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National Academy of Agricultural Science (NAAS) Rating : 3.03

Molecular, Biochemical and Pathogenicity Analysis of *Fusarium fujikuroi* Isolates Collected From *Bakanae* Disease of Indica Rice

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ABSTRACT: *Fusarium fujikuroi Niremberg (Gibberella fujikuroi [Sawada] Ito in Ito & K.Kimura] is an important rice pathogen and causes Bakanae disease in rice. It is the most abundant species isolated from Indica rice field and characterized with regard to morphological, biochemical, molecular analysis and pathogenicity test. The morphological study showed a close variation among the isolates that were distributed into three different genetic clades. The gibberelic acid, ascorbic acid and indole-3-acetic acid content showed close resemblance between the isolate 2 and 3. On the basis of pathogenicity test, the seed germination was reduced and possessed varying ability to cause symptoms of Bakanae disease by using three isolates. The affected plants had tall lanky tillers with longer internodes and aerial adventitious roots that were developed from the nodes of above ground level. The stem and leaves became yellowish and the stalks exhibit a reddish discoloration. A total of 116 fragments were scored that varied from 05 to 12 bands with an average of 7.73 bands per primer. The polymorphic bands ranged from 04 to 11 with an average of 5.86 bands per primer. All the isolates produce an approximately 700 bp DNA fragment in all the primer tested. Pair wise values of similarity coefficient ranged from a minimum of 0.42 between 'IS-3' and 'IS-1 to a maximum of 0.66 between 'IS-3' and 'IS-2'. The dendrogram was constructed based on the similarity matrix using UPGMA method and it indicates that the three isolates divided them into two clusters at 53% similarity. One group having only one isolate 'IS-1' and the other having the remaining two isolates i.e. 'IS-2', and 'IS-3'. This study will help the valuable genetic tools for analyzing genetic diversity of Fusarium fujikuroi species complex infection in Indica rice.*

Keywords: *Fusarium fujikuroi , RAPD profiling, Phylogenetic analysis, Pathogenicity test*

INTRODUCTION

Fusarium species is an economically important plant pathogenic fungus with a wide host range. It is considered to be one of the most important genera of plant pathogens, as it contains a large number of species that are distributed worldwide and attack many economically important agricultural and horticultural Crops (Leslie and Summerell, 2006; Kvas *et al.*, 2009). *Fusarium fujikuroi* (*Gibberella fujikuroi*) is seed-borne and casual agent of bakanae diseases of rice in a number of rice growing areas (Carter *et al.*, 2008). Although bakanae disease was first described over 100 years ago in Japan, the identification of species associated with the different symptoms like morphology is still not clear (Wulff *et al.*, 2009). Initially, the pathogen is identified as *F. moniliforme* in a broad sense (Ou, 1985); however, the taxon comprises a number of distinct species, now it is called as *Gibberella fujikuroi* species complex. Three mating populations of the *G.fujikuroi* complex have been associated with bakanae- disease in rice. The typical morphological symptoms of Bakanae are slender, chlorotic and elongated primary leaves that indicate the production of gibberellins by the pathogen. (Amoah *et al.*, 1995). This disease occurs at the seedling stage which causes the loss of crop production by 40% (Ou, 1987). Some of the authors reported the *G.fujikuroi* species having three mating populations which have been associated with Bakanae disease on rice (Desjardins *et al.*, 2000; Amoah *et al.*, 1995). There is very measure information on occurrence and population structure of *G.fujikuroi* species complex associated with rice seeds and seedlings from India, Nepal, China, Japan, Ghana and Africa (Desjardins *et al.*, 2000; Amoah *et al.*, 1995; Wulff *et al.*, 2009). In some countries, the seeds are soaked in fungicides like

