American-Eurasian J. Agric. & Environ. Sci., 8 (5): 588-596, 2010
ISSN 1818-6769

Antagonistic Activity of Some Bacterial Isolates Against *Erwinia amylovora*

[1]*Sanaa S. Kabeil,* [2]*M.I. Fayed,* [2]*M.A. Amar and* [2]*I.M. Al-Far*

[1]Genetic Engineering and Biotechnology Research Institute,
Mubarak City for Scientific Research and Technology Applications,
New Borg El-Arab, Postal code 12934, Alexandria, Egypt
[2]Agricultural Botany Department, Faculty of Agriculture (Saba-Basha),
Alexandria University, P.O. Box 21531-Bolkley, Alexandria, Egypt

Abstract: *Erwinia amylovora* is the causing agent of fire blight, a severe infectious disease of apple and pear fruit trees. Biological control by using epiphytic bacteria against *Erwinia amylovora* has been considered as a safe and environmentally sound alternative to chemical control methods for controlling the disease. Seventy bacterial isolates were obtained from soil and plant samples contaminated with such a pathogen. The preliminary identification of the enzymes producing isolates indicated that only two of them were classified as members of *Enterobacteriaceae* and *Bacilluseae*. These were the isolates *Bacillus mycoida* (B1), *Bacillus cereus* (B2). They were tested and compared with the previously known isolate *Pontoae agglomerans* (WX112), as biocontrol agents. They showed high biocontrol activity. Moreover, the ability of the isolates to produce the extra-cellular inhibitory substances in a liquid culture was examined. The three isolates *Bacillus mycoida* (B1), *Bacillus cereus* (B2) and *Pontoae agglomerans* (WX112) were resistible to heat, protease sensitive and producers of active proteins whose molecular weights were 52,000, 45,000 and 30,000 Daltons; respectively. The three isolates demonstrated superior activity against *E. Amylovora* and could be bacteriocin producers.

Key words: Antagonistic bacteria · Apple · Biocontrol · Fire blight

INTRODUCTION

Fire blight disease, caused by the Gram-negative enterobacterium *Erwinia amylovora*, is a major constraint to fruit production in many areas of the world [1-3]. It is most commonly initiated by epiphytic populations of *E. amylovora* development on blossoms [4], so infection occurs mostly through blossoms and less often through succulent shoots [5,6]. The economic importance of *E. amylovora* ranks high since it infects approximately 75 different species of plants, all in the family Rosaceae. The hosts for this bacterium include apple, blackberry, cotoneaster, crabapple, firethorn (*Pyracantha*), hawthorn, Japanese or flowering quince, mountain-ash, pear, quince, raspberry and serviceberry [3, 5]. Under relatively dry climatic conditions, the bacterium colonizes flower stigma and subsequent rain or heavy dew usually facilitates movement to the floral cup (hypanthium), where infection generally occurs [7]. In Europe, the disease was first detected in 1955 in England and since then it has spread to most European countries [8, 9]. In the Czech Republic, fire blight of rosaceous plants was first observed in 1986. In 2005, the disease spread to all fruit-growing areas [10].

Disease symptoms of *E. amylovora* were described by Aldwinckle and Beer [4], Vanneste [5] and Wilson and Wisniewski [6]. Briefly, during moist periods in the bloom season, spurs, blossoms and twigs may become infected, developing a darker, water-soaked appearance. They turn brown to black and rapidly wilt and die. Infected blossoms frequently are distorted. Tan-yellow bacterial exudate (ooze) often appears where infection occurs. The most obvious symptom on pear or apple trees is the scorched

Corresponding Author: Sanaa S. Kabeil, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab, Postal code 12934, Alexandria, Egypt.

appearance of leaves on affected branches [11, 12]. Fire blight is controlled mainly by burning and rooting out of affected tree [13]. These practices are very costly and can result in substantially reduced fruit production. Therefore, antibiotics have been used against this pathogen in some countries [14]. However, antibiotics pose the risk of inducing resistance to antibiotics in bacteria, which are also pathogenic to livestock [1, 14-16]. Microbial biocontrol of the blossom blight phase of fire blight has been proposed as an alternative to antibiotics [17]. El-Masry *et al.* [18] and Hattingh *et al.* [19] demonstrated that *E. herbicola* Eh252 could be used for the biocontrol of *E. amylovora*, where *E. hebicola* Eh252 was a non-pathogenic epiphytic bacterium that reduced fire blight incidence when sprayed onto apple blossoms before inoculation with *E. amylovora*. *Erwinia herbicola* Eh252 was found to produce an antibiotic-like compound that inhibits the growth of *E. amylovora*. Additional applications with other strains, i.e. A506 and C9-1, were made 24 hr after inoculation with *E. amylovora* in 2004 where the surfactant could breakthrough in 2005 [7,20-22].

