

Laboratory Study for Biodegradation of Oxymatrine Insecticide by Single and Mixed Cultures of Fungi Isolated from Agriculture Soils in Basrah Governorate, Iraq

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Abstract:

This study focuses on the biodegradation of oxymatrine insecticide by some soil fungi isolated from four agriculture stations. The results showed that the highest degradation rate 94.66% was recorded by *Ulocladium* sp. at 10 days and *A. niger* recorded the lowest degradation rate 45.86%, while at 20 days *Ulocladium* sp. also showed the highest degradation rate 94.98% and the lowest degradation rate reached to 82.49% with *A.niger*. The mix (*Exerohilum* sp.+*Ulocladium* sp.) recorded the highest degradation rate of oxymatrine insecticide 90.22%, 88.51%, 85.34% at 4, 8 and 12 ppm. The use of mixed isolates enhanced the biodegradation process. There is no study of oxymatrine biodegradation so this study is the first of its kind in the region which can be used as a baseline study for incoming studies.

Key words: Biodegradation, HPLC, Oxymatrine.

Introduction:

Different kinds of pesticides are used yearly in modern agriculture to increase the production through controlling harmful effects caused by the target organisms including insects, fungi, bacteria, viruses as well as grasses grown in between the economical crops and to enhance the quality of crops (1).

The widespread use of pesticides has resulted in the remaining of the pesticides residues in many environmental matrices, such as soil, water and air (2).

Currently, there has been a tendency towards the utilization of natural products for controlling the pest and among them the naturally occurring insecticides derived from plants that have been formulated specifically for their ability to control insects (3, 4).

Oxymatrine, with trade name (Levo 2.4) is a new botanical insecticide with contact and stomach mode of action, it is a tetracycloquinolizidine alkaloid derived from *Sophora flavescens* Aiton (Leguminosae) roots (5).

Usually, the materials derived from plant do not damage plants and many of them have low to moderate in toxicity to mammals, but some botanicals have toxic effect, for example nicotine

preparations can cause death through inhalation and skin exposure to it, rotenone has similar in toxicity to the common synthetic insecticides carbaryl and diazinon (6,7).

Biodegradation is a possible way to solve the problems of pesticide waste which contaminate the soil with pesticide degrading microorganisms. Recently there has been increasing interest by researchers in the use of microorganism (bacteria and fungi) for the biodegradation of pollutants such as pesticides (8).

Oxymatrine insecticide entered the country in 2015 .It has been used to control Dubas bug (*Ommatissus binotatus*), lesser date moth (*Batrachedra amydraula*), and it was used for a wide range of crops against a wide range of pests. The farmers used it to enhance the quality of agriculture product ,but the increase use of oxymatrine pesticide may contaminate soil and food. There is no study deals with the biodegradation of oxymatrine by fungi, so this study is the first of its kind in the area which can be as a base line study for incoming studies.

Material and Methods:

Samples Collection

Twenty four soil samples were collected during the period from August 2016 to January 2017 from four agriculture stations. Six soil samples form each agriculture area were collected according to the

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method of (9). Samples were stored at 4°C until processing.

Isolation and Identification of fungi

Dilution method was carried out for fungal isolation, the culture media Malt Extract Agar (MEA), Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA) were used for the isolation of fungi, the media were prepared according to the procedure of (Himedia company, India) then 250mg /L from the antibiotic chloramphenicol was added to inhibit the bacterial growth. The cultured media were inoculated and incubated at 25°C ± 2°C for 7 to 14 days, the appeared fungi were purified and transferred to PDA slant and stored at 4°C until use.

Czapeks Dox Broth Medium was used in the biodegradation experiment, the medium was prepared from the following material: FeSO₄.2H₂O: 0.1 g, MgSO₄.7H₂O: 0.1 g, KCl: 0.5 g, K₂ HPO₄: 1 g, NaNO₃: 3 g, Sucrose: 30g, D.W: 1 L. All the media were sterilized in the autoclave at 121°C under 15pounds/inch² for 15 minutes.

Chemical Materials:

Oxymatrine Standard Solution

Three mg of oxymatrine (Toronto company, Canada) were dissolved in 1ml of ethanol for the preparation of stock standard solution with 3000 ppm concentration.

