

Pathogenicity of *Alternaria* species Causing Tomato Blight in Agro-ecological Zones of Kirinyaga County in Kenya

ABSTRACT

Tomato (*Lycopersicon esculentum* Mill) is a highly consumed and produced vegetable crop with an annual global production of over 185 million tonnes, due to its nutritional and health benefits. However, tomato production is characterized by inconsistent quality and yields partly emanating from biotic constraints attributed to fungal foliar diseases. In Kirinyaga County, where tomato production is a significant source of livelihoods, there exists knowledge gap on the identity of *Alternaria* species causing blight in different Agro-ecological zones (AEZs) and on the susceptibility of locally grown tomato varieties to the infection. The objective of this study was to determine the characteristics of blight pathogen isolated from tomato leaves collected from different AEZs and assess the susceptibility level of some tomato varieties grown in Kirinyaga County to the blight pathogens isolates. Pathogen characterization was carried out using morphological and molecular approaches while susceptibility of tomato varieties to fungal pathogens was evaluated by artificial inoculation. Molecular characterization for fungal pathogen involved BLASTIN analysis of DNA amplicon Sanger sequenced nucleotides and use of NCBI databases. The susceptibility assay was carried out in the greenhouse using a completely randomised design (CRD). Data on the fungal conidia lengths (μm) were subjected to Analysis of Variance (ANOVA) and significant means separated by Least Significance Difference (LSD) at $\alpha = 0.05$. Data on the susceptibility (%) of tomato varieties to fungal pathogens isolates was subjected to ANOVA and significance means separated using LSD at $\alpha = 0.05$. Morphologies of *Alternaria* pathogens differed significantly ($p < 0.05$) with conidia lengths from 52.8 and 204.20 μm . Molecular characterization of some selected culture implicated *Alternaria solani*, *Alternaria cerealis*, *Alternaria arborescens* and *A. alternata* involvement in causing tomato blight. Tomato varieties differed in their susceptibility to early blight pathogens ($p < 0.05$). Kilele F₁ was the most susceptible variety (73.84%) while Terminator F₁ was least susceptible (56.48%) to early blight pathogens. It can be concluded that Terminator F₁ was the least susceptible among tomato variety cultivated by farmers in Kirinyaga County and can serve as source for genetic improvement of tomato varieties for Kirinyaga patho-system.

Keyword: AEZs, Early blight, characterization, tomato susceptibility, Kirinyaga County

I. INTRODUCTION

Early blight is a destructive and widely spread fungal diseases and may potentially causes tomato yield loses of up to 80% [1] [2]. Necrotrophic fungus in the genus *Alternaria* that include *Alternaria alternata*, *Alternaria solani* and *Alternaria tomatophila*, have been vindicated as the causative agent of early blight diseases [1] [2]. Plants diseases limit chlorophyll levels and enhances build-up of injurious reactive oxygen species such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which damage biomolecules such as lipids and nucleic acids [3] [4]. Pathogens such as *Alternaria solani* according to Sallam *et al.* [3], enhances synthesis of ascorbic acid while limiting synthesis of antioxidant (Glutathione and soluble phenolic compounds), salicylic acid a stimulant, and catalysts such as polyphenol oxidase and phenylalanine ammonialyase which are necessary in plant protection in times of stress.

Tomato production is practised in different agro-ecological zones (AEZs) of Kirinyaga County ranging from zone LM4 to UM2 [5]. These AEZs vary in climatic conditions [6] and tomato cultivation practices such as variety grown among other factors [7]. Variations in microclimate i.e. humidity, temperature, light duration is likely to influence pathogen selection, pathogen development and disease progress [8] [4]. Due to environment fluctuations and its impact on pathogens' behaviour, adaptations and morphologies, the burden of disease control requires accurate identification of causing pathogen [9]. Accuracy of pathogen identification is fundamental to farmers as it link the disease-causing pathogen with the already available information necessary to effect management [10]. Traditionally, identification of fungal pathogen entails an elaborate process that include pathogen isolation and characterization [11] [12] [13]. Phenotypic characterization has been predominantly used in identification of fungal pathogen which uses conidial shape, colour and septation among other traits [14] [15] [16]. However, phenotypic characterization provides variable results for different fungal phytopathogens. Kumar *et al.* [17] evaluated characteristics of *Alternaria solani* from different agro-climatic conditions and observed yellow, brown, black, brown to greenish black pigments on potato dextrose agar medium. The conidiogenous hyphae varied in thickness from 1.17 to 9.56 μm . In the majority of the isolates, mycelial growth was smooth having circular or irregular margins that did not form concentric rings. Regarding cultural traits, variation in *Alternaria solani* strains from Jordan valley was reported with mycelial width range of 0.8 to 1.5 μm , conidia breadth of 35-75 μm , length of 10-20 μm , 2 to 7 transverse septa and 1 to 4 longitudinal septa [18]. In Limpopo, Mphahlele [19] reported variability of *Alternaria solani* isolated from tomato leaves collected from four tomato production regions. Isolates were highly varied phenotypically with reference to the pigmentation of mycelial, conidia length, septa number and diameter of the colonies. In Kenya, despite reported cases of tomato early blight in Mwea [20], little work has been done on characterization of the causative pathogens from different AEZs. Use of morphologies for identification of fungal phyto-pathogens is tedious, require expertise and are hindered by fungal inconsistency and phenotypic plasticity [21] [22] [23] [24]. To resolve this inadequacy, there is need for using advanced identification criteria, such as molecular techniques that allows comparative genome analyses to distinguish closely-related pathogens [25]. A combination of phenotypic and molecular based identification approaches can provide more comprehensive knowledge of tomato foliar fungal pathogen in Kirinyaga County to facilitate management.

Improved tomato varieties such as Terminator F₁, Kilele F₁, Hansal F₁ and Riotinto F₁ have been introduced to farmers in Kirinyaga County. However, cases of early blight and other fungal diseases have persisted [26] [20] [5]. Success in breeding for the EB resistance have been slowed down by limited available effective genes for resistance particularly among the cultivated tomato [27]. Therefore, ssusceptibility tests of tomato varieties to early blight disease may serve as guidelines for breeding of disease resistant cultivars, hence improving tomato production and income to farmers. According to Odilbekov [28] and Adhikari *et al.* [25], knowledge gap exists on the susceptibility of varieties of tomato grown by farmers to disease that include early blight. Thus, screening of local tomato varieties against early blight diseases in different patho-systems such as AEZ of Kirinyaga County is necessary to bridge the existing knowledge gap on their susceptibility to the pathogen in the area.

II. MATERIALS AND METHODS

2.1 Study Area

The fungal pathogen used in this study were isolated from diseased tomato leaves collected from farms in Kirinyaga County (Figure 2). Kirinyaga County is located in the Southern Mt. about 100 km North East of Nairobi [29] at latitudes 0° 37'S and 0° 45'S, longitudes of between 37° 14'E and 37° 26'E with altitude ranges of 1,100 m to 1,200 m above sea level. The average annual rainfall is 940 mm with long and short rains occurring between April and May, and October to November, respectively [6]. Temperatures range from 12°C to 26°C with an average of 20°C. The AEZ in Kirinyaga County are grouped from Tea Dairy Zone LH 1 at the base of Mount Kenya National park, three coffee zones (UM 1, UM 2, UM 3), Marginal Cotton Zone in zones LM 3 and LM 4 (Table 1 [6]).

