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National Academy of Agricultural Science (NAAS)
Rating : 3. 03

Characterization of the Plant Pathogenic Isolates of *Alternaria Burnsii*

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Abstract: *Cumin is an important cash crop but it frequently suffers from destructive blight disease caused by the fungus* Alternaria burnsii. *The pathogen first attacks the aerial plant parts, develops necrotic spots, and eventually kills the affected areas. The isolates varied widely in appearance, colony margins, and growth pattern. At fourth and seventh day of incubation (28±1°C) the isolate AB-01 showed maximum growth on potato dextrose agar medium. Conidia and beak size ranged widely in length, width, and number of longitudinal (0-1 to 0-2) and transverse septa (0-3 to 1-5). The isolate AB-01 showed highest sporulation frequency (1.24 × 10^5/ml). The Maximum Composite Likelihood method placed these isolates into two groups. The first group in the phylogenetic grouping was represented by seven isolates of* A. burnsii *whereas rest of the isolates (AB-04, AB-09, AB-10) represented the second group. The principal coordinate analysis of the* A. burnsii *ITS gene sequences based on the dissimilarity matrix again grouped these isolates into two groups, which also varied from members of other group in cultural and spore characteristics.*

Keywords: *Alternaria burnsii; Blight; Conidia; ITS gene sequence; MEGA.*

INTRODUCTION

India is the largest producer (460 thousand tons) and consumer of cumin (*Cuminum cyminum* L.) seed in the world, which is nearly 73% to the global cumin production [1]. Cumin seed is mainly produced in the states of Gujarat and Rajasthan in India, more generally on the sandy loam soil by the resource poor farmers with minimal use of inputs like irrigation, chemicals, and fertilizers. Therefore, a wide gap exists between the potential and actual yield of the crop. Beside this, many other factors result in considerable qualitative and quantitative yield losses.

Among various factors affecting cumin yield, the blight disease caused by the fungus *Alternaria burnsii* is one of the most predominant. Under favorable climatic condition, the disease severity may go upto 65 per cent and the pathogen may cause severe damage [2] or almost complete failure of the crop.

Large number of studies exists on the morphological, cultural, and pathogenic characteristics of the various species of *Alternaria* [3,4]. However, very few of them have focused on the diversity studies of *A. burnsii*, the causative agent of cumin blight. They have selected few isolates in their studies and the findings thereof are not supported by genetic information. There exist a need of detailed study encompassing a reliable molecular tool for characterizing this fungus along with the pathological, cultural, and morphological attributes. Sharma and Pandey [3] have used RAPD marker for molecular characterization of *A. burnsii,* however this marker is considered unreliable as it suffers from severe lacunae like, non-reproducibility, low level of polymorphism, locus-unspecific, low sample throughput, and dominance. Recently, molecular approaches have increasingly been used in taxonomy and systematics of phytopathogens at the species and subspecies level [5].

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Sequence based information especially, ITS (Internally Transcribed Spacer) gene sequences are even able to discriminate among species, which appear identical, based on biochemical and morphological characters [6]. The ITS gene sequence has been useful in classification of fungi and in systematic and taxonomic studies due to its suitable size for PCR amplification, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well [7]; which may help in deciphering actual genetic variation existing within a species. In this background, the present study was undertaken to characterize the plant pathogenic isolates of *Alternaria burnsii* using cultural, morphometric and ITS gene sequence.

MATERIALS AND METHODS

Collection, Isolation, Maintenance, and Pathogenicity Test of Isolates

Cumin plants showing typical blight symptoms were randomly collected from the cumin-growing belt of North Gujarat. The blight pathogen was isolated from the diseased aerial plant parts by standard tissue isolation technique [8]. Single spore isolation under microscope followed by inoculation and culturing in potato dextrose agar medium was done to prepare pure culture of the isolates, which were identified following the cultural and morphological characteristics [9] and were maintained on slants containing PDA. In this way, we obtained ten isolates of the fungus *Alternaria burnsii*, which were named sequentially as AB-01 to AB-10. The isolates were subjected to pathogenicity test in pot experiments using *Cuminum cyminum* cultivar GC4 for authentication of causal agents and calculation of disease intensity.

