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Morphological, biochemical and molecular characterization of *Xanthomonas campestris* pv. *vesicatoria* of African nightshades (*Solanum scabrum* Mill.)

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Abstract

African nightshade *(Solanum scabrum* Mill.) is one important vegetable in terms of food security and nutritional balance. The vegetable is usually medicinal and also a source of income to many small scale farmers in Kenya. Productivity of this vegetable is affected by several factors including low soil fertility and bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* which reduces yield by up to 40-70%. Little information is available regarding diseases caused by *Xanthomonas campestris* pv. *vesicatoria* in African nightshades. The aim of the study was to Characterize *Xanthomonas campestris* pv. *vesicatoria* from infected African nightshades. Six isolates from diseased leaves were morphologically, biochemically and molecular genetically characterized. All the isolates produced yellow colonies on modified Tween B Media (mTBM) and Yeast Dextrose Calcium carbonate (YDC) media, were rod shaped, obligately aerobic, gram negative, catalase positive, Oxidase negative and produced hydrogen sulphide gas. On the phylogenetic tree, the isolates were 100% related to *Xanthomonas campestris* from NCB sequence database hence identified as *Xanthomonas campestris* one of the species produced when *Xanthomonas campestris* pv. *vesicatoria* strains were reclassified.

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Introduction

African nightshades are amongst the most popular and widely spread leafy vegetables in warmer and humid zones of Africa (Edmonds & Chweya 1997; Musyimi *et al.,* 2012). They are traditional leafy vegetables that continue to be cultivated by many communities in Kenya. African nightshades are useful in that they ensure food security and better nutrition as they are rich in calcium, iron, and vitamins A and C (Ondieki and Aguyoh 2011; Musyimi *et al.*, 2012). Nightshades are in the genus *Solanum* and family Solanaceae (Edmonds and Chweya, 1997). There are five common species grown in Kenya (Onyango *et al* ., 2016), which includes *Solanum nigrum* L., *Solanum villosum* Miller., *Solanum americanum* Miller., *Solanum scabrum* Miller and *Solanum physalifolium*. Although much is known about the importance of nightshades, little information is available regarding their diseases and causative agents of diseases.

Solanum scabrum Mill. is extensively cultivated for consumption and export in West, East and Central Africa. It is popularly consumed due to its high calcium (Ca) content, iron (Fe), methionine and vitamin A (Assaha *et al*., 2013; Musyimi *et a*l., 2012). Despite the numerous advantages attributed to *Solanum scabrum*, its productivity continues to decline. Optimal yield of *S. scabrum* has been reported to be as high as 20-40 tones/ha but farmers in Kenya have been recording low yield ranging between 1-3 tones/ha (Ashilenje *et al*., 2012; Onyango *et al*., 1999). Diseases, soil fertility, poor cultural practices and low quality seeds are some of the main causes of low productivity (Wanjekeche *et al.,* 2003). Diseases such as late blight caused by *Phytophthora infestans* and bacterial leaf spot caused by *X. campestris* pv. *veiscatoria* lower the yield of African nightshades (Edmonds and Chweya 1997).

Bacterial leaf spot of S*olanum* is caused by *Xanthomonas campestris* pv. *vesicatoria* bacterium, which is Gram negative, aerobic and rod-shaped (AL-Saleh, 2011). Infection by bacterial leaf spot results in 40-70% nightshades loss (Opara and Obani, 2010; Mbega *et al.,* 2012).

The disease has a wide range of hosts especially those from Solanaceae family including tomatoes and pepper. In tomatoes, it reduces seed germination, causes defoliation and fruit loss (Kebede *et al*., 2013). AL-Saleh (2010) reported that once present in the crop, it is difficult to control the disease and presents major fruit loss in tomatoes when environmental conditions remain favorable. The bacterium has been isolated from tomatoes in Saudi Arabia, Brazil and Tanzania (Shenge *et al.,* 2014, Kebede *et al*., 2013, and AL-Saleh, 2010). In Kenya particularly Bondo region of Siaya County, there is limited information on identification and characterization of *X. cpvvesicatoria* that causes leaf spot of *S. scabrum*. Farmers in Bondo have been experiencing low yield of Nightshades due to bacterial leaf spot. This study therefore, aimed at isolating and characterizing the causative agent of bacterial leaf spot using morphological biochemical and molecular characteristics.

