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Full Length Research Paper

Specific and Rapid Detection of Camellia oleifera Anthracnose Pathogen by Nested-PCR

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Camellia oleifera **is an economical important plant in southern China for edible oil production. Anthracnose is a serious disease that limited its development. The internal transcribed spacer (ITS) regions and the 5.8S rRNA gene of strain C1 of the pathogenic fungus *Colletetrichum gloeosporioides* were sequenced in order to design specific PCR primers for pathogen detection. Alignment of the sequence data of strain C1 and the other *Colletetrichum* species obtained from the Genbank were made using CLUSTAL W. Based on the aligned ITS sequences, specific primers for *C. gloeosporioides* were developed (YT1 and YT2). The infecting pathogens were successfully detected with our specific primer set and showed high specificity. The result showed that the nested-PCR reaction was at least 10,000-fold more specific than that of the simple PCR method. This new method provides a useful technique to further study disease cycle and for early prediction of anthracnose of *Camellia oleifera*.**

Key words: *Camellia oleifera*, anthracnose, taxon-specific primer, molecular detection, nested PCR.

INTRODUCTION

Camellia oleifera (teaoil camellia) is an important edible oil woody plant, native to China (Deng and Xie, 2008). It oil has rich vitamins and shows capacity of enhancing human immunity (Wang et al., 2007).In terms of oil production by woody species in the world, teaoil camellia valued equivalent to palm, olive, and coconut. Camellia oil is extracted from *C. oleifera* seeds and has a unique aroma. In southern China, it is regarded as the top edible oil. A related commercial product called Camellia oil is used for medical and cosmetical purposes but is originated from the leaves of a different plant. The seed oil can be used treat ringworm. The seed oil contains over 80% monounsaturated fat. As such, it can reduce LDL ('bad cholesterol') and prevent cardiovascular diseases. Camellia oil is also regarded as the ideal edible oil for diabetic patients (Wikipedia, 2008).

Anthracnose is a common disease of *C oleifera.* The symptoms include fruit drop, bud drop, leaf loss, dead branches, and sometimes death of the plants. The Pathogen causing anthracnose in *C. oleifera* is *Colletotrichum gloeosporioides*. This disease can spread very quickly and is difficult to control, often resulting in huge losses of the crop (Ji and Guo, 1992; Zhou et al., 2007). As a result, *C. gloeosporioides* is one of the most important plant pathogens in China. The current method used for the detection and identification of *C. gloeosporioides* of *C. oleifera* depends on isolation of pure cultures on nutrient media, followed by morphological examination of the isolates (Ji and Guo, 1992). Such a procedure is time consuming and not specific. The lack of a specific detection for *C. gloeosporioides* on *C. oleifera* has hindered our understanding of their life cycles and epidemiology. For rapid and specific detection, we applied specific nested-PCR assay for detecting *C. gloeosporioides* causing *C. oleifera* anthracnose.

MATERIALS AND METHODS

Fungal material

A total of 34 samples of strains were collected in China and listed in Table 1.

DNA extraction from mycelia

Total genomic DNA was isolated from fresh mycelia followed a miniprep protocol described by Cenis (1992). Briefly, 500 µl of liquid

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Table 1. The samples of strains in this study.

