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Pathogenicity, electrophoretic characterisation and *in planta* detection of the cocoyam root rot disease pathogen, *Pythium myriotylum*

James Tabi Tambong, Joseph Poppe and Monica Höfte*
*Laboratory of Phytopathology, Faculty of Agricultural and Applied Biological Sciences, University Gent, Coupure Links 653, B-9000 Gent, Belgium; *Author for correspondence (E-mail: monica.hofte@rug.ac.be; Fax: +32 9 264 6238)*

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Abstract

Cocoyam (*Xanthosoma sagittifolium*), an important staple food crop for many people in the tropics and subtropics, suffers great losses from a root rot disease which is most probably caused by *Pythium myriotylum*, although it has been claimed that a complex of three root pathogens is needed to cause the disease. In this study, we compared two *Pythium* isolates from diseased cocoyam roots, CRPm and Bokwai, with other putative *P. myriotylum* isolates from culture collections and from Cameroonian soil, with respect to host range and isozyme patterns. Pathogenicity was tested on tomato, bean, cowpea, tobacco and cocoyam. CRPm and Bokwai were only pathogenic to tobacco and cocoyam. On cocoyam, these isolates caused typical symptoms within 48 h on 100% of the inoculated plantlets. Only two other isolates of *P. myriotylum* from culture collections were moderately to weakly pathogenic to cocoyam. Isolates of *P. myriotylum* were very variable in their pathogenicity to bean, cowpea, tomato and tobacco. Isozyme patterns of α- and β-esterases were used to differentiate CRPm and Bokwai from all other isolates. Unlike the other *P. myriotylum* strains, cocoyam isolates were unable to grow at 37 °C. Malate dehydrogenase isozyme bands originating from CRPm were consistently detected in CRPm-infected cocoyam roots grown *in vitro* and *in vivo*. These findings indicate that CRPm can penetrate cocoyam roots and cause disease in the absence of other root pathogens. This study also indicates that *P. myriotylum* from cocoyam developed a certain degree of host specialisation.

Introduction

Cocoyam (*Xanthosoma sagittifolium*) is an important staple food for about 200 million people in the tropics and subtropics. *Xanthosoma* provides carbohydrate in the human diet, and also contains essential amounts of proteins, fat, and vitamins such as β-carotene, riboflavin, niacin, ascorbic acid and substantial amounts of thiamine (Cobley and Steele, 1976). Root rot, caused by *Pythium myriotylum* (Pacumbaba et al., 1992), is the most devastating disease of cocoyam and can cause yield reductions as high as 90% (Nzietchueng, 1983). Characteristic symptoms of the disease are stunting, yellowing of leaves and severe reduction of the root system.

The genus *Pythium* contains species that range from saprophytic, facultative parasites with extensive host ranges to highly pathogenic species with limited host ranges (Hendrix and Campbell, 1983; Van der Plaats-Niterinck, 1981). In sugarcane, *Pythium arrhenomanes* Drechs. is a highly pathogenic species (Hoy and Schneider, 1988), whereas variability in pathogenicity within *Pythium* species occurs in rice (Schneider et al., 1988). We wanted to investigate whether *P. myriotylum* isolates from cocoyam show or have developed host specialisation because of its very destructive nature on

this crop. It is often difficult to identify *Pythium* species and pathogenic strains on the basis of morphological traits. Therefore, we initiated a study to determine whether isozyme analysis could be used to identify and characterise the cocoyam root rot disease pathogen and to detect the presence of the pathogen *in planta*.

Isozyme analysis has been widely used to study fungal and Oomycete taxonomy, phylogenetics and populations (Oudemans and Coffey, 1991; Chen et al., 1992). However, the use of isozyme analysis to detect Oomycete pathogens *in planta* has not been reported. Kerssies et al. (1994) reported detection of *Fusarium oxysporium* f. *dianthi* in cyclamen and carnation using isozyme patterns of α-esterase and β-glucosidase. Herper et al. (1988) showed that the vesicular-arbuscular mycorrhizal fungi *Glomus caledonium* and *G. mosseae* could be detected in leek roots using isozyme banding patterns of esterase, glutamate oxaloacetate and peptidase.

The objectives of the present research were to determine if the cocoyam root rot pathogen is specific to cocoyam or pathogenic to other crops, to examine if *P. myriotylum* isolates from other crops are pathogenic to cocoyam, to distinguish or characterise the cocoyam root rot pathogen from other related *Pythium* strains using isozymes, and to use isozyme techniques to confirm pathogenicity and detect the presence of *Pythium* in plant tissue.

Materials and methods

Pythium *cultures and plant materials*

Table 1 provides information about the *Pythium* isolates used in this study. Isolates CRPm and Bokwai were obtained from diseased cocoyam roots. Cocoyam roots with necrotic spots were excised into sections of 3–4 cm, surface-sterilised with 1% commercial bleach (0.053% NaOCl) for 2–3 min and blotted on filter paper. The sections were trimmed and incubated at $28 \pm 2\,^{\circ}$C on 1.5% water agar supplemented with $150\,\mathrm{mg\,l^{-1}}$ of streptomycin sulfate. Three days later 5 mm agar plugs of actively growing cultures were transferred onto antibiotic-free water agar (1.5%) and incubated as above. Myt14 and NS2 were isolated by Dr. Nwaga, University of Yaounde, Cameroon and tentatively identified as *P. myriotylum*.

