Serological detection of latency incited by *Colletotrichum gloeosporioides* and *Lasidiplodia theobromae* in mango for indexing in storage

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Abstract
The postharvest pathogens Colletotrichum gloeosporioides and Lasidiplodia theobromae inciting quiescent infection caused huge economical loss during storage. Twenty-five pathotypes of C. gloeosporioides and sixteen of L. theobromae were isolated from different regions of Tamil Nadu, India. The SDS- PAGE assay showed the variation in protein profile analysis. The polyclonal antibodies (PCA) were raised against the unfractioned mycelial protein (UMP) of 40 and 70 kDa for C. gloeosporioides and 70 kDa for L. theobromae polypeptide present in all pathotypes. Standardization of antigen and antiserum dilutions revealed that the dilutions of antibodies were standardized by indirect ELISA as 1:1000 for the 40 kDa antibody of C. gloeosporioides and 1:3000 for the 70 kDa antibody of C. gloeosporioides and L. theobromae. The antigen dilutions were standardized as 1:3000 for C. gloeosporioides and 1:2500 for L. theobromae was found to be optimum for the detection of anthracnose and stem end rot pathogen. Both antisera detected the C. gloeosporioides and B. theobromae antigen in ELISAs and Western blots.

The PCA raised against 40-kDa exhibited the specific L. theobromae isolates in ELISA and blotting immunoassay techniques. By utilizing both PCA, the presence of latent infection was observed in healthy-looking leaves, flowers and fruits in orchard conditions. The fruit tissues recorded high absorbance values followed by flowers and leaves in all the detection methods. This technology is suggested for indexing the mango variety for the latency infection.

Keywords: Latent infection, Antigen, Antibody, ELISA, Western blot.

Introduction

Mango is the most important fruit crop in India having socio-economic significance and has been acclaimed as the 'King of fruits'. It occupies 35% of the total area under fruits comprising of 2.5 million hectares with a total production of 18 million tonnes. The post-harvest loss of mango fruits accounting to 25-40 per cent of the total production, worth billions of dollars is a cause of major concern worldwide. In India, during post-harvest handling, mango is subjected to infection by twenty different genera of fungi.[11] Among the diseases, anthracnose, caused by *Colletotrichum gloeosporioides* (Penz. and Sacc.) and stem-end rot caused by *Lasiodiplodia theobromae* (Pat.) Griffon and Maublanc, are of post-harvest significance.[8,15] Both *C. gloeosporioides* and *L. theobromae* cause infection in the field and are carried over to fruits wherein they remain latent and establish upon fruit ripening.

Thus unripe mango fruits that appear healthy during harvest may develop typical symptoms of the diseases upon ripening.[13] Although the extensive use of postharvest fungicide treatment ruins the key policy of disease control, it is less valuable due to the prolonged and latent existence of pathogen from the orchard onwards.[20] In this context, early diagnosis in the orchard may assist to surmount the quiescent infection in later stages. It is also indispensable for timely management practices to reduce the extent of postharvest loss. In the past, the presence of latent infection has been substantiated by isolating the fungus from surface sterilized fruits or by dipping the fruits in paraquat solution.[3]

In this technique, the anthracnose and stem end rot pathogen grow saprophytically on the senescent tissue and can be identified based on their morphological, cultural and spore ontogeny characteristics. But these methods comprise of demerits by causing damage to plant tissue and also time-consuming process. The pathogen may not display its typical morphology on paraquat- or fungicide-treated fruits due to systemic nature of chemicals.[14] They may suppress the growth and sporulation of the Colletotrichum due to the inhibitory effect. Hence, there is a need for swifter, precise techniques for the detection of latent infection in mango. Recently, the utilization of modern methods may provide positive diagnostic results than other conventional methods. The detection based on serological techniques has been developed for many pathogens of plants.[2,16,17]

They are more sensitive and specific for the detection of *C. gloeosporioides* and *L. theobromae* pathogen. Earlier study from Viswanathan et al[17] reported that the serological methods including ELISA and Western blot analysis were highly useful to detect the *C. falcatum* in sugarcane.

The polyclonal antibodies raised against pathogen protein (antigen) revealed the presence of dormant structures in sugarcane varieties. Similarly, Kratka et al[9] developed immunochemical methods to detect the quarantine pathogen of strawberry *C. acutatum*.

