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Isolation and Molecular Characterization of the Fire Blight Pathogen, *Erwinia amylovora,* Isolated from Apple and Pear Orchards in Egypt

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ABSTRACT
Eleven *Erwinia amylovora* isolates, the causal agent of the fire blight disease, were isolated from apple orchards adjacent to those of pears showing fire blight disease symptoms. Initial characterization concerning morphological and biochemical traits revealed that all isolates were typical to those of *E. amylovora*. Furthermore, all bacterial isolates showed typical symptoms of the fire blight disease upon infection of unripe pear fruits. Molecular characterization of these isolates via PCR utilizing specific primers based on a region of plasmid pEA29 and chromosomal DNA *ams*–region was performed. PCR products were positive and represented the expected length 1.1 and 1.6 kb, respectively. Moreover, PCR reactions utilizing the 16S rRNA universal primers were carried out. DNA sequence of PCR products and analysis via blast and Genbank data showed that, the bacterial isolates actually belonging to *E. amylovora*. Moreover, there are interference between these *Erwinia* isolates and identified *E. amylovora* strains based on constructed phylogenetic analysis. To the best of our knowledge this is the first report of fire blight of apples caused by *E. amylovora* in Egypt.

Key words: *Erwinia amylovora*, apple, pear, PCR, 16S rRNA, phylogenetic analysis

INTRODUCTION

Fire blight caused by the bacterium *Erwinia amylovora* (Burrill) (Winslow *et al*., 1920) is one of the most destructive diseases attacking pome fruit trees, with apple and pear being of special importance. Fire blight is undoubtedly the most serious disease that affects pear plantation in Egypt. The productivity of pears in Egypt has been seriously decreased due to the severe epidemic outbreaks of this disease in Alexandria and El-Behera Governorates starting from 1982 (Abo-El-Dahab, 1985; Abo-El-Dahab *et al*., 1983, 1984). The pathogen was progressively detected in different regions in Egypt, reaching pear orchards in Kafer El-Sheikh, Monofia and Nobaria and leading to severe damages in approximately all regions of Nile delta (Ashmawy, 2010).

Various methods have been described for the identification and detection of the *E. amylovora*, such as screening on semi selective media (Bereswill *et al*., 1998) or PCR based methods (Bereswill *et al*., 1992, 1995). The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently, it has also become important as a mean to identify an unknown bacterium at the genus or species level (Sacchi *et al*., 2002). The variable regions of the 16S rRNA gene provide unique signature that can be analyzed to provide an identification of bacterial species. The present study describes the isolation and characterization of eleven *E. amylovora* isolates, from apple and pear orchards in Egypt that have the ability to cause fire blight disease.

MATERIALS AND METHODS

Bacterial isolation and reference isolates: Isolation trials were carried out from infected apples (*Malus domestica*)

and pears (*Pyrus communis*) tissues (blossoms, leaves, spurs and stem with cankers), as well as, twig pieces with well developed symptoms. Isolate source was from two locations in Egypt (Kafr El-Dawar, Beheira Governorate and Borg El-Arab, Alexandria Governorate), during 2013.

Plant materials were washed with tap water, surface sterilized by soaking in 1% sodium hypochlorite for 3 min, rinsed three times in sterile distilled water and gently blotted dry on sterilized tissue paper. Samples taken from the internal tissues of plant materials were macerated in 3 mL sterile saline solution (0.8%) in a sterile mortar. A loop full of the resulting suspension was streaked on 5% Sucrose Nutrient Agar (SNA) plates (Billing *et al*., 1960). Single colonies observed after 48 h incubation at 27°C were isolated and consequently purified. Three *E .amylovora* isolates (Ea1, Ea2 and Ea3) were kindly provided by Prof. Alia Shoeib, Department of Plant Pathology, Faculty of Agriculture, Alexandria University. All isolates were kept on 2% glycerol nutrient agar slants (El-Helaly *et al.*, 1966) for later use.

Media and culture conditions: Sucrose Nutrient Agar (SNA) medium (Billing *et al*., 1961), King's medium B (King *et al*., 1954), MM2Cu medium (Bereswill *et al*., 1998) and glycerol nutrient agar were used.

Pathogenicity test: Immature pear and apple fruits were inoculated as described by Beer and Rundle (1983) with some modifications. The immature fruits were surface sterilized with 70% ethanol and cut in transverse slices (1 cm) thick. For each isolate, three slices were placed on a sterile moist filter paper in a sterile plastic dish. To each slice, 50 µL of bacterial suspension, at a concentration of approximately 1×10^8 CFU mL^{-1} was added at the center of each slice. The slices were maintained under humid conditions at 27°C for five days.

