Induction of phenolics and defense-related enzymes in coconut (*Cocos nucifera* L.) roots treated with biocontrol agents

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The effect of soil application of biocontrol agents (*Pseudomonas fluorescens*, *Trichoderma viride* and *T. harzianum)* in combination with chitin on induction of phenolics and defense enzymes in coconut roots infected with *Ganoderma lucidum*, the causal agent of *Ganoderma* disease, was investigated. Soil application of these biocontrol formulations in combination with chitin induced a significant increase in the activities of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), chitinase and β–1,3-glucanase in the *G. lucidum* infected palms. Activities of both PAL and PO reached maximum levels within 3 d while the activity of PPO reached the maximum level 6 d after application of a mixture of *P. fluorescens*, *T. viride* and chitin. Isozyme analysis revealed that unique PO3 and PPO2 isozymes were induced in coconut palms treated with *P. fluorescens* + *T. viride* + chitin. Accumulation of phenolics was recorded 3 d after treatment and reached maximum levels 9 d after treatment application. Activity of chitinase was significantly increased from the third day after treatment imposition and continued to increase up to 9 to 12 d in all treatments. Chitinase isozyme analysis revealed that a unique Chit3 isoform was induced in coconut roots treated with *P. fluorescens* + *T. viride* + chitin. The β-1,3-glucanase activity was maximum 9 d after treatment application. The mechanisms by which *P. fluorescens* + *T. viride* + chitin reduced the incidence of *Ganoderma* disease in coconut may be related to its ability to induce defense mechanisms in coconut palms.
Key words: Chitin, *Ganoderma* disease, induced systemic resistance, pathogenesis-related proteins, phenolics, *Pseudomonas fluorescens, Trichoderma*

Indução de fenóis e de enzimas relacionadas à defesa em raízes de coqueiro (*Cocos nucifera* L.) tratadas com agentes de biocontrole: Investigou-se o efeito da aplicação, no solo, de agentes de biocontrole (*Pseudomonas fluorescens*, *Trichoderma viride* e *T. harzianum,* em combinação com quitina), sobre a indução de enzimas de defesa e fenóis em raízes de coqueiros infectados com *Ganoderma lucidum*, o agente causal da enfermidade *Ganoderma.* A aplicação dessas formulações de biocontrole acarretou em aumento significativo nas atividades de peroxidases (PO), polifenol oxidase (PPO), fenilalanina amônia liase (PAL), quitinase e β-1,3-glicanase nas plantas infectadas com *G. lucidum*. Após a aplicação de uma mistura de *P. fluorescens*, *T. viride* e quitina, as atividades tanto da PAL como da PO foram máximas dentro de 3 d, ao passo que a da PPO foi máxima dentro de 6 d. Análises izoenzimáticas sugeriram que apenas as isozimas PO3 e PPO2 foram induzidas nas plantas tratadas com *P. fluorescens* + *T. viride* + quitina. Após a imposição dos tratamentos, o acúmulo de fenóis ocorreu dentro de 3 d, com concentrações máximas dentro de 9 d. A atividade da quitinase aumentou significativamente a partir do terceiro dia após a aplicação das formulações, com incrementos consistentes até 12 d de experimento, nas plantas de todos os tratamentos. A isoforma Chit3 foi a única isozima de quitinase induzida nos coqueiros tratados com *P. fluorescens* + *T. viride* + quitina. A atividade da β-1,3-glicanase foi máxima aos 9 d após a aplicação dos tratamentos. Os mecanismos pelos quais a combinação de *P. fluorescens* + *T. viride* + quitina reduziu a incidência da *Ganoderma* nas plantas podem estar relacionados à capacidade do coqueiro de induzir mecanismos de defesa.
Palavras-chave: fenóis, *Ganoderma*, quitina, proteínas relacionadas a patogênese, *Pseudomonas fluorescens*, resistência sistêmica induzida, *Trichoderma*

INTRODUCTION

Plants can acquire local and systemic resistance to diseases through various biological agents including necrotizing pathogens, non-pathogens and soil-borne rhizosphere bacteria and fungi (Van Loon et al., 1998). This type of resistance, known as induced systemic resistance, is mediated by a jasmonate/ethylene sensitive pathway (Van Loon et al., 1998). Fluorescent pseudomonads are non-pathogenic rhizobacteria and several isolates of *Pseudomonas fluorescens*, *P. putida, P. aeruginosa* and *P. aureofaciens* are known to suppress the soil-borne pathogens through rhizosphere colonization (Elad and Chet, 1987), antibiosis (Pierson and Weller, 1994) and iron chelation by siderophore production (Lemanceau et al., 1992). *Trichoderma* spp are non-pathogenic rhizosphere colonizing fungi. Several isolates of *T. viride, T. harzianum, T. longibrachiatum, T. reesi* and *T. pseudoconingi* suppress the soil-borne pathogens by diversified mechanisms *viz.,* production of a wide range of broad spectrum antifungal metabolites, mycoparasitism, competition with the pathogen for nutrient and for occupation of the infection court, induced resistance, production of protease and fungal cell wall degrading enzymes (Perello et al., 2003).