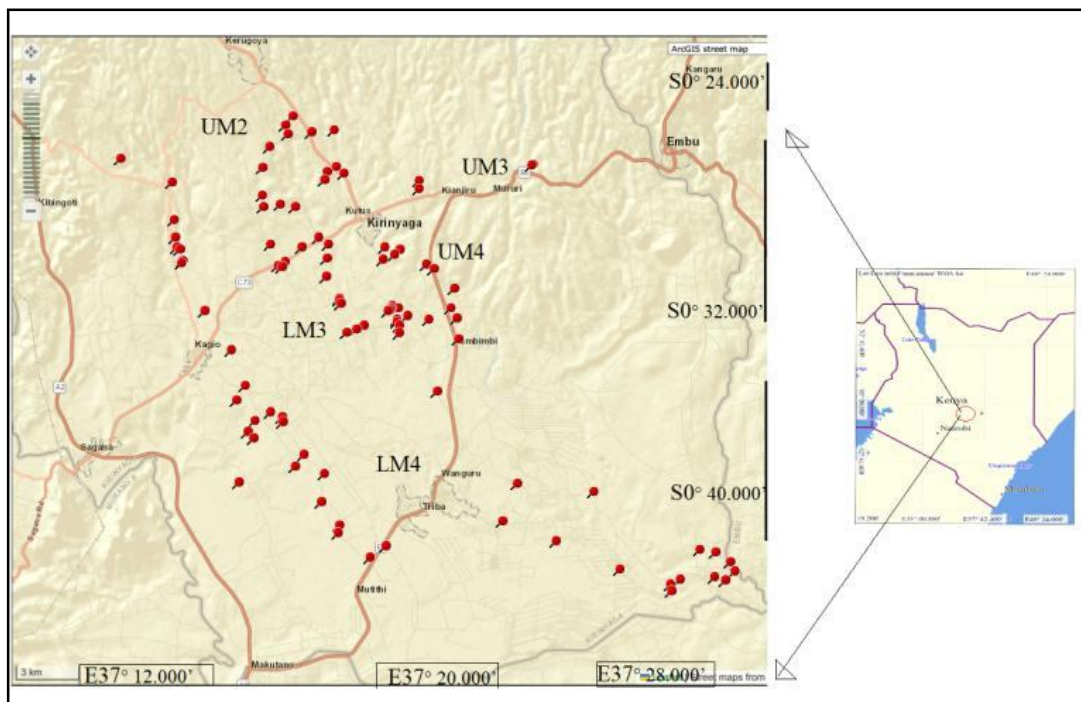


Figure 1: Map of Kirinyaga County showing agro-ecological zones (UM2, UM3, UM4, LM3 and LM4) surveyed for foliar diseases of tomato; where UM = Upper midland (1, 2 and 3), LM = Lower midland (3 and 4).

As shown in Table 1, soils types in Kirinyaga County differ within and across AEZs. For instance, whereas AEZ UM2 and UM3 comprises majorly of humic nitosols soil type, LM4 comprises of three soil types i.e. humic nitosols, eutric nitosols and pellic vertisols [6]. Specifically, the diseased tomato leaves used in this study were collected in five tomato growing AEZs of Kirinyaga namely LM 3, LM 4, UM 4, UM3 and UM 2 (Figure 2).

Table 1: Features of agro-ecological zones where diseased tomato leaves were collected in Kirinyaga County

*AEZs	Soil type	Altitude (m)	Temp (°C)	Subzone	Rainfall (mm)
UM2	Humic Nitosols	1400-1580	19.0-20.1	m/l + m/s	1220-1500
				m + s/m	1200-1250
UM3	Humic Nitosols	1340 - 1400	20.1-20.6	m/s + s	1100 - 1250
UM4	Humic Nitosols	1280 - 1340	20.4-20.9	s/m + s	950 - 1200
	Eutric Nitosols			s + s	350 - 960
LM3	Humic Nitosols	1220 - 1280	20.9-21.2	s /m+ s	950 -1200
				s + s	350 -960
LM4	Humic Nitosols, Eutric Nitosols Pellic Vertisols	1090 - 1220	21.2-22.0	s + s/vs	850 - 950

*AEZ = agro-ecological zones, in the subzones, *m*= medium rainfall, *s*= short rainfall, *l*= long rainfall, *vs*= very short rainfall, UM = Upper midland (1, 2 and 3), LM = Lower midland (3 and 4) Jaetzold *et al.* [6].

2.2 Experimental Designs

To determine the susceptibility of tomato varieties to foliar fungal pathogens a 5 × 4 factorial experiment was used and was laid out in complete randomized design (CRD) and replicated 6 times under greenhouse conditions. There were two factors, i.e., tomato varieties 5 levels (Hansol F₁, Kilele F₁, Rambo F₁, Riotinto F₁ and Terminator F₁) and fungal isolates 2 levels (Early blight and control). Control treatment comprised of un-inoculated tomato plant for each treatment.

2.2.1 Isolation of Blight Pathogen from diseased Leaf

A small piece of diseased leaf tissue (3 x 3 mm) at the margin of early blight lesion and healthy leaf part was cut using sterile blade. The cut lesion was surface disinfected with 0.3% sodium hypochlorite for 1 min, rinsed in 3 changes of distilled water to remove the detergent. The excisions were surface sterilized in 70% ethanol for 30 s, rinsed with sterile distilled water 3 times then blot dried using sterilized Whatman filter paper. Water free excised leaf parts were placed on PDA in the petri dishes and incubated for 1 week at 25°C to realize optimal fungal growth. Upon growth of the colonies were viewed under the microscope and pure cultures prepared.

2.2.2 Purification and Identification of Fungus

Pure fungal pathogens were obtained by sub culturing in fresh PDA media. A flame sterilized scapel was used to cut the culture media with grown mycelia at the periphery and transferred in fresh media and were re-incubated for 2 weeks. After the pure mycelia had completely covered the plates, phenotypic characterization was done based on external cultural morphologies and by use of microscopy following scotch tape method [30]. In culture plates fungal phenotypic traits such as spore colour (SP), culture pigmentation, colour of colony margins (M-col), growth rate (GR) and Mycelial fluffiness [(MF) to facilitate identification (Table 2). Scotch tape method involved picking of the spore using transparent adhesive tape where the spores were picked from the plates using the adhesive side of the tape then placing the tape on the lactophenol cotton blue stain on the slide [30]. The slide was then observed under LCD phase contrast microscope light microscope (Model: OMAX 40X-400X) at $\times 40$ objective lenses and microscopic details of the fungus observed, measured and the images photographed. Fungal characteristics assessed under the microscope were; conidial length in μm , number of septa, and existence of beak and arrangement of conidia [(C-Arr) Table 2].

Table 2: Aspects of blight pathogen isolates used for Phenotypic characteristic

Cultural characteristic blight pathogen isolates		
Growth rate (GR)	Colony margin (M-col)	Spore colour (SP)
1= Slow	1= Brownish	1= Greenish
2= Medium	2= Greyish	2= Greyish
3= Rapid	3= Whitish	3= Dark
Presence of Beak (PB)	No. transverse septor (T-Spt)	No. longitudinal septa (L-Spt)
1= Beak present		
2= Beak absent		
Conidia arrangement (C-Arr)	1=Single	
	2 = Chains	

The reference materials used for identification of blight pathogen isolates included published material from related work [18] [31] [32] [21]. Pathogenicity test was carried out for selected pathogen isolates for further identification and characterization. All the conidial measurements were taken in micrometer (μm).

