JOURNAL OF PLANT PROTECTION RESEARCH
Vol. 46, No. 3 (2006)

INFLUENCE OF *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* (E.F. SMITH) SNYDER AND HANSEN ON 2,4-DIACETYLPHLOROGLUCINOL PRODUCTION BY *PSEUDOMONAS FLUORESCENS* MIGULA IN BANANA RHIZOSPHERE

Thangavelu Saravanan[1], *Mari Muthusamy*[2]

[1] Tamil Nadu Agricultural University, Agricultural Research Station
Kovilpatti – 628 501, Tamil Nadu, India
pathsaran75@rediffmail.com
[2] Agricultural College and Research Institute, Killikulam – 628 252, India

Accepted: August 18, 2006

Abstract: Influence of *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder and Hansen on 2,4-diacetylphloroglucinol (DAPG) production in the rhizosphere of banana cultivar Rasthali by *Pseudomonas fluorescens* was investigated. The purified extracts of Pfm strain of *P. fluorescens* isolated from banana rhizosphere inhibited the growth and spore germination of *F. oxysporum* f. sp. *cubense* under laboratory conditions. DAPG extracted from the cultures of the strain was observed as distinct spots in thin layer chromatographic plates at Rf value of 0.88. The extracts of soil inoculated with *P. fluorescens* and challenge inoculated with *F. oxysporum* f. sp. *cubense* eluted at retention time ranges from 20.00 min to 21.30 min. The quantity of DPAG production was less in the extracts of soil inoculated with *P. fluorescens* and challenge inoculated with *F. oxysporum* f. sp. *cubense* as compared to *P. fluorescens* alone inoculated soil. The talc formulation of Pfm strain also reduced vascular discolouration due to the pathogen in banana plants when inoculated at 15 g/plant.

Key words: 2,4-diacetylpholoroglucinol, *Pseudomonas fluorescens*, banana, *Fusarium oxysporum* f. sp. *cubense*

INTRODUCTION

Fusarium wilt disease in banana is a serious destructive disease in many cultivars (Ploetz et al. 1990; Sebasigari and Stover 1988). The management of this disease by using *Pseudomonas fluorescens* was reported earlier (Raguchander et al. 1997). In crop plants, various mechanisms account for the ability of *Pseudomonas* strains to control plant pathogens, including competition for iron and other nutrients, niche

exclusion (Rioux et al. 1983) and production of inhibitory compounds or metabolites (Thomashow and Weller 1988). The ability of pseudomonads to suppress soil borne fungal pathogens depends on their ability to produce antibiotic metabolites such as pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol (DAPG) (Maurhofer et al. 1994; Sullivan and O'Gara 1992; Shanahan et al. 1992). Numerous strains producing DAPG were isolated from soils from diverse geographical regions and they were isolated mostly from rhizosphere regions of roots that are naturally suppressive to diseases such as black root rot of tobacco and Fusarium wilt of tomato (Keel et al. 1992, 1990). There are some reports of such cases in which control of specific root diseases is correlated with *in vitro* production of metabolites (Broadbent et al. 1976). Environmental factors influence the production of anti microbial compounds such as DAPG in fluorescent pseudomonads. Variation in the biological control performance of these agents has been attributed to changes of biotic and abiotic factors associated with field locations and cropping time (Duffy and Defago 1997; Thomashow and Weller 1996). In biotic environment, microbial metabolites produced by microorganisms play an important role in the regulation of synthesis of DAPG by *P. fluorescens* (Schnider-Keel et al. 2000). Notz et al. (2002) observed that fusaric acid produced by *F. oxysporum* strain repressed the production of DAPG by *P. fluorescens* strain CHA0 in wheat. The significance of fusaric acid produced by *F. oxysporum* f. sp. *cubense* in biological control of banana fusarium wilt with *P. fluorescens* in root regions has not been documented. Hence, the effect of *F. oxysporum* f. sp. *cubense* on DAPG production by *P. fluorescens* was studied in banana root regions. The DAPG production by isolated strain of Pfm of *P. fluorescens* was assessed in Pfm of *P. fluorescens* inoculated and challenged with *F. oxysporum* f. sp. *cubense* in banana root regions. Furthermore, vascular discolouration suppression by the strain was determined against *Fusarium* wilt of banana (*F. oxysporum* f. sp. *cubense*).

MATERIALS AND METHODS

Fusarium Isolate

Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense* Race 1) which affected banana plants (cv. Rasthali) were collected from the Horticultural Farm, Agricultural College and Research Institute, Madurai, India. The corm showing brown discolouration symptoms of the disease were washed in sterile water and cut into small pieces using a sterilized scalpel and the pieces were surface sterilized in 0.1 per cent mercuric chloride solution for 30 sec followed by washing in several changes of sterile distilled water. The sterile Petri plates were poured with sterilized potato dextrose agar (PDA) medium amended with 100 ppm of streptomycin sulphate (to avoid bacterial contamination) at 15 ml/plate and the surface sterilized three corm pieces were placed equal distance, in aseptic conditions. The plates were incubated at 28°C temperature for five days, observed for the presence of *F. oxysporum* f. sp. *cubense* based on description given by Snyder and Hansen (1940). The fungus was purified by single spore isolation technique of Ricker and Ricker (1936) by transferring a single spore to PDA medium. The pathogen was grown in sand maize medium at ratio of 19:1. The sterilized medium was inoculated with a pathogen disc and incubated for 12 days at room temperature (28±2°C). Then the well-grown fungal culture was used for this study.

