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Research Paper

SCANNING ELECTRON MICROSCOPY OF *Xanthomonas phaseoli* PV. *manihotis*, THE CAUSAL AGENT OF CASSAVA BACTERIAL BLIGHT DISEASE

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Abstract

A survey was carried out in eight regions of Ghana to assess the incidence of *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*), the causal agent of Cassava Bacterial Blight (CBB) disease of cassava. Samples of cassava plants showing suspected symptoms of CBB were collected for isolation of the putative pathogen. The pathogen was isolated by surface sterilizing the plant parts using 70% ethanol and cultured on semi selective Cefazolin trehalose agar (CTA) and incubated at 27°C for three days. The general colony morphology of the cultured bacterium was examined by using scanning electron microscope (SEM) after fixing the spacemen strips on the stubs and following standard procedure for biological sample preparations for SEM. Scanning electron micrographs revealed fine, smooth, straight rod structures with varied sizes of individual cells ranging from 1.3 – 2.0 µm long and a width of 0.3 – 0.8µm. To fulfil the Koch's postulate, pathogenicity test was carried out by inoculating healthy cassava seedlings and the results confirmed that, the isolated pathogen was responsible for causing CBB disease in the infected cassava samples collected from the various zones of Ghana.

Key words: Scanning Electron Microscope, *Xanthomonas phaseoli* pv. *manihotis*, Pathogenicity, Cassava Bacterial Blight, Cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) which is in the Euphorbiacae family is an important staple crop and plays a vital role as a food security player in drought and famine prone

areas of the world. It serves as a major source of staple food in which millions of low income consumers, especially in sub-Sahara Africa depend for their daily calories [1, 2].

The cassava crop's ability to provide high and stable yield depends on the ability of the farmer to produce disease free crop. Cassava Bacterial Blight (CBB) disease is one of the major threat to cassava production, especially in Ghana. The disease is caused by a vascular, Gram negative rod *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) [3], formerly known as *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) [4]. The disease was first described in Brazil in 1912 [5], but has now assumed widespread occurrence and can be found in almost every part of the globe, including Ghana. The pathogen has now been blacklisted as a quarantine pathogen by the international phytosanitary quarantine department. The bacterium has a single polar flagellum and shares common biochemical and physiological characteristics of the genus Xanthomonads, except that it does not produce pigmented colonies [6, 7]. Infection of cassava plant produce characteristic symptoms that include the formation of angular leaf spots, wilting and blighting of leaves and stems, necrotic tissues, cankers on the stem, oozing of exudates from stems and petioles as well as die-back of the stem [8]. Losses of 100% in cassava yield and planting materials as a result of this vascular pathogen has been reported [9, 10]. In order to minimize yield losses of cassava, proper identification of the causal agent of bacterial blight disease, which in this case, *Xanthomonas phaseoli* pv. *manihotis* (*Xpm*) is paramount.

There are a number of methods available for the identification and detection of *Xpm*. These methods include: PCR assays [11], amplified fragment length polymorphisms (AFLP) [12], dot-blot hybridization [13], ELISA test, random amplified polymorphic DNA (RAPDs) [14] and restriction fragment length polymorphism (RFLPs) [15] among others. Of all the available methods use in the identification and detection of *Xpm*, we did not come across any information regarding the use of scanning electron microscope (SEM) in the identification of this pathogen.

There are two basic modes of electron microscopy, these include scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and each of them utilizes specific developed microscopes. SEM is one of the most versatile tools available today for investigating the chemical composition and microstructure morphological development of outgrowth of fungi spores, yeast, bacteria among other organisms [16,17], as well as make some cell wall architecture comparism of surface features of

spores [18,19]. The tool is used to examine bacteria that have been dehydrated or dried and fixed. It helps to also reveal detail characteristics of bacteria in their natural habitat. The SEM technique is relatively easy to carry out and the results are also easy to interpret, because the image of the target spacemen is shown as a three-dimensional structure [20]. Due to the limited information regarding the use of SEM in the identification of *Xpm*, the objective of this study was to identify *Xpm*, the causal agent of CBB disease in cassava using SEM technique.

