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Molecular Detection of *bae16* and *bace16* of *Bacillus subtilis* and their Bio-control Efficiency Against Nematodes that Infect Cucumber Plants

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Abstract: This study aims to detect the qualitative detection of *Npb* and *Apb* genes responsible for producing neutral proteases (*Bea16*) and alkaline serine proteases (*Bace16*), which are among the main proteases produced by *Bacillus subtilis* that affect nematodes, and determine the extent of their effect on nematode cuticle degradation with increasing exposure time. *B. subtilis* is a wild type of bacteria present in the soil and can produce various proteases, some of which may be effective against nematodes. In the current study, *B. subtilis* was isolated from the soil and identified molecularly based on a conserved region in the *16S rRNA*. Real-time PCR amplified the *Npb* and *Apb* genes extracted from *B. subtilis* for the qualitative detection of their genes, and the results of the qualitative detection showed the presence of genes at C_t 27.91, 27.33 for *Npb*, and C_t 33.51, 35.09 for *Apb*, respectively. After nematodes (second-stage juvenile) isolates were treated with 10, 20, 30, 40, and 100% crude extracts at 24, 48, and 72 h, the in vitro experiments after 72 h of exposure showed that 30, 40, and 100% crude dilutions had the highest event mortality rates, with averages of 90.7%, 97.3%, and 96.7%, respectively. This is due to the crude extract containing some proteases produced by *B. subtilis* that can affect nematodes, including neutral and alkaline serine. This study found that among the various proteases produced by *B. subtilis* that can impact nematodes, *B. subtilis* can make two intriguing ones: *Bae16* and *Bace16*. These proteases, along with others, may degrade the nematode's cuticle.

Keywords: Proteases, Qualitative detection, Nematodes mortality, *16S rRNA* gene.

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Introduction

Cucumber (*Cucumis sativus L.*) is one of the most significant and extensively distributed vegetable crops in the *Cucurbitaceae* family, due to its fast development, early maturity, copious output, and persistent demand for fresh consumption (1). Nematode-caused plant root disease ranks first with an infection rate of up to 3,000 plant families; in Iraq, 120 plant families have been documented as having this disease, which makes it occupy the first place in the group of plant pathogens(2). It's a devastating cucumber crop disease that stunts development and decreases yields; in extreme cases, production losses of up to 50% have been recorded (3). Nematodes, which feed on plant roots, especially on cucumber, have caused extensive harm to crops and other

plants, unfortunately, chemical pesticides are now the only option for dealing with this issue (4). Today, environmental contamination and the depletion of water and soil resources are on the rise due to the improper use of chemical pesticides in farming (5). Consequently, contemporary agricultural systems are turning their attention to bio-farming, which utilizes beneficial bacteria (6). Bacteria that live in harmony with root systems improve growing circumstances in several ways and can even make plants more resistant to various environmental pressures, both living and nonliving (7). Previous studies had shown that *Bacillus spp.* and *Pseudomonas spp.* were the most common populations that colonized the rhizosphere and could effectively antagonize root-knot nematodes for their nematocidal activity (8). Isolates of *Bacillus spp*., and particularly *B. subtilis*, have long attracted the interest of several researchers among the probiotic bacteria found in the rhizosphere (9). Numerous species of *Bacillus* are present in the root system and are thought to be effective against nematodes. Nematicidal action is attributed to secondary metabolites generated by Bacillus spp., according to several studies (10). In addition, *B. subtilis B16* is volatiles have a variety of impacts, such as nematicide activity and the induction of plant resistance (11). The neutral protease *Bae16* and the extracellular alkaline serine protease *Bace16* are the kinds of extracellular proteases that bacteria release, and they kill nematodes by destroying their intestinal tissue (12). The bacterium *Bacillus B16* produces potent volatile organic compounds (VOCs) that are much more effective on worms than those from ordinary dietary bacteria and successfully attract its hosts. Then they turn into pathogenic factors on parasites., mainly including a neutral protease *Bae16* and an extracellular alkaline serine protease *Bace16*, which are responsible for the death of nematodes (13). Serine protease is linked to strong nematicidal activity, according to a study of the *Bae 16* and *Bace 16* proteins (14). This study aimed to qualitative detection of *Npb* and *Apb* genes isolated from *B. subtilis* and verify that their *Bae16* and *Bace16* proteases are involved in the degrading of nematode cuticles and that they have nematocidal activity.

