# DIVERSITY OF NEMATODES AND ENDOPHYTIC FUNGI ASSOCIATED WITH BANANAS CULTIVATED IN TAVETA SUB COUNTY

**Yusuf Salim Masud[1], James Gacheru[*1], Maurice Juma Ogada[2], Mwamburi Mcharo[1], Marianne Maghenda[1], Leah Andisi Akula[1] and Anne Kelly Kambura[1]**

[1]Department of Agricultural Sciences, School of Agriculture, Earth and Environmental Sciences, Taita Taveta University, P. O. Box 635-80300 Voi, Kenya.

[2] School of Business, Economics and social Sciences, Taita Taveta University

[*] Corresponding author E-mail: **jmark@ttu.ac.ke**

**Abstract**

**Plant-Parasitic Nematodes (PPN) are small wire-like multicellular soil-inhabiting living organisms. A great proportion of these PPN are capable of causing enormous destruction to almost all cultivated crops in the tropics and subtropics. Banana PPN is root invading thus, causing destruction to primary roots in form of lesions and knots. This could hamper water flow and nutrient uptake, compromising plant stability and overall crop performance and culminating in yield losses of up to 85%. Although there exist antagonistic fungal and bacterial endophytes that could suppress the population of PPN and reduce their negative impact, the occurrence and densities of these endophytes are yet to be studied and documented. In this study, a random survey of farmers' fields in the Taveta sub-county was carried out to evaluate the occurrence and distribution of nematodes and endophytes. Banana farms were randomly selected from the four wards Mahoo, Mboghoni, Bomeni, and Mata based on the willingness of the farmer to voluntarily participate in the research. On each farm, soil and banana roots samples were collected from three randomly selected stools where nematodes were quantified. In addition, endophytes diversity associated with these orchards were isolated and characterized with emphasis on the colony and cell morphology, and phylogenetic affiliation using partial sequences of the Internal Transcribed Spacer region (ITS) gene. The efficacy and effectiveness of biological control methods to suppress nematodes were evaluated in banana stools found to harbor nematodes above a certain threshold (index ≥ 10) using cowpeas intercrop and, application of Tithonian plant extracts and Nimbecidine® biopesticides. Cowpeas intercrop, Tithonian plant extracts and Nimbecidine were effective in suppressing the population of RKN, *Helicotylenchus, Tylenchus, Pratylenchus, Aphelenchoides, Aphelenchus* and *Tylenchorychus* while increasing the population of the free-living organisms, which further reduces the population of PPN. Results of partial sequence analysis indicated the fungal endophyte isolates that were recovered from the samples collected from selected banana orchards to belong to the fungal domain, 90 % being affiliated to *Ascomycota* phylum. The key genera identified as next neighbors in BLAST comprised *Penicillium*, *Mortierella*, *Aspergillus* and an *uncultured fungal genus* with a range of identity scores of 95 – 100%.**

**Keywords**: Banana, Nematodes, Endophytes, biocontrols

## Introduction

Banana is a valuable crop after maize, rice, and wheat in relation to food security particularly in the emerging nations (Petsakos et al., 2019; Scott, 2020). The crop is widely exploited as fruit, carbohydrate source, nutrition and income (Scott, 2020). In Taveta, banana is an excellent source of food, animal feed, minerals and livelihood for many rural households. The crop is grown as a mono-crop or intercropped with other annual crops. On small scale, the crop is grown with sweet potatoes, maize and beans. This ensures a continuous supply of food and cash flow throughout the year. Banana production in Taveta is likely to expand with the construction of a banana processing plant in the near future. Presently, the sub-county of Taveta has about 4,104 acres under banana production, with an estimated yield of up to 65,280 tons (TTC, 2021).

However, banana production in Taveta is threatened by various pests, diseases, and soil-related challenges. Among the risk factors, pests and diseases are expected to reduce banana yields and quality (Jones, 2000). In particular, banana parasitic nematodes are likely to contribute to a tonnage loss of between 30 to 60% (Luc et al., 2005; Nyang'au et al., 2021). These attack plant roots and in many cases, spend their entire life span burrowing in the root system (Fourie et al., 2017; Njenga, 2019; Luc et al., 2005). However, there exist some migratory endo-parasitic nematodes that attack and damage the banana root system, constricting the roots and thus, obstructing water and minerals uptake from the ground (Coyne et al., 2018). The damages caused by such root nematodes are not specific enough and often muddled to edaphic stresses such as poor soil nutrition and water stress (Coyne et al., 2018). For instance, chlorosis due to root nematode damage could easily be confused with nitrogen deficiency. But when a banana plant does not respond to nitrogen fertilization, it is predictable that root nematodes could be the cause of chlorosis. The banana plant may eventually topple or wilt as the root damage or constriction hinder growth, nutrients and water uptake as well as the plant's anchorage.

Naturally, nematode threat is often checked by use of beneficial endophytes that feed on parasitic nematodes thus reducing their potential damage to a crop. Some endophytes are characterized by hyphae that form associations with the host plant which promotes mineral and water uptake, besides reducing plant stresses and use of synthetic inputs (Khalil et al., 2021). Endophytes close association with plant have attracted researchers' attention resulting to commercial exploitation (Altieri and Nicholls, 2012; Hassan, 2017; Khalil et al., 2021). Previous studies have pointed towards endophytic *Actinomycetes* as being associated with planted crops, particularly within the tropics as main sources of functional metabolites (Strobe et al., 2004). Endophytes have been reported to produce novel metabolites with potential of application in several ranges of biotic activities and highly structurally diverse (Gao et al., 2018; Khalil et al., 2021).

Conversely, despite the importance of endophytic communities in the soils, the range distribution and composition of the endophytes in the soil ecosystem is not well understood. Endophytic microorganisms are commonly determined by grouping of host class characteristics such as plant genome of the host, edaphic factors, with extensive difference in taxonomic alignment across various groups (Edward et al., 2015). This study aimed at assessing the nematodes and endophytes associated with different cultivated banana varieties in Taveta and their impact on the production scale.

## MATERIALS AND METHODS

### Study site

The study was conducted in Taveta constituency, which is lies approximately -3.398 latitude and 37.68$^{\circ}$ longitudes in the southern he-

misphere. Its lies on the southern-west coastal region of Kenyan geographical map bordering Tanzania. Taveta ranges between 752 - 1,006 m above the seal level, with an average temperature of 23.3 °C. The area receives bimodal rainfall pattern with about 616 mm per year. The short rainfall is April to May and long rain in October to December. Banana is the leading crop in terms of production, as an important food crop as well as a cash crop at both subsistence level and commercial level. Taveta cub-county comprises of five wards, Mboghoni, Mata, Bomeni Challa and Mahoo, Mboghoni being the leading ward in terms banana production (Figure 1).

