A local *Bacillus spp.*: isolation, genetic improvement, nematode biocontrol, and nitrogen fixation

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Received: 20 June 2021
Revised: 8 August 2021
Accepted: 20 August 2021
Published: 24 December 2021

Egyptian Pharmaceutical Journal 2021, 20:352–363

Background
Increasing chemical nematicide and fertilizer application has disturbed the natural balance and increased human health risks, which in turn compelled and increased the urgency to discover novel and more safe and effective biological tools to control root-knot nematode (*Meloidogyne incognita*) distributed throughout Egypt.
Objective
Isolation and screening of rhizobacteria from soil samples was the first step. The second step was the evaluation of the isolated rhizobacteria capability as nematicides under laboratory and screenhouse conditions. The third was to employ protoplast fusion as genetic technique to improve the nematicidal effect of rhizobacterial bacteria strains and comparison between parent and fusant protein profiles to analyze the genetic recombination among them.
Materials and methods
Serial dilution method was used to isolate rhizobacteria on Tryptic Soy Agar plates. The evaluation test against *M. incognita* J_2 of bacterial isolates was achieved under laboratory conditions and nematode parameters under screenhouse. The most potent rhizobacteria were exposed to molecular identification using 16s rDNA sequencing. *Bacillus thuringiensis* subsp *tenebrionis* (Bt) was evaluated for the first time as a bioagent that acted as a nematicide. Protoplast fusion was employed between the most potent bacterial strain, *Bacillus cereus* (Bc) and *B. thuringiensis* subsp *tenebrionis* (Bt). Selection of bacterial fusants was achieved on complete media containing selectable antibiotics marker. Parental and fusant strains were evaluated against J_2 under laboratory and screenhouse conditions and their ability in plant-growth promotion by assessment of plant parameters. SDS-PAGE analysis was used to analyze protein profiles.
Results and conclusion
Out of 15 bacterial isolates, NRC12 recorded the highest percentage mortality 88.3% after 24 h compared with control. NRC12 was identified as *B. cereus* NRC12 (Bc) and deposited in Genbank under accession number MW548408. Protoplast fusion between *B. cereus* (Bc) and *B. thuringiensis* (Bt) resulted in formation of 10 stable bacterial fusants that gave higher nematicidal effect compared with parental strains. Bacterial fusant, F7, achieved the highest mortality and reduction in J_2 in soil by 98.3 and 87.19% under laboratory and screenhouse conditions, respectively. The highest reduction in the number of galls and egg masses on root system was also recorded by F7 with 77.18 and 72.35%, respectively, compared with its parents. Also, F7 registered the highest significant ($P \leq 0.05$) increment in length, fresh weight, and dry weights in the shoot system of eggplant in pot experiments. The bacterial fusant, F7, exhibited more ability to fix atmospheric nitrogen compared with bacterial parents. Protein profile of F7 proved that this fusant was the only one that acquired equal protein bands from each parent. The highest frequency of genetic recombination might occur in F7 and subsequently, induced effective nematicidal effect more than its parents.

Keywords:
16s rDNA sequencing, biocontrol, *Meloidogyne incognita* J_2, plant-growth promotion, protoplast fusion, SDS-PAGE

Egypt Pharmaceut J 20:352–363

1687-4315

Introduction

The main way for controlling root-knot nematode, *Meloidogyne incognita* and increasing crop production, was the use of chemical nematicides and fertilizers, however, their extensive use has disturbed the natural balance of delicate ecology and biology in

soil, contaminated groundwater, developed resistance races of pathogens, and increased human health risks [1]. This risk has compelled our attention toward using and developing of biological control measures as an alternative potential ecofriendly strategy for controlling diseases and pests.

Endophytic bacteria have been studied to control root-knot nematodes such as *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus mycoides*, *Bacillus megaterium*, *Bacillus licheniformis*, and *Bacillus thuringiensis* [2,3]. *Bacillus cereus* (Bc) BCM2 was reported to have great ability to inhibit *M. incognita* second-stage juveniles on tomato by reducing the number of galls and egg masses by 81.2 and 75.6%, respectively [4]. *B. thuringiensis* can produce toxic compounds of various chemical structures and properties, besides production of chitinase enzyme [5,6].

For good coincidence, application of ecofriendly agents, such as plant growth-promoting bacteria (PGPR) to avoid the hazardous effects of chemical nematicides, is now in progress to increase crop production, reduce production costs, and environmental impacts [7]. PGPR are a group of microorganisms capable of stimulating plant growth through direct mechanism production of plant enzymes, hormones, nitrogen fixation, and phosphate solubilization and/or indirect mechanisms of biological control such as resistance induction [8,9]. The most dominant known genera of PGPR are *Pseudomonas* and *Bacillus spp*. for controlling nematodes, among these, *Pseudomonas fluorescens* has been extensively exploited for the suppression of root-knot nematode *Meloidogyne* spp. and improving yield [10].

Protoplast fusion is considered an important tool in strain improvement for exhibiting genetic recombination by gene combination from different microorganisms and to obtain strains with desired properties. In our investigation, protoplast fusion will be employed for further improvement of bacterial strains that have been prized qualities for creating one strain that combines multiple mechanisms of action, such as biocontrol and plant-growth promotion. Many scientific investigations were conducted, depending on the efficiency of protoplast fusions in nematode control. From these studies, bacterial fusants were more effective than their parental strains, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* in reducing different parameters of nematode and enhancing the growth of plant [10]. Furthermore, bacterial fusants resulted from genetic combination between *B. licheniformis* and *P. aeruginosa* exhibited an increase in their nematicidal activity than their parents against root-knot nematode, *M. incognita* J_2 on eggplant under laboratory and screenhouse conditions [3].

As microbial organisms are extensively used as ecofriendly tools for controlling plant-parasitic nematodes (PPNs) as an alternative to chemical nematicides, our intension in this research was to use and examine the efficacy of bioagents and employing the protoplast fusion technique for obtaining bacterial fusants with higher nematicidal activity as biological control. This attempt has been made by the combination between two bioagents, *B. cereus* and *B. thuringiensis* subsp *tenebrionis* isolated from dead snails and was identified in Microbial Genetics Department, National Research Centre, Egypt. It is the first study that deals and examines the nematicidal effect of *B. thuringiensis* subsp *tenebrionis* on the root-knot nematode, *M. incognita*.

