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Antagonistic *Bacillus thuringiensis* isolates from citrus rhizosphere effective against *Phytophthora nicotianae*

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Abstract

Citrus root rot, caused by *Phytophthora nicotianae*, is one of the most important diseases affecting citrus trees worldwide. This study aimed to isolate, identify, characterize, and evaluate the antagonistic effect of bacilli rhizobacteria isolated from the citrus rhizosphere in Kerman province, Iran, against *P. nicotiana*. According to the *in vitro* dual culture bioassays, five out of 67 *Bacillus*-like isolates showing the highest antagonistic effect were selected and characterized using phenotypic and PCR-based molecular tests for identification. Phenotypic characteristics and nucleotide sequence analyses of the 16S rRNA gene revealed that the isolates were highly similar and belonged to *Bacillus thuringiensis*. Findings enhance our understanding of the importance and potential role of *B. thuringiensis* isolates as biocontrol agents against *P. nicotianae*, offering a promising alternative to chemical fungicides for managing citrus root rot disease in integrated management and sustainable agriculture programs. These five antagonist isolates could be further evaluated in greenhouse and field experiments for their commercial exploitation.

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Introduction

Horticultural products in Iran have been about 22.4 million tons and among them, the highest share is for apples, citrus, grapes, greenhouse cucumbers and date palm with 18%, 14.7%, 12.4%, 6.8% and 6.7%, respectively. Citrus grown in over 150 countries from five continents with the highest share (51.7 %) for Asia. Iran is among the top 5 citrus producing countries in Asia with 137.5 thousand hectares of bearing citrus orchards and 3.9 million tons citrus production (FAOSTAT, 2023). Mazandaran, Fars, Hormozgan, Kerman, Gilan, Khuzestan, Golestan and Bushehr are the most citrus producing provinces of Iran with more than 98% of Iran's citrus production (Ahmadi et al., 2022).

Citrus is affected by various soilborne pathogens, particularly *Phytophthora nicotianae* which causes severe root rot and gummosis worldwide and a 10-30% yield reduction. Using chemical pesticides in agriculture has led to environmental pollution, increased resistance in plant pathogens, and elevated production costs. Given these concerns, research efforts have shifted towards alternative strategies such as biological control (Nagorska et al., 2007). Biological control of plant diseases is considered more cost-effective, sustainable, environment-friendly, and safe for human health than chemical control. Among the biocontrol agents, *Bacillus* species have a prominent position due to their ability to form endospores, plant growth-promoting hormone and antibiotic production, and induce plant resistance. Several studies have demonstrated the biocontrol potential of *Bacillus* spp. against plant pathogens, making them promising candidates for controlling soilborne diseases (Mahadtanapuk et al., 2007). This study aimed to isolate *Bacillus* species from citrus rhizosphere in southern Kerman and evaluate their potential as biocontrol agents against *P. nicotianae*.

Materials and Methods

Isolation of citrus rhizosphere-associated bacilli

Soil samples were collected from healthy or symptomatic citrus rhizosphere of different citriculture area of southern Kerman Province, Iran. Ten grams of each soil sample were added to an Erlenmeyer flask containing 90 mL of distilled water and homogenized thoroughly by shaking at 120 rpm for 30 minutes. Subsequently, 4.5 mL of the suspension was incubated in a water bath at 80°C for 10 minutes to eliminate non-spore-forming bacteria. After being serially diluted up to 10^{-5}, 100 µL aliquots of each dilution were spread on nutrient agar supplemented with sucrose (NAS) and incubated at 30°C for 24 hours (Schaad et al., 2001). *Bacillus*-like single colonies were selected for purification, amplification, and subsequent experiments. A characterized *P. nicotianae* strain, previously isolated from symptomatic root rot citrus trees (kindly provided by Dr. Mousa Najafinia, IRIPP), was cultured on potato dextrose agar (PDA) and used in subsequent experiments.

In vitro screening against *P. nicotianae*

Preliminary screening of the isolated bacteria against *P. nicotianae* was determined using a dual culture method (Schaad et al., 2001). Bacterial isolates were spot-inoculated at four equidistant points, 4 cm away from the *P. nicotianae* mycelial plug placed at center of the petri plates containing Potato dextrose agar and Nutrient agar (50:50 w/w) medium and incubated at 25°C. Plates were assessed for the presence/absence of a growth inhibition, 5 days after inoculation. The isolates exhibiting antagonistic activity were selected for further analysis. Selected isolates were spotted in a triple-point pattern on the same medium and the size of growth inhibition zone was recorded after 5 days. The experiment was carried out in a completely randomized design test in 3 replicates. Data were analyzed using ANOVA with SAS software, and mean comparisons were performed using Duncan's multiple range test at a 5% significance level.

