

Association of *Pantoea ananatis* and *Pantoea agglomerans* with leaf spot disease on ornamental plants of Araceae Family

R. Yazdani · N. Safaie · M. Shams-Bakhsh

Accepted: 4 June 2017 / Published online: 23 June 2017
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2017

Abstract During a survey in 2011–2012, three ornamental plants of Araceae namely *Aglaonema nitidum*, *Syngonium podophyllum* and *Dieffenbachia amoena* showing foliar disease symptoms were collected from central region of Iran. Infected plants exhibited spots on their leaves which appeared as yellow and water-soaked with chlorotic haloes and necrotic center. To investigate the etiology of this disorder, symptomatic leaves were collected from affected plants and six bacterial strains (B2Y, J3Y, SY, E60Y, E68Y and E5MM) were isolated and identified as *Pantoea ananatis* or *P. agglomerans* based on morphological, physiological, biochemical and molecular characters. The pathogenicity tests of the isolates demonstrated that they were not host specific. Furthermore, 16S rRNA gene sequencing revealed that the strains were phylogenetically closely related to genus *Pantoea*. Multilocus sequence analysis (MLSA) of concatenated partial *atpD*, *gyrB* and *rpoB* gene sequences of the six isolates showed a high similarity of B2Y, J3Y, and SY strains to *P. ananatis* and also of E60Y, E5MM and E68Y strains to *P. agglomerans*. These results were confirmed by phylogenetic analysis. To the best of our knowledge, this is the first report of leaf spot and necrosis of *A. nitidum*,

S. podophyllum and *D. amoena* caused by the genus *Pantoea*.

Keywords 16S rDNA · Bacterial leaf spot · Biochemical · Pathogenicity

Introduction

Ornamental plants production is one of the growing sectors of commercial horticulture in the world. Most of Araceae ornamental plants are of tropical and subtropical origin, therefore the greenhouse conditions are markedly different from their native habitats. This may put the plants under stress and thereby predispose them to a variety of pathogens (Chase 1987). The occurrence of bacterial pathogens in Araceae ornamental plants; e. g. *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya chrysanthemi* that cause soft rot, stem rot and leaf spot (Alippi and Lopez 2009), *Xanthomonas campestris* pv. *dieffenbachiae* (McFadden 1962), *Acidovorax anthuri* (Gardan et al. 2000) and *P. agglomerans* (Romeiro et al. 2006) has been reported from different parts of the world.

Markazi (Mahallat) and Isfahan provinces are two main growing regions for ornamental plants in Iran. *Aglaonema nitidum*, *Syngonium podophyllum* and *Dieffenbachia amoena* are popular potted ornamental plants and that may get infected by *P. carotovorum* subsp. *carotovorum* and *D. chrysanthemi* in Iran (Baghaee-Ravari et al. 2011; Yazdani et al. 2015), while

Electronic supplementary material The online version of this article (doi:10.1007/s10658-017-1264-z) contains supplementary material, which is available to authorized users.

R. Yazdani · N. Safaie · M. Shams-Bakhsh (✉)
Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
e-mail: shamsbakhsh@modares.ac.ir

other pathogenic bacteria have not been reported in ornamental plants from Iran.

The genus *Pantoea* was established by Gavini et al. (1989) and classified in the family *Enterobacteriaceae*. *Pantoea* species are widely distributed in nature and have been isolated from numerous ecological niches, including plants, water, and soil, as well as from humans and animals (Walterson and Stavrinides 2015). They are frequently associated with plants as epiphytes or endophytes and some species have been reported as plant pathogens, while others are opportunistic pathogens of humans (De Baere et al. 2004). Furthermore, several species of the genus *Pantoea* are known as plant pathogens: bacterial wilt of maize and sweet corn caused by *P. stewartii* subsp. *stewartii* (Mergaert et al. 1993); it is also reported to be associated with seed and boll rot in cotton (Medrano and Bell 2007), russetting of pear fruits (Lindow et al. 1998), and necrosis of the leaf tips and margins of pearl millet (Frederickson et al. 1997); *P. ananatis* causes a range of diseases on a wide variety of agricultural crops the most recent ones include agave disease (Fucikovskiy and Aranda 2006), palea browning of rice (Cortesi and Pizzatti 2007), leaf blotch disease of sudangrass (Azad et al. 2000), brown stalk rot of maize (Goszczyńska 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).

Surveys of ornamental plants during 2011–2012 in the central provinces of Iran including Markazi (Mahallat) and Isfahan showed the leaf spot symptoms in plants of Araceae family. During the early stages of the disease, the spots appeared as small, yellow, and water-soaked with chlorotic haloes. In time, the spots enlarged and necrotized. Considering the importance of ornamental plants of Araceae family in Markazi (Mahallat) and Isfahan provinces in Iran, the alleged existence of some bacteria damaging them, and since only *P. carotovorum* subsp. *carotovorum* and *D. chrysanthemi* had previously been reported in Iran, a study was undertaken to identify the putative agent causing the disease and determine its taxonomic position through host range biochemical and genetic characterization.

Materials and methods

Media and cultural condition

Isolations of bacteria from the diseased leaves of *A. nitidum*, *S. podophyllum* and *D. amoena* were done

according to Perombelon and Van der Wolf (2002). Briefly; samples were washed under tap water to remove soil particles, dried and surface sterilized in 0.5% sodium hypochlorite for 30 s and then rinsed twice with sterile distilled water. The plant tissues were crushed in sterile water, suspensions were streaked on nutrient agar (NA) medium (Perombelon and Hyman 1986) and incubated at room temperature for five days to allow bacterial growth. Single colonies obtained on NA medium were selected for further characterization. All isolates were stored in sterile water at room temperature and/or in 30% (v/v) glycerol at -80°C .

Phenotypic tests

To characterize the selected isolates at the genus level, the following tests were performed using standard methods (Schaad et al. 2001): Gram reaction, levan production, Esculin hydrolysis, (an) aerobic growth, production of fluorescent pigment on KB medium, yellow pigment on YDC medium, urease and motility test, oxidase and catalase activity. Hydrolysis of gelatin and casein were tested on gelatin agar and on skimmed-milk agar, respectively, acid production from inulin in phenol red peptone water, production of reducing substances from sucrose, malonate utilization, indole production from tryptophan, anaerobic degradation of arginine (Moeller medium) and utilization of citrate (Simmons' medium) were studied. Bacterial isolates were also checked for production of phosphatase, sensitivity to erythromycin (Schaad et al. 2001), the ability to grow at 37°C in nutrient broth, and in 5% sodium chloride on nutrient agar at 28°C . Further characterization was performed with assimilation tests of carbon sources on the basal medium of Ayers supplemented with 0.1% carbohydrates including glucose, cellobiose, D-Sorbitol, L-Rhamnose, D-Melibiose, L-Tartrate, D-Tartrate, adonitol, maltose, L-Arabinose, erythritol (Schaad et al. 2001).

