

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. High consumption and production are 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, knowledge gaps exist regarding the identity of *Alternaria* species causing tomato blight in different Agro-ecological zones (AEZs) and the susceptibility of locally grown tomato varieties to the *Alternaria* pathogens. To address these gaps, we conducted a study to determine the characteristics of a *Alternaria* pathogen isolated from tomato leaves from different AEZs and assessed the susceptibility level of some tomato varieties grown in Kirinyaga County. Pathogen characterization was done using phenotypic and molecular approaches, and the susceptibility assay was evaluated by artificial inoculation. The molecular characterization involved BLASTIN analysis of Sanger sequenced DNA nucleotide amplicons, and the use of NCBI databases. The susceptibility assay was carried out in the greenhouse using a completely randomized design. Data on the fungal conidial lengths (μm) and the susceptibility (%) of tomato varieties to fungal pathogen isolates were subjected to Analysis of Variance (ANOVA) with significance means separated using LSD at $\alpha = 0.05$. The morphologies of *Alternaria* pathogens differed significantly ($p < 0.05$). Molecular characterization implicated *Alternaria solani*, *Alternaria cerealis*, *Alternaria arborescens*, and *Alternaria alternate* in causing tomato blight. Tomato varieties differed in their susceptibility to *Alternaria* pathogens ($p < 0.05$). Kilele F₁ (73.84%), and Terminator F₁ (56.48%) were the most and least susceptible tomato varieties, respectively. Thus, Terminator F₁ can serve as a source for the genetic improvement of tomato varieties in the Kirinyaga patho-system. Our study provides valuable information on the identification of *Alternaria* species causing tomato blight in different AEZs in Kirinyaga County and the susceptibility of locally grown tomato varieties to infection by *Alternaria* pathogen isolates. The findings can aid in the development of effective disease management strategies and the genetic improvement of tomato varieties in the area.

Keyword: *Alternaria solani*, *A. cerealis*, *A. arborescens*, *A. alternate*, tomato, Kirinyaga

I. INTRODUCTION

Early blight is a destructive and widely spread fungus disease that may potentially cause tomato yield losses of up to 80% [1][2]. Necrotrophic fungi in the genus *Alternaria*, which include *Alternaria alternate*, *Alternaria solani*, and *Alternaria tomatophila*, have been vindicated as the causative agents of early blight diseases [1][2]. Plant diseases limit chlorophyll levels and enhance the 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], enhance the synthesis of ascorbic acid while limiting the synthesis of antioxidants (Glutathione and soluble phenolic compounds), salicylic acid, a stimulant, and catalysts

such as polyphenol oxidase and phenylalanine ammonia-lyase, which are necessary in plant protection in times of stress.

Tomato production is practiced 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, and light duration, are likely to influence pathogen selection, pathogen development, and disease progress [8][4]. Due to environmental fluctuations and their impact on pathogens' behaviour, adaptations, and morphologies, the burden of disease control requires accurate identification of the causing pathogen [9]. Accurate pathogen identification is fundamental to farmers, as it links the disease-causing pathogen with the already available information necessary to effect management [10]. Traditionally, identification of fungal pathogens entailed an elaborate process that included pathogen isolation and characterization [11][12][13]. Phenotypic characterization has been predominantly used in the identification of fungal pathogens, 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, and brownish-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, with circular or irregular margins that did not form concentric rings. Regarding cultural traits, variation in *Alternaria solani* strains from the Jordan Valley was reported with a mycelial width range of 0.8 to 1.5 μm , conidial 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 the 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 the characterization of the causative pathogens from different AEZs. Use of morphologies for identification of fungal phyto-pathogens is tedious, requires expertise, and is hindered by fungal inconsistency and phenotypic plasticity [21][22][23][24]. To resolve this inadequacy, there is a need for using advanced identification criteria, such as molecular techniques that allow comparative genome analyses to distinguish closely-related pathogens [1]. A combination of phenotypic and molecular based identification approaches can provide more comprehensive knowledge of the tomato foliar fungal pathogen in Kirinyaga County to facilitate management. Improved tomato varieties such as Terminator F₁, Kilele F₁, Hansal F₁ and Riotinto F₁ among others have been introduced to farmers in Kirinyaga County. However, cases of blight i.e., early blight and other fungal diseases have persisted [25][20][5]. Success in breeding for EB resistance has been slowed down by the limited availability of effective resistance genes, particularly among the cultivated tomato [26]. Therefore, susceptibility tests of tomato varieties to early blight disease may serve as guidelines for the breeding of disease resistant cultivars, thereby improving tomato production and income to farmers. According to Odilbekov [27] and Adhikari *et al.* [1], knowledge gap exists on the susceptibility of varieties of tomato grown by farmers to diseases that include early blight. Thus, screening of local tomato varieties against early blight diseases in different patho-systems such as the 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 pathogens 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 side of the Mountains about 100 km North East of Nairobi [28] at latitudes of 0° 37'S and 0° 45'S and longitudes of 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 and 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, and UM 3), and the marginal cotton zone in zones LM 3 and LM 4 (Table 1 [6]).