The objectives of the present study were isolation of *E. amylovora* suppressive bacteria, especially the active strains against this pathogen. Moreover, characterization of the antimicrobial materials obtained from promising isolated bacteria was performed.

MATERIALS AND METHODS

Indicator Strain: An indicator strain of *E. amylovora* was used in this study. The strain was kindly provided by Prof. Hassan Abd-El-Khare, Department of Plant Pathology, National Research Centre, Giza, Egypt.

Specific Media for Indicator Strain Growth: *Erwinia amylovora* was grown on nutrient agar of composition (g/l distilled water): beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0; agar, 15.0; pH 7-4 and 1.5% (v/v) glycerol. The medium was autoclaved at 121°C for 20 min and *E. amylovora* grown aerobically on nutrient agar slants at 28°C for 36 to 48hrs.

Micro Organisms (Bio-control Agents): A previous survey was conducted aiming at isolating possible biocontrol agents against *E. amylovora* from soil and plant parts contaminated with *E. amylovora*. Seventy bacterial samples were collected from Alexandria, Monofyia and Gharbia governorates, Egypt. Two of these samples were identified and found to contain promising Biocontrol agents based on the following bioassays. *Bacillus mycoida* (B1) was isolated from Gharbia governorate. Biochemical identification was carried out according to Collee *et al.* [23] at faculty of medicine, Alexandria University. Another isolate identified as *Bacillus cereus* (B2) was also isolated from soil samples from Borg-El-Arab City, Alexandria governorate. This latter was identified using the 16S r RNA gene according to Sambrook et *al.* [24]. The sequence analysis was performed using DNA BLAST and the DNA nucleotides sequence and submitted into the Gen Bank under the accession No. GU113138 [25]. A previously identified strain [26], identified as *Pantoea agglomerans* WX112, was also used to test its activity against *E. amylovora*.

Medium for Selection and Purification: Beef peptone glucose (BPG) medium was used for growing the bacteriocin-producing bacteria and to maintain them for short and long term purpose; it contained per liter of distilled water: 3g beef extract, 5g peptone and supplemented with 2% glucose; in addition to 2% (v/v) agar. The medium was adjusted to pH 7.2, then autoclaved at 121°C for 20 min without glucose, which was separately autoclaved at 110°C for 10 min. The glucose at 40% concentration was then added to the medium at 2% (v/v).

Bioassay: The agar well-cut diffusion technique depended on the diffusion of bacteriocin randomly through the agar layer from circular cup cut out from the agar layer. Four wells were punched out from the deep agar medium using clean sterile cork borer (5 mm in diameter) [27]. The base of each well was sealed with a drop of melted sterile water agar (15g/L). A certain volume (100 µl) of cell free supernatant, filtered through a sterile micro-filter membrane (0.22 µm), was pipetted into each well [18]. The plates were left at room temperature for one hour and then incubated at 30°C for 48 h. After the incubation period, clear zone around each well (y) was measured. The square radius of the clear zone around each well (y^2) was divided over the square well radii (x^2) to obtain an arbitrary unit (AU) for the clear zone. In this method, sterile Petri-dishes were poured with a deep BPG agar medium inoculated with 1% (v/v) of *E. amylovora*.

Identification of the Bacterial Strain and Culture: The selected bacterial isolates were identified according to Bergey's Manual of systematic bacteriology [28, 29]. Morphological examination of individual colonies included colony and cell characterization, gram test, spore and acid fast staining. Motility was observed microscopically by the hanging drop technique since the young broth cultures of bacterial isolates were examined by using a high-power dry objective reduced illumination, motility was confirmed by using stab method in semi-solid media after 7 days of incubation. Diffusion of growth was recorded as positive result. Cell culture in liquid BPYG broth medium was inoculated with 1% (v/v) of 24 hours old culture of the enzyme producer strain. It was then incubated at 30°C overnight in a shaking incubator with agitation at 200 rpm. Bacterial cells were collected by centrifugation at 5000 rpm for 10 min at 4°C.

Conventional Methods for Bacterial Identification

Biochemical Testes: Tryptone broth was used as a basal medium for fermentation test. A phenol red 0.01 % was used as an indicator. Fermentation tubes with 1.0 m1 of medium provided with the indicator were prepared and pH was adjusted at 7.5 with NaOH. The medium was sterilized at 121°C for 15 min. One ml sterilized glucose, arabinose, xylose and manitol was transferred to 10-ml-sterilized-glass tubes and inoculated in duplicate with fresh culture for each of the tested bacterial isolates under sterilized conditions, then incubated at 37°C for 72 hrs. Biochemical and physiological identification were carried out as described in Bergey's Manual of Systematic Bacteriology [30].

