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Role of *P. aeruginosa* in Bioremediation of The Insecticide Chlorpyrifos in Soil Planted with Stevia

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Abstract

The first factor is The *P. aeruginosa* bacteria-based bacterial inoculate at two levels, B0 and B1. The second factor is white fungus waste, which was added at three levels and coded as Ab0, Ab1, and Ab2. Thirdly, Zinc and boron nanoparticles was incorporated into four levels coded N0 minus addition, N1 nano Zinc, N2 nano Boron, and N3, a combination of both nano boron and nano zinc. It was carried out under field conditions employing a randomized complete block design (RCBD). Its presence was ascertained by subjecting the initial soil sample to the estimation of the pesticide accumulated in it by GC-MS. They further identify the kind of pesticide accumulating in the soil and then select for that specific high-resistant bacterium strain against such high concentrations of that identified pesticide. From the rhizosphere of the sugar bean plant, *P. aeruginosa* was isolated and used as a bacterial inoculum during a series of laboratory and field experiments. All combinations with a bacterial inoculum were degraded entirely compared to their control; their control had 18.090 mg kg^{-1} soil.

Keywords: Chlorpyrifos, Bioremediation, *Pseudomonas aeruginosa*, White mushroom waste, Nano zinc.

دور بكتيريا *P. aeruginosa* في المعالجة الحيوية لمبيد كلوربيريفوس في تربة رايزوسفير نبات ورق السكر

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الخلاصة

نفذت التجربة بهدف دراسة إمكانية تحلل المبيدات المتبقية في التربة بعد الزراعة باستخدام بكتريا *P. aeruginosa* التي أضيفت على مستويين (B0 و B1) والتي عُدت كعامل أول، في حين تم استخدام مخلفات الفطر الأبيض على ثلاثة مستويات والمرمز لها (Ab0، Ab1 و Ab2)، فضلاً عن إضافة الأسمدة النانوية كعامل ثالث على أربعة مستويات تضمنت (N0، N1، N2، N3) وصُممت باستخدام التجربة العاملية ذات ثلاثة عوامل وفق تصميم القطاعات العشوائية الكاملة (RCBD). تم الكشف عن بقايا المبيدات المتعددة الفئات في التربة قبل الزراعة باستخدام جهاز الطيف الكروموتوغرافي الغازي، إذ ان مبيد Chloropyrifos كان الأكثر تركيزاً في التربة بتركيز (167 ملغم كغم$^{-1}$ تربة) وبعد تحديد نوع المبيد المتراكم في التربة أُختيرت السلالة البكتيرية الأكثر مقاومة للتركيزات العالية من هذا المبيد وهي بكتريا *P. aeruginosa* والتي تم عزلها من محيط جذور نبات ورق السكر. إذ حققت إضافة بكتريا *P. aeruginosa* تفوقاً معنوياً في تحلل بقايا المبيد، فضلاً عن دور مخلفات الفطر الأبيض والسماد النانوي في التحفيز الحيوي للبكتريا، كما ساهم نبات ورق السكر ايضاً بعملية التحلل من خلال افراز مواد محللة، مما أدى إلى تسريع عملية تحلل مبيد Chloropyrifos إذ لوحظ انخفاض تركيز المبيد حتى في معاملة المقارنة التي سجلت 18.090 ملغم كغم$^{-1}$ في التربة.

كلمات مفتاحية: Chloropyrifos، المعالجة الحيوية، *Pseudomonas aeruginosa*، البورون النانوي، الزنك النانوي.

Introduction

Pesticides are significant crop protectors when the crop is under siege from one or a cohort of resident or imported pests. This unbalanced use of pesticides, though, has most, unfortunately, contributed to increased pollution of soil, water, plants, and air in most parts of the world, notably in all the little thanks to the likes of chlorpyrifos that tend to linger long within the environment and are detected in soil and water (54), posing a risk not just to humans but also animals as well, for these are toxic compounds that bear significant, serious, and destructive damage. Despite this, farmers use them in excess of the required quantity since they offer a high monetary return and are highly beneficial and convenient (39 and 42). Therefore, there is a pressing need to intensify the efforts directed at diminishing these pollutants' negative impact through the use of

modern scientific techniques which may include bioremediation by microorganisms such as bacteria and fungi because bioremediation is a typical application of bacterial metabolism for the degradation of pollutants, On August 3, 2011, the US Environmental Protection Agency published in the Federal Register FR-3-000 a formal list wherein the triphosphate TCP compound is among the toxic chemical compounds that may cause several multiple diseases; hence, there is urgency to get rid of these residues from the soil 2 - Isolation and identification of bacteria most tolerant to Chlorpyrifos pesticide polluted soil, and bioremediation could take place spontaneously through dilution or eradication or the process of bioremediation can be enhanced through the addition of fertilizers, which increases the bioavailability of pollutants and supports biostimulation. This method involves the use of compatible microbial strains, referred to as augmentation, to improve the effectiveness of pollutant-degrading microbes. Scientific literature has shown promising results with this approach, demonstrating significant success in enhancing the degradation of contaminants (3). Overall, the combination of biostimulation and microbial augmentation presents a powerful strategy for improving bioremediation outcomes *Pseudomonas* bacteria is one of the key actors in bioremediation and biodegradation by producing metabolites acting as a biosurrogate role for antibiotics mainly involved in breaking down complex organics such as pesticides (44). Most food fungi are heterotrophic microorganisms that lead a saprophytic life, breaking down and analyzing the basic materials present in their environments from plant and organic waste. This is one of the essential treatments to reduce environmental pollution accumulating in agricultural waste, air pollution, and CO_2 gas if these wastes can be disposed of by burning (19). Moreover, the growth of fungal mycelium on the wastes increases their protein content while reducing the carbon-to-nitrogen ratio. It amends them with several enzymes to decompose organic matter (7 and 53), including cellulase and alkenase, which will feed the nutrients in a form ready for absorption and assimilation into the plant without much effect on its growth and productivity (10 and 24). As studies have indicated, the unique construction of nano fertilizers with targeted delivery or slow-release mechanisms can provide a more accurate responsive operational release of their active components concerning environmental stimuli and biological requirements. Nanofertilizers improve nutrient use efficiency, reduce soil toxicity, minimize potential adverse effects of overdosage, and reduce application frequency; thus, nanotechnology holds enormous potential for achieving sustainable agriculture, particularly in developing countries (4). Mainly, fertilizers provide macro elements usually found deficient in the soil; nearly 35-40% of crop productivity is attributed to fertilizers, but some fertilizers directly influence growth. Nanofertilizers can thus be applied as an alternative to mineral fertilization and conventional fertilization to solve these problems relating to low fertilizer efficiency (48).

