Isolation and Identification of Two Antagonistic Actinomycetes against pomegranate wilt pathogen

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Abstract. Pomegranate wilt is a soil-borne disease relatively difficult to prevent. In this study, actinomycetes with strong antagonistic activity against pomegranate wilt pathogen were separated and screened from the soil samples collected from Yunnan Mengzi Wanmu Pomegranate Garden and Jianshui Pomegranate Garden. The indoor bacteriostatic radius of strain MZ12 against pomegranate wilt pathogen was 11 mm while the indoor bacteriostatic radius of strain MZ1 against pomegranate wilt pathogen was 7.25 mm. Combined with the characteristics, morphological observation and 16S rDNA sequence analysis of the two strains on identification medium, MZ12 was initially identified as Streptomyces cinnamonensis, and MZ1 as Streptomyces fulvissimus.

1. Introduction
Pomegranate (Punica granatum L.), one of the plants of the punicaceae family, is one of the most important fruit trees in China. It has many nicknames such as the pearl pomegranate, An pomegranate, Ruo pomegranate, Shan Liye, Dan Ruo, Hai pomegranate, Xie Liu, Wodan, Tianjiang, Jinying. Yunnan Mengzi Wanmu Pomegranate Garden is known as the “Hometown of Chinese Pomegranate”. With the intensification of pomegranate cultivation and the growth of the age of the pomegranate, the harm of pomegranate disease is increasingly serious, especially the pomegranate wilt disease. China's pomegranate wilt disease has occurred in Mengzi County, Yunnan province [1], Panxi area and Huili county, Sichuan province[2]. In 2003, the rate of diseased plants in Mengzi Wanmao Pomegranate Garden reached 11.2%, causing huge economic losses. At the same time, people were taking active action on relevant research work on prevention and control [3-6]. Pomegranate wilt disease is soil-born fungus disease which causes by Ceratocystis fimbriata Ellis & Halsted, proved by experiments. Ceratocystis fimbriata Ellis & Halsted, a widely-distributed pathogen, causes ceratocystis black spot disease, pomegranate wilt disease, taro black rot [7] via the wounded root[8].

The control of pomegranate wilt disease depends on chemicals. However, long-term use of chemical agents is easy to cause resistance to pomegranate wilt pathogen and pollution to fruit and environment. Therefore, the use of biocontrol microorganisms or their secondary metabolites for disease control has become an effective measure to control the further spread of the disease. Previous studies show that indigenous microorganisms have a bright future in application in the control on
pomegranate wilt \cite{6}. This study screened antagonistic actinomycetes which has a strong inhibitory effect on pomegranate wilt pathogen, and provided a reference for better control of pomegranate wilt.

2. Materials and methods

2.1 Materials

Forty-five rhizosphere soil samples of healthy pomegranate trees and rhizosphere soil samples of pomegranate trees with pomegranate wilt disease were collected from Mengzi Wanmu Pomegranate Garden and Jianshui Pomegranate Garden. The standard sample of *Ceratocystis fimbriata* Ellis & Halsted was provided by Huang Qiong, a professor of the Plant Pathology Laboratory of Yunnan Agricultural University. The actinomycete separation medium was modified Gao's No. 1 medium, and PDA medium was used in the plate confrontation test.

2.2 Experiment Methods

2.2.1 Separation and Culture of Actinomycetes in Soil

The rhizosphere soil samples were collected from the triangle area, Shizilu, Hongtangzi, Sanludi, Liaowang Tower, Bandengqiao, Jiangtai, Shuijinao, Mashanchong of Mengziwanmu pomegranate Garden and Zhou Wu, Xiaoying, Li Wushan of Jianshui pomegranate Garden including pomegranate wilt disease tree and of the healthy pomegranate tree. (The tool should be disinfected with 75% ethanol after the collection). At each place, 3 strains and 3 different locations of each strain were selected. Using the hoe to dig a hole of 10 to 20 cm until the pomegranate roots are exposed. Then, shaking off the surrounding bulk soil slightly and collecting 500g soil which was within 1 cm from the root. Mixing 3 samples from each collecting place. Putting 500g soil into the sterile ziplock bag, numbering it. Carrying out the decontamination and 0.1 cm sieve treatment after bringing the soil sample back to the laboratory as soon as possible.

The actinomycetes were separated and cultured by using modified Gaoshi No.1 medium, and the actinomycetes in the soil were separated by a dilution coating flat plate method. The isolated actinomycetes with different morphology and color were Scribing and purification to obtain a single colony. The purified actinomycete strain was kept in a modified Gaoshi No. 1 medium and stored in a refrigerator at 4 °C.

