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Full Length Research Paper

Pseudomonads and symbiotic micro-organisms as biocontrol agents against fungal disease caused by *Pythium aphanidermatum*

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Experiments were undertaken to assess the antagonistic aptitude of *Pseudomonas* spp. alone or associated with mycorrhizal fungi on *Pythium aphanidermaturm*, the causal agent of seedlings damping-off and stem rot of cowpea (*Vigna unguiculata* L. Walp). Evaluation was made using selected strains of pseudomonad (*Pseudomonas fluorescens*, *P. putida* and *Pseudomonas* sp.) isolated from *V. unguiculata* and *Solanum tuberosum* rhizospheric soils collected in three agroecological zones of Cameroon. Cultures were conducted on agar plates and in liquid media to evaluate the antagonistic capacity of those strains against *Pythium aphanidermaturm* and to evaluate their biocontrol activity in protecting cowpea plants. The results showed a direct inhibition of the pathogenic fungus by these bacteria. *P. fluorescens* used alone against *Pythium aphanidermaturm*, provided a reduction of the disease index from 3.44 to 1.06. When the arbuscular mycorrhizal fungi *Glomus deserticola* is associated to *P. fluorescens*, the disease index dropped to 0.13, confirming the synergistic effect of those beneficial micro-organisms.

Key words: antagonism, beneficial micro-organisms, biological control, *Glomus deserticola*, *Pseudomonas* spp., *Pythium aphanidermaturm*.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important legumes in Cameroon (Mbouemboue, 1988). The importance of this plant is related to its leaves and seeds which are edible for their protein quality (Borget, 1989). The cultivation of this plant is extended from the humid forest zones to the soudano-sahelian ones (Thurston, 1984). The estimation of the world production of cowpea by F.A.O. in 1993 was 1,000,000 tones per year. But this estimation did not take into consideration the unequal distribution of the production in different areas where it is cultivated. Singh and Allen (1979) estimated the yield of cowpea in arid and sub-arid zones of Africa and Asia to 400 kg/ha. This value is very low compared to that of USA which is more than 1500 kg/ha. The low performances observed in tropical zones are generally linked to the irregularity of the rains (Farid et al., 1988), the non adapted cultural techniques (Mbouemboue, 1988) and particularly to pest and disease caused by insects and micro-organisms. The most severe diseases are damping-off and root rot caused by fungi such as *Fusarium*, *Pythium*, *Sclerotium rolfsii* and *Rhizoctonia solani* (Agrios, 1997). These pathogens are usually polyphagous and natural plant resistance to their infection does not exist (Georgakopoulos, 2002). It has been admitted that *Fusarium* sp and *Pythium* sp are the main

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fungal pathogens responsible of the damage observed on cowpea in southern Cameroon (Mbouemboue, 1988). To fight against those pathogens, fungicides such as methylthiophanate, thiram, carbendazyim, metalaxyl and others are either applied on seed with a variety of seed-coating methods or by drenching (Georgakopoulos, 2002). *Pythium* spp. is the most important pathogen infecting seeds or seedlings before emergence from the soil; they may reduce rhizosphere populations of beneficial micro-organisms such as fluorescent pseudomonas and arbuscular mycorrhiza and also cause significant economic losses to growers (Martin and Loper, 1999). However, control with fungicides has its limitations such as availability, environment pollution as well as resistance of *Pythium* spp. to many of these compounds (Becker and Cook, 1988; Ichitani et al., 1994). For these reasons, these compounds are being phased out from agriculture and alternative protection methods are urgently needed. Increasing attention has been paid to biological control through antagonistic micro-organisms such as bacteria (Artigues and Davet, 1982; Paulin, 1994, Thind and Ahmad, 1994), particularly pseudomonads (Rankin and Paulitz, 1994; Perdomo et al., 1995; Fedi et al., 1997; Hani and Mohamed, 1998; Trane et al., 2000). Preliminary observations showed that under cultural conditions fluorescent pseudomonads were closely associated with some *Pythium ultimum* hyphae, with the ability to inhibit their growth (Nwaga, 1988).

