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Identification and quantification of soil borne root rot pathogens communities in smallholder agro-ecosystems of Kenya

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Abstract

The root rot disease complex has continued to be a major constraint in the production of common beans (*Phaseolus vulgaris*) resulting in losses of up to 70% in Kenya. The aim of this study was to establish (i) the occurrence and quantification of root rot fungal pathogens of common bean in Western Kenya and (ii) the effect of farming practices on the populations of the pathogens. A survey was conducted in Western Kenya's LM1 LM2 UN1 and UM3 AEZ's to obtain data on different farming practices and soil characteristics. Pathogens were isolated and identified using morphological and molecular techniques. Soil pH ranged from 4.59 to 6.01, Percent carbon and nitrogen ranged from 9.8g/Kg0 to 19g/Kg and 0.8 g/Kg to 1.5g/Kg. All farms were infected with root rot fungi, including *Fusarium solani, Pythium ultimum, Rhizoctonia solani* and *Macrophomina phaseolina*. *Fusarium* spp. was the most abundant with the highest populations of 62 X 10^3 cfu/g soil recorded in lower midland zone 2. The isolation frequency of *Fusarium* spp., *Pythium* spp. and *Rhizoctonia* spp. was high in upper midland zone 1. Quantification of genomic DNA from soil by qPCR was highest for *Rhizoctonia solani* (2.23X10° pg μL^{-1}). Sand had a positive correlation with *Pythium ultimum* DNA and *Rhizoctonia solani* DNA while clay had a negative correlation with *Fusarium spp.* and *Rhizoctonia solani* DNA. In conclusion, soil properties, management practices and elevation affected root rot pathogen populations and should be considered when developing management strategies.

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Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes in the world and a key source of human dietary protein, calories and fiber (Katungi *et al*., 2011). It is also a component in the improvement of rural lively hoods in Eastern Africa through its production and marketing systems (Katungi *et al*., 2009; Birach *et al*., 2011). In Kenya it is a major source of protein for many households but its production has not kept pace with the demand due to severity of biophysical stress such as climatic variability, insect pests, diseases and declining soil fertility (Kimiti *et al*., 2009; Odendo *et al*., 2004). These stresses lead to bean productivity to less than 25% of the potential yield. In western Kenya, common bean is mostly grown by small holder farmers with limited resources leading to intensifying the production of common bean with low in put addition and elimination of regenerative fallowing (Sanchez, 2002) leading to increase in root rot diseases. The increase in importance of soil borne root rot diseases in these regions could be related to a decline in soil productivity found to be greatest in areas with low soil fertility status (Wortmann *et al.,* 1998).

Of the biophysical stresses, soil borne diseases are of great importance in the production of common beans in western Kenya with losses of up to 70% being reported due to root rot diseases (Otsyula *et al*., 2003). These diseases reduce both yield and quality of bean (Abawi *et al*., 2000) and are difficult to control due to the complex of pathogens involved as well as their ability to survive in the soil as saprophytes or as resting spores over long periods of time (Rani and Sudini, 2013).

Root rot diseases of common bean are caused by a complex of soil-borne fungal pathogens which include *Fusarium sp., Pythium sp*, *Macrophomina phaseolina*, and *Rhizoctonia* spp (Nzungize *et al.,* 2012; Mwang'ombe *et al*., 2008). These pathogens may occur in the fields at the same time there by resulting to synergistic interactions leading to higher disease incidences and severity (Ongom *et al*., 2012).

Management of soil borne diseases of common bean has been hindered by the ability of these pathogens to survive in soil for long periods as mycelia, conidia, oospores, sclerotia or chlamydospores. Continuous cultivation of the same crop in the same field for many years also leads to build up in soil borne pathogen inoculum leading to increased infections (Marzano, 2012). However, Meenu *et al*., 2010 reported that employing of agronomic practices such as crop rotation, deep tillage, fallowing and application of organic amendments reduces disease inoculum density in the soil, deprives the pathogen of its host and creates conditions that favour the growth and development of microorganisms that are antagonistic to plant pathogen. These practices have also been shown to have positive changes in the soil structure and root rot disease dynamics leading to increased yields (Bailey and Lazarovits, 2003). Farmers have been introduced to application of organic amendments (Medvecky *et al*., 2007) to address the decline in fertility levels though the relationships between organic input type, soil borne pathogen dynamics, and soil characteristics have however not been well understood (Medvecky, 2007) despite the efforts to evaluate root rot severity as influenced by organic inputs (Otsyula *et al*., 1999).

The objectives of this study were (i) to assess the prevalence of common bean root rot pathogens in different agro ecological zones of western Kenya, (ii) to characterize and quantify root rot pathogens using molecular techniques, and (iii) to establish the effect of different farming practices on root rot fungal populations.

Material and Methods

Study sites and field selection

The study was carried out in four agro ecological zones of western Kenya referred to as: lower

midland zone 1 (LM1), lower midland zone 2 (LM2), upper midland zone 1 (UM1) and upper midland zone 3 (UM3) (Fig. 1).

All these regions are characterized by a bimodal rainfall with a long rain season from March to July and short rains from September to November allowing for two cropping seasons per year (Jaetzold and Schmidt, 1983). Lower midland zone 1 is situated in an elevation ranging between 1300-1500 M ASL and characterized by mean temperatures of 21.0^{o}C -22.2^{o}C and rainfall of 750-850mm during the long rains and 550-730mm. The LM2 has mean temperatures of 21.4-22.3^{o}C and rainfall of 600-650mm during the long rains and 460-480mm during the short rains. Upper midland humid (UM1) situated at an elevation of 1500-1900M ASL is characterized by mean temperature of 18.5^{o}C-21.0^{o}C and rainfall of 700- 1000 mm during long rains and 650-800mm during the short rains. Upper midland humid (UM3) situated at an elevation of 1500-1900 M ASL is characterized by mean temperatures of 18.8-21.0^{o}C and rainfall of 550-650mm during long rains and 450-580mm during the short rains (Jaetzold and Schmidt, 1983).

The farms were located in the different agro-ecological zones with varying soil types. Lower midland humid had farms with predominantly ferrasols while LM2 had farms with both gleysols and acrisols as was the case with UM3. Upper midland humid was however predominated by both ferrasols and acrisols.

In March 2013 a survey was taken from all 60 farmers using a semi structured questionnaire to characterize the farming systems and input management as well as their knowledge of root rot disease on beans.

Soil sampling

In each farmer field a composite soil sample was taken from an area of 475m² up to a depth of 20cm by taking 250-300g of soil from 13 points randomly selected on two concentric circles using an auger with a diameter of 7cm. After homogenizing by hand a total of 1kg of soil was taken from each field and stored in a sealed plastic bag. The soil samples were transported to the laboratory in a cooler box and there stored at 4^{o}C. All sampling tools were thoroughly washed and then sterilized with 70% ethanol between sampling different fields to avoid contamination.

