Research Article *Journal of Plantation Crops, 2009, 37 (2): 134-137*

Activation of defense enzymes in arecanut (*Areca catechu* L.) seedlings upon inoculation with biocontrol agents

C. Kannan[1] and C.T. Jose

Central Plantation Crops Research Institute, Regional Station, Vittal-574243, Karnataka

(Manuscript Received: 05-08-08, Revised: 06-03-09, Accepted: 08-06-09)

Abstract

The effect of soil application of three biocontrol agents viz., *Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride* on arecanut seedlings were studied with respect to the induction, dynamics and persistence of activity of the defense enzymes viz., peroxidase, poly phenol oxidase, phenyl alanine ammonia lyase, catalase and chitinase. Results showed that the biocontrol agents differed in their ability to activate different enzymes and sustain their persistence in the seedlings.

Keywords: Arecanut, *Bacillus subtilis*, Induced resistance, *Pseudomonas fluorescens*, *Trichoderma viride*

Introduction

Arecanut (*Areca catechu* L.) is an important cash crop, involved in a trade of about Rs.15,000 million annually and is grown in an area of 0.396 million hectares producing 0.559 million tonnes in India. Arecanut is affected by a number of diseases and disorders among which, fruit rot, yellow leaf disease, basal stem rot and inflorescence dieback are the major ones of economic importance. Several improved chemicals are available to manage these diseases, but with a heavy price of environmental degradation. Host plant resistance has not been reported in arecanut against any of the diseases. Biological control of diseases, especially induction of systemic resistance (ISR) in host plants using native antagonistic organisms could be a viable alternative in the management of diseases. *Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride* which commonly survive in the rhizosphere have been established to induce systemic resistance in the plants (Borneman and Becker, 2007). Resistance build up is through activation of series of defense enzymes, most commonly peroxidase (PO), phenyl alanine ammonia lyase (PAL), polyphenol oxidase (PPO), catalase (CT) and chitinase (CH), systemically in the plant tissues. These enzymes, called pathogenesis related proteins (PR-proteins), have been related with definite roles in the host plant defense system (VanLoon, 1997). Quantification of these defense enzymes will give an estimate of the induced resistance in the plants. The present study aims at establishing the ISR activities of the three native rhizosphere microbes viz., *Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride* on arecanut seedlings.

Materials and Methods

The fungal and bacterial antagonists were isolated from the arecanut rhizosphere using selective media viz., *Trichoderma* selective medium (Elad *et al*., 1981) for *T. viride*, King's B for *P. fluorescens* and Nutrient agar medium for *B. subtilis* (Difco Manual, 1953). The organisms were identified based on their morphological characters given by Rifai (1969) for *Trichoderma* and Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) for *Pseudomonas fluorescens* and *Bacillus subtilis*. The isolates were maintained on their respective culture media under refrigerated condition and sub cultured periodically. Cement pots of the size 75 cm^3 were filled with 80 kg of pot mixture containing red soil, sand and decomposed FYM in the ratio of 1:1:1. Arecanut seedlings of variety Mangala were planted in the pots. The antagonists were multiplied in the respective broths

[1]*Present address: Sr. Scientist, Plant Pathology, NRCWS, Jabalpur - 482 004*

for six days (bacteria) and ten days (fungi) by incubating at 30^{0}C in a shaking water bath. The broth solution along with the biocontrol agent were collected, homogenized in a blender and applied at the rate of 10 ml per pot to the root zone of eight months old arecanut seedlings in the pots. The treatments were given as follows- T_1- *Pseudomonas fluorescens* (28 X 10^8 cfu /ml), T_2- *Bacillus subtilis* (12 X 10^8 cfu /ml), T_3-*Trichoderma viride* (30 X 10^4 cfu /ml) and T_4-Control (distilled water). Ten replications were maintained for each treatment. The treatments were repeated in the same seedlings, 15 months after planting. The activity of defense enzymes upon application of biocontrol agents were calorimetrically estimated from fresh leaf samples for different periods viz., immediately after the spraying (0^{th} day) and afterwards at an interval of 3 days upto 45 days from application, when the enzyme activity became static or started to decrease. Five key defense enzymes viz., CH, CT, PPO, PO and PAL were estimated. The homogenized tissues were stored in deep freezer (-70° C) until used for biochemical analysis. Calorimetric assay of enzyme CH was carried out according to the procedure developed by Boller and Mauch (1988). PAL activity was estimated as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al*. (1984). The procedure described by Hammerschmidt *et al*. (1982) was followed for the analysis of PO, Meyer *et al*. (2000) for PPO and Luck (1974) for CT.

