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THE FITNESS OF A RECOMBINANT STRAIN OF *PSEUDOMONAS AERUGINOSA* BACTERIA COMPARING WITH ITS PARENT STRAINS UNDER THE EGYPTIAN ENVIRONMENTAL CONDITIONS (MOWAS RIVER) IN 2020

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ABSTRACT

The present study aimed to study the fitness between a trans-conjugant (recombinant strain) of *Pseudomonas aeruginosa* bacteria with its parents after transferring genetic material by conjugation mechanism. Whereas, environmental fitness expresses the interaction of an organism with its environment therefore it is considered a good indicator for the assessment of genetically engineered microorganisms (GEM) released into nature. Incubation time was carried out *in vitro* and incubating time in situ in Mowas River Zagazig city during winter and summer. Accordingly, the fitness of the parents and the recombinant strain was studied. The three strains of *Pseudomonas aeruginosa* (PAO1, MAM2 and PU21) were tested on chloramphenicol and tetracycline. Strain MAM2 was resistant to chloramphenicol 1200 µg/ml while was sensitive to tetracycline and has been used as the recipient. While strain PAO1 was resistant to tetracycline 200 µg/ml and was sensitive to chloramphenicol and has been used as the donor. Results proofed the presence of the plasmid in the donor and trans conjugant strains. The donor was treated with acridine orange to match the results obtained with the results at the molecular level. It was observed that bacterial fitness continued for up to 35 days in vitro, while in situ during the summer it did not last at the site for only 21 days. While it lasted 28 days during the summer. So, the risks that may be caused by releasing the genetically modified microorganisms into environments have been canceled. In addition to its ability to preserve the new genetic material, it may be able to transfer this new genetic material to other strains and species that may be live in the same ecosystem, as it is largely stable in the environment. In genetically modified microorganisms that are added to the environments for agricultural uses such as increasing soil fertility (bio-fertilizer) or biodegradation for a harmful substance such as pesticides, the soil must be re-inoculated in winter every 21 days and during summer every 28 days due to the loss of the plasmid, which carries some important genes.

Keywords: *Pseudomonas aeruginosa,* conjugation, trans-conjugant, Ecological fitness, genetically engineered microorganisms (GEM).

INTRODUCTION

Horizontal gene transfer (HGT) is primarily responsible for the genetic diversity (evolution) in bacteria (Wiedenbeck and Cohan, 2011). Fully sequenced genome It reveals that a significant portion of the open reading frame (ORF) was horizontal Transfer (Nakamura *et al.,* 2004; McDaniel, *et al.,* 2010) Plasmids play an important role in this process *via* conjugation (Summers, 2009; Smalla, *et al.,* 2015). The most significant indication of the importance of plasmids as aides to bacterial adaptation is their role in the spread of antibiotic resistance genes among strains (Carattoli, 2013). It plays an important role in helping bacteria adapt to different conditions and in bacterial ecology (Smillie *et al*., 2010; Smalla *et al*., 2015; San Millan *et al.,* 2016).

Environmental applications of microorganisms in agriculture are wide and varied, ranging from bioremediation, biopesticides, nitrogen fixation, plant growth promoter, to biocontrol of plant diseases, and other agricultural practices. The application of recombinant DNA techniques demonstrated the ability of genetically modified microorganisms to be used to pollinate soil, seeds, or water. This is done by improving the genetically modified microorganisms as they are able to raise agricultural crops production and clean the environment from pollutants (Birkenhead *et al*., 1988; Schubert, 2002; Prakash *et al*., 2011).

Different incubation time *in vitro* and different incubation time in situ during winter and summer in Mowas River in Zagazig city Sharkia government in 2020. Then the fitness of the parents and the recombinant strain were studied.

The PAO1 strain of *Pseudomonas aeruginosa* bacteria as donor was resistant to tetracycline 200 µg/ml and sensitive to chloramphenicol. While MAM2 strain of *Pseudomonas aeruginosa* bacteria as recipient was resistant to chloramphenicol 1200 µg/ml and sensitive to tetracycline. The two parent strains (donor and recipient) and the transconjugant were tested after the occurrence of the conjugation process by isolation of plasmid DNA and using gel electrophoresis to show if there is any difference in the plasmid characters in them. Results show the presence of the plasmid in the donor and the transconjugant and the absence of it in the recipient on the molecular level.

