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Studies and Characterization of Bacterial Spot Pathogen of Tomato *Xanthomonas campestris* PV *Vesicatoria*

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Abstract: Investigation was conducted in the laboratory to characterize, identify and determine the strains of bacteria responsible for bacterial spot disease of tomato in humid tropics of South Eastern Nigeria. The bacteria were isolated from infected tomato plant; leaf, fruit and seed and subjected to some standard bacteriological tests. The results of the cultural, physiological and biochemical analyses obtained showed that the bacteria isolated from bacterial spot lesions of tomato fruits, leaves and seed were gram negative, yellow aerobic rod shaped bacteria with a polar flagella. The bacteria colonies exhibited strong starch hydrolysis, metabolized glucose and produced acid from arabinose, sucrose and cellobiose but not from ducitol or sorbitol. Also nitrite was not reduced to nitrite based on bacteriological characteristics, the bacteria strains were identified as *Xanthomonas campestris* pv. *vesicatoria* (ex Doidge). The pathogens were found to be influenced by temperature with optimum temperature of 30-32°C but little or no growth at 20 and 45°C.

Key words: Isolation, *Xanthomonas campestris* pv. *vesicatoria*, bacterial spot, physiological and biochemical analysis

INTRODUCTION

Xanthomonas campestris pv. *vesicatoria* (*Xcv*) the causal organism of bacterial spot of tomato (*Lycopersicon esculentum* Mill) and pepper (*Capsicum annuum* L.) is the most important bacterial disease of tomato and pepper in Nigeria especially in the humid South Eastern Nigeria. Bashan *et al.* (1982) isolated *X. campestris* pv. *vesicatoria* from pepper and tomato seed using Tween-A and Tween-B media. However, growth of non target bacteria can be a problem in the dictation and identification of bacteria from diseased plant materials, seed or soil samples.

Peixoto *et al.* (2006) reported that in bacterial disease of grapevine the isolation of *X. campestris* pv. *viticola* from infected plant tissues was impaired by the presence of bacterial contaminants including *Microbacterium barkeri*. In order to overcome this problem several selective and semi-selective media have been developed for target phytopathogenic bacteria that can aid in such differentiation and selection. Some of these media are specific for pathovars of *Xanthomonas campestris* and have been used in seed assays for determination of contamination (Beaulieu *et al.*, 1991). Selective isolation was also achieved by Ranahawa and Schaad (1984) using general plating medium such as nutrient starch cycloheximide agar in which 50% colonies of *X. campestris* were recovered from saprophytic and antagonistic bacteria associated with crucifer seeds. Mcguire *et al.* (1986) reported three differential selective media developed for isolation and detection of *X. vesicatoria* from soil and plant materials. These media contained Tween 80, Bacto peptone and Difco agar among other nutrients.

They reported that although total elimination of contaminating microorganisms was not possible however, identification of *X. campestris* was achieved through the use Tween 80 incorporated with Potassium Bromide. Similarly, Gram-negative yellow aerobic cellulolytic rod-shaped, bacteria were originally isolated in mixed culture along with other phytopathogenic rod shaped bacteria from tomato and pepper transplants. Based on fatty acid composition, presence of *xanthomonadin* pigment and standard bacteriological characteristics, the unknown bacteria strains were identified as *Xanthomonas campestris* pv. *vesicatoria* (Gitatis *et al.*, 1987). Nascimento *et al.* (2005) in addition to selective isolation reported that the growth of *X. campestris* pv. *vesicatoria* ranged from 5-35°C, with optimum growth from 27-29°C

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but no growth at zero and 40°C. In this investigation however, efforts were made to isolate, identify and determine the temperature growth of the bacterium that induces black spot on tomato in humid South Eastern Nigeria.

MATERIALS AND METHODS

Isolation from leaf spot: Leaves infected with bacterial spot disease were collected from the farms of Michael Okpara University Umudike, Abia state during the wet season in 2007 and taken to the university laboratory for isolation and characterization. The method of isolation adopted was according to Bradbury (1970) in which the infected parts were surface sterilized with 70% absolute alcohol and a small piece of the infected portions were cut from the advancing margin of the lesions with a sterile scalpel. The tissue was washed thoroughly in two or three exchanges of sterile distilled water and placed in a petri dish with a drop of sterile water, teased apart with sterile needles and allowed to stand for 30 min after which the resulting suspension was then streaked on Sucrose Peptone Agar (SPA) using flamed wire loop.

