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Specific Detection of *Colletotrichum musae* Inciting Anthracnose Disease in Banana

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Abstract: A survey conducted in various parts of Tamil Nadu for the collection of infected banana fruits and isolation *C. musae* was performed. Fourteen isolates of *Colletotrichum musae* were isolated purified and confirmed by morphological methods. The comparison of protein profile among them showed a coomon 65 kDa protein. The monospecific fractionated mycelial protein 65 kDa present in all the isolates were tested for the production of PCA. Standardization of optimum titre value for the detection represents antigen dilution of 1: 1000 and antigenic dilution at 1: 5000. The PCA produced against 65 kDa mycelial protein exhibited the specific reaction with *C. musae* which was confirmed by ELISA and western blotting. PCA was allowed to react with flowers, bracts, remnants under field conditions. The infected fruit tissues recorded highest absorbant values followed by inoculated fruits. By utilizing these PCA, presence of latent infection was detected in inoculated mature green fruits and matured ripened fruits.

Abbreviations: Polyclonal antisera (PCA) % Enzyme linked immuno-sorbant assay (ELISA)
Key words: Colletotrichum musae % 65 kDa mycelial protein % Polyclonal antisera % ELISA

INTRODUCTION

Banana is one of the most popular fruits in India, for national and international trading banana fruits are usually harvested before ripening and stored at relatively low temperature during transportation and market process. Long distance transport and extended storage period in the market may make them sensitive to diseases [1]. Some of important consequences of the diseases are (i) reduction of nutritive value [2] (ii) contamination of food stuffs by mycotoxins produced by pathogens (iii) toxic metabolites produced by diseased plant tissue in response to fungal attack (iv) unacceptable taste of products associated with diseased materials. Among the various factors responsible for postharvest losses in banana, especially anthracnose caused by *C. musae*, is a major one intended for local as well as distant markets [3,4].

Detection of specific fungus from the fruit surface of banana is difficult because of latency. Therefore, rapid, reliable and accurate diagnostic tools are required to detect and identify causal organism. Clark [5] emphasized the potential of enzyme-linked immunosorbent assays for detecting and identifying plant pathogen in much lower concentrations than was possible by classical methods. Majority of assays and experiments have been with plant viruses, but same technique has been attempted to detect bacterial and fungal plant pathogen. Immunological methods provide a promising tool for effective detection of specific pathogen in infected plant tissues [6-8].

Sible [9] showed that a Polyclonal antiserum raised against 65 kDa polypeptide from mycelial extracts of C. *musae* reacted specifically with *C. musae* and detected the latency of pathogen under field conditions. Similarly, Viswanathan and Samiyappan [10] purified and raised antiserum against 101 kDa monospecific protein from *Colletotrichum falcatum* which reacted specifically with all the isolates of *C. falcatum* causing red rot disease in sugarcane.

Sundaram *et al.* [11] reported that polyclonal Antibody based indirect ELISA, raised against purified mycelial proteins from *Verticillium dahliae*, reacted positively with 11 of 12 isolates from potato, cotton and soil; but negatively with one isolate from tomato and also with non-target pathogens *viz. Fusarium oxysporum, Colletotrichum lindemuthianum, Rhizoctonia solani, V. nigrescens* and *V. tricorpus*, the antiserum reacted strongly with *V. dahliae* and intensely with *V. albo-atrum.*

Velicheti *et al.* [12] used polyclonal Antibody based ELISA and attempted to develop a method for early detection of *Phomopsis phaseoli* and *P. longicolla* of

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soybean. Unfortunately, their assay system yielded cross-reaction with all *Phomopsis* sp. and *Colletotrichum truncatum*, although it discriminated *Phytophthora sojae*, *Rhizopus* sp. *Rhizoctonia solani*, *Septoria glycines* and *Cercospora kikuchii*, hence cross reactions are often a menace for serological based detections.

As an alternative, polymerase chain reactions (PCR) havebeen developed by using specific primer to pathogens [13]. The PCR assay is proved to be a highly sensitive method for detecting very low titers of pathogens. But enzyme-linked mmunosorbent assay (ELISA) is often preferred over PCR because a specific primer should be developed for PCR test and there are technical difficulties encountered in screening the DNA from conidia, which is time-consuming when testing a large number of samples [14].

