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Isolation and characterization of *Ustilaginoidea virens* and survey of false smut disease of rice in India

D. Ladhalakshmi · G. S. Laha · Ram Singh · A. Karthikeyan · S. K. Mangrauthia · R. M. Sundaram · P. Thukkaiyannan · B. C. Viraktamath

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Abstract The intensity of rice false smut disease in selected states of northwest and south India was studied. In northern Indian states as a whole, disease incidence (percentage of false smut-infected tillers) varied from 2% to 75%. In the state of Haryana, maximum infection was recorded on hybrids like PA 6444 and PA 6129 while in Punjab state, 10–20% disease incidence was recorded in popular inbred rice varieties like PR 114, PA 116 and PAU 201. In the southern state of Tamil Nadu, the disease incidence varied from 5% to 85%. A heavy incidence of the disease was noticed in variety BPT 5204 and due to this, the air above the infected field gave a black smoky appearance from a distance as a result of release of spore mass in the atmosphere. In severe cases the number of infected grains reached even more than 100 per panicle. The pathogen *Ustilaginoidea virens* was isolated in potato dextrose agar medium and was characterized by both pathogenicity test and molecular analysis. Under glasshouse conditions, when a conidial suspension of the pathogen was injected during boot leaf stage of the rice variety TN1, typical smut balls were observed. The identity of the pathogen was further confirmed through polymerase chain reaction (PCR) analysis using *U. virens*-specific internal transcribed spacer (ITS) primers. The primer pair US 1-5/US3-3 and US2-5/US4-3 amplified 380 bp and 232 bp product, respectively, which are typical for the *U. virens* fungus.

Keywords *Oryza sativa* · Paddy · Pathogenicity · Teleomorph *Villosiclava virens*

D. Ladhalakshmi (✉) · G. S. Laha · S. K. Mangrauthia · R. M. Sundaram · B. C. Viraktamath
Directorate of Rice Research,
Hyderabad, Andhra Pradesh, India
e-mail: ladhasavitha@gmail.com

R. Singh
CCSHAU Rice Research Station,
Kaul, Haryana, India

A. Karthikeyan
Tamil Nadu Rice Research Institute,
Aduthurai, Tamil Nadu, India

P. Thukkaiyannan
Krishi Vigyan Kendra, Coastal Saline Research Centre,
Ramanathapuram, Tamil Nadu, India

Introduction

False smut disease caused by *Ustilaginoidea virens* (Cooke) (Takahashi) is a common grain disease of rice around the world. The disease was first reported from Tirunelveli district of Tamil Nadu State of India (Cooke 1878). False smut was recognized as a symbol of a bumper harvest and was categorized as a minor disease due to its sporadic occurrence. 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 an apparent change in climate. In recent years, it has emerged as the most devastating grain disease in the

majority of the rice-growing areas of the world. In India the disease has been observed in severe form since 2001 in major rice-growing states, *viz*., Haryana, Punjab, Uttar Pradesh, Uttaranchal, Tamil Nadu, Karnataka, Andhra Pradesh, Bihar, Jharkhand, Gujarat, Maharastra, Jammu & Kashmir and Puducherry (Dodan and Singh 1996; Mandhare *et al.* 2008).

The symptoms produced by *U. virens* are visible after flowering only, when the fungus transforms individual grains of the panicle into a yellowish smut ball, which changes to yellowish orange, green, olive green and finally to greenish black. *U. virens* produces both sexual (ascospores) and asexual (chlamydospores) stages in its life cycle (Biswas 2001). Recently, *Villosiclava virens* has been proposed as the new name for the teleomorph of the false smut fungus (Tanaka *et al.* 2008).

