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A simple and cost-effective diagnostic of *Macrophomina phaseolina* on watermelon by direct PCR

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ABSTRACT. *Macrophomina phaseolina* (Tassi) Goid is the causal agent of charcoal rot and vine decline in cucurbits such as watermelon. Molecular methods have been used for rapid identification. However, a large number of steps used reduces its applicability. This study aimed to detect *M. phaseolina* in watermelon from producing areas in Northeastern Brazil by direct PCR. Plant tissue samples were collected from seven producing areas and the DNA was extracted using the CTAB method. Amplifications were performed by direct PCR using the MpKFI/MpKRI primers, then the PCR products were subjected to agarose gel electrophoresis and sequenced. Amplicons of 350 bp were observed in stem tissue samples from three areas. The identity of the samples was confirmed by sequencing. This study represents the first molecular diagnosis of *M. phaseolina* associated with watermelon in Northeastern Brazil. The methodology presented here can be applied for a reliable and simple diagnosis of the pathogen in other crops.

Keywords: charcoal rot; *Citrullus lanatus*; direct PCR; ITS primer.

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Introduction

Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) is the most produced cucurbit in the world, with 103 million fruits produced. Brazil ranks fifth, with a production of 22 million tons and 101 thousand hectares of planted area (Food and Agriculture Organization [FAO], 2018). The crop is well adapted to environments with high light intensity, low rainfall, and dry climate, such as the Northeastern Brazil (Sales Júnior, Senhor, Michereff, & Negreiros, 2019b). The region has high production potential, accounting for 26% of national production (IBGE, 2019).

The occurrence of diseases is among the main factors limiting watermelon productivity. *Macrophomina phaseolina* (Tassi) Goid, belonging to the phylum *Ascomycota*, has been reported as the causal agent of charcoal rot disease and vine declining in cucurbits grown in Asia and South America (Cohen, Elkabetz, & Edelstein, 2016). Also, the species was associated with crown rot and wilting in watermelon grown in Iran and Pakistan (Baloch et al., 2013; Mahdizadeh, Safaie, & Aghajani, 2011).

The disease causes concern among producers because the fungus can remain in the field, surviving for up to 15 years in the soil through resistance structures (Kaur et al., 2012) or in alternative hosts, such as weeds without symptoms (Negreiros et al., 2019). Also, the fungus takes advantage of hosts under water stress and in hot and dry environments, such as in the Brazilian semiarid (Cohen et al., 2014).

Taking into account that the amount of inoculum, and consequently its severity, increases with each cultivation cycle (Ambrósio et al., 2015; Lodha & Mawar, 2020), prior detection of the fungus in the producing areas is important to suggest effective control measures. *M. phaseolina* is generally identified by colony morphology under microscopy (Biswas et al., 2013). However, this method is not specific since the species has high morphological diversity (Iqbal & Mukhtar, 2014). Neither practical, since such procedure are often laborious and time-consuming (Biswas, Dey, Mandal, Satpathy, & Karmakar, 2014a).

In this context, molecular methods such as PCR (Polymerase Chain Reaction) has been used for fast and reliable detection of plant pathogens (Martinelli et al., 2015). Species-specific primers developed by

Babu, Saxana, Srivastava, and Arora (2007) allowed the detection of *M. phaseolina* in several crops around the world. However, the methodology is based on prior isolation of the fungus from soil or infected plants in culture medium, followed by DNA extraction and isolation, and finally detection by PCR (Babu, Babu, & Sharma, 2013).

On the other hand, the direct PCR method is based on the amplification of DNA from samples without prior isolation, reducing time and costs, and allowing the analysis of a large number of samples (Ben-Amar, Oueslati, & Mliki, 2017). Quickly and safely, phytopathogenic species of bacteria (Fujikawa, Miyata, & Iwanami, 2013), fungi (Ben Amar, Oueslati, Ghorbel, & Mliki, 2012), and viruses (Biswas et al., 2014a) were detected directly from plant tissues by direct PCR.

Despite being a simple technique, *in planta* detection requires optimizations for each type of tissue used to prevent the presence of PCR inhibitors in the DNA amplification reactions (Biswas et al., 2013). Currently, no direct PCR protocols exist for the detection of pathogens in cucurbits. In this work, we investigated and validated a methodology for direct detection of *M. phaseolina* from watermelon tissues collected in commercial areas in Northeastern Brazil.

