Original Research Article

RAPD Analysis of *Colletotrichum capsici* Causing Anthracnose of Chilli in Madhya Pradesh, India

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A B S T R A C T

In the present investigation an attempt was carry out to test the pathogenicity of the sixteen isolates of *C. capsici* obtained from different districts of Madhya Pradesh on ripe and green chilli by pin prick method and the genetically variation of these isolates was also detected by Random Amplified Polymorphic DNA (RAPD) technique. On ripe chilli, significantly maximum anthracnose incidence was recorded in BHP-1 (56.60 %) and minimum anthracnose incidence was recorded in CHN-2 (33.80 %) whereas on green chilli, significantly maximum anthracnose incidence was recorded in JHB-2 (89.60 %) while minimum anthracnose incidence was recorded in CHN-2 (61.40 %). For genetic variability, two primers were used which produced 32 scorable bands in which 23 bands were polymorphic and showed 72 % polymorphism. Cluster analysis using the unweighted pair-group method with arithmetic average (UPGMA) clearly separated the isolates into two clusters (I and II), confirming the genetic diversity among the isolates of *C. capsici*. The genetical similarity coefficient value ranged from 0.335 – 0.992 for OPD 07 primer across 7 isolates of *C. capsici*. RAPD analysis showed a clear difference in different isolates of *C. capsici*.

Introduction

In India, chilli is being grown in an area of 7,89,000 ha with the production of 1,38,9000 tonnes and yield of 1760 kg/ha (Anonymous, 2016). Chilli (*Capsicum annum* L.) suffers from many diseases caused by fungi, bacteria, viruses, nematodes and also by abiotic stress. Among the fungal diseases, anthracnose/die back/ fruit rot caused by *Colletotrichum*

capsici (Syd.) Butler and Bisby has become a most serious problem in all chilli-growing areas of India. Anthracnose reduces marketable yield from 10 to 80% (Poonpolgul, 2007). Although anthracnose appears on leaves and stems, it causes severe damage to mature fruits in the field, transit and storage (Mehrotra and Agrawal, 2003). Several species of *Colletotrichum viz., C. capsici* (Butler and Bisby), *C. gloeosporioides* (Penz.), *C. acutatum* (Sim- monds), *C. atramentarium* (Berk and Broome), *C. dematium* (Pers.) and *C. coccoides* (Wallr.), *Glomerella cingulata* (Stone- man) along with *Altrenaria alternata* (Keissler) have been reported as the causal agents of chilli fruit rot worldwide (Than *et al.,* 2008). Mahmodi *et al.,* (2014) reported the genetic diversity and differentiation of 50 *Colletotrichum* isolates from legume crops studied through multigene loci, RAPD and ISSR analyses. Prasad *et al.,* (2013) reported the genetic variability in 10 commercial pepper varieties using RAPD markers. Analysis of genetic diversity is one step towards understanding the pathogen population. The objective of this study was to assess the diversity of *Colletotrichum capsici* infecting chilli in Northern region of Madhya Pradesh, India.

Materials and Methods

Isolation and identification of the Pathogens from infected chilli

Total 16 isolates were collected from eight districts (two blocks of each district) of Madhya Pradesh *viz.*, Khandwa, Khargone, Burhanpur, Badwani, Dewas, Jhabua, Jabalpur and Chhindwara. Diseased samples were packed in sterile transparent polyethylene bags, labelled and transported to the Plant Pathology Laboratory. Three 5×5 mm pieces of tissue were taken from the margins of infected tissue, surface-sterilized with 2.0 % sodium hypochlorite solution for 30 seconds

and rinsed three times with sterile water then placed on the surface of PDA and incubated at 25±2°C temperature. The growing edges of the hyphal mycelium developing from the diseased tissue discs were transferred aseptically to potato dextrose agar for re-culturing. The identification of *Colletotrichum* was done based on the morphological and colony characteristics (Ellis, 1971; Barnett and Hunter, 1972).