Cultural and Morphological Characteristics

To test cultural variability, the centre of the PDA containing petriplates was inoculated with a 5 mm disc of actively growing fungal isolates and incubating them at 28±1°C. The colony characters, pigmentation, and growth habits were visually observed whereas sporulation intensity was calculated by counting total conidia/ml with the help of haemocytometer in a fungal spore suspension [10]. The morphological characters were studied by staining ten days old culture with Lactophenol-Cotton Blue and recording the size of conidia and number of transverse and longitudinal septa using a compound light microscope and the images were analyzed using the NIS-Elements Documentation software (Nikon, Japan).

Molecular Variability

Isolation of fungal genomic DNA

The genomic DNA was isolated from the log phase culture using genomic DNA purification kit (Merck, India) according to the manufacturer protocol. The quantity and quality of isolated DNA was confirmed spectrophotometrically and the concentration of DNA samples were adjusted to 20 ng/µL.

PCR amplification, electrophoresis and gene sequencing

The internally transcribed spacer region between the 18S and 28S rRNA gene was amplified using consensus primers ITS1 and ITS4 [11]. The PCR was carried out in a final volume of 50¼l having 1X assay buffer 5A, 10 mM dNTP, 100 ng each of forward and reverse primers, 3U of XT-5 DNA polymerase and 20 ng of genomic DNA. PCR cycle for ITS sequence amplification consisted of initial denaturation (94°C for 5 min) followed by 35 cycles of denaturation (94°C for 30 sec), annealing (56°C for 30 sec), extension (72°C for 1.0 min 30 sec) and a final extension of 10 min at 72°C.

Amplification products were resolved by electrophoresis on 1.0% agarose gel (stained with 1% ethidium bromide) in 1X TAE buffer for 2.5 h at 75 volts. The single band of about 1200bp obtained were cut and purified using QIAquick Gel Extraction Kit (Qiagen) and was sent for gene sequencing using Big Dye terminator cycle sequencing kit and the products were resolved on an Applied Biosystems model 3500XL automated DNA sequencing system (Applied BioSystems, USA). These sequences from all the isolates were aligned using ClustalW alignment algorithm, the evolutionary history was inferred using UPGMA method, evolutionary distances were computed

using Maximum Composite Likelihood method using MEGA5 [12] and factorial analysis was done using the DARwin5 software [13].

RESULTS

Cultural and Morphological Characteristics

Alternaria burnsii, the causal organism of cumin blight, survives in the crop debris and is transmitted through seeds or air stream. The disease occurs mostly under cool, humid, and cloudy weather conditions. The disease leads to development of small, isolated, whitish necrotic areas on the aerial plant parts, which gradually enlarge and coalesce with each other. Under favorable climatic conditions, the infection readily spreads all over the aerial plant parts, including stem and blossom, and result in death of the plant. It severely affects seed production and mostly the seeds produced may be shriveled, dark-colored and usually nonviable.

The isolates varied widely in appearance on the Potato Dextrose Agar medium. The colony appeared whitish brown, brownish black, greenish dark black, grey black, and dark black colonies whereas the colony margin was dirty white, light brown, brownish, and blackish in color and the growth pattern observed was plain or fluffy, regular or irregular radial growth, and sometimes knotting (Table 1). The isolate AB-01 (50.5mm) showed maximum growth at 4th day of incubation followed by AB-03 (42.2mm) and AB-10 (41.5mm) whereas at 7th day of incubation the isolate AB-01 (76.5) again showed maximum growth followed by the isolates AB-04 (69.5mm) and AB-03 (67.5mm). The conidiophores produced by *A. burnsii* were branched, erect, straight, irregularly bend, and geniculate whereas conidia produced by these isolates varied widely regarding their size, number of longitudinal or transverse septa, beak length, and sporulation frequency. The highest percent disease intensity (31.4) was observed with the isolate AB-08 (Table 2).