Treatment with these rhizosphere bacteria and fungi have been reported to sensitize plants to defend themselves against pathogen attack by triggering various defense mechanisms including production of phytoalexins, synthesis of phenolics (M'Piga et al., 1997; Chen et al., 2000), accumulation of pathogenesis-related proteins (Meena et al., 2000) and deposition of structural barriers (Benhamou et al., 1996). The defense gene products peroxidases (PO) and polyphenol oxidases (PPO) catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) is involved in the synthesis of phytoalexins and phenolics (Karthikeyan et al., 2005). Pathogenesis-related proteins (PRs) such as β-1,3-glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8, and PR-11) degrade the fungal cell wall and cause lysis of fungal cell walls. Furthermore, chitin and glucan oligomers released during degradation of the fungal cell wall by the action of lytic enzymes act as elicitors that elicit various defense mechanisms in plants (Karthikeyan et al., 2005).

Ganoderma disease of coconut incited by *Ganoderma lucidum* is a serious soil-borne disease in coconut. Earlier studies indicated that soil application of biocontrol agents prevented the entry of *G. lucidum* in the vascular tissue by strengthening of cell wall structures and accumulation of phenolic substances (Bhaskaran, 2000). Recently we demonstrated that soil application of a mixture of *P. fluorescens*, *T. viride* and chitin prevented the multiplication of *G. lucidum* in the previously infected plants (Karthikeyan et al., 2006a). However, studies on induction of defense mechanisms in coconut upon treatment with biocontrol agents are limited. The present study was carried out to assess the induction of phenolics and defense enzymes involved in the phenylpropanoid pathway and accumulation of PR-proteins in *Ganoderma* disease infected coconut palms in response to application of biocontrol agents in combination with chitin.

MATERIAL AND METHODS

Biocontrol agents: The fungal and bacterial antagonists *viz., Trichoderma viride*, *T. harzianum* and *Pseudomonas fluorescens* Pf1 isolates were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The *T. viride* and *T. harzianum* cultures were maintained on potato dextrose agar medium. The *P. fluorescens* Pf1 culture was maintained on King's B broth (KBB) (King et al. 1954).

Preparation of talc-based formulation: The talc-based formulations for *P. fluorescens* were prepared by following the method described by Vidhyasekaran and Muthamilan (1995). Briefly, a loopful of bacteria was inoculated into 400 μL of KBB in a conical flash and incubated on a rotary shaker at 150 *g* for 48 h at room temperature (25 ± 2°C). One kilogram of talc powder was taken in a sterilized metal tray and its pH was adjusted to neutral by adding $CaCO_3$ at the rate of 15 g kg^{-1}. Ten grams of carboxymethyl cellulose (CMC) were added to 1 kg of talc and mixed well and the mixture was autoclaved for 30 min on each of two consecutive days. Four hundred milliliters of a 48 h-grown bacterial suspension containing 9×10^8 cfu mL^{-1} were mixed with sterilized talc powder under aseptic conditions. After shade drying overnight, the formulation was packed in a polypropylene bag and sealed.

For preparation of the formulation for *Trichoderma*, a 5 mm mycelial disc of *Trichoderma* was inoculated into 500 mL of sterilized molasses yeast (molasses 2%, yeast 0.3%) broth and incubated at room temperature (28 ± 2°C)

for 15 d (Singh et al. 2001). One kilogram of talc powder was taken in a sterilized metal tray and its pH was adjusted to neutral as above. Ten grams of CMC were added to 1 kg of talc and mixed well and the mixture was autoclaved for 30 min on each of two consecutive days. Five hundred milliliters of spore suspension were mixed with sterilized talc powder under aseptic conditions and the spore concentration was adjusted to $1.5x10^8$ cfu g^{-1}. After shade drying overnight, the formulation was packed in a polypropylene bag and sealed.

Chitin amendments with talc-based formulations: Five grams of crab shell chitin (Sigma, USA) were slowly added into 100 mL of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glasswool into 200 mL of ethanol at 4°C with rapid stirring. The resulting chitin suspension was centrifuged at 10000 *g* for 20 min and the chitin pellet washed repeatedly with distilled water until the pH became neutral. The concentration was adjusted to 10 mg mL^{-1} and added to KBB or molasses yeast broth (1%, v/v). The medium amended with chitin was used for culturing *P. fluorescens* and *Trichoderma* spp and talc-based formulations were prepared as described above.