Recently, Faisal et al[2] also developed an immuno-based detection kit using PCA raised against 65-kDa polypeptide for early detection of latent post-harvest anthracnose pathogen in banana. Based on these important considerations, the present investigation was undertaken (i) to detect the latency in different mango varieties (ii) to develop PCA against *C. gloeosporioides* and *L. theobromae* pathogen protein and (iii) to standardize different serological techniques for prompt detection of post-harvest pathogens

Material and Methods

Isolation of pathogen from fruits: The survey was conducted in different agroclimatic zones comprising major mango-growing areas and markets in Tamil Nadu, India and collected anthracnose-and stem end rot infected fruit samples. The isolation was carried out from infected fruits by plating the portions showing typical symptoms of anthracnose and stem end rot on potato dextrose agar medium and used in the tests. Totally, twenty isolates of *C. gloeosporioides* and sixteen isolates of *L. theobromae* were isolated and pathogenicity was proved.

Extraction of UMP and specific polypeptide protein: Mycelial discs of actively growing cultures of *C. gloeosporioides* and *L. theobromae* isolates were inoculated on PDA broth and incubated on a rotary shaker (100 rpm at room temperature for 7 days). Biomass obtained 10 days after inoculation was filtered through Whatmann filter paper no. 1 and washed three times with sterile distilled water. The excess moisture was removed by blotting with filter papers. The biomass was homogenized in a prechilled mortar placed in an ice-bath with pestle using phosphate buffer, 0.1 M (pH 7.0). The homogenate was centrifuged at 10 000 g for 20 min at 4°C. The supernatant containing mycelial protein was collected and stored at-70°C.

Identification and comparison of specific polypeptide protein found in *C. gloeosporioides* isolates: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli[10] using a separating gel of 12% acrylamide and a stacking gel of 0.8% to separate the mycelia proteins for the identification of specific polypeptide protein. The protein extracts from high, medium and low virulent *C. gloeosporioides* pathotypes along with protein samples from other *Colletotrichum* spp and mango stem end rot pathogen were separated.

Purification of single protein of *C. gloeosporioides* and *L. theobromae*: Purification of the mycelial protein was carried out by ammonium sulphate fractionation followed by electroelution. Frozen mycelia of *C. gloeosporioides* isolate *Cg 4* and *Lt* 14 of *L. theobromae* were homogenized in 250 ml of 0.1 M potassium phosphate buffer (pH 7.0) using a pre-chilled pestle and mortar. The homogenates were centrifuged at 12,000 rpm for 20 minutes 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 overnight under constant stirring.

Each fraction was collected separately by centrifugation at 10,000 rpm for 20 minutes 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 of 12,000 to 14,000 Dalton (Spectra/Por, SPECTRUM Laboratories, CA, USA.). Each ammonium sulphate fraction was then analyzed by SDS-PAGE and the required protein was cut out from the gel and electroeluted for 3 h at 12 mA against 0.1 M potassium phosphate buffer.

Production of polyclonal antisera to UMP and 40-kDa protein: The purified proteins of *C. gloeosporioides* and *L. theobromae* were used for raising polyclonal antisera (PCA) in rabbits by intramuscular immunization method.[1] Adult New Zealand white rabbits weighing about 1.5 kg (3 months old) were purchased and used for the study. Around 500 µg protein was used for each immunization. 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 and a booster injection was given 4 weeks after the first injection, with the same quantity of antigen added with Freund's incomplete adjuvant. Fifteen days after the fourth injection, blood sample was collected from the marginal ear vein in sterile glass vials. The vials were immediately placed in a slanting position so as to clot the blood and separate the serum. The antiserum was carefully collected in sterile centrifuge tubes and the red blood cells were pelleted by centrifuging at 8000 rpm at 4°C for 10 minutes three times. Finally, the serum was transferred to sterile microfuge tubes and stored at -70º C until further use. The protein concentration of antisera was estimated as described by Jayaraman et al[7].

