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RESEARCH ARTICLE

Isolation and molecular detection of *Cephalosporium acremonium*: The Causative agent of black bundle disease in maize

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ABSTRACT

Several diseases affect maize plants and one among them is black bundle disease, causal organism of which is yet to be confirmed with conflicting reports appearing in literature. In the present work, an effort was made to record the disease incidence by carry out filed surveys in Southern most district of Karnataka state, India and also to isolate and identify the causal organism. The surface sterilized pith tissue from the infected plants was cultured on PDA medium to identify the organism in the affected vascular tissue. The isolated tissue produce *Cephalosporium acremonium* which was confirmed based on morphological characters. In addition, *Simplicillium sp.* and *Macrophomina phaseolina* were also isolated from the same infected plant material and suspected to have participated in the disease development. In accordance with several reports in literature, *Cephalosporium acremonium* was tested and confirmed as the causal organism of disease in green house experiments by following the Koch's postulates. This is in contrast with some earlier reports, wherein the exact symptoms of black bundle disease were reported as barren stalks in corn caused by *Fusarium sp*. The *simplicillium sp*. which had morphological similarity with *C. acremonium* and had an ambiguous taxonomic status in literature was confirmed as a distinct species through PCR based diagnosis.

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's leading annual crop, which belongs to the family Gramineae or Poaceae. It is one of the most important cereal crop grown extensively only after rice and wheat all around the world (AICRP on maize 2007). Maize is a versatile crop grown over a range of agro-climatic zones. In fact, the suitability of maize to diverse environment is unmatched by any other crop. The major maize production areas are located in temperate regions of the globe. The United States, China, Brazil and Mexico account for 70% of global production. India has 5% of corn acreage and contributes 2% of world production. The major maize growing states are Uttar Pradesh, Bihar, Rajasthan, Madhya Pradesh, Punjab, Andhra Pradesh, Himachal Pradesh, West Bengal, Karnataka and Jammu and Kashmir, jointly accounting for over 95% of the national production. Though, several resistant varieties have been developed against various diseases of maize, still these will be affected by many diseases. Black bundle disease is one such soil borne disease, which is complex in nature (El-Shafey and Claflin 1999). This disease is common in humid heavy soils in hot regions. When the plant gets affected, it shows the symptoms like wilting of topmost leaves and brown vascular bundles in lower portion of the stem. Black bundle disease kills the plants during flowering time hence it is also called as "Post flowering stalk rots". (Reddy and Holbert 1924). Reddish-purple colored leaves and stalks, barren stalks, multiple ears per node (commonly 3-5 ears per node) and excessive tillering are the other symptoms reported by McGee 1988, Nyvall 1999, Shurtleff 1980 and Shekhar and Kumar 2007. Reddy and Holbert (1924) first reported about black bundle disease of maize. Later Harris (1930) published a contradictory report stating that he was unable to repeat the work of Reddy and Holbert, placing in question their conclusion that the symptoms were caused by *Cephalosporium acremonium* Corda (1839) syn. *Acremonium strictum* (Reynolds 2001). The similar symptoms of black bundle disease were also reported as barren stalks in corn caused by *Fusarium* sp. (Reynolds 2001). Koehler *et al.* (1925) reported that the black bundle disease caused by *C. acremonium* is a systemic disease and seed infection by this organism was found to increase the percentage of broken stalks. From infected seed this organism may invade the vascular bundles throughout the whole length of the stalk during the active growing period. Affected vascular strands become disorganized. This causes a very pronounced weaking of the stalk. Tangne *et al.* (2002) studied different effect of *Acremonium strictum* from Cameroon on maize cultivars Nodak 8701, CMS 8704 and CMS 8501 and observed that the symptoms in leaves include vein necrosis, chlorosis, yellowing and wilt developing in the acropetal manner. These observations proved the pathogenic nature of *A. strictum* on maize of Cameroon. Molecular diagnostic techniques offer the potential screening and specific detection of pathogens. Information on DNA sequence of the plant pathogen of interest is obtained and primers are designed to amplify that pathogen DNA through PCR technique. Traditional methods to detect the pathogens involve plating infected plant parts on selective medium.

However this is limited by its lack of sensitivity and specificity, as *C. acremonium* shares morphology with certain other species such as *Simplicillium sp.* when grown on medium. The detection of *C. acremonium* is complex because of the existence of closely related species of this genus. Polymerase chain reaction (PCR) techniques offer advantages over traditional methods of detection and diagnosis because the fungi do not need to be cultured prior to detection by PCR and the technique is rapid and sensitive (Bonants *et al*. 1997; Lacourt and Duncan 1997; Frederick *et al*. 2002; Ippolito *et al*. 2002; Kong *et al.* 2003; Li and Hartman 2003; Mercado – Blanco *et al*. 2003; Hayden *et al*. 2004; Silver *et al*. 2005 and Zhang *et al*.

