Full Length Research Paper

Study on isolated pathogen of leaf blight and screening antagonistic bacteria from healthy leaves of *Camellia oleifera*

LI, He[1,2]*, ZHOU, Guoying[1,2], ZHANG, Huaiyun[1,2], SONG, Guangtao[2] and LIU, Junang[1,2]

[1]Central South University of Forestry and Technology, Hunan provincial key laboratory of forestry biotechnology, Changsha, 410004, China.
[2]Central South University of Forestry and Technology, Biotechnology Core Facilities, College of Forestry, Changsha, 410004, China.

Leaf blight of *Camellia oleifera* is one of the major diseases in *Camellia* producing areas of China. The pathogen infects leaves of the host plants and causes leaves to drop, resulting in early defoliation and new treetops death and eventually yield reduction. The pathogen of leaf blight was isolated from the infected leaves of *C. oleifera* (NO.CSUFT070160B). The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA from pathogen was sequenced and comprises 516 bp. By comparing them with the sequences in the Gene Bank, we found that the strain was highest homologous to *Pestalotiopsis microspora*; and the concordance ratio is 99%. The molecule phylogenetic tree indicated that the CSUFT070160B and DQ000995 were in the same branch with high confidence level. Combined with morphological characteristics, the strain was checked as *P. microspora*. For the control of the *C. oleifera* leaf blight, antagonistic bacteria were screened from 175 endophytic bacteria by confront culture method. The results showed that the antagonistic effect of YB18 strain was the best with 84.7% inhibitory rate. According to the results of the morphology, cultural characteristics, physiological and biochemical properties, 16S gene sequence and molecular phylogenetic tree, YB18 was identified as the strain belonging to *Bacillus subtilis.*

Key words: *Camellia oleifera,* leaf blight, pathogen, antagonistic bacteria, biological control.

INTRODUCTION

Camellia oleifera is a special woody plant for edible oil production in China which is rich in vitamins (Deng et al., 2008). It can not only enhance human immunity, but also has high economic value (Liu et al., 2009). The pathogen of leaf blight of *C. oleifera* mainly infects the leaves and new treetop, resulting in early defoliation, new treetop death, as well as yield reduction. Diseased spots of leaf blight often cause irregular shapes, reddish-brown to dust color, and often merge into a bigger spot. The color at the

edge of leaf blight spot is deeper than the spot itself; hence the boundary between sick and healthy parts is an important feature. From June to September every year, the cultivating area of *C. oleifera* witness more than 50% withered leaves. Now the chemical method is the main way to control the leaf blight of *C. oleifera*, but it has severely bad effect on our environment. Screening and application of antagonistic bacteria against plant pathogens has become a subject of general interest. Measures to control *C. oleifera's* leaf blight caused by antagonistic bacteria has not been reported before. The aim of this study was to isolate antagonistic bacteria from the healthy leaves of

*Corresponding author. E-mail: csuftlihe@163.com.

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C. oleifera in order to control leaf blight.

MATERIALS AND METHODS

Blight leaves of *C. oleifera*

The blight leaves of *C. oleifera* were picked from the demonstration base of *C. oleifera* in Hunan, China.

Isolation of pathogens

The tissues were selected from the diseased and healthy areas of leaves infected by pathogen and the surface of disease spots were washed with water and 75% alcohol. After culturing at the constant temperature of 20°C for 6 days on PDA plates medium, they were transferred to PDA plate and cultured for 4 days with inoculation needle, while screening the dominant strains and purifying the superior strains.

Pathogenicity testing

After being purified, the acquired isolates were kept cultured in PDA medium. On appearing, acervuli were picked out to the antiseptic distilled water, and the spores were released to obtain the suspending solution, the density was 107 - 108 cells mL^{-1}. We then cut a 10 mm wound on the healthy *C. oleifera* leaf with an antiseptic knife, got 10 μL spores suspending solution to the wound with pipette and replaced the spore suspending solution with the sterilized water to make the comparison. Moist cotton was used to cover the wound while the entire leaf was covered with a 1000-mL beaker to culture at 20 - 25°C. After 7days, the symptoms were observed.