Molecular characterization of *Colletotrichum capsici* in chilli

Fungal DNA extraction

For DNA extraction, each isolate of *C. capsici* was grown in 100-ml conical flasks, containing 30 ml of potato dextrose broth for seven days at room temperature (28 ± 2°C). The mycelia were harvested by filtration and frozen in liquid nitrogen. Freeze-dried mycelium (1 g) was ground to a fine powder using liquid nitrogen, and DNA was extracted according to standard protocols (Murray and Thompson, 1980) [8]. The genomic DNA was checked by agarose gel electrophoresis and stored at –20ºC for further use. In total, 10 random primers were used for RAPD analysis. PCR amplification was performed by using florescent labeled RAPD primers; (OPD-07: 5'TTGGCACGGG and OPL-05: 5'ACGCAGGCAC3') (Table 1). All the RAPD primers were purchased from Operon (Operon Biotechnologies, Cologne, Germany) and used as single primers. Amplification was performed in a 20 μl reaction volume consisting of 5 mM each dNTPs, 20 pmol of primer, 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt Ltd, Banglore, India) and 50 ng of template.

RAPD analysis

The amplified fragments of each isolate were scored as 1 (present) or 0 (absent).

Comigrating bands were considered homologous characters. Faint bands and bands showing variable levels of intensity were not considered for scoring.

Statistical analysis

A binary matrix was compiled using numerical system of multivariate analysis. The dendogram was constructed by the unweighted paired group method of arithmetic average (UPGMA) based on Jaccard's similarity coefficient with SHAN program of NT-sys, (Jaccard's, 1912).

Results and Discussion

Total 16 isolates were collected from eight districts (two blocks of each district) of Madhya Pradesh *viz.*, Khandwa, Khargone, Burhanpur, Badwani, Dewas, Jhabua, Jabalpur and Chhindwara (Fig. 1, 2 and Table 2).

Pathogenicity of the sixteen isolates of *C. capsici* obtained from different districts of Madhya Pradesh was tested on ripe and green chilli by pin prick method. On ripe chilli, significantly maximum anthracnose incidence was recorded in BHP-1 (56.60 %) except DWS-2 (56.40 %), JHB-2 (54.40 %), BRW-2 (53.40 %) and CHN-1 (53.00 %) followed by BHP-2 (52.00 %), KNW-2 (51.00 %) and KGN-1 (49.40 %), while minimum anthracnose incidence was recorded in CHN-2 (33.80 %) which was found at par with BRW-1 (35.60 %) and JBP-2 (36.40 %) followed by KGN-2 (38.20 %), KNW-1 (40.40 %), JBP-1 (43.80 %), DWS-1 (44.40 %) and JHB-1 (46.60 %). On green chilli, significantly maximum anthracnose incidence was recorded in JHB-2 (89.60 %) followed by CHN-1 (84.40 %) and DWS-2 (83.40 %) while minimum anthracnose incidence was recorded in CHN-2 (61.40 %) which was found at par with BRW-1 (62.20 %), JBP-2 (63.60 %), KNW-1(63.80 %), JBP-1 (64.40 %) and

DWS-1 (64.40 %). Anthracnose disease is responsible for major economic losses in chilli production worldwide, especially in tropical and subtropical regions (Pakdeevaraporn *et al.,* 2005). In this study, *C. capsici* was confirmed as the species responsible for chilli anthracnose in Northern Madhya Pradesh by pathogenicity test. The pathogenicity study showed that the behavior of *C. capsici* isolates were homogeneous with regard to disease symptoms. However, variation in virulence or the level of disease (measured quantitatively) within the isolates was observed. Differences in aggressiveness of *C. capsici* isolates have been reported previously by Taylor *et al.,* (2007).

