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Growth of *Pseudomonas* species isolated from Algerian agricultural soils in the presence of Abamectin

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ABSTRACT

Abamectin is a broad spectrum highly effective acaricide, insecticide, and antiparasitic agent. Studies about the microbial degradation of abamectin are still rare and only three abamectin-degrading bacterial strains were isolated until now. In this paper, we reported the isolation and molecular characterization of bacterial strains isolated from three soils located in eastern Algeria, and the evaluation of their ability to grow in the presence of abamectin. Out of a total of 48 Gram negative and Gram positive bacterial strains identified, seven strains were able to develop on agar in the medium of mineral salt different concentrations of the tested insecticide. The four bacteria showing the best growth capacity were identified as being *Pseudomonas mediterranea* isolate PSB47, *Pseudomonas* sp. OX1, *Pseudomonas migulae* strain OKB3, and *Pseudomonas* sp. clone 6A4 and were tested for their ability to grow in the presence of abamectin as the only energy and carbon sources. In general way, these strains showed a slow but a significant growth visualized by the monitoring of absorbance at 600 nm, suggesting they could be used for bioremediation of polluted soil with abamectin.

Key words: *Insecticides, Bioremediation, Algerian soils, Bacterial growth*

Introduction

The spread of industrialization, the birth of new technologies, the increase in population and the development of agriculture have forced Algeria to improve its agricultural production in order to solve the people's nutrition problems. This was strongly linked to the consumption of huge quantities of pesticides (Ayad Mokhtari, 2012). Avermectins are biological agents made up of a series of naturally occurring macrocyclic lactones, generated by *Streptomyces avermitilis* (Burg *et al*., 1979), with the structure of these compounds has been elucidated (Albers-Schonberg *et al.,* 1981). Abamectin (ABA), also known as avermectin B1a, is a commercial avermectin formulation which has been developed by Merck & Co. Inc. as a high-efficiency, wide-spectrum acaricide, insecticide and pest control agent applied in agriculture or veterinary medicine (Egerton *et al*., 1979; Fisher *et al*., 1992). It is commer-

cially distributed as a mixture of two homologues, avermectin B1a (>90%) and avermectin B1b (<10%). however, the extensive use of ABA has highlighted questions about its persistence in the environment and the hazard of toxicity to non-target organisms (Kolar *et al*., 2008). A study by Kozuh Erzen *et al*. (2005) on the physicochemical properties of ABA showed that once it enters the environment, it can persist for long periods of time at concentrations high enough to exert toxic effects. Abamectin can bind strongly to soil particles and persists for 14 to 70 days in soil. ABA can leach to surface and groundwater where it can cause adverse effects in the aquatic environment due to its toxicity, even at very low doses (Halley *et al*., 1993; Tisler and Kozuh Erzen, 2006).

Escalada *et al*. (2008) reported that ABA cannot be photo-degraded when exposed to ambient light, suggesting that ABA in the environment is therefore primarily due to the biodegradation process. Microbial degradation of ABA is still rare and only three abamectin-degrading bacterial strains have been described so far. The first was the LYH strain of *Bacteroidetes endosymbiote*, isolated by Li *et al*. (2008) from activated sludge from the Heibei Veyong Biochemical Co. Hebei (PRC) sewage treatment plant. The second was a *Burkholderia* sp.GB-01 (eventually identified as *Burkholderia diffusa* species) (Ali *et al*., 2010 a; Ali *et al*., 2013 b) isolated from a field of citrus orchards located in the Chinese province of Jiangxi, which was exposed to the use of Abamectin for many years. The last one was *Stenotrophomonas maltophilia* ZJB-14120 by Wang *et al*. (2015). Bioremediation is recognized as a major and economically viable approach for the elimination of pollutants in the environment. Therefore, among existing treatment techniques, bioremediation using soil micro-flora is of greatest importance for the removal of ABA from the environment because of its cost-effectiveness, the inherent nature of its ecological features, and its ability to completely breakdown ABA *in situ* (Burker and Schnoor, 1998).

The aim of this study was to isolate bacterial strains from agricultural soils located in eastern Algeria, and to evaluate their ability to grow in the presence of ABA as the sole source of energy and carbon.

