The Incidence, severity and aetiology of a bacterial canker disease of citrus in Ghana

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Abstract

A disease of citrus characterised by slightly raised spots with chlorotic halo was observed in the citrus orchard of the Soil and Irrigation Research Centre of the University of Ghana, located at Kpong in 2009. Samples of the diseased leaves and fruits were taken and a bacterium isolated and identified using cultural, morphological and polymerase chain reactions with three different sets of species specific primers. The pathogenicity of the bacterium was established using citrus seedlings. The bacterium isolated was found to produce yellow mucoid colonies and the cells were short rods and gram negative. It showed pectolytic activity on potato plugs and was pathogenic to artificially inoculated seedlings. The expected PCR product of 222 bp, 179 bp and 197 bp were obtained in the PCR using the primer pairs 2/3, J-RXg/J-RXC2 and J-pth1/J-pth2 respectively. It was concluded that the bacterium was *Xanthomonas campestris* pv *citri* A and the disease was a form of citrus canker. The disease incidence decreased from 100% to 13.3% in six years (2009-2014). Correspondingly, the disease severity declined from 88.3% to 1.7% in six years (2009-2014) without the application of any control measure. It was conjectured that the prevailing microclimate and environmental conditions might have influenced the survival of the pathogen. The practical implications of the findings are discussed and future studies suggested.

Introduction

Citrus, particularly sweet orange, is an important fruit crop in Ghana. Different cultivars of the crop are produced in the country mainly for local consumption. The crop serves as one of the cheapest sources of minerals and vitamins in the diet of the populace. Citrus is a major source of income to many people engaged in the production and marketing of the crop in Ghana.

The cultivation of citrus in Ghana is concentrated around the Eastern, Volta and Ashanti regions, where the rainfall distribution pattern favour the production of the crop. However, the cultivation of the crop is faced with many challenges including the incidence of pests and diseases. Diseases affecting the crop are numerous and occur both in the field and after harvest. As many as 13 different fungal species have been associated with different diseases on the crop (Offei *et al*., 2008). Notably among them is black spot caused by *Guinardia citricarpa* (Brentu *et al*., 2012). Largely, these fungal species have been controlled using well formulated control measures relying on the use of integrated control measures.

There is limited information on the incidence of diseases caused by bacteria on citrus in Ghana. Honger *et al*. (2007) isolated a yellow bacterium identified as *Xanthomonas spp*., on citrus using cultural, morphological and biochemical characterisation. Due to the limitations of the methods used for the diagnosis and the restriction of the disease to the nursery at that time, the disease was simply identified as citrus canker-like disease. Offei *et al*., (2008) also reported of a *Xanthomonas* leaf/fruit spot disease of citrus in Ghana. In 2009, a new disease of citrus, characterised by canker symptoms were observed on fruit bearing citrus trees planted at a citrus orchard of the Soil and Irrigation Research Centre (SIREC) of the University of Ghana located in the Coastal Savanna Zone of Ghana. The symptoms of the disease in the field were found not only on the leaves, but on the fruits as well. The infected fruits looked unsightly and most of them, at different stages of development, dropped from the infected trees.

The nature of the disease symptoms in the field suggested that the disease could be citrus canker, reported to be caused by *Xanthomonas campestris* pv. *citri* (Agrios, 2005; Gabriel, 2000). However, to date, citrus canker disease has not been reported in Ghana. The only diseases on citrus, attributed to *Xanthomonas* species in Ghana were citrus canker-like disease (Honger *et al*., 2007) and *Xanthomonas* leaf/fruit spot (Offei *et al*., 2008). While the former was found in the nursery, the latter was reported in the field. The latter disease was described as producing lesions on leaves, stems and fruits of citrus trees and leaves and fruits dropped prematurely (Offei *et al*., 2008). These disease symptoms were similar to what was observed on the citrus trees in the fields at SIREC, except that the raised nature of the spots observed in the SIREC, was not reported by Offei *et al*. (2008). The raised spot is a critical diagnostic feature for distinguishing between two important diseases on citrus, namely, the citrus bacterial black spot and the citrus canker, both caused by different strains of *Xanthomonas campestris* (Agrios, 2005; Gottwald and Graham, 2000). Hence the disease observed at SIREC may be different from what was reported by Offei *et al*. in 2008. On the other hand, citrus diseases such as scab caused by *Elsinoe fawcetti* and anthracnose caused by *Colletotrichum gloeosporioides* have been reported in Ghana (Honger *et al*., 2016; Offei *et al*., 2008) which produce symptoms akin to the disease observed in the field. However, the presence of water soaked margins accompanying the disease symptoms on the leaves and fruits at SIREC is normally associated with citrus canker. It stands to reason that the disease may be a new disease in Ghana and therefore require further studies to elucidate its aetiology and identity.

