

Full Length Research Paper

Rapid detection of *Ganoderma lucidum* and assessment of inhibition effect of various control measures by immunoassay and PCR

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Molecular and immunological methods have been applied for detecting the *Ganoderma* disease of coconut. Polyclonal antibodies (PABs) raised against basidiocarp protein of *Ganoderma* were used for detection. For polymerase chain reaction (PCR) test, the primer generated from the internal transcribed spacer region one (ITS 1) of ribosomal DNA gene of *Ganoderma*, which produced a PCR product of 167 bp in size is used for early detection. *Ganoderma* disease in apparently healthy palms in two coconut gardens was tested by ELISA test using basidiocarp protein antiserum. Field trials were laid out in these early-diagnosed palms for the management of the disease. Based on the ELISA results, *Pseudomonas fluorescens* + *Trichoderma viride* with chitin amended treatments arrested the multiplication of the pathogen and showed below the infection level of optical density (O.D) within six months. Integrated disease management (IDM) and fungicide tridemorph treated palms showed below infection level (O.D value) within seven months and *T. harzianum* and *P. fluorescens* + *T. viride* treated palms showed below infection level (OD value) of the disease in eighth months.

Key words: *Ganoderma*, early diagnose, PCR, ELISA, integrated disease management.

INTRODUCTION

Coconut is an important oil seed as well as plantation crop in India with an area of 1.8 million hectares and an annual production of 54 billion nuts (Rethinum, 2004). In India, basal stem rot disease (BSR) caused by *Ganoderma lucidum* (Leys) Karst is a major limiting factor in coconut production. The disease is also referred as Thanjavur wilt, bole rot, *Ganoderma* disease and Anabe (Vijayan and Natarajan, 1972; Nambiar and Rethinam, 1986; Bhaskaran et al., 1990).

The incubation period of this disease has been determined to be several years (Turner, 1981). The visible disease symptoms appear at a very late stage of infection when more than half of the root tissues have

been decayed, leaving no chance for the grower to cure the infected palms. Basal stem rot disease of coconut can be contained by management practices if the disease is detected in the early stages. A few methods have been reported to be useful to identify the diseased palms even before expression of symptoms, though the methods are non-specific for BSR (Natarajan et al., 1986; Vijayaraghavan et al., 1987; Samiyappan et al., 1996). The polymerase chain reaction (PCR) technology has revolutionized the field of plant pathology in diagnosing various plant pathogens (Henson and French, 1993). The internal transcribed spacer (ITS) regions of ribosomal RNA gene (rDNA) have been selected as specific targets for PCR detection of *Ganoderma* (Utomo and Niepold, 2000). Secondly, the development of polyclonal antibodies against the crude mycelial proteins of *Ganoderma* to serologically detect this fungus by applying indirect enzyme-linked immunosorbent assay

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Table 1. Field trials for the management of the *Ganoderma* disease of coconut.

Treatments	Description
T ₁ .	<i>Trichoderma harzianum</i> (500 g) + 50 kg farm yard manure (FYM)
T ₂ .	<i>T. harzianum</i> + chitin (500 g) + 50 kg FYM
T ₃ .	<i>Pseudomonas fluorescens</i> (200 g) + <i>T. Viride</i> (200 g) + 50 kg FYM
T ₄ .	<i>P. fluorescens</i> + chitin (200 g) + <i>T. viride</i> + chitin (200 g) + 50 kg FYM
T ₅ .	Phosphobacteria (200 g) + 10 kg FYM
T ₆ .	Fertilizer application - N – 0.35: P ₂ O ₅ – 0.25: K ₂ O – 0.45 kg tree ⁻¹
T ₇ .	CaSO ₄ (500 g) + MgSO ₄ (500 g).
T ₈ .	Ca(NO ₃) ₂ (25 g)
T ₉ .	Tapping – sweet toddy for six months.
T ₁₀ .	Banana intercrop
T ₁₁ .	Integrated Disease Management (IDM) - regular basin irrigation during summer months , application of 50 kg of FYM and 5 kg neem cake per palm per year, raising banana intercrop and root feeding of tridemorph (2 ml 100 ml ⁻¹) thrice a year at quarterly interval
T ₁₂ .	Root feeding – Tridemorph 2 ml in 100 ml water (thrice a year at quarterly interval)
T ₁₃ .	Untreated control

(ELISA) and dot immunobinding assay (DIBA) techniques is reliable.

