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Full Length Research Paper

Isolation and identification of endophytic bacteria antagonistic to *Camellia oleifera* anthracnose

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Anthracnose is a common *Camellia oleifera* disease caused by *Colletetrichum gloeosporioide*. This study was to isolate antagonistic bacteria from *Camellia oleifera* tissues to control the anthracnose. Antagonistic bacteria was screened from 175 endophytic bacteria with confront culture method. The results of the study showed that the antagonistic effect of YB128 strain was the best with 86.1% inhibitory rate. According to the results of the researching on morphology, cultural characteristics, physiological and biochemical propertices, 16S gene sequence and molecular phylogenetic tree, YB128 was identified as *Bacillus licheniformis.*

Key words: *Camellia oleifera*, anthracnose, antagonistic bacteria, biological control.

INTRODUCTION

Teaoil camellia (*Camellia oleifera* is a special woody plant for edible oil production which is rich in vitamins in Southern China. It is not only capable improve human immunity, but also has high economic value (Deng and Xie, 2008). Anthracnose is a common disease in *C. oleifera*. The symptoms include abscission of fruits, buds and leaves, death of branches, and sometimes death of the plants. The pathogen causing anthracnose in *C. oleifera* is *Colletetrichum gloeosporioides*. This disease can spread very quickly and is difficult to control, often resulting in huge economic losses (Ji and Guo, 1992; Zhou et al., 2007). Now the chemical method is the main way to control *C. oleifera* anthracnose, but it has severely side-effects on our environment. Biological control is ecologically safe and it will not pollute the environment. The tendency of controlling plant disease is to use biological fungicide as a substitute for chemical ones. Endophytic bacteria as a potential fungicide, which have stably living environment in plants and is not liable to be affected by the external condition, have been extensively studied (Opoku at al., 2007; Mann at al., 2008). However, there is no research yet reported of controlling *C. oleifera* anthracnose with antagonistic bacteria. The aim of this study was to isolate antagonistic bacteria from *C. oleifera* tissues to control anthracnose.

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MATERIALS AND METHODS

The pathogen of *C. oleifera* anthracnose is *C. gloeosporioides* stored in our lab and the approach of isolating endophytic bacteria from leaves of *C. oleifera* described by Fang (1997) was used. Antagonistic bacteria was screened with confront culture method (Li at al., 2006). Width of the inhibition zone on NA plates were recorded after 4 days. The strains with good inhibiting efficacy were individually inoculated to 250 ml erlenmeyer flask containing 150ml NB medium, and shaked for 48 h at 28℃, 150 rpm. Then the culture was filtered with a germ filter (d = 0.22 μm). The Culture filtrate was mixed with PDA in a ratio of 1:19 and mixture was poured into a petri dish. The mycelial mats of *C. gloeosporioides* (d = 6 mm) was transplanted directly to surface of mixed medium for secondary screening. The diameters of pathogen colony were measured and recorded after 4days. Medium which mixed sterilized distilled water with PDA in a ratio of 1:19 was used as a control. The strain having the best inhibition efficiency was chosen by the inhibitory rate that was calculated with the formula as follows.

$$A (\%) = 100 \times (B - C)/B$$

A: Inhibitory rate;
B: Diameter of colony on control culture medium surface;
C: Diameter of colony on mixture culture medium surface.

The fermentation filtrate of the best strain was diluted to 10, 100, 200,400, 800, 1000 times to determine the potency of the fermentation filtrate against the pathogen. According to the experimental design described by Li (2002), the best strain was studied on the morphology, cultural characteristics, physiological and biochemical propertices and Gram stain.

Total bacterial genomic DNA was isolated followed the lysozyme-SDS-protease method described by Zheng et al., 2006. The forward primer used for PCR amplification of 16S rRNA gene sequence was 27F . The reverse primer was 1492R (Martin and Collen, 1998).