The surface of the agar medium was kept dry of moisture by placing the plates inside an oven at 40°C for 15 min before streaking to avoid motile bacteria swimming in the surface moisture thereby forming a carpet of mixed growth instead of discrete, well separated colonies. After streaking, the plates were incubated at temperature of 27°C for a period of 48 h. The plates were examined after this period for the appearance of bacterial colonies. A pure culture of the colonies was later obtained by sub culturing each colony of the bacterium twice. The pure culture obtained was then used for pathogenicity test in order to fulfill the requirements of Koch's postulates of pathogenicity.

Isolation from infected fruit: The method of isolation was similar to that of the leaf stated above in which infected tomato fruits with bacterial spot lesions were collected from the field taken to the laboratory surface sterilized with 70% alcohol and washed thoroughly with sterile water before isolation. A small piece (3 mm) of the necrotic lesion on the fruit was cut from the boundary between the diseased and the healthy tissue with a sterile scalpel. The tissue washed thoroughly in sterile water thrice before placing it in a petri dish with a drop of sterile water, crushed with sterile needles and allowed to stay for 30 min. The suspension obtained was then streaked on Sucrose Peptone Agar (SPA). The culture media were placed upside down in the incubator and the temperature maintained at 27°C for period 48 h. A pure culture was obtained after sub culturing twice.

Isolation from infected seed: The procedure followed was that described by Mcguire *et al*. (1986) for isolation of the pathogen in seeds. Twelve grams of tomato seeds (approximately 500 seeds) were placed in 500 mL Erlenmeyer flask containing 100 mL of sterile peptone buffer. Peptone buffer was made by combining 5.30 g of KH_2PO_4 8.61 g of Na_2HPO_4 and 1.0 g of Bacto-peptone in I L of water. The seeds were incubated in the buffer, without shaking at 4°C for 3 h. Thereafter, they were agitated on a wrist shaker for 1 h at room temperature to dislodge bacteria. After shaking about 0.1 mL of the buffer solution was spread over the surface of the semi-selective culture medium, Tween B (Mcguire *et al*., 1986). Tween medium B contained (n g L^{-1}); Peptone 10.0, KBr 10.0, $Cacl_2$ 0.25, H_3BO_3 0.30, agar 15.0. After autoclaving, the following was aseptically added; Tween 80, 10 mL, cycloheximide 100 mg, cephalexin 65 mg, 5-fluorourcil 12 mg and tobramysin 9.4 mg.

The remaining buffer solution containing suspension of the bacterium was filtered through 0.4 micro membranes to concentrate the pathogen and the volume of the filtrate recorded. The filter membrane was centrifuged for 60 sec (in a 20 mm diameter test tube containing 5 mL of Tween 80) in 5 mL of peptone buffer. About 1 mL of this concentrated suspension was spread onto the surface of the Tween B medium on ten petri dishes. Dilutions of this suspension were also plated to ensure easy counting of the colonies.

The plates were incubated at 28°C for 5 days, after which the number of characteristic colonies formed were counted and compared with those from plates with suspension not centrifuged. The cfu g^{-1} of *X. vesicatoria* of tomato seeds tested was obtained by multiplying the average number of colonies on the 10 plates by 25 (corrected for the volume of buffer originally filtered) then divided by weight of seeds tested as follows:

$$\text{Cfu g}^{-1}\text{ seed} = \frac{\text{Mean of colonies x 25}}{12}$$

Bacterial inoculum preparation: Bacterial suspension of fresh colonies from SPA in distilled water was used. The inoculum was prepared by incubating bacterial cultures for 24 h and suspending the young active colonies from the culture in sterile distilled water to give a concentration of approximately 10^8 cells mL^{-1} (cfu mL^{-1}) adjusted with a haemocytometer.

