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Biocontrol of citrus canker with endophyte *Bacillus amyloliquefaciens* QC-Y

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Abstract: Citrus canker is an important disease caused by *Xanthomonas axonopodis* pv. *citri* that affects citrus species. We isolated a bacterium denominated QC-Y with a strong inhibitory effect on citrus canker from navel orange leaves. The isolate was identified as *Bacillus amyloliquefaciens* based on the morphological, physiological, and biochemical characteristics and the 16S rDNA sequence analysis. The inhibitory activity of the pathogen was significantly affected by environmental factors such as the medium, inoculation amount, media volume, and pH. The biocontrol strain QC-Y effectively colonised on navel orange leaves, and the colonisation gradually decreased with time. Twelve days after inoculation, the isolate maintained a certain population level in the leaves. Mancozeb demonstrated a strong inhibitory effect on the growth of QC-Y; Chlorpyrifos at high concentrations inhibited QC-Y. Thiophanate-methyl, Bordeaux mixture, Kasugamycin, Imidacloprid, amino acid, Difenoconazole, Etoxazole, Alphacypermethrin, Buprofezin, Spirodiclofen, Avermectin, and Pyraclostrobin had no effect on the growth of QC-Y. In the detached leaf assay, compared with the leaves inoculated with the pathogens only, the disease incidence of the leaves treated with QC-Y was reduced by 77.5% and the lesions were smaller. Our findings reveal that *Bacillus amyloliquefaciens* QC-Y can be used as a potential biocontrol agent against the citrus canker disease.

Keywords: *Xanthomonas axonopodis* pv. *citri*; endophytic bacteria; navel orange; colonisation

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* (Xac), is a serious disease on commercial citrus crops. This disease has resulted in heavy economic losses to the citrus industry worldwide and is a subject of quarantine at home and abroad (Sharma & Sharma 2009). Citrus canker affects the leaves, shoots, and fruits and causes severe damage to seedlings and young trees (Schoulties et al. 1987). In severe cases, it causes symptoms such as lightness, tree decline, leaf fall, and fruit drop, which affect the economic value of the citrus Graham et al. 2004). The pathogen is easily dispersed in wind-driven rain (Bock et al. 2005), and the optimal temperature for growth is 20–30 °C (minimum of 5–10 °C and a maximum of 35 °C) (Liao et al. 2019). Therefore, citrus canker often occurs in tropical and subtropical

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areas (Ference et al. 2018). The disease is widespread in the countries of Asia, the Americas, and Africa (Gottwald et al. 2002; Das 2003). Currently, in the countries where citrus canker is endemic, an integrated management approach, including chemical control and cultural practices, is used. These measures include use of disease-free seedlings, resistant varieties, windbreaks (to reduce the pathogen dispersion), systemic acquired resistance inducers, and insecticides [(to control citrus leafminer (*Phyllocnistis citrella* Stainton)] and copper sprays (to reduce the inoculum build up on susceptible leaves) (Gottwald et al. 2002). Cultural practices need a lot of manpower and material resources. Copper-based fungicide sprays have prevented infections (Menkissoglu & Lindow 1991). However, increased application has led to a copper toxicity in the soils (Fan et al. 2011) as well as resulting in resistant strains in the xanthomonad populations of copper-tolerant phytobacterial strains (Canteros et al. 2017). In addition, the repeated application of copper-containing fungicides has had adverse effects on the microbial biomass and bacterial community diversity of citrus soils (Zhou et al. 2011).

Nature is a biobank rich in biological resources, so disease management researchers have focused on antagonistic microorganisms for the control of plant diseases. Endophytic microbes enter and colonise plants and confer mutualistic benefits through plant-microbe interactions (Del Barrio-Duque et al. 2019). Endophytic bacteria promote plant growth by producing or inducing the host plants to produce growth hormones, and have the functions of endogenous nitrogen fixation, phosphorus dissolution, iron-producing carriers, etc. (Rangjaroen et al. 2019). Plant endogenous bacteria also confer stress resistance to their host plants (Bruisson et al. 2019). Endophytic bacteria multiply after entering the plant and occupy the niche quickly to reduce the pathogen growth. These endophytic bacteria produce antagonistic substances, which directly inhibit the growth of the existing bacteria in the host (Bacon & Hinton 2002). Under saline conditions, the endophyte *Bacillus subtilis* NUU4 stimulated plant growth and controlled root rot in the chickpea (Egamberdieva et al. 2017). Endophyte *Bacillus safensis* B21 demonstrated antifungal activity against *Magnaporthe oryzae*. B.C. Couch. This endophytic bacteria produced biologically active compounds that inhibited hyphal growth (an endophyte strain, B21, was isolated from *Osmanthus fragrans* Lour. fruits and identified as *Bacillus safensis* by analysis of its 16S rDNA gene sequence and its biochemical and physiological characteristics. The culture filtrate showed antifungal activity against *Magnaporthe oryzae*, which causes rice blast disease, and the IC_{50} of the methanol extract was 15.56 μg/mL, which was significantly lower than that of carbendazim (25.16 μg/mL; Rong et al. 2020).

