SHORT COMMUNICATION

FIRST REPORT OF *PANTOEA ANANATIS* ASSOCIATED WITH LEAF SPOT DISEASE OF MAIZE IN POLAND

K. Krawczyk, J. Kamasa, A. Zwolinska and H. Pospieszny

Virology and Bacteriology Department, Institute of Plant Protection, National Research Institute, Wladyslawa Wegorka 20, 60-318 Poznan, Poland

SUMMARY

Maize (*Zea mays*) is one of the most important crops in Poland. Its acreage and production have grown over the last ten years, also thanks to the increasing interest for the bioenergy sector in the country. Maize is used as a source of methanol and biomass, and for fodder. Studies on bacterial infections of maize are plentiful in the world literature, but not in Poland, were only two such reports exist. This prompted the present investigation, in which combined samples from plants growing in different Polish locations were tested. Leaf samples were collected from plants displaying symptoms resembling those of leaf spot disease of maize induced by *Pantoea ananatis*. Four bacterial isolates were identified as *P. ananatis* using pathogenicity, biochemical, serological and molecular tests. To our knowledge this is the first report of the presence of the *P. ananatis* on maize in Poland and in Europe.

Key words: bacterial disease, pathogenicity, diagnosis, cloning, sequencing.

Maize (*Zea mays*) is one of the most important crops in Poland. Its acreage (over 350,000 ha) and production (2,000,000 tons) have increased during the last ten years and are still on the rise, because of the continuous development of the bioenergy sector. In Poland, maize is used to produce biomass either for direct burning, or for production of biogas, or of liquid engine fuels, especially bioethanol (2,500 litres/ha). However, the agricultural significance of this crop is also growing, as a source of fodder for animals and grains for human consumption.

The occurrence of bacterial pathogens of maize is reported from different parts of the world. For instance, *Pseudomonas avenae* subsp. *avenae* (Manns), the causal agent of bacterial leaf blight (Giester *et al.*, 2004; Claflin, 2000; Pataky *et al.*, 1997), *Erwinia carotovora* subsp. c*arotovora*, and *Erwinia chrysanthemi* pv. z*eae* (Sabet), that elicit bacterial stalk and top rot are known to have a worldwide distribution (Giester *et al.*, 2004; Claflin, 2000; Dickey *et al.*, 1987). Stewart`s wilt disease caused by *Pantoea stewartii* (Lamka *et al.*, 1991; Mergaert *et al.*, 1993), was recorded from USA, Brazil, Italy, Guyana, Peru, Poland, the former Soviet Union, Romania, Thailand, Vietnam and former Yugoslavia (Giester *et al.*, 2004; Claflin, 2000), and bacterial stripe and leaf spot caused by *Pseudomonas andropogonis* (Smith) Stapp (syn. *Burkholderia andropogonis*) occurrs also in many places (Giester *et al.*, 2004; Claflin, 2000). The list of maize bacterial pathogens continues with *Clavibacter michiganensis* subsp. *nebraskensis* (Vidaver and Mandel) the agent of Goss`s bacterial wilt and blight (Smidt and Vidaver, 1986), and *Pseudomonas syringae* pv. *coronafaciens* (Elliott) (Ribeiro *et al.*, 1977), that causes chocolate spot, both of which were reported only from the USA, the same as holcus spot caused by *Pseudomonas syringae* pv. s*yringae* (Lindow *et al.*, 1982) which, however, may be present wherever maize is grown (Giester *et al.*, 2004). By contrast, bacterial leaf spot caused by *Xanthomonas campestris* pv. *zeae* (Elliott) Dye (Giester *et al.*, 2004; Claflin, 2000; Coutinho and Wallis, 1991) was recorded only from South Africa.

