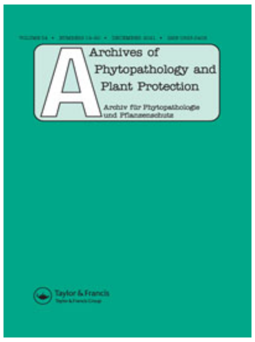

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RESEARCH ARTICLE

Molecular characterization, pathogenicity and copper sensitivity of *Xanthomonas citri* pv *mangiferaeindicae*, the causal agent of mango bacterial black spot disease in Ghana

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ABSTRACT

Recent outbreak of the mango bacterial black spot disease has resulted in massive damages to the Ghanaian mango industry. Research was carried out to confirm the etiology of the disease. Diseased mango fruits and leaves were collected from Greater Accra (Asutuare and Dodowa), Eastern (Akuse, Somanya) and Volta (Juapong) regions, and the suspected causal bacterium was isolated. The pathogen was identified as *Xanthomonas citri* pv *mangiferaeindicae* based on cultural and morphological characteristics and sequence analysis of the *cpn60* gene. The pathogenicity of the bacterial pathogen on mango and cashew was determined and its sensitivity to some selected copper based fungicides available in Ghana was evaluated by the agar diffusion bio-assay. All strains of the pathogen were able to cause disease symptoms on artificially inoculated mango and cashew leaves. The pathogen was highly sensitive to Copper oxychloride, Copper hydroxide and Coprous oxide at very low concentrations.

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Introduction

Mango (*Mangifera indica*), is one of the most important fruit crops cultivated in Ghana and serves as an important source of income to people engaged in its production and marketing in the country. One of the major diseases limiting mango production worldwide is mango

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bacterial black spot disease. The disease can cause heavy dropping of immature fruits and could leave rotten dark spots on fruits that go into maturity, therefore making such fruits unmarketable. In severe infestation, all fruits could be lost. Estimates show that yield loss due to the disease can range from 50% to 100% (Kishun 1982; Prakash and Misra 1992). In South Africa, it was estimated that the disease caused loss of revenue to a tune of 5 million Rands (approximately U.S. $1 million) in 1996 (Gagnevin and Pruvost 2001). Apart from fruit destruction, the disease causes canker symptoms on branches which weaken them and therefore, easily break with the slightest wind breeze. The disease also has the potential of restricting movement of mango from producer countries known to have the disease, since the causal agent is a quarantine pest in most countries.

Mango bacterial black spot is caused by the bacterium *Xanthomonas citri* pv. *mangiferaeindicae* (syn. *Xanthomonas campetris* pv *mangiferaeindicae*). Phenotypically, the strains of the bacterium causing the mango bacterial black spot were non-pigmented (Dye et al. 1980). Yellow-pigmented strains of *Xanthomonas*, causing black angular lesions on mango were however reported from Brazil (Robb et al. 1978), South Africa (Pruvost and Manicom 1993) and Reunion Island (Pruvost and Luisetti 1991). Reports however, suggest that the reported yellow-pigmented strains from Brazil and Reunion were less pathogenic than the non-pigmented isolates (Pruvost and Luisetti 1991). Morphologically, cells of the bacterium are gram negative, single, short and straight rods (0.4–0.7 × 0.7–1.6 µm) and motile with a single flagellum (Kwee and Chang 1985). The isolates of the bacterium grow well at a temperature range of 10–35 °C with growth mostly pronounced at 30 °C (Lakpale et al. 2006).

Mango bacterial black spot disease was first reported in Ghana in 2011 (Pruvost et al. 2011) on fruit bearing trees present in an orchard in the Northern Region of the country. Apparently, the orchard was established with planting materials imported from neighbouring Burkina Faso. The disease appeared to be confined in that single orchard as it was not observed anywhere else in the country. However, significant damage to mango orchards due to the disease began to manifest in the Southern portions of the country around 2016. Since then massive damages in the middle belt of the country has also been reported. Currently, studies showed that the disease is now present in all the major mango growing areas of the country (Honger; unpublished). The damages being recorded in areas especially, the Coastal savannah zone of the Southern parts of the region, are rendering the production of mangoes in these areas unprofitable. This area is one of the few in the world where two mango production seasons per calendar year exist, and many people in this area depend on the production and marketing of the crop for their livelihood. Currently, due to the disease outbreak, some farmers have stopped production in the major season, while others have put up their farms for sale to real estate developers (Osabutey 2019). Therefore, if not controlled, the disease has the potential of collapsing the mango industry in this area and of the entire country.

