**Isolation, Characterization and Pathogenesis of *Ustilaginoidea virens* Causing False Smut Disease in Rice (*Oryza sativa* L.)**

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**A B S T R A C T**

The present study was conducted in order to characterize the isolates of the fungus, *Ustilaginoidea virens* causing false smut disease, collected from different geographical regions of south India and cultured on Potato Sucrose Agar (PSA) medium. Morphological characterization exhibited that, the isolates produced well defined distinct colonies on PSA medium with colony growth ranging from 3.47 cm (TNUv5) to 5.57 cm (TNUv2), colony colour appeared as whitish to yellow except for TNUv5 wherein it showed yellowish to green. Higher mycelia dry weight was observed in TNUv2 (511.00 mg) followed by TNUv1 (503.62 mg) and least weight was recorded by TNUv5 (325.50 mg). Genetic diversity was estimated and dendrogram (UPGMA method) was constructed using 10 polymorphic RAPD markers. It produced two major cluster at 0.62 similarity coefficient, each cluster comprising of two (TNUv1 and TNUv6) and four (TNUv2, TNUv3, TNUv4 and TNUv5) isolates respectively. The divergence among the isolates depended on the geographical location. Beside morphological and molecular identification, the identity of the fungal pathogen was confirmed through ITS sequencing which showed 91 to 99 % identity with *U. virens* in NCBI-BLAST analysis. Pathogenecity test using TNUv2 isolates revealed that, injection of conidial suspension to the plants at booting stage develops symptoms within 12 days of injection and it will be useful for identification of resistance source and breeding resistant varieties against false smut. This study also emphasize that, different isolates of *U. virens* exhibit variation in the ITS (internal transcribed spacer) sequence which may be helpful to study the phylogenetic relationships among the isolates/races and seek the specific resistance genes.

**Keywords**  
False smut, *Ustilaginoidea virens*, Genetic diversity, Pathogenecity.

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**Introduction**

False smut (Green smut or pseudo smut) is a common grain disease of rice around the world, caused by *Ustilaginoidea virens* (Cooke) (Takahashi). Recently *Villosiclova virens* has been proposed as the new name for teleomorph of the false smut fungus (White *et al.*, 2000; Tanaka *et al.*, 2008; Ashizawa *et al.*, 2010). The fungus overwinters in soil by means of sclerotia and chlamydospores. *Sclerotia* produce ascospores, which are the primary source of infection to rice plants, whereas secondary infection comes from airborne chlamydospores (Ashizawa *et al.*, 2010). Infection by the pathogen transforms
individual grains of infected panicles into initially orange, becoming yellowish green or greenish black at maturity (Ou, 1972; Lee and Gunnell, 1992). The disease was first reported from Tirunelveli district of Tamil Nadu State of India (Cooke, 1878). Historically being an uncommon and minor disease by occurring sporadically in certain regions (Ou, 1972; Dodan et al., 1996), now epidemics of the disease is being reported frequently (Rush et al., 2000; Singh and Pophaly, 2010; Ladhalakshmi et al., 2012). This disease is considered as farmer's friendly and locally known as “Lakshmi” (meaning wealth) disease because it is found associated with bumper yields. However, the disease has been observed in severe form since 2001 due to widespread cultivation of high fertilizer-responsive cultivars and hybrids, heavy application of nitrogenous fertilizer and change in climate (Yaegashi et al., 1989; Sugha et al., 1993; Lu et al., 2009). 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 and Kashmir and Puducherry (Dodan, 1996; Mandhare et al., 2008; Ladhalakshmi et al., 2012).

Since U. virens is very slow growing fungus, it is very difficult to isolate in its pure form. It often gets contaminated by the fast growing saprophytes during isolation, incubation and also difficult to be created artificially under screen house or field conditions. The development of suitable medium for growth and sporulation would facilitate further studies. Practical stand-point, a medium capable of stimulating sporulation of the fungus would aid in the preparation of spore suspension required for inoculation of large populations in disease resistance breeding programs. The disease not only threatens rice production in yield and quality but the chlamydospores also contaminate the rice grains and straw with their antimitotic cyclic peptides (ustiloxin) which are dangerous to the health of human and livestocks (Nakamura et al., 1992; Luduena et al., 1994; Ashizawa et al., 2011). In India the research on false smut has been negligible because of its minor importance and partly due to a problem in the artificial culturing and inoculation of the pathogen. With all these points in mind, the present study was carried out to study/assess the variability of the pathogen collected from various geographical locations and standardization of artificial inoculation technique for large scale screening of rice genotypes in order to identify resistant source against false smut.

