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Improving Nematocidial Activity of Bacteria via Protoplast Fusion

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Abstract: Cucumber plants play an important role in economical field in Egypt. But they are infected by plant parasitic nematodes that leading to losses cucumber yield. This study attempted to construct more efficient bacterial strains may be able to controlling nematodes, as well as, maintenance the soil fertility using molecular biology method via induced protoplast fusion between different strains of *Pseudomonas* and *Serratia* to improve the biological control against the nematodes number in infected fields, in order to reduce the use of nematocidial agents. Four strains of *Serratia* and six *Pseudomonas* strains were used in this study. The results showed that more efficient strains in the production of indole acetic acid and antibiotic resistance were controlling plant parasitic nematodes such as *Meloidogyne* and some other type of nematodes. Mortality values were obtained by all strains of *Serratia* and *Pseudomonas*, as well as, the high values of mortality were obtained from *Pseudomonas* strains. Different bacterial strains could control the nematodes larva under green house condition which reduced the numbers of larva. per k.g soil, as well as, reduced galls formation and galling index. However, some bacterial strains were more efficient in controlling nematodes such as *Serratia* strains ESA.4, ESA12 and *Pseudomonas* strains NRRL-B 514, NRRL-B 694, NRRL-B.99, NRRL-B.51. On the other hand, fusants produced antibiotic chitinoltic enzymes, chitinases and bacteriocin which improved their ability to control the nematodes in the soil rhizosphere. Fusants induced high mortality levels against nematodes if compared with the parental strains, as well as, all the fusants obtained controlled nematodes and protected cucumber plants from nematode infection causing yield losses. This indicated that fusants evaluated against plant parasitic nematodes appeared high efficient nematocidial effect. The results of fusants documented using polyacrylamide gel electrophoresis and DNA fingerprinting appeared genetic variations between fusants and their parental strains.

Key words: Cucumber, DNA fingerprinting, fusants, nematocidial activity, *Pseudomonas*, *Serratia*,

INTRODUCTION

Cucumber was considered as one of the most important vegetable crops in Egypt. It was cultivated in 30 thousand feddans with an average production of 25.6 tons per feddan (2007/2008) recently. Plant parasite nematodes, especially root-knot nematodes are known among the most serious pests of cucumber in many countries, of some described species of *Meloidogyne*, only two parasitized cucumber plant *M. incognita*, *M javanica* essentially are hot weather organisms and most important , where cucumber plant are grown in regions with long hot summers and short, mild winter. In Egypt . *M. incognita* and *M. javanica* were reported as major nematode of cucumber (Maareg *et al.* 1988 a, and Ismail *et al.* 1996). The average annual loss in yield of cucumber plant due to *M. Incognita* in different states in the USA was estimated to be as high as 10-50% and in Italy as 5-15% (Altman and Thomson 1971). Yield loss estimates were based on nematode population, on curves derived earlier relating, nematode densities with crop yields and on the average in certain period. Importance of the biological control depends on bacteria, fungi , actinomycetes and another extracted products extracted from plants and also from microorganisms themselves. Bacteria was used as nematocide pesticides. Antibiotics produced by bacteria, actinomyces and fungi also used as nematocide products and some pharmacological compounds have been considered (Kerry and Bourne 1996 and Badr 2001). This study investigated the ability of some bacterial strains as a bioagent against plant parasitic nematodes which infected cucumber crop in green house grown in epidic soil. Many bacterial strains related to more kind of bacteria such as *Pseudomonas* strains obtained from USA culture collection, and other types obtained from local

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sources, as well as, some bacterial strains isolated from Egyptian habitat. All were examined for more one economical traits such as produce some antibiotic and nematocidial enzymes. These strains were used as a biological for controlling the plant parasite nematodes that infected cucumber crop to be reduced the losses in plant yield. Therefore, this study focuses on the antagonistic activity of different bacterial strains and their recombinants against nematode via nematode-antagonistic activity. Plant parasite nematocidial are playing an important role in induce crop slimy in agriculture sector therefore leading losses in crop yield all over the world in the last decade. This study aimed to induce bacterial recombinants via protoplast fusion technique to improve the nematocidial activity against nematode parasite to decreasing the damage resulted from infect different crops by these type of nematode.

MATERIALS AND METHODS

The present study is a result of co-operation between the Department of Genetics, Faculty of Agriculture, Mansoura University and Ain Shams Center of Genetic Engineering and Biotechnology (ACGEB), Faculty of Agriculture, ain Shams University and Microbiology Lab., Ministry of Agriculture.

1- Materials:

Bacterial Strains:

Bacterial strains used in this study and their sources are listed in Table 1. Pseudomonas strains NRRL-B.13 and *E.coli* DH5*a* (used as tester strains to evaluate bacterial strains in bacteriocin production) were also used in this study.

Table 1: Bacterial strains used in this study.

Strain	Source	Designation
Serratia marcescens	Egypt,. Hawamdia, Giza	ESA-1
Serratia marcescens	Egypt,. Hawamdia, Giza	ESA-4
Serratia marcescens	Egypt,. Hawamdia, Giza	ESA-5
Serratia marcescens	Egypt,. Hawamdia, Giza	ESA-12
Bacilluis subtlus	Giza, Agriculture Research Center ARC	ARC-BS.7
Bacilluis circulans	Giza, Agriculture Research Center ARC	BC-ATCC.1212
Bacilluis lichenformans	American Type Culture Collection U.S.A	BL-ATCC.1480
Pseudomonas fluorescens	National Regional Research Lab.	NRRL-B.12
Pseudomonas fluorescens	American Type Culture Collection U.S.A	NRRL-B.51
Pseudomonas fluorescens	American Type Culture Collection U.S.A	NRRL-B.99
Pseudomonas fluorescens	National Regional Research Lab.	NRRL-B.514
Pseudomonas fluorescens	American Type Culture Collection U.S.A	NRRL-B.694
Pseudomonas fluorescens	American Type Culture Collection U.S.A	NRRL-B.870
Pseudomonas putida	National Regional Research Lab.	NRRL-B.13
Escherichia coli	American Type Culture Collection U.S.A	DH5 *a*

Plant Material:

Cucumber plants variety Saaf and Tahreis-3 were used in this study as a host for nematode parasite. Their seeds were obtained from Horticulture Research Institute, Agriculture Research Center, Giza, Egypt.

Media:

Mineral Medium (MM):

This medium was used for testing the utilization of carbon sources according to Milnisky *et al.* 1997. PH was adjusted to 6.7, although, glycerin was replaced by appropriate carbon source at a final concentration of 1%.

Bantig Medium (BM):

This medium was used for testing chitinase activity via replacing glycerin by colloidal chitin 2.5 g/1 according to Yusupova *el al.* (1995).

Peptone Glycerol Medium (PG):

This medium was used for cultivation of *Serratia* isolates according to Palleroni 1984.

Nutrient Agar Medium: It was used for assaying enzyme activity according to Dowson 1957.

King B Medium:

This medium was used for cultivated *Pseudomonas* strains according to King *et al.* 1954.

