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Survey and Molecular Identification of *Xanthomonas euvesicatoria* Causing Bacterial Spot of Tomato in Blue Nile State in Sudan

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Abstract: This study aimed to survey, characterize, and identify the causal pathogen of tomato bacterial spots (TBS). The survey evaluated the disease incidence and severity on tomato cultivated in Blue Nile State during the autumn season 2015. The surveyed sites were in Alrosseris and Aldmazein localities. Four farms were selected from each locality. Symptomatic leaves were collected and sent to plant pathology laboratory, Department of Crop protection, Faculty of Agriculture, University of Khartoum for detection of the pathogen. Pathogenicity of the isolated bacteria was confirmed by relevant test. The pathogen was isolated and characterized morphologically, biochemically and at molecular level. The survey revealed that there was no significant difference in disease incidence within each locality, while there were significant differences ($P \leq 0.05$) among locations within both localities in disease severity. The disease incidence was remarkably high in Alrosseris and Aldamazein which recorded 49.27 and 52.17 %, respectively, while the disease severity was 28.28 and 30.32%, respectively. The results showed that the isolates were rod shaped, obligate aerobic, Gram negative, catalase

positive, oxidase negative, and were able to hydrolyse starch and gelatine, yellow colonies on nutrient agar and Yeast Dextrose Calcium carbonate (YDC) media. In the pathogenicity test, the bacterium known as *Xanthomonas vesicatoria* was confirmed to be as *Xanthomonas euvesicatoria* by PCR and sequence analysis of the 16S RNA gene (Gen Bank Accession No's MH047248, MH051263 and MG686236).These results also indicate that the tomato plants grown in Blue Nile State were highly infected by the bacterial leaf spots. Further studies should be considered to determine potential yield loss caused by the pathogen, as well as control strategies to limit the spread of this pathogen. To our knowledge, this is the first report of *Xanthomonas euvesicatoria* causing bacterial spot disease in tomato in Sudan.

Key words: *Xanthomonas*, tomato, leaf spot, survey, molecular identification, disease incidence, severity

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae, which includes more than 3000 species, occupying a wide variety of habitats (Knapp and Peralta 2016). In Sudan tomato is grown all around the country as a winter crop (main season) and as an off – season crop during autumn. An estimate of 124.5 million tons of tomatoes are produced annually throughout the world, making it one of the ten most important vegetables in modern history (Rashid *et al.* 2016). It is the second most important vegetable after onion. The tomato cultivated area in Sudan was about 42000 hectares in season 2014 and the total number of the greenhouses which grow tomato in Khartoum State are 800 houses in the year 2011. The total production of tomato in Sudan in 2014 was about 612000 tons (FAO 2014).

Tomato plants are attacked by many diseases and pests in Sudan. The important diseases of tomato in Sudan include: seedling damping-off, tomato yellow leaves curl virus (TYLCV), powdery mildew, late blight and Fusarium wilt (Juha 2009). More recently , it has been shown that bacteria belonging to four distinct groups (previously designated A , B ,

C , and D) cause bacterial spot on tomato and pepper (Jones *et al*. 2004),*i.e.* X. *euvesicatoria* group A, X. *vesicatoria* group B, X. *perforans* group C and X. *gardneri* group D. Group A is particularly more aggressive against pepper (Ignjatov *et al*. 2010). No strains in group C have been found in pepper. However, strains from all four groups have been isolated from tomato (Jones *et al*. 2004).

Bouzar *et al.* (1994) reported that *Xanthomonas campestris* pv. *vesicatoria* was the causal agent of bacterial spot of tomato and pepper in central Sudan. The bacterial spot affects all aerial parts of the plant. Yield reduction caused by the disease is a result of the direct infection on the photosynthetic leaf area, the drop of buds and flowers and reduction in commercial fruit quality (Araújo *et al.* 2012). Profits also decrease with the cost of chemical control. Infected seeds, volunteer crop plants and diseased plant debris may serve as inoculum sources of the disease. The estimated total marketable yield losses were 17 and 52 % due to late and early bacterial spot infections, respectively. The disease also reduces seed germination and causes defoliation and fruit losses (Kebede *et al.* 2013).

Tomato cultivated in Blue Nile State as one of the important autumn season crops, and due to spread of the bacterial leaf spot disease, this study was initiated to identify the causal agent. Therefore, the objectives of the study were to survey the main tomato production areas in Blue Nile State, to assess the incidence and severity of bacterial spot disease, isolate and identify the causal agent of bacterial leaf spots isolated from infected tomato plants using morphological, biochemical tests and molecular techniques.

