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Biocontrol potential of epiphytic bacteria against *Xanthomonas citri* pathotypes A and A*

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Abstract

Background Citrus canker caused by the bacterium *Xanthomonas citri* (Hasse 1915) is known as a dangerous disease that has serious economic impacts on citrus production in many tropical and subtropical countries. In Iran, Citrus canker disease has caused significant damage to various citrus fruits, particularly limes. Nowadays, employing antagonistic bacteria as an effective and environmentally friendly method for plant disease management has gained special importance.

Results Citrus leaf samples were collected from different regions of Iran, and after isolating epiphytic bacteria, *ex planta* and *in planta* investigations concerning antagonistic activities of the isolates against *Xanthomonas citri* pathotypes A and A* were performed. A total of 94 epiphytic bacterial isolates were isolated from citrus aerial parts. Based on biochemical, physiological, morphological, and genotypic tests, it was determined that these bacteria belong to the *Bacillus* and *Staphylococcus* genera. The highest inhibition activity against the pathogenic bacterium was related to isolates D4 and D5. Using the molecular method and the resulting dendrogram, it was found that these isolates were most similar to *Bacillus amyloliquefaciens*. The present findings demonstrated that pathogenicity test on key lime leaves infected with *X. citri* pathotypes A and A*, along with the application of the biocontrol strain *B. amyloliquefaciens*, resulted in a significant reduction in the number of canker lesions.

Conclusion The results strongly suggested that the identified antagonistic bacterial isolates hold promising potential as biocontrol agents for managing citrus bacterial canker disease.

Keywords *Xanthomonas citri*, *Bacillus*, Biological control, Citrus canker, Epiphyte, Phylloplane

Background

The genus *Citrus* belongs to the *Rutaceae* family and includes major fruits and crops such as grapefruits, orange, lemons, pummels and limes (Bora et al. 2020). As of 2022, Iran was ranked tenth worldwide in citrus fruit production, with an output of 4.38 million tons (source: http://faostat3.fao.org/home/E). Citrus canker disease caused by *Xanthomonas citri* is a dangerous, devastating disease that affects most citrus species worldwide (Rigano et al. 2010). In Iran, the presence of citrus canker was originally documented on *Citrus aurantifolia* in Kerman province and later confirmed in other southern provinces. In Iran, Citrus canker disease has caused significant damage to various citrus fruits, particularly limes. This widespread disease has resulted in a significant decrease in yield in the provinces of Hormozgan, Sistan and Baluchistan, Kerman, Fars, Bushehr, Ilam, and Kohgiluyeh and Boyer Ahmad (Khodakaramian and Swings 2011). The lime industry is of great importance in Iran and *X. citri* is a significant threat to citrus crops worldwide and is classified as a regulated organism in many countries, including those in the European Union. There are disagreements about the region where citrus canker originated and it is surmised that the emergence of this disease was from Southeast Asia (Thakre et al. 2017). The disease

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symptoms are observed on all aerial parts and include raised lesions on leaves, stems, and fruits. Lesions are surrounded by water-soaked margins and yellow chlorotic rings, and the tree is weakened due to bacterial attack and the fruits fall prematurely (Li et al. 2007). A range of biochemical, physiological, serological, and molecular tests has been performed to identify different types of the bacterium that causes citrus canker. At least three distinct forms of citrus canker have been identified, among which the Asiatic form (Canker A) is found to be the most destructive, and this form is of particular importance due to its wide host range and severe infection (Das 2003). As yet, no successful management program to control citrus bacterial canker disease exists, since only a few chemicals such as copper compounds and antibacterial agents have been found to be somewhat effective against this disease (Heydarpanah et al. 2020). However, applications of these chemicals would result in increased production costs, copper resistance of the pathogenic bacterium and environmental pollution (Fan et al. 2022). Biological control is a natural method that reduces the need for chemical compounds, resulting in lower risks and minimizing their harmful effects on the environment. Health and environmental concerns have prompted researchers to adopt solutions on the basis of the application of antagonistic agents that do not cause environmental pollution (Lahlali et al. 2022). In biocontrol method, antimicrobial properties of antagonistic agents, especially bacteria, are applied against various pathogenic agents such as pathogenic fungi; these properties are due to the ability of bacteria to produce antibiotics and antifungal peptides (Lee et al. 2023). One of the important and diverse components of microbial flora is epiphytic bacteria that are harbored by leaves of plants in temperate, tropical, and subtropical climates. These bacteria are capable of living on plant surfaces (Nongkhlaw and Joshi 2014). Entry points for endophytic bacteria include lenticels, plant wounds, and the exit zone of lateral roots; moreover, bacteria mainly gain entry into plant tissues through wounds (Mushtaq et al. 2023). Given the importance of diseases and the risks associated with chemical compounds, it is necessary to consider natural and eco-friendly methods that involve using microorganisms for controlling plant pathogens. In many citrus-growing regions across Asia, especially in Southwest Asia, both pathotypes A and A* are widespread and cause yield reduction in various citrus species including Mexican lime (*Citrus aurantifolia*), sweet orange (*Citrus sinensis*), grapefruit (*Citrus paradisi*), and tangerine (*Citrus reticulata*). This study aimed to identify epiphytic bacteria with antagonistic properties that could potentially control both pathotypes A and A*.

