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GENETICAL STUDIES AND ANTAGONISTIC EFFECTS OF A NEWLY BACTERIAL FUSANT AGAINST *MELOIDOGYNE INCOGNITA*, ROOT-KNOT NEMATODE, AND A PLANT PATHOGEN *FUSARIUM OXYSPORUM* INFECTING SUNFLOWER

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ABSTRACT:

All tested bacterial concentrations of *P.aeruginosa* and fusant strain (*Psa::Psf)*, between *Pseudomonas fluorescens and Pseudomonas aeruginosa*, affected *Meloidogyne incognita* juveniles J2 survival in *invitro.* The mortality percentages of nematode were dependent on the bacterial concentration and exposure time. The fusant strain *(Psa::Psf),* was used as soil drench or as seed soaking for controlling root-knot nematode *Meloidogyne incognita*, which was associated with sunflower plant (*Helianthus annus*) under greenhouse conditions. The fusant strain proved to be more effective than its parental strain, *P. aeruginosa*, in reducing different nematode parameters(give names) as well as enhancement of plant growth. Soil drench treatment was more effective in controlling root-knot nematode than the seed soaking treatment. The genetic stability of antagonist against *Fusarium oxysporum* of the fusant and its parental strain were 83% and 72%, respectively. RAPD-PCR studies indicated the same DNA-patterns among the fusant and its reisolated offspring. Three fusant isolates were selected, from which two isolates showed the same band when using two primers, while one isolate did not produce any band. Plasmid profile studies revealed the occurrence of one plasmid, of about 23 kb, in the fusant and its offspring while no plasmid has been found in the parental strain. The corresponding genes of antagonist against *Fusarium oxysporum* were located on the bacterial chromosome. From the present results, it could be advised to use modified genetically bacteria for management of the root-knot nematode.

INTRODUCTION:

Plant parasitic nematodes cause severe damage and economic loss to many crops. Plant-parasitic nematodes reduce the yield of the world's 40 major food staples and cash crops by an average of 12.3% (Sasser and Freckman 1987) while the root-knot nematodes (*Meloidogyne* spp.) being the main contributors to these losses. Collectively, the members of this genus have a host range of more than 2000 plant species. Their control is essential to maintain intensive crop production, but deregistration of chemical nematicides for environmental or human health reasons threaten to result in unchecked damage in many countries. Strategies for nematode problems are based on application of chemical compounds, use of resistant cultivars and specific agricultural practices. Currently, a number of approaches to engineer resistance against plant parasitic nematodes are being developed (Grundler *et al*., 1996).

Recently, research has focused on certain bacteria for the management of plant parasitic nematodes with the objectives of proposing methods for inhibition egg hatch (Kluepfel *et al*., 1993 and Siddiqui *et al.,* 2005), or

degradation of the hatching factor (Oostendorp & Sikora, 1990). *Pseudomonas fluorescens* has been found to have a potential in biocontrol of root-infecting nematodes (Cronin *et al*., 1997; Hamid *et al*., 2003; Siddiqui & Shaukat, 2004A and B) and also *Pseudomonas aeruginosa* have a potential in biocontrol of nematode (Larry & Manoil, 2001). In the light of the previous findings, the aim of this research is to study the effect of a fusant of two *Pseudomonas* species (i.e.*P.f* and *P.a*) for the management against *M. incognita* and *Fusarium oxysporu* infecting sunflower plants.

MATERIALS AND METHODS:

Materials: Strains:

a) ***Fusarium oxysporum. f. sp. sesami*** obtained from Dr. Saied Zedan, Plant Pathology Department National Research Centre.

b) Fusant (*Psa : Psf*) rif r, smr, protoplast fusant of *Pseudomonas aeruginosa* rifr and *Pseudomonas fluorescens* ATTC 17400 smr and antagonized against *Fusarium oxysporum f .sp. sesami* (El-Hamshary, 2002) .

c) *Pseudomonas aeruginosa* obtained from Dr. Ahmed Abu Eleinen, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt.

d) *Helianthus annus* Cu. Eurofler, obtained from Field Crops Research Institute, Agriculture Research Centre.

e) *Meloidogyne incognita*, Nematode was extracted from soil of pure culture of eggplant**.**

Media:

a) King's B agar complete medium (King *et al*., 1954) was used as selective medium for *Pseudomonas* strain.

b) Potato Dextrose Agar (PDA) medium (Burr *et al.*, 1978) was used for fungal growth.