Materials and Methods

Soil sampling

For this study three stations of agricultural in which they had been exposed to the usage of ABA for long periods; one sample has been taken from the top layer (0-25 cm) of each soil. These stations were located at the Technical Institute for Vegetable and Industrial Crops station (TIVIC), Wilaya El Tarf situated at the extreme east of Algeria (Fig. 1). Samples that served for microbiological analysis were collected in sterilized Erlenmeyer of 500 ml capacity conserved at 4 °C. The soil used for physico-chemical analysis was stored in plastic containers, transported to the laboratory without any special precaution. Samples were air-dried at room temperature, mixed thoroughly, and sieved to 2 mm in size. The soil was immediately used for experiments.

Physico-chemical analyses

The physicochemical analysis included: soil texture and water holding capacity (WHC), soil pH (in water), electrical conductivity (EC), organic matter (OM) and organic carbon (OC), as well as soil particle size. Based on texture analysis using saturation method, soil's water holding capacity has been determined and soils were classified according to

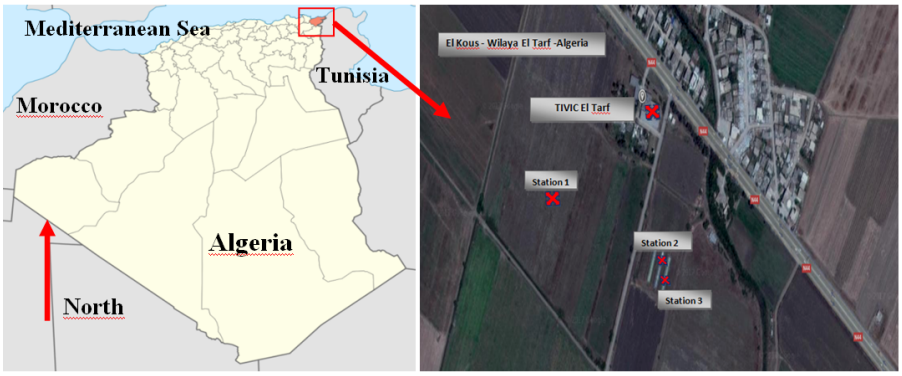

Fig. 1. Localization of sampling stations

Gauchers (1968). The electrical conductivity and pH value of aqueous soil extracts (1:5, w/v) were measured with a glass electrode by a HANNA instrument conductivity and pH-meter respectively at 25°C (Delaunois, 1976; Denis, 2000). The organic carbon and organic matter content were determined by the wet-oxidation method as described by Anne (1945, modified), while particle size of soil was determined using Robinson's pipette method (Dugain *et al*., 1961).

Isolation and, identification of soil indigenous bacterial strains

The isolation of soil indigenous bacteria method was carried out as described by Bhatt *et al*. (2016; modified). Ten grams of each soil sample was added to 0.9% (w/v) sterile NaCl medium (90 ml) and the suspension was shaken vigorously (150 rpm) at room temperature for 30 minutes. Serial dilutions (10^{-1} – 10^{-6}) were prepared using sterilized 0.9% (w/v) sterile NaCl medium. One ml aliquots were transferred into sterile Petri dishes and then kept for 10 min before pouring Nutrient agar or Lauria Bertani agar medium. Plates were incubated at 30 °C±2 °C for 24 hours, and colonies were purified by streaking them onto the respective agar medium from which colonies were isolated.

The identification of bacterial isolates was performed using standard methods such as morphological identification, Gram staining, oxidase, catalase as well as biochemical tests using API 20E, API 20NE, API Staph galleries (BioMérieux Inc., France), as well as molecular method.

The PCR amplification and sequencing of 16S rDNA gene with primers 1492r and 27f were realized as described by Batisson *et al*. (2009). Following multiple alignments of the sequence data using CLUSTAL_X (Thompson *et al.,* 1997), phylogenetic analysis was performed using the MEGA version 5.0 software package. Phylogenetic trees were generated using the neighbor-joining method according to the Maximum Composite Likelihood parameter model and evaluated by bootstrap analyses based on 1000 resembling (Tamura *et al*., 2011).