Methods

Sampling and isolation

In fall 2017, to isolate antagonistic bacteria, various citrus orchards in the southwest Iran were visited, and samples were collected randomly from symptomatic and asymptomatic plants (Fig. 1). The samples were kept in paper bags and transferred to the laboratory of Plant Protection Department for commencing tests. Each leaf sample was surface disinfected with sodium hypochlorite and washed in distilled water; then, 10 g of the sample was cut by a sterile scalpel and pounded in a sterile mortar with 25 ml of distilled water and its extract was prepared. Next, using a sterilized loop, 1 ml of the extract was cultured on a nutrient agar (NA) plate and was kept at 28 °C in an incubator for 5 days.

Purification and preservation methods

First, bacteria were cultured using the 16-streak method on NA plates. After incubation period was over, isolations were done based on colonies size, color and shape, and each single colony was transferred from the streak plate to a new NA plate. For long-term storage, the 24-h bacterial culture was dissolved in a glass container containing sterilized distilled water and stored in a refrigerator at a temperature of 4 °C. In this study, 94 epiphytic bacteria were obtained from citrus leaves collected from

Fig. 1 Infected Mexican lime (*Citrus aurantifolia*) trees in southwest Iran, showing symptoms of citrus canker disease

Table 1 Bacterial isolates, isolated from various citrus orchards of southwest Iran

Strain	Sampling site	Plant tissue	Halo formation	Plant
H6	Gachsaran	Leaf	+	*Citrus sinensis*
T7	Basht	Leaf	+	*Citrus aurantium*
H11	Gachsaran	Leaf	+	*Citrus aurantiifolia*
H12	Gachsaran	Leaf	+	*Citrus aurantiifolia*
A2	Dehdasht	Leaf	+	*Citrus limetta*
F1	Likak	Leaf	+	*Citrus aurantiifolia*
T19	Basht	Leaf	+	*Citrus limetta*
D4	Gousheh	Leaf	+	*Citrus aurantiifolia*
D5	Gousheh	Leaf	+	*Citrus aurantiifolia*
C2	Sarfaryab	Leaf	+	*Citrus aurantiifolia*

southwest regions of Iran. It was found that 10 out of 94 isolates could generate inhibition haloes in dual-culture assays (Table 1). Therefore, further studies were carried out on the inhibitory and antagonistic activities of these 10 isolates.

Pathogenicity test

To conduct this research, two strains of *X. citri* available in the laboratory of the Plant Protection Department were used: The M15 strain as the representative of pathotype A* and LMG9322 as the type strain of pathotype A, from which the M15 strain had been previously isolated from infected lime trees from southwest of Iran (Ebrahimi et al. 2020). To ensure the pathogenicity of the strains, the pathogenicity test was conducted on lime leaves. Young and fully expanded leaves of 2-year-old *Citrus aurantifolia* plants were used for inoculation. Each pathotype was inoculated into three plants by puncturing the underside of ten leaves with a sterilized needle at various points. A bacterial inoculum of 10^8 cfu/ml was applied to the pricked area by adding a drop of cell suspension. The plants were kept in a growth chamber at a temperature of 28 °C, receiving 16 h of light and 8 h of darkness each day. Symptoms were evaluated 12 days post-inoculation, and the causative agent of citrus bacterial canker was reisolated from leaf spots.

In vitro evaluation of the antagonistic potential of strains

Evaluations of the antagonistic potential of the isolated epiphytic bacteria were performed based on the diameter of inhibition halo using agar well diffusion method. This experiment was carried out using a nutrient-sucrose agar medium, and for each of the epiphytic bacteria and two pathogenic pathotypes A and A*, a suspension with a concentration of 10^7 CFU/ml was prepared and measured using a spectrophotometer. Then, 100 µl of the pathogen suspension was spread over the entire plate by a sterile loop. Following incubation, about 6-mm-diameter wells were made by inserting a cork borer into the media, and a total of 20 µl of the epiphytic bacteria suspension was pipetted into the wells (Nxumalo et al. 2020). For each antagonistic bacterium, three replicates were considered. All steps were performed under completely sterile conditions. The plates were incubated at 28 °C for 48 h. Then the inhibition halo diameter was measured using a ruler and in millimeters.

Investigation of phenotypic characteristics

For bacterial identification, some phenotypic and biochemical tests were carried out, including oxidase test and pigment production on YDC and KB media, aerobic and anaerobic growth (OF), carbohydrate fermentation test, gram staining (Schaad et al. 2001), bacterial growth at temperatures of 35, 37, and 39 °C, catalase, and sensitivity to antibiotics.

