International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; 8(2): 291-296
www.biochemjournal.com
Received: 15-11-2023
Accepted: 19-12-2023

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Molecular characterization of the *Alternaria alternata* causing leaf spot disease in Banana (*Musa* spp.)

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DOI: https://doi.org/10.33545/26174693.2024.v8.i2d.591

Abstract
Banana (*Musa spp*.) is a valuable horticultural crop worldwide. It grows primarily in hot, tropical areas from South and Central America to India, China, and Africa. Both as a table fruit and as a gourmet fruit, it is the most delicious. Banana plants, on the other hand, are more disease prone than other plants are due to their small genetic pool. The banana plantations in Maharashtra and other banana-growing areas in India are affected by *Alternaria alternata* species, which cause leaf spot disease. The purpose of this study was to ascertain the genetic diversity of *Alternaria alternata* species across a range of geographical regions. Isolates of 13 *Alternaria alternata* species were recovered from different Indian states. Mycelial color, pigmentation, and development pattern showed vast variability in the cultural and morphological characterization investigations. Thirteen isolates were classified into three groups based on their physical properties: fast, moderate, and slow developing. During molecular characterization, 29 (RAPD) random primers and 18 ISSR primers were evaluated, and 5 RAPD and 6 ISSR primers revealed repeatable banding patterns. The number of RAPD samples and ISSR marker data that were analyzed ranged from 67 to 87%. Thirteen *Alternaria alternata* isolates exhibited similar genetic characteristics. Based on similarity matrix data, the cluster analysis was able to divide 13 isolates into two basic groups. The presence of a genetically diverse population of *Alternaria alternata* species isolates was described in this study.

Keywords: *Alternaria alternata*, banana, genetic diversity, RAPD, ISSR

Introduction
Bananas are Musaceae family members that are grown in the humid tropics and subtropics. The banana plant is endemic to Southeast Asia. It is a perennial fruit crop that develops quicker than other fruit crops and produces fruit throughout the year. Carbohydrates, vitamins (particularly B vitamins), salt, potassium, calcium, and magnesium are all abundant in bananas (Dickinson, 2000) [6]. Bananas, the fourth most crucial global fruit crop, are cultivated across 130 countries and contribute significantly to India's fruit production, representing 37% of the total; with India being the predominant producer at 30.5 million metric tons, covering 866,000 hectares of banana cultivation (FAOSTAT 2022).

Alternaria is a widespread fungal genus that includes more than 250 different species and can be found in a variety of plant species. A warm, humid atmosphere is the most favorable environment for the spread and infection of Alternaria fungus. The fungus releases toxins after infection, which leads to the development of small, oval (0.5 to 1.0 mm in length and 0.3 to 0.9 mm wide) light brown patches on the leaf surface (Parkunan *et al*., 2013) [12]. Several *Alternaria* species, including *A. alternata*, have been identified as causal agents of banana leaf spot disease. *A. alternata* is particularly significant because it causes leaf diseases in a wide range of hosts.

Alternaria alternata is an *Alternaria* species that causes leaf disease on banana farms (Avagyan 2014; Fu *et al*. 2014) [1, 7]. Early studies by the National Banana Studies Center (NRCB) revealed that crop losses due to leaf spot disease ranged from 20% to 50%. Furthermore, *A. alternata* was isolated from leaves and fruits and found to cause leaf spot, necrosis, and rot (Amani and Avagyan 2014) [1].

Genetic variations in genomic DNA can be detected and compared using various techniques. One common approach is to analyze polymorphisms in the internal transcribed (ITS) and intergenic spacer (IGS) regions of ribosomal DNA to assess genetic diversity in *Alternaria.*

This amplification method can provide particular DNA fragments that are helpful for molecular biology applications, isolate identification, and genome mapping (Hadrys *et al*., 1992) [8]. In the interspecific sequence repeat (ISSR) technique, multiallelic markers are produced using microsatellite sequences as PCR primers. The advantages of microsatellites, AFLP markers, and random amplification polymorphic deoxyribonucleic acid (RAPD) markers are all combined in this method. The highly polymorphic ISSR gene can be utilized for molecular characterization, organic process biology, genetic diversity, and phylogenetic studies. This method has been effective in examining plant fungal populations. 2019 (Mohammadi and associates). The present study was conducted on 13 isolates of *Alternaria alternata* species obtained from various agroclimatic circumstances in India to explore the cultural and molecular heterogeneity among the thirteen isolates of Alternaria alternata species recovered from the banana crop.