Isolation of rhizosphere species of *Pseudomonas fluorescens*

Rhizosphere colonizing *Pseudomonas* species were isolated from fresh roots of banana (cv. Rasthali) collected in Horticultural Farm, Agricultural College and Research Institute, Madurai, India. After vigorous hand 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 S1 medium (Gould et al. 1985). The plates were incubated at 28°C for 36 h. After incubation, the colonies of *Pseudomonas* were identified according to Berg's manual of systematic bacteriology (Kreig and Holt 1984). *Pseudomonas fluorescens* were further identified based on experimental procedure of Blazevic et al. (1973) and Broadsky and Nixon (1973). The strain was inoculated separately in sterilized Petri plates containing Pseudomonas F agar and Pseudomonas P agar media. The sterilized Pseudomonas agar F (peptone from casein 10.0 g; peptone from meat 10.0 g; magnesium sulphate 1.5 g; dipotassium hydrogen phosphate 1.5 g; agar-agar 12.0; glycerol 10.0 m; water 1 000 ml) and Pseudomonas agar P (peptone 20.0 g; magnesium chloride 1.4 g; potassium sulphate 10.0 g; agar-agar 12.0 g; glycerol 10.0 ml, water 1 000 ml) was poured into sterilized Petri plates at 15 ml per plate. The isolated strain of *Pseudomonas* was streaked on the media and incubated for 7 days at 25°C. After incubation, the developed colonies were observed for growth and colours.

In vitro* extraction of DAPG produced by *P. fluorescens

The metabolite 2,4-diacetylphloroglucinol production by strain of *P. fluorescens* was determined using a modified method of Chang and Blackwood (1969). Pf1 strain of *P. fluorescens* was used as reference to confirm the metabolite production in this study. The Pfm strain of *Pseudomonas* was grown in 5 ml of PPM pigment production medium (PPM) (20 g peptone; 20 ml glycerol, 5 g NaCl and 1 g peptone; 20 ml glycerol, 5 g NaCl and 1 g KNO_3, 1 000 ml distilled water (pH 7.2)) broth for 24 hrs on a rotary shaker at 30°C. The ethyl acetate extracts were dehydrated *in vacuo.* The residue was dissolved in 1.5 m of 65% methanol. Aliquots were filtered (Pore size, 0.2 μm), analyzed using a thin layer chromatographic plates (TLC) and developed in acetonitrile/methanol/water (1:1:1). The plates were viewed by short wave length (254 nm) and sprayed with diazotized sulfanilic acid (DSA) and the Rf value of visualized spots was calculated and compared with reference stain.

Assay of DAPG against *F. oxysporum* f. sp. *cubense*

Developed TLC plates were viewed under UV light and then absorbing spots were scrapped from spot area of 0.5 cm^2 on clear sterilized plastic and dissolved with 1.0 ml of methanol. Then the silica dissolved methanol was centrifuged at 5 000 rpm for 10 min to separate silica from the eluant and then eluant was used for testing antifungal activity against *F. oxysporum* f. sp. *cubense* by paper disc assay and spore germination assay. The silica from areas where spots did not appear were also scrapped and dissolved in methanol at 1.0 ml. The silica-dissolved methanol was centrifuged and the eluants were used as check. Besides this, ten microliters of methanol was also used as check.

The paper disc assay was carried out according to a modified method of Deese and Stahman (1962). Under aseptic conditions, the eluants from the spots were applied into a filter paper discs (1.2 cm dia, Whatman 42) at 10 μl and dried using a hair drier. After drying, the filter paper was again treated with a respective eluant at the

same quantity. The control discs were dipped in eluant of non-spot area and methanol. 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 kept at room temperature for one hour and finally at 28°C for 5 days. After 5 days, inhibition zone was measured around the paper disc and compared with control. The experiments were repeated three times to confirm the results.

10 µl of the eluant were placed in the cavity of the each depression slide and allowed to air dry. A drop of the conidial suspension (4 x 10^6 spores/ml) of *F. oxysporum* f. sp. *cubense* prepared in sterile distilled water was added to the dried eluant and thoroughly mixed. The cavity slides were incubated in Petri dish glass bridge chamber. The control slides were dipped in eluant of non-spot area and methanol. Three cavity slides represented a replication and three replications were made. The spores of *F. oxysporum* f. sp. *cubense* were observed for germination after 48 h of incubation under microscope and per cent germination was calculated.

Development of antibiotic resistant strain of Pfm of *P. fluorescens*

In order to study the rhizosphere survival and endophytic nature, an antibiotic resistant strain of *P. fluorescens* was developed using a method described by Vidhysekaran and Muthamilan (1995). During the initial screening, rifampicin at 200 µg/ml, penicillin at 200 µg/ml, actinomycin D at 195 µg/ml inhibited the growth of Pfm of *P. fluorescens***.** The bacterium was grown in King's B medium (KMB) (King et al. 1954) with rifampicin at 200 µg/ml. Resistant colonies were selected and streaked on to KMB without antibiotic. From this, single colonies were selected and restreaked on rifampicin supplemented medium. Again, single colony observed in the rifampicin supplemented medium was restreaked on to medium containing penicillin at 200 µg/ml and actinomycin D at 195 µg/ml. Finally, the colony growing well in KMB containing all three antibiotics was reisolated and used for this study. An antibiotic resistant reference strain Pf1 of *P. fluorescens* was developed using this method.

