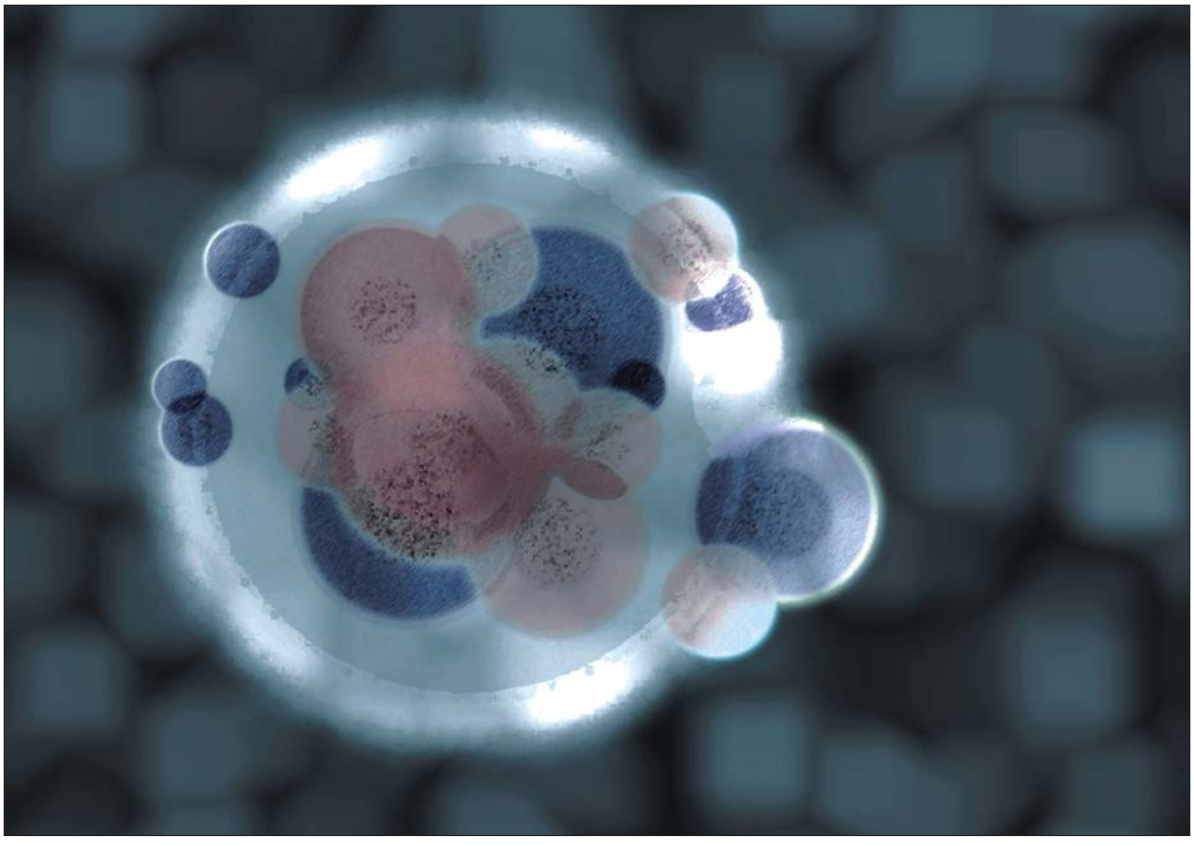

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Effect of *Pseudomonas fluorescens* on Fusarium Wilt Pathogen in Banana Rhizosphere