In this study, we isolated a QC-Y bacterial strain from navel orange (Newhall) leaves and young twigs to prevent citrus canker. The fermentative process was optimised by experimental design to improve the efficiency of the Xac inhibition. The colonisation dynamics were tested on the navel orange leaves. We also investigated the effect of several fungicides and insecticides on the QC-Y. Curative and preventive assays were performed against the citrus canker on navel orange trees in the field.

MATERIAL AND METHODS

Collection of plant materials and isolation of QC-Y

Leaves and young twigs were collected from the Navel Orange Garden of Gannan, Ganzhou, Autumn, 2018, and placed in plastic bags for isolation. These samples were washed under running tap water, cut into pieces (leaves, 4 × 4 cm; young twigs, 4 mm), and sterilised with 1% sodium hypochlorite for 3 min and 70% ethanol for 1 minute. The specimens were then washed three times with sterile distilled water and dried on sterile filter papers. The *Osmanthus fragrans* surface-sterilised samples were then ground with 2 mL of sodium chloride (NaCl, 0.9%; sterile) using a sterile mortar and pestle.

The bacterial suspension was diluted with a sterile NaCl solution (10^{-1}, 10^{-2}, and 10^{-3}), spread on a Luria-Bertani (LB) agar medium, and incubated at room temperature (28–30 °C) for two to three days to obtain the single bacterial colonies. All the bacterial colonies were streaked on new LB plates, and the whole process was repeated at least three times. The single colonies were stored in 50% glycerol at –20 °C until further use.

Testing inhibitory activity of bacteria on Xac

The paper plate method with minor modifications (Chen et al. 2008) was used to test the inhibition to Xac which was provided by Gannan Normal University. The cell suspension of Xac (100 μL) was swabbed on the LB agar, and three 6 mm diameter

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blank drug-sensitive paper pieces with a 10 µL bacterial specimen were placed evenly on each plate that was cultured in the LB for 24 hours. Sterile water was used as a negative control. All the LB agar plates were incubated at room temperature for 48 h, and the diameter of the inhibition zone was measured to determine the antagonistic activity of the bacteria against Xac.

Identification and selection of an effective bacteria

The endophytic bacteria QC-Y was selected as effective bacteria to inhibit Xac (Figure 1). The selected bacteria were identified and characterised on the basis of the morphology and 16S rDNA sequence analysis as well as the physiological and biochemical characters. The 16S rDNA sequence of QC-Y was entrusted to Shanghai Biotech Engineering Co., Ltd. (China) for determination. The DNA sequences were compared with the sequences in the GenBank using the Basic Local Alignment Search Tool (BLAST) program. The DNA sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (version 3.8.31). The phylogenetic tree was generated using the MEGA-X software (version 10.1) according to the neighbour-joining algorithm.

Evaluation of culture characteristics of selected entophytic bacteria

Culture Media. The selected QC-Y bacteria were cultured in six culture media, including corn flour (corn flour 30 g, 1 000 mL water), NYD (beef extract 8 g, yeast extract 5 g, glucose 10 g, water 1 000 mL, pH 7.0), LB (peptone 10 g, yeast powder 5 g, sodium chloride 10 g, water 1 000 mL, pH 7.0), NA (peptone 5 g, yeast extract 1 g, beef extract 3 g, sucrose 10 g, water 1 000 mL, pH 7.0–7.2), YPG (yeast extract 10 g , peptone 20 g, glucose 20 g, water 1 000 mL, pH 7.0), and SPA (sucrose 20 g, peptone 5 g, dipotassium phosphate 0.5 g, magnesium sulfate 0.25 g, water 1 000 mL, pH 7.2–7.4). The selected bacteria were cultured in these media at a constant temperature (30 °C) in a shaking incubator (HZQ-F100, Zhibo Rui Instrument Manufacturing Co., Ltd., China) at 180 rpm for 48 h, and the culture solution was centrifuged at 8 000 rpm, for 15 minutes. The supernatant was taken in order to test the inhibition of Xac by the paper plate method.

Effect of culture characteristics on the inhibitory activity of selected bacteria against Xac

Inoculation amount. The selected SPA medium was used as the fermentation medium. The selected isolate strain QC-Y was inoculated at 1, 2, 3, 4, and 5% concentrations, and these cultures were allowed to grow at a constant temperature (30 °C) in a shaking incubator (180 rpm) for 48 hours. The supernatant was obtained by centrifugation at 8 000 rpm for 15 min, and then applied to assess the inhibition of Xac by the paper plate method.