Detection of *C. gloeosporioides* and *L. theobromae* antigen by ELISA and Western blot techniques: Indirect ELISA (Enzyme Linked Immunosorbent Assay) was performed to fix the antigen-antibody titre according to the procedure given by Hobbs et al[6]. Antigen and antibody dilutions of 1:10 to 1:10,000, prepared with 0.1 M phosphate buffer (pH 7.0) were used for the standardization. Wells of polystyrene microtitre plate (Immunolon-2-Removawells; Dynatech Laboratories, Alexandria, Va.) were coated with 100 µl of each dilution of the antigen and incubated overnight at 4 ˚C. The plate was then washed with 0.01 M phosphate-buffered saline, pH 7.2 (PBS) containing 0.02 per cent (v/v) Tween 20 (PBS-T) thrice, at 3 minutes interval. After washing, 100µl of each dilution of antibody was added to each well and incubated at 37 °C for 4h.

The plate was then washed three times with PBS-T and 100µl of AP-labelled goat anti-rabbit immunoglobulin

(Genei, Bangalore) was added at 1:8000 dilution to each well. The plate was incubated at 37 °C for 4 h and then washed with PBS-T thrice. Finally, 100µl of freshly prepared *p*-nitrophenyl phosphate (1.0mg/ml) in 0.2M Tris buffer pH 7.0, (Sigma *FAST*, Sigma, USA) was added to each well and incubated at 37 °C for 30 minutes for colour development. The optical density was measured at 405nm using an ELISA reader.

Western blot: Crude mycelial proteins of *C. gloeosporioides* and *L. theobromae* isolates were freshly extracted and separated by SDS-PAGE (12 per cent) using Mini-Sigma gel unit (Sigma-Aldrich Techware system, Sigma, USA). Western blotting was carried out according to the method prescribed by Gallagher et al.[4] After SDS-PAGE, the gel was equilibriated with transfer buffer (15.15g Tris base, 72.1g glycine, 1.0l methanol in 3.5l distilled water). Nitrocellulose membrane (Protran BAS 5 Cellulose nitrate, Schleicher and Schuell, Germany) was cut to the size of the gel, immersed in the transfer buffer and used.

Fractionated proteins were transferred from the gel onto the nitrocellulose membrane 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 2 minutes to check the resolution, transfer quality and destained using 1X TBS buffer. Electroblotting was carried out at 140mA for 1 h. The membrane was then removed and incubated 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 unbound primary antibody was removed by washing the membrane with PBST buffer thrice at 10 minutes interval and incubated in primary antibody solution diluted with carbonate buffer for 3 h at room temperature on a rotary shaker.

After incubation, the membrane was washed as before and incubated in goat anti-rabbit IgG alkaline phosphatase conjugate (Genei, Bangalore) at 1:5000 dilution. The unbound secondary antibody was removed by washing with PBST (1X PBS 0.1 per cent Tween 20). Finally, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma-Aldrich Co., USA) at room temperature in dark condition.

Serological detection of latency by indirect ELISA: For testing the reactivity of the PCA for detection of the pathogen under field conditions, samples were collected from mango orchards at random when the fruits reached full maturity. The peel samples were homogenized in 0.1 M phosphate buffer pH 7.0 (1:1 w/v) and used as antigen. The ELISA and Western blot tests were performed as described earlier.

Statistical analysis: The data were statistically analysed[5] and treatment means were compared by Duncan's multiple range test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometric unit, the Philippines (IRRI, Los Baños, Laguna, the Philippines).

Results

Protein profiling of *C. gloeosporioides* and *L. theobromae*: The protein profile of the *C. gloeosporioides* isolates showed the presence of proteins of molecular weight ranging from 14.7 kDa to 97.4 kDa. While some of the isolates displayed few protein bands on the gel, rest of them produced several discrete bands. Among the various proteins obtained in the profile, two common protein bands of size 70 kDa and 40 kDa, pertaining to all the isolates were observed (Plates 1 and 2).