Chemicals

The following solvents were used for the extraction of pesticides from the liquid media after the end of the biodegradation experiments; dichloromethane and ethanol (Biosolve company, France), n- hexane (j.T.Baker company, Germany). Anhydrous sodium sulphate (Na₂SO₄) (Himedia company, India) was used to remove the reaming water from the extracting samples before measurement

Effect of Different Concentrations of Oxymatrine on the Fungal Growth.

The method of (10) was used to study the tolerance of fungi to oxymatrine in solid media, 23 fungal isolates were activated by culturing it on PDA for 7 days, petri dishes containing a solid culture medium PDA without insecticide (control) and a medium supplemented with different concentrations of oxymatrine (1, 2 and 3ppm), the plates were inoculated by 6 mm of fungal isolates by cork borer, duplicate were used for each fungal isolate, then the plates incubated at 25°C ± 2°C for 7 days. The growth and tolerance of fungi to oxymatrine was estimated by measuring the percentage of the inhibition rate according to (11) as follow:

$$\text{Inhibition \%} = \frac{\text{the growth in control (mm)} - \text{the growth in test (mm)}}{\text{the growth in control (mm)}} * 100$$

Biodegradation of Oxymatrine Insecticide in Liquid Medium by Fungal Isolates.

The Ability of Single Fungal Isolates to Biodegrade Oxymatrine Insecticide in Liquid Medium for 10 and 20 days.

The fungal isolates that showed the highest resistance against oxymatrine insecticide in the previous test were selected to test their ability to biodegrade oxymatrine in liquid medium according to (12) as follow: Czapeks Dox broth medium supplemented with 4 ppm of oxymatrine was used for biodegradation test at two period of time 10 and 20 days, each conical flask was inoculated by a piece of growth taken from the fungal isolate which was selected previously by using 6mm cork borer from the margin of the colony, duplicate for each isolates was used then they were incubated in shaker incubator at 25°C for 10days, another set was prepared and incubated for 20 days. Control was prepared by adding 4 ppm of oxymatrine pesticide only without any fungal isolates to exclude contamination.

The Ability of Mixed Fungal Isolates to Biodegrade Oxymatrine Insecticide in Liquid Medium at Different Concentrations

Three fungal isolates that gave the highest degradation rate depending on the results of the previous experiments were selected to study their ability to biodegrade oxymatrine in liquid medium amended with 4,8,12 ppm as a mixed cultures according to (13) all the mixtures possibilities were taken.

Extraction of Oxymatrine Residues from Liquid Culture Medium

The liquid culture medium was first filtrated using the filtration unit with G.F.F filter paper. The leachate was transferred to 500 ml separating funnel, 100 ml of dichloromethane was added, the funnel was shaken well several times for (5-10) minutes and left until the formation of two layers. The lower layer was taken and passed through column containing glass wool and sodium sulfate anhydrous to remove the residual water. The descending from the separating column stored in container until analysis by HPLC.

Analysis of Pesticides by HPLC

HPLC device type Shimadzu LC solution equip was used for the identification and quantification of the pesticides, the operation condition for HPLC were: the column was C18 (250 mm, 25 cm, 4.6 mm) the mobile phase was

acetonitrile /water (90:10 v/v) flow rate was 0.5 ml/min, the injection volume was 20µl, the wavelength of UV /visible was 254 nm. The oxymatrine degradation rate was calculated according to (14) as follow:

$$\text{Degradation}\% = \frac{\text{ppm of pesticide in control} - \text{ppm of pesticide in test}}{\text{ppm of pesticide in control}} * 100$$

Statistical Analysis

Minitab ver.16 software was applied and Relative Least Significant Differences (RLSD) values were calculated to identify the fungal degradation significant differences.

Results:

Twenty three fungal isolates were isolated from soil, fungi was isolated in pure cultures and glass slide was prepared using Lacto phenol cotton blue then examined under the compound

microscope to study the characters of each isolated fungi. Isolated fungi were identified and classified according to the following references: (15, 16, 17, 18, 19).

