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Systemic induction of defense enzymes by rhizosphere microbes in cocoa seedlings

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ABSTRACT: Certain rhizosphere organisms called plant growth promoting rhizobacteria (PGPR) are capable of inducing systemic defense in plants by enhancing the activity of defense enzymes produced in the plant system. In an experiment conducted in pot cultured seedlings of cocoa, three biocontrol agents, *viz., Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride*, were able to promote the activity of the defense enzymes (called PR proteins), *viz.,* peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase, catalase and chitinase in the plants when applied in the soil. The results showed that the biocontrol agents varied in their ability to activate different enzymes and sustain their persistence in the seedlings, *P. fluorescens* being the best in inducing the defense enzymes.

KEY WORDS: Induced resistance, PGPR, *Pseudomonas fluorescens, Bacillus subtilis, Trichoderma viride*, rhizosphere, biocontrol

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a perennial tree crop, which was introduced into India in the early 20th century, with limited cultivation on government owned farms. Systematic cultivation of cocoa in India started in 1970s, with the intervention of the state and central governments, mainly as an intercrop in the arecanut and coconut plantations. Cocoa is cultivated in India in an area of 34,049 ha producing 10720 Mg of dry beans, with Kerala contributing the major share (5532 Mg from 10,708 ha) (DCCD, 2009). It is grown mainly in the southern states of India, *viz.,* Kerala, Karnataka, Tamil Nadu and Andhra Pradesh and the cultivation is being extended to other states, where coconut and arecanut are grown. Diseases caused by microorganisms are one of the major constraints in the production of cocoa resulting in great economic losses to the farmers. *Lasiodiplodia* wilt disease in cocoa caused by *Lasiodiplodia theobromae* has recently been reported to be major threat to cocoa production in India (Kannan *et al.,* 2009).

In the present era of critical awareness about health hazards posed by excessive use of chemicals in the management of plant diseases, biological control and host plant resistance, especially induction of systemic resistance (ISR) in host plants using native antagonistic organisms could be a viable, environmentally safe, effective and economical alternative. Of the many natural defense mechanisms plants have evolved to survive in nature, only a few can be triggered by biological or chemical agents without any deleterious side effects on the yield or chemical nature of the produce and in a practically controlled way for desired use. The best known examples are systemic acquired resistance (SAR) activated by localized infections with necrogenic pathogens, and induced systemic resistance (ISR), which in nature is activated by non-pathogens including viruses, bacteria, or fungi present in the rhizosphere (Van Loon *et al.,* 1998).

Rhizosphere is the immediate zone of the soil surrounding the plant roots, which is often very active because of the mutual association between the plant roots and the microbes (Kerry, 2000). Resistance build up is through activation of a 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 (Karthikeyan *et al.,* 2006). These enzymes, called pathogenesis related proteins (PR-proteins), have been designated with definite roles in the host plant defense system (Van Loon, 1997). Quantification of these defense enzymes will give an estimate of the induced resistance in the plants. The present study was aimed at establishing the ISR activity of the three native rhizosphere microbes, *viz., Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride,* on cocoa seedlings.

MATERIALS AND METHODS

The antagonists were isolated from the cocoa rhizosphere using serial dilution technique (Dhingra and Sinclair, 1995) in their respective selective media, *viz., Trichoderma* selective medium (TSM) (Elad *et al*. 1981) for *T. viride*, King's B (KB) for *P. fluorescens* and Nutrient agar medium for *B. subtilis* (Difco Manual, 1953). The organisms were identified based on their morphological characters for *Trichoderma* (Rifai, 1969) and biochemical characters for bacteria (Krieg and Holt, 1984). They were maintained in their respective culture media under refrigerated conditions and recultured at an interval of 15 days to prevent attenuation and loss of viability. Cement pots of the size 2.5 ft^3 were filled with 80 kg of pot mixture containing red soil, sand and decomposed FYM in the ratio of 1:1:1. The antagonists were multiplied in their respective broths in 250 ml conical flasks for 6 days (*P. fluorescens* and *B. subtilis*) and 10 days (*T. viride*). The whole broth solutions along with the biocontrol agents were harvested, homogenized in a blender and applied at the rate of 10 ml per pot near the root zone of the seedlings, by maintaining the population of the microbes to above $2x10^9$ cfu/ml (bacteria) and $2x10^7$ conidia/ml (fungus) in the suspension. Treatments were given as follows – T_1 – *Pseudomonas fluorescens*, T_2 – *Bacillus subtilis*, T_3 – *Trichoderma viride* and T_4 – control (distilled water). Five replications per treatment and three plants per replication were maintained in the experiment. The activity of the defense enzymes was calorimetrically estimated in fresh leaf samples from 10 months old seedlings, on 0^{th} day and afterwards at an interval of 3 days till 45 days of application of the biocontrol agents from the same plants, when the enzyme quantity became less than or equal to the 0^{th} day values. Though the plants will trigger their own mechanism to heal the wounds upon removing the leaves, which will alter the enzyme activities, this effect will be uniform in all the treated plants and the control plants.

