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EFFICIENCY OF BIOLOGICAL CONTROL OF ROOT-KNOT NEMATODES IN INFECTED GRAPEVINES SEEDLING BY GENETIC IMPROVED BACTERIA

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Abstract

Root-knot nematodes, *Meloidogyne* spp cause several losses in agriculture world-wide. However, these losses have not been clearly estimated for grapevines in Egypt. The risk to humans and environments presented by using chemical nematicides emphasizes the need for alternative tools. Rhizosphere bacteria have been reported as eco-friendly approach biological control to be effective in improving plant growth and affect nematodes reproduction. Protoplast fusion technique has been utilized as helpful tools for developing more powerful bacterial strains that combine all desired properties in one organism. The present study was conducted to increasing efficiency of biological control of root-knot nematode; *M. incognita* infecting grapevines as nematicidal by using protoplast fusion technique. An attempt has been made to combine between two bio-agent *Bacillus thuringienesis* I977 as a biocontrol and *Pseudomonas aeruginosa* as inducer plant growth. Under laboratory condition, 10 fusants evaluated against *M. incognita* J_2 appearing their efficiency as nematicidal compared to parents and control. The percentages of mortality J_2 after 48 h of exposure were 83% and 84 by *B. thuringienesis* and *P. aeruginosa* suspensions, respectively, whereas the percentages of mortality caused by four fusants were achieved 100% compared with parental and control. Also, three fusants F36, F45 and F 46 and its parental were evaluated to control root-knot nematodes on grapevine seedling under greenhouse conditions. F45 was more effective on *M. incognita* nematode-related parameters in Superior and Thompson seedling as compared to the parents and untreated control. While F46 achieved the best reduction in Flame seedling. All fusant recorded significant ($P \leq 0.05$) increase in length, fresh and dry weight of shoot, also, length and fresh weight of root and decreased the nematode-related parameters as compared their the parent and untreated control. Generally, all fusant were achieved reduction on nematodes parameters and improved grapevine seedling growth.

Key words: Biological control, *Meloidogyne incognita* Protoplast fusion, Grapevines.

Introduction

Grapevine (*Vitis vinifera* L.) is considered as one of the most important and favorable fruit crops in Egypt. The production of grapes affected by several problems such as nematodes (Mervat, *et al*., 2012; Nicol, *et al*., 1999; El-Hady, *et al*., 2015). At the world scale, plant-parasitic nematodes continue to be one of the major problems affecting on grapevines with damage normally reflected in lower production and in some cases total crop loss (Nicol, *et al*., 1999). Estimates that plant-parasitic nematodes cause losses of US$157 billion in agriculture world-wide; however, these losses have not been clearly estimated for grapevines in Egypt since damage caused by nematodes varies depending on many factors, such as vine cultivar, climate, soil type, and crop management among others (Ferris and McKenry, 1975). The world estimated losses due to nematode damage ranges from 12.5% to 20.0% of vine growth and productivity (Kesba, 1999; Anwar and McKenry, 2000).

Some table grape cultivars, such as Red Globe and Flame Seedless, are also very sensitive to root-knot nematodes, which are frequently associated with the fungi responsible for black-foot disease and grapevine decline (Scheck, *et al*., 1998). In Egypt, severe nematode infestations forced grape growers to uproot their grapevines and obliged them to let the land lie fallow for

many years before replanting.

M. incognita and *M. javanica* cause significant economic losses to grapevines grown in sandy soil under the mild temperature conditions that prevail in most grapevine growing areas of the Mediterranean Basin (Nicol, *et al.*, 1999). Also, El-Gendy and Shawky, (2006) reported that *M. incognita* causes. The high cost of conventional nematicides; withdrawal of nematicides from the market due to detrimental side effects such as residual effect, contamination of groundwater, and their harmful effects on nontarget species, including humans (Abawi and Widmer, 2000). Biological control is one of as eco-friendly approaches the alternative tools to control plant nematodes. Rhizosphere bacteria, have been reported to be effective in improving plant growth and affect nematodes reproduction through different mechanisms. Plant Growth- Promoting rhizobacteria belonging to *Pseudomonas* and *Bacillus* spp. has also been used in bio-antagonism (Adesemoye, *et al.*, 2008; Aksoy and Mennan, 2004; Dey, *et al.*, 2004; Soliman, *et al.*, 2017). The genus *Pseudomonas* is one of the most diverse Gram negative bacterial genera, isolated from many sources. *P. aeruginosa* strain acting as plant growth-promoting rhizobacteria (PGPR) (Udai, *et al.*, 2003). Strains of the genus *Pseudomonas* have the ability to suppress a range of plant disease caused by soil born plant pathogenic including plant-parasitic nematodes.

B. thuringiensis can produce toxic compounds of various chemical structures and properties. *B. thuringiensis* have the ability to production chitinase enzyme (Abd – El-Bary, *et al.*, 2007). The effects of *B. thuringiensis* as nematicidal biocontrol agent have been investigated for controlling plant-parasitic nematode. On the other hand, mixtures of biocontrol agents may be useful for bio-control of different plant pathogens compared a single bio-agent, but the antagonistic between different bacterial species reduce its effect. To overcome the inconsistent results of the biological control under field conditions, we are used protoplast fusion.

