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First report of strawberry bacterial leaf blight caused by *Pantoea ananatis* in Egypt

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Abstract

Strawberry leaves showing leaf blight symptoms were collected from six different farms in Ismailia and Beheira Governorates in Egypt during the 2020–2021 growing season. Eight bacterial isolates, i.e., *Pa*₁, *Pa*₂, *Pa*₃, *Pa*₄ (Ismailia farms) and *Pa*₅, *Pa*₆, *Pa*₇, and *Pa*₈ (Beheira farms) were isolated. A pathogenicity test of bacterial isolates was carried out using detached strawberry leaf technique. All bacterial isolates produced leaf blight disease symptoms. Isolates *Pa*₂ and *Pa*₆ showed the highest pathogenic characteristics with clear symptoms on detached strawberry leaves. The phenotypic, biochemical and physiological characters of the highest pathogenic isolates were confirmed by PCR analysis using 16S rRNA gene. The two bacterial isolates were identified as *Pantoea ananatis* with similarity of 97.05% with accession number MH_127816.1 (isolate *Pa*₂, Ismailia), while the isolate (*Pa*₆, Beheira) with similarity of 97.03% with accession number NR_026045.1. The 16S rDNA sequences were deposited in the GenBank nucleotide databases under accession numbers OM258167 and OM279507, respectively. According to the pathogenicity test, morphological and physiological characteristics as well as molecular data (16S rRNA sequencing analysis), this finding is the first report of *P. ananatis* as a causal agent of strawberry leaf blight disease in Egypt.

Keywords: *Pantoea ananatis*, leaf blight, polymerase chain reaction, 16S rRNA, strawberry

Introduction

Strawberry (*Fragaria × ananassa* Duch.) is an economically important and famous vegetable crop that is consumed worldwide because of its great nutritional value and lovely flavor. Strawberries are very rich in antioxidants and useful compounds. The total world production of strawberries has reached up to 8,337,099 tons (Roshdy *et al.* 2021). Several pathogen microorganisms attack strawberry plants and cause severe diseases in different developmental stages in either nursery or field production such as root rot, wilt, leaf blight, spots

and fruit rot which reduce the quantity and quality of strawberry productivity, resulting in both yield and economic losses (Ragab *et al.* 2017)

Serrano (1928) first discovered *Pantoea ananatis* on pineapple, where it caused fruitlet rot in the Philippines. The bacterium causes a wide range of disease symptoms on both monocotyledonous and dicotyledonous plants. Since 1983, the host range of *P. ananatis* has increased and the pathogen has now been reported to cause plant disease symptoms in at least

11 countries (Coutinho and Venter 2009), where the bacterium could be widespread in nature and survive as epiphytes, saprophytes and pathogens on the surfaces of many plants (Monier *et al.* 2005). *Pantoea ananatis* could cause disease symptoms including leaf blotches and spots, die-back and the rots of stalks, fruits and bulbs in a wide range of economically important crops over the world (Frederick *et al.* 2001; Coutinho and Venter 2009). *Pantoea ananatis* was reported as the major causal agent of plant disease in pineapple, corn, rice, maize, sorghum, onions, cotton, Sudan grass, shallot, wheat, soybeans, eucalyptus, melons and strawberries (Cota *et al.* 2010; Bajpai *et al.* 2020).