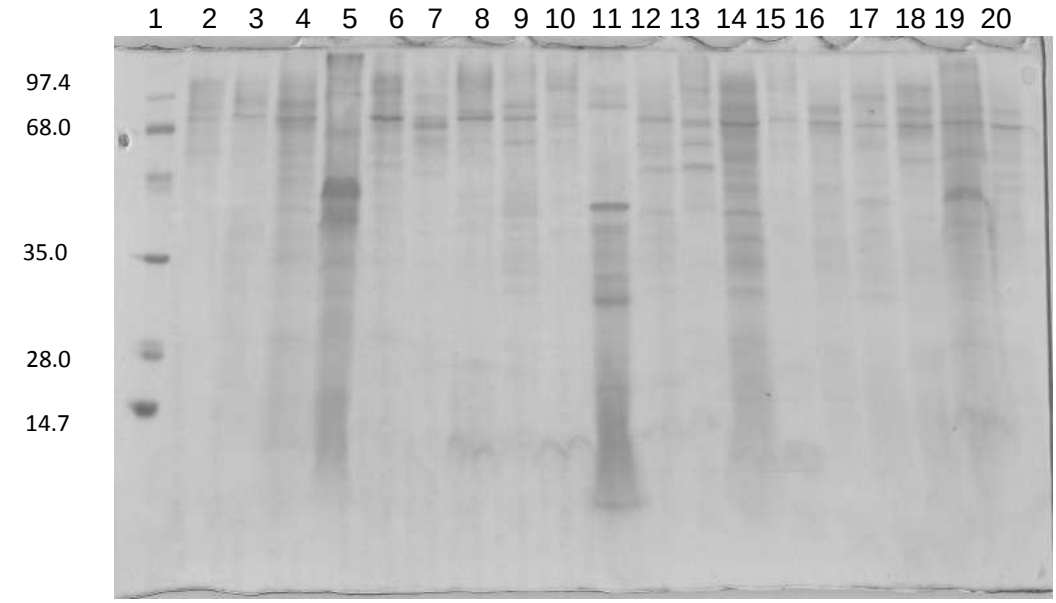
The crude mycelial proteins of the virulent isolate, *Cg* 4 were subjected to ammonium sulphate fractionation. Among the different fractions subjected to SDS-PAGE, the 80 per cent fraction showed the maximum expression of the required proteins. The two proteins were eluted from the gel, purified by dialysis and lyophilized. The purified proteins were then used for raising antibodies in New Zealand white rabbits.

The profile of the sixteen *L. theobromae* isolates revealed diverse proteins, most of which were in the range of approximately 30.0 to 97.4 kDa. Each isolate expressed several discrete protein bands on the gel. However, the common protein band identified in most of the isolates pertained to 70 kDa (Plate 2). Among the different fractions, the 80 per cent ammonium sulphate fraction produced clear bands on SDS-PAGE. The corresponding protein was eluted from the gel and dialysed as per the *C. gloeosporioides* proteins and used for raising antibodies.

Standardization of antigen-antibody titre by ELISA: Standardization of the titre revealed that an antigen dilution of 1:3000 and antibody dilution of 1:1000 for the 40 kDa antibody of *C. gloeosporioides* was optimum for the significant detection in ELISA. For the antibody raised against the 70 kDa mycelial proteins of *C. gloeosporioides*, the dilutions of both the antigen and antibody were optimized as 1:3000. The dilution of antibody and antigen of *L. theobromae* was standardized as 1:3000 and 1:2500 respectively. The reactions of the different dilutions of antibodies and antigens are furnished in tables 1 and 2.

