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ISOLATION AND PRELIMINARY IDENTIFICATION OF PATHOGENIC AND TOXIGENIC *FUSARIUM* SPECIES USING CONVENTIONAL AND MOLECULAR TECHNIQUE

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ABSTRACT

Wheat is infected by more than 50 pathogens in our country. Among the fungal diseases, stripe rust / yellow rust, karnal bunt, black point, loose smut, powdery mildew, head scab are the most important diseases affecting wheat crop. *Fusarium* head blight or head scab is a destructive disease in the humid and sub humid wheat growing areas of the world. It has been associated nearly with 17 causal organisms of which *Fusarium graminearum* is the principal pathogen responsible for head blight in many countries including India. This study was carried with the objective to isolate *F. graminearum* from infected wheat plants and proving pathogenicity by conventional and molecular technique during 2015-16 at Department of Plant Pathology, A.C. & R.I., Killikulum, Tamil Nadu. The *Fusarium* sp. was isolated from infected wheat plants successfully on PDA medium. The fungus produced macroconidia only which is the characteristics feature of *F. graminearum* and produced characteristic pinkish discolouration on PDA medium. The isolated *Fusarium* sp. was identified based on morphological and cultural characters as *F. graminearum* and was confirmed as *F. graminearum* based on ITS sequence anaylsis at molecular level. DNA of *Fusarium* strains when amplified with primers ITS-1 and ITS-4 produced amplified PCR fragments of approximately 700 bp in length. When the ITS sequence of the *Fusarium* sp. fasta format sequence was BLAST searched in the NCBI data base, the output data showed several matching sequences in which most of them belongs to the sequence of *F. graminearum* and the *Fusarium* sp. strain used in the present study was confined as *F. graminearum.*

(Key words: Wheat, *F. graminearum.,* isolation, pathogenicity, cultural characters)

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the most important cereal crop for the majority of world's population. It is the most important staple food of about two billion people (36 per cent of the world population). Worldwide, wheat provides nearly 55 per cent of the carbohydrates and 20 per cent of the food calories consumed globally (Breiman and Graur, 1995).The major wheat growing countries are Europe, China, India, United States and France (FAO, 2016). In India wheat is grown in an area of 31.19 million hectares with a production of 95.85 million tonnes and an average productivity of 3,075 kg ha^{-1} under irrigated condition during 2013-14 (Annonymous, 2016).

Wheat is infected nearly by fifty pathogens in our country (Weise, 1987). Among the fungal diseases, stripe rust / yellow rust (*Puccinia striiformis tritici*), karnal bunt (*Tilletia indica -Neovossia indica*), black point (*Alternaria alternata*), loose smut (*Ustilago nuda tritici*), powdery mildew (*Erysiphe graminis tritici*), head scab (*Fusarium graminearum*) are the most important diseases affecting wheat crop.

Head blight or Head scab is a destructive disease in the humid and sub humid wheat growing areas of the world. It has been associated nearly with 17 causal organisms, of which *F. graminearum* (Schwabe) is the principal pathogen responsible for head blight in many countries including India (Saharan *et al.,* 2002). Head blight or scab of wheat caused severe epidemics in many areas worldwide (Dubin *et al*., 1997). The disease has been especially destructive in the United States since 1991 and particularly caused severe epidemic during 1993 that accounted for a crop loss of up to $1 billion (McMullen *et al.,* 1997). Totally losses due to this disease in the USA during 1990s approached $ 2.6 billion (Windels, 2000). In India, wheat grain yield losses of 15.1-29.0 per cent have been reported from Arunachal Pradesh due to head blight incidence on different wheat varieties (Chaudhary *et al.,* 1991) and also in Punjab maximum yield loss of 21.6 per cent in wheat variety PBW 222 (Kaur *et al*., 2000).

The first symptom of *Fusarium* head blight occurs shortly after flowering. Diseased spikelets exhibit premature bleaching as the pathogen grows and spreads within the head. One or more spikelets located in the top, middle, or bottom of the head may be bleached and it progresses throughout the entire head. As symptoms progresses, the fungus colonizes the developing grains causing them to shrink and wrinkle inside the head. Often, the infected

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kernels have a rough, shriveled appearance, ranging in color from pink, soft-gray, to light-brown. This disease is otherwise known as "tombstone" kernels of wheat because of the chalky and lifeless appearance of the infected kernels (Tuite *et al.,* 1990). Head blight is a re-emerging disease of wheat (Champeil *et al*., 2004) that causes extensive damage through the grain yield and the quality by adversely affecting the grain size, weight, germination rate, protein content, baking quality of the flour and other technological parameters (Parry *et al*., 1995). In addition to the reduction in grain yield, *F. graminearum* produces trichothecene type of mycotoxins which accumulate in the infected grains and making them unsuitable for food and feed (Goswami and Kistler, 2004).

