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Genetic Variability among the Isolates of *Alternaria brassicae* in Mustard of Northern Madhya Pradesh

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ABSTRACT

Background: Alternaria blight is most destructive fungal disease of oilseed which cause severe losses 47% in seed quality and quantity. The current research aimed to identify the specific pathogen species and its aggressiveness prevailing in M.P. area.

Methods: Isolates of *A. brassicae* from five districts *viz*., Gwalior-A1, Morena-A2, Bhind-A3, Datia-A4 and Sheopur-A5 were cultured on potato dextrose agar medium to study their cultural characteristic and genetically variability in 7 days old culture. Genomic DNA from different fungal isolate culture having concentration of DNA to ~25 ng/µl was used for amplification of RAPD primers.

Result: The distance matrix analysis was drawn phylogenetic tree by PCR data showed genetic distance among the fungal isolates. The phylogenetic tree was clearly showed isolated of *A. brassicae* illustrated in dendogram framing three major cluster, in which Isolate A1 was completely different from rest of the isolates and made separate cluster. All other samples were also significantly different from each other *i.e.* A4 (78%), A5 (37%) and A2 and A3 (94%).

Key words: *Alternaria brassicae*, DNA, Fungal disease, Genetic variability, Oilseed crop, PCR, RAPD.

INTRODUCTION

The production of oilseed *Brassicas* is infected by several fungal diseases. Alternaria blight being one of the most devastating and pervasive diseases. It is reported over wide geographical areas in the world, causing an average yield loss of up to 70% (Kolte, 2002). Forty two fungal pathogens are associated with rapeseed mustard out of these alternaria blight caused by *Alternaria brassicae* (Berk) Sacc. and *Alternaria brassicicola* (Schw.). Wiltsh, are the most important diseases causing heavy losses throughout the country attacking all *brassica species*. Four species of *Alternaria viz., A. brassicae* (Berk) Sacc. *A. brassicicola* (Schw.), *A. raphani* and *A. alternata*. Out of which *A. brassicae* is most widely prevalent in India (Bilgrami *et al.,* 1981). Variability studies are important to document the changes occurring in populations and individuals as variability in morphological and physiological traits and indicate the existence of different pathotypes.

In northern M.P. very meager work has been done on the molecular variability among Indian mustard. The variability is a well-known phenomenon in genus *Alternaria.* Many reports on the existence of the variability among different hosts have been reported earlier (Pryor and Gilbertson 2002: Pryor and Michailidesnt *Alternaria* species from (Quayyum *et al*., 2005) as also with *A. brassicae.* DNA (RAPD) molecular marker has become powerful tool to study taxonomy and molecular genetics of a variety of organisms. The aim of present investigation is demonstrated the specific pathogen species and its aggressiveness prevailing in particular area.

MATERIALS AND METHODS

The laboratory work was done in the Department of Plant Pathology and Department of Agriculture Biotechnology, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya, College of Agriculture, Gwalior, Madhya Pradesh and 2016-17.

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Isolation, purification and identification

One hundred samples of Alternaria blight-infected leaves from various *Brassica* species were collected from the major northern MP 2106-17 in India. The diseased samples collected during survey from various locations and cultured in the laboratory. The surface of diseased leaves was sterilized with 0.1% mercuric chloride for 1 minute (Clarkson 2003). The sterilized leaves were cut into two-three pieces with the help of sterilized blade in sterilized petri plate and transferred to potato dextrose agar (PDA) culture and incubated at 25±1°C. The pathogen (*Alternaria brassicae*) mycelium disc (5 mm) taken from five days old culture and transferred into sterilized poured petri plates. Purification of the isolated fungus was carried out by single spore technique. Purified pathogen was used for examination at frequent intervals Identification was done based on its morphological and taxonomical characters. Pure cultures were obtained by repeated sub-culturing

followed by single-spore isolation. The purified *Alternaria brassicae* isolates were observed microscopically under 40x optical microscope and identified based on conidia shape.

DNA extraction from isolate

Five isolates of *A.brassicae* were grown on potato dextrose agar medium and incubated at 25± 2°C for 7 days. A vigorous margin mycelium disc were transferred to 250 ml flasks containing 100 ml potato dextrose broth (PDB) to allow to growth of mycelium mate. Mycelium mate was aseptically harvested by Whatman filter paper in laminar air flow chamber. Mycelium mate crushed in liquid nitrogen with the help of pre chilled pastle mortar. Modified CTAB (Cetyltrimethylammonium bromide) method was used for DNA isolation. Purified genomic DNA was stored at -20°C and quantification was done using Nano-Drop spectrophotometer (Weising *et al*., 1995).

