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PATHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF THE SEED-BORNE BACTERIA "*PANTOEA* SPP., *XANTHOMONAS* SPP. AND *PSEUDOMONAS* SPP." FROM SOLANACEOUS PLANTS IN EGYPT

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ABSTRACT

The seed-borne bacterial diseases of family solanaceae cause significant economic losses worldwide. In the present study, fourteen bacterial isolates were recovered from seeds of different varieties of tomato, eggplant, black nightshade and tobacco. The seed samples were collected from different locations in Egypt. Isolated bacteria were identified based on morphological, physiological and biochemical tests as well as colonial morphology aspects on the differential medium, yeast extract dextrose calcium carbonate (YDC) besides the selective media (PA 20, King's B and Tween B). Pathogenicity of the isolated bacteria was assessed for causing graywall, bacterial speck and bacterial spot symptoms on tomato seedlings. Disease symptoms were recognized on leaves and stems of the inoculated seedlings. Re-isolation from collected seeds of the inoculated tomato plants was performed. Results indicated that *Pantoea* spp. was an endophytic bacteria, *Pseudomonas* spp. was an epiphytic bacteria, while *Xanthomonas* spp. was both epiphytic and endophytic bacteria. Furthermore, cellular fatty acids composition were identified and quantified to give a profile for tested bacterial isolates. Moreover, the bacterial isolates were identified at the molecular level via PCR reactions utilizing the 16S rRNA gene. Partial DNA sequences were analyzed using BLAST tool revealed that the inferred 16S rRNA partial sequences of the 7 isolates showed similarity to *Pantoea ananatis* (3 isolates), *Pseudomonas syringae* pv. *tomato* (2 isolates) and *Xanthomonas vesicatoria* (2 isolates). To the best of our knowledge the bacterium associated with the graywall seems to be first report of *P. ananatis* isolated from tomato fruits and black nightshade seeds in Egypt.

Keywords: seed borne bacteria, solanaceae, fatty acids, 16S rRNA, PA20

INTRODUCTION

Seed production has been moved to semi-arid regions to escape seed-borne pathogens. Seed-borne bacterial diseases continue to be problematic and cause significant economic losses worldwide. Infested seeds are responsible for there-emergence of diseases of the past, movement of pathogens across international borders, or the introduction of diseases into new areas **(Gitaitis andWalcott, 2007)**.

Family Solanaceae, included important economical, medicinal crops and weeds in the world. One of them is Tomato (*Lycopersicon esculentum* Mill.) which cultivated for its fruits, having economic importance for domestic consumption, export and food industries. Egypt ranks fifth in the world for tomato production, 6.62 million metric tons (**FAO, 2018**).

Tomato seeds are subjected to infection by bacterial seed-borne diseases such as graywall disease caused by *Pantoea ananatis* **[**formerly, *Erwinia ananas***] (Stall *et al.*, 1970 and Boyle, 1994).** The genus *Pantoea* is a diverse group of yellow-pigmented, rod shaped Gram-negative bacteria in the family *Enterobacteriaceae*. Some of the first members were recognized as plant pathogens causing wilting, soft rot and necrosis in different host plants **(Walterson and Stavrinides, 2015)**. *P. ananatis* causes disease symptoms in a wide range of economically important agricultural crops and forest tree species worldwide, which associated with plants as an epiphyte and endophyte bacteria **(Coutinho and Venter, 2009 ; Gi Yoon Shin *et al.*, 2019)**.

Moreover, bacterial speck disease of tomato which caused by *Pseudomonas syringae* pv. *tomato* can also reduce yield when it severely affects leaves early in the growing season. The diseases is developed due to high humidity and low night temperatures (**Kolomiets *et al.*, 2017)** .The bacterium can be moved from plant to plant via splashing water or on hands and gardening tools causes a serious outbreak on tomato plants grown in commercial fields (**Sahin, 2001** ; **Shenge *et al.*, 2010)**.

Bacterial spot of tomato, caused by *Xanthomonas vesicatoria*, is a potentially devastating disease that can lead to unmarketable fruit and even plant death. Bacterial spots can occur wherever tomatoes are grown, but are found most frequently in warm, wet climates, as well as in greenhouses **(Shenge *et al.*, 2010; Kolomiets *et al.*, 2017)**. The pathogens enter plants through natural openings (e.g., stomates), as well as through wounds. Disease development is favored by warm, wet weather. Wind-driven rain can contribute to more severe disease as the pathogens are splashed and spread to healthy leaves and fruit. The species-specific PCR assays, biochemical and serological analyses were used to identify the *Xanthomonas* species associated with bacterial leaf spot of tomato as diagnostic protocols for this disease **(Manjula *et al.*, 2017; Roach *et al.*, 2018; Vancheva *et al.*, 2018)**.Therefore, the present study was conducted to achieve the following objectives: to isolate the seed-borne bacterial pathogens from solanaceous plants collected from different locations in Egypt, to identify and characterize the isolated bacteria through cultural, biochemical tests and fatty acid profiling ,as well as, molecular techniques through 16S rRNA gene sequences.

MARERIALS AND METHODS

Sampling collection

Seeds of different varieties of some selected vegetable crops and weeds which belonging to family Solanaceae were collected as following: 6 varieties of tomato seeds (*Solanum esculentum*) namely Castle Rock, Super strain B, Peto 86, Super Jakal, Gs 12 and local variety were purchased from local markets at Alexandria Governorate. Moreover, one tomato seed sample of Super Jakal variety was obtained from naturally infected fruits collected from Assiut Governorate. Two varieties of eggplant seeds (*S. melongena*) namely local variety and Black Beauty, as well as, one local variety of pepper seeds (*Capsicum annuum*) were purchased from local markets at Alexandria Governorate. Seeds of datura (*Datura stramonium* and *D. metel*), tobacco (*Nicotiana tabacum*) and black nightshade (*S. nigrum*) were obtained from Plant Pathology Institute, Agricultural

Research Center (ARC), Giza, Egypt. The above mentioned varieties were used for isolation trials.