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carbendazim, thiram and trifumezol to control the disease. In recent years, due to the reduction in pesticide availability, the disease incidence has increased also in European countries like Italy, France and Hungary. *F.fujikuroi* species complex are able to synthesize fumonisins, beauvericin, fusaproliferin, fusaric acid and fusarin (Baird *et al.,* 2008) and other bioactive secondary metabolites that are harmful to human and animal health (Wulff *et al.*, 2009; Gelderblom *et al.*, 1988). These compounds have also contaminated grains and contribute to pathogencity in plant (Ou, 1987; Desjardins and Hohn, 1997). Molecular techniques based on DNA analysis have been widely used to identify and discriminate among isolates within species and to develop rapid, sensitive and accurate detection methods. Molecular fingerprinting including randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP), could provide an efficient tool to analyze the genetic variations with high levels of detectable polymorphism (Paterson *et al*., 1991; Yin *et al.*, 2014). As the identification of the bakanae causal agent seems to be still unclear, the objective of the present investigation was to assess the variability of different isolates of *Fusarium fujikuroi* collected from naturally infected Indica rice plants in two seasons on the basis of morphological, biochemical, pathogenicity and molecular analysis.

MATERIAL AND METHODS

The investigation was carried out at the department of Plant Pathology and department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture & technology, Bhubaneswar. Bakanae–infected rice samples were collected during the growing season for two years (2012-14) in the major rice cultivation in the districts of Baragarh, Koraput and Central farm of OUAT, Bhubaneswar of India. The morphological symptoms were recorded before collection of stem from the diseased plants of different varieties of rice.

COLLECTION OF SAMPLES

The infected stem pieces (1 – 2 cm long) were brought to the laboratory and surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride, rinsed twice with sterile distilled water and cultured in the Potato Dextrose Agar (PDA) medium. The cultures were incubated at 28±2 °C for seven days under UV light. The fungus was isolated and identified through the reference of Booth (1977) and Nelson *et al.* (1983). The preliminary identification of the strains was based on habit and morphological characters of the fruiting bodies and spores/conidia Further, the fungus were subcultured once again on PDA before production of a single spore.

MAINTENANCE OF SINGLE SPORE CULTURES

Fungal culture (two-week-old) growing on PDA was flooded with 15 ml sterile water and gently scraped with a flamed steel spatula. The obtained inoculums suspension was filtered through a three layer sterilized gaze and 500 µl of the suspension was used for serial dilution. Two hundred microliters of each dilution was placed on petridishes with low nutrient agar and incubated at room temperature. Single spores with one to three germ hyphae were examined everyday under a stereo microscope (Nikon, Japan). Individual germlings were excised from the nutrient plates with a sterile needle and transferred to a new PDA plate. The plates were incubated under UV light at room temperature (28±2 °C) for one week. Suspension of single spore cultures were prepared with sterile water and kept at -80 °C for further use.

EXTRACTION OF TOTAL GENOMIC DNA

One ml of the single spore culture suspension of each isolates was inoculated with 50 ml of sterile potato dextrose broth (PDB) and incubated under agitation 100 rpm at room temperature for 4 days. The fungal cultures were then taken into a 15 ml centrifuge tube and centrifuged with 5000 rpm for 10 minutes. The supernatant was removed and the pellets were washed with sterile distilled water for two times and transferred to 2 ml Eppendrof tubes and centrifuged at 1000 rpm for 10 minutes. The pellets were lyophilized overnight (Wulff *et al.*, 2009). The lyophilized mycelium was then ground into a fine powder in liquid nitrogen using sterile pestle and mortar. DNA was extracted from each sample using the CTAB method (Doyle and Doyle, 1990). An aliquot of 2 µl of the preparation was checked on 1.0% (w/v) agarose gel for estimating the amount and purity of DNA isolated comparing it against a known weight of uncut lambda DNA.

RAPD Analysis of *Fusarium fujikuroi* Isolates

Fourty customs synthesized (M/s EMerk Bioscience, India) RAPD primers were chosen for the study of phylogenetic analysis and fingerprinting. Polymerase chain reactions (PCR) with single primer was carried out in a final volume of 25 ìl containing 20 ng template DNA, 100 µM of each deoxyribonucleotide