Molecular Characterization

Gel electrophoresis of the amplified ITS gene sequences of the fungal isolates yielded a band of approximately 1200 bp (Figure 1). These sequences of the isolates AB-01 to AB-10 were subjected to similarity search using NCBI-BLAST and multiple sequence alignment using ClustalW and were submitted in the GenBank with accession numbers respectively, KR604836, KR604837, KR604838,

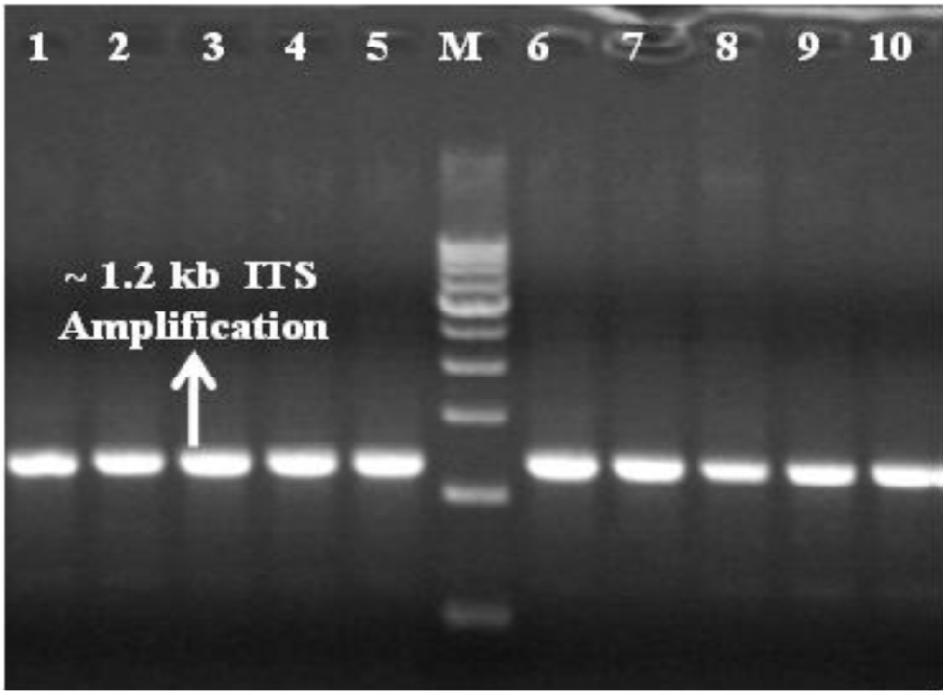
Table 1
Cultural characteristics of the cumin blight pathogen

Cultural characteristics						
			Colony growth (mm)			
Sr. No.	*Strains*	*Colony color*	*4th day*	*7th day*	*Color of colony margin*	*Growth pattern*
1.	AB-01	Whitish brown	50.5	76.5	Brownish	Fluffy radial
2.	AB-02	Dark black	27.5	43.2	Dirty white	Fluffy radial
3.	AB-03	Whitish brown	42.2	67.5	Dirty white	Fluffy radial
4.	AB-04	Grey black	40.4	69.5	Light brown	Plain irregular knotting
5.	AB-05	Dark black	32.5	45.5	Brownish	Fluffy irregular radial
6.	AB-06	Brownish black	37.4	56.5	Blackish	Fluffy irregular radial
7.	AB-07	Greenish dark black	37.2	48.5	Brownish	Fluffy knotting
8.	AB-08	Dark black	30.5	46.5	Brownish	Fluffy radial
9.	AB-09	Grey black	37.2	42.8	Light brown	Plain irregular radial
10.	AB-10	Grey black	41.5	59.5	Light brown	Plain irregular radial
	S.Em. ±		0.78	0.91		–
	C.D. (0.05)		2.20	2.59		–
	C.V. %		5.86	3.34		–