Protoplast fusion, scientists could improve the activity of such microorganisms (Rygielska, 2004; Soliman, *et al.*, (2018) found that fusants between *B. licheniformis* and *P. aeruginosa* exhibited increase in their nematicidal activity than their parents against root-knot nematode, *M. incognita* J_2, compared with parental and untreated control. All the tested fusants exhibited an increase in their nematicidal activity than their parents against root-knot nematode, *M. incognita* J_2, under laboratory and greenhouse conditions. Yari, *et al.*, (2002) reported that the concentration of δ-endotoxin of *B. thuringiensis* fusants were 1.48 times more toxic than the wild type. El-Nagdi, *et al.*, (2019).

El-Hamshary, *et al.*, (2006) found that, the fusant strain between *P. fluorescens* and *P. aeruginosa* was more effective than their parental strains in reducing different nematode parameters as well as enhanced plant growth.

Protoplast fusion can be applied to further improve the strains that have prized qualities by creating one strain that combine multiple mechanisms of action as biocontrol and plant growth-promoting.

Material and Methods

Bacterial strains

Two bacterial strains, were isolated from Egyptian soil and it's had nematicidal activity against nematode, *Pseudomonas aeruginosa* (Pa) (GenBank under accession number (LC215048) and *Bacillus thuringiensis* I977 (Bt). Bacterial strains were used in this study were obtained from of Plant Pathology Department and Microbial Genetic Department, National Research Centre, Cairo, Egypt and the two strains were used as parents in protoplast fusion experiment.

Protoplast fusion experiments

Antimicrobial susceptibility

Eleven antibiotics were used with final concentrations as follows: Rifampicin (Rif) 100 µg/ml, ampicillin (Amp) 100 µg/ml, amikacine (Amk) 30 µg/ml, streptomycin (Sm) 200 µg/ml, kanamycin (km) 40 µg/ml, tetracycline (Tc) 15 µg/ml, chloramephincol (cm) 35 µg/ml, gentamycin (Gm) 15 µg/ml, polymyxin (Pmx) 50 µg/ml, neomycin (Nm) 40 µg/ml and erythromycin (Erm) 20 µg/ml. the Kirby- Bauer disc diffusion method for antimicrobial susceptibility test was used (NCCLS, 1999).

Growth conditions and protoplast formation

The method of protoplast formation, regeneration and fusion were performed according to (El-Gaali, *et al.*, 1995). *B. thuringiensis* and *P. aeruginosa* strains were incubated Luria Broth medium was prepared of tryptone 10 g, Yeast Extract 5 g, NaCl 5 g, and agar 20 g up to 1000 ml of distilled water according to (Davis, *et al.*, 1980) at 30°C for 24 h with shaking at 120 rpm. The cells were harvested by centrifugation at (3913×g) for 10 min and washed with 1% N-laurylsarcosine, followed by washing three times with osmotic stabilizer buffer (30 mMTris-HCl buffer, pH 7.5 and 0.6 M MgSO4). Lysozyme was dissolved in osmotic stabilizer buffer and was added at a final concentration of 4 mg/ml. The cell suspensions were incubated at 37°C for 4 h. Protoplast

formation was confirmed by staining with methylene blue and then observed under light microscope. The viable cells were determined by spreading onto LB medium solidified by adding 2% agar where all protoplasts lysed and only the intact cells will grow after incubation.

Protoplast regeneration and fusion

The protoplasts in the mixture were collected by centrifugation at (3913×g) for 10 min. The precipitate was washed with Tris-HCl buffer with an osmotic stabilizer and the resulting precipitate was re-suspended in the same buffer to allow all protoplasts to regenerate. Protoplast suspension was diluted and overlaid on the LB medium solidified by adding 2% agar and the colonies were counted after 2-5 days. Aliquots (1.0 ml each) of the two parental protoplasts were mixed in the presence of 25% PEG 6000 and 100 mM $CaCl_2$ (Hopwood, *et al.,* 1985) and incubated at 30°C. Aliquots of 100 µl from the mixture were taken every 10 min and added to selective agar medium to screen the fusants.

SDS–PAGE protein analysis

The parental and fusion products strains were grown in suspensions following the method of (Von Tersch and Gonzalez, 1995). One hundred millilitres of nutrient broth in 500 ml flasks was inoculated with 1 loopful of bacteria and shaken for 3 days at 30°C (220 rpm). The suspension was centrifuged at (15 652×g) for10 min at 4°C, and the pellet was washed 2 times with high salt TNT-1 buffer (50 mM Tris–HCl (pH 7.5), 1.0 M NaCl, and 0.05% Triton X-100) followed by 2 washes with TNT-2 buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.05% Triton X-100). An aliquot was solubilized by heating in Laemmli buffer (10% glycerol, 5% β-mercaptoethanol, 1% SDS, 0.188 M Tris–HCl (pH 6.8), and 0.01% (v/v) bromophenol blue) at 100°C for 5 min. The aliquot was centrifuged at (26 452×g) for 5 min and the supernatant containing solubilized proteins was size fractionated by 12.5% SDS–PAGE, according to the method of (Laemmli, 1970), to compare the products secreted by the parental strains and those secreted by fusant strains. After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye. The protein marker (Biomatik Corp, (Wilmington, USA) with nine molecular weight bands (kDa). The gels were scanned using Gel Doc 2000 system, and molecular masses were determined using Total Lab version 1.10 software based on protein marker purchased from Biomatik Corporation (Wilmington, Delaware, USA).

Preparation of the root-knot nematode (*M. incognita*) culture

Pure culture of *M. incognita* was propagated on eggplant from stock of the nematode species. Newly hatched second-stage juveniles (J_2) from this culture were used as inoculum. Perennial patterns of adult females from eggplant roots were used to confirm the nematode species as described by (Taylor and Netscher, 1974). Liquid culture of strains was prepared on Luria broth (LB).

laboratory experiments

Nematicidal activity of *B. thuringiensis* and *P. aeruginosa* and their fusants on *M. incognita* J_2 mortality.