Therefore, aims of this work was to early diagnosis and manage the *Ganoderma* infected coconut before appearance visual symptom by applying the enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) technique by using specific primers with ITS 1 region as a target.

MATERIALS AND METHODS

Root sampling for early diagnostic tests

Roots from healthy and diseased palms were collected in the basin area of 1.8 m radius at 15 - 30 cm depth from all the four directions in the basin (Karthikeyan et al., 2002). All plant samples collected were washed with distilled water, weighed and ground in 0.1 M phosphate buffer (pH 7.0; 1:2 dilution) in a sterile pestle and mortar at room temperature (30 ± 2°C) and clarified at 12,000 rpm for 10 min at 4°C. The supernatant was stored at -70°C until use for the early diagnosis tests.

Indirect enzyme linked immunosorbent assay

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 for 2 h 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) (pH 7.4) (3 min each). The primary antibodies diluted in PBS-T (1:3000) containing 2 per cent polyvinylpyrrolidone and 0.2 per cent ovalbumin (PBS-TPO) were added (100 µl per well) separately. After incubation (37°C, 2 h), the plates were washed with PBS-T. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin (Bangalore Genei, India) (1:6000 with PBS-TPO) were added separately (100 µl per well). The plate was incubated for 2 h at 37°C. The plates were emptied and washed with PBS-T for three times and added 100 µl ALP substrate (1 mg/ml) solution of *p*-nitrophenyl phosphate (SD fine chemicals, India) dissolved in

diethanolamine (Sigma, USA) (pH 9.8). After incubation for half an hour at room temperature (28 ± 2°C), the reaction was terminated by adding 50 µl of 3 M NaOH. The colour developed was read at 405 nm with a Microplate reader (Bio Rad Model 3550, USA).

Molecular diagnosis

In polymerase chain reaction (PCR), the DNA region used for the molecular determination of the fungus is the gene cluster that codes for the ribosomal RNA gene, in which internal transcribed spacers region is used for the identification of *Ganoderma* fungus.

Template DNA was extracted from coconut roots by the method described by Moller et al. (1992). The PCR buffers, nucleotide mix and Taq polymerase were used as given by Niepold and Schober-Butin (1997). The Gan1 and Gan2 primers 18 mers chosen as primers are 5' – TTG ACT GGG TTG TAG CTG – 3' (forward primer) and 5' – GCG TTA CAT CGC AAT ACA – 3' (Reverse primer). These primers were designed from ITS region 1 of ribosomal DNA of *G. boninense* (Obtained from EMBL accession number X78749). The application of this primers generated from the ITS1 sequence proved to be useful for the specific detection of plant pathogenic *Ganoderma* (Utomo and Niepold, 2000). The expected DNA fragment product size is 167 bp. The thermocycler was programmed as follows; 5 min preheating at 95°C followed by 48 cycles consisting of denaturation at 94°C for 40 s, annealing at 52°C for 40 s and extension at 72°C for 45 s with a final 12 min extension at 72°C. The PCR products were analysed by electrophoresis on a 1.6% agarose gel followed by visualized under UV light, photographed and documented using AlphaImager (Alpha Innotech, California, USA).

Management trial in early-diagnosed palms

In two coconut gardens, palms infected by *Ganoderma* but yet to express symptoms were identified by employing immunoassay and molecular diagnosis. Field trials for the management of the disease were laid out at Lakshathoppu, Pattukottai and Nanjundapuram, Coimbatore, Tamil Nadu, India. The infected palms that are yet to express symptoms were identified and the treatments were imposed on these palms. The experiments were laid out in randomized block design (RBD) with three replications (Table 1.)