Catalase Test: One drop of 30% hydrogen peroxide was placed on a slide. One loopful of the fresh bacterial culture for each of the tested isolates was taken by a sterile needle and placed on the drop of hydrogen peroxide. Bubble production indicated a positive result [31]. The test was conducted under sterilized conditions.

Hydrolysis of Starch: 10 g soluble starch in 100 ml distilled water was heated in water bath until dissolved; 20ml of this solution was mixed with 100 ml of melted nutrient agar and poured in the Petri-dish after sterilization. A loopful of fresh bacterial culture was picked up by a sterile needle and stabbed onto the agar plate; after 24hrs of incubation at 37°C, the plate was flooded with dilute iodine solution according to Cowan and Steel [31].

Methyl Red Test: One ml of fresh culture for each bacterial isolate grown in glucose phosphate medium was aseptically transferred into a test tube. Five drops of methyl red reagent was added and read immediately. Positive tests indicated light red color and negative had yellow [30].

Indole Production Test: One loopful fresh bacterial culture (24hrs old) was inoculated in peptone broth and incubated at 37°C for 1-3 days, after incubation, Kovac's solution was added and shaken vigorously for one min. A red color in the reagent layer indicated positive reaction [31].

Nitrate Reduction Test: The freshly prepared bacterial cultures were inoculated in sterile nitrate broth in tubes and incubated at 37°C for 24hrs. At the end of incubation 0.1ml of solution (A) was added followed by solution (B) in equal volume [32]. The appearance of pink deep color showed that bacterial isolate reduced nitrate to nitrite.

Voges Proskauer Test (V.P.): One ml of fresh bacterial culture was grown in phosphate peptone medium. Then, 0.2 ml of 40% KOH, 0.6 ml of 5% alpha naphthol in absolute ethanol was added. After 10-15 minutes with vigorous shaking bright orange red color developed if acetyl methyl carbinol was present [32].

Citrate Utilization Test: Slope culture of the bacterium isolated from Alexandria with a 1 inch butt of Simmon's citrate agar was inoculated by streaking over surface with a wire needle and incubated at 37°C for up to 3 days [33].

Further Characterization of the Bacteria

Biochemical Test using api E 20 kit: The bacterial strains that proved to have the ability of inhibiting the indicator strain were identified according to standard international methods [34]. Biochemical test and confirmatory tests using api kits (20 and 50CH) were carried out for identification [35].

Identification using 16srDNA Amplified by Polymerase Chain Reaction (PCR): DNA extraction and PCR amplification of 156srDNA region were isolated from the selected isolates according to Sambrook *et al.* [24], the 16srDNA was amplified by PCR using primers designed to amplify 1500 bp fragment of the 16srDNA region. The forward primer was 5'AGAGTTTGATCMTGGCTCAG3' and the reverse

primer was 5'TACGGYTACCTTGTTACGACTT3'. The PCR mixture consisted of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94°C for 1 min, 55°C for 1 min and 72 °C for 2 minutes. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis [36] (Fig. 1) and the remnant was purified using QIAquick PCR purification reagents (Qiagen). DNA sequences were obtained using 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan), BigDye Terminator Cycle Sequencing. The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment. Molecular phylogeny was performed using BioEdit software [37].

DNA Sequencing: Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger *et al*. [38] was done using 3130 X DNA Sequencer. The sequencing reaction was performed with four different fluorescent labels identifying the ddNTPs, instead of the radioactive labels. The specific emissions were detected and the data were collected for analysis [39, 40]. The thermal cycling mixture was as follows: 8 µl of BigDye terminator mix, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product or plasmid), then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95°C, then 49 cycles of 30 sec at 95°C, 10 sec at 52°C and 4 min at 60°C. The products were purified using special column according to the instruction of the manufacturer. The elute were taken and added high dye formamide with (1:1) volume ratio, run at 95 °C for 5 min for denaturation, shock on ice, then the sample become ready for sequencing in 3130 X DNA sequence and analysis.

Purification and Characterization of Bacteriocin

Ammonium Sulfate Precipitation: BPG broth medium (250ml) was inoculated with 1% (v/v) of 24 h old culture of the tested bacterium; isolated from Gharbia governorate, then incubated at 30°C for 48hrs in a shaking incubator, with agitation at 150 rpm. Bacterial cells were collected by centrifugation at 4,000xg for 10 min at 4°C using high-speed centrifuge (Beckman model JA-20). The cell free supernatant was saturated with 60, 70 and 85% ammonium sulfate. Bacteriocin activity and SDS-PAGE were Determined using the supernatant at 60, 70 and 85% saturation; the precipitate was dissolved in a 25 mM phosphate buffer and then dialyzed against the same buffer overnight with gentle stirring at 4°C [41].