For bioassay test, *M. incognita* eggs were extracted from the infected tomato roots that carry egg masses (Hussey and Barker, 1973). To provide *M. incognita* J_2 the hatching eggs in plastic cups at laboratory temperature for 72 h. 1 ml of the freshly hatched surface-sterilized juvenile suspension (45 ± 5 juveniles/ml) with 1 ml of the culture suspensions were transferred to test tubes. Juveniles kept in 1 ml served as controls.

All test tubes were kept in incubator at 35°C. Test tubes were loosely covered to permit aeration and lessen evaporation. Number dead was counted after 24 h for 2 days using 1ml nematode counting slide. After the exposure periods, the nematodes in each treatment were transferred to distilled water and left for 24h to observe whether immobile nematodes resumed activity or not. The corrected percentages of nematode mortality were calculated according to the following equation: mortality (%) = (m–n)/ (100–n) ×100, where m and n indicate the percentages of mortality in treatments and control, respectively (Abbott, 1925).

Greenhouse experiments

During February 2018, roots of seedling grapevine cultivars were trimmed, and then dipped in fungicide (Rizolex T 50% WB). Also, scion of the seedling cultivars ones were trimmed and left only 2 buds of each. Seedlings were transplanted in plastic pots filled with virgin sandy soil mixed with Peatmoss 2:1 (V/V) 12.5 kg. After four months (during June), 5 vines (one vine/pot) the uniform of each grapevine variety seedling were selected. All vines were inoculated with the infective juvenile stage of *M. incognita* (2000 J_2/ pot). After one week adding 5 ml of bacterial suspension, one ml containing about 2 x10^7cfu/ ml. The vines were irrigated every 2-3 days with a compound fertilizer (19:19:19) at rate 2g/l (one liter). After (J_2) inoculation, nematode and vine growth.

Parameters were determined as following

Determination of vine parameters

Vine growth parameters *i.e.* vine height (cm), root

Table 1: Antibiotic resistance patterns of *B. thuringiensis* and *P. aeruginosa* strains.

Gm	Pmx	Nm	Amp	Rif	Erm	Sm	Cm	Amk	Km	Tc	Strain
-	+	+	+	+	-	-	+	+	-	+	Bt
-	+	+	-	+	-	+	+	+	-	+	Pa

+, Resistant -, Sensitive
Bt, *Bacillus thuringienesis*; Pa, *Pseudomonas aeruginosa*.

length (cm), vine fresh weight (gm), root fresh weight (gm) and vine dry weight (gm) were recorded during October 2018.

Chemical analysis

For the determination of nutrient content and chlorophyll a &b contents, samples from 5-7th nodes (Shawky, *et al.,* 1996) from vine top were picked then mixed together as a composite for carrying out the following chemical analysis:

Nitrogen and phosphorus in leaves were calorimetrically determined according to the methods described by **((**Bremner and Mulvaney, 1982), and (Olsen and Sommers, 1982), respectively. Potassium was determined flame photometrically according to the method advocated by (Jackson, 1970).

Chlorophyll a &b as mg/g were colormetrically determined in fresh leaf samples at wave length of 660 and 640 nm for a&b respectively (Wettestien, 1957).

Statistical methods

The data were subjected to analysis of variance and Duncan's multiple rang test was used to differentiate means at 5% (Duncan, 1955).

Results

Antibiotic resistance pattern of and *Bacillus thuringienesis* and *Pseudomonas aeruginosa*

The antibiotic resistance data of the *B. thuringiensis* **(**Bt) and *P. aeruginosa* (Pa) strains are presented in data in (Table 1) showed that two strains were resistant to Tetracycline (Tc^r), Amikacin (Amk^r), Chloramphenicol (Cm^r), Rifampicin (Rif^r), Neomycin (Nm^r) and Polymyxin (Pmx^r) respectively,While they are sensitive to three different antibiotics *i.e.* Gentamicin (Gm^s), Kanamycin (Kn^s) and Erythromycin (Erm^s) respectively. The strain (Bt) revealed antibiotic resistance pattern, where it was resistant to one antibiotics Ampicillin (Amp^r). While it is sensitive to one antibiotic Streptomyces (Sm^s). On the other hand Pa strain was resistance to Streptomyces (Sm^r), While it was sensitive to Ampicillin (Amp^s).

Protoplast fusion between *B. thuringiensis* and *P. aeruginosa*

Protoplast fusion technique used to produce genetically improvement microorganisms (new strains) can effect of biocontrol against plant parasitic nematodes and persistence for long time. Protoplast fusion technique used between the two selected parent strains. Protoplast formation of the two selected strains, Bt and Pa were tested periodically by microscopic examination Fig. 1.

Expression of the parental strains protein bands in their fusants

The SDS-PAGE protein banding patterns of the two parental strains; Bt and Pa and fusants are shown presented in Fig. 2. SDS-PAGE analysis of total proteins of the two parental strains, Bt and Pa revealed a total of 12 and 9 protein bands, respectively. Their molecular weights are ranged from 207 to 11kDa. While the 10 fusants showed variable number of protein bands ranged from 14 in one fusant to 19 in two fusants Table 2.