Analysis of soil properties

The particle size distribution of the three fractions (sand, silt and clay) for soil sample composite for each filed was determined using the hydrometer method (Bouyoucos, 1962).

Soil pH was determined for all samples by mixing 25g subsamples with 50mL of distilled water, and measuring with a glass membrane electrode (MRC Ltd., Tel-Aviv Israel).

All sixty soil samples were characterized for total nitrogen (% N), available nitrogen (NO_3^- and NH_4^+), organic carbon (% OC), available phosphorus (P_2O_5), exchangeable potassium (K_2O_5), calcium (Ca_2^+), magnesium (Mg^{++}) copper (Cu^{++}) zinc (Zn^{++}) and boron (B) ions. Soil organic carbon content was determined by acid digestion and titration according to Walkley and Black (1934). Total N was determined by the micro-Kjeldhal distillation method as described by Bremner (1996). Extractable NO_3^- and NH_4^+ were determined on 1M KCl extracts measured using a colorimetric assay described by Bremner *et al*. (1965). Extractable phosphorus (P) was determined on 0.1M bicarbonate extracts measured using a colorimetric assay described by Olsen *et al*. (1954).

Exchangeable Ca^{++} and Mg^{++} of soils were determined on 1M KCl extracts measured using an atomic adsorption spectrophotometer (Buck Scientific Inc., Norwalk, USA). Exchangeable K^+ was determined on 0.1M CaCl2 measured using a flame photometer (Sherwood Scientific Ltd, Cambridge, UK).

Isolation and quantification of fungal flora from the soils

Soil inhabiting fungi were isolated from soil samples collected from the sixty sites following storage at 4°C. Three sub samples each weighing 1g were taken from each 1 kilogram of soil, dissolved in 10ml sterile distilled water in three different universal bottles, mixed by shaking for 1 minute followed by a 10-fold serial dilution series for each sample to achieve a 10^{-4} dilution. One milliliter of 10^{-3} and 10^{-4} dilutions were plated on potato dextrose agar (PDA-HIMEDIA®) medium using pour plate method.

The PDA had been amended with 50ppm streptomycin sulphate antibiotic to suppress bacterial growth. Each dilution was replicated three times and incubated for 7 days at room temperature. Different fungal colonies were counted and quantified per gram of soil. These were then sub cultured on fresh PDA medium and upon identification, different genera of fungi were sub-cultured on different media. *Fusarium spp.* was sub-cultured on Spezieller Nährstoffarmer agar - SNA (Nirenberg, 1981) and PDA media. Cultures on SNA were incubated under UV light to facilitate sporulation while those on PDA were incubated at 25°C for 14- 21 d to study cultural characteristics. *Fusarium* isolates were identified to species level based on their morphological characteristics following Nelson *et al*. 1983 and the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Identification of other fungi was based on morphological and cultural features such as colour of the colony, growth type colour of mycelia and spore types (Zhou *et al.,* 2010).

The number of colony forming units of each fungal type per gram soil was also calculated by multiplying the number of colonies by the dilution factor. *Pythium* sp. were sub cultured on corn meal agar to observe for production of sporangia, oogonia and antheridia used in identification based on keys by Plaats-Niterink (1981) and Dick (1990).

Relative isolation frequency of each genus as well as each species was calculated using the formula by Gonzalez *et al.,* 1999. All the fungal isolates were stored on PDA slants at 4°C for further identification by gene sequencing.

$$\text{Frequency } (\%) = \frac{number\ of\ isolates\ of\ a\ genus}{total\ number\ of\ a\ genus} \text{ x } 100$$

Gene sequencing of soil borne fungi isolates

Extraction of DNA from soils and root rot pathogens

DNA extraction was carried out on all soil samples collected from the 60 farms during the survey period. Twenty grams from each of the sixty soil samples were stored at -20°C until they were processed. Total microbial DNA was extracted from 0.25 g (fresh weight) of each soil samples using a Power Soil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with minor modifications of using a bead beater (Bio Spec 1001 Mini-Beadbeater-96 Cell Disruptor, Bartlesville, OK, USA) at high speed for 10 minutes. The DNA was then lyophilized and stored at −20°C until it was used for further downstream processes (Fillion *et al*., 2003).

Fungal cultures of all known root rot isolates that were obtained from soil samples were grown for seven days on PDA (HIMEDIA®) in 9-cm diameter petri dishes and incubated at 25°C. Mycelia were gently scrubbed and collected from the surface of the medium with a sterile glass slide after adding sterile distilled water containing 0.05% (v/v) Tween 80. The mycelial suspension was then transferred to a 1.5ml micro tube and centrifuged at 3000g, at 4°C for 5 min. The supernatant was discarded and the resultant pellet used for DNA extraction. The DNA was extracted using a phenol and chloroform protocol, followed by isopropanol precipitation following the procedure by González-Mendoza *et al.* 2010 with minor modifications. The extracted DNA samples were then lyophilised and stored at -20°C at IITA-ICIPE, Nairobi Kenya before being used at a later stage.

The fungal and soil DNA was rehydrated with 50µL and 100µL of nuclease free water respectively. It was then quantified using the Qubit® 2.0 Flourometer at the Biotechnology Research Center of Cornell University Ithaca, NY. USA.

DNA amplification by Polymerase Chain Reaction and molecular identification of isolates

Conventional polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region of the fungal pathogens using universal primers ITS1 and ITS4. A reaction volume of 50ºL containing 10µL nuclease free water (IDT), 25.0ºL of IQ SYBR Green Super Mix 2X (Bio Rad 170-8880), 5µl of each primer (2µM) [ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCT TATTGATATGC-3')] White *et al*., 1990 and 5ºL of DNA template was used for all the pathogens. PCR amplifications were done as previously described by (White *et al*., 1999) in a BIO RAD T100 thermal cycler. The PCR program used for *Fusarium spp, Rhizoctonia spp, Macrophomina spp. and Paecilomyces spp* were an initial denaturation at 95ºC for 3 min, followed by 25 cycles of denaturation at 95ºC for 30s, annealing at 50ºC for 30 s and extension at 72ºC for 1 min with a final step of extension at 72ºC for 10 min at the end of the amplification reaction. For *Pythium spp,* the annealing temperature was changed to 58ºC with the other parameters being maintained. Electrophoresis and estimation of the size of the PCR amplicons was undertaken following the procedure by (Fillion *et al*., 2003). Thirty two (32) PCR amplicons were purified with the Wizard PCR Clean Up System (Promenga, USA) as per the manufacturer's instructions. Twelve and a half microlitres (12.5µl) of each amplicon was then mixed with 2.5µl of the forward primer (ITS 1) and then submitted to the Biotechnology resource center (BRC Genomics facility, Institute of Biotechnology Cornell University Ithaca, NY USA) for sequencing. ITS sequences of isolates of *Pythium, Rhizoctonia* and *Macrophomina* were compared with ITS sequences of known species available in the GenBank database by performing nucleotide blast search at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Gnbnk/). Whereas the sequences from *Fusarium* isolates were subjected to BLAST analyses in the FUSARIUM-ID v. 1.0 database (http://fusarium .cbio.psu.edu) (Geiser *et al*., 2004).

