Isolation and Molecular Identification of *Fusarium spp*., associated with Pokkah boeng disease of sugarcane

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Abstract
Pokkah boeng is a fungal disease and causes significant yield losses in sugarcane growing zones of the country. The disease is caused by the Fusarium species complex, however, scientist have different opinions regarding the species involved. A rapid detection and identification of the pathogen is urgently required to manage the Pokkah boeng disease in sugarcane keeping in view the damage caused by the disease. In our experiment, twenty five isolates were recovered on the basis of morphological observations from the Pokkah boeng infected sugarcane samples. Pathogenicity assay confirms two Fusarium isolate (F2 and F7) by giving typical Pokkah boeng symptoms.

These two isolates were identified by phylogenetic analysis based on the internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and large subunit ribosomal RNA gene, partial sequence. Thus, rDNA-ITS region study revealed that these two Fusarium strains are Fusarium fujikuroi strain F2 and Fusarium proliferatum strain F7 causing Pokkah boeng disease of sugarcane.

Keywords: Pathogenicity, *Fusarium fujikuroi, Fusarium proliferatum,* Internal Transcribed Spacer (ITS).

Introduction

Pokkah boeng (a Javanese term) was first described in Java by Walker and Went in 1896[38]. It is a economically important fungal disease worldwide[30]. It has been recorded that almost all cane growing countries are affected with this disease. Pokkah boeng caused severe damage where susceptible varieties are widely planted in hot dry season followed by wet season[4]. Vishwakarma et al[35] reported that disease severity increased from 5-90% in different sugarcane cultivars and it can cause significant quality reduction in high sugar yielding varieties reducing sugar by approximately 40.8% - 64.5% in infected crops[23].

The fungal propagules are dispersed by wind and cause the infection in 3-7 months old crop[30] and heavy infection was observed during monsoon season in July to September (known as primary infection) while it is also caused due to infected setts, irrigation water, soil and rains (known as secondary infection). For identification of this disease in sugarcane crop, there are certain characteristic symptoms such as young leaves possessing chlorotic patches near its base (preliminary symptom), stalk distortion, rottening of stalk apical part and knife like cut on stalks (acute symptoms)[35].

During our survey, it was observed that in chlorotic phase, wrinkling of leaves was found in almost all Pokkah boeng affected varieties while twisting of leaves was mostly visible in the Co-0238 variety of sugarcane. The disease incidences were noticed in India during 2017-2019 in different sugarcane cultivars namely Co0238, CoLk 8102, CoPk 05191, Co1148, CoJ 064, CoS 8436, CoS 8432 and CoLk 94184 and during this time Co 0238 and CoLk 8102 emerged as most susceptible varieties of sugarcane.

The development of symptoms has been observed as four phases namely Chlorotic phase I, Chlorotic phase II, Acute or Top rot phase and Knife cut phase[26,35]. Vishwakarma et al[35] reported that under field condition, Pokkah boeng may develop many variations in symptoms but the final result is usually a damaged top. Raid and Lentini[27] observed some ladder like or unusual growth externally as well as internally in the internodal region of sugarcane. A knife cut symptom in different sugarcane varieties namely CoS 767, CoC 671, CoC 8014, Co 1158, CoS 8315 and CoS 8436 is reported[25].

It is re-emerging disease of sugarcane and recently it has been found to cause major yield losses in most sugarcane producing countries such as India, South Africa, Malaysia and China.[17,18,31,32,35] The disease is caused by *Fusarium* spp. with various workers reporting *F. moniliformae, F. sacchari, F. verticillioides, F. proliferatum, F. fujikuroi* and *F. andiyazi* as the major species associated with this disease in different regions.[17,18,31,32,35,36] For the management of phytodiseases, along with the detection of phytopathogens, there should be an understanding of their density, changes in the distribution of pathogens and the understanding between its biotic and abiotic environments[16].

The present investigation was undertaken with an aim to isolate for molecular identification of *Fusarium spp*. associated with Pokkah boeng disease of sugarcane by rDNA internal transcribed spacer (ITS) sequence analysis.

Material and Methods

Sample collection: Field survey was conducted in different farm of ICAR-IISR, Lucknow, its regional centre (Motipur, Bihar) and different districts of Uttar Pradesh (Table 1). About 38 diseased plant samples were collected with higher

Pokkah boeng affected fields during month of June-August, 2017.