They operate uniquely compared to traditional fertilizers because of their nano-dimensions, which are attributed to two factors. First, the ratio of their surface area to volume has been increased beyond what it is in the natural state due to the small diameter of the molecules, while the second one varies in terms of properties like hardness, color, strength, chemical activity, thermal properties as well as electrical conductivity. This means that they have a substantial external surface, so more of their

molecules lie on external surfaces, so more interact, thereby showing higher chemical activity. Secondly, the effects of nanomaterials are quantitative. Having small diameters, they are not entirely under classical physics but under quantum physics (46 and 51).

hence giving them increased quantitative effect. Nanofertilizers are a substantial biostimulating factor for microorganisms in increasing the general decomposition of Pesticides. Hence, this study aimed to determine the role of *P. aeruginosa* bacteria in the breakdown of an active ingredient of an insecticide in soil, specifically in Chlorpyrifos, after 120 days of incubation with soil.

Materials and Methods

Collecting soil samples: Soil samples were taken at 0-30 cm depth related to plowing, avoiding special areas like piles of fertilizers and pesticides or where animals usually gather. The samples were collected from an experimental field of the Agricultural Research Department/Diwaniyah Research Station on 15th January 2024 and stored until pesticide residues were analyzed biologically, chemically, and physically (Table 1).

Table 1: Some physical-chemical-biological analyses of the study soil before planting.

Attribute		Value	Unit
pH 1:1		7.23	-
EC 1:1		4.78	ds m^{-1}
Cations	Ca+2	11.20	Meq mol^{-1}
	Mg^{+2}	6.58	
	Na^{+1}	4.76	
	K^{+1}	2.6	
Ions	Cl^{-1}	16.17	
	SO_4^{-2}	5.95	
	CO_3^{-2}	Nill	
	HCO_3^{-1}	3.60	
CEC		15.18	$Cmol_c$ Kg^{-1} soil
$CaCO_3$		201.00	g Kg^{-1} soil
Organic Matter		1.69	%
Total bacteria		$26.33*10^7$	CFU g^{-1} dry soil
Total fungi		$5.20*10^3$	CFU g^{-1} dry soil

Estimation of pesticides accumulated in soil using GC-MS: In the extraction process of pesticides from soil, a sample of 5 grams of air-dried and sieved soil is weighed and placed in a plastic box, with 1 ml of water added to aid in the extraction of both polar and non-polar insecticides. The extraction is performed using a mixture of 10 ml hexane and 10 ml dichloromethane, which is agitated in a vibrating device for 30 minutes at 250 rpm. Following this, the mixture is centrifuged at 3500 rpm for 5 minutes to separate the filtrate from the sediment, and the filtrate is further filtered through 0.45 micrometer filter paper (37). The organic phase is isolated by adding chlorobenzene and centrifuging again, after which the sample is prepared for analysis using a GC-MS device to detect residues of multi-class pesticides, while also identifying bacterial strains that exhibit resistance to soil conditions (17, 27 and 38).

Total bacteria in the soil: In this study, bacterial species were isolated using a dilution method and cultured on nutrient agar plates (20), with the most active species selected for further analysis. These selected bacteria were then cultured in a liquid broth containing Chlorpyrifos pesticide, and after incubation, the most opaque culture was identified using a VITEK device. Various tests, including Gram staining, movement assessment, and catalase and oxidase tests, were conducted to characterize the bacteria, along with tests for nitrate reduction, gelatinase activity, indole production, and sugar fermentation to determine their metabolic capabilities (20, 41 and 45).

P. aeruginosa isolates are preserved by transferring a portion of the bacterial colony to test tubes with slanted nutrient agar under sterile conditions, followed by incubation at 28˚C and storage at 4˚C, with monthly culture renewals (21). To prepare a biological inoculum of *P. aeruginosa*, pure isolates are cultured in nutrient agar medium within a flask, inoculated with a young culture, and incubated in a shaking incubator at 28 °C for four days to ensure proper aeration. This method ensures the viability and maintenance of the bacterial strains for further study (23).

Experimental design: The experiment was laid out in a Randomized Complete Block Design with 24 treatments, including control along three replicates, which were randomized to each plot. Cultivation was done in the experimental unit form of lines wherein three lines were drawn per experimental unit, line separation 50 cm and 25 cm plant distance. Thence 15 plants lines in total in each, and the rate is 80,000 plants h^{-1}.

The study factors were divided into three factors. The first factor is the biofertilizer represented by *P. aeruginosa* bacteria, symbolized by B, at two levels (not adding a inoculate of *P. aeruginosa* B0, adding *P. aeruginosa* B1) by dipping the seedling in 2 ml of liquid *P. aeruginosa* inoculate. As for the white fungus waste, the characteristics of which are shown in Table 5 and symbolized by Ab, it will be added at three levels, which are (without adding Ab0, 5 tons h^{-1} as a second level Ab1, 10 tons h^{-1} as a third level Ab2) added in one batch upon planting. The nanofertilizer symbolized by N will also be added at four levels, which are (without adding N0, 4 kg h^{-1} nanozinc N1, 2 kg h^{-1} nanoboron N2, 1 kg h^{-1} nanoboron + 2 kg h^{-1} nanozinc N3). Added in one batch simultaneously with the addition of white fungus waste. Samples were collected from the rhizosphere soil of the stevia sugar plant and the two genitals, i.e., after 120 days.

Estimation of the residual Chlorpyrifos pesticide using HPLC liquid chromatography device: The residual pesticide was estimated according to the method of (59) using the standard solution of the pesticide for qualitative detection by comparing the retention time of the standard compound of Chlorpyrifos pesticide 8.10 minutes.

