ISOLATION, IDENTEFECATION AND EFFECINCY OF *PSEUDOMONAS FLUORESCENS* BACTERIA TO TERMITE *MICROCEROTERMIS DIVERSUS*

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ABSTRACT
This study was conducted to isolate the bacteria *Pseudomonase fluorescens* from the termite, locust, and American cockroach in the Iraqi environment and to diagnose it based on morphological, biochemical, and molecular diagnosis using the polymerase chain reaction (PCR), as well as test its pathogenicity and efficacy to termites under laboratory conditions. The results of morphological , biochemical, and molecular diagnosis using polymerase chain reaction (PCR) tests showed the isolated bacterial isolates are similar to *P. fluorescens* .The results of efficiency of different isolates of *P. fluorescens* showed that they have a high pathogenicity towards termite workers in laboratory and incubation condition.

Key words: White ant , biocontrol , Iraqi environment , Locust

عزل وتشخيص وتقييم كفاءة البكتريا *Pseudomonas fluorescens* ضد الارضة *Microcerotermis diversus*

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مدرس مساعد استاذ باحث
1 قسم التقنيات الاحيائية ، كلية العلوم ، جامعة بغداد
2 قسم وقاية النبات ، كلية علوم الهندسة الزراعية ، جامعة بغداد
3 مركز المكافحة المتكاملة ،دائرة البحوث الزراعية ، وزارة العلوم والتكنولوجيا

المستخلص :
أجريت الدراسة لعزل بكتيريا *Pseudomonase fluorescens* من النمل الأبيض والجراد والصرصر الأمريكي في البيئة العراقية وتشخيصها على أساس الصفات المظهرية والكيموحيوية والجزيئية باستخدام تفاعل البلمرة المتسلسل (PCR)، وكذلك اختبار. الإمراضية والفعالية ضد شغالات النمل الأبيض في ظروف المختبر. أظهرت نتائج التشخيص المورفولوجي والكيميائي الحيوي والجزيئي باستخدام اختبارات تفاعل البلمرة المتسلسل (PCR) أن العزلات البكتيرية المعزولة تطابق *P. fluorescens* من حيث استجابتها وتفاعلها مع الاختبارات المختلفة، والتي تطابق نتائج الاختبار الجزيئي في تماثلها مع النوع المذكور أعلاه. أظهرت نتائج تقييم كفاءة العزلات المختلفة من *P. fluorescens* أنها ذات قدرة إمراضية عالية تجاه شغالات النمل الأبيض في ظروف المختبر والحاضنة .

الكلمات المفتاحية: النمل الابيض، مكافحة احيائية، البيئة العراقية، جراد

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INTRODUCTION

The termite is one of the most important social insects of economic importance in the tropics and subtropics, as it lives in special colonies consisting of thousands of individuals (4). Due to the role played by termites in the decomposition of organic matter, the cycle of elements and soil fertility in tropical and subtropical regions, it has great importance in the ecosystem (13,19) However, it also causes great damage to agricultural crops, forest trees, and timber (13) Some studies globally indicate that termites causes economic damage of $40 billion annually (31) Termites, particularly *Microceroterms diversus* silvestri, cause severe losses in old, new, or under construction buildings, fruit trees, field crops, and vegetable crops, making them one of Iraq's most important economic insects in the majority of governorates (7, 10).Given the high cost of chemical pesticides, in addition to the negatives resulting from their use, which are the effect on human and animal health, their pollution on the environment, and the emergence of insect resistance to them, as well as an imbalance in the natural balance, due to their impact on natural enemies, which encouraged interest in finding safer ways to preserve the environment. ,Bacteria are one of the most important microbial groups that have attracted interest as pathogens of insects (5, 27 ,30) Among them is *Pseudomonas fluorescens* a gram-negative aerobic bacteria, with polar flagella, which has an important role in pathogenesis. It does have the ability to produce substances such as hydrocyanic, lytic enzymes, and blood cell analyzers (2, 6, 32, 37). Pseudomonas imposes its biocontrol activity in phytopathogens such as *Phytophthora infestans* (3, 23, 25) *and Fusarium oxysporum*(12, 20, 26). Until recently, the role of *Pseudomonas fluorescens* bacteria strains against insects was not sufficiently clear, but much research indicated the importance of these bacteria strains against insects later, such as aphids and some ladybugs feeding on plants (22,28). and the termite (9). demonstrated that *P. fluorescens* has a high pathogenicity for different types of termites, and all species were sensitive to bacterial infection(17). The LT reached 50 (101-127) hours and LT90 (265-302) hours. (9) showed that the bacteria *P. fluorescens* affects the respiratory system, causing death to termites by producing hydrogen cyanide gas as well as its ability to inhibit the enzyme cytochrome c oxidase in the respiratory chain. This could be a promising way to use bacteria in pest control. The types of bacteria can be diagnosed using many methods, including biochemical, which depends on the response of bacteria types to specific reactions that distinguish them from other types (12). It is also possible to diagnose bacteria types by more accurate methods that depend on genetic genes, such as the polymerase chain reaction (PCR) technique, which can give an accurate diagnosis more efficiently than the aforementioned methods (8). From this point of view and considering the importance and role of the bacteria *Pseudomonas fluorescens* in controlling insects, the study is aimed at the possibility of isolating bacteria from different types of insects in the Iraqi environment and determining some of their biochemical ,phenotypic characteristics and partially diagnosing them using PCR technology as well as testing their pathogenicity and effectiveness in causing death to termites.