Inoculation of young seedlings: Young tomato seedlings of 2 weeks old after transplanting into pots i.e., young susceptible cultivars with immature, rapidly developing tissues and partially expanded leaves and emerging shoot

were used. This stage is important because plant tissues usually become resistant to bacteria infections as the tissue reaches maturity (Fahy and Hayward, 1983). Some seedlings were also sprayed with sterile distilled water instead of the bacterium inoculum. These served as the control plants. Leaf inoculation was conducted by spraying the inoculum prepared above containing 10^8 cells mL^{-1} over both surfaces of a turgid leaf to a run off without infiltration using a hand atomizer.

Damage to tissue at inoculation was not necessary for leaf pathogen; however, all the inoculation tests had five replicates. Immediately following inoculation, the plants were incubated at high relative humidity 90-100% by covering with moistened transparent plastic bags. The seedlings were kept in the laboratory at 27°C for 48 h. They were not exposed to direct sunlight immediately after covering with plastic bags to avoid damage or scorching by the sun's heat to the leaves. However, after 48 h of incubation in the laboratory the plastic bags were removed and plants placed inside screen house for symptoms to develop for a period of 14 days at 30°C.

Inoculation of tomato fruit: Seedlings containing immature-unripe fruits were inoculated with the suspension of the bacterial isolate by spraying the bacterial suspension (10^8 cfu mL^{-1}) on the fruits (few pricks were made on the fruit surface). In another test, immature unripe fruits were detached with cotton wool soaked in 70% ethanol. When dry, a drop of inoculum was placed on the surface of the fruit and pricked into the fruit by giving a mild surface puncture on the skin according to Fahy and Hayward (1983). The inoculated fruits were kept in a closed humid chamber lined with damp filter paper and incubated at 27°C for 14 days. An optimal method adopted was to seal the needle puncture holes with Vaseline to prevent drying. A metal spatula was flamed, dipped into Vaseline and quickly smeared over the punctured holes. The spatula was flame sterilized between treatments. The procedure for incubation was the same as stated earlier with puncture inoculation.

Seed inoculation: Three grams of seeds were artificially inoculated by contaminating the seed lot with the isolate obtained from the extracted seeds using 1 mL of the isolate inoculum containing 10^8 cfu mL^{-1}. The infected seeds were then allowed to dry for 48 h under ambient temperature after which the seeds were tested for infection by isolating the pathogens using the extraction method discussed above and the re-isolate compared with the original pathogen (Fahy and Hayward, 1983).

Microscopic examination: Pure cultures that were re-isolated from the treated leaf, seed and fruits were examined under a binocular Olympus microscope by collecting a loopful of the inoculum and then mounted in a drop of water under a cover slip on a glass slide using the hanging drop method and examined at X-40 magnification (Hayward, 1979).

Identification and confirmation tests

Pigmentation and colony colour: Plant pathogenic bacteria produce a variety of colours and pigments, which are characteristic features of the group (Gitatis *et al*., 1987). For this reason a non-selective and selective media were used to distinguish the pathogen from other similar rod shaped bacteria. The preferred non-selective medium for this test was sucrose peptone agar, SPA (12). SPA contains (in g L^{-1}): sucrose, 20.0; peptone, 5.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.25 and agar, 15.0, the pH adjusted to 7.2 with NaOH. The usefulness of SPA lies in the fact that the rate of growth and colony types of Xanthomonads on SPA is characteristic of the genus. In addition, the colonies were readily distinguishable from other pathogens of related biochemical characteristics such as the fluorescent and levan-producing Pseudomonads (Hayward, 1979).

The selective medium used in this study was the SX agar medium developed by Schaad and White (1974). Its selectivity depends on starch-hydrolysis. SX agar contains (mg L^{-1}); soluble potato starch 10, beef extract 1, ammonium chloride, 5; KH_2PO_4, 2, agar 15 and methyl violet B 1 mL of a 1% solution in 20% ethanol, methyl green 2 mL of a 1% solution and cycloheximide 250, pH was adjusted to 6.8. The plates were inoculated with the bacterial colonies and allowed for 2 days at 28°C. After which the colonies were examined for colour shape, size and their nature of the mucoid substance produced in culture.