Materials and Methods

Disease Investigation and Sample Collection

In the banana-growing regions of India, a survey was conducted from 2020–2021. Several samples of banana trees that produce the ailment known as leaf spot disease were collected from Maharashtra, Uttar Pradesh, Madhya Pradesh, and Tamil Nadu. A list of samples can be found in Table 1.

Isolation and purification of the pathogenic fungal strain

The isolation of the pathogen was carried out as per the protocol of Fu *et al*. (2014) [7] with modifications. Seven-millimeter leaf sections were excised from lesion margins, surface disinfected with 70% ethanol for 20-30 s, rinsed with sterile water for 30 s three times, cultured on potato dextrose agar (PDA, Himedia India) plates and incubated at room temperature (25 ± 2 °C). On potato dextrose agar medium, the fungus initially produced white mycelia, which later turned light gray after 4 to 5 days of incubation at room temperature (25 ± 2 °C). To obtain a pure culture, two three subcultures were performed.

Morphological Observations

Sterilized leaf portions were injected into glass petri dishes under artificial growth conditions, such as potato dextrose agar (PDA) culture. The infected plates were incubated at 25± 2 °C. The growth of the fungal cultures was monitored for five days, after which the cultures were observed.

The colony diameters were measured, and based on mycelial growth, the species were categorized as slow, moderate, or fast-growing. The spore morphology of the isolates was studied under a microscope. A bite of mycelium was teased with lactophenol dye, placed on a clean glass coverslip, and photographed under a microscope. The species were identified based on the shape of the spores.

Pathogenicity identification

To prove pathogenicity, Koch's postulates were verified on detached banana leaves according to the method described. To accomplish Koch's postulates, pieces of diseased leaves collected from the edge of blight lesions and healthy tissue were surface sterilized and placed on PDA. The fungus was reisolated consistently from all inoculated leaves with morphological characteristics identical to those described above. The fungal pathogen isolated from symptomatic banana leaves was characterized by morphological and microscopic observations, which were similar to those of *A. alternata*.

Genetic diversity analysis of *A. alternata* isolates

After culturing all *Alternaria alternata* species on PDA at 25± 2 °C for 5 days, the morphology and coloration of the colonies were observed and recorded under a microscope (Labomed, Vision-2000).

The genomic DNA of all thirteen isolates was extracted from the mycelial mat using a method developed and standardized by Chavan *et al*. (2008) [4] with some modifications.

For genetic diversity analysis of 13 isolates of *Alternaria alternata*, PCR was performed using RAPD and ISSR primers. The 25 µl PCR mixture contained 2.5 µl of 10X PCR buffer, 1.7 µl of 1.5 mM $MgCl_2$, 2.0 µl of dNTP mixture (2.5 mM each), 0.3 µl of Taq DNA polymerase (1.25 U/l), 1.5 µl of primers, and 1.0 µl of template DNA. The PCR conditions for the RAPD primer were set as follows: initial denaturation at 94 °C for 4 minutes; 35 cycles of denaturation at 94 °C for 1 minute, annealing at 37°C for 1 minute, and extension at 72 °C for 1.5 minutes. For the last extension, the temperature was set to 72°C for 10 minutes, and the PCR conditions and the sequence of the ISSR primer were set as follows: initial denaturation at 94°C for 4 minutes and 35 cycles at 94 °C for 1 minute. The primers were annealed at 56 °C for 1.30 minutes, followed by a 2-minute extension at 72 °C. This was followed by a final extension at 72°C for 10 minutes. The PCR products were detected via 1.2% agarose gel electrophoresis.

Scoring and data analysis

Each amplified product was evaluated as a RAPD and ISSR amplicon, and the results were calculated as an average across all samples. It was determined whether there were bands (+/1) or not (-/0). The molecular weights of the bands were calculated using a typical 1 kb DNA ladder. Only repeatable bands were taken into consideration for analysis after each amplification was verified at least twice. The data were entered into a binary matrix and analyzed using NTSYS-pc version 2.10. The Jacquard similarity coefficient was used to calculate the similarity coefficient.

Results and Discussion

In the present study, morphological analysis and genetic markers were used to study the diversity of *Alternaria alternata* species that cause banana leaf spot disease. The use of RAPD and ISSR markers was used to investigate the pathogenic diversity of several Indian banana-growing regions.