Tolerance of Fungal Isolates to Different Concentrations of Oxymatrine in Solid Medium.

The results showed that some fungal isolates could grow well at the three concentrations 1, 2 and 3 ppm without any inhibition rate and all concentration did not affect their growth and they grew well and filled the plates, the fungal isolates were *Aspergillus flavus*, *A. fumigatus*, *Exserohilum* sp., *Fusarium* sp. and *Ulocladium* sp. while *Cheatomium elatum* gave the highest inhibition rate 70.94%, other fungi gave variable inhibition rate ranged from 4.91% to 55.33% as shown in (Table1).

Table 1. Tolerance of Fungi at Different Concentrations of Oxymatrine in Solid Medium.

species	Control (mm)	Growth zone of colony(mm) at different concentrations of oxymatrine(ppm)			Inhibition % at different concentrations of oxymatrine			Inhibition mean %
		1ppm	2ppm	3ppm	1ppm	2ppm	3ppm	
<i>Aspergillus flavus</i>	85	85	85	85	0	0	0	0 ^a
<i>A. fumigatus</i>	85	85	85	85	0	0	0	0 ^a
<i>Fusarium</i> sp.	85	85	85	85	0	0	0	0 ^a
<i>Exserohilum</i> sp.	85	85	85	85	0	0	0	0 ^a
<i>Ulocladium</i> sp.	85	85	85	85	0	0	0	0 ^a
<i>Aspergillus niger</i>	78	78	74.5	70	0	4.48	10.25	4.91 ^a
<i>A. terreus</i>	75	70	70	70	6.66	6.66	6.66	6.66 ^a
<i>Cheatomium madransense</i>	41	39.5	35.5	33.5	3.65	13.41	18.29	11.69 ^a
<i>Humicola grisea</i>	47	41	40.5	39.5	12.76	13.82	15.95	14.17 ^a
<i>A. vesicolor</i>	77.5	53.5	75	71	30.96	3.22	8.38	14.18 ^a
<i>Penicillium</i> sp.1.	78	71.5	65	59	8.33	16.66	24.35	16.45 ^b
<i>Cladosporium herbarum</i>	26	22.5	22.5	18	13.46	13.46	30.76	19.11 ^{bc}
<i>Myrothecium gramineum</i>	54	49	45.5	35.5	9.25	15.74	34.25	19.66 ^{bc}
<i>A. wentii</i>	75	53.5	54.5	51	28.66	27.33	32	29.33 ^{bcd}
<i>A. candidus</i>	85	66	56	47.5	22.35	34.11	44.11	33.45 ^{cde}
<i>Penicillium</i> sp.2.	85	52.5	43	55	38.23	49.41	35.29	40.98 ^{def}
<i>Alternaria alternata</i>	25	41	31.5	11	18	37	78	44.33 ^{efg}
<i>Microascus trigonosporus</i>	50	27.5	26	25	45	48	50	47.67 ^{efg}
<i>Stachybotrys sansevieria</i>	30	20.5	13.5	8.5	31.66	55	71.66	52.78 ^{fg}
<i>Alternaria</i> sp.	50	27.5	23.5	16	45	53	68	55.33 ^g
<i>C.gloposum</i>	50	28	23	11	50	53	68	55.33 ^g
<i>C. semon-citrilli</i>	25	9	7	6.5	61.53	73.07	75	70.94 ^h
<i>C. elatum</i>	25	9	7	6.5	61.53	73.07	75	70.94 ^h

The similar letter means no difference and the different letter means there is differences between them (p<0.01), RLSD=14.36.

Biodegradation of Oxymatrine In Liquid Medium at 4ppm for 10 and 20 days by Single Fungal Isolates.

The results showed that the lowest residual concentration 0.131 ppm and the highest degradation rate 94.66% was appear in the liquid medium of *Ulocladium* sp. while the highest residual concentration of oxymatrine at 10 days 1.337 ppm was appear in the liquid medium of *A. niger* which recorded the lowest degradation rate 45.86%. The degradation rate of other isolates ranged from 50.01% to 91.31% (Fig1).