Phenotypic characteristics of the isolates

The five top antagonist isolates were subjected for some key phenotypic tests, including colony morphology, Gram reaction, KOH 3% solubility, catalase, oxidase, oxidative/fermentative, starch hydrolysis, gelatin hydrolysis, levan formation, and lecithinase production (Schaad et al., 2001). Surfactin production of the isolates was assessed by spot-inoculation on NA medium containing 7% fresh sheep blood, and recording the clear hemolytic zone after incubation at 28°C for 72 hours (Feignier et al., 1995; Fernandes et al., 2007). For protease production test, isolates were spot cultured on skim milk agar and evaluated for clear halo around the colonies 48 hours after incubation at 28°C (Phyu et al., 2015).

Molecular identification and *16S rRNA* gene sequencing

Selected isolates were cultured overnight on nutrient broth at 28°C and the genomic DNA was extracted using a CTAB protocol (Ausubel et al., 1995). The extracted DNAs were used as the template for PCR amplification of a part of the 16S rRNA gene using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'- GGGCGGWGTACAAGGC-3') universal primer pairs (Marchesi et al., 1998) in 50 µl reactions containing 100 ng of template DNA, 10 Pmol/µl of each primer, and ready-to-use 2X Taq master mix (Ampliqon, Denmark). Reactions were performed in a peqSTAR (PEQLAB, Germany) programmed as follows: initial denaturation for 5 min at 94 °C followed by 35 cycles of 94 °C for 45 s, 56 °C for 60 s, 72 °C for 2 min and final extension for 10 min at 72°C (Weisburg et al., 1991). PCR products were electrophoresed on 1% agarose gel using TBE buffer containing DNA safe stain (SinaClone, Iran) at 80 V and visualized using a Gel Documentation System (Cleaver Scientific, UK). Amplicons were directly sequenced for both forward and reverse directions (Bioneer, South Korea). The sequences were manually checked in BioEdit (Hall, T.A. 1999), and sequence homology searches were performed using the online BLASTn search engine on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using MEGA5 (Tamura et al., 2011), and the phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap. *Pseudomonas fluorescence* was used as outgroup**.**

Results

In total, 67 *Bacillus*-like colonies were isolated from 145 citrus-rhizosphere samples collected from different citriculture area across the province. Screening results by dual culture bioassays showed that eleven bacterial isolates induced growth inhibition of *P. nicotianae* (Fig. 1) with different levels of antagonistic effect. Out of

eleven antagonist isolates, five isolates DM1, NM1, KB3, FD4, and GA5 showed high antagonistic activity against *P. nicotianae* (Fig. 2). The mean comparison of inhibition zone among the isolates shows that DM1 and NM1 isolates exhibited the strongest inhibition zone (15 mm) and were therefore classified in group 'a' (Fig. 2). The colonies were circular, 2-5 mm in size, white color, opaque, dry, aerobic, positive for gram reaction, catalase, and starch hydrolysis, but negative for oxidase, levan formation, lecithinase production, and gelatin and casein hydrolysis, indicating the isolates are belonging to *Bacillus* sp. (Table 1).

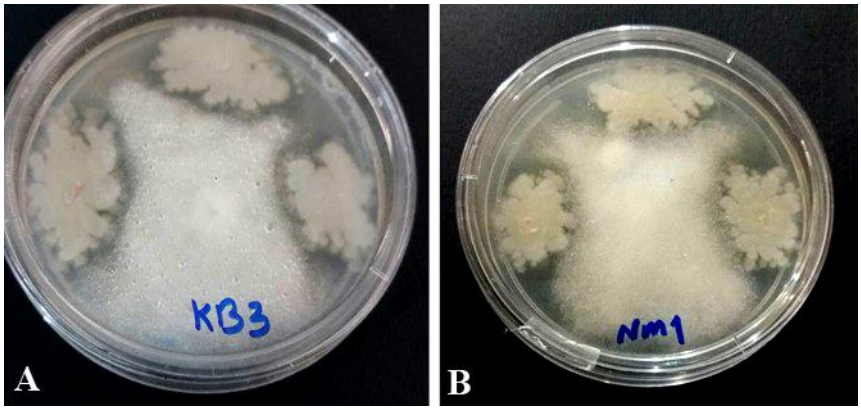

Fig. 1. Triple point dual cultures showing antagonistic activity of KB3 (A) and NM1 (B) isolates against *Phytophthora nicotianae*.