Morphological characterization of isolates of *Alternaria alternata* species

On potato dextrose agar (PDA) media, the morphological characteristics of thirteen isolates of *Alternaria alternata* species were noted. The mycelia color and pigmentation of all the examined isolates varied greatly, as shown in Table 2. The thirteen isolates produced green, greenish, and gray pigments, respectively, with rapid growth from isolates AA1, AA2, AA4, AA7, AA11, AA12, and AA13. Both AA3 and AA5 produced pigmentation that was light green or dark green, and both showed moderate growth. However, AA6, AA8, AA9, and AA10 slowly developed and

generated green coloration. The diversity among *Alternaria alternata* species isolates may be reflected in colony characteristics. The morphological features of all thirteen isolates were similar and agreed with those of *Alternaria alternata* described by Simmons (2007) [16].

Genetic diversity analysis by using RAPD and ISSR markers

The genetic diversity of 13 isolates of *Alternaria alternata* species from banana leaves was analyzed using RAPD and ISSR markers in this study. Twenty-nine RAPD and eighteen ISSR primers were screened for molecular characterization studies, among which 5 RAPD and 6 ISSR primers generated reproducible and scorable banding patterns, respectively. The DNA fingerprinting pattern generated by the RAPD primer OPB11 was unique and produced a maximum of 36 amplicons (Figure 4). The ISSR fingerprint profile generated by the primer UBC 835 produced a maximum of 47 amplicons (Figure 5). PCR products were resolved on a 1.2% agarose gel, and the fingerprint pattern was visualized using an Alpha Innotech Gel documentation system. The similarity coefficient was used to construct a UPGMA (unweighted pair group method with average) dendrogram using NTSYS-pc (version 2. 02i). The RAPD and ISSR marker data analysis revealed 67-87% genetic similarity among the 13 isolates of *Alternaria alternata* species. The cluster analysis of the marker data based on UPGMA using a similarity matrix showed a relatively wide genetic background. According to the cluster analysis, all 13 isolates were found to be distributed into two major groups. Group I included ten isolates, for which the average similarity ranged between 68% and 87%. The major group I comprised a maximum of 10 isolates of *Alternaria* species with six subclusters. Subcluster I included isolates AA1 and AA3, which exhibited 79% genetic similarity. Subcluster II included isolates AA5 and AA12, which exhibited 76% genetic similarity. The AA9 isolate showed unique identity and was assembled into separate subcluster III. Subcluster IV of major group I included isolates AA10 and AA11 together and exhibited 86.50 (i.e., 87%) genetic similarity. Subcluster V included isolates AA7 and AA8, which exhibited 73% genetic similarity to each other. However, the lone member isolate AA13 exhibited a separate subcluster within members of major group I. However, major group II included isolates AA2, AA6 and AA4 together with two subclusters. The member isolate AA4 showed 69% genetic similarity to the member isolates AA6 and AA2 of subcluster I of major group 2. However, isolate AA4 exhibited unique identity and was out-grouped as subcluster II of major group 2. Several molecular markers have been described by many workers (Kakvan *et al.*, 2012; Mohammadi *et al.*, 2019; Adhikari *et al.*, 2021; Bagherabadi *et al.*, 2015) [10, 11, 2, 3], and Chandra *et al.* (2013) [5] studied SSR molecular markers.

Table 1: List of *Alternaria alternata* species isolates utilized in this study

Sr. No.	Species	Host	Origin
1.	*Alternaria alternate*	*Musa spp.*	Pune (M.S.)
2.	*Alternaria alternate*	*Musa spp.*	Utter Pradesh
3.	*Alternaria alternate*	*Musa spp.*	Madhya Pradesh
4.	*Alternaria alternate*	*Musa spp.*	Madhya Pradesh
5.	*Alternaria alternate*	*Musa spp.*	Pune (M.S.)
6.	*Alternaria alternate*	*Musa spp.*	Nanded (M.S.)
7.	*Alternaria alternate*	*Musa spp.*	Tamilnadu
8.	*Alternaria alternate*	*Musa spp.*	Vai, (Nanded, M.S.)
9.	*Alternaria alternate*	*Musa spp.*	Mugat, (Nanded, M.S.)
10.	*Alternaria alternate*	*Musa spp.*	Indore, (M.P.)
11.	*Alternaria alternate*	*Musa spp.*	Nagpur, (M.S.)
12.	*Alternaria alternate*	*Musa spp.*	Rajwadi, (Nanded, M. S.)
13.	*Alternaria alternate*	*Musa spp.*	Pune, (M.S.)

