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Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1

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Abstract

Root rot of cocoyam (*Xanthosoma sagittifolium*) caused by *Pythium myriotylum* is the most devastating disease of this important tropical tuber crop with yield reductions of up to 90%. Bioassays were conducted *in vitro* and in sterile volcanic soil artificially infested with *Pythium myriotylum*, isolate CRPm, to test whether *Pseudomonas aeruginosa* PNA1 can control the cocoyam root rot disease. *P. aeruginosa* PNA1 (wild type) produces phenazine-1-carboxylic acid and phenazine-1-carboxamide (oxychlororaphin), while its tryptophan auxotrophic mutant FM13 is phenazine negative and secretes anthranilate *in vitro*. PNA1 and FM13 have previously been shown to control *Pythium debaryanum* and *Pythium splendens* on lettuce and bean. PNA1 and FM13 significantly inhibited growth of *P. myriotylum* in dual cultures, while their supernatants highly reduced mycelial dry weight in potato dextrose broth. However, in the presence of tissue culture derived cocoyam plantlets, only strain PNA1 strongly reduced root rot disease severity. Soil experiments involving strain PNA1 in comparison to phenazine-deficient mutants suggested that the biocontrol activity of PNA1 against *P. myriotylum* may involve phenazines. Phenazine involvement was further strengthened by the fact that FM13 fed with exogenous tryptophan (so that phenazine production is restored) significantly reduced disease severity on cocoyam. The efficiency of PNA1 to control *P. myriotylum* on cocoyam was significantly improved when the strain and the pathogen were allowed to interact for 24 h prior to transplanting cocoyam plantlets, while doubling the inoculum density of the pathogen negatively affected its efficiency.

Introduction

Xanthosoma sagittifolium, also called new cocoyam, tannia or yautia is a member of the *Araceae* family and originates from tropical America. During the nineteenth century, it was introduced to West Africa, which is now the major producer. In West Africa it has been displacing the 'old cocoyam' or taro (*Colocasia esculenta*) because of its better yield (Giacometti and León, 1994). *Xanthosoma* grows from a tuber that can be boiled, baked or mashed into a meal. It has traditionally been a subsistence crop, and even though it is the major staple food for about 200 million people in the tropics and subtropics, little information is available on its cultivation and requirements. The main limitations to the development of cocoyam as a crop are diseases, particularly a root rot disease caused by *Pythium myriotylum* (Pacumbaba et al., 1992) that can cause yield reductions as high as 90% (Nzietchueng, 1983). *P. myriotylum* from cocoyam has a restricted host range (Tambong et al., 1999) and can attack both young plantlets and mature plants. Characteristic symptoms of the disease are stunting, yellowing of leaves and severe reduction of the root system. In West Africa, symptoms in the field are mainly observed on 4–5 months old plants during the rainy season. Disease control is difficult and for the time being cultural practices such as soil drainage and crop rotation are recommended.

Pythium diseases can also be controlled by chemical pesticides (Martin and Loper, 1999) but they can have adverse effects on the environment and human health. Among alternative strategies, biological control exhibits unquestionable potentials. Interest in biological control has recently intensified because of imminent bans on effective chemical controls such as methyl bromide, widespread development of fungicide resistance in pathogens, and a general need for more sustainable disease control strategies (Duffy and Défago, 1999). Considerable progress is being made with regard to diseases of some 'major' plants while sustainable disease control strategies for research-neglected crops like cocoyam are still in their initial stages.

Fluorescent pseudomonads are ubiquitous soil micro-organisms and common inhabitants of the rhizosphere. They have emerged as the largest and potentially most promising group of plant growth-promoting rhizobacteria involved in the biocontrol of plant diseases (Dowling and O'Gara, 1994; O'Sullivan and O'Gara, 1992; Weller, 1988). In many crop-pathogen systems, the primary mechanism of biocontrol by fluorescent pseudomonads is production of antibiotics such as 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and phenazines such as phenazine-1-carboxylate (Thomashow and Weller, 1996). Phenazines, pigmented nitrogen-containing heterocyclic compounds, inhibit mycelial growth of several fungal plant pathogens (Haynes et al., 1956; Turner and Messenger, 1986). Phenazine-1-carboxylic acid (PCA), produced by *P. fluorescens* 2–79 (Ownley et al., 1991; Thomashow et al., 1990) and *P. aureofaciens* 30–84 (Hamdan et al., 1991) plays an important role in the control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Phenazine-1-carboxamide (oxychlororaphin) produced by *P. chlororaphis* PCL 1391 can control tomato root rot caused by *Fusarium oxysporum f.* sp. *radicis-lycopersici* (Chin-a-Woeng et al., 1998). Anjaiah et al. (1998) demonstrated the involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn*5* derivatives toward *Fusarium* spp., *P. debaryanum* and *P. splendens* on chickpea, bean and lettuce. Phenazines and anthranilate are synthesised from chorismic acid. In *P. fluorescens* 2–79, seven biosynthetic genes, *phzABCDEFG*, organised in an operon (Mavrodi et al., 1998) are sufficient for PCA production and introduction of this operon under the control of a P*tac* promoter into the chromosome of *P. fluorescens* SBW25 enhanced its ability to control *P. ultimum* on peas (Timms-Wilson et al., 2000). The phenazine gene cluster is very highly conserved and is also present in the chromosome of *P. chlororaphis* strain 30–84 and *P. aeruginosa* PAO1. Although the *phzABCDEFG* genes are sufficient for phenazine biosynthesis, anthranilate also seems to play a role in phenazine biosynthesis, at least in *P. aeruginosa*. In *P. aeruginosa* PAO1, synthesis of anthranilate from chorismate is catalysed by two distinctive anthranilate synthases (AS). AS I, encoded by *trp*E and *trp*G, is involved in tryptophan biosynthesis, whereas AS II, encoded by *phn*AB, is involved in phenazine biosynthesis (Essar et al., 1990a). Transcription of *trp*G and *trp*E occurs in early and late exponential phase, whereas *phn*AB are transcribed primarily in stationary phase (Essar et al., 1990b). Anthranilate has antifungal activity against *G. graminis* var. *tritici*, but has only a limited role in suppression of wheat take-all disease by strain 2–79 (Hamdan et al., 1991).