Mango bacterial black spot disease can be described as relatively new disease in the country and therefore the information about the causative organism is scanty. Apart from the original orchard where the disease was first described, no work has been done in any part of the country, to confirm the etiology of the disease. Therefore it is not known whether the same pathogen responsible for the disease in the original orchard where the disease was first described, is also responsible for the current outbreak in the major mango producing areas of the country as well. However, knowledge of the nature of a causative organism is essential for the formulation of control measures against the disease. For example, the ability of the causative agent to survive on other closely related species of the original host and the fungicide sensitivity of the pathogen would be useful information that can be used to formulate a sound control strategy against the disease. However, these information are lacking about the suspected causal agent of the current outbreak of mango bacterial black spot disease in the coastal savannah zone of Ghana.

Due to the scanty nature of information about the causal agent of the disease in the coastal savannah zone of the country, this work was carried out to isolate and characterize the causal agent of mango bacterial black spot disease, determine the pathogenicity of the pathogen on cashew and to evaluate the copper sensitivity of the pathogen in the coastal savannah zone of Ghana.

Materials and methods

Collection of diseased plant parts

Collection of diseased mango fruits and leaves were carried out in infected mango farms in the coastal savannah zone of Ghana, comprising parts of the Greater Accra (Asutuare and Dodowa), Eastern (Akuse, Somanya) and Volta (Juapong) regions (Table 1). Diseased fruits showing the typical black spot lesions characterized by star shaped cracks and sap oozing (Figure 1) were harvested and placed in polythene bags and transported in an ice chest to the Plant Pathology Laboratory, Department of Crop Science, University of Ghana, Legon for isolation of the causal organism of the disease. Also leaves showing the angular leaf spots

Table 1. Administrative districts and regions in the coastal savannah agro-ecological zone of Ghana where diseased mango fruits and leaves were collected for the study.

Location	District	Region
Asutuare	Shai-Osudoku	Greater Accra
Dodowa	Shai-Osudoku	Greater Accra
Akuse	Lower Manya Krobo	Eastern
Somaya	Yilo Krobo	Eastern
Kpong	Lower Manya Krobo	Eastern
Juapong	North Tongu	Volta

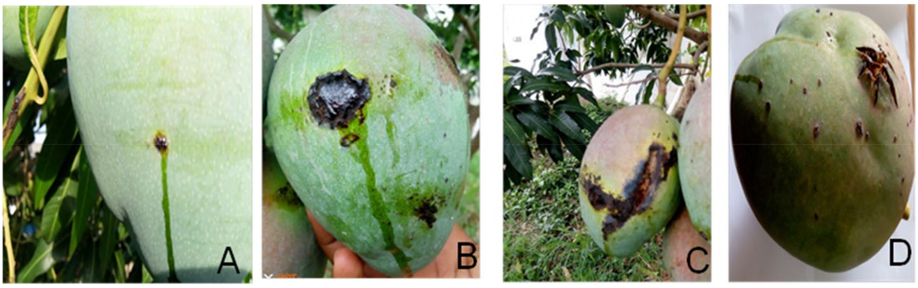

Figure 1. Typical black spot disease symptoms on mango fruits at different stages of the disease. (A) Tiny spot showing star crack, (B) Large solitary spot almost round in shape. (C) Large cracked longitudinal spot and (D) Large star crack spot.

Figure 2. Leaves of mango showing the angular leaf spot symptoms of mango bacterial black spot disease. Symptoms on upper leaf portion (left) and lower leaf portion (right).

(Figure 2), characteristics of the disease were also collected and sent to the laboratory for the isolation of the disease causing agents. The collected diseased samples were stored in a refrigerator at 4 °C in the laboratory until required.

Isolation of the causal bacterium from diseased mango leaves and fruit samples

The isolation of the suspected causal agent of the disease was done on nutrient agar (Oxoid, England). Nutrient agar (NA) was prepared following the manufacturers instruction. Thirty nine grams of the NA powder was dissolved in 1 L of water and autoclaved at 121 °C for 15 min. The molten medium was allowed to cool and poured into oven sterilized petri dishes to solidify. Diseased mango fruits and leaves exhibiting BBS symptoms were thoroughly washed under running tap water and blotted dry with sterile tissue paper. Pieces of the leaves and fruit tissues at the advancing edge of the disease lesions including some portions of the lesions were excised with sharp sterile scalpel and surface sterilized with 5% sodium hypochlorite and the chemical flushed off three times with sterile distilled water. The excised tissues were macerated in sterile mortar with a pestle and the preparation was allowed to stand for 10 min to allow the bacterium to ooze out. A loop full of bacterium suspensions were streaked on the NA in the Petri dish and incubated at 25 °C and 70% relative humidity on benches in the laboratory. Three days later single colonies of creamy white bacterium that grew were sub-cultured on separate NA plates to obtain pure cultures. A total of 30 creamy white bacterial isolates were obtained and maintained in pure culture.