F. graminearum does not produce microconidia and produces macroconidia only which is the characteristics features of this pathogen (Leslie and Summerell, 2006). It can survive as mycelium, ascospores, macroconidia and chlamydospores (Sutton, 1982). *F. graminearum* infects wheat spike tissues primarily through anthers that protrude from the glumes, then spreads to the epidermis of glumes and to the ovaries (Xu and Hideki, 1989). Anthesis is the period of greater susceptibility, because the high levels of choline and betaine produced in the extruded anthers stimulate the growth of *F. graminearum* and promote the infection of wheat spikes by the pathogen (Strange and Smith, 1978). *F. graminearum* causes infection mainly (ear heads) on aerial parts of the plant called phyllosphere.

Therefore, it should be wise to find various control measures for preventing loss due to *F. graminearum* . With these above said background, this study was carried out with the objective "Isolation of *F. graminearum* from infected wheat plants and proving pathogenicity by conventional and molecular technique" .

MATERIALS AND METHODS

The freshly infected wheat ear heads showing typical symptoms of *Fusarium* head blight disease collected from the field were used for isolation of the pathogen. The *Fusarium* infected ear heads were made into small pieces of three mm size cut along the edges of the lesions using a sterilized scalpel. The ear head pieces were surface sterilized with 0.1 per cent mercuric chloride solution for 30 seconds. These bits were then washed 3-5 times separately in repeated changes of sterile distilled water. The sterilized potato dextrose agar medium amended with 100 ppm of streptomycin sulphate (to avoid bacterial contamination) was prepared. The sterilized cooled (warm) medium (20 ml) was poured in to sterile petri plates (90mm) and allowed to solidify. The surface sterilized plant tissue bits were placed individually at equidistance at the rate of 3 bits plate^{-1}. All these works were carried out under aseptic conditions. The plates were incubated at room temperature (25 ± 2° C) for 5 days and observed for fungal growth. Identification of cultures was done by microscopic examination and by perithecial production. The growing fungal colony of each plant piece was sub cultured and purified by single hyphal tip method (Tutte, 1969). The pure culture of the pathogen was maintained on PDA slants for further use in this study. The stock cultures were maintained in PDA slants for long term storage under refrigerated condition at 4^0 C.

Isolation of total genomic DNA from *F. graminearum*: Total genomic DNA was isolated from *F. graminearum* as described by Lee *et al.* (1988) for fungi with slight modifications. To verify the quality of isolated DNA, 2.5µl of total DNA solution was resolved in the 1% agarose gel electrophoresis. The gel was examined under a UV light and the image was documented using Gel Doc™ apparatus (Bio-Rad). Amplification of ITS from *F. graminearum* was done in PCR. The PCR products were resolved by electrophoresis in 1% agarose gel. The PCR products were purified using FavorPrep GEL/ PCR purification kit. The primers used for amplification of ITS region were:-

ITS1 - 52 TCCGTAGGTGAACCTGCGG 32 (forward primer) and

ITS4 - 52 TCCTCCGCTTATTGATATGC32 (reverse primer)

Sequencing of ITS and identification of species *Fusarium* spp. by bioinformatics analysis:-Purified ITS PCR product was sequenced at Eurofins genomics India pvt. Ltd, Bangalore. The obtained DNA sequence were trimmed from 5'and 3' region where the sequencing chromatogram were not clear. Then DNA sequence in which clear chromatogram was obtained was made in Fasta format. This was used as input sequence (Query sequence) in nucleotide blast analysis program at NCBI database. The output was retrieved from the bioinformatics analyse and then, the organism showing major score from the output result is considered as the closely related species in the test fungus used in the study.

Pathogenicity test for *F. graminearum* : To confirm whether the isolated *F. graminearum* is pathogenic and also to prove the Koch's postulates, pathogenicity test was carried out by the method described by Engle *et al.* (2003). The soil mixture containing clay soil, sand and farm yard manure at the ratio of 1:1:1 was used in the pot culture experiments. This is referred as 'pot culture mixture'. The earthen pots of 30 cm diameter each with five kg of pot culture mixture were used in all the pot culture experiments. The wheat head blight disease susceptible Agra local variety was raised in all the pot culture experiments. Five seedlings of highly susceptible Agra local were planted in each pot and three pots were maintained for each replication in all the pot culture experiment of this study. The pots were maintained in the glass house with uniform, regular and judicious watering. Water congestion was provided to the inoculated plants both 24 h prior to and 24 h after inoculation. The inoculated plants were observed regularly for lesion development. The incidence of the disease was assessed at grain maturity.

