Journal of Biotechnology and Crop Science
4(4): 60-63, 2015

Genetic diversity of *Colletotrichum* species in chili using randomly amplified microsatellites (Ram)

Saket Kumar, Vineeta Singh, Rajesh Singh, Major Singh

Received: 24 February 2015; Revised Accepted: 08 Apri 2015

ABSTRACT

Present study was assessment genetic diversity of *Colletotrichum* species in chili pod, using Randomly Amplified Microsatellites (RAM). Eight RAM primers were used to amplify genomic DNA of 17 isolated *Colletotrichum* species. These samples were infected by *C. capsici*, *C. acutatum* and *C. gloeosporioides,* causing anthracnose disease. The DNA was isolated and was amplified by thermo cycler after DNA quality tests, using RAM primer. These DNA fragments ranged from 250 bp – 5000 bp. On an average 3-9 bands appeared in per sample of DNA. Estimation of genetic distances (GD) ranged from 16-63% was estimated.

Keywords: Anthracnose, Chili, *Colletotrichum species,* Genetic Diversity, RAM

Introduction

Anthracnose disease is an important chili disease, which infect on chilli pods (Bailey and Jeger,1992). Typical anthracnose symptoms appears on premature chili fruit with sunken necrotic tissues, with circular concentric rings of acervuli. Chili fruits blemishes structure affects the market ability of chili (Manandhar *et al.,* 1995). The anthracnose disease of chili is caused by *Colletotrichum* species (Cannon *et al*., 2000) including *Colletotrichum* sacc.,), *C.* species including *C. capsici* (syd.) Butler and Bisby, *C. gloeosporioides* (Penz, and *acutatum* (Simmonds). It has been reported that a part of post harvest losses due to anthracnose range from 21-47% (Rajapakse, 2009). The disease gets transferred either systematically or superficially from older part of plant to new part of plant including pod. Recently develop DNA based molecular marker that recognize of *Colletotrichum* species for best management of disease (Ratalski *et al*., 1996). RAM, studying *Colletotrichum* species help in identifying genes of agronomic interest and study genetic diversity. This molecular marker is based on the PCR amplification of random locations in the genome of the *Colletotrichum*. With this technique, a single primer of arbitrary nucleotide sequence by generated 8 primers. The PCR primers are designed to amplify a specific target sequence, through amplify loci and presumably scattered throughout the genome (Tinker *et al.,* 1993). The technique has been successfully used to distinguish accessions to evaluate genetic diversity among them (Daher *et a*l., 2002; Palomino *et al*.,2005). RAM is one such method of identifying polymorphism that can be used to elicit information on genetic differences among individuals of a population between lines or germplasm accessions in breeding materials (Williams *el al.,* 1990). In thiis study were conduct and analyze genetic diversity of *Colletotrichum species to* identification different species of *colletotrichum.*

Saket Kumar (✉), Rajesh Singh
Genetics and Plant Breeding, Institute of Agricultural Sciences (IAS), BHU, Varanasi

e-mail: saketjntradelink@gmail.com

Vineeta Singh
Dept. of Mycology and Plant Pathology, Institute of Agricultural Sciences (IAS), BHU, Varanasi

Major Singh
Division of Crop Improvement and Biotechnology, Indian Institute of Vegetable Research (IIVR), Varanasi

Materials and Methods

Collection and Isolation of Culture

Anthracnose affected chili pods were collected from different parts of India. The diseased parts of the fruits were cut at advanced margin of lesions into small pieces (5x3cm). The *colletotrichum* isolates were identified by using key that given by Sutton (1990), Gunnels & Gabbler (1992). The *C. capsici* produce grey white scattered, falcate conidia black acervuli, and non uniform shape of mycelium. This species formed smooth circular margin in the colony. The grey whitish mycelium of *C. capsici* gradually developed from the second day of culture of isolates. The spore measurement of this species varies between 13.21- 16.21 µm long & 1.79 – 3.28 µm wide (Yun *et al.*2009). Slides were made after 5 days & 10 days old culture and sporulation, presence of conidial masses were measured with hematocytometer (Thalhinhas *et al.*, 2005).

Pathogenecity Test

Pathogenecity tests were done separately for each isolate on host plant using plug Inoculation methods following a modified protocol by Sanders and Kirsten (2003). Data was collected as lesion diameter of tested fruits (mm) and the coefficient of variation was computed (Ratanacherdcherdchai *et al*. 2009).