Material and methods

Nematodes collection

Following the Sohrabi *et al.,* (15) method, a cucumber field infested with root-knot nematode populations was randomly sampled from Iraqi regions, such as Al-Sulaymaniyaha/Banjan, Bazian, and Shuan Kara regions, Wasit/Rahmaniyah, Ghanaia, and Saouira and Tikrit/Khizamiah regions by collecting several samples from diseased roots and soil. In the lab, a magnifying glass was used to examine the infected roots and pick out individual egg masses on the surface of the galls that had developed. After being moved to a 16 cm culture dish the eggs were placed in an incubator set at a constant 28 °C for three to four days to get second-stage juveniles (J2s). The freshly hatched *Meloidogyne spp.* J2s were used in subsequent experiments.

Isolation of *Bacillus subtilis*

The Isolation protocol were used as mentioned by Abdulhussein and Hussein (16). The *B. subtilis* bacteria utilized in this investigation was obtained from cucumber fields in the Banjan district of Al-Sulaimania city, Iraq 35°35'29.3"N 45°08'27.4"E. The 10 g of rhizosphere soil was centrifuged with a mixture of 90 mL of distilled

water for 15 minutes at 150 rpm. The 10-fold series approach was used to dilute the supernatant. The LB (Luria-Broth-Agar) plates were covered with 150 µL of each dilution and left to incubate at 37 °C for 24 h. Each isolate was grown in Difco Sporulation Medium (DSM) broth for 48 h, and the cultures were incubated at 80 °C for 20 min to form spores. After identifying individual colonies, streak purification was carried out on LB plates. For future research, three independent cultures were kept on LB slants at 4 °C. The strains were kept at -60 °C in LB with 25% glycerol (v/v). (Figure 1). shows the isolated *B. subtilis* from cucumber rhizosphere soil.

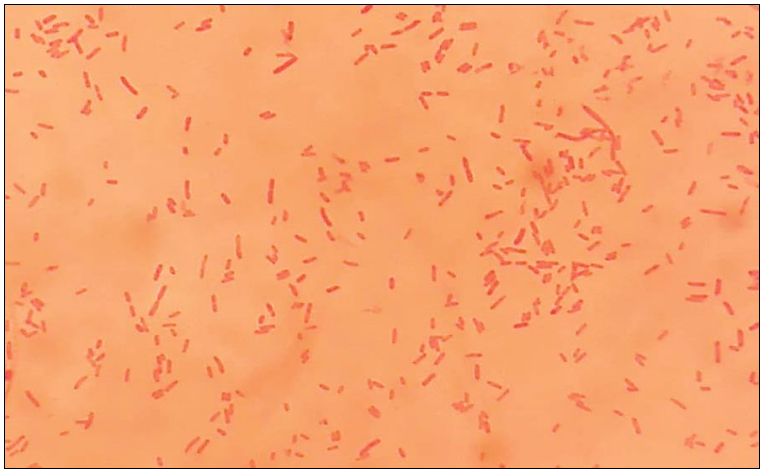

Figure (1): The exponential phase of *Bacillus subtilis* isolated from cucumber rhizosphere soil.

Bacterial DNA extraction

B. subtilis was cultured in LB medium overnight to extract total genomic DNA as described by Mezaal *et al.* (17). QIAprep® Spin Miniprep Kit: Quick-Start protocol of (18) was used to extract genomic DNA from *B. subtilis* bacteria. Pellet 1–5 ml bacterial culture was centrifuged at >10000 rpm for 3 min. Then, pelleted bacterial cells resuspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. After the transferred, 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times until the solution became clear. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube six times and centrifuged for 10 min at 12,500 rpm. The supernatant was transferred in the spin column and centrifuged for 30–60s, discarding the flow-through. The spin column was washed by adding 0.5 ml of Buffer PB and centrifuged for 30–60 s, discarding the flow-through. The spin column was washed with 0.75 mL of Buffer PE and centrifuged for 30–60s, discarding the flow-through. The residual wash buffer was removed by centrifugation. The QIAprep column was placed in a clean 1.5-ml microcentrifuge tube and 50 µl Buffer EB was added to elute DNA.