Fungal isolation: *Fusarium spp.* was isolated from different Pokkah boeng affected varieties (Table 1). The infected plant parts were cut in small pieces and surface sterilized with 4% sodium hypochlorite (NaOCl), 70% ethanol and final washing with sterile distilled water sequentially and inoculated on plates containing PDA. The colonies were selected on the basis of their cultural characteristics and the morphology of their vegetative and reproductive structures produced on PDA culture media according to different keys of identifications.[1,5,9,21] The purified cultures were preserved at -4^{0}C till further use.

Pathogenicity test: Pathogenicity test was performed by the method suggested by Lin et al[17] and Wang et al[38] with slight modification. A pot experiment was designed on the basis of randomized block designing (RBD). In each pot, 2 eyes of 3 setts of sugarcane variety Co 0238 which is highly susceptible for Pokkah boeng disease were planted. During and after plantation, all the agronomical practices were followed except the plant protection measures.

The sugarcane plants were wounded and then spore suspension of the *Fusarium* isolates (10^8 conidia ml^{-1}, 100 lL) was injected in third internode with a sterile needle into sugarcane stalk consisting of 6-7 internode stage. Wounded places were sealed with molten wax and progressive symptoms were regularly observed at 7 days intervals. Detached leaf assay was also performed by cutting five leaves with 8-10 cm length and inoculated in the conidial suspension of the isolates (10^8 conidia mL^{-1}, 100 lL) at SWPAM laboratory of ICAR-Indian Institute of sugarcane research, Lucknow. Symptoms were observed 48 hour post-inoculation and pathogen was re-isolated and morphologically identified.

Molecular identification of *Fusarium* spp.: Two *Fusarium* strains were given for DNA isolation and sequencing to Xcelris Labs Ltd. (An Abellion company, Ahmadabad). Accessions were collected for nineteen *Fusarium* species from NCBI and nucleotide comparisons were performed by using Basic Local Alignment sources (BLAST) against NCBI database. The BLAST search showed that *Fusarium fujikuroi* strain F2 and *Fusarium proliferatum* strain F7 was 99% similar to *Fusarium fujikuroi* and *Fusarium proliferatum* respectively. To infer the relationship, seventeen sequences of *Fusarium spp*. were obtained for the nuclear marker of the internal transcribed spacer 1, the gene 5.8S and internal transcribed spacer 2 (ITS) from the NCBI. Phylogenetic tree was constructed with a focus on the species *Fusarium fujikuroi* strain F2 and *Fusarium proliferatum* strain F7. Sequences for ITS region were aligned using Muscle alignment using default setting as implemented in Molecular Evolutionary Genetics Analysis Program (MEGA X) software[14].

Maximum Likelihood Estimates (MLE) was performed for 1000 bootstrap values to estimate the relationships among the *Fusarium* species. Table 2 contains the list of all the accession numbers obtained from the NCBI for different species.

Results and Discussion

Pokkah boeng is one of the most serious fungal diseases in the sugarcane growing regions of the world[39]. Disease significantly reduces the yield and quality of sugarcane, which is found in susceptible cultivars ranging from 40%-60%[11]. The aim of this study was efficient identification of causal organism of Pokkah boeng disease of sugarcane for further disease management strategies.

Fusarium is one of the most widely recognized plant pathogenic fungi causing a range of diseases in innumerable crops of agriculture and horticulture. *Fusarium* is now well established causal agent of Pokkah boeng of sugarcane but species clarification is still obscure and disputed amongst researchers. Recent studies have suggested *Fusarium moniliformae*[35], *Fusarium* sacchari,[19,22,37] *Fusarium verticillioides* and *Fusarium proliferatum*[17], *F. fujikuroi*[12] *Fusarium andiyazi*[3,37] as the causal agents of Pokkah boeng of sugarcane.