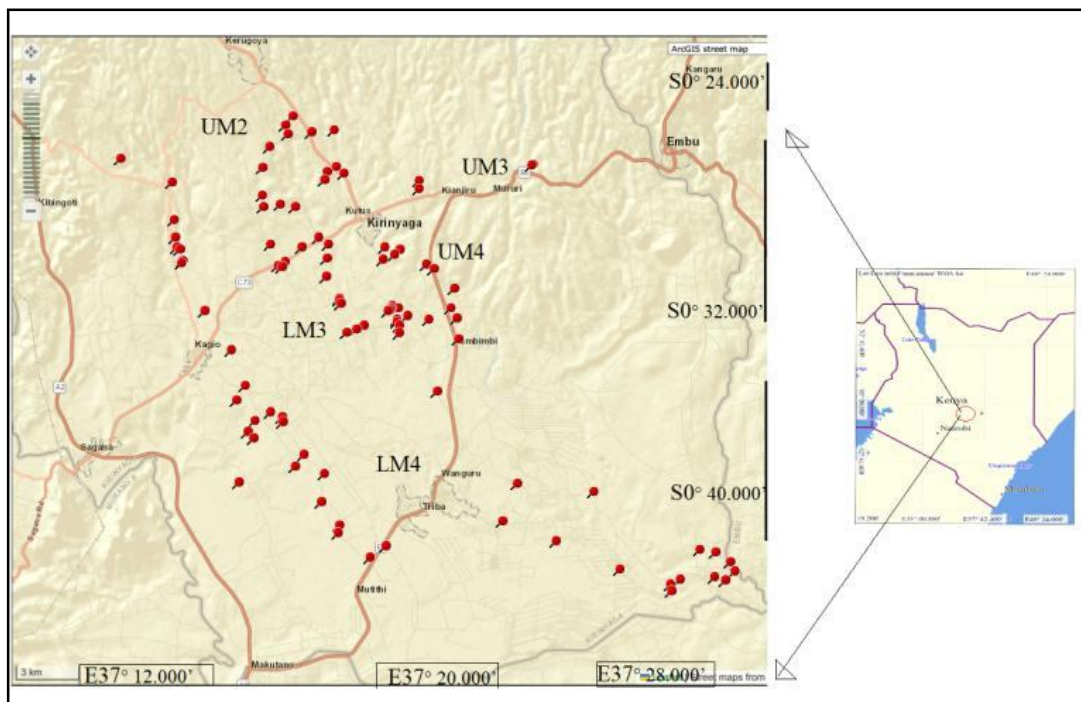


Figure 1: Map of Kirinyaga County showing agro-ecological zones (UM2, UM3, UM4, LM3 and LM4) surveyed for foliar diseases of tomato; where UM = Upper midland (1, 2 and 3), LM = Lower midland (3 and 4).

As shown in Table 1, soil types in Kirinyaga County differ within and across AEZs. For instance, whereas AEZ UM2 and UM3 are composed primarily of humic nitosols, LM4 is composed of three soil types, i.e., humic nitosols, eutricnitosols, and pellicvertisols[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 EutricNitosols	1280 - 1340	20.4-20.9	s/m + s	950 - 1200
				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, EutricNitosols PellicVertisols	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) Jaetzoldet al.[6].

2.2 Experimental Designs

To determine the susceptibility of tomato varieties to foliar fungal pathogens, a 5 × 5 factorial experiment was used, which was laid out in a complete randomized design (CRD) and replicated 6 times under greenhouse conditions. There were two factors, i.e., tomato varieties at five levels (Hansol F₁, Kilele F₁, Rambo F₁, Riotinto F₁ and Terminator F₁) and *Alternaria* pathogen isolates 5 levels (*Alternaria* pathogen isolates EB-KAM8, EB_WNG4, EB_NDM3, EB_KER7 and control). The control treatment consisted of an uninoculated tomato plant for each treatment.

2.2.1 Isolation of Blight Pathogen form diseased Leaf

A small piece of diseased leaf tissue (3 x 3 mm) at the margin of a blight lesion and a healthy leaf part were cut using a sterile blade. The cut lesion was surface disinfected with 0.3% sodium hypochlorite for 1 min, then rinsed in 3 changes of distilled water to remove the detergent. The excisions were surface sterilized in 70% ethanol for 30 seconds, rinsed with sterile distilled water three 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, the colonies were viewed under the microscope and pure cultures were prepared.