Molecular identification

The molecular characterization of isolated *B. subtilis* was based on conserved gene regions in the *16S* ribosomal RNA gene as mentioned by Koilybayeva *et al.* (19). PCR technique and OneTaq 2X Master Mix (Biolabs) were used to amplify the *16S rRNA* gene with PCR product size 478 bp, using newly designed primers by (Geneious R8.0 software), rpo F-

ATTACACGTGCCATTGCAGA and rpo R-ATTTGCGAAGTGCTTTTGCT. The PCR was performed as the following program: Initial denaturation at 94Cº for 3 min followed by 35 cycles of denaturation at 94Cº for 30 sec, annealing at 58Cº for 40 sec, and an extension at 72Cº for 35 sec. To amplify the specific gene sequence, the gold standard PCR technique. A 1.2 % gel electrophoresis was performed on the final result to determine the size of the produced fragments.

Qualitatively detection of *bae16* and *bace16* genes

Primers, reaction and program for RT-PCR

The qualitative detection technique was used, as according to Salh (20). The primer sequences used for the Real-time PCR reaction were F-Npb AAGCACGCTTCCTGGAAACAA, R-Npb TGTCCAGTTCGGGTGCTTAGA, and F-Apb AGCTACGACAATAAAGGCGGC, R-Apb GAACCGGAAAGAGGCGAGAAG. Each DNA sample was divided into doublecate reaction tubes for each gene. The Luna® Universal qPCR Master Mix was used to prepare the reaction and real-time PCR program as described by Maricic *et al.* (21). All PCR reactions are performed on a Bioer real-time PCR Linegene. The reaction components were thawed at room temperature. The assay mixer was aliquoted into PCR tubes. Then, the DNA template was added to the PCR tubes. The tube was quickly spin to remove bubbles. The real-time PCR reaction conditions for the *Npb* and *Apb* genes were as follows: 60-sec initial denaturation at 95°C by 1 cycle, 15 sec denaturation at 95°C, 30-sec extension at 60°C by 40 cycles, and a 7-min melt curve at 60°C by 1 cycle.

***In vitro* protease activity**

According to Niu *et al.* (14) method. *B. subtilis* was cultured in 2x Yeast Extract Tryptone medium for 70 h at 37 °C to reach a stationary face because of the way proteins are synthesized during this stage (22). Then, the bacterial culture was centrifuged at 8000 rpm for 15 min, the pellet was discarded while preserving the supernatant for further dilution preparations. 200 µl of bacterial crude at dilutions of 10, 20, 30, 40, and 100% were placed in 15 separate petri dishes. Egg masses of root-knot nematodes were collected from the infected cucumber roots and incubated in water for 3 days to allow eggs to hatch.

After 3 days, 50 of J2 was incubated at 30 °C for 3 days in a pitre dish with different crude dilutions from *B. subtilis*. A control group of juveniles-2 was placed in separate pitre dishes with distilled water. For every dilution, three separate replicates were conducted. Juvenile-2 mortality was monitored using an optical microscope at 24, 48, and 72 h intervals.

Statistical analysis

The SAS program, GenStat Release, Version: 12.1 software was employed to identify the impact of various crude dilutions on nematode mortality as mentioned by Alrikabi (23).

Results and discussion

Identification of *Bacillus subtilis* by *16S rRNA*

The sequence of the *16S rRNA* gene has been identified for *B. subtilis* to confirm bacterial isolation because the *16S rRNA* gene is universal in bacteria(24). The result showed that the PCR product for *16S rRNA* is 478 bp, as shown in (Figure 2).

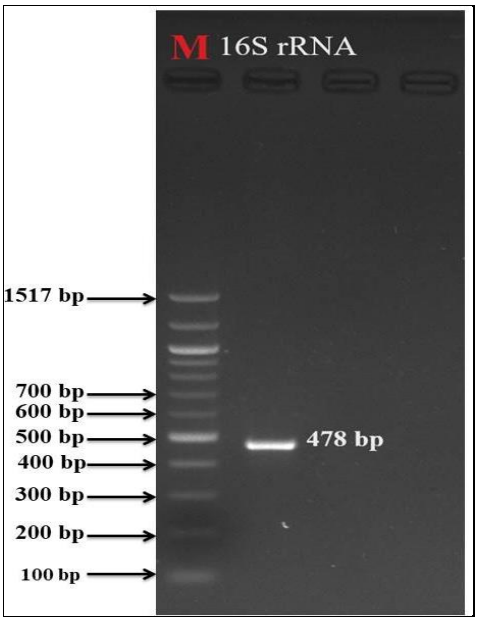

Figure (2): PCR product of the *16S rRNA* gene (478 bp) of the *Bacillus* isolate with Marker ladder 100–1517 bp.

