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Molecular identification of *Fusarium* species associated with bakanae disease of rice (*Oryza sativa*) in India

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ABSTRACT

Fifty isolates of *Fusarium* spp (*Gibberella fujikuroi* species complex) were obtained from rice seeds collected from major rice-growing regions of India. *Gibberella fujikuroi* species complex were detected in popularly grown rice varieties with infection ranging from 1% to 24%. Pathogenicity test of *Fusarium* spp was performed in susceptible rice variety Pusa 1121, which showed reduced seed germination and possessed varying ability to cause symptoms of bakanae on rice. Maximum incidence of slender and chlorotic leaves were produced by *F. fujikuroi* (90%), while maximum incidence of crown rot and stem rot was produced by *F. moniliforme* (50%). Use of ITS sequence gave reliable identification of pathogenic isolates of *Fusarium* spp associated with bakanae disease of rice. Three *Fusarium* spp, viz *Fusarium moniliforme, Fusarium fujikuroi* and *Fusarium proliferatum* were found associated with bakanae disease of rice in India. Phylogenetic studies showed a broad genetic variation among different species isolates that were distributed into three different clades. This finding provides new information on the bakanae disease and its distribution in India which could be helpful for the development of management strategies.

Key words: Bakanae, *Fusarium fujikuroi, Fusarium moniliforme, Fusarium proliferatum, Gibberella fujikuroi,* Rice

Bakanae disease is one of the emerging diseases of rice (*Oryza sativa* L.). The causal agent of bakanae disease of rice is *Gibberella fujikuroi* (Sawada) Wollenworth (teleomorph) (anamorph: *Fusarium moniliforme* Sheld.). Bakanae disease causes up to 40% losses in rice (Ou 1987). Bakanae disease of rice has been reported from the rice tracts of south Asia, European countries and America (Desjardins *et al.* 2000). It is emerging as a potential threat in Japan, Taiwan, Thailand and India (Webster and Gunnell 1992, Kini *et al.* 2002, Saremi 2005, Anonymous 2007). The typical symptoms of bakanae are slender, chlorotic and abnormally elongated primary leaves, however, not all infected seedlings show these symptoms, as crown rot is also seen, resulting in stunted rice plants (Ou 1987, Amoah *et al.* 1995).

Although bakanae disease was first described over 100 years ago in Japan, it is still not clear which *Fusarium* species are associated with different symptoms (Amatulli *et al. 2010*). Early work in Japan identified the pathogen as *Fusarium moniliforme* in a broad sense (Ou 1985); however, this taxon comprises a number of distinct species, now collectively termed the *Gibberella fujikuroi* species complex. Additional genetic studies have identified eight biological species or mating populations, designated A-H, within the *G. fujikuroi* species complex (Leslie 1995, Viljoen *et al.* 1997). Out of which three mating populations (A, C and D) of the *G. fujikuroi* complex have been associated with bakanae disease of rice. Mating population C (MP-C) (anamorph, *Fusarium fujikuroi*) (Nirenberg 1976) was first identified in 1977 among strains from rice from Taiwan (Hseieh *et al.* 1977). It has been found responsible for bakanae disease in Italy (Amatulli *et al.* 2010). Mating population A (MP-A) [anamorph, *Fusarium verticillioides* (*F. moniliforme*)] has been isolated from Asia and mating population D (MP-D) (anamorph, *Fusarium proliferatum*), has been isolated from rice from Africa, Australia, and the United States (Desjardins *et al.* 1997, Amoh *et al.* 1996, Voigt *et al.* 1995). Thus, more than one species of *Fusarium* may be able to infect rice and cause symptoms of bakanae disease.

Identification of these species, based on morphological and biological characters, has proved difficult and time-consuming. At least two of the species, *F. fujikuroi* and *F. proliferatum* are difficult to differentiate from each other morphologically (Leslie and Summerell 2006). Taxonomy of many species of the genus has been revitalized by

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application of biological species concepts based on reproductive compatibility and phylogenetic species concepts based on DNA sequence variation.

The elevation of the eight mating populations to a distinct species was supported by DNA sequence analysis of multiple unlinked loci; only the DNA sequence of the rDNA ITS2 region was incongruent with other gene genealogies (O'Donnell *et al.* 1998). Furthermore, it is not yet clear whether all the three *Fusarium* species are associated with the symptoms of bakanae or if *F. moniliforme* and *F. proliferatum* are present only as saprophytes.

The identity of the bakanae causal agent seems to be still unclear, therefore, present study was undertaken to assess the *Fusarium* spp associated with bakanae-infected rice, to identify species by amplification and sequencing of the ITS region, and to evaluate the pathogenicity of the Indian isolates on the susceptible rice variety.