2.2.2 Purification and Identification of Fungus

Pure fungal pathogens were obtained by subculturing in fresh PDA media. A flame sterilized scalpel was used to cut the culture media with grown mycelia at the periphery and transfer it into fresh media, which 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 [29]. 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) 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 [29]. The slide was then observed under LCD phase contrast microscope light microscope (Model: OMAX 40X-400X) at x 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 2= Medium 3= Rapid	1= Brownish 2= Greyish 3= Whitish	1= Greenish 2= Greyish 3= Dark
Presence of Beak (PB)	No. transverse sepor (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 [17][30][31][20]. 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

A pathogenicity test was carried out as required by Koch's postulate [32] on disease-causing organisms in a pot experiment in the greenhouse at Chuka University. Spore suspension (10^3 spores/mL) prepared from two-week-old pure cultures of the blight disease pathogen The spores on the plates were scraped gently with a scalpel, suspended in 100 ml distilled water, and filtered through three-fold cheesecloth. A hemocytometer (30 x 70 x 4mm) was used to standardize the inoculum to 10^3 spores/mL. A two-month-old Terminator F₁ tomato variety was used in the pathogenicity test (Koch's postulate experiment). The Terminator tomato variety was selected for the pathogenicity test because it is grown in most areas where the pathogens were obtained. The leaves of tomato plants were sprayed with respective fungal pathogen isolates and 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[33] and Tymon *et al.*[34]was used. Ten well-developed, mature, and disease-free

tomato leaves from Terminator F1 were aseptically collected from the 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, surface-sterilized with 70% alcohol for 5 seconds, and rinsed again in three changes of sterile water to remove the alcohol. The water on the leaf surface was wiped out using sterile blotting paper. The five sets of leaves were pricked using a sterile needle and inoculated with a pathogen suspension (20 µl) of the 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 a 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 the development of lesions. 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 [35].

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

2.2.4 Molecular Identification of Selected Isolates

The molecular analysis of fungal pathogens isolated in this study was done at the University of Nairobi's Centre for Biotechnology and Bioinformatics. The DNA was extracted from 20 mg of mycelia harvested by scraping the colony surface. The mycelia were ground into fine pieces using a 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 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% (w/v) polyvinylpyrrolidone, and 1% (v/v) β-mercaptoethanol [36]). 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). The 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 (10I 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 sets ITS1 [(50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) described by White *et al.* [37] and Lee and Taylor [36]. Amplification reactions were 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 with ethidium bromide. The DNA amplicon (1 µl) mixed with loading dye was assayed alongside a 1 kb 0 gene ruler plus ruler with an initial reading of 75 kb. The DNA loading bands were viewed on a gel documenter imager (Bio-Rad). Positive DNA PCR products were cleaned using the Quick cleaning kit supplied by Gene Script Company in readiness for Sanger sequencing. The sequencing of the DNA amplicons was done at the International Livestock Research Institute in Nairobi, Kenya, using a Sanger sequencer (Model: ABI 3730 genetic analyzer) 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 the Kenya Seed Company in Nairobi. The seedlings 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 daily for three weeks before transplanting. Calcium Ammonium Nitrate (CAN) and Diammonium phosphate (DAP) were used to supplement 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) [38]. The three-week-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 the respective fungi pathogen culture containing 10³ CFU/ml as determined by hemocytometer.

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 [39]. 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 [40]:

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

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 PCR fragment ITS rDNA sequences were compared with the record present in the NCBI/Gene Bank database (www.ncbi.nlm.nih.gov/blast/). The obtained fungal species sequences from the database were subjected to multiple sequence alignment using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program method available in the Mega X version 11[41]. 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 [42]. 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 [43].

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

The skewness value for the data on susceptibility, presented as percentages of symptom severity, was calculated to be 0.0119. Additionally, the results of the Shapiro-Wilk normality test indicated that the data followed a normal distribution, with a p-value greater than 0.05. Consequently, the susceptibility data was analyzed using the general linear model procedure in SAS version 9.4, with the significance of the means being determined through the least significant difference (LSD) test at an alpha level of 0.05.

