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Morphological and molecular characterization of *Fusarium* sp. causing Bakanae disease of rice (*Oryza sativa*) in western Uttar Pradesh, India

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ABSTRACT

Rice (*Oryza sativa* L.) is considered as one of the main staple food grains. The Asian countries lead in production and consumption of rice i.e. more than 90% rice is produced and consumed in Asia. India is considered as one of the leading exporters of rice. Rice is affected by number of diseases and pests and among the several diseases which infects the rice crop, a fungal disease commonly known as Bakanae disease or Foot rot of rice is caused by *Fusarium moniliforme* (*Gibberella fujikuroi*) fungus. It is one of the important diseases of rice and leads to severe losses in rice yield particularly in basmati rice. According to some previous reports, some other *Fusarium* species were also found to be causing the disease. Therefore, the following study was conducted in the year 2018–19 at Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India, to find out and identify the *Fusarium* sp. mainly associated with the Bakanae disease in western Uttar Pradesh. Twelve isolates of *Fusarium moniliforme* were collected and isolated from different locations of western Uttar Pradesh and their characterization of morphological and molecular characters was carried out. Morphological characters, like fungal growth type, pigmentation, radial growth, colony characters were studied and compared with the standard culture of *Fusarium moniliforme* obtained from ITCC, New Delhi. The molecular characterization was done with specific primers. The results indicated that Bakanae disease of rice in western Uttar Pradesh is primarily caused by *Fusarium moniliforme*. The study also confirms that identification based on morphological and culture characteristics must be coupled with molecular characteristics for proper identification of specific fungal sp. causing Bakanae disease.

Keywords: Bakanae*, Fusarium moniliforme,* Molecular characterization, Morphological characterization, Rice

Rice (*Oryza sativa* L.) is an important food grain throughout the world and it is consumed as the staple by nearly about three quarters of the Indian population. Basmati rice in India is produced mostly in Punjab, Haryana, Uttar Pradesh, Uttaranchal, and in some regions of J&K, Rajasthan, and Himachal Pradesh. There are several constraints which affect rice production. Rice diseases caused by fungi, bacteria, virus, and nematodes are the most major limitations contributing to low rice yields in the country, amongst several biotic and abiotic factors impacting rice production and quality (Kumar *et al.* 2013). Bacterial leaf blight, Brown leaf spot, sheath blight, blast, Bakanae etc. are the important diseases of rice. Bakanae disease caused by *Fusarium moniliforme* Sheldon (*Gibberella fujikuroi*) is emerging as an important disease affecting the rice crop. The Bakanae disease incidence has now become a reason for serious concern in basmati rice in the northern India, viz. Punjab, Uttar Pradesh and Haryana. Earlier Bakanae was a minor disease but now it is becoming a major disease particularly in basmati rice. The most common basmati rice cultivar, PB-1121, is most susceptible to the Bakanae disease in all basmati growing areas (Khilari *et al.* 2011). In all rice-growing states of India, the prevalence of Bakanae disease has progressively increased, wreaking havoc on aromatic rice varieties and causing severe yield losses (Bashyal *et al.* 2014, Gupta *et al.* 2014). The word Bakanae disease is derived from Japanese meaning "foolish seedling". It was first described in 1898 by Japanese scientist Shotaro Hori. Crown rotting, discoloration and aberrant elongation of diseased rice seedlings are perhaps the most noticeable symptoms of this disease, which is caused by the synthesis of Gibberellic acid (GA_3), which causes hypertrophy. Foot rot, seedlings rot, grain infertility, and grain discoloration are few other characteristic symptoms of this disease (Ou 1985). These severe symptoms ultimately affect the seed quality and grain yields. In India, Bakanae disease incidence leads to 15–25% of the yield losses, which have been reported from different states of Uttar Pradesh, Andhra Pradesh, Tamil

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Nadu, Haryana and Punjab (Rathaiah *et al.* 1991, Sunder *et al.* 1998 and Pannu *et al.* 2012). Disease incidence on different basmati rice cultivars was recorded as 2.1–3.2% in Uttarakhand, 1.2–11.7% in Uttar Pradesh, 2.1–2.8% in Haryana, 10.5–40.0% in Punjab, 1.8–8.7% in Bihar and 2.4–13.6% in Rajasthan (Gupta *et al.* 2014).

