Identification and screening apple endophytic bacteria applied at different times for biological control of Alternaria rot on apple in Turkey

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Research Article

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Abstract

In this study, an isolate of *Alternaria alternata*, the causal agent of apple fruit rot, was isolated from rotten cv Amasya Apple and identified based on ITS gene analysis and morphological analyses. Out of 58 different endophytic bacteria isolated from internal tissues of apples, twenty-six isolates caused the radial growth inhibition of *A. alternata* in dual cultures. Based on 16S rRNA sequencing analysis, these endophytic antagonistic bacteria were identified as *Bacillus* ssp. *Bacillus methylotrophicus*(ib17) exhibited the highest significant inhibitory effect on the mycelial growth of *A. alternata* at a rate of 79.45% under *in vitro* conditions. The *in vivo* analysis revealed that the biocontrol efficiency of the *Bacillus licheniformis*(ib20) against *A. alternata* reached 100% with a treatment duration of 24 h before the introduction of *A. alternata*. When the bacteria and *A. alternata* were introduced simultaneously, *Bacillus amyloliquefaciens*(ib1), *Bacillus licheniformis*(ib21), and *Endophytic bacterium* (ib16) showed the highest biocontrol effect against *A. alternata* at 81.8%. Moreover, when the bacteria introduced 24 h after *A. alternata*, *Bacillus licheniformis*(ib21) showed the highest biocontrol efficacy at 83.3% against the fungus compared to the controls. The endophytic bacteria could produce fungus cell wall hydrolyzing enzymes such as chitinase, cellulase, amylase, and protease. According to the results of this study, apple endophytic bacteria are a potential candidate to control apple rot caused by *A. alternata* due to cell wall hydrolyzing enzymes, as well as to reduce disease severity. However, further research needs to be carried out on the biochemical basis of their activity against *A. alternata*.

Introduction

Turkey is one of the major producers of apples (Malus domestica Borkh.) in the world with a production capacity of almost 3.6 tons per year. There are over 465 apple cultivars in Turkey, each with various qualities. Owing to fruit cultivars, the 'Amasya Apple' is one of the best among these local apple varieties. It has a deep red skin color, very crispy white flesh color, contained in an asterisk, pleasant taste, and long storage life without major loss of features. However, fungal pathogens are primarily responsible for major financial damage in the apple production sector. Black spot rot caused by *Alternaria alternata* is becoming a significant disease in Amasya apple fruits in Turkey (Erturk and Akcay 2010). This fungus could quickly grow at low temperatures and cause significant post-harvest damage during the storage period on apples (Yan et al. 2015). At the same time, *A. alternata* can produce extraneous metabolites considered both mycotoxins and phytotoxins, which can be harmful to humans, and it causes food safety problems (M. Wanget et al. 2017). To control this pathogen, the use of chemical fungicides is one of the main effective methods. The use of synthetic fungicides such as mancozeb, tebuconazole, and iprodione has environmental contamination effects (Yuan et al. 2019). Moreover, the use of these chemicals progressively induces the emergence of synthetic fungicide-resistant fungus strains and raises significant concerns regarding human health and environmental hazards (Zouari et al. 2016). For this reason, eco-friendly alternatives to chemical fungicides are necessary (Ghazanfar et al. 2016). Currently, the use of bacteria as biocontrol agents is an alternative to chemical fungicides (Liu et al. 2013). Endophytic bacteria are widely present in plant tissues such as the endosperm, roots, leaves, stem, flowers, and fruits. These bacteria have a non-pathogenic symbiotic life cycle associated with their host plant tissues. These endophytes can also be easily isolated from plant tissues (Costa et al. 2012). Endophytes take part in reducing the unfavorable effects of one or more phytopathogenic microbes or fungi. This effect occurs by the production of substantial antagonistic substances; for example, siderophores, hydrogen cyanide (HCN), antifungal compounds, and cell wall-hydrolyzing enzymes (chitinases, cellulases, amylases, and proteases) (Mahmood et al. 2009). The latest studies have reported that endophytic bacteria may play an important role in the biocontrol of phytopathogens. For instance, the endophytic bacterium *Bacillus velezensis* 8 − 4 could be considered a potential biocontrol agent for potato scab (Cui et al. 2020). Additionally, *Bacillus amyloliquefaciens* RWL-1 was found to be a biocontrol agent against *Fusarium oxysporum* f. sp. *lycopersici* in tomatoes (Shahzad et al. 2017). In another study, an endophytic antagonist bacterial isolate of *Pseudomonas putida* was determined to have a potential as a biocontrol agent for crown gall disease caused by *Rhizobium radiobacter* in apples (Bozkurt and Soylu 2019). For these reasons, the research and use of apple endophytic bacteria for biological control are significant for reducing or replacing the usage of synthetic pesticides and fertilizers for improving agroecosystems and maintaining biodiversity. This is particularly important for apple fruit spots because the pathogens that cause apple spots can survive in the skin for a long time, and chemical fungicides have relatively limited effects on them. Moreover, the endophytic bacteria of apples can colonize the apple for a long time, and their controlling effect on apple fruit spots will be more stable. However, there are very limited studies using apple endophytic bacteria as a biocontrol agent against phytopathogens including *A. alternata*. The aims of this study were as follows: (i) isolation and identification of *A. alternata* causing fruit rot on Amasya Apple and (ii) evaluation of the biological control potential of endophytic bacteria against *A. alternata* pathogen under *in vitro* and *in vivo* conditions.

Materials and methods

Isolation and identification of fungus

Amasya apples showing characteristic symptoms of brown rot were obtained from a tree nursery in Amasya, Turkey in August-October 2021. The causal agent of the disease was isolated from apple fruits as described by Kurt et al. (2020). The fruits were sterilized using NaOCl 1.5% for 6 min.. Rot tissue pieces were cut from the fruit samples using a knife and then divided into small parts (1.5 cm^2). These parts were placed on the surface of a potato dextrose agar (PDA) (potato: 200 g, glucose: 20 g, agar: 20 g, sterile water to 1000 ml, pH 7) medium supplemented with 250 mg/l of streptomycin and then incubated at 30°C for 5 d. The pathogenic fungus *A. alternata* was identified using microscopic observations and molecular methods with ITS1 and ITS4 primers spanning the ITS1, 5.8-S rRNA, and ITS2 regions, 5-TCCGTAGGTGAACCTGCGG-3 and 5-TCCTCCGCTTATTGATATGC-3 (White et al. 1990).The nucleotide sequences were assembled using the CAP program available on the NCBI website (http://www.ncbi.nlm.njh.gov/blast). The nucleotide sequences were deposited in the Gen Bank database, and the access numbers were obtained.

Pathogenicity tests of fungus

For pathogenicity testing, using healthy Amasya apples, three lesions (3 mm in width and 3 mm deep) were created at the lateral sides of each fruit with a sterile perforator. 100 µl conidia suspensions of *A. alternata* (2×10^2, 2×10^4, 2×10^6, and 2×10^8 spores/ml) were inoculated in each lesion, and sterilized distilled water served as the control. The test was performed in five repetitions. Apples were placed in sterilized plastic boxes and then incubated at 27°C for 7 day.

