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

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**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 Claffin 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 weakening 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.

*Key words:* Black bundle disease, *Cephalosporium acremonium*, *Simplicillium*, *Macrophomina phaseolina*, Maize.
In the present investigation the field survey was carried out around Chamrajanagar 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 Chamrajanagar district (Geographical coordinates are 11° 40’ and 12° 48’ North latitude, 74° 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 caused by *C. acremonium* -5 ears per node) and excessive tillering are the other 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 polyelectyline 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.

**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.

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

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

*Total Number of Plants in each Row \* Total Number of Rows in Field. **Percentage Disease Incidence.
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). Pathogenicity 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).
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.

![Image](image1)

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

**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.

![Image](image2)

**Fig. 5. Restriction site maps of amplified rDNA fragments in C. acremonium isolate.**
Macrophomina phaseolina is known to cause charcoal rot disease in maize during favorable environmental conditions.

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 greenhouse experiments by following the Koch’s postulates.

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REFERENCE


community college. Pearson education (Singapore) Pvt Ltd
Indian branch, 482 FIE Patparganj, Delhi.

Koehler B., Dungan G.H. and Holbert J.R. 1925. Factors influencing
loading in corn. University of Illinois, Agricultural experiment
station, Bulletin No 266, pp311-371.

Kong P., Hong C.X., Jeffers S.N. and Richardson P.A. 2003. A
species-specific polymerase chain reaction assay for rapid
detection of Phytophthora nicotianae in irrigation water.
Phytopathology 93:822-831.

Lacourt I. and Duncan J.M. 1997. Specific detection of Phytophthora
nicotianae using the polymerase chain reaction and primers based
on the DNA sequences of its elicitin gene Para A1. European

Li S. and Hartman G.L. 2003. Molecular detection of Fusarium
solani f.sp. glycines in soybean roots and soil. Plant pathology
52:74-83.

McGee D.C. 1988. Maize disease, a reference source for seed
technologists. The American Phytopathological Society, St Paul
MN, pp150.

Mercado-Blanco J., Collado-Romero M., Parrilla-Araujo S.,
monitoring of colonization of olive genotypes by Verticillium
dahlia pathotypes with real-time polymerase chain reaction.
Physiological and Molecular Plant Pathology 63:91-105.

based assay for rapid and reliable identification of pathogenic

Nagendra Prasad M.N., Bhat S.S., Charith Raj A.P. and Janardhana
G.R. 2006. Molecular detection of phomopsis azadirachtae, the
causative agent of dieback disease of neem by polymerase chain

University Press, Ames IA, pp1021.

Poza-Carrion C., Aguilar I., Gallego F.J., Nunez-Moreno Y., Biosca
E.G., Gonzalez R., Lopez M.M. and Rodriguez-Palenzuela P.
2008. Brenneria quercina and Serratia sp. isolated from Spanish
oak trees: molecular characterization and development of PCR

acremonium and Fusarium moniliforme with stalk rots of maize.
Indian Phytopathology 29:227-231.

University of Minnesota, Minnesota.

Comparison of 5.8S and ITS2 rDNA RFLP patterns among
isolates of Acremonium obclavatum, A. kiliense and A. strictum

Pathogenic behavior of Cephalosporium maydis and
Cephalosporium acremonium. Annals of Applied Biology
66:257-263.

Shelkar M. and Sangit K. 2007. Epidemiology and Management of
post flowering stalk rots of maize. In: Sustainable pest
management. Directorate of maize research, IARI, New Delhi.

The American Phytopathological society, St Paul MN, pp105.

Silvar C., Duncan J.M., Cooke D., Williams N.A., Diaz J. and
Merino F. 2005. Development of specific PCR primers for
identification and detection of Phytophthora capsici Leon.

interaction between maize and Acremonium strictum from

of Corn Diseases, American Phytopathology Society, St Paul,
MN, pp41.

Molecular detection of Phytophthora capsici in infected plant
tissues, soil and water. Plant pathology 55:770-775.

Molecular detection of Fusarium oxysporium f.sp. niveum and
Mycosphaerella melonis in infected plant tissue and soil. FEMS
Microbiology Letters 249:39-47.

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