2.2.3 Pathogenicity Test and Aggressiveness of Early Blight Fungal Pathogen Isolates

Pathogenicity test was carried out as required by Koch's postulate [31] on disease causing organism in a pot experiment in the greenhouse at Chuka University. Spore suspension (10^3 spores/ml) prepared from two weeks old pure cultures of blight disease pathogen. The spores on the plates were scraped gently with an aid of scalpel and suspended in 100 ml distilled water then filtered through threefold cheese cloth. Haemocytometer (30 x 70 x 4mm) was used to standardize the inoculum to 10^3 spores/ml. Two-month old Terminator F₁ tomato variety was used in the pathogenicity test (Koch's postulate experiment). Terminator tomato variety was selected for pathogenicity test because it is grown in most areas where the pathogens were obtained. The leaves of tomato were sprayed with respective fungal pathogen isolates and were monitored for two weeks for the occurrence of blight like disease symptoms.

To determine the aggressiveness of the blight pathogens, detached leaf method described by Pryor and Michailides [32] Tymon *et al.* [33] was used. Ten well developed mature and diseases free tomato leaves from Terminator F₁ were aseptically collected from green house. The leaves were transported to the laboratory in moist sterile zip lock bags. At the laboratory, the leaves were rinsed under running

tap water and surface sterilised with 70% alcohol for 5 seconds and rinsed again in three changes of sterile water to remove the alcohol. The water on leaf surface wiped out using sterile blotting paper. The five sets leaves were pricked using sterile needle and inoculated with pathogen suspension (20 µl) of respective disease pathogens. The suspension was dropped at the point of the lesion and one leaf that served as the control was inoculated with 20 µl distilled water. Tomato leaves were then placed on sterile clean grid in a transparent glass desiccator (Diameter 300 mm height 240 mm). The desiccator had 250 ml of distilled water underneath to maintain humidity. The inoculated leaf area was monitored for five days for development of lesion. The length (L) and width (l) of the lesion were measured for each leaflet inoculated. The lesion size (S in mm²) was calculated using the formula below [34].

$$S = \frac{\pi(L + l)^2}{4}$$

2.2.4 Molecular Identification of Selected Isolates

Molecular analysis of fungal pathogens isolated in this study was done at the University of Nairobi's Centre for Biotechnology and Bio-informatics. The DNA was extracted from 20 mg mycelia harvested by scraping the colony surface. The mycelia were ground into fine pieces using motor and pestle and placed into a 1.5 mL micro centrifuge tube containing 800 µL cetyltrimethyl-ammonium bromide (CTAB) extraction buffer (Final concentration: 2% (w/v) CTAB, 200 mMTris-HCl pH 8.0, 20 mMEDTA pH 8.0, 1.4 MNaCl, 1% (w/v) poly-vinylpyrrolidone, 1% (v/v) b-mercaptoethanol [35]). The DNA extracts were dissolved in 50 µL of sterile distilled water and stored at 20°C for further use.

Polymerase chain reaction (PCR) was done using Thermocycler (Model C1000, Touch Biorad). Polymerase chain reaction was done in a 0.5 ml Eppendorf tube containing 10.5 µL ddH₂O, 12.5 µL Premix Ex Taq (Containing 0.625 U Taq DNA polymerase, 200 µM dNTP and 2 mM MgCl₂), 0.5 µL each of the two primers (10l M) and 1 µL DNA template (100 µg mL⁻¹). The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) of the isolates was amplified using primer set ITS1 [(50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50- TCCTCCGCTTATTGATATGC-30) described by White *et al.* [36] and Lee and Taylor [35]. Amplification reactions was done with an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 40 s, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Polymerase chain reaction products were checked using gel electrophoresis on 1% agarose gel stained using ethidium bromide. The DNA amplicon (1µl) mixed with loading dye was assayed alongside 1kb 0 gene ruler plus ruler with initial reading at 75kb. The DNA loading bands were viewed on gel documenter imager (Bio- Rad). Positive DNA PCR products were cleaned using Quick cleaning kit supplied by Gene Script Company in readiness for senger sequencing. Sequencing of the DNA amplicons was done at the International Livestock Research Institute in Nairobi, Kenya using Sanger sequencer (Model: ABI 3730 genetic analyser) and The Big Dye Terminator v3.1.

2.2.5 Susceptibility of Tomato Varieties to Different Fungal Pathogen Isolates

Five tomato varieties used for susceptibility screening were obtained from Kenya Seed Company in Nairobi. The nurseries of the 5 tomato varieties (Kilele F₁, Hansol F₁, Rambo F₁, Riotinto F₁ and Terminator F₁) were germinated separately in sterile germination trays in the greenhouse at Chuka University. The trays were watered every for three weeks before transplanting. Calcium Ammonium Nitrate (CAN) and Diammonium phosphate (DAP) was used to supplement the tomato growth. The growth bags used for growing tomatoes were 45 x 30 x 30 cm in size. The containers were filled with oven sterilized soil made up of sand, silt and compost (Ratio 3:1:1), respectively [37]. The three weeks old tomato seedlings were individually transferred to the plant growth bags (One plant per container) with each treatment being replicated six times. The distance between each tomato plant and the next was 0.5 meter. Watering was done by sprinkler every morning and evening to maintain humidity. Three weeks after transplantation, each tomato variety was inoculated (By spraying) with 50 ml of respective fungi pathogen culture containing 10³ CFU/ml determine by haemocytometer.

After inoculation, the tomato plants were watered at alternative days and plants inspected for the development of disease symptoms. At the end of 28 days after inoculation, the diseases symptoms were scored using rating scale of 1 - 6 (Desta and Yesuf, 2015). The scores for different pathogens were used to calculate disease severity that represented the susceptibility of individual tomato variety using the formula below as used by Negesa and Ayana (2021):

$$\text{Percent Disease Severity} = \frac{\text{Number of individual ratings}}{\text{Total number of leaves assessed}} \times \frac{100}{\text{Maximum scale}}$$

2.5 Data Analysis

3.5.1 Characterization of Alternaria Fungal Pathogens Causing Foliar Diseases of Tomato in Different Agro Ecological Regions of Kirinyaga County in Kenya

Cluster analysis of data collected on the phenotypic traits was done using factextra R package in R studio version 4.0.3. The normality test evaluated using Shapiro-Wilk test. Given that the normality test for conidial length and breadth gave non-significance ($p > 0.05$) Shapiro-Wilk test results, their means were subjected to the ANOVA using general linear model and significance means were separated using LSD in SAS version 9.4 at $\alpha = 0.05$.

The sequences (PCR fragment ITS rDNA) were compared with the record at the NCBI/Gene Bank database (www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment of fungal species obtained from the database were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program method in the Mega X version 11.0 [38]. A phylogenetic tree of the pathogen sequences and those of related fungal species downloaded from the gene bank was generated using unweighted pair group method with arithmetic mean (UPGMA) program and bootstrapping performed with 10000 replication to estimate the stability the branches [38]. The evolutionary distances of the species in the phylogenetic tree was computed using the p-distance method and output presented in units of the number of base substitutions per site [38].

2.5.2 Susceptibility Test of Tomato Varieties to Different Foliar Fungal Disease Pathogens

Data on the susceptibility based on percentages of severity of symptoms had skewness value of 0.0119 and Shapiro-Wilk test had $p > 0.05$ for normality test indicating that the data was normally distributed. Therefore, the data on susceptibility were subjected to the analysis of variance using the general linear model procedure in SAS version 9.4. Significant means were separated using least significant difference (LSD) at $\alpha = 0.05$ in SAS.