triphosphate, 20 ng of primer, 1.5 mM $MgCl_2$, 1x *Taq* buffer (10 mM Tris-HCl [pH- 9.0], 50 mM KCl, 0.01% gelatin), and 0.5 U *Taq DNA polymerase* (M/S EMerk Bioscience, India). Amplification was performed in a thermal cycler (Peqlab Deutschland & Osterreich, UK) programmed for a preliminary 5 min denaturation step at 94 °C, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at required temperature (depending on nucleotide sequence of RAPD primer) for 30 s and extension at 72 °C for 1 min, final extension at 72 °C for 10 min. The separation of amplified DNA fragments was performed by electrophoresis in 1.5% (w/v) agarose gel along with a molecular weight marker (1.0 kb plus ladder, M/S EMerk Bioscience, India), pre stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through a Gel Documentation System (UVITECH, UK) and the amplification product sizes were evaluated using the software Quantity One (BioRad, California, USA). All the PCR reactions were repeated twice. The PCR amplification products were scored visually for presence (1) or absence (0) of band between samples and transcribed into binary format. The data was used to calculate Jaccard's coefficient of similarity. The similarity measures were subjected to unweighted pair group method for arithmetic averages (UPGMA) and plotted in a phenogram using NTSYS-pc Ver. 2.1 (Rolhf, 2002).

Pathogenicity Tests

Pathogenicity tests were conducted in seed inoculation assays. One hundred seeds of variety "Lalat' were taken and surface sterilized with 70% ethanol for 2 min and subsequently 15 min. for 0.1% (w/v) mercuric chloride. Further, the seeds were washed with sterile distilled water three times. Seeds were then left to dry in the Laminar-Air-Flow cabinet in petridishes containing sterile filter paper. Fungal inoculums suspension was prepared from the single spore cultures. The concentration of the inoculums suspension was determined using a haemocytometer and diluted to 1 $x10^5$ spores per ml with sterile water. Two hundred seeds were soaked in 15 ml of inoculums suspension for overnight at room temperature. Control seeds were soaked in sterile distilled water. Both inoculated and control seeds were sown into 10" earthen pots (Three pots per isolate/ twenty seeds per pot). Each pot contained 5 kg of autoclaved mixure of soil and sand (3:1). The watering was taken place in every one day interval. The pots were covered with white polyethylene and kept in the polyhouse at 28 ± 2 °C. Seven days after inoculation, the number of germinated seeds was assessed. The seedlings were observed for symptoms of Bakanae disease after 30, 45 and 60 days of inoculation.

Extraction & Determination of Gibberelic Acid

One ml of the single spore culture suspension of each isolates was inoculated with 50 ml of sterile potato dextrose broth (PDB) and incubated under agitation 100 rpm at room temperature for 7 days. Broth samples were centrifuged to remove biomass. An aliquot was transfered to a 100 ml separating flask. Fifteen ml of an aliquot were mixed with 2 ml of zinc acetate and kept for 2 minutes. Thereafter, 2 ml of potassium ferrocyanide was added and centrifuged at 8000 rpm for 15 minutes. The supernatant was collected and kept in a separate flask. Five ml of supernatant was mixed with 5 ml of 30% hydrochloric acid and incubated at 20 °C for 1.5 hours. The absorbance of the solution was measured at 254 nm. A calibration curve was made by using standard GA_3 solutions prepared by dissolving 40 mg GA_3 in absolute alcohol and diluted to 100 ml with absolute alcohol (Berrios *et al.*, 2004). Each experiment was repeated twice with three replications.

Estimation of indole-3-acetic Acid (IAA)

One ml single spore culture suspension of each isolates was inoculated with 50 ml of sterile potato dextrose broth (PDB) and incubated under agitation 100 rpm at room temperature for 7 days. Broth samples were centrifuged to remove biomass. One ml of fungal extract (supernatant) was taken and added to 2 ml of sulphur reagent drop wise with continuous agitation. The reaction mixture was incubated in the dark for 35 minutes till the pink colour development. The absorbance of the solution was measured at 535 nm against the solvent reagent as a blank. If the colour density became deep, the reaction mixture is to be diluted with the solvent. A calibration curve was then obtained by using standard IAA (Sigma, USA) solutions prepared by dissolving 50 mg IAA in absolute alcohol and diluted to 100 ml with distilled water. Each experiment was repeated twice with three replications.