Five key defense enzymes, *viz.,* chitinase (CH–nmol of GlcNAC $min^{-1}g^{-1}$), catalase (CT–nmol of H_2O_2 used/min/g of fresh leaf sample), polyphenol oxidase (PPO–increase in OD $min^{-1}g^{-1}$), phenylalanine ammonia lyase (PAL–nmol of transcinnamic acid/min/g of leaf tissue) and peroxidase (PO–changes in abs of PO activity/min/g of fresh leaf tissue), whose role in plant defense has been established, were estimated using a spectrophotometer. Colorimetric assay of the enzymes were carried out as per the standard procedures. CH, PAL, PO, and PPO were assayed as per the procedure developed by Boller and Mauch (1988), Dickerson *et al*. (1984), Hammerschmidt *et al*. (1982), and Meyer *et al*. (2000), respectively. The estimated values were subjected to statistical analysis using SPSS version 11. The enzymes were estimated for two consecutive years on the same seedlings in the post-monsoon season and analyzed. 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 the dynamics of the enzyme activity, a quadratic regression model of the form $y=ax^2+bx+c$, where, y-the amount of enzyme and x-time in days were fitted and the peak activity period derived by the formula - b/2a.

RESULTS AND DISCUSSION

Induced systemic resistance in plants has been mostly studied with annuals (Van Loon *et al.,* 1998). However, the establishment of such a self-sustaining mechanism in the perennials would be of immense use in the management of the diseases in these long standing crops. In this context, the study of systemic induction of defense enzymes in cocoa seedlings and the persistence of these enzymes in the system would help in the integration of the biocontrol agents with other disease management packages. The present study using the antagonists to elicit defense enzymes in cocoa indicates that the three antagonists are able to increase the activities of the defense enzymes in cocoa seedlings. Application of both fungal and bacterial antagonists triggered the activity of all the defense enzymes from the 3^{rd} day of application, significantly in the treated plants compared to untreated. Similar increase in the activity of the defense enzymes was observed upon application of *P. fluorescens* (Chen *et al.* 2000), *B. subtilis* (Utkhede, 1984) and *T. viride* (Roiger and Jeffers, 1991) in different crops. The results further indicate that the biocontrol agents vary in their ability to induce the defense enzymes in the seedlings (Table 1). *P. fluorescens* (T_1) 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* (T_2) was able to induce the the maximum activity of CT (2.76 nmoles of H_2O_2 used/min/g of fresh leaf sample) and *T. viride* (T_3) induced the 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 the interactions between these two factors result in the activation of defense mechanisms in the plants which results in the 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 defense chemicals in plants. Catalase is involved in the oxidation of phenols to produce phytoalexins and lignins (Karthikeyan *et al.,* 2006).

Table 1. Estimated treatment means for the activity of different enzymes on application of biocontrol organisms (average of two years)

Treatments	Peroxidase	PAL	PPO	Catalase	Chitinase
P. fluorescens	3.48	2911.38	2.21	1.17	0693.38
B. subtilis	2.39	2413.00	2.39	1.58	0792.13
T. viride	1.82	1515.25	2.86	0.82	1037.41
Control	0.79	0713.88	0.48	0.33	0307.63
CD (P = 0.05)	0.37	0439.37	0.35	0.21	179.69

Table 2. Peak activity period (number of days after application) of enzymes from the date of application of biocontrol organisms

Treatments	Peroxidase	PAL	PPO	Catalase	Chitinase
P. fluorescens	24.45	21.65	23.94	20.88	22.05
B. subtilis	24.06	22.76	27.42	22.50	21.88
T. viride	22.25	21.42	26.19	23.25	25.17

The results (Table 2) indicate that the peak activity of the enzymes is different for each enzyme and each of the microbes. The estimation of peak activity period helps in determining the persistence of the enzyme activity in the plants and when to apply the next dose of the microbial inoculum in order to make the plant resistant against the invading pathogens. In the present study, the enzyme activity followed a curvilinear path (Figs. 1), to reach a maximum value (peak activity) followed by a decline in the values / activity. The estimated values were shown till the 35th day, when the values declined; however, the actual values were observed till the 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 the plants may produce certain biochemical substances that may discourage the inducing activities of the microbes in the plants (Karthikeyan *et al.,* 2006), which was not estimated in the present case. The peak values differ with the 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, the 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 then compared to the other enzymes which indicate that they are responsible for the initial defense response of the cocoa seedlings (Van Loon, 1997). may produce certain biochemical substances that may discourage the inducing activities of the microbes in the plants (Karthikeyan *et al.,* 2006), which was not estimated in the present case. The peak values differ with the 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, the 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 then compared to the other enzymes which indicate that they are responsible for the initial defense response of the cocoa seedlings (Van Loon, 1997).

