Bacterial Strains from the Rhizosphere for Remediation of Certain Pesticides

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ABSTRACT

Laboratory and field trials were conducted by cultivating four plants namely Amaranth (*Amaranthus caudate*) Lettuce,*L(actuca sativa*) Water cress (*Nasturtium officinale)* and Kidney bean (*Phaseolus vulgaris)* for monitoring and detecting the probability degradation of some pesticides belonging to different groups and have different uses. These pesticides were carbofuran, abamectin (insecticides), fosthiazate (nematicide), and carbendazim (fungicide). Three bacterial strains were isolated from the rhizosphere area of the cultivated plants soil .These bacteria were characterized and identified as *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Bacillus megaterium*. Certain concentrations of the tested pesticides were added to the media (MSM) of each of the isolated bacteria. Microscopic examination showed that most of the bacterial cells in the used media were found to gather around carbendazim, carbofuran, abamectin and fosthiazate and aggregated in the aqueous phase. The results revealed that *Pseudomonas fluorescens* as a model of bioremediation organism exhibited potential efficacy for biodegradation of the used pesticides. Carbendazim and carbofuran exhibited the most deleterious effect with partial lysis of the bacterial cells. For abamectin and fosthiazate, it was clear that the surface of the bacterial cells became rough and swollen, but unlysed. The obtained results showed that the isolated bacteria from the soil could enhance the plants to increase their ability for the bio-degradation of pesticides.

INTODUCTION

Due to the extensive use of different agrochemicals, there are numerous anthropogenic contaminants today in soils and these agrochemicals are toxic to biological systems, and represent a substantive threat to human health and environmental quality (Philp and Atlas, 2005). Agrochemicals used for plant protection and pest control are ranked as the most widely distributed chemical contaminants for the environment in the twentieth century (Mishra *et al*., 2006). Pesticides are partially eliminated only by biological and chemical degradation (Jacques, 2001).

One of the methods that can remediate pesticide residues in soil is phytoremediation. This method utilizes green plants to clean up contaminants in the environment (Anonymous, 1999). Phytoremediation (Greek: *phyton* = plant; Latin: *remediare* = remedy) is the method that uses plants to clean up or remediate pollutants from the environment such as

soil (Sinsha *et al.*, 2003). Phytoremediation in the rhizosphere increases soil organic carbon, soil bacteria, and mycorrhizal fungi and all factors and others encourage the degradation of organic chemicals in soil. Rhizosphere bioremediation is also known as phytostimulation or plant-assisted bioremediation (Burton *et al.*, 1998).

Not only do certain plants uptake the unwanted materials, but such plants also limit the movement of materials within the soil, while other plants can convert the toxic material into a non-toxic or non-bioavailable form (Taha and Abdallah, 2004). Plants may also release exudates to the soil environment that help to stimulate the degradation of organic chemicals by inducing enzyme systems of existing bacterial populations, stimulating growth of new species that are able to degrade the wastes, and/or increasing soluble substrate concentrations for all micro organisms (Barkovskii *et al.*, 1996, Jordahl *et al.*, 1997).

Studies and research for the selection of certain types of plants to degrade chemical pesticides residues in soil deserves attention. Therefore, this study was conducted to select the plants that possess ability to degrade certain pesticide residues in soil as well as to explain their roles in remediating the soil from these pesticide residues. Moreover, the species of bacteria that plays a role in the bio-remediation precess were detected and identified.

MATERIALS AND METHODS

Experimental design

Laboratory and field trials were conducted by cultivating four plants namely Amaranth (*Amaranthus caudate)*, Lettuce (*Lactuca sativa)* Water cress, (*Nasturtium officinale)* and Kidney bean (*Phaseolus vulgaris)* for detecting the degradation of some pesticides namely carbofuran, fosthiazate, abamectin and carbendazim. The experimental trials were treated with pesticides according to the normal agricultural practices and recommendation guidance of the Egyptian Ministry of Agriculture.

