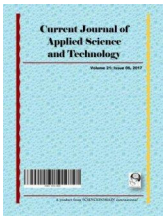

Current Journal of Applied Science and Technology

26(5): 1-8, 2018; Article no.CJAST.40414
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

Molecular Detection and Diversity Analysis of *Colletotrichum sp* from Chilli Using RAPD-PCR

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AK, SN, KV and DU designed the study, supervised and facilitated the research and wrote the first draft of the manuscript. Author VK performed the experiments and analyzed the results obtained in the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2018/40414
Editor(s):
(1) Zhi-Qiang Xiong, Professor, Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China.
Reviewers:
(1) Ilham Zahir, Sultan Moulay Slimane University, Beni Mellal, Morocco.
(2) Ashwani Kumar, Shri Ram College Muzaffarnagar (CCSU Meerut) UP, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/24013

Original Research Article

Received 19th January 2018
Accepted 26th March 2018
Published 6th April 2018

ABSTRACT

The molecular variability among the isolates of *Colletotrichum capsici* and *Colletotrichum gloeosporioides* that differed in virulence was analysed by means of random amplified polymorphic DNA (RAPD) using 10 random primers. Analysis of the genetic coefficient matrix derived from the scores of RAPD profile showed that maximum per cent similarities between species of *C. capsici* and *C. gloeosporioides* which were in the range of 92% respectively. 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* and *C. gloeosporioides* from chilli. RAPD analysis in the present study showed a clear difference between *C. gloeosporioides* and *C. capsici.* Furthermore, *C. capsici* isolates were found to be more closely related than *C. gloeosporioides* isolates.

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Keywords: C. capsici; C. gloeosporioides; molecular variability.

1. 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. [1]. 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% [2]. Although anthracnose appears on leaves and stems, it causes severe damage to mature fruits in the field, transit and storage [3]. 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 [4]. [5] reported the genetic diversity and differentiation of 50 *Colletotrichum* isolates from legume crops studied through multigene loci, RAPD and ISSR analyses. [6] 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 sp* infecting chilli in Tamil Nadu, India.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of the Pathogens from Infected Chilli

Chilli fruit showing the typical symptoms of fruit rot were collected from different places of Tamil Nadu state. The pathogen was isolated by plating on Potato Dextrose Agar (PDA) medium. The fungus was purified by single hyphal tip method and maintained on PDA slants [7]. The isolates were identified based on the of morphology under compound microscope.

2.2 Molecular Characterization of *Colletotrichum sp* in Chilli

2.2.1 Fungal DNA extraction

For DNA extraction,each isolate of *C. capsici* and *C. gloeosporioides* was grown in 100-ml conical flasks, containing 30 ml of potato dextrose broth for seven days at room temperature (28 ± 2ºC). 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 [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. 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.

2.2.2 RAPD analysis

The PCR was performed using Eppendorf – Master Cycler ep gradient S (Eppendorf, A G, Hamburg, Germany), with an initial denaturation step for 5 min at 94ºC, followed by 40 cycles of 1 min at 94ºC, 1 min at 37ºC and 2 min at 72ºC, with a final extension for 10 min at 72ºC. Following amplification, 10 µl of each PCR product was separated by electrophoresis in 2% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04 M Tris-ac etate, 0.001 M EDTA, pH 8.0). A 100- base pair (bp) and 1kp ladder (Bangalore Genei Pvt Ltd, Banglore, India) was used as a size standard. To visualise DNA, gels were stained with ethidium bromide (0.1 mg l^{-1}) and then photographed under transmitted ultraviolet light, using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). All RAPD analyses were repeated at least three times for each isolate.