Table 2
Conidial and pathogenic characteristics of the cumin blight pathogen

Conidial characteristics						
			Number of septa			
Sr. No.	*Conidia size* (L × B) (µm)*	*Beak Size (µm)*	*Longitudinal*	*Transverse*	*Sporulation/ml*	*Percent Disease Intensity*
1.	47.48 × 10.84	20.34	0-1	0-3	1.24×10^5	19.5
2.	55.18 × 22.28	28.37	0-2	1-3	9.15×10^4	14.1
3.	44.92 × 15.28	24.57	0-1	1-3	1.18×10^5	15.8
4.	51.98 × 19.70	41.01	0-1	1-4	1.04×10^5	17.8
5.	63.28 × 21.28	27.91	0-1	1-5	6.75×10^4	29.0
6.	56.52 × 24.36	32.64	0-2	1-4	$8.34x\ 10^4$	12.7
7.	58.90 × 22.82	32.68	0-2	0-5	7.64×10^4	23.2
8.	57.38 × 20.78	36.87	0-2	1-5	6.25×10^4	31.4
9.	60.80 × 23.00	47.85	0-2	1-5	8.33×10^4	21.5
10.	61.60 × 20.16	42.55	0-2	1-5	8.14×10^4	25.7
		S.Em. ±				0.36
		C.D. (0.05)				1.02
		C.V. %				3.01

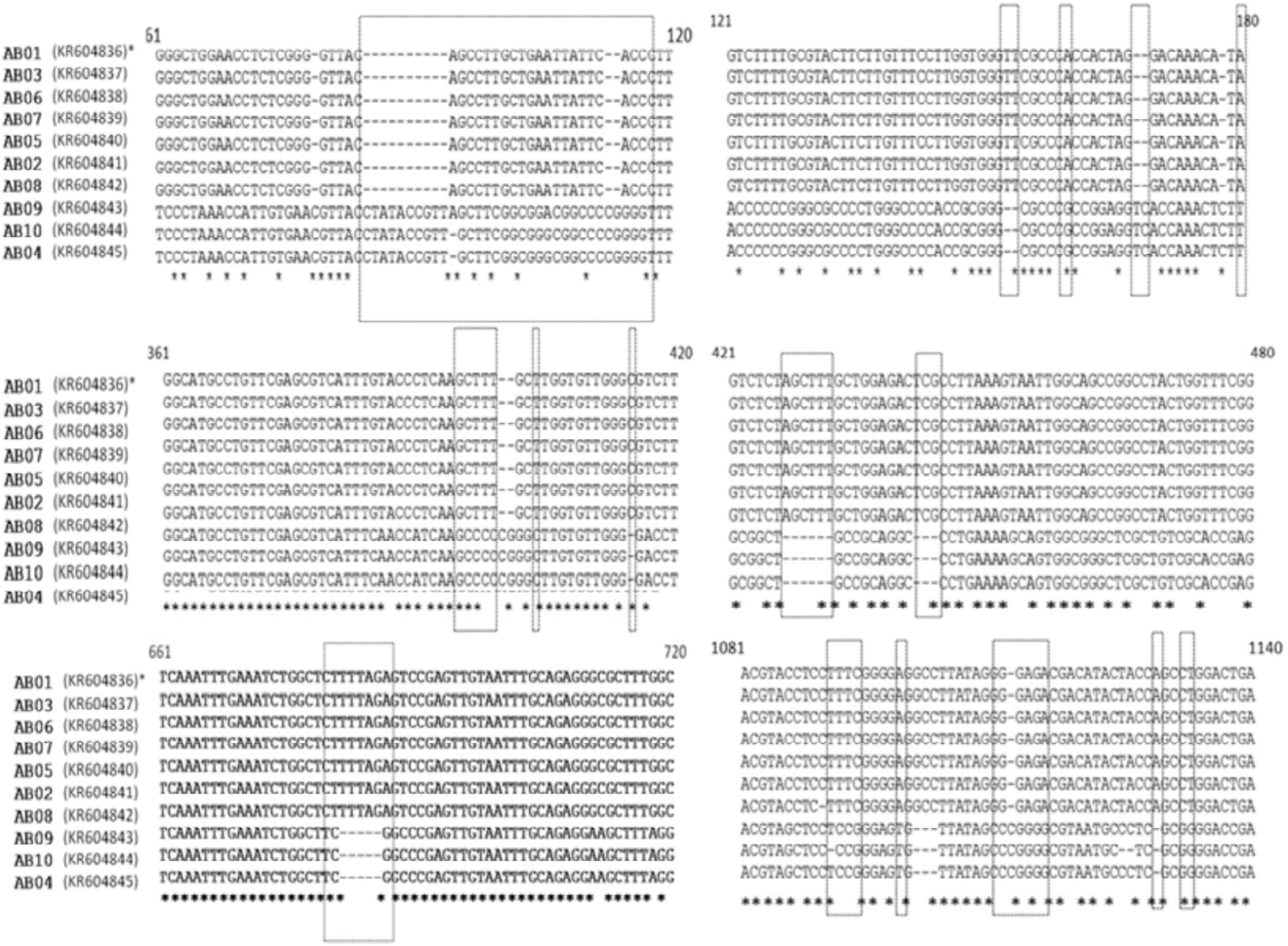
Note: Size of conidia and beak and number of longitudinal and transverse septa indicate average of fifty observations.