Pesticides used

1. **Nematicide:** Fosthiazate (Nemathorin® 10G)
2. **Fungicide:** Carbendazim (Kemazed® 50WP)
3. **Insecticides:**
    - **3.1.** Carbofuran: (Furadan® 50G)
    - **3.2.** Abamectin: (Vertimec® 1.8 EC)

Soil physic-chemical properties:

Physico-chemical properties of the used soil are shown in Table (1). The data discriminated the soil as an alkaline clay soil. The soil has a clayey texture (73.75%) and high level of soil salinity. The soluble cations and anions content agree with high salinity. The sodium ion content has a slight effect on soil properties. The clayey texture of soil reflects on soil properties especially soil permeability. As the clayey texture increased the soil permeability is restricted. The clayey texture resulted in low soil permeability value and water movement, but the high salinity level can decreased the bad effect of soil clayey texture.

Table (1): Physical and chemical properties of soil used in the experiment

Parameters	value
Particle-size distribution,%	
Sand	11.92
Silt	14.18
Clay	73.75
Textural class	**Clay**
EC (1:2, soil: water extract), dS/m	5.71
pH	8.01
Organic carbon (OC),%	2.32
Sodium Adsorption Ratio (SAR)	5.27
Soluble Cations, meq/l	
Calcium (Ca^{+2})	21.82
Magnesium (Mg^{+2})	12.78
Sodium (Na^{+})	21.25
Potassium (K^{+})	0.86
Soluble Anions, meq/l	
Carbonates ($CO_3^{=}$)	-
Bicarbonates (HCO_3^{-})	5.00
Chloride (Cl^{-})	39.00
Sulphate ($SO_4^{=}$)	13.10

Soil treatments

Approximately 100 kg soil samples were collected from the Faculty of Agriculture (Saba Basha) farm at 15-30 cm depth. To get rid of the pesticides residues before installation of the experiment, the soil was washed with a mixture of three organic solvents (Hexane, acetone and isopropanol) before washing again with distilled water for 6 hrs. Soil samples were air dried by exposing them to the sunlight directly for 12 hrs. Pots with capacity of one kg were filled with the dried soil. Four plants species were cultivated in 20 pots for each. Five treatments (four pesticides plus untreated check) were used upon the soil of each plant (4 pots x 5 treatments x 4 plant species). The pesticides treatments were applied according to the dose recommended by the Egyptian Agriculture Ministry at the same day of plants cultivation. The doses of pesticides were applied according to the calculated soil weight per feddan (the surface depth of 50 cm and area of 4000 m^3). The pesticides rates were 0.7 g of carbendazim, 0.5 g of carbofuran or nemathorin and 0.4 ml of abamectin/1 kg soil. The pots were put inside a green house at 25:27 °C. All the pots were watered daily during the experiment. Untreated checks were non-cultivated treated soil with the pesticide. After two months from cultivation, 80 g of soil were collected from each treatment (from 4 pots) from the rhizosphere area of the plant at 5-10 cm depth. The soil samples were implemented for the extraction and isolation of rhizosphere bacteria.

Extraction, isolation and cultivation of rhizosphere bacteria

The isolation and identification of the isolates were carried out in accordance with Bergey's Manual of determinative Bacteriology (Anonymous, 1974).

Two media were used for the enrichment and cultivation of the isolated bacteria. The media were Luria Betrani medium (LB) and Mineral Salts Medium (MSM). The usage purpose and components of both media were presented in Table (2). These media were used for culturing the bacteria in glass-tubes and in petri dishes. The media were sterilized in an autoclave at 120°C under 15 lbs pressure for 15 min. The enrichment and propagation of the isolates were carried out in sterilized Erlenmeyer flasks containing LB media. The cultivation was carried out in sterilized 100 ml flask containing 20 ml the Mineral Salts Media (MSM). The pH value of the culture solution was adjusted to 7.0 with NaOH. The flasks were tightly sealed with screw caps. Both solidified and liquid culture media were used for study of the bacteria.

