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Development of Polyclonal antibody based detection of *Curvularia lunata* causing Leaf blight of *Persea bombycina*

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Polyclonal antibody was raised in white rabbit against *Curvularia lunata,* causal agent of leaf blight of *Persea bombycina* Kos, coomonly known as 'Som'plant, a new record of foliar disease. Effectiveness of mycelial antigen raising antibody was confirmed by immunodiffusion test,Dot immunobinding assay. The titre value of PAb of *C. lunata* was determined by PTA-ELISA format. Leaf antigens from eight morphotypes of healthy and naturally infected som plant were also analysed by Dot Blot and PTA-ELISA to detect the presence of the pathogen in the leaves. Detection of pathogens in leaf tissue was also confirmed by indirect immunofluorescence test. Early detection of pathogens in leaf tissues after artificial inoculation with spores of *C. lunata* was also performed using PTA-ELISA where it was found that presence of pathogen could be detected as early as 24 hrs after inoculation whereas the symptoms of the disease was not established in the plant before 96 hrs at the earliest. Thus by using these immunoassays, early detection of the pathogens in the leaves can be done that would help to develop management strategies for the leaf blight disease of som plant.

Key words: *Persea bombycina*, *Curvularia lunata*, immunodiffusion, dot blot, PTA-ELISA, immunofluorescenece

INTRODUCTION

Persea bombycina Kost, commonly known as 'Som' plant is one of the economically important plant of Assam, India. Its leaves are the food for the silk-worms *Anthareae assama* which produces the golden yellow silk, Muga. *Litsea monopetala* (Sualu) is one of the primary host plant of muga silkworm (Acharya et al.,2013). Sericulture is an agro-based industry playing an important role in rural economy of the country. High demand of muga silk has led to the domestication of som plants and rearing of silkworms in closed area. These plants are now being grown in West Bengal, mainly in Coochbehar district. Since leaf quality has significant impact on quantity and quality of the silk fibre, for sustaining muga culture it is important to ensure availability of adequate quantity of qualitatively superior leaves.

A major problem in cultivation of healthy som plants are associated with various foliar fungal diseases that eventually reduce the quality of the leaves which directly affects the quantity and quality of the silk produced and in turn the economy of the state. The common foliar fungal diseases of this plant as reported earlier include grey blight caused by *Pestalotiopsis dessiminate* (Das et al, 2010), leaf spot caused by *Phyllosticta persea* and leaf blight caused by *Colletotrichum gleoesporioides* (Chakraborty et al, 2014). In the experimental field of Department of Botany, University of North Bengal, around 60-70% of the som plant leaves were found to be infected. Initial symptoms include the leaf tip turning black that eventually spreads towards the base and the whole leaf turns black, dry and withers away (Fig.1D-F). After completion of Koch's postulate, morphological characterization and molecular identification of this pathogen was done by 18S rDNA sequence followed by BLAST analysis for confirming the causal organism. A new fungal pathogen causing leaf blight of som plant was identified as *Curvularia lunata* (Fig. 1A-C) and the sequence was deposited to NCBI database (Accession no. KM491737).

Recent trends in detection of plant pathogens include the development of more rapid diagnostic

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techniques with high specificity for the target organism. These techniques can be used to detect fungi, bacteria and viruses present in low quantities and on plant tissues and therefore in many cases the pathogen can be detected at an earlier stage of disease development than was previously possible. (Chakraborty and Chakraborty, 2018). In the present investigation, polyclonal antibody based serological detection of *C. lunata* was developed using various immunological formats such as immundiffusion. Dot immunobinding assay, PTA-ELISA and immunofluorescence in order to develop management strategies of the leaf blight disease of som plant.

MATERIALS AND METHODS

Plant sample

Eight different morphotypes (S1, S2, S3, S4, S5, S6, S7 and S8) of som plants (*Persea bombycina*) were collected from Boko, Assam and maintained under glass house condition in the Department of Botany, University of North Bengal.

