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ETIOLOGY OF PHAEOSPHAERIA LEAF SPOT DISEASE OF MAIZE

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SUMMARY

Different fungal species and the bacterium *Pantoea ananatis* (*Pa*) have been reported as etiological agents of *Phaeosphaeria* leaf spot (PLS) disease. This work aimed at using molecular identification of the fungi and bacteria occurring in PLS and its etiology. Genomic DNAs from (i) pool of each of the four stages of PLS lesions; (ii) bacteria and fungi isolated from lesions of natural PLS (NPLS) and artificial injuries (AI); (iii) fungi isolated from lesions obtained from plants inoculated with *Pa* in greenhouse (GH) were used in PCR with universal primers for bacteria and fungal rRNA genes, and species-specific primers for *Pa*. Bacterial amplicons were observed at all stages of lesions and fungal amplicons in stages 3 and 4. Bacterial amplicons of pooled NPLS lesions were from *Pa* while fungal amplicons were from *Phaeosphaeria* sp. and *Phoma* sp. Bacteria from NPLS, GH and AI lesions were identified as *Pa, Pa* and *Bacillus subtilis*, respectively, while the fungi were *Epicoccum nigrum, Leptosphaeria sacchari*, *Cochliobolus geniculatus*, *Pithomyces chartarum*, *Alternaria alternata*, *A. ricini*, *Gibberella intricans*, *G. fujikuroi*, *Phaeosphaeria* sp., *P. avenaria*, *Phoma* sp., *Phyllosticta* sp., *Sarocladium strictum*, *Glomerella graminicola*, and *Cochliobolus heterostrophus*. Symptoms of PLS decreased by 90% with the use of oxytetracycline in foliar treatment of maize plants in the field, and its addition to culture medium completely inhibited the growth of *Pa*. The results strongly show that *Pa* is the causal agent of PLS disease and different species of opportunistic fungi appear late in the necrotic stages of lesions caused by *Pa*.

Key words: Maize leaf spot disease, etiology, *Pantoea ananatis,* diagnosis, PCR.

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INTRODUCTION

The maize foliar disease, commonly known as Phaeosphaeria leaf spot (PLS) as well as leaf spot of corn, maize leaf spot, maize white patch, white spot, and maize white spot (MWS) disease is widespread throughout Central and South America, Asia, and Africa (Rane *et al*., 1966; Shurtleff, 1984; Carson *et al*., 1991; Casela, 1998; Carson, 1999).

The early stage of PLS disease is characterized by small dark green water-soaked leaf spots which may be circular, oval, elliptic, slightly elongate to oblong, 0.3 to 2.0 cm in diameter (Rane *et al*., 1966; Fantin, 1994; Carson, 1999; Paccola-Meirelles *et al*., 2001). Lesions are scattered over the leaf surface and have a chlorotic appearance, which later turns pale green, straw-colored, bleached and necrotic (Rane *et al*., 1966; Shurtleff, 1984; Fantin, 1994; Paccola-Meirelles *et al*., 2001). These lesions may coalesce, become irregularly shaped, and blight the entire leaf. Perithecia and pycnidia may develop at the center of the necrotic lesions in the late stages of the disease (Casela, 1998).

PLS can cause premature leaf drying thus shortening the plant growth cycle and reducing the size and weight of the grains (Pinto and Fernandes, 1995; Pinto, 2004). Although maize plants are generally affected at the end of the growing season, young plants may suffer a premature desiccation following more severe attacks (Cervelatti *et al*., 2002). Currently, PLS is considered a widely distributed and major foliar disease of maize in Brazil and South Africa (Fantin, 1994; Casela, 1998; Sibiya, 2009). In many countries, however, PLS is of minor importance (Carson, 1999). In Brazil, yield losses may exceed 60%, and the severity of infection is dependent on the susceptibility of the cultivars and favorable environmental conditions (Pinto and Fernandes, 1995). In the USA yield losses of 13% were reported (Carson, 2005).

In the last decade, the etiological agent of PLS has been the subject of much controversy and discussion. It was initially described in India as being the necrotrophic fungus *Phaeosphaeria maydis* (Henn.) Rane, Payak and Renfro (sin. *Sphaerulinia maydis* Henn.), anamorph *Phyllosticta* sp. (Rane *et al*., 1966) Since then, several publications reported PLS in Brazil accepting *P. maydis* as its

causal agent without isolation and identification of the pathogen, pathogenicity tests and fulfillment of Koch's postulates (Fantin, 1994; Pinto, 2004; Brasil and Carvalho, 1998). Initially, disease diagnosis was made in Brazil based only on visual aspects of plant lesions in the field and visual description of the sexual and asexual reproductive structures found in PLS lesions (Fantin, 1994). Moreover, *P. maydis* has not been found in most maize-growing Brazilian areas known for the high PLS incidence (Paccola-Meirelles *et al*., 2001; Cervelatti *et al*., 2002; Amaral *et al*., 2004, 2005; Carli, 2008) and several attempts to reproduce PLS symptoms by inoculating *P. maydis* in maize plants under controlled conditions have failed (Paccola-Meirelles *et al*., 2001; Cervelatti *et al*., 2002; Amaral *et al*., 2005). All such information has caused much confusion and serious doubts on PLS etiology. In addition, characteristic structures of *P. maydis* (perithecia) or *Phyllosticta* (pycnidia) are hardly seen on PLS lesions.

In 2004, *Phoma sorghina* was described as a new fungal species associated with PLS on maize (Amaral *et al*., 2004). In another study (Amaral *et al*., 2005), *P. sorghina*, *Phoma* sp. (*Plenodomus* section), *Phyllosticta* spp., and *Sporormiella* sp. were postulated as the causal agents of PLS. However, the incidence of each of these species was restricted to a specific environmental condition and varied according to growing regions and seasons of the year (Amaral *et al*., 2005). This led Amaral *et al*. (2005) to hypothesize that various pathogens are involved in induction of PLS-like symptoms in maize and that the environmental conditions could influence the predominance of a particular causal agent. In agreement with Amaral *et al*. (2005), Carli (2008) reported the occurrence of *Phoma sorghina* and *Phoma* sp. (*Plenodomus* section) associated with PLS in six different environments. The species *P. sorghina* was predominant in four locations and *Phoma* sp. was highly frequent in two.