Gram stain reaction: An important preliminary guide in the identification of an unknown bacterial isolate is the application of Gram staining procedure. The advantage of this method is that it provides useful information about the shape and size of the cells and distinguishes between Gram-positive and Gram-negative species. The result of this staining reaction is essential for decision as to which criteria should be used in further identification of the strain. Numerous modifications of the Gram stain are available however, the following method based on Skerman (1967) was adopted and smears were prepared from young culture of about 24 h old on a sterile glass slide. The smear was first air dried and gently fixed by mild flaming on the under side of the slide. The slide was flooded with crystal violet for 1 min. Washed for 3-4 sec in a gentle stream of tap water. Followed by flooding with

iodine solution for one min and then washed again with tap water. It was decolourized by applying 95% ethanol drop-wise to the smear held at an angle against a, while background (e.g., white tile) until no more colour ran from the lower edge (about 10-20 sec). Finally, it was washed under tap water and counterstained with safranin, washed and dried. The slide was observed under oil immersion without a colour filter to observe the colour of well-separated cells. The reagents used for gram staining were as follows: crystal violet solution; crystal violet 2.0 g, ammonium oxalate 1.0 g; distilled water 100 mL. Iodine solution: iodine 1.0 g, potassium iodide 2.0 g, distilled water 100 mL. The iodine crystal were ground dry in a mortar and dissolved by slowly adding water and stored in a dark bottle. Aqueous safranin: Safranin 2.5 g in 95% ethanol diluted by 1 mL distilled water.

Motility test: The test was carried out by transferring actively growing cultures (24 h old) to moistened agar slopes of nutrient agar incubated at 28°C for two days. The slope was examined daily by gently removing a loopful of the growth from the water at the base of the slope using the hanging drop method (Schaad, 1980; Moffett and Croft, 1983). Also a sloppy agar plates were prepared by melting the nutrient agar (0.3%) and pouring into petri dish. On cooling, a colony of the pathogen was inoculated onto the center of the solidified medium and incubated for 24 h until obvious spreading of culture occurred.

Thereafter, a loopful of the active inoculum from the colony edge was transferred into a moistened agar slope and incubated until the medium became turbid and was examined using a hanging drop technique to observe the motility. The hanging method procedure involves use of a drop of the bacterial suspension onto the depressed cavity of a microscopic slide, covered with cover slip, inverted and mounted under high power objective (x40) to observe the movement of the bacterial cells (Fahy and Hayward, 1983).

Xanthan gum production test: Starr *et al.* (1977) and Gitatis *et al.* (1987) reported that most Xanthomonads produce yellow-pigmented colonies on SPA medium. The pigments have been called *xanthomonadins* a source of xanthan gum. This characteristic feature has become one of the important initial diagnostic characteristics of the genus *Xanthomonas*. The SPA medium was recommended by Starr *et al.* (1977) for xanthan gum production tests. The procedure was as follows. A well dried SPA medium was streaked with colonies of the test bacterium. The medium was incubated at 25-27°C for 24 h, after which the colonies were examined for production of xanthan gum. Another species such as *Pseudomonas syringae* was also streaked on the different plates containing the same medium to compare their results.

KOH solubility test: A rapid method known as solubility test was recently added to assist in the differentiation between Gram-negative and Gram-positive bacteria especially where gram stain smears gives a cause for doubt was employed. The first method is based on the resistance of the treatment with potassium hydroxide solution (3% KOH). In most cases, Gram-negative bacteria do not posses walls that resist solubility in KOH. The procedure was according to Gregerson (1978). Two drops of 3% KOH were placed on a glass slide. A colony of the test bacterium was picked from the surface of a solid medium with an inoculating loop. Another bacterium (*Corynebacterium michiganense*) known to be Gram-positive was also used as a control.

The bacterial culture was stirred in KOH for 5-10 sec and the inoculating loop raised from the drop. This is useful to determine if the KOH has become viscous or not. In addition, it was necessary to examine if a thread like slime followed the loop or not. Incase there was a slime or viscous culture then the result was positive i.e. a Gram-negative bacterium was involved but if a watery suspension did not follow the loop then the reaction was regarded as negative, i.e., the bacteria was a Gram-positive.