Culture media volume. The strain QC-Y was inoculated in 25, 50, 75, 100, 125, and 150 mL (the total capacity of the triangular flask is 250 mL) of the selected medium using an optimal inoculation amount, and the fermentation was cultured at a constant temperature (30 °C) in a shaking incubator (180 rpm) for 48 hours. The supernatant was obtained by centrifugation at 8 000 rpm for 15 min, and then applied to assess the inhibition of Xac by the paper plate method.

Initial pH. The selected bacteria QC-Y was cultured in the selected medium with an initial pH of 4, 5, 6, 7, 8, 9, and 10 at a constant temperature (30 °C) in a shaking incubator (180 rpm) for 48 hours.

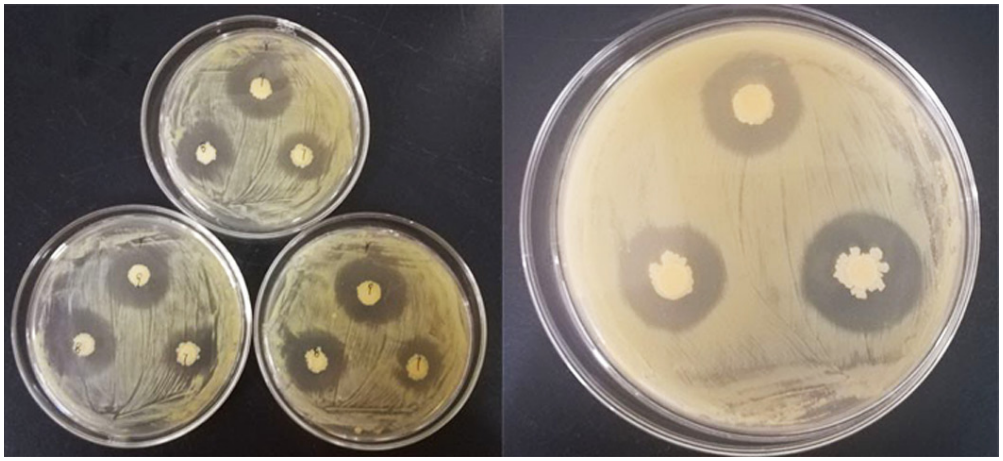

Figure 1. Antagonistic activity of QC-Y against *Xanthomonas citri* subsp. *citri*

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The supernatant was obtained by centrifugation at 8 000 rpm for 15 min, and then applied to assess the inhibition of Xac by the paper plate method.

Incubation time. The selected QC-Y bacteria was cultured for 12, 24, 36, 48, 60, and 72 h at a constant temperature (30 °C) in a shaking incubator (180 rpm). The supernatant was obtained by centrifugation at 8 000 rpm 15 min, and then applied to assess the inhibition of Xac by the paper plate method.

Colonisation of QC-Y in navel orange leaves

The strain QC-Y was labelled with antibiotics (Simons et al. 1996) the number of bacteria present on the root tip was analyzed. The system was optimized with respect to root morphology, inoculation of the seedling, and isolation of root tip bacteria. With this system, rhizosphere colonization on tomato, radish, wheat, and potato was analyzed. For detailed analysis of tomato rhizosphere colonization by some representative plant growth-promoting rhizo-bacteria, the colonization of known poor, moderate, and good potato root-colonizing *Pseudomonas* strains and of four *Rhizobium* strains was determined. All strains colonized the root tips when inoculated as single strains. When inoculated in competition with the efficient root colonizer *P. fluorescens* strain WCS365, many strains were outcompeted. Mutants of *Pseudomonas* biocontrol bacteria lacking flagella or the O-antigen of lipopolysaccharide (LPS). The mutant strain QC-Y1, resistant to 300 μg/mL streptomycin, whose colony morphology and anti-antibiotic activity were basically the same as those of the original strain, was screened. The labelled QC-Y1 was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of the LB with 300 μg/mL of streptomycin, and the medium was cultured at a constant temperature (30 °C) in a shaking incubator (180 rpm) for 48 h. The bacterial cells separated from the culture medium by centrifugation at 8 000 rpm for 10 min were suspended in sterile water, and the concentration was adjusted to 10^8 CFU/mL using a spectrophotometer (UV-5500, Yuan Analysis Instrument Co., Ltd., China).

The QC-Y1 bacterial strain suspension was sprayed evenly on both sides of the newly-formed navel orange leaves. These leaves were incubated at 28 °C. The first leaf samples were collected 1 h fter spraying, and the remaining samples were collected 1, 3, 5, 7, 9, 12 days after processing. The samples were washed under running tap water, cut into pieces (leaves, 4 cm × 4 cm), and sterilised with 1% sodium hypochlorite for 3 min and 70% ethanol for 1 min. The specimens were then washed three times with sterile distilled water and dried on sterile filter papers. The surface-sterilised samples were then ground with 2 mL of sterile NaCl (0.9%) using a sterile mortar and pestle and counted (CFU/cm^2) by the dilution coated plate method.

