Viability and dissemination of *Pantoea ananatis*, etiological agent of Maize White Spot disease

Maria Eugenia Escanferla[1], Philip Traldi Wysmierski[1], Walter Fernandes Meirelles[2], Luzia Doretto Paccola-Meirelles[1,3,*]

[1]Universidade Estadual de Londrina, Departamento de Biologia Geral, Londrina, PR, Brazil. [2]Embrapa Milho e Sorgo, Sete Lagoas, MG, Brazil. [3]Laboratório de Biologia Molecular, Universidade Paranaense, Umuarama, PR, Brazil. *Corresponding author, E-mail: paccola@uel.br

ABSTRACT

Maize white spot (MWS) is a disease widely spread in maize production regions in Brazil and causes serious economic damages to the culture. Little is known about the dissemination, growth and development conditions of the MWS causal agent, the bacterium *Pantoea ananatis*. The objective of this work was to determine the viability of this bacterium, as well as its distribution in the plant. *P. ananatis* after isolated was stored in both, Nutrient Broth (NB) medium and in mineral oil. For the viability tests, the cultures stored in NB were maintained at 12ºC and –6ºC and the cultures stored in mineral oil were maintained at room temperature. The isolate remained viable for seven months in NB medium at the two temperatures assessed, and four months when stored in mineral oil. The presence of the *P. ananatis* bacteria was analyzed in, 1) soil samples collected close to rhizosphere, 2) in seeds from plants severely attacked by MWS, 3) foliar segments of healthy plants and 4) the stem of healthy plants. *P. ananatis* was found surviving epiphytically on the leaf surface and its population increased with plant age, air relative humidity, and decreasing of temperature. This agent was not found neither rhizosphere nor in seeds, but it was isolated in low quantity in maize stem. *P. ananatis* resides epiphytically on leaves and, due to several factors, may trigger injuries to its host. All epiphytic isolates were characterized in regards to ice nucleation activity, a characteristic of this pathogen.

Key words: *Erwinia ananatis*, ice nucleation, epiphytic bacteria, MWS symptoms.

INTRODUCTION

The maize white spot disease (MWS) (Figure 1) was observed by first time in Brazil in the 80's, and its incidence and severity has increased significantly since 90's. Today it has been found in all maize producing regions of the country (Fernandes and Oliveira 1997; Costa et al., 2009).

Figure 1. Maize leaf with MWS lesions under natural infection conditions (A), MWS lesions reaching superior leaves (B), and corn ears with MWS lesions (C).

Disease incidence increases under high air relative humidity, and moderate temperatures, mainly when it increases after raining. Relative humidity above 60% and night temperatures around 14 °C are considered favorable for disease development (Costa et al., 2011). These conditions occur frequently during the maize cultivation period in several Brazilian regions (Sachs et al., 2011).

The disease initial stages are characterized by presence of small dark green water-soaked leaf spots, that later become necrotic, straw-colored, which may be circular, elliptical or oval in form and with a diameter varying from 0.3 to 2.0 cm (Fernandes and Oliveira 1997; Paccola-Meirelles et al., 2001; Lana et al., 2012) (Figure 2).

