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BIODEGRAELATOIN OF MALATHION BY FUNGI ISOLATED FROM CONTAMINATED SOIL THROUGHOUT THE USE OF MILLET AS BIOINDICATOR

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ABSTRACT

Malathion degradation was studied *in vitro* and *in vivo* during the period from 2002-2004 to investigate the biological removol of organophosphorus insecticide, malathion, by *A. niger, A. flavus* and *T. viride* as a sole phosphorus source and to determine its residues and its major metabolities. Results indicated that malathion was not toxic to tested fungi at concentration up to 500 ppm. *In vitro* tests showed that fungal growth and fresh weight was increased by increasing the concentration of insecticide applied to some extent. *A. niger* degraded malathion more efficiently than did *A. flavus* and *T. viride*. As a result of inoculation with biodegraders, % loss in complete Dox medium was 90.5, 92.2 and 95% compared to 91.3, 93 and 98% in P-free medium when inoculated with *T. viride, A. flavus* and *A. niger*, respectively. O,S, S-trimethyl phosphorodithioate was the major metabolite. Inoculation of malathion-treated sawdust, as a growing medium, with *A. niger* improved significantly millet growth and this effect was more pronounced when incubation period with the fungus was increased. These results suggest the use of these candidates for bio-disposal of malathion.

Keywords: Biodegradation, malathion, organophosphorus pesticide, millet, *Aspergillus niger, Aspergillus flavus, Trichoderma viride*

INTRODUCTION

The use of insecticides for controlling plant diseases and pests characterizes the modern agriculture. Malathion is an organophosphorus (OPPs) insecticide that has been used for the control of insects in field crops, fruits, vegetables and livestock and as a substitute for DTT (Rettich, 1980; Chambers, 1992 and Barlas, 1996).

As the case with all chemicals, malathion has some drawbacks in terms of their costs, ecological impacts (Menzer, 1987; Howard, 1991 and Kidd and James, 1991), risks to the operators and risks opposed to the consumer safety as a result of accumulation of theses compounds in the human body. It has been reported that malathion affects central nervous system of invertebrates, immune system of higher vertebrate wildlife, liver and blood of fish (Senanayake and Karalliedde, 1987; El-Dib et *al.*, 1996; Galloway and Handy, 2003). Also, it has been reported that three types of

cultured human cells, including white blood and lymph cells, are mutated by malathion (Gallo and Lawryk, 1991; U.S. Public Health Service, 1995).

After application, the insecticides may reach the soil by different ways such as run-off from leaves and stems (Hill and Wright, 1978), treated seeds, root exudates or waste degradation of treated plants (Sato and Tanaka, 1987). Incorporation of pesticides into the soil affects not only the target pests but also non-target beneficial groups of soil flora (Sahrawat, 1979, Omar, 1991, Omar and Abd-Alla, 1992, and Abdel-Mallek et *al.*, 1994).

Biological removal of OPPs pesticides became the method of choice, since microorganisms could use a variety of xenobiotic compounds including pesticides for their growth and mineralize and detoxify them (Kanekar et *al.*, 2004). Depending on the soil environment, it has been found that malathion degradation was faster in non-sterile than in sterile soil. The initial half-life of malathion in non-sterile and sterile soil samples was 7 and 21 days, respectively. A comparison of the residues remaining in the soil samples after 14 days of incubation showed that 50% of total insecticide loss was due to biological degradation (Anyanwu and Odeyemi, 2002). Also, it has been found that some fungal strains, including *Aspergillus niger*, efficiently degraded considerable amounts of some organophosphorus pesticides such as dimethoate (49%), malathion (48%), chlorpyrifos (54.5%), profenofos (81.8%) and cypermethrin (54.2%).

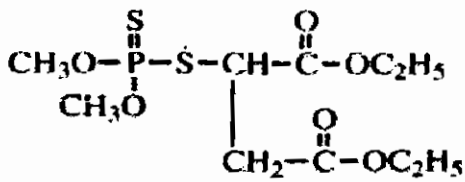
On the other hand, several studies have reported that millet is inexpensive and nutritionally comparable or even superior to major cereals (Pathak *et al.*, 2000). It is also important food cereal in many parts of Africa, Asia and semi-arid tropics worldwide (Sanaa *et al.*, 2006) where it has been found that it contains considerable levels of constituents of health benefits for humans such as antioxidants and anti-disease factors (Juntunen *et al.*, 2000; Karppinen, *et al.*, 2003 and Rieckhoff *et al.*, 1999).

