa by the then Arabian traders in1292 and through West Africa by the Portuguese around 1567 (Okwuowulu, 2005).

In Ethiopia, Ginger cultivation started during the 13th Century following its introduction to East Africa (Jansen, 198b; Okwuowulu, 2005). The major ginger growing areas in Ethiopia include wetter regions at altitude below 2000 m (Yohannes Wagesho, 2010). The producing areas in SNNPRS are said to be ginger belts in Ethiopia where much of the country's production and marketing activities are undertaken (Endrias Geta and Asfaw Kifle, 2011). Ginger is among the important spices used in every Ethiopian kitchen for the preparation of pepper powder and stew (Jansen, 1981b). It has also some use in traditional medicine for the treatment of flu and stomach ache (Jansen, 1981b; Girma Hailemichael and Digafie Tilahun, 2004). It has been reported that among the spices that are produced and exported from Ethiopia, ginger is leading both in volume and the value of foreign currency it generates. It has contributed 65% to the spice export value in the years 2008-2010 (ACP, 2010).

However, despite of its contribution to the life of the cultivating family and to the national economy, its large scale production is limited by various problems (Endrias Geta

and Asfaw Kifle, 2011; Belay Berza *et al.*, 2012; Biruk Ayenew, 2012). Among the reported problems, ginger wilt disease has become the merely threatening one. Knowledge on the causative agents of the disease is not well established. There are also arguments among professionals in the field whether the disease is of bacterial or fungal (Sinedu Abate, 2013). The problem is more aggravated since a sudden emergence of the disease occurred in the 2012 cropping season. Therefore, this study has been undertaken to examine if fungal infections are the causes for the ginger wilt disease which has largely affected ginger production in the southwestern parts of Ethiopia. The fungal genera *Fusarium* and *Pythium* are the most recognized pathogens having close intimacy with ginger (Sagar, 2006). Several genera of fungi, including *Fusarium*, *Penicillium*, *Aspergillus*, *Mucor*, *Eurotium* and *Rhizopus* have been isolated from ginger as causal agents of rhizome rot on the crop in Ethiopia (Belay Berza *et al*., 2012).

Fusarium is one of the most ubiquitous, abundant, and important genera of soil micro fungi. The genus contains many species of environmental, agricultural and human health importance (Maina *et al.*, 2009). The notoriety of this genus mainly results from pathogenicity towards a wide range of plants (Maina *et al.*, 2009). Many plants have at least one *Fusarium*-associated disease (Leslie and Summerell, 2006). Pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in over 100 plant species, among which there are several economically important crops (Lievens *et al.*, 2008). 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* (Senapati and Ghose, 2005; Pappalardo *et al*., 2009). *Fusarium solani* (Ramteke and Kamble, 2011), *Fusarium equiseti* (Senapati

and Ghose, 2005) and *Fusarium semitechtum* (Moreira *et al*., 2013) are also reported to cause ginger rhizome rot. These species can be recognized by their characteristic microscopic features.

Fusarium semitectum is characterized by the spindle shaped (look like rabbit ears) macroconidia on CLA and by the lack of microconidia (Leslie and Summerell, 2006). *Fusarium oxysporum* and *Fusarium solani* can be distinguished from one another by the length of microconidial bearing monophialides and the microconidia themselves. The monophialides of *Fusarium solani* are quite longer than the relatively short monophialides of *Fusarium oxysporum*. Microconidia of *Fusarium solani* tend to be wider, more oval in shape and to have thicker wall than that of the microconidia of *Fusarium oxysporum* (Leslie and Summerell, 2006). *Fusarium equiseti* is distinguished from all the above species for its long to very long macroconidia 5-7 septa, pronounced dorso ventral curvature, foot shaped basal cells and prolonged or even to whip like apical cells; and by the absence of microconidia (Leslie and Summerell, 2006).

Isolation and identification of pathogens is a precondition to control pathogenic diseases. Studying these pathogens and controlling them will help to increase the yield and quality of ginger product, thereby supporting the supply and finally quenching the demand. Knowledge about the pathogens provides a way to seek for prevention and treatment strategies.Therefore, the present research project has been undertaken to identify and study the cultural, morphological and molecular characterization of the causative agents of vascular wilt disease of ginger (*Fusarium* sp).

OBJECTIVES

2.1. General objective

The main objective of this study is to isolate, identify and study the cultural, morphological and molecular characters of *Fusarium* wilt pathogen (*Fusarium* sp) from South Western parts of Ethiopia

2.2. Specific objectives

- To isolate, identify and characterize *Fusarium* sp retrieved from diseased ginger samples
- To examine cultural and morphological characteristics of *Fusarium* sp isolated from diseased host
- To study the molecular variability among the identified *Fusarium* species

LITERATURE REVIEW

3.1. Vernacular names, Taxonomy, botanical description, geographical distribution and traditional uses of ginger

3.1.1. Vernacular names of ginger in different foreign languages

Ginger *(Zingiber officinale* R.*)* is worldwide known for its culinary and medicinal values; as such its names in most of languages in the world share some syllables as for instance, Ginger in (English), Gember in (Dutch), Gingembre in (French), Gyomber in (Hungarian), Gimbre/Gengiver in (Portuguese), Gingibre in (Spanish), Ghimber/Imbir in (Rumanian), Imbir in (Russian), Ingwer in (German), Ingifaer in (Scandinivian), Zenzero/Zenzebero in (Italian), Zanjabil in (Arabic), Shangabir/ Zangabil in (Persian), Ganjian (dried rhizome) in (Chinese), Mangaratia in (Brazilian) and Shoukya (dried rhizome) in (Japanese) (Pakrashi and Pakrashi, 2003).

3.1.2. Vernacular names of ginger in some Ethiopian languages

In Ethiopia the spice crop *Zingiber officinale* is recognized as Zingibil in (Amharic and Tigrigna); Zinjibillaa in (Afan Oromo); Jingibl in (Gurage Language) (Lock, 1995) and Yenjeluwa in (Wolayta) and Janjebelu in (Kembata) (Home Gardens of Ethiopia, 2010 unpublished). Among these, the name “ZINJIBIL” is the most popular name used by different ethnic groups from local to national market levels and considered as the common vernacular name of the product (Home Gardens of Ethiopia, 2010 unpublished).

3.1.3. Taxonomic position of ginger (*Zingiber officinale*)

Ginger belongs to Kingdom: Plantae, Division: Angiosperma, Class: Monocotyledoneae, Order: Scitaminaea, Family: Zingiberaceae, Genus: *Zingiber,* Species: *officinale* (Natural Remedies, 1999). The species name *Zingiber officinale* was given by the English botanist William Roscoe (1753-1831) in an 1807 publication (Ghosh *et al.* 2011) and that is why this scientific name is commonly annexed with the name Roscoe to give *Zingiber officinale* Roscoe.

3.1.4. Botanical descriptions

Zingiber officinale: is a rhizomatous herb; rhizomes thick; leafy shoots to 1 m tall (Fig.1A). Leaves linear to lanceolate, up to 20x2 cm, apex acuminate, narrowly cuneate at base; ligule up to 5mm long, bi-lobed, glaborous, later scarious. Inflorescence arising from rhizome at base of leafy shoots, peduncle 10-20 cm long; spike ellipsoid, 4-7 x 1.5-2.5 cm; bracts 2-3 x 1.5-2 cm, green with scarious margins, each sub tending a single flower. Calyx is whitish. Corolla is pale yellow. Labellum is 3-lobbed, the central lobe largest, dark purplish with yellow spots. Fruits are very rare in cultivation (Jansen, 1981a). Scraped rhizome with buff external surface showing longitudinal striations and occasional loose fibers, outer surface dark brown and more or less covered with cork which shows conspicuous, narrow, longitudinal and transverse ridges (Fig.1B); the cork readily exfoliates from lateral surfaces but persists between branches. Smoothed transversely cut surface exhibiting a narrow cortex separated by an endodermis from a much wider stele, numerous widely scattered fibro vascular bundles, abundant scattered oleoresin cells with yellow contents (Natural Remedies, 1999).

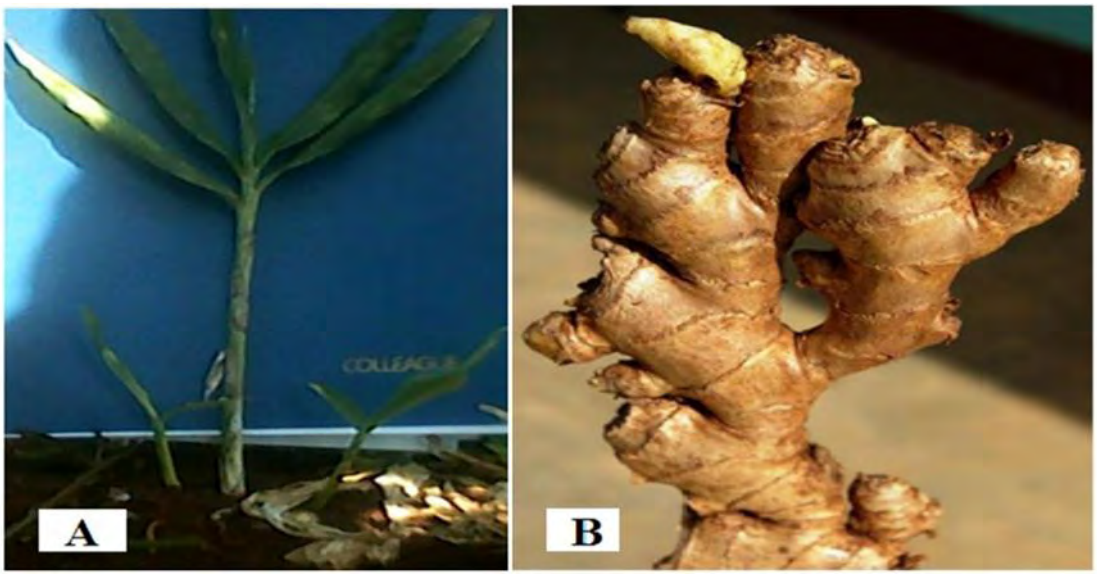


Fig.1. Ginger shoot showing leaves and pseudostem (A) Underground Ginger rhizome (B)

3.1.5. Geographical distribution and traditional uses of ginger

The plant is widely cultivated all over India, Bangladesh, Taiwan, Indonesia, Nigeria, the Philippines, Thailand, Jamaica, Mexico, Brazil, Australia, Haiti, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua and Ethiopia (Plotto, 2002; Okwuowulu, 2005; Girma Hailemichael and Kindie Tesfaye, 2008; Mendi *et al.*, 2009). It is a perennial that grows in warm climates (Natural Remedies, 1999).

Ginger is carminative, pungent, stimulant, used widely for indigestion, malaria and fevers. It has been chiefly used to cure diseases due to morbidity of Kapha and Vata in India (Natural Remedies, 1999). Ginger with lime juice and rock salt increases appetite and stimulates the secretion of gastric juices. It is said to be used for abdominal pain, anorexia, arthritis, atonic dyspepsia, bleeding, cancer, chest congestion, chicken pox, cholera, chronic bronchitis, cold extremities, colic, colitis, common cold, cough, cystic fibrosis, diarrhea, difficulty in breathing, dropsy, fever, flatulent, disorders of gallbladder,

hyperacidity, hypercholesterolemia, hyperglycemia, morning sickness, nausea, rheumatism, throat ache and vomiting (Natural Remedies, 1999). Ginger also forms an important constituent of many pharmacopoeia ayurvedic formulations (Natural Remedies, 1999; Ghosh *et al.*, 2011). Ethiopians chew fresh or dried ginger rhizome to get relieved from accidental constipation. Ginger is one of the principal constituents of the dry powdered chilly (Berbere) of the Ethiopians hot stew ingredient. Ginger is believed to heal from common cold when prepared in the form of tea locally known as "Keshir". Keshir can be prepared in home and is available in cafeterias and also in some petty business houses.

3.2. Agronomic characteristics of ginger

Ginger has low genetic diversity because it reproduces asexually by vegetative propagation in which the new plants are created from the rhizome of the parent plant. The stem generated from the plants' rhizome forms a bud that becomes a complete plant, a clone of the original plant. The new plant arises from no production of seeds (http://www. bioweb.uwlax.edu).

Ginger is a relatively long term crop taking 10-12 months from planting to harvest. The crop may be harvested from December to May of the following year. It takes about six weeks for shoots to emerge after seed set plantation.Vegetative growth is maximized until flowering begins in September-October. Flowering marks the beginning of rhizome maturity and increasing fibrous tissue development (Valenzuela, 2011).

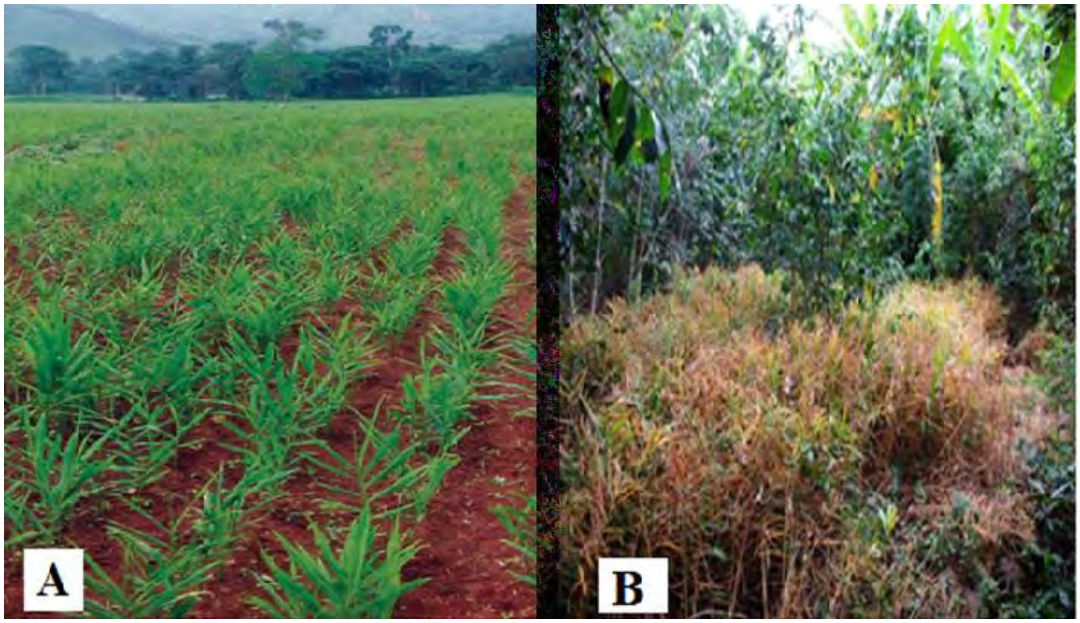
In Ethiopia specifically in SNNPR, farmers prefer to plant ginger in December-February. In this region, harvesting of ginger usually begins 8 to 9 months starting from late September when the leaves start turning yellow and the stems stop growing, which may extend to March or until the onset of the spring rain (Endrias Geta and Asfaw Kifle, 2011; Asfaw Kifle and Birhanu Sima, 2013). Sometimes ginger is grown as a perennial crop in SNNPR. In this case farmers deliberately leave the matured ginger rhizomes in the field for two or more successive seasons without harvesting (Endrias Geta and Asfaw Kifle, 2011; Asfaw Kifle and Birhanu Sima, 2013). In the pre-harvest management practices there are two ways of harvesting ginger; the one-season harvesting and perennated harvesting (Yadere or Yekereme) (Home Gardens of Ethiopia, 2010 unpublished). The one-season harvesting is the practice in which ginger is harvested after 8 to 10 months of planting time which is known to keep the natural quality of the ginger like, less fibrous, more essential oil and oleoresin content. Perennated harvesting is the practice of harvesting ginger after two years of planting time where such a practice is reported to affect the natural quality of ginger (Home Gardens of Ethiopia, 2010 unpublished; Asfaw Kifle and Birhanu Sima, 2013).

