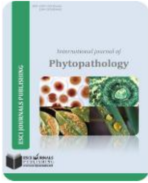

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ANTIBACTERIAL ACTIVITY OF ANTAGONISTIC BACTERIA AND PLANT EXTRACT ON *ERWINIA AMYLOVORA* THE PATHOGEN OF FIRE BLIGHT DISEASE IN EGYPT

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A B S T R A C T

The blossom blight phase of fire blight disease on pear trees, caused by the bacterium *Erwinia amylovora* (Burrill), was typically managed by applying the antibiotic, streptomycin sulfate and copper, to trees during blossom. Biological control agents of fire blight can be achieved by applying nonpathogenic bacteria, viz. *Bacillus subtilis* or *Pantoea agglomerans* and plant extract, viz. Harmel (*Peganum harmala* L.) during open flowers as spraying treatments. The objective of this study was to examine the alternative bactericides against bacterium *E. amylovora* in vitro and in vivo during two seasons (2013-2014) in Al-Gharbia governorate, Egypt. Our results revealed the ability of these antagonistic bacteria and plant extract can decreased fire blight severity on pear trees. Further studies at different locations in Egypt with large scale application would allow us to make stronger recommendations including their ability to prevent disease and used them as main component in integrated pest management program.

Keywords: Biological control agents, fire blight, *Erwinia amylovora*, antagonistic bacteria, plant extract.

INTRODUCTION

Pear trees cultivated area in Egypt are 3741 (Ha), with production 66403 (tones), according to FAO (2013). *E. amylovora* (Burrill) Winslow *et al*. (1920), the causal agent of fire blight disease (FBD) on fruit tree of pear was one of the most destructive pathogenic bacteria. The spreading direction of *E. amylovora* in Europe was from the south. In the early 1980s, rainy weather during flowering contributed to an epiphytotic outbreak in Egypt (where it occurred for the first time in 1962-1964). In Cyprus, it occurred in 1984 and in Israel and Turkey in 1985. Once *E. amylovora* had established in the triangle Egypt-Cyprus-Israel, it was only a matter of time before it appeared in neighboring countries. FBD was very soon observed in Lebanon, Jordan and Iran. From Turkey, it reached Greece (1986) over Crete, and from Greece, it arrived in the SFRY (Socialistic Federal Republic of Yugoslavia) (Bonn and Van der Zwet, 2000). According to EPPO (2012), *E. amylovora* is currently present in more than 50 countries in the world. Suppression of the blossom blight phase of fire blight is a key point in the management of the destructive and increasing important disease of pear (Sanaa *et al*., 2012). Chiriac and Ulea (2012) found that six of the eleven plant extracts tested in vitro were effective against *E. amylovora*. Biological control of fire blight can be achieved by applying bacterial antagonists onto the flowers before a sizable epiphytic population of *E. amylovora* is established. Biological control agents (BCAs) are now commercially available (Smits *et al*., 2010). However, the effective of BCAs products for fire blight control was generally low and highly variable (Ngugi *et al*., 2011; Sundin *et al*., 2009). Therefore, there is still a need of new species and strains of BCAs with novel mechanisms of action and fulfilling the current strict authorization requirements in most countries for microbial bio-bactericides (Montesinos and Bonaterra 2009). The objective of this research was develops the BCAs, as biotic (*P. agglomerans*; *B. subtilis*) and abiotic (Harmel seed extract) to investigate the capacity of bio-bactericides to control infection of blossoms by *E. amylovora* under commercial pear orchard conditions.

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MATERIALS AND METHODS

Plant Samples Collection: Diseased pear flowers, leaves and fruitlets showing necrotic/cankers characterizing symptoms of fire blight were collected from trees of Al-Gharbia governorate fields (Abo Serena village, Samannoud district, Al-Mahallah Alkubra city) during 2012 season and used for isolation of the causative bacterium.

Isolation, Identification and Confirmative Pathogenicity Test: The pathogen was isolated from samples of natural infected according to Miller and Schroth (1972). The plates were incubated at 28°C. Bacterial single colonies developed within almost 2-3 days of incubation then transferred to King's B agar slants. Unripe fruitlets of pear were surface sterilized with ethanol then distributed in sterilized plates and inoculated by pricking with needle dipped into the bacterial suspension (10^8 cell/ml) for each isolates tested. Each plate containing four inoculated fruitlets were maintained with high humidity with wet cotton and incubated at 28 °C for 3-5 days. Negative control pear fruitlets were inoculated with Sterile Distilled Water (SDW). Inoculated fruitlets were examined visually after 3-5 days for degree of necrosis and copious bacterial oozing (Schaad, 1980). In all treatments, re-isolation was carried out from the infected plant material for each pathogenic isolate.

