X. Campestris pv. *dieffenbachiae*, the Causative Agent of Common Leaf Blight Disease in Dasheen (*Colocasia* sp.) and Cocoyam (*Xanthosoma* sp.)

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Dasheen (*Colocasia* sp.) and cocoyam (*Xanthosoma* sp.) are nutritionally and medically rich tuber crops that grow readily in tropical and sub-tropical regions where favorable growing conditions exist. However, they are affected by numerous pests and diseases. In the present study *E. chrysanthemi* pv. *dieffenbachiae*, *X. campestris* pv. *dieffenbachiae*, *P. syringae* and a non-fluorescent *Pseudomonas*, are associated with the common leaf blight disease. However, the causative agent, *X. campestris* pv. *dieffenbachiae*, was found to be host specific producing symptoms of common leaf blight disease infection on dasheens and cocoyams and a hypersensitive response on the non-host pepper (*Capsicum chinense*). DNA fingerprinting analyses of *X. campestris* pv. *dieffenbachiae* indicated sequences from the hypersensitive response gene, *hrp*B6 and *hrp*G, and the ORF1-ORF2 of a pathogenicity gene of *X. campestris* pv. *glycines*. The *hrp* cluster is conserved among pathovars of *X. campestris* and is responsible for infection. The *hrp* cluster can be used as a molecular probe for assessing *X. campestris* pv. *dieffenbachiae* infections. The insertion of the avirulence (*avrBs*3) gene into *X. campestris* pv. *dieffenbachiae* increased the hypersensitive response of pepper and induced resistance in dasheen and cocoyam.

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INTRODUCTION

Dasheen (*Colocasia* sp.) and cocoyam (*Xanthosoma* sp.) grow readily in tropical and sub-tropical regions where they are used as a source of carbohydrate. However, they are affected by numerous pests and diseases including *Papuana* sp. which feeds on the roots of aroids, *Erwinia chrysanthemi*, *Pseudomonas* sp., and the common leaf blight disease [1]. The common leaf blight disease poses a serious threat in tropical and subtropical countries that experience heavy rainfall or dew and an average temperature of 20-30^{o}C. The disease is characterized by white flecks or discolored spots that develop into water-soaked lesions. The lesions often spread and coalesce to form extended chlorotic regions, which frequently degenerate into holes on older leaves and result in leaf dieback and corm reduction [2]. The disease affects the plants at any stage during growth; on young plantlets, necrosis appears along the margin of the cotyledon of the corm, which later dies. As the disease progresses, the veins on the leaves become prominent and the leaf becomes yellow or wilts and finally falls off. The pathogen is capable of surviving in the cormels until the next planting season where it passes from cotyledon to young leaves either directly or through the stomata. The infection occurs later at the margin of the leaves and is propagated though insect-feeding injuries and breathing pores. The pathogen spreads chiefly by running water, infected detached leaves and

contaminated material [2]. Control of the disease using copper-based bactericides has proven ineffective and inadequate [3-4]; therefore, this study was designed to identify the bacterial pathogen responsible for the common leaf blight disease, assess host specificity and the effect of the avirulence gene on pathogenicity.

METHODS

Sample collection

Leaves from dasheen and cocoyam plants showing symptoms of the common leaf blight disease were collected. Cultures of *Xanthomonas campestris* pv. *vesicatoria* strains T-55 and 85:10 were obtained from the Microbiology Laboratory, University of the West Indies and the Plant Pathology Laboratory, Wye College. *Xanthomonas campestris* pv. *vesicatoria* strain T-55, a non-pathogenic opportunistic strain of *X*. *campestris* was used as a negative control while pathogenic strain 85:10 was used as positive control.

Isolation of bacterial pathogen

A modified Paulraj and O'Garro method [2] was used for bacterial isolation from dasheen and cocoyam. Briefly, infected leaves were washed with ethanol and rinsed in hypochlorite solution. Sections of the leaf exhibiting chlorosis or water-soaked lesions were excised and crushed in sterile distilled water. The suspension was streaked onto nutrient yeast agar (NYA) plates, and incubated at 37°C for 3 days; pure colonies were obtained by re-streaking fresh NYA plates.

Biochemical characterization bacterial pathogen from infected dasheen and cocoyam

Catalase, urease, oxidase, and nitrate reductase activities; starch and gelatin hydrolysis and acid production from sugars were tested using Bergey's method [5]: *Catalase activity* – bacterial cultures were incubated with hydrogen peroxide; *oxidase activity* - bacterial cultures were incubated on bacto-oxidase plates; *urease activity* - bacterial cultures were incubated in urea broth; *starch hydrolysis* - bacterial cultures were incubated on bacto-starch agar plates; *gelatin hydrolysis* - bacterial cultures were incubated on bacto-gelatin plates; *acid production from glucose, arabinose, mannose and celloboise* - bacterial cultures were incubated in sugar-free medium prepared from carbohydrate solution; and *resistance to antibiotics* - bacterial cultures were incubated on LB plates containing 50 µg/mL of rifampicin, nalidixic acid and streptomycin.

Bacteria identification

Bacterial isolates were identified using the **Bact**eriological **Id**entification, BACTID, (Natural Resources Institute, UK) and confirmed using the Biolog Microstation System (Biolog Inc.). The biochemical composition of bacterial isolates was determined by standard determinative tests as outlined in the BACTID system, in which pre-selected media were incubated after inoculation with single bacterial colonies.

