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Molecular variability of *Ramularia areola* isolates causing grey mildew of cotton

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Abstract: Molecular variability of Ramularia areola pathogen was carried out in the laboratory, Department of Plant Pathology, College of Agriculture Dharwad, University of Agricultural Sciences, Dharwad by molecular methods using ITS primers and subsequently subjected to sequence analysis during 2015. Molecular variability in the pathogen served as a guideline for breeding the suitable cotton varieties against grey mildew disease of cotton which severely affects cotton productivity. Results revealed that, Phylogenetic analysis grouped Ramularia areola isolates into two main clusters with 0.12 similarity coefficient. The similarity coefficient ranged from 0.001 to 0.12 indicating good level of similarity. UPGMA cluster analysis based on genetic distance coefficients clearly separated the all isolates. Out of four isolates, Dharwad, Arabhavi and Naragund isolates were grouped under cluster A, where as Devihosur isolate formed separate cluster B. In cluster A, Dharwad and Naragund isolates were showed only 3 per cent of divergence, compared to Arabhavi isolate which showed 5.8 per cent divergence. In cluster B only one isolate Devihosur was present with nearly 12 per cent divergence with cluster A. This indicated there was 92.00 per cent of similarity among the isolates of *Ramularia areola.*

Key words: Cotton, Grey mildew, Primer, Variability

Introduction

Cotton is one of the most ancient and important commercial crops next only to food grains and is the principal raw material for a flourishing textile industry. India is the only country in the world where all the four cultivated species of cotton, *viz., G. hirsutum, G. arboreum, G. herbaceum* and *G. barbadense,* are cultivated on commercial scale, besides their hybrid combinations.

In world, cotton is grown on an area of 33.1 million hectares producing 118 million bales with a productivity of 766 kg/ha. In India, the crop is grown in an area of 12.65 million hectares producing 400 lakh bales with a productivity of 537 kg/ha. Maharashtra has the largest acreage in India (38.27 lakh ha) while, Gujarat leads with a production of 121.80 lakh bales. Tamilnadu ranks first with a productivity 659 kg/ ha. Karnataka has an area of 7.60 lakh hectares with a production and productivity of 26.9 lakh bales and 626 kg/ha, respectively (Anon., 2016).

The low productivity of cotton in Karnataka is attributed to many factors, one of which is the losses due to diseases, although insect pests continue to be a major production constraint. A large number of fungal, bacterial, viral and nematode diseases have been reported on cotton crop right from early stage to maturity. Among them, the economically most important ones are bacterial blight, alternaria leaf spot, grey mildew, rust and vascular wilts which occur throughout the world (Kotasthane and Agrawal, 1970).

Before the introduction of new world cottons, the desi types (*G. arboreum* L. and *G. herbaceum* L.) were mostly in cultivation in India and in still some pockets desi cottons are being cultivated. The important diseases of desi cotton include wilt (*Fusarium oxysporum* f. sp. *vasinfactum* (Atk.) and grey mildew (*Ramularia gossypii* (Speg.) Chif.). With the introduction of American and Egyptian varieties of cotton, diseases such as root rot (*Macrophomina phaseolina* (Tassi) Goid), wilt (*Verticillium dahliae* Kleb), angular leaf spots and black arm of cotton (*Xanthomonas campestris pv. malvacearum* (E F Sm) Dows) and leaf spots (*Alternaria microspora* Zimm.), became predominant. Among them "grey mildew" caused by *Ramularia areola* Atk. is gaining importance in recent years as it has been reported from all the cotton growing countries of the world. It has spread wide occurrence on crop grown under *kharif* in south India. It has proved to be a serious threat especially in the transitional zone of Dharwad, Belagavi and Haveri districts of Karnataka state.

Atkinson (1890) first reported the occurrence of Ramularia areola on cotton (*Gossypium herbaceum* L.) from Auburn, Alabama (U.S.A.). He described the symptoms produced by the pathogen in brief and named as Areolate mildew and its symptoms mainly on leaves. In India, occurrence of the disease was reported by Butler (1918). Govindrao and Subbaiah (1964) reported that the disease occurred sporadically at different places in Andhra Pradesh and Mysore state. They all agreed that the intensity of infection was more on cotton grown in low lying area and during years of heavy precipitation.

