Isolation and Identification of the Pathogen of Stem Rot

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Abstract

Anoectochilus (aka Anoectochilus fortunei) is a plant of the genus Orchidaceae in the family Orchidaceae. It is a precious Chinese herbal medicine plant. In this experiment, the suspected stem rot pathogens of Anoectochilus fortunei were isolated and identified. Then the plate confrontation method was used to verify the inhibitory effect of the four biocontrol bacteria, Stenotrophomonas maltophilia, Bacillus belesi, Trichoderma harzianum and Trichoderma viride. The results showed that the suspected stem rot pathogen of Anoectochilus was FusariumoxysporumSchl.

Keywords

Anoectochilus roxburghii, Stalk rot, Fusarium oxysporum.

1. Introduction

Anoectochilusroxburghii (Wall.) Lindl. Is a perennial herb of the genus Anoectochilus of the Orchidaceae family. It is a perennial rare Chinese herbal medicine with high ornamental value and medicinal value, mainly produced in Fujian, Taiwan [1]. Clematis is rich in flavonoids, Alkaloids, saponins, steroids and other ingredients, all herbs can be used as medicine, sweet and taste, has liver protection, lower blood pressure, lower blood sugar, anti-tumor, and improve immunity [2-4], due to natural The severe environmental disruption, and the long-term large-scale over-harvesting and wildlife infringement, the clematis has become an endangered plant. The clematis planted in the greenhouse at present is prone to white silk disease due to changes in cultivation techniques such as nutritional levels, light and temperature conditions, etc. Diseases such as gray mold, stalk rot and soft rot [5-7]. In this paper, a strain of A. aureus stem rot pathogen was isolated and identified.

2. Materials and Methods

2.1 Test materials

Diseased plant: Taken from Fujian Rulian Biotechnology Co., Ltd. The diseased plant of Anoectochilus spp. Fell down, the wilting at the base shrunk into a linear, brown, white moldy rot in the diseased part. Collect disease tissues for pathogen isolation.

Healthy plants: Taken from Fujian Rulian Biotechnology Co., Ltd., they are sterile healthy plants cultivated with culture medium in culture bottles.

2.2 Test method

2.2.1 Isolation and purification of pathogenic bacteria

Take the 5mm square stems at the junction of the disease and health, disinfect the surface with 75% alcohol for 1min, then rinse with sterile water three times, use sterilized filter paper to absorb the water on the surface, and use the sterilization inoculation ring to pick the diseased parts The white moldy rot was streaked in PDA medium and cultured at 28 ℃. If a colony is formed, pick the mycelium from the edge of the colony and transfer to a new PDA medium for cultivation. Repeat repeatedly to obtain pure culture..

2.2.2 Pathogenicity detection of pathogens

The four fungi were isolated and inoculated into healthy and sterile plants by means of back-linking, and cultivated in breathable sterile glass culture bottles. They were compared with each other, and the growth status of the plants was observed daily.

2.2.3 Morphological observation of pathogens

The strain was placed on a PDA plate and cultured, and the colony characteristics and the growth status were observed.

2.2.4 Molecular biology identification of pathogenic strains

(1) DNA extraction (kit extraction).

The strains were activated and cultured in PDA medium. After the hyphae were grown, they were picked up in the PDA liquid medium with an inoculating ring, and cultured with shaking at 150 r.min-1 in a shaker at 28℃ for 2 days. Draw 2ml of bacteria liquid in a mortar and grind it into powder under the condition of liquid nitrogen. Then follow the steps of the kit to extract DNA.

(2) PCR amplification of rDNA-ITS.

The ITS1 and ITS4 primers for fungal ITS were used for amplification. The PCR reaction system is 50 μL, and the components are as follows: template DNA 1 μL, gold MIX (green) (including DNA polymerase, containing Mg^{2+} buffer, dNTP and other components) primers ITS1 and ITS4 each 2 μL (0.4 μmol-1), the primer is BGI gene synthesis.

The PCR amplification was completed on a BIO-RAD type amplifier. The amplification procedure was: 98 ℃, pre-denaturation for 2min, then 98 ℃, 10S, 58 ℃ annealing 10S, 72 ℃ extension for 15S, 30 cycles, 72 ℃ extension for 5min , Store at 4 ℃.

(3) PCR product electrophoresis detection and sequencing

After the reaction was completed, 10 μL of the amplified product was taken and subjected to 1.5% agarose gel electrophoresis detection, observed, photographed, and stored in the gel imaging system. If the band is correct, send the PCR product to BGI for sequencing.

