A-5804
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RESEARCH ARTICLE Indian Journal of Agricultural Research, Volume Issue : ()

Investigation on the Severity of Alternaria Blight of Pigeonpea and its Characterisation in Northern Karnataka

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10.18805/IJARe.A-5804

ABSRACT

Background: The Alternaria blight of pigeonpea is considered as an important foliar disease and poses threat to the pigeonpea cultivation due to change in the climatic condition, cultivation practices and variation in pathogenic character. Though the disease is noticed in an alarming proportion after the post rainy season and causing economic yield, little is known about the severity of disease and its impact on crop production. The current study is aimed to study the disease severity and variability of the pathogen and which would helps us to understand the biology of the pathogen to develop suitable management strategies.

Methods: In the field and laboratory investigations during 2015 and 2017, different localities of Northern Karnataka were surveyed. Twenty sampling localities were selected based on geographical situation. In the laboratory, collected diseased samples were isolated and determined morphologically and culturally based on taxonomic and molecular characters.

Result: Our investigations revealed the varied disease severity in surveyed districts, Vijayapura district recorded the maximum disease severity with a range of 38.67 to 49.33 and this might be due to variation in pathogenic character, rainfall pattern and climatic conditions and also disease severity varied with varieties indicating the source of resistance. The present study contributed for identification of geographical distribution of the disease and its severity in different varieties and places of Northern Karnataka and their genetic and morphological variations.

Key words: *Alternaria alternata*, *Cajanus cajan*, Growth media, ITS region, PCR.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is one of the major resilient, widely adopted and drought tolerant legume crop commonly known as redgram or arhar and grown in the tropics and sub-tropics accounting 5 per cent of world legume production with India being the largest producer (Ae *et al*., 1990). Inset, the crop is grown on area of 6.99 million ha with 5.96 million tonnes of total production accounting 852 kg/ha of productivity (Anonymous, 2018). In India, this crop is grown in an area of 4.78 m. ha. with an annual production of 3.59 m. t. and productivity is 751 kg/ha, which accounts for 80 per cent of the pigeonpea area and production of the world and grown mainly in Maharashtra, Uttar Pradesh, Madhya Pradesh, Gujarat, Andhra Pradesh, Karnataka and Tamil Nadu (Anonymous, 2019).

Being an important pulse crop, enhances the economic and financial well-being of the farmers as it is a low input, rainfed crop where all the plant parts provides monetary benefits. Even though India is the largest producer of the crop, productivity of the crop is low and diseases and insect pests are the major constraints to high yield potential of pigeonpea cultivars (Nene *et al*., 1984). The crop is facing a greater yield loss due to fungal, viral and bacterial diseases *viz*., *Fusarium* wilt, *Phytophthora* blight and sterility mosaic disease and are countable in respect of disease impact. But recently due to change in the climate, leaf blight incited by *Alternaria alternata* emerged as one of the important pathogen of pigeonpea occurs from seedling to maturity stage. The fungus incites small circular, brown necrotic spots. Later these spots coalesced and cause blighting of leaves and severe infection leads to necrotic lesion on petiole, stem and flower buds (Kannaiyan and Nene, 1977).

Due to cursory literature on the severity of the disease and molecular method of identification of pathogen, present study was undertaken with an aim of mapping the disease severity in pigeonpea growing districts of Northern Karnataka and its characterisation to formulate an effective management strategy to mitigate the emerging threat to pigeonpea production.

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How to cite this article: Savitha, A.S., Ajithkumar, K. and Yenjerappa, S.T. (2021). Investigation on the severity of Alternaria blight of pigeonpea and its characterisation in Northern Karnataka. Indian Journal of Agricultural Research. DOI: 10.18805/IJARe.A-5804.