3.3. Ecological distribution and requirement of ginger

The ginger producing belt in the study areas is found between altitudinal ranges of 1100 to 1800 meters above sea level. This altitudinal range falls within the WEYNA DEGA Agro-Ecology setting of the country. The WEYNA DEGA agro-ecology is reported as very suitable agro ecology for production of ginger (Home Gardens of Ethiopia, 2010 unpublished). In addition, the annual rainfall amount and the warmer temperature are the

main environmental requirements associated with the production of ginger in the study areas. The altitudinal range between 1300 to 2000 meters above sea level is suitable for ginger cultivation if the average rainfall is greater than 1200 mm per annum in the region. In general, in Ethiopia ginger cultivation requires sub optimal conditions like, temperature range between 28 ^{0}C to 32 ^{0}C, altitudes up to 2000 m., and rain fall often less than 1500 mm per year (Home Gardens of Ethiopia, 2010, unpublished).

In the study areas ginger is cultivated mainly in the home garden both as a monocrop in separate plots (Fig. 2 A) and inter-cropped with coffee (*Coffea arabica*), enset (*Ensete ventricosum*), banana (*Musa paradisiaca*), papaya (*Carica papaya*), pepper (*capsicum annuum*), cabbages (*Brassica species.*) and taro (*Colocasia esculenta*) in which these plants withstanding the effect of light and providing shade for ginger plant (Fig. 2B). There is also some level of field cultivation in the form of strip cropping with maize (*Zea mays*) and taro which is usually not far from the house. In addition, the livestock component of the biodiversity has a direct link with respect to compost supply in which the cultivation of ginger in the study areas predominantly depend on animal manure, plant remains and household wastes.


A
B

3.5. Recommended storage conditions for ginger rhizome

Because ginger rhizomes are bulky and perishable, the storage of the seed rhizome for 3 to 4 months from harvesting to the next planting season is usually encountered with several problems, such as rotting, sprouting and shriveling, which can result loss of the important rhizome. Therefore, adopting storage techniques that can mitigate the loss coming as a result of improper storage is imperative (Alye Tefera, 2013). The temperature at which ginger rhizomes are to be stored, the relative humidity (HR) and ventilation conditions are important factors to be considered during ginger storage. The optimal temperature for storing ginger is 12°C (Alye Tefera, 2013). Storing ginger in the room temperature range (25-30°C) causes high moisture loss, surface shriveling, and sprouting of the rhizome whereas temperature below 12°C exposes the rhizome to chilling injury (Alye Tefera, 2013). Rhizomes stored in 100% gauze polythene bag with 3% ventilation covered by dry sand has been claimed to be the most effective storage condition (Pakrashi and Pakrashi, 2003).

3.6. History of ginger cultivation in Ethiopia and areas of production

Ginger is known to have been introduced to Ethiopia as early as in the 13th century (Jansen, 1981). It is cultivated in many places of the country than any other spices (EIA, 2010, unpublished). In Ethiopia it is limited mostly in the wetter regions of Southern Nations, Nationalities and Peoples Regional State (SNNPRS) and some parts of western Oromiya. Most of the commercial production is practiced in SNNPR by farmers within Kambata-Tambaro, Wolaita and Hadiya zones (Home Gardens of Ethiopia, 2010, unpublished, Endrias Geta and Asfaw Kifle, 2011). In this region the major ginger

producing weredas are found in Kembata-Tembaro and Welayta zones and the minor ginger producing weredas are found in Hadiya, Dawro, Kefa and Bench Maji zones (Home Gardens of Ethiopia, 2010, unpublished; Asfaw Kifle and Brihanu Sima, 2013). Other areas include East and West Gojam, Illubabor, Jima, North and South Omo, Bale, Sidamo and Wollega zones of the country (EIA, 2010, unpublished). The producing areas in SNNPRS are said to be Ginger belts in Ethiopia where much of the country's production and marketing activities are located (Endrias Geta and Asfaw Kifle, 2011). Ginger is produced as cash crop, and is among the important spices used in every Ethiopian kitchen for the preparation of pepper powder and stew. It has also some use in traditional medicine for the treatment of flu and stomach ache (Jansen, 1981; Girma Hailemichael and Digafie Tilahun, 2004).

3.7. Economic importance of ginger to Ethiopia

Meanwhile, Ethiopia generates a substantial amount of foreign currency from spice exports. In 2007/08 fiscal year, spice exports secured 11.6 million dollars from exporting 14 million tones of spices (ETS, 2009). In addition, within those years, export of spice has increased from 4.4 million tons to 14 million tons (ETS, 2009). Among the spices that are produced and exported from Ethiopia, ginger is leading both in volume and the value of foreign currency it generates (ETS, 2009). From 2003 to 2007, the value of export of ginger has increased from Birr 14 million to56 Million (ETS, 2009). In the year 2008 more than Birr 59 million was generated from ginger (ETS, 2009). Ginger is the second most widely cultivated spice in Ethiopia, next to chilies (Biruk Ayenew *et al.*, 2012). Ginger being a typical "cash crop" is the largest exported spice in the years, 2008-

2010. It contributed 65% to the spice export value in those years (ACP, 2010 unpublished; Home Gardens of Ethiopia, 2010 unpublished). The top destinations of ginger export from Ethiopia are Sudan, with a lion's share of 47%, followed by India, United Arab Emirates, Morocco, and Yemen with a share of 13%, 8.8%, 7.5%, and 7% respectively (Masresha Yimer, 2010; Home Gardens of Ethiopia, 2010 unpublished).

All this data can show the significant role that ginger is playing and its' potential to play in the life of the farmers in ginger producing areas. In addition, it plays a significant role in the national economy since the highest proportion of the product is for export market (Home Gardens of Ethiopia, 2010 unpublished).

3.8. Problems associated with the production of ginger in Ethiopia

Production and marketing of ginger in the ginger production areas is constrained by a lot of problems: low quality varieties, poor pre- and post-harvest handling practices, low product market prices, lack of access to markets (Home Gardens of Ethiopia, 2010 unpublished; Endrias Geta and Asfaw Kifle, 2011; Sinedu Abate, 2013), Crucial shortage of planting material (Biruk Ayenew *et al.,* 2012), Lack of awareness to determine the optimum seed rhizome size or seed sets (Girma Hailemichael and Kinde Tesfaye, 2008; Endrias Geta and Asfaw Kifle, 2011) and Post-harvest deterioration due to fungal invasion (Home Gardens of Ethiopia, 2010 unpublished; Belay Berza *et al.*, 2012; Sinedu Abate, 2013). During the time of investigation in the present study (2013), vascular wilt of ginger in the field has been a hot issue among the concerned parties; officials of the Ethiopian Institute of Agricultural Research (EIAR), Farmers, agricultural bureau officers and researchers in the field.

3.9. Plant diseases and insect pests associated with ginger

Plant diseases are of two general types, parasitic and non parasitic. The parasitic diseases are caused by living organisms which subsist in whole or in part on the plant, thereby making the natural development of the plant impaired. These diseases are caused by fungi, bacteria, viruses, and nematodes (Trujillo, 1964). There are also potential insect pests attacking ginger: Tissue borer including shoot borer, *Dichocrosis punctiferatis* and rhizome maggots (*Formosina flavipes*, *Chaloidomyia articornis)* are the most dangerous ones; leaf eating caterpillars (*udaspes folus)* sucking insects such as hard scale (*Aspidiotus hartii*)(Pakrashi and Pakrashi, 2003); Nigra scale, Hibiscus scale, and Florida Black Scale (*Parasaissetia nigra*) are found throughout the world (Susan, 2012).

Sagar (2006) stated that rhizome rot is the major constraint in the production of ginger. It is caused by a multitude of pathogens either alone or in combination and distributed worldwide wherever intensive ginger cultivation is pursued. Rhizome rot as the name indicates results ultimately in rotting of rhizome, which is economically valuable part of ginger. The term rhizome rot is loosely used for all the diseases affecting the rhizome irrespective of the pathogens involved. The major pathogens involved in rhizome rot are species of *Pythium* causing soft rot, *Fusarium* spp. causing yellows or wilt and *Pseudomonas solanacearum* causing bacterial wilt (Pakrashi and Pakrashi, 2003, Sagar, 2006, Sinedu Abate, 2013). All these pathogens are known to form complexes with nematodes aggravating and causing disease on the ginger. Nematodes by themselves are also important pathogens of ginger. Pathogens infecting in the field also may cause decay of rhizomes in storage as well (Sagar, 2006).

In Ethiopia, Belay Berza *et al.* (2012) have identified that post harvest management practices by the local growers also cause post harvest spoilage. Microorganisms comprising six fungal genera (*Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus*, *Eurotium* and *Mucor*) associated with the post-harvest decay of ginger from Hadaro-Tunto and Boloso Bombae areas of southern Ethiopia were isolated by these investigators.

Belay Berza *et al.,* (2012) observed that all the identified fungal genera were found to be pathogenic to fresh ginger rhizomes with pathogenicity indices ranging from 17.35 % to 27.35 %**.** *Rhizopus* and *Eurotium* species had relatively the highest percentage of pathogenicity indices (27.35%) and (27.20%) respectively at Boloso-Bombae followed by *Fusarium* (25.78%) at Hadaro-Tunto. *Mucor* species caused relatively the least spoilage and rot. In the survey done during the sample collection period of the present investigation (November, 2013), 80% -100% crop loss has been recorded in the study areas.

3.9.1. Rhizome rot by *Fusarium oxysporum*

Fusarium oxysporum is a soil borne fungal species that includes both pathogenic and non-pathogenic forms (Leslie and Summerell, 2006; Chakrabarti *et al.,* 2011). Overall, this species causes wilt disease on a wide range of plant species; however, individual isolates have narrow host ranges and can be classified depending on host range, at the subspecies level as formae speciales (f.sp.). The formae speciales that causes tomato wilt is known as *F. oxysporum* f.sp *lycopersici* (*Fol*) where as the causal pathogen of cotton wilt is *F. oxysporum* f.sp. *vasinfectum* (*Fov*) (Loganathan *et al.,* 2009). *Fusarium* causes vascular wilt of vegetables, flowers, ornamentals and other important crops (Loganathan

et al., 2009). Mostly, vascular wilt of ginger is caused by *Fusarium oxysporum* f.sp. *zingiberi* (Senapati and Ghose, 2005; Pappalardo *et al*., 2009; Chaithra, 2013; Gupta *et al.*, 2014; Refai *et al.*, 2015). *Fusarium solani* (Ramteke and Kamble, 2011), *Fusarium equiseti* (Senapati and Ghose, 2005) and *Fusarium semitechtum* (Moreira *et al*., 2013) are also reported to cause ginger rhizome rot.

3.9. 2. Symptoms of *Fusarium* wilt

Vascular wilt of ginger caused by *Fusarium oxysporum* f.sp. *zingiberi* is the most serious problem among various diseases affecting ginger (Gupta *et al.,* 2014). Initial symptoms are yellowing of the foliage, beginning with the lower leaves and progressing upward (Fig. 3A). Later, yellowing starts in only one side of a leaf midrib, one branch, or one side of the affected plant. Infected leaves later show downward curling, followed by browning and drying (Loganathan *et al.,* 2009). Vascular browning can be seen in infected stems and large leaf petioles. Affected plants and their root systems become stunted. The degree of stunting depends upon time of root infection; hence young plants suffered severely than the matured plants (Fig. 3 B). Plants infected with *Fusarium* have a brown discoloration of the vascular vessels of the rhizome (Loganathan *et al.,* 2009) and a prominent black dry rot of the tissues of the cortex (Gupta *et al.,* 2014). This dry rot is characterized by collapse of the cortical tissues, occasionally accompanied by a purpling of the infected areas of the rhizome and a white cottony mycelial growth on the cut surfaces of ginger pieces (Gupta *et al.,* 2014). This discoloration can be used for diagnosis (Loganathan *et al.*, 2009).

Fig. 3. Ginger plantlets with symptoms of *Fusarium* wilt: (A) aged crop with the symptom; (B) young wilted plantlet

3.10. The genus *Fusarium*

Fusarium is one of the most ubiquitous, abundant, and important genera of soil micro fungi. The genus contains many species of environmental, agricultural and human health importance (Maina *et al.*, 2009). The notoriety of this genus mainly results from pathogenicity towards a wide range of plants (Maina *et al.*, 2009). Many plants have at least one *Fusarium*-associated disease. A recent persual of the plant disease list maintained by the American Phytopathological Society (APS) revealed that over 81 among 101 economically important plants on the list had at least one associated *Fusarium* disease (Leslie and Summerell, 2006). Lievens *et al.* (2008) reinforced this stating that Pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in over 100 plant species, among which there are several economically important crops. As these fungi also may grow as apparently symptomless as endophytes under many conditions,

the claim that, “If it is green, there is some *Fusarium* that can grow on it, in it, or with it” probably is not too far removed from the truth (Leslie and Summerell, 2006).

3.10.1. Classification of the genus *Fusarium*

The genus *Fusarium* belongs to the *Ascomycota* phylum, *Ascomycetes* class, *Hypocreales* order, while the teleomorphs of *Fusarium* species are mostly classified in the genus *Gibberella*, and for a smaller number of species, *Hemanectria* and *Albonectria* genera (Leslie and Summerell, 2006; Moretti *et al.*, 2009). Teleomorphs (sexual stages) for the species *Fusarium oxysporum* is unknown (Leslie and Summerell, 2006).

3.10.2. Geographic distribution and host range of *Fusarium oxysporum*

Fusarium oxysporum is reported as cosmopolitan in its distribution being an important vascular wilt pathogen on a variety of plant species worldwide and also a common soil saprophyte (Leslie and Summerell, 2006). It is responsible for an enormous range of plant diseases, usually involving a vascular wilt syndrome (Leslie and Summerell, 2006). The majority of the isolates causing vascular wilts are specific strains that infect only on a small number of host plants and are differentiated on the basis of pathogenicity as *formae specials* (Leslie and Summerell, 2006).

3.10.3. Reported world prevalence of *Fusarium oxysporum* f. sp. zingiberi

Fusarium oxysporum f.sp. zingiberi is one among the crop damaging *formae specials* of *Fusarium oxysporum.* Like its sister formae specials do on other crops, it causes vascular wilt on ginger plant. Ginger vascular wilt caused by to *Fusarium oxysporum* f.sp. zingiberi has been reported from around the world. The earliest of those reports refers to

the USA, Hawai state (Trijullo, 1964) where as majority of the reports recur from India (Dake and Edison, 1989; Senapati and Ghose, 2005; Chaithra *et al.*, 2013; Gupta *et al.*, 2014). Australia follows India with two reports (Stirling *et al.*, 2004; Pappalardo *et al.*, 2009) where as single reports could have been accessed from Thailand (Trikarunasawat, 2008) and Brazil (Moreira *et al.*, 2013). The Chinese have also very recently reported the identification of this pathogen for the first time (Li *et al.*, 2014). This data is in accordance with the accessibility of such research outcomes in the internet.

3.11. Ambient growth conditions for *Fusarium oxysporum:* growth media, temperature and pH

Various agar media have been used as standards on which to grow cultures for the identification of *Fusarium species*. Carnation Leaf-piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA), and Potato Dextrose Agar (PDA) are the standard media used in the identification of *Fusarium species* (Leslie and Summerell, 2006). PDA is one of the most commonly used culture media because of its simple formulations and its ability to support mycelial growth of a wide range of fungi (Albert *et al.,* 2012). PDA is used for growing and studying cultural and morphological characters of *Fusarium species* (Gerlach and Nirenberg, 1982; Seifert, 1996; Leslie and Summerell, 2006).

Most authors of standard literatures and research outputs reported that 25 ^{o} C is optimum to grow and characterize cultures of *Fusarium species* (Khaled, 2006; Lesile and Summerell, 2006; Ramteke and Kamble, 2011) whereas some recent articles report a faster and vigorous growth of Fusarium cultures at temperatures above 25 ^{o}C, mostly (28-30 ^{o}C) (Gupta *et al.*, 2010; khilare and Ahmed, 2012). Gupta *et al.* (2010) established that

pH 5.5 is best for mycelial growth and pH 6.5 is best for maximum macroconidial production of *Fusarium oxysporum*. Khilare and Ahmed, (2012) reported pH 6 is highest in terms of high conidial production; They also stated that the pH range 4.5 to 8.0 is optimum for *Fusarium oxysporum* where they also reportedly stated that the foremost acidic and alkaline pH conditions are not suitable for the growth of *Fusarium oxysporum* f.sp. ciceris.