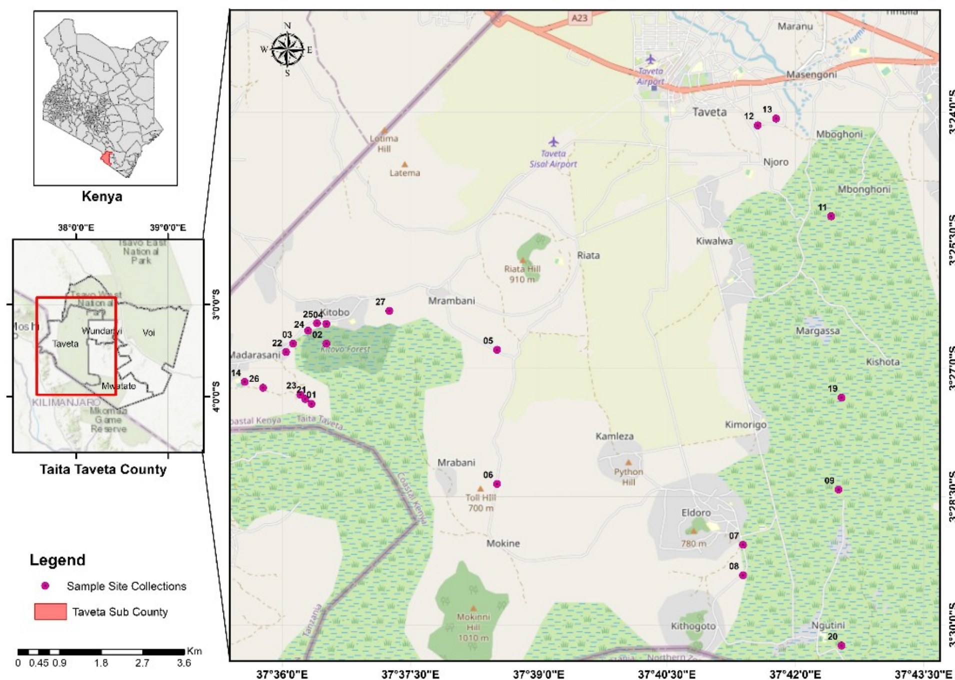


Figure 2: A map showing location of Taveta Sub County, the study area and locations of the sampled banana farms used in this study.

**Research survey design**

A cluster sampling procedure was employed in selecting banana orchard assessment based on a purposive selection of farms from each ward with 30 and above banana stools. The population was divided into wards, and a subset of the farms was randomly selected as described by Suresh et al. (2011). Data was collected using an open-ended organized questionnaire which was administered through face-to-face face interviews with smallholder farmers in Taveta Sub County. An aggregate of 90 banana orchard families was identified, and primary data was captured. The sample scope of households was acquired using the Fischer formula. A total of 60 banana farmers' household heads from the Mboghoni ward were exposed to the questionnaire: 10 from the Mata ward, 10 from the Mahoo and lastly 10 the from Bomeni ward.

**Samples collection**

Three distant banana stools from each farm were randomly selected (Figure 1) and samples were collected after scooping out 3-5 cm of the topsoil. Approximately 30g of banana roots and soil around the banana roots. Three samples from each stool were thoroughly mixed to constitute a composite representative from the root and soil respectively. Samples were immediately labeled sand stored in a cool box. The equipment were dipped into a solution of 70% antiseptic between specimen points for sterilization to prevent cross-

contamination. All geographical position satellites (GPS) were captured from each banana stool where the soil and roots sample were to be collected. The collected samples were ferried in a cool box to International Centre for Insect Physiology and Ecology (ICIPE) for extraction and identification.

**Extraction of nematodes**

**Root samples**

Roots samples were washed using tap water, to eliminate soil sediments from the roots and gently swabbed with paper towels. The roots were chopped into tiny pieces of around1cm each using a knife. The small pieces of roots will be mixed thoroughly. Approximately 50g of the chopped roots were weighed using a weighing scale. The weighed sub sample were blended into fine roots for 2-5 seconds bursts or if the roots are tougher for at least 10 seconds bursts. The suspension was left to settle, then poured into a beaker. The suspension was poured onto a paper towel, followed by overnight extraction. The liquid was decanted via small 28µl aperture before assessing the nematodes. The isolates were identified using under the microscope as per their characteristics.

**Soil samples**

Each soil sample was sieved to eliminate stones and debris from soil and break up soil lumps. The soil was then thoroughly mixed thoroughly then a representative soil sample about 100g was taken as a representative sample. Tissue paper was placed in the plastic bucket, then the soil sample was added on the tissue paper, water was added gently for the extraction. The solution was left to settle and carefully drained to a basin leaving the nematodes resting on the tray. The mixture was left to undisturbed for a period of 48hrs. The extra water was removed from the sieve or siphoned. Then the nematodes were then counted out.

**Killing and fixing nematodes**

Nematodes normally displays a particular shape when they are dead. Therefore, for easy identification it was necessary to kill and fix them. The material containing nematodes was immersed in hot water for at least 2 minutes to kill them and 2-3 drops of formaldehyde were added to fix them. The samples were kept at 20 °C to allow fixed nematodes to settle.

**Density estimation of nematodes**

Counting method was used to estimate the total number of nematodes, as per sample banana stool and farm. This was estimated from a known volume (10 ml) of liquid under a compound microscope X40. The economic index threshold was estimated as per Pattison et al. (2002).

**Nematodes identification**

The nematode samples were mounted on a glass slides wetted with water and then covered with a slip. The physiological features of nematodes were used to differentiate the plant-parasitic nematodes (PPN), in their mouth part according to their feeding nature. Free-living nematodes were categorized based on their feeding habits, predators, omnivores, bacterivores and entomopathogenic nematodes. The PPN were identified up to a genus level depending on the morphological and anatomical structures of each individual nematode. These features included shape, worm-like, pear-shape, spiral-shaped and mouth type.

**Field experiment to evaluate the efficacy of different nematode biological control**

This efficacy trial was conducted after a comprehensive soil sampling and laboratory nematodes count to determine occurrence and distribution of nematodes in the farms. Five farms were randomly selected based on banana stools that harbored threshold index ≥ 10 nematodes count as per Pattison et al. (2002) threshold of plant-parasitic nematodes. On each farm three stools were randomly selected

and were randomly allocated to one of the three treatments (cowpeas planting, Tithonian tea extracts or commercial product, Nimbecidine®, application). The treatments were applied around the banana stools. Cowpeas were planted around the stool while Tithonian tea extract and Nimbecidine® solution were irrigated at the base. Three months after treatment, soil samples were collected for nematodes extraction and population estimation from each treated stool soil as described above for the soil sampling.

**Isolation of Endophytic fungi from soil and root samples**

Soils and root samples were soaked in distilled water to disperse fungal endophytes. 1 gram of each sample was suspended in 10ml of sterile distilled water and thoroughly vortexed. One (1) ml aliquot of the sample suspension from this stock solution of 10 ml, were further diluted in 9ml sterile distilled water up to the $10^{th}$ dilution factor. About 0.1ml (100 µl) of each dilution were plated on sterile medium prepared using Malt extract agar (MEA), Potato dextrose agar (PDA) and Sabourand dextrose agar (SDA). The inoculated plates were incubated at 30°C for growth and colony formation. The colonies were further sub cultured into freshly prepared media in order to obtain pure cultures for further characterization. The pure endophytic fungal cultures were preserved under glycerol on agar slants and stored at 4°C.

