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Induction and characterization of pathogenesis-related proteins in roots of cocoyam (*Xanthosoma sagittifolium* [L] Schott) infected with *Pythium myriotylum*

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Although *Pythium myriotylum* is a very destructive root pathogen of cocoyam, the host defense response in this plant-pathogen interaction has not been fully studied. Four cocoyam germplasm accessions were inoculated with *P. myriotylum*, and their induced defense responses were characterized. The induction and spatio-temporal accumulation of chitinase and β-1,3-glucanase were determined by enzymatic activity assays of crude root extracts from inoculated and non-inoculated (control) plants, sampled at 0, 2, 4, 6 and 8 days post inoculation (dpi). Furthermore, induced proteins were extracted from roots of inoculated and control tolerant (RO1054 and RO3015) and susceptible (RO2063) accessions at 8 dpi, and characterized by isoelectric focusing (IEF), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. Chitinase and β-1,3-glucanase were consistently produced in high amounts in the roots of the tolerant accession RO1054, 8 days after inoculation. SDS-PAGE and immunoblotting showed that induced chitinases (37, 35 and 33 kDa) in the tolerant cocoyams were immunologically related to PR-3a purified from barley leaves inoculated with *Erysiphe graminis* f. sp. *hordei*, and induced osmotins (42-45 kDa) were immunologically related to osmotins purified from cultured NaCl-adapted tobacco protoplasts. These results suggest that tolerance in cocoyam infected with *P. myriotylum* may be associated in part with the production of pathogenesis-related (PR) proteins including one hydrolytic enzyme of known antifungal activity (PR-3).

Key words: β-1,3-glucanase, chitinase, cocoyam, PR protein, *Pythium myriotylum*, osmotin.

INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium* (L) Schott), in the family Araceae, is a staple food for millions of inhabitants in the humid tropics and subtropics where tubers and leaves are consumed as a source of carbohydrates, minerals and vitamins. Nigeria is the world's largest producer where production increased from 3.89 million

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Table 1. Morphological characteristics and putative levels of resistance of cocoyam accessions used for the study

Accession[a]	Color of petiole/petiole base	Tuber flesh color	Reaction to *P. myriotylum*[b]
"Local White"	Green/white	White	Susceptible
RO1054	Green/green	White	Tolerant
RO2063	Purple/pink	Pink	Susceptible
RO3015	Purplish/white	Yellowish/white	Fairly resistant

[a]Accessions RO1054, RO2063 and RO3015 were selections of the Cocoyam Breeding Program, Institute of Agricultural Research for Development (IRAD), Ekona, Cameroon. [b]Data obtained from Jay P. Johnson Biotechnology Laboratory, ROTREP Cocoyam Breeding Program, Institute of Agricultural Research for Development (IRAD), Ekona, Cameroon.

tons in 2000 to 5.068 tons in 2007 (Olagunju and Adesiji, 2011). The cocoyam root rot disease caused by *P. myriotylum* Drechs is the most damaging disease of cocoyam. As part of an ongoing research effort to improve cocoyam, notably in the development of cultivars that are resistant/tolerant to the root rot disease, the causal pathogen has been well characterized (Pacumbaba et al., 1992; Pacumbaba, 1996; Tambong et al., 1999). Although resistance or tolerance to the cocoyam root rot disease has been observed in some cocoyam types, however, the mechanism of tolerance/resistance is not known.

Plants possess various defense mechanisms that enable them to survive under biotic or abiotic stress. Among several defense tactics, induced defense mechanisms against plant pathogens which involve the hypersen-sitive response, reactive oxygen species, and pathogenesis-related (PR) proteins have received considerable research attention in many crop species (Hammond-Kosack and Jones, 1996; Rahimi et al., 1996; Reiss and Bryngelsson, 1996; Van Loon, 1999). Pathogenesis-related (PR) proteins are a group of plant proteins some of which are normally present in low amounts in the non stressed plant, but whose production becomes massively increased and new ones induced, when plants are subjected to biotic or abiotic stresses. Many of these proteins associated with host defense have been characterized as chitinases, ß-1,3-glucanases, peroxidases and thaumatin-like proteins (TLP). Among the PR proteins, chitinases, glucanases and osmotins expressed in plants attacked by fungi are thought to limit fungal growth. This antifungal biological function has been demonstrated *in vitro* against several fungi (Anfoka and Buchenauer, 1997; Ji and Kie, 1996; Lawrence et al., 1996; Yun et al., 1996; Zhang et al., 1996). The hydrolases, chitinase and glucanase which degrade the ß-1,4-linkage between N-acetylglucosamine residues of chitin and glucans in fungal cell walls, and thaumatin-like proteins are also known to be induced in plants by adverse environmental factors (Bowles, 1990; Cruz-Ortega and Ownby, 1993; Hincha et al., 1997; Yalpani et al., 1994).