In the present study, we investigated the use of *P. aeruginosa* PNA1 and its mutant FM13 to control the cocoyam root rot disease pathogen, *P. myriotylum*. FM13 is a *trpC* mutant of *P. aeruginosa* PNA1, which is auxotrophic for tryptophan and excretes anthranilate, an intermediate in the tryptophan biosynthesis pathway (Manch and Crawford, 1982). FM13 is deficient in phenazine production (Anjaiah et al., 1998). *Pythium* that infect cocoyam seems to have developed some degree of specialisation towards the plant (Tambong et al., 1999) and we were interested to investigate if the pathogen could be suppressed by *P. aeruginosa* PNA1 and FM13 which are effective against *P. debaryanum* and *P. splendens* (Anjaiah et al., 1998). A series of experiments were conducted *in vitro* and in sterile volcanic soils of Cameroonian origin. The effects of *Pythium* inoculum density and time of transplanting cocoyam plantlets on the biocontrol efficacy of PNA1 and FM13 were studied. The study also aimed at understanding if phenazines and anthranilate are involved in the biological control of this pathogen by PNA1 and FM13 respectively. This article provides the first detailed report on the possibilities of using biocontrol agents to reduce root rot disease severity on cocoyam.

Materials and methods

Chemicals and culture media

Pythium myriotylum, isolate CRPm was grown on potato dextrose agar (PDA, Oxoid Ltd, Basingstoke,

Hampshire, England) or potato dextrose broth (PDB, Difco, Becton Dickinson S.A. Le Pont de Claix, France). Bacterial strains were grown on King's medium B (KB, Buysens et al., 1996) or glucose-casamino acid-yeast extract (GCY, glucose 15 g L^{-1}, casamino acids 1.5 g L^{-1}, yeast extract 1.0 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 1.0 g L^{-1}, agar 15 g L^{-1}). Anthranilate was obtained from Sigma Chemical, St Louis, MO, USA and L-tryptophan from Merck KGaA, Darmstadt, Germany.

Bacteria, Pythium, plant material and soil

Table 1 gives the relevant characteristics of bacteria and transposon Tn*5* mutants used in this study. *P. aeruginosa* PNA1 was isolated from the roots of chickpea and produces phenazine-1-carboxylic acid and phenazine-1-carboxamide. *P. aeruginosa* FM13 is a *trpC* mutant of PNA1 which is tryptophan auxotrophic, phenazine-deficient and excretes anthranilate. *P. aeruginosa* FM29 is a phenazine-deficient mutant of PNA1 due to a mutation in *phnA*. *P. myriotylum* isolate CRPm was isolated from diseased cocoyam roots as described by Tambong et al. (1999). Tissue culture derived cocoyam plantlets were used. *In vitro* plantlets were produced as described by Tambong et al. (1998). Shoot-tips were excised from sprouting cormels, surface-disinfected in 15% commercial bleach (Clorox), rinsed three times with sterile demineralised water and incubated in Gamborg et al. (1968) micro- and macro-nutrients supplemented with 0.49 µM (Δ^2-isopentenyl)adenine. The cultures were incubated at 22 ± 3 °C with 12/12 photoperiod and light intensity of 75 µmol m^{-2} s^{-1}. Six- to eight-week-old plantlets were used. Production of *in vitro* plantlets was necessary to obtain a uniform population. Volcanic soil used in the study was collected from cocoyam growing fields of Ekona, Cameroon where root rot disease has been reported. Soil was steam-sterilised at 121 °C, 1 kg cm^{-1} for 2 h at least three days before transplanting cocoyam plantlets.