2.0. MATERIALS AND METHODS

2.1 Sample collection

Cassava plant samples, both symptomatic and asymptomatic leaves and stems were collected from different ecological zones of Ghana. The samples were collected during 2013/ 2014 farming season. In all, eight regions were visited. The GPS of the locations where the samples were collected and confirmed to be present were accurately mapped as follows: Volta (N 6°39′31.716″, E 0°33′ 29.0232″, N 6°33′6.3108″, E 0°39′39.9168″); Ashanti Region-Semi-deciduous forest (N 7°14′7.4616″, W 1°24′12.492″, N 7°14′5.8056″, W 1°24′10.854″); Eastern-Semi deciduous forest (N 5°57′16.2216″, W 0°6′54.4464″); Greater Accra-Coastal savannah (N 5°56′43.9368″, W 0°5′16.3464″, N 5°45′55.3212″, W 0°19′35.472″) and Brong-Ahafo Region-Transitional zones (N 7°44′ 49.56″, W 2°5′45.5532″, N 8°4′45.6492″, W 1°42′ 21.9168″). The samples were well packaged and sent to a laboratory for isolation of the suspected pathogen.

2.2 Isolation of putative bacteria from cassava samples

The putative pathogen was isolated from both symptomatic and asymptomatic tissues. The method of isolation of the pathogen was as described by [21]. About 3 mm^2 of the cassava tissues were surface sterilized in 70% ethanol and macerated in 1.5 ml Eppendorf tubes after allowing to settle for a few minutes, aliquot was then streaked on *Xpm* semi-selective medium - Cefazolin trehalose agar (CTA) with constituent components/liter of 3.0 g of K2HPO4, 1.0 g of NaH2PO4, 0.3 g of MgSO4·7H2O, 1.0 g of NH4Cl, 9.0 g of D (+) - trehalose, 1.0 D (+) - glucose, 1.0 g of yeast extract and 14.0 g of agar as described by [22]. The plates were incubated at 27 °C for the observation of the bacterial colony growth. The colonies were further purified and stored at – 80 ^oC for SEM analysis.

2.3 Morphology of bacterial isolates

2.3.1 Preparation of the bacterial isolates for SEM

Scanning electron microscope was the main tool employed for the morphological identification of *Xpm* from the infected cassava samples. The stored bacterial isolates extracted from the infected cassava tissues were freshly grown in CTA media and the bacterial strips fixed on stubs for SEM analysis prepared as described by Evrik *et al*. [23], with little modifications, using Hayat [24]; Haberman *et al*. [25, 26] protocols.

2.4 Data analysis

The scan SEM images were cropped to zoom the fine morphological structure of the pathogen with the appropriate measuring scale using Microsoft word for the storage of the captured images.

Pathogenicity confirmation of the scanned pathogen

The pathogenicity test of the putative isolates extracted from the infected cassava leaves and stems were tested on healthy Esam cassava seedlings sourced from Adidwan in the Ashanti region of Ghana. The cassava seedlings were inoculated with the prepared bacterial suspensions after checking the concentrations at approximately 1×10^8 CFU/ml- OD600 (OD = 0.5 at 600 nm) with a spectrophotometer (Eppendorf Biophotometer 6131, Germany). Water inoculated cassava plants served as positive control. The experiment was maintained in a greenhouse (30 °C), for observation of disease symptoms. The severity of the disease was scale according to [27] reference scale where disease severity (DS): 1 = no symptom; 2 = only angular leaf spots; 3 = angular leaf spots, wilting, blighting, defoliation, and sometimes exudates on stems/petioles;

4 = blighting of leaves, wilting, defoliation and exudates.

Beside the pathogenicity test, the isolates were also subjected to molecular characterization using Real-time PCR diagnostic tool with designed LNA probe and primer, based on the variable number tandem repeats (VNTRs) sequence of the pathogen (data not shown) to confirm the true identity of the scanned pathogen.

RESULTS AND DISCUSSION

In all, thirty-one (31) isolates were recovered from the collected field samples from five out of eight regions visited. The regions included: Ashanti, Brong- Ahafo, Eastern, Volta and Greater Accra regions of Ghana. The growth of the bacteria in the semi selective

CTA media after incubation at 27 °C produced whitish-grey to cream, raised, convex, mucoidal, smooth and shiny colonies, which initially look hyaline, then becomes turbid with time. The colony growth on the media ranges in diameter sizes of 1 to 3 mm as shown in Figure (1). This is similar to the findings as reported by [28].