Hydrogen sulphide production: Hydrogen Sulphide (H_2S) production from organic sulphur compounds is of differential value for *Xanthomonas* genus. H_2S production from peptone is usually used but cysteine has been found to give results that are more consistent. For this reason cysteine was employed in this test (Cerny, 1976). The procedure recommended by Dye (1968) was followed. The medium contained (in gL^{-1}): $NH_4H_2PO_4$ 0.5; K_2HPO_4 , 0.5; $MgSO_4$, $7H_2O$, 0.2; Ncl, 5.0; yeast extract (Difco) and 5; cysteine hydrochride, 0.1. The medium was thoroughly mixed before dispensing 5 mL quantities into tubes and then autoclaved and allowed to set. A lead acetate strip was prepared by immersing cut filter paper strips in 5% lead acetate, air dried and autoclaved. The lead acetate strip was suspended over the medium after inoculation and held by a screw cap and allowed to stand for 14 days. Thereafter which the medium was observed for change in colour or reaction of the paper strips.

Acid production from carbohydrates: The following two media developed by Dye (1968) which were modified by Hayward (1979) were employed. Medium A: 1% peptone water containing bromo-cresol purple (0.7 mL of a 1.5

ethanol solution per mL of the medium) filtered carbon source (sucrose, arabinose, cellobiose, rhmnose, innositol, salicin, or sobitol) was added asceptically at 1% concentration after autoclaving. Medium B: This contained (mg L^{-1}): $NH_4H_2PO_4$ 0.5:, KH_2HO_4, 0.5; $MgSO_4$. $7H_2O$, 0.2; NaCl, 5; yeast extract (difco), 1.0, agar, 12 and bromocresol purple (0.7 mL of 1.5% ethanol solution). The pH of medium adjusted to 6.8 with NaOH before autoclaving.

After autoclaving carbon source in form of sucrose (or any other) was added to the molten medium and sloped to set (Hayward, 1964; Dye, 1968). Inoculation of the test pathogen was carried out by having duplicate tubes of the medium stabbed to the base with the test bacterium picked on a straight inoculating wire; one of the two tubes was then sealed with about 3 mL of paraffin oil, whereas the second is left open; also another tube (control) without inoculation. The tube were incubated at 28°C and examined daily for 14 days and their pH (rise or decrease) recorded which might indicate either acid or alkali production occurred in the open tube.

Effect of varying temperature on the growth of bacteria: Yeast Salt (YS) containing (mg L^{-1}) $NH_4H_2PO_4$, 0.5; K_2HPO_4, 0.5; $MgSO_4$. $7H_2O$, 0.2; NaCl 5.0 and yeast extract (Difco), 5.0 was used. A loopful of the test bacterial suspension from a fresh culture of about 48 h was inoculated in tubes containing about pf YS broth as described before. The inoculated tubes were then incubated in water bath at diffeent temperature regimes (25, 30, 35 and 40°C) for 7 days, after which the tubes were assessed for growth using haemocytometer to determine the number of colony and thus the percentage growth of the bacterium.

RESULTS AND DISCUSSION

Isolation of bacterium: The preliminary studies of the bacterium isolated from the diseased leaf, seed and fruit under a phased contrast of the microscope showed that the bacteria isolated from leaf, seed and fruit were the same organisms; rod shaped motile polar flagellate bacteria. Examination of the motile nature of the bacteria under the microscope using the hanging method showed motility, which is characteristic of polarly flagellate bacteria (Hayward, 1983).

Seedlings inoculation: The inoculation of the tomato seedlings with inoculum prepared from the infected leaf, fruit and seed produced black spot symptoms on leaves, stems, branches and fruits. However, the control seedlings sprayed with distilled water did not show any black spot symptoms. On inoculation of the leaves with the isolate from the leaf spot after 7 days, the leaves produced bacterial spot symptoms. Infected leaves showed a typical symptom by the bacteria days of inoculation. The symptoms observed include small yellowish green, water-soaked spots on surface of young leaves after four days. Spot became slightly raised on the lower leaf surface with corresponding slight depression on the upper side of the leaf. On the older leaves, spots were faint dark green and water soaked. Lesions later darkened and eventually tuned brownish black. Later lesions appeared sunken and greasy on the upper leaf surface with translucent center and black margin. With time, the center of the spot dried and proved to tear under pressure of raindrops, subsequently leaf lets later turned yellow and dropped prematurely.