Fungal culture

Fungal pathogen was isolated from infected leaves of som plants (morphotype S5). Repeated isolation in Potato dextrose agar (PDA) produces white mycelia that turn black within 7 days at 28-30°C. Conidia produced were usually septate, bulged in the centre (Fig.1 A&B) and germinated within 12 hrs from the first septum (Fig 1C).Identification to species level was confirmed by sequence analysis of 18S rDNA of the fungus that was amplified using ITS1and ITS4 primers. Following BLAST analysis the sequence was deposited to NCBI database (Accession no. KM491737). The fungus was identified as *Curvularia lunata*.

Inoculation technique and disease assessment

The pathogen (*C. lunata*) was grown in 100 ml PDA medium in 250 ml flask for 10 days till sporulation was evident. Spores were suspended in sterile distilled water to prepare a spore suspension containing $3X10^4$ spores per ml. This suspension was mixed with few drops of Tween 20 and sprayed on to the leaves of potted som plants. The disease severity on the leaves was recorded on the basis of a 0-5 rating scale and calculated following the method of Chakraborty *et al* (2014).

Preparation of antigen

Antigens were prepared from mycelia of *C. lunata*, healthy and artificially inoculated leaf tissue of som plants following the methods as described by Chakraborty et al (2016). Fungal mycelia and leaf tissues were crushed separately in 0.05 M sodium bicarbonate buffer (pH 9.6) in a chilled mortar and pestle and centrifuged at 10,000 rpm. The supernatant was collected and used as antigen.

SDS-PAGE analysis of proteins

Total soluble protein content was determined following the method of Lowry et al (1951) and resolved on SDS-PAGE by a standard procedure (Sambrook et.al. 1989). The molecular weight of protein bands visualized after staining with coomassie blue in SDS-PAGE were determined from the known molecular marker. The band in the gels were further analyzed by IMAGE-LAB version 5.1 software.

Polyclonal antibody preparation

Polyclonal antibody was raised against mycelia antigen of *C. lunata* in New Zealand white male rabbit according to the method of (Acharya et al, 2015) . Before immunization, normal sera were collected from rabbit. Following injection schedule with antigen, blood samples were collected and kept at 37^0C for 1 h for clotting, followed by centrifugation at 5000 rpm for 10 min at room temperature. IgG was purified from the serum as described by Clausen (1988)

Immunodiffusion

Agar gel double diffusion tests were performed using PAb raised against *C. lunata* following the method of Ouchterlony (1967). Following the white precipitin reactions as evident in each gel plates were stained with coomassie blue and then excess stain was eluted with a solvent for destaining following the standard methods. This treatment allows the visualization of precipitin bands as blue bands on a clear background.

Dot immunobinding assay

Mycelial antigens prepared from *C. lunata* were loaded on nitrocellulose membrane filter using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay

was performed using PAb of *C. lunata* as outlined by Lange et al (1989).

Plate trapped antigen coated (PTA) ELISA

Optimization of ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Antigens from fungal pathogens as well as antigens from healthy and infected leaves were diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5% BSA. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (pNPP) (100mg/ml) were used for PTA-ELISA as enzyme substrate. Reaction was terminated after 60 min and the absorbance values were recorded as mean of five adjacent wells measured at 405nm essentially as described by Chakraborty and Sharma (2007).. Absorbance values were measured at 405 nm in an ELISA reader (Microplate Reader, Analytical technologies Ltd). Absorbance values in wells not coated with antigens were considered as blanks.

