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Identification of *Fusarium* species Associated with Bakanae Disease of Basmati Rice by using Molecular Marker

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ABSTRACT: Bakanae caused by *Fusarium fujikuroi* (Nirenberg), is emerging as a serious threat for rice (*Oryza sativa* L.) cultivation in India. It is supposed to be caused by *Fusarium fujikuroi* but other *Fusarium* species such as *Fusarium verticilloides* or *Fusarium proliferatum*, have also been linked to the development of this disease. Correct diagnosis and identification of the pathogen is the key to the successful management of plant disease. Identification that relies solely on cultural and morphological characteristics is unreliable for *Fusarium* isolates so this investigation was conducted to assess the efficiency of PCR analysis of Tef -1 alpha gene for identification purpose. From rice samples with bakanae infections, twenty six different *Fusarium* spp. strains were identified. Samples were collected from different districts of Haryana *viz*., Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra, from different basmati rice varieties namely PB 1121, PB 1401, PB 1509, PB 1718 and Basmati 521. Tef-1 genes of all isolates were successfully amplified using primers (EF 1 and EF 2) as forward and reverse, respectively. A PCR product of approximately 700 base pairs was observed for 22 *Fusarium* isolates, four isolates did not showed any amplification. Hence, it is recommended that Tef-1 alpha gene be analyzed to solve the limits of cultural and morphological methods in order to identify the *Fusarium* species linked to the bakanae disease of basmati rice.

Keywords: Rice, *Fusarium* spp, PCR, Tef- 1 , identification.

INTRODUCTION

Fusarium fujikuroi Nirenberg is responsible for this significant emerging seed and soil borne diseases known as bakanae or foolish seedling (Carter *et al*., 2008). The disease has become a significant issue in India's key rice-growing regions of North Indian states, particularly with regard to aromatic rice varieties (Bashyal *et al*., 2014; Gupta *et al*., 2014). Bakanae disease has gotten progressively worse as hybrid rice cultivation has expanded and new seedling-raising techniques have been used, particularly a greater use of dry seeding for hybrid rice (Yang *et al*., 2003). Plant pathologists formerly believed that *Fusarium moniliforme* was the only species to blame for this disease based on its morphological characteristics. But earlier research suggested that three *Fusarium* spp. recovered from seeds were connected to it in India (Bashyal and Aggarwal 2013). There are limitations on the use of morphological features for the identification of species since (*F. proliferatum* and *F. fujikuroi*, *F. verticillioides* and *F. andiyazi*) exhibit very similar morphologies (Rahjoo *et al*., 2008). Morphological identification of *Fusarium* spp. is ambiguous under light microscopy where diagnosis relies solely upon morphology

Numerous studies have demonstrated that molecular methods can enhance morphological diagnosis by providing a quick and accurate test for the routine confirmation of *Fusarium* spp. (Hafez *et al*., 2020). Some researchers have used species-specific PCR assays to identify *Fusarium* species such as *F. proliferatum, F. subglutinans*, and *F. verticillioides* (Mulè *et al*., 2004). The second biggest subunit of RNA polymerase II and the translation elongation factor 1-alpha genes are two additional frequently utilised PCR methods for *Fusarium* identification (Šiši *et al.,* 2018). The GenBank and FUSARIUM-ID databases both contain the sequences for the TEF 1-alpha gene, which is widely utilised for identification purpose (Geiser *et al*., 2004). For *Fusarium* isolates, identification solely based on morphological and cultural characteristics is unreliable. In view of the seed borne nature of the

pathogen and complexity of morphological identification, there is a need for developing a rapid detection assay for management of bakanae. Hence keeping all this in view, current investigation was carried out to assess the efficiency of PCR analysis of the Tef -1 gene for identification of different isolates.

