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DETECTION OF *Acidovorax citrulli* IN WATERMELON SEEDLINGS IN HOPELCHÉN, CAMPECHE, MEXICO

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ABSTRACT

In Mexico, there are no reports of the presence of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch and seedling blight in cucurbits. The introduction of *A. citrulli* is a latent risk due to the importation of seeds and seedlings. Fruits and plants with typical symptoms of this disease have been observed in watermelon (*Citrullus lanatus*) production fields of small growers and commercial nurseries in the municipality of Hopelchén, Campeche. The objective was to detect *A. citrulli* by PCR in watermelon seedlings in a production area in Hopelchén, with the hypothesis that the bacterium is present in this municipality. In December 2018, 51 samples of nine watermelon varieties were collected from two seedling production nurseries. Each sample contained ten symptomatic seedlings ranging in age from 12 to 41 days. End-point PCR analysis with primers WFB1 and WFB2 indicated the presence of *A. citrulli* in 68.6 % of the samples; immuno-PCR results confirmed the presence of the bacterium in 80.9 % of these samples. Consensus 16S rRNA gene fragment sequences from eight samples aligned with 98 % similarity on average to *A. citrulli* sequences deposited in the GenBank-NCBI database. The dark, sunken, elongated lesions on cotyledonal leaves, as well as the black, spreading lesions from the margin to the base of true leaves observed on watermelon seedlings, were caused by *A. citrulli*. This study constitutes a new report of *A. citrulli* in Campeche, which should be considered to delimit and prevent the spread of the bacterium to other cucurbit-producing areas in Mexico.

Keywords: *Citrullus lanatus*, bacterial fruit blotch, seedling blight.

INTRODUCTION

Acidovorax citrulli is absent in Mexico, as there have been no reports of it. However, the risk of introducing this regulated pest into the country is high (Elizalde-Jiménez *et al*., 2011). Some seed lots and seedlings used in the production of watermelon and other cucurbits are imported from countries such as the United States of America, which are not required to certify that the seed is free of the bacterium (SENASICA, 2022).

A. citrulli causes seedling blight and bacterial fruit blotch of cucurbits (Burdman and Walcott, 2012). It is a Gram-negative seed-borne bacterium with a high potential for

destruction, capable of causing losses of up to 100 % in watermelon nurseries and plantations under favorable environmental conditions of hot weather (temperatures between 28 and 32 °C, relative humidity above 60 %, and sunny days with heavy rains), which promote its multiplication and dissemination (Elizalde-Jiménez *et al*., 2011; Osdaghi, 2022).

Elizalde-Jiménez *et al.* (2011) conducted a NAPPFAST geospatial analysis, which indicated that the southeastern region of Mexico has favorable environmental conditions for the establishment of *A. citrulli.* According to these authors, if the bacterium enters the country, it could attack multiple species of cucurbits and cause economic damage, particularly in the states of Campeche, Yucatan, Quintana Roo, Tabasco, and the southern region of Veracruz.

In Mexico, in 2020, watermelon, melon (*Cucumis melo*), cucumber (*Cucumis sativus*), squash (*Cucurbita maxima*), and zucchini (*Cucurbita pepo*) crops covered an area of 109 494.65 ha and had an estimated production value of $ 20 511.97 million MXN (SIAP, 2022). Part of this area includes the southeastern zone reported by Elizalde-Jiménez *et al.* (2011).

In tours conducted between 2017 and 2018 through watermelon production fields of small producers and commercial nurseries in the municipality of Hopelchén, Campeche, freshly harvested fruits of the watermelon variety Charleston and watermelon plants with typical symptoms of bacterial fruit blotch were observed (Osdaghi, 2022). The fruits exhibited surface cracks surrounded by watery lesions, rotting, and release of effervescent exudate as a type of amber "foam", as well as "bursting" of the edible part. Necrotic spots and lesions were visible along the edges of true leaves. According to the people who cut and buy in those fields, at least five to eight fruits out of every 100 watermelons harvested have issues with "white material" or "watermelon juice" expulsion and internal rotting.

Therefore, the objective of this study was to detect *A. citrulli* by PCR in a watermelon seedling production area in Hopelchén, Campeche, with the hypothesis that the bacterium is present in that municipality.