Quantification of root rot fungal DNA in soils sampled using Real-time PCR

Quantitative PCR amplifications were performed using ABI ViiA7 Real-Time PCR system (Life Technologies, USA) in a total volume of 20ºL on a 96 well plate. The 20ºL reaction mixtures contained a final concentration of (2X) IQ SYBR Green Supermix (BioRad), 2µm each of forward and reverse primers for respective fungi, 1ºL of soil DNA template and sterile Nuclease free water. Primers used were; *F. solani*- AFP346 (5'GTATGTTCACAGGGTTGATG3') Lievens *et al.,* 2006 and ITS1f (5'CTTGGTCATTTAGAGGAAGTAA 3') Gardes & Bruns 1993; *P. ulimum* - AFP276 (5' TGTATGGAGACGCTGCATT3') Lievens *et al.,* 2005 and ITS4 (5'TCCTCCGCTTATTGATATGC3') White *et al*., 1990; *R. solani* - ST-RS1 (5'AGTGTTATGCTTGGTTCCACT3') Lievens *et al*., 2005 and ITS4 (White *et al*., 1990); *M. phaseolina* primers were designed based on the available *M. phaseolina* sequences' at NCBI database to give a product length of 218 base pairs. The sequences of the primers used was Upper Primer (5'TCCCGATCCTCCCACCCTTTG TAT3'), and Lower Primer (5'CATTTCGCTGCGT TCTTCATC3'). The samples were run in triplicate and the thermal-cycling conditions for amplification were; *F. solani* an initial denaturation at 95ºC for 3 min, followed by 40 cycles each consisting of a denaturation step at 95ºC for 15 s, annealing at 58ºC for 30 s and a final step at 72ºC for 30s; *P. ultimum and R. solani*, the thermal-cycling conditions were an initial denaturation of 95ºC for 3 min, followed by 40 cycles each consisting of a denaturation step at 95ºC for 15 s, annealing at 60ºC for 30 s and a

final step at 72°C for 30s. *M. phaseolina* thermal-cycling conditions were an initial denaturation of 95°C for 15 min, followed by 40 cycles each consisting of a denaturation step at 94°C for 15 s, annealing at 60°C for 30s and a final step at 72°C for 30s The amplification results were analysed with ABI ViiA7 Real-time PCR Software v 1.2 (Life Technologies, USA).

Standard curve and qPCR efficiency

The standard curves were generated using a sevenfold dilutions of each of the fungal DNA. The primers used for *F. Solani, P. ultimum, R. solani* and *M. phaseolina* were AFP 346 and ITS1f; AFP276 and ITS4; ST-RS1 and ITS4; and upper and lower primers respectively. Cycle threshold (Ct) values were calculated by the ABI ViiA7 Real-time PCR software v 1.2 (Life Technologies, USA). These values were used to generate standard curves by plotting them (ct) versus the logarithm of the concentration of each 10-fold dilution series of fungal genomic DNA. In every qPCR run, seven of the respective DNA dilutions (10;1; 0.1; 0.01; 0.001; 0.0001 and 0.00001ng) with three replicates of each were included in the plate to interpolate the amplification results to the absolute quantity of the target in each sample since Ct values may slightly vary between experiments (Fillion *et al*., 2003).

Data Analysis

Data collected at survey with the help of a semi-structured questionnaire was analyzed using IBM Statistical package for social science (SPSS) version 20 and for the laboratory data (soil particle size percentages, counts of different fungi, soil pH measurements, different soil nutrient counts) analysis of variance (ANOVA) was performed using GENSTAT version 16 and the Tukey test Least Significant difference (LSD) used for mean separation at 5% level of significance. Permutation multivariate analysis of variance of the "Biodiversity R" package was used to address the relative importance of edaphic characteristics, spatial distance among ago-ecological zones, the sites, farm management practices and fungal community composition. The fungal operational taxonomic unit abundances across samples were standardized following auto transformation in R. Bray–Curtis distances of the environment and fungal species communities were used for non-metric multidimensional scaling (NMDS) analyses. Confidence ellipses at 95% confidence interval for the successional stages were calculated with the function 'ordiellipse' in 'Biodiversity R' package.

The DNA quantified from the soils was subjected to a correlation analysis with soil properties and the fungal populations obtained from the laboratory. This was done using IBM Statistical package for social science (SPSS) version 20.

Results

Farming system characteristics

The total farm size and the proportion cultivated with common bean in the different agro-ecological zones exhibited pronounced differences. A majority (81.25%) of the farmers in all the AEZ's had less than 2 acres under bean production and 8.1% of the farmers produced beans on more than 5 acres of land across the four AEZ's. The duration of land use varied across the farmers and the different regions. Majority (47.32%) of the farmers had used their farms for cultivation for over 20 years.

Forty seven percent of the sampled farmers undertake crop rotation on their farms. The proportion of farmers who did not undertake crop rotation was 52.1% (Table 3). Manure application was undertaken by 63% of the respondents while 37% do not apply manure. Lower midland humid had the highest percentage of farmers who undertake manure application while UM3 had the lowest. Fifty three percent of farmers in UM3 do not apply manure accounting for the highest percentage across all the AEZ's. Tillage practices did not have a major influence on the root rot communities.

Table 1. Proportion (%) of farmers and the corresponding farm size and respective acreage under beans in different regions of Western Kenya.

Agro-ecological zone	Total farm Size (ha)				Acreage under beans (ha)		
	0.2-0.8	0.81-2	2.1-4	>4	0.2-0.8	0.81-2	2.1-4
LM1 (n=7)	29	71	-	-	100	-	-
LM2 (n=10)	10	60	30	-	60	30	10
UM1 (n=11)	46	55	-	-	90	9	-
UM3 (n=32)	22	47	22	9	75	19	6
Mean	26	58	26	9	81	19	8

LM1- Lower midland humid; LM2- Lower midland sub humid; UM1- Upper midland humid; UM3- Upper midland semi humid

Table 2. Duration of land use for cultivation of crops in different regions of Western Kenya.

Agro-ecological zone	≤5	>5 to 10 years	>10 to 20 years	>20 years
LM1 (n=7)	0	0	57	43
LM2 (n=10)	10	0	30	60
UM1 (n=11)	18	27	18	36
UM3 (n=32)	0	22	28	50
Mean	7	12	33	47

LM1- Lower midland humid; LM2- Lower midland sub humid; UM1- Upper midland humid; UM3- Upper midland semi humid.