Isolation procedures

Isolation and detection of presumptive bacterial isolates from the above mentioned seed varieties were carried out using either dilution plating of seed extracts on semi-selective media or direct seed plating methods, as well as, liquid assay (LA) method.
Symptomless and morphologically infected seed samples were divided into 2 groups (I, II) for isolation trials. In group I, seeds were non surface sterilized to detect epiphytic. In group II seeds were surface sterilized to detect only the endophytic bacteria. Then seeds were macerated in the phosphate buffer saline (PBS-Tween 20). All seed varieties in each group were incubated overnight for 14 hrs at 4ºC in the same buffer at a ratio of 3 mL of buffer to 1 gram of seeds (v:w). (www.wordseed.org/--/Tomato_Xanthomonas_spp_010).
Ten-fold serial dilutions of either seed suspension or its extracts in PBS buffer were prepared. The resultant dilutions (0.1 mL) was spread onto two semi selective media, PA 20 medium for isolation of *Pantoea* spp. **(Goszczynska*et al.*, 2006)** and Tween B medium for *Xanthomonas* spp.**(McGuire *et al.*, 1986 andGore and O'Garro, 1999)**. Colonies observed after 4-7 days at 28ºC were isolated and purified through the single colony isolation technique. Colonies were transferred on a yeast extract-dextrose-$CaCO_3$ (YDC) medium **(Gitaitis*et al.*, 1991)** for further identification. Cultures were kept on nutrient agar (NA) medium for further studies.

Direct plating on semi-selective media and liquid assay methods

These methods were used for detection of *Pseudomonas* spp. from non-surface sterilized seeds (Group I). In direct plating method, 100 seed of each variety were planted on King's B medium (King *et al.* 1954). Seeds were distributed in ten plates, 10 seeds in each plate then incubated at 28ºC for 4-5 days.
In liquid assay method, 0.1 gram of non-surface sterilized seeds of each variety was soaked in 10 mL sterile saline solution (0.85% NaCl) for 24 hrs at room temperature 25±2 ºC then, 0.1 mL of the suspension aliquots were spread on King's B medium. Isolated colonies were transferred on YDC medium for further identification.

Pathogenicity tests

Inoculation of tomato seedlings

Pure cultures of (P) and (X) bacterial isolates were cultivated on NA medium for 48 hrs. at 28ºC,whereas, (Ps) isolates was cultivated on King's B (KB) medium for 48 hrs at 28ºC. Bacterial cells were collected and suspended in sterile distilled water (SDW). In case of (P) isolates, bacterial suspension was adjusted to ca. 10^7 CFU/mL. Four weeks-old tomato seedlings (Alissa F1 variety) were inoculated by injecting bacterial suspension into the stem. Then, stem-inoculated plants were placed in plastic bags to increase the relative humidity (RH) and maintained in a greenhouse. The development of symptoms was recorded after 15 days. In case of (Ps) and (X) , bacterial suspensions (ca. 10^8 CFU/mL) were atomized with a hand-held sprayer until run-off. After inoculation, plants were placed in plastic bags and maintained in a greenhouse. Development of symptoms was observed daily **(Milijasevic *et al.*, 2009)**. Control plants were treated in a similar way using sterile water. All inoculation tests were replicated three times.

Inoculation of tomato fruits

Immature tomato fruits (Alissa F1 variety) were inoculated with the suspension of (P) isolates by injection the bacterial suspension (ca. 10^7 CFU/mL) into the fruits using sterile syringe. Disease symptoms were recorded after 14 days. Healthy fruits injected by sterile water were served as a control.

Re-isolation from artificially infected tomato

Tomato Seedlings and fruits

Re-isolation from tomato seedlings was performed after 6 weeks from artificial infection. Leaves and stems of infected tomato seedlings (Alissa F1variety) were gently washed with tap water, cut into small pieces and soaked into a few drops of SDW for 30 min. The resultant suspension was streaked on nutrient agar (NA) medium. Bacterial spots and speck lesions were excised and surface sterilized by dipping in 70% ethanol for 2 seconds followed by two successive rinses in SDW. Lesions were crushed in 0.5 mL SDW, and the resultant suspension was streaked on NA medium. All inoculated plates were incubated for 3 days at 28ºC and a single colony of the predominant colonies was selected and purified by repeated streaking on the same medium, then examined for further studies. Moreover, re-isolation was performed from fruits showing disease symptoms as previously mentioned.

Tomato seeds

Re-isolation from seeds which collected from tomato fruits was performed after 9 weeks from artificial infection of tomato seedlings as previously described **(EL-Meneisy, 2005)**.

Identification of bacterial isolates

Cultural, morphological, physiological and biochemical Tests

Bacterial isolates were identified using cultural, morphological, physiological and biochemical characteristics, which were conducted by performing the standard tests **(Abd-Alla, 2000; Brenner *et al.*, 2005; Brady*et al.*, 2009; Deletoile *et al.*, 2009 and Al-Saleh, 2011)**. The above mentioned tests were applied on the obtained bacterial isolates which included: cell shape, sporulation, motility, Gram staining, catalase, oxidase, starch hydrolysis, gelatin liquefaction, arginine dihydrolase, nitrate reduction, levan production and acid production from mannose, cellobiose, mannitol and sorbitol.
Cultural characteristics of the bacterial isolates were studied on different culture media such as PA 20 medium for *Pantoea* spp., King's B medium for *Pseudomonas* spp. and Tween B medium for *Xanthomonas* spp., glycerol agar, NA and YDC media were used, also bacterial growth of *Pseudomonas* spp. and *Xanthomonas* spp. isolates was tested on PA 20 medium **(Goszczynska et al., 2006a)**.

