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Genetic variability in *Albugo candida* pathogen isolates collected from Indian mustard in Northern Madhya Pradesh using RAPD marker analysis

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Abstract
Rapeseed-mustard group of crops are moist important rabi oilseed crops cultivated in Northern region of Madhya Pradesh. Indian mustard [*Brassica juncea* (L.) Czern and Coss] is the dominating crop occupying >80% share of the total area of rapeseed-mustard in the region. White rust incited by *Albugo candida* ((Pers. ex. Lev.) Kuntze) is one of the major biotic constraints of Indian mustard in the state. The white rust infected leaf samples were collected from five major mustard growing districts of Madhya Pradesh viz., Morena, Bhind, Sheopur, Gwalior and Datiya to find out the genetic variability occurring in the pathogen across the region. To show genetic distance among fungal isolates, banding pattern of RAPD marker was used to construct phylogenetic tree by distance matrix analysis. The phylogenetic tree was clearly showed genetic variation in different isolates Genetic variability showed by five isolated of *Albugo candida* illustrated in dendrogram, framing three major cluster, in which Isolate L5 was completely different from rest of the isolates and made separate cluster. All other samples were also significantly different from each other i.e. L4(25%),L2(35%) and L1 & L3(67%).

Keywords: *albugo candida*, Indian mustard and RAPD marker analysis

Introduction
Rapeseed-mustard group of crops are the major rabi oilseed crops of the India. The group comprising of Indian mustard (*Brassica juncea*). Indian mustard (*Brassica juncea*) is dominating oilseed crop of the Country. In Madhya Pradesh, *Brassica juncea* occupying more than 75% share in respect of total area and production of the state. The area, production and productivity of rapeseed-mustard in the country was 5.8 million ha, 6.3 million tonnes and 1083kg/ha, respectively (Anon. 2016) [2]. *B. juncea* is the most important oilseed crop of Northern Madhya Pradesh comprising Morena, Bhind, Gwalior, Sheopur and Datia districts contributing more than 80% share in the production of this crop in the state.

Disease such as White rust, Downy mildew, *Alternaria* blight and *Sclerotinia* stem rot are the major biotic constraint *of Brassica juncea* cultivated in state. White rust or white blister, caused by the oomycete *Albugo candida* (Pers.) Kuntze, is a disease of many *Brassicaceae* species, including the condiment mustard and oilseed crop *Brassica juncea*. The pathogen produces localized lesions on the leaves and systemic infection which results in distorted, sterile inflorescences (called stag heads). Resistance to the *B. juncea* attacking race 2 was introduced into Canadian *B. juncea* germplasm from brown and oriental *B. juncea* accessions (Petrie, 1988) [6]. However, in Canada in 1989 a pathotype of race 2 that attacked the three commonly grown resistant *B. juncea* cultivars was discovered (Petrie, 1994) [7]. The virulent isolate of race 2 was thereafter referred to as 2A and the "new" virulent pathotype was designated 2V (Petrie, 1994; Rimmer *et al*., 2000) [7, 9]. Resistance to pathotype 2V has not been identified in *B. juncea* germplasm, although resistance to 2V in *B. napus* has been introgressed into *B. juncea* (Franke *et al*., 1999) [4]. The pathogenic variability has not been studied in the Madhya Pradesh hence the studies was carried out to know to genetic variability among the white rust infected samples collected from major growing districts of the state.

Material and Methods

Plant material Leaf samples of Indian mustered Infected with *Albugo candida*, were collected from Datiya (L1), Morena (L2), Bhind (L3), Sheopur (L4) and Gwalior (L5) District of Madhya Pradesh. Infected leaf samples were collected at vegetative growth stage of plant and stored at -80°C.

Methodology

A. DNA Isolation

Genomic DNA was isolated from all the five fungal infected leaf samples by Minispin kit method (RKN9/10).