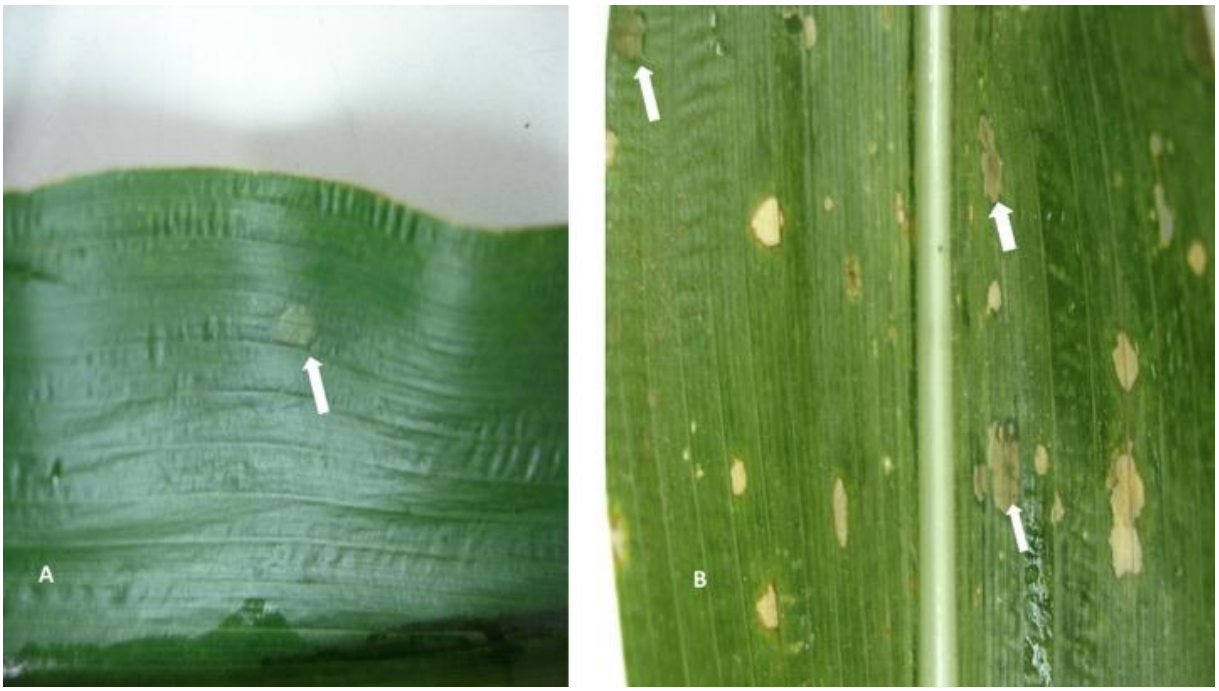

Figure 2. Young lesions of Maize White Spot according to the classification of Paccola-Meirelles et al. (2001). A: water-soaked leaf spot at the initial stage, Stage 1 (arrow); and B: water-soaked leaf spots at stage 2 (arrows).

The MWS symptoms begin during the flowering period, turning into more severe after tassel (Costa et al., 2009). The lesions appear first in the inferior leaves, in the distal portions, progressing rapidly towards the superior part of the plant (Fernandes and Oliveira 1997; Costa et al., 2011).

It has been observed that the disease has occurred alarmingly in younger plants, in a 40-day crop, affecting their superior leaves successively and sometimes affecting the whole plant, including the ear (Figure 1B). Under favorable conditions, the disease causes premature drying of the plant and serious damages to the grain filling process, with cycle reduction and strong decrease in grain size and weight (Balmer and Pereira 1987).

Paccola-Meirelles et al. (2001, 2004) described the bacterium *Pantoea ananatis* as being the etiological agent of the disease, later this was confirmed by Gonçalves et al. (2013). According to Sauer et al. (2014), isolates of de *P. ananatis*, obtained from MWS lesions have the capacity to catalyze the formation of nuclei or ice crystals in the presence of water, under temperatures where this phenomenon would never occur. This is a mechanism known as ice nucleation activity (INA). Active bacteria in ice nucleation are denominated INA+ (Lindow et al., 1982).

There is very little information about the biology of this pathogen, its dissemination in the plant and environment. So, the objective of this work was to evaluate the viability of *P. ananatis* and its distribution through the plant.

MATERIALS AND METHODS

Isolation of *P. ananatis*

The bacterium *P. ananatis* was isolated from young lesions of the disease also known as water-soaked spot (Figure 2). Bacterial isolation methodology (Figure 3) followed the technique described by Paccola-Meirelles et al. (2001). Maize leaves with water-soaked spot (Figure 2) were collected from maize plants growing in the field at the tasseling stage at Embrapa Soja Londrina – PR. The leaves were washed with neutral soap and dried with sterile paper.

The water-soaked spot lesions were detached from leaves with the aid of a scalpel, disinfected with alcohol 70% (1 min), chloramine T 0.25% (4 min) and washed three times with sterilized distilled water for 1 min (each washing). The water from the last washing was plated on Nutrient Agar (NA) medium to test the efficiency of the disinfection methodology.