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Abstract: A detailed study was conducted to find out the efficacy of *Pseudomonas fluorescens* on fusarium wilt pathogen in banana rhizosphere. All the strains of *P. fluorescens* isolated from banana rhizosphere had significant inhibitory action on the growth of *Fusarium oxysporum* f.sp. *cubense*. Among the strains, Pfm of *P. fluorescens* had higher inhibitory action while comparing with other strain. Talc formulation of Pfm of *P. fluorescens* was prepared and applied in three month old banana plants to study their efficacy on fusarium wilt disease. After three month of inoculation, Pfm of *P. fluorescens* inoculated at 10 and 15 g plant^{-1} showed lesser vascular discoloration index than the control. To study the rhizosphere colonizing ability in banana rhizosphere against fusarium wilt disease, a rifampicin resistant strain of Pfm of *P. fluorescens* was developed. The banana plants inoculated with a rifampicin resistant strain of Pfm of *P. fluorescens* showed gradual increase in population of *P. fluorescens* in the rhizosphere over the period of time upto 60 days after inoculation. The extracts of soil inoculated with rifampicin resistant strain of Pfm of *P. fluorescens* at 10 g plant^{-1} showed significant reduction in spore germination of *F. oxysporum* f.sp. *cubense*. Gradual increasing in the reduction of spore germination was observed in the soil extracts over the period of inoculation upto 60 days after inoculation. Similarly, the filter paper dipped in extracts of soil had higher inhibition of growth of *F. oxysporum* f.sp. *cubense* at 60 days after inoculation.

Key words: Fusarium wilt, banana, *Pseudomonas fluorescens*, vascular discoloration, rhizosphere colonization

INTRODUCTION

Fusarium wilt disease in banana is a serious and most destructive disease in the world[1]. Management of the disease by using currently practiced corm injection procedure with fungicide carbendazim is tedious[2]. The possibility of replacing chemical control of plant root diseases with a biological alternative remains an exciting and challenging objective. In 1988, Sivamani and Gnanamanickam found that biocontrol agent *Pseudomonas fluorescens* reduced the wilting and internal discolouration due to *Fusarium oxysporum* f.sp. *cubense*. Raguchander *et al.*[3] also found that dipping of suckers in the suspension of *P. fluorescens* (10^6cfu ml^{-1}) along with the application of 500 g wheat bran saw dust inoculum (1:3) at three months after planting effectively reduced the wilt incidence. Several mechanisms viz., production of siderophores[4], rhizosphere colonizing ability[5], production of antibiotics[6] and induced systemic resistance[7] have been identified in the disease suppression by various strains of *P. fluorescens*. The significance of metabolites production of *P. fluorescens* and more generally in microbial antagonism in natural disease suppressive soils, often has been questioned in the past because of the indirect nature of the supporting evidence and the perceived constraints to the metabolites production in soil environments[8]. However, the importance of metabolites in these interactions between bacteria and pathogens in the rhizosphere is much less clearly established, partly because antibiotics never have been detected directly in rhizosphere[9,10] and no experimental evidences has been reported so for on interactions between the pathogen and *P. fluorescens* in the rhizosphere of banana. A better understanding of root colonization and disease suppressive mechanisms of introduced *P. fluorescens* strains are needed before suitable strains can be directly selected for commercial use. So the study was taken on to find out interaction between *F. oxysporum* f. sp. *cubense* and *P. fluorescens* in the rhizosphere.

MATERIALS AND METHODS

Fusarium isolate: Fusarium wilt (Race 1) affected banana plants (cv. Rasthali) were collected from the Horticultural Farm, Agricultural College and Research Institute, Madurai, India. The suckers showing brown discolouration symptoms of the disease were washed in

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sterile water and cut into small piece using a 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 streptomycin sulphate (to avoid bacterial contamination) was poured into sterile Petri plate 15 ml plate^{-1} and the surface sterilized plant pieces were placed at 3 pieces plate^{-1} at equidistance. All these were carried out in aseptic condition. The plates were incubated at 28°C temperature for five days, observed for the presence of *Fusarium oxysporum* f.sp. *cubense* based on description given by Snyder and Hansen[11]. The fungus was purified by single spore isolation technique of Ricker and Ricker[12] by transferring a single spore to PDA medium. The pathogen was multiplied in sand maize medium at ratio of 19:1. The sterilized medium was inoculated with a pathogen disc and incubated for 12 days under room temperature (28±2°C). Then the well-grown fungal medium was used for this study.

Isolation of rhizosphere *P. fluorescens*: Rhizosphere colonizing *P. fluorescens* were isolated from fresh roots of banana (cv. Rasthali) collected from Horticultural Farm, Agricultural College and Research Institute, Madurai and 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[13]. 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 (1984). 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[14]. 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 9 x 10^8 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 talc (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[15] (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 a factorial experiment.