Genomic DNA isolation and PCR amplification

Quality and quantity of genomic DNA of all the five samples were checked by agarose gel electrophoresis. For quantification, 1 µl of DNA samples were loaded, along with known quantity of λ uncut DNA (100 ng, 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 (Weising *et al*., 1995).

The specific primers of the 3 RAPD marker described below was used for amplification of 5 fungal infected leaf samples of Indian mustard collected. Amplification carried out in 25 µl reaction volume containing 10 mM Tris-buffer (10X TaqPol Assay buffer), 10 mM of each dNTPs, 3 unit Taq DNA polymerase, 100 ng/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) and 72°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 3.5 h.

PCR amplification was performed by using three RAPD primers:

Primer 1: 5 CCCHGCAMCTGMTCGCACHC 3.
Primer 2: 5 AGGHCTCGATAHCMGVY 3.
Primer 3: 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.

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).

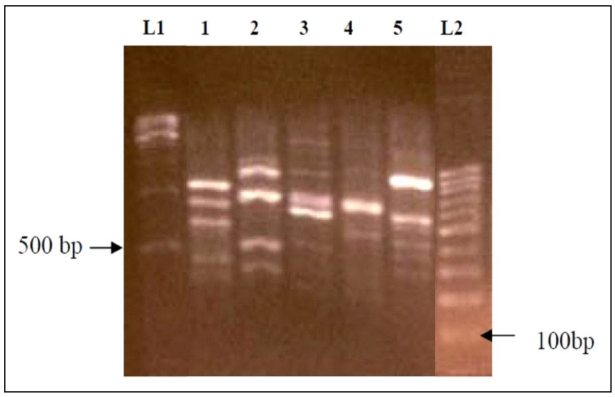
RESULTS AND DISCUSSION

Genetic variability through random amplified polymorphic DNA

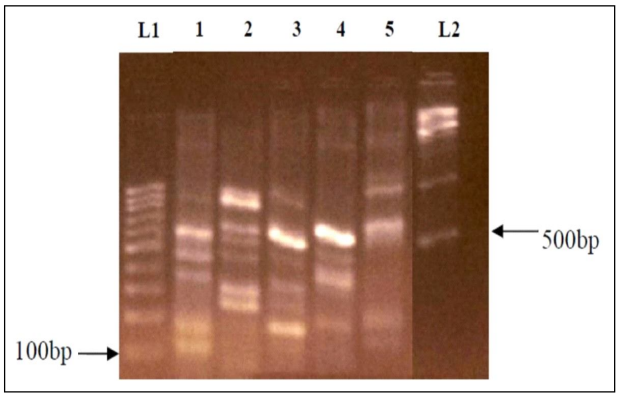
The current research provides the information about the genetic variability in different isolates of different district of Northern M.P. The result showed that the phylogenetic tree was clearly showed genetic variation in different isolates of Northern Madhya Pradesh. Genomic DNA for different fungal isolates extract having concentration of DNA to DNA to ~ 25 ng/µl was used for amplification of RAPD primers. The analysis of the similarity matrix data for *Alternaria brassicae* isolates revealed a high level of the diversity among all the isolates from different districts of Northern M.P. Molecular techniques like RAPD have been used to study the genetic variations in fungi at genus, species and/or subspecies levels (Gherbawy and Abdelzaher, 2002). Although some work has been reported on diversity of *Alternaria* species in other crops in India (Gherbawy and Abdelzaher, 2002) and Goyal *et al*., 2011). There exists only a single report on diversity of A. brassicae isolates pathogenic to Brassica species in India and that too with very small number of isolates (Kaur *et al.,* 2007).

Maximum genetic diversity was obtained in A1-Gwalior 100% followed by A4-Datia 78% and A5-Sheopur was 37%. Further A2-Morena and A3-Bhind were 94% similar to each other. The distance matrix analysis was drawn phylogenetic tree by PCR data showed genetic distance among the fungal isolates (Fig 1). Analysis of RAPD banding profiles also showed a high level of genetic diversity varying between 57-78%, 78-92% and 89-100%, among the *A. brassicae, A. brassicicola* and *A. alternata* isolates, respectively. Extensive variations were observed in isolates for all the parameters studied, but no correlation could be established. This study, thus indicates that a significant non-specific variation exist between isolates infecting different species and varieties of Brassica in India (Aneja *et al*. 2014).

The phylogenetic tree was clearly showed genetic variation in different isolates. Genetic variability showed by five isolated of *A. brassicae* illustrated in dendogram, framing in which isolate isolate 1 was completely different from other four of the isolates and form separate cluster. All other samples were also significantly different from each other *i.e*. isolate 4 (78%), isolate 5 (37%) and isolate 2 and isolate 3(94%) (Fig 2). Earlier studies based on symptoms, assumed that a quantitative form of resistance governed by polygenes or minor genes exists (Krishnia *et al.,* 2000). Further molecular analysis of the current isolates is to investigate order to make a reliable inference for the pathogen behavior a detailed study using large numbers of isolates of different *Alternaria* species from various *Brassica* species spread over a wide geographical location was lacking. Aggressiveness and genetic diversity of *Alternaria species* isolates infecting oilseed *Brassica* in Northern M.P.

