RESEARCH ARTICLE

Histopathological and molecular characterization of *Ustilaginoidea virens* in rice

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ABSTRACT: Histopathological studies of false smut balls on rice spikelet were conducted through SEM and microtomy. Detection and identification of the fungal culture of *Ustilaginoidea virens* from infected host tissue was done through polymerase chain reaction (PCR) using *U. virens* specific internal transcribed spacer (ITS) primer. The primer amplified 380bp product.

Keywords: False smut balls, microtomy, scanning electron microscopy, *Ustilaginoidea virens*

False smut of rice is caused by *Ustilaginoidea virens*, teleomorphic stage is *Villosiclava virens*. Earlier it was considered as a minor disease of rice (Padwick, 1950; Tanaka *et al.*, 2008) and considered to be a sign of 'bumper harvest' of the crop. False smut balls on rice panicles are the typical symptom of the disease (Pooja *et al.*, 2015). False smut balls are intertwined with the mycelium of *U. virens* at early stage and then chlamydospores are formed. The chlamydospores are initially smooth and later become warty at maturity (Kim and Park, 2007; Mathew and Sharma, 2015). Under bright-field light microscopy, the conidia are elliptical and warty on the surface with diameters ranging from 2 to 6 µm. In scanning electron microscopic study the conidia appeared to be globose to irregularly rounded and ornamented with prominent spines. The spines are either pointed or slightly curved at the apex and approximately 200-550 nm long. In transmission electron microscopy, both the spined conidia and hyphae along with lipid globules and vacuoles were seen in the cytoplasm of host cell.

The stigma, ovary, and even the young grains of the rice were assumed to be the site of primary infection (Padwick, 1950; Wang, 1992), but subsequently histological observation showed that the pathogen can attack the root of the rice plant at the seedling stage and may colonise symptomlessly in the entire plant (Schroud and TeBeest, 2005; TeBeest, 2010). The fungal life cycle of *U. virens* and primary infection site on the paddy crop in nature is still very unclear. SEM and TEM study of the ultrathin sections of false smut balls and colonies of *U. virens* on different media showed that the dense chlamydospores are piled around the inner hyphae. The fungal hyphae and endosperm were not distinguishable.

Large stretch of well-developed endosperm was present in the center which was filled with large amount of starch grains. This suggests that the infection of pathogen may also occur in host plant after the flowering and grouting, and the fungal pathogen may be saprophytic in nature. The aim of the present study was to find out the nature of the disease in plants.

MATERIALS AND METHODS

Spore observation

Chlamydospores of *U. virens* were assessed for their variation using compound and scanning electron microscope.

Compound microscope

Fungal spores were placed over the cleaned glass-slide having a drop of distilled water using a needle and covered with a cover slip and viewed under the compound microscope under 40X and oil immersion at 100X. The spores were observed for colour and shapes of conidia. The resulting image was then photographed. The cultures were also observed and characters of hyphae and septation were noted.

Scanning electron microscopy (SEM)

The spores of *U. virens* were dusted over the specimen stub and sputter coated with palladium (24 nm thick) under vacuum for an hour. The specimens were then observed under Zeiss EVOMA10 SEM in high vacuum. The surface morphology, size, hyphal width and spines of the conidia were measured and the photographs were taken under different magnifications ranging from 2.00-

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34.83 KX. For uniformity, besides other magnifications, photographs were taken at a magnification of 6.0 KX.

Anatomy of smut balls

The infected panicle samples were washed thoroughly and then with sharp tweezers lemmas and paleas were removed. These samples were then immersed in lactophenol solution having 0.1% trypan blue and then boiled for 10-12 min in a water bath. Thereafter destained in lactophenol for 5-6 min in the boiling water bath. Mounting and sample observations were done under a stereoscopic or light microscope to select the infected spikelet for further study. False smut balls were cut into small pieces with sharp blade in different directions to separate ovary, anthers and other parts for further sample preparation.