Pathogenicity and hypersensitivity assays

Pathogenicity was confirmed by infiltrating $100\ \mu\text{l}$ of bacterial suspension (1×10^5 CFU/ml) into the surface mesophyll of *A. nitidum*, *S. podophyllum* and *D. amoena* leaves and incubation under greenhouse condition (Paccola-Meirelles et al. 2001). Plants were inoculated with isolated bacteria and re-isolations of bacteria from inoculated plant leaves were performed

as described above. Two independent experiments were performed and each strain was inoculated separately into two leaves. Negative controls (water) were included in these experiments. Symptoms were recorded for up to 21 days post-inoculation.

Hypersensitive reaction on tobacco (*Nicotiana tabacum* cv. *Xanthi*) and geranium (*Pelargonium × hortorum*) was performed as described by Coplin and Kado (2001) and Lelliott et al. (1966). Two leaves were infiltrated with a suspension containing 2×10^8 CFU/ml. Inoculated tobacco and geranium plants were examined for hypersensitivity elicitation after 24 and 96 h.

Bacterial DNA extraction

To prepare DNA, isolates were grown in nutrient broth at 28 °C for 24 h, DNA was extracted using a commercial extraction kit as suggested by the manufacturer (Accuprep DNA extraction kit- k3032, Bioneer, Korea) and stored at –20 °C.

Polymerase chain reaction (PCR) and sequencing

16S rRNA gene sequences were determined for representatives of all strains, using the primers and conditions described by Hauben et al. (1997), and partial *gyrB*, *rpoB* and *atpD* gene sequences were determined for all strains (Brady et al. 2008). Amplified fragments were resolved by electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide at final concentration of 0.5 µg/ml. The PCR products were purified by QIAquick PCR purification kit (Qiagen), and were sequenced using an ABI 3730XL sequencer (Bioneer Corporation, South Korea).

16S rRNA gene sequencing

16S rRNA sequences of our isolates were compared with the type strains of species from the genera *Pantoea*, *Erwinia*, *Pectobacterium*, *Enterobacter* and *Tatumella* of the family *Enterobacteriaceae* using the BLAST homology search program. Our newly obtained DNA sequences, together with the GenBank sequences used for phylogenetic analyses, were aligned by Clustal X2 (<http://www.clustal.org/>) using the default parameters. The model of base substitution was selected using MrModeltest 2 (Nylander 2004). The Akaike-supported model, a general time reversible model, including among-site rate heterogeneity and

estimates of invariant sites (GTR + G + I) was used in phylogenetic analyses. Bayesian analysis was performed to infer a phylogenetic tree using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) running the chains for 10^6 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to determine equilibrium distribution and help estimate the posterior probabilities of the phylogenetic trees (Larget and Simon 1999) using the 50% majority rule. *Escherichia coli* (accession number X80725) was used as outgroup taxon (Cilia et al. 1996). Sequences were deposited into the GenBank database (accession numbers: KR361752 for E5MM, KR361753 for E60Y, KR361754 for B2Y, KR361756 for J3Y, KU679909 for SY).

GyrB, *rpoB*, *atpD* and *infB* sequences

Multi Locus Sequence Analysis (MLSA) based on partial sequences of *gyrB*, *rpoB* and *atpD*, three protein encoding genes, was performed on all strains as described previously (Brady et al. 2008). Sequence analysis and tree construction were performed as described above. Phylogenetic tree was based on concatenated *gyrB*, *rpoB* and *atpD* sequences of selected *Pantoea* strains. Sequences from *Shigella dysenteriae* M131649 was obtained from the genome sequencing database of the Sanger Institute (<http://www.sanger.ac.uk>) and included as outgroup.

Results

Identification of *Pantoea* spp. in ornamental plants

A survey of some greenhouses in Markazi (Mahallat) and Isfahan provinces in Iran was made in 2011–2012. Leaf samples of *A. nitidum*, *S. podophyllum* and *D. amoena*, *Epipremnum aureum* and *Dracaena marginata* showing leaf spot and soft rot symptoms were collected. A total of 84 different bacterial strains were isolated from the collected diseased specimens, of which, twenty-one bacterial strains were able to cause leaf spot and soft rot on host plants. Among these isolates, 15 strains were recognized as *P. carotovorum* subsp. *carotovorum* (Yazdani et al. 2015) and six of the isolated bacterial strains were identified as *Pantoea* spp.

(Table 1). Symptoms in these plants appeared as water soaked lesions with yellow margin, developing to necrotic leaf spot (Fig. 1). Fig 1 shows leaf of *A. nitidum* that was infected naturally by the SY strain. Infected spots enlarged and coalesced under wet conditions. *Pantoea* spp. were divided into two groups (Table 2) based on phenotypic assays. The biochemical tests indicated all six bacterial isolates were Gram-negative and facultatively anaerobic. These isolates produced entire, yellow colonies on NA, smooth, glistening colonies and yellow pigmentation on YDC, and were nonfluorescent on KB. These features agree with those expected for *Pantoea* spp. (Coplin and Kado 2001; Krawczyk et al. 2010). Reisolated bacteria from inoculated ornamental plants, showed the same characteristics as above. Isolates B2Y, J3Y and SY were determined as *P. ananatis* because of the results of oxidase test, indole production and ability to produce acid from D-sorbitol (Mergaert et al. 1993; Walcott et al. 2002) and E60Y, E5MM and E68Y isolates were diagnosed as *P. agglomerans* based on their ability to utilize malonate and D-tartrate (Gavini et al. 1989; Deletoile et al. 2009).

The 16S rDNA sequences of the six representative strains including SY, B2Y, J3Y, E60Y, E68Y and E5MM obtained in this study were deposited in the GenBank database. BLAST searches of the B2Y, SY and J3Y sequences showed that 16S rDNA of the three indole-positive strains had the highest homology (99.3 to 99.9%) with *P. ananatis* strains (accession number AF364847), LMG 20106 (AF364844) and PA4 (AY530796). The 16S rDNA sequence of the three indole-negative and D-tartrate-positive strains had the highest homology (99.6%) with *P. agglomerans* DSM 3493 T (AJ233423). The phylogenetic relationship



Fig. 1 Symptoms of bacterial leaf spot caused by *Pantoea ananatis* on *Aglaonema nitidum*, SY sample, as observed in nature

derived from Bayesian analysis of the 16S rDNA sequences of the six strains from present study with 32 sequences of other bacteria are shown in Fig. 2. The 16S rRNA gene phylogenetic tree showed that strains E68Y, E5MM and E60Y clustered with members of *P. agglomerans* with high posterior probability value, while the three strain B2Y, SY and J3Y clustered with the type strain of *P. ananatis* with a high posterior probability value (Fig. 2).