III. RESULTS

3.1 Phenotypic Characterization of Early Blight Pathogen

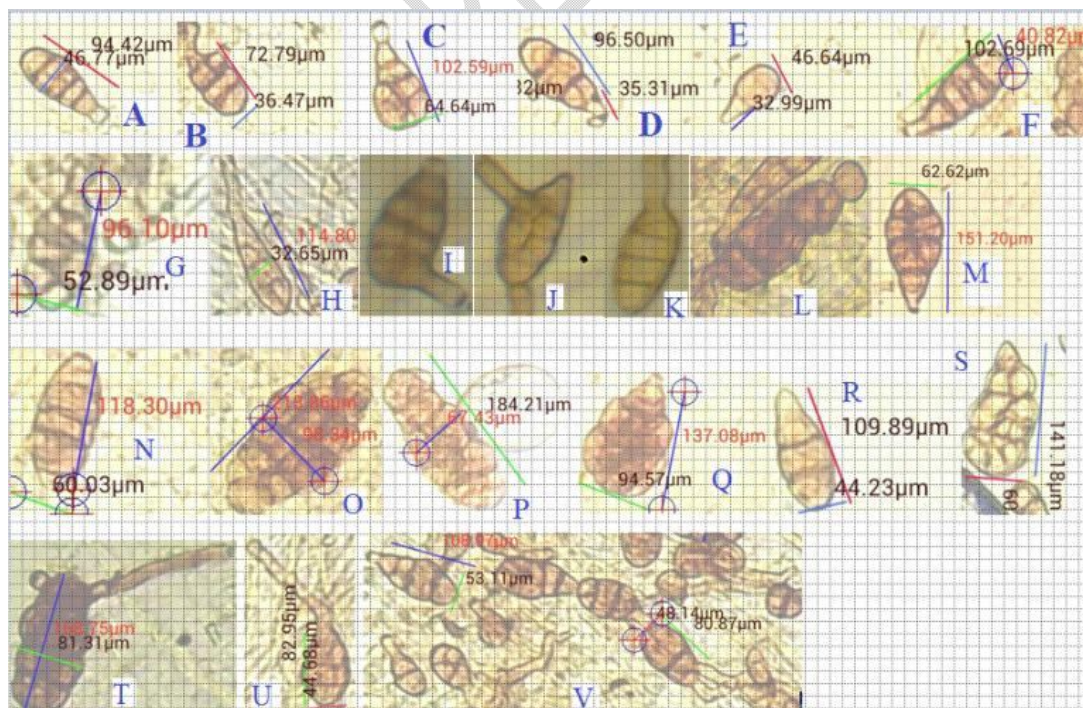
A total of 30 *Alternaria* pathogen isolates were obtained from diseases tomato leaves from different AEZ. Most on the isolates on potato dextrose agar were not different in terms of spore's colour on PDA. The isolates had green- dark grey spores and had dark pigmentation. The cultures had either regular 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
	EB_KGG9	2	1	2	1	0	3	1
UM4	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
	EB_GTH3	1	1	2	2	2	4	1
	EB_GTH5	1	2	2	2	2	3	1
UM3	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
	EB_GEO3	2	2	1	3	0	3	2
	EB_GEO6	2	3	2	2	0	4	1
UM2	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),



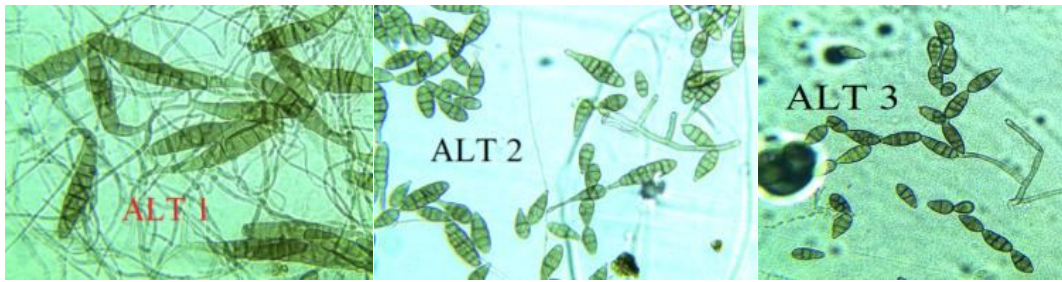


Plate 1: Selected conidia morphologies for the early blight pathogen (*Alternaria*spp) isolates from tomato leaves from Kirinyaga County, Kenya,

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

The study observed that the conidiophores of *Alternaria* spp isolates were either solitary or grouped and exhibited straight or flexuous morphologies. The conidia displayed variations in breadth, length, and the presence of beaks. The shape of the conidia was either straight or slightly curved, with some displaying muriform or ellipsoid morphology. The conidia tapered towards one end and were observed to be olivaceous brown in colour (Plate 1). Notably, the transverse and longitudinal septa of the conidia were found to vary, ranging from 1 to 6 and 0 to 2, respectively. The conidia appeared in chains or as single units, and some exhibited short, long, or no beaks (Plate 1).

