Rapid Detection of *Ganoderma* Disease of Coconut and Assessment of Inhibition Effect of Various Control Measures by Immunoassay and PCR

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Abstract

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Molecular and immunological methods were applied for detecting the *Ganoderma* disease of coconut. Polyclonal antibodies (PAbs) raised against basidiocarp protein of *Ganoderma* were used. For the polymerase chain reaction (PCR) tests, 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, was used. Apparently healthy palms in two coconut gardens were tested for *Ganoderma* disease 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 within 6 months showed an optical density (OD) below the level of infected plants. Integrated Disease Management (IDM) and fungicide tridemorph treated palms showed OD values below infection level within 7 months, and *T. harzianum* and *P. fluorescens* + *T. viride* treated palms showed OD values below infection level in 8 months.

Keywords: *Ganoderma*; early diagnosis; PCR; ELISA; integrated disease management

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

The incubation period of this disease has been determined to be several years (Turner 1981). Visible disease symptoms appear at a very late stage of infection when more than half of the root tissues have decayed, leaving no chance for the

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grower to cure the infected palms. Basal stem rot disease of coconut can be contained by management practices if the disease is detected in its early stages. A few methods have been reported to be useful to identify 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). Polymerase chain reaction (PCR) technology has revolutionised the field of plant pathology in diagnosing various plant pathogens (Henson & 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 & 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 (ELISA) and Dot Immunobinding Assay (DIBA) techniques is reliable.

Therefore, the goals of this study were to early detect and manage, by various treatments, *Ganoderma* infected coconut palms before appearance of visual symptoms 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.

MATERIAL 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 0.1M phosphate buffer (pH 7.0; 1:2 dilution) by sterile mortar and pestle 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 (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 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% polyvinylpyrrolidone and 0.2% 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) was 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 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 3M 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 the internal transcribed spacers region is used for the identification of *Ganoderma*.

DNA extraction from plant samples. Template DNA was extracted from coconut roots by the method described by Moller *et al*. (1992).

Polymerase Chain Reaction (PCR). The PCR buffers, nucleotide mix and Taq polymerase were used as given by Niepold and Schober-Butin (1997). The two 18 mers were chosen as primers:

5'-TTG ACT GGG TTG TAG CTG-3' (Forward primer),

5'-GCG TTA CAT CGC AAT ACA-3' (Reverse primer).

These primers were designed from ITS region 1 of ribosomal DNA of *Ganoderma boninense* (obtained from EMBL accession number X78749). The application of these primers generated from the ITS1 sequence proved to be useful for the specific detection of plant pathogenic *Ganoderma* (Utomo & Niepold 2000). The expected DNA fragment product size was 167 bp. The thermocycler was programmed for 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, visualised under UV light, photographed and documented using an AlphaImager (Alpha Innotech, California, USA).

Table 1. Treatments list

Treatments	Description
T_1	*Trichoderma harzianum* 500 g + 50 kg farm yard manure (FYM)
T_2	*T. harzianum* + chitin 500 g + 50 kg FYM
T_3	*Pseudomonas fluorescens* 200 g + *T. viride* 200 g + 50 kg FYM
T_4	*P. fluorescens* + chitin 200 g + *T. viride* + chitin 200 g + 50 kg FYM
T_5	Phosphobacteria 200 g + 10 kg FYM
T_6	Fertiliser application: N – 0.35: P_2O_5 – 0.25: K_2O – 0.45 kg/tree
T_7	Micronutrient: $CaSO_4$ 500 g + $MgSO_4$ 500 g
T_8	$Ca(NO_3)_2$ – 25 g
T_9	Tapping – neera 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) 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

Management trial with early-diagnosed palms. In two coconut gardens, at Lakshathoppu, Pattukottai and Nanjundapuram, Coimbatore, Tamil Nadu, India, palms infected by *Ganoderma* but yet to express symptoms were identified by employing immunoassay and molecular diagnosis (Karthikeyan *et al*. 2006).

These palms were used for field trials on the management of the disease, and the treatments applied (Table 1). The experiments were laid out in RBD with three replications.

Statistical analysis. The data were analysed independently for the studies under field conditions. The data were analysed as randomised block design (RBD) using the IRRISTAT version 92-1 programme developed by the biometrics unit at the International Rice Research Institute, Philippines.