There has been interest in studies investigating the fate of GEMs that were set off in the environment either to be used for a specific purpose or unintentionally (Williamson, 1992).

In most releases, the GEMs might fail to survive or even if a stable population developed, the recombinate strain may be not expressed under environmental conditions. The GEMs can induce a measurable sustained change in the ecosystem. So a study and comparison was made between the fitness of GEM strain that has been constructed by conjugation and their parents in vitro and in situ (Mowas River).

The current study amid to define the effect of incubation time on the fitness of the transconjugant and compare it with the parents to find out the ability of the genetically engineered bacteria to retain the genetic material transferred to it under controlled conditions in vitro and under uncontrolled conditions in situ. The aim is to conclude whether genetically modified organisms pose a threat to the environment or are they less harmful than we think.

The results in this study are useful in the field of microbial biotechnology that being applied for the solutions of agricultural and environmental problems. Microbial biotechnology often requires the release and dispersal of a GMM to different ecosystems. This requires an understanding the ecology of microorganism and their fitness associated with different ecosystems.

MATERIALS AND METHODS

All experiments were carried out at Microbial Genetics research Laboratory, Faculty of Agriculture, Genetics Department, Zagazig University.

Strains used

Pseudomonas aeruginosa bacteria strains PAO1, PU21 and MAM2 that used in this study were obtained from M. Day, university of wales, Cardiff, UK.

Nutrient media

Different culture media were used in this study namely Luria broth (LB), nutrient broth (NB) and nutrient agar (NA). NB medium was prepared by mixing 10 g peptone, 5 g yeast extract and 5 NaCl in 1 L distilled water (dH2O). Medium pH was adjusted at the range of 7.0–7.2. For semi-solid nutrient agar (NA) medium, 1.5 % agar was added. Then medium was autoclaved at 121ºC for 20 min. Phosphate buffer was prepared from 1/15 M potassium phosphate (KH_2PO_4) and 1/15M disodium phosphate ($Na_2HPO_4.2H_2O$).

The concentration of tetracycline was 200µg/ml and the concentration of Chloramphenicol was 1200 µg/ml. Both antibiotics are dissolved in distilled water.

Enumeration of bacteria

CFU/ml was calculated for both donor and recipient at zero time and 24 h. CFU/ml was calculated after incubation in each experiment for donor, recipient and trans-conjugants. After incubation, the two incubated filters were placed together in 10 ml phosphate buffer and then a series of dilutions were made. Three replicas for each of donor, recipient, and Trans-conjugant on the selected media for each of them 0.1 ml is taken from this donor, recipient, and Trans-conjugant for each plate.

Conjugation experiment under laboratory condition

Pseudomonas aeruginosa PAO1 strain was used as the donor strain and *Pseudomonas aeruginosa* MAM2 was used as the recipient strain in this experiment. They were inoculated in 25 ml liquid media, incubated for 24 hours at 37°C with shaking at 160 rpm, and then by syringe 1 ml of donor strain was taken and passed through a filter membrane filtered onto a 0.22 µm pore size so that bacterial cells are not passed through the holes. The same was done for recipient strain. The donor and the recipient strain filters were placed face to face on NA media.

Incubation time experiment

Both the donor strain and the recipient strain were prepared as previously mentioned. The donor and the recipient strain filters were placed face to face on NA media. The plates were incubated at different times (1 day, 7 days, 14 days, 21 days, 28 days, 35 days, 42 days, 49 days, and 56 days). After incubation, the incubated filters were taken and placed in a tube containing phosphate buffer pH 6.8 in a glass test tube for one minute.

Then a series of dilutions were made, and from each dilution three replicas was done to calculate the CFU/ml for donor, recipient and trans conjugants, to know the extent of the effect of high or low Incubation time on the gene transfer rate.