Effect of fungicides and insecticides on QC-Y

We further investigated the effect of six fungicides and seven insecticides on the biocontrol QC-Y strain by the inhibition zone method. The test agents were diluted with sterile water. The chemicals used in this study are as follows: 80% Mancozeb (Shandong United Pesticide Industry Co., Ltd. China; 1 000, 2 000, 3 000 mg/L); 70% Thiophanate-methyl (Zhejiang Weida Chemical Co., Ltd., China; 1 670, 3 340, 5 010 mg/L); 80% Bordeaux mixture (American Shannon Co., Ltd., USA; 1 250, 2 500, 3 750 mg/L); 6% Kasugamycin (Wuhan Keno Biotechnology Co., Ltd., China; 670, 1 340, 2 010 mg/L); 70% Imidacloprid (Shandong United Pesticide Industry Co., Ltd., China; 143, 286, 429 mg/L); Amino acid (Changsha Lvba Fertilizer Co., Ltd., China; 1 000, 2 000, 3 000 mg/L); 20% Difenoconazole (Qingdao Zhengdao Pharmaceutical Co., Ltd., China; 500, 1 000, 1 500 mg/L); 45% Chlorpyrifos (Shandong United Pesticide Industry Co., Ltd., China; 1 000, 2 000, 3 000 mg/L); 20% Etoxazole (Taizhou Dapeng Pharmaceutical Co., Ltd., China; 500, 1 000, 1 500 mg/L); 3% Alpha-cypermethrin (Hebei Saifeng Biotechnology Co., Ltd., China; 1 000, 2 000, 3 000 mg/L); 25% Buprofezin (Shandong United Pesticide Industry Co., Ltd., China; 670, 1 340, 2 010 mg/L); 29% Spirotetran (Jiangsu Jianpai Agricultural Chemical Co., Ltd. China; 400, 800, 1 200 mg/L); 5% Abamectin (Hebei Weiyuan Biochemical Co., Ltd., China; 400, 800, 1 200 mg/L); and 250 mg/L pyraclostrobin (Yong'an Bioscience Co., Ltd. China; 670, 1 340, 2 010 mg/L).

The biocontrol QC-Y strain was inoculated into 50 mL of the SPA medium and allowed to grow (30 °C, 180 rpm) for 24 hours. The biocontrol bacterial suspension (3 mL) was added into 300 mL of the LB media at 50 °C, mixed gently, and poured slowly and uniformly onto a Petri dish (about 20 mL). After the agar solidified, three blank, drug-sensitive papers (6 mm diameter) with 20 μL of the bactericide were placed evenly on each plate. All the plates were sealed and incubated at 30 °C for 24 hours. The diameter of the inhibition zone was measured using a digital calliper.

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Inhibitory activity of QC-Y against citrus canker of navel orange

Navel orange leaves (a leaf that turned green and attained a maximum leaf area) of an appropriate age were picked, and after cleaning, were put in a sterile petri dish, with a layer of sterile wet filter paper at the bottom, and then a row of inoculation points were pricked on the back of the leaves with a sterilisation pin. The 6 mm diameter filter paper sheet, containing a suspension of the pathogen with a concentration of about 10^8 CFU/mL, was attached to the inoculation site, and the petiole was wrapped with cotton containing the nutrient solution. Then the petri dish was sealed with a plastic film. After incubating at 30 °C for 24h, the filter paper was removed, and the QC-Y strain suspension was sprayed on the leaves to continue the cultivation. The leaves of the pathogen treatment alone were used as a negative control. The incidence of citrus canker at the inoculation site was observed every day after the inoculation. The symptoms, number of disease spots and incidence rate were recorded until the 7th day (Chen et al. 2014). The incidence rate and biocontrol efficacy were calculated as following Formulation (1):

$$\mathrm{BE}(\%) = \frac{IC - IBS}{IC} \times 100 \quad (1)$$

where: BE – biocontrol efficacy; *IC* – incidence rate in the control; *IBS* – incidence rate in the biocontrol strain.

Statistical analysis

The data were expressed as the mean ± standard deviation by measuring three independent replicates per experiment. The statistical analysis of the results was conducted by a one-way ANOVA followed by a homogeneity test of variance and an LSD test (least significant difference) at $P = 0.05$ using the SPSS 24 statistical software (version 24) (Islam et al. 2019).

RESULTS

Isolation of bacteria from citrus plants and screening for inhibitory activity against Xac

Thirty endophytic bacteria were isolated from the leaves and young twigs of Gannan navel oranges. The QC-Y isolate demonstrated the highest antagonistic activity against Xac with an inhibition zone of 23–25 mm in the agar well diffusion method (Figure 1). This endophytic bacterium was selected to inhibit the growth of Xac and control the citrus canker disease of the citrus plants.