Material and methods

Material collection and experiment location

Leaf and root samples were collected from seven watermelon production fields in Rio Grande do Norte State, Brazil (Figure 1). The climate of the region is a hot semiarid according to the Köppen classification. Leaves were collected for use as a negative control. The samples were placed in plastic bags and transported to the laboratory for disinfestation (in 10% sodium hypochlorite solution for 1 min, followed by washing in distilled water for 2 min), then storage in a freezer at -20°C. Collections were carried out after the approval of the Genetic Heritage Management Council in the Brazilian National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), and materials were registered under the access code: AA2E8D4.

The experiment was carried out at the Plant Biotechnology Laboratory and Phytopathology Laboratory II from the Department of Agronomic and Forest Sciences, Federal Rural University of Semiarid (UFERSA), located in Mossoró city, Rio Grande do Norte State, Brazil.

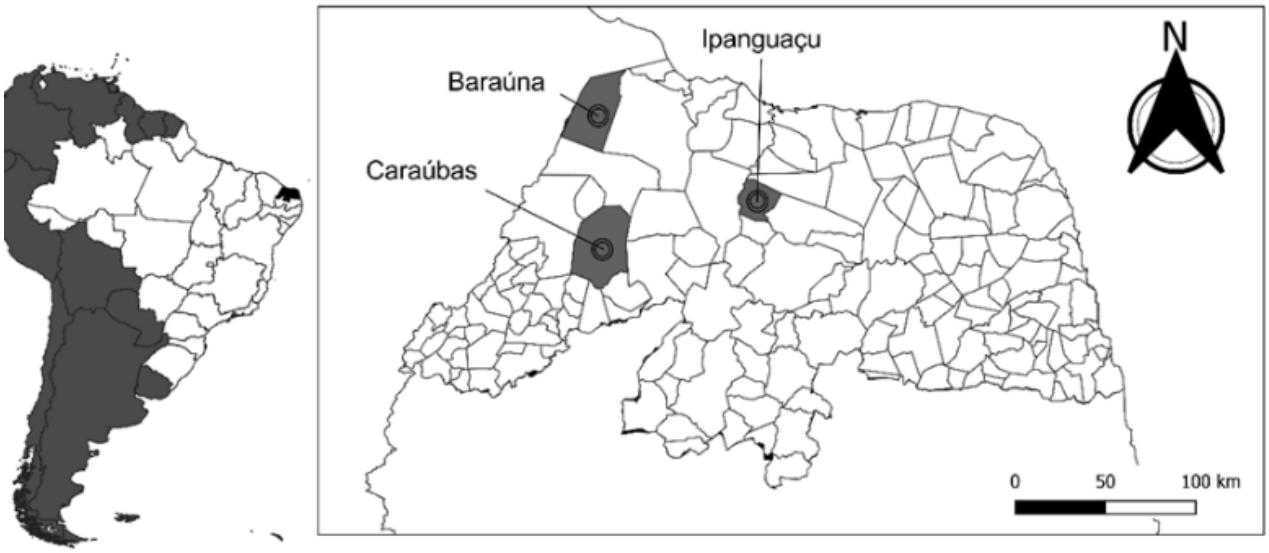

Figure 1. Geographical description of the collection areas of plant material used in this study.

DNA extraction

The genomic material was extracted using the CTAB method (Doyle & Doyle, 1990) adapted for direct PCR (Ben-Amar et al., 2017; Bellstedt, Pirie, Visser, Villiers, & Gehrke 2010) with modifications. 150 mg plant material was macerated in liquid nitrogen and transferred to sterile 2 ml microtubes. Then, 800 µL extraction

buffer solution (100 Mm Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-Mercaptoethanol, and Milli-Q water) previously heated to 65°C were added to the microtubes, which were then shaken and incubated in a water bath at 60°C for 40 min., shaking every 10 min. Afterward, 800 µL Chloroform:Isoamyl alcohol solution (24:1) were added to microtubes, which were shaken and then centrifuged at 10,000 g for 10 min. After centrifugation, the upper aqueous phase was transferred to a new microtube, and this step was repeated once more. Subsequently, the aqueous phase was transferred to a new tube and 2/3 volume of cooled isopropanol was added, homogenizing the solution until pellet formation. The tubes were incubated at -20°C for at least 2 hours, then centrifuged at 10,000 g for 10 min., and the supernatant was discarded. To clean the pellet, 1 mL ethanol (70%) was added to the microtube, which was then centrifuged at 10,000 g for 5 min., discarding the supernatant. 1 mL ethanol (90%) was added again to the microtubes for new centrifugation at 10,000 g for 5 min. The supernatant was discarded again, leaving only the pellet into the microtube. The samples were dry at room temperature, then 45 µL ultrapure water and 5 µl RNase (10 µg mL^{-1}) were added to the microtubes, which were incubated in a water bath at 37°C for 30 min. Finally, the DNA was quantified by agarose gel electrophoresis (Dash, Shrivastava, & Das, 2020).

