Australian Journal of Basic and Applied Sciences, 6(7): 425-429, 2012
ISSN 1991-8178

Molecular Monitoring of Gramonol Herbicide Biodegradation in Relation to *Streptomyces scabies* Isolates

Abbas, I.H.

Botany Department, Faculty of Science, Benha University, Egypt

Abstract: The aim of this study was to test the effect of *Streptomyces scabies* on the degradation of Gramonol herbicide in order to reduce its residual toxicity. Four isolates of *S. scabies* (St_1, St_2, St_3, and St_4) showed different ability to degrade the gramonol *in vitro* and in soil. They varied in their potential ability to degrade Gramonol in vitro after 5 days incubation, and after 35 days post herbicidal treatment. *S. scabies* isolate St_4 was the most active of the four isolates. To demonstrate this effect, PCR in DNA genome was used to detect dehalogenase gene in the 4 isolates and residual Gramonol was determined by GLC after 10, 20 and 30 days. The study showed the degradation rate of Gramonol increased with time and that less Gramonol residue was found in non sterile soil compared to sterile soil. However, Gramonol did not show an effect on the Streptomyces isolates growth. Therefore, Streptomyces scabies can be used to degrade Gramonol herbicide and reduce toxicity hazard to man.

Key words: Herbicide, Biodegradation, Dehalogenase, Gas liquid chromatography polymerase chain reaction.

INTRODUCTION

The biodegradation behavior of pesticides on and in various edible plants has been studied by numerous authors. These previous studies concluded that the current levels of the pesticides in surface water and plants did not constitute an acute toxicity hazard to man on a short term basis, but as a result of its accumulation in the body it causes hazard after long term exposure **(**Azhar, 2009**).** On the other hand some studies were published about the methods of removing pesticides from soil and water by biodegradation using micro-organisms **(**Yokota *et al*., 1987**;** Dahrog *et al*., 2006**;** Azhar, 2009**).** So far haloalkinase dehalogenase is the only enzyme known to be capable of direct hydrolytic dehalogenation of chlorinated and brominates hydrocarbons without the requirements for enzymes or oxygen. The enzymes of *Xanthomanas autotrophic* and *Rhizobium* sp. constitutively expressed 2 to 3 % of the solute cellular protein up to 30 % (Keuning *et al.*, 1985 and Azhar, 2009**).** They have a remarkably broad substrate range which includes terminally halogenated alkenes with chain lengths up to 4 carbons for chlorinated and up to at least 2 carbons for brominates alkenes. Other haloalkinase Dehalogenase of broad substrate range have been found in gram positive haloalkinase, utilizing bacteria **(**Janssen et al, 1989**).** The Objective of the present study was to clarify the degradation of gramonol herbicide by *Streptomyces* sp. under laboratory and pot- experiments.

MATERIALS AND METHODS

The degradation of gramonol herbicide by *Streptomyces* sp. was studied under laboratory and pot-experiments in the Botany Department, Faculty of Science, Benha University during the spring season of 2010 by (biodegradation) using micro-organisms **(**Dahrog *et al*., 2006 and Azhar, 2009**).**

Source of Herbicide:

Gramonol 24% liquid E.C. supplied by Imperial Chemical industries limited U.K. (ICI), containing frequent Ion, 100 g/L (1-1 dimethyl, 4-4-bipyridylum ion and Monolinuron), 140 g/L, 3- (4-Chloropenyl) -1- methoxy-1-methyl urea.

Source of Streptomyces:

Four *Streptomyces scabies* i.e. St_1, St_2, St_3 and St_4 were kindly obtained from Botany Dept. Fac. of Sci. Benha Univ.

Laboratory Experiment:

Gramonol Herbicide Clearing Zone:

The assay was done on starch- nitrate agar medium (containing 20g starch, 2g $KP0_4$, 1g $KH2P0_4$, 0.5g $MgS0_4$, 0.5g NaCl, 3g $CaC0_3$; 0.01 $FeS0_4$; 1g trace salt solution in one liter according to Tadashi (1975), and

Corresponding Author: Abbas I. H., Botany Dept. Faculty of Science, Benha University, Egypt.

supplemented with 0.5% (V/V) of emulsified gramonol (50%) . The assay was carried out on Petri dishes under aseptic conditions. The proper amount of herbicide was added using a pipette onto the surface of the individually labeled agar plates. Each of the four *Streptomyces* isolates was distributed at $1x10^5$ to the surface of the Petri dishes, in three replicates of each isolate. The Petri dishes were incubated at 37°C and 65 ± 1% relative humidity. After 5 days, the Petri dishes were inspected to estimate the efficacy of *Streptomyces* isolates on the degradation of Gramonol herbicide by using the methods according to (Dahrog *et al*., 2006).