Another indirect role of ß-1,3-glucanases and chitinases is that they act as elicitors of defense reactions, that is they release oligosaccharides from cell walls of both fungi and plants that in turn activate the accumulation of phytoalexins, extensins, proteinase inhibitors and lignin in the attacked host plant (Ham et al., 1991). PR proteins occur in many plants where they also play a developmental role (Leubner-Metzger and Meins, 1999).

Due to the involvement of PR proteins in host resistance, genes encoding such PR proteins could be exploited to engineer resistance in crops (Sharma et al., 1993). The root rot problem in cocoyam could be solved if genes conditioning resistance against *Pythium myriotylum* were identified and either bred in or cloned and genetically engineered into cocoyam. A logical first step would be to understand the host resistance response that is deployed during pathogen infection or attempted infection. Also, identified genes may be targeted for over-expression to develop resistant cultivars. Broglie et al. (1991) found that transgenic plants over-expressing a basic bean chitinase exhibited increased resistance against *Rhizoctonia solani*.

The objective of this study was to determine and characterize host-specific defense related proteins induced in cocoyam, their serological relationship to known PR proteins and if there is temporal accumulation of these PR proteins in response to *P. myriotylum* infection.

MATERIALS AND METHODS

Plant inoculation and protein extraction for enzymatic activity assays

Five plants from each of the cocoyam breeding accessions RO3015, RO2063, RO1054 and "Local White" (Table 1) were inoculated at the roots with a suspension of 1×10^4 mycelial fragments/ml or zoospores of *P. myriotylum* or distilled water in a split root system. *P. myriotylum* used for inoculation was a gift from Dr. Tambong. A hyphal tip was grown on lima bean sucrose agar (LBSA, pH 7.0) at 31°C in the dark for 5 days prior to being used for inoculum preparation. The inoculum was derived by macerating 5-day old flocculent mycelia cultures with distilled deionized water in a Waring blender. The final inoculum concentration was adjusted to 1×10^4 mycelial fragments per ml. The split root system was set up such that about 8-10 cocoyam roots in the potted soilless medium were carefully extended through the holes at bottom of the pot into
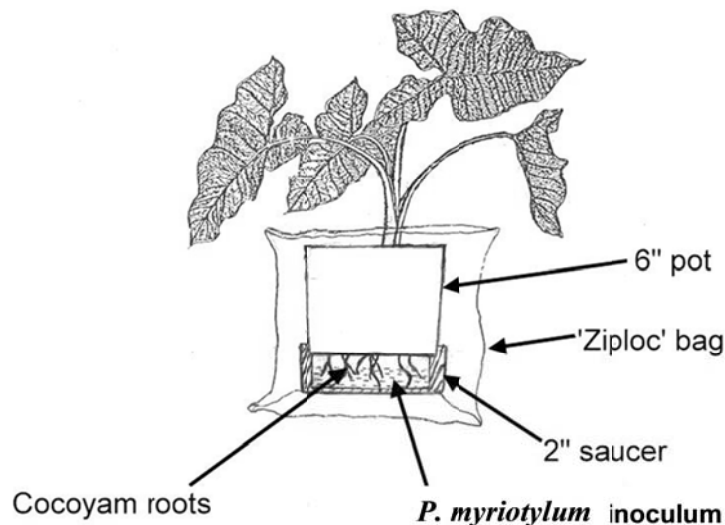
Figure 1. Schematic of the split root method used in inoculating cocoyam plants to study induced defense responses. The inoculated roots were maintained in the pathogen inoculum until time of sample collection.

a Pm inoculum containing saucer placed outside the bottom of the pot, while the rest of the roots remained undisturbed in the potted soil (Figure 1). This set up was used in order to exclude pathogen proteins in the analysis. During sample collection, only non-inoculated root samples from inoculated and control plants, were collected at 0, 2, 4, 6 and 8 days post inoculation for protein extraction and further analysis by specific enzymatic activity assays.

For SDS-PAGE, IEF and Immunobloting, non-inoculated root samples from control and inoculated RO1054, RO2063 and RO3015 plants, were collected 8 days after inoculation for protein extraction and analysis. Since there was no resistant cocoyam, these accessions were selected to represent tolerant (partially resistant) and susceptible cocoyams only. The eighth day after inoculation was chosen for sample collection because it represented the time point where the maximum enzyme activity for inoculated sample plants of these accessions was recorded in preliminary experiments. Root samples were stored at -70°C for 48 h followed by crude protein extraction. The frozen roots were ground (10:1; w/v) in extraction buffer (pH 6.8), containing 0.1n M Tris-HCl, 1% (w/v) polyvinylpyrrolidone (PVP:40) and 1% (v/v) 2-mercaptoethanol, with a mortar and pestle at 4°C. Samples were sieved through autoclaved cheesecloth and centrifuged at 15,000 rpm for 20 min at 4°C.