The disease causes both quantitative and qualitative losses. The losses in grain yield occur due to chaffiness, reduction in test weight, and sterility of the spikelets neighboring the smut balls. The yield losses in different states of the country have been estimated to vary between 0.2% and 49% depending on the disease intensity and rice variety (Dodan and Singh 1996). The chlamydospores also contaminate the rice grains and straws with their antimitotic cyclic peptides (known as ustiloxin), which are poisonous to both humans and animals (Koiso *et al.* 1994). A cursory perusal of the literature revealed that research on false smut has been negligible in India partly because of its minor importance and partly due to a problem in the artificial culturing and inoculation of the pathogen. In the present work, we report the occurrence and intensity of false smut disease in farmers' fields from some of the high-input rice production localities in India, and isolation and characterization of the pathogen.

Materials and methods

Survey A random survey was conducted in the districts of Ambala, Kurukshetra and Yamunanagar in the state of Haryana, and Ludhiana in the state of Punjab of north-western India during September 2009. During February 2010, Thanjavur, Cuddalore, Nagapattinam and Ramanathpuram districts of Tamil Nadu state in southern India were surveyed. These areas were selected as they represent high-input production localities in India and have high rice productivity. In each district, one to eight villages were randomly selected. In each village, three to five 1-m^2 areas were marked in fields of different rice varieties and observations on number of infected tillers/m^2 and number of smut balls/infected panicle were recorded; the data are presented as range and mean.

Isolation and artificial inoculation of U. virens The pathogen *U. virens* was isolated from the false smut-infected spikelets. Initially the smutted spikelets were surface sterilized with 0.1% mercuric chloride for 1 min and subsequently washed three times with sterile distilled water. Using a sterilized needle the chlamydospore mass was streaked onto petri dishes containing potato dextrose agar (PDA) medium. To check the bacterial contamination, the medium was incorporated with streptomycin (100 ppm). The plates were incubated at 27°C for 7 days. The morphological characters, *viz*., color, size (length and width) and shape of the chlamydospores and conidia were recorded. Spores were measured using an image analyzer (CETI, Antwerp, Belgium) and photomicrographed using the computer software BIOWIMPLUS at 100x magnification; the mean measurements were calculated.

Paddy variety TN1 grown under glasshouse conditions was used for pathogenicity studies. The plants were inoculated with conidial suspension as described by Fujita *et al.* (1989) with slight modifications. Pure culture of the *U. virens* was inoculated into 100 ml of potato dextrose broth (PDB) and incubated in an incubator shaker at 125 rpm at 28°C for 2 weeks. 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 (2×10^5 conidia ml^{-1}). The inoculated plants were kept at 95% r.h. in a humidity chamber for one week, followed by normal room temperature (27°C) on glasshouse benches. The plants were observed for symptom expression 15 days after inoculation of the pathogen. The rice panicles injected with sterile distilled water served as control.

Extraction of DNA and PCR detection of the pathogen Cetyl trimethyl ammonium bromide (CTAB) method was adopted to extract the total DNA from the mycelium of *U. virens*. A pure culture of *U. virens* mycelia was inoculated in PDB and incubated at 28°C for 2 weeks in a rotary incubator shaker at 125 rpm. The mycelium was harvested by filtration and dried under sterile conditions. Approximately 100 mg of the dried

mycelium was powdered using liquid nitrogen ; to the powdered mycelia 750 μl of CTAB buffer (2.0 g CTAB, 10.0 ml of 1 M Tris pH 8.0; 4.0 ml of 0.5 M EDTA pH 8.0; 28.0 ml of 5 MNaCl; 40.0 ml of H_2O; 1 g of PVP 40 Mw 40,000 and made up to 100 ml with H_2O) was added and incubated at 65°C for 45 min. During the incubation the tubes were vortexed for 5 s for complete mixing. After incubation, the tubes were spun at 13,000 rpm for 15 min and to the supernatant an equal volume of chloroform and isoamyl alcohol (24: 1) (v/v) was added and centrifuged at 13,000 rpm for 15 min. The aqueous phase was transferred to a new Eppendorf tube and the above step was repeated to extract the entire DNA. To the pooled supernatant an equal volume of chilled iso-propanol was added and incubated for 1 h at −20°C; DNA was precipitated by centrifugation at 13,000 rpm for 15 min. The DNA pellet was washed with 70% ethanol, air dried and finally dissolved in 50 μl of the sterile distilled water. The final concentration of DNA in the extract was checked by 0.8% agarose gel.