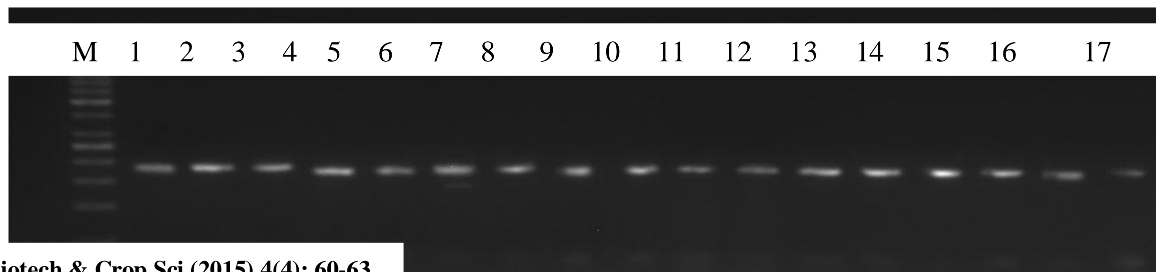
Table 2 The number of primers and there sequences, used in PCR amplification

Primer	Sequences (5'-3)
TG	HBH TGT GTG TGT GTG TGT
CGA	DHB CGA CGA CGA CGA CGA
CT	DYD CTC TCT CTC TCT CTC
CA	DBD ACA CAC ACA CAC ACA
GT	VHV GTG TGT GTG TGT GTG
AG	HBH AGA GAG AGA GAG AGA
CCA	DDB CCA CCA CCA CCA CCA
ACA	BDD ACA ACA ACA ACA ACA

Table 1 Characteristics of different group of *Colletotrichum* species on the basis of growth rate and mycelium colour on PDA media

Isolates Group	Casual Agent	Number of Isolates	Characteristics of Mycelium	Growth Rate	Frequency
I	*C. capsici*	12	Acervuli scattered,	5.4	
			Black mycelium with	5.8	63%
			gray and white	6.8	
			Falcate	6.3	
			Salmon colour	8.2	
			Falcate	6.7	
II	*C. acutatum*	2	Falcate white colour mycelium	4.9	
				5.2	16%
III	*C. gloeosporioides*	3	Fluffy, gray-white	7.4	
			cylendrical	6.8	21%
				6.6	

Note: Letter designation for degenerate primer sites are: H (A, T ór C); B (G, T or C); V (G, A or C) and D (G, A or T). Source: Gandley and Bradshaw (2001) **.**



J of Biotech & Crop Sci (2015) 4(4): 60-63

Figure 1 Analysis of DNA molecules with RAM primer for identification of *Colletotrichum* species

Harvesting of fungal mycelium and isolation of DNA

After 6 - 10 days this inoculation in Potato Dextrose Broth at 27±2ºC, fungal mycelium mat of *C. capsici* was harvested for molecular characterization. The mycelial mat was stored at -20°C or - 80°C for long term storage. The total genomic DNA was extracted using modified protocol originally developed by Doley and Doley (1988). After isolation of DNA quantification was done with the help of Nanophotometer.

Gel Electrophoresis as Quality Test

Amplification products were separated on 1.2 % agarose gels in 1 x TAE stained with ethidium bromide (EtBr) and photographed were taken by using gel documentation system (Sharma *et al., 2005*) 4 µl (10ng / ml) concentrated EtBr solution was added per ml melted agarose at the time of pouring agarose in the DNA quality test.

Molecular characterization

The molecular identification of *Colletotrichum* species involved in the infection of chilli fruit was conducted through polymerase chain reaction amplification (PCR) of sequences from the inter generic region of ribosomal DNA. The specific primers were used, ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) for *C. capsici,* CaInt2 (5´-GGTGGTAAGGCCTCTCGCGG-3') for *C. acutatum* and C-gInt (5´-GGCCTCCCGCCTCCGGGCGG-3´) for *C. gloeosporioides* and combined with as a universal primer by following the amplification conditions suggested by Freeman *et al.* (2000) and Afanador *et al.* (2003). For the isolates of *colletotrichum* species amplification was carried out in a final volume of 25 µL containing the Taq Polymerase Promega® buffer (1X) (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1% Triton® X-100); 1.5 mM de MgCl2; 200 µM from each dinucleotide (dATP, dCTP, dGTP, and dTTP (Promega®), 0.3 µM of each primer; one unit from the Taq Polymerase Promega® enzyme, and 40 ng from the DNA sample (Williams *et al.,* 1990). The amplification profile used consisted of an initial 5-min cycle at 95°C, followed by 40 cycles at 95°C for 30 s, at 58°C, and a final extension cycle at 72°C for 7 minutes.

