



Cultural, morphological and molecular variability in *Fusarium moniliforme* isolates causing bakanae disease of aromatic rice in Haryana, India

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Received: 31 May 2023; Accepted: 21 February 2025

ABSTRACT

Bakanae disease caused by *Fusarium moniliforme* has become a serious problem in the successful cultivation of aromatic rice under north-west plain zone of India. The present study was carried out during rainy (*kharif*) season of 2020 and 2021 at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana to assess cultural, morphological and molecular variability among 26 selected isolates of *Fusarium moniliforme* collected from different locations and 11 districts of Haryana from roving survey conducted which includes bakanae infected plants of aromatic rice varieties namely PB 1121, PB 1718, PB 1401, PB 1509 and *Basmati* 521. On potato dextrose agar (PDA) media, colony colour of different isolates varied from white, milky white to creamy white. The microscopic observations showed that only three isolates showed macro-conidia, whereas the others 23 had micro-conidia. The macro and micro conidia size ranged between $(22.36\text{--}28.09) \times (3.01\text{--}4.71)$ and $(4.80\text{--}9.92) \times (1.42\text{--}2.71)$ μm in length and breadth, respectively. The isolates were divided into 3 groups based on cultural characteristics, macro and micro conidial size range. The precision molecular characterization of different isolates was done using specific primers, viz. β -tubulin, Vertf and (rp 32 and rp 33) primers which were found positive for 20, 22 and 9 isolates, respectively. The present investigations confirm that, there is variability among 26 isolates on the basis of morphological characteristics and further precision technique molecular markers confirm the same in *Fusarium moniliforme* causing bakanae disease and they may be useful in breeding programme for bakanae resistant genotypes.

Keywords: Aromatic rice, Bakanae, Cultural variability, *Fusarium moniliforme*, Molecular variability

The bakanae is caused by *Fusarium moniliforme* (Sheldon) teleomorph: *Gibberella fujikuroi* Sawada, Wollenweber has gained substantial importance in Asian countries during recent years (Asmaul *et al.* 2020). The disease incidence is very high on the varieties Pusa Basmati 1121 and Pusa Basmati 1509 which is being growing throughout the country especially in Punjab and Haryana due to its grain quality. The typical and distinguished symptoms of the disease are elongation and rotting of rice plants. *Fusarium moniliforme* produces broad spectrum secondary metabolites, pigments and mycotoxins resulting quantitative and qualitative losses to rice crop. According to Gupta *et al.* (2014), disease prevalence on various varieties of basmati rice ranged from 2.1–3.2% in Uttarakhand, 1.20–11.7% in Uttar Pradesh, 3–95% in Haryana, 10.5–40.0% in Punjab, 1.83–8.7% in Bihar, and 2.4–13.6% in Rajasthan. Disease

is known to result in yield losses of 20–50% in Japan, 70–80% in Australia, 3.7–14.7% in Thailand, 5–23% in Spain, 3.0–95.4% in India, 40% in Nepal, 6.7–58.0% in Pakistan and up to 75.0% in Iran under ideal conditions (Singh and Sunder 2012).

Fusarium fujikuroi, *Fusarium concentricum*, *Fusarium proliferatum* and *Fusarium verticillioides* are some members of the *Gibberella fujikuroi* spp. complex that are thought to be connected to the bakanae disease of aromatic rice (Amoah *et al.* 1995, Desjardins *et al.* 2000). Numerous investigations have been conducted to find out the *Fusarium* species that cause bakanae disease, however it is still unclear which *Fusarium* species have been connected to the disease's symptoms. Precise understanding of the pathogen and variability on cultural, morphological and molecular characteristics is very crucial for its management. Molecular approaches are required to corroborate the phenotypic data since identification and variability studies based solely on morphological or cultural characteristics are unreliable. Bag *et al.* (2022) studied this pathogen morphologically and used specific primers, viz. rp32 and rp33, H3-1a and H3-1b and Bt2a and Bt2b for its characterization at genetics level. Mohiddin *et al.* (2021) characterized the isolates

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of *Fusarium* spp. Based on cultural and morphological characteristics and amplifications of different genes was done for confirming the variability among *Fusarium moniliforme* isolates. There is also very less information available on *F. moniliforme* population studies. Keeping in view the economic importance of disease and less information available on cultural, morphological and molecular aspects of this pathogen under Haryana conditions present study was undertaken to assess the cultural, morphological and molecular variability among different isolates of *F. moniliforme* from major aromatic rice growing areas of Haryana.