In diagnosis and control of a disease, accurate knowledge of the causative organism is essential. The causal agent of the suspected canker disease at SIREC is unknown, not excepting the incidence and severity of the disease at the time of the commencement of this present study. In view of the limited information about the new disease of citrus observed in the citrus orchards, this research work was carried out to isolate the causative agent and identify it using cultural, biochemical and molecular

characteristics. Data on the incidence and severity of the disease over a period of six years was also obtained to augment the information about the disease.

Materials and methods

Experimental site

The field studies to determine the disease incidence and severity were carried out

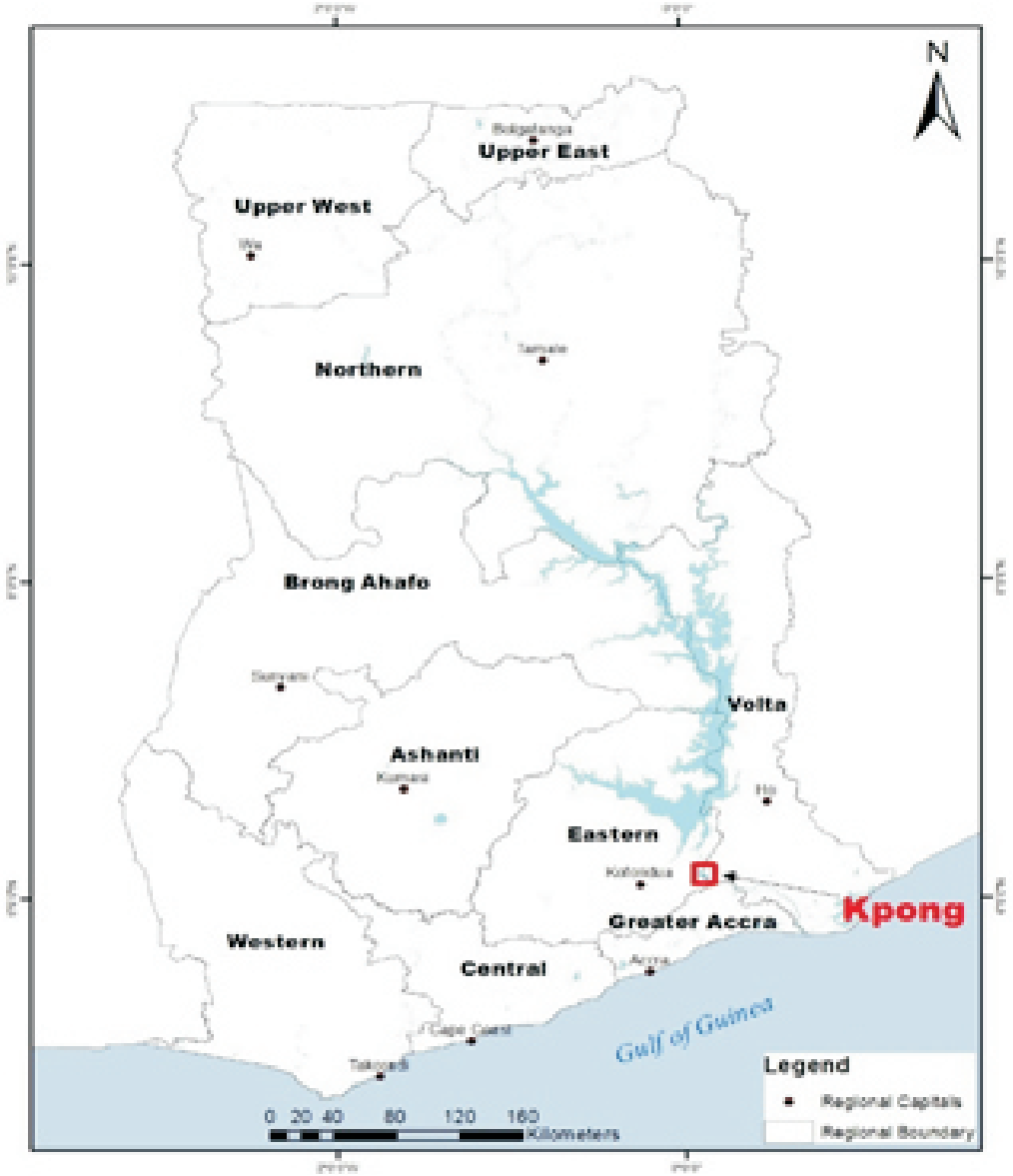

Fig. 1. Map of Ghana showing the experimental site, Kpong (indicated in red)

