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The effect of *Pseudomonas fluorescens* and *Fusarium oxysporum* f.sp. *cubense* on induction of defense enzymes and phenolics in banana

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Abstract

The effect of *Pseudomonas fluorescens* treatment and *Fusarium oxysporum* f. sp. *cubense* inoculation on induction of phenylalanine ammonia-lyase (PAL), peroxidase (POX), chitinase, β-1,3-glucanase and accumulation of phenolics in banana (*Musa* sp.) was studied. When banana roots were treated with *P. fluorescens* strain Pf10, a two-fold increase in phenolic content in leaf tissues was recorded 3 - 6 d after treatment. Challenge inoculation with *F. oxysporum*, the wilt pathogen, steeply increased the phenolic content in *P. fluorescens*-treated banana plants. Significant increase in POX activity was detected 6 - 9 d after *P. fluorescens* treatment. PAL, chitinase and β-1,3-glucanase activities increased significantly from 3 d after *P. fluorescens* treatment and reached the maximum 6 d after treatment. Challenge inoculation with *F. oxysporum* further increased the enzyme activities. These results suggest that the enhanced activities of defense enzymes and elevated content of phenolics may contribute to bioprotection of banana plants against *F. oxysporum*.

Additional key words: fluorescent pseudomonad, *Fusarium* wilt, induced resistance, lytic enzymes, *Musa* sp.

Introduction

Biological control of crop diseases with antagonistic bacteria is now considered as a promising alternative or complementary approach to chemical disease control. This approach will reduce our dependence on chemical fungicides. Several strains of *Pseudomonas fluorescens*, *P. putida*, *P. cepacia*, *P. aeruginosa* and *Bacillus subtilis* have been widely used for biological control of several plant pathogens (Leeman *et al.* 1995, Vidhyasekaran *et al.* 1997, Raupach and Kloepper 1998). In addition to their direct antagonistic activity against fungal pathogens, certain strains of fluorescent pseudomonads also promote plant growth by secreting auxins, gibberellins and cytokinins and hence they are also called as plant growth-promoting rhizobacteria (PGPR) (Dubeikovsky *et al.* 1993). These PGPR are also known to induce systemic resistance in plants to fungal (Wei *et al.* 1991), bacterial (Vidhyasekaran *et al.* 2001) and viral (Alstrom 1991) diseases. This type of resistance, known as induced systemic resistance (ISR) is mediated by a jasmonate/ ethylene sensitive pathway (Van Loon *et al.* 1998). A signal, translocated from the induced site to the entire plant was proposed to be involved in ISR (Kloepper *et al.* 1993). Treatment with the endophytic bacteria have been reported to sensitize plants to defend themselves against pathogen attack by triggering various defense mechanisms including production of phytoalexins (Van Peer *et al.* 1991), accumulation of pathogenesis-related proteins (Meena *et al.* 2000) and deposition of structural barriers (Benhamou *et al.* 1996). Zdor and Anderson (1992) reported increases in peroxidase activity and an increase in content of mRNAs encoding for phenylalanine ammonia-lyase and chalcone synthase in the interaction between bean roots and various bacterial endophytes. Meena *et al.* (2000) demonstrated that *P. fluorescens* strain Pf1 induced accumulation of a 23 kDa thaumatin-like protein and a 30 kDa glucanase in groundnut.

Recently, we isolated a *P. fluorescens* strain Pf10 from rhizosphere soil of banana which can inhibit mycelial growth of *Fusarium oxysporum* f. sp. *cubense*

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Abbreviations: CFU - colony-forming units; ISR - induced systemic resistance; KMB - King's medium B; PAL - phenylalanine ammonia-lyase; PGPR - plant growth-promoting rhizobacteria; POX - peroxidase.

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in vitro. Banana plants were protected from fusarium wilt when they were pretreated with *P. fluorescens* Pf10 before inoculation with *F. oxysporum* (Thangavelu *et al.* 2001). In the present study we evaluated the potential of *P. fluorescens* strain Pf10 in inducing defense-related enzymes such as phenylalanine ammonia-lyase (PAL), chitinase, β-1,3-glucanase, peroxidase and accumulation of phenolics in banana leaves.

Materials and methods

Fungal culture: The fungus, *Fusarium oxysporum* f. sp. *cubense* race 1 was isolated from rhizomes of wilt-infected banana (*Musa* sp.) plants (cv. Rasthali) using half-strength potato dextrose agar (PDA) medium. The culture was maintained on carnation leaf agar medium (Burgess *et al.* 1988) for immediate use and for long term use, the culture was stored as dried filter paper cultures according to Correll *et al.* (1986) at 4 °C.