Development of talc based formulation of *P. fluorescens*

Talc-based formulation of antibiotic resistant strain of Pfm and reference strain of *P. fluorescens* were prepared by the following method described by Vidhyasekaran and Muthamilan (1995). Briefly, a loopful of bacterial strain culture was inoculated into King' B medium broth (KMB) and grown in a rotary shaker at 150 rpm/min 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. Ten grams of carboxymethyl 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 was mixed with carrier – cellulose mixture under aseptic conditions. After air 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 of talc powder.

Plant material and growing conditions

Four-month-old banana cultivar Rasthali were purchased from Horticultural Farm, Agricultural College and Research Institute, Madurai, India. The cultivar has no resis-

tance to Race 1 of *F. oxysporum* f. sp. *cubense*. The plants were grown in a tank of size 70 x 40 cm containing 25 kg of soil. The tank filed with loamy organic soil containing CEC –10.5 c mol (p^+)/kg, organic C-2.9 g/kg, EC – 0.51 dS/m, low in available N (254 kg/ha), medium in P (13.4 kg/ha) and K (345 kg/ha), available Ca – 6.9 c mol/kg, Mg – 0.92c mol/kg, Zn – 2.8 g/kg, Mn – 0.70m g/kg and Cu – 1.23 g/kg. The tank was maintained in a glasshouse under natural light and day/night temperature of approx. 30/24°C.

Effect of *P. fluorescens* on vascular discolouration

Roots of healthy banana (cv. Rasthali) were dipped in conidial suspension of the pathogen (10^6 cfu/ml) for 30 min before planting. Ten days after planting in the tank, talc-based formulation of *P. fluorescens* at 10 g/plant and sand maize inoculum of *F. oxysporum* f. sp. *cubense* at 20 g/tank were applied in rhizosphere region. Plants treated with the pathogen alone or *P. fluorescens* or water alone constituted control object. The plants were grown in glasshouse under natural light and day/night temperature of approx. 30/24°C. After 90 days of incubation, the suckers were cut horizontally and observed for brown discolouration. The vascular discolouration index was calculated by using 1–6 grade given by Orjeda (1998) (grade 1 = corm completely clean, no vascular discolouration; 2 = isolated points of discolouration in vascular tissue; 3 = discolouration up to 1/3 of vascular tissue; 4 = discolouration up to 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 consisted of four plants and the experiments were performed in three replications. The vascular discolouration index was calculated using formula:

$$\frac{\text{Sum of all grades of observed suckers x 100}}{\text{Total number of suckers x maximum value of grades}}$$

Extraction of 2,4-diaetylphloroglucinol from rhizosphere soil

The metabolite 2,4-diacetyl phloroglucinol was extracted from *P. fluorescens* inoculated rhizosphere soil using the method described by Bonsall et al. (1997), in which, talc formulation of *P. fluorescens* inoculated in the banana rhizosphere at 10 g/plant. Ten days after inoculation, the soil samples were taken from the rhizosphere and used for extraction of DAPG. Thirty g of rhizosphere soil was mixed in a 250 ml flask with 40 ml of 80 per cent acetone acidified to pH 2.0 with tri-fluroacetic acid (TFA) and shaken (200 rpm) for 2 h at room temperature (28°C). Samples were subsequently filtered (Whatman No. 1) through a Buchner funnel and the filtrate was centrifuged at 12 000 rpm for 30 min at 4°C to remove soil particles. The supernatant was evaporated to a volume of 8 ml and acidified to pH 2.0 with 10 per cent acetic acid and then extracted twice with 10 ml of ethyl acetate and evaporated to dryness. Extracts were resuspended in 1 ml of 35 per cent acetonitrile and 0.1% acetic acid and centrifuged in an Eppendorf 5415 centrifuge at 16 000 rpm for 20 min at 4°C prior to separation and identification by high pressure liquid chromatography (HPLC). The known concentration of pure DAPG was added into root regions for quantification by standard curve by HPLC. The residual extracts from uninoculated soil were used as check.

Detection of DAPG by HPLC

HPLC used for this analysis consisted of a autosampler, solvent delivery system, controller and UV detector (Varian Model). The HPLC protocol described by Raai-

jmakers et al. (1999) was employed for the detection. The separation was achieved using reverse phase column chromatography (Varian, Microsorb C_{18}; 100 x 2.5 mm). Solvent conditions included a flow rate of 0.5 ml/min with a 2 min initialization at 10 per cent acetonitrile – 0.1% TFA followed by a 20 min gradient to 100% acetonitrile – 0.1% TFA using curve profile. HPLC gradient profiles were monitored at 270 nm and 330 nm, which represent the peak maxima of DAPG. The HPLC system in use up to 45 min for the detection. The standard curve was prepared from the extracts of soil spiked with pure DAPG only.

Statistical analysis

All analyses were performed using IRRISTAT version 92-1, which was developed by International Rice Research Institute Biometrics Unit, The Philippines. The per cent data was arc sine transformed, that analysed and back transformed to original value. The means were grouped using least significant difference (LSD).