Sample no.	Species	Host	Geographic origin
C1	*Colletotrichum gloeosporioi*	*Camellia oleifera*	China, Hunan
C2	*C. gloeosporioides*	*C. oleifera*	China, Hunan
C3	*C. gloeosporioides*	*C. oleifera*	China, Zhejiang
C4	*C. gloeosporioides*	*C. oleifera*	China, Yunnan
C5	*C. gloeosporioides*	*C. oleifera*	China, Guangxi
C6	*C. gloeosporioides*	*C. oleifera*	China, Guangdong
C7	*C. gloeosporioides*	*C. oleifera*	China, Jiangxi
C8	*C. gloeosporioides*	*C. oleifera*	China, Jiangxi
C9	*C. gloeosporioides*	*C. oleifera*	China, Guizhou
C10	*C. gloeosporioides*	*C. oleifera*	China, Guizhou
C11	*C. gloeosporioides*	*C. oleifera*	China, Sichuang
C12	*C. gloeosporioides*	*C. oleifera*	China, Hubei
C13	*C. gloeosporioides*	*C. oleifera*	China, Fujian
C14	*C. orbiculare*	*Citrullus lanatus*	Stock culture in our laboratory
C15	*C. lindemuthianum*	*Phaseolus vulgaris*	Stock culture in our laboratory
C16	*C. graminicola*	*Eulaliopsis binat*	Stock culture in our laboratory
C17	*C. higgisianum*	*Brassica chinensis*	Stock culture in our laboratory
C18	*C. truncatum*	*Glycin max*	Stock culture in our laboratory
C19	*C. capsici*	*Capsicum annuum*	Stock culture in our laboratory
C20	*C. musae*	*Musa nana*	Stock culture in our laboratory
C21	*C. coccodes*	*Capsicum annuum*	Stock culture in our laboratory
C22	*C. boninense*	*Anthurium andracanum*	Stock culture in our laboratory
C23	*C. destructivum*	*A. Andracanum*	Stock culture in our laboratory
C24	*Ascochyta gossypii*	*Gossyphum herbaceum*	Stock culture in our laboratory
C25	*Tilletia indica*	*Triticum aestivum*	Stock culture in our laboratory
C26	*Erwinia amylovora*	*Pyrus ussuriensis*	Stock culture in our laboratory
C27	*Pantoea stewartii*	*Zea mays*	Stock culture in our laboratory
C28	*Phytophthora colocasiae*	*Colocasiu esculenta*	Stock culture in our laboratory
C29	*Fusarium oxysporum*	*C. oleifera*	Stock culture in our laboratory
C30	*Verticillium alboatrum*	*G. herbaceum*	Stock culture in our laboratory
C31	*Botrytis cinerea*	*Vitis vinifera*	Stock culture in our laboratory
C32	*Rhizoctonia solani*	*Oryza sativa*	Stock culture in our laboratory
C33	*Pestalotiopsis sp*	*C. oleifera*	Stock culture in our laboratory
C34	*Magnaporthe grisea*	*O. sativa*	Stock culture in our laboratory

potato dextrose medium was inoculated with fungal hyphal threads and left at room temperature for 72 h. The resulting mycelial mat was pelleted by centrifugation at 13,000 rpm for 5 min and was washed with 500 µl of Tris-EDTA (pH 8.0).The mat was then homogenized by hand in 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) for 5 min. 150µl of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20℃ for 10 min. Fungal debris was pelleted by centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 13,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was resuspended in 50 µl Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Amplification of ITS regions

The Internal Transcribed Spacers (ITS) regions and the 5.8S rRNA was amplified from C1 strain using the universal fungal primers ITS1 and ITS4 as described by White et al. (1990). Each PCR reaction mixture contained 5 - 10 ng of genomic DNA, each of the primers ITS1 and ITS4 (1 µM), 5 µl of 10 X reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH 8.3] 0.1 mg/ml bovine serum albumin), 3 mM $MgCl_2$, 200 µM each of dNTP and 2.5 U of Taq DNA polymerase in a total volume of 50 µl. The PCR profile was denaturation at 95℃ for 5 min, followed by 36 cycles of 95℃ for 30 s, 56℃ for 30 s, and 72℃ for 1 min, then a final extension at 72℃ for 10 min.

Sequencing of ITS regions

The amplified PCR product of C1 strain was purified and then cloned into PMD18-T plasmids and transformed into competent cells of *Escherichia coli*. Positive colonies were selected using the white-blue-colony method (medium containing X-Gal, IPTG, Amp LB). The colonies were then cultured in liquid LB culture medium. Then PCR product was sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

GTTTACGCTCTACAACCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGTAGGG
TCTCCGTGACCCTCCC**GGCCTCCCGCCCCCGGGCGGG**TCGGCGCCCGCCGGAGG
ATAACCAAACTCTGATTTAACGACGT

YT1

TTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACGG**CTGAC**
GTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCG

YT2(Reverse complementary sequence)

TAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAA
TTTCCAAAG

Figure 1. Oligonucleotide primers, YT1 and YT2, specifs of PCR-amplified products with specific primers YT1 and YT2 for *Colletotrichum gloeosporioides.* Lane M: DNA marker; Lane 1: H_2O; Lanes 2-14. Isolates C1 to C13 of *C. Gloeosporioides* from China respectively.