Soil properties of agro-ecological zones

The fractions of clay and sand in soils showed significant differences between the four AEZ (Table 4). Farms in UM3 and LM2 were significantly different from farms in UM1 and LM1. Farms in UM3 had soils with the highest percentage of clay while farms in LM1 had soils with the lowest percent clay. The exact opposite was observed for clay particles. The fraction of silt in soils did not exhibit significant differences between AEZ, with UM1 having highest mean silt content and UM3 the lowest. The pH of soils demonstrated significant differences between the four AEZ (Table 4). All the four AEZ's were significantly different with farms in UM3 having the highest pH those in LM1 had the lowest pH. Significant differences ($p<0.05$) were also observed in percent total Carbon and percent Nitrogen.

Lower midland humid and upper midland humid had significantly higher percent carbon and nitrogen than farms in LM2 and UM3. Significant differences were also observed for potassium calcium copper and zinc where UM3 had significantly ($p<0.05$) higher concentrations.

Table 3. Proportion (%) of farmers undertaking crop rotation, manure application, and tillage practices.

Agro-ecological zone	Crop Rotation Practiced on farm		Manure application		Methods of cultivation used on the farm		
	Yes	No	Yes	No	Hand Tillage	Oxen plough	Tractor
LM1 (n=7)	29	71	71	29	43	57	0.0
LM2 (n=10)	70	30	70	30	10	90	0.0
UM1 (n=11)	27	73	64	36	46	55	0.0
UM3 (n=32)	66	34	47	53	3	94	3.
Mean	48	52	63	37	25	74	1

LM1- Lower midland humid; LM2- Lower midland sub humid; UM1- Upper midland humid; UM3- Upper midland semi humid.

Table 4. Selected properties of surface (0 – 20 cm) soils from smallholder farms taken from fields in western Kenya.

AEZ	Clay (g kg^{-1})	Sand (g kg^{-1})	Silt (g kg^{-1})	pH (H_20)	C (g kg^{-1})	N (g kg^{-1})	P (ppm)	K (ppm)	Zn (ppm)	Cu (ppm)	Bo (ppm)	Ca (ppm)	Mg (ppm)	Mn (ppm)	Fe (ppm)
LM1	372_b	467_a	160_a	4.59_d	19.0_a	1.5_a	5.5_a	68_b	2.6_{ab}	2.8_{ab}	0.1_a	497_b	107_a	268_a	104_b
LM2	603_a	253_b	143_a	5.67_b	10.1_b	0.8_b	10.2_a	140_a	1.5_b	2.4_{ab}	0.2_a	879_{ab}	129_a	77_b	170_a
UM1	447_b	386_a	165_a	5.06_c	16.0_a	1.3_a	9.8_a	108_{ab}	4.2_a	3.2_a	0.2_a	1010_a	204_a	249_a	138_{ab}
UM3	623_a	256_b	119_a	6.01_a	9.8_b	0.8_b	10.2_a	133_a	2.2_b	1.9_b	0.2_a	897_{ab}	125_a	114_b	123_b
Mean	511	341	147	5.33	13.7	1.1	8.9	112	2.6	2.6	0.2	821	142	177	134
LSD ($p \leq 0.05$)	102	137	48	0.31	4.7	0.4	6.7	42	1.3	0.9	0.2	361	76.9	38	26
CV %	40	29.4	42.1	11.3	46.8	45.7	145	73.2	98.4	70.9	176.2	85	104.	42.2	38.1

Means followed by same letter(s)within each column are not significantly different at $p \leq 0.05$. AEZ-Agro-ecological zone, LM1- lower midland zone1,LM2- lower midland zone2,UM1- Upper midland zone1, UM3-upper midland zone3. ,LSD: Least significance difference at 5% level, CV: Coefficient of variation.

Fungal communities in soils

All the soils sampled from the four agro-ecological zones in Western Kenya were infected with root rot causing pathogens (Table 5). Soil borne fungi isolated were; *Fusarium* spp*, Pythium* spp, *Macrophomina* spp*, Rhizoctonia* spp, *Trichoderma* spp, *Aspergillus* spp and *Penicillium* spp. Of all the pathogenic fungi isolated,

Fusarium spp. had the highest population mean per gram of soil while *Macrophomina* had the lowest population mean. There was no significant difference across AEZs in respect to fungi isolated at the beginning of the study with the exception of *Rhizoctonia spp.* and *Macrophomina spp.* However, lower midland humid (LM2) had the highest populations of *Fusarium spp* and *Pythium spp* while UM3 and LM1 had the highest populations of *Rhizoctonia spp.* and *Macrophomina spp* respectively. The interaction between AEZ's and time resulted in significant difference ($p<0.05$) being observed 49 days and 90 days after planting. High populations of *Fusarium spp* were recorded in UM1 and UM3 while the lowest was recorded in LM2. The populations of *Pythium spp* were observed to reduce from the initial isolation with the passage of time. Significant differences ($p<0.05$) were observed at 49 and 90 days with the highest being recorded in UM1 at day 49 while the lowest was recorded in LM1.

Table 5. Population of Soil borne root rot fungi (x 103 CFU g-1 in soil) isolated over a time (days) during the long rains of 2013.

AEZ	*Fusarium spp*				*Pythium spp*				*Rhizoctonia spp*				*Macrophomina spp*			
	0	49	90	Mean AEZ	0	49	90	Mean AEZ	0	49	90	Mean AEZ	0	49	90	Mean AEZ
LM1	44.8_{a}	21.3_{ab}	47.2_{ab}	37.8_{a}	35.2_{a}	24.8_{ab}	5.7_{b}	31.7_{a}	24.4_{abc}	19.5_{b}	43.97_{a}	31.6_{a}	12.2_{a}	25.3_{a}	2.7_{a}	13.4_{a}
LM2	62.0_{a}	10.0_{b}	35.6_{b}	35.9_{a}	39.7_{a}	28.7_{ab}	17.0_{ab}	28.5_{a}	41.0_{ab}	6.7_{b}	32.2_{a}	29.3_{a}	10.0_{a}	11.4_{c}	6.1_{a}	9.2_{a}
UM1	44.6_{a}	28.5_{a}	47.0_{ab}	40.0_{a}	33.9_{a}	36.1_{a}	25.2_{a}	25.1_{a}	22.4_{c}	31.8_{a}	29.5_{a}	27.9_{a}	1.8_{b}	21.7_{ab}	9.2_{a}	10.8_{a}
UM3	60.3_{a}	25.0_{ab}	56.4_{a}	47.2_{a}	39.2_{a}	21.3_{b}	14.7_{ab}	21.9_{a}	42.4_{a}	16.3_{b}	35.95_{a}	26.6_{a}	6.0_{ab}	15.3_{bc}	3.8_{a}	8.4_{a}
Mean time	55.4_{A}	22.6_{B}	49.8_{A}		37.7_{A}	25.7_{B}	15.6_{C}		35.8_{A}	18.0_{B}	35.3_{A}		6.8_{B}	17.3_{A}	5.0_{B}	
LSD AEZ	15.1				11.6				14.1				5.1			
LSD time	5.7				5				6.7				4.1			
%CV	36.5				52.2				62.1				113.7			

Means followed by same letter(s)within each column are not significantly different at p ≤0.05. AEZ-Agro-ecological zone, LM1- lower midland zone1,LM2- lower midland zone 2, UM1- Upper midland zone1, UM3-upper midland zone3. LSD: Least significance difference at 5% level, CV: Coefficient of variation.