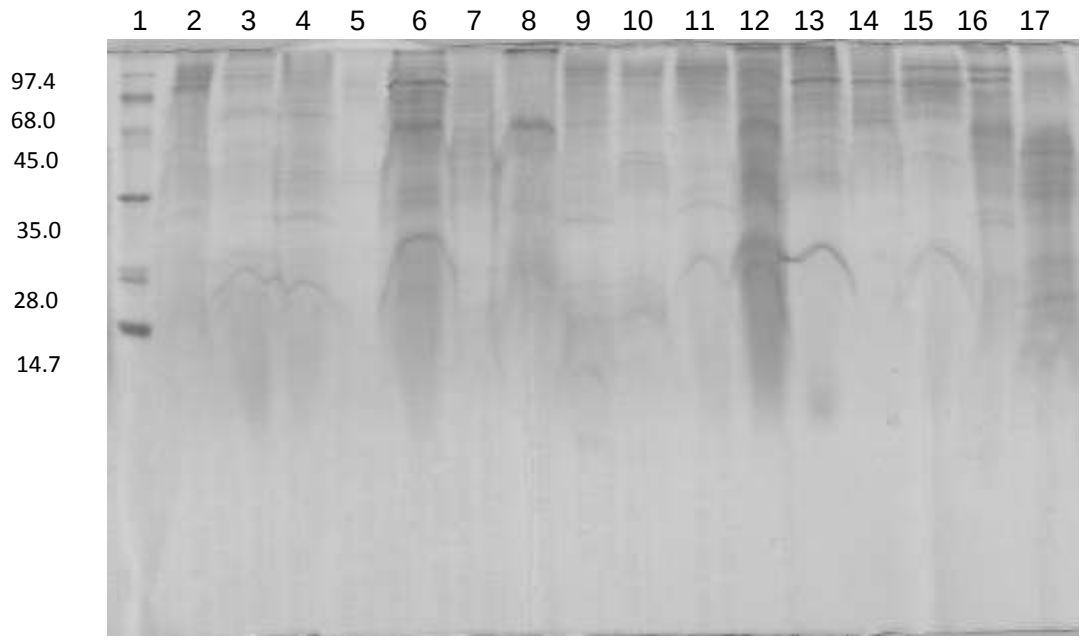
Western blot: Western blot analysis of the mycelial proteins of six isolates each of *C.gloeosporioides* and *L. theobromae* against the respective 70 kDa antibodies showed the appearance of a discrete band corresponding to 70 kDa on the nitrocellulose membranes (Plates 3 and 4).

Detection of latency in mango fruits by ELISA: The infected fruit samples recorded the highest absorbance in ELISA, when tested against the 40 kDa and 70 kDa antibodies of *C. gloeosporioides* and *L. theobromae.*



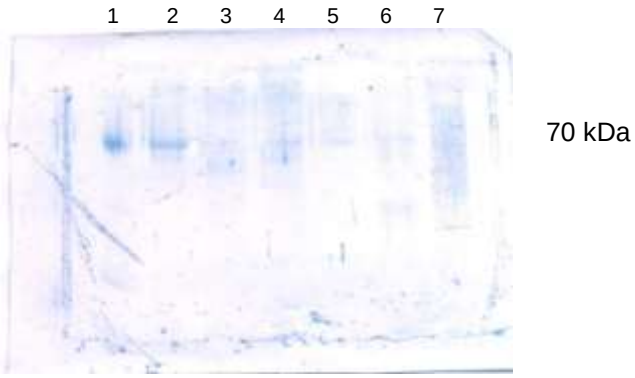
Lane 1: Marker: Lanes 2-20 : *C. gloeosporioides* isolates 1-19

Plate 1: Protein profile of *C. gloeosporioides* isolates



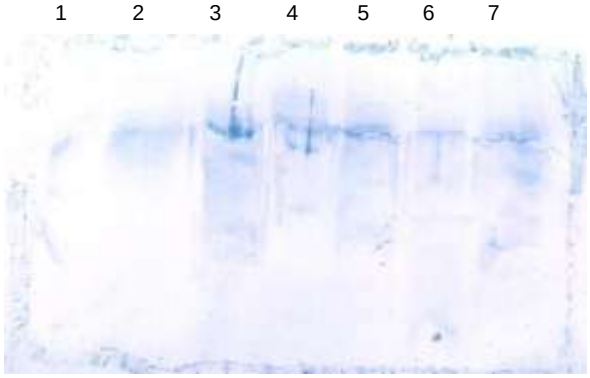
Lane 1: Marker; Lanes 2-17: *L. thoebromae* isolates 1-16

Plate 2: Protein profile of *L. theobromae* isolates



Lanes 1- 6: Cg 3, 4, 6, 7, 8, 11
Lane 7: Marker

Plate 3: Western blot of *C. gloeosporiodes* isolates against 70 kDa antibody



Lanes 2- 7 Lt 1, 2, 3, 6, 11, 14
Lane 1: Marker

Plate 4: Western blot of *L. theobromae* isolates against 70 kDa antibody

Plate 5: Detection of latency of *C. gloeosporioides* and *L. theobromae* in unripe mango fruits by ELISA

Table 1
Standardization of antigen dilution of *C. gloeosporioides* and *L. theobromae* by ELISA