The borders of each lesion were removed (approximately 1 mm) the aid of a sterilized scalpel and each segment transferred to NA medium added of cycloheximide (50 µg/mL of the medium). Plates were incubated at 30ºC $\pm$ 2ºC. Two days after incubation, bacteria with morphological characteristics similar to *P. ananatis* were isolated, purified on NA medium and stoked in this same medium. Isolate identity was confirmed by the technique described by Figueiredo and Paccola-Meirelles (2012).

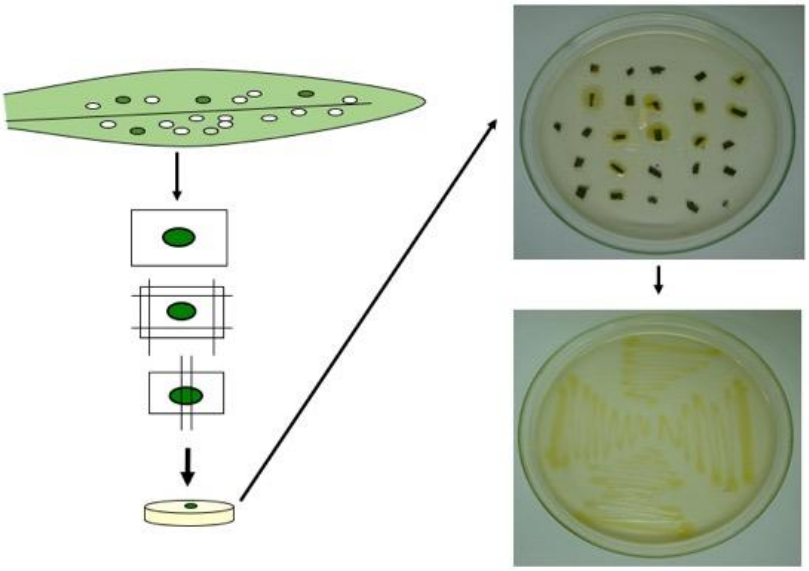

Figure 3. Isolation and purification of the causal agent of the MWS, the bacterium *P. ananatis*, from water-soaked spot on maize leaves.

Viability of *P. ananatis* under different storage conditions

Two experiments were carried out for evaluated the viability of *P. ananatis* from MWS disease. In the first experiment the isolates were maintained in NB medium at two temperatures, 12ºC and at -6ºC. For this experiment, the isolates were cultivated during 8 hours in Nutrient Broth (NB) medium at 30 ± 2ºC. Culture aliquots (100µl) were transferred to tubes containing 5 ml of NB and incubated at 30 ± 2ºC for 24 h. After this period, part of the culture was kept at 12ºC and the other part at -6ºC. The cellular viability was evaluated every 30 days through plating 100µl of each culture on Nutrient Agar (NA) medium. Three replications were realized for each evaluated temperature.

In the second experiment, the isolates were stored on NA medium and covered with mineral oil. For this, tubes containing inclined NA medium were inoculated with *P. ananatis* and incubated at 30 ± 2ºC for 24 h. After this period the cultures were covered with mineral oil. Monthly the culture was transferred to petri plates with NA medium to check the viability of *P. ananatis*. This procedure was repeated during five months. The isolates recovered of first and second experiment were evaluated in regards to ice nucleation activity.

Ice nucleation test

The isolates were cultivated at 30 ± 2ºC in NB and aliquots of 100µl of this culture were transferred to tubes containing 1ml of sterilized distilled water at -5ºC. The immediate development of ice crystals revealed the INA^+ phenotype of the isolate. For the control, was used 100µl of the NB without bacteria.

Distribution of *P. ananatis* in maize plants

I) In the rhizosphere: Soil samples from 12 randomized locations were collected from maize experimental field of the Londrina State University/ Londrina/PR. Samples were collected from the first 10 cm of soil, next to the adventitious roots. The samples were mixed, homogenized and 0.5 kg of each one of them was sieved. Ten grams of the material were added to 95 mL of sterilized saline solution and the suspension strongly agitated on vortex. Serial dilutions techniques in saline solution were used and 50µL of each suspension were plated on the NA medium and selective medium (Kado and Heskett 1970).

II) In maize seeds: Twenty seeds from plants infected by MWS were disinfected in alcohol 70% (1 min), chloramine T 0.25% (4 min) and washed three times with sterilized distilled water (1 min each washing). Water from the last washing was plated on NA medium for disinfection methodology control. Part of the disinfected seeds were deposited on NA medium and part was segmented with a sterilized scalpel. The segments were transferred to NA medium. The petri plates were incubated at 30ºC ± 2ºC.

