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Diversity of *Asperisporium caricae* (Speg.) Maubl. isolates causing papaya black spot disease

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ABSTRACT

Papaya black spot disease is an emerging concern in Karnataka, accompanied by a lack of knowledge regarding the associated pathogen. This study focuses on assessing the cultural and molecular variability of ten *Asperisporium caricae* isolates responsible for causing papaya black spot disease. The isolates underwent evaluation on twelve distinct solid media, with recorded observations on colony characteristics and sporulation. Among the ten isolates, three exhibited excellent radial growth, five displayed good growth, one had moderate growth, and one isolate demonstrated poor radial growth. All cultural media were subjected to sporulation testing, revealing that two isolates exhibited fair sporulation, five displayed sparse sporulation, and the remaining three showed no sporulation. PCR amplification using ITS 4 and ITS 5 resulted in a 590bp amplicon for all ten *A. caricae* isolates. Dendrogram clustering grouped the isolates into two clades, where the AcG isolate belonged to clade I, and AcH, AcKa, AcKu, AcMa, AcMU, AcMy, AcNa, AcR, and AcV formed clade II. Notably, the AcG isolate demonstrated lower similarity (62%) compared to other isolates. AcH and AcKu isolates exhibited the highest similarity (85%), followed by AcMa and AcMu (78.5%). The resemblance between AcH and AcKu was evident in certain cultural characteristics as well.

Key words: *Asperisporium caricae*, dendrogram, isolates, radial growth, sporulation.

INTRODUCTION

Papaya (*Carica papaya* L.), a member of the *Caricaceae* family, is a short-lived perennial with large palmate leaves, hollow stems, and rapid growth. Known for producing climacteric fruits, it reaches maturity within 9–12 months after planting (Gonsalves, 4). Widely cultivated and consumed, papaya stands as the most popular fruit in tropical and subtropical regions globally, appreciated for its nutritional, digestive, and medicinal benefits (Wall, 18).

Asperisporium caricae (Speg.) Maubl. is responsible for the black spot disease in papaya, posing a significant threat to crop yield as it diminishes plant vigor by reducing the photosynthetic area. This foliar disease also inflicts both quantitative and qualitative damages, leading to the commercial devaluation of fruits (Santos and Barreto, 12). In India, the disease was initially documented in 1977, affecting the Coorg Honey Dew variety in Chettali, Karnataka, and the Co 1 variety in the Palani hills of Tamil Nadu (Ullasa *et al*., 17). The symptoms of black spot, characterized by small, water-soaked spots that are sometimes angular, appear on the dorsal surface of young leaves and later turn greyish-white. Affected leaves undergo curling, necrosis, and extensive defoliation under severe disease pressure (Cumagun and Padilla, 1). This disease affects both leaves and fruits, recognizable by round, grey to black necrotic spots surrounded by a yellow halo, the disease exhibits seasonal incidence, with a peak in late winter and early spring (Patel *et al*., 6 & 7) resulting in a reduction in market value (Shantamma *et al*., 13), with a higher incidence during cool weather accompanied by rains (Gabrekiristos and Dagnew, 3).

Presumably, this fungus is prevalent in most papaya-growing regions in Karnataka. Southern Karnataka's 2018 survey highlighted diverse disease severity, showing the highest per cent disease index (PDI) in Ramanagar district and the lowest in Chamrajnagara. PDI fluctuated from 7.86% to 38.00% on leaves and 0.00% to 24.97% on fruits across the surveyed districts during September to November (Patel *et al*., 9). This black spot disease poses a severe challenge to global papaya production, causing post-harvest losses and hindering export-import activities. Effective management strategies involve soil health maintenance, *Trichoderma viride* application, and systematic fungicide use at specific intervals during kharif months (Shetty *et al*., 14). *In vitro* assessment of *T. harzianum*, *Prosopis juliflora* (15%), and Tebuconazole (75ppm) has been explored (Patel *et al*., 8), and the application of *Trichoderma viridae* along with the fungicide trifloxystrobin (25% WG) + tebuconazole (25% WG) has been suggested

