Survival of *Pantoea ananatis*, causal agent of maize white spot disease in crop debris

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ABSTRACT

Maize white spot, caused by the bacterium *Pantoea ananatis*, is one of the most detrimental diseases of maize in Brazil and has contributed to significant yield reduction. In this study, *P. ananatis* isolates were recovered from maize white spot lesions, healthy leaves, corn crop residues and *Digitaria horizontalis*. All the bacterial isolates were identified and characterized by morphological traits analysis, acid production from sorbitol and glycerol, ice nucleation activity, metabolic fingerprint similarities and molecular analysis by Polymerase Chain Reaction with species-specific primers for *P. ananatis* (ANAF/ANAR). Results showed that isolates recovered from maize white spot lesions were similar to those isolates collected from other sources. It suggests that corn crop residues, *D. horizontalis*and healthy leaf surface of the corn plant itself, could be a source of survival for *P. ananatis*.

Key words: epiphytic bacteria, *Digitaria horizontalis*, metabolic fingerprint, ice-nucleation, corn crop residues.

Maize (Zea *mays* L.) is one of the most important crops in the world. It is estimated that worldwide production was around 872 million ton in the 2012/2013 harverst. The United States, China and Brazil are the largest maize producers in the world, and they are responsible for 31, 24 and 8% of the world's production, respectively (FAO, 2013).

Several diseases bring economical and agronomic problems to this crop, and the foliar disease caused by the bacterium *Pantoea ananatis* is one of the most important. The disease has been termed maize white spot (MWS) or leaf spot disease of maize (Figure 1) (Paccola-Meirelles et al., 2001; Malagi et al., 2011). The MWS disease appeared in Brazil in the 1980s and rapidly spread over all corn producing regions. The symptomps include water-soaked lesions on the leaves, which become necrotic and straw-colored and can be disseminated to the entire leaf surface in advanced stages of the disease (Paccola-Meirelles et al., 2001). The disease can cause premature leaf dry, shortening plant cycle and reducing grain length and weight (Pinto, 2004).

P. ananatis associated with maize lesions has been also reported in South Africa (Goszczynska et al., 2007), Mexico (Pérez-y-Terrón et al., 2009); Poland (Krawczyk et al., 2010) and Argentina (Alippi and López, 2010) and it has caused diseases in a wide range of other economically important agricultural crops and forest tree species worldwide (e.g. onion, rice, pineapple, melon, sorghum, *Eucalyptus* and others) (Walcott et al., 2002; Kido et al., 2008; Coutinho and Venter, 2009; Cother et al., 2010; Cota et al., 2010). It is regarded as an emerging pathogen based on the increasing number of reports on MWS disease occurring in previously unrecorded hosts in different parts of the world (Coutinho and Venter, 2009).

Very little is known about the spread and survival of *P. ananatis*. The knowledge of this pathogen survival in cultures is important since control measures can be simpler and less expensive during the establishment phase of the primary inoculum than thereafter. This study is focusing on identifying the potential source of survival of *P. ananatis* in corn crops through a comparative study between *P. ananatis* isolates from MWS lesions, epiphytic isolates from healthy leaves, isolates from corn crop residues and *D. horizontalis*.

Leaves of the susceptible maize cultivars, HS200 and DAS657, at the tasseling stage with MWS lesions and *D. horizontalis* with lesions similar to MWS were collected from naturally infected plants in Londrina, Paraná (PR), Brazil. The samples were washed with neutral soap. The water-soaked lesions were removed from leaves and sterilized with 70% etanol (1 min), 0.25% chloramine T (4 min), 70% ethanol (30 s) and washed three times with sterilized water (30 s each). Water from the last wash was plated out on trypticase soy agar (TSA) culture media, to evaluate the efficiency of the disinfection procedure. The edges of each segment (approximately 1 mm) containing lesions were removed with a sterile scalpel, transferred to TSA medium and incubated at 30 ºC ± 2 ºC, during 24 h with photoperiod of 12 h light and 12 h dark. After 24 h, typical yellow colonies morphologically similar to *P. ananatis* were isolated in pure colonies and maintained in TSA medium.

