

Original Research Article

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## Molecular Characterization of *Alternaria alternata* Causing Fruit Rot of Chilli through RAPD Marker

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### ABSTRACT

#### Keywords

Random Amplification of Polymorphic DNA, Molecular characterization, *Alternaria alternata*, Chilli, fruit rot

#### Article Info

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Molecular variation in chilli fruit rot pathogen (*Alternaria alternata*) was analyzed by using six isolates collected from major chilli growing region of Maharashtra. The genomic DNA extracted from each isolate of *A. alternata* was subjected to polymerase chain reaction using 15 primers of OPA and OPB series off which 10 primers produced 83 scorable bands with size ranging from Among the RAPD primer 83 bands 80 bands were polymorphic and level of polymorphism was 96.38%. Molecular diversity using RAPD marker showed that the Aa2 (Satara) having higher similarity index with Aa4 (Akola). Dendrogram generated by pooled molecular data of 10 RAPD primers formed two clusters namely 'A' and 'B'. cluster A include Aa1 and cluster B include Aa2, Aa4, Aa3, Aa5 and Aa6. Thus, the molecular characterization of eight isolates of *A. alternata* by RAPD revealed existence of variations.

### Introduction

Chilli (*Capsicum annum* L.) is one of the most important commercial spice and export crop originated from Tropical America.

It is grown throughout the world for its green and red ripe fruit.

The crop is vulnerable to many diseases and pests due to its extreme delicacy and succulence. Diseases caused by fungi, bacteria and viruses are major constraints to chilli production. Among the fungal diseases, fruit rot is a major constraint in chilli causing several losses in terms of quality and quantity

(Sreekantiah *et al.*, 1973). In India, the first report of *Alternaria* sp. was made from Delhi by Dutt in 1937. Narain *et al.*, (2000) reported *Alternaria alternata* causing fruit rot of chilli on fruits. The present study carried out to ascertain molecular polymorphism in different isolates of *Alternaria alternata*.

### Materials and Methods

The experimental materials comprising eight *Alternaria alternata* isolates viz., Kolhapur (Aa1), Satara (Aa2), Sangli (Aa3), Akola (Aa4), Nagpur (Aa5) and Amravati (Aa6) collected from different chilli growing districts of Maharashtra.

### **DNA isolation of *A. alternata***

The DNA was extracted from eight isolates of *Alternaria alternata*. Potato dextrose agar medium was used to culture the isolates and mycelia were harvested after 7 days of incubation as described by Coddington and Gould (1992). DNA extraction of the isolates of *A. alternata* and their further molecular characterization were done by Cetyltrimethyl ammonium bromide (CTAB).

### **Protocol for isolation of fungal genomic DNA**

The pure culture of fungus grown on potato broth 200 ml in 500 ml conical flask for seven days at a temperature of  $27 \pm 2^\circ\text{C}$  in BOD incubator.

The mycelial mat was harvested after seven days.

It was wash thoroughly and repeatedly and then dried using blotter paper and crushed to powdered form in pre-chilled pestle and mortar with liquid nitrogen. The powdered mass was immediately homogenized by adding pre-warmed ( $65^\circ\text{C}$ ) 1 ml of CTAB extraction buffer (100 mM TrisHCl pH 8.0, 20 mM EDTA 1.4 M NaCl, 0.4 %  $\beta$ -mercaptoethanol and 2 % w/v CTAB) per tube and the content was mixed gently by inversion.

The mixture was incubated at  $65^\circ\text{C}$  for one hour in hot water bath with intermittent shaking by gently inverting the tube after 10 minutes.

The tubes containing homogenate were centrifuged at 8000 rpm for 15 minutes.

The supernatant was transferred into another 2 ml eppendorf tubes without disturbing the pellet of cell debris.

Then equal volume (1 ml) of chloroform isoamylalcohol (24:1) was added and mixed gently but thoroughly to emulsify both the components for five minute.

Centrifugation was carried out at 12000 rpm for 15 min.

The upper aqueous phase was transferred into another 2 ml eppendorf tube.

Equal volume of ice-cold isopropanol was added and mixed by inversions. CTAB-DNA complexes formed at the bottom of the eppendorf tubes.

After mixing with isopropanol, the sample were kept at  $4^\circ\text{C}$  for 10 minutes and then centrifuged at 10000 rpm for 10 minutes.

After centrifugation a pellet was formed at the bottom of the eppendorf tube.

The supernatant was removed and the pellet was washed with 70 % ethanol twice and centrifuged at 8000 rpm for 5 minutes.

The pellet was air-dried for 30-60 minutes and then dissolved in 0.5 ml of TE buffer.

The pellet was allowed to dissolve completely overnight at  $4^\circ\text{C}$  without agitation.