Some members of the *Gibberella fujikuroi* sp. complex (*Fusarium fujikuroi, Fusarium concentricum, Fusarium proliferatum,* and *Fusarium verticillioides*) are believed to be associated with the Bakanae disease in rice (Amoah *et al.* 1995, Desjardins *et al.* 2000). The ultimate goal of all phytopathological studies is to identify the pathogen causing the disease, control plant diseases and reduce yield losses. Several studies have been carried out to identify the *Fusarium* sp. causing the Bakanae disease, furthermore, it is not yet clear which *Fusarium* sp. is associated with the symptoms of Bakanae. Keeping all this in view, the present investigation including collection, and morphological and molecular characterization were carried out to determine morphological and molecular characters of the species causing Bakanae of rice in the western Uttar Pradesh region.

MATERIALS AND METHODS

Samples collection: The infected plant parts were collected from different regions of western Uttar Pradesh, and carried to laboratory for isolation of pathogens. The experiment was conducted during 2018–19 at Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh.

Isolation of pathogen from infected plant part of Rice: Small pieces of the diseased parts of stem were cut (0.5 mm) with a sterilized scissor. Then these cut-pieces were surface sterilized by dipping in 1% sodium hypochlorite solution for 20–30 sec and were washed thrice in sterilized water using sterilized forceps. The sterilized pieces were then transferred to the sterilized Petri-plates containing potato dextrose agar (PDA) medium, and were incubated at 26±2°C temperature, and then the pure cultures were maintained. The isolates were designated as Fm 1–Fm 12 (Table 1), and further morphological and molecular characterization was carried out and were compared for confirmation with the control culture of *Fusarium moniliforme* obtained from ITCC, IARI, New Delhi for confirmation of the fungus.

Morphological characterization: The morphological characteristics of *Fusarium moniliforme* was observed on PDA media. Fungal growth, diameter of the colony, pigmentation, radial growth, colony texture, growth pattern, spore characters of all the isolates were recorded after the 10^{th} day of inoculation (DAI). The slides were made and examined under a microscope. Microconidia and macroconidia sizes were measured at 40X for each isolate independently. All the observations, spore measurements, and imaging were done with a (Olympus) microscope.

Molecular characterization: For the extraction of the fungal DNA, the pure cultures were maintained on 100 ml Potato dextrose broth (PDB) medium flasks for about 10 days at 26±2°C. The fungal mycelial mat was obtained after 10 DAI, by filtering with filter paper (Whatman, 125 mm, Cat No.1442 125).

Extraction of DNA: By using the Cetyl Trimethyl Ammonium Bromide (CTAB) technique, genomic DNA was isolated from 12 pure cultures of *Fusarium* sp. (Murray and Thompon 1980). Filtered mycelium (5 g) was finely grinded in liquid nitrogen, then transferred to a 50 ml tube containing 10 ml of CTAB buffer (0.1 M Tris, 0.01M EDTA, 1.5 M NaCl, and 2% CTAB) and maintained at 65°C for one hour with intermittent stirring. All of the tubes were filled with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged for 10 min at 12,000 rpm. The uppermost aqueous layer was pipetted out into a new tube and mixed with 0.1 volume sodium acetate (3 M) and 0.6 volume ice-cold isopropanol, then centrifuged for 10 min at 4°C at 10,000 rpm. For further use, the pellet was resuspended with 70% ethanol, allowed to dry at room temperature, and then dissolved in TE (10 mM Tris–Cl, *p*H 8.0; 1 mM EDTA). The purity of the DNA was determined using 0.8% agarose gels and a Nano-drop (Thermo Scientific, USA). The Polymerase chain reactions were then performed using additional specified primers.

Primers used: Four specific primers (Table 2) were

Table 1 Samples collected for the study

Isolate	Area	Rice variety	Part of plant from where fungus isolated	Type of symptom
Fm 1	Shamli	Pusa Basmati 1509	Internode region	White fungal growth
Fm 2	Muzaffarnagar	Pusa Basmati 1121	Internode region	White fungal growth
Fm 3	Hapur	Pusa Basmati 1121	Internode region	White fungal growth
Fm 4	Moradabad	Pusa Basmati 1121	Internode region	White fungal growth
Fm 5	Hastinapur	Pusa Basmati 1121	Internode region	White fungal growth
Fm 6	Bagpat	Pusa Basmati 1121	Internode region	White fungal growth
Fm 7	Saharanpur	Pusa Basmati 1121	Internode region	White fungal growth
Fm 8	Bareilly	Pusa Basmati 1509	Internode region	White fungal growth
Fm 9	Meerut	Pusa Basmati 1121	Internode region	White fungal growth
Fm 10	Ghaziabad	Pusa Basmati 1121	Internode region	White fungal growth
Fm 11	Meerut	Pusa Basmati 1121	Internode region	White fungal growth
Fm 12	Muzaffarnagar	Pusa Basmati 1509	Internode region	White fungal growth

