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DETECTION AND BEHAVIOR STUDY OF SOME GENETICALLY ENGINEERED MICROORGANISMS RELEASED IN SOIL USING PLATE COUNTING, GENE TRANSFER ASSAY AND SPECIFIC PCR METHODS

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ABSTRACT

The objective of this study was monitoring and behavior study of genetically engineered microorganisms (GEMs) that might be accidentally or deliberately released into the environments. Three methods (plate count, gene transfer and specific PCR) were used with four genetically engineered *E. coli* strains (A:pJan25, B: pJan25, pGWB533 and pGWB404) harboring antibiotics resistance genes with additional *gfp* and *gus* genes as a molecular markers. The GEMs were added to soil microcosms at 10^7 cells/g soil and incubated at room temperature for 35 days. After intervals time 0, 7, 14, 21, 28 and 35 days, bacterial cells were recovered and CFU/g were estimated. The number of viable cells were decreased from 10^7 to 10^3 after 28 days with A: pJan25 and pGWB533 and reached to zero at 35 days. Strain B: pJan25 was survived up to 35 days (10^3 CFU/g), while pGWB404 was disappeared after 21 days. Comparing with sterilized soil, it was found that the viable cells were alive up to 35 days (CFU/g was 10^4).The variation of CFU and presence of a viable cells in soil microcosm may due to the effect of indigenous microbial populations and the type of strain.

Specific PCR was applied on the random selected colonies at 14, 21 and 28 days only, the target genes was *gfp* and *gus.* The results showed the presence of both genes in all tested colonies. This indicated that the tested GEMs could be maintained their constructed genes at long incubation time.

Horizontal gene transfer was also assayed using conjugation under laboratory and soil microcosm conditions to confirm that GEMs genes were transferred to other organisms and to monitor the persistence of GEMs genes in soil. The gene transfer was started at 14 days in sterilized soil and 21 days in soil microcosm. The conjugation frequency under laboratory and sterilized soil conditions was higher than under soil microcosm condition. The results showed that the used GEMs were able to transferred three genes to recipient cells. This indicated that these genes were plasmid harboring and it were transferred to a recipient

Keywords: Genetically engineered microorganisms (GEMs), horizontal gene transfer, PCR, *gfp* and *gus* genes.

INTRODUCTION

Genetically engineered microorganisms (GEMs) have been constructed for environmental applications such as, bioremediation of toxic chemicals, biological pest control, plant growth promotion **(**Wilson and Lindow 1993 and Viebahn *et al.,* 2009**)** and energy production. Biran *et al.,* (2009) were used genetically engineered bacteria for genotoxicity assessment.

Considerable research has been conducted on the use of molecular markers for detection of GEMs in the environments and reviewed up to 1994 (Greer *et al.,* 1993 and prosser, 1994). Prosser (1994) presented a comprehensive review of molecular systems including antibiotics resistance, Lac Z and others used for detection of GEMs in the environments. The green

fluorescent protein marker (GFP) became also available (Chalfie *et al.,* 1994). The *gfp* gene codes for a green fluorescence protein which exhibits bright green fluorescence without any exogenous substrate or energy requirements so, the *gfp* gene is a potentially useful marker for tracking GEMs in nature (Jansson 1995). Errampalli *et al.* (1999) presented the applications of GFP protein as a molecular marker in environmental microorganisms. The genetic contents of which have been altered by the integration of novel genes conferring new characteristics, frequently in combination with marker genes such as the *gfp* gene and LacZ (Elenis *et al.,* 2008). The LacZ gene codes for β- galactosidase which can generate a blue-colored product from a colorless modified galactose (x-gal) substrate (Yeom *et al.,* 2011). Both genes are frequently employed in genetics and molecular biology applications such as cloning and gene expression analysis (Atlas, 1992 and Errampalli *et al.,* 1999). The applications of GEMs require the release of them into the environment (Jansson, 1995, Glandorf *et al.,* 2001 and Yeom *et al.,* 2011) and so it requires detection and tracking.