No significant differences were observed in the populations of the beneficial fungi isolated from the soils (Table 6). *Trichoderma* spp had the lowest initial population while *Aspergillus* spp was highest. LM2 had highest populations of *Trichoderma* spp and *Penicillium* spp. Whereas the upper midland semi humid recorded high numbers of *Aspergillus* spp while UM1 recorded the least. No significant differences in mean abundance for all fungi between AEZ, was due to high variation between individual farmer fields

Table 6. Density (CFU g-1 soil) of beneficial fungi in soils of each agro-ecological zone isolated at different times. DAP = days after planting.

	Trichoderma spp (x 10^3 CFU g^{-1}_{soil})				*Aspergillus* spp (x 10^3 CFU g^{-1}_{soil})				*Penicillium* spp (x 10^3 CFU g^{-1}_{soil})			
DAP	0	49	90	$Mean_{AEZ}$	0	49	90	$Mean_{AEZ}$	0	49	90	$Mean_{AEZ}$
LM1	0.0_{a}	14.7_{a}	4.8_{a}	6.5_{a}	12.8_{a}	2.3_{a}	30.6_{a}	13.7_{a}	12.2_{a}	3.1_{a}	14.4_{a}	9.9_{a}
LM2	1.0_{a}	12.0_{a}	7.0_{a}	10.0_{a}	26.3_{a}	0.7_{a}	21.8_{a}	16.2_{a}	19.5_{a}	12.3_{a}	12.3_{a}	14.7_{a}
UM1	1.8_{a}	16.3_{a}	5.3_{a}	7.8_{a}	16.9_{a}	5.7_{a}	17.6_{a}	13.4_{a}	6.4_{a}	11.9_{a}	18.0_{a}	12.1_{a}
UM3	6.7_{a}	15.1_{a}	3.9_{a}	8.6_{a}	24.1_{a}	7.1_{a}	27.5_{a}	19.6_{a}	14.4_{a}	9.0_{a}	15.5_{a}	13.0_{a}
LSD AEZ	7.4				7.6				7.2			
LSD DAP	3				6				4.1			
LSD AEZ * Time	8.9				12.7				9.9			
%CV	97.8				96.5				89.1			

Lower case characters indicate significance of difference between AEZ for specific time after planting at p ≤ 0.05. CV: Coefficient of variation

The NMDS analysis revealed pronounced difference in the composition of soil fungal communities between AEZs at the initial sampling period (Fig. 1), manure application (Fig. 2) and frequency of crop rotation (Fig. 3). Findings show that: (i) UM3 had higher abundance of

Trichoderma spp and lower *Macrophomina* spp than all other AEZ; (ii) LM1 had higher abundance of *Rhizoctonia* spp, *Pythium* spp and *Fusarium* spp than LM2 and UM3; (iii) UM1 had higher abundance of *Aspergillus* spp than LM1 and LM2; and (iv) LM2 had higher abundance of *Macrophomina* spp than LM1 (v) Cattle manure and chicken manure.

Applications resulted in higher abundance of *Trichoderma* spp and low abundance of *Macrophomina* spp than when no application was done; (iv) seasonal rotation resulted in lower levels of *Macrophomina* spp and increased abundance of *Trichoderma* spp while no rotation resulted to an increase in abundance of *Rhizoctonia* spp*, Fusarium* spp and *Pythium* spp.

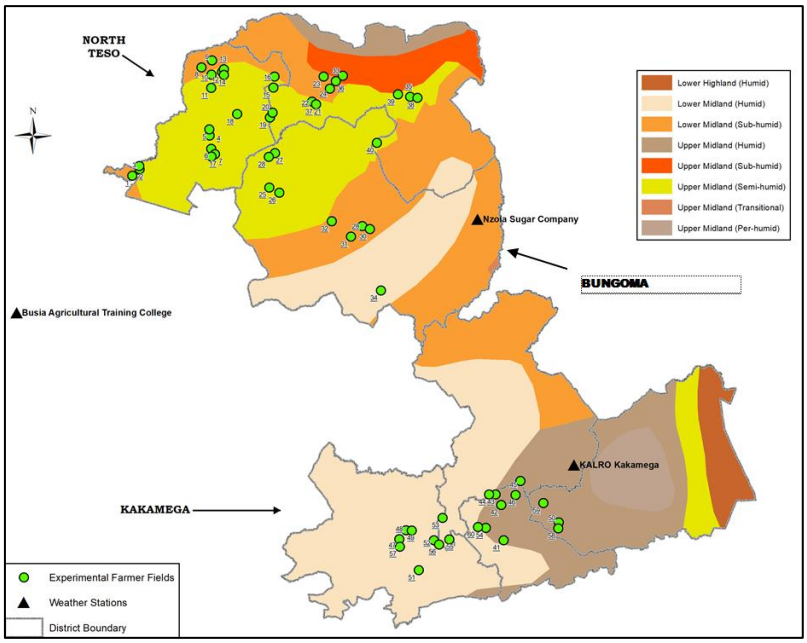

Fig. 1. Agro ecological zones of study sites in Western Kenya.

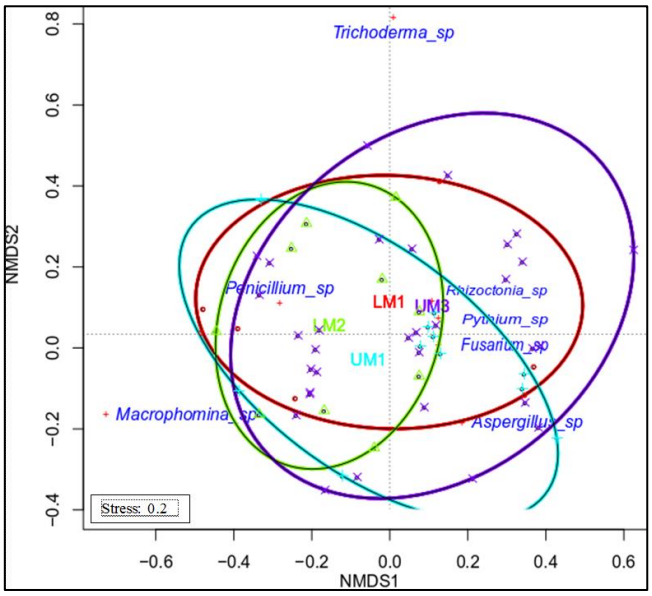

Fig. 2. Non-metric multidimensional scaling (NMDS) plot for soil inhabiting fungi. Each symbol represents one pooled soil sample. Ellipses represent ordination confidence intervals (95%). Agro-ecological zones indicated by color and fungal species placement by the + symbol.