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for improved black spot disease management (Taj and Kumar, 16). Plant extracts, a safe and eco-friendly alternative to traditional fungicides, were tested for papaya black spot disease control. *Allium sativum* proved highly effective, reducing infection to 9.87% at a 40% concentration during a ten-minute fruit dip, surpassing other extracts. *Azadirachta indica* exhibited the least efficacy with a 20.15% infection rate (Patel *et al*., 9). Recognizing the limited research on papaya black spot disease, with scarce literature available, it is imperative to bridge this gap to enhance our comprehension of the disease and the genetic diversity of *A. caricae* isolates. The current study aims to evaluate the cultural and genetic diversity of ten *A. caricae* isolates, providing valuable insights for the development of effective management strategies.

MATERIALS AND METHODS

The present study was conducted in College of Agriculture, V. C. Farm, Mandya during 2020-2021. Ten isolates of *A. caricae* were gathered from diverse regions across Karnataka. The designations and corresponding geographical locations of these isolates are outlined in Table 1.

To examine the cultural variability of various *A. caricae* isolates, twelve distinct solid media were employed. *viz*., Potato dextrose agar (PDA), Oat meal agar (OMA), Corn meal agar (CMA), Richard's synthetic agar (RSA), Sabouraud's dextrose agar (SDA), Yeast extract agar (YEA), Malt extract agar (MEA), Carrot agar (CA), Potato carrot agar (PCA), V-8 juice agar (VJA), Rye agar A (RAA), Rye agar B (RAB).

To assess variation in radial growth and sporulation on solid media, each of the twelve autoclaved media was individually melted and supplemented with streptomycin sulphate. Using a sterile inoculation loop, a 5 mm mycelial disc from the ten-day-old mother culture was transferred to the center of the respective solidified media and then incubated at 28±1°C for ten days. Radial growth of mycelia was recorded for each isolate on each cultural medium after the incubation period. The cultures were further examined for sporulation and classified based on the observed conidia counts: poor sporulation (+) (1-10 conidia); fair sporulation (++) (11-25 conidia); good sporulation (+++) (26-40 conidia); very good sporulation (++++) (>40 conidia); and no sporulation (-). The categorization was determined by counting the number of conidia/spores per microscopic field under 10x magnification.

Table 1. Isolates of *Asperisporium caricae* collected from different parts of Karnataka.

Sl. No.	Designation	District	Place
1	AcG	Bengaluru	GKVK
2	AcH	Hassan	Hassan
3	AcKa	Chikkamagaluru	Kadur
4	AcKu	Kodagu	Kushalnagara
5	AcMa	Mandya	Maddur
6	AcMu	Dakshina Kannada	Mudbidire
7	AcMy	Mysuru	Mysuru
8	AcNa	Mandya	Nagamangala
9	AcR	Ramanagara	Ramnagara
10	AcV	Mandya	V.C. Farm

A numerical code was assigned to represent various colony characteristics (including mycelial color, center of the colony, pigmentation, growth, margin, and topography) displayed by different isolates on diverse cultural media. This coding system was implemented to facilitate straightforward identification of similarities and differences among the colony characteristics of distinct isolates on various media, as outlined in Table 2.

The genomic DNA of the ten isolates of the papaya black spot pathogen was extracted using the Cetyl Trimethyl Ammonium Bromidemethod, as described by Doyle and Doyle (2) in 1987. To confirm the identification of the pathogen, the genomic DNA from the obtained isolates underwent PCR amplification using primers ITS 4 (5'-AAGTTTGATCCTGGCTCAG-3') and ITS 5 (5'-GGAAGTAAAAGTCGAACAAGG-3'), Table 3. The amplified bands from different *A. caricae* isolates were scored using binary values, with '1' indicating the presence of a band and '0' indicating its absence. This binary matrix was then input into the NTSYS software (Rohlf, 11) to calculate PIC (Polymorphic Information Content) values and gene diversity. The software was further utilized to construct a dendrogram representing the grouping of isolates based on variability.

