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Detection, isolation and baseline characterization of indigenous *PhlD*$^+$ fluorescent *Pseudomonas* species for the control of bean root rot and coffee wilt disease in Uganda

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ABSTRACT
Fluorescent *Pseudomonas* spp. which produces antibiotics such as 2,4-diacetylphloroglucinol (DAPG/phl) have biocontrol activity against many important soil-borne fungal pathogens. We isolated several *Pseudomonas* spp. carrying genes for DAPG synthesis (phlD) from coffee and bean rhizospheres from different sites in Uganda. RFLP analysis using *Hae* III and *Msp*I revealed one dominant genotype in the coffee fields and two dominant genotypes in the bean fields. The three genotypes were unique and novel. *In vitro* antifungal assay against *Fusarium xylaroides, Fusarium oxysporum, Fusarium solani* and *Pythium ultimum* revealed strong inhibition by *phlD*+ isolates against all four pathogens. However, some *phl*D-negative isolates from coffee showed strong inhibition against the *Fusarium* spp. Furthermore screen-house experiments showed good disease control against coffee wilt disease (*Fusarium xylaroides)* and bean root rot (*Pythium ultimum).* The bio-control potential of the *phl*D+ isolate against the two diseases appeared to increase with time compared to chemical control which reduced with time.

Keywords: 2,4-diacetylphloroglucinol, biocontrol, coffee wilt disease, fungal root rots, fluorescent *Pseudomonas* bacteria, *phl*D+ gene, Uganda

RÉSUMÉ
Les souches de Pseudomonas fluorescentes produisant des antibiotiques tels que le 2,4-diacétylphloroglucinol (DAPG/phl) ont une activité de lutte biologique contre de nombreux agents pathogènes fongiques du sol importants. Nous avons isolé plusieurs souches de Pseudomonas portant les gènes de synthèse du DAPG (phlD) dans les rhizosphères du café et des haricots provenant de différents sites en Ouganda. L'analyse RFLP à l'aide de Hae III et MspI a révélé un génotype dominant dans les champs de café et deux génotypes dominants dans les champs de haricots. Les trois génotypes étaient uniques et nouveaux. Les essais antifongiques in vitro contre *Fusarium xylaroides, Fusarium oxysporum, Fusarium solani et Pythium ultimum* ont révélé une forte inhibition par les isolats phlD+ contre les quatre agents *pathogènes*. Cependant, certains isolats phlD-négatifs du café ont montré une forte inhibition contre les Fusarium spp. De plus, des expériences en serre ont montré une bonne maîtrise des maladies, notamment la maladie de flétrissement du café (*Fusarium xylaroides*) et la pourriture des racines des haricots (Pythium ultimum), avec l'isolat phlD+. Le potentiel de lutte

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biologique de l'isolat phlD+ contre ces deux maladies semblait augmenter avec le temps, contrairement au contrôle chimique qui diminuait avec le temps.

Mots-clés: Bactéries Pseudomonas fluorescentes ,Lutte biologique, maladie de flétrissement du café, pourritures racinaires fongiques, , 2,4-diacétylphloroglucinol, gène phlD+, Ouganda

Introduction

Common bean (*Phaseolus vulgaris* L.) is a vital staple food and cash crop in Africa, providing the main source of affordable protein for the majority of resource poor rural and urban households, and for institutions such as hospitals, schools, and the army (Grisley, 1990; Pachico, 1993). *Pythium* root rot is the most destructive soil-borne disease of beans causing up to 70% yield loss in beans in eastern and central Africa (Buruchara *et al.,* 2002; Otsyula *et al.,* 2002). *Pythium ultimum* var. *ultimum* is the most prevalent *Pythium* species causing root rot in Uganda (CIAT, 2006).

Coffee is one of Uganda's main export commodities (Wasukira, 2004) and Uganda is ranked second in Africa and seventh in the world in coffee production. Two main types of coffee are grown in Uganda, that is robusta coffee (*Coffea robusta*) and arabica coffee (*Coffea arabica*). Robusta coffee accounts for about 90 percent of Uganda's coffee production and raises 80% of revenue (Musoli *et al.,* 2001). Coffee wilt disease (CWD) also known as tracheomycosis is the biggest threat to Robusta coffee production in Uganda since the early 1990s. CWD kills all infested plants and spreads at an alarming rate (NARO, 2001).

In the perspective of a reduction in the use of fungicides it is imperative to find alternatives (Adipala *et al.,* 2001; Delany *et al.,* 2001; CIAT, 2006). The development of commercially viable biocontrol agents against CWD and bean root rot is a promising approach and strains of fluorescent *Pseudomonas* spp have shown ability to inhibit a broad spectrum of plant pathogenicfungi and control a variety of root and seedling diseases in many agricultural crops (Bergsma-Vlami *et al.,* 2005). The biocontrol ability of these strains has been directly correlated with production of antibiotics such as 2, 4-diacetylphloroglucinol (DAPG), phenazines (Phz), pyrrolnitrin (PRN), pyoluteorin (PLT) and hydrogen cyanide (HCN) (Thomashaw and Weller, 1988; Raaijmakers *et al.,* 2002). The genetic loci responsible for the biosynthesis of these antibiotics have been well characterised and the sequences are available (Bangera and Thomashow, 1999; McSpadden *et al.,* 2000). It is therefore possible to detect and isolate candidate biocontrol agents based on the presence of antibiotic biosythesis genes in their genome.