Statistical analysis of the experimental data: The measured data for the study indicators were taken, and the results were statistically analyzed using the Genstat program. The averages were compared according to the least significant difference (LSD) test at a probability level of 5% (11).

Results and Discussion

Detection of pesticide residues in the soil before the study: The chromatogram is the detector's response plotted against time as the column components leave the solution (i.e., the sample compounds making up the solution). It shows many peaks; each peak corresponds to a soil solution compound.

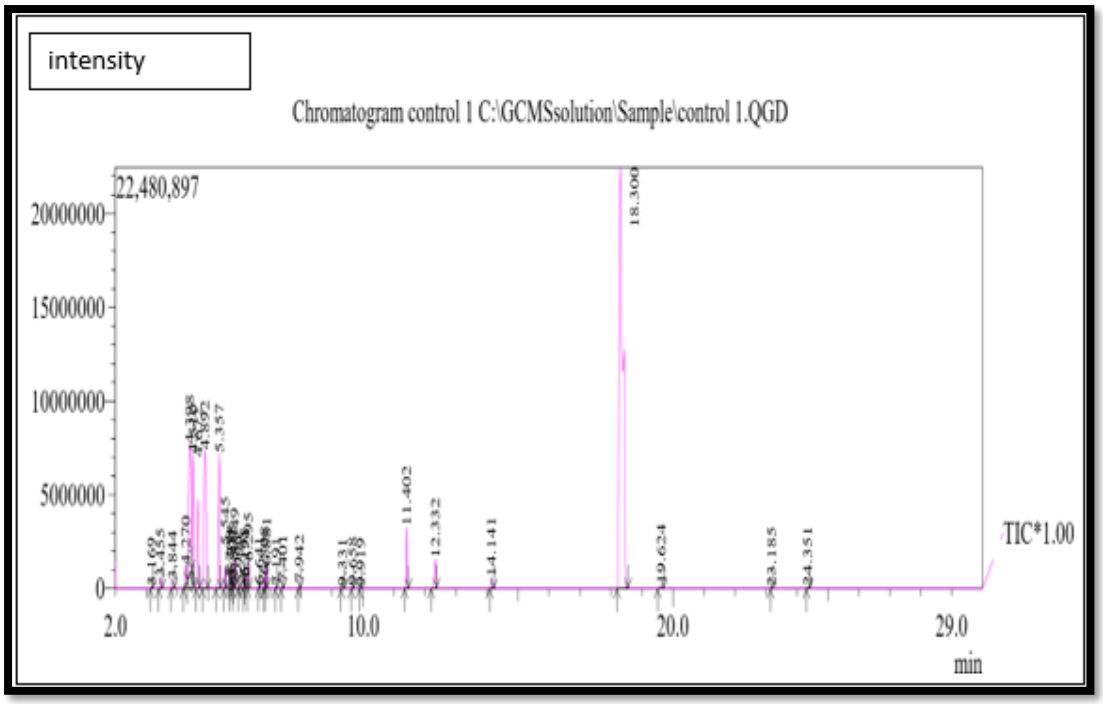

Figure 1: Chromatogram in the GC-MS device for the initial soil sample.

Thus, from Table 2 R.Time and Area present the peak of each curve in Figure 1 at the retention time of the compound R.Time and is indexed in a sequence from curve 1 to curve 5 in Table 2 as seen from Figure 1 that there is an 18.300 peak which represents the RT of this most concentrated component in position 31, and the Area % is 53.91 as it was the highest percentage area of the compound present in the soil sample and the sequence of this Peak was 31 (Table 2 as displayed as Fig. 2) It is culled Chlorpyrifos or Dursban or Phosphorothioic acid or O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) ester.

Table 2: Using the GC-MS device, The compounds' peak number, retention time, and concentration in the initial soil sample.