Pantoea ananatis was isolated from onion bulbs (*Allium cepa* L.) naturally infected with center rot disease in Georgia (Walcott *et al.* 2002), in South Africa (Goszczyńska *et al.* 2006) and in Morocco (Achnani *et al.* 2016). The bacterium can cause blight and chlorosis disease on garlic leaves (*Allium sativum* L.) and shallot leaves (*Allium cepa* L. aggregatum group) in Indonesia (Nurjanah *et al.* 2017). Many pathogenic symptoms caused by *P. ananatis* were recorded on rice plants (*Oryza sativa*) such as an unusual necrosis on stems in Australia (Cother *et al.* 2004), leaf blight and rice grains discoloration in Russia (Egorova *et al.* 2015) and discoloration and abortion of rice grains in the Republic of Korea (Min *et al.* 2017). The bacterium was also the causal agent of leaf blight disease on maize (*Zea mays* L.) in Poland (Krawczyk *et al.* 2010), on sorghum (*Sorghum bicolor* L.) in Brazil (Cota *et al.* 2010) as well as the white spot disease on maize in Brazil (Sauer *et al.* 2015). The bacterium was recorded as a causal agent of dieback disease on eucalyptus in Iran (De Maayer *et al.* 2010), leaf spot disease on ornamental plants of the Araceae family (Yazdani *et al.* 2018), fruitlet blight disease on Nagpur mandarin (*Citrus reiculata* L.) in India (Das *et al.* 2020), on white leaves in Poland (Krawczyk *et al.* 2020) and bacterial leaf blight disease on strawberry in Canada and rice in southeast China (Bajpai *et al.* 2020; Yu *et al.* 2021). Identification of *Pantoea* species was obtained by polymerase chain reaction (PCR) sequencing using the 16S rRNA gene fragment (Cother *et al.* 2004; Nurjanah *et al.* 2017; Azizi *et al.* 2020), where 16S rRNA gene sequence was found insufficient to assign the taxonomy of a new species owing to its polyphyletic nature not only in the same family, but also in the same genus (Liu *et al.* 2016). Analysis of the 16S rRNA gene sequence was applied to identify *P. ananatis* which infected rice in the Republic of Korea and Malaysia (Min *et al.* 2017; Azizi *et al.* 2020), onion bulbs in South Africa and Morocco (Goszczyńska *et al.* 2006; Achnani *et al.* 2016), eucalyptus in South Africa (De Maayer *et al.* 2010) and ornamental plants in Iran (Yazdani *et al.* 2018). Therefore, this work was aimed

to isolate and identify the causal agent of bacterial leaf blight disease of strawberry plants based on the standard procedures of isolation, pathogenicity, physiology and biochemical characters and molecular analysis using 16S rRNA fragment.

Materials and Methods

Field sites of strawberry samples

Strawberry leaves, showing bacterial leaf blight symptoms, were collected from six different farms in Ismailia and Beheira Governorates in Egypt during the 2020–2021 growing season. The Global Positioning System (GPS) of the six farms in Ismailia Governorate were 30°33'27.8"N31°57'38.7"E, 30°33'37.0"N32°00'30.8"E, 30°33'33.1"N32°00'30.3"E, 30°33'07.6"N32°02'50.0"E, 30°32'54.4"N32°02'28.3"E and 30°33'19.1"N32°02'09.9"E. The GPS of the six farms of Beheira Governorate were 30°30'04.0"N30°32'12.8"E, 30°32'27.1"N30°32'15.1"E, 30°33'43.4"N30°34'54.7"E, 30°33'36.3"N30°36'47.2"E, 30°33'05.6"N30°41'30.0"E and 30°33'05.1"N30°42'43.4"E as listed in Table 1.

Isolation of strawberry leaf blight pathogen

The infected strawberry leaves containing lesions were first washed with sterile water and surface sterilized with sodium hypochlorite (2.5%) solution for 5 min. Then, the surface sterilized plant leaves were carefully washed in sterile distilled water and the leaves blot dried between two sterile filter papers. Then, the surface sterilized plant leaves were cut into small pieces and mashed in 5 ml of sterile distilled water and left for a few minutes at room temperature. Loopfuls of leaves suspension were streaked onto plates of nutrient glucose (2%) agar medium (NGA) and then incubated at 30°C ± 2 for 48 h. After incubation, slimy yellow bacterial colonies were selected. The colonies were re-streaked on new fresh NGA plates and the dilution streak technique was used to obtain single colonies and kept at 4°C on slant agar to be used in the following studies.