Antibiotics were used at the following concentrations, rifampicin (rif) 100µg/ml, and streptomycin (sm) 200µg/ml.

Methods

1- Inoculum strains for bioassay: Three dilutions (S, S/2 and S/4) from cell suspension of parental and fusant *Psa::Psf* were used. About 1000 of *M. Incognita* juveniles (J2) were added to 10 ml of each previously described suspensions (S, S/2 and S/4) in 50 ml plastic tubes. Distlled water was used instead of bacterial cells as control treatment. Each treatment was replicated five times. The numbers of active and immobile nematodes were counted by using light microscope after 24, 48 and 72 hrs exposure periods at 25±5˚C. Nematodes after 72 hrs were transferred to other plastic tubes containing distilled water and were observed for 24 hrs to make sure that they either regain their activities or not. The nematode, which did not regain their activities were considered "dead". The mortality percentage was recorded for each treatment according to formula reported by Abbott (1925). Where % mortality = (m-n/100-n**) x** 100 as m and n stand for the percentages mortality in the treated and control; respectively. Percentages Net mortality = % mortality after 72 hrs - % recovery.

2- Nematode- antagonistic studies: A four-days culture of the tested strains, growing in King,s medium were used at 16x108 CFU and its half concentration (S/2) to inoculate seeds of sunflower plant, directly added to the soil, under greenhouse conditions. Seeds of sunflower, *Helianthus annus* L. cv. Euroflor were sown in 30cm-diam., pots filled with solarized sandy loam soil (1:1 w/w). After germination, the plants in each pot were thinned to one plant per pot. One week later, soil in each pot was inoculated with 500 newly hatched second stage juveniles (J2) of *M. incognita.* Fusant and parental *Pseudomonas* cultures were arbitrarily termed as standard (S) and another dilution (S/2) was prepared from (S) concentration with distilled water. Each concentration was added in each pot as soil drench (20 ml/pot). For seed

soaking, sunflower seeds were soaked in the tested concentrations for ½ hr. then, seeds were sown in pots and inoculated with nematode as mentioned previously. All treatments were replicated 5 times and 5 untreated pots were used as control treatment. In a greenhouse, all treatments were arranged on a bench in a completely randomized block design and watered as needed. Sixty days after nematode inoculation, plants were harvested. Numbers of galls, females and eggmasses were recorded. Number of galls and eggmasses were indexed according to Sharma *et al.,* (1994). Length, fresh weights of shoot and root and flowering disc were recorded.

3-Counting and reisolating the strains: To reisolate single colonies of fusant and parental strains from soil, one gram of soil inoculated in 100 ml saline buffer (0.9%NaCl), shaked at 120 rpm for 20 min. , spreaded on solidified medium of King,s sm, rif or without antibiotics, incubated at 30˚C for four days and counted. Several single colonies were isolated, purified and used further in genetic stability studies; antagonistic test study, plasmid isolation and RAPD-PCR.

4-Genetic stability of antagonistic properties of (Psa::Psf) fusant: Screening for antifungal activity was performed on King's medium without $FeCl_3$ using *Fusarium oxysporum f.sp sesami* according to Gauthier *et.al*.(1975). Several single reisolated colonies were grown on King's medium for one day at 28 ̊C, then one ml of chloroform was added to the plate cover for 20 minutes. Then the plate was opened for another 40 min. and overlayed with media of PDA, which was seeded with *Fusarium* and incubated for three days. Growth inhibition zone was measured.

5-Plasmid profile: Plasmids were isolated according to Marisa *et. al*. (1982) from fusant, parental and reisolated strains for characterizing these strains and comparing studies.

Genetic fingerprinting of fusant strain:

6- DNA fingerprinting: To follow a fusant at the molecular level, genotyping was performed with PCR- RAPD.

a) **Genomic DNA isolation**: Genomic DNA was isolated from three reisolated colonies and from the original fusant according to the manufacture of DNA isolation kit (DNASY from Qiagen).

b**) RAPD -PCR:** Genomic DNA was analyzed with RAPD PCR technique according to the method of Morais *et al*. (1996). Two random primers were used, number 113 (ATCCCAAGAG) and 123 (GTCTTTCAGG) (Roche Diagnostic, Gmbh, Manheim, Germany), which gave reproducible and sufficient amplification products with the original fusant. PCR amplification was performed in 25µl volume containing 60 ng of genomic DNA, 1 unit of Taq polymerase (fermentase), dNTP (200µM), MgCl2 (25 mM), 10x buffer, 3ng/µl primer. Amplification was performed using gene amplification system 9600 Perkin Elmer. The temperature profile was: 5 min at 94˚C followed by 40 cycles: 1min. at 94˚C, 1 min. 35˚C and 3 min at 72˚C.