III. RESULTS

3.1 Phenotypic Characterization of Early Blight Pathogen

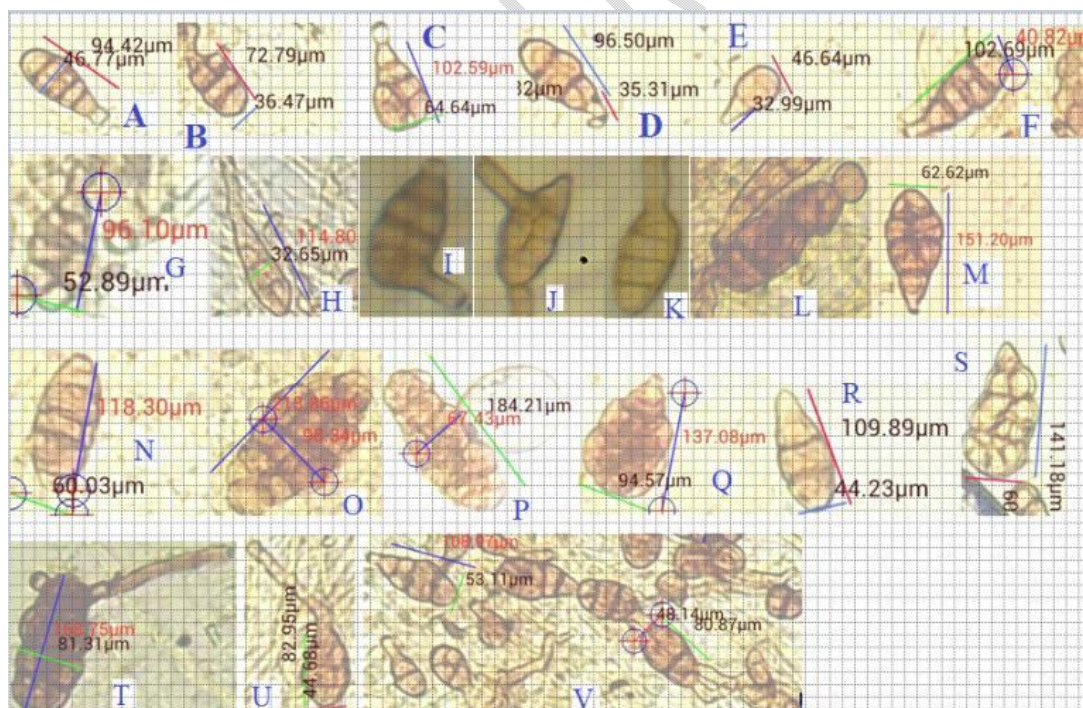
A total of 30 Alternaria pathogen isolates were obtained from diseases tomato leaves from different AEZ. Most on the isolates on potato dextrose agar were not different in terms of spore's colour on PDA. The isolates had green- dark grey spores and had dark pigmentation. The cultures had either regular or irregular margins (Table 3).

Table 3: Phenotypic characteristics of Alternaria pathogen isolated from different agro-ecological zones of Kirinyaga County

AEZ ¹	Isolate	GR ²	SP ³	PB ⁴	Mcol ⁵	L-Spt ⁶	T-Spt ⁷	C-Arr ⁸
LM4	EB_Gtg6	3	1	2	1	3	4	1
	EB_KBU3	2	3	1	2	0	1	1
	EB_ND1	2	2	1	1	0	5	2
	EB_ND4	3	3	1	1	2	4	2
	EB_ND7	2	3	1	2	0	3	1
	EB_WNG4	3	1	2	1	2	4	1
LM3	EB_KDG1	2	2	1	2	2	5	2
	EB_KDG9	3	1	2	1	1	4	1
	EB_KDO9	3	2	1	1	2	3	1
	EB_NYT4	2	3	1	2	0	6	1
	EB_NYT6	1	2	2	2	2	4	1
	EB_SRG2	1	2	2	2	1	5	1
	EB_KGG1	2	2	1	1	2	3	2

UM4	EB_KGG9	2	1	2	1	0	3	1
	EB_NDM3	3	3	1	3	3	5	1
	EB_NDM7	1	3	1	3	1	4	1
	EB_NJI2	2	1	2	1	1	4	2
	EB_NJI6	1	2	1	1	3	5	1
UM3	EB_GTH3	1	1	2	2	2	4	1
	EB_GTH5	1	2	2	2	2	3	1
	EB_KAM2	2	1	1	3	0	4	2
	EB_KAM3	2	1	1	3	0	4	2
	EB_KAM8	3	2	1	1	1	4	2
	EB_THR9	1	2	2	2	2	3	1
UM2	EB_GEO3	2	2	1	3	0	3	2
	EB_GEO6	2	3	2	2	0	4	1
	EB_KER2	2	1	1	3	2	3	1
	EB_KER7	3	1	1	1	1	3	1
	EB_KRG1	1	2	2	3	0	4	1
	EB_KRG9	3	3	2	3	2	3	1

¹Agro-ecological zones: UM = Upper midland, LM = Lower midland, ²Growth rate (1= Slow, 2= Medium, 3 =Rapid), ³Spore colour (1= Greenish, 2= Greyish, 3= Dark), ⁴Presence of beak (1= Beak present, 2= Beak absent), ⁵ Margin colour (1= Brownish, 2= Greyish, 3= whitish), ⁶Number of longitudinal septa, ⁷ Number of Transverse septa, ⁸ Conidia arrangement (1=Single, 2 = Chains),



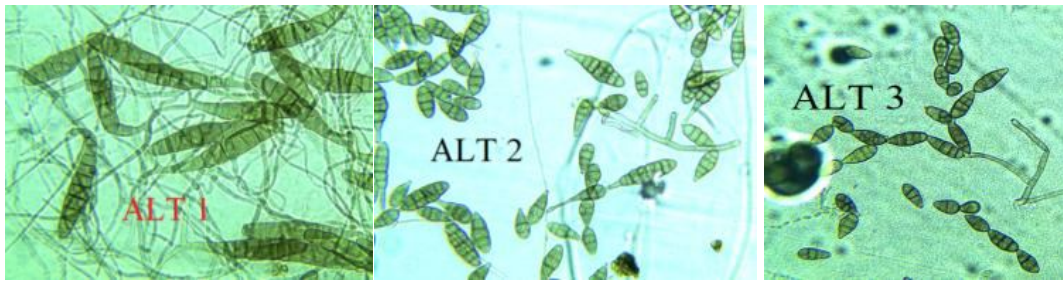


Plate 1: Selected conidia morphologies for the early blight pathogen (*Alternaria* spp) isolates from tomato leaves from Kirinyaga County, Kenya,

NOTE: Muriform shape (A, C, F, R, S), Pyriform (I, E, O, P), Cuneate shape (Q), Conidia with short beak (A, C, D, E), Medium curved beak (B and D), long beak (H and U) no beak (M, N, O, P, Q, R, S), ALT 1= Isolate EB-KAM8, ALT2= Isolate EB-NJ12-, ALT3= Isolate EB-ND4

Conidiophores of the *Alternaria* spp isolates were solitary or in groups and were either straight or flexuous. Morphologically, conidia differed in breath, length and presence of beak. The shape of conidia was straight or slightly curved muriform or ellipsoid. Conidia were tapering towards one end and were olivaceous brown in colour (Plate 1). The conidia's transverse and longitudinal septa varied ranging from 1 to 6 and from 0 to 2, respectively. The conidia appeared in chains or solely and some had beaks which were short, long or had no beak (Plate 1).