2.2.3 Analysis of RAPD profiles

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. A similarity matrix was constructed, using Jaccard's coefficient, and the resulting similarity data were used to construct a dendrogram, using UPGMA and the NTSYS-pc software version 2.02 developed by [9].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of the Pathogens from Infected Chilli

Colletotrichum sp was the most commonly isolated fungal species from infected chilli fruits. Amids the 25 chilli samples collected, *C. capsici* and *C. gloeosporioides* were predominant. *C. capsici* was isolated from the samples collected from Theni, Dindugal, Pudukottai, Virudhunagar and Kanchipuram districts. *C. gloeosporioides* was isolated from the collected from Madurai, Coimbatore, Dindugal, Virudhunagar, Pudukottai, Kanchipuram and Tirupur districts. The virulence of all the fungal isolates was determined by artificial inoculation on chilli fruits (Susceptible K1) (Table 1).

A total of 25 isolates of *C. capsici* and *C. gloeosporioides* were tested for their genetic variability by RAPD analysis using 10 random primers (Table 2). Of these, 10 random primers *viz*., OPA1,OPA2,OPA3,OPA4,OPA5,OPA6, OPA7,OPA8,OPA9,OPA10 produced scorable and consistent banding patterns, which were used for RAPD analysis of test (Fig. 2). Oligonucleotide primers generated a total of 1484 DNA amplification products. (Table.3). The number of bands generated by each primer varied from one to seven. Analysis of the genetic coefficient matrix (Table.4) derived from the scores of RAPD profile showed the maximum and minimum per cent similarities among *C. capsici* and *C .gloeosporioides* isolates which range upto 92%. (Fig.1). Cluster analysis using UPGMA clearly separated the isolates of *C .capsici* and *C .gloeosporioides* into two clusters (I,II) confirming the level of genetic diversity among the isolates of *Colletotrichum sp* infecting chilli. Cluster I consisted of *Cc*1, *Cc*3, *Cc*4, *Cc*2, *Cc*13, *Cg*14, *Cc*15, *Cg*6, *Cg*7, *Cg*24, *Cg*21, *Cg*25, *Cg*10, *Cc*23, *Cc*5. and Cluster II consisted of *Cg*8, *Cg*19, *Cc*20, *Cg*17, *Cg*18, *Cg*9, *Cg*12, *Cg*11,*Cg*22.

[10] reported that when inoculated into non-host, *C. gloeosporioides* could genetically adapt to a new host, and resultant isolates could not be distinguished morphologically from the wild type. Thus requires further investigation for the cross-infection of different hosts by *Colletotrichum* species. [11] studied on anthracnose basal rot caused by *C. graminicola* (Ces.) Wils., in annual bluegrass (*Poa annua* L.), creeping bentgrass (*Agrostis palustris* Huds.) golf course putting greens in North America and Europe. Cluster analyses of RAPD markers showed that, isolates from bluegrass and creeping bentgrass were separated into two distinct groups. [12] reported that RAPD markers were used to determine genetic relationships among isolates that recovered from non cultivated hosts and diseased strawberry plants. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from noncultivated hosts into two separate clusters. Isolates from strawberry were interspersed within the cluster containing the isolates recovered from noncultivated hosts. [13] 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*. [14] 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*. [15] 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.

Table 1. *Colletotrichum sp* isolates used in this study

Hosts	Isolates	
Chilli	***C. capsici***	***C. gloeosporioides***
	*Cc*1, *Cc*2, *Cc*3, *Cc*4, *Cc*5, *Cc*13, *Cc*15,*Cc*20, *Cc*23	*Cg*6, *Cg*7, *Cg*8, *Cg*9, *Cg*10, *Cg*11, *Cg*12, *Cg*14,*Cg*16,*Cg*17,*Cg*18,*Cg*19,*Cg*21,*Cg*22,*Cg*24,*Cg*25

The genetic variability among the isolates of *C. capsici* should be taken in to account when *C. capsici* isolates are used for screening of chilli genotypes for anthracnose resistance. [5] analysed genetic diversity among isolates of *C. gloeosporioides*, *C. truncatum and C. dematium* from Malaysia by RAPD and ISSR analysis and reported that there were three distinct groups of *C. gloeosporioides*, *C. truncatum* and *C. dematium*. 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*, *C. truncatum and C. dematium*.