DNA extraction, PCR and sequencing

To prepare DNA templates, a suspension with a concentration of 10^7 cells/ml was prepared from a 24-h culture of each bacterium in 1.5-ml vials. The suspensions were heated for 10 to 12 min in Bain-Marie, and then they were centrifuged for 10 min at 10,000 rpm. The supernatant phase was used as DNA template. The ingredients required for PCR were pipetted and mixed in 0.5-ml vials and transferred to 0.2-ml vials to add template DNA. The pair of primers 24f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- TACGGYTACCTTGTTACGACTT -3′) (Farris and Olson 2007) were used to amplify 16S-rDNA gene. PCR amplifications were performed in volumes of 20 µl comprising 0.05 µM of each forward and reverse primer (CinnaGen), 0.4 µM dNTPs (MBI 172 Fermentas), 1 × Dream Taq buffer (MBI Fermentas) and 0.5 U Dream Taq DNA polymerase 173 (MBI Fermentas). Thermal cycling was performed by using an initial denaturation step for 1 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing temperature step for 1 min at 56 °C elongation step for 2 min at 72 °C and a final elongation step for 8 min at 72 °C and then cooled to 4 °C (Zheng et al. 2018). The amplicons were analyzed on a 1% agarose gel, stained with ethidium bromide and viewed under UV light to check the amplification size and purity (GelDoc, Bio-Rad Laboratories). PCR products were purified from agarose gels using a PCR purification kit (Fermentas) and DNA sequencing was performed in both directions by Macrogen Company (South Korea).

Phylogenetic analysis

16S-rDNA sequences were blasted using Megablast to identify their closest neighbors. Then, 16S-rDNA sequences obtained in this study were compared with NCBI database to determine the taxonomic status of identified species. Nucleotide sequences were edited by BioEdit v. 7.2.5 (Hall 2012) and aligned using MAFFT v.7 program (Katoh and Standley 2013). Phylogenetic analyses were performed using the distance scale. The matrix distance of sequences was computed by Tamura 3-Parameters Model and a phylogenetic tree was constructed using maximum likelihood (ML) method from MEGA 6.06 software (Tamura et al. 2013). To ensure the stability of the branches in the phylogenetic tree, the bootstrap value was calculated based on 1000 replicates using this program. Bootstrap analysis of 1000 replicates was confirmed the support of the branches and shown next to the branches. *Streptomyces thermogenesis* was used as out-group of 1000 replicates was confirmed the support of the branches and shown next to the branches.

Evaluation of the antagonistic potential of strains

To assess the antagonistic activity of the strains, after selecting the best biocontrol agents, twenty-four seedlings were provided and a fresh culture suspension of epiphytic bacteria with a concentration of 10^7 CFU/ml was sprayed on the lime leaves. Twenty-four hours after foliar spray, using the needle-pricking method, lime leaves were inoculated with *X. citri* pathotypes A and A*. Two seedlings were inoculated with the pathogenic bacterium pathotypes as the positive controls and two seedlings were considered as negative controls, which were sprayed only with sterile water. The plants were kept in a greenhouse with 16-h light and 8-h dark photoperiod, at temperature of 28 °C and 80% humidity. The population of pathogenic bacterium was calculated at 1, 3, 5, 7, and 14 days' post-inoculation (dpi). To perform calculations in each period, three leaves were separated from each seedling, washed with tap water for 30 s., disinfected with sodium hypochlorite 0.5%, followed by three successive rinses in sterile water and extracted in a sterilized porcelain mortar. Ten milliliters of leaf extract with a dilution of 0.01 was prepared and pipetted in the center of NA plate, spread evenly over the entire plate by a loop and kept at 28 °C in an incubator. After 48 h, the number of grown colonies was counted (Zarei et al. 2018). At 20 days' post-inoculation, necrotic lesions were counted on leaves inoculated with different pathotypes. The disease severity of citrus bacterial canker caused by the different strains was expressed as the number of necrotic lesions per leaf (Kunwar et al. 2023). Furthermore, entophytic bacteria were isolated from infected citrus leaves 7 days after inoculation by plating them on NA medium in order to recover antagonistic bacteria. The recovered isolates were identified using phenotypic characteristics and 16S rDNA PCR amplification as mentioned above. The experiment was conducted in a completely randomized design with three replications of each treatment, and the experiment was performed twice.

Statistical analysis

Data of each experiment were analyzed separately using the ANOVA method by SAS statistical software. The homogeneity of error variances was tested using Bartlett's Chi-square test. For assays of the inhibitory capacity of bacterial epiphytes against *Xcc* pathotypes, data were subjected to a 10×2 (epiphytes × *Xcc*) factorial analysis of variance (two-way ANOVA) in a completely randomized design, using SAS statistical software. For the greenhouse experiment, data of necrotic spots were subjected to a 11×2 (epiphytes × *Xcc*) factorial analysis of variance (two-way ANOVA) and the data of population dynamics of *Xcc* pathotypes were subjected to a $2 \times 2 \times 5$ (epiphytes × *Xcc* × Time) factorial analysis of variance (three-way ANOVA) in a completely randomized design, using SAS statistical software (SAS Institute, Cary, NC). Treatment means were compared to the Duncan's multiple range test ($P < 0.01$).

Results

Phenotypic identification of isolates

D4, D5, A2, T7, T19, and C2 isolates were gram positive, and H6, H11, H12, and F1 isolates were gram negative. All isolates were oxidase-negative. None of the isolates produced yellow pigment on the YDC medium. Catalase tests were positive in all isolates and none of the isolates produced fluorescent pigment on KB medium. Also, isolates D4, D5, T7, T19, and C2 could not grow anaerobically. Most of the strains were able to ferment simple sugars such as glucose. Carbohydrate fermentation test was performed using four sugars: fructose, sucrose, galactose, and sorbitol, separately. All isolates except F1 were able to ferment fructose, isolates H6, D4, D5, H11, H6, T7, and F1 fermented galactose, isolates D4, D5, T19, H12, H11, H6, F1, T7, and A2 fermented sorbitol, and isolates D4, D5, A2, and H12 fermented sucrose. All isolates grew at 35, 37, and 39 °C.