Heavy Metal and Antibiotic Markers:

The ability of *Pseudomonas* and *Serratia* to grow on medium containing antibiotics and or heavy metals was tested according to Chan *et al.* 1988. All antibiotics used in this study are presented in Table 2, which were obtained from Sigma chemical-Co. St- Louis, Missauri, USA.

Table 2: Antibiotics and heavy metals used in this study.

Antibiotic and heavy metals	Abbreviation	Concentrations
Oxy. Tetracyclin	*Oxy. Tetra*	5, 10, 15, 20, 25, 30 µg/ml
Streptomycin	*Strp*	27, 40, 50, 75, 100 µg/ml
Rifampicin	*Rifm*	5, 10, 15, 25 µg/ml
Erythromycin	*Erth*	25, 50, 75, 100 µg/ml
Neomycin	*Neo*	27.5, 30, 36, 40, 100 µg/ml
Nalidixic acid	*Nali*	5, 6, 7.5, 10 µg/ml
Penicillin	*Pen*	100, 500, 1000 µg/ml
Methyl glyphaste	Meth. Gly	50, 90, 100 µg/ml
Copper sulphate	Cu^{++}	150, 200, 400, 500 µg/ml
Cobalt chloride	Co^{++}	25, 50, 75, 100, 150 µg/ml
Ferus sulphate	Fe^{++}	1000 µg/ml
Aluminum	Al^{+++}	10, 50, 100 µg/ml
Mercury oxid	Pb++	100, 150, 200, 400, 500 µg/ml

2- Methods:

Bacterial Protein Extraction:

The frozen samples were first quickly defrosted for 2 min in a warm heated water bath (30°C), and were then frozen again at minus marker70°C (20min). Such a procedure allows partly disrupted bacterial cells to be obtained. Each sample was centrifuged for 5 min at 10000g, and the pellet obtained was resuspended in 20ml lysozyme. The suspension was incubated for 30 min at 37°C. Afterwards, the sample was again recentrifuged and 80ml from each sample was transferred into a new 1.5-ml Eppendorf tube. Then, 20ml of samples treatment buffer (1x) was added and the whole mifor plaque formation was plated on different *Serratia* strains (Struffi *et al.* 1998).

Quantitation of IAA production:

Mineral medium was used to cultivating *Serratia and Pseudomonas* and incubated for 24 hours and then bacterial cells were removed from the culture by centrifugation (6000 rpm) at 4°C for 10 min. One ml aliquot of supernatant was mixed vigorously with 4 ml of Salkowaski's reagent (150ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5m $FeCl_3$. $6H_2O$ (Gordon and Weber, 1951) and allowed to stand at room temperature for 20 min before absorbance measured at 535 nm. The concentration of IAA in each culture medium was determined by comparison from the standard curve.

Rhizobiotoxin Production Assay:

This was determined for *Pseudomonas* and *Serratia* strains using Balasa mineral medium (Balasa 1963). Supernatant of each *Pseudomonas* and *Serratia* strains were filtered by filter paper in sterile test tube. Each tested strains streaked on YEM plates, the and supernatant was added in agar disk and the inhibition zones were measured.

Preparation of Colloidal Chitin:

Colloidal chitin was prepared by dissolving 10g of ground chitin in 180 ml of conc. HCl. The acid was added to the chitin with stirring and the mixture was allowed to stand at 20°C with stirring until dissolved (90 - 120 minutes). The solution was poured into 6 liter half filled with tap water. A suspension of chitin in water was formed and additional water was added to complete a final volume to 5 liter. The suspension as allowed to stand overnight to allow the chitin to settle and form a concentrated suspension. The liquid supernatant was slowly siphoned off and tap water was added to resuspend chitin. This process of washing with tap water was repeated 4 times, followed by 3 times washed with distilled water. After final wash, the suspension was passed through filter to remove large particles. The resulting chitin suspension had a pH of 5.5-6.0 was stored in dark at 4°C until used. Chitin content of the suspension was dried at 80°C for 24h. Three stock suspensions were used to prepare others of lower concentration by diluting with distilled water immediately prior to use (Rodriguez-Kabana *et al.* 1983).

Testing Chitinase Activity:

The activity of chitinase gene was validated on minimal agar medium supplemented with 0.5% colloidal chitin according to Jones *et al*. (1986).

Plant Protein Extraction:

Leaf samples (0.25 g) were frozen with liquid nitrogen and ground with 1 ml of water-soluble extraction with vortexes to ensure good homogenization. Such prepared samples were kept on boiling water bath for 4 min, and again recentrifuged (1000 rpm, 10s) in order to remove any accidentally- remaining whole cells or their fragments, and to obtain cell-free extracts. (Zarnowski *et al*. 2001).

Application of Samples:

A volume of 50µl of the samples was added to the same volume of buffer. 10µl Mercaptoethanol was added. Samples were boiled for 10 minutes and 100ml of samples was loaded on the gel after adding one drop of the loading buffer (Bromophenol blue and glycerin).

Dry Weight of the Plant:

The plants were dried at room temperature for two days, then put in oven with forced dry air circulation at 70ºC for two days and put in the desiccators before measurement of dry weight. The dry weight per plant was calculated for each inoculation treatment.

Photosynthetic Activity:

Chlorophyll a and b, as indicator for photosynthetic activity, were determined after 13 weeks of planting. Spectrophotometric (OD) method, using optical density measurements according to Markinney (1941) was to determine chlorophyll a, b and total.

Sampling, Extraction, Enumeration of Plant Parasitic Nematodes:

Soil and root samples collected from cucumber field at the west of Nubaryia and El. Bostan village at the deeps of 25.30 Cm according to Barker and Campbell (1981) and Wallace *et al*. (1995). Root fragments from samples were macerated and placed in Baseman-pan for three days for nematode separation. Tested soil samples were carefully mixed and from each sample 300 mL (three replicates) were processed for nematode extraction by wet sieving technique (Goody, 1957). Direct sieving through 100, 200, 250 mesh screens were also employed. Resulting suspension was cleaned by means of the Baseman-pan technique for separating active nematodes from fine soil particles. The nematodes recovered from each sample were examined and counted under a microscope using 1 ml eelworm counting slide. Average number of nematodes in one ml was recalculated as average number for final extract nematode suspension.

Effect of Soil Inoculating Bacteria on Nematodes Infected Cucumber Plant:

A. population of the root knot nematode, originally isolated from culture collection banks has been maintained continuously on cucumber plants of the cultivar on small plots. Newly hatched second stage juveniles were obtained by dissecting egg masses from fresh root and incubating them on a 50 µm sieve in water for two days at 20ºC as described by Den Belder and Jansen (1994).

Nematocidial Activity (IN vitro Bioassay Test):

To study the effects of the obtained (TMP) toxic metabolic products of the bacteria on *M. incognita* larva, 9 ml of each seven tested bacteria TMPs were added to 1 ml nematodes aqueous suspension containing 1000 second stage larvae (j2) of *M. incognita* in 10 cm diameter Petri-dish. Each treatment was replicated three times, 9 ml distilled water was added to 1 ml nematodes (1000 larvae) aqueous suspension as a control. The nematode mortality counts were recorded after different times.