Concentration and partial purification of samples

After centrifugation, the supernatant was mixed (1:5 v/v) with ice-cold acetone, incubated at -20°C for 2 min to precipitate proteins (Bollag et al., 1996) or was precipitated with ammonium sulfate at 85% saturation (Bollag et al., 1996). The precipitate was centri-fuged at 10,000 rpm for 10 min at 4°C. The protein pellet was re-dissolved in 0.1 M Tris-HCl, pH 6.8, containing 5% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol. Aliquots of 100 µl were placed in 1.5 ml microfuge tubes and stored at -70°C until used. Protein samples were desalted and further purified using Micro Bio-Spin® 6 chromatography columns (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein concentration of the purified sample was then determined.

Determination of protein concentration

The protein concentration in all samples was measured according to the method of Bradford, using a protein kit (Bio-Rad, Hercules, CA) and samples were used for electrophoresis without further purification.

Assays for enzymatic activity

Chitinase assay

The substrate used for this assay was carboxymethyl chitin remazol brilliant violet (CM-chitin RBV, 2 mg/ml; Lowe Biochemica, Germany). The reaction mixture in a 1.5 ml microfuge tube consisted of 100 µl of substrate, 200 µl of 0.1 M sodium acetate buffer, pH 5.0 and 100 µl crude protein extract from cocoyam roots. The mixtures were incubated for 1, 10, 20 and 30 min at 37°C. The reacted samples were centrifuged at 5000 g for 10 min and supernatant read at 550 nm. The log phase of the slope of the resultant curve was used to calculate the enzymatic activity, which was expressed as units/µg protein. One unit was equivalent to 1 µg CM-chitin hydrolyzed per minute.

ß-1,3-glucanase assay

Carboxymethyl-curdlan remazol brilliant blue (CM-curdlan RBB, 4 mg/ml; Lowe Biochemica, Germany) was used as glucanase substrate. The assay mixture contained 100 µl of substrate, 200 µl of 0.1 M sodium acetate buffer, pH 5.0 and 100 µl crude protein extract. The mixture was incubated at 37°C at 1, 15, 30 and 60 min, then centrifuged at 5000 g for 10 min and supernatant read at 595 nm (Microplate Reader Model 550; Bio-Rad, Hercules, CA). Enzymatic activity was determined as described for chitinase. One unit of activity was equal to 1 µg CM-curdlan hydrolyzed per minute.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was

carried out on 0.75 mm 18% (30:0.8 acrylamide/bis-acrylamide) slab gels to determine purity of the crude protein extracts, differences between samples, and resolve proteins for western blotting, according to the protocols of Bollag et al. (1996). The resolving gel was prepared by combining acrylamide (18.48%), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 50 µl 10% ammonium persulfate and 5 µl TEMED. The stacking gel (5%) contained 5.159% total acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.04% SDS, 30 µl of 10% ammonium persulfate and 5 µl TEMED. Mini gels (6 cm x 8 cm x 0.75 mm) were cast in slabs according to the BIO-RAD mini gel procedures (Bio-Rad, Hercules, CA). After gels were cast, protein samples were combined (4:1) with sample buffer (5x) containing 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue. The solution was mixed and heated at 95°C for 4 min, centrifuged briefly (1 s) and sample volumes corresponding to approximately 20 µg was loaded in each well. Samples were resolved at 150 V constant voltage for 1.4 h. Proteins were fixed and stained for 8-10 min in Coomassie gel stain solution containing 0.1% Coomassie Blue R-250, 45% methanol and 10% glacial acetic acid. Gels were destained overnight in a solution containing 10% each of methanol, and glacial acetic acid, photographed and image processed using the AlphaImager® computer software. The molecular masses of resolved proteins were estimated by coelectrophoresis of marker proteins (Bio-Rad) of molecular masses ranging from 6.5 to 200 kDa.

Isoelectric focusing and detection of chitinase and glucanase in overlay gels

Isoelectric focusing was carried out on 0.4 mm gels using the Bio-Rad Mini IEF Cell (Bio-Rad, Hercules, CA). The gels were prepared by combining 2 ml 24.25% acrylamide+0.75% bis-acrylamide, 0.5 ml water-free glycerol, 500 µl 3/10 ampholyte and 5.5 ml distilled water. After degassing the solution for 5 min, 15 µl ammonium persulfate, 50 µl 0.1 % riboflavin and 3 µl TEMED were added and gels cast according to the equipment manufacturer's protocol. Protein samples were loaded (5 µl, approx. 4.5 µg/well) directly on the gel. Samples were focused for 15 min at 100 V, 15 min at 200 V and 1 h at 450 V. Overlay slab gels (7.5%) were cast according to the protocol of Bollag et al. (1996). They contained either 0.04% CM-Chitin RBV or CM-Curdlan RBB in 0.2 M sodium acetate buffer, pH 5.0.

