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Pseudomonas fluorescens Induced Enzymological Changes in Banana Roots (Cv. Rasthali) against *Fusarium* Wilt Disease

T. Saravanan, R. Bhaskaran and M. Muthusamy
Agricultural Research Station, Tamil Nadu Agricultural University, Kovilpatti-628 501, India

Abstract: *Pseudomonas fluorescens* isolated from banana rhizosphere reduced the vascular discolouration associated with *Fusarium* wilt disease and induced the accumulation of resistance associated enzymes in roots. The banana roots inoculated with talc based formulation of *P. fluorescens* at 10 g / plant had minimum vascular discolouration index when compared to pathogen *Fusarium oxysporum* f.sp. *cubense* alone inoculated roots. The resistance associated enzymes viz., peroxidase and polyphenol oxidase activity increased two folds on 8 days in roots treated with *P. fluorescens* and challenged with *F. oxysporum* f.sp. *cubense*. Whereas phenylalanine ammonia lyase activity increased two folds on 6 days, later shows decreasing trend in the roots. In *F. oxysporum* f.sp. *cubense* alone inoculated plants, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase activities increased upto 4 days after inoculation. The induction was compared with other fungal biocontrol agent's viz., *Trichoderma viride* and *Trichoderma harzianum*. Same trend of enzymes induction were noticed in *T. viride* and *T. harzianum* inoculated roots. Further more, in Native Poly Acrlyamide Gel Electrophoresis analysis, two isoforms of peroxidase and five isoforms of polyphenol oxidase were observed in roots treated with *P. fluorescens* and challenged with *F. oxysporum* f.sp. *cubense*.

Key words: Banana, *Fusarium* wilt, *Pseudomonas fluorescens*, enzyme accumulation

INTRODUCTION

Fusarium wilt in banana is a serious and most destructive disease on many cultivars grown in different banana growing regions in the world[1,2]. The causative agent, *Fusarium oxysporum* f.sp. *cubense* (E.F. Smith) Snyder and Hansen is a highly variable soil borne fungus[3]. *Fusarium* wilt is a classic vascular wilt disease in which the fungus gains entry to the water conducting xylem vessels, then proliferates within the vessels causing water blockage. The typical symptoms include wilting and death of the leaves, followed by death of whole plant. There are no effective chemical control measures for the wilt disease and currently practiced corm injection procedure with the fungicide carbendazim is tedious[4]. Several workers have examined the possibilities of using antagonistic actinomycetes, arthrobactor, agrobacterium and other bacteria for the suppression of the wilt disease[5-9]. In recent years, there has been much success in obtaining effective control of plant pathogens by using beneficial bio control agents. Some bio control agents of Pseudomonas belongs to Plant Growth Promoting Rhizobacteria (PGPR) are known to induce systemic resistance to fungi, bacteria and viral diseases[10-13]. Besides this the biocontrol agents suppress the pathogens by various mechanisms namely competition for food, root colonization[14,15], antibiosis by producing antibiotics[16,17]. Moreover, the biocontrol agent, *P. fluorescens* produce plant growth promoting substances such as auxins, gibberelins and enhance plant growth and yield. Application of biocontrol agents enhanced the defense resistance has been studied in several crop plants. However, the actions of available biocontrol agents have not been documented in banana to fusarial wilt disease. In compatible interactions between plants and pathogens, Pathogensis Related (PR) related proteins are often induced in response to infection[18]. This PR proteins synthesis and accumulation have long been thought to play an important role in the plant defense responses. The study of enzymalogical changes is prerequisite for documenting the resistance mechanisms in the host. In the present study, effect of *P. fluorescens* on the enzyme induction in banana roots against *F. oxypsorum* f. sp. *cubense* was studied.

