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Monitoring of Variation among Faba Bean *Rhizobium* Isolates: 2. Biodegradation of Herbicide, 3(3,4 Dichlorophenyl) -1-methoxy-1-methylurea

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Abstract: The ability of 3 Faba bean *Rhizobium* isolates to degrade the herbicide Linuron (Afalon 50% WO), 3 (3,4-dichlorophenyl), 1-methoxyl-1-methylurea under sterilized and non sterilized soil was studied. Results showed that, two weeks earlier the herbicide treatment in soil resulted in the degradation after 9 days of treatment. *R. leguminosarum* isolate (F) was found to be highly effective which degraded the herbicide followed by isolate (IS) then isolate (M) specially under non sterilized soil conditions. Dehydrogenase gene was successfully detected in total DNA of 3 isolates (M), (1S) and (F) by PCR which showed a significant differences among isolates based on number of bands (isozymes) and base pairs gene 357.89 isolate (M), 1373.40, 921.05, 368.42 isolate (1S) and 1059.57, 936.84; 357.89 isolate (F) relative mobility and density. The partial nucleotide sequence of the PCR-amplified fragment for the dehalogenase gene of *R. leguminosarum* isolate (F) (EU693117 GenBank accession) was done to determent the relationship with other recommended bacteria registered in GenBank. The DNA sequence was performed using PCR produced when the specific primer. Nucleotides were found to be 2320 bp from dehalogenase gene sequence. A phylogenetic tree of gene revealed 80% a moderate degree of similarity to the other isolates sequences and enzyme gene.

Key words: *R. leguminosarum*, dehalogenase, herbicide, Biodegradation PCR.

INTRODUCTION

Nowadays, great quantities of fungicides and herbicides are consumed annually for the control of plant pathogenic fungi and woods during our chemical warfare against a multitude of noxious organisms in the soil. It is necessary to avoid the injury of these fugicides together with their various carriers diluents and solvents on the various beneficial soil microorganisms and their biological activities contributing to soil fertility. It is known that the actual degradation of fungicides by microorganisms is caused by the release of enzymes that breakdown of Linuron, Kitozin and dichlofunanid by *Pseudomonas, Bacillus* and *Streptomyces lavedulae* was studied by Mashish *et al*. (1990) and Dahrog *et al*. (2006).

So far, haloalkane dehalogeneses are the only enzymes known to be capable of direct hydrolytic dehalogenation of chlorinated and brominated hydrocarbons, without the requirement for coenzymes or oxygen. The enzyme of *X. autotrophicus* G110 is constitutively expressed to 2 to 3% of the soluble cellular protein (Keuning *et al*., 1985) up to 30% (Jansen *et al*., 1989). It has a remarkably broad substrate range which includes terminally halogenated alkanes with chain lengths up to 4 carbons for chlorinated and up to at least to carbons for brominated alkanes. Other haloalkane dehalogenases of broad substrate range have been found in gram-positive haloalkane-utilizing bacteria (Yokota *et al*., 1987 and Janssen *et al*., 1988).

Autotrophic organisms are nitrogen fixing bacteria that are able to grow with mixture of hydrogen and oxygen as an energy source. A number of organisms that is able to utilize several halogenated hydrocarbon as carbon sources has been isolated (Jenssen *et al*., 1984). The organism was obtained from an enrichment culture with 1,2-dichloroethane, which is an environmentally important compound with a production volume larger than that of any other industrial halogenated chemical. The 1,2-dichloroethane degrading bacterium, designated strain GJ10, was found to degrade 1,2-dichloroethane via 2-chloroethanol, 2-chloroacetaldehyde and chlora acetic acid to glycolate (Janssen *et al*., 1985).

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The dehydrogenation steps in this sequence were found to be catalized by two different hydrolytic dehalogenases (Keuning *et al*., 1985).

Conversion of 1,2-dichloroethane was mediated by a haloalkane dehalogenase. This was the first enzyme found to catalyze hydrolytic dehalogenation of chlorinated hydrocarbhons. The protein was been purified (Keuning *et al*., 1985) and crystallized (Rozeboom *et al*., 1988) and its three-dimensional structure is now under study.