MATERIALS AND METHODS

Isolation and identification of Gibberella fujikuroi *species complex*

Gibberella fujikuroi species were isolated from 100 seed samples of eleven rice varieties cultivated in India. Agar plate method of International Seed Testing Association (Miles 1963) was adopted for the isolation of pathogen from seed. Maximum of 10 seeds were aseptically and evenly placed onto the surface of each malt agar plate. Plates were incubated for 8 days at 25 ±1 ^{O}C under dark conditions and one colony per seed was re-isolated from a single spore and identified by morphology when grown on potato dextrose agar (Nelson *et al.* 1983). Number of *Gibberella fujikuroi* species associated with different rice varieties were scored.

Pathogenicity test

Pathogenicity tests were conducted in seed inoculation assays. The susceptible rice cultivar Pusa Basmati 1121 was used to assess the pathogenicity of the different species of *Gibberella fujikuroi*. Seeds were surface-disinfected by immersion in 70% ethanol for 1 min, transferred to 1% sodium hypochlorite for 3 min and rinsed three times consecutively in sterile distilled water. Seeds were then left to dry in the flow cabinet in Petri dishes containing sterile filter paper. Fungal inoculum suspension was prepared for each species of *Gibberella fujikuroi* species complex (*Fusarium moniliforme, Fusarium moniliforme* var *subglutinans, Fusarium lateritium, Fusarium pallidoroseum, Fusarium equisetti*) and another species *Fusarium oxysporum* associated with rice seed, from the single spore cultures. Fungal cultures were subcultured on PDA at room temperature (25°C). After 15 days plates were flooded with sterile water and scraped with a sterile spatula. The resulting suspensions were filtered through two layers of sterile cotton lint and brought to a final concentration of 10^6 spores/mL in sterile distilled water. Thirty rice seeds were soaked in 10 ml of inoculum suspension for 18 hr at room temperature. Control seeds were soaked in sterile water. Inoculated and control seeds were sown in pots (three pots per isolate/ten seeds per pot) containing autoclaved mixture of soil and sand in the ratio of 3:1. The greenhouse temperature was maintained at 24–26°C. Fifteen days after inoculation, the number of germinated seeds was assessed. The seedlings were observed for symptoms of bakanae as number of slender and chlorotic leaves and number of plants showing crown rot (Wulff *et al.* 2010) 15 and 30 days after inoculation. Similarly, pathogenicity test was conducted with eighteen different isolates of pathogenic species of *Gibberella fujikuroi* as described above.

Morphological characterization of pathogenic species

Sporulation of different pathogenic isolates of *Gibberella fujikuroi* was recorded by excising 5 mm^2 colony area of 8 days old colony grown on Potato Dextrose Agar media using a cork borer of 5 mm^2. It was shaken well for two minutes in magnetic stirrer, after mixing in 1 ml water to dislodge the conidia. Number of spores/cm was counted by haemocytometer (B S 748) of Rohem, India.

Microscopic studies for the presence of macroconidia were performed under light microscope (Olympus, OIC 37901). Mycelial disc of 5 mm^2 was excised from 8 days old colony grown on Potato Dextrose Agar media and its mycelium was scrapped out with the help of sterile needle and mounted in a glass slide after staining with cotton blue and lactophenol. Observations were taken for the presence of macroconidia in pathogenic isolates of *Gibberella fujikuroi*.

For spectral values different pathogenic isolates of *Gibberella fujikuroi* species were grown on PDA and incubated at 25 ^{O}C. After, seven days, growth of these isolates was photographed using a Sony digital Camera (12.1 mega pixels). These photographs were used for the spectral RGB (Red, Green and Blue) analysis of the individual isolates using Adobe Photoshop 7.0 versions (Chand *et al.* 2008). Vertical and horizontal grid lines were brought at a distance of 0.5 cm in Adobe Photoshop. Spectral tool was selected and placed in the centre of each rectangle. The spectral value displayed in the RGB box was recorded. Observations were drawn from all areas of the growing mycelium. Nine rectangles were covered for one replication and seven such replications were taken for every isolate. Experiment was conducted in completely randomized design for the analysis of variance.