Estimation of Ascorbic Acid

One ml of the single spore culture suspension of each isolate was inoculated with 50 ml of sterile potato dextrose broth (PDB) and incubated under agitation 100 rpm at room temperature for 7 days. Broth

samples were centrifuged to remove biomass. The 20 ml aliquot were mixed with 20 ml of 4% oxalic acid and centrifuged at 8000 rpm for 10 minutes. Transfer the 10 ml of aliquot to a conical flask and added bromine water drop wise with a constant mixing till the aliquot turn orange-yellow colouration. Then after, take 2 ml of brominated sample extract and make up the volume up to 5 mil of adding distilled water. Add 1 ml of 2% 2,4-dinitrophenylhydrazine (DNPH) followed by 1 -2 drops of 10% thiourea and incubate at 37 °C for 3 hours. After incubation, 7 ml of 80% of sulphuric acid was added and mixed thoroughly. The absorbance of the solution was measured at 540 nm against the solvent reagent as a blank. A calibration graph was then obtained by using standard ascorbic acid (Sigma, USA) solutions prepared by dissolving 50 mg in 100 ml of distilled water. Each experiment was repeated twice with three replications.

OBSERVATION & STATISTICAL ANALYSIS

Each experiment was repeated at least twice. The average data pertaining the mean percentage of showing response were evaluated statistical**ly** by the Post-Hoc Multiple Comparison test **(**Marascuilo and McSweeney**,** 1977). Among the treatments, the average figures followed by the same letters were not significantly different at $P < 0.05$ level.

RESULTS AND DISCUSSION

F. fujikuroi species complex is an important rice pathogen observed in all the rice production area in the world. Some of the members of the *G.fujikuroi* species complex have also been found associated with Bakanae disease. Fungal contamination of rice seed samples with *F.fujikuroi* causes a problem for farmers in the developing countries. The early and correct identification of this species is therefore very important for the rice industry to certify the disease free seeds. In the present study, out of twenty five isolates collected from different geographical regions, three isolates of *Fusarium fujikuroi* collected from different rice field of Odisha, India showed Bakanae diseases in Indica rice varieties. These isolates were characterized morphologically based on the mycelium growth and presented in Table 1. At the initial stage, the isolates collected from high altitudes were whitish and central pink colour and subsequently it became milky white and white with purple tinge. The isolate collected from coastal belt was initially milky white with central yellow colour and subsequently whitish with yellow tinge floppy growth (Table 1). Under microscopic examination of the colonies, differential diagnosis was initiated with the observation of oval microconidia with one or two septa and phialide structures exhibiting polyblastic features, with transparent hyphal structures and macroconidia having a needle like appearance, slightly bent two to five celled and lightly curved in places, conidiaferous structures and a large number of transparent chlamydospores were present and formed singly or in pairs. The bakanae disease symptoms and growth of the mycelium were observed in the infected plants typically wilt, leaves turn grayish-green in coloration. The mycelium growth was observed at lower stalk nodes. The root symptom is fibrous and bushy (Figure 1). Similar observation was obtained by various authors (Mathur and Manandhar, 2003; Wulff *et al*., 2009). DNA markers have been used successfully to evaluate the degree of relatedness among the group of accessions to identify the genetic structure or variation among isolates, populations and species. Fourty randomly amplified polymorphic DNA (RAPD) primers were tested to detect the genetic diversity among the *F.fujikuroi* isolates from different agro-climatic regions. Polymorphisms were identified by separating the PCR products using agarose gel-electrophoresis and visualizing the product by ethidium bromide staining. On the basis of number, intensity and reproducibility of banding pattern, fifteen primers were selected out of the forty primers tested in three *F.fujikuroi* isolates (Table 2). Bands with same mobility were considered as identical irrespective of their band intensity. Very weak band with negligible intensity and smear bands were excluded from the final analysis. RAPD profiles of three different isolates of *F.fujikuroi* are shown in Figure 2. A total of 116 fragments were scored that varied from 05 (OPA-09, OPC-04) to 12 (OPM-03) with an average of 7.73 bands per primer. The polymorphic bands ranged from 04 to 11 with an average of 5.86 bands per primer. All the isolates produce an approximately 700 bp DNA fragment in all the primer tested. The size of the fragment (700 bp) obtained in three isolates was similar with TEF sequences (Forward primer: 5-ATGGGTAAGGARGACAAGAC-3′) and (reverse primer:5′-GGARGTACCAGTSATCATGTT-3′) in NCBI database as reported earlier (O'Donnell *et al*., 1998; Geiser *et al*., 2004). Genetic similarity among the varieties was estimated using similarity coefficient matrix based on RAPD bands scored. Pair wise values of similarity coefficient ranged from a minimum of