The present study was formulated based with the objective to detect the latent infection of mycelia in the harvested fruits so as to derive at the appropriate management practices.

MATERIALS AND METHODS

Collection and Isolation of *C. Musae* Isolates: The infected fruits of banana showing typical anthracnose symptoms were collected in butter paper bags and later placed in polythene covers. The samples were used for recording the disease symptoms. The samples were first microscopically examined to confirm the presence of the fungus. The diseased tissues were teased with a sharp blade on a glass slide having a drop of clear water and covered with a cover slip to confirm the presence of fungal spores under the binocular research microscope (10 X). After confirming the presence of fungal spores, isolation was carried out in the laminar flow chamber under aseptic conditions following a standard tissue isolation method [15].

The infected tissue of fruits which showed typical symptoms were cut into small bits measuring about two mm, surface sterilized in 0.1 per cent mercuric chloride solution for one minute and washed repeatedly thrice in sterile distilled water to remove the traces of mercuric chloride. Surface sterilized tissues were transferred to sterile Petri plates containing PDA medium under aseptic conditions. The inoculated Petri plates and slants were incubated under sterilized bell jar at room temperature (28±2°C) and observations were taken at regular intervals. A total of 14 isolates were collected from different parts of Tamil Nadu (Table 1).

Table 1: Isolates of *C. musae* used in the study

Isolate	Variety	Place of collection
Cm1	Nendran	Coimbatore
Cm2	Nendran	Erode
Cm3	Nendran	Tirunelveli
Cm4	Karpooravalli	Erode
Cm5	Nadan	Madurai
Cm6	Rasthali	Coimbatore
Cm7	Robusta	Coimbatore
Cm8	Hill banana	Madurai
Cm9	Karpooravalli	Coimbatore
Cm10	Nadan	Erode
Cm11	Poovan	Kancheepuram
Cm12	Poovan	Coimbatore
Cm13	Red banana	Coimbatore
Cm14	Poovan	Trichirappalli

Identification of the Pathogen: The pathogen was identified up to species level based on their cultural and morphological characters. A loop full of fungal culture grown on PDA plates were taken on a glass slide and observed with image analyzer under 100 x magnifications for the presence of conidia and conidiophore. After confirming the spores, the cultures were purified by single spore isolation technique.

Protein Profile by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Extraction of Mycelial Protein: One gram of powdered mycelial sample was extracted with one ml of 0.1 M Potassium phosphate buffer (pH 7.0) under 4°C. The homogenate was centrifuged for 20 min at 10000 rpm. The supernatant served as protein source and was used for the SDS-PAGE [16]. The protein content of the sample was determined by Bradford method [17].

Forty microgram of protein from each isolate was taken and mixed with 10 µl of sample buffer in microfuge tube, boiled for four min and incubated at 4°C for 30 min.

Then the samples containing equal amount of proteins were loaded into the wells of polyacrylamide gels (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used. Electrophoresis was carried out at constant voltage of 75 volts. The gels were stained with 0.2 per cent Coomassie brilliant blue (R250) solution. Based on the R_m value of each protein band stained, the molecular weight was calculated.

Purification of Single Protein from Colletotrichum Musae: Purification of the mycelial protein of *C. musae* was carried out by ammonium sulphate fractionation followed by electroelution. Frozen mycelia of *C. musae* isolate *Cm7* (100 g) were homogenized in 250 ml of 0.1

M Potassium phosphate buffer (pH 7.0) using a pre-chilled mortar and pestle. The homogenates were centrifuged at 12,000 rpm for 20 min in a refrigerated centrifuge at 4°C and the supernatant solutions were collected. Ammonium sulphate was added to the supernatant to precipitate the proteins at different saturation levels viz. 0-20, 20-40, 40-60 and 60-80 per cent saturation and incubated at 4°C for overnight under constant stirring. Each fraction was obtained by centrifugation at 10,000 rpm for 20 min at 4°C. The precipitates were dissolved in 5 ml of extraction buffer and dialyzed against the same buffer using dialysis membrane with molecular cut off 12,000 to 14,000 Dalton (Spectra/Por, SPECTRUM Laboratories, CA, USA.). Each ammonium sulphate fraction was then analyzed by SDS-PAGE as described above. The 65 kDa protein from 40 to 60 per cent fraction was cut from the gel and electroeluted for 3 h at 12 mA against the 0.1M Potassium phosphate buffer. The electroeluted protein was analyzed by 12 per cent SDS-PAGE.