Detail Protocol used for DNA isolation is given below

1. Taken 500 mg of the fungal infected leaves.
2. 1X 500 µ suspension buffer was added into the pestle and crush with mortar.
3. Fungal infected leaves were crushed in the liquid with mortar for about 3-5 mins. Transfer the crushed liquid into 2 ml vial.
4. 250µl suspension buffer was added to rinse the mortar and ensured that entire ground material was taken into the vial.
5. It was mixed 5-6 times by inverting the vial. Placed it at 65°c for 10 min with intermittent mixing.
6. 1ml of lysis buffer was added in vials and mixed 5-6 times then Kept the mixture at 65°C for 15 min.
7. Spin at 10,000g for 1min at Room Temperature and the clear supernatant was collected in a 2ml vial.
8. Supernatant was loaded on the spin column (600 µl each time).
9. Spin at 10,000g for 1min at Room Temperature and the contents were discarded of the collection tube. It was placed the spin column back in the same collection tube.
10. 500 µl of 1X wash buffer was added to the column and spin at 10,000g for 1min at room temperature then discarded the contents of the collection tube. Placed the spin column back in the same collection tube.
11. Step 10 was repeated.
12. The empty column was spin with the collection tube at 10,000g for 3 min at Room Temperature.
13. The spin column was placed in a fresh vial.
14. 25 µl of warm elution buffer was added (already kept at 65°C) into the spin column. Ensure the elution buffer was added to the centre of the membrane
15. It was kept the vial along with the spin column at 65°C for 1min then spin at 10,000g for 1 min at room temperature.
16. Step 14 and 15 were repeated then elute and collect in the same vial.

DNA Quantification

Quality and Quantity of genomic DNA of all the five samples were checked by agarose gel electrophoresis using1%Agarose. The amount of DNA is a solution is proportional to the fluorescence emitted by ethidium bromide in that solution. For quantification, 1µl of DNA samples were loaded, along with known quantity of λ uncut DNA (100ng, 200 ng) on 0.8% agarose gel. Carry out the electrophoresis at 70-80 volts for 45-90 min. stained DNA with ethidium bromide and observed under UV. Determined the quantity of DNA by comparing the florescence of sample with that of standards after quantification, the diluted the DNA with TE buffer such that the final concentration of DNA to 20 ~ 25 ng/µl.

Polymerase chain Reaction

The specific primers of the 3 RAPD marker described below was used for amplification of 5 fungal infected leaf samples of Indian mustard collected from 5 different District. Amplification was be carried out in 25 µl reaction volume containing 10 mMTris-buffer (10XTaqPol Assay buffer), 10 mM of each dNTPs, 3-unitTaq DNA polymerase, 100ng/ul of primer,50 ng of genomic DNA and water. Amplification reactions was performed in Thermal Cycle with the following thermal profile: initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min (denaturation), 45°C for 1 min (primer annealing for RAPD) and72°C for 2 min (primer extension), with a final extension at 72°C for 5 min. PCR products will be resolved on 2% agarose gel at 80 V for 3.5h.

PCR amplification was performed by using three RAPD primers;
Primer1:5'CCCHGCAMCTGMTCGCACHC3'
Primer2:5'AGGHCTCGATAHCMGVY3'
Primer3:5'MTGTAMGCTCCTGGGGATTCHC -3'.
Genetic analysis of PCR products were conducted on ABIC3130 genetic analyzer. The Binary output was used to generate phylogenetic relationship between groups.
Reaction condition for PCR master mix preparation and PCR cycle condition, used for amplification of RAPD primer is given below.

Table 1: PCR Reaction conditions

gDNA (50ng)	0.5 µl
RAPD primer (100ng/ µl)	2.0 µl
dNTPs (10mM)	1.0 µl
Taq Pol Assay buffer (10X)	2.5 µl
Taq DNA Polymerase (3U/µl)	0.5 µl
Water	18.5 µl
Total	25.0µl

Table 2: PCR Cycle conditions

94 °C	94 °C	45 °C	72 °C	72 °C
5 min	1 min	1 min	2 min	5 min
	40 Ycles			

Statistical analysis of Molecular marker

A binary matrix was compiled using numerical system of multivariate analysis. The dendrogram 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

Genetic variability through Random amplified polymorphic DNA

Genomic DNA of different fungal infected leaves having concentration of

DNA 20 to ~ 25 ng/µl was used for amplification of RAPD primers (Fig. 1). All the three Primer set shows significant polymorphism among the fungal isolates (Fig 2A, B & C).

Ladder 100bp and 500bp was used as size standard.

100bp ladder: Contains 10 DNA fragments of size100bp, 200bp, 300bp, 400bp, 600bp, 700bp, 800bp, 900bp and 1kb (Fig. 3).

500bp ladder: Contains 10 DNA fragments of size 500bp, 1000bp, 1500bp, 2000 bp, 2500bp, 3000bp, 3500bp, 4000bp, 4500bp and 5000bp (Fig. 4).