Table 1. The tested strawberry cultivars from different governorates

Cultivars	Farm no.	Governorates
Safana	1	Ismailia
Amega	2	Ismailia
Festival	3, 4, 5 and 6	Ismailia
Fertona	1, 2, 3, 4 and 5	Beheira
Beauty	6	Beheira

Pathogenicity test

The pathogenicity of *P. ananatis*, was determined using the detached leaf technique. The healthy strawberry leaves were surface sterilized with 2.5% sodium hypochlorite solution for 5 min and then the leaves were rinsed three times with sterile distilled water and left to air dry. Each purified bacterial isolate was suspended in sterile 0.2 M phosphate buffer (K_2HPO_4 and KH_2PO_4 , pH = 7.2). The bacterial suspension was adjusted to a standard inoculum density [ca. 10^{7-9} colony forming unit CFU) $\cdot ml^{-1}$] by measuring the turbidity using a Prim light spectrophotometer at 610 nm and kept under cool conditions until used. The detached leaves were infiltrated with bacterial suspension and put in moisture plates/moisture chamber. The plates were immediately sealed with parafilm and incubated in a growth chamber. In the control, the strawberry leaves were infiltrated with sterile phosphate buffer. All inoculated leaves were incubated at $30 \pm 2^\circ C$ until symptoms appeared. This assay was repeated three times (Sherafati *et al.* 2014).

Cultural, morphological and biochemical characterizations of *Pantoea ananatis*

Using standard procedures, the cultural, morphological, and biochemical characteristics of the highest pathogenic isolates of *P. ananatis* (Pa_2 and Pa_6) were determined according to Schaad *et al.* (2001). The characteristics of the two bacterial isolates were tested with yellow pigment on NGA, King's Medium B (KB) and Yeast Extract Dextrose $CaCO_3$ (YDC) agar medium. Gram staining, cell shape, spore forming, reaction with 3% KOH, growth on 4% of sodium chloride solution, growth at $36^\circ C$ or $4^\circ C$, anaerobic conditions, motility, arginine dihydrolase, catalase production, gelatin liquefaction, H_2S production, Tween 80 lypolysis, nitrate reduction, starch hydrolysis and levan production were determined. The ability of acid production by tested bacteria from L-arabinose, cellobiose, galactose, glycerol, lactose, maltose, mannose, mannitol, rhamnose, salicine, starch and sucrose was determined (Schaad *et al.* 2001).

Polymerase chain reaction (PCR) sequencing

The identification of the two bacterial isolates (Pa_2 and Pa_6) isolated from Ismailia and Beheira farms were confirmed by extracting the total genomic DNA and sequence analysis of the 16S rRNA gene.

Total DNA extraction

The total genomic DNA was extracted from liquid media using thermo Scientific Gene JET Genomic DNA

Purification Kit (cat No.k0722,Thermo Fisher Scientific Inc., USA), according to the manufacturer's instructions. The quantity and purity of DNA were measured using Nano Drop 2000c (Thermo Fisher Scientific Inc., USA) at the absorbance of 260 nm and 280 nm, prior to being used as a template in PCR amplification (Vingataramin and Frost 2018).

Detection by direct PCR

The PCR amplification was done using the 16S rRNA gene from each bacterial isolate. This gene was amplified using the universal 16S rRNA gene bacterial primers F (5'-AGAGTTTGATCCTGGCTCAG-3') and R (5'-GGTTACCTTGTACGACTT-3') according to Ausubel *et al.* (1992) and Srinivasan *et al.* (2015). The analysis of PCR was carried out in 25 μl reaction mixture containing 2 μl genomic DNA ($50 ng \cdot \mu l^{-1}$), 12.5 μl master mix, 1 μl of 10 μM of each forward and reverse primers, then the total volume was completed with ddH_2O to 25 μl . The conditions were programmed with an initial denaturation step at $95^\circ C$ for 5 min, followed by 37 cycles of denaturation at $95^\circ C$ for 30 s, and an annealing step at $57^\circ C$ for 30 s then an extension step at $72^\circ C$ for 1 min. Finally, the analysis was completed with one cycle of a final extension at $72^\circ C$ for 10 min. The PCR reaction was carried out in the Applied Biosystems (Veriti[®] 96-Well Thermal Cycler, Singapore). The product of PCR was resolved by 1.5% (W/V) agarose gel in $1 \times$ TAE buffer stained with ethidium bromide. The molecular weight of the amplified fragment was determined by a 1 kb DNA ladder (Thermo Scientific Gene Ruler 1 kb DNA Ladder, ready-to-use, USA) and photographed under UV light using gel documentation system (Bio-Rad[®] Gel Doc-2000).