Table 1. Relevant characteristics of *P. aeruginosa* PNA1 and its Tn*5* derivatives

Strains	Relevant characteristics[1]	Reference or source
PNA1	Wild type; Phz^+ Trp^+ Ap^r	Anjaiah et al., 1998
FM13	*P. aeruginosa* PNA1 *trpC*::Tn*5*-Tc (Trp^-, Phz^-)	Anjaiah et al., 1998
FM29	*P. aeruginosa* PNA1 *phnA*::Tn5-Tc (Phz^-)	Anjaiah et al., 1998

[1]Phz: phenazine; Trp: tryptophan; Ap^r: ampicillin resistant; Tc: tetracycline.

Antagonism of PNA1, FM13 and their supernatants against Pythium isolate CRPm in vitro

Pseudomonas aeruginosa PNA1 and FM13 were screened for antagonism against the cocoyam root rot pathogen on GCY agar. In plate assay, an agar plug (4-mm diameter) was cut from an actively growing *Pythium* culture and placed on the surface of fresh agar medium at the centre of a petri plate. Simultaneously, PNA1 and FM13 grown on KB agar were streaked at opposite sides towards the edge of the petri dish about 3 cm away from the agar plug. Plates inoculated with a *Pythium* plug alone were used as controls. The plates were incubated at 28 °C. Radial growth and inhibition zones were measured after 48 h of incubation.

The effect of supernatants of PNA1 and FM13 on mycelial dry weight of isolate CRPm was evaluated in potato dextrose broth. PNA1 and FM13 were grown in liquid GCY medium overnight at 28 °C with shaking. The cultures were centrifuged at 5000 rpm for 10 min and the supernatant collected. Supernatants of PNA1 and FM13 were filter-sterilised using 0.22 µm Millipore filter units (Millex-GV, Millipore S.A. 67 Molshein, France). Potato dextrose broth (PDB, 50 ml) was amended with 1 ml and 5 ml of the sterilised supernatants in 100-ml flasks. The flasks were seeded with 4 *Pythium* plugs (5 mm diameter) and incubated at 24 °C for five days. Dry weights were determined by sieving mycelia from PDB, blotting on sterile filter paper and drying at 60 °C to constant weight. Six replicates were made and the experiment was repeated three times with similar results.

Suppression of Pythium CRPm *in nutrient solution in the presence of cocoyam plantlets*

Eight-week-old tissue culture derived cocoyam plantlets were acclimatised in Gamborg et al. (1968) micro- and macro-nutrients (pH 5.8) for two weeks in bottles covered with plastic foils. Bacterial strains PNA1 and FM13 were grown on KB agar plates overnight and plates were washed with sterilised distilled water. The bacterial suspension was adjusted to 2.4×10^8 cfu ml^{-1}. Liquid B5 medium

(80 ml, pH 5.8 ± 1) was dispensed into culture bottles, covered with aluminium foil and autoclaved at 121 °C, 1 kg cm^{-1} for 21 min. *Pythium* inoculum was produced by aseptically placing four 5-mm mycelial-agar plugs into 40 ml PDB and incubated at 24 ± 2 °C for seven days. The resulting mycelial mat was blended in 25 ml distilled water for 20–30 s using a Polytron macerator (Kinematic GmbH, Switzerland). The planting medium was infested with 64 mycelial strands ml^{-1} of nutrient solution. One milliliter of the bacterial suspension corresponding to 2.4×10^8 cfu was added to the set-up and incubated at 25 ± 2 °C overnight prior to seeding with cocoyam plantlets. Plantlets were observed daily for typical symptoms of the cocoyam root rot disease. The root rot severity was scored using a rating scale of 0: no visible symptoms to 4: dead of plantlet. These ratings were transformed to disease severity index (DSI) as described by Liu et al. (1996) as follows: DSI = Σ(rating number × number of plantlets in the rating)/(Total number of plantlets × highest rating). DSI ranges from 0: no visible symptoms to 1: dead of plantlet. The experiment was repeated twice.

Influence of anthranilate on growth of Pythium CRPm in vitro *and in the presence of cocoyam plantlets*

The effect of various concentrations of anthranilate on growth suppression of CRPm, and on DSI in the presence of cocoyam plantlets was investigated in potato dextrose broth, liquid B5 medium or volcanic soil. Anthranilate was tested because *P. aeruginosa* FM13 secretes high amounts of the chemical in culture medium. PDB was amended with filter-sterilised anthranilate concentrations of 0, 12.5, 25 and 50 μg ml^{-1} in flasks. CRPm plugs were produced, seeded, incubated for five days and dry weight was determined as described above. Four replicates were made for each treatment and the study was repeated twice. The effect of anthranilate on DSI was studied in liquid B5 and soil using 50 μg ml^{-1} and 100 μg ml^{-1} or μg g^{-1} of soil where applicable. *Pythium* inoculum used was 64 mycelial propagules ml^{-1} for nutrient solution studies and 750 mycelial propagules g^{-1} of soil. Plantlets were evaluated and scored for disease symptoms daily for five days in the nutrient solution system and 10 days in the soil . Disease severity indices were computed as mentioned earlier. Each treatment was replicated five times and the experiments repeated twice.