After the virulence of the fungus was defined, the growth of the brown rot lesions around the inoculated lesions was measured (in mm) and compared to the measurements of the control fruits (Mohamed and Saad 2009).

Isolation of endophytic bacteria

Different samples were collected from among healthy Amasya apples cultivated in the Amasya region of Turkey. The fruits were washed in running tap water and then surface-sterilized first in 70% ethanol for 5 min and then in 1% NaOCl for 20 min. Eventually, the fruits were fully washed thrice with sterilized distilled water. For sterility check, water collected from the final wash process was placed on a Nutrient Agar (NA) medium and incubated at 30°C for 24 h. The surface-sterilized apple fruits were dissected into 1-2-cm pieces, transferred to the NA medium and incubated at 30°C for 70 h for the development of colonies. Bacteria colonies were isolated based on phenotypic characteristics and purified using plate streaking techniques. Finally, each colony was stored at -20°C in 30% sterilized glycerol for testing antagonism (Sun et al. 2013).

Screening of antagonistic bacteria

The antagonistic properties of the endophytic bacteria against *A. alternata* were determined using *in vitro* dual culture confrontation assays on PDA plates following the method of Bektas and Kusek (2019) with some modifications. Briefly, 0.5-cm^2 discs of fungal mycelia of *A. alternata* were placed at the center of each 90-mm disposable plastic Petri dish containing PDA. A single colony of each bacterium was inoculated in 10 ml NB and incubated at 27°C for 72 h by continuous shaking (120 rpm). Pellets obtained by centrifuging the bacterial cultures at 5000 rpm for 20 min were re-suspended in sterile water at a concentration of 10^8 CFU ml^{-}. 10-µl endophytic bacterium suspensions were inoculated overnight around the fungal disc at a distance of approximately 25 mm from the fungus. For the control, a fungal disc was placed on PDA agar, but instead of endophytic bacteria, only 10 µl of sterile water was streaked. The experiments were conducted in triplicates. For comparison, the fungal growth inhibition effects of the endophytic bacteria against the pathogen were determined. All plates were incubated at 27°C for 10 day. After incubation, each antagonistic bacterium inhibition zone was measured, and the percent inhibition of radial/mycelial growth (PIRG) was calculated according to the following formula:

PIRG = (R1 − R2)/R1 x 100

Where R1 is the average diameter of the fungus on the control, and R2 is the average diameter of the fungus on the endophytic bacteria co-cultured plate.

Effects of antagonistic endophytic bacterium treatment on the control of black spot rot in apple fruits

The antifungal effect of the endophytic bacteria on black spot rot development on Amasya apple fruits was investigated based on the method reported by Ge et al. (2019) with minor changes. Healthy apple fruits (cv. Amasya) were harvested at commercial maturity from an orchard in Amasya and transported directly to the Suluova Vocational School's microbiology laboratory. The surfaces of the apple fruits were disinfected with 1.0% (v/v) NaOCl for 3 min, thoroughly washed with distilled water and then dried at room temperature (25°C). Then, 100 µl of the spore suspension of *A. alternata* at a concentration of 2×10^8 ml^{-} was injected into the uniformly sized lesions (7-mm deep, 3-mm-wide) of the fruit. The fruits were air-dried for 2 h, and the same volumes of bacterium solutions were injected into the same lesions (i) simultaneously, (ii) 24 h after *A. alternata*, and (iii) 24 h before *A. alternata.* The positive control apples were injected with *A. alternata* only, while the negative control apples were injected with distilled water only at the lesion sites. Each treatment included three replicates. Lastly, the fruits were incubated at 25°C in clean boxes by maintaining a humidity of 80–85%. After 10 day of incubation, the disease scores were measured. The disease severity index (DSI) was calculated according to the formula below:

(0 = no rot, 1 = rot area 0–20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, 5 = 80–100% with tissue rot), and the

Observations were converted to DSI values based on the method of Promwee et al.(2017) as follows: $DSI(\%) = [\sum(SXA)/MxT]/100$

S: Scale, A: Amount of fruit, M: Maximum level and T: Total number of fruits

The % of disease reduction (R) rate was calculated by using the Abbott formula: R(%) = (A − B)/Ax100

A = disease severity of positive control, B = disease severity of samples treated with endophytic bacteria against A. alternata

Genomic DNA extraction and 16S rDNA gene amplification of antagonistic bacteria

The genomic DNA of All bacteria was isolated using the enzymatic hydrolysis method (Y.-H. Liu et al. 2016). The bacteria grown in fresh cultures were sampled by approximately 1 ml in 1.5 ml Eppendorf tubes and centrifuged at high speed (14,000 rpm) to remove supernatants. Then, 50 mg of the bacterial samples was put into the pellet portions of the tube along with 480 ml of TE buffer and 20 µl of lysozyme solution (2 mg/ml). The bacterial suspension was incubated in a shaking hot water bath (37°C) for 2 h. The blend was then mixed with 50 ml of SDS solution (20%, w/v) and 5 µl of Proteinase K solution (20 mg/ml), and it was incubated for 1 h at 55°C in a water bath. Preparations were made for DNA extraction by treating the samples twice with a solution of phenol, chloroform, and isoamyl alcohol (25:24:1 v/v/v), followed by precipitation with 100 µl of sodium acetate (3 mol/l, pH 4.8–5.2) and 900 µl of absolute ethanol. After rinsing with 70% ethanol and allowing the samples to air dry, the resulting DNA precipitate was centrifuged at 5°C (12,000 rpm, 10 min. The extracted DNA was resuspended in 50 µl of sterile water and stored at -20°C for PCR reactions. Using the universal primers UB_16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and UB_16SR (5'-GTACGCTACCTTGTTACGAC-3'), bacterial endophyte isolates were identified by 16S rDNA gene partial sequencing (Mauti et al., 2013). For each reaction, amplification was carried out in 40-µl PCR tubes containing 1 µl MgCl2, 1 µl DNA, 4 µl Taq buffer, 0.5 µl Taq DNA Polymerase, 1 µl dNTPs, 2 µl of the primers, and 32 µl of nuclease-free dH_2O. The PCR instrument was optimized to operate at the following temperatures: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, elongation at 72°C for 1 minute, and final elongation at 72°C for 5 minutes. There were 35 iterations of the denaturation, annealing, and elongation cycles. The separation of the amplification products on 1% (w/v) agarose gel in 1X TBE buffer was followed by ethidium bromide dye staining and UV visualization.

