Study on isolation, identification and colonization ability of antagonistic bacterium M39 against pomegranate wilt pathogen

Zhou Yinli1, Guo Jianwei1, Yang Wei2, Bai Jianbo1, Kong qiong1, Hu Xianqi3*

1 Key Laboratory of Higher Quality and Efficient Cultivation and Security Control of Crops for Yunnan Province, Honghe University, Mengzi 661100, China
2 Commercial college, Honghe University, Mengzi 661100, China; 3Key Laboratory for Agri-biodiversity and Pest Management of Education Ministry of China
3 Yunnan Agricultural University, Kunming 650201, China
*Corresponding author’s e-mail: xqhoo@126.com

Abstract. In this study, the bacteria with strong antagonistic activity against pomegranate wilt pathogen were isolated and screened from the soil samples collected from Yunnan Jianshui Pomegranate Garden and Mengzi Pomegranate Garden. The indoor inhibition rate of bacterial strain M39 against pomegranate wilt pathogen was 81.17%. The study showed that strain M39 could be stably colonized in the rhizosphere of pomegranate, which had potential biological control value. Based on the cultural characteristics of strain M39, colony morphology observation, physiological and biochemical characteristics analysis (Biolog system), strain M39 was initially identified as Corynebacterium freneyi.

1. Introduction
In Yunnan Mengzi ten-thousand Mu Pomegranate Garden, which enjoys the reputation of “the hometown of Chinese pomegranates”, with the intensive management of cultivation and the growth of pomegranate age, various pomegranate pests and diseases have become increasingly serious. Thereinto, the wilt disease, called the ”cancer” of pomegranate by fruit growers, is particularly serious. Pomegranate wilt disease is a kind of soil-borne fungus disease, and its pathogen Ceratocystis fimbriata Ellis & Halsted is widely distributed, mainly causing taro black rot, sweet potato black spot disease, and pomegranate wilt disease in China [1]. Pomegranate wilt disease has occurred in Mengzi County of Yunnan Province and Panxi District of Sichuan Province [2-4], and pomegranate wilt pathogen mainly invades through root wounds [5]. It is difficult to survive for the replanted pomegranate in the pomegranate garden, and the pomegranate orchard will be ‘encroached on’ by the wilt disease, with the decreasing area. It has become an important limiting factor for the healthy and sustainable development of the pomegranate industry.

At present, the control of pomegranate wilt disease is mainly dependent on chemical pesticides. Some studies have reported that indigenous microorganisms show good application potential in the prevention and control of pomegranate wilt disease [6-9], and some also attempted to control diseases using biodiversity and plant-derived pesticides [10-12]. This study screened antagonistic bacteria with strong antibacterial activity against pomegranate wilt pathogen, providing a certain reference to more environmentally control the pomegranate wilt disease.
2. Materials and methods

2.1 Collection of rhizosphere soil samples
The types of soil samples collected were rhizosphere soils of pomegranate wilt disease and healthy pomegranate. The pomegranate garden was divided into districts, and by the five-point sampling method, 3 strains were sampled at each point. The rhizosphere soil at approximately 1 cm around the root system was collected from three different locations around each strain, and the rhizosphere soil of the three strains was mixed into one sample. A total of 45 samples were collected.

2.2 Screening of antagonistic bacteria against pomegranate wilt pathogen
Coarse screening of antagonistic bacteria: Spore suspension was prepared by the pomegranate wilt pathogen cultivated for 7 days, at a concentration of $10^3$/(pieces/mL), and 100μL was applied to a PDA plate and allowed to stand for 5 h. Single colonies of bacteria isolated from soil were inoculated onto the PDA plates coated with pathogens with sterilized toothpicks. At the time of inoculation, bacteria with different cultural characteristics were selected to obtain more antagonistic strains. After being cultured in a constant-temperature incubator at 28 °C for 3-5 days, the size of the inhibition zone was observed and determined.