3.12. Cultural characters of *Fusarium*: radial growth, aerial mycelium and pigmentation

Growth rates are used primarily to distinguish between the slowly growing species (less than 3 cm diameter) in sections *Eupionnotes* and *Arachnites*, from the more rapidly growing species (7-10 cm diameter) in other sections. There are a few species in sections *Discolor* and *Lateritium* that are characterized in part, by intermediate growth rates (Seifert, 1996). Aerial mycelium is the growth of hyphae above the agar surface, often forming a convex shape, with a cottony or somewhat ropey texture. This character is used to distinguish species in sections *Eupionnotes* and *arachnites*, which tend to have smooth colonies with little aerial mycelium, from those in other sections (Seifert, 1996). The lack of aerial mycelium tends to correlate with slower growth rates. The color of the aerial mycelium is dramatic amongst cultures of some species (Seifert, 1996).

Mycelial color varies from white to dull white with slightly yellowish to pinkish tinge (Gupta *et al*., 2014). Also white, cream, tan, salmon, cinnamon, yellow, red, violet, pink or purple (Fig. 4 A) and on the reverse, colorless, tan, red, dark purple or brown pigmentation are reported (Gerlach and Nirenberg, 1982, Dhoro, 2010).

Pigmentation/colony reverse which means the color of the back side of the colony tends to be highly variable within a species. But can be critical for a few species. Generally, a purple reverse is indicative of species in section *Leseola.* Red pigments are produced by species of several sections. The absence of red pigments is critical for recognizing some species (Seifert, 1996).

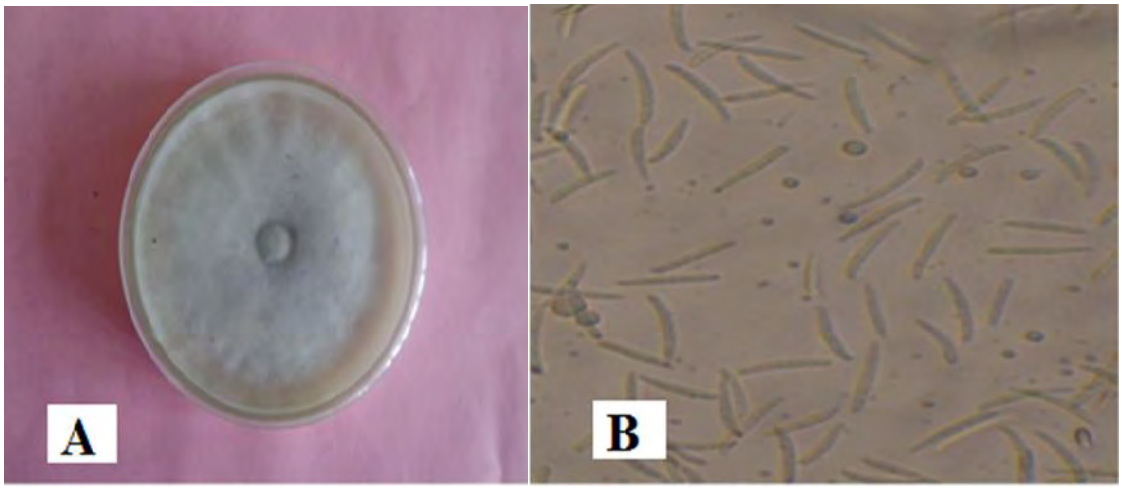


Fig. 4. Cultural and morphological appearance of *Fusarium*: Culture of *Fusarium* sp (A). Macro conidia of *Fusarium* (B), Source: Rifat *et al.* (2013).

3.13. Conidial morphology: macroconidia, microconidia, phialides and chlamydospores

Fusarium is reported to produce three types of asexual spores *viz*., microconidia, macroconidia and chlamydospores (Vicente *et al*., 2014). Microconidia are one or two celled while macroconidia are five celled and curved (Leslie and Summerell, 2006). Macroconidia are the single most important character in the identification of *Fusarium species* (Fig. 4 B). In many cases, the morphology of this spore alone is sufficient to identify a culture to species level (Seifert, 1996, Leslie and Summerell, 2006). Chlamydospores are one or two celled and thick walled round structures. Though all

three types of spores of the fungus can survive in soil, the chlamydospores have the capacity to survive for a long time (20 years +) (Vicent *et al*., 2014).

Fusarium species produce hyaline macroconidia (3-8 x 11-70 µm) which are septate and generally have a foot-shaped or notched base to the basal cell. These can be observed microscopically in different species. Macroconidia are produced from phialides on unbranched or branched conidiophores in the aerial mycelium (Li-Jun Ma *et al.*, 2013; Vicente *et al.*, 2014; Refai *et al.*, 2015). They are two or more celled, thick-walled, smooth, and cylindrical or sickle shaped with pointed distal ends (Dhoro, 2010). The different shape of macroconidia remains the most important feature for distinguishing the species (Moretti, 2009).

Microconidia are not produced by all *Fusarium* species, so their presence alone is an important character (Leslie and Summerell, 2006). The microconidia themselves, the conidiogenous cell on which they are borne, and the arrangement of the microconidia on and around the conidiogenous cell all are important and potentially diagnostic characters (Leslie and Summerell, 2006).

Chlamydospores are “storage” cells, usually thick walled, swollen cells, sometimes with roughened and / or pigmented walls (Seifert, 1996). They are resistant structures with thickened walls and high lipid content (Moretti, 2009). They may be produced in the agar, in the aerial mycelium, or in sporodochial macroconidia. Unfortunately, many *Fusarium* species are very slow to make chlamydospores (20-30 days) as of (Seifert, 1996). According to Leslie and Summerell (2006), even they often take a long time 6+ weeks to produce, and may not be produced in large numbers. Chlamydospores often form more

readily on SNA than on CLA (Leslie and Summerell, 2006). They may be formed singly, doubly, in clumps and in chains (Seifert, 1996, Leslie and Summerell, 2006; Moretti, 2009). Phialides are the cells of the hyphae on which the microconidia are borne (Leslie and Summerell, 2006). They are of two types based on the possession of either single or multiple openings through which microconidia are borne. The length of phialides bearing microconidia is useful character to distinguish between *Fusarium oxysporum* and *Fusarium solani*; Phialides of *Fusarium solani* being quite longer than that of *Fusarium oxysporum* (Leslie and Summerell, 2006).

3.14. Ancient trends of identifying plant pathogenic fungi and its constraints

Classically, plant pathogenic fungi were characterized by a set of morphological criteria including cultural characteristics on growth media, diagnostic symptoms on the host along with the presence of the fungus in the affected tissues (Baayen *et al.*, 2000) and pathogenicity test (Lievens *et al.*, 2008). Morphological methods have major limitations such as, reliance on the ability of the fungus to be cultured, time-consuming and laborious nature of identification process and the requirement for extensive taxonomical knowledge, which retards timely disease management decisions. As a result, attempts have been made to replace these methods with molecular identification techniques (Lievens *et al.*, 2008; Saikia and Kadoo, 2010).

3.15. Molecular identification of fungi

In the last three decades, molecular tools have got a major focus in the identification of plant pathogens. Molecular techniques can avoid many of the drawbacks associated with classical methods of pathogen identification and can improve the understanding of

pathogen detection in different conditions (Saikia and Kadoo, 2010). These techniques are more specific, sensitive, more quick and accurate than traditional methods, and do not demand specialized taxonomical expertise (Lievens *et al.*, 2008; Saikia and Kadoo, 2010). Today, a wide range of molecular techniques are being applied to accurately identify *F. oxysporum* isolates of which those based on detection of pathogen DNA or RNA are the most predominant (Saikia and Kadoo, 2010).

3.16. Genomic regions used in phylogenetic studies of fungi

Nuclear ribosomal DNA genes: The ribosomal DNA (rDNA) has an important function in protein synthesis and its variability can reflect the genomic evolution and also provides a useful intra and inter polymorphism in eukaryotic organisms. Within nuclear DNA, ribosomal genes are in multiple copies separated by non-codifier spacers (Parte *et al.*, 2014).

3.17. The principle of molecular identification- tracing to DNA barcodes

Short, standardized DNA regions or "barcodes" (Chase and Fay, 2009; Diaz *et al.*, 2012) have been used to identify biological materials from many groups of animals (Chase and Fay, 2009). DNA barcodes are regions in the DNA molecules of organisms that share common sequences in the genome of evolutionarily related organisms. The barcoding approach has great potential for identifying plants and fungi (Chase and Fay, 2009). The standard region of *coxI (Ctochrome Oxidase I)* which is used as a standard barcode for algae, insects and higher animals is not found suitable to be used as a DNA barcode in plants and fungi because the mitochondrial genes in these later groups evolve too slowly to allow discrimination between species (Chase and Fay, 2009; Diaz *et al.*, 2012). Thus,

other alternatives have been sought. In land plants, the two short coding regions of plastid DNA (*matK* and *rbcL*) have been selected (Chase and Fay, 2009) Whereas, the nuclear ribosomal RNA genes have been considered as promising candidates for fungal DNA barcoding because they have been the most widely used genes for phylogenetic studies for more than two decades (Begerow *et al.*, 2010). *COI* in fungi is difficult to amplify, insufficiently variable, and some fungal groups lack mitochondria (Kozel *et al.*, 2014).

3.18. ITS and IGS regions-the DNA barcodes of fungi

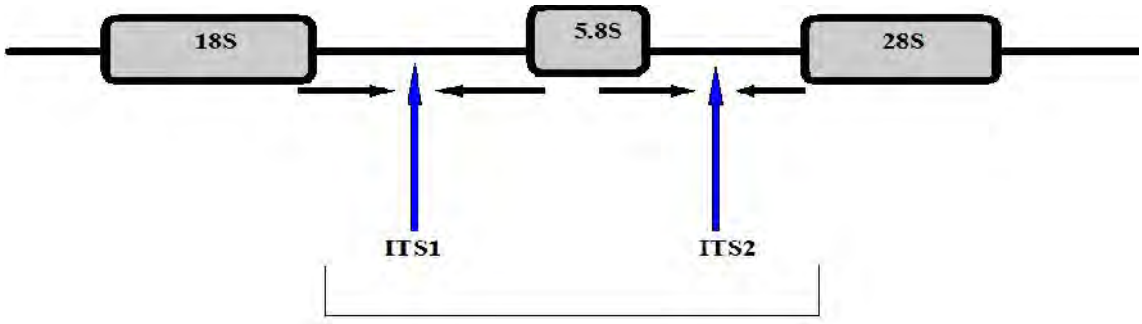
Each repetitive unit of rDNA consists of 18S, 5.8S and 28S genes of rRNA and two internal transcribed spacers (ITS1 and ITS2) that are located between these genes (Fig. 4). Major intergenic spacers (IGS) or non-transcribed spacers (NTS) are found between the regions codifying for larger and minor subunits of consecutive cistrons (Pecchia, 1998). The intergenic spacer (IGS) is located between the 28S and the 18S genes and separates the ribosomal repeat units. It is known to show differences at the intra-species level either as variation in the restriction patterns or as variation in the length of the amplification products (Pecchia, 1998). The internal transcribed spacers (ITS) of nuclear ribosomal DNA have been selected as the best alternative DNA region (Begerow *et al.*, 2010; Diaz *et al.*, 2012; Kozel *et al.*, 2014). It is routinely used to address research questions relating to systematics, phylogeny and identification of specimens at and below the species level (Begerow *et al.*, 2010; Diaz *et al.*, 2012). The sequence variation within the ITS region allows reliable and faster discrimination of the isolates at both the genus and species level (Oechsler *et al.*, 2009). Like organelle genes, cytochrome Oxidase gene in the mtDNA of animals and the *matK* and *rbcL*, in plastids of green plants, there are

many copies of *ITS* per genome of fungi as well as their variability in fungi allows for their identification (Selim and Zanaty, 2014).

3.19. Molecular markers used in identification of *Fusarium oxysporum*

Since the advent of molecular techniques, numerous works have been done on molecular characterization of *Fusarium oxysporum* (Singh *et al.*, 2006; Bayraktar and Dollar, 2010; Yang *et al.*, 2011). Saikia and Kado (2010) categorized the molecular detection and identification methods of *Fusarium oxysporum* into two; as anonymous markers (RFLP, RAPD, AFLP, SSR) and sequence specific markers (ITS and IGS, Transposons, other genes as TEF-1α). The suitability of ITS regions for the detection and identification of various *Fusarium species* have been reported by several authors from around the world (Chehri *et al.*, 2010; Bayona *et al.*, 2011; Parte *et al.*, 2014).

ITS1, 5.8S and ITS2 region has been the priority in PCR amplification by almost all researchers working with the ITS region in fungal molecular identification (Fig. 5). The ITS1, 5.8S and ITS2 region is amplified by the Universal fungal primer pairs, ITS1 and ITS4. However, 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 which amplifies the ITS1 region only (Osmundson *et al.*, 2013). PCR amplification of the ITS1 region alone confers the advantage of showing interspecies and intra species variability among isolates. This is possible due to the fact that ITS1 is a 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 (Santamaria *et al*., 2102). In the present study, ITS1 region is PCR amplified with primer pairs ITS1/ITS2 to



Internal Transcribed Spacer (ITS) Regions

Intergenic Spacer (IGS) regions are the well recognized ones (Iwen *et al.*, 2002; Schoch *et al.*, 2012). PCR amplification of these DNA barcoding regions with the optimal primers enables to visually distinguish organisms based on their genetic composition, either by the length of the amplicons size or using restriction digestion profile (Pechia, 1998).

In silco amplification of a DNA barcoding region is possible by using the artificial "molecular processing machine" called the PCR machine. Since its development in 1985, the specificity, sensitivity and speed of Polymerase Chain Reaction has led to the development of many methods for a wide range of biological research areas and for all classes of organisms (Edel, 1998). It can be put to a wide variety of uses (Schlief, 1993) as extensive applications have been found in many fields of mycology, including fungal genetics, systematics, ecology, soil microbiology, plant pathology, medical mycology, fungal biotechnology and many others (Edel, 1998). Moreover, it is certain that fungal studies will continue to progress with PCR, as numerous improvements and new applications are regularly being reported (Edel, 1998). PCR remains to be the fundamental step in the current and future approaches of molecular fungal identification strategies whereas there are insights which show the replacement of the downstream routine actual experimental steps including gel electrophoresis and restriction digestion with newly revolutionizing computerized technologies, *in sico* restriction digestion and *in silco* virtual gel electrophoresis (Wei *et al.*, 2007a).

3.21. Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP)

PCR-RFPL is a technique that involves the Polymerase Chain Reaction amplification of the DNA barcoding region of a taxon under consideration followed by cutting down of the amplified DNA section by the use of restriction enzymes having restriction site (cutting site) at certain locations of that amplified DNA section. Agarose gel electrophoresis of the restriction digested DNA will result in a band of DNA fragments that can be visualized with Uv-traslminator when observed after staining with a staining agent, Ethidium bromide. The PCR-RFLP gel band profile of different size in base pairs as compared to a DNA ladder of known size visualized in the gel helps to distinguish the organism from which the sample DNA is obtained (Kachuei *et al*., 2015).

MATERIALS AND METHODS

The study was carried out from December 2013 to August 2015 at the Institute of Biotechnology and Department of Microbial, Cellular and Molecular Biology (DMCMB), College of Natural (CNS), Addis Ababa University (AAU), Addis Ababa, Ethiopia.

4.1 Study areas

Sample materials were collected from the South western parts of Ethiopia: Sheka Zone (Yeki woreda and Teppi town administration), Bench Maji Zone, (Aman woreda and Semen Bench woreda), Kaffa Zone (Gimbo woreda and Bonga town administration) and Jimma Zone Shabe Woreda of the ginger growing areas (Fig. 6).