ITS amplification *of pathogens*

The total genomic DNA of pathogens was extracted with CTAB method. The fungi universal primers ITSI and ITS4 (synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd.) were used to amplify the internal transcribed spacers (ITS). The PCR reaction mixture (25 μL) contained: 10×buffer solution 2.5 μL, dNTP (each was 2.5 mmol·L^{-1}) 1 μL, primers 1 μL, Taq enzyme (5 μ·L^{-1}) 0.25 μL, template DNA 2 μL which was diluted 50 times (the negative control groups were administered with the same volume of distilled water, respectively) and 18.25 μL ddH$_2$O. The 35 cycles of PCRs were as follows: predenature for 5 min at 95°C; denature for 30 s at 95°C; anneal for 30 s at 56°C; extend for 1 min at 72°C. The last cycle was to extend for 10 min at 56°C. 2 μL PCR products were fetched, 1.0% agarose gel electrophoresis performed and gel imaging system was analyzed, tested and photographed after EB dyeing (JI et al., 2006).

Isolation and screening of antagonistic bacteria

Endophytic bacteria in healthy leaves of *C. oleifera* were isolated according to the method described by Fang (1997). Antagonistic bacteria was screened by confront culture method (Li et al., 2006). The inhibition zone width on NA plates were record after 4 days. The strains with good inhibiting efficacy were individually inoculated in 250-ml Erlenmeyer flask containing 150 ml NB medium, 28°C,

150 rpm shakily cultured for 48 h. Then the culture medium was filtered with a germ filter (d = 0.22 μm). A mixture of culture medium was poured into the petri dish prepared by mixing culture filtrate with PDA (1:19). The mycelial mats of *Pestalotiopsis microspora* (d = 6 mm) was transplanted directly to mixture culture medium surface for secondary screening. When it grew, we measured its diameter after 4days and ddH$_2$O was used for control. The best strain was screened based on the inhibitory rate. Inhibitory rate was calculated with the formula A (%) = 100 × (B-C)/B (A is Inhibitory rate; B is Diameter of colony on control culture medium surface; C is Diameter of colony on mixture culture medium surface).

Characteristics of antagonistic bacteria

According to the experimental design described by Li (2002), the best strain was studied on the morphology, cultural characteristics, physiological and biochemical properties and gram stain.

16S rDNA amplification and analysis of antagonistic bacteria

Bacterial DNA was extracted by the lysozyme-SDS-protease method (Zheng et al., 2006). Complete sequencing was done by the amplification of 16S rDNA with bacterial universal primers 27F and 1492R (Ludwig and Schleifer, 1994). Sequences of the primers were performed by the Shanghai Sangon Biological Engineering Technology and Service Co.,Ltd. The sequence was analyzed using the BLAST program and phylogenetic tree.

Construction of the molecular phylogenetic tree

The sequence was analyzed using the BLAST program (National Center for Biotechnology Information) to determine the closest available database sequences. The selected DNA sequences were aligned using the Clustal X program. Published sequences were obtained from GenBank. A phylogenetic tree was constructed using MEGA (Version 4.1) by distance matrix analysis and the neighbour-joining method. Bootstrap analysis was used to provide statistical confidence for the tree branch points.

RESULTS AND DISCUSSION

Morphology and cultural characters of pathogen

The colonies of the isolated pathogen were round, the surface was covered by the pink villous aerial hypha, and the colonies were orange-yellow from the back culture dish because of the secretion pigment. After being cultured for 5 days at 20°C, the acervuli looked like black dots spreading inside the colony and on the hypha surface. While observing the morphological characteristics of acervuli and spores under the microscope, conidia was like spindle, (18.2 - 20.8) μm × (6.1 - 7.2) μm length; 3 cells were present in the olive; 2 of 3 colored cells were deep in color; the length