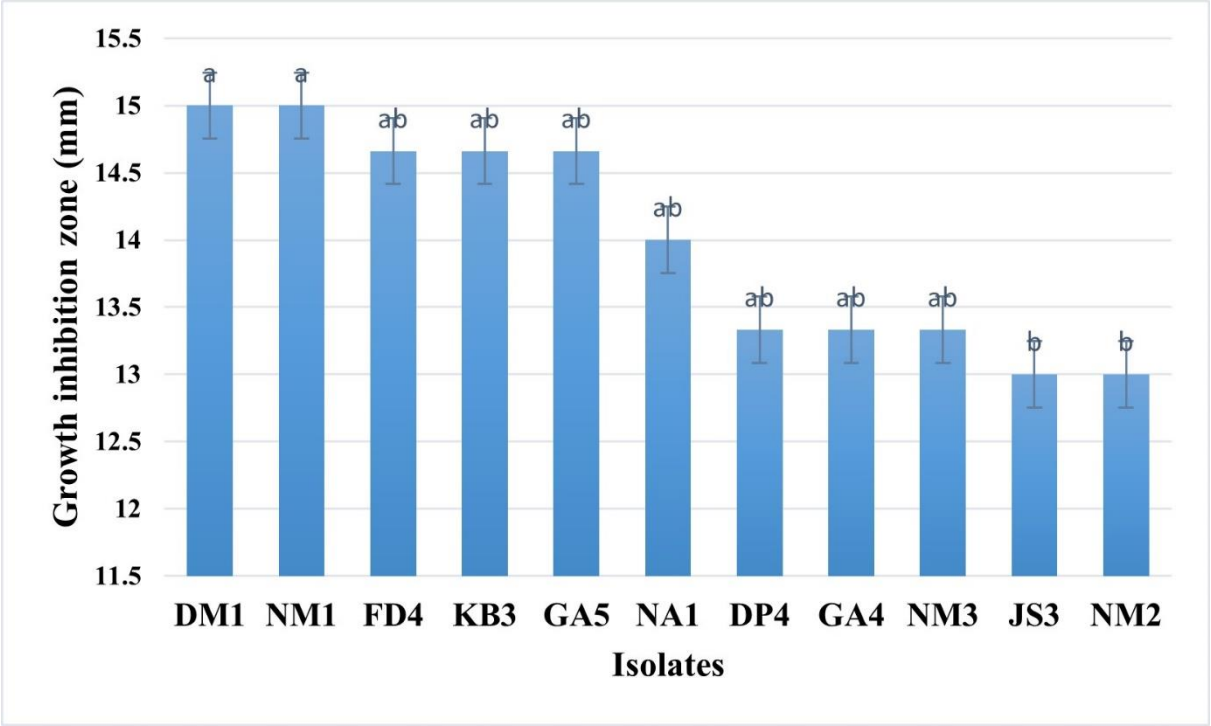

Fig. 2. Inhibitory effect of the eleven bacterial isolates against *Phytophthora nicotianae in vitro*. Each value is average of growth inhibition zone of three replicates. Values having the same letter are not significantly different according to the Duncan test ($p < 0.05$).

Hemolytic activity test indicated that all five isolates were able to produce surfactin leads blood cell lysis and a clearance zone. NM1 isolate with 15 mm clearance zone had the highest surfactin production (Table 1). Among the five selected antagonistic isolates, DM1 and

FD4 isolates were able to protease activity and showing inhibition zone (Table 1).

Table 1. Phenotypic and biochemical characteristics of the five selected isolates.

Bacterial isolates	Location	Sweet orange variety	Gram reaction	Catalase activity	Oxidase	Starch hydrolysis	Gelatin hydrolysis	Levan formation	Lecithinase production	Protease	Surfactin production clearance zone (mm)
DM_1	Jiroft-Dalfard	Washington Navel	+	+	-	+	+	-	-	+	15.0
NM_1	Jiroft-Narab	Washington Navel	+	+	-	+	-	-	-	-	15.0
KB_3	Anbarabad-Jabalbarez	Washington Navel	+	+	-	+	+	-	-	-	14.6
FD_4	Faryab-Faryab	Local variety	+	+	-	+	-	-	-	+	14.7
GA_5	Anbarabad-Jabalbarez	Washington Navel	+	+	-	+	-	-	-	-	14.6

+: Positive reaction, -: Negative reaction.