Tissue culture

Initiation: Koch's postulates stipulates that if a pathogen found associated with a disease is isolated, grown in pure culture, characterized and used to inoculate healthy plants of the same species or varieties from which it was originally isolated it should produce the identical symptoms [6]. Therefore, to test Koch's postulate, tissue culture plantlets were used to compare symptoms of infection to those found in field grown plants. Tissue-cultured plantlets were obtained by a routinely used method developed in the Biotechnology

Laboratory, University of the West Indies. Briefly, small dasheen and cocoyam cormels were washed with water and tween, and then sterilized with distilled water and ethanol. Small segments were placed on initiation media and incubated at 25°C under controlled lighting. *Acclimatization* - *In vitro* grown plantlets were washed with distilled water, planted in a peat:vermiculite mixture in Sorbarod Micro-propagation System (SMS). The SMS was incubated at 25°C with high moisture content and shade cover. The 1st ventilation window was partially opened after 3 days and fully opened after a week while the 2nd ventilation window was opened after 2 weeks. Following the onset of new leaves (~4 weeks) the lid was removed and the plantlets transferred to a greenhouse at 25°C with lower moisture content and shade cover.

Plant inoculations

Single colonies isolated from greenhouse-grown infected dasheen and cocoyam were used to inoculate LB medium and incubated for 24-48 hr at 28°C. Bacterial pellets were obtained from centrifugation was washed and re-suspended in $MgCl_2$ solution. Concentration of the resulting bacterial inoculum was adjusted to $5x10^8$ CFU/ml and injected into plantlets. The plant reactions were observed over 4 weeks.

Bacterial population assessment

0.5 cm^2 discs from the inoculated plantlets were washed with ethanol and homogenized in $MgCl_2$ solution. Serial dilutions were plated onto LA plates containing 50 µg/mL rifampicin and colony counts recorded 24-48 hr post inoculation.

Bacterial DNA isolation

DNA was isolated from *X*. *campestris* pv. *vesicatoria*, and bacterial isolates obtained from greenhouse-grown infected dasheen and cocoyam using a modified CTAB procedure [7]. Briefly, the pellet from the bacterial inoculum was suspended in Tris-EDTA (TE), SDS and proteinase K and incubated. After initial incubation, CTAB-NaCl mixture was added and the mixture re-incubated. Chloroform extraction was repeated 3 times, ice-cold isopropanol added to the aqueous phase. The pellet was dissolved in TE at room temperature and the DNA stored at 4°C.

Amplification of bacterial DNA

Reaction mixture contained 10X PCR buffer, $MgCl_2$, dNTP, primer pair, *Taq DNA* Polymerase, 50 ng purified DNA and sterile de-ionized water. Amplification consisted of initial denaturation at 95°C for 10 min; 30 cycles consisting of 30 sec of denaturation at 95°C, 30 sec of annealing at 62°C and 45 sec of extension at 72°C. Final extension for RST2-RST3 primer pair was performed at 72°C for 5 min. The annealing temperatures for the other primer pairs used are shown in Table 1.

Restriction endonucleases analysis

Amplified fragments were *Hae*III and *Sau*3AI digested using the Sambrook *et al*. method [8].

Southern Analyses

Transfer of Restricted Bacterial DNA to Nylon Membrane: A modified Southern protocol [9] was used for DNA transfer. Briefly, gel containing amplified DNA was depurinated and placed on NaOH-soaked Whatman 3MM filter paper. Nylon membrane soaked with water, 3 sheets of NaOH-soaked filter paper and paper towels were carefully placed on the gel to facilitate transfer. The membrane was washed with 2X SSC and stored at –20°C. *Probe Labeling*: Qiaex purification kit was used to purify the amplified fragments. The purified fragments

Table 1. Chloroplast- and mitochondria- specific primers

Primer	Sequences (5' – 3')	Primer Pair	Anneal Temp/^{o}C
RST2	AGG CCC TGG AAG GTG CCC TGG A	RST2/RST3	62
RST3	ATC GCA CTG CGT ACC GCG CGC GA		
RST9	GGC ACT ATG CAA TGA CTG	RST9/RST10	54
RST10	AAT ACG CTG GAA TGA CTG		
RTS21	GCA CGC TCC AGA TCA GCA TCG AGG	RST21/RST21	40
RST22	GGC ATC TGC ATG CGT GCT CTC CGA		
XCP1	CCT GGC GCT GCT ACT GAA CG	XCP1/ XCP2	49
XCP2	CCA ACG TCA GGC ATC TGC AT		
XCG1	CCA ATC CCC AGG CAC AGT GG	XCG1/ XCG3	55
XCG2	GCG GCA CTC GCC GTG CGA GC	XCG2/ XCG3	61
XCG3	CAG GCG GCT GCG TGA TGT GC		

were boiled and cooled. 5X OLB, Klenow fragment and ^{32}P dCTP were added to the cooled fragment. The mixture incubated to facilitate denaturation. *Southern Hybridization*: A modified Lanzillo protocol [10] was used to hybridize the membrane to the prepared probe. The hybridized membrane was washed with 2X SSC and exposed to film.