Microscopic and transmission electron microscopy (TEM)

The selected samples including root, stem, leaf and grain of infected plant were cut into small pieces of 2 x 2 x 3 mm, then washed in PBS buffer (pH 7.2, 100 mM) for one hour with several buffer changes. These were then fixed in 1% osmium tetroxide (OsO₄) for 2 h at room temperature. These were then washed several times with double distilled water. Later dehydrated serially in 30, 50, 70, 80, 90 and 100% ethanol solution for 20 min at each step. Subsequently, samples were immersed and embedded in resin. Polymerization was done at 72°C for 15-16 h. The intact embedded samples were cut into 2 mm sections and then mounted on glass slides using stain 0.05% methylene blue (wD v), for light microscopy study.

For TEM study the samples screened through light microscopy (LM) were cut into ultrathin sections of 6-7 nm. After using an established standard procedure, they were visualized with a Hitachi H-7650 transmission electron microscope. The stem, as well as pedicels of severely infected panicles were also cut into 1 x 2 mm size. These were treated as mentioned above to visualize if there were pathogen hyphae in them.

Molecular characterization

In the glasshouse, conidial suspension of the pathogen was injected at boot leaf stage typical smut balls were observed in rice variety PRH-10. Pathogen identity was checked through polymerase chain reaction (PCR) analysis using *U. virens*-specific internal transcribed spacer (ITS) primer US 1-5/US3-3 (Zhou *et al.*, 2003).

Production of mycelium

Mycelia of *U. virens* isolates were cultured in potato dextrose broth. Conical flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with four 0.5cm discs taken from actively growing monospore culture of *U. virens* fungus in petriplate. The cultures were put into rotary shaker (120 rpm) and incubated at 26±1°C

for 20 days. Mycelia were harvested by filtration using filter paper, blotted dry with sterile blotting paper and used immediately for DNA extraction.

DNA extraction

Single culture of *U. virens* isolates was transferred in 100 ml potato dextrose broth (PDB) in the 250 ml conical flasks and incubated at 26±2°C in shaking BOD incubator for 20-30 days (Mathew *et al.*, 2014). Harvesting by filtering of fungal mycelium mat using Whatman filter paper no. 1, washed regularly with distilled water, blot dried and stored in Al-foils at -80°C for genomic DNA extraction. Genomic DNA was extracted from mycelium harvested from broth media based on Cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980) with partial modification. About 0.2 gm of mycelium of each isolate was ground separately in a sterile pre-chilled mortar and pestle using liquid nitrogen to a fine frozen powder after which DNA extraction buffer (100mM TrisHCl, pH 8.0, 1.4 M NaCl, 50mM EDTA, pH 8.0 and 2% CTAB) was added. The finely ground tissue was put into sterile 1.5 ml centrifuge tube and mixed well and incubated in a water bath at 65°C with occasional gentle shaking for an hour. After incubation, an equal volume of phenol: chloroform (24: 1 v/v) was added and mixed slowly and was centrifuged at 14000 rpm for 30 minutes at 4°C and repeated for 3-4 times. The total nucleic acid RNA present with DNA was removed by RNase treatment. Nano drop (nano drop, USA) spectrophotometer was used to quantify the DNA and working solutions having 25 ng/μl were prepared for optimization of polymerase chain reaction (PCR) by adding double sterilized nuclease free water.

Ustilagoidea virens - specific internal transcribed spacer (ITS) region analysis

The specific internal transcribed spacer (ITS) primers US1-5, Forward (CCGGAGGATACAACCAAAAAA ACTCT) and US3-3, Reverse (GCTCCAAGTGCAGG ATAAGTGAAT) were used for confirmation of *U. virens* (Zhou *et al.*, 2003). The Polymerase Chain Reaction (PCR) mixture of 20 μl consisted of 10 pmol of each primer (US1-5/US3-3), dNTPs 2.5 mM, DNA template 20 ng and Taq polymerase 14. PCR reaction was carried out with an initial denaturation step at 96°C for 2 min, and 30 cycles of amplification which includes, (denaturation at 96°C for 20 s, primer annealing at 53°C for 30 s and extension at 70°C for 30 s) and single cycle of final extension was done at 72°C for 7 min. PCR amplification was performed on a Thermocycler (Applied Biosystems). Analysis of PCR-amplified products were done by standard agarose gel electrophoresis (Sambrook *et al.*, 1989).