Phylogenetic analysis

PCR amplification and sequencing were successful for six antagonistic strains and PCR fragments with the size of 1500 bp were amplified from all the studied antagonistic strains using the general primers 24F and RP2. The topology and branch lengths of the phylogenetic inferences are shown in Fig. 2.

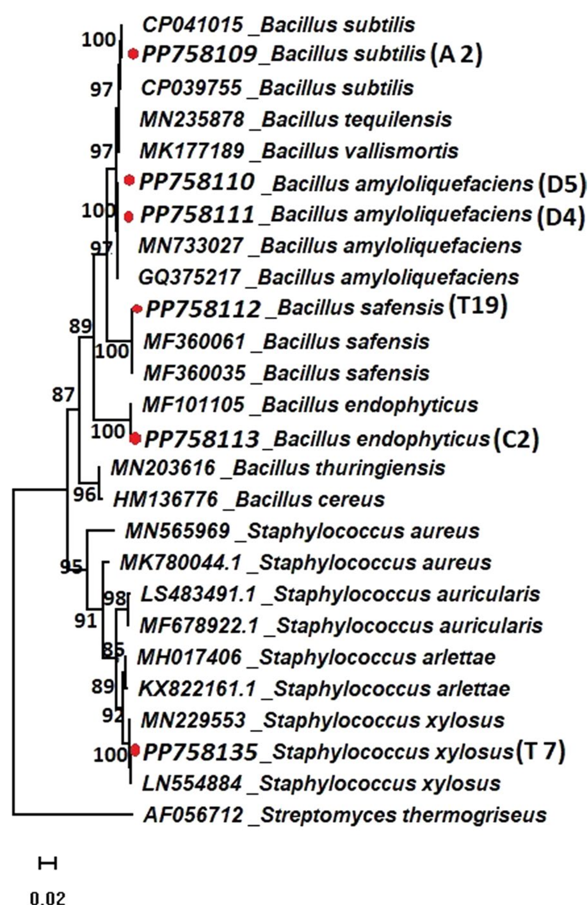

Fig. 2 Phylogenetic tree constructed using maximum likelihood (ML) method based on 16S-rDNA dataset of antagonistic strains. Bootstrap values (in %) are indicated above the branches. *Streptomyces thermogenesis* is used as out-group taxon (6 antagonistic strains are indicated by *). Scale bars indicate 0.02 changes per site per branch

For molecular studies, 6 out of 10 antagonistic strains were selected as biocontrol representatives of citrus orchards located in southwest Iran. *Streptomyces thermogenesis* was also chosen as an out-group. The constructed phylogenetic tree using maximum likelihood (ML) method based on the 16S-rDNA gene sequences, showed that isolate T7 with a bootstrap value of 100% in the clade is related to the *Staphylococcus xylosus* species with the accession number MN229553. Strain A2 is grouped in the clade related to *Bacillus subtilis* species with the accession number CP039755. Isolates D4 and D5 with a bootstrap value of 100% are grouped with the *Bacillus amyloliquefaciens* species with the accession number GQ375217 in the same clade. Isolate T19 with a bootstrap value of 100% belonged to the same clade with *Bacillus safensis* species and the accession number of MF360061, and the last isolate named C2 with a bootstrap value of 100% was placed in a separate clade with *Bacillus endophyticus* species with the accession number of MF101105 (Fig. 2).

Inhibitory capacity of bacterial epiphytes against *Xcc* pathotypes

The ANOVA table showed that all tested antagonists, *Xanthomonas* pathotypes, and their combination had a statistically significant effect on the diameters of inhibition halos (Table 2).

Among the isolates, only in the isolates D5, D4, and A2 there was a significant difference between the diameter of the generated inhibition halo against A and A*. In these mentioned isolates, the diameter of generated inhibition halo against pathotype A*was greater than that of A, but in other bacterial isolates, there were non-significant differences in the diameters of the inhibition haloes generated against A and A*. Isolate D5 showed the highest inhibition halo diameter against A* compared to all other isolates tested and it was significantly higher than the other isolates (Figs. 3 and 4) ($P \leq 0.01$).

Evaluation of antagonistic activity of the isolates

The ANOVA table showed that all tested antagonists, *Xanthomonas* pathotypes, and their combination had a statistically significant effect on the number of necrotic spots (Table 3).

