Cultural, Morphological and Biochemical Identification of *Xanthomonas Spp* the Causative Agent of *Bacteria Leaf Spot in Tomatoes* in Wanguru, Mwea, Kirinyaga County, Kenya

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Abstract: **- Bacteria diseases are major constraints in commercial agriculture. Bacteria diseases have continuously caused huge loss to both conventional and organic farming systems globally. Bacteria leaf spot is a tomato disease of concern whose management remains a challenge across the globe. The pathogen *Xanthomonas campestris pv vesicatoria* the causative agent of leaf spot in tomato has been isolated and positively identified in many countries to enable control. In Kenya, tomato farmers have experienced losses associated with bacterial diseases despite using available control strategies. Application of control mechanisms by majority of farmers heavily rely on symptoms on plants other than laboratory facts on pathogen identification. As such wrong chemicals have been applied which have led to development of resistance due to pathogen evolution. Scientific identification of pathogen is necessary to enable application of accurate and effective management strategy. Nonetheless, this has scarcely been done for tomato bacteria diseases in Kenya.**

The objective of this study was to isolate and identify bacteria leaf spot pathogen of tomato from Wanguru area in Mwea, Kirinyaga county in Kenya using nutrient agar, differential staining and selected biochemical methods. A total of ten tomato heavily infected leaves were selected randomly from different farms. The colonies were generally yellow on the surface of nutrient agar. Results of differential staining showed gram negative rods while biochemical tests slightly varied. Based on totality of cultural, morphological and biochemical tests results, we concluded that *Xanthomonas campestris pv vesicatoria* is responsible for leaf spot tomato disease in Wanguru. However, we recommend the inclusion of molecular tool for proper identification.

Key words: **Xanthomonas_campestris, Cultural, Morphological, Biochemical, Tomatoes, Wanguru, Mwea, Kenya**

I. INTRODUCTION

Bacteria species of the genus *Xanthomonas* are important group of plant pathogenic that are known to cause leaf spot disease affecting both monocotyledonous and dicotyledonous plants. *Xanthomonas Spp* pathogen is major concern in tomato production [1]. Leaf spot disease can affect and diminish tomato yield significantly [2]. The economically important crops affected by *Xanthomonas* leaf spot among others include *Oryza sativa*, *Solanum lycopersicum*, *Capsicum annuum* and *Brassica oleracea* [3], [4], [5]. *Xanthomonas Spp* have been reported to be more prevalence in region experiencing warm and humid climate [4]. Pathogenicity of *Xanthomonas Spp* is attributed to its wide range of plant cell wall degrading enzymes [3]. These enzymes include endoglucanases, polygalacturonases, pectinases and xylanases [3], [6]. Pathogenicity enzymes are secreted by the Xps type II secretion system (T2SS) which is conserved in *Xanthomonas Spp* [7], [6].

The major constraints in commercial agriculture are plant diseases such as bacteria diseases causing huge loss to both conventional and organic farming systems. However, the major challenge in the study of bacterial pathogens lies in pathogen proper identification [8]. Pathogens have constantly changed into new strains hence a challenge to farmers [9]. Different researchers have reported success of identification of *Xanthomonas Spp* based on cultural, morphological and biochemical reactions [10], [11].

Nutrient agar (NA) have been successful used for cultural study and identification of *Xanthomonas Spp* in different countries. Researchers who have used NA include; Bouzar *et al*., [12] in Central Sudan in 1993; Manjula and Khan, [13] in Karnataka; Srabani, [14] and Vanlalruata, [11] in West Bengal, India; Basim *et al*., [15] in Turkey and El-Ariqi *et al.,* [16] in Yemen. The current study was done to isolate and identify bacteria leaf spot of tomato in Wanguru area in Mwea, Kirinyaga county in Kenya using nutrient agar, differential staining and selected biochemical tests.

II. MATERIALS AND METHODS

Study Area

Wanguru area is located in Mwea irrigation scheme in Kirinyaga county, Southern outskirts of Mt. Kenya and approximately 100 Kilometers North East of Nairobi [17] (Figure 1). The area lies between latitudes 0° 37'S and 0° 45'S and between longitudes 37° 14'E and 37° 26'E and between 1,100 m and 1,200 m above mean sea level. Area under scheme receives an average annual rainfall of 940

mm. The long and short rains occur from April to May and October to November respectively.