Table 1
Morphological Characteristics of Three Isolates of *F.fujikuroi* Species Complex Associated with Indica Rice grown in Odisha, India

Name of the isolates	*Source of Origin*	*Cultural growth*	*Cultural growth*	*Percentage of Fusarium infection*
		At Initial stage	After 10 days	33.5%
IS-1	Bargarh, Sambalpur, Odisha	Whitish growth with central purple colour	Whitish with purple tinge	
IS-2	Bhubaneswar Central Farm, Khurda, Odisha	Milky white with central yellow colour	Whitish with yellowish tinge fluppy growth	27.5%
IS-3	Jeypore , Koraput, Odishs	Milky white with pink boarder	Milky white	21.5%

Table 2
Total Number of Amplified Fragments and Number of Polymorphic Bands Generated by PCR using Selected Random Decamers in three Isolates of *F.fujikuroi* Species Complex Associated with Indica Rice

Name of Primer	*Sequence of the primer*	*Total No. amplification products*	*No. of polymorphic products*	*Size range (kb)*
OPD-02	5′-GGACCCAACC-3′	08	06	700 - 5000
OPM-03	5′-GGGGGATGAG-3′	12	11	100- 4500
OPA- 07	5′-GAAACGGGTG-3′	06	05	500 - 3500
OPA- 09	5′-GGGTAACGCC-3′	05	04	100- 4000
OPN- 03	5′-GGTACTCCCC-3′	07	05	200 - 3000
OPN- 06	5′-GAGACGCACA-3′	08	07	250-2000
OPN-11	5′-TCGCCGCAAA-3′	11	07	100-3000
OPB-04	5′-GGACTGGAGT-3′	06	05	150-3000
OPB-16	5′-TTTGCCCGGA-3′	08	05	500 - 3500
OPC-04	5′-CCGCATCTAC-3′	05	03	700 - 3000
OPC-12	5′-TGTCATCCCC-3′	07	04	600 -3500
OPK-06	5′-CACCTTTCCC-3′	08	07	500 -4000
OPK-12	5′-TGGCCCTCAC-3′	07	05	100 - 3000
OPK-20	5′-GTGTCGCGAG-3′	10	08	200 -2500
OPK-16	5′-GAGCGTCGAA-3′	08	06	100- 3500

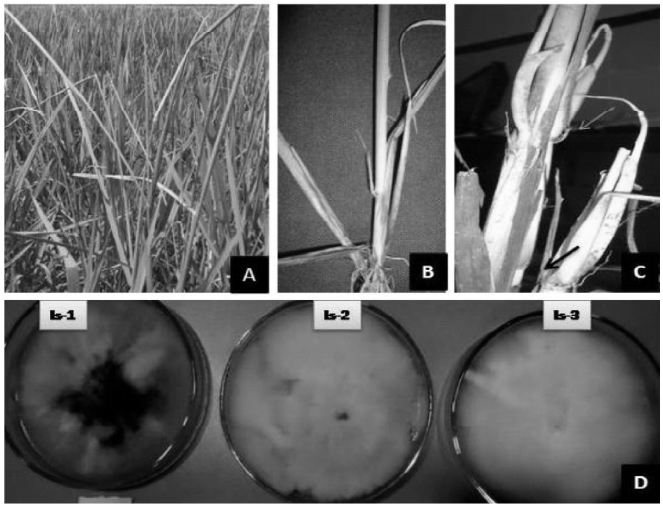

Figure 1: A. Disease occurs in the rice field. B & C. Morphological observation *F.fujikuroi* grown on rice plants at initial stage (Fig. B) and later stage (Fig. C) D. Mycelium growth development in PDA of three isolates (Is-1, Is-2, Is-3) collected from different locations