Isolation of *Pseudomonas fluorescens*: *Pseudomonas fluorescens* strain Pf10 was isolated from the rhizosphere of banana with King's medium B (KMB) (King *et al.* 1954) and demonstrated to be effective in suppression of *F. oxysporum* in banana (Thangavelu *et al.* 2001) was used in this study.

Induction of defense mechanisms in banana by treatment with *P. fluorescens*: Tissue culture derived banana cv. Rasthali plants, susceptible to *Fusarium* wilt were obtained from Southern Petrochemical Industries Corporation, Coimbatore. Plants were grown in earthen pots (30 cm in diameter) filled up with 7 kg of sterilized soil and arranged on the greenhouse benches. *P. fluorescens* Pf10 were grown at 27 °C for 48 h on KMB, then centrifuged at 3 000 *g* for 15 min, and the pellet was resuspended in sterile distilled water. Three-months-old banana plants were bacterized by pouring 250 cm^3 of bacterial suspension (10^9 CFU per cm^3) per pot. *F. oxysporum* was multiplied on the sand-maize medium for 15 d. Plants were inoculated with *F. oxysporum* 3 d after bacterization by applying the sand-maize inoculum at the rate of 10 g per pot. At various times after application, leaf samples were collected and various analyses were made. Samples were stored at -80 °C until analyzed. Samples from each treatment were analyzed thrice.

Estimation of phenolic content: Banana leaves (1 g) were homogenized in 10 cm^3 of 80 % methanol and agitated for 15 min at 70 °C (Swain and Hillis 1959). One cm^3 of the methanolic extract was added to 5 cm^3 of distilled water and 0.250 cm^3 of Folin-Ciocalteau reagent (1 M) and the solution was kept at 25 °C. After 3 min 1 cm^3 of saturated solution of Na_2CO_3 and 1 cm^3 of distilled water were added and the reaction mixture was incubated for 1 h at 25 °C. The absorption of the developed blue colour was measured using a *Beckman DU64* spectrophotometer (*Beckman Instruments Inc.*, Fullerton, California, USA) at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with phenol.

Peroxidase activity: Leaves (1 g) were homogenized in 0.1 M phosphate buffer, pH 6.5 at 4 °C. The homogenate was filtered through four layers of muslin cloth and the filtrates were centrifuged at 12 000 *g* at 4 °C for 20 min. The supernatant served as an enzyme source. To a spectrophotometer sample cuvette 1.5 cm^3 of 0.05 M pyrogallol and 0.1 cm^3 of enzyme extract were taken up. In reference cuvette inactivated enzyme (by boiling) extract (0.1 cm^3) was taken along with 1.5 cm^3 of 0.05 M pyrogallol. The reading was adjusted to zero at 420 nm in a *Hitachi 200-20* spectrophotometer (*Hitachi*, Tokyo, Japan). To initiate the reaction 0.1 cm^3 of 1 percent H_2O_2 (v/v) was added to the sample cuvette and the changes in absorbance were recorded at 30 s interval.

Determination of PAL activity: Leaves (1 g) were homogenized in 5 cm^3 of 0.1 M sodium borate buffer, pH 7.0 containing 0.1 g insoluble polyvinylpyrrolidone (PVP). The extract was filtered through cheese cloth and the filtrate was centrifuged at 20 000 *g* for 35 min and the supernatant was used in the enzyme assay. PAL activity was determined as the rate of conversion of L-phenyl-alanine to *trans*-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984). Samples containing 0.4 cm^3 of enzyme extract were incubated with 0.5 cm^3 of 0.1 M borate buffer, pH 8.8 and 0.5 cm^3 of 12 mM L-phenyl-alanine in the same buffer for 30 min at 30 °C (Dickerson *et al.* 1984). In reference cell, 0.4 cm^3 of enzyme extract was taken along with 1.0 cm^3 of borate buffer. The amount of *trans*-cinnamic acid synthesized was calculated as described by Dickerson *et al.* (1984).

Assay of chitinase: Leaves (1 g) were homogenized in 5 cm^3 of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10 000 *g* at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared according to Berger and Reynolds (1958) from crab shell chitin (*Sigma*, St. Louis, USA). The commercial lyophilized snail gut enzyme (helicase, obtained from *Sepracor*, Villeneuve la Garenne, France) was desalted as described by Boller and Mauch (1988). For the colorimetric assay of chitinase 0.01 cm^3 of 1 M sodium acetate buffer (pH 4.0), 0.4 cm^3 of enzyme extract and 0.1 cm^3 colloidal chitin (1 mg) were pipetted

into a 1.5 cm^3 Eppendorf tube. After 2 h at 37 °C, the reaction was stopped by centrifugation at 1 000 *g* for 3 min. An aliquot of the supernatant (0.3 cm^3) was pipetted into a glass reagent tube containing 0.03 cm^3 1 M potassium phosphate buffer (pH 7.1) and incubated with 0.02 cm^3 desalted snail gut enzyme for 1h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig *et al.* (1959) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as µmol(GlcNAc equivalents) g^{-1}(f.m.) s^{-1}.