A different organism, the bacterium *Pantoea ananatis* (*Pa*), was postulated as being the causal agent of PLS disease (Paccola-Meirelles *et al*., 2001). These authors isolated only this bacterium from young PLS lesions of maize plants growing in the field. In addition, plants inoculated with *Pa* under controlled conditions showed typical symptoms of the disease 5 to 7 days post inoculation and the bacterium was re-isolated, suggesting its involvement in the early stages of the disease (Paccola-Meirelles *et al*., 2002). These results strengthened the conclusions reached by Paccola-Meirelles *et al*. (2001), demonstrating the role of *Pa* in the etiology of the disease. In that study, the authors coined a more appropriate name for this disease calling it maize white spot (MWS) in substitution of PLS. Subsequent studies were inconclusive because they considered separately the bacterium or fungi present in PLS lesions (Pinto, 2004; Bomfeti *et al*., 2007, 2008; Duarte *et al*., 2009). Recently, the interaction between *Pa* and fungi was proposed as necessary for reproducing PLS symptoms (Vieira *et al*., 2009).

Because of the conflicting information on the etiology of PLS, more studies were deemed necessary so that, in the present investigation, both bacteria and fungi present in PLS lesions were taken in to account for the first time. Experiments were carried out for: (i) establishing the molecular identity of bacterial and fungal species present in different stages of PLS lesions in the field and greenhouse; (ii) discriminating the organisms that colonizing natural PLS lesions from those colonizing artificial maize lesions induced by liquid nitrogen damages under field conditions; (iii) evaluating the effect *in vitro* of a bactericide on *Pa* growth and its efficacy for the disease control in the field.

MATERIALS AND METHODS

Four experiments were carried out for isolation and molecular identification of bacterial and fungal species associated with PLS disease. The first experiment addressed PCR-based diagnosis of bacterial and fungal species in four different developmental stages of PLS disease in field-grown plants. In the second experiment, leaves with PLS symptoms were collected from maize plants growing in the field and used for isolating bacteria and fungi associated with the four stages of PLS lesions. The third experiment was conducted in greenhouse and aimed at reproducing PLS symptoms in plants inoculated with *Pa*, and the isolation and molecular identification of fungi species colonizing the lesions induced by the bacterium. The fourth experiment aimed at identifying fungal species colonizing natural PLS lesions and artificial necrotic lesions in field-grown maize plants.

A fifth experiment was conducted to assess the effect of bactericide treatment for the suppression of PLS symptoms in field-grown maize plants showing a high disease incidence. All experiments were repeated twice.

Characterization of PLS lesions and sampling. In the first experiment, leaves with PLS symptoms were collected from maize plants growing in the field at the tasseling stage. The leaves were washed with neutral soap and dried with sterile paper. PLS lesions were detached from leaves with the aid of a scalpel and classified in four different disease stages according to Paccola-Meirelles *et al*. (2001): stage 1, dark green, water-soaked spot; stage 2, greyish lesion; stages 3 and 4, necrotic lesion without and with fungal reproductive structures, respectively. The differentiation between the third and the fourth stages was made using a magnifying glass. Pieces of symptomless tissues excised from infected leaves were used as control. Samples were superficially disinfected with 70% ethanol (1 min), 2% chloramine-T (4 min), 70% ethanol (30 sec) followed by three rinses with sterile distilled water (30 sec each) (Paccola-Meirelles *et al*., 2001). Total genomic DNA was isolated from approximately 150 mg of leaf tissue of each stage by the hexadecyltrimethylammonium bromide (CTAB) method plus 5% polyvinylpolypyrrolidone (PVPP).

Isolation of microorganisms from PLS lesions. In the second experiment, leaves with PLS symptoms were collected from maize plants growing in the field at the tasseling stage and superficially disinfected as described. Water from the last wash was plated out on a suitable culture medium (trypticase soy agar, TSA) to test the efficiency of the disinfection procedure. The edges of each individual PLS lesions (approximately 1 cm) were removed, transferred to TSA and incubated at 30°C in a moist chamber for 48 h with a photoperiod of 12 h light and 12 h dark. Leaf segments containing lesions at stage 4, showing fungal reproductive structures (pycnidia and perithecia), were washed with neutral soap, dried with paper, and placed in a moist chamber for 48 h with a photoperiod as above. The pycnidia/perithecia exudate containing spores were transferred to potato dextrose agar (PDA) and incubated at 25(±2)ºC for fungal isolation. For genomic DNA isolation, approximately 200 mg of mycelium of each fungus grown on potato dextrose broth (PDB) were used.

Reproduction of PLS symptoms re-isolation of *Pa* and isolation of fungi under greenhouse conditions. In the third experiment, the reproduction of PLS symptoms under greenhouse conditions was obtained by inoculating *Pa* on susceptible maize plants of the Brazilian hybrid HS200. Three pots with two plants each were used. A suspension of *Pa* (1×10^6 CFU/ml) from a pure culture grown for 12 h at 30°C on TSB (trypticase soy broth) was inoculated by spraying maize leaves pre-injured with a sponge, until the point of dripping. Plants sprayed only with sterile TSB served as controls. The plants were kept in a moist chamber for 72 h, then transferred to a greenhouse at 30°C and 70% relative humidity. After appearance of symptoms, *Pa* was re-isolated from typical water-soaked lesions (stage 1). The plants were kept in the greenhouse until the lesions become necrotic, and fungi were isolated from pycnidia/perithecia from those lesions that progressed to stage 4. These fungi were used for molecular identification.