between 2009 and 2014 in The soil and Irrigation Research Centre of the University of Ghana located at Kpong in the Eastern Region of Ghana (Fig. 1). The site is located on latitude 6 09'N and longitude 00 04'E at an altitude of 22 m above mean sea level. The study site is within the coastal savanna zone ecology of Ghana, characterised by a bimodal rainfall pattern. The soil type in the area is classified as Calcic Vertisol, Typic Calciustert or tropical black clay of the Akuse series. The soil contains a large amount of montmorillonite clay, swells and becomes sticky when wet and shrinks and cracks when dry. The area is bounded by the Volta lake from which fresh water is pumped for the irrigation of citrus plants in the area.

Disease incidence and severity determination

During the period of the study, three major citrus orchards located at different parts of the centre were present. Each plot had different varieties of citrus, namely, Late Valencia, Washington, Tangerine and Rough lemon. In each plot, a total of 30 trees, irrespective of variety, were sampled at random for the determination of the disease incidence and severity. Each selected tree was observed and the presence or absence of the disease on either the leaves or fruits was noted. The number of trees showing the disease symptoms was then recorded. The disease incidence (DI) per plot was calculated using the formula, $DI = \frac{n}{N} \times 100$

where

n = number of trees with diseases symptoms

N= total number of trees inspected.

After that 100 fruits from each tree was selected at random and inspected for the symptoms of the disease. The number of fruits showing the disease symptoms was determined and used to calculate the disease severity using the modified formulae of Cardoso *et al*., (2004), ie.,

$DS = \frac{f}{F} \times 100$

where

f=number of trees with diseases symptoms

F= total number of trees inspected

Data analysis

Data on the disease incidence and severity were arcsine transformed and subjected to analysis of variance using Genstat version 11(Lawes Agricultural Trust; Rothamstead Experimental Station). Means were separated using L.S.D at 5%.

Collection of diseased plant parts and isolation of causal agent

Diseased leaves and fruits from the diseased trees were harvested, placed in black polyethene bags, labelled and sent to the Plant Pathology Laboratory of the Department of Crop Science, University of Ghana for the isolation and identification of the causal agent. The symptoms were similar to what was caused by a bacterium on citrus in the nursery in Ghana (Honger *et al*., 2007) and elsewhere (Gottwald *et al*., 1991). The method of dilution after Tuite, (1969), was used for the isolation of the causal agent. Yeast nutrient agar was prepared by dissolving 23 g of nutrient agar in 2.5 g of yeast extract in 1litre of distilled water. The mixture was then sterilised at a temperature of 121 ^{o}C for 15 minutes and 20 ml aliquots were poured into oven-sterilised glass petri dishes to set. The diseased leaves and fruits were rinsed three times with sterile distilled water after which a scalpel was used to dissect the raised spots and yellow chlorotic areas on the fruits and

leaves. The pieces of the tissue were then sterilized in 1% sodium hypochlorite for 1 minute and the rinsed three times with sterile distilled water. The tissues were pulverised in few drops of sterile water and allowed to stand for 10 minutes for the bacterium to ooze out. The suspension was then streaked on yeast nutrient agar in plates using a heat-sterilized inoculation loop. The plates were covered, sealed in cellophane plastic bags and incubated in the laboratory at 23 ^{o}C and 50–60% Relative Humidity (RH) for 3 days. The bacterium which grew on the plates was reinoculated on slants of Yeast Nutrient Agar in MaCartney Tubes and kept for future use.

Morphological and biochemical characterization of the isolated bacterium

Growth characteristics of the bacterium on a glucose medium

The method of Schaad (1988) was used. A glucose nutrient agar was prepared by dissolving 2.5 g of glucose and 23 g of nutrient agar in 1litre of distilled water. The mixture was autoclaved at 121 ^{o}C for 15 minutes, allowed to cool and poured into sterile petri plates to set. Using a sterile inoculation loop, the bacterium was streaked onto the glucose nutrient agar. The plates were covered and then sealed in cellophane plastic bags and incubated in the laboratory at 23 ^{o}C and 50–60% Relative Humidity (RH). After two days the nature of the growth characteristics of the bacterium on the medium was recorded to aid in its identification.