(4) Analysis of sequencing results

After obtaining the gene sequence, the rDNA-ITS sequence of the strain was compared with the known sequence in the Genbank database, and homology analysis was carried out, combined with the morphological observation and pathogenicity determination of the pathogen to determine the type of pathogen.

3. Results and Discussion

3.1 Isolation and identification of pathogenic fungi

The four fungi shown in the figure below were isolated and purified from the diseased part of the plant, from left to right, named No. 1, No. 2, No. 3, No. 4 respectively,as shown in Fig. 1.

Fig.1. Four fungus flat pictures

3.2 Pathogenicity test results

Figure 2 shows the growth of healthy plants 12 days after inoculation with bacteria 1, 2, 3, and 4 from left to right. It can be seen from the picture: the plant infected with No. 4 bacteria fell down, the decay was the most serious, and white mold rot similar to the diseased plant appeared, while the other three plants all grew better, and the No. 4 bottle was infected The pathogenic bacteria of Anoectochilus fortunei can be isolated from pathogenic bacteria to obtain the same pathogenic bacteria. Therefore, it was determined that the fungus 4 was the most pathogenic bacterium for stem rot of Anoectochilus fortunei.

Fig.2. Growth of healthy plants after inoculation with 4 strains of fungi

3.3 Morphological characteristics of pathogens

When cultivated on a PDA plate, the pathogenic bacteria produce a large amount of aerial hyphae in the medium in the form of cotton wool, and the hyphae produce purple pigment in the medium.

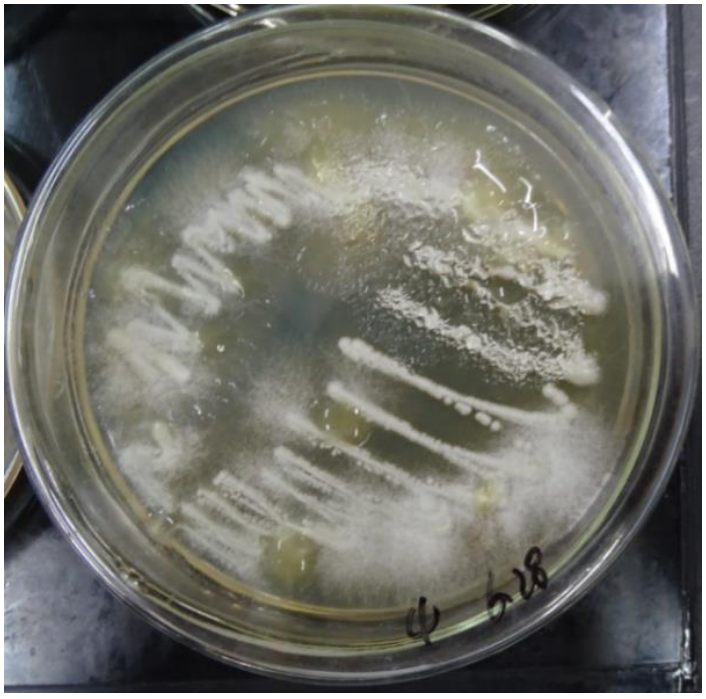

Fig.3. Schematic diagram of PDA tablet of pathogenic fungi

3.4 Molecular biology identification of pathogenic fungi

3.4.1 rDNA-ITS segment amplification and sequencing

The rDNA-ITS sequence of the stem rot pathogen was amplified with universal primers. Using the genomic DNA of the pathogen as a template, a single band of 550-600bp was amplified ,as shown in Fig. 4.

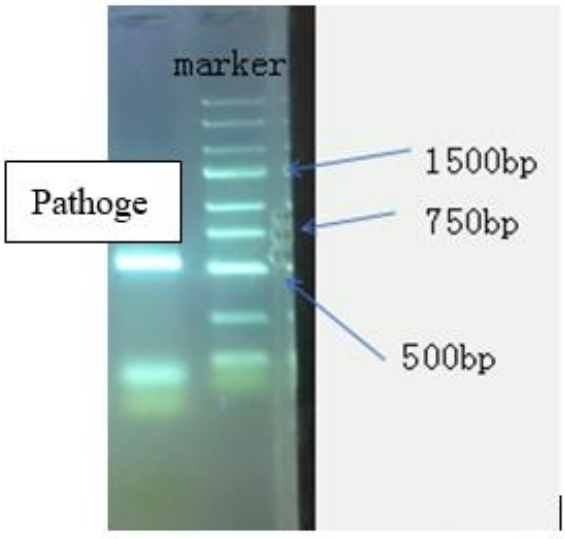

Fig.4. Pathogen bacteria rDNA-ITS pcr electrophoresis

3.4.2 Blast sequence alignment to determine the strain type of pathogenic fungi

The rDNA-ITS sequence of the stem rot pathogen was amplified with universal primers, and a specific fragment of 518 bp was amplified. The sequence obtained by sequencing is shown in Fig. 5. The rDNA-ITS (GenBank) sequence of the strain was compared with the existing DNA sequence in GenBank. The data showed that the strain with higher homology was FusariumoxysporumSchl. (Fusarium oxysporum), the similarity reached 100%.