Other bacterial pathogens are polyphagous and widespread. Some are epiphytic or endophytic and non phytopathogenic, others are plant or opportunistic pathogens of humans (De Baere *et al.*, 2004; Stock *et al.*, 2001; Rezzonico *et al.*, 2009). These species are: (i) *Pantoea ananatis* associated with leaf spot disease reported from South Africa (Goszczynska *et al.*, 2007), Brazil (Paccola-Meirelelles *et al.*, 2001), and Mexico (Pérez-y-Terrón *et al.*, 2009); (ii) *Enterobacter cloaceae* subsp. *dissolvens* (Hoffman *et al.*, 2005; Rosen, 1922) the agent of bacterial stalk rot and (iii) *Pantoea agglomerans* causing leaf blight and vascular wilt of maize and sorghum, present in Mexico (Morales-Valenzuela *et al.*, 2007). Since there are only two reports concerning bacterial diseases of maize in Poland (Lisowicz, 1995; Claflin, 2000) this issue was investigated in more detail.

Samples each made up of 15-20 symptomatic maize plants were collected from twenty different Polish loca-

Corresponding author: K. Krawczyk
Fax: 0048.61.8676301
E-mail: K.Krawczyk@ior.poznan.pl

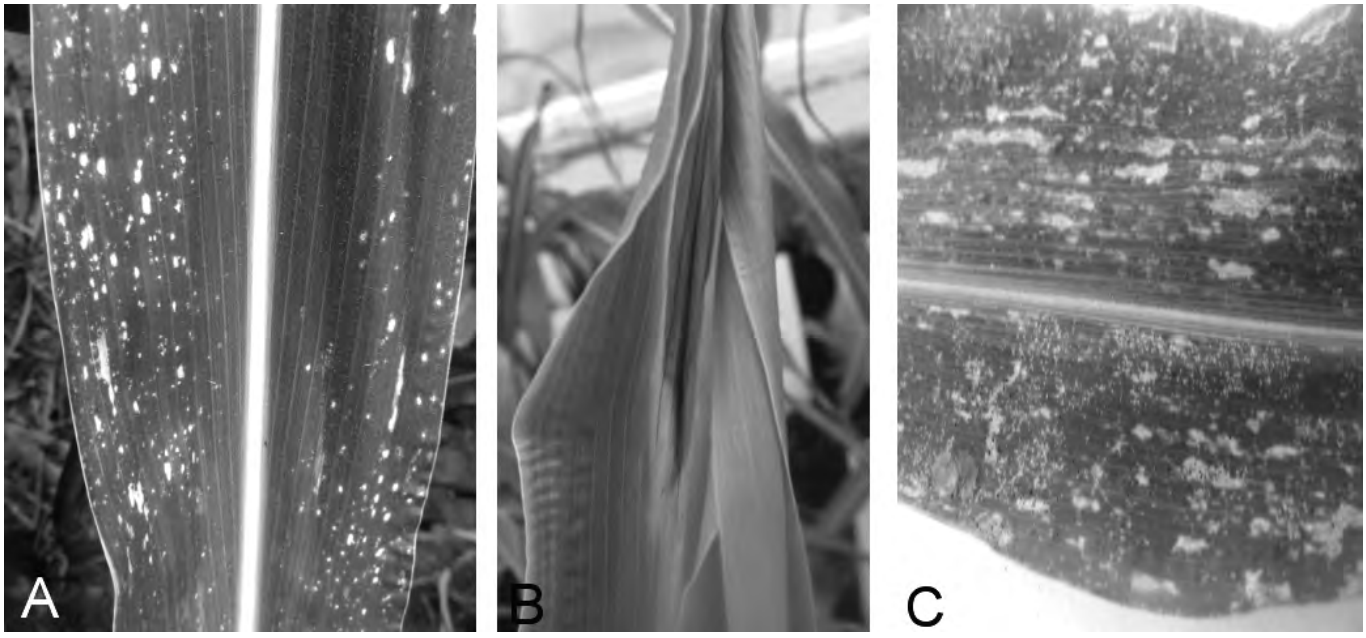

Fig. 1. A. Maize leaf naturally infected by *Pantoea ananatis*. B. A large brown lesion developed at the place of inoculation by infiltration using a syringe (Lamka *et al.*, 1991). C. Necrotic lesions following bacterial inoculation on carborundum-abrades leaves (Paccola-Meirelles *et al.*, 2001).

tions to check for the presence of possible bacterial diseases. Four of these samples, each from a different location (Smolice, Swadzim, Winna Gora, Pleszew), showed tiny dark-green water-soaked leaf spots that quickly developed into necrotic lesions (Fig. 1A) resembling very much those caused by *P. ananatis* (Paccola-Meirelles *et al.*, 2001).