Sequencing of amplified fragment

Purified amplified DNA was sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The sequence mix contained Terminator Ready Reaction mix, primer, 20 g DNA/100 bp PCR product, and sterile de-ionized water. The mixture was amplified using initial denaturation at 95°C for 10 min; 25 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 2 min; and final extension at 60°C for 5 min. Sodium acetate and sterile de-ionized water were added prior to ethanol precipitation. The pellet was washed with ethanol, re-suspended in Template Suppression Reagent and centrifuged. The sample was heated, rapidly cooled and sequenced.

Transformation of bacterial isolates using pD36 plasmid carrying *avrBs*3 gene

avrBs3 transformation of isolates was achieved using tri-parental mating, a procedure developed at Wye College, University of London. The procedure involved the transfer of *avrBs*3 from a donor (*X. campestris* pv. *vesicatoria* with pD36 plasmid carrying *avrBs*3) to a receptor (bacterial isolates) using a pRK2013 helper plasmid. *Isolation of pD36 Plasmid*: *X. campestris* pv. *vesicatoria* containing pD36 plasmid carrying *avrBs*3 gene was grown on rifampicin/streptomycin LB plates. Single colonies were used to inoculate LB broth containing rifampicin and streptomycin prior to incubation. The mixture was centrifuged and the pellet suspended in chilled GET buffer. The mixture incubated on ice after adding freshly prepared SDS/NaOH. Sodium acetate was added to the chilled mixture followed by centrifugation. RNase was to the supernatant which was incubated at 37°C. An equal volume of chloroform was added and the mixture centrifuged. The aqueous was separated and chilled sodium acetate and ethanol added. The mixture was centrifuged and the pellet suspended in de-ionized water. *Transformation of Competent E. coli Cells*: Plasmid DNA and competent *E. coli* cells were chilled on ice and incubated at 42°C. LB broth was added and the sample re-incubated. The transformed cells were grown on LB plate containing

streptomycin. *Tri-parental Mating*: Bacterial cultures grown in rifampicin, *E. coli* transformed with pD36 carrying *avrBs*3 in streptomycin, and *E. coli* carrying pRK2013 in kanamycin were separately incubated at 37°C. Each culture was centrifuged and the pellet suspended in LB broth. Isolates, *E. coli* containing pD36 carrying *avrBs3* and *E. coli* carrying pRK2013 were cultured on King's B media containing rifampicin/streptomycin to select for transformed bacterial isolated.

Assessment of the effects of *avrBs*3 transformed isolates

Bacterial isolates carrying the pD36 plasmid-borne *avrBs*3 gene were used to inoculate LB medium containing rifampicin/streptomycin. The mixture was centrifuged and re-suspended in $MgCl_2$ before being used to inoculate plantlets.

RESULTS AND DISCUSSION

Identification of bacterial isolates

Pathogenic bacteria are found in infected leaves of dasheen and cocoyam plants where evidence of water-soaking, white flecks, die-back of leaf apex and/or chlorosis existed. 43 of 50 bacterial isolates assessed were yellow-pigmented obligate aerobes, gram (-), oxidative (-), catalase (+) and produced acid from glucose, arabinose, mannose and cellobiose. They bore polar flagellum, did not utilize asparagine as the sole source of carbon and nitrogen, and were resistant to streptomycin and nalidixic acid similar to the positive control, *X*. *campestris* pv. *vesicatoria* (Table 2). BACTICD analysis was used to eliminate non-pathogens and further identify the bacterial isolates. Bacterial isolates 2 and 4–7 were gram (-), produced positive reactions with 0.02% TTC but unreactive with 0.1% TTC. Furthermore, they were unable to break down starch, produce a cytochome-c oxidase (+) or convert nitrate (NO_3^-) to nitrite (NO_2^-), dinitrogen gas (N_2) or dinitrogen oxide (N_2O) (Table 3). *Xanthomonas* sp. is unable to convert NO_3^- to NO_2^-, N_2 gas or N_2O and cannot grow on 0.1% TTC. Isolates 1, 3 & 8–10 produced colonies on 0.1% TTC and silver nitrate and therefore were not *Xanthomonas* sp. Bacterial isolates 3, 8 and 9 produced pearly-white shiny high domed and mucoid colonies indicating that they were levan (+). Additionally, these isolates produced a blue-green fluorescence with King's B medium; however, they were unable to produce the pectolytic enzyme that causes potato soft rot, or produces colonies in the presence of 0.1% TTC and NO_3^- which are typical of *Pseudomonas* sp. Bacterial isolates 1 and 10 hydrolyzed starch, was oxidase [+], caused potato soft rot and produced colonies in the presence of 0.1% TTC and NO_3^- which is indicative of *Erwinia* sp. [5, 11]. BACTID and Biolog Microstation System analyses identified bacterial isolates 2 & 4–7 as *X*. *campestris* pv. *dieffenbachiae*, 1 & 10 as *E*. *chrysanthemi* pv. *dieffenbachiae*, 3 & 9 as *P*. *syringae* and 8 as a non-fluorescent *Pseudomonas* (Table 3).