Peak Report										
Peak	**R.Time**	**I.Time**	**F.Time**	**Area**	**Area%**	**Height**	**Height%**	**A/H**	**Mark**	**Name**
1	3.169	3.125	3.208	212161	0.06	74677	0.10	2.80	MI	**Benzene**
2	3.455	3.400	3.517	1750055	0.51	564482	0.79	3.10	MI	**o-Xylene**
3	3.844	3.800	3.908	1301736	0.38	419557	0.59	3.10	MI	**2,2,3,3-Tetrafluoro-1-propanol**
4	4.270	4.192	4.325	3878912	1.13	980658	1.38	3.87	MI	**4-Chlorobutanoic anhydride**
5	4.398	4.325	4.467	36937472	10.77	6955376	9.75	5.30	MI	**Toluene**
6	4.510	4.467	4.542	15703054	4.58	6692742	9.39	2.37	MI	**Urea**
7	4.672	4.608	4.725	12543485	3.66	4740483	6.65	2.65	MI	**Peroxide, dimethyl**
8	4.892	4.808	4.983	38366934	11.19	7373742	10.34	5.20	MI	**3-(Bromomethyl)picolinonitrile**
9	5.357	5.242	5.400	19878226	5.80	7166711	10.05	2.77	MI	**2-Ethyl-3,5-dimethylpyridine**
10	5.545	5.500	5.575	1891776	0.55	1113531	1.56	1.70	MI	**Phenylalanine**
11	5.708	5.675	5.717	310938	0.09	139694	0.20	1.39	MI	**Propyl-2-iden-5-amino-1,2,4-triazole**
12	5.738	5.725	5.758	364654	0.11	363421	0.51	1.00	MI	**1,2-Benzenediol**
13	5.807	5.775	5.825	525326	0.15	284555	0.40	1.66	MI	**Hydratropic acid**
14	5.849	5.825	5.875	1888264	0.55	1386264	1.94	1.39	MI	**1,3-Difluoro-2-propanol**
15	5.999	5.975	6.025	256573	0.07	166690	0.23	1.54	MI	**2-Tolyloxirane**
16	6.158	6.125	6.175	537909	0.16	316188	0.44	1.56	MI	**2-Chloro-N-methoxy-N-methylacetamide**
17	6.191	6.175	6.217	309482	0.09	310729	0.44	1.08	MI	**alpha.-Chloroethyl chloroformate**
18	6.295	6.258	6.325	2377750	0.69	1377641	1.93	1.73	MI	**3,4-DIETHYLHEXANE-3,4-DIOL**
19	6.641	6.617	6.667	184340	0.05	118721	0.17	1.55	MI	**4-Ethylbenzoic acid**
20	6.808	6.767	6.833	1083260	0.32	632517	0.89	1.70	MI	**Peroxide, dimethyl**
21	6.881	6.842	6.908	1900581	0.55	1141532	1.60	1.66	MI	**Peroxide, dimethy**
22	7.191	7.158	7.225	147348	0.04	80688	0.11	1.80	MI	**2-Propenenitrile, 3-phenyl-**
23	7.401	7.342	7.433	557992	0.16	228168	0.32	2.44	MI	**2-(3-Aminopropyl)-pyridine**
24	7.942	7.900	7.975	438269	0.13	235907	0.33	1.85	MI	**5-Benzylidene-3-(4-morpholinylmethyl)-2,4-thiazolidinedione**
25	9.331	9.283	9.392	135862	0.04	52649	0.07	2.57	MI	**6-Chloro-4-mercaptobenzo-1,2,3-triazine**
26	9.658	9.617	9.692	219539	0.06	121153	0.17	1.81	MI	**1-Methyl-1-(2,2-dimethylpropyl)oxy-1-silacyclobutane**
27	9.919	9.875	9.958	92646	0.03	49503	0.07	1.87	MI	**Naphthalene**
28	11.402	11.333	11.467	6626337	1.93	3286834	4.61	2.02	MI	**Molybdenum**
29	12.332	12.192	12.375	5113171	1.49	1558107	2.18	3.28	MI	**2,3,5-Trichloropyridin-4-amine**
30	14.141	14.092	14.225	1519796	0.44	583077	0.82	2.61	MI	**Molybdenum, tricarbonylchloro(.eta.5-2,4-cyclopentadien-1-yl)-**
31	18.300	18.192	18.558	184895043	53.91	22472325	31.51	8.23	MI	**Chlorpyrifos**
32	19.624	19.508	19.708	705189	0.21	190509	0.27	3.69	MI	**E,E,Z-1,3,12-Nonadecatriene-5,14-diol**
33	23.185	23.150	23.217	106576	0.03	59987	0.08	1.79	MI	**Glutaric acid**
34	24.351	24.308	24.417	179300	0.05	73127	0.10	2.43	MI	**i-Propyl 9-octadecenoate (Z)**
				342939956	100.00	71311945	100.00			

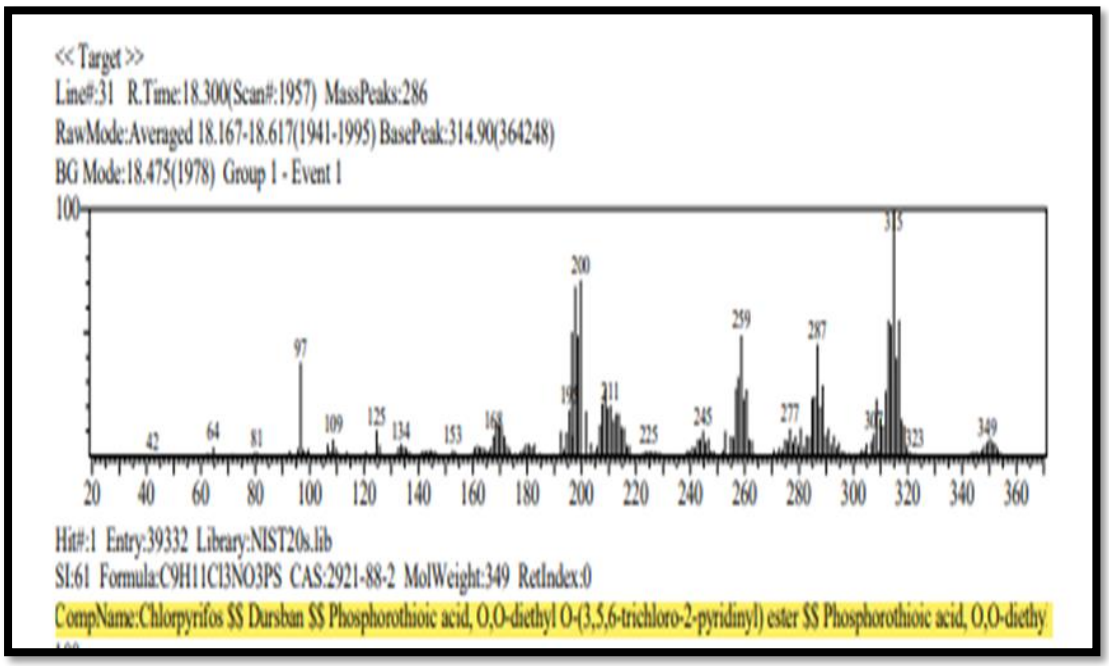

Figure 2: Peak 31, the pesticide Chloropyrifos.

The primary soil has the insecticide Chlorpyrifos at a concentration of (167 mg kg^{-1} soil) most probably since this pesticide is found in many agricultural and food products, and winged insects, immune insects, etc, attack all types of soil and plant pests. The field site is associated with the Agricultural Research Department - Diwaniyah Research Station and has been cultivated with consecutive barley crops since 2008 AD. Various agricultural pesticides were used in it, including insecticides, thin and broad weed killers, and others. After the crisis period in 2020, there was a water shortage crisis for three years; hence, the land was lying barren. The present study experiment was conducted only after three years. Among the commonly and broadly used organic pesticides for crops globally, Chloropyrifos is used at 3-15 kg h^{-1} (52), while its half-life is 120-360 days (15).

The extensive use of Chloropyrifos in the soil has caused high-level soil contamination with Chloropyrifos pesticide. Also, there is a high abundance of Chlorpyrifos pesticide in the subject soil because it stays for a long while within the soil. This pesticide negatively affects the population by killing or inhibiting certain groups of microorganisms and increasing the numbers of some resistant and dominant microorganisms (22 and 59).

