CHARACTERISATION OF AN EMERGING BANANA WILT DISEASE ON EAST AFRICAN HIGHLAND BANANAS (*MUSA-AAA*) IN CENTRAL UGANDA

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DECLARATION

I, Phillip Kinyera, declare that this is my original work, except where acknowledged by reference, and that it has never been submitted to any other institution of higher learning for any award. I am directly responsible for any omissions, corrections, or mistakes in this thesis.

Sign…….

Date……...

APPROVAL

This dissertation has been submitted to the Directorate of Research and Graduate Training of Kyambogo University with our approval as university supervisors.

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DEDICATION

I dedicate this work to my dear parents Mr. & Mrs. Tanga for raising me into a wonderful man, because of their steadfast encouragement and intercession during this academic journey, made me reach this far. To my dear fiancée Adie J. and my sweet daughter Briella, thanks for loving me and standing by me throughout this academic path. To my dear classmates, students, and close friends; who added their valuable time, support, and effort to help me achieve this work. Thank you all and God bless.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EAHB	East African Highland Banana
FOC	*Fusarium Oxysporum Cubense*
FocSTR4	Foc subtropical race 4
FocTR4	Foc tropical race 4
FON	*Fusarium oxysporum f. sp. niveum*
HR	Highly resistant
HS	Highly susceptible
ID1	Index of disease
ID2	Incidence of disease
IITA	International institute of tropical agriculture
ITC	International Transit Center
MR	Moderately resistant
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
TC	Tissue culture
TR4	Tropical Race four (4)
UV	Ultra violet light
VCG	Vegetative compatibility group
YS	Yellow-sigatoka

OPERATIONAL DEFINITIONS

Fusarium wilt Disease: Fusarium wilt is a significant soil-borne fungal disease that causes water-conducting vessels to become blocked, causing plants to wilt and frequently die.

Sequencing: DNA sequencing is the process of sorting out the arrangement of the four "bases," or chemical building blocks, that make up the DNA molecule.

Amplicons: Represent dynamic structures that have the potential to cause higher-order amplification, duplication, or deletion of the intervening DNA sections, as well as genomic rearrangements.

Cavendish banana: Cavendish, or dessert, bananas can be fried, mashed, and chilled in pies or puddings, but they are almost always consumed fresh. They were named after William Cavendish, 6th Duke of Devonshire, as their name.

Abiotic: Abiotic stress is the negative effects of non-living substances on living organisms in a specific environment.

Biotic: An organism experiences "biotic stress" when it is impacted by other living entities, such as weeds, naturally occurring or artificially grown plants, bacteria, viruses, fungi, parasites, and both beneficial and harmful insects.

Syndrome: A syndrome is a group of related medical signs and symptoms that are frequently connected to a specific illness or condition.

Invitro: Research is conducted using biological molecules, cells, or microorganisms that are not in their natural biological environments.

Variety: In taxonomic nomenclature, variety (botany) is formally ranked below subspecies.

Genotype: An organism's genotype is made up of all of its genetic components.

Rhizome: The primary stem of a plant that extends horizontally below ground is called a rhizome.

Underproductivity: The production of less than what is typically produced or not enough to meet demand

Severity: Conventionally, the degree of organ system dysfunction or physiological decompensation is used to determine the severity of an illness; on the other hand, the probability of dying is referred to as the risk of mortality.

Resistance: The capacity to stop or lessen the occurrence of diseases in hosts who would otherwise be vulnerable is known as immune resistance.

ABSTRACT

The genus *Musa spp.* is a crucial food to humans in the world, and bananas play a significant role in Uganda's socio-economic fabric. However, emerging pests and diseases threaten sustainable banana cultivation. A study was conducted in Ugandan central districts of Wakiso, Mpigi, and western districts of Mbarara, Isingiro to understand the causative agent of an emerging golden yellow leaf syndrome similar to symptoms of *FOC* Race 4 in Uganda between October 2023 to May 2024. The key objectives; survey banana gardens associated with golden yellow-leaf syndrome on EAHBs, collect and segregate the pathogens from the selected study districts; diagonize the pathogen related to golden yellow-leaf syndrome on EAHB in the selected districts; determine the pathogenicity of isolated pathogens on selected EAHB cultivars. Questionnaires were administered to 25 farmers to understand indigenous knowledge about the disease in the study areas. Symptomatic plant samples were collected and delivered to the laboratory for pathogen diagnosis using molecular markers. The characterized isolates were screened against selected EAHB varieties to confirm Koch's postulates. About 88% of respondents had ever seen 'golden leaf' syndrome in their banana gardens but did not know the cause. Farmers reported symptoms starting within two years, with 64% having shared planting materials, and the disease symptoms were reported prevalent during rainy seasons. Of 21 isolates purified, 6 were positive for the 28S - 18S rRNA inter-spacer region using FocTR4 primers, and after sequencing, five of them were verified to be *FON* and one was *FOC* Race 1. Screening results showed that Sukali ndizi, and EAHB varieties were vulnerable to *FON.* Thus *FON*, a devastating pathogen of watermelons, is now pathogenic to EAHBs, a threat to farmers' livelihoods and food security in Uganda. This is the first report of this pathogen in Uganda affecting bananas, Further research is needed to map its spread and resistant sources in the banana germplasm.

Keywords: Pathogenicity, molecular markers, *Fusarium oxysporum f. sp. niveum*, symptoms, susceptible.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

The genus *Musa* is significant to the food basket of man; they produce among the greatest consumed foods in the world today (ranked 4th, after rice, wheat, and maize) (FAO, 2018). Banana (*Musa acuminata L.)* is one of the key tropical fruits consumed worldwide majorly as a cash crop for a few nations engaged in trading internationally. In 2019, global banana exports rose to 24.7 million tons valued at 13 billion USD, which presents the highest export value for international trade in fruits (Rashad *et al*., 2021). In 2019, the export of bananas in Asia hit 4.5 million tones and later increased by 18% compared to 2018 (FAO, 2018). The plantain (Musa sp.) and dessert banana are among the most common staple food crops in the world (Woldu *et al*., 2015). It's commonly noted for its rich nutritional value in potassium and is a common cuisine in Asia. Bananas have key nutrients that can contribute to a protective impact on healthy living. Consuming bananas can help bring down high blood pressure and also reduce cancer risks (Sidhu & Zafar, 2018). Green bananas contain vital minerals; potassium, phosphorus, magnesium, zinc, fiber, vitamins (Vit B6, provitamin A, C), and resistant starch, which are all essential for a healthy being (Falcomer *et al*., 2019).

In Africa, the top banana-producing countries are; Cameroon, Côte d'Ivoire, Ethiopia, Ghana, Madagascar, Uganda, and Zimbabwe, which feed over eighty (80) million people and also provide income (FAO, 2018). Bananas are a very important income source and food security crop in Uganda with 65% of Ugandan households known to depend on bananas for food and income (Kikulwe *et al*., 2008). EAHB cooking bananas (AAA) are part of eighty percent (80%) of the banana cultivars found in regions of the Great Lakes and have been a key source of income and food to over forty million people in the region.

In Uganda, the EAHB is cultivated majorly as a common household food and source of money. At the time of maturity, the green fruits are peeled then wrapped in banana leaves and steamed using a firewood oven or over a charcoal stove. The steamed bananas are later mashed (Matooke) and enjoyed with several pastes, soups, and sauces such as; beef, groundnut paste, and fish among others (Industries, 2008). Banana plantations also capture pockets of carbon dioxide (also other combined carbons in the air) from the atmosphere through photosynthesis, which is then converted into food substrates (Priver *et al*., 2020).

The sustainable production of bananas has, however, been affected by a wide range of environmental stresses that significantly reduce their productivity as crops (Kubiriba *et al*., 2016). These two types of environmental stresses are can be categorized as abiotic stress and biotic stress. Abiotic stress leads to the major loss of crop productivity worldwide and these are brought about predominantly by factors such as; radiation, salinity, drought, extreme temperature, floods, heavy metals, etc. On the other hand, biotic stresses come as a result of various pathogens such as fungi, bacteria, nematodes, and herbivores. Some pests and diseases target banana cultivars specifically, making mono-crops or even backyard plantings difficult to manage and needing a lot of care to keep them healthy and productive.

Some of the common diseases of bananas that are the leading cause of underproductivity of this crop globally include: Yellow-sigatoka (YS) caused by fungus - *Mycosphaerella* species; black sigatoka traceable to the pathogen fungus - *Mycosphaerella fijiensis*; then fungal-Fusarium wilt- ascribed to *Fusarium oxysporum f sp. cubense*, the nematode to *Radopholus similis* and *Pratylenchus spp*). Some disease-resistant hybrids have been developed by banana research scientists in recent years (Achparaki *et al*., 2012). However, these hybrids are not preferred by consumers due to taste disparities from the original clone sets of EAHB (Madalla *et al*., 2023).

Banana Fusarium wilt is not new to the African continent, Foc race 1 was first been reported from West Africa in 1924, then on a farm in northern Mozambique, Foc-TR4 was previously reported in Africa. A second introduction occurred in 1951 when Foc Race 1 entered Tanzania. Foc race 1 strain is now widely spread in most banana-producing countries in Africa. The majority of bananas grown on the continent, such as East African Highland Banana (EAHB), plantains, and Cavendish bananas, however, are resistant /immune to Foc Races 1 and Foc Race 4 (Karangwa e*t al*., 2016).

Recently, typical symptoms of Foc-like infections in EAHB cultivars have been observed and reported in several banana-growing areas of Uganda, The EAHB varieties have for a long time been presumed to be immune to Fusarium infection (Karamura, *et al*., 2016).

1.2 Statement of the Problem

The emergence of Fusarium-like symptoms in EAHB, a key staple food for Uganda and the East African region, raises a serious concern since the EAHB were presumed to be resistant to Foc Race 1 (Zuo *et al*., 2018). Several countries around the world have reported the occurrence of a deadly strain of Foc-TR4 (Magdama *et al*., 2020). There is little information on which banana genotypes have resistance or tolerance to the pathogen and which could be used in prone areas as a long-term breeding intervention if, for instance, resistance is planned. It is therefore urgent to identify and confirm the cause of the golden yellow-leaf syndrome on the EAHBs and to rule out or confirm the existence of TR4 in Uganda.

1.3 Objectives of the study

1.3.1 General Objective

This research aimed at contributing to sustainable banana production and productivity through early detection of an infectious agent that causes a golden yellow-leaf syndrome on the East African Highland Banana (EAHB) in selected districts in central and southwestern regions of

Uganda.

1.3.2 Specific Objectives

i. Determine the prevalence of golden yellow-leaf syndrome on EAHB in the selected study districts of Uganda.

ii. To identify the causative agent of golden yellow-leaf syndrome on EAHB in the selected districts of Uganda.

iii. To determine the potential sources of resistance to the causative agent of golden yellow-leaf syndrome amongst popular EAHB cultivars.

1.4 Research Questions

i. What is the prevalence of the yellow-leaf syndrome in the EAHB?

ii. What is the causative agent of yellow-leaf syndrome in EAHB?

iii. Are there any sources of resistance to the causative agent of yellow-leaf syndrome among EAHB cultivars?

1.5 Significance of the Study

This study is crucial for the sustainable productivity of the EAHB, which is a significant staple food and income source for Ugandan farmers. Early detection of the infectious agent could help save thousands of farms across the country and pave a way forward on how best to contain its spread.

Precise determination of the disease-causing agent/condition is the basis for designing and recommending effective control measures. This is in congruence with Uganda's Vision 2040

that aspires for the transformation of Ugandan societies from peasant to modern developed society, and as further enshrined in the National Development Plan III (NDPIII) that promotes Agro-Industrialization to transform the subsistence agriculture sector to a commercial and competitive sector (Uganda National Planning Authority, 2020). This project was in alignment with Kyambogo University's strategic research agenda to increase agricultural production, productivity, food security, and value addition also in line with United Nations Department of Economic and Social Affairs (17 SDGs) (For *et al*., 2019).

1.6 Scope of the Study.

In this research, only symptomatic samples were collected from the districts of Wakiso, Mpigi, Isingiro, and Mbarara in Uganda between October 2023 to May 2024. Pathogen isolation and identification were conducted at the Kyambogo University Biology Research Laboratory. Pathogenicity assessment was done using four EAHB varieties; Mpologoma, Mbwazirume, Kibuzi,and Sukali Ndizi as susceptible control all under the greenhouse conditions to screen for sources of resistance to the causative agent.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification and importance of the EAHB

Edible banana belongs to the family *Musaceae* of the order Zingiberales. The genus does not pose as an invasive species as it tolerates well with other plant species. Musa species can grow in several different environmental settings depending on the variety and purpose of human use. They range from the easily consumable plantains and bananas of the tropics to the fiber bananas and decorative plants that can withstand freezing temperatures. Banana plants can measure from about 2–9 m (6.6–30 ft) in height. They evolved in areas of New Guinea Asia, Southeast, and the Indian subcontinent, developing in modern times to its secondary location of genetic diversity in Africa, Latin America, and the Pacific. Indigenous or local varieties typically provide better results with less input (compost, fertilizer, and water) and are more useful and tolerant of local circumstances. In terms of banana production in the world, India ranks first followed by China, Indonesia, and Brazil (Singh & Atre, 2018).

Musa species gained central importance within Pacific societies, Africa, and the world over. Since the beginning of time, humans have used bananas as a food source. A banana plant is a source of fermentable sugars, food, flavorings, beverages, silage-making, medicines, shelter, fiber, clothing garments, cordage, smoking material, and other many uses (Bandaranayake, 1998). The EAHB also known as ‘Matooke’ bananas are a big boost to the livelihoods of about thirty million people, mostly village/upcountry farmers found in East Africa near the Great Lakes. Only about seventy percent (70%) of the matooke produced is for the household level, making the remaining thirty percent (30%) as sold produce for income (Akankwasa *et al*., 2021). In common cases, men manage the banana farms with the intention of sales for money while the women manage directly for household food.

At full maturity, matooke bunches are gathered from gardens as mature-green-sized fruits. Depending on how the final consumer wishes to consume it, it can be peeled, then shrouded in banana leaves, which are steamed or boiled, and finally either squashed or not, it can be eaten as desired. The fruits may also be dinned directly after sizzling (Nowakunda *et al*., 2000). The EAHB's significance to consumers include; it is a natural/traditional fruit, it is believed by some to be medicine to help treat ulcers, aids digestion (softens stomach); easy to prepare, children like it as a meal, source of pro-vitamins; source of roughage, it is a local delicacy, aids in recovery from conditions such as high blood pressure and diabetes (Marimo et al., 2019).

2.2. Constraints to EAHB production in Uganda

Sustainable production of the EAHB has been affected by a wide range of environmental stresses that significantly reduce their productivity (Kubiriba *et al*., 2016). In this case, they can be categorized as abiotic stress and biotic stress. The abiotic stresses have been the primary factor contributing to low crop productivity globally and these are brought about predominantly by factors such as; radiation, salinity, drought, extreme temperature, floods, heavy metals, winds, global warming, etc. On the other hand, biotic stresses as a result of various pathogens/animals such as fungi, bacteria, nematodes, and herbivores. Certain pests and diseases specifically target certain banana cultivars, making planting them difficult and requiring a lot of care to keep them in good, productive shape (Nyombi, 2013).

Some of the common disease attacks on bananas that are the leading cause of underproductivity of this crop globally include Yellow Sigatoka caused by the fungus - *Mycosphaerella species*, .The lack of formal reporting methods and limited confirmed information makes it difficult to estimate the disease's current and potential impact. Race 1 of the fungus is believed to have caused over $2.3 billion in damage over the past century.

Recent breakouts in areas already afflicted by TR4 are grounds for alarm. According to unofficial statistics, damaged regions include 15 500 ha in the Philippines, 40,000 ha in China, and 80% of production area in the Jordan Valley. Scientists estimate that TR4 has infected around 100,000 ha1 globally(FAO, 2017).

2.3. Banana wilt disease

The Banana wilt is caused by the *Fusarium oxysporum f sp cubense* (Foc) races, a fungus that likes habiting in soil. This disease is also commonly known as Banana Wilt, a devastating disease of bananas. This disease has spread all over the tropics and can be located anywhere where suscetible banana cultivars are planted. delay in detection must have paved the way for the further spread of the pathogen both to commercial and small-homestead farms mostly Cavendish and Grand Nain banana cultivars(Aguayo *et al*., 2021).

2.4. Races of Fusarium

There are three main races in Foc: one (1), two (2), and four (4). "Gros Michel" is impacted by Race 1, while "Bluggoe," the hybrid triploid, is vulnerable to attack; Additionally, race four can be separated into subtropical Race four (Foc STR4) and tropical Race four (Foc TR4) (Ploetz, 2006).

Following further research, twenty four (24) vegetative compatibility groups (VCGs) have been established for Foc races (Fourie *et al*., 2009). Since its discovery in Southeast Asia in 1990, Foc TR4 (VCG 01213/ VCG 01216) has drawn significant attention due to its strong pathogenicity and broad host range (Ploetz, 2006). The Philippines, South China, Pakistan, Malaysia, Indonesia, and other major banana-growing regions of Asia are all experiencing this very contagious pathogen.

The three currently recognized pathogenic *Foc* races were classified based on the reference varieties that they infect under field conditions, i.e. Dessert banana cultivars like Bogoya (AAA) and Sukali Ndiizi (AAB) are sensitive to Race 1, while Bluggoe, Kayinja, and Kivuvu are at risk from Race 2. Meanwhile, Cavendish and all other genotypes that are vulnerable to Races 1 and 2 are at risk from Race 4 (TR4) (Zuo *et al*., 2018). While the EAHB (EA-AAA) is not known to be disturbed by Race 1 and 2, preliminary tests have shown it to be susceptible to Race 4 (Karamura *et al*.,2016).

Recently scientists in banana research have made admirable efforts in banana crop variety developments through mostly plant-breeding techniques giving rise to productive banana hybrids. However, due to the final consumer acceptance, it is much less likely for these products to be adopted by farmers. Despite efforts to enhance crop varieties, bananas remain vulnerable to common pests and diseases as Fusarium wilt, nematodes, banana bacterial wilt (BXW), and weevils. As a result, between 60% and 100% of output losses are attributed to Foc (Madalla *et al*., 2023).

