Egyptian Journal of Biological Pest Control, 25(3),2015,739-746
Proceedings of 4th International Conference, ESPCP2015, Cairo, Egypt, 19-22 October 2015

Isolation and Identification of Entomopathogenic Bacteria from Algerian Desert Soil and their Effects against the Migratory Locust, *Locusta* migratoria (Linnaeus, 1758) (Orthoptera: Acrididae)

Oulebsir-Mohandkaci*, H.; S. Khemili-Talbi; F. Benzina and F. Halouane
Laboratory of Valorization and Conservation of Biological Resources, Department of Biology, Faculty of Sciences, University of M'hamed Bougara, Boumerdes, BP35000, Algeria, mohandkacihakima@yahoo.fr
(*Received: October 19, 2015 and Accepted: December 25, 2015*)

ABSTRACT

After isolation and characterization of some bacteria in the rhizosphere soil of the Algerian desert plants, a total of 17 bacterial strains were isolated. They are mobile, aerobic, with a positive catalase. From these bacterial strains, four were selected for their entomopathogenic potential and identified based on their genetic traits. The rDNA16S sequences of these named strains (B3, B4, B5 and B6) were recorded in the EMBL/EBI data bank and their phylogenetic analysis revealed that they belong to the genera; *Pseudomonas*, *Bacillus* and *Enterobacter*. This study was highlighted on their entomopathogenic activity against the 5th instar larvae of the migratory locust, *Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae). The results obtained, one week after treatment, showed that the treated nymphs were highly sensitive to the tested isolates of bacteria, with mortality rates of 100, 98, 71 and 65% for *Pseudomonas* sp. strain B3 (HF911369), *Pseudomonas* sp. strain B4 (HF911366) *Enterobacter* sp. strain B6 (HF911368) and *Bacillus* sp. strain B5 (HF911367), respectively, in comparison to the control (3.33%).

Key words: Isolated bacteria, Entomopathogenic activity, *Locusta migratoria*, soil, mortality.

INTRODUCTION

The soil has long been considered only as a mineral substrate in which the plants are rooted. It was not until the late nineteenth century that we began to see the soil as a living environment (Alvarez *et al.*, 2002). Living fraction of the soil is extremely varied. It is composed of both simple organisms like bacteria and evolved organisms like microfauna (Horner-Devine *et al.*, 2003 and Leake *et al.*, 2004). Soil bacteria are of particular importance for crop production. Various bacteria are able to fix atmospheric nitrogen, while others play major roles in the cycles of certain fertilizing elements (Kowalchuk and Stephen, 2001). Other bacteria protect plants against pests. Most of these bacteria produce antibiotics or toxins that seem to play a significant role in the mechanisms of plant protection (Fuchs, 1999). Moreover, the plant rhizosphere is the soil region nearest to plant root system and directly influenced by roots, it represents the essentially exchange place between the plant and soil. It contains an amount of microorganisms at least 1000 times greater than in the ambient soil not influenced by roots. The microbiological activity in this region is of a great importance and has various consequences for plants (Fuchs, 1999 and Van Loon, 2007).

Indeed, access to these microorganisms in biological control has important ecological benefits deriving from their high selectivity, their infectious or lethal action being limited to only a few target pests and their rapid biodegradation (Greathead *et al.*, 1994). Biological control also allows improvement of the quality of life and health of agricultural workers (Lefort, 2010) and from a commercial point of view, it constitutes a viable alternative to the use of chemical insecticides (Dunphy and Tibelius, 1992). On the other hand, the first difficulty encountered for protecting plants with antagonists is the selection of effective strains. This requires relatively tedious research and applications of bio-tests under controlled and natural conditions.

In this context, the present study focused on isolation and identification of some bacterial strains from land to valorize the best entomopathogenic effectiveness against the locust migratory, *Locusta migratoria* (Linnaeus, 1758) in order to broaden their spectrum of action.

MATERIALS AND METHODS

Sampling:

Three soil samples were taken from the Adrar region in the Algerian desert at the rhisosphere layer of the date palm. Preparation of these samples was performed by the method of Tamietti and Pramotton (1990). After drying and sieving, 10 grams of soil were introduced into an Erlenmeyer flask containing 90 ml sterile physiological water and a sterile glass bead of 2 mm in diameter. The sample was homogenized by agitation

for 15 minutes to retrieve soil bacteria. The resulting suspension is the mother suspension used to make decimal dilutions.