**Morphological characterization of the isolates**

Colonies of the obtained fungal endophytic isolates were described based on shape, colour, form, elevation, and pigmentation on both sides of the Petri dish. Cellular characterization of the isolates was performed by simple staining using lacto phenol cotton blue dye. A glass slide was placed on spaced pair of toothpicks in a petri dish, partially submerged with water. A sterile block of PGA culture media was placed on the upper surface of the glass slide. This culture medium was inoculated with endophyte isolates and a cover slip was placed on the inoculated medium. The cultures were incubated for 3-7 days, after which, the cover slip with mycelia was placed on a clean slide, stained and used for examination under a microscope. The stained colonies were observed under a light microscope from the lowest (X10) to the highest (X100) magnification.

**DNA extraction from fungal endophytes cells**

Fungal endophyte isolates were cultured on PDA medium and incubated for 4-7 days. Genomic DNA of the isolates was extracted from endophyte cells in triplicates using lysis buffers; solution A (50mM Tris pH 8.5, 50mM EDTA pH 8.0 and 25% sucrose solution) and solution B (10mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1% SDS). Precisely, each of the selected fungal endophyte isolate cells were scrapped into a sterile eppendorf tube using a sterile surgical blade. The cells were crushed in 200 µl solution A using a sterile mortar and pestle, and re-suspended in 100 µl of lysis buffer solution A. 30 µl of Lysozyme (20mg/l) and 15µl of RNase A (20mg/l) enzymes were added, vortexed and incubated at 37°C for two hours to allow for lysis of the cell wall as well as removal of RNA contaminants. 600µl solution B were added to the mixture, gently vortexed, and 10µl of Proteinase K (20mg/l) were added. The mixture was incubated at 60°C for 1 hour. The DNA pellet was precipitated overnight using 3M NaCl and Isopropanol, washed using 70% ethanol and air dried. The presence and quality of DNA were checked on 1% agarose gel electrophoresis. The DNA bands were visualized under ultraviolet light after staining with 0.3µl ethidium bromide dye to determine the relative intensity of bands.

**PCR amplification and sequencing of ITS gene region**

Polymerase Chain Reaction (PCR) amplification of the ITS gene region was carried out using fungal primers ITS 1 (5′TCCGTAGGTGAACCTTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) as described by White et al. (1990). Amplification was carried out using DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) PCR machine in a 40 µl mixture containing PCR buffer (×10) 5µl, dNTP's (2.5mM) 3µl, ITS1 forward primer (5 pmol) 1µl, ITS4 reverse primer (5pmol) 1µl, Taq DNA polymerase 0.3 µl, sample DNA template 1.5 µl and 28.2 µl molecular grade PCR water. The control contained all the above except the DNA template. PCR reaction mixtures were subjected to 35 repeated temperature cycles comprising of initial enzyme activation at 95°C for 5 minutes, DNA double helix denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, extension of DNA chain at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The quality of PCR products was assessed on 2% agarose gel and visualized under ultraviolet by staining with ethidium bromide. The PCR products were purified using multiscreen filter plate (Millipore Corp and sequenced by a Big Dye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Bio systems) according to manufacturer's instructions. Sequencing of the PCR products was performed at Macrogen, Korea using a commercial service provider.

**Phylogenetic analysis**

Partial sequences obtained from fungal endophyte isolates analyzed using Basic Local Alignment Search Tool (BLAST) hosted in the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST/). The sequences were blasted against the GenBank 18S rDNA database. Nearest neighbours in blast were selected for further comparison with the available endophyte sequences. Sequence alignments for both endophyte isolates were checked and corrected based on conserved gene regions. The partial gene sequences were compared to sequences in the public database using BLASTn in order to determine similarity to sequences in the Gene bank database. The 18S rDNA gene sequences with high similarities to those determined in this study were retrieved based on BLASTn results and added to the alignment. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021) where history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and phylogenetic tree drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. The partial sequence analysis comprised 38 nucleotide sequences which included 1st+2nd+3rd+Noncoding Codon positions. All ambiguous positions were removed for each sequence pair using pairwise deletion option inbuilt in Mega 11, giving a total of 1565 positions in the final dataset. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site.

**Statistical Data analysis**

Nematode counts from each of the collected soil and root samples were quantified and scored per each identified species. Recorded data collected was exposed to analysis of variance (ANOVA) using R version 3.6.1 (2019-07-05) program. Nematodes population before and after treatment were subjected to statistical tool to evaluate population change before and after the three biological treatments. Where the means were statistically different, population means were separated by the "Tukey" test at α = 0.05.

## RESULTS

The nematodes population from the sample collected revealed a number of different genera of nematodes. Among the recovered genera, free-living nematodes were in abundance when compared to other identified genera in both soil and root samples (Table 1). The nematode pool was similar irrespective of the farming system adopted by the farmers. There were other nematodes species within the pools of the extracted samples that were not identified. Eight (8) nematode genera were identified within the soil samples while six (6) genera were identified colonizing the root samples. Five (5) root samples (12, 17, 18, 24 and 26) were "free" of the identified nematodes (Table 1).

In the evaluation of the efficacy of bio suppressor (cowpeas), organic product (Nimbecidine), and crude Tithonia tea extracts, five farms were randomly selected, demarcated, and treated. The results indicated that all the three treatments were effective against other types of parasitic nematodes and boosted the population of free-living nematodes (Table 2 and Table 3)

### Fungal endophytes isolates and morphological characterization

A total of 7 fungal endophyte isolates were recovered from the samples collected within selected banana orchards. The isolates colony characteristics displayed differences in form, margin, color and elevation as shown in Table 4 while cellular characteristics were as shown in Figure 2.

### Phylogenetic analysis of sequences

The partial sequence analysis of endophyte isolates recovered from the soils and banana roots in this study revealed that the isolates were affiliated to the domain fungi, with 90% of the isolates being linked to *Ascomycota* phylum. The sequences clustered closely to nearest neighbors in blast with scores ranging between 95 – 100% identities as shown in Table 5. Major taxa were associated with members of *Penicillium*, *Aspergillus*, *Mortierella* and *Uncultured* fungal genera and Figure 3.

## DISCUSSION

Banana play a crucial role towards achievement of Kenya's big 4 agenda and Sustainable Development Goal 2; *Zero Hunger*: Providing food and relief, to Kenyan population and serve as a source of income to alleviate poverty (SDG 1 – No poverty). Bananas are rich in minerals that could cushion the population from malnutrition, contributing to well-being of rural communities. To safeguard these values, proper crop agronomic practices aimed at safeguarding and increasing production is paramount.

Approximately, 25.1% of the banana farmers were informed about nematodes while about 74.9% of the farmers in Taveta are not aware of nematodes as banana pests in the field. However, majority were informed of the toppling banana disease although, they associated it with other abiotic factors such as water stress and poor soil nutrition among others (Brooks, 2004; Wang and Hooks, 2009). Therefore, there is a need to create awareness to farmers on which crop families could form excellent intercrops, nematodes as a biotic factor affecting banana production, and encourage the use of clean planting materials, preferably obtained from certified/registered suppliers (Coyne et al., 2018).