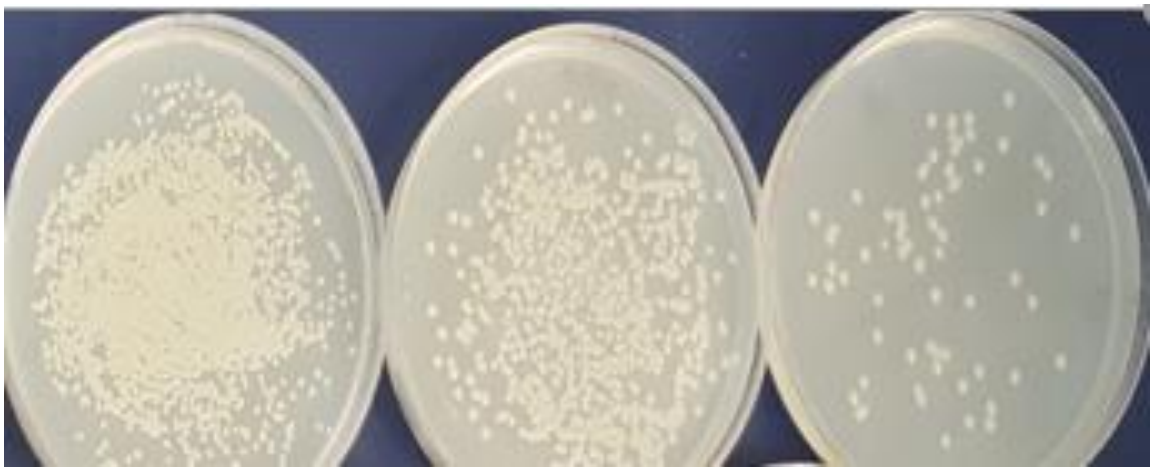

Figure 1. *X. phaseoli* pv. *manihotis* colony growth on Cefazolin trehalose agar

The resultant SEM micrographs revealed fine, smooth, straight rod structures with varied sizes of individual cells, ranging from 1.3 – 2.0 µm long and width of 0.3 – 0.8µm. A scale bar on the image shows the size of the target pathogen with great accuracy. SEM captured images are usually shown as monochrome grayscale digital images consisting of varying black and white colours depending on the pixel intensity, Figure (2). The SEM as an identification tool has widely been employed in the field of microbiology in investigating surface structures of biological materials as well as measure microbial cell attachment and their morphology. The tool can be used for viewing things that are perceived invisible in the microspace world by utilizing beams of electrons that can expose the complex structure of material that is not readily accessible by light microscopy. It can magnify a mounted object from about 10x up to 300,000x. SEM as a diagnostic tool has comparative advantage over the light microscope (LM) in three key areas as follows: 1. Resolution at high magnification: the possible best resolution for LM is about 200 nm whereas SEM has a resolution of better than 10 nm; 2. Depth of field: SEM can be used to view spacemen images in three dimensional (3D) appearance, which is not possible with LM. 3. Microanalysis: SEM can also be used to analyze the chemical composition of the sample as well as other parameters such as electrical, crystallographic and magnetic characteristics of the target pathogen.

SEM uses two types of signals in viewing the surface of materials. These signals include secondary and backscattered electrons. These two signals are continually being generated from the exterior of the spacemen when subjected to electron beams, but they appear as two independent types of interaction. Secondary signals are produced as a result of inelastic collision and scattering of incident signals with target specimen signals. They are commonly distinguished by having energies less than 50 eV. The signals are used to show the exterior structure of the target material using a resolution of ~10 nm or better [29]. The backscattered electrons are products of an elastic collision and scattering action between incident signals and the target specimen nuclei or electrons. The signals under this category can also originate from the target material surface and can play a vital role in resolving topographical and atomic number disparity with a resolution of >1 micron.

The only major limitation of SEM method is its inability to provide genetic information about the target organism and for that reason, Genetic analysis of the pathogen was taken care of by the Real-time PCR using *Xpm*-VNTR specific primer and LNA probe (Results not shown) to confirm the scanned pathogen.