c) **Agarose gel electrophoreses:** A 1.5 % agarose gel containing 89 mM Tris-HCl buffer pH 8.0, 89 mM boric acid, 2mm EDTA and 1.34 mM ethidium bromide was used to assay the amplification products. The gel was run for 3hr at 60 V and photographed.

RESULTS:

1. The effects of bacterial inoculum on nematode and sunflower growth: As illustrated in Table 1, all tested bacterial concentrations of *P.aeruginosa* and fusant *Psa::Psf*, affected survival of *M. incognita* juveniles J2. The mortality percentages of nematode were dependent on the bacterial concentration and exposure time. Complete nematode mortality was found after 24 hr exposure due to parental or fusant at concentration of 16x108 CFU (Colony forming units) and 8x108 CFU. At

concentration of 4x108 CFU, 95.9% mortality, was induced by the parental strain while the same concentration induced 98.9% when using the fusant. After 48 and 72 hr exposure, complete mortality was achieved by both tested strains.

The antagonistic effects of the fusant and *P. aeruginosa* on the development of the root-knot nematode, *M. incogmita*, are present in Table 2. Data presented in Table 2 showed that higher nematode reduction percentages were obtained by the fusant than the parental strain either as soil drench or seed soaking at the two concentrations used. Soil drench treatment was more effective in reducing the nematode than seed soaking treatment. In other words, by using soil drench, fusant *Psa:Psf* at (16x108 CFU) concentration achieved the highest percentages of nematode reduction i.e., 82.7, 79.2 and 92.6 for number of the galls, females and eggmasses; respectively. The same trend was noticed at the lowest concentration (S/2) of fusant strain. As for seed soaking treatment, fusant. *(Psa:Psf)* at (16x108 CFU) concentration achieved the highest nematode reduction percentages 59.8, 79.7 and 80.0 % for number of the galls, females and eggmasses, respectively. At the lowest concentration (S/2), fusant behaved the same trend.

Concerning plant growth, all sunflower growth criteria increased non-significantly by using either fusant or parental strain except for the weight of flowering disc, (Table 3). The degree of increase was concentration dependent. However, fusant strain increased the plant growth criteria more than those by parental strain.

Table (1): Survival of *Meloidogyne incognita* juveniles (J2) as affected by different concentrations of parental *P. aeruginosa* or the fusant *Psa::Psf* after 24, 48 and 72 hrs.

Treatment		% Nematode mortality			% Recovery	% Net mortality
		24 hr	48 hr	72hr		
Parental	s	100.0	100	100	0.0	100
P. aeruginosa	s/2	100.0	100	100	0.0	100
	s/4	95.9	100	100	0.0	100
Fusant	s	100.0	100	100	0.0	100
Psa::Psf	s/2	100.0	100	100	0.0	100
	s/4	98.9	100	100	0.0	100
Control		13.0	18.0	21.0	0.0	21.0

Values are averages of 5 replicates, S= 16x 108 CFU; S/2= 8x 10 8 CFU; S/4= 4x 10 8 CFU; % Mortality= m-n/100-n x 100 % Net mortality = % mortality after 72 hrs - % recovery (Abott, 1925).

Genetic stability: After nematode /bacterial treatment, the parental *P. aeruginosa* and the fusant were reisolated from the soil were prepared to study their genetic stability**.**

a-Plasmid patterns analysis: Plasmids were isolated from *P. aeruginosa* original strain and six reisolated single colonies, and from the original Psa::Psf fusant and five reisolated single colonies and electrophoresised. Plasmid patterns of the tested colonies (Fig.1) indicated that both *P. aeruginosa* had no plasmid while the fusant harbors one large plasmid (~23 Kb). All the five reisolated fusant were still containing this plasmid.

Table (2): Effect of fusant *Psa::Psf* and parental *Pseudomonas aeruginosa* on the development and reproduction of *Meloidogyne incognita* root-knot nematode, infecting sunflower.