Materials and methods

Isolation of rhizobacteria

For isolation of bacteria from the rhizosphere, three soil samples were collected from eggplant fields in Giza governorate and each stored in sterile plastic bags at 4°C until isolation in the laboratory. Standard microbiological methods were used to isolate bacteria from the rhizosphere [11]. Bacterial suspensions were separately diluted in 1% (v/v) peptone water and then plated onto Tryptic Soy Agar (TSA; Acumedia Lab, United States of America). After incubation at 28°C for 48 h, single colonies with different morphology were subcultured onto TSA slants and stored at 4°C for further studies. Another bacterial strain, *B. thuringiensis* subsp *tenebrionis* (Bt) strain was used [12].

Nematode assay on second-stage juveniles (J_2) by using rhizobacterial cell suspensions in the laboratory

Rhizobacterial isolates were evaluated as bionematicide as follows: bacteria were grown on Tryptic Soy Broth (TSB) at 28°C in darkness. After 3 days on a rotary shaker at 120 rpm, bacterial cell suspension of 2×10^7 CFU/ml was used as stock concentration (S). A second concentration of the bacterial cell suspension was made by addition of bacterial stock concentration to distilled water (1 : 1 v/v) to adjust the final concentration to $S/2=1\times10^7$ CFU/ml. Briefly, a 2.5 ml of each concentration was added to 100-ml plastic tubes. Afterward, each tube received 1 ml of water containing 200±5 freshly individuals of hatched *M. incognita* juveniles and was completed to the final volume of 5 ml. Tubes were then placed in an incubator at 25°C in darkness. A 5-ml distilled water

containing the same number of nematodes served as control. All treatments were replicated five times. The numbers of survived and dead individuals were counted for 2 days. From each treatment, all nematodes were transferred to distilled water and left for 24 h to see whether immobile nematodes would resume activity or not. Then, nematodes were collected and transferred to the counting slide under light microscope. The percentages of nematode mortality were calculated according to Abbott's formula [13] as follows:

Juvenile mortality (%)=(m–n)/(100–n)×100, where m and n indicate the percentages of mortality in treatments and control, respectively.

Molecular identification of bacterial isolate

Genomic DNA extraction

A single colony of each bacterium was cultured in 20 ml of TSB in a 100 ml conical flask (Pyrex, United States of America) by shaking in an orbital shaker (Thermo Fisher Scientific, United States of America) at 120 rpm for 18 h at 30°C. Bacterial culture was subjected to genomic DNA extraction using GeneJET Genomic DNA Purification Kit (Thermoscientific, United States of America) according to the manufacturer's instructions. Genomic DNA was used for genes of 16s rDNA amplification.

16s rDNA amplification and sequencing

The fragment of 16s rDNA gene was amplified in a reaction volume of 25 µl containing 12.5 µl of PCR Master Mix 2x conc. (Thermoscientific, United States of America). One microliter of each forward and reverse primer sets 8 F/1492 R, 9.5 µl of sterile deionized water, and 1 µl of the bacterial genomic DNA. The reaction was achieved in GeneAmp PCR system 9600 (Applied Biosystems, Bedford, MA, United States). The temperature cycling program used was modified slightly from that used by El-Sayed *et al.* [14], by adding an initial hot start and was as follows: one cycle of 95°C for 10 min; 40 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. The 16s rDNA nucleotide sequence was determined using the same primers with the dideoxy-chain termination method using 3500 Genetic Analyzer (Thermo Fisher Scientific) in colors laboratory, Clinilab, Egypt. Nucleotide sequence was compared with available sequences using the BLAST program from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and submitted for revision and getting an I.D accession number.

Bacterial protoplast fusion

To employ the technique of protoplast fusion, genetic marker must be present on which the selection of bacterial fusants can be achieved. Antibiotic susceptibility was done to obtain antibiotic marker.

Antibiotic-susceptibility pattern

Antibiotic-resistance pattern of parental stains was tested for obtaining selectable markers on which bacterial fusions were selected. Eleven antibiotics with specific concentrations (µg/ml) listed in Table 1 were used. About 5 ml of each overnight culture of the tested strains was transferred to a 250-ml conical flask containing 45 ml of TSB. The antibiotics were added to these flasks separately with specific concentrations. All flasks were incubated at 30°C for 24 h. Growth of bacteria was recorded as (+), and no growth was recorded as (–). Based on antibiotic susceptibility, genetic marker was determined.

Protoplast formation, regeneration, and fusion

Protoplast formation, regeneration, and fusion were conducted based on the combination among particular methods employed by Chen *et al.* [15] and El-Gaali *et al.* [16]. Briefly, in a 250-ml flask containing 50 ml of LB broth medium, parental bacterial strains were separately inoculated and incubated at 30°C for 18 h at 120 rpm. The next morning, 1 ml of each bacterial culture was added to 20 ml of LB broth and incubated in the same conditions for 4 h. By centrifugation at 10 000 rpm for 5 min, bacterial cells were harvested and then washed once with 1% N-lauryl sarcosine, followed by washing three times with an osmotic stabilizer buffer (30 mM Tris-HCl, 0.6 mM $MgSO_4$, pH 7.5). The bacterial cells were then pelleted by centrifugation. Lysozyme was dissolved in P medium [LB medium containing 10% (w/v) lactose, 20 mM $CaCl_2.2H_2O$, and 10 mM $MgCl_2.7H_2O$) with a final concentration of 5 mg/ml, sterilized by a 0.2-µm Millipore sterilized

