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Sensitive detection of *Xanthomonas axonopodis* pv. *dieffenbachiae* on *Anthurium andreanum* by immunocapture-PCR (IC-PCR) using primers designed from sequence characterized amplified regions (SCAR) of the blight pathogen

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Abstract

One of the most devastating *Xanthomonas* diseases affecting the *Anthurium* cut flower industry worldwide is the bacterial blight caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (*Xad*). The disease can be spread through latently infected tissue-cultured plants that are used for the propagation of *Anthurium* worldwide. Current disease diagnostic techniques involve the use of semi-selective media and serological tests. This study describes the development of a PCR tool combined with a genus-specific monoclonal antibody for the sensitive detection of the pathogen directly from plants. It was demonstrated that the immunocapture PCR (IC-PCR) was more sensitive than the conventional PCR and even more sensitive than indirect ELISA for the detection of the pathogen. Latently infected plants could be positively screened for the presence of the pathogen. Three sets of primers were designed from DNA probes that were reported to show some specificity to the pathovar *dieffenbachiae*. The use of all three sets of primers in a single reaction successfully amplified the three individual loci when bacterial DNA was used as a template. The multiplex PCR generated PCR profiles that could differentiate between the reference strains of *X. axonopodis* pv. *dieffenbachiae* from other control bacteria. The new primers could therefore be used both for the diagnosis of *Anthurium* blight in single PCR reactions and also for the profiling of *Xanthomonas*. pv. *dieffenbachiae* strains using the multiplex PCR technique.

Introduction

Anthurium (*Anthurium andreanum*) has been cultivated for many decades for cut flower production in Mauritius and the country is now the third world producer of this ornamental. *Anthurium* blight, caused by *Xanthomonas axonopodis* pv. *dieffenbachiae* (Vauterin et al., 1995) (= *X. campestris* pv. *dieffenbachiae*, McCulloch and Pirone, 1939), is by far the greatest challenge to the *Anthurium* grower worldwide and in Mauritius outbreaks have never been reported. The disease has been devastating in many world production areas such as Hawaii and Brazil (Hayward, 1972; Nishijima and Fujiyama, 1985; Lipp et al., 1992), Venezuela (Guevara and DeBrot, 1987), California (Cooksey, 1985), The Philippines (Natural, 1990), Tahiti (Mu, 1990), Jamaica (Young, 1990), Guadeloupe and Martinique (Rott and Prior, 1987) and recently in the Mediterranean region of Turkey (Aysan and Sahin, 2003).

While some chemicals are effective as a preventive measure, none of the bactericides on the market today can eradicate the pathogen from

infected fields (Norman and Alvarez, 1994b). The pathogen can persist in crop debris for more than 4 months (Duffy, 2000) and can be disseminated via aerosols (Venette et al., 1992; Alvarez et al., 1994). Considerable progress has been made in the biological control of blight since it was first studied by Fernandez et al. (1989). Fukui et al. (1999) have used sprays of non-pathogenic bacteria and bacterial mixtures to successfully control the infections but this method has remained experimental. Tolerant varieties that maintain flower production in the presence of the disease have been developed but these tend to latently harbour high concentrations of the pathogen for extended periods of time (Norman and Alvarez, 1996; Fukui et al., 1998). In addition, infected tolerant varieties eventually succumb to the disease (Fukui et al., 1996). Management strategies for *Anthurium* blight focus on the use of axenically-propagated tissue-cultured plants, sanitation, and preventive cultural practices (Nishijima and Fujiyama, 1985; Cook, 1988; Nishijima, 1989; Tanabe et al., 1990). The latency period and the rapidity with which the plants are killed after the appearance of symptoms make the surveillance of *Anthurium* blight difficult (Norman and Alvarez, 1994a). In the absence of an efficient indexing system, latently infected plants can be shipped or acquired, thus providing inoculum sources for a new disease epidemic. *Xanthomonas axonopodis* pv. *dieffenbachiae* strains have been introduced through tissue-cultured plants in India (Sathyanarayana et al., 1997) and Reunion Island (Soustrade et al., 2000).

