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Molecular diversity and pathogenic variability in *Colletotrichum capsici* of chilli (*Capsicum annuum*) in Haryana

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ABSTRACT

The fruit rot of chilli (*Capsicum annuum* L.), caused by *Colletotrichum capsici*, is the most serious disease of chilli in India. Fourteen isolates of *C. capsici* collected from different locations in Haryana state were investigated for molecular variations along with their virulence pattern. Different levels of virulence were expressed dividing isolates into four groups. Out of 33 primers, 22 exhibited DNA polymorphism producing a total of 108 bands with an average of 4.90 bands per primer. At an arbitrary cut-off at 45% similarity level of dendrogram, the isolates were clustered into two main clusters. The grouping of the isolates based on RAPDs was in general neither in agreement with their pathogenicity on different host cultivars nor with the geographical location of the isolate. The information may be helpful in improving chilli fruit rot resistance and varietal deployment strategies essential for effective disease management.

Key words: Chilli, *Colletotrichum capsici,* Fruit rot, RAPD

Chilli (*Capsicum annuum* L.) is an important tropical and subtropical vegetable, low in sodium, rich in vitamins A and C and good source of potassium, folic acid and vitamin E. Anthracnose fruit rot caused by *Colletotrichum capsici* (Sydow) Butler and Bisby, is one of the most destructive disease causing severe damage on red fruits. Symptoms include dark, sunken necrotic tissues, with concentric rings of acervuli on leaves, stems and fruits. Sharma *et al.* (2005) reported existence of 15 pathotypes of *C. capsici* based on disease symptoms on inoculated fruits. Sangdee *et al.* (2011) classified 10 isolates of *C. capsici* into highly resistant (<40% inhibition) and highly sensitive group (>90% inhibition).

It is noteworthy that there is not a single resistant pepper variety available commercially, anywhere in the world (Oh *et al*. 2007) and AVRDC started the well-organized efforts on this disease. New races of *C. capsici* have caused severe breakdown of resistance in many chilli varieties and Khirbat *et al.* (2004) reported four different strains on the basis of their pathogenic behavior on four chilli varieties.

Application of molecular tools promises great opportunity to clarify the genetic relationships of phytopathogens. SSR are robust markers for molecular characterization but lack of sequence information in *C. capsici* does not allow use of these markers. Diversity studies using internal transcribed spacer region have been done in the past (Sanders and Korsten 2003, Lee *et al*. 2007). Shin *et al*. (2000) used RAPD markers to detect variation among four species of *Colletotrichum* isolated from chilli growing areas in Korea and China. RFLP were also used to study variation among *Colletotrichum* species (Balardin *et al*. 1999, Martin and Garcia-Figueres, 1999 and Sheu *et al.* 2007. Ratanacherdchai *et al*. (2010) used ISSR for comparison of cross-inoculation potential of *C. capsici.*

The Haryana state has vast scope for chilli growing and marketing. The present study was conducted to assess genetic diversity of fungus isolates collected from different regions of Haryana state using RAPD analysis and reaction on host genotypes.

MATERIALS AND METHODS

Fourteen isolates of *Colletotrichum capsici* were sampled from different locations of Haryana. Fresh chilli fruits showing symptoms of fruit rot were examined microscopically and diseased portions were cut into small pieces. Spore isolations were done after surface sterilizing the pieces with 0.1% mercuric chloride for 30 seconds and than rinsing 3-4 times in sterile distilled water. These were then placed on oatmeal agar slants and incubated at 28 $\pm 1^0$ C for 7 days. Cultures were purified through single spore isolations.

For testing the pathogenicity of isolates, healthy red chilli fruits of variety Pusa Jawala were surface sterilized with 0.1% mercuric chloride and kept at 28° C for 48 hours.

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Fruits were then inoculated by pin prick method (Muthulakshmi 1990) and incubated at 28 ±1^{0}C. The pathogen was reisolated and characters were compared with the original one.