MATERIALS AND METHODS

Fungal isolate: *Fusarium* wilt (Race 1) affected banana plants (Cv. Rasthali) were collected from the Horticultural Farm, Agricultural College and Research Institute,

Corresponding Author: Dr. T. Saravanan, Research Associate, Agricultural Research Station, Tamil Nadu Agricultural University, Kovilpatti-628 501, India

Madurai, India. The suckers showing brown discoloration symptoms of the disease were washed in sterile water and cut into small piece using s sterilized scalpel and surface sterilized in 0.1% mercuric chloride solution for 30 sec followed by washing in several changes of sterile distilled water. Sterilized Potato Dextrose Agar (PDA) medium amended with 100 ppm of strephtomycin sulphate (to avoid bacterial contamination) was poured into sterile Petri plate 15 mL per plate and the surface sterilized plant pieces were placed at 3 pieces per plate at equidistance. All these were carried out in aseptic conditions. The plates were incubated at 28°C temperature for five days, observed for the presence of *Fusarium oxyporum* f.sp. *cubense* based on description given by Snyder and Hansen[9]. The fungus was purified by single spore isolation technique of Ricker and Ricker [20] by transferring a single spore into Potato Dextrose Agar Slant (PDAS) and incubated at 28°C temperature for five days. Then the culture slant was flooded with 20 mL of sterile distilled water. Fifty mL of the conidial suspension (10^6 cfu mL^{-1}) was poured into each banana-growing tank for the pathogen challenge treatment.

Isolation of rhizosphere *P. fluorescens*: Rhizosphere colonizing *P. fluorescens* were isolated from fresh roots of banana (Cv. Rasthali) collected Horticultural Farm, Agricultural College and Research Institute, Madurai, Central Farm, Agricultural College and Research Institute, killikulam, India. After vigorous shaking of excised roots to remove all but slightly adhering soil, root segments (1 g) were shaken in 100 mL of sterile distilled water for 15 min. One mil of the suspension was poured into a sterilized petri plate and 15 mL of sterilized King's B medium[21]. The plates were incubated at 28°C for 36 h. After incubation, the colonies of *P. fluorescens* were identified according to Bergy's manual of systematic bacteriology[22]. Cell suspension of *P. fluorescens* was prepared by streaking them from in nutrient broth +10% glycerol stored at -80°C into Tryptic Soy Agar (TSA) plates and incubating at 25°C for 36 h to activate it and check for purity.

Development of talc based formulation of *P. fluorescens*: The talc-based formulation of *P. fluorescens* was prepared by following the method described by Vidhyasekaran and Muthamilan[23]. Briefly, a loopful of bacterial strain was inoculated into King' B Medium Broth (KMB) and growth in a rotary shaker at 150 rpm min^{-1} for 48 h at room temperature (25±2°C). One kg of talc powder (montmorillonite) was taken in a metal tray under aseptic conditions and its pH was adjusted to pH 7.0 adding $CaCO_3$ at the rate of 15 g kg^{-1}. Ten grams of carboxy methyl cellulose were added to 1 kg of talc and mixed well and the mixture was autoclaved for 30 min at 120°C on each of 2 days. Then 400 mL of the bacterial suspension containing $9x10^8$ cfu mL^{-1} mixed with carrier-cellulose mixture under aseptic conditions. After drying (35% moisture content) overnight under aseptic conditions, the mixture was packed in a polypropylene bag, sealed and stored at room temperature (28±2°C). At time of application, the population of bacteria in the formulations was 10^8 cfu g^{-1} of talc powder.

Effect of *P. fluorescens* on vascular discolouration: The healthy banana (Cv. Rasthali) dipped in conidial suspension of the pathogen (10^6 cfu mL^{-1}) for 30 min before planting. Ten days after planting in the pot, talc-based formulation of rifampicin resistant strain of *P. fluorescens* was applied in rhizosphere region of the plants of 5, 10 and 15 g $plant^{-1}$. The plants treated pathogen alone or *P. fluorescens* or water alone kept as control. The plants are grown in glass house at a temperature of 30°C. After 90 days of bacterial application, the suckers were cut horizontally and observed for brown discolouration. The vascular discolouration index was calculated by using the scale 1-6 given by Orjeda[24] (scale 1= corm completely clean, no vascular discolouration; 2 = isolated points of discolouration in vascular tissue; 3 = discolouration upto 1/3 of vascular tissue; 4= discolouration upto between 1/3 and 2/3 or vascular tissue; 5-discolouration more than 2/3 of vascular tissue 6 = total discolouration of vascular tissue). Each replication contains four plants and three replications were kept for this experiment in an factorial experiment.