Disease severity on the inoculated fruit estimated depending on the grade of fruits necrosis and oozing, using scale of four grades as show: (0= no necrosis, no oozing, 1=1-20% weak, slight necrosis and oozing, 2= 21-40% moderate necrosis and oozing,3= 41-60% high necrosis and oozing and 5= 61-100% blackening necrosis and oozing). Disease severity index was estimated according to Westwood (1978) as follows:

$$\text{DSI\%} = \Sigma \frac{(\text{Class} \times \text{No. of fruits in class}) \times 100}{\text{Total No. of fruits} \times 4}$$

The bacterial isolates had been selected which display a high virulence test inoculation in laboratory experiments were performed with mixture of these isolates at concentration of 10_8 cfu/ml. The isolates of *E. amylovora* were identify according to their morphological cultural and biochemical characteristics (Krieg et al., 1994).

Preparation of Plant Extracts: The dried seeds of Harmel plant (*Peganum harmala* L.) were obtained commercially from markets of herbs. Two methods of extraction were used. In method (i) the ground powder from seeds (10g) was soaked in sterile distilled cold water (100 ml) for 18 hr on a shaker at room temperature (Meyer *et al*., 2006). The extract was filtered through filter paper Wattman No. (1), then sterilized through (0.2 µl) porosity mill pore filter (Glman Sciences Inc). The sterile aqueous extract was kept in sterilized flask under a refrigerator conditions for experimental purpose. In method (ii) this was carried out by boiling the ground powder from seeds (10g) with 100 ml of sterile distilled cold water. The flasks then put in a water bath at 100 °C for 20 min. The extract was filtered, sterilized and kept as mentioned before.

Screening Harmel Plant Extract as Antibacterial Activity *In-vitro:* The antibacterial assay was performed using the agar disc diffusion method (Bauer *et al*., 1966). The suspension of both most virulent pathogenic bacteria isolates 1 and 3 (10^8 cfu/ml) were mixed with sterilized King's B agar medium (2% v/v) and poured into 9 cm diameter Petri dishes. After solidification, the treated paper discs were placed in the center of plates, with three replications for each treatment. Sterilized distilled water discs were served as control. The plates were kept at room temperature for 1h to allow diffusion of extract into the agar. The plates were incubated at 28 °C for 48 hrs and the inhibition zone diameter was measured to the nearest mm (Ruddock *et al*., 2005).

Isolation and Identification of Biotic Agents: Phylloplane and rhizosphere samples were collected from healthy pear tree grown in infested pear orchards at Al-Gharbia governorate. The method of isolation was according to Shaheen (2010). The bacterial cultures isolated from phylloplane and rhizospher of pear tree were used to assay their antagonistic effect against *E. amylovora*. These cultures were identified according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), Aly *et al*. (1996).

Effect of Biotic Agents on Growth of *E. amylovora:* All isolates were tested for their ability to inhibit the growth of *E. amylovora* on KBA medium. A loopful of bioagents (24 hr old culture) was placed at the center of the plates containing medium previously seeded with *E. amylovora* using appropriate amounts of 24 hr old broth culture as inoculum. The plates were incubated at 28°C for 72 hr. The diameter of inhibition zone was measured as mentioned before.

Field Trials: Field evaluation of biotic bioagents *B. subtilis*, *P. agglomernas* and plant extract Harmel as abiotic agent for control of fire blight disease in pear caused by *E. amylovora* were established at Al- Gharbia governorate. Disease severity was frequent during 2013-

2014 seasons, on fifteen years old common pear trees cultivar Le-Conte in Egypt. The bioagents cell suspension was adjusted at 10^8 cfu/ml; while harmel adjust at 10%. Bioagents were sprayed at blooming period with four spraying (two weeks between each other) and three replicates of trees were included in each treatment. Control trees were treated with water as negative treatment (-). While, Starner 20% WP, applied at 1.50 g/L rates (Oxolinic acid, Sumitomo Co.) as positive treatment (+). The severity of infection was recorded two weeks after the last application of the treatments according to the number of pear fruits stablished on trees compared with untreated trees. Disease severity Index (DSI %) was calculated as following: (Westwood, 1978).

$$\text{DSI\%} = \Sigma \frac{(Class\ \times No.of\ fruits\ in\ class) \times 100}{Total\ No.of\ fruits\ \times 4}$$

Disease severity was rated according to a class from 0 to 4 as following: 0: > %75 fruits stablished, 1: 75% fruits stablished, 2: 50% fruits stablished, 3: 25% fruits stablished and 4: < 25% fruits stablished. The percentage of fruits stablished, disease severity and the effective of treatments were calculated. Experimental design was in randomized complete blocks and the differences between treatments of DS% were determined at 5% significance level.