The molecular technique, RAPD was used to detect the genetical variation among the 16 isolates of *C. capsici* causing anthracnose in Chilli. Total 02 primers falling in OPD07 and OPL 05 were used to accesses molecular variation. Both primers produced 32 scorable bands. Among 32 bands 23 bands were polymorphic and level of polymorphism was 72 % (Table 3).

The dendrogram constructed from the pooled data of primer OPD 07 indicated that, there were two major clusters I and II. Cluster I consist of five isolates sample 6, 7, 8, 9 and 2 while cluster II consist of two isolates sample sample 1 and 3 which is distinct and showed least similarity with all other isolate of *C. capsici*. The genetical similarity coefficient value ranged from 0.335 – 0.992 for OPD 07 primer across 7 isolates of *C. capsici* (Fig. 3 and Table 4).

The dendrogram constructed from the pooled data of primer OPL 05 indicated that, there were two major clusters I and II. Cluster I consist of five isolates sample 6, 7, 8, 9 and 2 while cluster II consist of two isolates sample sample 1 and 3 which is distinct and showed least similarity with all other isolate of *C.*

capsici. The genetical similarity coefficient value ranged from 0.634 - 0.995 for OPL 05 primer across 9 isolates of *C. capsiciI* (Fig. 4, 5 and Table 5).

Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from non-cultivated hosts into two separate clusters. Isolates from strawberry were interspersed within the cluster containing the isolates recovered from non-cultivated hosts.

Sharma *et al.,* (2005) reported considerable pathogenic variation in *C. capsici* isolates collected from chilli-growing areas of Himachal Pradesh (HP), India.

Pathological and RAPD grouping of isolates suggested no correlation among the tested isolates of *C. capsici*. Ratanacherdchai *et al.,* (2007) analysed 18 isolates of two species, *C. gloeosporioides* and *C. capsici* isolated from three varieties of chilli i.e. Chilli pepper (*C. annuum*), Long cayenne pepper (*C. annuum var acuminatum*) and Bird's eye chilli (*C. frutescens*) using RAPD analysis and reported a clear difference between *C. gloeosporioides* and *C. capsici*.

Ratanacherdchai *et al.,* (2010) analysed the genetic diversity among isolates of *C. gloeosporioides* and *C. capsici* from Thailand by Inter simple sequence repeat (ISSR) analysis and reported that there were two distinct groups of *C. gloeosporioides* and *C. capsici*.

Furthermore, genetic diversity was correlated with geographical distribution, while there was no clear relationship between genetic diversity and pathogenic variability among the isolates *of C. gloeosporioides* and *C. capsici*. Pathogen diversity plays a major role in disease dynamics and consequently, in the success of disease management strategies, including the development of cultivars resistant to diseases. The results of the present study demonstrate that there is a high level of genetic diversity among isolates of *C. capsici* in Tamil Nadu. Pathogenicity tests revealed that these isolates expressed different levels of virulence.

The existence of molecular variability among isolates of *C. capsici* that differed in virulence was earlier established by Srinivasan *et al.,* (2010) using RAPD markers.

The result of RAPD in the present investigation was further confirmed by sequence analysis of the ITS region which revealed 100% sequence similarity between the two *C. capsici* isolates of chilli and CI isolate of soybean. *C. gloeosporoides*, on the other hand, revealed significant sequence difference with *C. capsici* isolates CI. Freeman (2008) used sequence analysis of ITS region to establish similarity between *Colletotrichum acutatum* isolates from almond and strawberry.

Colletotrichum spp. causing anthracnose of chilli was identified as *C. capsici*. The phylogenetic grouping based on RAPD showed a relationship between clustering in dendrogram and geographical distribution of isolates.

However, the pathological and RAPD grouping of isolates was suggested on correlation among the tested isolates.

Therefore, RAPD markers are a useful method of studying genetic diversity in *Colletotrichum* spp. PCR-based technique like RAPD used in this study are rapid, reproducible and produce a large number of polymorphic bands.