Detection of enzyme activity on overlay gels

After IEF was complete, the focused gels were overlaid with the overlay gels and the gels sandwich incubated under moist conditions at 37°C overnight (12 h). Chitinase was detected by incubating the overlay gel in freshly made 0.01% (w/v) fluorescent brightener 28 (Calcofluor white M2R) in 0.5 M Tris-HCl (pH 8.8) at 26°C for 10 min. The gels were rinsed in distilled deionized water at 26°C for 10-15 min. Chitinase isozymes were visualized as clear zones on a UV light source. Gel pieces representing different bands were cut and incubated in distilled deionized water overnight after which the pH of each piece was determined.

Western blotting

SDS-PAGE was carried out using 18% polyacrylamide gels as described earlier. After SDS-PAGE, proteins were transferred onto Immun-Blot® PVDF or nitrocellulose membranes using the Bio-Rad Mini Transblot® electrophoretic transfer cell (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Electroblotting was completed in 1 h at 100 V, constant voltage. After electro-transfer,

the membrane was blocked for 1 h with 1% (w/v) BSA in Tris buffered saline (TBS; 20mM Tris, 500mM NaCl, pH 7.4) containing 0.05% Tween 20 (TBS-T) and washed with TBS-T twice for 15 min each. The primary polyclonal antibodies for chitinase (PR-3) and glucanase (PR-2) were gifts from Dr. Tomas Bryngelsson; the antibodies were raised from barley leaves inoculated with the powdery mildew fungus, *Erysiphe graminis* f. sp *hordei* syn. *Blumeria graminis* f. sp *hordei*. Anti-osmotin antibody raised against osmotin purified from NaCl- adapted cultured tobacco cells, a gift from Dr. Meena Narsimhan, Purdue University, USA, was also used. Anti-PR-3 anti-chitinase and anti-PR-2 anti-glucanase antibodies were diluted 1:250, while anti-osmotin was diluted 1:3000, in blocking buffer. The antibodies were incubated in this solution overnight (11 h) at room temperature. The next day, the membranes were washed in two changes of TBS-T each for 15 min and subsequently incubated for 2 h in the secondary antibody diluted 1:1000 (goat anti-rabbit IgG [H+L] alkaline phosphatase conjugate in 10mM Tris, 150mM NaCl, 1mM $MgCl_2$, pH 8.0) (Bio-Rad, Hercules, CA) for chitinase and glucanase, and 1:5000 (rabbit anti-chicken IgG [H+L] alkaline phosphatase conjugate in 10mM Tris-HCl, 250 mM NaCl, pH 8.0) (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for osmotin, with blocking buffer, followed by two washes with TBS-T as above. The protein bands were revealed by incubating the membranes with constant shaking in 25 ml of alkaline phosphatase substrate containing 250 µl nitroblue tetrazolium (NBT) in aqueous dimethyl formamide [DMF] containing $MgCl_2$ and 250 µl BCIP (5-bromo-4-chloro-3-indoyl phosphate in DMF). Color development was stopped, by rinsing the membranes three times in distilled water.

Statistical analysis of data

Enzymatic activity measurements were subjected to the analysis of variance procedure. Treatment means were compared using Tukey HSD test ($p \leq 0.05$). The SAS statistical software was used in all data analyses.

RESULTS

Chitinase activity

There was a significant increase in chitinase activity in roots of all inoculated plants, compared to the controls. Constitutive levels of chitinase activity ranged from 10-60 units/µg of protein. Chitinase activity increased up to 4-fold in roots of inoculated RO1054 (tolerant) plants at 8 dpi (Figure 2A). In the susceptible "Local White", chitinase activity also increased significantly (up to 6-fold) in roots of inoculated plants at 6 dpi (Figure 2B), and then sharply declined. The time course of chitinase activity in the tolerant RO3015 showed significant activity in roots of inoculated plants at 4, 6 and 8 dpi (Figure 2C), while in the susceptible RO2063, high and significant chitinase activity was observed at 6 and 8 dpi (6-fold increase) (Figure 2D). These results show there was a systemic increase in chitinase activity, in cocoyam roots resulting from inoculation with *P. myriotylum*.