Pathogenicity test

X. *campestris* pv. *dieffenbachiae*, *P*. *syringae* and *E. chrysanthemi* pv. *dieffenbachiae* were individually used to infect greenhouse-grown plantlets. Symptoms observed on X*. campestris* pv. *dieffenbachiae* inoculated plantlets were similar to field-grown plants. Dasheen plantlets inoculated with bacterial isolates 2 and 4-7 revealed water-soaked lesions with a yellow halo and discoloration on day 3 which expanded covering more of the leaf surface before going from yellow to brown over 7 days (Figure 1). Necrotic and chlorotic leaves died 20 days post inoculation. Symptoms of pathogenicity, leaf discoloration, and water-soaked lesions

Table 2. Biochemical and physiological characterization of pathogenic bacteria isolates

Test		Characteristic of isolates	
		Isolates	Control (*X. campestris* pv. *vesicatoria*)
Number of isolates		50	1
Color of colonies		43 - yellow 4 - cream 2 - pale yellow 1 was beige	yellow
Monotrichous flagellation		+	+
Gram staining		Gram (-)	Gram (-)
Starch hydrolysis		+	-
Gelatin hydrolysis		+	+
Asparagine utilization		-	-
Catalase activity		-	-
Urease activity		-	-
Oxidase activity		-	-
Acid production:	Arabinose	+	+
	Cellobiose	+	+
	Glucose	+	+
	Mannose	+	+
Utilization of glucose		-	-
Resistance to antibiotic:	Nalidixic acid	+	+
	Rifampicin	+	+
	Streptomycin	+	+

Key: +: positive reaction; -: negative reaction. Ten representative isolates were selected for identification Table 3.

Table 3. BACTID analysis of the bacterial samples isolated from infected leaves

Isolate no.	KOH	TTC	Oxidase	KMB	Starch	Gelatin	Nitrate	O/F	Leavan	Potato rot
E. chrysanthemi pv. *dieffenbachiae*										
1	+	-	(+)	-	(+)	+	+	-/-	n	n
10	+	-	-	-	(+)	+	+	+/+	n	n
X. campestris pv. *dieffenbachiae*										
2	+	-	-		-	+	-	+/-	n	n
4	+	-	-	-	-	+	-	+/-	n	n
5	+	-	-	-	-	+	-	-/-	n	n
6	+	-	-	-	-	+	-	+/-	n	n
7	+	-	-	-	-	+	-	+/-	n	n
Pseudomonas										
3	+	-	-	+	-	+	-	+/-	+	-
8	+	+	-	-	-	+	-	+/-	n	n
9	+	-	-	+	-	+	-	+/-	+	-

KEY: +: strong positive reaction; (+): weak positive reaction; -: negative reaction; n: not done.
TTC: Triphenyl tetrazolium chloride. KMB: Kings Medium B. O/F: Oxidation/Fermentation. KOH: Potassium hydroxide

developed 5 days post inoculation of cocoyam (Figure 1C). The water-soaked lesions, yellow to brown discoloration and chlorotic regions slowly expanded over the next 7 days. The leaves developed necrosis after 14 days and died in 20 days. Screening of *in vitro* plantlets using *X. campestris* pv. *dieffenbachiae* did not produce any resistant or tolerant variety. According to Koch's postulates, if a pathogen found associated with a disease is isolated,

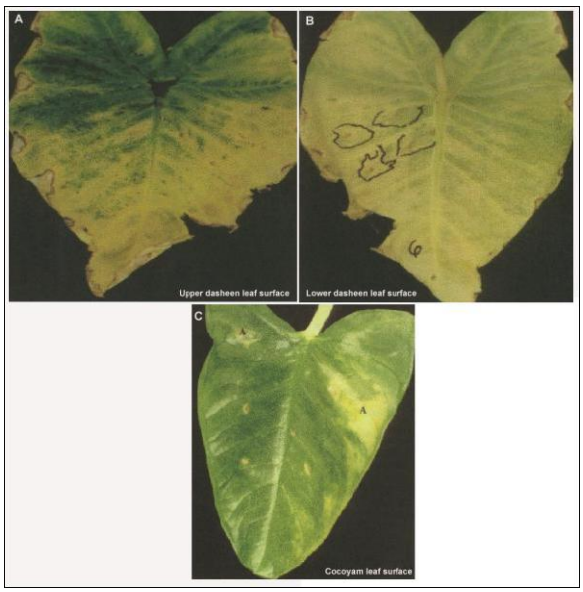

Figure 1. Effects of isolates on dasheen and cocoyam: A/B shows the areas on the dasheen leaf surface that were inoculated using bacterial isolate; the plant died 14 days after inoculation. Image C shows cocoyam leaf 14 days after inoculation with bacterial isolate.

grown in pure culture, characterized and used to inoculate healthy plants of the same species or varieties from which it was originally isolated it should produce the same symptoms [6]. Symptoms of pathogenicity observed on greenhouse-grown plantlets inoculated with bacterial isolates 1, 3 and 8–10 were different from field grown plants, therefore these were discredited as the causal agent of the common leaf blight disease in dasheen and cocoyam. However, those inoculated with 2 and 4–7 had symptoms similar to field grown plants. It was reasonable to assume that the common leaf blight disease in these two aroids was caused by bacterial isolates 2 and 4-7 which were previously identified as *X*. *campestris* pv. *dieffenbachiae*.

