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OCCURRENCE OF BACTERIAL FRUIT BLOTCH ON WATERMELON (*CITRULLUS VULGARIS*) CAUSED BY *ACIDOVORAX CITRULLI* IN THE REPUBLIC OF NORTH MACEDONIA

Sasa Mitrev✉, Emilija Arsov, Biljana Kovacevik

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Abstract

In late summer 2019, *Acidovorax citrulli* the causative agent of bacterial seedling blight and fruit blotch (BFB) of cucurbit plants was first detected in a commercial watermelon production area in the village of Sopot, Kavadarci, North Macedonia. Later, in the summer of 2019 and also in 2020, *A. citrulli* was observed on watermelon fruits (*Citrullus lanatus* L., varieties "Bibo" and "Olakala") in the Strumica region in the Eastern part of North Macedonia. Initial symptoms included water-soaked, irregular spots on the fruits that appeared greasy and dark olive green, eventually developing into brown lesions. Round, cream-coloured, Gram-negative bacterial isolates were obtained from symptomatic fruit tissue, which were slow-growing, nonfluorescent, aerobic, and oxidase-positive.

In recent years, the disease has spread globally, primarily through the inadvertent distribution of contaminated commercial seeds, emerging as a significant pathogen of cucurbitaceous plant species worldwide. Due to costly lawsuits filed by growers against seed companies and the lack of effective management methods, BFB poses a serious threat to the cucurbit industry, particularly to watermelons and melons. *Acidovorax citrulli* is a seed-borne pathogen and is listed as an A1 quarantine pathogen by EPPO in 2021. Despite the economic importance of the disease, little is known about the basic aspects of *A. citrulli* pathogenesis in our country.

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Based on the fruit symptoms, pathogenicity tests, biochemical tests, serological and PCR analyses, the pathogen was identified as *Acidovorax citrulli*. To our knowledge, based on the current findings, including symptomatology, pathogenicity tests, biochemical characterization and PCR analyses, this is the first detailed study of *A. citrulli* causing bacterial fruit blotch on watermelon in North Macedonia.

Key words: *Acidovorax citrulli*, blight, fruit blotch, biochemical tests, PCR

Introduction. *Acidovorax citrulli* is the causative agent of bacterial seedling blight and fruit blotch of cucurbits [1]. It has become one of the most serious diseases threatening watermelon production since its first outbreak in the late 1988's in United States [2]. It has been reported as epidemic disease in the last 20 years, particularly on watermelon and on susceptible melon genotypes [3].

Acidovorax citrulli pathogenic bacterium developed disease symptoms on several cucurbit hosts including musk melon [4], honeydew [5], citron melon [6], melon [7], rock melon [8], cucumber [9], pumpkin [10], and Hami melon [11].

Seeds represent the most important source of primary inoculum for BFB outbreaks. This confirms what has been observed in field, that expanded leaves and stems are the main inoculum sources for melon blossoms and fruit [12]. Bacteria landing on uninfected seedlings penetrate cotyledons and leaves via stomata and multiply rapidly in intercellular spaces [13].

Acidovorax citrulli is biotrophic, Gram-negative, economically important seed-borne pathogen, showing typical symptoms on watermelon and melon worldwide, with the ability to affect other cucurbits, such as cucumber, squash, and pumpkin. Seed disinfestation treatments, seed health testing, and chemical control in the field are limited in their ability to reduce the yield losses associated with bacterial fruit blotch (BFB) [14].

In 2005, Burdman et al. [15] characterized the *Acidovorax citrulli* isolates from different cucurbit hosts according to DNA fingerprinting profiles and their study indicated the two differentiated groups such as: watermelon group and non-watermelon cucurbits group of *Acidovorax citrulli*.

Since the beginning of this century, *A. citrulli* has been reported for the first time in Guadeloupe [16], California [17], Serbia [3], and the Republic of North Macedonia [18].

In this article, the main purpose was to isolate and identify the causative agent of watermelon bacterial fruit blotch in North Macedonia, by using morphological, physiological, biochemical, serological and molecular studies.