Field trials: A field trial was conducted during May 2003 at Nanjundapuram village of Coimbatore District, Tamil Nadu, India. The infected palms that were as yet to express symptoms were identified by an ELISA test using basidiocarp protein antiserum (Karthikeyan et al., 2006b). The experiments were arranged in a randomized block design with three replications and the following treatments: T_1- *T. harzianum* talc-based formulation (500 g); T_2- Chitin amended *T. harzianum* talc-based formulation (500 g); T_3- *P. fluorescens* talc-based formulation (200 g) + *T. viride* talc-based formulation (200 g); T_4- Chitin amended *P. fluorescens* talc-based formulation (200 g) + Chitin amended and *T. viride* talc-based formulation (200 g); and T_5- Control. The formulations were mixed with 50 kg of farmyard manure and incubated for 30 d before applying to each coconut palm.

Sample collection for biochemical analysis: The roots of treated palms and untreated control palms were collected at 0, 3, 6, 9, 12, 15, 18 and 21 d after treatment application, washed in running tap water and stored in a deep freezer (-70°C) until used for biochemical analyses.

Estimation of PAL (EC 4.3.1.5) activity: Root samples (1g) were homogenized in 3 mL of ice-cold 0.1 M sodium borate buffer, pH 7.0, containing 1.4mM 2-mercaptoethanol and 0.1g insoluble polyvinyl-pyrrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16000 *g* at 4°C for 15 min. The supernatant was used as the enzyme source. Activity of PAL was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid as described by Dickerson et al. (1984). A sample containing 0.4 mL of enzyme extract was incubated with 0.5 mL of 0.1 M borate buffer, pH 8.8, and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. The optical density (O.D.) value was recorded at 290 nm and the amount of *trans*-cinnamic acid formed calculated using its extinction coefficient of 9630 M^{-1} (Dickerson et al., 1984). Enzyme activity was expressed as µmol *trans*-cinnamic acid min^{-1} g^{-1} protein.

Assay of PO (EC 1.11.1.7): Root samples (1 g) were homogenized in 2 mL 0.1 M phosphate buffer, pH 7.0, at 4°C. The homogenate was centrifuged at 16000 *g* at 4°C for 15 min and the supernatant used as the enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2°C). The changes in O.D. at 420 nm were recorded at 30 s intervals for 3 min. The enzyme activity was expressed as changes in the O.D. min^{-1} g^{-1} protein (Hammerschmidt et al., 1982).

To study the expression pattern of different PO isoforms, activity gel electrophoresis was carried out. For native anionic polyacrylamide gel electrophoresis, a resolving gel of 8% acrylamide concentration and a stacking gel of 4% acrylamide concentration were prepared. After electrophoresis, PO isoforms were visualized by soaking the gels in staining solution containing 0.05% benzidine (Sigma, USA) and 0.03% H_2O_2 in acetate buffer (20 mM, pH 4.2) (Nadlony and Sequira, 1980).

Assay of PPO (EC 1.14.18.1): PPO activity was determined as in Mayer et al. (1965). Root samples (1 g) were homogenized in 2 mL 0.1 M sodium phosphate

buffer (pH 6.5) and centrifuged at 16000 *g* for 15 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µL of 0.01 M catechol were added and the change in O.D. was recorded at 30 s interval up to 3 min at 495 nm. The enzyme activity was expressed as changes in O.D. at 495 nm min^{-1} g^{-1} protein.

An aliquot of the enzyme source was used to detect PPO isozymes. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol followed by gentle shaking resulted in the appearance of discrete dark brown protein bands (Jayaraman et al., 1987). After staining, the gel was washed with distilled water and photographed.

Estimation of phenolics: Root samples (1 g) were homogenized in 10 mL of 80% (v/v) methanol and agitated for 15 min at 70°C (Zieslin and Ben-Zaken, 1993). One milliliter of the methanolic extract was added to 5 mL of distilled water and 250 µL of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25°C. The absorbance of the developed blue colour was measured at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg^{-1} FW.

Assay of chitinase (EC 3.2.1.14): Root samples (1g) were homogenized in 2 mL of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 16000 *g* for 15 min at 4°C and the supernatant was used in the enzyme assay. The colorimetric assay of chitinase was carried out as described by Boller and Mauch (1988). Colloidal chitin was prepared according to Berger and Reynolds (1958) from crab shell chitin (Sigma, USA). The reaction mixture consisted of 10 µL of 1 M sodium acetate buffer (pH 4.0), 0.4 mL of enzyme extract and 0.1 mL of colloidal chitin (10 mg). After 2 h incubation at 37°C, the reaction was stopped by centrifugation at 8000 *g* for 3 min. An aliquot of the supernatant (0.3 mL) was pipetted into a glass reagent tube containing 30 µL of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µL desalted snail gut enzyme (Helicase). Finally, the mixture was incubated with 2 mL of dimethylaminobenzaldehyde for 20 min at 37°C and O.D. was measured at 585 nm. The enzyme activity was expressed as nmol N-acetylglucosamine $min^{-1}g^{-1}$ protein.