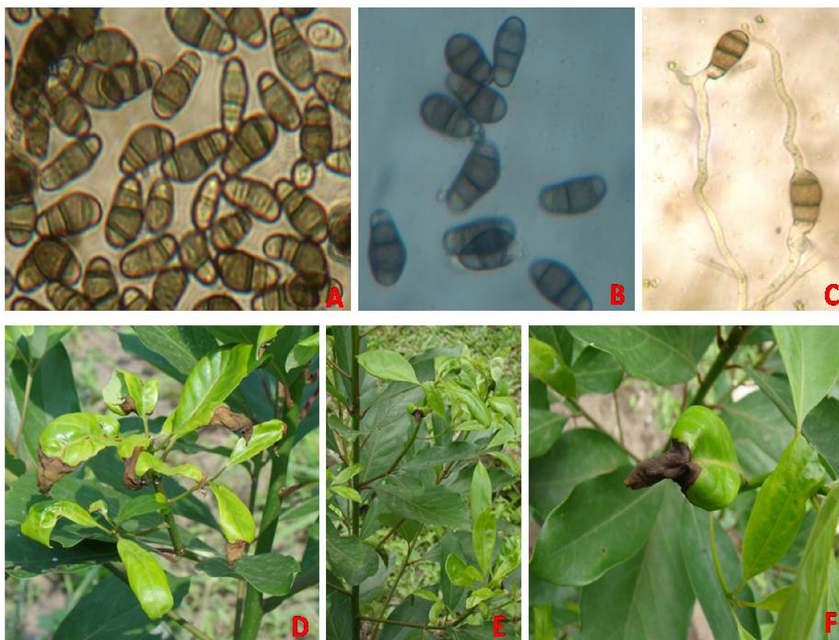

Fig. 1 : (A and B): Spores of *Curvularia lunata*; (C): Germinating spores of *C. lunata*; (D-F): Disease symptoms showing leaf blight of som plant.

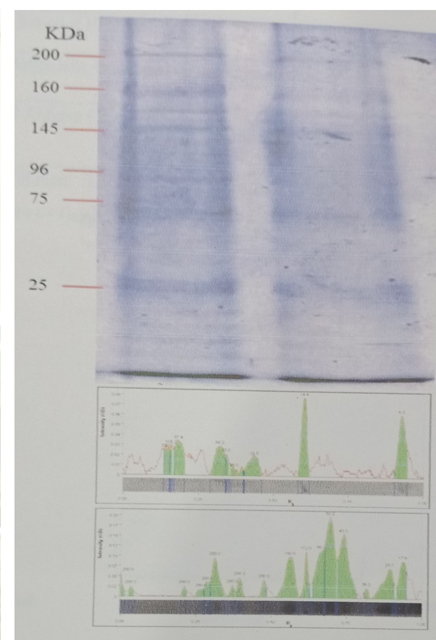

Fig. 2 : SDS-PAGE analysis (top) and gel image scanning through Image-Lab version 5.1 software(below) of mycelia antigen of *Curvularia lunata*

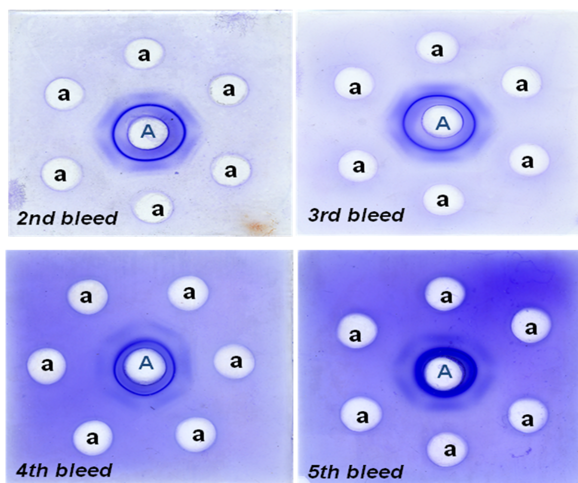

Fig. 3 : Immunodiffusion test with mycelia antigen(a) in peripheral well reacted with antibody of *Curvularia lunata* (A) in central well.

Fluorescence antibody staining and microscopy

Five days old mycelia of *C. lunata* was treated with PAbs of *C. lunata* and goat antisera specific to rabbit globulins conjugated with fluorescein isothiocyanate (FITC). As described by Acharya et al.(2015). At the same time thin cross sections of healthy and leaf blight infected leaf tissues were also treated in similar manner. Observations were made using a Biomed microscope (Leitz) equipped with an I-3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Moticam Pro 285B.

RESULTS ANS DISCUSSION

Curvularia lunata has been reported earlier as pathogen causing leaf spot of maize (Akinbode, 2010), *Amaranthus spinosa* (Sharma et al, 2011) and leaf blight of Rice (Kamaluddeen et al, 2013). However *Curvularia lunata* has not been reported as a leaf pathogen for som plant earlier which was identified and the sequence was deposited to NCBI database (Accession no. KM491737). SDS-PAGE analysis of soluble proteins of *C. lunata* revealed protein band of molecular weight ranging from 20 to 200 KDa (Fig.2). Effectiveness of mycelial antigen of *C. lunata* in raising antibody was assessed using immunodiffusion test. Strong precipitin reactions were observed when mycelia antigen was reacted with antibody of 2nd, 3rd, 4th and 5th bleedings. However, intensity was higher from 4th and 5th bleeding. (Fig.3).