Many studies were focused on the reversal effect of specific pesticide induced phytotoxicity on the growth, yield quality of specific crop. So objective of the present work was to study the biodegradation of the herbicide linuron (Afalon 50% WP) in soils inoculated with *R. leguminosarum* isolates cultivated with faba bean plant. The hatoalkane dehalogenase gene detected by PCR and sequenced.

MATERIALS AND METHODS

Rhizobium leguminosarum isolates:

Three *Rhizobium leguminosarum* isolate namely M, IS and F (Amar, 2008) were grown in/on yeast extract mannitol broth medium (YEM).

Herbicide, Linuron (Afalon 50% WP):

3- (3,4-dichlorophenyl)-1- Methoxy-1-methyl urea, it was applied at a rate of 4 g/liter of water. Emulsifiable concentration of 50% of this Linuron was used in this investigation. These compounds were supplied by Kumia Chemical Industries Co. Ltd., Japan.

Laboratory Experiment:

The effect of Linuron on *R. leguminosarum* isolates under laboratory conditions were on Petri dishes containing YEM agar. The Linuron was pipetted onto the surface of approprially labeled agar plate. *R. legumonosarum* isolates were distributed of 1 x 10^5 to the surface of Petri dishes. Three replicates of Petri dishes were used to each isolate as well as three Petri dishes were used without Linuron as control. The Petri dishes were incubated at 27ºC. After 4 days, were inspected to estimate the efficacy of Linuron against *Rhizobium* isolates by using the method adopted by Kirby-Bauer Antimicrobial susceptibility test procedure, 1990.

Potted Experimentally:

The soil sample was taken from the surface layer (30 cm depth) of clay loamy soil. The chemical and physical analyses were determined according to Amar (2008). Twenty pots of 250 ml capacity were filled with clay loamy soil of Fayium Governorate (200 g/pot). Fifteen pots were sterilized after raising the soil moisture content up to 60% of WHC at 15 ibs for 2 hrs. during 3 days. Another half of pots were left without sterilization. All pots were supplemented with 50 ml broth medium YEM and Linuron 40 ppm were added to all pots. Ten ml of each *Rhizobium* wild type (M), (IS) and (F) grown on YEM media for 15 days at 28ºC was used for inoculation of the pots. Soil samples were taken periodically after 1, 15 and 30 days for the determination of residual Linuron using Gas liquid chromatography (GLC) according to Vogeler (1968).

Determination of Linuron Residues in Soil

1. Extraction and Clean Up:

One gram of each treated soil and untreated soil for each isolates were transferred into 250 ml separating funnel and added 60 ml at 15% methylene chloride/hexane (V/V). The funnel was stoppered and shaken vigorously for 2 min and vented the pressure during shaking. The two layers were separated. The aqueous layer was drawn off into separating funnel. This procedure was repeated using 30 ml of 15% methylene chloride/hexane (V/V). The aqueous layer was discarded and the solvent extracts in both separating funnels were combined and poured on top of anhydrous sodium sulphate column. Before the solution recedes the top of sodium sulphate layer, three 10 ml rinses of 15% methylene chloride/hexane were added. The filtrates were collected for clean up by using the US. EPA (1988) procedures.

For clean up a florisil column chromatography (300 mm long X 25 mm) internal diameter (i.d) with a small glass plug in the bottom was prepared by adding an activated florisil (130ºC/overnight) in small portions, while tabbing until about 10 cm high. About one and a half cm layer at anhydrous sodium sulphate was added to the top carefully without mixing with florisil.

Mature solvents of 6% diethyl ether/petroldum ether was added then 10 ml of 15% diethyl ether/petroleum ether and 50% diethyl/petroldium ether were used for elution.

The filtered elution mixture was evaporated using rotary evaporator 40ºC for gas chromatographic determination.

2. Determination:

Separating of the residues was done on a borosilicate glass column 2 meters long, 3 mm internal diameter containing 3% oV-17 (phenyl methyl silicone) on chromosorb W,H.b. 100/120 mesh. The operating conditions were: column oven temperature (250ºC) carrier gas (Nitrogen) (1.2 kg/cm), Burner gas (Hydrogen) (1.0 kg/cm) and air (1.0 kg/cm). Phillips PU4410, computerized Gas Chromatography with FPD was used.