The PCR products of approximately 1.3 kb were amplified for all five isolates (Fig. 3). BLAST analysis of the sequences using the NCBI database showed that all isolates were identical nucleotide sequences and 100% similarity with the 16S rRNA gene sequences of different *Bacillus thuringiensis* strains. Therefore, all of the isolates were identified as *B. thuringiensis*.

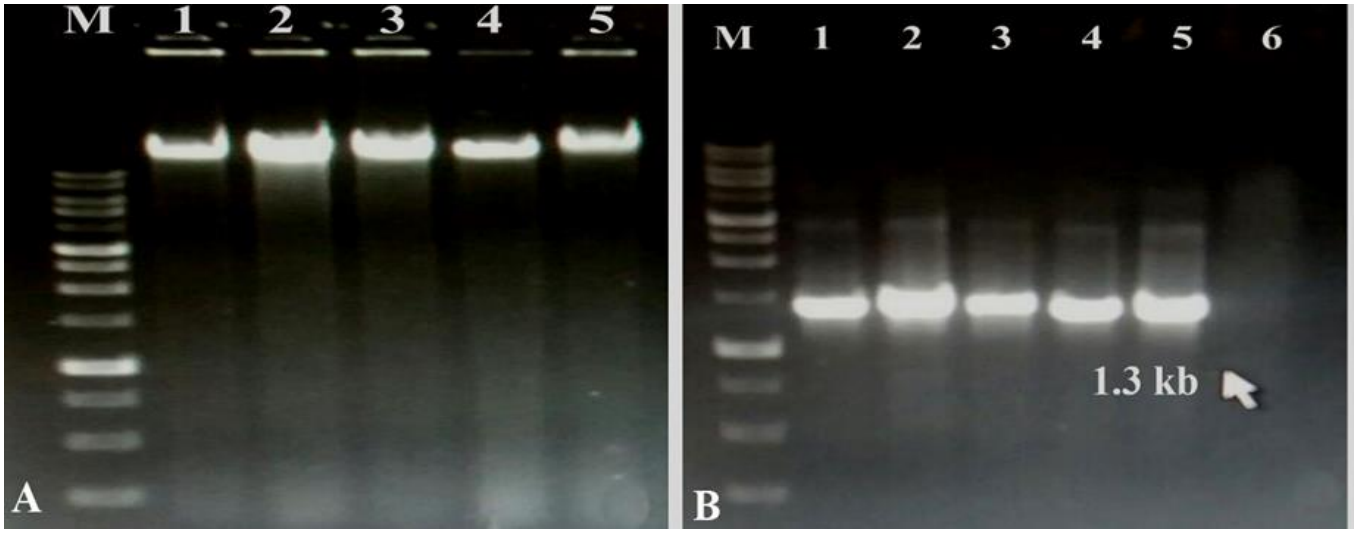

Fig. 3. Agarose gel electrophoresis of the extracted DNAs (A) and the PCR products (B) of the 16S rRNA gene. The isolates 1. DM1, 2. NM1, 3. KB3, 4. FD4, 5. GA5, 6. Negative control, M. 1 kb DNA size marker.

Phylogenetic tree constructed with the 16S rRNA gene sequences of DM1, NM1, KB3, FD4, and GA5 isolates confirmed all of the isolates were related to and clustered with *B. thoringiensis* strains (Fig. 4).

