INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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SEROLOGICAL DETECTION OF *PESTALOTIOPSIS DISSEMINATA* IN *PERSEA BOMBYCINA* CAUSING GREY BLIGHT DISEASE

[a]Amrita Acharya, [b]Shilpi Ghosh & [a]Bishwanath Chakraborty
[a]Immuno-Phytopathology Laboratory, Department of Botany
[b]Department of Biotechnology, University of North Bengal, Siliguri, Darjeeling – 734013

ABSTRACT
Polyclonal antibody was raised in white rabbit against *Pestalotiopsis disseminata*, causal agent of grey blight of *Persea bombycina* Kost. Effectiveness of mycelial antigen raising antibody was confirmed by Dot immunobinding assay as well as Western Blot analysis. The titre value of the antibody 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 *P. disseminata* 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 96hrs at the earliest. Thus by using these serological tools easy and early detection of the pathogens in the leaves can be done that would help to develop better and faster management strategies for the grey blight disease.

KEYWORDS: *Persea bombycina*, Grey blight, early detection, PTA-ELISA, DIBA, Immunofluorescence.

INTRODUCTION

Muga silk is an exclusive prerogative of the North East India and more particularly in the Brahmaputra valley of Assam. The people of more than 38,000 villages in this region fully depend upon sericultural activities. The muga silkworm is a multivoltine and polyphagous insect. Som *(Persea bombycina* Kost) and Soalu *(Litsea monopetela* (Roxb.) Pers, are the primary food plants of muga silkworm (Choudhury, 1970). Som leaves improves silk producing ability whereas, soalu leaves enhances egg laying capacity of muga silkworm. The nutrition of silkworm entirely depends upon the quality of leaves. The food plants (leaves) have significant effect on health and survival of silkworms. Better the quality of leaves greater the possibility of obtaining good quality cocoons (Khanikar and Unni, 2006). Growth of silkworm, cocoon quality and quantity of raw silk entirely depends upon the quality of leaves (Chakravorty *et al*., 2006). Diseases, unfavorable weather conditions, insect pests, poor agronomical practices, un-wanted weeds are the main reasons for low productivity. The muga food plant som is vulnerable to many foliar diseases that affect the normal growth of the plant, quantity and quality of leaves and ultimately cocoon yield production (Das *et al*., 2003). Grey blight is one of the major foliar fungal diseases of som caused by *Pestalotiopsis disseminata* (Thum) Stey (Bharali, 1969). The symptoms of the disease are the appearance of small, oval, and discolour lesions which are irregularly scattered on the leaves. The brown or grey spots develop irregularly in the subsequent days after infection (Das *et al*., 2010). As the disease development is in progress, the spots get collapsed, malformed and ultimately the entire leaf dries up (Keith *et al*., 2006). This has been reported as a major epidemic disease of muga host plant, som, causing 13.8-41.6% leaf yield loss (Bharali, 1969; Das and Benchamin, 2000). The disease is so severe that it leads to shortage of quality leaves for rearing of muga silkworm finally causing severe economic loss to farmers.

Recent trends in detection of plant pathogens include the development of more rapid diagnostic techniques with high specificity for the target organism (Chakraborty, 1988). 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, 2003). Since *P. disseminate* causes huge losses to the farmers, the present study deals with the early detection of the disease by the help of various serological methods that might lead to certain degree of management of grey blight disease.

MATERIALS & METHODS

Plant material

Eight different morphotypes of som plant *viz*. S1 to S8, was collected from Central Muga and Eri Research and Training Institute (CMER&TI), Jorhat Assam and maintained in experimental field condition of Immuno-phytopathology lab, Department of Botany, University of North Bengal

Fungal culture

The pathogen (Som/P/01) was isolated in PDA media from naturally infected som leaves of S1 morphotype and the

fungal culture was purified using hyphal tip method in PDA slants. These slants were maintained for further studies. For light microscopic studies, hyphae and spores were stained with Lactophenol-cotton blue.

Inoculation technique and disease assessment

Artificial inoculation of som leaves were done following the method of Chakraborty *et al*. (1996). Conidial suspension of *P. disseminata* (3 X 10^6 conidia ml^{-1}) was placed on adaxial surface of each leaf (2-6 droplets/ leaf) with a hypodermic syringe. In control sets drops of sterile water were placed on the leaves. Humid conditions were maintained by covering each tray with a glass lid and sealing with petroleum jelly. Trays were kept at 25°C.