Table 2. Sequence of RAPD primers used to study the genetic variability among the isolates of *Colletotrichum sp*

Primer code	Base sequence (5'-3')
OPA 1	CAGGCCCTTC
OPA 2	TGCCGAGCTG
OPA 3	AGTCAGCCAC
OPA 4	AATCGGGCTG
OPA 5	AGGGGTCTTG
OPA 6	GGTCCCTGAC
OPA 7	GAAACGGGTG
OPA 8	GTGACGTAGG
OPA 9	GGGTAACGCC
OPA10	GTGATCGCAG

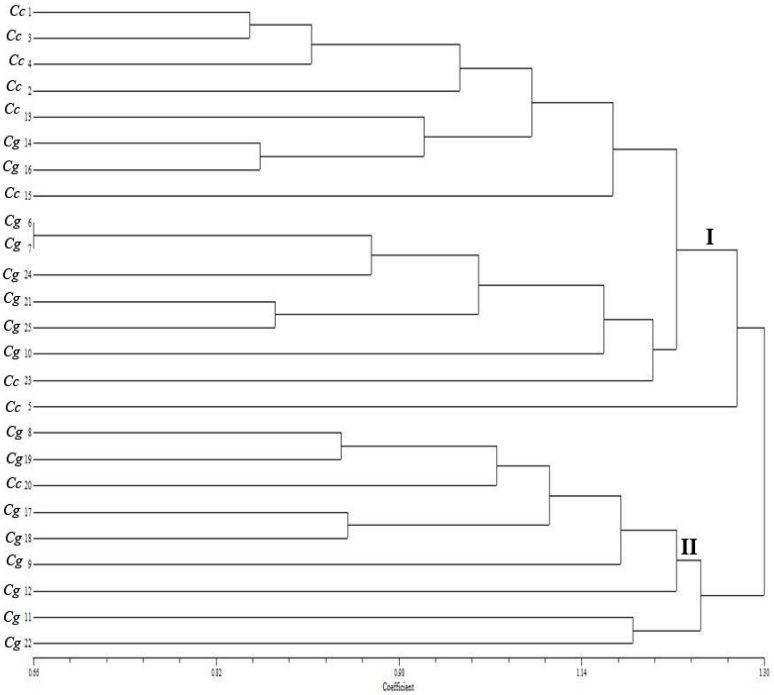


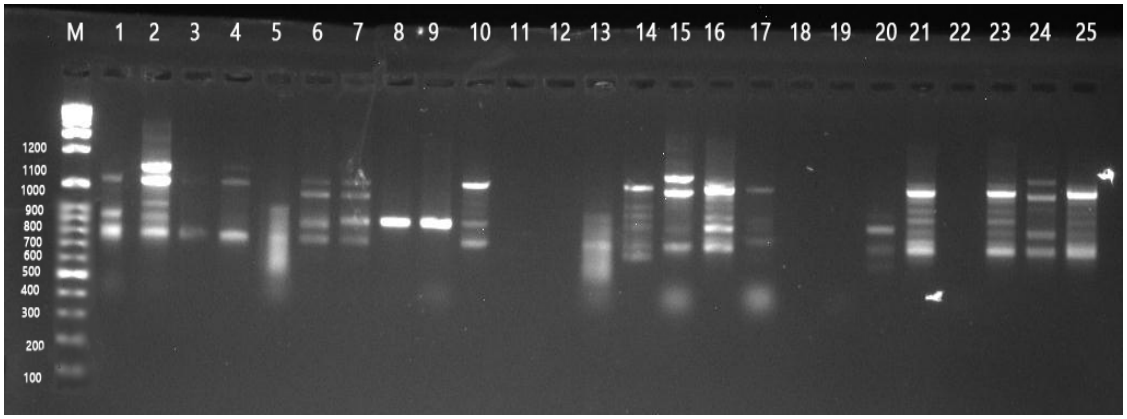
Fig. 1. Unweighted pair group method arithmetic average dendrogram constructed from RAPD data indicating the relationship among the isolates of *Colletotrichum sp* from chilli