Phenotypic identification: The morphology of the bacterial isolates (cell and colony morphology, arrangements and motility) was investigated by light microscopy. Gram reaction, oxidase and catalase tests, growth at 36-39°C, growth in 5% NaCl and nitrate reduction test were carried out according to protocols described by Jones and Geider (2001). Fermentation of glucose with gas formation was tested using the medium of Board and Holding (Smibert and Krieg, 1981).

DNA extraction protocol: Bacterial isolates were grown overnight in LB medium at 28°C with constant shaking at 200 rpm. Cells from 3 mL culture were pelleted by centrifugation at 6000 g for 5 min using a microcentrifuge. Cells of each culture were washed in TE buffer (10 mM Tris-HCl, 1 mM E DTA, pH 8.0), then resuspended in a mixture of 567 µL Tris EDTA, 30 µL of 10% Sodium Dodecyl Sulphate (SDS) and 3 µL proteinase K (20 mg mL^{-1}). After incubation at 37°C for 1 h, 100 µL 5M NaCl and 80 µL of CTAB/NaCl solution were added and the tubes were inverted well before incubation for 10 min in a water bath at 65°C. Phenol/chloroform/isoamyl alcohol (0.8 mL) were then added, mixed thoroughly and the tubes were centrifuged at 11000 g for 5 min. The aqueous supernatant was then taken and the phenol/chloroform step was repeated one more time. DNA was precipitated by adding equal volume of isopropanol and washed with 70% ethanol. DNA pellets were suspended in 100 µL sterilized distilled water (Ausubel *et al*., 1995).

PCR analyses: Two pairs of primers (Invitrogen Life Technologies Ltd, Renfrew, UK) were used: A (5`-CGGTTTT TAACGCTGGG-3`) and B (5`-GGGCAAATACTCGGA TT-3`), based on the plasmid pEA29 DNA (Bereswill *et al*., 1992); AMSb1 A (5`-GCTACCAGCAGGGTGAG-3`) and AMSb1 B (5`-TCATCACGATGGTGTAG-3`), based on chromosomal *ams* gene (Bereswill *et al*., 1995). The PCR reactions were carried out in a volume of 25 µL each containing 2 µL of template DNA, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 0.5 unit Dynazyme TM II DNA Polymerase (Finnzymes and Finland). Amplifications were performed with a thermal cycler (Techne, UK). The PCR programme consisted of an initial denaturation at 94°C for 3 min followed by 35 cycles, each with 30s at 94°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for elongation. Reaction mixtures were then incubated at 72°C for 10 min for final extension. PCR products were separated on a 1.5% agarose gel in TBE buffer (Maniatis *et al*., 1982), stained with ethidium bromide and photographed under UV light.

Moreover, full length of 16S rRNA gene (1550 bp) was amplified for all bacterial isolates using two universal primers P0 (5`-GAAGAGTTTGATCCTGGCTCAG-3`) and P6 (5`-CTACGGCTACCTTGTTACGA-3`). The PCR amplification was carried out in a total volume 25 µL containing 2µL of template DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pmol of each primer and 0.5 unit Dynazyme TM II DNA Polymerase. PCR amplification was performed as one cycle at 95°C for 5 min followed by 34 cycles each with 45 s at 95°C for denaturation, 1 min at 50°C for annealing and 2 min at 72°C for elongation. Reactions were then incubated at 72°C for 10 min for final extension. The PCR products were analyzed as above.

Sequencing of 16S rRNA gene and alignment: The amplified products of 16S rRNA gene (1550 bp) were purified using Centri-Sep spin columns. The products were sequenced by the use of a Big Dye terminator cycle sequencing kit and resolved on an ABI PRISM model 310 automated DNA sequencer at Sigma Company. Pair-wise and multiple DNA sequence alignment were carried out using CLUSTAL W (1.82) http://www2.ebi.ac.uk/clustalw (Thompson *et al*., 1994). Bootstrap neighbor-joining tree was generated using MEGA version 5.1 (Tamura *et al*., 2011) from CLUSTAL W alignments. 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 16S rDNA sequences have been deposited in the GenBank database under the accession numbers shown in Table 1.

Sequences used in the phylogenetic analysis: The following *E. amylovora* sequences were collected from the GenBank database and used in the construction of the phylogenetic tree and the similarity matrix. Ea7/74 (X83265), Ea-Apricot Po1 (HF546214), Ea-PlumBo1 (HE610678) and YPPS200 (AB546196).