According to this research, a significant difference between the pathotype A* and pathotype A control was observed. Moreover, non-significant differences were observed among the other isolates. The results demonstrated that pathotype A* generated more lesions than

Table 2 Two-way ANOVA results for the effect of different bacterial isolates and *Xanthomonas* pathotype on the diameters of inhibition haloes

Sources	Degrees of freedom	Sum of squares	Mean square	F-value	*p*-value
Antagonists	9	4765.4000	529.4888	252.14	<.0001
Xanthomonas citri pathotypes	1	135.0000	135.0000	64.29	<.0001
Antagonists × *Xanthomonas citri* pathotypes	9	397.3333	44.1481	21.02	<.0001
Error	40	84.0000	2.1000		
Corrected total	59	5381.7333			

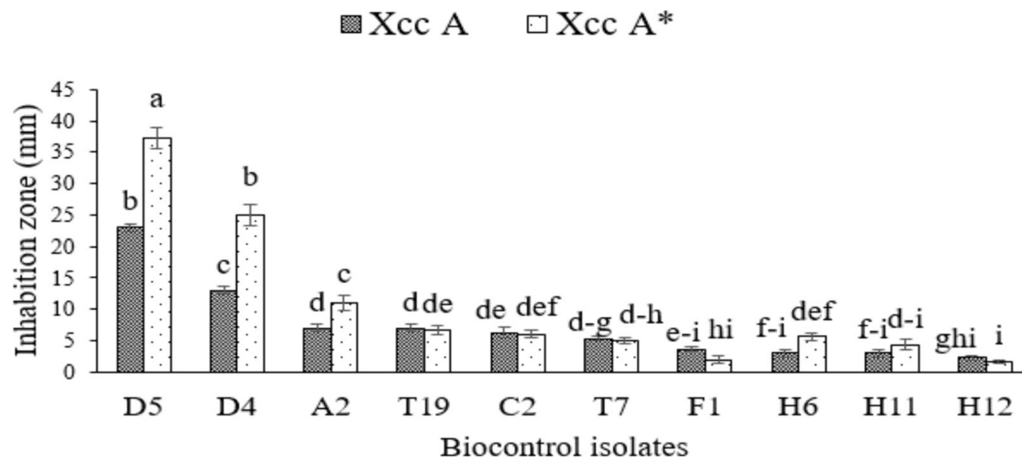

Fig. 3 Diameters of inhibition haloes generated by antagonistic bacteria against *Xanthomonas citri* using well diffusion method. Data are means, and error bars indicate standard error of the mean. Means followed by the same letter(s) are not different according to Duncan's multiple range test ($P < 0.01$)

pathotype A (Fig. 5). All antagonistic strains reduced the occurrence of lesions caused by the pathogenic bacterium pathotypes. The greatest decrease in disease severity was observed in D4 and D5 treatments (*Bacillus amyloliquefaciens*) (Figs. 6 and 7).

Population dynamics of *Xanthomonas citri* pathotypes

To investigate the inhibitory effect of the antagonistic bacteria, ten antagonistic bacterial strains were sprayed on key lime leaves; after 24 h, leaves were inoculated with two pathogenic bacterial strains (pathotypes A and A*). Following leaf extraction, 100 µl of a 100-fold dilution was spread over the entire NA plates. Pathotypes A and A* colonies were counted on day 1, 3, 5, 7, and 14 post-inoculation (dpi). In lime leaves treated with D5 (*Bacillus amyloliquefacien*) and T19 (*Bacillus safensis*), the population of pathotypes A and A* decreased from 5 dpi onward (Fig. 8). Moreover, in leaves treated with T7 (*Staphylococcus xylosus*) the population of pathotypes had a descending trend from 7 dpi onward, while pathotypes A and A* in treatments without antagonists exhibited an increase in population up to 14 dpi. In lime leave treated with H6, H11 and F1, population of pathotypes A and A* had a descending trend from 7 dpi onward, while in treatments without antagonists, pathotypes A and A* also showed an increase in population up to 14 dpi (Fig. 8). In lime leaves treated with H12, similar to those treatments without antagonists, the population of pathotypes A and A* showed an upward trend up to 14 dpi. Moreover, the population of pathotypes A and A* had a descending trend from 5 dpi onward. In lime leaves treated with A2 (*Bacillus subtilis*) the two pathotypes population started to decrease, while in treatments without antagonists, pathotypes A and A* population had an upward trend up to 14 dpi. In lime leaves treated with C2 (*Bacillus endophyticus*), the population of pathotypes A and A* started to decrease from 5 dpi onward, while population of pathotypes A and A* in treatments without antagonists had an ascending trend up to 14 dpi.

Discussion

The entry of citrus bacterial canker into southern Iran and its rapid spread is considered a serious threat to infected provinces and other citrus growing regions of the country. Strains of the causative agent of the disease have been subdivided to different pathotypes and there are clear differences in pathogenicity and host range. The results of other researchers' studies have also shown that the most prevalent form of the pathogen causing bacterial citrus canker in Iran is the Asiatic form (pathotype A) (Khodakaramian and Swings 2011). Based on genotypic tests, it was found that A* strain in Saudi Arabia, India, Oman, and Iran which generates lesions similar to the A-Like form on Mexican lime, was distinct from A strain (Pruvost et al. 2015; Izadiyan and Taghavi 2024). Considering the importance of citrus fruits in the country, it seems necessary to investigate and determine their limiting factors. Since there is no effective and sustainable way to control the disease in the short term, researchers focus is on applying safe and low-risk methods which can lead to successful results in the long term. Among these methods, using antagonists against plant pathogens for biological control has long been the subject of numerous research investigations. Many studies have shown that beneficial microorganisms found in the environment can suppress phytopathogens via various mechanisms. Our research focused on the isolation of epiphytic bacteria and the evaluation of their inhibitory effect against the