Fusarium of the genus Fusarium is a soil fungus disease with a wide range of hosts, which can cause more than 100 plant diseases such as melons, solanaceae, bananas, cotton, legumes and flowers [8]. The traditional classification of fungi is based on morphological characteristics and physiological and biochemical indicators, but the morphological characteristics of fungi are affected by the environment during the growth process, or there are intermediate species, which makes the morphological identification sometimes inaccurate. The use of modern molecular biology technology can directly reflect the gene itself, which has unparalleled advantages of traditional morphological methods [9]. The classification of pathogenic bacteria caused by Fusarium oxysporum is common in other plants [10-12]. For the molecular identification of the pathogen of Anoectochilus stem rot, I used the 16S-rDNA-ITS sequence to analyze and identified the pathogen causing the stem rot as Fusarium oxysporum, which was the gold thread caused by Fusarium oxysporum The screening research of biocontrol agents for lotus stem rot provides a research basis and has certain guiding significance for agricultural production.

CGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACC
ACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCG
CCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCA
TAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT
GCCTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACT
CGCGTTAATTCGCGTTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCA
TAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGT
TAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCT
GAACTTAAGCATATCAAT

Fig.5. Pathogen rDNA-ITS sequencing results

4. Conclusion

In this experiment, the isolation and purification of the stem rot department of Anoectochilus fortunei and the pathogenicity verification, molecular biology identification proved that the pathogenic fungus that caused a large number of stem rots of Anoectochilus was Fusariumoxysporum Schl.

References

[1] Cai Wenyan, Xiao Huashan, Fan Xiuzhen. Research progress of Anoectochilus (review) [J]. Subtropical Plant Science, 2003 (03): 69-73.

[2] Wang Yong, Chen Shuo, Lu Duanping, et al. Study on the chemical constituents of Anoectochilus fortunei [J]. Chinese Herbal Medicine, 2017 (13): 36-41.

[3] Zeng B, Su M, ChenQ, et al. Antioxidant and hepatoprotective activities of polysaccharides from Anoectochilus roxburghii[J]. Carbohydrate Polymers, 2016, 153:391-398.

[4] Jer-minlin, Chun-chinglin, Hui-fenchiu, et al. Evaluation of the Anti-inflammatory and Liver-protective Effects of Anoectochilus formosanus, Ganoderma lucidum and Gynostemma pentaphyllum in Rats[J]. The American Journal of Chinese Medicine, 2012, 21(1).

[5] Shao Qingsong, Liu Hongbo, Zhao Xiaofang, et al. Biological characteristics of the stem rot fungus of Anoectochilus formosanus and the inhibitory effect of five fungicides on it [J]. China Journal of Chinese Materia Medica, 2014 (08): 50-54.

[6] Marika, Rossi, Mattia,等. Genetic Diversity of Flavescence Dorée Phytoplasmas at the Vineyard Scale.[J]. Applied & Environmental Microbiology, 2019.

[7] Bing L, Jinliang L, Xianghui Z, et al. Control of maize stalk rot by bacterial strain GS2 from Ginseng rhizosphere soil [C] // Proceedings of the Chinese Academy of Plant Pathology 2019 Annual Conference. 2019.

[8] Ruan Xingye, Luo Wenfu. Species identification of Fusarium in Yunnan Province [J]. Journal of Yunnan Agricultural University, 1986, V1 (1): 1-13.

[9] Lei Zhengyu. Application of 16SrDNA sequence analysis technology in microbial classification and identification [J]. Journal of Hubei Ecological Engineering Vocational Technology College, 2006 (1).

[10] Fravel D, Olivain C, Alabouvette C. Fusarium oxysporum and its biocontrol[J]. New Phytologist, 2003, 157(3):493-502.

[11] Kai-Cheng W. Pathogen identification of watermelon Fusarium oxysporum and its synergistic effect of fungicides[J]. Hub Agricultural ences, 2019.

[12] Moosa A, Farzand A, Sahi S T, et al. First report of post-harvest Fusarium rot caused by Fusarium oxysporum on Citrus reticulata Blanco cv. 'Kinnow' in Pakistan[J]. 2020.