Plant material and growing condition: The banana cultivar Rasthali was purchased from Horticultural Farm, Agricultural College and Research Institute, Madurai, India. The cultivar has no resistance to race 1 of the *F. oxypsorum* f. sp. *cubense*. the plants were collected from the disease free field and four months old. the plants were grown in a tank size of 70x40 cm containing 25 kg of soil. the tank filled with loamy organic soil containing CEC-10.5 c mol (p^+), kg^{-1}, organic C- 2.9 g kg^{-1}, EC-0.51 ds m^{-1}, low in available N (254 kg ha^{-1}), medium in P (13.4 kg ha^{-1}) and K (345 kg ha^{-1}), available Ca-6.9 c mol kg^{-1}, Mg-0.92 c mol kg^{-1}, Zn-2.8 g kg^{-1}, Mn-0.70 mg kg^{-1} and Cu-1.23 g kg^{-1}. the tank was maintained in a glass house under natural lighting and day/night temperature of approx. 30/24°C.

Enzyme activity: Fresh banana roots and leaves were washed in running tap water after sampling and

homogenized with liquid nitrogen mortar and pestle. The homogenized tissue was rinsed with the same volume of 10 mM sodium phosphate buffer (pH 6.0) at 4°C and filtered through a 0.20 mm nylon filter into a centrifuge tube. The tissue extracts were centrifuged at 12000 g for 20 min at 4°C the supernatant to be used for the enzymatic activity assay was transferred to a 1.5 mL vial and stored at -80°C. A colorimetric assay for enzymatic activity was performed with a Hitachi 200-20 spectrophotometer.

Phenylalanine Ammonia Lyase (PAL): PAL assay was conducted as per the method described by Ross and Sederoff[25]. The assay mixture containing 100 µL of enzyme, 500 µL of 50 mM Tris HCL pH 8.8 and 600 µL of 1 mM L-phenylalanine was incubated for 60 min the reaction was arrested by adding 2 N HCL. Later 1.5 mL of toluene was added, vortexed for 30 sec centrifuged (1000 rpm, 5 min) and toluene fraction containing transcinnamic acid was separated. The toluene phase was measured at wavelength 290 nm against the blank of toluene. St and ard curve was drawn with graded amounts of cinnamic acid in toluene. The enzyme activity was expressed as n moles of cinnamic acid min^{-1} g $protein^{-1}$.

Peroxidase: Peroxidase activity was assayed spectrophotometrically[26]. The reaction 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 to start the enzyme reaction. In reference cuvette, inactivated enzyme (by boiling extract) was taken along with 1.5 mL of pyrogallol (0.05 M). The reading was adjusted to zero at 420 nm in a Hitachi 200-20 Spectrophotometer. To initiate the reaction, 100 µL of 1% H_2O_2 v/v was added to the sample cuvette and the absorbance values were read at 420 nm over 3 min. The enzyme activity was expressed as units PO mg $proteins^{-1}$ in each sample[27].

Polyphenol Oxidase: Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.*[28]. The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µL of the enzyme extract. To start the reaction, 200 µL of 0.01 M catechol was added and the rate of increase in absorbancy at 420 nm was measured for one min after addition of 200 µL of 0.01 M catechol. The activity was expressed as change in the absorbance of reaction mixture min^{-1} g protein.