Bean (*Phaseolus vulgaris* cv. prelude), cowpea (*Vigna unguiculata*), tomato (*Lycopersicon esculentum* cv. marmande), tobacco (*Nicotiana tabacum* xanthi NN) and cocoyam (*Xanthosoma sagittifolium* cv. white) were used in this study. Seeds were used for all the materials except cocoyam. Tissue culture-derived cocoyam plantlets were used for cocoyam. *In vitro* plantlets were produced as described by Tambong et al. (1998). Shoot-tips were excised from sprouting cormels, surface-disinfected in 15% commercial

Table 1. Host/habitat and geographic origin of *Pythium* isolates used in the study

Pythium isolate	Host/habitat	Origin/reference
P. splendens 706	*Monstera* roots (Swiss cheese plant)	Buysens et al. (1996)
P. myriotylum		
CRPm	Cocoyam roots (*Xanthosoma sagittifolium*)	Ekona, Cameroon
Pm16166*	Ground nut (*Arachis hypogea*)	Israel
Bokwai	Cocoyam roots (*Xanthosoma sagittifolium*)	Bokwai, Cameroon
CBS 315.33**	Tobacco (*Nicotiana tabacum*)	CBS
IMI 248096***	Sorghum (*Sorghum bicolor*)	Katerine, Australia
IMI 061432R***	Pineapple (*Ananas* sp.)	Brisbane, Australia
IMI 135212***	Groundnut pods (*Arachis hypogea*)	Libya
Pythium spp. myt14	Soil	Batouri, Cameroon
Pythium spp. NS2	Soil	Akonolinga, Cameroon

*Isolate obtained from MUCL, Louvain-La-Neuve, Belgium.
**Isolate obtained from Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands.
***Isolates obtained from International Mycological Institute, Surrey, United Kingdom.

bleach (Clorox, 0.79% NaOCl), 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.

Pathogenicity experiments

Pathogenicity studies were carried out in a potting mixture (dry matter at least 25%, organic matter at least 20%, pH (water) 6.0–6.5, water capacity 80%, resistivity 2000 ohm cm^{-1}; Klasmann-Deilmann GmbH, The Netherlands) and *in vitro* on steam sterilised 2-mm thick 90 mm diam filter papers (Schliecher & Schuell GmbH, Drassel, Germany) in Petri dishes. The potting mixture was steam-sterilised for 1 h at 121 °C 15 psi and used 3 days after sterilisation. Inoculum was produced by aseptically placing four 5-mm mycelial-agar plugs into 40 ml potato dextrose broth that was incubated at 24 ± 2 °C for 7 days. The resulting mycelial mat was blended in 25 ml distilled water for 20–30 s using a Polytron macerator (Kinematica GmbH, Switzerland). Inoculum was adjusted to 300 propagules ml^{-1} for all the isolates except *P. splendens*. Inoculum preparation for *P. splendens* was as described by Buysens et al. (1996) with 250 swelling hyphae g^{-1} potting mixture or ml^{-1} nutrient solution. The planting medium was infested with 250 propagules g^{-1} and mixed thoroughly. Seeds were soaked for 4 min in sterile water prior to sowing. Each treatment (200 g substrate) included 20 seeds and was replicated three times. The substrate was irrigated daily to 300 g with water. For *in vitro* experiments, 1 ml of inoculum (300 propagules) was added to 9 ml nutrient solution (Gamborg et al., 1968 without sugar) and 20 surface-disinfected (15% Clorox for 3–4 min) seeds were sown per Petri dish with three replicates per isolate per plant type. Petri dishes with seeds were incubated at 27 °C and observed daily. Test-tubes containing 6–8 week-old cocoyam plantlets were opened in a laminar flow hood, the medium poured-off, and the plantlets rinsed with sterile water before replacing the medium with Gamborg et al. (1968) mineral solution. One ml of prepared inoculum (100 propagules) was added to the test-tube, and plantlets were incubated at 28 °C for 48 h with 3 replicates per *Pythium* isolate. All experiments were repeated at least once.

Characterisation of Pythium *isolates using isozyme analysis*

Isolates were grown on water agar (1.5%) for 72 h to obtain actively growing mycelia. Four plugs were inoculated into 40 ml of Schitthenner's medium (2.5 g sucrose, 150 mg KH_2PO_4, 100 mg $MgSO_4 \cdot 7H_2O$, 150 mg K_2HPO_4, 55 mg $CaCl_2 \cdot 2H_2O$, 4.4 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $FeSO_4 \cdot 7H2O$, 270 mg L (−) asparagine, 2.0 mg thiamine-HCl, 10 mg cholesterol, and 1000 ml of water) and incubated at 26 °C for 7 or 10 days depending on the enzyme. Mycelial mats were sieved from the medium, blotted between sterile filter paper and reduced to a fine powder in liquid nitrogen. The powder (0.5 g) was weighed into 1.5 ml eppendorf tubes and sample buffer (20 ml extraction buffer, 200 µl mercapthethanol and 200 mg polyvinylpyrrolidon 40) was added in a 1 : 1 (w/v) proportion. Extraction buffer contained 0.056 M Tris-HCl (pH 6.8) and 20% water-free glycerol. The mixture was vortexed for about 15 s and then centrifuged at 14000 rpm ($4375 \times g$) for 30 min at 4 °C. The supernatant was collected and total protein content determined with a commercial kit (Bio-Rad, Hercules, California, USA), using bovine serum albumin as standard. Sample protein was adjusted and aliquots of 10–15 µl (6–9 µg protein) were applied to each well, depending on the enzyme to be localised. There were at least two runs per enzyme. Six enzyme reactions were used to compare the cocoyam root rot pathogens (CRPm and Bokwai) to eight other *Pythium* isolates.

Native PAGE. Native polyacrylamide gel electrophoresis of enzyme extracts was done on 0.75 mm thick gels using Biorad Mini Protean II (BioRad, Hercules, California, USA) equipment. Polyacrylamide resolving gels were prepared by mixing 3 ml of 40% acrylamide (acrylamide/Bis; 40 : 0.8), 3 ml of 1.5 M Tris-HCl (pH 8.6), 7.2 µl of TEMED, 2.4 ml of 0.2% ammonium persulfate, and 4.8 ml of distilled water. Acrylamide was filtered through a 0.22 µm Millipore filter (Millipore, France) before addition of the other components. The stacking gel included 1.2 ml of 40% acrylamide, 0.75 ml of Tris-HCl (pH 8.6), 10.8 µl TEMED, 5.55 ml of distilled water and 0.2% ammonium persulfate. The electrode buffer contained 0.031 M Tris and 0.25 M glycine (pH 8.6). The gels were run for 20 min at a constant voltage of 80 V and then for 1.2 h at 100 V with a BioRad 3000 electrophoretic power unit.

