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PCR based detection of *Phomopsis vexans* (Sacc. & Syd.) - The causative agent of leaf blight and fruit rot disease of Brinjal (*Solanum melongena* L.)

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**ABSTRACT**

Leaf blight and fruit rot disease of brinjal (*Solanum melongena*) caused by the fungal pathogen *Phomopsis vexans* is the most prevailing diseases responsible for the significant yield loss in many Brinjal growing regions of India. The survey conducted during 2008 - 2010, seven (07) isolates of *Phomopsis vexans* causing leaf blight and fruit rot disease in different areas of Karnataka were isolated, identified and confirmed by Koch's postulates. Identification of *Phomopsis vexans* by conventional culture method was time consuming and laborious. Hence, PCR based detection assay was used to detect the pathogen from DNA samples obtained from fungal isolates. Universal primer pairs designed from internal transcribed spacer regions ITS1 (5'-CGG ATC TCT TGG TTC TGG CA-3') and ITS4 (5'-GAC GCT CGA ACA GGA ATG CC-3') of the ribosomal DNA (rDNA) of the genus *Phomopsis* sp. were used for PCR. The study revealed the amplification of expected 553 bp PCR products in all the DNA samples isolated from different isolates of *Phomopsis vexans* confirming their association in leaf blight and fruit rot disease of Brinjal.

**INTRODUCTION**

Brinjal (*Solanum melongena* L.) is a common and popular vegetable crop grown in the tropics and subtropics. It is also extensively grown in China, India, Bangladesh, Pakistan and the Philippines. It is commonly known as egg plant and one of the most important vegetable crops being cultivated in India for the last 4000 years. The global area under brinjal cultivation has been estimated to be at 1.85m ha with total production of about 32 million metric tonnes. It is grown on nearly 550,000 hectares in India, making country as the second largest producer after China. India accounts for about 8.7 million metric tonnes with an area of 0.53 million hectares under cultivation (Anon 1998; Sidhu 1998). Brinjal is susceptible to a wide range of pests and pathogens which causes severe loss in all stages of growth and development. The most significant and widespread diseases are leaf blight and fruit rot (*Phomopsis vexans*), leaf spots (*Alternaria melongenae* and *Cercospora melongenae*), damping off (*Pythium aphanidermatum*), wilt (*Verticillium dahliae*), bacterial wilt (*Pseudomonas solanacearum* and *Ralstonia solanacearum*), little leaf (*Mycoplasma candidatus*) and root knot of brinjal (*Meloidogyne incognita*) (Rangaswamy and Mahadevan 2002). *Phomopsis vexans* (Sacc and Syd.) Harter, (teleomorph: *Diaporthe vexans* Gratz) is a major constraint in the production and productivity of Brinjal. Fruit rot and leaf blight disease caused by *Phomopsis vexans* is a major concern in brinjal production as it reduces yield and marketable value of the crop nearly 20-30% (Jain and Bhatnagar 1980; Kaur et al 1985). Seed is the infection source of *P. vexans* and may serve as a substrate for pathogen survival. The pathogens remain on the seed coat and the cotyledons causing various degrees of seed discoloration (Chaudhary and Hasija 1979). Detection, transmission, location of seed-borne inoculum of *Phomopsis vexans* and its effect on seed quality in Brinjal has been studied (Vishunavat and Kumar 1993, 1994). Recently, the use of molecular marker techniques has improved the accuracy and speed of identification. Hence in the present investigation, primers designed from the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) of the genus *Phomopsis* were used as rapid and reliable markers for the detection of *P. vexans* (Vincelli and Tisserat 2008; Shishido et al. 2010).
Keeping in view of the economic importance of the crop plant and the amount of destruction caused by Phomopsis vexans, in the present study field survey was conducted to collect Phomopsis vexans samples from Mysore and Mandya district regions. Molecular detection of the pathogen was done by using gDNA of Phomopsis vexans and amplified using ITS1 and ITS4 primer pairs and detected using 1.5% agarose gel electrophoresis.