The red ripe fruits were collected from 105 days old plants of six varieties (Table 1). These were surface sterilized with absolute alcohol, washed with sterile distilled water and inoculated with the spore suspension of different isolates (3×10^4 spores/ml) by pin prick method as suggested by Muthulakshmi (1990). Inoculated fruits were incubated at 28± 1°C with 100% relative humidity for fifteen days. The percentage of diseased area of fruits was determined and disease rating scale (Jeyalakshmi 1998) was used for differentiation of the isolates. The disease ratings were then categorized into disease reaction as listed below-

0-1, Resistant (R); 1.1-2.0, Moderately resistant (R^+); 2.1-3.0, Moderately susceptible (S^1); 3.1-4.0, Susceptible (S); 4.1-5.0, Highly susceptible (S^+).

Further to distinguish the isolates clearly, these were grouped into Resistant (R, R^+) and Susceptible (S^1, S, and S^+) categories.

The total genomic DNA of different isolates of *C. capsici* was extracted from mycelia using Murray and Thompson method (1980). Genomic DNA was purified by RNase A treatment. The extracted DNA was run on 0.8% agarose gel to check the quality and was quantified using spectrophotometer.

Thirty three random decamer primers from Operon Technologies Inc. CA, USA were screened to compare genetic variability among fourteen isolates of *C. capsici.* PCR reaction mixture consisted of 20 ng of template DNA, 1 U Taq polymerase, 25 mM $MgCl_2$, 10 mM dNTPs and 10 mM of primer. Reactions were run in Eppendorf Thermocycler using following programme: initial denaturation of 3 min at 94°C following 35 cycles at 94°C for 30 seconds, 35°C for 30 seconds, 72°C for 1 minute and a final extension step of 72°C for 10 minutes.

The amplified PCR products were resolved on 1.5 per cent (w/v) agarose gels. The bands were visualized under UV Trans-illuminator and photographed using Bio-Rad Gel Documentation system.

RAPD analysis was based on the binary values '1' for presence or '0' for absence of bands. The mean similarity values of the fourteen isolates were calculated from the total amplified products of 33 primers by using NTSYS-PC version 2.01. Jaccard's coefficient was used to construct a dendrogram using unweighted pair group method with arithmetic means (UPGMA).

RESULTS AND DISCUSSION

Pathogenicity studies

Virulence studies provide a basic insight into fungal identification and evolutionary development. Six different chilli varieties were inoculated with different Haryana isolates of *C. capsici* and pathogenic variability was evaluated on standard differentials (Sharma 2005). Data on differential interaction between host genotypes and isolates of *C. capsici* (Table 1) indicated that isolate C14 collected from Faridabad area of Haryana state constituted a distinct group as it gave susceptible reaction on varieties Hisar Vijay, Hisar Shakti, CH-1, CH-3, Pusa Jawala and Punjab Surkh. The isolates C1, C2 and C3 showed susceptible reaction on varieties Hisar Vijay, Pusa Jawala and Punjab Surkh. However, the isolates C4, C5, C6, C7, C8 and C9 showed susceptible reaction on varieties Hisar Shakti, Punjab Surkh and Pusa Jawala. The remaining isolates C10, C11, C12 and C13 exhibited resistant reaction on all the six varieties. Although these isolates were collected from wide apart locations of Haryana state, still they showed same pathogenic behavior indicating no correlation between geographical location and virulence pattern.

Based on the reaction of two varieties, viz Hisar Vijay and Hisar Shakti, which showed resistance under disease stress conditions, all isolates were divided into 4 groups (Table 2). Isolate C14 constituted a distinct group as it gave susceptible reaction on varieties Hisar Vijay and Hisar Shakti. In second group C1, C2 and C3 induced susceptible reaction on Hisar Vijay and resistant on Hisar Shakti. Whereas, isolates C4, C5, C6, C7, C8 and C9 which gave susceptible reaction on Hisar Shakti and resistant on Hisar Vijay constituted third group. Remaining isolates (C10, C11, C12 and C13) induced resistant reaction on all the six varieties and constituted fourth group. Two isolates from Karnal, viz. C7 and C8 showed same virulence pattern, whereas Jind isolates (C4 and C11) exhibited different virulence pattern, showing susceptible and resistant reactions respectively. Than *et al*. (2008) studied pathogenic variation among 10 isolates of *C. acutatum* against seven Thai cultivars of *Capsicum*. They revealed that two pathotypes were based on qualitative differences in infection. Of the 10 isolates assayed, 5 showed complete resistance, whereas other 5

Table 1 Reaction of chilli varieties to different *Colletotrichum capsici* isolates.