Thirty early blight pathogen isolates from five AEZ (Table 3) were grouped in three clusters by euclidean distance constructed using phenotypic characteristics. The first and third clade comprised of 13 isolates each of which were further divided into two sub clades. The smallest clade comprised of isolates EB_KBU3 isolated from AEZ, isolates EB_LM4, EB_KD09 isolated from AEZ UM2 and isolate EB_KER2 and EB_KER7 both isolated from AEZ LM3 and AEZ LM4 and lastly, isolate NDM7 isolated from AEZ UM4 [(Table 3) Figure 2]. Isolate EB_KD09 and EB_KER7 had similar distance while EB_KBU3 formed a separate sub sub clade. Isolates EB_KD09 and EB_KER7 had similar growth rate, had beak, had similar colony margin, same conidia arrangement and number of transverse septa as outline in preceding Table 3.

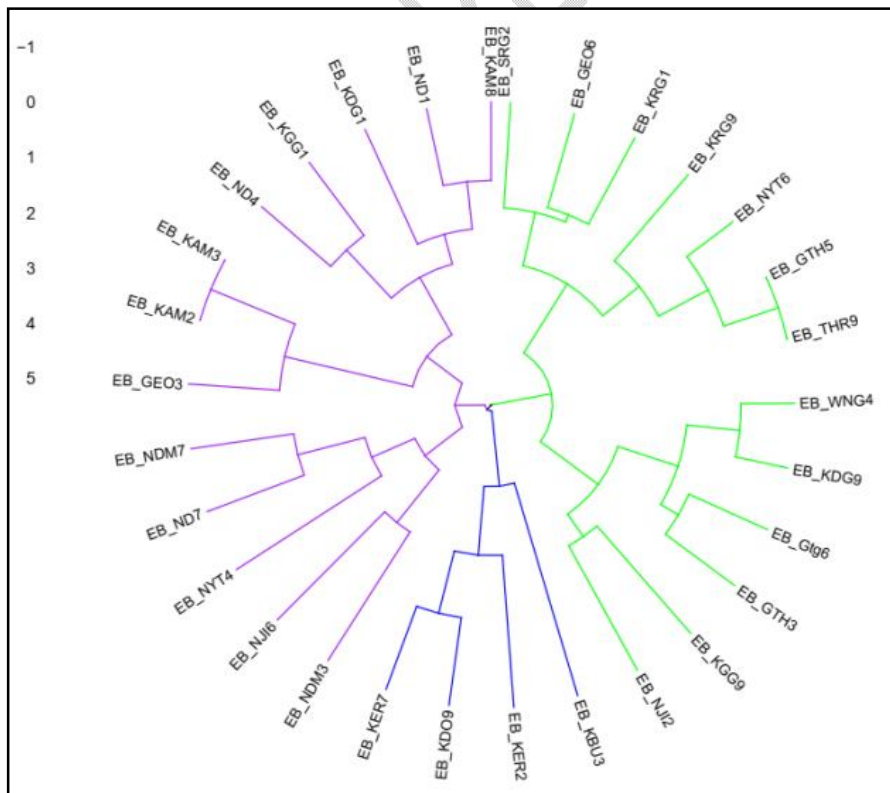


Figure 2: Cluster membership based on phenotypic traits of early blight pathogens isolated from tomato leaves from different agro-ecological zones of Kirinyaga County

There was a significant ($F(29, 210) = 8.25, p < 0.0001$) difference among the conidial lengths of pathogen isolates from infected tomato leaves from different AEZ. In LM4, the lengths of pathogen isolates were significantly ($F(5, 42) = 22.31, p < 0.0001$) different with the minimum and maximum conidial lengths measured being 52.8 and 194.2 μm , respectively (Table 4). Isolate EB_ND4 had longer conidia (148.37 μm) while isolate EB_KBU3 had smallest length of the conidia at 78.52 μm (Table 4). In LM3, the lengths of the conidia of the pathogen did not differ significantly ($F(5, 42) = 2.05, p < 0.0909$). However, conidia lengths ranged from 95.60 μm to 204.6 μm , respectively. In AEZ UM4, the lengths of the conidia of the pathogen were significantly ($F(5, 42) = 3.93, p < 0.0051$) different where the minimum and maximum length of conidia lengths measured were 85.2 and 184.2 μm , respectively. Isolate EB_NJI2 had longer conidial length (131.17 μm) while isolate EB_NDM7 had short conidial length (101.17 μm). The length of conidia for early blight pathogen were significantly ($F(5, 42) = 5.30, p < 0.0007$) different in UM3 where the minimal and maximum conidia lengths measured were 64.2 and 159.4 μm , respectively. Isolate EB_KAM3 had longer conidial length (123.62 μm) while isolate EB_GTH5 had short conidial length (92.75 μm) (Table 4). Conidia lengths in AEZ UM2 differed significantly ($F(5, 42) = 2.59, p < 0.0398$) with the minimum and maximum lengths being 64.6 μm and 138.4 μm , respectively. Isolate EB_KER7 was significantly longer measuring 112.37 μm (Table 4).

Table 4: Conidia length and breadth of *Alternaria* pathogens isolated from diseased tomato leaves collected from different agro-ecological zones of Kirinyaga County

AEZ ^a	Isolate	N	Length (μm)	Breadth (μm)	Length (μm)		Length Anova Statistics
					Min ^b	Max ^c	
LM4	EB_Gtg6	8	123.72 ^{edf}	39 ^h	99.00	149.00	Cv = 17.86 Mean= 115.71 $p < 0.0001$ LSD = 20.85
	EB_KBU3	8	78.52 ^m	38.7 ^h	66.00	94.40	
	EB_ND1	8	83.95 ^{km}	48.83 ^{de}	52.80	109.00	
	EB_ND4	8	148.37 ^{abc}	42.65 ^{etgh}	104.20	194.20	
	EB_ND7	8	97.97 ^{hijkl}	42.68 ^{etgh}	84.40	114.80	
	EB_WNG4	8	161.72 ^a	45.6 ^{etgh}	133.20	207.20	
LM3	EB_KDG1	8	139.85 ^{abcd}	41.1 ^{igh}	104.80	180.40	Cv = 20.89 Mean= 130.49 $p = 0.0246$ LSD = 27.52
	EB_KDG9	8	138.50 ^{abcd}	41.77 ^{tgh}	108.40	174.60	
	EB_KDO9	8	150.3 ^{ab}	41.3 ^{tgh}	96.60	204.60	
	EB_NYT4	8	124.5 ^{edf}	40.5 ^{tgh}	95.60	156.20	
	EB_NYT6	8	126.77 ^{cdef}	40.58 ^{tgh}	89.40	171.80	
	EB_SRG2	8	103.05 ^{ghijh}	44.15 ^{etgh}	81.60	137.80	
UM4	EB_KGG1	8	102.65 ^{ghij}	47.05 ^{def}	88.40	141.60	Cv = 17.33 Mean= 114.58 $p = 0.0051$ LSD = 20.04
	EB_KGG9	8	102.72 ^{ghij}	46.23 ^{etg}	85.20	130.20	
	EB_NDM3	8	121.8 ^{edfg}	62.43 ^b	89.80	161.00	
	EB_NDM7	8	101.17 ^{hijk}	46.78 ^{ectr}	89.80	112.60	
	EB_NJI2	8	131.17 ^{bcde}	71.1 ^a	91.80	184.20	
	EB_NJI6	8	128.0 ^{bcde}	58.75 ^{bc}	114.60	167.80	
UM3	EB_GTH3	8	99.2 ^{ijkl}	45.07 ^{etgh}	77.80	159.40	CV= 15.28 Mean= 107.93 $p = 0.0007$ LSD = 16.65
	EB_GTH5	8	92.75 ^{klm}	40.65 ^{tgh}	64.20	111.00	
	EB_KAM2	8	107.0 ^{fghij}	45.43 ^{etgh}	95.80	125.20	
	EB_KAM3	8	124.9 ^{bcdef}	60.58 ^b	109.40	158.20	
	EB_KAM8	8	123.62 ^{cdef}	69.97 ^a	113.20	152.80	
	EB_THR9	8	100.15 ^{hijk}	40.83 ^{tgh}	85.20	128.60	
UM2	EB_GEO3	8	100.07 ^{hijk}	44.58 ^{etgh}	80.80	138.40	
	EB_GEO6	8	91.5 ^{klm}	39.43 ^{gh}	64.60	132.80	

