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Full Length Article

Molecular Characterization of *Alcaligenes faecalis* and *Pseudomonas aeruginosa* causing Root Rot of Date Palm

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Abstract

A sudden death syndrome was observed on one-year-old date palm plants cv. Zaghlol during 2016 summer season in Egypt. Wilt symptoms began in outer leaves, then whole leaves were dried within several days. In addition, yellowish, brown discoloration, soften rot of shoot base and root were developed. Bacterial isolates were isolated and purified on Cetrimide's agar medium. Pathogenicity test was verified on 6-months old date palm seedlings and the bacterial isolates were re-isolated from diseased tissue. They were identified based on the morphological and molecular characteristics. Four bacterial isolates caused wilt symptoms on shoot and brown discoloration on root of date palm seedlings. Based on 16S rRNA gene sequencing, the identity of the bacteria were *Alcaligenes faecalis* and *Pseudomonas aeruginosa*. The 16S rRNA nucleotides sequences were conserved in the GeneBank with specific accession numbers. Concerning genetic relationships among the four isolates and the cluster analysis, the dendrogram illustrated that the genotypes Z-Kh-B1and Z-Kh-B3 which assumed to be highly related of genetic distance were grouped together in one cluster, but the other genotypes Z-Kh-B2 and Z-Kh-B4 were highly diverged and located in another cluster. This is first world report about *A. faecalis* and *P. aeruginosa* as the causal agents of bacterial root rot of date palm plants. © 2020 Friends Science Publishers

Keyword: 16S rRNA gene; *Alcaligenes faecalis;* Date palm; Root rot; *Pseudomonas aeruginosa*

Introduction

Date palm (*Phoenix dactylifera* L.) are attacked by several pathogenic bacteria such as *Bacillus* spp. that caused soft rot of tissue culture off shoot (Leary *et al*. 1986; Leary and Chun 1989; Benjama 1994). *Erwinia chrysanthemi* caused sudden decline in Al-Qassim region, Saudi Arabia on date palm tree Cv. Succary (Abdalla 2001). In addition, inflorescence pink rot on date palm caused by *Serratia marcescens* in Kuwait (Riaz *et al*. 2009). *Pseudomonas aeruginosa* caused soft rot bulbs of onion during storage in Egypt (Abd-Alla *et al*. 2011), root rot of ginseng in China (Gao *et al*. 2014), leaf spot of soybean in Cuba (Plasencia-Márquez *et al*. 2017). *Alcaligenes faecalis* is associated with bacterial blast disease in North of Iran that caused a high level of loss in citrus (Alimi and Darzi 2012). Molecular tool is a powerful method that confirm the identification of bacterial species in addition to their morphological and physiological features. The ribosomal 16S rRNA has confirmed to be a stable and specific molecular marker for the identification of bacteria (Patel 2001; Yamamura *et al*. 2007; Park *et al*. 2010). This study aimed at molecular identification of new bacteria causing root rot disease of date palm in Egypt.

Materials and Methods

Isolation of the bacterial isolates

The discolored rotten roots of date palm plants were cut into small pieces (1-cm-long). The small pieces were washed three times with sterilized water, then they were surface sterilized by 1% sodium hypochlorite solution for three minutes and rinsed in sterile distilled water. One g of the sterilized pieces were crushed in sterilize pestle and mortar with 5 mL of sterile 0.05 *M* potassium phosphate buffer and was streaked on Cetrimide's agar medium (Baron *et al*. 1994). The emerged individual bacterial colonies were taken it with sterile loop and re-cultured onto fresh nutrient agar medium supplemented with glycerol 10 mL/L. Pure cultures were obtained by re-streaking the colonies three times on the same medium and they were stored at 4°C for further use. The bacterial isolates were identified by sequencing of 16S rRNA gene.