Optimization of ELISA

Optimization of ELISA was done considering two variables, dilutions of antiserum and antigen to obtain the maximum sensitivity. Antiserum dilutions ranging from 1:125 to 1:16000 were tested against homologous antigen at a concentration of 5mg/L. Absorbance values in ELISA decreased from the dilution of1:125 to 1:2000 after which it levelled off. Dilutions of antigen concentration in two fold series ranging from 25 to 1600mg/L were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigen concentrations. Concentration

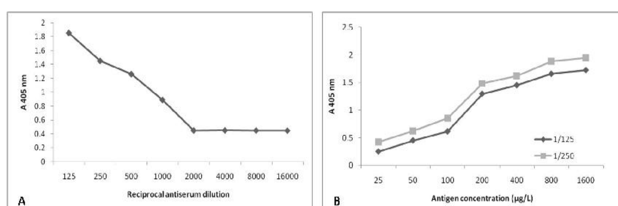

Fig. 4 :Optimization of ELISA by considering two variable, (A) dilution of the antiserum and (B).dilution of the antigen extract.

as low as 25ig/L can be easily detected by ELISA at both antisera dilutions (Fig. 4).

Detection of pathogen using DIBA and PTA-ELISA format

Effectiveness of mycelial antigen of *C. lunata* in raising antibody was assessed using Dot immunobinding assay (DIBA). Development of deep violet colour following homologous reaction with antigen and antibody confirm its identity (Fig. 5). Effectiveness of mycelial antigen of *Macrophomina phaseolina* (Chakraborty *et al.* 2012), *Bipolaris sorokiniana* (Chakraborty et al , 2016), *Drechslera oryzae* (Khati and Chakraborty, 2019) in raising antibody against these fungal pathogens were also evident as a positive reaction between the antigen and antibody on nitrocellulose membrane.

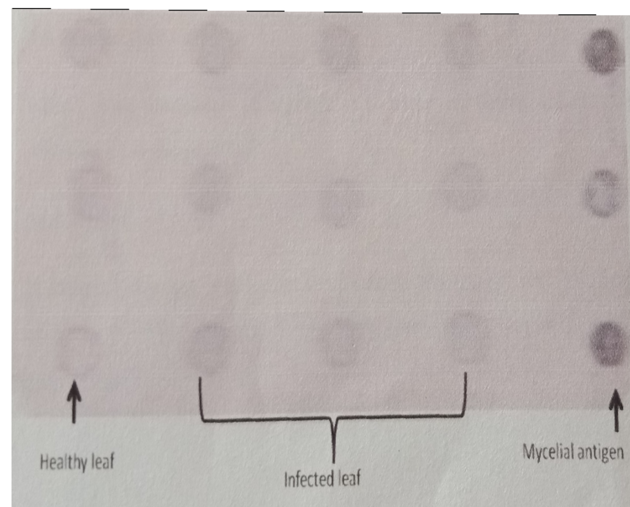

Fig. 5: Dot immunobinding assay with mycelia antigen of *Curvlaria lunata*, healthy and *C. lunata* infected leaf antigen of som plant (morphotype S5) probed with PAb of the pathogen.

Antigens prepared from healthy and infected leaf samples of S5 morphotype were reacted with PAb of *C. lunata* on nitrocellulose membrane. Results revealed development of violet colour in infected leaf samples indicating the presence of leaf blight disease in som plants. In the next set of experiments eight different morphotypes (S1-S8) of som plants were used for the detection of infection. Antigen preparations from both healthy and naturally infected leaves of som plants were tested against the 1:125 dilution of antiserum of *C. lunata*. Results are presented in the Table 1. Absorbance values for healthy leaf samples were significantly lower than corresponding infected leaf samples. This technique can therefore be used to detect the presence of the fungal pathogen in the plant easily and fast. Early and rapid diagnosis of brown spot of rice disease caused by *Drechslera oryzae* and spot blotch of wheat caused by *Bipolaris sorokiniana* were also performed using DIBA technique where infected samples depicted dark