Seeding, purification and storage of bacterial isolates

Ordinary nutrient agar (GN), pH 7.2 was used as isolated bacterial strains environment. 0.1ml of 10^{-1} to 10^{-5} dilutions was seeded on the surface in the isolation medium. The Petri dishes were incubated subsequently at 32°C for 48 hours. The bacterial colonies obtained underwent purification, by performing the necessary successive subcultures. The strains obtained were stored under three different conditions: i) conservation in agar slant at 4°C for a period of about two months, ii) in a freezer at -20°C after liquid culture and in the presence of glycerol (20%, v/v) used as cryo-protector for a longer period, and iii) by lyophilization.

Identification techniques

Identification of bacterial genera was based primarily on the study of several macroscopic characters corresponding mainly to the colonies type. In addition, other microscopic examinations were conducted to determine the form of bacterial cells and their mobility. The identification of isolated species needed physiological traits (highlighting the respiratory type, presence and position of spores and type of Gram) and biochemical (Research enzyme catalase, oxidase, levansucrase and arginine di hydrolase, highlighting the Voges Proskauer reaction (VP), degradation of certain substrates, gelatin, starch, casein, urea, indole, sorbitol, nitrate, mannitol, citrate, tartrate, and the production of lipase) (Guiraud, 2003). These physiological and biochemical tests were performed as described by Bergey *et al.* (1974) and Dong & Cai (2001).

Study of physiochemical properties

Variations in physical factors (pH of the medium and temperature) and chemical factors (salinity) may accelerate, delay or stop the microbial growth (Davet, 1996). Temperature influence on the growth and bacterial metabolism was studied using 4 temperatures 10, 32, 45 and 55°C. The pH was the measure of activity of ions hydrogenes of a solution (Guiraud, 2003). It is known to have a strong influence on the thermal resistance of microorganisms (Davet, 1996). The pH chosen for this study were: 3.5, 4, 6, 7, 8 and 9.5. For salinity, 6 different concentrations of NaCl were performed: 0 mµ (nutrient agar without added NaCl), 100, 300, 500, 1000 and 1500mµ.

Molecular identification technique

The 16S rRNA gene was amplified in three overlapping fractions with the following universal bacterial primers:

Primer	Sequence	Fragment	Size
16S1FOR_0008MF	AGAGTTTGATCATGGCTCAG	16S1	832bp
16S1REV_0800MR	ACCAGGGTATCTAATC		
16S2FOR_0514MF	GTGCCAGCAGCCGCGGTA	16S2	599bp
16S2REV_1073MR	CACGAGCTGACGACA		
16S3FOR_0775MF	GAGCRAACAGGATTAG	16S3	805bp
16S3REV_1540MR	AAGGAGGTGATCCAACCGCA		

The forward primers (FOR) were tailed by the M13-20FOR (CTGTAAAACGACGGCCAGT) primer whereas the reverse ones (REV) by the M13-26REV (AGGAAACAGCTATGACCATG). Two colony PCR trials were performed per sample and per fragment and the obtained amplicons were run on agarose gel, cleaned-up and Sanger sequenced. Sanger sequencing was performed using the BigDye v3.1 chemistry on 3730XL machines (Applied Biosystems; www.appliedbiosystems.com). All obtained amplicons were sequenced from the forward and reverse directions using the universal primer-tailing of the 16S amplification primers. The sequences were base called status with the software program Sequencing Analysis v.5.2 (Applied Biosystems; www.appliedbiosystems.com). All generated chromatograms were assembled per sample using the TraceEditPro software (Ridom Bioinformatics; http://www.ridom.de/traceedit/). The consensus sequences were analyzed by human expert for base calling errors and IUPAC code was applied for any mixed position (originating from the presence of several copies of the 16S rRNA gene for one species). The primer sequences chromatograms were manually trimmed and the resulting consensus sequences were exported in FASTA format. The obtained FASTA consensus sequences were analyzed by similarity searches with BlastN/Megablast against the non-redundant nucleotide database of GenBank retaining the 50 best hits per sample (NCBI GenBank; http://blast.ncbi.nlm.nih.gov). Further on, the degree of statistical support for the branches was determined by 1000 bootstrap replicates. The maximum likelihood trees were estimated by using the rapid bootstrap option (100 replicates) (Tamura *et al.*, 2011).