2.5 General growth of *Fusarium* strains

Fusarium oxysporum strains may not be easily distinguished morphologically as there exist different races of Foc (Table 2.1) (Thilagam *et al*., 2018). Incubated colonies grow between 4 to 7 millimeters per day while employing potato dextrose agar media at 24°C (room temperature) treated with streptomycin gives a characteristic appearance of whitish to purplish mycelium color. Sporodochia appears orange in color and sclerotia blue in color. Microconidia as well as macroconidia develop on branched and unbranched mono-phialides. Microconidia appear in lengths of about 5 μm (16 × 2.4) to 3.5 micrometers (16 × 2.4), either double-celled or single even in some cases oval-shaped. Macroconidia are between 27 μm to 55 × 3.300 to

5.500 µm (micrometers), and the sickle-shaped basal cells are four to eight cells long, with foot-shaped basal cells (Rodriguez *et al*., 2014). Final-intercalary chlamydospores typically form singly or in pairs (doubles) within hyphae or rather conidia, with an apparent diameter of 7.0 to 11.0 µm. It is rare for isolates in the vegetative compatibility group (VCG) to produce chlamydospores (Rana et al., 2020).

Table 2. 1: Typical races of Foc and banana varieties they commonly invade.

Race	**Genome affected**	**Cultivars affected**
Race 1	ABB group, AAB group, and AAA group of Musa	Gros Michel, Pisang Awak, Lady finger, Bluggoe.
Race 2	Musa (the ABB group)	Close cooking cultivars and bluggoe
Race 3	Heliconia species, and Gros Michel and seedlings to a lesser degree	*Musa balbisiana*
Race 4	Musa (AAA), Musa (AAB), Musa (ABB)	Impacts all cultivars, each of which is equally impacted by Races 1 and 2.

Young roots or root bases are attacked by the Foc fungus, usually through damaged areas. Occasionally, infections move to the rhizome, which resembles a root, and then quickly spread to the rootstock and leaf bases. Vascular bundles, which first turn brown, then turn dark red, and last turn purplish or black, are how the disease spreads. Mature leaves have a yellow hue along their outer edges.

In addition, in a month or two, every leaf—aside from the youngest—turns yellow and eventually wilts, falling and hanging downward to cover the pseudostem (trunk) in finally dead brown leaves. Although new shoots may appear at the base of the plant, they usually wilt later and the entire plant dies; in this scenario, the fungus continues to grow and the spores will either survive in the soil or other hosts. Eventually, all of the plant's above-ground components perish (Zuo *et al*., 2018).

2.6. Symptoms of Fusarium wilt and damages

There are two common exterior symptoms of Fusarium wilt, often called Panama disease of bananas: "Yellow-leaf syndrome" and "Green-leaf syndrome." The most obvious and typical sign of Fusarium wilt in bananas is yellow-leaf syndrome. It is typically identified by the border of elder leaves turning golden yellow; occasionally, especially in dry and chilly climates, it is mistaken for a potassium deficit in the soil (Segura *et al*., 2018). The yellowing in leaves proceeds from the mature leaves to the youthful ones. The leaves gradually collapse, bow at the petiole, mostly not far to the midrib of the leaf, and hang downwards near the pseudostem. Unlike the yellow-leaf syndrome, the green-leaf syndrome causes some cultivars of affected plants to eventually retain their predominant green color until the petioles bend and collapse.

Younger leaves typically exhibit clear indications of remaining upright and looking brittle. Even diseased plants continue to grow, despite their newly developing pale leaves. The lamina of the developing leaves can look somewhat withered, deformed, and diminished. Eventually, at the base of the plant, the pseudostem divides lengthwise to form the comb. Regarding the banana plant's fruits, there doesn't seem to be any indication of any symptoms. When a banana plant contracts Fusarium wilt, it frequently shows poor growth, a low chance of recovery, and a large number of infected suckers before dying. Internally, explants of infected plants exhibit vascular discoloration, which starts with a yellowing of the vascular tissue in the rhizome and roots and then constantly develops into strands that are yellow, red, or brown in the pseudostem. As shown in (Fig. 2.1) these symptoms are quite typical of the disease that cultivars experience. Petioles are similarly speckled with reddish-colored vessels.

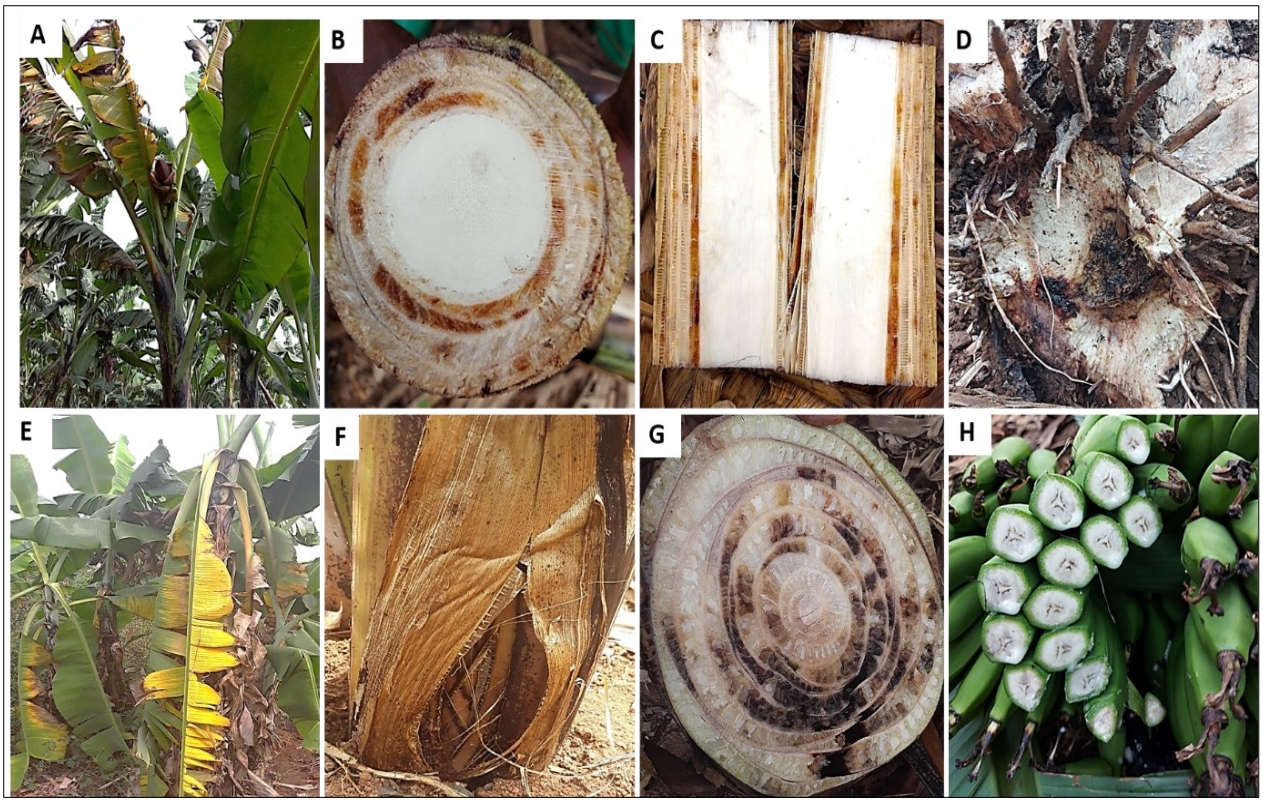

Figure 2. 1: Adverse symptoms showed by bananas infected by Foc- pathogen (figures **A**-Yellowing in leaves, **B&C**- Chlorosis and vascular darkening, **D**-Darkening and necrosis of rhizome **E**- leaf wilting, **F**-Bursts in combs, **G**- Advanced Chlorosis and vascular darkening, **H**-Premature banana.

2.7 Relationship between soil abiotic factors and Fusarium wilt races

Previous research has demonstrated a correlation between soil management techniques and the successful eradication of Fusarium wilt disease in banana plants. Nevertheless, as a crucial containment tactic for Fusarium wilt, this has gotten little attention. A wealth of information from the literature indicates that the primary cause of the spread and severity of plant diseases is related to soil conditions, specifically soil fertility, and texture (Teixeira *et al*., 2021). However, the attention given to soil management techniques in crop disease management is hampered by the inconsistent outcomes of case studies. The goal of the current study was to determine how soil abiotic variables affected plant tissue nutrient concentrations, biomass production, and the frequency of Fusarium wilt (Foc race 1) in bananas (‘Gros Michel, AAA)

grown in fields. The effects of soil pH and nutrients (nitrogen, calcium, magnesium, and manganese) were investigated in this study through the setup of a field trial. In the first season, almost 30% of the plants displayed Fusarium wilt signs during flowering. However, the incidence of Fusarium wilt did not change across treatments (Segura *et al*., 2018).

Research findings indicate that there are notable correlations between the pH of the soil and the amounts of magnesium and nitrogen in the soil. These interactions result in decreased banana bunch weight production and growth, but elevated micronutrient concentrations in the pseudostem. Bunch weight rose when pH rose, however this beneficial effect was countered by greater manganese concentrations. Lower micronutrient concentrations in the pseudostem and a higher bunch weight were the results of interactions between high soil pH and calcium and magnesium. Even in farms where banana disease is present, the findings can be utilized to create soil management plans that will increase banana yield (Segura *et al*., 2018).

2.8 Common Methods known to control Fusarium wilt Disease

There is no effective treatment for fusarium wilt once a plant is infected, from literature currently since the soil-borne pathogen stays in the soil for decades. However, methods such as crop rotation, avoiding sharing of planting materials, and sterilizing garden tools apply. Quarantining off the affected gardens and non-sharing of planting material such as banana suckers from garden to garden can be helpful. Also planting EAHBs and other resistant banana cultivars has proven useful (Blomme *et al*., 2024).

2.9 Conceptual Framework

The food basket of Uganda depends very much on a variety of bananas, with the EAHB locally called Matooke (*Musa AAA*), considered the major consumed banana variety. The precise presence of the fatal Tropical Race 4 of Fusarium wilt in Uganda and its impact on the East African Highland Banana have not been sufficiently determined by available data. An understanding of whether TR4 is genuinely existent in Uganda or if EAHB cultivars have become vulnerable to the country's Race 1 will come from the characterization of an emergent banana wilt disease among the cultivars of the EAHB grown in the chosen locations of Uganda. This will pave hope for local small-scale, large scale banana farmers and concerned Government Agricultural Authorities as described by the conceptional framework (Fig. 2.2). Occurrences and regeneration of different strains of FOC have been attributed to climate change, farming practices, as well as human migrations and settlements.

1. The Dependent Variables were: Cooking Banana variety belonging to the EAHB group.

2. The Independent Variables were: Isolated Fusarium wilt Strains affecting banana varieties in the selected districts.

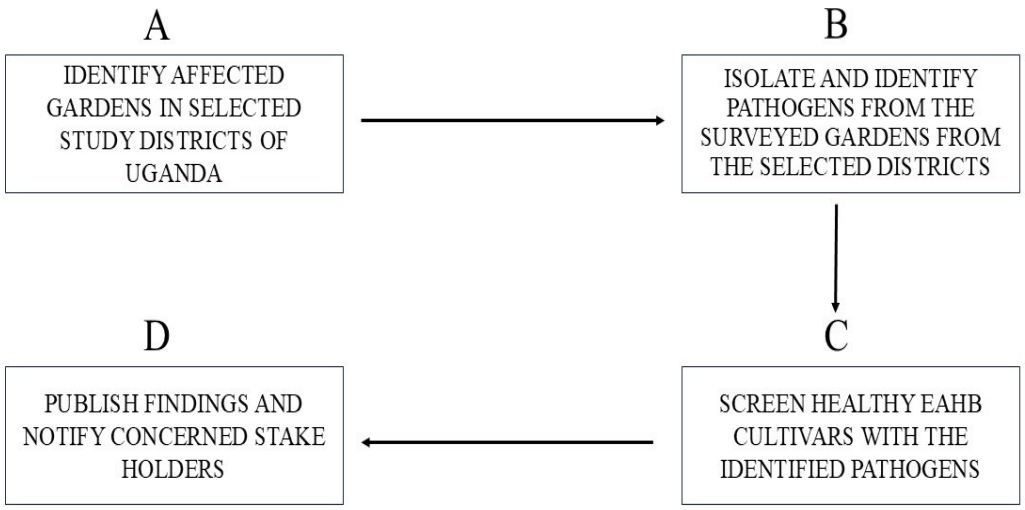

Figure 2. 2: Framework and conceptual flow of the research study. Adopted from (Ploetz, 2006)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study areas and their description

The survey on FOC occurrence and incidence in EAHB was conducted in the Ugandan districts of Wakiso, Mpigi, Mbarara, and Isingiro to represent the banana growing areas and based on reports of disease occurrences by extensive workers (Fig. 3.1).

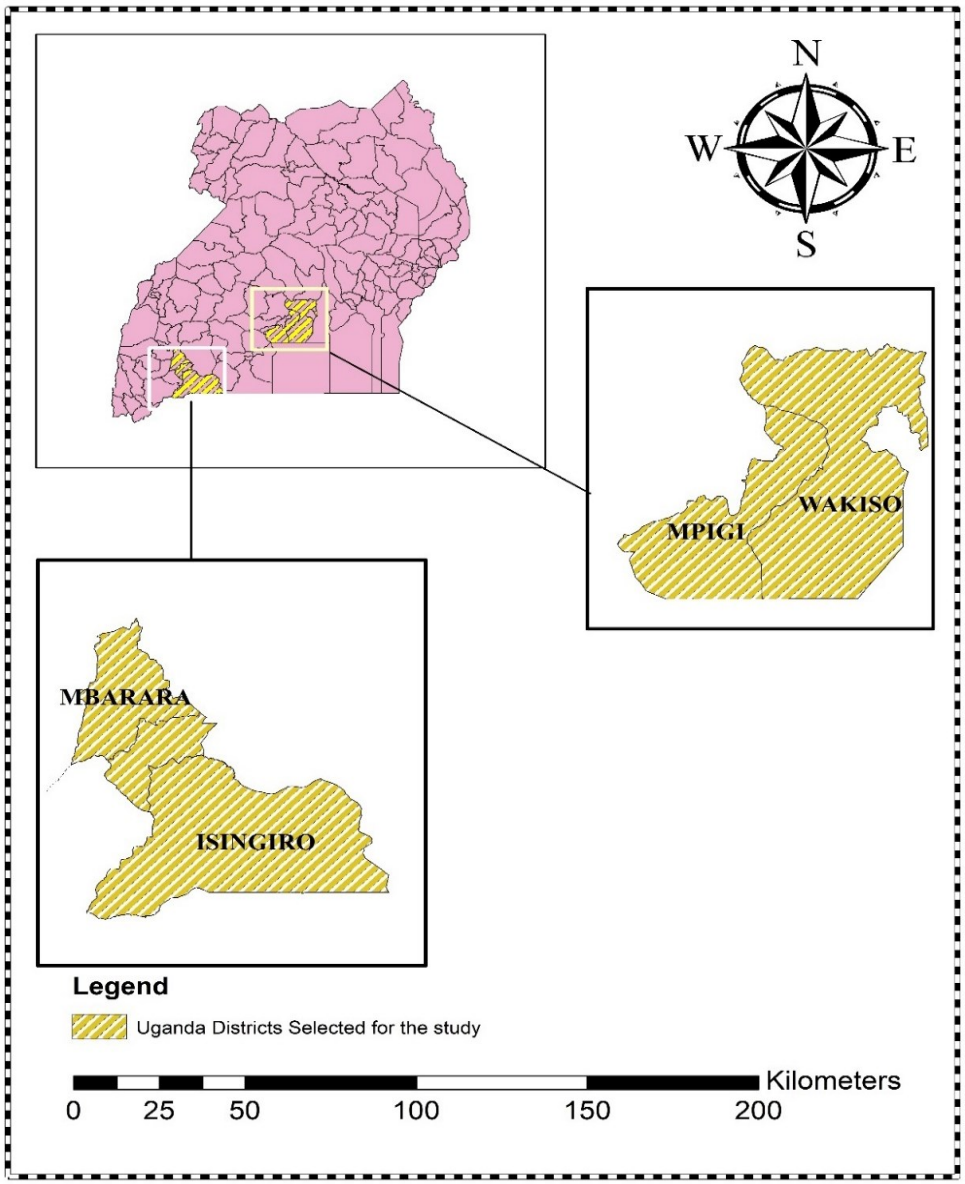

Figure 3. 1: A map of Uganda showing the location of the selected study districts; Wakiso, Mpigi, Mbarara, and Isingiro.

Wakiso and Mpigi in central Uganda have a comparatively poor banana output, while Mbarara and Isingiro districts in western Uganda were chosen to represent high-producing banana areas (Roz, *et al*., 2017).

3.1.2 Location of Wakiso, Mpigi, Mbarara and Isingiro Districts

Wakiso District is situated in Uganda's Central Region. To the south is Kalangala District, which borders Lake Victoria; to the southwest is Mpigi District; to the northwest is Mityana District; to the north is Nakaseke District and Luweero District; and to the east is Mukono District. The district headquarters are situated at Wakiso, northwest of Kampala, Uganda's capital and largest city, some 20 kilometers (12 miles) away by car. The district is located between 900 and 1340 meters above sea level, with coordinates of 00 24 N and 32 29 E (UBOS, 2017a).

Uganda's Mpigi district is a large municipality 45 miles west of the capital, Kampala. The city of Entebbe is located about forty (40) kilometers south of the town. The district is situated 1,217 meters (3,993 feet) above mean sea level on average, with latitude and longitude coordinates of 0.227353 and 32.324924, respectively (UBOS, 2017b).

Mbarara is located west of Masaka on the Kabale road, a busy transportation hub, and adjacent to Lake Mburo National Park. It is around 270 kilometers (168 miles) southwest of Kampala on the route. The coordinates of Mbarara are latitude -0.6132 and longitude 30.6582, or 00 36 48S, 30 39 30E. The city is situated at 3,763 feet (1,147 meters) above sea level (UBOS, 2017b).

Situated around 42 kilometers (26 miles) southeast of Mbarara, Isingiro is the principal city within the Ankole sub-region. The town of Isingiro is surrounded by the settlements of

Nyakigera, Gayaza, Rwembwa, Mabona, Kibwera, and Rumira. Isingiro and Kampala are around 310 kilometers (193 mi) apart by car. It is located at a height of 1455.72 meters (4775.98 ft) above sea level. Latitude: -0.795000, Longitude: 30.815278 or 0°47'42.0"S, 30°48'55.0" E are the coordinates of Isingiro Town Council (UBOS, 2017a).

