EARLY DETECTION OF *Ganoderma* CAUSING BASAL STEM ROT DISEASE IN COCONUT PLANTATIONS

RAJENDRAN, L*; KANDAN, A*; KARTHIKEYAN, G*; RAGUCHANDER, T* and SAMIYAPPAN, R*

ABSTRACT

Basal stem rot disease of coconut caused by Ganoderma lucidum *(Leys) Karst is prevalent throughout the world, causing significant damage and losses to coconut plantations. The pathogen is soil-borne in nature. For early diagnosis, different techniques* viz., *enzyme-linked immunosorbent assay, dot immunobinding assay and polymerase chain reaction have been used in modern era of plant pathology. In this study, we have tried ELISA and DIBA using the monospecific antibodies raised against a distinct common protein with a molecular weight of 62 kDa. The protein was observed in all the isolates of* Ganoderma *collected throughout Tamil Nadu, India, by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and variations in the protein patterns were found between* Ganoderma *isolates. The polyclonal antisera were developed against the purified single protein of* Ganoderma. *The presence of the 62 kDa protein in the crude mycelial extract was confirmed by western blot analysis. Using the antisera, the pathogen was detected by Indirect ELISA and dot immuno binding assay (DIBA) which resulted in positive reactions for infected samples and negative reactions for apparently healthy palms. These serological techniques gave better results in early diagnosis of field samples, and it could be adopted for large-scale application.*

Keywords: *Ganoderma*, monospecific antibody, basal stem rot.

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INTRODUCTION

Coconut is an important oilseed as well as a plantation crop in India, occupying 1.9 million hectares with an annual production of 12 141 million nuts. Among some 50 diseases affecting the coconut palm, basal stem rot (BSR) disease, caused by species of *Ganoderma*, is the most devastating disease on numerous perennial, coniferous and palmaceous hosts. The genus *Ganoderma* has worldwide distribution, and cause root and stem rots of many plantation crops. In India, *G. lucidum* was first recorded in coconut in Karnataka state by Butler (1913). In Tamil Nadu, it was reported in Thanjavur district during 1952 (Bhaskaran *et al*., 1994). BSR of coconut is a serious disease in India and in severely infected areas, the incidence as high as 80% was recorded (Ramdoss, 1991). BSR is also called Thanjavur wilt, bole rot, *Ganoderma* disease and Anabe roga in different states of India. This disease can be managed effectively by an integrated disease management (IDM) package, if the disease is diagnosed before the external symptoms are produced (Samiyappan *et al*., 2006). While cultural studies and microscopic observations of fungal structures are highly accurate for diagnosis, these techniques are too slow and not amenable for large-scale application (Miller and Martin, 1988). Even when single palm is infected in a plantation, the disease spreads to neighbouring palms by root contact (Turner, 1965). Immunoassay and molecular probes are more specific, rapid and sensitive than conventional methods (Leach and White, 1990), and many serological and molecular methods have been developed for the early diagnosis of BSR disease. Among the different techniques,

* Department of Plant Pathology,
Centre for Plant Protection Studies,
Tamil Nadu Agricultural University,
Coimbatore – 641 003,
Tamil Nadu, India.
E-mail: rucklingraja@rediffmail.com

monospecific protein detection gives more accurate results. With this background, the present research was undertaken to address the possibility of early diagnosis of *Ganoderma* in coconut.

MATERIALS AND METHODS

Isolation of the Pathogen

The *Ganoderma* pathogen from the sporophores or infected roots of diseased palms and forest trees was isolated on potato dextrose agar (PDA) medium. The cultures were maintained on PDA slants throughout the period of study.

Growth of *Ganoderma* Isolates

Potato dextrose broth was used for the growth of *Ganoderma* (Moncalvo *et al*., 1995). A fungal disc (8 mm diameter) was placed in a 250-ml Erlenmeyer flask containing 100 ml of broth and incubated at room temperature (27 ± 2ºC) under static conditions for the growth of the fungus.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The fungal mycelia were harvested and dried on filter paper. One gram of powdered mycelial sample was extracted with 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4ºC. The homogenate was centrifuged for 20 min at 10 000 rpm. The supernatant was used to estimate the protein content of the sample by the Bradford method (Bradford, 1976). For SDS-PAGE (Laemmli, 1970), 40 μg of protein from each isolate were taken and mixed with 10 μl of sample buffer in a microfuge tube, boiled for 4 min and incubated at 4ºC for 30 min. Then the samples containing equal amounts of proteins were loaded into the wells of polyacrylamide gels (Sigma-Aldrich Techware system, Sigma, USA). Medium range molecular weight markers (Bangalore Genei, India) were used. Electrophoresis was carried out at a constant voltage of 75 volts for 2 hr. The gels were stained with 0.2% Coomassie brilliant blue (R250) solution. Based on the R_m value of each stained protein band, the molecular weight was determined. All the experiments were repeated at least two times to get consistent results.