Chitinase activity was also detected by PAGE according to the method of Trudel and Asselin (1989) with modification. Chitinase was extracted by homogenizing 1g of tissue in 0.01 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 16000 *g* for 15 min at 4°C and the supernatant used as the enzyme source. After native electrophoresis, gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min, and then in 100 mM sodium acetate buffer at pH 5.0 containing 0.01% (w/v) glycol chitin for 30 min at 37°C. The gels were finally transferred into a solution containing 0.01% (w/v) Calcofluor white M2R (Sigma) in 500 mM Tris HCl (pH 8.9). After 5min the brightener solution was removed and the gels were rinsed with distilled water for more than 1h. Lytic zones were visualized and photographed under UV light in an alpha imager (AlphaImager®, Alpha Innotech Corporation, San Leandro, California, USA).

Assay of β-1,3-glucanase (EC 3.2.1.39): Root samples (1 g) were extracted with 2 mL of 0.05 M sodium acetate buffer (pH 5.0) and centrifuged at 16000 *g* for 15 min at 4°C. The supernatant was used in the enzyme assay. The activity of β-1,3-glucanase was determined by measuring the release of reducing sugars by using laminarin as substrate and glucose as standard. The reaction mixture consisted of 0.25 mL of dialyzed enzyme solution, 0.3 mL of 1M sodium acetate buffer (pH 5.3) and 0.5 mL of 4% laminarin (Pan et al., 1991). The reaction was carried out at 40°C for 2 h. The reaction was stopped by adding 375 µL of dinitrosalicylic acid and heating for 5 min on a boiling water bath, vortexed and its O.D. measured at 500 nm. Protein concentration was determined by the method of Bradford (1976). The specific activity of β-1,3-glucanase was expressed as µg glucose released min^{-1} g^{-1} protein.

Statistical analysis: The data were analyzed by using the IRRISTAT version 92-1 programme developed by the biometrics unit at the International Rice Research Institute, The Philippines.

RESULTS

A field trial was conducted to study the induction of biochemical defense mechanisms in coconut roots in

response to treatment with biocontrol agents in combination with chitin. The infected palms that were as yet to express symptoms were identified by an ELISA test using basidiocarp protein antiserum and O.D. values higher than 0.717 were taken to indicate an infected palm (Kartikeyan et al., 2006b). These palms were treated with talc-based formulations of biocontrol agents and root samples were collected at different time intervals and analyzed for induction of defense enzymes.

A significant increase in PAL activity was noticed in all the treated palms. Application of *P. fluorescens* + *T. viride* + chitin showed significantly higher PAL enzyme activity than other treatments. The activity of PAL reached the highest level within 3 d after treatment and then slowly declined. However, treated palms exhibited significantly higher PAL activity than the control plants at all time intervals. Application of *P. fluorescens* + *T. viride* + chitin and *T. harzianum* + chitin led to an increase in PAL activity of up to 50% and 40%, respectively, 3 d after treatment imposition, relative to control plants (Figure 1).

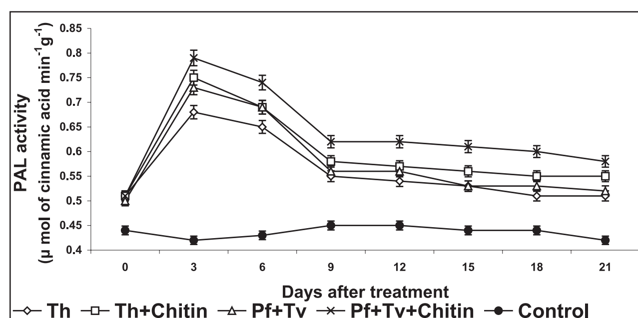

Figure 1. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the activity of phenylalanine ammonia-lyase (PAL) in coconut roots. Values are the mean of three replications and bars represent SE.

A significant increase in PO activity was detected in coconut roots upon treatment with biocontrol agents and chitin. However, treatment with *P. fluorescens* + *T. viride* + chitin recorded significantly higher enzyme activity compared to other treatments. The activity of PO reached the highest level in all the treatments at 3 d after treatment and then slowly decreased. Soil application of *P. fluorescens* + *T. viride* + chitin and *T. harzianum* + chitin

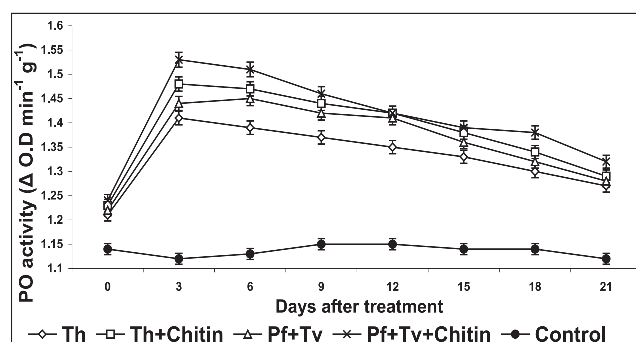

Figure 2. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the activity of peroxidase (PO) in coconut roots. Values are the mean of three replications and bars represent SE.

resulted in a 25% increase in PO activity 3 d after treatment compared to control. Significantly increased PO activity was also observed in coconut palms upon treatment with *P. fluorescens* + *T. viride* and *T. harzianum* (Figure 2).