Artificial lesions and fungal isolation. In the fourth experiment, leaves of maize plants growing in the field were injured with liquid nitrogen splashes. Thirty artificial injuries looking like stage 1 of PLS lesions were labeled and monitored for approximately 30 days. Slices of 0.5 cm of thirty necrotic lesions with fungal reproductive structures (pycnidia/perithecia) were superficially disinfected, as described, plated in PDA and incubated at 25(±2)°C for fungal isolation and later used for molecular identification.

DNA extraction, molecular identification and data analysis. Total genomic DNA from leaf lesions was extracted by the modified hexadecyltrimethylammonium bromide (CTAB) protocol (Porebski *et al*., 1997). Genomic DNA of bacterial and fungal isolates were extracted according to methods used by Gürtler and Stanisich (1996) and Shaolan *et al*. (2002), respectively. Genomic DNA of the fungus *Neurospora crassa* and FTA cards (Whatman, USA) with preserved DNA from bacterial cultures of *Pa* reference strains (PNA 08-2, PNA 97-5 and PNA 99-13) were used as positive PCR controls. The bacterial 16S rRNA gene was amplified with the forward 16F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse 16R1542 (5'-AAGGAGGTGATCCAAGCCGCA-3') universal primers (Gürtler and Stanisich, 1996). The bacterial internal transcribed spacer (ITS) region of 16S-23S rRNA gene was amplified with the following species-specific pair of primers designed for *Pa* (Figueiredo and Paccola-Meirelles, 2012): ANAF (forward) (5'-CG TGAAACTACCCGTGTCTGTTGC-3') and ANAR (reverse) (5'-TGCCAGGGCATCCACCGTGTACGCT-3'). Fungal ITS region of 18S-28S nuclear ribosomal rRNA gene was amplified with the forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') universal primers (White *et al*., 1990). Genomic DNA of the fungus *Neurospora crassa* and genomic DNA of reference strains of *Pa* were used as positive controls and PCR reactions without DNA template were used as negative controls. PCR reactions were performed with 25 ng of genomic DNA plus 2.5 µl of 10× 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, Brazil) in a total volume of 25 µl. PCR was performed in a model PTC-100 thermolcycler (MJ Research, USA) with the following conditions: one cycle for denaturation of DNA samples at 94ºC for 1 min, 30 cycles of 1 min at 94ºC, 1 min at 55ºC (annealing) and 1 min at 72ºC (extension). Finally, reactions were incubated for 10 min at 72ºC. Amplified DNAs were analyzed by horizontal gel electrophoresis at 6 V/cm^2 in 1.0% agarose gel (wt/v) in 1× TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) plus ethidium bromide (0.5 mg/l). Gels were visualized under UV light, photographed, and fingerprints were compared visually with the overview gels. The amplicons were cut out from gels and purified with the GeneClean kit II (BIO 101, USA). PCR products were directly sequenced at least two times in both directions using the PCR primers. The 16S internal primers 16F518 and 16R928 (Lane, 1991) or 968F (5'-AACGCGAAGAACCTTAC-3') and 1401R (5'-CGGTGTGTACAAGACCC-3') primers designed by Nübel *et al*. (1996) were used as an extra sequencing primers for bacteria. Sequencing reactions were performed with the BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems, USA), and DNA sequencing was resolved on an Applied Biosystems automatic sequencer ABI-377. Sequence editing and contig sequences were generated with ClustalW (Thompson *et al*., 1994) and CAP3 programs (Huang and Madan, 1999) and similarity was evaluated by the BlastN program (Altschul *et al*., 1997) run against

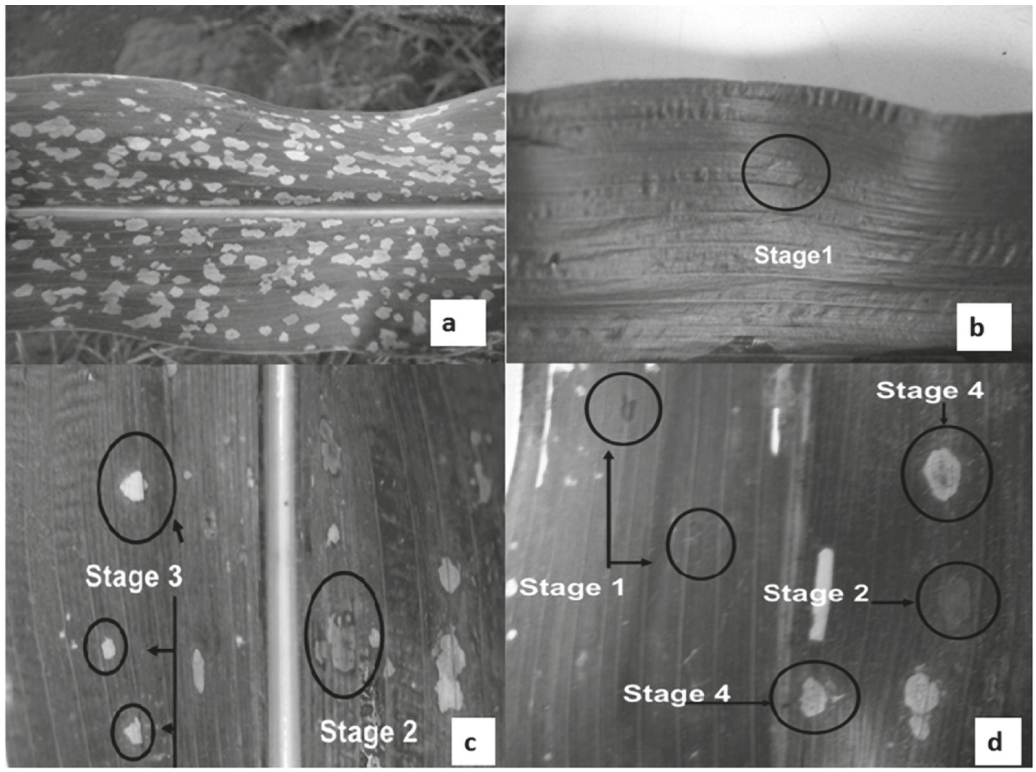

Fig. 1. Phaeosphaeria leaf spot (PLS) disease of maize. **a.** general appearance of the disease at advanced stage, and **b**, **c**, and **d**: lesions at different stages. Stage 1: dark green water-soaked leaf spots; stage 2: greyish lesions; stage 3: lesions become necrotic and look like straw without visible fungal structures; and stage 4: necrotic lesions displaying fungal reproductive structures (pycnidia and perithecia).