Nematocidial Activity (Green House Test):

Pots experiment was carried out to evaluate the relative efficacy of the bacteria against *M. incognita* infecting cucumber plants grown in green houses. Stock liquid culture of each bio-agent were prepared on suitable medium for each organism. Fifteen days-plant old were inoculated with 1000 larvae of *M. incognita* was originally propagated on cucumber plant. The larva were prepared as described by Ehwaeti *et al*. (1998). Three days after nematode inoculation, 150 ml of each bioagent were added to 2 cm deep around each plant and covered with soil.

Protoplast Fusion:

Pseudomonas and *Serratia* strains were grown in starved nutrient broth medium, 1 : 10 dilution rate, one ml from each parent was transferred into ependrof. Then treated each bacterial strain by 10 µl of lysozyme for 15 min after protoplast processes in completely 400 ul of protoplasted parental strain then transferred into new ependrof and added 10 ul of PEG, 400,(25%) to bacterial suspension and 100 mM of $CaCl_2$ were added to protoplast mixing. After 60 min of incubation at 30ºC, 100 µl of mixing suspension were added to flasks containing 100 ml nutrient medium for regeneration of fused cells. This method was described before by Ganguli and Bhaleros (1965). Then planting appropriated dilution of bacterial cell in nutrient agar medium supplemented with collide chitin and soluble casein and 0.11 % $CaCl_2$. The fused formed showed clear zone around colony formation cells. Fusions conducted and the resulted fusants are listed in Table 3.

Table 3: Fusants obtained between different bacterial strains.

Parental fusion	Recombinant fused isolates
ESA. 4 × NRRL. B 514	ESA 4 NRRL 514
ESA. 4 × NRRL. B 99	ESA 4 NRRL 99
ESA. 4 × NRRL. B 694	ESA 4 NRRL 694
ESA. 4 × NRRL. B 51	ESA 4 NRRL 51
ESA. 12 × NRRL. B 514	ESA 12 NRRL 514
ESA. 12 × NRRL. B 99	ESA 12 NRRL 99
ESA. 12 × NRRL. B 694	ESA 12 NRRL 694
ESA. 12 × NRRL. B 51	ESA 12 NRRL 51

Genomic DNA Preparation:

It was conducted according to King *et al*. (1954) using cultures grown for 4 days at 28°C in nutrient broth medium.

Oligonucleotide Primers and PCR Amplification Conditions:

This experiment was conducted at the centre of Agri. Biotech., Fac. of Agric, Ain Shams Univ., Cairo. according to Sikora *et al*. (1997).

Statistical Analysis:

The data were subjected to the analysis of variance using the computer Statistical Analysis System (SAS). The least significant difference (LSD) value was applied to make comparisons among the means when the differences between treatments were significance (Steel and Torrie, 1980).

RESULTS AND DISCUSSIONS

Intrinsic Antibiotic and Heavy Metal Resistance:

Minimum inhibitory concentration (MIC) of antibiotics and resistance or sensitivity to heavy metals were evaluated in this study. All bacterial strains appeared high resistance to 1000 µg of pencillin (Table 4). However, some strains revealed degree of sensitive. Pencillin resistant strains showed high degree of tolerance to Fe^{+++} which used as a ferrous sulphate. In addition, strain ESA-4 revealed high resistance to AL^{+++} ions while some strains showed moderate heavy metal resistance. Neomycin, streptomycin, Cu^{++} and cobalt except melodox revealed higher sensitivity in most strains. These results agreed with Stewers and Eagleshman. (1984), who found that intrinsic antibiotic resistance (IAR) was effectiveness for evaluating isolates of *Pseudomonas*, *Serratia* and *Bacillus*. The variation in response may be attributed to spontaneous mutation (Kingsley and Bohlool, 1983). However many antibiotic concentrations didn't provide a limit patterns of resistance, some isolates were resistance to high concentration of antibiotic such as streptomycin ($\leq$ 200 µg/ml), penicillin ($\leq$ 1000 ug/ml) erythromycin ($\leq$100 ug/ml) and Rifangain ($\leq$ 106 µg/ml) (Chao 1987).

Bacteriocin Production:

Bacteriocin produced by different bacterial types and different strains from the same type was used to describe any inhibitory effect due to antagonism between closely related strains (Table 5) were examined by disc diffusion agar plate methods. All strains showed high to moderate bacteriocin production, However, *Pseudomonas* strains NRRL-B 870 was more extremely antibiotic producing but the other ones showed a moderate production of antibiotic.

Table 4: Minimun inhibitory concentration (MIC) of bacterial strains against antibiotics and heavy metals.

Antibiotic -------- Strains	Oxy. Tetra	Stryp	Rihm	Ertro	Neomy	Melodox	Pen	Methyl glypho	Cu^{++}	CO^{+}	Fe^{+++}	AL^{+++}	Pb^{+++}
ESA-1	10	75	25	50	70	5	100	100	400	50	1000	10	100
ESA-4	25	100	25	75	53.7	5	1000	80	200	50	1000	100	500
ESA-5	20	100	25	100	63.3	5	1000	100	150	100	1000	100	500
ESA-12	25	100	10	75	102	6	1000	90	200	50	1000	50	200
ARC-BS.7	15	50	5	50	100	10	1000	100	400	10	1000	50	500
BC-ATCC.1212	10	100	10	75	40.6	5	100	50	506	75	1000	50	406
BL-ATCC.1480	25	40	5	25	40.6	10	100	50	206	100	1000	50	500
NRRL-B.12	50	100	10	75	36	75	1000	50	200	25	1000	100	100
NRRL-B.51	5	40	10	75	40	5	500	100	1000	150	1000	100	100
NRRL-B.99	15	27	15	50	27.2	10	1000	10	50	25	1000	100	100
NRRL-B.514	30	75	25	50	30	7.5	1000	50	300	25	1000	10	150
NRRL-B.694	20	40	15	100	40	5	1000	50	150	50	1000	50	100
NRRL-B.870	30	106	10	75	36	7.5	1000	50	206	25	1006	106	106

Table 5: Bacteriocin production by different bacterial strains.

Inoculants	Bacteriocin (µg/ml)	Pro bacteriocin (µg/ml)
$C\ v^{+\ \dagger}$	0.0	0.0
$Cv^{-\ \dagger\dagger}$	0.0	0.0
ESA-1	1.25	2.33
ESA-4	1.34	4.3
ESA-5	4.4	3.84
ESA-12	1.3	2.0
ARC-BS.7	2.81	4.23
BC-ATCC.1212	0.0	2.31
BL-ATCC.1480	0.0	4.34
NRRL-B.12	2.28	2.34
NRRL-B.51	3.43	3.1
NRRL-B.99	3.43	4.1
NRRL-B.514	3.32	2.34
NRRL-B.694	3.43	2.1
NRRL-B.870	2.82	3.3

†, †† = Positive control treated with *Pseudomonas putida* and negative control treated only with mixed bacteriocin from all strains, respectively

In addition, different strains from *Bacillus*, *Serretia* and *Pseudomonas* such. B.S7, ESA-4, ESA-12 and NRRL-B 51 were produced bacteriocin. These agents consist of amino glycoside or terpenoid and polyketide. The results obtained here agreed with Joseph *et al.* (1993), who examined various strains of bacteria for production of bacterial bacteriocin. Like substances reached maximum on day 7 of growth. The production of bacteriocin by species has been reported by various investigators (Roslycky 1967, Schwinghamer, 1971 and Schwinghamer *et al.* 1973).