Treating the fifth instar nymphs (L5) of *Locusta migratoria* by bacterial isolates

Application of biological treatments took place versus the 5th nymphal instar (L5) aged of 48 hrs.

Individuals were injected 20µl of the identified bacterial suspension under metathoracic paw. Controls received the same volume of sterile saline. The nymphs were treated by four bacterial strains selected according to the results obtained after preliminary tests performed on all isolates. Three concentrations were used for each strain; 1.70 mg/ml; 0.72 mg/ml and 0.30 mg/ml; at 30 individuals per concentration, divided into 3 groups, each containing 10 individuals (continuous photoperiod and constant temperature around 30°C). Daily control was performed to determine the number of fatalities. Finally, percentage of observed mortality was corrected relatively to the control according to the Abbot formula (1925).

RESULTS AND DISCUSSION

Macroscopic and microscopic characterization of isolates

A total of 17 bacterial strains were isolated in the collected samples. Appearance of the colonies obtained on agar was very variable. The 17 isolates were aerobic, mobile and with a positive catalase. Microscopic observation permitted their division into two groups, the first consisted of long rods, gram positive, and form a propertied sporal shape and the second contained short gram negative rods and which non sporulating.

Molecular and biochemical characterization

In the present study, three bacterial genera *Bacillus, Pseudomonas* and *Enterobacter* were selected where the main members of the entomopathogenic bacteria belong to these genera (Greathead *et al.,* 1994). Among the 17 isolates, four strains named B3, B4, B5 and B6 were identified as belonging to the three genera according to their molecular analyses of 16S rRNA sequences. The rDNA 16S sequences of these named strains B3, B4, B5 and B6 were recorded in the EMBL/EBI data bank under the accession numbers; HF911369, HF911366, HF911367 and HF911368, respectively. The phylogenic analysis showed that the strains B3 and B4 had a sequence identity higher than 98% with those of the genus *Pseudomonas* (Fig. 1). A significant similarity (based on partial 16RNAr sequence) for possible species relatedness (99%) was found with the validly described species *P. fragi* strain *JCM5420*, *P. syringae* isolate *Lz4W* and *P. psychrophila* strain *HA-4*. The distance matrix indicated that strains B3 and B4 might belong to one of these species. Otherwise, the strain B5 showed a high sequence identity (99%) with the closely related type strains of *Bacillus* species (*B. anthrancis* strain *Ames*, *B. cereus* strain *HN* and *B. thuringiensis* strain *CMBLBT-5)*, which indicated that this strain can belong to one of these species. Further, the strain B6 was similarly closed to the species *Enterobacter ludwigii* strain *EN-119* and *E. aerogenes* strain *MDAZTVIIIR105a* with 99% of sequence identity. However, the complete 16S rDNA sequence analysis and DNA: DNA hybridizations are suggested for an extensive study for the identification of the obtained isolated strains (Table 1).

Cultural characteristics on GN of the strains B3, B4 and B6 showed generally bacterial colonies whose contour was with a regular staining often beige cream. The elevation was convex for all strains with a glossy smooth appearance and varying diameters 1 to 2 mm after 24 hrs of culture. The bacterial strain B5 had smooth medium-sized colonies with an irregular edge. Moreover, the strains B5 and B6 showed positive results for some tests like nitrate reductase and mannitol. The strain B3 showed a positive result for the tests of mannitol and citrate. However, almost all strains B3, B4, B5 and B6 expressed negative results towards hydrolysis test of indole (Table 2).

Presence of *Pseudomonas* bacteria in the rhizosphere can be explained by their rapid growth, ubiquitous nature and ability to adapt to various environmental conditions, which was reported in many studies (Bossis *et al*., 2000). The facility of *in vitro* culture and the ability of some species of this genus of bacteria to produce antibiotics and siderophores in case of iron deficiency confer special characters in the biological control (Mamoun and Olivier, 1989).