3.1.3 Climate of Wakiso, Mpigi, Mbarara and Isingiro Districts

Wakiso has a warm on top of that wet climate with relatively soaring humidity. These conditions favor the rapid growth of plants such as bananas but also encourage disease outbreaks. This is due to its proximity to Lake Victoria, thus rendering it very productive. Wakiso has a bi-modal rainfall distribution, with two wet seasons in the year from October through November and April to May. January to February and July to August are the months with a dry spell. Although it is generally significantly higher, ranging from 1750 mm to 2000 mm, in most regions close to the lake zone, the average annual rainfall is 1320 mm. One of the main sources of rainfall in this district is Lake Victoria, a body of warm water covering around 63,000 square kilometers at an elevation of 1134 meters above sea level. In Wakiso, average surface air temperatures are approximately 11.0 °C, with a maximum of approximately 33.3 °C. The district experiences two distinct temperature peaks, one occurring between January and May and the other between July and September. Typically, February experiences the greatest temperatures, while July typically has the lowest temperatures (UBOS, 2017a).

The district of Mpigi receives a bi-modal rainfall pattern, with an average rainfall amount of 1320 mm falling between September and November. The first rains arrive between March and May. On the other hand, it is typical for the Lake Victoria basin to have rainfall of between 1750 and 2000 mm. Additionally, the district sees maximum yearly temperatures of 22.5 to 27 °C on average. Particularly in forested areas, the average relative humidity falls between 80%

and 95%. The Mpigi district is best characterized as evergreen, with little savanna vegetation and a variety of seasonal ponds (UBOS, 2017a).

Mbarara's weather is characterized as tropical savanna. The difference in precipitation between the wettest and driest months is 94 millimeters. There is a 2.4 °C yearly temperature variance. November has 76.93% relative humidity, the highest of any month. At 53.76%, July has the lowest relative humidity of any month. With 22.37 rainy days, October has the rainiest days of the month. Of all the months, July gets the fewest rainy days—3.67 days. Mbarara is in the middle, and it's easy to tell which is the summertime location (UBOS, 2017a).

The Isingiro district experiences a savanna-like tropical climate that is both wet and dry. The annual temperature in the city is 22.01 ºC, which is -1.46% cooler than the Ugandan average. On average, Isingiro receives 357.72 millimeters (14.08 inches) of precipitation per year and 282.18 rainy days (77.31% of the time) (UBOS, 2017b).

3.1.4 Demographics of Wakiso, Mpigi, Mbarara and Isingiro Districts

Wakiso district's population is about 1,997,418 million people as per statistics from the National Population and Housing Census (NPHC) 2014 (UBOS, 2017). Wakiso majorly produces the cooking type bananas at 24,547 metric tons per year followed by cassava at 21,712 metric tons per year (UBOS, 2017a). Mpigi has a population of about 250,548 people as per statistics from the NPHC 2014 (UBOS, 2017a).

Mpigi district has an estimated 299,100 people living in the area, with 150,300 men and 148,800 women (UBOS, 2017a). The average household size is 4 people, and the yearly population growth rate is 0.244%. Seventy-nine percent (79%) of the households in the country make their living from agriculture, which employs the vast bulk of the population. Along with neighboring areas like Wakiso and Mukono, Mpigi District continues to have a significant impact on the economy of Kampala, in the capacity of a partial dormitory. The district supplies Kampala with a sizable labor force in terms of both labor and market (UBOS, 2017b).

Mbarara district is made up of two counties and nineteen rural sub-counties arranged into one municipality (Mbarara Municipality). Mbarara District's economy is based mostly on agriculture, as is the case with most districts in Uganda (UBOS, 2017a). Mbarara District is also known for being one of Uganda's major banana-growing districts (Roz, *et al*., 2017).

During the mid-year population census of 2020, Isingiro population was projected by the population agency to be 34,300 people, 16,800 (49%) of whom were men and 17,500 (51%) were women. According to UBOS, between 2015 and 2020, the population of Isingiro Town Council grew at an average yearly rate of 3.6%. In Isingiro district, banana growing is one of the most well-earning cash crops and food baskets for bananas in Uganda (Lee, 2023). Rugaaga, a sub-county in Isingiro, is considered one of the most productive of cooked bananas in the nation (Rietveld *et al.,* 2021).

3.2 Field Survey and Sample Collection

A total of twenty-five gardens were surveyed. Each farmer from a respective garden was interviewed and the gardens were sampled purposively. Field data was obtained by recording GPS coordinates of every garden where samples were collected using a hand-held GPS.

A structured questionnaire was utilized to gather information about the cultivated bananas as well as demographic data (Appendix II). Throughout the entire field survey, purposive sampling was applied by (Duan & Hoagwood, 2013). Sample size determination was derived from Mead's resource equation (Israel, 1992). The equation for sample size determination was expressed as;

$$E = N - B - T$$

where B is the blocking component, which indicates environmental influences permitted by the design, (minus 1); N is the total number of persons (units employed in the research study); T is the treatment component equal to the number of questions posed (minus 1) or the number of experimental groups (including the control group) that are being employed; The error component's degrees of freedom, or E, will be found in the range of 10 and 20. With this formula, twenty-five (25) samples were arrived at.

Permission was obtained from farmers for destructive sampling based on Kish and Frankel's method (Kish & Frankel, 1974).

In each garden, banana plants were observed for golden leaf syndrome characteristic of Foc.

The symptomatic plant was uprooted pseudo-stem cut using sterile tools transversely and then split longitudinally to look for characteristic necrotic tissue (Fig. 2.1). Plants with clean pseudo-stems were not sampled and presumed to be suffering from potential nutrient deficiencies. A sample collection protocol is described in (Appendix III).

Table 3. 1: Data Collection Instruments that were used in the study

S/N	Data Collection Instrument	Purpose of Instrument
1	Questionnaires	The main purpose was to collect data from the respondents
2	Portable Global Positioning System (GPS) device	To get accurate GPS coordinates of gardens where samples were collected during actual field activities.
3	Digital camera	Was used for still photography of both field and lab activities.
4	Sterile Knives, surgical blades,	Were used for trimming and cutting necrotic tissues into thin slices
5	Hoes and pangas	Was used for uprooting the selected diseased plants
6	Brown paper bags and markers	These were used to store trimmed slices of necrotic tissues later labeled.

Necrotic tissue of the explants was aseptically harvested and wrapped in sterile tissue paper and carefully placed into a paper bag duly labeled with details of the date, time, place (location and coordinates), collector, and purpose of the collection before inserting inside a cool box. The tools used during the exercise as in Table 2 were sterilized using a disinfectant (JIK™) containing 65% Sodium Hypochlorite poured into a 20-liter plastic jerrican with a lid for easy mobility. Other pieces of equipment and tools used in this research are listed in (Appendix VI). Used tools were rinsed in a basin to avoid cross-contamination with other gardens during the field activity. The motor vehicle that was used was always parked away from the main gardens and at times left on the main road (minimum of 50 meters) to avoid picking up pathogens and spreading to far places and farms. After all daily garden visits, the vehicle was disinfected and washed thoroughly. All samples collected were dispatched to the laboratory and placed in a sterile incubator at 24^{o}C (room temperature) for further processing. The resulting wastes from the sampled plants were incinerated to void transmission of infections to other surroundings.

3.2.1 Isolation of pathogens from explants

Collected samples were aseptically inoculated into potato dextrose agar (PDA) (full-strength) medium. This media had been previously prepared autoclaved at 121 °C for 15 mins then on cooling to 40°C, supplemented with streptomycin (antibiotic strength of 0.25 mg/1000 ml. After cooling down, the media was transferred into sterile, 90 x 15 mm disposable plastic Petri dishes, and left overnight to solidify completely. Inoculation was then done on the sterile plates inside a biosafety cabin by aseptically cutting pieces of the necrotic tissue (4 mm) using sterile scalpel blades and introducing them into sterile PDA plates. The plates were incubated at 24 °C until the growth of fungal mycelia was seen. Sub-culturing was then proceeded by further cutting freshly seen mycelia using sterile scalpel blades and placing them into fresh sterile PDA plates. The plates were incubated at 24 °C until fungal mycelial growth covered the entire plate. The resulting pure culture was observed under a microscope for structures related to FOC as described by (Nelson *et al*., 2006; Ploetz, 2006). Cultures that showed FOC-like chlamydospores and pigmentation related to FOC were preserved on PDA slants at 4 °C and all resulting wastes of experiments were autoclaved in the laboratory for proper decontamination and to prevent the spread of any potential pathogens to surrounding environments and gardens.

3.3 Identification of the pathogen causing the golden leaf syndrome on EAHB

Two methods of identification were employed in this study, namely; Direct amplification of TR4 genes using PCR and Microscopic Observation.

3.3.2 DNA extraction from isolates

The CTAB technique was utilized to extract DNA from the fungal isolates using the procedure described by (Mahuku, 2004). Fresh mycelium 150 mg was weighed into a sterilized 1.5 mL

Eppendorf (micro-centrifuge) tube. About 300µL–500µL of TES extraction buffer and acid-washed, sterilized sea sand were added into the tube. Using a hand-held, disposable homogenizer that fits the 1.5 mL microcentrifuge tube, the mycelium was macerated for 40 seconds. Following a 30-second vortex, 200 µL of TES extraction buffer containing proteinase K (final concentration of 50 µg/mL) was added to the samples. The centrifuge tube contents were thoroughly mixed using a vortex machine. The tubes were incubated in a water bath at 65°C for 30 minutes, thereafter, 250µL of 7.5 M cold ammonium acetate was added. The samples were vortexed and allowed to stand on ice for ten minutes. The samples were centrifuged for 15 minutes at 13400 RPM. The supernatant was transferred to fresh tubes and to it was added 500µL of ice-cold isopropanol and incubated at -20^{o}C for 20 minutes. To pellet the DNA, the tubes were centrifuged for 10 minutes at 13400 RPM. The supernatant was poured off, 800µL of cold 70% ethanol was used to wash the resultant DNA pellet, and it was centrifuged for an additional three minutes. For 10 to 15 minutes, the tubes were inverted on sterile, clean paper towels to allow the DNA to air dry. To dissolve the pelleted DNA, 50µL of double distilled water was added and left overnight to dissolve the DNA at 4^{o}C. Extracted DNA from all isolates was stored at -20 °C for PCR. DNA extraction protocol is elaborated in (Appendix VII).

3.3.3 DNA quality analysis

3.3.3.1 Agarose Gel Preparation

To analyze visually the quality of the crude DNA extracts, agarose gel (1%) was freshly prepared by dissolving 0.5g of agarose in 50 ml of 1X TAE buffer in a 250 mL flask. The flask was covered with a cotton wool plug and microwaved for 2 minutes while checking visually to see that all the agarose had melted. The gel was allowed to cool to 60°C on the bench and then 0.5 µg/ml (5µl) ethidium bromide was added to it before pouring into the gel tray. The agarose

gel, combs were placed inside the tray, and pouring was done slowly to avoid bubbles. The gel was left to set for 30 min. On cooling and setting, the comb was removed after loading enough 1X TAE buffer into the buffer chamber to cover the top surface of the gel while inside the electrophoresis tank.

3.3.3.2 Genomic DNA electrophoresis.

The gel was loaded with extracted DNA by adding 2µl of loading buffer to 10 µl/ (10 ng) of the sample. The power supply unit was set at 80V, 400 mV for 45 mins. The gel was stopped when bromophenol blue had run ¾ of its length. The gel was viewed under UV light, and a photo was captured using a digital camera.

In terms of purity (RNA contamination runs as a diffuse smear at the bottom of the gel) and integrity (size range). The quality of good genomic DNA was observed when bright bands formed with uniform molecular weights and band size.

3.3.3.3 Direct amplification of TR4 genes using PCR

Housekeeping primers EF1-5'ATGGGTAAGGARGACAAGA-3' (19mer) and EF2-5'GGARGTACCAGTSATCATGT-3' (20mer) were used to determine the quality of the extracted DNA for PCR amplification and diagnosis.

After the DNA quality check was conducted, a diagnostic PCR was conducted using the Foc-TR4 primer genes to identify the pathogens and produced a predicted 463-bp amplicon that was confirmed by gel electrophoresis. The sections of these primer genes used were FocTR4-R 5'-GCCAGGACTGCCTCGTGA-3' (18mer) and FocTR4 - 5'CACGTTTAAGGTGCCATGAGAG-3' (22mer) that were reported to be specific for FOC TR4 (Dita *et al*., 2010). The components of the PCR reaction were 10 ng of DNA, 200 µmoles of each primer, and PCR 1X Master Mix. Double-distilled water was used to top off the reaction

to 20 µl. The PCR cycle conditions comprised; five minutes of initial denaturation at 95 °C followed by 30 cycles of one-minute denaturation at 95 °C, one-minute primer annealing at 64 °C, one-minute primer extension at 72 °C, and a five-minute final extension at 72 °C. The amplicons were separated on 1.5 percent agarose gel run at 80 V for 45 minutes. The gel was examined in a UV light source and captured digitally with a camera (Appendix VIII). PCR results were used to determine which cultures should be maintained for further study.

3.3.3.4 Preparation of PCR products for Sequencing

Selected PCR amplicons were purified using the ethanol precipitation method Ramaciotti Center for Genomics (RAMAC) (RAMAC, 2019). Briefly, the PCR product was precipitated using absolute ethanol in a tenth of the initial volume (10x) to concentrate it in vitro as guided in Incubation was then done for 1.5 hours at -20°C followed by centrifuging for 10 mins at a maximum speed of 14 000 rpm. Washing was done three times with 70% ethanol. The dried pellets were shipped to the sequencing facility at Macrogen Europe in Amsterdam using a courier. The amplicons were sequenced using the Sanger method (RAMAC, 2019). Results were transmitted electronically inform of (“. ab1” and “.txt” files). Assembling and editing were done using Mega 11 software (Tamura *et al*., 2011).

3.3.3.5 Molecular identification of the pathogen

To establish the identity of the causative agent at the species level, DNA sequences were subsequently blasted through the National Center for Biotechnology Information website (https://www.ncbi.nlm.gov). The relationship between the isolates and other published sequences from the Gen Bank was clarified by constructing a phylogenetic tree using MEGA 11 software. By applying the Maximum Likelihood method and the Tamura-Nei model, the evolutionary history of isolates was inferred. To automatically build the first tree or trees for

the heuristic search, the Neighbor-Join and Bio-NJ algorithms were used to a matrix of pairwise distances estimated with the Tamura-Nei model (Tamura *et al*., 2011).

3.4.4 Pathogenicity testing of the fungal isolates

3.4.4.1 Soil sterilization

Black soil (two-wheel barrows) was harvested from the Kyambogo University Demonstration farm. The soil was spread on tarpaulin of 6 × 3 feet for 5 hours in sunlight. After drying, the soil was graded and sorted using a 12mm stainless screen to remove non-uniform particles from the soil such as roots, large stones, and plastics. The soil was prepared for sterilization by stacking halfway in woven sacks made of sisal fibers. The soil was sterilized based on a method by (Wolf & Skipper, 2018). The sacks were loaded into a precleaned soil steaming tank. The soil was sterilized for 6 straight hours and allowed to cool overnight without opening or unloading.

3.4.4.2 Selection of Plantlets for Screening

Four-month-old cooking-type post-hardened tissue culture banana plantlets (cultivars Mpologoma, Mbwazilume, and Kibuzi) and one susceptible variety (Sukali Ndizi) were acquired from the laboratory of Royal Trees and Nurseries, situated in the Kenjojo district in Uganda. The plantlets were carefully transported to a greenhouse at Kyambogo University Faculty of Agriculture. The plantlets were repotted into five-liter (5l) plastic buckets containing the sterilized soil. The experiment was conducted in a screen house at room temperature and relative humidity of 70–80%. Before the inoculation, the plants were maintained in the screen house by drenching with 200ml of tap water per pot every day. A screening protocol was developed as described in (Appendix IX).

3.4.4.3 Inoculum production

To produce inoculum, mung bean broth was prepared in flasks of 100 ml. Two grams of mung beans were placed in 500ml of distilled water. Autoclaving was done for 1 hour at 121 °C and allowed to cool before being distributed into sterile 100ml flasks. The now-sterile mung-bean broth was inoculated with newly grown Fusarium isolates from PDA plates (6 days old) of sample strains in accordance to (García-Bastidas *et al*., 2019). After five days of incubation at 25 °C and 150 rpm on an orbital shaker incubator, the concentration was adjusted to 1×10^6 chlamydospores per ml in the flasks.

3.4.4.4 Microscopic Observation

PDA plates containing fully grown sporulating isolates were selected. Flasks containing 100 mL of mung beans broth each prepared initially by weighing two grams of mung beans and placing them in 500ml of distilled water. Autoclaving was done for 1 hour and allowed to cool before being distributed in sterile 100ml flasks. The fully and freshly grown Fusarium from PDA plates (5-7 days old) of sample strains were inoculated into the now sterile mung-bean broth according to (García-Bastidas *et al*., 2019) and after three days were observed microscopically for Foc-related cellar structures by use of oil emersion microscopes at x250 and x100 magnifications, with no staining agent used (Kalman *et al*., 2020). Identified cultures were further confirmed by PCR. Resultant identified isolates were preserved on PDA agar slants at 4 °C. All waste materials were destroyed by incineration and autoclaving to avoid any potential transmission of the pathogen to surrounding places.

3.4.4.5 Quantification of spores

After five days, a hemocytometer was used to count the number of spores that had grown in the mung bean broth (Abcam, 2015). Trypan Blue solution was added to the spore suspension

at a ratio of 1:5, or 1 milliliter of Trypan Blue for every 5 milliliters of culture media, to determine the number of viable cells per milliliter. After loading the mixture onto the hemocytometer, a compound light microscope was used to count the spores. The average number of cells in each of the 16 corner square sets was recorded for each count, and this number was then multiplied by 10,000 (10^4). To account for the 1:5 dilution ratio of the trypan blue additive, the result was additionally multiplied by 5. The number of viable cells/mL in the initial cell suspension was the end figure. Ultimately, the concentration of 1×10^6 spores per milliliter was adjusted. To determine the appropriate spore concentration, the formula $C_1V_1=C_2V_2$ was utilized.