Assessment of inoculums infectivity and symptom development were done on the basis of percentage of drops that resulted in lesion production after 72hrs (Chakraborty & Saha, 1994). Observations were based on 50 inoculated leaves for each morphotype.

Preparation of antigen

Antigens were prepared from mycelia of *P. disseminata*, healthy, naturally infected and artificially infected leaves of som plant following the method described by Chakraborty and Purkayastha (1983). They were stored at -20°C till further use.

Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelia antigen of *P. disseminata* following the method of Chakraborty & Purkayastha (1983). Normal sera were collected by ear vein puncture from the rabbits before immunization. The antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant was injected subcutaneously at weekly interval for six consecutive weeks. The blood samples were collected after six weeks following injection and kept for 1h at 37°C. The clots were loosened and stored at 4°C. The antisera was clarified by centrifugation and then stored at -20°C till further use. IgGs were purified by DEAE-Sephadex column chromatography following the protocol of Clausen (1988).

Dot immunobinding Assay

Mycelial antigens prepared from *P. disseminata* were loaded on nitrocellulose membrane filters using Bio-Dot Apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *P. disseminata* as outlined by Lange *et al*. (1989).

Western Blotting

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electro transferred to NCM using semi-dry Trans-blot unit (Bio-Rad) and probed with PAbs of *P. disseminata* following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indoylphosphate (NBT-BCIP) as substrate. Immuno-reactivity of the proteins was visualized as violet-coloured bands on the NCM.

PTA-ELISA

Optimization of ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100mg/ml) were used for PTA-ELISA as enzyme substrate (pNPP), reaction was terminated after 60 mins and the absorbance values were recorded as mean of five adjacent wells measured at 405nm essentially as described by Chakraborty & Sharma (2007). 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 (100mg/ml) was used for PTA-ELISA as enzyme substrate (pNPP) in PTA-ELISA test. 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.

Fluorescence antibody staining and microscopy

Five days old mycelia and conidiospores of *P. disseminate* were treated with PAbs of *P. disseminata* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC). At the same time thin cross sections of healthy and grey blight infected leaf tissues were also treated in similar manner. Observations were made using a Biomed microscope (Leitzz) 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 & DISCUSSION

Identification of the pathogen

The pathogen was isolated in Potato Dextrose Medium (PDA) from infected leaves. It was purified using hyphal tip method and further grown in petriplates. The pathogen grew as a sheet of white mycelium and after 7 days black acervulus was formed. Identification of the pathogen was based on the microscopic view of the spores present in the acervuli. The spores contained dark septate cell and transparent setulae and appendages, characteristic feature of *Pestalotiopsis* sp. (Fig 1).

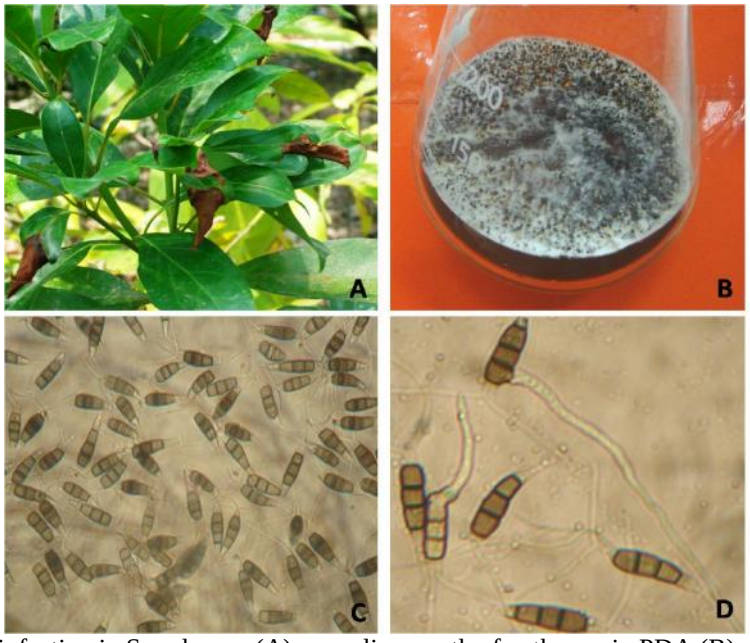

FIGURE 1: Grey blight infection in Som leaves (A), mycelia growth of pathogen in PDA (B), spores of pathogen (C-D)

Dot Immunobinding Assay (DIBA)

Effectiveness of mycelia antigen of *P. disseminata* in raising antigen was assessed using Dot immunobinding assay (DIBA). Development of deep violet colour following homologous reaction with antigen and antibody confirm its identity (Fig. 2) Chakraborty *et al.* (2012) studied the effectiveness of mycelia antigen *Macrophomina phaseolina* in raising antibody against the pathogen by confirming a positive reaction between the antigen and antibody on nitrocellulose membrane.