Further analysis was carried out by MLSA. PCR amplification and partial sequencing of three house-keeping genes, *gyrB* (610 bp), *atpD* (790 bp) and *rpoB* (830 bp) was carried out. These partial sequences were used for comparing the *P. agglomerans* and *P. ananatis* *gyrB*, *atpD* and *rpoB* gene sequences with those of other *Pantoea* deposited in GenBank. The same sequences were used for construction of one combined phylogenetic tree (Fig. 3). In all phylogenetic analyses, regardless of whether the gene sequences were utilized in combination (Fig. 3) or separately (Supplementary Fig. S1-S3), two groups of three were generated; one group harbouring the *P. ananatis* and one group consisting of the *P. agglomerans* isolates. Although the basic topologies derived by the use of the three genes separately were preserved in the generated trees, minor differences were observed. Phylogenetic evaluation confirmed that the six strains reported in this study from ornamental plants were divided into two groups. The trees demonstrated clearly that the sequences of the three indole-positive isolates from ornamental plants were clustered with *P. ananatis* and three indole-negative and D-tartrate-positive strains were grouped with *P. agglomerans*.

Table 1 *Pantoea* spp. strains isolated from ornamental plants in Iran

No. of strain(s)	Host species	Geographical origin in Iran	Plant part
B2Y	<i>Syngonium podophyllum</i>	Markazi	Leaf
J3Y	<i>Aglaonema nitidum</i>	Markazi	Leaf
SY	<i>S. podophyllum</i>	Markazi	Leaf
E68Y	<i>A. nitidum</i>	Isfahan	Leaf
E5MM	<i>A. nitidum</i>	Isfahan	Leaf
E60Y	<i>Dieffenbachia amoena</i>	Markazi	Leaf

Table 2 Comparison of phenotypic features of field isolates with those of *Pantoea* spp. type and reference strains

Test	Type strain: <i>P. ananatis</i>	B2Y	J3Y	SY	Type strain: <i>P. agglomerans</i>	E68Y	E5MM	E60Y
Gram	- ^a	-	-	-	-	-	-	-
Yellow pigment	+ ^b	+	+	+	+	+	+	+
Hypersensitive reaction	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
Pectolytic activity	-	-	-	-	-	-	-	-
Levan	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-
Fluorescent pigment on KB	-	-	-	-	-	-	-	-
Taupe yellow on YDC	-	-	-	-	-	-	-	-
O/F ^c	F	F	F	F	F	F	F	F
Esculin hydrolysis	d	-	-	-	+	+	+	+
Growth at 37 °C	+	+	-	+	+	+	+	+
Growth at 41 °C	-	-	-	-	-	-	-	-
Growth in 5% NaCl	+	+	-	+	+	+	+	+
Nitrate reduced	D^d	-	-	-	+	+	+	+
Indole production	+	+	+	+	-	-	-	-
Starch hydrolase	-	-	-	-	-	-	-	-
H ₂ S from cysteine	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Malonate	-	-	-	-	+	+	+	+
Utilization of: Sucrose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Cellobiose	+	+	+	+	D	+	-	+
D-Sorbitol	+	+	+	+	-	-	-	-
L-Rhamnose	D	+	+	-	+	+	-	+
D-Melibiose	+	+	+	+	-	-	-	-
L-Tartrate	-	-	-	-	-	+	+	-
D-Tartrate	-	-	-	-	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	-

a (-) Negative, b (+) Positive, c Oxidative fermentative test, d differs among strains

Pathogenicity and hypersensitivity assays

Most isolates caused hypersensitive reaction in geranium (*Pelargonium × hortorum*) and tobacco (*Nicotiana tabacum*) plants 2–5 days after infiltration of 5×10^5 CFU/ml bacteria (Figs. 4 and 5). As a result necrotic areas formed on tobacco and geranium leaves: necrotic areas in geranium leaves were surrounded by

chlorotic halos and tended to enlarge, albeit, very slowly but in tobacco leaves only necrotic areas were formed. The results showed that *P. ananatis* and *P. agglomerans* strains induced a delayed hypersensitive reaction in geranium and tobacco.

Preliminary results suggested that host specificity was not observed in the two *Pantoea* spp. on the three ornamental hosts and all six isolates were pathogenic on