Gram staining

The Gram staining method (Bradbury, 1970) was carried using freshly prepared reagents. A thinly spread film of the bacterium was made on a clean slide, air dried and the lower side of the slide lightly flamed to fix the bacterium on the slide. The smear was then flooded with crystal violet solution for 1 minute, washed in tap water for 10 seconds and excess water drained off. It was carefully blotted dry with a paper towel and flooded with iodine solution for 1 minute, washed in tap water for 15 seconds and blotted dry. The smear was then decolourised with ethyl alcohol for 30 seconds and rinsed in tap water for 2 seconds. Safranin solution was then used to counter stain for 10 seconds, washed briefly in water and blotted dry. The smear was then observed under oil immersion of the compound microscope. A culture of *Escherichia coli (E. coli*), a bacterium which is gram negative, was obtained and given the same treatment as the test bacterium to serve as a positive control.

Catalase test

Yeast nutrient agar was prepared by adding 5 g of yeast extract to 23 g of nutrient agar. The mixture was dissolved in 1L of distilled water, dispensed into test tubes and autoclaved at 121 ^{o}C for 15 minutes and allowed to set as slants. Two of the slants were inoculated with the bacterium; two uninoculated slants served as control. The slants were incubated for two days at 23 ^{o}C and 50–60% RH. Few drops of 3% hydrogen peroxide were poured gently along the walls of the tubes. The production of gas bubbles in the tubes was recorded to aid in the identification of the bacterium.

Pectolytic activity

The method employed was that of Lelliot and Stead (1987). An Irish potato was scrubbed thoroughly with soap water. A 1cm cork-borer was pushed into the tuber at one end until the end appeared at the other

side. The plug of the tuber in the cork borer was removed using a sterile inoculating pin and placed immediately into sterile distilled water contained in 9 cm petri dish. Using a scalpel, the peel was removed from both ends and a slope was made at one end of the plug. The plug was then placed in a test tube with the diagonal end, uppermost. Sufficient distilled water was used to cover half of the plug in the tube, and capped. The preparation was autoclaved at 121 ^{O}C for 15 minutes and allowed to cool. Using a sterile loop, the slanted end of the plug was inoculated with the test bacterium and the set up was incubated in the laboratory at 23 ^{O}C and 50–60% RH for three days. Control was an autoclaved plug that was not inoculated with the bacterium. After three days the rounding of the edges of the plug and the growth of the bacterium was recorded to further characterize the bacterium.

Polymerase chain reaction

Extraction of total DNA from cultured bacteria cells. A two-week old culture of the pathogen was sub cultured twice in glucose nutrient agar medium to obtain single colonies. A well separated colony was picked with a sterile inoculating loop into a 10ml sterile YP medium (yeast: 7g/l, Peptone : 7 g/l) and was incubated for 24 hrs on a rotary shaker at 28 °C and 250 rpm. 1ml of the bacterial suspension was transferred into a sterile 1.5 ml tube. This was then centrifuged at 8000 g to obtain pelleted bacteria. Total genomic DNA was extracted using a modified protocol developed by Llop *et al*. (1999). Pellets were resuspended in 500 µl of extraction buffer (0.2 mM/l Tris HCl *p*H 7.5, 0.25 mM/l Na Cl, 0.025 mM/l EDTA, 0.5% PVP and 0.5% SDS) and incubated at room temperature of 23 ^{O}C at 200 rpm in orbital shaker for 1 hour. The suspension was then centrifuged at 1000 g for 5 min; 450 µl of the supernatant was transferred into a new tube and an equal volume of isopropanol was added, gently mixed and left at room temperature (23 ^{O}C) for 45 minutes. Pellets formed at this stage were concentrated by centrifugation at 8000 g for 10 min after which the supernatant was discarded. Pellets were then air dried at room temperature for one hour and then resuspended with 100 µl of molecular grade sterile distilled water. 5 µl of RNase was then added and incubated at 65 °C for 15 minutes. The presence of DNA was confirmed by electrophoresis on 1% agarose gel stained with gel red. A 1 in 5 dilutions of extracted DNA was used in polymerase chain reaction (PCR).

