Isolation, identification, and improving nematotoxicity of rhizobacterial strains against *Meloidogyne incognita*

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Background and objectives
Root-knot nematodes, *Meloidogyne* spp., have been reported to cause severe losses to field, horticultural, and medicinal plants throughout the world. Chemical nematicides are routinely applied for protection of the aforementioned crops. Residues of these toxic nematicides are carried to the final consumed products, which can be quite hazardous to human health on consumption. Biocontrol by using antagonistic organisms to inhibit the pathogens and reduce diseases is an ecofriendly management tool.
This study was planned to isolate, identify, and improve or increase the activity of two isolated rhizobacterial strains known to have nematicidal properties – based on preliminary studies – using protoplast fusion technique and assessing the nematicidal potential of the fusants against root-knot nematode, *Meloidogyne incognita* J_2.
Materials and methods
Certain rhizobacterial colonies isolated from the rhizosphere soil of cucumber and banana plants were identified by 16S rDNA to *Pseudomonas aeruginosa* (accession number LC187271) and *Bacillus licheniformis* (accession number LC187270). They were manipulated using protoplast fusion technique to improve their nematicidal potentials. The fused protoplasts generated and 10 fusant isolates were assessed against *M. incognita* J_2.
Results and conclusion
All the tested fusants exhibited increase in their nematicidal activity than their parents against root-knot nematode, *M. incognita* J_2, under laboratory conditions. The percentages of nematode mortality after 72 h of exposure were 85 and 86% by *B. licheniformis* and *P. aeruginosa* suspensions, respectively, whereas the percentages of mortality caused by fusants ranged between 91 and 99% as compared with control. The obtained results indicated that protoplast fusion technique is a successful tool to enhance the lethal effect of the isolated rhizobacteria strains against root-knot nematode. The genetically engineered bacteria can play a dual role under field conditions as a biocontrol agent against nematode and subsequently improve plant productivity.

Keywords:
bioassay, identification, isolation, *Meloidogyne incognita*, protoplast fusion, rhizobacteria

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Introduction

Field and horticultural plants are cultivated principally for food and fruits productivity. On the contrary, medicinal plants are cultivated for traditional herbal remedy and extraction of phytochemicals for drugs. All plants are affected by many diseases and pests including plant-parasitic nematodes, resulting in great losses in quality and quantity of their constituents. Root-knot nematodes, *Meloidogyne* spp., are serious problem affecting plants, and their infestation results in root galling, stunted growth, and low productivity of the plant. Chemical nematicides are routinely applied for protection of plants. Residues of these toxic compounds remaining in/on harvested plant parts may be further carried to final products which are sold and subsequently consumed by consumers, leading to risks of contamination, because they are quite hazardous to human health and polluting to water basins. This incites scientists for working on the alternatives to nematicides. Biological control using antagonistic microorganisms appears to be a promising alternative strategy in the management of root-knot nematodes. Plant growth-promoting rhizobacteria, which are the first defense line against nematodes attack, are gaining importance in controlling nematodes and promoting plant growth [1–7]. Now, through biotechnological approaches,

like protoplast fusion, scientists could improve the activity of such microorganisms [8]. The concentration of δ-endotoxin of *Bacillus thuringiensis* fusion was 1.48 times more than the wild type [9]. The intrageneric fusants between *P. fluorescens* and *P. aeruginosa* strains were more effective than their parental types in reducing nematode as well as enhancing plant growth [10]. Moreover, the intergeneric fusants between *Serratia* spp. and *Pseudomonas* spp. induced high mortality levels against *M. incognita* [11].

The objectives of this study are (a) to identify two isolated rhizobacterial strains have nematicidal activity by 16S rDNA, (b) improve their nematicidal efficiency through protoplast fusion technique, and (c) to evaluate the biocontrol potency of the fusants against *M. incognita* J_2 in comparison with their parents under laboratory conditions.

Materials and methods

Collection of samples and bacterial strains isolation

Soil samples were collected at 10–15 cm depth (with help of sterile spatula in a sterile plastic bag) from rhizosphere region of cucumber and banana plants from Gazirat El-Dahab, Giza, Egypt. Bacterial colonies were isolated from soil samples as previously described in the literature [12].