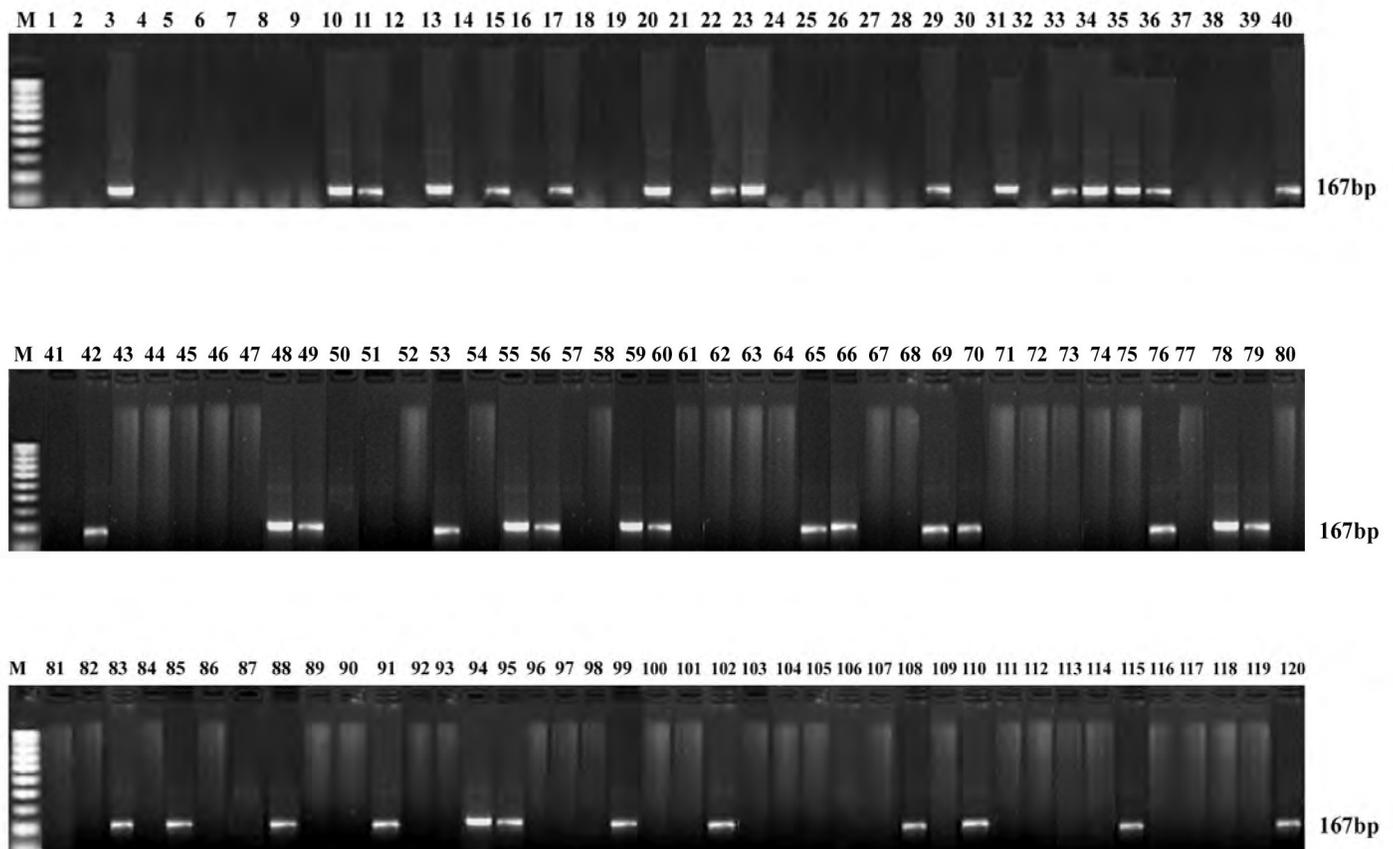


Figure 1. Field testing of PCR technique for early diagnosis of *ganoderma* disease at lakshathoppu, pattukottai field.

Statistical analysis

The data were analyzed independently for studies under field condition.