**Materials and Methods**

**Sample collection**

The samples of typical false smut on rice (Fig. 1) were collected from different geographical regions viz., Hyderabad (IIRR), Paddy Breeding Station (Tamil Nadu Agricultural University, Coimbatore), Department of Agronomy (Wetland, TNAU Coimbatore), Hybrid Rice Evaluation Centre (HREC, Gudalur), farmers fields of Thanjavur, and Gobichettipalayam during 2016. Latitude, longitudes and annual mean temperature of site of collection were depicted on map (Fig. 2).

**Isolation, purification and maintenance of the pathogen**

The collected smut balls were surface sterilized by dipping them in 70% ethanol followed by 0.1% mercuric chloride and subsequently washed with sterile distilled water. Using a sterilized inoculation loop, the mass of chlamydospores were streaked onto Petri dishes containing potato sucrose agar medium (PSA) under aseptic condition. To avoid bacterial contamination, Streptomycin
@ 100 ppm per litre was added in the medium at lukewarm stage before pouring into Petri plates. The Petri dishes were incubated at 27 ± 2°C until the appearance of mycelium. A single, well isolated colony of the fungus was picked up using sterilized needle and transferred to the fresh PSA slants and maintained as a pure culture. Six isolates were purified and maintained on PSA medium at 4°C. Thus, the purified cultures were maintained by periodical transfers on PSA slants, they were serially numbered (TNUv1 to TNUv6) and used for further studies (Table 1).

**Cultural and morphological characterization**

In order to study the detailed cultural characteristics of the pathogen, 5 mm mycelial discs were collected from ten days old culture of *U. virens* and inoculated at the center of PSA plates in three replicates under aseptic cultural condition (27 ± 2°C temp). After 30 days of inoculation (DAI) the observations were recorded on growth of the pathogen on the solid media viz., colony colour, colony diameter, growth type and mycelia dry weight. Radial mycelium growth was measured at 10 days interval (10th, 20th, 30th DAI). Mycelial dry weight of each isolate was observed by culturing the fungus on potato sucrose broth. Two mycelial discs of 5 mm of each isolates were inoculated in the broth and the flasks were incubated at 27 ± 2°C temperature for 30 days. Culture filtrate along with mycelial mats were filtered on Whatman No. 1 filter paper and dried in the oven at 60 °C for 3 hours, the weight of the mycelial mats were recorded.

**Diversity analysis**

**DNA extraction**

All the six isolates were cultured in 50 ml liquid potato sucrose broth in a 100 ml conical flask at 26°C and shaken at 200 rpm. After 7 days, the culture was transferred to 50 ml liquid potato sucrose broth in a 250 ml conical flask and incubated under the same conditions for 3 days. The mycelia were harvested and immediately ground into a fine powder by liquid nitrogen for DNA extraction following the protocol described by Nakada *et al.*, (1994). Concentrations of DNA were estimated by measuring absorbance at 260 nm with a Nanodrop (GENOVA NANO, UK). DNA samples were diluted to working solutions of 30 ng/ μl and stored at 4 °C until use.

**RAPD amplification and data analysis**

Fourteen random decamer RAPD primers (Table 2) were used to survey the isolates. Amplifications were performed in a total volume of 25 μl containing 0.5 μl dNTPs mix (10 mM), 1 μl primer (12.5 pmol), 2 ml of DNA (30 ng/ μl), 1.2 μl MgCl2, 1.5 μl reaction buffer (10X), 0.3 μl of *Taq* polymerase (1.5 U) and 18.5 μl deionized water. The DNA amplification was done in a thermal cycler (BioRad, USA) using the following PCR cycles. The first denaturation step of 2 minutes at 94 °C, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 1 min at 40°C, extension at 72°C for 2 min and final extension at 72 °C for 5 min with holding temperature at 4°C for 10 min. Reaction products (8 μl) were resolved by electrophoresis in 1.2% agarose gels stained with ethidium bromide in 1X TBE buffer at 100 V for 90 min. Differences in banding patterns among isolates were assessed visually using gel documentation system (BioRad, USA). Polymorphisms including faint bands that could be scored were included in the analyses. Polymorphic RAPD markers were manually scored as binary data with presence as (1) and absence as (0). The similarity matrix was then subjected to the unweighted pair group method with arithmetical mean (UPGMA)
cluster analysis by using NTSYSpc version 2.02i.