Detection of Pythium *spp. in plant material*

Isozyme patterns of diseased seeds/seedlings or plantlets were analysed in the tomato and cocoyam models for the presence of bands originating from *P. myriotylum* CRPm and *P. splendens*. Two enzyme systems: β-glucosidase and malate dehydrogenases were used. *Pythium splendens*, which is pathogenic to tomato (Buysens et al., 1996), was used as a control pathogen. Tomato seeds and cocoyam plantlets were inoculated in Petri dishes or test-tubes, respectively, as described above. Nine days after incubation, tomato seedlings and seeds inoculated or not inoculated with *P. splendens* and *P. myriotylum* CRPm were harvested. Cocoyam roots were collected 48 h after inoculation with CRPm. Harvested plant materials were surface-disinfested in 0.1% commercial bleach (Clorox) for 2 min and rinsed in sterile water prior to enzyme extraction. Cocoyam plantlets acclimatised for 14 days in pots containing volcanic soil were plug-inoculated as described by Xue et al. (1998). Plantlets were incubated at 28 °C, irrigated as required, and examined daily for typical symptoms of the root rot disease. Four days after inoculation, roots were collected, surface-disinfested as above, blotted on tissue paper, and immediately frozen in liquid nitrogen before storing at −80 °C. Roots were ground to a fine powder in liquid nitrogen and enzymes were extracted and separated electrophoretically as indicated above.

Enzyme detection. After electrophoresis, gels were incubated for 5 min in their corresponding buffers followed by immersion into their respective staining mixture given below: Alcohol dehydrogenase (ADH, EC 1.1.1.1): 100 ml of 0.03 M Tris, 0.005 M citric acid pH 8.5, 10 mg MTT, 3 mg phenazine methosulphate (PMS), 1 ml ethanol, 15 mg NAD; esterases (EST, EC 3.1.1.1): 100 ml of 0.05 M phosphate buffer pH 7.2, 10 mg each of α- and β-naphthylacetate dissolved in 1 ml acetone, 50 mg Fast Blue RR, and 4 ml of 4% formaldehyde; β-glucosidase (β-GLU, EC 3.2.1.21): 100 ml of 0.2 M sodium acetate pH 5.0, 100 mg esculine and 30 mg ferric chloride as described by Kwon et al. (1994); malate dehydrogenase (MDH, EC 1.1.1.37): 85 ml buffer, NAD, MTT, PMS as for ADH, 15 ml 0.5 M malate pH 7.0 as described by Micales et al. (1986); malic enzyme (ME, EC 1.1.1.40): 90 ml buffer, MTT, PMS as for ADH, 10 ml malate pH 7.0, 10 mg NADP; 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44): 50 ml 0.1 M Tris buffer pH 8.0, 20 mg 6-phosphogluconate barium salt, 100 mg $MgCl_2$, 10 mg NADP, 6 mg MTT, and 1 mg PMS as described by Oudemans and Coffey (1991). All staining was done at 37 °C for 20–25 min.

Data collection and statistical analyses. The number of healthy seedlings of tomato, bean, cowpea and tobacco were recorded 7–10 days after sowing. The counts were transformed into percentage survival by the relationship: (number of healthy seedlings/total number of seeds sown) multiplied by 100. Arcsine transformations were performed where necessary before analysing the data. SPSS (1996) statistical software was used to evaluate possible differences at the 5% level of probability and the Duncan Multiple Range Test was used to separate means. The stained gels were immediately photographed on an illumination table with a video camera module (Vilbert Lourmat, Marne-la Vallé, France). The number of bands was recorded and relative mobilities (R_f values) were calculated as the ratio of the distance moved by the band over the distance moved by the tracking bromophenol blue dye. For characterisation of CRPm and Bokwai with respect to the other isolates, data matrices were generated by scoring for the absence or presence of an electrophoretic band as 0 or 1 respectively as described by Chen et al. (1992). Jaccard's coefficients were computed (SPSS, 1996) and used to evaluate possible similarities between the different *Pythium* isolates. To focus on the isolates from Cameroon, gels stained for esterase were projected into a personal computer linked to a video camera module (Vilbert Lourmat, Marne-la Vallé, France) and densitometric profiles were generated using Gelcompar software (1998, Version 4.1, Applied Maths Kortrijk, Belgium).

Results

Pathogenicity tests

Seeds of tomato, bean, cowpea and tobacco, and tissue culture-derived cocoyam plantlets were infected with 10 *Pythium* isolates: two isolates (CRPm and Bokwai) from diseased cocoyam plants, five *P. myriotylum* isolates from culture collections, and two isolates obtained from soils of Cameroon. An isolate of *P. splendens* that has been used extensively in our laboratory was used as a control. Figure 1 shows the percentage healthy seedlings of bean, tomato, cowpea and

tobacco. The cocoyam root rot isolates (CRPm and Bokwai) were both pathogenic to tobacco seedlings but did not affect the survival of bean, tomato or cowpea seedlings. *P. splendens* was highly pathogenic to bean, tomato, tobacco and cowpea. Myt14 was pathogenic to bean, tomato and tobacco; NS2 was only pathogenic to tomato; IMI 248096 affected both bean and tobacco; IMI 135212 affected tobacco and cowpea, while IMI 061432R was only pathogenic to cowpea. CBS 315.33 significantly affected seedling survival of cowpea; Pm 16166 had no adverse effects on seedling survival. Pathogenicity to cocoyam was restricted to 4 of the 10 isolates studied. *Pythium myriotylum* CRPm and Bokwai, isolated from diseased cocoyam roots, were highly pathogenic to tissue culture-derived cocoyam plantlets (Table 2). Symptoms were evident about 30 h after inoculation with 100 propagules. The plantlets showed typical and severe root rot disease symptoms 48 h after inoculation. Isolate IMI 248096 caused moderate symptoms while CBS 315.33 revealed mild symptoms after 48 h. Even though CBS 315.33 showed mild symptoms after 48 h, the disease did not progress.