Table 2: Description of morphological characters of *Alternaria alternata* species isolates

Sr. No.	Isolate Code No.	Pigmentation	Mycelia Growth Pattern	Diameter in cm
1	AA-01	Green	Fast	9.1 cm
2	AA-02	Green	Fast	9.2 cm
3	AA-03	Light green	Moderate	3.1 cm
4	AA-04	Greenish	Fast	5.9 cm
5	AA-05	Dark green	Moderate	2.1 cm
6	AA-06	Greenish	Slow	1.3 cm
7	AA-07	Grey	Fast	9.0 cm
8	AA-08	Light green	Slow	4.5 cm
9	AA-09	White	Slow	2 cm
10	AA-10	Light green	Slow	1.3 cm
11	AA-11	Green	Fast	9 cm
12	AA-12	Green	Fast	9 cm
13	AA-13	Light green	Fast	9 cm

Table 3: Percent polymorphism among isolates of *Alternaria alternata* species on the basis of RAPD analysis

Sr. No.	RAPD primers	Sequence of primer 5' 3'	Total no. of bands	Polymorphic bands	Polymorphism (%)
1	OPB1	GTTTCGCTCC	27	27	100%
2	OPB3	CATCCCCCTG	19	19	100%
3	OPB11	GTAGACCCGT	36	36	100%
4	OPD3	GTCGCCGTCA	19	19	100%
5	OPD5	TGAGCGGACA	33	33	100%
Total			134	134	

Table 4: Percent polymorphism among isolates of *Alternaria alternata* species on the basis of ISSR analysis

Sr. No	ISSR Primers	Sequence of Primer 5'3'	Total no of bands	Polymorphic bands	Polymorphism (%)
1	UBC818	CACACACACACACACAG	30	30	100%
2	ISD20	GAGAGAGAGAGAGAGACG	38	38	100%
3	UBC826	ACACACACACACACACC	38	38	100%
4	UBC827	ACACACACACACACACG	30	30	100%
5	UBC835	AGAGAGAGAGAGAGAGC	47	47	100%
6	UBC899	CATGGTGTTGGTCATTGTTCCA	46	46	100%
Total			229	229	

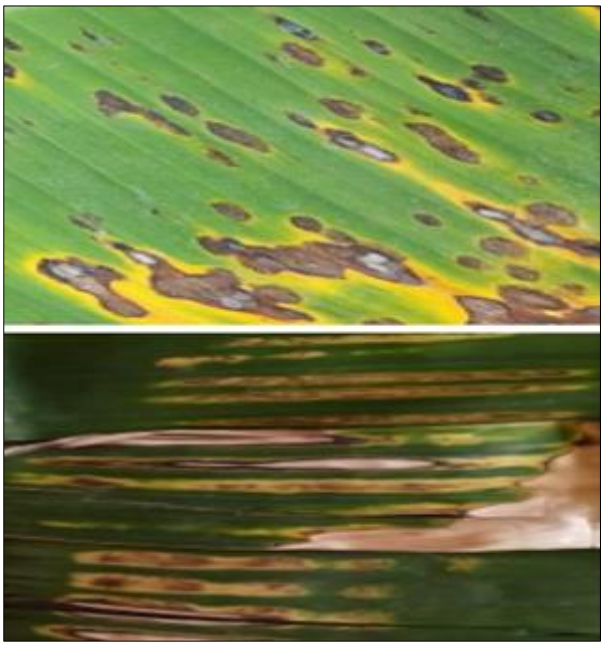

Fig 1: Leaf spot disease of banana

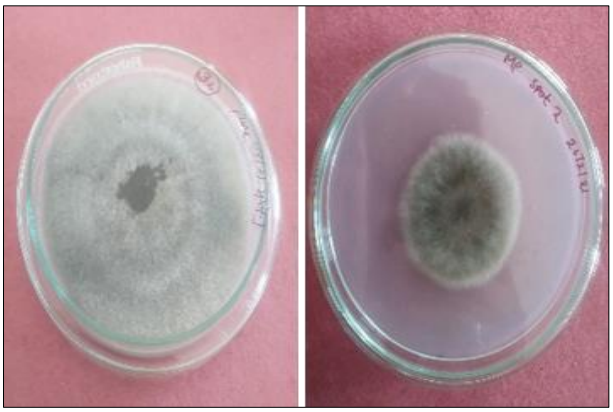

Fig 2: Colony characteristics of *Alternaria alternata* species

Fig 3: Spores of *Alternaria alternata* isolates *Alternaria* species

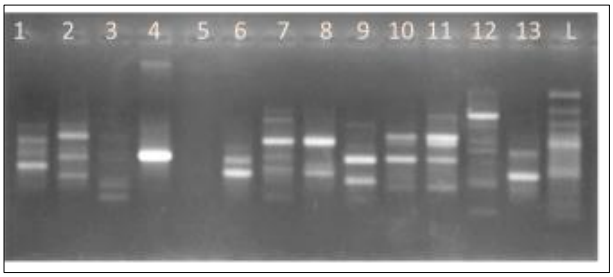

Fig 4: RAPD fingerprint profile of isolates of Alternaria species generated by primer OPB11: M, 1kb DNA ladder; Lane 1-13; Alternaria isolates 1-13 respectively