**PCR amplification and sequencing of ITS region**

Based on the RAPD diversity study, four diverse isolates were selected out of six and examined for the amplification of ITS region, which is having gene cluster of ITS1, 5.8S and ITS2. ITS region specific primers ITS1 F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 R (5'-TCCTCCGCTTATTGATATGC-3') designed by White et al., (1990) were used for PCR amplification. Reaction products (8μl) were resolved by electrophoresis in 1.2% agarose gels stained with ethidium bromide in 1X TBE buffer at 100 V for 90 min. After electrophoresis the gel slice along with PCR fragments were excised with a clean, sharp scalpel. Then, they were purified using QIAquick Gel Purification Kit (QIAGEN, California) according to the manufacturer’s protocol. The purified DNA from ITS-PCR was given for sequencing to Bioserve solutions, Hyderabad. The partial sequence of rDNA was obtained and it was submitted to the National Centre for Biotechnology Information (NCBI), Gene Bank, New York, USA and accession numbers were obtained (KX421100, KX421101, KX421102 and KX421103) (Table 3).

**Pathogenicity test**

Paddy variety BPT 5204 (Samba Mashuri) plants were brought to the screen house from main field at booting stage. The plants were inoculated with conidial suspension as described by Fujita et al., (1989). Pure culture of fast growing (TNUv2) isolate was inoculated into 100 ml of potato sucrose broth (PSB) and kept in an incubator shaker (125 rpm) at 28°C for 2 weeks for incubation. The conidia were harvested and suspended in sterile distilled water. Plants at booting stage were selected for inoculation and injected with 2 ml of conidial suspension (1×10⁵ conidia ml⁻¹). The inoculated plants were kept in a humidity chamber with an RH of 95% and 24-26°C temperature in screen house. The rice panicles injected with sterile distilled water served as control.

**Results and Discussion**

**Morphological characterization of U. virens**

The isolates produced well defined colonies on PSA medium. Maximum colony growth of 5.57 cm was observed in TNUv2 followed by TNUv3 (5.07 cm), whereas minimum colony growth of 3.47 cm in TNUv5. The colony colors in most of the isolates were initially white and turned to yellow. However, TNUv5 isolate alone showed greenish yellow even after incubation period of 30 days. Most of the isolates produced raised hat like structures except TNUv1 and TNUv2, which showed flat growth in culture media. The highest mycelial dry weight was observed in TNUv2 (511.00 mg) followed by TNUv1 (503.62 mg) and the lowest was in TNUv5 (325.50 mg) (Table 4).

**Molecular characterization of U. virens using RAPD markers**

Genomic DNA was isolated from six different isolates and subjected to RAPD-PCR amplification using 14 random decamer primers. Out of 14 primers, 10 RAPD (Table 2) primers produced reproducible and scorable polymorphic bands. The size of RAPD fragments on 1.2% agarose gel ranged from 100 to 2000 bp (Fig. 3). The Jaccard similarity coefficient values among U. virens isolates ranged from 0.51 to 0.96. The dendrogram constructed through UPGMA analysis clearly grouped the isolates into two major clusters at 0.62 similarity coefficient (Fig. 4). Dendrogram consist of two major
clusters A and B. The isolates TNUv1 (Hyderabad) and TNUv6 (Gobichettipalayam) coming under cluster A and TNUv2 (PBS, Coimbatore), TNUv3 (Wetland, Coimbatore), TNUv4 (Gudalur) and TNUv5 (Thanjavur) belongs to cluster B. There were two sub clusters (BI and BII) were observed under major cluster B, BI consist of three isolates TNUv2 (PBS, Coimbatore), TNUv3 (Wetland, Coimbatore), TNUv4 (Gudalur), where isolates TNUv3 and TNUv4 showed a relative genetic similarity value of 0.96. The isolate TNUv5 (Thanjavur) belongs to BII sub cluster. Although, major cluster supported the geographical origin of isolates in general, critical analysis revealed that fine clustering does not support exact geographical origin of isolates and their genetic properties. The information obtained from sequencing of isolates viz., TNUv1, TNUv2, TNUv4 and TNUv5 had 678 (95%), 612 (99%), 674 (97%) and 649 (91%) base pairs respectively. Inside the parenthesis says per cent coverage identity in BLAST search http://www.ncbi.nih.gov/index.html (Table 3).

The rice variety BPT 5204 was used to test the pathogenicity of the isolated fungus by artificially injecting the conidial suspension of TNUv2 to the tillers at the booting stage. Out of ten inoculated plants, three plants produced typical false smut balls on the panicles with 30 percentage of diseased hill (PDH) after 12 days of inoculation. U. virens infected the young ovary of the individual spikelets and transformed them into large, velvety green balls. Young smut balls were fleshy inside and became hard after some time. Initially the smut balls were small, visible between glumes and were covered with a membrane that burst at a later stage (Fig. 5).