Development of rifampicin resistant strain of *P. fluorescens*: In order to study the rhizosphere survival, a rifampicin resistant strain of *P. fluorescens* Pfm was developed as per the method described by Vidhysekaran and Muthamilan[14]. During the preliminary screening, rifampicin (90 μg ml^{-1}), penicillin (90 μg ml^{-1}), actinomycin D (90 μg ml^{-1}) and strephtomycin D (90 μg ml^{-1}) inhibited the growth of Pfm strain of *P. fluorescens*. The bacterium was grown in KMB medium with rifampicin at 90 μg ml^{-1}. Resistant colonies were selected and streaked on to KMB without antibiotic. From this, single colony was selected and restreaked on rifampicin supplemented medium. Again, single colony was selected and restreaked onto medium containing penicillin (90 μg ml^{-1}), actinomycin D (90 μg ml^{-1}) and strephtomycin D (90 μg ml^{-1}). Finally the colony grown well in KMB containing all four antibiotics was isolated and used for this study.

Assessment of survival of rifampicin resistant strain of *P. fluorescens*: The talc based formulation of rifampicin resistant strain of *P. fluorescens* was prepared and applied

to pot soil (sterilized) at 10 g plant^{-1} containing three month old banana plant (cv. Rasthali). At 20 days intervals, the roots adhered soil were collected from the rhizosphere of banana plants and used for assessing the population of *P. fluorescens* by serial dilution technique. 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 ml of the suspension was poured into a sterilized petri plate and 15 ml of sterilized King's B medium[13]. KMB media amended with the antibiotics was used for estimating the population and the results were expressed as colony forming units g^{-1} of soil.

Extraction of metabolites of rifampicin antibiotic resistant strain of *P. fluorescens* Pfm strain: The metabolites were extracted from *P. fluorescens* inoculated rhizosphere soil as per the method described by Bonsall *et al.*[16]. For which, talc formulation of *P. fluorescens* inoculated in the banana rhizosphere at 10 g plant^{-1}. At 0, 30, 60 and 90 days after inoculation, the soil samples were drawn from the rhizosphere and used for extraction of metabolites. Thirty g of rhizosphere soil was mixed in a 250 ml flask with 100 ml of 80% acetone acidified to pH 2.0 with acetic acid and shaken (200 rpm) for 2 h at room temperature (28°C). Samples were subsequently filtered through a Buchner funnel and the filtrate was centrifuged at 12000 rpm for 30 min at 4°C to remove soil particles. The supernatant was evaporated to a volume of 8 ml acidified to pH 2.0 with 10% acetic acid and then extracted twice with 10 ml of ethyl acetate and evaporated to dryness. Extracts were resuspended in 1 ml of sterile distilled water. The residual extracts from uninoculated soil kept as check.

Assay of extracts on *F. oxysporum* f. sp. *cubense*: One drop of the suspension was placed in the cavity of the depression slide and allowed to air dry. A drop of the conidial suspension (4 x 10^6 spores ml^{-1}) of *F. oxysporum* f. sp. *cubense* prepared in sterile distilled water was added to the dried eluant and thoroughly mixed. The cavity slide was incubated in Petri dish glass bridge chamber. Three cavity slides represent a replication and three replications were maintained. The spores were observed for germination after 48 h of incubation under microscope and percent germination was calculated. The paper disc assay was carried out as per modified method of Deese and Stahman[39]. Under aseptic conditions, filter paper disc (1.2 cm dia, Whatman No. 42) used for testing their efficacy were dipped in the suspension and dried using a hair drier.

This procedure was repeated twice. The control disc was dipped in sterile water. Each disc was placed on *F. oxysporum* f. sp. *cubense* seeded agar in a sterilized Petri plate and two drops of sterile distilled water were placed on each dry disc. Each disc was placed at room temperature for 1 h and finally at 28°C for 5 days. After 5 days, inhibition zone was measured around the paper disc and compared with control. Three plates kept as a replication and three replications were maintained.

Statistical analysis: All analyses were performed using IRRISTAT version 92-1, which was developed by International Rice Research Institute Biometrics Unit, The Philippines. The percent data was arc sine transformed, than analyzed and back transformed to original value. The results were studied by using Duncan multiple range test (DMRT) at p=0.05% level.