RESULTS AND DISCUSSION

The results of mycelial growth are represented in Table 4. Among the ten tested *A. caricae* isolates AcMy (89.30 mm), AcNa (88.48mm), AcKu (88.02 mm) showed excellent growth, AcMa (85.76 mm), AcH (85.82 mm), AcKa (85.28 mm), AcV (83.96 mm), AcR (83.81mm) showed good growth, AcG (80.60 mm) showed moderate growth and poor growth was reported by AcMu (77.92 mm) on an average on all tested media. AcMu isolate showed good sporulation on PDA and PCA media, fair sporulation on OMA and RSA media, poor sporulation on RAB media. AcG isolate showed fair sporulation on PDA and

Table 2. Numerical codes assigned for colony characters of *Asperisporium caricae.*

Numerical code	Character of fungus	Numerical code	Character of fungus
1	White	19	Moderate filamentous
2	Dull white	20	Profuse velvety
3	Greyish white	21	Moderate velvety
4	Whitish grey	22	Scanty cottony
5	Grey	23	Scanty filamentous
6	White and black intermixed	24	Slow cottony
7	Greyish black	25	Slow filamentous
8	Blackish grey	26	Slow velvety
9	Blackish brown	27	Regular
10	Reddish brown	28	Irregular
11	Black	29	Flat and thick
12	Pale yellow	30	Flat and very thick
13	Yellowish white	31	Flat, thick and concentric
14	Yellow	32	Flat, thin and concentric
15	No pigmentation	33	Flat and thin
16	Profuse cottony	34	Flat and very thin
17	Profuse filamentous	35	Raised and thick
18	Moderate cottony	36	Raised and very thick

Table 3. Sequence of ten random primers used in RAPD-PCR analysis.

Sl. No	Primer code	Primer sequence (5'→ 3')
1	A-07	GAAACGGGTG
2	A-10	GTGATCCCAG
3	A-18	AGGTGACCGT
4	B-05	TGCGCCCTTC
5	C-11	AAAGCTGCGC
6	E-08	TCACCACGGT
7	K-12	TGGCCCTCAC
8	K-14	CCCGCTACAC
9	M-15	GACCTACCAC
10	P-09	GTGGTCCGCA

PCA, poor sporulation on OMA, CMA, RSA, CA and VJA. AcNa isolate showed poor sporulation on CA and PCA, AcKu, AcRand AcV isolates showed poor sporulation on PCA. Poor sporulation was detected on PDA by AcMa isolate (Table 5).

Colony characters varied from isolate to isolate on different cultural media. Mycelial type ranged from cottony to filamentous. Mycelial color exhibited dull white, white, yellowish white, grey, greyish white, whitish grey, blackish grey, white and black intermixed, greyish black forms.

Pigmentation type variations found were pale yellow, yellow, blackish brown, reddish brown and black, white, yellow, grey and black. Centre of the colony had variations like white, greyish white, grey, whitish grey, greyish black, blackish grey, black. Margin of the isolates observed were regular and irregular, topography variations of different isolates found on various cultural media were flat and thick, flat and very thick, flat and thin, flat and very thin, raised and very thick, raised and thick.

Results obtained are in accordance with findings of several workers who mentioned different colony character of *A. caricae*. (Reddikumar *et al*., 10) witnessed that initially the mycelium of papaya black spot fungi was pale white later the mycelia became grey color (Patel *et al*., 8) and observed variations in radial growth, sporulation and colony characters of different isolates of *A. caricae*. (Shreedevasena *et al.,* 15) reported that the colonies of *A. caricae* on PDA were near dark green to black in color.

The ten isolates of *A. caricae* were subjected to PCR amplification by using ITS 4 and ITS 5 primers. Upon visualizing the amplified product on agarose gel, the anticipated single fragment of 590 bp was observed (Fig. 1) in all the isolates. Results were in conformity with findings of Sreedevasena *et al.* (15) who got an amplicon size of 560bp of 18S rRNA gene sequencing of *Asperisporium caricae.*

Table 4. Radial growth of different isolates of *Asperisporium caricae* on different cultural media.