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2005). In the present investigation the field survey was carried out around Chamarajanagara district of Karnataka state in order to record the symptoms and isolate the pathogen from the infected tissue of the plant and we report a method to detect *C. acremonium* using polymerase chain reaction.

MATERIALS AND METHODS

Field survey and collection of infected plant materials

The field survey was carried out to record symptoms of the post flowering stalk rot disease. Maize growing areas were surveyed around Chamarajnagar district (Geographical coordinates are 11° 40' and 12º 48' North latitude, 74º 54' and 76° 07' East longitude), during August to February of 2008 and 2009 (Kharif and Rabi seasons). The fields were of both irrigated and non-irrigated croplands and the soil was red to black in color. Temperature recorded was around 25-30°C during winter seasons and relative humidity was 70-80%. During field survey the major symptoms of black bundle disease were spotted, which were used as disease diagnostic criteria, such as wilting of topmost leaves and brown vascular bundle in the stalk region of the maize plant. The diseased plant produced ears with undeveloped shrunken kernels. The fungi grew systemically into the crown and internodal region, producing the brown or black colored vascular bundles in the affected plants.
Reddish-purple colored leaves and stalks, multiple ears per node (commonly 3-5 ears per node) and excessive tillering are the other symptoms appears on the black bundle disease infected maize plant caused by *C. acremonium*. The plants showing typical black bundle disease symptoms were uprooted and the rhizospheric soil was collected along with the roots and stalk. The infected stem portion showing brown color was cut and collected separately in different polyethylene bags and carried to the laboratory. The sample material was stored at 4°C for further study. Samples were subjected to subsequent analysis for detection and isolation of pathogen.

Estimation of disease incidence

Based on the typical symptoms, the disease incidence in the field was calculated by using the following formula.

$$\text{Percentage of disease incidence} = \frac{\text{Total no. of plants showing disease symptoms}}{\text{Total no. of plants observed in field}} \text{ X } 100$$

For the sake of convenience, the disease incidence ≤ 5 % was considered as mild, ≤ 10 % and > 10 % was recorded as moderate and high incidence respectively. Based on the morphological symptoms of the disease, the diseased and healthy plants were counted to estimate the percentage disease incidence (PDI).

Isolation and identification of pathogen

A freshly collected infected plant material is cut into small pieces and surface sterilized by immersing in 0.1 percent mercuric chloride solution for 3 minutes, then washed in sterile distilled water several times to remove traces of mercuric chloride. The surface sterilized plant tissues is transferred on to the Potato dextrose agar (PDA) medium in petri dishes and incubated at 25-30° C for about 10 days. The colonies thus obtained from the diseased plant tissue were studied to observe their morphological characters. Further the confirmation of pathogenicity of the isolated fungus was recorded by following Koch's postulates, conducting pot experiment under *in vivo* condition. The experiment was repeated three times and the results obtained in all three experiments were similar. The seed variety Renuka G-25 was used in the present experiment.

Molecular characterization

Genomic DNA Extraction of pathogenic fungi

After cultivation in Potato dextrose Broth at 120 rpm, 28°C for 5–7 days, the culture was centrifuged at 5,000×g for 5 min and ground using a mortar containing 600 µL CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 1% PVP) at 65°C. The mycelial mixture was transferred into a 1.5-mL Eppendorf tube and heated to 65°C for 30 min, extracted twice with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) and washed with chloroform/isoamyl alcohol (24:1). After centrifugation at 10,000×g for 5 min, the supernatant was transferred to a new microtube and precipitated by adding equal volume of isopropanol at −20°C for 1 h. Finally, the DNA pellets were collected by centrifugation (12,000×g, 15 min), washed with 70% ethanol twice and re-suspended in 40 µL TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNA was removed by adding 2 µL of RNase A (10 mg/ml; Merck Pvt. India Ltd., Bangalore) at 60°C for 10 min. The Purity of DNA was quantified at 260 nm using UV-visible double beam Spectrophotometry (Shimadzo UV 1800, Japan).