3.4.4.6 Inoculation procedure

The roots of the selected plants were uniformly injured with a sterile knife at two opposite sides. All EAHB and control banana genotype Sukali Ndizi was set up in three replications. For the negative control plantlets were drenched with 200 ml of tap water while for test plants, each pot of plantlets was drenched with 200 ml of respective inoculum followed by 200 ml of distilled water.

3.4.4.7 Experimental design and management

A completely randomized design (CRD) using a factorial arrangement was used to set up the experiments (Table 3). After being inoculated, plantlets were kept in the greenhouse and watered every day with 200 ml of tap water at a temperature of 25 °C and a relative humidity of 70–80%. At the screen house, the potted plants were maintained for 84 days. Pictorials of the experimental setup are illustrated in (Appendix X)

Table 3.2: Layout of the Completely Randomized Design setup that was used.

RANDOMISED TREATMENTS*				
B^{II}	A^{II}	C^{I}	A^{IV}	D^{III}
D^{II}	B^{IV}	A^{III}	D^{I}	C^{III}
A^{I}	B^{C}	C^{II}	A^{IV}	A^{C}
C^{C}	C^{IV}	D^{IV}	B^{IV}	C^{II}
D^{C}	B^{II}	C^{C}	A^{II}	A^{II}
B^{I}	A^{I}	D^{I}	B^{IV}	B^{C}
C^{I}	A^{III}	C^{III}	C^{IV}	D^{II}
A^{III}	C^{II}	A^{C}	C^{IV}	B^{I}
C^{I}	B^{C}	D^{C}	B^{II}	B^{III}
D^{I}	A^{I}	D^{III}	B^{III}	B^{I}
B^{III}	D^{II}	D^{III}	D^{IV}	D^{C}
A^{IV}	C^{C}	A^{C}	D^{IV}	C^{III}

*__Varieties__: A = Mbwazirume, B = Mpologoma, C = Kibuzi, D = Sukali Ndizi; **Fungal isolates**: I = MPO-WAK-01, II = NDI-WAK-13, III = KIV-MPIG-23, IV = MFU-MPIG-24, C = Control

3.4.4.8 Pathogenicity Evaluation

The rhizome discoloration index, was used to rate the disease severity as described in (Table 5). After uprooting and splitting the rhizomes longitudinally, discoloration and comb were graded accordingly to the scale described by (Arinaitwe *et al*., 2019). Score 1 means there is no discoloration visible, 2 means there are a few spots on isolated points, 3 means there is 1/3 discoloration, 4 means there is 1/3 to 2/3 discoloration, and 5 means there is 90% discoloration throughout the inner rhizome. The soil and plant components were destroyed in an incinerator after the data was collected.

3.4.4.9 Statistical Analysis

3.4.5.0 Survey Data Analysis

Table 3.3: Interpretation of DSI from RDI in resistance groups (Arinaitwe *et al*., 2019)

DSI (RDI)	Translation
1	Highly resistant (HR)
2	Resistant (R)
3-4	Susceptible (S)
5	Highly susceptible (HS)

Survey data obtained from the structured questionnaire used in the field was analyzed using SPSS software ver.27.0 and Microsoft Excel 2021 to analyze the descriptive statistical significance in data such as P-values and Chi-square values amongst others.

3.4.5.1 Experimental Data Analysis

Results of Rhizome Disease Index (RDI) data obtained during screening were analyzed using data analysis software, Graph Pad Prism version 10 and SAS ver. 9.4M7.

Different banana cultivars' different degrees of resistance to the Fusarium isolates under evaluation in the screen house were computed using a Two-Way Analysis of Variance (ANOVA). Turkey’s studentized range test was applied at an alpha level of 5% where significant differences between means were observed.

3.4.5.2 Quality Control and Quality Assurance

Following the completion and termination of screening experiments in the screening house, all leftover material including; old isolate plates, and clean control plantlets were decontaminated by incineration, and those used in the laboratory by autoclaving (Appendix XI). The workbenches were potted plants once sat were disinfected and cleaned with 65% sodium hypochlorite solution. The used soils from the experiment were decontaminated by sterilizing in the soil steaming tank for 6 hours and left overnight to cool before unloading.

CHAPTER FOUR

RESULTS

4.1 Determine the prevalence of golden yellow-leaf syndrome on EAHB in the selected study districts of Uganda

This study was undertaken to investigate the causative agent of the golden yellow leaf syndrome on EAHB that had been severally reported and observed in several districts of Uganda. The map of Uganda shows the selected study districts together with actual surveyed gardens and their georeferenced GPS coordinates (Fig. 4.10).

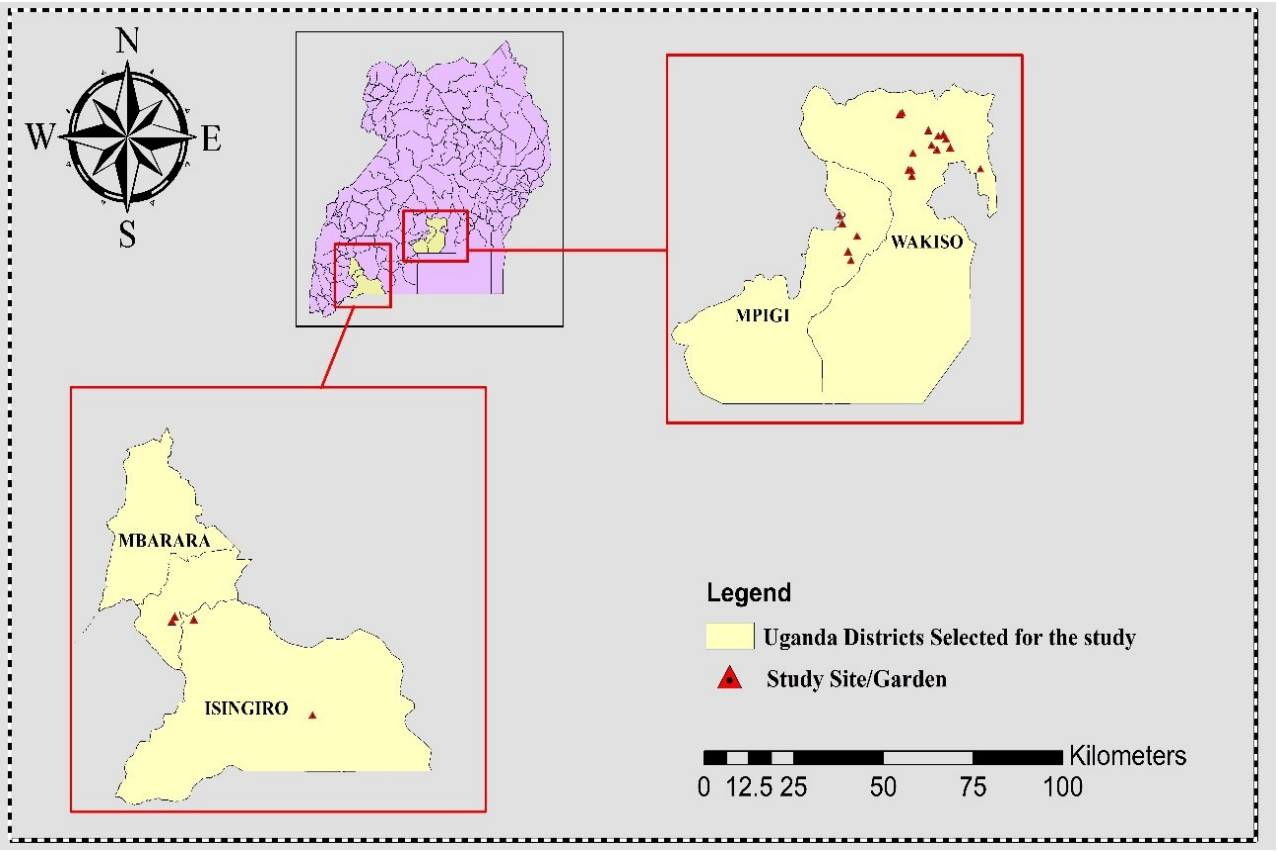

Figure 4. 10: A map of Uganda showing the selected districts of Wakiso, Mpigi, Mbarara, and Isingiro where samples were collected.

4.1.1 Demographic information results

Samples were collected from Wakiso (60%), followed by Mpigi 20%, Isingiro 12% and Mbarara 8% in a total of 25 gardens. Pictorials of the field survey are captured in (Appendix IV). In regards to the most affected banana variety noted by all farmers interviewed, Sukali ndizi was noted to be more prone to suffer golden yellowing of banana leaves than other varieties planted by most farmers. This was the key reason most farmers suggested they avoided planting them on a large scale to avoid losses.

Regarding awareness of the golden yellow disease, most respondents (88%) had seen the golden yellowing of leaves in their banana gardens. They were not aware of what disease this was how it spreads or how it should be controlled from spreading. Only a few of the respondents (12%) had not seen the golden yellow leaf syndrome in their banana gardens. The Chi-square computed was X^2=0.518 and P=0.0874 (Fig. 4.11).

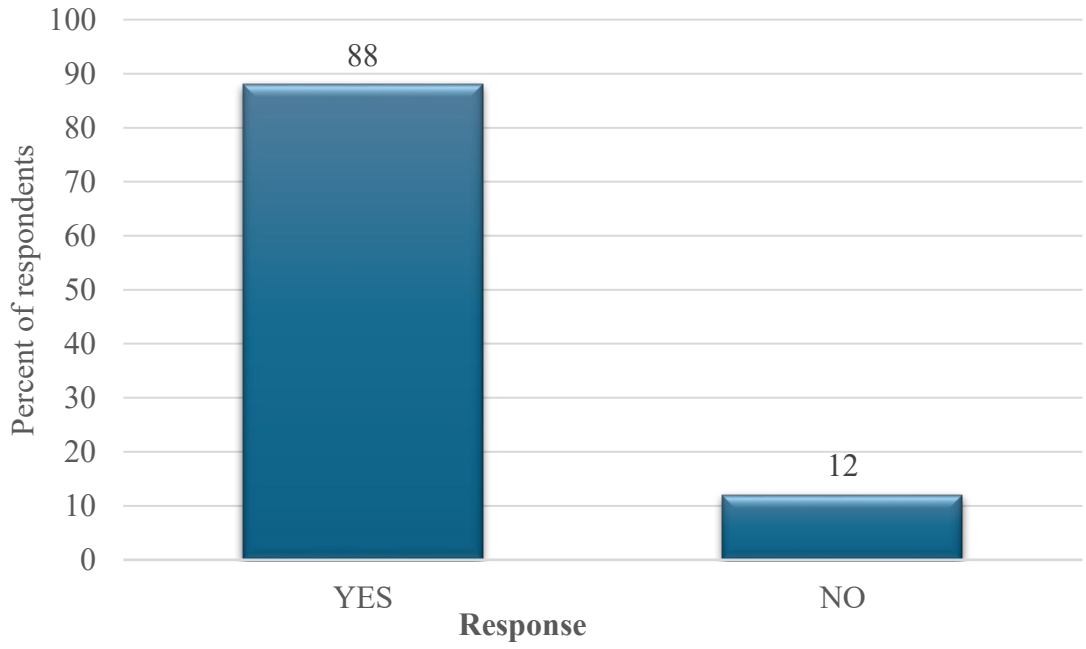

Figure 4.11: Response by farmers if they had ever seen symptoms of the yellow golden leaf syndrome on EAHBs in their gardens.

Regarding farmer' knowledgeability about how long they had sighted the signs and symptoms of Foc-related disease in their banana gardens, they indicated that the sightings varied from 1 year which was the lowest disease prevalence observation to many years. Farmers in Wakiso, Mpigi, and Isingiro specifically reported seeing the highest prevalence of yellow-leaf symptoms on the EAHBs during the preceding two years. The Chi-square computed was X^2=0.366 (Fig. 4.12).

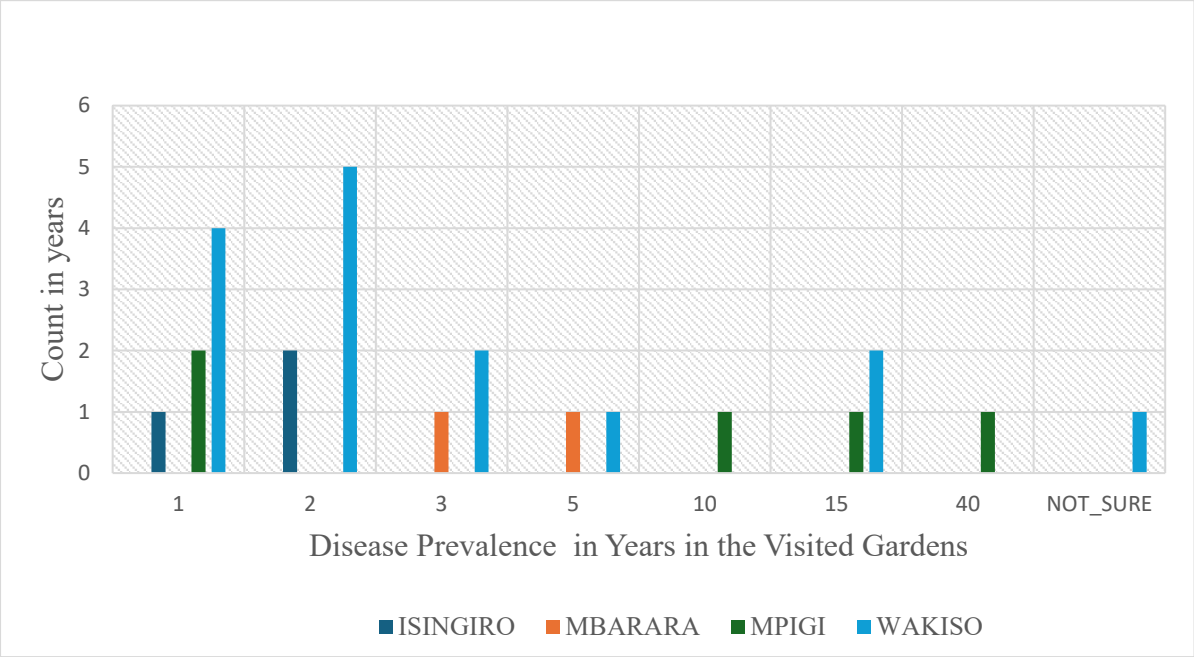

Figure 4.12: The period within which the farmers first noticed and kept track of the golden-yellow leaf syndrome in their EAHB gardens.

4.2 Identify the causative agent of golden yellow-leaf syndrome on EAHB in the selected districts of Uganda.

4.2.1 Morphological Characterization

Following a seven-day incubation period on the PDA, a total of 48 isolates were obtained (Appendix II). The isolates were examined and categorized into three distinct groups according to their cultural characteristics (Table 4.21).

Table 4.21: Colony characteristic and growth rate of selected Fusarium isolates 15 days after incubating at 25 °C

Group	Colony characteristics on PDA	Growth rate (cm/day)
1	White colony with floccose and sparse mycelia and purple pigmentation	0.52 ± 0.12
2	Cottony with magenta color and floccose mycelia	0.57 ± 0.20
3	Whitish cottony with floccose mycelia	0.47 ± 0.13

A representative isolate was chosen randomly for morphological analysis (KIV-MPIG-23) after 15 days of growth on PDA. The front and back of the plates were covered with whitish cottony floccose mycelia. (Fig. 4.20)

Figure 4.20: Colony characteristic (whitish cottony with floccose and sparse mycelia) of Fusarium isolates III obtained from KIV-MPIG-23, 15 days after plating. Front (A) and back of plates (B) fully grown isolates on PDA after 15 days

Fusarium-related cellular structures of microconidia and macroconidia for fungal isolate KIV-MPIG-23 were examined under a compound-light microscope at x1000 magnification (Fig. 4.21).

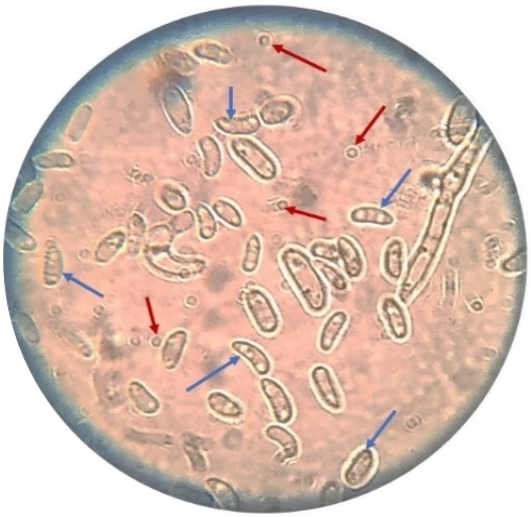

Figure 4.21: Conidial characteristics of the fungal pathogen isolated from the KIV-MPIG-23 under a compound-light microscope at x1000. (Red Arrows-Microspores and Blue Arrows-Macrospores)

4.2.1 Quality Assessment of DNA extracts of Fusarium-like isolates

The quality of extracted DNA of all isolates analyzed using the gel electrophoresis technique showed that they were of desired concentration, undegraded, and of the right molecular weights, suitable for a diagnostic PCR as described by the method in Chapter three (3) subsection 3.3.3.3 shown in (Fig. 4.22).