Submitted: 24-04-2021 **Accepted:** 15-09-2021 **Online:** 13-10-2021

MATERIALS AND METHODS

Survey

A random survey was conducted in eight districts (Raichur, Kalaburgi, Bidar, Koppal, Ballari, Yadgir, Vijayapur and

Bagalkot) of Karnataka state representing mono-cropping system with high productivity during October to Novermber of *Kharif* 2015-2017. In each district, two to eight villages, in each village, three to five fields were selected and observations on disease intensity were scored using 0-5 scale (Balai and Singh, 2013) and the per cent disease index (PDI) was calculated by using the formula given by Wheeler (1969). The data are represented as range and mean.

$$PDI = \frac{\text{Sum of individual ratings}}{\text{No. of leaves assessed}} \times \frac{100}{\text{Maximum disease grade}}$$

Collection of samples and isolation

Blight infected pigeonpea samples were collected and the pathogen was isolated by following standard tissue isolation on Potato dextrose agar (PDA) medium. In total, 20 isolates (two from Raichur, seven from Kalaburgi, one from Bidar, two from Koppal, two each from Ballari, Koppal, Yadgir, Vijayapur and Bagalkot) were isolated and maintained as pure culture by following hyphal tip isolation. The fungus was morphologically were identified as *Alternaria* spp., based on standard mycological parameters such as colony characters and dimensions of conidia and further the pure culture was maintained on PDA slant at 4°C for further study. The isolated *Alternaria* spp., representing Raichur district (PLS-5) was studied on eight different media *viz*., host leaf extract agar, host stem extract agar, malt extract agar, Sabouard's agar, Asthana Hawker's agar, carrot agar, potato carrot agar and Czapek agar media to identify the best media for growth and sporualtion. Further, the observations were taken on parameters such as colony diameter, colony colour, colony shape, type of growth, type of margin and number of spores per microscopic field (10 X) and the results were analyzed statistically.

Diversity in cultural characters

The diversity in cultural characters of 20 isolates of *Alternaria* spp., collected from surveyed fields of Northern Karnataka were studied on the standard PDA media, as it was identified as best media for growth. Twenty ml of PDA medium was poured into 90 mm diameter Petri-plates. After solidification, 5 mm disc of the *Alternaria* were cut using a cork borer and a single disc placed at the center of Petridish. Each set of experiment was replicated twice and the plates were incubated at 28±1°C for nine days. The cultural characters such as the colony diameter, colony color, type of margin and sporulation were recorded.

Proving pathogenicity

Ten days old seedlings of pigeonpea variety (TS-3R) grown under glasshouse was inoculated with 250 ml of *Alternaria* spore suspension as described by Sharma *et al.* (2013). Seven day old pure culture of the *Alternaria* spp., grown in 100 ml of potato dextrose broth (PDB) was harvested and suspended in sterile distilled water. Seedlings were inoculated by spraying the spore suspension (5×10^6 conidia ml^{-1}).The inoculated seedlings were incubated at 28±1° with alternate day and night photoperiod and un-inoculated plants served as control.

Extraction of DNA and PCR detection of the pathogen

Cetyl trimethyl ammonium bromide (CTAB) method was adopted to extract the total DNA from the mycelium of *Alternaria* (Murray and Thomson, 1980). Approximately 0.5 g of mycelium was powdered using liquid nitrogen and transferred to 750 µl pre warmed CTAB extraction buffer and incubated at 65°C for 45 min. During the incubation, tubes were vortexed for five seconds for complete mixing. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently to denature proteins and centrifuged at 12,857 rpm for 10 min. DNA was precipitated with 0.6 volume of chilled ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) and centrifuged at 18,514 rpm for 15 min. The pellets were washed twice in chilled 70% ethanol, dried at room temperature, re-suspended in 100 µl sterile TE buffer and stored at -20°C deep freezer.

Sequencing of rDNA-ITS region

The universal internal transcribed spacer primers (ITS) were used to confirm the *Alternaria* species (White *et al*., 1990). The reaction mixture (50 µl) consisted of 50 ng genomic DNA, IX PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each primer and 1.0 unit *Taq* DNA Polymerase was amplified with an initial denaturation step of 94°C for 2 min, 35 cycles of amplification (30 s for denaturation at 94°C, 30 s for primer annealing at 55°C and 30 s for extension at 72°C) and one cycle of final extension at 72°C for 5 min. The amplification was carried out on a thermal cycler. PCR amplified products were analysed by agarose gel electrophoresis.

The amplified PCR products (ITS 1 and ITS 4) were sent for purification through gel elution using gel extraction kit and sequenced at Eurofins Genomics Pvt. Ltd., Bengaluru. The sequence data was assembled and analysed using the programme Cap3 online software. Homology search was done using BLAST algorithm available at the http://www.ncbi.nlm.nih.gov. The sequences were compared with the previously published database sequences and were deposited in the NCBI GenBank, Maryland, USA, to get accession numbers. The phylogenetic tree was constructed by using MEGA 4.0 online software version using the default parameters of one character based algorithm. The bootstrap consensus phylogenetic tree was constructed for each algorithms with 500 replication.