**Table 1**: Relative abundance of nematodes in 100 ml of soil (S+) l and root (R+) extracts under different farming systems

| Sample | Latitude (S) | Longitude (E) | Free-living | *Pratylenchus* | Root Knot | *Tylenchus* | *Aphelenchus* | *Helicotylenchus* | *Aphelenchoides* | *Tylenchorynchus* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | -3.4569 | 37.6057 | S+ | S+ | S+ | --- | --- | S+ | --- | --- |
| 2 | -3.44518 | 37.608592 | S+ | R$^{+}$ | --- | --- | --- | --- | --- | --- |
| 3 | -3.44518 | 37.602116 | S+ R+ | --- | --- | --- | --- | --- | --- | --- |
| 4 | -3.44132 | 37.608592 | S+R+ | --- | S+ | --- | --- | --- | S+ | --- |
| 5 | -3.446375 | 37.641874 | S+ | S+ | S+ | --- | --- | --- | S+ | --- |
| 6 | -3.472514 | 37.641874 | S+R+ | S+ | S+ | --- | --- | --- | --- | --- |
| 7 | -3.484385 | 37.68981 | S+R+ | S+R+ | S+ | --- | --- | --- | --- | --- |
| 8 | -3.49032 | 37.68981 | S+ | R$^{+}$ | --- | --- | --- | --- | --- | --- |
| 9 | -3.473635 | 37.68981 | S+ | --- | --- | --- | S+ | --- | --- | --- |
| 10 | -3.473635 | 37.708423 | S+ | S+ | --- | --- | --- | --- | S+ | --- |
| 11 | -3.378723 | 37.71556 | S+ | --- | S+ | --- | --- | --- | --- | --- |
| 12 | -3.420339 | 37.707 | S+ | --- | --- | --- | --- | --- | --- | --- |
| 13 | -3.402616 | 37.692685 | S+ | --- | --- | --- | S+ | --- | S+ | --- |
| 14 | -3.401295 | 37.696253 | S+ | S+ R+ | S+ | S+ | --- | --- | R$^{+}$ | --- |
| 15 | -3.452628 | 37.592698 | S+R+ | S+ | S+ | S+ | --- | R$^{+}$ | --- | --- |
| 16 | -3.44101 | 37.1267 | S+ | --- | --- | --- | --- | --- | --- | --- |
| 17 | -3.4215 | 37.3547 | S+ | --- | --- | S+ | S+ | --- | --- | --- |
| 18 | -3.32271 | 37.17294 | S+ | --- | R$^{+}$ | S+ | --- | --- | --- | --- |
| 19 | -3.44511 | 37.28383 | S+ | --- | --- | S+ | --- | --- | --- | --- |
| 20 | -3.455699 | 37.709022 | S+R+ | R$^{+}$ | S+ | --- | --- | --- | --- | --- |
| 21 | -3.504044 | 37.709022 | S+ | --- | --- | --- | --- | --- | --- | --- |
| 22 | -3.456 | 37.604 | --- | S+ | --- | R$^{+}$ | --- | --- | R$^{+}$ | --- |
| 23 | -3.447 | 37.6 | S+ | --- | S+R+ | --- | --- | --- | --- | --- |
| 24 | -3.455 | 37.604 | S+ | --- | --- | --- | --- | S+ | --- | --- |
| 25 | -3.443 | 37.605 | S+ | R$^{+}$ | --- | S+ | R$^{+}$ | --- | --- | --- |
| 26 | -3.441 | 37.607 | S+ | --- | --- | --- | --- | --- | --- | S+ |
| 27 | -3.453 | 37.595 | S+ | --- | S+R+ | S+ | --- | S+ | --- | --- |

Table 2: Abundance of nematodes extracted from 10 ml aliquot from soil samples under different banana cropping systems

| | | | After Treatment | | |
|---|---|---|---|---|---|
| Nematode type | Banana cropping system | Abundance before treatment | Tithonia tea | Nimbecidine | Cowpea |
| Free Living | Mono + intercrop | 92a | 180b | 120b | 201b |
| *Pratylenchus* | Mono + intercrop | 7a | 5a | 3b | 3b |
| *Root Knot* | Mono + intercrop | 15a | 7b | 6b | 6b |
| *Tylenchus* | Mono + intercrop | 7a | 3b | 5a | 6a |
| *Helicotylenchus* | Mono + intercrop | 0 | 1 | 0 | 0 |
| *Aphelenchus* | Mono + intercrop | 15a | 6b | 7b | 6b |
| *Helicotylenchus* | Mono + intercrop | 15a | 6b | 7b | 6b |
| *Aphelenchoides* | Mono + intercrop | 16a | 8b | 5b | 5b |
| *Tylenchorynchus* | mono | 0 | a | 2a | 0 |
| Total | | 2322 | 2762 | 1733 | 2937 |

Table 3: Abundance of different nematode genera within 10 ml aliquot samples under different banana cropping systems

| | Total No including non-identified | Free-living | *Pratylenchus* | Root-knot | *Tylenchus* | *Aphelenchus* | *Helicotylenchus* | *Aphelenchoides* | *Tylenchorynchus* |
|---|---|---|---|---|---|---|---|---|---|
| Population before treatment | 435 | 105 | 7 | 8 | 210 | 5 | 6 | 6 | 0 |
| | 105 | 56 | 7 | 8 | 5 | 5 | 6 | 6 | 0 |
| | 323 | 105 | 7 | 7 | 3 | 5 | 6 | 6 | 0 |
| | 375 | 105 | 7 | 8 | 0 | 5 | 6 | 6 | 0 |
| | 518 | 266 | 7 | 8 | 0 | 5 | 6 | 6 | 0 |
| After Tithonian treatment | 3045 | 735 | 2 | 3 | 1 | 1 | 1 | 1 | 0 |
| | 735 | 392 | 0 | 2 | 1 | 1 | 1 | 1 | 1 |
| | 2261 | 735 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| | 2625 | 735 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 3626 | 1862 | 1 | 0 | 1 | 1 | 1 | 3 | 1 |
| After Cowpeas treatment | 3045 | 1127 | 2 | 1 | 1 | 1 | 1 | 2 | 1 |
| | 581 | 392 | 2 | 2 | 1 | 1 | 1 | 1 | 1 |
| | 1736 | 735 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| | 3885 | 1470 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 1106 | 392 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| After Nimbecidine treatment | 476 | 392 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| | 1841 | 392 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| | 1946 | 392 | 0 | 1 | 1 | 1 | 1 | 2 | 1 |
| | 5831 | 1127 | 2 | 2 | 1 | 1 | 1 | 1 | 1 |
| | 1155 | 735 | 2 | 2 | 1 | 1 | 1 | 2 | 1 |