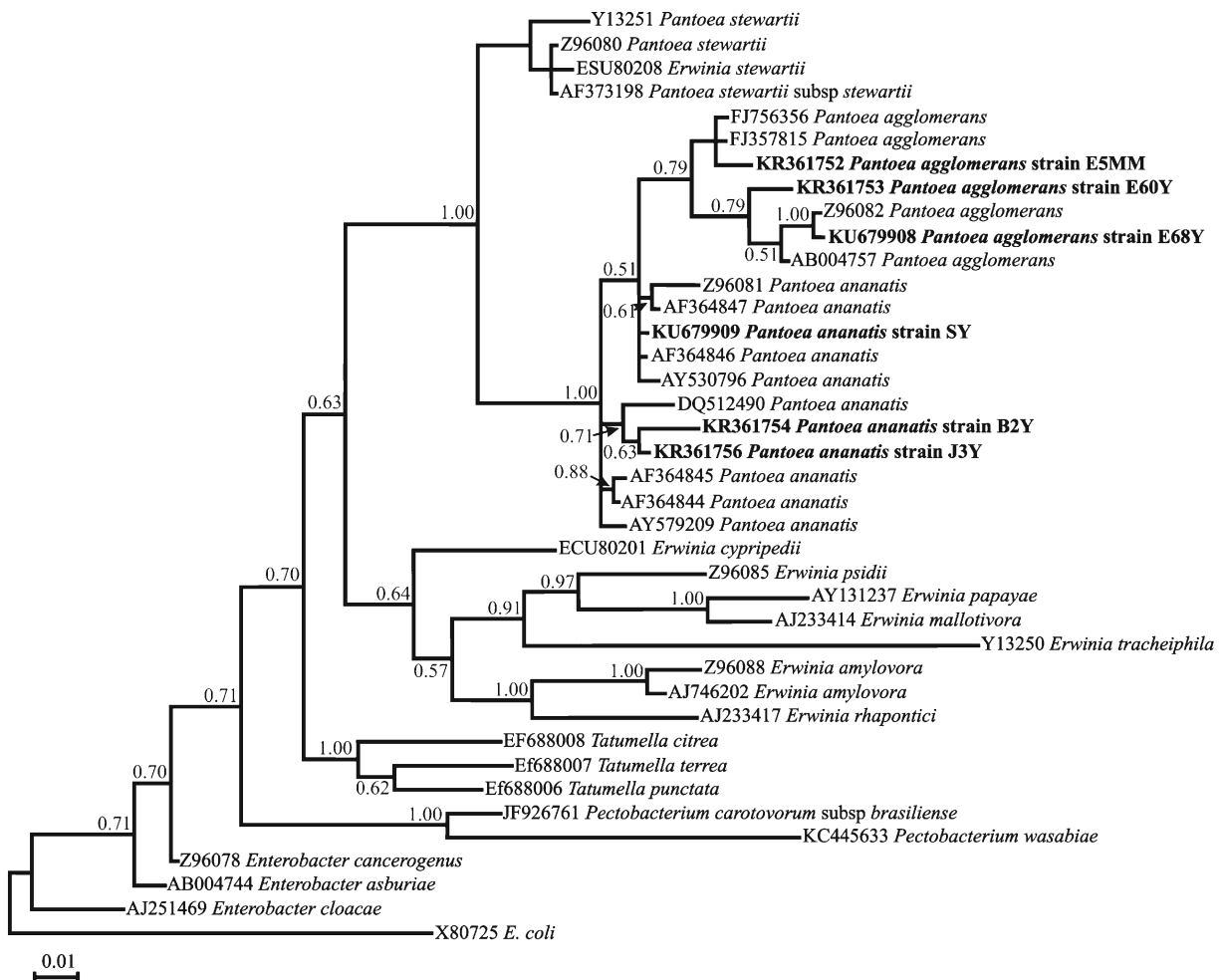


Fig. 2 Bayesian 50% majority rule consensus tree inferred from 39 sequences of partial 16 s rDNA under the GTR + I + G model. At nodes are given posterior probabilities values >50%. The new sequences are in bold

A. nitidum. Water-soaked yellow spots developed on leaves of *A. nitidum* inoculated with each of the six strains 7–10 days after inoculation (dai). After 12–14 days, the center of lesions became necrotic with yellow halo around the lesions. After 14 days, large necrotic lesions had developed on the leaves with no apparent extension of symptoms from these spots into the main vein and petiole. Symptoms in inoculated *D. amoena* consisted of water soaked lesions with yellow surrounding after 7–10 days that enlarged and became oval shaped to necrotic leaf spots after 14–16 days. Symptoms produced on *D. amoena* by E60Y were similar to *A. nitidum* symptoms. All isolates also created leaf spots on *S. podophyllum*. Initial symptoms appeared as water-soaked spots on the leaves within 7 or 8 days, which expanded into brownish yellow areas after 12–14 days (Fig. 6).

Based on phenotypic specifications, the *Pantoea* sp. isolated from inoculated plants was shown to be identical with the main strains used in these pathogenicity examinations. Recovered colonies from plants showing disease symptoms were yellow, gram negative rods, oxidase negative, and utilized glucose as facultative anaerobes. Uninoculated plants did not develop any symptoms, and no yellow bacterial colonies were isolated from their leaves or stems.

Discussion

In this study, two species of bacteria belonging to the genus *Pantoea*; i. e. *P. ananatis* and *P. agglomerans*, associated with the leaf spot disease of ornamental

Fig. 3 Bayesian 50% majority rule consensus tree inferred from 112 sequences based on the concatenated nucleotide sequences of *gyrB*, *rpoB* and *atpD* under the GTR + I + G model. At nodes are given posterior probabilities values >50%. The new sequences are in bold

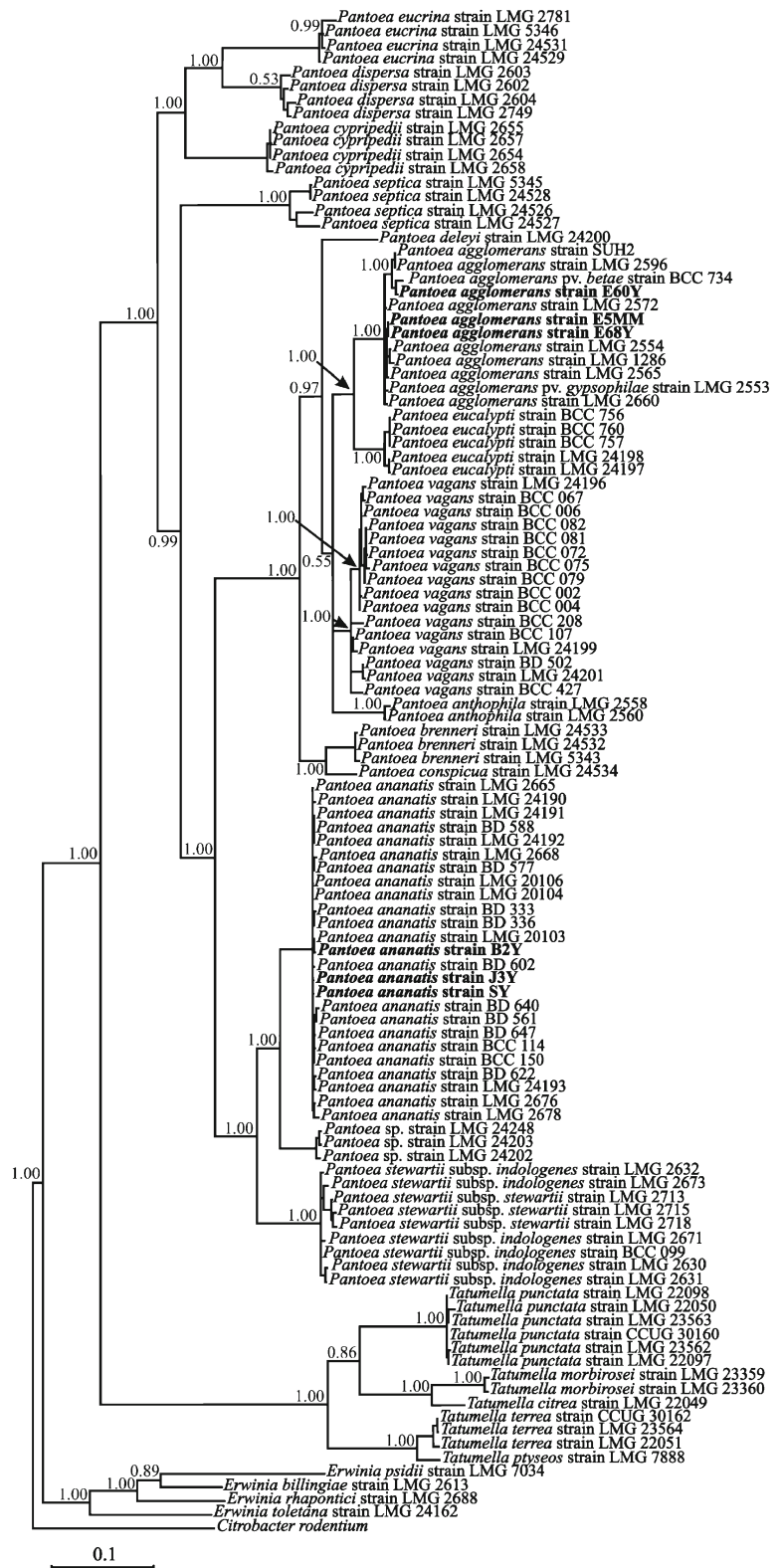




Fig. 4 The results of hypersensitive response (HR) by infiltration of geranium (*Pelargonium × hortorum*) leaves with *P. ananatis* strain SY