III) In maize leaves and stem: The presence of the bacterium *P. ananatis* on leaves and stem was verified on healthy corn plants at three different stages of development. The stages were based on the number of leaves on the plant, V10, V12 and V16. Three repetitions were made for each stage. One leaf of each stage was removed from

median portion of the plant. The same was made for the stem. The leaves collected were segmented in small segments and samples of two grams were incubated in 100ml of buffer phosphate 0,1M pH 7.0 added of 0.1% of bacteriological peptone at room temperature for 2 h at 60 rpm. The same procedure was also performed with foliar segments previously disinfected [alcohol 70% (1 min), chloramine T 0.25% (4 min) and three washings in sterilized distilled water (1 min each washing)]. Aliquots of 100µl were removed from these incubation buffers and plated on NA medium and NAG medium (NA medium with 2.5% of glycerol). The plates were incubated at 30 ± 2ºC, and the bacterial colonies were then counted after 24 to 48 h in order to quantify the number of bacterial colonies with similar morphology to *P. ananatis*. These colonies were isolated and the INA activity verified according to the methodology described previously.

Segments of the stem were disinfected externally, according to the procedure used during the leaves disinfection, and chopped with a sterilized scalpel. Small segments were deposited on NA medium and NAG medium and incubated under the same conditions described above, with three replications for each treatment.

RESULTS AND DISCUSSION

P. ananatis Isolation

The bacterial isolation procedure from water-soaked leaf spots (Figure 3) was successful at obtaining 20 bacterial isolates from 52 lesions. Foliar disinfection during the isolation process was adequate, which is evidenced by the lack of bacterial growth in the media where the water of the last washing was plated, showing that the bacterial isolates have been originated from the lesion and not from the leaf surface. The isolate PaE2 of P. ananatis was selected to be used for the tests of viability.

P. ananatis viability

The isolate PaE2 remained viable for a period corresponding to seven months when maintained on NB medium at 12ºC and at -6ºC. However, when this isolate was stored in oil it remained viable by a shorter period of time, only four months. After the stocking, the PaE2 isolate maintained its ice nucleation capacity expressing its INA^+ phenotype. Preservation methods that allow the stability of the morphological, physiological and genetic characteristics of bacterial isolates for longer periods are necessary (Sola et al., 2012). The Figure 4 shows the results of the ice nucleation test for the PaE2 isolate where it was possible to see the development of an ice block after the deposition of bacterial cells in the cold water.

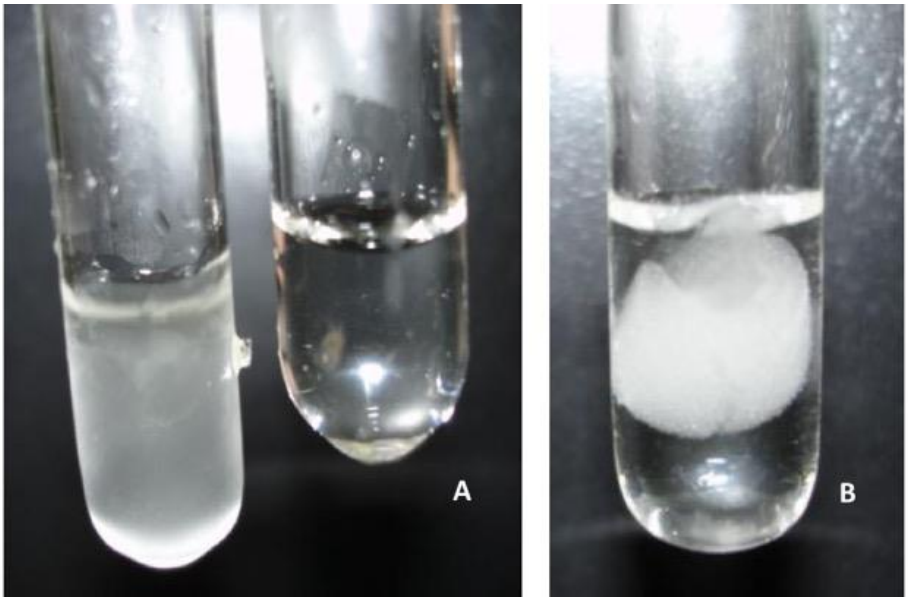

Figure 4. Ice nucleation test with the PaE2 isolate of *P. ananatis* from MWS. A: Left test tube contains cold water inoculated with the PaE2 INA+ isolate, which can be checked for ice formation. The right test tube shows the control (without bacteria); B: Visualization of the block of ice of the tube A during process of defrosting.