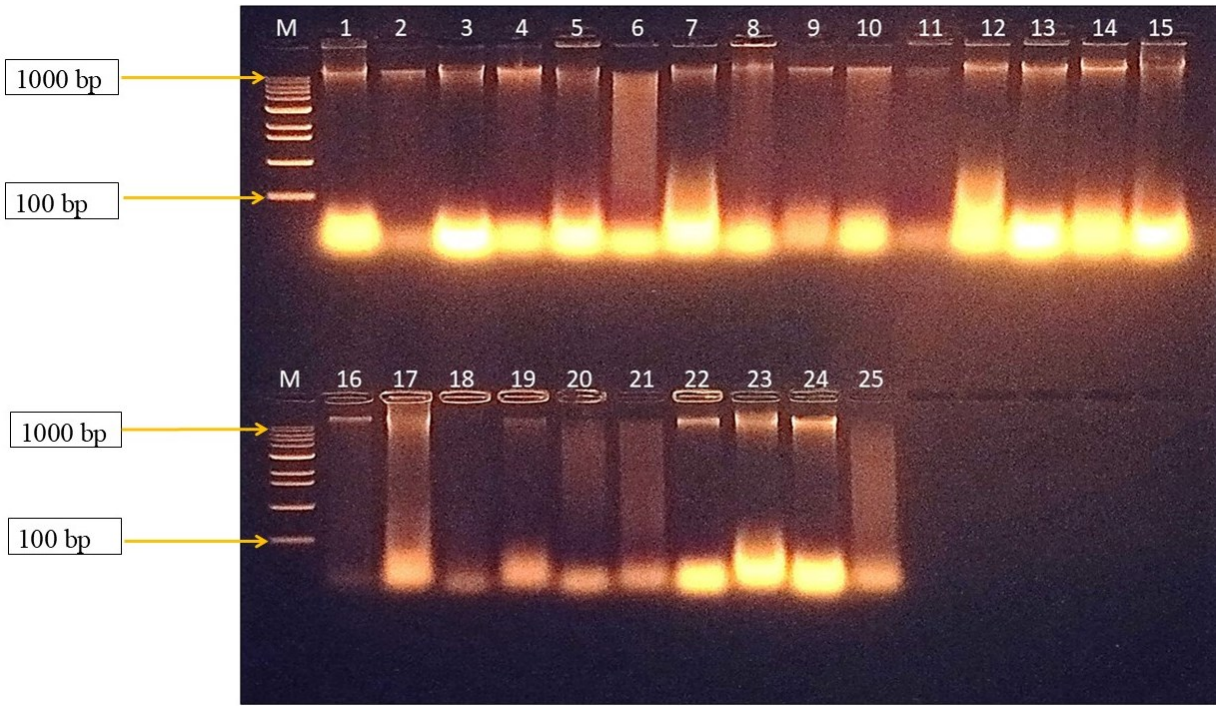

Figure 4.22: Agarose-gel electrophoresis for DNA of 25 isolates with equal masses loaded 10ηg. (M= 100bp Gene Ruler (Direct load Cat. No. D-1030 AccuLadder™ made by BIONEER Korea), 1 to 25 = DNA isolates from different surveyed gardens -Appendix V)

4.2.2 Diagnostic PCR validation

Isolated DNA was amplified using housekeeping primers EF1 and EF2. Of the 25 isolates, DNA extracts of 4 samples did not yield any amplicons with the EF1 and EF2 primers corresponding to conserved regions within the EF1 (elongation factor 1-alpha) and EF2 (elongation factor 2) genes of fungi (Fig. 4.23). For the other 21 isolates, the DNA extracts gave amplicons with EF1 and EF2 at about 680bp. They were therefore advanced to the next step of diagnostic PCR.

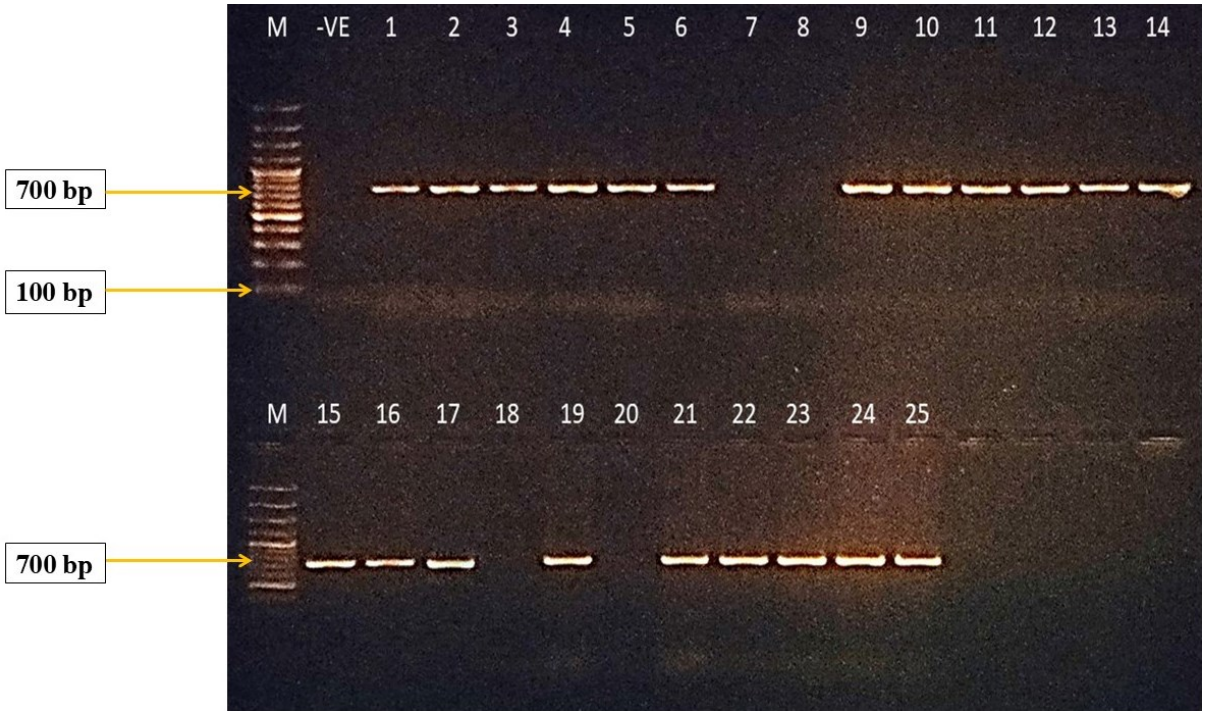

Figure 4.23: PCR outcome for EF1 and EF2 primers on all isolated DNA. (M= 100bp Gene Ruler (Direct load Cat. No. D-1030 AccuLadder™ made by BIONEER Korea), 1 to 25 = isolated DNA from surveyed gardens-Appendix V), -VE= negative control.

4.2.3 Pathogen Identification with Foc -TR4 Primers

Results showed that out of the 21 isolates, six (6) were positive for the 28S - 18S rRNA inter-spacer region giving amplicons with the FocTR4-F and FocTR4-R primers at approximately 460 bp in size (Dita *et al*., 2010) (Fig.4.24). This shows that out of the 21 EF- positive samples, six (6) were positive for Foc TR4, although sample 2 (NKI-WAK-02) amplified at the wrong size (Fig. 4.24). All six isolates were treated as positive for Foc TR4 and subjected to subsequent sequencing.

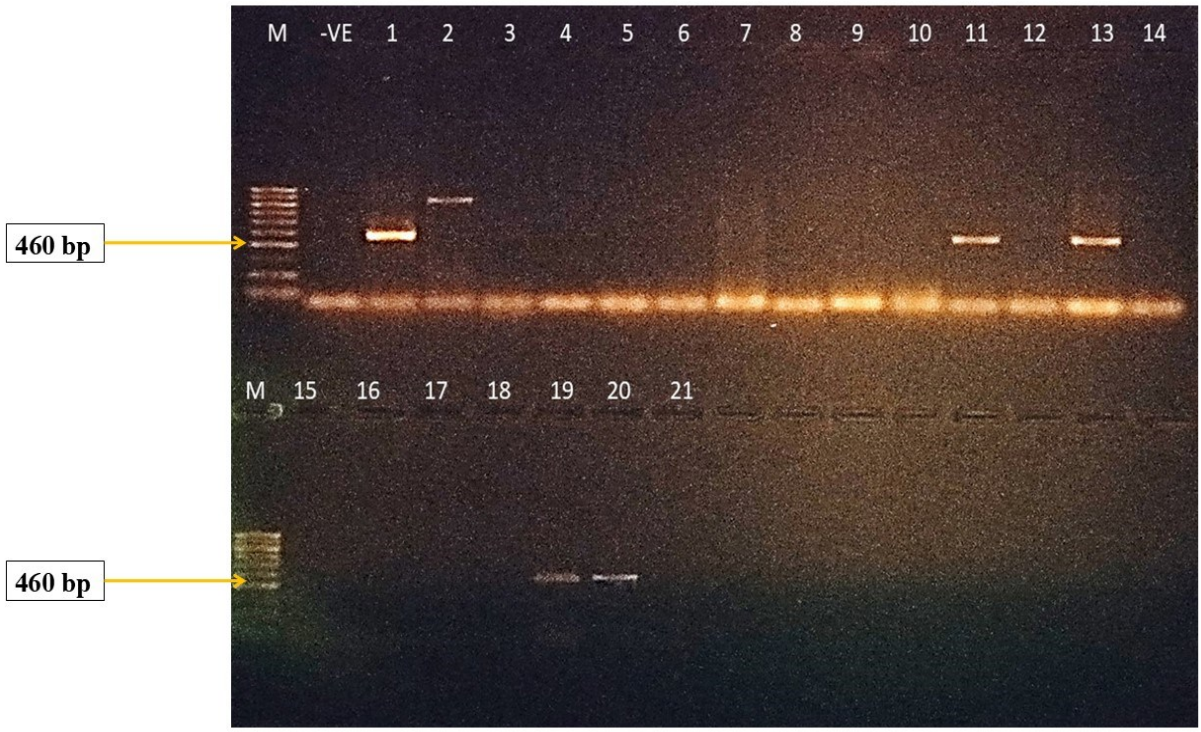

Figure 4.24: PCR outcome for Foc-TR4 primer reactions with the 21 samples successfully amplified giving amplicons with six isolates. PCR outcome was run on 1.5% Agarose-gel and viewed under UV light. (M= 100bp Gene Ruler (Direct load Cat. No. D-1030 AccuLadder™ made by BIONEER Korea),1 to 25 = isolates of DNA from different visited gardens visited-Appendix V), -VE= negative control.

The six (6) positive isolates were reassessed individually with FocTR4-F and FocTR4-R primers. The wells were arranged in order from left to right as; M- 100 bp Gene Ruler, -VE Negative control, 1-MPO-WAK-01, 2-NKI-WAK-02, 3-NDI-WAK-13, 4 MBW-WAK-15, 5-KIV-MPIG-23, 6-NFU-MPIG-24. These final positive isolates were shipped for DNA sequencing (Fig. 4.25).

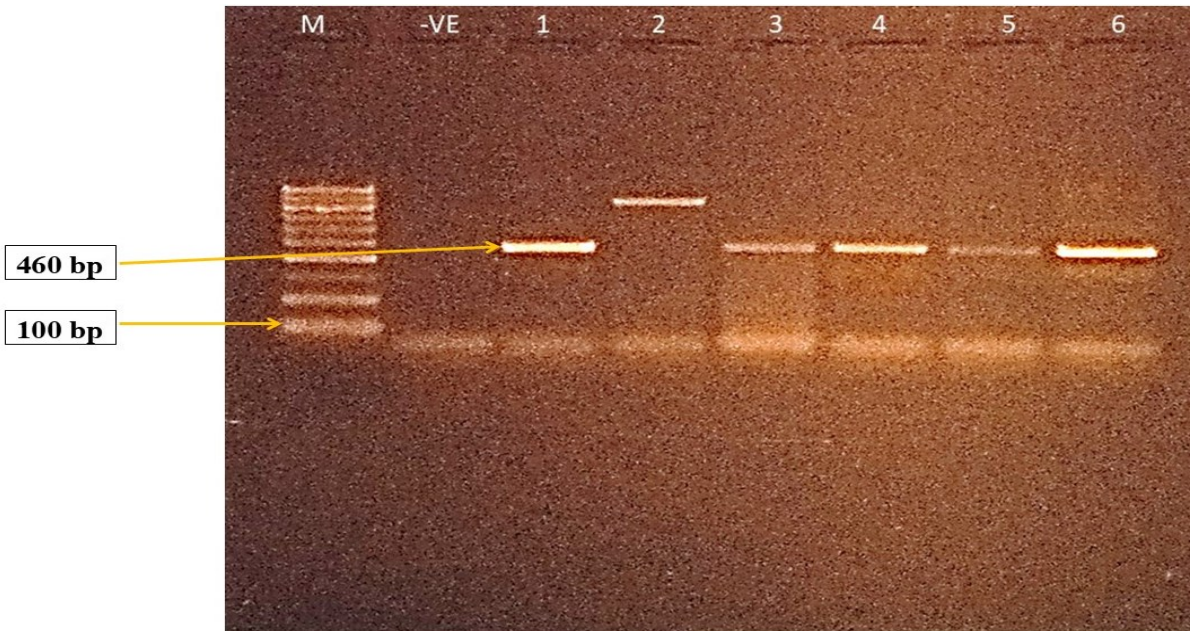

Figure 4.25: Isolated DNA positive with Foc-TR4 primers. (M= 100bp Gene Ruler (Direct load Cat. No. D-1030 AccuLadder™ made by BIONEER Korea), 1 to 6 = 1-MPO-WAK-01, 2-NKI-WAK-02, 3-NDI-WAK-13, 4-MBW-WAK-15, 5-KIV-MPIG-23, 6-NFU-MPIG-24), -VE= negative control.

4.2.4 DNA sequencing results

A nucleotide blast search of the sequences showed that four of the sequences were FON. One was *Fusarium fujikuroi* and the other Foc Race 1. *Fusarium fujikuroi* (NKI-WAK-02) was the one observed to have amplified at a wrong size. The confirmed identities have been recorded in (Table 4.22).

Table 4.22: List and identities of sequenced isolates

Isolate No.	Isolate Sample code	Identity of the pathogen	Max Score	Query Cover	Percentage Identity	Accession No. of related Subject from NCBI
1	MPO-WAK-01	*Fusarium oxysporum f. sp. niveum* strain 110407.1-1. B2-F8 28S ribosomal RNA gene	317	82%	77.32%	MN428314.1 (FON)
			317	82%	77.32%	MN428313.1 (FON)
2	NKI-WAK-02	*Fusarium fujikuroi* strain	133	44%	76.24%	CP023093.1
			133	44%	76.24%	CP023105.1 (*Fusarium fujikuroi*)
3	NDI-WAK-13	*Fusarium oxysporum f. sp. cubense* isolate Race 1	682	95%	97.49%	KU577995.1 (FOC RACE 1)
4	MBW-WAK-15	*Fusarium oxysporum* f. sp. *niveum*	228	42%	88.83%	KJ159632.1 (FON)
5	KIV-MPIG-23	*Fusarium oxysporum* f. sp. *niveum*	414	76%	92.18%	MN428302.1 (FON)
			414	76%	92.18%	MN428300.1 (FON)
6	NFU-MPIG-24	*Fusarium oxysporum f. sp. niveum* strain 150513 28S ribosomal RNA gene	708	93%	97.58%	MN428302.1 (FON)
			708	93%	97.58%	MN428300.1 (FON)

4.2.5 A phylogenetic tree of the identified fungal isolates

A total of five (5) reported TR4 sequences from the GenBank were retrieved for purposes of comparison to other isolates obtained in the study. After multiple sequence alignment and trimming, there were 436 positions in the final dataset. The tree topology selected was the one with the highest log likelihood value. The number of substitutions made at each location was used to express the branch lengths of the scaled-up tree. (Fig. 4.26).

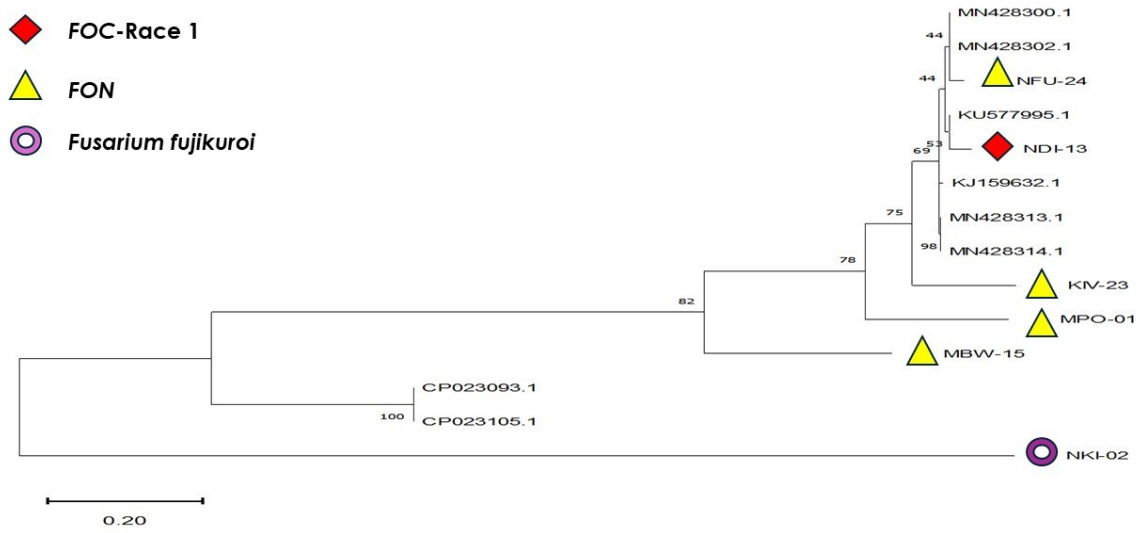

Figure 4.26: A phylogenetic tree elucidating the relationship between the identified isolates and Foc-TR4 from published sequences in the Gen Bank. The top clade contains TR4 isolates from the GenBank represented by Accession Numbers. Triangles represent FON isolated in this study. NDI-13 is Fusarium *oxysporum f. sp. cubense* Race 1 and NKI-02-*Fusarium fujikuroi.*

4.3 To determine the potential sources of resistance to the causative agent of golden yellow-leaf syndrome amongst popular EAHB cultivars

4.3.1 Data analysis

The rhizome discoloration index data was subjected to the Analysis of variance (Two-way ANOVA). The dataset was initially tested for normality and homogeneity of variance, but the untransformed data was used. Where significant differences were observed, the means were separated using Tukey's studentized range test at an alpha level of 5% (Table 4.30).

Table 4.30: The susceptibility of the East African Highland banana cultivars to different Fusarium fungal isolates, eighty-four (84) days after inoculation.

Fungal treatments	Banana varieties							
	Kibuzi		Mbwazirume		Mpologoma		Sukali Ndizi	
Control	1.0 ± 0.0	B	1.0 ± 0.0	b	1.0 ± 0.0	b	1.0 ± 0.0	c
MPO-WAK-01	1.3 ± 0.3	B	1.0 ± 0.0	b	1.0 ± 0.0	b	4.0 ± 0.5	ab
NDI-WAK-13	2.7 ± 0.9	Ab	3.0 ± 0.6	ab	1.3 ± 0.3	b	4.3 ± 0.3	a
KIV-MPIG-23	3.7 ± 0.3	a	3.7 ± 0.7	a	3.3 ± 0.3	a	4.3 ± 0.3	a
NFU-MPIG-24	1.0 ± 0.0	b	2.7 ± 0.7	ab	1.0 ± 0.0	b	2.3 ± 0.3	bc

For each banana variety, means in a column followed by similar letters are not significantly different at a 5% confidence level, Tukey's studentized range test (Table 4.30).