all available DNA sequences deposited in GenBank database (http://www.ncbi.nlm.nih.gov). Nucleotide sequences of bacteria and fungi were deposited in EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession numbers shown in Tables 1, 2, and 3.

Antibiotic oxytetracicline for *Pa* and PLS disease control. The fifth experiment was conducted to test the inhibitory effect of the antibiotic Mycoshield (17% oxytetracycline) for controlling the growth of *Pa* in the laboratory and PLS disease in the field. In the laboratory, the *Pa* strain E19, isolated from maize plants with PLS lesions (stage 1) were cultured in the presence of different concentrations of the antibiotic (0.5, 1, 2, 3 and 3.5 g/l of culture medium). The bacterium growing in the same medium without the antibiotic was used as control. In the field, two maize hybrids (HS200 and DAS657), highly susceptible to PLS disease were sown in two rows of 10 plants each. After 30 days of growth, plants were sprayed three times with the antibiotic (2 g/l) with fifteen days intervals between applications. Control plants were sprayed with water. Disease evaluations were made when the plants reached phenological stage 7, about 24 days after pollination. The first eight leaves of each plant were evaluated by counting the number of PLS lesions. Twenty plants of each hybrid were evaluated and the data were submitted to analysis of variance (ANOVA) in a 2×2 factorial design.

Table 1. Bacterial and fungal species identified by DNA sequencing of PCR-amplicons from pool of each of the four stages of natural PLS lesions in field-grown maize plants of Embrapa Maize and Sorghum, Sete Lagoas, MG, Brazil.

Current name of species	Synonymy	Genome sequence	Disease stage	GenBank accession Nos.
Pantoea ananatis	*Erwinia herbicola* (syn. *Erwinia uredovora*) *Pantoea ananas* *Erwinia ananatis* corrig. Serrano 1928	16S ANAF/ANAR	1 to 4 1 to 4	JX985034 to JX985041 JX985042 to JX985049
Phaeosphaeria sp.	————————	ITS	3	JX294910 and JX294911
Phoma sp.	————————	ITS	4	JX294912 and JX294913

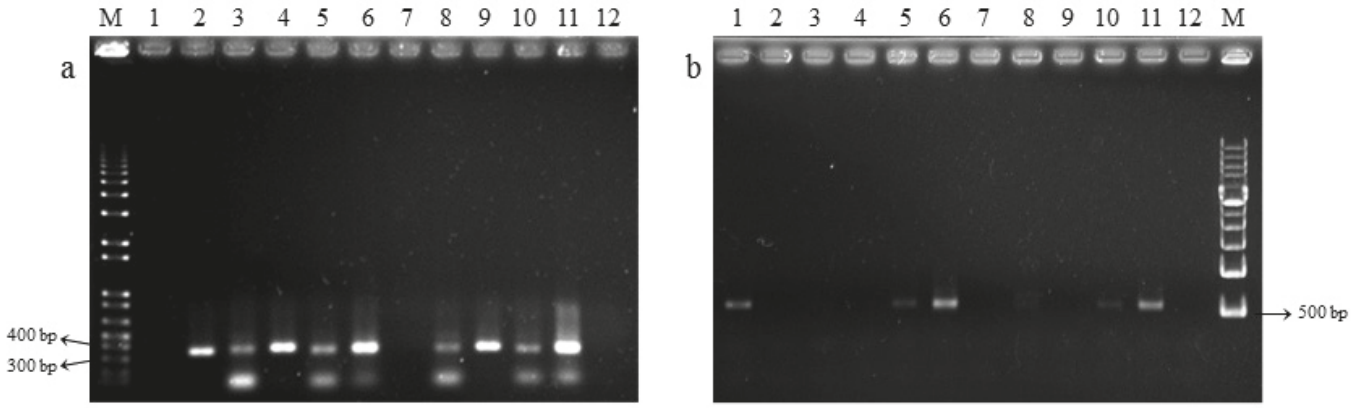

Fig. 2. Agarose gels of PCR products of DNA extracted from PSL lesions at different stages of development. Amplicons obtained with: **a**) species-specific primer pair ANAF and ANAR for *P. ananatis*; **b**) fungal universal primer pair ITS1 and ITS4. Lanes 3: stage 1 (soaked lesions); lanes 4: stage 2 (gray lesions); lanes 5: stage 3 (necrotic lesions without fungal reproductive structures); lanes 6: stage 4 (necrotic lesions with visible fungal reproductive structures); and lanes 7: asymptomatic leaf tissue.
Lanes 1 and 2 are negative and positive control in (**a**), and positive and negative control in (**b**) of PCR, respectively. Lanes M in **a** and **b** are 1 kb plus molecular marker (Invitrogen) and 1 Kb molecular marker (Amresco), respectively. Lanes 8 to 12: duplicated PCR reactions of DNA extracted from samples 3 to 7 presented in the same order.

RESULTS

The first of the five experiments carried out in the course of this study, encompassed the molecular identification of bacteria and fungi associated with PLS, a disease that, as reported above, has a distinctive pattern of progression and may be separated into four distinct stages (Fig. 1). In stage 1, the lesions have dark green color and look like water-soaked spots; in stage 2, lesions are grayish; in stage 3, lesions become necrotic and look like straw; in stage 4, lesions are necrotic displaying fungal reproductive structures.