MATERIALS AND METHODS

Survey and samples collection

Eight tomato fields were surveyed in autumn 2015 in two localities in the Blue Nile State. Four fields (Alrosseris, Algrif, Tiba and Bdoos) in Alrosseris locality and four fields (Alseriow, Abormad, Alregiba and Haroon) in Aldamazein locality were chosen. Fifty plants were randomly chosen from each field and inspected for the disease. Ten leaves were taken from each plant to calculate the severity. The diseased materials were sent to the laboratory of plant pathology, Department of Crop Protection, Faculty of

Agriculture, University of Khartoum for isolation of the pathogen and its characterization.

Disease incidence and severity

The disease incidence (DI) was calculated based on the following formula:

$$\text{Disease Incidence} = \frac{Number\ of infected\ plants}{Total\ number of\ plants\ assessd} x100$$

Disease severity was estimated using scale from 1-5according to Rao *et al*. (2016) as follows (Table 1):

Table 1. Rating scale used for assessment of the disease severity:

Rating Scale	(%) of Disease severity
1	1-5
2	5-25
3	25-50
4	50-75
5	75-100

The percentage of disease severity (PDS) was then calculated as follows: -

Percentage of disease severity (PDS) =

$$\frac{Number\ of\ individual\ Rating}{Number\ of\ plants\ assesed} \times \frac{100}{Maximum\ Scale}$$

Isolation and maintenance of the pathogen

Symptomatic tomato leaves were collected and sent to Plant Pathology Laboratory, Faculty of Agriculture, University of Khartoum. All materials used in this experiment were sterilized using ethyl alcohol. The diseased parts of leaves were washed under running water and cut into small pieces. They were surface sterilized with 0.5 % NaOCl for 30 sec, and rinsed three times in sterilized distilled water and blotted dry on sterilized filter paper. Specimens were soaked for 2 hours in normal saline and serial dilutions were made. One ml was taken from the last three dilutions and spread over Nutrient agar medium (NA). The inoculated plates were incubated at 25-30°C for 5 days. Well isolated single colonies were picked and streaked slant on nutrient agar and kept in the refrigerator for further use.

Identification of the pathogen

Morphological and Cultural Characteristics

The bacterium was isolated and characterized morphologically, biochemically and physiologically according to (Rafi *et al.* 2013), viz (Gram staining reaction, motility test, colony characteristics on nutrient agar (NA) and yeast dextrose calcium carbonate (YDC) media, potassium hydroxide (KOH) solubility test, aerobic and anaerobic test, oxidase test, catalase test, starch and gelatin hydrolysis.

Pathogenicity tests

This test was carried out on young tomato plant with 4–5 true leaves. Susceptible cultivar Gold was used. Six days-old bacterial culture was suspended in sterile water. Inoculum concentration was estimated by the spectrophotometer and adjusted to 10^8cell/ml. Plants were spray-inoculated with bacterial suspension until runoff, and by injection into the leaves using sterile syringe. Equal numbers of plants were left uninoculated, or treated with sterile distilled water as a control. Inoculated plants were covered with plastic bags to maintain high humidity for 48 h after inoculation. Symptoms were checked within 3 weeks at 25°C. Symptoms developed were then observed, and recorded. The bacterium was re -isolated and identified to confirm positive pathogenicity test.

DNA extraction, PCR amplification and sequencing

The bacteria were cultured in NA for 48-72 h at 28°C. DNA extraction was performed following the protocol CTAB (Kebede *et al.* 2014). A loopfull of each isolate in 800µl of 2% CTAB (DNA extraction buffer) was added to each tube separately. Extraction buffer (per 100ml) contained1ml CTAB (2.0g), NaCl (8.9g), EDTA (0.744g), with sterile distilled water (100ml), together with 5µl of mercaptanol was added separately to each tube. This mixture was incubated at 65ºC in a water bath for 5 min with intermittent shaking. After adding 600µl of chloroform: simile alcohol (24:1) the mixture was centrifuged at 13000 rpm for 15 min. The supernatant was transferred to other microtubes with equal volume of isopropanol and incubated at 20°C overnight. Next day, the mixture was centrifuged again at 14000 rpm for 20 min, the supernatant was discarded and the pellet was kept at room temperature to dry. Finally, the pellet was suspended in 100-200µl of distilled water and incubated at -20 °C for further usage. DNA concentrations were estimated by Nano drop apparatus.