Pieces of plant tissue with many necrotic spots were cut with a sterile scalpel and ground in a mortar with 1 ml of sterile water. The homogenate was then spread on the following media: tryptic soy agar (TSA), yeast extract dextrose calcium agar (YDC), King B (KB) and potato-dextrose agar (PDA). After incubation for 48 h at 27ºC, the morphology of bacterial colonies was observed. Representative colonies were selected and kept in a Bacto-Protect system (TSC, UK) at -20ºC.

The pathogenicity of the isolates was tested to fulfil Koch`s postulates based on hypersensitivity assays on tobacco plants (*Nicotiana tabacum* L. Xanthi) using the standard procedure (Coplin and Kado, 2001), inoculation of maize plants using two techniques (Lamka *et al.*, 1991; Paccola-Meirelles *et al.*, 2001) and reisolation of bacteria from mechanically infected maize plants. With the first inoculation method (Lamka *et al.*, 1991) a bacterial suspension was injected with a syringe into the pseudostem of the plant, approximately 1-2 cm above the soil line, whereas with the second inoculation method the bacterial suspension was spread on leaves that had been slightly abraded with carborundum just before inoculation (Paccola-Meirelles *et al.*, 2001). With both methods three maize plants of cv. Waza (F1) were inoculated. at the 4-5 leaf stage. Experiment were repeated five times. In each case a turbid aqueous bacterial suspension of pure isolated cultures (*ca.* $1x10^6$ CFU/ml from cultures grown for 24 h at 27ºC on TSA medium) was used. Sterile distilled water and the ATCC 33243 reference strain of *P. agglomerans* were used as negative controls. The positive control was the ATCC 33244 reference strain of *P. ananatis*. Four bacterial isolates (M241, M304, M408 and M471) that registered positive in pathogenicity tests were used for further biochemical, serological and molecular investigations

To characterize the tested isolates at the genus level, the following tests were performed using standard techniques (Coplin and Kado, 2001): determination of Gram reaction, (an)aerobic growth, production of fluorescent pigment on KB medium, yellow pigment on YDC medium, urease and motility test and testing for the presence of fungi on PDA medium. Identification at the species level was done with the API 20E test (BioMerieux, France) and the BIOLOG GN system (GN database v. 4.20.05 Micro Log 2) (BIOLOG, USA), utilizing 95 carbon substrates according to the manufacturer's instructions. Results were read automatically with a spectrophotometer after 24 h incubation at 28ºC. Numerical analysis of the results was made using GN Microlog 2N software, which calculates Microlog distances derived from the number of differences between strains. The four tested isolates were checked by ELISA for the presence of *Pantoea stewartii* subsp. s*tewartii* antigens using a commercial kit (Agdia, USA).

The 16S rRNA gene was amplified using the universal primers: 5`-AGTTTG ATCCTGGCTCAG-3` (forward) and 5`-AAGGAGGTGATCCAGCCGCA-3` (re-

verse) (Edwards *et al*., 1989). Amplicons (1.5 kb) were cloned in *Escherichia coli* using the pGEM-T Easy Vector System II (Promega, USA) according to manufacturer's directions. Recombinant plasmids were screened for insertions based on blue/white selection, PCR using the universal primers, and digestion with the restriction enzyme *Eco*RI. Plasmids containing the insert were sequenced using the universal M13 primers. A BLAST search of the four sequences against the GenBank database was performed.

The sequences of the four isolates were deposited in the GenBank under the accession Nos GQ332197 (iso-