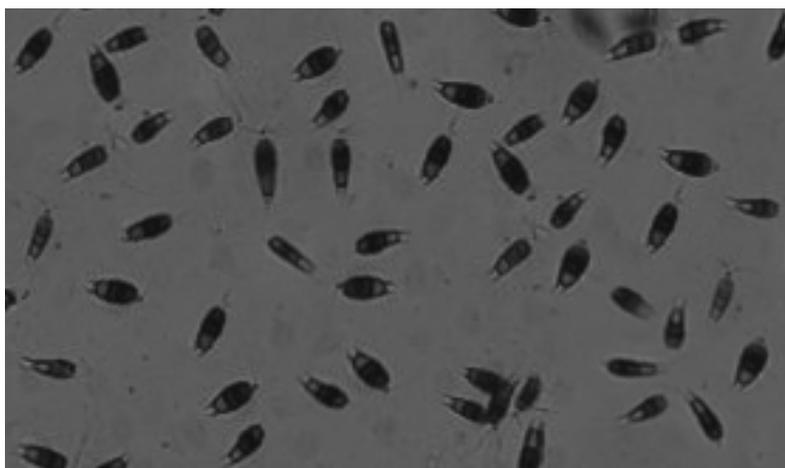**Figure 1.** Symptom of *C. oleifera* leaf blight.

Figure 2. Morpha of pathogen conidia.

was about 12.8 - 16.4 µm; the color of cells at the both ends was light; 4 septum were real septum; there were 2 - 3 attached mycelium about 15 - 17 µm length and was not branched at the top of spores. The sporephore is long with light color, no branches, no septum. According to the isolated host, the disease symptom and the morphological characteristics of the colony and the spore, we can appraise that it is *P. microspora*; the access

number in our lab is CSUFT070160B (Figures 1 and 2).

Pathogenicity testing

After 15 days, the vaccinated leaves had the same symptom as the initially infected oil-tea tree leaves, the

Table 1. Inhibitory effects of fermentation filtrate against *P. microspora.*

Serial number	Colony diameter (mm)	Inhibitory rate (%)
YB7	17.2	73.9
YB10	16.8	74.5
YB18	10.1	84.7
YB23	15.7	76.2
YB38	20.2	69.4
YB45	19.8	70.0
YB67	12.4	81.2
CK	66	—

comparison did not appear in the symptom. Isolated disease had spots once more, the obtained colonial morphology was consistent with the first time it was isolated and this is true of the morphology of the spores.

Sequence analysis of rDNA ITS region of pathogen

The rDNA ITS region of the strain CSUFT070160B was sequenced by ABI PRISM 3730 and with BigDay terminator v3.1 reagent. The total of ITS spaces is 517 bp, ITS1 region comprises 193 bp, 5.8s ribosomal RNA comprises 158 bp, and ITS2 region comprises166 bp. Phylogenetic tree indicated that CSUFT070160B and DQ000995 (*P. microspora*) were in the same branch with high confidence level and the concordance ratio is 99%. Compared with the sequence of DQ000995 and combined with morphological characteristics of the strain CSUFT070160B, we could draw a conclusion that it has a closer relationship with *P. microspora*. This lays the foundation for researching on how to prevent and control the harms and infection caused by *P. microspora* of *C. oleifera* leaf blight. The method used to identify species by the phylogenetic tree was according to the standard of using PSR (phylogenetic species recognition) to identify the phylogenetic species (Taylor et al., 2000) (Figure 3).

Screening of antagonistic bacteria

There were 175 endophytic bacterias isolated from *C. oleifera* tissue, 26 strains could antagonize the *P. microspora* in different degree. And among these 26 strains, 19 strains of the inhibition zone width each were smaller than 4 mm. Out of the 19 strains, 4 strains each were between 4 - 7mm, each of the two strains each was between 7 - 10mm, and one strain (No.YB18) was larger than 10mm. Seven strains whose inhibition zone width

was larger than 4mm were chosen for secondary screening. The fermentation filtrate of 7 strains restricted pathogen growth. The inhibiting zone method adopted in the experiment indicated that the control effect of YB18 strain was the best one with 84.7% inhibitory rate (Table 1), and it had the best antibacterial effect against *P. microspora* (Figure 4).

Physiological and biochemical characteristics of YB18

YB18 strain was bacillus, gram-positive, and its colony has more rapid speed at 32°C. The color of the strain's clone was creamy, and the edge was smooth, round or oblong, wrinkled, lawn grew thicker, opaque, non-pigment. Biolog physiological and biochemical test of YB18 strain indicated V.P-positive, contact enzyme-positive, M.R-negative, Starch hydrolysis and gelatin.

