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Cultural, morphological and molecular diversity of *Ustilaginoidea virens* (Cooke) Tak. isolates causing false smut of rice in different agroclimatic regions of Karnataka

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The ascomycete fungal pathogen *Ustilaginoidea virens* (Cooke) Takahshi cause false smut in rice and considerable yield loss. In this study, we collected isolates of *U. virens* from the rice growing ecosystems of Karnataka and characterized for cultural, morphological and molecular characters. The isolates of the fungus on Potato Sucrose Agar media exhibited distinct colonies with colony growth ranging from 21.50 mm (Uv-20) to 70.00 mm (Uv-15). The colony colour appeared as whitish to yellowish with varied growth pattern from flat, raised flat to fluffy and raised fluffy colonies with sectoring in Uv-1, Uv-3, Uv-6 and Uv-9 isolates. The isolates of *U. virens* also showed variation in the morphology of spores, where the conidia were globose, irregularly round to elliptical and warty on the surface with spore radius ranging from 2.91 to 5.36 µm. The scanning electron microscopy revealed hyaline globose to irregularly rounded ornamented chlamydospores with prominent spines. Besides cultural and morphological characters, molecular identification of false smut isolates was confirmed through ITS sequencing which showed 91 to 99% identity with *U. virens* in NCBI-BLAST analysis. Dendrogram constructed using ITS sequence data broadly separated the isolates into two major clusters with divergence among clusters. This ITS (internal transcribed spacer) sequencing of isolates should help better understanding of the phylogenetic relationships among these isolates.

Keywords: Chlamydospores, Disease severity, Growth characters, *Oryza sativa*, Paddy

Rice (*Oryza sativa* L.) is one of the most important, perhaps the oldest cultivated crop, which plays a greater role in food security with high economic importance as primary source of food for more than half of the world's population with 509.87 million metric tons during 2021-22[1]. Despite an impressive progress in increasing food grain production in general and rice in particular, the productivity of rice in India is very low. The prime factors limiting rice yield includes both the biotic and abiotic factors. Among the biotic factors affecting rice production, false smut of rice caused by *Ustilaginoidea virens* (Cooke) Tak. is known to cause significant quantitative and qualitative yield losses in grain. In India, the disease was first reported from Tirunelveli district of Tamil Nadu[2]. False smut was previously categorized as a minor disease due to its sporadic occurrence and considered as symbol of good harvest in old times[3]. However, due to the introduction of high yielding semi-dwarf varieties, change in climate and as associated change in the rice growing technology (higher planting density and dose of fertilizers, increase in irrigation) contributed to a gradual shift in the disease. Hence, the false smut of rice became devastating disease in majority of the rice-growing areas of the world[4]. In India the disease has been observed in major rice-growing states, *viz.*, Haryana, Punjab, Uttar Pradesh, Uttaranchal, Tamil Nadu, Karnataka, Andhra Pradesh, Bihar, Jharkhand, Gujarat, Maharashtra, Jammu & Kashmir and Puducherry[4,5].

The symptoms produced by *U. virens* become visible only after flowering and occurs in the field at the hard dough to mature stages of the crop. A few spikelets in the panicle transforms into globose, yellowish green and velvety spore balls[6]. The pathogen is known to overwinter as sclerotia and chlamydospores in soil, debris and as admixture with seed for several months. The sclerotia produce ascospores, which act as primary source of infection for the disease, whereas secondary infection may

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come from air borne chlamydospores[7,8]. The disease is known to cause significant quantitative and qualitative yield losses in grain and that might be due to sterile spikelets adjacent to the smut balls and makes panicle chaffy[6,8, 9]. The disease losses have been estimated in different states, where the disease incidence was found to be 2 to75% for North West states and 5 to 85% from Southern state of Tamil Nadu[4].

The development of effective disease management strategies demands a comprehensive understanding of the causal agent with reference to cultural, morphological, physiological and molecular characters[10]. Cursory literature review revealed that, research work on basic and molecular characters of false smut is limited. The differences in disease development on different rice cultivars in various localities of the state may be due to the variability in the isolates of pathogen, which includes the morphological and physiological variations. However, genetic diversity in plant pathogens may be of great importance. Hence, objectives of the present study was to examine the cultural, morphological and molecular variability in *U, virens* population in rice growing regions of Karnataka and to understand the genetic structure of the pathogen population in the region with a view to provide useful information for breeding programme and improved disease management.

Materials and Methods

Collection, isolation, identification and maintenance of isolates

The false smut infected rice panicles showing typical smut balls were collected from different ecosystems of Karnataka *viz*., irrigated and direct seeded Tunga-Bhadra and upper Krishna project command area (TBP and UKP), hilly upland, coastal ecosystem, irrigated Bhadra and irrigated Kaveri ecosystem during *Kharif* 2017. The pathogen *U. virens* was isolated on potato sucrose agar media (PSA) from typical rice smut ball using standard tissue isolation and subsequently purified by hyphal tip isolation method. The purified cultures were incubated at 25 ± 2°C and used for further studies. The cultures were identified according to cultural descriptions[11,12]. The obtained 20 isolates from different ecosystems of Karnataka were stored in the refrigerator at 4°C and renewed once in two months.