Production of Polyclonal Antisera: The polyclonal antisera (PCA) against the fractionized 65 kDa specific protein were raised in rabbit by intramuscular immunization method. The protein content was assessed and 500 µg protein was used for each immunization. Adult New Zealand white rabbits weighing about 1.5 kg (3 months old) were used. Freund's complete adjuvant (1.0 ml) was added to 1.0 ml of antigen (500 µg protein/ml). The contents were mixed thoroughly and taken in a sterile syringe with 22G needle and administered intramuscularly in the rabbits. Four injections were given at weekly intervals (7 days) and booster injection was given 4 weeks after first injection with the same quantity of antigen added with Freund's Incomplete adjuvant. After fourth injection, blood was collected from the vein transferred to sterile glass vials allowed to stand in a slanting position till the blood clotted. The antiserum was transferred to sterile centrifuge tubes and the red blood cells were pelleted by repeated centrifuge (8000 rpm at 4°C for 10min) three times finally the serum was transferred to sterile microfuge tube and stored at -70° C for further studies.

Western Blot Analysis

SDS-PAGE: Crude mycelial protein of *C. musae* and other mycelial proteins were fractionated by SDS-PAGE (12 per cent) using Mini-Sigma gel unit (Sigma-Aldrich Techware system, Sigma, USA) according to a standard procedure [16].

Western Blotting: Western blotting was carried out according to the method prescribed by Gallagher *et al*. [18]. Fractionated proteins were transferred onto a nitrocellulose membrane (Protran BAS 5 Cellulosenitrat, Schleicher and Schuell, Germany) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, USA).

The membranes were then stained with Ponceau S stain (Sigma, USA) for two min to check the resolution and transfer quality and destained using 1X TBS buffer. Then, the membrane was kept in blocking buffer (0.1 per cent Ovalbumin in 1X TBS containing 1 per cent Tween 20) for 2 h at room temperature with a gentle shaking (50 rpm) on a rotary shaker. The blot was, then, incubated for 2 h at room temperature with a gentle shaking in an Carbonate buffer (pH 9.6) containing polyclonal antibodies raised in rabbits against the 65 kDa purified protein(1:1000 dilution).

The unbound primary antibody was removed with 2 to 3 washings with antiserum buffer and the blot was incubated for 2 h at room temperature in goat anti-rabbit IgG Alkaline Phosphatase conjugate (Bangalore Genei Pvt. Ltd. Bangalore, India) diluted (1:5000) in antiserum buffer. The unbound secondary antibody was removed with 2 to 3 washings with TBST (1X PBS + 0.1 per cent Tween 20). Polypeptides which are recognized by the specific antibody were visualized by incubating the membrane in dark with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma-Aldrich Co. USA).

Indirect ELISA (DAC-ELISA): Wells of polystyrene microtiter plate (Immunolon-2-Removawells; Dynatech Laboratories, Alexandria, Va.) were coated with 100 µl mycelial protein of *C. musae* isolate with (1:50, 1:100, 1:500, 1:1,000, 1:3,000 and 1:5,000) dilutions and incubated at 37°C for 4 h. The plate was then washed with 0.01M Phosphate- buffered saline, pH 7.2 (PBS) containing 0.02 per cent (vol/vol) Tween 20 (PBS-T) for 3 times at 1 min interval. After washing, 100 µl of antibody raised against the 65 kDa *C. musae* mycelial protein (1:50, 1:100, 1:500, 1:1,000, 1:3,000, 1:5,000 and 1:10,000 dilutions) was added to each well and incubated at 37°C for 4h. Then the plate was washed three times with PBS-T and 100 µl of AP-labelled goat anti-rabbit IgG (1:8000 dilution) (GeNei, Bangalore, India) was added to each well and incubated at 37°C for 4 h. Finally the wells were washed 3 times with PBS-T and 100 µl of freshly prepared *p*-nitrophenyl phosphate (1.0 mg/ml) in 0.2M Tris buffer

pH 7.0, (Sigma *FAST*, Sigma, USA) was added to each well. The plate was incubated at 37°C for 30 min for colour development and the optical density was measured at 405 nm with an ELISA reader. The reaction was stopped by addition of 50 µl of 3 M NaOH.