**MATERIALS & METHODS**

**Sample collection and identification of the pathogen**

The leaf blight and fruit rot samples of Brinjal were collected from different areas of Mysore and Mandya Districts, Karnataka (India) (Figure 1). Collected leaf and fruit samples were examined visually and microscopically in the laboratory to identify the symptoms and associated pathogens.

![Map showing sampling sites of Phomopsis vexans isolates collected during the investigation](image)

**Figure 1. Sampling locations of Phomopsis vexans isolated from Mysore and Mandya districts of Karnataka.**

**Isolation of Phomopsis vexans**

The causative agent of the leaf blight and fruit rot disease was isolated from all the seven infected leaf and fruit samples. The fungal isolates were cultured on Potato Dextrose Agar (PDA) medium. All the isolates were subjected to pathogenicity test on 30 days’ old Brinjal plants under green house conditions (Dhingra and Sinclair 1995; Mathur and Kongsdal 2003).

**Pathogenicity test (Koch’s postulates)**

To know the association of fungal pathogen with the leaf blight and fruit rot disease of Brinjal, Koch postulates were conducted. Brinjal seed samples collected from healthy fruits from farmer’s field were used. Each tray containing soil: sand: compost (2:1:1) was sown with seeds of Brinjal. Seedlings of 30 days old were sprayed with conidial suspension of P. vexans (1x105 conidia/ml) using a sprayer. High relative humidity condition was maintained for each inoculated Brinjal plant for 24 hrs by covering with plastic bag. Appearance of leaf blight symptoms was assessed after 15 days of post inoculation.

**Isolation of genomic DNA from isolates of Phomopsis vexans**

The genomic DNA was prepared from the seven (07) different isolates of P. vexans by following the procedure of Zhang et al. (1998) with slight modification (Sreenivasa et al. 2006; Shishido et al. 2010). Approximately 200mg mycelial mat of 12 days old P. vexans isolates grown in potato dextrose broth was taken in microfuge tubes along with 500 µl of lysis buffer (0.13M Tris-HCl, pH 8.9; 0.017M EDTA pH 7.0; 0.83% SDS, 5% PVP and 1M LiCl) (preheated at 65ºC). The samples were ground with blunt end of disposable pipette tips and incubated at 65ºC for 15 minutes followed by vortexing several times. Supernatant was treated with 700µl of phenol: chloroform (1:1) and vortexes for one minute and centrifuged at 2000 rpm for 8 minutes. DNA was re-extracted with an equal volume of ice-cold 99.5% isopropanol and incubated at -20ºC for 60 minutes and again centrifuged at 8000 rpm for 8 minutes and pellet was rinsed with 80% ethanol, air dried, re-suspended in 40µl of nucleic acid free water and used for PCR directly. DNA from Fusarium moniliformae was used as negative control.

**Amplification of isolated DNA using PCR**

The primer pairs ITS1- 5’-CGG ATC TCT TGG TTC TGG CA-3’ and ITS4 - 5’-GAC GCT CGA ACA GGC ATG CC-3’ were used for r-DNA amplification. The primers were synthesized and obtained from Bangalore, Genie, India. PCR was performed using Advanced Thermus 25 Thermocycler (Peqlab, Germany). The PCR amplification was carried out in 50µl reaction mixture containing 1µl of DNA sample with 2.5µl of 10 X PCR buffer, 2.5mM MgCl2, 1.0µl of 2 mM dNTPs, 20pmol of each forward and reverse primer (0.5µl) and 0.2µl of Taq DNA Polymerase and made up to 25µl with 19.3 µl of nuclease free water. The PCR conditions include initial denaturation at 94ºC for 3 min, 35 cycles of denaturation at 94ºC for 30 sec, primer annealing at 55ºC for 30 sec, followed by primer extension for 30 sec, at 74ºC and final extension at 74ºC for 10 min. 1.5µl of each PCR mixture was separated on 1.5% agarose containing 4µl of ethidium bromide and visualised and documented in a Biorad UV transilluminator.
RESULTS & DISCUSSION