Enhanced induction of PO was also evident in isozyme analysis where higher levels of induction of PO1, PO2, PO3 and PO4 isoforms were detected in the treatment involving *P. fluorescens* + *T. viride* + chitin. Treatment with *T. harzianum* in combination with chitin or *T. harzianum* alone resulted in induction of PO1, PO2 and PO4 isoforms (Figure 3). Protein extracts from control palms exhibited only two isozymes (PO2 and PO4).

An increase in PPO activity was also observed in coconut palms upon treatment with biocontrol agents and chitin. As shown in Figure 4, the PPO activity increased significantly within 3 d after treatment and reached the highest level at 6 d. The activity of PPO nearly doubled in plants treated with *P. fluorescens* + *T. viride* + chitin, *T. harzianum* + chitin, and *P. fluorescens* + *T. viride*. Increases in such activity were also found in palms treated with *T. harzianum* alone (Figure 4). Enhanced induction of PPO was also evident by activity gel electrophoresis where higher levels of induction of PPO1, PPO2, PPO3 and PPO4 isoforms were detected in treatments involving *P. fluorescens* + *T. viride* + chitin or *P. fluorescens* + *T. viride*. Treatment with a combination

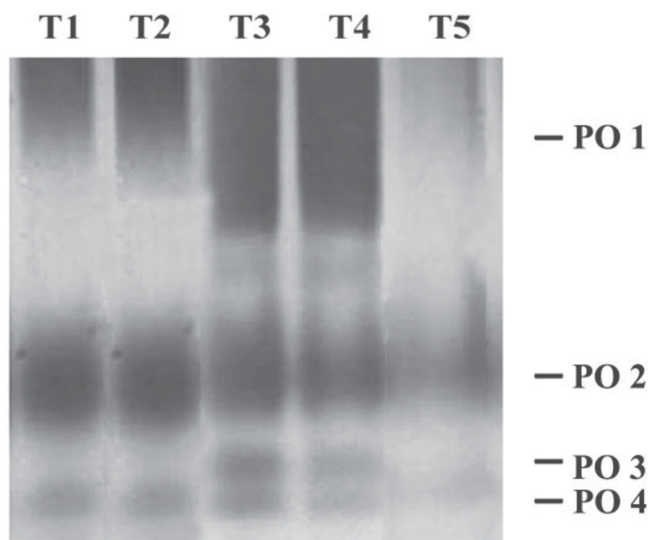

Figure 3. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the peroxidase (PO) isozyme pattern in coconut roots. T1 = Th; T2 = Th + Chitin; T3 = Pf + Tv; T4 = Pf + Tv + Chitin; T5 = Control.

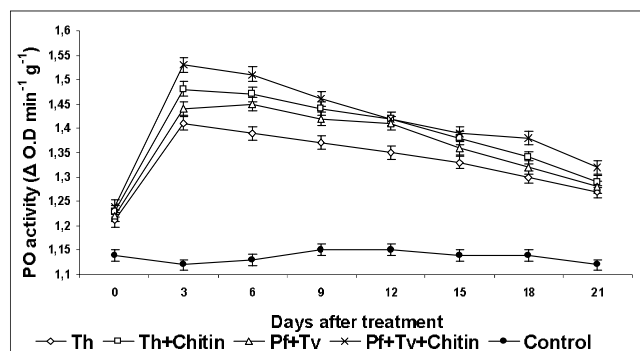

Figure 4. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the activity of polyphenol oxidase (PPO) in coconut roots. Values are the mean of three replications and bars represent SE.

of *T. harzianum* and chitin resulted in induction of PPO1, PPO2 and PPO4 (Figure 5). In the control PPO1 and PPO2 isoforms were expressed with lower intensity than the treatments.

Treatment of coconut palms with biocontrol agents and chitin resulted in a high phenol accumulation in coconut roots. Soil application of *P. fluorescens* + *T. viride* + chitin resulted in the maximum accumulation of phenol (389 µg g^{-1} FW) when compared to the control (295 µg g^{-1} FW). Treatment with *T. harzianum* + chitin, *P. fluorescens* + *T. viride* and *T. harzianum* alone recorded 374, 366 and 356 µg g^{-1} FW, respectively (Figure 6). The accumulation of phenol increased from 3 d after treatment application and reached the maximum level at 9 d and thereafter decreased even though the concentration was significantly higher than the initial level throughout the sampling period.