The ice nucleation phenomenon described for bacteria of *Pseudomonas*, *Pantoea* and *Xanthomonas* genus (Maki et al., 1974; Turner et al., 1991; Lindow and Andersen 1996) may cause serious damages to the host due to freezing the water at temperatures under which it would never occur. According to Sauer et al. (2014) the occurrence of this phenomenon in *P. ananatis* isolated from MWS foliar lesions suggests that the ice crystals formed by bacteria in the intercellular spaces of the foliar epidermis might be responsible by rupture of the cell

wall of the infected tissue. Whenever such fact occurs, the cell membrane collapses, resulting in a tissue disorganization with further development of a soaked lesion (Figure 2), which later becomes necrotic due to the death of tissues by freezing (Figure 1). Bomfeti et al. (2008) detected this cell disorganization inside MWS lesions through optical and electron microscopy.

Distribution of *P. ananatis* in maize plants

In this study *P. ananatis* was found neither in the soil samples nor in corn seeds. Rijavec et al. (2007) and Mamed et al. (2018) succeeded in isolating *P. ananatis* from maize seeds. Rijavec et al. (2007) evaluated seeds of four maize cultivars but detected the presence of *P. ananatis* in only one of the cultivar. Similar results were obtained by Mamed et al. (2018). These authors evaluated three corn seed lot samples obtained from plants with MWS symptoms. The bacterium *P. ananatis* was detected only in two corn seed lot samples, demonstrating a variability regarding the presence of the bacterium in the seeds. This specie might be associated with specific maize cultivars, causing this variability. The bacteria isolated of maize seeds by Mamed et al. (2018) reproduced MWS symptoms when inoculated on maize seedlings. *P. ananatis* has also been reported in seeds of others cultures, such as onion (Goszczynska et al., 2006a, 2006b) and sudan- grass (Azad et al., 2000).

According Coutinho and Venter (2009) and De Maayer et al. (2014), *P. ananatis* may occupy a wide variety of environments such as the air, insects, water, soil and plants. Depending of the host and ecological niche, *P. ananatis* strains may be mutualists, saprophytes or pathogenics. It was described as epiphytic on leaves of healthy maize plants by Bomfeti et al. (2008) and on maize crop debris by Sauer et al. (2015). Our results showed that *P. ananatis* can be isolated from health maize leaves surface at different stages (10, 12 and 16 leaves). When the foliar segments were previously sterilized, the amount of *P. ananatis* was reduced to zero or close to zero (Figure 5).

With the growth of the maize plant was possible to observe also an increase of the bacterial population on the foliar surface (Figure 5). In addition, there was better growth when the isolation was done on NAG medium compared to the medium without glycerin (Figure 5). According Lindow et al. (1977) INA+ bacteria show greater ice nucleation activity when cultivated on medium with this additional source of carbon.

The foliar epiphytic *P. ananatis* were purified and evaluated in regards to the INA+ characteristic. All isolates were INA+. A low amount of *P. ananatis* was isolated from maize stem of plants in stage V10 and V12. No bacterium was recovered from corn stem at V16 stage. Environmental conditions of high humidity and milder temperature contributed to increase the bacterial epiphytic population (Figure 5).