Antigen dilution ⇨ / **Antibody ⇩**	**1:50**	**1:100**	**1:200**	**1:500**	**1:700**	**1:1000**	**1:1500**	**1:2000**	**1:2500**	**1:3000**	**Buffer**
***Cg* 40 kDa**	0.890	0.941	1.028	1.191	1.113	1.086	1.241	1.148	0.918	0.902	0.328
***Cg* 70 kDa**	0.899	0.831	0.898	1.088	1.209	1.012	1.082	1.172	1.041	0.935	0.365
***Lt* 70 kDa**	0.568	0.607	0.617	0.757	0.761	0.755	0.786	0.842	0.562	0.463	0.287

Table 2
Standardization of antibody dilution of *C. gloeosporioides* and *L. theobromae* by ELISA

Dilution factor ⇨ / **Antibody ⇩**	**1:50**	**1:100**	**1:500**	**1:1000**	**1:3000**	**1:5000**	**1:7000**	**1:10000**	**Buffer**
***Cg* 40 kDa**	0.794	0.867	0.633	0.816	0.692	0.419	0.392	0.305	0.249
***Cg* 70 kDa**	0.632	0.670	0.710	0.755	1.028	0.747	0.585	0.450	0.191
Reactivity with other pathogen	0.296	0.293	0.290	0.250	0.267	0.293	0.260	0.252	0.239
***Lt* 70 kDa**	0.778	1.018	1.103	0.860	0.808	0.584	0.351	0.300	0.284
Reactivity with other pathogen	0.274	0.272	0.371	0.210	0.297	0.270	0.276	0.253	0.189

The OD values generated for the samples tested against the 40 kDa antibody of *C. gloeosporioides* ranged from 0.206 to 0.368, while that of the infected fruits were 0.418 indicating that the samples might be healthy.

In case of the 70 kDa antibody of *C. gloeosporioides*, sample 1 was found to show an OD value of 0.462 while the infected samples recorded an OD value of 0.474 suggesting that there might be possible infection in the sample.

In case of *L. theobromae*, the samples number 2 and 7 recorded OD values of 0.519 and 0.491 respectively while the infected samples showed an OD value of 0.527 indicating the possible presence of the pathogen in the fruits. All the other samples tested were assumed to be apparently healthy (Plate 5).

Discussion

In recent years, post-harvest pathogens anthracnose and stem end rot proved to be a major challenge to both the pathologists working with the pathogen and to mango researches in general. The pathogens are widely prevalent in virulent forms in many parts of India. The severe pre- and postharvest fruit losses of up to 90 per cent have been recorded in many parts of Tamil Nadu, India. The pathogen rapidly adapts to new environments and develops new strains. The frequent occurrence of variation in *C. gloeosporioides* and *L. theobromae* has been well documented in this country. The screening test for mango anthracnose concluded that nearly all commercial varieties are sensitive to the disease.[12] As it causes initial infection in the orchard (latency), the use of bioprotectants or chemicals in the stored condition is less effective.[20]

Detection of the infection of *C. gloeosporioides* and *L. theobromae* at the early stages of fruit development is difficult, as they remain latent in the young fruits and express only upon ripening. Serological methods such as Enzyme Linked Immunosorbent Assay (ELISA), Dot Immunobinding Assay (DIBA), Tissue Immunobinding Assay (TIBA) and Western blotting are widely used for detection of latent infections as they allow sensitive, specific and simultaneous analysis of many samples in a single microplate.

Studying the protein profiles of pathogens would provide information about the variation in protein expression levels of the isolates. Besides, it would also help in the identification of common proteins within the population of the pathogen which can be used as antigens to raise polyclonal antibodies. This would further enable to detect the presence of latent infections in young fruits much earlier than the natural expression of symptoms and accordingly suitable disease management strategies can be devised. Several workers have studied the morphological and genetic diversity among the isolates of *C. gloeosporioides* and *L. theobromae* in various crops.

However, not much work has been reported on the protein profiles of these pathogens. In the present study, the protein profiles of the isolates of the two pathogens were analysed by SDS-PAGE. Results showed that although certain isolates were morphologically similar in culture, the proteins expressed on SDS gels were different. These variations in the protein profile suggest a possible reason for the variation in the level of pathogenicity of the isolates.