Conjugation experiment under situ condition during the winter and summer

The experiment was conducted in the winter season during the period from 26/2/2020 to 25/3/2020 while in the summer season was from 17/8/2020 to 29/9/2020 in front of the Administrative Control building next to the Sharkia Governorate building.

The donor (PAO1) and recipient (MAM2) cells were grown in NB at 37°C for an overnight, then 1 ml of donor and recipient was taken by a sterile syringe and placed on a membrane filter (0.2 µm whatman) held in a Swinney

filter holder. By the previous method, 10 membrane filters were prepared from the donor and also the recipient. So that both the donor and recipient membrane filters were in separate dishes from NA. CFU/ml was also calculated for the developing culture.

Shortly, donor and recipient membrane filters were at the sampling site in the river. With forceps, the filter carrying the donor cells was placed face-to-face with the filter that holds the recipient cells. The membrane filters were then held face-to-face by bull dog clips and suspended by a nylon line from a tree branch hanging down the riverbank. A weight was tied at the end of the nylon line to keep the filter membrane 20-40 cm below the water surface. The water temperature and PH have been calculated.

After 24 hours, the filter is removed from the river water and placed in a jar containing 10 ml phosphate buffer, then the jar is placed in a box containing ice and then we return to the laboratory. Sampling was repeated at constant weekly intervals.

In the laboratory, the jar was placed on a shaker for 60 sec. A series of dilutions were made and then a counting experiment was performed to account CFU/ml for donor, recipient and trans-conjugants on the selective media of each strain.

Isolation of plasmid DNA

The LB media supplemented with specific antibiotics were individually inoculated with a single colony from each donor, recipient, and trans-conjugants. The cultures were incubated at 37°C overnight (24 h), with constant shaking at 160 rpm. The bacterial culture was harvested (10 ml) by centrifugation at 4000 rpm for 5 min at room temperature. The supernatant was discarded; 1 mL of sterile distilled water was added followed by centrifugation at 4000 rpm for 5 min.

10 µl of RNase was added and the plated cells were re-suspended in 250 µl of resuspension solution in an Eppendorf tube with a vortex. 250 µl of the lysis solution was added, turning the tube 5 to 6 times until it becomes clear, 350 µl of the neutralization solution was added with vortexing well 4 to 6 times to avoid sedimentation of the destroyed bacterial cells, centrifugation at 12000 rpm for 5 minutes. Then, the supernatant was transferred to the GeneJET-spin column by pipetting without touching the precipitated particles. Centrifugation for one minute at 12000 rpm with the leachate removed from the bottom of the tube. 500 µl of the washing solution was added to the GeneJET-spin column followed by centrifugation at 12000 rpm for one minute. Washing was repeated to remove the residues. The GeneJET spin column was transferred into a clean 1.5 mL Eppendorf tube. 40 µl of the elution buffer was added to the center of GeneJET spin column membrane to elute the plasmid DNA, The tube was left for 2 minutes at room temperature and then centrifuged at 12000 rpm for two minute and . The purified plasmid DNA was stored in the freezer until future use.

DNA electrophoresis

Isolated plasmid products were then separated on 1.6% agarose gel in Tris-boric acid – EDTA (TBE) buffer supplemented with ethidium bromide for the detection of amplified plasmid DNA bands. 100 kbp and 10000 kbp DNA ladder were used.

Statistical analysis

The average and standard deviation was calculated using Excel 2010 program for all analyzed data.

RESULTS AND DISCUSSION

Isolation of antibiotic resistant bacteria

Tetracycline and chloramphenicol

The three strains of *Pseudomonas aeruginosa* bacteria (PAO1, MAM2 and PU21) were tested on tetracycline where a series of concentrations were made, ranging from 20µg/ml, 30µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml and 200 µg/ml. The three strains were resistant to this antibiotic to a concentration of 100µg/ml but at concentration 200 µg/ml it gave resistant result with PAO1 while it was negative with MAM2 and PU21. Depending on these results, PAO1 was used as a donor and MAM2 as a recipient. The data also showed in table 1. As well, the three strains of bacteria (PAO1, MAM2 and PU21) were tested on chloramphenicol where a series of concentrations were made, ranging from 20µg/ml, 30µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml, 600µg/ml, 700µg/ml, 800µg/ml, 1000µg/ml and 1200 µg/ml. The three strains were resistant to this antibiotic up to concentration of 1000 µg/ml, but at concentration 1200 µg/ml it gave positive result with MAM2 and PU21 while it was negative with PAO1. The data are shown in Table 2. (Dean *et al.*, 2003; Amina and Amin, 2010).