Pathogenicity test of the bacterial isolates

Six-months old date palm seedlings Cv. Zaghlol were grown in sterilized plastic pots with a sterilized loam sandy soil. Four plants in each pot were infested after injured in stem base with ten milliter of each bacterial suspension (1 x 10^8 cell/mL) grown in nutrient broth medium for 2 days at 28 ± 2°C. Three pots were used as replicates for each bacterial isolate and three pots free from bacteria served as a control. Plants were watered well, with avoided wetting the foliage. The experiment was developed in completely randomized design. At the experimental end after 6 months according to (Ziedan *et al*. 2015), percentage of root rot of date palm plants was determined. The disease severity on shoot was determined based on linear scale from (0–3), whereas: 0 = no wilt for any leave, 1= wilt one leave, 2= wilt two leaves, 3= wilt three leaves, and whole leaves wilted. Meanwhile, disease severity on root system was determined based on brown discoloration using linear scale from (0–4) whereas: 0= no discoloration on root, 1= 1–25% brown discoloration, 2=26–50% brown discoloration, 3=51–75% brown discoloration and 4=76–100% brown discoloration as follows:

$$\text{Disease intensity} = \Sigma (n \times r) \times 100/N$$

Where: n= the number of plants in each numerical disease degree; r = Number of the disease degree and N= The total number of plants multiplied by the maximum numerical disease degree.

Molecular identification of the bacterial isolates

DNA extraction from the bacterial isolates was done by genomic DNA purification Kit (Thermo K0721, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, U.S.A.) according to the manufacturing protocol. PCR amplification of 16S ribosomal gene was done using PCR Master Mix (Thermo K1051) and 16S primers: forward primer, 5' AGAGTTTGATCCTGGCTCAG -3'; reverse primer, 5'-GGTTACCTTGTTACGACTT -3'. PCR product was clean up by PCR Purification Kit (Thermo K0701). The sequencing of the PCR-DNA product was carried out by ABI 3730 x l DNA sequencer (GATC Company, Germany) using specific forward and reverse primers.

Phylogenetic analysis

The sequences of 16S rDNA products of the bacterial isolates were compared with the sequences available in the NCBI, GenBank database by BLAST search. The sequences were aligned together and the evolutionary degree were calculated according to (Jukes and Cantor 1969). The phylogenetic tree was designed by the method initiated by Saitou and Nei (1987).

Nucleotide sequence accession number

The 16S rDNA sequence of the identified bacteria was saved in the NCBI GenBank database with accession number MH698835, MH698836, MH698837and MH698838.

Molecular analysis of the isolated bacterial strains by PCR

Genomic DNA was extracted using i-genomic BYF (iNtRON Biotechnology Inc., South Korea; Cat. No. 17361) using illustrated protocol D. HOT start PCR Master Mix blend (Solis BioDyne, Estonia, Cat. No. 04-27-02025) was used for PCR analysis. The Master Mix includes all useful reagents, except primer and DNA template, for performing 25 μL PCR amplification reaction. Three primers were applied. First primer sequence was 5'-GTGTTGTGGTCCACT-3'. Second primer sequence was 5'- AACCTCCCCCTGACC-3'. Third primer sequence was 5'- TGAGTGGTCTACGTG-3'. All primers were supplied by Operon Technologies Company, Netherlands. 12 ng of random primer and 40 ng of the purified DNA sample were added to each PCR sample and completed the total volume to 25 μL using sterile distilled water. The amplification process included thirty-five cycles each consists of the following steps: denaturation at 95°C for one min; primer annealing at 55°C for two min. Then, 72°C for 10 min, at the end, hold the PCR at 4°C till analysis. Finally, DNA products obtained after PCR amplification were loaded on 1.5% agarose gel and 1X TBE buffer at consistent 100 volt for about 2 h and the obtained bands were measured against 100 bpDNA-ladder (Pharmacia Biotech.) after staining with 0.5 μg/mL ethidium bromide and photographed using Gel Documentation System with UV-Transeliminator.

Analysis of RAPD-data

The amplification profiles for the four bacterial isolates as a result of RAPD application were compared with each other as well as the standard DNA marker. The DNA bands were recorded as a binary data. Each band was recorded as absent (0) or, present (1) and pairwise comparisons between individuals were made to calculated according Jukes-Cantor coefficient using PAST program adapted by Hammer *et al*. (2001). To produce a dendrogram using neighbor-joining (NJ) trees, the cluster analysis was used (Saitou and Nei 1987).

