Environment and Ecology 41 (1B) : 513—518, January–March 2023
ISSN 0970-0420

Comparative Assessment of Different Isolates of *Fusarium moniliforme* Sheldon Causing Bakanae Disease of Aromatic Rice using Beta-Tubulin Gene by PCR Analysis

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Received 1 November 2022, Accepted 18 January 2023, Published on 6 March 2023

ABSTRACT

Bakanae disease of basmati rice is known for causing huge economic losses in all the basmati growing areas every year. It is caused by *Fusarium moniliforme* (syn. *F. verticilloides*) and has become a serious bottleneck in the successful cultivation of basmati rice in Haryana and its adjoining areas. Differentiation based on cultural and morphological features only is not reliable for differentiating *Fusarium moniliforme* isolates so this study was carried out to evaluate the efficiency of PCR analysis of the beta (β)-tubulin region. A total of 26 strains of Fusarium spp. were studied from bakanae-infected rice samples collected from different districts of Haryana viz., Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra. Samples were collected from different basmati rice varieties namely PB 1121, PB 1401, PB 1509 and Basmati 521. Twenty pure cultures of pathogen were used to isolate DNA using the mini-prep Cetyl Trimethyl Ammonium Bromide (CTAB) technique. The β-tubulin genes of all isolates were successfully amplified with primer pairs (Bt2a and Bt2b) as forward and reverse, respectively. A PCR product of approximately 360 base pairs was observed for 20 *Fusarium* isolates, 6 isolates did not showed any amplification for (β)-tubulin region. Hence, it is recommended that other genes must be evaluated to overcome the limitations of the β-tubulin gene in differentiating the *Fusarium moniliforme* species.

Keywords Rice, *Fusarium moniliforme,* PCR, β-tubulin, Bakanae disease.

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INTRODUCTION

The bakanae disease caused by *Fusarium fujikuroi* Nirenberg [*F. moniliforme* (Sheld.) sexual stage: *Gibberella fujikuroi* Sawada, Wollenweber] of aromatic rice is well known for generating significant economic losses in all basmati growing regions every year and has turned into a significant barrier to the successful production of basmati rice in Haryana and

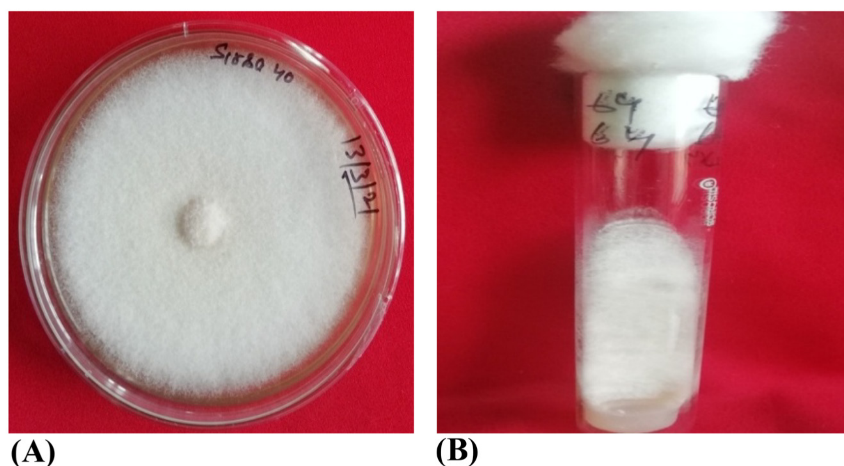
its neighboring areas. The Pusa Basmati 1121 and 1509 types, which are produced all over the country but primarily in Punjab and Haryana, are known for their outstanding grain quality, and as a result, the disease prevalence is very high. Other well-known cultivars, such as Pusa Basmati 6, CSR 30, Pusa Basmati 2511 and sarbati, are also affected by this disease in northern India, which covers Punjab and Haryana. Eastern Uttar Pradesh, Haryana, and Punjab have all reported yield losses of between 15 and 25% and it has been getting worse over time (Pannu *et al.* 2012). At the moment, it is unknown how to distinguish the species connected to the many symptoms, such as morphology (Wulff *et al.* 2010). The disease was formerly referred to by the generic name *F. moniliforme* (Ou 1985), however the taxon really consists of a number of distinct species, and is now known as the *Gibberella fujikuroi* species complex. There is an association between three mating populations of the *G. fujikuroi* complex and the rice bakanae disease. The distinguishing morphological characteristics of Bakanae include slender, chlorotic, and elongated primary leaves (Fig. 1), which illustrate the pathogen's production of gibberellins (Amoah *et al.* 1995).