The broad-spectrum antibiotic DAPG is one of the most important antibiotics produced for biocontrol *Pseudomonas* and is implicated in the suppression of several fungal root diseases in suppressive soils (Raaijmakers *et al.,*2002; Ramette *et al.,*2003; Weller *et al.,*2003). One of the genes responsible for the biosynthesis of DAPG in *Pseudomonas* is *phl*D and is used widely as a genetic marker for identifying and studying DAPG-producing pseudomonads (Bangera and Thomashow, 1999; McSpadden *et al.,* 2000).

Antibiotic-producing Pseudomonas have been extensively studied for their use as biocontrol agents in agriculture. Yet little is currently known about their presence and diversity in Uganda. Also their ability to control major crop diseases in Uganda has not yet been investigated. The objective of this study was to test the hypothesis that antibiotic producing *Pseudomonas* spp. are present in Uganda and have potential for use as biological control agents of *Pythium* root rot of beans and CWD. Specifically we tested the

presence of 2,4 DAPG-producing *Pseudomonas* spp. (*phlD*+) fluorescent *Pseudomonas* spp. in Ugandan soils and investigated their genetic diversity. We tested the inhibitory potential of those isolates against *Fusarium xylaroides, Fusarium oxysporum, Fusarium solani* and *Pythium ultimu in vitro* and against CWD *(Fusarium xylaroides)* and bean root rot (*Pythium ultimum)* in the screen house.

Materials and Methods

Sites for coffee and bean rhizosphere sampling. All pseudomonas strains in this study were isolated from rhizospheres of coffee and beans from sites around central Uganda because of their proximity to the laboratory and relatively high incidences of the CWD and root rots of beans in these areas. For coffee, three of the sampling sites were located at the Coffee Research Institute (CORI) at Kituuza in Mukono District, and included; Block 13 where CWD was first observed at CORI, a tetraploid coffee field with mild incidences of CWD and a germplasm trial block with differential infection of CWD

One site was in an infested area at a second coffee research site located at Kawanda Agricultural Research Institute (KARI) in Wakiso district. Eight (8) of the sites were farmers' fields located in Kampala and Wakiso districts. Most of these were intercropped with beans and/or bananas. The last two sites were at the Makerere University Agricultural Research Institute at Kabanyolo (MUARIK) where coffee has been grown as a monoculture for several years. Thus, a total of 14 coffee fields were sampled.

Similarly for beans, root samples were harvested from both experimental and farmers' fields located around Kampala and Wakiso Districts. The experimental site chosen was at MUARIK and consisted of bean plots planted with the variety K132 in newly opened fields not previously occupied by beans. Two bean crops were grown at this site with plantings in October 2004 and March 2005. All laboratory procedures were carried out at the biotechnology laboratories of the Department of Crop Science in Makerere University and the National Biotechnology Centre in KARI.

Bacterial isolation culture conditions. Roots samples from either coffee or bean fields were obtained randomly. For beans, most of the root sampling took place at flowering and involved careful digging out of the intact root system which was quickly placed in plastic bags. Similarly for coffee, 15cm of the lateral root system were carefully dug and 500mg of small sized lateral roots with several root hairs placed in plastic bags. All samples were transported to the laboratory within three hours after sampling. A total of 10 to 30 root samples of beans and coffee were collected fortnightly over a 10 month period (September 2004 and July 2005) from these study fields.

In the laboratory, the roots were separated manually from loosely adhering soil and individual roots cut with a clean razor blade and placed into test tubes with 7.5ml sterile distilled water. Thereafter, the tubes were vortexed eight times every 15sec. to make rhizosphere root washes. Samples were prepared for PCR processing according to a protocol by McSpadden *et al.* (2001). Briefly, two 96-well microtiter (MT) plates were prepared and labeled as follows; A wash plate, where MT wells were filled with 200 µl of sterile distilled water using a micropipettor, or as Replica media plate filled with 200 µl of King's Media B broth containing 40µg/ml ampicillin and 13µg/ml chloramphenical (KMB++) per well. One hundred microlitres of each rhizosphere wash was transferred from the test tube into a MT well in the first column to form a dilution of each sample. A serial dilution was done by pipetting 100 µl of rhizosphere wash in column 1 and transferring it to the next well (column 2), this was repeated until the 12^{th} dilution (column 12) per sample. Fifty microlitres of rhizosphere wash from each dilution (well) was then transferred into the corresponding

well in the media MT plate. The wash plates were sealed and stored at -80°C, while the media MT plates were incubated at room temperature (RT) for 48hr. Replica glycerol MT plates were prepared with 100µl of 35% glycerol in each well. One hundred microlitres of culture was transferred from wells in the media plates into corresponding wells in the glycerol MT plates. Glycerol MT plates were sealed and stored at -80°C. Culture MT plates were frozen at -80°C for a minimum of 1hr and then stored in a -20°C freezer. All strains were routinely maintained on KMB agar.

Detection of *phlD*$^+$ gene using PCR amplification. The *phlD* gene was amplified with specific primers BPF2 and BPR4 described previously (Raaijmaker *et al.*,1997; McSpadden Gardener *et al.*,2001) (Table 1).

Table1. Primer sequences used for PCR

PRIMER	SEQUENCE	Tm °C	Product length (bp)
B2BF	5'-ACC CAC CGC AGC ATC GTT TAT GAG C-3'	65.6	629
BPR4	5'-CCG CCG GTA TGG AAG ATG AAA AAG TC-3'	63.4	

McSpadden *et al*., 2001

Thawed culture from the media/culture MT plates was used as template for whole cell PCR according to the protocol (McSpadden Gardener *et al.*,2001). PCR amplification were carried out in 25 µl of reaction mixture containing 2.5 µl of culture template, 1X DNA polymerase buffer, 1.5 mM $MgCl_2$, 2mM each of dGTP, dATP, dCTP and dTTP, 25 pmoles of each primer and 1.5 units of *Taq* DNA polymerase. The PCR conditions consisted of a 35-cycle event performed using the following cycling parameters: 94°C for 1 min. denaturation, 60°C for 1 min. annealing and 72°C for 1 min. elongation, with a soak temperature of 4°C. Amplification products were electrophoresed through a 1.5% agarose gel in 1x TAE for 1 hr at 100V. The gel was then stained with ethidium bromide, visualized under UV light and gel images taken. Wells which gave a positive PCR product were recorded and percentage of plants which gave positives calculated.