Materials and methods

Isolation of Xanthomonas campestris pv. *vesicatoria from diseased leaves of S. scabrum*

Infected leaves of *S. scabrum* showing water-soaked and necrotic areas were collected from farmer's fields around and taken to Jaramogi Oginga Odinga University of Science and Technology for isolation and characterization. Opara and Obani (2010) method was used. The infected leaves were washed with sterile water, surface-sterilized in 70% ethanol and rinsed in several exchanges of sterile water. Small pieces of the infected portions were cut from advancing margin of the lesion on leaves using sterile scalpel then teased apart with sterile dissecting needle in 1ml sterile water and left to stand for 30 minutes. The suspension was then streaked onto the surface of modified Tween B media.

Media preparation and bacterial inoculation

Opara and Odipo (2009) procedure with slight modification was used. Tween B media was modified by using boric acid and cephalexin as inhibitors of contaminants and leaving out cycloheximide, 5-fluorourcil and tobromysin.

The media for isolation of *X. campestris* pv. *vesicatoria* was modified Tween B media (mTBM) which was prepared from Peptone 10.0g, Potassium bromide 10.0g, Boric acid 0.10g, Calcium chloride anhydrous 0.25g and Agar 15.00g. 35.35gm of the mixture was weighed and then dissolved in 990ml of sterile water containing 10ml Tween 80 in a conical flask.

The mixture was melted on a hot plate for complete dissolution then autoclaved at 121°C for 15 minutes at a pressure of 15psi. The media was then cooled to about 45-50°C before adding cephalexin 65mg. About 20ml of the media was dispensed in Petri-plates in a lamina flow hood. The media was allowed to set before incubating at 30°C to dry the surface. The surface of the media was then inoculated by a loop full of bacterial suspension using a sterile wire loop and incubated for 48 hours at 30°C.

Morphological, Biochemical and Molecular identification of the bacteria

Morphological characterization

Characteristic colonies based on size and colour were identified, isolated and purified on yeast dextrose calcium carbonate (YDC) to obtain pure culture. Thin smear was prepared from the colonies, heat fixed by passing the slide over the flame, then methylene blue stained and observed microscopically under oil of emulsion objective lens to reveal the shape and arrangement of the bacterial cells. The cells could be either cocci or bacilli.

Biochemical characterization

Gram's staining

A thin fixed smear was prepared using characteristic colonies and stained using gram's reagents. The reagents were applied in the following order. Crystal violet for 2 minutes, gram's iodine for 1 minute, absolute ethanol for 30 seconds and lastly safranin for 1 minute. Washing with tap water was after every step as described by Rafi *et al.* (2013). The smear was then allowed to dry in air and observed microscopically using oil of emulsion objective lens. Purple coloured cells would indicate Gram positive while red or pink coloured cells would be Gram negative.

Oxidase test

On a piece of Whatman No. 2 filter paper placed in a Petri dish, several drops of oxidase test reagent (1% solution of dimethyl *p*- phenylenediamine hydrochloride) were added then a loop full of the bacteria from the colonies smeared on the paper and the reaction observed. The isolates were rated as oxidase-positive if a purple color developed within 10-60 seconds, and negative if no color developed within 60 seconds (Rafi *et al.*, 2013).

KOH solubility test

Two drops of 3% KOH were put on a glass slide then a colony of the test bacterium picked from the surface of a solid medium with an inoculating loop and mixed vigorously in the KOH for 5-10 seconds. Thread-like slime formation when picked by wire loop indicated presence of G- bacterium but no slime or thread formation indicated G+ bacterium (AL-Saleh, 2011).

Catalase test

A few drops of 3% hydrogen peroxide were placed on a clean slide and two colonies mixed in it to observe the reaction. Presence of bubbles indicated positive results while no bubbles indicated negative results (Gracelin *et al.*, 2012).

Hydrogen sulphide production.