The references fungal isolates were marked as L5 (sample-5) among all the other samples. The value of L5 was indicated as 100%. The genetic distance showed for L4 (sample-4) was 25% and L2 (sample-2) was 35%. Further L1 and L3 (sample-1 and sample-3) were 67% similar to each other. To find out the genetic variability occurring in the pathogen across the region. To show genetic distance among fungal isolates, banding pattern of RAPD marker was used to construct phylogenetic tree by distance matrix analysis. The phylogenetic tree was clearly showed genetic variation in different isolates Genetic variability showed by five isolated of *Albugo candida* illustrated in dendrogram, framing three major cluster, in which Isolate L5 was completely different from rest of the isolates and made separate cluster. All other samples were also significantly different from each other i.e. L4 (25%),L2(35%) and L1 & L3(67%).

Table 3: Similarity Distance Matrix of RAPD analysis

	L1	L2	L3	L4	L5
LI		0.80556	0.63380	0.89796	0.89041
L2	0.80556		0.79592	0.97368	0.84314
L3	0.63380	0.79592		0.97333	0.96000
L4	0.89796	0.97368	0.97333		0.89610
L5	0.89041	0.84314	0.96000	0.89610	

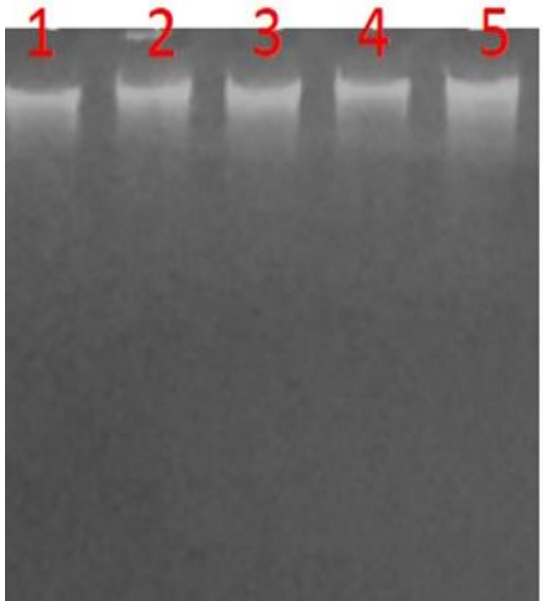

Fig 1: Genomic DNA concentration of five different leaf samples (L1; 2, L2; 3, L3; 4, L4; 5,)