M. phaseolina isolate MCBR5 (CMM 4737) belonging to the Coletânea Maria Menezes (Federal Rural University of Pernambuco, Pernambuco State, Brazil) was used as a positive control. The isolate was cultured in PDA (Potato Dextrose Agar) medium at ± 28°C for 5 days and its genomic material was isolated using the same protocol.

PCR amplification and sequencing

Species-specific primers for *M. phaseolina*, MpKFI (5'-CCGCCAGAGGACTATCAAAC-3') and MpKRI (5'-CGTCCGAAGCGAGGTGTATT-3'), based on conserved internal transcribed spacer (ITS) regions in DNA, adjacent to the 5.8 S gene (Babu et al., 2007), were used for direct PCR amplification. Amplification reactions were performed in 20 µL containing 10x reaction buffer (Cellco, Brazil), 2 mM of each dNTP, 1 U Taq DNA polymerase (Cellco, Brazil), 5 pmol of each primer, 10 ng DNA, and q.s.p. ultrapure water). PCR was achieved in a thermocycler (Amplitherm, USA) programmed to operate an initial denaturation at 94°C for 1 min., followed by 35 cycles at 94°C for 30 seconds, 62.4°C for 30 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for 5 min. Then, the amplified products were separated by electrophoresis at 110 V for 70 min. on 1.5% agarose gel with 1x TBE buffer and stained with ethidium bromide (1 µg mL^{-1}). Subsequently, the gel was visualized under ultraviolet (UV) light in a photocumenter (ProteinSimple, USA). A 100 bp molecular weight marker (BioLabs, USA) was used as a standard.

Also, the PCR products from some of the samples were sequenced to confirm the identity of the amplified samples and validate the diagnostic method (Macrogen Co., Seoul, South Korea). The nucleotide sequences were then registered on GenBank. Search for similar sequences was performed in the NCBI (National Center for Biotechnology Information) database using the BLAST (Basic Local Alignment Search Tool) software. Phylogenetic analyzes were performed using the Neighbor-Joining clustering method with 1000 bootstrap replicates, and *Botryosphaeria dothidea* isolate (MN731269) was used as an outgroup. Analyzes were performed in MegaX software version 10.1.7 (Tamura, Stecher, Peterson, Filipski, & Kumar 2013).

Results

M. phaseolina was detected in three of the seven production fields where watermelon tissues were collected, two in Baraúna and one in Ipanguaçu. Profile of PCR products after amplification using the species-specific primers are shown in Figure 2a. Amplification of the stem tissues generated amplicons of 350 bp, which was expected according to the methodology described by (Babu et al., 2007). The same pattern was obtained with the control sample (MCBR5 isolate). However, no PCR products were obtained from leaf tissue samples.

To confirm identity and validate diagnosis, the PCR products of the samples with positive results were sequenced. By sequence analysis through BLASTn, the identity of all the obtained PCR products was confirmed (>99%) as *M. phaseolina* isolate MN061079.1, based on the amplification of the internal transcribed spacer 1 (ITS-1), partial sequence of the 5.8 S ribosomal RNA, and complete sequence of internal transcribed spacer 2 (ITS-2) (Figure 2b).