Table 1 The antibiotics used in this study and their concentrations

No	Antibiotic	Abbreviations	Final concentration (µg/ml)
1	Chloramphenicol	Cm	35
2	Streptomycin	Sm	200
3	Amoxicillin	Amox	50
4	Rifampicin	Rif	100
5	Kanamycin	Km	40
6	Tetracycline	Tc	15
7	Clarithromycin	Cla	100
8	Erythromycin	Erm	20
9	Amikacine	Amk	30
10	Ceftriaxone	Cef	200
11	Gentamicin	Gm	15

filter. About 1 ml of P medium containing lysozyme was added to the cell pellet of each parental strain and mixed thoroughly to make a suspension that was then incubated in the water bath at 30°C with shaking at 100 rpm up to 4 h. Protoplast formation was checked by direct observation under light microscope. Protoplast fusion was accomplished by mixing equal volumes of a protoplast suspension of each parent, and 0.1 ml of the protoplast mixture was added to 0.9 ml of freshly prepared 40% (w/v) polyethylene glycol. The fusion mixture was gently mixed and incubated for 10 min at 30°C. The fused protoplasts were collected by centrifugation at 1000 rpm for 5 min at room temperature and gently suspended in the same volume of P medium, and samples (0.1 ml) were plated on R medium (P medium containing 0.8% Bacto-Agar, pH 7).

Cells from R-medium plates were transferred to 3 ml of LB medium mixed with a vortex stirrer, and collected by centrifugation. The cells were suspended in 3 ml of LB medium and diluted, and samples (0.1 ml) were plated on the LB agar medium. After incubation for 14–16 h at 30°C, single colonies were transferred to the same medium with sterile toothpicks to form master plates. After incubation at 30°C, the master plates were replicated onto appropriate selective media with appropriate antibiotics to score the selected phenotypes (the fusion recombinants). Colonies that grew on selective LB medium were considered to be fusion products. All bacterial fusants were evaluated for nematicidal activity under laboratory and greenhouse conditions the same as their parents.

Determination of *Meloidogyne incognita* parameters on eggplant by using bacterial cell suspensions in screenhouse

An experiment was carried out in plastic pots (20 cm in diameter) containing 2 kg of nonsterilized mixture of loamy soil and sandy (1 : 1 w/w) in the screenhouse of Plant Pathology Department. In each pot, 1-month-old eggplant cv. Baladi seedlings were transplanted independently. Each pot was inoculated with 2000 newly hatched J_2s of root-knot nematode, *M. incognita*, pipetted in four holes around the roots one week later. Afterward, two volumes, 10 or 20 ml/pot of bacterial cultures (2×10^7 CFU/ml), were added. The seedlings were cultivated in a screenhouse at a temperature of 30±5°C and a relative humidity of 85%. The pots were watered and maintained to 50% water-holding capacity and plant growth was observed every week. Treatments were achieved as follows: parental bacterial strains were applied in single and consortium; however, bacterial fusants were applied separately. Control pots were inoculated with nematode but not bacteria. Pots were arranged in a completely randomized design with five replicates for each treatment. Plants were gently plucked and roots were properly cleansed with flowing tap water 3 months following nematode inoculation. Data on nematode criteria included the number of J_2s in soil, galls and egg mass numbers per root system were scored. The second-stage juveniles (J_2s) were extracted from soil using sieving and decanting technique and counted [17]. For each treatment, roots (5 g) were stained for the number of galls and egg masses [18]. For each parameter, the percentage of nematode reduction was calculated in comparison with the untreated control.

Assessment the effect of bacterial strains on plant-growth parameters

After 3 months of treatment, parameters, such as length of shoots (cm), fresh weights of shoots and roots, and dry weight of shoots (g) of eggplant, were measured using standard procedure. The percentage of plant-growth increase for each criterion was calculated compared with untreated control.

Screening for biofertilization activities

In vitro nitrogen-fixing activity

Nitrogen-fixation activity was assessed on glucose nitrogen-free mineral agar medium using bromothymol blue (BTB) as an indicator [19]. BTB was prepared by dissolving 0.5 g into 100 ml of distilled water and filter-sterilized. Onto glucose nitrogen-free mineral plates, each strain was inoculated and incubated at 28°C for 7 days. After flooding with BTB solution, the examination for color change in the agar from green to dark blue or bluish green was considered as am indication of nitrogen-fixation activity.

In vitro phosphate solubilization

Parental strains and their fusants were screened for inorganic phosphate solubilization by culturing them on Pikovskaya's agar medium, this medium contained 0.5% (w/v) of tricalcium phosphate (Ca_3PO_4) as a complex insoluble phosphate source [20]. The bacterial strains were inoculated separately and incubated at 37C±2°C for 10 days. Formation of yellow halos and/or clearing zones is considered an indication for phosphate solubilization. Solubilization index (SI) was measured using the following formula [21]:

S1 colony diameter + halozone diameter/ Colony diameter.

Statistical analysis

Using the Computer Statistical Package (CO-STAT) User Manual, Version 3.03 (Barkley Co., United States of America), data on nematode and plant-growth criteria were subjected to analysis of variance. Duncan's multiple-range test was used to compare the means [22].

Analysis of protein profile for wild and fusant bacterial strains

Protein analysis of parental and fusants has been achieved by SDS-PAGE. A loopful of fusion recombinants, which grew on LB plates containing selectable marker (selected antibiotics) and bacterial wild types, were inoculated in a 100-ml conical flask containing 10 ml of LB liquid medium with appropriate antibiotics at 120 rpm for 3 days at 30°C. Bacterial cultures were centrifuged and subjected to protein extraction [23]. Protein separation was done by 15% SDS-PAGE [24], to compare the protein profile of the parental strains with their fusant. After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye. The gels were scanned using Gel Doc 2000 system, and molecular masses were determined using total laboratory, version 1.10 software based on prestained protein marker purchased from Thermoscientific.

Results

Isolation and evaluation of the nematicidal potential of rhizobacterial isolates against *Meloidogyne incognita*

Based on different morphology on TSA plates, fifteen bacterial colonies were selected and tested for their ability against *M. incognita* juveniles. The highest mortality percentage noticed after 24 h as compared with control was 88.3% by bacterial isolate NRC12 under laboratory conditions. Also, *B. thuringiensis* subsp *tenebrionis* recorded 88.3% mortality for *M. incognita* juveniles after 24 h.