The specific internal transcribed spacer (ITS) primers were used to confirm *U. virens* (Zhou *et al.* 2003). The polymerase chain reaction (PCR) mixture (20 μl) consisted of 0.5 μl of 2.5 mM of dNTPs, 10 pmol of each primer (US1-5/US3-3 or US2-5/US4-3), one unit of *Taq* polymerase and DNA template (20 ng). PCR reaction was performed with an initial denaturation step at 96°C for 2 min, 30 cycles of amplification (20 s for denaturation at 96°C, 30 s for primer annealing at 53°C and 30 s for extension at 70°C) and one cycle of final extension at 72°C for 7 min. The amplification was carried out on a Thermocycler (Applied Biosystems). PCR-amplified products were analyzed by standard agarose gel electrophoresis (Sambrook *et al*. 1989).

Results

Survey A survey on the occurrence of false smut disease of rice in the states of Haryana, Punjab, during September 2009 and Tamil Nadu during February 2010 revealed that the disease incidence varied widely from region to region (Table 1). Within a region, the intensity of the disease varied depending on the cultivars. In Haryana, the incidence of infected tillers was found to range between 2% and 75% in different varieties, the maximum being in hybrids like PA 6444 and PA 6129. In Punjab the intensity of false smut was comparatively less in inbred varieties which are cultivated by the farmers. The percentage of infected tillers ranged from 10% to 20% in rice varieties like PR 114, PR 116 and PAU 201. In Tamil Nadu, the disease incidence varied from 5% to 85% (infected tillers), causing a substantial reduction in grain yield. A heavy incidence of the disease was recorded on rice varieties CR 1009 and BPT 5204 in Kunnakudi village of Ramanathapuram district, and in Thiyagasamuthiram and Maharajapuram villages of Thanjavur district. Due to this heavy incidence of the disease and due to the discharge of smut spores in the atmosphere, the air above the infected fields gave a black smoky appearance from a distance. Among the districts surveyed, disease incidence was high in Yamunanagar and Kurukshetra of Haryana. Incidentally, a large part of these districts is under cultivation with rice hybrids (POS 2009). However, in Tamil Nadu state, where inbred rice varieties dominate, the incidence was very high to the tune of 80% in Ramamnathapuram and Thanjavur, especially on the rice varieties CR 1009 and BPT 5204.

Isolation and characterization of the false smut pathogen U. virens is a very slow grower on PDA plates, the fungus appeared as a tiny white colony 7 days after inoculation. The single colony of the fungus was picked up and was maintained as pure culture. On the culture plate, the white mycelium of the fungus changed to yellow in about 15 days and produced a mass of chlamydospores which were initially yellow in color and turned olive green upon maturity, similar to the gradual change of color morphology of smut balls under field conditions (Fig. 1). The chlamydospores of this fungus were spherical to elliptical, thick and double-walled, which are borne laterally on minute sterigmata. Chlamydospores germinated by short germ tubes and formed conidiophores, bearing conidia at the tapering apex. The conidia were minute and ovoid. In total, nine *U. virens* isolates (three from Haryana, two from Punjab and four from Tamil Nadu) were isolated and maintained as pure culture. The pathogenicity of the isolated fungus was confirmed on rice variety TN1 by artificially injecting the tillers at the booting stage with the conidial suspension. The inoculated plants produced typical false smut balls on the panicles 15 days after inoculation. *U. virens* infected the young ovary of the

Table 1 A preliminary survey on incidence of false smut of rice in some selected high-input rice production localities in India