MATERIALS AND METHODS:

Isolation and culture of *Pseudomonas fluorescens* from locusts, cockroaches and termites: *P.fluorescens* bacteria were isolated from different insects, including termites, locusts, and American cockroaches, each separately, using the method described by 15, 18) with some modifications, . Insects were collected from greenhouses, and the inactive and immobile individuals were chosen, sterilized with 70% ethyl alcohol, dried, then cut the ends of the legs, wings, and tentacles, then crushed in a ceramic mortar containing 10% water, then filtered the resulting mashing solution using a boring cloth, and for the purpose of culturing insect extracts on nutrient medium, a series of dilutions were made. Then, at a rate of three replications for each dilution, 18 sterile Petri dishes with a diameter of 9 cm and a depth of 12 mm were prepared, and a quantity of King B culture media was applied to each dish. bacteria Isolates were diagnosed according to morphological and biochemical properties (14).

Morphological and biochemical characterization: For morphological, cultural, and biochemical characterization of *P. fluorescens*, pure cultures of each isolate were streaked on fresh King's B agar Petri plates separately for colony growth and gram staining, tested for shape, colony elevation, colony edge and pigment production. series of biochemical tests to diagnose the bacterial isolates under study were conducted, which included: Citrate utilization, Methyl red, Indol production, Voges-Proskauer, Oxidase test, Catalase, (24, 35) Hydrogen cyanide production capacity, growth at $42C^0$ (9).

Molecular characterization of *Pseudomonas flourescens* bacteria: Separately, DNA was extracted from bacteria isolated from termites, locusts, and cockroaches using the Genomic DNA extraction kit), supplied by the Korean PROMEGA company. Bacterial strains were inoculated into sterile 3 ml of King Agar B medium and incubated overnight at 27±2 ◦C in a rotary incubator for 24 h. 2 ml of the resulting bacterial suspension was pelleted at 10,000 rpm for 3 min .Extracted DNA was amplified using primers that target the specific sequence of the 16s RNA gene. Forward primer5'- AGAGTTTGATCCTGGCTCAG- 3' and Reverse primer5'- GGTTACCTTGTTACGACTT- 3'. Polymerase chain reaction (PCR) amplification was carried out by the Maxime PCR PreMix kit (i-Taq) 20µlrxn (Cat. No. 25025) supplied by (Intron) Korea and according to the company's instructions. Prepare a 25 µl PCR reaction mixture containing 1.5µl extracted DNA , 10 picomols/µl (1 µl)of each primer, and 5µl Taq PCR PreMix Taq DNA polymerase and 16.5 µl sterile deionized water. PCR amplification was performed with an Eppendorf Master Cycler Gradient using the following program: Initial denaturation at 95 ◦C for 5 min, followed by 35 cycles of denaturation at 95 ◦C for 45 sec, annealing temperature at 58 ◦C for 45 sec, and extension at 72 ◦C for 45 sec, with a final extension at 72 ◦C for 7 min. The PCR products were electrophoresed in a 1% (w/v) agarose gel containing ethidium bromide and the amplicons were visualized under a gel documentation system. The gel section with the desired band was carefully excised under UV light and subjected to extraction using a gel band purification . The amplification products were purified and sent with the primers to Macrogen Company in South Korea for the purpose of conducting a DNA sequencing assay. Sequences were subjected to BLAST search at NCBI (www.ncbi.nlm.nih.gov) to assign putative identities and designations of operational taxonomic units based on sequence similarity measures and phylogenetic inference. Partial nucleotide sequences were deposited in the NCBI Gen Bank and authentic accession numbers were obtained. A phenogram was constructed with MEGA 6 software by grouping the isolates deposited at GenBank to reveal the relationships of the identified strain with taxonomically similar bacteria by constructing a phylogenetic tree. Alignments were manually edited wherever necessary, and a phylogenetic analysis was performed to assess phylogenetic affiliation.