16S rRNA sequencing and analysis

The sequencing of purified amplified products (1,500 bp) of 16S rRNA gene for each isolate was carried out by MacroGen Laboratory (MacroGen Co., Spain) using Sanger methods. The DNA sequences obtained for both forward and reverse 16S rRNA gene sequencing were submitted to NCBI GenBank, USA. The sequences of the isolates were subjected to the standard Basic Local Alignment Search Tool (BLASTn) of the NCBI database online tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), to check the sequence similarity against sequences in the nucleotide collection (nr/nt) database. Alignments of sequence were achieved by CLUSTALW algorithm. Phylogenetic trees for the isolates were generated to visualize the distance between the query sequence and the highest similar sequences available on the GenBank database (www.ncbi.nlm.nih.gov) (Thompson *et al.* 1994).

Results

Bacterial isolates

Results revealed that eight bacteria isolated from naturally infected strawberry leaves showed characteristics of their morphology and physiological reactions. The colonies produced yellow pigment in the selective medium (NGA medium), were circular in shape, convex, entire, smooth, viscid and all bacterial isolates were short rod shaped and Gram negative. The bacterial isolates grown on YDC medium produced yellow colonies, while those grown on KB medium did not have fluorescent pigment (Fig. 1).

Pathogenicity test

Results of pathogenicity revealed that all bacterial isolates showed characteristic leaf blight disease symptoms. The isolate *Pa*₂ (obtained from farm 2 in Ismailia Governorate) and the isolate *Pa*₆ (obtained from farm 2 in Beheira Governorate) showed high pathogenicity and had clear symptoms in the detached strawberry leaf test, where chlorosis spread throughout leaves (Fig. 2). No leaf blight symptoms were recorded on the control leaves. The two bacteria had a positive hypersensitive reaction on tobacco leaves (Fig. 1). The two bacterial isolates were re-grown on slant agar and used in the following studies.

Biochemical and physiological characters of *P. ananatis*

Pantoea ananatis isolates (*Pa*₂) and (*Pa*₆) could grow on nutrient glucose 2% agar medium producing yellow pigment. The bacterium also was short rod, Gram negative and non-spore forming. The bacterial isolates had a positive reaction with KOH (3%), growth on NaCl (4%), growth under anaerobic conditions, growth at 36°C, growth at 4°C, motility, catalase production, gelatin liquefaction, H₂S production, Tween 80 hypolysis and levan production, while there was a negative reaction with arginine dihydrolase, and starch hydrolysis. *Pantoea ananatis* isolates could utilize L-arabinose, cellobiose, galactose, glycerol, lactose, maltose, mannose, mannitol, starch and sucrose producing acid only after 48 h, while the same isolates produced acid only from salicine after 1 week (Table 2).