Rescreening of antagonistic bacteria: The antagonistic bacteria with obvious antibacterial effect were selected and rescreened by the plate confrontation method to preserve the Bacillus strains with good antagonistic effect for subsequent experiments.

The pomegranate wilt pathogen in this experiment was provided by professor Huang Qiong in the Plant Pathology Laboratory of Yunnan Agricultural University.

2.3 Colonization ability of antagonistic bacteria against pomegranate wilt pathogen
Pomegranate seedlings potted with sterile soil were prepared in the greenhouse, fertilized and watered on schedule, and the pomegranate seedlings grown for one year. The antagonistic strain was subjected to rifampicin resistance labeling, and the rifampicin-resistant strain was inoculated into LB nutrient medium, cultured to a concentration of $1\times10^7$ CFU/mL under the conditions of 150 rpm in a shaker and 37° C. 100 mL of the suspension was taken and inoculated in the rhizosphere soil of pomegranate seedlings by watering, with three repetitions, which was watered and fertilized regularly, with conventional management.

Determination of colonization ability: After the rifampicin-resistant strain suspension was applied, the number of antagonistic strains in the root soil was sampled on the 5th, 10th, 15th, 20th, 25th, 30th and 35th day, and the rhizosphere soil samples were taken. The rhizosphere soil samples were taken to prepare serial dilutions of soil samples $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$. The diluted solution of $10^4$, $10^5$, and $10^6$ were selected, and 200μL of the samples was transferred and uniformly coated on a LB plate containing rifampicin (200μg/mL), respectively. Each gradient was repeated 3 times, and the numbers of colonies on the plates were counted separately after cultivation at 37° C for 48 hours. The colonization density was calculated according to the following formula: Colonization density (CFU/g) = average number of colonies repeated three times at the same dilution × dilution factor × 5

2.4 Biochemical identification of antagonistic bacteria against pomegranate wilt pathogen
Identification procedure: It was conducted according to the standard procedure for bacterial identification of GENIII MicroPlate™.

3. Results and analysis

3.1 Screening and colonization ability study of antagonistic bacteria against pomegranate wilt pathogen
In this study, 45 soil samples were collected from Yunnan Jianshui Pomegranate Garden and Mengzi Ten-thousand Mu Pomegranate Garden. 26 strains of Bacillus antagonism against pomegranate wilt
disease were screened and further screened by plate confrontation method, to totally obtain 10 strains of Bacillus with obvious antagonistic activity against pomegranate wilt pathogen. Thereinto, the inhibitory rate of antagonistic bacterium M39 against pomegranate wilt pathogen was 81.17%.

![Image of plate confrontation method](image)

**Fig.1** Antifungal plate of M39 against *Ceratocystis fimbriata* A and the control B

The numbers of recovered rifampicin-resistant strain M39 on the 5th, 10th, 15th, 20th, 25th, 30th and 35th day in the rhizosphere of pomegranate seedlings are shown in Table 1. The first-time recovered number of strain M39 in the rhizosphere of pomegranate was 7.98. ×10⁷, and the number of recovered bacteria decreased to 1.00×10⁷ in the second sampling. The number of recovered bacteria in the third sampling increased slightly, but decreased to 2.70×10⁶ in the fourth sampling. At the fifth sampling, there was a decrease again, and then the number of recovered bacteria in the rhizosphere of the pomegranate stabilized, indicating that the antagonistic bacterium M39 against pomegranate wilt pathogen could stably colonize the rhizosphere of the pomegranate.

| Bacterial strain | Sampling period and Recovery of bacteria amount (CFU/g) |
|------------------|--------------------------------------------------------|
| M39              | 7.98 E+07 1.00 E+07 1.10 E+07 2.70 E+06 4.80 E+05 4.10 E+05 1.20E+05 |

### 3.2 Identification of strain M39

#### 3.2.1 Observation on cultural characteristics and morphological characteristics of strain M39

Culture characteristics of antagonistic bacteria M39: The colonies were nearly circular, the surface was rough, the bulge was dry, the edges were not irregular, and the colonies were white. Antagonistic bacteria M39 were blunt at both ends and rod-shaped, without spores formed, without motility, and the Gram reaction was positive.