Molecular identification of bacterial isolate

The universal primers of 16s rDNA gene amplified ~1500 bp (Fig. 1) for the bacterial isolate. Partial DNA sequence was subjected to BLAST search on https://blast.ncbi.nlm.nih.gov/ against the available sequences. 16s rDNA gene sequence of bacterial isolate scored 99% with *B. cereus* strain IAM 12605 16s ribosomal RNA. This isolate was recorded to genbank database as *B. cereus* NRC12 under accession number MW548408.

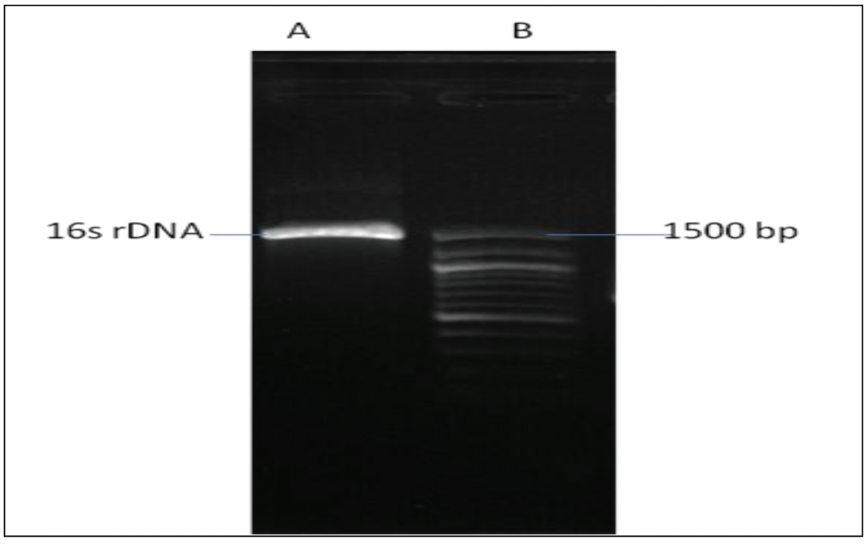
Antibiotic-resistance pattern of *Bacillus cereus* and *Bacillus thuringiensis* subsp *tenebrionis*

The antibiotic susceptibility was done with 11 antibiotics to obtain selective markers based on which bacterial fusants can be selected. From Table 2, the antibiotic marker of Bc NRC12 was taken as Sm^r, Km^S, and *Bt* was Sm^S, Km^r. Antibiotics of streptomycin and kanamycin were used as selectable markers in the growth media.

Protoplast formation, regeneration, and fusion

Protoplast formation of the parental strains, Bc and Bt, was tested periodically every 10 min after incubation with lysozyme by microscopic examination. A 90% of vegetative cells for parental strains was converted to protoplast after 3 h (Fig. 2). After transferring the colonies that grew on the master plate containing selective media with streptomycin and kanamycin, all

Figure 1



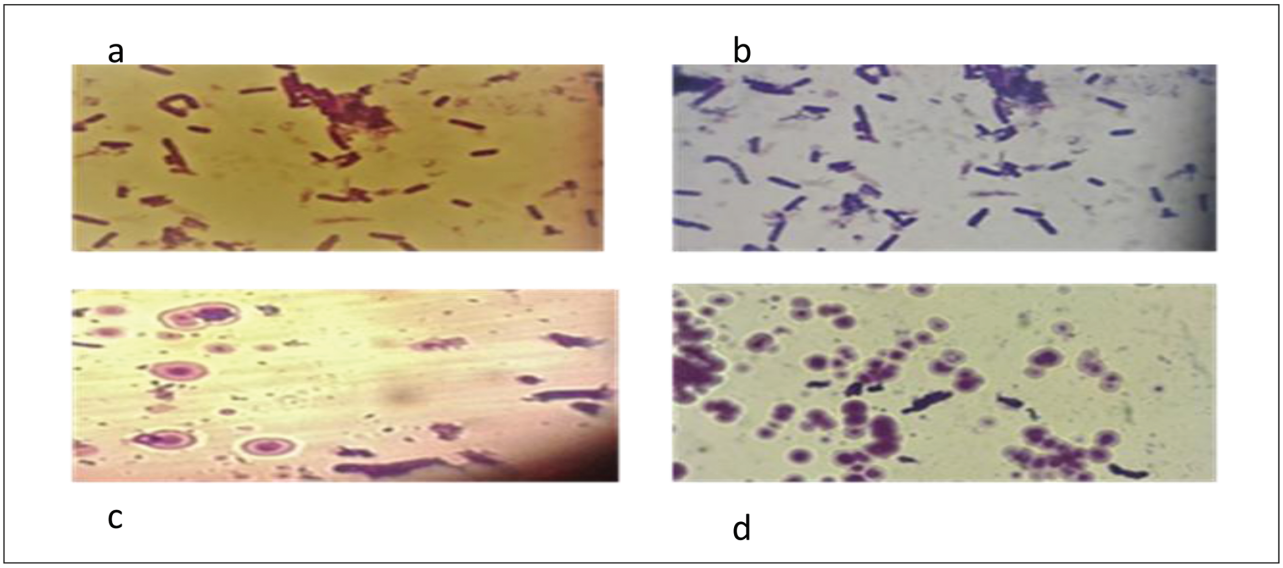
Agarose gel electrophoresis for PCR product of 16s rDNA in NRC12 bacterial isolate (a), and 100-bp DNA ladder, enzynomics, Biovision (b).

Table 2 Antibiotic susceptibility of *Bacillus cereus* and *Bacillus thuringiensis* subsp tenebrionis (Bt)

Strains	Sm	Antibiotics									
		Cef	Amk	Erm	Amo	Cla	Km	Gm	Cm	Rif	Tc
Bc	+	–	–	+	–	+	–	–	–	–	–
Bt	–	–	–	–	–	–	+	–	–	–	–

Bc, *Bacillus cereus*. +Resistant. –Sensitive.