4.3.2 Rhizome discoloration index

Results of the analysis for rhizome discoloration index revealed that the EAHB infection by the pathogen was dependent on the isolate ($F=29.41$, $df=4$, $P<0.0001$) and cultivar ($F=16.24$, $df=3$, $P<0.0001$). Overall, isolate KIV-MPIG-23 exhibited the highest discoloration index followed by isolate NDI-WAK-13 and Isolate MPO-WAK-01 (Fig. 4.31). On the other hand, Sukali Ndizi exhibited the highest susceptibility to the pathogens with a discoloration index that was statistically higher than the other cultivars, while Mpologoma recorded the least index (Fig. 4.31).

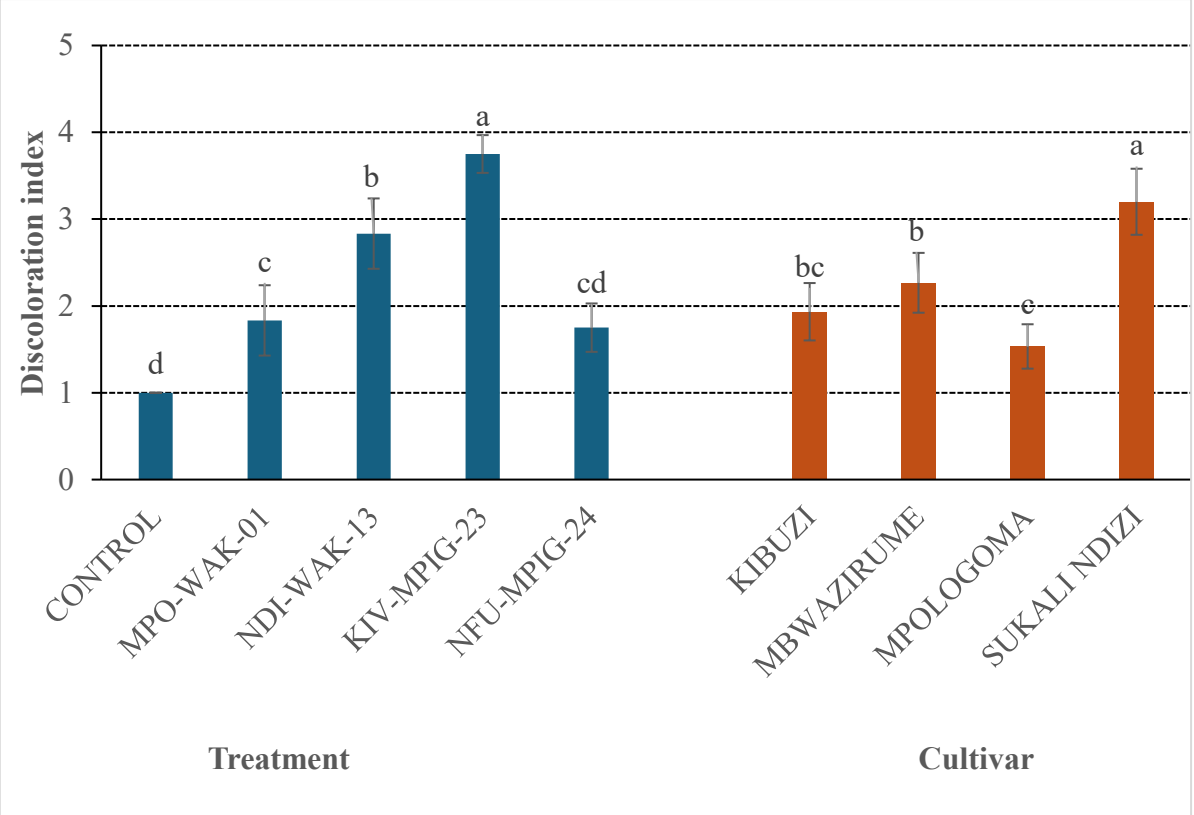

Figure 4.31: The pathogenicity of isolates on the EAHB and Sukali Ndizi. Standard errors represent the standard error of the mean. For each category, bars were followed by similar letters that are not significantly different at a 5% confidence level, using Tukey's studentized range test.

However, there was an interaction between the fungal treatments and the banana cultivars (F=3.29, df=12, P=0.0022) whereby the rhizome discoloration index attributed to the different pathogens was noted to depend on the cultivar. For the Kibuzi banana cultivar, the discoloration index caused by Isolate KIV-MPIG-23(FON) was significantly higher than that of the water control treatment as well as that of the Fusarium Isolates MPO-WAK-01 (FON) and NFU-MPIG-24 (FON) pathogens (F=7.06, df=4, P=0.0058). Equally for Mbwazirume, Isolate KIV-MPIG-23(FON) caused the highest discoloration index that was significantly higher than that of Isolate MPO-WAK-01 (FON), and the control treatment (F=6.0, df=4, P=0.01). For the Mpologoma cultivar, the water control treatment and the pathogen isolate MPO-WAK-01 (FON), NDI-WAK-13 (FOC) and KIV-MPIG-23(FON) registered similar susceptibility and

discoloration index that was significantly higher than that caused by isolate KIV-MPIG-23(FON) (F=23.35, df=4, P<0.0001). However, for the control cultivar, Sukali Ndizi, the discoloration indices attributed to Isolates NDI-WAK-13 (FOC) and KIV-MPIG-23(FON) were statistically similar but significantly different from the indices attributed to the control and the pathogenic Isolate NFU-MPIG-24 (FON) treatments (F=16,5, df=4, P=0.0002).

Based on results from the characterized pathogen KIV-MPIG-23(FON) screening of all plant varieties, the disease incidence was noted to be most virulent to all varieties, leading to cases of colored rhizomes among the plantlets (Fig. 4.32). As observed, it was noted that isolate KIV-MPIG-23 (FON) caused the highest disease index on susceptible control Sukali Ndizi and a relative tolerance in Mpologoma variety with slightly fewer affected discolored rhizomes (Fig. 4.32).

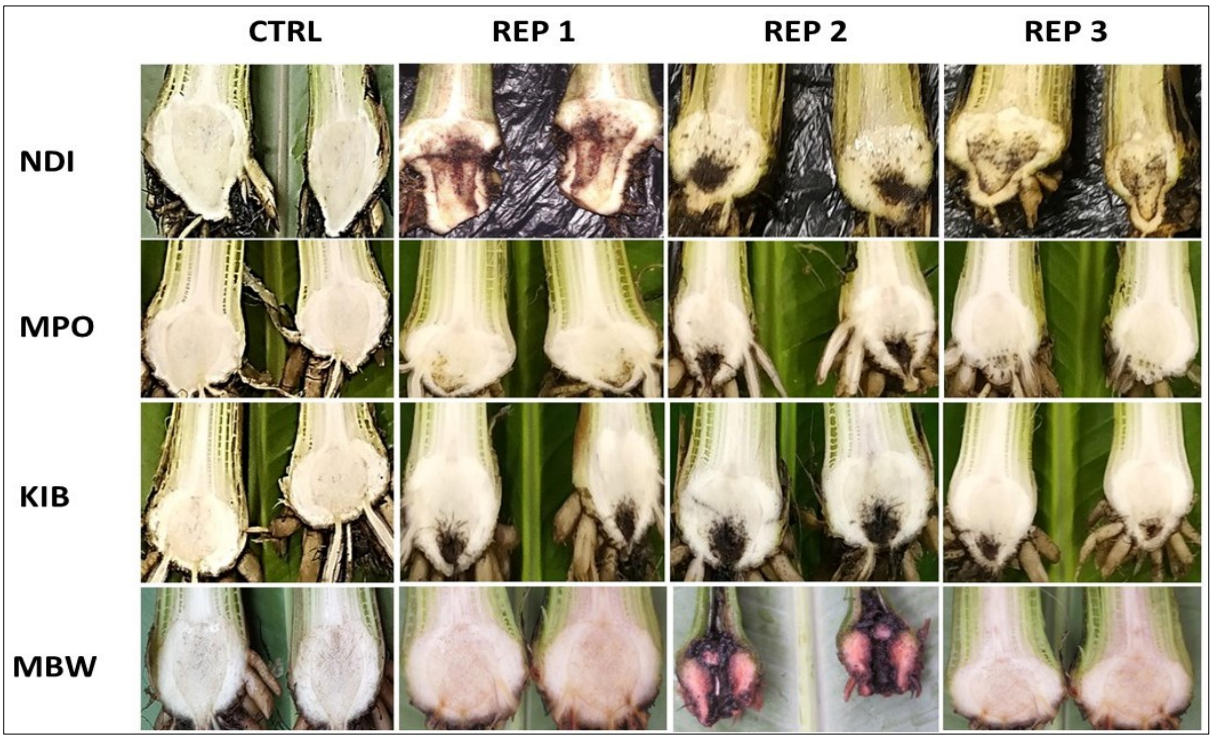

Figure 4.32: Pathogenicity screening of Sukali Ndizi and three EAHB cultivars (MPO – Mpologoma; KIB –Kibuzi; MBW -Mbwazirume) against isolate KIV-MPIG-23(FON)

All Plantlets (NDI – Sukali Ndizi; MPO –Mpologoma; KIB –Kibuzi; MBW -Mbwazirume) that were screened with isolate KIV-MPIG-23 (FON) were noted to suffer adverse symptoms around the psuedostems and leaves. Cracking of psuedostems and golden yellowing of leaves was a predominant symptom observed (Fig. 4.33).

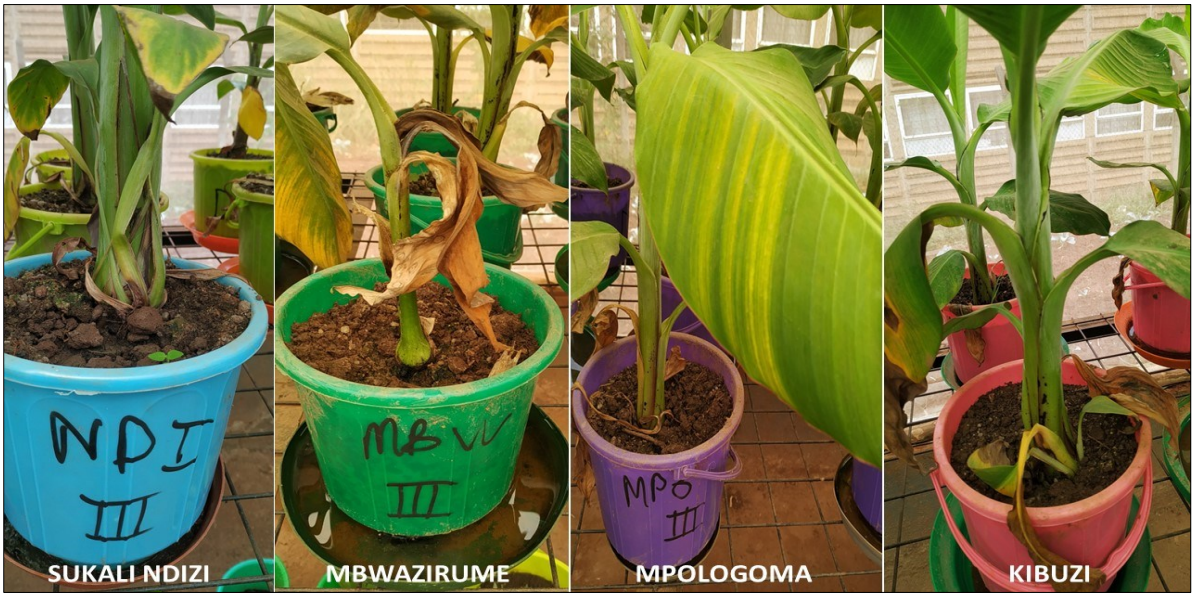

Figure 4.33: Disease severity observed on leaves and stems of; all Plantlets (NDI – Sukali Ndizi; MBW -Mbwazirume MPO –Mpologoma and KIB –Kibuzi;), after screening to isolate KIV-MPIG-23 bearing a FON strain.

4.4.1 Confirmation of Koch's Postulates

Using the EAHB variety Mbwazirume infected plantlets and control as a case study to confirm this hypothesis, isolates of pathogens were obtained from necrotic tissue of the severely affected respective treatments by inoculating in PDA and after 15 days when plates were covered with suspected Fusarium growth, DNA was extracted from and confirmed using specific FOC-TR4 primers using PCR (Fig. 4.34). The methods are described in Chapter 3, sections 3.3.2 and 3.3.4. This affirms that what was initially introduced through inoculation into the plantlets is what caused the disease symptoms observed in the screen house experiments (section 4.3.2, Fig. 4.32 and Fig. 4.33).

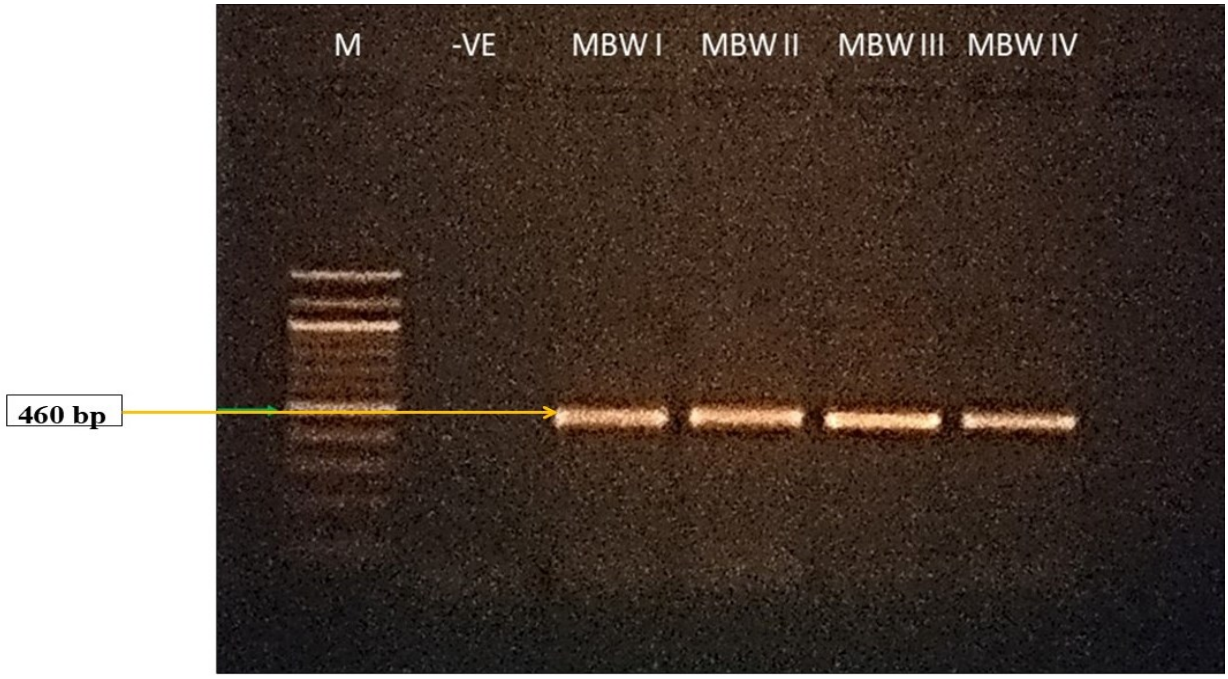

Figure 4.34: Agarose gel of PCR products of four isolates DNA tested against Foc-TR4 primers. (M= 100bp Gene Ruler (M= 100bp Gene Ruler (Direct load Cat. No. D-1030 AccuLadder™ made by BIONEER Korea), 1 to 4, 1=MPO-WAK-01, 2= NDI-WAK-13, 3=KIV-MPIG-23, 4-NFU-MPIG-24, -VE= negative control).

CHAPTER FIVE

DISCUSSION

5.1 Determine the prevalence of golden yellow-leaf syndrome on EAHB in the selected study districts of Uganda

The cause of the golden yellow leaf syndrome in cooking bananas was investigated by visiting and sampling 25 infected gardens in Wakiso (60%), Mpigi (20%), Isingiro (12%) and Mbarara (8%). About 40% of sampled gardens were owned by female respondents, whereas 60% were male-owned. According to a case study in Uganda, the male population still dominates farms over the female gender in ownership (Elias *et al*., 2021).

During the field survey, farmers reported the emergence of golden-leaf syndrome more than what they knew many years ago. Particularly, during the previous two years, farmers in Wakiso, Mpigi, Mbarara, and Isingiro reported the highest occurrence of goldens-leaf symptoms on EAHBs and related this occurrence of the disease to be rampant in the rainy seasons. According to earlier research, the primary temperature rise and altered rainfall patterns are two consequences of climate change, which might affect the frequency of pests and diseases and, in turn, affect crop yield. Waterlogging or an abundance of moisture in the soil would make the banana plants more vulnerable to pathogen invasion (Olivares *et al*., 2021). Another finding suggested that *Fusarium* wilt disease was usually more intense in the year's warmer, rainier months (Rodriguez *et al*., 2014).

All farmers interviewed had no prior information about the occurrence of the disease, nor its symptoms and how to control it. However, 88% concurred that they have seen the unfamiliar golden-yellow leaf syndrome in the past 1-2 years on their farms. It is not uncommon for an infectious disease to enter new territories unnoticed. For instance, the recent discovery of Foc

TR4 in the Caribbean and Mozambique has sparked intense worry since it could have a direct impact on the food basket of millions of people in Africa, which is bananas. (Zuo *et al*., 2018). Another case registered for TR4 was on the Island of Mayotte by (Aguayo *et al*., 2021)

During sampling, it was observed that most of the sick banana plants had symptoms related to Foc-pathogen infections of golden yellow leaves and cracked pseudo-stems to having internal reddish-brownish necrotic tissue which related to findings in Ecuador and where similar observations were noted (Magdama *et al*., 2020).

Fully grown isolates on PDA plates underwent keen examination on morphology (front and back of plates) and it was generally noted that most isolates had related whitish-cottony colonies with purple pigmentation and some with magenta color then floccose sparse mycelia and in some cases had only floccose mycelia. These observations were registered in an earlier related finding, where Foc had whitish-cottony colonies with purple pigmentation while some colonies had a magenta color (Rodriguez *et al*., 2014).