Quantification and purity of DNA was checked on 0.8% agarose gel at voltage of 60V/cm by using 1X TBE buffer and ethidium bromide (0.5 mg/ml) staining. After completion of 5 cm run, the gel was observed under UV light and the DNA yield and quality was confirmed.

### **Internal Transcribed Spacer (ITS) amplification**

Genetic variability of *Alternaria alternata* was evaluated by using ITS primers (Table 1).

### **Procedure for PCR reaction**

Sterile PCR tubes were numbered and placed on PCR tube stand. At first 2 µl of DNA was added to each PCR tube followed by master mix given in Table 2 and 3.

The samples were mixed by brief centrifugation to bring down the content of tube. PCR were run on the programmable thermal cycler given in Table 4.

PCR products were separated by electrophoresis in 2 per cent agarose gels run in 1x TBE, stained with ethidium bromide and visualized with a UV transilluminator.

### **Random Amplification of Polymorphic DNA analysis**

A total of 15 OPA and OPB series were screened for RAPD analysis. The list of RAPD primers was used for the analysis of random amplification of polymorphic DNA (Table 8) to study the polymorphism present in the isolates of *A. alternata*.

### **Procedure for PCR reaction**

Random Amplified Polymorphic DNA (RAPD) polymerase chain reaction (RAPD-PCR) procedure was performed as previously described method by Williams *et al.*, (1990) with some modification in a reaction mixture given in Table 5 and 6. Amplification products were separated on 1.2% agarose gel in 1x TBE buffer at 70 V for about 2 hour.

The PCR tubes containing reaction mixture were placed in the thermal cycler for 40 cycles with the following profiles:

### **Data analysis**

The gel images were captured and visualized in gel documentation system. The data was

scored as the presence (1) or absence (0) of individual band for each isolates in RAPD-PCR analysis of isolates of *Alternaria alternata*.

The data was used to generate similarity coefficient using simple matching coefficient based on RAPD bands scoring. The Dice coefficient between each pair of accessions was then used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetic Average (UPGMA).

### **Results and Discussion**

The isolates of *Alternaria alternata* were obtained from different agro climatic regions of Maharashtra. The six isolates viz., Kolhapur (Aa1), Satara (Aa2), Sangli (Aa3), Akola (Aa4), Nagpur (Aa5) and Amravati (Aa6), were selected for the analysis. The *Alternaria alternata* specific ITS primers pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used for molecular characterization of the isolates. All the isolates of *Alternaria alternata* yielded the 440 bp band with the ITS marker, therefore the results are confirmed with Zhao *et al.*, (2016).

A total 15 primer screened for RAPD analysis. The PCR (Polymerase Chain Reaction) amplified product of each primer were resolved on 1.2 % agarose gel electrophoresis and the size of the amplified product was compared with 100+500 bp DNA ladder. Out of 15 primer screened 10 primer produced 83 scorable bands. Among 83 bands 80 bands were polymorphic and level of polymorphism was 96.38%. The primer OPA-13 amplified maximum 15 bands within the size 6009 to 237bp. While OPA-6 amplified minimum 4 bands within the size 5895 to 205bp. OPA-1, OPA-4, OPA-5, OPA-9 and OPA-15 observed no banding pattern for the set of six isolates of *Alternaria alternata*.

**Table.1** List of ITS primers used with their sequences

Oligo Name	Primer Sequence (5'-3')	GC %
ITS 1	TCCGTAGGTGAACCTGCGG	63
ITS 4	TCCTCCGCTTATTGATATGC	50

**Table.2** PCR reaction mix for 1x of 12.5 µl reaction

Sr. No.	Master Mix	1x
1	10x <i>Taq</i> buffer	1.25 µl
2	MgCl <sub>2</sub> (25 mM)	1.25 µl
3	dNTPs (10 mM)	0.3 µl
4	<i>Taq</i> polymerase (5 U/µl)	0.3 µl
5	Sterile distilled water	5.4 µl
	<b>Total Volume</b>	<b>8.5 µl</b>

**Table.3** Constituents of PCR reaction for ITS

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	8.5 µl
2	Primer (Forward)	1.0 µl
3	Primer (Reverse)	1.0 µl
4	Template DNA (37.5 ng)	2.0 µl
	Total Reaction Volume	12.5 µl

**Table.4** Steps used for PCR-ITS reaction

Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
30 Cycles	- Denaturation	94°C
	- Annealing	55°C
	- Extension	72°C
Final Extension	72°C	10 min.