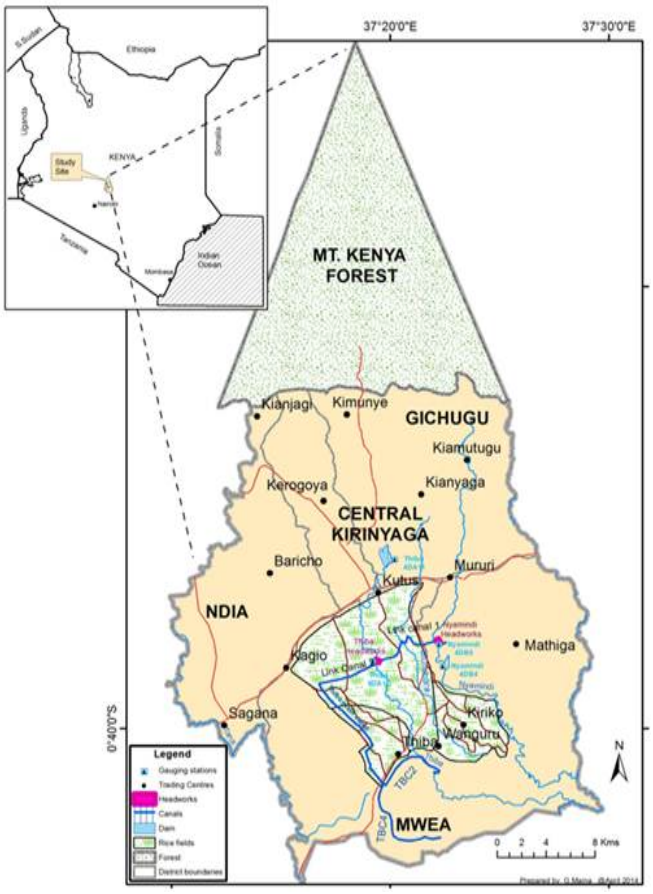


Figure 1: Map of Kirinyaga county showing Wanguru where the study was conducted

Kirinyaga county has temperatures ranging from a minimum of 12 °C to a maximum of 26°C with an average of 20 °C [18]. However, the mean monthly temperature in the area around the scheme is approximately 22.2 ^{o}C with a minimum and maximum of 21.8^{o}C and 24.0 ^{o}C in January and March respectively [17]. Higher temperatures are experienced during the rainy season. The soils have been classified previously as Vertisols. Rice, Maize and horticultural crops among them being tomatoes are grown under furrow irrigation in the scheme [19].

Preparation of Media

Seven grams of nutrient agar (HIMEDIA) was weighed and dissolved in 200 ml of distilled water in 250 ml capacity conical flask by heating while stirring then filled up to 250 ml mark. Dissolved media was then autoclaved at 121ºC for 15 minutes at a pressure of 15 psi in an autoclave model (Model X280A). The media was then cooled to 50 ºC in a water bath. Culture media 15 ml was dispensed in Petri dishes and allowed to solidify.

Sample Collection and Inoculum Preparation

Ten tomato leaves severely diseased with bacteria leaf spot samples were collected from tomato farms at Wanguru area in Mwea in Kenya in February 2019. Symptoms considered positive for bacteria leaf spot were dark and circular or brown-black and angular shaped, water-soaked and smaller [20]. The leaves were aseptically collected in ten different sterile zip lock bags. Collected leaf samples were brought to Chuka University botany laboratory for pathogen isolation. Symptomatic leaf parts infected by bacteria spot were cut, washed in tween twenty for 5 second to remove debris and rinsed in distilled water thrice to remove the detergent. The leaf sections were then passed in 70% ethanol for surface sterilization for 5 seconds and passed through three changes of distilled water to rinse off alcohol. Cleaned and surface sterilized leaf sections were blot dried and crushed using sterile motor and pestle in addition to a drop of distilled water forming a bacteria suspension. This procedure was repeated to prepare ten inoculums from ten different leaf samples collected from the field.

Inoculation and Incubation

Inoculation was done using streak plate method. Dried media surface was streak using bacterial suspension (inoculum) prepared above. Wire loop was sterilized on the flame to redness then cooled. A loop of the inoculum was picked and a line streaked on media surface on one end of the plate. From the initial streak four parallel streaks at an angle of 90^{0} was made. The plate was again turned at 90^{0} and four streaks made from the initial streaks made earlier. The third 90^{0} turn of the plate was done and four streaks originating from the previous 4 streaks made and ended with a zigzag line. A total of ten plates were inoculated each plate being individual sample. Inoculated plates were incubated for 48 hours at 27 ºC in the incubator (model - Memmert TYP INB200).