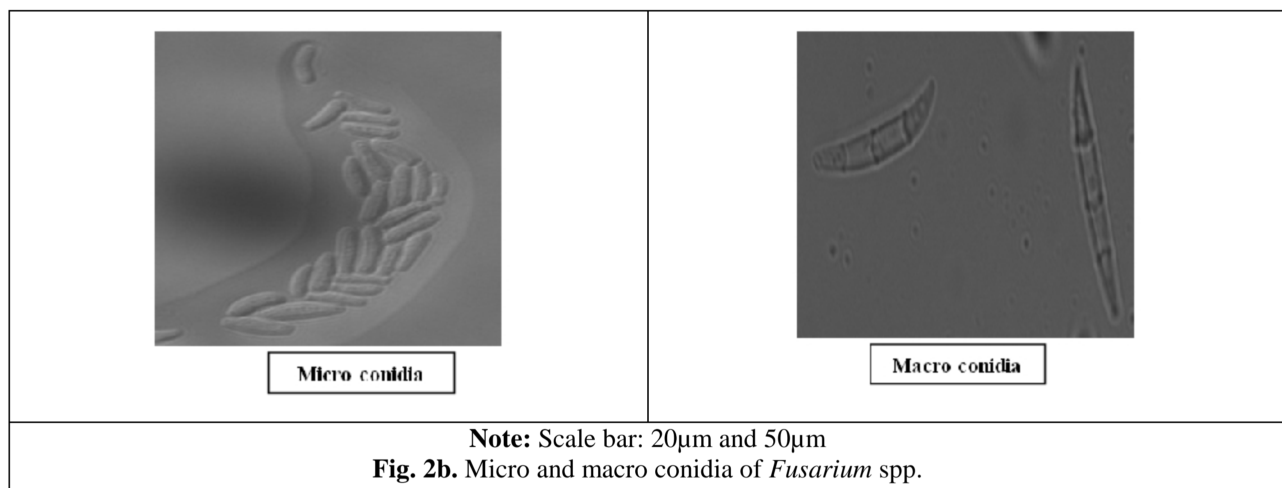
Fig. 1. Symptoms of bakanae disease in rice field.

MATERIAL AND METHODS

Bakanae infected plant samples (66) from commonally grown aromatic rice cultivars *viz*., PB 1121, PB 1401, PB 1718, PB 1509 and Basmati 521, were collected from Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra districts of Haryana during *Kharif* season. From these samples, finally twenty six different isolates of *Fusarium* spp. were selected and maintained for this study.

Isolation, purification and maintenance. The infected samples were excised, cut into pieces around 3 and 4 millimeters and surface sterilized with $MgCl_2$ (0.1 %). Under completely sterile and aseptic conditions, the excised pieces were distributed evenly over potato dextrose agar media in each petri dish. In a BOD incubator, plates were incubated at 25±2°C. Purified cultures were obtained, and the related fungus was identified through microscopic examination. *Fusarium* spp. isolates were purified using the single spore culture method and replicated on potato dextrose agar for additional research.

Fig. 2a. Pure culture of pathogen.



Note: Scale bar: 20µm and 50µm

Fig. 2b. Micro and macro conidia of *Fusarium* spp.

Table 1: Isolates collected from different locations of Haryana.

Sr. No.	Isolates id	Locations (Districts)	Variety grown
1.	FM 3	Dhad (Hisar)	PB 1121
2.	FM 7	Gurana (Hisar)	PB 1121
3.	FM 10	Kheri jalab (Hisar)	PB 1121
4.	FM 12	Intal Khurd (Jind)	PB 1121
5.	FM 16	Ikkas (Jind)	PB 1121
6.	FM 18	Saniana 1 (Fatehabad)	PB 1121
7.	FM 20	Saniana 2 (Fatehabad)	PB 1401
8.	FM 25	Pirthala 1 (Fatehabad)	PB 1121
9.	FM 28	Pirthala 2 (Fatehabad)	PB 1121
10.	FM 31	Kungar (Bhiwani)	PB 1509
11.	FM 34	Alakhpura (Bhiwani)	PB 1121
12.	FM 36	Barsi (Bhiwani)	PB 1121
13.	FM 37	Patli dabar (Sirsa)	PB 1401
14.	FM 40	Mochi wali (Sirsa)	PB 1121
15.	FM 44	Bajekan (Sirsa)	PB 1121
16.	FM 50	Naiwala (Sirsa)	PB 1718
17.	FM 51	Bapoli (Panipat)	PB 1509
18.	FM 52	Panipat 1	PB 1718
19.	FM 53	Panipat 2	PB 1718
20.	FM 56	Sonipat	PB 1121
21.	FM 59	Tarawari (Karnal)	PB 1121
22.	FM 60	Sikri (Karnal)	PB 1718
23.	FM 62	Kartarpur (Yamunanagar)	PB 1509
24.	FM 63	Sandhala (Yamunanagar)	PB 1121
25.	FM 64	Kaithal	Basmati 521
26.	FM 66	Babain (Kurukshetra)	PB 1509