A Mineral Salt Medium (MSM) adapted from Rousseaux *et al.* (2001) was used for both the strains selection and the biodegradation assays of Abamectin-degrading strains. This medium contained per liter of distilled water: 1.6 g K_2HPO_4, 1.6 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 1 g NaCl, 0.02 g $CaCl_2$, 3.5 g $(NH_4)_2SO_4$, 1 ml of $FeSO_4.6H_2O$-EDTA stock solution (5 g and 7gl^{-1} respectively). $FeSO_4.6H_2O$-EDTA was prepared, autoclaved separately, and then added to the medium after sterilization. The pH was adjusted at 7.5. A solid MSM medium obtained by adding 15 gl^{-1} agar was used to test the capacity of the strains to grow on such solid medium.

Selection of bacterial strains growing in the presence of ABA

The selection test consisted of evaluating the bacterial growth on MSM agar plates supplemented with concentration of Abamectin (MSMA) as the only carbon and energy source. Abamectin was sterilized by 0.45 µm membrane filtration and introduced into medium to get the desired concentrations of 12.5, 25, 50, 100 and 200 mgl^{-1}. The plates were incubated in the dark at 30 °C±2 °Cfor 24 hours to 15 days. The strains were then evaluated as negative and positive growth. Bacterial strains that exhibited a higher growth on MSMA agar media were then, subjected to biodegradation test in MSMA liquid media (Bhatt *et al*., 2016).

Tests of bacterial growth in MSM liquid media

A bacterial suspension of each isolates of interest was realized by transferring the pure colonies from the LB agar dishes to 0.9% sterile NaCl medium (Ali *et al*., 2010). Growth experiments with abamectin as a unique carbon and energy source were performed in 500 ml sterile Erlenmeyer flasks containing 100 ml of sterile MSM broth. Abamectin was introduced in a form of a solution to give the final concentration of 50 mgl^{-1}. The flasks were incubated at 30°C ± 2°C in a shaking incubator at 150 rpm under aerobic conditions and protected from light. Samples of liquid culture medium are periodically removed aseptically every 24 hours to measure the bacterial concentration which has been determined spectrophotometrically by measuring the optic density (OD) at 600 nm using a UV spectrophotometer (Secomam Uviline, 9000). Samples not inoculated with the bacterial suspension served as abiotic controls. All measurements were made in triplicate (Calvayrac, 2011).

Results

Physico -chemical parameters

The stations 1 and 3 have physico chemical properties very close comparing to the second one (Table

Table 1. General characteristics of the soil samples used in the study

Sampling Stations Soil texture classification	Station 1 Loamy Sand	Station 2 Sandy Loam	Station 3 Loamy Sand
Water holding capacity (WHC) (%)	28.10	23.59	28.28
pH (in water)	7,77	7,39	7,81
Conductivity (iSimens/cm)	221	454	223
Organic Carbon (%)	1,42	1,26	1,42
Organic Matter (%)	2,44	2,17	2,44
Clay (<2 µm) (%)	21.44	31.63	20.34
Loam (<2 –50 µm) (%)	71.42	53.97	69.52
Sand (50 – 2000 µm) (%)	7.17	14.40	10.14

1). Unlike the second site which has a sandy-loamy texture (Gauchers, 1968) with a not negligible percentage of sand (Dugain *et al*., 1961), the other two stations have a loamy-sandy texture, a fairly high percentage of silt and poor in the sand. The three sampling stations have an alkaline pH based on the pH range proposed by Denis (2000) and are relatively poor in the organic matter according to Anne (1945). For the conductivity, the three sites are classified as unsalted according to the soil salinity scale by Delaunois (1976).

Isolation, characterization and strain selection

A total of 48 Gram-negative and Gram-positive bacterial strains were isolated from the three stations. Among them, seven strains were able to grow up on

Table 2. Microscopic and biochemical characteristics of isolated bacterial strains