The IRRISTAT version 92-1 programme developed by biometrics unit at the International Rice Research Institute, Philippines was used.

RESULTS

Field testing of early diagnostic tests and management

In two coconut gardens, apparently healthy palm roots were tested for infection by *Ganoderma* by ELISA test. A total of 255 palms were tested in two fields (120 palms from Pattukottai field and 135 palms from Coimbatore field). The field evaluation was carried out with basidiocarp mycelial protein antiserum and the O.D values of more than 0.717 were recorded as infected. This value was fixed based on the O.D value obtained by ELISA test of 20 healthy coconut root samples. The results of the ELISA test in 255 palms revealed that 85 palms were infected, while the rest were free from

infection (Data are not shown here). These results were further confirmed with the PCR technique using Gan1 and Gan2 primers with the amplification product of 167 bp (Figure. 1). From these infected palms that were diagnosed early before expression of symptoms, 39 palms were selected from each field for disease management trial.

Management

Treatments as listed in Table 1 were imposed on the early diagnosed palms and diagnostic tests were continued at monthly interval in the treated palms. Banana intercropped, neera tapped and micronutrients treated palms did not respond to the treatments in the initial stages and showed delayed response in ELISA tests. Banana intercrop and neera tapped palms showed response only after 2 months. The micronutrients treated palms responded only 3 months after the treatment imposition. In control palms, symptoms were observed on 4th, 6th and 7th months on 4 palms. Remaining two trees in control did not show any symptom till the completion of the experiment.