Identification of the effective strain QC-Y

The colonies of QC-Y were irregular in shape with a rough and dry surface on the LB agar. QC-Y, a gram-positive bacterium, produced acid from sugars (e.g., glucose, sucrose, lactose, and mannitol) other than lactose. The isolate was catalase-positive and Volt-Pulse-positive; however, it was negative for citrate utilisation, methyl red production, and hydrogen sulfide production. A phylogenetic tree was constructed using the 16S rRNA sequences of QC-Y and its close relatives' species. The phylogenetic analysis of the 16S rRNA gene sequences revealed 100% identity of QC-Y with the *Bacillus amyloliquefaciens* strain MPA1034 (Figure 2).

Culture medium of QC-Y for efficient inhibition of Xac

The fermentation of different media components has an effect on the production of antibacterial substances. It was found that the metabolic products of the QC-Y strain fermented in the NYD medium and the metabolites fermented in the other mediums have significant differences in the inhibitory activity on the citrus canker. However, the SPA medium had the largest inhibition zone diameter compared to the other five mediums. The components in the SPA medium may favour the production of some bioactive compounds, which inhibit the growth of Xac (Table 1).

Effect of culture conditions of QC-Y for efficient inhibition of Xac

Inoculation amount. We recorded the highest antagonistic activity against Xac when QC-Y was in-

Table 1. Effect of the culture media on the antibacterial activity of QC-Y against Xac

Culture media	Inhibition zone (mm)
Corn flour	18.92 ± 1.74^a
NYD	17.83 ± 2.42^b
LB	20.00 ± 1.48^a
NA	20.33 ± 1.21^a
YPG	20.08 ± 2.54^a
SPA	21.00 ± 1.34^a

The lowercase letters following the data represent a significantly difference at 0.05

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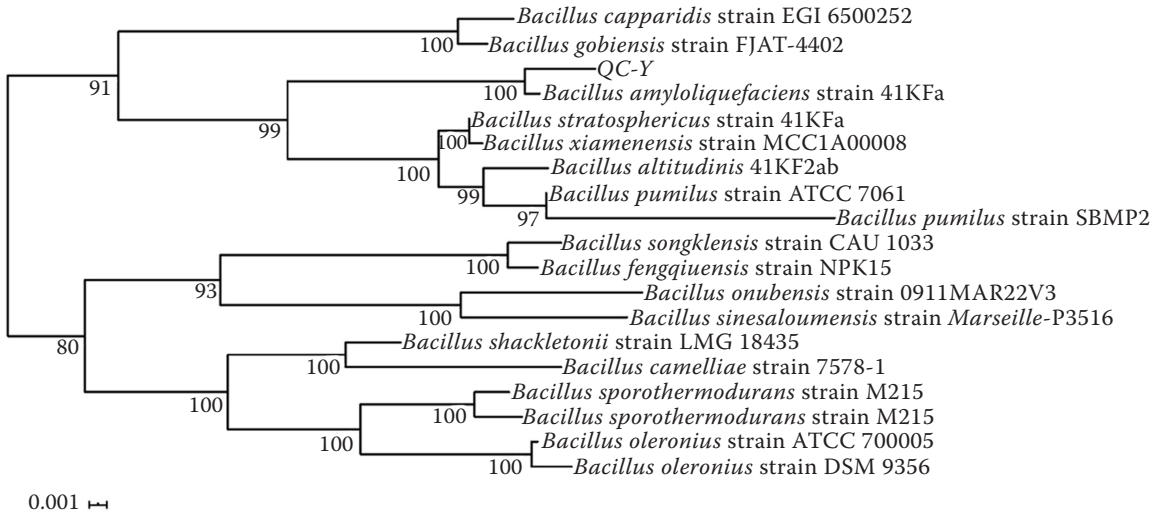

Figure 2. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences showing the relationship among the selected QC-Y strains

The evolutionary distances were computed using the maximum composite likelihood method; the numbers at the nodes are the bootstrap values based on 1 000 replicates (%)

oculated at a 2% concentration. With an increase in the inoculation amount, the antibacterial activity of QC-Y decreased (Table 2). These findings indicate that a higher concentration of the inoculation inhibits the production of bacteriostatic agents.

Media volume. During the shake flask fermentation, the smaller the media volume was, the greater the oxygen transfer coefficient was. When QC-Y was cultured in 25–75 mL of the culture media, the bacteriostatic activity increased sustainably. In 75 mL, QC-Y had highest the bacteriostatic activity against Xac. With an increase in the volume of the culture media, the antibacterial activity decreased (Table 3).

Initial pH. At an initial pH of 4, QC-Y demonstrated a slight bacteriostatic activity, which indicates an acid resistance. However, when the pH was 5–10, the difference in the antibacterial effect of QC-Y strain was not significant. When the pH was 8, the bacteriostatic activity was the highest. This indicates the strong acid and alkali resistance of QC-Y (Table 4).