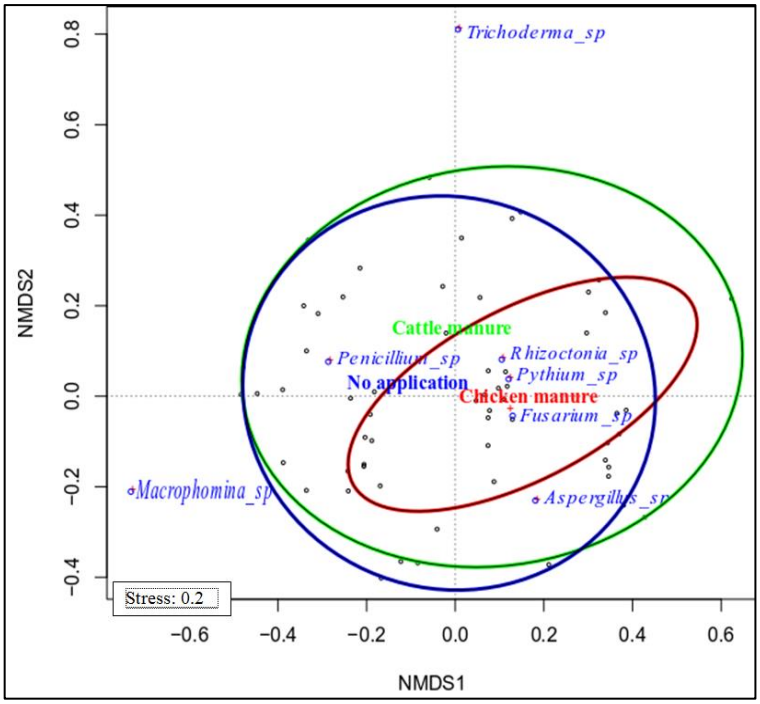

Fig. 3. Non-metric multidimensional scaling (NMDS) plot for soil inhabiting fungi. Each symbol represents one pooled soil sample. Ellipses represent ordination confidence intervals (95%). Manure application indicated by color and fungal species placement by the + symbol.

Correlation of soil properties, isolation of root rot fungi and quantification of the root rot fungal pathogens by molecular techniques

A number of relationships can be observed from the results (Table 8). The correlation between soil particle size of percent sand and the quantification of *P. ultimum* DNA in the soil was positive and significant (r = 0.256, p<0.). Significant correlation (r = 0.268, p<0.05) was also observed between percent sand particle size and *R. solani* soil DNA. The correlation between *Macrophomina phaseolina* soil DNA and percent sand soil particle size was however negative and highly significant (r = -0.398, p<0.01). Percent clay content showed a significant negative correlation (r = -0.265, 0.05) with *Fusarium sp.* isolated from soils and quantification of *R. solani* DNA in the soil (r = -0.37, p<0.01). Correlation between percent silt and quantification of *R. solani* DNA was negative and significant (r = -0.366, p<0.01). Soil pH and isolated *Trichoderma spp* was found to have a positive and significant correlation (r = 0.312, p<0.05). The correlation between *Fusarium* sp. isolated from the different soils of Western Kenya and *Pythium* sp. isolated was positive and highly significant (r = 0.602, p<0.01). A positive and significant correlation was also observed between *Fusarium* sp. and *R. solani* DNA (r = 0.256, p<0.05). The correlation between *Pythium* sp. and *Rhizoctonia* sp as well as between *Pythium sp.* and *Trichoderma sp.* was positive and significant (r = 0.342, p<0.01) and (r = 0.287, p<0.05) respectively. Altitude had a negative significant correlation with *F. solani* DNA and *R. solani* DNA (r = -0.321, p<0.05) and (r = -0.274, p<0.05) respectively. The other correlations were not significant.

Gene sequence of soil borne fungi isolated from Western Kenya soils

Twenty five isolates were identified following successful gene sequencing (Table 8). A total of 12 isolates were identified as *Fusarium* with 8 being *F. oxysporum*, 4 *F. solani* and 2 *F. equisetti.* Five *Pythium spp* were identified with 3 being *P. ultimum* and 2 *P. irregulare.* Other fungi identified include 2 *R. solani*, 2 *M. phaseolina* and 2 *Paeciliomyces lillacinus*.

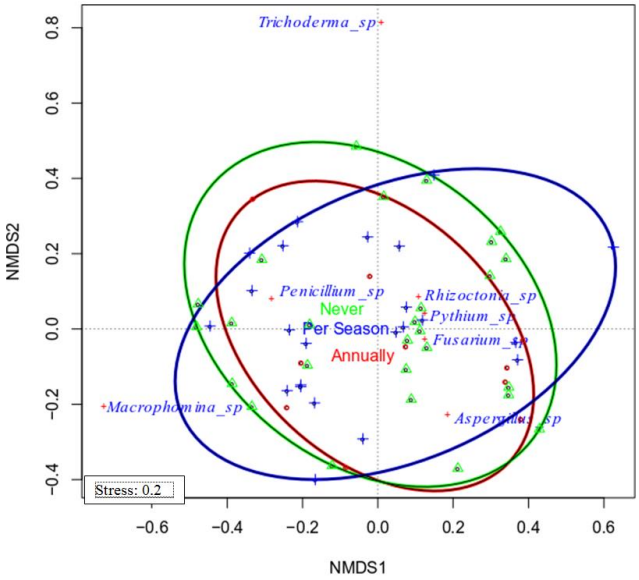

Fig. 4. Non-metric multidimensional scaling (NMDS) plot for soil inhabiting fungi. Each symbol represents one pooled soil sample. Ellipses represent ordination confidence intervals (95%). Frequency of crop rotation undertaken are indicated by color and fungal species placement by the + symbol.

Table 7. Correlation coefficients (r) between soil characteristics, common bean root rot fungal populations and DNA quantity of root rot fungal pathogens from soils in western Kenya.