Soil free from Linuron was used to estimate rate of recovery by using the previous producers. The mean of the obtained recovery was 88%. All the obtained data for the residues of Linuron on treated soil were corrected by using such rate of recovery.

A series of concentration 10, 20, 30, 40, 50, 60, 70 mg of A falon analytical strandard to 10 and toluene were prepared for obtaining the standard curve (Fig. 1). A suitable aliquot (5 µl) was injected from each concentration.

Enzyme Assay:

Cultures were harvested at the end of the experimental growth phase and extracts were prepared as described previously (Janssen *et al*., 1987).

Isolation of Genomic DNA:

DNA isolation was preformed using the CTAB method of Dayle and Doyle (1990). The cells of three *R. leguminosarum* isolates were collected from colony by pipeting 50 µl of triton X 100 up and down several times over the same spot on the plate. The cells/triton-X100 mixed to 500 µl of CTA buffer to 1.5 ml tubes using vortex (Disruptex Genie) for 2 mins and inoculated at 65ºC for min. The suspension was then mixed with 237 µl of isopropanol and 7.5 M NH_2OAC into a new 1.5 ml tube using vortex 1 min and then inoculated at 65ºC for 15 mins. Then added 500 µl of chloroform: 1.50 µl amyl alconol and mixed by shaking and centrifuged 5 min at max. spped. The upper aqueous layer was transferred to a new sterilized tube 2/3 volume of 150 propanol and NH_4 OAC was added and mixed by shaking the centrifugated for 5 min at max. speed. The pellet was washed carefully twice with 500 µl of cold 70% ethanol, dried at room temperature and resuspended in 20 µl distilled water. DNA was purified by inoculation the resuspended sample at 37ºC for 30 min with RNase (Boehringer Mannheim). DNA concentration was determined using electrophoresis of 5 µl of sample in 0.8 agarose (Antoni *et al.,* 1994).

PCR Amplification:

Amplification of DNA was performed in 10 µl react mixture containing 20 µl template DNA (25 mg), 0.2 µl tag. DNA polymerase (unit), 3.0 µl DNTPs (25 not of each dNTP, dCTP, dTTP, d GTP), 3.0 µl $MgCl_2$ (25 mM), 30 µl PCR buffer (10X), ml 20 µl specific primers (dehalogenase encoding gene, Table (1) and 16.8 µl H_2O (d.w). The mixture was assembled on ice, overlaid with 2 drop of mineral oil. The amplification was carried out in DNA thermal cycler (MWG-Bio TECH Primuse) programmed as follows: One cycle at 94ºC for 2 min and then 30 cycles at 94ºC for 2 min and then 30 cycles at 94ºC for 30 sec., 55ºC for 30 sec and 72ºC for 20 sec. One cycle at 72ºC for 5 min then store in 4ºC final mix of PCR reaction 25 µl.

Gel Electrophoresis Analysis:

All electrophoresis was carried out using a pharmacia GN-100 submarine gel electrophoresis apparatus 1% agarose gel in TAE buffer was prepared and a total sample volume of 6 µl (1 µl of miniprep, 4 µl at H_2O and 1 µl 6X loading

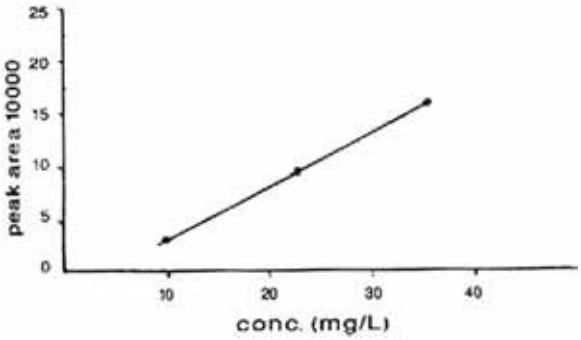

Fig. 1: Standard calibration curve of Kinuron.