The 16S rRNAgene was amplified using the universal primers fd1 5'AGAGTTTGATCCTGGCTCAG3'and rp2 5'ACGGCTACCTTGTTACGACTT 3' as described by (Weisburg *et al.* 1991). The PCR reaction was performed in a total volume of 100 µl reaction mixture containing 1X PCR Master Mix, 2µ M of each primer and 1 µl of template DNA. Lysates were incubated on ice for 5 min and centrifuged for 5 min at 8000 rpm The temperature profile was as follows: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 3 min; and final extension at 72°C for 15 min. PCR reaction was conducted in a Thermal Cycler 2720. .

PCR product was electrophoresed through a 1.5 % agarose gel (containing 3 µl ethidium bromide (10 mg/ml)) suspended in 1x TAE buffer (40mM Tris-HCl, 20 mm NaOAc and 1 mm EDTA, pH 8.5) for 30 minutes at a current of 42 Amps and a voltage of 100 V. The gel was assessed under UV for the presence of bands. Sequencing was done by BGI / Hong Kong, using

universal primer (FD1, rp2), sequence identity was determined using the BLAST from the NCBI website.

Statistical analysis

Data were subjected to Analysis of Variance (ANOVA) and means were separated by Duncan Multiple Range Test (DMRT) at 0.05 % level of significance. All statistical analyses were carried out using SPSS.20 computer software program.

RESULTS

Field Survey

The survey, conducted in the main fields of tomato in Blue Nile State displayed some characteristic symptoms on foliage and fruits. Typical symptoms were observed upon examination of tomato plants infected with bacterial spot TBS (Fig.1). The characteristic external symptoms of TBS observed on tomato plant consisted of a unique syndrome ranging from pistols to necrotic lesions consisting of rampant corky tissues on leaves, stems, fruits and twigs. The spot lesions on leaves and fruits were surrounded by water-soaked tissues and yellow haloes. Raised corky or erumpent lesions may appear on leaves with a prominent yellow halo surrounding the spot lesion.

Incidence and severity of TBS

The results shown in Figs. 2 and 3 indicate considerably, high TBS incidence was recorded in both localities. In the four sites of Alrosseris locality, the incidence percentage recorded was in Alrosseris (52.17 %), AL-Grif (46.45 %), Tiba (51.55 %) and Bdoos (46.92 %). In Aldamazein locality, TBS were in AL-seriow (56.86 %), Abormad (55.00 %), AI-regiba (50.40 %), and Haroon (45.42 %). No significant differences in disease severity were observed between localities as well as between sites.