Confirmation of conjugation occurrence and conjugation frequency under controlled laboratory

Donor and recipient filter membranes were incubated together for 1 min and 24h. The membranes then transferred into phosphate buffer under shaking at 160 rpm for 1 min, after that CFU/ml of transconjugants, donor, and recipient were calculated. It was always found that CFU/ml for transconjugants after one minute of incubation was zero. The conjugation experiment was repeated three times, and in each experiment three replicas were made to confirm the occurrence of the conjugation process. The data are shown in Table (3).

Table 1: Bacterial strains of *Pseudomonas aeruginosa*

Strain	Genotype	Reference
PAO1	Prototrophic	Holloway and Morgan (1986)
	Tet^r, Chl^s, Amp^s	This study
MAM2	Auxotrophic, Met^-	Amin *et al.,* (1987)
	Chl^r, Amp^s, Ter^s	This study
Pu21	Auxotrophic, Val^-	Amin and Day (1988)
	Chl^r, Amp^s, Ter^s	This study

Table 2. Effect of different concentrations of tetracycline and chloramphenicol on *Pseudomonas aeruginosa* strains PAO1, MAM2 and PU21

Antibiotics / Strains	Tetracycline				Chloramphenicol				
	20 µg	80 µg	100 µg	200 µg	20 µg	100 µg	600 µg	1000 µg	1200 µg
PAO1	+	+	+	+	+	+	+	+	–
PU21	+	+	+	–	+	+	+	+	+
MAM2	+	+	+	–	+	+	+	+	+

Table 3: Confirmation of conjugation occurrence and conjugation frequency under controlled laboratory

No. of experiments		Donor (CFU/ml) at zero time	Donor (CFU/ml) at 24 hours	Recipient (CFU/ml) at zero time	Recipient (CFU/ml) at 24 hours	No. of trans-conjugants cells ($x10^2$) per plate			Mean CFU/ml ($x10^3$) ± SD	Conjugation Frequency ($x10^{-6}$) per recipient at zero time
						R1	R2	R3		
Experiment 1	R1	9.1×10^8	8.4×10^7	7.2×10^8	8.9×10^7	1.81	1.76	1.65	1.74 ± 0.08	2.42
	R2		7.8×10^7		8.5×10^7	1.88	1.93	2.04	1.95 ± 0.08	2.7
	R3		8.1×10^7		8.3×10^7	2.2	2.26	2.44	2.3 ± 0.12	3.19
Experiment 2	R1	8.3×10^8	7.4×10^7	8.5×10^8	7.7×10^7	2.43	2.56	2.63	2.54 ± 0.10	2.99
	R2		7.1×10^7		7.9×10^7	2.74	2.88	2.93	2.85 ± 0.10	3.35
	R3		7.5×10^7		8.2×10^7	1.89	2.01	2.04	1.98 ± 0.08	2.33
Experiment 3	R1	9.5×10^8	8.3×10^7	6.6×10^8	1.4×10^8	0.39	0.35	0.49	4.1 ± 0.07	6.21
	R2		7.9×10^7		9.9×10^7	2.79	2.97	2.93	2.9 ± 0.09	4.39
	R3		8.5×10^7		1.1×10^7	2.63	2.76	2.81	2.73± 0.09	4.1

Table 4. Effect of incubation time on the fitness of parents and recombinant strain of *Pseudomonas aeruginosa in vitro*