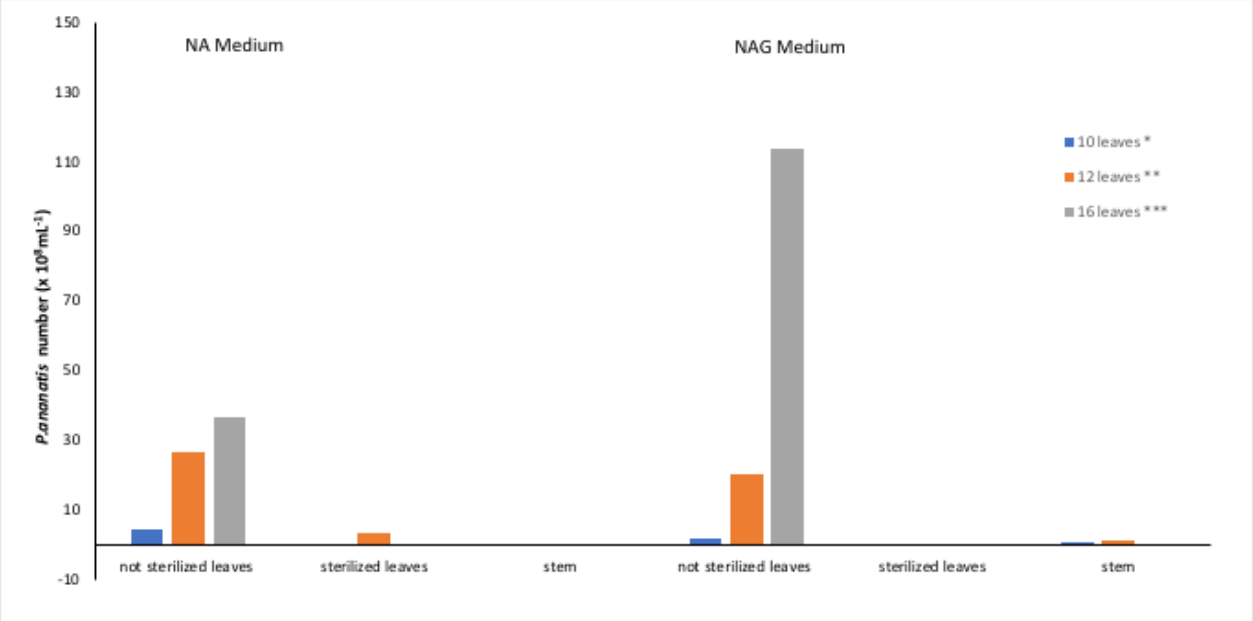

Figure 5. Number of *P. ananatis* bacteria in foliar and stem segments collected from maize plants of three different stage of development (10, 12 and 16 leaves).
Rainfall and temperature data (respectively) on the collection day:
* = 0.0 mm and 26.1ºC
** = 0.0 mm and 23.9 º C
***= 4.8 mm and 16.7º C

CONCLUSIONS

P. ananatis is a microorganism that resides epiphytically in maize leaves and that, by factors not yet clarified, may cause injuries to its host.

P. ananatis was not found neither in the maize rhizosphere nor in seeds, but it tends to be low in maize stem.

REFERENCES

Azad HR, Holmes GJ and Cooksey DA (2000) A new leaf blotch disease of sudangrass caused by *Pantoea ananas* and *Pantoea stewartii*. Plant Disease 84: 973-979.

Bomfeti CA, Souza-Paccola EA, Massola Jr NS, Marriel IE, Meirelles WF, Casela CR and Paccola-Meirelles LD (2008) Localization of *Pantoea ananatis* inside lesions of maize White Spot Disease using transmission electron microscopy and molecular techniques. Tropical Plant Pathology 33: 1-6.

Coutinho TA and Venter SN (2009) *Pantoea ananatis*: an unconventional plant pathogen. Molecular Plant Pathology 10: 325-335.

Balmer E and Pereira OAP (1987) Doenças do milho. In: Paterniani E and Viegas GP (eds) Melhoramento e Produção do milho. 2. ed. Fundação Cargil, Campinas, p. 595-634.

Costa RV, Casela CR and Cota LV (2009) Doenças. In: Cruz JC (ed.) Cultivo do milho. 5. ed. Embrapa Milho e Sorgo, Sete Lagoas.

Costa RV, Cota LV, Silva DD and Lanza FE (2011) Recomendações para o controle químico da mancha branca do milho. Circular Técnica da Embrapa 167: 1-6.

De Maayer P, Chan WY, Rubagotti E, Venter SN, Toth IK, Birch PR and Coutinho TA (2014) Analysis of the *Pantoea ananatis* pan-genome reveals factors underlying its ability to colonize and interact with plant, insect and vertebrate hosts. BMC Genomics 15:404.