The 10 fusants revealed variable number of Bt protein bands. The highest number of Bt bands (10 bands) was displayed in 4 fusants while, the lowest number (7 bands) was revealed by F74. This represented a percentage ranged from 46.7% (F74) to 62.5% (F18). The total Bt expressed bands in the rest of the fusants were classified in descending order as follow: 8 bands in two fusants, 9 bands in two fusants, 7 bands in two fusants and 6 bands

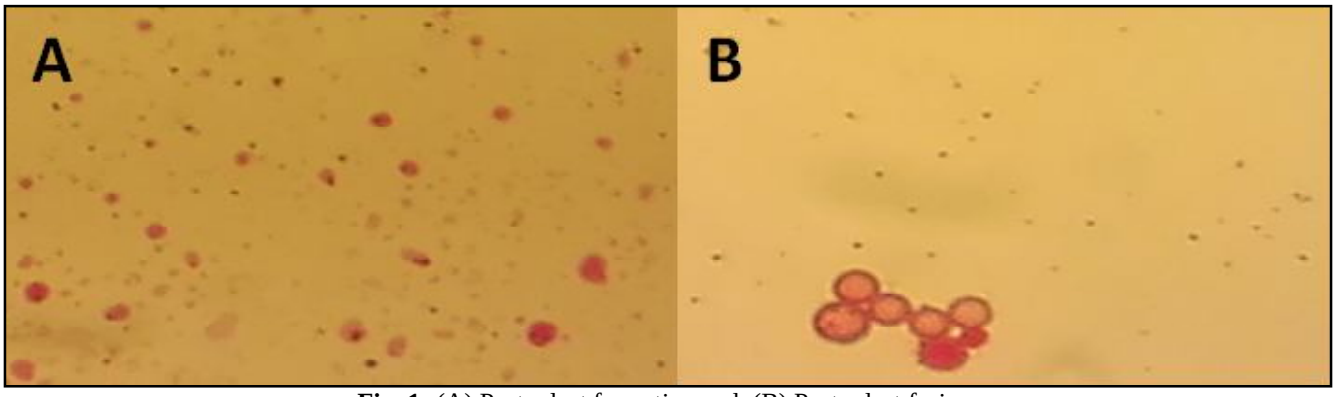

Fig. 1: (A) Protoplast formation and (B) Protoplast fusion.

in four fusants. On the other hand variable number of Pa protein bands as showed in fusants. The highest number of Pa bands (9 bands) was displayed in two fusants while, the lowest number (6 bands) was revealed by four fusants. This represented a percentage ranging from 53.3% (F74) to 37.5% (F18).

The 10 fusants were characterized based on the absence and presence of the 12 Bt protein bands. A total of 12 Bt protein bands with different molecular weights were expressed in all the 10 fusants. The presence of the remaining 12 bands was distributed in descending order as follows: one band with molecular weights of 51 kDa and three bands was molecular weights 189, 64 and 23kDa in 6 fusants and two bands with molecular weights 19 and 11kDaexisted in 7 fusants, three bands with molecular weights of 148, 33 and 29 kDa in 8 fusants, two bands with molecular weights of 45 and 15kDa in one fusant and one band with molecular weights of 88 kDa in all fusants respectively.

The 10 fusants were characterized based on the absence or presence of the expression of the 9 Pa protein bands. A total of 9 protein bands with different molecular weights were found in all fusants. The presence of the remaining 4 bands with molecular weights of 73, 60, 39 and 21 kDa was distributed in descending order among the 9 fusants ,three bands with molecular weights of 207, 163 and 17 kDa were displayed in 8 fusants, while two bands with molecular weights of 117, 13kDa was detected in 6 fusants.

Table 2: SDS-PAGE analysis of total proteins of the two parental strains and their fusants.

Band No.	MW KDa MW	Parental strains Bt	Fusants Pa	F18	F29	F36	F45	F46	F50	F55	F74	F77	F82
1	207		•	•	•	•		•	•	•	•		•
2	189	+		+	+	+	+				+		+
3	163		•			•	•	•	•	•	•	•	•
4	148	+		+	+	+	+	+	+	+		+	
5	117		•	•		•	•	•				•	•
6	88	+		+	+	+	+	+	+	+	+	+	+
7	73		•		•	•	•	•	•	•	•	•	•
8	64	+		+		+		+	+	+			+
9	60		•	•	•	•	•	•	•	•	•	•	
10	51	+		+	+	+		+			+		
11	45	+			+	+	+	+	+	+	+	+	+
12	39		•	•	•	•	•	•	•	•	•	•	
13	33	+		+			+	+	+	+	+	+	+
14	29	+		+	+	+	+	+	+	+		+	
15	23	+					+		+	+	+	+	+
16	21		•	•	•	•	•	•	•	•	•		•
17	19	+		+	+	+	+	+			+		+
18	17		•			•	•	•	•	•	•	•	•
19	15	+		+	+	+	+	+	+	+		+	+
20	13		•	•	•	•	•	•			•		
21	11	+		+	+	+	+	+				+	+
Total no.of protein bands	12	9	16	15	19	18	19	15	15	15	14	15	
Numbers of Bt bandsexpressed in fusants			10	9	10	10	10	8	8	7	8	9	
Bt bands %			62.5	60	57.8	55.6	57.8	53.3	53.3	46.7	57.1	60	
Numbers of Pa bandsexpressed in fusants			6	6	9	8	9	7	7	8	6	6	
Pa bands (%)			37.5	40	42.2	44.4	42.2	46.7	46.7	53.3	42.9	40	

(+) Refers to presence of protein bands of *B. thuringienesis* (Bt).
(•) Refers to presence of protein bands of *P. aeruginosa* (Pa).