Table (1): Percentage identity to sequence of 16S rDNA of the strains B3, B4, B5 and B6 with some species

Bacterial species (Genbank Accession code)	Percentage identity	
	With the strain -B3 (%)	With the strain - B4 (%)
Pseudomonas fragi strain JCM5420 (AB685632)	99	99
Pseudomonas syringae isolate Lz4W (AJ576247)	99	99
Pseudomonas psychrophila strain HA-4 (JQ968688)	99	99
Bacterial species (Genbank Accession code)	Percentage identity with the strain - B5 (%)	
Bacillus thuringiensis strain CMBLBT-5 (AM292032)	99	
Bacillus anthrancis strain Ames (NR 074453)	99	
Bacillus cereus strain HN (JF705198)	99	
Bacterial species (Genbank Accession code)	Percentage identity with the strain – B6 (%)	
Enterobacter ludwigii strain EN-119 (NR 042349)	99	
Enterobacter aerogenes strain MDAZTVIIIR105a (JF513188)	99	

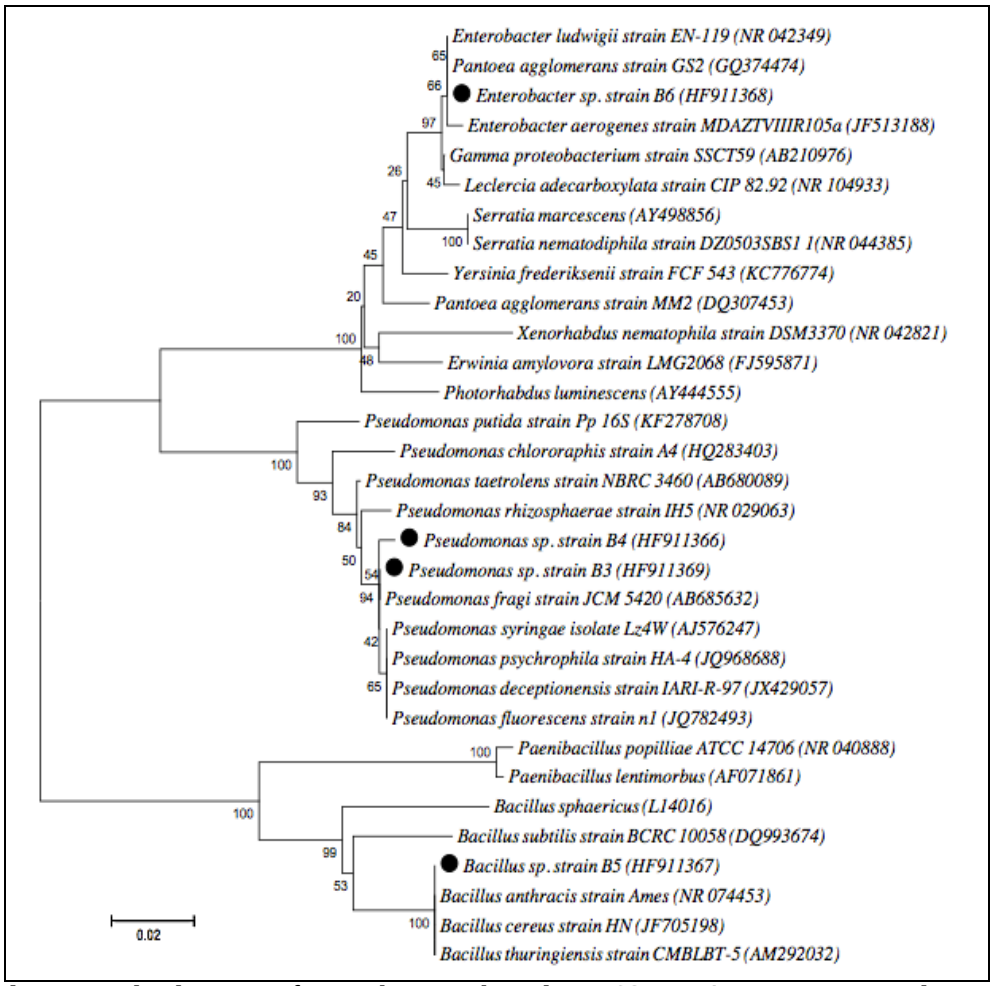

Fig. (1): Phylogenetic dendrogram of some bacteria based on 16S rRNA gene sequence data, indicating the position of the isolated strains B3, B4, B5 and B6 (labeled by✹). The tree was constructed using the neighbor joining method.

