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CHARACTERIZATION OF BACTERIAL ISOLATES (*Rhizobium leguminosarum and Bacillus thuringiensis)* ABLE TO DEGRADE OF MALATHION AND METHOMYL PESTICIDES .

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ABSTRACT

The symbiotic nitrogen fixing soil bacterium, *Rhizobium leguminosarum bv. vicieae,* is well known for its ability to interact with the leguminous plant *Vicia faba*. It has, however, not been reported that this species possesses the capability to degrade pesticides that are commonly associated with the agricultural processes. In this study the ability of *R.leuminosarum bv.vicieae* and the bioinsecticide *B.thuringiensis* bacteria to degrade malathion and methomyl was investigated. Some mutants were have been isolated. Three mutants of Rhizobia were found to be resistant to malathion up to 7000 ppm(RF12 mal^r, RZ11 mal^r and RH11 mal^r). Bt mutants (Bt3 mal^r, Bt7 mal^r and Bt8 mal^r) were also resistant up to 7000 ppm. Methomyl resistant mutants (RF12 lan^r, RZ11 lan^r and Bt7 lan^r) were resistant up to 10.000 ppm. All these mutants were able to utilize malathion or methomyl as a sole carbon source. The yeast extract was observed to enhance bacterial growth rate in the presence of the two pesticides, Whereas, Cfu/ml was reached up to 9.78×10^{16} in Rhizohium and 9.84×10^{16} in Bt after 10 days of incubation time. Bacterial mutants did not lose the property to grow on media containing 7000 ppm malathion or 10.000 ppm methomyl after acridine orange mediated curing. Gene transfer by conjugation mechanism showed that degradative gene(s) was able to transfer among not only between strains but also between different genera. Transconjugants were able to resist and grow in malathion or methomyl containing media. Some transconjugants were found to be higher response to these pesticides than their parents. It has been suggested that malathion and methomyl degradation gene(s) of *Rhizobium leguminosarum bv. vicieae* and *Bacillus thuringiensis* are chromosomal harboring not plasmid genes.
Keywords: Bt, conjugation,mutants, pesticides, Plasmid curing, Rhizobium.

INTRODUCTION

The excessive use of pesticides leads to an accumulation of a huge amount of residues in the environment, thereby posing a substantial health hazard for the current and future generations due to uptake and accumulate of these toxic compounds in the food chain and drinking water. Organophosphate pesticides such as malathion, diethyl (dimethoxythiophosphorylthio) succinate, are still extensively used world wide despite their high toxicity (Kumar *et al.,* 1996 and Zeinat *et al.,* 2008). Too, methomyl [S.methyl N.(methylcarbamoyloxy) thioacetimidate] is widely used for the control of insects and nematode, although, it is very toxic and hazardous pesticides. Methomyl belongs to a class of compounds known as oxim carbamates and it is highly soluble in water, so it can easily cause contamination of both ground and surface water resources (Mervat, 2009). At

the same time, microorganisms are thought to play an important role in the removal and detoxification of these toxicants from the environment.

Many bacteria that are able to degrade malathion and methomyl have been isolated from soil around the world, from the rhizosphere of different plants and from agricultural waste water (Abou-Shosha *et al.,* 2005; Zeinat *et al.,* 2008 and Mervat, 2009).These bacteria including *Bacillus sp, Bacillus thuringiensis, Pseudomonas sp, Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Rhizobium sp*. Many pesticide degradation genes reside on plasmids (Chung and Ka, 1998; Laemmli *et al.,* 2000). These plasmids known as catabolic plasmids, which can give the organism containing them the ability to degrade certain compounds. However, some of these genes are located on the chromosome (Abo-Amer, 2007 and Ajaz *et al.,* 2009).

This study aimed to select some of bacterial mutants from *R. leuminosarum* and *B. thrunigiensis*, to assess their abilities to degrading malathion and methomyl and determining the location of these degradative genes. *Rhizobium leguminosarum* is useful for legminous plants in the fixation of atmospheric nitrogen in root nodules *as well as B. thrunigiensis* (Bt) was recently used in insects control and for many purposes.

MATERIALS AND METHODS

This study was carried out in Microbial and Moleculer Genetics Lab., Genetics Dept., Fac. Agric., Zagazig Univ.

Bacterial isolates:

Two different bacterial genera were used in this study to assess their efficiency for degrading pesticides. *Rhizobium leguminosarum bv. vicieae* isolates were obtained from Agric. Microbiol. Dept., Fac. Agric., Zagazig Univ. These isolates were isolated from different location in Sharkia Governorate (Salem *et al.,* 2006). *Bacillus thuringiensis* (Bt) isolates were obtained from stocke Lab.Genetics Dept. Fac. Agric. Zagazig Univ. In addition *Pseudomonas aeruginosa* strain (MAM_2) used in this study was obtained from M. Day Wales Univ. College of Cardiff, UK.

Pesticides:

Two pesticides malathion and methomyl (Lannate) have been obtained from Plant Protection Dept., Fac. Agric., Zagazig Univ.