After the incubation period of 24 hrs on a rotary water bath shaker at 37°C and 200 rpm, growth was observed in both media. To observe the growth of the colonies on agar media, agar plats were inoculated with 10 µl of the isolates containing medium. Agar dishes were sealed with tape and incubated upside down at 37°C for 24 hrs till the colonies were observed. Well-grown bacterial colonies were picked up with a sterile wire loop and cultured separately in liquid culture tubes. These were numbered numerically. Streaking method was used for making bacterial cultures in plates. After significant cell growth was achieved in the enrichment culture, the bacteria were sub-cultivated in 100 ml Erlenmeyer flasks containing the same media as previously described.

For treating the bacteria isolates, single purified colony of each of *Pseudomonos aeruginosa and Pseudomonos fluorescens* was picked and cultivated again with each pesticide (0.5 g/l of carbendazim, 0.7 g/l of carbofuran, 0.4 g/l of abamectin and 0.5 g/l of nemathorin) containing MSM to evaluate their effect on these pesticides and to carry out the scanning with the electron micrograph. Before adding the pesticides, the isolates were purified for many times until identical colonies in shape and morphological characteristics were obtained. The effect of these isolated strains on pesticides was characterized and identified depending on the changes occurred in the cell wall composition, substrate selectivity and the growth.

Table (2): The purpose and components of both media used in the enrichment and propagation of the bacteria isolates.

Bacterial medium type				
LB*		MSM**		
Usage purpose	Components	Usage purpose	Components	
Enrichment	Yeast extract 0.5 % NaCl 0.5 % Agar 1.0 % Peptone 1.5 %	Bacterial cultivation under stress condition without carbon source (Yeast extract), but with the pesticide itself.	0.30 % KH2PO4 1.28 % Na2HPO4 0.10 % NH4Cl 0.05 % NaCl	

* **LB** = Luria Betrani medium ** **MSM** = Mineral Salts Medium

Scanning electron microscopy for *Pseudomonas fluorescen* as a model treated by abamectin, fothiazate, carbendazim and carbofuran

After the cells were treated with abamectin, fothiazate, carbendazim and carbofuran, the shape of the cells was examined by electron microscopy (Amray Model 1820 Scanning Electron Microscope, UK). The cells were fixed at 24°C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), dehydrated with a serial concentration of ethanol, and then dried on a critical point dryer (HCP-2; Hitachi Co.). The dried cell samples were coated with gold, and examined using a scanning electron microscope (S-4100; Hitachi Co.). For transmission electron microscopy, dehydrated cells were embedded in a medium type LR white resin (Sigma Chemical Co., St. Louis, Mo.), which was polymerized at 60°C for 24 h. The polymerized samples were sliced with an ultra-microtome and observed using a transmission electron microscope (Hitachi Co.).

RESULTS AND DISCUSSION

Isolation and characterization of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Bacillus megaterium* from *rhizosphere* zone

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. The rhizosphere is a centre of intense biological activity due to the food supply provided by the root exudates. Bacteria, actinomycetes, fungi, protozoa, slime moulds, algae, nematodes, enchytraeid worms, earthworms, millipedes, centipedes, insects, mites, snails, small animals and soil viruses compete constantly for water, food and space. Soil chemistry and pH can influence the species mix and functions of microbes in the rhizosphere. Three bacterial strains were isolated from the rhizosphere of the cultivated plants. They were characterized and identified as *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Bacillus megaterium* according to Bergy's manual (Sambrook *et al.*, 1989). Table (3) presents more details about the morphological and phenotypic characterization.