Figure 2



Released protoplasts from parent isolates observed after 3 h of incubation with lysozyme. (a, b) Vegetative cells of Bc and Bt before lysozyme treatment, respectively. (c, d) Protoplast formation of Bc and Bt after lysozyme treatment for 3 h under light microscope. Bc, *Bacillus cereus*.

colonies that grew on it were recultured on selective medium to select the stable bacterial fusions. Twenty-five fusants could grow on the first plate with selective media, and then only ten could regrow on the same selective medium. It was noticed that 10 fusants could grow on selective media for five generations and were marked as F1–F10. Afterward, the bacterial fusants and their parents were cultured for 3 days to evaluate their nematicidal potential against *M. incognita* J_2 under laboratory and then, nematode parameters under screenhouse conditions.

Effect of bacterial fusants on root-knot nematode juveniles compared with their parents under laboratory conditions

Stock concentration (S) and diluted concentration (S/2) of bacterial cell suspension for parents and fusants were used to evaluate their potential against root-knot nematode juveniles. From Table 3, both concentrations achieved high nematicidal effect. It is noticeable that all bacterial suspension concentrations yielded 100% mortality against nematode juveniles after 48 h; therefore, the evaluation of nematicidal effect depended on the mortality percentage after 24 h compared with control treatment. It was noticed that all bacterial suspensions of fusants of both employed concentrations achieved higher juvenile mortality, especially at concentration S compared with their parents. The most potent bacterial fusants were F6, F7, and F10 with 95.0, 98.3, and 96.7% mortality at S and 93.3, 95.0, and 95.0% by S/2 compared with their parents, 88.3% by S for both, and 81.7 and 81.2% by Bc and Bt by S/2. From the previous mentioned results, bacterial suspension of the three fusants F6, F7, and F10 as promising candidates for the control of J_2, was applied under screenhouse conditions.

Effect of *Bacillus cereus*, *Bacillus thuringiensis*, and the best fusants on *Meloidogyne incognita* reproduction in eggplant under screenhouse conditions

As the most potent nematicidal effects were achieved by bacterial fusants F6, F7, and F10 under laboratory conditions, they were applied against the root-knot nematode, *M. incognita* infesting eggplant under screenhouse conditions accompanied with the parental strains, Bc and Bt. Simultaneously, all the

bacterial suspensions of parents in separated and consortium form (1 : 1) and their fusants were added in separated form as a soil drench. When compared with parental and untreated controls, the collected data in Table 4 revealed that all treatments had a high significant ($P \leq 0.05$) reduction potential against root-knot nematode infectivity and reproduction. Three parameters, including number of J_2 in soil, number of galls, and egg masses on root system, were determined after bacterial suspension addition. It was worth mentioning that all bacterial suspensions of 10-ml volume yielded the best reduction percentages in all nematode parameters higher than those by 20 ml of bacterial suspensions. The highest reduction that occurred in all parameters was recorded by F7 with 87.19, 77.18, and 72.35% in number of J_2/soil, galls, and egg masses in the root system, respectively, compared with its parents that achieved lower reduction in all nematode parameters as compared with untreated control treatment.

Table 3 Effect of *Bacillus cereus* and *Bacillus thuringiensis* subsp *tenebrionis* and their fusants on *Meloidogyne incognita* J_2 mortality under laboratory conditions

Treatments	Dilution	% mortality of	
		24 h	48 h
Bacillus cereus	S	88.3	100
	S/2	81.7	100
Bacillus thuringienesis subsp *tenebrionis*	S	88.3	100
	S/2	81.2	100
F_1	S	95.0	100
	S/2	91.7	100
F_2	S	93.3	100
	S/2	91.7	100
F_3	S	95.0	100
	S/2	93.3	100
F_4	S	90.0	100
	S/2	87.0	100
F_5	S	91.7	100
	S/2	88.3	100
F_6	S	95.0	100
	S/2	93.3	100
F_7	S	98.3	100
	S/2	95.0	100
F_8	S	95.0	100
	S/2	91.3	100
F_9	S	92.5	100
	S/2	88.3	100
F_{10}	S	96.7	100
	S/2	95.0	100
Control (water only+nematodes)		0	0

Protein profile of parental strains and their fusants

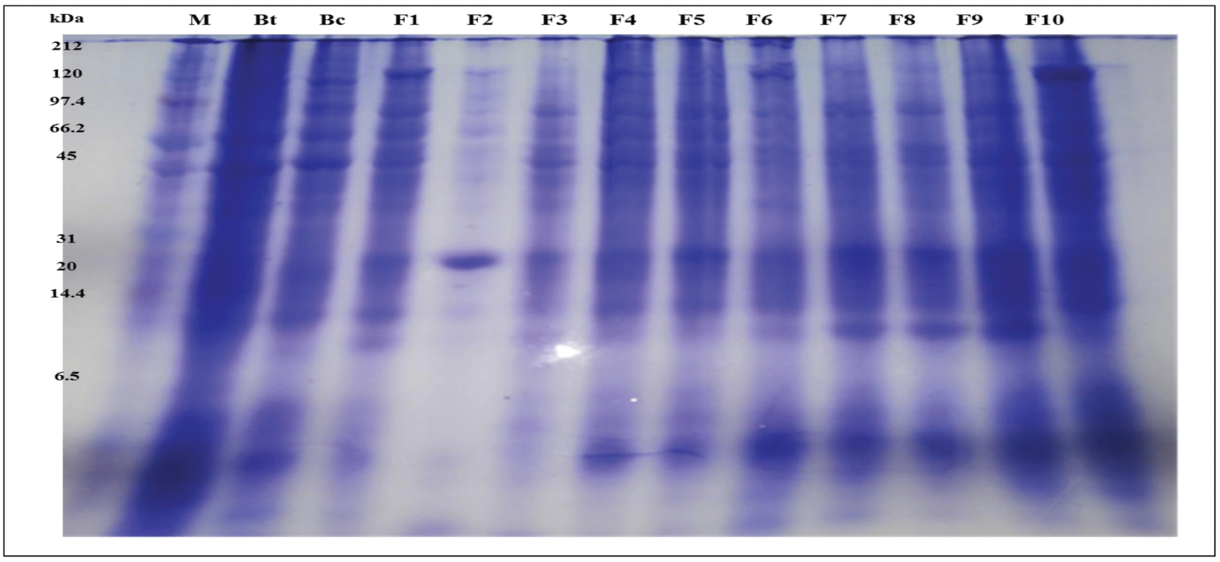
From Fig. 3 and Table 5, SDS-PAGE was utilized to analyze the protein profile of Bc and Bt and their fusants. The polypeptide profile was noticed with reference to a protein ladder (prestained protein ladder, Thermoscientific), with 17 bands between 5 and 171 kDa. SDS-PAGE protein analysis of the 10 selected fusants and their parental strains confirmed that all fusant strains acquired and expressed many specific protein bands from the parental strains. F1 acquired four protein bands from Bc and six bands from Bt parent strain, the highest number of protein bands acquired from Bt was recorded by F5 with nine bands and F6–F8 with eight bands. Simultaneously F5 and F6 acquired

