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Detection of *Pantoea ananatis*, causal agent of leaf spot disease of maize, in Mexico

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Abstract. Bacterial isolates from maize plants showing a leaf spot disease were identified through molecular and phenotypic traits, showing that the isolates belong to *Pantoea ananatis*. Maize plants inoculated with those isolates showed a pathogenic reaction. This is the first report of a disease of Mexican maize caused by *P. ananatis*.

Additional keywords: Enterobacteriaceae, *Zea mays*, phytopathogen.

Maize is the most important cereal for human nutrition in Mexico. Therefore, detection and control of diseases of this plant is particularly important since world prices for maize have risen rapidly.

Species of the genus *Pantoea* have clinical, economic and ecological significance. Several *Pantoea* strains display plant growth promoting activity (Sergeeva *et al*. 2007). Paradoxically, phytopathogenic species are also included in this genus. For example, *P. ananatis* is the causative agent of agave disease (Fucikovsky and Aranda 2006), and also of palea rice browning (Cortesi and Pizzatti 2007), blight and dieback of *Eucalyptus* (Coutinho *et al*. 2002), leaf blotch disease of Sudangrass (Azad *et al*. 2000), brown stalk rot of maize (Goszczynska *et al*. 2007), leaf spot disease of maize (Paccola-Meirelles *et al*. 2001), and also a post-harvest disease in melon (Bruton *et al*. 1991).

In 2007 in the Mexican states of Puebla and Tlaxcala, we detected maize plants showing a leaf spot disease. This paper reports on the isolation and identification of the causative agent of this syndrome in Mexican maize.

Plant samples of the native maize cultivar 'Criollo' showing signs of a leaf spotting condition (Fig. 1), including roots and soil were obtained from twelve maize fields in the States of Tlaxcala and Puebla.

Nonsterile leaf surfaces were sampled with sterile cotton plugs soaked with 1% sucrose. In addition, leafs and stem pieces were surface sterilised with 0.25% tosylchloramide sodium (Cloramine T), washed with sterile water, homogenised and serially diluted in a 1% sucrose solution.

Fig. 1. Field grown maize plants showing leaf spot disease.

Dilutions of sterile and non-sterile parts of the plant parts were plated on modified MESMA agar (Fuentes-Ramírez *et al*. 1999).

Rhizospheric soil was diluted and plated as described above. The plates were incubated at 30°C for two days and any pale and slightly yellow colonies were selected.

Isolates were Gram stained and colony morphology observed on YDC plates (Lakso and Starr 1970). The isolates were tested

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for: urease, arginine dehydrolase, ornithine decarboxylase, lysine decarboxylase, β-galactosidase, catalase, oxidase, indole production, gelatin liquefaction, and citrate utilisation. Acid production was determined from D-mannose, sorbitol, L-rhamnose, melibiose, sucrose, arabinose, xylose, and *meso*-inositol in media supplemented with Bromothymol Blue.

Motility was determined by microscopic observation in tryptic soy broth.

Table 1. Phenotypic tests of the bacterial isolates

Test	Isolates	*P. ananatis*[a]
Production of yellow pigment	+	+
Citrate utilisation	+	+
Indole production	+	+
Gelatin liquefaction	–	–
Catalase	+	+
Oxidase	–	–
Urease	–	–
Arginine dehydrolase	–	–
Ornithine decarboxylase	–	–
β-Galactosidase	+	+
Lysine decarboxylase	–[b]	–
Motility	+	+
Acid production from		
D-Mannose	+	+
Sorbitol	+[c]	+
L-Rhamnose	+	+
Melibiose	+	+
Sucrose	+	+
Arabinose	+	+
Xylose	+	+
meso-Inositol	+[d]	+

[a]According to Mergaert *et al*. (1993), Cha *et al*. (1997), and Coplin and Kado (2001).
[b]80% of the isolates.
[c]90% of the isolates.
[d]80% of the isolates.