Characterisation of detected *phlD* gene using Restriction Fragment Length Polymorphism (RFLP). RFLP was performed using the enzymes *HaeIII* and *MspI (*McSpadden *et al.,* 2001). Eight microlitres of positive PCR product was digested in a total volume of 20µl of 1x sample buffer with 5 units of a single restriction enzyme. Reactions were incubated at 37°C for 4 hrs and digestion products were separated on 2.5% agarose gels in 1x TAE for 1 hr at 80 V. Gels were then stained with ethidium bromide and banding patterns visualized under UV light. Gel images were then taken and printed out. The banding patterns obtained were compared to those previously published by McSpadden *et al.* (2000; 2001; 2005) and McSpadden and Weller (2001).

Recovery, isolation and purification of *phlD*$^+$ fluorescent pseudomonads. Isolation and purification of *phlD*$^+$ fluorescent pseudomonads was done using the streak plate method described by McSpadden (unpublished 2002). Petri dishes were filled with KMB++ and pre-labelled with sample name/number and date. Glycerol stocks were removed from -80 freezer and wells which gave positive PCR products identified. Ten microlitres of glycerol from a positive well was scraped onto a plate and streaked out with a sterile loop. The plate was incubated at room temperature (RT) for three days. Using a sterile tip, small clear bacterial colonies were picked and streaked on KMB media without antibiotics and incubated at RT for three days. About 14 colonies were picked from each plate, subcultures were made on KMB until pure single colonies whose morphology and colour was consistent with the initial colony were obtained. The isolated colonies were then amplified through whole cell PCR with the primers described in

Table 1. Positive products were digested with *Hae*III, and *Msp*I to confirm their genotypes. Some of the colonies isolated were also stored in 80% glycerol as glycerol stocks at -80^{o}C.

***In vitro* inhibition assays to determine bio-control ability of indigenous p*hlD*$^{+}$ *Pseudomonas* spp.** In order to evaluate the antagonistic activity of the recovered *phl*D+ pseudomonas against key *Fusarium* species on coffee and *Pythium ultimum* root rot pathogen, growth inhibition assays were carried out. For coffee, inhibition assays were against the CWD pathogen (*Fusarium xylaroides)* and two other related Fusarium species, namely, *F. oxysporum* and *F. solani (*Rutherford *et al.,* 2003). All the Fusarium isolates were obtained from a collection used for epidemiological studies by the National Coffee Research Institute (CORI).

For beans, ability to inhibit *Pythium ultimum* was investigated using strain MS 61 provided by Dr. Robin Buruchara of CIAT-Kawanda. Prior to all experiments, sub-cultures of *P. ultimum* were maintained on water agar for one week while *Fusarium* species were sub-cultured on Special Nutrition Weak agar (SNA).

Inhibition assays were performed using the central disk method (McSpadden *et al.,* 2005). Agar plugs (6mm) containing the fungal mycelium, taken from the growing edge of each culture were placed in the centre of fresh potato dextrose agar (PDA) plates and incubated at room temperature for 24 hours. Ten microlitre aliquots of the *phl*D+ bacterial inoculum from three different bacterial strains were inoculated at three equidistant positions along the perimeter of the assay plate. Two plates with no bacteria inoculum were included as controls for each pathogen. Assay plates were incubated at room temperature. Each treatment was replicated three times and the entire assay was repeated three times.
Fungal growth was assayed when the growth on the control plates extended the full radius of the plate. Two measurements were made: distance from the edge of the plug to the growing edge of the fungus (X), and the distance from the edge of the bacterial growth to the growing edge of the fungus (Y). Inhibition index (I) was calculated as: I=Y/X+Y (McSpadden *et al.,* 2005). Inhibition indices were recorded ranging from -1 (no inhibiton and fungus overgrew the bacterial inoculum) to 1 (total inhibition of fungal growth**).** Inhibition indices were subjected to analysis of variance (ANOVA) using GenStat Release 9.1, ninth edition computer package.

Evaluation of the *phl*D+ *Pseudomonas* spp. antagonistic biocontrol activity in the screenhouse. The screen house experiment against coffee wilt disease (*Fusarium xylaroides)* was carried out at CORI while the one against pythium root rot was done at KARI. The procedures for the two screen house experiments are briefly described below:

Bean experiment. *Pythium ultimum* (MS61) was incubated on PDA for two days and then inoculated into sterile millet grains. One kilogram of millet was mixed with 1/2 MS61culture plates and left incubated at room temperature for two weeks in the dark. Ten wooden trays (40cm x 60cm) were filled with sterile soil. Two trays were assigned per treatment and labeled as follows; MS61 for *Pythium ultimum* (MS61)-infested soil, MS61+pseudo., for MS61-infested soil inoculated with *phl*D+ isolates, MS61+chem-MS61-infested with Ridomil (50g/ml) and *phl*D+ isolates. Two controls were included, i.e., a sterilized soil and one inoculated with *phl*D+ isolates only. Inoculum for *phl*D+ isolates was prepared by culturing on KMB^{++} and incubating at RT for four days. The experimental trays were then inoculated with the respective pathogens. A fungicide was also added in trays where this treatment was required. Pythium was added, at a ratio of 1:8 (inoculum: soil) mixed thoroughly and left for one week. The bacterial suspension concentration was adjusted to 3x10^{6} cfu ml^{-1}

using a hemocytometer before introduction into the soil in targeted trays (marked pseudo and MS61+pseudo). They were incubated for one week before planting. Ridomil was mixed and drenched into the soils marked MS61+chem.