Diversity in cultural and morphological characters

The 20 isolates were grown on PSA at 25 ± 2°C for three weeks to study the cultural characteristics and the colony characters like colony diameter, colour, growth pattern, sectoring, backward appearance of colony colour and shape of the chlamydospores. The morphological characters, *viz*., colour, size (length and width) and shape of the chlamydospores were recorded on 21st day of incubation. Dimensions of the chlamydospore were measured using binocular microscope fitted with the Gryphax image analyser and capture software.

Genetic diversity analysis

Extraction of DNA and PCR detection

Twenty monosporic isolates of *U. virens* isolates were grown on potato sucrose broth for mycelial production to be used for DNA extraction. Mycelial mats were harvested by filtering through Whatman filter paper no.1 and washed repeatedly with distilled sterilized water, blot dried and cetyl trimethyl ammonium bromide (CTAB) method was used to extract genomic DNA from the harvested mycelium. The quality and quantity of DNA was analysed by running 2 µL of each sample mixed with 2 µL of 1 0X loading dye in agarose gel (1%). The DNA from all the isolates produced clear sharp bands in one per cent agarose gel indicating good quality of the DNA. The DNA was quantified by Nanodrop (Denovix, USA)[4].

The universal internal transcribed spacer primers such as ITS1 F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 R (5′-TCCTCCGCTTATTGATATGC-3′) were used for specific amplification of ITS region of 20 isolates of *U. virens* isolated from different rice growing ecosystems of Karnataka. The amplification was performed in a total volume of reaction mixture containing 2.0 µL dNTPs mix (2 mM), 1 µL primer (5 pmol), 1 µL of DNA (40 ng/ µL), 2 µL *Taq* buffer A (10X with 2.5mM $MgCl_2$), 0.3 µL of *Taq* polymerase (3U) and 12.7 µL deionized water. The DNA amplification was done in a thermal cycler (Eppendorf, Germany) using the following PCR cycles. The first denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation for one min at 94°C, annealing for 1 min at 54°C, extension at 72°C for 2 min and final extension at 72°C for 10 min with holding temperature at 4°C for 20 min. Reaction products (8 µL) were resolved by electrophoresis in 1.4% agarose gel stained with ethidium bromide in 1X TBE buffer at 65 V for 90 min. The gel was observed under UV light and documented using gel documentation unit.

The amplified PCR product of both the regions (ITS1and ITS4) were sent for purification through gel elution using gel extraction kit (Qiagen gel extraction kit) and sequenced at Eurofins Genomics Pvt. Ltd., Bengaluru from both the ends (ITS1and ITS4). 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 sequences were deposited in the NCBI GenBank, Maryland, USA, to get accession numbers. Multiple alignments for homology search were performed using the clustral W algorithm software and the phylogenetic tree was constructed using MEGA 7.0 online software version[13]. The experimental data were analysed using IBM-SPSS statistics versison-28 software.

Results

Cultural characters

The false smut samples collected from farmer's field of different rice growing ecosystems of Karnataka (Fig. 1) showed varied symptoms different stages of infection. The symptoms incited by *U. virens* were recorded after blooming, where it transformed the floret into a large velvety yellow to orange pulverulent mass (pseudomorph) and changed to olive-green colour at the later stage of infection. The 20 isolates collected from different rice growing ecosystems of Karnataka were identified as *U. virens* based on the cultural, morphological and molecular characters and they were designated as Uv-1 to Uv-20 (Table 1). All the isolates produced clear colonies on PSA with colony diameter ranging from 21.50 to 70.00 mm. Maximum colony diameter was observed in Uv-15 (70.00 mm) followed by Uv-17 (64.50 mm), Uv-11 (62.50 mm), whereas minimum colony diameter of 21.50 mm was noticed in the isolate Uv-20. Based on the growth characteristics the six

Table 1 — Source and identity of 20 isolates of *Ustilaginoidea virens*

Eco-system	Taluk	Place	Isolates	GenBank accession no. deposited in Genbank, Maryland, USA
Irrigated and DSR eco-systems of TBP and UKP command	Raichur	Burdipad	Uv-1	MN340255
		Kasabe camp	Uv-2	MN340256
	Sindhanur	Hanumapur	Uv-3	MN340257
	Manvi	Neermanvi	Uv-4	MN340258
	Gangavathi	Siddapur	Uv-5	MN340259
		ARS, Gangavathi	Uv-6	MN340260
	Siruguppa	Malapur	Uv-7	MN340261
	Shahpur	Gonal	Uv-8	MN340262
Hilly upland	Sirsi	Boppanahalli	Uv-9	MN340263
	Sirsi	Bommanahalli	Uv-10	MN340264
	Dharwad	Kalakeri	Uv-11	MN340265
	Virajpet	Ponnampet	Uv-12	MN340266
Coastal	Brahmavara	Charitharu	Uv-13	MN340267
	Karkala	Andaru	Uv-14	MN218699
Irrigated Bhadra	Shivamogga	Kumsi	Uv-15	MN218700
	Harapanahalli	Nittur	Uv-16	-
	Harihara	Dittur	Uv-17	MN218701
	Harihara	Hulaginahole	Uv-18	MN218702
	Bhadravati	Holehonnur	Uv-19	MN218703
Irrigated Kaveri	Mandya	VC Farm	Uv-20	MN218704