On examining the spores of the isolates under a compound-light microscope, reproductive structures such as; macroconidia (basal cells with a foot-like and somewhat falcate form) and microconidia (oval) structures related to *Foc* pathogens. A similar study that was observed by (Rana *et al*., 2020).

5.2 To identify the causative agent of golden yellow-leaf syndrome on EAHB in the selected districts of Uganda.

Out of 21 DNA samples, the findings indicated that 6 were positive for the 28S - 18S rRNA inter-spacer region, yielding amplicons with the FocTR4-F and FocTR4-R primers at a size of about 460 bp when employing a 100 bp gene ruler which is the same as the amplicon size reported by (Dita *et al*., 2010). This result was due to the FocTR4 primers being specific in nature and binding to only DNA with 28S - 18S rRNA inter-spacer regions thus only 6 DNA isolates had these corresponding regions, ruling out the remaining 15 DNA isolates. It is interesting to note that during the time of sampling, plants identified from gardens had common signs and symptoms relating to *Foc*-pathogen disease. However certain cases could have been symptoms arising from other causes such as nutrient deficiency or other diseases such as bacterial wilt as reported by (Segura *et al*., 2018). The sequences of the Foc TR4 positive isolates were identified as; *Fusarium oxysporum f. sp. niveum*, *Fusarium fujikuroi*, *Fusarium oxysporum f. sp. cubense isolate Race 1* strains.

The evolutionary background of *F. oxysporum* is intricate. The phylogenic tree drawn showed that *Fusarium oxysporum f. sp. cubense isolate* Race 1 has a very close relationship with *Foc* TR4 subjects compared with from the GeneBank. In contrast, *Fusarium oxysporum f. sp. niveum* and *Fusarium fujikuroi* had a close ancestral origin with *Foc* TR4 subjects being compared. Studies show that in one way or another, FON attacks its host in watermelon xylem vessels similar to Foc in bananas causing severe harm such as necrosis, chlorosis, and immature death (Rahman *et al*., 2021).

Specific primers for FOC TR4 proposed by Dita *et al*., (2010), amplified non-TR4 pathogens. This un-specificity has been reported in a number of studies and literature states there are four (4) PCR-based molecular techniques for identifying and detecting TR4 4 strains, including the Tropical Race 4 (Foc STR4) and Subtropical Race 4 that cause Fusarium wilt in bananas. As described in the findings by Magdama *et al* (2019), using the known four markers, a total of 302 isolates were screened. For some isolates, pathogenicity tests were conducted, sequence comparison, phylogenetic analyses, and Vegetative Compatibility Group (VCG) testing. Findings showed it is not possible to identify Foc STR4 and TR4 using three of the four markers that were evaluated (Magdama *et al*., 2019).

5.3 To determine the potential sources of resistance to the causative agent of golden yellow-leaf syndrome amongst popular EAHB cultivars.

All four identified isolates of *Fusarium oxysporum f. sp. niveum* and *Fusarium oxysporum f. sp. cubens*e Race 1, were tested for pathogenicity against selected EAHB cultivars; Mpologoma, Kibuzi, Mbwazirume and Sukali Ndizi as a susceptible control in the greenhouse.

Screening results showed that Sukali Ndizi (susceptible control) had no resistance to all isolated pathogens. The highest incidence of disease noted for Sukali Ndizi was against isolate KIV-MPIG-23, causing total necrotic rhizome on this cultivar leading to death of the plantlets during screening. According to earlier research findings, Sukali Ndizi has been and is always used as a screening susceptible control and used in multiple banana pathogen screening experiments (Ndayihanzamaso e*t al*., 2020; Zuo *et al*., 2018). Sukali Ndizi however showed some tolerance to isolated pathogens *Fusarium oxysporum f. sp. niveum*. Accordingly, Sukali Ndizi's susceptibility to all pathogens varied across isolates, with isolate NFU-MPIG-24 (FON) exhibiting less virulence, and isolate KIV-MPIG-23 (FON) registering the highest virulence to

this Sukali Ndizi. It is possible that this pathogenic strain (isolate KIV-MPIG-23-FON) possesses the avirulent genes that are capable of wiping out the entire Sukali Ndizi plantations if its spread is not contained.

Variety Mpologoma demonstrated the highest level of tolerance to all the pathogen isolates, except for isolate KIV-MPIG-23(FON). Previous reports have shown that Mpologoma was tolerant to Foc race 1 but could now be susceptible due to new suspected variants of the disease (Tushemereirwe *et al* ., 2000). Similarly, early findings also revealed that the EAHB was resistant to Foc Race 1 (Diseases Threatening Banana in Uganda) (Gold & Rutherford, 2000).

Kibuzi variety had strong tolerance to the infections in Isolates MPO-WAK-01 (FON), isolate NDI-WAK-13 (FOC), and isolate NFU-MPIG-24 (FON) except isolate KIV-MPIG-23 (FON). According to findings, Kibuzi was earlier found to be resistant to Foc Race 1 and Foc TR4. (García-Bastidas *et al*., 2019; International *et al*., 2014).

Mbwazirume variety showed some tolerance to infections from *Fusarium oxysporum f. sp. niveum* in isolate MPO-WAK-01 (FON) as compared to the control treatment but it was also susceptible to isolates NDI-WAK-13 (FOC), NFU-MPIG-24 (FON) and the highest incidence of disease was noted with isolate KIV-MPIG-23 (FON). Mbwazirume from earlier findings shows that it has resistance to most Foc strains including TR4 (Ndayihanzamaso *et al*., 2020; Zuo *et al*., 2018).

To confirm Koch's postulates, the pathogen was re-isolated from an infected Mbwazirume plant. DNA was extracted from the re-isolated pathogen and confirmed using specific PCR primers for TR4. This affirms that what was initially introduced through inoculation into the

plantlets is what caused the disease symptoms observed in the screening house experiments (Kalman *et al*., 2020).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

FON has for the first time been recorded in Uganda. FON has never been known to affect bananas. EAHB varieties; Mpologoma, Kibuzi, and Mbwazirume have all shown a significant level of susceptibility to *Fusarium oxysporum f. sp. niveum*. This study demonstrated the development of disease symptoms arising from FON infections under screen house conditions.

The deadly banana pathogen Foc-TR4 was not encountered in this study, although this finding does not rule out its absence in Uganda at large since sampling was done on a few studied districts of Wakiso, Mpigi, Mbarara, Isingiro, and in a relatively short time. Many farmers and garden owners in Uganda growing bananas don't know about the deadly pathogen Foc-TR4 and its adverse effects, signs and symptoms, containment, and detection on their farms. TR4-specific primers were not only a unique target to FOC TR4 as they as well amplified FON from the isolates. Hence pathologists need to be keen on the primer sets they intend to use.

6.2 Recommendations

i) The new findings on FONs and EAHBs in Uganda and how the affected banana farmers and other stakeholders can prepare to mitigate this disease need to be published.
ii) Need for development & design of more accurate primers for precise detection of *FOC*-TR4 and other *Fusarium* strains to achieve correct results by plant pathologists.
iii) Literature has noted that FON affects watermelon and now EAHBs, both essential cash crops in Uganda. A wider research survey on mechanisms of spread, hosts, and containment is urgently needed.

6.3 Suggestion for further research

i) There is a need to perform a wider pathogen risk assessment to determine the potential harm of the newly identified FON infections in banana plants.

ii) To improve diagnostic tools, its strongly recommend to take a more comprehensive approach to the research of *F. oxysporum* as a plant pathogen, taking into account the biology and diversity of the species.

iii) There is a need to conduct a wider epidemiological survey and investigation to determine the source of the pathogen, hosts, mechanisms of spread, and pathological effects on affected plants, why it affects xylem vessels mostly.

iv) Provide countermeasures to prevent the infection from spreading to unaffected areas. There is a need to develop effective detection measures for rapid identification of affected areas or plants.

v) There is a need to assess a wider coverage of banana germplasm for sources of resistance that can be used in conventional breeding or genetic engineering.

vi) Further research on the identified pathogen *Fusarium oxysporum f. sp. niveum* is necessary, particularly on the primary host watermelon that is another essential cash crop in Uganda and world at large.

vii) Need for further research on why is the KIV -MPIG-23(FON) more virulent compared to others.

viii) Need for research works on Fusarium wilt fungicides (ZO groups) and agents that can suppress its growth and reproduction.

ix) There is a need to reach out to banana farmers on how best to control the spread of this pathogen FON and FOC as data gathered showed less knowledgeability about them.

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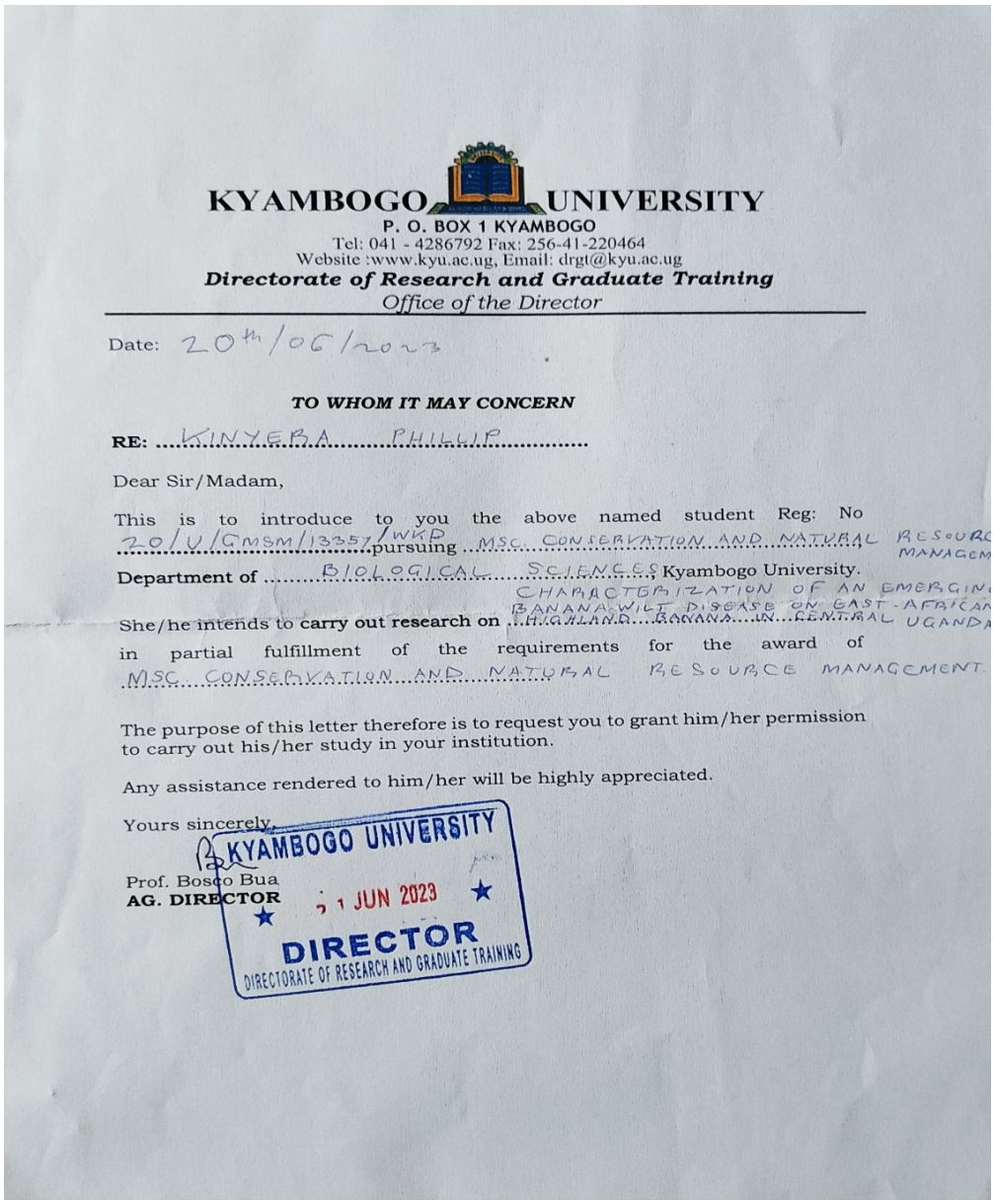
APPENDICES

APPENDIX I: Introductory Letter



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Date: 20th/06/2023

TO WHOM IT MAY CONCERN

RE: KINYERA PHILLIP

Dear Sir/Madam,

This is to introduce to you the above named student Reg: No 20/U/GMSM/13357/WKP pursuing MSC. CONSERVATION AND NATURAL RESOURCE MANAGEM

Department of BIOLOGICAL SCIENCES Kyambogo University.

She/he intends to carry out research on CHARACTERIZATION OF AN EMERGING BANANA WILT DISEASE ON EAST-AFRICAN HIGHLAND BANANA IN CENTRAL UGANDA

in partial fulfillment of the requirements for the award of MSC. CONSERVATION AND NATURAL RESOURCE MANAGEMENT.

The purpose of this letter therefore is to request you to grant him/her permission to carry out his/her study in your institution.

Any assistance rendered to him/her will be highly appreciated.

Yours sincerely,

Prof. Bosco Bua
AG. DIRECTOR

KYAMBOGO UNIVERSITY
1 JUN 2023
DIRECTOR
DIRECTORATE OF RESEARCH AND GRADUATE TRAINING

APPENDIX II: Questionnaire used for field survey

QUESTIONNAIRE FOR FORMAL FIELD SURVEY

SECTION A: Identification

Date..... Questionnaire Number.....
District..... Sub-county.....
Parish..... Village.....
GPS Reading.....N .....E
Enumerator.....

Respondent
Name of farm owner .....
Sex: M □ F □
Age..... (Years)

Highest level of Education:
1. No formal education □
2. Primary □
3. Secondary □
4. Certificate/Diploma □
5. Degree □
6. Post Graduate Degree □

Marital Status
1. Single □
2. Married □
3. Widow/widower □
4. Separated/Divorced □
5. Other (specify) ……

Respondent's phone number:
……

Primary occupation:
1. crop production; □
2. Livestock keeping; □
3. Business; □
4. Salaried employment; □
5. Wage work; □
6. Technician; □
7. Artisan/ hand craft; □
8. Fishing; □
9. Unemployed; □
10. Other (specify) _____

Farming experience:
1 = less than 5 years; □

2 = 5–10 years; □
3 = more than 10 years □

SECTION B: Socio-economic characteristics

1. How long have you lived farming in this village.....(Years)

2. Which crops do you grow? *(First list and then rank in descending order of importance)*
i).....
ii).....
iii).....
iv).....

3. Which 3 crops give you the highest income? (*(list and rank in descending order*)
i).....
ii).....
iii).....

Emerging banana disease
[Request to assess the field and collect diseased samples for identification]

3 (a). Have you ever observed *yellow syndrome disease* on your farm? (*Tick one*)

Yes □
No □

4. (b). If yes, which symptoms did you observe?
.....
.....
.....
4. (c). Which other plant part(s) are you aware to be affected by the disease?
.....
.....
.....
4. (d). How much of your banana mats are affected by the disease and what is the estimated loss you are incurring per month/season?
.....
.....
.....
NB: For estimated loss:
i. Negligible loss (< 2%)
ii. small loss (3-10%)
iii. Significant loss (11-20%)
iv. Major loss (21-40%)
v. Unacceptable loss (> 41%)

5. For how long have you seen this disease on your farm (years)? .....

6. What name do people call this new disease? .....

7. What is your major source of banana planting materials?
   i) Suckers from own field □
   ii) Friends/neighbours □
   iii) Markets □ Market name (s).....
   iv) Borrowed/exchanged □
   v) NGOs □ Name it.....
   vi) Others (*Specify*).....

8. What banana varieties do you have in this field? *(List and rank most important first)*
   i).....
   ii).....
   iii).....
   iv).....

9. What is your most important attribute for the choice of a banana variety to plant? *(List and rank most important first)*
   i).....
   ii).....
   iii).....
   iv).....

10. Name the varieties in your garden most affected by the new disease. *(Examine, take a sample and label with the name given by the farmer)*
    i).....
    ii).....

11. How do you manage *this new disease* on your farm?
    i) Use resistant varieties □
    ii) Crop rotation □
    iii) Manure □
    iv) Fertilizers □
    v) Do nothing □
    v) Others (*Specify*).....

APPENDIX III: Sampling protocol

This research was conducted in Ugandan central districts of Wakiso and Mpigi in Uganda. This guide described how samples were harvested from diseased plants and prepared in the field prior to further laboratory investigations.

Purposive sampling was done on a total of Ten (21) Gardens growing EAHB in all selected district. They were visited by observing gardens with related signs and symptoms of the diseases caused by Fusarium wilt.

Assessing EAHB Cultivars with symptoms of yellowing leaves and Harvesting explants

A. A questionnaire was used to acquire information from every; head/ supervisor/owner of a garden visited.
B. The questionnaire was filled by the lead researcher during the interactive session with the farmers before proceeding with other activities.
C. Access to visit the gardens were on request and through liaising with local farm owners and local council authority (LCs' of visited places) or District Agricultural Officers on **acceptance**.
D. Before harvesting explants, **only** plants observed and identified with severe symptoms showing, golden yellow leaves followed by burst psuedostems were selected.
E. A selected plant was then put down (cut), especially towards the psuedostem. Cutting was done using a clean sterile knife to expose brownish colored patterns along the psuedostems (Necrotic tissue).
F. Further cuts were made into Necrotic tissues in vascular bundles longitudinally in sections of about 10cm long and 0.5cm thin.
G. Necrotic tissue of the explants was aseptically harvested and wrapped in sterile tissue paper and put in sugar paper bag duly labelled with details of the **date, time, place (location and coordinates), collector, and purpose of collection** then placed in a cool-box before being transported to the laboratory and kept at 24ºC for further processing. The wastes from the sampled plants were collected and incinerated within the same garden to void transmission of infection to other cultivars.
H. Tools used during cutting processes were all disinfected with 70% ethanol and packed and all gloves/papers used incinerated.

Recommendations that were taken up during sampling

- Avoiding of excess moisture that accelerates tissue degradation processes of explant tissue
- Avoiding samples with dark high state of oxidation or advanced decomposition.
- Avoiding samples with too much sap or fluid.