Testing the Reactivity of the Pca with C. Musae and Common Phytopathogenic Fungi by Indirect ELISA: The reactivity of PCA raised against 65kDa polypeptide was tested against mycelial proteins extracted from *C. musae, C. gloeosporioides, C. capsici, C. falcatum, Rhizoctonia solani, Macrophomina phaseolina, Fusariium oxysporum* f.sp. *cubense, Alternaria solani* and *Pythium aphanidermatum* by Indirect ELISA. The mycelial proteins of the above fungi were extracted by following the procedure mentioned in section 2.7.

Testing the Reactivity of the PCA with Filed Residues and with the Field Samples of Banana Cv. Poovan by Indirect ELISA: For testing the reactivity of the PCA for detection of the pathogen under field conditions, samples were collected from banana fields at random during last hand emergence. The samples included tissues from bract, fruit, floral remnants and flowers. In addition, samples were also drawn from artificially inoculated tissues of ripe banana fruit and naturally infected fruit cv. poovan. The tissue samples were homogenized in 0.1 M Phosphate buffer pH 7.0 (1:1 w/v) and used as antigen for indirect ELISA as mentioned in section2.7.

ELISA for Green and Ripened Inoculated Fruits for Detection of Latency: For testing the reactivity of the PCA for detection of the pathogen under latent conditions, samples (Matured green fruits and ripened fruits) were used, samples were artificially inoculated with *C. musae* spore, protein were extracted at different intervals (0 h to 36 h with an interval of 6 h). The tissue samples were homogenized in 0.1 M Phosphate buffer pH 7.0 (1:1 w/v) and used as antigen for indirect ELISA as mentioned in section above section.

RESULTS

Collection and Isolation of Pathogen: Banana fruits showing typical symptoms of anthracnose were collected during the survey from different banana growing areas of Tamil Nadu. Four isolates of *C. musae* were isolated and purified. The isolates were maintained on PDA slants for further studies.

Identification of the Pathogen: Pathogen associated with anthracnose disease was isolated on PDA medium and it was identified as *C. musae* (Berk. and Curt.) Arx based on the morphological and cultural characteristics of the fungus.

SDS-PAGE Analysis of Protein Profile of *C. Musae:* SDS-PAGE profile of 14 isolates of *C. musae* is given in (Fig.1). A polypeptide of 65 kDa was selected after ammonium sulphate fractionation (Fig. 2). Presence of single band in the purified protein extract was confirmed by SDS-PAGE (Fig. 3).

Reactivity of the Polyclonal Antiserum with the Other Organism Using Western Blot Analysis: Results of western blotting showed that the *C. musae* mycelial protein reacted strongly with its antiserum which was exhibited by the presence of discrete bands at the 65 kDa position in the blot. The reaction didn't amplified the protein extracts of other organism (Fig. 4).

Reaction of Pca by Elisa: Standardisation of titre for polyclonal antiserum (PCA) raised against 65 kDa polypeptide of C. musae Standrardization of the titre of PCA raised against 65 kDa revealed that an antigen dilution of 1:1,000 and antiserum dilution of 1:5,000 was required for the significant difference to be detected in ELISA. The reactions of the different dilutions of antisera against the respective dilutions of antigen are furnished in Table 2.

Reactivity of the Pca with Mycelial Proteins of Certain Fungal Pathogens: In, Indirect ELISA, the PCA gave highest mean absorbance for mycelial protein extract of *C. musae* compared to other fungal pathogens tested. Among the mycelial proteins tested the maximum mean absorbance at 405 nm was recorded in *C. musae* (0.453), followed by *C. gloeosporioides* (0.188). The least absorbance was recorded with mycelial protein extract of *Rhizoctonia solani* (0.159) (Fig 5).