Pseudomonas aeruginosa

This bacterial strain was isolated from rhizosphere of the cultivated plants and further identification and characterization were carried out on

mineral salts medium (MSM) with carbendazim, carbofuran, abamectin and fosthiazate as a carbon source. It was identified as a member of the genus *Pseudomonas* based on physiological characteristics and the morphology of the cells. This gram negative, rod shaped bacterium was 0.3 - 0.7 µm in diameter and 1.2 - 4.0 µm long (Fig. 1). The colonies were smooth on LB media. During the growth on abamectin and fosthiazate, a frothy emulsion was observed in the growth media. Furthermore, one or more protuberances were observed on many colonies. Moreover, the colonies seemed relatively small in case of growth on carbofuran mineral salt medium compared to the normal growth on LB medium.

Fatty acids composition indicated that the isolate belongs to the genus *Pseudomonas* and 16S rDNA sequence was 98 % identical to *Pseudomonas aeruginosa*.

Table (3): Morphological and physiological properties of three bacterial strains

Physiological characteristic	*P. aeruginosa*	*P. fluorescens*	*B. megaterium*
Shape of the cells	rod-shaped	rod-shaped	rod-shaped
Gram reaction	-	-	+
Aminopeptidase	+	+	+
Caprate	+	+	+
Catalase	+	-	+
Citraconate	+	+	+
Citrate	+	+	+
Denitrification (to N2)	-	-	-
D-xylose	+	+	+
Erythritol	-	-	+
Gluconate	+	+	+
Glucose	+	-	+
Hydrolysis of gelatine	+	+	+
Pigments fluorescent	+	+	-
Pyocyanin	-	-	-
Sorbitol	+	+	+
Trehalose	+	-	+
Oxidase	+	+	+
Phenylacetate	+	-	+
NO_2 from NO_3	-	-	-
Arabinose	+	+	+
Benzylamine	+	-	+

+: growth -: no growth

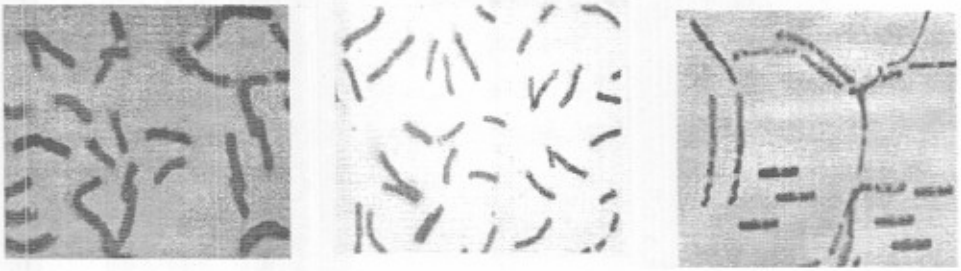
Pseudomonas fluorescens

The Gram-negative bacteria *Pseudomonas fluorescens* were isolated from soil samples collected from rhizosphere region of *Amaranthus caudate, Lactuca sativa, Nasturtium officinale and Phaseolus Vulgaris*. Colonies that showed fluorescence at 570 nm were selected and further purified and characterized. The fluorescent Pseudomonads were identified as gram negative, rod shaped and all produced yellowish green pigment on King's B medium. Fatty acids composition indicated that the isolate belongs to the genus Pseudomonas and 16S rDNA sequence was 99.5 % identical to *Pseudomonas a fluorescens*. These bacteria were successfully enriched in mineral salt medium II.

Pseudomonas fluorescens cells are rods (Fig. 1) and have been classified as strict aerobes. Growth characteristic exhibited the formation of bio-films. It was noticed that significant number of cells can produce surface active agents that can be described as bio-films. Secretion of these bio-surfactants facilitates to assimilate the substrate. Combined with the ability to form bio-films, they are thus able to survive in a variety of unexpected places. This fact have supported our results for the assimilation of carbendazim, carbofuran, abamectin and fosthiazate as carbon sources