Incubation time. The incubation time has a great influence on the secondary metabolites of the

Table 3. Effect of the media volume on the antibacterial activity of QC-Y against Xac

Media volume (mL)	Inhibition zone (mm)
25	16.83 ± 1.26^{b}
50	17.00 ± 0.50^{b}
75	19.67 ± 0.58^{a}
100	15.00 ± 0.71^{b}
125	14.50 ± 0.71^{b}
150	15.75 ± 1.06^{b}

The lowercase letters following the data represent a significantly difference at 0.05

Table 2. Effect of the inoculation amount on the antibacterial activity of QC-Y against Xac

Inoculation amount (%)	Inhibition zone (mm)
0.5	21.33 ± 1.53^{b}
1	20.67 ± 0.29^{b}
2	24.67 ± 1.26^{a}
3	21.67 ± 1.89^{b}
4	21.83 ± 1.04^{b}
5	21.50 ± 0.50^{b}
6	21.42 ± 1.39^{b}

The lowercase letters following the data represent a significantly difference at 0.05

Table 4. Effect of the initial pH on the antibacterial activity of QC-Y against Xac

Initial pH	Inhibition zone (mm)
4	18.50 ± 3.28^{b}
5	21.50 ± 2.65^{a}
6	22.83 ± 0.76^{a}
7	23.00 ± 1.00^{a}
8	24.00 ± 1.80^{a}
9	22.83 ± 1.89^{a}
10	20.50 ± 0.71^{a}

The lowercase letters following the data represent a significantly difference at 0.05

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Table 5. Effect of the incubation time on the antibacterial activity of QC-Y against Xac

Incubation time (h)	Inhibition zone (mm)
12	13.67 ± 0.73[b]
24	12.25 ± 1.77[b]
36	13.75 ± 0.35[b]
48	19.50 ± 0.71[a]
60	15.50 ± 0.87[b]
72	16.67 ± 0.58[b]

The lowercase letters following the data represent a significantly difference at 0.05

microorganisms, which directly affects the antagonistic activity of the fermentation broth. The antagonistic activity of the fermentation broth after 48 h of culturing was significantly higher than that of the other fermentation broths. Therefore, 48 h was selected as the best fermentation time (Table 5).

Colonisation of QC-Y in navel orange leaves

The colonisation of QC-Y1 on the leaves decreased gradually with time. Many colonies were observed on the navel orange leaves on the day of treatment, and the number gradually decreased. On the day of inoculation, the bacterial population on the navel orange leaves was 2.413 × 10^5 CFU/cm^2. After three days, the population on the navel orange leaves dropped sharply to 61.1% compared with that at the time of inoculation. Later, the decline rate was slightly slower, and it showed no change after nine days (Figure 3). After 12 days of treatment, bacteria were still found colonised on the leaves.

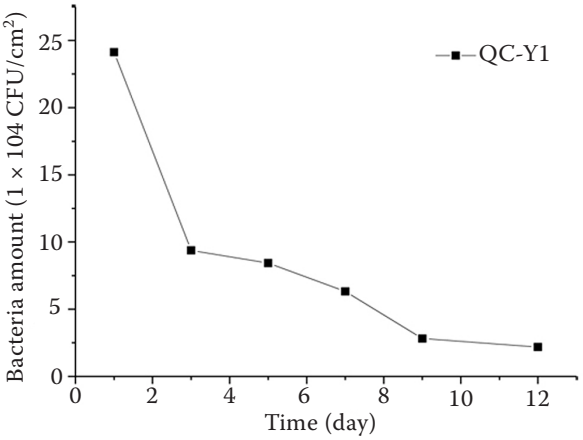

Figure 3. Colonisation dynamics of QC-Y1 in the navel orange leaves

Effect of fungicides and insecticides on QC-Y

The filter paper with Mancozeb had an obvious inhibition zone on the plate coated with the QC-Y bacterial suspension. It showed that Mancozeb had a significant inhibitory effect on the growth of QC-Y. In addition, Chlorpyrifos at concentrations of 2 000 mg/L and 3000 mg/L demonstrated a weak inhibitory effect on QC-Y. Thiophanate-methyl, Bordeaux mixture, Asugamycin, Imidacloprid, amino acid, Difenoconazole, Etoxazole, Alphacypermethrin, Buprofezin, Spirodiclofen, Avermectin, and Pyraclostrobin had no effect on the growth of QC-Y (Table 6).