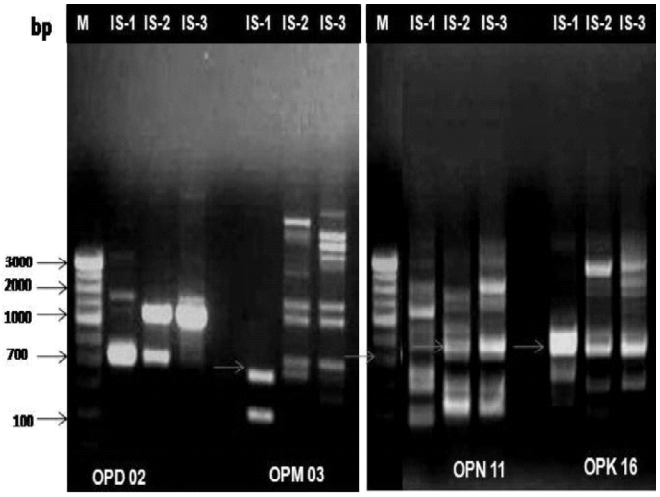

Figure 2: Amplification profile of three isolates (Is-1, Is-2 & Is-3) of *F.fujikuroi* employing RAPD primers OPD-2; OPM-3; OPN-11 and OPK-16 M: Medium range DNA ruler, Numbers on the margin represent molecular weight of DNa ruler in base pairs (bp)

0.42 between 'IS-3' and 'IS-1 to a maximum of 0.66 between 'IS-3' and 'IS-2' (Table 3). The dendrogram was constructed based on the similarity matrix using UPGMA method and it indicates that the three isolates collected from different agroclimatic regions divided into two clusters at 53% similarity. One group having only one isolate 'IS-1' and the other having the rest two isolates i.e. 'IS-2', and 'IS-3'. In the second cluster, 'IS-2' and 'IS-3' have been grouped together sharing a similarity of 66%. (Figure 3). Wulff *et al.* (2009) used TEF DNA sequences to characterize four different Fusarium species associated with Bakanae disease of rice and phylogenetic analysis. The genetic analysis of the different isolates of *Fusarium* species by using various molecular markers have been reported (Geiser *et al*., 2004; Amatulli *et al*., 2010; Wulff *et al*., 2009). Moretti *et al*. (2004) used AFLP markers to produce the genetic profile of *Fusarium verticillioides* from banana fruits. The present result indicates that the clustering pattern formed within the three isolates was similar in morphological characteristics as well as genetic analysis which causes the Bakanae disease in rice. Wulff *et al.* (2009) reported that the *Fusarium* species like *F.verticillioides*, *F.fujikuroi* and *F.proliferatum* are commonly found associated with Bakanae disease of rice. They also reported that the *F.fujikuroi* strains were only detected in seed samples originating from Asia, all the other *Fusarium* species identified were found in both Africa and Asian seed samples.

Table 3
Similarity Matrix based on RAPD Profiles of three Isolates of *F. fujikuroi* Species Complex Associated with Indica Rice (Rohlf, 2002)

	IS-1	IS-2	IS-3
IS-1	1.00		
IS-2	0.53	1.00	
IS-3	0.42	0.66	1.00

IS-1 : Isolate -1, IS-2 : Isolate -2: IS-3 : Isolate -3

All the three *F.fujikuroi* isolates showed pathogenicity on Indica rice. The treated seeds were germinated less as compared with control treatment that was set to 100% germination . The germination of the seeds treated with a fungal isolate ranged from 63 to 72% in different isolates. The average number of germinated seed was 68.5%, 72% and 63%, respectively. Bakanae symptoms were not seen on healthy control plants. The *F.fujikuroi* isolates were ranked based on their pathogenicity and biochemical analysis. All the tested fungal isolates were able to induce leaf chlorosis. Most of the plants has chlorotic and slender leaves indicating varying pathogenic potentiality which are confirming the observations of Ou, (1985) and Zainudin et al (2008). Amoah et al (1995) reported that the pathogenicity of *G.fujikuroi* species complex on rice may depend on the balance of toxin and growth regulators. Wulff et al (2009) reported that the African and Asian populations of *Fusarium* species (*G.fujikuroi* species complex) were able to reduce seed germination and possess varying ability to cause symptoms on rice, *F.fujikuroi* being more pathogenic than other strains. On the basis of biochemical analysis, it was shown that the gibberellic acid content was 1.21 g/l in Isolate 1 & 3 and 0.5 g/l in Isolate-2, respectively (Table 4). The ascorbic acid and IAA content varied in isolates collected from rice field in different regions. The ascorbic acid and IAA content were varied from 2.3 – 3.5 mg/l and 3 .0 – 8.6 mg/l, respectively (Table 4). The gibberelic acid content was higher in isolate 1 & 3 with higher percentages of chlorotic and slender leaves in Indica rice compared with isolate-2. Similar observation has also been reported in rice (Ou, 1987). Gibberellins might represent the expression of Bakanae disease in Indica rice. Further studies are essential to clarify if the variation in aggressiveness among the isolates was caused by physiological features of the isolates or by temperature and humidity or photoperiod.