PCR amplification

Three primer pairs (Table 1) were used in PCR for the identification of the isolated bacterium. The reaction was performed in 25 µl reaction mixture containing 1xTaq buffer, 1.5 mM Mg Cl, 0.04 mM primer *J-RX*g, 0.04 mM primer *JRX*c2, 0.2 mM of each dNTP's, and 1U of Taq polymerase. For primer pairs *J-pth*1 and *J-pth*2 and 2 and 3, the 25 ul PCR contained 1x Taq buffer, 3 mM $MgCl_2$, 0.1 mM of each primer pair, 0.2 mM of each dNTP's and 1U Taq polymerase. Amplification conditions for all three primer pairs were as follows: initial denaturation step at 94 ^{O}C for 5 minutes. This was followed by 40 cycles of denaturation at 93 ^{O}C for 30 seconds, annealing at 58 ^{O}C for 30 seconds and extension at 72 ^{O}C for 45 seconds. The final extension step was 72 ^{O}C for 10 minutes. Exactly 8 µl of PCR products were run on a 2% agarose gel matrix (stained with gel red

TABLE 1
Primers used in the study and their sequences

Primer name	*Primer sequence, 5'-3'*	*Reference*
2	CAC GGG TGC AAA AAA TCT	(Hartung *et al*., 1993
3	TGG TGT CGT CGC TTG TAT	
J-pth1	CTT CAA CTC AAA CGC CGG AC	Cubero and Graham, 2002
J-pth2	CAT CGC GCT GTT CGG GAG	
JRXg	GCG TTG AGG CTG AGA CAT	Cubero and Graham, 2002
J-RXc2	CAA GTT GCC TCG GAG CTA TC	

nucleic acid stain) using TAE buffer. Amplification products were visualized with the aid of a UV transilluminator.

Pathogenicity test of isolate

The pathogenicity of the isolated bacterium was tested on young expanding leaves of seedlings of the late Valencia variety of citrus, using the method of Gabriel, (2002). A nutrient broth containing the bacterium cultured for 2 days was sucked by drawing into a syringe without a needle. The syringe containing the inoculum was then pressed gently, but firmly to the lower leaf surface and the slurry introduced into the leaf through the stomata until about 2 cm^2 of the leaf became water congested. A few minutes after the congestion has cleared, the inoculated seedlings were arranged in a completely randomized design in a green house. The treatment was replicated three times with controls made up of seedlings inoculated with a broth without the bacterium. The inoculated seedlings were observed daily till typical symptoms were recorded. The bacterium was re-isolated to confirm pathogenicity and to ascertain the fact that the causal agent was able to elicit the disease in the test plants.

Results

Mean monthly rainfall and temperature figures for the study area

The mean monthly rainfall and temperature Figures of the research area are stated as Fig. 2. The mean monthly temperature ranged from a minimum of 26.5ºC in August to the highest of 29.9 ºC in February and March. On the other hand, rainfall figures were low, ranging from 3.1 mm in January to 13.3 mm in December.

Disease symptoms

The disease symptom was characterized by raised spots. In very severe cases, these spots have coalesced forming larger spots which in most cases became blister-like growing into yellow spongy pustules which darkened and thickened into a light tan to brown corky canker with rough surface (Fig. 3). In freshly developed and expanding spots, the raised spots were surrounded by a clear water soaked margin and large yellow chlorotic halos (Fig. 3)

Disease incidence and severity

The disease incidence varied from 100% in 2009 to 13.3% in 2014 (Table 2). The highest incidence of 100% was recorded in

