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SHORT COMMUNICATION

Detection of *Pantoea ananatis* in corn seeds on semi-selective medium

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Abstract

Maize white spot, caused by the bacterium *Pantoea ananatis*, is one of the main leaf diseases of maize in Brazil. However, until now, its presence in corn seeds has not been detected. In this study, *P. ananatis* was detected in corn seeds using a semi-selective culture medium. A bacterial suspension of *P. ananatis* was serially diluted ten-fold and plated on 523, YDC or semi-selective PA20 media, with or without the addition of methyl thiophanate. The detection of bacteria in seeds was evaluated in three seed lot samples, collected from field maize plants with white spot symptoms. Seed washings were prepared, ten-fold serially diluted and plated on the same three culture media. Addition of methyl thiophanate to 532, YDC and PA20 culture media did not inhibit bacterial growth. *Pantoea ananatis* was detected in two corn seed samples on YDC and PA20 media, which was confirmed by biochemical, physiological and molecular characterization as well as by pathogenicity tests. This is the first report of *P. ananatis* detection in corn seeds.

Keywords Etiology · PCR · White spot · *Zea mays*

Maize white spot, caused by the bacterium *Pantoea ananatis* (Serrano) Mergaert et al. 1993 (Paccola-Meirelles et al. 2001), is considered one of the major leaf diseases of maize in Brazil (Costa et al. 2013). Leaves with 10 to 20% disease severity exhibit about 40% reduction in photosynthetic rate (Godoy et al. 2001). The disease can cause over 60% yield losses (Casela et al. 2006).

Maize white spot incidence and severity have increased over the years in Brazil (Costa and Cota 2009). However, *P. ananatis* has not been detected in corn seeds yet. Seeds deserve special attention in regard to bacterial dissemination, since they can transport the pathogen over long distances and are important primary sources of inoculum in the field (Neergaard 1979), thus justifying the need to run seed phytosanitary tests before their commercialization or sowing.

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The detection of *P. ananatis* in seeds and its transmission has been reported for anions (Goszczynska et al. 2006a) and rice (Azegami et al. 1983). Control of bacterial diseases in plants is difficult, warranting the use of healthy seeds, certified by laboratory analyses. Immunological and molecular techniques have been described for bacteria detection in seeds (Tebaldi et al. 2010). Also, the semi-selective medium PA20 has been used for detection of *P. ananatis* in onion seeds (Goszczynska et al. 2006b). The advantages of using semi-selective culture media for bacterial detection are that expensive equipment is not required and that they can be used routinely in the laboratory. Therefore, the objective of this study was to detect *P. ananatis* in corn seeds using a semi-selective medium.

The experiment was conducted in the Laboratory of Plant Bacteriology (LABAC) and in the greenhouse of the Institute of Agricultural Sciences at the Universidade Federal de Uberlândia, Minas Gerais, from March to June 2015. The isolate UFU E43 of *P. ananatis,* previously obtained from maize leaves in Uberlândia, and deposited in the culture collection of the LABAC, was grown on solid 523 medium (Kado and Heskett 1970) for 48 h. Subsequently, a bacterial suspension was prepared in 0.85% NaCl and adjusted to an $OD_{550} = 0.5$, corresponding to approximately 1×10^9 CFU/mL. The bacterial suspension was serially diluted (10^{-1} to 10^{-7}) and plated on solid 523, YDC or semi-selective PA20

media (Goszczynska et al. 2006b), with or without methyl thiophanate (0.04 mg/L). Three Petri dishes were used for each dilution, and were incubated at 28 °C for four days, when the number of colony forming units (CFU)/mL were counted. The data were subjected to analysis of variance and the averages compared by Tukey's test at 5% probability (Ferreira 2008).

Bacteria detection in seeds was evaluated in three corn seed lot samples obtained from plants with white spot symptoms collected in seed production fields located in Formosa, Goiás, Brazil. One hundred grams of seeds were soaked in 200 mL of 0.85% saline solution for 18 h in a refrigerator. Subsequently, the seed washing was serially diluted (10^{-1} and 10^{-2}) and plated on the culture media previously described, with the addition of methyl thiophanate. The Petri dishes were incubated at 28 °C for 4 days, time at which yellow colonies, typical of *P. ananatis*, were counted to determine the number of CFU/g of seed. Bacterial colonies were then transferred onto solid 523 medium.