The purpose of this work was to investigate the rate and metabolites produced as a result of biodegradation of malathion by fungi commonly isolated from soil environment. The bioassay test on millet plants was also considered.

MATERIALS AND METHODS

Pesticide

Organophosphorus insecticide, malathion [O,O-dimethyl S-1, 2-bis (ethoxy carbonyl) ethyl phosphorothioate], commonely used in Egypt's modern agriculture was used in this investigation and purchased from Kafr-El-Zyat Company for Chemicals and Pesticides. Its chemical structure is as follows:



$C_{10}H_{19}O_6PS_2$

Soil

Samples of clay loam soil that has history of previous exposure to malathion insecticide were collected from Mostorod Farm, Faculty of Agriculture, Al-Azhar University. The soil had the following physical and chemical properties: pH, 8.5; E.C, 2.2 dS cm^{-1}; organic matter, 0.26%; total $CaCo_3$, 2.1%. The soil contained Ca^{++}, Mg^{++}, Na^{++} and K^{+} of 3, 8, 11.7 and 0.35 meq/l whereas Co_3^{-}, HCo_3^{-}, Cl^{-} and SO_4^{-} were 0.00, 9.0, 9.5 and 4.55 meq/l, respectively.

Organisms and culture media

The soil samples were taken from the top 25 cm, air dried and passed through a 2-mm sieve where 500 g portions were placed in plastic containers. Freshly prepared pesticide was applied at rates of 10, 50, 100 and 500 ppm to the soil in addition to the control untreated soil. After 4 weeks of incubation at 25±1°C, where the moisture content of the soil was kept at 60% of water holding capacity (WHO), 10 g portions of each treated soil were used for isolation of fungi using serial dilution plate technique (Johnson *et al.*, 1959) as 1 mL aliquot was plated into Petri dishes contained Potato Dextrose Agar (PDA) medium as isolation medium. Rose Bengal was added to the medium at a rate of 65 ppm as a bacteriostatic agent. The plates were then incubated at 23-25°C for 3-5 days. Following incubation, the developed colonies were sub cultured on PDA slants and single spore technique was used for purification of fungal isolates. Isolated fungi colonies were identified by Plant Pathology Section, Department of Botany, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.

Utilization of malathion as phosphorus source

Fungal isolates were screened for their ability to utilize malathion as a sole P-source on Czapek's-Dox liquid medium with and free of P source. The medium contained (per liter): Dextrose, 20.0 g; $NaNO_3$, 2.0 g; $KHPO_4$ (P source) 1.0 g; KCl, 0.5 g; $MgSO_4$, 0.5 g and $FeSO_4$, 0.01 g. The pH of the medium was adjusted at 7.0. Aliquots of 100 ml of the medium were dispensed in 250 ml flasks and autoclaved for 20 min. After cooling, freshly prepared malathion was added aseptically at final concentrations of 0, 10, 50, 100, 200, 300, 400 and 500 ppm. Inoculation of the media was carried out, in triplicates, by introducing a disk (1.0 cm diameter) of 3-4 days old of each

individual fungus grown at 28±1°C on PDA agar medium into insecticide-amended media. Flasks were then incubated in dark with shaking at 28±1°C for 4 weeks. Following incubation, mycelial growth of each individual fungus was harvested by filtration on filter paper (Watman No. 1) and washed several times with sterile distilled water. The mycelial yield was plotted with filter paper to remove excess water where mycelial fresh weight of each fungus was recorded.

Determination of malathion residues

Fungal mycelial was mixed in a mortar with an approximately equal weight of clean sand, pulverized and extracted with 70% ethanol. The obtained slurry was centrifuged at 6000 rpm for 10 min. as the supernatant was decanted and used for colorimetric determinations of total P content by ascorbic acid method according to Murphy and Riley (1962).