DNA extraction. Twenty six pure cultures of *F. moniliforme* were used to extract genomic DNA according to the mini-prep Cetyl Trimethyl Ammonium Bromide technique (Murray and Thompon 1980). The fungal mycelium was in liquid nitrogen using sterilized pestle and mortar. The resulting powder was gathered in two ml centrifuge tubes. DNA extraction buffer of 800 µl containing 1% mercaptoethanol was mixed to the powder and and stored at 65°C for 1 h with occasional stirring. After each fifteen minutes, the tubes were gently turned over to mix up the contents. Each tube received an equal quantity of chloroform: isoamyl alcohol (24:1) after incubation. The samples were cooled, and the tubes were shaken at 70 rpm for 30 to 45 minutes to ensure appropriate mixing and centrifuged at 10,000 rpm for fifteen min. The supernatant was gathered in new 1.5 ml centrifuge tubes, and every tube undergone RNAse treatment by being added 10 µl of RNAse and then being incubated at 37°C for 30 min. It was then followed by the addition of 800µl of cold isopropyl alcohol, mild inversions, and a 15-minute incubation period at 4°C and centrifuged at 10,000 rpm for ten min. The supernatant was discarded and pellet was washed with seventy percent ethanol. After that it was air dried and dissolved in 50 µl of (Tris EDTA) buffer. Each one of the isolates' DNA was kept at -20°C to be used later.

PCR amplification. Tef-1 alpha genes of isolates were amplified using primer sets (EF 1 and EF 2) as forward and reverse, respectively described previously (Bashyal *et al.,* 2015). Gene amplification was carried out in a total volume of 25 µl with every tube containing 12.5 µl of the master mix (Promega corporation, USA) 7.5 µl water, 1.5 µl each primers (EF 1-5'- ATGGGTAAGGAAGACAEAGAC-3', EF 2-5'-GGAGGTACCAGTGATCATGTT-3') and 2 µl DNA of each isolates. Primers were synthesized by IDT (USA). The PCR was performed as follows: pre-incubation at 94ºC for 4 min, followed by amplification for 35 cycles, including denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 1 min and a final extension step of 7 min at 72ºC. Amplicons were observed by electrophoresis on 2% agarose gels using the EtBr dye and documented with a (Bio-Rad, Philadelphia, PA, USA) gel documentation system.

RESULTS AND DISCUSSION

A total of twenty six strains of *Fusarium* spp. were recovered from bakanae-infected rice samples collected from different districts of Haryana from popular basmati rice varieties. Tef-1 alpha genes of all isolates were successfully amplified with primer sets (EF1 and EF 2) as forward and reverse, respectively.

Table 2: Details of *Fusarium* Isolates collected from different locations of Haryana for positive/negative of Tef-1 alpha gene.

Sr. No.	Isolates id	Isolates positive/negative
1.	FM 3	-
2.	FM 7	+
3.	FM 10	+
4.	FM 12	+
5.	FM 16	+
6.	FM 18	+
7.	FM 20	+
8.	FM 25	+
9.	FM 28	+
10.	FM 31	-
11.	FM 34	+
12.	FM 36	+
13.	FM 37	+
14.	FM 40	+
15.	FM 44	+
16.	FM 50	+
17.	FM 51	-
18.	FM 52	+
19.	FM 53	+
20.	FM 56	+
21.	FM 59	+
22.	FM 60	+
23.	FM 62	-
24.	FM 63	+
25.	FM 64	+
26.	FM 66	+

Note: Isolates positive for Tef -1alpha gene shown by symbol (+) and negative by symbol (-)