RESULTS AND DISCUSSION

Isolation and initial characterization: Eleven bacterial isolates were isolated from infected pear and apple plant materials as stated in materials and methods. Distinct colonies that possessed typical morphological characteristics of *E. amylovora* were purified. High level of homogeneity among isolates was observed where colonies were typically white, domed, shiny, mucoid (levan type) with radial striations and possessed dense flocculent centre (Billing *et al*., 1961; Ashmawy, 2010). All isolates (four isolates from apple and seven isolates from pear orchards) were oxidase-negative, catalase-positive and consisted of gram-negative motile rods. They were able to ferment glucose without gas formation and did not reduce nitrate (Table 1). Moreover, all the isolates did not grow at 36-39°C and were not able to grow in the presence of 5% NaCl.

Based on the aforementioned morphological and biochemical tests, all the bacterial isolates possessed the characteristics of *E. amylovora* (Holt *et al*., 1994; Bereswill *et al*., 1997; Jones and Geider, 2001).

Pathogenicity test: All bacterial isolates showed typical symptoms of the disease upon infection to unripe pear fruits such as necrosis accompanied by oozes, brownish and blackish colorization of the fruits.

Identification of the bacterial isolates through PCR amplification of pEA29 plasmid: It was reported that the pEA29 plasmid plays a quantitative role in pathogenicity (Falkenstein *et al*., 1989; Laurent *et al*., 1989). Consequently, the presence of this plasmid in all tested *E. amylovora* isolates allowed a mean for detection of *E. amylovora* by PCR based technique (Bereswill *et al*., 1992). In the present work, all the *Erwinia* isolates from apple and pear produced PCR products utilizing pEA29 plasmid specific primers. The PCR product was approximately 1,100 kb in length as shown in Fig. 1. Data is in agreement with earlier reports stating that, the amplified fragments were of same length (Bereswill *et al*., 1993; McManus and Jones, 1995; Ashmawy, 2010).

Identification through PCR amplification of chromosomal *ams* gene: *E. amylovora* produces a complex exopolysaccharide with high molecular weight named amylovoran. It is a key factor of pathogenicity. The genes coding for this polysaccharide (*ams*) are located in the chromosome and are arranged in clusters of one or more transcriptional units (Whitfield and Valvano, 1993). A cluster of 12 genes coding for *E. amylovora* amylovoran was described earlier Bernhard *et al*. (1993) and Bugert and Geider (1995). In the present study, chromosomal *ams* gene specific primers (AMSb1 A and AMSb1 B) were used to direct PCR

Table 1: Isolate code, accession number and host plant of *Erwinia amylovora* isolates used in this study

Isolate code*	Accession No.	Host plant
Ea1	HG423347	Pear
Ea2	HG423348	Pear
Ea3	HG423349	Pear
Ea4	HG423350	Apple
Ea5	HG423351	Pear
Ea6	HG423352	Pear
Ea7	HG423353	Pear
Ea8	HG423354	Apple
Ea9	HG423355	Pear
Ea10	HG423356	Apple
Ea11	HG423357	Pear
Ea12	HG423358	Pear
Ea13	HG423359	Pear
Ea14	HG423360	Apple

*Isolates Ea1, Ea2 and Ea3 are reference isolates while isolates Ea4-Ea8 and Ea10-Ea12 as well as Ea14 were isolated from Behera, Kafr El-Dawar. Isolates Ea9 and Ea13 were isolated from Borg Al-Arab

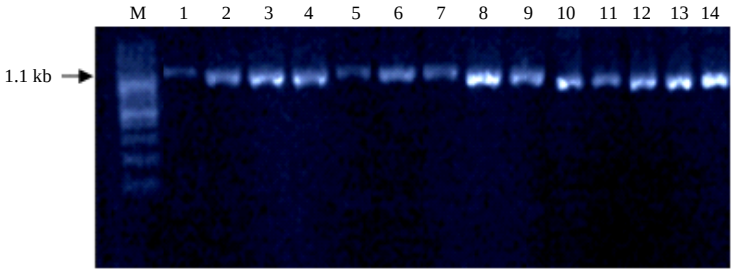

Fig. 1: Agarose gel (1.5%) electrophoresis illustrating PCR products based on pEA29 plasmid specific primers for 14 *Erwinia amylovora* isolates. Lane 1-3: Reference pear isolates, (Ea1, Ea2 and Ea3, respectively). Lane 4-7: Correspond to the apple isolates, (Ea4, Ea8, Ea10 and Ea14, in the order given). Lanes 8-14: Pear isolates, (Ea5, Ea6, Ea7, Ea9, Ea11, Ea12 and Ea13, respectively). M: A-100 bp DNA marker ladder. The position of 1.1 kb is indicated