Cultural characteristics of the leaf isolates of *Xanthomonas Spp* was observed on the nutrient agar growth media. Colonies with mucoid yellow pigmentation were considered positive species of *Xanthomonas*.

Positive *Xanthomonas* colonies (yellow and mucoid colonies) were selected and subjected to differential staining (grams stain), for colony morphology determination. A thin smear was prepared on a clean slide from pure colonies of isolates for gram staining.

Differential Staining

On a slide, a drop of normal saline was added then a loop of pure colony added, homogenized and air dried by passing over the air. The air-dried slide was heat fixed by rapidly passing the slide over the flame. Following smear preparation, a drop of primary stain (crystal violet) was added for 1 minute then rinsed in gently flowing tap water. A drop of Gram's iodine (mordant) was then added for 30 seconds then rinsed off. The cells on slide were then cleared using 95% ethanol for 30 seconds then rinsed off. In order to stain gram negative bacteria, a drop of safranin (counter stain) was added to the cleared cells on the slide and held for 1 minute before

washing the stain off. The slides were blot dried and observed microscopically under SLD inverse light microscope (Model A33.1005). Gram negative bacteria isolates were identified by their ability to stain pink reddish while those retaining the purple colour of crystal violate were considered Gram positive.

Biochemical tests; KOH hydrolysis test, Catalase test, Hydrogen sulphide production, hydrogen sulphide production, catalase production was carried out.

Catalase Test

A loopful of 24 hours old culture grown on the nutrient agar slant was smeared on a slide and covered with two drops of 3% hydrogen peroxide. The reactions producing gas and bubbles were considered to be positive for *Xanthomonas spp.*

Hydrogen Sulphide (H_2S) Production

Broth media for this test was prepared using; 2.5 g of peptone, 1.25g NaCl dissolved in 250 ml of water and adjusted to pH 7.0. 5ml of prepared broth was then dispensed into test tubes, autoclaved and inoculated with 48 h old cultures. lead acetate test strip was suspended in the tube held with cotton plug. Inoculated tubes were incubated at 27 ^{o}C for two weeks and observed for H_2S gas production by blackening of test strip.

KOH Solubility Test

Potassium hydroxide solubility test was conducted by placing two drops of 3% KOH on a glass slide then a colony bacterium isolates from pure cultures were picked with an inoculating loop and homogenized in the KOH for 10 seconds. Isolates were considered KOH positive by occurrence of thread-like slime when picked by wire loop.

Indole Production

Five ml of the medium containing tryptophan 10g, *L-tryptophan* 1g and distilled water poured in test tube and sterilized by autoclaving at 15lbs. at 121 ^{o}C for 15 min. the tubes were thenInoculated test tubes were incubated at 27 ^{o}C for five days. After incubation 0.5 ml of Kovacs indole reagent was added and shaken well. A positive test was indicated by the development of dark red colour in the surface layer.

Methyl red test

Methyl red test was carried out using Methyl red indicator (0.1g methyl red dissolved in 300 ml of 95% ethanol and made up to 500 ml with distilled water). The indicator was added to test culture and change from yellowish to red colour was considered positive reaction. Where there was no colour change the reaction was considered negative.

Starch Hydrolysis

Starch hydrolysis was carried out using starch agar. To prepare this media, Potato starch (1 g) was added to 100 ml of 2.8 g nutrient agar dissolved by heating and sterilize in autoclave before cooling. 10 ml of sterilized cool medium was dispensed in to each of the Petri plates and allowed to solidify, starch agar plates were streak with loopful culture of the bacterium and incubated for 5 days at 28 ^{o}C. After five days, the plates were flooded with Lugol's iodine solution and observations made for starch utilization by the bacteria isolates.