Native Polyacrylamide Gel Electrophoresis (PAGE) analysis: *P. fluorescens* treated plants were used for Native PAGE analysis. The isoform profiles of PO and PPO were examined by discontinuous Native PAGE[29]. Root samples were collected on the 8 days after pathogen challenge, at which time the activity of PO and PPO was the maximum. The protein extract was prepared by homogenizing 1 g of root samples in 2 mL of 0.1 M sodium PO_4 buffer pH 7.0 and centrifuged at 16,000 g for 20 min at 4°C. The protein content of the sample was determined[30]. Samples (50 µ protein) were loaded onto 5% polyacrylamide gels in staining solution containing 0.05% benzidine (Sigma, USA) and 0.03% H_2O_2.

Statistical analysis: All analyses were performed using IRRISTAT version 92-1, which was developed by International Rice Research Institute Biometrics Unit, The Philippines. Data from experiments with a common design were pooled for analysis when the intensity between experiment and treatment was not significant and variances were homogenous ($p>0.05$). The percent data was arc sine transformed, than analyzed and back transformed to original value.

RESULTS AND DISCUSSION

The strain of *P. fluorescens* isolated from rhizosphere of banana used for assessing their antifungal efficacy against *F. oxysporum* f. sp. *cubense in vitro.* The results of the study demonstrated that all four strains isolated form the rhizosphere of banana had significant inhibitory action on the growth of *F. oxysporum* f. sp. *cubense* (Table 1). In the strains, Pfm strain had higher inhibitory action on the growth of the pathogen.

In green house experiments conducted talc based formulation of *P. fluorescens* (Pfm strain) inoculated plants showed a significant reduction in vascular discolouration in the rhizome of the plant after three month of inoculation (Table 2). The strain applied at 10 g $plant^{-1}$ had lesser discolouration index and which was followed by plants inoculated with *P. fluorescens* at 5 g $plant^{-1}$. The bacteria treated *Fusarium* inoculated plants did not show wilting or death.

The bio control agent *P. fluorescens* has been used to study the resistance enzyme induction in banana against the fusarium wilt disease. The fungal agents viz., *Trichoderma viride* and *T. harzianum* also used in the study to compare the induction. The higher activity of enzymes viz., peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were observed in *P. fluorescens* treated banana plants and challenged with *F. oxysporum* f. sp. *cubense.* The level of induction of PO, PPO and PAL was more in *P. fluorescens* and challenged with *F. oxysporum* f. sp.*cubense* when compared to *P. fluorescens* alone inoculated plants (Fig. 1, 2 and 3). The significantly higher activity and two folds increase in activity of PO (Fig. 1) and PPO (Fig. 2) were observed at

Table 1: *In vitro* efficacy of *Pseudomonas fluorescens* against *Fusarium oxysporum* f.sp. *cubense*

Strains of *Pseudomonas fluorescens*	*Colony diameter of *F. oxysporum* f.sp. *cubense* (mm)	Percent inhibition of mycelial growth over control
Pf1	24.00^{e}	73.19
Pf2	25.75^{b}	71.25
Pf3	22.33^{d}	75.06
Pf4	21.33^{e}	76.18
Pfm	18.50^{f}	79.34
Control	89.55^{a}	–

*Mean of four replications, values in parenthesis are arc sine transformed values, Means followed by common letter are not significantly different at DMRT (0.05%) level

Table 2: Efficacy of *P. fluorescens* on vascular discoloration in banana roots due to *Fusarium oxysporum* f.sp. *cubense*

Strains of *Pseudomonas fluorescens*	Doses of the agents (g/plant)	*Percent vascular discolouration
Pf1	5	71.33(57.63)^{bc}
	10	60.67(57.16)^{e}
Pf2	5	73.00(58.69)^{b}
	10	66.00(54.33)^{d}
Pf3	5	60.33(50.69)^{ef}
	10	56.00(48.45)^{g}
Pf4	5	55.67(48.26)^{gh}
	10	53.33(46.91)^{i}
Pfm	5	53.33(46.91)^{i}
	10	50.83(45.48)^{ij}
Control	-	97.67(81.21)^{a}

*Mean of four replications, values in parenthesis are arc sine transformed values, Means followed by common letter(s) are not significantly different at DMRT (0.05%) level

8 days after inoculation of bacteria in the *P. fluorescens* treated and challenged with *F. oxysporum* f. sp. *cubense* and PAL activity (Fig. 3) was maximum at 6 days after bacteria treatment in the roots. There after it shows decreased trend in its level. The pathogen inoculation also stimulated the enzymes but the level was less than that of *P. fluorescens*. The induction was also observed in *T. harzianum* and *T. viride* inoculated plants but the level is lesser than that of *P. fluorescens* inoculated plants in all three enzymes.