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 the Pattukottai field and 135 from the Coimbatore field). The field evaluation was carried out with basidiocarp mycelial protein antiserum and OD values higher than 0.717 were taken as indicating infection; this value was arrived at after ELISA tests of 20 samples from healthy coconut roots. The results of the ELISA tests on 255 palms revealed that 85 palms were infected, while the others were healthy (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, with infection diagnosed early before expression of symptoms, 39 palms were selected from each field for the disease management trial.

Management trial

Treatments as listed in Table 1 were applied on the early diagnosed palms and diagnostic tests were continued at monthly intervals on the treated palms. Trees receiving the treatments banana intercropped, neera tapped and micronutrients, viz., $CaSO_4$, $MgSO_4$ and $Ca(NO_3)_2$, did not respond in the initial stages and showed a delayed response in ELISA tests. Banana intercrop and neera tapped palms responded only after 2 months, the micronutrients treated palms only 3 months after treatment. In palms of the healthy control,

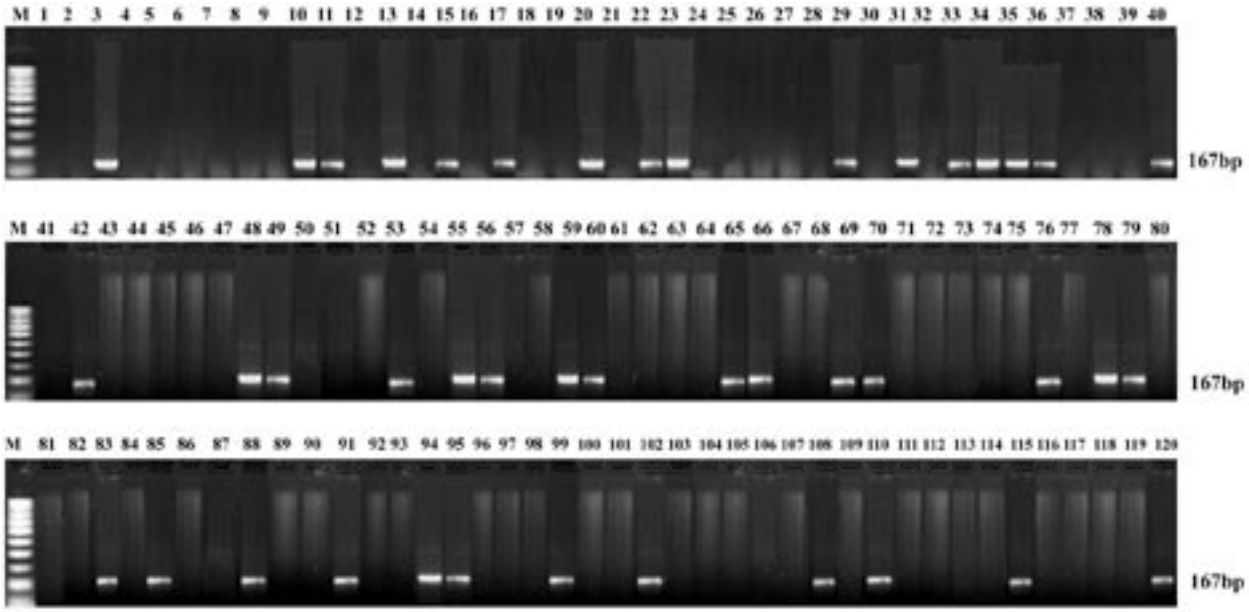

Figure 1. Field testing of coconut palms by PCR technique for early diagnosis of *Ganoderma lucidum* at Lakshathoppu, Pattukottai field

symptoms were observed on four palms in months 4, 6 and 7. The remaining two trees of this control did not show any symptoms till the completion of the experiment.

Based on the ELISA results, *P. fluorescens* + *T. viride* with chitin amended treatment showed a reduced infection level within 6 months (Figure 2). IDM and Tridemorph treatments showed reduced infection level in 7 months, while *T. harzianum* and *P. fluorescens* + *T. viride* treatments were effective after 8 months. These results were also confirmed by PCR (Figure 3). In this test also, infection was present in fertiliser, micronutrients ($CaSO_4$ + $MgSO_4$, Ca $(NO_3)_2$) treated and neera