16S rRNA sequencing and analysis

Data of 16S rRNA sequencing and analysis in Figure 3 showed that the PCR products of 16S rRNA gene amplification were as follows: Lane M: 1 Kb DNA ladder, Lane 1: fragment of isolate *Pa*₂, Lane 2: fragment of isolate *Pa*₆ and Lane 3: negative control. Thus, the total genomic DNA was extracted and used as a template to amplify partial 16S rRNA gene sequence using universal primers. Sharp bands with molecular weight 1,500 bp were observed. Phylogenetic

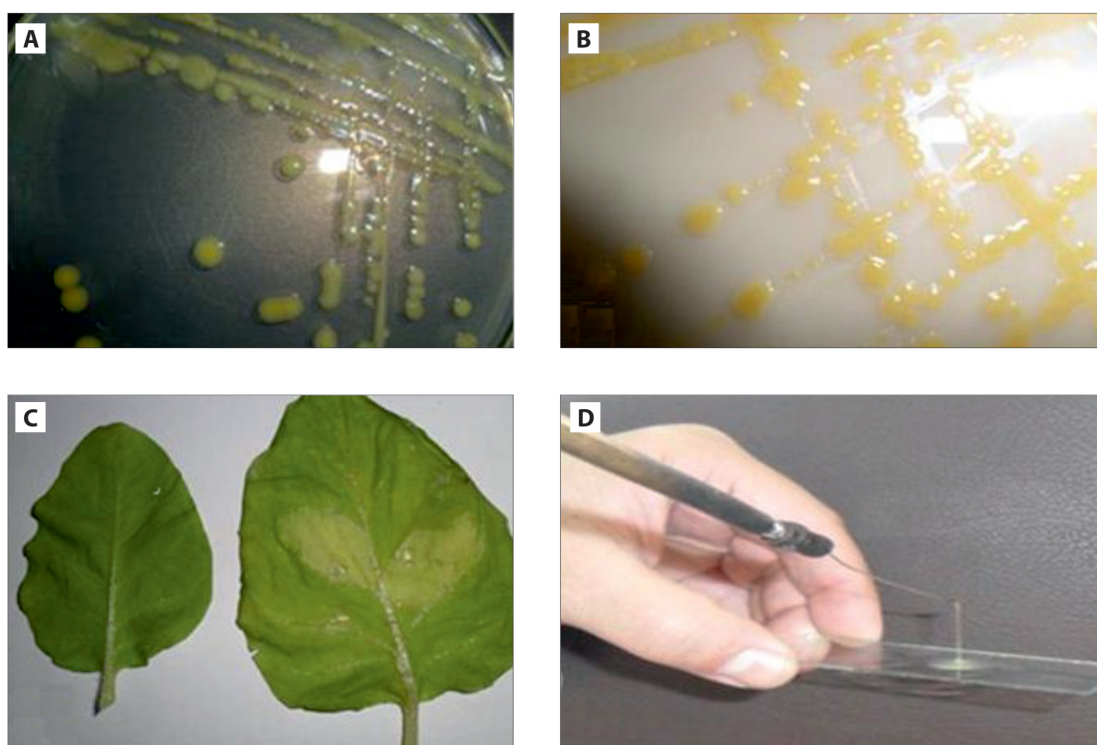


Fig. 1. The cultural characteristics of *Pantoea ananatis* colonies on nutrient agar (A) and yeast extract dextrose CaCO₃ medium (B); hypersensitive reaction on tobacco (C) and reaction on 3% KOH (D)