Few studies have investigated the ability of symbiotic microorganisms such as arbuscular mycorrhiza, rhizobia and combinations to improve the growth and the protection against a plant disease. Therefore, the main objective of this study is to test the efficiency of some fluorescent pseudomonad associated with arbuscular mycorrhiza and rhizobia in the protection of *V. unguiculata* against *Pythium aphanidermaturm*. To achieve this objective, we assessed the *in vitro* and the *in vivo* aptitude of theses beneficial micro-organisms as antagonists against *Pythium aphanidermaturm*, the main causal agent of cowpea root rot disease.

MATERIALS AND METHODS

Seeds

Seeds of *Vigna unguiculata* (L) Walp. used in our experiments were kindly provided by the Department of Plant Protection of the University of Dschang, Cameroon. They were from seeds of the local variety 222.

Isolation and identification of *Pythium* sp. from rhizosphere soil

Isolation of *Pythium* sp. was made from soils and roots sampled in three agroecological zones of Cameroon, Dschang and Foumbot in the West province, Ekona in the South West and Yaoundé in the Centre. Samples were collected mainly in the rhizosphere of infected cowpea showing systemic symptoms of root rot disease. Isolation was made according to the method described by Ricci et al. (1976). Identification of the fungal pathogen was made using morphological parameters such as sporangial shape and sexual organs through a light microscope (Velna Dare, 1931; Plaat-Niterink, 1981; Dick, 1990). Inoculum production was performed in Petri dishes containing PDA (Potato Dextrose Agar) medium. The culture obtained was either used directly or after some treatments to induce the liberation of zoospores (Tsao, 1974).

Biocontrol microorganisms

***Pseudomonas* strains:** Four strains of *Pseudomonas fluorescens* and two strains of *Pseudomonas pudida* were used. Among *P. fluorescens* strains, two strains ($VuPf_6$ and $VuPf_4$) were isolated from *V. unguiculata* rhizosphere soil while the two others ($StPf_2$ and $StPf_6$) were obtained from *Solanum tuberosum* rhizosphere soil. Strains of *P. putida* were also obtained from *V. unguiculata* rhizosphere soil. All the *Pseudomonas* strains were isolated and grown on King B medium according to the method described by Bisen and Verma (1994), and identified using the dichotomous chart for identification of *Pseudomonas* sp. by Randrianangaly (1995).

Rhizobia strains: The rhizobia strains used in our survey was isolated from cowpea (*V. unguiculata*) soil rhizosphere collected in Yaoundé in the centre province (Mbenoun, 1992; Nwaga and Ngo Nkot, 1998) and from soil collected in Douala, in the Littoral province.

Mycorrhizal fungi: Local strains of *Glomus deserticola*. were isolated from maize (*Zea mays*) in our laboratory according to the method described by Cooper and Gordon (1987). For plant inoculation, 1.5 g of scattered air dried roots was thoroughly mixed with 100 g sand containing approximately 160 spores/g and further to 2 kg of sterilized soil representing plant rhizosphere.

Preparation of bacteria antagonist

Bacterial culture was performed in liquid King B medium for two days at 124 rpm agitation. Centrifugation was undertaken at 1000 rpm at 20°C for 10 min and the pellet consisting of bacteria particles was then collected and re-suspended in 10 ml sterile physiological water. A second centrifugation was done at the same conditions and the resulting pellet re-suspended in 10 ml sterile water. Bacterial concentration was adjusted to 10^8 cfu/ml at 650 nm according to the method for correspondence described by Prior and Beramis (1990). The bacteria suspension was then used for a series of dilutions until 10^1 cfu/ml obtained.

In vitro antagonism between *Pythium aphanidermatum* and pseudomonads

The evaluation of antagonistic biocontrol activity was performed both in liquid culture and on agar plates.