Table 4 Effect of *Bacillus cereus*, *Bacillus thuringiensis* subsp. *tenebrionis* (Bt) and their three fusants on *Meloidogyne incognita* reproduction in eggplant under screenhouse conditions[a]

Treatments	Number of J_2 in soil	% reduction	Number galls/root system	% reduction	Number of egg-masses/ root system	% reduction
10 ml/pot						
Bc	132.67[#]bc	56.79	38.33bc	51.71	116.33a	19.38
Bt	138.33bc	54.94	34.00cd	57.67	144.33a	0.23
Bc+Bt	145.33b	52.55	24.00fg	70.12	64.33bc	55.53
F6	115.67cd	62.32	22.33 g	72.20	49.67bc	65.67
F7	39.33 g	87.19	18.33 g	77.18	40.00c	72.35
F10	109.33de	64.39	22.33 g	72.20	41.33c	71.43
20 ml/pot						
Bc	143.33b	53.31	47.00b	41.49	118.67a	17.97
Bt	140.67bc	54.20	32.67cd	59.33	129.67a	10.37
Bc+Bt	143.67b	53.20	35.33cd	56.02	71.33b	50.69
F6	115.35cd	62.42	22.00g	72.61	60.67bc	58.06
F7	78.33f	74.49	24.33ef	69.71	44.00bc	69.86
F10	115.33cd	62.43	25.67df	68.04	47.33bc	67.28
Control (inoculated with nematode only)	307.00a	–	80.33a	–	144.67a	–

Bc, *Bacillus cereus*. [a]Means followed by the same letter(s) are not significantly ($P \leq 0.05$) different according to Duncan's multiple range test. [#]Values are average of five replicates.

Figure 3



SDS-PAGE protein profiles of the two parental strains; *Bt* and *Bc* and their fusants (F1–F10). M: protein marker (prestained, Thermoscientific). Bc, *Bacillus cereus*.

Table 5 SDS-PAGE analysis of total proteins of the two parental strains; *Bt* and *Bacillus cereus* and their fusants, F1 to F10

Band No.	MW KDa	Parental strains		Fusants									
		Bt	Bc	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	171		•	•			•	•	•	•	•	•	•
2	134	+		+	+	+	+	+	+	+	+	+	+
3	110	+		+	+	+	+	+			+	+	+
4	90	+						+	+	+	+		
5	89		•	•	•	•	•	•	•	•	•	•	•
6	67	+						+	+	+		+	
7	66		•	•	•	•	•	•	•	•	•	•	•
8	65		•	•		•	•		•	•	•	•	•
9	64	+		+				+	+	+	+		
10	56	+		+	+	+	+	+	+	+	+	+	+
11	48		•	•		•	•	•	•	•	•	•	
12	33	+						+	+	+	+	+	+
13	32		•	•		•	•	•	•	•	•	•	
14	15	+		+	+	+	+	+	+	+	+	+	+
15	11		•	•	•	•		•	•	•	•	•	•
16	7		•	•		•	•	•		•	•	•	
17	5	+		+		+	+	+	+	+	+	+	+
Total no. of protein bands	9	8	14	7	13	13	16	15	16	16	15	11	
Numbers of protein bands acquired from Bt	9	8	6	4	6	6	9	8	8	8	7	6	
Numbers of protein bands acquired from Bc	0	8	8	3	7	7	7	7	8	8	8	5	

•Presence of protein band acquired from Bc (+) presence of protein band acquired from Bt.

seven bands but F7 and F8 acquired eight bands. The lowest number of acquired protein bands from Bc and Bt was recorded by F2 with total seven bands, four bands from Bt and three bands from Bc.

Effect of *Bacillus cereus*, *Bacillus thuringiensis*, and their best fusants on plant-growth traits

Observation on growth parameters, such as length, fresh, and dry weights of shoots in eggplant after application, reflected noticeable results. From Table 6, all bacterial suspensions of 10-ml volume yielded the best increases in plant-growth parameters. In comparison, 20 ml of bacterial suspensions of all treatments did not achieve any increase compared with 10-ml volume. The best significant increases in all parameters were achieved by F7 that recorded the highest mean of total nematode reduction (Table 4). Treatment of 10 ml for F7 yielded

increases of 40.20, 92.80, and 53.85% in length, fresh, and dry weights of shoot, respectively, and 44.90% increase in root length. Due to the previous results, parental strains and their fusants were evaluated for biofertilization activitie, including nitrogen fixation and phosphate solubilization.