ß-1,3-glucanase

ß-1,3-glucanase activity was only significantly induced in
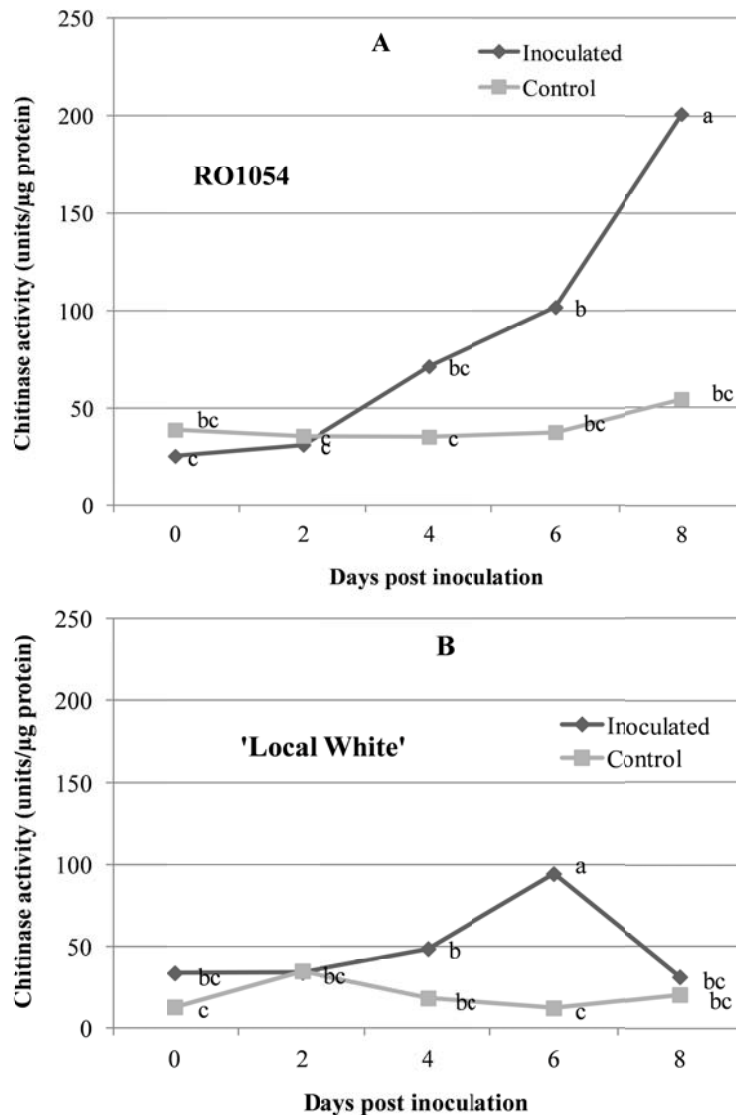
Figure 2. Chitinase activity in non inoculated roots of cocoyam accessions RO1054 (**A**), 'Local White' (**B**), RO3015 (**C**) and RO2063 (**D**) infected with *P. myriotylum*.

roots of inoculated tolerant RO1054 at 8 dpi (Figure 3). No significant increase in activity occurred in roots of inoculated RO2063, "Local White" and RO3015 (data not shown). Based on these results, it could be concluded that the induction of ß-1,3-glucanase activity was mainly associated with roots of the tolerant cocoyam RO1054 as roots of inoculated susceptible plants showed no apparent change in ß-1,3-glucanase activity.

IEF

Two basic chitinase isozymes (pI 8.5 and 8.6) exhibiting activity in overlay gels (visualized as clear zones in UV light), were detected in roots of inoculated RO3015,

RO2063 and RO1054 (Figure 4). The isozymes were constitutively present in RO2063 and RO1054, but their activity increased in inoculated plants as shown by the intensity of the bands. Evidently, more of the protein was expressed in RO2063 and RO1054 compared to RO3015. Another isozyme (pI 8.3) was weakly expressed in roots of inoculated RO2063 and RO1054 only. β-1,3-glucanase activity was not detected in overlay gels using the substrate CM-Curdlan RBB.

SDS-PAGE and Western blot analysis

Polypeptide profiles of protein extracts from roots of inoculated and control RO3015, R02063 and RO1054
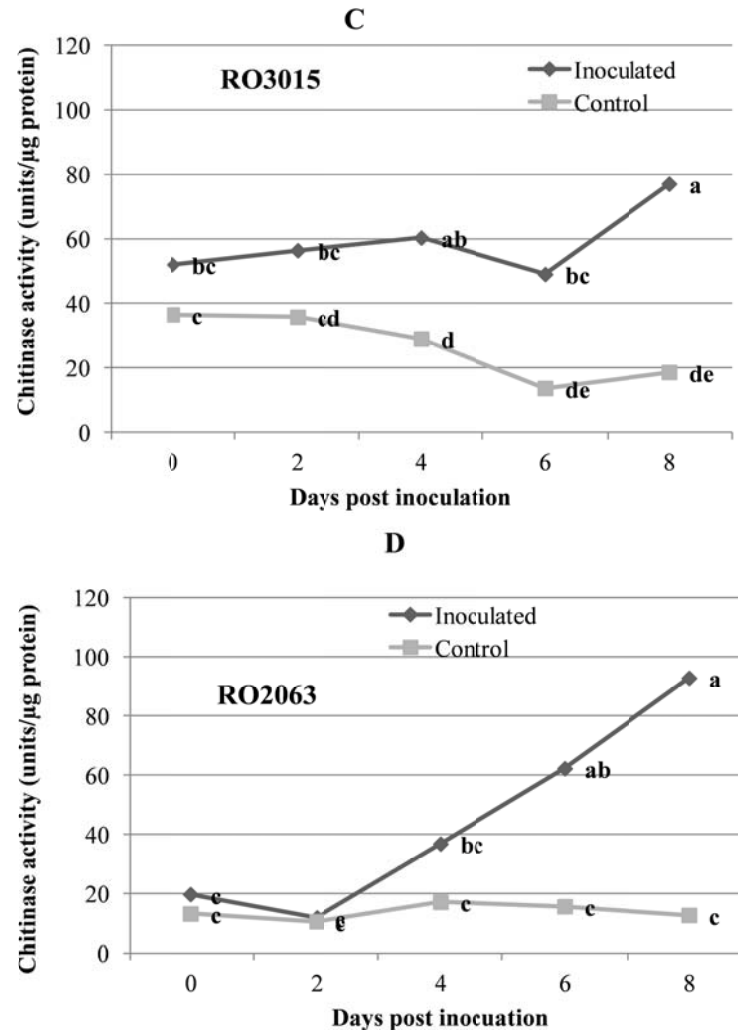
Figure 2. Contd.