Nematocidial Activity Against Plant Parasite Nematodes:

Antagonistic activity of bacterial strains against plant parasite nematodes were studied by many researcher since plant parasite nematode has been causing a large damage in a cucumber plant special by that grown under green house condition and causes to losing a large amount from the yield of cucumber plant. Data shown in Tables 6, 7 appeared the effect of different bacterial strains on the behavior of plant parasite nematodes against cucumber plants under green house.

The data appeared that different bacterial strains affected to reduce the number of galls formed by nematodes. This leading to increase biomasses production by cucumber plant treated with different bacterial strains. This was due to the decrease a number of larva in plants treated by bacterial strains in soil rhizosphere and plant roots.

Since *Pseudomonas* strains showed high decrease in the number of galls and number of larva/ kg soil, as well as, the number of larva/plant root, strains; NRRL-B 514, B.L-ATCC 1480, NRRL-B 99, ESA-5 and ESA-12 appeared highly decrease in the number of galls and nematode larva, as well as, increased accumulated biomasses in shoots and roots (Table 6). This agreed with Jatala (1985), who reported that the *P. Liacinus* was highly effective against root knot nematode and suggested it use as a biological tool against nematode. The present study didn't support his findings because of the reverse effect of high soil temperature on biocontrol agent. Root knot nematode can cause significant losses in cucumber yield if not treated with nematocides. Considerable efforts has been made to bread for nematode resistance in vegetable crops and it was successful with tomatoes but not in cucumber. Several authors reported on small scall, pot experiment which indicated that mites brought about considerable reduction in nematode number, Sharma (1971) showed that *Lasioseius penecilliger* reduced the population of plant parasite nematodes by 44% and nematofungus by 68%.

Table 6: Nematocidal activity of bacterial strains against plant parasite nematodes.

Treatment		No. of galls/ plant	No. of larva/ kg. soil	No. of larva/ plant	Eggs. number/ plant	Shoot DW/plant	Root DW/ plant
Cv-		0.0	0.0	0.0	0.0	126.4	117.2
Cv+		60	1200	180	20	139.33	121.667
ESA-1		30	115	10	33	150	132.6
ESA-4		35	90	5	5.0	132.33	140
ESA-5		14	53	17	2.7	129.4	136
ESA-12		12	108	8	7	123.3	88.6
ARC-BS.7		16	50	0.0	4.0	121.6	181.3
BC-ATCC.1212		23	280	19	3.7	124.3	130
BL-ATCC.1480		10	65	36	4.3	136.3	108.3
NRRL-B.12		18	123	84	7.2	200.8	119.3
NRRL-B.51		14	60	36	4.3	125.3	108.3
NRRL-B.99		8	110	76	4.0	226.3	93.8
NRRL-B.514		0.0	72	3	3.3	117.2	125.3
NRRL-B.694		10	43	10	4.3	123.3	92.3
NRRL-B.870		4	60	6	3.0	138.3	68.8
F-Test		0	**	0	**	**	**
LSD	0.05	2.31	3.93	5.3	0.9	6.53	3.1
	0.01	4.2	9.3	8.4	1.3	8.6	9.5

Cv^- , Cv^+ = Plants grown in autoclaved soil and infected soil with nematode larva, respectively, DW = Dry weight
** = Significant at 0.01 probability level.

Table 7: M ortality percentage of *Meloidogyne incognita* juveniles larva treated with different bacterial strains *in vitro*.

Bacterial filtrates		Mortality (%)
Cont (v^-)		0.0
Cont (v+)		90
ESA-1		46
ESA-4		47
ESA-5		40
ESA-12		55
ARC-BS.7		45
BC-ATCC.1212		78
BL-ATCC.1480		60
NRRL-B.12		59
NRRL-B.51		53
NRRL-B.99		53
NRRL-B.514		57
NRRL-B.694		40
NRRL-B.870		42
F-test		**
LSD	0.05	2
	0.01	9.3

v^-, v^+ = Nematode larva grown in petri dishes treated with all 13 bacterial strains and without bacterial treatment, respectively ** = Significant at 0.01 probability level.

Mankau and Imbriani (1978) found that incorporation of biocontrol agents into the soil infected with the nematode resulted in a marked decrease in root-gull index and the number of gulls were suppressed compared to the control. Also results obtained by these authors revealed that introducing antagonistic bacteria prior to nematodes was more effective than when introduced by simultaneously with nematodes. The reduction was as 95.7, 97.5 and 98.4% in treatments of *P. fluorescence* and *B. thuringiensis*, respectively, compared with the simultaneous introduction of both organisms. Mousa *et al.* 1989 showed some bacteria and actinomycetes have antagonistic effect on plant parasitic nematodes. The influence of rhizobacteria on nematode invasion and their ability to reduce gull formation by *M. incognita* is clearly demonstrated. The mechanisms were explained by the production of nematocidial compounds (Bacher *et al.* 1988) or by host root exudate to prevent the penetration of the host tissues by nematodes (Sikora *et al.* 1997). On the other hand, many soil bacterial species are capable of decomposing plant residue , and the products released by the metabolic of the bacteria vary from the complex to the simplest of molecular. Some of these products accumulate in the soil and may be toxic.

Data shown in Table 8 appeared protoplast fusion between *Serratia* sp. ESA-4 and *Pseudomonas fluorescens*. The results indicated that higher number of fusants were obtained at 10 hours and over night incubation period. However, the more efficient protoplastsi were obtained from the fusion between ESA-4 *Serratia* strain and NRRL-B 51 at all incubation times.

Table 8: Protoplast fusion between *Serratia* and *Pseudomonas fluorescent* NRRL-B.99 at different incubation time.