Effect of inoculum density and time of transplanting of plantlets on the biological control activity of PNA1 and FM13 in volcanic soil

The effect of inoculum potential and time of transplanting plantlets on biocontrol activity of PNA1 and FM13 in volcanic soil was investigated. *Pythium* inoculum was prepared as described above. Steam-sterilised soil was infested with 750 and 1500 mycelial propagules g^{-1} and 3.0×10^6 cfu g^{-1} of soil of bacterial suspension was added to the mixture. Plantlets were transplanted 0 (immediately after mixing bacteria and CRPm) and 24 h after incubating *Pythium* and bacterial strains into the soil. After each treatment combination, the set-up was incubated at 25 ± 2 °C and plantlets were observed daily for typical root rot disease symptoms. DSI was calculated as indicated above. The study was repeated twice with five replicates per treatment combination.

Effect of P. aeruginosa *PNA1 and its Tn5 mutants (FM13 and FM29) on biocontrol of* Pythium CRPm *on cocoyam*

PNA1 and its mutants FM13 and FM29 (for characteristics see Table 1) were grown overnight as indicated earlier. Bacterial suspensions (3×10^6 cfu g^{-1}) and *Pythium* inoculum (750 propagules g^{-1}) were thoroughly mixed into steam-sterilised soil. The mixture was incubated at 28 ± 3 °C for 24 h prior to planting seven-week-old cocoyam plantlets. Tissue culture derived plantlets were rinsed twice with sterile distilled water (to remove sucrose from culture medium) and blotted on paper towels. The roots were dipped into the various bacterial suspensions before transplanting cocoyam plantlets in the soil. The experiments were repeated three times with five replicates per treatment combination. Root rot disease severity were scored and calculated as indicated above.

Effect of tryptophan on the biocontrol activity of mutant FM13 against Pythium CRPm *in the presence of cocoyam plantlets*

Strain FM13 was grown on KB medium amended with filter-sterilised tryptophan (100 μg ml^{-1}) incubated at 28 °C overnight. Bacterial suspension was made in one-fourth strength of tryptophan solution (25 μg ml^{-1}) to be applied to soil. Soil was also amended with

60 μg g^{-1} tryptophan prior to addition of bacterial suspension (3×10^6 cfu g^{-1}) and *Pythium* inoculum (750 mycelia propagules g^{-1}). The mixture was incubated as indicated earlier after which seven-week-old plantlets were planted. Disease severity and percentage surviving plantlets were recorded 10 days after inoculation. The experiment was repeated three times with five replicates.

Statistical analysis

Data collected were analysed using SPSS (1996) statistical software. In all experiments except for the effect of inoculum density and time of transplanting, the design was a simple randomised complete block. The effect of inoculum density and transplantation time was analysed as a randomised complete block design with a split-plot arrangement of treatment. Where necessary, arcsine transformation was performed on data before statistical analysis. When analysis of variance was significant, Duncan Multiple Range Test was used to separate means at 5% level of probability.

Results

Antagonism of PNA1, FM13 and their supernatants against Pythium CRPm in vitro

CRPm was incubated in dual cultures with *P. aeruginosa* PNA1 or FM13 on GCY agar or inoculated with the supernatants of *P. aeruginosa* PNA1 and FM13 in potato dextrose broth (PDB). *P. aeruginosa* PNA1 and FM13 significantly influenced radial growth of CRPm and created clear inhibition zones when streaked parallel to the CRPm plugs. Supernatants of PNA1 and FM13 (grown overnight in GCY broth) significantly reduced mycelial dry weight of CRPm in PDB. Amendment of 50 ml PDB with 1 ml of supernatant of PNA1 or FM13 reduced mycelial dry weight from 83 mg in control treatments to 27 mg or 38.7 mg. Increasing the volume of supernatants to 5 ml drastically reduced mycelial dry weight to 2.3 mg or 7.1 mg for PNA1 or FM13 respectively. In GCY broth, FM13 produces about 20 mg L^{-1} of anthranilate (Anjaiah et al., 1998) while PNA1 produces about 82 mg l^{-1} of phenazine-1-carboxylate and 20 mg L^{-1} of phenazine-1-carboyxamide (Anjaiah, 1998). This means that the final concentration of anthranilate in PDB amended with FM13 supernatant was respectively, 0.4 and 2 μg ml^{-1}, while the final concentration of phenazines in PDB amended with PNA1 was respectively 2 and 10 μg ml^{-1}.