Specific primer design

The nucleotide sequence of the ITS regions of C1 strain was 480 bp long (Figure 1). Other nucleotide sequences of Colletetrichum species were obtained either from our previous study or from the Genbank nucleotide databases. An alignment of the nucleotide sequences of the ITS regions were made by CLUSTAL W (Thompson et al., 1994). The aligned ITS rDNA sequences were analyzed using DNA Star (5.01) Megalign program. Targeting the variable regions, a pair of internal primers was designated as YT1: 5'GGCCTCCCGCCCCCGGGCGGG 3'; YT2: 5'ACCTTTGAGGGCCTACGTCAG 3' (Figure 1). We followed the guideline on PCR primer designing (Singh and Kumar, 2001; Kamel, 2003).

Plant tissue DNA extraction

Top extract plant tissue DNA for evidence of *C. gloeosporioides*, we placed a few milligrams of young tissue into a 1.5 ml tube and, to every mg of tissue, add 10 µL 0.5 N NaOH. The tissues were thoroughly ground and centrifuged at 12000 rpm for 5 min. The supernatant (~ 5µL) was quickly transferred to a new tube containing 495 µL 100 mM Tris pH 8. 0 and mixed thoroughly through vortex (Wang and Qi, 1993). The solution was then used directly in PCR.

Specificity of PCR amplification

We tested the specificity of the primer pair YT1 and YT2 in PCR amplification *C. gloeosporioides* and other similar and related species collected in China. Amplification of the ITS rDNA with the taxon-specific PCR primer pair and the original ITS1 and ITS4 universal primers were performed by the PCR method described above, except that the annealing temperature was increased to 60℃. PCR fragments amplified with the two taxon-specific primer pairs were size-fractionated in 1.0% agarose gels.

Nested-PCR amplification

Our nested PCR amplification was performed in two rounds. The first round PCR amplification used primers ITS1 and ITS4 with the reaction conditions the same as those described in section of amplification of rDNA ITS regions. Then, 1 µL PCR product was used as templates for the second round of PCR amplification with primers YT1 and YT2, and the reaction conditions followed that in section of specificity of PCR amplification.

RESULTS

C. oleifera anthracnose

The progression of anthracnose in *C. olerfera* generally starts with small brown spots on the fruits and leaves, then gradually expands to form dark brown circular lesion, and finally becomes whorled acervulus on the circular lesion. After rain or dew, the acervulus becomes a sticky pink conidia heap. Overall, the symptoms of *C. oleifera* anthracnose in the leaves can be at the margin or tip, semi-circular or irregular, dark brown, with a purple edge (Figure 2).

Specificity of the designed primers

Oligonucleotide primers YT1 and YT2 were designed from the ITS sequences collected and assembled. The primers showed high specificity for the species. A 330bp unique band was obtained from each of the 13 strains of *C. gloeosporioides* collected from various areas in China (Figure 3). However, in other species, we were unable to get any product using this primer pair (Figure 4).

Sensitivity of the one-step simple PCR for detection

To determine the minimum amount of fungal DNA that can be detected by the simple PCR assays, variable

Figure 2. Symptom of anthracnose on the fruit and leaf of *Camellia oleifera*.