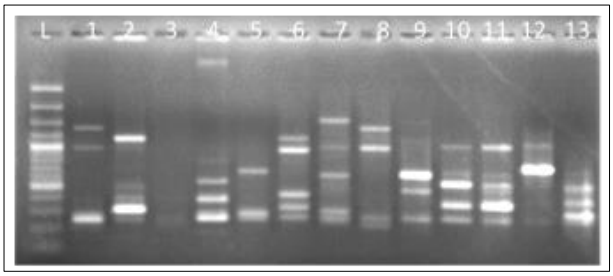

Fig 5: ISSR fingerprint profile of isolates of Alternaria species generated by primer UBC 835: M, 1kb DNA ladder; Lane 1-13; Alternaria isolates 1-13 respectively

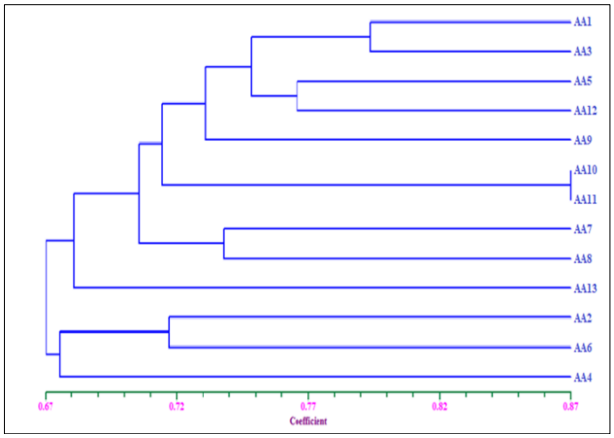

Fig 6: Dendrogram generated using NTSYS-PC based on combine RAPD and ISSR marker data showing genetic relationship among 13 isolates of Alternaria alternata species of banana

Table 5: Similarity matrix coefficient values obtained from combine RAPD and ISSR marker data of 13 isolates of *Alternaria alternate*

Isolates	AA1	AA2	AA3	AA4	AA5	AA6	AA7	AA8	AA9	AA10	AA11	AA12	AA13
AA1	1												
AA2	0.65	1											
AA3	0.79	0.67	1										
AA4	0.65	0.67	0.70	1									
AA5	0.74	0.71	0.76	0.71	1								
AA6	0.65	0.71	0.69	0.67	0.72	1							
AA7	0.71	0.65	0.75	0.65	0.71	0.67	1						
AA8	0.67	0.64	0.68	0.61	0.70	0.64	0.73	1					
AA9	0.70	0.67	0.77	0.68	0.73	0.65	0.72	0.70	1				
AA10	0.70	0.67	0.76	0.67	0.74	0.67	0.73	0.67	0.69	1			
AA11	0.69	0.68	0.71	0.67	0.70	0.64	0.71	0.69	0.67	0.86	1		
AA12	0.69	0.69	0.78	0.71	0.76	0.69	0.71	0.68	0.71	0.72	0.73	1	
AA13	0.66	0.69	0.73	0.67	0.70	0.60	0.69	0.59	0.67	0.70	0.66	0.69	1

Conclusion

Leaf spot disease caused by *Alternaria alternata* affects banana plantations. This study aimed to assess the genetic diversity of *Alternaria alternata* across different geographical areas in India. Thirteen isolates of *Alternaria alternata* were collected from various states, exhibiting significant variability in cultural and morphological characteristics, including mycelial color, pigmentation, and development patterns. The isolates were categorized into three groups based on their physical properties: fast, moderate, and slow developing. Molecular characterization using 29 random amplified polymorphic DNA (RAPD) primers and 18 inter-simple sequence repeat (ISSR) primers revealed repeatable banding patterns, with a genetic similarity ranging from 67% to 87%. Cluster analysis based on similarity matrix data divided the 13 isolates into two main groups, demonstrating the presence of a genetically diverse population of *Alternaria alternata* species isolates.

Acknowledgments

First Author would like to thanks Associate Dean and Principal, V.D. College of Agricultural Biotechnology, Nanded Road, Latur for providing necessary facility for conducting this study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare there are no competing interests.

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