Fernandes FT and Oliveira E (1997) Principais doenças na cultura do milho. Circular Técnica da Embrapa 26: 7-8.

Figueiredo JEF and Paccola-Meirelles LD (2012) Simple, rapid and accurate PCR-Based detection of *Pantoea ananatis* in maize, sorghum and *Digitaria* sp. Journal of Plant Pathology 94: 663-667.

Gonçalves RM, Figueiredo JEF, Pedro ES, Meirelles WF, Leite Junior RP, Sauer AV and Paccola-Meirelles LD (2013) Etiology of phaeosphaeria leaf spot disease of maize. Journal of Plant Pathology 95: 559-569.

Goszczynska T, Moloto VM, Venter SN and Coutinho TA (2006a) Isolation and identification of *Pantoea ananatis* from onion seed in South Africa. Seed Science Technology 34: 655-668.

Goszczynska T, Venter SN and Coutinho TA (2006b) PA 20, a semi-selective medium for isolation and enumeration of *Pantoea ananatis*. Journal of Microbiological Methods 64: 225-231.

Kado CI and Heskett MG (1970) Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. Phytopathology 60: 969-976.

Lana UGP, Gomes EA, Silva DD, Costa RV, Cota LV, Parreira DF, Souza IRP and Guimarães CT (2012) Detection and molecular diversity of *Pantoea ananatis* associated with white spot disease in maize, sorghum and crabgrass in Brazil. Journal Phytopathology 160: 441-448.

Lindow SE, Arny DC and Upper CD (1977) *Erwinia herbicola* ice nucleus active in increasing frost injury to corn. Phytopathology 68: 523-527.

Lindow SE and Andersen GL (1996) Influence of immigration on epiphytic bacterial populations on navel orange leaves. Applied and Environmental Microbiology 62: 2978-2987.

Lindow SE, Hirano SS, Barchet WR, Arny DC and Upper CD (1982) Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiology 70:1090-1093.

Maki LR, Galyan EL, Chiwn MC and Caldwell DR (1974) Ice nucleation induced by *Pseudomonas syringae*. Applied Microbiology 28: 456-459.

Mamede MC, Tebaldi ND, Mota LCBM, Martins OM and Coelho L (2018) Detection of *Pantoea ananatis* in corn seeds on semi-selective medium. Tropical Plant Pathology https://doi.org/10.1007/s40858-017-0203-z. Accessed 22 February 2018.

Paccola-Meirelles LD, Ferreira AS, Meirelles WF, Marriel IE and Casela CR (2001) Detection of a bacterium associated with a leaf spot disease of maize in Brazil. Journal of Phytopathology 149: 275-279.

Paccola-Meirelles LD, Marriel IE, Meirelles WF, Casela CR and Ferreira AS (2004) Reações sorológicas evidenciando a presença de bactéria no interior das lesões da mancha foliar de *phaeosphaeria* em milho**.** In: Proceedings 37th Congresso Brasileiro de Fitopatologia, Gramado, v. 29, p. 246.

Rijavec T, Lapanje A, Dermastia M and Rupnik M (2007) Isolation of bacterial endophytes from germinated maize kernels. Canadian Journal of Microbiology 53:802-808.

Sachs PJD, Neves CCSVJ, Canteri MG and Sachs LG (2011) Escala diagramática para avaliação da severidade da mancha branca em milho. Summa Phytopathologica 37:202-204.

Sauer AV, Rocha KR, Pedro ES, Meirelles WF and Paccola-Meirelles LD (2014) Ice nucleation activity in *Pantoea ananatis* obtained from maize white spot lesions. Semina Ciências Agrárias 35: 1659-1666.

Sauer AV, Rocha KR, Gonçalves RM, Meirelles WF, Figueiredo JEF, Marriel IE and Paccola-Meirelles LD (2015) Survival of *Pantoea ananatis*, causal agent of maize white spot disease in crop debris. Agronomy Science and Biotecnology 1: 21-24.

Sola MC, Oliveira AP, Feistel JC and Rezende CSM (2012) Manutenção de microrganismos: conservação e viabilidade. Enciclopédia Biosfera 8: 1398-1418.

Turner MA, Arellano F and Kozloff LM (1991) Components of ice nucleation structures of bacteria. Journal of Bacteriology 173: 6515-6527.