At 20 days *Ulocladium* sp.also recorded the highest degradation rate (94.98%) with the lowest residual concentration 0.124 ppm whereas *A. niger* recorded the highest residual concentration 0.432 ppm with the lowest degradation rate 82.49% , the degradation rates of other isolates ranged from 82.93% to 93.45% (Fig 2).

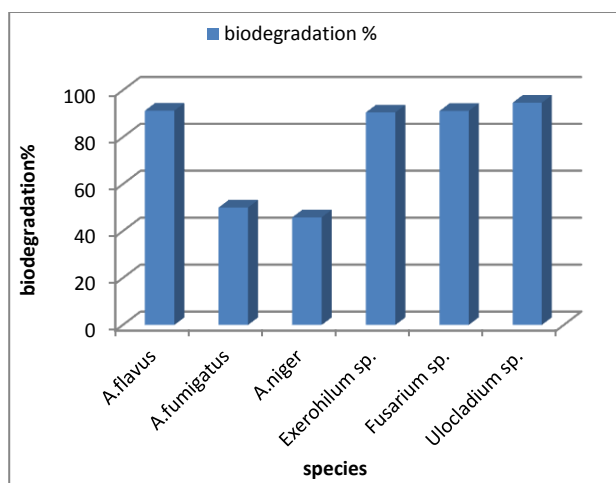


Figure 1. Biodegradation of Oxymatrine Insecticide at 4ppm after 10 days.

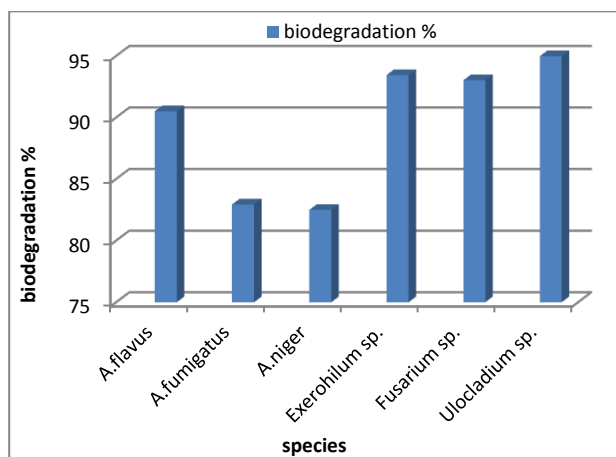


Figure 2. Biodegradation of Oxymatrine Insecticide at 4ppm after 20 days.

Biodegradation of Oxymatrine by Mixed Fungal Isolates in Liquid Medium at Different Concentrations for 10 days.

The fungal isolates A: *Exerohilum* sp., B:*Ulocladium* sp. and C: *Fusarium* sp. which gave the highest degradation rate in the previous experiment were used in this experiment and all the expected possibilities were taken for mixing the isolates. The results of the 4ppm concentration showed that the mix AB recorded the lowest residual concentration 0.266 ppm, and the highest degradation rate 90.22% while the mix AC recorded the highest residual concentration 0.655 ppm, and the lowest degradation rate 75.90%.

At 8ppm, the mix AB recorded the lowest residual concentration 0.599 ppm, and the highest degradation rate 88.51% while the mix BC recorded the highest residual concentration of the oxymatrine in the liquid medium 2.532 ppm and the lowest degradation rate 51.46%.

At 12 ppm, the mix AB recorded the lowest residual concentration 1.019 ppm, and the highest degradation rate 85.34%, whereas the mix ABC recorded the highest residual concentration 4.010 ppm, and the lowest degradation rate 42.28% .(Fig 3-5).

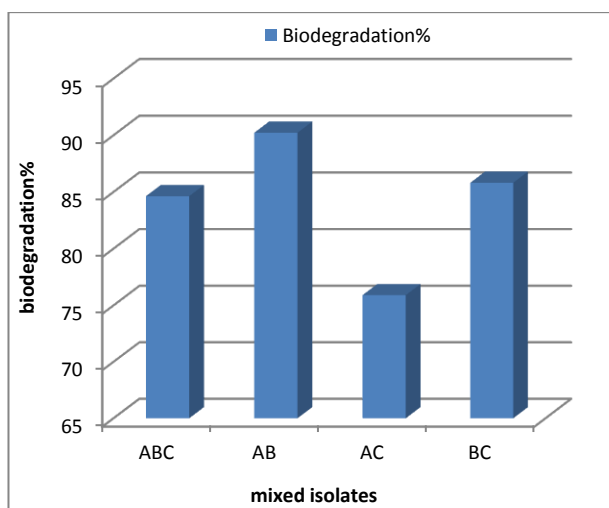


Figure 3. The Biodegradation of Oxymatrine Insecticide at 4ppm for 10 days.