Growth media:

Yeast extract mannitol (YEM) agar and YEM broth media were used for Rhizobium. Nutrient agar (NA), nutrient broth (NB) and M9 minimal salt media were used for Bt. M9 minimal salt medium containing: 0.64% Na_2HPO_4, 0.15% KH_2PO_4, 0.0 25% NaCl, 0.05% NH_4Cl. To 800 ml sterile deionized water, 200 ml of M9 salts was add (Zeinat *et al.,* 2008).

Antibiotic:

Streptomycin was used as a selective marker in conjugation .The concentrations were 5mg/ml for strain RH11 A and 10 mg/ml for strain MAM2A.

Isolation of malathion and methomyl resistant mutants:

Different concentrations of the two pesticides (100 – 10000 ppm) were added to growth media. One ml from each liquid culture was spreaded on plates. The plates were then incubated at 28ºC for 2 5 days. The formed colonies on the highest concentration were selected and streaked across the plates containing the same concentration of pesticides to ensure from their tolerance.

Growth of bacterial isolates in different media supplemented with pesticides:

To determine if the bacterial mutants are able to use the pesticide as a sole carbon source, the bacterial mutants were allowed to grow on different media lacking carbon source and supplied with pesticide.

Determination of growth rate in different media at different times:

Bacterial mutants which used the pesticides as a carbon source were inoculated in different liquid media (YEM, YEM + pesticide – mannitol and YEM + Pesticide – Yeast extract) for Rhizobium, (M9 + Yeast extract, M9 + Pesticide and M9 + Yeast extract + Pesticide) for Bt. The cultures were incubated at 28ºC. Bacterial growth was estimated based on determination of viable cells counted per ml (Cfu/ml) at different times (1, 5, 10, 15, 20, 25, 30 days).

Plasmid curing:

In order to determine the location of pesticide resistance gene(s), the experiments were performed using acridine orange, ethidium bromide and SDS. As curing agents. The pesticide resistant mutants were grown overnight in 5ml liquid media containing 7000 ppm of malathion, 10000 ppm of methomyl. Next day, 200 µl of cultures were added in 5 ml liquid media containing the same previous concentrations and incubated at 28ºC in shaking incubator for 2 hours. 200 µl cultures were added to 2 ml liquid media containing (10 mg/ml) acridine orange, (50 µg/ml) ethidium bromide (Guha *et al.,* 1997) and (10 mg/ml) SDS (Chin *et al.,* 2005). All the tubes incubated in dark at 28ºC for overnight. Next day, 100 µl volumes from these tubes were spread over plain YEM agar plates for Rhizobium and NA plates for Bt and incubated at 28ºC for overnight. A total of 148 colonies were selected, replicated on to complete media and complete media supplied with pesticides by replica plate technique (Chin *et al.,* 2005; El-Deeb, 2009 and Ajaz *et al.,* 2009).

Plasmid isolation and agarose gel electrophoresis:

Plasmid DNA was isolated from bacterial treated with acridine orange according to Kado and Liu (1981). All samples were running by agarose gel electrophoresis according to the standard procedure of Sambrook *et al.* (1989). Agarose gel electrophoresis through horizontal slab gel of 0.8% agarose submersed in TBE (Tris-Hcl, Boric acid, EDTA) running buffer at 70 V for 30 min. were berformed. DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

Conjugation gene transfer:

Overnight cultures of donor and recipient were mixed (0.5 : 0.5 ml) and spreading onto plates. Incubated at 28ºC for overnight, after overnight growth, the bacteria were suspended in buffer,diluted and plated on selective

media for determined donor, recipient and transconjugants (Dutt *et al.,* 2003; Amin *et al.,* 2008; El-Deeb, 2009 and Amina and Amin, 2010).

Comparison between parents and transconjugants growth rate in liquid media supplemented with pesticides:

All of donors and transconjugants inoculated in liquid media supplemented with pesticides. Cultures were incubated at 28ºC for 24h. Serial dilutions were prepared and spreading on plates. The plates were incubated, bacterial growth rate was estimated based on determination of viable cells counted per ml (Cfu/ml).

RESULTS AND DISCUSSION

Determination tolerance to pesticides:

The ability of *Rhizobium lguminosarum bv. vicieae* and *Bacillus thuringiensis* for malathion and methomyl tolerance was investigated. Three mutants of Rhizobium were tolerant to malathion up to 7000 ppm, one mutant ($RH_{11}A$) was tolerant up to 1000 ppm, but it was sensitive to the higher concentrations. The Bt isolates (Bt3, Bt7, Bt8) were tolerant also up to 7000 ppm (Table 1).

Table (1): Isolation of malathion tolerant mutants.