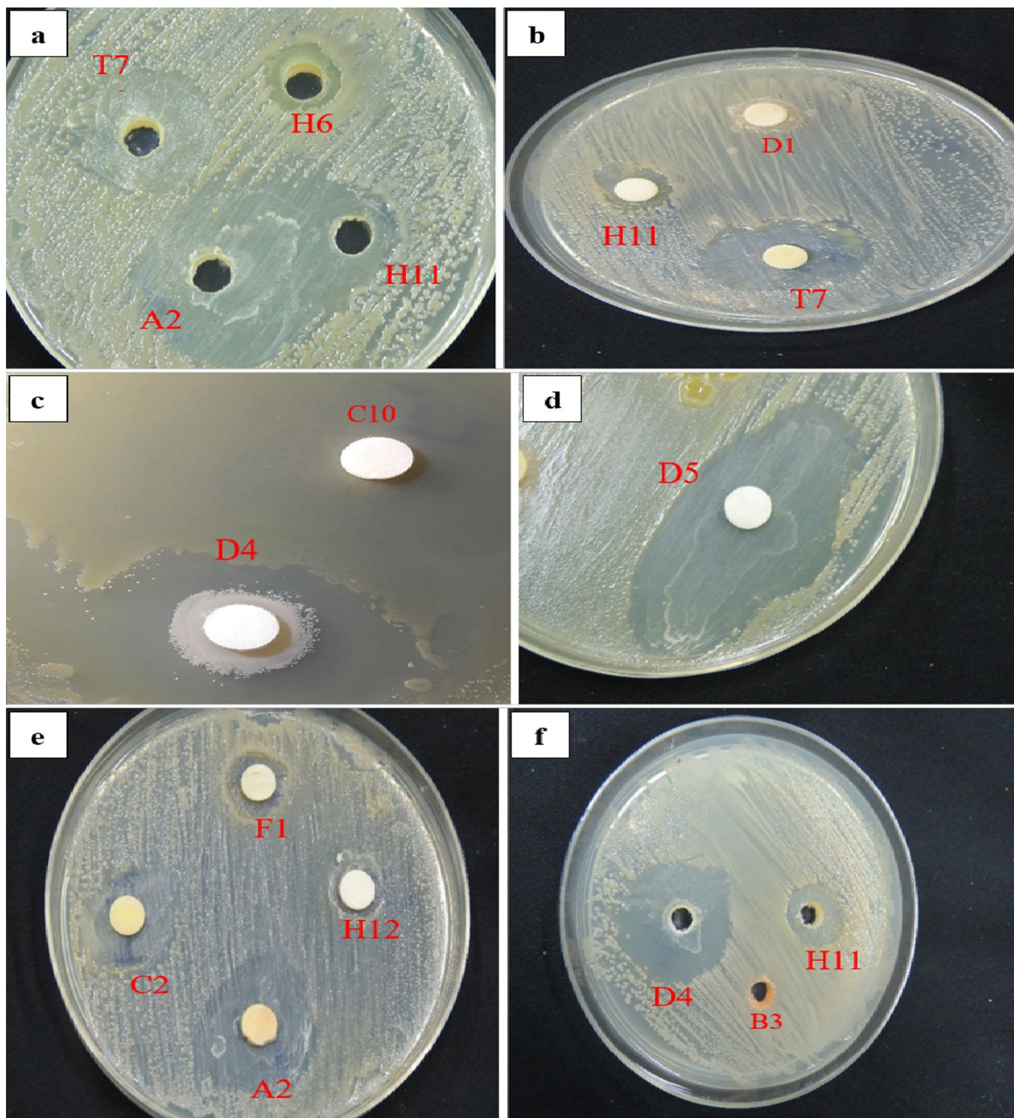

Fig. 4 The inhibition haloes generated by antagonistic bacteria against the pathogenic bacterium pathotypes: **a** generated inhibition haloes against pathotype A, **b** generated inhibition haloes against pathotype A*, **c** generated inhibition halo against pathotype A, **d** generated inhibition halo against pathotype A*, **e** generated inhibition haloes against pathotype A*, **f** generated inhibition haloes against pathotype A

Table 3 Two-way ANOVA results for the effect of different bacterial isolates and *Xanthomonas* pathotype on the number of necrotic spots

Sources	Degrees of freedom	Sum of squares	Mean square	F-value	*p*-value
Antagonists	10	5710.7575	571.0757	188.45	< .0001
Xanthomonas citri pathotypes	1	203.8787	203.8787	67.28	< .0001
Antagonists × *Xanthomonas citri* pathotypes	10	99.7878	9.9787	3.29	0.0029
Error	44	133.3333	3.0303		
Corrected total	65	6147.7575			