Sequence analysis of 16S of YB18

rRNA - based analysis is a central method in microbiology used not only to explore microbial diversity but also as a method for bacterial strain identification (Sivparsad and Gubba, 2008; Adolphe et al., 2008). In our study, the phylogenetic tree indicated that YB18 and *Bacillus subtilis* share a cluster (Figure 5). The comparative sequence analysis of 16S rDNA, gene (1475 bp) of isolated YB18 that gave the best antibacterial effect, revealed that this isolated one was a member of genus *Bacillus* exhibiting similarity values up to 99% to *B. subtilis*. By studying the morphology, cultural characteristics, physiological and biochemical properties, 16S gene sequence and phylogenetic tree, according to Bergeys Manual of Determinative Bacteriology (1984), YB18 strain was identified as the
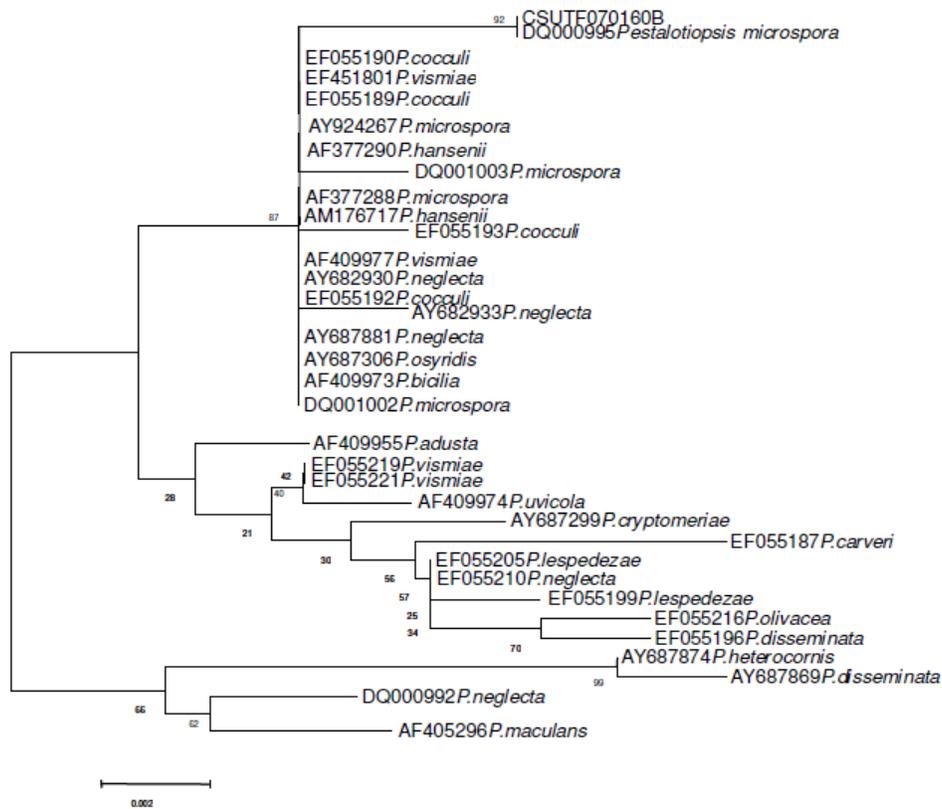
Figure 3. Phylogenetic tree based on the rDNA ITS1 sequences of *Alternaria* and relating species.

Figure 4. Inhibitive effect of YB18 strain against *P. microspora.*

strain belonging to *B. subtilis.* Endophytic bacteria and fungicide have been extensively studied (Opoku et al., 2007).