Such techniques aid in the understanding of pathogen population dynamics, which can facilitate the development of effective control strategies.

Table.1 Sequence of RAPD Primers used to study the Genetic variability among the isolates of *Colletotrichum* sp.

S.N.	Primers	Base sequence (5' ⟶ 3')	Sample No.
1.	OPD 07	TTGGCACGGG	1, 2, 3, 6, 7, 8 & 9
2.	OPL 05	ACGCAGGCAC	4, 5, 10, 11, 12, 13, 14, 15 & 16

Table.2 Pathogenic variability among the isolates of *C. Capsici*

District	Block/Place	Isolates code	Disease incidence (%)	
			Ripe Fruit	**Green Fruit**
Barwani	Barwani	BRW-1	35.60 (36.63)*	62.20 (52.07)*
	Thikari	BRW-2	53.40 (46.95)	73.20 (58.85)
Khargone	Khargone	KGN-1	49.40 (44.66)	67.20 (55.08)
	Kasrawad	KGN-2	38.20 (38.17)	71.60 (57.84)
Jabalpur	Jabalpur	JBP-1	43.80 (41.43)	64.40 (53.38)
	Shahpura	JBP-2	36.40 (37.03)	63.60 (52.90)
Burhanpur	Burhanpur	BHP-1	56.60 (48.79)	71.20 (57.57)
	Khaknar	BHP-2	52.00 (46.15)	75.40 (60.29)
Dewas	Dewas	DWS-1	44.40 (41.78)	64.40 (53.38)
	Khategaon	DWS-2	56.40 (48.69)	83.40 (66.01)
Jhabua	Rama	JHB-1	46.60 (43.05)	74.40 (59.66)
	Petlawad	JHB-2	54.40 (47.53)	89.60 (71.24)
Khandwa	Chegaon Makhan	KNW-1	40.40 (39.46)	63.80 (53.02)
	Pandhana	KNW-2	51.00 (45.57)	74.80 (59.93)
Chhindwara	Sausar	CHN-1	53.00 (46.72)	84.40 (66.77)
	Pandhurna	CHN-2	33.80 (35.54)	61.40 (51.60)
SEm±			0.84	0.94
C.D. at 5 %			2.42	2.70

Table.3 UPGMA average dendrogram constructed from RAPD data indicating the relationship among the isolates of *C. capsici* from chilli.

Primers	Monomorphic bands	Polymorphic bands	Total no. of bands	% Polymorphism
OPD 07	04	05	09	56 %
OPL 05	05	18	23	78 %
Total	09	23	32	72 %

Table.4 Genetic similarity coefficient matrix for *C. capsici* from chilli based
on RAPD analysis for primer OPD 07

Strain Number	S_3	S_6	S_7	S_2	S_8	S_9	S_1
Sample_3	0						
Sample_6	0.5851	0.0000					
Sample_7	0.5487	0.0175	0.0000				
Sample_2	0.6200	0.0218	0.0185	2.220E-16			
Sample_8	0.5553	0.0112	0.0076	1.747E-02	2.220E-16		
Sample_9	0.6646	0.0281	0.0284	3.192E-02	2.631E-02	-2.220E-16	
Sample_1	0.3172	0.3424	0.3249	3.742E-01	3.426E-01	3.953E-01	0

S- Sample

Table.5 Genetic similarity coefficient matrix for *C. capsici* from chilli based on RAPD analysis
for primer OPL 05

Strain Number	S_4	S_5	S_10	S_11	S_12	S_13	S_14	S_15	S_16
Sample_4	1.000								
Sample_5	0.913	1.000							
Sample_10	0.845	0.949	1.000						
Sample_11	0.634	0.744	0.809	1.000					
Sample_12	0.644	0.757	0.833	0.802	1.000				
Sample_13	0.906	0.947	0.965	0.776	0.794	1.000			
Sample_14	0.867	0.957	0.995	0.801	0.826	0.973	1.000		
Sample_15	0.788	0.878	0.974	0.805	0.826	0.932	0.968	1.000	1.000
Sample_16	0.781	0.862	0.958	0.790	0.806	0.917	0.953	0.994	1