Bacteria	*Pseudomonas mediterranea* isolate PSB47	*Pseudomonas* sp. OX1	*Pseudomonas migulae* strain OKB3	*Pseudomonas* sp. clone 6A
Gram staining	-	-	-	-
Mobility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+/-	+/-
Shape	Short rods, isolated	Short rods, isolated	Short rods, isolated	Short rods, isolated
Biochemical tests	-	-	+	-
Nitrite reduction	-	-	-	-
Tryptophanase	+	-	+	+
Glucosidase	+	+	-	-
Arginine dihydrolase	-	-	-	-
Urease	-	-	-	-
Esculin hydrolysis	-	-	-	-
Gelatin liquefaction	-	-	-	-
β-galactosidase	-	-	-	-
Assimilation of				
Arabinose Mannose	+	+	-	+
Mannitol	+	-	-	-
Maltose	-	-	-	-
N-acetyl-glucosamine	+	-	-	+
Potassium gluconate	-	-	-	+
Capric acid	+	+	+	+
Adipic acid	+	+	-	+
Malic acid	-	-	+	-
Trisodium	+	+	+	+
Citrate	+	+	+	+
Phenylacetic acid	-	-	+	-

+: positive reaction; - : negative reaction; +/-: weak reaction;

MSMA agar supplemented with 50 mgl^{-1} of ABA as the only carbon and energy source. However, four bacteria showing the best growth capacity with 50 $mg.L^{-1}$ of ABA were also able to grow on 200 $mg.l^{-1}$ of ABA. They were thus selected to grow test on MSMA liquid media.

The microscopic and biochemical characterization (Table 2), and the 16S rDNA of these four strains allowed us to identify them as *Pseudomonas mediterranea* isolate PSB47, *Pseudomonas migulae* strain OKB3, *Pseudomonas* sp. OX1, and *Pseudomonas* sp. clone 6A4. Their 16S rDNA sequences were deposited in GenBank database under accessing number: MK792954, MK792955, MK792957, and MK792958 respectively.

The molecular identification allowed us assessing the evolutionary relationship between the four selected strains. The evolutionary distances were calculated using the maximum composite likelihood method and are in units of the number of base substitutions per site (Tamura *et al*., 2011). The positions of codons were included first + second + third + no coding. The optimal tree with the sum of branch length was 0.65428571 (Fig. 2). The phylogenetic tree shows a close relationship between *Pseudomonas* species, with the exception of *Pseudomonas* sp OX1. There were a total of 625 positions in the final data set. Phylogenetic analyses were conducted in 5 MEGA (Tamura *et al*., 2011).

Bacterial growth in MSM liquid media supplemented with ABA

Only three bacterial strains degrading-abamectin have been reported till date: *Bacteroidites endosymbiont* strain LYH (Li *et al*., 2008), *Burkholderia* sp. GB-01 (eventually identified as *Burkholderia diffusa* species) (Ali *et al*., 2010; Ali *et al*., 2013) and *Stenotrophomonas maltophilia* ZJB-14120 by Wang *et al*., 2015. In our study, four strains belonging to the genera *Pseudomonas* capable of using abamectin as the only carbon and energy source by selection on MSMA agar technique were isolated from three soil samples collected from three agricultural fields situated in Algerian east.

Pseudomonas mediterranea isolate PSB47

The growth of *Pseudomonas mediterranea* isolate strain PSB47 with ABA as the only energy source, showed two distinct phases in its kinetics: a latency period of growth of about 144 h, where the OD decreased from 0.157 to 0.064 presumably due to a difficulty in adapting bacteria followed by a second exponential phase whose growth is steadily evolving to reach a maximum absorbance value of 1.155 after 384 h of inoculation (Fig. 3).

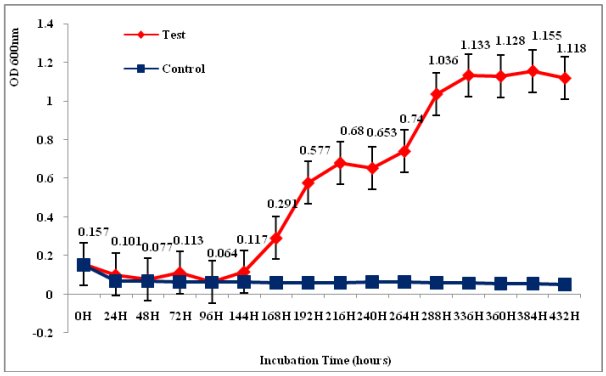

Fig. 3. Growth of *Pseudomonas mediterranea* isolate strain PSB47 in the presence of 50 mgl^{-1} of ABA as a sole source of energy and carbon.