Activation of ISR is an effective strategy for protection against systemic pathogens in perennial plants. Time of application of the bioagents and sustenance of the activity of the defense enzymes are important factors in the effectiveness of ISR (Krause *et al.,* 2003). The microbes used in the present study, *viz., P. fluorescens, B. subtilis* and *T. viride*, that commonly survive in the rhizosphere, have been reported to possess several antimicrobial activities against the plant pathogens and the most important among them is their ability to elicit defense response in the plants (Borneman and Becker, 2007). This study has revealed the induction of defense enzymes in cocoa seedlings, their peak activity period and persistence. The results may help in establishing that applying the antagonists in the seedling stages may improve the overall health of cocoa at the later stages in addition to reducing the effect of seedling diseases. Further, the time interval between two applications of the biocontrol agents can be determined based on the results of the studies on the persistence of enzyme activity in the seedlings. The goal is to integrate all the available genetic, cultural, biological, and chemical methods for disease control in a way to optimize their benefits and minimize their risks for producers, consumers, and the environment. To achieve this, the choices have to be based on the efficiency

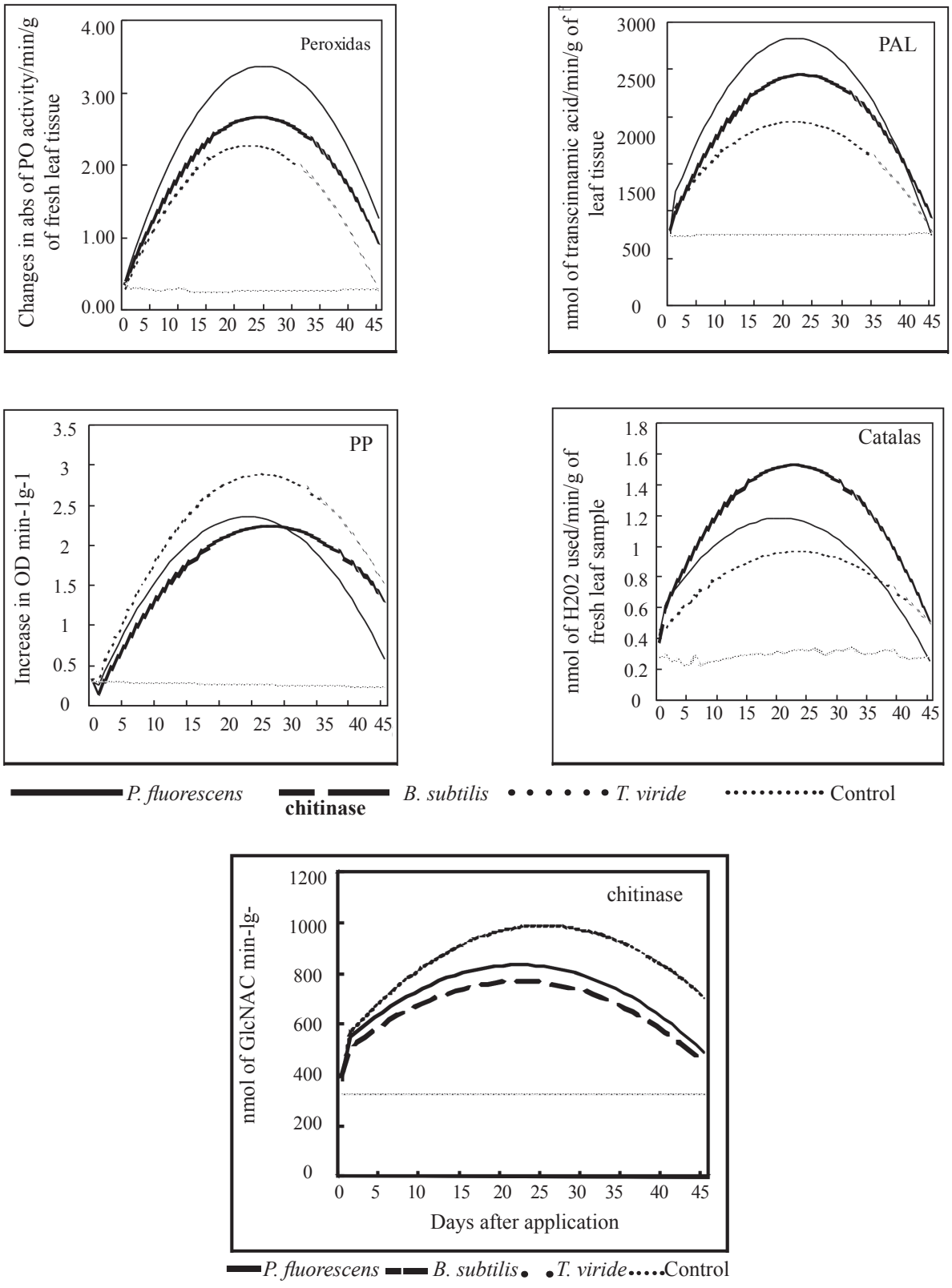

Fig. 1. The fitted quadratic regression model for peroxidase, phenyl alanine ammonia lyase, polyphenol oxidase, catalase and chitinase enzyme activity at different time intervals

and reliability of the disease control method, demonstrated safety for the environment and the consumers.

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