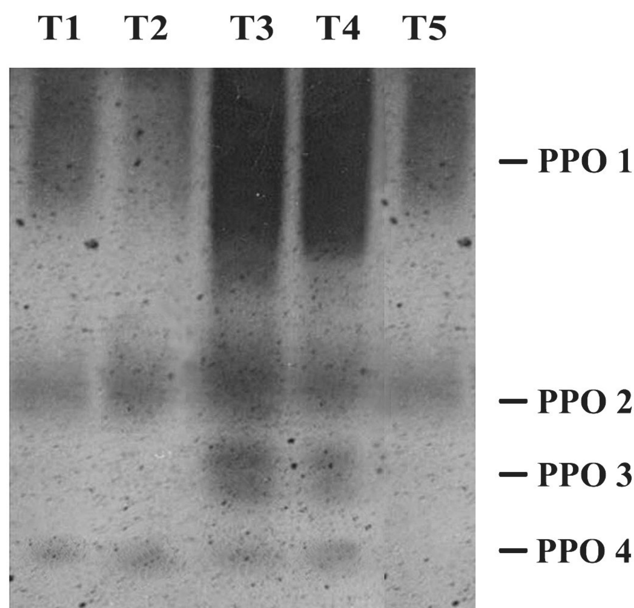

Figure 5. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the polyphenol oxidase (PPO) isozyme pattern in coconut roots. T1 = Th; T2 = Th + Chitin; T3 = Pf + Tv; T4 = Pf + Tv + Chitin; T5 = Control.

Treatment of coconut palms with *P. fluorescens* + *T. viride* + chitin significantly enhanced the chitinase activity in root tissues. The activity of chitinase significantly increased from 3 d after treatment application and continued to increase up to 12 d at which time a 30% increase in enzyme activity over the control was noticed. Relative to control plants, averaged increases in chitinase activity were observed in palms treated with *T. harzianum* + chitin (29%), *P. fluorescens* + *T. viride* (27%), and *T. harzianum* alone (23%) (Figure 7).

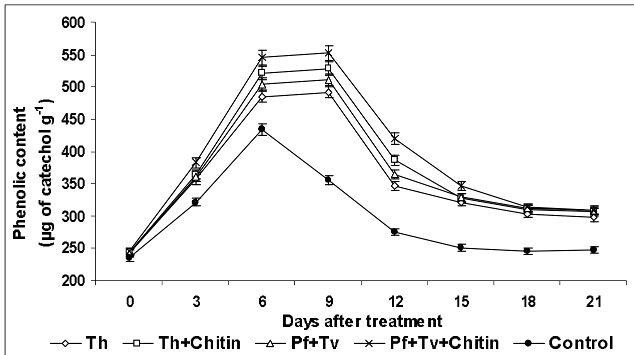

Figure 6. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the cathecol concentration in coconut roots. Values are the mean of three replications and bars represent SE.

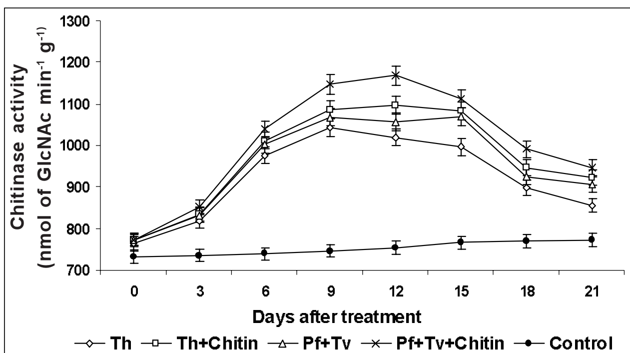

Figure 7. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the activity of chitinase in coconut roots. GlcNAc = acetylglucosamine. Values are the mean of three replications and bars represent SE.

Chitinase activity gels revealed that extracts from control palms exhibited the expression of three chitinase isoforms (chi1, chi2 and chi5). Treatment of palm with *T. harzianum* induced a new chitinase isoform (chi4), whereas treatment with *T. viride* + *P. fluorescens* + chitin or *T. viride* + *P. fluorescens* induced the expression of two new isozymes (chi3 and chi4; Figure 8).

An increase in β-1,3-glucanase activity was also observed in coconut root tissues treated with biocontrol agents and chitin. The β-1,3-glucanase activity increased within 3 d after treatment, reached the highest level at 9 d and declined thereafter. Application of *P. fluorescens* + *T. viride* + chitin resulted in a 30% increase in β-1,3-glucanase activity compared to the control 9 d after treatment application whereas treatment with *T. harzianum* + chitin, *P. fluorescens* + *T. viride* and *T. harzianum* also could increase β-1,3-glucanase activity (Figure 9).

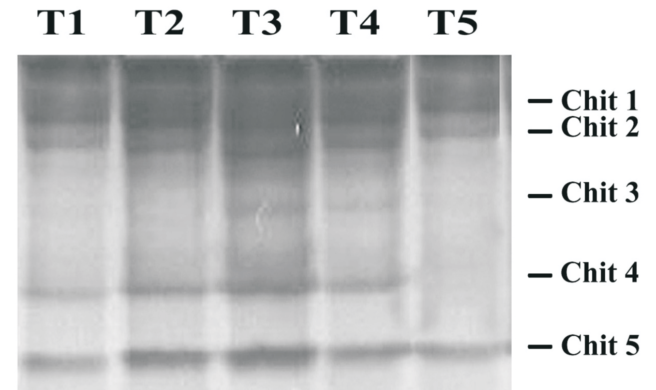

Figure 8. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the chitinase (Chit) isozyme pattern in coconut roots. T1 = Th; T2 = Th + Chitin; T3 = Pf + Tv; T4 = Pf + Tv + Chitin; T5 = Control.