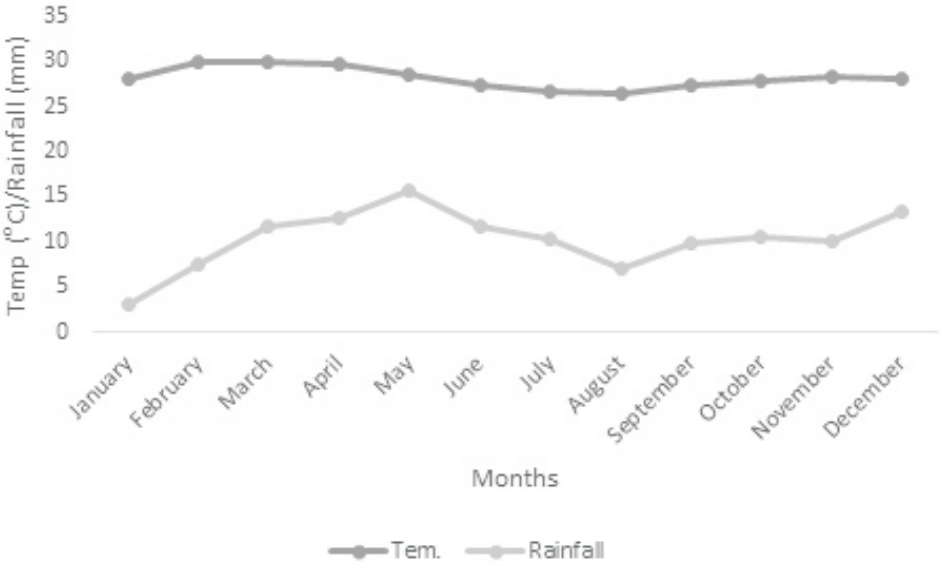

Fig 2. A Ten year's mean monthly rainfall and temperature for SIREC (2004-2014)

Fig. 3. Symptoms of diseased fruit harvested from ARC, Kpong. (Note the raised nature of spots and the large chlorotic area surrounding the spots on fruits)

TABLE 2

Incidence and severity of citrus canker disease in the field of Soil and Irrigation Resarch Centre (SIREC) during a 6-years citrus production seasons (2009-2014). (Fig. represent means from the four different varieties of citrus grown at the centre)

Years	*Disease incidence (%)*	*Disease severity (%)*
2009	100.0[a]	88.3[a]
2010	96.7[a]	66.7[b]
2011	70.0[b]	50.0[c]
2012	33.3[c]	20.0[d]
2013	20.0[d]	3.3[e]
2014	13.3[e]	1.7[e]

Means followed by the same alphabet in a column are not significantly different at L.S.D of 5%.

2009 which was not significantly different (P > 0.05) from the 96.7% recorded in 2010. The lowest incidence of 13.3% was recorded in 2014. The incidence was higher in 2011 (70.3%) than in 2012 (33.3%) and decreased with time till 2014 (Table 2).

Similarly, there was significant difference in disease severity recorded in the different years. The highest severity of 88.3% was recorded in 2009 while the lowest of 1.7% was recorded in 2014 (Table 2). The disease severity declined with time reaching the lowest level of 1.7 in 2014. Thus both disease incidence and severity declined to very low levels during the six years of observation.

Identification of the isolated bacterium

Cultural, Morphological and bio-chemical characteristics of the bacterium. The colonies of the bacterium were shiny yellow, mucoid and convex. The cells were gram negative short rods. The bacterium was also catalase positive producing gas bubbles in few seconds after the introduction of the hydrogen peroxide. It also grew on the plug of the potato on which it was inoculated resulting in the rounding of the edges of the potato plug. These characteristics of the isolated bacterium were similar to those of *Xanthomonas campestris* spp. (*Xanthomonas axonopodis*) on citrus (Schaad, 1988; Lelliot and Stead, 1987; Gottwald and Graham, 2000; Gabriel, 2000) and was therefore provisionally identified as *Xanthomonas campestris* pv. *citri.*

Polymerase chain reactions

The expected PCR product of 222, 179bp and 197bp were obtained when PCR was carried out using primer pair 2/3, J RXg/J-RXc2 and *J-Pth* and *J-pth2* (Fig. 4a and b).

Pathogenicity test

The isolated bacterium was able to induce the disease symptoms on the inoculated seedlings of citrus used in the study. First disease symptoms on the inoculated leaves was evident 7 days after inoculation. It consisted of a yellow area on the upper leaf surface around the point of inoculation. Twenty-one days later, raised spots characteristic of the disease, were formed at the lower portion of the leaves at