Molecular approaches based on DNA analysis have been extensively employed to differentiate between isolates within a species as well as to develop quick, sensitive, and accurate detection methodologies. Molecular fingerprinting, which encompasses the techniques of randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP), may be a useful method for evaluating genetic variants with high levels of visible polymorphism (Yin *et al.* 2014). *Fusarium* species were also identified using the histone H3 (H3) and β -tubulin genes (Young-Ah *et al.* 2013). Nosratabadi *et al.* (2018) used the beta-tubulin gene in addition to PCR-RFLP analysis to differentiate between various *Fusarium* species. β-tubulin gene encodes for the structural proteins of microtubules and other structural components in eukaryotes. Differentiation based on cultural and morphological features only is not reliable for differentiating *Fusarium moniliforme* isolates so in current study beta(β)-tubulin gene was evaluate to identify and differentiate *Fusarium*

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

Table 1. Isolates collected from different locations of Haryana.

S.l 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



Figs. 2 (A, B). Pure cultures of *Fusarium moniliforme.*

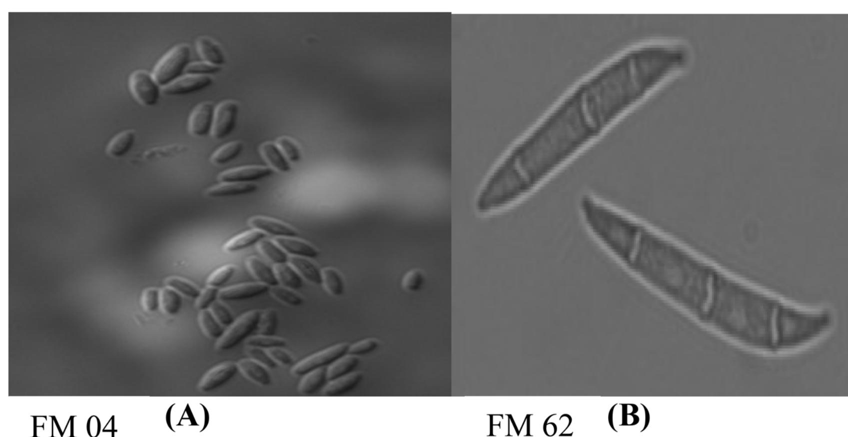
moniliforme isolates by PCR analysis.

MATERIALS AND METHODS

Fungal isolates collection - A total of 26 strains of *Fusarium* spp. were studied from bakanae-infected rice samples collected from different districts of Haryana viz., Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra. Samples were collected from different basmati rice varieties namely PB 1121, PB 1401, PB 1718, PB 1509 and Basmati 521(Table 1).

Isolation, purification and maintenance

The infected samples were excised, cut into pieces around 3 and 4 mm in size, and surface sterilized with mercuric chloride (0.1 percent). 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 (Figs. 2 A, B) and the related fungus was identified through microscopic examination (Fig. 3 A, B). *Fusarium* spp. isolates were purified using the single spore culture method and replicated on PDA



Figs. 3 (A, B). Micro (FM 04)-Macro (FM 62) conidia of the pathogen.

for additional research.

DNA extraction

Genomic DNA was extracted from 26 pure cultures of *Fusarium moniliforme* by using mini-prep Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompon 1980). Using a sterile pestle and mortar and liquid nitrogen, the fungus mat was ground into a fine powder. In 2 ml centrifuge tubes, the powder that was so obtained was collected. The powder was mixed with 800 µl of CTAB buffer containing 1% mercaptoethanol, and the tubes were incubated in a water bath at 65 °C for 1 hr. After every 15 minutes, the tubes were turned upside down to gently mix the contents. Immediately following incubation, 800 µl of chloroform: Isoamyl alcohol (24:1) were added.The samples were cooled to room temperature, and the tubes were shaken at 70 rpm for 30 to 45 minutes to ensure good mixing. After shaking, the samples were centrifuged in a micro-centrifuge at 10,000 rpm for 15 min. Using a pipette, the supernatant was extracted from the tissue debris and placed in new 1.5 ml centrifuge tubes. The supernatant was then given the RNAse treatment by adding 10 µl of RNAse to each tube, which was then incubated at 37°C for 30 min. Following the addition of 800 µl of cold isopropyl alcohol, moderate inversions were performed. Following this, tubes were incubated for 15 minutes at 4°C. Once more, the tubes were centrifuged for 10 minutes at 10,000 rpm. The pellet was rinsed with 70% ethanol after the supernatant was discarded. The pellet was then dried by air drying before being dissolved in 50 µl of Tris-EDTA buffer. All of the isolates' DNA was kept in storage at -20°C until usage.

PCR amplification

The β-tubulin genes of isolates were amplified with primer pairs (Bt2a and Bt2b) as forward and reverse, respectively described previously (Young-Ah *et al.* 2013). Gene amplification was performed in a total volume of 25 µl with each tube containing 12.5 µl of the master mix,7.5 µl water,1.5 µl each primers (Bt2a-5'- GGTAACCAAATCGGTGCT-GCTTTC-3',Bt2b-5'-ACCCTCAGTGTAGTGAC-CCTTGGC-3'forward and reverse respectively) and 2 ul DNA of each isolates. The primers were synthesized by IDT (USA). The reagents were purchased from Promega corporation (USA). The PCR conditions used included pre-incubation at 94^{0}C for 4 mins, followed by amplification for 35 cycles, including denaturation at 94^{0}C for 1 min, annealing at 60^{0}C for 1 min, extension at 72^{0}C for 1 min and a final extension step of 7 mins at 72^{0}C. Amplicons were visualized by electrophoresis on 2% agarose gels using the EtBR stain and documented with a (Bio-Rad, Philadelphia, PA, USA) gel documentation system.

