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Molecular Identification of Bacteria Spot of Tomato Incited by *Xanthomonas campestris* pv. *vesicatoria*, from Fields in the Ashanti, Brong Ahafo and the Upper East Regions of Ghana

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Abstract* – Recently, tomato farmers in some areas in the Ashanti, Brong Ahafo and the Upper East Regions of Ghana reported the occurrence of a strange disease affecting their crop. Symptomatic plant samples were collected from some infected fields in the affected areas for proper identification of the causal organism. Preliminary test on isolates using potassium hydroxide (KOH) and Gram reaction revealed the causal organism to be gram negative bacterium belonging to the genus *Xanthomonas*.*Xanthomonas* genus and species specific primers were used in a polymerase chain reaction (PCR) assay to confirm the genus and identify the species as*Xanthomonas campestris* pv. *vesicatoria.

Keywords* – Bacteria Spot, PCR, *Xanthomonas campestris.

I. INTRODUCTION

Tomato (*Solanum esculentum* Mill) in Ghana, is a very important crop widely cultivatedfor the preparation of many dishes [1]. The industry offers employment and descent income not only to urban and rural dwellers directly involved in its production, but, to transporters, retailers' agro input dealers and a host of other actors [1][2][3].The crop is also a source of foreign exchange for the country as 340,000 metric tons was produced in 2011 generating an estimated income of $125,652.00 for the country [4].Despite the importance of the crop, challenges such as high pest and disease incidence bedevil its successful production, thereby making it difficult for farmers to realize the full benefit of their investments[5][6]. This phenomenon has also greatly affected the ability of the country to meet local demand thereby resulting in the constant importation of about 100,000 tons of fresh tomato each year from neighboring Burkina Faso [6].

In 2010, farmers in some important tomato producing areas in the Ashanti and Brong Ahafo reported high incidence of a tomato leaf and fruit spotting disease. The disease was also observed in some tomato fields in the Upper East region. Though there were no statistics on yield losses due to the disease, it raised enough concern prompting some farmers to report it to agricultural extension agents (AEAs') in some of the affected communities (Abdallah Salifu (AEA, Tanoso) Personal communication).The disease was observed to affect the whole tomato plant revealing spotting on the leaves and dark brown cankerous spots on immature and ripe tomato fruits. Examination of the leaves showed water soaking as well as yellow chlorotic halos. A preliminary investigation revealed that the causal organism of the disease was a bacterium. Due to the similarity of the reported disease symptom to that expressed by septoria leaf spot disease of tomato caused by *Septoria lycopersici*, most farmers often mistook it as such often resulting in unsuccessful control attempts with various fungicides.

The current study was therefore conducted toidentify the genus and species of the causal organism.

II. MATERIALS AND METHODS

2.1 Collection of symptomatic plant samples from infected fields

Diseased fruit and plant samples were collected from randomly selected farms in six tomato growing districts where the disease was previously reported, from August to September, 2012. The districts included, Offinso North (Lat. N 7°29'24'', Long. W 1°57'25") in Ashanti region, Techiman North (Lat. N 7°37'60", Long. W 1°53'60") and Techiman Municipality (Lat. N 7°16'43", Long. W 2°14'27") in Brong Ahafo, and Bongo (Lat. N 10°54'28", Long. W 0°48'29"), Binduri (Lat. N 10°58'19", Long. W 0°18'24") and Kassena Nankana West (Lat. N 10°50'32", Long. W 1°19'40") in the Upper East region.The diseased plant and fruit samples were placed in labeled sterile transparent polythene sample bags and temporarily preserved in an ice chamber box. The samples were transported to the plant pathology laboratory in the Department of Crop Science, University of Ghana, Legon, where they were kept in a refrigerator at 5 °C (approx.).

2.2 Isolation of the causal bacterium from diseased plant samples

Symptomatic diseased leaves and fruits samples were washed thoroughly under running tap water to remove soil and other debris. The plant materials were blotted dry with

tissue paper following which pieces of plant tissue were excised from advancing lesions. The excised tissues were surface sterilized by dipping in 1% sodium hypochlorite (Sumo thick bleach: 10% NaOCl) solution for 30 sec after which they were rinsed thoroughly in sterile distilled water (SDW). The tissue segments were then immersed in 70% ethanol for 10 sec and rinsed thoroughly in sterile distilled water. The tissue segments were then teased in a sterilized Petri dish containing a few drops of sterile distilled water (SDW) and allowed to stand for 15-20 min in a laminar flow chamber. Loopfulls of the resulting suspension were streaked on sterilized 9 cm Petri plates containing Oxoid nutrient agar (NA) prepared according to the manufacturer's specification using disposable inoculation loops and incubated for 3-5 days at 25 - 30°C. Dailyobservations were made for colony growth during the period. Pure cultures were obtained by sub culturing single yellow colonies onto plates of nutrient agar and yeast dextrose carbonate medium (YDC) (Yeast extract, 10 g; $CaCO_3$, 20 g; D-glucose, 20 g; Bacto Agar, 17 g per liter of distilled water, autoclaved at 12^o C, 15 psi for 15 minutes).