Epiphytic bacteria were isolated from healthy leaves collected from three maize cultivars HS200, DAS657 (both susceptible to MWS) and 2B710 (resistente to MWS). Leaves were collected 50 days after seeding, cut in pieces and 2.5 g were incubated in 100 mL phosphate buffer 0.1M (pH 7.0) supplemented with peptone 0.1 (w/v) at 30 ºC for 2 h in agitation (140 rpm) using glass beads. Subsequently, they were centrifuged at 4,500 rpm for 10 min. The pellet was resuspended in 9 mL saline solution (0.85% NaCl) and 0.1 mL was incubated at 30 ºC for 48 h in TSA medium supplemented with erytromycine (40 µg/mL) and cycloheximide (5 µg/mL) (Sauer et al., 2009). The bacteria were purified and maintained in TSA medium.

Bacteria from crop debris were recovered from two cultivars (HS200 and DAS657) which plants were naturally infected by *P. ananatis* causing MWS. Crop debris with MWS were collected 60 days after harvesting. Plant material was cut in pieces and 0.5 g incubated in 100 mL phosphate buffer 0.1M (pH 7.0) supplemented with peptone 0.1 (w/v) at 30 ºC for 2 h in agitation (140 rpm) using glass beads. Subsequently, it was centrifuged at 4,500 rpm for 15 min. The pellet was resuspended in 9 mL saline solution (0.85% NaCl) and 0.1 mL was incubated at 30 ºC for 48 h in TSA medium supplemented with erytromycine (40 µg/mL) and cycloheximide (5 µg/mL) (Sauer et al., 2009). The bacterial isolates were purified and maintained in TSA medium.

Figure 1. Maize white spot (MWS) symptoms. A) maize plants severely attacked by MWS; B) detail of the symptoms of MWS on maize leaf.

The bacterial isolates showing similar morphological characteristics to *P. ananatis* were characterized by Gram staining, sorbitol/glicerol test (Schaad et al., 2001). The expression ice nucleation activity (INA) was also evaluated according protocol defined in Romeiro (2001). The bacterial isolates were incubated in 5 mL of TSB medium at 30 ºC for 24 h with agitation (60 rpm). Subsequently samples were mixed by vortex to homogenize the samples and 100 µl were added to 1 mL of sterilized distilled water (cold at -10 ºC), to evaluate ice formation . Negative controls were 100 µl of TSB bacteria free medium.

The metabolic fingerprint of isolates from lesions and from crop debris was performed using the biolog identification system (BIOLOG GN2 MicroPlate, Hayward, CA, USA). The metabolic patterns were read and identified using the MicroLog™ software. A similarity matrix among isolates was constructed using the Jaccard coefficient and the UPGMA method (Unweight Pair Group Method with Arithmetic Average) with NTSYS-Pc software (Applied Biostatistics Inc., NY, US).

For molecular analyses, the genomic DNA was purified according Gürtler and Stanisich (1996). The identity of the isolates was determined by partial sequencing of the 16S rRNA gene or the species-specific pair of primers designed for *P. ananatis*: ANAF (forward) (5'-CGTGAAACTACCCGTGTCTGTTGC-3') and ANAR (reverse) (5'-TGCCAGGGCATCCACCGTGTACGCT-3') (Figueiredo and Paccola-Meirelles, 2012). FTA cards (Whatman Inc., Clifton, USA) with preserved DNA from reference strains *P. ananatis* (PNA 08-2, PNA 97-5 and PNA 99-13) were used as positive controls. Specific PCR was performed to confirm *P. ananatis* isolates according Paccola-Meirelles et al. (2001). PCR was performed using 25 ng of genomic DNA as template. The reaction cocktail consisted of 2.5 µl 10x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 2.0 µM of each primer, 25 mM dNTP, 2,5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Phoneutria, Belo Horizonte, Brazil) in a final volume of 25 µl. The amplification protocol consisted of previous denaturation step of 94 ºC for 1 min followed by 30 cycles of amplification (94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min) with a final extension step of 72 ºC for 10 min. PCR was performed in a model PTC-100 thermolcycler machine (MJ Research, MS, USA). Amplicons were analyzed by horizontal gel electrophoresis at 6 V/cm^2 in 1.0% agarose gel (wt/v) in 1x TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) and subsequent staining with EtBr (0.5 mg/L) and visualized under UV light.