UM2	EB_KER2	8	94.0 ^{ijkl}	44.43 ^{etgh}	84.60	100.60	CV= 14.93 Mean= 101.16 $p = 0.0398$ LSD 15.24
	EB_KER7	8	112.37 ^{efgh}	52.98 ^{cd}	93.20	126.40	
	EB_KRG1	8	98.4 ^{hijk}	44.8 ^{etgh}	84.80	121.60	
	EB_KRG9	8	110.62 ^{efghi}	40.23 ^{tgh}	78.20	124.60	
Means (μm)			113.97	46.91			
LSD ($p < 0.05$)			20.034	7.02			
Cv (%)			17.832	15.18			

^aAgro-ecological zones: UM= Upper midland, LM = Lower midland, ^b Minimum conidia lengths, ^c Maximum conidia lengths, ^d Means followed by the same letter in columns is not significantly different at $\alpha = 0.05$

There was a significant ($F(4, 235) = 8.25, p < 0.0001$) difference in the length of conidia of the *Alternaria* pathogen isolated in the five AEZs. Conidia of the isolates in AEZ LM3 were significantly longer (130.5 μm) while those from AEZ UM2 were significantly shorter measuring 101 μm (Table 5).

Table 5: Overall lengths of conidia of *Alternaria* pathogens isolated in different agro-ecological zones of Kirinyaga County

AEZ ^a	N	Length (μm)	Minimum (μm)	Maximum (μm)
LM3	48	130.5 ^a	81.60	204.60
LM4	48	115.71 ^b	52.80	207.20
UM2	48	101.16 ^c	64.60	138.40
UM3	48	107.94 ^{bc}	64.20	159.40
UM4	48	114.59 ^b	85.20	184.20
Means (μm)		113.97		
LSD ($p < 0.05$)		10.59		
Cv (%)		23.11		

^aAgro-ecological zones, UM = Upper midland, LM = Lower midland, N = Sample size,

^b Mean followed by the same letter in columns is not significantly different at $\alpha = 0.05$

Pathogenicity test using isolate EB_WNG4 (Fast growing isolate) produced symptoms identical to those observed in tomato leaves leaf samples from farmers' fields (Lesion with visible concentric rings) on the tomato inoculated with the pathogen isolates. The pathogens re-isolated from the lesion were phenotypically similar to the initial pathogen isolates in terms of spores' colour and conidia shape.

Inoculation of the tomato leaves under detached leaf experiment with selected 10 isolates resulted in significantly ($F(13, 36) = 12.48, p < 0.0001$) different lesion sizes formed by the isolates (Table 6). Isolate EB_KAM8 was highly aggressive with lesion size of 122.17 mm^2 and was followed by isolate EB_NDM3 110.17 mm^2 . Isolate EB_KDG1 had smaller lesion size of 17.10 mm^2 and was followed by isolate EB_ND4 with lesion size 20.09 cm^2 (Table 6).

Table 6: Aggressiveness of *Alternaria* pathogen isolates from tomato leaves in Kirinyaga County

Isolate	N	Aggressiveness (mm^2)
EB-KAM8	5	12.27 ^a
EB_NDM3	5	110.17 ^a
EB_KER7	5	49.85 ^b
EB_WNG4	5	46.01 ^b
EB_KAM3	5	45.15 ^b
EB_KRG9	5	43.20 ^b
EB_KDO9	5	37.31 ^b
EB_NJI2	5	20.73 ^c
EB_ND4	5	20.09 ^c
EB_KDG1	5	17.10 ^c
Mean (cm^2)		41.27
LSD ($p < 0.05$)		1.67
Cv (%)		24.33

^a Means followed by the same letters are not significantly different at $\alpha = 0.05$

3.2 Molecular Characterization of *Alternaria* Pathogen Isolates from infected tomato leaves

The agarose gel analysis of DNA amplicon PCR products (ITS rDNA) of *Alternaria* pathogens isolated from infected tomato leaves produced bands at different locations between 500 to 700 bp based on 1kb gene ruler plus ladder (Plate 2).

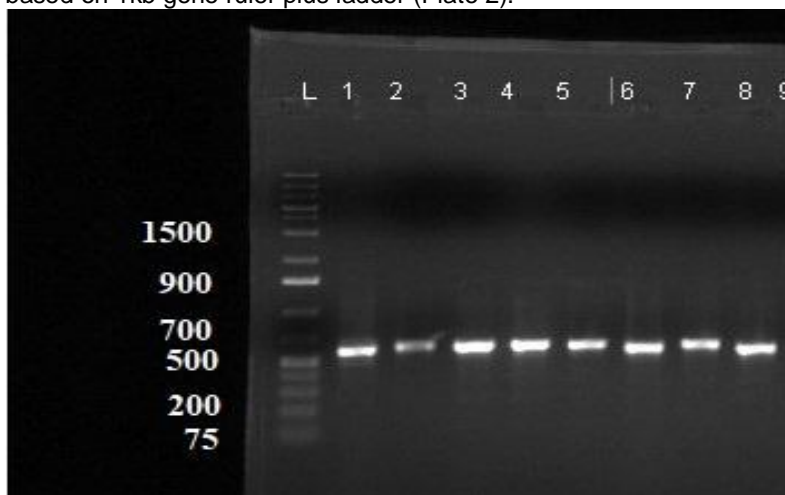


Plate 2: The agarose gel analysis of DNA amplicon PCR products (ITS rDNA) of tomato foliar pathogen isolates isolated from diseased leaf samples from Kirinyaga County where L= ladder (1kb gene ruler plus), 2= KRG-F02, 3= EB-KAM8., 4= EB-WNG4, 5= KRG-F05, 6= EB_NDM3., 7= KRG-F07, 8= KRG-F08, 9= EB-KD09, 10= KRG-F13, 11= EB-NJ12, G1= KRG-G01. 12= EB_KER7, 13= KRG-F06, 14= KRG-F08, 15= EB_KER7, 16= EB-KAM8; Isolates EB_KER7, EB-KAM8 were assayed twice

The blast analysis of DNA amplicon Sanger sequenced nucleotides results (ITS rDNA sequences) revealed the involvement of different pathogens in causing early blight like symptoms. These pathogens included *A. solani*, *A. alternata* among others (Table 7). The similarity identities of the nucleotide from pathogen isolates and fungal species in the NCBI genbank ranged from 97 for *Alternaria solani* to 100% for *Alternaria alternata* and *Alternaria cerealis* (Figure 3).