	Altitude	pH	% sand	%Clay	%Silt	% N	% C	*F. s*	*P. u*	*R. s*	*M. p*	*Tricho*	*F.s D*	*P. u D*	*R. s D*	*M.p D*
Altitude	1															
pH	0.031	1														
% Sand	-0.154	**0.394****	1													
% Clay	0.146	**-0.367****	**-0.608****	1												
% Silt	0.129	-0.100	**-0.465****	**0.699****	1											
% N	0.271	-0.135	**-0.643****	**0.868****	**0.763****	1										
% C	0.240	-0.149	**-0.643****	**0.873****	**0.723****	0.991	1									
F. s	-0.116	0.173	0.200	**-0.265***	-0.089	-0.174	-0.176	1								
P. u	0.212	0.063	0.087	-0.200	-0.113	-0.029	-0.032	**0.602****	1							
R. s	0.082	0.192	0.082	-0.107	-0.179	-0.034	-0.018	0.044	**0.342****	1						
M. p	-0.012	-0.135	-0.056	0.008	-0.028	0.094	0.111	-0.132	-0.092	0.088	1					
Tricho	0.064	**0.312***	0.097	-0.035	0.054	-0.016	-0.037	0.163	**0.287***	0.213	-0.064	1				
F. s DNA	**-0.321***	0.072	0.004	0.066	0.044	-0.041	-0.046	0.121	-0.039	-0.019	-0.125	-0.051	1			
P. u DNA	0.034	0.036	**0.256***	-0.218	-0.215	-0.213	-0.211	0.169	0.230	0.133	0.134	-0.103	-0.096	1		
R. s DNA	**-0.274***	0.198	**0.268***	**-0.370****	**-0.366****	**-0.349****	**-0.338****	**0.256***	0.243	-0.100	0.012	0.093	-0.184	0.204	1	
M. p DNA	0.052	0.052	**-0.398****	-0.171	-0.147	0.051	0.053	0.102	0.057	0.031	0.170	-0.034	-0.066	-0.060	0.165	1

R. s- Rhizoctonia sp , *P. u- Pythium* sp, *F.s- Fusarium* sp, *M. p- Macrophomina* sp, *Tricho- Trichoderma* sp, *F.s* DNA - *Fusarium solani* DNA , *P. u* DNA- *Pythium ultimum* DNA, *R. s* DNA-*Rhizoctonia solani* DNA, *M. s DNA Macrophomina phaseolina* DNA , %Clay- Percent clay, %Sand–Percent Sand, %Silt- Percent Silt, pH–Soil pH.

Table 8. Identified isolates based on gene sequence and nucleotide BLAST fungal flora from Western Kenya.

AEZ's	Fungi from accession (counts)							
	F. oxy	*F. sol*	*F. equis*	*P. ulti*	*P. irreg*	*R. sol*	*M. phase*	*Pa. lilla*
LM1	2	-	1	-	-	-	1	1
LM 2	3	2	-	-	1	-	-	-
UM 1	1	-	-	-	-	1	-	-
UM3	2	2	1	3	1	1	1	1

Quantity of soil genomic DNA for selected root rot fungi in Western Kenya

There was no significant difference in the total microbial DNA extracted from the soils across the AEZ's with the highest being recorded in UM1 (Table 9). No significant differences were observed for quantification of the root rot pathogens with the exception *R. solani* which was observed to be significantly different ($p<0.05$) across the AEZ's. The lowest quantification was recorded for *F. solani* while *R. solani* was the highest. Upper midland semi-humid had the highest quantity of *R. solani genomic* DNA which was significantly different from UM1 which had the lowest. A similar trend was observed for the other root rot fungi though the difference was not significant.

Table 9. Total microbial DNA and root rot fungal and Oomycete genomic DNA in soil samples from different agro-ecological zones.

AEZ's	DNA (ug/ml)	*F. solani* (ng/µL)	*P. ultimum* (ng/µL)	*R. solani* (ng/µL)	*M. phaseolina* (ng/µL)
LM1	13.6a	$2.5X10^{-5}$a	$1.0X10^{-5}$a	$16.8X10^{-3}$ab	$148.5X10^{-3}$a
LM2	13.1a	$16.5X10^{-5}$a	$1531X10^{-5}$a	$354.3X10^{-3}$ab	$56.1X10^{-3}$a
UM1)	14.9a	$7.6X10^{-5}$a	$2.0X10^{-5}$a	$7.3X10^{-3}$b	$58.3X10^{-3}$a
UM3	10.7a	$16.4X10^{-5}$a	$16225X10^{-5}$a	$1133.9x10^{-3}$a	$185.4X10^{-3}$a
LSD	5.7	$21.0X10^{-5}$	$35600X10^{-5}$	$95.1\ X10^{-1.}$	$4.15\ X10^{-1}$
%CV	132.5	211.4	477.5	170.6	364.5
F. Pr.	0.859	0.568	0.569	0.011	0.837

Discussion

This study found that root rot fungal pathogens were present in all the sixty farms surveyed in the four agro-ecological zones of Western Kenya. More than one root rot pathogen occurred in each farm albeit at different populations and frequencies. The highest populations of root rot pathogens occurring in the soil were *Fusarium* spp. followed by *Pythium* spp and *Rhizoctonia* spp in that order. *Macrophomina spp* was also isolated from the farms though it was not widely spread. This confirms the importance of these root rot fungi in Western Kenya. Otsyula *et al.,* 1998 had earlier reported the importance of *Fusarium solani; Pythium* spp and *Rhizoctonia solani* as the main causal agents of common beans root rot in western Kenya. Other root rot fungi such as *Sclerotinia sclerotiorum* were not found to occur in the area of study.

Variations in populations of root rot pathogens occurred in all the AEZs. Upper midland humid (UM3) and LM2 had the highest number while UM1 had the lowest. These Agro-ecological zones are characterized by mean temperatures of 18.8-20.6^{o}C and rainfall of 550-650mm during long rains and 450-580mm during the short rains. The lower midland sub humid has mean temperatures of 21.4-22.3^{o}C and rainfall of 600-650mm during the long rains and 460-480mm during the short rains (Jaetzold and Schmidt, 1983). These characteristics result in moderate soil moisture in the farms. The findings are similar to earlier findings by Mwang'ombe *et al.,* 2007 on root rot pathogens of common bean in Embu. They observed that higher fungal pathogen populations occur in areas with moderate soil moisture content which encourages bean root rot establishment. Naseri, 2014 also reported *Fusarium* spp to be a major root rot pathogen at moderate soil moistures, hot weather, acidic and poorly fertilized soil conditions. *Fusarium* spp had the highest isolation frequency in all the AEZ's. In the humid zones (LM1 and UM1), *Pythium* spp was the second highest in frequency of isolation whereas in the sub humid (LM2) and semi humid (UM3) zones, *Rhizoctonia* sp. was second highest followed by *Pythium* sp. Naseri, 2015 reported high frequency of isolation for *Fusarium* spp in soils with high levels of root rot disease of common beans.