Determination of *Pythium* biomass in liquid medium: A mixture made of 40 ml containing Malt (1.2%) and 40 ml King B medium was distributed into 250 ml sterile flasks. A quantity of 80 µl of different bacterial concentration/dilution was added in each flask and thoroughly mixed. The mixture was then sowed

in a three days fungal culture consisting of four mycelial explants. For each concentration/dilution, four replicates and two controls were performed. All the flasks were incubated in the dark at room temperature for six days. Thereafter, fresh fungal (mycelia) biomass was evaluated after vacuum filtration and evaporation of the liquid culture.

Evaluation of mycelial growth of *Pythium* on agar plates: Interactions between *Pythium aphanidermatum* and biocontrol bacteria were assessed using PDA medium at 20 ml per Petri dish. Each plate was divided into two equal halves. One half was inoculated with 30 µl of each bacteria concentration/dilution and left to incubate at room temperature for two days to allow the development of bacterial colonies. Thereafter, a 5 mm diameter disc of *Pythium aphanidermatum* mycelia picked on a 36 h culture was transferred to the centre of the other half of the plate, at exactly 4.5 cm opposite the zone containing bacteria colonies. The mycelia growth was measured according to a method consisting of sampling daily the invading outline of the Petri dish by the fungal mycelia using a constant weight tracing paper (1 mg corresponding to 14.28 mm^2).

Measurement of *Pythium* inhibition by *Pseudomonas*

The percentage of inhibition was evaluated using the following formula; I (%) = $(P_o - P_c)/P_o$ where P_o is control fungi biomass, P_c is fungi biomass at c concentration and I (%) is percentage of inhibition.

Effect of beneficial micro-organisms on *Pythium* disease of *V. unguiculata*

The biocontrol effect of beneficial micro-organisms was assessed in pots experiments filled with a mixture made of sterilized field soil and sand (3/1, v/v) and in which seeds of *V. unguiculata* were sown. Inoculation with *Pythium* was done according to the method described by Rouxel and Regnault (1985). The pathogenicity of *Pythium* was evaluated according to the Koch postulates (symptoms and microscopic structures of the fungi). Many treatments were applied: sterilised soil (SS), the plant pathogenic fungi *Pythium aphanidermatum* (PAVE), the beneficial micro-organisms *P. fluorescens* (Pf),: *Bradyrhizobium* sp. (B) and *Glomus deserticola* (M). For evaluation of the parameters the mean of 16 plants/treatment or 8 plants/treatment were used respectively for disease index (DI from the symptoms) or plant growth. The evolution of some parameters such as disease index was follow up every week (minimum 0 and maximum 4) till 7 weeks after sowing. Root colonisation by mycorrhizal fungi was evaluated using an index from 0 to 4 for a maximum colonisation (75-100% frequency) after staining the roots according to Merryweather and Fitter (1991).

RESULTS

Occurrence of *Pythium* spp. in *V. unguiculata* rhizosphere soil

Results of fungal isolation from *V. unguiculata* rhizosphere indicated the presence of *Pythium aphanidermatum* and *Pythium myriotylum*. In general, from the eleven isolates obtained throughout the investigated areas only *Pythium aphanidermatum* was pathogenic to *V. unguiculata* (Table 1).

Pythium-Pseudomonas dual culture inhibition assays

The two methods used to evaluate the antagonistic aptitude of the five *Pseudomonas* sp. strains generally showed that pseudomonads were able to inhibit fungal growth in both liquid media and agar plates. Concerning the first method, all the five isolates displayed the antagonistic aptitude on *Pythium* during the period of incubation ranging from 8 to 12 days and these results were observed independent of bacterial concentration / dilution. For the second method, all the five isolates reduced *Pythium* growth, compared to control and the percentage of inhibition varied between 10 and 93% as indicated in Table 2. Data observed in Table 2 showed that the percentage of inhibition varies with bacterial concentration and with the type of strain. Statistical analysis showed significant differences between bacterial type at 5%. The use of Student test allowed a classification of *Pseudomonas* according to their efficiency in inhibiting *Pythium aphanidermatum* in the following decreasing order: $VuPf_1 > StPf_2 = VuPf_6 > VuPf_4 > StPf_6$. The inhibitory activity observed against *Pythium* strain $PAVE_1$ was also observed with strain $PSVF_1$, as indicated in Table 3. Here, the percentage of inhibition varies between 6.8 and 92.9% with bacterial concentration and also from one strain to another.