Phylogenetic analysis and DNA sequencing of isolated endophytic bacteria

Using the Sanger sequencing method of PCR products, DNA sequencing was performed bidirectionally with the help of the 3130xl Genetic Analyzer (Applied Biosystems, USA). At 84–100% similarity on the species level, 16S rDNA sequence data were subjected to Basic Local Alignment Search Tool (BLAST) analysis on the National Center for Biotechnology Information (NCBI) and selected as the nearest phylogenetic neighbor (Hentschel et al. 2001). These sequences were then aligned using Clustal X v.2.1 (Larkin et al. 2007). Phylogenetic dendrograms based on the 16S rDNA gene sequences were then generated using the MEGA 11 software with the Maximum Likelihood (ML) method and the Tamura-Nei model, with a p-distance matrix for nucleotides with the pair-wise gap deletion option selected and with 1,000 bootstrap repetitions (Tamura et al. 2021). The resulting phylogenetic trees were converted to the Newick format, and a more detailed visualization of the phylogenetic trees was performed using the Interactive Tree of Life (iTOL) (https://itol.embl.de/) server (Letunic and Bork 2021).

Production of extracellular enzymes

Chitinase activity: Chitinase enzyme antagonistic bacteria isolates were detected in the colloidal chitin agar medium (g/l: Na_2HPO_4, 6; KH_2PO4, 3; NH_4Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar, 15, and colloidal chitin 1% (w/v), pH 7). Extracellular chitinase activities of the bacteria were observed after 7 d of incubation at 27°C, and the observation of a clear zone around the colony was considered positive for chitinase production (Souza et al. 2009).

Cellulase activity

Cellulase activities of the antagonistic bacteria were tested using the Bushnell and Hass carboxymethyl cellulose (CMC) medium. The antagonistic bacteria isolates were spot-inoculated and incubated at 27°°C for 10 day. Then, the plates were flooded with 0.5% Congo red for 15 min, followed by 0.5-M NaCl wash for 10 min. The observation of a clear halo zone around the colony was considered positive for cellulase production (Singh et al. 2013).

Amylase activity: The amylase production capacity of the bacteria isolates was tested using starch agar medium ((l): raw starch, 20 g; $NaNO_3$, 1 g; K_2HPO_4, 1 g; $MgS0_4$, 0.5g; $FeS0_4$, 0.01 g; agar ,15 g). The isolates were spot-inoculated and incubated at 27°C for 72 h. Then, the plates were flooded with iodine solution for 8 min. The observation of a clear zone around the colony was considered positive for amylase production (Priest, 1977).

Protease activity

The protease activity of the isolates was tested in skim milk agar medium. The bacterial isolates were spotted onto plates and incubated for 48 h at 30°C, and the observation of a clear zone around the colony was considered positive for protease production (Gardini et al. 2006).

Statistical analysis

The data were statistically analyzed using the SPSS 20.0 program. The mean values of the control and treatment samples were compared using Duncan's multiple range test at the significance level of 5% ($p < 0.05$).

Results

Isolation and identification of pathogenic fungus

The fungal pathogen, which is the causal agent of brown rot, was isolated from the collected apple fruits(Fig. 1). Based on the screening of cultural and microscopic characteristics, this fungal culture was identified as *A. alternata* according to the description in a previous report (Simmons, 2007).

Morphological observations of *A. alternata* were recorded using the light microscopic culture technique. The fungus showed substantial mycelial growth in the PDA medium.At first, the fungal mycelium was hyaline, followed by a transition to a black-brown color, as well as a septate, multicellular, and irregularly branched appearance. In the primary growth phase, the hyphae were slim (2.04 µm in diameter), narrow, and hyaline, but they then became slightly thicker (3.92 µm in diameter) as they got older. Conidiophores arose separately and in groups, generally groups of 1–4, and they were occasionally long and occasionally short. These conidiophores measured 27.30–112 µm in length and 3.12–8.43 µm in diameter. Conidia formed in the conidiophores in sequences consisting of up to 7–10 members. They were light to dark brown, varying in shape but mostly ellipsoidal, having a tapered apex with 2 to 4 longitudinal and 2–12 transversal septa. The muriform conidia inclusive of the beak measured 21.82–96.40 mm x 8.26–16.52 µm. The length of each conidium was 2.64 to 5.84 times higher than its width. The beak measured 22.62–58.69 µm in length. The chlamydospores were formed in the old culture of *A. alternata*. They were intercalary, thick-walled, roundish to oval in shape, and dark brown, and they measured 4.92–9.89 µm in diameter. The molecular characterization of the fungus based on the sequence analysis of the nuclear internal transcribed spacer (ITS) was identified as *A. alternata*. Based on the analysis of ITS 404 bp sequence data, the isolate (GenBank accession No. OK175668.1) showed 96% pair-wise similarity with the *A. alternata* strain SBT-21 (GenBank accession no. JX971037.1).

Pathogenicity tests of fungus

The inoculation of *A. alternata* on Amasya apples at different inoculum concentrations caused different diameters of brown rot around the inoculated lesions. It was observed that the diameters of these rot areas were related to different spore concentrations. At the lowest concentration (2×10^2 spores/ml) of the fungal samples, the diameter of the rot lesion on the fruit was 1.3 cm. Depending on the concentration of *A. alternata*, 2×10^4, 2×10^6, and 2×10^8 spores/ml, the diameters of the rot lesions were 2.2, 3.15, and 3.8 cm, respectively. No typical signs of rot appeared on the control fruit inoculated with sterile water (Fig. 2). The results of the pathogenicity tests on the fruits indicated that the fungus might be endophytic and could be found on apples as a major pathogen.

Isolation of endophytic bacteria and screening of antagonistic bacteria

Initially, a total of 78 endophytic bacteria isolates were obtained from the healthy apple tissues based on different colony morphologies and growth ratios in the medium. To select potential *in vivo* biocontrol agents, the *in vitro* antagonistic activities of all endophytic bacterial isolates were tested against A. alternata. Out of the 78 bacterial isolates, only 26 showed antagonistic effects against *A. alternata* at different ratios (Fig. 3).

The data presented in Table 1 show that compared to the control, all 26 antagonistic endophytic bacterial isolates could reduce the growth of the A. alternata mycelium. The endophytic bacteria named ib-17 caused a significant inhibition of mycelium growth (79.45%) ($p < 0.05$). However, the lowest reduction percentage was observed for the bacterial isolate 25 at 54.26% (Table 1).