RESULTS AND DISCUSSION

Fusarium moniliforme genomic DNA was isolated using the mini-prep Cetyl Trimethyl Ammonium Bromide (CTAB) method from 26 pure cultures. All isolates' β-tubulin genes were amplified effectively using the primer pairs Bt2a and Bt2b as forward

Table 2. Details of *Fusarium* isolates collected from different locations of Haryana for positive/negative of beta-tubulin gene.

Sl 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 beta-tubulin gene shown by symbol (+) and negative by symbol (-).

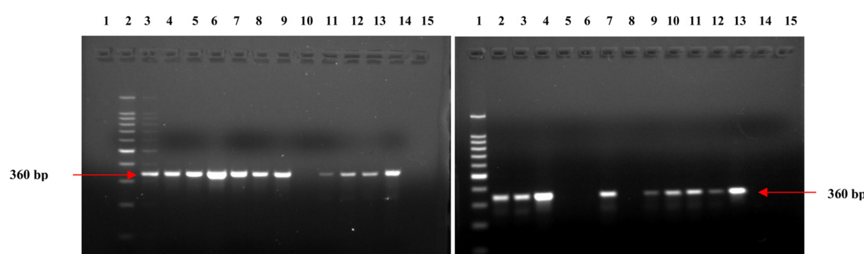


Fig. (4a) lane 2 M 100 bp lanes (3-14) isolates FM 3 FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 size 360 bp

Fig. (4b) 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 360 bp

Figs. (4a), **(4b).** Showing positive/negative of beta-tubulin gene for different isolates.

and reverse, respectively. For 20 *Fusarium* isolates a PCR result of about 360 base pairs was seen. Six isolates were unable to show any β - tubulin region amplification.

β-tubulin PCR products Fig. (4a) lane 2 M 100 bp lanes (3-14) isolates FM 3 to FM 36 (Table 2) , Fig. (4b) lane 1 M 100bp lanes (2-15) isolates FM 37 to FM 66 (Table 2) in sequence. Size approximately 360 bp related to previous study (Young-Ah *et al.* 2013). *Fusarium* spp. were commonly characterized at species level by translation elongation factor 1-α (TEF) gene sequencing (Wulff *et al.* 2010), internally transcribed spacer regions in the ribosomal repeat region (ITS1 and ITS2) and β-tubulin (tub2) (Bashyal and Aggarwal 2013). From rice plants and seeds infected with the bakanae disease in the main rice-growing regions of Italy, 144 isolates of the fungus *Fusarium* spp. were collected. An analysis of the translation elongation factor (EF-1a) sequence allowed for the identification of these isolates (Amatulli *et al.* 2010).

19 polymorphic SSRs were found by (Valente *et al*. 2017) while analyzing the genetic diversity of 334 isolates of *F. fujikuroi* from eight distinct rice-growing regions in Italy. Eight populations were scored with 107 unique haplotypes, showing a sizable amount of haplotype-level diversity. Analysis of molecular variance (AMOVA) found that 98% of genetic variation occurred among *F. fujikuroi* Italian populations, as shown by the allelic Shannon index, which varies from 0.56 to 1.06. A 1:1 ratio of mating type alleles is present in six of the eight Italian fungal populations, which raises the potential of sexual reproduction in the wild.

Additionally, various techniques have been employed to differentiate the individuals that make up the *G. fujikuroi* species complex, such as RAPD DNAs (Amoah *et al.* 1995), Amplified Fragment Length Polymorphism (AFLPs) (Petrovic *et al.* 2013), and CHEF gel karyotypes. Using RAPD, AFLP, and RFLP (Restriction Fragment Length Polymorphism) analyses, the molecular characterization of *F. verticillioides* has been completed (Patino *et al.* 2004).

CONCLUSION

It is challenging to identify and differeciate *Fusarium* isolates using conventional approaches such as morphological methods. As a result, quick molecular techniques like PCR have been developed to overcome the drawbacks of conventional techniques. For differentiation and evaluation of the genetic relationship among closely related *Fusarium* species, analysis of DNA sequences also have been performed in previous studies. In current study the β-tubulin genes of 20 isolates were successfully amplified, a PCR product of approximately 360 base pairs was observed, remaining six isolates showed no amplification. Therefore, it is advised that additional genes be investigated in order to overcome the β-tubulin gene's limitations in distinguishing the *Fusarium moniliforme* species.

ACKNOWLEDGMENT

For providing the resources needed to carry out these experiments, we are grateful to Professor and Head

Department of Plant Pathology, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

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