The plants that were treated with a combination of four treatments were selected to analyze the PO and PPO patterns in Native Poly Acrylamide Gel Electrophoresis (PAGE) (Fig. 4 and 5). Two isoforms of PO (Fig. 4) could be detected in *P. fluorescens* treated plants and challenged with the pathogen. The pathogen alone-inoculated roots had only one isoforms of PO. Five isoforms of PPO (Fig. 5) appeared in *P. fluorescens* inoculated upon challenged with the pathogen. The isoform PPO4 was observed in all four treatments and the intensity was higher in *P. fluorescens* alone treated plants. However PPO5 isoform could be detected only in *P. fluorescens* and pathogen inoculated roots.

In the present investigations, among the five strains of *P. fluorescens* tested against *F. oxysporum* f. sp. *cubense*, Pfm of *P. fluorescens* was the most inhibitory to the pathogen. Several strains of *P. fluorescens* have been reported to control various *Fusarium* wilt pathogens including *F. oxysporum* f. sp. *dianthi* in carnation[31], *F. oxysporum* f. sp. *ciceris* in chickpea[23] and *F. oxysporum* f. sp. *radicis lycopersici* in tomato[32]. Raguchander *et al.*[33] also found that among the eight antagonistic organisms tested for their efficacy against *F. oxysporum* f. sp. *cubense*, *P. fluorescens* recorded the maximum inhibition of growth of the pathogen. Thangavelu *et al.*[34] screened eleven isolates of *P. fluorescens* and among them Pf10 was the most effective in inhibiting the mycelial growth of *F. oxysporum* f. sp. *cubense*. Sivamani and Gnanamanickam[35] reported that application of *P. fluorescens* as seedling dip reduced the severity of wilting and internal discolouration. In the present study, the application of *P. fluorescens* as a talc formulation reduced vascular discolouration severity under glass house conditions.

The introduced bacteria was identified in the rhizosphere and it has maintained its initial level population upto 90 days after inoculation in banana plants. The favored hypothesis on how *P. fluorescens* types inhibit the growth of *F. oxysporum* f. sp. *cubense* and reduced the vascular discolouration is that their aggressive colonization results in the displacement of roots[36,37]. A threshold population density of *P. fluorescens* has shown to be required for significant suppression of *Fusarium* wilt of radish[14].

In effect, all disease suppressive mechanisms exhibited by *P. fluorescens* are essentially of no real value unless these bacteria can successfully establish themselves at the root environment[38]. It is well known that different strains of *P. fluorescens* have abilities to colonize a particular niche[39-41].

Plants endowed with various defense related genes. Inducing the plants own defense mechanisms by prior application of biological inducers is thought to be a novel plant protection strategy. In recent years, use of non pathogenic saprophytic PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated[13,42]. Biological control with *P. fluorescens* provide an effective tool for managing soil borne diseases. Several *P. fluorescens* have been reported to induce systemic resistance. Due to Induced Systemic Resistance (ISR), disease reduction and increased plant growth were observed in many crops[11,40,43,44]. In these investigations, *P. fluorescens* strain significantly induced plant defense enzymes both locally and systemically in banana roots. *P. fluorescens* had shown to suppress