RESULTS

Isolation, Identification and Confirmative Pathogenicity Test: The colonies of the bacterial isolates showed reddish-orange colored with deep orange in the center on Miller and Schroth (MS) medium. The isolates obtained from infected pear trees were used in inoculation of pear fruitlets; all tested isolates were able to infect pear fruitlets after 4 days. Symptoms appeared as necrotic black area with ooze drops on inoculated wounds. Results in Table 1. Indicated that, isolates Ea1 (60% necrosis and 60% oozing) and Ea3 (60% necrosis and 40% oozing) the most virulent on fruitlets, respectively. Followed by Ea6 (60% necrosis and 20% oozing), Ea4 (40% necrosis and 40% oozing), Ea5 (40% necrosis and 20% oozing) and Ea2 (40% necrosis and 20% oozing), respectively.

Table 1. Effect of *E. amylovora* isolates on fruitlets of pear.

Isolates	Symptoms	
	Necrosis%	Oozing%
Ea1	60	60
Ea2	40	20
Ea3	60	40
Ea4	40	40
Ea5	40	20
Ea6	60	20
Control	00	00

Identification of the Pathogens: The isolated organism's confirmed to the characteristics of *E. amylovora* were designated as Ea1 to Ea6. No morphological variation between isolates was recorded. Results of the physiological and biochemical characteristics are shown in Table 2 which indicated that all isolates of *E. amylovora* were short rod, non-sporulated and gave negative results for gram staining, nitrate reduction, oxidase test, indole formation, produce H_2S from cysteine and growth at 36°C.

Table 2. Morphological and biochemical Characteristics of *E. amylovora* isolates.

Characteristics	Isolates					
	Ea_1	Ea_2	Ea_3	Ea_4	Ea_5	Ea_6
Cell shape	SR	SR	SR	SR	SR	SR
Sporulation	-	-	-	-	-	-
Gram staining	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Indole formation	-	-	-	-	-	-
H_2s production	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+
Growth at 36°C	-	-	-	-	-	-
Levan production	+	+	+	+	+	+
Acid production from:						
Glucose	+	+	+	+	+	+
Lactose	-	-	-	-	-	-

(+): Positive reaction- Acid from sugars, (-): Negative reaction- no acid was produced from sugars.

All isolates could liquefy gelatin, levan production from sucrose. On the other hand, all isolates could produce acid within 7 days from glucose, but acid was not produced from lactose.

Testing Harmel Plant Extract against Growth of *E. amylovora In-vitro:* Effect of the two concentrations of harmel plant extracts on growth of *E amylovora* are shown in Table 3.

Table 3. Inhibitory effect from two concentrations of harmel plant extract by different methods against growth of *E. amylovora*.

Solvents	Inhibition zone (mm)	
	Concentrations of harmel plant extract	
	5%	10%
Hot water	3	4
Cold water	6	10
Control	0	0

Cold water extracts at two concentrations (5 and 10%) were the most effective against *E. amylovora* (inhibition zone 6 and 10 mm, respectively). While, the hot water extracts at the same concentrations were less effective (inhibition zone 3 and 4 mm, respectively). In general, cold water extract was showed highest effect compared with hot water extract of harmel in both concentrations.

Isolation and Identification of Antagonistic Bacteria: Forty and thirty isolates of bacteria were isolates from rhizosphere and phylloplane of healthy pear trees, respectively. All isolates were tested against growth of the pathogen in vitro. We collected four bacterial isolates from rhizosphere and phylloplane were the most effective against growth of *E. amylovora*. Data in Table 4 showed that, isolates no. 1 and 2 were identified as *Pantoea agglomerans* isolated from phylloplane. While, isolates no. 3 and 4 were *B. subtilis* isolated from rhizosphere.

Effect of some Bioagents against *E. amylovora:* Data in Table 5 represented the effect of some bacterial isolates on *E. amylovora* growth on agar plates. It was clear that, the highest inhibition zone diameters (18, 14, 10 and 6 mm) were recorded by isolates *B. subtilis* (no. 3), *P. agglomerans* (no. 2), *P. agglomerans* (no. 1) and *B. subtilis* (no. 4), respectively on the pathogen.

Table 4. Cultural morphological and physiological characteristics of four bacterial isolates collected from rhizosphere and phylloplane of pear trees.