Potted Experimentally Mottling System:

The soil samples were taken at 15 cm depths and mixed with organic fertilizer at the ratio 2:5 (w/w) and sterilized at 121°C/1 air pressure for 20 min. The sterilized soil was transferred into pots and injected with $5.2x10^4$ of each *Streptomyces* isolates in each pot according to Blazevic and Ederer (1975). Water (200ml) was poured into each pot to raise the moisture of the soil. After three days the pots were sprayed with Gramonol 50% WP solution at the recommended concentrations, and then 25ml urea (30%) was added in each pot for the purpose of adjusting pH and to activate *Streptomycetal* growth. *Streptomyces* isolates were counted in treated and untreated soil samples at 10, 20 and 30 days post soil inoculation. Part of the soil was kept in freezer up till analysis in order to determine the concentration of Gramonol herbicide.

Determination of Gramonol Residues:

Gramonol herbicide residues were extracted cleaned up and the concentrations were determined according to Dahrog *et al.* (2006). Soil, free from the tested herbicide, was used to estimate the rate of recovery by using the previous procedures. The mean of the obtained recovery was 90.5%. All the obtained data for the residues of Gramonol on treated soil was corrected by using such rate of recovery.

A series of concentrations (10, 20, 30, 40, 50 and 60 mg) of Afalon analytical standard in 10ml Toluene were prepared to obtain the standard curve. A suitable aliquot (5ml) was injected from each concentration.

Determination of Dihalogenase Activity:

Cells of *Streptomyces scabies* isolates were cultured in starch nitrate broth medium **(**Tadashi, 1975**)** and incubated at 28°C with shaking at 200 rpm for 24h. The cell cultures were harvested at the end of the experimental growth phase. The portion was extracted from cell cultures as described by Bradford (1976). The enzyme activity was assayed according to Janssen *et al.* (1987).

Isolation of Genomic DNA:

From *Streptomyces scabies*, cell cultures were harvested by centrifugation. The DNA was extracted using CTBA method as described by (Owen and Borman 1987).

Detection of Dihalogenase Gene DNA:

The dihalogenase gene was amplified by polymerase chain reaction (PCR) in 100 ml mixture containing: 20ml template DNA (25 mg), 0.2ML taq polymerase, 12.0µl dNTPs (25 mM of dATP, dTTP, dCTP and dGTP), 3.0µl $MgCl_2$ (25MM), 30µl PCR reaction buffer (10X), 20 µl specific primers (Dehalogenase encoding gene) (TF primer tgggcggatttttgggget and TR primer: gtacgaaatggccagcgtcc) and 23.8µl d H_2O. The PCR program included one cycle at 94°C for 2 min and then 30 cycles at 94°C for 30 sec.; 55°C for 30 sec. and 72°C for 20 sec. followed by staining with Ethidium bromide and visualized on UV Tran illuminator and photographed.

Results:

Potent Biodegradation of Gramonol:

In vitro, *Streptomyces scabies* population under study showed a great ability to degrade gramonol herbicide. Differences in the degradation levels were noted. The result showed that the levels of Gramonol degradation were 11.66, 8.33, 7.50 and 12.50 ppm with 21.20 , 16.6 , 15.0 and 25.0 % of *S. scabies* isolates St_1, St_2, St_3 and St_4 respectively (Table1).

Table 1: The potency of Streptomyces scabies on biodegradation of Gramonol herbicide.

Streptomyces isolates	Diameter cleaning Zone (cm)	Amount of gramonol degradation (ppm)	Percent of Degradation (%)
St_1	3.50	11.66	21.20
St_2	2.50	8.33	16.6
St_3	2.25	7.50	15.0
St_4	3.75	12.50	25.0

Plate diameter 15 cm; Gramonol amount in plate 5% (50 ppm/plate), Plate diameter 15 cm.

In vivo, the data regarding the degradation of Gramonol in the presence of *S. scabies* (St_1, St_2, St_3 and St_4) after 10, 20 and 30 days soil treatment showed that the concentration of Gramonol decreased faster in non-sterilized soil than sterilized soil. The residual amounts of Gramonol were 1050, 1600, 1550 and 750 ppm in

sterilized soil after 30 days soil treatment inoculated with St_1, St_2, St_3 and St_4 respectively. The total count of *S. scabies* isolates decreased in the first few days then increased gradually under sterilized and non-sterilized soil. The count of *S. scabies* isolates was 3.1; 2.2; 2.3 and 4.5 in sterilized soil, and was 2.1, 7.1, 7.2 x 8.7 and $x10^5$ in non-sterilized soil at 30 days of St_1, St_2, St_3 and St_4 respectively (Table 2).