Bacillus megaterium

Bacillus megaterium was also isolated from rhizosphere of the *Amaranthus caudate, Lactuca sativa, Nasturtium officinale and Phaseolus Vulgaris* (Fig. 1) and further identification and characterization were carried out on mineral salts medium with supplemented with carbendazim, carbofuran, abamectin and fosthiazate as carbon sources. This bacterial strain was a gram positive, oxidase negative, catalase positive rod and produced creamy secretions on MSM medium. *Bacillus megaterium* is known to be able to survive in some extreme conditions such as desert environments and polluted areas due to the spores it forms. *Bacillus megaterium* has the ability to grow at ambient temperature and its large size facilitates the direct microscopic observation. It is a Eubacteria and is found in the soil, where there are favourable conditions the spores can survive. Colonies form in chains due to sticky polysaccharides on the cell wall. Furthermore, the colonies were beige-red on TSB agar, salmon-red on GYM agar and shiny. The strain produced pigmented circular colonies on pesticides MSM. The isolated strains grew at a range of temperature of 15:25°C. The characterization showed that the 16S rDNA of the isolates had 97.6% identity to the 16S rDNA sequence of *Bacilli*.



P. Fluorescens *P. aeruginosa* *Bacillus megaterium*

Fig. (1) Isolated bacteria from the *Rhizosphere regions*

Growth characteristics of *Pseudomonas aeruginosa* on carbendazin, carbfuran, abamectin and fosthiazat

Microscopic examination exhibited the ability of *Pseudomonas aeruginosa* in assimilating carbendazim, carbofuran, abamectin and fosthiazate (Fig. 2). This microscopic micrograph showed the direct interfacial accession represented in the close direct contact of the cells to carbendazim, carbofuran, abamectin and fosthiazate. This led to the increase in bioavailability and subsequent biodegradation of the pesticides. Microscopic examination showed that most bacterial cells were found around carbendazim, carbofuran, abamectin and fosthiazate or as aggregates in the aqueous phase.

The growth characteristics of *Pseudomonas aeruginosa* on carbendazim, carbofuran, abamectin and fosthiazate were peculiar. After the bacterial attack, the medium and the pesticides droplets became darker with increase of the culture age. Furthermore, carbendazim was totally degraded after 5 days post treatment. Moreover, the microscopic examination showed only the growth of the colony on the pesticides droplets with dark colours and the droplets totally disappeared after 10 days (Fig. 2).

One of the observations that should be taken into consideration was that during the subcultivation of the pure strain, the capabilities of attaching, biodegradation and utilizing carbendazim, carbofuran, abamectin and fosthiazate were not lost. Moreover, the sub-cultivation on complex medium and cultivation again on the previously mentioned pesticides MSM did not affect the activity and efficiency of *Pseudomonas aeruginosa*.