All the leaf blight and fruit rot disease samples collected from H.D. Kote (Mysore), Hunsur (Mysore), K.R. Nagar (Mysore), D.M.G. Hally (Mysore), Nagamangala (Mandya) and Srirangapattana (Mandya) showed association of leaf blight causing fungal pathogen *P. vexans*. Field study showed 5-23% and 30-60% disease incidence of leaf blight and fruit rot disease in Mysore and Mandya district. The leaf blight disease of brinjal develops as small necrotic areas and decolorized and later appeared as blighted spots. On adaxial leaf surface, presence of black pycnidia was detected. The infected fruit showed typical zone with concentric rings with extensive rotting on its surface. Upon close examination, pycnidia were also observed as the disease advances.

Cultures were examined for fungal growth and identified based on cultural and microscopic characteristics (Harter, 1914). The fungal pathogen produced characteristic white profuse mycelium with dark coloured abundant pycnidia all over the surface. The pycnidia were dark, ostiolate, immersed, erumpent and globose and the number of pycnidia varied between each isolate. The conidiophores were simple, hyaline, one celled, bigittulate with ovoid to fusoid type conidia. Based on the cultural and microscopic features, the fungal pathogen was identified as *P. vexans* (Figure 2).

Figure 2. A - *Solanum melongena* plant severely infected by leaf blight disease, B - Leaf showing the characteristic blight symptoms, C and D - Different varieties of Brinjal fruits severely infected by *P. vexans*, E - *P. vexans* growing on Potato Dextrose Agar (PDA) medium, F - α-conidia of *P. vexans* (20 µm) under light microscope (45X).
The results of artificial inoculation on 30 day old Brinjal plants showed characteristic leaf spot which later turned blight symptoms by all isolates of P. vexans. In the present study all the seven isolates of P. vexans incited the leaf blight symptoms on 30 day old Brinjal plants under green house conditions. The symptoms persisted throughout the crop season and fruits were also got infected and showed typical fruit rot symptoms after 60 days of post inoculation.

The PCR study showed the amplification of expected 553 bp PCR products in all the seven isolates of P. vexans (Figure 3). Such amplification was not detected in DNA samples isolated from Fusarium oxysporum. This clearly indicated the association of the fungal pathogen P. vexans with leaf spot disease of Brinjal collected from different areas of Mysore and Manadya districts. The objective of study was to use a PCR assay to detect naturally occurring P. vexans in place of conventional culture methods. PCR based techniques have been used to detect large number of plant pathogenic fungi both qualitatively and quantitatively (Kageyama et al. 2003; Chen et al. 2006; Vincelli and Zhao et al. 2007; Tisserat, 2008). All such techniques commonly utilises ITS r DNA to construct PCR primers because of the species specificity of this region. PCR based detection assay has been used for characterization of the fungal pathogen such as Colletotrichum gloeosporioides and C. capsici causing anthracnose in Papaya (Tussell et al. 2008). Shishido et al. (2010) employed PCR to detect and quantify Phomopsis sclerotoides in plants and soils. In the present study the PCR technique was successfully used to amplify the target DNA sequences of seven isolates of P. vexans. The study demonstrated that, PCR assay could detect as little as 0.5 pg of DNA from P. vexans and the detection could be completed within six hours. The availability of such a rapid detection method for this pathogen has great practical applications for an easy and quick detection in place of long and laborious cultural morphological methods. Since the fungal pathogen, affects the host plant at various stages of its development early detection of the pathogen by means of molecular methods certainly help pathologists to look for appropriate management practices.

**REFERENCES**


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**Figure-3. Agarose gel (1.5%) showing amplified PCR products.**

M: 1000 bp marker and Lanes: 1-7 showing amplified products of 553bp from genomic DNA isolated from P. vexans isolates, Lanes: 8 - 9 negative control (Fusarium oxysporum).