Detection of malathion-derived degradation products

The culture filtrate was analyzed for pesticides residues and their degradation products by a modified method of Jarczyk (1983). Aliquots of 50 ml liquid medium were dispensed into 250 ml separator funnel and 100 ml of dichloromethane: ethylacetate mixture (9:1) was added followed by shaking for 3-5 min. The organic layer was separated whereas the aqueous layer was re-extracted twice by two volume of dichloromethane: ethylacetate mixture. The collected organic layers were passed down in a column containing granular anhydrous sodium sulphate and the solvent was evaporated in rotary evaporator. The remaining sample of culture filtrate was used to determine malathion residues by GLC (Hewlett-Packared, USA serial 6890) equipped with Flame Photometric Detector (FPD) and the column was PAS-1701, 30m x 0.32mm (internal diameter) x 0.25 film thickness. The operating conditions were as follows: injector temperature, 240°C; detector temperature, 250°C; oven temperature, 230°C; injector volume, 2 µl; nitrogen carrier gas flow rate, 3 ml/min; air flow rate, 100 ml/min and retention time, 2.850 min. Structures of malathion metabolites were identified by GC-mass spectrometry. GC-MS analysis was performed with an Agilent 6890 gas chromatograph equipped with a mass spectrometric detector (MSD) model Agilent 5973. A fused silica capillary column (HP-5MS) and phenyl polysiloxane as non-polar stationary phase (30m x 0.25mm internal diameter) x 0.25 µm film thickness were used. The operating conditions were as follows: injector port temperature, 250°C; Helium as carrier gas at a flow rate of 1 ml/min pulsed split less mode; column temperature, 80°C for 3 min then 260°C for 15 min; total analysis time 40.5 min; 1 µl volume injected split less. The mass spectrometric detector (MSD) was operated in electron impact ionization mode with an ionizing of 70 eV, scanning from m/z 60 to 500 at 3.62 per scanning. The ion source and quadrupole temperatures were 230 and 150°C, respectively. The electron multiplier voltage (EM voltage) was maintained at 1000 V above auto tune, and solvent delay of 2 min was employed. The instrument was manually tuned using perfluorotributylamine (PFTBA).

Bioassay

To confirm the biodegradation of malathion by *A. niger*, millet plant (*Millet bicolor* L.) was used as an indicator for biodegradation of malathion as our preliminary screening (data not published) of some plants, i.e. barnyard grass (*Echnichola crusgalli*), radish (*Raphamus sativus*) and common purslane (*Portulaca oleraca* L.) indicated that millet was the most sensitive plant to the tested insecticide. The experimental design was completely randomized block with 5 replicates per treatment. Sawdust, as a growing medium, was divided into four treatments: (1) control treatment, (2) sawdust treated with 5% w/w fungal mat, (3) sawdust treated with malathion at concentrations of 1, 10, 50, 100 and 200 ppm, (4) sawdust treated with same malathion concentrations and 5% fungal mat. Following treatment, 30 g portions of sawdust were placed into plastic pots (5x7 cm) and incubated at 28°C±1 where the moisture content was kept at 60% of WHC with sterile distilled water. Each pot was then seeded with 5 millet seeds after 0, 7, 14 and 21 day of incubation, where fresh and dry weights as well as length of plant shoots and roots were recorded after 7 days of sowing at each interval.

Statistical analysis

Data were statistically analyses using Analysis of Variance using computer program (PC Stat).

RESULTS AND DISCUSSION

Three fungal species, that showed high frequency, were isolated from the contaminated soil and identified as *Aspergillus flavus* Link ex Fries, *Aspergillus niger* van Tieghem and *Trichoderma viride* Persoon ex Fries.

Utilization of malathion as sole P source

Czapek-Dox medium was adjusted at pH 7.0 and incubated at 28°C as these conditions are the most preferable for the majority of fungi species (Martelleto *et al*, 1998 and Kodama *et al*, 2001). Preliminary observations showed that malathion was not toxic to *A. flavus*, *A. niger* or *T. viride*. at concentrations up to 500 ppm either in complete or P-free medium as all tested species grew successfully.

When P was omitted from the medium and replaced by different concentrations of malathion, the highest fresh weight of *A. flavus* and *A. niger* was obtained at concentration of 100 ppm while the highest fresh weight of *T. viride* was recorded at concentration of 400 ppm (Table 1). Accordingly, *in vitro* biodegradation of malathion by tested soil fungi could be arranged in the following order: *A. niger*> *A. flavus*> *T. viride*. This is in agreement with the findings obtained by Abou El-Hawa *et al.* (1993) who found that *Aspergillus* species were the most efficient isolates in degrading malathion into indol-3-acetic acid. Moreover, Omar (1998) stated that *Aspergillus* species had greater potential to degrade malathion than *Trichoderma* species. It has been pointed out that *A. flavus* capable of utilizing organophosphorus pesticides as a sole P source by releasing P through the action of their phosphatases

(Dave *et al.*, 1994 and Jauregui *et al.*, 2003). They found that the secreted enzymes responsible for pesticide degradation were directly proportional to both population and growth of the microorganisms.