Table 6. Effect of the different bactericides on QC-Y under greenhouse conditions

Bactericides	Concentration (mg/L)	Inhibition zones (mm)	Concentration (mg/L)	Inhibition zones (mm)	Concentration (mg/L)	Inhibition zones (mm)
Mancozeb	1 000	23.83	2 000	26	3 000	30
Thiophanate-methyl	1 670	–	3 340	–	5 010	–
Bordeaux mixture	1 250	–	2 500	–	3 750	–
Kasugamycin	670	–	1 340	–	2 010	–
Imidacloprid	143	–	286	–	429	–
Amino acid	1 000	–	2 000	–	3 000	–
Difenoconazole	500	–	1 000	–	1 500	–
Chlorpyrifos	1 000	–	2 000	10	3 000	11.5
Etoxazole	500	–	1 000	–	1 500	–
Alphacypermethrin	1 000	–	2 000	–	3 000	–
Buprofezin	670	–	1 340	–	2 010	–
Spirodiclofen	400	–	800	–	1 200	–
Avermectin	400	–	800	–	1 200	–
Pyraclostrobin	670	–	1 340	–	2 010	–

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Figure 4. Disease symptom development at 7 days post-infiltration (dpi) on the leaves of the navel orange inoculated with the pathogen *Xanthomonas citri* subsp. *citri* and the bacteria *Bacillus amyloliquefaciens* QC-Y
A, B – leaves treated with the pathogens only; C, D – leaves inoculated with the pathogen and the QC-Y strain

Biocontrol efficacy of QC-Y against citrus canker in navel orange

The QC-Y strain was tested for a biocontrol efficacy against Xac on the navel orange leaves. In the detached leaf assay, the leaves inoculated with Xac showed disease symptoms. In the leaves treated with the pathogens only, water-stained lesions appeared near the inoculation point on the 2nd day. The acupuncture point swelled and a white spongy substance appeared on the lesion on the 3rd day. With the passage of time, the white spongy material accumulates on the dorsal lesions, the lesions on the front of the leaves were sunken, and the lesions gradually expanded. Nevertheless, only a few inoculation spots appeared as lesions on the leaves infected with QC-Y, and the lesion incidence was reduced by 77.5% and the lesions were smaller compared with the leaves inoculated with the pathogens only. It showed that the QC-Y strain can effectively inhibit the formation and expansion of lesions (Figure 4).

DISCUSSION

Thirty endophytic bacteria were isolated from the leaves and young twigs of Gannan navel oranges. The QC-Y strain was found to exhibit a significant positive antibacterial activity according to the diameters of the inhibition zone (Figure 1). The isolated bacterial strain was identified and characterised as *Bacillus amyloliquefaciens* based on its morphology and the 16S rDNA sequence analysis as well as the physiological and biochemical characters (Figure 2). Therefore, it is commercially used as a biological fertiliser and a biological control agent in agriculture (Paul et al. 2015) FZB42 is used commercially as biofertilizer and biocontrol agent in agriculture. Genome analysis of FZB42 revealed that nearly 10% of the FZB42 genome is devoted to synthesizing antimicrobial metabolites and their corresponding immunity genes. However, recent investigations in planta demonstrated that – except surfactin – the amount of such compounds found in vicinity of plant roots is relatively low, making doubtful a direct function in suppressing competing microflora including plant pathogens. These metabolites have been also suspected to induce changes within the rhizosphere microbial community, which might affect environment and plant health. However, sequence analysis of rhizosphere samples revealed only marginal changes in the root microbiome, suggesting that secondary metabolites are not the key factor in protecting plants from pathogenic microorganisms. On the other hand, adding FZB42 to plants compensate, at least in part, changes in the community structure caused by the pathogen, indicating an interesting mechanism of plant protection by beneficial Bacilli. Sub-lethal concentrations of cyclic lipopeptides and volatiles produced by plant-associated Bacilli trigger pathways of induced systemic resistance (ISR) (Zhou et al. 2020). *Bacillus amyloliquefaciens* protect plants from pathogens through mechanisms related to an

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induced system resistance (ISR) or a system acquired resistance (SAR) (Li Yunlong et al. 2015; Cheng et al. 2016). In addition, *Bacillus amyloliquefaciens* produce a variety of antagonistic substances that have inhibitory effects on pathogens, such as antibiotic substances (mainly lipopeptide antibiotics and polyketides and some other antibiotics) and antibacterial proteins or cell wall degrading enzymes (Siti Nur Azizah et al. 2015; Li et al. 2016; Asma Ait Kaki et al. 2017).

In this study, we found SPA optimal for QC-Y with a wider zone of inhibition (Table 1). The ingredients in this medium may be suitable for *Bacillus* species to produce biologically active compounds, which may inhibit the growth of Xac. SPA contains peptone, sucrose, dipotassium phosphate, and magnesium sulfate. Peptone is rich in organic nitrogen compounds and contains vitamins and sugars. Vitamin B plays an important role in bacteria as a coenzyme involved in many metabolic processes (Daungfu et al. 2019). Sucrose protects microorganisms from the stress-induced inactivation of important cellular components and has an important effect on the enzyme gene expression and storage protein accumulation (Iraqi et al. 2005; Richard et al. 2016). In addition, the composition of the growth medium directly affects the production of secondary metabolites by inhibiting biosynthetic enzymes (Gallo & Katz 1972).