RESULTS AND DISCUSSION

Survey

A survey on the prevalence of Alternaria blight of pigeonpea carried out in Raichur, Kalaburgi, Bidar, Koppal, Ballari, Yadgir, Vijayapur and Bagalkot districts of Karnataka during *Kharif* 2015-2017 revealed the circular necrotic spots on leaves and later coalesced to form blighting of leaves (Plate 1). The disease scoring revealed the disease severity varied in

Table 1: Prevalence of *Alternaria* leaf blight of pigeonpea in Northern Karnataka during *Kharif* 2015 and 2017.

Districts	Locality	Disease severity (%) in 2015				Locality	Disease severity (%) in 2017				Cultivars
		Range	Mean	SD*	S.Em**		Range	Mean	SD*	S.Em±**	grown
Raichur	MARS, Raichur	14.33-25.33	21.10	5.67	3.27	MARS, Raichur	14.00-26.00	21.11	6.30	3.64	TS-3R
	MARS, (E and F)					Sath mile					
	Nandewadgi					Narakaladinni					
Kalaburgi	Mandewala	10.00-48.67	28.67	15.48	5.05	Balwandgi	12.00-48.67	25.56	13.21	4.40	TS-3R
	Needalgi					Harval					Maruthi
	Jeratagi					Handoor					Local
	Sirnur					Sirsagi					
	Naddisinnur					Siradgi					
	Halsultanpur					Harsoor					
	ARS, Gulbarga					ARS, Gulbarga					TS-3R
	Buddavihar					Gobburwadi					Local
	Ganjalkhed					Hesur					
Bidar	Hallikhed	15.33-18.67	17.00	2.36	1.67	Gurdal	12.00-14.67	13.33	1.89	1.34	Local
	Halhalli						Hargota				
Koppal	Hosahalli	15.33-35.33	25.33	14.14	10	Hirekhed	14.00-35.33	24.67	15.08	10.67	Maruthi
	Benathkallu					Chikkabenkal					Local
Ballari	Kolagallu	20.00-20.67	20.33	0.47	0.34	Homnahalli	20.00-22.67	21.33	1.89	1.34	TS-3R
	Kurugodu					Ibrampura					Local
Yadgir	Halisagar	20.00-21.33	20.67	0.94	0.67	Kakkebennihalli	21.33-22.67	22.00	0.95	0.67	Local
	B'Gudi					Belgera					TS-3R
Vijayapura	Aheri	41.33-46.67	43.33	2.91	1.68	Algur	38.67-49.33	42.89	5.67	3.27	TS-3R
	Bommanajogi					Budhihal AC,					Maruthi
	AC, Vijayapur					Vijayapur					
Bagalkot	Muddehoudagi	18.00-34.67	26.33	11.79	8.34	Manahalli	20.67-32.00	26.33	8.01	5.67	Local-Red
	Khalavada					Bommanagi					Maruthi

SD*-Standard deviation. S.Em±**- Standard error of mean

the surveyed areas (Table 1). The intensity of the disease varied depending on the cultivars. During, 2015 the highest disease severity (46.67 %) was recorded in the Aheri village of Vijayapur district and the district disease severity ranged from 41.33 to 46.67 per cent on different varieties, the maximum being noticed on varieties like TS-3R and ICP 8863. This was followed by Kalaburgi district, where the disease severity was in the range of 10.00-48.67 per cent and the maximum being recorded in Agriculture Research Station, Kalaburgi on the variety TS-3R with 48.67 per cent. The observations on the disease severity of surveyed places revealed that the severity was least on local cultivars and among the different districts; Koppal, Raichur, Ballari and Yadgir recorded the low disease incidence.

Similar trend of disease severity was recorded during *Kharif* 2017 survey (Table 2). The maximum severity was being recorded in the Bommanajogi village of Vijayapura district in the TS-3R cultivar and it was in the range of 38.67 to 49.33 per cent. Among the different districts surveyed, the least disease severity was recorded Bidar district with the range of 12.00 to 14.67 per cent on the local cultivar. The survey results of Kalaburgi, Koppal and Bagalkot districts data showed the higher standard deviation indicating the diversity of distribution of the disease in *Kharif* 2015 and 2017, respectively.