plants of Araceae family were described from Iran. The isolated bacterial strains were identified using a combination of phenotypic and pathogenesis assays as well as phylogenetic analysis of 16S rDNA. Three isolates, B2y, J3Y and SY, were identified as *P. ananatis* and

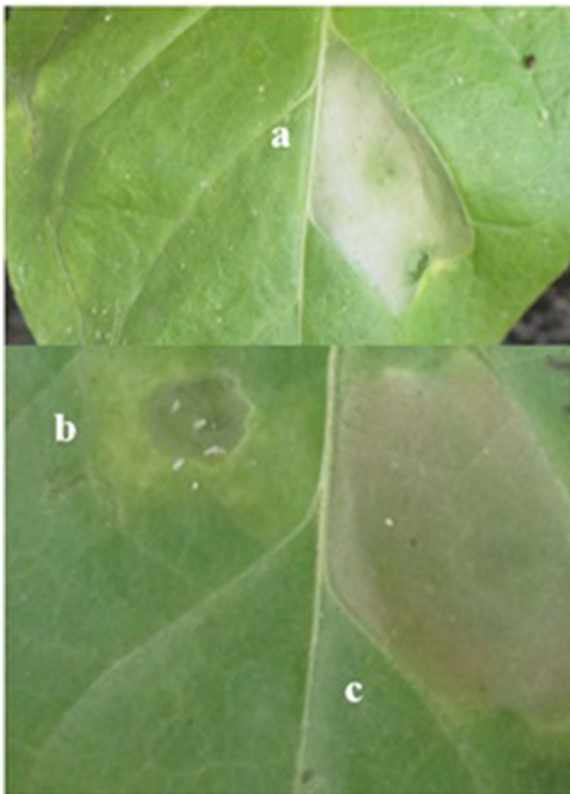


Fig. 5 The results of hypersensitive response (HR) by infiltration of tobacco (*Nicotiana tabacum* cv. *Xanthi*) leaves with *Pantoea ananatis* and *Pantoea agglomerans*, a: *P. ananatis* strain SY; b, c: *P. agglomerans* strains E5MM, E60Y respectively

three isolates, E60Y, E5MM and E68Y, were identified as *P. agglomerans*. To the best of our knowledge, this is the first report of a *Pantoea* sp. associated with leaf spot of *A. nitidum*, *S. podophyllum* and *D. amoena*.

Formerly the genus *Pantoea* contained seven valid species, namely: *P. agglomerans*, the type species of the genus, *P. ananatis*, *P. stewartii* (divided into the two subspecies *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*), *P. dispersa*; *P. citrea*, *P. punctata*, and *P. terrea*. Recently, polyphasic taxonomic studies resulted in the description of 18 novel species (*P. brenneri*, *P. cypripedii*, *P. septica*, *P. conspicua*, *P. eucrinea*, *P. gaviniae*, *P. calida*, *P. allii*, *P. coffeiphila*, *P. anthophila*, *P. vagans*, *P. eucalypti*, *P. deleyi*, *P. rwandens*, *P. wallisii*, *P. rodasii*) (Brady et al. 2010; Popp et al. 2010; Brady et al. 2011; Gueule et al. 2015; Brady et al. 2009; Brady et al. 2012) and the transfer of *P. citrea*, *P. punctata* and *P. terrea* to the genus *Tatumella* (Brady et al. 2009, 2010). MLSA based on *gyrB*, *rpoB* and *atpD* sequences was used in these studies as a supporting phylogenetic technique, since these genes were previously shown to be useful phylogenetic markers for *Pantoea* (Brady et al. 2008).

Since these bacteria are usually isolated from soil, fruit, and vegetables (Grimont and Grimont 2005). Determination of pathogenicity is a decisive step in the recognition of plant pathogenic bacteria. In this study, pathogenicity was confirmed by inoculation of bacterial suspension into the host plants leaf. In these tests, all strains caused leaf spots on the leaves of *A. nitidum*. All strains created symptom of leaf spot with yellowing on *S. podophyllum*. Only E60Y strain caused leaf spot symptoms on *D. amoena* leaves (Fig. 6).

Results of the pathogenicity tests of the isolates revealed that *P. agglomerans* and *P. ananatis* were not host specific. *P. agglomerans* has been altered from a saprophytic form to gall-forming bacterium on some plants (Weinthal et al. 2011). This transformation is caused by acquisition of a pathogenicity plasmid (pPATH) containing a pathogenicity island (Barash and Manulis-Sasson 2009). Reports on the virulence of genus *Pantoea* in different plants, indicate that this bacterium may have gained some genes related to virulence from other original pathogens.

Our results showed that strains identified as *P. agglomerans* and *P. ananatis* using biochemical methods were identical with description of species in Bergey's manual of systematic bacteriology (Grimont and Grimont 2005). Some chemical characteristics