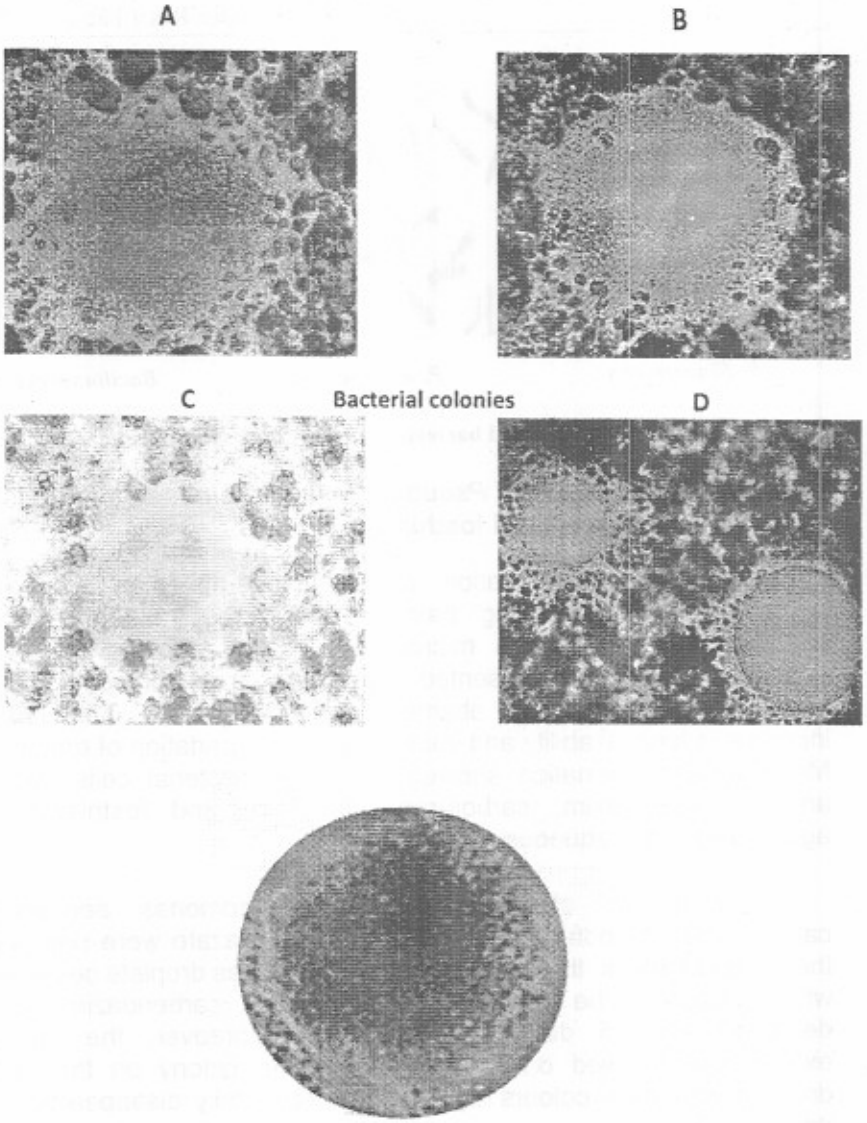


Fig. (2): Fluorescent photomicrograph of *Pseudomonas aeruginosa* growth after 5 days on pesticides mineral salt media (MSM): A= Abamectin, B= Fothiazate, C= Carbendazim, D= Carbofuran, E= untreated.

Scanning electron micrograph of the *Pseudomonas fluorescens* with the used pesticides