DNA extraction and ITS amplification

Nineteen pathogenic isolates of *Gibberella fujikuroi* species complex one non-pathogenic isolate of *Fusarium equisetti* and one isolate of *Fusarium oxysporum* were selected and DNA was isolated using CTAB method with minor modifications in the protocol given by Saghai Maroof *et al.*

(1984). DNA was quantified with a nanodrop 2000 spectrophotometer and quality analysis done on 0.8% agarose gel. The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S rDNA, were amplified by PCR using universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990) synthesized by Sigma Aldrich House, Suffolk, UK. PCR amplification reaction was carried out in the final volume of 25 µL, which consisted of 1X PCR assay buffer, 200 mM dNTPs mixture, 2.0 mM $MgCl_2$, 0.1 µM primer, 1.5 U Taq DNA Polymerase (Bangalore Genie, India), and 50 ng template DNA. All of the reactions were carried out in icycler, Bio-rad (96 well system), under following thermal cycler parameter conditions: initial denaturation 94°C for 4 min, annealing at 57°C for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min. PCR products were separated by electrophoresis in 1.4% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator.

Sequencing of ITS region and analysis of data

The PCR fragments were sequenced and used to identify the strains using the *Fusarium* ID database (Geiser *et al.* 2004) and blast searches. ITS1 and ITS2 sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.* 1994). Phylogenetic analyses were performed using MEGA 4.0 (Tamura *et al.* 2007). A bootstrap analysis was performed using 1 000 resamples of the data using maximum-parsimony as the criterion.

RESULTS AND DISCUSSION

Gibberella fujikuroi species complex in seed of different rice varieties

Different species of *Gibberella fujikuroi,* viz. *Fusarium moniliforme, Fusarium moniliforme* var *subglutinans, Fusarium pallidoroseum* were isolated from rice seeds. Percentage of infection was maximum in rice variety Pusa basmati 1121 (24%) followed by Tarori Basmati (20%).

Table 1 Rice varieties infected with *Gibberella fujikuroi* species complex from India

Rice variety	Infection (%)
Pusa Basmati 1121	24
Pusa Basmati 1	4
Tarori Basmati	20
Samba Mahsuri	2
Jaya	10
Ajaya	4
Vikramaraya	6
Swarnadhan	1
IR 50	4
IR 64	4
MTU 1010	4

Infection percentage was minimum for Swarndhan (1%) and Samba Mahsuri (2%) (Table 1).

Six species of *Fusarium*, namely *F. moniliforme, F. proliferatum, F. fujikuroi, Fusarium moniliforme var subglutinans, Fusarium equisetii* and *Fusarium pallidoroseum* were isolated from seed of different rice varieties. However maximum incidence of *Fusarium moniliforme* was observed in Pusa 1121 which shows its susceptibility to *Fusarium moniliforme* and carries inoculums to the next season crop. Indeed, bakanae has proved to be primarily seed-borne and high levels of incidence have been found in rice seed samples in this and previous studies (Desjardins *et al.* 2000, Mathur and Manandhar 2003, Anderson 2005). Therefore, disease control strategies that focus on clean seed are important for effective disease management.

Pathogenicity of isolates

Significant reduction in germination was observed with different isolates of *Fusarium* spp (Table 2). Minimum germination was observed in isolate F20 (40%) while maximum germination was present in isolates FM11, FM04, FM07 and FM21 (93%).

Out of 6 species belonging to different mating population of *Gibberella fujikuroi* three species *Fusarium moniliforme, Fusarium proliferatum* and *Fusarium fujikuroi* were found

Table 2 *Fusarium* spp showing different germination and pathogenicity inoculated to PB 1121 variety of rice.

Isolate no.	Place of collection	Germination (%)	No. of slender and chlorotic leaves (%)	Crown rot and stem rot incidence (%)
FM03	Rajasthan	60.00	20.00	20.00
FM10	Delhi	50.00	20.00	40.00
FM11	Tamilnadu	93.00	30.00	50.00
FM13	Andhra Pradesh	60.00	10.00	50.00
FM14	Uttar Pradesh	50.00	10.00	30.00
FM16	Delhi	80.00	30.00	40.00
FM17	Delhi	60.00	30.00	40.00
FM22	Delhi	60.00	10.00	30.00
FM23	Delhi	80.00	10.00	40.00
FM04	Andhra Pradesh	93.33	50.00	20.00
FM05	Uttaranchal	60.00	80.00	00.00
FM12	Haryana	80.00	40.00	10.00
FM24	Haryana	80.00	90.00	10.00
FM25	Delhi	50.00	50.00	30.00
FM29	Delhi	93.00	60.00	10.00
FM21*	Delhi	93.00	00.00	00.00
F09**	Delhi	80.00	00.00	00.00
F01	Uttar Pradesh	86.00	30.00	30.00
F07	Andhra Pradesh	93.33	10.00	20.00
F20	Haryana	40.00	10.00	20.00
F18	Haryana	80.00	30.00	30.00

**Fusarium oxysporum*; ***Fusarium equisetti*

responsible for bakanae disease. These produced three distinct symptoms, which were elongation, stunting and rotting, and elongation and stunting both (Fig 1). Number of slender and chlorotic leaves were maximum in isolates F24 (90%). Slender and chlorotic leaves were absent in isolates F21 and F09. Crown rot and stem rot incidence were maximum in isolate F11 and F13 (50%). Crown rot and stem rot incidence was absent in isolates F04, F09 and F21 (Table 2).