Reactivity of PCA with Anthracnose Infected Banana Tissues, Field Residues and Infected Fruits: ELISA reaction with the PCA gave highest mean absorbance for mycelial protein extract of *C. musae* (positive control) compared to banana tissues and residues tested. Among the banana tissues tested, infected fruit tissues and inoculated fruit were recorded highest absorbances with 0.370 and 0.369 respectively at

Table 2: Standardization of ELISA for the polyclonal antiserum raised against 65 kDa polypeptide of *C.musae*

	Antigen Dilution							
Antiserum Dilution	BUFFER	1:50	1:100	1:500	1:1000	1:3000	1:5000	1:10000
1:50	0.201	0.869	0.743	0.893	1.486	1.151	0.463	0.443
1:100	0.189	0.535	0.471	0.542	0.535	0.483	0.454	0.443
1:500	0.187	0.681	0.507	0.566	0.734	0.62	0.635	0.342
1:1000	0.208	0.775	0.719	0.506	0.495	0.551	0.657	0.243
1:3000	0.201	0.612	0.512	0.494	0.59	0.49	0.385	0.184
1:5000	0.192	0.429	0.67	0.416	0.5	0.266	0.085	0.245

Table 3: ELISA for the banana field samples with PCA raised against 65 kDa polypeptide of *C. musae* RESIDUES AND FRUITS

	Buffer	Bract	Flower	Remnants	Healthy Fruit	Infected Fruit	Inoculated Fruit	Mycelial Protein
R1	0.180	0.187	0.200	0.194	0.207	0.356	0.373	0.452
R2	0.177	0.198	0.214	0.185	0.187	0.398	0.349	0.487
R3	0.196	0.214	0.192	0.198	0.178	0.395	0.373	0.464
R4	0.159	0.205	0.205	0.177	0.197	0.345	0.378	0.395
R5	0.140	0.204	0.204	0.196	0.176	0.356	0.374	0.369
Mean	0.170	0.201	0.203	0.190	0.189	0.370	0.369	0.433

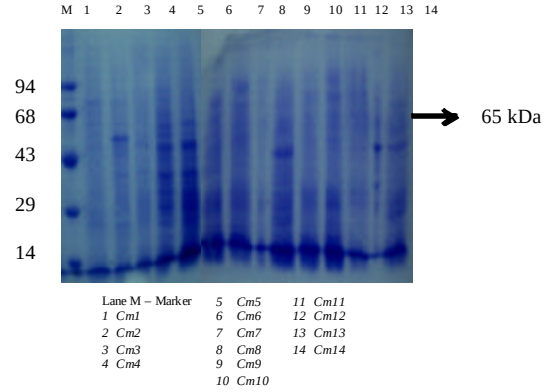


Fig. 1: Protein profile of C. musae isolates

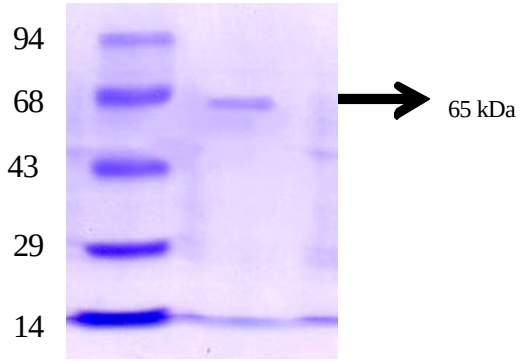


Fig. 3: 65 kDa purified protein

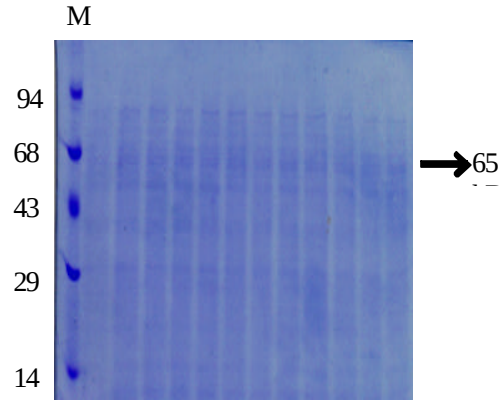
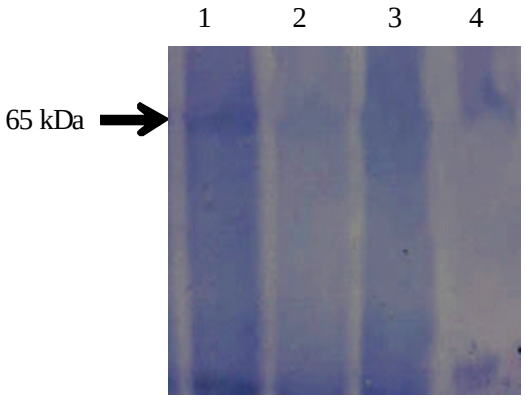