The method developed by Gupta *et al.* (2007) with slight modification of not incorporating bile salt was used. The medium was formulated by combining 20.0g peptone, 1.5g dipotassium hydrogen phosphate, 0.75g ferric ammonium citrate, 1.0g sodium thiosulfate 3.5g, agar and 5.0 yeast extract. 30g of the mixture was suspended in distilled water, mixed thoroughly by stirring and then heated for complete dissolution. The media was then dispensed into universal bottles and autoclaved at 121°C for 15 minutes. After cooling, the media was inoculated with the test organisms by stab method using inoculating wire. The bottles were then incubated with loose caps at 28°C for 10-14 days and observed for hydrogen sulphide gas production. Colour change to black indicated H_2S production by the bacteria while no colour change indicated inability of the bacteria to produce H_2S from media.

Molecular characterization of Xanthomonas isolates

DNA extraction and amplification

Purified colonies on YDC media from six isolates were used for DNA extraction and molecular characterization using the 16S rRNA, genes. Genomic DNA was extracted using the ZR Bacterial DNA Mini Prep™ kit according to the manufacturer's specifications (Zymo Research Corp, South Africa). The concentration and purity of DNA was estimated using a Nanodrop™ Lite Spectrophotometer (Thermo Scientific Inc, USA) at 260-280 nm and by horizontal gel electrophoresis (Thistle Scientific Ltd, USA) on a 0.8% (w/v) agarose gel at 100V for 30min and visualized under UV after staining with Gel Red™ (Thermo Scientific, USA) according to Lee *et al* (2012). For 16S rRNA genes, *Escherichia coli* was used as a positive control. The PCR primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACG ACT T) targeting the 16S rRNA gene was used according to White *et al* (1990). BioneerAccu Power® PCR Premix (BioneerInc, USA) was used to perform PCR. To each 20µl Bioneer tube 1µl of 50ng template DNA, 0.5µl of 10 picomole of each primer and 18µl of nuclease free water were added and mixed. Amplification was performed in a programmable Mastercycler thermocycler (C1000-Bio Rad, USA). The PCR conditions included denaturation, annealing, initial and final extension at temperatures of 94°C for 30 sec, 58°C for 1 min, 72°C for 2min respectively before cooling off at 15°C. PCR products were separated by horizontal gel electrophoresis on 1.5% (w/v) agarose gel at 100V for 45mins and visualized under UV after staining with 2µl Gel Red™ (Thermo Scientific).

Purification of PCR products

PCR amplicons were purified using the Thermo Scientific® Gene JET Purification Kit (EU Lithuania). A ratio of 1:1 volume of binding buffer was added to the completed PCR mixture and vortexed to mix properly. 10µl of 3M sodium acetate (pH 5.2) was added to alter the colour to yellow. 800µl of the solution was transferred to the Gene JET purification column and centrifuged at 10,000rpm for 30 sec and the flow-through discarded. 700µl of the buffer (diluted with ethanol) was added and centrifuged at 10,000rpm on a rotor for 30 sec and the flow-through discarded. Additional centrifugation was done to completely remove any residual buffer. The purification column was transferred to clean 1.5ml micro-centrifuge tubes and 50µl of elution buffer added followed by centrifugation at 10,000rpm for 1 min to obtain pure DNA amplicons.

Sequencing and molecular data analysis

Aliquots of purified PCR products were pipette into 10µl epperndoff tubes and submitted to the Segolip Sequencing Unit, BecA-ILRI Hub, for capillary sequencing on a 3730*xl* DNA Analyzer (Thermo Fisher Scientific Inc. USA). Forward and reverse sequences were assembled and trimmed on CLC Main Workbench (CLC Bio, Version 6.8.3). Assembled sequences were transferred to MEGA Version 6.0 software and aligned using CLUSTAL W according to Tamura *et al*., (2013). Sequences were submitted to the NCBI BLAST portal (www.ncbi.blast.lnm.nih.gov) for a sequence similarity search, and sequences with greater than 97% similarity were retrieved for phylogenetic analysis. Evolutionary histories were inferred using the Neighbor-Joining method and distances computed using the Maximum Composite Likelihood (Tamura *et al* 2011). Bootstrap tests (1000 replicates) were used to cluster associated taxa and replicate trees with above 50% likelihoods indicated on the branches.

Results and discussion

Morphological characteristics

Colonies of the six isolates on Tween B media (mTBM) were yellow, slimy, glistening and round in shape while on yeast dextrose calcium carbonate (YDC) colonies were deep yellow, highly viscous, slimy and round in shape. Microscopic analysis of the bacterial isolates showed that they were rod shaped occurring in singles with polar flagella.