Nature of growth on yeast dextrose carbonate medium (YDC)

Yeast dextrose carbonate agar was prepared by dissolving 10 g of yeast extract, 20 g of $CaCO_3$, 20 g of D-glucose and 17 g of water agar in 1 l of distilled water. The mixture was pre-heated and autoclaved at 121 °C for 15 min and allowed to cool. The mixture was mixed thoroughly by shaking and then poured in 6 mm Petri plates. The creamy white bacteria single colonies were streaked using sterile disposable inoculation loops in a lamina flow chamber on the YDC medium. The plates were then covered, sealed in cellophane plastic bags and incubated at 23 °C and 50–60% Relative Humidity (RH). Observations of growth characteristics of the bacterium colonies were recorded after 2 days of incubation. The nature of the growth on of the bacterium on the YDC was recorded to aid its identification.

Gram staining

Gram staining reaction done by using a method developed by Bradbury (1970) using freshly prepared reagents. A drop of sterile distilled water was first placed on a clean slide and single colony of the bacterium was carefully picked onto the slide. Smear was made by robbing the sterile inoculating loop on the surface of the slide and the bacterium was fixed on the slide by heat by flaming the slide through a light flame. The bacterium smear was covered with crystal violet solution for 60 s, the specimen was washed under tap water for 10 s and excess water drained off. This was carefully blotted dry with tissue paper and flooded with iodine solution for 1 min, washed in tap water for 15 s and blotted dry. Ethanol (absolute) was used to decolourised the smear for 30 s and quickly rinsed with tap water for 2 s. Counterstain was done by spreading Safranin solution on the smear for 10 s and it was gently washed with water and blotted dry. Observation of the specimen was done using immersion oil and viewed compound light microscope. Morphological characteristics such as the shape and colour of the bacterium was observed and recorded for further characterization. A culture of *Escherichia coli* obtained from the Noguchi Memorial Institute for Medical Research in Ghana served as the reference bacterium and was treated the same way as the bacterium isolates.

Catalase test

Yeast nutrient agar was prepared by adding 23 g of nutrient agar and 5 g of yeast extract in a litre of distilled water. The mixture was dispensed into test tubes and autoclaved for 15 min at a temperature 121 °C and the tubes were placed gently to set as slants. Slants were inoculated with two days old bacterium colonies of isolates whiles the control treatment was inoculated with only water. The preparations were incubated for 48 h at 50–60% RH and temperature of 23 °C. Three percent (3%) of fresh hydrogen peroxide was placed gently on the walls of the test tube and left for few seconds and same was repeated for the control treatment. The presence of gas bubbles in the test tubes was observed and recorded to aid the identification of the casual organism.

Pathogenicity test

Detached mango leaves of the Keitt variety, harvested from a mango orchard free of the disease were first surface sterilized with 5% sodium hypochlorite and the chemical was flushed off by rinsing three times with sterile distilled water. Micro wounds were created on the surface of the mango leaves using sterile inoculating pins. The inoculum of each of the isolated bacterium strains was prepared by scraping the bacterial growth on the surface of nutrient agar into a test tube containing sterile distilled water. The mixture was agitated till the water became cloudy. The inoculum was poured into a hand-held sprayer and applied on the surface of the leaves at the points where the wounds were created. The control was prepared using leaves inoculated with sterile distilled water only. Inoculated leaves were arranged in transparent polyethene bags and incubated in the laboratory at 23 °C and 65% RH. The set-up was observed daily until symptoms developed. Isolated strains that were able to elicit the symptoms on the inoculated leaves around the points of inoculation were considered pathogenic to the crop. Pathogens were re-isolated from the artificially inoculated leaves and their cultural and morphological characteristics compared to the original isolate used in the inoculation to confirm pathogenicity. An isolate was identified tentatively as an isolate of *Xanthomonas citri* pv *mangiferaeindicae* if it was able to cause the disease. Five isolates, three from the Eastern Region and two from the Volta Region, were selected and used for the phylogenetic study, cross-infection potential and fungicide sensitivity tests.