Fatty acids analysis

Extraction of total cellular lipids

Total lipids were extracted from seven bactrerial isolates grown on NB medium for 48 hrs as described by **Kates (1972)**. Bacterial cells were harvested after growth for 24 hrs at room temperature with occasional shaking by centrifugation at 3000 g for 10min. Bacterial pellet (0.5 g) was transferred to screw cape tube capacity 20 mL and extracted with 5 mL methanol-chloroform mixture (2:1 v/w). The organic layer was transferred to another tube, and 5 ml of methanol-chloroform mixture was added to re-extract the pellet for 2 hrs.
The organic solvent was decanted and collected to the first extract 2.5 mL chloroform and 2.5 mL of distilled water were add to each tube, mixed well, and left for phases separation. The chloroform lower layer containing total lipids was withdrawn into a screw cape tube 20 mL capacity. Chloroform was evaporated at 40ºC under stream of nitrogen and the traces of water were removed by drops of benzene. Total lipids extract was kept refrigerated under nitrogen (N_2) gas.

Preparation of methyl esters of fatty acids

The methyl esters of fatty acids (MEsFA) were prepared from total lipids as described by **Radwan (1978)**. The total lipids extract in the screw cape tube was dissolved in 2.0 mL benzene and 10 mL of the esterification reagent (1% sulphuric acid in absolute methanol) were added. The tubes were caped under nitrogen and heated at 90ºC for 90 minutes. Ten mL of distilled water were added to the cooled tubes and the MEsFA were extracted with 5 mL benzene and the extracts were dried using anhydrous sodium sulfate (5 g), received in 10 mL capacity vials, kept under N_2 and used for gas liquid chromatography (GLC) analysis.

Gas liquid chromatography of MEsFA

The MEsFA were analyzed using Shimadzu-8 A, GLC equipped with flam ionization detector and ordinary glass column (ID 3 mm X 2.5 m.) of 5 % diethylene glycol succinate on chromosorb Q 80/100 mesh. The following conditions were used for GLC analysis:column temperature 160ºC, detector temperature 270ºC, flow rates of nitrogen 20 mL/ min, H_2 75 mL/ min, air 0.5 mL/ min, chart speed 2.5 mm/ min. Standard MEsFA and their retention times were used for identification. The area under each peak was measured by the triangulation methods and expressed as percentage of each fatty acid with regard to the total area.

Molecular identification

Extraction of genomic DNA

Bacterial genomic DNA was extracted by boiling one mL of a suspension containing ca. $1x10^{10}$ CFU/mL for 10 min. DNA was separated by centrifugation for 5 min at 11.000 x*g* **(Yahiaoui-Zaidi*et al.*,2003)**.

PCR amplification of 16S rRNA gene

Full length (1550 bp) of 16S rRNA gene was amplified from 3 isolates of *Pantoea* spp. (P_1, P_2 andP_3),2 isolates of *Pseudomonas* spp. (Ps_1 and Ps_2), and 2 isolates of *Xanthomonas* spp. (X_1 and X_2) using the universal primers P0(F)

(**5'**GAAGAGTTTGATCCTGGCTCAG**3'**), P6(R) (**5'**CTACGGCTACCTTGTGTTACGA**3'**). PCR amplification was carried out in a total volume 50 µL containing 5 µL 10 x buffer, 4 µL 25 mM $MgCl_2$, 4 µL 2.5 mMdNTPs, 2 µL 10 pmol forward primer, 2 µL 10 pmol reverse primer, 2 µL 50 ng of bacterial genomic DNA and 0.4 µL (5 units/ µL) Taq DNA polymerase - Promega, Germany**.** PCR amplification was performed in a thermal cycler (Techne,UK) programmed for one cycle at 95ºC for 5 min followed by 34 cycles each with 45 s at 95ºC for denaturation, 1 min at 50ºC for annealing and 2 min at 72ºC for elongation. Reaction mixture was then incubated at 72ºC for 10 min for final extension **(Ashmawy *et al*., 2015)**.

PCR product of electrophoresis and visualization

Two µL of loading dye was added prior to loading of 10 µl per gel slot. Electrophoresis was performed at 100 volt with 0.5 x Tris-EDTA-borate (TBE) (Tris base, 108 g/L boric acid, 55 g/L and 0.5 M EDTA with a pH of 8, 40 ml for 10 x) as running buffer in 1.5% agarose gel cast in 0.5 x TBE gel and then the gel was stained in 0.5 µg/mL (w/v) ethidium bromide solution and distained in deionized water. Finally, the gel was visualized with a UV transilluminator at 254 nm.

Purification of PCR products

QIAquick PCR purification kit (Qiagen, Germany) was used to purify the amplified products of 16S rRNA gene.

Sequencing of 16S rRNA gene

The amplified product (1550 bp) of 16S rRNA was sequenced by Big Dye terminator cycle sequencing kit. Sequencing products were purified using Centri-Sep spin columns and were resolved on an ABIPRISM model 310 automated DNA sequencer at the Sigma Scientific Services Company.

Alignment and phylogenetic analysis

Pair-wise and multiple DNA sequence alignment were carried out using CLUSTALW (1.82) http://www.ebi.ac.uk/clustalw **.** Bootstrap neighbor-joining tree generated using MEGA version 7.0 **(Kumar *et al*., 2016)** from CLUSTALW alignment. Comparisons with sequences in the GenBank database were achieved in BLASTN searches at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov). The obtained sequences in the current study were deposited in European Nucleotide Archive (ENA) under accession numbers from LN880270 to LN880276.