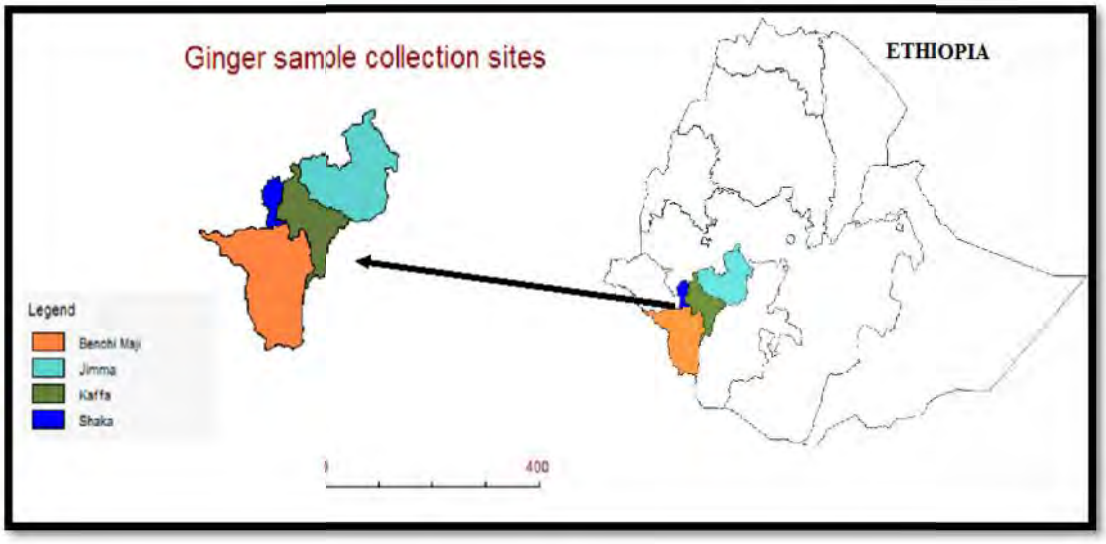


Fig. 6. Map of sample collection sites

4.2. Sample collection

Samples of diseased ginger parts (Rhizome, pseudostem, leaf) and soil were collected from the study areas (Table. 1). Rhizome and soil samples were kept in plastic sample bags where as leaf and pseudostem samples were handled in postal sample bags until transported to the Mycology laboratory of Department of Microbial Cellular and Molecular Biology, AAU.

4.3. Preparation of culture media and equipment sterilization

Commercial Potato Dextrose Agar (PDA, Himedia) medium was prepared with stock proportion of 39 g in 1000 ml distilled water. Spezieller Nahrstof farmer Agar (SNA) was prepared with a proportion of 1 g KH_2PO_4, 1g KNO_3, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.2 g Glucose, 0.2 g Sucrose, 20 g Agar in 1000 ml of distilled H_2O (Leslie and Summerell, 2006). Carnation leaf agar (CLA) was prepared by aseptically placing 5-8 mm oven dried (70^oC for 3 hours) sterile carnation leaf (*Dianthus caryophyllus*) pieces into a sterile petridish and adding 2% sterile water agar and used to enhance macroconidial production (Leslie and Summerell, 2006). The carnation leaves were obtained from Piazza fresh-flower selling shops, Addis Ababa. The Petri dishes and the culture media were sterilized with moist heat autoclave sterilizer at 121^0 C for 15 minutes. Chloramphenicol was introduced to the boiled media before autoclaving cooling the media to $45\text{-}50^0C$ to prevent bacterial contamination (Burgess *et al.*, 2008).

Table 1. Study areas

Zone	Woreda	Kebele	Altitude (m)	GPS Coordinates
Sheka	Yeki	Kubito	1405	UTM0801714 36N0762107
		Beko	1163	UTM0803003 36N0761578
		Beya -02	1224	UTM 0793029 36N0765547
Benchi Maji	Aman	Shesheka	1262	UTM: 0769097 36N0779716
	Mizanaman town administration	Hibret	1438	UTM: 0775225 36N0788312
	Semen Bench	Addis ketema	1441	UTM0775205 36N0788261
		Temenja yazhi	1520	UTM 0781471 56N0790456
Kaffa	Ginbo	Keykeila	1748	UTM 0810490 37N0194172
		Ufudo	1766	UTM 0816481 37N0192811
Jimma	Shabe	Kishene	1563	UTM0833107 37N0229729

UTM: Universal Transverse Mercator, coordinate system is following Bartelett's guide (Bartelett, 2007).

4.4. Isolation of the *Fusarium* wilt pathogen from samples

Diseased plant parts (rhizomes, leaves and pseudostems) were thoroughly washed with running tap water. The washed samples were allowed to air dry. The air dried rhizomes and leaves were cut into 5 mm^2 sized slices using sterilized scissors. The slices were then surface disinfected using 1% sodium hypochlorite for 5 minutes (Trikarunasawat, 2008). The slices were washed five times with sterile distilled water to remove the disinfectant. Infected ginger rhizomes, leaves and pseudo stems were used for isolation of fungal pathogens by using tissue-transplanting technique (Trikarunasawat, 2008). Ginger tissues at the boundaries of lesion and healthy tissue were selected from the sample and placed on the surface of potato dextrose agar (PDA) plates supplemented with 0.05g/l chloramphenicol (Himedia Laboratories technical data, 2013). Fungal isolates from soil samples were obtained by means of serial dilution agar plating technique (Aneja, 2005). All the inoculated plates were kept in an incubator at 25 ± 1^0C.

The cultures were periodically checked for mold growth starting from the 24^{th} hour after inoculation and emerging mycelial hyphae were transferred to PDA medium by means of hyphal tips transfer technique for purification (Leslie and Summerell, 2006). From the PDA cultures a portion of the pure isolate was sub cultured to slants and stored in refrigerator at 4^oC. From the initial 24 isolates, 14 isolates were identified as *Fusarium* genus. These Fusarium isolates were selected and subjected to pathogenicity, cultural, morphological, molecular and antagonistic effect studies.

The Fusarium isolates were designated systematically with an acronym AAUFG, meant to stand for Fusarium isolated from ginger at Addis Ababa University and numeral suffices from 1-16. Numbers 2 and 9 were omitted.

4.5. Sporulation study and inoculum preparation

Spore production (sporulation) on was undertaken. Three Petridishes full of mycelia from 10 days old culture grown on PDA were soaked with 20 ml sterile distilled water (per each plate) and scraped with sterile scalpels and filtered through muslin cloth to filter out mycelial fragments. Ten (10 µl) of the spore suspension was placed on a heamocytometer using micropipettes. The number of spores in five larger square grids in the heamocytometer was counted. The number of spores per milliliter of the suspension was calculated (Gupta *et al*., 2010). The concentration was adjusted to$1X10^6$ spores/ml (Stirling, 2004) and the spores so obtained were used for the pathogenicity test of isolates under *in vitro* condition.

$$\text{No. of Spores per ml} = \frac{N \times 100}{X}$$

Where: N = Total No. of spores counted/No. of square grids

X = Volume of mounting solution between the cover glass and above the squares counted.

4.6. Pathogenicity test

Spore suspension of the Fusarium isolates were put into the hole made on fresh ginger rhizome and incubated at 30 ± 1^0C. A periodic check up has been undertaken for *Fusarial*

mold growth. Pathogenicity test of the *Fusarium* isolates was evaluated (Salami and Akintokun, 2008). Pathogenicity index (PI) value of each isolate was assessed by evaluating the ability of the isolate to induce rot in healthy (surface disinfected) test rhizomes.

Fresh rhizomes were obtained from Piazza vegetables market, Addis Ababa by looking at vigorous and healthy looking tubers. Rhizomes were washed in sterile distilled water and surface sterilized using 1% sodium hypochlorite for five minutes and then washed in three successive changes of sterile distilled water. Holes of approximately 2cm deep were dug in the rhizomes by using 5 mm diameter sterile cork-borer; the plug was pulled out and 1mL spore suspension ($1x10^6$ spores) was injected at the bottom of the hole on the rhizome. The wounded areas were capped with about 5 mm thick sterile agar discs to cover the openings.

Infected rhizomes were then kept in screw capped air tight bottle and weighted. The weighted 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. Five rhizomes were used for pathogenicity test per each isolate. The control experiment was done by using sterilized distilled water of equal volume placed in to the holes of similarly disinfected rhizomes. At the end of the 15 day, the rhizomes were reweighed to determine the pathogenicity index of each *Fusarium* isolate

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

Where: WD=weight of the diseased rhizome and WH= weight of healthy rhizome.

The rhizomes were cut along the plane of inoculation by means of sterilized scalpel-blade 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 that if the rhizome rotting was due to the inoculated pathogens.

4.7. 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 characterization. The isolates were grown on PDA plates at 30±1◦C for 4 and 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 milli meter calibrated ruler.

Slide cultures for the pathogenic *Fusarium* isolates were prepared (Aneja, 2005). Morphological species identification was done by referring to the illustrative literature (Gerlach and Nierenberg, 1982; Leslie and Summerell 2006). 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 QImaging retiga camera system. Phase contrast images of the macroconidia and microconidia were captured using the Olympus System Microscope at 400X magnification.

4.8. Culture preparation and DNA extraction for Molecular study

The *Fusarium* 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 days old *Fusarium* colony with sterile cork borer and placed into 500 ml Erleinmeyer 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 kept under water bath shaker incubator (Temperature adjusted at 30 ^{0}C) at 200 rpm for three to seven 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 a Clorox treated and sterilized white porcelain mortar and pestle under excess liquid nitrogen (Siddiquee *et al.*, 2010). The fine powder of the mycelia so 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 following the manufacturer's instructions of HIMEDIA (HiPurA Fungal DNA Purification Kit, MolBio^{TM}). The DNA extraction kit was imported and accessed in care of "NEWAY P.L.C"., the agent of Himedia Laboratories in Ethiopia and the experiment was conducted at the Plant Genetics Laboratory.

4.9. Determination of DNA concentration and purity

Concentration and purity of DNA was measured using NanoDroP Spectrophotometer 2000c (Thermo Scientific, UK) as well as in 1% agarose-gel electrophoresis. After

Primer Name	Direction	Sequence (5' ⟶ 3')	GC (%)	TM^0	Reference
ITS1	Forward	TCCGTTGGTGAACCAGCGG	63.1	71.8	Cheri *et al.* 2011
ITS2	Reverse	GCTGCGTTCTTCATCGATGC	55	68.2	White *et al.* 1990

2% agarose gel in 1X TBE buffer; stained with ethidium bromide and photographed in gel doc system with Uv-translluminator (BioSens 750, Japan). 100 bp DNA ladder (HIMEDIA) is used as a size reference marker. PCR products were then kept in a refrigerator at 4°C for 16 hours until subjected to restriction digestion experiment.

4.11. Restriction endonuclease digestion

The amplified rDNA regions were treated with four restriction enzymes of which two were hexa cutters (*Hind* III, *Pst*I) and the other two were tetra cutters (*Hha*I, *Msp*I) with the following set of conditions. For *Hind* III (recognition sequence, AAGCTT) and *Pst*I (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 ^{0}C for 1 hour. A 6X loading buffer was used as a tracking dye for the agarose gel electrophoresis following the restriction digestion experiment.

For *Hha*I and *MspI* with recognition sequences, (GCGC) and (CCGG), respectively, restriction digestion reaction was 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 minutes at 37 ^{0}C in water thermostat. The 10X FastDigest Green buffer included in the reaction also served as a loading buffer to track the migration of amplicons during the gel electrophoresis run.

The product was immediately loaded to 2% agarose gel and subjected to electrophoresis at 100V for 1.5 hours in 1X TBE buffer using horizontal gel electrophoresis tank (BIO-RAD) connected to Biometra standard power pack (Biometra).

4.12. *Insilco* restriction digestion simulation of ITS1 sequences of *Fusarium oxysporum* retrieved from the ITS1 database (ITSoneDB)

One hundred (100) sample ITS1 region sequences were retrieved from the ITS1 data base (ITSoneDB) which has been developed by Santamaria *et al.* (2012) and is online available at http://itsonedb.ba.itb.cnr.it/ containing a collection of seven hundred eleven (711) ITS1 sequences of *Fusarium oxysporum* alone. The retrieved ITS1 sequences were downloaded in FASTA format for *in silco* RFLP analysis. The FASTA format sequences so obtained were digested *in silco* with restriction enzymes (*HhaI, HindIII, PstI and MspI*) using online restriction mapper software (RestrictionMapper version 2, Blaiklock, (2000) availed at :http://www.restrictionmapper.org/.)

The FASTA format ITS1 sequences were copied and pasted from word pad into the online restriction mapper software form one by one; restriction enzymes used in the actual PCR-RFLP experiment *(HhaI, HindIII, PstI and MspI)* were selected from the list provided in the software under the menu, “Select individual enzymes”, Genbank accession number of the particular sequence was entered to the tab labeled “Name your sequence” and the “map sites” tab was pressed to generate the result of the *in silco* restriction digestion.

4.13. Data analysis

Pathogenicity Index (PI) of isolates on ginger rhizome was analyzed using Statistical Packages for Social Sciences (SPSS, version 20). Comparison of mean PI values of isolates against mean PI value of the control experiment was done by Dunets test of mean comparison and mean comparison of the PI values of every isolate against one another was done by the Duncans 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*) which showed genotypic polymorphism between the isolates were analyzed using NTSYSpc (Numerical Taxonomy System, Applied Biostatistics, Jefferson, 2000) computer program version 2.02h. The data (presence or absence of bands) were coded in the form of a binary matrix, and a pair wise similarity matrix was constructed with the Jaccards similarity coefficient. UPGMA (Unweighed Pair-Group Method with Arithmetical averages) dendrogram was generated with the (sequential agglomerative hierarchal nested cluster analysis) SAHN module of the NTSYSpc software (Rohlf, 2004).

RESULTS

5.1. Isolation of the fungal genera from ginger and soil samples

Hairy to cottony mold growth was observed on PDA emerged from the sample specimens (leaf, pseudostem and rhizome) upon incubation at 25 ± 1^0C for 4 to 7 days (Fig. 7). The genus level identification of the isolates revealed about four genera of fungi: *Fusarium*, *Trichoderma*, *Aspergillus*, *Penicillium* (Table 3). 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 incubated at 25 ± 1^0C.

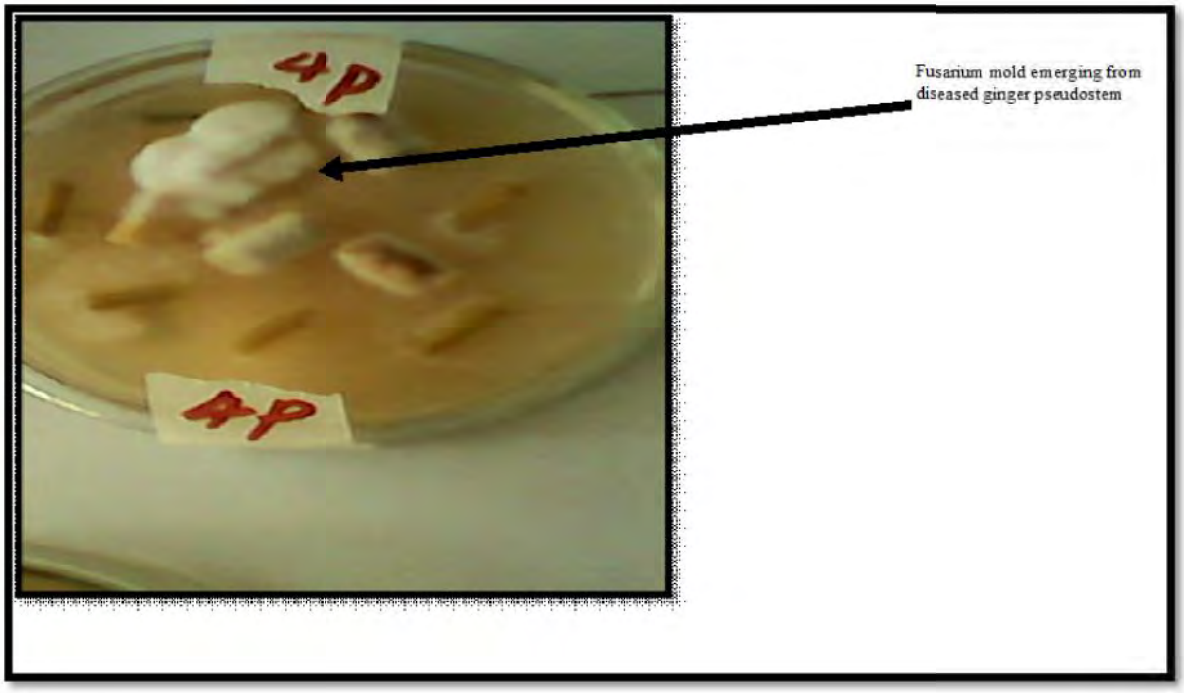


Fig. 7. *Fusarium* isolate emerging from pseudostem samples of ginger on PDA

Table 3. List of Fungal genera isolated from Rhizome, Leaf, Pseudostem and Soil samples

Zone	Woreda	Kebele	Isolate	Source sample	Identified Genera
Sheka	Yeki	Kubito	AAUFG1	Leaf	*Fusarium*
			AAUTG1	Rhizome	*Trichoderma*
			AAUFG12	Soil	*Fusarium*
			AAUpenG	Rhizome	*Penicillium*
			AAUFG13	Soil	*Fusarium*
		Beko	AAUFG3	Pseudo-stem	*Fusarium*
			AAUTG3	Leaf	*Trichoderma*
		Beya -02	AAUFG4	Pseudo-stem	*Fusarium*
Benchi maji	Aman	Shesheka	AAUFG5	Rhizome	*Fusarium*
			AAUTG5	Rhizome	*Trichoderma*
			AAUFG6	Leaf	*Fusarium*
			AAUFG11	Rhizome	*Aspergillus*
			AAUFG14	Soil	*Fusarium*
			AAUFG16	Soil	*Fusarium*
	Mizan aman	Hibret	AAUFG7	Rhizome	*Fusarium*
		Addis ketema	AAUFG8	Rhizome	*Fusarium*
	Semen Bench	Temenja yazhi	AAUFG10	Rhizome	*Fusarium*
			AAUTG8	Rhizome	*Trichoderma*
Kefa	Ginbo	Keykeila	AAUTG9	Leaf	*Trichoderma*
			AAUFG15	Soil	*Fusarium*
		Ufudo	AAUTG10	Rhizome	*Trichoderma*
Jimma	Sheben	Kishene	AAUFG10	Rhizome	*Fusarium*
			AAUTG11	Rhizome	*Trichoderma*

5.2. In vitro pathogenicity test of *Fusarium* isolates on ginger rhizomes

The pathogenicity index data is presented in Table 4. The highest pathogenicity index (PI) values are recorded from isolates AAUFG6 and AAUFG7 with 45.35% and 38.6 % respectively; followed by AAUFG1 and AAUFG8 with PI values 32.46% and 28.4% respectively which are relatively higher than the rest of the isolates. 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 similar; pinkish white dense mycelia covering the external surfaces of the injured rhizomes were observed (Fig. 8). Pigmentations with reddish pink color were also developed on the rotting rhizome beneath the externally proliferated mold (Appendix A). When transversely split, the internal part showed pale to dark brown discoloration. The rotting usually gave off nasty foul odor.