Purification of the Single Protein from *Ganoderma*

The monospecific 62 kDa protein extracts from whole mycelia of *Ganoderma* were electrophoresed and electroblotted onto Nitrocellulose membrane (NC) (western blot) using all available slots of the SDS polyacrylamide gel. After staining of the left and right borders of the western blot, the location of the 62 kDa protein band from the protein extract of *Ganoderma* was determined within the untreated middle part of the NC, cut out, macerated and dissolved in dimethyl sulfoxide (DMSO) until the solution was pipettable. Addition of an equal volume of 10 mM phosphate buffer/0.15 M NaCl (pH 7.2) to the DMSO-NC solution precipitated the protein-containing NC. After centrifugation the NC pellet was washed twice with 10 mM phosphate buffer/0.15 M NaCl (pH 7.2), resuspended in 1 ml of the same buffer, mixed well, lyophilized and stored at -70ºC (Niepold and Huber, 1988).

Raising of Polyclonal Antibodies

Polyclonal antiserum to the *Ganoderma* protein (62 kDa) was raised by the intramuscular immunization in rabbit as described by Shanmugam *et al*. (2002) with slight modification. The *Ganoderma* protein content was assessed (Bradford, 1976), adjusted to 100 μg ml^{-1} and used for immunization. Adult New Zealand white rabbits (Courtesy: Pasteur Institute, Coonoor, India) weighing about 1.5 kg each were used. One millilitre of antigen (0.5 g of freshly harvested *Ganoderma* mycelia suspended in 0.1 M phosphate buffer pH 7.0 and clarified at 12 000 rpm for 10 min at 4°C) was mixed with 1 ml of Freund's complete adjuvant, and emulsified in a cyclomixer. The emulsion was administered intramuscularly into the rabbit with a sterile syringe and 22G needle. Three injections were given at 10-day intervals, and a booster injection was given one week after the third injection. Bleeding was done 10 days after the booster injection. Immediately after bleeding, the blood was transferred into sterile glass vials and allowed to stand in a slanting position for a few minutes for coagulation to take place. The sera were transferred into sterile centrifuge tubes and the red blood cells were pelleted by centrifuging (10 000 rpm at 4°C for 10 min) three times. The antisera were stored at -70°C after adding sodium azide (0.01%) for use in further studies.

Western Blotting

Western blotting was carried out according to the method prescribed by Gallagher *et al.* (1995). 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 2 min to check the resolution and transfer quality, and destained using 1X PBS buffer. Then, the membrane was kept in a blocking buffer (5% milk powder in 1X PBS containing 1% Tween 20) for 2 hr at room temperature with gentle shaking (50 rpm)

on a rotary shaker. The blot was then incubated for 2 hr at room temperature with gentle shaking in an antiserum buffer (5% milk powder in 1X PBS + 0.1% Tween 20) containing polyclonal antibodies raised in rabbits against the 62 kDa purified protein (1:700 dilution). The unbound primary antibody was removed with two or three washings with antiserum buffer, and the blot was incubated for 2 hr at room temperature in a 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 two or three washings with PBST (1X PBS + 0.1% Tween 20). Polypeptides which were recognized by the specific antibodies were visualized by incubating the membrane in the dark with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma-Aldrich Co., USA).

Indirect - ELISA

A standard indirect ELISA method as described by Hobbs *et al*. (1987) was used with slight modifications. Microtitre plates (Tarson, India) were coated with 100 µl of samples, left for 2 hr at 37°C, and then incubated at 4°C overnight. The plates were emptied and washed three times with Phosphate Buffer Saline-Tween (PBS-T) (3 min each). The primary antibodies diluted in PBS-T (1:700) containing 2% polyvinylpyrrolidone and 0.2% ovalbumin (PBS-TPO) were added (100 µl per well) separately. After incubating the plates at 37°C for 2 hr, they were washed with PBS-T. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin (Bangalore Genei, India) (1:5000 with PBS-TPO) was added separately (100 µl per well). The plates were incubated for 2 hr at 37°C. After washing in PBS-T, 100 µl ALP substrate (1 mg ml^{-1}) solution 4-nitrophenyl phosphate (SD fine chemicals, India) dissolved in diethanolamine (Sigma, USA) (pH 9.8) were added. The reaction was terminated by adding 50 µl of 3 M NaOH after incubation for 0.5 hr at room temperature (28 ± 2°C). The colour development was read at absorbance 405 nm with a Microplate reader (Bio Rad Model 3550, USA).