Depending on the host plant two reactions are possible: if the plant is susceptible, symptoms of the bacterial infection will be visible and the pathogen-host interaction is compatible; however, if the plant is resistant a hypersensitive response is induced indicating an incompatible interaction. Resistance is determined by gene-for-gene relationship and pathogenic genes for avirulence are matched by genes for resistance in the host. If the host lacks the dominant alleles for resistance it will be susceptible to infection resulting in water-soaked lesions and necrosis which indicates a compatible interaction. However, if matching genes for avirulence is present, a hypersensitive response occurs. The hypersensitive response is a local defense reaction accompanied by rapid necrosis which prevents multiplication of the bacteria [12]. *X*. *campestris* pv. *vesicatoria* inoculated pepper leaf became discolored after 24 hr and died within 2 days, indicating a compatible interaction. Within 24 hr dasheen and cocoyam plantlets inoculated with *X*. *campestris* pv. *vesicatoria* strain 71:21 developed chlorosis and revealed signs of necrosis in 3 days (Figure 2A). The rapid death of the necrotic areas indicated a hypersensitive response to infection and hence an incompatible interaction. Pepper plantlets infected with strain 71:21 manifested symptoms of chlorosis and water-soaked lesions with a pale yellow halo >24 hr post inoculation and died within 3 days (Figure 2B). Symptoms of infection manifested slower with strain 85:10 with the chlorotic and water-soaked leaf dying in 6 days.

Identification of pathogenicity gene

Identification of *X*. *campestris* strains at the pathovar level has been investigated using techniques such as fatty acid analysis and metabolic profiling, serology and SDS-PAGE; however, these methods have proven ineffective [13-14]. PCR amplification of the *hypersensitive response* (*hrp*) gene cluster using primer designed from the conserved and variable regions has been effective for

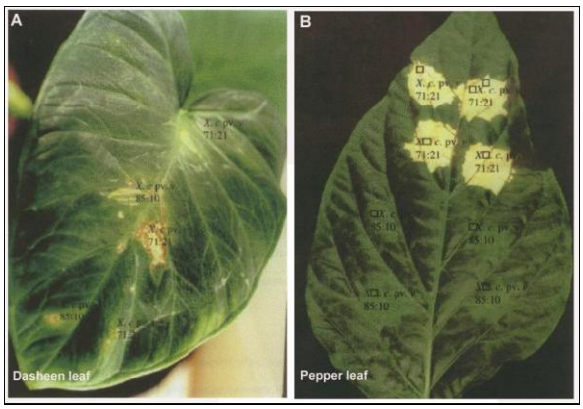

Figure 2. The effect of *X. campestris* pv. *vesicatoria* strains 71:21 and 85:10 on cocoyam and pepper: A shows the effect of *X. campestris* pv. *vesicatoria* strain 85:10 on cocoyam; symptoms of hypersensitive response were visible on the leaf 24 hr post inoculation; B illustrates the pepper leaf 36 hr post inoculation. Strain 71:21, a non-pathogen of pepper, elicited a strong hypersensitive response.

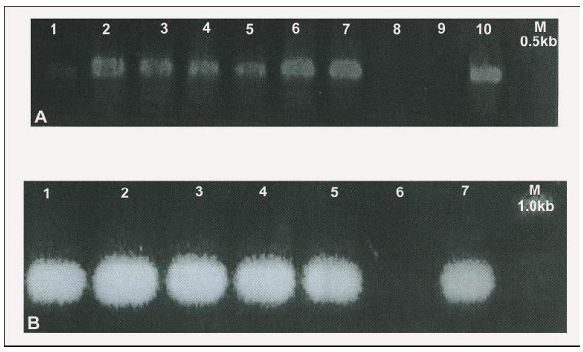

Figure 3. Amplification of *hrp* cluster: In image A, lanes 1–8 represent isolates 2, & 4-10; lanes 9 & 10 represent *X. campestris* pv. *vesicatoria* strains T-55 and 85:10 respectively. *P. syringae* does not contain a homologue of *X. campestris hrp*B gene; therefore, it was not amplified. In B lanes1–5 represent isolates 2, 4, 5, 6 & 7 respectively; 6 & 7 represents *X. campestris* pv. *vesicatoria* strains T-55 and 85:10 respectively. There was no amplification of *X. campestris* pv. *vesicatoria* strain T-55 by either primer pair RST2/3 nor RST9/10; it is an opportunistic bacterium and does not contain the *hrp* gene.

pathogen identification. Therefore, RST2/RST3 primer pair corresponding to the *hrpB* operon that encodes a putative ATPase and RST9/RST10 primer pair corresponding to *hrpB* from *X*. *campestris* amplified a 0.8- and 0.4-kb fragment, respectively, from isolates 2 & 4-7 and the positive control, *X*. *campestris* pv. *vesicatoria* strain 85:10 (Figure 3). However the negative control, *X*. *campestris* pv. *vesicatoria* strain T-55, an opportunistic non-pathogen, was not amplified. Additionally, primer RST21 was designed outside the variable non-coding ORF1 and primer RST22 was designed within the highly conserved ORF2 of the pathogenicity gene of *X*. *campestris* pv. *glycines* thus the RST21/RST22 primer pair was unable to amplify bacterial isolates 2 and 4–7. Bacterial isolates 2 and 4–7 contained sequence homology to the *hrp* cluster responsible for the pathogenicity of *X*. *campestris* which is conserved across pathogenic strains but lacks homology to non-pathogenic forms [15-18]. Insertion of transposon into the *hrp* cluster eliminates pathogenicity and induces a hypersensitive response; modification of the *hrp* loci can be used to engineer resistance and identify genes linked to hypersensitive response.