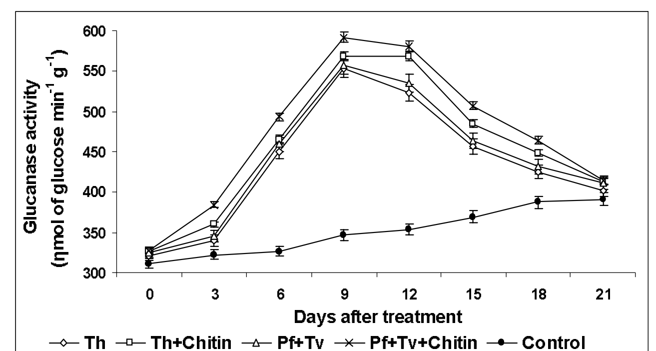

Figure 9. The effect of soil application of biocontrol agents *Trichoderma harzianum* (Th), *Pseudomonas fluorescens* (Pf), and *T. viride* (Tv) in combination or not with chitin on the activity of β-1,3-glucanase in coconut roots. Values are the mean of three replications and bars represent SE.

DISCUSSION

The present study gives direct evidence on the induction of plant defense mechanisms in the control of *Ganoderma* disease by a mixture of *P. fluorescens*, *T. viride* and chitin under field conditions. Application of the biocontrol agents, *P. fluorescens*, *T. viride* in combination with chitin induced accumulation of high levels of phenols and activities of PAL, PO, PPO, chitinase and β-1,3-glucanase in coconut palm compared to other treatments and control.

Induction of systemic resistance has been established as a new mechanism by which plants defend themselves against pathogen attack (Van Loon et al., 1998). Various reports confirm the induction of systemic resistance by *Pseudomonas* spp and *Trichoderma* spp against fungal pathogens (Yedidia et al., 1999; Meena et al., 2000).

Phenylalanine ammonia-lyase plays an important role in the biosynthesis of various defense chemicals in phenylpropanoid metabolism. Activity of PAL could be induced in plant-pathogen interactions and fungal elicitor treatment. De Meyer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots and increased the salicylic acid levels in leaves. In the present study, increased activity of PAL was recorded in bioformulation and chitin-treated palms. The time required to activate the defense mechanisms is important for the suppression of the invading pathogen. Earlier and higher levels of expression of defense enzymes and accumulation of inhibitory compounds at the infection site certainly prevent the fungal mycelial colonization. In the treated palms, the activity of PAL reached a maximum on the third day after treatment while the enzyme activity in control palms remained constant. The activity of PAL was maintained at a higher level throughout the experimental period in all treated palms. Several studies have shown that PAL activity is induced in plants upon treatment with *P. fluorescens* (Chen et al., 2000; Sundaravadana, 2002; Saravanakumar et al., 2003).

Peroxidase represents another component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West, 1989). The products of this enzyme in the presence of a hydrogen donor and hydrogen peroxide have antimicrobial activity and even antiviral activity (Van Loon and Callow, 1983). Increased activity of cell wall bound peroxidases has been elicited in different plants such as cucumber (Chen et al., 2000) and tobacco (Ahl Goy et al., 1992) due to pathogen infection. In bean, rhizosphere colonization by various bacteria induced PO activity (Zdor and Anderson, 1992). In the present study, a rapid increase in PO activity was recorded in palms treated with a mixture of *P. fluorescens*, *T. viride* and chitin. Chen et al. (2000) reported higher PO activity in cucumber roots treated with *P. corrugata* challenged with *P. aphanidermatum*. Yedidia et al. (1999) reported that the root inoculation of *T. harzianum* induced increased peroxidase activity in leaves of cucumber seedlings. The present study also indicates that expression of four PO isoforms, PO1-PO4 was more prominent in coconut roots treated with *P. fluorescens* + *T. viride* + chitin. The unique PO3 isoform induced in coconut root upon treatment with the mixture of *P. fluorescens*, *T. viride* and chitin might have contributed to induced resistance against invasion by *Ganoderma*.