Genetically modified *Escherichia coli*, *Pseudomonas putida* and *Acinetobacter* species are routinely generated in a variety of biological laboratories. Despite the increase in the commercial applications of GEMs and possibility of accidental release, their ecological safety continues to be a matter of some controversy. This issue has driven a variety of studies regarding to survival of GEMs in the environment and the putative horizontal gene transfer of recombinant DNA from GEM to indigenous bacteria, (Pontiroli *et al*., 2007 and Keese, 2008). Another consideration is the fate of the DNA in the environment and the stability of the DNA relative to the time required for a competent cell (a cell capable of taking up DNA) to take in the genetic material (Fink and Moran, 2005).

Several detection methods have been designed for the detection and enumeration of GEMs in the environment, including the direct plate counting method, most-probable-number (MPN) method, direct microscopy, serology, immunofluorescence and immunoradiography (McCormick, 1986; Fredrickson *et al.,* 1988; Henschke and Schmidt, 1990; Michelini *et al.,* 2008). Owing to their high specificity, PCR based detection methods are generally accepted as the most sensitive and reliable methods for the detection of genetically modified microorganisms (GMMs) (Steffan and Atlas, 1988; De Leij *et al.,* 1995; Le´vy *et al.,* 1996).

The present study aimed to detect genetically engineering *E.coli* strains harboring either the *gfp* , *gus* genes and tetracycline or streptomycin resistance genes as a selectable markers using direct plate-counting and amplification of specific sequences of DNA by the polymerase chain reaction (PCR) methods. In addition, study of GEMs behavior under environmental conditions.

MATERIALS AND METHODS

This study was performed in Microbial Genetics and Molecular Genetics Lab. Dept. Genetics, Fac. 0f Agric. Zagazig Univ.

Bacterial strains

Four genetically engineered *E. coli* strains were used in this study. These strains and plasmids map were obtained from: Matthews lab, Soybean Genomics Laboratory, Beltsville, USDA, USA. These strain named; A: pJan25, B: pJan25,pGWB404 and pGWB533. All vectors were designed for testing the spatial and temporal expression of promoters. Fig.1 shows the maps of plasmids containing strains. A:pJan25 and B:pJan25 two different stress promoters with both enhanced green fluorescent protein (*gfp*), encoding β- glucuronidase (*gus*) marker genes and gene encoding tetracycline resistance. Strain pGWB404 contain *gfp* gene only, strain pGWB533 contain *gus* only, both strains contain the spectinomycin/ streptomycin adenylytransferase. *Rhizobium leguminosarum* streptomycin resistant strain obtained from Microbial Genetics Lab. Genetic Dept., Fac. Of Agric. Zagazig Univ.