Table (2): Physiological and biochemical characteristics of the bacterial strains

Test	Strain			
	B3	B4	B5	B6
Cellshape	BC	BC	BL	BC
Spore	-	-	+	-
Sporale position	ND	ND	T	ND
Gram	-	-	+	-
Respiration	Aer	Aer	Aer	Aer
Catalase	+	+	+	+
Nitrate	-	-	+	+
VP	ND	ND	-	+
Mannitol	+	-	+	+
Motility	+	+	+	+
Starch	ND	ND	-	ND
Casein	ND	ND	+	ND
Gelatin	-	+	-	ND
Urease	ND	ND	+	-

Test	Strain			
	B3	B4	B5	B6
Indole	-	-	-	-
Citrate	+	+	-	+
Acid on Glucose	ND	ND	-	-
Delayedgrowth on glucose	ND	ND	-	ND
Gaz on Glucose	ND	ND	-	+
Culture at 41°C	+	ND	+	ND
Culture at 55°C	ND	ND	+	ND
Culture at 65°C	ND	ND	-	ND
Oxydase	+	+	ND	-
ADH	+	+	ND	+
Levan sucrase	-	-	ND	+
Tartrate	ND	ND	ND	+
Lipase	+	+	ND	ND
Sorbitol	ND	ND	ND	+

+: Positive Response, -: Negative response, BL: long stick, BC: short stick
ND: Not determined, T: Terminal, C: Central, Aer: Aerobic

Several such bacteria are associated with roots, and have been isolated from various plants, for instance the sample isolated from *P. brassicacearum* isolated from garlic plant (Achouak *et al*., 2000) and *P. rhizosphaerae* and *P. lutea* isolated from rhizosphere of grass (Peix *et al*., 2004). Benzina (2004) noted that while a bacterial isolation performed in Jordan soil, the predominance of the species *Pseudomonas fluorescens* and the distribution of species and biovars in the soil were related to the location and nature of crops. In addition, the majority of bacteria of the genus *Bacillus* live in the soil or they persist thanks to their spores. These are bacteria from land as they derive their nutritional needs from organic matter nitrogen and minerals present in the soil (Davet, 1996 and Ashnaei *et al.*, 2009).

Various bacteria of the genus *Enterobacter* were isolated from the soil (Grimont and Grimont, 2006a), among them *E. soli*, Gram-negative bacteria isolated from a soil collected in the Reserve Tambopata in Peru (Manter *et al*., 2011), *E. cloacae* which was able to use the L- asparagine as sole carbon source and nitrogen isolated from soil by Nawaz *et al.* (1998) and *E. sakazakii* (Kim *et al*., 2011).

Physico-chemical properties of selected bacterial strains

Effect of temperature

Results of the effect of different temperatures (10, 32, 45 and 55°C) on the growth of the four strains B3, B4, B5 and B6 (Fig. 2) showed an increase in growth rate at the temperature 32°C. At 45°C, a slight decrease was observed at B5 strain. The three remaining strains, growth decreased significantly at that temperature. Moreover, at 10 and 55°C, bacterial growth was reduced. Heat causes an increase in the rate of metabolic reactions and growth rate, then quickly denaturation of the microbial compounds. The cold causes the slowdown in growth of microbial transformations (Guiraud, 2003). Optimum temperature for growth of most of *Pseudomonadaceae* and *Enterobacteriaceae* was between 30 and 37°C (Larpent, 2000). However, *Bacillaceae* strains exhibited a variation of temperature tolerance. The strain *B. sphaericus* had an optimal growth temperature of 28-35°C (Brossard *et al*., 2008). *B. thuringiensis* could not grow at temperatures below 7°C. *B. subtilis* strain was classified as thermo-tolerant that can develop between 20 and 50°C (Larpent, 2000).

Effect of pH

Results showed an increase in growth rate in the four bacterial strains B3, B4, B5 and B6 till peaking at pH6 for B3 and at pH7 for the other strains (Fig. 3). In pH9.5 there was a remarkable decrease in growth rates of the four strains. Moreover, it turns out that the strain B5 preferred relatively basic media, in contrast to B3 which was tolerant to pH variations. Furthermore, for a given microorganism, the growth rate depending on the pH passed through an optimum. They are often sensitive to pH enzymatic activities which were the limiting factors of microbial growth (Cuq, 2007). In fact, most bacteria can grow on media of pH varies from 4.5 to 9 but reached the optimum with pH typically between 6.5 and 7.5. At pH values below 4 the growth is slow (Guiraud, 2003). Davet (1996) reported that the soil bacteria predominate in neutral soils or slightly alkaline soils. According to Leveau *et al*. (2001) and Brossard *et al*. (2008), the optimum pH for the *Bacillus* species is between 6.8 and 7.5, but these bacteria can withstand a minimum pH of 5.6 and a maximum pH of 9.4 to 10.0. *B. thuringiensis* grows well on nutrient agar at pH 6. It is well known that vegetative cells cannot thrive in an environment of high pH (condition prevailing in the digestive tract of certain insects. Over a PH 10, the alkaline environment was indispensable for activation of toxins (Joung and Cote, 2000). However, *Enterobacteriaceae* including species belong to the genus *Enterobacter*, can grow in the pH range 3.5 to 9 (Guiraud, 2003).