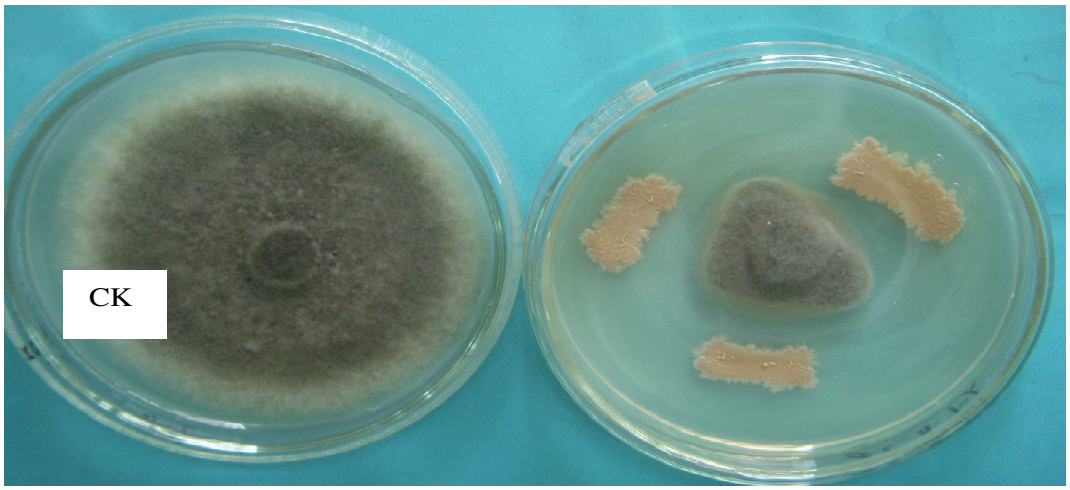

Figure 1. Inhibitive effect of the YB128 strain against *Colletotrichum gloeosporioides*.

A DNA thermal cycler (ABI 9700, USA) used for amplification was programmed as following: an initial denaturizing for 5 min at 94℃ followed by 35 cycles of denaturation for 1 min at 94℃, annealing for 1 min at 55℃ and extending for 1.5 min at 72℃, finally reactions were held at 72℃ for 10 min (Ma et al., 2008). PCR products were purified by PCR Cleanup Kit (Tiangen, Biotech Ltd, Beijing, China) and sequenced with ABI 3730 DNA Analyzer (Shanghai Sangon Biotechnology Co., Shanghai, China).The sequence was analyzed using the BLAST program (National Center for Biotechnology Information) to determine the closest available database sequences. Selected DNA sequences were aligned using the Clustal W program (Thompson et al., 1994). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using MEGA (Version4.1) by distancematrix analysis and the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to provide statistical confidence for the tree-branch points.

RESULTS AND DISCUSSIONS

There were 175 endophytic bacteria isolated from *Camellia oleifera* tissues, 30 strains could inhibite *Colletetrichum gloeosporioides* in different degrees. And among these 30 strains, diameters of inhibition zone of 15 strains were less than 4 mm. Those of 9 strains were between 4~7mm, and those of 5 strains were between 7 ~ 10 mm. Only that of one strain No. YB128 was more than 12 mm. 15 strains which diameters of inhibition zone was more than 4mm were chosen for secondary screening. The fermentation filtrate of 15 strains restricted pathogen growth. The inhibiting zone method adopted in the experiment indicated that: the inhibition effect of YB128 strain was the best one with 86.1% inhibitory rate, and it had the best antibacterial effect against *C. gloeosporioides* (Figure 1).

The experimental results showed there is little difference when the fermentation filtrate was diluted 200 times and 100 times (inhibitory rate 34.8%). The inhibiting effect was not good when fermentation filtrate was diluted more than 400 times. Considering reduces production cost, we determined the potency of the fermentation filtrate of YB128 strain against the pathogen was 200 times (Figure 2).

YB128 was a Gram- positive bacillus and the edge was smooth, round or oblong, wrinkle, lawn grows thicker, opaque, non-pigment. Physiological and biochemical test of YB128 indicated: Simmons Citrate-positive, V.P-positive, contact enzyme-positive, M.R- positive, starch and gelatin hydrolysis.

Bacteriological approach is widely used in bacterial identification, whereas, with the development of molecular biology techniques, homology analysis based on16S rRNA gene sequences have became the “golden index” in the field of bacterial identification (Shen and Feng, 2004; Ma et al., 2008; Rani et al., 2008; Ojo et al., 2008). In our study, the phylogenetic tree (Figure 3) indicated that YB128 and *Bacillus licheniformis* shared a cluster. Comparative sequence analysis of 16S rDNA gene (1366bp) of YB128 which has the best antibacterial effect, revealed that this bacterium was a member of genus *Bacillus* and exhibited similarity values up to 99% to *Bacillus licheniformis*. Based on the morphology, cultural characteristics, physiological, biochemical properties, 16S gene sequence, phylogenetic tree and Bergeys Manual of Determinative Bacteriology (1984), YB128 strain was identified as *B. licheniformis.*

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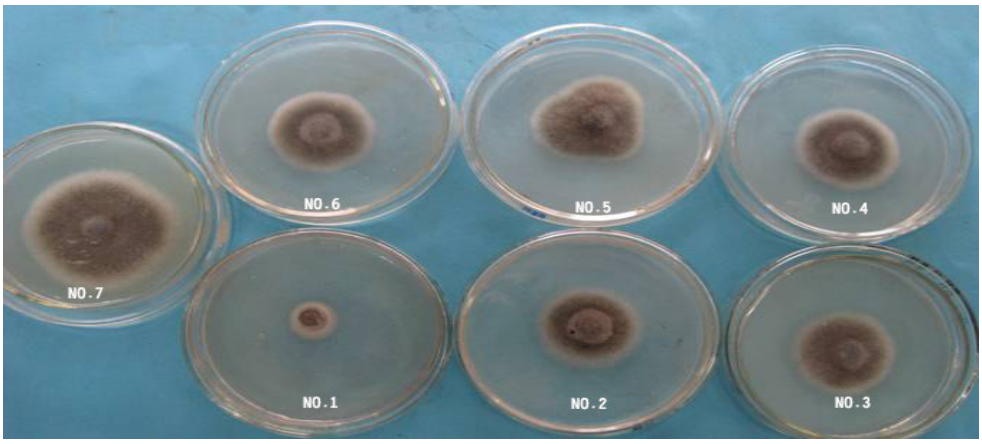