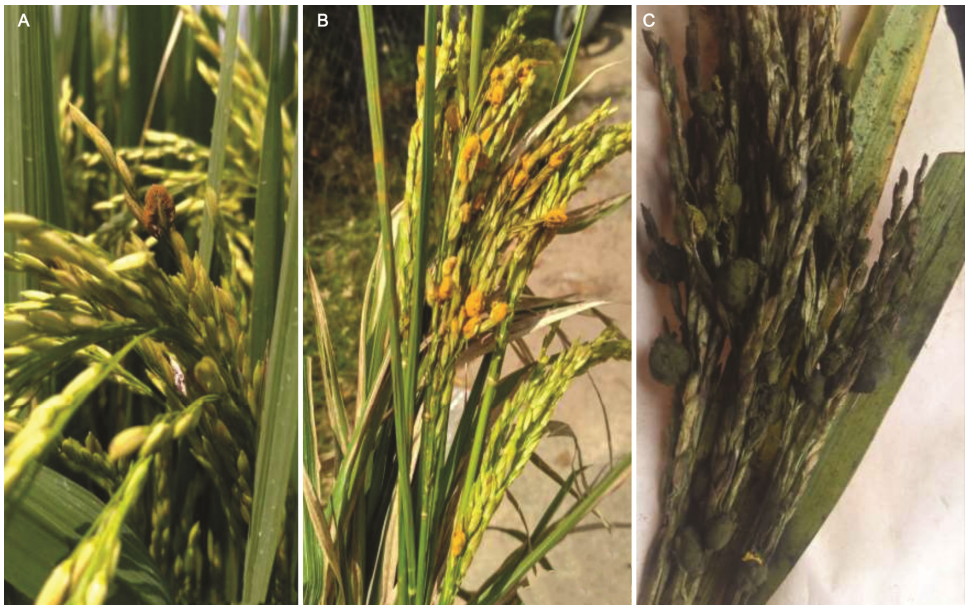

Fig. 1 — Symptoms of False smut of rice. (A) Infected grain with mycelia; (B) Development of yellow smut ball; and (C) Formation of dark green smut ball

isolates Uv-6, Uv-7, Uv-8, Uv-14, Uv-19, Uv-20 showed poor growth, nine isolates *viz*., Uv-1, Uv-2, Uv-3, Uv-4, Uv-5, Uv-10, Uv-12, Uv-13, Uv-18 revealed moderate growth and four isolates *viz*., Uv-11, Uv-15, Uv-16, Uv-17 showed good growth on PSA medium (Table 2 and Fig. 2).

Colony colour in most of the isolates was initially white which changed to greenish white (Table 2 and Fig. 2). However, two isolates *viz.*, Uv-17 and Uv-11 showed grey and greyish black colonies, respectively and the isolate Uv-14 depicted yellowish white colour. The colour of the colony changed with time and final colour recorded after three weeks of incubation for most of the isolates was white, greenish white, yellowish white and dark.

On the basis of mycelial colour, all the isolates were grouped in to five group such as white, white to yellow, white to green, grey to black and dark green. The isolates also differ in their growth pattern from flat, raised flat to fluffy and raised fluffy. The isolates Uv-2, Uv-4, Uv-5, Uv-8, Uv-11, Uv-12 and Uv-17 showed flat colonies, whereas Uv-1, Uv-6, Uv-7, Uv-9, Uv-14, Uv-15, Uv-16, Uv-19 and Uv-20 exhibited raised flat colony character. The isolates Uv-10 and Uv-13 showed fluffy, but on the contrary isolates Uv-3 and Uv-18 showed raised fluffy character. Sectoring was observed in four isolates *viz*., Uv-1, Uv-3, Uv-6 and Uv-9.

Morphological characters

Bright field light microscopy of the fungus showed that the colour of the chlamydospore varied in different isolates from light brown, brown and dark brown which were borne laterally on minute sterigmata on radial hyphae and are echinulate or bear warty spore wall (Table 2 and Fig. 3). Isolates such as Uv-9, Uv-10, Uv-11, Uv-12, Uv-13 Uv-17 and Uv-18 produced light brown coloured chlamydospores, but those of isolates *viz.*, Uv-1, Uv-2, Uv-4, Uv-5, Uv-6, Uv-7, Uv-14, Uv-16, Uv-19 and Uv-20 were brown in colour, whereas three isolates such as Uv-3, Uv-8 and Uv-18 were dark brown. Shape of the chlamydospores also varied among the isolates from globular (Uv-1, Uv-2, Uv-6, Uv-7, Uv-12, Uv-13, Uv-16 and Uv-17) to ovoid (Uv-2, Uv-8 and Uv-11) and irregular (Uv-4, Uv-9, Uv-10, Uv-12 and Uv-13).