Statistical analysis

Data was statistical analysis of variance (ANOVA) among the means of the treatments. The least significant difference (LSD) test was used at $P < 0.05$ to identify the significant differences between the means (Snedecor and Cochran 1980).

Results

Bacterial root rot symptoms of date palm plant

Symptoms on the infected date palm plants were observed as sudden death of shoot and wilting of leaves. Stem base and roots of the infected plants were showing a vascular discoloration (Fig. 1). Bacterial ooze was observed when the infected tissue was incubated at 28°C and high relative humidity after 2 days.

Bacterial isolation and identification

Pure cultures of the bacterial isolates shown in (Fig. 2) were identified. The pure cultures of the bacterial isolates belonged to two different species. Three bacterial isolates of *A. faecalis* and one isolate of *P. aeruginosa* were isolated on Cetrimide's agar medium then purified and identification was done on molecular basis, *i.e*., Z-Kh-B1, Z-Kh-B2, Z-Kh-B3 and Z-Kh-B4.

Pathogenicity test

Pathogenicity test approved that the four bacterial isolates caused wilt of leaves and discoloration of the roots in various degrees on date palm seedlings. Percentage of root rot of date palm plants, disease severity on leaves and brown discoloration of roots were calculated (Table 1 and Fig. 3 and 4). The highest percentage of root rot disease and disease severity of shoot and root was shown by isolate Z-Kh-B4 followed by isolate Z-Kh-B1, meanwhile isolates Z-Kh-B2 and Z-Kh-B3 recorded moderate incidence of root rot disease and disease severity of date palm plants.

Molecular identification of bacterial isolates

The 16S primers F and R were applied to amplify the region of the 16S ribosomal RNA gene from the genomic DNA of the four bacterial strains (Z-Kh-B1, Z-Kh-B2, Z-Kh-B3 and Z-Kh-B4). After the amplification by PCR, a product of approximately 1500 bp was obtained. The BLAST analysis of the amplified 16S ribosomal RNA gene sequence revealed 99% similarity to the partial 16S ribosomal RNA gene of *A. faecalis* strain (accession number: KM502543.1). Based on this result, bacterial isolates Z-Kh-B1, Z-Kh-B2 and Z-Kh-B3 were identified as *A. faecalis* (Fig. 5–7). However, bacterial isolate Z-Kh-B4 was identified as of *P. aeruginosa* (Fig. 8).

Molecular analysis of bacterial isolates

To evaluate the genetic variations based on the DNA nucleotide sequence of the four bacterial isolates, three random primers obtained from Operon Technologies were used to identify the genetic variability among them. All primers successfully amplified specific fragments of the genomic DNA.

Fig. 1: Wilt symptoms and healthy date palm plants respectively (left), moderately and sever brown discoloration of stem base and root (middle and right) respectively

Fig. 2: Cultures of *P. aeruginosa* (**1**) and *A. faecalis* (**2**) bacterial isolates on nutrient agar medium supplemented with glycerol (10 mL/L)

Fig. 3: Root rot disease symptoms on date palm plants under artificial infestation with bacterial isolates *A. faecalis* (1, 2, 3) and *P. aeruginosa* (4) compare control (left)

The RAPD method was used to test if any genetic markers could be correlated with the isolates. The primers successfully generated reproducible polymorphic and scorable bands. The polymorphic patterns of the scorable three RAPD primers among the four isolated bacteria are shown in Table 2 and Fig. 9, 10 and 11. The fragment patterns of RAPD fragments using these primers exhibited 43 amplified fragments varied among the used three primers, with 13 fragments out of total bands were polymorphic and 16 fragments were unique loci (Table 2

Table 1: Pathogenicity test of bacterial isolates on date palm seedlings

No.	Isolate code	Isolate name	Root rot %	Diseases severity	
				Shoot	Root
1	Z-Kh-B1	*A. faecalis*	75.0 b	3.0 b	3.0 b
2	Z-Kh-B2	*A. faecalis*	50.0 c	2.0 c	1.0 c
3	Z-Kh-B3	*A. faecalis*	50.0 c	1.0 d	1.0 c
4	Z-Kh-B4	*P. aeruginosa*	100.0 a	4.0 a	4.0 a
	----------	Control	00.0 d	0.0 e	0.0 d