Fig.1. Symptoms of bacterial spot of tomato on leaves and fruits

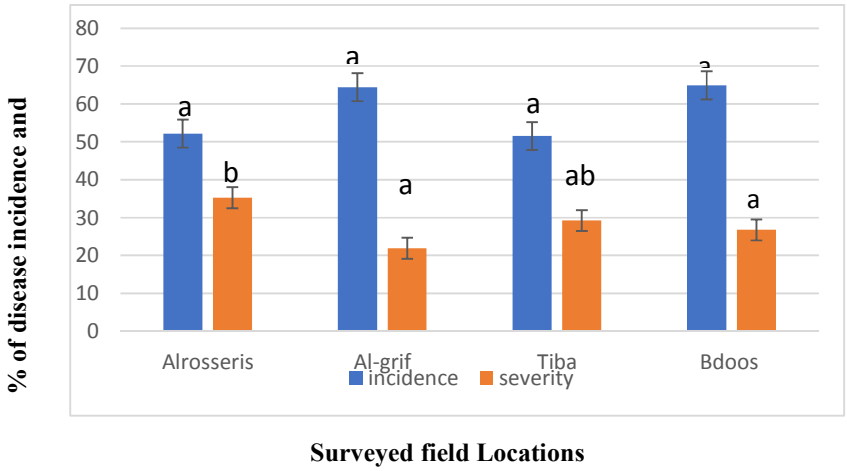

Fig .2. Mean disease incidence and severity in Alrosseris locality

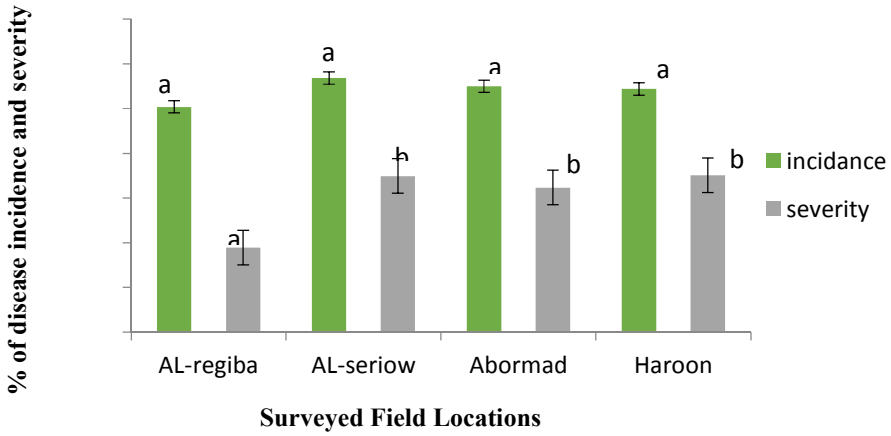

Fig. 3. Mean of disease incidence and severity in Aldamazein locality.

The TBS severity ranged from low to high values in the surveyed areas. The same trends in TBS severity levels were observed with respect to variations among localities. The disease incidence was 49.27 and 52.17 respectively in Alrosseris and Aldamazein, while severity was 28.28 and 30.32, respectively in Alrosseris and Aldamazein. In Aldamazein the least mean incidence of 45.42 % was recorded for Haroon, while the highest mean of 56.86 % was noted at Alseriow. Similarly, the least mean severity of TBS was 18.91 % recorded for Alregiba, while the highest (35.10 %) was recorded at Haroon. Significant differences in disease severity were observed between the sites.

Morphological and cultural characteristics of the pathogen

Three bacterial isolates were recovered from the diseased tissues and were characterized morphologically and biochemically. The results showed that the three isolates were rod-shaped, obligate aerobic, Gram negative, catalase positive, oxidase negative, hydrolyse starch and gelatine and show yellow colonies on nutrient agar medium and yeast dextrose calcium carbonate (YDC) medium (Table 2).

Pathogenicity tests

Leaf symptoms similar to those of the original plant symptoms were reproduced on the inoculated tomato plants within 3-14 days of inoculation. Symptoms on the leaves started as lesions and then developed to leaf spot (Fig. 4). No symptoms were developed on the uninoculated and those injected with sterile water only. Yellow bacterial colonies were reisolated from symptomatic plant tissues and they demonstrated identical cultural characteristics of the original culture of the isolates.

Molecular characteristics

Genomic DNA was isolated from the three *Xanthomonas euvesicatoria.* Purity and Quantity were checked by Nano Drop.

Polymerase Chain Reaction (PCR)

The DNA was amplified using universal primers (fd1, rp2), to confirm the identification of the pathogen. The PCR products of the isolates were run in

1.5 % agrose gel. Three bands were visualized from each of the samples. These were estimated to be in the region of 1500 bp (Fig.5).

Table 2. Biochemical and physiological characteristics of the bacterial isolates

Biochemical tests	Isolate1	Isolate 2	Isolate 3	*Xanthomonas vesicatoria* (reference)
Growth on NA	+ve	+ve	+ve	+ve
Gram reaction	-ve	-ve	-ve	-ve
KOH Solubility	+ve	+ve	+ve	+ve
Motility test	+ve	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve
Oxidase test	-ve	-ve	-ve	-ve
Starch hydrolysis	+ve	+ve	+ve	+ve
Gelatin hydrolysis	+ve	+ve	+ve	+ve
Anaerobic growth test	-ve	-ve	-ve	-ve
Growth on YDC	+ve	+ve	+ve	+ve
Pathogenicity	+ve	+ve	+ve	+ve

Sequencing

Based on the closest match of 16SrRNA sequences, all isolates from bacterial spot of tomato were compared with the sequence in Genbank. According to the BLAST search tool, the pathogen identified from the matched sequences is tabulated. Sequence analyses of the16S RNA gene remains one of the most reliable indicators for revealing the identity of the Pathogen. In this study, the generated sequencing data of the studied isolates, yielded unique matches for most isolates, with the Genbank sequence database. However, sequence similarity was observed of all isolates 100 % as *Xanthomonas euvesicatoria* (GenBank accession NOs MH047248, MH051263 and MG686236).