False smut disease is commonly occurring in the wet season all over the world. Flowering stage coinciding with rains and high humidity, many of the high yielding rice varieties succumb to varied level of infection. Limited information is available on the pathogen diversity and hence this study becomes relevant. The pathogen was characterized based on its colony and spore morphology, which produced pure white coloured, raised, smooth colony margin and on continuous incubation, the colour converted to light yellow and in some cases green. Similar morphological characteristics of the pathogen were described by Hegde et al., (2000) and Ladhalakshmi et al., (2012) in rice. The isolates produced well defined colonies on potato sucrose agar medium with colony growth ranging from 3.47 to 5.57 cm after 30 days of incubation. Initially the colony growth was creamy white colour resembling straw hat shape with undulations, which turned yellowish after 20-25 days of incubation. In the culture medium the chlamydospore burst and appears after 30- 40 days after incubation at 27 °C. The colour of the mycelium changed white to yellow and later greenish yellow resembling symptoms in the field. These results agreed with Ladhalakshmi et al., (2012) and Baite et al., (2015). In culture medium U. virens proliferated and produced creamy-white colony, flat or rose with slight undulations, mycelium fluffy, compact and leathery. Chlamydospores were observed at the centre or margin of the colony at later stage of growth.

RAPD markers were used to estimate genetic variation in U. virens and to determine if any of population structure occurred with regard to geographical origin. The UPGMA algorithm constructed a neighbor joining tree which grouped the isolates into two major clusters at 0.62 Jaccard similarity coefficients (Fig. 4). Cluster A comprised of two isolates TNUv1 (Hyderabad) and TNUv6 (Gobichettipalayam). Cluster B comprised of four isolates which were again subdivided into two subgroups. The first subgroup (BI)
comprise of three isolates, out of which two isolates TNUv3 (Wetlands, Coimbatore) and TNUv4 (Gudalur) with 0.96 similarity coefficient and TNUv2 (PBS, Coimbatore) different from other isolates of same subgroup, the only isolate TNUv5 (Thanjavur) belongs to second subgroup (BII). In earlier studies RAPD marker system has been used successfully to characterize molecular variation in *U. virens* (Li et al., 2004; Xiao-ping et al., 2008; Mathew et al., 2014). Our results suggest that RAPD markers can be used to evaluate genetic diversity within the species *U. virens* through estimates of variation at multiple loci across the genome.

The advent of molecular biology has caused a significant shift in the types of approaches used to identify and characterize plant pathogens and to device management strategies. The polymerase chain reaction (PCR) method has been developed for the *in vitro* amplification of nucleic acid sequence and has been used to detect a number of plant pathogens based on the specific nucleotide sequences. This method is highly sensitive and capable of detecting even a single copy of DNA molecule (Henson and French, 1993). PCR based technique was used for detection and characterization of *U. virens* based on the Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA).

**Table.1** Isolates collected from different locations

| Isolates | Place of collection |
|----------|---------------------|
| TNUv1    | Indian Institute of Rice Research, Hyderabad |
| TNUv2    | Paddy Breeding Station, Coimbatore |
| TNUv3    | Wetland Farm, Coimbatore |
| TNUv4    | Hybrid Rice Evaluation Centre, Gudalur, The Nilgiris |
| TNUv5    | Farmer field, Thanjavur |
| TNUv6    | Farmer field, Gobichettipalayam |

**Table.2** Primers used for RAPD-PCR amplification of *U. virens* isolates

| Primer name | Primer sequence 5'-3' | Nature of primer |
|-------------|-----------------------|-----------------|
| OPK07       | AGCGAGCAAG             | Polymorphic     |
| OPF03       | CCTGATCACC             | Polymorphic     |
| OPG02       | GCCACTGAGG             | Polymorphic     |
| OPD20       | ACCCGGTAC              | Polymorphic     |
| OPA-08      | GTGACGTAGG             | Monomorphinic   |
| OPB-09      | TGGGGGACTC             | Monomorphinic   |
| OPG17       | ACGACCAGCA             | Polymorphic     |
| OPE03       | CCAGATCGAC             | Polymorphic     |
| OPL17       | AGCCTGAGCC             | Polymorphic     |
| OPC10       | TGTCTGCTG              | Polymorphic     |
| OPG06       | GTGCCTAAC              | Polymorphic     |
| OPC-11      | AAAGCTGCGC             | Monomorphinic   |
| OPE-14      | TGCCGGCTG              | Monomorphinic   |
| OPM16       | GTAACCAGCC             | Polymorphic     |
Table 3 Fungal identification based on NCBI-BLAST search (for ITS1-5.8s-ITS4 region)