Table 1
Effects of selected antagonistic endophytic bacteria on spore germination of *A. alternata*

Bacterial isolate	Diameter of the pathogen in treatment (cm) ± standard error	Percentage of inhibition (%)	Bacteria lisolate	Diameter of the pathogen in treatment (cm) ± standard error	Percentage of inhibition (%)
Control(-)	5.166 ± 0.88^{a}	-	ib14	1.933 ± 0.333^{cde}	62.59
ib1	1.566 ± 0.666^{fg}	69.76	ib15	1.233 ± 0.176^{hı}	76.16
ib2	1.400 ± 0.577^{gh}	72.86	ib16	1.766 ± 0.88^{def}	65.89
ib3	1.866 ± 0.120^{cde}	63.95	ib17	1.066 ± 0.333^{ı}	79.45
ib4	1.733 ± 0.666^{ef}	66.47	ib18	1.566 ± 0.881^{fg}	69.76
ib5	1.933 ± 0.666^{cde}	62.59	ib19	2.066 ± 0.333^{c}	60.07
ib6	1.366 ± 0.666^{gh}	73.64	ib20	1.600 ± 0.577^{fg}	68.99
ib7	2.100 ± 0.57^{c}	59.30	ib21	1.600 ± 0.577^{fg}	68.99
ib8	1.600 ± 0.577^{fg}	68.99	ib22	1.933 ± 0.666^{cde}	62.59
ib9	1.700 ± 0.577^{ef}	67.05	ib23	1.566 ± 0.333^{fg}	69.76
ib10	1.733 ± 0.333^{ef}	66.47	ib24	2.000 ± 0.577^{cd}	61.24
ib11	2.000 ± 0.577^{cd}	61.24	ib25	2.366 ± 0.88^{b}	54.26
ib12	2.066 ± 0.333^{c}	60.07	ib26	1.400 ± 0.115^{gh}	72.86
ib13	1.600 ± 0.577^{fg}	68.99			

Data are presented as mean ± standard error. Mean values followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$). C: no bacterial treatment against fungi, control.

Efficacy of antagonistic endophytic bacteria in controlling brown rot in apple fruit

The data shown in Table 2 indicate that compared to the positive control treatment, all of the tested antagonistic endophytic bacteria could reduce the rot on the fruit samples caused by *A. alternata* at different rates. The diameters of the rot lesions on the fruits were measured by a ruler. Then, the Disease Severity Index (DSI) values were calculated. These rot lesions occurred at a low rate in the antagonistic endophytic bacteria inoculate treatment compared to the inoculation with pathogenic *A. alternata* alone. The diameters of the rot lesions treated with most of the endophytic bacteria were significantly lower, particularly compared to the diameters of the positive controls (Table 2).

Table 2
Effects of inoculation of fruit in vivo with *A. alternata* and antagonistic endophytic bacteria

Bacteria applied 24 h before *A. alternata*				**Bacteria applied 24 h after *A. alternata***				**Bacteria applied simultaneously with *A. alternata***			
Bacterial Treatment	Lesion diameter (cm) ± standard error	DSI	% Disease reduction	Bacterial Treatment	Lesion diameter (cm) ± standard error	DSI	% Disease reduction	Bacterial Treatment	Lesion diameter (cm) ± standard error	DSI	% Disease reducti
C	2.66 ± 0.16^{a}	73.3	-	C	3.16 ± 0.16^{a}	80	-	C	2.83 ± 0.16^{a}	73.3	
İb1	0.63 ± 0.63^{efg}	5.2	81.8	ib1	1.13 ± 0.40^{ef}	26.6	66.7	ib1	0.93 ± 0.033^{g}	13.3	
ib2	1.66 ± 0.44^{abcde}	40	45.4	ib2	2.2 ± 0.47^{bcd}	33.3	58. 3	ib2	1.33 ± 0.33^{cdefg}	26.6	
ib3	2 ± 0.28868^{abc}	46.6	36.4	ib3	1.83 ± 0.12^{cde}	33.3	58. 3	ib3	1.36 ± 0.18^{cdefg}	26.6	
ib4	0.73 ± 0.43^{efg}	20.0	72.7	ib4	2.2 ± 0.05^{bc}	60	25	ib4	1.23 ± 0.12^{efg}	26.6	
ib5	0.5 ± 0.28^{fg}	13.3	92.9	ib5	2.1 ± 0.15^{bcd}	60	25	ib5	1.93 ± 0.033^{bcde}	33.3	
ib6	1.16 ± 0.60^{cdef}	33.3	54.5	ib6	2.33 ± 0.16^{abc}	73.3	8	ib6	1.9 ± 0.1^{bcde}	33.3	
ib7	1.9 ± 0.05^{abcd}	33.3	54.5	ib7	3 ± 0^{ab}	66.6	16. 7	ib7	1.93 ± 0.06^{bcde}	33.3	
ib8	0.93 ± 0.58^{cdefg}	13.3	81.8	ib8	2.7 ± 0.15^{abc}	66.6	16. 7	ib8	1.53 ± 0.03^{bcdefg}	26.6	
ib9	1.43 ± 0.46^{bcdef}	46.6	36.4	ib9	2.33 ± 0.33^{abc}	73.3	8	ib9	1.16 ± 0.60^{fg}	33.3	
ib10	1.03 ± 0.03^{cdefg}	33.3	54.5	ib10	2.13 ± 0.08^{bcd}	60	25	ib10	1.26 ± 0.13^{defg}	26.6	
ib11	0.83 ± 0.44^{defg}	20	72.7	ib11	2.5 ± 0.057^{abc}	66.6	16. 7	ib11	1.5 ± 0.05^{bcdefg}	26.6	
ib12	1.66 ± 0.16^{abcde}	40	45.4	ib12	2.33 ± 0.14^{abc}	66.6	16. 7	ib12	1.23 ± 0.37^{efg}	26.6	
ib13	0.86 ± 0.06^{defg}	20	72.7	ib13	1.8 ± 0.36^{cde}	60	25	ib13	2.1 ± 0.05^{b}	46.6	
ib14	1.33 ± 0.16^{cdefg}	20	72.7	ib14	1.33 ± 0.66^{def}	20	75	ib14	1.56 ± 0.06^{bcdefg}	40	
ib15	1.66 ± 0.16^{abcde}	33.3	54.5	ib15	2.43 ± 0.47^{abc}	73.3	8	ib15	1.83 ± 0.16^{bcdef}	33.3	
ib16	0.66 ± 0.33^{efg}	13. 3	81.8	ib16	0.6 ± 0.17^{fg}	33.3	58. 3	ib16	0.26 ± 0.26^{h}	13.3	
ib17	1 ± 0.28^{cdefg}	13. 3	81.8	ib17	2.4 ± 0.23^{abc}	73.3	8	ib17	1.96 ± 0.033^{bcd}	33.3	
İb8	1.03 ± 0.33^{cdef}	26.6	63.7	ib18	2.2 ± 0.2^{bcd}	60	25	ib18	1.66 ± 0.12^{bcdef}	40	
ib19	2.5 ± 0^{ab}	60	181	ib19	2.3 ± 0.25^{abc}	66. 6	16. 7	ib19	1.66 ± 0.08^{bcdef}	40	
ib20	0.16 ± 0.16^{g}	0	100	ib20	1.96 ± 0.20^{cde}	26. 6	66.7	ib20	1.16 ± 0.08^{fg}	33.3	
ib21	0.83 ± 0.16^{defg}	13.3	81.8	ib21	0.16 ± 0.16^{g}	13.3	83.3	ib21	0.23 ± 0.23^{h}	13.3	
ib22	1.53 ± 0.03^{bcdef}	26.6	63.7	ib22	1.36 ± 0.18^{def}	33. 3	58. 3	ib22	1.73 ± 0.14^{bcdef}	40	