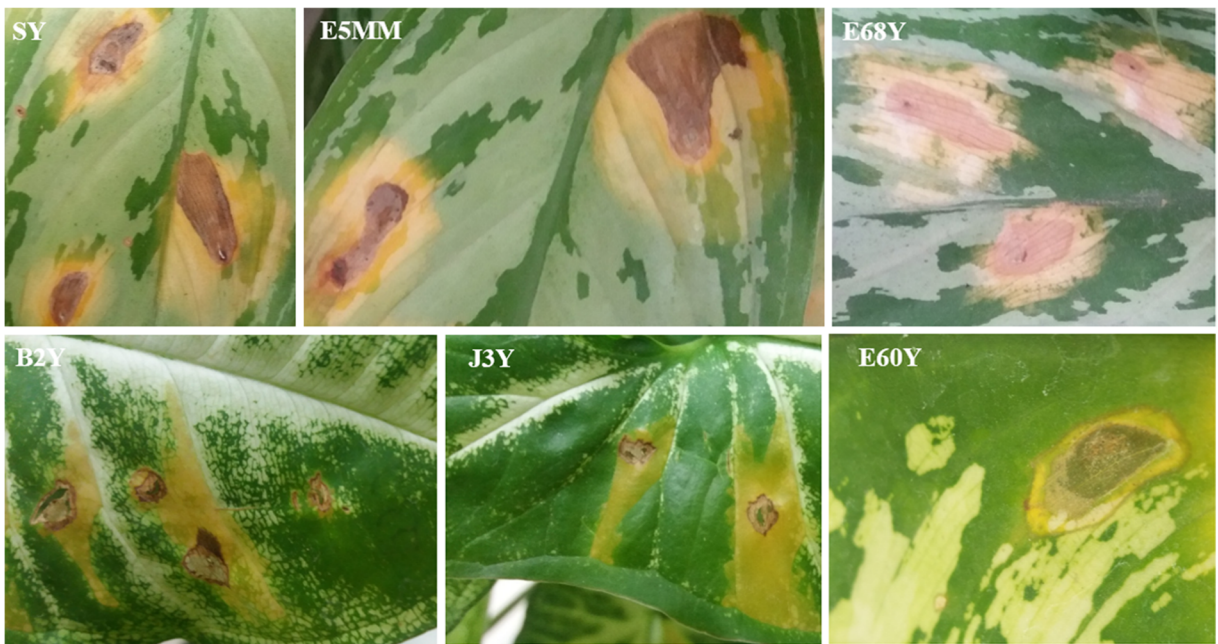


Fig. 6 Symptoms of inoculation on *Aglaonema nitidum* by *Pantoea agglomerans* and *Pantoea ananatis*. **a, b, c:** *P. agglomerans* strains E68Y, E60Y and E5MM and **d, e, f:** *P. ananatis* strains B2Y, J3Y and SY

could be used to distinguish these species. Deletoile et al. (2009) have demonstrated that the key characteristic differentiating *P. agglomerans* from other species of *Pantoea* is utilization of D-tartrate. D-tartrate was not used by other species of *Pantoea*, whereas *myo*-inositol and *meso*-tartrate were used only by *P. agglomerans* and closely related *Pantoea* species. Our results were consistent with this data, just three strains, E60Y, E5MM and E68Y, were able to utilize D-tartrate. Gavini et al. (1989) have distinguished *P. agglomerans* and *P. dispersa* by only two characters, the ability to use malonate and erythritol; *P. agglomerans* was able to use malonate and unable to use erythritol, our results are also consistent with these data. *P. ananatis* and *P. stewartii* subsp. *indollgense* are two species of the genus that are able to produce indole, and which can hardly be differentiated by biochemical and nutritional properties, but are only differentiated by the ability to produce acid from D-sorbitol and α -methyl-D-glucose, *P. ananatis* is able to use D-sorbitol and unable to use α -methyl-D-glucose (Mergaert et al. 1993). Based on these key phenotypic tests, the strains SY, B2Y and J3Y were identified as *P. ananatis*.

Sequencing of the 16S rRNA gene is considered a standard method for the description of bacterial taxa such as *Pantoea* genus (Clarridge 2004; Noller et al.

2005; Coutinho et al. 2002). The sequence analysis of the 16S rDNA also clearly showed that our bacteria are members of genus *Pantoea*, and confirmed biochemical characters. The six isolates formed a distinct well-supported cluster in phylogenetic tree based on 16S rRNA gene sequences that closely related to *P. ananatis* and *P. agglomerans*. The phenotypic characterization and 16S rDNA analysis have been used in identification of *P. ananatis* that caused stem necrosis of rice (Azad et al. 2000) and *P. ananatis* isolated from leaf spot disease of maize in Poland (Krawczyk et al. 2010). Coutinho et al. (2002) have shown that *P. ananatis* is the causal agent of bacterial blight and dieback of Eucalyptus by analysis of 16S rRNA gene along with other methods. Sherafati et al. (2014) also have used 16S rRNA sequences together with biochemical tests for determination of the agent associated with the citrus canker in Iran.

Although SY, B2Y, J3Y, E68Y, E60Y, E5MM strains clustered closely with members of the genus *Pantoea* in the 16S rRNA gene phylogenetic tree with high support, this study also used partial sequences of housekeeping genes which have been shown to be more reliable genetic markers for identification and phylogenetic analysis (Brady et al. 2008). The six isolates formed two

distinct well-supported clusters that were closely related to *P. ananatis* and *P. agglomerans*, not only in the phylogenetic tree based on the concatenated sequences (Fig. 3), but also in each of the single gene-based trees (Supplementary Fig. S1–S3). In concatenated tree, E68Y, E60Y and E5MM strains grouped with strains isolated from onion in South Africa (LMG 2596) as well as cereal (LMG 2565) and wheat (LMG 2572) in Canada, and strains infecting beet in South Africa (BCC734). In all of the nucleotide sequence trees (Fig. 3 and Supplementary Fig. S1–S3), these strains clustered in close proximity to *P. agglomerans* strains that were isolated from different plants. B2Y, J3Y and SY strains clustered closely with isolates of the agent of Eucalyptus blight and dieback in South Africa (BD 602) and agent of maize brown stalk rot in South Africa (LMG 20103).

The concatenated nucleotide sequence tree had similar groupings to those of the 16S rRNA sequence tree, with one exception. In the 16S rRNA nucleotide sequence tree, *P. agglomerans* and *P. ananatis* formed a cluster. The reasons for these groupings can be explained by the high level of homoplasy in the 16S rRNA gene sequences of members of the Enterobacteriaceae (Stephan et al. 2007). It is apparent that a concatenated tree, based on the nucleotide sequences of the four housekeeping genes, appeared to be most reliable for determining phylogenetic relationships amongst SY, B2Y, J3Y, E5MM, E68Y and E60Y strains.

P. ananatis and *P. agglomerans* are ubiquitous bacteria occupying diverse and unusual ecological niches. They are associated with plants as epiphytes, endophytes, saprophytes, pathogens or symbionts. Environmental conditions determine if a bacterial disease will occur. Some factors such as temperature and moisture on the plant surface also soil nutrients affect the initiation and development of bacterial plant diseases (Agrios 2005). High humidity and low temperature conditions and may be imbalance of nutrients in the ornamental plant greenhouses affect disease development through their influence on the susceptibility of the host, on the penetration, multiplication and activity of the bacteria. An explanation of how *P. ananatis* and *P. agglomerans* have become so broadly adapted to these different habitats will probably only be determined once species-specific genes have been identified (Coutinho and Venter 2009).