Two bean varieties, K132 (susceptible) and RWR719 (resistant) were planted in all the trays at a spacing of 10 cm between rows and 7 cm within rows, giving 10 seeds per row with three rows per variety per tray giving a total of 60 plants per tray. The trays were randomly arranged on the screen house bench. At nine days the germination counts for each treatment were observed and recorded. After three weeks, all the plants were carefully uprooted washed and scored for *Pythium* root rot damage using a 1-9 pythium root rot scale (Table 2) developed by CIAT scientists (Dr. Robin Bruchara, 2006 *personal communication*).The procedure was repeated three times. Data were recorded and subjected to ANOVA using GenStat Release 9.1, ninth edition computer package.

Coffee experiment. *Fusarium xylaroides* was incubated on PDA for two days and then inoculated into a mixture of sterile forest soil and lake sand (ratio 3:1). The soil was incubated for four weeks at room temperature. Sterile forest soil/sand mixture was divided into five equal amounts and marked F.xy for *Fusarium xylaroides*-infested soil, F.xy+pseudo for *Fusarium xylaroides*-infested soil inoculated with *phl*D+ isolates, F.xy+chem-*Fusarium xylaroides* –infested soil drenched with Benomyl (5g/l), pseudo- *phl*D+ isolates only. Soil marked F.xy and F.xy+pseudo was inoculated with the *Fusarium* -infested soil mixture at a ratio of 1:5 (infested soil: sterile soil), mixed thoroughly. All soil was potted into labeled black polyethene pots (5cm radius x 18cm height). The pots with *Fusarium*-infested soil were left to establish for one week. Indigenous *phl*D+ isolate inoculum was raised on KMB for four days. Six month old robusta seedlings clone (1s/6) were selected for the study. Healthy seedlings were carefully uprooted and the soil carefully removed from their roots and gently washed under running water. The seedlings were then carefully transplanted into the experimental pots. Thirty –six (36) seedlings were potted per treatment, with one seedling per pot. The bacterial suspension ($3x10^6$ cfu ml^{-1}) was drenched into pots labeled F.xy+pseudo and pseud. F.xy+chem. marked pots were drenched with Benomyl. The pots were then randomly arranged on the floor of the screen house and monitored for symptoms of CWD at 30-day intervals for 90 days (Hakiza *et al.*, 2001). Disease severity was scored based on a scale of 1 to 4 (Table 2) developed by CORI scientists (Dr. Hakiza J.G., 2005 *personal communication*). Data were recorded on incubation period that is time from inoculation to the appearance of the first symptoms. The CWD incidence and severity were also recorded.

Table 2. Assessment scale to determine extend Coffee wilt disease caused by *Fusarium xylaroides* in screen house experiments carried out at the Coffee Research Institute, Uganda

Scale	Description
0	No disease or no detectable leaf or other symptoms
1	Initial disease stage, leaves curl inwards some wilting or yellowing may occur. Up to 25% of foliage is affected
2	Die back and defoliation/yellowing affecting up to 50% of the canopy
3	Die back and defoliation/yellowing affecting up to 51-99%
4	Seedlings drying (100%) or dead

Table 3. Estimated recovery rate of *phlD*$^+$ pseudomonas populations in the rhizosphere of coffee and beans

Year	Crop	Site	Number of plants sampled	% roots detected with *phlD*$^+$ pseudomonas
2004	Beans	Kibuli (KAMPALA)	12	16.70
	Beans	Matuga (WAKISO)	30	6.67
	Beans	Mpererwe (KAMPALA)	20	6.30
	Beans	MUARIK (WAKISO)	30	0.00
	Coffee	Matuga (WAKISO)	16	0.00
	Coffee	Mpererwe (KAMPALA)	12	0.00
	Coffee	Western field MUARIK; (WAKISO)	12	16.70
	Coffee	Eastern field.MUARIK (WAKISO)	12	25.00
	Coffee	Germplasm under field trial (CORI)	20	60.00
	Coffee	Block 13 (CORI,MUKONO)	12	58.33
	Coffee	Tetraploid coffee field trial (CORI,MUKONO)	12	33.33
	Coffee	Infested Coffee (KARI,WAKISO)	10	20.00
2005	Beans	Kibuli (KAMPALA)	16	25.00
	Beans	Matuga (WAKISO)	30	3.33
	Beans	Mpererwe (WAKISO)	20	5.00
	Beans	MUARIK (WAKISO)	40	7.50
	Coffee	Matuga (WAKISO)	20	15.00
	Coffee	Mpererwe (WAKISO)	15	13.33
	Coffee	Eastern field (MUARIK)	20	50.00
	Coffee	western field (MUARIK)	20	25.00
	Coffee	Germplasm under field trial (CORI, MUKONO)	20	75.00
	Coffee	Block 13 (CORI, MUKONO)	12	50.00
	Coffee	Tetraploid coffee field trial (CORI, MUKONO)	12	66.67
	Coffee	Infested Coffee (KARI,WAKISO)	15	46.67

RESULTS

Detection of *phlD*$^+$ pseudomonads in coffee and bean rhizospheres. Strains of *Pseudomonas* carrying the *phlD* gene were detected by the presence of 629 bp band indicative of *phl*D+ pseudomonas isolates.