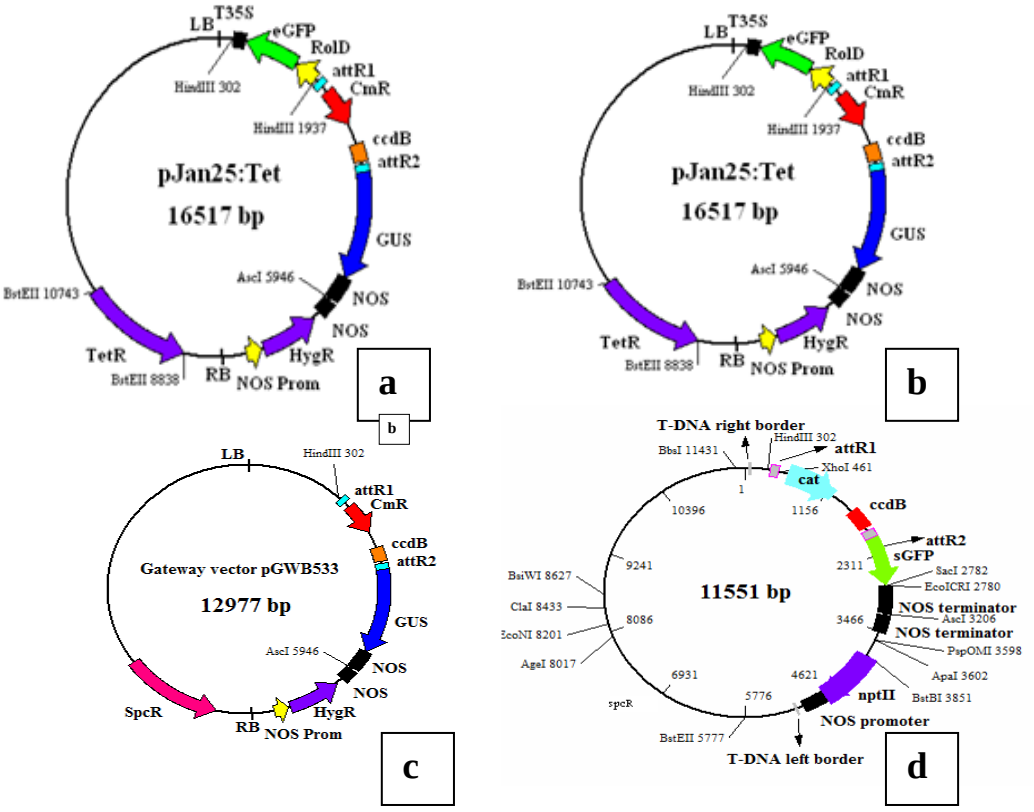

Fig. 1. Maps constructed plasmids used: (a,b) shows the A:pJan25 and B:pJan25, (c) shows the pGWB533 and (d) shows pGWB404.

Media and soil inoculum conditions.

Nutrient agar (NA), Nutrient broth (NB), Yeast extract mannitol (YEM) agar, YEM broth media and phosphate buffer (1/15 M KH2PO4 and 1/15 M Na2 HPO4.H2O) were used (Hassan, 2010). Tetracycline concentration was 50µg/ml and streptomycin was 100µg/ml. Antibiotics were added to autoclaved media as sterilized solution after filtration through 0.2µm filter membrane. *E. coli* cells were grown in NB at 37°C with shaking. Bacterial cultures were grown overnight and inoculated at defined numbers (10^7 cells/g soil) into 70 g of air-dried clay soil in a sterilized glass Jar (300 ml). Sterilized soil samples were used as a positive control. In addition, non-inoculated soil was used as a used negative control. The soil samples were collected from different location of Zagazig City at 10 cm depth. Physical and chemical properties of soil used in this study were shown in Table(1) according to Abo-Hashim (2002). Deionized sterile water was added to soil samples at soil moisture 30% (v/w) (Trevors *et al.,* 1990). The soil samples were incubated at room temperature (Yeom *et al.,* 2011) for 35 days.

Enumeration of GEMs using plate counting technique.

Soil samples were collected at 0 , 7 , 14 , 21 , 28 and 35 days. Five grams of each soil sample were suspended in 20 ml of phosphate buffer for 1h with vigorous shaking. Suspended samples were diluted and spreaded onto agar plates which were either amended with or without antibiotics. The plates were incubated for 3-5 days at 30 and 37°C. Colonies were enumerated for total cells, tetracycline resistant and streptomycin resistant cells, and CFU/g were calculated (Yeom *et al*., 2011).