RESULTS AND DISCUSSION

Isolation of *F. graminearum* from infected wheat plants

The causal agent of wheat head blight disease *Fusarium* sp. was isolated from the diseased wheat plant (ear head), using PDA medium and sub cultured by the single hyphal tip method. The morphology of the mycelium initially appeared white colour. Two days later, mycelia turned pink and entire agar medium became typically reddish pink colour. Analysis of asexual spore showed that the pathogen produced only macroconidia and no microconidium was produced. This is the typical characteristics feature of *F. graminearum.* (Fig. 1, 2, 3 and 5).

The *Fusarium* sp. was isolated from infected wheat plants successfully on PDA medium. The fungus produced macroconidia only which is the characteristics feature of *F. graminearum* and produced characteristic pinkish discolouration on PDA medium. The isolated *Fusarium* sp. was identified based on morphological and cultural characters as *F. graminearum.* It was again confirmed as *F. graminearum* based on ITS sequence analysis.

Identification and confirmation of *Fusarium* sp. by molecular technique

Though *F. graminearum* was confirmed by morphological and cultural characters, in the present study this strain needs to confirmed by molecular technique such as ITS sequence analysis which is one of the commonly used molecular methods for the identification of fungi at species level. DNA from *Fusarium* strain were isolated using CTAB method. Single bands of intact DNA were visualised on the agarose gel. DNA of *Fusarium* strains was amplified with primers ITS-1 and ITS-4 using a thermocycler and the products produced were visualised as a single band in agarose gels stained with ethidium bromide. The size of the PCR fragments was approximately 700 bp in length (Fig. 6).

ITS products of the *Fusarium* strain obtained by PCR were cleaned with PCR cleanup kit to remove the residual primers, polymerase and salts in the PCR product. Cleaned up product was sequenced at Eurofins genomics Pvt. Ltd. The full length ITS sequences obtained for *Fusarium* strain were BLAST searched in the database of National Center for Biotechnology Information [NCBI], U.S.A. When the ITS sequence of the *Fusarium* sp. fasta format sequence was BLAST searched in the NCBI data base, the output data showed several matching sequences in which most of them belongs to the sequence of *F. graminearum*. Thus, the *Fusarium* sp. strain used in the present study was confined as *F. graminearum.*

In this modern genomic era, DNA sequences have been used to identify various unknown organisms. In fungus, the sequences of ITS region are typically the most useful for molecular systematics at the species levels (Op De Beeck *et al*., 2014). For identification of specific genera and species, the rDNA repeat unit, consisting of the subunits 18S, 5.8S, and 28S rDNA interrupted by the internal transcribed spacer (ITS) and the intergenic spacer (IGS) are employed due to their specific sequences as target regions. Though a number of DNA based identification methods are available, the specific advantage of ITS sequencing is, the identification of any fungal isolate using the database containing the corresponding sequence of previously identified fungal species (Schmidt *et al.,* 2012).Preliminary identification and typing or pathogenic and toxigenic *Fusarium* species ITS region analysis has been used for *Fusarium* spp. that infects wide range of crop plants (Mirhendi *et al.,* 2010).

Pathogenicity test of *F. graminearum*

The isolated *F. graminearum* was tested for pathogenicity by inoculating the pathogen in the healthy wheat plants during anthesis stage of the plant. The inoculated and uninoculated wheat plants were observed for symptoms of the disease and growth of fungal colonies. After four days of inoculation, fungal colonies started growing on inoculated wheat ear heads (Fig 6). Initial infections appeared as small water soaked brownish spots at the middle of the glume. A salmon pink mycelial growth on the edge of the glumes was seen. One or more spikelets in the middle of the head bleached and premature bleaching of spikelets progressed throughout the entire head. The infected kernels are become smaller, shrivelled in appearance and pale pink in colour. After eight days the grains become chaffy. Uninfected grains remain healthy even after 10 days (Fig. 5).

In the present study, *F. graminearum* was pathogenic and produced symptoms under greenhouse conditions. At the time of mid anthesis stage of wheat crop, conidial suspensions containing 5 x 10^4conida ml^{-1} was inoculated into the floret of the selected spikes by hypodermic syringe method (Engle *et al.*, 2003). *Fusarium* head blight disease incidence was noticed after 7 days of inoculation. There are strong debates concerning the most effective inoculation method for FHB assessment. Inoculation techniques differ between programmes internationally (Rudd *et al*., 2001). Some of the more general inoculation techniques include single floret inoculation, cotton wool method, spray inoculation of the wheat head with a liquid spore suspension, the distribution of infected grain (grain spawn) or other plant material and solid media. Single floret inoculation is used to control the method of inoculation so that the initial inoculation point is limited to that single floret within one wheat head (Engle *et al.*, 2003).