These growth pattern and microscopic observation provided preliminary information on the identification of the *Xc.* Pv *vesicatoria* as similar characteristics had been described by Lee *et al.* (2009) and Gracelin *et al.,* (2012).

Biochemical identification of the bacteria

The isolates were gram negative rods, catalase positive, Oxidase negative and produced hydrogen sulphide gas (Table 4.1).

Table 4.1. Biochemical characteristics of *X.* cpv. *vesicatoria* isolated from diseased *S. scabrum* leaves

Test	Result
KOH solubility	+
Catalas test	+
Hydrogen sulphide production	+
Oxidase	-
Gram stain	Gram negative

All the isolates retained secondary stain (safranin) and appeared red (gram negative) and rod shaped after treatment with Gram's reagents. These results were in agreement with those obtained by AL-Saleh (2011), Gracelin *et al* (2012) and Rafi *et al.* (2013) who performed gram staining on *Xanthomonas* isolates from tomato, *Centellaasiatica* and rice respectively and found that all were Gram negative rods. Gram staining is an important technique used for preliminary identification of unknown isolate. It divides bacterial isolates into two broad groups' gram positive and gram negative, based on the composition of the cell wall and also providing information on the shape and size of the isolate. Gram negative bacteria contain lipolysaccharide layer which dissolve in alcohol during decolourization making the cells to lose crystal violet used as primary stain therefore appearing red while gram positive appear purple as they retain crystal violet colour.

There was a positive catalase reaction on all the six isolates tested and this results concurred with those obtained by Opara and Odipo (2009) and Gracelin *et al.* (2012) who tested for catalase reaction on *Xanthomonas* isolates from tomatoes and *Centella asiatica* respectively. Catalase test differentiate aerobic and anaerobic bacteria. Aerobic bacteria produce hydrogen peroxide during metabolism which must be broken down immediately as it is toxic to the cell for it damages the cell's DNA. Aerobes have the ability to synthesize enzyme catalase which breaks down H_2O_2 into water and oxygen.

All the six isolates were oxidase negative which was in line with the result obtained by AL-Saleh (2011)and Rafi *et al* (2013) whose *Xanthomonas* isolates were oxidase negative. Oxidase test identifies bacteria that produce cytochrome c oxidase enzyme important in bacterial electron transport chain. The enzyme is able to hydrolyze tetramethyl-p-phenylenediamine reagent to indophenols which is purple in colour. When the enzyme is absent the reagent is not hydrolyzed and therefore it remains colourless.

KOH solubility test

The KOH solubility was positive which was in agreement with studies carried out by Muneer *et al,* (2007) and Rafi *et al.* (2013) to test for solubility of the bacterial cell wall of *Xanthomonas* isolates. KOH solubility test is a rapid method used to identify gram negative bacteria when gram stain procedure seem not to give exact difference between gram negative and gram positive. When bacterial colonies are emulsified in 3% KOH, the gram negative cell wall dissolves in the solution to form a thread like slime which is identified by lifting the loop used to emulsify and the slime is seen following the loop a few millimeters high.

Hydrogen sulphide gas production

The isolates produced hydrogen gas an indication that they were *Xanthomonas*. The results were in line with those obtained by Opara and Odipo (2009) and Gracelin *et al,* (2012) on *Xanthomonas* isolates from different plants. Hydrogensulphide gas production from organic compound is important in differentiating organisms of genus *Xanthomonas* from other gram negative plant pathogens. Ferric Ammonium Citrate and Sodium thiosulfate are used to detect hydrogen sulfide production. Sodium thiosulphate is utilized by bacteria, with the production of hydrogen sulfide which is colorless. Ferric ammonium citrate reacts with H_2S, producing a black ferrous sulphide that turns the media black.

Molecular Characterization

Phylogenetic analysis of 16S rRNA gene sequences of Xanthomonas isolates

The 16S rRNA gene sequences of six *Xanthomonas* campestris sequences isolates and 16 sequences of the genus *Xanthomonas* obtained from the NCBI database produced a phylogenetic tree with three distinct clades I, II and III (Fig. 1).