FIGURE 2: DIBA showing reaction of *P. disseminata* antigen with PAb of *P. disseminata* (Homologous reaction)

Optimization of ELISA

Optimization of ELISA was done considering two variables, dilution of the antigen extract and dilution of the antiserum 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 of 1:125 to 1:2000 after which it levelled off. Dilutions of antigen concentration in two fold series ranging from 25 to 1600µg/L were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigen concentrations. Concentration as low as 25µg/L can be easily detected by ELISA at both antisera dilutions (Fig 3).

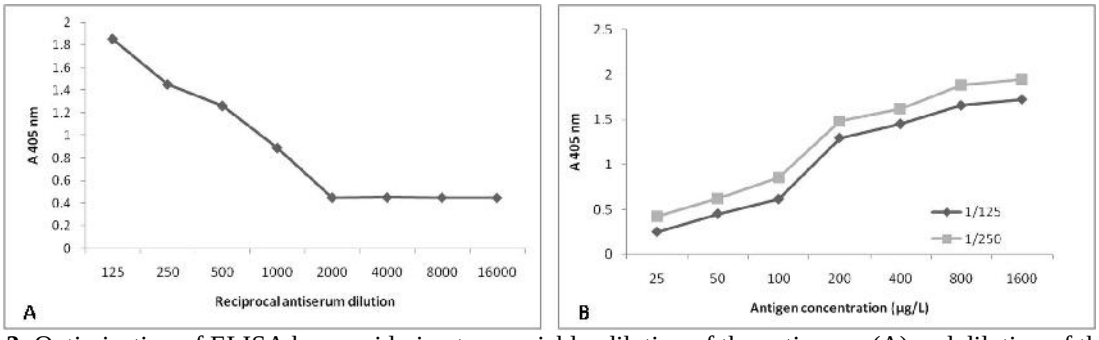

FIGURE 3: Optimization of ELISA by considering two variable, dilution of the antiserum (A) and dilution of the antigen extract (B).

Detection of pathogen using PTA-ELISA format and DIBA

Leaf antigen of both healthy and infected samples of eight different morphotypes was reacted with PAb of *P. disseminata* on nitrocellulose membrane. Results revealed development of deep violet colour in infected leaf samples indicating the presence of grey blight disease in som plants. In the next set of experiments eight different morphotypes of som plants, viz. S1 to S8 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 *P. disseminata*. 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 red rot disease in sugarcane caused by *Colletotrichum falcatum* Went was also performed using DIBA technique where infected samples depicted dark blue precipitate on the nitrocellulose membrane due to the antigen-antibody reaction (Hiremath *et al*., 2004).

TABLE 1: ELISA values showing reaction of antiserum of *P. disseminata* with antigens of healthy and naturally infected som leaf samples

Morphotype	Antigen concentration at 40µg/L		Colour intensity on Nitrocellulose membrane	
	Healthy	Infected	Healthy	Infected
S1	0.084±0.021	0.890±0.015	+	++
S2	0.084±0.004	0.989±0.005	+	++
S3	0.080±0.011	0.898±0.011	+	++
S4	0.076±0.008	0.988±0.014	+	++
S5	0.082±0.011	1.108±0.002	+	+++
S6	0.095±0.03	1.123±0.009	+	+++
S7	0.087±0.003	0.998±0.006	+	++
S8	0.081±0.004	0.890±0.008	+	++