Isolates	Hisar Vijay	Hisar Shakti	CH-1	CH-3	Pusa Jawala	Punjab Surkh
C1	4	1	2	2	4	3
C2	3	2	1	2	4	4
C3	4	1	2	2	3	4
C4	1	3	2	1	3	3
C5	1	3	2	1	3	4
C6	1	4	1	2	3	4
C7	1	4	2	2	4	4
C8	1	4	2	2	4	3
C9	2	4	2	2	4	3
C10	1	1	1	1	1	1
C11	1	1	1	1	1	1
C12	1	1	1	1	2	2
C13	1	1	1	1	2	2
C14	4	4	4	4	4	4

1=Resistant, 2=Moderately resistant, 3=Moderately susceptible, 4=Susceptible, 5=Highly susceptible.

Table 2 Grouping of *Colletotrichum capsici* isolates on the basis of disease reaction on two chilli varieties**.**

Variety	Isolate													
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
Hisar Vijay	S	S	S	R	R	R	R	R	R	R	R	R	R	S
Hisar Shakti	R	R	R	S	S	S	S	S	S	R	R	R	R	S

R: Resistant reaction,S: Susceptible reaction

showed susceptible reaction. Sharma *et al*. (2005) studied the pathogenic variability in *C. capsici* in India and proposed that 15 pathotypes of *C. capsici* existed among 37 isolates from from Himachal Pradesh. The studies revealed that there is continuous evolution of the fungus and its variation study is important for managing this devastating disease.

RAPD polymorphism

Out of the 33 random decamer primers screened, 22 primers generated a total of 108 bands where 103 were polymorphic and 5 were monomorphic. The total number of amplified bands varied between 1 (OPB-04) and 9 (OPQ-18) with an average of 4.9 bands per primer. Size of PCR amplified products ranged between 2200-4000 bp (Table 3). Sharma *et al.* (2007) studied the molecular diversity in *C. lindemuthianum* isolates from Himachal Pradesh using RAPD primers. Consistent with our results, they also obtained 8-12 bands with size ranging from 0.2 to 3.5 kb. Ratancherdchai *et al.* (2007) used 13 decamer primers for RAPD analysis of 18 test isolates including 2 species *C. gloeosporoides* and *C. capsici*, producing 429 bands. Their study showed the clear difference between the two species.

In the present investigation, range of polymorphism among 14 isolates was 80 to 100%. The majority of primers (19) displayed 100% polymorphism. The maximum numbers (7) of polymorphic bands were observed with primer OPA-04 and OPD-05. A representative RAPD profile obtained with the primers OPD-05 and OPQ-18 is given in Fig 1. Mohanraj *et al.* (2002) observed that out of 80 primers used, only 61 resulted in well-defined DNA polymorphism among the different isolates of *C. falcatum.* Isolate specific RAPD fingerprints were obtained by Gupta *et al.* (2010), who studied 25 isolates of *C. gloeosporioides*.

Table 3 DNA polymorphism in fourteen isolates of *Colletotrichum capsici* with twenty two amplified RAPD primers