Isozyme characterisation of the cocoyam root rot pathogen

Isozyme characterisation was carried out on the ten *Pythium* isolates presented in Table 1. Six enzymes produced distinct and consistent banding patterns. Forty-five discrete bands were resolved with these six enzymes (Figure 2). Figure 2 also shows the relative mobility values (R_f) of each band. A similarity matrix of Jaccard's coefficients among *Pythium* isolates is presented in Table 3. The highest similarity coefficient, 0.94, was obtained between CRPm and Bokwai. Isolates NS2 and myt14 exhibited the lowest similarity index (0.09 and 0.08) to CRPm and Bokwai. CBS 315.33 (0.63 and 0.68), IMI 248096 (0.72 and

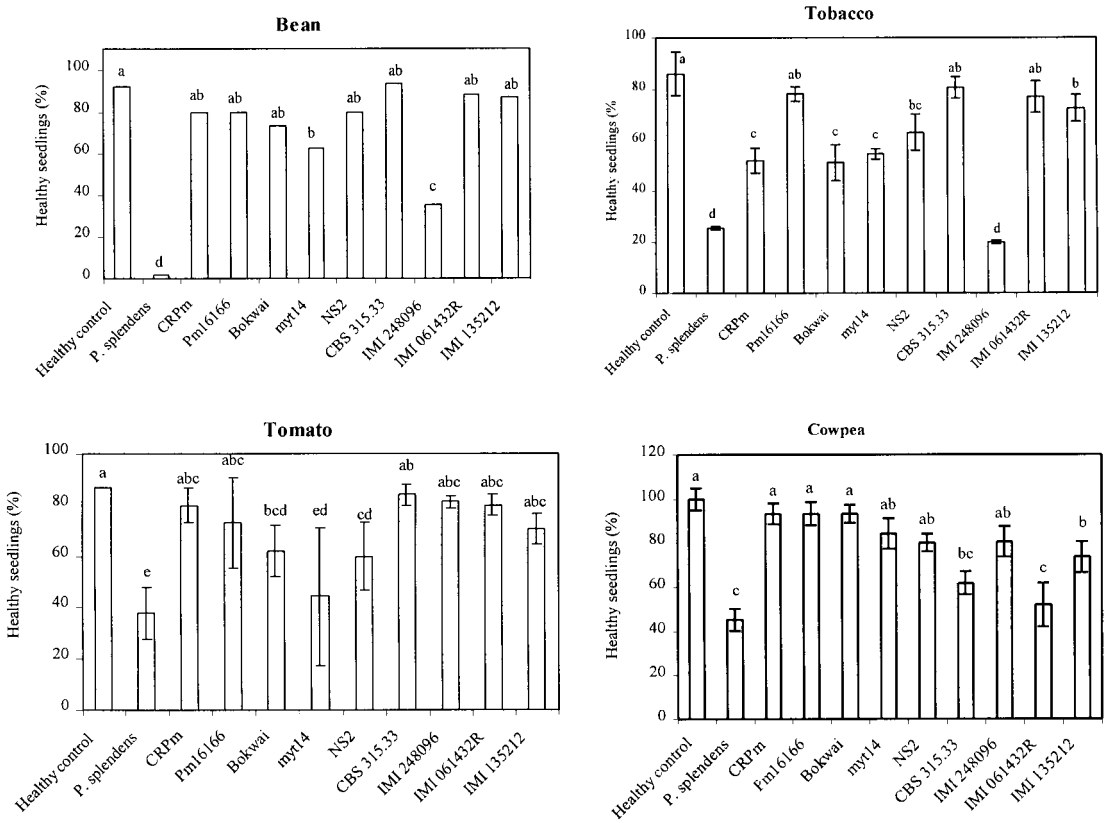

Figure 1. Percentage healthy seedlings of bean, tomato, tobacco and cowpea sown in the presence of 10 *Pythium* isolates. Bars are means of 3 replicates. The experiment was repeated at least once. Bars within the same plant type with the same letter(s) are significantly different at 5% level of probability using Duncan Multiple Range Test. Arcsine transformation of data was done where necessary.

Table 2. Pathogenicity of 10 *Pythium* isolates on tissue culture-derived cocoyam plantlets in test-tube assays[1]

	Mean disease severity scores[2]	Severity remarks
P. splendens	0.0 c	Non-pathogenic
P. myriotylum		
CRPm	3.0 a	Highly pathogenic
P m 16166	0.0 c	Non-pathogenic
Bokwai	2.8 a	Highly pathogenic
CBS 315.33	1.0 c	Weakly pathogenic
IMI 248096	2.0 b	Moderately pathogenic
IMI 061432R	0.0 c	Non-pathogenic
IMI 135212	0.0 c	Non-pathogenic
Pythium spp. myt14	0.0 c	Non-pathogenic
Pythium spp. NS2	0.0 c	Non-pathogenic

[1]Observations were made on 4 replicates (plantlets) per *Pythium* isolate *in vitro* using liquid Gamborg et al. (1968) macro- and micro-nutrients; 6–8 weeks-old tissue culture-derived cocoyam plantlets were used. The experiment was repeated twice. [2]Score ratings: 0: no root rot disease symptoms after 72 h; 1: slight disease symptoms after 48 h; 2: moderate disease symptoms after 48 h; and 3: severe root rot disease symptoms after 48 h. Values followed by the same letters are not significantly different at $P = 0.05$.