Horizontal gene transfer

To confirm that the GEMs able to transfer their genetic material, gene transfer by conjugation was carried out under laboratory, sterilized soil and soil microcosm conditions. Donor and recipient strains were inoculated in liquid Media for 24 h., using equal volumes (1ml) of donor and recipient cells were added on the surface of complete media plates and then incubated for 24 h. Growth was washed by 10 ml phosphate buffer and removed by spreader to sterile flasks. Serial dilutions were prepared, from each 0.1 ml was spreaded on selective media. transconjugants appeared on selective media were picked up and maintained on NA (Hassan and Amin 2010).Under soil microcosm conditions, donor and recipient cells were added to non-sterilized soil and incubated at room temperature, also sterilized soil was used to know the effect of indigenous microbial population on GEMs survival by comparing between sterilized and non-sterilized soil. At intervals time 0, 7, 14, 21, 28 and 35 days, the cells were collected and enumerated at selective to be appeared transconjugants which picked up and saved on NA.

Table 1. Physical and chemical properties of soil used in this study(Abo-Hashim (2002)).

Properties	Value
• Particle size distribution (%)	
Coarse sand	7.12
Fine sand	27.10
Silt	12.97
Clay	52.82
Texture	Clay
• Densities (Mgm^{-3})	
Bulk	1.43
Particle	2.54
• Soil moisture %	
Saturation point	90
Field capacity	45
Wilting point	22.5
Hygroscopic water	11.9
pH	7.82
electric conductivity ($ds.m^{-1}$)	2.67
Sodium adsorption ratio	6.4
Organic mater %	2.49
CaCO3	1.89

Detection of GEMs using specific PCR.

Colonies appeared on selective media were picked and subjected to specific PCR using specific primers for *gfp* and *gus* genes.

PCR amplification for testing colonies

Individual colonies were selected from plates with sterile and non-sterile soil and grown overnight in 5ml LB culture supplemented with tetracycline, 50 µg/mL, for bacteria (A: pJan25) and (B:pJan25) or with streptomycin 100 µg/mL for bacteria pGWB533 and pGWB404. Cells from 20 µl solution from overnight cultures were collected by centrifugation and resuspended in 20 µl ddH2O and heated at 100°C for 10 min. The resulted solution containing all cells components were subjected to colony PCR as from each culture was used as a template in a 20 µl PCR reaction to confirm the presence of all genes harboring plasmids in right orientation using recombinant *Taq* polymerase following manufacturer instructions (Invitrogen, Carlsbad, CA). Polymerase chain reaction was started with an initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 35 seconds, annealing at temperature based on the primers used, Table (2) for 35 seconds, and extension at 72°C for 2 minute per kb of PCR product. Final extension of the amplification was for 10 minutes denaturation at 72°C. All PCR reactions were performed using DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, USA).

Gel electrophoresis

Ten µl of PCR products were separated on agarose (1.2 %) gel electrophoresis, stained and loaded in 2 µl EZ-VISION™ ONE (Amresco, USA), at 100 Volts in 1X SB (10mM NaOH solution with Boric acid, pH = 8.5) and photographed on a UV transilluminator (Pharmacia) by a Canon S5 digital camera with UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included in PCR run .

Table 2. Primers of *gfp* and *gus* used for detection GEMs obtained.

Primer name	Sequence
RolD-eGFP-T35s ClaI Forward	5 ` ATCGATGCATGCCTGCAGGTTAG 3`
RolD-eGFP-T35s ClaI Reverse	5`ATCGATGCAGGTCACTGGATTTTGG 3`
GUS Forward	5`AGGAAGTGATGGAGCATCAG 3`
GUS Reverse	5 `CATCAGCACGTTATCGAATCC 3`

RESUTLS AND DISCUSSION

Detection of GEMs in soil microcosm using the plate counting technique:

Bacterial establishment in an environment depends on the ability of introduced bacteria to survive. There are several environmental factors that can affect bacterial survival in an environment such as soil texture, moisture content, temperature, pH, the presence of plant roots, minerals, organic matter competition and antagonism by other microorganisms and predation by protozoa. In microcosm experiments, these parameters should be as close as possible to the natural situation (Mashreghi, 2007).