The Alternaria blight of pigeonpea might be causing 20-24 per cent loss to the farmers by infecting crop at all the stages and majorly at vegetative to flower formation stage. Prominent high yielding varieties like TS-3R and Maruti were showed more infection compared to the local cultivars. Even though the disease was widely prevalent in all the varieties observed during survey but varied in the range of moderate to high severity of infection in the surveyed fields and varieties and which might be due to rain pattern prevailed in the cropping season, soil factor and cultivars grown in those areas and they were significantly contributed for the change in the disease severity pattern. Several workers observed similar observations of the disease severity, where 20 to 80 per cent of disease incidence was recorded in 60 per cent of surveyed fields by Sharma *et al.* (2013).

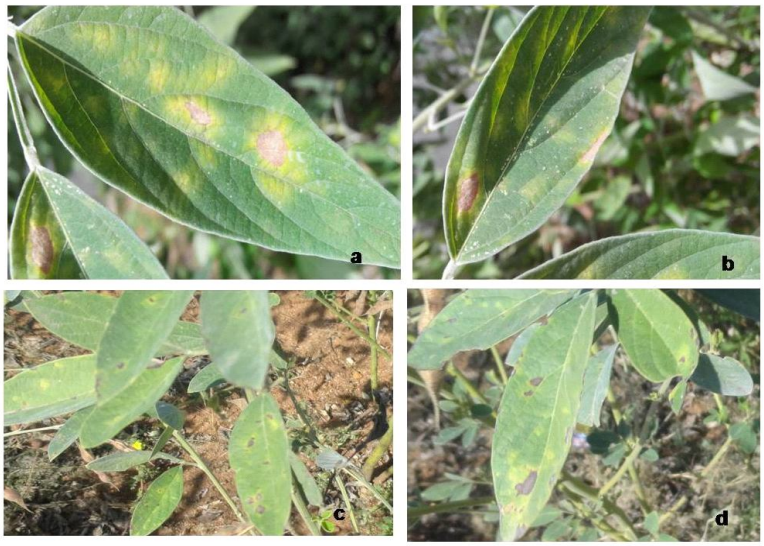

Plate 1: Symptoms of Alternaria blight on pigeonpea.

Table 2: Cultural characters of *A. alternata* (PLS 5) on different solid media.

Media	Colony diameter (mm)	Colony colour	Type of growth	Type of margin	Spores/10X microscopic field
Malt extract agar	38.30	Black	Partially aerial	Uniform	Nil
Host stem extract agar	28.30	Light grey	Immersed mycelia	Uniform	150
Sabourad's agar	36.30	Dark grey	Immersed mycelia	irregular	80
Carrot agar	90.00	Black	Partially aerial	Uniform	100
Potato carrot dextrose agar	90.00	Black	Partially aerial	Uniform	120
Czapeks agar	61.67	Greyish black	Partially aerial	irregular	10
Host leaf extract agar	88.30	Light grey	Partially aerial	Uniform	300
Asthana hawker's media	32.33	Black	Immersed mycelia	Regular	100
Potato dextrose agar	90.00	Black	Partially aerial	Uniform	Nil
S.Em±	1.414				
CD at 1%	4.234				

Isolation and characterisation of pathogen

The fungus *Alternaria* produced greyish brown to black coloured colony with cottony to velvety texture and raised to flat mycelial growth. The fungus produced abundant branched septate brownish mycelia. The conidia were solitary or in short chains produced on simple conidiophores.

The conidial mean measurement is in the range of 171.54- 312.02 X 64.65-141.22 µm with beak and beakless, ovate to ellipsoidal and some are elongated and branched in chains. The conidia were having 2 to 8 horizontal septa and 1 to 5 vertical septa (Plate 2). Based on the morphological characters the fungus was identified as *Alternaria alternata*.

The pathogenicity test revealed that, the inoculated plants produced typical symptoms at seven days of incubation as light brown concentric circular ring spots and later they turned to dark brown coloured and coalesced to cause blighting of leaves.