PCR Amplification of 18S rRNA Gene

The resulting genomic DNA was used as templates to amplify the fungal 18S rRNA gene fragment using 22 mer primer nu-SSU-Forward (AAGGCATGGAATAATAATAGGA) and 21 mer primer nu-SSU-Reverse (TTGCAAT GCCT ATCC CCAGGA). The PCR mixture (50 µL) contained 2 µg DNA template, 5 µL 10×Taq buffer (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer, and 1.25 U Taq DNA Polymerase. The PCR program was carried out with 3 min at 94°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58.6°C for 30 seconds and elongation at 72°C for 30 seconds and final elongation at 72°C for 10 min.

Table 1. Survey of maize fields to calculate PDI around Chamarajnagar district of Karnataka State

Place	Field condition Infected/Un-infected	Maize Variety	Total No. of plants in a field R × R_1*	Total No. of plant expressing symptoms	PDI**
Ramapura	Infected	Proagro-62	50×21=1050	10±2	0.95%
Ramapura	Infected	Proagro-62	65×21=1365	14.3±1.5	1.04%
Ramapura	Infected	Local variety	40×20=800	0.9±1	1.12%
Ramapura	Un-infected	---	---	---	---
Ramapura	Un-infected	---	---	---	---
Alur	Infected	BISCO-740	58×22=1276	10.6±3.0	0.83%
Begooru	Infected	Kaveri-225	38×19=722	10.3±1.5	1.42%
Kagalavadi	Un-infected	---	---	---	---

*Total Number of Plants in each Row × Total Number of Rows in Field. **Percentage Disease Incidence.

Fig. 1 (A). Photograph shows the symptoms of black bundle disease infected maize plant. (B). T.S of the infected plant stem.

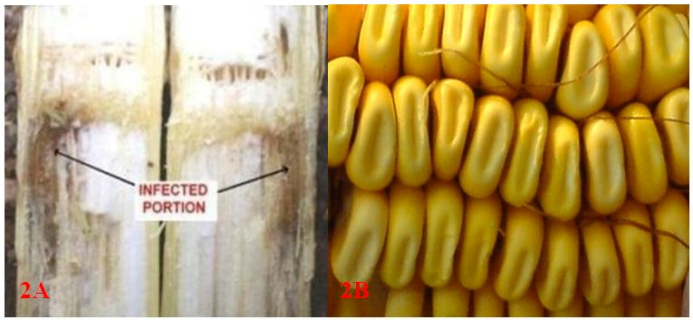

Fig. 2 (A). L.S of infected stem show brown spots. (B). Wrinkled kernels in black bundle disease infected plant.

Fig. 3 (A). Photograph shows the symptoms of black bundle disease infected maize plant. (B). Formation of more than single ears at each node.

PCR products were purified using PCR Cleanup Kit (Merck Pvt. India Ltd., Bangalore). The amplicons were analysed on 1% agarose gel electrophoresis using Ethidium Bromide (EtBr) as the staining dye and was documented using Biorad gel documentation device (Chemi Doc XRS, Biorad, USA).

RESULTS

Disease incidence

The observations made during the field survey showed the similar symptoms as described by Reddy and Holbert (1924). The symptoms like vein necrosis, chlorosis, yellowing and wilting in the acropetal manner were found. At advanced stage, the mature plant showed loss of healthy green color in the lower portion of the stalk, becoming dry and eventually killed the plant prematurely after flowering (Fig.1A). When split opened the diseased stalk, it showed brown vascular bundles (Fig.1B). Out of the eight fields surveyed, five fields showed mild incidence of Post flowering stalk rot disease during Kharif and Rabi seasons (Table 1). The diseased plant produced ears with undeveloped shrunken kernels (Fig. 2B). The fungi grew systemically into the crown and internodal region, producing the brown or black colored vascular bundles in the affected plants (Fig. 2A). Reddish-purple colored leaves and stalks, multiple ears per node (commonly 3-5 ears per node) and excessive tillering are the other symptoms appears on the Post flowering stalk rot or black bundle disease infected maize plant were recorded during the survey (Fig. 3A and 3B).

Identification of pathogen

Study of morphological characters confirmed that the pathogen was *Cephalosporium acremonium* (Fig. 4 A, B). In addition to this, *Simplicillium sp.* and *Macrophomina phaseolina* were also isolated from the same infected plant material, *Macrophomina phaseolina* is known to cause charcoal rot in maize (White D.G. 1999). The identification of *Cephalosporium acremonium* was carried out based on cultural characteristics on media, morphological characters under microscopic observation and also consulting suitable identification manuals and keys (Domsch and Gams 1972; Gilman 2001). The characters fit the description of *Cephalosporium acremonium* made by Domsch and Gams (1972). Pathogenicitic nature of *Cephalosporium acremonium* was confirmed by following all the four steps of Koch's postulates in green house experiments (Fig. 4 C, D, E and F).