Incubation Time	Donor (CFU/ml) at 24 hours	Percent survival for donor	Recipient (CFU/ml) at 24 hours	Percent survival for recipient	No. of trans-conjugants cells ($x10^2$) per plate			Mean CFU/ml ($x10^3$) ± SD	Conjugation Frequency ($x10^{-6}$) per recipient at zero time
					R1	R2	R3		
After 1 day	$5.3x10^8$	37.86	$1.9x10^8$	86.36	1.83	1.76	1.96	1.85 ± 0.10	8.4
After 7 day	$6.5x10^8$	46.4	$1.6x10^8$	72.7	2.49	2.40	2.69	2.53 ± 0.15	11.5
After 14 day	$7.1x10^8$	50.7	$1.4x10^8$	63.6	0.35	0.42	0.38	3.8 ± 0.04	17.3
After 21 day	$9.3x10^7$	6.64	$3.1x10^7$	14	1.81	1.65	1.7	1.72 ± 0.08	7.8
After 28 day	$1.8x10^6$	0. 129	$9.2x10^6$	4.18	0.55	0.46	0.52	0.51 ± 0.05	2.32
After 35 day	$3.7x10^3$	0.0002	$1.2x10^5$	0.054	0.33	0.38	0.30	0.34± 0.04	1.54
After 42 day	0	0	0	0	0	0	0	0	0

Recipient at zero time = 2.2×10^8 CFU/ml
Donor at zero time = 1.4×10^9 CFU/ml

Table 5: Effect of incubation time in situ (Mowas River) in the winter season on the fitness of parents and recombinant strain of *Pseudomonas aeruginosa*

Incubation Time	°C	PH	Donor (CFU/ml) at 24 hours	Percent survival for donor	Recipient (CFU/ml) at 24 hours	Percent survival for recipient	No. of transconjugants cells ($x10^2$) per plate			Mean CFU/ml ($x10^3$) ± SD	Conjugation Frequency ($x10^{-6}$) per recipient at zero time
							R1	R2	R3		
After 1 day (25/2/2020)	17	7.2	$3.4x10^6$	0.44	$5.3x10^7$	40.77	0.41	0.48	0.43	0.44±0.04	3.38
After 7 day (2/3/2020)	19	7.2	$6.1x10^5$	0.08	$4.8x10^6$	3.69	0.55	0.83	0.74	0.71±0.14	5.46
After 14 day (8/3/2020)	18	7.3	$6.8x10^4$	0.009	$7.1x10^5$	0.55	1.09	1.02	1.06	1.06±0.09	8.15
After 21 day (14/3/2020)	21	7.2	$8.2x10^2$	0.0001	$1.3x10^2$	0.0001	0.59	0.62	0.68	0.63±0.06	4.8
After 28 day (20/3/2020)	20	7.2	0	0	0	0	0	0	0	0	0

Recipient at zero time = 1.3×10^8 CFU/ml
Donor at zero time = 7.7×10^8 CFU/ml

Table 6. Effect of different incubation time in situ (Mowas River) in the summer season on the fitness of parents and recombinant strain of *Pseudomonas aeruginosa*

Incubation Time	°C	PH	Donor (CFU/ml) at 24 hours	Percent survival for donor	Recipient (CFU/ml) at 24 hours	Percent survival for recipient	No. of trans-conjugants cells ($x10^2$) per plate			Mean CFU/ml ($x10^3$) ± SD	Conjugation Frequency ($x10^{-6}$) per recipient at zero time
							R1	R2	R3		
After 1 day (13/8/2020)	32	7.1	1.4×10^6	1.27	1.2×10^6	0.67	1.12	1.06	0.97	1.05 ± 0.08	5.8
After 7 day (19/8/2020)	30	7.2	1.6×10^5	0.15	9.5×10^5	0.53	1.51	1.46	1.59	1.52 ± 0.07	8.4
After 14 day (25/8/2020)	29	7.2	1.1×10^4	0.01	8.1×10^4	0.045	0.69	0.73	0.80	0.74 ± 0.6	4.1
After 21 day (31/8/2020)	33	7.2	2.5×10^2	0.0002	6.7×10^3	0.004	2.01	2.13	2.31	0.215 ± 0.15	1.19
After 28 day (6/9/2020)	30	7.3	2.3×10^2	0.0002	2.2×10^2	0.0001	1.09	1.18	1.15	0.114 ± 0.05	0.63
After 35 day (12/9/2020)	28	7.1	0	0	0	0	0	0	0	0	0