Ammonia Production

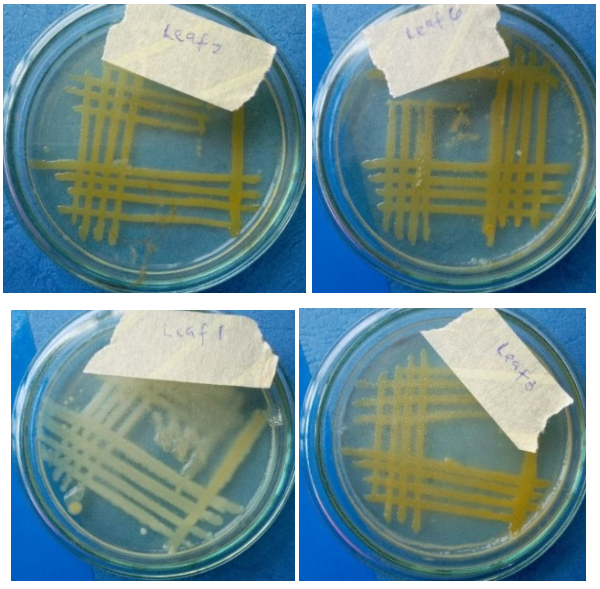
Ammonia production test was performed using nutrient broth. In each test tube 8 ml of sterile nutrient broth was added. The tubes were then inoculated using bacteria isolates and sealed with sterile cotton. Inoculated tubes and one uninoculated tube (control) were incubated at 27 ^{o}C for 48h. after incubation cotton was removed and red litmus paper strip was inserted inside the wall of the tube sealed again with cotton holding litmus paper. No change in the colour indicated negative result.

Voges-Proskauer Test

Test was performed using glucose phosphate broth made up of 0.5% glucose, 0.5% peptone and 0.5% K_2HPO_4). Bacteria isolates were re-cultures in the broth and incubated at 27 ^{o}C for 5 days. On the fifth day,1 ml of culture was added to a test tube containing 0.6ml of naphthol and 0.2 ml of 40 % KOH and shaken vigorously. The tubes were allowed to settle for ten minutes and formation of red colour was considered positive reaction.

III. RESULTS

Cultural characteristics of the leaf isolates of *Xanthomonas* was observed on the nutrient agar growth media. Biochemical results indicated that the isolates differed slightly on cultural traits. Isolate LF1 and LF5 had pale yellow colour and slightly mucoid while isolates LF10 colonies were dark yellow and non mucoid. Isolates LF1, LF7, and LF5 formed yellow/ white colonies (Table 1: Plate 1).



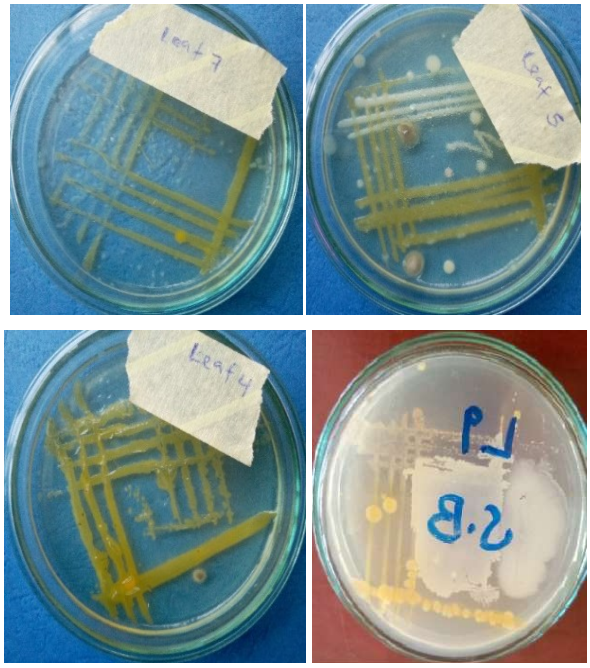


Plate 1: Cultural Characteristics of Xanthomonas Leaf Spot Tomato Isolates Collected from Wanguru Area in Mwea.

Table 1: Cultural Characteristics of *Xanthomonas Spp* Tomato bacteria leaf spot Isolates Collected from Wanguru Area in Mwea

Trait / Isolate	Colour on Nutrient agar	Appearance of colony surface	Mucoidness
LF1	Pale yellow/ whitish	shiny surface/ translucent	Slightly mucoid
LF2	Dark yellow	shiny surface/ translucent	mucoid
LF3	Dark yellow	shiny surface / translucent	mucoid
LF4	Dark yellow	shiny surface / translucent	mucoid
LF5	Yellow/ whitish	shiny surface / translucent	Slightly mucoid
LF6	Dark yellow	shiny surface / translucent	mucoid
LF7	Medium yellow/ whitish	shiny surface/ translucent	mucoid
LF8	Pale yellow	shiny surface / translucent	mucoid
LF9	Dark yellow	shiny surface / translucent	mucoid
LF10	Pale yellow	shiny surface / translucent	Non mucoid

Biochemical Identification

Biochemical results showed that bacteria isolates were Gram-negative, catalase positive, indole formation and methyl red test were conducted to all the bacteria leaf isolates obtained in this study (Table 2).