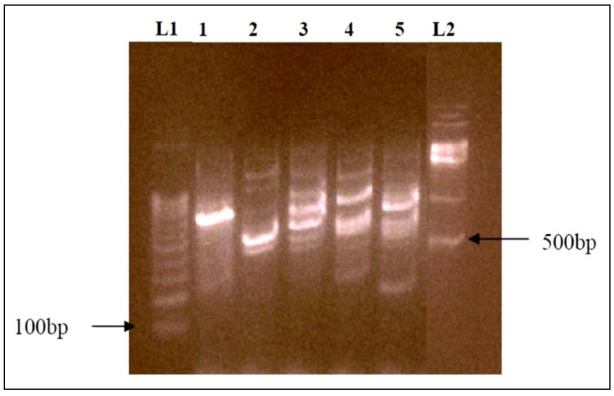


RAPD primer 1 polymorphism expressed by isolate of *Altemaria brassicae.*
Lane description-L1 500 bp ladder, Isolate 2, Isolate 3, Isolate 4, Isolate 5, L2 100 bp ladder.

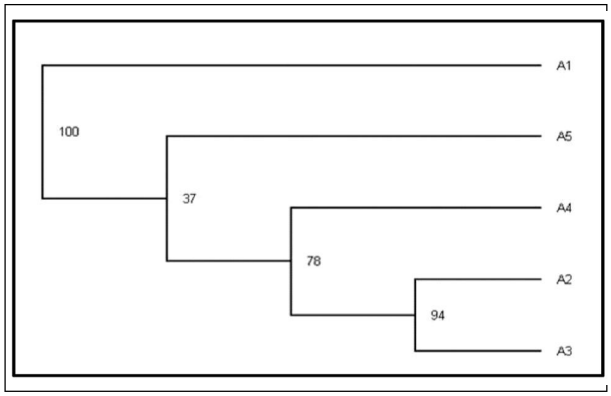


RAPD primer 1 polymorphism expressed by isolate of *Altemaria brassicae.*
Lane description-L1 500 bp ladder, Isolate 2, Isolate 3, Isolate 4, Isolate 5, L2 100 bp ladder.

Fig 1: Analysis of RAPD Primer 1 and 2 polymorphism expressed by isolates of *Alternaria.*



Dendogram showing grouping of Alternaria brassicae based on RAPD Polymorphism.



RAPD primer 1 polymorphism expressed by isolate of *Altenaria brassicae.* Lane description-L1 500bp ladder, Isolate 2, Isolate 3, Isolate 4, Isolate 5, L2 100bp ladder.

Fig 2: Analysis of RAPD Primer 3 polymorphism expressed by isolates of *Alternaria brassicae.*

The current research provides the information about the genetic variability in different isolates of different district of Northern M.P. The result showed that the phylogenetic tree was clearly showed genetic variation in different isolates of Northern Madhya Pradesh. Genetic variability showed by five isolated of *A.brassicae* illustrated in dendogram, framing in which isolate 1 was completely different from other four of the isolates and form separate cluster. All other samples were also significantly different from each other *i.e.* isolate 4 (78%), isolate 5 (37%) and isolate 2 and isolate 3 (94%). Extensive variations were observed in isolates for all the parameters studied, but no correlation could be established. This study indicates that a significant non-specific variation exist between isolates infecting different species and varieties of *Brassica* in India (Aneja *et al.,* 2013). The *Alternaria* isolates of all the three species in this study showed extensive variations in their morphological and cultural characters. This is in agreement with the earlier reports on *A. brassicae* (Kumari *et al*., 2012; Goyal *et al*., 2011).Although some work has been reported on diversity of *Alternaria* species in other crops in India there exists only a single report on diversity of *A. brassicae* isolates pathogenic to *Brassica* species in India and that too with very small number of isolates (Kaur *et al*., 2007).

CONCLUSION

In order to investigate ,five isolates of *Alternaria brassicae* infected leaves were collected from the different five district of Northern M.P. Based on culture and molecular characterization pathogen were identified. The result indicate that the phylogenetic tree was clearly showed genetic variation in different isolates of Northern Madhya Pradesh. Genetic variability showed by five isolated of *A .brassicae* illustrated in dendogram, framing in which isolate A1 was completely different from other four of the isolates and form separate cluster. The present study may

also suggest similar inference, but conclusively showed that the diversity observed between and among two *Alternaria* species (*brassicae* and *brassicicola*) infecting Brassica crops in M.P.

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Conflict of interest: None.

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