The estimated values were subjected to statistical analysis using the standard software SPSS version 11. The enzyme values were estimated for two consecutive years on the same seedlings and analysed. Since there was no significant difference between the two years data, the values were averaged and presented. The replication wise maximum values of enzyme activities were used for ANOVA test to compare the treatments. For the estimation of dynamics of the enzyme activity, quadratic regression model of the form $y=ax^2+bx+c$, where, y is the quantity of enzyme and x is the time (days) was fitted. Then the peak activity period is given by - b/2a.

Results and Discussion

The results indicate that induction by the biocontrol agents increased activities of the defense enzymes in arecanut seedlings. Application of both fungal and bacterial antagonists triggered the activity of all defense enzymes significantly in the treated plants from the 3^{rd} day of application, compared to untreated control. Similar increase in the activities of the defense enzymes were observed upon application of *P. fluorescens* (Chen *et al*., 2000), *B. subtilis* (Utkhede, 1984) and *T. viride* (Roiger and Jeffers, 1991) in different crops. Results indicate that the biocontrol agents vary in their ability to induce the defense enzymes in the seedlings (Table 1). *P. fluorescens* was able to induce the maximum activity of PO (6.58 changes in abs of PO activity/min/g of fresh leaf tissue) and PAL (5067.5 nmole of transchinnamic acid/min/g of leaf tissue), while *B. subtilis* was able to induce the maximum activity of CT (2.76 nmoles of H_2O_2 used/min/g of fresh leaf sample) and *T. viride* induced maximum activity of PPO(5.81 increase in OD $min^{-1}g^{-1}$) and CH (3924.00 nmol of GlcNAC $min^{-1}g^{-1}$). This may be due to the presence of various elicitor sites in the microbes and receptor sites in the plants to induce particular enzymes in large quantities. Similar observations were recorded by Hammerschmidt (1982), who stated that interactions between these two factors result in the activation of defense mechanisms in plants which result in plants becoming resistant against the invading pathogen. However, in the plant system as a whole, these interactions are never independent of each other and there always exists cross talk between these reactions (Bostock, 1999). Chitinase is a key hydrolytic enzyme, which helps in the release of the elicitors from the pathogen cell wall and thus induces the series of defense reactions in the plant (Viswanathan and Swamiyappan, 2001). PPO and PAL are prominent enzymes of the phenyl propanoid pathway, which produces the defense chemicals in the plants. Catalase is involved in the oxidation of phenols to produce phytoalexins and lignins (Karthikeyan *et al.,* 2006).

Table 1. Treatment means for different enzymes

Treatments	Peroxidase	PAL	PPO	Catalase	Chitinase
T_1	6.58	5067.50	4.67	1.88	3455.00
T_2	4.72	4546.50	4.63	2.76	2810.60
T_3	4.94	3907.20	5.81	1.82	3924.80
Control	1.55	1049.10	3.16	0.45	546.30
CD (P = 0.05)	0.17	34.54	0.10	0.07	21.31

T2 - P. fluorescens, T2 - B. subtilis, T3 - T. viride

Units of the enzymes

Peroxidase	Changes in abs of PO activity/min/g of fresh leaf tissue
PAL	nmole of transcinnamic acid/min/g of leaf tissue
PPO	increase in OD $min^{-1}g^{-1}$
Catalase	nmoles of H_2O_2 used/min/g of fresh leaf sample
Chitinase	nmol of GlcNAC $min^{-1}g^{-1}$

Results from Table 2 indicate that the peak activity of enzymes is different for each enzyme and these differ for each of the microbes. The estimation of peak activity period helps in determining the persistence of the enzyme activity in plants and when to apply the next dose of the microbial inoculum in order to make the plant resistant

Table 2. Peak activity period (No. of days after application) of enzymes

Treatments	Peroxidase	PAL	PPO	Catalase	Chitinase
T_1	22.10	21.66	23.94	20.88	22.05
T_2	24.07	22.77	27.42	22.50	21.88
T_3	22.26	21.42	26.19	23.25	25.17

Period of peak activity of the enzyme obtained by fitting the quadratic regression model $y = ax^2 + bx + c$, where, y is the quantity of enzyme and x is the time (days) Peak activity period = -b/2a

against the invading pathogens. In the present study the enzymes activity followed a curvilinear path (Figure 1) to reach a maximum value followed by a decrease. The estimated values are shown till 35th day, when the values declined. The actual values were observed till 45th day when the enzyme values reached lower than the 0th day value. This may be due to the decline in the activity of the microbes in the rhizosphere due to various factors or