The one sided (> Control) Dunnet test of the PI values of each isolate against the PI value of the control group showed that isolates AAUFG1, AAUFG6 and AAUFG7 showed statistically significant difference ($\alpha = 0.05$) with regard to their PI values (Table 4); The mean PI values of the rest of the isolates falling between 7.94 ± 10.32 and 25.38 ± 10.32 did not show statistically significant difference ($\alpha = 0.05$) from the mean PI value of the control group (3.02). However, the qualitative data 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 and inside the test rhizomes. All the *Fusarium* isolates showed cottony mycelial growth over the surface of the test rhizome; on the contrary, no mold growth was observed on control rhizomes (treated with sterile dis tilled water).

Table 4. Pathogenicity index of *Fusarium* isolates as tested on ginger rhizomes

S. No	The test (Isolate)	Mean Pathogenicity index (PI)(%) ± Standard error (SE)	Mean difference in PI of isolate from control (I-J)	Sig.
1	AAUFG1	32.46 ± 10.66^{abc}	29.44*	0.028
2	AAUFG3	14.71 ±4.53^{bcd}	11.69	0.534
3	AAUFG4	19.15 ±11.52^{bcd}	16.13	0.331
4	AAUFG5	18.45 ±6.94^{bcd}	15.43	0.361
5	AAUFG6	45.35 ±11.57^{a}	42.33*	0.001
6	AAUFG7	38.69 ±12.28^{ab}	35.67*	0.006
7	AAUFG8	28.40 ±9.62^{abc}	25.38	0.070
8	AAUFG10	14.84 ±6.41^{bcd}	11.82	0.528
9	AAUFG11	16.47 ±6.81^{bcd}	13.45	0.451
10	AAUFG12	13.27 ±3.21^{cd}	10.25	0.602
11	AAUFG13	10.96 ±1.55^{cd}	7.94	0.704
12	AAUFG14	14.65 ±1.43^{bcd}	11.63	0.537
13	AAUFG15	11.19 ±1.71^{cd}	8.17	0.694
14	AAUFG16	12.96 ±1.21^{cd}	9.94	0.616
15	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 siginificant differnence with the PI value of the control group, letters indicate homoginioues subsets of Duncans multiple range test.

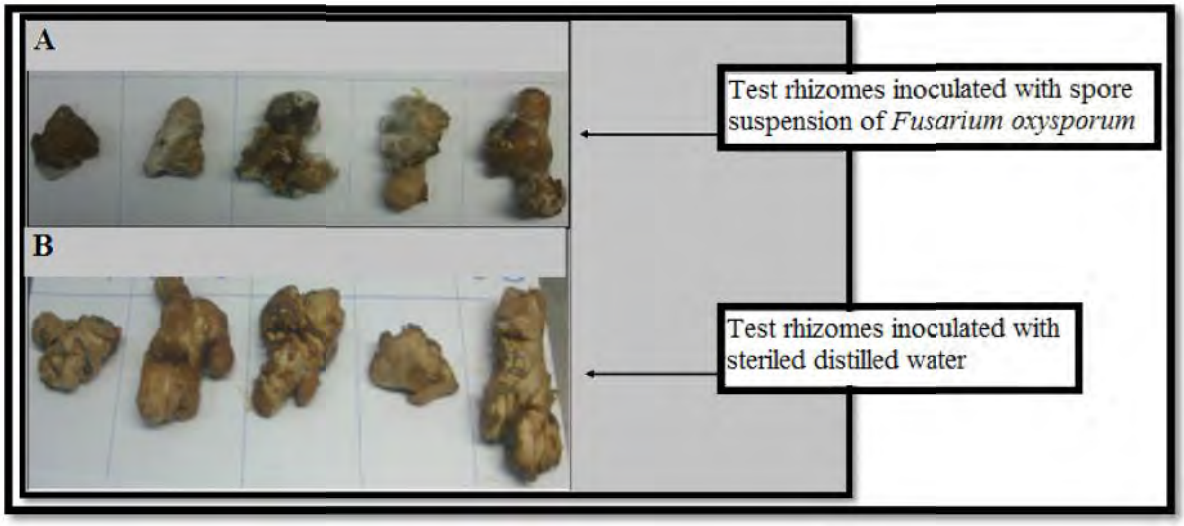


Fig. 8. A comparison of rhizomes treated with spore suspension of *Fusarium sp* (A) and a control group treated with sterile distilled water (B).

5.3. Cultural and morphological identification

5.3.1. Cultural characters (colony color, aerial mycelium, pigmentation)

Based on cultural characters on PDA, the isolates showed different colony colors including dull pink, pink, creamy white and white (Fig. 9). Specifically, the colony color of the isolates on PDA medium varied between white (AAUFG8), creamy white (AAUFG6 and AAUFG 7) and dull pink (AAUFG3, AAUFG4, AAUFG10, AAUFG12, AAUFG13, AAUFG14, AAUFG15, AAUFG16) to pink (AAUFG 1, AAUFG 5, AAUFG 11), respectively. The aerial mycelium of the isolates resulted in three different appearances which could be described as fluffy growth (AAUFG6, AAUFG7), adherent smooth growth (AAUFG1, AAUFG3, AAUFG4, AAUFG7, AAUFG11, AAUFG12, AAUFG13, AAUFG14, AAUFG15, AAUFG16) and adherent smooth growth with concentric circle (AAUGF5 and AAUFG10). Most of the isolates showed adherent growth while some isolates revealed fluffy growth on the medium (Fig. 9). The isolates

imparted dull white, orange, light reddish purple, intense reddish purple and dark reddish purple pigmentations (Fig. 10).

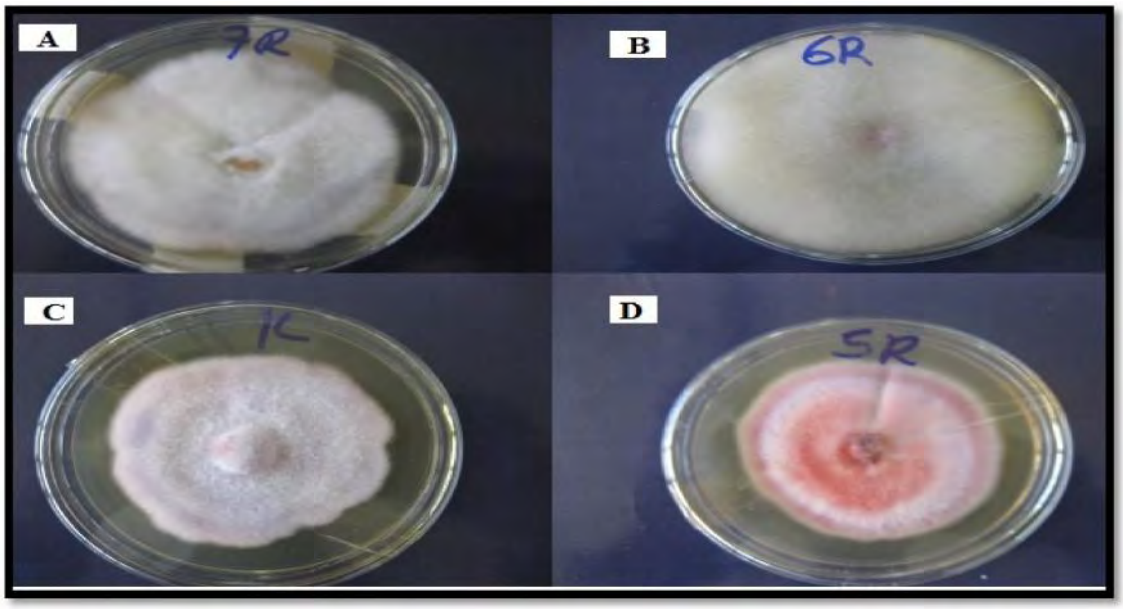


Fig. 9. Colony color of *Fusarium* isolates on PDA (A) white, (B) Creamy white , (C) dull pink, (D) Pink with concentric circles.

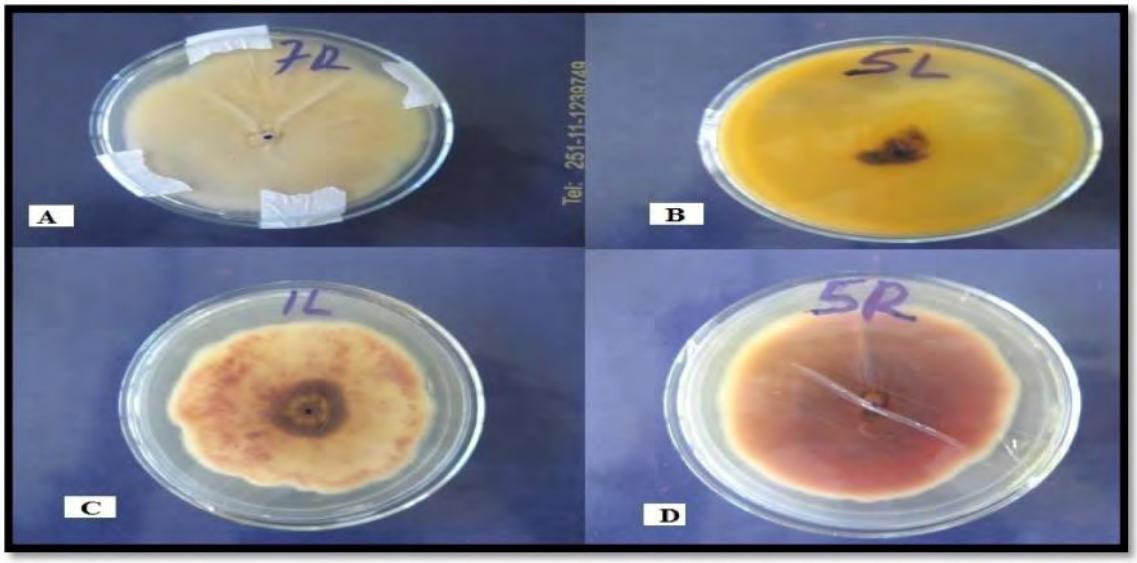


Fig. 10. Colony pigmentations of *Fusarium* isolates on PDA: (A) dull white, (B) Yellow/orange, (C) light reddish purple with dark purple at center (D) intense reddish Purple

5.3.2. Radial growth of *Fusarium oxysporum*

5.3.2.1. Radial growth of Fusarium oxysporum after four days of incubation

A record of radial growth of the isolates is presented in Table 5. Mean radial growth of the isolates after 4 days of incubation at 30 $\pm$ 1^0 C lied in the range 33.40 – 90 mm. Isolates AAUFG6 and AAUFG7 showed the highest mycelial growth with fluffy aerial mycelium (90 mm all). These isolates were extraordinarily fast in their growth rate covering the entire PDA Petridish within four days.

Moderate mycelial growth was showed by isolates (AAUFG 12, AAUFG11, AAUFG15, AAUFG14, AAUFG16, and AAUFG5) with mean radial growth 52.30, 52.73, 54.27, 56.00, 57.77, 59.80, respectively where as the lowest mycelial growth records were attained by isolates AAUFG3, AAUFG4, AAUFG1, and AAUFG 10 with mean radial growth 33.40, 36.73, 43.53, and 45.27 mm respectively.

5.3.2.2. Radial growth of Fusarium oxysporum after seven days of incubation

Mycelial growth of isolates was recorded after seven days of incubation period at 30 ± 1^0 C as presented in Table 5. The radial growth data ranged from 54.20 to 90 mm. Isolates AAUFG5, AAUFG6, AAUFG7, AAUFG8, AAUFG12, AAUFG13, AAUFG14, AAUFG16, AAUFG11 and AAUFG15 showed the highest mycelial growth (90.00, 90.00, 90.00, 90.00, 81.83, 81.17, 80.83, 78.67, 75.53, 75.00) followed by AAUFG10, AAUFG1, AAUFG4 and AAUFG3 showed moderate growth with mycelial growth of 67.40, 66.80, 55.80 and 54.20) in mm, respectively.

Table 5. Cultural characteristics of *Fusarium oxysporum* (radial growth, colony color and colony reverse) grown at 30 $\pm$ 1^0 C on PDA

No	Isolate	Mean radial growth 4 DAI (mm)	Mean radial growth 7 DAI (mm)	Colony color	Colony reverse
1	AAUFG1	43.53^{ef}	66.80^{cd}	pink	dark reddish purple in the center with the rest red tinged
2	AAUFG 3	33.40^{g}	54.20^{e}	pink	reddish purple tinge
3	AAUFG 4	36.73^{fg}	55.80^{de}	dull pink	orange
4	AAUFG 5	59.80^{c}	90.00^{a}	pink with concentric circle	intense reddish purple
5	AAUFG 6	90.00^{a}	90.00^{a}	creamy white	orange with dark purple at the center
6	AAUFG7	90.00^{a}	90.00^{a}	creamy white	dull yellow with purple at the center
7	AAUFG8	74.80^{b}	90.00^{a}	white cottony	dull white
8	AAUFG10	45.27^{de}	67.40^{cd}	dull pink with concentric circle	orange
9	AAUFG11	52.73^{cd}	75.53^{bc}	Pink	lite reddish purple
10	AAUFG12	52.30^{cd}	81.83^{ab}	dull Pink	lite reddish purple
11	AAUFG13	59.20^{c}	81.17^{ab}	dull Pink	lite reddish purple
12	AAUFG14	56.00^{c}	80.83^{ab}	dull Pink	lite reddish purple
13	AAUFG15	54.27^{c}	75.00^{bc}	dull Pink	lite reddish purple
14	AAUFG16	57.77^{c}	78.67^{abc}	dull Pink	lite reddish purple

Values are means of 3 replicates, DAI= Days after Inoculation, letters indicate Duncans homoginous subsets for multiple mean comparison

5.3.3. Morphological characters

Microscopic morphology of the *Fusarium* isolates is presented in Table 6. Macroconidia were formed on mycelial conidiophores (Fig. 11A) and were straight with 1-5 septated and of medium length (Fig. 11 B). Microconidia are small to large and mostly non septated (Fig. 11 C). Chlamydospores were observed in all of the isolates in single, in pairs and occasionally in clumps at intercalary and terminal positions (Table 6). Phialides were short in all isolates (Fig. 11 D). Microconidial size between the isolates ranged from 6.56 µm X 2.50 µm to 16.25 µm X 6.50 µm where as macroconidial size was in the range 14.79 µm X 3.98 µm to 55.26 X 10.67 µm (Table 7).