Genomic DNA extraction from the isolated bacteria

Genomic DNA was extracted from a single bacterial colony cultured on Luria-Bertani (LB) media [13] at 35°C for 18 h on a rotary shaker at 180 rpm, and the culture was centrifuged at 13 000 rpm for 5 min at 4°C. The pellet was subjected to genomic DNA extraction using the G-pin TM Genomic DNA Extraction kit (Promega, USA). The extracted DNA was used as a template for PCR for amplifying 16S rDNA gene.

Bacteria identification

According to the method of Gooma and Momtaz [14] with some modifications, the obtained DNA was amplified with universal primers, forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACG-ACTT-3′ producing amplicon of 1500 bp. In this reaction, amplification was carried out in a 50 µl reaction mixture by using a PCR master mix kit (Promega, USA) according to manufacturer's instruction using a Gene Amp PCR system 400 thermal cycler (Perkin-Elmer Corp., Norwalk, CN, USA). The following program was used: 94°C for 3 min as initial denaturation step followed by 30 cycles at 94°C for 1min of denaturation, annealing at 55°C for 1min and 30 s and extension at 72°C for 1 min. PCR product was purified by wizard Sv Gel and PCR cleaning up system kit (Promega, USA). The purified product was sequenced using the same primers nucleotide. Sequence was determined with the dideoxy-chain termination method using the PRIM Ready Reaction Dye terminator/Prime cycle sequencing kit (PerkinElmer Corp., Norwalk, CN, USA). The obtained sequences were analyzed for similarities with other known sequences found in the GenBank database using BLAST program of the NCBI database. Isolated bacterial colonies were identified by 16S rDNA to *Pseudomonas aeruginosa* (accession number LC187271) and *Bacillus licheniformis* (accession number LC187270).

Antibiotic susceptibility/resistance of isolated bacteria

Nine antibiotics used at a certain concentration of each antibiotic were listed in Table 1 for determination of the susceptibility/resistance of the isolated bacterial strains to antibiotics. For protoplast fusion, the antibiotic marker was used as genetic marker; the two parental strains must have different antibiotic susceptibility/resistance.

Protoplast formation and fusion

Protoplast were formed and fused between the two isolated bacterial strains known to have nematicidal potential from preliminary investigation and termed as GAZ1 and GAZ2 [15,16].

Protoplast regeneration

The cells were re-suspended in 0.5 ml LB medium containing 0.5 M sucrose, and serial dilutions were immediately spread on soft agar LB plates containing 8 g/l agar and 0.5 M sucrose. After growth on these plates, individual colonies were counted and patched onto plates containing LB agar, with kanamycin (40 µg/ml) and neomycin (40 µg/ml) as markers to isolate fusants. Colonies growing on these plates were obtained after growth

Table 1 The antibiotics and their concentrations used in this study

Number	Antibiotics	Final concentration (µg/ml)
1	Ampicillin (Amp)	100
2	Kanamycin (Km)	40
3	Streptomycin (Sm)	200
4	Neomycin (Nm)	40
5	Tetracycline (Tc)	15
6	Erythromycin (Erm)	20
7	Rifampicin (Rif)	100
8	Gentamicin (Gm)	15
9	Chloramphenicol (Cm)	35

at 30°C for 48 h, counted and patched onto the same plates twice more to confirm their ability to grow in the presence of the two antibiotics. Control experiments were carried out using the aforementioned procedures but without either addition of PEG6000 or mixing of the protoplasts formed from the two mutants [15].

Evaluation of the nematicidal effect of *Pseudomonas aeruginosa* and *Bacillus licheniformis* and their fusants on *Meloidogyne incognita* J_2 mortality under laboratory conditions

For bioassay test, *M. incognita* eggs were extracted from the infected tomato roots that carry egg masses [17], and then incubated in egg hatching plastic cups at laboratory temperature of 24±3°C for 72 h. to provide *M. incognita* J_2. Petri dishes 6 cm in diameter were supplied separately with one ml from the bacterial suspensions (parents and fusants) which had the same OD of 0.9 at 600 nm on spectrophotometer plus 4 ml of nematode suspension in distilled water containing 100±5 freshly hatched *M. incognita* J_2. A volume of 5 ml of distilled water containing 100±5 freshly hatched *M. incognita* J_2 served as control. All treatments and control were replicated five times. All dishes were kept in incubator at 35°C. Dishes were loosely covered to permit aeration and lessen evaporation. Number of live and dead individuals was counted after 24 h for 3 days using 1 ml nematode counting slide. After the exposure periods, the nematodes in each treatment were transferred to distilled water and left for 24 h to observe whether immobile nematodes resumed activity or not. The corrected percentages of nematode mortality were calculated according to the following equation: mortality (%)=$(m–n)/(100–n)\times100$, where m and n indicate the percentages of mortality in treatments and control, respectively [18].