Among the 20 isolates, the representative isolate from Raichur (PLS 5) district studied on eight different media revealed that (Table 3 and Plate 3), the isolate showed varied growth of partially aerial to immersed mycelia and colour from light, dark grey to black colour on different media. On potato carrot dextrose, potato dextrose and carrot agar, the isolate exhibited the maximum colony diameter of 90 mm and statistically significant over other media tested, whereas good sporulation was recorded on host leaf (300/) and host stem extract agar (150) followed by potato carrot agar with 120 conidia/microscopic field indicating potato dextrose, potato carrot dextrose and carrot agar were best for growth and host extract agar was more suitable for sporulation.

The present study has indicated that, the good growth and sporulation of the fungus was achieved by employing the potato carrot agar media followed by carrot agar media. The good growth and sporulation is attributed to inherent complex nature of natural media supporting good fungal growth and sporulation. These observations are in conformity with the findings of earlier worker (Savitha *et al*. 2013), where they used PDA supplemented with $CaCo_3$ for growth and sporulation of *Alternaria sesame* causing blight of sesame and the description of the fungus was agreed with *A. alternata* on pigeonpea and other crops as reported by several workers (Sharma *et al.* 2013 and Nidhika *et al*. 2019).

Diversity in cultural characters

Diversity studies of twenty isolates of *A. alternata* obtained from different geographical regions (Table 4 and Plate 4) revealed that the maximum colony diameter of 90 mm was recorded in the isolates of PLS7, PLS8, PLS10, PLS11, PLS12 and PLS18 and which was followed by the isolate PLS 15 recorded the colony diameter of 89.70 mm and showed at par results. The rest of the isolates differed significantly with the above mentioned isolates. The mycelial colour showed striking differences, five isolates of *A.*

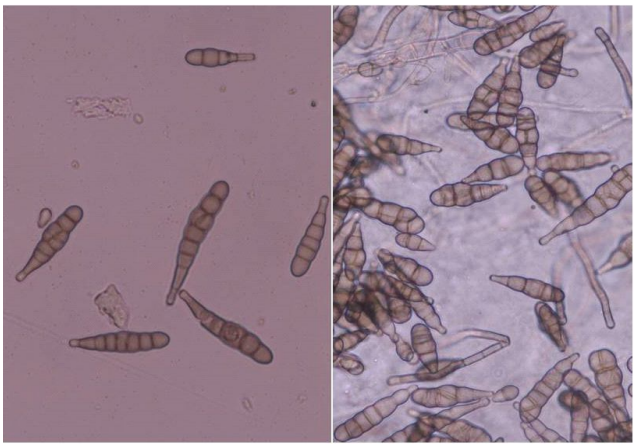

Plate 2: Morphology of *Alternaria alternata*.

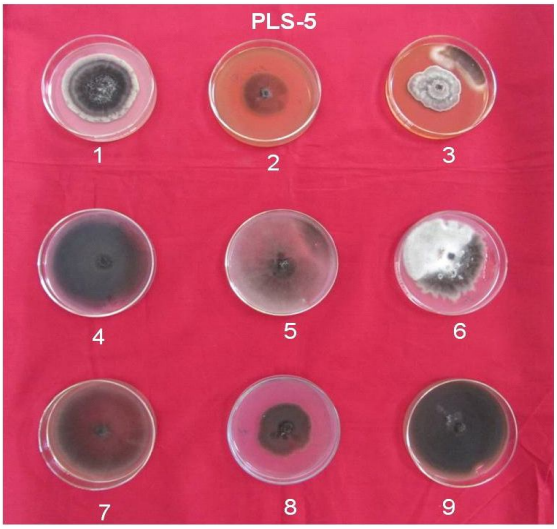

Plate 3: Cultural diversity of *A. alternata* on different solid media.

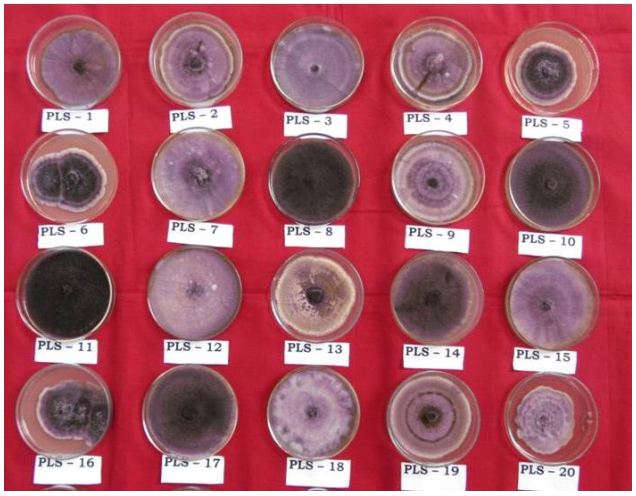

Plate 4: Cultural diversity of 20 isolates of *A. alternata* on PDA media.