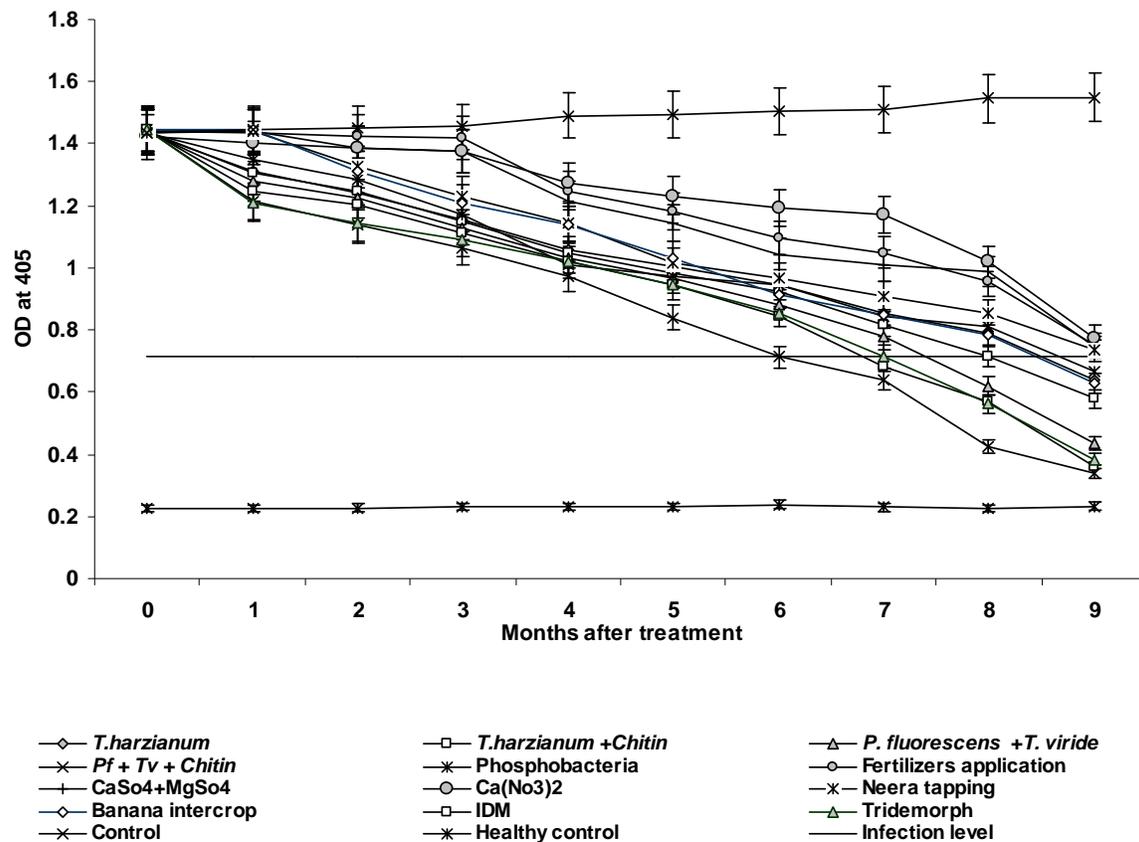


Figure 2. Field evaluation of ELISA test at monthly interval (combination of two trials).

Based on the ELISA results, *P. fluorescens* + *T. viride* with chitin amended treatment showed reduced infection level within six months (Figure 2). Integrated disease management (IDM) and Tridemorph treatment showed reduced infection level in seven months while *T. harzianum* and *P. fluorescens* + *T. viride* treatment responded in eighth months. These results were also confirmed by PCR (Figure. 3). In this test also, infection was present in fertilizer-, CaSO_4 + MgSO_4 -, $\text{Ca}(\text{NO}_3)_2$ -treated and neera tapped palms as observed in ELISA. One palm in each of fertilizer-, $\text{Ca}(\text{NO}_3)_2$ -, CaSO_4 + MgSO_4 -treated and neera tapped treatments showed infection in Pattukottai field.

DISCUSSION

In the present study, work was mainly concentrated on the detection of *Ganoderma* at early stages of infection by applying ELISA and PCR technologies, and management of the disease by using various treatments. Infected roots and *Ganoderma* isolates showed 167 bp amplification, which confirms *Ganoderma* presence. ITS1 region of *Ganoderma* is flanked by highly conserved sequences (Moncalvo et al., 1995) and no variation was observed from generated amplificate within the other pathogenic *Ganoderma* species surveyed. Based on the

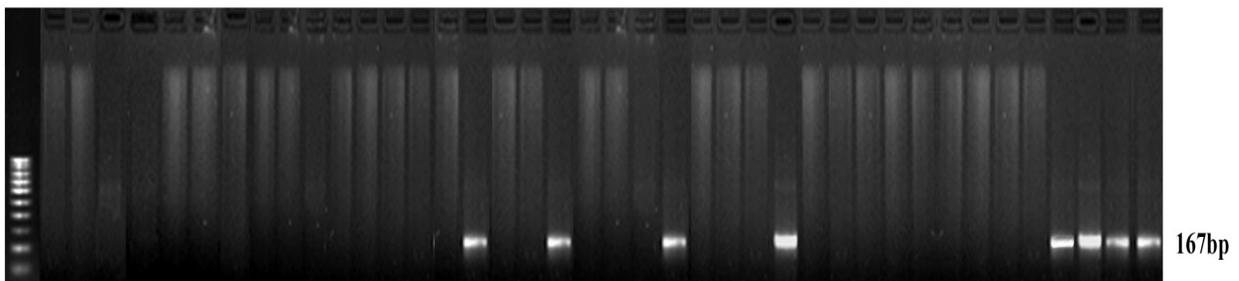
above findings, *Ganoderma* detection in apparently healthy palms in two coconut gardens was done by immunoassay and molecular diagnosis in the early diagnosed palms

The progress of infection was arrested in all the treated palms as assessed by ELISA and PCR tests. Among the treatments, mixture of *P. fluorescens* + *T. viride* amended with chitin showed the best results. Early responses were obtained in palms with IDM practice and fungicide treatments also. The O.D value in ELISA was below the infection limit in *P. fluorescens* + *T. viride* + chitin treated palms within 6 months of treatment followed by IDM practice and fungicide treated palms, where the O.D value below infection level was obtained on 7th month of treatment.