PCR reactions using genomic DNA extracted from pool of superficially disinfected lesions of each disease stage using universal primers, 27F and 1542R, yielded a DNA fragment *ca.* 1500 bp in size corresponding to the bacterial 16S rRNA gene in all samples of the four stages of PLS. DNA sequencing analysis of the four PCR products revealed 100% identity with 16S rRNA gene sequence of *Pa* and 99% identity with other *Pantoea* species. PCR using species-specific primers for *Pa,* ANAF/ANAR (Figueiredo and Paccola-Meirelles, 2012) and sequencing of these amplicons confirmed the identity of the bacterial species present in all stages of PLS disease as *Pa* (Fig. 2). Moreover, PCR using the universal primers ITS1 and ITS4 for fungal species produced amplicons only with DNA samples of the necrotic stages of PLS lesions (stages 3 and 4) (Fig. 2). DNA sequencing and search for similarity with nucleotide sequences from GenBank using the BLASTN program showed a high degree of sequence similarity (99-100%) with *Phoma* sp. and *Phaeosphaeria* sp. (Table 1).

In the second experiment, leaves with PLS symptoms were collected from maize plants growing in the field at the tasseling stage and used to isolate bacteria and fungi species associated with the four stages of PLS lesions. In the early stage of lesions (stages 1 and 2) yellow bacterial colonies were consistently recovered. The majority of necrotic PLS lesions at stage 4 showed pycnidia and perithecia, and different fungal species were isolated from those lesions. Morphological analysis of bacterial colonies, biochemical tests (Gram reaction, production of acid from adonitol and sorbitol), characterization by BIOLOG GN2 MicroPlate system for diagnosis of Gram-negative bacteria based on reactions to a series of 95 carbon sources, and ice nucleation activity (INA) indicated that all isolates forming yellow colonies belong to *Pantoea* spp. (data not shown). DNA sequencing of 16S rDNA from 12 yellow-pigmented colonies confirmed the identity of the bacterial isolates as *Pantoea* spp. (Table 2). PCR using species-specific primers for *Pa* (Figueiredo and Paccola-Meirelles, 2012) were positive for all isolates, and DNA sequencing of amplicons confirmed the identity of all isolates as *Pa* (Table 2).

Eighteen fungi were isolated from necrotic lesions of stages 3 and 4, and identified by direct sequencing of PCR products of the ITS region of 18S/23S rRNA genes. The degree of sequence similarities ranged from 99-100% with fungal sequences from different species of the order Pleosporales deposited in GenBank (Table 2).

In the third experiment, reproduction of PLS symptoms under greenhouse conditions was observed within 10 days from inoculation of maize plants with *Pa*. The PLS symptoms were similar to those observed in the field and the control plants did not develop symptoms. After a few days, the water-soaked spot (stage 1) progressed to necrotic straw-colored lesions (stages 3 and 4). In some necrotic lesions it was possible to observe the occurrence of fungal structures (pycnidia/perithecia). These fungi were isolated to determine their molecular identity and to compare with fungi isolated from PLS affected maize plants growing in the field. Fourteen fungi were isolated

Table 2. Bacteria and fungi isolated from natural PLS lesions in maize plants grown in the field of the State University of Londrina (SUL) and Embrapa (ES) and from necrotic lesions of PLS induced by inoculation of maize plants with *P. ananatis* under greenhouse conditions (GH).

Current name of species	Synonymy	Locality	Isolate	GenBank accession Nos.
Pantoea ananatis	*Erwinia herbicola* (syn.*Erwinia uredovora*) *Pantoea ananas* *Erwinia ananatis* corrig. Serrano 1928	SUL / ES	PA01 to PA22 16S ANAF/ANAR	 KC200048 to KC200069 KC200026 to KC200047
Epicoccum nigrum	*Phoma epicoccina* Punith., M.C. Tulloch et C.M. Leach, (1972)	SUL/ES/GH	PLSFUN01 PLSFUN02 PLSFUN03 PLSFUN06 PLSFUN07 PLSFUN08 PLSFUN09 PLSFUN10 PLSFUN11 PLSFUN12 PLSFUN13 PLSFUN15 PLSFUN16 PLSFUN17 PLSFUN18 PLSFUN21 PLSFUN25 PLSFUN26 PLSFUN27 PLSFUN31 PLSFUN32	KC005650 KC005651 KC005652 KC005653 KC005654 KC005655 KC005656 KC005657 KC005658 KC005659 KC005660 KC005661 KC005662 KC005663 KC005664 KC005665 KC005666 KC005667 KC005668 KC005669 KC005670
Leptosphaeria sacchari (Breda de Haan, 1892)	*Phoma sorghina* (Sacc.) Boerema, Dorenb. et Kesteren, (1973) *Phoma* sp. *Phoma annulata* N. Pons [as 'annullata'], (1991) *Phaeosphaeria sacchari* (Breda de Haan) Shoemaker et C.E. Babc., (1989) *Epicoccum sorghinum* (Sacc.) Aveskamp, Gruyter et Verkley [as 'sorghi'], (2010) *Phyllosticta sorghina* Sacc., (1878) *Phyllosticta glumicola* (Speg.) Hara [as 'glumicola'], (1918)	ES	PLSFUN22 PLSFUN28 PLSFUN29	KC005678 KC005679 KC005680
Cochliobolus geniculatus (R.R. Nelson, 1964)	*Pseudocochliobolus geniculatus* (R.R. Nelson) Tsuda, Ueyama et Nishih, (1977) *Helminthosporium geniculatum* Tracy et Earle, (1896)	ES	PLSFUN23	KC005681
Pithomyces chartarum (Berk. et M.A. Curtis) M.B. Ellis, 1960	*Leptosphaerulina chartarum Cec.* Roux, (1986)	SUL	PLSFUN04	KC005671
Alternaria alternata (Keissl. 1912)	*Alternaria fasciculata* (Cooke et Ellis) L.R. Jones et Grout, (1897)	GH / SUL	PLSFUN14 PLSFUN24	KC005673 KC005674
Alternaria ricini (Yoshii) Hansf. 1943	*Alternaria ricini (Sawada)* (1959)	GH	PLSFUN30	KC005675
Gibberella intricans (Wollenw. 1930)	*Fusarium chlamydosporum* var. *fuscum* (1977) *Fusarium equiseti/Fusarium chlamydosporum* Wollenw. et Reinking, (1925) var. chlamydosporum	SUL	PLSFUN05 PLSFUN19	KC005672 KC005676
Gibberella fujikuroi var. *fujikuroi* (Sawada) Wollenw. 1931	*Gibberella fujikuroi* var. *moniliformis* (Wineland) Kuhlman, (1982)	ES	PLSFUN20	KC005677