Table 2. Protection of cocoyam plantlets against *P. myriotylum* isolate CRPm by *P. aeruginosa* PNA1 incubated in nutrient solution

Treatment	Disease severity index[1,2]	Surviving plantlets (%)[1,3]
Pythium alone	0.95 ± 0.05 a	0.0 c
Pythium + PNA1	0.35 ± 0.06 c	80.0 a
Pythium + FM13	0.77 ± 0.07 b	20.0 b

[1]Values within a column with different letters were significantly different at 5% level of probability using Duncan Multiple Range Test. Nutrient solution used contained Gamborg et al. (1968) macro- and micro-nutrients at pH 5.8 ± 0.1. The experiment was repeated three times with five replicates per treatment combination.
[2]DSI was calculated from score ratings (0: no visible symptoms, to 4: dead) as described by Liu et al. (1996): DSI = ∑(rating number × number of plantlets in the rating)/(Total number of plantlets × highest rating).
[3]Values are proportions of surviving plantlets over initial number of plantlets transplanted into soil infested with CRPm. Arcsine transformation was performed prior to statistical analysis.

Suppression of Pythium CRPm *in nutrient solution in the presence of cocoyam plantlets*

Gamborg et al. (1968) liquid medium was infested with CRPm and inoculated with *P. aeruginosa* PNA1 or FM13 incubated for 24 h prior to transplanting tissue culture derived cocoyam plantlets. *P. aeruginosa* PNA1 and FM13 affected the disease severity differently (Table 2). PNA1 significantly reduced root rot DSI from 0.95 in cocoyam plantlets treated with only CRPm to 0.35. The DSI of plantlets incubated in the presence of CRPm and FM13 was lowered to 0.77 after five days of incubation. The percentage number of surviving plantlets increased considerably in treatments inoculated with PNA1 (80%) compared to CRPm-control treatments (0%) or FM13 treatments (20%).

Influence of anthranilate on growth of Pythium CRPm in vitro *and in the presence of cocoyam plantlets*

Since FM13 produces about 20 mg l^{-1} of anthranilate in GCY broth and since it was shown that the supernatants of FM13 inhibited the growth of CRPm

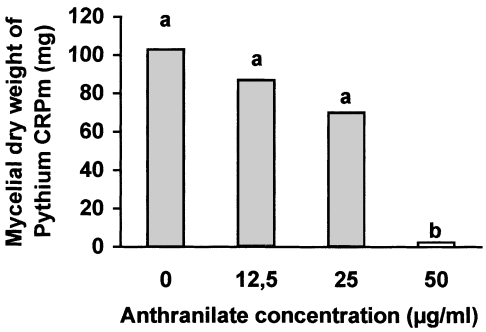

Figure 1. Effect of synthetic anthranilate on mycelial dry weight of *P. myriotylum* CRPm in potato dextrose broth. Values are means of four replicates and bars with different letter are significantly different at 5% level of probability using Duncan Multiple Range Test.

in PDB, we investigated the effect of the synthetic chemical anthranilate against CRPm in PDB and in the presence of cocoyam plantlets. Various anthranilate concentrations were evaluated against CRPm in PDB, liquid nutrient medium and in the soil in the presence of plantlets. Anthranilate concentrations influenced mycelial dry weight of CRPm (Figure 1). Mycelial dry weight was significantly reduced in PDB amended with 50 $\mu g\,ml^{-1}$ of anthranilate compared to unamended PDB. There was a steady decrease in mycelial dry weight as the concentration of anthranilate increased from 0 to 50 $\mu g\,ml^{-1}$. In liquid medium, plantlets inoculated with CRPm and treated with 50 $\mu g\,ml^{-1}$ or 100 $\mu g\,ml^{-1}$ anthranilate showed a significantly reduced DSI after three days of incubation (Table 3). However, this significant effect was lost when the treatments were incubated for five days. In soil experiments, the DSI was significantly reduced in anthranilate-treated plantlets to 0.6 and 0.5 for respectively 50 and 100 $\mu g\,g^{-1}$ of soil compared to 0.8 in plantlets treated with CRPm alone seven days after incubation. After 10 days of incubation, the DSI of plantlets treated with 50 $\mu g\,g^{-1}$ or 100 $\mu g\,g^{-1}$ of anthranilate were not significantly ($p = 0.05$) different from that of CRPm alone (Table 3).

Table 3. Effect of anthranilate on cocoyam root rot DSI in liquid Nutrient solution and volcanic soils of Cameroonian origin

Treatment	Disease severity index[1]			
	Nutrient solution[2,3]		Volcanic soil[3,4]	
	Days after transplanting plantlets			
	3	5	7	10
P. myriotylum alone	0.80 a	0.95 a	0.80 a	1.00 a
P. myriotylum + 50 µg/ml or g soil of anthranilate	0.60 b	0.90 a	0.60 b	0.85 a
P. myriotylum + 100 µg/ml or g soil of anthranilate	0.55 b	0.90 a	0.50 c	0.80 a

[1]Disease severity indices were calculated from ratings scores (0: no visible symptoms, to 4: death of the plantlets) as described by Liu et al. (1996): DSI = Σ(rating number × number of plantlets with the score)/(Total number of plantlets × highest rating).
[2]Liquid solution used was Gamborg et al. (1968) with pH 5.8 ± 0.1.
[3]Values within a column followed by the same letter are not significantly different at 5% level of probability, and are means of five replicates. The experiments were repeated once.
[4]Soil was steam-sterilised for 2 h at 121 °C, and allowed to stand for at least five days before using.