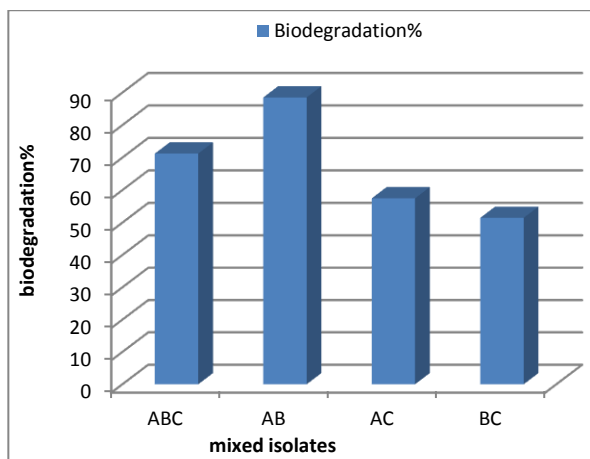


Figure 4. The Biodegradation of Oxymatrine Insecticide at 8ppm for 10 days.

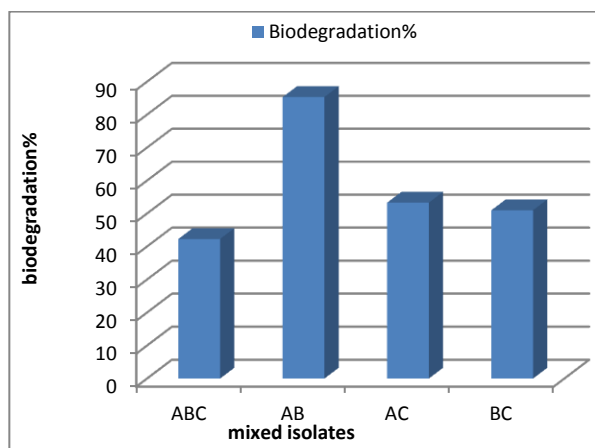


Figure 5. The Biodegradation of Oxymatrine Insecticide at 12ppm after 10 days.

Discussion:

The ability of fungi to tolerate oxymatrine insecticide

At the concentration 1,2 and 3ppm of oxymatrine insecticide some fungal species were grew well in the medium supplemented with pesticide without any inhibition rate . this may be attributed to the capability of fungi to degrade a different kinds of pesticides and they isolated from different contaminated soil with pesticides, tolerance nature of *Aspergillus* sp. were suspected to be influenced by adaptability to environmental stress caused by pesticide application and the production of extracellular enzymes which degrade wide range of pesticides, this results are in similarity with the findings of (20, 21). Other fungi could not tolerate the different concentrations of oxymatrine used and their growth was affected and gave a high inhibition rate such as *C. elatum* , this may be due to many factors such as the culture media used which are not suitable , the period of incubation was short and the concentrations of pesticide (22, 23 ,13).

The results agreed with the finding of numerous laboratory studies indicated that the various fungal species vary in susceptibility to pesticides (24, 14, 25, 10).

Biodegradation of oxymatrine in liquid media as single cultures.

At 4 ppm of oxymatrine insecticide for 10 days *A.flavus*, *Exerohilum* sp., *Fusarium* sp. and *Ulocladium* sp., showed higher degradation rate and this indicate that these fungi were not affected by insecticides and also they proved their ability to degrade wide range of insecticides and they used oxymatrine insecticide as a carbon and energy source under aerobic conditions (26, 27) while *A. niger* and *A. fumigatus* showed the lowest degradation rate and that not mean they unable to degraded oxymatrine insecticide but may be they need more time or the conditions may be not suitable for them (28).