Figure 2. Inhibitory effect s of the fermentation filtrate.
NO.1: Fermentation filtrate was diluted 10 times, the inhibitory rate was 76%; NO.2: Fermentation filtrate was diluted 100 times, the inhibitory rate was 36.0 %; NO.3: Fermentation filtrate was diluted 200 times, the inhibitory rate was 34.8%; NO.4: Fermentation filtrate was diluted 400 times, NO.5: Fermentation filtrate was diluted 800 times, NO.6: Fermentation filtrate was diluted 1000 times, the inhibitory rate of these three dilution all were 29.6%; NO. 7: CK.

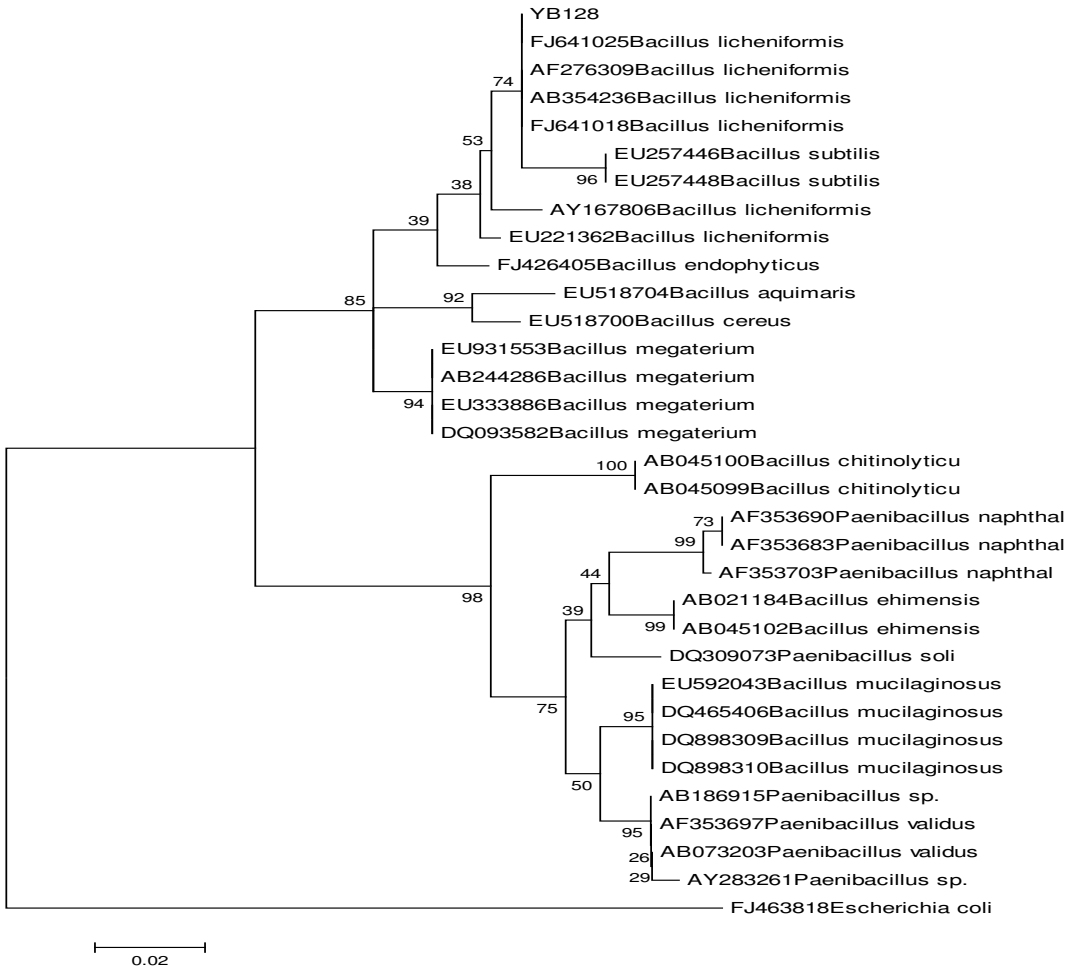

Figure 3. Phylogenetic relationship of the selected isolate (YB128) with other 16S rDNA sequences of published strains. The Escherichia coli strain (FJ463818) was used as out group. In the phylogenetic tree the YB128 and the *Bacillus licheniformis* were clusterd together as one clade.

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