RESULTS

The antibiotic production by Pfm strain of *P. fluorescens* was assessed and their antifungal effects on Fusarium wilt pathogen *F. oxysporum* f. sp. *cubense* were evaluated. The chromatographic characteristics of the DAPG were compared with those from reference strain Pf1 of *P. fluorescens*. Obtained data are summarized in Table 1. The compounds extracted from culture of Pfm strain behaved similarly to those of reference strain Pf1 of *P. fluorescens* when compared by thin layer chromatographic plates. The metabolite DAPG extracted in ethyl acetate fraction of the Pfm strain produced bright yellow colour spot at a Rf value of 0.88 in the TLC plates. While extraction of reference strain Pf1 produced yellow colour spot at the same Rf value in the TLC plates.

Table 1. Rf values of DAPG metabolite of *P. fluorescens* visualized in Thin Layer Chromatographic Plates

Strain	*Colour of the spots in TLC plates	Mean Rf
Pfm strain (isolated strain)	bright yellow	0.88
Pf1 strain (reference strain)	yellow	0.88
Control (methanol)	no colour development	0.00
Control (distilled water)	no colour development	0.00

* metabolites visualized in TLC plates after spraying with diazotized sulfanilic acid

Bioassay of the compound (DAPG) extracted from the cultures and eluted from the spots observed in the TLC plates were carried out to tests its action against *F. oxysporum* f. sp. *cubense*. The results presented in Table 2. showed that filter paper dipped in the eluant isolated from the TLC plates significantly inhibited the mycelial growth as well as spore germination of *F. oxysporum* f. sp. *cubense*. Eluants of spots produced by Pfm strain had the maximum inhibitory action on mycelial growth as well as spore germination of the pathogen when compared to reference strain and eluant of the spots observed in the plates.

Table 2. Antifungal effects of DAPG against *Fusarium oxysporum* f. sp. *cubense*

Strains of *P. fluorescens*	*Inhibition zone diameter in paper disc assay [mm]	**Per cent spore germination	
		Microconidial germination	Macroconidial germination
Pfm strain	8.1	10.20 (18.65) a	14.30 (22.04) a
Pf1 strain	8.0	14.56 (22.43) b	18.95 (25.81) b
Control (methanol)	0.2	86.56 (68.50) c	88.63 (70.29) c
Control (distilled water)	0.0	90.12 (71.68) d	92.53 (74.15) d

* mean of four replications, each replication contains five plates

** mean of five replications, each replication contains five cavity slides, from each cavity slide ten observations were made. Vaues in parenthesis are arc sine transformed values. Means followd by different letters are signficantly differnt at LSD 0.05%

Table 3. Quantity of DAPG produced by *Pseudomonas fluorescens* in banana rhizosphere

Strains of *P. fluorescens*	Doses of *P. fluorescens* [g/plant]	*Quantity of DAPG in root soil [ng/g of root soil]
Pfm +FOC	5	14.2 kl
Pfm +FOC	10	24.1 h
Pfm +FOC	15	38.0 f
Pf1 +FOC	5	15.2 k
Pf1 +FOC	10	30.2 g
Pf1 +FOC	15	43.4 e
Pfm strain alone	5	20.1 j
Pfm strain alone	10	56.4 d
Pfm strain alone	15	81.8 b
Pf1 strain alone	5	22.2 i
Pf1 strain alone	10	60.2 c
Pf1 strain alone	15	100.1 a
Untreated soil	–	ND

ND: DPAG not detected, *Average values of two experiments are given, in each experiment, treatments were replicated five times and six tanks were used for each replicate. In all plants, sand maize inoculum of *F. oxysporum* f.sp. *cubense* at 20 g/tank was applied in rhizosphere region of the plants. Means followed by different letters are significantly different at LSD 0.05%

Purified extracts of soil inoculated with Pfm (or) Pf1 strain and challenge inoculated with *F. oxysporum* f. sp. *cubense* were used for HPLC analysis. The chromatograms of HPLC indicated that peaks from all eluted samples at retention times ranging from 20.00 min to 21.30 min, were in the same area where as for pure DAPG elutes under the same conditions and the same trend of peak was observed in the extracts of reference strain (Pf1 strain) inoculated soil. The results also revealed that a peak was observed approximately at 20.428 retention time in the chromatogram of soil in

which Pfm of *P. fluorescens* alone was inoculated. The reference strain also had a peak at 21.025 retention time. DPAG was not detected in rhizosphere soil extracts collected from untreated banana grown soil. The results presented in Table 3. indicated that the quantity of DAPG production was higher in the extracts of soil inoculated with *P. fluorescens* alone than that of extraction of soil inoculated with the strains and challenged with *F. oxysporum* f. sp. *cubense*. Reference strain had higher DAPG production in the extracts of soil than that of isolated strain. Experimental results presented in Table 4 showed that talc formulation of *P. fluorescens* inoculated at 15 g/plant as soil application resulted in least level of vascular discoloration index when compared with other doses of application. However, the effect of Pfm strain of *P. fluorescens* inoculated at 10g/plant is on par with the strain inoculated at 15 g/plant.