Evaluation of antagonist biocontrol activity

The evaluation focused on the effects of beneficial microorganisms on plant growth. Parameters such as plant height, fresh and dry weight of roots and shoots were considered as indicated in Table 4. Statistical analysis revealed that *Pythium aphanidermatum* significantly reduced plant growth (height, weight) in the absence of biocontrol microorganisms. The plant growth reduction by the disease was 60% on dry weight basis. When present, all the treatments showed the effects of biocontrol agents on plant growth. Otherwise, root weight was greatly and differently influenced according to the microorganisms used. The treatment containing the pathogenic fungi *Pythium aphanidermatum* showed the lowest root weight. Conversely, treatments containing dual inoculation of mycorrhizal fungi (*Glomus* sp.) associated with *P. fluorescens* displayed the highest root weight. When compared with the *Pythium* treatment control, *Pseudomonas* and *Pseudomonas* + *Glomus* sp. combined treatment gave a 100 and 213% plant biomass

Table 1. Identification and characterisation of *Pythium* sp. isolates used in the study.

Origin of isolates	Aspect of colonies	Growth on PDA (mm^2)	Mycelia diameter (µm)	Oospores diameter (µm)	Number of isolates	Isolate codes	Pathogenicity*	Species
Foumbot	Coton-like	2795	7.0	27.0	2	$PSVF_1$ and $PSUF_2$	+	*Pythium* sp
Ekona	Coton-like	3518	5.0	28.0	2	$PAVE_1$ and $PAVE_2$	+	*Pythium aphanidermatum*
Dschang	Flat	1983	4.3	17.7	1	PMVD	-	*P. myriotylum*
Dschang	Coton-like	3329	5.1	19.3	1	PAVD	-	*P. aphanidermatum*
Foumbot	Flat	2059	5.0	22.6	3	$PMVF_1$ $PMVF_2$ $PMVF_3$	-	*P. myriotylum*
Ekona	Coton-like	2450	8.0	23.0	2	$PSVE_1$ $PSVE_2$	-	*Pythium* sp.

*Using cowpea (*Vigna unguiculata*) as host plant.

Table 2. Inhibition of *Pythium aphanidermatum* (strain $PAVE_1$) in liquid media according to *Pseudomonas* strains and bacterial concentration.

Pseudomonas	Pythium inhibition by 5 pseudomonad strains (%)				
Concentration (cfu/ml)	$VuPF_6$	$VuPF_4$	$StPF_2$	$StPF_6$	$VuPF_1$
0	00.00	00.00	00.00	00.00	00.00
10^1	04.74	04.07	01.003	-10.57	23.05
10^2	33.82	12.64	29.93	01.05	50.98
10^3	54.57	28.41	56.07	08.34	65.87
10^4	72.59	29.23	81.48	10.77	81.04
10^5	83.91	36.67	86.20	12.61	90.30
10^6	84.21	40.34	89.91	14.84	91.61
10^7	89.53	43.15	91.86	27.38	93.42
10^8	89.64	43.67	91.31	29.08	93.25

Data in the table are mean of three replicates per treatment (bacteria concentration).

Table 3. Inhibition of *Pythium aphanidermatum* (strain $PSVF_1$) on agar plates according to *Pseudomonas* strains and bacterial concentration.