Table 1
Pokkah boeng infected samples collected from different sugarcane growing areas

Area Surveyed	Affected sugarcane varieties	No. of affected varieties/genotype	No. of samples collected
Farm of ICAR-IISR, Lucknow	Co-0238, CoLk-8102, CoPk-05191, Co-1148, CoJ-064, CoM-0265	6	14
Regional centre of ICAR-IISR, Motipur, Bihar	Co-0238, CoJ-064	2	4
Sitapur	Co-0238, CoLk-8102, CoS-8432, CoLk-94184, CoC-8014, Co-1158, CoS-8315	7	9
Lakhimpur kheri	Co-0238, CoS-8436, CoS-8432, CoLk-8102, CoS-8315, CoS-767, CoC-671	7	11

This is due to the opacity in taxonomy of Liseola section of *Fusarium*[15]. *Gibberella fujikuroi* or section Liseola includes eight known mating populations which are capable of causing disease on a wide range of crops such as maize, sorghum, rice and sugarcane.[2,6,13,33,42]

For isolation and identification of causal organism, there is need of Pokkah boeng infected samples of sugarcane. A survey was conducted in different sugarcane growing areas, in agricultural land of ICAR-IISR Lucknow and its Regional Centre, Motipur (Bihar), Sitapur and Lakhimpur district of Uttar Pradesh and 38 diseased plant samples were collected (Table 1) and analyzed with Pokkah boeng symptoms. Later followed standard surface sterilization and pathogen isolation protocols and thus heavy mixtures of fungal species in petri plates were recognised. So, there was a need of further single spore isolation. After that we isolated fourty seven fungal isolates.

To identify *Fusarium* strains, we screened these forty seven strains based on phenotypic characters such as culture colour, colony morphology and aerial mycelia abundance. The isolates were confirmed as *Fusarium* spp. based on their cultural and morphological characters designated as F-1 to F-25.

The pathogenicity test has proved that strains F2 and F7 are the most predominant and aggressive fungal microorganism associated with sugarcane Pokkah boeng. These symptoms were first observed in pot trials three weeks post inoculation. Initially, symptoms were noted in underlying areas of young growing leaves. These symptoms noted as wrinkling of leaf sheath (Fig: 2A), longitudinal red stripe in midrib (photo1B) and rhomboid shape holes on leaf sheath (photo 2B), characteristic leaf twisting (photo 2A) and in severe cases a top rot symptom (photo 1C) was also noticed. This may be due to the pathogen directly infecting the tissue without any natural barrier of leaf sheath and thus increased the degree of disease, leading to higher incidences.[38]

These studies found similar as described by Vishwakarma et al[35]. In later stages, an unusual growth at internodes (photo3 C&D), shortened internodes (photo 3b) and short cane length (photo 3c) was recorded followed by Raid and Lentini[27]. A knife cut symptom was also found in sugarcane cultivar Co0238 (photo 2C).

Raid and Lentini[27] suggested that the unusual growth at internodes is due to splitting of diseased cells which were unable to growth with healthy tissues. Detached leaf assay of pathogenicity test started showing symptoms 72 hours post inoculation (photo 4). Conidial suspension of *Fusarium* strains produced lesions on leaves that can be compared with negative water control. These studies on pathogenicity confirm that if weather is favorable and pathogen gets access into the host, it causes severe damage to the crop. All these experiments were conducted in three replications and resulted in consistent outcomes.

Table 2
List of Accession numbers of isolates of the study and strains obtain from NCBI.

S.N.	Fusarium strains	Accession numbers
Isolates of the study		
1	*Fusarium fujikuroi* strain F2	MG965881
2	*Fusarium proliferatum* strain F7	MG965882
Strains obtain from NCBI		
3	*Fusarium culmorum*	MG274304
4	*Fusarium pseudograminearum*	MH333077
5	*Fusarium graminearum*	MK828118
6	*Fusarium langsethiae*	MG274309
7	*Fusarium sporotrichioides*	MK274314
8	*Fusarium poae*	MG274313
9	*Fusarium equiseti*	MG274305
10	*Fusarium avenaceum*	MG274299
11	*Fusarium tricinctum*	MG274296
12	*Fusarium subglutinans*	MG274315
13	*Fusarium napiforme*	MK072719
14	*Fusarium sacchari*	MK072727
15	*Fusarium andiyazi*	MG557862
16	*Fusarium oxysporum*	MK828120
17	*Fusarium verticillioides*	MN698249
18	*Fusarium fujikuroi*	KJ000433
19	*Fusarium proliferatum*	MH707085