Table 3. Variability observed in *Colletotrichum sp* isolates by RAPD

Primers	Monomorphic bands	Polymorphic bands	Total no of bands	%Polymorphism
OPA1	53	72	125	58%
OPA2	66	109	175	62%
OPA3	131	144	275	52%
OPA4	08	92	100	92%
OPA5	37	113	150	75%
OPA6	34	116	150	77%
OPA7	48	127	175	72%
OPA8	8	101	109	92%
OPA9	20	55	75	73%
OPA10	68	82	150	54%
Total	**473**	**1011**	**1484**	

Table 4. Genetic similarity coefficient matrix for *Colletotrichum sp* isolates from chilli based on RAPD analysis

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	0.00																								
2	9.82	0.00																							
3	8.52	1.11	0.00																						
4	9.06	1.01	9.06	0.00																					
5	1.19	1.24	1.19	1.00	0.00																				
6	1.02	1.17	1.33	1.09	1.11	0.00																			
7	1.11	1.24	1.33	1.09	1.23	6.62	0.00																		
8	1.06	1.21	1.23	1.30	1.21	1.21	1.01	0.00																	
9	1.24	1.42	1.35	1.48	1.43	1.38	1.38	1.04	0.00																
10	1.23	1.38	1.17	1.21	1.32	1.10	1.10	1.35	1.26	0.00															
11	1.33	1.44	1.26	1.33	1.43	1.48	1.32	1.21	1.24	1.24	0.00														
12	1.42	1.30	1.36	1.27	1.29	1.45	1.45	1.29	1.28	1.24	1.31	0.00													
13	1.05	1.08	9.84	1.13	1.33	1.28	1.35	1.18	1.23	1.32	1.28	1.37	0.00												
14	1.02	1.07	1.09	1.06	1.23	1.08	1.08	1.03	1.30	1.20	1.30	1.29	9.13	0.00											
15	9.74	1.07	1.22	1.22	1.39	1.21	1.34	1.37	1.41	1.40	1.47	1.42	1.24	1.27	0.00										