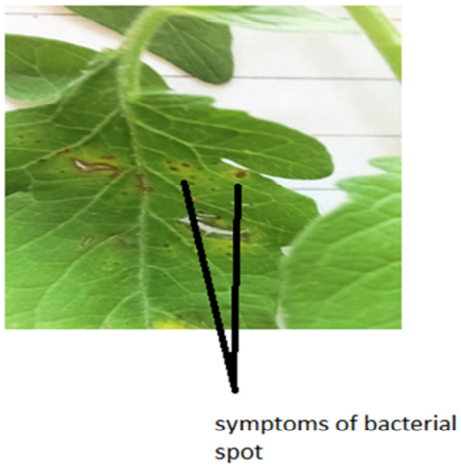

Fig. 4. Tomato leaf showing typical symptoms of bacterial spot.

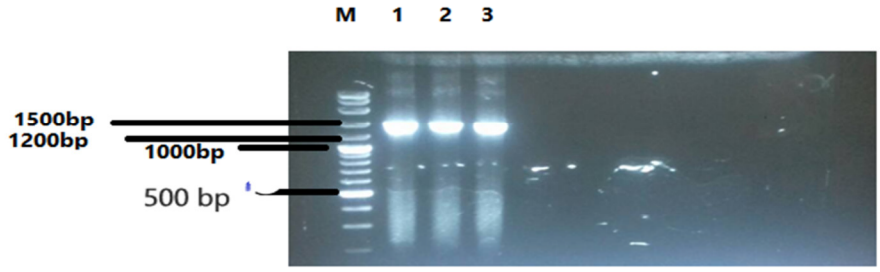

Fig.5. Gel electrophoresis (Agarose 1.5 %) of PCR products using universal primer fd1 rp2 designed to amplify a fragment of 1550bp. Lane M, 100bp DNA ladder marker; lanes1-3 tested samples.

DISCUSSION

The study was carried out to survey, isolate and identify the causal agent of leaf spot disease on tomato crop in Blue Nile State. The survey results of season 2015 (September – October) detected the occurrence of Tomato bacterial spot (TBS) in Blue Nile State. In the present study there was no significant difference in incidence between the locations (Alrosseris and Aldamazein). However, high severity was shown in Aldamazein locality compared with Alrossires. These results were probably due to the high humidity in Aldamazein which enhances the development of the disease. The same result was found by Bouzar *et al.* (2000) and Kebede *et al.* (2014) who found high severity of TBS in some fields in central part of Ethiopia from July to September 2011 and in September 2012. The detection, diagnosis and identification of TBS were investigated based on symptomatology, isolation,

pathogenicity test and molecular techniques. The symptoms of (TBS) displayed on tomato plants in different locations in Blue Nile State were typical of those described in the literature (Aiello *et al.*2013). According to our study based on the laboratory investigations and the molecular techniques the causal agent was identified as *Xanthomonas euvesicatoria*. *X.euvesicatoria* caused small circular dark brown spots, which were water-soaked, and approximately 3mm in diameter. The same symptoms were also mentioned by Jones *et al.* (2000). Similar symptoms were also observed by Itako *et al.* (2011) when tomato plants were inoculated with *X. euvesicatoria.* All isolates of bacteria from infected leaves, showed yellow, convex circular and mucous colonies, rod shape, and motile cells. These growth characteristics and microscopic observations provided preliminary information on the identification of *X. euvesicatoria*. Also these results were in agreement with a number of researchers (Rafi *et al.* 2013) who performed gram staining on *Xanthomonas* isolates from tomato, and found that all were Gram negative rods. In this study potassium hydroxide (KOH) solubility was positive which was in agreement with studies carried out by Muneer *et al.* (2007) and Rafi *et al.* (2013). Aerobic bacteria produce hydrogen peroxide during metabolism which must be broken down immediately as it is toxic to the cell as it damages the cell's DNA. Aerobes have the ability to synthesize enzyme catalase which breaks down H_2O_2 into water and oxygen. There was a positive catalase reaction on all the isolates tested which also concurred with those obtained by Gracelin *et al.* (2012) who tested for catalase reaction on *Xanthomonas* isolates from tomatoes. All isolates were oxidase negative, which was in line with the result obtained by Rafi *et al.* (2013). Also, all the isolates exhibited starch and gelatine hydrolysis property, which is in line with Mbega *et al.* (2012).