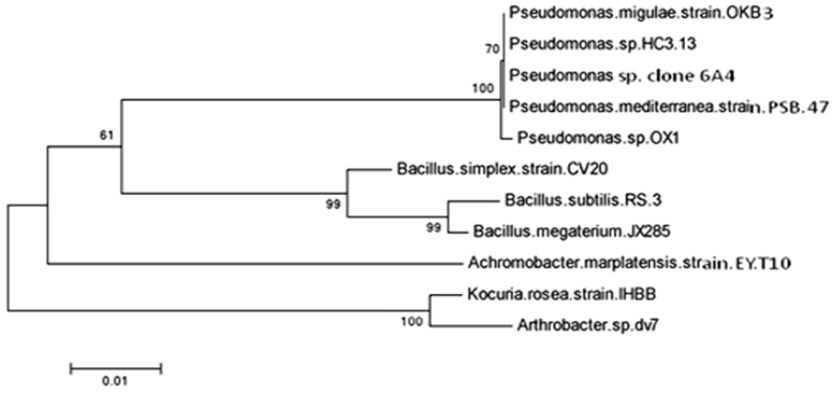

Fig. 2. Phylogenetic tree and Evolutionary relationships between the selected species

Pseudomonas sp.OX1

The latency phase of *Pseudomonas* sp. OX1 with our pesticide as the sole source of carbon and energy was very long (216 h), in which there is a remarkable decrease in turbidity from 0.153 to 0.07 due to a difficulty of adaptation of bacteria. This interval was then followed by an accelerated exponential phase resulting in the significant increase of the absorbance during 72 h, thus reaching a maximum value of 1.029 at 336 h. Then, a decrease was observed until the end of the experiment (480 h) to reach to 0.353 (Fig. 4).

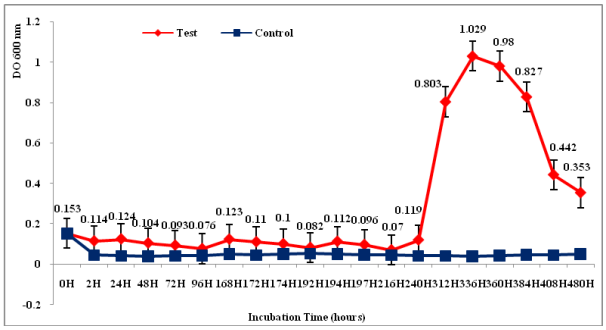

Fig. 4. Growth of *Pseudomonas* sp. OX1 in the presence of 50 mgl^{-1} of ABA as the sole source of energy and carbon.

Pseudomonas migulae strain OKB3

As we can see in Fig. 5, this strain has the slowest growth phases; with latency phase of120 h and an exponential phase in which the growth evolves slowly to reach a maximum value of 0.743 units of OD 600 nm 552 h later.

Pseudomonas sp. clone 6A4

Pseudomonas sp. clone 6A4 revealed relatively short growth phases compared to other tested strains (Fig. 6). We can distinguish a reduced latency phase of the order of 72 h followed by an accelerated exponential division phase whose the absorbance reached 0.777 after 192 h. Then a very slow growth was observed until 408 h of incubation, an $OD_{600\ nm}$ of 0.909 before the start of the decline phase.

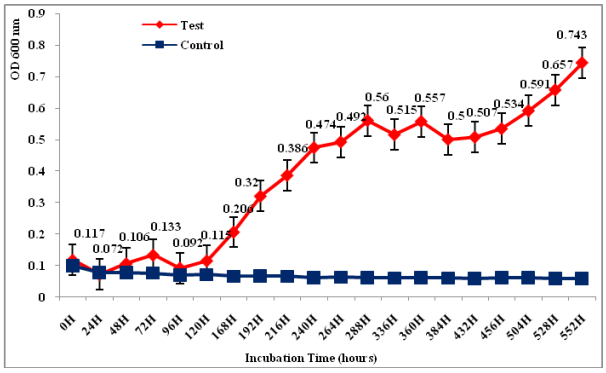

Fig. 5. Growth of *Pseudomonas migulae* strain OKB3 in the presence of 50 mgl^{-1} of ABA as the sole source of energy and carbon.