Table 1: Oligonucleotide sequences of the primers.

TF	TGGGGCGGATTTTTGGGGCT
TR	GTACGAAATGGCCAGCGTCC

dye) of each DNA extract for 3 isolates was loaded in each well the gel was electrophoresed in 65V for 1.5 hr and then stained with ethrdium bromide solution (10 mg/ml) for about 10-15 min. DNA was visualized on UV transilluminator (1 = 254 nm) and photographed.

RESULTS AND DISCUSSION

Results: Date concerning the inhibition percent in the population of *R. leguminosarum* isolates was differed of linuron herbicide the obtained results clearly indicate negligible effects of linuron herbicide on *R. leguminosarum* isolates. The inhibition percent reached 0, 0, 1.25 and 1.75% of F, M, IS isolates respectively.

R. leguminosarum isolates from different soils under study showed a great ability to degrade herbicide Linuron as a sole source of carbon. They differed in their ability to Linuron degradation. Residues analyzed by GLC after 15 days of inoculation at 28ºC are recorded in Table (2), it is clear that *R. leguminosarum* (F) was the most active isolate degrading linuron, this isolate accumulated 75.25% of linuron as a source of carbon followed by (M) 60.20% then (IS) 50.75% after 15 days (Table 2).

The other isolated of *R. leguminosarum* showed loss assimilation percentage of carbon of herbicide 40.75 and 45.21% for isolates M and IS respectively (Table 2). Therefore 3 isolates were shosen for further studies of biodegrading process of Linuron. The biodegradation of Linuron in sterilized and non-sterilized soil inoculated with *R. leguminosarum*, M, IS and F isolates (Table 2) showed that the amount of herbicide was decreased after 30 days in sterilized soil than uninoculated soil. The residual concentrations were 95.25; 85.75 and 75.95 and 725.25 ppm, respectively. While the concentration of herbicide was completely disappeared in inoculated non-sterilized soil with 3 isolates, but it was only 2.5 ppm in uninoculated soil after 30 days of inoculation (Table 2).

Biochemical Variabit

Dehalogenase Activity:

R. leguminosarum M, IS and F isolates altered in dehalogenase activity. Data in Table (3) showing that, the level of the dehalogenase activity was found to be considerably higher in F isolate followed by IS isolate and M isolate (Fig. 2).

Molecular Variation:

Total DNA was extracted from 3 isolates of *R. leguminosarum*. The integrity and quantity of the purified DNA were confirmed by gel electrophoresis and UV spectrophotometer. The concentration of DNA was about 25 µg/0.5 gm of cells. The purity of DNA was measured by on 260/280 absorbance ratio 1.8 for 3 isolates indicating high yield and purity of the extracted DNA.

Table 2: Biodegradation of Linuron under sterilized and non-sterilized soils inoculated with M, IS and F. *R. leguminosarum* isolates.

		Sterilized soil				Non-sterilized soil			
		Uninoculated		Inoculated		Uninoculated		Inoculated	
Parameters Isolates	Carbon* accumulation	Linuron(ppm)	In(%)	Linuron(ppm)	In(%)	Linuron(ppm)	In(%)	Linuron (ppm)	In(%)
Control 0 time**	0.0	2000	100	2000	100	2000	100	2000	99.5
Menofia (M) 7th	20.25	1960	980	815	40.75	315	15.75	110	5.5
15th	60.20	1940	970	604	3020	115	5.72	70	3.5
Ismilia (IS) 7th	10.20	1980	99.0	904.2	45.21	145	7.25	50	2.5
15th	50.75	1980	99.0	515	25.75	105	5.25	40	2.0
Fayom (F) 7th	25.10	1960	98.0	210	10.25	105	5.25	25	1.25
15th	75.25	1900	950	00	0.0	100	5.0	00	00

** After one hour addition.

Table 3: Dehalogenase activity in *R. leguminosarum* isolates.