Detection of bacteria most adapted to soil contaminated with the pesticide Chlorpyrifos: Soil samples were taken from the greenhouse soil, supplemented with Pseudomonas chlorpyrifos strain BAM5 at a concentration of 10^9 colony-forming units g^{-1} to the soil. It gave about approximately 26.33 x 10^7 (CFU g^{-1} dry soil). Incubated plates were incubated for 24-48 hours at 37 °C. After incubation, the bacteria strain having the serial number (1) was selected based on the dense growth in the plates, and this recorded several 13.64 x 10^7 (CFU g^{-1} dry soil). The ideal soil to detect resistant bacteria is one in which the species exist, and it is evident that isolated species do not affect microbial activities elsewhere (36 and 38). The results of testing the bacterial isolate using the VITEK system came out to be *P. aeruginosa*, as in Table 3; this was

supported by the morphological examinations of the bacterial isolate under a light microscope, where almost a single rod-shaped bacterium was observed post staining with Gram stain in red (Gram-negative) and on nutrient culture medium exhibited a convex with an irregular edge colonial shape that produced a yellowish-green pyocyanin dye, Table 4. The findings from morphological and biochemical tests agreed with what was reported by (25). The colonies were sub-inoculated onto King's B and incubated; they were then inoculated to test for growth on Cetrimide Agar (selective medium for this pathogen) and Simon citrate medium by checking the color of the medium from green to blue. All six isolates turned out positive to both oxidase and catalase tests, motile rods negative to capsule and spore forms, aerobic, well grown on King A and Mannitol media, and had an optimal temperature of growth range. A few isolates had a temperature of optimum growth between 4-42°C, a key diagnostic characteristic of a few isolates.

They were singly on rods seen under the microscope, and the stain did not produce any similarity in staining to those in any group. These characteristics agree with the morphological, microscopic, and biochemical characteristics of *Pseudomonas* spp. The same results have been repeated in other studies, such as (58). As shown in Table 4, the results show that the bacteria is *Pseudomonas aeruginosa*. They are small to medium-sized, smooth, convex colonies. They also appear mannitol positive and pigmented on both King B and King A media, with different colors of the pigmentation and always a greenish pigment typical of this particular species, at 42°C. These features are characteristic features and confirmatory tests for the species *P.aeruginosa*. That also agrees with what (50) said. (T) The reason for the dense presence of *P. aeruginosa* in primary soil is that this organism is ubiquitous and primarily survives in colonies in the root area around several plants (9). Since it also does not have a reasonable nutritional requirement, representing the nature of adaptability to most conditions, this organism would have to thrive in most places instead of the commons's abundance in different areas. The organism can utilize many carbon sources for energy and has fast growth rates (56).

Table 3: Diagnosis of bacteria using the VITEK device.

Bionumber:0043051303500240 **Organism Quantity** **Selected Organism:** ***Pseudomonas aeruginosa***					
Identification Information	Card: GN	Lot Number: 2412586503		**Expires: 25,12,2023 CST**	
	Status: Final	Analysis Time*: 5.82 hours*		**Completed: 27,1,2024 CDT**	
Organism Organ	**VITEK 2**				
Selected Organism	**% Probability** ***Psedomonas aeruginosa*97** **Bionumber:0043051303500240**				
Analysis Organisms and Tests to Separate:					
Analysis Message:					
Contraindicating Typical Biopattern(S) ***Psedomonas aeruginosa*** **URE(16),**					
Susceptibility Information	Card: AST-N419	Lot Number: 0442809204		**Expires:5,1,2025 CDT**	
	Status: Final	Analysis Time: 14.37 hours		**Completed: 27,1,2024 CDT**	
Antimicrobial	MTC	Interpretation	Antimicrobial	MTC	**Interpretation**
Ampicillin/Sulbactam			Meropenem	1	**S**
Piperacillin/Tazobactam	8	S	Amekacin	4	**S**
Cefotaxime			Gentamicin	2	**S**
Ceftazidime	2	S	Ciprofloxacin	0.25	**S**
Ceftazidime/Avabactam	2	S	Tigecycline		
Ceftolozane/Tazobactam	0.5	S	Colistin	2	**S**
Cefepime	2	S	Trimethoprim/ Sulfamethoxazole		
Imipenem	2	S			
AES Findings:	**Last Modified: Dec 23,2023 CST Parameter Set: Global CLSI-based +Phenotypic 2023**				
Confidence Level:	**Consistent**				

Table 4: Biochemical tests for *P. aeruginosa* bacterial inoculate.

S.	Test name	Culture medium	Result	Notes
1.	Gram stain	**-**	-	Single red rod (stain negative)
2.	Pyocyanin dye production	Nutrient Aqar	+	The presence of yellowish-green pyocyanin dye on the surface of the medium
3.	Growth at 4°C	Nutrient Aqar	-	No bacterial growth because it is unable to grow at 4°C Presence of clear bacterial growth
4.	Growth at 42°C	Nutrient Aqar	+	Change of the color of the bacterial colony to purple
5.	Oxidase	**-**	+	Appearance of a clear transparent halo around the colony
6.	Hemolysin	Blood Agar with 6% human blood	+	Change of the color of the medium from green to blue
7.	Citrate consumption	Simmon citrate	+	Appearance of pink color
8.	Urease	Christensen	+	Change the consistency of the solid gelatin to the liquid state.
9.	Gelatinolysis	Nutrient gelatine	+	Appearance of red color using the a-Naphthol indicator
10.	Methyl red	medium containing dextrose as well as K_2PO_4 is called Protease Broth.	+	Appearance of pale bacteria on the medium due to their
11.	Lactose fermentation	MacConkey Agar MCA	-	inability to decompose lactose sugar

The results of Table 5 indicate that *P. aeruginosa* bacteria isolated from the primary soil were able to degrade Chlorpyrifos pesticide in solid culture media, as the highest value of bacterial density at a concentration of 10 mg L^{-1} of Chlorpyrifos pesticide after 48 hours of incubation was 27.9 x 10^7 (CFU g^{-1} dry soil), while the highest concentration of the pesticide 50 mg L^{-1} during the same incubation period gave the lowest bacterial number of 9.3 x 10^7 (CFU g^{-1} dry soil). Table 5 shows that gradually increasing the concentration of pesticides reduces the number of *P. aeruginosa* bacteria.