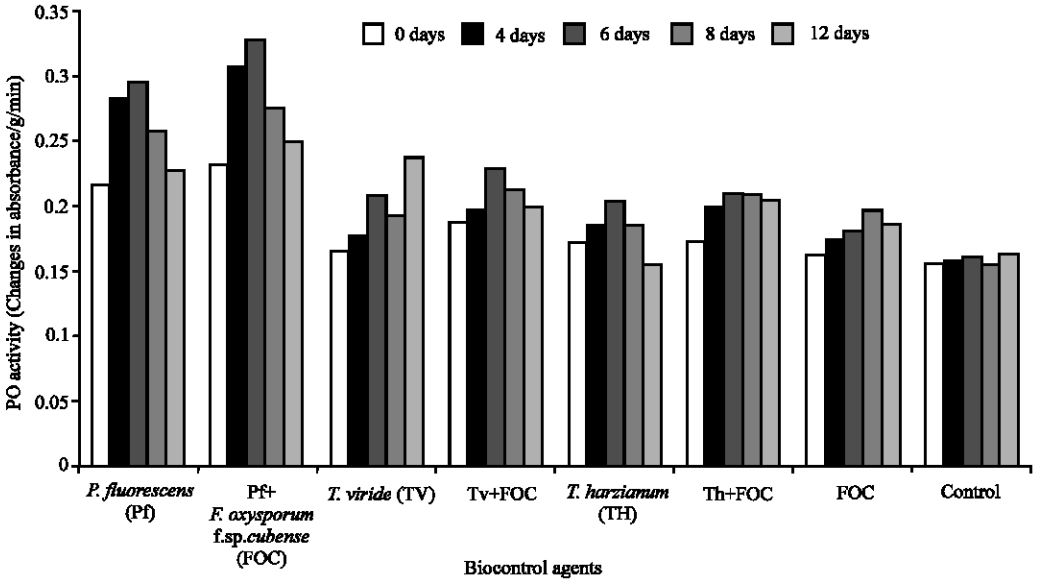

Fig. 1: Effect of Pseudomonas fluorescens on peroxidase enzyme induction in banana root

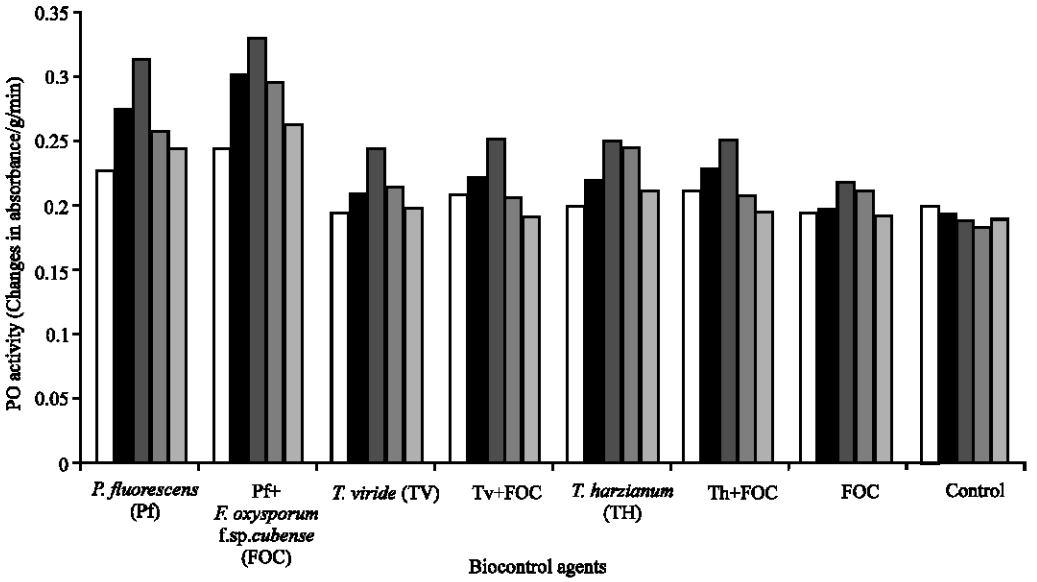

Fig. 2: Effect of biocontrol agents on polyphenol oxidase enzyme induction in banana root

Fusarium wilt in banana[33]. The resistance inducing enzymes stimulated when banana roots infected with *F. oxysporum* f. sp. *cubense* and increased locally or systemically later when *P. fluorescens* or root pathogen penetrated the banana root system. In the study, *P. fluorescens* treatment and challenge inoculation with the pathogen stimulated more enzymes accumulation in roots.