Deoxyribonucleic acid (DNA) extraction

Total DNA was extracted from the selected five bacterial strains obtained from the mango leaves and fruits. The strains were first sub-cultured onto nutrient agar to obtained single colonies. After two days, single colonies from the bacterial cultures were transferred to LB broth, using a sterile inoculation loop, and incubated overnight at 28 °C on a rotary shaker set at 250 rpm. The broth cultures were centrifuged at 8000 g for 5 min (MicroCL 17 Centrifuge, Thermofisher), to collect the bacterial cell pellet. Total genomic DNA was extracted from the cell pellet following the modified protocol developed by Llop et al. (1999). DNA pellets were re-suspended in 500 mL in extraction buffer consisting of 0.25 mM NaCl, 0.2 mM Tris HCl pH 7.5, 0.5% PVP, 0.5% SDS and 0.025 mM EDTA. This was further incubated at 23 °C and 200 revolutions per minute in orbital shaker for 1 h. Suspension samples were then spinned at 1000 g for 5 min; 450 mL of the supernatant was gently pipetted into different tube which isopropanol of equal volume of was added, this was mixed gently and placed at room temperature of 23 °C for 45 min. Pellets were again centrifuged at 8000 g for ten min of which the supernatant was decanted. The final pellets were air dried at room temperature for 1 h and it was re-suspended with 100 µl in molecular grade sterile distilled water. One percent agarose gel stained with gel red was used to check the presence of DNA on electrophoresis. Extracted DNA was diluted in 1 µl in 8 µl of PCR water and used as template for the polymerase chain reaction (PCR).

PCR amplification

The primer pair, H1594/H1595, attached at the 5′ ends with M13 primer (Tian et al. 2016) designed to amplify the cpn60 gene with sequences

Table 2. Primers used in the PCR and sequencing of cpn60 genes in the study.

Name	Sequences (5′-3′) with attached M13	Gene
H1594	CGCCAGGGTTTTCCCAGTCACGACGACGTCGCCGGTGACGGCACCACCAC	*Cpn60*
H1595	AGCGGATAACAATTTCACACAGGACGACGGTCGCCGAAGCCCGGGGCCTT	*Cpn60*
M13F	CGCCAGGGTTTTCCCAGTCACGA	-
M13R	AGCGGATAACAATTTCACACAGGA	-

NB. Underlined sequences are the M13 portion of each primer sequence.

Table 3. Isolates collected and used in this study and their GenBank accession numbers.

Host	Bacterial species	Strain identification	*Place of collection	GenBank Accession number (cpn60)
Mangifera indica	*X. c.* pv. *mangiferaeindicae*	XamGH1	Ghana (ER)	MT274314
Mangifera indica	*X. c.* pv. *mangiferaeindicae*	XamGH2	Ghana (ER)	MT274315
Mangifera indica	*X. c.* pv. *mangiferaeindicae*	XamGH3	Ghana (ER)	MT274316
Mangifera indica	*X. c.* pv. *mangiferaeindicae*	XamGH4	Ghana (VR)	MT274314
Mangifera indica	*X. c.* pv. *mangiferaeindicae*	XamGH5	Ghana (VR)	MT274315

*ER = Eastern region, VR = Volta region.

stated in Table 2, was used in the PCR. Polymerase chain reaction was conducted in a volume of 50 µl. Each mixture comprised of 25 µl master mix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 5% Glycerol, 0.08% IGEPAL® CA-630, 0.05% Tween® 20, 25 units/ml Taq DNA Polymerase, pH 8.6@25 °C) (New England Biolabs, UK), 2 µl each of both forward and reverse primers, 8 µL of target DNA and 13 µl of PCR grade water. The amplification conditions used for the PCR are as follows: the initial denaturation was done at temperature 94 °C for 5 min, 35 cycles of denaturation at 93°Cfor 30 s, annealing temperature of 57 °C for 30 s and extension at 72 °C for 45 s. The final extension step was 72 °C for 10 min. A volume of 10 µl of polymerase chain reaction products was run on a 2% agarose gel matrix which was stained with gel red nucleic acid stain using TAE buffer. Amplified products were viewed with the aid of a UV transilluminator (Syngene Bio-imager, Fredrick Maryland, US).

Sequencing and phylogenetic analysis

The products of the cpn60 gene obtained were sent to Inqaba Company in South Africa for purification and sequencing. The universal primer M13 was used to sequence the gene region from both forward and reverse positions. For the phylogenetic analysis, the sequences of the *cpn 60* gene of the five putative *X. citri* pv. *mangiferaeindicae* (*Xcm*) isolates obtained in this study (Table 3) were compared with those of 30 *Xanthomonas* species/pathovars and *Pseudomonas aeruginosa*, which was selected as an out-group, that were retrieved from the stable public repository Figshare (DOI: 10.6084/m9.figshare. 4083378) (Table 4). ClustalW was used for the alignment of different sequences

Table 4. Isolates whose cpn60 gene sequences were downloaded from figshare public depository and used in the study.