P. agglomerans and *P. ananatis* have high genetic flexibility (Manulis and Barash 2003), for example *P. agglomerans* and *P. ananatis* are common epiphytes

on host and non-host plants (Gitaitis et al. 2002). In many such cases, the occurrence of *P. ananatis* and *P. agglomerans* on the plant surface have not been linked to a specific disease on the host whence it was isolated as an epiphyte. However, these asymptomatic non-hosts could provide a source of inoculum, causing disease outbreaks in susceptible hosts grown in their vicinity or in contrast these strains have been used for biological control of other plant pathogens (Vantomme et al. 1989; Pusey et al. 2011). Application of *P. agglomerans* and *P. ananatis* in research as agents of biological control should be done with great caution, because these bacteria and other members of Enterobacteriaceae have the potential for acquisition of some pathogenicity specific genes.

Acknowledgements We gratefully acknowledge financial support from Tarbiat Modares University, Tehran, Iran.

Author contributions Razieh Yazdani carried out all experiments, drafting the manuscript;

Naser Safaie gave advice;

Masoud Shams-bakhsh supervised the project, conception and design of study, edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards The result of this work has not been published previously and is not under consideration elsewhere.

Funding This research was funded by Tarbiat Modares University, Tehran, Iran.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Agrios, G. N. (2005). *Plant Pathology* (5th ed.). New York: Academic Press.
- Alippi, A. M., & Lopez, A. C. (2009). First Report of *Pectobacterium carotovorum* subsp. *carotovorum* on *Spathiphyllum wallisii* in Argentina. *Plant Disease*, 93, 842.
- Azad, H. R., Holmes, G. J., & Cooksey, D. A. (2000). A new leaf blotch disease of sudangrass caused by *Pantoea ananas* and *Pantoea stewartii*. *Plant Disease*, 84, 973–979.
- Baghaee-Ravari, S., Rahimian, H., Shams-Bakhsh, M., Lopez-Solanilla, E., Antúnez-Lamas, M., & Rodríguez-Palenzuela, P. (2011). Characterization of *Pectobacterium* species from

- Iran using biochemical and molecular methods. *European Journal of Plant Pathology*, 129, 413–425.
- Barash, I., & Manulis-Sasson, S. (2009). Recent evolution of bacterial pathogens: The gall-forming *Pantoea agglomerans* case. *Annual Review of Phytopathology*, 47, 133–152.
- Brady, C. L., Cleenwerck, I., Venter, S. N., Vancanneyt, M., Swings, J., & Coutinho, T. A. (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.
- Brady, C. L., Venter, S. N., Cleenwerck, I., Engelbeen, K., Vancanneyt, M., Swings, J., & Coutinho, T. A. (2009). *Pantoea vagans* sp. nov., *Pantoea eucalypti* sp. nov., *Pantoea deleyi* sp. nov. and *Pantoea anthophila* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 59, 2339–2345.
- Brady, C. L., Cleenwerck, I., Venter, S. N., Engelbeen, K., de Vos, P., & Coutinho, T. A. (2010). Emended description of the genus *Pantoea* and description of four novel species from human clinical samples, *Pantoea septica* sp. nov., *Pantoea eucrina* sp. nov., *Pantoea brenneri* sp. nov. and *Pantoea conspicua* sp. nov., and transfer of *Pectobacterium cyripedii* (Hori 1911) Brenner et al. 1973 emend. Hauben et al. 1998 to the genus *Pantoea* emend. as *Pantoea cyripedii* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 60, 2430–2440.
- Brady, C. L., Goszczynska, T., Venter, S. N., Cleenwerck, I., De Vos, P., Gitaitis, R. D., & Coutinho, T. A. (2011). *Pantoea allii* sp. nov., a novel species isolated from onion and onion seed. *International Journal of Systematic and Evolutionary Microbiology*, 61, 932–937.
- Brady, C. L., Cleenwerck, I., van der Westhuizen, L., Venter, S. N., Coutinho, T. A., & De Vos, P. (2012). *Pantoea rodasii* sp. nov., *Pantoea rwandensis* sp. nov. and *Pantoea wallisii* sp. nov., isolated from Eucalyptus. *International Journal of Systematic and Evolutionary Microbiology*, 62, 1457–1464.
- Bruton, B. D., Wells, J. M., Lester, G. E., & Patterson, C. L. (1991). Pathogenicity and characterization of *Erwinia ananas* causing a postharvest disease of cantaloupe fruit. *Plant Disease*, 75, 180–183.
- Chase, A. R. (1987). *Compendium of ornamental foliage plant diseases (no. 635.923/C487)*. St. Paul: APS press.
- Cilia, V., Lafay, B., & Christen, R. (1996). Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Molecular Biology and Evolution*, 13(3), 451–461.
- Clarridge, J. E. (2004). Impact of 16SrRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17, 840–862.
- Coplin, D. L., & Kado, C. I. (2001). Gram-negative bacteria *Pantoea*. In N. W. Schaad (Ed.), *Laboratory guide for identification of plant pathogenic bacteria* (pp. 73–83). St. Paul: APS Press.
- Cortesi, P., & Pizzatti, C. (2007). Palea browning, a new disease of rice in Italy caused by *Pantoea ananatis*. *Journal of Phytopathology*, 89, S76.
- Coutinho, T. A., & Venter, S. N. (2009). *Pantoea ananatis*: An unconventional plant pathogen. *Molecular Plant Pathology*, 10(3), 325–335.
- Coutinho, T. A., Preisig, O., Mergaert, J., Cnockaert, M. C., Riedel, K. H., Swings, J., & Wingfield, M. J. (2002). Bacterial blight and dieback of Eucalyptus species, hybrids, and clones in South Africa. *Plant Disease*, 86, 20–25.
- De Baere, T., Verhelst, R., Labit, C., Verschraegen, G., Wauters, G., Claeys, G., & Vanechoutte, M. (2004). Bacteremic infection with *Pantoea ananatis*. *Journal of Clinical Microbiology*, 42, 4393–4395.
- Deletoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G., & Brisse. (2009). Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multilocus gene sequencing. *Journal of Clinical Microbiology*, 47, 300–310.
- Frederickson, D. E., Monyo, E. S., King, S. B., & Odvody, G. N. (1997). A disease of pearl millet in Zimbabwe caused by *Pantoea agglomerans*. *Plant Disease*, 81, 959–964.
- Fucikovskiy, L., & Aranda, S. (2006). *Pantoea ananatis* a new pathogen of agave in Mexico. *Phytopathology*, 96, S37.
- Gardan, L., Dauga, C., Prior, P., Gillis, M., & Saddler, G. S. (2000). *Acidovorax anthurii* sp. nov., a new phytopathogenic bacterium which causes bacterial leaf-spot of anthurium. *International Journal of Systematic and Evolutionary Microbiology*, 50, 235–246.
- Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., & De Ley, J. (1989). Transfer of *Entrobacter agglomerans* (Beijerinck 1888) Ewing and fife 1972 to *Pantoea* gen. Nov. as *Pantoea agglomerans* comb. Nov. and description of *Pantoea dispersa* sp. Nov. *International Journal of Systematic Bacteriology*, 39, 337–345.
- Gitaitis, R. D., Walcott, R., Culpepper, S., Sanders, H., Zolobowska, L., & Langston, D. (2002). Recovery of *Pantoea ananatis*, causal agent of center rot of onion, from weeds and crops in Georgia, USA. *Crop Protection*, 21, 983–989.
- Goszczynska, T., Botha, W. J., Venter, S. N., & Coutinho, T. A. (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.
- Grimont, P. A. D., & Grimont, F. (2005). Genus XXIII. *Pantoea*. *Bergey's Manual of Systematic Bacteriology*, 2, 713–720.
- Gueule, D., Fourny, G., Ageron, E., Le Flèche-Matéos, A., Vandenbogaert, M., Grimont, P. A., & Cilas, C. (2015). *Pantoea coffeiphila* sp. nov., cause of the 'potato taste' of Arabica coffee from the African Great Lakes region. *International Journal of Systematic and Evolutionary Microbiology*, 65, 23–29.
- Hauben, L., Vauterin, L., Swings, J., & Moore, E. R. B. (1997). Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology*, 47(2), 328–335.
- Krawczyk, K., Kamasa, J., Zwolinska, A., & Pospieszny, H. (2010). First report of *Pantoea ananatis* associated with leaf spot disease of maize in Poland. *Journal of Plant Pathology*, 92(3), 807–811.
- Larget, B., & Simon, D. L. (1999). Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution*, 16, 750–759.