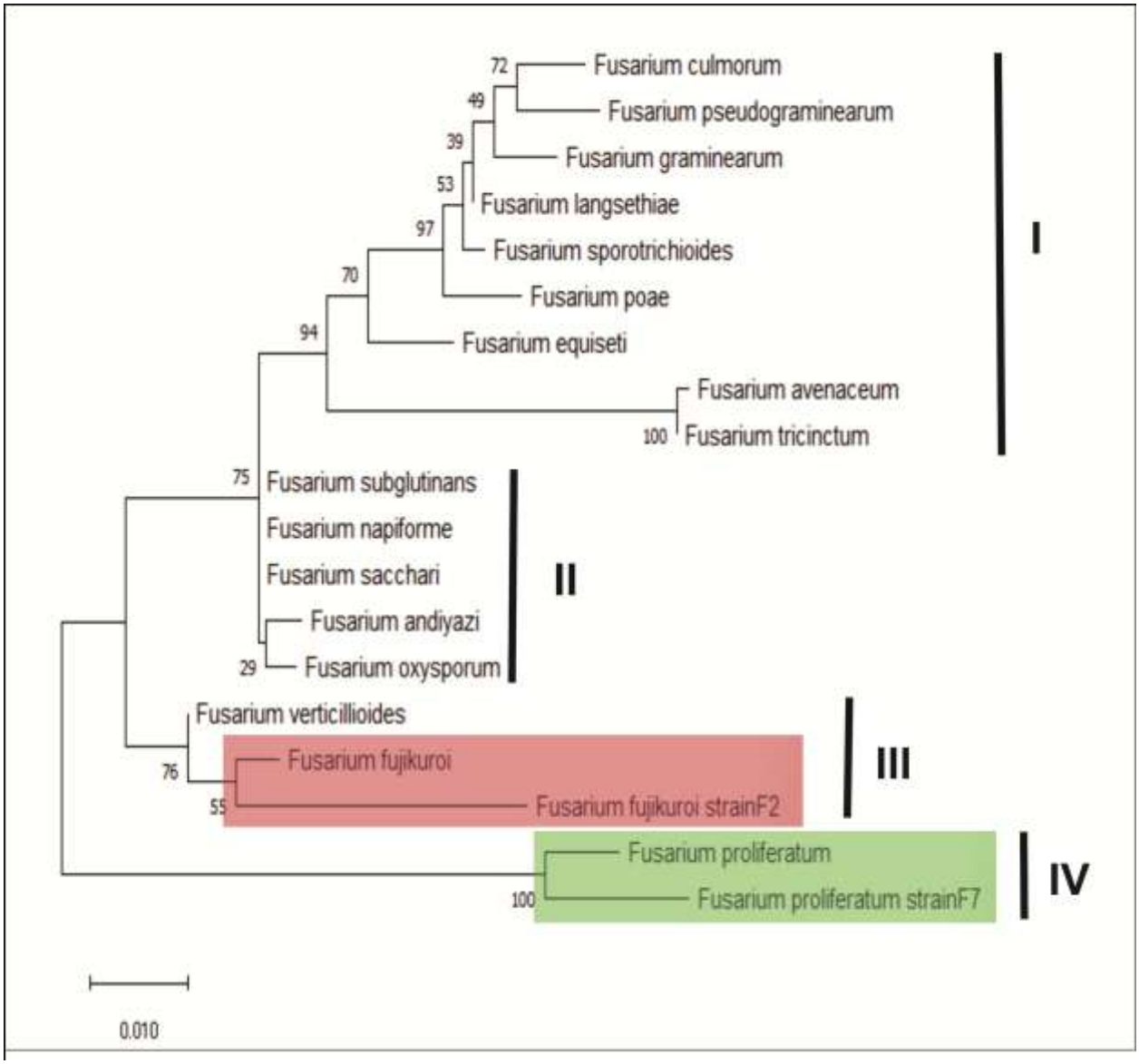

Fig. 1: Phylogenetic analysis of maximum likelihood tree was constructed and aligned with partial sequences of the Internal transcribed spacer-ITS (ITS-1, 5.8 S and ITS2) for 19 taxa (two isolated itself and 17 taken from NCBI) belonging to the Fusarium species.

F. fujikuroi and *F. proliferatum* belong to *Fusarium fujikuroi* species complex (FFSC) closely related to one another and it is not easy to identify them exclusively on morphology.[24] Rice disease of bakane is the hall mark of *F. fujikuroi* pathogenicity where the disease is caused through the overproduction of gibberellic acid[40]. *F. proliferatum* is also a known widespread phyto-pathogen causing diseases ranging hosts from rice to corn and sorghum[41]. *F. fujikuroi* and *F. proliferatum* are worldwide occurring fungal pathogens of several agricultural crops. These phyto-pathogens releasing mycotoxins may be cause of high occurrences of Pokkah boeng symptoms. The most hazardous impact on agricultural crops is by mycotoxin deoxynivalenol (DON) and its derivatives[20].