Pesticide concentration (ppm) / Mutants	100	500	1000	2000	3000	4000	5000	6000	7000	10000
RF12	+	+	+	+	+	+	+	+	+	-
RZ11	+	+	+	+	+	+	+	+	+	-
RH11	+	+	+	+	+	+	+	+	+	-
RH11A	+	+	+	-	-	-	-	-	-	-
Bt3	+	+	+	+	+	+	+	+	+	-
Bt7	+	+	+	+	+	+	+	+	+	-
Bt8	+	+	+	+	+	+	+	+	+	-
MAM2A	-	-	-	-	-	-	-	-	-	-

+ = resistances **- = sensitive**

For methomyl, the RF12, RZ11, Bt7 were resistant up to 10.000 ppm, but RH11A was resistant up to 2000 ppm (Table 2). The *Pseudomonas aeruginosae* strain (MAM2A) was sensitive to all concentrations of both pesticides used in this study.

Table (2): Isolation of methomyl tolerant mutants.

Pesticide concentration (ppm) / Mutants	100	500	1000	2000	3000	4000	5000	6000	7000	10000
RF12	+	+	+	+	+	+	+	+	+	+
RZ11	+	+	+	+	+	+	+	+	+	+
RH11A	+	+	+	+	-	-	-	-	-	-
Bt7	+	+	+	+	+	+	+	+	+	+
MAM2A	-	-	-	-	-	-	-	-	-	-

Growth of these bacterial isolates in the presence of high concentration of pesticides suggested that these mutants may degrade these pesticides.

Ability of bacterial isolates to utilize pesticides as a sole carbon source:

Data in Tables (3 and 4) showed that mutants can grown in different media supplemented with pesticides. Data in Tables (5and 6) indicating that pesticides can be used as a sole source of carbon for Rhizobium and Bt mutants.

Table (3): Growth of *Rhizobium* mutants in different media supplemented with malathion or methomyl.

Media / Mutants	YEM	YEM without mannitol	YEM without yeast	YEM without mannitol and yeast
RF12 mal^r	+	+	+	+
RF12 lan^r	+	+	+	+
RZ11 mal^r	+	+	+	+
RZ11 lan^r	+	+	+	+
RH11 mal^r	+	+	+	+

Table (4): Growth of *B.thuringiensis* isolates in different media supplemented with malathion or methomyl.

Media / Mutants	NA	NA without yeast	NA with peptone	M9	M9 + yeast
Bt3 mal^r	+	+	+	+	+
Bt7 mal^r	+	+	+	+	+
Bt7 lan^r	+	+	+	+	+
Bt8 mal^r	+	+	+	+	+

Table (5): Growth of *Rhizobium* pesticide tolerant mutants in different liquid media at different times.

Mutants / Time (days) and media	RF12 malr	RF12 lanr	RZ11 malr	RZ11lanr	RH11malr
One day					
YEM only	$1.64x10^{12}$	$5.81x10^{11}$	$1.07x10^{11}$	$1.54x10^{11}$	$6.92x10^{11}$
YEM-mannitol+Pesticide	$0.52x10^{11}$	$7.36x10^{11}$	$3.32x10^{11}$	$3.02x10^{11}$	$3.64x10^{11}$
YEM- yeast+pesticide	$0.45x10^{7}$	$2.38x10^{11}$	$0.31x10^{7}$	$0.27x10^{11}$	$0.32x10^{11}$
5 days					
YEM only	$1.28x10^{16}$	$4.47x10^{15}$	$1.25x10^{15}$	$1.7x10^{15}$	$1.25x10^{15}$
YEM-mannitol+Pesticide	$4.92x10^{15}$	$7.25x10^{15}$	$1.04x10^{16}$	$9.84x10^{15}$	$2.16x10^{15}$
YEM- yeast+pesticide	$0.36x10^{6}$	$1.51x10^{15}$	$2.2x10^{5}$	$0.84x10^{15}$	$1.5x10^{8}$
10 days					
YEM only	$2.41x10^{16}$	$4.42x10^{16}$	$7.97x10^{16}$	$0.91x10^{16}$	$1.92x10^{15}$
YEM-mannitol+Pesticide	$3.96x10^{16}$	$4.74x10^{16}$	$9.78x10^{16}$	$3.8x10^{16}$	$3.16x10^{15}$
YEM- yeast+pesticide	-	$1.92x10^{16}$	-	$1.1x10^{15}$	$2.8x10^{6}$
15 days					
YEM only	$1.52x10^{15}$	$2.21x10^{15}$	$5.91x10^{15}$	$0.85x10^{15}$	$1.23x10^{15}$
YEM-mannitol+Pesticide	$0.72x10^{15}$	$3.98x10^{15}$	$6.49x10^{15}$	$2.9x10^{15}$	$1.2x10^{15}$
YEM- yeast+pesticide	-	$1.65x10^{15}$	-	$1.5x10^{15}$	-
20 days					
YEM only	$0.57x10^{15}$	$2.16x10^{15}$	$2.07x10^{15}$	$0.78x10^{15}$	$0.52x10^{15}$
YEM-mannitol+Pesticide	$0.54x10^{15}$	$3.21x10^{15}$	$5.81x10^{15}$	$2.51x10^{15}$	$0.43x10^{15}$
YEM- yeast+pesticide	-	$1.20x10^{15}$	-	$0.76x10^{15}$	-
25 days					
YEM only	$0.40x10^{15}$	$0.79x10^{15}$	$0.99x10^{15}$	$0.29x10^{15}$	$0.18x10^{15}$
YEM-mannitol+Pesticide	$0.36x10^{15}$	$2.19x10^{15}$	$3.48x10^{15}$	$2.11x10^{15}$	$0.31x10^{15}$
YEM- yeast+pesticide	-	$0.37x10^{15}$	-	$0.21x10^{15}$	-
30 days					
YEM only	$2.04x10^{11}$	$0.30x10^{11}$	$0.53x10^{11}$	$0.72x10^{11}$	$3.6x10^{10}$
YEM-mannitol+Pesticide	$0.38x10^{11}$	$0.78x10^{15}$	$0.86x10^{15}$	$4.0x10^{13}$	$9.3x10^{10}$
YEM- yeast+pesticide	-	$0.2x10^{10}$	-	$0.17x10^{13}$	-