(M-marker, Lane 1-10 sequentially indicates the isolates AB-01 to AB-10)

Figure 1: Amplification of ITS gene of *A. burnsii*

KR604839, KR604840, KR604841, KR604842, KR604843, KR604844 and KR604845. The multiple sequence alignment reflects several addition, deletion, and substitution in the nucleotide sequence of the isolates (Figure 2). The phylogenetic tree prepared using Maximum Composite Likelihood method using MEGA5 placed these isolates into two clusters A and B. The cluster A was



*** Indicate Accession number of the ITS gene sequences submitted to the GenBank**

Figure 2: Multiple sequence alignment of ITS gene sequences using ClustalW

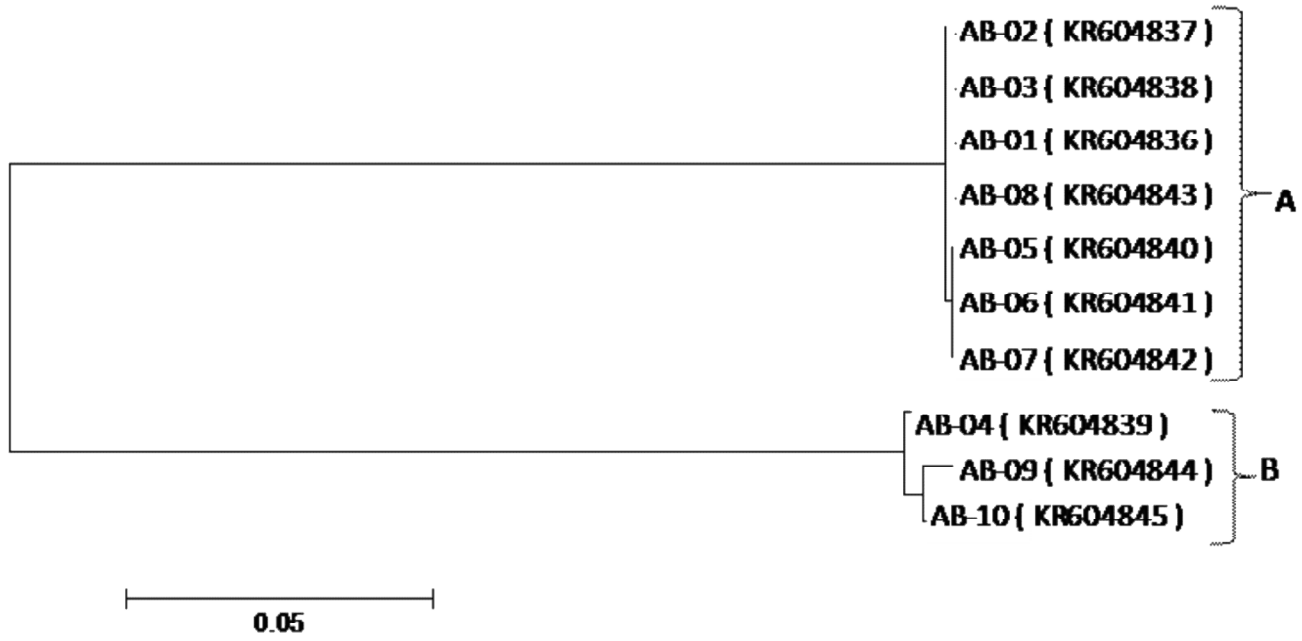


Figure 3: Phylogenetic grouping of *A. burnsii* isolates using MEGA 5.6