Species/Pathovar	Strain identification	Source
X. axonopodis pv. *citri*	1	CAIQ
X. axonopodis pv.*citri*	2	CAIQ
X. axonopodis pv. *betlicola*	ICMP 312	ICMP
X. axonopodis pv. *betlicola*	ATCC 11677	ATCC
X. axonopodis pv. *begoniae*	LMG553	LMG
X. axonopodis pv. *begoniae*	LMG550	LMG
X. axonopodis pv. *axonopodis*	LMG539	LMG
X. axonopodis pv. *axonopodis*	DSM3585	DSMZ
X. arboricola pv._*celebensis*	DSM50853	DSMZ
X. arboricola pv._*celebensis*	ATCC19045	ATCC
X. arboricola pv. *pruni*	ATCC15924	ATCC
X. albilineans	ICMP196	ICMP
X. albilineans	DSM3583	DSMZ
X. fragariae	NCPPB1469	NCPPB
X. fragariae	ATCC29076	ATCC
X. cucurbitae	NCPPB3168	NCPPB
X. cucurbitae	NCPPB2597	NCPPB
X. citri subsp._*malvacearum*	DSM3849	DSMZ
X. citri subsp. *malvacearum*	DSM1220	DSMZ
X. citri pv. *cajani*	LMG558	LMG
X. citri pv. *bauhiniae*	LMG548	LMG
X. cassavae	LMG671	LMG
X. cassavae	ICMP204	ICMP
X. campestris pv. *musacearum*	ICMP2870	ICMP
X. campestris pv. *musacearum*	ATCC4908	ATCC
X. campestris pv. *mangiferaeindicae*	ICMP5740	ICMP
X. campestris pv. *mangiferaeindicae*	ATCC11637	ATCC
X. campestris pv. *euphorbiae*	NCPPB1828	NCPPB
X. campestris pv. *campestris*	NCPPB528	NCPPB
P. aeruginosa		

ATCC = American Type Culture Collection; NCPPB = National Collection of Plant Pathogenic Bacteria; DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen; LMG = BCCM/LMG Bacteria Collection, Laboratory for Microbiology; ICMP = International Collection of Micro-organisms from Plants; CAIQ = Chinese Academy of Inspection and Quarantine.

and gene regions. The aligned sequences were optimized manually to ensure positional homology and gaps were treated as missing data. The multiple sequence alignment obtained was used to construct a phylogram.

The Neighbor-Joining method (Saitou and Nei 1987) was used to infer the evolutionary history of the isolates using the molecular evolution genetic analysis (MEGA) software. The percentage of replicate trees in which the associated taxa clustered together was evaluated with a bootstrap analysis with 1000 replicates. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Determination of cross infection potential of isolates on cashew

The cross infection potential of isolates was determined on detached leaves of the Brazilian cultivar of cashew. Young leaves of the crop were harvested and surface sterilized with 10% dilution of household bleached and rinsed three times with sterile distilled water. Inoculum of five of the isolates used in the molecular characterization were produced separately by scraping the growth of the bacterium on nutrient agar into sterile distilled water and adjusting the concentration to approximately 1×10^5 CFU/ml. A sterile inoculation pin was used to create micro wounds at three places on each leaf after which the leaf was sprayed with the inoculum using a hand held sprayer. Five leaves were inoculated with each bacterium isolate to serve as a replicate. There were four replicates per isolate. Negative control, made up of cashew leaves inoculated with sterile distilled water only and positive controls made up of leaves of the Keith variety of mango, inoculated with the inoculum of the isolates, were maintained. Inoculated leaves were tied in transparent polyethene bags and incubated in the laboratory. Five days after incubation, the incidence of the disease was calculated by expressing the number of leaves diseased as a percentage of the number of leaves incubated.

Fungicide sensitivity test

Bacteria are known to be susceptible to copper based fungicides. Three concentrations (the manufacturers recommended concentration, half of the manufacturers recommended concentration and double of the manufacturers recommended concentration) each of five copper based fungicides and an organic fungicide (bamboo distillate) (Table 5) obtained from chemical shops in Ghana were evaluated to determine their effect on the growth of the isolated bacterial strains. A simple agar diffusion method was used in the trial. The five bacterial isolates subjected to the molecular characterization were used as the test isolates individually. Each of the selected bacterial strains was cultured on nutrient agar in

Table 5. Fungicides and their concentrations evaluated for their inhibitory activity against the bacterial isolates.