Preparation of bacterial suspensions for local and commercial *P. fluorescens* isolates: Bacterial suspensions were prepared on Nutreint broth liquid media and for each isolate separately. The cultures were incubated at 27 °C for 48 hours. The Plate Count Method was adopted to calculate the number of bacterial colonies by preparing a series of dilutions. after incubating the dishes at 27 ± 2 °C for 48 hours. Then count the developing bacterial colonies by adopting the dilutions in which the number of colonies is between 30-300 and calculate the number of colony-forming units (CFU) using the equation (18) as follows: Colony formation unit (concentration)/ml = average number of colonies x dilution inverse / Sample volume On the basis of calculating the unit of colony formation for each isolate, three concentrations of isolates of local bacteria *P. fluorescens* were prepared for use in the subsequent research steps, based on the equation: C1 × V1 = C2 × V2 as: C1 = the original concentration, V1 = the volume required to be taken from C1 To obtain a volume of C2 with a volume of V2, C2 = the desired concentration, V2 = the final volume of the desired concentration. For the purpose of obtaining active colonies of commercial *P. fluorescens* bacteria, 1 gm of the bacterial powder was taken and 9 ml of

sterile distilled water was added to it. 3 sterile Petri dishes containing King b solid culture medium were prepared, then cultured and incubated at a temperature of 27 ± 2 °C for 48 hours. After an hour, they followed the same steps mentioned in the preparation of the concentrations of the isolates above.

Efficiency of local and commercial *Pseudomonas fluorescens* isolates in causing death to termites in incubator and laboratory conditions: *P.fluorescens* bacteria strains isolated from termites, locusts, American cockroaches, and commercial *P.fluorescens* isolates were tested for their ability to kill termite individuals when treated with termite media (cellulose 4 gm and agar 4%) in incubator conditions at a temperature of 27 2 °C. All treatments were administered at three different concentrations: 5 $\times 10^9$, 10 $\times 10^9$, and 15$\times$ 10^9 Cfu/ml. Three ml of each concentration for each treatment e was added to the food medium in the dishes and left for two hours until the food medium was saturated with the treated solution (34). 100 termites worker were transferred by a soft brush to the treated dishes. After that, the treated dishes were closed and covered with cellophane paper to provide conditions of complete darkness. They were placed in the incubator at a temperature of 27 °C ± 2 °C and a relative humidity of 60-70% three replicate done for each treatment. The examination was conducted for the third day after treatment for all the dishes, and the number of dead individuals was recorded at each examination. Then the examination was conducted every three days until all the individuals died. The mortality rates were corrected according to the Abbott equation (1) The same steps were repeated when studying the efficiency of isolates in laboratory conditions.