PAL is one of the key enzyme in the phenyl propanoid pathway and the flavonoid pathway, was increased in both compatible and incompatible interactions between plants and pathogens and plays an important role in the biosynthesis of phenolics that are effective chemical barriers against pathogen infection[45]. In cucumber roots inoculated with *Pythium aphanidermatum* but root treated with *P. corrugata* had initially higher level of PAL and the levels decreased after challenging the plant with *P. aphanidermatum*[10].

Peroxidase (PO) is a multipurpose enzyme that catalyses the condensation of phenolics into lignin[46] and

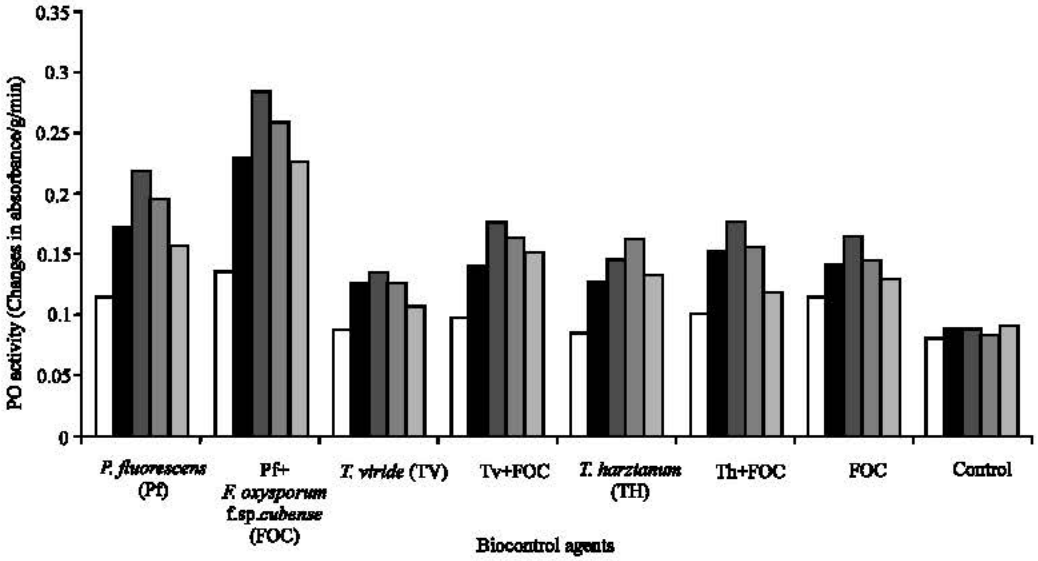

Fig. 3: Effect of biocontrol agents on phenyl ammonia lyase enzyme activity in banana root

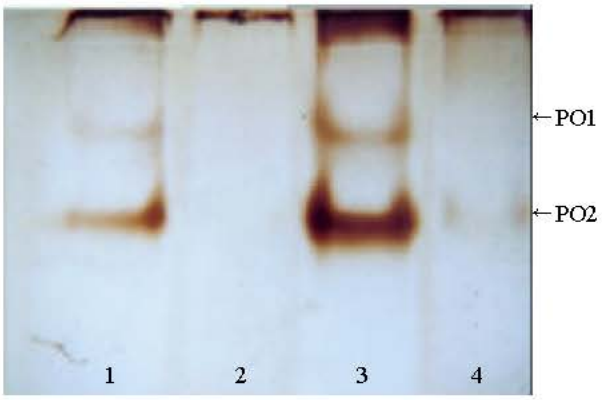

Fig. 4: Peroxidase isoforms separated by native poly acrylamide gel electrophoresis of soluble proteins from banana roots inoculated with *Pseudomonas fluorescens*
Lane. 1: Roots were treated with *Fusarium oxysporum* f.sp.*cubense* only
Lane. 2: Uninoculated control
Lane. 3: The roots bacterized with *Pseudomonas fluorescens* two days later challenged with *Fusarium oxysporum* f.sp. *cubense*
Lane. 4: The roots were bacterized with *Pseudomonas fluorescens* only