RESULTS

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 15 g plant $^{-1}$ had lesser discolouration index and which is on par with index of plants inoculated with Pfm strain of *P. fluorescens* at 10 g plant $^{-1}$. The bacteria treated Fusarium inoculated plants did not show wilting or death.

So the strain Pfm of *P. fluorescens* was used for testing their colonizing ability in the rhizosphere. A rifampicin resistant strain of Pfm was developed *in vitro* for this study. When the plants inoculated with the strain under green house condition, the bacteria could be detected in the rhizosphere (Table 3). The results showed that a significant increase in population of introduced rifampicin antibiotic resistant strain of *P. fluorescens* as well as Pfm of *P. fluorescens* in the rhizosphere of inoculated banana plants. The population showed increasing trend up to 60 days after inoculation later shows a decreasing trend.

The soil samples was drawn from rhizosphere regions of the banana plants at different intervals in which Pfm strain was inoculated at 10 g plant^{-1} and used for extracting metabolites of Pfm$^-$ strain of *P. fluorescens*. The suspension of soil extracts showed a significant declined

Table 1: Efficacy of strains of *P. fluorescens* on the growth of *Fnsarium oxysporum* f.sp. cubense

Strains	*Mycelial growth of *F. oxysporum* f.sp. cubense(mm)
Pfm	16.50d
Pf1	23.67d
Pf2	26.73c
Pf3	29.67b
control	89.55a

* Mean of three replications, each replications contain three plates of observation. Mean followed by common letters are not significantly differed at Duncan Multiple Range Test (0.05%) level

Table 2: Efficacy of rifampicin strain of *P. fluorescens* on vascular discolouration

Doses of rifampicin strain of *P. fluorescens*	*Percent vascular discolouration index
5	45.55(42.45)cd
10	32.22(34.59)c
15	31.11(33.90)cd
control	93.50(75.33)a

* Mean of three replications, each replications contain four plants of observation, values in parenthesis are arc sine transformed value. The discolouration was measured at three month after inoculation by using the scale 1-6. Mean followed by common letters are not significantly differed at Duncan Multiple Range Test (0.05%) level

Table 3: Rhizosphere colonizing ability of rifampicin antibiotic resistant strain of *P. fluorescens* against *Fnsarium oxysporum* f.sp. cubense in banana

	*Population (cfu g^{-1} of soil) at days after inoculation			
Treatment	0	30	60	90
Antibiotic resistant strain of *P. fluorescens*	20.63b	33.71ab	36.07b	22.67b
P. fluorescens	27.30a	34.62a	39.71a	26.52a
Control	6.21c	7.03c	5.81c	2.31c

* Mean of four replications, each replication contain observation of four plates. Mean followed by common letters are not significantly different at Duncan Multiple Range Test (DMRT) at p=0.05% level

Table 4: Efficacy of soil extracts inoculated with rifampicin strain of *P. fluorescens* on spore germination and growth of *Fnsarium oxysporum* f.sp. cubense

Soil collected at	*Percent spore germination of *Fnsarium oxysporum* f.sp. *cubense*	*Inhibition zone in paper disc assay
0 days	65.10(53.79)b	0.02d
30 days	12.04(20.29)d	0.89b
60 days	7.40(15.78)e	0.96a
90 days	22.43(28.26)c	0.86c
Control	88.89(70.53)a	0.00de

*Mean of three replications, each replication contain three cavity slides observation for spore germination assay and three plates for inhibition zone observation, Values in parenthesis are arc sine transformed value, Mean followed by common letters are not significantly differed at Duncan Multiple Range Test (0.05%)level

in spore germination of *F. oxysporum* f. sp. *cubense* (Table 4). The reduction was more pronounced at 30 days after inoculation. After that the germination slightly increased up to 90 days after inoculation. The spore germination was reduced from 88.89 to 7.40% at 60 days after inoculation. The extracts also showed significant inhibition of growth of the pathogen around the filter paper disc dipped in the extracts. Greater inhibition zone was observed in the filter paper disc dipped in the extracts of soil inoculated with Pfm⁻ strain of *P. fluorescens* at 60 days after inoculation.