Incubation time	Total	ESA-4 x NRRL 514		ESA-4 x NRRL99		ESA -4 x NRRL 694		ESA.4 x NRRL 51	
(h)	No of cells	Fused No.	Fused frequency	Fused No.	fused frequency	Fused No	Fused frequency	Fused No	Fused frequency
2	6×10^{8}	0.0	0.0	0.0	0.0	0.0	0.0	1×10^{2}	0.16×10^{-6}
4	3×10^{8}	0.0	0.0	0.0	0.0	6.0	6.0	43×10^{2}	14.3×10^{-6}
6	4.2×10^{8}	0.0	0.0	0.47×10^{-4}	0.0	0.0	0.0	96×10^{2}	22.8×10^{-6}
8	6.3×10^{8}	3×10^{3}	47×10^{-5}	7.1×10^{-4}	0.0	0.0	0.0	3.2×10^{3}	0.5×10^{-5}
10	3×10^{9}	4×10^{3}	1.3×10^{-6}	21.6×10^{-5}	8×10^{2}	8×10^{2}	2.6×10^{-7}	8×10^{4}	2.66×10^{-5}
12	25×10^{9}	13×10^{4}	0.52×10^{-5}	0.12×10^{-4}	17×10^{3}	17×10^{3}	0.68×10^{-6}	9×10^{6}	0.36×10^{-3}

Table 8: Continued

Incubation time	Total	ESA-4 x NRRL 514		ESA-4 x NRRL99		ESA -4 x NRRL 694		ESA.4 x NRRL 51	
(h)	No of cells	Fused No.	Fused frequency	Fused No.	fused frequency	Fused No	Fused frequency	Fused No	Fused frequency
2	8×10^{7}	0.0	0.0	0.0	0.0	6.0	0.0	0.0	0.0
4	50×10^{7}	0.0	6.0	22×10^{2}	0.44×10^{-5}	0.0	0.0	1×10^{2}	0.02×10^{-5}
6	3.6×10^{8}	0.0	6.0	3.5×10^{2}	0.97×10^{-6}	13×10^{3}	3.6×10^{-5}	5×10^{2}	1.38×10^{-6}
8	9.6×10^{8}	0.0	0.0	6.6×10^{3}	0.68×10^{-6}	28×10^{3}	2.9×10^{-5}	13×10^{2}	1.85×10^{-6}
10	13.5×10^{9}	4×10^{2}	03×10^{-7}	90×10^{3}	6.66×10^{-6}	50×10^{3}	3.67×10^{-6}	15×10^{2}	1.11×10^{-7}
12	15×10^{9}	3×10^{3}	0.2×10^{-8}	90×10^{3}	6×10^{-6}	5.5×10^{4}	0.36×10^{-5}	23×10^{2}	1.53×10^{-7}

The results obtained from the fusion between ESA-12 *Serratia* strains with the following *Psendomonas fluorescence*; NRRL-B 514, NRRL-B 99, NRRL-B 694, and NRRL-B 51, using lysozyme and polyethylene glycal at different incubation times 2, 4, 6, 8, 10 and 12 hours showed high efficiency in fusing which taken a place between ESA-12 and NRRL-B 99 *Pseudomonas* strains, in contrast to the number of fused cell obtained from ESA-12 with NRRL-B 514 which also formed a decrease number of fused cell and fused frequency in all strains number incubated for 4 hour but all other strains didn't give any transformation at incubation time of two hours. This agreed with the results obtained by Abdel-Salam (2007), who found that twelve hybrid fusants were successful and isolated fifty colonies.

Chitinase activity (Table 9) produced by different fusants compared with the parental strains; ESA-4, ESA-12, NRRL-B 514, NRRL-B 99, NRRL-B 694, and NRRL-B 51 appeared a significant variation in the activity of chitinase and the same results were also obtained in antibiotic production by fused bacteria in relation to the control.

Table 9: Evaluation of bacterial strains and their fusants for antibiotic production and chitinase activity.

Fused strain		Antibiotic	Chitianse activity
Control		0.0	0.0
ESA. 4		0.0	5.92
ESA. 12		0.0	8.51
NRRL B 514		121.8	9.0
NRRL B. 99		127	10.0
NRRL B. 694		109.2	0.0
NRRL B 51		118.3	0.0
ESA. 4 NRRL. 514		78.3	6.63
ESA. 4 NRRL. 99		24.6	7.75
ESA.4 NRRL 964		32.6	8.33
ESA.4 NRRL 51		134.1	6.29
ESA. 12 NRRL 514		10.2	4.78
ESA. 12 NRRL 99		57.33	9.52
ESA. 12 NRRL 694		112.2	4.93
ESA. 12 NRRL 51		39.3	0.41
F. test		**	0
L.S.D	%1	34.1	0.97
	%5	42.5	1.33

Control = Petri dishes including antibiotics and chitinase activities without bacterial strains

*, ** = Significant at 0.05 and 0.01 probability levels, respectively

The results indicated that while the parental strains; *P. aeruginosa* and *P. fluorescence* induced a clear holas, all eight fusants showed clear holas greater than that of the parental strains, all lysates indicating different level of gene expression among fusants. Evaluation of the fusants to producing antinematodes agent named antienthlement agent into growth medium by assaying chitinase enzyme activity into growth medium via titration method depending on the concentration of N acetyl (Zhang *et al.* 2001).

These results agreed with Abdel-Salam (2007), who determined the capacity of both parents and their fusants to inhibit the growth of *fusarum*, where the presence of hals surrounding a colony is an indication of antifungal compounds.

The results obtained in Table (10) appeared that the recombinant isolates affect to decreased the number of nematode larve when treated with bacterial strains if compared with the control. Significant variations were also obtained among all the fused isolates, as well as, the parental strains. This indicated that fusants caused nematode disease leading to decreasing the larval population.

Table 10: Nematocidial effect of different bacterial strains against nematode.

Treatment		Total number of larva / kg
Cont		1000
ESA 4		43.7
ESA 12		45.7
NRRL. B 514		26.7
NRRL. B 99		44
NRRL. B 694		17
NRRL. B 51		48.3
ESA-4 NRRL 51		23
ESA-4 NRRL 99		43
ESA-4 NRRL. 694		20
ESA-4 NRRL 51		63
ESA-12 NRRL 514		49
ESA-12 NRRL. 99		39
ESA-12 NRRL 694		46
ESA-12 NRRL 51		35
F. test		**
L.S.D	0.05	14.8
	0.05	16.7

Cont = Petri dishes including nematode larva without any bacterial treatment ** = Significant at 0.01 probability level.

These results agreed with Bacher *el al.* (1988), who found that about 1% of more than 5000 bacterial strains isolated from rhizosphere produced chemical compounds that affect the viability of *M incagnita* juveniles and that 20% of there significantly reduced the number of larva in the rhizosphere of cucumber roots, when applied as seed treatment. These results showed that the effect was greater on *Meloidogyne* than on *R. similes*. The results obtained were related to the reduction of nematode invation of root system first inoculated with bacterial strains which led to the reduction of nematode number in plant and demonstrated a storage repellant effect on *Meloidogyne* spp.

Nematocidial Efficiency of Transfusants Against Nematodes:

The results obtained from specificity tests using different bacterial fusants against the infection of cucumber plants with nematode larva grown in pots under green house condition were presented in Table (11). By direct inoculation with a constant number from nematodes larve juvenile, the results showed a different abilities of bacterial agents in controlling the plant parasite nematodes *Meloidogyne*, which infect cucumber plants under open door and out door. The results appeared a significant variation in shoot length between all treatments but didn't affect on the growth of cucumber plants at 28 days plant-old after treatment, as well as, on galling root knot nematodes.

Table 11: Nematocidial effect of different bacterial isolates on growth parameters of cucumber plants.

