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Organophosphorus pesticides biodegradation by three bacterial genera and their protoplast fusants

Sarah Aggag1*. Ramy Shoman1. Mounir Moussa1 . Mohamed Yacout1

[1]Department of Genetics, Faculty of Agriculture, Aflaton St., El-Shatby, P.O.Box 21545, Alexandria University, Alexandria, Egypt.

*Corresponding Author
E-mail: sarah.aggag@alexu.edu.eg

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Abstract
Among the various groups of pesticides the major and most widely used group all over the world, is Organophosphorus. This group is rating more than 36% of the total world market. Chlorpyrifos as an Organophosphorus pesticides (OP) used for agriculture crop protection in the world, although it was prevented, it is used in some developed countries. The objective of this work was to degrade these pesticide using three common bacteria from farmlands that showed susceptibility for OP degradation (*Pseudomonas fluorescens* (Ps), *Bacillus megaterium* (Ba) and *Rhizobium legumino-sarum* (Rh)). The promising strains had been chosen for Intergeneric protoplast fusion. The protoplast fusion abilities for Chlorpyrifos biodegradation were examined and compared with their parental bacteria. Molecular study using randomly amplified polymorphic DNA (RAPD) and Sodium Dodecylsulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) were applied to identifying the genetic interrelationship between fusants and their parents. These fusions showed a highly increase in Chlorpyrifos biodegradation and were very stable. Our results indicated that these fusants strains could be environmentally friendly bacterial strains and present some important characteristics effective for biodegradation of pesticides.
Keywords Chlorpyrifos · fusant strains · RAPD-PCR · pesticide degradation

INTRODUCTION

Organophosphates (OPs) compounds are widely used in developing countries causing major public health problems. Especially in the control of agricultural, industrial and domestic pests which case a toxic effect on humans [1] could be estimated 200,000 people every year [2]. One of the popular organophosphorous pesticide used in the agricultural market is Chloropyrifos. EPA estimated the food residue per day of chlorpyrifos in people per kilogram of their body weight in 2011 is about 0.009 micrograms [3].

One of the efficacious technologies to construct multifunctional genetic strains is protoplast fusion. This technology has been applied to improve the efficiency of bacterial degradation in environmental organic pollution control.

Successfully protoplast fusion between *Agrobacterium tumefaciens* and *Bacillus thuringiensis* has been performed to produce the fusants bacteria [4]. Also, protoplast fusion technique has been used in *Saccharomyces cerevisiae*, *Phanerochaete chrysosporium* and native bacterium YZ strains to degrade contaminated wastewater by terephthalic acid [5].

A functional strain improves the ability of degradation for bensulfuron-methyl and butachlor with protoplast fusion [6]. As well as, degradation of lower molecular weight of phenanthrene that was achieved by protoplast fusion between *Sphingomonas sp*. and *Pseudomonas sp*. [7].

High biodegradation capability demonstrated by scanning electron microscope (SEM) and randomly amplified polymorphic DNA (RAPD) in fusant strain F14 which obtained by protoplast fusion between *Sphingomonas sp.* and *Pseudomonas sp.* [7].

The objective of this study is to endowing intergeneric protoplast fusion with the ability to efficiently degrade Organophosphorus pesticides using three fusant from *Pseudomonas fluorescens*, *Bacillus megaterium* and *Rhizobium* sp. then compare them with their parental strains. The fusants were characterized to its morphology and physio-biochemical characteristics. While the RAPD marker and SDS PAGE Protein analysis were chosen to evaluate the genetic relationship and detect the genetic diversity between the protoplast fusants and its parent's strains. by the protoplast fusion technique

MATERIALS and METHODS

Pesticides with technical purity grade, Chloropyrifos® 48 % (0.0- diethyl 0-(35.6- Trichloro-2- Pyridyl) Phosphoro-thioate, OrganoPhosphorus group was purchased from DOW Agrosciences company, UK. Three bacterial strains were used (*Pseudomonas fluorescens, Bacillus megaterium and Rhizobium leguminosarum).* Strains used in the study had been previously obtained from department of microbiology, Ain shams university and department of soils, Alexandria university and have been maintained on LB slap medium.