APPENDIX IV: Fieldwork and sample collections

A-Cutting down an identified plant, **B-** Questionnaire interview with a farmer, **C**-Severely infected banana plant psuedostem with suspected Fusarium wilt, **D**-Banana with bursting combs, **E-**Wilted banana leaves due to fusarium wilt, **F**-Brown rings of infections along xylem vessels, **G**-Harvested infected-necrotic pieces of psuedostem destined for the laboratory, **H**-Inspection for necrosis on explants.

APPENDIX V: Samples from surveyed gardens in the selected Ugandan Districts

Sample code	Variety local name	Garden Location	GPS Coordinates
MPO-WAK-01	Mpologoma (cooking banana/EAHB)	Wakiso-Garden 1	(32.53568,0.43516)
NKT-WAK-02	Nakitembe (cooking banana/EAHB)	Wakiso-Garden 2	(32.55167,0.470545)
NDI-WAK-03	Sukali Ndizi	Wakiso-Garden 3	(32.53866,0.46638834)
NKT-WAK-04	Nakitembe (cooking banana/EAHB)	Wakiso-Garden 4	(32.514233,0.47981668)
NKT-WAK-05	Nakitembe (cooking banana/EAHB)	Wakiso-Garden 5	(32.443207,0.5170683)
NDI-WAK-06	Sukali Ndizi	Wakiso-Garden 6	(32.44845,0.51996166)
GON-WAK-07	Gonja	Wakiso-Garden 7	(32.475094,0.427395)
NKT-WAK-08	Nakitembe (cooking banana/EAHB)	Wakiso-Garden 8	(32.474266,0.38934332)
BOG-WAK-09	Bogoya	Wakiso-Garden 9	(32.472965,0.3738)
BOG-WAK-10	Bogoya	Wakiso-Garden 10	(32.47545,0.38430834)
MBW-WAK-11	Mbwazirume (cooking banana/EAHB)	Wakiso-Garden 11	(32.53103,0.440839)
NKGU-WAK-12	Nakitegu (cooking banana/EAHB)	Wakiso- Garden 12	(32.522117,0.445336)
NDI-WAK-13	Sukali Ndizi	Wakiso-Garden 13	(32.558947,0.46)
NDY-WAK-14	Nyabalangira (cooking banana/EAHB)	Wakiso- Garden 14	(32.569138,0.440001)
MBW-WAK-15	Mbwazirume (cooking banana/EAHB)	Wakiso-Garden 15	(32.644407,0.391123)
BOG-WAK-16	Bogoya	Wakiso-Garden 16	(30.67513, -0.646839)
BOG-ISING-17	Bogoya	Isingiro-Garden 17	(30.674464, -0.647379)
MRA-WAK-18	Nakitembe (cooking banana/EAHB)	Mbarara-Garden 18	(30.627537, -0.639444)
MRA -WAK-19	Nakitembe (cooking banana/EAHB)	Mbarara -Garden 19	(30.620288, -0.651068)
MBW-ISING-20	Mbwazirume (cooking banana/EAHB)	Isingiro-Garden 20	(30.972246, -0.8668563)
NFU-MPIG-21	Nfuka (cooking banana/EAHB)	Mpigi-Garden 21	(32.33605,0.235946)
KIV-MPIG-22	Kivuvu	Mpigi-Garden 22	(32.313163,0.200682)
KIV-MPIG-23	Kivuvu	Mpigi-Garden 23	(32.320521,0.180029)
NFU-MPIG-24	Nfuka (cooking banana/EAHB)	Mpigi-Garden 24	(32.291348,0.284381)
NDI-MPIG-25	Sukali Ndizi	Mpigi -Garden 25	(32.298324,0.265285)

APPENDIX VI: List of equipment used in the research project available at Biology Laboratory (West End) Kyambogo University

S/N	Equipment	Purpose of Equipment	Model
1	Thermal cycler/ PCR machine	This instrument was used to amplify DNA and RNA samples by the polymerase chain reaction.	Biometra-T professional Basic (96)
2	Electrophoresis apparatus	Electrophoresis equipment applies an electric charge to molecules, causing them to migrate towards their oppositely charged electrode. This aids in separating charged DNA molecules as per pole of attraction.	Consort (EV2650)
3	Microcentrifuge	Microcentrifuge was used to spin down samples at high speeds in order to separate particles in suspension.	Eppendorf-mini spin (22331 Hamburg)
4	U.V-light transilluminator	This UV transilluminator was used to view DNA or RNA that had been separated by electrophoresis through an agarose gel.	Fisher Bioblock Scientific (MO337498)
5	Oven	Heating and drying ovens was used for simultaneously heating and drying samples.	Memmert (E07086)
6	Microwave oven	A microwave oven is an electric oven that heats and cooks' food by exposing it to electromagnetic radiation in the microwave frequency range. This was be used for some warming purposes such as preparing Agarose Jel.	Sayonapps (SMO-2314)
7	Computers and printers	The purpose of a printer is to accept typed text and graphic images from a computer and transfer it to paper.	Lenovo X280
8	Automatic Pipettors	Theses Automatic pipets were used in the biological, and research laboratories to accurately transfer small liquid volumes.	BIO-RAD, SPINREACT, LAMBDA PLUS-CORNING
9	Fume Hoods	The purpose of a chemical fume hood was to prevent the release of hazardous substances into the general laboratory space by controlling and then exhausting hazardous and/or odorous chemicals.	Captair-SMART 714

10	Vortexer	Is a laboratory instrument that is typically used to vigorously spin while vibrating to stir and solutions. This was for homogeneous mixing during preparation of reagents and experimental solutions.	Techno Kartell (TK3S)
11	Incubators	The incubators were be used to grow and maintain microbiological cultures or cell cultures of micro-organisms in Petri dishes.	Stuart-orbital incubator (S1500)
12	Autoclave	An autoclave is a machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores on items that are placed inside a pressure vessel. This tool was used to disinfect used materials, samples, media to effect sterility.	Asco (since 1948)-portable steam Autoclave(18-21P0)
13	Freezes and Fridges	Laboratory Freezers are refrigerated cabinets useful for storing biological specimens and volatile reagents at temperatures ranging between -40 ºC and 10 ºC. these was be used for sample storage and reagent storage.	LG (GN-F702HLHU)
14	Microscope	A microscope is a laboratory instrument used to examine objects that are too small to be seen by the naked eye. This was used for observing structures of micro-organisms and cellular organelles.	XSZ-107BN
15	Water bath	A water bath is a laboratory equipment that is used to incubate samples at a constant temperature over a long period of time. This was used during warming of samples, reagents, media.	BIBBY (RE100B)
16	Analytical balance	Analytical balances are highly sensitive lab instruments designed to accurately measure mass. It was used to weigh masses of reagents to be used in experiments.	BIOBASE (BA2204B)
17	PH meter	pH meter is an instrument used to measure acidity or alkalinity of a solution. This was used for measuring PH of media and buffer solutions used in experiments.	ATC

18	Biological safety Cabins	A biological safety cabinet (BSC) is a primary engineering control used to protect personnel against biohazardous or infectious agents and to help maintain quality control of the material being worked with as it filters both the inflow and exhaust air. Much of culturing transfer activities was done in this environment.	Bio-II-Advance 3 (#522631)
19	Thermometers	A thermometer is a device that is used to measure the temperature of a body. In this case it was used to measure temperature of different work cabins, media.	ZEAL-England (76mm-imersion)

APPENDIX VII: A simple DNA extraction protocol that was used for extracting good-quality DNA from suspected fungi

This research was conducted in the Ugandan central districts of Wakiso and Mpigi. This guide will describe how good quality and pure DNA was extracted from isolated samples suspected to contain the fungal pathogen causing the golden yellowing syndrome on banana leaves in gardens.

List of Requirements

1. Potato Dextrose Agar Media (PDA)
2. Streptomycin sulphate Antibiotic (Concentration of 0.05g/liter for every 200ml of media prepared)
3. 10cm Sterile-disposable petri dishes (10 packs containing 100 pieces for all the experiments)
4. TES extraction buffer consists of 10 mM Tris HCl, 100 mM NaCl, and 1.0 mM EDTA at a pH of 7.8 i.e. (0.2 M Tris-HC1 at pH 8, 10 M EDTA pH 8, 0.5 M NaC1, 1% SDS- Sodium Dodecyl Sulphate (SDS) - (where mM is Millimolar)
5. Acid-washed, sterilized sea sand.
6. Sterilized 1.5-mL Eppendorf (microcentrifuge) tubes (1 pack)
7. 99.8% Ethanol (to prepare 70% ethanol)
8. Sterile surgical blades
9. Forceps
10. Surgical gloves (2 packs/boxes)
11. Micro-pipette tips (p1000 and p200)
12. PCR tubes (1 tube)
13. Paper towel (2 rolls)
14. Micro- centrifuge tubes 1.5ml (1 pack)
15. Aluminum foil
16. Primers, ladders and tracking dye
17. Enzymes (Polymerase enzyme)
18. Nuclease free water to dissolve DNA
19. Running Buffer.
20. Agarose

Procedure for Production of fungal mycelium

Isolation of fungus from explant

i) The harvested infected tissues along with adjacent small unaffected tissue were cut into small pieces (2–5 mm squares) using a surgical blade and a flame-sterilized forceps.

ii) These small cut pieces of the explant were then aseptically inoculated in potato dextrose agar (PDA) medium containing streptomycin (antibiotic).

iii) The cultures on the Inoculated plates were incubated at 24 °C room temperature in a rotary shaker (115 rpm) incubator and kept at room temperature until growth of fungal mycelia was observed for within 5-7 days for the complete growth of fungi (Thilagam *et al*., 2018)

iv) The fungal growth from the explant were sub-cultured further by aseptically cutting a piece of the medium with mycelia and introducing them in sterile PDA containing the antibiotic.

v) Sub-cultured plates were then be incubated at 24 °C until fungal growth was observed.

vi) The resulting spores grown were observed under a microscope (Ploetz, 2006). Positive cultures were preserved on PDA at 4 °C and all resulting wastes of experiments were incinerated onsite to avoid spread of potential pathogens to surrounding environments and gardens

DNA extraction stage

i) Fresh mycelium was transferred (150 mg) to a sterilized 1.5mL Eppendorf (micro-centrifuge) tube containing 300µL of TES extraction buffer (0.2 M Tris-HC1 [pH 8], 10 mM EDTA [pH 8], 0.5 M NaC1, 1% SDS) and acid-washed, sterilized sea sand.

ii) Macerating of mycelium for 2 min with a hand-held disposable homogenizer that fits the 1.5-mL microcentrifuge tube was done

Vertexing of samples was done for 30 s and add an additional 200µL of TES extraction buffer containing proteinase K (final concentration of 50µg/mL).

iii)

iv) Vertexing was done to thoroughly mix and place tubes in a water bath at 65ºC for 30 min.

v) One-half volume (250µL) of 7.5 M cold ammonium acetate was added.

vi) Mix and incubate the samples on ice or at 5ºC in the refrigerator for 10 min.

vii) Centrifuge for 15 min at 13,400rpm.

viii) Transfer the supernatant to a new tube and add an equal volume (500µL) of

ice-cold isopropanol.

ix) Incubation of the tubes was done at -20ºC for 1-2 hours.

x) Centrifuging was done for 10 min at 13,400rpm.to pellet the DNA.

xi) Decanting of the supernatant and washing of DNA pellet with 800µL of cold 70% ethanol was done.

xii) Thereafter the tubes were turned upside-down on clean sterile paper towels for 10-15 min to air-dry

DNA.

xiii) Elution of DNA from the pellet with twice-repeated extractions with 250µL of 1x

TE buffer (10 mM Tris-HC1 [pH 8], 1 mM EDTA), where every time centrifuging was done to avoid collecting pelleted polysaccharides.

xiv) DNA solution was transferred to a 1.5mL microcentrifuge tube, 5µL (20 mg/mL) of RNase was added and incubated at 37ºC for 60 min.

xv) DNA was recovered and air-dried as described above.

xvi) The dry DNA pellets were to be used in further PCR activities

Note: DNA extracted was stored between -20 °C and -80 °C to prevent degradation by chemical

and enzymatic processes, DNA is often stored as a precipitate in ethanol, at -80 °C (Mahuku,

2004).

APPENDIX VIII: Laboratory activities

A-Isolated pathogens growing on PDA plates, **B-c**rushing of harvested mature mycelia for DNA extraction work, **C**-Setting of agarose gel outcome for observation on UV light apparatus, **D**-Isolation of pathogens in a biosafety cabin, **E**- electrophoresis in progress during separations of PCR outcome-DNA extraction work.

APPENDIX IX: Pathogenicity screening protocol for Fusarium isolates

Materials and Methods

Plant Materials

The germplasms of banana cultivars used in the greenhouse screening experiments were selected from only sterile tissue culture plantlets from a confirmed tissue culture lab.

Evaluation under Greenhouse Conditions

The strains for the greenhouse experiments were selected based on identity results of PCR and sequencing.

The identified strains were cultured on potato dextrose agar (PDA) medium for 5 days, and then, the mycelium was inoculated into mung beans broth to grow for another 5 days at 25°C in a rotary incubator with a rotor speed of 180 rpm.

Inoculum production and inoculation methods

To produce inoculum conidial suspensions of the reference strains, they were produced in flasks containing 100 mL of mung beans broth.

Method

- In 500ml of distilled water, 2 grams of mung beans were added.
- Auto-claving was done for 1hour and allowed to cool and distributed in sterile 100ml flasks
- Freshly grown Fusarium from PDA plates (5-7days old) were inoculated into sterile mung-bean broth García-Bastidas et al., (2019).
- The flasks are incubated at 25°C, 150 rpm for five days, after which the concentration is adjusted to 1×10^6 conidia $^{-1}$ ml.

Counting-cells-using-a-hemocytometer

After 5 days, count the number of cells grown in the mung bean broth using a hemocytometer ; a (Abcam, 2015)

To calculate number of viable cells per mL:

- Take the average cell count from each of the sets of 16 corner squares.
- Multiply by 10,000 (10^4).

- Multiply by five to correct for the 1:5 dilution from the trypan blue addition. The final value is the number of viable cells/mL in the original cell suspension.

Example

If the cell counts for each of the 16 squares were 50, 40, 45, 52, the average cell count would be

- (50 + 40 + 45 + 52) ÷ 4 = 46.75
- 46.75 x 10,000 (104) = 467,500
- 467,500 x 5 = 2,337,500 live cells per mL in original cell suspension

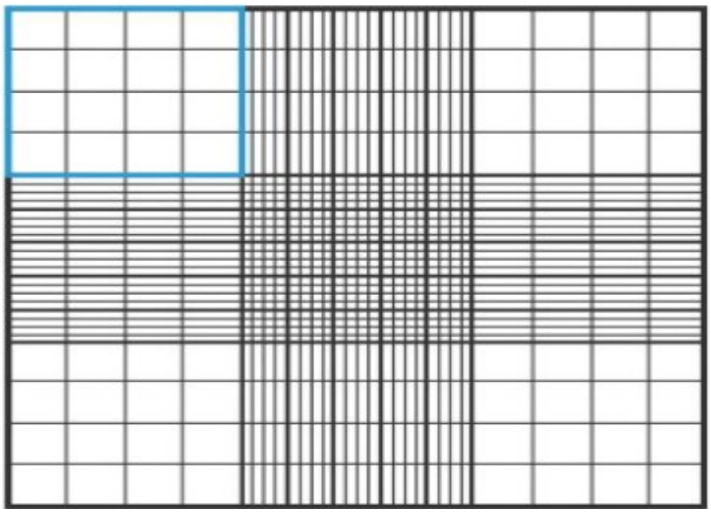

Figure of Hemocytometer gridlines diagram indicating one of the sets of 16 squares that should be used for counting.

Adjust the concentration to 1×10^6 spores per ml.

- Apply $C_1V_1=C_2V_2$ to arrive at correct spore concentration

Inoculation procedure

Four-month-old sterile plantlets from tissue culture lab were selected for each genotype to be screened.

Black soil used in the experiments was autoclaved for six hours and allowed to cool a day before.

The plantlets were then uniformly wounded on a few roots before being placed into clean pots loaded with sterile black soil. No water was added at this stage.

Three replications (six plantlets for each replication) were used for each genotype.

For negative controls, each potted plantlet was drenched with 200 ml of tap water after damaging the roots.

For test plants, each potted plantlet was drenched with 200 ml of respective inoculum followed by 200 ml of distilled water.

Management of the experiments

The experimental plot was arranged as a completely randomized (CRD) design with three replications (blocks) and 10–15 plants within each block for each genotype (can be readjusted as desired). After inoculation, plantlets were maintained in the greenhouse by watering daily with at least 200ml of tap water and at 25–28°C, with 70–80% relative humidity. No chemicals were applied to control pests and diseases.

Evaluation of the experiment

Disease evaluation was performed at 84 days after inoculation when typical Fusarium wilt disease symptoms were observed in the susceptible control selected with >80% of Fusarium wilt incidence. The external symptoms of Fusarium wilt, such as leaf yellowing on the oldest leaves and occasional pseudostem splitting appeared on the susceptible control.

The disease severity was assessed basing on the Rhizome Discoloration Index (RDI) according to the rhizome discoloration (internal symptom) ratio (rating scale) as below:

1: the absence of internal symptoms;

2: the occurrence of several internal spots;

3–5: <1/3, 1/3–2/3 and >1/3 areas discolored;

6: the whole inner rhizome discolored.

Additionally, the genotypes were classified as;

1. ‘susceptible’(S)
2. (S, RDI > ‘3’),
3. ‘intermediate’ (I, RDI = ‘2’– ‘3’)
4. or ‘resistant’ (R, RDI < ‘2’)

Once plants were dead or harvested, internal symptoms on the rhizome were evaluated. The incidence of disease (ID) was calculated when plants were dead or harvested as follows:

ID (%) = [diseased plants/total plants] 100.

According to the ID values, the following categories of disease reaction to the plantlets were established:

1 a 0% ID 20%: highly resistant (HR)
2 20% < ID 40%: resistant (R)
3 40% < ID 60%: intermediate (I)
4 60% < ID 80%: susceptible (S)
5 80% ID: very susceptible (VS)

APPENDIX X: Greenhouse Screening Experiments

A setup of plantlets in the greenhouse during the screening experiments to confirm the effect of pathogens on EAHBs.

APPENDIX XI: Containment of Experimental Waste

A-Old and used-up PDA plates with active pathogens ready for decontamination, **B**-Old plates loaded carefully in an autoclave for destruction, **C**-Outcome of decontaminated plates, **D**-Decontaminated plates placed in a biohazard waste bin for safe disposal.