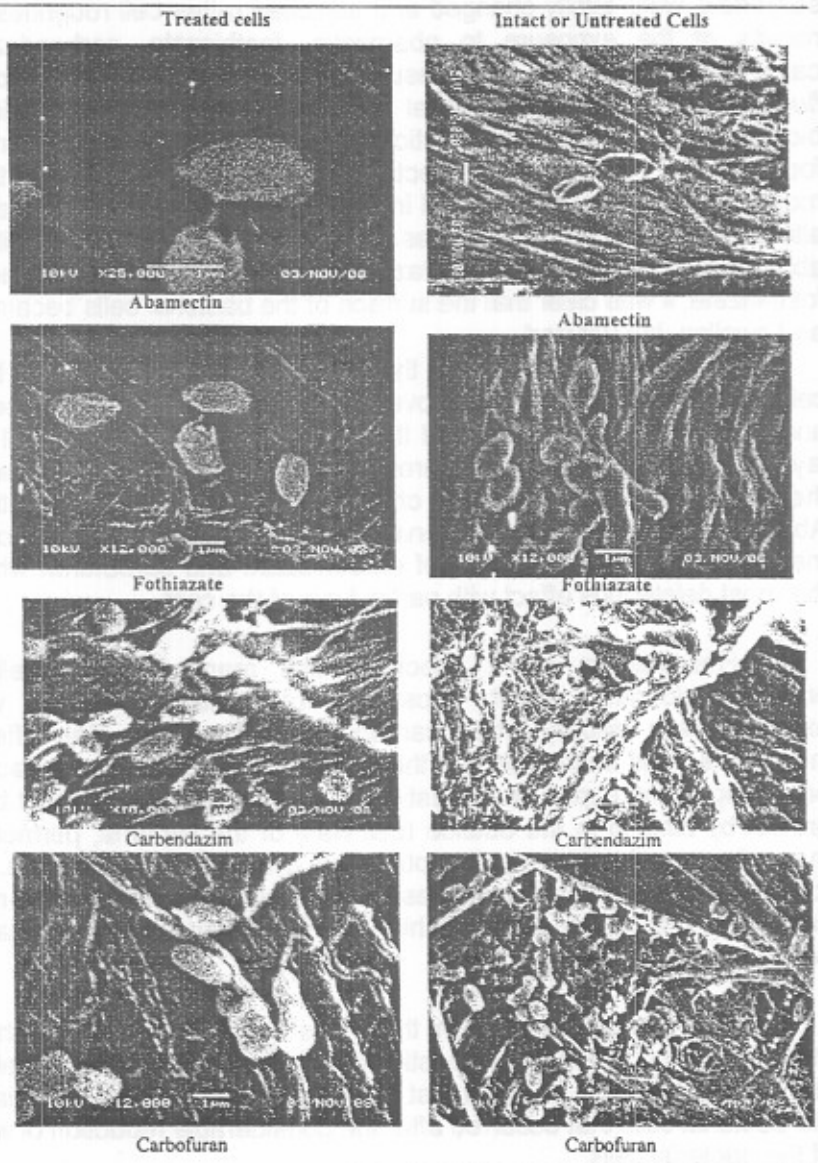
It was obvious that the morphological micrograph of the examined *Pseudomonas fluorescens* cells using scanning electronic microscope, somehow, were totally changed and exhibited rather cell roughness as the results of the exposure to abamectin, fosthiazate, carbendazim and carbofuran (Fig., 3). The results also revealed that *Pseudomonas fluorescens* exhibited potential efficacy for the assimilation and biodegradation of the used pesticides. From the first observation, it was found that the scanning electronic microscope revealed that the morphological changes occurred in the bacterial cells nature caused by the alteration in cell permeability as a direct result of the assimilation of abamectin, fosthiazate, carbendazim and carbofuran. For abamectin and fosthiazate, it was clear that the surface of the bacterial cells became rough and swollen, but unlysed.

In contrast, it was found that intact cells of the untreated bacterial cells had a smooth surface with overall intact morphology. For carbendazim and carbofuran, it was observed that the structure of the cell wall surface layer was wrinkled, and round pores were partially deformed, indicating that there were cytoplasmic structure changes which led to flush out of the cells. Abnormal cell division was observed at high frequencies among cells that tried to divide in the presence of carbendazim and carbofuran which had the most deleterious effect with partial lysis of the cells.

However, in some special cases many bacterial cells were enlarged, elongated, empty ghosts, or fragmented, consistent with the extremely low viability when carbendazim was used. The effect was investigated and compared with the untreated bacterial cells. In fact, in the beginning of the exposure to pesticides, certain pesticides inhibit bacterial growth by binding to the outside (cell wall) of the bacteria, permeabilizing the outer membrane and disrupting the cytoplasmic membrane. It was found that the efficacy of some pesticides increased against Gram-negative bacteria and acts in a similar fashion to other cationic biocides (Rawlinson *et al.*, 2010).

As a general observation, there was a pronounced visible shrinkage of the bacterial cells with all pesticides used. These shrinking cells were significantly reduced, but the most important result was the high viability of the bacterial cells that occurred after the considerable reduction of wrinkling of the bacterial cells.