plants, sampled 8 days post inoculation, showed only one new protein band (26 kDa) in RO3015, one new band (24 kDa) in RO2063, and three bands (24, 31, 37 kDa) in RO1054. Some protein bands however, were not clearly visible in the gel photographs after processing.

Immunoblotting of proteins using anti-PR-3 raised against chitinase induced in barley leaves inoculated with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, revealed one protein band with a molecular mass of 35 kDa and detected in both inoculated and control plants of RO1054 and RO3015. In addition, one new chitinase band (37 kDa) was detected in inoculated RO1054 and one band (35-36 kDa) detected in inoculated RO3015 and RO2063 plants (Figure 5). The protein band of 33kDa also detected in inoculated plants of RO1054 was detected only in control plants of RO3015. These chitinase bands are isoforms of the PR-3 protein. In tomato, Joosten et al. (1995), purified four chitinase isoforms with molecular weights 26, 27, 30 and 32 kDa belonging to PR-3a, -3d, -3b and -3c respectively. Lawrence et al. (1996), reported similar findings in tomato. The PR-3 protein isoforms detected in cocoyam did not share identical molecular weight with the published isoforms detected in tomato. Anti-osmotin polyclonal antibodies purified from NaCl-adapted tobacco cells, detected a 42-45 kDa protein band in inoculated RO1054, RO2063 and RO3015. This protein was absent in the controls Yc and Rc but present in Wc (RO1054) (Figure 6). This indicates that the protein maybe constitutively expressed in RO1054, an accession which exhibited significant tolerance to *P. myriotylum*.
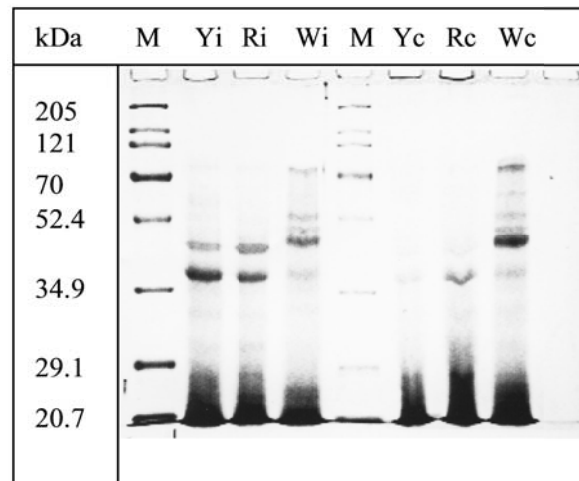
DISCUSSION

The main objective of this study was to investigate host defense mechanisms in susceptible and tolerant cocoyams inoculated with *P. myriotylum*. We observed
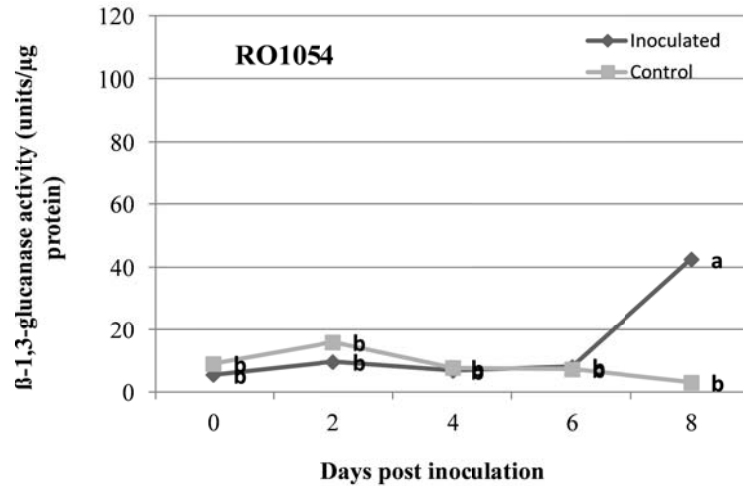
Figure 3. β-1,3-glucanase activity in non inoculated roots of cocoyam accessions RO1054 infected with *P. myriotylum*.
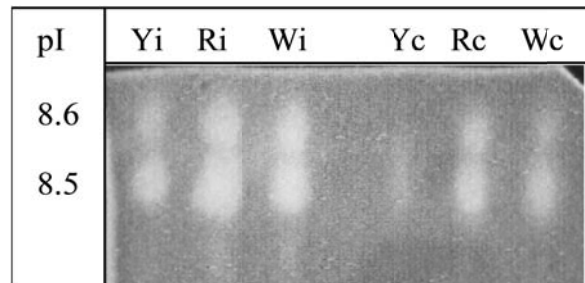