	Isolates		
	Dehyalogenase activity		
Time sec.	M-isolate	IS-isolate	F-isolate
0	0.75	1.05	1.10
30	1.58	1.25	1.250
60	1.85	1.50	1.75
90	1.90	1.50	1.95
120	1.90	1.95	1.95
150	1.90	1.95	1.95
180	1.90	1.99	1.95
210	1.90	1.95	1.95
270	1.90	1.95	1.95
300	1.90	1.95	1.95

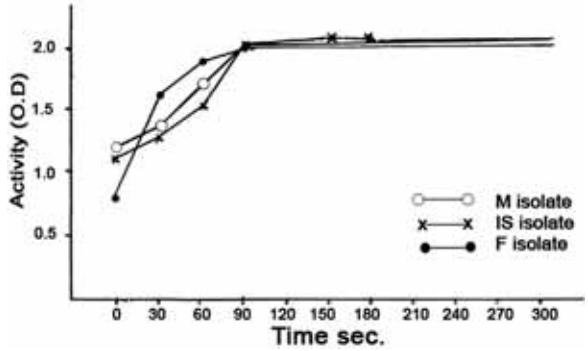

Fig. 2: Illustrates the dehalogenase activity in *R. Leguminosarum* idolates (M. IS and F).

Table 4: Electrophoretic of PCR products banding patterns of Dehalogenase isozymes of *Rhizobium leguminosarum* isolates.

	M isolate					IS isolate					F isolate					Ahl clone				
RF	% of fraction	Width mass	Height	Area calc.	Molecular weight (bp)	% of fraction	Width mass	Height	Area calc.	Molecular weight (bp)	% of fraction	Width mass	Height	Area calc.	Molecular weight (bp)	% of fraction	Width mass	Height	Area calc.	Molecular weight (bp)
0.2171	-	-	-	-	-	9.2	12	67	545	1373.40	-	-	-	-	-	-	-	-	-	-
0.2196	-	-	-	-	-	-	-	-	-	-	14.5	33	75	1213	1054	-	-	-	-	-
0.2972	-	-	-	-	-	-	-	-	-	-	26.3	25	118	2204	936.84	-	-	-	-	-
0.2997	-	-	-	-	-	14.6	14	82	858	921.05	-	-	-	-	-	-	-	-	-	-
0.3786	-	-	-	-	-	76.2	33	184	4489	368.42	-	-	-	-	-	-	-	-	-	-
0.3773	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	61	92	3677	373.68
0.3811	100	14	189	1851	357.89	-	-	-	-	-	59.2	44	174	4955	357.89	-	-	-	-	-

(*): Width mass x Height.
Fraction % Relative to the total DNA contents in each isolate.
M-- isolate: Monofia *R. leguminosarum*. IS = Isolate: Ismailia *R. leguminosarum*. F = Isolate: Fayoum *R. leguminosarum*.

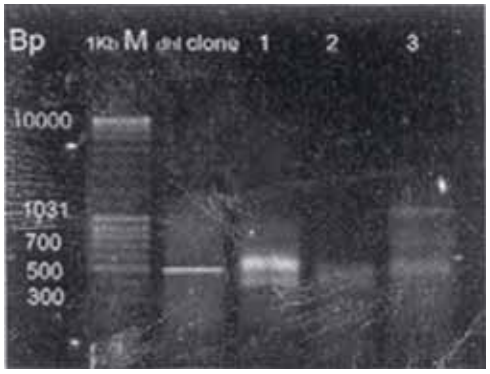

Fig. 3: Agarose gel electrophoresis stained with cthidium bromide showing the PCR product of total DNA isolated from three *R. Leguminosarum* isolates. Arrow indicated the correc size of the amplified product by using downstream and upstream primers desigend in the dehalogenase gene.

Lane M: DNA molecular weight marker
Lane *dhi* clone: Dehalogenase clone.
Lane 1 F1: *R. Leguminosarum* wild type.
Lane 2 Fs: *R. Leguminosarum* sensitive to streptomycin.
Lane 3 FR: *R. Leguminosarum* resistance to streptomycin.

The dehalogenase gene of 3 isolates were amplified from DNAs extracted from cells of 3 *R. leguminosarum* isolates using one step PCR technique. The DNA (5 µl) was mixed with PCR reaction mixture, taq DNA polymerase and homologous and complementary primers directly.