Pseudomonas	Pythium inhibition by 5 pseudomonad strains (%)				
concentration (cfu/ml)	$VuPF_6$	$VuPF_4$	$StPF_2$	$StPF_6$	$VuPF_1$
0	00.00	00.00	00.00	00.00	00.00
10^1	10.30	09.83	19.67	02.57	19.06
10^2	36.01	-06.80	33.17	-01.05	39.08
10^3	49.43	26.01	73.04	06.28	68.93
10^4	73.38	28.80	85.28	06.16	87.34
10^5	84.80	39.80	88.18	13.90	90.82
10^6	85.53	44.39	88.22	16.81	90.29
10^7	86.54	44.12	87.73	26.03	90.32
10^8	90.59	45.22	88.75	26.57	92.90

Table 4. Effects of beneficial micro-organisms on *Vigna unguiculata* performances against root rot disease caused by *Pythium aphanidermatum* (Strain $PAVE_1$).

Soil treatment[1]	Plant height (cm)[2]	Disease index[2]	Fresh weight (g)[3,4]		Dry weight (g)[3,4]		Mycorrhizal
			Shoots	Roots	Shoots	Roots	colonisation index (MCI)[5]
SS	37.12 ab	0.00 a	3.98 cd	1.18 a	0.56 b	0.24 a	0
PAVE	13.77 c	3.44 e	1.63 e	0.48 e	0.23 d	0.09 e	0
PAVE+Pf	32.66 ab	1.06 bc	3.45 cd	0.93 bc	0.47 bc	0.17 bc	0
PAVE+B	31.93 ab	1.81 cd	2.28 e	0.53 e	0.30 d	0.09 de	0
PAVE+M	37.06 ab	0.75 ab	4.40 b	0.98 abc	0.61 b	0.17 bc	4
PAVE+M +Pf	39.26 a	0.13 a	5.50 a	1.10 ab	0.80 a	0.20 ab	4
PAVE+M+B	31.18 ab	1.81 cd	4.48 b	0.80 cd	0.58 b	0.13 cd	3
PAVE+Pf+B	28.91 b	1.94 d	4.30 bc	0.93 bc	0.54 b	0.15 c	0
PAVE+Pf+B+M	33.19 ab	0.63 ab	3.93 cd	0.85 cd	0.35 cd	0.17 bc	4
LSD 5 %	10.25	0.76	0.93	0.21	0.14	0.04	-
SD	4.98	0.37	0.45	0.10	0.07	0.02	-
CV %	17.70	28.80	11.90	12.00	13.00	13.00	-

Values followed by the same letter(s) do no differ significantly at $P > 0.05$ using t test of Student-Fischer.
[1]SS: Sterilised soil, PAVE: *Pythium aphanidermatum,* Pf: *Pseudomonas fluorescens*, B: *Bradyrhizobium* sp, M: *Glomus deserticola*.
[2]Mean of 16 plants/treatment, Disease index minimum 0 and maximum 4 after 7 weeks. [3]Mean of 8 plants/treatment.
[4]Parameter per plant.
[5]Root colonisation index: 0-4: Maximum colonisation (75 - 100% frequency).

Table 5. Effects of beneficial microorganisms on the control of *Pythium aphanidermatum* ($PAVE_1$) roots rot disease of *Vigna unguiculata* (results are average of 16 plants/treatment and 4 replicates).

Soil treatment[1]	Disease index (DI)[2]			
	1 week after treatment	3 weeks after treatment	5 weeks after treatment	7 weeks after treatment
SS	0	0.00	0.00	0.00a
PAVE	0	1.94	2.75	3.44e
PAVE+Pf	0	0.50	0.63	1.06bc
PAVE+B	0	0.75	1.38	1.81cd
PAVE+M	0	0.75	0.75	0.75ab
PAVE+M +Pf	0	0.00	0.00	0.13a
PAVE+M+B	0	0.50	0.50	1.81cd
PAVE+B+Pf	0	1.06	1.31	1.94d
PAVE+M+B+Pf	0	0.25	0.50	0.63ab

Values followed by the same letter(s) do no differ significantly at $P > 0.05$ using t test of Student-Fischer.
Mean of 16 plants/treatment.
[1]SS: Sterilised soil, PAVE: *Pythium aphanidermatum,* Pf: *Pseudomonas fluorescens*, B: *Bradyrhizobium* sp, M: *Glomus deserticola*.
[2]Disease index (DI), scale from 0 to 4 (maximum),

increase respectively. No nodule formation was observed on *Pythium* treatment, while those associated with *Glomus* sp. or *P. fluorescens* showed the greatest nodules formation.