Considerable variation in conidial morphology was observed among the isolates of *U. virens.* Among the isolates tested (Table 3 and Fig, 3), Uv-19 recorded maximum mycelial width of 6.37 µm followed by Uv-6 (5.43 µm), Uv-1 (5.17 µm) and Uv-16 (5.10 µm). Least mycelia width was recorded in the isolate Uv-15 (3.85 µm). Chlamydospore morpho-metrics also revealed considerable variation among the isolates. The size of the chlamydospores ranged from 28.82 (Uv-20) to 90.16 μm^2 (Uv-19), whereas the radius

Table 2 — Cultural diversity of *U. virens* isolates collected from different rice ecosystems of Karnataka

Isolates	Colony diameter (mm)	Colony colour after			Sectoring	Growth pattern	Appearance on reverse side of plate	Chlamydospore character	
		7 days	14 days	21 days				Colour	Shape
Uv-1	41.50	White	Greenish white	Greenish white	Present	Raised flat	Greenish white	Brown	Globular
Uv-2	40.00	White	White	White	Absent	Flat	Yellowish	Brown	Globular
Uv-3	52.50	White	White	Greenish white	Present	Raised fluffy	Blackish white	Dark brown	Irregular
Uv-4	42.50	White	White	Greenish white	Absent	Flat	Light brown	Brown	Ovoid
Uv-5	46.50	White	White	White	Absent	Flat	Creamish white	Brown	Irregular
Uv-6	47.00	White	White	White	Present	Raised flat	Whitish brown	Brown	Globular
Uv-7	41.50	White	White	Greenish white	Absent	Raised flat	Creamish white	Brown	Globular
Uv-8	40.00	White	White	White	Absent	Flat	Light green	Dark brown	Ovoid
Uv-9	41.00	White	Creamish white	White	Present	Raised flat	Creamish white	Light brown	Ovoid
Uv-10	55.00	White	Greenish white	Greenish white	Absent	Fluffy	Greenish white	Light brown	Irregular
Uv-11	62.50	Greenish white	Greyish black	Greyish black	Absent	Flat	Black	Light brown	Ovoid
Uv-12	42.50	Grey	Creamish white	White	Absent	Flat	Creamish white	Light brown	Globular
Uv-13	51.00	Creamish white	White	White	Absent	Fluffy	Light brown	Light brown	Globular
Uv-14	35.50	White	Yellowish white	Yellowish white	Absent	Raised flat	Light yellowish	Brown	Irregular
Uv-15	70.00	White	Green	Dark green	Absent	Raised flat	Black	Brown	Ovoid
Uv-16	57.50	White	Greenish white	Greenish white	Present	Raised flat	Dark green	Brown	Globular
Uv-17	64.50	Grey	Grey	Grey	Absent	Flat	Black	Light brown	Globular
Uv-18	51.50	White	White	Greenish white	Absent	Raised fluffy	Greenish	Dark brown	Ovoid
Uv-19	39.00	White	White	White	Absent	Raised flat	Whitish brown	Brown	Irregular
Uv-20	21.50	Greenish white	Greenish white	Dark green	Absent	Raised flat	Dark green	Brown	Irregular
S. Em ±	2.37								
C.D. at 1%	7.02								