Table 1: ELISA values showing reaction of antiserum of *C. lunata* with antigens of healthy and naturally infected som leaf samples

Morphotype	Antigen concentration at 40 µg/L		Colour intensity on Nitrocellulose membrane	
	Healthy	Infected	Healthy	Infected
S1	0.084±0.023	0.899±0.014	+	++
S2	0.084±0.007	0.975±0.009	+	++
S3	0.080±0.011	0.898±0.011	+	++
S4	0.076±0.009	0.988±0.015	+	++
S5	0.082±0.014	1.127±0.007	+	+++
S6	0.095±0.031	1.129±0.009	+	+++
S7	0.097±0.003	0.998±0.008	+	++
S8	0.088±0.004	0.890±0.006	+	++

*Antisera used at 1:125 dilutions, absorbance taken at 405nm, '±' Standard error
Colour reaction '+' – Pink, '++' – Violet, '+++' – Deep violet.

Table 2: ELISA values showing reaction of antiserum of *C. lunata* with antigens of healthy and artificially inoculated som plants at different time intervals

Morphotype	Time interval (h)	Healthy	Inoculated
S5	24	0.055±0.003	0.167±0.010
	48	0.058±0.001	0.399±0.008
	72	0.047±0.006	0.887±0.009
	96	0.042±0.008	1.198±0.120
S6	24	0.030±0.002	0.155±0.160
	48	0.039±0.001	0.542±0.007
	72	0.036±0.004	0.777±0.018
	96	0.042±0.002	1.189±0.016

*Antisera used at 1:125 dilution, Antigen concentration at 40ìg/ L,absorbance taken at 405nm, '±' Standard error

blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Chakraborty *et al,* 2016, Khati and Chakraborty, 2019).

Natural infected leaves of two different morphotypes (S5 and S6) showing highest absorbance values in PTA-ELISA test were further artificially inoculated with *C. lunata*. Antigens were extracted at 24hr interval for 4 days from both healthy and inoculated leaves. These antigens (40ìg/L) were tested against *C. lunata* PAb at 1:125 dilution. Infections could be detected from 24hrs onwards in PTA-ELISA format based on higher absorbance values of infected leaf extracts in comparison to healthy leaf extracts as presented in Table - 2. Here we could see that detection of infection using PTA- ELISA was possible before the appearance of symptoms which was evident on leaves after 3days of inoculation In order to facilitate implementation of disease management strategies effectively, early and reliable detection of pathogen is important. Indirect PTA-ELISA format was employed for the detection of *Macrophomina phaseolina* (Chakraborty *et al*., 2012), *Bipolaris sorokiniana* (Chakraborty et al. 2016) and *Glomerella cingulata* (Som and Chakraborty, 2016). causing root rot disease of mandarin, spot blotch disease of wheat and brown blight disease of tea respectively.

Indirect immunofluoresecence assay

In the present study indirect immunofluorescence of young hyphae of *C. lunata* was carried out with homologous antibody and reacted with fluorescein isothiocyanate (FITC) labelled antibodies of goat specific for rabbit globulin. Strong apple green fluorescence was seen in mycelia which was confirmation of the homologous reaction of the pathogen and the antibody (Fig 6A). Chakraborty *et al*. (2012) conducted indirect immuno fluorescence with young hyphae and sclerotia of *M. phasolina* with homologous PAb to obtain apple green fluorescence confirming the pathogen.

In case of *P. disseminata* spores only the setulae and appendages showed apple green fluorescence as the conidia are dark septate, confirming the identity of the pathogen (Acharya *et al.* 2015). Similarly, treatment of mycelia of