S- Sample

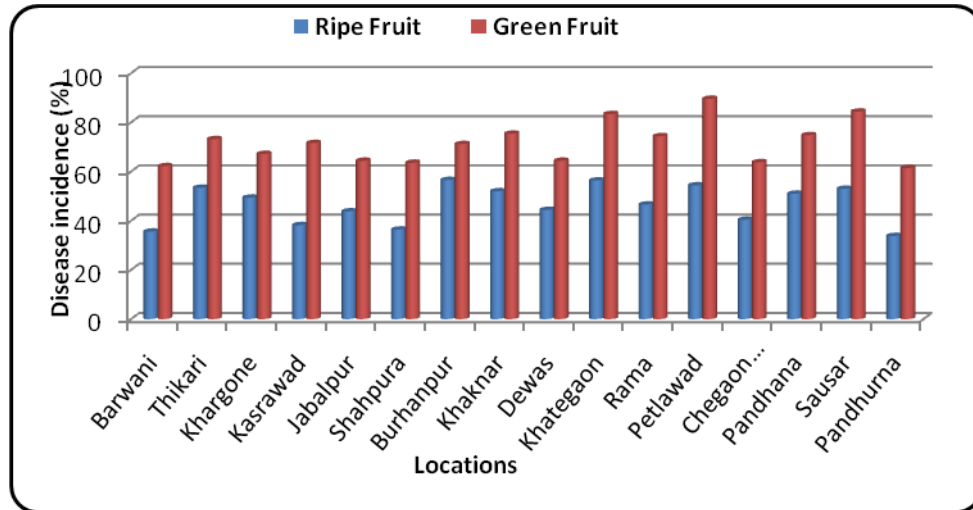**Fig.1** Pathogenic variability among the isolates of *C. Capsici*