Evaluation of biofertilization abilities of parental strains and their fusants under controlled conditions

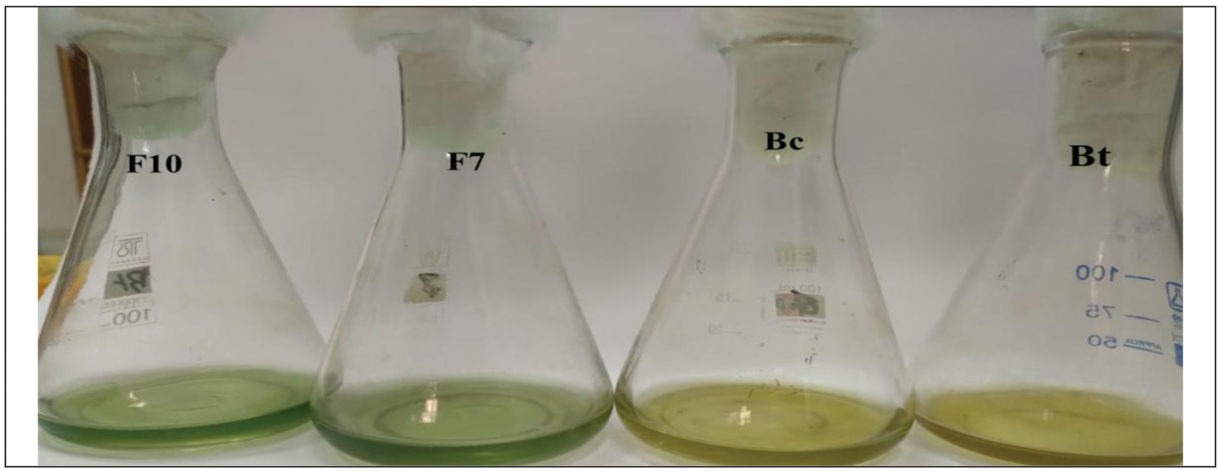
Growth percentage of eggplant, including shoots and roots in screenhouse pots supplemented by F7, can be explained by the ability of parental strains and the three tested fusants to fix the atmospheric nitrogen. All the inoculated tested strains showed blue color on nitrogen-free medium (Fig. 4). Plates inoculated with F7 showed dark-blue color as compared to that produced by parental strains and F6 and F10. Parental strains, Bc and Bt, gave negative results in phosphate solubilization, where they could grow on Pikovskaya's agar medium, but did not give clear zones around the growth and therefore, their fusants also gave negative results in phosphate-solubilization test.

Table 6 Growth promotion effects of *Bacillus cereus*, *Bacillus thuringiensis* on eggplants, with three best fusants having the highest nematicidal activity

Treatments	Shoot						Root			
	Length (cm)	% increase	Fresh weight (g)	% Increase	Dry weight (g)	% increase	Length (cm)	% increase	Fresh weight (g)	% increase
10 ml/Pot										
Bc	31.75[#] ab	24.51	11.59bc	41.51	3.73ab	36.63	30.00bc	22.45	12.01cd	–
Bt	25.75e	0.98	9.67 bc	18.07	3.21bc	17.58	27.00de	10.20	9.44fg	–
Bc+Bt	30.75bc	20.59	11.20bc	36.75	3.13bc	14.65	30.50bc	24.49	11.23cd	–
F6	33.00ab	29.42	11.93b	45.67	3.38ab	23.81	32.00ab	30.61	14.39ab	–
F7	35.75a	40.20	15.79a	92.80	4.20a	53.85	35.50a	44.90	13.96ab	–
F10	31.00bc	21.57	9.10cd	11.11	3.49ab	27.84	31.50ab	28.57	15.32ab	
20 ml/Pot										
Bc	25.75e	0.98	7.95e	–	2.84bc	4.03	28.25cd	15.31	11.85cd	–
Bt	27.00de	5.88	7.94e	–	2.37e	–	27.00de	10.20	8.29 g	–
Bc+Bt	27.00de	5.88	8.43de	2.93	2.46de	–	30.50bc	24.49	10.36ef	–
F6	28.25cd	10.74	10.61bc	29.55	2.92bc	6.96	31.00bc	26.53	10.91de	–
F7	31.25bc	22.55	11.78b	43.83	3.47ab	27.11	34.00ab	38.78	12.86bc	–
F10	30.75bc	20.59	10.00bc	22.10	3.54ab	29.67	31.00bc	26.53	10.04ef	–
Healthy plants only)	27.75cd	8.82	8.33de	1.71	2.97bc	8.79	25.25e	3.06	7.97 g	–
Control (treated with nematode only)	25.50e	–	8.19de	–	2.73cd	–	24.50e	–	16.05a	–

Means followed by the same letter(s) are not significantly ($P \leq 0.05$) different according to Duncan's multiple range test. #Values are average of five replicates.

Figure 4



The ability of nitrogen fixation for parental strains and its fusants on glucose nitrogen-free mineral media. Bc: *Bacillus cereus* strain NRC12, Bt: *Bacillus thuringienesis* subsp *tenebrionis*. F7 and F10: the most potent bacterial fusants in nitrogen fixation.

Discussion

PPNs infect a wide range of agricultural crops, causing serious damage and yield losses. Control strategies with low environmental impact are needed in light of the revision of EU legislation on the use of pesticides on agricultural crops. The bacteria-based approach appears to be especially promising because it helps to minimize the amount of chemicals used. In this investigation, bacterial isolate of *B. cereus* NRC12 and *B. thuringiensis* subsp *tenebrionis* demonstrated high nematicidal potential against *M. incognita* J_2 with 88.3% for each after 24 h. It has been known that more than one mode of action can be utilized to create antagonistic interactions between rhizobacteria and PPNs. Bacteria may have both direct and indirect effects on PPNs. Colonization, parasitism, and antibiosis (production of lytic enzymes, antibiotics, toxins, and volatile organic compounds) are considered direct modes of action; however, induction of system resistance and food supply for bacterivorous species (protozoa, nematodes) are examples of indirect [25]. Rhizobacteria nematicidal compounds, such as proteases, chitinases, collagenases, lipases, and complexes of enzymes that generated by Bacillaceae members and affect different stages of the PPNs life cycle, were recently studied [26]. A similar study that reported 83% *in vitro* nematicidal activity against the second-stage juveniles (J_2) of *Meloidogyne javanica* and *Heterodera filipjevi* by cultural filtrates of *B. cereus* 09B18 was conducted [27]. The production of complex chitosanase, alkaline serine protease in *B. cereus*, was also demonstrated [28]. *B. cereus* strain Jdm1 produced strong protease, chitinase, and siderophores, which probably have an important virulence role against *M. incognita* J_2s or eggs by inhibition in egg hatching and killed J_2s [29]. Also, *B. cereus* strain Jdm1 in the rhizosphere may also have increased the levels of hydrogen peroxide as defense compound in the tomato plants, or induced the plants' own systemic resistance. It was reported that the effect of *B. cereus* on root-knot nematode, *M. incognita* may be due to the repellent effect on nematode by changing the composition of root exudates [30]. However, in case of *B. thuringiensis*, it was demonstrated that the production of crystal toxins and chitinase is responsible for nematicidal activity against a broad range of nematodes as it reduced the number of eggs of root-knot nematode, *M. incognita* [30,31].