Diversity of *phlD*$^+$ genotypes. Restriction analysis of the *phl*D+ isolates from beans using *Msp1* enzyme revealed fragments of approximately 100bp, 200bp and 300bp in samples 1, 2, 4, 5, 6, 7 and 8. Digestion with *Hae* III produced two different band patterns. Sample 1 fragment of size 200bp was observed, whereas for samples 2, 3 and 4, fragment sizes of 200bp and 400bp were seen. These patterns do not correspond to any previously published genotype profile (McSpadden *et al.*, 2000; 2001; 2005). Thus, the isolates appear to be unique genotypes. Similarly, digestion of all 20 *phl*D$^+$ isolates from coffee with *HaeIII* revealed a uniform finger-print made of fragments approximately 300bp, 200pb,100bp and one fragment less than 100bp.

Digestion with *MspI* showed three different band patterns. The pattern observed in sample 1 had approximately 200bp and 400bp fragments, sample 2, 8 and 9 had one fragment less than 100bp while samples 3, 6 and 7 had fragments of approximately 200bp, 150bp and below 100bp. These also did not correspond to any known genotype profile (McSpadden *et al.,* 2000; 2001; 2005). Therefore, the isolates from coffee also appear to be unique.

Recovery of *Phl*D+ *Pseudomonas* isolates . In 2004, between 0 to 60% of harvest coffee root samples harboured measurable of *phl*D+ pseudomonas isolates while in 2005, slightly higher numbers of coffee roots (13.33 to 75%) were positive for *phl*D+ isolates. The highest number of samples detected with *phl*D+ occurred in 2005 in an experimental coffee field at CORI (Table 3). The plot was a germplasm field trial with mild occurrence of CWD (data not presented). No *phl*D+ pseudomonas was detected in coffee roots from the two farmers' gardens at Matuga and Mpererwe in 2004. But in 2005, up to 15% of the coffee roots were observed to harbour high populations of *phl*D+ pseudomonas isolates (Table 4). The farmers' fields are typical subsistence coffee farms found in central Uganda where the plant is grown as an intercrop with beans and bananas. In contrast, almost all bean gardens harboured much lower populations of *phl*D+ isolates. The percent detection of *phl*D+ isolates ranged from 0 to 25% (Table 4). Also, like coffee higher numbers of roots tested positive for *phl*D+ pseudomonas in 2004 than in 2005 (Table 4). In both years, the bean farm in Kibuli (sub-urban location) consistently gave the highest roots testing positive with *phl*D+ *Pseudomonas* isolates (Table 3).

In vitro inhibition assays. All antibiotic producing strains were tested *in vitro* against *P. ultimum, F. xylariodes, F.oxysporum* and *F.solani* (Table 4).

Table 4. Inhibition of *Fusarium xylaoides, F. oxysporum and F.solani* by representative isolates of the Pseudomonas from the rhizospheres of coffee tree

		Inhibition Index		
Isolate	*phl*D+[a]	*F.xylaroides*	*F.oxysporum*	*F.solani*
1	-	0.24	0.15	0.22
2	+	0.31	0.24	0.21
3	+	0.27	0.16	0.07
4	+	0.31	0.27	0.21
5	-	0.48	0.33	0.11
6	-	0.26	0.03	0.07
7	-	0.42	0.12	0.17
8	-	0.57	0.17	0.13
10	-	0.46	0.14	0.11
11	+	0.56	0.19	0.19
13	+	0.51	0.06	0.13
14	+	0.58	0.13	0.18
15	-	0.45	0.04	0.09
16	+	0.48	0.09	0.18
17	-	0.42	0.12	0.14
18	+	0.44	0.11	0.28
19	+	0.50	0.11	0.24
20	+	0.56	0.12	0.23
Mean		0.43	0.14	0.17
LSD $_{(5\%)}$		0.2108	0.1351	0.128
CV%		29.5	56.7	46.8
F. prob.		0.011	0.008	0.054

[a] Presence (+) or absence (-) of the phlD gene

Some *phl*D- isolates were included for comparisons. The *in vitro* assays showed variable antagonistic ability of *phl*D+ isolates against the four *Fusarium* species. The *phl*D+ isolates from coffee were highly effective against all the *Fusarium* spp with high inhibition indices observed for isolates 11, 14 and 20 (Table 4). Isolates 11, 14 and 20 that are *phl*D+ had the highest inhibition indices, while six and three showed very low indices. Interestingly, one *phl*D- isolate from coffee also displayed quite a high inhibitory activity against the *Fusarium* spp. This isolate numbered 8 displayed an inhibition index of up to 0.57 (Table 5). Nonetheless, the inhibition effect of *phl*D+ isolates against *F. xylaroides* and *F. oxysporum* ($P<0.05$) against *F. solani* ($P= 0.05$) were generally higher than for *phl*D- isolates.

Generally, all the *phl*D+ bacteria isolated from beans displayed very low inhibition indices when used to inhibit *P. ultimum.* This ranged from 0.061 to 0.184 (Table 5). The highest inhibition index was shown by isolate 1 recovered from a bean field in Kibuli, a sub-urban area. Unlike in coffee, none of the phlD-negative isolates displayed any inhibitory activity and were completely overgrown by *P. ultimum. F. xylariodes, F. oxysporum* and *F. solani* (Table 5).

Effect of *phl*D$^+$ isolates on Pythium root rot and CWD. There were significant differences ($P<0.05$) in percentage germination at three and nine weeks among treatments in variety CAL96 (Table 6).

There was no significant difference among treatments in varieties RWR at three weeks, CAL96 and RWR718 at six weeks and RWR718 at nine weeks. Germination percentage was significant between the two varieties. Interaction between treatments, weeks and days after inoculation was also significant ($P<0.05$). Significant differences ($P<0.05$) in disease severity were observed among treatments and in the three varieties CAL96 and RWR718 at three weeks after inoculation (Table 7).