Table (6): Growth of *B.thuringiensis* - pesticide tolerant mutants in different liquid media at different times.

Mutants / Time (days) and media	Bt3 malr	Bt7 malr	Bt7 lanr	Bt8 malr
One day				
M9 + Yeast	$5.99x10^{11}$	$3.93x10^{11}$	$5.82x10^{11}$	$6.40x10^{11}$
M9 + Pesticide	$4.36x10^{11}$	$5.96x10^{11}$	$5.68x10^{11}$	$4.32x10^{11}$
M9 + Yeast + Pesticide	$3.42x10^{11}$	$2.39x10^{11}$	$6.12x10^{11}$	$7.68x10^{11}$
5 days				
M9 + Yeast	$1.71x10^{16}$	$1.99x10^{16}$	$1.51x10^{16}$	$1.95x10^{16}$
M9 + Pesticide	$5.31x10^{15}$	$1.91x10^{16}$	$1.2x10^{16}$	$1.68x10^{16}$
M9 + Yeast + Pesticide	$1.96x10^{16}$	$2.56x10^{16}$	$8.62x10^{16}$	$2.79x10^{16}$
10 days				
M9 + Yeast	$1.35x10^{16}$	$1.6x10^{16}$	$9.82x10^{16}$	$6.53x10^{16}$
M9 + Pesticide	$6.47x10^{15}$	$3.65x10^{16}$	$6.58x10^{16}$	$7.2x10^{16}$
M9 + Yeast + Pesticide	$9.84x10^{16}$	$6.97x10^{16}$	$9.83x10^{16}$	$9.41x10^{16}$
15 days				
M9 + Yeast	$1.24x10^{16}$	$4.89x10^{15}$	$1.46x10^{16}$	$6.53x10^{16}$
M9 + Pesticide	$1.72x10^{15}$	$4.32x10^{15}$	$2.73x10^{16}$	$6.76x10^{16}$
M9 + Yeast + Pesticide	$7.36x10^{16}$	$6.51x10^{15}$	$3.18x10^{16}$	$9.19x10^{16}$
20 days				
M9 + Yeast	$9.39x10^{15}$	$1.88x10^{15}$	$1.23x10^{15}$	$5.98x10^{15}$
M9 + Pesticide	$0.58x10^{15}$	$1.11x10^{15}$	$1.69x10^{15}$	$5.09x10^{15}$
M9 + Yeast + Pesticide	$3.32x10^{16}$	$3.6x10^{15}$	$2.15x10^{15}$	$8.51x10^{15}$
25 days				
M9 + Yeast	$1.54x10^{15}$	$1.23x10^{15}$	$0.33x10^{15}$	$0.22x10^{15}$
M9 + Pesticide	$0.21x10^{15}$	$0.56x10^{15}$	$0.04x10^{15}$	$0.19x10^{15}$
M9 + Yeast + Pesticide	$2.11x10^{15}$	$2.68x10^{15}$	$1.03x10^{15}$	$4.6x10^{15}$
30 days				
M9 + Yeast	$0.17x10^{15}$	$0.26x10^{15}$	$9.0x10^{13}$	$0.12x10^{15}$
M9 + Pesticide	$0.47x10^{11}$	$0.09x10^{15}$	$2.0x10^{13}$	$0.09x10^{15}$
M9 + Yeast + Pesticide	$1.63x10^{15}$	$1.3x10^{15}$	$1.4x10^{13}$	$2.06x10^{15}$

As shown from the results, the highest growth was observed at 10 days in YEM without mannitol and supplemented with pesticides media. The Cfu/ml was 3.96×10^{16}, 4.74×10^{16}, 9.78×10^{16}, 3.8×10^{16}, 3.16×10^{15} for RF12 mal^r, RF12 lan^r, RZ11 mal^r, RZ11 lan^r and RH11 mal^r, respectively for Rhizobium isolates. Data appered that longer incubation time didn't increase bacterial growth.