Table 4: Fungal endophytes isolates and morphological characterization

| Isolate | Top | Bottom | Margin | Elevation | Form | others |
|---|---|---|---|---|---|---|
| 5 | Light pink | brown | Entire | Raised | Circular | sporulation |
| 6 | Fluffy white cotton with rings | white | Filamentous | Convex | Circular | None |
| 8 | Creamy /grey with brown rings | Brown | Entire | raised | Circular | None |
| 11 | White | Creamy | Undulate | umbonate | Irregular | None |
| 12 | Pink | Black | Highly fila-mentous | Raised | Circular | None |
| 13 | Black with white rings | Creamy wrinkled | Entire | Raised | Circular | Sporulation |
| 14 | Pink/white | Black | Undiluted | Raised | Circular | None |

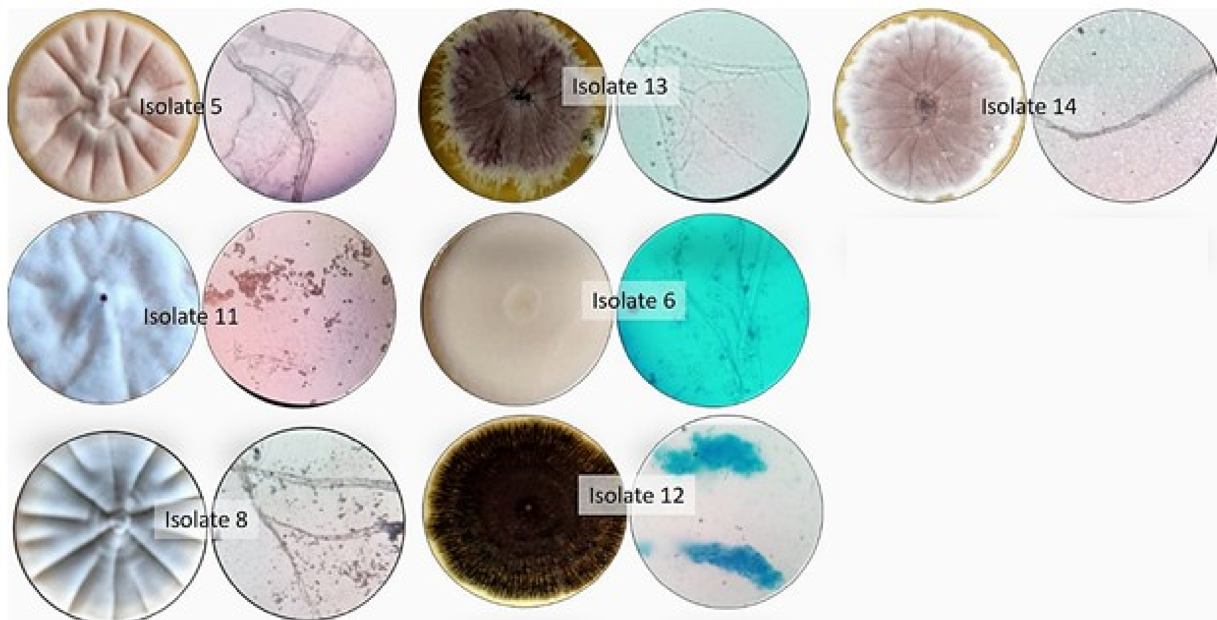


Figure 2: Colony and cell characteristics of some of the 7 isolates from banana orchards

Table 5: Nucleotide blast results of nearest neighbors of endophyte isolates recovered from banana orchards

| Isolate | Number of base pairs | Accession Number | Nearest neighbor in Blast | Identity (%) |
|---|---|---|---|---|
| 5 | 852 | KJ956023.1 | *Fungal sp*. | 98 |
| | | MK267452.1 | *Penicillium christenseniae* | 98 |
| | | KF938402.1 | *Penicillium commune* | 98 |
| | | *MT561884.1* | *Ogataea polymorpha* | 99 |
| 6 | 596 | MH062890.1 | *Aspergillus awamori* | 98 |
| | | GQ153049.1 | *Eurotiomycetes sp*. DC482 | 98 |
| | | MN839772.1 | *Aspergillus tubingensis* | 98 |
| | | *MN413688.1* | *Rhizopus stolonifer* | 98 |
| 8 | 553 | KR016777.1 | *Fungal endophyte* isolate 7157 | 98 |
| | | MH084714.1 | *Penicillium griseofulvum* strain PSF6 | 99 |
| | | KC143756.1 | *Uncultured Penicillium* clone CEobese401 | 98 |
| | | MW885868.1 | *Penicillium solitum* isolate EFA 214.14 | 99 |
| 11 | 1404 | KX058073.1 | *Marasmius tricolor* strain 3 | 96 |
| | | ON038715.1 | *Mortierella sp*. strain L3 | 95 |
| | | KC965591.1 | *Uncultured fungus* clone 87_NA10_P31_K10 | 96 |
| | | *DQ420854.1* | *Uncultured soil fungus clone 151a7* | 96 |
| 12 | 558 | MH248271.1 | *Fusarium nygamai* isolate AMUFN-1 | 99 |
| | | KY949602.1 | *Fusarium oxysporum* strain GR5F70 | 100 |
| | | GQ923973.1 | *Hypocreales sp*. O4i85H | 99 |
| | | FJ362205.1 | *Uncultured root-associated fungus* clone YL0c3P | 99 |
| 13 | 587 | EU002982.1 | *Uncultured Trichocomaceae* clone 7e | 99 |
| | | JQ697533.1 | *Penicillium janthinellum* | 98 |
| | | MK450712.1 | *Penicillium raperi* | 98 |
| | | MF351535.1 | *Penicillium simplicissimum* | 99 |
| 14 | 600 | JQ736021.1 | *Alternaria tenuissima* | 99 |
| | | JQ759881.1 | *Dothideomycetes sp.* | 99 |
| | | KU898065.1 | *Corynespora sp.* | 99 |
| | | KT290973.1 | *Fungal endophyte* strain DC-3 | 98 |