Recipient at zero time = 1.8×10^8 CFU/ml
Donor at zero time = 1.1×10^8 CFU/ml

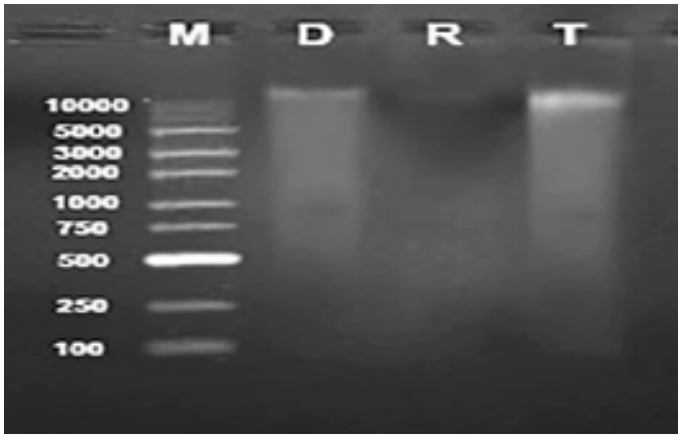

Fig. 1: Agarose gel electrophoresis showing plasmid DNA isolated from *P. aeruginosa* PAO1 as a donor, *P. aeruginosa* MAM2 as a recipient and the trans conjugant strain which received the plasmid from the donor

Molecular confirmation of conjugation

The two strains (donor and recipient) and the trans conjugant were tested for the occurrence of the conjugation process by the existence of the Isolation of plasmid DNA. The concentrations of the extracted plasmids were 58.5 µg/ ml, 0 µg/ ml, and 50µg/ml for the donor, recipient and trans conjugant respectively.

The previous data show the presence of the plasmid in the donor and the trans conjugant and the absence of it in the recipient on the molecular level as shown in figure 1. The molecular weight of the plasmid was greater than 10,000 bp.

Effect of incubation time on the fitness of parents and trans conjugant strains of *Pseudomonas aeruginosa* in vitro

The mating experiments were performed using PAO1 donors and MAM2 recipients on filter membranes on NA plates where conjugation results were calculated after 1, 7, 14, 21, 28, 35 and 42 days.

The recombinant strain was stable up to incubation time of 35 days increased at 7 days (11.5×10^{-6}), and was high at 14 days (17.3×10^{-6}), the decreased slowly at 21 days (7.8×10^{-6}). Then it decreased significantly at 28 days (2.32×10^{-6}), and its lowest value was at 35 days (1.54×10^{-6}). No trans-conjugants have been observed of the 42 days of incubation. These results are agreed with others (Fry and Day, 1990; Johnsen and Kroer, 2007). The data are shown in Table 4.

Effect of incubation time in situ (Mowas River) in the winter season on the fitness of parents and recombinant strain of *Pseudomonas aeruginosa*

A very significant decrease was observed in the fitness and stability of recombinant strain and its parents at the same times when compared to the experiment conducted in vitro where the conjugation frequency was after one day (3.38×10^{-6}), while the conjugation frequency increased after 7 days (5.46×10^{-6}) and reached the highest level at 14 days (8.15×10^{-6}). Then decreased at 21 days (4.8×10^{-6}), while it did not give any results at 28 days neither for recombinant strain nor for parents. No donor, recipient and transconjugants have been detected after 28 days incubation in the Mowas River. These results do agree with others (Richaume *et al.,* 1989: Fry and Day, 1990; Hassan, 2011).