A total of 30 early blight pathogen isolates from five distinct Agro-Ecological Zones (AEZ) were subjected to cluster analysis based on their phenotypic characteristics, resulting in the formation of three clusters. The first and third clusters consisted of 13 isolates each, which were further subdivided into two sub-clusters. The smallest cluster comprised of isolates EB_KBU3 (from AEZ), EB_LM4, and EB_KD09 (both from AEZ UM2), isolate EB_KER2 and EB_KER7 (both from AEZ LM3 and AEZ LM4), and isolate NDM7 (from AEZ UM4), as depicted in Table 3 and Figure 2. It is worth noting that isolates EB_KD09 and EB_KER7 had similar distances while isolate EB_KBU3 formed a separate sub-sub clade. Isolates EB_KD09 and EB_KER7 exhibited similar growth rates, beak morphology, colony margins, conidia arrangement, and the number of transverse septa, as outlined in Table 3.

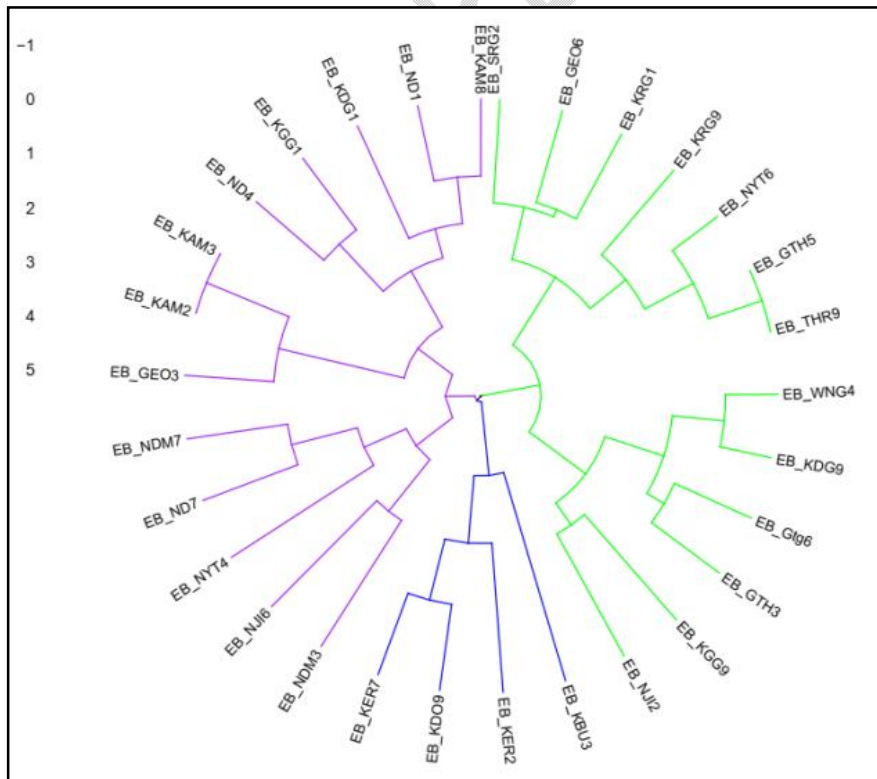


Figure 2: Cluster membership based on phenotypic traits of early blight pathogens isolated from tomato leaves from different agro-ecological zones of Kirinyaga County

There was a significant ($F(29, 210) = 8.25, p < 0.0001$) difference observed in the conidial lengths of pathogen isolates from infected tomato leaves collected from different Agro-Ecological Zones (AEZ). Within AEZ LM4, the lengths of pathogen isolates were significantly ($F(5, 42) = 22.31, p < 0.0001$) different, with the minimum and maximum conidial lengths being 52.8 and 194.2 μm , respectively (Table 4). Isolate EB_ND4 had longer conidia (148.37 μm), while isolate EB_KBU3 had the smallest length of conidia at 78.52 μm (Table 4). In AEZ LM3, the lengths of the conidia of the pathogen did not differ significantly ($F(5, 42) = 2.05, p < 0.0909$); however, the conidia lengths ranged from 95.60 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, with the minimum and maximum conidia lengths measured being 85.2 and 184.2 μm , respectively. Isolate EB_NJI2 had longer conidial length (131.17 μm), while isolate EB_NDM7 had shorter conidial length (101.17 μm). The length of conidia for the early blight pathogen was 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 a shorter 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 ^{tgh}	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 ^{edf}	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 ^{fg hij}	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, ^bMinimum conidia lengths, ^cMaximum conidia lengths, ^d Means followed by the same letter in columns is not significantly different at $\alpha = 0.05$

Analysis of the length of conidia of the *Alternaria* pathogen isolated in the five agro-ecological zones (AEZs) revealed a statistically significant difference ($F(4, 235) = 8.25, p < 0.0001$). Specifically, conidia length 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 $\alpha = 0.05$