0.78) and Pm 16166 (0.61 and 0.58) showed significant resemblance to the cocoyam root rot isolates (CRPm and Bokwai), according to Jaccard's coefficients (Table 3). IMI 06143R showed the highest similarity (0.58, Table 3) to isolate myt14.

Isozyme data were used to characterise the cocoyam root rot disease pathogens (CRPm and Bokwai) with respect to the other isolates using six isozyme reactions. Isozyme patterns of α- and β-esterases differentiated CRPm and Bokwai from all other isolates (Table 4, Figure 3). 6PGD and β-glucosidase gave a positive differentiation of CRPm and Bokwai from *P. splendens*, IMI 061432R, myt14 and NS2. With regards to relative mobility values of bands of MDH, the cocoyam root rot pathogens discriminated themselves from all other isolates except Pm 16166 and IMI 248096. Isozyme bands of malic enzyme discriminated CRPm and Bokwai against *P. splendens*, Pm 16166, IMI 061432R, NS2 and myt14. ADH separated the cocoyam root rot pathogens from *P. splendens*, IMI 061432R, IMI 135212, myt14 and NS2.

Figure 4 shows densitometric profiles of α- and β-esterases of isolates obtained from Cameroon with *P. splendens* as a control pathogen. *P. splendens* showed three major peaks at the extremes while CRPm and Bokwai showed three major peaks. Myt14 showed two major peaks while NS2 showed only minor peaks.

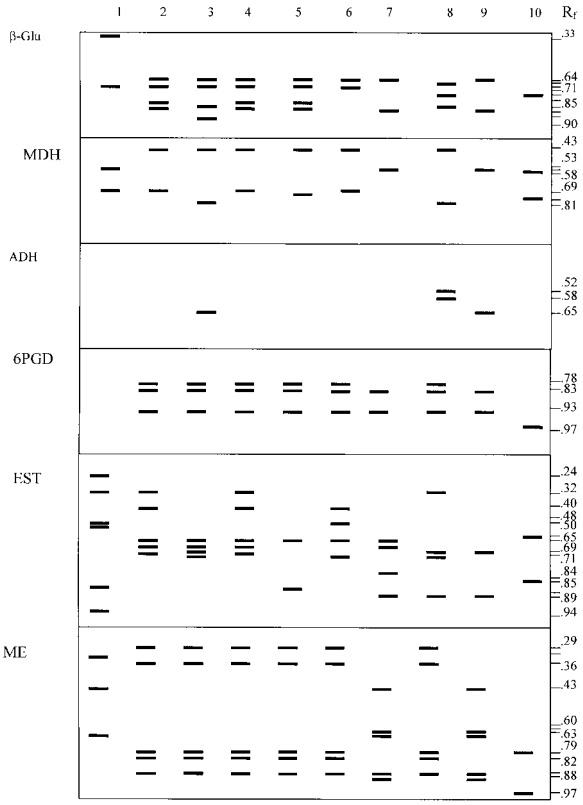

Figure 2. Isozyme patterns of 45 bands of 10 *Pythium* isolates represented as the relative mobility values (R_f is the mobility of each enzyme band that travelled from the origin divided by the distance travelled by the front tracking dye). The enzymes are β-glucosidase (β-GLU), malate dehydrogenase (MDH), alcohol dehydrogenase (ADH), 6-phosphogluconate dehydrogenase (6PGD), α- and β-esterases (EST), and malic enzyme (ME). *Pythium* isolates: lanes: 1. *P. splendens*, 2. *P. myriotylum* CRPm, 3. *P. myriotylum* Pm16166, 4. *P. myriotylum* Bokwai, 5. *P. myriotylum* CBS 315.33, 6. *P. myriotylum* IMI 248096, 7. *P. myriotylum* IMI 061432R, 8. *P. myriotylum* IMI 135212, 9. *Pythium* spp. myt14, 10. *Pythium* spp. NS2. Band intensity information was not utilised.

Detection of P. splendens *and* P. myriotylum *CRPm in plant material*

Detection of CRPm was done in the tomato and cocoyam models with *P. splendens* as control pathogen. Enzymes were extracted from CRPm-inoculated tomato and cocoyam seedlings/plantlets and electrophoretically separated using native PAGE. β-glucosidase and malate dehydrogenase were stained in an attempt to localise isozyme bands of the pathogen in plant tissues. Tomato seedlings inoculated with

Table 3. Jaccard's similarity coefficients between 10 *Pythium* isolates using six enzyme reactions with 45 discrete bands

		1	2	3	4	5	6	7	8	9	10
1	*P. splendens*		0.12	0.04	0.12	0.08	0.13	0.05	0.04	0.05	0.00
	P. myriotylum										
2	CRPm			0.61	0.94	0.63	0.72	0.18	0.41	0.09	0.09
3	Pm 16166				0.58	0.56	0.65	0.21	0.47	0.10	0.10
4	Bokwai					0.68	0.78	0.17	0.46	0.08	0.08
5	CBS 315.33						0.58	0.19	0.36	0.09	0.14
6	IMI 248096							0.19	0.43	0.09	0.09
7	IMI 06143R								0.14	0.58	0.06
8	IMI 135212									0.09	0.09
9	*Pythium* spp. myt14										0.00
10	*Pythium* spp. NS2										

Coefficients were calcuated using SPSS (1996) software. Enzymes were stained at least twice as described in the Materials and Methods section.

Table 4. Differentiation of the cocoyam root rot isolates *Pythium myriotylum* CRPm and *Pythium myriotylum* Bokwai from 8 other *Pythium* isolates using 6 isozyme reactions

	EST	6PGD	MDH	ME	β-GLU	ADH
P. splendens	+	+	+	+	+	+
P. myriotylum						
P m 16166	+	−	−	+	−	−
CBS 315.33	+	−	+	−	−	−
IMI 248096	+	−	−	−	−	−
IMI 061432R	+	+	+	+	+	+
IMI 135212	+	−	+	−	−	+
Pythium spp. myt14	+	+	+	+	+	+
Pythium spp. NS2	+	+	+	+	+	+

Each enzyme was stained at least twice; staining mixtures are given under Materials and Methods section (+: positive differentiation and −: negative differentiation). EST: esterase, 6PGD: 6-phosphogluconate dehydrogenase, MDH: malate dehydrogenase, ME: malic enzyme β-GLU: β-glucosidase, and ADH: alcohol dehydrogenase.