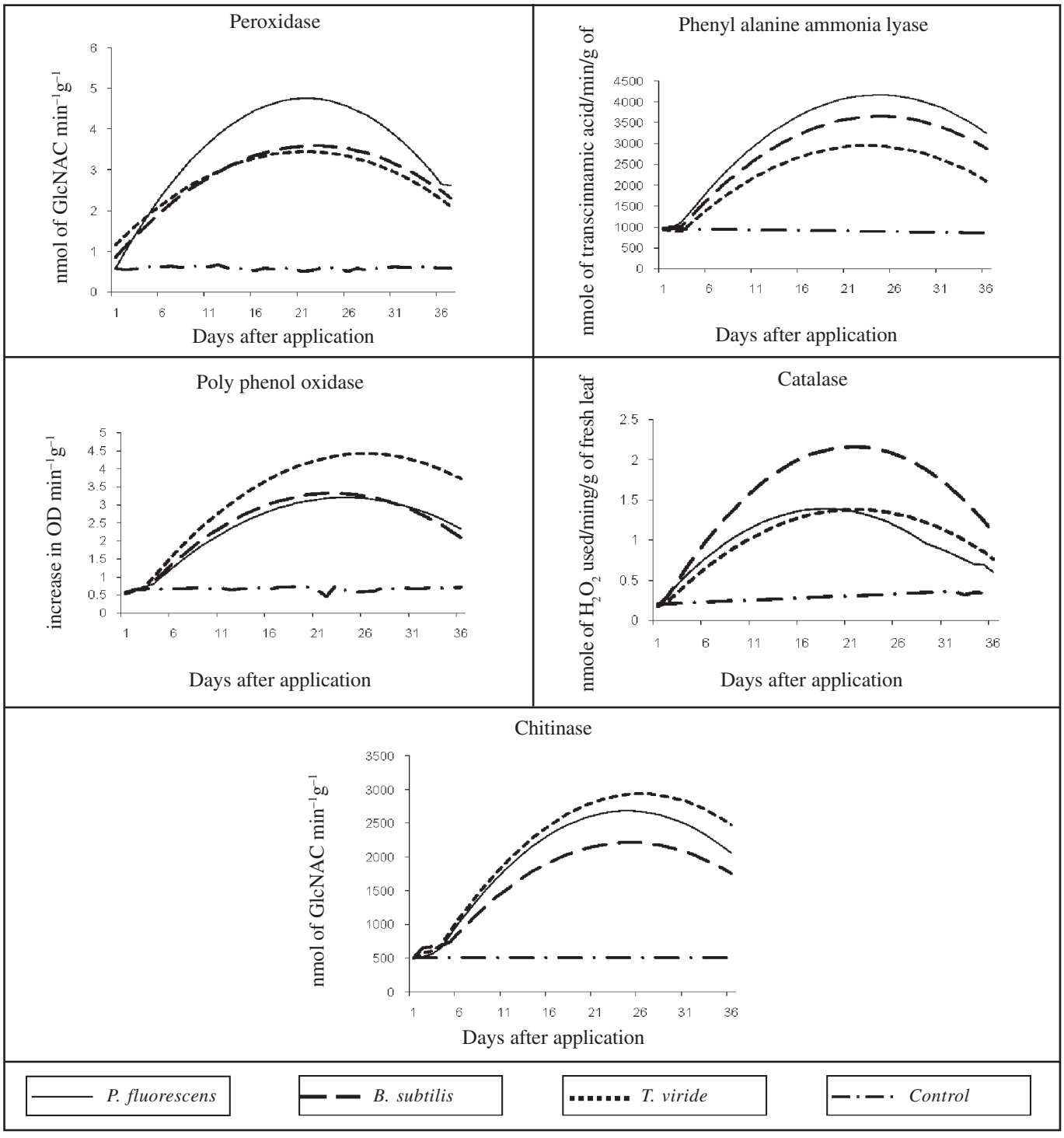

Fig. 1. The fitted quadratic regression model of activities of defense

the plants may produce certain biochemical substances that may discourage the inducing activities of microbes in plants (Karthikeyan *et al.,* 2006), which however was not estimated in the present case. The peak values differ with different biocontrol agents. *T. viride* produces peak activity of CH on the 25th day, when compared to *B. subtilis* (21st day) and *P. fluorescens* (22nd day). Similarly peak activity of PPO was maximum on 27th day of application of *B. subtilis*. The enzymes PO and PAL were found to have relatively shorter peak periods than compared with other enzymes, which indicate that they are responsible for the initial defense response of arecanut seedlings (VanLoon, 1997).