Determination of Protein by Bradford Method: The protein concentration was determined in cell free supernatant and in the precipitate after dialysis using bovine serum albumin standard according to Bradford [42].

SDS-PAGE and Molecular Weight Determination: The purified protein was subjected to SDS-PAGE [43]. Approximately 2 µg to 10µg of purified protein were analyzed by SDS-PAGE under reducing conditions on pre cast Bio-Rad 4 to 20% Tris-HCl gradient gels, using a Mini PROTEAN II electrophoresis system (Bio-Rad) following the manufacturer's instructions. Bio-Rad SDS-PAGE broad-molecular-weight-range proteins were used as a standard. Each sample was applied to a separate well in the slab gel along with a pre-stained SDS molecular weight marker (14-205 kDa).

RESULTS AND DISCUSSION

Bacterial Isolation and Identification: The preliminary identification through morphological, biochemical and physiological tests according to Holt and Krieg [28] and Claus and Berkeley [30] indicated their classification as members of *Enterobacteriaceae* and *Bacilluseae*. Using the methodology of Collee *et al.* [23], a bacterial isolate designated as B1 was motile, short rod, gram negative, positive for tests of catalase, Nitrate reduction, Methyl red, Casein hydrolysis, Vogas proskauer [32], Starch hydrolysis, citrate utilization but negative for lactose, sucrose, indole and urease tests (Table 1). Also, it was non-fermenting for glucose, sucrose, lactose and glycerol. The previous results indicated that the B1 isolate can be identified as a species of the genus *Bacillus*. It is well known that beneficial *Bacillus* antagonize pathogens by producing one or more of a variety of metabolites that induce antibiotics, siderophores and other substances such as cyanide [44]. Species of these bacteria are also efficient spermosphere and rhizosphere which may be considered as an added advantage for potential biocontrol agent [21, 45]. Eventually, such bacteria suppressed the growth of many important plant pathogens [35, 44, 46]. For further characterization, this isolate was subjected to identification to species level using the api 20 E kit as well (Table 2).

Table 1: Biochemical characteristics of *Bacillus mycoida*

Catalase test	Indole test	Nitrate Reduction test	Urease test	Methyl Red test	Lactose fermentation	Oxidase	Sucrose fermentation	Casein Hydrolysis	Vogas proskauer test	Starch Hydrolysis	Citrate utilization test
Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Positive	Positive	Positive

Table 2: Identification of api 20 E kit of *Bacillus mycoida*

Tests using API kit	Glucose	Glycerol	Fructose	Maltose	Lactose	Sucrose
Bacillus mycoida	Positive	Positive	positive	Positive	Positive	Positive

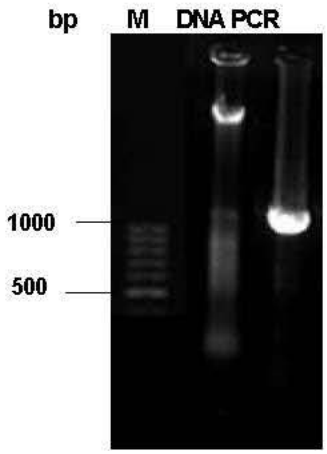

Fig. 1: Gel electrophoresis of fragment 500 bp of *Bacillus mycoida* (B1)

Identification Using 16s rRNA Method: Confirmatory test based on 16S rRNA phylogeny and the sequencing of 500 bp fragment assigned the isolate as *Bacillus mycoida* (B1) Fig. 1 and 2.

Production of the Antibacterial Agent in Liquid Culture: Four different agar diffusion tests were conducted to investigate antagonistic effects of *Pantoea agglomerans* strain WX112 against *E. amylovora* under laboratory conditions. The well-cut diffusion technique was used [27, 47] to detect and determine the induction of the antagonistic agent in the liquid culture [48, 49]. *P. agglomerans* strain WX112, isolated and identified by Kabeil [26] was used as a test organism against brown rot disease, since it has a well defined bacteriocin [21, 26, 50]. Thus, the same tests were carried out using *Bacillus cereus* and *Bacillus mycoida* against *E. amylovora* (Table 3 and Fig. 3). Our results are similar to those reported by Hatice and Bora [51] since our isolates suppressed *E. amylovora* activity using agar well-cut diffusion technique. The isolates were selected and tested for their ability to produce an extra-cellular inhibitory substance in a liquid culture. Thus, the two new isolates showed a positive inhibitory effect when their supernatants were tested against the indicator bacterium *E. amylovora* (Fig. 3); we used the previous isolate