RESULTS

Isolation trials from collected seeds

Seed samples of different varieties of tomato, eggplant, tobacco, black nightshade, pepper and datura were collected in this study. Only one seed sample was obtained from naturally infected tomato fruits (Super jakal variety) showed typical symptoms of gray wall disease.

Different bacterial colonies were observed on glycerol agar, nutrient agar, yeast extract dextrose-CaCo3 (YDC), as well as, semi selective media, PA20, King's B and Tween B (Table 1). According to their colonies characteristics on the previous semi selective media, three types of bacterial isolates were detected: Type I [typical colonies of *Pantoea* spp. (P_1 , P_2 , P_3 , P_4 and P_5)] on PA20 medium, colonies were yellow, 3–4 mm in diameter, shiny, drop shaped with small, granular, darker inclusions inside and yellow lighter zones around colonies, Type II [Typical colonies of *Pseudomonas* spp. (Ps_1, Ps_2, Ps_3, Ps_4, Ps_5 and P_6)] on King's B medium, colonies were white, circular and mucoid and Type III [typical colonies of *Xanthomonas* spp. (X_1, X_2 and X_3)] on Tween B medium, colonies were raised, circular and yellow surrounded by zones of white crystals giving a fried-egg appearance (Fig.1).

Pathogenicity tests

Tomato seedlings

All the bacterial isolates Type I (P_1,P_2,P_3,P_4 and P_5), Type II (Ps_1, Ps_2, Ps_3, Ps_4, Ps_5 and P_6) and Type III (X_1, X_2 and X_3) were tested for their pathogenicity on tomato seedlings Alissa F1 and Gs Nada varieties. Inoculation with isolates of type I, stems appeared shrivel and wither, moreover, discolored water-conducting tissue and chlorosis was appeared on leaves (Figs. 2) which were suspected to belong to *Pantoea* spp. Inoculation with isolates of type II showed necrotic spots surrounded by a chlorotic halo appearing on leaves (Fig. 3) which were suspected to belong to *Pseudomonas* spp. In case of inoculation with type III bacterial isolates, leaves appeared water soaked lesions and became brown color (Fig. 4) which was suspected to belong to *Xanthomonas* spp.

Table 1 Isolates and colony type of seed-borne bacteria isolated from different seed cultivars on semi-selective media

Seed samples	Media	Colony type	Isolate code
Tomato cultivars			
Castle rock	PA 20	I*	P_4
Super strain B	King's B	II	Ps_1
Peto 86	Tween B	III	X_1, X_2
Gs 12	King's B - Tween B	II-III	Ps_2, X_3
	PA 20	I	P_2
Local variety 1	King's B	-	-
	Tween B	-	-
Local variety 2	PA 20	-	-
	King's B	-	-
	Tween B	-	-
Super jakal	PA 20	I	P_1
Eggplant cultivars			
Black beauty	King's B	II	Ps_3, Ps_4, Ps_5, Ps_6
	PA 20	-	-
Local variety	King's B	-	-
	Tween B	-	-
Black nightshade	PA 20	I	P_3
Tobacco	PA 20	I	P_5
Pepper cultivar			
	PA 20	-	-
(Local variety)	King's B	-	-
	Tween B	-	-
Datura			
	PA 20	-	-
	King's B	-	-
	Tween B	-	-

* Type I, yellow colonies, 3–4 mm in diameter, shiny, drop shaped, Type II, white colonies, circular and mucoid and Type III, yellow colonies surrounded by zones of white crystals. (-), no colonies were detected.

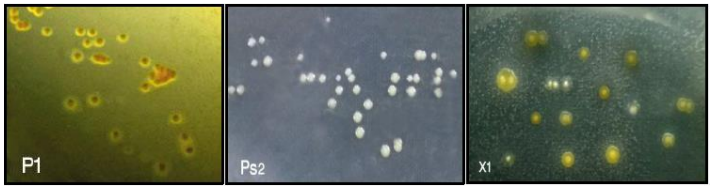

Figure 1 Typical colonies of *Pantoea* spp. (P_1 isolate) on PA 20 medium, *Pseudomonas* spp. (Ps_2 isolate) on King's B medium and *Xanthomonas* spp. (X_1 isolate) on Tween B medium.

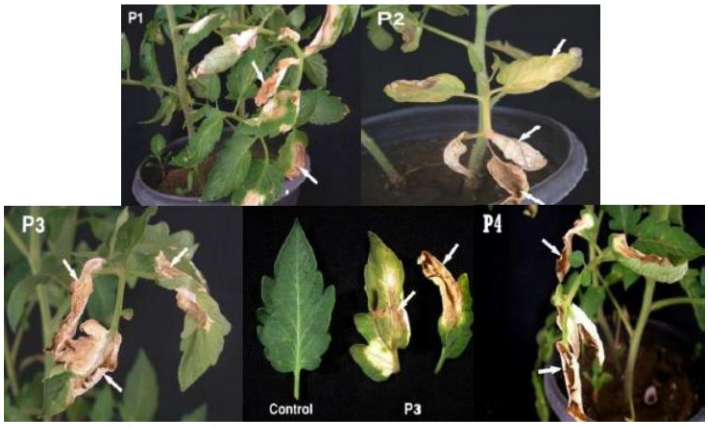

Figure 2 Artificially infected tomato seedlings "Alissa F1" with Type I isolates (P_1, P_2, P_3 and P_4) of *Pantoea* spp. Showing yellowness on leaves (arrows).