Recently it is reported that mycotoxin Fumonisin released by *F. fujikuroi* can inhibit the growth of shoots and roots, while causing wilting, chlorosis and necrosis in several plant crops. However, some studies revealed that lack of fumonisin production did not affect ability of strains causing maize ear rot[8]. *F. proliferatum* producing broad range of toxins such as fusaric acid, implicated in the pathogenesis of tomato wilt symptoms[10], moniliformin, toxic to the tobacco plants[7]. Fusaproliferin is another mycotoxin, a sesterterpene, produced by *F. proliferatum* and *F. subglutinans*.[20,28] Due to the similar morphological traits, *F. proliferatum* and *F. verticillioides* often misidentified although have different toxin profiles[29].

Fusarium strain F2 and F7 were subjected to 18S rDNA gene sequencing-based identification. The 18S rDNA gene amplicons were sequenced and received nucleotide sequences were submitted in GenBank with accession numbers (MG965881, MG965882). Phylogenetic tree (Fig: 1) resulting from the maximum likelihood method of ITS region corresponds to the accession number isolated by the

authors compared with the accession numbers from the same sp. available at the NCBI.

Phylogenetic analysis for the ITS (nuclear) region recovered three major clades. Clade 1 comprises of 9 species namely *F. culmorum, F. pseudograminearum, F. graminearum, F. langsethiae, F. sporotrichioides, F. poae, F. equiseti, F. avenaceum and F. tricinctum.* Clade 2 comprises of 5 species such as *F. subglutinans, F. napiforme, F. sacchari, F. andiyazi and F. oxysporum*. Clade 3 comprises of 3 sp. viz., *F. verticillioides, F. fujikuroi* and *F. fujikuroi* strain F2 in which *Fusarium fujikuroi* and *Fusarium fujikuroi* strain F2 form a sister clade showing two sp. are closely related to each other with >50% bootstrap support. Clade IV comprises of 2 species, *Fusarium proliferatum* and *Fusarium proliferatum* strain F7 sharing the same sister clade indicating high resemblance with each other with 100% bootstrap support. The evolutionary history was inferred by using the maximum likelihood method and Tamura-Nei[34] model. The tree with the highest log likelihood (-4890.69) is shown.

The percentage of trees in which the associated taxa clustered together are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei[34] model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2365 positions in the final dataset.

Conclusion

Our study concludes that two species of *Fusarium* are prominent **(***Fusarium fujikuroi* strain F2 and *Fusarium proliferatum* strain F7), causing sugarcane Pokkah boeng disease in different parts of India. This study will be very useful for a broad range of research as well as in the management and prevention of Pokkah boeng disease of sugarcane. We are testing efficacy of different strains of biocontrol *Trichoderma* for the management of the disease and the promising results are being obtained.