Regardless of fungal species, application of malathion to culture medium resulted in greater fungal growth as fresh weight of most tested species was increased with increasing concentrations of pesticide to certain extent. Thus, higher fungal growth was recorded with intermediate concentrations while too low or too high concentrations of pesticide lowered fungal growth (Table 1). Similar results were recorded by Abou El-Hawa *et al.* (1993) and Hasan and Omar (1993) with dimethoate, malathion and selecron. They believed that the respiration depression at higher concentration might be a contributing factor.

Table (1):Fresh weight (g) of tested fungi grown on either complete or P-free medium treated with different malathion concentrations

Conc. (ppm)	Complete medium				P- free medium			
	Fresh weight			LSD (5%)	Fresh weight			LSD (5%)
	A. flavus	*A. niger*	*T. viride*		*A. flavus*	*A. niger*	*T. viride*	
0.0	3.600	4.050	3.900	0.340	0.100	0.470	0.300	0.013
10	3.620	4.230	4.800	0.250	0.200	1.550	0.700	0.160
50	3.700	4.250	4.900	0.310	1.700	4.150	1.000	0.240
100	3.900	4.410	4.500	0.230	2.600	4.700	0.800	0.310
200	4.340	4.370	4.100	0.360	1.280	3.700	1.100	0.240
300	3.970	4.210	4.400	0.150	1.310	3.700	1.300	0.250
400	3.720	4.130	4.000	0.370	1.570	3.660	1.700	0.220
500	0.900	3.340	3.910	2.160	0.450	3.110	0.000	
Average	3.450	4.130	4.370		1.300	3.510	0.940	
LSD (5%)	0.430	0.520	0.660		0.140	0.280	0.160	

When P was omitted from the medium and replaced by different concentrations of malathion, the average total P content recorded was 0.86, 0.26 and 0.19 mg g^{-1} fungal fresh weight for *A. niger*, *A. flavus* and *T. viride*, respectively, (Table 2). This result is in harmony with the findings listed in Table (1), thus, the fungus that had greater ability to metabolize malathion as a sole source of P showed higher growth (as fresh weight) and higher P content. It has been found that application of malathion at high concentration increased both DNA and RNA content as well as insoluble P content within the mycelium (Hasan, 1999). However, when malathion was added to the medium as a sole P source at 500 ppm, metabolism of *T. viride* was not accompanied by detectable release of inorganic P which may suggest that soluble P was incorporated into the fungal biomass (Hasan, 1999).

Table (2): Total P content in cell free-extract of fungal mycelial grown on either complete or P-free medium treated with different concentrations of malathion

Conc. (ppm)	Complete medium				P- free medium			
	Total P mg g^{-1} fresh weight			LSD (5%)	Total P mg g^{-1} fresh weight			LSD (5%)
	A. flavus	*A. niger*	*T. viride*		*A. flavus*	*A. niger*	*T. viride*	
0.0	1.427	1.024	1.88	0.182	0.041	0.0283	0.0187	0.003
10	1.645	1.062	2.615	0.321	0.0602	0.1297	0.0749	0.103
50	1.736	1.092	3.328	0.556	0.326	0.9354	0.118	0.057
100	2.863	1.620	2.58	0.494	0.499	1.2568	0.247	0.083
200	2.561	1.039	2.66	0.261	0.391	1.1383	0.244	0.074
300	2.091	1.035	2.989	0.554	0.266	1.0917	0.255	0.067
400	1.428	1.030	2.717	0.315	0.246	0.7212	0.369	0.055
500	0.176	0.751	0.493	0.244	0.059	0.7467	0.00	0.268
Average	1.79	1.09	2.48		0.26	0.86	0.19	
LSD (5%)	0.22	0.13	0.282		0.031	0.093	0.017	

Determination of malathion residues

The disappearance of malathion (100 ppm) in inoculated liquid medium, either complete or P-free, was taken as an evidence of biodegradation. All tested fungi species exhibited higher potential of malathion degradation in P-free medium than in complete medium where *A. niger* was the most active one in both cases (Table 2). The loss percent of malathion in complete medium inoculated with *T. viride*, *A. flavus* and *A. niger* recorded 90.5, 92.2 and 95.0%, compared to 91.3, 93.0 and 98.0% in P-free medium, respectively. Similar results were reported by Abou El-Hawa *et al.* (1993) and Omar (1998) as they stated that *Aspergillus* species was the most efficient in malathion degradation. Moreover, Matsumura and Boush (1966) reported that *T. viride* metabolized nearly 90% of applied malathion to other hydrolytic metabolities which is in accordance with the present results.