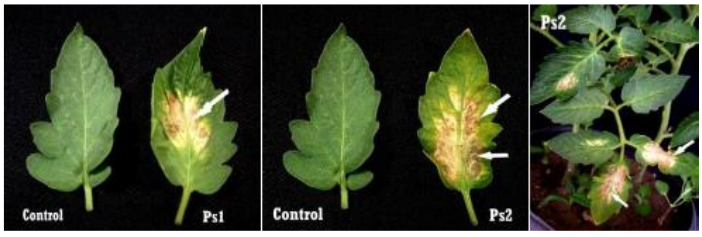

Figure 3 Artificially infected tomato seedlings "Alissa F1" with Type II isolates (Ps_1 and Ps_2) of *Pseudomonas* spp. showing necrotic spots surrounded by a chlorotic halo appearing on leaves (arrows).

Figure 4 Artificially infected tomato seedlings "Alissa F1" with Type III isolates (X_1 and X_2) of *Xanthomonas* spp. showing water soaked lesions on leaves which became brown colour (arrows).

Tomato fruits

All isolates of *Pantoea* spp. (P) were tested for their pathogenicity on tomato fruits (Alissa F1) and showed grey areas around the inoculation site (Fig. 5).

Re-isolation from artificially infected seedlings

Tomato seedlings and fruits

Re-isolation from infected seedlings with isolated bacteria appeared identical to those colonies of *Pantoea* spp., *Pseudomonas* spp. and *Xanthomonas* spp.
Re-isolation from inoculated fruits on selective media produced yellow colonies typical to *Pantoea* spp.

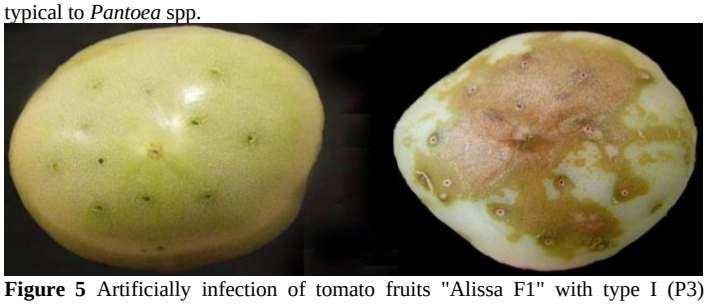

Figure 5 Artificially infection of tomato fruits "Alissa F1" with type I (P3) isolate of *Pantoea* spp. showed typical graywall symptoms (right), compared with control treatment (left).

Tomato seeds

Re-isolation from non-surface sterilized seeds from infected seedlings with P_1 isolate appeared typical graywall disease symptoms didn't revealed colonies identical to *Panteoa* spp. Otherwise non surface-sterilized seeds from infected seedlings with Ps2 isolate and X3 isolate showed typical bacterial speck and spot diseases symptoms and produced colonies identical to *Pseudomonas* spp. and *Xanthomonas* spp. respectively.
Re-isolation from macerated surface-sterilized seeds revealed colonies identical to *Pantoea* spp. (P_1 isolate) and *Xanthomonas* spp. (X_1 isolate), otherwise didn't revealed colonies identical to *Pseudomonas* spp. (Ps_2 isolate).

Table 2 Morphological traits, physiological and biochemical tests of *Pantoea* spp.(P, type I), *Pseudomonas* spp. (Ps, type II) and *Xanthomonas*spp.(X, type III)

Characteristics	*Pantoea* spp. (Type I)	*Pseudomons* spp. (Type II)	*Xanthomonas* spp. (Type III)
Cell shape	short rods	short rods	short rods
Gram Stain	-	-	-
Spore formation	-	-	-
Growth at 36℃	+	+	-
Growth at 4℃	+	+	+
Motility	+	+	+
Catalase production	+	+	+
Oxidase activity	-	-	-
Hydrolysis of: Starch	-	-	-
Casein	-	+	+
Gelatine	+	+	+
Urea	-	+	-
Lipid (Lipase activity)	-	+	-
Arginine dehydrolase	-	-	-
Nitrate reduction	-	-	-
Levan production	+	+	+
Acid production from: Mannitol, Mannose, Sorbitol, Cellobiose	+	+	+
Growth in the presence of: NaCl 4%	+	-	-
Streptomycin (10 µg)	-	+	-
Pathogenicity	+	+	+

+ = Positive reaction -= Negative reaction

Identification of the seed borne bacterial isolates

Isolation process revealed three types of bacteria, type I, II and III which were short rods and non-spore formers and Gram Negative (Table 2). Data presented in Table (2) showed that all tested bacterial isolates *Pantoea* spp.(P, type I), *Pseudomonas* spp.(Ps, type II) and *Xanthomonas* spp. (X, type III) were positive for catalase, gelatin hydrolysis, levan production, negative for oxidase, starch hydrolysis, arginine dihydrolase and nitrate reduction. Whereas, all isolates were positive for acid production of mannitol, mannose, sorbitol and cellobiose. Some tests were revealed differentiation in their reaction among tested isolates.
Data presented in Table (3) and Figs. (6, 7 and 8) showed differences among colonies characteristics of *Pantoea* spp. (P, type I), *Pseudomonas* spp. (Ps, type II) and *Xanthomonas* spp.(X, type III) on different culture media.
Tested isolates of *Pseudomonas* spp. and *Xanthomonas* spp. couldn't grow on PA 20 medium compared with *Pantoea* spp. (Fig. 9).