Statistical analysis

Factorial experiments were conducted using a completely randomized design, and the differences between treatment means were compared using the value of the least significant difference at the 0.05 probability level. The results were analyzed by statistical program Genstat

RESUITS AND DISCUSSION

Morphological and biochemical characterization: The morphological characteristics of *Pseudomonas fluorescens* isolated from different insects were studied after they were cultured on King Agar B (King b) solid media for 24 hours. The cultures spread on the medium were characterized by their small size and circular shape with different edges, regular and irregular, and the color contrast between cream and yellowish green, as in Table (1). Microscopic investigations revealed that they are rod-shaped, gram-negative cells with a pink color that do not form spores. Table (2) shows the results of biochemical tests for bacterial isolates of *Pseudomonas fluorescens* isolated from different insects. It shows that bacterial isolates are able to consume citrate as the only carbon source and ferment glucose sugar ,produce cytochrome oxidase and produce hydrogen cyanide, catalase enzyme, which showed the test positive. The ability of two isolates of *P. fluorescens* bacteria isolated from termites and locusts to partially ferment glucose was inferred from the positive test, while the test did not show the ability of bacteria isolated from the American cockroach to partially ferment glucose sugar . The inability of the bacterial isolates to analyze the amino acid tryptophan and, consequently, their inability to produce indole were inferred from the negative results. . The results indicated that the bacterial isolates were unable to grow at 42°C, which indicates that the bacterial isolates are *P. fluorescens.* This test is one of the important differential tests through which it is possible to distinguish between *P. fluorescens* bacteria and the rest of the isolates, such as *P. aeruginosa*. Similar results were shown by several studies that dealt with the diagnosis of *P. fluorescens* bacteria based on morphological and biochemical characteristics Suman et al (35) Manasa et al(21).

Table 1. morphological characteristics of *Pseudomonas fluorescens* isolated from various insects

Isolation	spores formation	shape of bacteria	Gram stain	Pigment color	edge colony	colony surface	Colonyl color	colony shape	Size
P. fluorescens Termite1	Negative	Rod	Negative	yellow green	Regular	Smooth	yellow green	circular	Small
P. fluorescen Trmite 2	Negative	Rod	Negative	light green	Irregular	soft glossy	yellow cream	circular	Small
.P. fluorescens locust 1	Negative	Rod	Negative	yellow green	Regular	Smooth	yellow green	circular	Small
P. fluorescens locust 2	Negative	Rod	Negative	light green	Regular	soft glossy	yellow cream	circular	Small
P. fluorescens cockroach 1	Negative	Rod	Negative	yellow green	Regular	Smooth	yellow green	circular	Small
P. fluorescens cockroach 2	Negative	Rod	Negative	light green	Irregular	soft glossy	cream	circular	Small

Table 2. Biochemical characteristics of *Pseudomonas fluorensces* isolated from different insects

isolation	growth at 42	Catalase test	Indol production test	HCN Test	Oxidase test	Voges – Proskauer test	Methyl red test	Citrate utilization test
P. fluorescens Termite	_	+	_	+	+	+	+	+
P. fluorescens Locust	_	+	_	+	+	+	+	+
P. fluorescens cockroach	_	+	_	+	+	_	+	+

Molecular identification of *Pseudomonas fluorescens* isolate using 16S rRNA genetic sequencing: The sequences of nitrogenous bases of the 16SRNA gene produced by amplification of bacterial isolates were determined after they were sent to the Korean company Macorogen, , as 3 pure isolates isolated from termites, locusts and American cockroaches were selected to perform sequence analysis of the sequences amplification outputs, then the different sequences of bacterial isolates were compared based on the information in the NCBI gene bank, using the Blast program. the results showed that the isolates isolated from the termite, locust and cockroach were similar with the standard strains of *Pseudomonas fluorescens*, with an identical percentage of 99% for all isolates of insects mentioned in succession, which indicates that all bacterial isolates isolated from insects belong to the type *Pseudomonas fluorescens*, and the results are identical to the phenotypic and biochemical tests. The three isolates were recorded for the first time in Iraq in the gene bank with the numbers MT889677.1, MT889678.1 for *Pseudomonas fluorescens* . fig(1) (www.ncbi.nlm.nih.gov).