In present study, in order to estimate the survival of GEMs, the indigenous bacterial populations in non-sterile soil were assessed. Number of viable cells from indigenous soil bacteria was decreased from 10^8 to 10^7 CFU/g soil after 7 days, and remained constant over time (up to 35 days) at the absence of antibiotics (Fig. 2 a). Antibiotics resistant populations in soil were very low at zero time which reached to (10^2) for tetracycline and(10^1)for streptomycin. This number was decreased to zero in used volume (0.1 ml) after 21 and 14 days for tetracycline and streptomycin, respectively. In order to assess survival of GEMs in soil, approximately 10^7 CFU/g of soil-harboring bacterial strains carrying genetic marker genes were added to the soil microcosms and enumerated over time. Figure (2 b) shows survival of A: p Jan25 (*gfp* and *Tet*^{r}), cells number of this strain remained constant (10^7) up to 21 days and decreased to 10^3 and zero at 28 and 35 days respectively. It has been demonstrated that the numbers of total populations remained high when GEMs were added, because the numbers of total cells with inoculation were higher than in control experiment. The same results were obtained with strains B: pJan25 and pGWB533 (Fig. 2 c and d).When soil microcosms were inoculated with pGWB404, the total bacterial count was high, but the viable cells of pGWB404 were disappeared after 21 days (Fig. 2 e). Figure (2 f) shows the survival of genetically engineered strains in sterilized soil, all strains were persisted up to 35 days, except for pGWB404 strain. The viable cells were higher than in non–sterilized soil, This indicated that the survival of *E. coli* in soil was inversely related to the complexity of soil microbial community (Liang, *et al.,* 2011). These results consistent with other previous studies, Liang *et al.* (2011) found decreased CFU at 10 days and although no obvious growth of *E. coli* was detected, all three *E. coli* strains could persist in soil over an extended period of time. These results agreed with Yeom *et al.* (2011) who found that, the number of viable cells of indigenous soil bacteria remained constant over time (28 days) at the absence of antibiotics and the survival of GMMs was decreased by increasing the incubation time.

Fig. (2): Cell counting using plate counting method.
(a) Control experiments for counting indigenous bacteria.
(b) A: pJan25 strain (*gus, gfp* and *tet*^{r}).
(c) B: pJan25 (*gus, gfp* and *tet*^{r}). **(d) pGWB533 (*gus* and *str*^{r}).**
(e) pGWB404 (*gfp* and *str*^{r}). **(f) Cell counting of GEMs is sterilized soil.**

In contrast, Sogin *et al*., (2006) found that engineered microorganisms would survive for 3 years in their introduced environment. Layton *et al*., (2012) found recombinant *Pseudomonas fluorescens* HK44 was survived for 14 years after released into subsurface soil environment of soil lysimeters.

Detection of GEMs in a soil microcosm using specific PCR method.

The increased introduction of GEMs into the environment has stimulated the focus of research into the development of sensitive methods for detecting specific genetically defined microorganisms within the complex microbial communities of natural ecosystems. The most widely used technique is the amplification of specific sequences of DNA by the polymerase chain reaction (PCR). This method has been successfully applied for detecting microorganisms that are difficult to culture *in vitro* as well as tracking the fate of GEMs and particular genes that disseminate by transfer to indigenous microbes(Peng *et al.,* 2007).

Amplification of specific sequences (*gfp* and *gus* sequences) of DNA by the polymerase chain reaction (PCR) method was applied on the samples at 14, 21 and 28 days to detect the GEMs bacteria added into soil and estimate its stability of their constructed genes. The grown colonies of all GEMs on selective media of both sterilized and non-sterilized soil were tested. The *gus* primers were used with strains A: pJan25, B: pJan25 (which contain *gus* and *gfp* genes) and pGWB533 (which contain *gus* gene only) but, the *gfp* primers were used with the first tow strains and pGWB404 which contain *gfp* gene only. The results in Figure (3) shows the presence of *gus* and *gfp* genes in tested colonies at all tested time periods. This indicated that the tested GEMs were persisted in soil microcosms up to 28 days and it was maintained their constructed genes for a long time. The viability of GEMs and stability of constructed genes at a long time is very important for the apply of GEMs in the environments. So, the tested strains can be useful for carrying some genes for environmental applications.