Evaluation of disease control by beneficial microorganisms

In our experiments, diseases caused by *Pythium aphanidermatum* were expressed during the first four

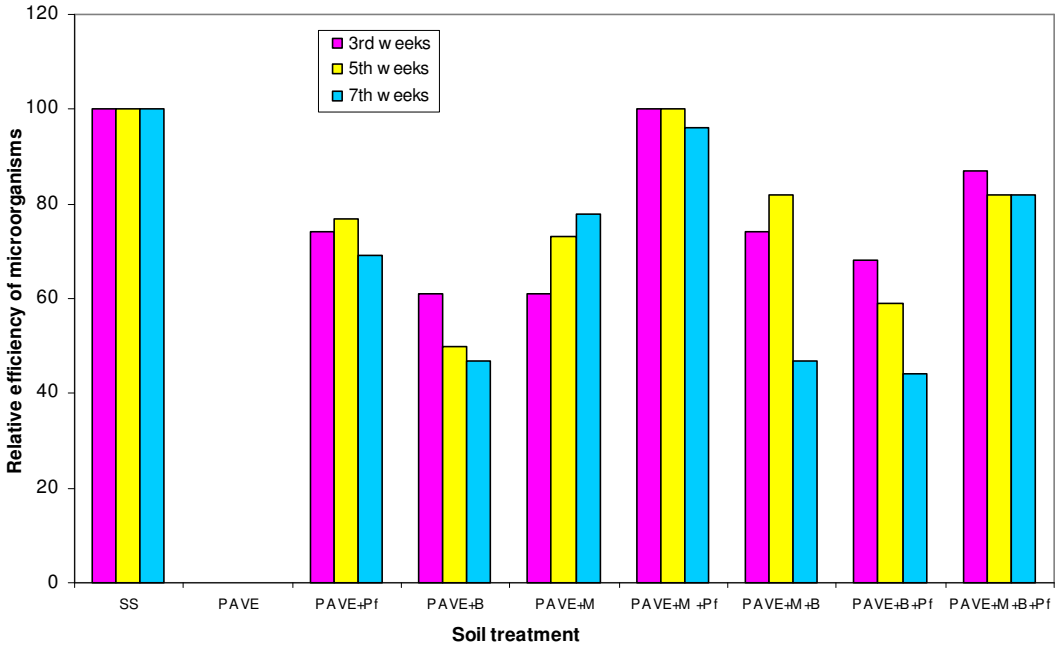

Figure 1. Effects of beneficial microorganisms on the control of *Pythium aphanidermatum* ($PAVE_1$) roots rot disease of *Vigna unguiculata* (relative efficiency of microorganisms).

weeks. Symptoms expressed were generally plant stem and root rot, general stunting, leaf fall or plant died. The biocontrol effects on *Vigna unguiculata* infected with *Pythium aphanidermatum* are reported in Table 5 and Figure 1. Statistical analysis of disease index (DI) showed a significant effect of all the beneficial microorganisms treatments when compared to the control (PAVE) *Pythium aphanidermatum.* The efficiency increased in treatment made of *Pythium aphanidermatum* and *Glomus* sp., while treatment made of *Pythium aphanidermatum* associated with *Bradyrhizobium* sp. and *Pythium aphanidermatum*, *Bradyrhizobium* sp associated with *Glomus* sp. showed decreased efficiency with time. At the seventh week, the values of disease index of the different treatments (except that of the sterilised control soil) varied between 44 and 96%. The highest efficiency was observed in the treatment *Pythium aphanidermatum* associated with *Pseudomonas fluorescens* and *Glomus* sp. (PAVE+M+Pf). Some treatments or association of treatments was able to reduce or increase the disease index of *Pythium* root rot.