PCR amplification of dehalogenase genes appeared differences among 3 isolates of *R. leguminosarum* (Table 4 and Fig. 3) i.e, number and size of PCR products. The size of PCR product (band) was estimated by comparing its electrophoretic mobility with those of standard DNA markers shown in Fig. (3). It was found that, 6 total bonds of dehalogenase isozymes in all isolates (M, IS and F). The *dhl* clone has one pattern (373, 68 bp) of dehalogenase isozyme. The M-isolate has one pattern (357.89 bp.) isozyme, IS isolate has 3 pattern (1373, 40, 921.05 and 368.42 bp.) isozymes and F isolate has 3 pattern (1359.57, 436.84 and 357.89 bp.) isozymes (Fig. 3). On the other hand, then pattern were differed in RF mobility and density of 3 isolates. Where as, M-isolate near to F isolate in one fraction (isozymes) 357.89 bp but it was differed in density of isozyme. In addition, it was appeared different in height of peak and area, under peak (Table 4). This different among *R. leguminosarum* isolates reacted to genetic variation.

Nucleotide Sequence Analysis:

The partial nucleotide sequence of the PCR amplified fragment for the dehalogenase gene of *R. leguminosarum* (accession number, EU693117) was done to determine the relationship with other recommended

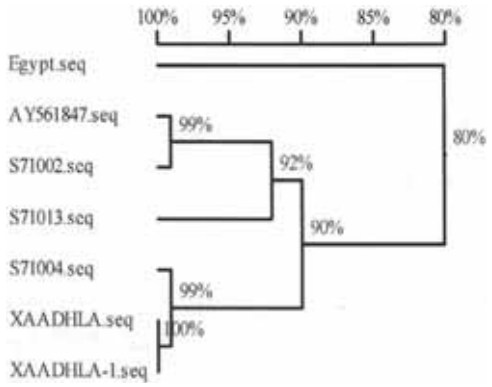

Fig. 5: Phylogenetic three of *R. leguminosarum*/dehalogenase gene based on the nucleotide sequences of the dehalogenase gene. The dendrogram displaying the percentage of sequence homology between the *R. leguminosarum* and the other six isolate of bacteria published in GenBank.

dehalogenase genes of bacteria registered in GenBank. The sequencing was done from the forward direction at Macrogen 3730XL6-1518-009, Korea. The nucleotide sequence revealed the highest content for adenine, thymine, guanine and cytosine. It was aligned by using DNAMAN program (Wisconism, Madisson, USA) with 6 isolate to bacteria which are AY 56, 847 sec, S71002 sec; 571004. sec; S71013 sec.; XAA-DHL A-soc and XAADHLA-1 sec. (Fig. 6).

The nucleotide sequence similarity of *R. leguminosarum* with 6 published bacteria was shown in Fig. 6. A phytogenetic tree of *R. leguminosarum* reduced 80% a moderate degree of similarity to the other bacteria sequences (Fig. 5).

Discussion: The present study reveals that the genus *Rhizobium* was the main inhibitant of the old manuscript. This observation further substantiales the results formerly reported by Hashish *et al*., 1990 and Jonssen *et al*. (1989) who reported that bacteria among others, deteriorating factors causing damage of library materials. *Rhizobium* isolates from the different soils under study showed ability to degrade herbicide, Linuron assimilation as a sole source of either C and N. Residues analysed by GLC after 15 days of incubation at 28ºC. The biodegradation of herbicide was decreased after 30 days in sterilized soil and increased after 15 days in non-sterilized soil.

We can conclude from the previous results that *R. leguminosarum* isolates can be used as degradative factor, for the harmful herbicide residues in the soil, specially Linuron. The inoculation of soil with *Rhizobium* spp., one week earlier the application of pesticides gives a high security in using certain herbicides. In this case, the produced crops obtained from the treated soil by *Rhizobium* spp. Would be free from of the harmful herbicide residues for man and animal use.