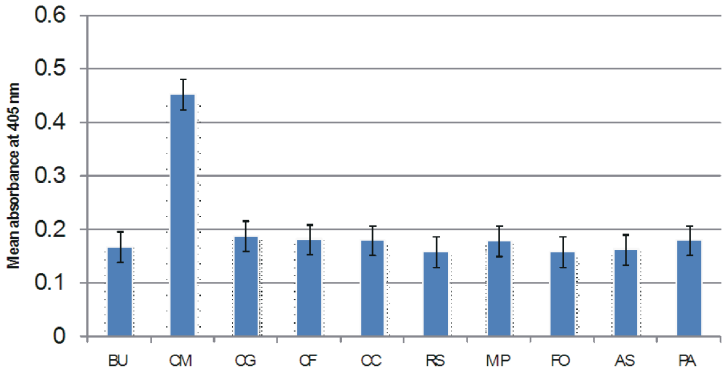
Fig. 2: 60% protein precipitation by Ammonium Sulphate fractionation

405 nm. All the other tissues *viz*. bract, flower, remnants and healthy fruits were on par and found to be apparently healthy (Table 3).



1. *Colletotrichum musae*
2. *C. gloeosporioides*
3. *C. capsici*
4. *C. falcatum*

Fig. 4: Western blot analysis of 65 kDa protein antisera



BU	:	Buffer	RS	:	*Rhizctonia solani*
CM	:	*Colletotrichum musae*	MP	:	*Macrophomina phaseolina*
CG	:	*C. gloeosporioides*	FO	:	*Fusarium oxysporum f.sp. cubense*
CF	:	*C. falcatum*	AS	:	*Alternaria solani*
CC	:	*C. capsici*	PA	:	*Pythium aphanidermatum*

Fig. 5: Reaction of PCA with various mycelial proteins of other plant pathogens

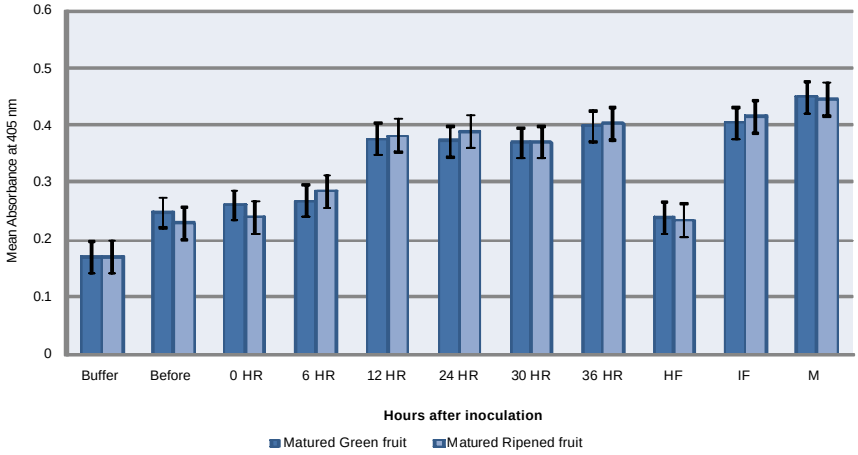


Fig. 6: ELISA reactions of Green and matured inoculated fruits at various intervals

ELISA Reaction for the Detection of Latency: Increased activity of PCA was observed significantly from 12 h after inoculation with *C. musae* both in green and ripened fruits. The mean absorbance value at 12 h after inoculation was 0.377 and 0.382 in green and ripened fruited respectively. The maximum absorbance was noticed at 36 h after inoculation. But a marked increase in absorbance was noticed from 6 h to 12 h [Fig. 6].