At 20 days of incubation all the fungi recorded high degradation rate at 4ppm than in 10 days this is due to the fact that the biodegradation rate increase with the increasing of the incubation period (29, 30, 31), the results do not agree with the finding of (32).

Biodegradation of oxymatrine by mixed isolates

The results of the biodegradation of oxymatrine insecticide showed that at 4ppm the mixtures showed good degradation rate (AB 90.22%, BC 85.81%, ABC 84.63%, AC 75.9) this is attributed to the fact that these fungi were effective because of an extracellular enzyme system that catalyzes reaction that can be capable of degrading various chemicals including pesticides and made them useful for remediation of pesticides. In addition these species have been adapted to grow in pesticides contaminated soil as where they isolated and the presence of these fungi together enhanced the degradation of the oxymatrine insecticide (33, 34, 35).

While at the concentration 8 and 12 ppm only the mixed AB (*Exerohilum* sp.+ *Ulocladium* sp.) recorded a high degradation rate (88.51%, 85.34%) respectively , this may be due to the adaptation ability of these fungi in soil contaminated with pesticides in addition to the fact that the species which grow well with high mass may increase the contact with the pesticide in the medium and leads to increasing the biodegradation rate (36).

The decrease in the biodegradation of other mixed isolates may be attributed to many reasons, first the concentration of oxymatrine has an important effect on their degradation rate in liquid medium, in general the higher concentration cause

the lower degradation rate (23). Oxymatrine play an inhibitors to microorganism such as fungi (32), the metabolites that arise from the degradation of parental compound may be toxic and prevent the proliferation resulted incomplete degradation of parental compound(37). The loss of degradative capabilities of the isolates and the inhibition of their growth by the toxic intermediates that occur during the degradation of parental compound was another reason for decreasing the biodegradation rate (38, 39). However, it was thought that abiotic factor such as competition for nutrients and niches also a most important factor that influences the success of the biodegradation rate (36,40).

Conclusion:

As a conclusion , the fungal isolates tolerate oxymatrine insecticide at different concentrations in solid medium, and have a good ability to degrade oxymatrine insecticide at high concentration in liquid medium when they used as single or mixed cultures, the use of mixed isolates were enhanced the process. It increased with increasing the incubation time. The process was decreased with the increasing of the concentration. The fungal isolates was powerful for biodegradation of oxymatrine at different concentration and as a single or mix isolates.

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Conflicts of Interest: None.

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دراسة مختبرية للتكسير الحيوي للمبيد الحشري الأوكسي ماترين بواسطة مزارع مفردة ومختلطة من الفطريات المعزولة من ترب زراعية في محافظة البصرة، العراق

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الخلاصة:

تركزت الدراسة الحالية على دراسة التكسير الحيوي للمبيد الحشري oxymatrine بواسطة بعض الفطريات المعزولة من الترب الزراعية التي تعود لأربع محطات زراعية، أظهرت النتائج أن أعلى نسبة تكسير سجلت من قبل الفطر *Ulocladium* sp. وبنسبة 94.66% في تجربة العشرة أيام، بينما الفطر *A. niger* سجل أقل نسبة تكسير 45.86%، أما في تجربة العشرين يوم فأن الفطر *Ulocladium* sp. سجل أعلى نسبة تكسير 94.98% وأقل نسبة تكسير سجلت 82.49% من قبل الفطر *A. niger*. أما الفطريات الممزوجة مع بعضها (*Exerohilum* sp.+*Ulocladium* sp.) فقد سجلت أعلى نسبة تكسير للمبيد الحشري في كل التراكيز (4, 8, 12 ppm) وبنسبة 85.34%, 88.51%, 90.22% على التوالي، أن استخدام العزلات المختلطة ساعد في تحسين عملية التكسير الحيوي. لا توجد دراسات تهتم بدراسة التكسير الحيوي للمبيد الحشري الأوكسي ماترين لذا تعد هذه الدراسة الأولى من نوعها في المنطقة والتي يمكن الاستفادة منها في الدراسات اللاحقة.

الكلمات المفتاحية: التكسير الحيوي، مبيد الأوكسي ماترين، HPLC.