2.2.2 Screening of antagonistic actinomycete against pomegranate wilt pathogen

Screening of antagonistic actinomycete: The antagonistic actinomycete were screened with PDA medium. The 7-day pomegranate wilt pathogen was made into a spore suspension via a concentration of $10^3$/mL with sterilized water. 100 μL of spore suspension was applied to the PDA plate, letting it stand for 5h. Activated the isolated actinomycetes and inoculated onto a plate coated with the pathogen of pomegranate wilt. Each plate was cross-hatched, and four actinomycetes were inoculated at a distance of 3 cm from the center of the crosshair. After being cultured for 5 days in a constant temperature at 28 ° C in incubator, the radius (r) of the antagonistic circle was observed and measured. The grading and criteria of antagonistic strength: Strong antagonistic actinomycetes, $r\geq10$mm; moderate antagonistic actinomycetes, $10mm>r\geq7.5$mm; weak antagonistic actinomycetes, $r<7.5$mm; no antagonistic actinomycetes, $r=0$mm \cite{9}.

Antagonistic actinomycetes rescreening: According to the results of the initial screening, the actinomycetes with obvious antibacterial effect were selected. Rescreening the biocontrol bacteria via the plate confrontation test. The criteria for determining the bacteriostatic effect is the same as above.

2.2.3 Identification of Antagonistic Actinomycetes against Pomegranate wilt pathogen.

The strains of actinomycetes were cultured by interpolation method and on Gaoshi No.1 medium plate at 28 °C for 6-10 days. The morphology of the mycelium was observed by an optical microscope, and the morphological color of the aerial hyphae and spore silk in the colony morphology were recorded.
Then studied whether soluble pigment was produced etc. Preparation for actinomycetes identification culture: Czapek's medium, Glycerine-asparagine medium, Malt-yeast extract medium, Inorganic starch medium, Glucose-asparagine medium, potato medium, Oatmeal Agar medium, Nutrient Agar Medium. The growth situation of the strain on it was observed [10-11].

The DNA of the actinomycete strain was extracted by promega bacterial genome extraction kit, and the PCR amplification was performed by using 16S rDNA universal primers (27F: 5’-AGA GTT TGA TCC TGG CTC AG-3’; 1492R: 5’-GGT TAC CTT GTT ACG ACT T-3’). The obtained sequences were used for homology comparison with BLAST in GenBank. Different strains with high similarity were selected. The phylogenetic tree was constructed and analyzed using the neighbor-joining method in MEGA 5.0 software.

3. Results and analysis

3.1 Screening Antagonistic Actinomycetes against pomegranate wilt pathogen

45 soil samples were gathered from Mengzi Pomegranate Garden and Jianshui Pomegranate Garden, and 168 strains of actinomycetes with different colony characteristics were isolated. 38 strains of actinomycetes against pomegranate wilt pathogen were sieved, with six strains of antagonistic actinomycetes of pomegranate wilt pathogen were further screened by plate confrontation test method. The MZ12 strain has strong antagonistic activity against pomegranate wilt pathogen with 11mm antibacteriostatic radius. However, the antagonistic activity of MZ1 strain against pomegranate wilt pathogen was weak with 7.25mm anti-bacteriostatic radius. The antagonistic effect of the strain on pomegranate wilt pathogen is shown in Figure 1.

![Fig. 1 Antifungal plate of MZ12, MZ1 against Ceratocystis fimbriata](image)

3.2 Identification of Antagonistic Actinomycetes against Pomegranate wilt pathogen

3.2.1 Identification of Antagonistic Actinomycetes MZ12 against Pomegranate wilt pathogen

Morphological characteristics of MZ12 on Gao's No.1 agar medium are as follows: The aerial hyphae are white; the hyphae in the base are milky white; and spore silks are long, soft or straight without real spiral; the spores are oval to elliptical with smooth surface. The morphological characteristics of MZ12 on PDA medium are as follows: the aerial hyphae are white; the hyphae in the base are dark to creamy; there is no soluble pigment; later, melanin is produced around the center of the hyphae in the base; and the hyphae on the opposite side is light yellow-brown. The morphological characteristics of MZ12 on each identification medium are shown in the table.

| Culture medium                  | Color of aerial mycelium | Color of substrate mycelium | Soluble pigments |
|---------------------------------|--------------------------|-----------------------------|------------------|
| Glucose asparagine medium       | Not rich white - Lilac grey | Light yellow               | None             |
| Gaos no. 1 AGAR medium          | White                     | Milk white                  | None             |
| Nutrient agar medium            | Not rich white            | Fawn - Light yellow         | None             |
| Inorganic salt starch agar medium| Light orchid - Milky white | Yellowish gray              | None             |
| Oatmeal agar medium             | Lilac grey has oil droplets | Fawn                        | None             |
| Glycerin asparagine agar        | Lilac grey                | Ecru - Light brown yellow   | None             |
The 16S rDNA of strain MZ12 was obtained through PCR amplification method, and the near-full length sequence of 16SrDNA was obtained by sequencing. Blast similarity analysis shows that the 16S rDNA sequence homology of actinomycete MZ12 and *Streptomyces cinnamonensis* is 100%. When the phylogenetic tree was established, the actinomycete MZ12 was in the same branch as the *Streptomyces cinnamonensis* (Fig. 2). The strain MZ12 was identified as *Streptomyces cinnamonensis* by a combination of the culture characteristics on various mediums, morphological characteristics and phylogenetic analysis.