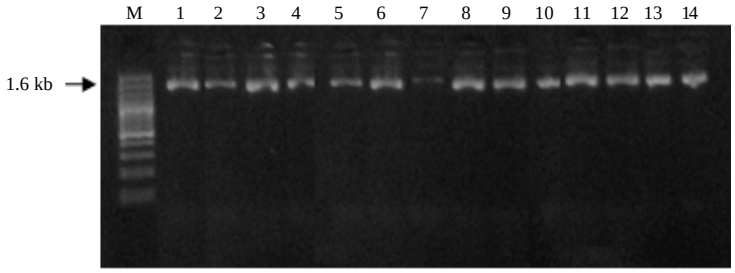

Fig. 2: Agarose gel (1.5%) electrophoresis showing PCR products utilizing *ams* gene specific primers for 14 *Erwinia amylovora* isolates. Lane 1-3: Reference pear isolates, (Ea1, Ea2 and Ea3, respectively). Lane 4-7: Correspond to the apple isolates, (Ea4, Ea8, Ea10 and Ea14, in the order given). Lane 8-14: Pear isolates, (Ea5, Ea6, Ea7, Ea9, Ea 11, Ea12 and Ea13, respectively). M: A 100 bp DNA marker ladder. The position of 1.6 kb is indicated

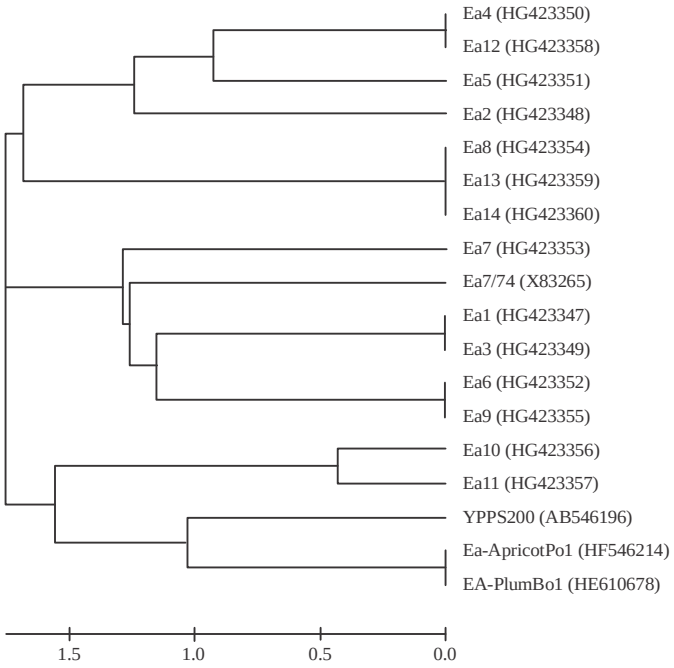

Fig. 3: Phylogenetic tree constructed upon bootstrap neighbor-joining tree method based on 16S rRNA gene partial sequence of *Erwinia amylovora* isolates. The scale at the bottom indicates linkage distance

reactions. All bacterial isolates from apple and pear orchards produced PCR products that have same length 1.6 kb (Fig. 2). Data is in agreement with earlier reports (Bereswill *et al*., 1995).

Identification of *E. amylovora* through PCR amplification of the 16S rDNA gene: The region of the 16S rRNA gene (approximately 1550 bp) was amplified for all the 14 *Erwinia* isolates utilizing the universal primers P0 and P6 as stated in materials and methods. The obtained amplicons were purified and sequenced using ABI PRISM model 310 automated DNA sequencer. The BLAST search (http://www.ncbi.nlm.nih.gov) revealed that, the nucleotide sequences of all apple and pear isolates were identical to those of *Erwinia amylovora*. The homology of the Egyptian *Erwinia* isolates to the Genbank strains reached 98% or above. All the sequences were submitted to Genbank with accession numbers as illustrated in Table 1.

Alignment and phylogenetic analysis: Alignment of the 16S rRNA sequences of *E. amylovora* isolates with the 16S rRNA sequences of other *Erwinia* strains collected from the GenBank was carried out utilizing CLUSTAL W (1.82) (http://www2.ebi.ac.uk/clustalw; Thompson *et al*., 1994) at which MEGA version 5.1 (Tamura *et al*., 2011) was used to generate the Bootstrap neighbor-joining tree (Fig. 3). Data illustrate that, there are interferences between the present *E. amylovora* isolates and the identified *E. amylovora* strains based on constructed phylogenetic analysis.

CONCLUSIONS

Our study was carried out to investigate fire blight disease of apple and pear and its causal pathogen, *Erwinia amylovora*. Symptoms were recognized and eleven isolates were obtained from different locations in Egypt. All isolates were shown to belong to *E. amylovora* based on morphological, cultural, biochemical, pathological and molecular characteristics including PCR analyses and sequencing of 16S rRNA gene. This study is the first identification and characterization of *E. amylovora* isolated from apple in Egypt.

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