The results indicate that the degradation of Linuron residues in the untreated soil, reached 0.075, 0.065, 0.025 and 0.010 ppm at 0, 3, 7 and 14 days respectively. This may be attributed to the concentration of soil from previous application or from drift occurrence of this herbicide. Linuron residues were detected in potted soil with bacterial population, but in different values because of bacterial type and time after treatment. The Linuron amount in ppm reached 0.089, 0.046, 0.029, 0.016 (M); 1.065, 0.166, 0.092, 0.027 (IS) and 1.151, 0.159, 0.035, 0.026 ppm (F) isolates at 0, 3, 7 and 14 days respectively. The percentage of the loss in Linuron residues from the initial deposit (0-time) was 0.0 and then increased to reach 85.05, 43.67 and 20.68% after 3, 7 and 14 days respectively, M was reached to 51.68; 32.58; 17.97; IS was 13.81, 3.04, 2.25% and F was 15.58, 8.6, 2.53% at 2, 7 and 14 days respectively.

These results are in agreement with those obtained by Tapp and Stotzky (1997) that the toxins from *B. thuringiensis* subsp. tendebrionis and Kurstaki were responsible for degradation of herbicides, therefore they were it bound on clay or silt sized particles of amended to 6% v/v with the clay minerals montmorillonite or kadoliite (as an internal control). The role of herbicides on bacterial population in treated soil was intensively studied and reported by Berger (1998) who stated that *Pseudomonas* sp. biotransformed Linuron was only bacteria capable of significant biotransformation and reduced phenylurea concentrations in liquid culture. Shin and Cheney (1984) conducted a trial to determine the effects of alcohol, Linuron, Simazine and nonselective paraquat on *Bradyrhizobium japonicum* bacteria Alachlor and Linuron were decreased significantly by 27.4 and 57.8% respectively. While little effect was observed in simazine and marked

reduction of survival was observed in 200 ppm of parquet. The effect of alachlor differed with isolates of the nodule bacteria, isolate 1-122 was relatively resistant and 1-145 was more sensitive than other isolates tested. Inermediate K-5 strain was most resistant at the recommended dose and the survival dose was drastically reduced with increament of concentration. Nodulation of the soybean was different with the varieties and isolates. Most nodulation was observed in the resistant isolates 1-122 and K-5 strains for which the nodulation may be related to the resistance to environmental stress factors.

The degradation of Linuron by *R. leguminosarum* proceeds through the concerted action of enzymes that are specific for halogenated compound and enzymes that are involved in the metabolism of natural compounds. The former are the dehalogenases that catalyze hydrolysis of Linuron. The enzymes show a broad substrate range and one only produced by isolates that utilize halogenated substrates and thus can be considered enzymes that are acquired by this specific strain of *R. leguminosarum* during genetic adaptation to degradation of chlorinated hydrocarbons. This paper describe present a further one lysis of the data alkane dehalogenase encoding gene *dhlA*. The absence of dehalogenase activities in natural of *R. leguminosarum* allowed the identification of clones containing the dehalogenase gene. Isolation of genes involved in methanol-dehalogenase and chloroacetaldehyde dehydrogenase activity was possible by screening for complementation of mutants lacking the dehalogenase activities. In this way; harboring genes were identified and the genes were localized on different DNA segments.

The D NA segment harboring the dehalogenase gene was further characterized by DNA-sequencing. The dehalogenase gene sequence that are in agreement with date that were previously with the purified enzyme (Keuning *et al*., 1985) which was compared to the others sequence data bank.

The efficient expression of the halokinase dehalogenase gene in other gram-negative bacteria is not surprising in view of the fact that two regions with the consensus *E. coli* promoter sequence were present. Copy number probably also plays a role, since expression levels were higher in *Xanthobacter autotrophicus* GJ10 ($_{p}$p120) than in the wild-type isolates GJ10. The *E. coli* consensus promoter sequence is known to stimulate transcription in *Pseudomonas* spp. (Jeenes *et al*., 1986) and our data suggest that it might also do so in *Rhizobium* spp. In order to determine which of these sequences is the actual cause of the high expression and whether the promoter can be used for expression of the others genes in *Rhizobium* spp., it will be necessary to identify the transcription start site of the gene and to study expression of different genes linked to the promoter regions.

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