Dot Immunobinding Assay (DIBA)

DIBA assays for coconut samples, *Ganoderma* mycelia samples and *Trichoderma viride* were performed on a nitrocellulose membrane (NCM) (Sigma, USA) following the method of Hampton *et al*. (1990) with slight modification. Different samples and antigen diluted in TBS buffer (0.02 M Tris, 0.5 M NaCl, pH 7.5) were spotted on to the membrane and, after drying, the loaded membrane was incubated in a blocking solution (5% spray-dried milk in TBS). After subsequent washings, the membrane was first incubated in *Ganoderma* antisera 62 kDa (1:1000) and then in enzyme conjugate (1:5000) (Sigma). The substrate solution contained 0.33 mg ml^{-1} nitroblue tetrazolium (Sigma) and 0.175 mg ml^{-1} 5-bromo 4-chloro, 3-indolyl phosphate (Sigma) in 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2.6H_2O$, pH 9.5 buffer. Immediately after colour development the membranes were washed in distilled water, dried and stored. The experiment was repeated twice for consistency of results.

RESULTS

Isolation and Growth of *Ganoderma* Isolates

The pathogen *Ganoderma* sp. was isolated and maintained on PDA slants. Twenty-five isolates of *Ganoderma* spp. were isolated from the samples collected from different hosts in Tamil Nadu and they were maintained on PDA slants. The lists of different *Ganoderma* isolates from different parts of Tamil Nadu are given in *Table 1*. The *Ganoderma* isolates took nine to 15 days to cover the entire surface of the potato dextrose broth in 250 ml conical flasks. Two isolates *viz*., SV and CRS-1, produced more mycelial growth when compared to the other isolates (*Table 2*).

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Variations in the protein pattern of *Ganoderma* isolates were studied by SDS-PAGE. All the 25 isolates tested shared most of the proteins with molecular weights ranging from 14 to 97 kDa. Although, there was not much variation among the isolates, a distinct common band with a molecular weight of 62 kDa was observed irrespective of the isolates.

Purification of 62 kDa Protein

The common protein with a size of 62 kDa was eluted from the SDS PAGE and purified. The purified protein was lyophilized and run again on a SDS-PAGE. A single band was observed with the same molecular weight of 62 kDa. This single band indicated the purified nature of the protein (*Figure 1*).

Raising of Polyclonal Antiserum

The polyclonal antiserum was raised on New Zealand white rabbits intra-muscularly against the single purified 62 kDa protein of *Ganoderma*.

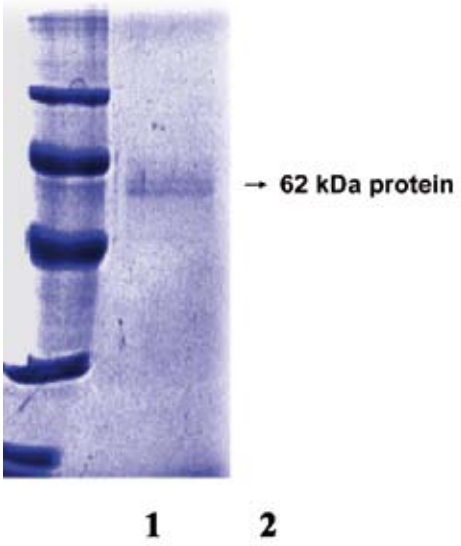
TABLE 1. ISOLATES OF *Ganoderma* SPECIES AND THEIR SOURCES