As illustrated in Table 3, the nematicidal effect of *P. aeruginosa* and *B. thuringienesis* as parental strains and 10 fusants to kill root-knot nematode, *M. incognita* second-stage juveniles (J_2), were investigated under laboratory conditions. The reduction in the movement was irreversible, and the mortality of the juveniles was confirmed when they were transferred to distilled water for 48h. All tested fusants had achieved nematicidal effect on *M. incognita* J2 as shown by the greater mortality percentage compared with the control (water). The superior fusant on mortality percentage of *M. incognita* J_2 were recorded in fuasnt no.(F18, F36, F45, F46) which achieved 100% in *M. incognita* J_2 mortality after exposure period 48 h compared to the two parental strains *B. thuringienesis* and *P. aeruginosa* induce 83% and 84% mortality, respectively and control. The increase in the juveniles mortality positively correlated with the length of the exposure period.

Greenhouse experiment

The nematicidal effect of *B. thuringiensis* and *P. aeruginosa* and three fusants *viz.*, F36, F45 and F46 on the root knot nematode, *M. incognita* infesting grapevine seedling (*Vitis vinifera* L.) *cv.* Superior under greenhouse condition the parental and its fusants adding to soil as soil drench after one week nematodes inoculation was recorded in Table 4. The obtained data showed that all treatments had the potentiality to reduce the root–knot nematode infectivity and reproduction, to a great extend

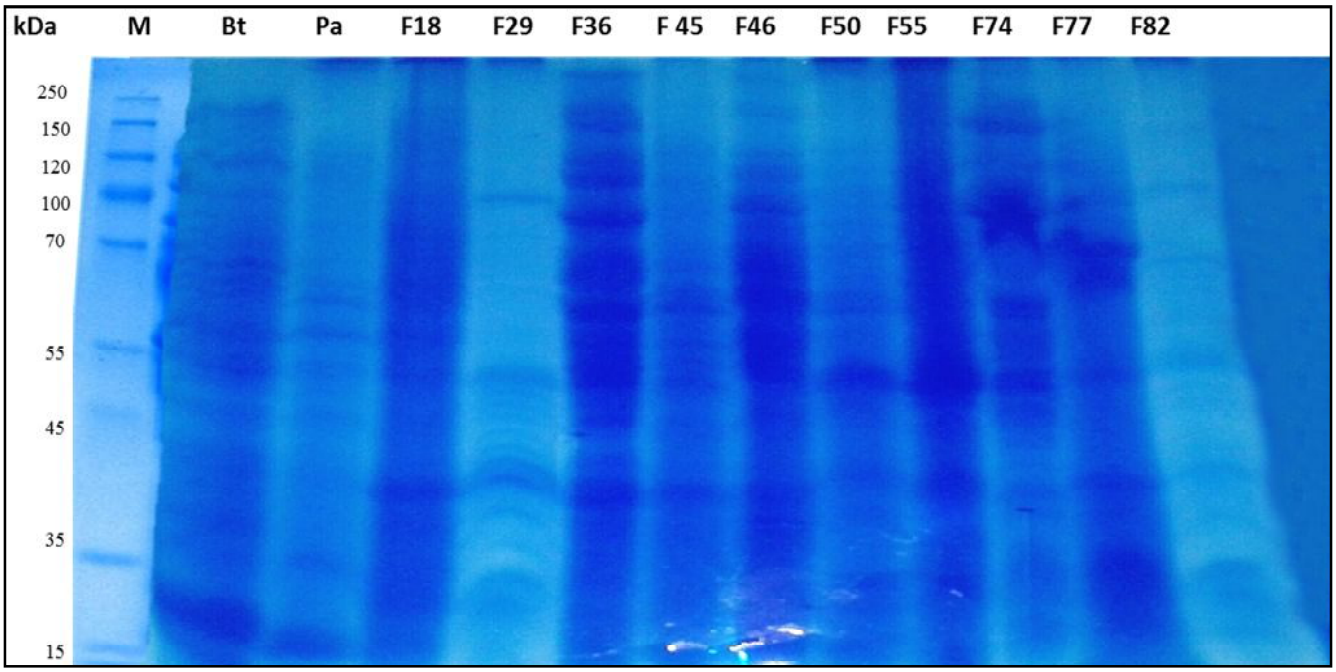

Fig. 2: SDS-PAGE protein profiles of two parental strains; *B. thuringiensis* (Bt) and *P. arugenosa* (Pa) and their 10 fusants. M refer to protein marker.

Table 3: Effect of *B. thurenginsis, P. aeruginosa*, parentals and their 10 fusants on *M. incognita* J_2 mortality under laboratory conditions.

Treatments	Mortality% of *M. incognita* J_2		% Recovery	% Net Mortality**
	24 h	48 h		
B. thurin gienesis	80.00*h	83.00f	0.00	83.00
P. aeru ginosa.	82.00g	84.00ef	0.00	84.00
F18	94.00a	100.00a	0.00	100.00
F29	85.00f	93.00bc	0.00	93.00
F36	95.00a	100.00a	0.00	100.00
F45	95.00a	100.00a	0.00	100.00
F46	92.00b	100.00a	0.00	100.00
F50	89.00cd	86.66d	0.00	86.66
F55	86.00ef	95.00b	0.00	95.00
F74	87.33de	92.00bc	0.00	92.00
F77	90.00c	95.00b	0.00	95.00
F82	86.00ef	90.00cd	0.00	90.00
Control (water)	1.00i	1.67g	0.00	1.67

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test. **Net mortality = % mortality after 48 h - % nematode recovery in distilled water.

as compared to parental and untreated control and significantly $P \leq 0.05$ suppressed J_2 in soil, J_2 in 10 gram/ root, root galls and egg masses /root system as compared to untreated control. The best percentages reduction achieved when applied F45 recorded 69.75%, 78.05%, 72.63% and 54.42% in No. of J_2 in soil, No. of J_2 /10g of root , no. of galls/root system and no. of egg- masses, respectively compared with their parental which was achieved the lowest reduction in all nematodes parameters compared to untreated control.

