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Molecular characterization of *Colletotrichum capsici* causing anthracnose of chilli

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Abstract
The Random Amplified Polymorphic DNA (RAPD) technique was employed to detect the genitical variation among the four isolates of *Colletotrichum capsici.* Out of 20 randomly selected RAPD primers screened, 16 primers produced 117 scorable bands. Among the RAPD primer 117bands 100 bands were polymorphic and level of polymorphism was 85.47%. The result from the UPGMA analysis based dendrogram generated for *Colletotrichum capsici* isolated revealed that they were divided into two main clusters, cluster I and II. The highest genetic similarity to an extent of 49.50% was recorded between Cc1 and Cc2 isolates and the least genetic similarity that is 29.30% noticed between Cc2 and Cc3.Cc3 is distinct and showed least similarity with all other isolates of *Colletotrichum capsici*. Cc3 may be present in temprate region (Western Himalayan Region).

Keywords: Chilli, *Colletotrichum capsici,* RAPD, isolates

Introduction
The Chilli (*Capsicum annuum* L.) is one of the most popular and widely grown vegetable in the world and most popular in Asia (Makari *et al.,* 2009) [5] originated from tropical America belongs to solanaceae family. Anthracnose disease is one of the most economically important diseases reducing marketable yield from 10 to 88% of the crop production in some developing countries (poonpolgul and Kumphai, 2007) [9]. Anthracnose causing severe losses due to both pre- and post-harvest fruit decay is caused by *Colletotrichum* species. Chilli can be cultivated year round however; kharif crop is most probably grown. Therefore, seasonal disease occurrence is an essential for management strategies. The isolates of *C. capsici* collected from different geographical situations may be helpful for understanding the pathogenic nature existed within the isolates of *C. capsici* for management of disease. The genetical variation among the isolates of *C. capsici* may also be detected with the help of molecular technique RAPD using specific primers. These studies will show the genetical variability among the isolates of *C. capsici* collected from different geographical areas. The molecular characterization of *C. capsici* is necessary for plant breeding purposes and also for developing effective disease control strategies. To overcome taxonomical problems associated with traditional identification methods, molecular markers have been applied to *C. capsici* diversity studies. Molecular approaches such as random amplified polymorphic DNA (RAPD), and sequence analysis of the internal transcribed spacer (ITS), have proved useful in studying phylogenetic relationships of *Colletotrichum* species because of their comparative variability (Photita *et al.,* 2005) [8].

Materials and Methods
Isolation and identification
The culture of the isolates of *Colletotrichum capsici* thus obtained from the IARI, New Delhi were maintained on Potato Dextrose Agar (PDA) media. The mycelium hypha picked up from the fungal growth was transferred on fresh PDA media and incubated at 27 ± 2°C for 7 days to obtain pure culture The 4 isolates were designated as cc1, cc2, cc3 and cc4. Pure cultures of isolates were stored at 4°C for storage and subsequent studies. *Colletotrichum capsici* was confirmed in all isolates by comparing their morphology characters and molecular confirmation was done by using Internal Transcript Spacer (ITS) forward (ITS 1) and reverse (ITS 4) markers.

DNA isolation

For DNA isolation, Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with two 1-cm discs of actively growing cultures of *Colletotrichum capsici*. The cultures were placed on a rotary shaker (100 revs min-1) and incubated at 27°C for 2-3 days. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction. Total genomic DNA was isolated from the fungus following the CTAB method (Murray and Thompson 1980) [7]. The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel (containing ethidium bromide @ 0.5 mg/ml) in a horizontal gel electrophoresis system.

PCR amplification and electrophoresis

Twenty Random Amplified Polymorphic DNA (RAPD). Primers of OPA and OPB series which indicated a higher degree of polymorphism, were selected for the diversity analysis studies and ITS1 and ITS4 were used for Molecular confirmation of *Colletotrichum capsici.* Sterile PCR tubes were numbered and placed on PCR tube stand. At first 2 µl of DNA was added to each PCR tube followed by master mix. The samples were mixed by brief centrifugation to bring down the content of tube. PCR were run on the programmable thermal cycler Amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for 35 cycles at a temperature regime of 94^0C for 30 seconds, 50^0C (Annealing temperature is varied from primer.) for 45 seconds, 72^0C for 1 minute after an initial denaturation at 94^0C for 2 minutes. Following the cycling, the mixture was incubated at 72^0C for 10 minutes and then kept at 4^0C hold till electrophoresis. PCR products were separated by electrophoresis in 2 per cent agarose gels run in 1X TBE, stained with ethidium bromide and visualized with a UV trans illuminator.