DISCUSSION

In vitro antibiosis was the criterion used in this study to select efficient strain of *P. fluorescens* for use in assessing their rhizosphere colonizing ability and metabolites production in green house condition as few other earlier workers have done. Efficient strains of *P. fluorescens* were selected for suppression of severity of wilt disease in rice from among those, which inhibited mycelial growth and sclerotial germination of Rhizoctonia solani *in vitro*[17]. The data on suppression of *F. oxysporum* f. sp. *cubense* induced internal discolouration of the rhizome and roots of banana plants reveal that bacterization with *P. fluorescens* protected banana plants against *F. oxysporum* f. sp. cubense. The vascular discoloration and wilting due to Fusarium wilt disease in banana is suppressed by *P. fluorescens* when applied as soil application[3]. The application of *P. fluorescens* as dipping also reduced the wilting and discolouration by *F. oxysporum* f. sp. cubense in banana[18].

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[19,20]. 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[21]. It is well known that different strains of *P. fluorescens* have abilities to colonize a particular niche[22-24].

Besides Panama wilt suppression, the other beneficial effect due to bacterization realized in this study is the production of antifungal metabolites. The disease suppressive nature of the rifampicin resistant strain introduced rhizosphere soil was assessed *in vitro* as spore germination and paper disc assay. The metabolite production in the rhizosphere of banana by rifampicin strain of *P. fluorescens* had shown the antifungal activity against *F. oxysporum* f. sp. cubense. The metabolites extracted from the rhizosphere soil could be responsible for the inhibition of growth of *F. oxysporum* f. sp. cubense. The metabolite could be responsible for the inhibition of several fungal pathogens. Among the various mechanisms identified from *P. fluorescens* against various diseases in crop plants, certain fluorescent

pseudomonades isolated from soil have been shown to promote plant growth by inhibiting bacteria and fungi that are deleterious to plants[25,26,27,19] by the production of antibiotic substances by the strains in the rhizosphere and this has been recognized as a major factor in the suppression of many root pathogens.

Until recently there was no direct evidence that the production of antibiotics or secondary metabolites *in vivo* contributed to the biological control ability of fluorescent pseudomonads. Typically a correlation was made between the inhibition of pathogens by the production of antibiotics *in vitro* and the *in vivo* protection of plants from disease by selected strains. There are numerous reports of such cases in which control of specific root disease is correlated with the *in vitro* production of antibiotics inhibitory to particular pathogen[28,25,29].

The antibiotic viz., phenazine, pyocyanine, pyrrolnitrin and phoroglucinol were produced by some strains of *P. fluorescens* against various diseases. Earlier, a number of strains of Pseudomonas have been shown to produce phloroglucinol[30,31]. The severity of tobacco black root rot was reduced when soil was added with phloroglucinol[26]. Phloroglucinol metabolites are phenolic and produced by bacteria with broad-spectrum antibacterial, antifungal and phytotoxic properties[6]. Maurhofer *et al.*[32] reported that phlorogluicinol producing strains of *P. fluorescens* have been shown to be effective against root pathogens viz., Pythium ultimum, Thielaviopsis basicola, Gauumannomyces graminis var. tritici in wheat. Beside this, phloroglucinol induced defense mechanism against fungal infection[33]. The metabolites phenazine and pyrrolnitrin also had antifungal effect[34,35]. Earlier, Elander *et al.*[36] identified pyrrolnitrin in the medium from two fluorescent strains. Pyrrolnitrin, a potent antifungal compound was isolated from two strains of P. cepacia originally isolated from rice seeds[37]. Beside this, the bacterium has also been reported to reduce southern maize leaf blight[38]. Phenazine from *P. fluorescens* suppressed the take all disease of wheat[33]. In the study, the maximum root colonizing ability at 60 days after bacterization may be attributed that to higher production of the metabolites which are identified at 60 days as higher inhibition of spore germination and growth of the pathogen *in vitro*.

In conclusion, *P. fluorescens* Pfm strain effectively inhibited the growth of the pathogen and suppressed the vascular discolouration induced by fusarial wilt disease in banana. The bacteria could be identified in the rhizosphere of banana plants. However, the existing biological ecosystem in the rhizosphere may influence the root colonizing ability and metabolites production. The complexity of soil ecosystem is a constraint that makes biological control of these root pathogens by introduced antagonists a challenge[40]. Hence it needs an elaborate study on influence of biotic and abiotic niche in the rhizosphere on disease suppression ability of *P. fluorescens*.

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