Nematicidal effect *B. thuringiensis* and *P. aeruginosa* and three fusants *viz.,* F36, F45 and F46 against root knot nematodes *M. incognita* galls and egg-masses formation on Flame seedling were recorded in Table 5. The obtained data showed that all treatments had the potentiality to reduce the root–knot nematode infectivity and reproduction, to a great extend as compared to untreated control and significantly $P \leq 0.05$ suppressed J_2 in soil, J_2 in ten gram/root, root galls and egg masses/root system as compared to untreated control. The best percentages reduction was achieved when applied F46 recorded74.96%, 65.81%, 74.44% and 59.51% in No. of J_2 in soil, No. of J_2/10g of root, no. of galls/root system and no. of egg- masses, respectively compared with their parental was achieved the lowest reduction in all nematodes parameters compared to untreated control.

The nematicidal effect of *B .thuringiensis* and *P. aeruginosa* and three fusants *viz.,* F36, F45 and F46 on the root knot nematode, *M. incognita* infesting grapevine seedling (*Vitis vinifera* L.) cv. Superior under greenhouse condition the parental and its fusants adding to soil as soil drench after one week nematodes inoculation Table 6. The obtained data showed that all treatments had the

Table 4: Effect of *B. thuringiensis* and *P. aeruginosa* parentals and their three fusants on *M. incognita* reproduction on Superior under greenhouse conditions.

Treatments	No. J_2 in Soil	R %	No. of J_2 in 10 root	R %	No. galls/ root system	R %	No. of eggmass	R %
F36	110.00* c	66.05	165.00d	49.70	111.00cd	65.74	13.33c	54.55
F45	98.00 c	69.75	72.00f	78.05	88.67d	72.63	13.67 c	54.42
F46	238.00b	26.54	123.33e	62..40	125.67c	61.21	14.00c	52.27
B. thuringiensis	309.67a	4.42	277.67b	15.34	276.67b	14.61	27.66a	5.69
P. aeruginosa	309.33a	4.53	205.00c	37.50	278.33b	14.10	23.00b	21.58
Untreated control	324.00a	---	328.00a	---	324.00a	---	29.33a	---

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's R%.=Reduction over untreated control.

Table 5: Effect of *B. thuringiensis* and *P. aeruginosa* parental and their three fusants on *M. incognita* reproduction on Flame seedling under greenhouse conditions.

Treatments	No. J_2 in Soil	R %	No. of J_2 in 10 root	R %	No. galls/ root system	R %	No. of eggmass	R %
F36	66.00*d	68.62	85.00d	45.16	138.00d	54.00	17.33c	57.03
F45	72.00c	65.66	80.00e	48.39	123.33d	58.89	16.33c	59.51
F46	52.67e	74.96	53.00f	65.81	76.67e	74.44	16.33c	59.51
B. thuringiensis	191.00b	8.19	115.00b	25.81	257.00b	14.33	22.33b	44.63
P. aeruginosa	189.33b	9.98	95.00	38.71	215.67c	28.11	25.33b	37.19
Untreated control	210.33a	---	155.00a	---	300.00a	---	40.33a	---

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's R%.=Reduction over untreated control

Table 6: Effect of *B. thuringiensis* and *P. aeruginosa* parental and their three fusants on *M. incognita* reproduction on Thompson seedling under greenhouse conditions.

Treatments	No. J_2 in Soil	R %	No. of J_2 in 10 root	R %	No. galls/ root system	R %	No. of eggmass	R %
F36	41.67*d	83.56	137.33d	50.90	76.00d	52.30	19.33cd	40.83
F45	45.33d	81.96	130.00d	53.52	64.00d	59.83	14.33d	56.14
F46	73.33c	70.82	155.00cd	44.58	77.00d	51.67	16.00cd	51.03
B. thuringiensis	124.00b	50.66	202.00b	27.77	74.00d	53.56	21.00bc	35.72
P. aeruginosa	106.33b	57.69	180.00bc	35.64	155.67a	2.30	25.67b	21.43
Untreated control	251.33a	---	279.67a	---	159.33a	---	32.67a	---

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's **R%.=Reduction over untreated control

potentiality to reduce the root–knot nematode infectivity and reproduction, to a great extend as compared to parental and untreated control and significantly Pd"0.05 suppressed. The best percentages reduction was achieved when applied F 45 recorded 53.52%, 59.83 % and 56.14% in no. of J_2 /10g of root , no. of galls/root system and no. of egg- masses, respectively with few expiation compared with their parental which achieved the lowest reduction in all nematodes parameters compared to untreated control.

Plant physical parameters

Data in Table 7 indicated that some plant physical parameters were positive significantly affected by different fusants and their parental strains treatments compared with seedling untreated control in all cultivars under study.