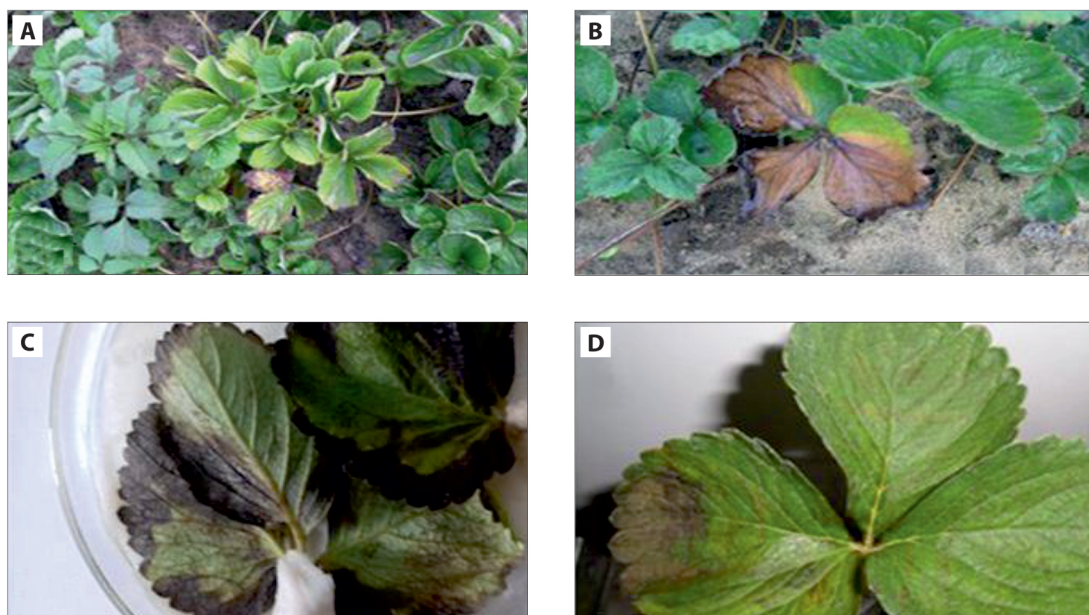


Fig. 2. Natural bacterial leaf blight on strawberry in field (A) and artificial leaf blight symptoms of *Pantoea ananatis*: MH_127816.1 (B), Pa_2 (C) and NR_026045.1 Pa_6 (D)

Table 2. Phenotypic, physiological and biochemical characters of *Pantoea ananatis* isolates isolated from strawberry showing natural bacterial blight leaf symptoms

Characteristics	Reaction of <i>P. ananatis</i>	Characteristics	Reaction of <i>P. ananatis</i>
Growth on NGA	+	H ₂ S production	+
Yellow pigment on NGA medium	+	Tween 80 lypolysis	+
Yellow pigment on YDC medium	+	Levan production	+
Fluorescent pigment on KB medium	-	Starch hydrolysis	-
Pathogenic effect on strawberry leaves	+	Acid production from:	
Hypersensitive reaction	+	L-arabinose	A ⁺
Gram staining	G ⁻	galactose	A ⁺
Cell shape	short rod	cellobiose	+
Spore formation	non-spore	glycerol	A ⁺
Gram reaction with 3% KOH	+	lactose	A ⁺
Growth on NaCl 4%	+	maltose	A ⁺
Growth at under anaerobic conditions	+	mannose	A ⁺
Growth at 36°C	+	mannitol	A ⁺
Growth at 4°C	+	rhamnose	A ⁺
Motility	+	salicine	A ⁽⁺⁾
Arginine dihydrolase	-	starch	A ⁺
Catalase production	+	sucrose	A ⁺
Gelatin liquefaction	+		

"+" = positive reaction, "-" = negative reaction

A = acid production after 48 h, A⁽⁺⁾ = acid production after 1 week

NGA = Nutrient Glucose (2%) Agar medium; KB = King's Medium B; YDC = Yeast Extract Dextrose CaCO₃ agar medium

relationships of the isolated bacteria were analyzed with other partial 16S rRNA sequences of related bacterial species present in GenBank, where the DNA sequences of partial 16S rRNA gene of studied bacteria were aligned and compared using the standard Basic

Local Alignment Search Tool (BLASTn). Our results showed that the isolate Pa_2 was similar to *P. ananatis* with 97.05% [accession number MH_127816.1] isolated from Ismailia Governorate, while the isolate Pa_6 was similar to *P. ananatis* with 97.03% [accession number

Table 3. BLAST analysis of 16S rRNA gene sequence of *Pantoea ananatis* isolates

Bacterial isolates	Closest relative	% Identity	Accession no.
Isolate <i>Pa</i> ₂	<i>P. ananatis</i> strain EB370 16S ribosomal RNA gene, partial sequence	97.05	MH_127816.1
Isolate <i>Pa</i> ₆	<i>P. ananatis</i> strain 1846 16S ribosomal RNA gene, partial sequence	97.03	NR_026045.1

NR_026045.1] isolated from Beheira Governorate (Table 3 and Figs 3, 4, 5). Moreover, the 16S rDNA sequences (*Pa*₂ and *Pa*₆) were deposited in the NCBI Gene Bank nucleotide sequence database under accession numbers OM258167 and OM279507, respectively.