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

Leaves of two different morphotypes, S5 and S6, showing highest absorbance values in case of natural infection, were artificially inoculated with *P. disseminata* as described in materials and methods. Antigens were extracted at 24hr interval for 4 days. These antigens (40µg/L) were tested against *P. disseminata* antisera at 1:125 dilution Infections could be detected from 24hrs onwards in ELISA on the basis of higher absorbance values of infected leaf extracts in comparison to healthy leaf extracts (Table 2). Here we could see that detection of infection by ELISA was possible before the symptoms generally appeared on detached leaves after 3days of inoculation. In order to facilitate implementation of disease management strategies effectively, early and reliable detection of pathogen is important. Indirect ELISA format was employed for the detection of *Phytophthora infestans* causing the potato late blight disease even before the first appearance of visible symptoms (Narayanasamy, 2010), *Pestalotiopsis theae* causing grey blight disease in tea (Chakraborty *et al.*, 1996), *Exobasidium vexans* causing blister blight disease in *Camellia sinensis* (Chakraborty and Sharma, 2007) as well as *Macrophomina phaseolina* causing root rot disease of *Citrus reticulate* (Chakraborty *et al*., 2012). Chakraborty *et al*. (2009) also reported that PTA ELISA format could easily detect pathogen *Exobasidium vexans* in susceptible variety of *Camellia sinensis* (AV-2) as early as 24h after artificial inoculation whereas the disease symptoms were not visible before 12 days. Similar strategy can be used to detect grey blight pathogen in som plants at an early stage using this ELISA format.

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

Morphotype	Time interval	Healthy	Inoculated
S5	24hrs	0.045±0.003	0.152±0.010
	48hrs	0.049±0.001	0.369±0.008
	72hrs	0.041±0.006	0.877±0.009
	96hrs	0.042±0.008	1.189±0.120
S6	24hrs	0.029±0.002	0.135±0.160
	48hrs	0.030±0.001	0.448±0.005
	72hrs	0.036±0.004	0.767±0.014
	96hrs	0.040±0.002	1.172±0.014

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

Western Blot analysis

Western blot analysis using PAb of *P. disseminata* was also performed to develop strategies for rapid detection of the pathogen. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed followed by probing with alkaline phosphatase conjugate. The bands on nitrocellulose membrane were compared with bands on SDS-PAGE. Bands of varying molecular weights was seen in SDS-PAGE but two bands of molecular weights around 68 and 65KDa respectively were seen on nitrocellulose membrane suggesting these two to be the respective epitope of the antibody (Fig 4). Hence the results suggest that Western Blot format can be used as a refined tool for detection of the pathogen. Kitagawa et al (1989) also used Western blot analysis to identify *F. oxysporum* f. sp. *cucumrium* among other *Fusaria* by first analysing the mycelia antigen of the pathogen on SDS-PAGE and then performing Western blot analysis using homologous PAb.

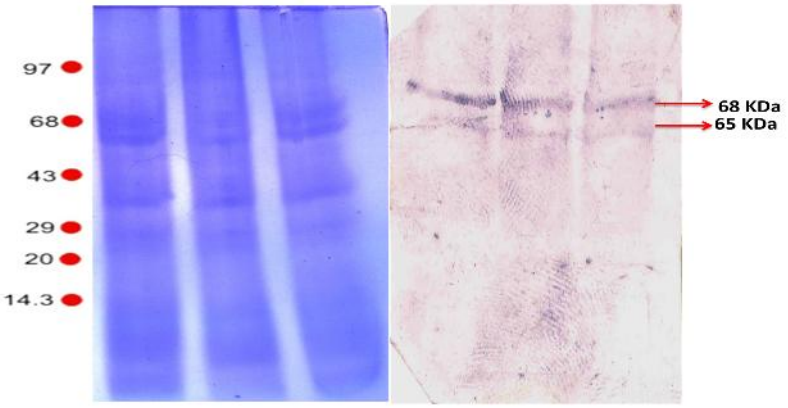

FIGURE 4: Western Blot analysis of protein profile (SDS-PAGE) of *P.disseminata* antigen with homologous PAb

Indirect immunofluoresecence assay

In the present study indirect immunofluorescence of young hyphae and spores of *P. disseminata* 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 both mycelia and spores which was confirmation of the homologous reaction of the pathogen and the antibody (Fig 5).