For Bt bacteria , growth was increased in the presence of yeast extract and pesticides if compared to growth in media containing only yeast extract or pesticides. The highest Cfu/ml was observed after 10 days, whereas reached 9.84×10^{16}, 6.97×10^{16}, 9.83×10^{16} and 9.41×10^{16} for Bt3 mal^r, Bt7 mal^r, Bt7 lan^r and Bt8 mal^r respectively. As in Rhizobium, longer incubation time didn't increase bacterial growth. The disappearance of pesticide was due to complete utilization by the tested organisms. The results showed that the presence of yeast extract in growth media supplemented with pesticides enhanced bacterial growth. Also, the highest number of bacterial colonies was detected after 10 days. Similar results have been reported, since, Zeinat *et al.* (2008) found that bacterial growth rate of Bt was increased after 12 days ,however the presence of yeast extract in culture media supplemented with malathion increased the bacterial growth. Kannan and Vanitha (2005) found that malathion degradation via *Serratia marcescens* was high in nutrient broth than in mineral salts medium.

These results are in agreement with those of Digrak and Ozel (2002) and Abou-Shosha *et al.* (2005).

Increase in number of colonies in the presence of pesticides may due to one or more product(s) upon the degradation of pesticide(s)formed which causd high growth rate of bacterial cells as it has been suggested by (Abou-Shosha *et al.,* 2005).

Zeinat *et al.* (2008) noticed that, the growth of Bt in M9 poor media was due to the biodegradation of these pesticides by the enzyme machinery that already exists inside the cell. These enzymes are largely hydrolytic to such compounds and play major role in utilization of these materials as nutrient sources.

Malathion was used as a sole carbon source by *Micrococcus sp.* (Guha *et al.,* 1997) and *Pseudomonas sp., Pseudomonas Putida, Micrococcus lylae* (Goda *et al.,* 2010).

Methomyle was also used as the sole carbon or nitrogen source by *Paracoccus sp.* (Xu, 2009) and by *Stenotrophomonas maltophilia* M1 (Mervat, 2009).

Consistent with these results, different bacteria have shown to grow on other pesticides as the sole carbon source: carbaryl (Chapalamadugu and Chaudhry, 1991), atrazin (Struthers *et al.,* 1998), parathion (Shimazu *et al.,* 2001; Zhongli *et al.,* 2001 and Liu *et al.,* 2005), dursban and stomp (Abou-Shosha *et al.,* 2005), endosulfan (Verma *et al.,* 2006) chloropyrifos (Li and Li 2007) and diazinon, ethion (Sabdono and Radjasa, 2008).

Genetic localization of malathion and methomyl degradation genes:

These experiments were carried out to detect the degradation gene(s) of *Rhizobium leguminosarum* and *Bacillus thuringiensis* whether are plasmid or chromosomal mediated. The isolates were subjected to plasmid curing via acridin orange, ethidium brmide and SDS. All selected colonies were growth on complete media (CM) and complete media supplemented with pesticides (Table 7).

Table (7): Ability of bacterial mutants to grown on media supplemented with pesticide after plasmid curing by different agents.

Curing agents and number of tested colonies / Mutants	Acridin orange		Ethidium bromide		SDS	
	CM	CM + Pesticide	CM	CM + Pesticide	CM	CM + Pesticide
Rhizobium						
RF12 mal^r	148	148	148	148	148	148
RF12 lan^r	148	148	148	148	148	148
RZ11 mal^r	148	148	148	148	148	148
RZ11 lan^r	148	148	148	148	148	148
RH11 mal^r	148	148	148	148	148	148
Bt						
Bt3 mal^r	148	148	148	148	148	148
Bt7 mal^r	148	148	148	148	148	148
Bt7 Lan^r	148	148	148	148	148	148
Bt8 mal^r	148	148	148	148	148	148

Curing experiment was repeated again via acridine orange to comparison between isolates before and after curing. The Cfu/ml of all cured isolate was nearly 50% from non cured isolates (Table 8).

Table (8): Comparison between counts of mutants before and after curing by acridine orange.

Mutants	Before curing		After curing	
	CM	CM +Pesticide	CM	CM+ Pesticide
RF12 mal^r	$6.94x10^{12}$	$6.27x10^{12}$	$6.19x10^{6}$	$6.17x10^{6}$
RF12 lan^r	$8.73x10^{12}$	$8.67x10^{12}$	$6.23x10^{6}$	$6.18x10^{6}$
RZ11 mal^r	$8.96x10^{12}$	$8.31x10^{12}$	$7.96x10^{6}$	$7.91x10^{6}$
RZ11 lan^r	$9.78x10^{12}$	$9.52x10^{12}$	$8.87x10^{6}$	$8.82x10^{6}$
RH11 mal^r	$7.81x10^{12}$	$7.82x10^{12}$	$7.63x10^{5}$	$7.67x10^{6}$
Bt3 mal^r	$9.65x10^{12}$	$9.64x10^{12}$	$5.26x10^{5}$	$5.17x10^{6}$
Bt7 mal^r	$9.84x10^{12}$	$9.76x10^{12}$	$9.36x10^{5}$	$9.27x10^{6}$
Bt7 lan^r	$8.89x10^{12}$	$8.75x10^{12}$	$2.36x10^{5}$	$2.23x10^{6}$
Bt8 mal^r	$6.89x10^{12}$	$6.72x10^{12}$	$2.52x10^{5}$	$1.14x10^{6}$

All samples (cured and non-cured) were subjected to plasmid screening, as shown in Figure (1) . The results obtained from agarose gel electrophoresis for *Rhizobium* mutants appeared that, lane 1, 3, 5, 7, 9: non cured mutants (RF12 mal^r, RF12 lan^r, RZ11 mal^r, RZ11 lan^r and RH11 mal^r respectively), from these, it was observed that presence of plasmid bands. Lane 2, 4, 6, 8, 10 cured isolates at the same previous order, observed the absence of plasmid bands. This indicated that acridine orange was able to cure plasmid from all isolates.