Table 4
Biochemical Analysis of three Isolates of *F.fujikuroi* Species Complex Associated with Indica Rice.

Different isolates of F.fujikuroi species complex associated with Indica rice collected from different agroclimatic regions.	*Biochemical parameter*		
	*Gibberelic acid content (gm/l) (Mean ± SE)**	*Ascorbic acid content (mg/l) (Mean ± SE)**	*Indole-3-acetic acid (mg/l) (Mean ± SE)**
Isolate -1 (IS-1)	1.21 ± 0.3 b	3.5 ± 0.3 b	3.0 ± 0.6 a
Isolate-2 (IS-2)	0.5 ± 0.03 a	2.4 ± 0.2 a	8.6 ± 0.8 c
Isolate-3 (IS-3)	1.21 ± 0.08 b	2.3 ± 0.3 a	7.3 ± 0.7 b

*Three replications; repeated twice.
Among the mean average figures followed by the same letters were not significantly different at $P < 0.05$ level.

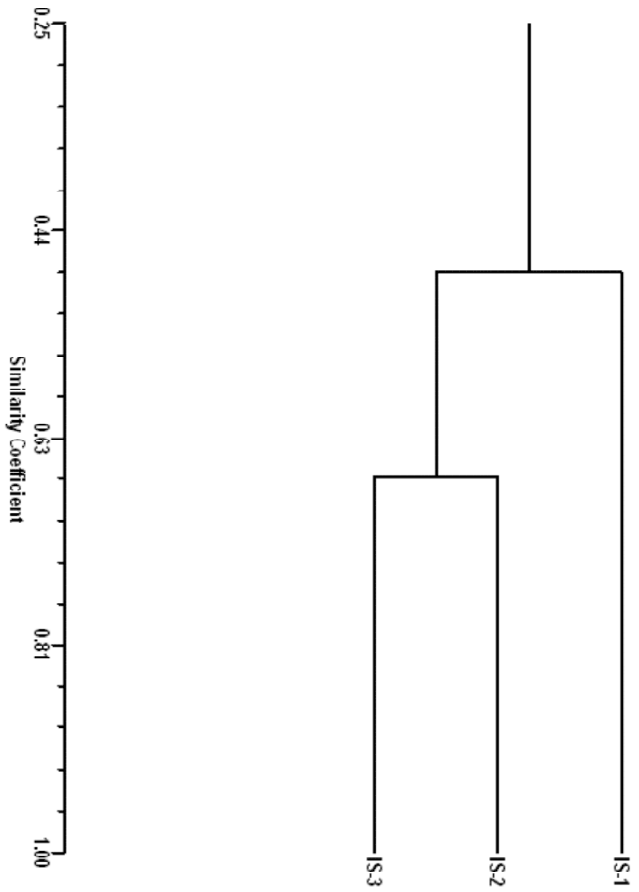

Figure 3: Dendrogram based on RAPD profiles of three isolates of *F.fujikuroi* species complex associated with Indica rice depicting genetic relationship

In conclusion, the markers developed in this study have been used successfully on isolates causing Bananae disease in Indica rice and originating from different geographical regions. Therefore, these markers provide valuable genetic tools for analyzing genetic diversity of *Fusarium fujikuroi* species complex. These markers will also enable a greater understanding of the movement of this important pathogen around the world.

ACKNOWLEDGEMENT

The authors wish to acknowledge to Professor & Head, Department of Agricultural Biotechnology, College of Agriculture for providing laboratory facility.

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