Assay of β-1,3-glucanase activity: β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan *et al.* 1991). Leaves (1 g) were extracted with 5 cm^3 of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4 °C using a pestle and mortar. The extract was then centrifuged at 10 000 *g* for 15 min at 4 °C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 0.06 cm^3 of 4 % laminarin and 0.06 cm^3 of enzyme extract. The reaction was carried out at 40 °C for 10 min. The reaction was then stopped by adding 0.37 cm^3 of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 cm^3 of distilled water, vortexed and its absorbance at 500 nm was determined.

Statistics: The analyses were made with three independent samples and the experiment was repeated once with similar results. Statistical calculation was performed using *IRRI STAT* version *92-1* developed by the International Rice Research Institute Biometrics Unit, Philippines.

Results

When banana plants were treated with *P. fluorescens*, phenolic content, PAL, chitinase and β-1,3- glucanase activities were significantly increased from 3 d after treatment and reached the maximum 6 d after treatment and then declined (Fig. 1). A two-fold increase in phenolic content, three-fold increase in PAL activity and four-fold increase in chitinase activity were recorded 6 d after treatment with *P. fluorescens* as compared to

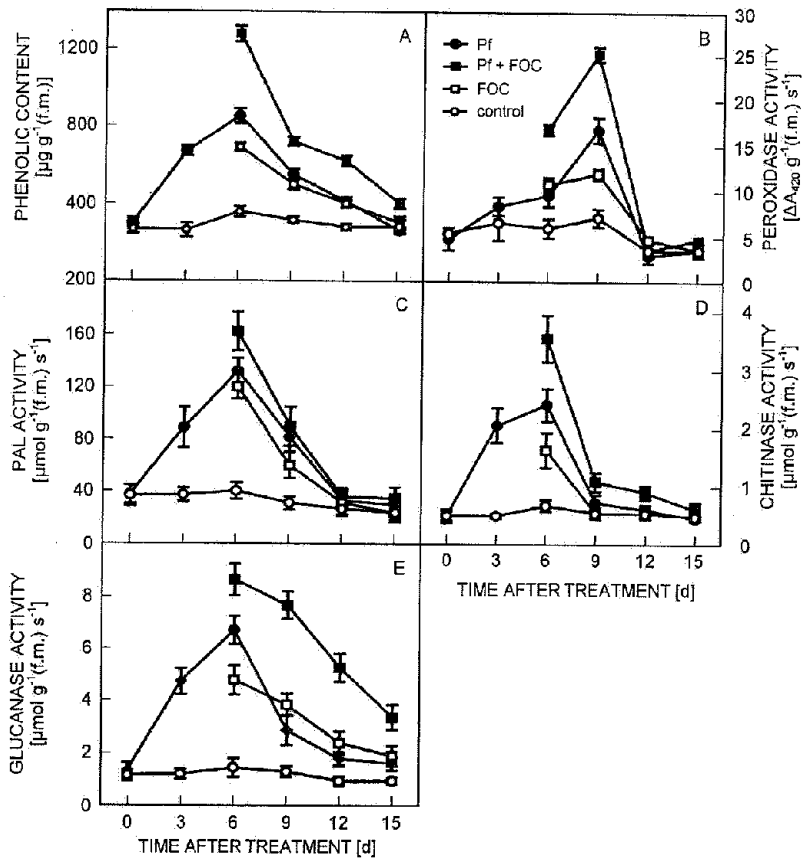

Fig. 1. Changes in phenolic content (*A*), peroxidase (*B*), phenylalanine ammonia-lyase (*C*), chitinase (*D*) and β-1,3-glucanase (*E*) activities in banana leaves in response to treatment with *Pseudomonas fluorescens* strain Pf10 (Pf) and inoculation with *Fusarium oxysporum* f. sp. *cubense* (FOC). Data are means from three independent samples. *Bars* indicate standard error.

untreated control. *P. fluorescens*-treated plants had higher phenolic content and defense enzyme activities than the untreated control at all time intervals. Significant increase in POX activity was also detected 6 - 9 d after *P. fluorescens* treatment.