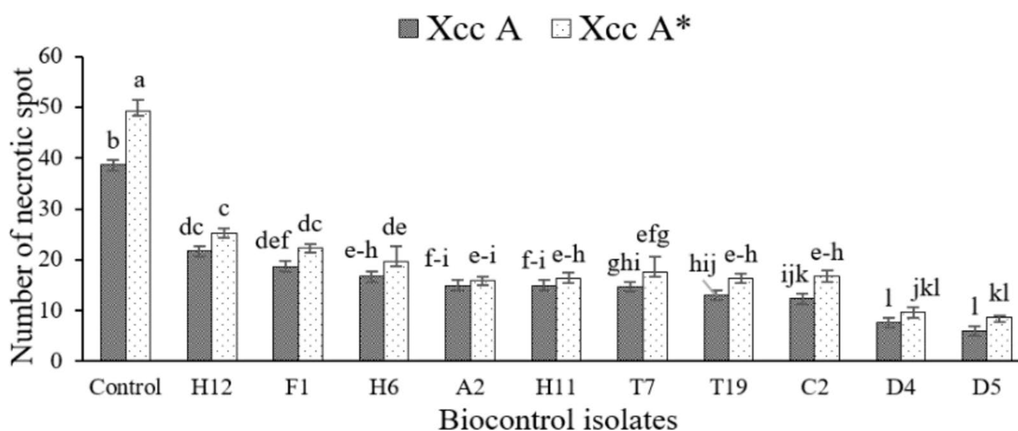

Fig. 5 The number of lesions generated by *Xanthomonas citri* pathotypes in various treatments under greenhouse conditions using the needle-pricking method. Control = *Xcc* A, *Xcc**A Data are means, and error bars indicate standard error of the mean. Means followed by the same letter(s) are not different according to Duncan's multiple range test ($P < 0.01$)

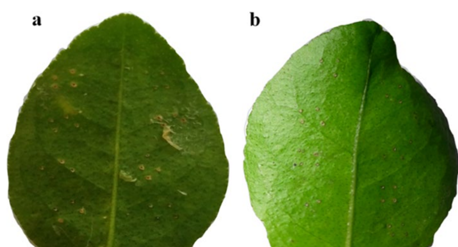

Fig. 6 Pathogenicity test of pathotype A (LMG9322) on key lime leaf: **a** the pathogenic *Xanthomonas citri* pathotype A control, **b** the antagonistic isolate D5 and the pathogenic *Xanthomonas citri* pathotype A

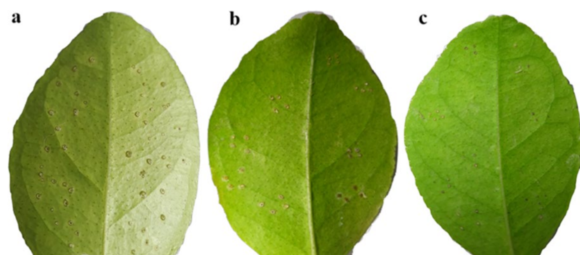

Fig. 7 Pathogenicity test of pathotype A* (M15) on key lime leaf: **a** the pathogenic *Xanthomonas citri* pathotype A* control, **b** the antagonistic isolate T19 and the pathogenic *Xanthomonas citri* pathotype A*, **c** the antagonistic isolate D4 and the pathogenic *Xanthomonas citri* pathotype A*

pathogenic bacterium *X. citri* under in vitro and in vivo conditions. The results of 16S-rDNA sequence analysis illustrated that isolates D4, D5, C2, A2, and T19 were classified in the genus *Bacillus* sp. and the isolate T7 in the genus *Staphylococcus* sp. Among the bacterial biocontrol agents, *Bacillus* species are known as the most widely used bacteria (Hou et al. 2006). Different species of *Bacillus* such as *B. cereus*, *B. amyloliquefaciens*, *B. lichiformis*, *B. megateria*, *B. mycoides* and *B. pumilus* possess antagonistic ability against phytopathogens and induce systemic resistance in host plants (Choudhary and Johri 2009). Studies have demonstrated that *Bacillus* sp., especially *B. subtilis*, produces a variety of antimicrobial peptides, such as surfactin, lantibiotics and iturin (Stein 2005). Three species, namely *Bacillus atrophaeus*, *B. mojavensis*, and *B. vallismortis*, were previously classified as *B. subtilis* (Roberts et al. 1996) and currently strains of *B. subtilis* have been divided into two subspecies, namely *B. subtilis* subsp. *subtili* and *B. subtilis* subsp. *zizenii* (Reva et al. 2004). Many species of *Bacillus* sp. have been isolated from soil or leaf surfaces and these strains can produce antimicrobial compounds. According to the results found in the present study, among ten antagonistic strains, isolates D4 and D5, identified as *B. amyloliquefaciens* strains, showed the highest inhibitory activity against both A (LMG9322) and A* (M15) pathotypes and were successful in attenuation of disease severity. Li et al. (2016) affirmed that AXLP14, a lipopeptide derived from *B. amyloliquefaciens*, possesses antagonistic effects on *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). According to their research, it was determined that AXLP14 consists of surfactin homolog and with a concentration of 0.613 mg/ml, strongly inhibits *Xoo* colonization. Furthermore, with an increase in the concentration of this lipopeptide, the diameter of generated inhibition haloes increased and under greenhouse conditions, reduced disease development was achieved (Li et al. 2016). According to a study, it has been determined that controlling citrus canker disease is feasible through employing *Bacillus* species. Application of *Bacillus subtilis* and *B. amyloliquefaciens* strains resulted in reduced symptom development. Furthermore, *B. subtilis* reduced the