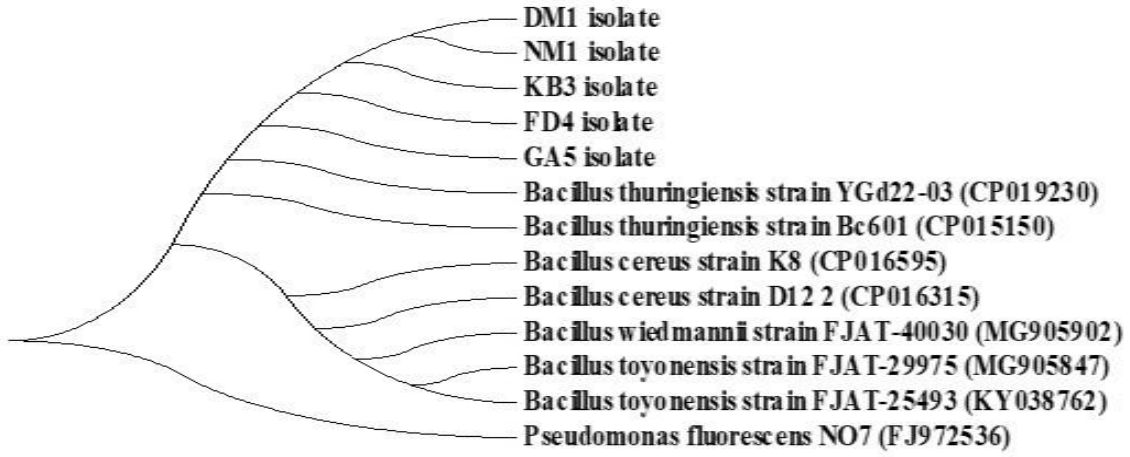

Fig. 4. Neighbor-Joining Phylogenetic tree of partially sequenced 16S rRNA gene of *Bacillus* isolates using MEGA 5.0 software.

Discussion

Biological control of soil-born plant pathogens using rhizobacteria has emerged as an effective and eco-friendly alternative to chemical fungicides for plant disease management (Emmert & Handelsman, 1999; Shoda, 2000; Bais et al., 2004; Cazorla et al., 2007; Ajilogba et al., 2013; Sethi & Mukherjee 2018). Rhizobacteria may inhibit plant pathogens through multiple mechanisms, including production of antimicrobial substances such as antibiotics, siderophores, HCN, and toxins, competition for space and nutrients, parasitism by extracellular cell-wall-degrading enzymes, promoting plant growth, and inducing plant defense mechanisms (Glick, 1995; Singh et al., 2008; Chung et al., 2008; Sharma et al., 2009).

Phenotypic tests revealed that all of the five top isolates with high inhibitory potential on mycelia growth of citrus root rot pathogen, isolated in Kerman Province were belonged to *Bacillus* sp. (family *Bacillaceae*). Among the different rhizobacteria, *Bacillus* species have widely been used in microbial control of pests mainly due to produce a variety of antimicrobial compounds and endospore formation, which provide resistance to harsh environmental conditions (Jacobsen et al., 2004; Grover et al., 2010; Ongena & Jacques, 2008). 16S rRNA gene sequence and phylogenetic analyses confirmed that these isolates were related to *B. thuringiensis*. Antagonistic activity of various *B. thuringiensis* strains against a wide range of plant pathogens, including *P. nicotianae*, have previously demonstrated in the other countries (Chung et al., 2008; Liu et al., 2010; Ashwini & Srividya, 2014; Rabinovitch et al., 2017). To the best of our knowledge, this is the first report of *B. thuringiensis* as a biological control agent on *P. nicotianae*, citrus root rot pathogen, in Iran. Further studies should be aimed on biocontrol activity of the isolates under greenhouse and field experiments.

Bacillus species produce various secondary metabolites, including antifungal antibiotics such as surfactin with a broad spectrum of antibiotic activity against phytopathogens and inhibit the growth and germination of fungal and bacterial plant pathogens (Ongena & Jacques, 2008; Liu et al., 2010). These antibiotics offer several advantages over traditional pesticides by low toxicity, high biodegradability, and environmental compatibility. In the current study, surfactin production and protease activity could have an important role in *P. nicotianae* mycelial growth inhibition. The bacillus strains isolated in our study could be considered as potential candidates for biological control of citrus root rot disease and its causal agent. In addition, *Bacillus* species also enhance plant growth by secreting phytohormones that stimulate root regeneration, allowing plants to recover more quickly from pathogen-induced damage, thereby reducing disease impact (Sadfi et al., 2002; Kloepper et al., 2004; Dworkin et al., 2006; Kumar et al., 2012). Applying biopesticides reduces the need for pesticides and chemical fertilizers (Ohno et al., 1995).

This study is considered to be the first step in identifying potential *B. thuringiensis* for biocontrol of citrus root rot disease caused by *P. nicotianae* We suggest that *B. thuringiensis* DM1, NM1, FD4, KB3 and GA5 strains isolated from citrus rhizosphere in Kerman

Province are feasible to be used for development of commercial biopesticide.

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Conflict of interest

The authors declare that they have no conflict of interest.

CRediT author statement

M. Azavdar: Supervision, methodology, writing & reviewing. **P. Mirzaee:** Laboratory works & sampling.

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