Fruit inoculation: Initial water soaked spots were found on green immature fruits, which later appeared as black raised spots surrounded by narrow greenish white halo in time, the raised spots developed into scabby, tan to brownish black and sunken lesion although, spots occurred on green immature fruits, no such symptoms were found on the mature or ripe fruits in this investigation.

Seed inoculation: Tomato seeds artificially inoculated evidence by bacterial spot bacterium. The bacterium re-isolated showed symptoms of bacterial spot when inoculated on young seedlings.

Pigmentation and colour of colony: Several different colony pigments and colour were encountered in the non-selective medium (SPA) used. However, the most frequent and consistent were the yellow, water-soluble pigmented colonies, which were circular, smooth, domed and mucoid. These pigments according to Starr *et al.* (1977) have been identified as *xanthomonadins*. The mucilaginous extra cellular slime consists of an unusual hetero-polysaccharide known as xanthan gum. The mucoid nature of the colonies of *Xanthomonas* on SPA or other media with high percentage of available carbohydrate is a characteristic feature of the genus and differs from other colonies of other genera such as *Pseudomonas* on the same medium (Table 1).

On the selective medium (SX), the test bacterium developed as small translucent colonies (0.1 mm) surrounded by a clear zone after 3 days at 30°C. By the fifth day, colonies were about 3 mm in diameter mucoid; glistering, convex circular surface with entire margins became translucent with purple centers. This served as an additional diagnostic feature of the group xantomonads.

Table 1: Cultural and biochemical test for identification of *Xanthomonas campestris* pv. *vesicatoria*

Test	Result*
Cultural characteristics	
Colony colour	Yellow
Gram reaction	-
Motility	+
Xanthan gum production	+
Biochemical characteristics	
KOH solubility	+
H_2S production	+
Acid production from carbohydrate sources arabinose	+
Sucrose	+
Cellobiose	+
Trehalose	+
Rhmnose	-
Inulin	-
Adonitol	-
Ducitol	-
Inositol	-
Salicin	-
Sorbitol	-

* + = Positive result; - = Negative result

The gram reaction: The result obtained from young cultures after the necessary staining and careful examination under the microscope showed that the test bacterial colonies stained red purple but the reference culture, which was the control, strained blue-to blue black. Therefore, the red/purple stain was an indication that the test bacterium was a Gram-negative bacterium (Table 1).

Motility test: Examination of motile (active) bacteria in hanging drop preparation under microscope showed that the cells were polarly flagellate with single long polar flagella. The cells underwent a rapid, darting motility, which is a characteristic of polar flagellate bacteria. In Xanthomonads group a single flagellum is often present which may be several times the length of the cell from which it arises which is not true of Pseudomonads (Hayward, 1960; Fuerst and Hayward, 1969). With the Pseudomonad groups the cells are usually two to several flagella at one or both polar ends of the cells (Macnab, 1976).

Xanthan gum test: On SPA (sucrose peptone agar) medium, which was the preferred medium for this test, colonies produced some extracellular bacterial polysaccharides containing mannose. This was the characteristic constituent of xanthan gum, which was recognized by its characteristic yellow pigments, which have been reported to be due to the presence of a pigment, xanthomonadin which is diagnostic characteristic of the genus *Xanthomonas* (Starr *et al*., 1977).

KOH solubility test: The treatment of the test organism with potassium hydroxide solution (3% KOH) resulted in the production of a viscous solution, which was a slimy substance and when stirred with a loop followed the loop for about 2 cm when raised up from the solution. This was regarded as a positive reaction and is another characteristic of Gram-negative bacteria. This is a supplementary test to Gram-stain confirming that the test bacterium was a Gram-negative bacterium.

Hydrogen sulphide production: In the test for H_2S production, it was observed that after 3-7 days of inoculation the edges of the acetate paper strips turned black in time the whole paper strip turned to black. This reaction was a result of H_2S reacting with lead acetate forming a black lead sulphide, which discoloured the paper strips. This is an indication that H_2S was produced from cystein.

Acid production from carbohydrates: The tests conducted with various forms carbohydrates and their results are shown in Table 1. The change in colour from blue to yellow was regarded as a positive result since acid was produced from such carbon source. The carbohydrate sources tested that proved positive included arabinose, sucrose, cellobiose and trehalose. However, no acid was produced from rhmnose, inculin, adonitol, adonitol, ducitol, salicin and sorbitol in addition, no acid production from the control tubes, which had no carbon source or the tube sealed with paraffin.