		*Concentration (/l of water)		
Product	Active ingredient	C1	C2	C3
Curenox	500 g/kg Copper oxychloride	1.5 g	3.0 g	6.0 g
Coprous super	850 g/kg Copper oxychloride	1.5 g	3.0 g	10.0 g
Cuprofix disperss	120 g/kg Copper+ 300 g/kg Mancozeb	1.5 g	5.0 g	10.0 g
Yellow gold	Bamboo distillate	15.0 ml	30.0 ml	45.0 ml
Funguran	770 g/kg Copper hydroxide	1.25 g	2.5 g	10g
Nordox	750 g/kg Cuprous oxide	1.25 g	2.5 g	5.0 g

*C1 = ½ of recommended concentration, C2 = recommended concentration, C3 = double recommended concentration.

9 cm Petri dishes for 3 days. A suspension of the organism was produced by scraping the growth on NA into sterile distilled water and the concentration adjusted to 1.0×10^6 CFU/ml. Two hundred microliters of each suspension was streaked uniformly on fresh solidified nutrient agar in plates using a sterile bent glass rod and the plates were allowed to stand for 3 min. A solution of each of the selected copper based fungicides and the bamboo distillate was prepared by dissolving the required amount of each separately in a litre of water. Two sterilized filter paper disc each of approximately 5 mm diameter were treated with a fungicide mixture by dropping 20 µl of the mixture on the disc. These were allowed to dry for 3 s and placed on the media in the plates, using sterile forceps. A third disc which served as control was treated with 20 µl of sterile distilled water only. The set-up was replicated three times and incubated in the laboratory for 3 days under temperature of 23 C and RH of 65%. The presence of a clear zone around the treated disc was an indication that the chemical was lethal to the growth of the bacterium.

Results

Cultural characteristics and pathogenicity of bacterial strains isolated in the study

A total of 45 strains of the bacterium were isolated from the diseased leaves and fruits of mango collected in the study. A total of 30 isolates were creamy while 15 were yellow. All Colonies of creamy white isolates nutrient agar were shallow and convex with complete margins on nutrient agar and produced yellow, shiny and mucoid colonies after six days of incubation at 25–30 °C on YDC. All were gram negative short rods and were also catalase positive. All were able to cause the disease on inoculated detached mango leaves (Figure 3) and were tentatively identified as strains of *Xanthomonas citri pv mangiferaeindicae*. The yellow isolates which could not cause any reactions on the inoculated leaves were considered saprophytes and therefore, they were not included in the molecular characterisation and cross-infection studies.

Phylogenetic analysis

The cpn60 gene was successfully amplified and sequenced from the selected isolates obtained in the study. The assembled sequences were 555 bp long and were deposited in the GenBank under accession number of MT274314-MT274318. The aligned sequences of the strains obtained from this study and those downloaded from Figshare was approximately 600 bp. The phylogram (the optimal tree) generated with the multiple

Figure 3. Symptoms of mango bacterial black spot on mango leaves inoculated with inoculum of *Xanthomonas campestris pv mangiferaeindicae* (XamGH1). Control (left) was inoculated with sterile distilled water only.

sequence alignment generated is shown as Figure 4. The sum of branch length is 0.48891922. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 35 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. There were a total of 554 positions in the final dataset. In the tree, several clades were formed and in most cases were supported by high bootstrap figures. From the phylogram, it can be seen that strains belonging to the same species/pathovars, clustered together in the same clade. All the strains from Ghana clustered together in a clade which includes the two strains of *Xanthomonas citri* pv. *mangiferaeindicae*. The clade was supported by a high bootstrap value of 96%.

Pathogenicity of bacterial isolates on cashew

All bacterial strains tested in the study were able to cause the disease symptoms on all the 20 cashew leaves inoculated. The disease began as a translucent area around the point of inoculation and becomes darker in colour as the lesion aged. The symptom was not observed on the control leaves

Figure 4. Phylogram generated based on the multiple sequence alignment of the *cpn 60* gene obtained from the 35 *Xanthomonas* strains used in the phylogenetic analysis. The strain designation and species of strains are indicated in Table 3. The five putative *Xanthomonas citri pv. mangiferaeindicae* collected in this study were designated as Xam GH1-5. Sequences of the other strains were obtained from Figshare stable repository. Type species of *Xanthomonas c. pv. mangiferaeindicae* are indicated in red.

inoculated with sterile distilled water only (Figure 5). The same symptoms were elicited also on the mango leaves inoculated with the bacteria.