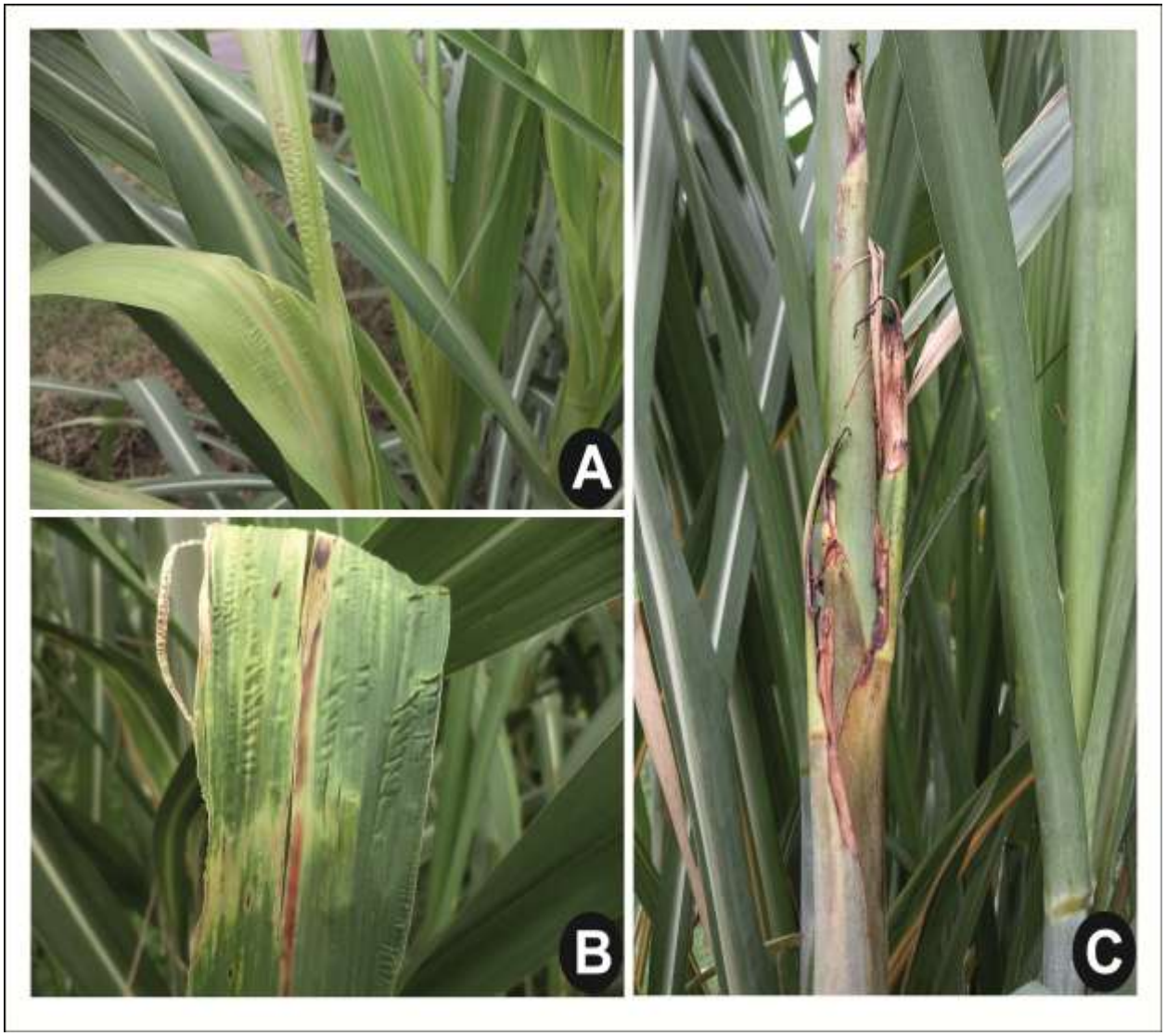

Photo 1: (A) Wrinkling on young leaf sheath, (B) Red strip in midrib and (C) Top rot symptom

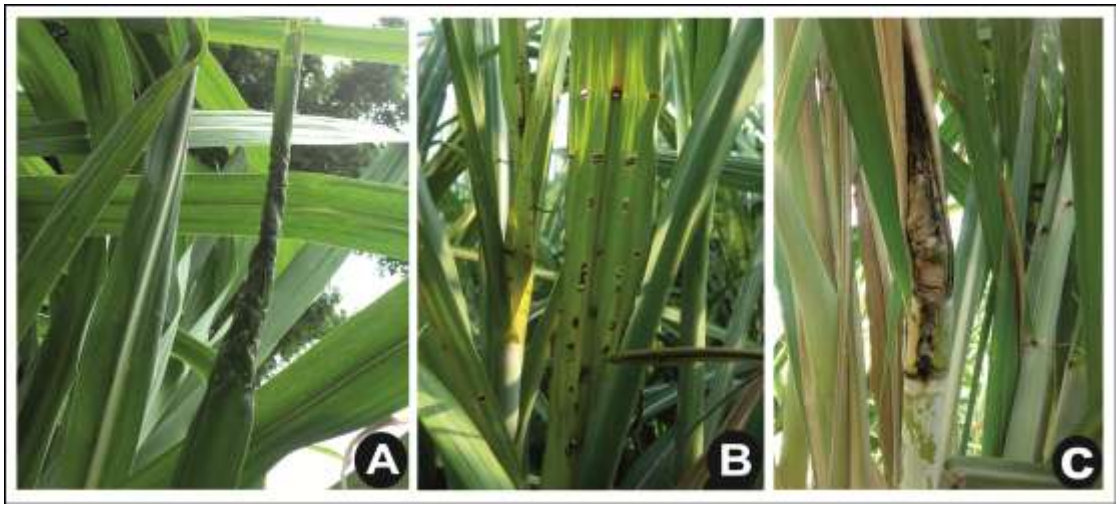

Photo 2: (A) Twisting of leaf, (B) Rhomboid shape holes on leaf sheath and (C) Knife cut