DISCUSSION

C. musae is the most important pathogen on wounded green and ripe banana fruits [19-20]. The infection usually starts as quiescent infections on green fruit in the field. However, successful penetration of the fungus is restricted by accumulation of phytoalexins as the fruits ripen [21]. Therefore, symptoms generally can be seen only in overripe fruits. Anthracnose becomes a serious problem when bananas are shipped as bunches for a long time and ripened under high temperature [22].

In the present study, at the early stages of infection, it is difficult to recognize the presence of the disease in the field as the infection is profound latent, the characteristic symptoms of the disease develops on fruit at later stages during ripening. Serological methods (Enzyme Linked Immunosorbent Assay) are widely used for these purposes because they allow sensitive, specific and simultaneous analysis of many samples in a single microplate. Kratka *et al*. [23] diagnosed the latent infection caused by *C. acutatum* in strawberry plants and the latent form of pathogen was detected in apparently healthy roots, crowns, petioles and fruits by using the ELISA and DIBA techniques. However, molecular methods based on polymerase chain reaction

(PCR) are being used more frequently for detecting fungal pathogens in plant tissues and fruits [24,9], owing to their increased specificity and sensitivity compared with more traditional techniques. Also, large numbers of samples can be processed in a short time by these methods.

In the present study, in order to develop immunological method of detection of *C. musae* in banana fruits, protein extracts obtained from mycelia of *C. musae* isolates were analyzed by SDS-PAGE to identify a specific protein for each isolate. However, the results indicated that all the *C. musae* isolates tested share a common protein profile, similar results were obtained by Sible [25]. Hence step-wise ammonium sulphate fractionation followed by electroelution was performed to yield a monospecific protein with molecular weights of 65 kDa from *C. musae* isolate *Cm*7.

The 65 kDa purified protein from *C. musae* isolate *Cm*7 was used as antigen source to raise polyclonal antibodies in New Zealand white rabbit. The developed polyclonal antibody was tested for detection of *C. musae* by ELISA and immunoblot analysis. The polyclonal antibodies specifically detected *C. musae* in infected fruits, when tested with ELISA. In Western blot analysis, a protein band with a molecular weight of 65 kDa reacting to *C. musae* antiserum was detected in protein samples from mycelial protein extract of *C. muase* whereas no bands where detected in other organisms. The ELISA results also revealed that the developed antibody was very sensitive and could detect *C. musae* proteins even at a dilution of 1:10,000. The optimal working dilution of the antiserum was found to be 1:5,000. The raised antiserum can able to detect the inoculum in green fruits as early as 12 h upon inoculation before any symptom production. This result confirms the finding of Postmaster *et al*. [26] who observed that the appresorium formation takes place between 6 and 12 h after inoculation in banana anthracnose. Hence, the raised antisera can able to detect the latent infection. The high specific reactivity of the antiserum towards the infection of *C. musae* indicates its potential suitability for ELISA-based detection of anthracnose disease of banana.

These results are in accordance with the findings of Viswanathan *et al.* [27] who had detected red rot pathogen in sugarcane by ELISA using polyclonal antisera produced against unfractionated protein and a monospecific 101 kDa polypeptide protein.

They concluded that the polyclonal antisera raised against 101 kDa polypeptide were found to be highly specific in detecting *Colletotrichum falcatum* in sugarcane.

Shanmugam *et al*. [28] developed PCA in rabbit and chicken against rice sheath blight toxin. The degree of sheath blight infection in rice tissues was correlated with toxin produced by *Rhizoctonia solani*. The polyclonal antisera have been used for screening *R. solani* isolates for toxin production. Polyclonal antisera are widely used to detect the fungal pathogens.

From the current study, we conclude that the PCA produced against 65 kDa mycelial protein exhibited the specific reaction with *C. musae* isolates. By utilizing these PCA, presence of latent infection was detected in inoculated mature green fruits and matured ripened fruits, flowers and bracts, remnants in field conditions. Hence immunological tools are powerful for the detection of pathogens.

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