The amplification products were visualized through electrophoresis in 2% agarose gels stained with ethidium bromide from a 1 mg mL-1 solution at 90 V/5 h for 30 min and visualized gel documentation system. *Colletotrichum* species isolates with which a similarity analysis was performed by using the NTSYS program version 2.1 (Rohlf, 2000). The estimations of the similarity analysis were calculated with dice coefficient, also known as the Nei-Li coefficient. The resulting similarity matrices were then analyzed by the SAHN program to construct dendrogram using the Unweighted Pair Group Method with Arithmetic Mean "UPGMA". Complementarily, a multiple correspondence analysis (MCA) was performed via the SAS statistical program (SAS Institute Inc., 2000) to visualize the multidimensional representation of individuals. The analysis of molecular variance (AMOVA) was also done to establish the relationship among the isolates collected from different place of India, examine the genetic distances among the corresponding isolates to each host, and determine the relationship among the isolates belonging to each species involved. This statistical program was also used to determine the overall diversity among the populations.

Results and discussion

The sampling method used in this study represented the geographic diversity of fruit production for whole of Uttar. A total of 17 isolates resulted from this sampling, which was morphologically typified for their preliminary assignment to the *C. capsici*. *C. acutatum*, *C. gloeosporioides*. Molecular identification of *Colletotrichum* isolates amplified with specific primers revealed a 490-pb DNA fragment with the CaInt2/ITS4 primer combination for the *C. acutatum* species, and 450 bp for *C.*

gloeosporioides using CgInt/ITS4 primers (Chakraborty *et al.*, 2013). The amplification products were visualized through electrophoresis in 1.5% agarose gels treated with ethidium bromide (Afanador *et al.*, 2003). Eight RAM primers were used to amplify specific sequences (Table 2) using amplification conditions described by Ganley and Bradshaw (2001). The reactions of amplification consisted in a final 25µL volume composed of 1.25 µL of Taq Polymerase Promega® buffer (10X) (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1%. Triton® X-100); 1 µL of MgCl2 25 mM; 0.75 µL of the RAM primer 10 µM, 2 µL of dNTP 1.25 mM (dATP, dCTP, dGTP, and dTTP Promega®), 0.5 µL of the Taq polymerase enzyme Promega®, and 1 µL of the DNA sample at a 5-ng concentration, and carried out in a thermocycler programmed with different cycles, specific for each of the RAM primers (Hantula *et al*., 1996).

The morphological tests made for the same isolates permitted unequivocally assigning each isolate to the corresponding species. According to these tests, isolates *Colletotrichum species* were identified as 63% *C. capsici,* 16% *C. acutatum* and 21% as *C. gloeosporioides* (Reyes *et al*., 2007).

Acknowledgement

We duly acknowledge, Division of Crop Improvement & Biotechnology, Indian Institute of Vegetable Research (IIVR) Sahansahpur Jakhni, Varanasi, for support.

References

Bailey JA, Jeger MJ (1992) Colletotrichum: Biology, Pathology and Control. CAB International. Wallingford, UK.

Cannon PF, Bridge PD, Monte E, Linking the Past, (2000) Present, and Future of *Colletotrichum* Systematics. In: Prusky D, Freeman S, Dickman M, editors. *Colletotrichum:* Host specificity, Pathology, and Host-pathogen Interaction. St. Paul, Minnesota: APS Press. pp. 1–20.

Chakraborty S, Vhora Z, Trivedi R, Ravikiran R, Sasidharan N (2013) Molecular studies of *Colletotrichum capsici*. Genotypes for quality traits using microsatellite markers. The Bioscan. 8(2): 359-362.

Daher RF, Pereira MG, Tupinamba EA, Amaral-Júnior AT, Aragão WM, Ribeiro FE, Oliveira LO, Sakiyama NS (2002) Assessment of coconut tree genetic divergence by compound sample RAPD marker analysis. Crop Breed. App. Biotech. 2: 431-438.