P. splendens and *P. myriotylum* CRPm showed significant differences in number of β-glucosidase bands localised after staining (Figure 5) compared to non-inoculated plants. Healthy control seedlings did not show any β-glucosidase bands (Figure 5, lane 1). Seedlings inoculated with *P. splendens* showed a β-glucosidase enzyme band with R_f 0.68 (Figure 5, lane 2). Mycelia of pure cultures of *P. splendens* exhibited two isozyme bands with one band ($R_f = 0.68$) having the same R_f value as that observed in tomato seedlings inoculated with *P. splendens*. This suggests that the isozyme band localised in the *P. splendens*-inoculated tomato seedlings was from the pathogen. Tomato seedlings derived from seeds inoculated with *P. myriotylum* CRPm did not show any β-glucosidase bands (Figure 5, lane 4), which is similar to non-inoculated plants (lane 1). None of the bands observed in mycelia of pure cultures of CRPm could be localised in CRPm-inoculated tomato seedlings.

Analysis of roots of cocoyam plantlets inoculated with *P. myriotylum* CRPm gave significant differences in MDH banding patterns compared to non-inoculated cocoyam plantlets (Figure 6A). In test tube assays, roots of non-inoculated plantlets revealed one MDH isozyme band at $R_f = 0.65$ while inoculated plantlets showed two MDH bands. None of the bands localised

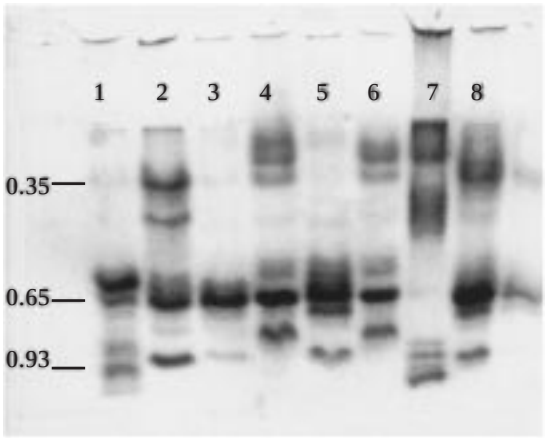

Figure 3. α- and β-Esterase patterns on Native PAGE of 8 *Pythium* isolates. Total protein was the same. Lanes 1. *P. myriotylum* IMI 061432R; 2. *P. myriotylum* IMI 248096; 3. *P. myriotylum* CBS 315.33; 4. *P. myriotylum* Bokwai; 5. *P. myriotylum* 16166; 6. *P. myriotylum* CRPm; 7. *P. splendens*; 8. *P. myriotylum* IMI 135212. (esterase staining solution: 100 ml of 0.05 phosphate buffer (pH 7.2), 10 mg α-napthyl acetate + 10 mg β-napthyl acetate dissolved in 1 ml acetone, and 50 mg Fast Blue RR).

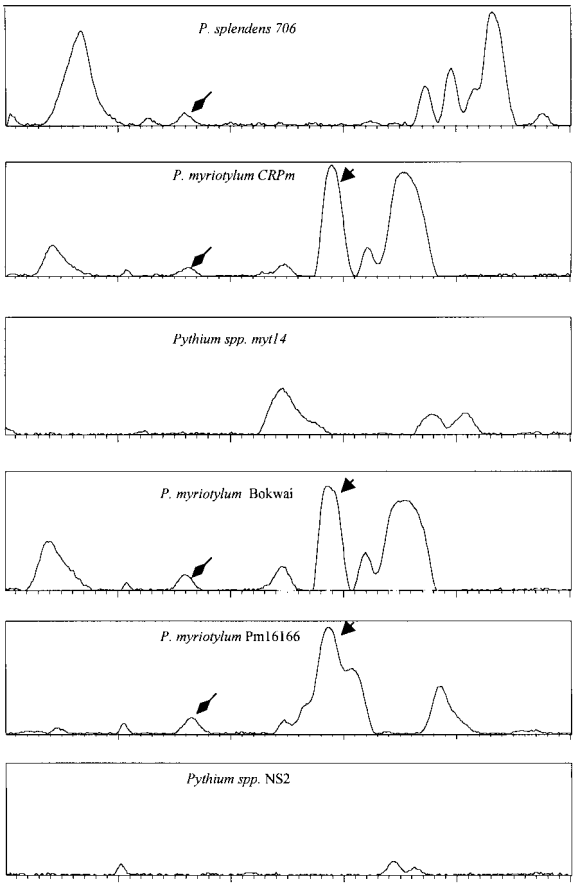

Figure 4. Densitometric analysis of α- and β-esterase isozyme patterns of *Pythium* isolates obtained from Cameroon with *P. splendens* and *P. myriotylum* 16166 as standards. Native PAGE electrophoresis was run at least twice with similar bands. Profiles were generated using Gelcompar software (1998, Version 4.1, Applied Maths, Kortrijk, Belgium). Potato dextrose broth was used as growth medium. Similar arrow shapes depict isozyme band shared by these isolates.

in roots obtained from CRPm-inoculated plantlets corresponded to the single band ($R_f = 0.65$) observed in non-inoculated plantlets (Figure 6A). MDH bands in CRPm-inoculated plantlets were localised at $R_f = 0.35$ and 0.69 (Figure 6A, lanes 2 and 4) corresponding to bands stained from mycelia of pure cultures of CRPm. The staining was more intense when plantlets were inoculated with the pathogen for 3 days compared to 2 days. Figure 6B shows MDH isozyme bands of tissue culture-derived cocoyam plantlets inoculated in volcanic soil of Cameroonian origin. Results show a similar banding pattern as described in the test-tube assay. Again the isozyme technique detected MDH bands originating from the cocoyam root rot pathogen ($R_f = 0.36$) in plantlets plug-inoculated with CRPm. Localisation of β-glucosidase isozyme bands of CRPm in CRPm-inoculated plantlets was negative.