Detection of malathion-derived degradation products

The major metabolites produced as a result of biodegradation of malathion by any of our tested fungi species were the same (Table 3).

Table (3). Major metabolites of fungal degradation in liquid medium contained 100 ppm malathion

Metabolite	Retention time (min)	Molecular formula	Molecular weight
Parent compound	16.6.42	$C_{10}H_{19}O_6PS_2$	330.3
O,S,S-trimethyl- phosphorodithioate	7.00	$C_3H_9O_2PS_2$	172.0

Obtained from GC-MS spectral data

The obtained results indicated that decarboxylated product was the major identified metabolite as a result of malathion degradation by *A. flavus*, (Figs. 1 & 2). Matsumura and Boush (1966) reported that *T. viride* degraded malathion to carboxylic acid derivatives due to the presence of powerful

carboxyesterases. Also, Jauregui *et al.* (2003) studied the enzymztic mechanism of the transformation of five organophosphorus pesticides including malathion by different white-rot fungi strains. They stated that malathion was enzymatically converted to O, O, S-trimethyl phosphorodithioic acid, butanedioic acid and dimethoxy phosphinothiol-4-ethyl ester. They believed that at least two different reactions could be involved, the cleavage of S-C bond and the hydrolysis of one of the ethyl ester bonds, with the concomitant release of one ethanol molecule.

Bioassay

Growth characteristics of millet plant in control treatment, where no malathion was applied, either inoculated or non-inoculated with *A. niger* showed insignificant differences among each other, which suggest that the fungus had no significant pathogenic effect on plant growth (Table 4). However, growth parameters were significantly reduced when malathion was applied to the growing medium compared to control treatment. Thus, there was a positive correlation between the concentration applied and the reduction of growth parameters, which reflected the phytotoxic action of the tested pesticide. Application of *A. niger* significantly improved plant growth compared to control treatment. Also, under different concentrations of malathion, plant growth was significantly improved due to inoculation with *A. niger*. Moreover, an imporovement in growth characteristics was recorded when fungus incubation period was increased (data not published), which was in accordance with findings obtained by Khalifa (2004). Interestingly, in presence of *A. niger*, maximum growth characteristics was achieved when malathion was applied at concentration of 100 ppm, which confirms the obtained results where the maximum fresh weight of *A. niger* was recorded at this particular concentration.

It can be concluded that *A. niger* degraded malathion and reduced the phytotoxic action of this pesticide and that the resulted biodegradation products were consumed as nutrients either by plant or biodegrader or by both.

Table (4). Effect of inoculation with *A. niger* on growth characteristics of millet plant grown in sawdust treated with malathion

Conc. (ppm)	Fresh weight g^{-1}		Dry weight g^{-1}		Shoot length (cm)		Root length (cm)	
	A	B	A	B	A	B	A	B
control	2.300	2.330	0.450	0.480	12.33	12.800	4.800	4.830
1	2.010	2.390	0.400	0.500	12.13	13.030	4.500	4.900
10	2.000	2.500	0.370	0.610	11.17	13.200	4.100	4.930
50	1.800	2.900	0.330	0.750	9.600	13.300	3.600	5.100
100	1.650	3.000	0.290	0.800	7.600	13.500	2.600	5.300
200	1.500	2.420	0.220	0.490	5.600	12.900	2.130	4.860
Average	1.792	2.642	0.322	0.630	9.220	13.186	3.386	5.018
LSD (5%)	0.187267		0.076303		0.499359		0.416987	