Screening and application of antagonistic bacteria against plant pathogens has become a subject of general interest. Tapping and application of antagonistic bacteria and their metabolic products, and production of new - type, highly effective, steady, well - fitting antagonistic bacteria by means of genetic engineering, are the major tasks in this field. The tendency of controlling plant disease is with biological fungicide as a substitute for chemical (Bhanumathi et al., 2007). Morphological classification is a traditional classification method and it has many shortcomings. Although molecular biology methods take genetic material as study object directly or indirectly, the experimental results often vary because of the different DNA fragments. Therefore how to choose the representative DNA sequence has become a difficult problem in biochemistry and molecular biology methods. The sequence of the coding region is relatively conservative, but ITS of fungi and 16S of bacteria sequence evolves very quickly between two generations, often mutatesand has various degrees of
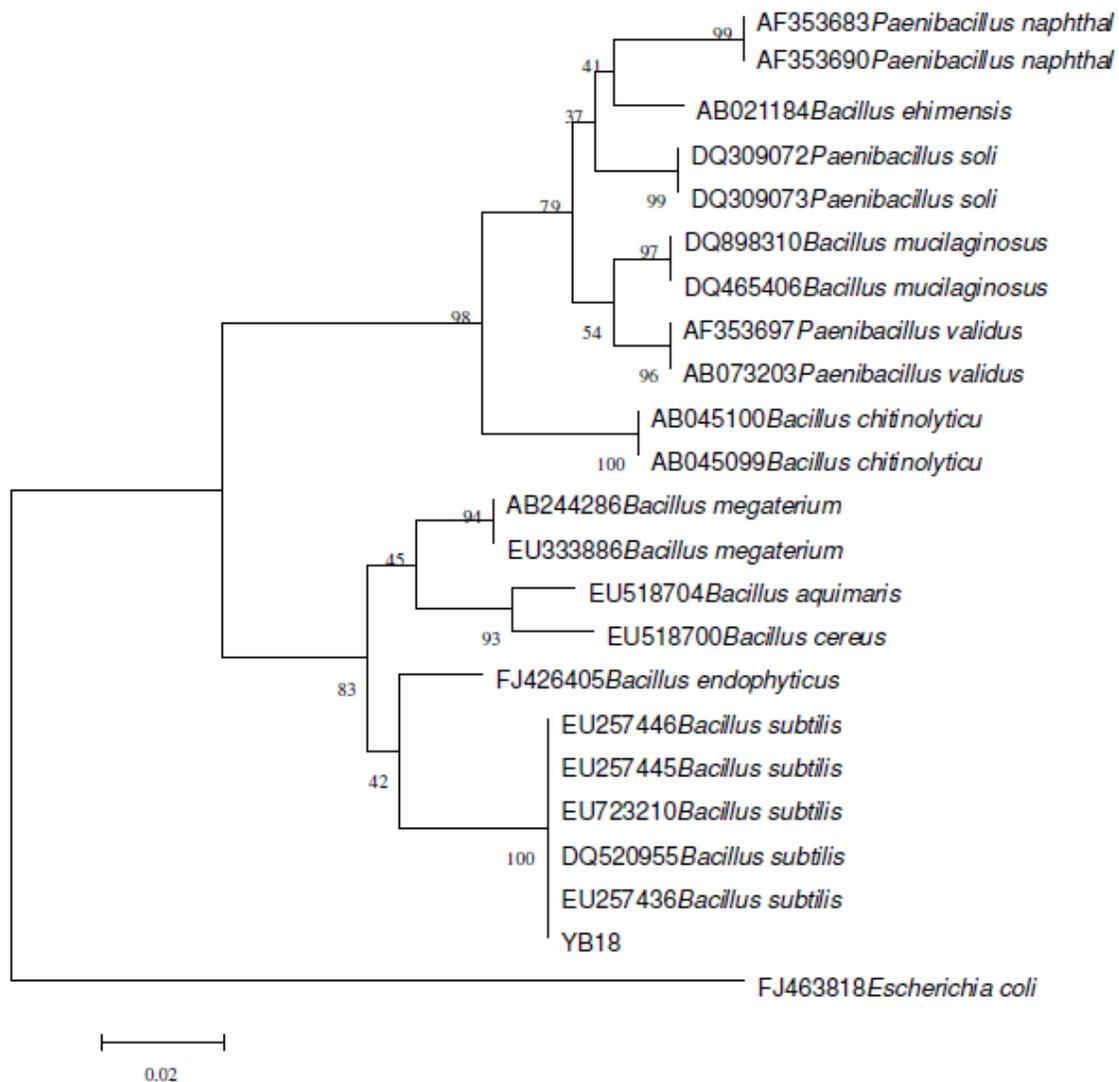
Figure 5. Phylogenetic relationship of the selected isolate (YB18) with other 16S rDNA sequences of published strains. The *E. coli* strain (FJ463818) was used as out group. In the phylogenetic tree, the YB18 and the *B. subtilis* were clusterd together as one clade (Bootstrap value is 99).

difference in the same interspecific even in the intraspecific. Nevertheless, it is not good enough to identify microbe species correctly based only on molecular sequences; it also should be combined with the morphological of strains and spores.

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