Discussion

This study demonstrates that *P. myriotylum* isolates from cocoyam may not have a wide host range. The cocoyam isolates CRPm and Bokwai, which appear to be very similar, were only able to infect tobacco seeds and cocoyam plantlets. Both cocoyam isolates were able to infect 6–8 week-old cocoyam plantlets *in vitro* with severe typical root rot disease symptoms evident within 48 h. Under field conditions in Cameroon, severe disease incidence caused by *P. myriotylum* was observed 3–4 months after planting (July–August) which coincides with the rainy season. This suggests that isolates of *P. myriotylum* that attack cocoyam *in vitro* and under field conditions, may have developed some specialised structures enabling them to parasitise mature plant organs. This possible specificity is evident in the traditional farming systems where cocoyam is grown. Intercropping of cocoyam,

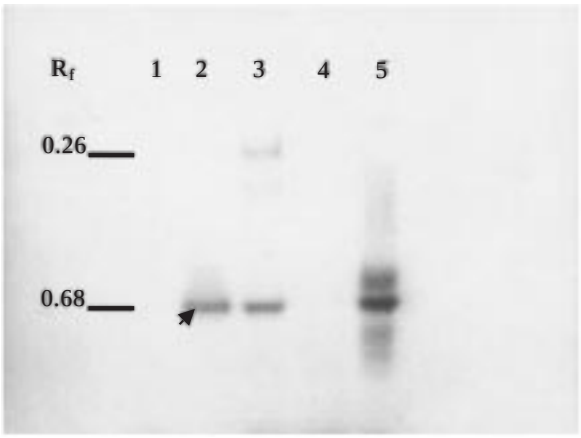

Figure 5. Detection of β-glucosidase isozyme bands of *P. splendens* (Ps) but not *P. myriotylum* CRPm in tomato seedlings inoculated with these pathogens. Lanes 1. Non-inoculated tomato seedlings; 2. Ps-inoculated; 3. pure culture of Ps; 4. CRPm-inoculated; and 5. pure culture of CRPm. The enzyme was detected on Native PAGE with esculin as substrate as described by Kwon et al. (1994). R_f value is the ratio of the distance moved by the band over distance moved by tracking dye (bromophenolblue). Total protein in sample was same. Arrow indicates corresponding Ps band localised in Ps-infected tomato seedlings.

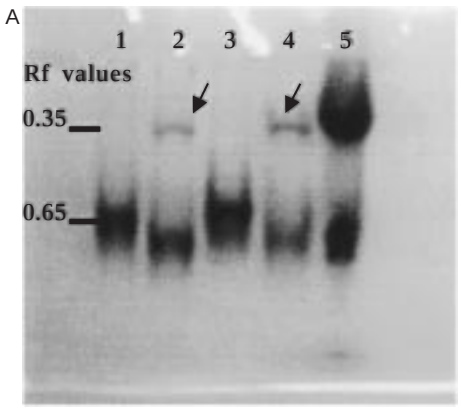

Figure 6A. Detection of malate dehydrogenase (MDH) isozyme bands of *P. myriotylum* CRPm in roots of infected cocoyam plantlets. Lanes: 1. healthy plantlets (2 days); 2. cocoyam-inoculated with CRPm for 2 days; 3. healthy control plantlets (3 days); 4. plantlets inoculated with CRPm for 3 days; and 5. pure culture of CRPm. R_f values were calculated as a ratio of the distance moved by the band over distance moved by tracking dye (bromophenolblue). Total protein in plant extracts was the same. Note slight increased staining of isozyme band with $R_f = 0.35$ from inoculation for 2–3 days. Non-inoculated plantlets did not stain for this band.

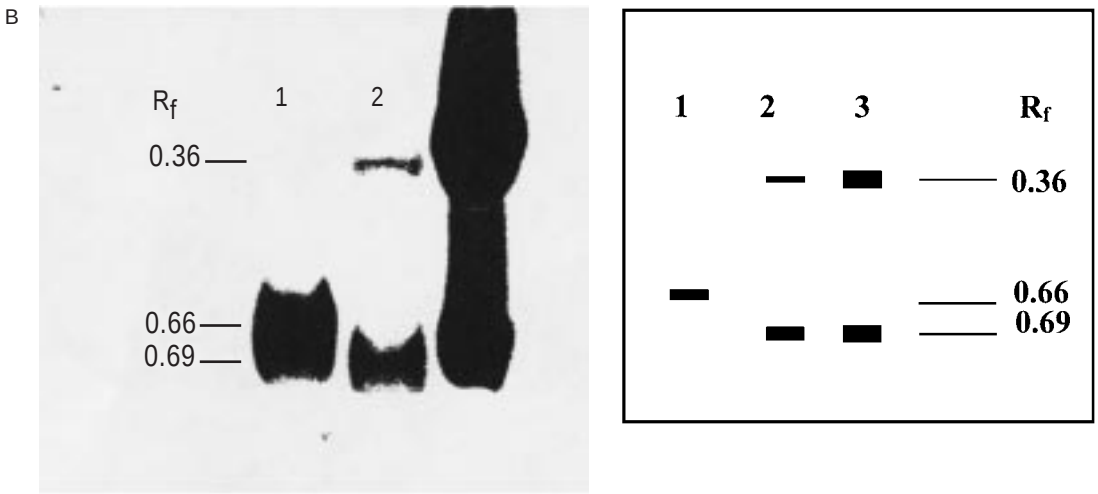

Figure 6B. Detection of malate dehydrogenase (MDH) isozyme bands of *P. myriotylum* CRPm in roots of cocoyam plants grown in volcanic soils of Cameroon. Plants were plug-inoculated as described by Xue et al. (1998). Lanes: 1. non-inoculated cocoyam plants; 2. roots of plants inoculated with *P. myriotylum* CRPm; 3. pure mycelia cultures of CRPm. Note the appearance of isozyme band $R_f = 0.36$ on plants inoculated with CRPm and the non-existence of band $R_f = 0.66$ observed in non-inoculated plants. Roots were collected four days after inoculation.