Character	1	2	3	4
Shape of cells	SR	SR	SR	SR
Sporulation	-	-	+	+
Colony colour	Y	Y	C	C
Colonal appearance	Slimy	Slimy	Rough	Rough
Gram reaction	-	-	+	+
Fluorescent pigments	-	-	-	-
Hydrolysis of starch	-	-	+	+
Gelatinliquefaction	-	-	-	-
Growth in NaCl				
2%	+	+	+	+
7%	-	-	+	+
Growth at:				
30° C	+	+	+	+
40°C	-	-	+	+
Acid production from:				
Glucose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Fructose	-	-	+	+

Isolates: 1 = *Pantoea agglomerans,* 2 = *Pantoea agglomerans,* 3 = *Bacillus subtilis,* 4 = *Bacillus subtilis*

SR = single rods, C = Creamy, Y= Yellow

Table 5. Effect of some bioagents on growth of *E. amylovora*.

Bioagents	Zone of inhibition (mm)
Pantoea agglomerans (1)	10
Pantoea agglomerans (2)	14
Bacillus subtilis (3)	18
Bacillus subtilis (4)	6
Control	0

Field Trials: Data in Table 6 indicated that, DS% was decreased with the treatment by *P. agglomerans* (36.67 and 33.33% in the first and second season, respectively with % of fruits stablished 63.33, 66.67% and mean of effective 52.27%) compared with control treatment (78.33 and 68.33% DS, respectively with % of fruits stablished 21.67, 31.67%), followed by Harmel treatment (48.33 and 36.67% DS, respectively with % of fruits stablished 51.67, 63.33% and mean of effective 42.04%). While *B. subtilis* was the latest one for decreased disease severity (53.67 and 48.33% DS, respectively with % of fruits stablished 46.33, 51.67% and mean of effective 30.45%). On the other hand, the control positive Starner as bactericide was the most effective for decreased disease severity (21.67 and 18.33% DS, respectively with % of fruits stablished 78.33, 81.67% and mean of effective 72.73%). On the contrary, no significant was found between seasons and the interaction between them.

Table 6. Effect of bioagents and harmel extract on fire blight of pear tree in the field.

Treatments	% of fruits	DS%	% of fruits	DS%	Mean of DS%	Effective %
	Season1		Season2			
Control (C^-)	21.67	78.33	31.67	68.33	73.33 a	--
Control (C^+)	78.33	21.67	81.67	18.33	20.00 e	72.73
Harmel	51.67	48.33	63.33	36.67	42.50 c	42.04
B. subtilis	46.33	53.67	51.67	48.33	51.00 b	30.45
P. agglomerans	63.33	36.67	66.67	33.33	35.00 d	52.27
Mean	--	47.734	--	40.998	44.366	--

DISCUSSION

Fire blight bacterial disease destroyed Le-conte pear cultivar in Egypt year to year (where it occurred for the first time in 1962-1964). All isolates of *E. amylovora* were able to infection pear fruitlets with variation of symptoms. Two isolates Ea1 and Ea3 were the most virulent on fruitlets, respectively. These results are in agreement with (Shaheen, 2010). Abiotic agent *viz*., haramel (aqueous plant extract) was the high effective against *E. amylovora in vitro* at concentration 10%. In fact, the seeds of *P. harmala* L. are rich of some alkaloids like harmaline, harmine and harman which possess antimicrobial properties (Shahverdi, *et al*., 2005). Nassima and Thoraya (2013) mentioned that, the evaluation of the antimicrobial effect of the vegetable extracts of *P. harmala* L. seeds has permitted to affirm that it has an inhibitor power against some pathogenic bacteria tested. In general, cold water extract method was the highest effect compared with hot water method of plant extracts (Arafat, *et al*., 2013). Biotic agents, viz., *B. subtilis* was the most effective *in vitro* against *E. amylovora* compared with *P. agglomerans*. In the field Treatment of *P. agglomerans* was the most effective to decreased disease severity and increased fruit set on pear trees, followed by harmel plant extract. While, *B. subtilis* was the latest one. These results are in agreement with (Sanna *et al*., 2012), they mentioned that, the use of some strains of nonpathogenic bacteria as a biological control against fire blight proved to be useful, cheap and safe methods to reduce shoot, blossom and immature fruit infection. Also, improving fruit quality. Wright *et al.* (2006) found the same result with pantocin antibiotic produced by *Pantoea* isolates and this antibiotic could inhibit enzymes for histidine and arginine biosynthesis in *E. amylovora*. Also, prevention of blossom infections by *E. amylovora* is a key in fire blight administration because the cankers and bacterial ooze originating from blossom infections provide more of the inoculum for secondary phase of the disease, including the infection of shoots and fruits (Stockwell *et al*., 1998). In the recent years, there has been considerable pressure in pest management to reduce chemical pesticides and to look for their better alternatives. (Dubey *et al*., 2011). Our results confirmed the ability of these non-pathogenic bacteria and plant extract can decreased fire blight severity on pear trees. Further studies at different locations in Egypt with large scale application would allow us to make stronger

recommendations including their ability to prevent disease and used them as main component in integrated pest management program.

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