Soil background of *gus* and *gfp* genes was tested, no PCR amplification was detected from soil DNA using the *gus* and *gfp* primers . Thus, the *gus* and *gfp* primers which used in this study appeared to be useful for detect of genetically engineered strains .

Green fluorescent protein (GFP) which encoded by *gfp* gene was used widely as a molecular detection marker for GEMs in the environmental applications, in plant- microbe interactions. Bloemberg *et al.* (1997) constructed a *gfp* plasmid which was maintained in *P. flourescens* cells for 7 days. A *gfp* labeled *P. putida* has been used to study bacterial survival in activated sludge (Eberl *et al.,* 1997). The chromosomally *gfp*- labeled *Moraxella sp* strain G21 had been constructed using a mini- Tn5 – *gfp* suicidal plasmid (Tresse *et al.,* 1998) to degraded PNP.

Fig. (3): Detection of *gus* and *gfp* gene markers in genetically engineered *E. coli* bacteria after 14 (a,b), 21 (c,d) and 28 (e,f) days incubation in soil. A: pJan25, B: pJan25 and pGWP533 used with *gus* primers. A: pJan25, B:pJan25 and pGWP404 used with *gfp* primers. Control(c): purified DNA plasmid with target gene.

Horizontal gene transfer assay using conjugation mechanism.

When GMMs are introduced into the environment, indigenous bacteria function as recipient cells for genetically modified DNA, which can subsequently induce physiological alterations and perturbations of the indigenous soil microbial community (Miyakoshi *et al*., 2007).

This experiment was included two parts, the first was horizontal gene transfer under laboratory conditions. Diparental mating was used intra-GEMs strains and inter-GEMs strains and *Rhizobium Leguminosarum* (Table 3). Conjugation frequency ranged from 1.7×10^{-7} to 3.8×10^{-7} between *E. coli* strains and from 6.5×10^{-8} to 1.08×10^{-7} between *E. coli* strains and *R. leguminosarum*. The second part was under sterile soil conditions and non-sterile soil microcosm (Tables 4 and 5). The cells were collected from soil as previous demonstrated at intervals 0, 7, 14, 21, 28 and 35 days. The results shown that gene transfer was started at 14 days in sterilized soil and at 21 days in non- sterilized soil, and it was persisted up to 35 days in sterilized soil, but it was stopped at 28 days in non- sterilized soil. Conjugation frequency in sterilized soil was higher than in non- sterilized soil.

Horizontal gene transfer between microorganism in the environment was studied by Normander *et al.* (1998) who used *P. putida* donor cells with a derivative of the TOL plasmid conferring kanamycin resistance and had the *gfp* gene. The *P. putida* recipient had a chromosomal tetracycline resistance marker. The number of transconjugants was 3×10^{3}.

DNA molecules, released into the soil by microorganisms, are closely associated with soil constituents such as clay minerals, sand, and humic substances. Being partially protected against degradation by nucleases, such DNA molecules retain the capacity to transform competent bacterial cells (Cai *et al*., 2006). The persistence of recombinant DNA and its potential transfer to indigenous microorganisms have raised concerns about the deliberate or accidental release of genetically engineered microorganisms (GEMs) into the environment (Peng *et al.,* 2007).

Previous study shown that the transforming ability of chromosomal and plasmid DNA bound on Ca-montmorillonite could persist for 15 days in non-sterile soils under moist conditions (Peng *et al.,* 2007).

Gene transfer assay was used as a method for monitoring of released genes in the environments. LO *et al.,* (2007) used transformation assay to monitor the persistence and bioavailability of transgenic genes released from genetically modified papaya expressing npt II and PRSV genes in the soil. So, conjugation assay was used in this study.