MATERIALS AND METHODS

Nursery seedling collection

In December 2018, fifty-one seedling samples of nine watermelon varieties were collected from two nurseries located in Ich-EK and Hopelchén, in the Municipality of Hopelchén, Campeche. These towns are located at 19° 48′ 29.86″ and 19° 44′ 56.07″ N, 89° 56′ 58.27″ and 89° 50′ 16.44″ W, respectively. The nurseries were constructed with plastic and earthen floor, without controlled environmental conditions, and sprinkler irrigation.

Each sample's seedlings were individually wrapped in absorbent paper. They were placed in a Kraft paper bag (15.8 cm wide by 33 cm high) and then placed in a Ziploc® plastic bag (23 cm wide by 32 cm high). Each sample, consisting of 10 seedlings, was

labeled with site and variety information. They were shipped in a styrofoam cooler with refrigerant gels to the Bacteriology Laboratory of the Directorate of the National Center of Phytosanitary Reference in Tecámac, State of Mexico (SENASICA, 2018).
Symptoms were photo-documented on seedlings in the laboratory, and small portions of cotyledonal leaves, stems, and true leaves were obtained. The sectioned samples were stored in Ziploc® plastic bags and identified with an alphanumeric key, where the letter corresponded to the variety and the number to the sample. They were refrigerated at 4 ± 2 °C until analysis, which began within a few days of arrival.

Molecular detection and identification of *A. citrulli*

The protocol reported by SENASICA (2021) was used for bacterium detection, and the EPPO (2016) recommendations for the diagnosis of *A. citrulli* were followed. In general, we proceeded as described below.

DNA extraction

DNA (deoxyribonucleic acid) was extracted from the 51 plant samples using the 2 % CTAB (hexadecyltrimethylammonium bromide) method. DNA purity and quantity were estimated by spectrophotometry (Nanodrop 2000, Thermo Scientific®, Waltham, MA, USA) prior to PCR (polymerase chain reaction) amplifications.

PCR endpoint

A region of the 16S rRNA gene was amplified with the primer pairs WFB1 (5′-GAC CAG CCA CAC CAC TGG GAC-3′) and WFB2 (5′-CTG CCG TAC TCC AGC GAT-3′) (Walcott and Gitaitis, 2000). The reaction mixture was prepared in a final volume of 12.5 µL with 1.25 µL of 10x PCR buffer, 0.375 µL of 50 mM $MgCl_2$, 0.2 µL of 10 mM dNTP's, 0. 5 µL per 10 µM primer, 0.15 µL of Taq DNA polymerase (5 U μL^{-1}, Invitrogen®, USA), 2.5 µL of DNA (100 ng μL^{-1}) and 7.025 µL of PCR grade water. For the controls, mixtures were prepared with DNA from the *A. citrulli* strain ATCC® 29625 for the positive and water without template DNA for the negative.
Reactions were set up on a Thermal Cycler (BioRad® / T100 Thermal Cycler, USA) with the following thermal cycling program: an initial cycle at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final cycle at 72 °C for 5 min. The amplification product was separated by 1.5 % agarose gel electrophoresis. A positive sample was considered when the DNA band of the expected size (360 bp) was visualized in a photodocumenter (Gel Doc EZ Imager, BioRad®, USA).

Immuno-PCR

The test was established with 21 of 32 plant samples that were positive by endpoint PCR. First, the DAS-ELISA test was developed using the diagnostic protocol and reagents recommended by Agdia® for the detection of *A. citrulli* (catalog number: SRA 14800), modifying the addition of the PNP (P-nitrophenylphosphate) substrate. According to a chart designed for sample distribution, 100 µL of the sample's tissue macerate extract,

as well as 100 µL of each of the controls: negative (healthy watermelon tissue macerate extract and buffer solution) and positive (lyophilized bacterial suspension from the AacReagent Set kit), were deposited in the 96 wells of a polystyrene plate. Each plant sample and control were included in duplicate. In place of the PNP substrate, 100 µL of PCR-quality water was added to each well. The plate was heated for 45 s to release the cell contents. Subsequently, the PCR reaction was set up with the crude DNA, with a final volume of 12.5 µL and preparation as described in the previous paragraph. The reactions were set up in a Thermal Cycler (Bio-Rad / T100 Thermal Cycler) using the program described above. The expected DNA band (360 bp) was visualized in a photodocumenter (Gel Doc EZ Imager, BioRad®, USA).

Band sequencing

Sequences from both directions of the band obtained from 17 positive samples were analyzed, edited and assembled using BioEdit® v. 7.0.9.0 (Hall, 1999). The consensus sequences were compared with *A. citrulli* sequences stored in the NCBI GenBank to verify the identity of the bacterium.