Table 2: Selected Biochemical Reactions of *Xanthomonas* Bacteria Leaf Spot of Tomato Isolates Collected from Wanguru Area in Mwea

Isolate / Test	*Xanthomonas* leaf isolates									
	Lf 1	Lf 2	Lf 3	Lf 4	Lf 5	Lf 6	Lf 7	Lf8	Lf9	Lf10
Gram negative	+	+	+	+	+	+	+	+	+	+
KOH 3% test	+	+	+	+	-	+	+	-	+	+
H_2S production	+	+	+	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+	+	+	+
Indole test	+	+	+	-	+	-	+	+	-	-
Methyl red reaction	-	-	-	-	-	-	-	-	-	-
V-P test	-	-	-	-	-	-	-	-	-	-
Starch Hydrolysis	+	+	+	-	+	+	-	+	+	+
NH_3 production	+	+	+	+	+	+	+	+	+	+

IV. DISCUSSION

Cultural Characteristic

Bacteria leaf spot isolates were generally yellow in colour with isolated cases of whitish-yellow colonies (Table 1; Plate 1). The yellow colonies of *Xanthomonas Spp* when cultured on nutrient agar is supported by other studies [20], [21]. Even in nutrient agar, *Xanthomonas* leaf isolates cultured showed variation in terms of colony colour density and brightness. Observation on colour density and brightness corresponds with the report by Ali, [22], [11]. Growth of yellow colonies on suitable agar medium such as nutrient agar among others is unique to the genus xanthomonads. This characteristic is due to production of yellow xanthomonadin pigment and is among cultural criterial used to distinguish *Xanthomonas Spp* [23]. Differences of isolates

surface brightness can be as a result of Xanthomonas isolates race difference [24].

Biochemical tests

Isolates obtained in this study stained redish, the colour of safranin after clearing crystal violate-iodine complex with 85 % ethanol and appeared as thin rod shapes thus, isolates were gram negative rods. Gram negative cell has its outer membrane and small peptidoglycan layer with no teichoic acid unlike gram positive bacteria [25]. This thin layer is completely washed off by alcohol, remains colourless and is counter stained with safranin [26] giving it red or pinkish colour.

Biochemical test results showed that all the isolates were positive for hydrogen sulphide productions test, Catalase test and NH_3 production test. Mixed results were observed in Indole test and KOH test. Voges-proskaure (V-P) and methyl red test were all negative (Table 2). Similar reaction has been reported by Kolomiets *et al.,* [27]. The results on V-p test differed with observation of Ali, [22] who reported positive V-P test. Positive results of ammonia production and hydrolysis of starch of bacteria leaf spot isolates was observed in this study. Similar observations have been made and reported for xanthomonads in other studies [28]. Negative Methyl red test can be attributed to the failure of bacteria leaf spot isolates to metabolize glucose and produce acetoin to be oxidized to diacetyl thus, no red colour was formed with naphtol at alkaline pH [29].

Catalase test on the bacteria leaf spot bacteria were positive (Table 2) and immediate production of bubbles was observed when bacteria isolates were brought in contact with hydrogen peroxide (H_2O_2). The findings on catalase reaction are similar with the observation by Kolomiets *et al.,* [27]. Formation of red ring was observed when kovac reagent was added to bacteria isolates grown in broth in test tube. Production of bubbles during catalase test is brought about by activities of catalase enzyme that expedites the breakdown of hydrogen peroxide into water and oxygen. Enzyme catalase is useful for the bacteria activity. It is produced by microorganisms to help neutralize the toxic effects of hydrogen peroxide [30].

Indole reaction was positive for isolates f1, f2, f3, f5 and f8 (Table 2). Positive indole reaction demonstrates bacteria ability to produce the endoenzyme tryptophanase. Tryptophanase enzyme hydrolyses the amino acid tryptophan into indole, pyruvic acid and ammonia [31]. Indole combines, in the presence of a tryptophan rich medium, with pDimethylaminobenzaldehyde at an acid pH in alcohol resulting in reddish ring [29].

V. CONCLUSION

Cultural, Morphological and Biochemical test indicate that the bacteria leaf spot isolates were majorly *Xanthomonas campestris*. Nonetheless, we recommend use of molecular tool for more accurate characterization of bacteria leaf spot pathogen for better management.

ACKNOWLEDGEMENT

We acknowledge the contribution of Mr. Sioma Mulambula of Plant Science department, Chuka University for his assistance during the manuscript write up. Lastly, we acknowledge Chuka University particularly the department of Biological Science for facilitation of this study.

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