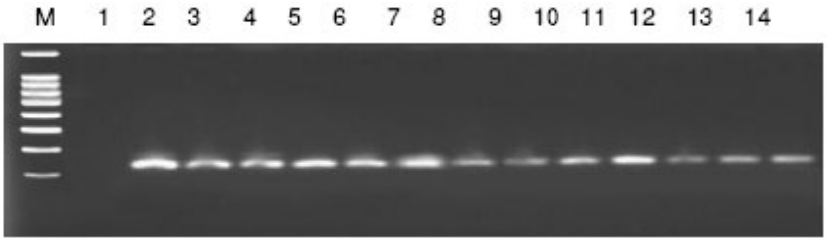

Figure 3. Electrophoresis of PCR-amplified products with specific primers YT1 and YT2 for *Colletotrichum gloeosporioides.* Lane M: DNA marker; Lane 1: H_2O; Lanes 2-14. Isolates C1 to C13 of *C. Gloeosporioides* from China respectively.

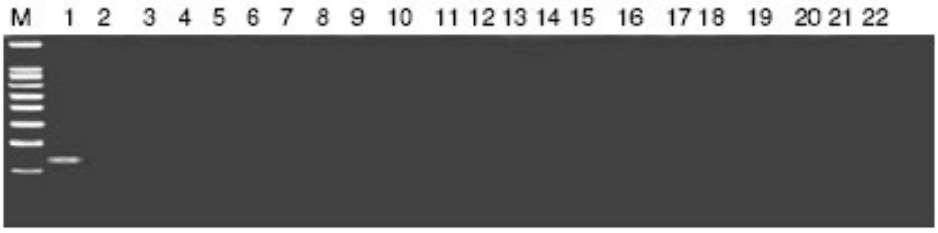

Figure 4. Electrophoresis of PCR-amplified products with specific primers YT1/YT2 for other isolates from diverse species.
Lane M: DNA marker; Lane 1:*Colletotrichum gloeosporioides*; Lanes 2-22: correspond to the following species respectively *C. orbiculare*; *C. lindemuthianum*; *C. graminicola*; *C. higgisianum*; *C. truncatum*; *C. capsici*; *C. musae*; *C. coccodes*; *C. boninense*; *C. destructivum*; *Ascochyta gossypii*; *Tilletia indica*; *Erwinia amylovora*; *Pantoea stewartii*; *Phytophthora colocasiae* ;*Fusarium oxysporum*;*Verticillium alboatrum*; *Botrytis cinerea*; *Rhizoctonia solani*; *Pestalotiopsis* sp.; *Magnaporthe grisea.*

quantities of genomic DNA from strain C1 ranging from 100 ng to 1 ag were used as DNA template. We identified that 1 pg to 100 ng all generated reliable amplification product with the primer combination YT1 and YT2 (Figure 5).

Sensitivity of nested-PCR for detection

The same range of genomic DNA concentration of C1 strain was tested to determine the sensitivity by the nested-PCR assay. For DNA from the mycelia of C1 strain, 10 ag to 100 ng was sufficient for reliable amplification of the nested PCR with primers ITS1 and ITS4 for the first reaction and primers YT1 and YT2 for the second amplification. The result showed that the nested-PCR reaction is at least 10,000-fold more sensitive than the simple one-step PCR meth. (Figure 6).

Detection of *C. gloeosporioides* from *C.oleifera* tissue

The plant materials were classified into two groups based on the results of histological inspection: the “+” group includes materials with obvious symptoms of anthracnose and the “-” group includes materials with no visual symp-

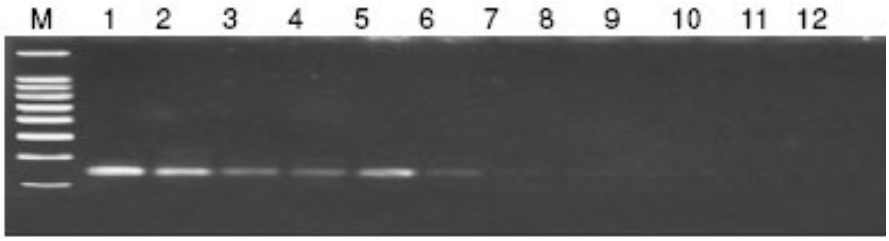

Figure 5. Sensitivity of simple PCR for the detection of *Colletotrichum gloeosporioides* with primers YT1 and YT2 using different quantities of genomic DNA.
Lane M: DNA marker; Lane 1-12: Amplified DNA products at quantity of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10 ag, 1 ag in a 25μL PCR reaction system, respectively.