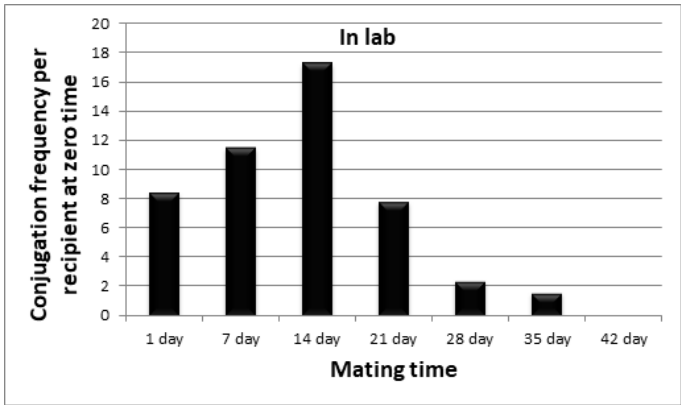

Fig. 3: Effect of incubation time in vitro on the fitness of recombinant strain of *Pseudomonas aeruginosa*

Previous studies shown that, recombinant strain can transfer their new genetic information to the other microbial populations in the winter season in situ (Awong *et al,*. 1990). The processes for genetic exchange and uptake of DNA within and between species are widespread in nature and have been documented (Colwell, 1986).

This decrease in the fitness of bacteria compared to the experiment that was conducted in vitro is due to the decrease in the water temperature during this time of the year, reaching 17 degrees Celsius. It may also be due to the lack of the nutrient present in the environment. All these factors may cause a decrease in the stability and fitness of both. The recombinant strain and its parents *in situ*. The data also showed in Table 5.

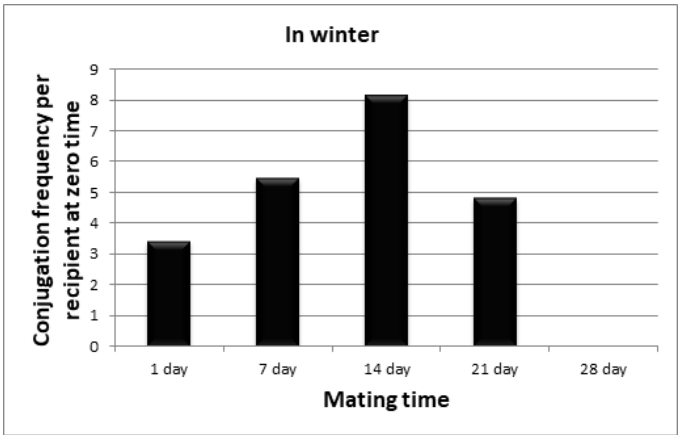

Fig. 4: Effect of incubation time in situ (Mowas River) in the winter season on the fitness of recombinant strain of *Pseudomonas aeruginosa*

Effect of different incubation time in situ (Mowas River) in the summer season on the fitness of recombinant strain of *Pseudomonas aeruginosa*

A very significant decrease was observed in the fitness of the recombinant strains and their parents at the same times compared to the experiment conducted in the laboratory, where the recombinant strains were after one day (5.8×10^{-6}) and the recombinant strains reached the highest level of fitness after 7 days ($8.4x10^{-6}$). Then it decreased about 3 times at 14 days ($4.1x10^{-6}$), while the results were very weak at 21 days ($1.19x10^{-6}$) and at 28 days ($0.63x10^{-6}$) and there were no results after 35 days neither for recombinant strain nor for parents (Fry and Day, 1990; Headd and Bradford, 2018).

These results indicated the incidence of conjugation very much in the summer experience about the winter experience, and it is certain that this increase is due to the high temperature.

This decrease in the fitness and stability of bacteria compared to the experiment that was conducted in vitro is due to the decrease in the water temperature during this time of the year, reaching 33 degrees Celsius. It may also be due to the lack of the nutrient present in the environment. All these factors may cause a decrease in the stability and fitness of both. The recombinant strain and its parents in situ in the summer. The data are shown in Table 6.

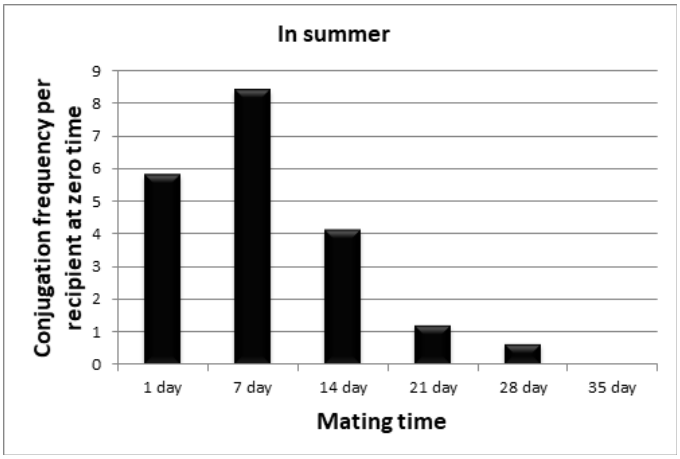

Fig. 5: Effect of different incubation time in situ (Mowas River) in the summer season on the fitness of recombinant strain of *Pseudomonas aeruginosa*