Table 3 Colonial characteristics of *Pantoea* spp. (P, type I), *Pseudomonas* spp. (Ps, type II) and *Xanthomonas* spp. (X, type III) on different culture media

Media	*Pantoea* spp. (Type I)	*Pseudomons* spp. (Type II)	*Xanthomonas* spp. (Type III)
Glycerol agar	Yellow, circular and smooth	Beige, circular and smooth	Yellow, circular and mucoid
Nutrient agar	Pale yellow, smooth and translucent	White, circular and smooth	Pale yellow circular and smooth
Yeast extract dextrose-CaCo3 (YDC)	Yellow,regularly round and mucoid surrounded by clear ring	White,circular, domed and smooth	Yellow, circular, smooth, domed and mucoid – fluidal surrounded byclear ring
PA 20	Yellow, 3–4 mm in diameter, shiny drop shaped with small, granular and darker inclusions inside	-	-
Tween B	-	-	Yellow surrounded with halo, raised and circular
King's B	-	white, circular and mucoid	-

(-) = No growth

Figure 6 Typical colonies of *Pantoea* spp. (P, Type I) on Glycerol Agar (A), Nutrient Agar (B) and Yeast Extract Dextrose calcium carbonate agar (C).

Figure 7 Typical colonies of *Pseudomonas* spp. (Ps, type II) on Glycerol Agar (A), Nutrient Agar (B) and Yeast Extract Dextrose calcium carbonate agar (YDC) (C).

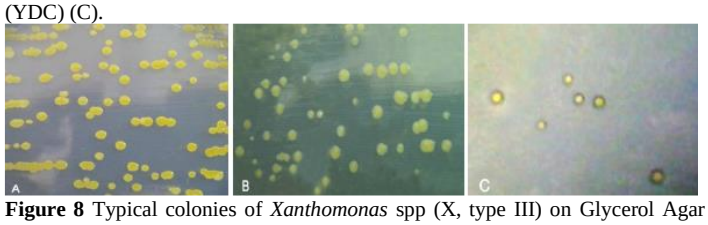

Figure 8 Typical colonies of *Xanthomonas* spp (X, type III) on Glycerol Agar (A), Nutrient Agar (B) and Yeast Extract Dextrose calcium carbonate agar (YDC) (C).

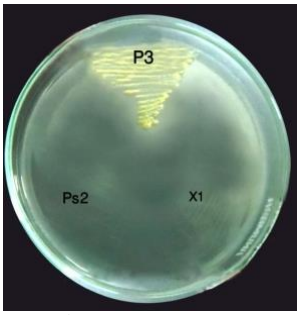

Figure 9 Bacterial growth of *Pantoea* spp. (P, Type I) on PA 20 selective medium. No growth appeared of *Pseudomonas* spp. (Ps, Type II) and *Xanthomonas* spp. (X, Type III) on PA 20 medium.

Determination of cellular fatty acids composition

Fatty acids methyl esters (FAMEs) were identified and quantified to give a profile for tested isolates. Cellular fatty acid contents of isolates of *Pantoea* spp. (P_1, P_2, P_3), *Pseudomonas* spp. (Ps_1, Ps_2) and *Xanthomonas* spp. (X_1, X_2) were determined and summarized in Table (4) as following:

The main data analyses of fatty acid compositions measured by percentage of total area of a fatty acid to all fatty acids in percentage (P) were used to obtain the ratio between the fatty acids values.The considered method for explanation of the quantities and distribution of cellular fatty acids among all bacterial isolates, showed in presence of 18 fatty acids.

The major fatty acids detected in *Pantoea* spp. isolates were Dodecanoic (12:0),Tetradecanoic (14:0),Hexadecanoic(16:0) and Octadecanoic acids (18:0).whereas, the primary fatty acids found in *Pseudomonas* spp. isolates were Tetradecanoic (14:0) and Hexadecenoic acids (16:1). In case of *Xanthomonas* spp. isolates, the major fatty acids detected were Tetradecanoic(14:0), Pentadecanoic (15:0) and Hexadecanoic acids (16:0).

The fatty acids Tetradecenoic (14:1), Tetradecanoic (14:0) and Pentadecenoic **(**15:1) were found in of *Pantoea*spp. (P_1), in high percentage 24.97%, 23.07% and 24.39% respectively, while these fatty acids were found in P_2 and P_3 isolates of *Pantoea* spp. in low percentage. The number of fatty acids found in of *Pseudomonas* spp. (Ps_1) were less than the number of fatty acids which found in Ps2 isolate.

The fatty acids Tridecanoic (13:0), Tetradecanoic (14:0) and Pentadecenoic (15:1) were found in *Xanthomonas* spp. (X_1) in high percentage 35.85%, 23.06% and 26.78% in order given, while they were found in X_2 isolate in low percentage 10.06%, 0.77% and 6.39% respectively. Tridecanoic acid (13:0) and Pentadecenoic acid (15:1) were found in all previous isolates. Undecanoic (11:0), Heptadecanoic (17:0) , Linoleic (18:2c) and Eicosatrienoic acids (20:3w6) were absent in P_1, P_2, P_3, Ps_1 and X_2 isolates, while they were detected in Ps_2 and X_1 isolates. Dodecanoic acid (12:0) and Octadecanoic acid (18:0) were found in all isolates except Ps_1 and X_1 isolates, respectively.

Table 4 Relative fatty acid compositions of tested bacterial isolates.