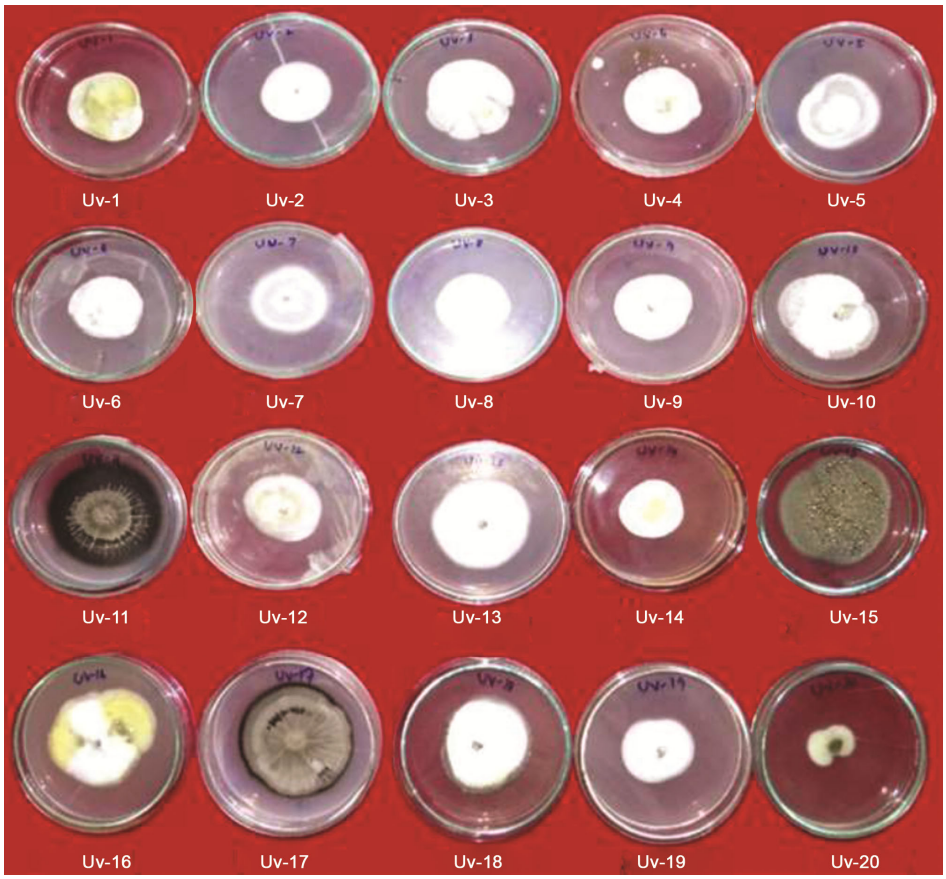

Fig. 2 — Growth of twenty isolates of *Ustilaginoidea virens* on Potato sucrose agar media

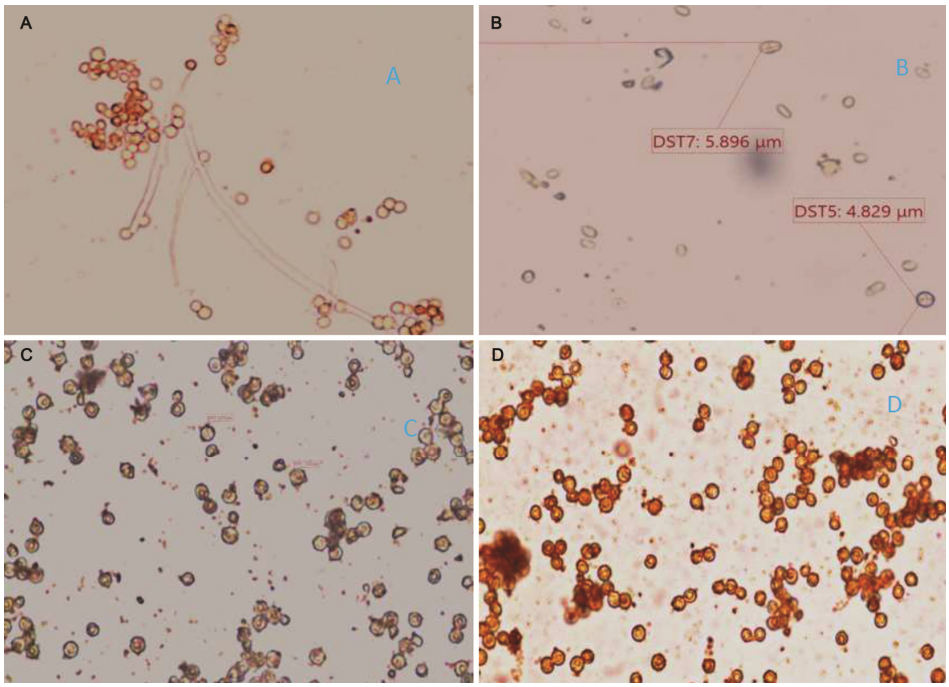

Fig. 3 — Spores of *Ustilaginoidea virens*. (A) Chlamydospores borne on minute sterigmata of lateral hyphae; and (B-D) Chlamydospores of UV-16, UV-18 and Uv-6 isolates

Table 3 — Morphological diversity of *Ustilaginoidea virens* isolates collected from different rice ecosystems of Karnataka