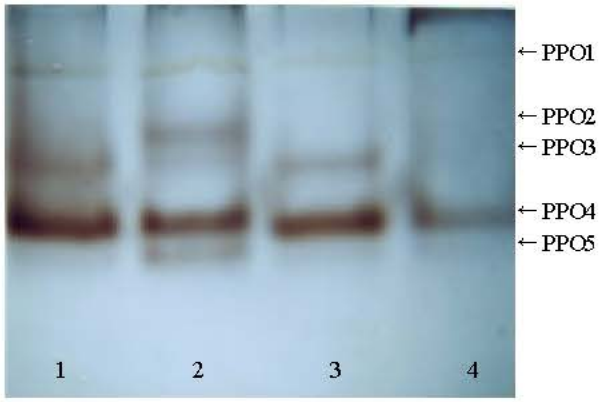

Fig. 5: Polyphenol oxidase isoforms separated by native poly acrylamide gel electrophoresis of soluble proteins from banana roots inoculated with *Pseudomonas fluorescens*
Lane. 1: The roots were bacterized with *Pseudomonas fluorescens* only
Lane. 2: The roots bacterized with *Pseudomonas fluorescens* two days later challenged with *Fusarium oxysporum* f.sp.*cubense*
Lane. 3: Roots were treated with *Fusarium oxysporum* f.sp. *cubense* only
Lane. 4: Uninoculated control

play specific role in the hypersensitive containment of the pathogen[47]. PO is a part of the PR-9 family is of the lignin forming types of plant disease responses[18]. The activity is associated with disease resistance in plants[40] and increase in host plants following pathogen infection[48]. *P. fluorescens* strain WCSH 171 afforded resistance to carnation plant against *F. oxysporum* f.sp. *dianthi*. The resistance was due to increase in the activity of peroxidase[49].

PPO is copper containing enzyme, which oxidizes phenolics to highly toxic quinines and involved in the terminal oxidation of diseased plant tissue, which was attributed for its role in disease resistance[50]. Recent

studies implies prior application of *P. fluorescens* strengthen host cell wall structures resulting in restriction of pathogen invasion in the host tissues[51]. Enzyme accumulation could be involved not only in plant defense response but may also be associated with induced systemic resistance of *P. fluorescens* against wilt disease in banana caused by *F. oxysporum* f. sp. *cubense*.

Detection of greater activity of PO and PPO in treated plants suggests that difference in the isoforms associated with induced resistance is quantitative and not qualitative. A similar quantitative type of ISR was observed in cucumber[29]. Although both *P. fluorescens* and the pathogen could induce PO in banana, roots bacterized and challenged with the pathogen. The intensity of PO1 and PO2 isoforms were high in roots bacterized and challenged with the pathogens. Five isoforms of PPO were observed in roots bacterized and challenged with the pathogens. However, PPO5 pattern was only in *P. fluorescens* treated and challenged with the pathogens. This may be associated with resistance mechanisms by *P. fluorescens* in banana.

In conclusion, the ability of *P. fluorescens* to induce high levels of three key defense enzymes in the roots of banana and this induction was systemic. The levels of induction was high in *P. fluorescens* treated roots have impaired the vascular discolouration in banana roots by the *F. oxysporum* f. sp. *cubense* triggered the activities of defense enzymes. Accumulation of PAL, PO and PPO by *P. fluorescens* in banana roots have contributed to induced resistance in banana against *F. oxysporum* f. sp. *cubense*.

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