Effect of inoculum potential and time of transplanting of plantlets on the biological control activity of PNA1 and FM13 in volcanic soil

Tissue culture derived cocoyam plantlets were transplanted into volcanic soil infested with different levels of *Pythium* inoculum. DSI and surviving plantlets were recorded after 10 days of incubation. The inoculum density of CRPm and time of transplanting of plantlets affected the efficiency with which strains PNA1 and FM13 protected cocoyam against the pathogen (Table 4). Incubation of *Pythium* and bacterial strains for 24 h prior to transplanting cocoyam plantlets significantly enhanced the biocontrol activity of PNA1. Disease severity decreased from 0.9 in treatments with CRPm alone (750 propagules g^{-1} soil) to 0.31 in treatments inoculated with CRPm and PNA1 (Table 4). Percentage number of surviving plantlets increased significantly in the presence of PNA1 (Table 4). The presence of *P. aeruginosa* PNA1 in soil with 750 *Pythium* propagules g^{-1} improved the number of surviving plantlets to 75% from 0% in treatments with CRPm alone. Doubling the inoculum density to 1500 propagules g^{-1} of soil decreased the number of surviving plantlets in treatments with *P. aeruginosa* PNA1 to 20%. The highest number of surviving plantlets in treatments involving FM13 (20%) was observed for treatments with 750 propagules g^{-1} soil and incubated for 24 h (Table 4).

Table 4. Effects of inoculum densities and time of transplanting plantlets on the biocontrol activity of *P. aeruginosa* PNA1 and its mutant, FM13, against the cocoyam root rot disease in volcanic soil

Treatment	Inoculum[1] (prgules/g)	Transplanting time (h)	Disease severity index [2,3]	Surviving plantlets (%)[3,4]
P. myriotylum alone	750	0	1.00 a	00.0 e
		24	0.90 a	00.0 e
	1500	0	1.00 a	00.0 e
		24	1.00 a	00.0 e
P. myriotylum + PNA1	750	0	0.50 e	40.0 b
		24	0.31 f	75.0 a
	1500	0	0.60 d	10.0 d
		24	0.50 e	20.0 c
P. myriotylum + FM13	750	0	0.80 b	00.0 e
		24	0.75 c	20.0 c
	1500	0	1.00 a	00.0 e
		24	0.95 a	10.0 c

[1]prgules/g: propagules of *P. myriotylum* isolate CRPm per gram of soil.
[2]Observations were made daily for 10 days with score ratings of 0: no visible symptoms to 4: dead. DSI was calculated from scores as described by Liu et al. (1996): DSI = Σ(rating number × number of plantlets in the rating)/(Total number of plantlets × highest ratings). Experiments were repeated two times with five replicates per treatment combination.
[3]Values within a column followed by the same letter are not significantly different at 5% level of probability using Duncan Multiple Range Test.
[4]Values are proportions of surviving plantlets over initial number of plantlets transplanted into soil infested with CRPm.

Effect of PNA1 and phenazine-deficient mutants (FM13 and FM29) on biocontrol of Pythium CRPm *on cocoyam*

Soil artificially infested with 750 propagules g^{-1} of soil was inoculated with *P. aeruginosa* PNA1 or its phenazine-deficient mutants FM13 and FM29 and incubated for 24 h prior to transplanting cocoyam plantlets to investigate their effects on disease severity and percentage number of surviving plantlets. Inoculation of soil with *P. aeruginosa* PNA1 significantly reduced the incidence of *Pythium* root rot observed on cocoyam plantlets 10 days after transplanting (Table 5). The mutant strains deficient in phenazine production (FM13, FM29) suppressed the disease to a much lower extent than the wild-type strain. In soils that were not infested with CRPm, no significant difference in plant growth was observed between non-inoculated and inoculated plantlets. Therefore, bacterial inoculation had no detectable adverse effect on the plantlets. Percentage number of surviving plantlets increased from zero in CRPm-alone treatments to 75% in plantlets inoculated with PNA1 (Table 5).