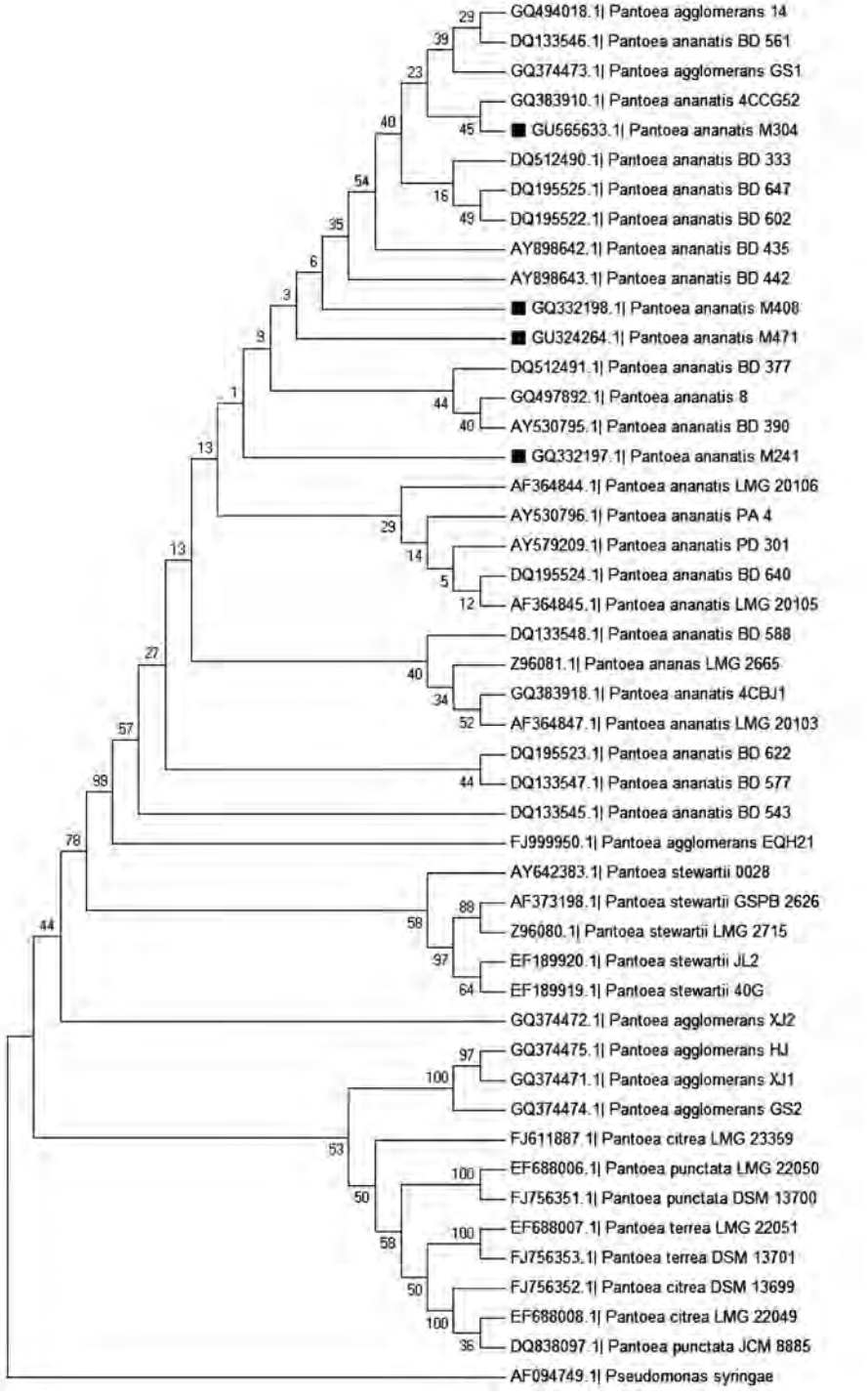

Fig. 2. Dendrogram constructed by neighbor joining analysis of the 16S rRNA gene sequences from different *Pantoea* species and a *Pseudomonas syringae* strain sequence (AF094749) as an outgroup. The Polish isolates are marked with black squares. The nucleotide sequences were analyzed using the BioEdit and Mega 4.0 software. Multiple sequence alignments were performed using the ClustalW program. Phylogenetic analysis was carried out by the neighbor joining algorithm implemented with Mega 4.0. Bootstrap values for phylogenetic comparisons were based on 1000 pseudoreplicates.