MATERIALS AND METHODS

The present study was carried out during rainy (*kharif*) season of 2020 and 2021 at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana

Survey and collection of diseased samples: Total 64 bakanae infected plant samples were collected from commonly grown aromatic rice varieties, viz. PB 1121, PB 1401, PB 1718, PB 1509 and Basmati 521 from i.e. Karnal, Kurukshetra, Kaithal, Sirsa, Jind, Fatehabad, Sonapat, Yamunanagar, Panipat, Hisar and Bhiwani districts of Haryana (Table 1).

Isolation, purification and maintenance: The infected samples were cut into fragments of 3 and 4 mm in size, and were surface sterilized with $MgCl_2$ (0.1%). The cut fragments were equally spread over potato dextrose agar medium in each petri dish under totally sterile and aseptic conditions. Plates were kept at $25 \pm 2^\circ C$ in a BOD incubator. Using the single spore culture approach, purified cultures were produced, and pathogen was identified by microscopic analysis and preserved for subsequent study.

Cultural and morphological characterization: The isolates were cultured and multiplied on potato dextrose agar (PDA) in petri dishes for comparisons of cultural characteristics, each treatment was replicated thrice. The medium was made up and sterilized in autoclave, for 30 min at 15 psi. Ninety millimeters diameter petri dishes were filled with 20 ml of media. With the cork-borer, 5 mm mycelium

discs from each isolate's actively growing colony were cut and put in the petri dish's centre, and kept at $25 \pm 2^\circ C$. The observations were recorded at 8 days after incubation. The cultural characteristics i.e. colour on surface and reverse, elevation and texture of purified isolates were studied. The morphological characteristics i.e. micro-macro conidia length, breadth, shape and septa were measured with the help of phase contrast microscope (Zeiss) using image analyzer software Zen 2.0 (blue edition) with objectives 20X and 40X zoom.

Molecular variability: Molecular variability in *Fusarium moniliforme* isolates selected (26) was done using three specific primers, viz. β -tubulin (Bt2a and Bt2b), Vertf (Vertf 1 and Vertf 2) and (rp 32 and rp 33). β -tubulin gene was amplified using (Bt2a-5' GGTAACCAAATCGGTGCTGCTTTC-3') and (Bt2b-5' ACCCTCAGTGTAAGTACCCTTGCC-3') primers reported by (Procter *et al.* 2004). Vertf (Vertf 1-5' GCGGGAATTCAAAGTGGCC-3') and (Vertf 2-5' GAGGGCGCGAAACGGATCGG-3') as described by (Patino *et al.* 2004) and (rp 32-5' ACAAGTGTCTTGGGGTCCAGG-3' and rp 33-5' GATGCTCTTGGGAAGTGGCTACG-3') as reported by (Procter *et al.* 2004) used for the amplification of *F. moniliforme* isolates.

Culture preparation and DNA extraction: The isolates pure cultures were raised in 250 ml flasks with 100 ml of potato dextrose broth (PDB) medium for 10 days at $25 \pm 2^\circ C$. The fungal mycelial mat was collected by filtering through Whatman paper No. 1 after being grown for 10 days. Utilizing the mini-prep cetyl-trimethyl ammonium bromide method, the genomic DNA was isolated from 26 pure cultures of *Fusarium* spp. (Murray and Thompson 1980). With a sterile pestle and mortar, the mycelium mat of the fungus was broken down in liquid nitrogen, and the resulting powder was collected in 2 ml centrifuge tubes. The powder was added with 800 μ l of DNA extraction buffer containing 1% mercaptoethanol, and the mixture was kept at $65^\circ C$ for one hour while being stirred continuously. The tubes were carefully turned over to mix the contents every 15 min.