Table 5. Biocontrol of *P. myriotylum* CRPm by *P. aeruginosa* PNA1 in volcanic soil of Cameroonian origin compared to its phenazine-deficient mutants[1]

Treatment	Disease severity index[2,3]	Surviving plantlets (%)[3,4]
P. myriotylum + PNA1	0.38 d	75.0 a
P. myriotylum + FM13	0.77 b	20.0 b
P. myriotylum + FM29	0.75 b	20.0 b
P. myriotylum alone	1.00 a	00.0 c

[1]Soil was steam-sterilised at 121 °C for 2 h and *P. myriotylum* CRPm was applied at 750 propagules g^{-1} of soil. Plantlets were transplanted 24 h after incubating CRPm and the appropriate bacterial strains. Each treatment combination was replicated five times and experiment repeated twice.
[2]DSI was calculated from score ratings (0: no visible symptoms, to 4: dead) as described by Liu et al. (1993): DSI = Σ(rating number × number of plantlets in the rating)/ (Total no. of plantlets × highest rating). DSI were recorded 10 days after transplanting cocoyam plantlets.
[3]Values within a column followed by the same letter are not significantly different at 5% level of probability using Duncan Multiple Range Test. Healthy control and treatments with bacteria only showed no visible symptoms of any disease.
[4]Values are percentages of surviving plantlets over initial population transplanted recorded 10 days after transplanting cocoyam plantlets.

Effect of tryptophan on the biocontrol activity of mutant FM13 against Pythium CRPm *in the presence of cocoyam plantlets*

Plantlets were transplanted in tryptophan-amended soil infested with CRPm and inoculated with FM13 fed with exogenous tryptophan to investigate the effect of tryptophan on the biocontrol activity of this bacterial strain and to confirm the role of phenazine in the antagonism of PNA1 against CRPm. We have shown before that phenazine production is completely restored when FM13 is grown in the presence of 100 $\mu g\,ml^{-1}$ tryptophan and that under these conditions, anthranilate is no longer excreted (Anjaiah et al., 1998). FM13 fed with tryptophan significantly reduced the incidence of the disease on cocoyam plantlets after 10 days (Table 6). Infested soil inoculated with FM13 not fed with tryptophan exhibited a DSI similar to treatments with CRPm alone or CRPm and tryptophan. Plantlets fed with tryptophan in soil not infested with *Pythium* did not show any significant difference in plant growth. Split-root experiments did not show any evidence of induced resistance elicited by the application of tryptophan (data not shown). Percentage surviving plantlets (60%) increased significantly in plantlets inoculated with tryptophan-fed FM13 compared to treatments with CRPm (0%) and FM13 without tryptophan (20%) (Table 6).

Table 6. Effect of tryptophan on the biocontrol activity of *P. aeruginosa* FM13 against *P. myriotylum* CRPm in volcanic soil

Treatment[1]	Disease severity index[2,3]	Surviving plantlets(%)[3,4]
P. myriotylum alone	0.95 a	00.0 d
P. myriotylum + FM13	0.90 a	20.0 c
P. myriotylum + FM13 + tryp	0.33 b	60.0 b
P. myriotylum + tryp	0.90 a	00.0 d
Tryp alone	0.00 c	100.0 a

[1]tryp: L-tryptophan; FM13: *P. aeruginosa* FM13.
[2]DSI was calculated from score ratings (0: no visible symptoms, to 4: dead) as described by Liu et al. (1996): DSI = Σ(rating number × number of plantlets in the rating)/(Total number of plantlets × highest rating). DSI were recorded 10 days after transplanting cocoyam plantlets.
[3]Values within a column followed by the same letter are not significantly different at 5% level of probability using Duncan Multiple Range Test. Healthy control and treatments with bacteria only showed no visible symptoms of any disease.
[4]Values are percentages of surviving plantlets over initial population transplanted recorded 10 days after transplanting cocoyam plantlets.

Discussion

Pseudomonas aeruginosa PNA1 suppressed *P. myriotylum* isolate CRPm *in vitro* and in soil in the presence of cocoyam plantlets and phenazine antibiotics (mainly phenazine-1-carboxylate and minor amounts of phenazine-1-carboxamide) contributed to the antagonistic effect. *P. aeruginosa* FM13, a tryptophan mutant of PNA1 that is deficient in phenazine production and secretes anthranilate (Anjaiah et al., 1998), only slightly reduced disease severity on cocoyam plantlets in nutrient solution or soil. Both PNA1 and FM13 significantly inhibited CRPm *in vitro*. This *in vitro* growth inhibition of CRPm by FM13 cannot be explained by anthranilate production alone. Five milliliter of FM13 supernatant, containing 20 $\mu g\,ml^{-1}$ of anthranilate (Anjaiah et al., 1998) strongly reduced the growth of CRPm when added to 50 ml of potato dextrose broth resulting in a final anthranilate concentration of 2 $\mu g\,ml^{-1}$ in the potato dextrose broth. However, Figure 1 shows that about 50 $\mu g\,ml^{-1}$ of synthetic anthranilate has to be added to potato dextrose broth to obtain a comparable growth inhibition. These results indicate that the supernatant of FM13 contains other unknown substances that have an inhibitory effect against CRPm *in vitro*.