Fungicide sensitivity test

All isolates used in the study were sensitive to the all the copper based fungicides but not to the bamboo distillate, and reacted to each of the fungicides in the same way. There was significant difference ($p > 0.05$) in the diameter of the zone of inhibition of the growth of the bacterial isolates around the filter paper discs treated with the recommended concentrations of the different fungicides. The recommended concentration of Curenox (3 g/l), Coprous Super (3 g/l) and Nordox (2.5 g/l) produced a significantly higher zone of inhibition of the growth of the bacterium around the filter paper discs compared to the recommended concentration of Funguran (2.5 g/l) and Cuprofix disperss (5 g/l). On the other hand, there was no significant difference in the zone of inhibition at double

Figure 5. Disease symptoms induced on detached cashew leaves by *Xanthomonas citri* pv. *mangiferaeindicae* strain XamGH1isolated from mango in this study. Note; control (left) was cashew leaf inoculated with sterile distilled water only.

Table 6. Effect of different fungicidal treatments on the diameter of the clear zone around treated filter paper discs on growth of an isolate of *Xanthomonas citri pv mangiferaeindicae* on nutrient agar.

	Concentration of fungicides*/Diameter of zone of inhibition (cm)		
Fungicides (recommended concentration)	C1	C2	C3
Curenox (3 g/l)	4.0	4.2	4.3
Coprous super (3 g/l)	3.9	4.1	4.0
Cuprofix disperss (5 g/l)	0.0	0.9	3.8
Yellow gold (30 ml/l)	0.0	0.0	0.0
Funguran (2.5 g/l)	0.0	2.5	3.7
Nordox (2.5 g/l)	3.5	3.7	3.9
LSD (5%)	0.1	0.2	0.4

*C1, C2 and C3 = half, full and double of manufacturers recommended concentrations of fungicides.

the recommended concentration of all copper based fungicides. Also, half of the manufacturers recommended concentration of Curenox (1.5 g/l) and Nordox (1.25 g/l) inhibited the growth of the isolates, while the half concentration of other treatments did not. On the other hand, all concentrations of the bamboo distillate used in the trial could not inhibit the growth of the bacterial isolates (Table 6 and Figure 6).

Discussion

Recent outbreak of mango bacterial black spot disease in the coastal savannah areas of Ghana caused massive destruction to the mango

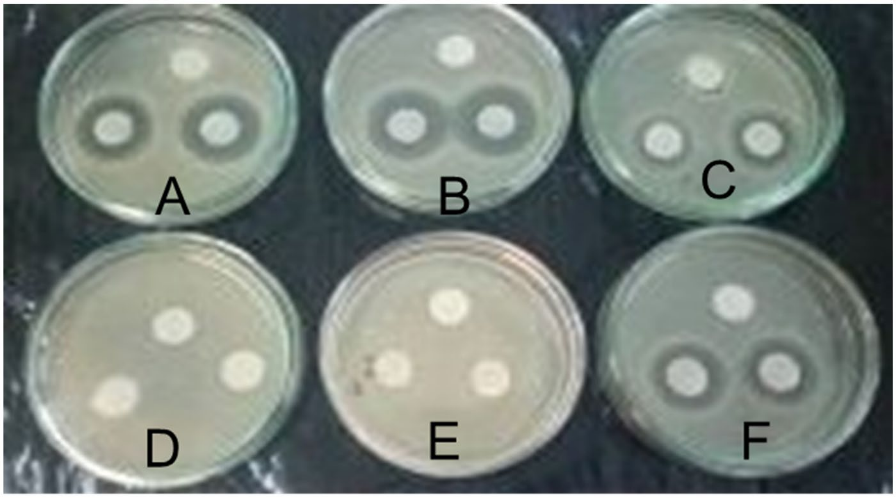

Figure 6. Inhibitory effect of the recommended rates of some copper based fungicides on the growth of a strain of *Xanthomonas citri* pv *mangiferaeindicae* (XamGH1) obtained in this study after 3 days of incubation. A = Curenox (1.5 g/l), B = Coprous Super (3.0 g/l), C = Funguran (2.5 g/l), D = Cuprofix disperss (5 g/l), E = Yellow gold (15 ml/l) and F = Nordox (1.25 g/l). Note: the 2 discs placed side by side (below) in each plate, were treated with Copper based fungicide or yellow gold while the lone disc (above) was control dipped in sterile distilled water.

industry in the area as compared to the damage caused in the Northern Region, where the disease was first reported. Secondly, several copper based fungicides available in the area have failed to control the disease as reported by farmers. These have led to questions as to whether the causal agent was a bacterium as suspected. The isolation of a bacterium and confirmation of its pathogenicity on detached mango leaves in this study have confirmed that the disease was caused by a bacterium. The nature of the disease symptom also showed that, the disease was the much dreaded bacterial black spot reported in several mango growing areas including the Northern part of the country (Manicom and Wallis 1984; Pruvost et al. 2011).