Thus, it is inferred from this study that the causal agent of *Fusarium* head blight (FHB) disease can be isolated from the diseased wheat plant (ear head) using PDA medium and sub cultured by the single hyphal tip method. Single band of intact DNA were visualised on the agarose gel. The size of the PCR fragments was approximately 700 bp in length and the isolated pathogen was identified and confirmed as *F. graminearum*.

Fig.1

Fig.4

Fig. 2

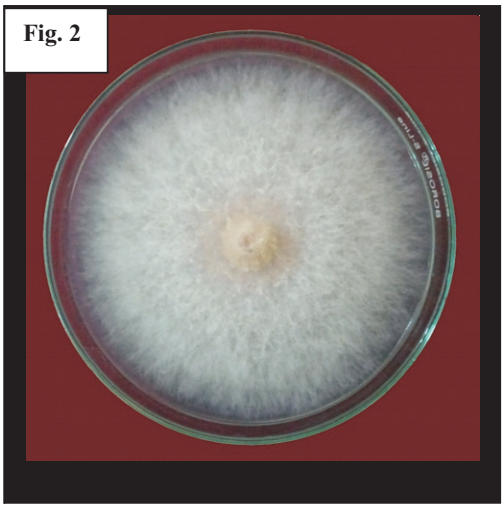


Fig.5

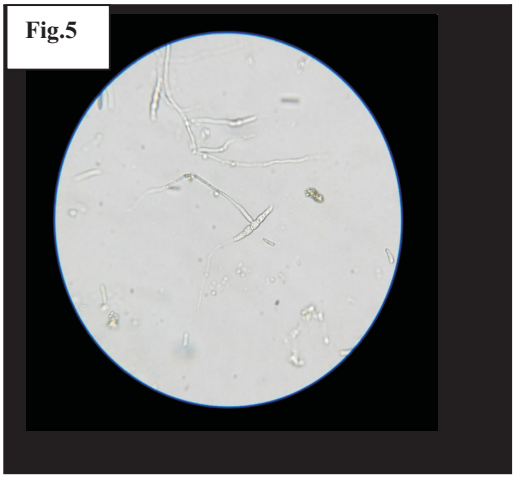


Fig. 3

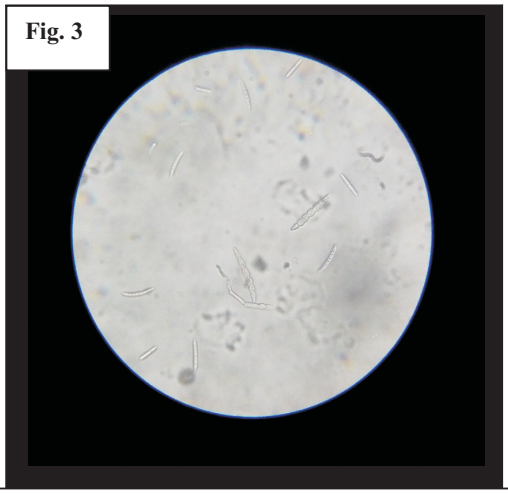
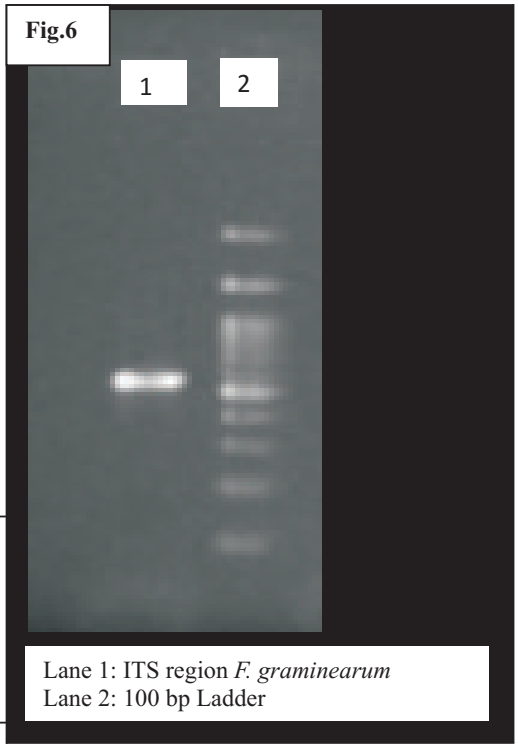


Fig.6



Lane 1: ITS region *F. graminearum*
Lane 2: 100 bp Ladder

Fig.1 Symptom of wheat head blight
Fig.2 *Fusarium graminearum* culture
Fig.3. Conidia and chlamydospores of *F. graminearum*
Fig.4 Pathogenicity of *F. graminearum*
Fig.5 Conidial germination of *F. graminearum*
Fig.6. ITS region of *F. graminearum*

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