Physical characteristics

Effect of varying temperature on growth of bacterium: The results obtained from the effect of four temperature regimes on the growth of the black spot bacterium are presented in Fig. 1. The best colony growth (95.67%) was attained at temperature 30°C, which was significantly higher than the percentage growth obtained at 25°C (33.33%). While the value (20.00%) obtained at temperature 35°C was significantly lower than that at 30 and 25°C. However, the minimum value (5.33%) was recorded at 40°C.

Cultural tests: The initial diagnosis made was based on the microscopic studies which indicated that the organism was a rod shaped, non-spore forming Gram negative and motile with a single flagellum. Further investigations revealed that the pathogen on Sucrose Peptone Agar (SPA) produced colonies that are circular, smooth, doomed and mucoid. These are distinguishing characteristics of the *Xanthomonas* from other related group such as the pseudomonad (Dye and Lelliot, 1974). Further pigmentation and colour test revealed a non water-soluble yellow pigments which agree with

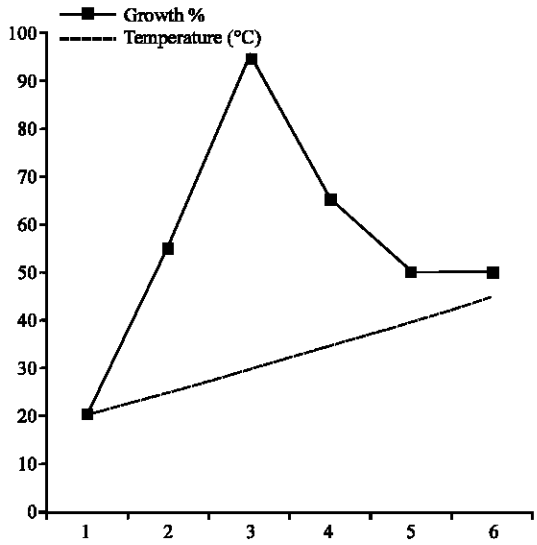

Fig. 1: Effect of varying temperature on growth of *Xanthomonas* campestris

Starr *et al.* (1977) report that *Xanthomonas* in SPA media produced yellow pigmented substance consisting of a diffusible brominated aryl octanes, which they identified as *xanthomonadins* a unique diagonostic feature of the *X. campestris*. These pigments according to Hayward (1983) are not carotenoids as was once thought. Pigmentation in the genus *Xanthomonas* is found to be variable with age of culture and nutritional status and sometimes with the organism. For instance, a yellow diffusible pigment is produced optimally by *Xanthomonas camprestris* pv. *vesicatoria* on GYCA and SPA than in other media. Similarly, the intensity of the yellow colour vary with organism. For instance, X. *campestris* pv. *pruni* and pv. v*itians* are frequently paler than pv. *vesicatoria*. Albino forms also occur, in which colonies are white e.g., *X. campestris* pv. *mangiferaeindicae* and pv. *manihotis* (Moffet and Croft, 1983).

Yellow or orange pigmentation are also characteristic feature of the saprophyte, *Erwnia herbicola* but the pigments do not contain hetro-polysaccharides instead they contain carotenoids. However, a unique characteristic and diagonistic feature of the *Xanthomonas* is the production of this mucilaginous extracellular slime consisting of an unusual hetero-polysaccharide known as xanthan gum (Starr *et al.*, 1977); this was found to be positive in the isolate under study. Xanthan gum has a large-scale application in the food, cosmetic and pharmaceutical industries. For instance xanthan gum is used in sugar industry as a gelling agent and as an important polysaccharide in production of mannose and some phosphate sugars (Bilanovic *et al.*, 1999). The rapid darting motility of the test organism as was observed in the motility and flagellation studies is a characteristics of single polar flagellate bacteria (Schaffer, 1965). These are distinguished from the peritrichous flagellate bacteria based on a relative slow transitional movement accompanied with chaotic tumbling in the peritricious flagellate bacterium. Another feature of the polar flagellate bacteria that distinguished them from the peritricious flagellate cells is the abrupt double reversal, i.e., they briefly reverse direction before returning along the same path. This abrupt reversal does not appear within the peritrichous bacteria. Instead according to Macnab (1976), the peritrichous flagellate cell motility involves many changes in direction due to vibration from different side flagella. Macnab (1976) further observed that the mean velocity of two polar flagellate bacteria such as *Xanthomonas* and peritrichous flagellate cell such as S*almonella typhimurium* was 50-60 and 30 μ sec^{-1}, respectively. Again in polar flagellate bacteria the number of flagella per cell is of diagnostic significance (9; 15). In this study *Xanthomonas* was found to be polar flagellated. According to Hayward (1983) when single flagellated, the flagellum be several times the length of the cell from which it arises, while in pseudomonades the cell usually have one several flagella at one or both poles of the cell. If single flagellum the pseudomonad is said to posses as sheathed flagellum (Fuerst and Hayward, 1969; Hayward, 1983).