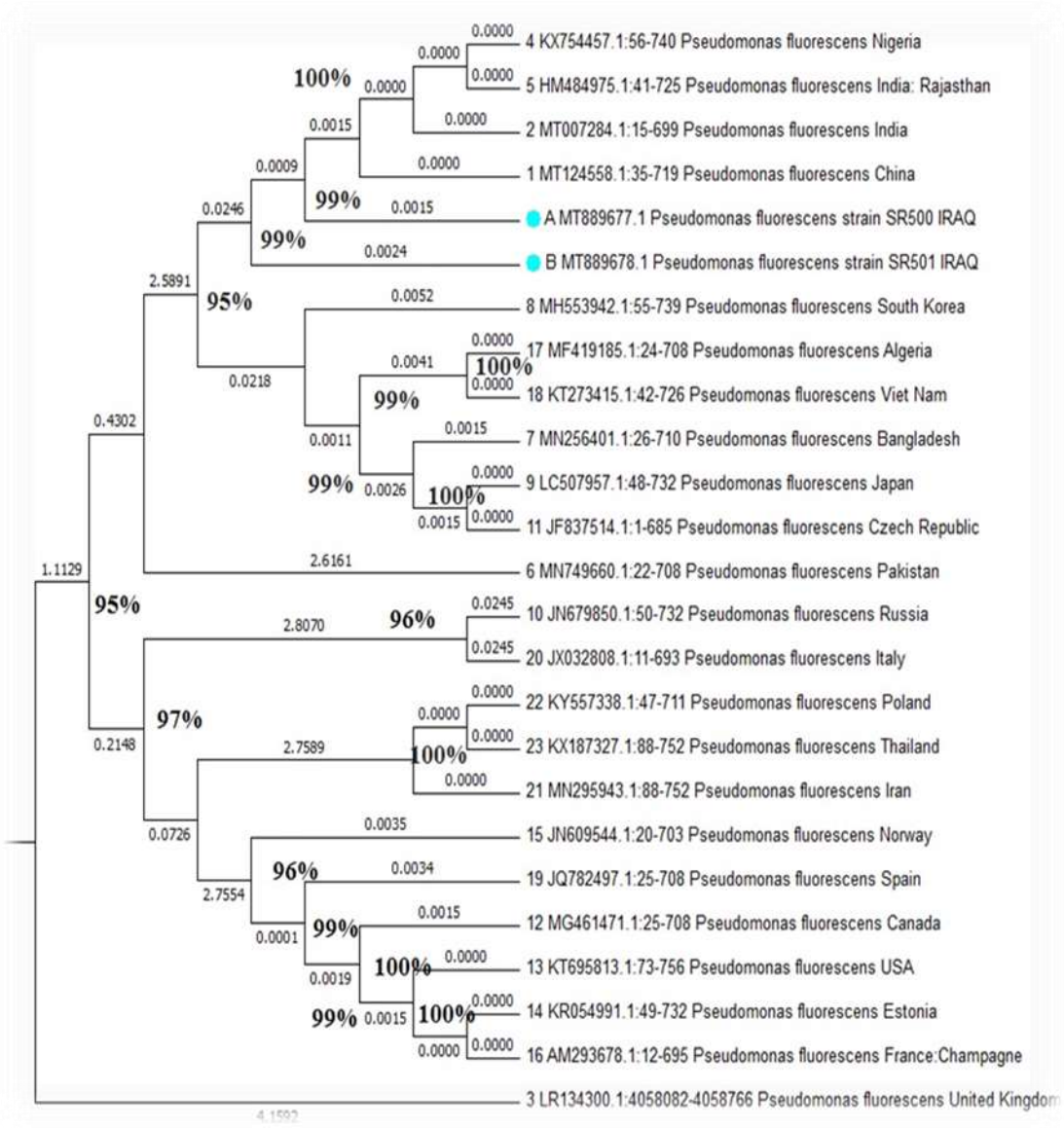

Figure 1. Phylogenetic tree and relationships between bacterial isolates

Efficiency of local and commercial *Pseudomonas fluorescens* isolates in inducing death of the members of the earth at a temperature of 27 ± 2 °C in the incubator and under normal laboratory conditions: The results in Table (3) showed that all bacterial isolates were highly effective in killing termites, as the mortality rate of the isolates of *Pseudomonas fluorescens* isolated from termites , locusts, cockroaches, and commercials had the highest mortality rates at the concentration $^{9}10\times15$, which amounted to 99.16%, 100%, 91.88%, and 87.07%, respectively, after 27 days of treatment at a temperature of 27±2°C. There were no significant differences in the mortality rates at this concentration for isolates of bacteria isolated from termites, locusts, and cockroaches, which in turn differed significantly from the commercial isolates of bacteria. The results of the statistical analysis also showed that there were significant differences between mortality rates at different concentrations, . The results showed that the time factor had an effect on increasing the effectiveness of the isolates against the termite with a significant difference. The reason may be attributed to the fact that with the passage of time, the number of bacterial colonies increases and their arrival in the digestive system by continuing to feed on the treated medium, thus increasing their virulence through the abundance of secondary compounds produced, such as hydrogen cyanide and others, which exposes the insect host to high concentrations of these products compared to the beginning of the treatment, and this is consistent with what was reached

(34). Also, the effectiveness of the isolates is greatly affected by the interaction of the studied factors, with a significant difference. From these results, it can be concluded that the isolates of *Pseudomonase fluorescens* isolated from different insects are better in their effect than the commercial isolation of the same bacteria above. The results in Table (4) showed that isolates of *P. fluorescens* isolated from termites, locusts, and cockroaches, and the commercial isolate of the bacteria produced the highest mortality rates at the 15 x 10^9 concentration, reaching 100%, 98%, 95%, 92%, respectively, after 36 days of treatment at laboratory conditions. The results of the statistical