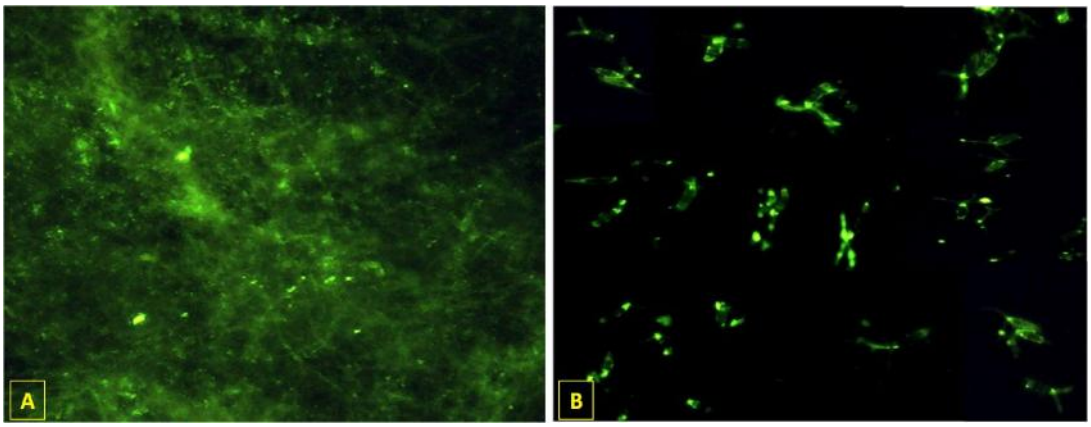

FIGURE 5: Indirect immunofluorescence of *P. disseminata* mycelia (A) and spores (B)

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. Chakraborty *et al*. (2012) conducted indirect immune fluorescence with young hyphae and sclerotia of *M.phasolina* with homologous PAb to obtain apple green fluorescence confirming the pathogen. Similarly, treatment of mycelia and conidia of *P. theae* with its own antiserum followed by FITC labelling developed a general fluorescence that was more intense on young hyphal tips and on the setulae and appendages of the conidia (Chakraborty *et al*., 1995). In this study indirect immunofluorescence of healthy leaf tissue segement with PAb of *P. disseminata* showed autofluorescence of the cuticle layer of the segment that indicates that pathogen is not present in the healthy section. 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 6).

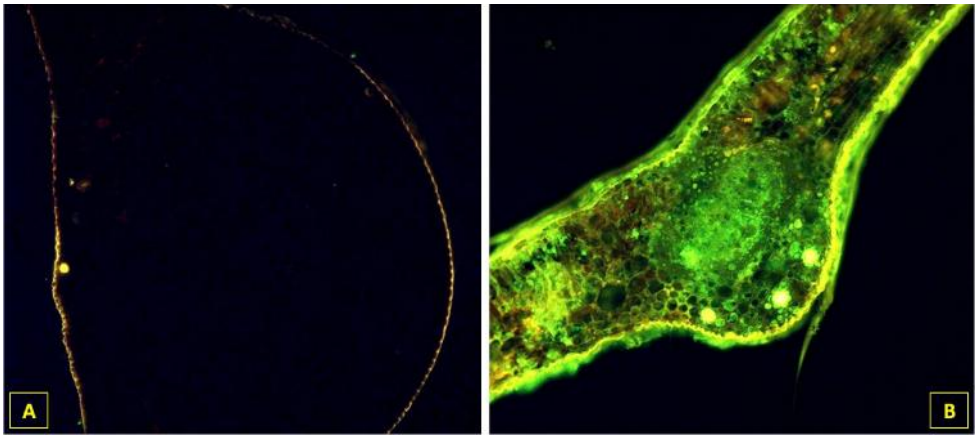

FIGURE 6: Indirect immunofluorescence of healthy leaf tissue (A) and blight infected leaf tissue (B) treated with PAb of *P. disseminata*

CONCLUSION

From the present study it can be concluded that serological techniques are easy, fast and reliable methods to detect grey blight pathogen in som plants. Early detection of pathogen will help in developing of management strategies against grey blight disease that will in turn help to improve the leaf quality and health status of som plant.

ACKNOWLEDGEMENT

Authors would like to acknowledge the financial help received from Department of Biotechnology, Ministry of Science and Technology, Govt. of India for carrying out this study.

REFERENCES

Bharali, N. (1969) Grey blight disease of Som. *Plant Sci. Cult* **35**, 573-574.

Chakraborty B.N. & Sharma, M. (2007) Serological detection and immunogold localization of cross-reactive antigens shared by *Camellia sinensis* and *Exobasidium vexans*. *J. Appl. Microbiol* **103**, 1669–1680.