In our study, the mixture of two antagonists (*P. fluorescens* + *T. viride*) suppressed the *Ganoderma* disease development. Numerous modes of action have been postulated and demonstrated for the antagonistic effects of *P. fluorescens* in controlling diseases which include synergistic effects observed on fungal pathogens with a combination of antifungal compounds (Dowling and O'Gara, 1994; Dunne et al., 1998), competition for nutrients (O'Sullivan and O'Gara, 1992), production of cell wall lytic enzymes (Singh et al., 1999) and induced systemic resistance (Dalisyay and Kuc, 1995;

A. Lakshathoppu, Pattukottai field trial

M T1 T1 T1 T2 T2 T2 T3 T3 T3 T4 T4 T4 T5 T5 T5 T6 T6 T6 T7 T7 T7 T8 T8 T8 T9 T9 T9 T10 T10 T10 T11 T11 T11 T12 T12 T12 T13 T13 T13 PC



B. Nanjundapuram, Coimbatore field trial

M T1 T1 T1 T2 T2 T2 T3 T3 T3 T4 T4 T4 T5 T5 T5 T6 T6 T6 T7 T7 T7 T8 T8 T8 T9 T9 T9 T10 T10 T10 T11 T11 T11 T12 T12 T12 T13 T13 T13 PC

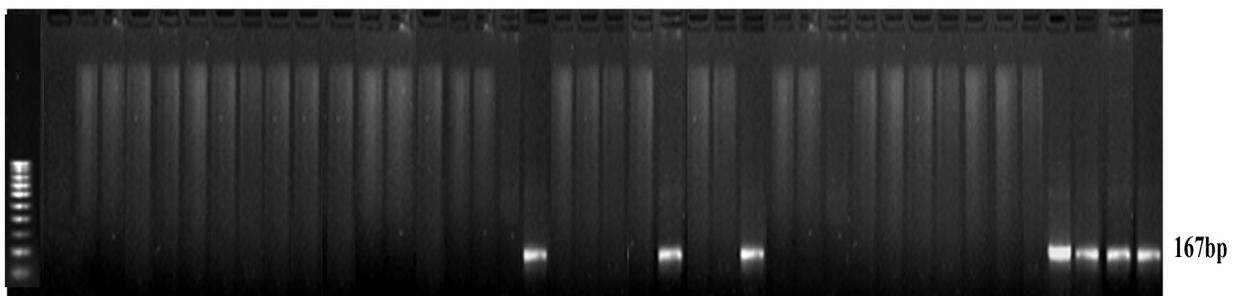


Figure 3. Field evaluation of PCR technique for the treated early diagnosed coconut palms from the field trials.

Nandakumar et al., 2001). Several antibiotics have been reported to be produced by bacteria (Raajimakers and Weller, 2001)

In case of *Trichoderma* spp. the diversified mechanism include production of wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with pathogen for nutrient and for occupation of the infection court, induced resistance, production of protease and fungal cell wall degrading enzymes have been reported (Denis and Webster, 1971; Elad, 2000; Perello et al., 2003).

Antagonist-host interaction may involve any of these mechanisms individually or more than one of them acting simultaneously in synergistic manner to suppress the disease. Moreover, the antagonists used in this study are best inducers of plant chitinase and peroxidase, which are some of the important components of the induced systemic resistance (ISR) (Dalisay and Kuc, 1995; Yedidia et al., 1999; Ramamoorthy et al., 2002).

In the present study, the antagonists selected for field studies, *P. fluorescens* Pf1 + *T. viride* and *T. harzianum*, were prepared as talc based formulation for field application. The effect of chitin amendment with the bioformulations was also studied. Talc based formulations of biocontrol agents have been reported to

be effective against various plant diseases under greenhouse and field conditions (Vidyasekaran and Muthamilan, 1995; Vidhyasekaran et al., 1997a,b; Nandakumar et al., 2001; Ramamoorthy et al., 2002). Chitin amendment has already been used with bioformulations (Radjacommare et al., 2002). Kokalis-Burelle et al. (1991) found that the insoluble polymer chitin could selectively enhance the growth and antagonism of chitinolytic bacteria. Chitin, a polymer of N-acetyl glucosamine, is a structural polysaccharide present in fungi (Cabib, 1987). Viswanathan and Samiyappan (2001) have confirmed the production of chitinases by fluorescent pseudomonad strains grown in chitin containing medium, which in turn resulted in enhanced inhibition of *C. falcatum* by the bacterial strains *in vitro*.