Table 2: Potent degradation of *Streptomyces scabies* isolates of gramonol herbicide in soil.

Period soil inoculation (days)	Sterilized soil			Non-sterilized soil		
	Gramonol (ppm)	residual (%)	total count	Gramonol (ppm)	Residual (%)	total count
10 St_1	3700	74	$6.0x10^4$	2700	54	$6.7x10^4$
St_2	4000	80	$4.1x10^4$	3000	60	$6.5x10^4$
St_3	3750	75	$3.7x10^4$	3000	60	$5.2x10^4$
St_4	2600	52	$5.7x10^4$	2100	42	$7.3x10^4$
20 St_1	2650	53	$7.7x10^4$	2000	40	$7.1x10^4$
St_2	2250	45	$7.5x10^4$	1750	35	$7.2x10^4$
St_3	2600	52	$6.2x10^4$	2050	41	$6.5x10^4$
St_4	1500	30	$80x10^4$	1000	20	$8.5x10^4$
30 St_1	1050	21	$3.1x10^5$	900	18	$8.1x10^5$
St_2	1600	32	$2.2x10^5$	1050	21	$7.1x10^5$
St_3	1550	31	$2.3x10^5$	1250	25	$7.2x10^5$
St_4	750	15	$4.5x10^5$	500	10	$8.7x10^5$

Inject inoculums 5.2x104 CFU *S. scabies*; Gramonol recommended at zero time 5000 ppm.

Dehalogenase Activity:

The Protein content was determined in *Streptomyces* isolate relative to BSA standard protein. *Streptomyces scabies* isolate St_1 induced high protein content (2.15) followed by St_4 (1.95), St_3 (1.85) and St_2 1.75 mg/1g cells. The dehalogenase specific activities were 285.2; 290.2; 275.6 and 235.7 unit/mg protein. The units per gram growth were 499, 413.3; 425.3 and 357.5 for St1, St2, St3 and St4 isolates respectively (Table 3).

Table 3: Protein content and dehalogenase activity in *Streptomyces scabies* isolates.

Streptomyces isolate	Protein content (mg/g growth)	Dehalogenase Specific activity	(Units/g growth)
S. scabies without gramonol	1.12	125.1	235.5
S. scabies (St1)	2.15	285.2	499
S. scabies (St2)	1.75	290.2	413.3
S. scabies (St3)	1.85	275.6	425.3
S. scabies (St4)	1.95	255.7	357.5

Specific activity (Unit/mg protein)

Dehalogenase rate activity of *Streptomyces scabies* were tabulated in Table (4). Its data demonstrated that *S. scabies* isolates showed variations in dehalogenase rate activity. St_1 showed a higher rate activity followed by St_4, St_2 and St_3. The ranges were between 1.25 to 4.00, 1.15 to 3.50, 0.95 to 3.00 and 0.75 to 2.00 respectively. The data in the same table also showed increased dehalogenase rate activity of all *Streptomyces scabies* isolates with increasing time used from zero to 7min respectively.

Table 4: Dehalogenase rate activity of *Streptomyces scabies* isolates.

St_4	St_3	St_2	St_1	Time
1.15	0.75	0.95	1.25	0 time
1.50	0.95	1.49	1.75	1.0 min
2.25	1.25	1.75	2.50	2.0 min
2.50	1.50	2.10	2.75	3.0 min
3.15	1.75	2.25	3.25	4.8 min
3.50	2.00	3.00	4.00	5.0 min
3.50	2.00	3.00	4.00	6.0 min
3.50	2.00	3.00	4.00	7.0 min

Dehalogenase Activity Genetically:

The amplification of Dehalogenase gene of *S. Scabies* isolates showed variation among isolates, i.e. number of amplified DNA fragment, size and density of bands. The data in Table (5) and Fig. (1) show 11 amplified bands (dehalogenase isozymes) for 4 isolates as well as one amplified band of dh1 clone (510 bp) of dehalogenase.

S. scabies St1 isolate has 3 bands (950and 600 bp), St_2 has 2 bands (1350, 510 bp). St_3 has 3 bands (1350, 880 and 510) and St_4 has 3 bands (1350, 880 and 510). On the other band, these isozymes bands were different in density (percent of Fraction). The variability among the 4 isolates showed one monomorphic amplified band 510 bp (common in 4 isolates), two polymorphic (specific simplified bands 1350 and 880 bp and two unique

bands (genetic marker) in St_1 isolate 950 and 600 bp. The variation between the isolates in their degradation of Gramonol herbicide is linked to the genetic variability among them.