State/District	Villages/surveyed unit	Varieties/Hybrids	Percentage of infected tillers			Number of smut balls per infected tiller		
			Range	Mean	SEM[z]	Range	Mean	SEM
Punjab								
Ludhiana	PAU plots	PR 114 (I[y]), PAU 201 (I), KRH-2 (H[y]), PR 120 (I), PR 115 (I), PR 116 (I), Signet 5050 (H), NK 6704 (H), HRI 107 (H)	10–20	15.33	2.90	10–15	12.67	1.45
Haryana								
Kurukshetra	Lotni, Chhodpur, Thol	PA 6129 (H), RH 257 (H), PR 114 (I)	5–65	34.25	16.34	2–10	5.75	1.93
Ambala	Badola, Sadhapur, Naraingarh	PAU 201 (I), PHB71(H), Hybrid 832 (H), PHB 71 (H)	2–10	5.33	1.25	1–3	2.17	0.31
Yamunanagar	Sadhora, Bal Chhappar, Uncha Chandlana, Sadikpur	RH 257 (H), PA 6444 (H), Sudha 999 (H), Hybrid 748 (H)	4–75	24.75	10.63	1–10	5.5	1.36
Tamil Nadu								
Ramanathapuram	Thiruvadani, Konnakudi, Keelakottai, R.S. Mangalam, Nainar Kovil, Uppoor, Solanthur	ADT-38 (I), CR 1009 (I), BPT 5204 (I) and CO 43 (I)	70–85	76.67	4.41	5–30	16.67	7.26
Thanjavur	Aduthurai, Thiruvaiyaru, Thirumandakkudi, Thiyagasamuthiram, Maharajapuram, Ullikadai, Patteeswaram, Swamimalai	Co 43 (I), ADT-38 (I), CR 1009 (I) and BPT 5204 (I)	20–80	50.00	12.91	10–15	12.5	1.04
Cuddalore	Vridhachalam, Komangalam, Thoravalur	CR 1009 (I) and BPT 5204 (I)	18–30	23.00	2.65	8–12	10.00	0.91
Nagapattinam	Thirukkadaiyur, Thalainayar, Pattavarthi	BPT 5204 (I) and CR 1009 (I)	5–20	10.00	3.53	4–5	4.5	0.829

[z] *SEM* standard error of mean

[y] *H* hybrid; *I* Inbred

Fig. 1 Mass of chlamydospores on the *Ustilaginoidea virens* culture

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. The range in size of the smut ball was 4.3–9.2 mm x 3.2–6.5 mm. The smut balls were yellow in color, and later turned to olive green and to greenish black.

The identity of the fungus was further confirmed through PCR using *U. virens* specific primers, *viz*., US1-5/US3-3 and US2-5/US4-3 (Zhou *et al.* 2003). *U. virens* DNA was isolated from the mycelium by the CTAB method. The primer pairs amplified a product of 380 bp (Fig. 2) and 230 bp, respectively, from all the nine isolates which are specific to the

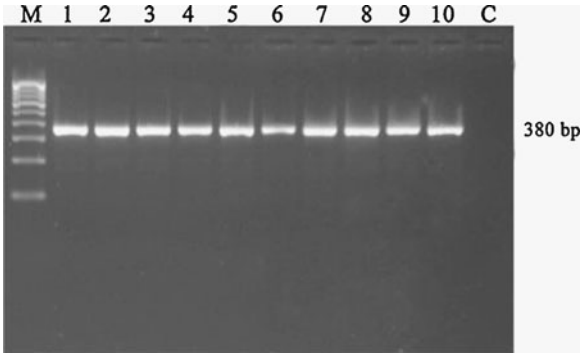

Fig. 2 Polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) regions of *Ustilaginoidea virens* with specific ITS primers (US1-5/US3-3). Lane M – 100 bp DNA marker; lanes 1 to 3 - *U. virens* isolates collected from Haryana; lanes 4 & 5 – Punjab isolates; lanes 6 to 9 – Tamil Nadu isolates; lane 10 – positive control; lane C – negative control

false smut fungus. A positive control was kept with the DNA isolated from the smut spores of young false smut balls.