Similar to other enzymes, PPO activity also increased by treatment with *P. fluorescens* + *T. viride* + chitin. Expression of four PPO isoforms, PPO1, PPO2, PPO3 and PPO4 was very clear in coconut palms treated with *P. fluorescens* + *T. viride* amended with chitin. Two new isoforms PPO3 and PPO4 with a high degree of expression were observed in *P. fluorescens* Pf1 + *T. viride* treated root tissues. The induced PPO3 and PPO4 isoforms and a higher level of expression of the other isoforms were implicated in induced defense responses against *Ganoderma* in coconut. Inductions of PPO activity and PPO isozymes by biological agents have been reported in several host pathogen combinations. Meena et al. (2000) reported that *P. fluorescens* induced the activities of PPO in response to infection by *Cercospora personatum* in groundnut. Chen et al. (2000) reported that various rhizobacteria and *Pythium aphanidermatum* induced the PPO activity in cucumber root tissues.

The phenolic compounds as constituents of lignin may contribute to enhance the mechanical strength of the host cell wall and may also inhibit fungal growth as they are fungitoxic in nature. The induction of phenols might be due to the activation of the shikimic acid pathway, through which the aromatic amino acids phenylalanine and tyrosine are formed and channeled for the synthesis

of phenolics. M'Piga et al. (1997) found that the hyphae of the pathogen surrounded by phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. In the present study accumulation of phenolics was observed in palms treated with biocontrol agents, especially in *P. fluorescens* + *T. viride* + chitin-treated palms. Similar findings were reported for blackgram against dry root rot (Sundaravadana, 2002) and groundnut against *C. personatum* (Meena et al., 2000). Benhamou et al. (2000) reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots and offered resistance to *P. ultimum* infection. Singh et al. (1998) found that seed and soil application of *T. viride* enhanced the phenolics concentration in chickpea plants that led to induced resistance to *Machrophomina phaseolina*. Zdor and Anderson (1992) observed an increase of PO activity as well as an increase in the level of mRNAs encoding for PAL and chalcone synthase in the early stages of interaction between bean roots and various bacterial endophytes. Since the production of phenolic compounds depends upon PAL activity (Graham and Graham, 1991), increased phenolic synthesis in treated coconut plants may be due to increased activity of PAL.

Pathogenesis-related proteins are host coded proteins that are induced by pathogens and abiotic stresses (Van Loon et al., 1998). The PR-proteins have been classified into 14 families based on amino acid sequences, serological relationship, and/or enzymatic or biological activity. Some of the PR proteins such as chitinases (PR-3) (Legrand et al., 1987) and β-1,3-glucanases (PR-2) (Kauffmann et al., 1987) have the potential to hydrolyze chitin and β-1,3-glucan, respectively, which are major components of fungal cell walls, leading to direct inhibition of growth of several fungi (Leah et al., 1991). In addition chitinases and glucanases play an indirect role in stimulating plant defense by releasing oligosaccharides from the fungal cell walls by their enzymatic action which act as "elicitors" or inducers of several defense genes (Ryan, 1987). In the present investigation chitinase and glucanase activities significantly increased in the treated palms when the antagonists *Trichoderma* and *P. fluorescens* were applied either alone or in combination. Significantly increased glucanase activity was recorded in palms treated with biocontrol agents and the highest activity was observed in coconut palms treated with the formulations of *P. fluorescens* + *T. viride* amended with chitin. Karthikeyan et al. (2005) demonstrated that soil and foliar application of *P. fluorescens* enhanced the glucanase activities in onion plants. Synthesis and accumulation of PR-proteins have been reported to play an important role in plant defense mechanisms (Van Loon, et al., 1998). Colonization of bean roots by rhizobacteria was correlated with induction of PR proteins resulting in induced systemic resistance against *Botrytis cinerea* (Zdor and Anderson, 1992). Similarly in tobacco, induction of two PR-proteins, namely β-1,3-glucanase and chitinase, was observed following application of the *P. fluorescens* isolate CHAO and in response to infection by tobacco necrosis virus (TNV). Induction of these hydrolytic enzymes was also reported in pea against *P. ultimum* and *Fusarium oxysporum* f. sp. *pisi* (Benhamou et al., 1996) and in tomato against *F. oxysporum* f. sp. *radicis-lycopersici* (M'Piga et al., 1997). Yedidia et al. (1999) reported that the root inoculation of *T. harzianum* induced increased chitinase activity in leaves of cucumber seedlings. Lorito et al. (1993) observed enhanced production of chitinases by *Trichoderma*. There has been a spate of publications over the past two decades on the production of glucanases and chitinases by species of *Trichoderma* involved in mycoparasitism (Viterbo et al., 2001).

In our earlier studies we demonstrated that bioformulation treated palms did not exhibit any disease symptom even two years after treatment (Bhaskaran, 2000). From the present study it can be postulated that rapid and higher accumulation of enzymes involved in phenylpropanoid metabolism and of PR-proteins in coconut roots in response to treatment with biocontrol agents in combination with chitin might have induced resistance against *Ganoderma* in coconut palm. One of the emerging strategies for managing plant diseases is the use of microbial biocontrol agents with the aim of reducing pesticide residues. In this context, the findings of the present investigation that biocontrol formulations amended with chitin can be effectively utilized for managing *Ganoderma* disease in coconut is of great significance.

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