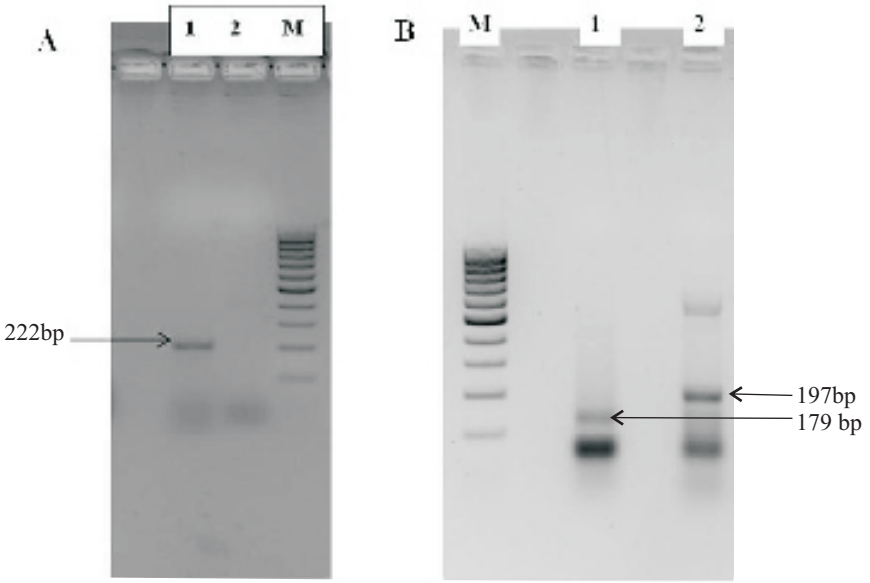

Fig 4. Amplicons for *X. axonopodis pv. citri* generated by primer 2 and 3 (**a**) with expected band size of 222 bp. (b) amplicons generated by primer pair JRXg and JRXc2 (lane 1) and primer pair *J-pth*1 and *J-pth*2 (lane 2) with expected and sizes of 179bp and 197bp respectively. Products were run alongside a 100bp DNA ladder (M).

the point of inoculation. The disease symptoms were similar to what was found on the both the leaves and fruits on the field. This confirmed the bacterium as the causal agent of the disease on the leaves as well as the fruits on the field.

Discussion

Currently, two bacterial diseases of citrus have been reported in Ghana. These are the citrus canker-like disease, reported on foliage of nursery seedlings (Honger *et al*., 2007) and *Xanthomonas* fruit/leaf spot (Offei *et al*., 2008). Symptoms of both diseases have been attributed to *Xanthomonas* species. In this study, the disease symptoms found on the diseased fruits, particularly, the raised, corky spots with brown sunken centres and the yellow halo surrounding some of the spots have been reported as a diagnostic feature of citrus canker. (Agrios, 2005; EPPO, 2005). Thus the disease under study could be citrus bacterial canker, a disease of quarantine importance in all citrus growing regions of the world (Gottwald, 2001).

Symptoms of Citrus Canker could be easily confused with other citrus diseases such as scab caused by *Elsinoe fawcetti*, anthracnose caused by *Colletotrichum gloeosporioides* and melanose caused by *Diaporthe citri* (EPPO, 2005). In this study only *C. gloeosporioides* isolates were obtained from few diseased samples. These isolates were confirmed to be existing as saprophytes on the citrus fruits (Honger *et al*., 2016). On the contrary, the bacterium in

this study was consistently isolated and was shown to be pathogenic to the citrus leaves, indicating that the disease was not caused by a fungus. The isolated pathogen produced a shiny yellow mucoid growth on the glucose medium, a characteristic of *Xanthomonas* species attributed to the production of the xanthomonadin pigment by members of the species (Lelliot and Stead, 1987). Members of the genus *Xanthomonas* have rod shaped, Gram negative cells (Schaad, 1998). According to Lelliot and Stead (1987), the ability of a bacterium to show pectolytic activities on potato plugs such as found in this study separates it from other non-pathogenic yellow bacteria that may frequently be isolated on citrus. The bacterium isolated in this study was therefore the pathogenic type on citrus plants. The morphological and physiological characteristics of the isolated bacterium was an indication that it could be a pathovar of *Xanthomonas campestris* (Lelliot and Stead, 1987). Currently, on citrus, the two pathogenic *Xanthomonas campestris* pathovars are *citrumelo* and *citri*. The former is associated with citrus bacterial spot disease characterised by flat spots and restricted to the nursery (Civerolo, 1984, Gottwald and Graham, 2000), while the latter is associated with citrus canker, characterised by raised spots surrounded by chlorotic halo (Gabriel, 2000). The raised spots characteristic of the disease symptoms observed in this study were therefore, an indication that the disease was not the citrus bacterial spot but may be citrus canker.

It has been recommended that the use of disease symptoms must be complemented with PCR with species specific primers for accurate diagnosis of canker disease of citrus (EPPO, 2005). Several primers are available for the identification of *Xanthomonas axonopodis* pv. *citri* (EPPO, 2005). The primer pair, *J-pth*1 and *J-pth*2, (which was based on the nuclear localization signal in virulence gene *pthA*, allowed amplification of a 197 bp PCR fragment in A, B and C strains of *Xanthomonas axonopodis pv. citri* (Cubero and Graham, 2002). In this study, the expected PCR product was obtained when the primer pair was used in the polymerase chain reaction. This showed that the bacterium isolated in the study contained the virulence gene *pthA* and could therefore possibly cause canker in citrus. Similar findings were made by Agbetiameh (2007), who isolated and identified a canker causing bacterium from citrus leaves in Ghana using PCR with kingsley primers. However, this method was limited in identifying the specific strain of the bacterium.