TGCTGCGCACGCCGAGGGGTGATGTCGAGCGAGTCTCTTCGGAGGCTAGCGGCGGACGGG
TGAGTAACACGTAGGCAACCTGCCTCTCAGACTGGGATAACATAGGGAAACTTATGCTAA
TACCGGATAGGTTTTTGGATCGCATGATCCGAAAAGAAAAGGCGGCTTCGGCTGTCACTG
GGAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGC
GTGAACGATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGGGACGAATAAGTACCGTT
CGAATAGGGCGGTACCTTGACGGTACCTGTTTTTTTAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGGATTTATTGGGCGTAAAGCGCGC
GCAGGCGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGGCTCAACCCCGGTTTCGCATCGG
AAACTGTGTAGCTTGAAGTGCAGAAGAGGGAAAGCGGTATTCCACGGTGTAGCGGTGAAA
TGCGTAGAGATGTGGTAGGAACACCGAGTTGACGAAGGCGCTTTCTGGGTCGTGTAACTG
ACGCTGAGCGCGAAGCGTGGGAGCAAACAGGAATAGATACCCTGGTAGTCCGCGCGTAAA
CGATGACGTGCTAGGTGTTGCGGGATTCCATAGCATCAGTGTCTGACCTAAGCCATTAGC
ACTCGCTGGAATGCCTCGAGAAGTGACTA

Fig. 2: Sequence of the fragment 500 bp of *Bacillus mycoida* (B1)

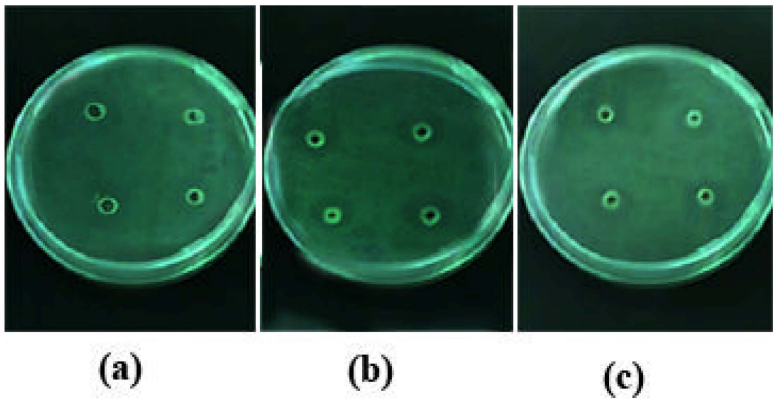

Fig. 3: The inhibitory effect of bacterium-produced substance against *Erwinia amylovora* (a) *Bacillus mycoida* (b) Bacillus *cereus* (c) *Pantoea agglomerans* strain WX11

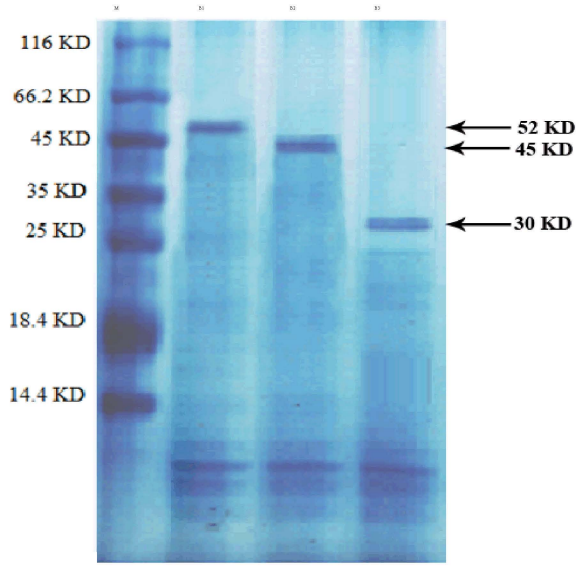

Fig. 4: SDS-PAG of the bacteriocin at 85% saturation ammonium sulfate precipitation level. Lane 1, protein pattern M, molecular mass standard Lane 2, protein pattern at 85% saturation ammonium from *Bacillus mycoida* (B1); Lane 3, protein pattern at 85% saturation ammonium from, *Bacillus cereus* (B2) and Lane 4, protein pattern at 85% saturation ammonium from *Pontoae agglomerans* (B3).