Results and discussion

PCR amplification of the 16S rDNA gene of the isolated bacterial strains

PCR amplification of the 16S rDNA gene has been used extensively to study prokaryote diversity and allow identification of prokaryotes as well as the prediction of phylogenetic relationships [19–21]. The two isolated bacteria, which have nematicidal potential from preliminary bioassay study and different morphological characteristics, were selected and directed for PCR amplification of the 16S rDNA gene. Nucleic acids extracted from the formed bacterial colonies were used to amplify a region of the 16S rDNA gene, and all produced an expected size (ca. 1520 bp).

16S rDNA gene sequence analysis of isolated GAZ1 and GAZ2 strains

Molecular identification of the two isolated strains, GAZ1 and GAZ2, was carried out based on 16S rDNA sequence analysis. The partial sequence of 16S rDNA obtained from the isolated bacterial strains was aligned with all the presently available 16S rDNA sequences in the GenBank database nucleotide. Sequence of 16S rDNA gene of GAZ 1 had revealed that strain GAZ1 had a similarity of 98% with *B. licheniformis*, and its nucleotide sequence was deposited in GenBank under accession number LC187270. Moreover, strain GAZ2 of 16S rDNA gene nucleotide sequence had a similarity of 97% with *Pseudomonas aeruginosa*, and its 16S rDNA nucleotide sequence was deposited in GenBank under accession number, LC187271.

Antibiotic susceptibility/resistance of bacterial parental types

Table 2 indicated that *P. aeruginosa* strain was resistant to kanamycin and *B. licheniformis* strain was resistant to neomycin. Kanamycin and neomycin were used as genetic markers for selecting and isolating the bacterial fusants.

Formation of protoplast from the bacterial parental types

The two bacterial parental types were treated with lysozyme for protoplast formation, fused and regenerated by using PEG, and then added to nutrient agar (NA) plates supplemented with antibiotics (kanamycin and neomycin) for fusants selection (Fig. 1).

Fusants selection

In this experiment, kanamycin and neomycin were added in nutrient medium as genetic markers, where kanamycin will prevent the growth of *B. licheniformis* and neomycin will prevent the growth of *P. aeruginosa*. Only the fusants which combined the two characters will grow in this selected medium.

Fusant stability studies

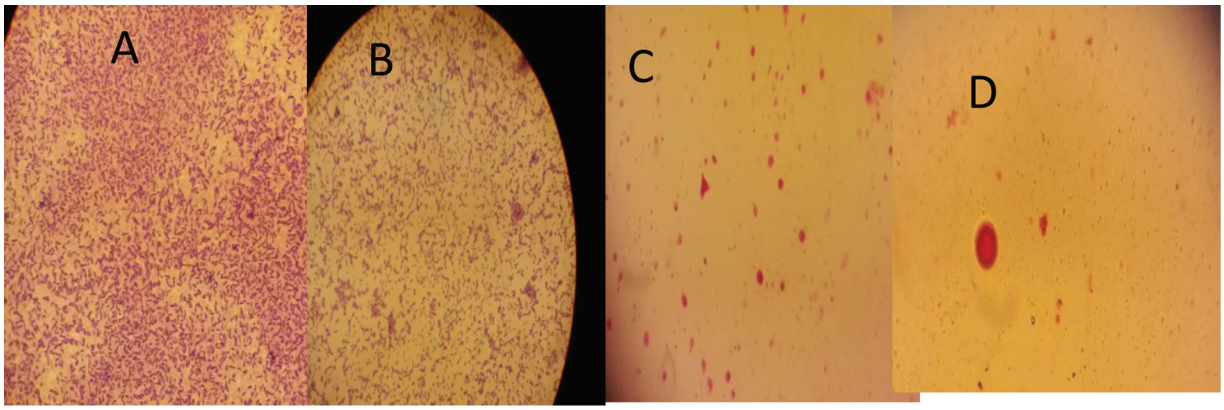
Table 3 indicated that 10 fusants were stable, where they behaved with the same growth efficiency (++), that

Table 2 The obtained antibiotic resistant patterns of *Pseudomonas aeruginosa* and *Bacillus licheniformis* strains

Bacterial strains	Antibiotic resistance	
	Km	Neo
PA	+++	–––
BL	––––	+++

BL, *Bacillus licheniformis*; PA, *Pseudomonas aeruginosa*.