Fig. (3): Scanning electron microphotograph of bacterial cells treated with different pesticides after 10 days post-treatment in Mineral Salt Media (MSM)



The results clearly indicate that the activity of *Pseudomonas fluorescens* against abamectin, fosthiazate, carbendazim and carbofuran vary with the rate of mutation occurred against the bacterial cells. These morphological changes and the mutation occurred in the bacterial cells explained somehow high efficiency of *Pseudomonas fluorescens* in assimilate and use the previously mentioned pesticides as a source of carbon and energy source.

These results are in agreement with those results obtained by Al-Qurainy and Abdel-Megeed (2009) who reported that the isolated *Pseudomonas frederiksbergensis* were effective in the degradation and assimilation of dimethoate and malathion (organophosphorous insecticide).

The obtained results show the isolated bacteria from a soil could assist the plants to increase their ability to degrade abamectin, fosthiazate, carbendazim and carbofuran. In the analysis of abamectin, fosthiazate, carbendazim and carbofuran degradation in plants, soil and growth media, there was a reduction of total pesticide residues in the cultivated soil compared with the non cultivated one. Similar results were obtained by Cho *et al.*, (2000) who studied the natural activity of the microorganisms in the rhizospheric region together with the cultivated plants and concluded that these plants evolved interactions and association with microorganisms that could cause accelerated breakdown or transformation of certain pesticides in the plant root zones to non-hazardous products. It was reported also that the understanding pesticide metabolism in plants and microorganisms is necessary for pesticide development, for safe and efficient use, as well as for developing pesticide bioremediation strategies for contaminated soil and water (Brimecombe *et al.*, 2001). Pesticide biotransformation may occur via multistep processes known as metabolism or cometabolism (Sharaf *et al.*, 2006).

The results of scanning electronic microscope could help in understanding the mechanism of the biodegradation of abamectin, fosthiazate, carbendazim and carbofuran by and microorganisms, as well as to design efficient biocatalyst allowing transformation of pesticides into non toxic compounds. On the other hand, the isolation of the previously mentioned bacteria has a great significance in understanding the role played together with plants in rhizospheric area. However, bacteria could

be used very effectively for in situ bioremediation in an environment, which is highly contaminated with pesticides.

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الملخص العربي

سلالات بكتيرية من الريزوسفير للتنقية من بعض المبيدات الكيميائية

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أجريت تجارب معملية و حقلية بزراعة أربعة نباتات هي أمارانس ، الخس، الجرجير و الفاصوليا من أجل عزل سلالات بكتيرية من منطقة ريزوسفير النباتات المنزرعة لرصد و كشف قدرتها

في تحطم بعض المبيدات من مجاميع كيميائية وذات أغراض استخدامية مختلفة مثل كاربوفيوران وأبامكتين (مبيدات حشرية)، فوسيازيت (مبيد نيماتودي)، و كربندازيم (مبيد فطري). و تم تصنيف و تعريف هذه البكتريا على أنها سيدوموناس اريوجنوزا، سيدوموناس فلوريسينس و باسيلس ميجاتيريام .

تم تنقية السلالات المعزولة ومعاملتها بالمبيدات المختبرة و أوضح الفحص الميكروسكوبي أن معظم الخلايا البكتيرية تواجدت حول المبيدات المختبرة في صورة مجموعات في المرحلة المائية. و أظهرت النتائج أن استخدام نموذج بكتريا سيدوموناس فلوريسينس أظهر كفاءة عالية في تحطيم المبيدات المستخدمة.

كما أظهر كل من مبيد كربندازيم و كاربوفيوران التأثير الأكثر ضرراً مع حدوث تحلل جزئي للخلايا. بينما كان واضحاً في حالة المبيدين أبامكتين و فوسيازيت أن سطح الخلايا البكتيرية أصبح خشن و منتفخ و لكنه غير متحلل. و على هذا توضح النتائج ان البكتريا المعزولة من التربة يمكنها تشجيع النباتات لزيادة قدرتها على تحطيم المبيدات الكيميائية و يمكن الإستفادة بها تطبيقيا للتخلص من ملوثات التربة الكيموزراعية.