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Assessment of genetic diversity in Colletotrichum capsici using Randomly Amplified Polymorphic DNA

KEYWORDS: Colletotrichum capsici, Pathogenicity test, DNA isolation, RAPD analysis.

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ABSTRACT *Present study was undertaken to estimate genetic diversity in Colletotrichum capsici causing anthracnose disease on chilli fruits, using Randomly Amplified Polymorphic DNA (RAPD). In this study collect 12 isolates of Colletotrichum capsici from different sites and grow in PDA media. After pathogenicity test fungal pathogens were inoculate in PD broth. Mycelium mat form that harvest after 14-21 days and DNA was isolate. RAPD analysis after PCR amplification. Six RAPD primers were use to amplify genomic DNA of 12 Colletotrichum capsici isolates. DNA fragments of various sizes ranging from 250 to 4000 bp were amplified. The isolates were divided into two major clusters based on dendrogram construction from UPGMA clustering. Some pathogen cluster were to be pathogenically diverse and the diversity seems to be due to continuous generation of molecular characterization. The analysis of pathogenic cluster according to geographic distribution of the pathogenic population.*

Introduction

Colletotrichum capsici is one of the most important plant pathogen of chilli pods in India (Bailey and Jeger,1992). Colletotrichum capsici is a plant pathogen causing anthracnose disease in chilli fruits in tropical and subtropical conditions, resulting in qualitative and quantitative losses (Cannon et al., 2012, Noireung et al.,2012). The anthracnose of chilli has been shown to be caused by Colletotrichum capsici. The anthracnose is one of serious diseases on chili to cause the yield loss and to reduce the quanlity of marketable fruits. Typical anthracnose symptoms seen on premature chilli fruit include sunken necrotic tissues with circular concentric rings of acervuli. Fruits showing blemishes have reduced marketability (Manandhar et al., 1995). The use of differential hosts were a viable option for the evolution of pathogenic variability and morphological diversity. The symptoms of anthracnose invasion are sunken necrotic lesions on fruit (Waller et al. 2012; Agrios 2005). The anthracnose lesions on chilli fruit reduced their market able value (Manandhar et al., 1995).

Colletotrichum capsici infecting different hosts have a high degree of pathogenic variability that has to be assessed for effective development of resistance. Colletotrichum capsici appeared to be the most severe being able to infect a range of Capsicum species and resistant genotypes (Taylor, 2007). Colletotrichum capsici infecting diverse hosts have a high degree of pathogenic variability (Sharma et al., 1999). The use of differential hosts is a viable option for the evaluation of pathogenic variability. Disease symptoms developed only on fully ripened pods or wound chilli fruits. The symptoms typically occur on fruits at maturation under wet autumn conditions appear as dark sunken lesions with abundant production of orange masses of conidia (Williams et al. 1990). Colletotrichum capsici is cosmopolitan with either multiple species occurring on a single host or a single species occurring on multiple hosts (Sander & Korsten, 2003). Fungus-host relationships are broad, imprecise and often overlapping (Freeman & Shabi; 2000). Colletotrichum capsici is infect many hosts and may adapt to new environments (Sanders&Korsten 2003, Photita et al. 2004), leading to serious cross infection problems in plant production.

The development technologies have revolutionized the utilization of molecular markers in molecular study (Ratalski et al., 1996). Molecular methods have been employed successfully to differentiate between populations of Colletotrichum capsici from many hosts in general, according to the study of Agwanda et al. (1997), which the RAPD (random amplified polymorphic DNA) technique has been used to identify of RAPD markers for resistance in chilli. Furthermore, Sharma et al. (2005) studied on the genetic relationship between five morphological groups recognized within Colletotrichum capsici by using RAPD analysis. Molecular polymorphism generated by RAPD confirmed the variation in virulence of Colletotrichum capsici the cause of anthracnose of chillies, and different isolates were grouped into two major clusters (Sharma et al., 2005). Present study was conducted to analyze genetic diversity in Colletotrichum capsici isolates using RAPD molecular marker.

Materials and methods

Isolation of Pathogens and identification of Colletotrichum capsici

Anthracnose infected chilli pods were collected from different fields situated in Uttar Pradesh and Bihar in India(Table 1.1). The diseased part of the fruits were cut at advanced margin of lesions into small pieces (5mm diameter). The small pieces were surface sterilized in aqueous solution of mercuric chloride (0.1:100w/v), streptocycline and transferred in PDA after washout. Spore masses were picked up with a sterilized wire loop and streaked onto the surface of water agar (WA) plates which were incubated over night. The spore of Colletotrichum capsici 13.21- 16.21 µm long & 1.79 – 3.28 µm wide. A single germinating spore was picked up with a sterilized needle and transferred onto Potato Dextrose Agar (PDA). Pure culture was stored at 4°C (Yang et al.,2009).

