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Genetic Variation in *Corynespora cassiicola*, the Target Leaf Spot Pathogen

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Abstract: Random Amplified Polymorphic DNA (RAPD) analyses was undertaken to assess genetic relationships among isolates of *Corynespora cassiicola,* a destructive fungal pathogen of many economically important crop plants including tomato, cucumber, perilla, rubber, etc. from different host plants. Of the 8 random primers tested, two were selected that generated reproducible amplification pattern using three isolates representing each pathogen. RAPD-PCR also amplified reproducible polymorphic bands. A total of 64 bands were scored, 12 of which were polymorphic. RAPD analyses revealed that the amplification products were similar among isolates of each pathotype from the different host plants.

Key words: Phylogenetic, target leaf spot, *Corynespora cassiicola*

INTRODUCTION

Target leaf spot of the leaves and fruits of caused by *Corynespora cassiicola* (Berk. and Curt.) Wei, is a common disease in almost all the tomato, cucumber and perilla crops growing regions of the world[1,2]. The disease occurs on a wide range of host plants in both tropical and subtropical countries. It was first described in Europe on cucumber in 1906, in Korea in 2003[3] on tomato (*Lycopersicon ecsulentum* Mill.) by F.C. Deighton in Sierra Leone[4] followed by India[5], Queensland, Australia[6], the United States[7] and southern Nigeria[8] and on perilla[9]. These plants appear to be most susceptible at the seedling stage and just before and during fruiting[8]. Initially, symptoms consist of small, brown, pinpoint, water-soaked lesions on the upper surface of the leaves. They become circular and pale brown lesions surrounded by conspicuous yellow halos. The coalescence of the lesions may result in a rapid collapse of tissue, but the leaves remain attached to the petiole. Petiole and stem lesions are brown and oblong to elongate. On fruits, the lesions appear as dark, sunken, pinpoint, brown spots with a pale brown center, which crack[10]. The rapid development of the symptoms led researchers to the hypothesis that a toxin may be involved in the disease syndrome[11,12].

The early, rapid, reliable and consistent detection of *C. cassiicola* pathotypes infecting different crops would be of importance for the management of target leaf spot disease. On the other hand, an understanding of the genetic variation among *C. cassiicola* pathotypes from different crops would contribute to the improvement of management schemes for this important disease. In recent years, Internal Transcribed Spacer (ITS) regions of the ribosomal genes (rDNA or rRNA) have been used to detect and differentiate fungal pathogens. The Polymerase Chain Reaction (PCR)-mediated random amplified polymorphic DNA (RAPD) technique has been shown to be useful as a diagnostic tool for numerous fungal pathogens which are difficult to differentiate using conventional methods[2,13-15]. However, *C. cassiicola* pathotypes of host crops such as tomato, cucumber and perilla have not been extensively characterized at the molecular level. The main objective of this research was to analyze the genetic relatedness of isolates of *C. cassiicola* obtained from different hosts.

MATERIALS AND METHODS

Fungal isolates: Highly virulent isolates of *C. cassiicola* were recovered from diseased cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* L.) and perilla (*Perilla frutescens* (L.) Britton) plants, showing typical target leaf spot symptoms from Japan, which represent different geographical regions. In previous years, these isolates were identified as *C. cassiicola* by morphological criteria[1]. A single microconidial culture was prepared from each isolate and maintained on Potato Dextrose Agar (PDA) medium (potato, 250 g; dextrose, 20 g; agar, 15 g; and distilled water, 1000 mL; pH 7.0).

Pathogenicity tests: In this test, conidial suspensions (10^4 and 10^5 conidia mL^{-1}) of *C. cassiicola* were prepared from 3-week-old cultures grown on PDA. Detached, young leaves of tomato, cucumber and perilla plants were slightly wounded crosswise at the center of the lower surface with a needle and treated with 25 μL droplet of the spore suspension. The leaves were kept on sponge mats in a moist chamber for 3 days at 25°C and were assessed for necrosis.

RAPD-PCR analysis: From the resulting sequence, the following primers were developed. 5' GGAAGTAAAAGTCGTAACAAGG 3' and 5' GCTGCGTTCTTCATCGATGC 3'(Operon Technologies, Inc., Alameda, CA) which had been found to generate banding patterns capable of differentiating *C. cassiicola* isolates (cucumber pathotype, C95; perilla pathotype, PC90; and tomato pathotype, LC93).

Genomic DNA extraction: Total genomic DNA from *C. cassiicola* isolates was prepared using the mini-prep method of Lee *et al.*[16]. DNA was extracted from 30 mg of freeze-dried mycelium in 400 μL of Yoder' s extraction buffer (100 mM LiCL, 100 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.5 L Sodium Dodecyl Sulphate (SDS), 60 mL sterile distilled water) at 45°C for 1 h in 1.5 mL Eppendorf tubes. The solution was extracted twice with 200 μL of (1:1:1) phenol: Yoder's buffer: chloroform and 200 μL chloroform was added, mixed and centrifuged at 13 000 x g for 10 min. The supernatant was removed and DNA precipitated by the addition of 0.1 volume of 3 M sodium acetate and 750 μL of 120°C absolute ethanol followed by centrifugation (HIMAC CT13, HITACHI Co., Jp) as above for 20 min at 4°C. DNA pellets were washed in 70%ice-cold ethanol and resuspended in 100 μL of TE buffer (Tris-HCL 10 mM, EDTA 1 mM, pH 8).