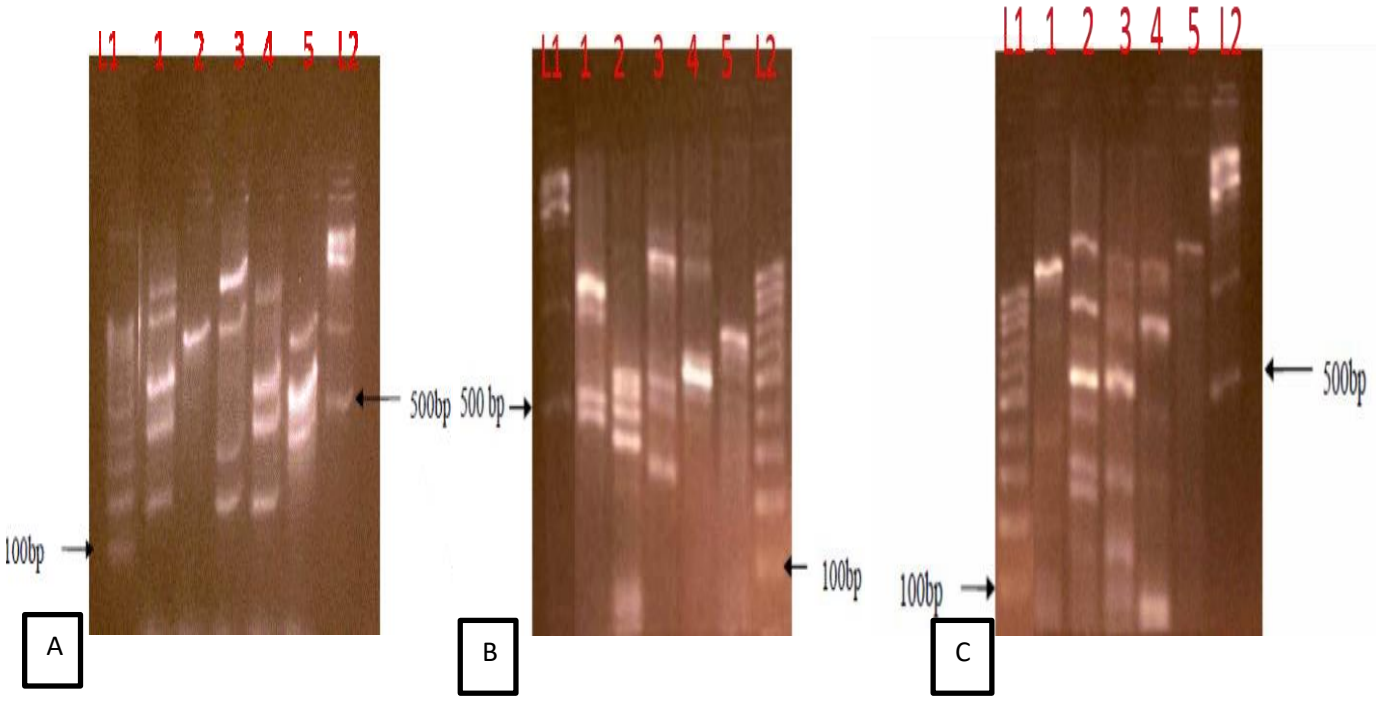

Fig 2: Gel pictures showing RAPD profile of five samples with Primer 1(A), Primer 2 (B) and Primer 3 (c) (L1: 500 bp ladder, 1: sample from L1, 2: Sample from L2, 3: Sample from L3, 4: Sample from L4, 5: Sample from L5).

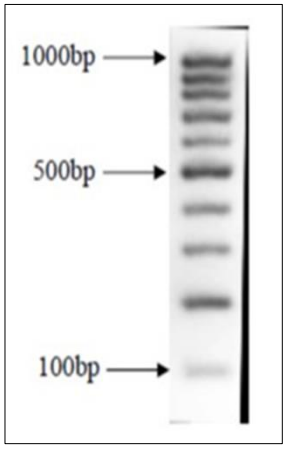

Fig 3: ladder 100 bp

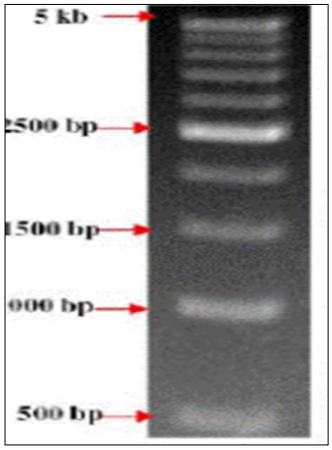

Fig 4: ladder 500 bp

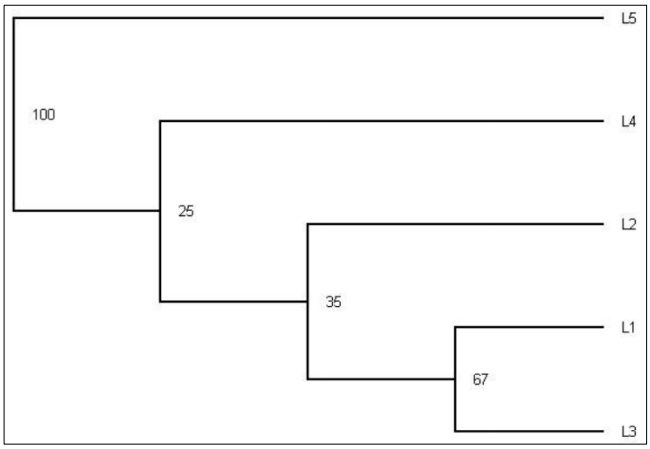

Fig 5: Phylogenetic tree of RAPD profile showing correlation between the Fungal infected leaf samples.