Treatment	Bacterial conc.	No. of Females / plant	% Red.	No. of galls / plant	% Red.	Gall index	No. of eggmass / plant	% Red.	Eggmass Index
Soil drench									
Parantal P. aeruginosa	S	51	75.4	23	81.9	5	9	90.5	3
	s/2	68	67.2	47	63	6	15	84	4
Fusant Psa::Psf	S	43	79.2	22	82.7	5	7	92.6	3
	s/2	56	73	41	67.7	6	12	87.4	4
Seed soaking									
Parantal	S	73	64.7	56	55.9	7	24	74.7	5
P. aeruginosa	s/2	154	25.6	70	44.9	7	43	54.7	6
Fusant	S	42	79.7	51	59.8	7	19	80	4
Psa::Psf	s/2	151	27.1	54	57.5	7	33	65.3	5
Control	-	207	-	127	-	9	95	-	8
L.S.D. 5 %	-	46.39	-	29.6	-	-	29.14	-	-
L.S.D. 1 %	-	66.47	-	40.6	-	-	39.97	-	-

Values are averages of five replicates, Red. = Redudtion, S= 16x 108 CFU, S/2= 8x 10 8 CFU. G I/E I = gall / egg masses indices: 1=no galls or egg mass, 2=1-5, 3=6-10, 4=11-20, 5=21-30, 6=31-50, 7=51-70, 8=71-100 and 9>100 gall or egg masses per plant (Sharama *et al*., 1994).

Table (3): Effect of *Pseudomonas* and fusant strains on growth and yield of sunflower infected by *Meloidogyne incognita*.

Treatment	Bacterial conc.	Length of shoot (cm)	% Inc.	Weight of shoot (gm)	% Inc.	Weight of root (gm)	% Inc.	Weight of flowering disc	% Inc.
Soil drench									
Parantal P. aeruginosa	S	82.2	12.7	35.6	10.9	3.4	3.0	22.2	52.1
	s/2	73.3	0.7	33.1	3.1	2.9	0.0	16.3	11.6
Fusant Psa::Psf	S	83.2	14.3	36.9	14.9	5.7	72.7	23.2	58.9
	s/2	79.2	8.8	33.4	4.0	3.4	3.0	18.6	27.4
Seed soaking									
Parantal	S	87.5	19.8	37.9	18.1	4.2	42.4	21.1	44.5
P. aeruginosa	s/2	74.5	2.3	24.8	0.0	4.7	27.3	16.5	33.6
Fusant	S	91.0	25.0	44.2	37.7	5.4	63.6	30.6	109.6
Psa::Psf	s/2	83.3	14.4	36.4	12.5	5.1	54.5	26.0	78.1
Control	0.0	72.8	0.0	32.1	0.0	3.3	0.0	14.6	0.0
L.S.D. 5 %	-	N.S.	-	N.S.	-	N.S.	-	13.11	-
L.S.D. 1 %	-	N.S.	-	N.S.	-	N.S.	-	N.S.	-