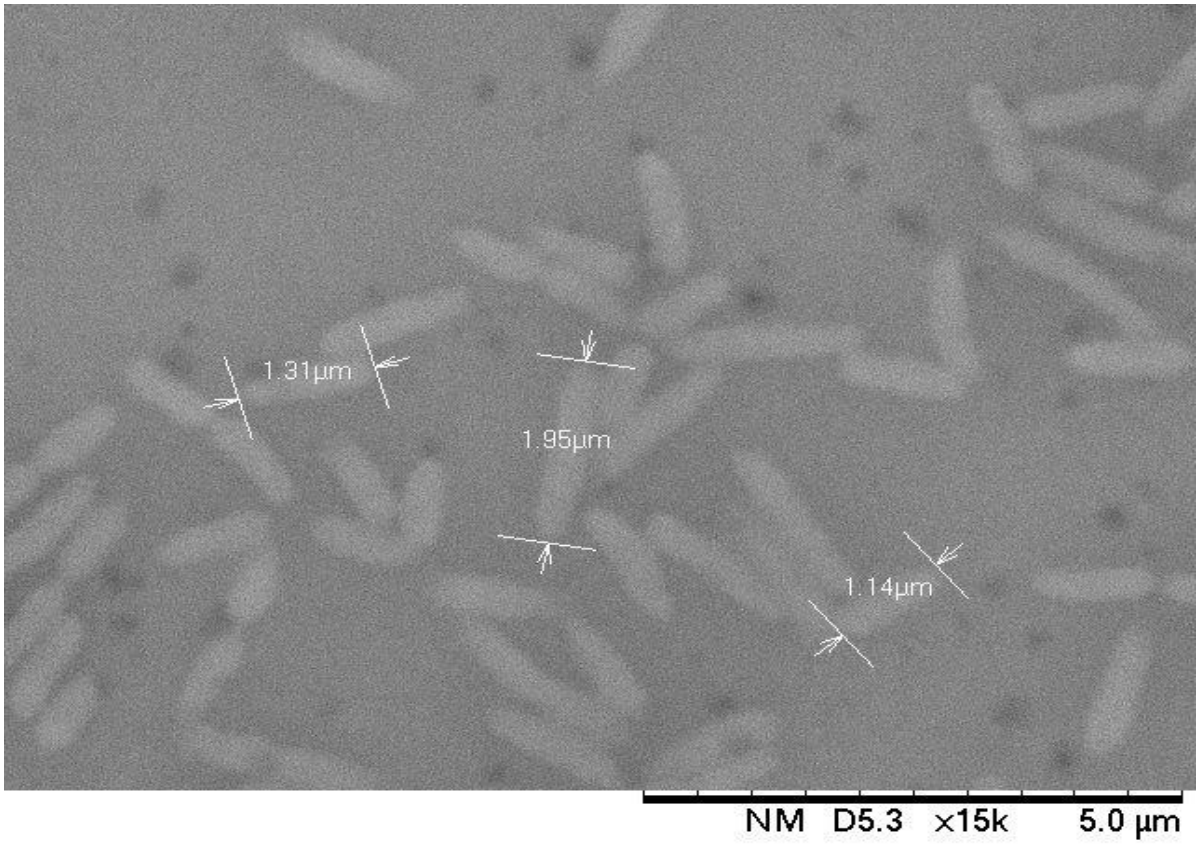

Figure 2. Scanning electron micrograph of *Xanthomonas phaseoli* pv. *manihotis* structure

To further confirm whether the scanned pathogen isolated from the cassava tissues using SEM, was the actual causal agent of the blight disease in cassava, and also to satisfy the Koch's Postulate, pathogenicity test of the isolates was confirmed on the susceptible Esam cassava variety. The isolates produced a number of symptoms on the inoculated cassava seedlings that included angular leaf spots, blighting of leaves, necrosis of the stem and leaves, wilting and exudates on the petioles and stems as shown in Figure (3). These symptoms are similar to those reported by Leuschner *et al*. [30]; Lozano [31] and Maraite [8] on inoculated cassava plants. Based on the reference scale used, there were no differences in the severity among the various isolates. The pathogen was successfully re-isolated from the infected cassava parts and thereby satisfying the above disease postulate.

Figure 3. Symptoms of cassava bacterial blight disease on inoculated cassava seedlings

In a similar work, pathogenicity test of *Xanthomonas phaseoli* pv. *manihotis* was used to confirm this pathogen as the causal agent of blight disease in cassava cultivars in DR-Congo [32]. This vascular pathogen is a serious threat to cassava production in Ghana, where farmers have little information about their control. Control and management of this pathogen are paramount to increase cassava yield production with a corresponding

increase in the livelihood of poor farmers. Before these control measures are initiated, proper identification using good and reliable diagnostic tool is needed and in this case SEM tool was employed in this study in revealing the fine structure of *Xpm*, which is widely known to limit tuber yield and planting materials to Ghanaian farmer. Although cassava yield losses as a result of this pathogen has been remarkably reduced due to the recent introduction of cassava resistant varieties, the problem is far from over because, farmers in Ghana have a high preference to the local cassava varieties which are pruned to this disease. By the way of recommendation, Ministry of Agriculture (MoFA) of Ghana must intensify their education to farmers about the dangers of this pathogen and farmers must also be advised to use resistant variety in place of susceptible local variety.

CONCLUSION

Morphological characteristics of *Xanthomonas phaseoli* pv. *manihotis* causing blight disease in cassava in various agro-ecological zones of Ghana have been examined using scanning electron microscope. The analysis of the pathogen by SEM showed fine, smooth and rod-shaped structure, which was later confirmed as the true causal agent of cassava blight disease in Ghana using follow-up methods such as pathogenicity test and Real-time PCR. To the best of our knowledge, this is the first study of *Xpm* using scanning electron microscope.

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