Sl. No.	Solid media	Radial growth (mm)									
		Isolates									
		AcG	AcH	AcKa	AcKu	AcMa	AcMy	AcMu	AcNa	AcR	AcV
1	PDA	89.47	90.00	90.00	90.00	90.00	90.00	89.13	90.00	90.00	89.40
2	OMA	85.75	79.73	90.00	90.00	90.00	90.00	89.33	78.27	90.00	90.00
3	CMA	73.53	73.60	90.00	90.00	90.00	85.23	77.23	87.23	90.00	90.00
4	RSA	81.00	90.00	83.57	88.40	90.00	90.00	69.47	90.00	90.00	90.00
5	SDA	65.36	75.65	89.20	86.80	90.00	90.00	56.70	90.00	90.00	90.00
6	YEA	87.06	90.00	81.29	90.00	58.40	90.00	61.23	90.00	55.40	58.07
7	MEA	88.83	90.00	67.51	88.87	90.00	86.41	74.20	89.03	90.00	85.67
8	CA	89.12	90.00	90.00	90.00	90.00	90.00	89.40	87.30	90.00	83.78
9	PCA	72.33	90.00	88.83	90.00	90.00	90.00	82.53	90.00	90.00	90.00
10	VJA	66.57	82.45	73.20	72.20	71.43	90.00	67.67	90.00	50.43	60.63
11	RAA	88.23	90.00	90.00	90.00	89.33	90.00	90.00	90.00	90.00	90.00
12	RAB	80.05	88.50	89.37	90.00	90.00	90.00	88.23	90.00	90.00	90.00
F		**	**	**	**	**	**	**	**	**	**
SEm ±		0.19	0.21	0.16	0.09	0.10	0.08	0.21	0.09	0.12	0.17
CD @1%		0.73	0.84	0.62	0.34	0.40	0.30	0.81	0.35	0.48	0.68

Table 5. Sporulation of different isolates of *Asperisporium caricae* on different solid media.

Sl. No.	Solid media	Sporulation									
		Isolates									
		AcG	AcH	AcKa	AcKu	AcMa	AcMu	AcMy	AcNa	AcR	AcV
1	Potato dextrose agar	++	-	-	-	+	+++	-	-	-	-
2	Oat meal agar	+	-	-	-	-	++	-	-	-	-
3	Corn meal agar	+	-	-	-	-	-	-	-	-	-
4	Richard's synthetic agar	+	-	-	-	-	++	-	-	-	-
5	Sabourard's dextrose agar	-	-	-	-	-	-	-	-	-	-
6	Yeast extract agar	-	-	-	-	-	-	-	-	-	-
7	Malt extract agar	-	-	-	-	-	-	-	-	-	-
8	Carrot agar	+	-	-	-	-	-	-	+	-	-
9	Potato carrot agar	++	-	-	+	-	+++	-	+	+	+
10	V-8 juice agar	-	-	-	-	-	-	-	-	-	-
11	Rye agar A	-	-	-	-	-	-	-	-	-	-
12	Rye agar B	+	-	-	-	-	+	-	-	-	-

Poor sporulation (+) (1-10 conidia); Fair sporulation (++) (11-25 conidia); Good sporulation (+++) (26-40 conidia); Very good sporulation (++++) (>40 conidia); No sporulation (-); Number of conidia per microscopic field under 10x considered for categorization

The UPGMA based dendrogram (Fig. 2) was obtained from the data deduced from the DNA profiles of the samples analyzed. It grouped ten isolates into two clades (CI and CII). The clade CI consist of one isolate and CII consists of nine isolates. The clade CI consist of AcG (Bengaluru) isolate with similarity index 0.62, which means it is 62% similar with the other isolates of CII clade. CII clade consists of AcH (Hassan), AcKu (Kushalnagar), AcMy (Mysuru), AcNa (Nagmangala), AcR (Ramanagara), AcKa (Kaduru), AcMa (Madduru), AcMu (Mudbidire) and AcV (Mandya). CII is further divided into two

Table 6. Grouping of ten *Asperisporium caricae* isolates based on similarity co-efficients.