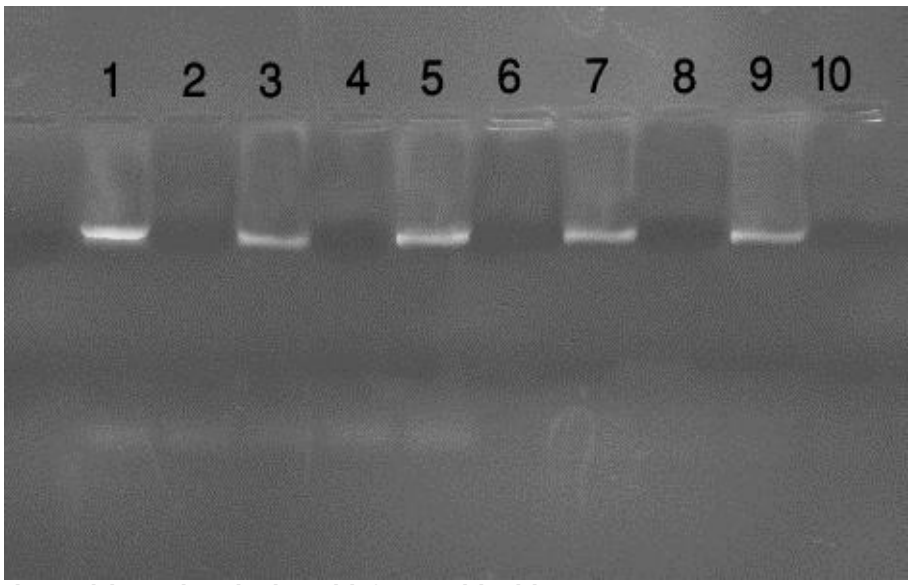

Figure (1): Isolated plasmid from Rhizobium mutants. Lane 1, 3, 5, 7 and 9 represent the presence of plasmid in non cured

isolates. Lane 2, 4, 6, 8, 10 represent plasmid absence in cured isolates.

Figure (2) shows agarose gel electrophoresis of Bt, which appeared Similar results.

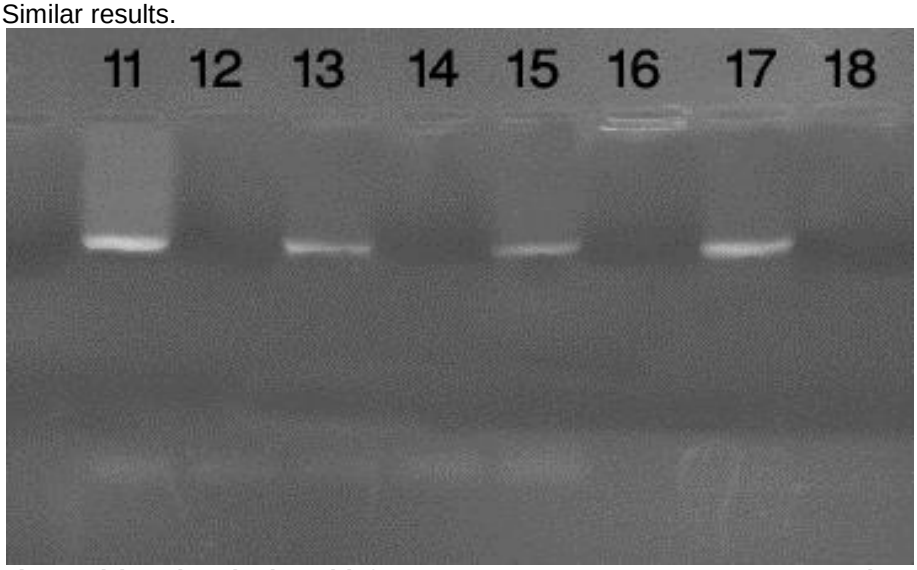

Figure (2):Isolated plasmid from Bt mutants. Lane 12, 14, 16 and 18 represent plasmid absence in cured mutants.

It is clear that plasmid could not be extracted from the acridine orange treated bacterial isolates, while the plasmids are visible in the uncured isolates. Guha *et al.* (1997) and Ajaz *et al.* (2009) have also reported the absence of plasmid in acridine orange treated strains of *Micrococcus sp.* and *Pseudomonas putida* MAS-1, respectively.