The bacteriostatic effect of QC-Y was influenced by the inoculation amount, pH, media volume, and incubation time. Bacteria produce a variety of antagonistic or competitive metabolites during their growth and development to block or kill pathogenic bacteria through direct or indirect effects (Chen et al. 2019) we isolated 12 strains resistant to *Magnaporthe oryzae* from western Sichuan subalpine soil. Among them, CQ07 exhibited remarkable activity against *M. oryzae*. The result of 16S rRNA sequence analysis revealed that CQ07 is approximately 99% similar to *Bacillus australimaris*. The sterilized culture filtrate of CQ07 inhibited the growth of *M. oryzae*, which motivated us to deduce the influence of CQ07 on the pathogenicity of *M. oryzae*. As shown by experimentation, sterilized culture filtrate (10 µL/mL). The study of the culture characteristics showed that an inoculation amount of 2%, a pH of 8.0, a media volume of 0.3%, and an incubation time of 48 h were optimal for the production of bacteriostatic substances (Tables 2–5).

Bacillus effectively colonise plant tissues and soils and produce a variety of biologically active compounds that promote plant growth and act against plant pathogens (Abdallah et al. 2019; Nascimento et al. 2020). Effective bacterial colonisation on plants determines the stability and long-lasting biocontrol effect on the plant diseases (Ji et al. 2008). Studies have reported that *Bacillus amyloliquefaciens* effectively colonised plants such as the banana (Yuan et al. 2015), the tomato (JunQing et al. 2013), lettuce (Chowdhury et al. 2013), and peppers (Lee & Ryu 2016). Ours is a preliminary study on the colonisation dynamics of a biocontrol agent (bacteria) on navel orange leaves. We observed a gradual decrease in the colonisation of QC-Y on navel orange leaves with time. Twelve days after inoculation, the strain maintained the colonisation in the leaves (Figure 3). Therefore, we assume that a reinoculation 10 days after the first inoculation may help maintain the population, which can have a long-term protective effect on plants.

The citrus leafminer (*Phyllocnistis citrella* Stainton) (Heppner 1993), citrus psyllids, the citrus red mite (*Panonychus citri*) (Hare et al. 1989), and the citrus whitefly (Jamieson et al. 2009) are the most common insect pests of citrus trees in the orchard and are controlled by fungicides and pesticides (Jeppson et al. 1962; Sétamou et al. 2010). Mancozeb, a fungicide widely used for plant disease control in the field, demonstrated a strong inhibitory effect on the growth of the biocontrol QC-Y strain; Chlorpyrifos at high concentrations inhibited QC-Y. Thiophanate-methyl, Bordeaux mixture, Kasugamycin, Imidacloprid, amino acid, Difenoconazole, Etoxazole, Alphacypermethrin, Buprofezin, Spirodiclofen, Avermectin, and Pyraclostrobin had no effect on the growth of QC-Y (Table 6). Therefore, spraying these fungicides and pesticides for pest control along with QC-Y to control citrus canker can be a good integrated management strategy. This study provides a basis for the application of QC-Y for the control of citrus canker in the field.

The detached leaf assay showed a significant inhibitory effect of QC-Y on the citrus canker. The leaves of the navel oranges infected with QC-Y showed a significantly reduced lesion incidence and disease symptoms in comparison to the leaves inoculated with Xac alone (Figure 4). There are reports on microorganisms with an antagonistic activity against citrus canker. The application of *Bacillus subtilis* (S-12) in West Bengal (India) effectively controlled citrus canker of limes (Das et al. 2014). Under greenhouse conditions, *Acinetobacter baumannii* Bt8

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demonstrated a 55% control of citrus canker (Tan et al. 2006). *Bacillus thuringiensis* TbL-22 reduced the incidence of canker disease by 64.05% (Islam et al. 2019). The combined application of *Pseudomonas fluorescens* and salicylic acid significantly reduced the number of lesions per leaf by 72% and the severity of citrus canker disease by 84% (Al-Saleh et al. 2015). Experiments using isolated leaves from susceptible citrus species showed that *Bacillus velezensis* EB-39 significantly reduced the incidence of citrus canker on infected leaves by 38% (Rabbee et al. 2019). *Burkholderia territorii* A63, *Burkholderia metallica* A53, and *Pseudomonas geniculata* 95 significantly reduced the canker symptoms of the Duncan grapefruit (Riera et al. 2018). As shown by the experimental results, it is obvious that the biological efficacy of QC-Y against citrus canker in navel oranges is measured by the pin prick method. However, a population dynamics analysis can better understand the interactions between the bacterial populations and further research is needed to explore this.

In short, *Bacillus amyloliquefaciens* QC-Y has high activity against Xac and can be used as a biological control agent or as a source of bioactive compounds. It is a promising, eco-friendly source of antibacterial compounds that can protect citrus trees from citrus canker. Further field studies with QC-Y may help develop sustainable control measures.

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