Sl. No.	Isolate	Geographical origin*	Host	Source of material	External symptoms
1	CRS-1	Veppankulum, TN	*Cocos nucifera*	Sporophore	BSR lesion
2	SV	Silent Valley, KL	Unknown	Sporophore	Dead plant
3	SLP	Sultan pet, TN	Unknown	Sporophore	Dead plant
4	PR	Paiyur, TN	*Cocos nucifera*	Infected root	BSR lesion
5	PY	Pondicherry, TN	Tamarind	Sporophore	Dead plant
6	OTY-1	Ooty, TN	Silveroak	Sporophore	Dead plant
7	CRS-2	Veppankulum, TN	*Cocos nucifera*	Infected root	Dead palm
8	TKT- 5	Thambikottai, TN	Tamarind	Sporophore	Dead plant
9	TKT-2	Thambikottai, TN	*Cocos nucifera*	Infected root	BSR lesion
10	UDP	Udumalpet, TN	*Cocos nucifera*	Sporophore	Dead palm
11	OTY-2	Ooty, TN	*Pinus* spp.	Sporophore	Dead plant
12	SM	Salem, TN	*Cocos nucifera*	Sporophore	Dead palm, BSR lesion
13	MDU	Madurai, TN	*Cocos nucifera*	Sporophore	Dead palm, BSR lesion
14	CUD	Cuddalore, TN	*Cocos nucifera*	Infected root	BSR lesion
15	CRS-3	Veppankulum, TN	*Palmyra*	Sporophore	BSR lesion
16	CBE-1	Coimbatore, TN	*Cocos nucifera*	Sporophore	BSR lesion
17	CBE-2	Coimbatore, TN	*Cocos nucifera*	Infected root	Dead palm
18	CBE-3	Coimbatore, TN	*Cocos nucifera*	Sporophore	Dead palm
19	PK	Pudukottai, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
20	VPM-1	Veppankulum, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
21	TKT-1	Thambikottai, TN	*Cocos nucifera*	Infected root	BSR lesion
22	TKT-4	Thambikottai, TN	*Prosopis julifera*	Sporophore	No discoloration
23	TKT-3	Thambikottai, TN	*Cocos nucifera*	Infected root	Foliar, BSR lesion
24	MTP	Mettupalayam, TN	*Areca catechu*	Sporophore	BSR lesion
25	CBE-4	Coimbatore, TN	Red gloom over	Sporophore	Lesion

Note: *TN and KL indicates Tamil Nadu and Kerala state of India.

TABLE 2. GROWTH AND CULTURAL CHARACTERISTICS OF *Ganoderma* SPECIES

Isolate	Nature of growth	Morphology in culture
CRS-1	Fast	Light yellow, dense mycelial growth
SV	Fast	Pure white, dense mycelial growth
SLP	Slow	White to pale yellow, sparse mycelial growth
PR	Slow	Pale yellow, dense mycelial growth
PY	Medium	Light white, powdery growth
OTY-1	Slow	Pure white, dense mycelial growth
CRS-2	Medium	Light white, dense mycelial growth
TKT-5	Slow	Dirty white, sparse mycelial growth
TKT-2	Slow	Pale yellow, dense mycelial growth
UDP	Slow	Light white, sparse mycelial growth
OTY-2	Slow	Light white, sparse mycelial growth
SM	Slow	White to yellow, powdery growth
MDU	Medium	Light white, sparse mycelial growth
CUD	Slow	White to pale yellow, dense mycelial growth
CRS-3	Medium	Light yellow, powdery growth
CBE-1	Medium	Pure white, sparse mycelial growth
CBE-2	Fast	Pure white, dense growth
CBE-3	Medium	Dirty white to pale yellow, dense mycelial growth
PK	Medium	Pure white, dense mycelial growth
VPM-1	Fast	White to pale yellow, dense mycelial growth
TKT-1	Slow	Pale yellow, powdery growth
TKT-4	Fast	Pure white, dense mycelial growth
TKT-3	Medium	Dirty white, sparse mycelial growth
MTP	Fast	White to yellow, dense mycelial growth
CBE-4	Medium	Dirty white, powdery growth



Lane 1. Protein molecular weight marker medium range (97 to 14 kDa).
Lane 2. Purified 62 kDa protein band.

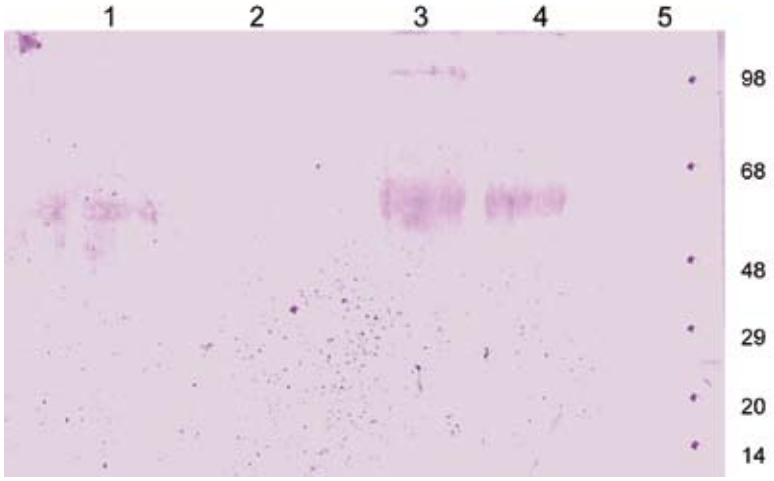
Figure 1. Purification of 62 kDa protein from Ganoderma.