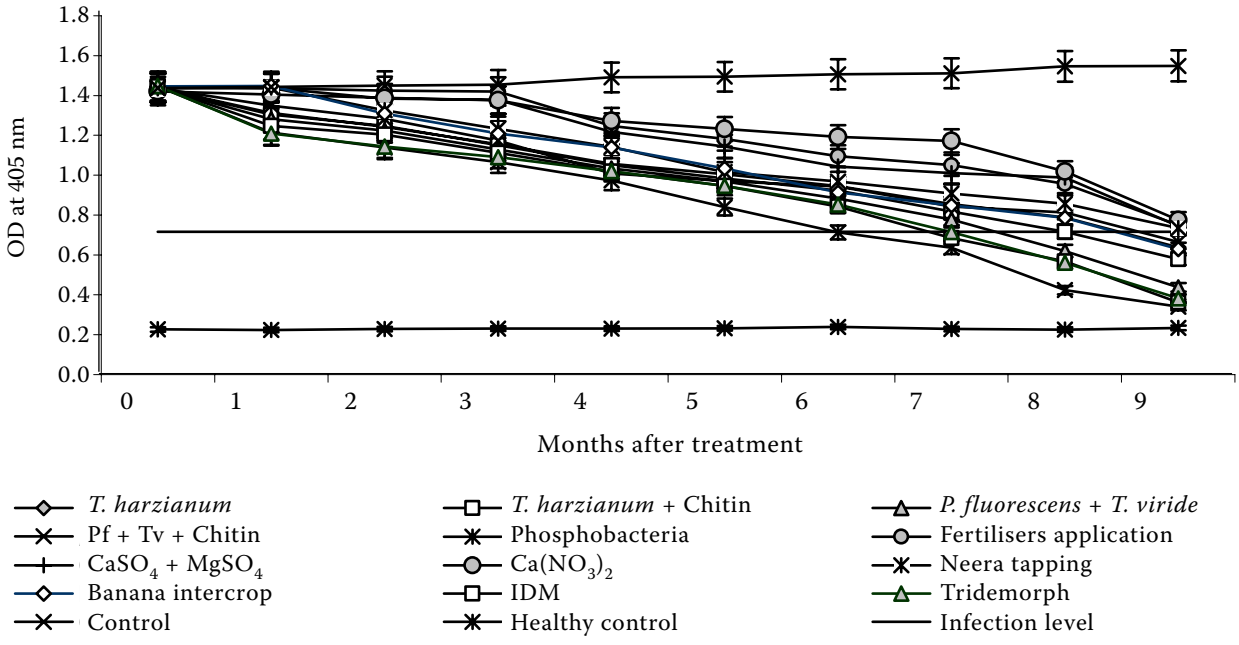

Figure 2. Field evaluation, at monthly intervals, by ELISA test of 13 treatments of coconut palms to manage *Ganoderma lucidum* (combination of two trials)