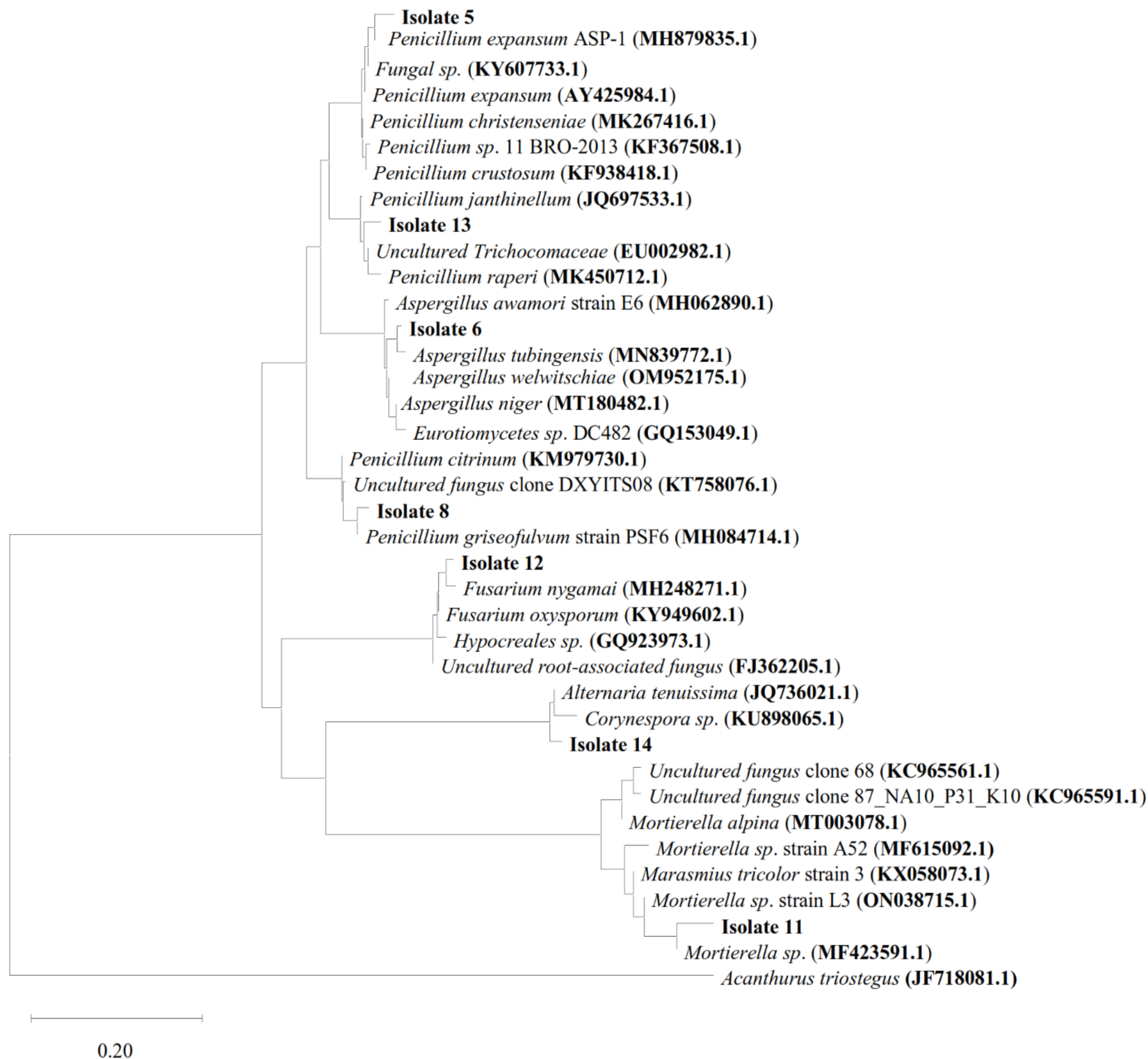


Figure 3: Evolutionary relationships of endophyte isolates associated with bananas to other fungal groups.

Since many banana growers were not aware of nematodes presence and if they were, they lack of knowledge on applicable control methods or attribute nematodes symptoms with other abiotic factors (Brooks, 2004). However, a small portion (7%) of banana farmers in the area were using synthetic Nematicides as a nematode management strategy. Unfortunately, most of them use the chemicals indiscriminately, some of them applying the chemicals without putting protective gears. Synthetic chemicals have become a primary part of present day agriculture, and play a notable role in increasing agricultural output. However, the excessively and wide use of chemicals characterizes one of the major ecological and public health complications all over the biosphere (Keikotlhaile and Spanoghe, 2011; Bechem and Afanga, 2018). About 31.2% farmers were reported to have vast knowledge and skills and practiced Good Agricultural Practices (GAP) as the main strategy to control pests and diseases in their farms, also reported by Wachira *et al*. (2013). Some farmers (16.6%)

were reported to mix charcoal and ashes together grind them until form a powder then pour the powder into the affected banana stool as a nematode control. Ashes is an excellent soil amendment to overly acidic soils. Application of a mixture of ashes and charcoal helps to moderate soil PH, which in turns buffers the beneficial microorganisms in the soil which are antagonistic to nematodes hence suppress their population. Furthermore, adding ashes as a soil amendment could raise the pH of the soil habitat. Due to lack of Good Agricultural Practices in the Taveta, soils have lower pH which increases gall formation by root-knot nematodes. Therefore, adding ashes will raise the pH hence reducing the activities of nematodes in the soil and decrease the infection caused by cyst nematodes.

The nematode pool was similar irrespective of the farming system adopted by the farmers. The distribution and spread of nematodes in the banana farms has been catalyzed by several agronomic practices by the famer. The type of irrigation applied in the farm, source of planting materials tools or equipment's used in the farm. Among the identified nematodes were *pratylenchus* which have been discussed as among the major pests in banana fields and other crops in the field (Coyne et al., 2018). *Pratylenchus* acts by penetrating the roots of the plant and therefore making a secondary way for other microbes which they cause primary infections to plants, which then favor secondary infections by fungi and bacteria (Fourie et al., 2017; Nyang'au et al., 2021; Pusztahelyi et al., 2015). Other identified species includes Root knots nematodes, both present in the soil samples and soil samples. Root knots nematodes generally spend ample time living in the roots of the plants. They cause significance loss and destruction of roots architecture and cause tissue cavities, high density of RKN cause root galls, which impede the flow of water and nutrients resulting in reduced growth of banana bunches, and poor quality fruit yields (Aravind et al., 2010). Free-living nematodes were the most abundant found in all the samples both soil and roots. Free living are a very important organisms in the soil niche that help to maintain soil ecological dynamics especially in soil with poor nutrients. They help in microbial colonization of substrates and mineralization of nutrients by disseminating microbial propagules in the soil. The free living feeds on other soil microorganisms including plant pathogens (bacteria, fungi and plant parasitic nematodes). Other identified species include *Tylenchus*, *Aphelenchus Aphelechoides* and *Tylenchorynchus* both in the soil samples and roots samples.

Plant parasitic nematodes management has relied on chemical soil fumigants and synthetic Nematicides (Luc et al., 2005; Lambert and Bekal, 2002). Despite the fact that several demerits have been linked with the utilization of these chemicals, such as pollution of water, soil, killing both targeted and non-targeted organisms (Achparaki et al., 2012). In this study, application of bio control was shown to have a positive impact on suppression of nematodes in banana stools. There was a significant change in population of RKN and free living in the plots treated with Tithonian tea and Nimbecidine. Tithonian tea had the greatest effect on the nematodes and free living, therefore considered as the most effective in controlling nematodes, followed by Nimbecidine and lastly cow pea. The difference on the effects could be attributed to mode of action and/or due to difference in active chemical compounds and concentration of toxic compounds found in the treatments. Application of Tithonian tea brought variations in soil features which possibly adversely affected the survival and reproduction of RKN while encouraging multiplication of free living organisms. In a previous study, PPN population can be suppressed by use of botanicals as reported by literature (Gao et al., 2018; Khalil et al., 2021). The significance of biological compounds in production, evades application of synthetic chemicals, intensified the investigation on botanical pesticides with potential use for nematode control (Mishra et al., 2018). In this study application of bio pesticides was found to suppress the population of RKN, *Helicotylenchus, Tylenchus, and pratylenchus, Aphelenchoides, Aphelen-*

*chus* and *Tylenchorychus* while increasing the population of the free living organisms, which in turns reduce the population of PPN.
Molecular characterization revealed taxa associated with members of *Penicillium*, *Aspergillus*, *Mortierella* and *Uncultured* fungal genera as nearest neighbors in BLAST. In this study, sequence contigs of Isolate 5, Isolate 8 and Isolate 13 were closely related to *Penicillium* clusters of the *Aspergillaceae* family within Eukaryota. Isolate 5 nearest neighbours includes; *Penicillium commune, Penicillium expansum, Penicillium sp.* and *Uncultured Penicillium* species with up to 99% similarity. Isolate 8 was closely related to *Penicillium citrinum and Penicillium sp*., with identity of 100% similarity while Isolate 13 was affiliated to *Penicillium janthinellum scoring* 99% similarity.