2.3 Identification of bacterium

Isolated bacterial colonies were initially identified using Gram reaction and 3% Potassium hydroxide (KOH) tests [7] [8]. Bacterial isolates were cultured on yeast dextrose carbonate (YDC) agar and their morphological characteristics observed [9]. Hypersensitive reaction test on tobacco (*Nicotina tabaccum*) was conducted to determine the pathogenicity potential of the bacterial isolates[10]

2.4 Pathogenicity test

Pathogenicity tests were conducted on young tomato seedlings to assess the pathogenicity potential of the isolates in order to satisfy Koch's postulates.

A local tomato cultivar susceptible to the disease, *Wosowoso*, was used in the confirmation of Koch's postulates involving 21 bacterial isolates.

Ten (10 g) of the*Wosowoso* tomato seed was sanitized to remove any potential seed borne pathogen by soaking in 2% sodium hypochlorite solution for 3 min after which it was rinsed thoroughly with distilled water and allowed to dry at room temperature (25-30^{o}C) for 48 hours [11]. The seeds were then nursed in a 60 cm x 60 cm seed box containing heat sterilized weed free sandy loam soil. The seeds were nursed in rows 5cm apart and covered lightly. Nitrogen: phosphorus: potassium fertilizer (NPK15:15:15) was broadcasted on the nursery to encourage rapid growth. The seed box nursery was watered lightly and placed in a screen house. Subsequent watering and other nursery practices were conducted when necessary until the seedlings were at the 3-leaf stage at 3 three weeks after sowing.

The tomato seedlings were then transplanted into 9 cm black polythene bags (one seedling per bag) containing heat sterilized sandy loam soil amended with compost manure (3:1). Six seedlings were used as test plants with six seedlings as control. Leaf inoculation was done by spraying, with a hand atomizer, the prepared bacterial inoculumof approximately $1x10^8$ cells ml^{-1}, on the seedlings a week after transplanting, till run off. Control plants were sprayed with sterile distilled water using a hand atomizer. The inoculated plants were then covered with moistened 10 x 30cm transparent polythene bags for 48 hours at 25-30^{o}C in order to obtain high humidity of about 85-100%. The polythene bags were removed and the seedlings transferred to the screen house and maintained at a temperature 25-30°C and RH of >80% for 14 days, during which time daily observations were made for symptom development.

Bacteria pathogens were re-isolated from symptomatic tissue sampled from infected tomato and pepper seedlings used in pathogenicity test to conclude Koch's postulates.

2.5 Polymerase chain reaction (pcr) identification of bacterial isolates

Polymerase chain reaction procedure (PCR) was used to accurately determine the genus and species of the bacterial isolates using optimized genus and species specific primers by Melanie L. Lewis Ivey of the Ohio State University.

DNA was extracted from the bacterial isolates using a modified CTAB genomic DNA isolation protocol described by [12]. A twenty four (24) hour single colonies grown on YDC agar medium were transferred to a falcon tube containing 5 ml prepared Luria Bertani (LB) broth. The cultures were placed on a shaker set at 200 revolutions per minute (rpm) for16 hours at 28ºC. The cultures were then centrifuged at 10,000 rpm for 5 min following which the supernatant was discarded and the resulting pellet suspended in 740 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A 20 µl lysozyme (conc. 100 mg/ml) was added and incubated for 30 min at 37°C after which 10% SDS and 18 µl of Proteinase K (20 mg/ml) were added to the mixture and incubated for 1 hour. 100 µl of 5 M NaCl and 100 µl of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65ºC and then kept on ice for 15 min. 0.5 ml chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10000 rpm for 10 min. 0.6 ml volume isopropanol was added and incubated at -20°C for 2 hours after which it was centrifuged at 10000 rpm for 15 min at 4°C. The pellets were washed with cold 500 µl of 70% cold ethanol, air dried at room temperature for approximately 3 hours and finally dissolved in 50 µl of TE buffer. 7 ul of DNA was run on a 1% agarose gel matrix to check DNA quality. Extracted DNA was stored at -20^{o}C for later use.