Table 4. Effect of *Pseudomonas fluorescens* on vascular discolouration in banana cv. Rasthali

Biocontrol agent	Doses [g/plant]	*Per cent vascular discolouration index
Pseudomonas fluorescens Pfm strain	5	55.56 (48.19) g
	10	31.14 (33.92) bc
	15	30.20 (33.33) b
Pseudomonas fluorescens Reference Pf1 strain	5	48.75 (44.28) f
	10	35.56 (36.61) e
	15	32.18(34.56) bcd
Control (pathogen alone inoculated)	–	88.89 (70.53) h
Uninoculated control	–	0.00 (0.91) a

* mean of five replications, each replication contains six plants raised in tank of size 70 x 40 cm. In each tank, *P. fluorescens* was inoculated in the rhizosphere regions before planting. 90 days after bacterization, the suckers were removed and cut horizontally and observed for discolouration. Values in parenthesis are arc sine transformed values. Mean followed by the same letter are not significantly different at LSD 0.05%

DISCUSSION

A group of root-associated bacteria, plant growth-promoting rhizobacteria (PGPR) intimately interact with the plant roots and consequently influence plant health environment in soil. Among these PGPR, fluorescent pseudomonads occur commonly in the rhizosphere of plants and help suppress disease establishment and spread. Certain fluorescent pseudomonads from soil have been shown to promote plant growth and are supposed to inhibit deleterious bacteria and fungi (Howell and Stipanovic 1980; Keel et al. 1992; Schippers et al. 1987; Schroth and Hancock 1982). The production of antibiotic substances by some strains has been recognized as a major factor in the suppression of many root pathogens.

P. fluorescens produces a variety of metabolites (Dowling and O' Gara 1994), many of which are inhibitory to other microorganisms and some of them are implicated in the biological control of plant pathogens. A metabolite responsible for antifungal activity of the strain of *P. fluorescens* was isolated and identified using standard chromatographic methods. In past experiments, the DAPG production by *P. fluorescens* has been detected by using bioassays (Reddi et al. 1969; Ricker and Ricker 1936).

However, they only indirectly indicate the presence of an inhibitory substance, and do not specify the nature of the compound. In our investigations, the metabolite of *P. fluorescens* viz., DAPG extracted from the culture raised in pigment production medium produced the distinct spots at Rf value 0.88, respectively, in the TLC plates. Rosales et al. (1995) extracted the metabolites from various strains of *P. fluorescens* in KMB medium and pigment production medium and identified them in the TLC plates. The metabolite DAPG extracted from the strain Pfm could be responsible for growth inhibition of *F. oxysporum* f. sp. *cubense*. In our investigations, metabolite DAPG showed significant inhibition of growth and spore germination of *F. oxysporum* f. sp. *cubense*. This metabolite could be responsible for the inhibition of several fungal pathogens. DAPG is an antibiotic known to be active against a broad spectrum of microorganisms and is involved in the suppression of many plant diseases (Defago 1993; Keel et al. 1990; Keel et al. 1992; Weller and Thomashow 1993). Earlier, a number of strains of Pseudomonas have been shown to produce phloroglucinol (Broadbent et al. 1976; Garagulya et al. 1974). The severity of tobacco black root rot was reduced when soil was amended with phloroglucinol (Keel et al. 1992). Phloroglucinol antibiotics are phenolic metabolites produced by bacteria with broad antibacterial and antifungal spectrum and phytotoxic properties (Thomashow and Weller 1996). Keel et al. (1990) and Raaijmakers et al. (1997) reported that phloroglucinol – producing strains of *P. fluorescens* have been shown to be effective against root pathogens viz., *Fusarium oxysporum* in tomato, *Thielaviopsis basicola* in tobacco and *Gaeumannomyces graminis* var. *tritici* in wheat. Beside this, phloroglucinol induced defense mechanism against fungal infection (Tomas Lorente et al. 1989). Chang and Blackwood (1969) indicated that some strains produce more than one metabolite. Hence to detect DAPG production in the rhizosphere, a single HPLC based system was developed. The results of HPLC show the presence of DAPG in *P. fluorescens* inoculated rhizosphere soil. Phloroglucinols have previously been identified chromatographically by using gas liquid chromatography (Pyysalo and Widen 1979) and HPLC (Widen et al. 1980). However, in this study, we identified DAPG production in Pfm strain *P. fluorescens* inoculated soil against *Fusarium* wilt disease in banana using HPLC. Raaijmakers et al. (1999) also identified DAPG production in the rhizosphere soil of wheat inoculated with *P. fluorescens* using HPLC and they also indicated that DAPG production in the rhizosphere of wheat is related strongly to the ability of introduced strain to colonize the roots. Detection of DAPG at the site of disease suppression by introduced strain in the rhizosphere also demonstrated the role of the metabolite in disease suppression (Bonsall et al. 1997; Keel et al. 1992; Maurhofer et al. 1992; Raaijmakers et al. 1997). In our experiments, DAPG production was less when *P. fluorescens* inoculated rhizosphere of banana plants and challenged with *F. oxysporum* f. sp. *cubense* when compared to sole *P. fluorescens* inoculated rhizosphere of banana plants. Various secondary metabolites viz., phytotoxins, pigments and mycotoxins produced by species of *F. oxysporum* may cause serious responses in plants, animals, humans and microorganisms (Marasas et al. 1984). Similarly, Regina Notz et al. (2002) found that Phl A gene expression for DAPG was repressed by addition of fusaric acid in the culture. Duffy and Defago (1997) observed that fusaric acid mycotoxin produced by *Fusarium* spp. repressed DAPG production in strain CHA0 *in vitro* and on tomato roots.