from the necrotic lesions produced by inoculation with *Pa* under greenhouse conditions. The isolates were identified by direct sequencing of the PCR products of the ITS region of 18S/28S rRNA genes. The degree of sequence similarities ranged from 99-100% with fungal sequences from different taxonomic species of the order Pleosporales deposited in GenBank (Table 2). *Epicoccum nigrum* (*Phoma epicoccina*, Punith.) was predominant among the isolated fungi (68.75%).

In the fourth experiment, 30 necrotic artificial lesions induced by liquid nitrogen and 60 natural PLS lesions, 30 of stage 1 and 30 of stage 4, from plants growing in the same field were collected at the same time and analyzed. *Pa* was isolated from naturally occurring PLS lesions at

Table 3. Bacterial and fungal species isolated from natural PLS lesions (NPLS), and from artificial necrotic injuries (AI) induced by splashing liquid nitrogen in the leaves of maize plants growing in the field of Embrapa Maize and Sorghum.

Current name of species	Synonymy	NPLS isolates	GenBank accession Nos.	AI isolates	GenBank accession Nos.
Pantoea ananatis corrig. (Serrano 1928) Mergaert *et al.* 1993	*Erwinia ananas* (synonym, *Erwinia uredovora*) *Pantoea ananas* *Erwinia ananatis* corrig. Serrano 1928	PA01 to PA30 16S ANAF/ANAR	 KC188675 to KC188704 KC188705 to KC188734	–––	
Bacillus subtilis	–––	–––	–––	BS01 BS02	KC188673 KC188674
Leptosphaeria sacchari Breda de Haan, (1892)	*Epicoccum sorghinum* (Sacc.) Aveskamp, Gruyter et Verkley [as 'sorghi'], (2010) *Phoma annulata* N. Pons (1991) *Phaeosphaeria sacchari* (Breda de Haan) Shoemaker et C.E. Babc. (1989) *Phoma sorghina* (Sacc.) Boerema, Dorenb. et Kesteren *Phyllosticta sorghina* Sacc.	NLF01 NLF06 NLF08 NLF09 NLF12 NLF14 NLF15 NLF21 NLF22 NLF23 NLF26 NLF29	KC106688 KC106699 KC106689 KC106700 KC106690 KC106691 KC106692 KC106701 KC106702 KC106703 KC106704 KC106705	ALF33 ALF34 ALF35 ALF36 ALF39 ALF45 ALF47 ALF50 ALF54 ALF57 ALF59 ALF60 ALF62 ALF63 ALF64	KC106693 KC106694 KC106706 KC106707 KC106708 KC106709 KC106695 KC106696 KC106697 KC106713 KC106714 KC106698 KC106715 KC106716 KC106717
Phaeosphaeria sp./ *Phaeosphaeria avenaria* f. sp. triticae	–––	NLF04 NLF10 NLF11 NLF16 NLF17 NLF18 NLF25 NLF30	KC005690 KC005691 KC005692 KC005693 KC005694 KC005695 KC005696 KC005697	ALF37 ALF40 ALF41 ALF42 ALF43 ALF44 ALF51 ALF52 ALF58	KC005698 KC005699 KC005700 KC005701 KC005702 KC005703 KC005704 KC005705 KC005706
Phoma sp.	–––	NLF02	KC005682	ALF46 ALF49 ALF56	KC005683 KC005684 KC005685
Phyllosticta sp.	–––	NLF27 NLF31	KC005686 KC005687	ALF48 ALF53	KC005688 KC005689
Gibberella fujikuroi var. *fujikuroi (Sawada) 1931*	*Gibberella fujikuroi* var. *moniliformis* (Wineland) Kuhlman, (1982)	NLF19 NLF20 NLF24	KC106718 KC106719 KC106720	ALF38	KC106721
Sarocladium strictum (W. Gams) Summerb., (2011)	*Acremonium strictum* W. Gams, (1971)	NLF05	KC175286	–––	–––
Glomerella graminicola D.J. Politis 1975	*Colletotrichum graminicola (Ces.)* G.W. Wilson, 1914	NLF03 NLF13 NLF32	KC106722 KC106723 KC106724	ALF55	KC106725
Cochliobolus heterostrophus (Drechsler) 1934	*Bipolaris maydis* (Y. Nisik. et C. Miyake) Shoemaker, (1959)	NLF07 NLF28	KC005707 KC005708	ALF61	KC005709

stage 1 and *Bacillus subtilis* was isolated from two artificial lesions induced by liquid nitrogen (Table 3). Sixty-four fungi were recovered from natural and artificial lesions. Fungi of the order Pleosporales (85.94%) were identified in the stages 3 and 4 of naturally occurring PLS lesions (39.06%) and in the artificial necrotic injuries (46.88%). *Leptosphaeria sacchari* (syn: *Phoma sorghina*, *Epicoccum sorghinum*, *Phaeosphaeria sacchari*) was the prevalent fungus, both in the natural (18.75%) and artificial lesions (23.44%). The second most prevalent fungal species was *Phaeosphaeria* sp. with the frequencies of 12.5% in natural PLS lesions and 14.1% in artificial injuries, followed by *Phoma* sp. with a frequency of 1.56% and 4.69% in natural and artificial injuries, respectively; and *Phyllosticta* sp. (3.2% in both, natural and artificial lesions). Other fungi identified in natural PLS lesions or artificial injuries were *Cochliobolus heterostrophus* (Pleosporales) with frequency of 4.69%, *Gibberella fujikuroi* (6.25%) and *Sarocladium strictum* (1.56%) (order Hypocreales), and *Glomerella graminicola* (6.25%) (order Glomerellales) (Table 3).