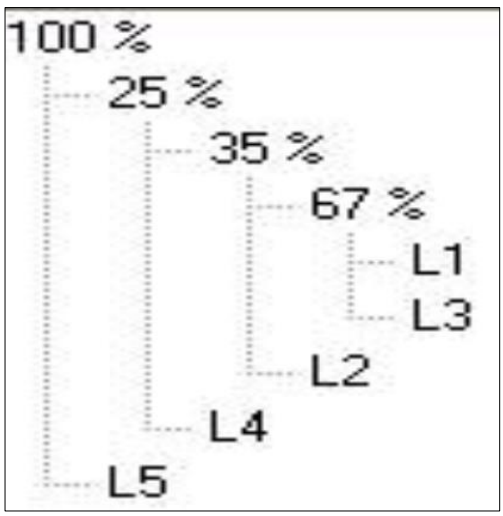

Fig 6: Reference Tree for fungal infected leaf samples

Discussion

In study of Choi *et al.* (2007) [3] A study of white rust pathogens on *C. bursa-pastor is* was performed on 36 specimens, using sequence analysis from the *cytochrome oxidase* subunit II (COX2) region of mt DNA, the internal transcribed spacer (ITS) region of the r DNA, and D1 and D2 regions of the nr DNA. Specimens were obtained from Asia (India, Korea, Palestine), Europe (England, Finland, Germany, Hungary, Ireland, Latvia, Netherlands, Romania, Russia, Sweden, Switzerland), North and South America (Argentina, Canada, USA), and Oceania (Australia). The molecular data indicated strong support for species partition separating Korean and other continental specimens. There were 7.25, 17.4, and 4.8% sequence dissimilarity between the two groups in COX2, ITS, and 28S regions, respectively. In our study, we have analyzed five isolates of *Albugo candida* from five different locations *i.e.* Datiya (L1), Morena (L2), Bhind (L3), Sheopur (L4) and Gwalior (L5). The references fungal isolates were marked as L5 (sample-5) among all the other samples. The value of L5 was indicated as 100%. The genetic distance showed for L4 (sample-4) was 25% and L2 (sample-2) was 35%. Further L1 and L3 (sample-1 and sample-3) were 67% similar to each other. Genetic variability showed by five isolated of *Albugo candida* illustrated in dendogram, framing three major cluster, in which Isolate L5 was completely different from rest of the isolates and made separate cluster. All other samples were also significantly different from each other i.e. L4 (25%), L2 (35%) and L1 & L3 (67%).

Similarily Petkowski *et al.* (2010) examined the genetic diversity within Australian collections of *A. candida* from various *Brassicaceae* species in a range of geographic locations. Phylogenetic analysis of 31 Australian *A. candida* collections from 11 hosts using the rDNA ITS region, rDNA LSU region and cox2 mtDNA showed that the majority of Australian *A. candida* collections were the common form of *A. candida.* One collection from a common weed host, hairy bitter cress (*Cardamine hirsuta*), was found to belong to a previously reported but undescribed species, while three collections, also from *C. hirsuta*, were found to belong to a new undescribed species.

Adhikari *et.al.* (2003) [1] studies the inheritance of a virulence and polymorphic molecular markers in *Albugo candida*, the cause of white rust of crucifers, and of race 2 (Ac2), using isolates MiAc2-B1 or MiAc2-B5 (metalaxyl in sensitive and virulent to *Brassica juncea* cv. *Burgonde*) with race 7 (Ac7), using isolate MsAc7-A1 (metalaxyl-sensitive and virulent to *B.rapa*cv. *Torch*). The parents and F1 progeny were examined for virulence on the differential cultivars *B. juncea* cv. *b urgonde* and *B. rapa* cv. *Torch*. Putative F1 hybrids were confirmed by random amplified polymorphic DNA markers specific for each parent. Torch suggested 3:1 in each of three populations, supporting the hypothesis of a single dominant a virulence gene.

Studied identify RAPD markers for white rust resistance in an F1 -derived doubled-haploid (DH) population originating from a cross between white rust-susceptible and white rust-resistant breeding lines of *B. juncea.* The DH population was used to screen for RAPD markers associated with white rust resistance/ susceptibility using bulked segregant analysis (Prabhu *et al.* 1998) [8]. We have used RAPD marker (Bharti 2016) for characterization of different isolates. All five isolates were significantly differ from each other by giving (25%, 35%, and 67%) dissimilarity. Genetic analyses using markers confer that these isolates are genetically different from each other.

Summary

The isolates of *Albugo candida were* collected from Morena, Bhind, Gwalior, Sheopur and Datia and in dendogram the fungal isolates were marked as L5 (Gwalior) among all other samples. The value of L5 was indicated as 100%. The genetic distance showed for L4 (Sheopur) was 25% and L2 (Morena) was 35%. Further L1 and L3 (Datiya and Bhind) were 67% similar to each other.

Conclusion

The isolates significantly differed from each other but the isolate (L5) was distinguished completely from other isolates (L1, L2, L3 and L4).

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