Table 1 Diseased samples collected from different districts of Haryana (2020 and 2021)

Isolates Id. No.	Districts	Locations (Villages)	Varieties
FM 2 to FM 11	Hisar	Dhad, Kharak Punia, Gurana and Kheri Jalab	PB 1121
FM 12 to FM 17	Jind	Intal Khurd, Intal Kalan and Ikkas	PB 1121
FM 18 to FM 28	Fatehabad	Saniana and Pirthala	PB 1121 and PB 1401
FM 29 to FM 36	Bhiwani	Kungar, Durjanpur, Alakhpora and Bhiwani	PB 1121 and PB 1509
FM 37 to FM 50	Sirsa	Patli Dabar, Vaidwala, Mochiwala, Jodhkan, Bajekan, Fatehpuria, Rasulpur, Rania, Ootu, Ramsinghpuria and Naiwala	PB 1121, PB 1401 and PB 1718
FM 51 to FM 55	Panipat	Bapoli, Panipat, Samalkha and Chhichhrana	PB 1121, PB 1509 and PB 1718
FM 56 to FM 58	Sonipat	Sonipat, Kumasapur and Mukinpur	PB 1121
FM 59 to FM 61	Karnal	Taraori and Sikri	PB 1121 and PB 1718
FM 62 to FM 63	Yamunanagar	Kartarpur and Yamunanagar	PB 1121 and PB 1509
FM 64	Kaithal	Kaul	Basmati 521
FM 66	Kurukshetra	Babain	PB 1509

After incubation, the same amount of chloroform:isoamyl alcohol (24:1) was added to each tube. After cooling the samples, the tubes were centrifuged for 15 min @10,000 rpm after being shaken for 30–45 min @70 rpm to make sure proper mixing. New 1.5 ml centrifuge tubes were used to collect the supernatant, and each tube received RNase treatment (10 µl of RNase added) and 30 min of 37°C incubation. After adding 800 µl of cold isopropyl alcohol, doing some gentle inversions, letting it remain at 4°C for 15 min, and centrifuging it for 10 min @10,000 rpm. Once the pellet was washed with 70% ethanol, the supernatant was discarded. Following that, 50 µl of (Tris EDTA) buffer was used to dissolve it after it had been air dried. To be used subsequently, the DNA from each isolate was stored at -20°C. The spectrophotometer NanoDrop™ 1000 (Thermo Scientific, Wilmington, USA) was used to quantify DNA.

PCR amplification: The polymerase chain reactions (PCR) showed good amplification for specific primers. The amplification was carried out in a total volume of 25 µL, with each tube holding 2 µl of each isolate's DNA, 1.5 µL of primers, 7.5 µL of water, and 12.5 µL of the master mix (Promega corporation, USA). Integrated DNA Technologies (USA) produced the primers. The PCR conditions included a 4-min pre-incubation period at 94°C, followed by 35 cycles of amplification, which included denaturation for 1 min at 94°C, annealing for 1 min @52°C to 60°C, extension for 1 min @72°C, and a final 7 min extension step @72°C. Amplicons were produced by dissolving agarose powder in 1XTBE buffer, staining with EtBR, and electrophoresis on 2% agarose gels. The gel documentation system (Bio-Rad, Philadelphia, PA, USA) was used to record the results.

RESULTS AND DISCUSSION

Collection of diseased samples: 64 samples were collected from major aromatic rice growing districts of Haryana i.e. Karnal (3), Kurukshetra (1), Kaithal (1), Sirsa (14), Jind (6), Fatehabad (11), Sonapat (3), Yamunanagar (2), Panipat (5), Hisar (10) and Bhiwani (8) from 5 basmati varieties, viz. PB 1121, PB 1509, PB 1401, PB 1718 and Basmati 521 during *kharif* 2020 and 2021. Maximum samples (14) were collected from Sirsa district and a total of 11 villages were surveyed in this district followed by Fatehabad (11) and Hisar (10).

Cultural and morphological characterization of *Fusarium moniliforme* isolates: Out of all isolates which were characterized, 26 isolates (selected) characteristics mentioned here white (14), milky white (7) and creamy white (5) colour on surface. Isolates were also characterized on the basis of texture i.e. cottony (22) and cottony fluffy (4) and elevation of colony i.e. flat (6) slightly raised (8) slightly raised, centre raised (5) medium raised (5) medium raised, centre raised (1) and highly raised (1) on PDA. The different isolates showed colony colour on reverse as creamish (22) orangish (2) and orangish at centre, creamy at periphery (2) (Table 2 and Fig. 1). The microscopic observations revealed that among all purified cultures three isolates i.e. FM 51 Bapoli (Panipat), FM 60 Sikri (Karnal) and FM 62 Kartarpur (Yamunanagar) showed macro-conidia whereas, rest of isolates had micro-conidia. The macro-conidia size ranged between (22.36–28.09) µm × (3.01–4.71) µm in length and breadth, respectively and showed typical long slender falcate or straight shape with septation ranging from 1–5.