Discussion

Our results revealed that the bacteria isolated from strawberry leaves of Ismailia and Beheira farms, showing natural leaf blight symptoms, was Gram negative and yellow pigmented as well as able to produce clear symptoms of bacterial leaf blight disease in the

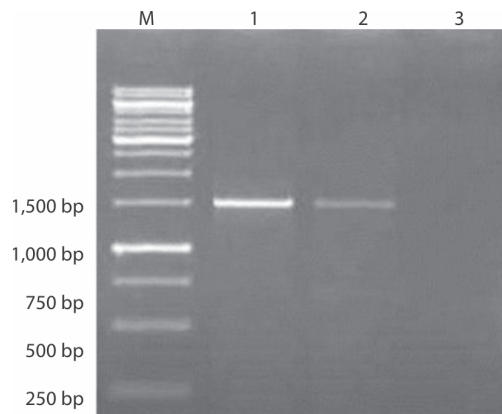


Fig. 3. PCR products of 16S rRNA gene amplification. Lanes: M – 1 Kb DNA ladder, 1 – fragment of isolate *Pa*₂, 2 – fragment of isolate *Pa*₆ and 3 – negative control

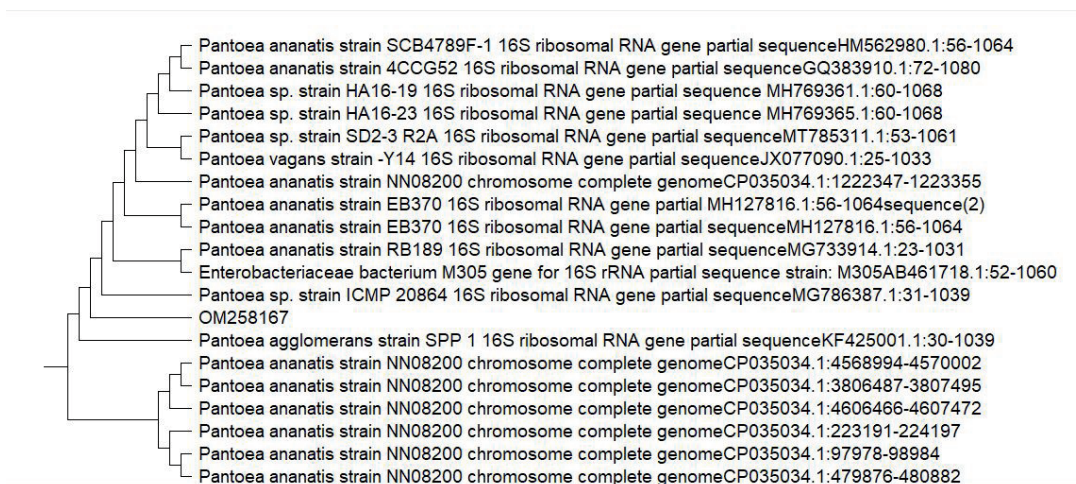


Fig. 4. Phylogenetic tree based on partial 16S rDNA sequence representing the isolate *Pa*₂ (Accession no. SUB10951609H201202_012_E01_2B_SH1.ab1 OM258167) compared to the whole bacterial database