![Fig2. Phylogenetic tree of MZ12 based on 16S rDNA sequence.](image)

### 3.2.2 Identification of Antagonistic Actinomycetes MZ1 against Pomegranate Fusarium

Morphological characteristics of MZ1 on Gao's No. 1 agar medium are as follows: The aerial hyphae are pink or slightly creamy; the hyphae in the base are yellow or slightly rust, and the soluble pigment is slightly yellow; The spores are long, straight or flexible, and elliptical or oblong, with a smooth surface. Morphological characteristics of MZ1 on starch agar medium: The aerial hyphae are pale pink and the mycelium was slightly brown without soluble pigment. The morphological characteristics of MZ1 on each identification medium are shown in Table 2.

| Culture medium         | Color of aerial mycelium | Color of substrate mycelium | Soluble pigments |
|------------------------|--------------------------|------------------------------|------------------|
| Glucose asparagine medium | Light pink               | Red orange                   | None             |
| Agar-agar one medium    | Pink or Light pink cream | Yellow or light rust         | None             |
| Nutrient agar medium    | Light yellowish pink     | Orange                       | None             |
| Inorganic salt starch agar medium | Rich pinky white        | Orange red, Light rose red   | None             |
| Oatmeal agar medium     | Not rich light pink      | Orange yellow                | None             |
| Glycerin asparagine agar | Light pink               | Orange yellow                | None             |
| Potato glucose medium   | Milky white              | Spring red, Isabelline       | None             |
| Malt extract medium     | Rich light pink yellow   | Light pink yellow, wine red  | None             |
| Czapek's medium         | Milky white, Light orange| Orange                       | None             |
The 16S rDNA of strain MZ1 was obtained by PCR amplification method while the near-full length sequence of 16SrDNA was obtained by sequencing. Blast similarity analysis shows that the 16S rDNA sequence homology of actinomycete MZ1 and Streptomyces fulvissimus is 100%. When the phylogenetic tree was established, the actinomycete MZ1 was in the same branch as Streptomyces fulvissimus (Fig.3). According to the culture characteristics on various mediums, morphological characteristics and phylogenetic analysis, the strain MZ1 was identified as Streptomyces fulvissimus.

4. Conclusions and discussions

The actinomycete MZ12 separated in this study has strong antagonistic effect on Ceratocystis fimbriata with 11mm bacteriostatic radius. The isolated actinomycetes MZ1 has weak antagonism effect against Ceratocystis fimbriata with 7.25mm bacteriostatic radius. Based on the culture characteristics, morphological observation and 16S rDNA sequence analysis, the MZ12 was initially identified as S. cinnamonensis while the MZ1 as S. fulvissimus.

Actinomycetes can produce abundant antibacterial active substances and agricultural antibiotics. It is an important microbial resource for controlling plant pathogenic organisms, with extensive use in the filtering of microbial pesticides and the prevention and control of plant fungal diseases. Some kinds of actinomycetes can produce a nematicidal active substance. For example, avermectin comes from Streptomyces has been applied to the prevention and treatment of Meloidogyne incognita [12]. Li Tao et al. screened a strain of high insecticidal active substance GX-29 from the soil. On a basis of its physicochemical characteristics and 16SrDNA sequence analysis results, the strain was confirmed to be Streptomyces fulvissimus [13]. Using isolation, Zhang Bei Bei et al. obtained marine actinomycetes NZ1203 which was identified as Streptomyces fulvissimus. Studies show that its metabolites have significant antibacterial and antitumor effects [14]. Chen Dan et al. isolated and identified a strain of CZB40 of Streptomyces fulvissimus. Studies indicate that the 5-fold dilution of the sterile filtrate of the actinomycete can be against the growth of the indicator mycelium up to 90.45% [15]. The MZ12 S. cinnamonensis isolated from this study has strong antagonistic effect on Ceratocystis fimbriata but the antagonistic effect of MZ1 Streptomyces fulvissimus on Ceratocystis fimbriata is relatively weaker. Yet, further research of these two strains in pot and field control effect on Ceratocystis fimbriata is needed.

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