Fig.2 Symptoms produced by different isolates of C. capsici on green and red ripe fruits of chilli.
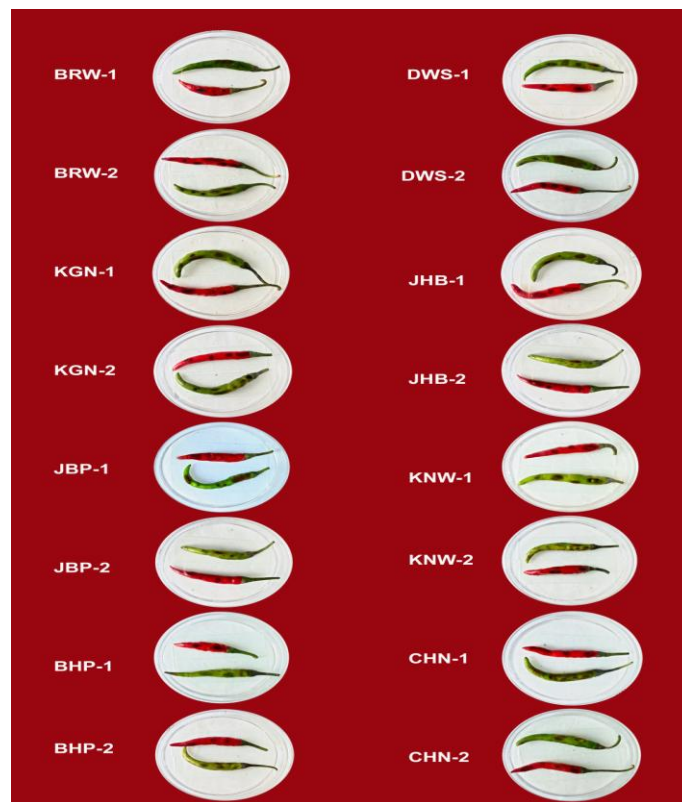
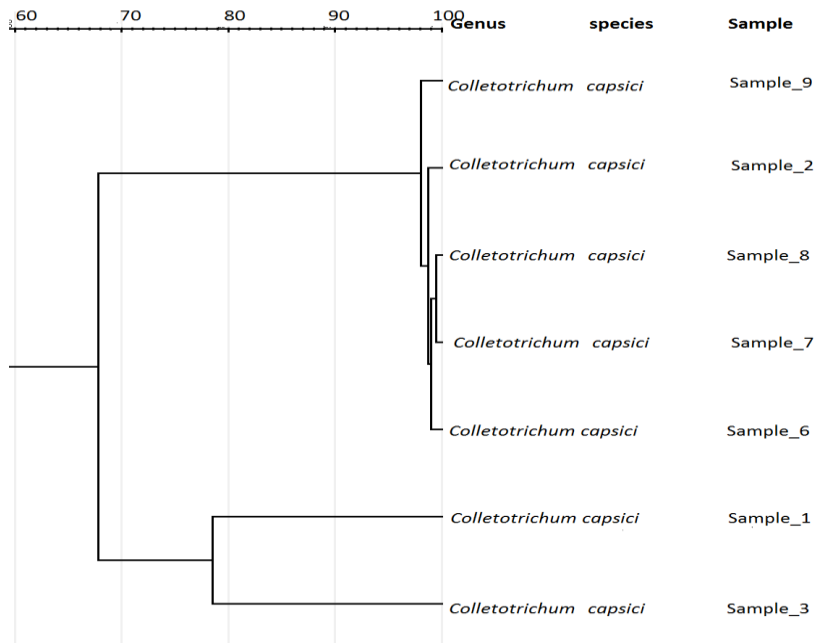**Fig.3** Dendrogram generated using Jaccard's similarity distance obtained from RAPD primers OPD 07.