Bacteria applied 24 h before *A. alternata*				Bacteria applied 24 h after *A. alternata*				Bacteria applied simultaneously with *A. alternata*		
ib23	1.2 ± 0.2^{cdef}	26.6	63.7	ib23	2.3 ± 0.25^{abc}	53.3	33. 3	ib23	2 ± 0.057^{bc}	33.3
ib24	1.66 ± 0.16^{abcde}	40	45.4	ib24	2.63 ± 0.06^{abc}	66. 6	16. 7	ib24	1.46 ± 0.14 ^{cdefg}	46.6
ib25	0.5 ± 0.5^{fg}	13.3	81.8	ib25	1.13 ± 0.33^{ef}	20	75	ib25	0.93 ± 0.18^{g}	20
ib26	1.3 ± 0.3^{cdef}	26.6	63.7	ib26	2.63 ± 0.18^{abc}	66. 6	16. 7	ib26	1.26 ± 0.39^{defg}	26.6

Data are presented as mean ± standard error. Different letters mean significantly different results according to Duncan's multiple range test ($p < 0.05$).

The screening process for the effectiveness of the antagonistic endophytic bacteria applied 24 h before *A. alternata* showed a percentage of inhibition range of 36.4–100%. From among these, 20 isolates significantly inhibited the growth of *A. alternata* compared to the controls ($p < 0.05$). Especially the antagonist ib20 completely inhibited the growth of *A. alternata* when it was used 24 h before *A. alternata* (Fig. 4).

The data presented in Table 2 show that compared to the control treatment, in the treatments where the bacteria were introduced 24 h after the introduction of the fungus, all antagonistic bacteria could reduce the growth of *A. alternata* rots on the apples. Fourteen isolates exhibited a significant antagonistic effect on *A. alternata*. Here, the greatest antagonistic effect was recorded for the ib20 isolate at 83.3%. When the ib26 endophytic bacterium was applied simultaneously with the fungus, it showed biocontrol activity against *A. alternata* at various rates (36.4–81.8%). All bacteria isolates caused a significant inhibition of *A. alternata* rot ($p > 0.05$). Here, the highest growth reduction percentages were recorded for the bacteria isolates named ib1, ib16, and ib21 at 81.8% .

Molecular characterization and phylogenic analysis

The collection of bacterial endophytes isolated from local apples in the Amasya province of Turkey resulted in the identification of 26 different bacterial strains. The 16S rDNA gene amplification sequencing procedure was used to identify the generated isolates on a molecular level. The BLAST search tool program was then used to compare the isolates to their closest matches. Closely related bacterial species from among 12 different bacterial species (*Bacillus* sp., *B. amyloliquefaciens*, *B. halotolerans*, *B. subtilis*, *B. siamensis*, *B. licheniformis*, *B. tequilensis*, *B. rugosus*, *B. atrophaeus*, *B. methylotrophicus*, and *B. velezensis*) were discovered by the analysis of the 16S rDNA gene sequence (Table 3). There were five dominant species: *B. amyloliquefaciens* (23.07%*), B. licheniformis* (19.23%), *B. siamensis* (15.38%), *B. subtilis* (7.69%), and *B. atrophaeus* (7.69%).

Table 3
16S rRNA gene sequences of bacterial endophytes isolated from Amasya apple (*Malus domestica*), obtained using the NCBI Blastn analysis tool, showing the maximum sequence similarity values of 84%-100% to other strains in the NCBI database.

Endophyte code	The best NCBI database matches		
	Blastn results (accession number)	e-value	Identity similarity (%)
ib1	*Bacillus amyloliquefaciens* strain MG1 16S ribosomal RNA gene, partial sequence (JX854556.1)	0.0	97.70
ib2	*Bacillus halotolerans* strain PL-3 16S ribosomal RNA gene, partial sequence (MK517597.1)	0.0	99.50
ib3	*Bacillus subtilis* strain GZUB23 16S ribosomal RNA gene, partial sequence (FJ434648.1)	0.0	99.10
ib4	*Bacillus siamensis* strain MER_24 16S ribosomal RNA gene, partial sequence (KT719604.1)	0.0	99.01
ib5	*Bacillus amyloliquefaciens* strain YFV015 16S ribosomal RNA gene, partial sequence (OK444818.1)	0.0	97.15
ib6	Bacillus siamensis strain HoB-1 16S ribosomal RNA gene, partial sequence (OM074300.1)	0.0	98.26
ib7	*Bacillus* sp. (in: Bacteria) strain Z4 16S ribosomal RNA gene, partial sequence (MG470658.1)	0.0	98.73
ib8	*Bacillus subtilis* strain N012 16S ribosomal RNA gene, partial sequence (MG396985.1)	0.0	97.97
ib9	*Bacillus licheniformis* strain ML104A 16S ribosomal RNA gene, partial sequence (KC692185.1)	0.0	97.56
ib10	*Bacillus amyloliquefaciens* strain UK2 16S ribosomal RNA gene, partial sequence (MT491100.1)	0.0	99.82
ib11	*Bacillus tequilensis* strain RSUCC0161 16S ribosomal RNA gene, partial sequence (OK056324.1)	0.0	97.31
ib12	*Bacillus amyloliquefaciens* strain YP6 chromosome, complete genome (CP032146.1)	0.0	98.46
ib13	*Bacillus rugosus* strain MAA07 16S ribosomal RNA gene, partial sequence (OP782677.1)	0.0	100
ib14	*Bacillus amyloliquefaciens* strain RESI-50 16S ribosomal RNA gene, partial sequence (MT542326.1)	0.0	100
ib15	*Bacillus atrophaeus* strain CNY01 chromosome, complete genome (CP073265.1)	0.0	99.83
ib16	Endophytic bacterium strain LZH-F25 16S ribosomal RNA gene, partial sequence (MN099151.1)	0.0	98.60
ib17	*Bacillus methylotrophicus* strain GBPI_CDB76 16S ribosomal RNA gene, partial sequence (KT887215.1)	0.0	96.45
ib18	*Bacillus siamensis* strain MER_24 16S ribosomal RNA gene, partial sequence (KT719604.1)	0.0	98.83
ib19	*Bacillus velezensis* strain GMU231 16S ribosomal RNA gene, partial sequence (OP364986.1)	0.0	97.80
ib20	*Bacillus licheniformis* strain JBRI-MO-2019-0038 16S ribosomal RNA gene, partial sequence (MN865950.1)	0.0	99.09
ib21	*Bacillus licheniformis* strain CICC10094 16S ribosomal RNA gene, partial sequence (AY842873.1)	0.0	87.28
ib22	*Bacillus amyloliquefaciens* strain WB16 16S ribosomal RNA gene, partial sequence (OL636049.1)	0.0	99.83
ib23	*Bacillus atrophaeus* strain D171 16S ribosomal RNA gene, partial sequence (MK713585.1)	0.0	100
ib24	*Bacillus licheniformis* strain P6 16S ribosomal RNA gene, partial sequence (KC255386.1)	0.0	97.04
ib25	*Bacillus licheniformis* strain wx1 16S ribosomal RNA gene, partial sequence (KF963618.1)	0.0	84.92
ib26	*Bacillus siamensis* strain HoB-1 16S ribosomal RNA gene, partial sequence (OM074300.1)	0.0	99.77