Protoplast formation, fusion and regeneration

Bacterial strains were grown for 24 hours in 10 ml of L.B medium. The cells were centrifuged, washed and resuspended in 10 ml TES buffer, containing Lysozyme (1 mg / ml). Then the suspension incubated at 26ºc for 3 hours with shaking and 0.05M $MgCl_2$ was added. Protoplasts were regenerated on L.B agar plates and mixed in selective medium (SM) for isolation of fusants. The fusants were purified and tested by gram stained.

Strains and fusants were characterized and identified using morphological, physiological features under light microscope [5].

Biodegradation experiments

Five different concentrations of the Organophosphorus

pesticide compound (90 µl, 75 µl, 60 µl, 45 µl and 30 µl). For about five days (first day, second day, third day, fourth day, sixth day and seventh day). The concentration pesticide (Chlorpyrifos 48 %) were determined by measuring the absorbance at maximum wave length (290 nm), using UV-visible light Spectrophotometry (Thermo Scientific- VISIONpro SOFTWARE V4.30) [8].

RAPD-PCR analysis

Bacterial genomic DNA was extracted by PureLink™ Microbiome DNA Purification Kit. Amplifications were performed in a Thermo-cycler Rudolf-Wissell-StraBe, Goettingen, (Germany) programmed for random amplified polymorphic DNA PCR (RAPD-PCR). DNA of fusant strains and its parental strains was amplified using a set of primers [9, 10] described in Table (1). Five RAPD primers were used in this study in 25 µl total volume with the following steps: 2µl from Genomic DNA, 2 µl from each primer, 12.5 µl from the Master Mix (promega®) and 9.5 µl H2O.

Table 1 Sequences of Random primers used in the present study

Primer	Nucleotide Sequence 5`→3`	Reference
OPC03	5` CACTGGCCAC 3`	[9]
OPA04	5` AGGACTGCTC 3`	
OPX11	5` GGAGCCTCAG 3`	[10]
OPU15	5` ACGGGCCAGT 3`	
OPW20	5` TGTGGCAGCA 3`	

Subsamples of the PCR products (10 µl) were migrated on 1% agarose gel electrophoresis for 1.5 hours at 100 volt and visualized with 0.5µg/ cm3 (w/v) ethidium bromide. Finally, the gel was trans-illuminated under short-wave UV light, photographed by gel documentation system and the length of each band was estimated via the DNA marker 100 bp ladder (Thermo Scientific). All RAPD reactions were repeated twice in order to ensure reproducible results.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using discontinuous buffer system [11]. Ten percentage slab gel was prepared by mixing acrylamide, bisacrylamide, (10 ml) resolving gel buffer stock, (3.7 ml) 10 % SDS, (0.3 ml) freshly prepared, 1.5% ammonium per sulphate (1.5 ml), distilled water (14.45 ml) and TEMED (0.015). Stacking gel was prepared using acrylamide-bisacrylamide (2.5ml), stacking gel buffer stock, (5.0 ml), SDS 10 % (0.2ml) freshly prepared, 1.5% ammonium per sulphate (1.0 ml), distilled water (11.3 ml) and TEMED (0.015 ml).

The samples were loaded and electrophoresis was performed at 75 Volt through stacking gel. The gel were stained with 0.1% Commassi Blue R-250 for 2 hours.

Data analysis

RAPD and protein data were scored for presence (1) or absence (0), and used to create a data matrix. The Phoretix ID image analysis system (Phoretix International, London) was used to analys the data. A similarity dendrogram among the bacteria and the fusants was produced using UPGMA cluster analysis program [12].