Inc. = Increase S= 16x 108 CFU S/2=8x 10 8 CFU

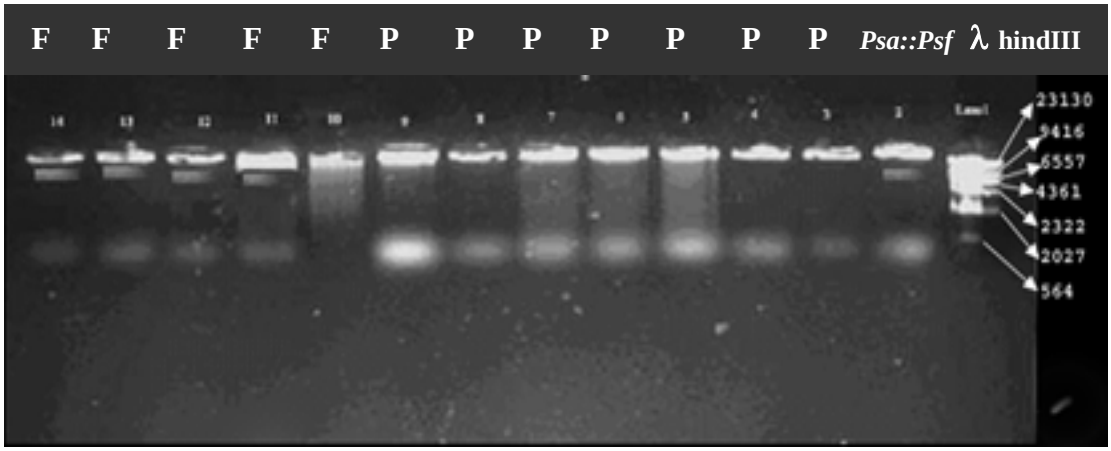


Figure-1: Plasmid Patterns of fusant, wilde type and reisolated parental, fusant from soil, lane (1) λ hind III, lane (2) original fusant, lane (3) parental strain, lane (4) parental strain (1.1), lane (5) parental strain (1.5), lane (6) parental strain (1.6) lane (7) parental strain (2.1), lane (8) parental strain (2.2), lane (9) parental strain (2.3), lane (10) fusant strain (3.1), lane (11) fusant strain (3.4), lane (12) fusant strain (4.1), lane (13) fusant strain (4.2), lane (14) fusant strain (4.3).

b-Genetic stability of antagonistic properties: To evaluate the genetic stability of the antagonistic genes of fusant and parental strains , a total of 30 reisolated strains were tested for *in vitro* antagonism towards the soilborne pathogen *Fusarium*. by using an overlay technique of Gauthier (1975). The results (figure 2 and table 4), indicated different degrees of *Fusarium* growth inhibition levels among the tested strains. Results showed that the fusant *Psa::Psf* was two times more efficient than parental strain in inhibition of *Fusarium*. All the tested reisolated fusant single colonies had the same activity as the original fusant except the two isolates 8.4 and 8.8 which had less antagonistic ability. This results indicated that the fusant *Psa::Psf* had 83% antagonistic stability.

Table 4, showed that five isolates out of, eighteen isolates of the parental strain had lost their antagonism activity against *Fusarium.* This results , indicated that the parental *P. aeruginosa* strain had 72% antagonistic stability.

Table-4 Antagonistic effects of reioslated and parental strain against Fusarium

Reisolated Parental strain	Inhibition effect	Isolate	Inhibitio n effect
1.1	+	6.1	+
1.2	+	6.2	+
1.5	-	6.4	-
1.6	+	6.5	-
2.1	+	6.6	-
2.2	+	6.7	-
2.3	+	6.14	+
2.4	+	6.18	+
2.5	+		
2.6	+		

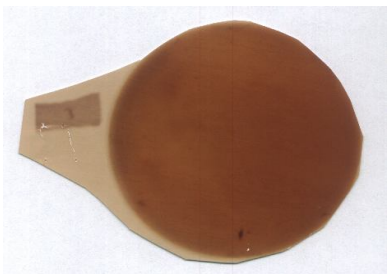
Reisolated fusant	Inhibition effect	Reisolated fusant	Inhibition effect
3.1	++	8.2	++
3.4	++	8.3	++
3.6	++	8.4	+
4.1	++	8.7	++
4.2	++	8.8	+
4.3	++		
4.4	++		

++=2cm

original fusant 4.1 4.2 *P. aeruginosa*

4.3 P fluorescens

original fusant 3.1 3.4 *P. aeruginosa*



3.6 *P. fluorescens*

Figure (2): Antagonism effects of different reisolated single colonies of fusant 4.1, 4.2, 4.3, 3.1, 3.4 and 3.6 against *Fusarium oxysporum .f.sp.sesami.*

Genetic fingerprinting of fusant strain:

DNA fingerprinting: RAPD-PCR finger printing was used for analyses of the fusant's genomes. Nine primers out of twenty-seven, produced an informative banding pattern within parental genomes. Two random primers used, number 113 (ATCCCAAGAG) and 123 (GTCTTTCAGG)(Roche Diagonistic Gmbh, Manheim, Germany) which gave reproducible and sufficient amplification products with the original fusant. The primer 123 produced DNA band with *P. fluoresces* genome and not with *P. aeruginosa* genome. The primer 113 was able to produce a DNA band with *P. aeruginosa* and not with *P. fluorescens* genome.Three fusant isolates (i.e., 4.1, 4.2, 4.3) were selected according to their performance. They harbor the same plasmid**,** have the same morphological character of the original fusant and have the best inhibition to both nematode and *Fusarium*. Their DNAs were analyzed with RAPD-PCR technique using two random primers No.113 (ATCCCA AGAG) and No.123 (GTCTTTCAGG), which gave reproducible and sufficient amplification products with the original fusant. Isolates 4.1, 4.2 showed that they have the same band when using the two primers (Fig. 3) while the isolate 4.3 did not produce any band.