The amplified PCR product was purified through gel elution using gel extraction kit (QIAGEN gel extraction kit) and was subsequently sent for sequencing (Eurofins Genomics India Pvt. Ltd, Bangalore, India - 560 066) using the forward as well as reverse primers. The sequence data were assembled and analyzed using the

programmer, BIOEDIT version 7.0.5. Gene products were predicted using GENERUNNER. Multiple nucleotide sequence alignments were done in BIOEDIT. (The NCBI <http://www.ncbi.nlm.nih.gov>) *Genebank* sequence database.

Data analysis

The program Clustal X (version 1.81) was used to carry out Multiple sequence alignments. Gene Doc (version 2.6.002) was used for comparison of pair-wise sequence identities among the *U. virens* isolates. The maximum likelihood phylogenetic relationships were determined with the aid of program MEGA4 (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Histopathological and molecular characterization indicated that the pathogen was confined only in the infected spikelet which shows that rice plants have the localized infection in the floral parts.

Microtomy of infected grain in transmission electron microscope (TEM)

In TEM it was found that more than 80% grid area were covered with the host membranes which were ruptured during the experiment. It is suggested that the fungal mycelium has role to rupture host membrane. With careful examination, it was observed that some area of the grid showed the fungal mycelium progressing to the host tissue was embedded with the fat bodies (Fig. 1, 2, 3).

Infected grain observed under scanning electron microscope (SEM)

In Scanning Electron Microscope (SEM), it was observed that the diseased grain surface was fully smeared with fungal chlamydospores and the fragmented mycelium. These chlamydospores have an echinulate surface (Fig. 4, 5).

Fig. 1. Transmission electron microscopy showing mycelium of *Ustilaginoidea virens* (UV-4) in infected rice grain at 3000X magnification

Fig. 2. Transmission electron microscopy showing mycelium of *Ustilaginoidea virens* (UV-4) in infected rice grain at 5000X magnification

Fig. 3. Transmission electron microscopy showing mycelium of *Ustilaginoidea virens* (UV-4) in infected rice grain Healthy grain (A): at 2500X magnification; diseased grain (B): at 2500X magnification

Specific ITS analysis and sequencing

The identity of the present seven isolates was confirmed through PCR test using the primers US1-5/US3-3 used earlier (Ladhalakshmi *et al.*, 2012; Zhou *et al.*, 2003; Zhou *et al.*, 2008). The desired specific length of 380 bp bands was observed in all the seven isolate taken for the study. No such band was observed in two controls (Fig. 6). Phylogenetic analysis of ITS sequences clearly grouped the isolates into two categories. All *U. virens* grouped in one cluster with 97-100% sequence similarity (Fig. 7).

In the present study under scanning electron microscope (SEM), the large number of chlamydospores were observed, which may be due to presence of some biochemicals secreted by the grain tissue that stimulated the fungus sporulation at higher rate. The fact should be confirmed by molecular techniques. The data showed that the present fungal isolates are the member of the fungal species *U. virens*. It is concluded that US1-5 and

Fig. 4. Scanning electron microscopy showing surface feature of conidia of *Ustilaginoidea virens* (UV-4) in infected rice grain at 1000X magnification

Fig. 5. Scanning electron microscopy showing surface feature of conidia of *Ustilaginoidea virens* (UV-4) in infected rice grain at 8000X magnification

Fig. 6. Gel electrophoresis showing ITS region of *U. virens* with specific ITS primer (US1-5/US3-3). Lane M-1000bp DNA marker; 1: UV-1 (Pantnagar), 2: UV-2 (Bulandshahr), 3: UV-3 (Haridwar), 4: UV-4 (Sonapat), 5: UV-5 (Delhi), 6: UV-6 (Varanasi), 7: UV-7 (Bagpat); Lane C: as two control one with water and another with healthy rice grain DNA

US3-3 are the specific primers to identify the fungus *U. virens*. These primer pair, US1-5 and US3-3 are very specific as it was shown earlier also by Zhou *et al.* (2008). The present study could provide a useful technique that will help to study the disease cycle and disease infection of *U. virens* which would lead in formulation of epidemiological model for crop improvement through early prediction of disease arrival in the field condition of rice growing areas in India.