Clades	Clade composition and similarity							
CI	62%	CI	AcG (Bengaluru)					
CII		CII	64%	SCI	69%	SCI a	77%	85% AcH (Hassan) and AcKu (Kushalnagara) isolate
								AcMy (Mysuru) isolate
						SCI b	76.5%	AcNa (Nagamangala) and AcR (Ramanagara)isolate
				SCII	64.5%	SCII a	AcKa (Kaduru)	
						SCII b	69.3%	78.5% AcMa (Maddur) and AcMu (Mudbidire) isolates
								AcV (Mandya) isolates

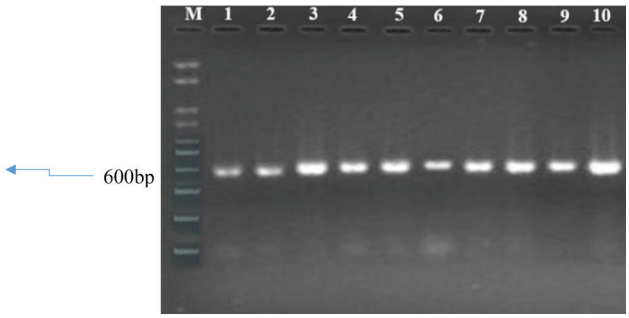

Fig. 1. PCR amplification of *Asperisporium caricae* (AcV) using ITS 4 and ITS 5 primer Lane index: M- DNA ladder (100kb), 1- AcG, 2-AcH, 3-AcKa, 4-AcKu, 5-AcMa, 6.AcMy, 7.AcMu, 8. AcNa, 9. AcR, 10. AcV.

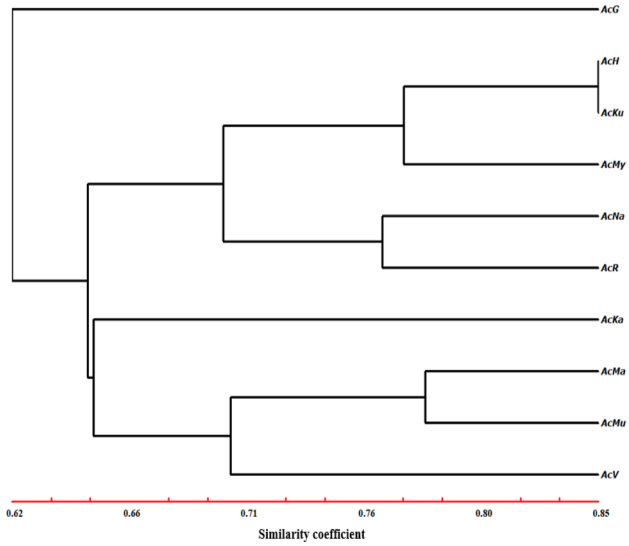

Fig. 2. Dendrogram showing clustering of ten isolates of *A. caricae* for ten RAPD markers.

subclades (SC) with similarity index 0.64. SCI (Subclade I) consists of five isolates *viz*., AcH, ACKu, AcMy, AcNa and AcR isolates. SCII (Subclade II) consists of four isolates AcKa, AcMa, AcMu and AcV (Table 6).

Similar study was conducted by Joshi *et al.* (5) who studied polymorphism of eleven isolates of *Cercospora canescens* by random amplified polymorphic DNA (RAPD) marker technique at molecular level and variation in the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). RAPD profiling clustered all the isolates into three clusters. Considerable genetic diversity was observed in the isolates from the same and different location.

To conclude, this study infers that there is substantial cultural and genetic variability among the isolates of *A. caricae*. The insights of this study about the variability of *A. caricae* isolates step towards better understanding about papaya black spot disease and pathogen also about the genetic similarity of isolates which may contribute to the virulence of isolates. These findings could be referred to advance the studies on papaya black spot disease.

AUTHORS' CONTRIBUTIONS

Execution of field/lab experiments, Designing of the experiments and data collection (SSY); Conceptualization of research, Designing of the experiments and supervised (SKVB); Contribution of experimental materials and critical revision of the article (KKN, MH, AKR and PSP).

DECLARATION

The authors declare that there is no conflict of interest.

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