RESULTS AND DISCUSSION

The endpoint PCR analysis, using primers WFB1 and WFB2, revealed the presence of *A. citrulli* in 32 of the 51 watermelon seedling samples collected in the Ich-Ek and Hopelchén greenhouses. By analyzing 21 of these positive samples using immuno-PCR, the presence of the bacterium was confirmed in 17 samples. However, only the 16S rRNA gene fragment consensus sequences of eight positive samples aligned with *A. citrulli* sequences deposited in the GenBank-NCBI database (Table 1).

The eight samples that aligned with *A. citrulli* had similarity indices ranging from 100.00 to 98.59 %. Sequences from two samples aligned with *A. avenae* and *A. oryzae* with 93.66 and 96.77 % similarity, respectively. Also, five samples aligned with an unidentified species of *Acidovorax* with similarities between 93.62 and 98.38 %, while two aligned with bacteria of other genera (Table 1).

In this study, the detection of other bacterial genera and species in addition to *A. citrulli* (Table 1) indicates the lack of overall specificity of the WFB1 and WFB2 primers. Although Walcott and Gitaitis (2000) note that false positive detection is low with these primers, 52.9 % of the samples sequenced here did not align with *A. citrulli*. In turn, Wang *et al.* (2012) note that the *A. citrulli* and *A. oryzae* species are closely related and are not easy to differentiate.

The comparison of the consensus sequences of the 360 bp band allowed verifying the identity of *A. citrulli*; therefore, it is concluded that the dark sunken and elongated lesions on the cotyledonal leaves, as well as the black lesions extended from the margin to the base on the true leaves observed in the seedlings of the samples that aligned with *A. citrulli*, were caused by this bacterium (Osdaghi, 2022) (Table 1, Figure 1), while the dry, yellow and dark lesions are attributed to the other *Acidovorax* species identified here (Figure 2).

Table 1. Sequence accession number, aligned species and result of molecular analysis of 18 watermelon seedling samples collected in the municipality of Hopelchén, Campeche.

Greenhouse Variety/Sample	PCR	Immuno-PCR	Aligned species¶	Accession number
In Ich-Ek				
Super Crisp N, A3	Yes†	Yes	*Acidovorax citrulli* M6	MT003980
Crisp, B1	Yes	Yes	*Acidovorax citrulli* M6	MT003983
Montreal				
C2	Yes	Yes	*Xylophilus ampelinus*	
C7	Yes	Yes	*Acidovorax citrulli* M6	MT004764
C8	Yes	Yes	*Acidovorax* sp.	
C9	Yes	Yes	*Acidovorax citrulli* M6	MT004779
C11	Yes	Yes	*Acidovorax citrulli* M6	MT004767
C12	Yes	Yes	*Acidovorax citrulli* M6	MT004790
In Hopelchén				
Santa Matilde				
D1	Yes	Yes	*Acidovorax* sp.	
D2	Yes	Yes	*Acidovorax* sp.	
Polimicer				
E1	Yes	Yes	*Acidovorax avenae*	
E4	Yes	Yes	Bacterial clone OTU3-Control WM1_BS57271	
800 G	Yes	Not analyzed		
Next				
F2	Yes	Yes	*Acidovorax* sp.	
F4	Yes	Yes	*Acidovorax* sp.	
Prime, H2	Yes	Yes	*Acidovorax citrulli* M6	MT004793
Policenter				
I1	Yes	Yes	*Acidovorax oryzae*	
I2	Yes	Yes	*Acidovorax citrulli* M6	MT004814

†360 bp band amplification. ¶Deposited at GenBank-NCBI.

The presence of watermelon seedlings infected with *A. citrulli* in the production nurseries of Ich-Ek and Hopelchén indicates the introduction of bacterial blotch of cucurbits into Campeche (Elizalde-Jiménez *et al*., 2011). It is possible that this is a localized sporadic outbreak resulting from the inadvertent introduction of *A. citrulli* through commercial seed contaminated with the bacterium (Burdman and Walcott, 2012). However, the presence of the bacterium in this area serves as a warning to take immediate action to contain the disease and prevent it from spreading.