Table 5. Inhibition of *Pythium ultimum* by representative isolates of the *Pseudomonas* from the rhizospheres of bean plants.

Isolate	*Phl*D+[a]	**Inhibition index against *P ultimum***
1	+	0.18
2	+	0.07
3	+	0.01
4	+	0.06
5	-	-0.63
6	-	-0.67
7	-	-0.59
8	-	-0.60
9	-	-0.44
10	-	-0.37
11	-	-0.58
12	-	-0.15
13	-	-0.13
Mean		-0.29
$LSD_{5\%}$		0.3137
CV%		100.8
F. prob		<0.001

[a] Presence (+) or absence (-) of the phlD gene

Table 6. Effect of *Phl*D+ *Pseudomonas* on germination count of bean varieties RWR719 and CAL96) in the presence of Pythium MS61

	%Germination						Mean
	3 weeks		6 weeks		9 weeks		
Treatment	CAL96	RWR719	CAL96	RWR719	CAL96	RWR719	
ms61	58	72	72	77	59	62	67
ms61+chem	82	82	80	76	54	60	72
ms61+pseud	61	73	67	88	49	48	64
Pseud.	80	77	72	82	57	60	71
Sterilized soil alone	72	81	81	83	66	61	74
Mean	71	77	74	81	57	58	69.7
CV%	14.4	14.1	16.3	11.7	14.3	19.3	14.9
$LSD_{5\%}$	12	NS	NS	NS	10	NS	NS
F-probability	<0.001	0.416	0.235	0.183	0.025	0.191	0.130

NS-Not significant at 5% level.

There was no significant difference among treatments in varieties RWR at three weeks, CAL96 and RWR718 at six weeks and RWR718 at nine weeks. Germination percentage was significant between the two varieties. Interaction between treatments, weeks and days after inoculation was also significant ($P<0.05$). Significant differences ($P<0.05$) in disease severity were observed among treatments and in the three varieties CAL96 and RWR718 at three weeks after inoculation (Table 7).

Table 7. Cumulative effect of *Phl*D+ pseudomonas on severity of bean root rot (*Pythium* MS61) on varieties RWR719 and CAL96

	Disease severity (0-9scale)						Mean
	3 weeks		6 weeks		9 weeks		
Treatment	CAL96	RWR719	CAL96	RWR719	CAL96	RWR719	
ms61	3.	1	6	1	8	1	3
ms61+chem	3	1	8	1	9	1	4
ms61+pseud	2	1	3	1	3	1	2
Pseud.	1	1	3	1	3	1	2
Sterilized soil alone	1	1	1	1	1	1	1
Mean	2	1	4	1	5	1	2
CV%	70.00	26.20	40.70	16.30	31.10	18.50	46.80
$LSD_{5\%}$	0.514	0.104	0.614	NS	0.524	NS	0.394
F-probability	<0.001	<0.001	<0.001	0.006	<0.001	0.304	<0.001

ms61= Pythium MS61, ms61+chem.= Pythium MS61+ chemical control (Ridomil), ms61+pseudo= Pythium MS61+ *phl*D+ , solates from beans , pseudo= *phl*D+ isolates from beans, Control=No Pythium MS61 or *phl*D + isolates from beans. NS-Not significant at 5% level.

There was a significant difference between treatments in CAL96 at both six and nine weeks. At six and nine weeks disease severity was highest (7 and 8) in trays where chemical treatment was applied. The resistant variety RWR719 did not develop any *Pythium* root rot symptoms. The root system of RWR719 remained intact and white in colour which was typical of the healthy root system. CAL96 roots from trays treated with MS61 and *Pseudomonas* isolates were healthier than CAL96 roots from trays treated with MS61 with no *Pseudomonas* isolates. Good control of *Pythium* rot was achieved in treatments where *phl*D+ pseudomonas had been incorporated in the soil. Typically, the roots of variety CAL96 showed dark brown lesions at three weeks which increased at six weeks, and were severe at nine weeks. At nine weeks CAL96 planted in MS61 alone were severely macerated, and had little or no root hairs and nodules. Treatments where the *phl*D+ isolates were incorporated in soil exhibited lower root rot severity than those with a fungicide (Table 8). The disease severity in the chemical treatments appeared to reduce with time. The effectiveness of *phl*D+ Pseudomonas was particularly consistent at six and nine weeks compared to treatments where a fungicide had been added. Overall, the Pseudomonas gave a good control of the pythium root rot compared to fungicide treatment in the nine weeks. After six and nine weeks, the disease severity in CAL96 in the fungicide treatments increased significantly whereas where *Pseudomonas* was added severity stabilised, giving an indication that the fungicide effect was wearing off or degrading with time.

For coffee, symptoms of wilting (CWD) first appeared after 40 days. The disease incidence was highest in the seedlings treated with *Fusarium xylaroides* alone. Disease incidence was higher in seedlings treated with fungicide in comparison to seedlings treated with *Pseudomonas*. Over the 100-day trial period, disease incidence increased at a faster rate in the fungicide treatments than in pseudomonas treatments. The effect of the treatments on the disease incidence was significant at days 90 and 100 implying that time influenced the performance of various treatments. Generally, the *phl*D+ *Pseudomonas* spp. appears to control CWD (Table 8).