Table 2 Grouping of isolates on the basis of cultural characteristics, micro and macro conidial size range

Category	Colour on PDA	Isolates Id. No.	Micro-conidial size range (µm)	Macro-conidial size range (µm)
Group 1	White (14)	FM 10, 12, 16, 18, 25, 28, 34, 37, 44, 50, 53, 56, 60, 64	(5.98–9.23) × (1.42–2.71)	(28.09 × 4.71)
Group 2	Milky white (7)	FM 3, 20, 36, 40, 52, 63, 66	(5.20–7.92) × (1.87–2.52)	----
Group 3	Creamy white (5)	FM 7, 31, 51, 59, 62	(7.99–8.50) × (2.38–2.45)	(22.36–22.70) × (3.01–3.84)
Isolates Id. No.			Colour on reverse	
FM 3, 10, 12, 16, 18, 20, 25, 28, 31, 36, 37, 40, 44, 50, 52, 53, 56, 59, 60, 63, 64			Creamish (22)	
FM 62, 66			Orangish (2)	
FM 7, 34			Orangish at centre, creamy at periphery (2)	
Isolates Id. No.			Colony elevation	
FM 10, 20, 28, 36, 44, 52, 56, 63			Slightly raised (8)	
FM 3, 7, 12, 18, 59			Slightly raised, centre raised (5)	
FM 16, 37, 51, 64, 66			Medium raised (5)	
FM 34			Medium raised centre raised (1)	
FM 25, 31, 40, 50, 53, 60			Flat (6)	
FM 62			Highly raised (1)	
Isolates Id. No.			Texture	
FM 3, 7, 10, 12, 16, 18, 20, 25, 28, 31, 36, 37, 40, 44, 50, 51, 52, 53, 56, 59, 60, 63			Cottony (22)	
FM 34, 62, 64, 66			Cottony fluffy (4)	



Fig. 1 Variability in cultural characteristics of different isolates.

Among the oval shaped micro-conidia with no septa observed and the size ranged between $(4.80-9.92) \times (1.42-2.71) \mu\text{m}$ in length and breadth, respectively. Among all isolates, FM 64 and FM 6 showed the largest and smallest macro-conidial length and FM 10 and FM 28 showed the largest and smallest macro-conidial breadth in size. There were 3 groups based on cultural characteristics, micro and macro conidial size range. Group 1 having 14 isolates with white colour on surface and micro-conidial size ranged between $(5.98-9.23) \times (1.42-2.71) \mu\text{m}$ and macro-conidial

size range $28.09 \times 4.71 \mu\text{m}$ in length and breadth, respectively. Group 2 having 7 isolates with milky white colour on surface and micro-conidial size ranged between $(5.20-7.92) \times (1.87-2.52) \mu\text{m}$ in length and breadth, respectively. Group 3 having 5 isolates with creamy white colour on surface and micro-conidial size ranged between $(7.99-8.50) \times (2.38-2.45) \mu\text{m}$ and macro-conidial size ranged between $(22.36-22.70) \times (3.01-3.84) \mu\text{m}$ in length and breadth, respectively (Table 2). The isolates were recognized as *F. moniliforme* on the basis of morphological traits according to the book by Leslie and Summerell (2006) "The *Fusarium* Laboratory Manual".

Similarly (Bag *et al.* 2022) characterized the isolates on the basis morphological characteristics and divided into two groups. Group I isolates Fm (1–6) and Fm 12 showed white colored cottony colony with micro-conidial size ranged between $(7.4-8.8) \times (2.8-3.7) \mu\text{m}$ and the size of macro-conidia ranged between $(24.24-35.70) \times (3.2-3.87) \mu\text{m}$ with no chlamydospores. Group II comprising isolates Fm (7–11) had creamy white colony with micro-conidial size ranged between $(7.3-9.1) \times (3.2-3.9) \mu\text{m}$ and macro-conidial between $(29.54-35.73) \times (2.92-4.8) \mu\text{m}$, with no chlamydospores. Similar to our studies, Zidan *et al.* (2020) identified 17 isolates of *Fusarium* spp. out of 105, 13 isolates belong to *F. proliferatum*, two of *F. verticillioides* and two of *Fusarium andiyazi* based on their morphological characteristics. Mohiddin *et al.* (2021) also divided the 119 isolates of *Fusarium* spp. into 4 groups on the basis of cultural and morphological characteristics. Bashyal *et al.* (2015) also categorized

isolates, broadly into three groups based on morphological characters studied. Egerci *et al.* (2022) done morphological characterization of *Fusarium andiyazi* associated with the bakanae disease of rice in Turkey. Aris *et al.* (2020) characterized the twenty-six isolates of *Fusarium* spp. based on microscopic and macroscopic morphologies from Malaysia. Puyam *et al.* (2017) studied cultural and morphological characteristics of *F. moniliforme* isolates and grouped into five groups. Bashyal *et al.* (2020) categorized the 63 isolates of *Fusarium moniliforme* from different