RESULTS

Screening and identification of bacterial fusant

There are differences between fusants and each other and differences between fusants and their parents in terms of shape, color, margin and elevation shown in Fig. (1). *Pseudomonas fluorescens* is a common gram negative, rod-shaped bacterium, with smooth edges, and convex surface. *Bacillus megaterium* is a gram positive, endospore forming, rod shaped bacteria, smooth margin and texture. *Rhizobium leguminosarum* is a gram-negative, motile, rod-shaped, aerobic bacterium, smooth margin, elevation is slightly domed and moderately mucoid, white color and smooth texture. *Pseudomonas fluorescens*Bacillus megaterium* fusant formed in individual and grouped bacterial colonies with soft and smoothly edge, it is slightly domed, dark beige and smoothly texture. *Pseudomonas fluorescens*Rhizobium leguminosarum* fusant formed in little colonies and individual, soft and straight margin, smoothly and flat elevation, transparent and white with smoothly texture. *Bacillus megaterium*Rhizobium leguminosarum* fusant formed in a large network colonies, wavy and soft edge, with flat margin, beige and texture is not smooth.

RAPD fingerprint analysis

In this study, five arbitrary primers (OPC03, OPA04, OPX11, OPU15 and OPW20) gave a total of 60 amplified detected bands with an average of about 12 % bands per primer, ranging from 1000-70 bp. Primer OPU15 had the highest total number of different PCR bands (20 bands), while primer OPC03 showed the lowest total number of bands (1 band). However, the results showed that Primer OPU15 had the highest polymorphic number of different PCR bands (12 bands), while primer OPC03 showed the lowest polymorphic number of different PCR bands (1 band), respectively.

The calculation of similarity coefficients based on the amplified DNA band profiles of the parents and their fusants in Fig. (2). The studied genotypes formed two main clusters A and B, the first main cluster separated was at genetic similarity of 0.10 which created two sub clusters A1 with one bacterium (Ps) and A2 the second sub cluster included A21 and A22 at genetic similarity of 0.11. The second sub cluster A22 contain individual genera (Ba*Rh). While, the first sub cluster implicate A211 and A212 at genetic similarity of

Table 2 Degradation percent of chlorpyrifos for fusant and parents

Concentrations	30 µl	45 µl	60 µl	75 µl	90 µl	SD
Pseudomonas	45.0434	27.753	33.1445	42.3761	58.022	±11.67074
Bacillus	48.0652	45.8771	40.0208	40.81173	59.13911	±7.689629
Rhizobium	52.9978	40.5998	49.6121	43.1606	47.943	±4.982348
*(Ps*Ba)*	80.784	65.6917	31.0291	58.5814	36.0863	±20.75263
*(Ps*Rh)*	85.209	74.082	67.9639	56.902	37.7616	±18.06433
*(Ba*Rh)*	41.1963	53.868	34.44	70.362	30.5788	±16.20104
SD	±19.12447	±16.91802	±14.06389	±11.83582	±11.98275	

0.12, the second sub cluster A212 contain individual genera (Ps*Rh). The A211 sub cluster included A2111 and A2112 at genetic similarity of 0.23. The A2112 sub cluster have individual genera (Ps*Ba) and the A211 sub cluster have individual genera (Rh). Where, the second main cluster B included individual genera (Ba).

SDS -PAGE analysis

The protein banding patterns showed a maximum number of 34 bands which characterized with molecular weight (MW) ranging from 100 to 25 KDa. Our data revealed that the fusant *Pseudomonas fluorescense* Bacillus megaterium* (Ps*Ba) has two unique bands at 49 and 59 KDa, which doesn't exist in its parents. Fusant *Pseudomonas fluorescense* Rhizobium (Ps*Rh)* three unique bands 40, 50 and 96 KDa. For the last fusant *Bacillus megaterium * Rhizobium* (Ba*Rh) showed 2 unique bands 49 and 36 KDa. Similar results have been found in 3 Egyptian isolates of *Rhizobium* [13].

According to the cluster analysis showed in Fig. (3), all molecular data combined reveled a high relation between (Ps*Ba) and (Ba*Rh), which was 0.68 similarity, than that between (Ps*Rh) and (Ps) 0.35 genetic similarity. So those four types are highly related than the last sub group (Rh) and (Ba) at 0.2 genetic similarity.

Degradation ability of chlorpyrifos

The comparison between parents (Table 2 and Fig. 4) showed that *Rhizobium* was the highest efficiency to degrade chlorpyrifos pesticide. Then *Bacillus* and finally *Pseudomonas* is the lowest degradation. However, *Rhizobium* bacteria were degraded the largest number of different concentrations including the recommended concentration.