Superior seedless

Showed data in Table, (7a) seedling treatment with F36 recorded that highest value in plant high, plant fresh weight and plant dry weight (77.79, 123.10 and 100.43%), respectively compared with parental strains and untreated control. On the other hand, F46 was achieved 179.19% over untreated control. Generally, F 36 was achieved the best improving in Superior Seedless growth parameters with few exceptions. While F46 was achieved parental strains and he highest value of root length (179.19%) over untreated control. Concerning root fresh weight, the while all fuasnts treatments were gave a highly significant

Table 7: Effect of *B. thuringiensis* and *P. aeruginosa* parentals and their three fusants on some plant physical parameters on grapevine seedling cvs. Superior, Flame and Thompson infected with *M. incognita* under greenhouse conditions.

Treatments	Plant high (cm)	Inc. %	Plant fresh weight (gm)	Inc. %	Plant dry weight (gm)	Inc. %	Root length (cm)	Inc. %	Root fresh weight (gm)	R %
a) Superior Seedless										
F36	58.67* a	77.79	33.13 a	123.10	14.07 a	100.43	25.33 c	58.31	10.75 e	74.37
F45	53.00 b	60.61	23.16 c	55.96	11.08 c	57.83	29.00 bc	81.25	22.40 d	46.59
F46	52.67 b	59.61	25.51 b	71.78	12.13 c	72.79	34.67 a	179.19	22.74 d	45.78
B. thuringiensis	45.67 c	38.39	26.54 b	78.72	13.88 b	97.72	30.33 b	89.56	32.88 c	21.60
P. aeruginosa	42.33 c	28.27	18.7 d	25.94	9.23d	31.48	28.00 bc	75.00	37.03 b	11.71
Untreated control	33.00 d	---	14.85 e	---	7.02 e	---	16.00 d	---	41.94 a	---
b) Flame seedless										
F36	61.33 b	82.15	27.88 a	150.49	12.37 ab	108.95	30.33 c	62.45	19.27 d	67.78
F45	56.33 bc	67.30	22.51 b	102.25	10.05 cd	69.76	34.33 b	83.88	18.79 d	68.58
F46	85.33 a	153.43	26.42 a	137.38	13.38 a	126.01	42.33 a	126.73	9.71 e	83.76
B. thuringiensis	49.33 c	46.51	25.22 ab	126.59	11.45 bc	93.41	37.67 b	101.77	27.73 c	53.63
P. aeruginosa	36.00 d	6.92	16.70 c	50.04	8.55 d	44.43	23.00 d	23.19	39.36 b	34.18
Untreated control	33.67 d	---	11.13 d	---	5.920 e	---	18.67 e	---	59.80 a	---
c) Thompson seedless										
F36	70.67 b	37.68	57.84 a	66.06	21.51 ab	64.95	35.33 a	76.65	40.88 d	55.14
F45	82.00 a	59.75	54.07 ab	55.24	24.10 a	84.82	32.33 a	61.65	41.33 d	54.65
F46	55.67 d	8.46	46.34 bc	33.05	18.40 bc	41.10	25.00 b	25.00	45.65 d	49.91
B. thuringiensis	62.33 c	21.43	52.91 ab	51.91	17.84 bc	36.81	23.33 b	16.65	53.50 c	41.29
P. aeruginosa	55.00 d	7.15	41.43 cd	18.95	16.05 cd	23.08	23.67 b	18.35	64.32 b	29.42
Untreated control	51.33 d	---	34.83 d	---	13.04 d	---	20.00 b	---	91.13 a	---

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's. Inc.% =Increase over untreated control R%. =Reduction over untreated control.

value comparing with parental strains and untreated control.

Flame seedless

Data in Table (7b) stated that plant high, plant fresh weight, plant dry weight, and root fresh weight were increased with treated F46 was recorded (153.43, 137.38, 126.01 and 126.73), respectively compared with parental strains and untreated control.

Thompson seedless

Data are given in Table (7c) revealed that the plant physical parameters for seedling treated with F36 and F45 were gave the highest values compared to parental strains and untreated control.

Chlorophyll (a & b).

Data presented in Table 8, showed that effect of fusants and their parental strains treatments on leaves chlorophyll (a & b) contents of Superior, Flame and Thompson seedless cvs. It could be noticed that the best increasing in on chlorophyll (a & b) contents were recorded in F46 compared with parental strains and untreated control.

Mineral percentage

Data tabulated in Table 8 showed that the effect of fusants and their parental strains treatments on seedling of Superior, Flame and Thompson seedless cvs. General, N, P and K% were increased with leaves of seedling in three cultivars under studied.

Discussion

Grapevine plant like most other crops and especially horticultural crops, suffer from attacks by plant-pathogenic nematodes. (Nicol, *et al.*, 1999). Root-knot nematodes, Meloidogyne spp., have been reported to cause severe losses to horticultural especially, grapevine (El-Hady, *et al.*, 2015). Currently, Plant Growth-Promoting rhizobacteria are using biological control against plant parasitic nematodes due to their multiple modes of action (Tian, *et al*., 2007). These findings were an agreement with (Ismail, *et al.*, 2010; Zuckerman and Dicklow, 1993). The treatment with such isolate gave a significant reduction in nematode parameters due to *Meloidogyne* infestation on the plant than in the untreated controls.

The recombinant fusant strains obtained from

Table 8: Effect of *B. thuringiensis* and *P. aeruginosa* parental and their three fusants on chlorophyll (a&b) and some mineral percentage on grapevine seedling cvs. Superior, Flame and Thompson infected with *M. incognita* under greenhouse conditions.