analysis showed that there were no significant differences in the mortality rates in the isolated isolates of locusts, cockroaches, and commercial ones, which differed significantly from the isolates of termites. Therefore, the bacteria *P. fluorescens* isolated from the termites. The results also showed that there were no significant differences in the mortality rate at the different concentrations. Also, the efficiencies of bacterial isolates increase with the progression of the treatment time in causing death to the termite workers, a significant difference. It is noted from the cumulative statistical analysis of the death rates of the different isolates in the temperature of the incubator and the natural laboratory conditions that there are no statistical differences between the mortality rate , where the overall rate was 62.91 ±5.63 in laboratory conditions and 55.36 ± 0.94 in the conditions of the incubator. Table (5). In similar studies, it was observed that many types of *P. fluorescens* bacteria are effective on many insects, as they caused a death rate of up to 70% in the larvae of the citrus leaf borer. They were also very influential on the fifth instar nymphs of the migratory locust, as they caused death rates ranging from 98-100% Mohandkaci et al (22) While Khan et al. (17) demonstrated that *P. fluorescens* has a high pathogenicity to different types of termites, the half-life of the killer is 101-127 hours, while the time that kills 90% is 265-302 hours. The bacteria *P. fluorescens* can affect insects, including termites, by producing hydrogen cyanide gas, which affects the inhibition of the cytochrome c oxidase enzyme in the respiratory chain and thus leads to the death of the insect by suffocation (10). The bacteria *P. fluorescens* can also be affected by secreting the chitinase enzyme, which plays an important role through the analysis of the important chitin layer in the insect's body wall, as well as the bacteria's production of the enzyme protease, which is one of the most important metabolic byproducts and has an important role in causing toxicity and death to the insect (22). The bacteria also attacks the stomach tissues in termites, causing the destruction of fatty tissue, and the death rate can reach 100% after 10-12 days (17).

Table 3. mortality rate of termite workers when treating the nutrient medium with local and commercial isolates of *Pseudomonase fluorescens* under incubator conditions 27±2 Cº