Activation of ISR is an effective strategy for protection against systemic pathogens in perennial plants. Time of application of bioagents and sustenance of the activity of defense enzymes are the important factors in the effectiveness of ISR (Krause *et al.,* 2003). The present study has revealed the induction of defense enzymes in arecanut seedlings, the peak activity period and persistence of the activity in arecanut seedlings. The results may help in establishing that applying the antagonists in seedling stages may improve the overall health of palm and in reducing the effect of seedling diseases. Further the time interval between two applications of biocontrol agents can be determined based on the results of studies on persistence of enzyme activity in seedlings. Since the causal organism cannot be artificially cultured, we have not studied the induction of defense enzymes upon challenge inoculation of the causal organism, instead the experimental seedlings have been planted in farmers field and regular observations are recorded for their resistance activity.

References

Boller, T. and Mauch, F. 1988. Colorimetric assay for chitinase. *Meth. Enzymol.* **161**: 430-435.

Borneman, J and Becker J.O. 2007. Identifying microorganisms involved in specific pathogen suppression in soil *Ann. Rev. Phytopathol*. **45**: 153-172.

Bostock, R.M. 1999. Signal conflicts and synergies in induced resistance to multiple attackers. *Physiol. Mol. Pl. Pathology* **55**: 99-109.

Chen, C., Belanger, R., Benhamou, N. and Paultiz, T.C. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiol. Mol. Plant Pathol*. **56**: 13-23.

Dickerson, D.P., Pascholati, S.F., Hagerman, A.E., Butler, L.G. and Nicholson, R.L. 1984. Phenylalanine ammonia-lyase and hydroxy cinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiol. Plant Pathol*. **25**: 111-123.

Diffco Manual 1953. Diffco laboratories, Inc., Detroit Michigan.

Elad, Y., Chet, I. and Henis, Y. 1981. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* **9**: 59-67.

Hammerschmidt, R., Nuckles, E.M. and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol*. **20**: 73-82.

Karthikeyan, M., Radika, K., Mathiyazhagan, S., Bhaskaran, R., Samiyappan, R. and Velazhahan, R. 2006. Induction of phenolics and defense related enzymes in coconut (*Cocos nucifera* L) roots treated with biocontrol agents. *Brazilian J. Plant Pathology* **18**: 367-377.

Krause, M.S., De Ceuster, T.J.J., Tiquia. S.M., Michel, F.C., Jr., Madden, L.V. and Hoitink, H.A.J. 2003. Isolation and characterization of rhizobacteria from composts that suppress the severity of bacterial leaf spot of radish. *Phytopathology* **93**: 1292-1300.

Krieg, N.R. and Holt, J.G. 1984. *Bergey's Manual of Systematic Bacteriology*, 9th ed., Vol. I. The Williams and Wilkins Co., Baltimore.

Luck, H. 1974. *Methods in Enzymatic Analysis* **2** (Ed Bergmeyer) *Academic Press,* New York p. 885.

Meyer, U.M., Spotts, R.A. and Dewey, F.M. 2000. Detection and quantification of *Botrytis cinera* by ELISA in pear stems cold storage. *Plant Dis.* **84**: 1099-1103.

Rifai, M.A. 1969. A revision of the genus *Trichoderma*. *Mycological Papers* **116.** Commonwealth Mycological Institute, Assoc. of Applied Biologists, Kew, Surrey, England.

Roiger, D.J., and Jeffers, S.N. 1991. Evaluation of *Trichoderma* spp. for biological control of *Phytophthora* crown and root rot of apple seedlings. *Phytopathology* **81**: 910-917.

Utkhede, R.S. 1984. Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Can. J. Bot*. **62**: 1032-1035.

VanLoon, L.C. 1997. Induced resistance in plants and the role of pathogenesis – related proteins. *Eur. Journal of Pl. Pat.* **103**: 753-765.

Viswanathan, R. and Samiyappan, R. 2001. Antifungal activity of chitinases produced by some fluorescent pseudomonads against *Colletotrichum falcatum* Went causing red rot disease in sugarcane. *Microbiol. Research* **155**: 309-314.