The information generated here is relevant for decision making for the design of delimitation strategies for *A. citrulli* that allow the establishment of phytosanitary measures for the prevention, control, and eradication of the bacterium, as well as for the implementation of practices to prevent the spread of the pest to other cucurbit-producing areas in southeastern Mexico. Likewise, it could serve as a basis to review and update the phytosanitary measures established for the importation of watermelon seedlings and seeds, especially from countries where the bacterium is present, and

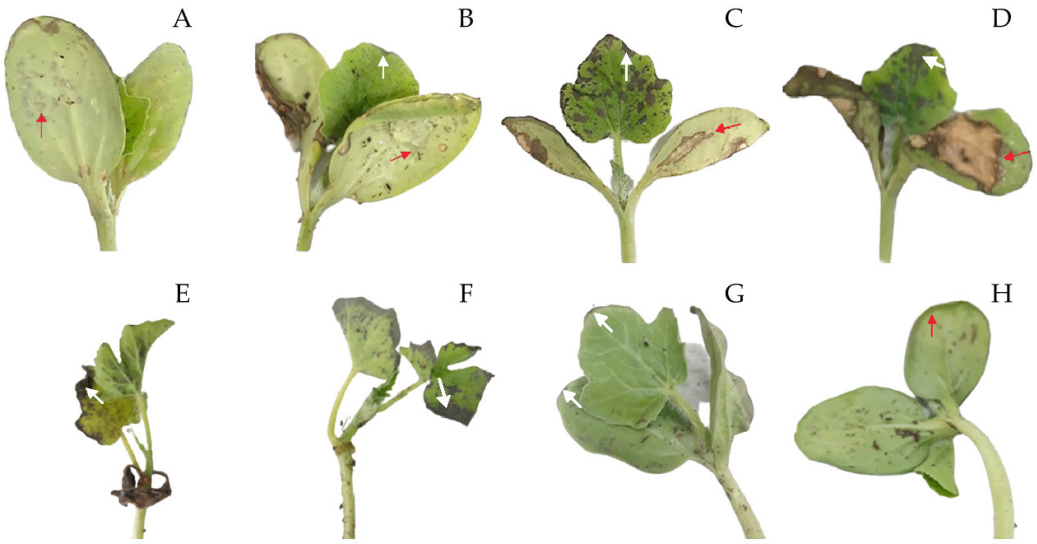

Figure 1. Seedlings of watermelon varieties with characteristic symptoms of seedling blight caused by *A. citrulli.* A, B, C, D: Montreal; E: Super Crisp N; F: Crisp; G: Policenter; H: Prime. The red arrow indicates sunken, elongated dark lesions on cotyledonal leaves, while the white arrow indicates extended black lesions, from margin to base, on true leaves.

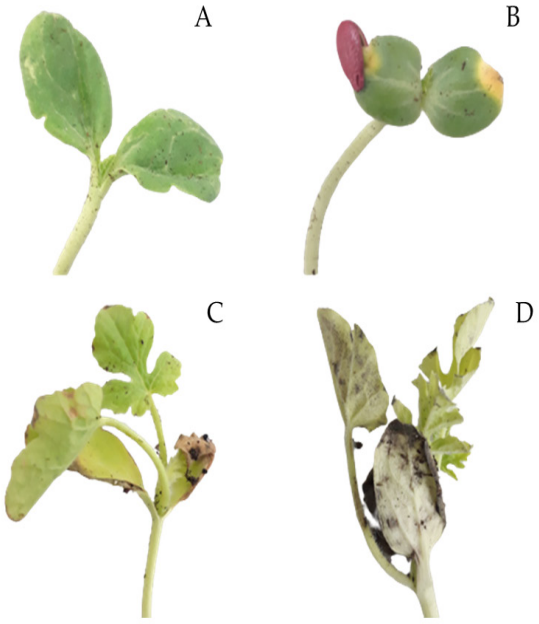

Figure 2. Seedlings of watermelon varieties with symptoms. A: dry lesions by *Acidovorax* sp. in Proxima; B: yellowing by *A. oryzae* in Policenter; C: *A. avenae* in Polimicer; D: dark spots by *Acidovorax* sp. in Santa Matilde.

likewise, to update the molecular diagnostic protocol for the detection of *A. citrulli* with more specific primers (SENASICA, 2021).

CONCLUSIONS

A. citrulli was detected and molecularly identified in watermelon seedlings with typical seedling blight symptoms in two production nurseries in Hopelchén, Campeche, Mexico. This work is a new report on the presence of *A. citrulli* in the Hopelchén municipality.

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