The vascular discoluration due of *Fusarium* wilt disease in banana is suppressed by *P. fluorescens* when used as soil application. A higher inhibition was observed in

P. fluorescens inoculated plants at 15 g/plant. The application of *P. fluorescens* reduced wilting and discolouration by *F. oxysporum* f. sp. *cubense* in banana (Raguchander et al. 1997; Sivamani and Gnanamanickam 1988; Thangavelu et al. 2001). In this plant species, precolonization with *P. fluorescens* was signalled to mobilize a number of defense mechanisms for preventing the spread of *F. oxysporum* f. sp. *cubense* in the root tissue (Sukhada et al. 2004). In our study, soil application of *P. fluorescens* in the rhizosphere cause the colonization of roots and in consequence may prevent the entry of pathogen in the root system. Beside this, Thangavelu et al. (2001) detected an increased level of phenyl alanine ammonia lyase, peroxidase, chitinase and ß-1,3-glucanase following treatment of roots with *P. fluorescens* and challenge inoculation with *F. oxysporum* f. sp. *cubense*. Identifying mechanisms of introduced biocontrol agent in the rhizosphere is an important requisite in the disease suppression. In our study, DAPG production in the rhizosphere is one of the mechanisms by introduced *P. fluorescens* strain in the disease suppression.

In conclusion, the DAPG production by the Pfm strain of *P. fluorescens* effected the antifungal activity against *F. oxysporum* f. sp. *cubense* and *P. fluorescens* suppressed the vascular discolouration due to fusarial wilt disease in banana. DAPG production was suppressed by *F. oxysporum* f. sp. *cubense* in the rhizosphere of banana plants. Most biocontrol agents, including strains of antibiotic-producing *Pseudomonas* spp., are still too variable in their performance in natural conditions to be successfully used as a common practice in plant protection (Raaijmakers and Weller 2001). However, existing biological complexity and abiotic environments in the rhizosphere and the DAPG production may be inconsistent. Beside this, the stage of plant growth appeared also to affect significantly the level of genetic diversity of the Pseudomonas DAPG producing population and seemed to be related to the level of root exudates production (Picard et al. 2001). These factors are strongly related to the need of prior knowledge of various compounds produced by the biological complexity and the host plant and role of nutrients in the metabolite production in the rhizosphere in developing a suitable strain of *P. fluorescens* for management of the disease.

ACKNOWLEDGEMENTS

We thank B.A. Subramanian, Textiles Committee, Govt. of India, Madurai for analysing the metabolites by using chromatography techniques and we also thank Dr. R.F. Bonsall, Department of Plant Pathology, Washington State University, Washington, USA for his helpful advice. This work was supported in part by grants from ASPEE Agricultural Research and Development Foundation, Mumbai, India.

REFERENCES

Blazevic D.J, Koepcke M.H., Matsen J.M. 1973. Incidence and identification of *Pseudomonas fluorescens* and *Pseudomonas putida* in the clinical laboratory. J. Appl. Microbiol. 25: 107–110.

Bonsall R.F., Weller D.M., Thomashow L.S. 1997. Quantification of 2,4-diacetyl phloroglucinol produced by fluorescent *Pseudomonas* spp. *in vitro* and in the rhizosphere of wheat. Appl. Environ. Microbiol. 63: 951–955.

Broadbent D., Mabelis R.P., Spencer H. 1976. C-acetylphloroglucinols from *Pseudomonas fluorescens*. Phytochemistry 15, p. 1785.

Brodsky M.H., Nixon M.C. 1973. Rapid method for detection of *Pseudomonas aeroginosa* on McConkey-agar under ultra violet. J. Appl. Microbiol. 26: 219–220.

Chang P.C., Blackwood A.C. 1969. Simultaneous production of three phenazine - 1- carboxylic acid pigments by *Pseudomonas aureofaciens*. Mac 436. Can. J. Microbiol. 15: 439–444.

Deese C.D., Stahman A.M. 1962. Pectic enzymes and cellulase formation by *Fusarium oxysporum* f. sp. *cubense* of stem tissues from resistant and susceptible banana plants. Phytopathology 52: 247–255.

Defago G. 1993. 2,4-diacetylphloroglucinol, a promising compound in biocontrol. Plant Pathol. 42: 311–312.

Dowling N.D., O'Gara F. 1994. Metabolites of pseudomonas involved in the biocontrol of plant diseases. Tibtech 12: 133–141.

Duffy B.K., Defago G. 1997. Zinc improves biocontrol of Fusarium crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic synthesis. Phytopathology 87: 1250–1257.

Garagulya A.D., Kiprianova E.A., Boiko O.I. 1974. Antibiotic effect of bacteria from the genus *Pseudomonas* on phytopathogenic fungi. Microbiology (*Kiev*) 36: 197–202.

Gould W., Hagedorn C., Bardinelli T., Zoblotowicz R. 1985. New selective media for enumeration and recovery of fluorscent pseuodomonads from various habitats. Appl. Environ. Microbiol. 49: 28–32.

Howell C.R., Stipanovic R.D. 1980. Suppression of *Pythium ultimum* induced damping off seedlings by *Pseudomona fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70: 712–715.

Keel C., Wirthner P., Obeshansli T., Voisard C., Burger U., Hass D., Defago G. 1990. *Pseudomonas* as antagonists of plant pathogens in the rhizosphere, role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis 9: 327–341.