DISCUSSION

In the present study, we have identified *Pythium aphanidermatum* from cowpea roots as a pathogen of *V. unguiculata* causing a root rot disease in West and South-West provinces of Cameroon. Conversely, strains obtained from soil samples were not able to infect *V. unguiculata*. This is a general phenomenon with *Pythium* disease, as exemplified by disease suppressiveness, where suppressiveness of soil is primarily correlated with a high general microbial metabolic activity (Lumsden et al., 1987; Chen et al., 1988). In some studies, Weststeijn (1990) realized that in autoclaved soil, root rot was more severe than in natural soil, indicating that the natural soil microflora is of importance in limiting the disease. In these cases, the suppressive effects are probably brought about by nutrient competition, depleting *Pythium* of necessary ingredients for germination, growth and infection (Hani and Mohamed, 1998).

In this study, the antagonism of beneficial microorganisms showed no relation with height, shoots weight, nodule number and disease index (DI). However, fresh root and dry weights were associated with DI. This indicates that the biocontrol agents used greatly act against *Pythium* development in the roots, protecting them against the damping-off disease. The results obtained showed the normal growth of *V. unguiculata* on sterile soil without *Pythium* sp., and the disease index in that case was zero (0). Conversely, in the presence of *Pythium aphanidermatum*, *V. unguiculata* showed typical symptoms of root rot, with a high DI (3.44/4 the seventh day). When, biocontrol agents are associated with the pathogenic fungus, the consequence is the decrease of the DI. This was the case with all the three beneficial micro-organisms and biocontrol agents used independently. Such a result may be interpreted as a direct antagonistic interaction between the micro-organisms and the

pathogen, e.g. by antibiotic production, competition for nutrients or release of cell wall degrading enzymes (Olivier and Guillaumes, 1983; Camprota, 1982; Paulits, 1994; Thrane et al., 2000). *Pseudomonas* spp. seem to be the most successful biocontrol agent against *Pythium ultimum* in a number of reports (Hagerdorn et al., 1993; Mathre et al., 1994; Liang et al., 1996; McCullagh et al., 1996; Williams and Asher, 1996; Trane et al., 2000; Georgakopoulos et al., 2002), In the present research, the arbuscular mycorrhiza *Glomus deserticola* a symbiotic fungi was the most consistent biocontrol agents against *Pythium aphanidermatum*. However, the biocontrol activity was much more important when *P. fluorescens* was associated to *G.deserticola*. Other researchers have also observed a synergistic effect of two antagonists combined into a single plant treatment (Dunne et al., 1998; Mao et al., 1998). The importance of *Pseudomonas* spp., mycorrhiza and rhizobia in protecting plants against soil diseases has been recognised for sometime now (Schenck, 1981; Lemanceau, 1992; Perdomo et al., 1995), even if most researcher still prefer single isolate antagonists. The biocontrol activity of *Pseudomonas* antagonists *in vitro* was positively correlated with the *in vivo* inhibition results. Even in a relatively low concentration of *Pseudomonas,* most strains were able to show significant control of root rot disease by *Pythium aphanidermatum* on *V. unguiculata*. The rhizosphere proficiency have been use to explain the biocontrol activity of fluorescent pseudomonads on soil pathogens by Weller (1988). The concept of the mycorhizosphere have been proposed to describe the rhizosphere of mycorrhizal plants which differ from the one of non nonmycorrhizal plants (Linderman, 1994). The low number of fluorescent pseudomonads in the soil could be attributed to their possible migration through plant roots to find carbon exudates.

These results support the idea that an integrated approach involving the use of diverse functional groups of selected beneficial soil micro-organisms such as nitrogen fixers, plant growth promoting rhizobacteria and mycorrhizal fungi could be very useful in improving the protection and the sustainable production of tropical crops such as cowpea.

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