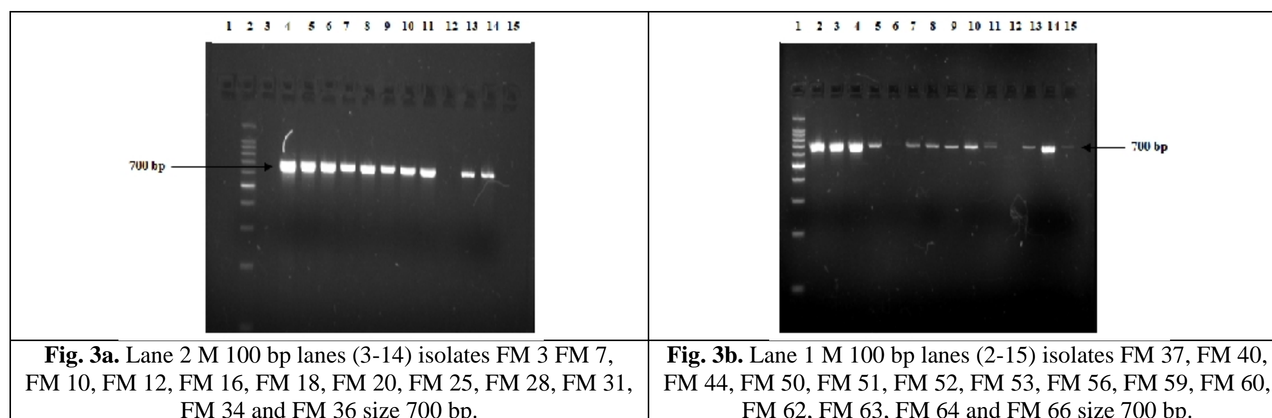


Fig. 3a. Lane 2 M 100 bp lanes (3-14) isolates FM 3 FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 size 700 bp.

Fig. 3b. Lane 1 M 100 bp lanes (2-15) isolates FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 size 700 bp.

A PCR product of size approximately 700 bp was observed for primers (EF1 and EF 2) in Fig. (3a) lane 2 M 100 bp lanes (3-14) isolates numbers as FM 3, FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 (Table 2) and in Fig. (3b) lane 1 M 100 bp lanes (2-15) isolates FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 (Table 2) in sequence. Twenty two isolates were showing positive results and four isolates (FM 3, FM 31, FM 51 and FM 62) were found negative.

Size approximately 700 bp product related to previous study (Bashyal *et al.,* 2015) was observed. Currently, internal transcribed spacer regions in the ribosomal repeat region and translation elongation factor 1-alpha gene sequencing are commonly used to characterise *Fusarium* spp (Bashyal and Aggarwal 2013). In the main paddy areas of Italy, 144 isolates of the fungus *Fusarium* spp. were taken from rice plants and seeds that had the bakanae infections. Examination of the translation elongation factor (TEF-1a) sequence allowed for the identification of these isolates (Amatulli *et al*., 2010). Similarly EF 1 and EF 2 primers were used for identification of pathogen associated with bakanae disease of rice (Bag *et al.,* 2022).

Bashyal *et al.* (2015) isolated 126 strains of *Fusarium* spp. from symptomatic plants and visually evaluated all of them, and then used translation elongation factor 1 (TEF-1) to identify 42 of them and of these 41 were found to be *Fusarium fujikuroi.*

Similarly Raghu *et al.* (2018) generated approx 700 bp bands by tef-1 alpha gene. Two major categories were created overall based on the tef-1alpha gene region. Laila *et al*., 2020 identified seventeen isolates from the

G. fujikuroi species complex as *F. proliferatum*, *F. verticillioides*, and *F. andiyazi* based on morphological characteristics and TEF 1-alpha gene sequence.

CONCLUSION

Fusarium isolates are difficult to identify and separate using traditional methods, such as morphological techniques. Therefore, to overcome the drawbacks of conventional procedures, fast molecular techniques like PCR have been developed. *Fusarium* species have been more easily identified using differences in gene nucleotide sequences (Bashyal *et al*., 2015). In current study the Tef-1 genes of 22 isolates were successfully amplified, a PCR product of approximately 700 base pairs was observed, remaining four isolates showed no amplification. Hence, to avoid the shortcomings of cultural and morphological methods in the identification of *Fusarium* species linked to the bakanae disease of basmati rice, it is therefore advised that the Tef-1 gene must be assessed.

FUTURE SCOPE

Tef-1 gene is a crucial tool for identifying *Fusarium spp*. associated to rice bakanae disease, which is challenging to identify using conventional approaches like morphological and cultural studies. It can be a significant substitute for a species-specific primer in the case of *Fusarium* spp. for identification purposes.

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Conflict of Interest. No.

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