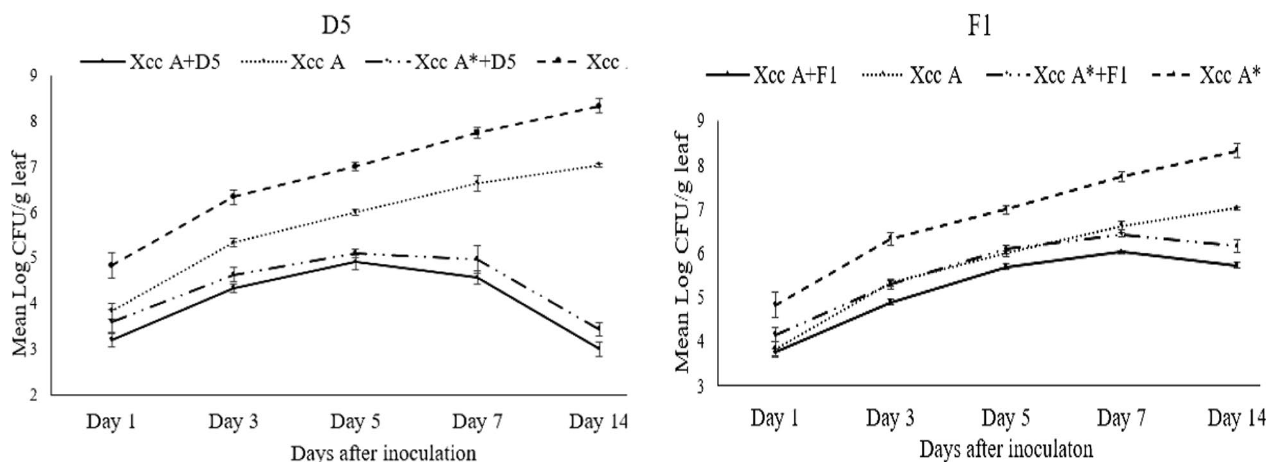

Fig. 8 Effects of the different epiphytic isolates on the growth of *Xanthomonas citri* pathotypes A and A*

occurrence of colonization on lime leaves (Huang et al. 2012). Results obtained in the present study revealed that among these ten isolates, strain A2 identified as *B. subtilis* after *B. amyloliquefaciens* strains showed the highest in vitro inhibitory activity against both pathotypes A (LMG9322) and A* (M15). In a study, various *Bacillus* strains were isolated from different plants; then, their antagonistic effects and their efficiencies as biocontrol agents against two pathogenic bacteria, *Xanthomonas campestris* pv. *campestris* and *Pectobacterium carotovorum* subsp. *carotovorum*, were investigated. Results indicated that *Bacillus cereus*, *B. subtilis*, *B. megaterium*, and *B. pumilus* strains exhibited great antagonistic activity against the mentioned pathogens (Issazadeh et al. 2012). Jung et al. (2014) determined that *B. subtilis* has strong antagonistic ability against tomato pathogen *Clavibavter michiganensis* subsp. *michiganensis*. In Wulff et al. (2002), the biocontrol efficiency of the antagonistic *B. subtilis* against *X. campestris* pv. *campestris* was evaluated in four crops (cabbage, cauliflower, broccoli, and canola) during three growing seasons and on two soil types and different areas in Zimbabwe and *B. subtilis* strain inhibited black rot disease caused by *Xcc* in all *Brassica* crops during dry seasons. According to Monteiro et al. (2005), *B. subtilis* showed the best antagonistic activity against *Xanthomonas campestris* pv. *campestris*, but compared to *B. megaterium*, this bacterium (*B. subtilis*) showed less hemolytic activity. The present study results determined that among ten isolates, isolate C2 identified as *B. endophyticus* showed in vitro inhibitory activity against both A (LMG9322) and A* (M15) pathotypes. According to the results of the present study, the other epiphytic bacterium which exhibited satisfactory antagonistic effects against the causative agent of citrus canker was *B. safensis*. Rong et al. (2020) mentioned that in vitro and in vivo antifungal efficacies of a strain of *B. safensis* against *Magnaporte oryzae* the causative agent of rice blast was affirmed, and these properties were ascribed to the synthesis of iturin A2 and iturin A6 as antifungal peptides. The present study indicates that citrus epiphytic bacteria have the potential to effectively manage the spread of *Xcc*. Therefore, the next steps would involve identifying the antibacterial compounds produced by these bacteria and studying their regulatory genes. Furthermore, these bacteria could be utilized as a biofertilizer to promote more sustainable agricultural practices.

Conclusion

Considering the importance of citrus yields, the negative impact of reduced crop quantity and quality on agricultural economics, and the relative dependence of citrus protection on chemical compounds, controlling citrus bacterial canker as one of the most destructive citrus diseases seems necessary. Moreover, taking into account the negative impact of chemical compounds on humans and the environment, using biocontrol agents and antagonists is considered as one of the most effective control methods. It was concluded that among the isolated epiphytic bacterial strains, *B. amyloliquefaciens* strains, which exhibited satisfactory in vitro and in vivo results, are promising microbial biocontrol agents against citrus bacterial canker and have the potential to be considered as a part of integrated disease management.

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Author contributions

ZF performed the experiments. RR supervised the study and edited the manuscript. HC analyzed the experimental data. FG revised the data. MGG edited the manuscript. All authors read and approved the final manuscript.

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Not applicable. This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by all authors.

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