Genomic DNA was extracted with a commercial kit (Wizard Genomic DNA Purification kit, Promega Co.) following manufactures' procedures. Amplified rDNA restriction analysis (ARDRA) 16S rRNA genes were amplified according to Weisburg *et al*. (1991). PCR conditions consisted of an initial denaturing cycle (95°C, 3 min), 35 amplification cycles (95°C, 1 min; 55°C, 1 min; 72°C, 2 min) and a final extension cycle (72°C, 3 min). The PCR mixture contained 150 µM dNTPs, 3 mm $MgCl_2$, 3 µM of each primer fD1 and rD1 (Weisburg *et al*. 1991), 10× Taq buffer, and 1.2 U Taq polymerase (Altaenzymes, Co.). The amplification products were digested with the endonucleases *Dde*I, *Hha*I, and *Hae*III and the DNA fragments separated by electrophoresis in a 3% agarose gels. In addition, the 16S rRNA gene was sequenced with primers fD1 and rD1 at the Institute of Biotechnology, UNAM, Mexico (Weisburg *et al*. 1991). The gene coding the ATPase domain of DNA gyrase was also partially sequenced, because this locus provides reliable phylogenetic relationships among *Enterobacteriaceae* (Dauga 2002). *gyrB* genes were amplified with *gyrB* 01-F and *gyrB* 02-R primers, and sequenced with *gyrB* 01-F and *gyrB* 08-R (Brady *et al*. 2008). The DNA fragments were sequenced. The sequences were analysed using BLAST (Altschul *et al*. 1997) and Clustal W (DNA Star, v. 5.08).

Maize seeds of the native cultivar 'Criollo' were surface sterilised with 0.25% tosylchloramide sodium (Cloramine T)

Table 2. Sequence similarity with *Pantoea ananatis* (% identity)[a]

	UAPB01001	UAPB01002	UAPS07100
	(GQ267502)[b]	(GQ267501)	(GQ267500)
	(GQ325305)[c]	(GQ325307)	(GQ325306)
16SrRNA (Z96081)	99.0	99.0	99.2
gyrB (FJ617371)	98.8	99.3	99.1

[a]Partial gene sequences.
[b]16S rDNA accession numbers.
[c]*gyrB* accession numbers.

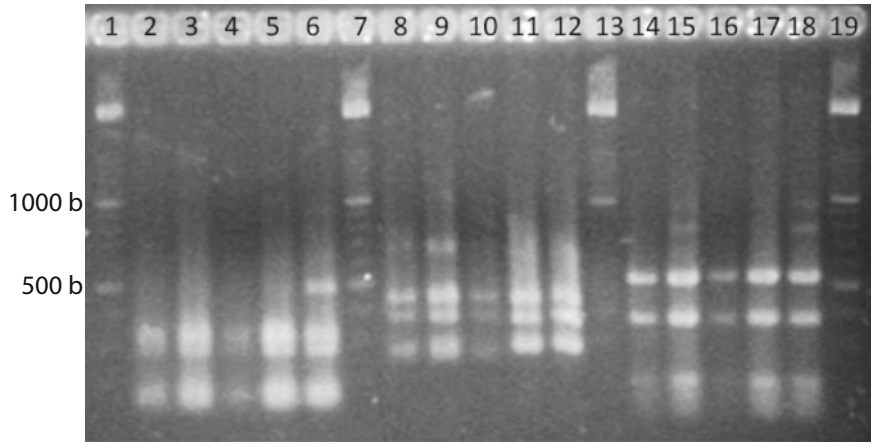

Fig. 2. 16S rDNA profiles of maize isolates. Lines 1, 7, 13, and 19, molecular weight marker; 16S rDNA PCR products digested with *Hae*III, lines 2–6; with *Dde*I, lines 8–12; with *Cfo*I, lines 14–18. Lines 2, 8, and 14, strain UAPB01001; lines 3, 9, and 15, strain UAPB01002; lines 4, 10, and 16, strain UAPS07100; lines 5, 11, and 17, strain UAPB01003; lines 6, 12, and 18, strain UAPB01003.