Values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test

Table 2: Number, different types of the amplified DNA bands as well as the total percentage of polymorphism obtained by three random primers

Primer No.	Total bands	polymorphic Loci	monomorphic Loci	Unique loci	Polymorphic bands	Polymorphic percentage
1	11	4	0	2	9	81.82
2	19	7	0	5	14	73.68
3	13	2	0	9	4	30.77
Total	43	13	0	16	27	62.79

Fig. 4: Degree of root rot discoloration of infested plant with bacterial isolates compared with healthy (left)

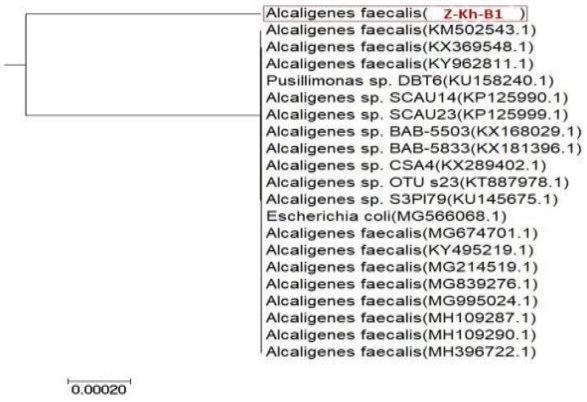

Fig. 5: Phylogenetic tree of *A. faecalis* Z-Kh-B1 (accession number MH698835) compared with the most related bacterial database

and Fig. 9, 10 and 11). The application of primer No.1 with the genomic DNA of the four isolates clearly showed that lanes 1 and 3 of Z-Kh-B1 and Z-Kh-B3 had new distinct band with size of 450 bp (Fig. 9).

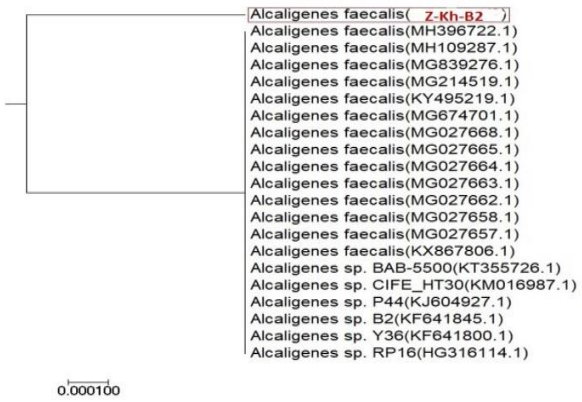

Fig. 6: Phylogenetic tree of *A. faecalis* Z-Kh-B2 (accession number MH698836) compared with the most related bacterial database

Fig. 7: Phylogenetic tree of *A. faecalis* Z-Kh-B3 (accession number MH698837) compared with the most related bacterial database

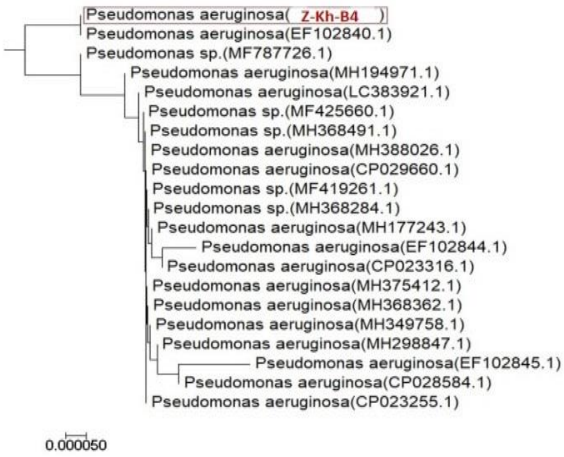

Fig. 8: Phylogenetic tree of *P. aeruginosa* Z-Kh-B4 (accession number MH698838) compared with the most related bacterial database

A distinct band with size of 1250 bp was detected in isolate Z-Kh-B3 (lane 3). A new band with size of 2100 bp was exhibited in the isolate Z-Kh-B2. A distinct band with size of 400 bp was detected in isolates Z-