Where (A) non-inoculated and (B) inoculated

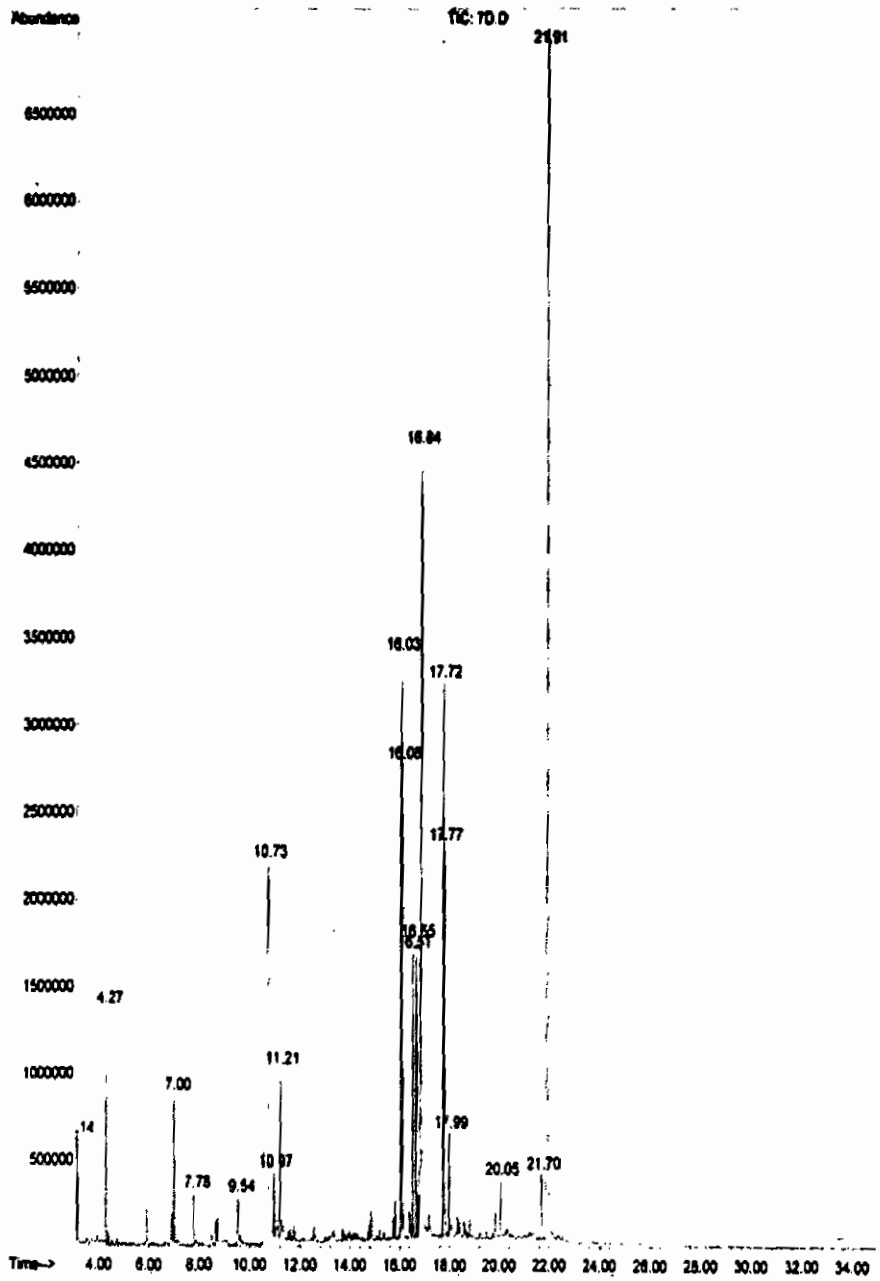
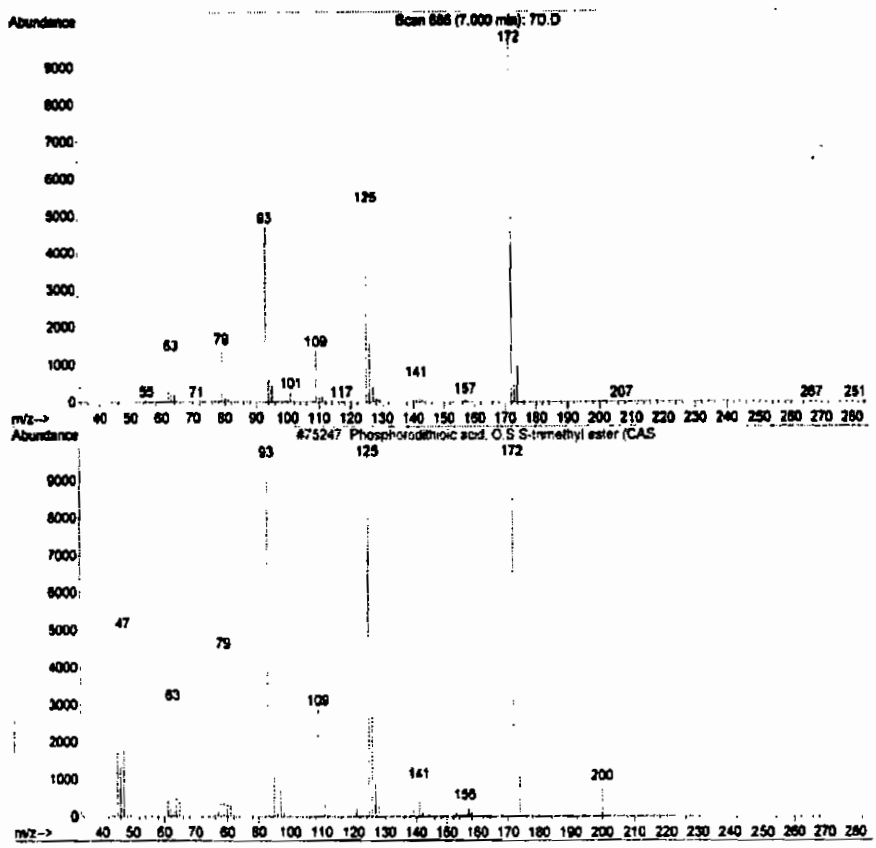