Table 3: Diversity in cultural characters 20 isolates of *A. alternata* on PDA media.

District	Locality	Isolates	Colony diameter	Shape	Growth pattern	Texture	Colour	Mycelial growth
Kalaburgi	Mandewala	PLS1	81.33	Regular	Circular	Cottony	Greyish black	Flat
Kalaburgi	Needalgi	PLS2	70.00	Regular	Circular	Cottony	Greyish black	Flat
Kalaburgi	Jeratagi	PLS3	70.00	Regular	Circular	Cottony	Greyish black	Raised
Kalaburgi	Sirnur	PLS4	71.33	Irregular	Feathery	Velvetty	Greyish black	Flat
Raichur	MARS, Raichur,	PLS5	55.33	Irregular	Feathery	Cottony	Black	Raised
Raichur	Nandewadgi	PLS6	60.33	Irregular	Feathery	Cottony	Black	Raised
Kalaburgi	Naddisinnur	PLS7	90.00	Regular	Feathery	Cottony	Grey	Raised
Kalaburgi	Halsultanpur	PLS8	90.00	Regular	Circular	Feathery	Pure Black	Flat
Kalaburgi	ARS, Kalaburgi	PLS9	89.33	Regular	Feathery	Cottony	Blackish white	Raised
Bidar	Hallikhed	PLS10	90.00	Regular	Circular	Feathery	Black	Flat
Koppal	Hosahalli	PLS11	90.00	Regular	Circular	Feathery	Black	Flat
Koppal	Benathkallu	PLS12	90.00	Regular	Circular	Cottony	Grey	Flat
Ballari	Kolagallu	PLS13	86.67	Irregular	Circular	Velvetty	Grey white	Flat
Ballari	Kurugodu	PLS14	86.00	Regular	Feathery	Velvetty	Black	Flat
Kalaburgi	Halisagar	PLS15	89.67	Regular	Circular	Cottony	Grey	Raised
Kalaburgi	Bheemrayangudi	PLS16	52.67	Irregular	Feathery	Cottony	Black	Raised
Vijayapura	Aheri,	PLS17	89.67	Regular	Circular	Velvetty	Black	Flat
Vijayapura	Agriculture College, Bijapur	PLS18	90.00	Regular	Feathery	Cottony	Blackish white	Raised
Bagalkot	Muddehoudagi	PLS19	85.67	Irregular	Circular	Velvetty	Greyish black	Flat
Bagalkot	Khalavada	PLS20	51.33	Irregular	Feathery	Cottony	Light Pista	Raised
		S. Em±	0.42					
		CD at 1%	1.25					

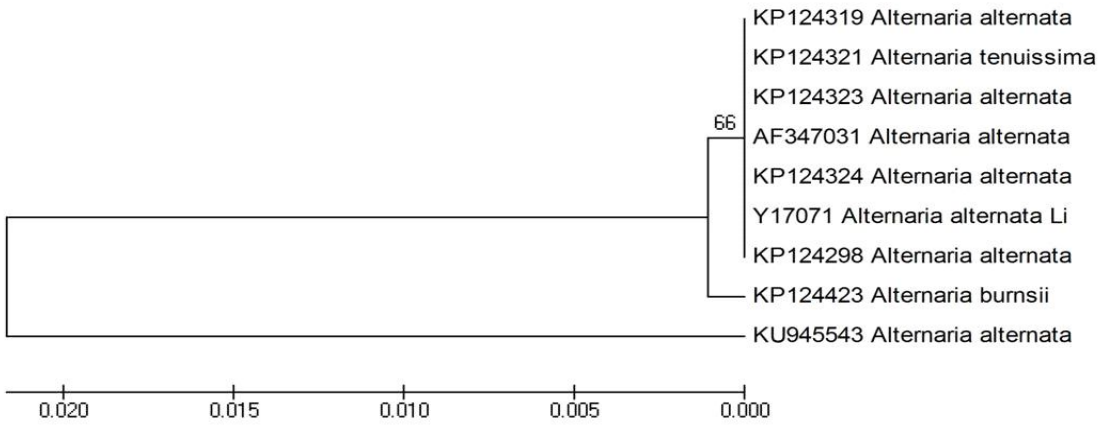

Fig 1: Phylogeny showing relationship among the *Alternaria alternata* CBS strain sequences with *A. alternata* (KU945543) of Raichur based on their ITS sequences