Table 3: Proximity Matrix between four bacterial isolates according of RAPD assays

Strain code	Z-Kh-B1	Z-Kh-B2	Z-Kh-B3	Z-Kh-B4
Z-Kh-B1	1.000			
Z-Kh-B2	0.111	1.000		
Z-Kh-B3	0.741	0.000	1.000	
Z-Kh-B4	0.154	0.286	0.000	1.000

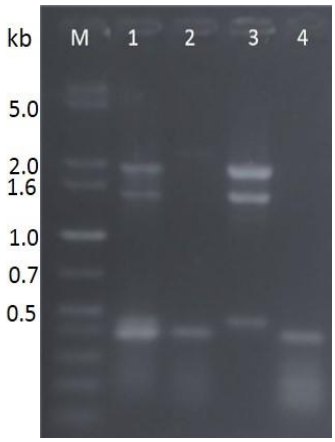

Fig. 9: DNA amplified banding patterns based on RAPD for the four isolates and ladder DNA marker 100 bp (lane M) by primer No.1. (lane 1 to 4: Z-Kh-B1, Z-Kh-B2, Z-Kh-B3 and Z-Kh-B4)

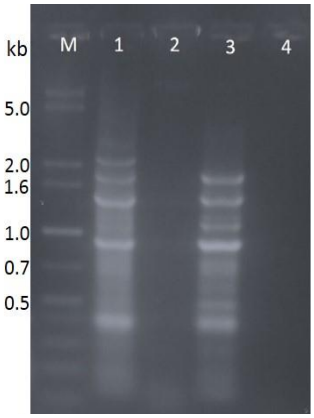

Fig. 10: DNA amplified banding patterns based on RAPD for the four isolates and ladder DNA marker 100 bp (lane M) by primer No. 2 (lane 1 to 4: Z-Kh-B1, Z-Kh-B2, Z-Kh-B3 and Z-Kh-B4)

Kh-B1, Z-Kh-B2 and Z-Kh-B4 (lanes 1, 2 and 4). On the other hand, the primer No.2 induced distinct bands with size of 2070 and 1800 bp in the isolate Z-Kh-B1 (lane 1). Distinct bands with size of 1850, 1200, 1100, and 500 bp were detected with isolate Z-Kh-B3 (lane 3) (Fig. 10). Primer No.3 involved in induction of specific band in isolates Z-Kh-B1and Z-Kh-B3 in the lanes1 and 3 with size of 900bp. Three distinct bands with size 4000, 2750, 1750 and 1100 bp in the lane 2 were detected in the Z-Kh-B2. Three different bands with size of 3000, 2900, 2100, 1400 and 200 bp were detected in the lane 3 were detected in isolate Z-Kh-B3 (Fig. 11).

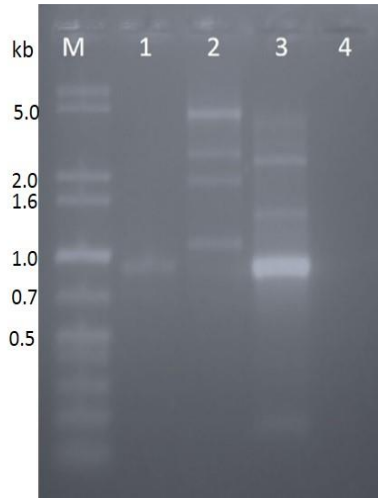

Fig. 11: DNA amplified banding patterns based on RAPD for the four isolates and ladder DNA marker 100 bp (lane M) by primer No.3 (lane 1 to 4: Z-Kh-B1, Z-Kh-B2, Z-Kh-B3 and Z-Kh-B4)

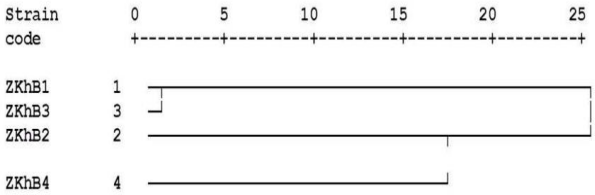

Fig. 12: Dendrogram using Average Linkage of the four isolates based on RAPD analysis