Chakraborty B.N., Sengupta, D. & Sharma, M. (2009) Serological relationship between *Exobasidium vexans* and *Camellia sinensis*. *Ind phytopathol* **62(1)**, 19-26

Chakraborty, B.N., Chakraborty, U., Rai, K., Sunar, K. and Dey, P.L. (2012) Serological and molecular detection of *Macrophomina phaseolina* causing root rot of *Citrus reticulate*. *NBU J Pl Sci*. **6(1)**, 77-86.

Chakraborty, B.N. (1988) Antigenic disparity. In Experimental and Conceptual Plant Pathology, Editors, Singh, U.S., Heiss, W.M. and Weber, D.J. Oxford and IBH Publishing Company, India, 477–484.

Chakraborty, B.N. & A. Saha (1994) Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. *Physiol Mol Plant Pathol.* **44,** 403–416.

Chakraborty, B.N. & Purkayastha, R.P. (1983) Serological relationship between *Macrophomina phaseolina* and soybean cultivars. *Physiol Plant Pathol.* **23**, 197–205

Chakraborty, B.N. & U. Chakraborty (2003) Immunodetection of plant pathogenic fungi. In Frontiers of Fungal Diversity in India. Editors, Rao, G.P., Manoharachari, C., Bhatt, D.J., Rajak, R.C. and Lakhanpal, T.N. International Book Distributing Company, India, 23–41.

Chakraborty, B.N., Basu, P., Das, R., Saha, A. & Chakraborty, U. (1995) Detection of cross reactive antigens between *Pestalotiopsis theae* and tea leaves and their cellular location. *Ann Appl Biol,* **127**, 11–21.

Chakraborty, U., Basu, P., Das, R., Saha, A. and Chakraborty, B.N. (1996) Evaluation of antiserum raised against *Pestalotiopsis theae* for detection of grey blight disease of tea by ELISA. *Folia Microbiol* **41**, 413–418.

Chakravorty R., Barah, A., Neog, K., Das, K., Kakati, P.K. (2006) Variability in muga silkworm *Antheraea assamensis* Helfer. *Indian Silk*, 16-17.

Choudhury, S. N. (1970) Muga Silk Industry, Directorate of Sericulture, Govt of Assam, Dispur Guwahati, 23.

Clausen, J. (1988) Immunochemical techniques for the identification and estimation of macromolecules. In Laboratory Techniques in Biochemistry and Molecular Biology. Editors, Burdon, R.H. and Van Knippenberg, P.H. The Netherlands: Elsevier, 464.

Das R. & Benchamin, K.V. (2000) Diseases of muga and eri food plants: Incidence pattern, Intensity and control measures. Proceedings of National Seminar on Sericulture, R & D in Muga and Eri, CMER&TI, Jorhat Assam, 34-45.

Das R., Das, K., Benchamin, K.V. (2003) Diseases of muga food plant som. *Indian Silk* **41**, 23-24.

Das R., Chutia, M., Das, K. & D.K. Jha (2010) Factors affecting sporulation of *Pestalotiopsis disseminata* causing grey blight disease of *Persea bombycina* Kost., the primary food plant of muga silkworm. *Crop Protect* **29,** 963-968.

Hiremath L. & G.R. Naik (2004) Rapid diagnosis of sugarcane red rot by Dot-immunobinding assay (DIBA) technique. *Ind J Biotech* **3**, 542-545.

Khanikar, D. & Unni, B.G. (2006) Food plant Biochemistry and its role in growth and development of muga silkworm, *Antheraea ammamica* W.w. In: Muga silkworm Biochemistry Biotechnology Molecular-biology. Editor, Unni, B.G., RRL, Jorhat, 88-101.

Kitagawa, T., Sakamoto, Y., Furumi, K. and Ogura, H. (1989) Novel enzyme immunoassays for the detection of *Fusarium oxysporum* f. sp. *cucumerinum* and for general detection of various *Fusarium* species. *Phytopathol* **79**, 162–165

Laemmili, U.K. (1970) Cleavage of structural proteins during assembly of the head of bactiriophage T_4. *Nature* **227,** 265-275.

Lange, L., Heide, M. and Olson, L.W. (1989) Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reaction by scanning electron microscopy. *Phytopathol* **79**, 1066–1071.

Narayanasamy, P. (2001) Plant pathogen detection and diagnosis. 2nd Ed. Marcel Dekker, Inc. publication.

Wakeman, A.J. & White, J.G. (1996) Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physio. Mol. Pl Pathol* **48**, 289-303.