- Lelliott, R., Billing, E., & Hayward, A. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Microbiology*, 29(3), 470–489.
- Lindow, S. E., Desurmont, C., Elkins, R., McGourty, G., Clark, E., & Brandl, M. T. (1998). Occurrence of indole-3 acetic acid-producing bacteria on pear trees and their association with fruit russet. *Phytopathology*, 88, 1149–1157.
- Manulis, S., & Barash, I. (2003). *Pantoea agglomerans* pvs. *gypsophilae* and *betae*, recently evolved pathogens. *Molecular Plant Pathology*, 4, 307–314.
- McFadden, L. (1962). Two bacterial pathogens affecting leaves of *Aglaonema robelinii*. *Phytopathology*, 52, 20.
- Medrano, E. G., & Bell, A. A. (2007). Role of *Pantoea agglomerans* in opportunistic bacterial seed and ball rot of cotton (*Gossypium hirsutum*) grown in the field. *Journal of Applied Microbiology*, 102, 134–143.
- 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 Bacteriology*, 43, 162–173.
- Noller, H. F., Hong, L., & Fredrick, K. (2005). The 30s ribosomal P site: A function of 16SrRNA. *FEBS Letters*, 579, 855–858.
- Nylander, J. A. A. (2004). *MrModeltest Version 2. Program distributed by the author*. Uppsala: Evolutionary Biology Centre: Uppsala University.
- Paccola-Meirelles, L. D., Ferreira, A. S., Meirelles, W. F., Marriel, I. E., & Casela, C. R. (2001). Detection of a bacterium associated with a leaf spot disease of maize in Brazil. *Journal of Phytopathology*, 149, 275–279.
- Perombelon, M. C. M., & Hyman, L. J. (1986). A rapid method for identifying and quantifying soft rot erwinias directly from plant material based on their temperature tolerance and sensitivity to erythromycin. *Journal of Applied Bacteriology*, 60, 61–66.
- Perombelon, M. C. M., & Van der Wolf, J. M. (2002). Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*) on potatoes: A laboratory manual. Dundee: Scottish Crop research institute occasional publication no. 10.
- Popp, A., Cleenwerck, I., Iversen, C., De Vos, F., & Stephan, R. (2010). *Pantoea gaviniae* sp. nov. and *Pantoea calida* sp. nov., isolated from infant formula and an infant formula production environment. *International Journal of Systematic and Evolutionary Microbiology*, 60, 2786–2792.
- Pusey, P. L., Stockwell, V. O., Reardon, C. L., Smits, T. H. M., & Duffy, B. (2011). Antibiosis activity of *Pantoea agglomerans* biocontrol strain E325 against *Erwinia amylovora* on apple flower stigmas. *Phytopathology*, 101, 1234–1241.
- Romeiro, R. S., Macagnan, D., Mendonca, H. L., & Rodrigues Neto, J. (2006). Bacterial spot of Chinese taro (*Alocasia cucullata*) in Brazil induced by *Pantoea agglomerans*. *New Disease Reports*, 14, 51.
- Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.
- Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for identification of plant pathogenic bacteria* (3rd ed.). St. Paul: APS: The American Phytopathological Society.
- Sherafati, F., Khodaygan, P., Azadvar, M., Sedaghati, E., Saberi-Riseh, R., & Baghaee-Ravari, S. (2014). Association of *Pantoea agglomerans* with the citrus bacterial canker disease in Iran. *Journal of Crop Protection*, 3 (3), 345–355.
- Stephan, R., Van Trappen, S., Cleenwerck, I., Vancanneyt, M., De Vos, P., & Lehner, A. (2007). *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov., isolated from fruit powder. *International Journal of Systematic and Evolutionary Microbiology*, 57, 820–826.
- Vantomme, R., Mergaert, J., Verdonck, L., & De Ley, J. (1989). Antagonistic effect in vitro of *Erwinia uredovora* LMG 2678 against some other bacteria. *Journal of Phytopathology*, 124, 372–376.
- Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders Jr., F. H., & Diaz-Perez, J. C. (2002). Natural infestation onion seed by *Pantoea ananatis*, causal agent of center rot. *Plant Disease*, 86, 106–111.
- Walterson, A. M., & Stavrinides, J. (2015). *Pantoea*: Insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiology Reviews*, 39(6), 968–984.
- Weinthal, D. M., Barash, I., Tzfira, T., Gaba, V., Teper, D., Sessa, G., & Manulis-Sasson, S. (2011). Characterization of nuclear localization signals in the type III effectors *HsvG* and *HsvB* of the gall-forming bacterium *Pantoea agglomerans*. *Microbiology*, 157, 1500–1508.
- Yazdani, R., Shams-Bakhsh, M., & Safaie, N. (2015). Identification of bacterial leaf spot and soft rot agent on ornamental plants in Isfahan province. *Plant Protection*, 38(1), 91–100.