Discussion

Cursory perusal of the literature revealed that false smut disease incidence varied widely from region to region depending upon the cultivars, cultivation practices and variation in environmental conditions. The survey data imply that false smut disease is emerging as one of the major diseases of rice in India. The infected tillers were found to vary between 2% and 85% in both the northern and southern parts of India. In Haryana state where hybrids occupy a large area under rice cultivation, false smut incidence was very high on hybrids like PA 6444 and PA 6129. In Punjab state, the incidence of the disease on varieties like PR 114, PR 116 and PAU 201 ranged between 10% and 20%. In Tamil Nadu state, the disease spread extensively during 2009–10. It was estimated that areas of approximately 8000 ha in district Ramanathapuram, 2000 ha in district Thanjavur and 150–200 ha in district Cuddalore were affected with false smut disease during 2009–10 (Source: Local newspaper *Dinamalar* dated 25.1.10). The Production Oriented Survey (POS) conducted by the All India Coordinated Rice Improvement Program (AICRIP) also revealed the gradual increase in the incidence and spread of the disease over the years (POS 2000-2009). In the past few years, severe disease incidence of false smut has been noticed in Madurai, Tirunelveli, Theni and Dindugal districts of Tamil Nadu state (A. Maheswari, M.Sc. thesis, TNAU, 2003). Singh and Pophaly (2010) reported that an area of more than 600 ha of rice was severely affected by false smut in Raigarh district of Chhattisgarh in 2007. Yield loss due to this disease depends upon the environmental conditions, the genetic make-up of the cultivar and the virulence of the pathogen. The disease also causes economic losses to farmers due to a lower market price for their produce owing to the presence of black chlamydospores masses on healthy rice grains.

One of the major problems in studying this disease is the artificial culturing of the fungus in a medium. In the present study, we report a methodology of isolation of the fungus in the artificial culture medium, on which the fungus grew very slowly and produced colonies which were initially whitish in color, gradually turning into yellow and then greenish black. The gradual changes in the color of the colony in the culture plate more or less resembled the transformation of the smut balls in the rice plants. In addition, the identity of the fungus was confirmed by molecular analysis. The PCR method is highly sensitive and capable of detecting even a single copy of DNA molecule (Henson and French 1993). Detection of *U. virens* was done based on the Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA). Zhou *et al.* (2003) developed *U. virens*-specific primers US l-5/US 3-3; US 2-5/US 4-3 based on the comparison of sequence alignments from the ITS region of *Claviciptaceae* spp. In this study the specific ITS primers amplified fragments of 380 bp and 230 bp and confirmed the identity of the false smut pathogen isolated on the culture plate. A positive control, *i.e.,* the DNA isolated from the smut spores of young false smut balls, also produced a specific amplified product with those specific primers.

For artificial inoculation, Fujita *et al.* (1989) 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. 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. In our study also, injection of the conidial

inoculum produced by artificially culturing the fungus, led to typical false smut symptoms under glasshouse conditions. The inoculated conidia infected the ovary and transformed individual grains of the panicle into false smut balls. The smut balls were initially flattened, smooth, whitish yellow in color confined between glumes, covered by a membrane. At a later stage the membrane burst and the size of the smut ball increased considerably and its color changed to yellowish orange and finally to greenish black. We are in the process of standardizing the mass screening technique which will help in identifying the sources of genetic resistance to this emerging disease. In the present work, we have demonstrated the successful isolation of the false smut fungus in the culture medium, its mass multiplication and artificial inoculation on rice plants. We have also confirmed the identity of the pathogen using specific ITS primers.

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