16	1.08	1.16	1.20	1.21	1.38	1.20	1.20	1.15	1.28	1.16	1.20	1.17	1.09	8.61	1.18	0.00									
17	1.20	1.35	1.20	1.35	1.40	1.32	1.32	1.22	1.17	1.13	1.26	1.20	1.29	1.10	1.22	1.14	0.00								
18	1.34	1.43	1.23	1.33	1.32	1.44	1.38	1.13	1.20	1.22	1.24	1.04	1.27	1.14	1.40	1.16	9.39	0.00							
19	1.14	1.28	1.14	1.35	1.25	1.34	1.26	9.32	1.20	1.40	1.17	1.40	1.16	1.12	1.32	1.24	1.08	9.87	0.00						
20	1.15	1.29	1.15	1.16	1.25	1.23	1.15	1.02	1.25	1.18	1.23	1.13	1.14	1.02	1.32	1.07	1.14	1.11	1.11	0.00					
21	1.25	1.35	1.46	1.35	1.40	1.03	1.19	1.42	1.44	1.24	1.45	1.38	1.29	1.16	1.10	1.01	1.27	1.35	1.41	1.26	0.00				
22	1.19	1.32	1.19	1.28	1.30	1.37	1.37	1.16	1.25	1.32	1.18	1.34	1.22	1.23	1.40	1.32	1.25	1.24	1.11	1.39	1.39	0.00			
23	1.40	1.24	1.49	1.38	1.56	1.22	1.28	1.44	1.37	1.29	1.53	1.35	1.32	1.32	1.23	1.08	1.37	1.44	1.55	1.29	9.67	1.54	0.00		
24	9.76	1.14	1.18	1.04	1.26	9.12	1.00	1.28	1.33	1.14	1.31	1.46	1.29	1.12	1.13	1.17	1.34	1.47	1.34	1.13	1.23	1.33	1.32	0.00	
25	1.13	1.33	1.38	1.16	1.30	8.98	1.00	1.30	1.36	1.21	1.33	1.46	1.33	1.13	1.16	1.21	1.35	1.44	1.35	1.16	8.75	1.35	1.14	9.61	0.00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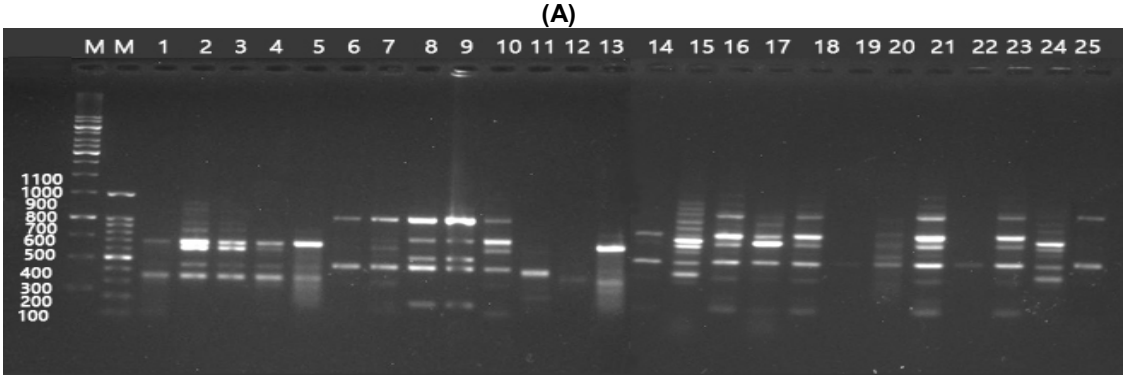
(A)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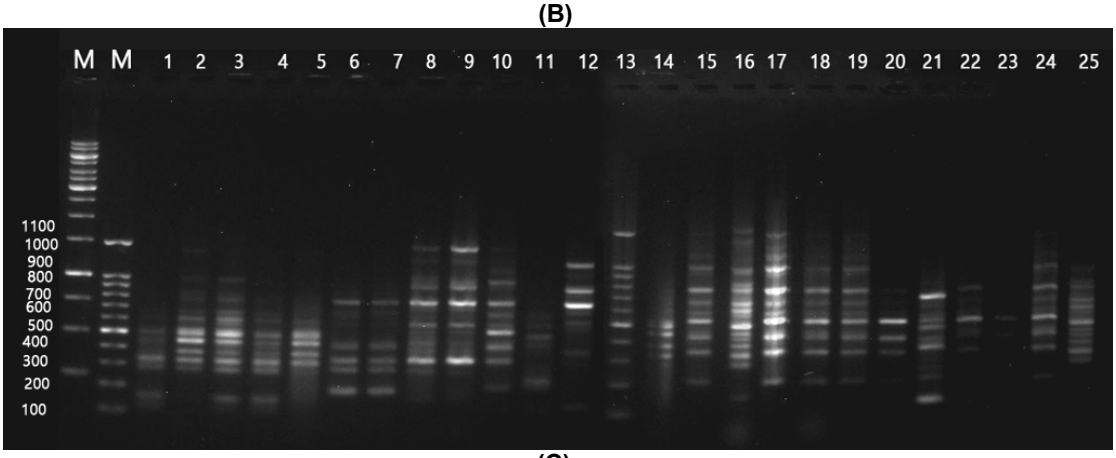
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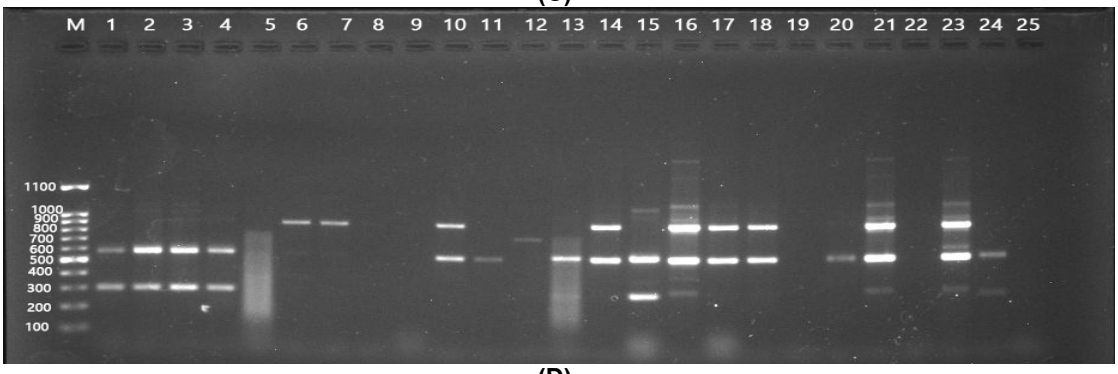
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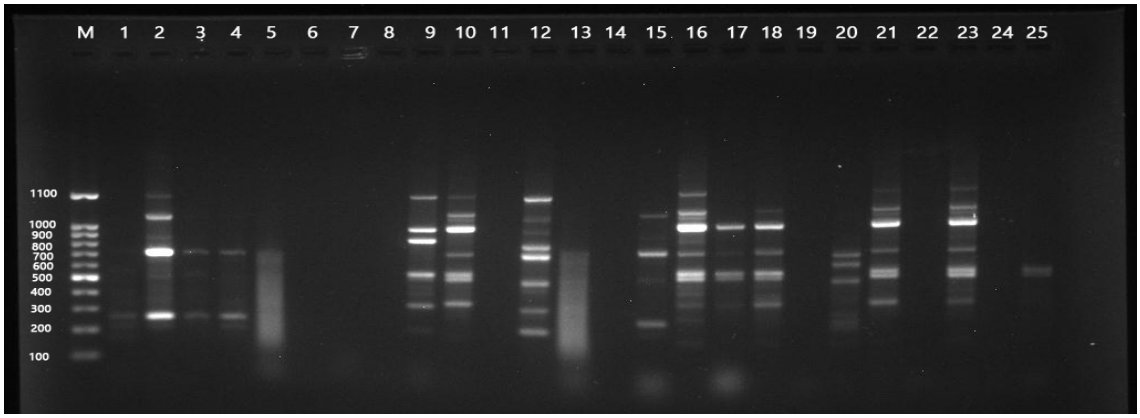