P. Agglomerans for comparison. The three bacterial isolates produced larger and clear inhibition zone demonstrating positive inhibitory effect against the indicator strain *E. amylovora* at AU = 2.2 ± 0.2; AU = 2.6 ± 0.2; and AU = 2.8 ± 0.2 for *Bacillus mycoida, Bacillus cereus* and *P. agglomerans*, respectively (Fig. 3). Wenneker *et al.* [22] and El-Goorani *et al.* [52] also found that the bacterium *P. agglomerans* had a biocontrol activity against *E. amylovora*. From characterization of the antagonistic substance produced by the new two isolates, we found that they are resistible to heat and protease sensitive according to Gross and Vidaver [47]. These two criteria indicated that the previous isolates could be bacteriocin producers [53, 54]. Such results are in agreement with those obtained by Heissenberger *et al*. [21] and Johnson and Stockwell [55]. Van der Zwet and Beer [1] and Zeller and Wolf [56] indicated that another strain of *P. agglomeran* was also capable of protecting blossoms of *Cotoneaster salicifolius* from fire blight and later Laux *et al.* [57] and Grondona and Junge [58] demonstrated that it was also able to significantly reduce the level of fire blight on apple flowers. Such observations were documented in field experimentation under natural and/or artificial infection conditions [14, 57]. Their data corroborate the economic importance of our *P. agglomerans* strain WX11 in the biological control of fire bilge disease in Pear and Apple. On the other hand, the use of *Erwinia herbicola* Eh252 in biocontrol of *Erwinia amylovora* was successful, especially because its specific effect; i.e. *E.herbicola* Eh252 is a non-plant-pathogenic epiphytic bacterium that reduces fire blight incidence when sprayed onto apple blossoms before inoculation with *E. amylovora*. It was found that *E. herbicola* Eh252 produces on minimal medium an antibiotic-like compound that inhibits the growth of *E. amylovora* [18, 19, 59].

Determination of the Molecular Weight of Bacteriocin: In order to determine whether these substances are related to bacteriocin group, the molecular weight of the purified substances was determined by SDS polyacrylamide gel electrophoresis. Thus, the purified

material, after dialyses was applied to 12% SDS polyacrylamide gel [43]. The molecular weight of two bands were calculated from the relation between molecular weight of the standard marker and the relative mobility of the targeted bands from the three isolates *B. mycoida; B.* cereus and *P. agglomerans*. They were found to be 52,000, 45,000 and 30,000 Daltons, respectively (Fig. 3). These weights confirm their relation to bacteriocin group. In practice, most work on the biological control of bacterial plant diseases has aimed to limit the growth and activity of phytopathogenic bacteria at the plant surface (aerial or subterranean), using strains of bacteria that are antagonistic to the pathogen. For a particular disease, development of a successful biological control agent involves initial selection of a suitable antagonist by laboratory (and small-scale field testing at the further investigations) followed by formulation of an effective strategy of application (including both timing and mode of application) with final large-scale field trials to establish cost-effectiveness of the new control measure under agricultural conditions. In conclusion, the three bacterial isolates extracted herein demonstrated superior activity against *E. Amylovora* and could be bacteriocin producers.

REFERENCES

1. Van der Zwet, T. and S.V. Beer, 1991. Fire Blight: Its nature, prevention and control-A practical guide to integrated disease management. US Dep. Agric. Inform. Bull., pp: 631.
2. Zeller, W., 2005. Status of biocontrol methods against fire blight. International Conference on: Biological and pro-ecological methods for control of diseases in orchards and small fruit plantations. Skierniewice, August, pp: 29-31.
3. Gavrilović, V., S. Milijašević and S. Živković, 2005. Characterization of epiphytic bacteria originated from quince and medlar trees and its antagonistic effect against *Erwinia amylovora* (*in vitro*) Berichte Biol. Bundesanst. Land-Forstwirtsch., pp: 128.
4. Aldwinckle, H.S. and S.V. Beer, 1979. Fire blight and its control. Hort. Rev., 1: 423-474.
5. Vanneste, J.L., 2000. Fire Blight the Disease and its Causative Agent, *Erwinia amylovora* Research. Hamilton New Zealand pp: 358. CABI Publishing.
6. Wilson, C.L. and M.E. Wisniewski, 1994. Biological Control of Postharvest Diseases-Theory and Practice. CRC Press, Boca Raton
7. Thomson, S.V., 1986. The role of the stima in fire blight infections. Phytopathol., 76: 476-482.
8. Bonn, W.G. and T. Van der Zwet, 2000. Distribution and Economic Importance of Fire Blight. In Vanneste J. (ed.) Fire Blight: The Disease and its Causative Agent *Erwinia amylovora* (pp: 37-53) CABI Publishing, New York, Wallingford, Oxon.
9. Hdyta, H., C. Bazzi, Susanne Jock, G. Klaus, D. Dermic and B. Cvjetkovic, 2006. Characterization of *Erwinia amylovora* strains from Croatia. European J. Plant Pathol., 114: 435-440.
10. Josef and Šillerová, 2005. Evaluation of epiphytic bacteria for potential control of the fire blight pathogen. Berichte Biol. Bundesanst. Land-Forstwirtsch., pp: 28.
11. Wilson, M. And S. Lindow, 1993. Interactions between the biological control agents *Pseudomonas fluorescens* A 506 and *Erwinia amylovora* in pear blossoms. Acta Hortic., 338: 329-330.
12. Vanneste, J.L. and J. Yu, 1996. Biological control of fire blight using *Erwinia amylovora* Eh 252 and *Pseudomonas fluorescence* A 506 separately or in combination. Acta Hortic., 411: 351-353.
13. Vanneste, J.L., 1995. Pathogenesis and Host-Parasitic Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Bases, Vol. 1: Prokaryotes. Pergamon Press, Oxford, pp: 21-46.
14. Laux, P. and W. Zeller, 2005. Mode of action of the bacterial antagonist *Rahnella aquatilis* against *Erwinia amylovora* 38 Berichte Biol. Bundesanst. Land-Forstwirtsch. 128, 2005. 1st International Symposium on Biological Control of Bacterial Diseases in Darmstadt, Germany, 23rd-26th October, 2005.
15. Beer, S.V., J.R. Rundle and R.S. Wodzinski, 1984. Interaction between *E. amylovora* and E. herbicola "*in vitro*", in immature pear fruits and in apple blossoms. Acta Horticulturae, 151: 203-204.
16. McManus, P.S. and A.L. Jones, 1994. Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathol., 84: 627-633.
17. Beer, S.V., 1990. Fire Blight. In: Compendium of apple and pear diseases. Eds. APS Press, St. Paul. Minnesota, pp: 61-63.
18. El-Masry, M.H., T.A. Brown, H.A.S. Epton and D.C. Sigee, 1997. Transfer from *Erwinia herbicola* to Escherichia coli of a plasmid associated with biocontrol of fire Blight. Plant Pathol., 40: 865-870.