#### 3.2.2 Routine physiological and biochemical determination of strain M39

Antagonistic bacteria M39: which could produce acid by utilizing glucose, maltose and sucrose. It could produce a reduction reaction with nitrate, but could not liquefy gelatin, hydrolyze urea and ferment xylose, lactitol, lactose and glycogen. the antagonistic bacteria M39 could use D-turanose and succinic acid carbon substrates to ferment glucose at 42 °C and grown well at 20 °C.

#### 3.2.3 Identification of strain M39 by Biolog microbial identification system

The statistical results of the utilization ability of strain M39 on 95 kinds of carbon substrates on Biolog plate: Saccharides: 19 kinds could be fully reacted, while 6 kinds undergone semi-reaction; Hexose phosphate: 2 kinds could be semi-reacted; Amino acid: There were 9 kinds subjected to complete reaction; Hexose carboxylic acid: 6 kinds undergone complete reaction, while 3 kinds were subjected to semi-reaction; Acid, ester and fatty acid: 11 kinds were completely reacted, while 5 kinds undergone semi-reaction, and 2 kinds of unreacted; The final identification results were that the similarity value with the *Corynebacterium freneyi* was 0.697, (if similarity value >0.500, it is validly identified). According to the reaction of strain M39 on the biochemical identification plate, strain M39 was initially identified as *Corynebacterium freneyi*. 
Table 2 Utilization Ability of 95 kinds carbon substrates in Biollog plate by bacterial strain M39