A total of 8 isolates were recovered (3 isolates from lessions, 2 from crop debris, 2 from healthy leaves and 1 from *D. horizontalis*). All of them were Gram negative with colony morphology similar to *P. ananatis* and yellow pigmentation. All isolates were confirmed as *P. ananatis* species by PCR using the specie-specific primers ANAF/ANAR. Ice nucleation and sorbitol/glicerol tests are summarized in Table 1, showing that crop debris isolates maintain the ice nucleation activity 60 days post-harvesting corn grains. Metabolic fingerprinting of all isolates was obtained and the similarity dendogram is shown in Figure 2. Isolates 1 and 3 were the most divergent and they were both collected from lesions (0.53 coefficient). Isolates from crop debris were included in a cluster that grouped one isolate from lesions (isolate 2) and other from healthy leaves (isolate 7) with 0.89 similarity coeffi-

Table1. Ice nucleation activity and sorbitol/glicerol tests of isolates recovered from lesions, corn crop debris, maize healthy leaves and *Digitaria horizontalis*.

Isolate	Origin	Ice Nucleation Activity	Acid production from Sorbitol/ Glicerol
1	MWS lesion	+	+/+
2	MWS lesion	+	+/+
3	MWS lesion	+	+/+
4	Corn crop debris	+	+/+
5	Corn crop debris	+	+/+
6	Maize healthy leaves	+	+/+
7	Maize healthy leaves	+	+/+
8	*D. horizontalis lesions*	-	+/+

cient. *D. horizontalis* isolate (8) was clustered with 0.71 coeffient along with isolates 2, 4, 5, 6 and 7.

In this study the majority of isolates were positive to ice-nucleation, including those recovered from crop debris. This feature allows the bacterium to invade the host tissue more efficiently (Lindow et al., 1978) causing important agronomic and economical problems. In addition, the metabolic pattern of isolates recovered from crop debris were similar to those recovered directly from lesions or from healthy leaves, suggesting that, in crop debris, these bacteria could act similarly to epiphytic isolates, being a source of inoculum for further infections (Escanferla et al., 2006). The recovery of viable and culturable forms of *P. ananatis* from crop debris 60 days after harvest supports the hypothesis that the pathogen can survive under adverse conditions, minimizing its nutritional requirements (Wilson et al., 1999). Results from this study have contributed to increase knowledge on *P. ananatis* survival, which has also been reported to survive in other species of *Digitaria spp*. (Gitaitis et al., 2002).

In conclusion, *P. ananatis* may survive as epiphytic in the leaves of healthy maize plants, non-host plants and in crop debris, and possibly multiply there. Thus, knowledge on how this pathogen's survival will help to improve the control of this important and serious foliar disease in maize.

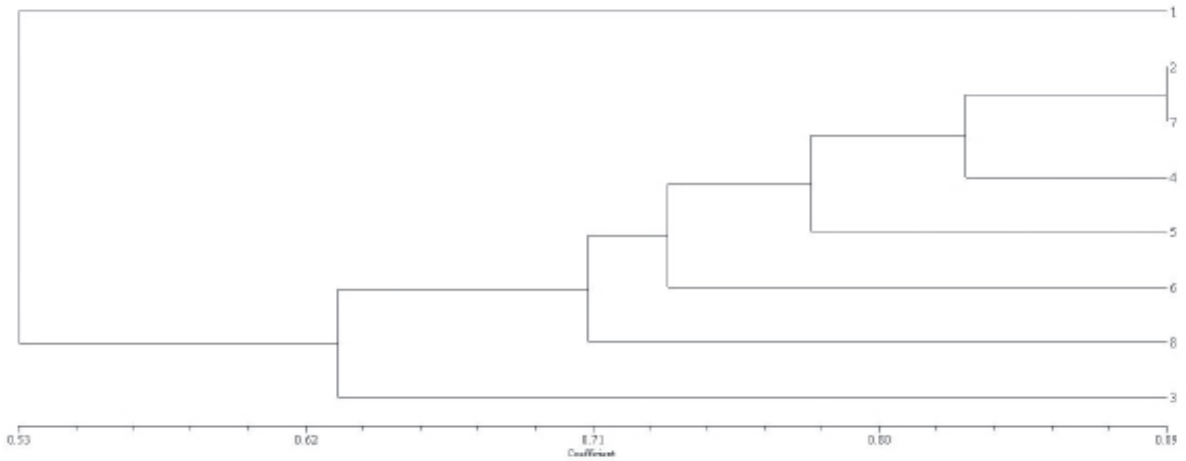

Figure 2. Cladogram tree among *Pantoea ananatis* isolates applying the Jaccard coefficient of similarity and the UPGMA method using NTSYS-Pc software.

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