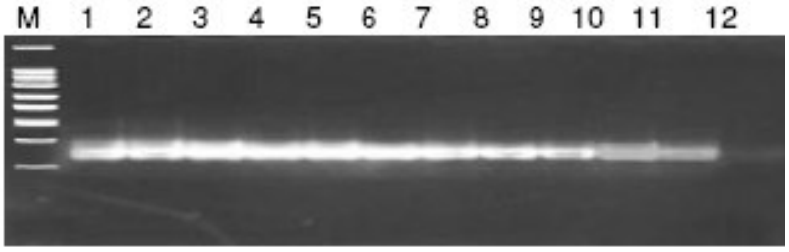

Figure 6. Sensitivity of nested-PCR for the detection of *Colletotrichum gloeosporioides*.
Lane M: DNA marker; Lane 1-12: Amplified DNA products at quantities of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, 10 ag, 1 ag in a 25 μL PCR reaction system, respectively.

toms of anthracnose. Simple PCR could detect the pathogen from the specimens classified into the + group (Lane 3; Figure 7). No amplification product was obtained from specimens cataloged into the "-" group (Lanes 5 and 6).However, the nested-PCR detected the pathogen in one material with no obvious symptoms (that is, the same DNA of used for Lane 5) (Lane 4). The negative control of no DNA (just water) processed simultaneously was consistently negative (Lane1).The positive control processed simultaneously was consistently positive (Lane 2).

These results indicated that the specific primer pairs are useful in detecting *C. gloeosporioides* directly from infected host tissues, even from the plant materials that have likely been exposed to the pathogen but before the onset of visible symptoms. Compared with histological examination, the nested PCR-based detection technique is more rapid and sensitive.

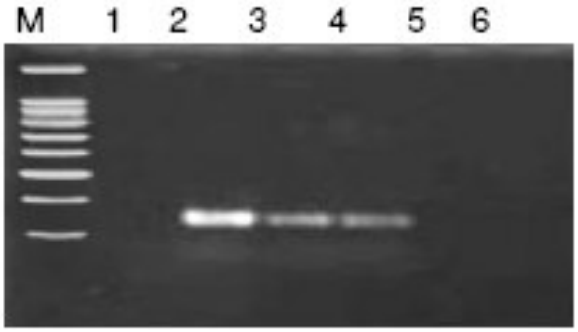

Figure 7. Detection of pathogen DNA by the simple PCR or nested-PCR from C. *oleifera* tissues.

Lane M: DNA marker; Lane 1 : negative control (H_2O); Lane 2: Positive control (*C. Gloeosporioides* mycelial DNA). Lane3: Simple PCR for specimen with visible disease symptoms; Lane 4: Nested-PCR for plant materials with no visible disease symptoms; Lanes 5 and 6 : Simple PCR for plant materials without any symptoms.

DISCUSSION

Traditional classification of plant pathogens are based on their macroscopic and microscopic morphological and structural characteristic. However, phenotypic characteristics can vary significantly depending on the specific environmental factors that they are exposed to. Such variations make the identification of many pathogens difficult and unreliable. As a result, it is difficult to monitor and control many pathogens in a timely and effectively way, especially during epidemics. Amplification of target DNA through PCR with taxon-specific primers is a potentially more sensitive and accurate approach than microbiologic techniques (Kamel et al., 2003; Farid et al.,

2006; Kawther, 2008). Unlike identification based on culture techniques, PCR does not require the presence of viable organisms and can work even when there is a limited amount of sample (Schubert et al., 1999).

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). In this study, the taxon-specific primers of YT1 and YT2 showed high specificity for the species of *C. gloeosporioides* isolated from *C. oleifera.* And the detection sensitivity was 10 ag of genomic DNA by nested-PCR reaction. Our results provide a useful technique to study disease cycle and early prediction of anthracnose on *C. oleifera*. In combination with the simple method of preparing plant samples for PCR (Wang and Qi, 1993), a detection system can be easily and broadly adapted to meet the actual demands.

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