Keel C., Schnider U., Maurhofer M., Voisard C., Laville K., Burger U., Wirthner U. P., Haas D., Defago G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Inter. 5: 4–13.

King E.O., Ward M.K., Raney D.E. 1954. Two simple media for demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 14: 301–307.

Kreig N.R., Holt J.G. 1984. Bergy's Manual of Systematic Bacteriology, 9th Edition, Vol. I. The Williams and Wilkins Co., Baltimore, 324 pp.

Marasas W.F.O., Nelson P.E., Tousson T.A. 1984. Toxigenic *Fusarium* Fusarium species: Identity and Mycotoxicology. The Pennsylvania State University Press, University Park, Pa, 114 pp.

Maurhofer M., Keel C., Schnider U., Voisard C., Hass D., Defago G. 1992. Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology 82: 190–195.

Maurhofer M., Hase C., Meuwly P., Metraux J.P., Defago G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonization *Pseudomonas fluorescens* strain CHA0: influence of the *gac A* gene and of pyoverdine production. Phytopathology 84: 139–146.

Notz R., Maurhofer M., Dubach H., Haas D., Défago G. 2002. Fusaric Acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expressions in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of Wheat. Appl. Environ. Microbiol. 68: 2229–2235.

Orjeda G. 1998. Evaluation of *Musa* germplasm for resistance to sigatoka diseases and *Fusarium* wilt. INIBAP Technical Guidelines 3. International Plant Genetic Resources Institute, Rome, Italy, p. 29.

Picard C.F., Di Cello F., Ventura M., Fani R., Guckert A. 2000. Frequency and biodiversity of 2,4--diacetylpholoroglucinol producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Appl. Environ. Microbiol. 66: 948–955.

Ploetz R.C., Herbert J., Sebasigari K., Hernandez J.H., Pegg K.G., Ventura J.A., Mayato L.S. 1990. Importance of *Fusarium* wilt in different banana-growing regions. p. 9. In "*Fusarium* Wilt of Banana" (R.C. Ploetz, ed.). The APS Press, St. Paul.

Pyysalo H., Widen C.J. 1979. Glass capillary gas chromatographic separation of naturally occurring phloroglucinols. J. Chromatography 168, p. 246.

Raaijmakers J., Wellers D., Thomashow L. 1997. Frequency of antibiotic producing *Pseudomonas* spp. in natural environments. Appl. Environ. Microbiol. 63: 881–887.

Raaijmakers J.M., Bonsall R.F., Weller D.M. 1999. Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89: 470–475.

Raaijmakers J.M., Weller D.M. 2001. Exploiting Genotypic diversity of 2,4-diacetylphloroglucinol producing *Pseudomonas* spp.: Characterization of superior root colonizing *P. fluorescens* strain Q8r-96. Appl. Environ. Microbiol. 67: 2545–2554.

Raguchander T., Jayashree K., Samiyappan R. 1997. Management of *Fusarium* wilt of banana using antagonistic micro-organisms. J. Biol. Control 11: 101–105.

Reddi T.K., Khudyakov P.Y.A., Borovkov A.V. 1969. *Pseudomonas fluorescens* strain 26 –0 a producer of phyotoxic substances. Mikrobiologia 38: 909–913.

Ricker A.J., Ricker R.S. 1936. Introduction to Research on Plant Disease. Johns. Swift Co. Mc., New York, 117 pp.

Rioux C., Jordan D.C., Rattray J.B.M. 1983 Colorimetric determination of catechol siderophores in microbial cultures. Ann. Biochem. 133: 163–169.

Rosales A.M., Thomashow L., Cook R.J., Mew T.W. 1995. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology 85: 1028–1032.

Schippers B., Bakker W., Bakker P.A.H.M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Ann. Rev. Phytopathol. 25: 339–358.

Schnider-Keel U., Seematter A., Maurhofer M., Blumer C., Duffy B., Gigot-Bonnefoy C., Reimmann C., Notz R., Défago G., Haas D., Keel C. 2000. Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J. Bacteriol. 182: 1215–1225.

Schroth M.N., Hancock J.G. 1982. Disease-suppressive soil and root colonizing bacteria. Science 216: 1376–1381.

Sebasigari K., Stover R.H. 1988. Banana diseases and pests in East Africa. Report of a survey in November 1987. INIBAP, Montpellier, France: 15–17.

Shanahan P., Sullivan D.O., Simpson P., Glennon J., O' Gara F. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonads and investigation of physiological parameters influencing its production. Appl. Environ. Microbiol. 58: 353–358.

Sivamani E., Gnanamanickam S.S. 1988. Biological control of *Fusarium oxysporum* f. sp. *cubense* in banana by inoculation with *Pseudomonas fluorescens*. Plant Soil 107: 3–9.

Snyder W.C., Hansen H. 1940. The species concept in *Fusarium*. Am. J. Botany 27: 64–67.

Sukhada M., Mohan M., Rawal R.D., Chakraborty S., Sreekantappa H., Manjula R., Lakshmikantha H.C. 2004. Interaction of *Fusarium oxysporum* f. sp. *cubense* pre colonized to banana roots. World J. Microbiol. Biotechnol. (NLD) 20: 651–655.

Sullivan D., O'Gara F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant pathogens. Microbiol. Rev. 56: 662–676.