In this study, DNA was extracted by CTAB protocol from the bacterium causing TBS. DNA extraction resulted in pure DNA suitable for PCR amplification. The PCR produced 1500 bp. using the universal primers fd1 and rp2 (Weisburg *et al.* 1991).Such result is typical to that of Rashid *et al.* (2016) and Moretti *et al.* (2009) which were confirmed with PCR amplification with primers 16S of all bacteria isolated from the infected tomato plants and produced 1500- 1600bp.In this study, the generated

sequencing data of the studied isolates, yielded unique matches with GeneBank sequences database for most isolates. However, sequence similarity of all isolates was 100 %. These results were in agreement with (Rashid *et al.*2016).Several publications reported sequences of PCR primers for identification of *Xanthomonas* pathogens for tomato (Moretti *et al.*2009; Park *et al.* 2009).

This study, therefore, confirmed the presence of *Xanthomonas euvesicatoria* causative pathogen for leaf spot disease in the infected leaves and fruits of tomato in Blue Nile State, Sudan. These results also indicate that the tomato plants grown in Blue Nile State localities, were highly infected by the bacterial leaf spots.

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مسح والتعرف الجزيئي لزانثوموناس أيوفيزكتوريا*Xanthomonas euvesicatoria* المسببة لمرض التبقع البكتيري للطماطم بولاية النيل الأزرق في السودان

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[2]قسم الحجر الزراعي، ادارة وقاية النبات، وزارة الزراعة الاتحادية، ولاية النيل الازرق. السودان

[3]قسم التقانة الزراعية، معهد أبحاث النباتات الطبية والعطرية والطب الشعبي، المركز القومي للبحوث، الخرطوم. السودان

المستخلص: هدفت هذه الدراسه الى مسح و وصف والتعرف مسبب مرض التبقع البكتيري في الطماطم .أجري المسح لتقييم نسبة وشدة الاصابة على الطماطم المزروعة فى ولاية النيل الأزرق خلال موسم الخريف 2015 .مواقع المسح كانت محليتي الروصيرص والدمازين. اختيرت اربع مزارع من كل محلية. جمعت وارسلت الاوراق المصابة الى معمل أمراض النبات، قسم وقاية المحاصيل كلية الزراعة ، جامعة الخرطوم، للكشف عن الممرض. تم التاكد من إمراضية سلالة البكتريا المعزولة بواسطة اختبار الامراضية. تم عزل المسبب المرضي و تمييزه ظاهريا، وعلى مستوي الاختبارات الكيموحيوية و الجزيئية. أظهر المسح عدم وجود فروق معنوية في نسبة الاصابة بالمرض بين المناطق الممسوحة في كل محلية، بينما كان هناك فروق معنوية ($P \leq 0.05$) في شدة المرض بين المناطق الممسوحة في كل محلية. كانت نسبة الاصابة عالية في كل من الروصيرص والدمازين بلغت 49.27 و 52.17%، على التوالي، بينما كانت شدة الاصابة 28.28 و 30.32%، على التوالي. أشارت النتائج الى أن العزلات كانت عصوية الشكل و هوائية اجبارية وسالبة لصبغة الجرام وموجبة الكاتاليز وسالبة الاكسديز و تحلل النشأ والجلاتين وتكوَن مستعمرات صفراءنامية على وسط الآجار المغذي, و بيئة الخميرة وكربونات الكالسيوم.

أظهراختبار الامراضية أن كل العزلات كانت ممرضة على نباتات الطماطم. وتم تأكيد هوية السلالات بواسطة تفاعل البلمرة المتسلسل وتحليل تتابعات الجين 16sRNA(رقم ايداع في بنك الجينات:MH047248و MH051263 و MG686236) على انها*Xanthomonas euvesicatoria*. وليس .X. vesicatoria. أشارت النتائج أن نباتات الطماطم المزروعة بولاية النيل الأزرق كانت مصابة بمرض التبقع البكتيري في الأوراق ،يوصى بالمزيد من الدراسات في تقدير قيمة الفاقد من الانتاج بسبب هذا الممرض, كذلك استراتيجيات للحد من انتشار المسبب.على حسب معرفتنا، هذا التقرير هو الاول عن بكتيريا *Xanthomonas euvesicatoria* المسببة لمرض التبقع البكتيري في الطماطم في السودان.