Biochemical and physiological characteristics: In the physiological test, the bacterium hydrolyzed starch by forming digestion zones. The ability of *Xanthomonas* to digest starch is a diagnostic characteristic of the *campestris* pathovars. Glucose and other carbonhydrates are metabolized oxiadatively, nitrite was not produced from nitrite (nitrite not reduced to nitrite), test for catalase are positive and oxidase negative and hydrogen sulphide is produced from cystein.

Although, these characteristics are not considered sufficient enough to differentiate members of the genus *Xanthomonas* from the genus pseudomonas (Dye and Lelliott, 1974). However, the mucoid nature of colonies of *Xanthomonas* on SPA or other media with a high percentage of available carbonhydrate is characteristics of these organisms and differs from colonies of pseudomonas on the same medium other identification test include inability to produce acid from rhamnose, inulin, adonitol, dulcitol, inositol or salicin and acid not produced in purple milk (Dye and Lelliott, 1974). The *X. campestris* parthovar isolated from tomatoes was found to be pathogenic. The parthogenicity test carried out of the organism were positive on tomatoes seedlings. Typical bacterial spot symptoms, which were not rapid at the on set but took several days or more than a week to appear was observed on the seedlings. This positive

pathogenicity test was therefore regarded as a proof that the pathogen causing bacterial spot is *Xanthomonas campestris* pv. *vesicatoria* (Doidge) (Dye, 1968).

Enzyme assay tests conducted showed that the pathogen produced aminopeptidase enzymes and hydrolases (which hydrolyzed starch) and this is considered as diagnostic characteristics of campestris parthovars however, nitrite was not reduced to nitrite by the bacterium, which suggests that the tests bacterium does not contain the enzymes nitrite reductase (Stanier *et al*., 1966). Respiration was found to be oxidative or respiratory metabolism but protein digestion and oxidase tests are negative.

Growth of *Xanthomonas* at varying temperature: Subjecting the bacterial spot bacterium to four different temperature regimes (25, 30 and 40°C) in attempt to determine the optimum and the minimal temperature of the bacterium showed that increasing the temperatures from 25-40°C the bacterium growth increased from 33.33% at 25°C to 75.67% at 30°C, however, increasing the above 30°C resulted in sharp decline in the growth of the bacterial colony to 20% at 35°C and finally to 5.33% at 40°C this shows that optimum growth of the bacterium occurred at 33°C and where as the minimum growth at 40°C *Xanthomonas campestris* in this study was found to be temperature specific maintaining an optimum temperature of 30°C below or above. This agrees with the research of Nascimento *et al.* (2005) in which they observed the growth of *Xcv* from 5-35°C with optimum growth from 27-29°C and that *Xcv* did not grow at zero and 40°C.

CONCLUSION

This study shows that the bacterium isolated from bacterial spot disease of tomato is a Gram-negative yellow aerobic rod with single polar flagellum. The bacterium exhibited strong starch hydrolysis, metabolized glucose oxidatively but nitrate was not reduced to nitrite and acid production was positive for arabinose, sucrose and cellobiose but negative for nulin, ducitol or sorbitol. The identity of the pathogen based on some cultural, physiological and pathogenic tests conducted was identified as *Xanthomonas campestris* pv. *vesicacatoria* (Doidge) (Dye and Lelliot, 1974) and it is temperature specific (27-29°C).

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