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The Colletotrichum capsici isolates were identified by using key given by Gunnels & Gabbler (1992). Colletotrichum capsici identification was based on morphological characters such as size and shape of conidia and aspersoria, existence of setae or presence of a teleomorph, and cultural characters such as colony colour, growth rate and texture (Smith & Black, 1990). These criteria alone are not always adequate to differentiate c. capsici because of variations in morphology and phenotype among c. capsici under different environmental conditions. The C. capsici produce grey white scattered, falcate conidia black acervuli, and non uniform shape of mycelium. The C. capsici formed smooth circular margin in the PDA and OMA. The grey whitish mycelium of C. capsici gradually developed from the second day in culture of isolates. (Yun et al., 2009).

Pathogenicity tests:
Pathogenicity tests were done separately for each isolate on host plant using plug inoculation method following a modified protocol by Sanders and Kirsten (2003). Plugs (5 mm diameter) were cut from actively sporulating areas near to colony periphery by using a sterilized cork borer & inoculate on fruit. The inoculated fruits were kept in moist chamber at room temperature (27±2°C) for 10 days.

Harvesting of fungal mycelium and DNA isolation:
Fungal mycelium mat of C. capsici was harvested after 6 - 10 days of inoculation in Potato Dextrose Broth at 27 ±2oC, for molecular characterization. The mycelium mat was stored at - 80°C for long term storage. The total genomic DNA were extracted using modified protocol originally developed by Sambrook et, al 2001.

0.5g mycelium was ground with liquid nitrogen using mortar & pestle to form powder. Mycelium was suspended with DNA extraction buffer (1000 µl) shaken properly in eppendorf tube & incubated it at 65°C in water bath for 1 hrs with shaking time to time. It was centrifuged for 10 minutes at 8000 rpm at room temperature. Taken supernatant & added equal amount of chloroform isopropoamyl (CI) then centrifuged for 10 minutes at 10000 rpm. Isolate aqueous layer & added ethanol 100%. Centrifuged 15 minutes 12000 rpm at 4°C. Supernatant discord & added chilled ethanol 70% in equal amount for wash. Discord supernatant & dry pallet in laminar air flow for 1-3 hrs added 100 µl sterilized distilled water in dry DNA pallet & for dissolve then quality check to 0.8 % agarose gel.

Selection of primers:
Selection of primer after quality test of DNA. A total of 6 primers (Operon Tech. Inc., USA) were used for RAPD analysis(figure1.2). DNA fragments of various sizes ranging from 250 – 4000 bp were amplified in different isolates.

PCR amplification
PCR amplification of DNA polymerase was followed by using a reaction mixture of 25 µl volume consisting of 2.5 µl of 10x PCR buffer, 1.0 µl of dNTPS, mix (dATP, dTTP,dGTP, and dCTP), 1.0 µl Taq DNA polymerase, 1.0 µl of primer, 1.0 µl $MgCl_2$, 1.0 µl (10 ng) genomic DNA and 17.5 µl of sterilized double distilled water. The reaction mixture was vertex and centrifuged for 30 seconds in microfuge for proper amplification. Amplification was carried out in a thermal cycler by using three temperature profiles programmed for initial DNA denaturation at 94°C for 5 minutes followed by 40 cycles consisting of DNA denaturation for 1 minutes at 94°C, Primer annealing at 37°C for 1 minutes and polymerization for 2 minutes at 72°C with a final extension period for 5 minutes at 72°C. PCR mixture supplemented with sterilized distilled water instead of template DNA was kept as control (Sambrook et, al 2001).

Initially,90 10 mer random primers (Shanghai Sangon Biological Engineering Tech. & Service Co. Ltd.) were screened to select 6 primers of them which produce easily scorable and consistent banding patterns and were used for RAPD analysis of test isolates. 1.2 % agarose [stained with ethidium bromide @ 4µl (10 µg) and photographed were taken by using gel documentation system 4 µl (10ng/ml)] in 1x TAE buffer(Sharma et al., 2005).

RAPD analysis :
RAPD analysis conducted in this study showing less variation in C. capsici isolates. Result from the dedrogram showed a clear distinction between thereby supporting the study was made by Sharma et al. (2005) which reported that the phylogenetic grouping of C. capsici causing anthracnose of chillies in the north-western region of India based on RAPD data did not appear to be congruent with morphological and virulence pattern. RAPD-based DNA fingerprinting methods of studying genetic diversity in C. capsici in the absence of a definite differential set in the RAPD analysis. Bands were scored as present (1) or absent (0) to create a binary matrix. The bivariate 1–0 data were used for estimation of genetic distance (GD) following unweighted pair group of Arithmetic mean (UPGMA) procedure described by Nei and Li (1979). Estimation of average genetic distances (GD) using RAPD primers ranged from 0 –100.