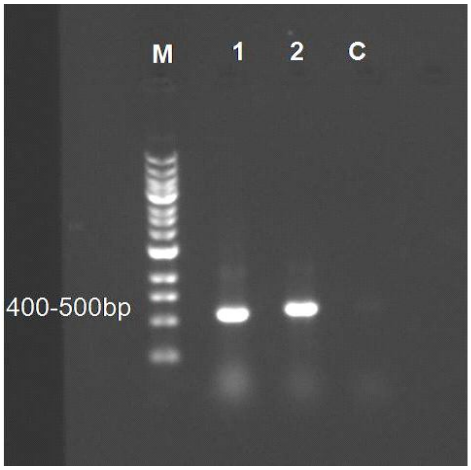

Plate 5: PCR amplification of ITS regions of *A. alternata*
Lane M – 1 Kb DNA marker; lane 1and 2 – *Alternaria alternata* isolates from Raichur district and Lane C; Control.

alternata PLS1, PLS2, PLS3, PLS4 and PLS9 showed greyish black colour whereas other eight isolates PLS5, PLS6, PLS8, PLS10, PLS11, PLS14, PLS16 and PLS17 exhibited black colour but isolates PLS7, PLS12, PLS13, PLS15 and PLS20 showed grey colour. Diversity in cultural characteristics among different isolates of *Alternaria* spp., infecting tomato and sesame was noticed by several workers (Perez and Martinez, 1996 and Savitha *et al*. 2013).

Sequencing of rDNA-ITS Region

The rDNA-ITS region was sequenced and sequences were deposited in the NCBI, GenBank database under the accession number KU945543 (481 bp) and KU945541 (545 bp) (Plate 5). The size of the PCR product was 480 to 550 bp and the blast analysis of the amplicon showed 97.19 per cent similarity with the *A. alternata* sequences (MK 518438) reported from other host from India and confirmed the identity of the fungus as *A. alternata* causing blight of pigeonpea. The phylogenetic tree was constructed by using Mega 4.1 online software to compare the pigeonpea isolate, *A. alternata* with standard CBS strain sequences of *Alternaria alternata* in the gene bank from different hosts to show the distinctness of isolate (Fig 1). The results revealed that the isolated *A. alternata* infecting pigeonpea grouped separately from the other host species. The rDNA-ITS analysis revealed the divergence of *A. alternata* isolate with the other isolates of *Alternaria alternata* infecting other host. The isolates also showed strong genetic similarity within the range of 97 to 100 per cent indicating high level of identity among the isolates irrespective of the host.

The cultural, morphological and molecular identification of the fungus acts as preliminary step for understanding the biology of the pathogen, to develop suitable disease management strategy either through chemical methods or by developing resistance breeding programmes. In this view, the sequencing of ITS amplified region of genome revealed the sequence similarity to *A. alternata*, acting as major cause for blight disease in pigeonpea. The sequence phylogeny of our isolate showed deviation from the *Alternaria* sequences from other hosts drawn from genbank submission, indicating the diversity of pigeonpea isolate of Karnataka with the isolate of *Alternaria* of different hosts (Sharma *et al*. 2013).

CONCLUSION

As we know that the pigeonpea is an important pulse and rich in protein, which helps to combat the protein malnutrition. The present experimental study revealed the varied mean severity in different geographical regions ranging from 13.33 to 42.89 per cent on different varieties and maximum mean disease severity was noticed in Vijayapura district and also he isolates collected from Vijayapura district showed maximum colony growth with varied growth characters on media plates indicating diversity in causing disease and that could be correlated to the maximum disease severity in that district. The cultural, morphological and molecular identification through rDNA-

ITS region sequencing showed the pathogen distinctness from the *Alternaria* sp., infecting other hosts. The confirmation of pathogen itself acts as foundation for understanding further aspects of the disease such as epidemiology and to develop suitable management stratergy and indirectly helps in mitigating the protein malnutrition.

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