*Penicillium* is a diverse genus of ascomycetous fungi which is among the most common groups that occur in a wide range of habitats such as soil, vegetation, indoor environments and numerous food products (Visagie et al., 2014). *Penicillium* genus has been impactful in several industries, especially upon discovery of its role in production of penicillin antibiotic that transformed medical industry through development of new approaches to bacterial disease management (Gao et al., 2018; Khalil et al., 2021; Pusztahelyi et al., 2015). Since then, species have been screened for production of new enzymes owing to their ability degrade organic matter in search for carbon resources (Li et al., 2007; Terrasan et al., 2010). Some *Penicillium* species play significant roles as organic matter decomposers and act as pathogens causing rots during preharvest and postharvest food handling (Frisvad and Samson, 2004; Samson et al., 2010), besides producing a wide range of mycotoxins (Frisvad et al., 2004). However, some species have been utilized in production of fermented sausages (López-Díaz et al., 2001; Ludemann et al., 2010) and cheese making (Giraud et al., 2010). These uses are among a wide range of applications, hence genus suggested as having affected every present human livelihood, either as *Penicillium* or one of its products (Frisvad et al., 2004; Visagie et al., 2014).

Sequence contigs of Isolate 6, were closely related to *Aspergillus* cluster of the *Aspergillaceae* family within Eukaryota. Its nearest neighbors in BLAST includes; *Aspergillus niger, Aspergillus sp. and Penicillium sp. Aff. Aspergillus* species with up to100% similarity. *Aspergillus* comprises a diverse group of species that have a significant impact in human health, indoor environments, and food production as fermentation agents, besides production of diverse metabolites such as antibiotics, organic acids, medicines, and enzymes with various applications in biotechnology industries (Samson et al., 2014).

Sequence contigs of Isolate 11, were closely related to *Mortierella* cluster of the *Mortierellaceae* family within Eukaryota. Its nearest neighbors in BLAST includes; *Mortierella alpina, Mortierella sp.* and *Uncultured fungal* species with up to100% similarity. *Mortierella* refers to a soil fungal *genus* of the order *Mortierellaceae* within Eukaryota commonly found in the rhizosphere, soil, and plants tissues besides being present in extremely hostile environments (Berg and Smalla, 2009). *Mortierella* sp. are saprotrophic microorganisms, hence valuable decomposers important in management of nutrients in agricultural ecosystems (Shi et al., 2014). This is enabled by their ability to survive under unfavorable conditions besides ability to utilize carbon sources within biopolymers such as cellulose, hemicellulose and chitin which present them as efficient agricultural inoculants. *Mortierella* sp. have been reported to improve access to the biologically available forms of soil phosphorus and iron and are known to protect of plants agricultural pathogens. In addition, they have been reported to promote synthesis of plant hormones and 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme useful in plant metabolism (Ozimek et al., 2021). There is a marked rise in applications of *Mortierella* spp. due its potential in promoting nutrient uptake efficiency, crop protection, and reduce reliance on nematicides (Ozimek et al., 2021). Contigs of Isolate 12, were closely related to *Fusarium oxysporum*, *Uncultured Asco-*

*mycota, Uncultured fungal* species with up to 99% similarity. This was among the suspected novel taxa that require further investigation to elucidate their possible application in the day to day life.

## CONCLUSION

Banana farmers in the Taveta sub-county were not aware of nematodes as a biotic pests affecting production; and those informed have been using Nematicides manage nematodes. In this study application of Cow peas intercrop, Tithonia plant extracts and Nimbecidine bio pesticides were found to suppress the population of RKN, *Helicotylenchus, Tylenchus, and pratylenchus, Aphelenchoides, Aphelenchus* and *Tylenchorychus* while increasing the population of the free living organisms, which further reduces the population of PPN. The partial sequences analysis showed that endophytes were closely associated with members of *Penicillium*, *Aspergillus*, *Mortierella* and *Uncultured* fungal genera with a range of identity scores of 95 – 100%. The unique endophytes isolated found to be closely related to uncultured groups need to be further confirmed and characterized by fatty acid analyses and DNA-DNA hybridization methods.

### Competing interests

The authors declare that they have no conflict of interest.

### Acknowledgements

The authors would like to thank the Taita Taveta University (TTU) and International Center for Insect Physiology and Ecology (ICIPE) for Financial and in-kind support during the study period. We also thank the farmers in Taveta, for contributing valuable information that was used in this study and allowing the selected farms to be used as experimental sites.

### References

Achparaki, M., Thessalonikeos, E., Tsoukali, H., Mastrogianni, O., Zaggelidou, E., Chatzinikolaou, F., Vasilliades, N., Raikos, N., Isabirye, M., Raju, D. V. ., Kitutu, M., Yemeline, V., Deckers, J., and J. Poesen Additional. (2012). Impacts of Agricultural Toxicity on Non-Target Organisms in Aquatic Ecosystem. *Intech*, 1-13.

Altieri, M. a, and Nicholls, C. I. (2012). Sustainable Agriculture Reviews. In Sustainable Agriculture Reviews (Vol. 11). https://doi.org/10.1007/978-94-007-5449-2

Aravind R, Eapen SJ, Kumar A, Dinu A and Ramana KV (2010). Screening of endophytic bacteria and evaluation of selected isolates for suppression of burrowing nematode (*Radopholus similis* Thorne) using three varieties of black pepper (*Piper nigrum* L.). Crop Prot 29:318–324.

Bechem, E. T., and Afanga, Y. A. (2018). Morphological and molecular identification of fungi associated with corm rot and blight symptoms on plantain (*Musa paradisiaca*) in macro-propagators. International Journal of Biological and Chemical Sciences, 11(6), 2793.

Berg, G.; Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol. Ecol., 68, 1–13

Brooks, F. E. (2004). Plant-parasitic nematodes of banana in American Samoa. Nematropica 34:65-72.

Coyne, D. L., Cortada, L., Dalzell, J. J., Claudius-Cole, A. O., Haukeland, S., Luambano, N., and Talwana, H. (2018). Plant-parasitic nematodes and food security in Sub-Saharan Africa. Annual Review of Phytopathology, *56*(August), 381–403.