Effect of salinity

Results of different salinity levels (0, 100, 300, 500, 1000 and 1500mμ) on the growth of the four tested strains showed a continuing decline in the growth rate when the content of the NaCl culture medium increased (Fig. 4). This decrease was more remarkable at the strain B3. However, it was noted that the strains studied had a high degree of salt tolerance. According to Dromigny (2008), most bacteria, except those that live in marine waters, grow in the presence of low concentrations of salts. Bacteria, except *Mycoplasmatales*, are insensitive to changes in osmotic pressure because they are protected by their wall. However, some marine species were adapted to media containing about 35g of NaCl per liter (Euzéby, 2007). *B. subtilis* is moderately halophilic, the optimum NaCl concentration in the medium was between 5 and 20% (Guiraud, 2003). Contrary to a nutrient broth containing 4% NaCl, incubated between 15 and 20°C, allowed the multiplication of the genus *Serratia* (a member of the Enterobacteriaceae) but not of *E. cloacae* (Grimont and Grimont, 2006_b). In addition, these isolates had varying degrees of tolerance for the three important physical factors (temperature, pH and salinity) that characterized the Algerian desert environment in which the migratory locust is subservient.

Effect of bacterial isolates on *Locusta migratoria* L5 nymphs

The effect of the strains; *Pseudomonas* sp. strain-*B3* (HF911369), *Pseudomonas* sp. strain-*B4* (HF911366),

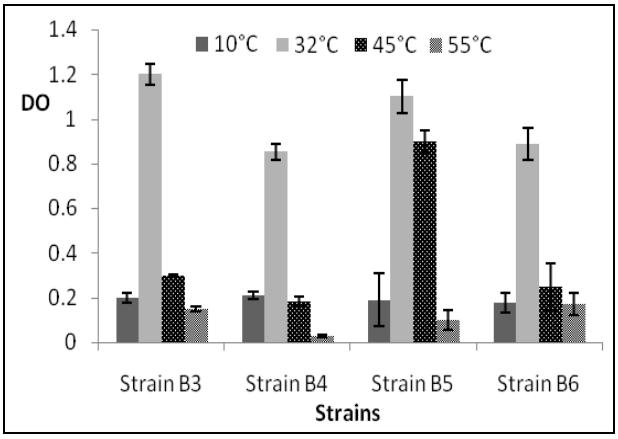

Fig. (2): Effect of temperature on the growth of four bacterial strains B3, B4, B5 and B6.

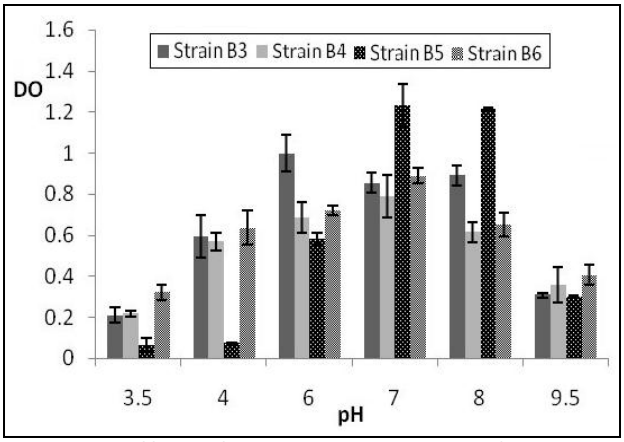

Fig. (3): Effect of pH on the growth of bacterial strains B3 4, B4, B5 and B6.