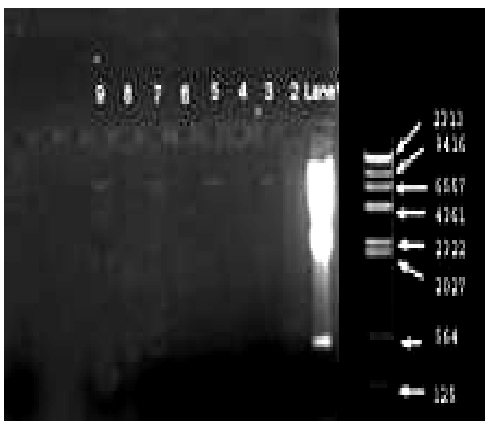


Fig (3) RAPD-PCR of reisolated fusant. Lane 1, λ Hind ш; primer number 113: Lane 2, negative control; Lane 3, isolate 4.1; Lane 4, isolate 4.2; Lane 5, the fusant *Psa::Psf* ; primer 123: Lane 6, negative control; Lane 7, isolate 4.1; Lane 8, isolate 4.2' Lane 9, the fusant *Psa::Psf.*

DISCUSSION

Microorganisms that can grow in the rhizosphere are ideal as biocontrol agents, since rhizosphere provides the front line defence for root against pathogens attack (Weller, 1988). The rhiziospheric microorganisms encounter the pathogens during the primary infection and also during

secondary spread on the roots. In the present study, the use of the modified genetically bacterial fusant Psa::Psf showed significant control of *M. incognita* reproduction and development and increased the plant growth better than original bacterial strain. The effect may be, presumably, due to produce iron chelating siderophores (Kloepper *et al.*, 1980; De Meyer & Hofte, 1997), antibiotics and hydrogen cyanide (Ahl *et al.*, 1986 and Larry & Manoil, 2001) which inhibited certain root-infecting fungi and with the release of certain bacterial metabolites, they reduced hatching with attraction and degradation of specific root exudates which control nematode behavior (Kluepfel *et al.*1993 and Oostendorp and Sikora, 1990). Besides production of certain antimicrobial compounds, plant growth promoting-bacteria are also known to release plant growth regulators such as auxion derivatives and gibberellin- like substances (Hussain & Vancura, 1970 and Brown, 1972), which play a significant role in the enhancement of plant growth. In the present study, the effect of fusant and parental strain on *M. incognita* when used as soil drench was better than those by seed soaking. It is possible that long period of sunflower seed germination enabled the tested bacteria to evoke changes in seeds before the germinating seeds were infected by the nematodes. In other words, by using seed soaking, the bacterial strain had no enough time to evoke changes in soaked seeds. At the same trend, Korayem and Salem (2000) had constructed genetically modified strain of *Azotobacter chroococcum*, a free living and nitrogen fixing bacterium, with the ability of producing saponin. The natural occurrence of both nitrogen fixation and saponin producing ability in a single organism may reflect on better control of plant parasitic nematodes and increased plant growth. Oostendorp and Sikora (1990) reported that, a 68% reduction in sugar beet cyst nematode root invasion was obtained by application of *Pseudomonas fluorescens P523* to beet seeds. Other studies showed that such antagonistic rhizobacteria can function directly by competition and antibiosis (Bucheaur, 1998). It can also act indirectly by inducing systemic resistance in the plant towards soil-borne pathogen and plant parasitic nematodes (Hasky- Gunther, *et.al*. 1998; Siddiqui and Shaukat, 2002a and b).This study focused on evaluation of a fusant *Psa::Psf*, which inhibits *Fusarium oxysporum. f. sp. sesami* under laboratory conditions for control of nematode and identifying the strains at the molecular level. Results indicate that the genetic stability of antagonistic properties against *Fusarium oxysporum* for fusant and parental strain were 83%, 72%; respectively.

The band pattern of RAPD-PCR reactions represents a “genetic fingerprint” that can used to characterize particular bacterial strain (Welsh and McClelland, 1990). The RAPD-PCR of the fusant and its isolates, yielded a products of the same size. The primer 113 was able to produce a DNA band with *P. aeruginosa* and not with *P. fluorescens* genome**.** RAPD-PCR studies indicated the same DNA-patterns among the fusant and its reisolated offspring. Three fusant isolates were selected, from which two isolates showed that they have the same band when using the two primers, while one isolate did not produce any bands.

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