The protein profile of the pathogens was carried out in order to develop immunological methods for detection of the presence of *C. gloeosporioides* and *L. theobromae* infection in unripe mango fruits. Results revealed the presence of common bands corresponding to 40 and 70 kDa for *C. gloeosporioides* and 70 kDa for *L. theobromae* isolates. Similar results were obtained by Vivekananthan[18] who reported a common protein band corresponding to 40 kDa in *C. gloeosporioides* isolates of mango. The common proteins of *C. gloeosporioides* and *L. theobromae* identified in the present study were isolated and purified and used as antigen to raise polyclonal antibodies in New Zealand white rabbits.

Since the proteins used for raising antibodies were common and represented all the isolates collected from the major mango growing regions of Tamil Nadu, the antibodies so raised would be useful to detect the races of the two pathogens from the different mango growing regions of the state

ELISA revealed that the antibodies were sensitive in pathogen detection and the dilutions were standardized as 1:1000 for the 40 kDa antibody, 1:3000 for the 70 kDa antibody of both *C. gloeosporioides* and *L. theobromae*. The optimal working dilution of the antigen was found to be 1:3000 for *C. gloeosporioides* and 1:2,500 for *L. theobromae*. These results are in accordance with that of Vivekananthan[18] who standardized the dilution of the 40 kDa antibody of *C. gloeosporioides* as 1:2000.

In Western blot analysis, a protein band corresponding to a molecular weight of 70 kDa reacting to *C. gloesporioides* antiserum raised against the particular protein was detected in the mycelial protein extract of the pathogen. Similarly, for *L. theobromae*, a 70 kDa band was obtained on Western blot against the mycelial proteins of the isolates used in the study. A clear and distinct band corresponding to 40 kDa was obtained by Vivekananthan[18] on Western blot against four different isolates of *C. gloeosporioides* of mango.

Generally, during ripening of fruits, physiological changes occur such as reduction in the phenol and tannin content and increase in sugar content that are favourable for the pathogen, thereby resulting in the breaking of dormancy and expression of symptoms. When fruits are treated with paraquat, similar physiological changes are induced in the fruits and thereby the pathogen establishes and symptoms are expressed.

As evident from the present study, in all the four cultivars tested, the fruits exposed to paraquat expressed typical symptoms of anthracnose, although they remained green, while the untreated fruits remained symptomless. Hence, paraquat can be employed as a successful aid in early detection of pathogens that survive in dormant forms in apparently healthy samples.

Over the past two decades, ELISA has been widely accepted as a highly sensitive immunodetection technique for detection of latent infections as it allows for specific detection and simultaneous analysis of many samples in a single microplate. ELISA involves the binding of an antigen to a specific antibody against which the antibody was raised, thus enabling very specific and reliable detection. The reactions are made visible using a "tag" enzyme which acts upon its substrate bound to a secondary antibody and generates a coloured product, the intensity of which is read at 405 nm using an ELISA reader.

Many workers have used ELISA for the specific detection of latent infections. Viswanathan et al[17] reported that the polyclonal antiserum raised against 101 kDa polypeptide was highly specific in detecting the red rot pathogen, *C. falcatum* in sugarcane setts. Kratka et al[9] diagnosed the latent infection of the strawberry anthracnose pathogen, *C. acutatum* in apparently healthy roots, crowns, petioles and fruits using ELISA and DIBA techniques. Vivekananthan[18] detected the latent infection of *C. gloeosporioides* in mango using polyclonal antibodies raised against a 40 kDa protein of the pathogen.

In the present study, ELISA was employed to detect the presence of *C. gloeosporioides* and *L. theobromae* in mango fruit samples in comparison with infected samples using the specific antibodies raised against the respective pathogen proteins. Although the infected samples recorded the highest absorbance, few of the test samples also recorded high OD values, thereby revealing the probable presence of the pathogen in the tissues. Thus, it is evident from these results that ELISA can be a promising immunodetection technique for the early diagnosis of latent infections of the two important pathogens of mango.

Conclusion

The quiescent infection was detected from this study using the ploy clonal antibody raised against mycelial protein of *C. gloeosporioides* and *L. theobromae*. The immunological techniques are widely used for early detection of viral and fungal infection. In future, the specific antibody would be helpful for indexing the mango to sort out the latent infection while exporting or stooging the harvested fruits.

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(Received 06th May 2020, accepted 09th July 2020)