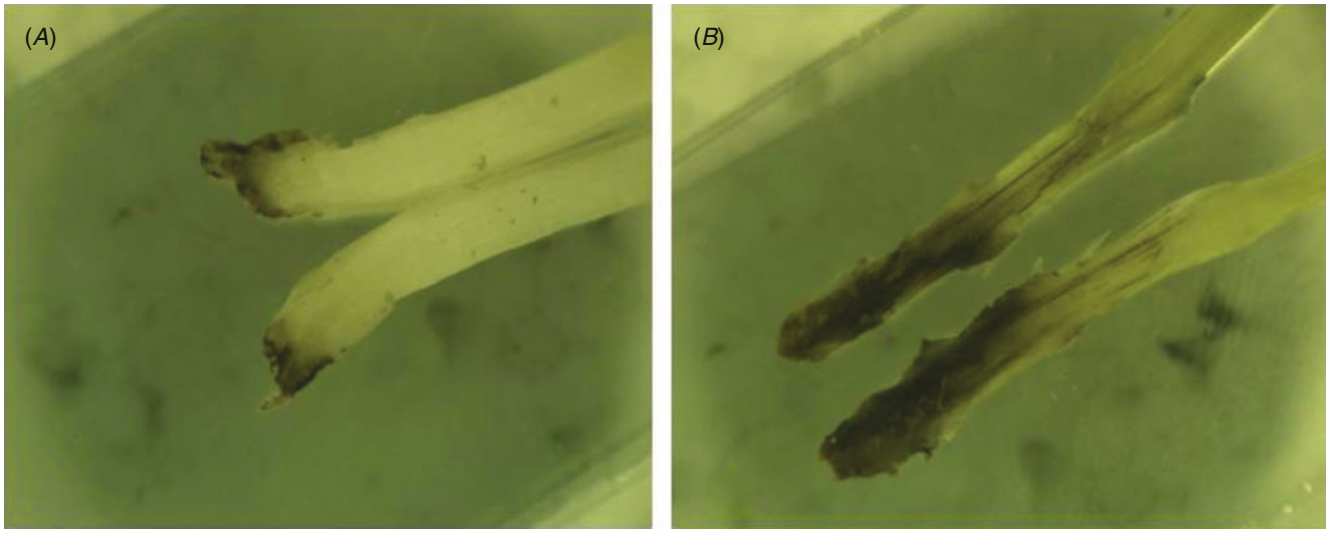

Fig. 3. Maize stems of inoculated plants and control. *A*, non-inoculated plant; *B*, inoculated with strain UAPB01001.

for 5 min and washed with sterile distilled water. Disinfected seeds were planted in sterilised vermiculite. Plants were irrigated with water four times per week, and two times in total over the experiment with 20 mL of Hoagland's solution. The stems of four week old plantlets were inoculated in the stem with 10^6 colony forming units (CFU) of the bacterial strains UAPB01001, UAPB01002, and UAPS07100, using a sterile syringe. Each strain was replicated five times. Inoculated plants were grown for four more days, and the stems cut and observed. Bacterial strains were re-isolated from the inoculated plants in order to evaluate the authenticity of the inoculated strain and to ensure that cross contamination had not taken place.

A limited amount of bacterial diversity was observed from disinfected surface sterilised leaves and stems, which were plated on in MESMA agar. Isolates showing yellow pigmentation were obtained from the rhizosphere isolations and from maize plants showing the leaf spot symptom. ARDRA tests and phenotypical characterisation of four isolates from surface sterilised tissue (including leaf tissue close to spots, and one from the rhizosphere) showed phenotypes similar to those reported for *P. ananatis* (Table 1). The production of yellow pigmentation, citrate utilisation, indole production, and motility also matched the profile for *P. ananatis*. Those strains shared the same ribotype after digestion with *Dde*I, *Hae*III and *Hha*I (Fig. 2). BLAST and Clustal W analysis of the 16S rRNA and *gyrB* gene from isolates UAPB01001, UAPB01002, and UAPS07100 showed an identity higher than 99% to the corresponding gene sequences for *P. ananatis*, indicating that these maize isolates belong to *P. ananatis* clade (Table 2). Inoculation of maize plants with isolates UAPB01001, UAPB01002, and UAPS07100 caused symptoms of stem rotting, including the vascular bundle (Fig. 3). Isolations from these inoculated plants yielded bacteria with phenotypic and ARDRA patterns identical to the inoculated bacteria. *P. ananatis* is capable of colonising diverse environments (Coutinho and Venter 2009) and its presence in the rhizosphere suggests that soil could provide an inoculum source. This work is the first report of *P. ananatis* causing disease of maize in Mexico.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402. doi: 10.1093/nar/25.17.3389