This study suggested that the degradation gene(s) of malation and methomyl are chromosomal harboring not on the plasmids in these isolates might not carry any degradation activity genes of malathion and methomyl. So the cured isolates could grow in the presence of pesticides at the same efficiency in the absence of it. Corresponding to Jamal *et al.* (2005) the plasmid in *Pasteurella multocida* was not carrying any virulence gene.

These results agreed with(Abo-Amer, 2007),who found that the degradation gene of malthion in *Pseudomonas aeruginosa* was chromosomal located. Ajaz *et al.* (2009) found that, another organophosphorous pesticide Chloryrifos, degradation gene in *Pseudomonas putida* MAS-1 was accomplished by the combined action of plasmid and chromosomal genes. As well as chromosome mediated degradation of polychlorinated biphenyls by *Pseudomonas pseudoalcaligenes* (Furukawa and Miyazaki, 1986).

In contrast, Guha *et al.* (1997) suggested that the probability of plasmid involvement in malathion degreadation by *Micrococcus sp.* For methomyl, it was referred previously that, degradation of methomyl by *R. leguminosarum* and Bt isolates due to chromosomal not plasmid genes. Mervat (2009) found

that, the degrading gene(s) of methomyl in *Stenotrophomonas maltophilla* were plasmid harboring.

Some studies, on the other hand, were estabiliched that bacterial degradation of some pollutants (aromatic compounds) were attributed to clusters of genes located in mobile genetic elements (Transposons) (Diaz, 2004) which facilitate their mobilization from one site to anther on the chromosome, to plasmid or from plasmid to chromosome.

As well as, Liu *et al.* (2005) found that Hin-dIII fragment revealed that the methyl parathion degrading gene (mph gene) was physically located in a typical transposon.

Transfer of pesticides degrading gene(s) by conjugation:

Conjugation studies were carried out in attempt to transfer pesticides degrading genes. Conjugation was used, between mutants from the same species (between Rhizobium isolates), as intraspecific conjugation (Abou-Shosha *et al.,* 2005) or interspecies conjugation (Amina and Amin, 2010) or between different genera (between Rhizobium and Bt), as intergeneric conjugation (Dutt *et al.,* 2003).

Data showed that, the number of transconjugants in interspecies conjugation was higher than in intergeneric. Transconjugants in Rhizobium were ranged from 2.0×10^3 to 1.73×10^7, 1.37×10^3 to 2.11×10^7 in Bt (Table 9).The highest frequency of conjugation was 10^{-6}, these results agreed with Liu *et al.* (2005). This indicated that horizontal gene transfer enables the microorganisms to show a rapid adaptation to pollutant environments (Diaz, 2004).

Table (9): Ability of pesticides degrading gene(s)to transfer by conjugation mechanism.

Parental mutants		Cfu/ml after 24 h.			Conjugation Frequency
Donor	Recipient	Donor	Recipient	Transconjugants	
RF12 mal^r	RH11A	$9.6x10^7$	$3.21x10^7$	$1.54x10^7$	$6.6x10^{-6}$
RF12 lan^r	RH11A	$7.63x10^7$	$7.68x10^7$	$2.0x10^3$	$8.6x10^{-10}$
RZ11 mal^r	RH11A	$1.69x10^5$	$3.50x10^5$	$2.1x10^5$	$9.05x10^{-8}$
RZ11 lan^r	RH11A	$8.52x10^7$	$1.37x10^5$	$9.0x10^5$	$3.9x10^{-8}$
RH11 mal^r	RH11A	$9.84x10^7$	$3.15x10^5$	$1.73x10^7$	$7.4x10^{-6}$
RF12 mal^r	MAM2A	$5.6x10^3$	$3.96x10^3$	$2.15x10^3$	$9.5x10^{-10}$
RF12 lan^r	MAM2A	$6.84x10^7$	$7.66x10^5$	$1.51x10^7$	$6.7x10^{-6}$
RZ11 mal^r	MAM2A	$9.85x10^7$	$3.98x10^7$	$1.29x10^3$	$5.7x10^{-10}$
RZ11 lan^r	MAM2A	$8.69x10^7$	$5.91x10^7$	$2.15x10^5$	$9.5x10^{-8}$
RH11 mal^r	MAM2A	$8.5x10^5$	$1.21x10^7$	$2.3x10^3$	$1.02x10^{-9}$
Bt3 mal^r	MAM2A	$2.23x10^7$	$9.76x10^7$	$2.11x10^7$	$9.3x10^{-6}$
Bt7 mal^r	MAM2A	$7.2x10^7$	$6.75x10^7$	$3.6x10^5$	$1.6x10^{-7}$
Bt7 lan^r	MAM2A	$8.62x10^7$	$5.61x10^7$	$1.37x10^3$	$6.08x10^{-10}$
Bt8 mal^r	MAM2A	$8.8x10^7$	$1.96x10^7$	$1.23x10^5$	$5.6x10^{-8}$

Cfu/ml of RH11A at zero time = $2.32x10^{12}$
Cfu/ml of MAM2A at zero time = $2.25x10^{12}$

Comparison between parental strain and their transconjugants for the degradation of pesticides:

Transconjugants were selected randomly to determine their response to use pesticides as nutrients compared with their parental strain. The obtained results in Table (10) revealed that all transconjugants were tolerant to pesticides. Six transconjugants (RH11A1, RH11A3, RH11A5, MAM2A2, MAM2A6 and MAM2A7) were higher than their parents to utilize pesticides during growth. Therefore, these transconjugants were able to degrade the used pesticide. These recombinants may be received one or more of degradative gene(s) from the donor parent. This gene(s) were responsible to degrade the pesticides to its precursors or primary substrates. These substrates can be utilized by new transconjugants, enhanced their growth and given large number of bacterial cells.