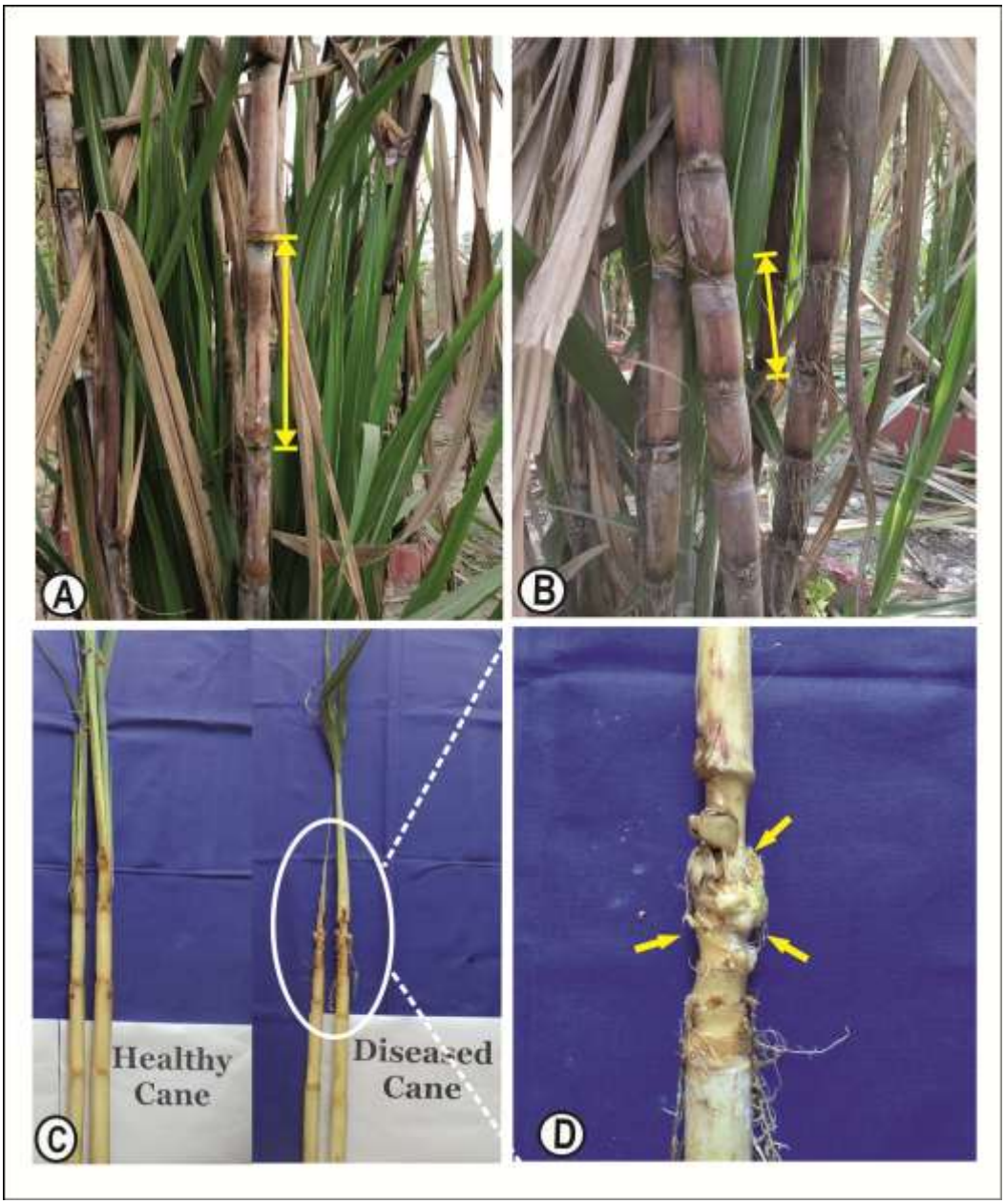

Photo 3: A) Normal internode (B) Pokkah boeng affected shortened internodes, (C) Diseased cane bi-furcated through unusual growth with healthy cane (D) Unusual/ladder like growth on internode

Photo 4: Detached leaf assay

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