Qualitative detection of *Npb* and *Apb* genes

The qualitative detection result of *Npb* and *Apb* genes appeared as C_t 27.91, 27.33, 33.51, and 35.09, respectively, and that means the responsible genes are present in the *B. subtilis* genome to produce the neutral protease (*Bae16*) and the extracellular alkaline serine protease (*Bace16*) in perfectly, This aligns with Deng *et al.* (13). The qualitative detection of *Npb* and *Apb* genes by Real-time PCR amplification is shown in (Figure 3).

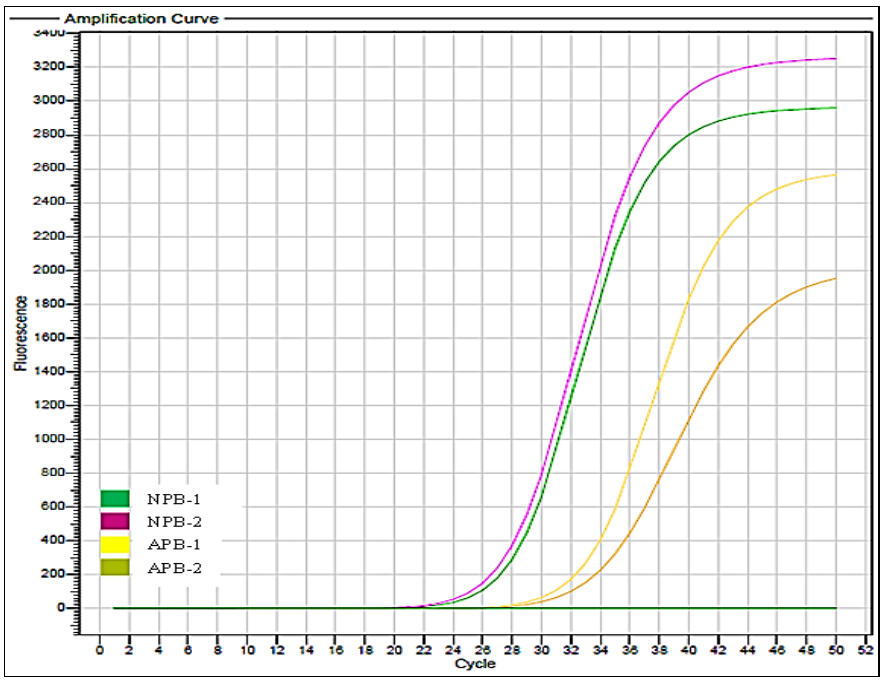

Figure (3): The amplification curve of *Npb* and *Apb* genes from *B. subtilis* by Real-time.

***Meloidogyne* mortality frequency**

The strain *B. subtilis*, with *Npb* and *Apb* genes that express the neutral protease (*Bae16*) and the extracellular alkaline serine protease (*Bace16*), respectively, was found to be promising in the degradation of root-knot nematodes cuticle and their deaths. The crude extract from *B. subtilis* exerted lethal effects on juveniles of the root-knot nematode as outlined by Kavitha *et al.* (25). The root-knot nematodes, *Meloidogyne*, were cultured with different crude dilutions extracted from *Bacillus* bacteria. This analysis demonstrated *Meloidogyne* juvenile mortality of 11.67 with a 100% crude dilution after 24 h, while no mortality was recorded with a 10% crude dilution after 24 h. That is, 10 and 20% crude dilutions at 24 h had no protease activity. A significant augmentation in mortality (an average of only 42.8 juvenile deaths) was observed at all dilutions after 72 h of exposure, as illustrated in (Figure 4). This result is in agreement with Kavitha *et al.* (25).