Data analysis

Data were scored as the presence (1) or absence (0) of the individual band for each isolate. This binary data was used to compute the similarity coefficient using the Jaccard similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc). The similarity matrix was used to construct the dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarchal Nested) cluster analysis module (Rohlf, 2000) [10].

Results and Discussion

The investigation was carried out in the laboratory of the Department of Plant Pathology, Dr. PDKV, Akola. The four isolates of *Colletotrichum capsici* were collected from ITCC (IARI New Delhi.).

Molecular confirmation with ITS-1 and ITS-4

The isolates of *Colletotrichum capsici* were obtained from different location of India. The four isolstes viz., Cc1, Cc2, Cc3 and Cc4 were selected for the analysis. The *Colletotrichum capsici* specific ITS primer pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used for molecular confirmation of the isolates. All the isolates of *Colletotrichum capsici* yielded the 520 bp band with the ITS marker, hence confirm the culture of *Colletotrichum capsici* were as per the result of Adhipath *et al*. (2013) [1].

Molecular characterization with RAPD primers

The molecular technique, RAPD was employed to detect the genetical variation among the four isolates of *C. capsici* causing anthracnose in chilli. Total 20 primers falling in OPA and OPB series group were used to asses molecular variation. Among 20 randomly selected primers screened, 16 primer produced 117 scorable bands. Among 117 bands 100 bands were polymorphic and level of polymorphism was 85.47%. The genetic similarity coefficient value ranged from 0.29 to 0.49 across four isolate of *Colletotrichum capsici*. The highest genetic similarity to an extent of 49% was recorded between Cc1 and Cc2 isolates followed by Cc1 and Cc4 isolates 44.20% similarity. Least genetic similarity was observed that is 29% between Cc2 and Cc3.

The dendogram constructed from the pooled data indicated that, there were two major clusters I and II. Cluster I consisted of two sub-cluster i.e IA and IB. IA grouped into two isolates Cc1 and Cc2 which were in one cluster showed 49% similarity. IB includes one isolates Cc4. While cluster II consist of only one isolate Cc3 which is distinct and showed least similarity with all other isolate of *C. capsici.* As the Cc3 was present in temprate region (Western Himalayan Region.)

Conclusions

The molecular variability was studied among the 4 isolate of *Colletotrichum capsici* by using 20 RAPD primers of OPA and OPB series. Among 20 primers screened 16 primers produced reproducible and scorable bands with high degree of polymorphism. Out of these 16 primers, 8 primers showed all polymorphic bands, whereas remaining 8 primers namely OPA-2, OPA-4, OPA-5, OPA-9, OPA-10, OPA-12, OPA-13 and OPB-20 each showed on monomorphic band. A total 117 amplicons were obtained with the 16 primers. Out of 117 bands, 100 were found to be polymorphic and the level of polymorphism was 85.47 per cent.

Acknowledgement

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Table 1: Details of the different isolates of *Colletotrichum capsici* from ITCC

S. No.	ITCC No.	Source	Agro ecological zone	Code Name
1.	2041	Nagpur	9 Western Plateau and Hills	Cc -1
2.	6307	Navsari	13 Gujrat Plains and Hills	Cc -2
3.	6078	Jammu	1 Western Himalayan Region	Cc -3
4.	6938	Umiam	2 Eastern Himalayan Region	Cc -4

Table 2: Primers selected for PCR with their sequence code

Sr. No.	Primers	Primer code
1	OPA 1	CAGGCCCTTC
2	OPA 2	TGCCGAGCTG
3	OPA 3	AGTCAGCCAC

4	OPA 4	AATCGGGCTG
5	OPA 5	AGGGGTCTTG
6	OPA 6	GGTCCCTGAC
7	OPA 7	GAAACGGGTG
8	OPA 8	GTGACGTAGG
9	OPA 9	GGGTAACGCC
10	OPA10	GTGATCGCAG
11	OPA11	GGACTGGAGT
12	OPA 12	TCTGTGCTGG
13	OPA 13	CAGCACCCAC
14	OPA15	TTCCGAACCC
15	OPA18	AGGTGACCGT
16	OPA19	CAAACGTCGG
17	OPB 6	TGCTCTGCCC
18	OPB 15	TGGGCCCTTC
19	OPB-17	AGGGAACGAG
20	OPB-20	ACTTCGCCAC

Table 3: Similarity coefficient for RAPD analysis

Isolates	Cc-1	Cc-2	Cc-3	Cc-4
Cc-1	1			
Cc-2	0.495	1		
Cc-3	0.391	0.293	1	
Cc-4	0.442	0.409	0.305	1