The six isolates from this study, classified as *Xanthomonas campestris* clustered into a monophyletic sub lade within Group I at 63% bootstrap support value. Two minor sub-clades occurred within Clade I with the second group consisting of NCBI sequences of *X. vesicatoria* strains CNPH345 and XV1111 (AYY288080, AF123088 respectively) together with *X. curcubitae* strain NBRC 13552 (AB680436) and *X. dyei* strain ICMP 12167 (NR_104949). The phylogenetic tree showed very close evolutionary relationship of the six isolates with *Xanthomonas dyei* strain (ICMP 12167) and *Xanthomnas vesicatoria* strain (CNPH 345). However, an evaluation of the sequence showed it had 100% identity with several species of *Xanthomonas vesicatoria*.

Indeed, the latter sequence (*Xanthomnas vesicatoria* strain CNPH 345) had 100% identity with all the six isolates from this study. According to Lue et al., (2010) when *Xanthomonas campestris* pv. *vesicatoria* strains were reclassified, they were separated into two species of *Xanthomas vesicatoria* and *Xanthomn asaxonopodi* spv. *vesicatoria*.

Thus, it is plausible to state that the *X. campestris* isolates from this study may validly belong to the recently created species *X. vesicatoria*. Studies by Lue *et al* (2010) and Obradovic *et al* (2004) showed that *Xanthomonas vesicatoria* and *Xanthomonas axonopodis* pv. *vesicatoria* have several strains that cause bacterial leaf spot disease in tomatoes and pepper. Therefore, the isolates were confirmed to be *Xanthomonas campestris* pv. *vesicatoria* the causative agent of bacterial leaf spot in night shades.

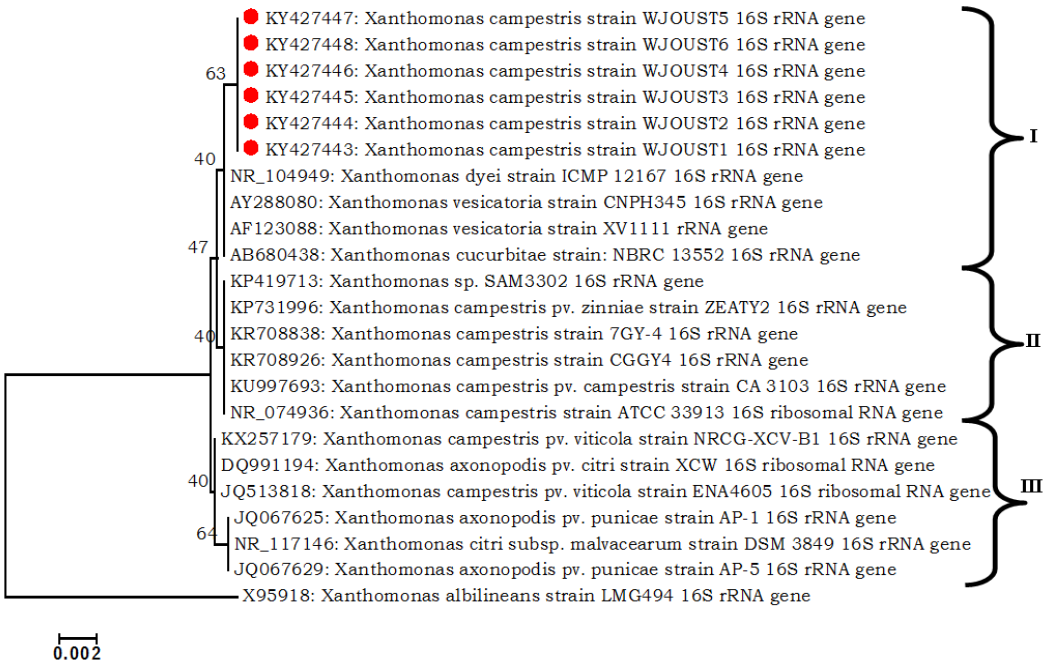

Fig. 4.1. Phylogenetic analysis of 16S rRNA gene sequences of *Xanthomonas* isolates.

Note: Isolates from this study are labeled with a bullet. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.02645044 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches [2]. Evolutionary analyses were conducted in MEGA5 [4].

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