Table 2 Primers used

Primer	Sequence	Reference
EF1	(5'-ATGGGTAAGGAGGACGACAAGAC-3')	(Bashyal *et al.* 2015)
EF-2	(5-'GGAAGTACCAGTGATCATGTT-3')	(Bashyal *et al.* 2015)
rp32	(5'-ACAAGTGTCCTTGGGGTCCAGG-3')	(Proctor *et al.* 2003)
rp33	(5'GATGCTCTTGGAAGTGGCCTACG-3')	(Proctor *et al.* 2003)
H31a	(5'-ACTAAGCAG ACCGCCCGCAGG-3')	(Glass NL *et al.* 1995)
H3-1b	(5'-GCGGGCGAGCTGGATGTCCTT-3')	(Glass NL *et al.* 1995)
Bt2a	(5'-GGTAACCAAATCGGTGCTGCTTTC-3')	(Glass NL *et al.* 1995)
Bt2b	(5'ACCCTCAGTGTAGTGACCCTTGGC-3')	(Glass NL *et al.* 1995)

selected specific for *Fusarium moniliforme* and used for identification The primer sequences were synthesized by Bangalore Genei.

PCR amplification: The PCR reaction was performed in BioRad Biometra thermal cycler program (CFX96). Total reaction volume was made up to 20 µl using sterile double distilled water. The reaction mixture composed of Sybergreen 10 µl , NFW 8.0 µl , forward primer 0.5 µl , reverse primer 0.5 µl , template DNA 1.0 µl. The master mix with template DNA was distributed equally into microcentrifuge tubes. Specific gene amplification was carried out using Real-Time PCR system (BioRad) with Sybergreen mastermix (Qiazen) and *Fusarium* specific primers. At the end of each cycle, melting curves were generated to ensure that the PCR result was pure. Tubes were placed in thermal cycler for 36 cycles of PCR. The program consisted of three distinct steps: Denaturation 94°C 5 min , denaturation 94°C 30 sec, annealing 58-60°C 1 min, extension 72°C 2 min , final extension 72°C 10 min. After completion of cycles, the samples were stored at 4°C till gel electrophoresis.

Gel electrophoresis: In a 1.2% agarose gel electrophoresis, the amplified products were seen. A gel documentation system (BioRad, USA) (GeNeiTMH06USA) was used