Primer	Band size (bp)	Total no. of bands	No. of polymorphic bands	Percentage of polymorphism
OPB-4	2200	1	1	100%
OPB-6	1000-2500	3	3	100%
OPB-7	600-2500	5	4	80%
OPB-8	1000	1	1	100%
OPB-10	250-100	3	3	100%
OPB-11	600-3000	6	5	83.33 %
OPB-14	650-4000	5	5	100 %
OPB-17	1500-3000	3	3	100 %
OPB-18	300-3000	5	5	100 %
OPB-20	600-300	4	4	100%
OPA-3	500-200	5	5	100%
OPA-4	400-2000	7	7	100%
OPA-5	400-3000	4	4	100%
OPA-7	300-2000	4	4	100%
OPA-9	300-3000	5	5	100%
OPA-11	400-2000	6	6	100%
OPA-13	400-2000	6	6	100%
OPD-2	400-2000	6	6	100%
OPD-5	250-3000	7	7	100%
OPD-7	500-2000	6	6	100%
OPD-13	350-2500	7	7	100%
OPQ-18	300-2500	9	6	66.66%
Total		108	103	
Mean		4.90	4.68	96.81%

Cluster analysis

Cluster analysis, at similarity coefficient of 0.56, grouped the isolates into A and B groups (Fig 2). C4 from Jind had highest distance from other isolates. Wijesekara *et al.* (2005) grouped twenty *C. capsici* isolates in two clusters at 57% similarity coefficient. Madhvan *et al.* (2010) used

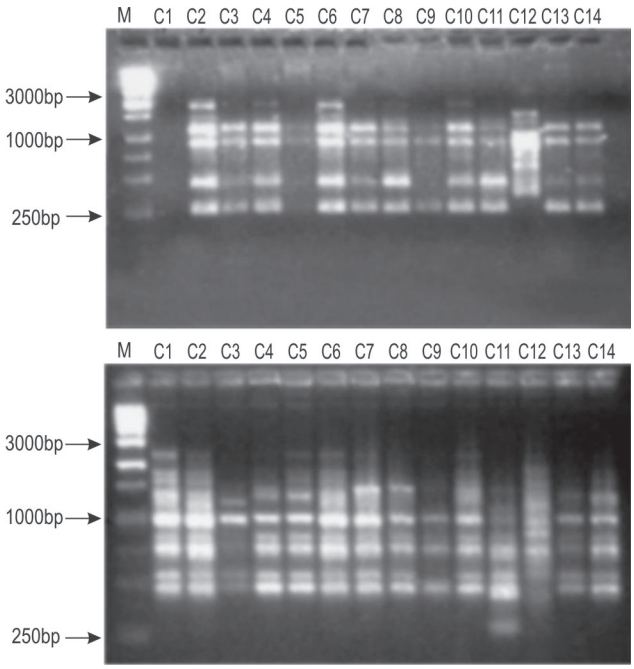

Fig 1 Electrophoretic pattern of PCR- amplified products of selected *Colletotrichum capsici* isolates using OPD-05 and OPQ-18 primers.