**Table.5** PCR reaction mix for 1x of 20 µl reaction

Sr. No.	Master Mix	1x
1	10x <i>Taq</i> buffer	2.0 µl
2	MgCl <sub>2</sub> (25 mM)	2.0 µl
3	dNTPs (10 mM)	0.5 µl
4	<i>Taq</i> polymerase (5 U/µl)	0.2 µl
5	Sterile distilled water	12.3 µl
	<b>Total Volume</b>	<b>17 µl</b>

**Table.6** Constituents of PCR reaction for RAPD

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	17 $\mu$ l
2	Primer	2.0 $\mu$ l
3	Template DNA (37.5 ng)	1.0 $\mu$ l
	Total volume	20 $\mu$ l

**Table.7** Steps used for PCR-RAPD reaction

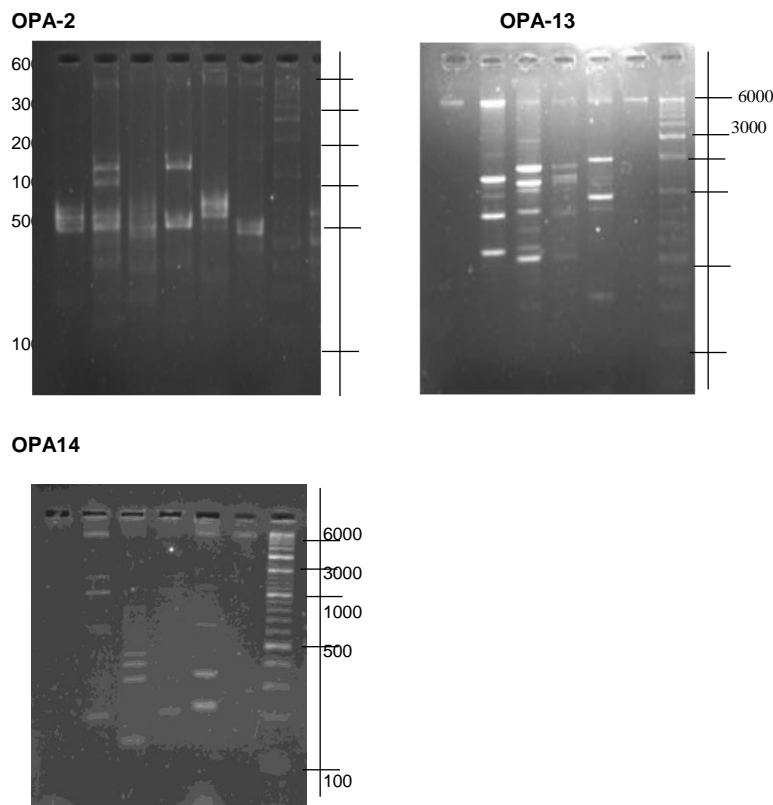
Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
30 cycles	- Denaturation	94°C
	- Annealing	34°C
	- Extension	72°C
Final Extension	72°C	10 min.

**Table.8** Per cent polymorphism observed in RAPD primers

Sr. No.	Primer	Total bands	Polymorphic bands	% polymorphism
1	OPA-2	8	7	87.5%
2	OPA-3	7	7	100%
3	OPA-6	4	4	100%
4	OPA-10	7	7	100%
5	OPA-12	5	5	100%
6	OPA-13	15	14	93.33%
7	OPA-14	12	11	91.66%
8	OPA-18	7	7	100%
9	OPA-19	11	11	100%
10	OPB-11	7	7	100%
<b>Total</b>		83	80	96.38%

**Table.9** Similarity coefficient for RAPD analysis

	Aa1	Aa2	Aa3	Aa4	Aa5	Aa6
Aa1	1.0000000					
Aa2	0.0303030	1.0000000				
Aa3	0.0689655	0.2978723	1.0000000			
Aa4	0.0312500	0.5365854	0.3043478	1.0000000		
Aa5	0.0400000	0.3658537	0.2619048	0.2500000	1.0000000	
Aa6	0.0625000	0.3428571	0.2222222	0.2432432	0.3928571	1.0000000



### Binary similarity matrix for RAPD analysis

The genetic similarity coefficient value ranged from 0.030 to 0.536 across six isolates of *A. alternata*. In this Dendrogram higher value of similarity coefficient 0.536 was between Aa2 and Aa4, whereas 0.030 was between Aa1 and Aa2 found to have lower value of similarity coefficient. Two major clusters were obtained on the basis of analysis. First group is named as cluster –A includes Aa1 (Kolhapur). Second group is named as cluster –B which include Aa2 (Satara), Aa4 (Akola), Aa3 (Sangli), Aa5 (Nagpur) and Aa6 (Amravati). The Aa2 was found to have a higher similarity index with Aa4. The Aa1 with Aa2 and Aa1 with Aa4 was found to have a lower similarity index.

In a parallel study Ginoya and Gohel (2016) RAPD analysis of the eight isolates of *A. alternata* analyzed by 10 random primers

produced 99 loci. Out of which, 98 loci were polymorphic. On an average, 98.98 per cent polymorphism was observed. Out of these, ten primers were found useful for amplification of DNA of *A. alternata*. Among the 10 primers, all primers gave 100% polymorphism of the DNA, which helped to ascertain variability except, OPF-1 primer having 85.71% polymorphism.

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