To confirm on gene transfer and monitor the persistence of constructed genes, different shapes and colors colonies (12) selected on selective media were subjected to amplification of specific sequences(*gfp* and *gus* sequences) of DNA by the polymerase chain reaction (PCR) for detection of constructed genes. Fig.(4) shows the presence of *gfp* and *gus* genes in 11 transconjugants from 12. The results shown presence of 3 genes (Tet^{r}, *gfp* and *gus*) from donor genes in transconjugants cells, these genes located on constructed plasmid though, this indicated that GEMs were able to transfer their plasmids to another bacterial cells.

Table 3. Gene transfer ability of GEMs under laboratory conditions.

Mating	Donor	Recipient	Transconjugants	Conjugation frequency/ recipient
A: pJan25 x pGWB533	7.9×10^8	8.9×10^8	6.0×10^2	1.7×10^{-7}
A: pJan25 x pGWB404	6.7×10^8	8.3×10^8	1.1×10^2	3.8×10^{-7}
B: pJan25 x pGWB533	8.5×10^8	9.2×10^8	1.3×10^3	3.8×10^{-7}
B: pJan25 x pGWB404	3.4×10^8	3.9×10^8	7.0×10^2	2.4×10^{-7}
A:pJan25 x *R.Leguminosarum*	3.9×10^8	2.4×10^8	7.8×10^1	6.5×10^{-8}
B:pJan25 x *R.Leguminosarum*	1.8×10^8	6.2×10^7	1.3×10^2	1.08×10^{-7}

PFU/ml of pGWB533 at zero time =3.4×10^9
PFU/ml of pGWB404 at zero time =2.9×10^9
PFU/ml of *R. leguminosarum* at zero time =1.2×10^9

Table 4. Ability of transfer genes from GEMs in sterilized soil.

Mating	No. of transconjugants and conjugation frequency/recipient					
	0	7	14	21	28	35
A: pJan25 x pGWB533	0	0	1.5×10^1 0.44×10^{-8}	4.4×10^2 1.29×10^{-7}	1.0×10^1 0.29×10^{-8}	1.0×10^1 0.29×10^{-8}
A:pJan25 x pGWB404	0	0	3.6×10^2 1.2×10^{-7}	1.8×10^2 0.62×10^{-7}	4.0×10^2 1.38×10^{-7}	3.2×10^2 1.1×10^{-7}
B:pJan25 x pGWB533	0	0	1.0×10^1 $.029 \times 10^{-8}$	3.3×10^2 0.97×10^{-7}	2.4×10^2 0.7×10^{-7}	6.0×10^1 1.8×10^{-8}

PFU/ml of pGWB533 at zero time =3.4×10^9
PFU/ml of pGWB404 at zero time =2.9×10^9

Table 5. Ability of transfer gene from GEMs to indigenous bacteria in non-sterilized soil.

Donor	No. of transconjugants and conjugation frequency/donor					
	0	7	14	21	28	35
A: pJan25 x pGWB533	0	0	0	2.0×10^1 0.83×10^{-8}	3.0×10^1 1.25×10^{-8}	0
A:pJan25 x pGWB404	0	0	0	1.3×10^1 0.45×10^{-8}	4.0×10^1 1.4×10^{-8}	0
B:pJan25 x pGWB533	0	0	0	1.7×10^1 0.5×10^{-8}	1.0×10^1 0.29×10^{-8}	0

PFU/ml of A: pJan25 at zero time =2.4×10^9
PFU/ml of pGWB404 at zero time =2.9×10^9
PFU/ml of pGWB533 at zero time =3.4×10^9

Fig.(4): Detection of *gfp*(a,b) and *gus*(c,d) genes indifferent bacterial transconjugants isolated from soil microcosms.

In conclusion, it was found that, three used methods in this study were successful for detection and behavior study of GEMs in soil, in addition, *gfp*, *gus* and antibiotics were useful as a molecular selectable markers in microbial soil.