Azad HR, Holmes GJ, Cooksey DA (2000) A new leaf blotch disease of sudangrass caused by *Pantoea ananas* and *Pantoea stewartii. Plant Disease* **84**, 973–979. doi: 10.1094/PDIS.2000.84.9.973

Brady C, Cleenwerck I, Venter S, Vancanneyt M, Swings J, Coutinho T (2008) Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). *Systematic and Applied Microbiology* **31**, 447–460. doi: 10.1016/j.syapm.2008.09.004

Bruton BD, Wells JM, Lester GE, Patterson CL (1991) Pathogenicity and characterization of *Erwinia ananas* causing a postharvest disease of cantaloupe fruit. *Plant Disease* **75**, 180–183.

Cha J-S, Pujol C, Ducusin AR, Macion EA, Hubbard C, Kado CI (1997) Studies on *Pantoea citrea*, the causal agent of pink disease of pineapple. *Journal of Phytopathology* **145**, 313–319.

Coplin DL, Kado CI (2001) *Pantoea*. In, Laboratory guide for identification of plant pathogenic bacteria. (Eds NW Schaad, JB Jones, W Chun) pp. 73–82 (APS Press: Minnesota, MN)

Cortesi P, Pizzatti C (2007) Palea browning, a new disease of rice in Italy caused by *Pantoea ananatis. Journal of Phytopathology* **89**, S76–S76.

Coutinho TA, Venter SN (2009) *Pantoea ananatis*: an unconventional plant pathogen. *Molecular Plant Pathology* **10**, 325–335. doi: 10.1111/j.1364-3703.2009.00542.x

Coutinho TA, Preisig O, Mergaert J, Cnockaert MC, Riedel KH, Swings J, Wingfield MJ (2002) Bacterial blight and dieback of *Eucalyptus* species, hybrids, and clones in South Africa. *Plant Disease* **86**, 20–25. doi: 10.1094/PDIS.2002.86.1.20

Dauga C (2002) Evolution of the *gyrB* gene and the molecular phylogeny of Enterobacteriaceae: a model molecule for molecular systematic studies. *International Journal of Systematic and Evolutionary Microbiology* **52**, 531–547.

Fucikovsky L, Aranda S (2006) *Pantoea ananas* a new pathogen of agave in Mexico. *Phytopathology* **96**, S37–S37.

Fuentes-Ramírez LE, Caballero-Mellado J, Sepúlveda J, Martínez-Romero E (1999) Colonization of sugar cane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. *FEMS Microbiology Ecology* **29**, 117–128.

Goszczynska T, Botha WJ, Venter SN, Coutinho TA (2007) Isolation and identification of the causal agent of brown stalk rot, a new disease of maize in South Africa. *Plant Disease* **91**, 711–718. doi: 10.1094/PDIS-91-6-0711

Lakso JU, Starr MP (1970) Comparative injuriousness to plants of *Erwinia* spp. and other enterobacteria from plants and animals. *Journal of Applied Bacteriology* **33**, 692–707.

Mergaert J, Verdonck L, Kersters K (1993) Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora*) and *Erwinia stewartii* to the genus *Pantoea emend* as *Pantoea ananas* (Serrano 1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *indologenes* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology* **43**, 162–173.

Paccola-Meirelles LD, Ferreira AS, Meirelles WF, Marriel IE, Casela CR (2001) Detection of a bacterium associated with a leaf spot disease of maize in Brazil. *Journal of Phytopathology* **149**, 275–279. doi: 10.1046/j.1439-0434.2001.00614.x

Sergeeva E, Hirkala DLM, Nelson LM (2007) Production of indole-3-acetic acid, aromatic amino acid aminotransferase activities and plant growth promotion by *Pantoea agglomerans* rhizosphere isolates. *Plant and Soil* **297**, 1–13. doi: 10.1007/s11104-007-9314-5

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697–703.

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