Treatment		Shoot Length	Shoot DW (g)	Root DW (g)	Total No Galls	Larva number	Egg Number
Control (+)		13.58	7.61	2.96	0.0	0.0	1000
Control (-)		15.41	13	3.6	3	800	90
ESA 4		14.16	7.65	2.8	10	90	50
ESA 12		16.08	12.45	4.5	2	26	39
ESA-4 NRRL 514		15.91	18	3.30	0.0	44	76
ESA-4 NRRL 99		14.5	10.92	3.96	18	17	21
ESA-4 NRRL 694		17.16	7.82	3.21	9	48	43
ESA-4 NRRL 88		14.6	12.32	4.46	0.4	43	21
ESA-12 NRRL. 514		18.16	17.07	3.17	0.0	20	11
ESA-12 NRRL. 99		18.58	12.53	4.8	0.0	63	29
ESA-12 NRRL 694		16.11	11.34	2.84	0.8	48	20
ESA-12 NRRL 51		13.91	11.83	3.76	0.0	50	53
F. test		**	**	**	NS	0	NS
L.S.D	0.05	0.98	1.3	0.46	0.0	8.3	0.0
	0.01	1.63	1.64	0.83	0.0	16.7	0.0

+, - = Plants grown in infected soil with nematode larva and in autoclaved soil, respectively
NS, *, ** = Insignificant and significant at 0.05 and 0.01 probability levels, respectively

Significant differences were obtained between the fusants concerning the decrease of larva number compared with positive and negative control. These results agreed with Andreoglou *et al*. (2003), who found that *Pseudomans anyzbatitans* culture filtrates contain compounds that inhibit hatching of root knot nematodes in vitro, Aslo, *P. orgzhabitans* cells decrease the number of female nematodes and egg masses when applied to soil at the time of nematode inoculation further demonstrating that *P. argzhabitans* produces metabolites used as a biological agent against plant- parasite nematodes.

Fusants obtained between *Serratia* and *Pseudomonas* produced chitinase enzyme which was antinematode agents leading to reducing a total number of nematodes larva in the soils. Fused bacteria were replated in a complete medium containing chitin substance and selection of recombinant isolates induced a visible marker green fluorescent and red pigment production, which indicating the ability of the recombinant fusants to hydrolysis chitin on mineral medium supplemented with collider chitin.

DNA Fingerprinting of Different Bacterial Fusants:

The results presented in Table (12) showed a genetic distance was obtained after protoplast fusion between *Serratia merscens* (ESA-4 and ESA.12) with *Psoudomans* (NRRL-B 514, NRRL.B 99, NRRL.B 694 and NRRL.B. 51). The data indicated a number of bands suitable for determining similarity percentage. Molecular weight of bands was ranged between 127 up to 1557 bp as a high molecular weight for all strains. The type number of bands was ranged between 2 up to 18 bands. The molecular weight of bands for ESA-4 was ranged between 959 to 128 (bp) , NRRL-B 514 (1195 to 129), NRRL-B 99 (1496 to 604), NRRL-B 694 (1527 to 239), and NRRL-B 51 (1597 to 211). On the other hand the molecular weight of bands in fusants compared with the parental strains were as the following ESA-12 NRRL 514 (1527 to 242 bp), ESA-12 NRRL 99 (1096 to 178 bp), ESA-12 NRRL 694 (604 to 131 bp) and ESA-12 NRRL 51 (605 to 137 bp).

Table 12: RAPD-PCR fingerprinting of parental strains and their fusants.

DNA number px 174		ESA-4		NRRL.B 514		NRRL.B 99		NRRL.B 694		NRRL.B 51		ESA.12 NRRL 514		ESA.12 NRRL 99		ESA.12 NRRL 694		ESA12 NRRL 51	
BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W
2	1353	6	959	2	1195	1	1496	2	1527	1	1597	2	1527	1	1096	3	604	3	605
3	1078	7	751	3	950	2	1377	3	981	2	910	3	949	2	42	4	576	5	603
4	603	12	610	4	746	3	900	4	725	3	605	4	709	3	619	5	281	6	279
5	272	22	605	8	605	4	648	5	634	5	603	5	605	4	605	6	267	7	314
6	271	23	128	16	281	5	604	6	507		211	8	280	5	134	7	242	9	118
7	221			17	226			7	279			9	242	8	211	8	131	17	137
8	194			18	129			17	239					17	178	9			
9	118																		
10																			

BN = Band number MW = Molecular weight of bands (bp)

Table 12: Continued:

DNA number px 174		ESA-4		NRRL.B 514		NRRL.B 99		NRRL.B 694		NRRL.B 51		ESA.12 NRRL 514		ESA.12 NRRL 99		ESA.12 NRRL 694		ESA12 NRRL 51	
BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W	BN	M.W
7	1353	1	1294	4	1075	5	1377	2	1102	7	1592	2	1440	2	1634	7	1571	4	1172
8	1078	2	1070	5	939	6	1070	3	991	8	1132	3	109	3	1014	8	1184	5	1031
9	872	3	935	6	818	7	885	4	481	9	940	4	406	4	805	9	949	6	818
10	610	4	596	7	593	8	602	5	381	10	617	5	584	5	588	10	787	7	593
11	603			8	421	9	428	6	309	11	473	6	43	6	339	11	640	8	421
12	281			9	306	10	366	7	281	12	417	7	346			12	485	9	306
15	271					11	309	8	254	13	249	8	309			13	427	10	242
14	234					12	288	9	200	14	268	9	296			14	323		
								10	123	15	127	10	262						

The molecular weight of bands was as the following; in ESA-12 was ranged between 1294 to 596 (bp), NRRL-B 514 was ranged between 1075 to 306, NRRL-B 99 was ranged between 1377 to 288, NRRL-B 694 was ranged between 1102 to 123 (bp) and NRRL-B51 was ranged between 1592 to 127 (bp). The fusant isolates obtained from these parents were as the following; ESA-12 NRRL 514 (ranged between 1440 to 262 bp), ESA-12 NRRL 99 (ranged between 1639 to 339 bp), which distributed to five bands, as well as, ESA-12 NRRL 694 (ranged between 1571 to 323 bp) which distributed to eight bands, ESA-12 NRRL 51 (ranged between 1172 to 242 bp) which distributed to seven bands.

RAPD-PCR Fingerprinting of Fusants Between Serratia Merscenes and Pseudomonas Strains:

The results shown in Figures 1 and 2 appeared a higher total number of bands ranged between five to eleven bands. Most strains showed a high matching or common bands among all strains used herein.

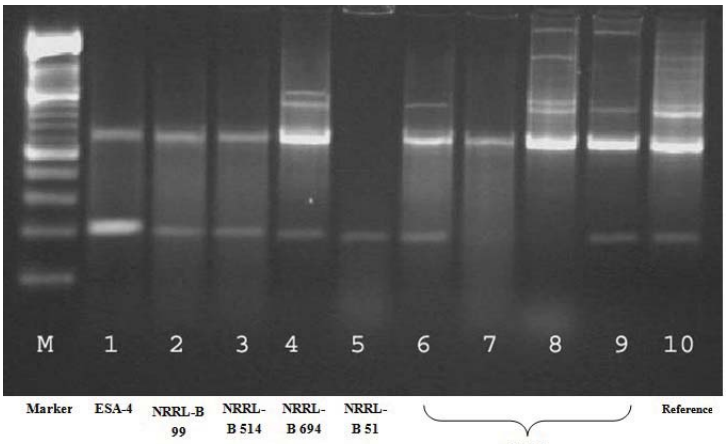

Fig. 1: RAPD-PCR. analysis of genomic DNA for the fusion between *Serratia* ESA-4 and the four *Pseudomonas* strains.