Only one method is used to describe how closely a set of species is related: rootless phylogenetic trees. Because the endophytes acquired in this study belonged to a single genus, rootless phylogenetic trees were used for tree construction. The obtained species were all Bacillaceae. Phylogenetic analyses were performed on all strains with at least 80–100% nucleotide sequence similarity using the maximum likelihood method with a bootstrap value of 1000. MEGA 11 was used to generate two separate phylogenetic trees, which would reveal their taxonomic similarity to other microbes. A phylogenetic tree was generated based on the molecular analysis, indicating the evolutionary link between the isolates and other taxonomically similar bacteria. The sequences acquired in this study are colored differently in Fig. 5-A, whereas other sequences from the NCBI database were used to compare the results. The phylogenetic tree built using the ML technique classified the isolates into five clades (Fig. 5-B).

The extracellular enzymes featured in the antagonistic endophytic bacteria isolates are shown in Table 4. We found that 24 bacterium strains showed protease activity. It was determined that ib-2 (*Bacillus halotolerans* strain PL-3) and ib-12 (*Bacillus amyloliquefaciens* strain YP6) showed the highest protease activity. Moreover, 24 isolates were determined to have cellulase activity.

Table 4
Protease, cellulase, amylase, and chitinase enzyme activities of endophytic bacteria

No	Isolate	Protease activity	Cellulase activity	Amylase activity	Chitinase activity
ib1	*Bacillus amyloliquefaciens* strain MG1	+	+	+	+++
ib2	*Bacillus halotolerans* strain PL-3	+++	+++	+++	+++
ib3	*Bacillus subtilis* strain GZUB23	+	+	+	+++
ib4	*Bacillus siamensis* strain MER_24	++	+	+	+++
ib5	*Bacillus amyloliquefaciens* strain YFV015	+	+	++	+++
ib6	Bacillus siamensis strain HoB-1	++	+	+	+
ib7	*Bacillus* sp. (in: Bacteria) strain Z4	+	+	++	+++
ib8	*Bacillus subtilis* strain N012	+	+	+++	++
ib9	*Bacillus licheniformis* strain ML104A	+	+	+	+++
ib10	*Bacillus amyloliquefaciens* strain UK2	+	+	-	+++
ib11	*Bacillus tequilensis* strain RSUCC0161	-	-	-	+++
ib12	*Bacillus amyloliquefaciens* strain YP6	+++	+++	+	+++
ib13	*Bacillus rugosus* strain MAA07	-	+	++	-
ib14	*Bacillus amyloliquefaciens* strain RESI-50	+	+	+++	+++
ib15	*Bacillus atrophaeus* strain CNY01	++	+	+++	+++
ib16	Endophytic bacterium strain LZH-F25	+	+	-	+++
ib17	*Bacillus methylotrophicus* strain GBPI_CDB76	++	-	+	+
ib18	*Bacillus siamensis* strain MER_24	+	+	+++	+++
ib19	*Bacillus velezensis* strain GMU231	+	+	+	++
ib20	*Bacillus licheniformis* strain JBRI-MO-2019-0038	++	+	++	+
ib21	*Bacillus licheniformis* strain CICC10094	++	+++	+++	+
ib22	*Bacillus amyloliquefaciens* strain WB16	+	+	+	+++
ib23	*Bacillus atrophaeus* strain D171	+	+	++	+++
ib24	*Bacillus licheniformis* strain P6	+	+	+++	+++
ib25	*Bacillus licheniformis* strain wx1	+	+	+++	+++
ib26	*Bacillus siamensis* strain HoB-1	++	+	+	+++

The highest cellulase activity was shown in the isolates of ib-21 (*Bacillus licheniformis* strain CICC10094), ib2 (*Bacillus halotolerans* strain PL-3), and ib12 (*Bacillus amyloliquefaciens* strain YP6). Twenty-one bacterium isolates were capable of in vitro amylase activity, where ib2 (*Bacillus halotolerans* strain PL-3), ib8 (*Bacillus subtilis* strain N012), ib14 (*Bacillus amyloliquefaciens* strain RESI-50), ib15 (*Bacillus atrophaeus* strain CNY01), ib18 (*Bacillus siamensis* strain MER_24), ib21(*Bacillus licheniformis* strain CICC10094), ib24 (*Bacillus licheniformis* strain P6), and ib25(*Bacillus licheniformis* strain wx1) showed the highest amylase activity. All isolates except ib-13 (*Bacillus rugosus* strain MAA07) were positive for chitinase activity. The ib-6 (Bacillus siamensis strain HoB-1), ib-17 (*Bacillus methylotrophicus* strain GBPI_CDB76), ib-20 (*Bacillus licheniformis* strain JBRI-MO-2019-0038), and ib-21 (*Bacillus licheniformis* strain CICC10094) isolates had the lowest levels of chitinase activity. It was determined that the other 19 isolates produced quite high amounts of chitinase .

Discussion

Apple fruit is one of the major fruit products worldwide, but post-harvest rot caused by pathogenic fungi leads to substantial economic losses. This study investigated the antifungal efficacy of endophytic bacteria against *A. alternata* rot and the possible mechanisms of this effect. We found that the *in vitro* colony growth and spore germination of *A. alternata*, as well as rot lesions on Amasya apple fruits, could be significantly inhibited by endophytic bacteria. The results showed that the healthy apples could carry antagonistic endophytic bacteria, which had antagonistic activities against the pathogenic fungus. Moreover, the severity of rot signs was dose-dependent, and the most severe results were obtained at a concentration of 2×10^8 spores/mL of *A. alternata*. The creation of rot lesions by the pathogenic fungus was observed based on its growth, which destroyed the apple tissues necrotrophically. The pathogen fungus then derived nutrition from these tissues, leading to the decay of the fruit and thus the induction of rot (Dukare et al. 2019). The selected twenty-six antagonistic bacteria isolates belonged to the closely related genera of *Bacillus*. The biocontrol agents of *Bacillus* ssp. were found highly effective in controlling *A. alternata* when applied 24 h before the pathogen compared to the application of the agent 24 after *A. alternata* and the simultaneous application of the agent and the pathogen. Similar studies (Madbouly et al. 2020) revealed that *Monilinia fructigena* is a causal agent for brown rot in apple fruits. In in vivo biocontrol assays, fruits were inoculated with endophytic yeast isolates simultaneously, 24 h before, and 24 h after the pathogen. The