Table 5: Dehalogenase isozymes of *Streptomyces scabies* isolates detected using Polymerase chain reaction.

	Dhl Clone		St_1		St_2		St_3		St_4		
Rf	%	bp	%	bp	%	bp	%	bp	%	bp	Polymorphism
0.30	_	_	_	_	25	1350	15	1350	10	1350	Polymorphic
0.30	_	_	50	950	_	_	_	_	_	_	Unique
0.40	_	_	_	_	_	_	35	880	30	880	polymorphic
0.45	_	_	20	600	_	_	_	_	_	_	unique
0.50	100	510	30	510	75	510	50	510	60	510	Monomorphic

Rf = Relative mobility, % = percent of amplified band; Bp = size of expect band; Monomorphic = common amplified fragment band; Polymorphic = specific amplified fragment band; Unique = Genetic marker.

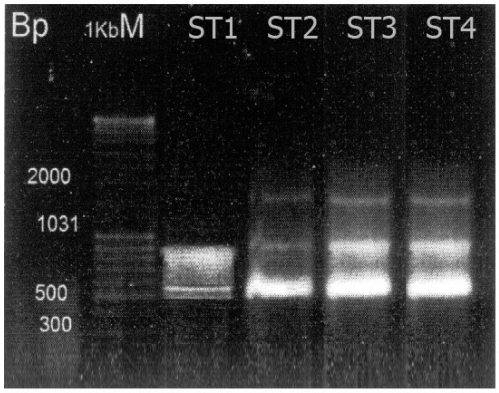

Fig. 1: Agarose gel 1.5% stained with ethidium bromide showing the PCR products of amplified dehalogenase gene of four S. *scabies* isolates using specific primers. (M. DNA leader 100 bp, St_1, St_2, St_3 and St4: *Streptomyces scabies* isolates).

Discussion:

Herbicides are consumed annually with greater quantities for the control woods. The behavior of herbicide in soil is regulated by herbicide properties, soil microbiology and climate conditions. The herbicide enters the plant seedling through the root and shoots (Workman *et al*., 1995). In our chemical warfare against a multitude of noxious organisms in the soil, it is necessary to avoid the harmful effect of these herbicides, together with their various carrier's diluents and solutes, on the various beneficial soil micro-organisms with their biological activities contributing to soil fertility.

Streptomyces scabies showed potential variability to degrade Gramonol herbicide *in vitro* and *in vivo*. The analysis of Gramonol residues by GLC at 10, 20, and 30 days post – inoculation showed the gradual reduction of Gramonol residues to be higher in non– sterilized soil than sterilized soil. This difference may be due to the presence of microbial flora in the soil and its role in the biodegradation of Gramonol.

The rate of herbicide application on the population density of soil microbes was studied by Roberts *et al*. (1993) and reported that *Pseudomonas* Sp. were important components of the population responsible for degradation of Limuron. It showed that the culture mineralized Limuron completely. No intermediated degradation products were detected in the medium. These results are in agreement with those obtained by Tapp and Stotzky (1997) and Dahrog *et al*. (2006).

This Study showed that the degradation of Gramonol was detected in vitro with the use of *Streptomyces scabies* and with different values among its isolates. These results are in agreement with those obtained by Hashish *et al*. (1990**)** and Dahrog *et al*. (2006). Azhar (2009) studied the ability of soil micro-organisms to degrade Limuron, Diuron, Ktozin and dichlofunoid. The actual degradation of the herbicide and fungicide by micro-organisms is caused by the release of enzymes that break them down or that because of bacteria capable of significant biotransformation and reduction of phenyl urea concentrations in liquid culture.

The Gramonol degradation by *Streptomyces scabies* isolates is done through the converted enzyme action that are specific for halogenated compounds and enzymes that are involved in the metabolism of natural compounds. The dehalogenase enzymes show a broad substrate range and one only produced by the isolates that utilize halogenated substrate. It can be considered that these enzymes are acquired by this specific strain of *Streptomyces scabies* during genetic adaptation for degradation of chlorinated hydrocarbons. The present study describes a further analysis of the data alkenes dehalogenase encoding gene dh1A. The presence of dehalogenase activities in natural *S. scabies* allowed the identification of clones containing the Dehalogenase

gene. Isolation of the gene involved in methanol-dehalogenase and chloro-acetaldhyde dehalogenase activity was possible by screening for complementation of mutants lacking the Dehalogenase activities. In this way, the harboring genes were identified and the genes were localized in different DNA segments.