Amplification of the *hrp*G cluster of *Xanthomonas* sp. and *Hae*III and *Sau*3AI restriction were used for strain identification; primer XCG1 was designed outside the variable non-coding ORF1 and primers XCG2 and XCG3 within the highly conserved ORF2 coding region of *hrp*G. XCG1/XCG3 amplification produced a 0.9- and 1.0-kb fragment from bacterial isolates 2 & 7 and 4 & 6; respectively, but isolate 5 was not amplified (Figure 4A). XCG2/XCG3 amplified a 1.0 kb fragment from isolates 4 & 6 and a 0.6 kb fragment from *X. campestris* pv. *vesicatoria* strain 85:10 (Figure 4B). Though similar size fragments were obtained for the *Hae*III and *Sau*3AI digestion of the *hrp*B6 and pathogenicity gene fragments, differences were observed in the digested XCG1/XCG3 fragments. The differences observed in the XCG1/XCG3 fragments indicated differences in the *hrp*G cluster. XCP1/XCP2 amplification produced a 0.8 kb fragment from bacterial isolates 2 & 4-7 and *X*. *campestris* pv. *vesicatoria* strain 85:10 but not strain T-55 (Figure 4C). The variability in the *hrp*G sequence lend credibility to the

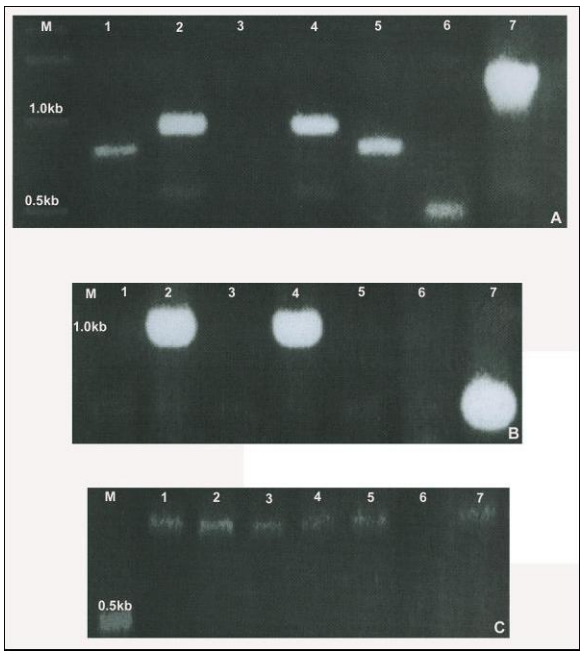

Figure 4. *hrp* gene amplification: A B and C indicate XCG1/3, XCG2/3 and XCP1/2 amplification respectively; lane M represents the DNA markers; lanes 1-5 represent isolates 2, 4, 5, 6 & 7, respectively; 6 & 7 represents *X. campestris* pv. *vesicatoria* strains T-55 and 85:10, respectively. The opportunistic bacterium, *X. campestris* pv. *vesicatoria,* strain T-55 produced a 0.55kb fragment with XCG1/3; only isolates 4 and 6, produced an amplified fragment with XCG2/3.

assumption that bacterial isolates 2 and 4-7 represents 3 distinct strains of *X*. *campestris* pv. *dieffenbachiae*. The PCR data was substantiated by hybridization of bacterial isolates 2 & 4-7 and *X*. *campestris* pv. *vesicatoria* strain 85:10 using RST2/RST3, RST9/RST10 and XCP1/XCP2 fragments as probes (Figures 5 & 6). The RST2/RST3- and RST9/RST10-derived fragments from bacterial isolates 2 and 4-7were ≥95% homologous to the *hrp*B6 region of *Xanthomonas* sp. and the XCP1/XCP2 fragment was ≥90% identical to the ORF1-ORF2 region which suggested a high degree of conservation [15-16, 18-19]. Amplification using the primer pair PZR1/PZF1 amplified a 1.0 kb fragment from isolates 3 and 9; however, isolate 8 was not amplified.

Effects of the avirulence gene on pathogenicity

E. *coli* transformed with pD36 plasmid carrying

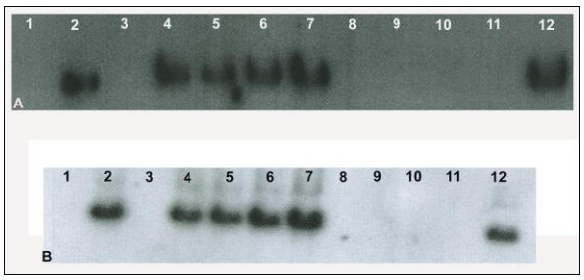

Figure 5. Hybridization of the *hrpB6* fragment from isolates: Lanes 1–10 represent isolates 1-10; 11 & 12 represent isolates of *X. campestris* pv. *vesicatoria* strains T-55 and 85:10, respectively. Non-pathogenic strains of *X. campestris* were not amplified.