Previously, the traditional methods such as shape of cells, gram staining and presence of appendages and ability of a bacterial strain to macerate potato has been used for the identification of a *Xanthomonas* infecting citrus in Ghana (Honger 2004). However, the inadequacies associated with the method led to some doubts about the diagnosis especially when the pathogen was being reported for the first time on the crop (Honger et al. 2007). It was therefore imperative that molecular methods were employed in identifying the causal agent of the disease in this study.

Molecular characterization of pathogens in general is widely accepted as the most appropriate especially when pests of quarantine importance are concerned. Sequence analysis of diagnostic genes has been found to be very useful in this regard. In the diagnosis of bacterial disease, commonly used diagnostic genes include the 16S rRNA, gyrB, and avrBs2 (Zeigler, 2003; Tian et al. 2016). Work done by Tian et al. (2016), has shown that the cpn60 was a more dependable DNA barcode which could provide a more reliable and effective means for the species and pathovar-level identification of the quarantine plant pathogen *Xanthomonas*. Therefore, the sequence of the *cpn 60* gene in the *Xanthomonas* isolates obtained from mango in Ghana was used to identify the causal agent of the black spot disease of mango in the coastal savannah region. In the phylogram generated based on the sequences of the *cpn 60* gene, the five isolates from Ghana were found in the same clade together with two species of *Xanthomonas citri* pv. *mangiferaeindicae*. The clade was supported by a high bootstrap value of 96%, thus, confirming the identity of the Ghanaian isolates as *Xanthomonas citri* pv. *mangiferaeindicae*. The phylogram shows that the clustering of the strains corresponded to their species or pathotype. Therefore, the results of this study have shown without doubt that *Xanthomonas citri* pv. *mangiferaeindicae* was responsible for the bacterial black spot disease and its associated damages to the mango fruits in the coastal savannah zone of Ghana.

Xanthomonas citri pv. *mangiferaindicae* on mango could be differentiated into three pathogenic clonal groups. Group I strains reproduced significantly in tissues of mango and cashew leaves and produced the characteristics black lesions associated with the original pathovar, Group II reproduced significantly in the tissue of cashew but not in mango, while Group III produce symptoms only on mombin (*Spondias mombin*) and ambarella (*Spondias dulcis*) (Ah-You et al. 2007). In this study, the isolates obtained were pathogenic to cashew and caused the characteristic symptoms associated with the disease on mango. It can therefore be conjectured the strains of the bacterium in Ghana belongs to the pathogenic clonal grouping 1. This means the pathogen is a threat as well to the production of cashew in Ghana. It also means that when cashew and mangoes are planted in the same area, control of the disease will be difficult since the pathogen would be cross-infecting the two crops in the field.

Control of bacterium in general is difficult, however, it has been reported that copper is detrimental to most bacteria and can be included in any integrated control measure against the pathogen. Copper fungicides used for controlling bacterial functions in different ways such altering bacterial protein synthesis, altering membrane integrity and being detrimental to microbial DNA (Warnes et al. 2010; Grass et al. 2011; Chaturvedi and Henderson 2014). In this study, the selected copper based fungicides were able to inhibit the growth of the bacterium in vitro. Elsewhere the use of copper based fungicides has been evaluated in the field against the disease and found to be effective (Vermuelen and Cimler 1992). Therefore copper based fungicides such as Coprous super, Curenox and Nordox can be useful in the control of the disease caused by the bacterium in the field in Ghana as they have demonstrated the ability to impair the growth of the bacterium. However, there will be the need for field trials to confirm this.

Conclusion

The bacterium *Xanthomonas citri* pv *mangifeareaindicae*, was confirmed the causal agent of mango bacterial black spot in the coastal savannah zone of Ghana, where the bulk of the country's mango for export is produced. The pathogen was not being reported for the first time in the country, however, this was the first time that the cpn60 gene sequence was being used to identify it in the country. The pathogen was found to be pathogenic to cashew and could therefore be a threat as well to the production of the crop in Ghana. Active ingredients such as Copper oxychloride and Copper oxide were found to be detrimental to the growth of the bacterium and could therefore be used to manage the disease in the field. Future studies must focus on whether the pathogen that was reported on the crop in the Northern region of Ghana was what has spread to the coastal savannah zone of the country.

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