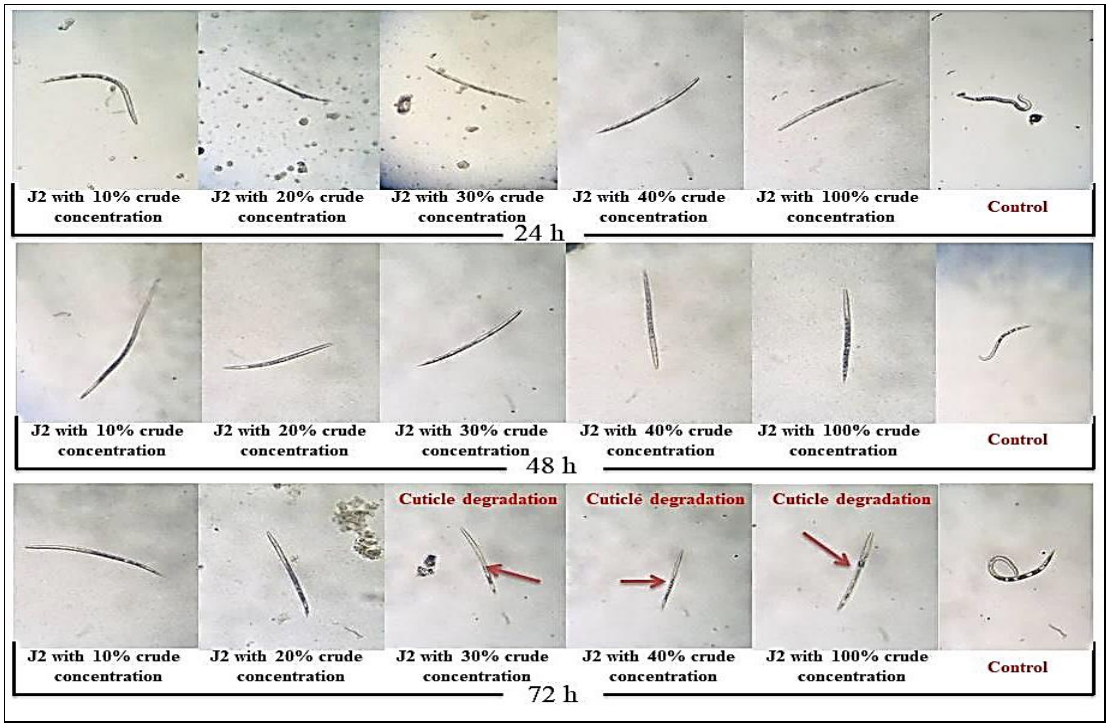

Figure (4): The effect of a crude *B. subtilis* extracellular protease on *Meloidogyne*: after 48 h, the nematode was dead and the cuticle was degraded. After 72 h, the cuticle was degraded specifically with 30, 40, and 100 crude dilutions by the action of *Bae16* and *Bace16* and other proteases.

As demonstrated by the decreased mobility and increased mortality rate with increasing exposure duration compared to the control, the crude extract also had a fatal impact on the nematode juveniles. Longer exposure times and higher dilutions of the crude extract were associated with a steady rise in the premature death rate, specifically with 40 and 100 crude dilutions. The greatest juvenile mortality (in averages of 90.7%, 97.3%, and 96.7%, respectively) was recorded in the 30, 40, and 100% dilutions of the crude extract after 72 h of exposure. Furthermore, after 72 hours of

exposure, the data demonstrated a very converging fatality rate at 30%, 40% and 100% crude dilutions, that may be result of increase in extracellular protease content. This finding is in agreement with Gautam and Azmi (26), who found that after 48 h of collagenase treatment, the worm bodies were fully digested. The result also show that there is no significant variance between the mortality rates in the 30, 40, and 100% crude dilutions at 48 h and the mortality rate in the 10% crude dilution at 72 h, as shown in (Table 1).

Table (1): The impact of different crude extracts from *B. subtilis* on nematode mortality at significant (P> 0.05).

Exposure time (hrs)	Control	Crude dilutions %/ 50 juveniles dead					Mean Exposure time
	0	10	20	30	40	100	
24	0(0%)	0.33(0.7%)	2.33(4.7%)	3.67(7.3%)	4.00(8%)	11.67(23.4%)	**4.40**
48	0(0%)	13.00(26%)	20.33(40.7%)	33.00(66%)	31.67(63.4%)	32.67(65.4%)	**26.13**
72	0(0%)	31.67(63.4%)	40.00(80%)	45.33(90.7%)	48.67(97.3%)	48.33(96.7%)	**42.8**
Cont-24	**0.00**						
Cont-48	**1.00**						
Cont-72	**1.33**						
LSD	**1.85**						**0.832**
Mean crude	0	15.00	20.89	27.33	28.11	30.89	
LSD	**1.074**						

Conclusion

Biological pesticides are an effective alternative to chemical synthetic pesticides, this research provides the ability of proteases as a biological control to kill nematodes and reduce their mobility. This study underscores that the neutral protease (*Bae16*) and the extracellular alkaline serine protease (*Bace16*) are two of the main proteases produced by *B. subtilis*, which cause the degradation of the nematode cuticle.

The longer these proteases are exposed, the more damage they may do to the nematode cuticle. One strategy to keep root-knot nematodes from infecting cucumber crops is to grow them in soil that is high in *B. subtilis* which can make proteases.

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