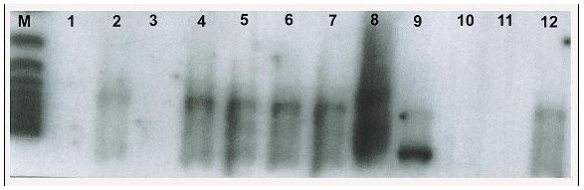

Figure 6. Hybridization of the pathogenicity gene fragment from bacterial isolates: Lane M represents the DNA markers; 1–10 represent isolates 1-10; 11 & 12 represent isolates of *X. campestris* pv. *vesicatoria* strains T-55 and 85:10, respectively. Non-pathogenic strains of *X. campestris* were not amplified.

the *avrBs*3 gene grown on LB medium containing streptomycin was used to transform bacterial isolates 4 and 5. Transformed bacterial isolates infected greenhouse-grown plants at a faster rate with infection localized to inoculated leaves. Water-soaked lesions and discoloration appeared on the dasheen leaf surface in 2 days with the untransformed bacterial isolates and after 3 days with untransformed bacterial isolates. The chlorotic water-soaked lesions with a yellow halo surrounding the outer edges were more pronounced on the lower surface and as the disease progressed, vascular wilting and necrosis were visible (Figures 7A & 7B). Leaves inoculated with transformed bacterial isolates died within 10 days compared to 20 days for untransformed bacterial isolates.

The effects were less pronounced on cocoyam; plantlet leaves inoculated with transformed bacterial isolates 4 and 5 showed symptoms of chlorosis and water-soaking within 3 days. The chlorotic regions covered the leaf surface in 5

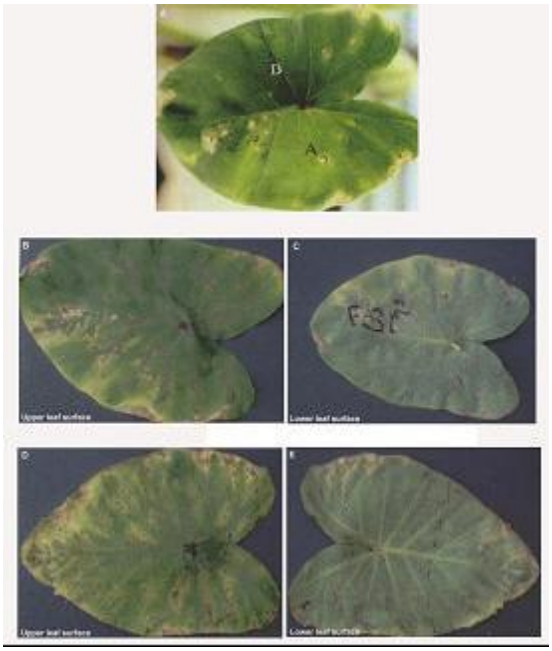

Figure 7: Effects of bacterial isolates carrying the *avrBs*3 gene in pD36 plasmid on dasheen: In A, 1-5 show the areas on the leaf surface that were inoculated with transformed isolate 2; symptoms of infections were visible on the leaf surface 5 days post inoculations. Images B/C illustrates dasheen leaf 10 days post inoculation with transformed isolate 4; infection resulted in vascular wilting of the leaf which suggests attacking of the vascular system of the plant. Images D/E shows dasheen leaf 10 days post inoculation with isolate 4. The infection resulted in vascular wilting of the leaf and was accompanied by yellowing and necrosis around the outer edges of the leaf.

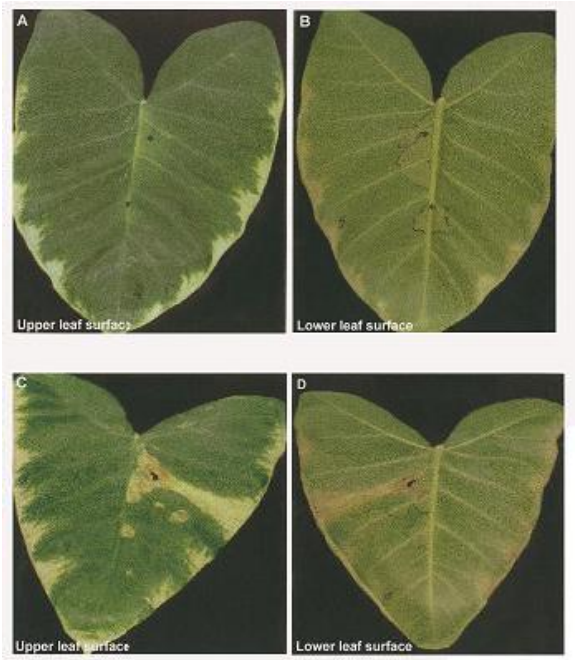

Figure 8: Effect of isolates containing pD36 plasmid carrying *avrBs*3 gene on cocoyam: Images A/B illustrates inoculation of the leaf of cocoyam with transformed isolate 2 resulted in a infection; the symptoms of the infection first appeared 3 days post inoculation. C/D shows the effect of transformed isolate 4.