In the fifth experiment, all doses of the antibiotic Mycoshield completely inhibited *Pa* growth, *in vitro*. In the field, the antibiotic was effective against PLS disease in both HS200 and DAS657 susceptible maize hybrids (Fig. 3) reducing by more than 90% the number of PLS lesions per leaf with no significant difference between them (Fig. 4).

Fig. 3. Maize hybrids HS200 (**a**) and DAS657 (**b**) growing in the field. Plants treated (**a** and **b**) and untreated (**a1** and **b1**) with the antibiotic oxytetracycline.

DISCUSSION

Although Phaeosphaeria leaf spot of maize is an important disease in many countries, its etiology has been the subject of protracted debate and controversy. In the scientific literature, different species of fungi (Rane *et al*., 1966; Carson *et al*., 1991; Fantin, 1994; Carson, 1999, 2005; Amaral *et al*., 2004, 2005) and the bacterium *Pantoea ananatis* (Paccola-Meirelles *et al*., 2001) have been proposed as causative agents of this disease. Also the reciprocal interaction among *P. maydis/Pa*, was reported as being necessary for the development of PLS disease (Vieira *et al*., 2009). In this study, five experiments were carried out to clarify controversial issues on the etiology of the PLS, which yielded results which clearly demonstrated that *Pa* is the causal agent of disease.

Since the etiological agent of PLS disease was identified as the fungus *Phaeosphaeria maydis* (Rane *et al*., 1966), PLS symptoms in maize have been attributed to *P. maydis* with no experimental stemming from isolation and characterization of the pathogen (Carson *et al*., 1991). The same has occurred in Brazil, where the disease was identified in the 1990s (Fantin, 1994; Pinto, 2004; Pinto and Fernandes, 1995; Brasil and Carvalho, 1998; Pegoraro *et al*., 2001; Souza and Duarte, 2002). However, as pointed out by Carson (1999), the majority of PLS reports were based only on anecdotal evidence.

A consensus exists among the proponents of *P. maydis* or other fungal species as the etiological agent of PLS disease that fungal structures are not present in all PLS lesions and their frequency varies according to climate and geographic region. *P. maydis* has been reported with high frequency in maize plants with PLS disease only in São Paulo state (Fantin, 1994). However, *P. maydis* has never been found in various surveys for the presence of fungi in PLS lesions of maize grown in different Brazilian areas (Amaral *et al*., 2005; Carli, 2008). Only two studies (Paccola-Meirelles *et al*., 2001; Amaral *et al*., 2004) reported the presence of *P. maydis* in PLS lesions and, even in these cases, its occurrence was restricted, and the frequency low (10 and 7%, respectively).

Authors performing identification of microorganisms in PLS lesions have focused their attention only on fungal species (Rane *et al*., 1966; Carson *et al*., 1991; Fantin, 1994; Amaral *et al*., 2004, 2005; Carson, 2005; Carli, 2008), obtaining results biased by the use of antibiotics, such as neomycin and streptomycin, for preventing bacterial growth during isolation (Amaral *et al*., 2004, 2005; Carli, 2008). In contrast, *Pa* has been consistently isolated from PLS of maize on PDA without addition of antibiotic (Paccola-Meirelles *et al*., 2001, 2002; Bomfeti *et al*., 2008; Pérez-y-Terrón *et al*., 2009; Alippi and López, 2010; Krawczyk *et al*., 2010; Figueiredo and Paccola-Meirelles, 2012; Lana *et al*., 2012).

To date, Koch's postulates were fulfilled and PLS symptoms in plants infected with *P. maydis* under controlled conditions were reproduced only in India (Rane *et al*., 1966). In the USA, successful infection of maize plants with *P. maydis* cultured in sorghum seeds and used as vehicle for inoculation has been reported only in the field (Carson, 1999, 2001, 2005), whereas in Brazil, attempts to fulfill Koch's postulates and reproduce PLS symptoms in plants infected with *P. maydis* have failed (Pinto and Fernandes, 1995; Fernandes and Oliveira, 1997; Casela, 1998; Paccola-Meirelles *et al*., 2001; Cervelatti *et al*., 2002; Carli, 2008).

Another fact that draws attention to PLS disease diagnosis is the report of other fungal species (*Phoma sorghina*, *Phoma* sp. (*Plenodomus* section), *Phyllosticta* spp., and *Sporormiella* sp.) as responsible for PLS-like symptoms (Amaral *et al*., 2004, 2005; Carli, 2008). However, the symptoms induced by fungal inoculations presented by Amaral *et al*. (2005) are very different from the true PLS symptoms. Amaral *et al*. (2005) and Carli (2008) postulated that PLS is caused by a fungal complex and that environmental conditions may influence the prevalence of a particular agent, in some climatic regions. In the present study, different fungal species of the order Pleosporales and of other orders have also been isolated from maize plants cultivated in different regions and climates. Our hypothesis is that these different fungi are opportunistic species that install themselves in the pre-existing lesions, and they do not responsible for PLS or PLS-like symptoms. By