inoculation process showed a noticeably different reduction in disease occurrence by 84.02–89.5%, 80.1–86.9%, and 56.3–86.9%, respectively, compared to the controls. The findings of our study also indicated that endophytic bacteria producing enzyme activities and bioactive components may suppress the effects of *A. alternata* on infected apples. Recent research has also shown the antifungal activity of Bacillus spp. against several species causing post-harvest fungal infections (Madriz-Ordeñana et al. 2022; Liang et al. 2023; Shao et al. 2023). Moreover, the suppression of mycelial growth of fungi was primarily due to diffusible substances. These diffusible antifungal substances can be antibiotics, hydrolytic enzymes, bacteriocins, or some other secondary metabolites (Beneduzi et al. 2012). Moreover, research should define the antifungal substances secreted by these endophytic bacterial antagonists and identify their modes of action. The cell wall is a characteristic structure of fungi and is composed mainly of glucans, chitin, and glycoproteins (Garcia-Rubio et al. 2020). In this study, twenty-six endophytic bacteria demonstrated different potentials for producing extracellular hydrolyzing enzymes that could hydrolyze components of the fungal cell wall, thus facilitating their antagonistic modes of action. It was found that some fungal wall-degrading enzymes such as chitinase, protease, cellulase, amylase, and β-glucanase produced by *B. velezensis* NKG-2 could adversely affect the growth of several plant pathogenic fungi such as *Fusarium* spp., Botrytis cinerea, and *A. alternata* (Myo et al. 2019). Abdelmoteleb et al. (2017) showed that *Bacillus* sp. significantly lowered the growth of *A. alternata* by producing volatile compounds. Additionally, the results of our study also confirmed and exhibited similar results to previous reports that the activities of protease, cellulase, amylase, and chitinase, as some of the several compounds produced by *Bacillus* species, can help protect apple fruits from the disease. In the taxonomy procedures of today for the complete identification of bacteria, the employment of classical methods in conjunction with current genetic/molecular techniques is commonly preferred (Kawaka, 2022). In this study, all isolates were identified to belong to the genus *Bacillaceae* based on the 16S rDNA gene sequence that was obtained. This was interpreted to be because of their critical involvement in plant growth, which includes the creation of plant growth hormones and protection against invading pathogens (Ek-Ramos et al. 2019). In the case of *Bacillus* sp., it should be mentioned that the majority of these species live in the plant rhizosphere and have the capacity to boost plant development as well as preventing infections with fungi and bacteria that cause various plant diseases (Zendehdel et al. 2021). In particular, *B. subtilis* is easily isolated from natural environments and has been used as a model organism to study its biocontrol mechanism worldwide. It has been seen as a promising bacterial strain for the control of plant diseases (Morikawa, 2006; Kovács et al. 2009; X. Wang et al. 2018). *Bacillus* sp. isolates in apples have been considered an adaptation of the fruit to disease factors. It has been determined in previous studies that *Bacillus amyloliquefaciens, B. badius, and B. gibsonii*, some of the epiphytic and endophytic bacteria isolated from apple shoots, have significant antagonistic activity against phytopathogenic fungi such as *A. alternata, Aspergillus tubingensis, Fusarium incarnatum, Fusarium tricinctum*, and *Phoma fungicola* isolated from the same plant (Vankova et al. 2021). The phylogenetic statuses of the endophytic bacteria isolated from Amasya apple samples revealed that each isolate formed a monophyletic line, which was supported by bootstrap values higher than 50%. The overall results of this study suggested that twenty-six *Bacillus* ssp. showed a strong biocontrol activity against *A. alternata*, and this is the first report on the growth inhibition of *A. alternata* by the usage of endophytic bacteria on apples under *in vivo* and *in vitro* conditions. Moreover, further research should be carried out to examine the antifungal membrane-binding sites of lytic enzymes produced by endophytic bacteria in more detail and understand their underlying molecular mechanisms.

Conclusion

In summary, apple endophytic bacteria could effectively inhibit A. alternata rot growth on fruits by the mode of enzyme activities. The endophytic bacteria induced the disturbance of membrane permeability, disruption of membrane integrity, and cell wall integrity in Alternaria alternata, resulting in the disintegration of the pathogen, and consequently, showing direct antifungal activity. These *Bacillus* ssp. endophytic bacteria are therefore possible candidates for the biocontrol of apple fruit rot caused by A. alternata and could be used as an alternative to chemical fungicides.

Declarations

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author Contributions

The conceptualization and design of the study were performed by Idris Bektas. Material preparation and investigation were performed by Idris Bektas,Mustafa Kusek and Ferit Can Yazic. Formal analysis and writing were performed by Idris Bektas. Manuscript review and editing were performed by all authors. Idris Bektas supervised the study. All authors read and approved the final manuscript.

Data availability

Data used in the researh are available from the corresponding author.

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Figures

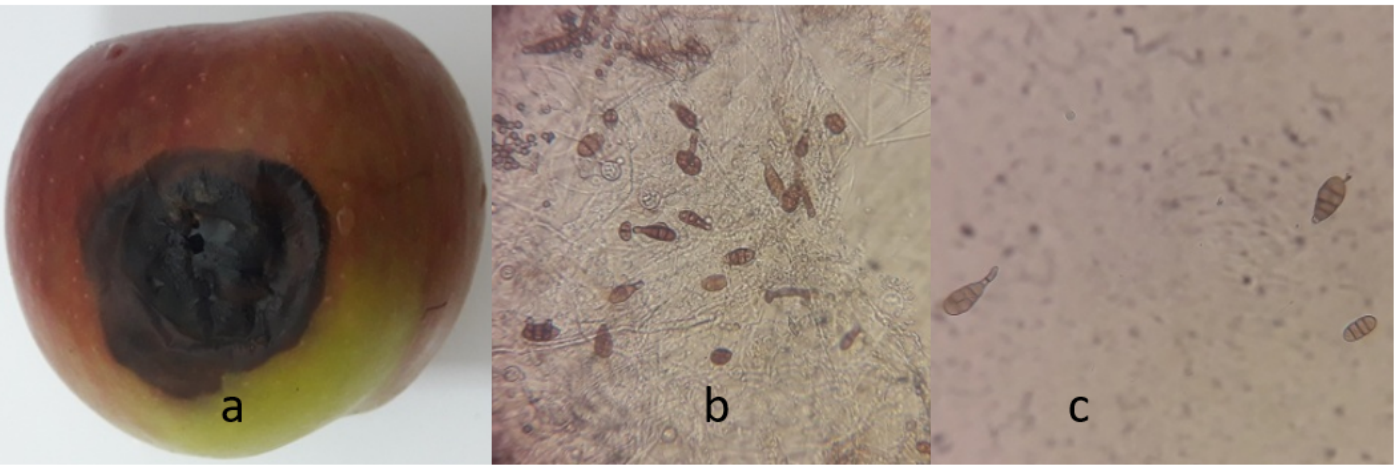

Figure 1

The effect of *A. alternata* on Amasya Apple: (a) inside the rot tissue, (b,c) conidiophores and conidia

Figure 2

Virulence of *A. alternata* isolates during the inoculation of different conidial suspensions into Amasya apple fruits with induced lesions (a: sterile water, b: 2×10^2, c: 2×10^4, d: 2×10^6, and e: 2×10^8 spores/ml)