Fig. 1. GLC – chromatogram of dichloromethane: ethyl acetate (9:1) extract from pure culture of *A. flavus* with malathion after 4 weeks incubation time at 28°C.



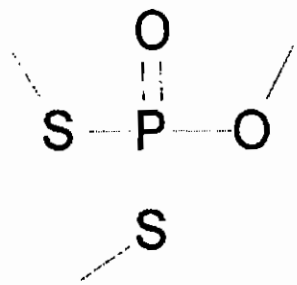


Fig. 2. GC – MS spectrum of malathion major metabolite (O, S, S-trimethyl phosphorodithioate).

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التحليل الحيوى للملاثيون بواسطة الفطريات المعزولة من تربة ملوثة مع استخدام نبات الذرة الرفيعة كدليل حيوى

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تم دراسة تحليل الملاثيون حيويا *In vitro* ، *In vivo* ما بين عامى ٢٠٠٢ – ٢٠٠٤ بواسطة فطريات *A. niger*، *A. flavus* ، *T. viride* واستخدامها للمبيد كمصدر وحيد للفوسفور ولتقدير متبقيات المبيد ونواتج التحليل الحيوى. وقد أوضحت نتائج الدراسة عدم سمية الملاثيون للفطريات المستخدمة حتى ٥٠٠ جزء فى المليون. وقد أظهرت دراسات *In vitro* زيادة النمو الفطرى وكذلك الوزن الحى للفطر مع زيادة تركيز المبيد الى مستوى معين. كما أوضحت الدراسة أن فطر *A. niger* قد تمكن من تحليل الملاثيون بكفاءة أكبر من كل من A. flavus، T. viride. وكنتيجة للتلقيح بواسطة الفطريات الثلاثة محل الدراسة، فقد كانت النسبة المئوية للفقد فى بيئة Dox الكاملة هى ٩٠,٥%، ٩٢,٢%، ٩٥% مقارنة بنسب فقد وصلت الى ٩١,٣%، ٩٣%، ٩٨% وذلك فى البيئة الخالية من الفوسفور عندما تم تلقيح البيئات بفطريات *T. viride*، *A. flavus*، *A. niger* على الترتيب. هذا، وقد كان مركب *O،S،S-Thosphorodithioatep rimethyl* هو الناتج الرئيسى تحليل الحيوى للملاثيون. كما ادت نشارة الخشب المعاملة بالملاثيون والملقحة بفطر *A. niger* كبيئة نمو الى تحسين معنوى فى نمو نباتات الذرة الرفيعة وقد كان هذا التأثير أكثر وضوحا عندما زادت فترة التحضين فى وجود الفطر. وبناء على النتائج المتحصل عليها من هذه الدراسة، فانه ينصح باستخدام هذه الفطريات فى أغراض التحليل الحيوى لمبيد الملاثيون.