In the pathogenicity test using isolate EB_WNG4, typical symptoms similar to those observed in tomato leaves from farmers' fields were observed, characterized by concentric rings visible in the lesion. Re-isolation of the pathogens from the lesions showed similar phenotypic features to the initial pathogen isolates, including spore colour and conidia shape. In the detached leaf experiment, inoculation of tomato leaves with selected 10 isolates showed a significant difference in lesion size formed by the isolates ($F(13, 36) = 12.48, p < 0.0001$), as presented in Table 6. Isolate EB_KAM8 demonstrated high aggressiveness with a lesion size of 122.17 mm^2 , followed by isolate EB_NDM3 with a lesion size of 110.17 mm^2 . Isolate EB_KDG1 exhibited a smaller lesion size of 17.10 mm^2 , followed by isolate EB_ND4 with a lesion size of 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

^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

Agarose gel electrophoresis of DNA amplicons (ITS rDNA) from *Alternaria* pathogens isolated from tomato leaves with early blight symptoms showed distinct bands ranging from 500 to 700 bp, as visualized using a 1kb gene ruler plus ladder (Plate 2).

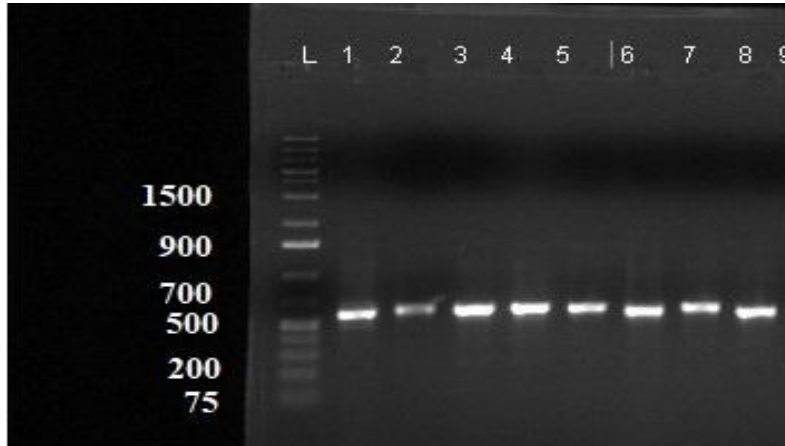


Plate 2: The agarose gel analysis of DNA amplicon PCR products (ITS rDNA) of tomato foliar pathogen isolates isolated from diseases leaf samples from Kirinyaga County

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 analysis of DNA amplicon Sanger sequenced nucleotides, specifically ITS rDNA sequences, using the BLASTn tool, identified multiple pathogens involved in causing blight-like symptoms. Notably, *Alternaria solani* and *Alternaria alternata* were found to be among the causative agents (Table 7). Sequence similarities between the 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

^aPercentage similarity

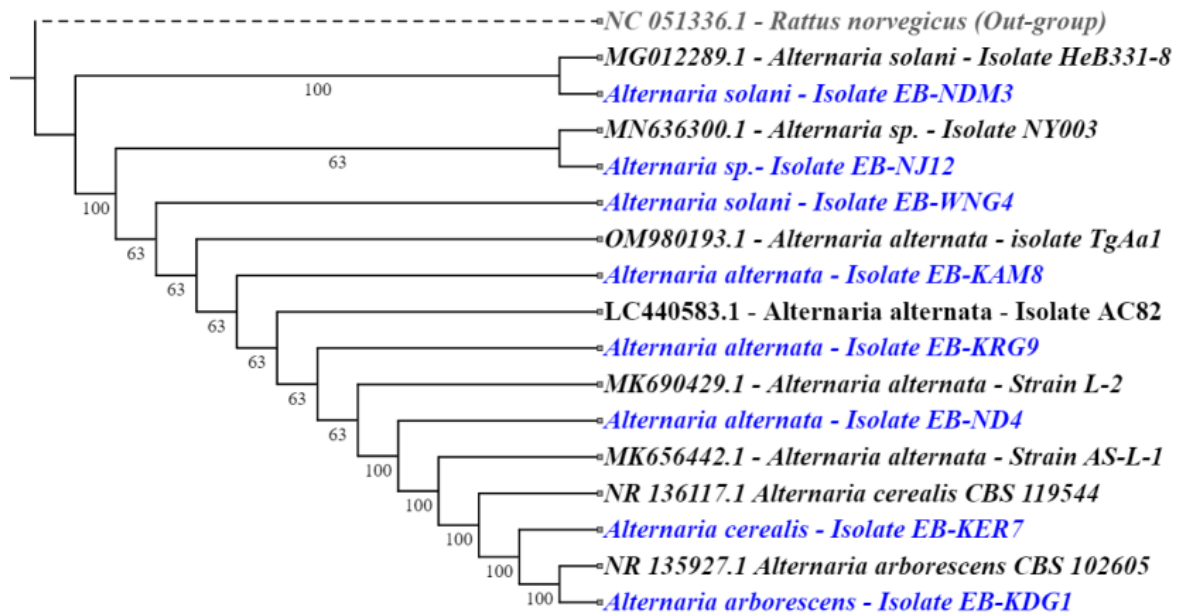


Figure 3: Phylogenetic analysis of sequences of some *Alternaria* pathogens isolated from tomato foliar and similar sequences in the gene bank using MEGA X 11 software, UPGMA method and 10000 boot-strapped replications. The species in blue font are the fungal pathogens species isolated in the current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