As protoplast fusion is an effective tool for bringing genetic recombination and developing superior hybrid strains in bacteria, in this present study, interspecific protoplast fusion program between Bc and Bt was done for both with the aim of enhancing the nematicidal activity. Antibiotic-susceptibility test was done to obtain antibiotic marker between parental strains, based on data presented in Table 2, streptomycin and kanamycin were selected as antibiotic markers. Protoplast fusions between *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* based on the antibiotic marker were constructed to improve the nematicidal activity for fusants compared with their parental strains [32]. The increase in nematicidal activity by F7 compared with its parental strains may be due to the increase in gene copy encoding the hydrolytic enzyme present in both parental strains, where protease-encoding genes are present in both strains [28]. Moreover, the presence of crystal toxins acquired from Bt and other hydrolytic enzymes acquired from Bc in one fusant fostered the nematicidal efficacy of all fusants, especially F6, F7, and F10, which yielded higher mortality percentages compared with those occurred by Bc and Bt as parental strains and other fusants. After application of cultural suspension of selected fusants in pot experiment, also F7 exhibited the highest nematicidal effect against *M. incognita* J_2 in soil, galls, and egg mas numbers on root system. In pot experiment, it was noticed that addition of 10 ml of all bacterial suspensions reduces *M. incognita* J_2 more than those by 20 ml, and that can be explained as follows: in this study, soil in screenhouse pots was taken from unsterilized soil field, so the addition of excess bacterial suspension related to the amount of soil to which it was added, might alter the organic matter that negatively affected bacterial growth and their nematicidal activity beside interspecific competition that depends on demand of all living microorganisms on the same nutrients so that the growth and efficiency of added bacterial suspension might be reduced [33]. The same situation by these bacteria in promotion of plant growth occurred, F7 promotes the growth of eggplant and that by significantly increasing the length, fresh, and dry weights of shoot system compared with the other treatments.

Rhizobacteria can influence plant growth by multiple indirect and direct mechanisms [34], these mechanisms can be active simultaneously at different stages of the plant development. The indirect mechanisms include the inhibition of various pathogens on plant growth by producing antagonistic substances or by induction of the resistance to pathogens and development in the forms of root colonizers and environmental protectors. However, the direct effect includes their ability for nutrient supply, such as nitrogen, phosphorus, potassium, and essential minerals, or

modulating plant hormone levels [35]. Since nitrogen is involved in proteins, nucleic acids, and other essential biomolecules' formation, it is considered one of the most common nutrients required for the plant growth. More than 80% of nitrogen is present in the atmosphere but is unavailable to plants in that time, the role of microorganisms is needed to convert nitrogen into ammonia by biological nitrogen fixation using a complex enzyme system. Under this scenario, this study concentrated on the *in vitro* tests for direct growth-promotion traits such as nitrogen fixation and phosphate solubilization. These tests demonstrated the ability of Bc and Bt and the most efficient fusant in nitrogen-fixation activity, but their inability in phosphate solubilization. The higher ability of F7 in plant promotion in pot experiment can be explained by higher capacity of this fusant in nitrogen fixation than those of parental strains. The sequences of the nifH gene responsible for nitrogen fixation from *B. thuringiensis* and *B. cereus* were first reported [36]. Another study was on disagreement, for example, *B. cereus* CUAMS116 isolated from soil, showed moderate level of phosphate-solubilization activity by appearance of a narrow clear zone on Pikovskaya's medium [37]. Moreover, *B. thuringiensis* isolated from acidic soil (Ultisols) in the field of a tea garden showed good capacity of *in vitro* phosphate solubilizing on Pikovskaya's medium containing $AlPO_4$ instead of $Ca\ (PO_4)_2$ [38], but in our investigation, bacterial isolate *B. cereus* NRC12 and *B. thuringiensis* subsp *tenebrionis* could grow on Pikovskaya's medium but did not yield a clear zone. Protein profile of the best fusants F6, F7, and F10 demonstrated that these fusants acquired more protein bands from their parents, especially F7 acquired eight bands from each parent. This profile is considered a documental tool for interpretation and explanation for the higher capacity against root-knot nematode juveniles and higher plant-growth promotion occurred by F7 bacterial culture. Using SDS gel electrophoresis to characterize and analyze the protein profile of bacterial fusants and their parents revealed that the most potent fusant no. 45 in reduction of *M. incognita*, acquired the higher numbers of protein bands from its parents, *B. thuringiensis* and *P. aeruginosa* [39], so the efficacy of protoplast fusion as biotechnological approach is promising in creating a single hybrid combined different desired characters from different bacterial strains.

Conclusion

To overcome the hazards resulted from overuse of chemical nematicides and fertilizers, we need to discover new bionematicides. So this research that focused on isolation and evaluation of local rhizobacteria with nematicidal effect has succeeded in obtaining an effective bioagent against *M. incognita* J_2. Protoplast fusion has been considered a promising biotechnological tool for production of more effective fusants compared with their parents in reduction of J_2 and promotion of plant growth. *B. thuringiensis* subsp *tenebrionis*, *B. cereus* NRC12, and their fusants can be considered good candidates for controlling *M. incognita* J_2 and consequently promoting the eggplant growth.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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