Materials and methods. ***Collection of symptomatic material.*** In late August, 2019/2020, watermelon fruits (*Citrullus lanatus* L., variety "Bibo", 1.5 ha and "Olakala", 3.5 ha) collected from a field located in Strumica region (Eastern part of North Macedonia) and Sopot Kavadarci, N. Macedonia exhibited typical disease symptoms resembling bacterial fruit blotch (BFB) caused by *Acidovorax*

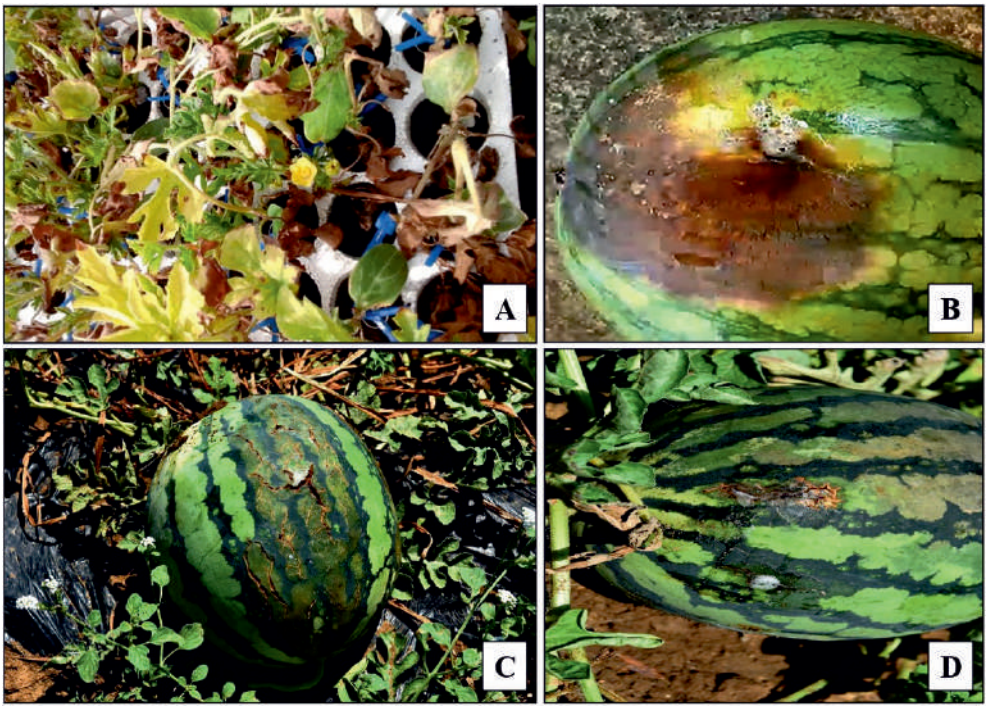

Fig. 1. Symptoms of bacterial fruit blotch on watermelon seedlings (A) and fruits (B, C, D)

citrulii. In seedling production and field observation, watermelon leaves were generally not symptomatic (Fig. 1A). On fruit, first symptoms observed were small cracks with some water soaking, and then cracks extended to the entire fruit, and water soaking and rotting areas appeared all around (Fig. 1B, C, D). Lesions usually become apparent shortly before fruit ripening.

Symptomatic watermelon fruit samples were collected in late summer (August 2019) following a request from growers in the village of Sopot (Kavadarci, North Macedonia). In total, 20 symptomatic fruit samples were obtained from this region. Additionally, 15 symptomatic watermelon fruit samples were collected in August 2019/20 from the Strumica region (Eastern North Macedonia).

Isolation and pathogenicity testing. Fruit samples were collected from the field, washed with running water, and the bacterium was isolated from the margin of lesions from within the mesocarp, within 24–48 h to allow maximum recovery of the pathogen and to avoid multiplication of saprophytes. The solution of macerated tissue was plated onto King's medium B and non-fluorescent colonies were visible after a few days. The plates were incubated at 28 °C for 2–3 days. The colonies were first checked for Gram reactions by the lysis of bacteria in 3% potassium hydroxide (KOH). Bacterial colonies on NAS media (Nutrient Agar with 2% sucrose) were cream-coloured with smooth margins, and convex, and individual cells were rod-shaped (Fig. 2).

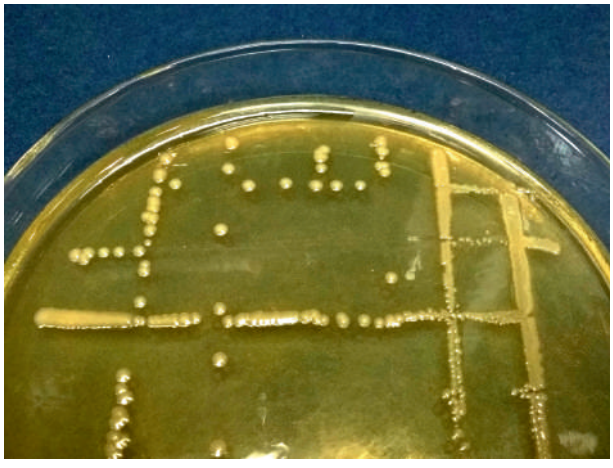

Fig. 2. *Acidovorax citrulli* on NAS, showing 4 days old colonies, grown at 28 °C in control temperature condition in UNILAB laboratory

Biochemical and physiological tests including LOPAT (levan production, oxidase reaction, potato soft rot, arginine dihydrolase and hypersensitivity on tobacco leaves) characteristics, were used for identification of the bacterial isolates.