Gel electrophoresis:
PCR products were monitored by staining with ethidium bromide on 1.2% agarose electrophoresis gels in 1×TAE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) at 70 V for 45 min. at room temperature and visualized under UV light and photographed using gel documentation system (GeneSnap Ver 6.03, Syngene Laboratories, Cambridge, United Kingdom). as described by Sambrook (Sambrook et al., 1989). The size of amplified DNA regions was estimated by comparison with Gene Ruler 1 Kb DNA ladder and Gene RulerTM 100 bp kb DNA ladder (Fermentas Co. Biosyntech Sdn Bhd, Selangor, Malaysia). DNA was quantified by ethidium bromide fluorescence on a UV transilluminator with known quantities of lambda DNA (Sambrook et al., 1989).

Results and Discussion
PCR amplifications of total genomic DNA using primer pairs listed in Table1.2 produced a single PCR product with specific size for C. capsici.

Molecular characterization
The RAPD-PCR was performed using selected primers based on the results of initial screening of a group of primers against a set of representative studied isolates. The RAPD primers were used and found to produce good quality banding patterns. RAPD primers produced multi band patterns for each of the isolates, and examples of RAPD markers generated by the primers are shown in Figure1.1.Most of the amplification products were reproducible and a total of 106 score-able markers were generated from the amplified products, 2 of which were monomorphic for all taxa. All implications were repeated twice, and both faint and intense bands were scored if shown to be reproducible in separate runs. The average number of

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bands per primer was 15 which ranged in size from 100 to 2700 bp.

Jaccard's similarity coefficient ranged from 0.12 – 0.25. RAPD markers have been used for the interspecific characterization of a number of pathogen. A total of 429 bands were obtained with 6 primers, out of 38 primers and dendrogram was drawn from the RAPD data using UPGMA (Unweighed Pair Group Method with Arithmetic Mean).

Cluster Formation

At the similarity coefficient value of approximately 72% all of the 12 isolates can be divided into two major clusters (major cluster I and major cluster II) for cluster formation. According to the dedrogram of Colletotrichum capcici that can be divided into two main groups (Figure1.2). The first group includes seven isolates while second group contains the other 3 isolates of Colletotrichum capsici.

Major cluster I

The major cluster-I has seven isolates (Cc1,Cc2,Cc3Cc4,Cc5,Cc6 and Cc7) grouped together. These cluster have 45 % similarities coefficient which characterized in two minor cluster viz. minor cluster-I and minor cluster-II. The minor cluster-I have out of seven cluster in which three isolates were include while in the minor cluster-II have out of seven isolates four isolate include. All the isolates were Colletotrichum capsici.

Major cluster II

The major cluster-II has five isolates (Cc8, Cc9,Cc10,Cc11,Cc12) grouped together. These isolates characterized in four cluster in which first and second cluster having two isolate but the minor clusterCc5 have single isolate.

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Table.1.1:Different location where's sample uses in genetic diversity identification

Sr No	Isolates No	Districts
1	Cc1	Banaras
2	Cc2	Ambedkar Nagar
3	Cc3	Bhuwaneswer
4	Cc4	Azamgarh
5	Cc5	Bihar
6	Cc6	Mirzapur
7	Cc7	Nagpur
8	Cc8	Sultanpur
9	Cc9	Faizabad
10	Cc10	Barabanki
11	Cc11	Jaunpur
12	Cc12	Gazipur

Table.1.2:Name of 6 primer which use in RAPD analysis

Sr.No	Name of Primers	Length of Primers
1	OPS-3	(5′-CAGAGGTCCC-3′)
2	OPS-4	(5′-CACCCCCTTG-3′)
3	OPS-7	(5′-TCCGATGCTG-3′)
4	OPS-10	(5′-ACCGTTCCAG-3′)
5	OPS-18	(5′CTGGCGAA CT-3′)
6	OPV - 12	(5′-ACCCCCCACT-3′)

Fig.1.1: Agarose gel showing RAPD fragments with OPV-12 RAPD primers and 12 isolates of C. capsici

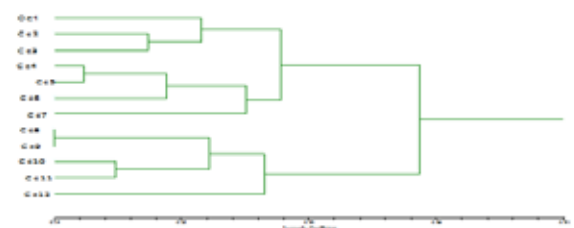

Fig.1.2: Dendrogram constructed (Jaccord similarity coficients) for 12 C. capsici genotypes using data obtained from PCR using to RAPD primers.

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