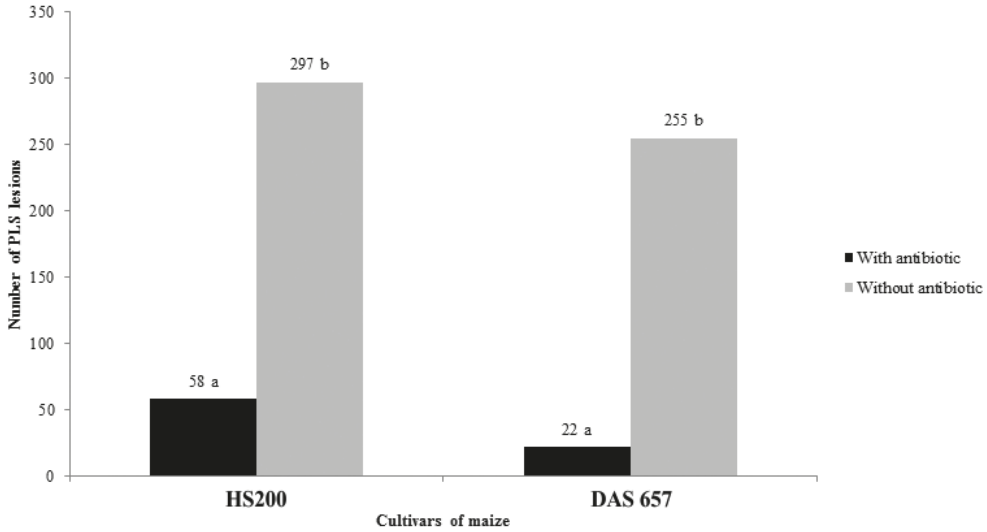

Fig. 4. Inhibitory effect of the antibiotic Mycoshield® (17% oxytetracycline) on PLS disease in two highly susceptible maize hybrids (HS200 and DAS 657) growing in the field. (Tukey test p≤0.05).

contrast, the bacterium *Pa* was always isolated from PLS lesions regardless of the ecogeographic regions or climate.

The manner of PLS disease progression is another source of controversy. PLS has at least four well-defined stages of development (Paccola-Meirelles *et al*., 2001; Bomfeti *et al*., 2008). In the first stage, lesions with the aspect of water-soaked leaf spots can be observed (Fig. 1). In the following two stages, the color of lesions changes to dark green-brown, then turns to straw color due to lack of chlorophyll in the injured tissues. In these three developmental stages, fungal reproductive structures cannot be viewed in the lesions. Only in the last stage, these structures become visible in the majority of the lesions.

The predominance of *Pa* in early stages (1 and 2) of the disease, and the prevalence of different species of fungi in more advanced stages (3 and 4) has been reported as a hallmark of PLS disease (Paccola-Meirelles *et al*., 2001; Bomfeti *et al*., 2008) a notion confirmed by experiments 1, 2 and 3 of the present study. Alternative explanation for the occurrence of *Pa* and different fungal species in PLS lesions is that a reciprocal interaction among them, or between *P. maydis/Pa*, is necessary for the development of PLS disease (Vieira *et al*., 2009). However, in the present study, different fungal species, including those described by some authors as the causal agent of PLS, were isolated from artificial necrotic lesions caused by liquid nitrogen, indicating that *Pa* is not necessary for the growth of those fungal species. As fungal species from natural PLS lesions and artificial lesions were isolated with similar frequencies, this may be taken as an indication that the fungi identified in this study are simply colonizing dead tissues of maize leaves (Table 3).

Finally, oxytetracycline reduced PLS lesions in field-grown maize plants by 80-90% (Fig. 4) and 100% of *Pa* growth of *in vitro*, whereas it had no effect on fungal growth (data not shown).

Pinto (2004), Pinto and Fernandes (1995) and Duarte *et al.* (2009), evaluated the effectiveness of some fungicides to control PLS disease. The efficacy of fungicides like Mancozeb, was retained an evidence that fungi could be the etiological agents of this disease (Pinto, 2004; Carli, 2008). However, Bomfeti *et al.* (2007) have shown that Mancozeb completely inhibits the growth of *Pa in vitro*, which explains the PLS disease control in the field.

Maize plants treated with Benomyl under natural conditions of infection, showed all symptoms of PLS but no reproductive structures of any fungal species developed on the lesions (Pinto and Fernandes, 1995). In addition, in South Africa, Benomyl did not provide adequate control of PLS (Flett *et al*., 1996; Kloppers and Tweer, 2009). Benomyl is selective towards fungal microtubules and causes disruption of the cytoskeleton during the meiotic cell cycle (Hochwagen *et al*., 2005). Interestingly, Benomyl did not show any inhibitory effect of the growth of *Pa in vitro* (Bomfeti *et al*., 2007). According to Bomfeti *et al.* (2007), the absence of fungal reproductive structures in PLS lesions, as described by Pinto and Fernandes (1995) after treatment of the plants with Benomyl, was due to inhibitory activity of the fungicide on opportunistic fungi that colonize PLS lesions caused by the bacterium. As Benomyl did not inhibit the *P. ananatis* growth, the disease symptoms persisted in the field.

The results of this study show that the bacterium *Pa* is present since the earliest stages of PLS lesions while different fungal species appear only in the late stages of the disease. Moreover, differently from what observed with different fungal species, the isolation of *Pa* from PLS lesions is independent of the region or climate.

In conclusion the fact that: (i) PLS symptoms were reproduced in maize plants inoculated with *Pa* under greenhouse conditions; (ii) different fungal species often found in PLS lesions occur also in artificial injuries; (iii) PSL

disease is controlled by the oxytetracycline, strongly reinforce the hypothesis that the PLS disease is caused by *Pantoea ananatis* as postulated by Paccola-Meirelles *et al.* (2001), rather than a specific fungus or a complex of fungal species as previously reported (Rane *et al*., 1966; Carson *et al*., 1991; Fantin, 1994; Amaral *et al*., 2004, 2005; Carson, 2005; Carli, 2008).

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