Different soil types were found to have an effect on the soil pathogen populations. *Fusarium* spp*, Pythium* spp*, Rhizoctonia* spp and *Macrophomina* spp populations were highest in loamy fine sand followed by sandy clay. The findings concur with Naiseri 2014 who observed high levels of *F. solani* in soils with high sand content. Other findings by Gill *et al.,* 2000 and Bliar 1943 have also shown the rapid growth of *R. solani* in nutrient deficient sandy soils. Other soil inhabiting fungi such as *Aspergillus* spp*, Penicillium* spp and *Trichoderma* spp were also isolated in the four AZE's. *Aspergillus spp* was the highest followed by followed by *Penicillium spp* while *Trichoderma* spp was the least isolated. Lower midland humid (LM1) had the highest populations of the beneficial microorganisms while LM2 had the lowest populations. This study observed farm management practices including manure application and frequency of crop rotation

influenced soil fungal populations. *Trichoderma* spp was greatly influenced by manure application. This was observed in farms that undertook application of cattle and chicken manure which showed increased populations of *Trichoderma* spp and decreased populations of the root rot fungal pathogens. Consequently seasonal crop rotation was observed to suppress pathogen populations as well as increasing the population of the beneficial soil borne fungi as compared to annual rotation and no rotation at all. The findings are similar to Zaidi and Singh 2004 who observed increased populations of *Trichoderma harzianum* and other root rot bio control agents on farm yard manure. Okoth *et al.,* 2009 and Sun *et al.,* 2012 also reported that soil moisture and carbon promote growth and populations of *Trichoderma* spp factors that are influenced by the application of manure.

Molecular techniques employed in identification of root rot fungi isolated from different AEZ's in western Kenya confirmed the presence of six fungi of importance in bean root diseases. These were *Fusarium solani, Fusarium oxysporum, Pythium ultimum, Pythium irregulare, Rhizoctonia solani* and *Macrophomina phaseolina.* The same were also positively identified by conventional methods where morphology and cultural characteristics were used.

Molecular quantification of root rot fungi in Western Kenya was observed to reflect similar findings as the conventional quantification methods used. This is in relation to the distribution of each fungus across the agro-ecological zones. The quantity of *F. solani* and *P. ultimum* were highest in LM2 and UM3 while *R. solani* was highest in UM3. The same findings were recorded for the conventional methods of quantification. However the two techniques greatly varied in relation to hierarchical quantification of different pathogens in the same AEZ's. The quantity of *Fusarium solani* genomic DNA from soil was the lowest of four root rot fungal pathogens occurring in Western Kenya. The concentrations ranged from 2.51 x 10^{-5} ng/µL to 16.4 x 10^{-5} ng/µL of soil DNA. *Rhizoctonia solani* on the other hand had the highest quantity of the genomic DNA from the soils at 113390 x 10^{-5} ng/µL which was the greatest of the four pathogens. Genomic DNA for *M. phaseolina* was second highest ranging from 5830 x 10^{-5} ng/µL to 18540 x 10^{-5} ng/µL. *Pythium ultimum* was also detected at low concentrations of 1.0 x 10^{-5} ng/µL to 16225 x 10^{-5} ng/µL which were higher than those of *F. solani* in two AEZ's of LM2 and UM3. Lievens *et al.,* 2006 observed that it was difficult to accurately distinguish target pathogens from non-target pathogens in naturally infested soils using the plating techniques on semi-selective medium. They however found that there was a high correlation between calculated DNA and inoculum density of *F. solani* and *R. solani* in artificially infested soils. This demonstrates how the technique can accurately quantify occurrence of pathogens in complex samples. Other findings, Fillion *et al.,* 2003 were not able to correlate colony forming units of *F. solani* with qPCR quantification data. They however demonstrated a consistent expression of *F. solani* DNA to symptom expression which showed that any detection in soil may lead to disease in weakened or stressed plants. Studies by Lievens *et al.,* 2006 also found that *R. solani* complex is pathogenic to different hosts largely based on the anastomosis groups (AG). Different AGs of the fungus are usually detected in mixed soil samples. Upon detection in soil, pathogenic capacity of the isolates needs to be tested since not all the AGs of *R. solani* cause disease to all plants. Lievens *et al.,* 2006 also made similar observations for *Pythium* species which are virtually present in all cultivated soils and can be detected easily using the DNA quantification.

The low detection of *F. solani* using molecular quantification can be attributed to the fact that the method was specific to *F. solani* only and was unable to pick the other *Fusarium* species.

At the same time high concentrations of *R. solani* and *M. phaseolina* can be attributed to their presence in the soil in form of mycelium over longer periods. This makes it possible for the pathogens' DNA to be extracted in higher quantities leading to higher quantification. Time of sample collection may also have an impact on the molecular quantification of the pathogens. *Pythium* and *Fusarium* do not thrive in dry soil and form resting spores which may yield lower DNA than their vegetative state.

These findings do not however lower the importance of *Fusarium solani* and *Pythium ultimum* in root rot diseases of common bean but rather emphasizes that even if the genomic DNA is found to be low, it may still cause serious infections greatly reducing bean yields. This was also observed Fillion *et al.,* 2003 when working with root rot of beans who found a consistent statistical trend between expression of symptoms in plants and soil genomic concentration of the *F. solani.* Lievens *et al.,* 2006 while working with wilt of tomato also found that *P. ultimum* was the major cause of root rot disease where it was quantified using molecular techniques.

In this study, root rot fungal populations were observed to be influenced by soil type, AEZ's, farm management practices and ecological factors in the soil microcosm. Positive and significant ($p<0.05$) correlation was observed between sand, *P. ultimum* DNA and *R. solani* DNA. Correlation between sand and *M. phaseolina* DNA quantity was however observed to be significantly ($p<0.001$) negative. These results confirm previous findings by Gill *et al.,* 2000. They observed that *R. solani* grew more rapidly in well-aerated soil than in moist soil with limited aeration. Blair 1943 also observed that *R. solani* was more aggressive in nutrient deficient sand.

There was also a significant ($p<0.05$) negative correlation between clay content and populations of *Fusarium spp* in this study. Similar observation was also made between clay and *R. solani* DNA. The findings concur with earlier experiments by Naseri, 2014 who observed high levels of *F. solani* in soils having high silt and sand content.

Positive significant ($p<0.05$) correlation in the populations of *Pythium spp; Fusarium spp; and Rhizoctonia spp* were observed in the area of study. From this study it shows that the pathogens operate synergistically to enhance root rot in the soils which concurs with observations by Paparu *et al.,* 2017 reported of similar findings in Western Uganda. Abawi and Pastor Corrales (1990) also reported of a synergistic interaction between *Fusarium solanif* sp *phaseoli* and *Pythium ultimum* resulting in higher damage to plants than when each pathogen acts alone.

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