Table 8. Cumulative effect of *Phl*D+ *Pseudomonas* on incidence of Coffee wilt disease (*F.xylaroides*)

	Cumulative Disease incidence								Mean
Treatment	30 days	40 days	50 days	60 days	70 days	80 days	90 days	100 days	
F.xy	0.00	12.50	15.62	21.88	25.00	28.12	28.12	34.38	20.70
F.xy+chem	0.00	3.12	3.12	12.50	18.75	15.62	37.50	46.88	17.19
F.xy+pseud	0.00	0.00	3.12	3.12	12.50	21.88	34.38	40.62	14.45
Pseud.	0.00	0.00	0.00	0.00	0.62	1.88	0.62	1.25	0.55
Mean	0.00	3.9	5.5	9.4	14.2	16.9	25.2	31.1	13.22
CV%	0.0	240.0	230.9	141.4	91.6	53.6	48.7	43.8	85.0
$LSD_{5\%}$		14.44	19.46	20.43	14.20	13.93	18.88	20.99	15.78
F-probability		0.243	0.350	0.138	0.105	0.009	0.004	0.003	0.005

F.xy=Fusarium xylaroides, F.xy+chem.= Fusarium xylaroides+chemical control (Benomyl), F.xy+pseudo= Fusarium xylaroides+ indigenous *phl*D+ isolates, pseudo= indigenous *phl*D+ isolates

Disease severity was highest in seedlings treated with *Fusarium xylaroides* alone (Table 8), followed by fungicide treated seedlings. The results indicate control of both CWD incidence and severity was achieved by the *phl*D+ *Pseudomonas* isolates.

Table 9. Cumulative effect of *Phl*D+ *Pseudomonas* on severity of Coffee wilt disease (*F.xylaroides*)

	Cumulative Disease severity								Mean
Treatment	30 days	40 days	50 days	60 days	70 days	80 days	90 days	100 days	
F.xy	0.00	0.25	0.25	0.75	1.75	1.25	1.25	1.25	0.844
F.xy+chem	0.00	0.00	0.25	0.25	0.50	0.75	1.25	1.50	0.562
F.xy+pseud	0.00	0.00	0.00	0.25	0.25	0.50	1.00	1.25	0.406
Pseud.	0.00	0.00	0.00	0.00	0.25	0.75	0.25	0.25	0.188
Mean	0.00	0.063	0.125	0.31	0.69	0.81	0.94	1.12	0.50
CV%	0.0	400.0	282.8	190.4	140.8	64.1	46.2	48.0	105.1
$LSD_{5\%}$		0.39	0.54	0.92	1.49	0.80	0.67	0.83	0.74
F-probability		0.426	0.588	0.380	0.141	0.274	0.020	0.103	0.142

Discussion and Conclusion

Strains of antibiotic-producing Pseudomonas have been isolated from the rhozosphere of a large number of agricultural crops (Mazzola *et al.,* 1991; Keel *et al.,* 1996; McSpadden *et al.,* 2001; Georgakopoulos *et al.,* 2002; Bergsma-Vlami *et al.,* 2005) yet to our knowledge, this is the first report of antibiotic-producing pseudomonas isolated from agricultural crops in Uganda. This study was an example of the usefulness of using primers targeting antibiotic producing genes for the isolation of biocontrol agents rather than the time-consuming isolation of strains and screening for antifungal activity. We targeted DAPG producing genes on the rhizosphere of coffee and bean roots. The *phlD* + *Pseudomonas* revealed a DNA fragment of 629 bp indicative of the presence of *phl*D. No PCR product was observed in *phlD* negative *Pseudomonas* isolates. These findings show that agricultural soils in Uganda harbour populations of *Pseudomonas* species with potential to elicit anti-biotic activity against CWD and *Pythium* root rot of beans.

More *phl*D+ *Pseudomonas* were recovered from coffee rhizospheres compared to bean rhizospheres (Table 3). This concurs with reports by Picard *et al.* (2004), Bergsma-Vlami *et al.* (2005), and Mazzola *et al.* (2005) that plant species and plant genotypes influence the abundance of *phlD*+ *Pseudomonas* spp. within the rhizospheres. Furthermore, coffee rhizosphere samples from sites under monoculture, for example, those located at MUARIK, CORI and KARI, were observed to harbour higher percentage of $phlD^+$ pseudomonas populations compared to samples from sites under intercropping such as those in Mpererwe and Matuga. This possibly suggests that fluorescent *Pseudomonas* spp. that produce 2, 4-DAPG are enriched or multiply in soils during monoculture. A similar finding has also been reported by others (Weller *et al.,* 2002; Mathieu, 2005). The study results also suggested that coffee rhizospheres from fields infested with *Fusarium xylaroides* harboured a higher percentage of *phlD*+ pseudomonas (50-75%) isolates compared to fields where healthy plants were found, an observation that is in agreement with Mazzola and Cook **(**1991). These authors reported that the population of 2, 4-DAPG producers increase considerably in the presence of target root pathogens compared to in healthy roots

(Charigkapakorn and Sivasithamparam**,** 1987; Sarniguet *et al.,* 1992; Weller *et al.,* 2002; de Souza *et al.,* 2003). Similarly, previous work by Weller (1983) and Sarniguet *et al.* (1992) showed that the sizes of bacterial populations, especially *Pseudomonads,* were larger in the rhizospheres of wheat diseased/infected with take-all of wheat (*Gaeumannomyces graminis* var. *tritici*).

Another important finding was that bean rhizospheres from the sub-urban areas of Kibuli appear to contain a higher percentage of positive *phlD*+ than those from rural areas. This is in line with Wang *et al.* (2001) who noted that *phlD*+ populations of fluorescent *Pseudomonads* in the rhizosphere of dicotyledonus crops appeared to be widely cosmopolitan at a worldwide scale. However, the findings from this study are based on a few randomly selected fields and sometimes under uncontrolled conditions on farmers' fields. More conclusive evidence will need to be gathered from better designed on-station experiments to verify these hypotheses.