Pathogenicity tests. All representative isolated samples were grown on NAS and King's B Medium for 48 h. Ten days old healthy watermelon seedlings (*Citrullus lanatus* L., variety "Anguria", sugar baby) at 2–3 true leave stage grown in UNILAB, and baby watermelon fruits obtained from healthy fields, were used for the pathogenicity tests. Both tests were prepared and done in sterile laboratory conditions and conducted with two replicates. Bacterial suspension was adjusted to 10^6 CFU/ml using a spectrophotometer, and it was injected into young watermelon seedlings and fruits.

Serological test by ELISA. The serological identification of the bacterial isolates was performed as two replications according to the manufacturer's instructions with Complete Kit Standard DAS-ELISA LOEWE, Biochemica GmbH, Germany. From the commercial kit, positive and negative controls were used in DAS-ELISA reactions. Absorbance values were read using ELISA micro plate reader at 405 nm after 45 min.

Molecular test by PCR. *Nucleic acid extraction* – fresh pure culture (24 h growing culture on NAS at 28 °C) is suspended in 0.9 mL of PCR-grade water, supplemented with 0.1 mL of a 0.5 M NaOH solution. Then, the suspension is heated for 4 min at approximately 95 °C and immediately put on ice for 10 min. The molecular identification of *Acidovorax citrulli* isolates was confirmed using specific primer pairs SEQ ID no. 3: 5′-GGA AGA ATT CGG TGC TAC CC-3′ and SEQ ID no. 4: 5′-TCG TCA TTA CTG AAT TTC AAC A-3′ (PM 7/127 (1) described by Schaad et al. [19]). PCR reaction was prepared in total volume of 25 µL in each reaction tube. PCR master mix contained reaction buffer, 4 mM

$MgCl_2$, 0.4 mM of each dNTPs, 0.05 U/µl of *Taq* DNA polymerase, 20 µM working concentration of each primer and 2 µl of each genomic DNA per reaction.

PCR cycling conditions were:

	Temp. (°C)	Time
Hold	94	10 min
Repeat 35 cycles	94	30 s
	56	45 s
	72	60 s
Final extension	72	7 min
Hold	4	

PCR products were analyzed after 1 hour gel-electrophoresis at 90 V in a 1.5% agarose gel stained with ethidium bromide (0.1 µl/ml). A 1 Kb DNA Ladder (Ready-to-Use) (NIPPON Generics EUROPE GmbH) was used as a size marker. KBF 0520 was used as a positive control; (collection of A. Obradovic, University of Belgrade, Serbia) and *Acidovorax avenae* subsp. *citrulli* No. 08154PC (commercial control from LOEWE).

Results. ***Disease symptoms on naturally infected plants.*** Samples of watermelon (*Citrullus lanatus* L., variety "Bibo" and "Olakala"), were taken from fruit material showing symptoms that initially appeared as small cracks with slight water-soaking. These cracks later extended over the entire fruit, and water-soaked and rotting areas developed across the surface. Samples were collected also from watermelon leaves, which showed no symptoms. Isolation was carried out during the two growing seasons (August 2019–2020) in the Strumica and Kavadarci regions.

Isolation and identification of the pathogen. The colonies were cream-coloured, convex, and smooth with regular margins, and the cells were rod-shaped and Gram-negative. The isolates were oxidase-positive, aerobic, and arginine dihydrolase-negative. A total of 15 representative bacterial strains were selected for complete characterization. All tested strains were negative for levan production, pectolytic activity on potato slices, and hypersensitive reaction (HR) on tobacco leaves.

Pathogenicity tests. All representative isolates, were artificially inoculated to the healthy watermelon seedlings (*Citrullus lanatus* L., variety "Anguria", sugar baby), caused lesions with dark-brown spots on inoculated leaf tissue, after 5 days (Fig. 3A). Disease symptoms on watermelon small fruits were observed 5 days after inoculation and symptoms started with brown necrosis, cracks and after 10 days, a lot of necrosis spots were shown (Fig 3B, C). Control plants and fruits were negative.

Serological test by DAS ELISA. In DAS-ELISA tests, all watermelon isolates were positive. The absorbance values using ELISA plate reader (read

Fig. 3. Symptoms after artificial inoculation on watermelon seedlings (A) and fruits (B, C). Water-soaked lesions on cotyledons (A); Water-soaked, sunken browning in fresh fruit followed by cracks on watermelon (B, C)

after 1 and 2 h), were 0.476 for positive control, 0.154 for negative control, and 0.535 for our representative samples, read at 405 nm wavelength.