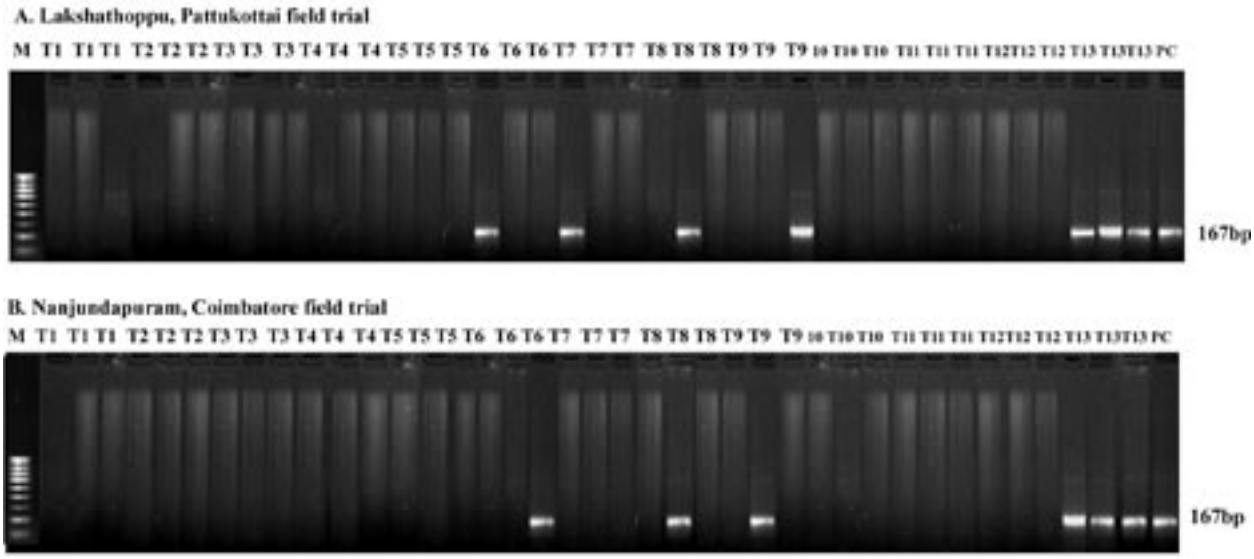

Figure 3. Field evaluation, by PCR technique, of early diagnosed coconut palms which were then treated in field trials

tapped palms as observed in ELISA. One palm in each of the fertiliser, Ca $(NO_3)_{2,}$ $CaSO_4$ + $MgSO_4$ treated and neera tapped treatments showed infection in the Pattukottai field.

DISCUSSION

The present study mainly concentrated on the detection of *Ganoderma* at early stages of infection and on managing the disease by using various treatments; both aspects were monitored by ELISA and PCR technologies. Infected roots and *Ganoderma* isolates showed 167 bp amplification, which confirms the presence of *Ganoderma*. The 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 these findings, infection by *Ganoderma* was successfully detected early by immunoassay and molecular diagnosis in apparently healthy palms in two coconut gardens.

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

In our study, the mixture of two antagonists (*P. fluorescens* + *T. viride*) suppressed *Ganoderma* disease development. Numerous modes of action have been postulated and demonstrated for the antagonistic effects of *P. fluorescens* in controlling diseases; they include synergistic effects observed on fungal pathogens with a combination of antifungal compounds (Dowling & O'Gara 1994; Dunne *et al*. 1998), competition for nutrients (O'Sullivan &d O'Gara 1992), production of cell wall lytic enzymes (Singh *et al*. 1999) and induced systemic resistance (Dalisay & Kuc 1995; Nandakumar *et al*. 2001). Several antibiotics have been reported to be produced by bacteria (Raajimakers & Weller 2001).

In case of *Trichoderma* spp., diverse mechanisms have been reported such as production of a wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with the pathogen for nutrients and for occupation of the infection court, induced resistance, production of protease and fungal cell wall degrading enzymes (Denis & 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 induced systemic resistance (ISR) (Dalisay &d Kuc 1995; Yedidia *et al.* 1999; Ramamoorthy *et al*. 2002).

In the present study, the antagonists selected for the field trials, i.e. *P. fluorescens* Pf1 + *T. viride* and *T. harzianum,* were prepared as talc based formulations 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 & 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 the present investigation, the integrated approach with cultural, chemical and biological methods (IDM) show OD values below infection level within 7 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 results when IDM practices were followed since they reduced the disease intensity and increased the yield by 132%.

In early diagnosed palms treated with the fungicide tridemorph, the disease intensity was reduced below infection level in the seventh month. This finding is also in agreement with previous experiments 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. The necessity 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 &d Mukherjee 1996). As a result, in recent years, the focus has been shifted to finding safer alternatives like biocontrol agents.

The infection level was low in the phosphobacteria treated palms in the 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*) gave lower disease intensity and higher nut yield. In the diseased coconut garden, growing banana intercrop, tapping for neera in diseased palms and micronutrients application, though 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; Karthikeyan *et al.* 1998b; Anonymous 1990; Bhaskaran 1990; Karthikeyan & Bhaskaran 1993). In the present study it was found that the induction of these chemicals took at least two months with the above treatments, and hence a delayed response.

Banana intercropped plots showed an OD below infection level in the ninth 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 populations of these components of the microflora would have inhibited the growth of *G. lucidum* and reduced the disease severity (Bhaskaran *et al.* 1993). A delayed response may be due to the time lag for building up the antagonists population and also the time required for accumulation of defence chemicals in the palms.

Fertiliser treated palms showed response from third month after treatment was implemented. 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 a lower disease index in plots that had received a lower dose of fertiliser. Calcium sulphate + magnesium sulphate treated

palms reduced the positive reaction in ELISA, but *Ganoderma* infection was detected in the PCR test; this treatment may thus 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) suggested another reason for the reduction in disease severity by calcium application. They observed that calcium enhanced the population of the 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 the healthy control plot, symptom expression in the palms began 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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