19. Hattingh, M.J., S.V. Bear and E.W. Lawson, 1986. Scanning electron microscopy of apple blossom colonized by *Erwinia amylovora* and *Erwinia herbicola*. Phytopathol., 76: 900-904.
20. Thomson, S.V., S.C. Gouk, J.L. Vanneste, C.N. Hale and R. Clark, 1993. The presence of streptomycin resistant strains of *Erwinia amylovora* in New Zealand. Acta. Hort., 338: 223-225.
21. Heissenberger, B., A. Spornberger, M. Keck, I. Loncaric and H. Foiby, 2005. *In vitro*-studies on fire blight control by bacterial antagonists. 1st International Symposium on Biological Control of Bacterial Diseases in Darmstadt, Germany, pp: 258.
22. Wenneker, M., B. Heijne, T. Deckers, I. Holb, S. Kunz and P. Oosterkamp, 2005. BACTOFRUCT-Development of a biological pesticide against fire blight 1st International Symposium on Biological Control of Bacterial Diseases in Darmstadt, Germany. 78 Berichte Biol. Bundesanst. Land-Forstwirtsch., pp: 128.
23. Collee, J.G., A.G. Fraser, B.P. Marmion and A. Simmons, 1996. Practical Medical Microbiology, 14th Edition. Tests for the Identification of Bacteria. Churchill Livingstone, pp: 131-149.
24. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular cloning A. Laboratory Manual. Cold Spring Harbor Laboratory, NY.
25. Kabeil, S. Sanaa A.A. Sawsan and A.Z. Sahar, 2010. Screening for Hydrolytic Enzymes Produced by Indigenous Bacterial Isolates from Egyptian Soil. IOBC Bulletin: In Press.
26. Kabeil, S. Sanaa, 2005. Production of potent bacteriocin from some soil bacteria and its biological use in controlling *Ralstonia solanacearum Pseudomonas solanacearum* Smith. Ph.D. Thesis, Biotechnology Department, Institute of Graduate Studies and Research, Alexandria University, Egypt. pp: 105.
27. Kekessy, D.A. and J.D. Piguest, 1970. New method for detecting bacteriocin production, Appl. Microbiol., 20(2): 282-283.
28. Holt, J.G. and N.R. Krieg, 1984. Berge's Manual of Systematic Bacteriology, Vol.1, Williams and Wilkins.
29. Holt, J.G., P.H.A. Sneath, N.S. Mair and M.E. Sharpe, 1986. The 8th Edition of Bergey's Manual of Systematic Bacteriology, Volume2, Williams and Wilkins.
30. Claus, D. and R.C.W. Berkeley, 1986. Genus *Bacillus* Cohn 1972, 174A.
31. Cowan, S.T. and K.J. Steel, 1974. Manual for the Identification of Medical Bacteria. Cambridge University Press, pp: 166.
32. Cheesbrough, M., 1985. Medical Laboratory Manual for Tropical Countries. Vol. 2. Butterworrth and Co. London.
33. Hugh, R. and E. Leifson, 1953. The taxonomic significance of fermentative metabolism of carbohydrates by various Gram- negative bacteria. J. Bacterial., 66: 24.
34. Barefoot, S.F., Y. Chen, T.A. Hughes, A.B. Bodine, M.Y. Shearer and M.D. Hugdes, 1994. Identification and purification of a protein that induces production of the Lactobacillus acidophilus bacteriocin lactacin B. Appl. Environ. Microbiol., 60(1994): 3522-3528.
35. Willey, J., L. Sherwood and C. Woolverton, 2008. Prescott, Harley and Klein's Microbiology 7th Edition. McGraw Hill.
36. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. More, J.G. Seidam, J.A. Smith and K. Struhl (eds), 1999. Short Protocols in Molecular Biology. John Willey and Sons, Inc. NY.
37. Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acid. Symp. Ser., 41: 95-98.
38. Sanger, F., S. Nicklen and A.R. Coulson, 1977. DNA sequencing with chain terminating inhibitors. Proc. Nat. Acad. Sci., 74: 5463-5467.
39. Prober, J.M., G.L. Trainor, R.J. Dam, F.W. Hobbs, C.W. Robertson, R.J. Zagursky, A.J. Cocuzza, M.A. Jensen and K. Baumeister, 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. Sci., 238: 336-341.
40. Freeman, M., C. Baehler and S. Spotts, 1990. Automated laser fluorescence sequencing. Biotechnol., 8: 147-148.
41. Spackman, D.H., W.H. Stein and S. Moore, 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., 30: 1190-1205.
42. Bradford, M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". Anal. Biochem., 72: 248-254.
43. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the Head of bacteriophage T4. Nature, 227: 680-5.