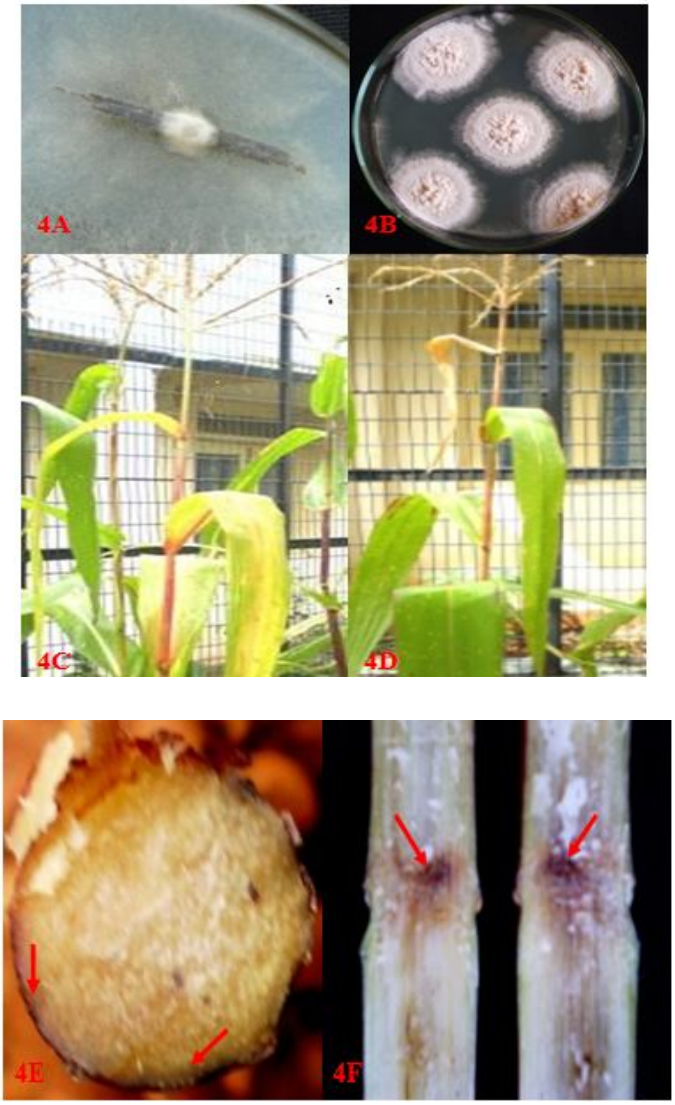

Fig. 4 (A). Culture of infected plant material for the isolation of pathogen. (B). Pure culture of the *Cephalosporium acremonium*. (C and D). Appearence of symptoms of Black bundle disease on maize under *in vivo*. (E and F). Vascular discolouration of infected plant under *in vivo*.

Molecular characterization

The lysate of *Cephalosporium acremonium* has resulted in an amplicon size of approximately 285- 300bp in length; on the other hand, *Simplicillium* lysate did not yield any amplicon indicating no amplification (Fig.6). The PCR products obtained for amplification of the rDNA region (Fig.5) containing the conserved 18S rRNA gene and the more variable internal transcribed spacer regions (ITS 1) in the primary DNA preparations ranged from approximately 285 to 300bp. The rDNA amplified fragments were approximately 285 to 300bp in size.

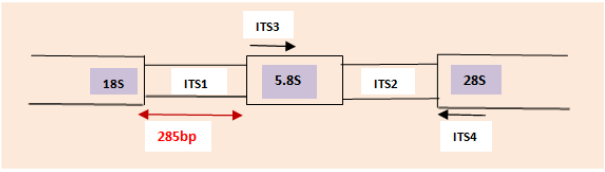

Fig. 5. Restriction site maps of amplified rDNA fragments in *C. acremonium* isolate.

DISCUSSION

Black bundle disease caused by *Cephalosporium acremonium* is one of the important diseases of maize crop. The symptoms exhibited by the *C. acremonium* infected plants are almost similar to wilt disease and barren stalks disease caused by *Fusarium* sp (Reynolds 2001). The symptoms like formation of reddish purple coloured leaves, formation of brown vascular bundle in the stalk region and leaf necrosis of black bundle disease are common in wilt disease (El-Shafey and Claflin 1999) and barren stalk disease (Reynolds 2001). The diseases can be distinguished only by the formation of wilting of topmost leaves. The present study was undertaken to record the disease incidence of black bundle disease of maize crop growing in Chamarajnagar district of Karnataka state. The results of field survey revealed that the percentage disease incidence (PDI) was less than 5% in all the fields surveyed. Hence the incidence was considered as mild infection. National Plant Disease Recovery System (NPDRS, 2008) reported that black bundle disease, poses a moderate to severe threat to corn production in Egypt and India, with yield losses approaching 40-70% in non-resistant cultivars. Efforts are also made to isolate and identify the *C. acremonium* from the infected tissue of the plant. The other fungus, *Macrophomina phaseolina* and *Simplicillium* sp. were isolated in the same infected plant materials.