Freeman S, Katan T, Shabi E. (2000) Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. Plant Disease. 82(6):596–605

Ganley RJ, Bradshaw E (2001) Rapid identification of polymorphic microsatellite loci in a forest pathogen, Dothistroma pini, using anchored PCR. Mycol. Res. 105, 1075-1078.

Gunnell PS, Gubler WD (1992) Taxonomy and morphology of Colletotrichum species pathogenic on strawberry. Mycologia 84: 157-165.

Hantula J, Dusabenyagasani M, Hamelin R (1996) Random Amplified Microsatellites (RAMS) – a novel method for characterizing genetic variation within fungi. Eur. J. Plant Pathol. 26, 159-166.

Kelemu S, Badel J, Moreno C, Miles J, Chakraborty S, Fernandes C, Charchar M (1994) Biodiversity, epidemiology and virulence of *Colletotrichum gloeosporioides*. I. Genetic and pathogenic diversity in *Colletotrichum gloeosporioides* isolates from Stylosanthes guianensis. Trop. Grasslands 31, 387-392.

Kim KD, Oh BJ, Yang J *(*1999) Differential interactions of a *Colletotrichum gloeosporioides* isolate with green and red pepper fruits. *Phytoparasitica* 27:1–10.

Mnadhar JB Hartman GL, Wang TC (1995) Anthracnose development on pepper fruit inoculated with *C. gloeosporioides* disease 79:380-383.

Park KS, Kim CH (1992) Identification, distribution, and etiological characteristics of anthracnose fungi of red pepper in Korea. Korean Journal of Plant Pathology. 8:61–69.

Palomino EC, Mori ES, Zimback L, Tambarussi EV, Moraes CB (2005) Genetic diversity of common bean genotypes of Carioca commercial group using RAPD markers. Crop Breed. App. Biotech. 5: 80-85.

Ratanacherdchai K, Wang HK, Lin FC, Soytong K (2010) ISSR for comparison of cross-inoculation potential of Colletotrichum capsici causing chilli anthracnose. African J. Microbiol.Res. 4: 76-83.

Rohlf FJ (1993) NTSYS-pc: Numerical taxonomy and multivariate analysis system, v. 2.0. Exeter Software. Setauket, New York.

Royle DJ, Butler DR (1986) Epidemiological Significance of Liquid Water in Crop Canopies and its Role in Disease Forecasting. In: Ayres PG, Boddy L, editors. Water, Fungi and Plants. Cambridge: Cambridge University Press; pp. 139–156.

RGAS, Rajapakse (2007) Mode of anthracnose development in chilli (*C. annum* L) pods ,Fild /2

Ratanacherdchai HK, Wang FC Lin, Soytong K (2009) RAPD analysis of *Colletotrichum* species causing chilli anthracnose disease in Thailand. Journal of Agricultural Technology.; (**3**):211–219.

Sanders GM, Kirsten L (2003) Comparision of cross inoculation potential of South African avocado and mango island of *Colletotrichumgloeosporioides*. Microvial.Res.158(2);143-150.

Sharma PN, Kaur M, Sharma OP, Sharma P, Pathania A (2005) Journal of Phytopathology. AB Fruit rot of chillies (*Capsicum annuum* L.), caused by *Colletotrichum capsici* under tropical and subtropical conditions, Department of Plant Pathology, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur 176 262(HP), India. 153: (*4*), 232-237.

Sutton BC (1990) The Coelomycetes. Commonwealth Mycological Institute. Kew, UK

Sreenivasaprasad S, Talhinhas P (2002) Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. Molecular Plant Pathology.;**6**(*4*):361–378.

Talhinhas P, Sreenivasaprasad S, Neves-Martins J, Oliveira H (2005) Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. Microbiology. 71: 2987- 2998.

Tinker NA, Fortin MG, Mather DE (1993) Random amplified polymorphic DNA and pedigree analysis in spring barley. Theor. Appl. Genet. 85: 976-984.

Williams JG, Kubelik AR., Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18: 6531-6535.

Yun HK, Ahmad AH, Muid S, Selan JSS (2009) First of *Colletotrichum* spp. B causing disease on capsicum spp. In Saah, Borneo, Malasia.