The efficient expression of the halkinase dehalogenase gene in other gram positive bacteria is not suppressing 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 autotropicus* G10 (p120) than in the wild type isolated G110 (Dpyle *et al*., 1984 and Jeenes *et al*., 1986) and the obtained data suggest that it might also do so in *Pseudomonas* sp. In order to determine which of these sequences is the actual cause of the higher expression and whether the promoter can be used for expression of other genes in *Pseudomonas* sp.; it will be necessary to identify the transcription short site of the gene and to study the expression of different genes linked to the promoter regions.

Conclusion:

Streptomyces scabies was found to have a great ability to degrade pesticides from the soil. It can be used to degrade Gramonol herbicide and reduce toxicity hazardous to man. The power of degradation varied among Streptomyces scabies isolates. Isolate St_1 was the most effective of the four isolates studied resulting in lower concentration of Gramonol in the soil sample. The degradation rates of Gramonol increased with time producing an optimum effect after 35 days post treatment. Gramonol did not show an effect on Streptomyces scabies growth in the soil.

REFERENCES

Azhar, A.E.S., 2009. Biodegradation of Duron herbicide in relation to *Pseudomonas* sp. Egypt. J. of Appl. Sci., 24(5): 225-240.

Blazevic, D.J. and G.M. Ederer, 1975. Principles of Biochemical Tests in Diagnostic Microbiology. John Wiley and Sons Inc., New York, USA.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Analytic. Biochemist., 72: 248-254.

Dahrog, S.M.A., W.M.A. El-Sayed, A.M. Abd-Allah and H.S.I. El-Masselati, 2006. Degradation of Linuron and metribuzi herbicides in relation to microbial population of *Bacillus* and *Pseudomonas* sp. J. Environ. Sci., 13(1): 131-149.

Dpyle, J.L., D.N. Nunn and M.E. Ldstrom, 1984. Molecular cloning of malyl coenzyme A lyase gene from *Pseudomonas* sp. strain AM1-A facultative methyltroph. J. Bacteriol., 160: 718-723.

Hashish, R.M., M.A. Azazy and A.N. Ibrahim, 1990. Biodegradation of fungicide Kilazin–p by soil microorganisms in relation to its residues in rice grains. Ann. Agric. Sci., 2: 653-665.

Janssen, D.B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra and B. Witholt. 1989. Cloning of 1, 2-dichloroethane degradation genes of Xanthobacter autotrophicus GJ10 and expression and sequencing of dh1A gene. J. Bacteriol., 171: 6791-6799.

Janssen, D.B., C. Kaining and B. Wrihold, 1987. Involvement of a quinoprotein alcohol dehydrogenase and an NAD development aldehyde dehalogenase in 2-chloroethanol metabolism in *Xanthobacter autotrophicus* GJ10. J. Gen. Microbiol., 133: 85-92.

Jeenes, D.J., L. Soldatri, H. Baur, J.M. Walson, A. Mercenler, C. Relammann, T. Leisinger and D. Haus, 1986. Expression of biosynthetic gene from *P. aeruginosa* and *E. coli* in the heterlogus host. Mol. Genet., 203: 241-429.

Keuning, S., D.B. Janssen and B. Witholt, 1985. Purification and characterization of hydrolytic halokalane dehalogenase from *Xanthobacter autotrophicus* G110. J. Bacteiol., 163: 635-669.

Owen, R.J. and P. Borman, 1987. A rapid biochemical method for purifying high molecular weight bacterial chromosomal DNA for restriction enzyme analysis. Nucleic acids Res., 15: 3631.

Roberts, S.J., A. Walker, N.R. Parekh, S.J. Welch and M.J. Waddington, 1993. Studies on a mixed bacterial culture from soil which degrades the herbicide Linuron. Pesic Sci., 39(1): 71-78.

Tadashi, A., 1975. Availability of phosphorus in manipulated rhizosphere of Faba-bean. Japan National Agricultural Lab., 1: 1-13.

Tapp, H. and G. Stotzky, 1997. Monitoring the insecticidal toxins from *Bacillus thuringiensis* in soil with flow cytometry. Cand. J. Microbiol., 43(11): 1074-1078.

Workman, S.R., N.R. Fausey and S.E. Noke, 1995. Pesticide dissipation and transport in the root zone of the Ohio MESA- Clean water clean environment 21st nature: Team agriculture working to protect water resources. Vol. 1: Pesticides proceedings Kansas City, Missouri, USA 5-8, March. 1995.

Yokota, T., T. Omori and T. Kodama, 1987. Purification and properties of haloalkane dehalogenase from *Corybacterium* sp. strain ml5-3. J. Bacteriol., 169: 4049-4054.