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N.K., Bhatnagar, S., and Sundaresan, V. (2015). Structure, variation, and assembly of the root- associated microbiomesofrice.Proceedings of the National Academy of Sciences, 112, E911–E920.from west Bengal, India. Journal of plant protection research Vol.50, No.3

Fourie, H., Spaull, V. W., Jones, R. K., Daneel, M. S., and De Waele, D. (2017). Nematology in South Africa: A View from the 21st Century. Nematology in South Africa: A View from the

21st Century, 1–558. https://doi.org/10.1007/978-3-319-44210-5

Frisvad J.C., Samson R.A. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne *Terverticillate Penicillia* and their mycotoxins. Studies in Mycology; 49:1–174.

Frisvad J.C., Smedsgaard J., and T.O Larsen (2004). Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Studies in Mycology; **49**:201–241.

Gao, H., Li, G., and Lou, H. X. (2018). Structural diversity and biological activities of novel secondary metabolites from endophytes. Molecules, *23*(3).

Giraud F., Giraud T., Aguileta G. (2010) Microsatellite loci to recognize species for the cheese starter and contaminating strains associated with cheese manufacturing. International Journal of Food Microbiology; 137:204–213.

Hassan, S. E. D. (2017). Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium* L. Journal of Advanced Research, *8*(6), 687–695.

Jones, D.R. (2000). Diseases of banana, Abaca and Enset. CABI, London, UK

Keikotlhaile, B. M., and Spanoghe, P. (2011). Pesticide Residues in Fruits and Vegetables. Intech, 13, 243-252.

Khalil, A. M. A., Hassan, S. E. D., Alsharif, S. M., Eid, A. M., Ewais, E. E. D., Azab, E., Gobouri, A. A., Elkelish, A., and Fouda, A. (2021). Isolation and characterization of fungal endophytes isolated from medicinal plant ephedra pachyclada as plant growth-promoting. Biomolecules, 11(2), 1–18.

Lambert, K., and Bekal, S. (2002). Introduction to Plant-Parasitic Nematodes. In The Plant Health Instructor. https://doi.org/10.1094/phi-i-2002-1218-01

Li Y., Cui F., Liu Z. (2007). Improvement of *xylanase* production by *Penicillium oxalicum* ZH-30 using response surface methodology. Enzyme and Microbial Technology, 40:1381–1388.

López-Díaz T-M, Santos J-A, García-L opez M-L, et al. (2001). Surface myco- flora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates. Intern. Journal of Food Microbiology 68: 69 -74.

Luc, M., Sikora, R. A., and Bridge, J. (2005a). Plant parasitic nematodes in subtropical and tropical agriculture: Second Edition. In M. Luc, R. A. Sikora, and J. Bridge (Eds.), Plant Parasitic Nematodes in Subtropical and Tropical Agriculture: Second Edition (2nd ed., Issue May 2005). c-A·B International 1990.

Ludemann V., Greco M. and Rodríguez M.P. (2010). Conidial production by *Penicillium nalgiovense* for use as starter cultures in dry fermented sausages by solid state fermentation. LWT – Food Science and Technology, 43:315–318.

Mishra, S. D. (2018). Effects of an aqueous extracts of green leaves of neem, madar and tulsion the hatching of eggs of root-knot nematode *Meloidogyne incognita*. Current Nematology, 7(2), 133-13

Njenga Joshua Kinene. (2019). Efficacy of Neem, Tithonia And Tephrosia Leaf Extracts In Management Of Root-Knot Nematodes In French Beans (*Phaseolus vulgaris* L.) In Tharaka Nithi County. Chuka University.

Nyang'au, D., Atandi, J., Cortada, L., Nchore, S., Mwangi, M., and Coyne, D. (2021). Diversity of nematodes on banana (*Musa spp*.) in Kenya linked to altitude and with a focus on the pathogenicity of Pratylenchus goodeyi. Nematology, *32*(48), 1–11.

Pattison, A. B., Stanton, J. M., Cobon, J. A., and Doogan, V. J. (2002). Population dynamics and economic threshold of the nematodes Radopholus similis and pratylenchus goodeyi on banana in Australia. International Journal of Pest Management, 48(2), 107–112.

Ozimek, Ewa, and Agnieszka Hanaka. 2021. "*Mortierella* Species as the Plant Growth-Promoting Fungi Present in the Agricultural Soils" Agriculture 11, 1: 7.

Petsakos, A., Prager, S. D., Gonzalez, C. E., Gama,

A. C., Sulser, T. B., Gbegbelegbe, S., Kikulwe, E. M., and Hareau, G. (2019). Understanding the consequences of changes in the production frontiers for roots, tubers and bananas. Global Food Security, 20(October 2018), 180–188.

Pusztahelyi, T., Holb, I. J., and Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. Frontiers in Plant Science, 6(AUG), 1–23. https://doi.org/10.3389/fpls.2015.00573

Scott, G. J. (2020). A review of root, tuber and banana crops in developing countries: past, present and future. International Journal of Food Science and Technology, *56*(3), 1–22.

Suresh, K., Thomas, S. V., and Suresh, G. (2011). Design, data analysis and sampling techniques for clinical research. Annals of Indian Academy of Neurology, 14(4), 287–290.

TTC. (2021). The County Government of Taita Taveta 2021 County Fiscal Strategy Paper (Issue February).

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Samson R.A., Houbraken J., Thrane U. (2010). CBS KNAW Biodiversity Center; Utrecht: Food and indoor fungi.

Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S. B., Hubka, V., Klaassen, C. H., Perrone, G., Seifert, K. A., Susca, A., Tanney, J. B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T., and Frisvad, J. C. (2014). Phylogeny, identification and nomenclature of the genus Aspergillus. Studies in mycology, *78*, 141–173.

Shi, L.L.; Mortimer, P.E.; Ferry Slik, J.W.; Zou, X.-M.; Xu, J.; Feng, W.-T.; Qiao, L. (2014). Variation in forest soil fungal diversity along a latitudinal gradient. Fungal Divers, 64, 305–315.

Strobel G, Daisy B, Castillo U and Harper J (2004). Natural products from endophytic microorganisms. J. Nat. Prod. 67: 257-268.

Tamura K., Nei M., and Kumar S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030-11035.

Tamura K., Stecher G., and Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution https://doi.org/10.1093/molbev/msab120.

Terrasan C.R.F., Temer B., Duarte M.C.T. Production of *xylanolytic* enzymes by *Penicillium janczewskii*. Bioresource Technology, **101**:4139–4143

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H., Perrone, G., Seifert, K. A., Varga, J., Yaguchi, T., and Samson, R. A. (2014). Identification and nomenclature of the genus *Penicillium*. Studies in mycology, *78*, 343–371.

Wachira, P. M., S., Okoth, J., Kimenju and R. R. Mibey, (2009). Influence of land use and soil management practices on the occurrence of nematode destroying fungi in Taita Taveta. Kenya. Tropical and subtropical agroecosystems, 10; 213-223.

Wang, K.H. and C. R. R. Hooks. (2009). Plant-parasitic nematodes and their associated natural enemies within banana (*Musa spp*.) plantings in Hawaii. Nematropica 39:57-73.

White T.J., Bruns T., Lee S. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Shinsky T.J., White T.J., editors. PCR protocols: a guide to methods and applications. Academic Press Inc; New York: pp. 315–322.

**Blank Page**