Table 7: Molecular identification of selected *Alternaria* pathogens isolated from tomato from Kirinyaga County in Kenya

Isolate	Place of isolation	Close match	GenBank Accession No.	% Sim ^a	County
EB_KER7	Kidaruni	<i>Alternaria cerealis</i>	NR_136117.1	100	New Zealand
EB_KDG1	Kiangunga	<i>Alternaria arborescens</i>	NR_135927.1	99.62	USA
EB_WNG4	Wanguru	<i>Alternaria solani</i>	KT354939.1	87.98	Egypt
EB-KAM8	Kiumbu	<i>Alternaria solani</i>	MG012289.1	99.82	China
EB_NDM3	Thumaita	<i>Alternaria alternata</i>	LC440583.1	99.64	Japan
EB_KRG9	Kerigo	<i>Alternaria alternata</i>	MK690429.1	99.33	China
EB-NJ12	Thumaita	<i>Alternaria sp.</i>	MN636300.1	99.82	Kenya
EB-ND4	Ndindiruki	<i>Alternaria alternata</i>	MK656442.1	100	China

^a Percentage similarity

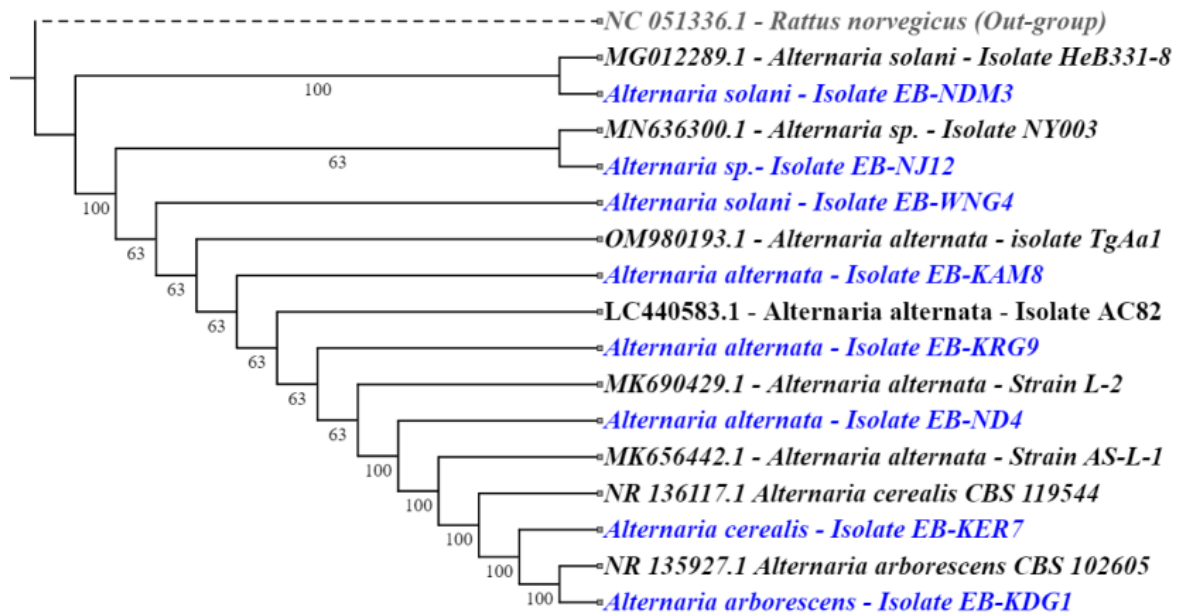


Figure 3: Phylogenetic analysis of sequences of some *Alternaria* pathogens isolated from tomato foliar and similar sequences in the gene bank using MEGA X 11 software, UPGMA method and 10000 boot-strapped replications. The species in blue font are the fungal pathogens species isolated in the current study.

3.3 Susceptibility of Tomato Varieties grown in Kirinyaga County to Early Blight Foliar Fungal Disease Pathogen Isolates

There was a significant [$F(19, 100) = 4.73, p < .0001$] difference on the susceptibility of different tomato varieties to *Alternaria* pathogen isolates. Kilele F_1 variety expressed most severe symptoms of early blight (78.24%) to isolate EB-KAM8 followed by Riotinto F_1 (72.22 %) while Terminator F_1 had least (58.33%) severe symptoms. Inoculation with isolate EB_KER7 triggered severe symptoms in Kilele F_1 variety (69.91%) followed by Rambo F_1 (67.59%) and the symptoms was less severe (54.17%) in Terminator F_1 . Isolate EB_NDM3 caused severe symptoms in Kilele F_1 variety (70.83%) followed by Riotinto F_1 (68.06%) and least in Terminator F_1 (58.33%). Inoculation with isolate EB_WNG4 induced severe symptoms in Kilele F_1 variety (76.39 %) followed by Riotinto F_1 (62.2%) and the symptoms was less severe (58.33 %) in Hansol F_1 (Table 8).

Table 8: Susceptibility of selected tomato varieties to *Alternaria* pathogen isolated from tomato leaves in Kirinyaga County

Isolates	Varieties	N	Severity (%)	Lsd ($p < 0.05$)	Cv (%)	Mean (%)
EB-KAM8	Hansol F_1	6	64.81 ^{bc}	8.43	10.44	67.96
	Kilele F_1	6	78.24 ^a			
	Rambo F_1	6	66.20 ^{bc}			
	Riotinto F_1	6	72.22 ^{ab}			
	Terminator F_1	6	58.33 ^c			
EB_KER7	Hansol F_1	6	64.81 ^a	8.57	11.18	64.44
	Kilele F_1	6	69.91 ^a			
	Rambo F_1	6	67.59 ^a			
	Riotinto F_1	6	65.74 ^a			
	Terminator F_1	6	54.17 ^b			
EB_NDM3	Hansol F_1	6	61.11 ^{bc}	8.49	10.98	65
	Kilele F_1	6	70.83 ^a			
	Rambo F_1	6	66.67 ^{abc}			
	Riotinto F_1	6	68.06 ^{ab}			
	Terminator F_1	6	58.33 ^c			
EB_WNG4	Hansol F_1	6	58.33 ^c	7.57	9.83	64.72
	Kilele F_1	6	76.39 ^a			
	Rambo F_1	6	61.57 ^{bc}			

Riotinto F ₁	6	66.20 ^b
Terminator F ₁	6	61.11 ^{bc}
Mean (%)		65.53
LSD ($p < 0.05$)		3.99
Cv (%)		10.62

^a Means followed by the same letters are not significantly different at $\alpha = 0.05$

Rambo F₁ tomato variety did not exhibit significantly [($F(3, 20) = 0.56, p = 0.649$] difference susceptibility towards *Alternaria* pathogen isolates (Table 9). Riotinto F₁ variety exhibited significantly [($F(3, 20) = 4.36, p = 0.0162$] different susceptibility towards pathogen isolates where isolate EB_NDM3 caused higher severity mean of 55.86 % and lower 50.31% in isolate EB_KER7. Hansol F₁ variety did not exhibit significantly [($F(3, 20) = 1.57, p = 0.229$] difference susceptibility towards pathogen isolates. Kilele F₁ did not exhibit significantly [($F(3, 20) = 2.16, p = 0.124$] difference susceptibility towards pathogen isolates. Terminator F₁ variety did not exhibit significantly [($F(3, 20) = 1.36, p = 0.283$] difference susceptibility towards pathogen isolates (Table 9).