The rapid increase in the use of insecticides is negatively related to environmental disturbances, especially the microbial community, as the insecticide inhibits the number of bacterial colony-forming units at high concentrations, which ranged between 30-50 mg 1, that the aim of adding cultured microorganisms obtained from previously contaminated sites is to have a high level of adaptation to break down Chlorpyrifos molecules using resistant and dominant bacterial strains, which is an important factor for enhancing soil biomass (1 and 30). (55) stated that soil loses fertility when contaminated with complex pesticides and that bioremediation using environmentally friendly techniques is paramount. Based on the results obtained from Table 5, concluding that *P.aeruginosa* bacteria effectively decomposed Chlorpyrifos pesticide in the laboratory. Hence, this study evaluates the bioremediation of Chlorpyrifos pesticide using *P.aeruginosa* bacteria isolated locally from contaminated soil, and *P.aeruginosa* bacteria was resistant over some time in this soil. Therefore, this

bacteria was nominated for the bioremediation of Chlorpyrifos pesticide by bioinoculation of plants grown in contaminated soil.

Table 5: The efficiency of *P. aeruginosa* bacteria isolated from the initial soil sample in degrading different concentrations of Chlorpyrifos pesticide.

Pesticide concentration mg L^{-1}	Number of live bacterial cells (cfu ml^{-1})
10	27.9×10^7
20	24.3×10^7
30	19.4×10^7
40	11.5×10^7
50	9.3×10^7
Control	3.27×10^7

The effect of *P. aeruginosa* bacteria, white fungus waste, and nano fertilizer in the soil bioremediation of the residual Chlorpyrifos pesticide: The results of the tabulated statistical analysis (Table 6) indicated that after 120 days of application, bioinoculation with P. aeruginosa caused a significant difference in the levels of chlorpyrifos residues across the different treatments. Figure 3 shows that chlorpyrifos was present as residues in the treatment B0Ab0N0 , While the results of HPLC analysis in Figure 4 show that there are no residues of the pesticide chloropyrifos in the soil after 120 days of biological treatment B1Ab2N3 of HPLC analysis of soil samples about chlorpyrifos, where it was found that no chlorpyrifos pesticide residue was reported in the soil of the treatment bio-inoculated with B1 compared to the control that had a concentration of 4.401 mg kg^{-1} soil. Due to the biodegradation process of the inoculated *P. aeruginosa* bacteria and the effectiveness of the roots of the sugar bean plant, Chlorpyrifos pesticide is lost from the soil inoculated with *P. aeruginosa*. These bacteria can transform pesticide molecules so they are no longer toxic and harmless through complete mineralization of organic pesticides or decomposition into small, nontoxic molecules by various metabolic processes. This ends with the environment's purification action (33 and 57).

The treatment of adding white mushroom waste at its three levels, coded (Ab0, Ab1, and Ab2), achieved a noticeable decrease in the amount of Chlorpyrifos pesticide remaining in the soil, but it did not wholly decompose it, as it recorded 4.275, 1.908 and 0.417 mg kg^{-1} soil, respectively. The reason for the decrease in the amount of Chlorpyrifos pesticide in the soil when adding white mushroom waste is that mushroom waste can decompose large amounts of soil pollutants, especially pesticides, into less toxic forms or non-toxic metabolites through mineralization and decomposition processes with the help of many oxidative enzymes, as lignocellulose enzymes present in mushroom waste compost participate in the decomposition of complex compounds. This activity is attributed to the increase in the amount of organic carbon in mushroom waste and the increase in surface area, which increases the bio-absorption process and, thus, the decomposition of polluted compounds (27, 28, 40 and 43). The Chlorpyrifos residues were traversed by statistically substantial differences in the levels of nano-fertilizer addition at the four levels (N0, N1, N2, and N3) in the averages. Comparatively, the lowest average residue of Chlorpyrifos pesticide was 0.815 mg kg^{-1} soil under treatment N3 where (1 kg h^{-1} nano-boron + 2 kg h^{-1} nano zinc)

was applied, which was higher with the highest value of 4.328 mg kg^{-1} soil obtained under control treatment.

The reduction of residual Chlorpyrifos pesticide is significantly enhanced by the addition of mixed boron and nano-zinc, which activate various hydrolytic enzymes, including urease and phosphatase. This degradation process occurs in four steps, beginning with the conversion of Chlorpyrifos into Oxon chlorpyrifos through the action of mixed-function oxidases. Subsequent transformations lead to the formation of TCP and diethyl thiophosphate, ultimately resulting in the breakdown of Oxon Chlorpyrifos into TCP via hydrolytic enzymes (5, 29 and 32).

The effect of the two-way interaction between bio-inoculation and white fungus waste was highly significant in recording variations in Chlorpyrifos pesticide residue values. This was confirmed by the HPLC analysis, wherein no treatment with bio-inoculants carried Chlorpyrifos pesticide residues, whereas the Ab0B0 comparison showed the highest pesticide residue of 8.550 mg kg^{-1} soil.

This is due to the development of the bioremediation process through biostimulation using organic fertilizers in addition to bio-inoculations such as white fungus waste, which is a good source for the biostimulation process, as these organic wastes act as an effective stimulating factor to enrich the biological community in the soil and increase its activity, thus working synergistically, which helps accelerate the biological decomposition of the pesticide in the soil (8, 13, 16 and 36).

Data in Table 6 indicated that the combined effect of bio-inoculant and nano-fertilizer is very significant since all treatments with *P. aeruginosa* bacteria had no residue of Chlorpyrifos pesticide when compared to the comparison B0N0, which recorded the highest residue of Chlorpyrifos pesticide at 8.657 mg kg^{-1} soil. This is why, post-bio-inoculant and nano-fertilizer application, Chlorpyrifos was decomposed due to the effect of zinc and nano-boron boosting the activity of soil-borne microorganisms as well as the inoculation of *P. aeruginosa bacteria*, which are mainly involved in breaking down pesticides minus toxic metabolites (18, 31 and 33).