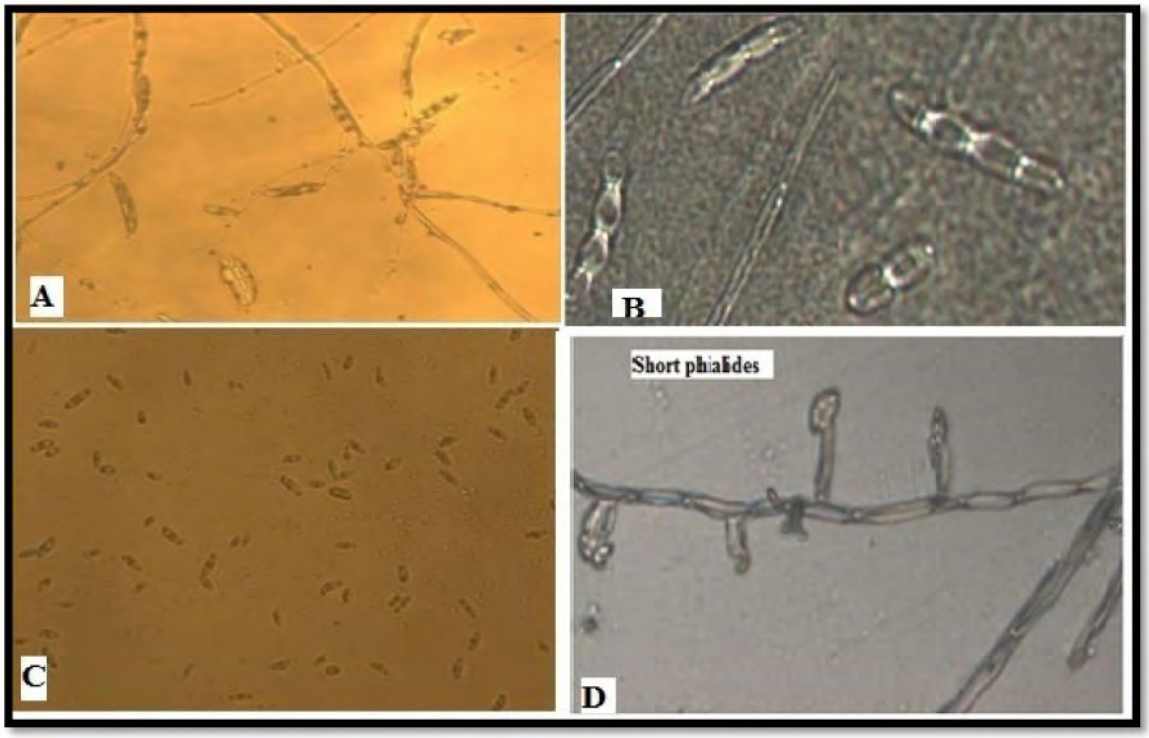


Fig.11. Morphological features of *Fusarium oxysporum*: (A) Macroconidia on mycelial conidiophores, (B). Macroconidial shape and septation, (C). Shape and septation of Microconidia, (D). short phialides branching from mycelium.

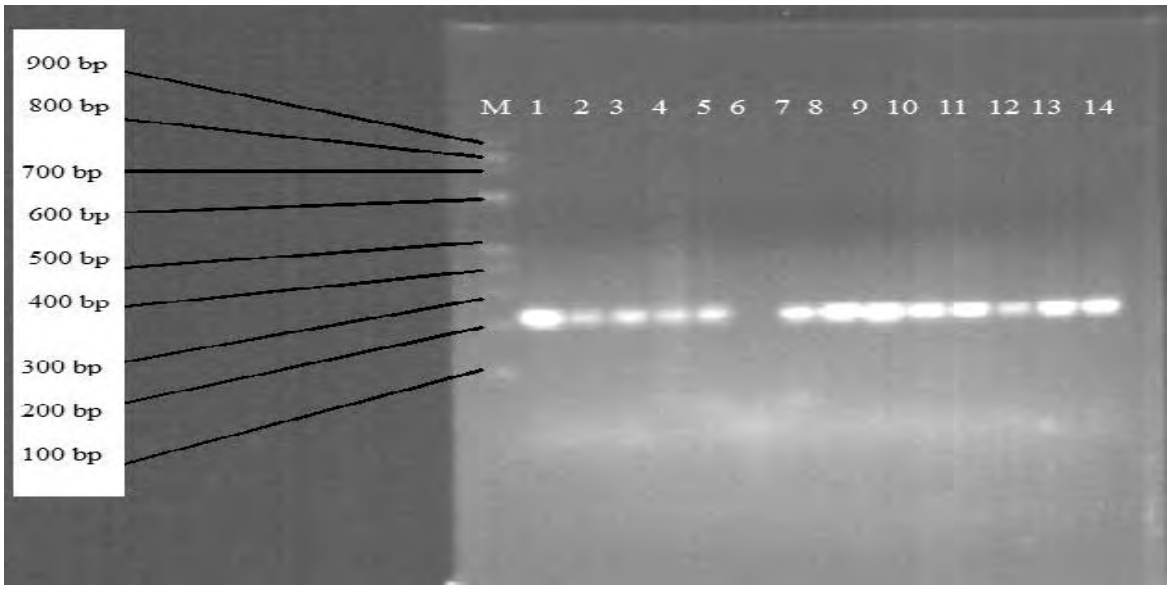
Table 6. Macro and microconidial shapes, septation, phialides, chlamydospores presence

Isolate	Macroconidial general shape	Septa	Microconidial shape	Septa	Phialide	Chlamydospores Present/absent
AAUFG1	Straight	3	Ovoid	0	Short	+ singe, in pair, at intercalary and terminal
AAUFG3	Straight with bent basal cells	1-3	Ovoid to elliptical	0	Short	+ pairs, clumps at terminal & 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 with broad at the middle	3	Elliptical to fusiform	0-1	Short	+ in pairs
AAUFG11	Straight with blunt apical & basal cells	3	Ovoid to elliptical	0-1	Short	+ in pairs
AAUFG12	Straight with bent apical & 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

Table 7. Microscopic measurements of macroconidial and microconidial size dimensions

Isolate	Spore count	Macroconidial spore size (µm)		Microconidial spore size (µm)	
	Number of spores/ml	Minimum Macroconidial size (Length X Breadth)	Maximum Macroconidial size (Length X Breadth)	Minimum Microconidial size (Length X Breadth)	Maximum Microconidial size (Length X Breadth)
AAUFG1	2.12 x 10^6	25.45 X 6.81	38.32 X 8.47	9.87 X 4.45	13.16 X 5.60
AAUFG3	1.16 X 10^6	23.94 X 5.46	39.78 X 6.32	7.55 X 3.36	14.26 X 5.60
AAUFG4	1.42 X 10^6	32.24 X 6.23	56.44 X 5.79	10.23 X 4.88	16.08 X 5.62
AAUFG5	1.32 X 10^6	23.85 X 4.71	45.99 X 3.88	7.85 X 2.65	14.50 X 5.55
AAUFG6	2.20 X 10^6	17.54 X 3.59	32.32 X 5.12	10.34 X 4.0	16.25 X 6.50
AAUFG7	1.09 X 10^6	29.50 X 7.12	47.43 X 6.45	10.55 X 4.53	15.50 X 6.56
AAUFG8	2.01 X 10^6	33.50 X 12.19	55.26 X10.67	8.42 X 3.57	13.31 X 5.36
AAUFG10	1.35 X 10^6	27.69 X 4.46	35.58 X 6.99	6.56 X 2.50	12.6 X 4.41
AAUFG11	1.04 X 10^6	26.44 X 4.61	36.2 X 5.25	7.55 X 2.81	11.54 X 6.12
AAUFG12	1.71 X 106	17.17 X 3.37	30.46 X 4.82	9.53 X 4.70	12.89 X 5.73
AAUFG13	1.92 X 10^6	27.69 X 2.96	35.98 X 5.56	6.56 X 3.25	8.94 X 6.51
AAUFG14	1.95 X 10^6	14.79 X 3.98	25.48 X 5.96	9.55 X 3.88	12.12 X 5.23
AAUFG15	2.70 X 10^6	19.97 X 3.91	29.25 X 4.52	8.45 X 3.82	12.52 X 5.60
AAUFG16	2.12 x 10^6	15.33 X 5.03	30.55 X 6.14	8.92 X 3.88	13.23 X 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. The species is confirmed to be *Fusarium oxysporum*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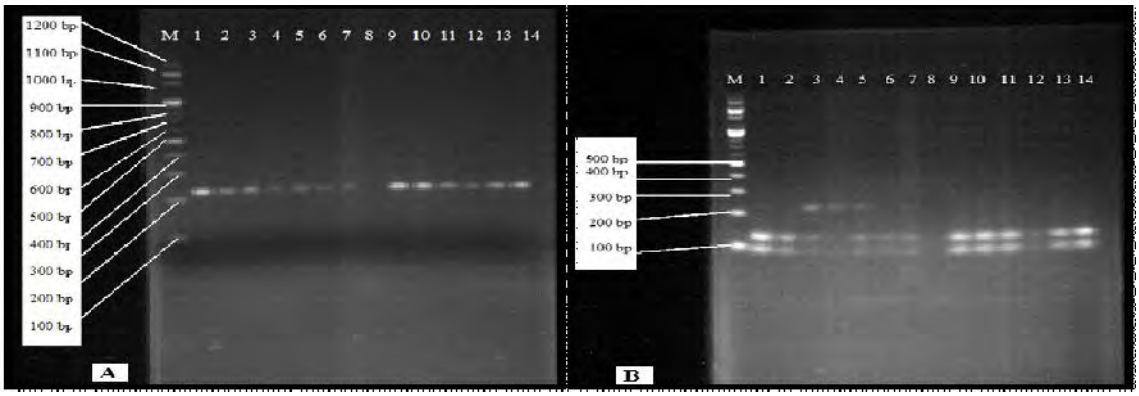
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100 bp
A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
500 bp
400 bp
300 bp
200 bp
100 bp
B

Table 8. PCR-RFLP band patterns generated by restriction digestion of ITS1 region of *Fusarium oxysporum* with the restriction enzymes (HhaI, HindIII, PstI and MSPI)

No	Isolate	Restriction Enzymes used					
					MSPI		
		HhaI	HindIII	PstI	digestion	Cut frequency	Band pattern
1	AAUFG1	**X**	**X**	**X**	✓	1	120bp, 100bp
2	AAUFG3	**X**	**X**	**X**	✓	1	120bp, 100bp
3	AAUFG4	**X**	**X**	**X**	✓	0,1	220bp,120bp,100bp
4	AAUFG5	**X**	**X**	**X**	**X**	0	220bp only
5	AAUFG6	**X**	**X**	**X**	✓	0,1	220bp,120bp, 100bp
6	AAUFG7	**X**	**X**	**X**	✓	1	120bp, 100bp
7	AAUFG8	**X**	**X**	**X**	✓	1	120bp, 100bp
8	AAUFG10	**X**	**X**	**X**	**X**	0	220bp only
9	AAUFG11	**X**	**X**	**X**	✓	1	120bp, 100bp
10	AAUFG12	**X**	**X**	**X**	✓	1	120bp, 100bp
11	AAUFG13	**X**	**X**	**X**	✓	1	120bp, 100bp
12	AAUFG14	**X**	**X**	**X**	✓	1	120bp, 100bp
13	AAUFG15	**X**	**X**	**X**	✓	1	120bp, 100bp
14	AAUFG16	**X**	**X**	**X**	✓	1	120bp, 100bp

✓ = There is digestion, **X**= There is no digestion

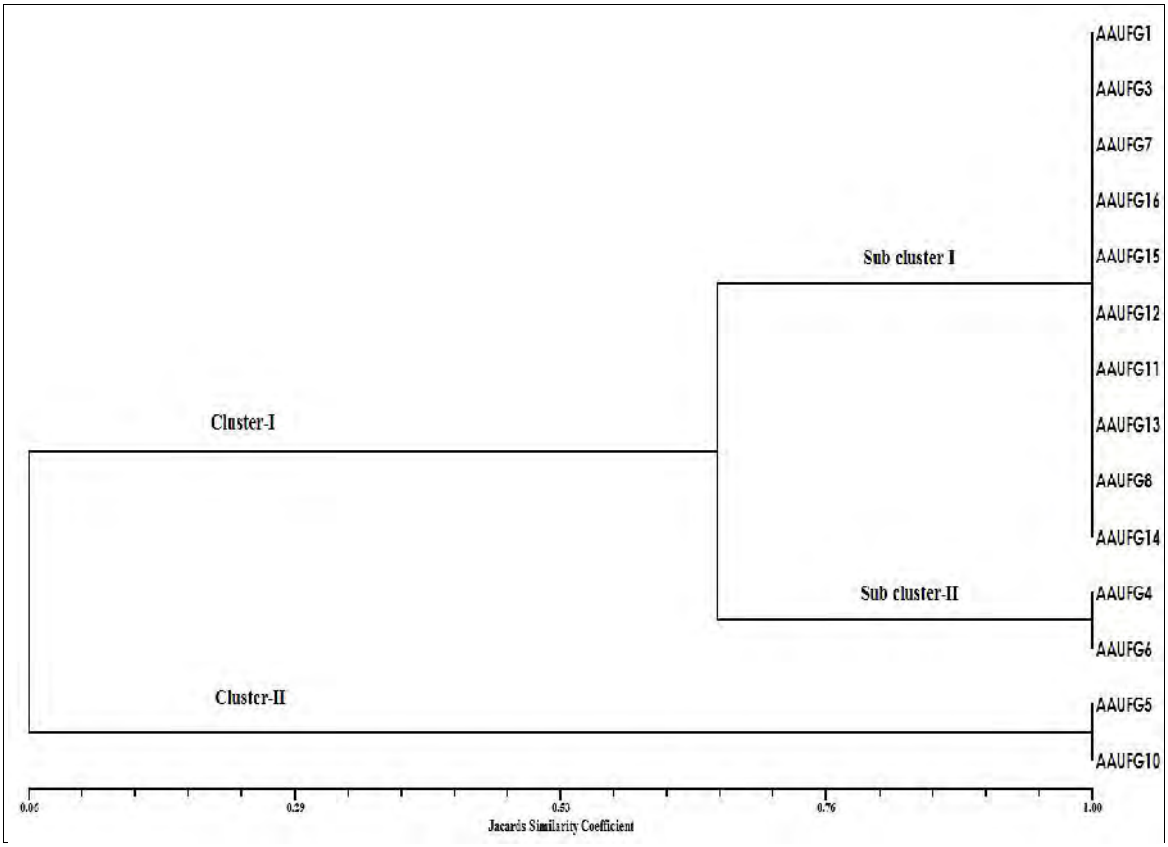
Table 9. Summary table grouping isolates according to similarity in band patterns

No	Band pattern	Isolates which attained the band pattern	No. of isolates	Percent (%)
1	220bp only	AAUFG5 and AAUFG10	2	14.29%
2	120bp,100bp	AUFG1,AAUFG3,AAUFG7, AAUFG8,AAUFG11,AAUFG12, AAUFG13, AAUFG14, AAUFG15, AAUFG16	10	71.43%
3	220bp,120bp,100bp	AAUFG4 and AAUFG6	2	14.29%
		Total =	14	100%

5.4.3. 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. 14). The overall similarity existing among the isolates ranged between 0% and 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.14).