Rice ecosystems of Karnataka	Place of collection	Isolates	Mycelial width (µm)		Chlamydospore radius (µm)		Chlamydospore size (µm)^2		Disease severity (%)
			Width	Mean	Width	Mean	Size	Mean	
Irrigated and DSR ecosystems of TBP and UKP command	Burdipad	Uv-1	4.01-5.85	5.17	3.70-4.21	4.01	43.03-55.70	50.49	5.84
	Kasabe camp	Uv-2	4.3-5.85	4.99	2.86-3.20	3.03	25.71-32.18	28.93	5.37
	Hanumapur	Uv-3	3.99-5.86	4.84	2.89-3.65	3.24	26.25-41.87	32.95	9.40
	Neermanvi	Uv-4	3.82-5.85	4.88	3.00-4.92	3.81	28.29-76.08	45.72	8.02
	Siddapur	Uv-5	3.25-5.45	4.28	2.40-3.25	2.91	18.10-33.20	26.58	4.19
	ARS, Gangavathi	Uv-6	4.26-6.59	5.43	3.95-5.26	4.61	49.04-86.96	66.91	14.68
	Malapur	Uv-7	4.12-5.47	4.91	3.05-4.25	3.87	29.24-56.77	47.07	2.02
	Gonal	Uv-8	4.65-5.49	5.00	3.50-4.89	4.26	38.50-75.15	57.04	2.32
	Boppanahalli	Uv-9	3.99-5.89	5.01	2.65-5.10	3.95	22.07-81.75	49.04	5.50
Hilly upland	Bommanahalli	Uv-10	3.90-6.10	4.75	3.70-5.02	4.11	26.43-79.20	53.04	10.71
	Kalakeri	Uv-11	3.89-6.91	5.04	3.70-5.25	4.63	43.03-86.63	67.26	8.27
	Ponnampet	Uv-12	3.37-5.85	4.84	3.90-5.89	4.97	47.80-109.03	77.69	17.76
Coastal	Charitharu	Uv-13	3.33-4.56	3.95	2.89-3.57	3.25	26.25 - 40.06	33.24	2.19
	Andaru	Uv-14	3.78-6.12	5.02	3.98-5.69	4.89	49.78-101.75	75.09	25.19
Irrigated Bhadra	Kumsi	Uv-15	3.44-4.20	3.85	4.00-4.27	4.17	50.29-58.11	54.76	11.74
	Nittur	Uv-16	3.99-6.47	5.10	3.68-6.49	5.00	42.56-132.38	78.45	10.11
	Ditur	Uv-17	3.44-5.23	4.54	3.70-4.95	4.00	43.03-78.57	50.29	19.04
	Hulaginahole	Uv-18	3.26-4.69	3.91	3.00-4.19	3.62	28.29-55.18	41.19	7.15
	Holehonnur	Uv-19	5.12-7.10	6.37	4.02-6.79	5.36	50.79-123.55	90.16	4.40
Irrigated Kaveri	VC Farm	Uv-20	3.10-4.69	4.02	2.20-4.00	3.03	15.21-40.73	28.82	2.70
S. Em ±				0.31		0.24		6.84	
C.D. at 1%				0.88		0.68		19.28	

Table 4 — Scanning electron microscopy based morphological diversity among five representative isolates of *Ustilaginoidea virens*

Isolates	Shape	Colour	Chlamydospore diameter (µm)		Spine length (nm)	Mean (nm)
			Width	Mean		
Uv-1	Round and echinulate	Hyaline	4.27-5.76	5.15	562.50-981.00	626.02
Uv-12	Round and echinulate	Hyaline	4.89-5.46	5.26	720.20-820.30	785.68
Uv-14	Round and echinulate	Hyaline	5.02-6.36	6.06	730.00-738.20	730.10
Uv-15	Round and echinulate	Hyaline	4.27-5.47	5.14	639.40-1084.00	771.70
Uv-20	Round and echinulate	Hyaline	4.70-4.90	4.66	495.00-689.10	573.55
S.Em ±				0.19		42.52
C.D. at 1%				0.57		127.49

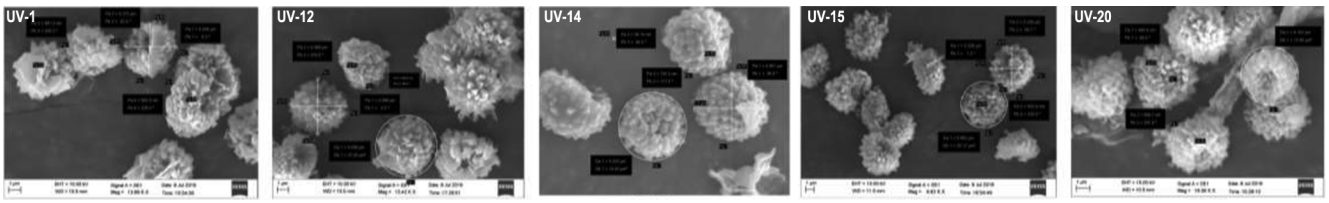

Fig. 4 — Surface features of five representative isolates conidia of *Ustilaginoidea virens* seen in the scanning electron microscope at magnification of 6 KX and well defined spines at magnification of 16.32 KX

varied from 2.91 µm (Uv-5) to 5.36 µm (Uv-19). The varied size was noticed among the chlamydospores of isolates collected from rice growing ecosystems of Karnataka. The chlamydospore size of the isolates from irrigated Tunga-Bhadra command area was from 26.58-66.91 µm^2, while the size of the chlamydospores from all other ecosystems ranged from 28.82 to 90.16 µm^2. The maximum chlamydospore size was noticed in the isolate Uv-19 with 90.16 µm^2 next in size were the isolate Uv-16 from Nittur with 78.45 µm^2 and Uv-12 from Ponnampet (77.69 µm^2). The maximum chlamydospore size was noticed among the isolates collected from coastal, hilly upland and irrigated Bhadra ecosystem. The isolate collected from irrigated Kaveri ecosystem revealed smaller size of 28.82 µm^2.