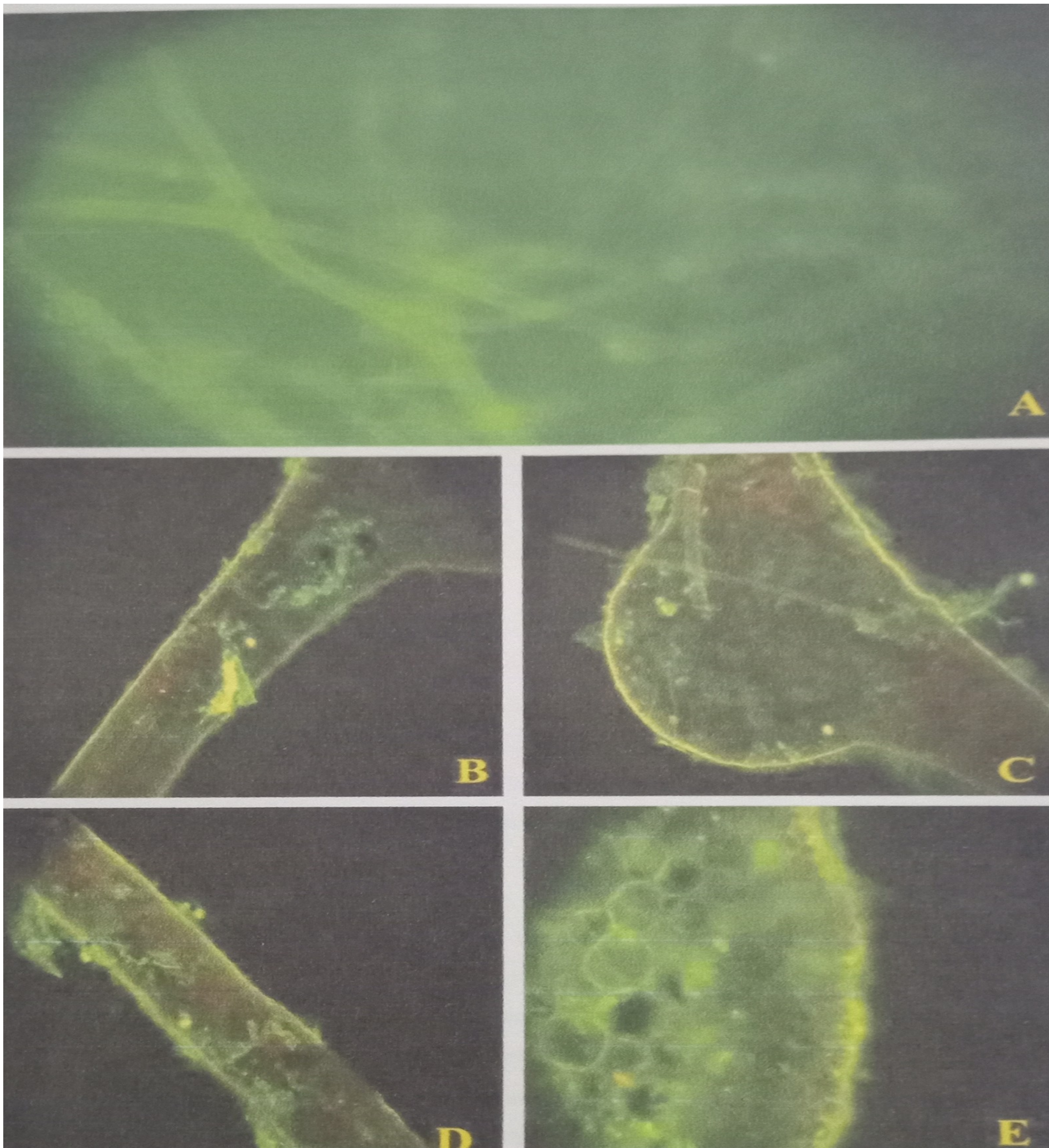

Fig. 6 : Immunofluorescence of (A) mycelia (*C. lunata*), healthy leaf tissue (B-D) and infected leaf tissue (E) reacted with PAb of the pathogen and labelled with FITC conjugate showing bright apple green fluorescence.

Drechslera oryzae with its own antiserum followed by FITC labelling developed a general fluorescence that was more intense on young hyphal tips (Khati and Chakraborty, 2019).In this study indirect immunofluorescence of healthy leaf tissue treated with PAb of *C. lunata* showed autofluorescence of the cuticle layer of the segment that indicates that pathogen is not present in the healthy section (Fig.6B-D). Whereas in case of infected leaf section, apple green fluorescence is seen at the centre at the same time present in small quantities throughout indicating the spread of infection in the leaf (Fig 6E). Based on these observation, early detection of the pathogen (*C. lunata*) in the leaves can be done that would help to develop management strategies for the leaf blight disease of som plant.

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