Notes : (M) Marker represented the marker used (Phage ϕ 174 Ban III).

Parental strain *Seratia marcescens* ESA-4. Parental strain NRRL-B 99.
Parental strain NRRL-B 514. Parental strain NRRL-B 694.
Parental strain NRRL-B 51. Fused strain ESA-4 NRRL 99.
Fused strain ESA-4 NRRL 514. Fused strain ESA-4 NRRL 694.
Fused strain ESA-4 NRRL 51. Reference Genomic DNA.

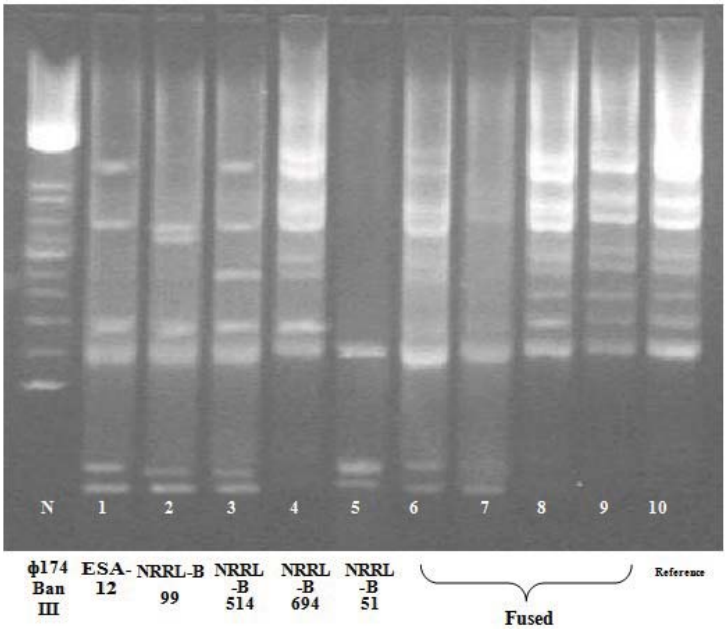

Fig. 2: RAPD-PCR. analysis of genomic DNA for the fusion between *Serratia* ESA-12 and the four *Pseudomonas* strains.

Notes. (N) Marker represented the marker used (Phage ϕ174 Ban III).

Parental strain *Seriatia marcescens* ESA-12. Parental strain NRRL-B 99.
Parental strain NRRL-B 514. Parental strain NRRL-B 694.
Parental strain NRRL-B 51. Fused strain ESA-12 NRRL 99.
Fused strain ESA-12 NRRL 514. Fused strain ESA-12 NRRL 694.
Fused strain ESA-12 NRRL 51. Reference Genomic DNA.

Protein Fingerprinting:

Electrophoresis separation of straved bacterial strains was done by extraction of total protein according to Laemmli 1970. This is a more sensitive methods for identification of bacteria.. Elias and Schneider (1992) suggested that SDS-PAGE was used because these methods evaluate the need for cultivating sample to be analyzed in more direct manner. Many samples can be analyzed at the same time, it is also cheaper than DNA fingerprinting. Moreover, the results obtained by SDS-PAGE of whole cell protein can be discriminated at match the same level as DNA fingerprinting in some cases. Results in figures 3, 4, 5 and 6 illustrated protein banding patterns as biochemical fingerprinting of different fusants obtained from protoplast fusion protocols. The similarity between different parental strains; *Serratia merscenes* ESA-4 and *Serratia merscenes* ESA-12 which fused with the following *Pseudomonas* strains NRRL-B 614, NRRL-B 99, NRRL-B 51 was determined. The results indicated high similarity between most of parental strains and their fusants obtained from protoplasting. The electrophoresis separation of total protein give a total numbers of main bans ranged between 6-10 bands in all isolates. The most frequent bands in all fusants were ranged between high molecular weight 102.3 K.D. and low molecular weight 27.3 K.D. The other bands could be representing 50% of the parental and fusant isolates distributed randomly. On the other hand, two specific bands in different strains named ESA-4 and NRRL-B 99 were found between both of them, which have a molecular weight 1.1-2 K.D, another specific band was found in recombinant ESA-4 NRRL 514, ESA-4 NRRL 99 and ESA-4 NRRL 964. But the following strains have a bands molecular weight 13.8 K.D and 13.3, these strains are ESA-4 NRRL 51 and ESA-4 NRRL 99, all of them containing most of bands found in each other.