maize, beans, cowpea, and other crops is common, but no occurrence of damping-off attributed to *Pythium* spp. has been reported for other crop species except cocoyam. Schneider et al. (1988) reported the existence of intraspecific variability in pathogenicity within a *Pythium* species in rice. Exposure of geographically isolated populations to non-preferred hosts or a variable array of hosts would result in new selection pressure and added genetic diversity (Bultin, 1987) leading ultimately to fragmented or new species (Schneider et al., 1988). Following this discussion, it could be postulated that the cocoyam root rot disease pathogen is associated with *P. myriotylum* but probably exhibits special characteristics. *P. myriotylum* isolates have an optimum growth temperature of 37 °C (CMI description No. 118) but both CRPm and Bokwai grew best at 28 °C with no growth at 37 °C; de Cock (unpublished) observed differences in growth rate of isolate Bokwai with differences in the ornamentation of its oogonia compared to other *P. myriotylum* isolates. However, the restriction pattern of the ribosomal intergenic spacer of isolate Bokwai was not different from other *P. myriotylum* isolates (de Cock, unpublished).

Another primary objective of this investigation was to determine whether isozyme banding patterns of the isolates could be specific and as such used for their characterisation. Clare et al. (1968) suggested that variation in protein and isozyme banding patterns could be used for identification. Contrarily, Chen et al. (1992) were unable to identify *Pythium* species using isozyme techniques. The use of isozyme techniques to characterise or identify an isolate depends on the choice of the enzyme(s) and on the initial population studied. Of all the enzymes studied, esterase provided a base for identification of isolates CRPm and Bokwai from the other isolates (Table 4) supporting Clare et al. (1968). This is not surprising because esterases are non-regulatory and as such display more genetic variation than do regulatory enzymes involved with energy metabolism. Micales and Bonde (1995) suggested that enzymes with high degrees of variability, such as esterases, are useful 'fingerprinting' tools. Jaccard's coefficients distinguished *P. splendens*, IMI 06143R, myt14 and NS2 from the population studied with a range of similarity values from 0.00 to 0.21. It is logical that *P. splendens* was distinguished because it belongs to another species, but it is difficult to explain the high dissimilarity of myt14, NS2 and IMI 06143R with respect to the other isolates of *P. myriotylum* species. Densitometric analyses of esterases of *Pythium* isolates from Cameroon revealed completely different profiles. None of the profile peaks were shared by CRPm, myt14 and NS2. Profiles of CRPm and Bokwai were identical for esterases, which generally exhibit very high variability. This supports the fact that isolates myt14 and NS2 may not be of the same species. Myt14 and NS2 were re-identified by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands as *P. inflatum* and *P. vexans*. Isolate IMI 06143R exhibited the highest similarity coefficient (0.58) to myt14 (*P. inflatum*) which indicates that the former isolate might not have been correctly identified.

A third objective of this study was to assess whether isozyme analysis could be used to detect *Pythium* pathogens *in planta*. Localisation of β-glucosidase bands in infected tomato roots corresponding to that of the pure culture of *P. splendens* suggests that this enzyme probably plays an important role in its pathogenicity mechanism. *Pythium splendens* probably produces these glucosidases for metabolism of sugars present within the plant.

We were able to detect isolate CRPm in inoculated cocoyam plantlets via isozyme analysis *in vitro* and *in vivo* (volcanic soil). β-glucosidase and malate dehydrogenase (MDH) were used to confirm pathogenicity of CRPm on cocoyam. Isozyme bands of β-glucosidase were not detected in plant samples, probably because of their relative low concentrations or because the mechanism of pathogenicity of CRPm does not directly involve this enzyme. However, consistent and reproducible MDH bands were localised in roots of plantlets inoculated with CRPm. Plantlets inoculated with CRPm exhibited two bands which corresponded to those of pure mycelia of CRPm (Figure 6A and B). The cocoyam band ($R_f = 0.65$, Figure 6A, or $R_f = 0.66$, Figure 6B) did not appear on zymographs of cocoyam inoculated with CRPm. This biochemical approach supports the pathogenicity results of Nzietchueng (1983) and Pacumbaba et al. (1992) who reported that *P. myriotylum* is the sole causal agent of the cocoyam root rot disease. On the contrary, Agueguia et al. (1991) suggested that the root rot disease of cocoyam is caused by a consortium of three pathogens: *Rhizoctonia solani* Kühn, and *Fusarium solani* (Mart.) Sacc., which are always associated with *P. myriotylum* in rotted roots. This blight complex theory suggested that *P. myriotylum* could not attack mature tissues and as such required assistance from more aggressive pathogens. Detection of isozyme bands of isolate CRPm in root tissues is evidence that

this *Pythium* isolate is able to penetrate cocoyam tissues and can itself cause the root rot disease.

In conclusion, this investigation has shown that the cocoyam root rot disease pathogen may not have a wide host range. This is also supported by field observations in Cameroon in traditional farming systems where cocoyam is intercropped with a variety of other crops. The use of isozyme techniques to characterise the cocoyam root rot disease pathogen was achieved with esterase, a non-regulatory enzyme, being the most effective. A biochemical (isozyme) approach was used for the first time to support the theory that *P. myriotylum* alone can cause the cocoyam root rot disease rather than the blight complex involving *P. myriotylum*, *R. solani* and *F. solani*.

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