Pathogenicity of the *G. fujikuroi* species complex strains on rice was seen in form of inhibition of seed germination and symptoms in the seedlings. Furthermore, the different species isolates inhibited seed germination differently and caused symptoms of chlorotic and/or slender leaves with and without induction of crown and stem rot, indicating varying pathogenic ability. According to Amoah and colleagues (1995), pathogenicity of *G. fujikuroi* species complex on rice (and maize) may depend on the balance of toxins and growth regulators that are, on the other hand, affected by the strain of the pathogen, the environment and the nutritional status of the plant.

Morphological characterization of pathogenic isolates

Significant differences were observed for sporulation (Table 3). Maximum sporulation was present in isolate F04 (2 488 spores/cm) followed by isolate F14 (2 493.33 spores/cm). Sporulation was minimum in isolate F18 (8.0 spores/cm). On the basis of presence or absence of macro conidia *Gibberella fujikuroi* spp were divided in two groups. Macroconidia were absent in isolates F03, F13, F14, F16, F04, F05, F24 and F01. Macroconidia were present in other isolates (Table 3). Isolates differed significantly for spectral values (Table 3). Spectral value was maximum for isolate F18 (203.28) and it was minimum for isolate F22 (104.50). However significant correlation was absent between spectral values and sporulation of isolates. Traditional morphological traits, such as colony colour, length of microconidial chains, or the production of macroconidia, may vary only slightly between species or be variable within species of *Gibberella* (Leslie 1995).

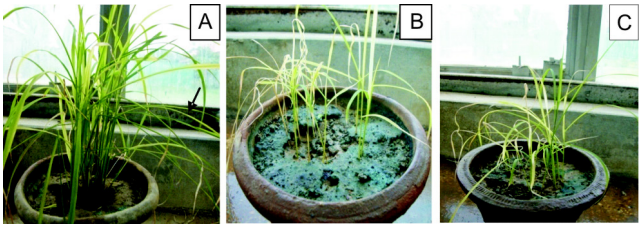

Fig 1 Symptoms produced by *Fusarium* spp. in rice. A, slender elongated primary leaves; B, chlorotic and stunted symptom; C, stunted and elongated plants

Table 3 Morphological characters of different pathogenic isolates of *Fusarium* spp

Isolate	Accession no.	Identification	Spectral values*	Macroconidia	Sporulation (cm^{-2})
F03	HQ995663	*Fusarium moniliforme*	190.61	–	45.67
F10	JF499681	*Fusarium moniliforme*	152.49	+	45.00
F11	HQ995666	*Fusarium moniliforme*	192.54	+	486.66
F13	HQ995667	*Fusarium moniliforme*	196.12	–	800.00
F14	JF499682	*Fusarium moniliforme*	178.24	–	2493.33
F16	HQ995669	*Fusarium moniliforme*	179.87	–	333.33
F17	JF499684	*Fusarium moniliforme*	135.39	+	41.67
F22	JF499685	*Fusarium moniliforme*	104.50	+	286.67
F23	JF499675	*Fusarium moniliforme*	135.44	+	152.33
F04	JF499678	*Fusarium fujikuroi*	159.09	–	2488.00
F05	HQ995664	*Fusarium fujikuroi*	188.71	–	540.00
F12	JF499683	*Fusarium fujikuroi*	123.68	+	216.00
F24	JF499677	*Fusarium fujikuroi*	135.44	–	152.33
F25	JF499679	*Fusarium fujikuroi*	136.49	+	1413.33
F29	JF499680	*Fusarium fujikuroi*	136.72	+	158.33
F21	JF499686	*Fusarium oxysporum*	175.41	+	73.33
F9	HQ995668	*Fusarium equisetti*	187.37	+	44.33
F01	JF499687	*Fusarium proliferatum*	125.39	–	257.33
F07	HQ995665	*Fusarium proliferatum*	192.36	+	148.33
F20	JF499675	*Fusarium proliferatum*	159.09	+	44.33
F18	JF499688	*Fusarium proliferatum*	203.28	+	8.33
LSD 0.001			18.71		132.79