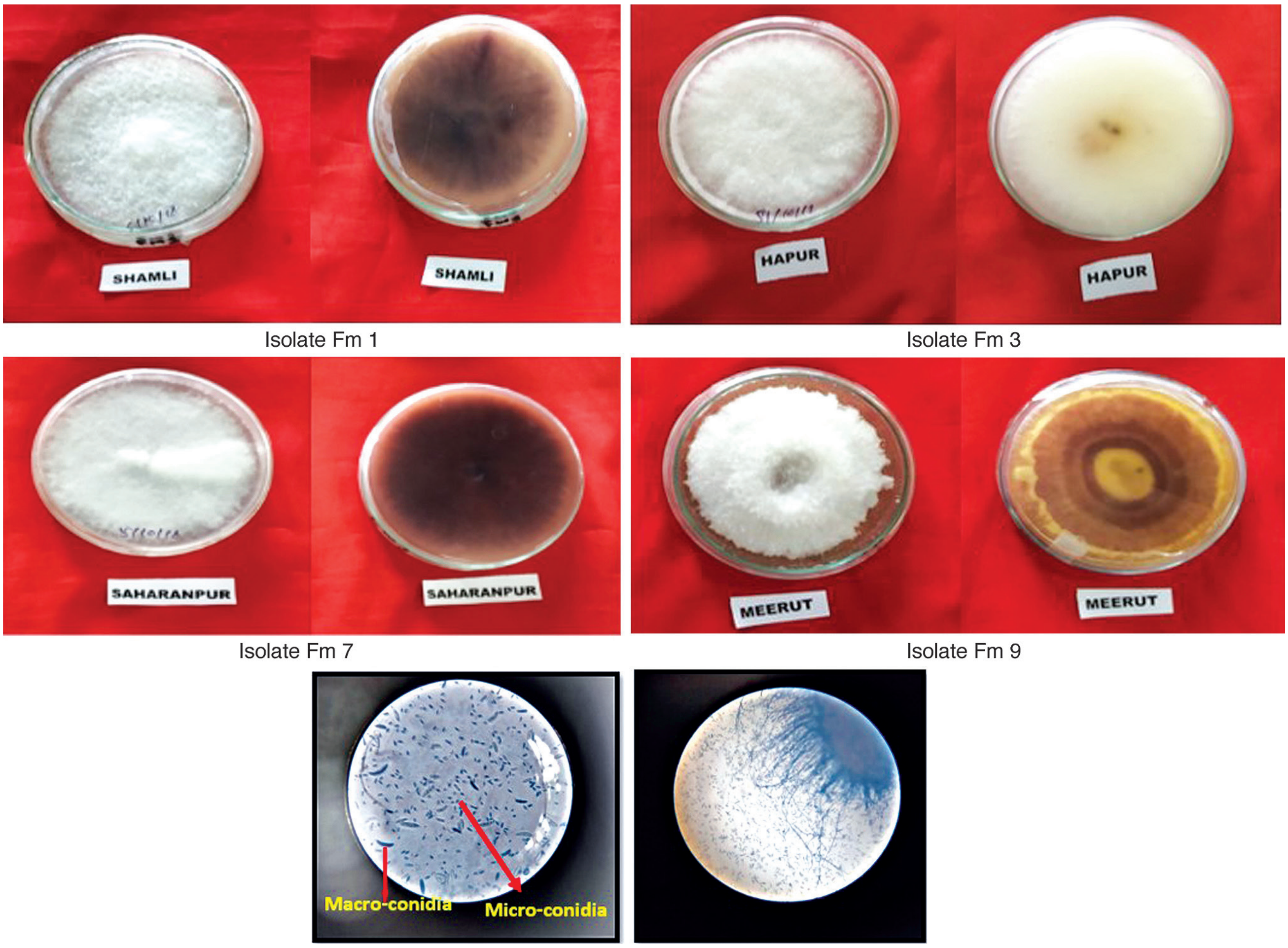

Fig 1 Colony characters of *Fusarium* sp. on PDA media and spores (Macro-conidia & Micro-conidia) of *Fusarium* sp.

Table 3 Isolates of *Fusarium* sp. are divided into groups based on cultural characteristics, macro-conidial characteristics, and micro-conidial characteristics

Group	Isolate	Colony characteristic	Macro-conidia size (µm)	Micro-conidia size (µm)	Chlamydospores
GRP 1	Fm (1–6) and Fm 12	White colored cottony growth	(24.24–35.70) × (3.2–3.87)	(7.4–8.8) × (2.8–3.7)	absent
GRP 2	Fm (7–11)	Creamy white colored growth	(29.54–35.73) × (2.92–4.8)	(7.3–9.1) × (3.2–3.9)	absent

to acquire the digital photos. The amplified products were resolved by electrophoresis in 1.2% agarose gels run at 80 V for 1 h in TAE buffer (1X), and stained with ethidium bromide at (4 ug/100 ml). The digital images were captured by gel documentation system (Bio-Rad, USA) (GeNeiTMH06USA).

RESULTS AND DISCUSSION

Twelve bakanae-infected rice plant samples were taken from various locations across western Uttar Pradesh (Table 1). The pathogen was cultured on Potato dextrose agar media after the samples were collected. The isolated and purified cultures were further used for morphological and molecular characterization.

Morphological Characterization of Fusarium sp.: After 10 days of incubation at 26±2°C, different cultural properties such as colony character, texture, pigments due to metabolites production and colony diameter on both the surface and reverse of the culture plate were recorded (Fig 1).