Isolates obtained were biochemically and physiologically characterized by the following tests: Gram reaction by means of the solubility of colonies in 3% KOH solution; growth on YDC medium; glucose oxidation or fermentation; motility; acid production from glucose, inositol, sorbitol, sucrose and D-arabinose; production of arginine dihydrolase, oxidase, and catalase; gelatin liquefaction; growth at 37 °C; and ability to cause the hypersensitive reaction (HR) in tobacco leaves (Mariano and Silveira 2005).

The molecular characterization of the bacterial isolates was performed by amplifying the 16S rRNA gene sequence from genomic DNA using the universal primers 27f (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1492r (5’ TAC GGY TAC CTT GTT ACG ACT T 3) (Lane 1991), which was compared with sequences of *P. ananatis* deposited in the GenBank. In addition, the genomic DNA of the bacterial isolates was used to PCR amplify a 389-bp species-specific fragment using the primers ANAf (5’CGT GAA ACT ACC CGT GTC TGT TGC 3′) and ANAr (5’ TGC CAG GGC ATC CAC CGT GTA CGC T 3′) (Figueiredo and Paccolla-Meireles 2012; Sauer et al. 2015).

The pathogenicity of bacterial isolates obtained from the seed washings was evaluated in maize seedlings grown in 500-g plastic pots containing soil, coarse sand and vermiculite (3:1:1), with two seedlings per pot. Inoculation was performed ten days after sowing (when plants had three to four leaves) using a cotton swab moistened in a bacterial suspension at $OD_{550} = 0.5$ (approx. 10^9 CFU/mL). The seedlings were maintained under high humidity 24 h before and after inoculation by covering them with 5-L plastic bags. The temperature in the greenhouse ranged from 18 to 30 °C during the experiment. Four days after inoculation, symptoms of white spot were observed as light green, water-soaked-like, round lesions.

The number of CFU/mL of *P. ananatis*, which varied from 3.7×10^9 to 4.9×10^9 CFU/mL, did not significantly differ among the culture media 523, YDC and semi-selective PA20, with or without the addition of methyl thiophanate. Moreover, methyl thiophanate did not inhibit bacterial growth. Similarly, Mehta et al. (2005) did not find significant differences in the number of CFU/mL during detection of *Xanthomonas axonopodis* pv. *malvacearum* in cotton seeds when comparing standard and semi-selective growth media.

The colonies of *P. ananatis* presented the following cultural characteristics on 523 and YDC media: yellow color, shiny, circular, flat and with smooth edges. In contrast, on the semi-selective PA20 medium there was a reduction in the diameter of the colonies, which were circular, shiny, with light-yellow haloes and dark yellow centers, resembling a “fried egg”. Size reduction of bacterial colonies was also observed when *Xanthomonas axonopodis* pv. *vignicola* from grapevine was detected on semi-selective CCM medium (Peixoto et al. 2006). According to Goszczynska et al. (2006b), the reduction in colony diameter is due to a pH reduction of the medium caused by the bacteria.

P. ananatis was detected in two corn seed lot samples, on both YDC and PA20 media with addition of methyl thiophanate. The number of bacterial cells recovered from seeds varied from 2×10^2 to 1.6×10^3 CFU/g of seed. Diameter reduction, light yellow haloes, and dark yellow centers were observed in colonies formed on semi-selective PA20 medium. The same morphological characteristics were observed when *P. ananatis* was recovered from onion seeds on PA20 medium (Goszczynska et al. 2006b). The addition of methyl thiophanate to the semi-selective PA20 medium was essential for preventing the growth of saprophytic microorganisms, which facilitated the detection and identification of *P. ananatis* in corn seeds.

Bacteria detected in corn seeds were Gram negative; positive for formation of yellow colonies on YDC medium; glucose fermentation; oxidase; catalase; and arginine dihydrolase; acid production from inositol, sorbitol, sucrose, and D-arabinose; motility; gelatin liquefaction; growth at 37 °C; and causing the hypersensitive reaction in tobacco leaves. They were also pathogenic to maize seedlings, in which they reproduced the water-soaked lesions, characteristic of white spot. Sequencing of the 16S rRNA gene region and amplification of the 389-bp fragment with the species-specific primer pair ANAf/ANAr confirmed them as *P. ananatis*. The bacterial isolates obtained from seed washings were preserved in glycerol and maintained in the LABAC collection, at the Universidade Federal de Uberlândia.

In conclusion, the semi-selective PA20 medium, with the addition of methyl thiophanate, was effective for the detection of *P. ananatis* in corn seeds, and is recommended for routine laboratory analysis. To our knowledge, this is the first report on the detection of *P. ananatis* in corn seed lots.

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