To determine the genotypes of all recovered *phlD*+ *Pseudomonads* from coffee and bean rhizopheres, a restriction enzyme analysis was undertaken. This was initially done with *Hae*III since previous findings revealed the greatest number of easily discernible restriction patterns from a single *HaeIII* digest (McSpadden *et al.,* 2001). The results revealed identical banding patterns for all coffee isolates suggesting that a single dominant *phlD* genotype exists in the rhizosphere studied. Picard *et al.,* (2000) also observed similar findings and concluded that in a single soil sample, the large majority of *phlD*+ Pseudomonads are nearly identical. It thus appears that under a given set of environmental conditions a single genotypically distinct population of *phlD*+ *Pseudomonas* strains will dominate in the root rhizosphere (McSpadden *et al.,* 2000; Picard *et al.,* 2000; Mavrodi *et al*., 2002). It has been shown that strains of one genotype will normally be dominant on the roots of crops subjected to many years of monoculture (Weller *et al.,* 2002). In this study, two unique restriction patterns were revealed in the bean rhizospheres on digestion with *HaeIII*. This, therefore, indicated the existence of two different *phlD*+ genotypes both new and unidentified in beans. Wang *et al.* (2001) also reported different *phlD* genotypic clusters co-existing in the rhizosphere of dicots at a given geographic location. The existence of distinct genotypes of fluorescent *Pseudomonads* in beans in Uganda raises the possibility of using different antibiotic-producing strains all adapted to the same local environment and plant host. The restriction profiles generated from a RFLP analysis of all *phl*D+ *Pseudomonas* recovered in this study appeared to be unique and not corresponding to any of the previously described *phlD*-RFLP profiles (Mcspadden and Weller, 2001; McSpadden *et al.,* 2001, 2005). However, since this is the first study to investigate the presence and diversity of *phl*D+ *Pseudomonas* in Uganda, wider sampling in other parts of Uganda would be necessary in order to draw more clear conclusions. This is important since natural populations of biocontrol agents such as the fluorescent Pseudomonds are an enormous resource for identifying beneficial candidates for biological control of plant diseases (Thomashow and Weller, 1996; McSpadden *et al.,* 2001; McSpadden and Weller, 2001; Landa *et al.,* 2002; Ramette *et al.,* 2003).

A major objective of this study was to test the capacity of *phlD*+ isolates to inhibit the growth of fungi known to be important pathogens either in beans or coffee. The pathogens tested were *F. xylaroides, F.oxysporum, F. solani,* and *P. ultimum. In vitro* tests revealed variable but significant inhibition potential among *phlD*+ isolates. For coffee, significant inhibition indices were recorded against *F. xylaroides* and *F. oxysporum*. Similarly, *P ultimum* was significantly inhibited by *phlD*+ isolates from beans against *P. ultimum*. Only *phlD*+ isolates from beans were observed to inhibit *P. ultimum*. This suggests that a specific

inhibitory mechanism exist in bean isolates. Surprisingly some *phlD*-negative isolates from coffee tested against the *Fusarium* spp. displayed inhibitory activity. However, only *phl*D+ isolates could inhibit *P. ultimum.* Since these isolates are not likely to produce 2, 4-DAPG, their inhibitory mechanism is different and could either be related to competition (Elad and Chet, 1987; O'Sullivan and O'Gara, 1992) or the production of other secondary metabolites like phenazines, pyolutorin, pyrrolnitrin, tropolone (O'Sullivan and O'Gara, 1992), related antifungal lipopeptide (O'Sullivan and O'Gara, 1992; de Souza *et al.,* 2003) or release of extracellular lytic enzymes like chitinase (O'Sullivan and O'Gara, 1992; Nielsen *et al*., (2002). None of these mechanisms was tested in this study. It would, therefore, be interesting to further investigate the mechanism of action of these isolates and their possible use in biocontrol, since they could have an effect complementary to antibiotic producing strains.

The best performing *phlD*+ *Pseudomonas* isolates in the *in vitro* plate assays were identified. Two of these, one each from beans and coffee, were used in further validation of bio-control potential in screen-house experiments. The results showed significant differences among treatments. Disease severity and incidence were highest in treatments where the pathogen was incorporated but without any control applied (chemical or *phl*D+ competent bacterial isolate) against it. In contrast, low disease incidence and severity were achieved in treatments that included *Pseudomonas* isolates. The results also showed that effectiveness of *phl*D+ *Pseudomonas* spp. as a bio-control agent against *Pythium* root rot severity increased with time. In contrast, the effect of fungicide (Rodmil) application decreased over time. In fact nine weeks after inoculation (WAI), *phl*D+ *Pseudomonas* isolates provided even better control of *Pythium* root rot probably because it continued to multiply and successfully establish itself at the root surface. On the other hand, the fungicide applied could have leached through or degraded with routine maintenance activities such as watering. These findings are important as they show the importance of timing of antagonistic activity *in situ* in relation to infection in order to prevent seed and root rot. All *Pseudomonas* isolates will need time to stabilize and express antifungal activity to counter the stimulatory effect of the seed on oospore germination and hyphal development on seeds or seedlings. The dynamics of *in-situ* production of antifungal compounds of indigenous *phlD*+ *Pseudomonas* isolates in relation to pathogen infection need to be studied further.

This study as a whole not only confirmed the presence of antibiotic-producing Pseudomonas in Ugandan soils but also demonstrated the potential of indigenous *phl*D+ *Pseudomonas* for use as bio-control agent. It also demonstrated that diversity exists. However, development of these isolates into commercial biofungicides still requires additional work. For example, to ensure commercial viability, long shelf-life and survival in the soil, types of formulation that could be used as carrier materials need to be tested.

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