(A)

(B)

Figure 4. Chitinase activity in overlay gel following isoelectric focusing of proteins extracted from roots of inoculated cocoyam accessions RO3015 (Yi), RO2063 (Ri), RO1054 (Wi), and those of their respective controls (Yc, Rc, and Wc). The IEF gels contained 4.5 µg protein/well. SDS-PAGE profile of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc).
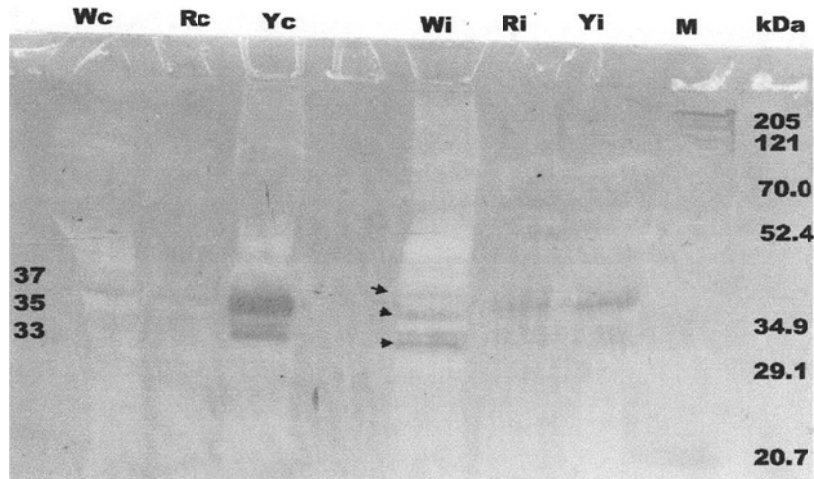
Figure 5. Western blot analysis of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc). Proteins were blotted on PVDF membranes following SDS-PAGE and probed with antibodies specific for PR-3 (chitinase).
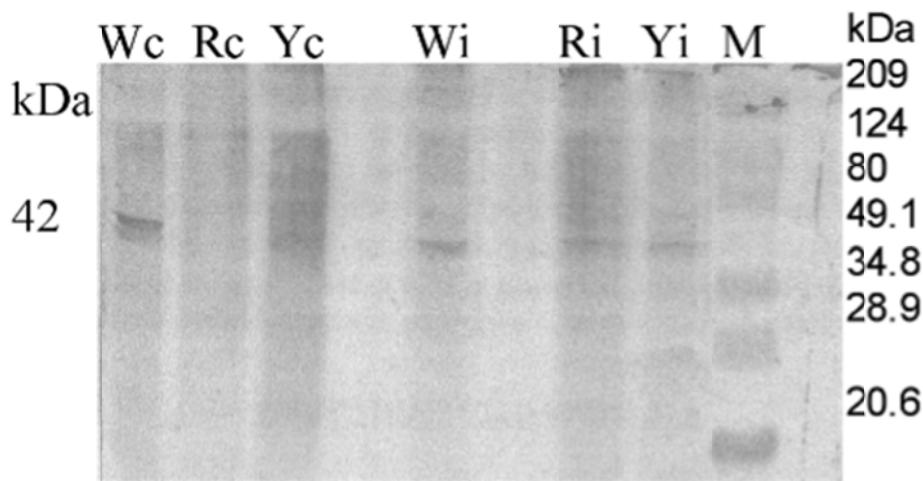
Figure 6. Western blot analysis of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc). Proteins were blotted on PVDF membranes following SDS-PAGE and probed with antibodies specific for osmotins.

and characterized the hydrolytic antifungal cell wall degrading enzymes, chitinase and ß-1,3-glucanase including the thaumatin-like PR protein osmotin, induced in cocoyam roots in response to *P. myriotylum* infection.