In recent years, the disease has become a potential threat to cotton cultivation. Therefore the present investigation on variability of pathogen have greater significance in breeding for resistance as well as for identification of the resistant sources. Most of the hybrids, germplasm and varieties are susceptible because of the presence of the variability in the pathogen. For the molecular variability study, the PCR based technique that is ITS (Internal Transcribed Spacer) was used in the present investigation.

Material and methods

Variability of the pathogen was carried out under in vitro at Department of Plant Pathology, College of Agriculture Dharwad, University of Agricultural Sciences, Dharwad by molecular

methods using ITS primers and subsequently subjected to sequence analysis.

During *kharif* 2015, *Ramularia areola* isolates were collected from 10 locations of major cotton growing areas of northern Karnataka and these collected isolates were assessed for molecular variability. Isolation of DNA from *R. areola* was done by following standard CTAB method with minor modifications (Cuervo-Parra *et al.,* 2011). The method is as follows.

2-3 g of fungal mat grown on modified Kirchoffs broth was taken and homogenized using pestle and mortar in liquid nitrogen. To the above solution 1 ml of lysis buffer was added. The suspension in pestle and mortar was extracted with equal volume of phenol: Chloroform: isoamyl alcohol (1:1 W/V) in centrifugation tube, centrifuged at 6000 rpm for 20 minutes at 10^0C. Supernatant was taken in fresh centrifuge tube and 2.5 µl RNase and 2.5 µl protienase-k was added and incubated at room temperature for 30 minutes. Cooled isopropanol of about 1/3rd volume (300-400 µl) was added. Centrifuged @ 6000 rpm for 20 minutes at 10^0C. Wash buffer about 500 µl was added and centrifuged at 6000 rpm for 20 minutes at 10^0C. For the necessary of DNA, pellet was washed with 70% ethanol, air dried and resuspended in 500 µl of $T_{10}E_1$. This DNA obtained was further quantified by agarose gel electrophoresis.

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of copy of 18S, 5.83S and 28S like rDNA and its spacer like internal transcribed spacer (ITS). The rDNA have been employed to analyse evolutionary events because it is highly conserved, whereas ITS rDNA is more variable hence, it was used for investigation. forward ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and reverse primers ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used. Amplification reaction mixture was prepared in 0.2 ml thin walled PCR tubes containing following components. The total volume of each reaction mixture was 20 µl. The following reaction mixture was found to be optimum for PCR amplification.

Except template the master mix was distributed to PCR tubes (19 µl/tube) and later 1 µl of template DNA from the respective isolates was added making the final volume of 20 µl.

Amplification were performed in a thermal cycler using the following temperature profile: Initial denaturation step at 94°C for five min, then 35 cycles at 94°C for one min of final denaturation, 54°C for one min annealing and 72°C for one minute as extension followed by final extension step at 72°C for 5 min (Cuervo-Parra *et al.,* 2011). Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent agarose in 1X TBE (Tris Borate EDTA) buffer, 0.5 µ1 ml^{-1} of Ethidium bromide and loading buffer (0.25 % Bromophenol Blue in 40% sucrose). Four µ1 of the loading dye was added to 20 µ1 of PCR product and loaded to the agarose gel. Electrophoresis was carried at 75 V for 1.5 hr. The gel was observed under UV light and documented using gel documentation unit. The ITS region was sequenced for five isolates of *R. areola* belonging to different geographical regions of to confirm organism and to know the variability present in them. UPGMA ("Unweighted Pair Group Method with Arithmetic Mean") cluster analysis based on genetic distance coefficients clearly separated the all isolates. By using this method construct the dendrogram.