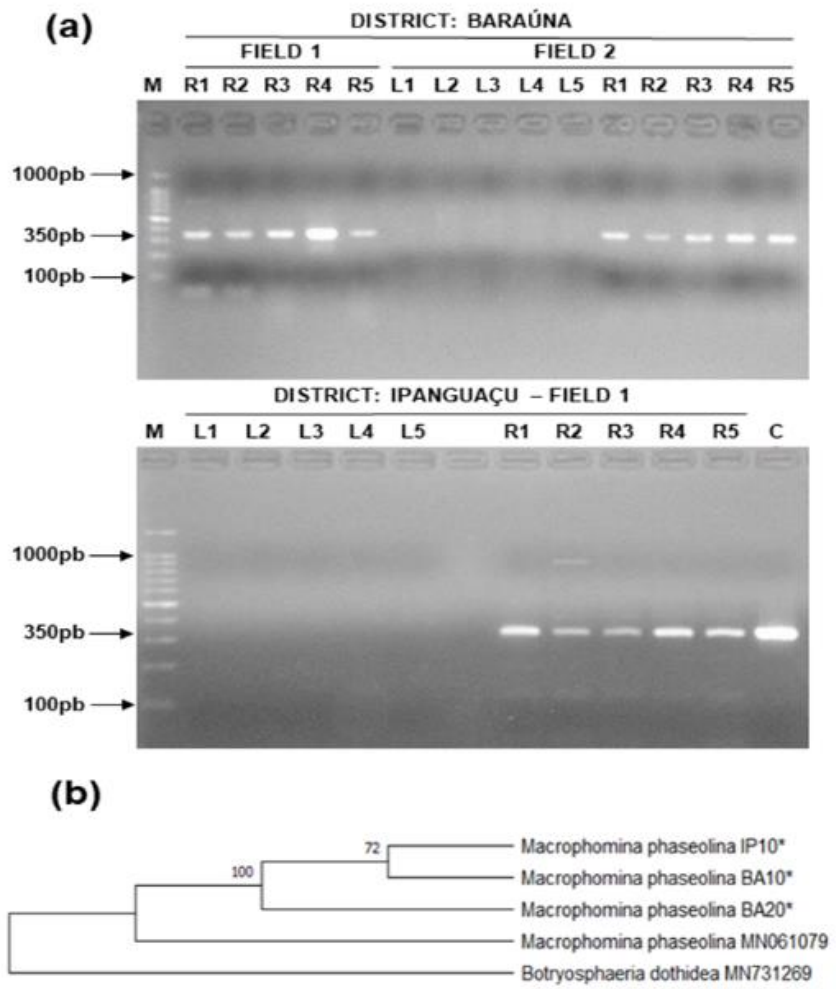

Figure 2. (a): PCR products of watermelon tissue samples after amplification by agarose gel electrophoresis using the MpFI/MoKRI primers. 'R': Root; L: leaf; 'M' in the first channel of the gel represents the 100 bp molecular weight marker; 'C': control (MCBR5 isolate). (b): Phylogenetic tree generated by the Neighbor-joining clustering method using 1,000 bootstrap replicates.

Discussion

M. phaseolina could be detected by direct PCR from infected stems of watermelon from producing areas in the state of Rio Grande do Norte. On the other hand, there was no amplification of leaf tissue samples, which was expected, since the fungus is a soil inhabitant and infects roots (Ambrósio et al., 2015).

Possibility of detecting *M. phaseolina* without prior isolation of the pathogen in culture medium was proposed by Biswas et al. (2013). By optimizing the DNA extraction protocol, the authors were able to detect the fungus in artificially infected seeds of Junta (*Corchorus olitorius*) plants. In another study, the research group detected the species by direct PCR in tissues from naturally infected Junta plants (Biswas et al., 2014b).

Other pathogenic species were also detected by direct PCR. *Fusarium culmorum*, associated with mycotoxins in cereals, was detected without prior isolation (Ben Amar et al., 2012). Also, Fujikawa, Miyata, and Iwanami (2013) observed that the detection of *Candidatus Liberibacter asiaticus* in lemon leaves by direct PCR has equivalent specificity to the conventional and real-time PCR. The PCR technique allows specific amplification using low concentration or purification level of the target DNA (Biswas et al., 2014a). In this study, the identity of the PCR products was confirmed by sequencing the positive samples.

This is the first molecular diagnosis of *M. phaseolina* associated with watermelon in Northeastern Brazil. Negreiros et al. (2019) and Sales Júnior et al. (2019a) reported the pathogen associated with weeds infesting melon and watermelon production fields in the region. In watermelon, charcoal rot caused by *M. phaseolina* occurs at the end of the crop cycle and symptoms are directly influenced by the infestation level in the growing areas (Cohen et al., 2016).

Implementing a management system to prevent the disease is a sustainable and effective way to control the pathogen. In this sense, the detection of *M. phaseolina* by direct PCR described here reduces the cost and time of diagnosis and is an accessible way, especially with handle a large number of samples. With appropriate modifications to the protocol, this methodology can be applied to different crops and pathogens worldwide.

Conclusion

By direct PCR it was possible to detect *M. phaseolina* in roots of watermelon plants from west of Rio Grande do Norte State, Brazil. The diagnostic method was validated by confirming the identity of the amplified samples.

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