Western Blotting

The monospecific antibody raised against the single purified protein (62 kDa) showed the presence of the 62 kDa protein band on a western blot for purified protein extracts of different *Ganoderma* isolates. The results confirmed that the purified protein (62 kDa) used as antigen for immunization was of a pure quality for the detection of *Ganoderma*. *Ganoderma* isolates showed a thick prominent protein band (62 kDa) on the western blot, and also two other protein bands were found at low molecular weight due to the same protein homology by the polyclonal antisera developed against the single purified protein (*Figure 2*).

ELISA

The experiment on standardization of the concentration of antiserum revealed that an antigen dilution of 1:50 and an antiserum dilution of 1:700 were optimum for the detection of *Ganoderma* in diseased plant samples through indirect ELISA (*Table 3*).



Lane 1. CRS-1 isolate
Lane 2. *T. viride*
Lane 3. Silent valley (SV) isolate
Lane 4. TKT isolate
Lane 5. Protein molecular weight marker

Figure 2. Western blot analysis of 62 kDa protein in G. lucidum.

TABLE 3. STANDARDIZATION OF ANTIGEN AND ANTIBODY DILUTIONS IN ELISA

Absorbance at 405 nm		Antiserum dilution						
		1:200	1:500	1:700	1:1000	1:2000	1:5000	Control (buffer)
Antigen dilution	1:10	0.100	0.101	0.102	0.092	0.073	0.044	0.022
	1:50	0.105	0.104	**0.128**	0.101	0.075	0.041	0.018
	1:100	0.035	0.048	0.090	0.045	0.029	0.024	0.008
	1:200	0.023	0.037	0.038	0.021	0.020	0.015	0.012
	1:500	0.022	0.026	0.020	0.015	0.014	0.011	0.007
	1:1000	0.017	0.020	0.022	0.016	0.013	0.010	0.006
	Protein 1:500	0.286	0.385	0.406	0.391	0.210	0.185	0.009

DIBA

Immunoblotting assays have been found to be useful in overcoming problems with non-specific interference in ELISA procedures. In the DIBA, at a 1:50 dilution of antigen, MS antisera at a 1:700 dilution and 1:5000 dilution of the secondary antibody gave clear distinctions in colour development between healthy and diseased samples. In the cross-reaction test, MS antisera showed no cross-reaction with *Trichoderma*. MS antisera showed positive reactions for the purified protein and infected palm samples (*Figure 3*).

DISCUSSION

Ganoderma species are widespread polyporous fungi causing basal stem rot or white rot of hardwoods, conifers and palms. BSR of coconut is a serious disease in India and in few of the severely infected areas of Tamil Nadu, the incidence is as high as