days (Figure 8) and the leaf died within 28 days. Pepper plantlets exposed to transformed bacterial isolates 4 became discolored in 2 days; chlorosis and water-soaking were evident in 3 days. The effects of bacterial isolate 4 were more pronounced than isolate 5, the infection did not spread to un-inoculated leaves. Leaves inoculated with transformed bacterial isolate 5 became discolored after 2 days; the infected areas became soft, showing evidence of chlorosis and cell collapse, turning brown and died within 4 days (Figure 9). Death of the infected area was the hypersensitive response to the invading pathogen indicating that the *avrBs*3 gene had mated into the isolates. Bacterial isolates carrying *avrBs*3 gene inoculated into greenhouse-grown plantlets had a profound impact on pathogenicity. The transformed bacterial isolates caused the death of the infected area, a hypersensitive response, which indicated that *avrBs*3 gene had been successfully mated into the isolates. Genes requiring compatibility with hosts have been identified in a wide variety of plant pathogens, including *Erwinia amylovora* [20], *Pseudomonas* sp. [21-22] and *X. campestris* [22-23]. These genes have been classified into three groups: the *disease specific* (*dsp*) genes, *hypersensitive response* (*hrp*) genes, and the *avirulence* (*avr*) genes [21-22, 25]. The *dsp* genes are involved in the development of the disease in the host plant but they are unable to induce a hypersensitive response in the non-host plants. The *hrp* genes are required for the initiation of the disease symptoms on the host plants and the induction of the hypersensitive reaction on non-host plant. Insertion of transposons into the *hrp* loci eliminates pathogenicity and enhances the ability to induce the

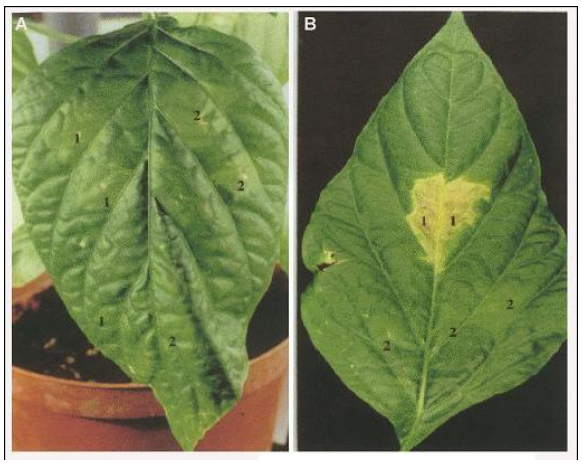

Figure 9: Effect of isolates carrying the pD36 plasmid-borne *acrBs*3 gene on pepper: A shows the effect of 2 pepper 4 days post inoculation; the plant was inoculated with *X. campestris* pv. *dieffenbachia* with (indicated by 1) and without (indicated by 2) the pD36 plasmid-borne *avrBs*3 gene. The presence of the *avrBs*3 gene did not alter the effect of the pathogen on the plant. In both instances, the plant appears to be susceptible to infection. B shows pepper leaf inoculated with with transformed (indicated by 1) and untransformed (indicated by 2) isolate 4; the transformed isolate elicited a strong hypersensitive response from the plant.

hypersensitive response in resistant host and non-host plants [16, 19]. The *avr* genes are involved in the control of pathogen/cultivar specificity, investigation of the regulation of the *avr* gene indicates a link between *hrp* genes of the pathogen and avirulence expression [25-27]. Pepper, a non-host of *X*. *campestris* pv. *dieffenbachiae*, exhibited a hypersensitive response to isolates 2 and 4-7 indicating host specificity. Non-hosts posses host resistance genes (*Bs*) which interact with the *avr* genes of the invading organism producing the hypersensitive response that denotes resistance [28-30].

CONCLUSION

X. campestris pv. *dieffenbachiae, P*. *syringae, E*. *chrysanthemi* pv. *dieffenbachiae* and non-fluorescent *Pseudomonas* were found to be associated with the common leaf blight disease in dasheen and cocoyam. *X*. *campestris* pv. *dieffenbachiae*, the causative agent of the disease belonged to 3 strains and is host specific thus producing hypersensitive response in non-hosts. The *hrp* gene which is responsible for the pathogenicity of *X. campestris* pv. *dieffenbachiae* is conserved among pathovars of *campestris* and served as a molecular diagnostic tool for assessing infection.

GENERAL SIGNIFICANCE

Expression of resistance phenotype requires the interaction of *Bs* and *avr* genes; the host resistance genes *Bs*1, *Bs*2 and *Bs*3 are each linked to specific *avr* genes and have been identified in some pathovars of *X*. *campestris*. The *avrBs*1, *avrBs*2 and *avrBs*3 correspond to *Bs*1, *Bs*2, and *Bs*3, respectively [12, 28-30]. These resistance genes are inherited independently and the phenotype of the hypersensitive response associated with each resistance reaction can be easily distinguished by the intensity and the timing of the response of the host to infection [30-31]. A hypersensitive response was induced in dasheen and cocoyam using isolates carrying the *avrBs*3 avirulence gene. Screening of infected hosts to identify genes linked to resistance is vital to developing transgenic plants resistant to infection. The results demonstrated that the *hrp*B, *hrp*G and the pathogenicity genes can be used to develop molecular markers for reliable pathogen identification. This is particularly useful for assessing and screening plants that are tolerant to the pathogenic infection caused by the common leaf blight disease since reliable pathogen identification will facilitate disease diagnosis even before symptom expression.

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