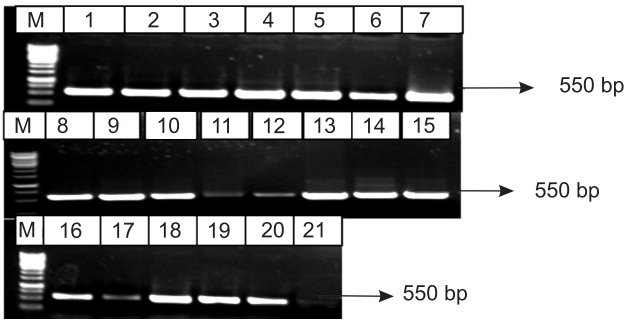

Fig 2 PCR amplification of ITSI and ITS2 region of *Fusarium* spp. M is molecular ladder 1 kb (MBI fermentas)

Sequence analysis of the ribosomal DNA spacer sequence (ITS)

All isolates yielded an approximately 550 bp fragment after PCR amplification with the ITS1 and ITS4 primer pair (Fig 2). The species identified were *F. moniliforme, F. fujikuroi* and *F. proliferatum* (Table 3). Phylogenetic analysis using MEGA 4.0 generated one phylogenetic tree from 550 bp of aligned DNA sequences (Fig 3). This tree consisted of three different clades (Fig 3). Clade I comprised *F. moniliforme* isolates. The bootstrap value for this clade indicated 98% unity. Clade II, with bootstrap value of 93%, consisted of *F. fujikuroi.* Clade III was formed by *F. proliferatum* strains with 100% bootstrap support (Fig 3).

ITS sequences clearly grouped the different species of *Fusarium* with high bootstrap values, confirming that the ITS region is a good marker for species identification among these *Fusarium* spp. It grouped pathogenic *Fusarium* spp in three different clades of *Fusarium moniliforme, Fusarium fujikuroi* and *Fusarium proliferatum.* Further it confirmed that three *Fusarium* spp (*Fusarium moniliforme, Fusarium fujikuroi* and *Fusarium proliferatum*) responsible for bakanae disease of rice and these species were not confined in a particular place, rather they are distributed throughout India. Based on translation elongation factor sequence Wulff *et al.* (2010) observed *Fusarium moniliforme, F. andiyazi, Fusarium proliferatum* and *Fusarium fujikuroi* were responsible for bakanae disease. Similarly based on TEF sequence *Fusarium fujikuroi* was found associated with bakanae disease in Italy (Amatulli *et al.* 2010). The discovery of DNA sequence variation within and between natural populations has provided new taxonomic characters and revolutionized fungal population genetics, especially among genera such as *Gibberella* with large numbers of species and often limited morphological diversity (Samuels and Seifert 1995).

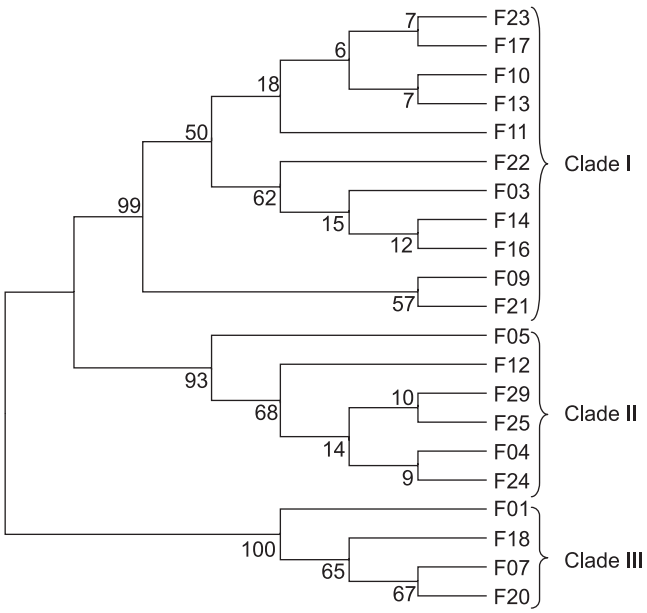

Fig 3 Maximum parsimony tree based on ITS sequences of *Fusarium* spp. Bootstrap values based on 1000 replications are indicated as percentages in the internodes when replication frequencies exceed 50%.

Present study identified three *Fusarium* spp viz. *Fusarium moniliforme, Fusarium fujikuroi* and *Fusarium proliferatum* were associated with bakanae disease of rice in India, which will be very useful for understanding and developing strategies to control the bakanae disease of rice.

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