Genetic relationships and phylogenetic tree generated from the RAPD analysis

The distance matrices resulting from the RAPD products data were performed to generate correct relationships based on RAPD analysis (Table 3). The highest percentage of genetic distance was detected between Z-Kh-B1and Z-Kh-B3 (74%) followed by 29% between Z-Kh-B2 and Z-Kh-B4, however, the genetic distance between Z-Kh-B1 and Z-Kh-B4 was 15%. On the other hand, no genetic distance was obtained between Z-Kh-B2, Z-Kh-B3, Z-Kh-B3 and Z-Kh-B4. The dendrogram based on RAPD distance separated the four isolated isolates into two main clusters (Fig. 12). The first cluster contained the Z-Kh-B1and Z-Kh-B3, whereas, the second cluster contained Z-Kh-B2 and Z-Kh-B4, which they were highly diverged than the others. Concerning genetic relationships among the four isolates and the cluster analysis used to divide the studied genotypes into groups, which could reflect the genetic diversity of the four isolated pathogenic bacterial isolates. The dendrogram illustrated that the genotypes Z-Kh-B1and Z-Kh-B3, which was assumed to be highly related of genetic distance, were grouped together in one cluster, but the other genotypes were highly diverged and located in another cluster.

Discussion

A. faecalis and *P. aeruginosa* are multi-host opportunistic pathogenic bacteria, which cause skin and soft tissue infection of human (Tena *et al*. 2015). *A. faecalis* attacks a wide range of hosts, bacterial and fungal pathogens of cyclamen *i.e*., *Pantoea agglomerans* and *Collettrichum gloeosporioides* due to their antimicrobial hydroxylamine production (Yokoyama *et al*. 2013), food and seed borne (Ethica *et al*. 2017), kills more than 70% of *Galleria mellonella* larvae (Quiroz-Castañeda *et al*. 2015). Recently it was recorded a new pathogen on silkworm (Anusha and Bhaskar 2018). Pathogenic *P. aeruginosa* is antagonistic agents against *Pythium myriotylum* the causal pathogen of cocoyam root rot (Tambong and Achuo 1997). It caused tail and fin rot disease in spotted snake head (Saikia *et al*. 2018) and infected larvae of giant mealworm (*Zophobas morio*) (Maciel-Vergara *et al*. 2018). Pathogenic isolates of *P. aeruginosa* PAO1 and PA14 were capable of infecting the roots of Arabidopsis and sweet basil (*Ocimum basilicum*) *in vitro* and in soil and were capable of causing plant mortality after 7 days post-inoculation (Walker *et al*. 2004). Our results approved that the four bacterial isolates were isolated of stem base and discolored roots of one- year-old date palm plants. Pathogenicity test of bacterial isolates indicated that all isolates caused root rot of date palm plants. These results are in agreement with investigation on *P. aeruginosa* contaminated onion bulbs during harvest by moving through wounds caused by topping, finally causing soft rot (Gupta *et al*. 1986; Cother and Dowling 1986) and caused soft rot of onion bulbs in storage in Egypt (Abd-Alla *et al*. 2011). They caused root rot of ginseng in China (Gao *et al*. 2014), leaf spot of soybean in Cuba (Plasencia-Márquez *et al*. 2017). *A. faecalis* is one of most common bacteria associated with bacterial blast disease of citrus in North Iran that caused high level of loss in citrus (Alimi and Darzi 2012). Bacterial isolates were identified based on 16S sequences that showed 98–100% similarity with the related bacteria recorded in the GenBank. The 16S regions of the bacterial pathogenic isolates have many nucleotide substations in comparison with the strains from GenBank and identified three isolates as *A. faecalis* and one isolate as *P. aeruginosa*. In agreement with our findings, Renders *et al*. (1996), Vos *et al*. (1997), Pethannan *et al*. (2018) utilized the RAPD protocol for fingerprinting and phylogenetic relationship of *P. aeruginosa* strains and exhibited usefulness of this protocol in laboratory in combination with other protocols used to detect the phylogenetic relationships between the strains under the same species.

Conclusion

As far as the authors are aware and according to the available literature, this is the first record of *A. faecalis* and *P. aeruginosa* causing bacterial root rot on date palm in Egypt and in the world.

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