To identify the bacterium to the strain level, two other primer pairs were included in the study. These were primer 2 and 3 (Hartung *et al*., 1993) and primers *J-Rxg*1 and *J-Rxc*2 (Cubero and Graham, 2002). While both primers are specific to the A strain of citrus canker bacterium, the former pair is most frequently used in assays on plant material. On the other hand, *J-Rxg*1 and *J-Rxc*2, which was designed based on the internally transcribed spacer region between the 16S and 23S genes, have been used for identification of pure cultures of A strain of the pathogen. In this study, both primer pairs resulted in the amplification of the expected PCR product of 222 bp and

179 bp respectively. These results were consistent with results obtained elsewhere by Hartung *et al*., (1993) and Cubero and Graham, (2002) and confirmed that the bacterium isolated in this study was *Xanthomonas axonopodis pv. citri* A. It could therefore be conjectured that the disease under study was Asiatic citrus canker.

Different cultivars of citrus were present at the Research Centre where the diseased fruits were collected for the study. All the different cultivars of citrus including sweet orange, tangerines and lemons were found to be susceptible to the disease. The disease severity of 83.3% recorded in 2009 was an indication of the heavy tole the disease took of the crop that year. The diseased fruits either dropped or were harvested and discarded due to the unsightly nature of the fruit exocarp. These observations about the citrus canker disease have been associated with the A strain of *Xanthomonas citri*, which has been reported as being very destructive and not restricted to any citrus cultivar (Gabriel, 2000: Lelliot and Stead, 1987) such as occurred under the Ghanaian tropic conditions at the Research Centre.

One observation about the disease is its rapid decline in disease incidence and severity as the trees aged. Several reasons could be adduced for this observation. Firstly, the environmental conditions at the Centre may not be favourable to the proliferation of the disease. It has been reported that when high rainfall coincides with high temperatures, citrus canker disease is exacerbated (Gottwald *et al*., 2002). At SIREC where the disease was found, though temperatures were high in most parts of the year, rainfall was very low throughout the year (Fig. 1) and this may not be favourable to the disease. The persistent unfavourable weather for successive years may have impacted negatively on the growth and survival of the pathogen resulting in reduced incidence and severity of the disease with time. Secondly, it could also be that the virulence of the pathogen in Ghana is related at some point to the vigour of growth of the plants and that virulence declines with the age of the host plant. The latter suggestion is supported by reports that several variants of the same pathotype of the bacterium could exist. For example, group of strains of the pathogen of pathotype A designated as A* and A^w with restricted host ranges have been reported (Verniere *et al*., 1998; Sun *et al*., 2000). It could therefore be that the strains in Ghana could be deviants of the pathotype A whose virulence decreases with age of host tissues. The use of more robust molecular methods such as sequence analysis of phylogenetic genes, would be useful in confirming this assertion.

The rapid reduction in the disease incidence within 5 years could be an indication that the disease may be more virulent only in the first few years after infection of the host. A survey of citrus orchards currently in Ghana for the presence of the citrus canker disease would be useful to determine the level of infection and the microclimate and environmental parameters which would be crucial for decision making on the disease control.

Results in this paper have helped in the confirmation of the causal agent of canker disease of citrus, though it was previously unknown in the country. This is an indication that compromised quarantine measures might have led to inadvertent

introduction of the pathogen from plant materials, which remain the major source of long distance transfer of the pathogen (Agrios, 2005). The strengthening of quarantine processes in the country should be the rule rather than the exception.

Conclusion

The disease symptoms observed on the diseased citrus leaves and fruits in this study was no doubt the slightly raised spots characteristic of citrus canker disease reported elsewhere. The pathogen associated with the disease was identified as *Xanthomonas campestris* pv *citri*. However, the decreasing disease incidence and severity with age of the citrus trees raised questions as to whether the interactions between the pathogen and the prevailing environmental and climatic conditions might have ameliorated the disease. Since the causal agent was identified as the canker causing *Xanthomonas*, it could be possible that the strain of the pathogen may differ from what has been reported elsewhere. Comparison of the strain obtained in this study with several others reported elsewhere through sequence analysis of phylogenetic informative genes would be very useful, Also, a field study to determine the incidence of the disease in the major citrus growing areas in Ghana would be very important before further decisions are taken for the control of the disease.

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