Contrastingly, the most satisfactory results in a binary interaction, as those obtained after the incorporation of white mushroom waste and nano fertilizer, are denoted by combinations N1Ab2, N2Ab2, N3Ab2, and N3Ab1 because the subsequent HPLC analysis did not manifest any residue of Chlorpyrifos pesticide compared to the reference treatment, registering the highest concentration at 9.045 mg kg^{-1} soil. This is attributed to the complementary effect of white mushroom waste and nano fertilizer since white mushroom waste applied to the soil right after the cultivation step under aerobic conditions acts as an enzyme source, initiating specific reactions with zinc and nanoboron that further enhance the mineralization of organic pollutants in the soil (34 and 49).

The triple interaction between inoculation with *P. aeruginosa*, white fungus waste and nano fertilizer showed that the following treatments B0Ab1N3, B0Ab2N1, B0Ab2N2, B0Ab2N3, B1Ab0N0, B1Ab0N1, B1Ab0N2, B1Ab0N3, B1Ab1N0, B1Ab1N1, B1Ab1N2, B1Ab1N3, B1Ab2N0, B1Ab2N1, B1Ab2N2, B1Ab2N3 did not have any Chlorpyrifos residue after 120 days of adding the fertilizer compared to the control treatment B0Ab0N0 which recorded the highest Chlorpyrifos residue of 18.090 mg kg^{-1} soil. The reason for the decomposition of the remaining Chlorpyrifos pesticide

in the soil is due to the joint synergistic role between the three study factors, as the addition of zinc and nano-boron led to an increase in the activity of microorganisms in general and the activity of the added *P. aeruginosa* bacteria in particular, which performs the biodegradation process as a result of restoring its biological activity in the soil, decomposing the pesticide into quickly metabolized materials, in addition to the role of white mushroom waste in the biostimulation of *P. aeruginosa* bacteria added to the soil, which increases the rate of decomposition of the remaining Chlorpyrifos pesticide (2, 6, 12 and 47).

Table 6: The effect of *P. aeruginosa* bacteria, white mushroom waste, and nano-fertilizer in the bioremediation of Chlorpyrifos pesticide residues (mg kg^{-1} soil) in the soil after 120 days of cultivation.

B1			**B0**		**Inoculation in *P. aeruginosa* (B)**
0.000			4.401		
		0.125			**LSD 0.05**
Ab2	**Ab1**		**Ab0**		**White fungus levels (tons h^{-1}) (Ab)**
0.417	1.908		4.275		
		0.153			**LSD 0.05**
N3	**N2**	**N1**	**N0**		**Nano fertilizer (N) (kg N h^{-1})**
0.815	1.747	1.911	4.328		
		0.176			**LSD 0.05**
Bilateral interaction between inoculation with *P. aeruginosa* and white mushroom waste					
Ab2	**Ab1**		**Ab0**		
0.835	3.817		8.550		**B0**
0.000	0.000		0.000		**B1**
		0.216			**LSD 0.05**
Bilateral interaction between *P. aeruginosa* inoculation and nano fertilizer					
N3	**N2**	**N1**	**N0**		
1.630	3.493	3.822	8.657		**B0**
0.000	0.000	0.000	0.000		**B1**
		0.249			**LSD 0.05**
dual interaction between white mushroom waste and nano fertilizer					
N3	**N2**	**N1**	**N0**		
2.445	2.578	3.032	9.045		**Ab0**
0.000	2.662	2.702	2.270		**Ab1**
0.000	0.000	0.000	1.670		**Ab2**
		0.305			**LSD 0.05**
Triple interaction between study factors					
N3	**N2**	**N1**	**N0**		
4.890	5.157	6.063	18.090	**Ab0**	**B0**
0.000	5.323	5.403	4.540	**Ab1**	
0.000	0.000	0.000	3.340	**Ab2**	
0.000	0.000	0.000	0.000	**Ab0**	**B1**
0.000	0.000	0.000	0.000	**Ab1**	
0.000	0.000	0.000	0.000	**Ab2**	
		0.432			**LSD 0.05**