Cluster-I bearing 85.71% of the isolates diverts into two sub clusters; Sub cluster-I aggregates 71.43 % of the total isolates with 100% similarity between each other whereas sub cluster-II holds two isolates (AAUFG4 and AAUFG6) with 100 similarity between them too. Cluster-II bears only two isolates (AAUFG5 and AAUFG 10) with 100% similarity between the two. The cladogram resulted in two groupings that agree with the cultural characters on PDA (colony color, adherent growth with and without concentric


AAUFG1
AAUFG3
AAUFG7
AAUFG16
AAUFG15
AAUFG12
AAUFG11
AAUFG13
AAUFG8
AAUFG14
AAUFG4
AAUFG6
AAUFG5
AAUFG10
Sub cluster I
Sub cluster-II
Cluster-I
Cluster-II
0.05
0.29
0.53
0.76
1.00
Jacards Similarity Coefficient

5.4.5. *Insilco* restriction digestion analysis

None of the retrieved sample sequences showed *in silco* digestion with restriction enzymes *HindIII* and *PstI* giving 100% similar result with the present study. 94% of the retrieved sequences exhibited digestion with the restriction enzyme *MspI*. This is also in line with the 85.71% of isolates digested with *MspI* in the present study (Table 10). The detail of *in silco* restriction digestion data is presented in table (Appendix I).

Table 10. Summary of *in silco* restriction digestion of sample100 ITS1 sequences of *Fusarium oxysporum* retrieved from ITSoneDB and digested with restriction enzymes (*HhaI, HindIII, MspI* and *PstI*) using online restriction mapping software.

No	Selected restriction enzymes	Number of sequences showed *in silco* digestion (out of 100 sequences)	Percentage (%)
1	*HindIII*	0	0%
2	*PstI*	0	0%
3	*HhaI*	3	3%
4	*MspI*	94	94%
5	Samples undigested with all restriction enzymes	3	3%
6	Total number of samples	100	100%

Three (3%) of the sample sequences did not show digestion with any of the enzymes as was observed in 2 (14.29%) of isolates in the present study. Generally 100% of the digestion results observed in the PCR-RFLP experiment of the present study have been exhibited in the 97% of the sampled *in silco* simulation i.e. 94% samples digested with *MspI* and 3% remained undigested with all of the selected restriction enzymes. In the actual experiment, 85.71% of isolates were digested with *MspI* and 14.29% remained

undigested with any of the enzymes applied in the experiment; altogether accounting 100%. The only difference observed between the actual experiment and the *in silco* simulation was that 3% of the retrieved sample sequences showed *in silco* digestion with the enzyme *HhaI* where as none (0%) of the isolates in the present study showed digestion with this enzyme.

DISCUSSION

Genus level identification revealed five genera of fungi: *Fusarium*, *Trichoderma*, *Aspergillus*, *Penicillium* and *Mycelia sterilia*. Belay Berza *et al*. (2012) have also isolated *Aspergillus, Eurotium, Fusarium, Mucor, Penicillium* and *Rhizopus* from ginger rhizome from southern Ethiopia. From the total 24 isolates identified in the present study, only 14 isolates were identified as *Fusarium oxysporum*. Belay Berza *et al*. (2012) reported a PI value of 25.78 % for *Fusarium species* which is less than the maximum PI value of the current report (45.35%). The method of inoculation of the *Fusarial* pathogen to the test rhizome may have impact on the efficiency of infecting and disease progression thereby contributing to the difference in the PI value. In the present study spore suspension of the isolates was used for the pathogenicity test whereas Belay Berza *et al*. (2012) used the agar block inoculation method.

Qualitative observation of the pathogenicity test of the *Fusarium* isolates on to ginger rhizome showed white and pink mycelial growth on the surface of rhizome with red pigmentations imparting underneath the moldy surface. Transverse cutting of the rotten rhizome along the plane of inoculation evidenced growth of mold along the vascular system of the test rhizome. Brown discoloration of the tissues near the site of inoculation was similar with Moreira *et al*. (2013).

Visual inspection of the cultural characters of isolates on PDA resulted in white, creamy white, dull pink and pink colony colors. Similarly, Gupta *et al.* (2014) reported white and dull white mycelial colors of *Fusarium oxysporum* f.sp. zingiberi. Two creamy white isolates in the present study have more fluffy aerial mycelium than that of Chaithra *et al.*

(2013) who identified *Fusarium oxysporum* with cream colored culture on PDA from ginger. Though some authors reported that colony color is dramatic (Seifert, 1996; Nirmaladevi and Srinivas, 2012) that it could not be used as an identification criterion, a consistent colony color of the *Fusarium oxysporum* between subsequent batch cultures has been 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 pigmentations described by Gerlack and Nirenberg (1982). It is also similar to the findings of Nirmaladevi and Srinavas (2012) who identified *Fusarium oxysporum* f.sp. *lycopersi*. Leslie and Summerell (2006) reported pigmentation to be a prominent secondary character that can be used in the identification of *Fusarium species*.

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 (2006).

Radial growth of mycelium incubated for 4 days at $30^{o} \pm 1^{0}$ C on PDA varied from 33.40mm to 90 mm diameters on 90mm diameter Petri plates. Hussain *et al.* (2012) reported a radial growth of 3.2 cm to 4.5 cm at 4 days of incubation 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 $26\pm1\ ^{0}$C in case of Hussain *et al.* (2012) where as the growing temperature in the present study was 30 ± 1^{0}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 the isolates of the former study were from guava wilt being a different *formae speciels*.

Microconidial size between the isolates ranged from 6.56 µm X 2.50 µm to 16.25 µm X 6.50 µm where as macroconidial size was in the range 14.79 µm X 3.98 µm to 55.26 µm X 10.67 µm. Both micro conidial and macro conidial size in the present study are quite broader covering a wider range of size when compared to a report from India who identified *Fusarium oxysporum* f.sp. zingiberi with microconidial size ranging from 5.20×4.00 µm to 12.30×5.70 µm and macroconidial size ranging from 16.20×4.70 µm to 32.0×5.7 µm (Gupta *et al.*, 2014), whereas it becomes narrower in terms of microconidial size but broader in terms of macroconidial size when compared with a recent report by Abreham Chebte (2015) who measured microconidial length ranging from 1.02 µm to 30.47 µm and macroconidial length of 47.51 µm to 79.63 µm for *Fusarium oxysporum* isolated from onion basal rot.

Comparison of these pathological, cultural and morphological records with previous works (Moreira *et al.*, 2013; Gupta *et al.*, 2014) and standard literature (Gerlach and Nirenberg, 1982; Leslie and Summerell, 2006), these features of the isolates complied with the descriptions of *Fusarium oxysporum* f. sp. zingiberi. Accordingly, the isolates are identified as *Fusarium oxysporum* f. sp. zingiberi.

To support the cultural and morphological identification, a molecular identification study PCR-RFLP of the Internal Transcribed Spacer1 (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.* (1998) who reported a 220 bp PCR amplicons of *Fusarium oxysporum* f.sp. vasinfectum employing the same primer pair ITS1/ITS2 and different from that of Mishra *et al.* (2013) who reported 230 bp ITS1 region from *F. oxysporum* f.sp. psidi. The primers used by (Mishra *et al.*, 2013) (ITS1F and ITS1R) were different in sequence from that used in the current study. Some authors subtract the length of the primers they employed from the amplicon size they observed on the gel band in reporting PCR amplicon size (White *et al.*, 1990; De Beek, *et al.*, 2014) but in the current study PCR amplicon sizes are reported without subtracting the length of the primers employed in the PCR amplification which otherwise would result in a decrease by 39 nucleotides.

The length of the amplified ITS1 region was in the range reported by (Santamaria *et al.*, 2012) 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 135bp to 183 bp in length. In this regard, our isolates 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.

The PCR amplified ITS1 region of the isolates was subjected to restriction digestion experiment with two hexa-cutters (*HindIII* and *PstI*) and two tetra-cutter (*HhaI and MspI*)

restriction enzymes. Treatment of the ITS1 region PCR product with three of the enzymes (*HhaI, HindIII* and *PstI*) resulted in a single band of equal size with the PCR product; indicating that the amplified region was not cut with these restriction enzymes and that these enzymes do not have cutting sites in the ITS1 region.

Digestion of the amplified ITS1region 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 bp 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 bp, 120 bp and 100 bp on the gel. As explained by Mishra *et al.* (2014), 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 study of *Fusarium oxysporum* f. sp. zingiberi was studied using DNA Amplification Fingerprinting (DAF) technique from Australia (Pappalardo *et al.*, 2009). This polymorphism study was conducted based on the PCR amplification of different loci employing five different primers. They found 17 different fragments from 29 isolates. Within *Fusarium oxysporum* f. sp. zingiberi (Foz) isolates, three haplotypes were identified based on 17 polymorphic bands generated with the five different primers. Even though, the methodology employed by Pappalardo *et al*. (2009) differs from the present study, the identification of three genetic haplotypes is similar to the finding of the present study (Table 10).

Another molecular study conducted on *Fusarium oxysporum* f. sp. zingiberi has been recently reported from India using RAPD technique (Gupta *et al*., 2014). In that study, screening was made among 40 "OPAD" primers for maximum polymorphism detection. One primer named "OPAD15" was selected for its maximum number of amplified loci from 19 isolates. PCR amplification of sample DNA of one isolate with that primer pair amplified more than one locus increasing the level of detection of polymorphism. Similarity matrix was constructed from 228 fragments generated from the RAPD-PCR amplification using the DICE coefficient of similarity. UPGMA dendrogram clustered the 19 isolates in to two major groups with 20% similarity between the two groups where by the UPGMA dendrogram generated in the present study tended a similar bifurcating node with 6% Jaccards similarity coefficient.

Higher level of genetic polymorphism has been detected between *Fusarium oxysporum* f.sp. zingiberi identified in the present study. Generally, The *Fusarium* isolates were diverse in the pathological, cultural, morphological and molecular characters considered in the present study. This finding goes in line with the idea of Gerlach and Nirenberg, (1982) who stated that *Fusarium oxysporum* is one of the most variable *Fusarium species* regarding macroscopic and microscopic characteristics. Leslie and Summerell (2006) have also explained that *F. oxysporum* and *F. solani* from the tropics are polyphyletic and are likely to be quite different from similar strains from temperate regions.

In Ethiopia, two studies on *Fusarium species* involving PCR-RFLP techniques have been reported. The first of these was reported by Mesfin Bogale *et al*. (2007) who worked on PCR amplification of Translation elongation factor 1 alpha (TEF-1α) gene and restriction

digestion of the amplicon using restriction endonucleases, *AluI* and *MseI.* The PCR-RFLP pattern of the amplified TEF1- α was in correspondence with another finding by O'donnell *et al*. (1998) confirming the identification of *Fusarium oxysporum.* Similarly, Abreham Chebte (2015) identified *Fusarium oxysporum*, *Fusarium solani* and *Fusarium proliferatum* from onion basal rot.

In that study, PCR amplified ITS+ 5.8S region of the *Fusarium* isolates was subjected to restriction digestion with restriction enzymes *Ecor I* and *Pst I* which resolved the isolates into two major groups and five sub groups whereas digestion with *Hind III* left the PCR products undigested. Restriction digestion with *HindIII* in the present study also gave a similar result with that of Abreham Chebte, (2015) which enables to deduce that this restriction enzyme does not have restriction site in the ITS1 region of *Fusarium oxysporum*. Contrary to Abreham Chebte (2015), *PstI* did not show digestion on any of the isolates in the present study.

The actual PCR-RFLP finding was supported with *in silco* RFLP simulation of *Fusarium oxysporum* ITS1 sequences retrieved from the database (ITSoneDB) where the employed restriction enzymes showed similar cutting frequencies. This was done to examine whether the *in silco* restriction digestion of the ITS1 sequences of *Fusarium oxysporum* species retrieved from the ITSoneDB will result in a similar restriction digestion pattern with that of the actual experiment of the present PCR-RFLP study or will it result in a different one. The use of this approach to support actual PCR-RFLP experiments has also been practiced by previous investigators some of which are briefly reviewed here. In an experiment aimed at identifying and differentiating dermatophyte species, PCR products

of the (ITS rDNA) were digested by a single restriction enzyme, *Mva*I. The enzyme was evaluated in both *in silico* and practical PCR-RFLP assay to find the differentiating restriction profiles for each species (Mirhendi *et al*., 2012). They found a similar PCR-RFLP profile in both the actual electrophoresis and the *in-silico* analysis. In USA, Wei *et al.* (2007b) employed computer-simulated RFLP analysis of 16S rRNA genes to identify new phytoplasma groups where they produced *in silco* RFLP patterns by mimicking actual restriction enzyme digestions and subsequent gel electrophoresis. The computer-simulated 16S rRNA gene analysis allowed differentiation and identification of phytoplasma strains. Wei *et al.* (2007a) identified new subgroups of phytoplasma in clover by virtual RFLP analysis of 16S rDNA sequences.

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. The virulence level of the *Fusarium* isolates was directly proportional to their respective radial growth value; the fast growing the isolate is the highly pathogenic.

Based on cultural characters (colony color and revers color) the *Fusarium* isolates are categorized as white, creamy white and pink on the abveres side and dull white, lite reddish purplke, 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 variability between the isolates. The *In silco* RFLP digestion simulation of the ITS1 region of *F. oxysporum* retrieved from ITSoneDB showed similar cutting frequency with the actual RFLP experiment supporting the identification of the isolates as *Fusarium oxysporum*. The UPGMA dendrogram generated from the similarity matrix data of ITS1/ITS2 PCR-RFLP band profiles indicated genetic polymorphism between the species of *Fusarium oxysporum* in the study areas.

RECOMMENDATIONS

From the outcome of this study, the following recommendations are forwarded.

Ginger is economically important crop both at the household level in the ginger cultivating areas in SNNPR and to the Ethiopian national economy. Therefore, if either continued benefits from the current production capacity or intensive productions are pursued in the future, the comprehensive pathological aspect of ginger shall be extensively studied including insect pests, nematodes, fungal and bacterial pathogens.

Morphological identification of fungal plant pathogens is time taking and also potentially erroneous as it depends on nuance structural differences. while molecular identification methods are more accurate and quick, there are limitations in laboratory facilities and access to the molecular recipes in Ethiopia. Therefore, adequate budget should be allocated to equip life science research laboratories with molecular laboratory facilities and chemicals.

When molecular approaches involving PCR-RFLP techniques are intended, virtual *in silco* restriction digestion on sequences from database collections must be performed on putatively suggested epithet of the specimens before purchasing restriction enzymes for such experiments. This is a critical step which can make an RFLP experiment both economical and successful.

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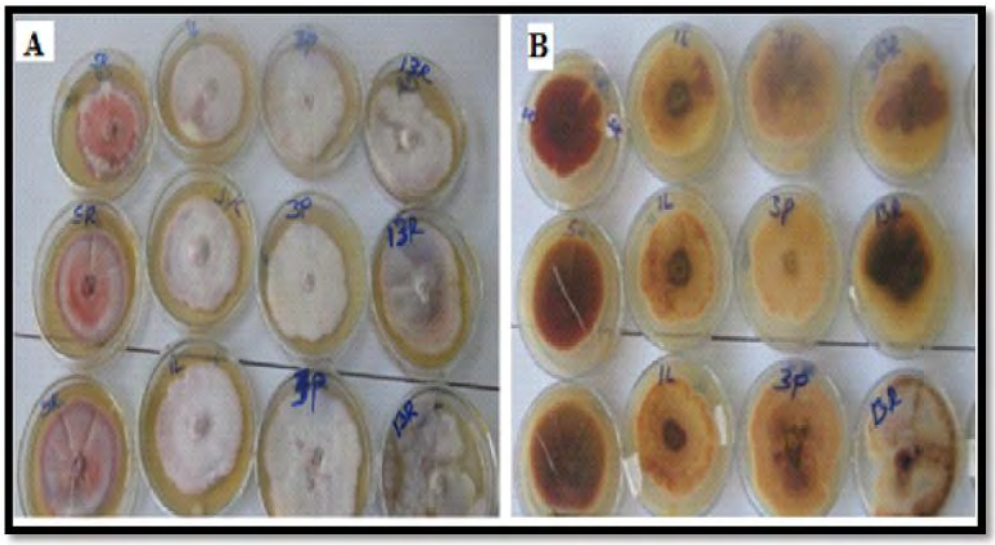
APPENDICES

Appendix A: Ginger rhizome rot by *Fusarium oxysporum*

Ginger rhizome rot by *Fusarium oxysporum* as seen on the external surface (A), Fusarium mold growth in the internal part of the rhizome as seen split along the plane of inoculation (B).