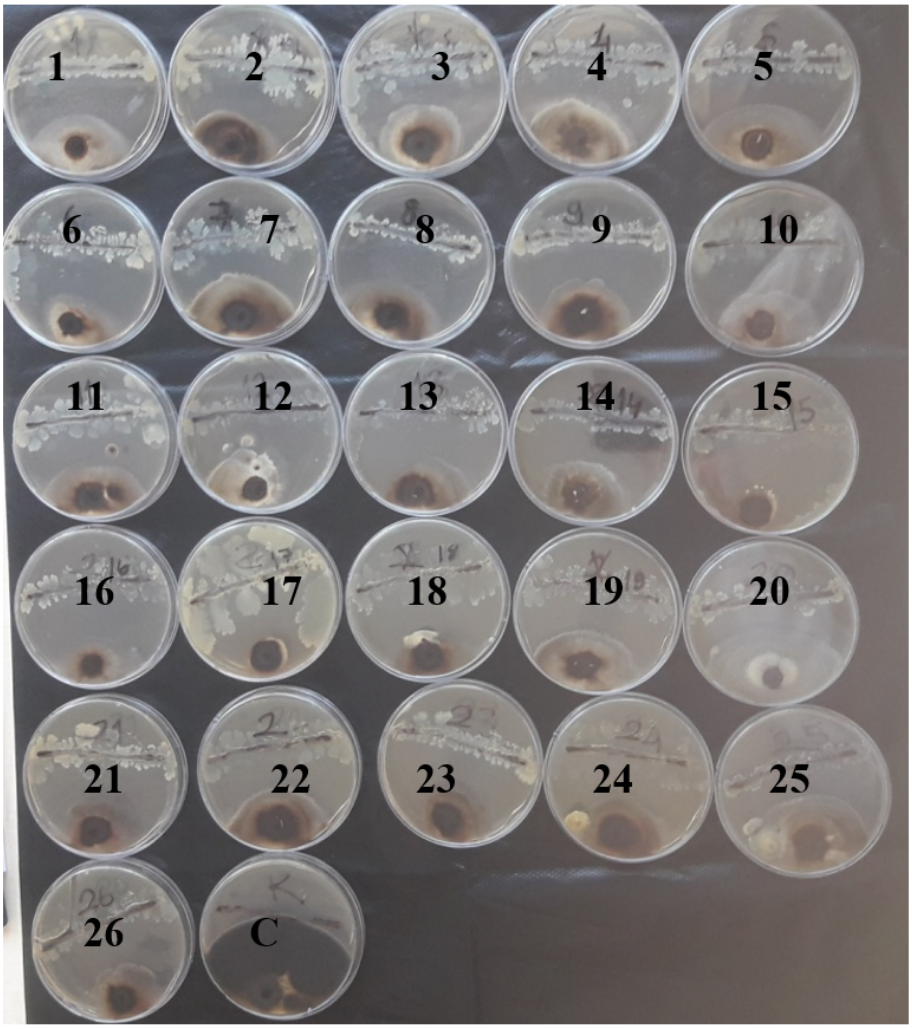

Figure 3

Antifungal effect of the endophytic bacteria strain ib1-26 against *A. alternata* at 10 days after culturing. C: *A. alternata* in the control plate and the limited growth of the fungus in the plate on the lower side

Figure 4

Biocontrol effect of the endophytic bacteria strains ib8 and ib21 applied 24 h before *A. alternata*; ib20 strains applied 24 h after *A. alternata*, and ib2 strains applied simultaneously with *A. alternata* K(+) :Control- only *A. alternata*; K(-):Negative control- only distilled water

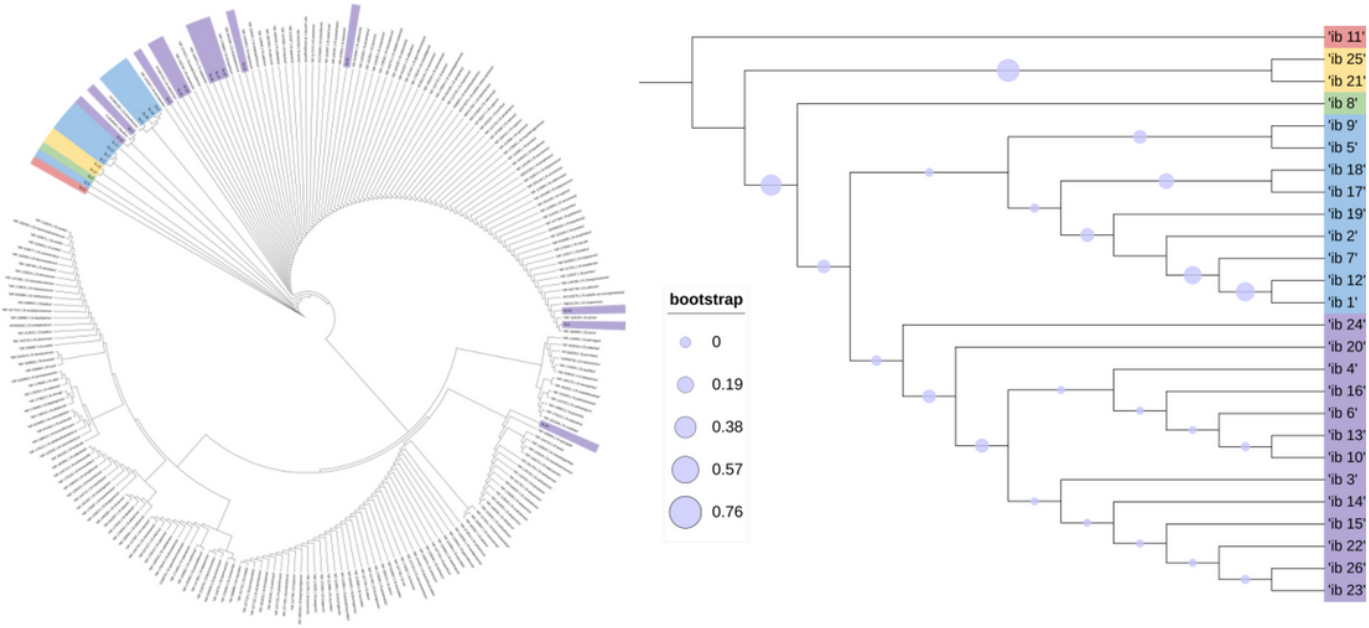

Figure 5

Maximum likelihood phylogenetic tree (A) was constructed using 16S rDNA gene sequences showing the association of 26 endophytic bacteria in this study with closely related species. The maximum likelihood phylogenetic tree (B) based on the analysis of the partial 16S rRNA nucleotide sequence of *Bacillus* sp.. Phylogenetic trees were constructed using MEGA version 11.0 with 1000 replications, a bootstrapping value above 50%, and visualized with Interactive Tree of Life (iTOL).