Fig.4 Dendrogram generated using Jaccard's similarity distance obtained from RAPD primers OPL 05.
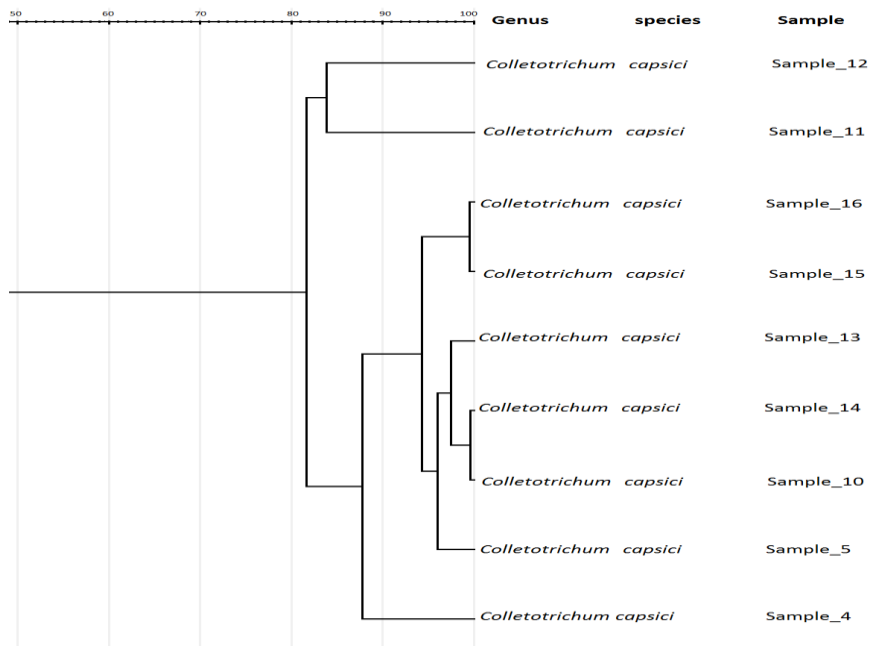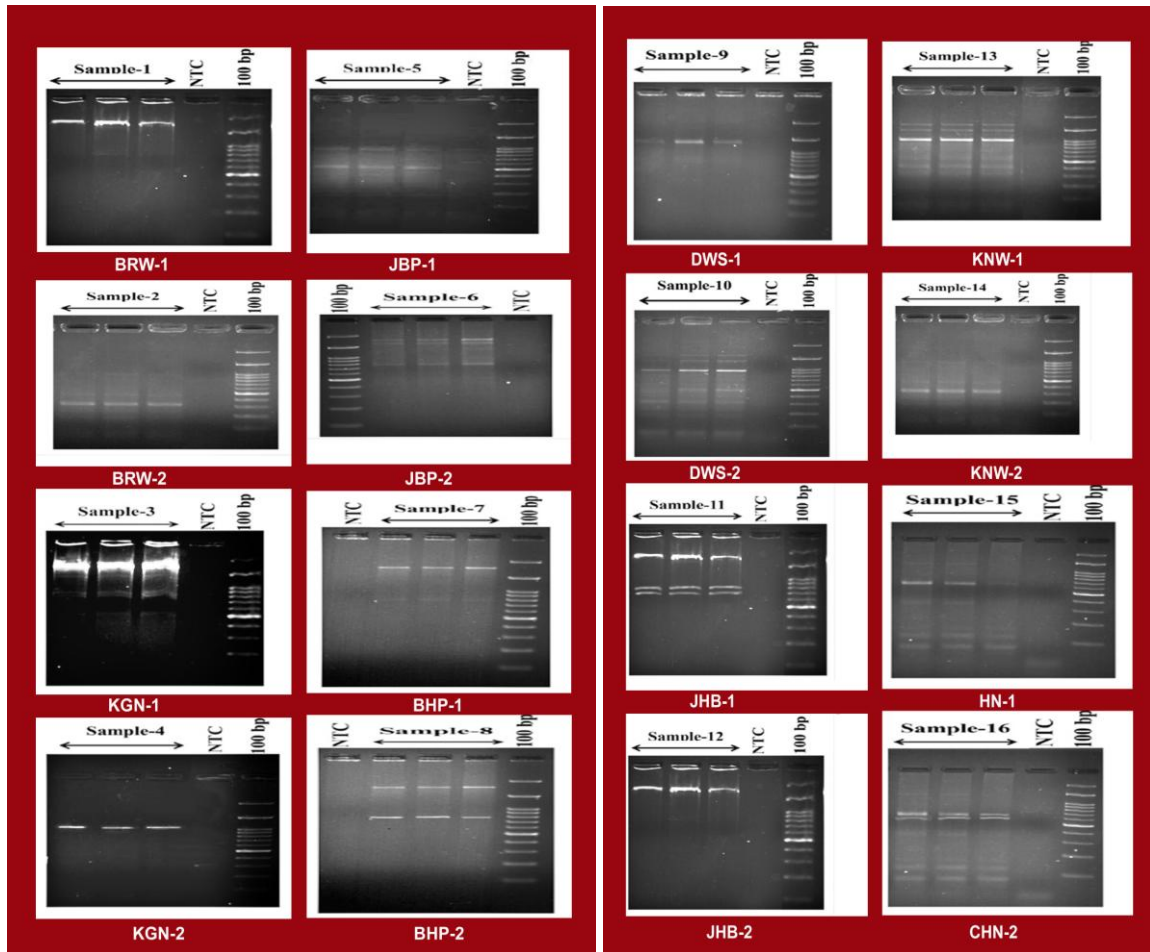
Fig.5 RAPD amplification banding pattern of primers OPL 05 and OPD 07 in *Colletotrichum capsici* isolates.

Competing Interests

Authors have declared that no competing interests exist.

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