Isolate	Concentration	time (days) 1	3	6	9	12	15	18	21	24	27	mean	General mean
	5×10^9	0.23	2.64	14.19	33.08	34.13	63.41	84.88	86.56	91.60	96.63	50.07	56.03
P.f Termite	10×10^9	5.75	14.87	32.34	51.54	50.88	60.16	86.56	89.08	92.44	95.77	57.93	
	15×10^9	6.21	12.23	26.12	43.86	54.76	59.35	97.48	97.48	79.48	99.16	57.61	
P.f	5×10^9	6.22	4.56	14.19	38.45	45.24	57.72	60.51	75.63	89.92	93.70	48.64	
Locust	10×10^9	12.42	11.51	26.13	42.31	50.80	50.40	64.71	100	100	100	55.82	55.63
	15×10^9	6.90	15.68	22.39	49.23	68.25	73.98	79.80	100	100	100	61.62	
P. f	5×10^9	24.86	33.10	38.07	38.47	45.24	59.35	63.87	96.75	72.27	82.40	52.73	
cockroaches,	10×10^9	40.63	44.61	54.48	56.16	64.29	68.29	68.07	69.75	72.27	82.12	62.06	.
	15×10^9	20.69	23.02	23.14	39.23	51.39	59.35	70.59	71.43	72.09	91.88	52.28	55.7
P.f	5×10^9	4.37	7.92	13.44	30.00	32.36	39.84	41.18	46.22	51.27	75.39	34.19	
Commercial	10×10^9	1.61	9.36	9.21	22.31	31.75	39.03	42.86	43.70	44.54	72.25	31.66	
	15×10^9	4.83	9.12	15.92	29.74	44.44	61.79	73.95	75.63	75.63	87.07	47.81	37.89
	Mean	11.23	15.72	24.14	39.53	47.79	57.72	69.53	77.10	78.46	89.70		
L.S.D 0.05	Isolations = 5.65	Concentrations = 4.81	time = 8.78	Isolates x concentrations = 9.62	isolates x time = 17.60	Concentrations × Time = 15.22	Isolates × Concentration × Time = 30.43						

Table 4. mortality rate of termite workers when treating the nutrient medium with local and commercial isolates of *Pseudomonas fluorescens* under normal laboratory conditions

Isolate	Conce	time (days													mean	total
		1	3	6	9	12	15	18	21	24	27	30	33	36		
	5×10^9	7.4	35.4	45.8	48.5	52.1	57.8	67.8	72.86	82.1	92.1	95	98.5	100	65.83	
P.f	10×10^9	8.1	38.1	45.8	55.7	62.1	65.7	66.4	70	71.4	74.2	78.5	83.5	89.8	62.2	61.41
Termite	15×10^9	7.4	27.08	38.8	40.7	42.1	44.2	50	53.5	56	73.5	90	79.1	100	56.1	
P.f	5×10^9	1.8	26.3	36.1	41.4	45.7	48.5	54.2	57.1	60	62.8	75.7	82.8	87.1	52.3	
Locust	10×10^9	2.2	37.5	50	51.4	52.8	58.5	60	62.8	75.7	77.1	80	82.8	84.2	59.6	53.73
	15×10^9	1.8	13.8	30.5	30	37.1	42.5	47.1	50	54.2	64.2	78.5	91.4	98.5	49.2	
P. f	5×10^9	0	31.9	38.8	48.5	51.4	58.5	62.8	65.7	81.4	90	94.2	95.7	95.7	62.6	
cockroaches›	10×10^9	10.8	33.3	45.8	45.7	45.7	48.5	50	52.8	60	62.8	64.2	64.2	77.1	50.8	57.86
	15×10^9	4.05	38.8	50	55.7	57.1	57.1	58.5	61.4	62.8	70	78.5	90	95.7	60	
P.f	5×10^9	3.1	5.5	27.7	35.7	42.8	47.1	55.7	55.7	55.7	57.1	60	62.8	68.5	44.4	
Commercial	10×10^9	0.9	22.2	40.2	55.7	60	62.8	67.1	68.5	72.8	74.2	75.7	75.7	75.7	57.8	56.69
	15×10^9	16.6	41.6	58.3	64.2	71.4	71.4	71.4	72.8	74.2	77.1	81.4	87.1	92.8	67.7	
	mean	5.3	29.3	42.3	47.7	51.7	55.2	59.2	57.4	67.9	72.9	79.4	84.3	88.7		

L.S.D 0.05 Isolations =4.66 Concentration = 4.04 time = 8.4 Isolates x concentrations = 8.07 isolates × time = 16.8 Concentrations × Time = 16.55 Isolates × Concentration × Time = 29.1

Table 5. cumulative mortality of termites when treating with local and commercial isolates of *Pseudomonase fluorescens* in the different conditions

Conditions	% mortality
Bacterial isolates under laboratory conditions (non-constant temperature)	**62.91 a ± 5.63**
Bacterial isolates in incubator conditions (constant temperature 27 ± 2 °C)	**55.36 a ± 0.94**
P * = 0.268 not significant	
t = 1.52	

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