Table 9: Susceptibility of selected tomato varieties isolates of *Alternaria* pathogen isolated from tomato leaves in Kirinyaga County

Isolate	Susceptibility level (%)				
	Riotinto F ₁	Hansol F ₁	Rambo F ₁	Kilele F ₁	Terminator F ₁
EB-KAM8	51.23 ^{bc}	64.82	66.20	78.24	34.87
EB_WNG4	54.32 ^{ab}	58.33	61.57	76.39	37.96
EB_NDM3	55.86 ^a	61.11	66.67	70.83	40.12
EB_KER7	50.31 ^c	64.81	67.59	69.91	37.96
Mean (%)	68.06	62.26	65.51	73.84	57.98
LSD ($p < 0.05$)	3.67	7.43	10.60	8.22	7.23
Cv (%)	9.73	9.91	13.44	9.24	10.35

^a Means followed by the same letters in column are not significantly different at $\alpha = 0.05$

IV. DISCUSSION

4.1 Characterization of Early Blight Pathogen

Morphological, biochemistry and metabolites feature of fungal pathogens are useful in identification and diagnosis of disease pathogen [12] [41] [14]. Conidial shape, colour, and septation are predominant phenotypic characteristics used in identification of fungal pathogens [15] [16] [17]. The spores of early blight pathogen isolates appeared greyish to greenish in colour and darkened as the culture aged, margins of the cultures were either regular or irregular pattern. These characteristics were consistent with those reported by Mugao *et al.* [21] in a study carried out in Mwea in Kirinyaga County in Kenya. Pigmentation and colony texture of early blight isolates in this study corroborated with other studies where greenish, dark and brownish pigmentation have been observed [18] [42] [21]. Surface texture of the colonies exhibited slight variations with zonation at margins observation which were similar to those observed in other studies involving early blight pathogens [18] [42] [43].

Microscopic observation revealed variation of pathogen isolates in their conidia morphology and coincides with the findings of Mugao *et al.* [21] where variability of tomato early blight pathogen in Mwea was reported. Further, the observation on the morphological traits are in agreement with other studies where conidia have differed in breadth, length and beak and have reported the conidia to be straight, slightly curved, muriform or ellipsoid [44] [45] [46] [47]. The conidial transverse and longitudinal septa varied ranging from 1 to 6 and from 0 to 2, respectively which was in tandem with other studies [44] [45] [46] [48]. The length of conidia of the early blight pathogen ranged from 78.52 μm to 161.72 μm . Conidia lengths observed in this study were within range of 105-220 μm reported earlier by Nolla [49]. However, conidia lengths were longer than those reported earlier by Neergaard [50] and Abubakar and Ado [51] ranging from 10.26 - 77.52 μm .

4.2.3 Evaluation of Aggressiveness of *Alternaria* Fungal Pathogens of Tomato

Knowledge on the aggressiveness level of different pathogen isolates may be useful in the evaluation on the magnitude of crop damage that can be caused by individual isolate [54]. Blight pathogens isolates in this study differed significantly in their aggressiveness level. Differences observed in the aggressive level of early blight pathogens may be attributed to the effect of factors such as environmental conditions influenced by AEZs, variation in farming practices and a possible occurrence of different strains of pathogens. Indeed molecular analysis revealed involvement of four *Alternaria* species. Further, according to reports aggressiveness of early blight pathogens such as

Alternaria solani is influenced by environmental conditions such as variation in temperature and moisture and farm practices like the cropping system [55] [33] [56]. For example, because of the impact on pathogen structure, mono-cropping or mixed cropping may influence pathogen aggressiveness. Differences in the aggressiveness of the isolates may be an indication of existence of different races of the pathogen. Occurrence of different races of pathogen may circumstantially cause synergistic interactions leading to elevated level of crop damage [55] [56]. Variation in the level of aggressiveness of blight pathogen isolates in this study is in tandem with the finding of van der Waals *et al.* [55]. Variation in aggressiveness of pathogen in an area influences the level of susceptibility of the crop varieties [54].

4.4 Susceptibility of Tomato Varieties to Foliar Fungal Disease Pathogen Isolates from Kirinyaga County

Cultivation of crop varieties that are tolerance/resistant to diseases lowers cost of diseases management in addition to protecting the environment against continuous use of harmful fungicides. Screening of tomatoes against disease pathogen in different niches may aid in identification and selection of varieties that may be explored in breeding for resistance and as well as adoption by farmers to lower mitigate high production cost associated with fungicide use. The tomato varieties screened against various *Alternaria* disease pathogens in this study showed significant variation on their susceptibility level. Kilele F₁ variety was found to be the most susceptible to *Alternaria* pathogens isolates (73.84%) while Terminator F₁ was least susceptible (57.99%). Difference in the susceptibility of tested tomato variety depicts variation in their genetical makeup. Varieties with high susceptibility rating may lack genes that confer resistant to early blight infection Chaerani *et al.*, [60] [61]. These results may be compared to the findings of Mphahlele *et al.* (2020) who observed variation in the susceptibility of Money-maker and Rodade tomato variety grown in South Africa to early blight isolates. The findings indicate that tomato varieties grown in different regions are susceptible to early blight infection. There was however no significant difference in the effect of isolates on the susceptibility in individual tomato varieties screened in this study. This may be attributed to the narrow genetic diversity of isolates used as they were selected on the basis of being the most aggressive during the detached leaf experiment. The findings of this study imply that tomato varieties screened do not have resistance towards *Alternaria* pathogens in Kirinyaga County. However, Terminator F₁ variety displayed reduced susceptibility when compared to the rest of varieties screened. The results deviate from the findings of Mphahlele *et al.* [20] in Limpopo Province where a significance effect of early blight isolates on the susceptibility of individual tomato varieties was observed. Further the results of this study differed to those of Bessadat *et al.* [54] and Ragupathi *et al.* [47] which reported variation in the susceptibility of tomato variety to early blights in Algeria and India respectively.

V. Conclusion and Recommendations

Early blight pathogen isolated from tomato leaves from different AEZ differs in their phenotypic characteristics such as aggressiveness and on the conidia aspects such as number of transverse and longitudinal septa. For instance, conidia lengths ranged from 52.8 µm for isolate EB_ND1 to 207.2 µm for isolate EB_WNG4 both isolated in AEZ in LM4. Further, morphological and molecular characterization revealed involvement of different species of *Alternaria* including *Alternaria solani*, *Alternaria cerealis*, *Alternaria arborescens* and *A. alternate* as tomato foliar pathogens in different AEZ of Kirinyaga County. Susceptibility screening showed that none of the tomato varieties screened was resistant towards fungal pathogens under artificial inoculation in the greenhouse setup. However, Kilele F₁ and Terminator F₁ were found to be the most and least susceptible varieties, respectively to the early blight pathogens. Thus, Terminator F₁ may be adopted by farmers in Kirinyaga County due to its low susceptibility to early blight infection. Further studies are also necessary to determine physiological and biochemical response of tomato varieties towards different isolates of early blight fungal pathogens in Kirinyaga County.

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