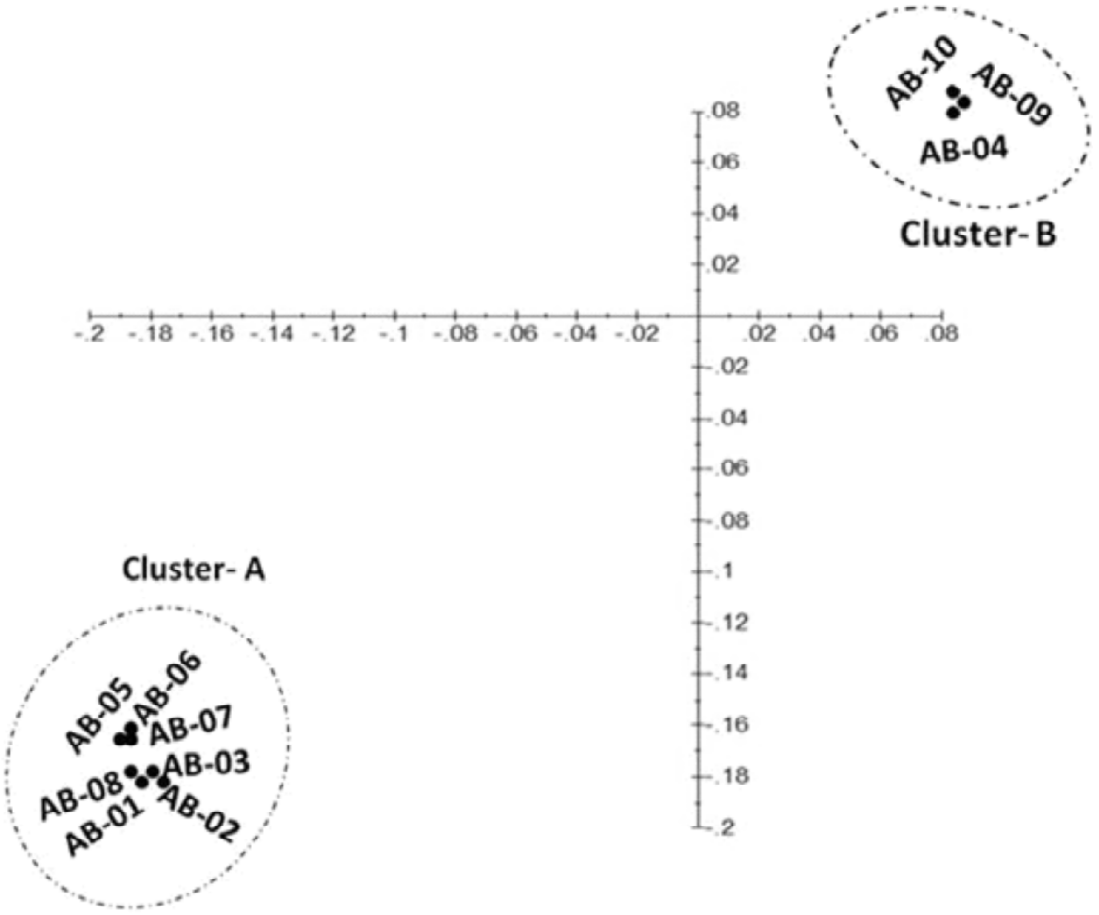


Figure 4: Factorial analysis of the *A. burnsii* isolates using DARwin5

represented by the seven isolates whereas the cluster B includes three isolates (AB-04, AB-09, and AB-10) (Figure 3). The bootstrap values ranged from 46-100 which indicates high degree of confidence for the phylogenetic grouping of the isolates. The factorial analysis based on the dissimilarity matrix grouped the isolates of *A. burnsii* again into two clusters (Figure 4). The factorial analysis showed a result near similar to that of clustering pattern. The members of the cluster-A comprised of seven isolates, which showed an average similarity coefficient of more than 0.98 whereas the members of the second group consisted of three isolates with a similarity of about 0.99.