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

The susceptibility of different tomato varieties to *Alternaria* pathogen isolates was found to differ significantly [$F(19, 100) = 4.73, p < .0001$]. Kilele F_1 exhibited the highest severity of blight symptoms (78.24%) when inoculated with isolate EB-KAM8, followed by Riotinto F_1 (72.22%). Conversely, Terminator F_1 exhibited the least severity at 58.33%. Inoculation with isolate EB_KER7 resulted in severe symptoms in Kilele F_1 (69.91%) and Rambo F_1 (67.59%), while Terminator F_1 exhibited the least severity (54.17%). Similarly, isolate EB_NDM3 induced severe symptoms in Kilele F_1 (70.83%) and Riotinto F_1 (68.06%), while Terminator F_1 exhibited the least severe blight symptom (58.33%). Additionally, inoculation with isolate EB_WNG4 resulted in severe symptoms in Kilele F_1 (76.39%) and Riotinto F_1 (62.2%), while Hansol F_1 exhibited the least severity [(58.33%) 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 $\alpha = 0.05$

The results of the experiment showed that the Rambo F₁ tomato variety did not demonstrate a significant difference in susceptibility towards *Alternaria* pathogen isolates, as indicated by the statistical analysis ($F(3, 20) = 0.56, p = 0.649$). In contrast, the Riotinto F₁ variety showed a significant difference in susceptibility towards pathogen isolates ($F(3, 20) = 4.36, p = 0.0162$), with isolate EB_NDM3 resulting in a higher severity mean of 55.86%, while isolate EB_KER7 resulted in a lower severity mean of 50.31%. However, the Hansol F₁ and Kilele F₁ varieties did not exhibit a significant difference in susceptibility towards pathogen isolates, as indicated by the statistical analysis ($F(3, 20) = 1.57, p = 0.229$ and $F(3, 20) = 2.16, p = 0.124$, respectively). Similarly, the Terminator F₁ variety did not demonstrate a significant difference in susceptibility towards pathogen isolates ($F(3, 20) = 1.36, p = 0.283$). Results are presented in 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

Fungal morphology, biochemistry, and metabolites are essential in the identification and diagnosis of plant pathogenic fungi [11][44][13]. Phenotypic characteristics such as conidial shape, colour, and septation are key features in the identification of fungal pathogens [14][15][16]. In this study, the conidia of *Alternaria* blight pathogen isolates appeared greyish to greenish in colour and darkened as the culture aged. The margins of the cultures were either regular or irregular in pattern, which is consistent with previous study conducted in Mwea in Kirinyaga County, Kenya[20]. Pigmentation and colony texture of the *Alternaria* blight pathogens in this study were similar to previous reports where greenish, dark, and brownish pigmentation have been observed [17][30][20]. The surface texture of the colonies exhibited slight variations with zonation at the margins, which were similar to those observed in other studies involving *Alternaria* blight pathogens [17][30][31].

Microscopic observation revealed variation in the conidia morphology of the pathogen isolates, which is consistent with the findings of Mugao *et al.*[20] where variability of the tomato early blight pathogen in Mwea was reported. Furthermore, the observations on the morphological traits of the conidia are in agreement with other studies where conidia have differed in breadth, length, and beak and have been reported to be straight, slightly curved, muriform or ellipsoid [45][46][47][48]. 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[45][46][47][49]. The length of conidia of these *Alternaria* pathogen isolates ranged from 78.52 μm to 161.72 μm . The observed conidia lengths in this study were within the range of 105-220 μm reported earlier by Nolla[50]. However, the conidia lengths were longer than those reported earlier by Neergaard[51] and Abubakar and Ado [52] ranging from 10.26 - 77.52 μm . These findings suggest that the pathogen populations in each AEZ are distinct and may have different virulence and pathogenicity levels.