Thangavelu R., Palaniswami A., Ramakrishman G., Doraiswamy S., Muthukrishnan S., Velazhahan R. 2001. Involvement of fusaric acid detoxification of *Pseudomonas fluorescens* strain Pf_{10} in the

biological control of *Fusarium* wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense*. J. Plant Dis. Protection 108: 433–445.

Thomashow L., Weller D.M. 1988. Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaummannomyces graminis* var. *tritici*. J. Bacteriol 170: 3499–3508.

Thomashow L.S., Weller D.M. 1996. Current concepts in the use of introduced bacteria for biological disease control: Mechanisms and antifungal metabolites. p. 187–235. In "Plant-Microbe Interactions" (G. Stacey, M. Keen, eds.). Chapman and Hall, New York, Vol. I.

Tomas-Lorente F., Iniesta-San Martin E., Tomas Barberan F.A., Trowitzsch-Kienast W., Wary V. 1989. Antifungal phloroglucinol derivatives and lipophilic flavanoids from *Helichrysum decumbens*. Phytochemistry 28: 1613–1615.

Vidhyasekaran P., Muthamilan M. 1995. Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. Plant Dis. 79: 782–786.

Weller D.M., Thomashow L.S. 1993. Use of rhizbacteria for biocontrol. Curr. Opin. Biotechnol. 4: 306–311.

Widen C., Pyysalo H., Salovaara P. 1980. Separation of naturally occurring acetylphloroglucinols by high performance liquid chromatography. J. Chromatography 188: 213–220.

POLISH SUMMARY

WPŁYW *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* (E.F. SMITH) HANSEN AND SNYDER NA WYTWARZANIE PRZEZ *PSEUDOMONAS FLUORENSCENS* 2,4-DIACETYLFLOROGLUCINOLU W RIZOSFERZE BANANA

Przedmiotem badań było określenie wpływu *Fusarium oxysporum* f. sp. *cubense* na wytwarzanie toksycznego związku 2,4-diacetylfloroglucinolu (DAPG) przez antagonistyczny szczep *Pseudomonas fluorescens*. Oczyszczone ekstrakty z kultur szczepu *P. fluorenscens* wyizolowanego z rizosfery banana inhibowały w warunkach laboratoryjnych wzrost i kiełkowanie zarodników *F. oxysporum* f. sp. *cubense*. Na cienkowarstwowych płytkach chromatograficznych wartość współczynnika Rf dla toksyny DAPG wyizolowanej z kultur bakterii *P. fluorenscens* i dającej wyraźne plamy na chromatogramach wynosiła 0,88.

Ekstrakty gleby inokulowanej bakterią *P. fluorenscens* i grzybem *F. oxysporum* f. sp. *cubense* eluowały w czasie retencji od 20.00 do 21.30 minut.

Ilość wytworzonego DAPG była mniejsza w ekstraktach z gleby inokulowanej bakterią *P. fluorenscens* i *F. oxysporum* f. sp. *cubense*, w porównaniu do ekstraktu z gleby inokulowanej jedynie bakterią *P. fluorenscens*. Zastosowanie formulacji inokulum *P. fluorenscens* z talkiem powodowało również zmniejszenie przebarwienia wiązek naczyniowych chorych roślin banana w przypadku inokulacji dawką wynoszącą 15 g na jedną roślinę.

BOOK REVIEW

Kirk P.M., Cannon P.F., David J.C., Stalpers J.A. 2001. Ainsworth & Bisby's Dictionary of the Fungi. CABI Publishing, CAB International. Wallingford, UK. 655 pp. ISBN 0-85199-377 X.

It is the ninth edition of the Ainsworth & Bisby's Dictionary of the Fungi. The efforts of the authors are focused on three main fields.

First part concerns the revision of the Ascomycota classification. It is mainly done on the basis of sequence analysis, especially of ribosomal DNA. Placement of families in higher taxa is in many cases based on such information. As the result six classes of Ascomycota, and 55 orders and 291 families are created. However, many groups, for example saprobiotei fungi and biotrophs that do not grow in culture lack necessary molecular information.

The classification of Basidiomycota is revised next. The gastromycetous hymenomycetous fungi are integrated in one system. A total of 32 orders are consolidated into 16. The classification of basidiomycetous yeasts has changed; they belong to three separate groups. That necessitated alterations: delimitation of the Urediomycetes and Ustilagomycetes.

In the third part all fungi, which are not known to produce sexual spores, are integrated. One organism with all its morphological forms has one name. Based on the available evidence, as many anamorphs as it is possible are placed in the teleomorph system. That is why many are only listed at class level.

Such placements might accelerate more research in the future. Hopefully this system will probably be accepted not only by mycologists involved in a pure taxonomy but also by wide range of applied biologists.

Generally, the style of the Dictionary is similar to the style of previous editions. However, in this last edition the references are included under each entry. Moreover, most abbreviations disappeared from the text of the Dictionary, which makes reading more comfortable.

There is information concerning more data available on the Internet too. This database is on the Internet since 1999 (www.indexfungorum.org).

I recommend this new edition of the Ainsworth & Bisby's Dictionary of the Fungi to all mycologists and plant pathologists working with fungal diseases too.

Maria Rataj-Guranowska
Institute of Plant Protection
Miczurina 20, 60-318 Poznań, Poland