Reaction mixture

Reagents	Volume/tube (µl)
Template DNA	1
Taq assay buffer (10x) (with $MgCl_2$)	2
dNTP mix	1
Forward Primer- F	1
Reverse Primer- R	1
Taq Polymerase	0.3
Sterile distilled water	12.7
Total	20

Results and discussion

The full length ITS rDNA region was amplified with ITS-1 (5'TCCGTAGGTGAACCTGCG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers for ten isolates of *R. areola viz.*, Ra-1 Dharwad, Ra-2 Arabhavi, Ra-3 Nargund, Ra-4 Navalagund, Ra-5 Haveri, Ra-6 Devihosur, Ra-7 Itagi, Ra-8 Bailahongal, Ra-9 Tadas, Ra-10 Hiremutthur. DNA amplicon was observed at the region 560 bp by checking the amplified products on 1.2 per cent agarose gel electrophoresis and representative samples were sequenced and by using the NCBI BLAST programme, these isolates were confirmed as *R. areola.* Cuervo-Parra *et al.* (2011) obtained the similar results where the DNA of three strains of R. areola electrophoresised and amplicon was observed at the region of 560 bp with universal ITS fungal primers (Plate 1). By using the NCBI-BLAST programme confirmed the isolates as *R. areola*. The list of isolates, accession number, per cent homology and name identified are given in Table 1.