| number | nutrition medium   | reaction type | number | nutrition medium   | reaction type | number | nutrition medium   | reaction type |
|--------|--------------------|---------------|--------|--------------------|---------------|--------|--------------------|---------------|
| A1     | Negative Control   | -             | C9     | Inosine            | +             | F5     | D-Glucaric Acid     | -/+           |
| A2     | Dextrose           | +             | C10    | 1% Sodium Lactate  | +             | F6     | Glucuronamide       | -/+           |
| A3     | D-Maltose          | +             | C11    | Fusidic Acid       | -             | F7     | Mucic Acid          | +             |
| A4     | D-Trehalose        | +             | C12    | D-Serine           | -             | F8     | Quinic Acid         | -/+           |
| A5     | D-Cellulose        | +             | D1     | D-Sorbitol         | +             | F9     | D-Saccharic Acid    | +             |
| A6     | Gentriobiose       | +             | D2     | D-Mannitol         | -             | F10    | Vancomycin          | -             |
| A7     | Sucrose            | +             | D3     | D-Arabitol         | -/+            | F11    | Tetrazolium Violet  | -/+           |
| A8     | D-Turanose         | +             | D4     | myo-Inositol       | +             | F12    | Tetrazolium Blue    | -/+           |
| A9     | Stachyose          | +             | D5     | Glycerol           | +             | G1     | p-Hydroxy-Phenylacetic Acid | -/+ |
| A10    | Positive Control   | +             | D6     | D-Glucose-6-PO4    | -/+            | G2     | Methyl Pyruvate     | +             |
| A11    | pH 6               | +             | D7     | D-Fructose-6-PO4   | -/+            | G3     | D-Lactic Acid/Methyl Ester | -/+ |
| A12    | pH 5               | +             | D8     | D-Aspartic Acid    | +             | G4     | L-Lactic Acid       | +             |
| B1     | D-Raffinose        | +             | D9     | D-Serine           | -             | G5     | Citric Acid         | +             |
| B2     | D-M-Lactose        | -             | D10    | Troleandomycin     | -             | G6     | α-Keto-Glutamic Acid | +             |
| B3     | D-Melibiose        | +             | D11    | Rifamycin SV       | -             | G7     | D-Malic Acid        | -             |
| B4     | β-D-Methyl-D-Glucose | +       | D12    | Minocycline        | -             | G8     | L-Malic Acid        | +             |
| B5     | D-Salicin          | +             | E1     | Gelatin            | +             | G9     | BromoSuccinic Acid  | +             |
| B6     | N Acetyl-D-Glucosamine | +     | E2     | Glycyl-L-Proline   | +             | G10    | Naldixic Acid       | -             |
| B7     | N N-Acetyl-D-E-DMannosamine | -/+   | E3     | L-Alanine          | +             | G11    | Lithium Chloride    | +             |
| B8     | N Acetyl-D-Galactosamine | -/+   | E4     | L-Arginine         | +             | G12    | Potassium Tellurite | +             |
| B9     | N-AcetylNeuraminic Acid | -/+       | E5     | L-Aspartic Acid    | +             | H1     | Tween 40            | +             |
| B10    | 1% NaCl            | +             | E6     | L-Glutaric Acid    | +             | H2     | γ-Amino-Butyric Acid | +             |
| B11    | 4% NaCl            | +             | E7     | L-Histidine        | +             | H3     | α-D-HydroxyButyric Acid | -/+ |
| B12    | 8% NaCl            | +             | E8     | L-Pyroglutamic Acid | +             | H4     | β-Hydroxy-DLButyric Acid | -/+ |
| C1     | α-D-Glucose        | +             | E9     | L-Serine           | +             | H5     | α-Keto-Butyric Acid | -             |
| C2     | D-Mannose          | +             | E10    | Lincomycin         | -             | H6     | Acetoacetic Acid    | +             |
| C3     | D-Fructose         | +             | E11    | Guanidine HCl      | -/+            | H7     | Propionic Acid      | -/+           |
| C4     | D-Galactose        | +             | E12    | Niaproof 4         | -             | H8     | Acetic Acid         | +             |
| C5     | 3-Methyl Glucose   | -/+            | F1     | Pectin             | +             | H9     | Formic Acid         | +             |
| C6     | D-Fucose           | -/+            | F2     | D-Galacturonic Acid | +             | H10    | Aztreonam           | +             |
| C7     | L-Fucose           | -/+            | F3     | L- Galactonic AcidLact one | +   | H11    | Sodium Butyrate     | +             |
| C8     | L-Rhamnose         | +             | F4     | D-Glucic Acid      | +             | H12    | Sodium Bromate      | -/+           |

Note: “+” positivity; “-” negative; “-/+” Means the reaction is between positivity and negative

4. Conclusions and discussions

This study utilized dilution plate coating and plate confrontation method, to totally obtain 10 strains of Bacillus with strong antagonistic activity against pomegranate wilt pathogen. Thereinto, the inhibitory rate of antagonistic bacillus M39 against pomegranate wilt pathogen was 81.17%, and the strain could be stably colonized in the rhizosphere of pomegranate, which had potential biological control value. Based on the cultural characteristics, biochemical reaction and the identification results of strain M39 on bacterial biochemical identification plates, strain M39 was identified as Corynebacterium freneyi.

There are few reports on the Corynebacterium freneyi. Zhang E et al. obtained eight strains of Co(II)-tolerant bacteria by plate screening and rescreening with a shaker, and one of them was Corynebacterium freneyi, which was expected to be developed and utilized in microbial remediation of heavy metal pollution [13]; Liu Mei et al. isolated Corynebacterium freneyi from surface sediments, and studies shown that the strain had strong radiation tolerance [14]; Some studies had reported the isolation of Corynebacterium freneyi from clinical samples [15]. This study demonstrated that Corynebacterium freneyi had strong indoor inhibitory effect on pomegranate wilt pathogen, with the
inhibition rate of 81.17%. The strain could be stably colonized in the rhizosphere soil of pomegranate and had better development and utilization value. The effective prevention and control of pomegranate wilt disease can not rely on one or two biocontrol bacteria stains. Finding more biocontrol bacteria and combining them reasonably to make a composite microbial agent to improve the unbalanced soil micro-ecological environment is a direction of efforts to effectively prevent and control pomegranate wilt disease.