Macrophomina phaseolina is known to causes charcoal rot disease in maize during favorable environmental condition.

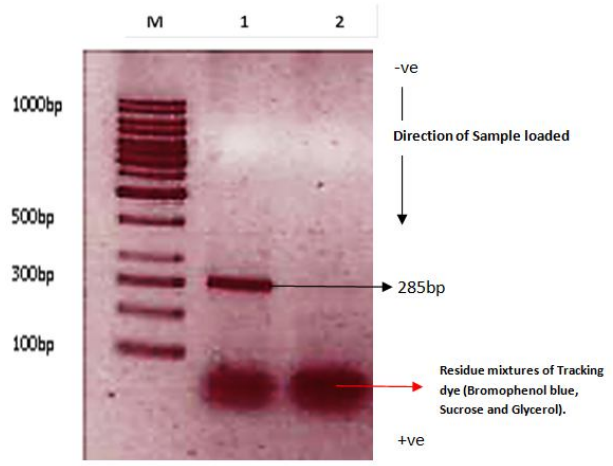

Fig. 6. Results of PCR amplicons on Agarose gel electrophoresis M - 1 Kb DNA ladder, *Cephalosporium acremonium* – lane 1 and *Simplicillium* – lane 2.

Simplicillium sp. is morphologically analogous to that of *C. acremonium*. Black bundle disease is often associated with infection by secondary invaders like *Macrophomina phaseolina* and *Simplicillium* sp. Symptoms may be modified by these secondary invaders and make identification of the black bundle disease in the field more difficult since some of these pathogens produce the similar symptoms of black bundle disease and similar reports were recorded on the late wilt disease of maize caused by *Cephalosporium maydis* (El-Shafey and Claflin 1999). *Cephalosporium acremonium* is considered to be very doubtful taxa and some species of which have been transferred to taxon *Simplicillium* sp. However, the PCR analysis clearly indicates that both isolates genetically divergent by producing unamplified amplicon. The *Simplicillium* sp. suspected to be realizing as endophyte or secondary invaders of maize. Further work need to be carried out to confirm the infectious ability of *Simplicillium* sp. The results of PCR analysis on DNA samples isolated form the *C. acremonium* and *Simplicillium* sp. revealed that expected, 285-300bp amplified DNA product was detected in the *C. acremonium* isolates and not in *Simplicillium* sp. The organization of the structural ribosomal genes is conserved in fungi. The three structural rRNA genes are separated by the ITS 1 and ITS 2 sequences. These ITS regions generally are more variable than ribosomal structural genes. Differences in the nucleotide composition of the variable ITS regions have been successfully employed to design primers to amplify (Abd-Elsalam *et al*. 2003; Bluhm *et al.* 2004; Grim and Geisen 1998 and Mishra *et al*. 2003). Regrettably less works has been done on *C. acremonium* for the preparation of primers to amplify. However the present work was carried out by using species-specific primers entitled nu-SSU-Forward and nu-SSU-Reverse to discriminate the pathogenic isolates. In contrast, representative *Simplicillium* sp. scored negative with the same set of primers.

Therefore, the ITS region represents a good choice for finding specific sequences to differentiate closely related strains at the intraspecific level (Gonzalez-Jaen *et al*. 2004 and Bryan *et al.* 1995). The PCR assay described in this work provides a useful tool for rapid and sensitive detection and differentiation of *C. acremonium* and *Simplicillium* sp. The result revealed that the symptoms observed during the survey confirmed the observations made by Reddy and Holbert (1924). The pathogen isolated from the infected plant tissue also identified as *C. acremonium* based on the morphological characters. Further, the observations made by us contradict the statement of Harris (1936) that, he was unable to repeat the work of Reddy and Holbert, placing in question their conclusion that the symptoms were caused by *Cephalosporium acremonium*. In accordance with several reports in literature, *Cephalosporium acremonium* was tested and confirmed as the causal organism of disease in green house experiments by following the Koch's postulates.

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