DISCUSSION

Cumin *(Cuminum cyminum* L.) is one of the most important cash crops and is an important ingredient in culinary items. However, *Alternaria burnsii* inflict widespread damage to the cumin plants under favorable climatic conditions and result in major yield losses of this crop [14]. Since, the first report of *Alternaria* blight of cumin in India [2], it has become one of the most common diseases all over the cumin growing areas. The plants become susceptible to this disease mostly during the flowering stage when the resistance of the cumin plant to this pathogen gets compromised. The disease development is further accelerated under the cool, humid and persistent cloudy weather [15]. Small necrotic spots first appear on the aerial plant parts, which enlarge, coalesce and turn brown to black. Under severe infestation, the stem and flowers may also be killed and interfere with the seed yield. Even if, the seeds are produced, they remain small, shriveled, light and blackish in color [16].

Several workers have tested various chemical fungicide, botanicals, and biological agents for control of diseases caused by *Alternaria*. The

pathogen *Alternaria burnsii* is seed borne and seed transmitted, however, it also survives in crop debris. The survivability of this fungus on seeds remains high during April-May but it reduces in October-November [17]. Several workers have reported cultural variability in *A. burnsii*, but it is not supported by extensive data and genetic information. Sharma and Pandey [3] have observed dark grayish black, light grayish black, and dirty greenish white colony colour of this fungus whereas the colony margin was white and dirty white. They observed the highest radial growth of 30 mm and least radial growth of 27 mm at 4th day of incubation. Similarly, cultural variability in *A. burnsii* has also been reported by Shekhawat *et. al.* [4] and Pipaliya and Jadeja [18]. Shekhawat *et. al.* [4] observed a significant variation in symptoms and latent period by the isolates on blight disease development. The virulent fungal isolates show more number of protein bands with high molecular weight. Such isolates are generally fast growing and cause high disease incidence. The more number of protein bands in more virulent isolates may probably be due to requirement and presence of more number of enzymes/proteins responsible for pathogenicity.

In the present study, multiple sequence alignment of the ITS gene sequence indicates variability present in the genetic makeup of these isolates of *A. burnsii*. The phylogenetic grouping of these isolates group them into two clusters, which may be due to transfer of genes from related or distant species during their survival on the crop debris. The members of the cluster- B show grey black colony color, produce long beaked conidia, and possess high sporulation. The morphological and cultural variation indicates the diversity present among the isolates of this fungus; however, it is not self sufficient to correctly characterize the isolates. Moreover, the cultural, morphological and genetic variation in the attributes of the second group indicates existence of horizontal gene transfer from various species of fungus existing in the agroecosystems; which may lead to stable integration of genetic material following transfer between individuals [19]. Several plant-pathogenic *Alternaria* species are also known to carry Conditionally dispensable chromosomes which may be transmitted horizontally between isolates in a population, potentially conferring new pathogenic attributes to the receiving isolate [20].

Although, cultural, morphological and conidial characteristics are valuable in preliminary identification of the plant parasitic fungus, they do not give clear indication of genetic variation or systematic position of very closely related isolates. However, a polyphasic approach including cultural, morphological and pathogenic characteristics along with the molecular data especially sequence based information is more helpful in deciphering the diversity present among the members/isolates of a particular plant pathogen. Therefore, in the present study, the molecular characterization based on internally transcribed spacer gene sequence clearly differentiated these plant parasitic fungal isolates into two distinct cluster, which is also supported by factorial coordinate analysis. Thus, ITS gene sequence data deciphered the differences between the closely related isolates, which were not visible in the morphological and cultural characteristics.

ACKNOWLEDGEMENTS

The authors humbly acknowledge the facilities and assistance provided by the Hon'ble Vice Chancellor, S.D. Agricultural University (Gujarat, India) and the Government of Gujarat (India) for providing financial assistance under the Plan Budget Head 12131 (Research on Eco-friendly Biological Fertilizers) for carrying out the research work and preparation of the manuscript.

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