Scanning electron microscopic studies

Five representative isolates of false smut of rice collected from different rice growing ecosystems of Karnataka were visualised under scanning electron microscope to study the variability among the isolates with reference to the morphological characters (Table 4 and Fig. 4). The isolates exhibited a great amount of polymorphism with regard to morphological

characters like, spore size, length of spine, colour and shape of chlamydospores. Scanning electron microscopy revealed hyaline globose to irregularly rounded and ornamented chlamydospores with prominent spines. The spines were pointed at the apex or irregularly curved and 495-1084 nm long. The diameter of spores ranged from 4.27-6.36 µm. The mean maximum spore diameter of 6.06 µm was recorded in Uv-14 and Uv-12 isolate recorded maximum spine length of 785.68 nm, whereas, the mean minimum spore diameter and spine length was recorded by Uv-20 with 4.66 µm and 573.55 nm, respectively.

Molecular characterisation

The molecular diversity of *U. virens* isolates was carried out using ITS-1 and ITS-4 universal primer pair. The ITS gene sequences of 20 isolates of *U. virens* were amplified at a size of 550-600 bp regions. The amplified products of 20 isolates were observed using agarose gel electrophoresis. The PCR products were sequenced based on their locality and the sequences obtained were compared with the available NCBI database in the Basic Local Alignment Search Tool (BLAST). Sequence data of *U. virens* submitted in gene bank were matched 91-99% with the reference strains of NCBI (Table 1). The results were agreed with Mathew *et al*.[14] who observed sequence length of Uv-2 (Bulandshahr, Uttar Pradesh) and Uv-3 (Haridwar, Uttarakhand) with 645 and 634 bp, respectively and the identity was 98-99%. It is thus indicated that identity of the fungus could be confirmed by sequencing PCR products of ITS regions using primers[15].

Dendrogram for the isolates of *U. virens* was constructed using MEGA 7.0 online software with bootstrap method indicated variation among the isolates among ecosystems as well as within the ecosystem. All the isolates were distinct from one another in the cluster but identical to each other in homology and divergence with different nodes (Figs. 5 and 6). The dendrogram broadly separated the 20 isolates into two major clusters, the cluster-I was comprised of 12 isolates and cluster-II with eight isolates. The cluster-I was further divided into four sub clusters, where the sub cluster-I comprised of Uv-12, Uv-18, Uv-19, Uv-17, Uv-20, Uv-15, Uv-9, Uv-14 and Uv-16 isolates showed divergence from Uv-10 (Bommanahalli) in sub cluster-II and Uv-18 (Gonal) from sub cluster-III` and Uv-11 (Kalakeri) from sub cluster IV and these three isolates formed a separate

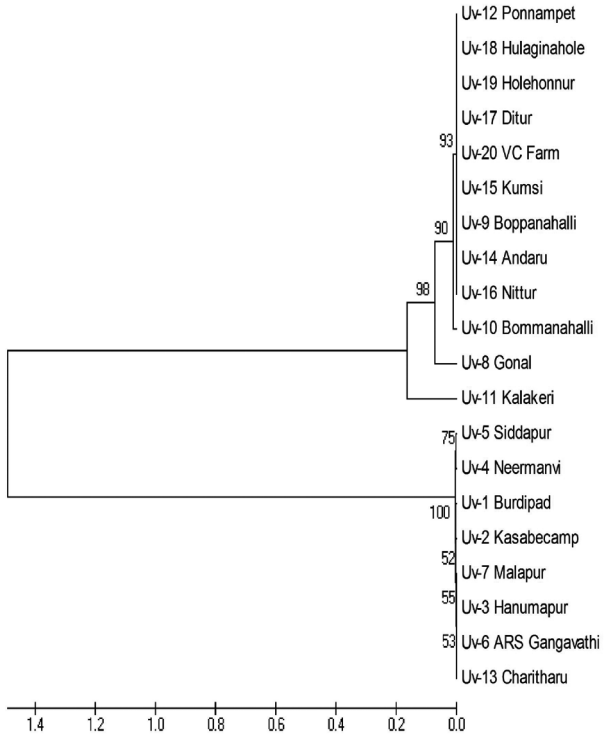

Fig. 5 — Dendrogram depicting relationship of *Ustilaginoidea virens* isolates based on ITS sequence data (Bootstrap method)

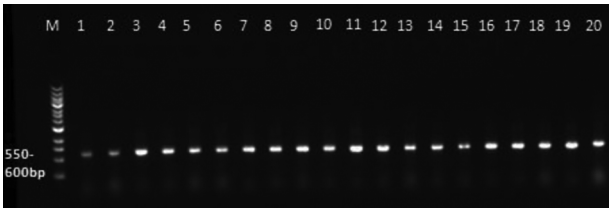

Fig. 6 — Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) regions of *Ustilaginoidea virens* with universal ITS primers (ITS 1 and ITS 4). [Lane M – 100 bp DNA marker; lanes 1 to20 - *U. virens* isolates collected from different rice growing ecosystems of Karnataka]