The results of *Pseudomonas fluorescens* showed highly significant differences and highly degradation for 90 µl and 30 µl concentrations than 70 µl concentrate. As well as, *Bacillus megaterium* have a good ability to degrade chlorpyrifos pesticide specially 90 µl concentration the degradation efficiency found to be 59%. While the concentrations of 30 µl and 45 µl was 48% and 45.8%, respectively. Other concentrations 60 µl and 75 µl were almost equal. *Rhizobium* presented high in concentrations 30 µl, 60 µl and 90 µl, where the percentage of the concentration of 30 µl was 52.9 %.

Fusants bacteria come from protoplast fusion technique had been exposed to five different concentrations from chlorpyrifos pesticide and the result showed more differences between fusants. The fusant *(Ps*Rh*) have high efficiency to degrade chlorpyrifos pesticide more than other two fusants in concentrations 30 µl, 45 µl, 60 µl and 90 µl. While, fusant *Bacillus megaterium*Rhizobium* revealed high degradation percent in concentration 75 µl.

*Pseudomona fluorescence * Bacillus megaterium* gives high percentage 80.7% for 30 µl concentrate. This percent decreased to 65.6% for 45 µl and down to 58% for 75 µl concentrate. High biodegradation capability of *Pseudomona fluorescence * Rhizobium.* The percentage of degradation was 85%, 74%, 68% and 60% for 30µl, 45µl, 60µl and 75 µl respectively. While was very low in 90 µl (38%). *Bacillus megaterium *Rhizobium* was different, the highest degradation was at 75 µl concentrate (70 %) then at 45 µl was 53 % down to 41% at 30 µl last was 30.5 % at 90 µl concentrate .

DISCUSSION

During microstructure analysis, the structure of fusants was studied under magnification. The properties of fusants determine their performance under the given application, and these properties are dependent on the intergenic structures. Their characteristics under microscope showed that the cell morphology of those fusants is differed from their bacterial parents.

In previous studies, PCR-RAPD assays are reliable and appropriate method for calculation of genetic distance between a fusant and its parents [14, 15]. The success of the protoplast fusion technique was through the appearance of novel fusant specific bands found. Polymorphisms of the bands in the gels indicated genomic differences between fusants and the parental strains. Their cluster data featured a high relation between (Ps* Ba) and (Rh) than (Ps*Rh). Which are more related than (Ba*Rh) and (Ps). While those five Bacteria not so far in similarity with (Ba). SDS-PAGE analysis revealed high relation between (Ps*Ba) and (Ba*Rh) than that between (Ps*Rh) and (Ps).

Generally, the degrees of inheritance of fusant strains from its parental strains using RAPD and protein analysis varied slightly. Likewise, similar results had been reported by Chakraborty and Sikdar [15], mentioning that predominat inherited was in inter-kingdom fusant with its parents. The ambivalence of these results might be due to recombination of genomes from all parental strains.

Protoplast fusion enhanced chlorpyrifos degradation for bacterial fusants, than their parents. Except 90 µl concentration in *Bacillus* showed strong superiority. Similar biodegradation studies by Mansee et al. [16] and Yacout et al. [13] have been conducted and reported that fustants could be powerful in degrade pesticides. Others provided the ability of biodegradation potential of microorganisms such as (*Pseudomonas* and *Sphingomonas*) [17]. Also, Zhao et al. [5] and Feng et al. [6] have reported the enhancement of fusants than their bacteria parent in different characters.

In conclusion, the three fusant strains produced by intergeneric fusion between (*Pseudomonas fluorescens*, *Bacillus megaterium* and *Rhizobium legumino-sarum*) could be arranged according to their ability to degrade chlorpyrifos followed; *Ps*Rh*, *Ps*Ba* and *Ba*Rh*. All results demonstrate an effective approach in order to construct high activity chlorpyrifos degradation by using protoplast fusion strains. This study implies a promising role for fusant strains in developing novel strategies for enhancing our environment.

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