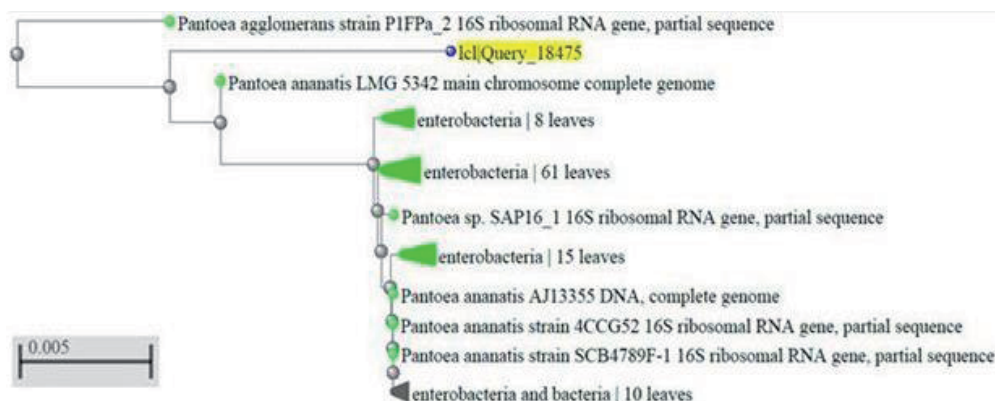


Fig. 5. Phylogenetic tree based on partial 16S rDNA sequence representing the isolate *Pa*₆ (Accession no. NR_026045.1) compared to the whole bacterial database (1c1 Query_18475)

detached strawberry leaf test. These results are in agreement with those recorded by Rugienius and Toldi (2005). They reported that several endophytic bacteria, including genus *Pantoea*, were isolated from internal tissues of *in vitro*-grown strawberries, in field-grown garden and wild strawberries. *Pantoea* and *Pseudomonas* were common genera.

The biochemical and physiological characteristics of isolated *P. ananatis* are in agreement with those recorded by Nurjanh *et al.* (2017). They reported that *P. ananatis* is Gram-negative, non-spore-forming, facultative anaerobic, motile, produce catalase, indole production and acid production from D-glucose, D-mannitol, sucrose and lactose. Furthermore, *P. ananatis* grew on nutrient agar medium and produced bacterial colonies which were yellow, shiny, translucent, convex and circular with entire margins (Das *et al.* 2020). The identification of *P. ananatis* was confirmed by PCR analysis using 16S rRNA (Yumoto *et al.* 2001; Suryani *et al.* 2012). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships between bacteria, but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level (Satchi *et al.* 2002).

Our obtained results are in agreement with those recorded by González *et al.* (2015). They found that *Pantoea* was the causal agent of leaf blight in many plants for example, on leaves of Nagpur mandarin, The symptom of *P. ananatis* infection were irregular water-soaked with *P. ananatis* infection the leaves became water-soaked and quickly turned dark brown or black (Das *et al.* 2020). In wheat there were brownish lesions with clear margins and yellow leaves (Krawczyk *et al.* 2020).

The pan-genome incorporates a large number of genes encoding proteins that may enable *P. ananatis* to colonize, persist in and potentially cause disease symptoms in a wide range of plant and animal hosts. The genome of a virulent strain of *P. ananatis* was recently sequenced from eucalyptus, as well as the type strain from pineapple, using 454 pyro-sequencing (De Maayer *et al.* 2014). Bajpai *et al.* (2020) showed that strawberry cv. Florida Radiance showing leaf blight-like symptoms were observed in commercial fields in Canada. The causal pathogen was identified as *P. ananatis* based on pathogenicity tests, morphological and physiological characteristics as well as molecular data (sequence analysis of ribosomal DNA and multilocus gene sequence). It was the first report of *P. ananatis* as the causal agent of leaf blight on strawberry in Canada. Partial DNA sequences were analyzed using 16S rRNA partial sequences. These revealed that *P. ananatis* was isolated from tomato fruits and black nightshade seeds and it was the first report in Egypt (Ashmawy *et al.* 2020). Therefore our obtained results concluded that

based on pathogenicity tests, morphological and physiological characteristics and characterization of pathogens based on 16S rRNA sequence, the causal pathogen of leaf blight was identified as *P. ananatis*. According to our data it is the first report of *P. ananatis* causing leaf blight on strawberry in Egypt.

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