sub clusters and isolate from Gonal showed more divergence from other isolates in the cluster-I. Further, cluster-II was divided into two sub clusters with sub cluster-I comprised of Uv-5 and Uv-4 isolates and showed divergence from the isolates Uv-1, Uv-2, Uv-7, Uv-3 Uv-6 and Uv-13 in sub cluster II. The majority of the isolates in cluster-I and cluster-II showed close relationship among them. Phylogenetic analysis revealed that isolates of *U. virens* clustered into different groups, the geographic differentiation of these isolates has no limit on the formation of cluster. The present findings are in accordance with the reports of previous workers, where phylogenetic profile was constructed based on the ITS sequence data sets resulted in well separated clades in the

method of neighbour joining. The 61 Indian isolates were grouped into two major clusters. However, most Indian isolates (n = 54) were grouped (in Cluster-I) along with Chinese and Japanese isolates indicating the shared evolutionary history. Three isolates from Tamil Nadu, two each from Karnataka and Telangana state, were diverged into a separate cluster-II. The analysis revealed that, the clade relationship existed among the isolates collected from different geographical origin[13,16].

Discussion

Colony diameter is generally used as a parameter to differentiate slow and fast growing fungal isolates in morphological characterization of a fungus. Similar observations made by Mathew *et al*.[14] showed that the colony diameter of *U. virens* isolates ranged from 25 to 40 mm after 30 days of incubation at 27 ± 2°C on PSA and based on the colony character the isolates of *U. virens* were grouped into three groups. Similarly, Rani *et al*.[17] found well defined colonies on PSA medium. Maximum colony growth of 68.74 mm and good sporulation were observed in PSA medium followed by potato dextrose agar medium, whereas faster rate of mycelial growth was noticed on potato sucrose broth. Similar observations were also recorded by different scientists indicating PSA as best media for growth of *U. virens*[18].

Thirty-fve isolates of *U. virens* were collected from different areas of Punjab to study the variability among the isolates. Isolates produced colony diameter ranging from 26 to 90 mm after 2 weeks of incubation on PSA medium. Colony colour in most of the isolates was white which changed to yellow and finally became green. The conidia were spherical, hyaline and warty with a size ranging from 4.05-4.94 to 6.10-6.36 µm. Isolates also differed in the growth pattern from oppressed, fluffy and less fluffy to raise[19].

The gradual changes in the colour of the colony in the culture plate were more or less in line with the transformation of the smut balls in the rice plants as noticed by Ladhalakshmi[4] on PDA medium. Where the fungus grew very slowly and produced colonies which were initially whitish in colour, gradually turning into yellow and then greenish black like transformation of smut balls in the field condition.

With respect to the growth characteristics, the observations of the present study were similar to the work of Mathew *et al*.[14] reported that on culture medium, *U. virens* produced creamy white colony, flat or raised with slight undulations, fluffy mycelium, compact and leathery characters.

Mathew *et al*.[14] recorded variation among the isolates of *U. virens* with respect to size, colour and shape of the chlamydospore and width of the hyphae. The chlamydospores were globose, irregularly round to elliptical and warty on the surface with diameters ranging from 4.20 to 6.54 µm. Variation in shape of the chlamydospores was not observed among the isolates of different ecosystems since the chlamydospores of all the isolates were globular, ovoid and round irregular in shapes. The morphological characters of the *U. virens* such as mycelial width and size of chlamydospore were recorded using fluorescent microscope at 40X magnification.

Present study revealed that the isolates of Karnataka showed maximum mycelial width (3.85-6.37 µm) compared to the North India isolates (1.26-2.81 µm)[12]. The possible reason for this variation between the North and South Indian isolates are to be understood. During the course of the research work, we observed positive correlation between the size of the chlamydospore and the disease severity, where isolates collected from irrigated Bhadra, coastal and hilly upland ecosystem showed maximum chlamydospore size of 33.34-90.16 μm^2 and the disease severity observed during the survey ranged from 2.19 to 25.19%[10]. The increased disease severity in the ecosystem where pathogen can produce bigger sized chlamydospore leading to the better survival of the pathogen in dormant stage to cause disease in the subsequent *Kharif*, when the summer crop was not grown. This could have led for the higher disease severity in irrigated Bhadra and hilly ecosystem compared to TBP and UKP.

The results agreed with work carried out by Kim & Park[20] who found that natural chlamydospores of *U. virens* had prominent spines and the spines were pointed at the apex or irregularly curved, and these spines are known to play a role in cell adhesion and aggregation phenomenon for spore ball formation.

Conclusion

In the current study, we have collected and isolated the pathogen from different rice growing agroclimatic regions of Karnataka and identified the variability of cultural and morphological characters of the

pathogen. The variability was evident among the isolates with regard to mycelia, colony colour and chlamydospore characters. We observed positive correlation between the size of the chlamydospore and the disease severity, where the isolates collected from irrigated Bhadra, coastal and hilly upland ecosystem showed maximum chlamysospore size and disease severity. We have also confirmed the identity of the pathogen using specific ITS primers.

Conflicts of interest

Authors declare no competing interests.

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