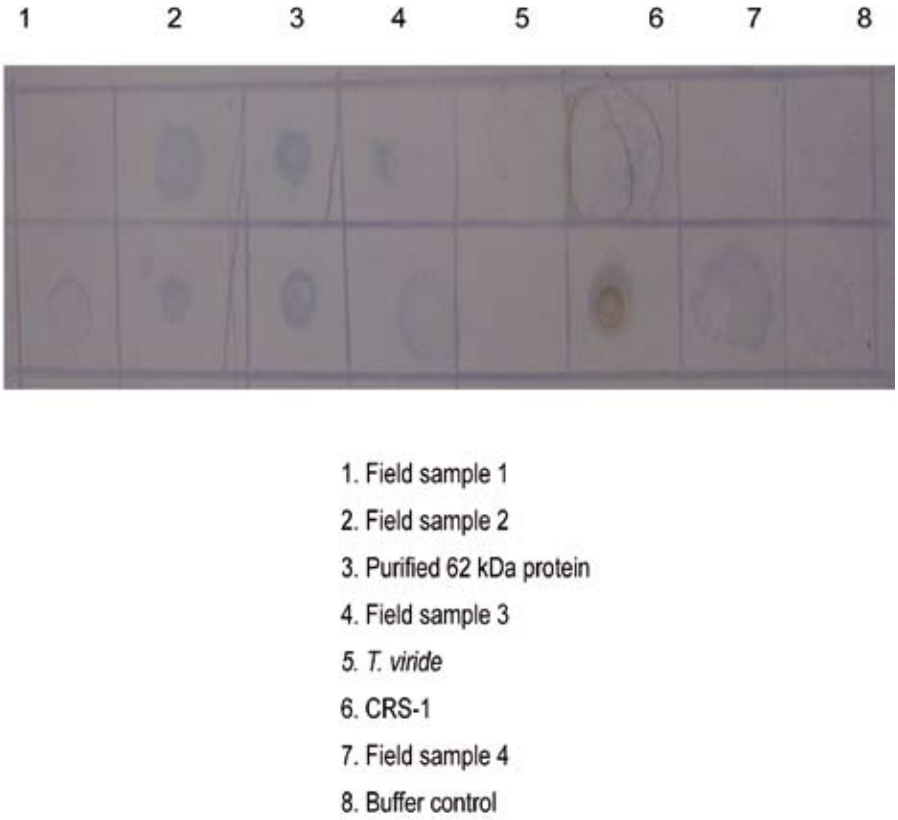


Figure 3. Dot immuno binding assay (DIBA) of Ganoderma *and other pathogens in different samples using a monospecific antibody.*

80% (Ramdoss, 1991). In Western blotting, the detection of a 62 kDa purified *Ganoderma* protein using monospecific antisera was achieved by loading different purified samples of *Ganoderma* protein, indicating that the antigen was pure for immunization. However, in the cross-reaction test, the same monospecific antisera showed different protein bands at different molecular weights for saprophytic organisms. The 62 kDa protein signals alone for *Ganoderma*, while the remaining high molecular weight protein bands indicate the other saprophytic organisms and these results are in agreement with Sundaram *et al*. (1991) and Velichetti *et al*. (1993). Sundaram *et al*. (1991) reported that there was no cross-reaction of *Verticillium dahliae* Klebahn polyclonal antiserum with *Fusarium*, *Colletotrichum* and some species of *Verticillium* except *V. albo-atrum* Reinke et Berthold. Velichetti *et al*. (1993) reported that a strong cross-reaction was observed in *Phomopsis phaseoli* (Dasmaz.) Sacc., and *Colletotrichum truncatum* (Schwein), but weak reactions occurred against *Aspergillus* sp., *Chaetomium* sp., and *M. phaseolina*. Over the past decade, advances in the fields of molecular plant pathology and immunology have greatly increased the accuracy, rapidity and sensitivity of nucleic acid and protein detection (Lamb *et al*., 1992; Samiyappan *et al*., 1996; Martin *et al*., 2000). In the present study, we concentrated only on the detection of *Ganoderma* infections at early stages of the infection process on palms through ELISA and DIBA techniques. Monospecific protein (62 kDa) paved the way for the detection of the pathogen at any form or stage in the plant samples. Numerous immunoassays have been developed for fungal pathogens utilizing polyclonal antisera against whole cells (Kraft and Boge, 1994), crude mycelial extracts (Harrison *et al*., 1990; Viswanathan *et al*., 1998), extracellular culture filtrates (Kim *et al*., 1991; Brill *et al*., 1994) and crude or partially purified soluble proteins (Velichetti *et al*., 1993; Viswanathan *et al*., 1998) in varying degrees of specificity for the target fungus. Low cross-reaction was found in the MP antisera when compared to other antiserum against related saprophytic organisms, especially *Trichoderma*, and this result is in agreement with previous findings of Wakeham and White (1996), Viswanathan *et al*. (1998), and Utomo and Niepold (2000).

DIBA is another sensitive serological assay that can be used to detect the pathogen other than by indirect ELISA. In the current investigation, *Ganoderma* was positively detected in samples from infected roots. This result is in agreement with earlier work by Mitchell (1988) and Velichetti *et al*. (1993). The present study indicates that both ELISA and DIBA would be useful for screening a large number of samples as already reviewed by Samiyappan *et al*. (2006). In future, provision of immunoassay-based kits would be helpful in the detection of infection at the earliest stage of disease development at field level and this would certainly help the adoption of suitable management strategies against *Ganoderma* disease in palms in advance.

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