In the tolerant cocoyam accession RO1054, inoculated plants showed higher chitinase activity in the roots. Both RO1054 and RO3015, which were more tolerant to *P. myriotylum*, also had higher constitutive levels of chitinase in their roots (Figure 2A and C) compared to the susceptible "Local White" and RO2063 (Figure 2B and D). Roots of inoculated RO2063 plants also showed an increase in chitinase activity, however, the increased chitinase activity in this accession did not lead to

increased resistance.

ß-1,3-glucanase activity was induced only in RO1054. This activity was relatively low compared to chitinase induction. ß-1,3-glucanase activity was significantly expressed in RO1054 only at 8 dpi. This could be due to a slow accumulation that could possibly increase beyond 8dpi. The low levels of ß-1,3-glucanase activity observed in the tolerant cocoyam (RO1054) suggest that this enzyme may not have a very significant role in cocoyam host defense against *P. myriotylum*. The strong induction of chitinase in the inoculated plants of the same type of cocoyam at 8 dpi compared to the control, could be defense-related in that it may be associated with expres-

sion of induced systemic resistance to any subsequent nonspecific or specific pathogen of cocoyam capable of attacking an already pathogenically challenged cocoyam plant. Tolerance (partial resistance) in RO1054 could be contributed by the synergistic induction of these two hydrolases. In many host-pathogen interactions involving active defense or induced resistance, the pathogenesis-related (PR) endohydrolases with chitinase or β-1,3-glucanase activity, including the thaumatin-like proteins, have been detected in the attacked plant (Anfoka and Buchenauer, 1997; Rahimi et al., 1996; Reiss and Bryngelsson, 1996; Van Loon, 1999). β-1,3-glucanase and chitinase are produced in many active defense responses involving fungi. The substrates for these enzymes are present as structural components of several fungal pathogens. Evidence from *in vitro* studies shows that the action of these enzymes leads to inhibition of fungal growth or hyphal lysis (Mauch et al., 1988; Woloshuk et al., 1991). However, it is unclear why chitinase accumulates in plants attacked by oomycetes since the oomycetes lack chitin in their cell wall. It appears therefore, that the accumulation of chitinase in this host-pathogen interaction is either induced by nonspecific elicitors or part of the induced resistance repertoire, which mainly helps to strengthen the plant's defense system in preparation for subsequent pathogen attack.

In Western analysis, using anti-osmotin polyclonal antibodies from NaCl-adapted tobacco cells, we detected a single protein band (42 kDa) commonly expressed in the roots of the inoculated accessions. Proteins of the PR-5 family have been placed into two major groups based on their cellular localization. Among these proteins, the osmotin-like proteins, which are said to be basic and mainly vacuolar (Jacobs et al., 1999; Kombrink and Somssich, 1995), are known to exhibit antifungal activity (Velazhahan et al., 1999).

This is the first time a study examining induced defense response in cocoyam has been undertaken. Apart from work done on a related aroid (*Colocasia esculenta*) in which cultivars were screened for induction of PR protein in response to infection by *Phytophthora colocasiae* (Ho and Ramsden, 1998), no attempts at understanding the mechanism of cocoyam resistance to the root rot disease have been undertaken at the molecular level.

In this study, we observed a significant increase in chitinase activity in the roots of both tolerant and susceptible cocoyam infected with *P. myriotylum*, however, multiple chitinase bands were expressed only in roots of the tolerant cocoyam. Our results also show that the closely related thaumatin-like (TL) protein, osmotin (PR-5), is induced in cocoyam in response to *P. myriotylum* attack. The increased chitinase and β-1,3-glucanase activity in healthy roots of RO1054 infected with *P. myriotylum* compared to the control, shows that these PRs were systemically induced. Infected roots of inoculated plants were generally avoided in order to

exclude pathogen proteins in the analysis. It is apparent, however, that induction of these proteins including osmotins, does not afford complete protection from the invading pathogen. Although osmotins have been shown to be inhibitory to several fungi including the oomycete *Phytophthora infestans* (Woloshuk et al., 1991), it seems members of the PR5 group may be specialized in their functions, for example, possession of antifungal activity against only selective pathogens (Yun et al., 1996). Complete protection from infection would probably require high and rapid accumulation of hydrolytic enzymes and antifungal proteins or accumulation of yet other unidentified antimicrobial compounds in the cocoyam roots. This work only sets the stage for one approach to understanding disease resistance/tolerance in cocoyam to *P. myriotylum*. Revealing the relatively weak defense capability of cocoyams attacked by a virulent isolate of *P. myriotylum* is of interest not only because it increases our understanding of the nature of cocoyam defenses but could offer researchers more rational and viable strategies for the effective control of this pathogen on cocoyam. For example, genetic studies in future research could identify genes in resistant plants expressing high levels of constitutive antifungal proteins that could be engineered into the susceptible consumer preferred cultivars to enhance disease resistance.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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