Consitent with these results, Liu *et al.* (2005) found that transconjugants were capable of utilizing methyl parathion and p-nitrophenol as a sole source of carbon and nitrogen. Abo-Shosha *et al.* (2005 and 2007) found that transconjugants and transformants were able to degraded herbicide stomp and insecticide malathion as their donor parent.

Table (10): Comparison between transconjugants and their parents grown in liquid media supplemented with pesticides.

Donor		Transconjugants	
mutants	**Cfu/ml**	**Strain**	**Cfu/ml**
RF12 mal^r	$6.91x10^{11}$	RH11A1	$1.38x10^{12}$
RF12 lan^r	$9.96x10^{11}$	RH11A2	$9.84x10^{11}$
RZ11 mal^r	$8.21x10^{9}$	RH11A3	$2.2x10^{10}$
RZ11 lan^r	$1.08x10^{11}$	RH11A4	$1.10x10^{11}$
RH11 mal^r	$1.6x10^{10}$	RH11A5	$1.39x10^{12}$
		MAM2A1	$7.0x10^{7}$
		MAM2A2	$1.20x10^{12}$
		MAM2A3	$1.1x10^{10}$
		MAM2A4	$9.76x10^{11}$
		MAM2A5	$6.5x10^{8}$
Bt3 mal^r	$8.69x10^{11}$	MAM2A6	$1.24x10^{12}$
Bt7 mal^r	$7.64x10^{11}$	MAM2A7	$1.59x10^{12}$
Bt7 lan^r	$1.20x10^{12}$	MAM2A8	$1.13x10^{12}$
Bt8 mal^r	$1.32x10^{12}$	MAM2A9	$9.82x10^{12}$

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توصيف عزلات بكتيرية من بكتيريا *Rhizobium leguminosarum and Bacillus thuringiensis* لقدرتها على تكسير مبيدات المالاثيون والميثومايل

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بكتيريا التربة المثبتة للنيتروجين تكافليا *Rhizobium leguminosarum bv. Vicieae* يعرف عنها جيدا مقدرتها على التفاعل مع النبات البقولى - الفول البلدى – ولكن لم يلاحظ أن هذا النوع يمتلك القدرة على تكسير المبيدات التى يشيع ارتباطها بالعمليات الزراعية.

فى هذه الدراسة تم اختبار قدرة *Bacillus thuringiensi* و *Rhizobium leguminosarum bv. Vicieae* على مقاومة مبيدات المالاثيون والميثومايل ، كما تم عزل بعض الطفرات المقاومة منهما .

تم عزل ثلاث طفرات من *Rhizobium* كانت مقاومة للمالاثيون حتى 7000 ppm وهى RF12mal^r,RZ11mal^r,RH11mal^r ، أيضا تم عزل ثلاث طفرات من Bt وهى Bt3mal^r,Bt7mal^r,Btmal^r وكانت مقاومة حتى 700ppm . الطفرات المقاومة للميثومايل من الجنسين هى RFlan^r, RZ11lan^r, Bt7lan^r والتى كانت مقاومة حتى 10000 ppm.

كل هذه الطفرات كانت قادرة على استخدام المالاثيون والميثومايل كمصدر وحيد للكربون، وقد لوحظ أن وجود مستخلص الخميرة يزيد من معدل النمو البكتيرى فى البيئة الموجود بها المبيدات حتى وصل Cfu/ml إلى $9.78X10^{16}$ فى *Rhizobium* ، $9.84X10^{16}$ *فى Bt وذلك* بعد 10 أيام من التحضين ، كما لوحظ أن الطافرات البكتيرية لم تفقد صفة النمو فى بيئة محتوية على 7000ppmمالاثيون أو 10000 ppmميثومايل وذلك بعد المعاملة بالأكريدين البرتقالى.

أوضحت ميكانيكية النقل الجينى Conjugation أن جين أو جينات التكسير لها القدرة على الإنتقال ليس فقط بين سلالات نفس النوع ولكن أيضا بين أجناس مختلفةمن البكتريا ، كما استطاعت الخلايا المولفة أن تقاوم وتنمو على البيئة المحتوية على المبيدات ، و كان البعض منها أكثر استجابة للمبيد من الآباء.

توصلت النتائج المتحصل عليها من هذه الدراسة أن جين أو جينات تكسير المالاثيون والميثومايل فى *Rhizobium leguminosarum bv. Vicieae, Bacillus thuringiensis* هى جينات كروموسومية وليست بلازميدية.

قام بتحكيم البحث

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