Primers, RST 65-2 (5'GTCGTTGGCACCATGCTCA 3') and RST 69 (5' TCGCCCAGCGTCATCAGGCCATC 3') were used inPCR to confirm the genus of *Xanthomonas* associated with the bacterial fruit spot disease, following an optimized protocol described by [13]

PCR reaction was conducted in labeled 0.2ml PCR tubes containing a final volume of 25 µl. The reaction mix composed of 1 µl genomic DNA template, 12.5 µl GoTaq Green Master Mix, 2X (GoTaq DNA Polymerase, Reaction Buffer pH 8.5, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2.), 1.25 µl RST 65-2, 1.25 µl RST 69 and 10 µl nuclease free water. The PCR conditions performed in a thermo cycler (BIO-

RAD, Icycler, USA), followed an initial denaturation phase of 95 ^{o} C for 5 min which was followed by 30 cycles involving a denaturation step of 95 ^{o} C for 30 sec, annealing at 63^{o}C for 30 sec, extension at 72^{o}C for 40 sec and a final extension at 72^{o}C for 5min.

Samples confirmed to belong to the genus *Xanthomonas* were used in further test to determine the specific species of *Xanthomonas*. This was conducted using a multiplex PCR reaction technique following an optimized protocol described by [14].

The following forward and backward primers (Table 1) were used;

Table 1: Forward and backward primers used in PCR amplification to identify species of *Xanthomonas campestris* pv. *vesicatoria*

Xanthomonas species	Primer
Xanthomonas vesicatoria	Bs-XvF: 5'- CCA TGT GCC GTT GAA ATA CTT G -3'
	Bs-XvR: 5'- ACA AGA GAT GTT GCT ATG ATT TGC -3'
Xanthomonas gardneri	Bs-XgF: 5'- TCA GTG CTT AGT TCC TCA TTG TC -3'
	Bs-XgR: 5'- TGA CCG ATA AAG ACT GCG AAA G -3'
Xanthomonas euvesicatoria	Bs-XeF (5'-CAT GAA GAA CTC GGC GTA TCG-3')
	Bs-XeR (5'-GTC GGA CAT AGT GGA CAC ATA C-3')
Xanthomonas perforans	Bs-XpF: 5'- GTC GTG TTG ATG GAG CGT TC -3'
	Bs-XpR: 5'- GTG CGA GTC AAT TAT CAG AAT GTG G -3'

A reaction mix composed of 12.5 µl Master Green mix, 10.5 deionized water and 1 µl of genomic DNA template was used in PCR. 10 µl of each primer at 100mM were mixed and 1 µl primer mix added to the mixture to obtain a final volume of 25 µl.

The PCR conditions performed in a thermocycler (BIO-RAD, Icycler, USA), followed an initial denaturation phase of 95 ^{o} C for 5 min which was followed by 30 cycles involving a denaturation step of 94^{o} C for 30 sec, annealing at 63^{o}C for 60 sec, extension at 72^{o}C for 60 sec and a final extension at 72^{o}C for 7 min.

PCR product analysis was performed in 1.5% and 3% agarose gel matrix (stained with ethidium bromide) for genus and species identification respectively and electrophoresed at 100 volts, in 1 x TBE buffer for 2 hours alongside a 1 kb plus ladder. Amplicons were then viewed with the aid of a UV transluminator and photographed.

III. RESULTS

3.1 Preliminary identification of bacterium.

Preliminary identification using 3% KOH treatment on bacterial colonies produced a viscous, mucoid, slimy substance that stretched to about 3 inches high when stirred and picked with an inoculation loop which indicates a bacterium Gram negative. Gram stain reaction of the bacterial isolates further revealed purplish/red stain of short rods shaped bacteriathereby confirming the bacterial isolate to be Gram negative. Cultured isolates on yeast dextrose carbonate (YDC) agar aftersix (6)days of incubation at 25-30^{o}C, produced yellow, shiny, mucoid colonies about 3mm in diameter. They were convex shaped, circular with entire margins. These features and reactions are all characteristic of *Xanthomonas campestris* pv. *vesicatoria.*

Tobacco plants used for hypersensitivity test produced a positive hypersensitive reaction on tobacco four (4) days after inoculation eliciting yellow chlorotic symptoms at the point of inoculation which later turned brown seven(7) days after inoculation.

3.2 Pathogenicity test

The 5 weeks old tomato seedlings inoculated with the bacterial isolates, expressed water soaked symptoms 8-14 weeks after inoculation which later turned brown with yellow chlorotic halos. The SDW inoculated plants however, expressed no visible symptoms.

Similar symptoms were also observed on the pepper seedlings which also elicited water soaked, brown lesions, 4-14 days after inoculation, whiles no visible symptoms were observed on control plants treated with sterilized distilled water over the same period. These symptoms are consistent with that of bacterial spot disease of tomato [15]. Koch's postulates was confirmed when the pathogen was re-isolated and found to be Gram negative following a Gram reaction and 3% KOH test.