late M241), GU565633 (M304), GQ332198 (M408), and GU324264 (M471) (Table 1). Twenty one *P. ananatis* 16S rDNA sequences were retrieved from database to estimate the similarity and phylogenetic relationships of the Polish isolates with other *P. ananatis* strains (Fig. 2). All accession numbers of *P. ananatis* 16S rDNA sequences derived from the GenBank and employed in the phylogenetic analysis, are listed in Fig 2. Multiple sequence alignments of all *P. ananatis* sequences were performed using ClustalW software. A phylogenetic tree was constructed using the maximum parsimony method with bootstrapping (1000 repeats). The tree was rooted with a *Pseudomonas syringae* 16S rDNA sequence (AF094749). All phylogenetic analyses were performed using Mega 4.0 software. Names and GenBank accession numbers of the strains used in the analysis, are listed on the phylogenetic tree (Fig. 1).

In pathogenicity tests the four Polish isolates and the *P. ananatis* reference strain gave a positive hypersensitivity reaction on tobacco leaves and caused brown water-soaked lesions in maize around the place of infiltration (Lamka *et al*., 1991) (Fig. 3). Tiny, dark-green water-soaked leaf spots that quickly turned into necrotic lesions developed on the leaves inoculated by the carborundum method. These reactions correspond to those described for leaf spot disease caused by *P. ananatis* (Paccola-Meirelles *et al*., 2001).

In biochemical tests, the four bacterial isolates proved to be Gram-negative, and facultatively anaerobic. They produced entire, smooth and glistening colonies on TSA and yellow pigmentation on YDC, were nonfluorescencent on KB and motile on semi-solid medium (tryptone 10 g, sodium chloride 5 g and agar 5 g in 1 l of water) (Coplin and Cado, 2001). Bacteria reisolated from mechanically infected maize plants showed the same characteristics as above.

The metabolic profile of the four Polish isolates and the *P. ananatis* reference strain determined using API 20 E test was characteristic for *P. ananatis* (Mergaert *et al*., 1993) (Table 1). The only difference was in the indole test, which was negative for isolates M241, M304 and M408, and positive for the reference strain (Table 1). The BIOLOG biochemical fingerprint of all tested strains was comparable to that of *P. ananatis* reference and the Microlog similarity was 3 for isolates M241, M408, M471, and 4 for isolate M304. These results are retained as evidence that the bacterial isolates recovered form Polish maize belong to *P. ananatis*. This likelihood was confirmed by BLAST and phylogenetic analyses of 16S rDNA sequences of the four isolates under study which clustered with a number of *P. ananatis* strains in the phylogenetic tree (Fig. 2).

To our knowledge, this is the first report of the occurrence of *P. ananatis* on maize in Poland and Europe.

Table 1. Comparison of API 20E profiles of reference strains and tested isolates.

Species name	Isolate name	API 20E 7-digit code	Reference
Erwinia ananas	LMG 2665, LMG 2672	1245573	Mergaert *et al*., 1993
Erwinia ananas	LMG 2666, LMG 2667, LMG 2664	1245773	Mergaert *et al*., 1993
Erwinia ananas	LMG 2668, LMG 2669	1245563	Mergaert *et al*., 1993
Erwinia ananas	LMG 2670	1245373	Mergaert *et al*., 1993
Erwinia ananas	LMG 2671, LMG 2673, LMG 2674	1245163	Mergaert *et al*., 1993
Erwinia ananas	NCPPB 1846	1245573	Mergaert *et al*., 1984
Erwinia ananas	BG2, BG1	1245773	Mergaert *et al*., 1984
Erwinia ananas	Y1, MF28	1245563	Mergaert *et al*., 1984
Pantoea ananatis	M241	1205173	GQ332197
Pantoea ananatis	M304	1205573	GU565633
Pantoea ananatis	M408	1205573	GQ332198
Pantoea ananatis	M471	1245573	GU324264
Pantoea ananatis	ATCC 33244	1245573	GSMZ collection-Germany

Table 2. BLAST sequence similarity (%) of the 16S rDNA sequences of the tested isolates with analogical *Pantoea ananatis* sequences derived from GenBank.

Tested strain	Amplicon size (bp)	Identity (%)	*P. ananatis* GenBank accession No.
M241	1477	99	EU331415
M304	1508	99	GQ383910
M408	1508	98	GQ332197
M471	1464	99	CP001875

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