Appendix B: colony color and pigmentations

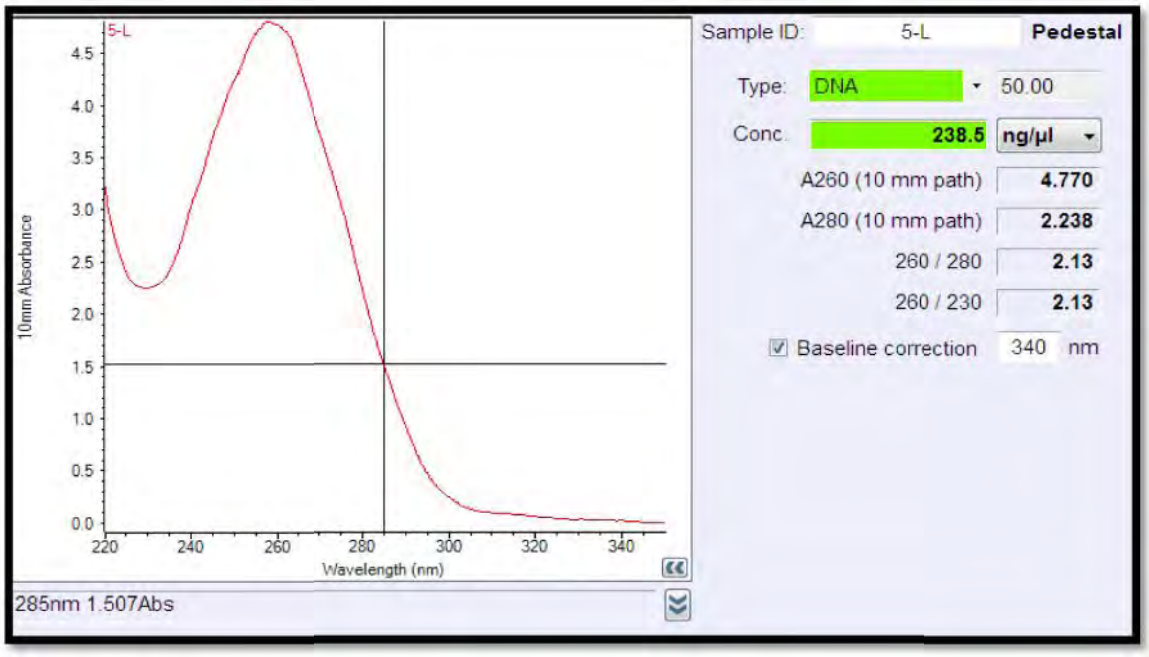


Colony color of *Fusarium* isolates (A), Colony reverse/pigmentation (B)

Appendix C. Spore suspension of *Fusarium oxysporum*

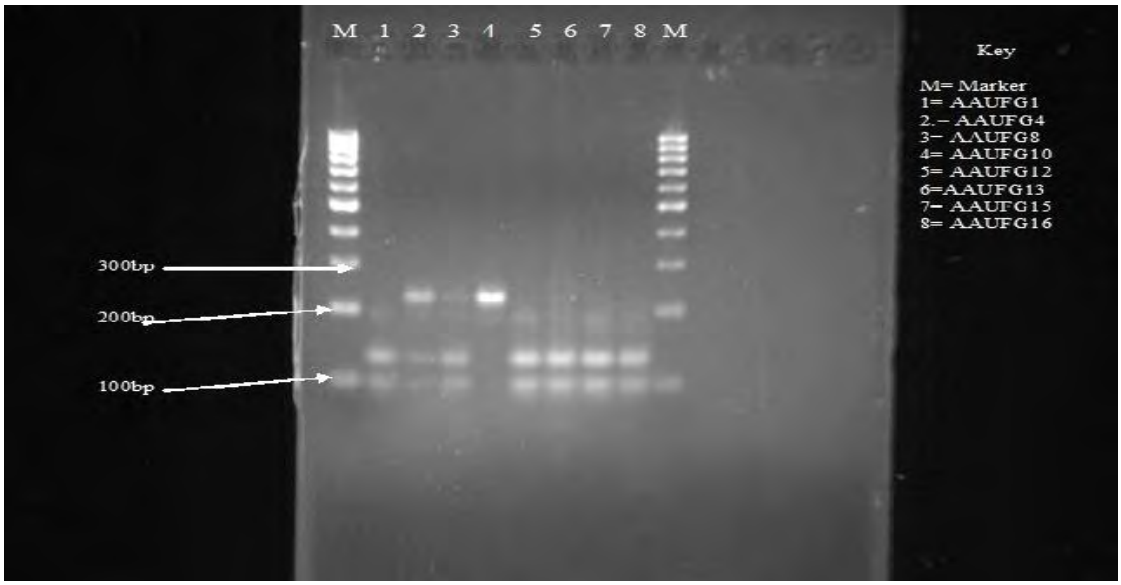
Appendix D. Growth of *Fusarium oxysporum* in liquid media (PDB)

Appendix E. DNA absorbance peak



DNA absorbance peak generated by NanoDrop Spectrophotometer

Appendix F. ITS1 PCR-RFLP band patterns restricted with *Msp*I



Restriction digestion of PCR amplified ITS1 region with restriction enzyme *MspI*; M=100bp DNA ladder; lanes 1-8, *Fusarium oxysporum* isolates.

Appendix G. NanoDrop readings of DNA concentration and Absorbances at 260 and 280

No	Sample ID	DNA (ng/µl)	Concentration	Absorbance		Ratio
				A260	A280	260/280
	BLANK (ET Elution Buffer)		0.1	0.002	-0.015	-0.16
1	AAUFG1		154.2	0.853	0.484	1.76
2	AAUFG3		100.5	1.004	0.549	1.83
3	AAUFG4		44.6	0.892	0.491	1.82
4	AAUFG5		32.1	0.642	0.37	1.73
5	AAUFG6		341.1	0.325	0.18	1.81
6	AAUFG7		546.8	0.914	0.504	1.81
7	AAUFG8		700.5	0.349	0.213	1.63
8	AAUFG10		274.5	0.744	0.419	1.78
9	AAUFG11		591.8	0.716	0.395	1.81
10	AAUFG12		180.7	0.423	0.247	1.71
11	AAUFG13		512.2	0.752	0.426	1.76
12	AAUFG14		129.5.	0.426	0.240	1.78
13	AAUFG15		1086	0.282	0.172	1.64
14	AAUFG16		183.2	0.446	0.27	1.65

Appendix H: Matrix table generated from PCR-RFLP data of rDNA ITS1 region of *Fusarium oxysporum*

Rows\Cols	AAUFG1	AAUFG3	AAUFG4	AAUFG5	AAUFG6	AAUFG7	AAUFG8	AAUFG10	AAUFG11	AAUFG12	AAUFG13	AAUFG14	AAUFG15	AAUFG16
AAUFG1	1.0000000													
AAUFG3	1.0000000	1.0000000												
AAUFG4	0.6666667	0.6666667	1.0000000											
AAUFG5	0.0000000	0.0000000	0.3333333	1.0000000										
AAUFG6	0.6666667	0.6666667	1.0000000	0.3333333	1.0000000									
AAUFG7	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000								
AAUFG8	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000							
AAUFG10	0.0000000	0.0000000	0.3333333	1.0000000	0.3333333	0.0000000	0.0000000	1.0000000						
AAUFG11	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000					
AAUFG12	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000	1.0000000				
AAUFG13	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000	1.0000000	1.0000000			
AAUFG14	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000	1.0000000	1.0000000	1.0000000		
AAUFG15	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	
AAUFG16	1.0000000	1.0000000	0.6666667	0.0000000	0.6666667	1.0000000	1.0000000	0.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000	1.0000000

Appendix I: *In silco* restriction digestion profile of rDNA ITS1 sequences of *F. oxysporum* retrieved from ITSoneDB

No	Accession number	ITS1 sequence length	Digestion with *MSPI*			Digestion with *HhaI*			Digestion with *HindIII*	Digestion With *PstI*
			Digestion	Frequency	Cut positions	Digestion	Frequency	Cut positions		
1	EU721723	155bp	✓	1	74	**X**	0	-	**X**	**X**
2	JN859441	150bp	✓	1	68	**X**	0	-	**X**	**X**
3	DQ016192	149bp	✓	1	66	**X**	0	-	**X**	**X**
4	AJ853769	148bp	✓	1	66	**X**	0	-	**X**	**X**
5	JN859448	150bp	✓	1	68	**X**	0	-	**X**	**X**
6	AY555719	150bp	✓	1	68	**X**	0	-	**X**	**X**
7	JQ301897	147bp	✓	1	66	**X**	0	-	**X**	**X**

8	HQ608012	150bp	✓	1	68	**X**	0	-	**X**	**X**
9	KC787030	150bp	✓	1	68	**X**	0	-	**X**	**X**
10	DQ016235	149bp	✓	1	66	**X**	0	-	**X**	**X**
11	EU721682	155bp	✓	1	74	**X**	0	-	**X**	**X**
12	EU326215	150bp	✓	1	68	**X**	0	-	**X**	**X**
13	HQ649819	141bp	✓	1	60	**X**	0	-	**X**	**X**
14	FJ172291	150bp	✓	1	67	**X**	0	-	**X**	**X**
15	EF488405	150bp	✓	1	68	**X**	0	-	**X**	**X**
16	DQ016193	149bp	✓	1	66	**X**	0	-	**X**	**X**
17	HQ649826	150bp	✓	1	68	**X**	0	-	**X**	**X**
18	AY462580	150bp	✓	1	68	**X**	0	-	**X**	**X**

19	DQ016217	149bp	✓	1	66	**X**	0	-	**X**	**X**
20	EU285552	147bp	✓	1	66	**X**	0	-	**X**	**X**
21	HM346537	209bp	X	0	-	✓	1	167	**X**	**X**
22	HQ451891	181bp	✓	1	98	**X**	0	-	**X**	**X**
23	JF779674	237bp	✓	1	134	**X**	0	-	**X**	**X**
24	AY667488	315bp	✓	3	45,84,234	**X**	0	-	**X**	**X**
25	GU934524	190bp	✓	1	107	**X**	0	-	**X**	**X**
26	JX077060	183bp	X	0	-	✓	1	162	**X**	**X**
27	JN942837	178bp	✓	1	96	**X**	0	-	**x**	**x**
28	U28160	196bp	X	0	-	✓	3	78,90,120	**X**	**X**
29	AY667489	319bp	✓	3	49,88,238	**X**	0	-	**X**	**X**

30	AJ301728	171bp	✓	1	75	**X**	0	-	**X**	**X**
31	EU839376	150bp	✓	1	68	**X**	0	-	**X**	**X**
32	AY928419	150bp	✓	1	68	**X**	0	-	**X**	**X**
33	JN859445	150bp	✓	1	68	**X**	0	-	**X**	**X**
34	HQ649821	150bp	✓	1	68	**X**	0	-	**X**	**X**
35	AB470850	151bp	✓	1	65	**X**	0	-	**X**	**X**
36	EU073196	148bp	✓	1	67	**X**	0	-	**X**	**X**
37	JN859460	148bp	✓	1	66	**X**	0	-	**X**	**X**
38	EU839389	150bp	✓	1	68	**X**	0	-	**X**	**X**
39	EU839402	150bp	✓	1	68	**X**	0	-	**X**	**X**
40	JQ809656	147bp	✓	1	66	**X**	0	-	**X**	**X**

41	EU285554	147bp	✓	1	66	**X**	0	-	**X**	**X**
42	GU136491	150bp	✓	1	68	**X**	0	-	**X**	**X**
43	AF440560	148bp	✓	1	66	**X**	0	-	**X**	**X**
44	AF322074	150bp	✓	1	68	**X**	0	-	**X**	**X**
45	FN598931	147bp	**X**	0	-	**X**	0	-	**X**	**X**
46	AF440547	147bp	✓	1	66	**X**	0	-	**X**	**X**
47	GU445364	150bp	✓	1	68	**X**	0	-	**X**	**X**
48	DQ016236	149bp	✓	1	66	**X**	0	-	**X**	**X**
49	JF817270	147bp	✓	1	66	**X**	0	-	**X**	**X**
50	AY928413	150bp	✓	1	68	**X**	0	-	**X**	**X**
51	JN005749	147bp	✓	1	66	**X**	0	-	**X**	**X**
52	GU445377	152bp	**X**	0	-	**X**	0	-	**X**	**X**

53	KC787031	150bp	✓	1	68	**X**	0	-	**X**	**X**
54	EU839383	150bp	✓	1	68	**X**	0	-	**X**	**X**
55	KJ620978	151bp	✓	1	69	**X**	0	-	**X**	**X**
56	JQ219941	146bp	✓	1	64	**X**	0	-	**X**	**X**
57	HM143731	146bp	✓	1	66	**X**	0	-	**X**	**X**
58	EU364848	151bp	✓	1	68	**X**	0	-	**X**	**X**
59	EU625403	153bp	X	0	-	**X**	0	-	**X**	**X**
60	JN232191	150bp	✓	1	68	**X**	0	-	**X**	**X**
61	HQ530550	163bp	✓	1	46	**X**	0	-	**X**	**X**
62	KC577177	143bp	✓	1	61	**X**	0	-	**X**	**X**
63	JN232157	150bp	✓	1	68	**X**	0	-	**X**	**X**
64	JX486823	150bp	✓	1	68	**X**	0	-	**X**	**X**

65	EF488408	150bp	✓	1	68	**X**	0	-	**X**	**X**
66	AF132800	147bp	✓	1	66	**X**	0	-	**X**	**X**
67	JQ301898	144bp	✓	1	71	**X**	0	-	**X**	**X**
68	DQ016227	149bp	✓	1	66	**X**	0	-	**X**	**X**
69	JF807393	137bp	✓	1	64	**X**	0	-	**X**	**X**
70	AY904065	147bp	✓	1	66	**X**	0	-	**X**	**X**
71	AY354397	147bp	✓	1	66	**X**	0	-	**X**	**X**
72	KC787025	150bp	✓	1	68	**X**	0	-	**X**	**X**
73	HQ379659	150bp	✓	1	68	**X**	0	-	**X**	**X**
74	GQ376117	150bp	✓	1	68	**X**	0	-	**X**	**X**
75	KF313101	147bp	✓	1	66	**X**	0	-	**X**	**X**

76	HQ530544	164bp	✓	1	46	**X**	0	-	**X**	**X**
77	AF443071	151bp	✓	1	69	**X**	0	-	**X**	**X**
78	KC339769	150bp	✓	1	68	**X**	0	-	**X**	**X**
79	JQ969044	148bp	✓	1	66	**X**	0	-	**X**	**X**
80	FJ196767	147bp	✓	1	66	**X**	0	-	**X**	**X**
81	DQ655731	147bp	✓	1	66	**X**	0	-	**X**	**X**
82	AY928420	150bp	✓	1	68	**X**	0	-	**X**	**X**
83	KJ466112	143bp	✓	1	62	**X**	0	-	**X**	**X**
84	JN859446	150bp	✓	1	68	**X**	0	-	**X**	**X**
85	HQ649822	150bp	✓	1	68	**X**	0	-	**X**	**X**
86	AB470894	147bp	✓	1	66	**X**	0	-	**X**	**X**

87	EU082779	149bp	✓	1	67	**X**	0	-	**X**	**X**
88	JN232148	151bp	✓	1	68	**X**	0	-	**X**	**X**
89	EU161241	147bp	✓	1	70	**X**	0	-	**X**	**X**
90	KC787018	150bp	✓	1	68	**X**	0	-	**X**	**X**
91	JN232186	150bp	✓	1	68	**X**	0	-	**X**	**X**
92	EU364857	150bp	✓	1	68	**X**	0	-	**X**	**X**
93	AY904065	147bp	✓	1	66	**X**	0	-	**X**	**X**
94	HQ530562	165bp	✓	1	46	**X**	0	-	**X**	**X**
95	KF278962	150bp	✓	1	68	**X**	0	-	**X**	**X**
96	GU056168	150bp	✓	1	68	**X**	0	-	**X**	**X**
97	JN254792	150bp	✓	1	68	**X**	0	-	**X**	**X**

98	EU721712	155bp	✓	1	74	**X**	0	-	**X**	**X**
99	HQ379658	150bp	✓	1	68	**X**	0	-	**X**	**X**
100	JN232167	150bp	✓	1	68	**X**	0	-	**X**	**X**

✓ = There is digestion; **X**= No digestion; "-", No cutting site