In this present investigation, integrated approach with cultural, chemical and biological methods show O.D values below infection level within seven months. This finding confirms the previous work done by various workers in the management of the disease in palms expressing visible symptoms (Bhaskaran et al., 1989; Karthikeyan et al., 1998a; Srinivasulu et al., 2002). Bhaskaran (1993) obtained better result when IDM practices were followed as these practices reduced the

disease intensity and increased the yield by 132%.

In early diagnosed palms treated with the fungicide tridemorph, the disease intensity reduced below infection level on seventh month. This finding is also in agreement with the previous experiment carried out by Anbalagan (1979), Bhaskaran et al. (1984), Lim et al. (1990) and Ramadoss (1991). However, chemical fungicides are not thought of as a long term solution to crop health management. Requirement for repeated application, residue problems, health and environmental hazards and development of fungicide resistance in the pathogen are the major problems associated with the use and overuse of chemical fungicides (Mukhopadhyay and Mukherjee, 1996). As a result, in recent years, the focus has been shifted in finding out safer alternatives like biocontrol agents.

The infection level was low in the phosphobacteria treated palms also on ninth month. Bhaskaran (1994) has reported that the treatment of *Ganoderma* affected coconut palms with phosphobacteria (200 g of peat based inoculum with 10 kg FYM either alone or in combination with *Azotobacter*) recorded lesser disease intensity and higher nut yield. In the diseased coconut garden, growing banana intercrop, tapping for neera in diseased palms and micronutrients application, though were effective in the long run, showed slow response in ELISA test in the initial stage of the experiment. Karthikeyan and Bhaskaran (1993) have already reported that tapping of sweet toddy in mildly diseased palms reduced the disease index. The reason for the reduction in disease intensity was attributed to the alteration of host physiology by means of increased level of phenol, starch, amino nitrogen, potassium and zinc and reduced level of sugars (Vijayaraghavan et al., 1987; Anonymous, 1990; Bhaskaran, 1990; Karthikeyan and Bhaskaran 1993; Karthikeyan et al., 1998b). In the present study, it was found that the induction of these chemicals took at least two months after treatments in the above treatments and hence a delayed response.

Banana intercropped plots showed below infection level O.D on the 9th month. Bhaskaran et al. (1993) also found that banana was the ideal intercrop for managing the disease. When intercropped, rhizosphere population of fungi, actinomycetes and the antagonist *Trichoderma* increased. The increased population of these microfloras would have inhibited the growth of *G. lucidum* and reduced the disease severity (Bhaskaran et al., 1993). Delayed response may be due to the time lag for building up of the antagonists population and also the time required for accumulation of defense chemicals in the palms.

Fertilizer treated palms showed response from third month after the treatment implementation. Soil nutrition can influence disease development, but the effect appears to be related to the nature of the soil and its chemical properties. Bhaskaran et al. (1989) observed lower disease index in lower dose of fertilizer applied

plots. Calcium sulphate + magnesium sulphate treated palms reduced the positive reaction in ELISA but in PCR test, *Ganoderma* infection was detected, so this treatment may take a few more months to give complete control. The reason for the reduction in disease intensity was attributed to strengthening the cell wall by calcium and thus enhancing resistance to *Ganoderma* attack (Muchorej et al., 1980; Spiegel et al., 1987). Kommedahl and Windels (1981) attributed another reason for the reduction in disease severity by calcium application. They observed that calcium enhanced the population of soil microflora, which in turn suppressed the pathogen. Sariah et al. (1997) applied calcium nitrate as a prophylactic measure against BSR and found slow establishment of the pathogen in the host's tissues.

In control plot, symptom expression in the palms commenced from the fourth month of initiation of the experiment and by the seventh month, four out of six palms selected exhibited visible symptoms of the disease.

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