Figure 1



(a) *Pseudomonas aeruginosa*, (b) *Bacillus licheniformis*, (c) protoplast formation, (d) protoplast fusion.

Table 3 Growth of *Pseudomonas aeruginosa* and *Bacillus licheniformis* parental strains and 32 of their fusants in NA plates with antibiotic markers

Fusants code	Growth in markers	Fusants code	Growth in markers	Fusants code	Growth in markers	Fusants code	Growth in markers
F1	++	F9	+	F17	+	F25	++
F2	+	F10	+	F18	+	F26	+
F3	+	F11	+	F19	++	F27	+
F4	+	F12	++	F20	+	F28	++
F5	+	F13	++	F21	+	F29	++
F6	+	F14	+	F22	+	F30	+
F7	+	F15	++	F23	+	F31	++
F8	+	F16	+	F 24	+	F 32	++
PA	–	BL	–				

++, good growth; +, low growth; –, no growth; BL, *B. licheniformis*; F, fusion; PA, *P. aeruginosa*.

is good growth, and 22 fusants showed low growth (+), using ++ and + to illustrate the difference in growth between the obtained fusants.

Nematicidal activity of *Bacillus licheniformis* and *Pseudomonas aeruginosa* strains and their fusants against root-knot nematode *Meloidogyne incognita* J_2 under laboratory conditions

The nematicidal effect of *P. aeruginosa* and *B. licheniformis* as parental strains and 10 protoplast fusants to immobilize or kill root-knot nematode, *M. incognita* second-stage juveniles (J_2), was investigated under laboratory conditions. As illustrated in Table 4, all tested bacterial strains had a lethal effect on *M. incognita* J_2 as shown by the greater mortality percentage than in the control. The reduction in the movement was irreversible, and the mortality of the juveniles was confirmed when they were transferred to distilled water for 24 h. The fusants were found to be more effective than their parental strains in killing *M. incognita* J_2. The increase in the juveniles' mortality positively correlated with the length of exposure period. The two parental strains *B. licheniformis* and *P. aeruginosa* induce 85 and 86% mortality, respectively, as compared with control, whereas the fusants recorded 91–99% mortalities after 72 h of exposure as compared with control (Table 4).

Table 4 Effect of *Bacillus licheniformis*, *Pseudomonas aeruginosa*, and 10 fusants on *Meloidogyne incognita* J_2 mortality under laboratory conditions

Treatments	% mortality of *Meloidogyne incognita* J_2		
	24 h	48 h	72 h
BL	85	85	85
PA	85	85	86
F1	87	93	94
F13	83	85	91
F16	76	91	95
F17	91	94	95
F22	88	92	94
F28	96	98	99
F40	92	95	98
F45	88	93	96
F46	86	91	99
F50	87	91	92
Control (water only+nematodes)	0	0	0

BL, *B. licheniformis*; F, fusion; PA, *P. aeruginosa*.

Rhizobacteria act against plant-parasitic nematodes by producing metabolic by-products, enzymes, and toxins

which suppress nematode reproduction, egg hatching, gall formation, and juvenile survival [22]. Some rhizobacteria can also act indirectly through stimulating the plant development and as a result, decreasing the damage related to nematode infection [23]. The obtained data showed that the recombinant DNA of *B. licheniformis* and *P. aeruginosa* in a single organism reflected on better killing of *M. incognita* J_2; the fusants were more potent in killing nematodes than their parents [9–11]. The hydrogen cyanide from *P. aeruginosa* in combination with the nematicidal activity of protease and chitinase produced by *B. licheniformis* enhanced the nematicidal effect of the fusants as compared with their parents [24–26].

Conclusion

From the present experiment, it might be concluded that protoplast fusion is considered as a classical or fundamental method of gene transfer in which multiple genes can be introduced, and it allows recombination to take place not only between related species but also between unrelated genera to improve bacterial strains activity. Recombinant DNA of *B. licheniformis* and *Pseudomonas aeruginosa* in a single organism can greatly reduce the effect of root-knot nematodes, *Meloidogyne* spp., on the host plants and improve their productivity under field conditions without depending on agrochemical products.

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Conflicts of interest

There are no conflicts of interest.

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