Acknowledgments
This work was financially supported by Applied Basic Research plan of Yunnan Province, P. R. China (NO:2013FZ125); Doctor Special Research fund of Honghe University(XJ17B10); Cooperative cultivation plan for excellent agricultural and forestry talents of plant protection specialty; Construction projects of plant protection First-Level discipline Master's degree in Honghe University.

Reference
[1] Guo J W, Guo J, Yang J, et al. (2014) Screening and Identification of Antagonistic Fungi against the Pathogen of Pomegranate Wilt and Taro Black rot. Acta Agriculturae Universitatis Jiangxiensis, 36(4):772-775+781.
[2] Liu Y L, He Y H, Wang X Z. (2003) A new fruit tree disease in china—the pomegranate wilt disease. Plant Quarantine, 5:206-208.
[3] Huang Q, Zhu Y Y, Chen H R, et al. (2003) First report of pomegranate wilt caused by Ceratocystis fimbriata in Yunnan, China. Plant Disease, 87(9): 1150.
[4] Xu B, Zheng X H, Guo W X, et al. (2011) First report of pomegranate wilt caused by Ceratocystis fimbriata in Sichuan Province. Plant Disease, 95(6): 776-776.
[5] Zhou Y L, Hu X Q, Wang W J, et al. (2010) The role of Root knot nematodes in the pomegranate wilt occurrence process of yunnan Province. Jiangsu Agricultural Sciences, 1:149-150.
[6] Pan J, Mao Z S, Li X, et al. (2013) Preliminary Study on Pomegranate Wilt Management by Employing Bacillus subtilis and Pseudomonas florescence Isolates. Journal of Yunnan Agricultural University, 28(1): 27-31.
[7] Zhou Y L, Yang Y L, Yuan S J, et al. (2016) Nematicidal activity of actinomycete antagonistic to pomegranate wilt pathogen. Plant Protection, 5:58-64.
[8] Zhou Y L, Yuan S J, Pan Y M, et al. (2016) Studies on the Toxicity of Actinomycete JS2 to Pomegranate Wilt Pathogen and Root Knot Nematode. Acta Agriculturae Universitatis Jiangxiensis, 38(2):268-274.
[9] Zhou Y L, Guo J W, Yang W, et al. (2018) Impact of intercropping peach tree in pomegranate wilt disease field on soil microbial carbon metabolism diversity. Jiangsu agricultural science, 46(14):106-109.
[10] Zhou Y L, Guo J W, Yang W, et al. (2018) Isolation and Identification of Pomegranate Wilt Pathogen Antagonism Bacillus A4 and Its Colonization Ability. Acta Agriculturae Universitatis Jiangxiensi, 40(01):168-173.
[11] Zhou Y L, Hu X Q, Wang W J, et al. (2010) The indoor toxicity of five plant extracts to different growth stages of Pomegranate Wilt Pathogen. Jiangsu agricultural science, 5:177-178.
[12] Zhou Y L, Hu X Q, Wang W J, et al. (2009) The indoor toxicity of rhizoma coptidis etc. six plant extracts to Pomegranate Wilt Pathogen. Jiangsu agricultural science, 6:182-183.
[13] Zhang E. (2014) Preliminary study on cobalt interaction microbial breeding and related mechanism. Southwest university of science and technology.
[14] Liu H. (2014) Distribution and identification of microorganism in beishan preselected area of gansu High-level waste disposal repository. Southwest university of science and technology.
[15] Renaud FN, Aubel D, Riegel P, et al. (2001) Corynebacterium freneyi sp nov. alpha-glucosidase-positive strains related to Corynebacterium xerosis. International Journal of Systematic & Evolutionary Microbiology, 51(5):1723-1728.