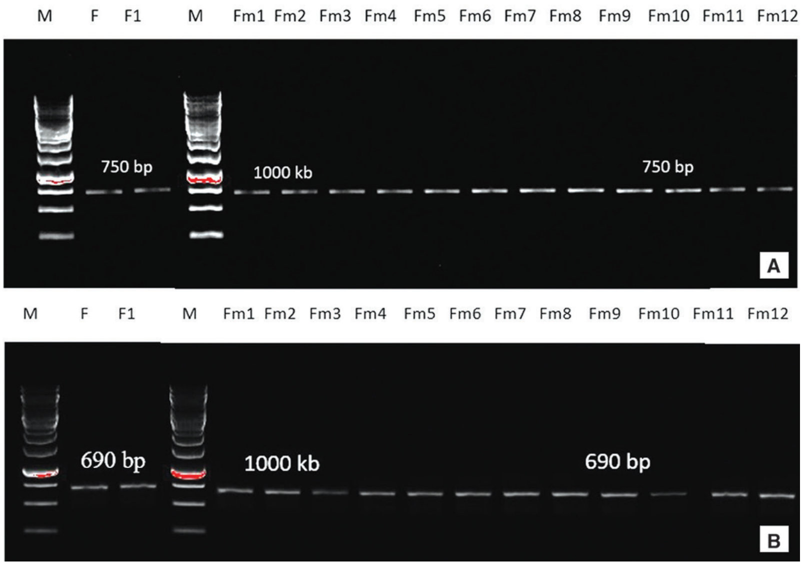

Fig 2 Gel electrophoresis of PCR amplified products with A. Ef-1 and Ef-2, B. rp-32 and rp-33. Lane M: 1 kb ladder; Control: F, F1; Samples: Fm1–Fm12.

Isolates were divided into two groups based on the investigated physical and cultural traits (Table 3). Group I (GRP 1) isolates Fm (1–6) and Fm 12 were white colored cottony colony producing isolates with micro-conidia size (7.4 to 8.8) × (2.8–3.7) µm and the size of macro-conidia (24.24–35.70) × (3.2–3.87) µm with no chlamydospores. Group II (GRP 2), comprising isolates Fm (7–11), had creamy white colony-forming isolates with micro-conidia size (7.3–9.1) × (3.2–3.9) µm and size of macro-conidia (29.54–35.73) × (2.92–4.8) µm, with no chlamydospores.

The isolates were identified as *F. moniliforme* based on morphological characteristics according to the book by Leslie and Summerell (2006) "The *Fusarium* Laboratory Manual" and a control culture obtained from ITCC, New Delhi, for comparison and confirmation of the isolates. The further confirmation was performed using molecular techniques.

Molecular Characterization of *Fusarium moniliforme* with the use of specific primers: As only the morphological and cultural traits alone are insufficient for reliable identification and characterization, species identification and characterization were confirmed using species-specific primers. The twelve isolates were recognized as *F. moniliforme* based on morphological and cultural characteristics. Using species-specific primers and a control culture of *Fusarium moniliforme*, the morphological identification was further validated. DNA from 12 isolates and one control culture with two replicates were amplified with the four sets of specific primers (Fig 2).

Molecular characterization of all the 12 isolates (Fm1–Fm12) was carried out and one control culture and four specific primers were used for the characterization, viz. Ef-1 and Ef-2, rp32 and rp33, H3-1a and H3-1b and Bt2a and Bt2b. With primer Ef-1 and Ef-2, all the 12 isolates showed single amplicon of 750 bp, with another set of primers, i.e. rp 32 and rp33, all the 12 isolates and the control culture gave single amplicon of 690 bp, with primers H3-1a and H3-1b; Bt2a and Bt2b, all the 12 isolates and the control culture gave single amplicon of 450 bp. All the isolates along with the single control culture of *Fusarium moniliforme* showed amplification with the specific primers and produced single amplicon bands at different product sizes, therefore it confirms all the isolates to be *Fusarium moniliforme*.

Bakanae, in the recent past has become a major threat to the rice production and productivity (Gupta *et al.* 2015). In the above study, 12 *Fusarium* isolates were studied and identified on the basis of their morphological and molecular characteristics. On the basis of their morphological characters, all the isolates were grouped into two groups where both the conidial sizes and other cultural characters were in the same range and showed similar kinds of variation as shown in some other reports (Zainudin *et al.* 2008, Bashyal *et al.* 2020), and then further identification was done on the basis of their molecular characters with the help of four specific primers (O'Donnell *et al.* 1998). All the isolates were identified as *Fusarium moniliforme* on the basis of their characters.

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