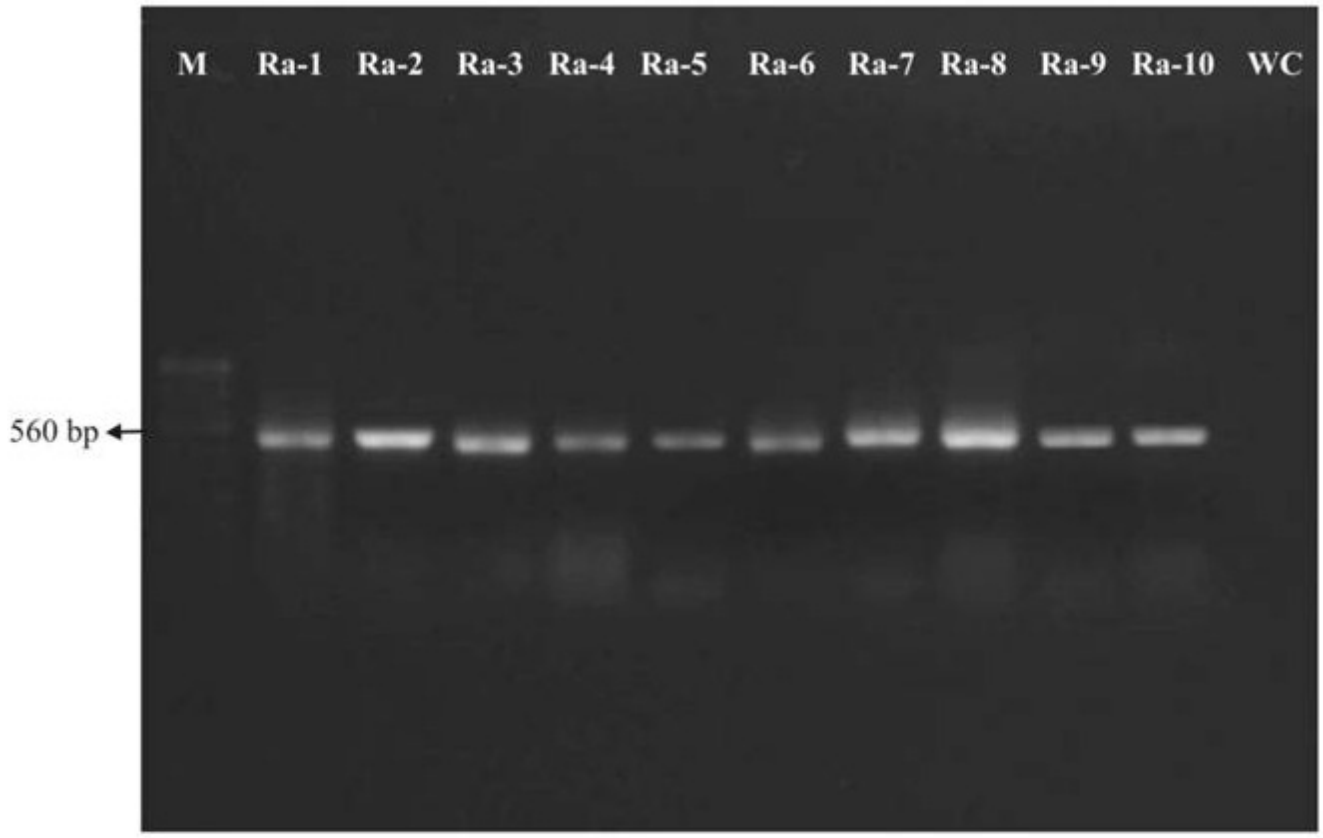
Phylogenetic analysis

The ITS rDNA region sequence was used in these analysis because it has been shown to be more informative and closest phylogenetic relative in *R. areola*, isolates. In order to evaluate whether the grouping pattern obtained on the basis of the ITS sequences would be useful frame to identify and align, these isolates and were identified at the species level by morphological characters using the existing taxonomic criteria analysis and analysis of their ITS rDNA region gene sequences (Fig. 1).

In the present case, isolates of *R. areola* were used as an out group vice versa to interpret the clustering of isolates as distinct or related out group of genus. Phylogenetic analysis grouped *R. areola* isolates into two main clusters with 0.12 similarity coefficient. The similarity coefficient ranged from 0.001 to 0.12 indicating good level of similarity. UPGMA cluster analysis based on genetic distance coefficient clearly separated all the isolates. Out of four isolates, Dharwad, Arabhavi and Naragund isolates were grouped under cluster A, where as Devihosur formed separate cluster B. The above result indicated there was 97 per cent similarity between Dharwad

Table 1. BLAST results of nucleotide sequence of *Ramularia areola* ITS region

Isolate code	Place	Accession number	NCBI BLAST Hit results	Location	Host	Max. Ident.
Ra1 Dha	Dharwad	DQ459082.1	Mycosphaerella areola strain Her clone CICR-1 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	Nagpur	Asiatic cotton	92
Ra-2 Arb	Arabhavi	KR265338.1	Mycosphaerella areola isolate RA44 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	Brazil	cotton	93
Ra-3 Nar	Naragund	DQ459075.1	Mycosphaerella areola strain Hir clone Dwd-3 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2	Nagpur	cotton	82
Ra-6 Dev	Devihosur	KR265336.1	Mycosphaerella areola isolate RA1403019 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	Brazil	Cotton	88



M - Marker
Ra-1: Dharwad
Ra-2: Arabhavi
Ra-3: Naragund
Ra-4: Navalgund
Ra-5: Haveri
Ra-6: Devihosur
Ra-7: Itagi
Ra-8: Bailhongal
Ra-9: Tadas
Ra-10: Hiremutthur
WC : Water control

Plate: Amplified ITS-1 and ITS-4 region of R. areola Atk. Isolates

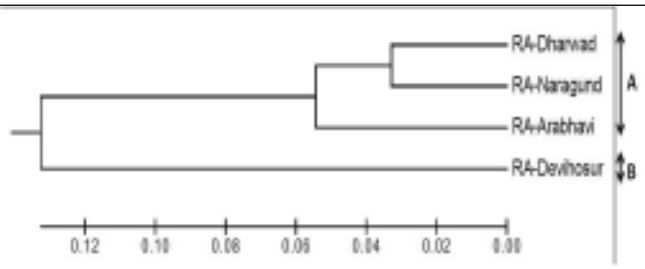


Fig. 1. Phylogenetic relationship for *Ramularia areola* isolates revealed by ITS marker

and Naragund, compared to Arabhavi isolate which showed 94.2 per cent similarity. Whereas cluster B of isolate Devihosur compared with the cluster A showed the 88 per cent similarity coefficient (Fig 1). These results are in conformity with the reports of Kadam (2005) who studied that the amplified products of ITS region of 11 fungal species from different crops, including strains of *R. solani, R. bataticola, A. macrospora* and *R. areola* reported in the present study, ranged between 569-575 bp, coinciding with the sizes obtained from similar fungal pathogens from other strains of the same species. Molecular techniques, if not alone, can be used in conjunction with classical methods where the latter approaches can at least narrow pathogen diagnosis to genus level. Once genus is narrowed by morphology, symptomatology, host-specificity, *etc.,* then PCR can be used to differentiate species (Chakrabarty *et al.,* 2007).

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