Comparison of conjugation frequency in the laboratory and in the Mowas River during winter and summer

The conjugation rate increased significantly in the laboratory trial compared to the winter and summer trials, and the results also lasted for 35 days. The highest coupling rate values were at the same time in the laboratory, then during the summer, and then almost during the winter, respectively. The data are shown in fig. (6).

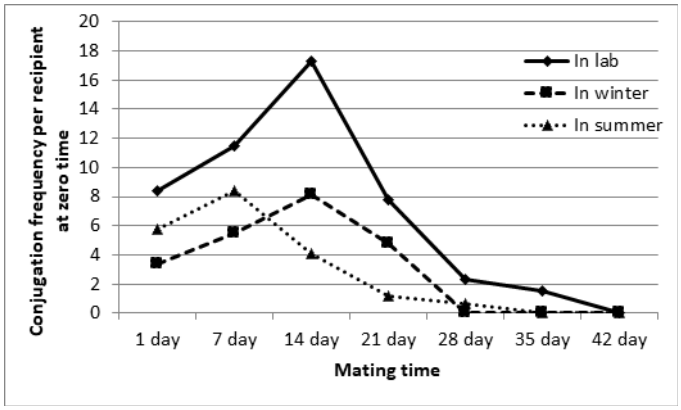

Fig. 6: Comparison of incubation time in vitro and in situ (through winter and summer) on the fitness of recombinant strain of *Pseudomonas aeruginosa*

CONCLUSION

Through the three previous experiments, we can confirm the risks that may be caused by genetically engineered microorganisms. In addition to its ability to preserve new genetic material, it can transfer this new genetic material to other strains and species, as it is stable to a large extent in the environment.

In genetically engineered microorganisms that are added to the environment to increase soil fertility (biofertilizer) or biodegradation for a harmful substance such as pesticides, the soil must be re-inoculation in winter every 21 days and during summer every 28 days due to the loss of the plasmid, which carries some important genes.

The results of this study are important especially in the field of microbial biotechnology. This field can be applied in the solutions for agricultural problems. These fields depend on the release and dispersal of genetically modified microorganisms (GMM) that have been constructed by tools of genetic engineering technology. The estimation of both the efficacy and the potential risk associated with the use of GMM require an understanding of the ecology fitness and stability of the modified gene of the GMM in different ecosystems. The environmental fitness of an organism will differ according to the environmental fitness into which it is released (Bentjen *et al.,* 1989).

There is a lack of data about the environmental fitness of the GMM. The wider environmental consequences of such release need to be determined (Bentjen *et al.,* 1989; Smith *et al.,* 1992).

There is an argument that recombinant organisms would have reduced fitness for survival and growth in the environment due to increased metabolic load imposed by maintenance and expression of the foreign genes (Brill, 1985).

To overcome the problem of unpredictable behavior of GMM in the environment, a fundamental understanding of the effect of conditions on the GMM behavior is required. So, in this study a number of growth conditions have been applied to assess the effect of such parameters on the survival percents as an indication to the fitness of recombinant strain when compared with its parent strains. Experiments using mixture of GEM and wild type organisms show that GMM are less fit than the wild type organisms from which they are derived (Bale *et al.,* 1987; Kandel *et al.,* 1992; Ryder *et al.,* 1994).

In some cases, however, no effects have been observed ref. and even enhanced survival of the GEM have been reported (Orvos *et al.,* 1990; Keil *et al.,* 1998). The data of this study do agree with these studies. Especially up on applied under the Egyptian environmental conditions (Mowas River).

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