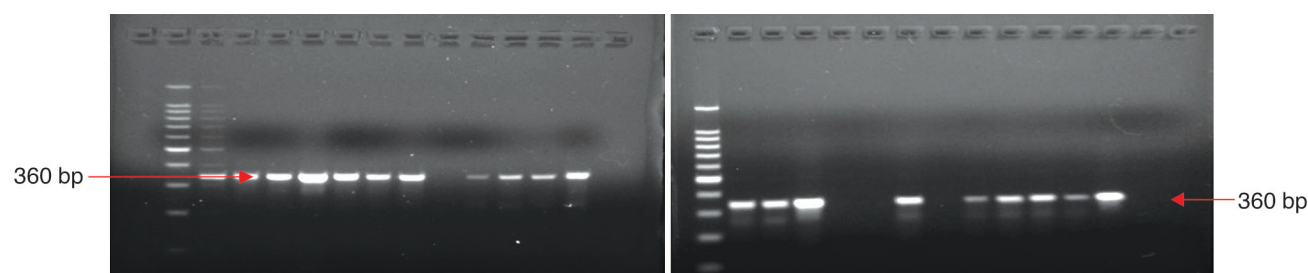


Fig. 2 Amplified products of *F. moniliforme* isolates obtained using β -tubulin primer.

locations of India based on their cultural and morphological characteristics.

Molecular variability of *F. moniliforme* isolates using different markers: In present study total 3 primers, viz. β -tubulin (Bt2a and Bt2b), Vertf (Vertf 1 and Vertf 2) and (rp 32 and rp 33) were used for assessment of molecular variability among 26 (selected) *Fusarium moniliforme* isolates. A polymerase chain reaction product of about 360 base pairs was obtained for 20 *Fusarium* isolates and 6 showed negative results for (β)-tubulin region (Fig 2). Two specific primers namely Vertf (Vertf 1 and Vertf 2) and (rp 32 and rp 33) were used to discriminate fumonisin producing and non-producing strains. Approximately 400 base pairs PCR product was observed for twenty-two isolates which were positive for Vertf primer proved to be potential fumonisin producers and four isolates did not showed any amplification. Nine isolates were positive for (rp 32 and rp 33) primers and approximately 680 base pairs product was observed, seventeen isolates show negative results.

Similar to our studies (Bag *et al.* 2022) used primers, viz. rp32 and rp33, H3-1a and H3-1b and Bt2a and Bt2b for differentiation among different isolates of *F. moniliforme* at genetic level. Egerci *et al.* (2022) done molecular detection of *Fusarium* spp. through PCR analysis of the internal transcribed spacer (ITS) region. Aris *et al.* (2020) characterized the 26 isolates of *Fusarium* spp. using β -tub genes from Malaysia. Jeon *et al.* (2013) also amplified H3, β -tubulin and FUM 1 genes in *F. moniliforme* isolates using specific primers. Several works also used these primers for molecular characterization *F. moniliforme* isolates (Glass *et al.* 1995, Bashyal *et al.* 2015 and Proctor *et al.* 2003). Nosratabadi *et al.* (2018) evaluate the efficiency of PCR-RFLP analysis of the beta (β)-tubulin region for differentiating a total of 107 isolates of *Fusarium* sp, with primer pairs (T1 and T22). Qiu *et al.* (2020) detected the fumonisins among different strains of *Fusarium* sp. using PCR analysis. Ouattara *et al.* (2021) used polymerase chain reaction (PCR) method for molecular identification of *Fusarium* strains and the detection of the presence of the Fum13 gene involved in the fumonisins biosynthesis.

Based on cultural and morphological characteristics all isolates were identified as *F. moniliforme*. All isolates were divided into 3 groups based on cultural characteristics, micro and macro conidial size range. The variability among the isolates was also confirmed using three specific primers. The present study concludes that differentiation among

the isolates was clearly visible. Precise identification and variability studies of this pathogen will be very useful for the management of this disease under field conditions also.

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