In another set of treatments, plants treated with *P. fluorescens* were challenge inoculated with *F. oxysporum* 3 d after *P. fluorescens* treatment. Challenge inoculation with *F. oxysporum* further increased the phenolic content, POX, PAL, chitinase and β-1,3-glucanase activities in *P. fluorescens*-treated banana plants. The maximum increase in phenolic content and enzyme activities were recorded 3 - 6 d after pathogen challenge in *P. fluorescens*-treated plants. When compared to non-bacterized, pathogen-inoculated control plants a two-fold increase in phenolic content, POX, PAL, chitinase and β-1,3-glucanase enzyme activities were detected 3 - 6 d after pathogen challenge in *P. fluorescens*-treated plants.

Discussion

ISR in plants is associated with increases in activity of chitinases, β-1,3-glucanases, POX and other pathogenesis-related proteins (Lawton and Lamb 1987), accumulation of phytoalexins (Kuc and Rush 1985) and accumulation of lignin, callose and hydroxyproline-rich glycoproteins (Hammerschmidt *et al.* 1984). In the present study it was observed that treatment of banana roots with *P. fluorescens* induced the accumulation of phenolics and increased the activities of PAL and POX in banana leaves. PAL, the first enzyme of phenylpropanoid metabolism plays a significant role in the regulation of biosynthesis of phenols in plants (Lawton *et al.* 1980). POX have been implicated in the last enzymatic step of lignin biosynthesis, that is, the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into the lignin polymer (Gross 1980). Furthermore, POX itself was found to inhibit the spore germination and mycelial growth of *Pseudocercospora abelmoschi* and *P. cruenta* (Joseph *et al.* 1998). Sariah *et al.* (2001) reported that the increase in phenolic compounds and POX in banana were positively correlated with resistance to fusarial wilt. Morpurgo *et al.* (1994) reported that the activity of POX was at least five times higher in the roots and corm tissues of the *F. oxysporum* resistant banana cultivar than in the susceptible cultivar. Inoculation of resistant cultivar with *F. oxysporum* resulted in a ten-fold increase in POX activity after 7 d of inoculation whereas, the susceptible cultivar exhibited only a slight increase in POX activity. The authors concluded that POX activity could be used as a parameter to discriminate between susceptible and tolerant clones of banana (Morpurgo *et al.* 1994). Since the production of phenolic compounds depends upon PAL activity (Graham and Graham 1991), increased phenolic synthesis in *P. fluorescens*-treated banana plants may be due to increased activity of PAL. The increased POX activity in *P. fluorescens*-treated banana might have involved in the lignin biosynthesis which in turn might have contributed for disease resistance.

Several studies have shown that pathogenesis-related (PR) proteins are also induced in plants upon treatment with *P. fluorescens* (M'Piga *et al.* 1997, Meena *et al.* 2000). Accumulation of PR-proteins is associated with systemic acquired resistance (SAR) in plants (Ryals *et al.* 1996). Chitinase (PR-3) and glucanase (PR-2) are thought to be implicated in the defense system of plants against fungal pathogens by degrading chitin and β-1,3-glucans, major components of the cell wall of many fungi (Boller and Mauch 1988). In the present study it was also observed that soil application of *P. fluorescens* induced the accumulation of chitinase and β-1,3-glucanase in banana. Challenge inoculation with *F. oxysporum* in *P. fluorescens*-treated plants augumented the expression of defense mechanisms. Maurhofer *et al.* (1994), while studying the bioprotection of tobacco leaves against tobacco necrosis virus by application of *P. fluorescens*, also found a positive correlation between bioprotection and expression of PR-1 proteins, endochitinases and β-1,3-glucanases. Using cytochemical labelling of chitin, M'Piga *et al.* (1997) demonstrated disorganization of *Fusarium oxysporum* f. sp. *radicis-lycopersici* hyphae colonizing bacterized tomato roots due to chitin degradation suggesting accumulation of chitinolytic enzymes in bacterized plants. Hence, it is possible that increased resistance in banana to fusarium wilt after application of *P. fluorescens* may also be related to the accumulation of PR-proteins.

Fluorescent pseudomonads are known to produce lipopolysaccharides (LPS) and salicylic acid which act as local or systemic signal molecules in inducing resistance in plants (Van Peer and Schippers 1992, Chen *et al.* 1999). Plant defense mechanisms can also be induced by factors present in the cell walls of fungi known as "elicitors". Elicitor molecules have been detected in various species of *Fusarium* which induced phytoalexins in plants (Hadwiger and Beckman 1980, Ren and West 1992). Similar elicitor molecule may be present in *F. oxysporum* which may induce expression of defense genes in banana. The present studies suggest that the *P. fluorescens* may release a signal and this signal in combination with a signal released by *F. oxysporum* may enhance the defense related activities conferring resistance.

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