| Isolates | Source     | Accession number | Base pair length | Species identified as | Coverage identity (%) |
|----------|------------|------------------|------------------|-----------------------|-----------------------|
| TNUv1    | Hyderabad  | KX421100         | 678              | U. virens             | 95                    |
| TNUv2    | Coimbatore | KX421101         | 612              | U. virens             | 99                    |
| TNUv4    | Gudalur    | KX421102         | 674              | U. virens             | 97                    |
| TNUv5    | Thanjavur  | KX421103         | 649              | U. virens             | 91                    |

Table 4 Cultural and morphological characteristics of different isolates of U. virens on PSA medium

| Isolates | Radial mycelium growth (cm) | Dry weight (mg) 30 (DAI) | Colony colour | Growth type         |
|----------|-----------------------------|---------------------------|----------------|---------------------|
|          | 10th day | 20th day | 30th day |               |                    |
| TNUv1    | 1.3 (1.14)c | 3.07 (1.75)c | 5.00 (2.24)b | 503.62 (22.44)a | Creamy white | Flat, Fluffy and Circular |
| TNUv2    | 1.88 (1.37)a | 3.93 (1.98)a | 5.57 (2.36a) | 511.00 (22.61)a | White to yellow | Flat and Circular |
| TNUv3    | 1.61 (1.27)b | 3.46 (1.86)b | 5.07 (2.25)b | 421.75 (20.54)b | Yellowish white | Raised, Fluffy and Circular |
| TNUv4    | 1.17 (1.08)c | 2.8 (1.67)d | 4.5 (2.12)c | 479.75 (21.90)a | White to yellow | Flat, Fluffy and irregular |
| TNUv5    | 0.88 (0.94)d | 2.35 (1.53)c | 3.47 (1.86)e | 325.50 (18.04)c | Yellow to green | Raised, irregular |
| TNUv6    | 1.00 (1.00)d | 2.3 (1.52)e | 3.92 (1.98)d | 339.25 (18.42)c | White to yellow | Raised, fluffy irregular |
| SE (d)   | 0.03 | 0.03 | 0.03 | 0.42 | - | - |
| CD(P=0.05)| 0.07 | 0.07 | 0.06 | 0.90 | - | - |

Figures in parentheses represent square root transformation. Means in a column followed by same superscript letters are not significantly different according to DMRT

Results revealed that all the four isolates amplified a fragment of 625 bp for universal ITS primer (ITS1/ ITS 4). The results agreed with Baite et al., (2015) who observed sequence length of Uv2 (Bulandshahr, Uttar Pradesh) and Uv3 (Haridwar, Uttarakhand) with 645 and 634 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. It is necessary to develop an efficient inoculation method for investigation of the rice-U. virens interaction. Up to date, quite a few reports consistently conclude that inoculation by injection of spores into the sheath during booting stage is sufficient and efficient to induce disease symptoms. Wang et al., (2008) obtained a white smut strain that is virulent to rice plants, with this strain, it was found that disease severity is higher by injection than by spraying. Low temperature exposure after inoculation has a strong stimulatory effect on disease development (Kulkarni and Moniz, 1975; Fujita et al., 1989;
Hegde et al., 1998; and Xiu et al., 2011; Ladhalaxmi et al., 2012). Lu et al., (2009) studied the pathogenic diversity of *U. virens* by injecting the conidial inoculum of the fungus during the booting stage under field conditions. Ashizawa et al., (2010) developed a modified method of artificial inoculation by injecting the conidial inoculum of the fungus. Haiyong et al., (2015), injected a conidial suspension (1 x 10^6 conidia ml^-1) of *U. virens* at the booting stage and incubated the plants in a moist cabinet initially at 15°C for 2 days, and then at 26°C for 5 days. Similar results were observed by injection of the conidial inoculum produced by artificially culturing the fungus, led to typical false smut symptoms under glasshouse conditions.

Genetic improvement of rice for false smut resistance is in need of a robust and moderate throughput screening methods for identification of genes and genotypes conferring resistance to false smut. The study has brought out some leads on the pathogenesity and isolates diversity that would carry forward the research in the right context.

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**Conflict of Interest**

Authors certify that there is no financial commitment to the sponsor. Authors also state that they have full control of all primary data and that they agree to allow the journal to review their data if requested.

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