Fatty acid(FAMEs)		Bacterial isolates						
Shorthand designation	**Systematic name**	**P_1**	**P_2**	**P_3**	**Ps_1**	**Ps_2**	**X_1**	**X_2**
		Fatty acids (% of the total fatty acids)						
C 11 : 0	**Undecanoic**	-	-	-	-	0.10	0.70	-
C 12 : 0	**Dodecanoic**	8.56	0.23	0.13	-	0.18	6.15	0.22
C 13 : 0	**Tridecanoic**	8.97	7.48	8.05	2.78	0.06	35.85	10.06
C 14 : 1	**Tetradecenoic**	24.97	4.27	4.95	1.60	6.32	22.45	5.74
C 14 : 0	**Tetradecanoic**	23.07	0.59	0.68	0.12	6.11	23.06	0.77
C 15 : 1	**Pentadecenoic**	24.39	3.88	5.28	1.64	6.63	26.78	6.39
C 15 : 0	**Pentadecanoic**	21.63	0.31	-	-	5.67	21.36	5.24
C 16 : 1	**Hexadecenoic**	10.20	0.23	0.12	0.51	2.91	2.01	0.93
C 16 : 0	**Hexadecanoic**	15.80	3.53	3.53	-	5.33	10.32	1.85
C 17 : 1	**Heptadecenoic**	3.77	-	-	-	0.09	0.57	-
C 17 : 0	**Heptadecanoic**	-	-	-	-	0.46	0.58	-
C 18 : 3	**Octadecatrienoic**	-	-	-	-	0.09	2.82	0.07
C 18 : 2 c	**Linoleic**	-	-	-	-	0.61	1.66	-
C 18 : 2 t	**Linolelaidic**	2.03	0.42	-	0.14	4.04	-	0.44
C 18 : 1 c	**Vaccenic**	-	-	0.41	-	-	0.56	-
C 18 : 1 t	**Octadecenoic**	5.57	1.09	0.51	0.19	-	-	-
C 18 : 0	**Octadecanoic**	1.53	0.37	0.39	0.14	1.27	-	0.51
C 20 : 3w6	**Eicosatrienoic**	-	-	-	-	0.13	3.96	-

-, Not detected; P_1, P_2, P_3, *Pantoea* spp. isolates; Ps_1, Ps_2, *Pseudomonas* spp. isolates; X_1, X_2, *Xanthomonas* spp. isolates

Molecular identification through 16S rRNA gene

The following partial sequences were obtained for *Pantoea* spp. isolates P_1, P_2 and P_3,*Psendomonas* spp. isolates Ps_1, Ps_2 and *Xanthomonas* spp. isolates X_1 and X_2**.** Search in databases to identify the bacteria was achieved in BLAST search at the NCBI. The search revealed that the sequence corresponding to each individual isolate of P_1, P_2 and P_3 was almost identical (99% homology) to that of *Pantoea ananatis* whereas, the sequence of each of the Ps1 and Ps_2 was almost similar to that of *Psendomonas syringae* pv. *tomato.* The Genbank accession numbers of the bacterial isolates were listed in Table (5). While the sequence of each of the X_1 and X_2 was almost similar (97% homology) to that of *Xanthomonas vesicatoria*.

Table 5 Accession number of partial 16s rRNA gene of *Pantoea ananatis*, *Pseudomonas syringe* pv. *tomato* and *Xanthomonas vesicatoria* isolates in the GenBank

Isolate code	Bacterial isolates	Accession no.
P_1	*Pantoea ananatis*	LN880270
P_2	*Pantoea ananatis*	LN880271
P_3	*Pantoea ananatis*	LN880272
Ps_1	*Pseudomonas syringae* pv. *tomato*	LN880273
Ps_2	*Pseudomonas syringae* pv. *tomato*	LN880274
X_1	*Xanthomonas vesicatoria*	LN880275
X_2	*Xanthomonas vesicatoria*	LN880276

Alignment and phylogenetic analysis

The phylogenetic tree generated in this study for the *P.ananatis* isolates P_1, P_2 and P_3 revealed that two main clusters do exist. Cluster I included the two out-group isolates *Pectobacterium carotovorum* subsp. *carotovorum* "Pcc" (AB680280) and *Erwinia tracheiphila* "E.trach" (NR044924). Cluster II divided into two Sub-clusters: Sub-cluster 1 divided further into two Groups: Group 1 contained the two isolates of *P.ananatis* isolated from tomato P_1(LN880270) and P_2 (LN880271) besides the *P.ananatis* isolates collected from Genbank. However, Group 2 included only one isolate P_3 (LN880272) which isolated from Black nightshade. Sub-cluster 2 included only one isolate KJ670108 (Fig.10).

While, the phylogenetic tree generated for the *Pseudomonas syringae* pv.*tomato* isolates revealed that two main clusters do exist. Cluster I contained our two isolates Ps_1 (LN880273) and Ps_2 (LN880274). Whereas Cluster II included the other isolatesof *Ps. Syringae* pv. tomato collected from Genbank (Fig.11).

In case of phylogenetic tree generated in this study for the *Xanthomonas vesicatoria* isolates revealed that two main clusters do exist. Cluster I included only one isolate KP84443. Cluster II divided into two Sub-clusters: Sub-cluster 1 divided further into two Groups: Group 1 contained the two isolates contained isolates collected from Genbank KU301883, HF585549, AF123088 and NR026388. Group II included the two isolates X_1 (LN880275) and X_2 (LN880276) (Fig.12)

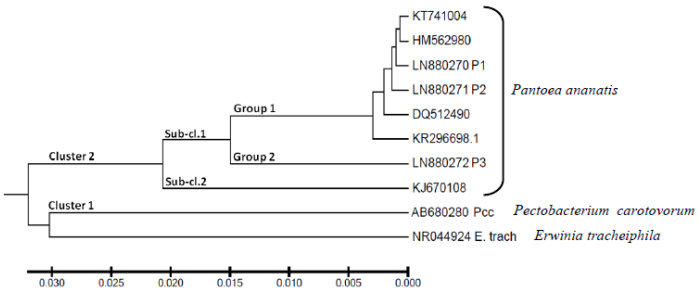

Figure 10 phylogenetic tree of *Pantoea ananatis* (P1, P2 and P3 isolates) obtained in this study and validly related bacteria from the alignment of 16S rRNA sequences. The scale appearing at the bottom indicates linkage distance.