PCR amplification: PCR amplification was performed in 19 μL volumes containing; 11 μL sterile distilled water; 2 μL of Ex *Taq* DNA polymerase buffer (Takara, Jp); 1.8 μL of deoxyribonucleoside triphosphate (dNTP); 1 μL of previous amplified primer; 0.2 μL of Ex *Taq* DNA polymerase (Takara, Jp); and 3 μL of template DNA. Incubation was carried out on a Thermal cycler (Takara, Jp) at 94°C for 5 min followed by 35 cycles of 0.20 min at 95°C (melt), 0.30 min at 62°C (anneal) and 1 min at 72°C (extension) with a final cycle at 94°C for 5 min. The amplification products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized by a digital imaging system (Image Saver AE-6905C, ATTA Bioinstrument, Seikosha, VP1500). RAPD tests were repeated twice and confirmed to be reproducible. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band, respectively. Dendrogram was constructed from the similarity coefficient data using the BLASTX 2.1.1 program by the Unweighted Pair Group Method with Aritmetic Average (UPGMA).

RESULTS AND DISCUSSION

Pathogenicity tests: In this trial, infection tests confirmed the virulence of all *C. cassiicola* isolates which produced slight symptom (±) and severe symptom (++) on different host plants under controlled conditions (Table 1). High inoculum density (10^5 conidia mL^{-1}) exhibited more typical target leaf spot symptoms compared with other inoculum density (10^4 conidia mL^{-1}). Water–soaked lesions beneath the inoculum droplet were observed 48 h after inoculation of different host plants. All isolates of *C. cassiicola* were pathogenic. *C. cassiicola* was also successfully re-isolated on PDA.

Table 1: Pathogenicity of *C. cassiicola* isolates on different hosts in a moist chamber

	Inoculum density (conidia mL^{-1})	
Hosts	10^4	10^5
Cucumber	++[1]	++
Tomato	+	++
Perilla	±	+
Control	NT[2]	NT

[1]Toxicity was evaluated as 0 = no toxicity (-), 1 = slight symptoms (±), 2= moderate symptoms (+) and 3=severe symptoms (++), [2]NT, No toxicity

RAPD-PCR analysis: Results based on RAPD analysis provide clear genetic evidence of at least three well characterized groups among the tested isolates of *C. cassiicola* from tomato, cucumber and perilla plants. This is the first characterization of target leaf spot of different host plants. This study also characterized isolates of *C. cassiicola* causing target leaf spot on various species of host plants. Of the 8 random primers tested, two were selected that generated reproducible amplification pattern using three isolates representing each pathogen. All amplified fragments were scored and given numbers based on their sizes. RAPD-PCR also amplified reproducible polymorphic bands. A total of 64 bands were scored, 12 of which were polymorphic. The number of polymorphic bands generated by each primer varied from one to four. Amplification was identical in two separate experiments. Typical amplification results from two primers for these isolates are shown in Fig. 1. RAPD analyses revealed that the amplification products were similar among isolates of each pathotype from the different host plants. Identification of this economically important pathogen is a lengthy and time-consuming process involving a combination of pathogenic testing and conidial morphology analysis. In many phytopathogenic fungi, RAPD analysis has proved useful for detecting genetic polymorphism directly related to host specialization. Cluster analysis separated the *C. cassiicola* isolates into 3 groups. Figure 2 represents the resulting dendrogram showing relationships among *C. cassiicola* isolates. RAPD-PCR analysis has already been used effectively to characterize plant pathogenic fungal isolates from different host plants[17]. Results obtained for *C. cassiicola* were in good agreement with those reported for other host of *C. cassiicola*[2], by means

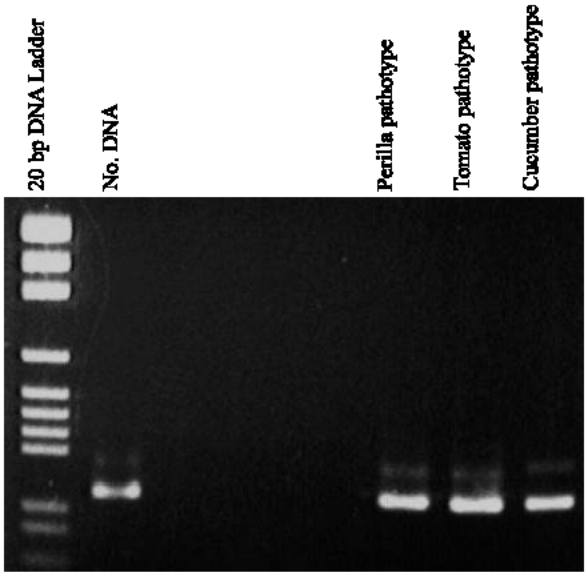

Fig. 1: Random amplified polymorphic DNA (RAPD) patterns of *C. cassiicola* isolates including Lane 1=20 bp ladder, perilla pathotype, PC90; tomato pathotype, LC93 and cucumber pathotype, C95, left to right

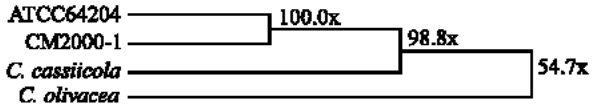

Fig. 2: Dendrogram derived from unweighted pair-group method with arithmetic average cluster analysis (UPGMA) of random amplified polymorphic DNA (RAPD) data obtained with two primers of 3 *C. cassiicola* isolates, using software BLASTX 2.1.1. Top scale is percentage similarity based on the Jaccard similarity coefficient

of a PCR-based quantitative approach. On the other hand, minor results contradict with other reports. However, because of the random nature of RAPD markers, the reproducibility of the technique might be influenced by several factors including the source and procedure used for DNA isolation, the occurrence of contaminants, the amplification of different DNA sequences of the same size, etc.[18]. Consequently, these studies reveal that RAPD analysis may be a useful molecular tool in detection and phylogenetic studies of pathogenicity studies of *C. cassiicola* isolates.

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