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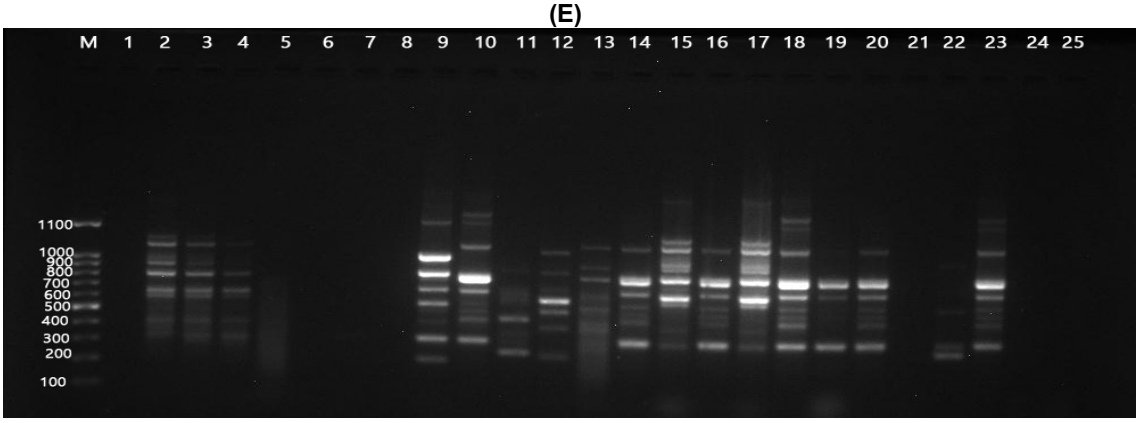

(F)

Fig. 2. RAPD profiles of *Colletotrichum sp* isolates using random primer OPA1 (A), OPA2 (B), OPA3 (C), OPA6 (D), OPA 9(E), OPA10 (F). M=100bp DNA ladder

4. CONCLUSION

Colletotrichum spp. causing anthracnose of chilli were identified as *C. gloeosporioides* and *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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/24013