SEM studies of the chlamydospores of the false smut balls of infected rice spikelet revealed that chlamydospores appeared to be globose to irregularly rounded and ornamented with prominent spines. The spines present on the wall were either pointed or slightly curved at the apex. Histopathological examination of the smut balls showed that hyphae of the *U. virens* were compact and arranged orderly in the interior, with lot of fat bodies filled in hyphae of the pathogenic fungus. Host cells were deformed by the fungal pathogen but pathogen

Fig. 7. Dendrogram illustrating UV-Hapur KJ195462, *Epichloe* sp. Rnj3202DQ234807, *Neotyphodium uncinatum* AB102783, JQ828996 (UV2), JQ828997 (UV3), UV2 and UV3 are previously reported *Ustilaginoidea virens*

was not able to penetrate the host cell wall. The identification of the fungi was confirmed from pure fungal culture and false smut infected rice plants. Histopathological and molecular characterization indicated that the pathogen was confined only in the infected spikelet which shows the localized nature of the disease.

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REFERENCES

- Kim KW and Park EW (2007). Ultra structure of spined conidia and hyphae of rice false smut fungus (*Ustilaginoidea virens*). *Micron*. 38: 626-631.
- Pooja Kumari, RK Sharma and Deebea Kamil (2015). Viability studies and evaluation of inoculation techniques of false smut (*Ustilaginoidea virens*) in rice. *Indian Phytopath.* 68: 254-259.
- Ladhalakshmi D, Laha GS, Singh R, Karthikeyan A, Mangrauthia SK, Sundaram RM, Thukkaiyannan P and Viraktamath BC (2012). Isolation and characterization of *Ustilaginoidea virens* and survey of false smut disease of rice in India. *Phytoparasitica* 40: 171-176.
- Mathew SB and RK Sharma (2015). Isolation technique and cultural conditions of false smut pathogen (*Ustilaginoidea virens*) on rice. *Indian Phytopath.* 68: 50-55.
- Mathew SB, Sharma RK, Prameela Devi T, Sharma Pratibha and Kamil Deebea (2014). Morphological and molecular characterization of *Ustilaginoidea virens* isolates, causing false smut of rice in India. *Indian Phytopath.* 67: 222-227.
- Murray MG and Thompson WF (1980). Rapid isolation of high molecular weight DNA. *Nucleic Acids Res.* 8: 4321-4325.
- Padwick GW (1950). *Manual of Rice Diseases*. Kew, UK: Commonwealth Mycological Institute.
- Sambrook J, Fritsch EF and Maniatis T (1989). Molecular cloning. In: *A laboratory manual*. 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
- Schroud P and TeBeest DO (2005). Germination and infection of rice roots by spores of *Ustilaginoidea virens*. *AAES Research Series* 540: 143-151.
- Tamura K, Dudley J, Nei M and Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
- Tanaka E, Ashizawa T, Sonoda R and Tanaka C (2008). *Villosiclava virens* gen. nov., comb. nov., teleomorph of *Ustilaginoidea virens*, the causal agent of rice false smut. *Mycotaxon* 106: 491-501.
- TeBeest D (2010). Infection of rice by *Ustilaginoidea virens*. *Phytopathology* 100: 125.
- Wang GL (1992). Studies on the infection period and the infection rate of the chlamydospores of *Ustilaginoidea virens* (Cooke) Tak. on rice. *Acta Phytophylacica Sinica* 19: 97-100 (in Chinese).
- Zhou L, Pan J, Xie W, Zhu H, Xu L, Wang S and Li K (2008). Genetic diversity of rice false smut fungus, *Ustilaginoidea virens* and its pronounced differentiation of populations in North China. *J. Phytopathol.* 156: 559-564.
- Zhou YL, Izumitsu K, Sonoda R, Nakazaki T, Tanaka E, Tsuda M and Tanaka C (2003). PCR-based specific detection of *Ustilaginoidea virens* and *Ephelis japonica*. *J. Phytopathol.* 151: 513-518.