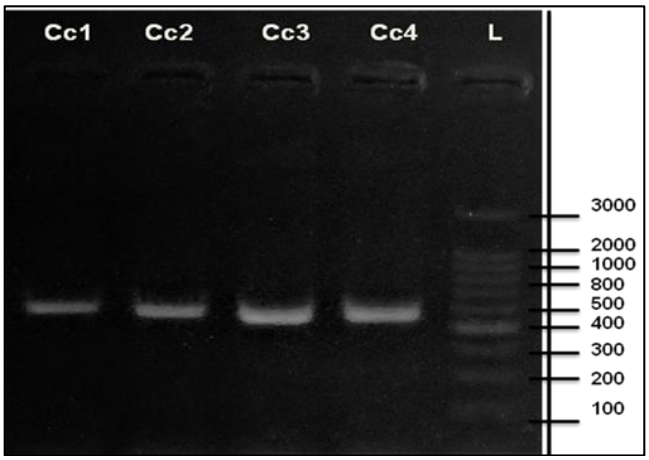

Plate 1: Banding pattern of ITS primer

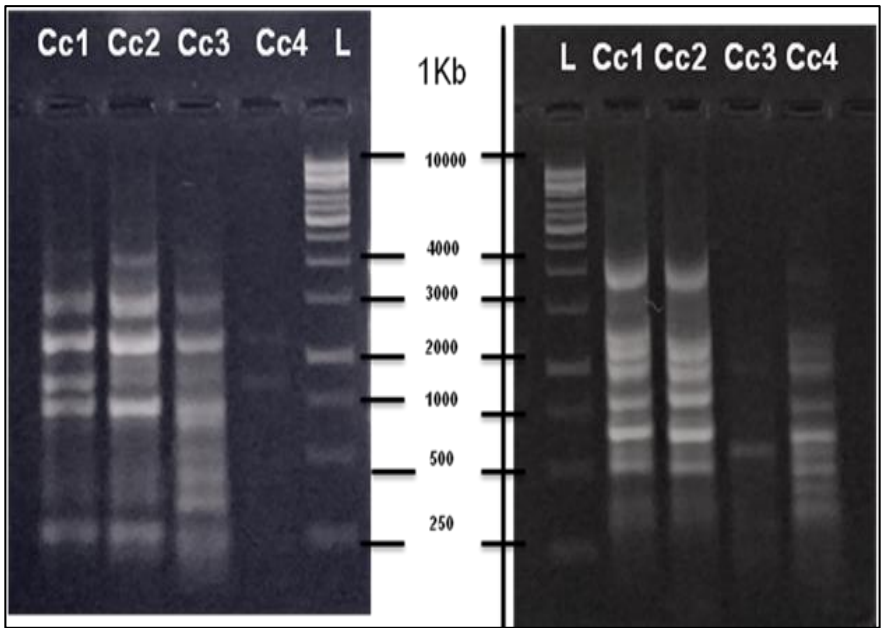

Plate 2: RAPD banding pattern of primers OPA9 and OPA10

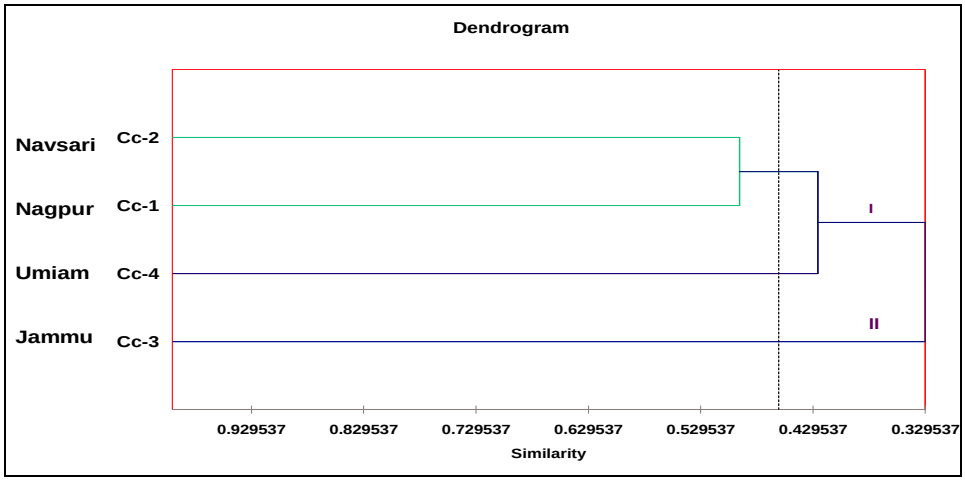

Fig 1: Dendogram based on RAPD analysis of four isolates of *C. capsici* causing anthracnose of chilli

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