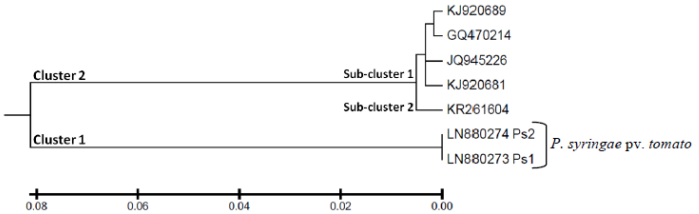

Figure 11 Phylogenetic tree of *Pseudomonas syringae* pv. *tomato* (Ps1 and Ps2 isolates) obtained in this study and validly related bacteria from the alignment of 16S rRNA sequences.The scale appearing at the bottom indicates linkage distance.

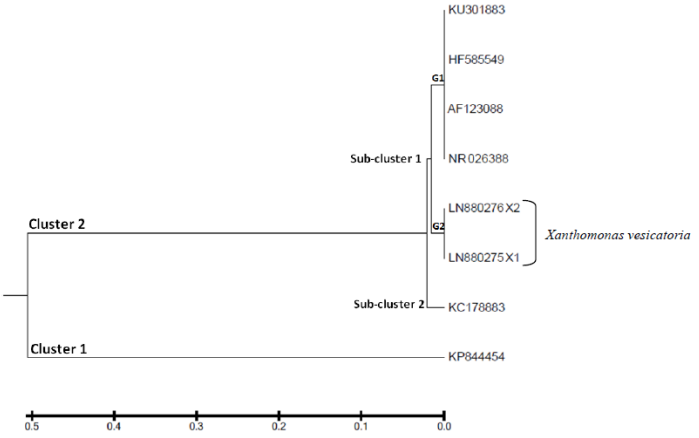

Figure 12 Phylogenetic tree of *Xanthomonas vesicatoria* (X1 and X2 isolates) obtained in this study and validly related bacteria from the alignment of 16S rRNA sequences. The scale appearing at the bottom indicates linkage distance.

DISCUSSION

Characterization of the population structure, diversity, and evolution are the main factors for understanding the pathogen biology and providing information necessary for the development of effective means for disease control **(Vancheva *et al.*, 2018)**. Results of the pathological behaviors of the isolated tomato seed-borne bacteria, their cultural, morphological and physiological characters, as well as molecular techniques (16S rRNA gene sequence) were indicated that *Pantoea ananatis* was associated with graywall symptoms **(Stall *et al.*, 1970)**, *Pseudomonas syringae* pv. *tomato* was the causal agent of bacterial speck disease and *Xanthomonas vesicatoria* was associated with bacterial spot disease **(Abd-Alla and Bashandy, 2008**; **Milijasevic *etal*., 2009 and Opara and Odibo 2009; Kolomiets *et al*., 2017; Roach *et al*., 2018)**.

Re-isolation of artificially infected tomato plants indicated that *P.ananatis* was an endophytic seed-borne bacteria, these results were consistent with findings of **Rijavec *et al*., (2007)**, while previous results were in contrast with **Cota *et al*., (2010)**. Whereas re-isolation of *Ps.syringae* pv. *tomato* from seeds proved that it's an epiphytic seed-borne. These results were matched with findings of **Hirano and Upper, (2000)**, while it's were in disagreed with **Ferrando *et al*., (2012)**. Results discovered that *X.vesicatoria* was an epiphytic and endophytic seed-borne, these results were fixed with **Kniskern *et al*., (2007)**.

Fatty acids profiling is a useful tool for identification and classification of plant pathogenic bacteria. It is potentially an extremely powerful predictive tool **(Stead 1992 and Weller *et al.* 2000)**. Isolates of *P.ananatis* were distinguished by the ratios of fatty acids (12:0,14:0 and 18:0), that similarly mimics to the findings of **Nischwitz *et al*., (2007)**. As well as, isolates of *Ps. s.* pv. *tomato* were distinguished by the ratios of fatty acids (14:0 and 16:1), that similarly mimics to the findings of **Denny, (1988)**. In case of isolates of *X. c.* pv. *vesicatoria* were distinguished by the ratios of fatty acids (14:0, 15:0 and 16:0), that similarly corresponded with**Vauterin *et al.*, (1996) and Obradovic*et al*., (2004)**.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker **(Ashmawy *et al*., 2020)**. 16S rRNAgene was used to identify the tested isolates and study the genetic variability among 3 isolates of *Pantoea* spp., 2 isolates of *Pseudomonas* spp. and 2 isolates of *Xanthomonas* spp. Results obtained of all tested isolates gave one band in the right expected molecular length. DNA sequences of tested isolates revealed that the sequences belong to *P.ananatis*, *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria*. Such findings agreed with data obtained with **Krawczyk*et al*., (2010) and Mbega*et al*., (2012).**

The bacterium associated with the tomato (*Solanum esculentum*) and the alternative host black nightshade (*S. nigrum*) seeds appeared to be *P. ananatis* on the basis of 16S rRNA gene sequence. This apparently is the first report of *P. ananatis* as a bacterial pathogen isolated from tomato and black nightshade seeds in Egypt. Alternative hosts such as several crop and weed species have been suspected to potentially play a role in the spread or survival of bacterial diseases **(Ocimati *et al*., 2018)**. The current study determined the potential risk posed by black nightshade (*S. nigrum*) as alternative hosts to *P. ananatis*.

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