Anjaiah et al. (1998) implicated phenazines in the antagonism of PNA1 against *Fusarium* wilt on chickpea and *Pythium* damping-off, but also found that FM13 was as effective as the parental strain PNA1 in suppressing *P. splendens* on bean (*Phaseolus vulgaris*). It was suggested that the effect of FM13 on *Pythium* could be explained by anthranilate production since synthetic anthranilate effectively controlled damping-off caused by *P. splendens* or *P. debaryanum*, but a definite role for *in situ* anthranilate production in the biocontrol activity of FM13 could not be established. The present study shows that FM13 is less effective than PNA1 in controlling *P. myriotylum* on cocoyam. FM13 had only a slight effect on disease severity, which is probably not due to anthranilate, since the same effect was observed with mutant FM29, which is deficient in phenazine production and does not excrete anthranilate. The residual protection observed for FM29 and FM13 might be due to unknown substances produced by both strains or could be explained by the fact that the strains were added to sterile soil. The reason why FM13 suppressed growth of *P. splendens* on bean but did not affect *P. myriotylum* on cocoyam is not clear. Anjaiah et al. (1998) suggested that secreted anthranilate antagonised *P. splendens* directly or that

sufficient quantities of tryptophan are present in the rhizosphere of bean to restore phenazine production by FM13. In the case of *P. myriotylum* on cocoyam, lack of enough tryptophan in the cocoyam rhizosphere to stimulate phenazine production could have affected the performance of FM13. Synthetic anthranilate added to the nutrient solution or volcanic soil at 50 or 100 $\mu g\ ml^{-1}$ or g^{-1} had a significant, but transient, effect on disease severity, indicating that anthranilate is less effective in controlling *P. myriotylum* root rot on cocoyam than in controlling damping-off caused by *P. splendens* or *P. debaryanum*. This is probably due to the fact that *P. splendens* and *P. debaryanum* only attack young plantlets while *P. myriotylum* is a much more aggressive pathogen which can also infect older plants. Our findings regarding efficiency of anthranilate to control plant pathogens, support results of Hamdan et al. (1991) and Ownley et al. (1991). Anthranilic acid inhibited growth of *G. graminis* var. *tritici in vitro* (Hamdan et al., 1991), but made only a minor contribution to the residual suppressiveness of phenazine-deficient mutants *in situ* (Ownley et al., 1991).

Experiments using PNA1 and its Tn*5* derivatives FM13 and FM29 demonstrated the involvement of phenazines in the biocontrol activity of PNA1 on the cocoyam root rot disease. The involvement of phenazines in biocontrol by PNA1 was further strengthened by the fact that FM13 strongly reduced disease severity when fed with tryptophan and applied to a soil treated with tryptophan. It was shown by Anjaiah et al. (1998) that addition of tryptophan to culture medium reduced the amount of anthranilate accumulated by FM13 and concurrently stimulated phenazine-1-carboxylate and phenazine-1-carboxamide production. Our results suggest that the tryptophan added to soil stimulated *in situ* phenazine production by FM13 and that this increased phenazine production resulted in a reduced disease severity. However, this can only be really proved by measuring *in situ* phenazine concentrations in tryptophan-amended soil. The addition of tryptophan to FM13 may positively regulate phenazine production (Anjaiah et al., 1998) or may simply increase cell density to a point that is sufficient to transcriptionally induce phenazine synthesis via homoserine lactone-mediated, quorum-sensing (Pierson et al., 1995; Stead et al., 1996; Latifi et al., 1996).

In addition to the involvement of phenazine in biocontrol activity of PNA1, this strain also affects the development of morphological structures such as sporangia by *P. myriotylum* CRPm when incubated in nutrient solution in the absence of cocoyam plantlets (data not shown). How PNA1 does this remains to be determined, but nutrient competition, parasitism or surfactant production cannot be excluded. Incubation of bacterial strains and *Pythium* CRPm for 24 h prior to planting cocoyam plantlets significantly enhanced the biocontrol activity of PNA1 against the pathogen in soil experiments. This probably allowed bacteria to interact with the pathogen in the absence of the cocoyam. Doubling of the inoculum density of CRPm significantly reduced the efficiency of PNA1 to control the pathogen. This is not surprising given the dose-response relationships established by Montesinos and Bonaterra (1996). This *Pythium* inoculum density probably surpassed the maximum proportion of pathogen that the biocontrol agent (PNA1) at the concentration used could inactivate as represented by the hyperbolic saturation relationship reported by Montesinos and Bonaterra (1996).

In conclusion, this investigation has shown that biological control of the most devastating disease of cocoyam is a possibility, but our results need to be confirmed in non-sterile naturally infected volcanic soil. Studies with phenazine-negative mutants indicated that the biocontrol activity of PNA1 against *P. myriotylum* in the presence of cocoyam plantlets may be due to phenazines. In addition, FM13 which significantly suppressed *P. splendens* and *P. debaryanum* on bean and lettuce inhibited *P. myriotylum in vitro* but only slightly reduced disease severity in soil. The study also showed that a high inoculum concentration of *P. myriotylum* could significantly affect the biocontrol efficiency of PNA1 against the pathogen. It is not known, however, at what concentrations *P. myriotylum* is present in naturally infected soil.

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