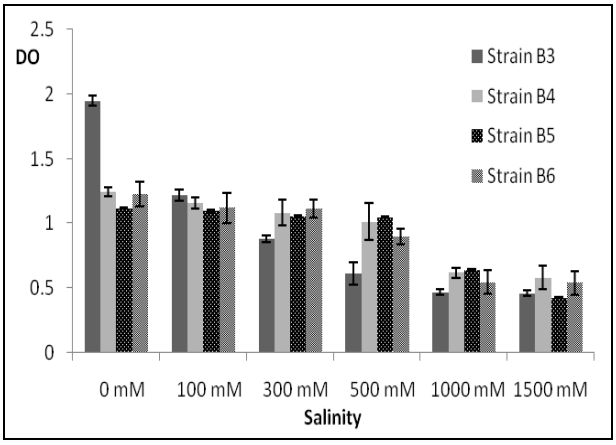

Fig. (4): Effect of salinity on the growth of four bacterial strains B3, B4, B5 and B6.

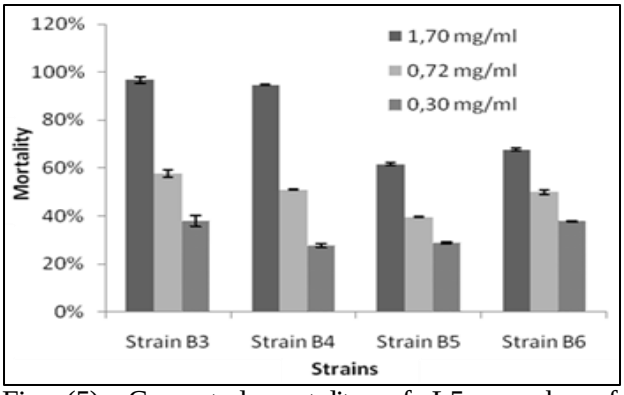

Fig. (5): Corrected mortality of L5 nymphs of *Locusta migratoria* treated by strains B3, B4, B5 and B6 within concentrations C1 = 1.70 mg/ml, C2 = 0.72 mg/ml and C3 = 0.30 mg/ml.

Bacillus sp. strain-*B5* (HF911367) and *Enterobacter* sp. strain-*B6* (HF911368) on the 5th instar nymphs of *L. migratoria* were studied. Results obtained one week after treatment indicated that the treated nymphs were highly susceptible to bacterial suspensions, with mortality rates of 100, 98, 71 and 65% for the strains B3, B4, B5 and B6, respectively after treatment with high concentrations compared to the control (3.33%) (Fig. 5). Also, it is noted that mortality was strongly linked to the applied bacterial concentration. In a subsequent study on treated L5 nymphs of *L. migratoria* by ingestion using *Bacillus* sp. (HE799656), mortality reached 100% after 12 days in opposition to 86.67% for *Bacillus* sp. (HE805963) treatments after 22 days (Oulebsir–Mohand Kaci, 2012). However, Histomorphological altérations were observed in the midgut of Moroccan locust *Dociostaurus maroccanus* larvae after treatment with γ-endotoxins from *B. thuringiensis* (Quesada-Morga and Santiago-Alvarez, 2001). Moreover, under a concentration of 0.50 mg/ml, exotoxins of the strain *Bt* A9 of the species *B. thuringiensis* increased mortality of *Ceratitis capitata* larvae compared to other concentrations tested (Aboussaid *et al.*, 2009). Works achieved by Elzbieta *et al.* (2001) demonstrated that the endotoxin had a significant larvicidal effect which varied from 41 to 81% of mortality according to the *Bt* strain used. The toxicity of the endotoxin observed in *Bt* A9 strain might be due to gene expression (Cry). These genes are responsible for the synthesis of such toxins during the sporulation and influence the activity of *Bt* towards insects (Martinez and Caballero, 2002). According to Abouelghar (2013), Spinosad (a natural substance made by a soil bacterium) had sublethal effects on *Spodoptera littoralis* (Lepidoptera: Noctuidae) that may affect population dynamics in the field via reductions in survival and reproduction.

Finally, obtained isolates could find then their places in biotechnological applications aiming to improving yields and preserving the environment for sustainable development. Although biopesticides based on bacterial

strains are already on the market, their large-scale application has not yet been materialized. Difficulties in formulating and use are among the obstacles that delayed their vulgarization.

ACKNOWLEDGMENT

The authors thank Prof Amina Khemili for her advice and careful reading.

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