3.3 PCR amplification using Xanthomonas genus specific primer

PCR results of fifteen(15) bacterial genomic DNA isolatesusing *Xanthomonas* genusspecific primers RST 65-2 (5'GTCGTTGGCACCATGCTCA 3') and RST 69 (5' TCGCCCAGCGTCATCAGGCCATC 3') banded at the expected amplicon size of approximately 430 bp as specified for *Xanthomonas* genus [13]. This showed that all the samples tested belonged to the genus *Xanthomonas* (Fig 1).

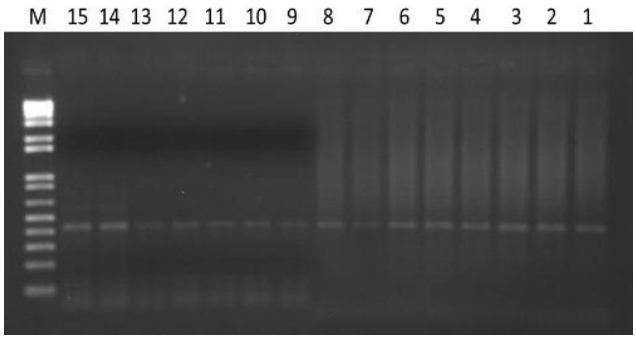

Fig.1. PCR Identification of Xanthomonas genus by species specific primers (RST 65-2 and RST 69). M= molecular marker (1Kb plus). isolates from symptomatic plant tissue.

3.4 Multiplex PCR amplification using Xanthomonas species specific primers

Further screening of the fifteen (15) bacterial DNA belonging to the genus *Xanthomonas* in a multiplex PCR reaction using four species specific primer pairs: Bs-XeF, Bs-XeR, Bs-XvF, BsXvR, Bs-XpF, Bs-XpR, Bs-XgF, Bs-XgR produced an approximate amplicon size (138 bp).This corresponds with the expected band size of *Xanthomonas vesicatoria* [14]thereby confirming the bacterium to belong to the species *Xanthomonas vesicatoria* (Fig. 2).

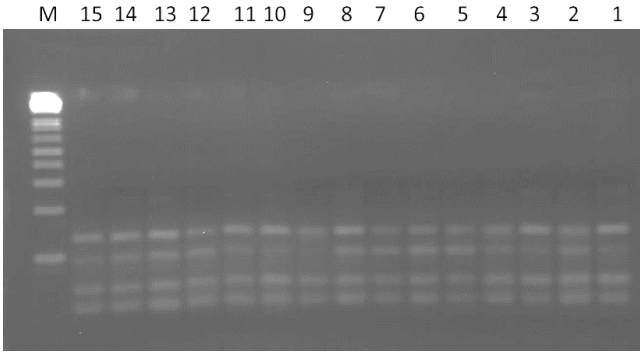

Fig.2. Multiplex PCR identification of Xanthomonas species by species specific primes: Bs-XeF, Bs-XeR, Bs-XvF Bs-XpF, Bs-XpR, Bs-XgF, Bs-XgR. M= molecular marker (1Kb plus), isolate from symptomatic plant tissue.

IV. Discussion

Preliminary identification of the bacterial isolates from symptomatic tomato tissue showed that the isolates were rod shaped, Gram negative phyto pathogenic bacteria. Genome analysis of the bacterial isolates using PCR, confirmed the causal organism of the leaf spotting disease to be caused by *Xanthomonas.* The bacterium was confirmed to belong to the genus *Xanthomonas* and the species *vesicatoria*. This confirmation is consistent with the identification of the same pathogen causing the similar disease on tomato and pepper in Zimbabwe [16], Tanzania [15] and worldwide [17]. The ability of the bacterium to elicit necrotic symptoms on tobacco leaves revealed the bacteria to be plant pathogenic with pathogenic potential. This was further confirmed when a pathogenicity test on young tomato seedlings to confirm Koch's postulates revealed symptoms that are consistent with bacteria spot disease of tomato [15][18]. These symptoms were also consistent with the disease symptoms observed on some of the infected tomato crops on fields in the Ashanti, Brong Ahafo and Upper East regions of Ghana. The disease was previously described as an unimportant tomato disease in [19], its occurrence in the three regions coupled with the recent concerns of farmers however, indicates the disease is widespread and measures should be taken to control it.

V. Conclusion

Ambiguity surrounding the causal organism of a reported bacterial disease of tomato was unraveled with the identification of *Xanthomonascampestris* as the causal agent of bacterial spot of tomato. The specific species responsible for the disease in all the study areas was also found to be *vesicatoria*.

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