Due to the intricate nature of *Alternaria* species isolates, molecular characterization was deemed essential for accurate identification. The analysis of Internal Transcribed Spacer (ITS) rDNA sequences revealed that the causal agents of tomato Kirinyaga County in Kenya are *Alternaria solani*,

Alternaria alternata, *Alternaria cerealis*, and *Alternaria arborescens*. While there is limited documentation of *Alternaria cerealis* and *Alternaria arborescens* as tomato pathogens in Kenya, these pathogens have been reported in tomato crops in other regions. For instance, *Alternaria arborescens* has been documented as a tomato pathogen in Egypt, Algeria, Iraq, and Iran, causing leaf blight, stem canker, tomato collar rot, and fruit rot [53], [54], [55], [56], [57]. Similarly, *Alternaria cerealis* has been reported as a tomato pathogen in Yemen [58]. It is possible that our study may constitute the inaugural documentation of *Alternaria cerealis* and *Alternaria arborescens* as a pathogenic organism for tomato crops within the geographical confines of Kenya. Basing on these results, it is essential to consider the variability of pathogen populations in the management of *Alternaria* pathogens in tomato crops. Our results also highlight the importance of conducting regular surveillance and monitoring of the *Alternaria* pathogen populations in tomato crops in different AEZs. This information can be used to develop appropriate control strategies and management practices that are tailored to specific pathogen populations. Further, it is a need for further studies to investigate the genetic and molecular basis of the observed differences in conidial lengths among the pathogen isolates.

4.2.3 Evaluation of Aggressiveness of Alternaria Fungal Pathogens of Tomato

Understanding the aggressiveness level of different pathogen isolates is critical for assessing the potential crop damage caused by individual isolates [60]. In this study, the blight pathogen isolates displayed significant differences in their aggressiveness level, which could be attributed to environmental conditions, farming practices, and the occurrence of different pathogen strains. Molecular analysis revealed the involvement of four *Alternaria* species in this study. According to reports, environmental factors such as temperature and moisture variations, as well as cropping systems, can influence the aggressiveness of early blight pathogens such as *Alternaria solani* [61], [34], [62]. For instance, monoculture or mixed cropping can impact pathogen structure and affect aggressiveness. Variation in the aggressiveness of isolates may indicate the existence of different pathogen races, which can result in synergistic interactions and increased crop damage [63], [64]. These findings are consistent with those of van der Waals *et al.* [65]. Differences in the aggressiveness of blight pathogen isolates may influence the susceptibility of crop varieties in the region [54]. Further studies are needed to investigate the factors that influence the aggressiveness of *Alternaria* blight pathogens in the study area.

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

Cultivation of crop varieties that are tolerant or resistant to diseases can decrease the cost of disease management and protect the environment from the continuous use of harmful fungicides. To identify and select resistant varieties that can be adopted by farmers in order to lower the high production costs associated with fungicide use, screening of tomatoes against disease pathogens in different niches may be helpful. The tomato varieties screened against various *Alternaria* disease pathogens in this study showed significant variation in their susceptibility level. Kilele F₁ variety was found to be the most susceptible to *Alternaria* pathogen isolates (73.84%), while Terminator F₁ was the least susceptible (57.99%). The differences in the susceptibility of the tested tomato varieties suggest variation in their genetic makeup. Varieties with a high susceptibility rating may lack genes that confer resistance to early blight infection [66], [67]. These results can be compared to the findings of Mphahlele *et al.* [66], who observed variation in the susceptibility of Money-maker and Rodade tomato varieties to early blight isolates in South Africa. However, there was no significant difference in the effect of isolates on the susceptibility of individual tomato varieties screened in this study. 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 low susceptibility when compared to the rest of the varieties screened. The results deviate from the findings of Mphahlele *et al.* [19] in Limpopo Province, where a significant effect of early blight isolates on the susceptibility of individual tomato varieties was observed. Furthermore, the results of this study differed from those of Bessadat *et al.* [60] and Ragupathi *et al.* [48], which reported variation in the susceptibility of tomato varieties to early blights in Algeria and India, respectively.

V. Conclusion

Cultural analysis of *Alternaria* pathogen isolates from tomato plants with early blight-like symptoms revealed the presence of different species, including *A. solani*, *A. alternata*, and *A. cerealis*. The morphological analysis of the isolates showed variations in colony colour, texture, and spore

morphology. Microscopic examination of the spores revealed distinct morphological features such as the shape, size, and septation pattern. Molecular analysis revealed involvement of different *Alternaria* pathogen in causing tomato blight in Kirinyaga County. In terms of susceptibility, the study found that different tomato varieties had varying levels of susceptibility to *Alternaria* pathogen isolates. Kilele F₁ variety expressed the most severe symptoms of early blight to isolate EB-KAM8, while Terminator F₁ had the least severe symptoms. In conclusion, this study highlights the importance of using a combination of cultural, morphological, microscopic, and molecular techniques in identifying and characterizing *Alternaria* pathogen isolates from tomato plants. It also underscores the need to select tomato varieties that exhibit resistance to *Alternaria* pathogen isolates, such as Terminator F₁.

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