Molecular analysis. The molecular identification of *Acidovorax citrulli* strains was confirmed by using *A. citrulli* specific primer pairs SEQ ID no. 3/SEQ ID no. 4. Representative isolates (based on their biochemical characteristics) selected for molecular identification (a total of 15; isolates 1–7 from the Kavadarci region and 8–15 from the Strumica region) were PCR-positive, producing amplicons of approximately 450 bp. All obtained fragments, corresponded to those of the two reference positive controls, KBF 0520 and K2 (Fig. 4).

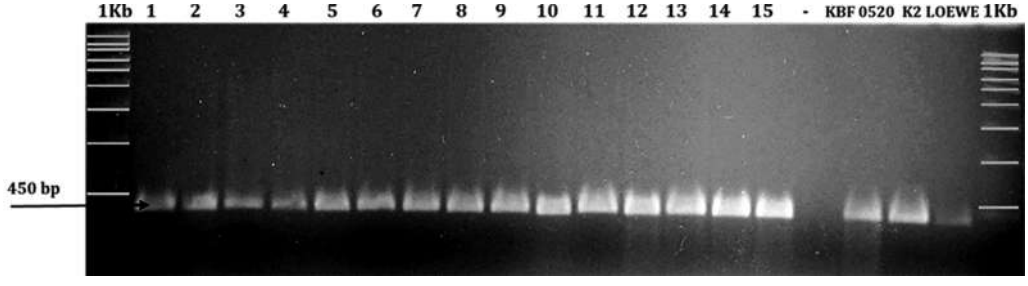

Fig. 4. PCR profile using *A. citrulli* specific primer pairs SEQ ID no. 3/SEQ ID no. 4. 1–15 Macedonian positive samples; KBF 0520 and K2 (collection from A. Obradovic, University of Belgrade, Serbia) and *Acidovorax avenae* subsp. *citrulii* No. 08154PC (commercial control from LOEWE) 1 Kb DNA Ladder (Ready-to-Use) (NIPPON Generics EUROPE GmbH)

Discussion. Previous studies indicate that watermelon cultivars exhibit varying levels of tolerance to *Acidovorax citrulli*, with susceptibility potentially associated with rind colour and ploidy level, where diploid cultivars tend to be more susceptible than triploid ones [8]. However, even the most tolerant cultivars currently available on the market do not provide complete resistance [8]. The study of Hopkins et al. [21] compared diploid and triploid watermelons for susceptibility to BFB in field trials and found that severe symptoms such as open wounds or rot were rarely observed in triploid fruits. Field observations in this study showed that the commercial seedless triploid varieties "Bibo" and "Olakala"

exhibited severe symptoms like fruit cracking and rot, suggesting that BFB can be devastating even for triploid commercial varieties, which shows that identifying resistant germplasm, understanding host–pathogen interactions, developing methods for rapid detection of contaminated seeds, and exploring sustainable biological control strategies are of utmost importance. Findings from the field observations also suggest that plants remain asymptomatic during the early stages of development, which additionally impedes effective management of the disease at these stages.

Because of the epidemiology of the disease, the chemical control is limited, and the effective management largely depends on the production of *A. citrulli*-free watermelon seed. This in turn requires a detailed understanding of the seed infection process. Although the study of Dutta et al. [20] has made significant advances in understanding seed infection, important gaps remain in understanding how the bacterium infects the plants and spreads within production systems. Since the disease continues to persist in the fields, the spread of the disease to other commercial fields especially in seedling production and nurseries, relies primarily on the implementation of appropriate phytosanitary measures, given the current stage of knowledge about the pathogen.

Conclusion. In our country, this is the first study about the occurrence of *Acidovorax citrulli* on watermelon in the commercial nurseries and fields in North Macedonia regions. Based on the fruit symptoms, pathogenicity tests on young watermelon fruits and seedlings, biochemical tests, serological and PCR analyses, the pathogen was identified as *Acidovorax citrulli*.

The ideal control strategy for preventing *A. citrulli* outbreaks is using disease free seeds to reduce and limit the pathogen introduction into the field. Implementing proper cultural practices is essential. These practices include minimizing manipulation of cultivated plants, decontaminating hands, seedling containers, tools, and machinery, keeping greenhouse doors closed during windy conditions, and destroying infected plant material and inoculum.

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Department of Plant Protection and Environment, UNILAB Laboratory, Faculty of Agriculture, Goce Delcev University, 10-a Krste Misirkov St, 2000 Stip, Republic of North Macedonia
e-mails: sasa.mitrev@ugd.edu.mk, emilija.arsov@ugd.edu.mk, biljana.kovacevik@ugd.edu.mk