44. Marta, Pujol, E. Badosa and M. Emilio, 2006. Epiphytic fitness of a biological control agent of fire blight in apple and pear orchards under Mediterranean weather conditions Published Online Federation of European Microbiological Societies. Blackwell Publishing Ltd., 59(1): 186-190.
45. Broggini, G., B. Duffy, H.J. Holliger, C.G. Schaerer and A. Patocchi, 2005. Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro (R) in a Swiss apple orchard European J. Plant Pathol., 111(2): 93-100.
46. Jock, S., B. Volksch, L. Mansvelt and K. Geider, 2002. Characterization of Bacillus strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora*. FEMS Microbiol. Lett., 211: 247-252.
47. Gross, D.C. and A.K. Vidaver, 1990. Bacteriocins, pp: 245-249, In "Method in phytophology", (eds).) Z. Klement, K. Rudolph and D.C. Sand, Budapest Akademia Kiado.
48. Fravel, D.R., 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol., 26: 75-91.
49. Julien, M. and S.E. Lindow, 2001. Field Performance of Antagonistic Bacteria Identified in a Novel Laboratory Assay for Biological Control of Fire Blight of Pear Biol. Control, 22: 66-71.
50. Kabeil, S.S., E. Hafez, A.S. Daba and M. El-Saadani, 2009. Recombinant Protein for Biocontrol of Brown Rot Disease in Potato. American-Eurasian J. Agric. Environ. Sci., 5(1): 14-19.
51. Hatice, O. and T. Bora, 2004. Biological control of fire blight in pear orchards with a formulation of *Pantoea agglomerans* strain Eh 24. Braz. J. Microbiol., 35(3): 224-229.
52. El-Goorani, M.A., F.M. Hassanein and A.A. Shoeib, 1992. Antibacterial and antifungal spectra of antibiotics produced by different strains of *Erwinia herbicola* (= *Pantoea agglomerans*). J. Phytopathol., 136: 335-339.
53. Vidaver, A.K., 1983. Bacteriocins: The lure and the reality. Plant Disease, 67(5): 471-75.
54. Helmy, M. and G. Pieroni, 2000. RCA60: Purification and characterization of Ricin D isoform from *Ricinus sanguineus*. J. Plant Physiol., pp: 477-482.
55. Johnson, K.B. and V.O. Stockwell, 1998. Management of fire blight: A case study in microbial ecology. Ann. Rev. Phytopathol., 36: 227-248.
56. Zeller, W. and B. Wolf, 1996. Studies on biological control of fire blight. Acta Hort., 411: 314-345.
57. Laux, P., J. Wesche and W. Zeller, 2003. Field experiments on biological control of fire blight by bacterial antagonists.Zeitschriftfur-Pflanzenkrankheiten-Und-Pflanzenschutz, 110: 401-407.
58. Grondona, B., H. Eilers and Junge, 2005. Characterization of an inhibitory strain of *Pantoea* sp. with potential as a biocontrol agent for bacterial plant pathogens. Beate VölkschBerichte Biol. Bundesanst. Land-Forstwirtsch, pp: 128.
59. Vanneste, J.L., J. Yu and S.V. Beer, 1992. Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. J. Bacterial., 174: 2785-2796.