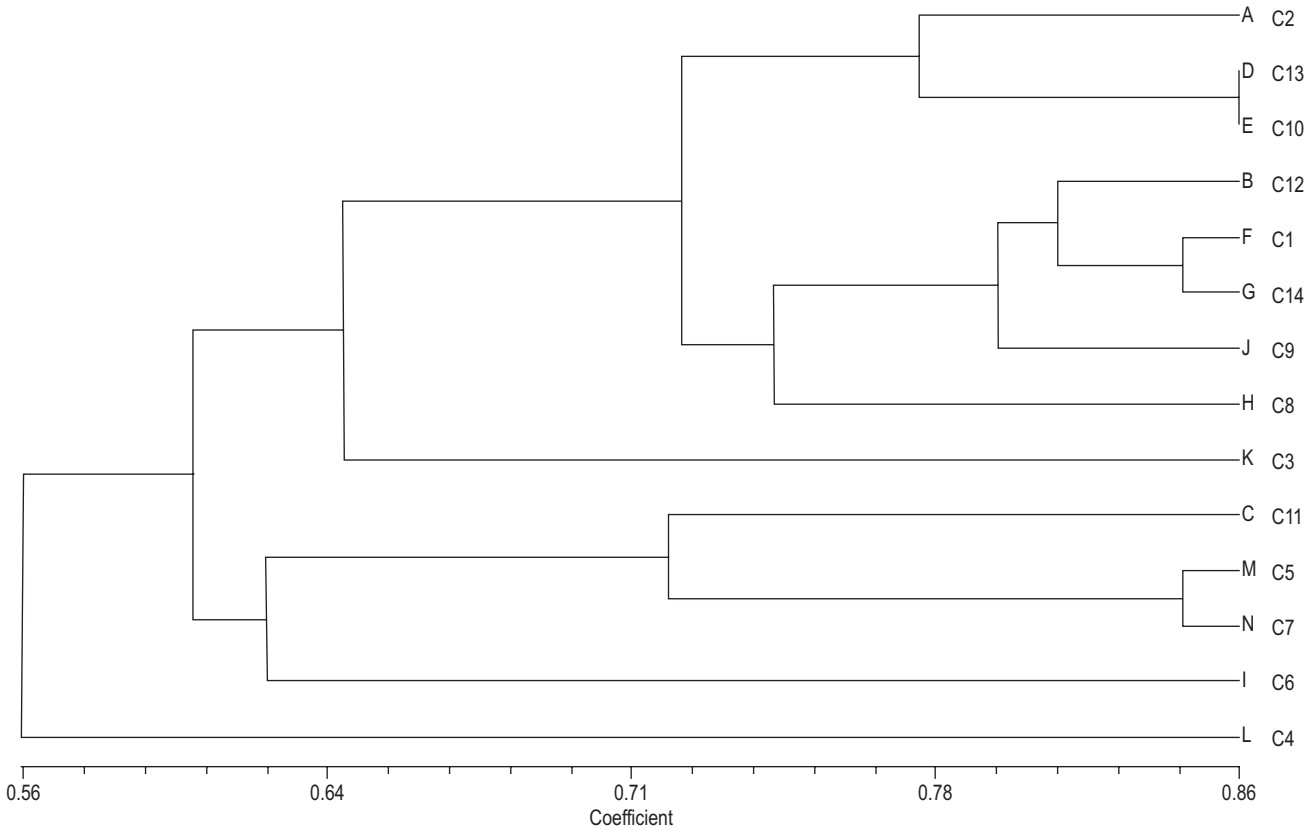

Fig 2 UPGMA dendrogram for 14 isolates of *Colletotrichum capsici* based on RAPD analysis using Jaccard's similarity coefficient.

22 RAPD primers and found 16 to 81% molecular variability among isolates of *C. capsici, C. gloesporioides* and *Alternaria alternate.* Three groups were evident while clustering and virulence of isolates was not found related.

In this study, Cluster A contained 9 isolates with genetic similarity ranging from 64 to 86 % making two sub groups. Sub group A_1 was occupied by three isolates while six isolates made sub-group A_2. The isolates C13 and C10 in cluster A showed the maximum similarity coefficient of 0.86 but were dissimilar geographically. Four isolates of Cluster B had identical virulence but were dissimilar in cluster analysis. Only C1, C2 and C3 isolates of group A with identical virulence clustered together. Sharma *et al.* (2005) did not observe any correlation among pathological and RAPD groupings but Mohanraj *et al*. (2002) found good correlation between diversity and virulence pattern in *C. falcatum* isolates of sugarcane.

Our results indicate high genotypic diversity not associated with the geographic localities. The only geographic tendency was observed for isolates C1 and C2 from Hisar in cluster A. Molecular analysis was not congruent with either geographic location or host gene pool (Balardin *et al.* 1997) while Singh *et al.* (2002) reported that pathogenic variation is related to the distribution of isolates in different climatic regions,

Accurate diagnosis and identification of plant pathogens is a pre-requisite of disease management (Sharma 2003) and genetic recombination may likely lead to variation (Agrios 2005). Molecular markers are more versatile and highly informative in pathogen identification and characterization. Combined with virulence data, they can evaluate genetic diversity. Several workers have used RAPDs for diversity analysis in this pathogen (Sharma *et al*. 2005, Ratanacherdchai *et al*. 2007, Sangdee *et al*. 2011).

The molecular diversity and pathogenic variability are important for exploring diversity of this pathogen in Haryana state. The study may find application in improving chilli fruit rot resistance and varietal deployment strategies essential for effective disease management.

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