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الكشف عن و دراسة سلوك بعض الكائنات الدقيقة المهندسة وراثيا المنطلقة فى التربة باستخدام طرق العد بالأطباق و تقييم النقل الجينى و تفاعل البلمرة المتسلسل المتخصص

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الهدف من هذه الدراسة هو الكشف عن ودراسة سلوك الكائنات الدقيقة المهندسة وراثيا والتى يمكن أن تنطلق فى البيئة عن قصد أو بدون قصد.

تم إستخدام ثلاث طرق هى : العد بالأطباق و النقل الجينى و تفاعل البلمرة المتسلسل المتخصص مع 4 سلالات من بكتيريا إيشيريشيا كولاى مهندسة وراثيا هى : A:p Jan25, B:p Jan25, pGWB533, pGWB404 والتى تحمل جينات المقاومة للمضادات الحيوية بالاضافة الى جينات الـ *gfp, gus* كمعلمات جزيئية. تم إضافة البكتيريا المهندسة وراثيا الى ميكروكوزم تربة بمعدل 10^7 خلية /جم وتم تحضينها على درجة حرارة الغرفة لمدة 35 يوم. بعد فترات زمنية مختلفة (0 ،7 ،14 ،21 ، 28 ، 35 يوم) كان يتم إستعادة البكتيريا من التربة و عدها ، لوحظ أن عدد الخلايا الحية قد تناقص من 10^7 الى 10^3 بعد 28 يوم وذلك لسلالات A:p Jan25، pGWB533، وقد وصل هذا العدد الى صفر عند 35 يوم .أما السلالة B:p Jan25 فقد إستمرت حتى 35 يوم ، بينما السلالة pGWB404 فقد إختفت تماما عند 21 يوم.

و قد اتضح اختلاف سلوك السلالات المهندسة وراثيا وذلك بالمقارنة مع تربة معقمة حيث وجد أن جميع السلالات إستمرت حتى 35 يوم وكان أقل عدد للخلايا هو 10^4 / جم وذلك فى التربة المعقمة. هذا التباين أو الاختلاف فى عدد الخلايا و إستمرار الخلايا الحية فى ميكروكوزم التربة يمكن أن يعزى الى تأثير العشائر الميكروبية المحيطة و كذلك نوع السلالة المهندسة وراثيا.

تم تطبيق تفاعل البلمرة المتسلسل المتخصص على مستعمرات تم إختيارها عشوائيا من على البيئة الإنتخابية وذلك على الفترات الزمنية 14 ،21 ، 28 يوم فقط وذلك للكشف عن جينات *gfp, gus* . أظهرت النتائج وجود كلا الجينين فى كل العزلات المختبرة و هذا يدل على أن السلالات الدقيقة المهندسة وراثيا التى تم إختبارها إستطاعت الإحتفاظ بجيناتها طوال فترة التحضين .كما اتضيح أن الطرق المستخدمة إستطاعت بنجاح الكشف عن البكتيريا المهندسة وراثيا فى ميكروكوزم التربة.

تم أيضا دراسة النقل الجينى الأفقى بإستخدام ميكانيكية التزاوج و ذلك تحت ظروف المعمل وباستخدام تربة معقمة وأيضا تحت ظروف الميكروكوزم ، وقد تم توضيح أن النقل الجينى بدأ عند 14 يوم فى التربة المعقمة وعند 21 يوم فى التربة غير المعقمة. كما أن معدل النقل كان أعلى تحت ظروف التربة المعقمة منها تحت ظروف الميكروكوزم.

أوضحت النتائج أن السلالات المهندسة وراثيا المستخدمة فى هذه الدراسة كانت قادرة على نقل ثلاث جينات من خلايا المعطى ، وهذه الجينات كانت محمولة على البلازميد وهذا يدل على إنتقال البلازميد.

قام بتحكيم البحث

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