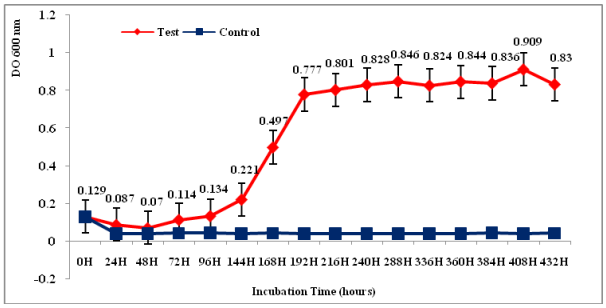

Fig. 6. Growth of *Pseudomonas* sp. clone 6A4 in the presence of 50 mgl^{-1} of ABA as the sole source of energy and carbon.

Discussion

As described above, all four strains exhibited long growing phases in the presence of 50 mgl^{-1} of abamectin as the sole carbon and energy source. Our findings are in line with those of Calvayrac (2011), who, in his study on the biodegradation of sulcotrione, was able to pick a single degrading strain from the seven strains he isolated, *Pseudomonas* sp. 1OP, which, in growth tests on liquid MSM with a 30 mgl^{-1} concentration of herbicide, exhibited greater biodegradation capacity. The isolate showed significant growth visualized by absorbance monitoring. The kinetics appeared in two phases: a lag period of about six days where the OD was stable at 0.3, and a continuous growth phase reaching an absorbance of 0.700, thirty days after seeding. Our data have been validated by the literature concerning the potential of the genus Pseudomonas to degrade a variety of xenobiotics found in the environment, particularly in soils (Calvayrac, 2011). Indeed, several strains belonging to this bacterial genus have been reported to be capable of degrading a number of plant protection products from different families. For example, *Pseudomonas aeruginosa, Pseudomonas fluorescens*, were able to degrade 46-72% of chlorpyrifos as the sole carbon source in an aqueous medium after a 20-day incubation period (Lakshmi *et al*., 2008). According to Hussain *et al*. (2007), *Pseudomonas aeruginosa* was isolated from agricul-

tural soil sites with a history of endosulfan application (Pakistan) and was able to remove 93.7% and 87.2% of α and β-endosulfan, respectively, within 14 days. In addition, *Pseudomonas cepacia* reported its ability to degrade phenol in the presence of toxic substances such as thiocyanate and cyanide (Arutchelvan *et al*., 2005).

The slow growth periods showed by our strains could be explained by the difficulty of adaptation with the active substance, little performing enzymes/genes owned by the four bacteria and the insufficient application of ABA at the level of the land from which it was isolated the bacterium. To more elucidate the obtained results, a study by Devers-Lamrani (2008) explained the general pattern to get an enhanced biodegradation as follows: the first contact of a pesticide with agricultural land translates into an elimination of the active substance after a time period that varies according to the chemical nature of the product; this delay is known as the latency phase. This initial phase represents the capacity of soil microbial populations to adapt physiologically and/or genetically in order to synthesize the set of enzymes required for decontamination. During the second step, a degradation of this molecule is often shown by a significant decline in the initial concentration of the pesticide. A condition for measuring this phase is to have a largely sufficient cell density. The successive and broadly repeated application of the same phytosanitary product, the presence of a large mass of well-adapted microorganisms leads to a shortening or even the elimination of the lag period. On these fields known as "adapted ", 80% of the sprayed pesticide can thus be biodegraded over a two-week period.

Conclusion

In the present study, four *Pseudomonas* degrading-abamectin strains have been isolated and identified from Algerian soils that have been exposed to different families of pesticides as well as ABA. The strains *Pseudomonas mediterranea* isolate PSB47, *Pseudomonas* sp. OX1, *Pseudomonas migulae* strain OKB3 and *Pseudomonas* sp. clone 6A4 were able to grow up on mineral salt agar in the presence of abamectin as the only energy and carbon source at various doses. However, the best growth curves were obtained in liquid medium containing 50 $mg.l^{-1}$ of abamectin indicating that this pesticide could be degraded. Given their growth in the presence of abamectin, these strains could be useful for the bioremediation of soil polluted by abamectin.

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