Treatments	chlorophyll (a) mg/g fresh wt.	chlorophyll (b)mg/g fresh wt.	N %	P %	K %
a)Superior seedless					
F36	0.38* b	0.22 a	1.84 b	0.464 a	1.76 a
F45	0.37 b	0.17 b	1.67 c	0.449 a	1.78 a
F46	0.50 a	0.22 a	2.03 a	0.445 a	1.68 a
B. thuringiensis	0.41 b	0.16 b	1.48 d	0.405 b	1.63 a
P. aeruginosa	0.30 c	0.13 c	1.33 e	0.364 c	1.03 b
Untreated control	0.22 d	0.10 d	1.16 f	0.235 d	0.84 c
b) Flame seedless					
F36	0.40 bc	0.19 ab	2.02 ab	0.438 ab	1.48 b
F45	0.42 ab	0.15 bc	2.13 a	0.454 a	1.60 a
F46	0.46 a	0.20 a	2.08 a	0.417 b	1.64 a
B. thuringiensis	0.37 cd	0.16 bc	1.92 bc	0.431 ab	1.44 b
P. aeruginosa	0.34 d	0.16 bc	1.80 cd	0.350 c	1.36 c
Untreated control	0.33 d	0.13 c	1.71 d	0.261 d	1.05 d
c) Thompson seedless					
F36	0.60 b	0.26 a	2.16 b	0.384 ab	1.02 c
F45	0.54 bc	0.24 a	2.01 c	0.353 bc	1.15 b
F46	0.74 a	0.26 a	2.44 a	0.405 a	1.41 a
B. thuringiensis	0.53 bc	0.20 b	1.98 c	0.354 bc	1.03 c
P. aeruginosa	0.51 c	0.21 b	1.83 d	0.347 c	0.92 d
Untreated control	0.38 d	0.18 b	1.73 d	0.249 d	0.87 e

Values are average of five replicates. *Means followed by the same letter(s) are not significantly different according to Duncan's

protoplast fusion experiment between *B. thuringiensis* and *P. aeruginosa* revealed higher nematicidal than the parental strains compared with untreated control. The recombinant fusants have more efficient and potential values for agricultural application more than the nematicidal parental strains against root knot nematodes the obtained results agree with the results of (Mohamed, *et al.*, 2016; Soliman, *et al.*, 2017 and 2018). Mohamed, *et al.*, (2016) found that increase the effectiveness of biological control against the Lepidoptera insects specially *Tuta absoluta* up for the production of toxins resistance to these pests damned using the methods of biotechnology to produce genotypes new from some bacteria that grow on the outer surface of the shoots and roots of the plants under study and have the ability to produce toxins with the same efficiency of the original bacteria (Liang, *et al*., 2011).

The 10 fusants from *B. thuringiensis* and *P. aeruginosa* were evaluated nematicidal effect against root-knot nematode, *M. incognita* J_2 under laboratory conditions. All tested fusants had achieved a nematicidal effect on *M. incognita* J_2 mortality as compared with their parental strains and the control. These findings were an agreement with (Soliman, *et al.*, 2018) refer that all protoplast fusion between *B. licheniformis* and *P. aeruginosa* product fusants exhibited an increase in their nematicidal activity than their parents and control against root-knot nematode, M. incognita J_2. (Soliman, *et al.*, 2017 and 2018) three fusant from *P. aeruginosa* and *B. Licheniformis* and could kill *M. incognita* J_2 in bioassay test and the percentages mortality ranged from 94-96% compared to control. These may be referring to the ability of *P. aeruginosa* to produce hydrogen cyanide toxin. All the tested fusants exhibited an increase in their nematicidal activity than their parents against root-knot nematode, *M. incognita* J_2, under laboratory. Moreover, The results show that *B. thuringienesis*, and *P. aeruginosa* their fusion significantly suppressed M. incognita and reduced nematodes parameters and improved plant growth parameters *viz*. shoot length, leaves, shoot fresh & dry weights and root fresh weight as a result of the application of fusion product compared to control. Similar observations were reported (Abdel-Salam, *et al*., 2018; Amin *et al*., 2014; Soliman, *et al*., 2017 and 2018). El-Nagdi, *et al*., (2019) found that the fusant were suppressed *M. incognita* reproduction on tomato and eggplant. Also, Zaied, *et al.*, (2009) who found that fusant are more powerful in biocontrol activity than their parents as it contains more than one of the most distinguishable and important biocontrol activity from the parents.

Protoplast fusion technique has opened new tools to obtain new biocontrol agents with increasing in their production from enzymes, toxins, and antibiotics against plant pathogens. This improving in plant growth of grapevine plant due to suppressing plant nematodes damage and the bacteria produced secondary metabolite affected in plant growth of grapevine plant. Similar observations were reported by Amin *et al.*, (2014) refer to the potency of *P. aeruginosa* to inhibit nematode reproduction to its ability to induce systemic resistance against nematode infection. Salicylic acid production by bacteria act as an endogenous signal for the activation of plant defense response. Biotechnology had been paved the approach for the development of a biological control methods of plant-parasitic nematodes.

Conclusion

The current experiment was conducted to control the root-knot nematode; *M. incognita* infecting grapevine seedless by using some genetically modified bacteria by protoplast fusion technique. Genetically improved bacterial strains (F36, 45 and 46) more efficient than its parents in reducing root-knot nematode, *Meloidogyne incognita* reproduction. So, protoplast fusion technique could be recommended as a promising tool for biocontrol of root-knot nematode (*M. incognita*) over the chemical nematicides and an alternative to harmful chemical nematicides.

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