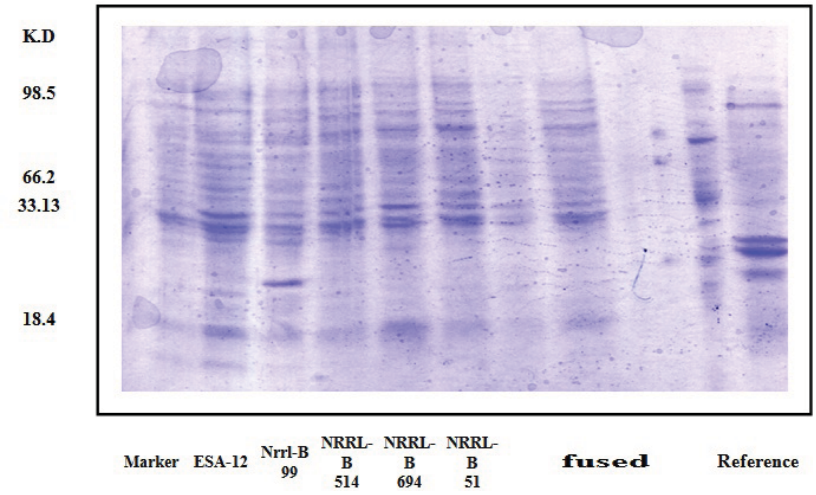

Fig. 3: Protein fingerprinting of Serratia ESA-4 and four Pseudomonas strains

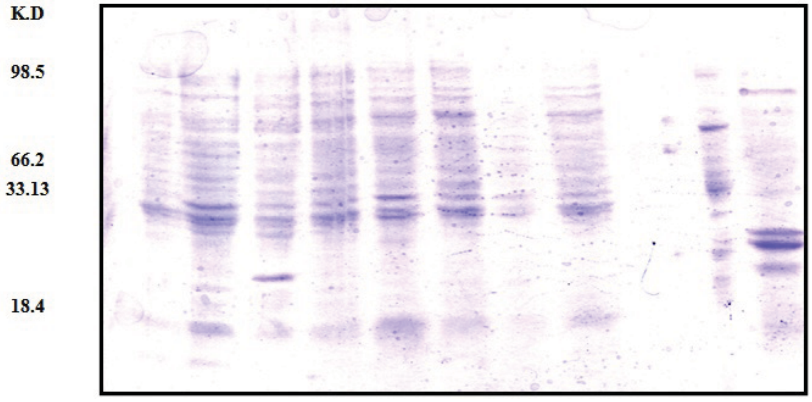

Fig. 4: Typical copy of Fig 10 used for more declaration of the above figures

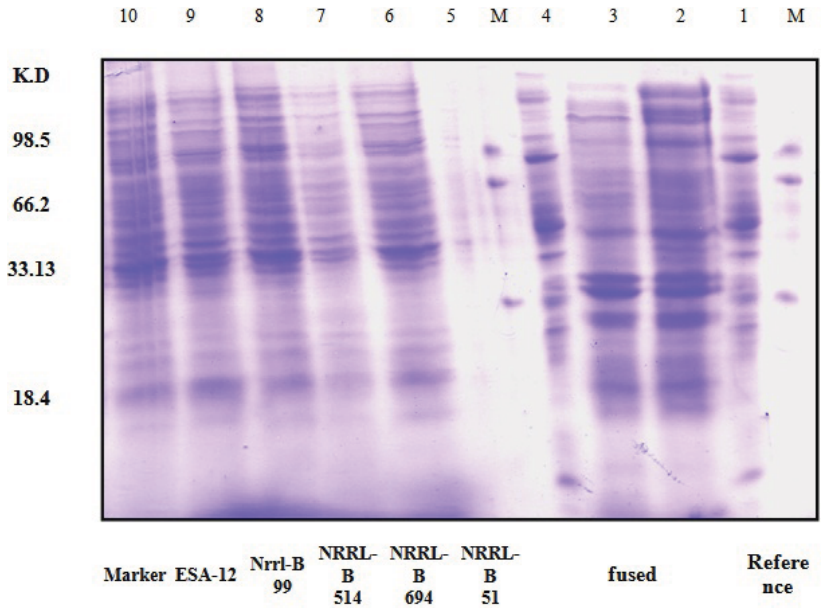

Fig. 5: Protein fingerprinting of Serratia ESA-12 and four Pseudomonas strains

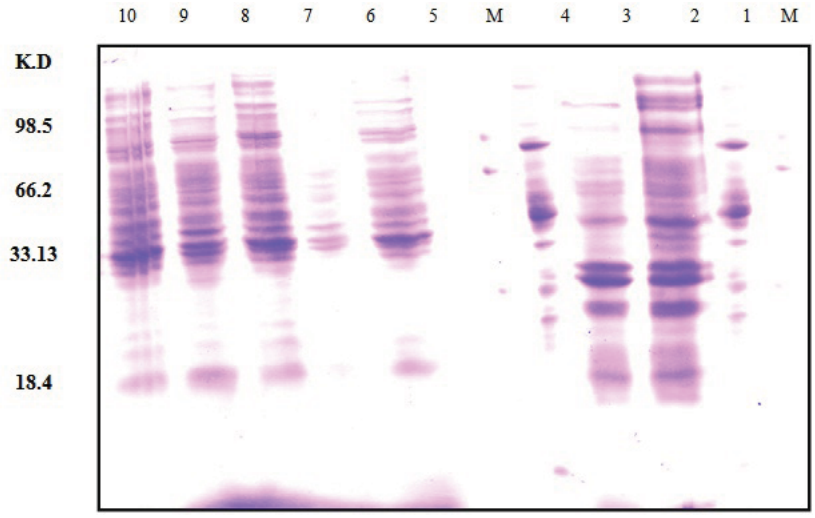

Fig. 6: Typical copy of Fig 12 used for more declaration of the above figures

The results obtained herein appeared that the molecular weight of band was ranged between 150 KD to 12.5 K.D which obtained in fusants ESA-12 NRRL 99, as well as, strain ESA 12 and their fusants ESA-12 NRRL 514, ESA-12 NRRL 99 and ESA-12 NRRL 964 which contain three common band that have a molecular weight 75.3 K.D, but the fusant named ESA-12 NRRL 964 have a three specific bands with a molecular weight 13.5, 12.3 and 4.3 K.D. These bands refer to fusant process, therefore it can be used as a molecular marker for the isolates carrier these bands. The data obtained in both Figures are in harmony with the results obtained by Ibrahim *et al.* (2000), who studied the protein banding patterns of *S. fibuliera* , *S. cerevisiae* and the modified *S. cerevisiea* strains. These results could be illustrated by the fact that there are high similarity of genomes belong to the same genetic groups. Therefore, it could be suggested that isolates included one master genome. The success of fusants formation between *Pseudomonas* and *Serratia* was proved to develop new genetically stable strains with high efficiency in producing chitinase.

The present study demonstrated that *Serratia* and *Pseudomonas* strains could be easily differentiated by RAPD fingerprinting, thus supporting the validity of this fast and accurate technique for studying diversity of *Serratia* and *Pseudomonas* population. The results obtained herein are in agreement with Sikora *et al.* (1997), who found that RAPD is a very discriminative and efficient method for differentiating and studying genetic diversity of *B. Japonica* strains. In addition modern techniques apply the polymerase chain reaction (PCR) method for strain characterization by amplifying DNA region between specific primer molecules. Differences between particular isolates as shown in this study revealed a DNA polymorphisms resulting from the amplification of different segments of genomic DNA using short oligonucleotide primers of arbitrary sequence. Salamone and Wodzinski (1997) revealed that purification of the extracellular metalloprotease (SMP 6.1) by fractionation and preparative iso-electric focusing showed a single isoform of the protease produced by a soil isolate of *Serratia marcescens* NRRL-B-23112. Iso-electric focusing of *Serratia* peptidase (SIGMA) clearly reveals two distinct bands that have iso-electric points differ from that of SMP 6.1 Comparison of published data (Salamone and Wodzinski, 1997) with those obtained in this study indicates some similarities, including different number of bands between fusants if compared with their parental wild type isolates. Therefore these results suggested that protein polymorphism of *Serratia* isolates can be described essentially to genetic variation at a structural gene locus The present results are in accordance with those reported by Treshow and Anderson (1989), who found that human protein E (APO E) has been recognized by one- or two-dimensional electrophoresis analysis of normal plasma to consist of several bands easily differentiated by RAPD-PCR fingerprinting thus supporting the validity of this fast and accurate technique in studying diversity of *Serratia* population.

In conclusion, cucumber plants play an important role in agriculture of vegetable crop which is high sensitive crop to infection by plant parasitic nematodes. Egyptian soil more effected by nematodes leading to high losses from crop yield. To improving nematocidial effect of biocontrol agents protoplast fusion was done between two *Serratia* and four *Pseudomonas* strains, from which eight isolates were obtained. All the resulted fusants induced high levels of mortality against nematodes because of their efficient in producing chitinase and bacteriocin. In addition, randomly amplified polymorphic (RAPD) DNA analysis can be successfully applied to identification of various bacterial strains and of their fusants. Using this technique, reproducible and characteristic fingerprints of complexes genomes can be obtained without any previous knowledge about the genomes being studied. Differences between particular strains and their fusants were obtained on the bases of DNA polymorphisms resulted from the amplification of DNA.

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