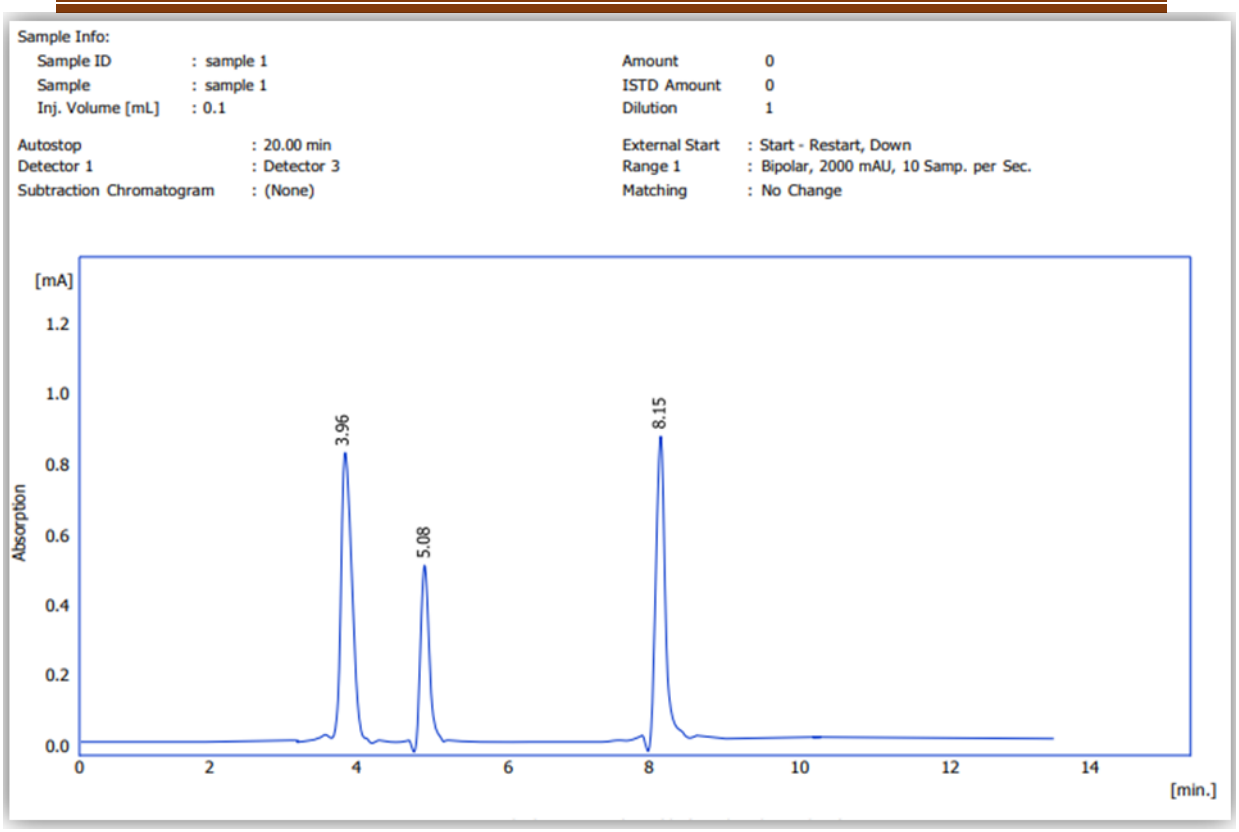

Figure 3: The estimation of Chlorpyrifos pesticide using HPLC in coefficients (B0Ab0N0).

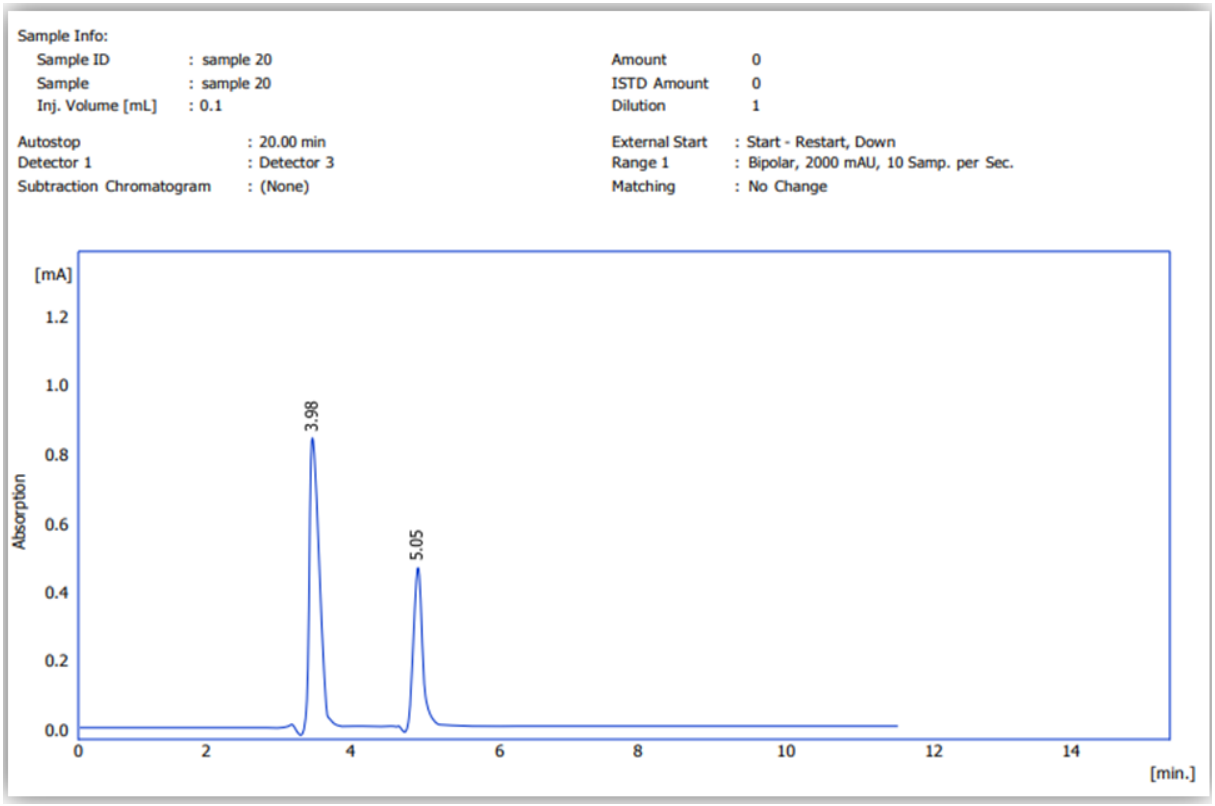

Figure 4: The estimation of Chlorpyrifos pesticide using HPLC in coefficients (B1Ab2N3).

Conclusions

It can be concluded that the triple interaction between inoculation with *P. aeruginosa* bacteria, white fungus waste, and nano fertilizer, the following treatments B0Ab1N3, B0Ab2N1, B0Ab2N2, B0Ab2N3, B1Ab0N0, B1Ab0N1, B1Ab0N2, B1Ab0N3, B1Ab1N0, B1Ab1N1, B1Ab1N2, B1Ab1N3, B1Ab2N0, B1Ab2N1, B1Ab2N2, B1Ab2N3 did not have any Chlorpyrifos pesticide remaining after 120 days of adding the fertilizer compared to the control treatment B0Ab0N0, which recorded the highest Chlorpyrifos pesticide residue of 18.090 mg kg^{-1} soil. All the residues measured in the plant and soil for all treatments are within the minimum-maximum limits allowed according to the European Union rule for determining the maximum limits for pesticide residues.

A combination of bacterial inoculate, white fungus waste, and nano fertilizer can be used to eliminate pesticide residues and their toxic effects. In addition, experiments must be conducted on using other microorganisms with pesticides for various crops in collaboration with researchers from various agricultural specialties to reach objective and integrated results.

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No Supplementary Materials.

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