The detection of pathogens and their identification are essential for evaluating disease progress. Common isolation and identification techniques for bacterial plant pathogens involve the use of semi-selective media (Norman and Alvarez, 1989; Jones et al., 1990; Gitaitis et al., 1991) followed by confirmatory bacteriological techniques such as Biolog and fatty acid profiling which necessitate the prior purification of the pathogen (Alvarez et al., 1991; Chase et al., 1992; Bouzar et al., 1994; Jones et al., 1998). The applications of these diagnostic techniques on a large-scale are limited because of the costs involved in handling large numbers of samples and the time needed to achieve conclusive results. In the case of *Anthurium* blight, this is complicated since several epiphytic and saprophytic bacteria often overgrow the pathogen even when semi-selective media are used (Norman and Alvarez, 1994b). A rapid detection of *X. axonopodis* pv. *dieffenbachiae* has been reported by the use of an enrichment ELISA assay (Norman and Alvarez, 1994b) which consists of growing the bacterial cells in a semi-selective broth before detection. The enrichment procedure in microtiter plates found applications in the detection of latent infections. The monoclonal antibody Xcd 108 used in the study does not react with all strains of the pathogen, for example strains of serotype 11 and 12 that are pathogenic on aroids such as *Dieffenbachia*. This limits the use of the enrichment ELISA for the detection on *Anthurium* only.

The polymerase chain reaction is now an essential tool in pathogen diagnosis because of its sensitivity (Bereswill et al., 1992; Prosen et al., 1993). A cloned and sequenced pathogenicity gene of *X. campestris* pv. *manihotis* was successfully used to develop a highly specific PCR test for this pathogen (Verdier et al., 1998) using either cells or leaf extracts and stem lesions. A sensitive and specific detection of *X. campestris* pv. *perlargonii* was devised using random amplified polymorphic DNA (RAPD-PCR) analysis to generate SCAR primers and probes (Manulis et al., 1994). Detection of *X. campestris* pv. *vesicatoria* in pepper and tomato seeds has also been done by PCR primers developed from the sequence of *hrp* genes (Leite et al., 1994).

A recent RAPD-PCR study of the genetic diversity of the *Anthurium* blight pathogen revealed probes that could differentiate among different groups of *X. axonopodis* pv. *dieffenbachiae* (Khoodoo and Jaufeerally-Fakim, 2004). This study describes the development of a sensitive PCR tool from the sequences of DNA probes for an early and quick diagnosis of *Anthurium* blight in plant material.

Materials and methods

Bacterial strains

Table 1 indicates the origin of the strains used in this study. The DNA extracts of strains of *X. axonopodis* pv. *dieffenbachiae* were obtained from cell collections in Hawaii (University of Hawaii, Manoa) and New Zealand (Landcare Research Centre, Auckland). Three non-pathogenic *Xan-*

Table 1. Origins of bacterial strains used in the study

Isolate/strain	Host	Location	Source or reference
X. axonopodis pv. dieffenbachiae			
JV502	*Anthurium*	Venezuela	I. Soustrade[a]
JV505	*Anthurium*	Hawaii	I. Soustrade
JV511	*Anthurium*	Puerto Rico	I. Soustrade
JV589	*Anthurium*	Reunion	I. Soustrade
JR579 (type strain LMG695)	*Anthurium*	Brazil	I. Soustrade
D15-3, D93, D108, D147, D194, X1, D99	*Anthurium*	Hawaii	A. Alvarez[b]
ICMP 9564, ICMP 9565	*Anthurium*	USA	J. Young[c]
ICMP 9841	*Anthurium*	Tahiti	J. Young
ICMP 7461, ICMP 4656	*Anthurium*	Brazil	J. Young
ICMP 9569, ICMP 576	*Dieffenbachia*	USA	J. Young
ICMP 9570	*Epipremnum*	USA	J. Young
ICMP 9566, ICMP 9586	*Philodendron*	USA	J. Young
T60, X210, X201, X204, X261, X263, X271, X272, X273	*Anthurium*	Turkey	This study
HK56 (non-pathogenic)	*Dieffenbachia*	Mauritius	M. H. R. Khoodoo[d]
DK02 (non-pathogenic)	*Anthurium*	Mauritius	M. H. R. Khoodoo
DK04 (non-pathogenic)	*Aglaonema*	Mauritius	M. H. R. Khoodoo
X. axonopodis pv. *allii* BS6	Onion	Mauritius	S. Soomary[e]
X. axonopodis pv. *begoniae* HK40	Begonia	Mauritius	This study
X. axonopodis pv. *carotae* HK46	Carrot	Mauritius	This study
X. axonopodis pv. *perlargonii* Sardunya	Geranium	Turkey	Y. Aysan[f]
X. axonopodis pv. *poinsetticola* HK38	Poinsettia	Mauritius	This study
X. axonopodis pv. *phaseoli* HK50,	Bean	Mauritius	This study
405	Bean	Turkey	Y. Aysan
X. axonopodis pv. *phaseoli fuscans* 266	Bean	Turkey	F. Sahin[g]
X. axonopodis pv. *raphani* DC91-1	*Raphanus* sp	Turkey	F. Sahin
X. axonopodis pv. vasculorum R1,	Sugar cane	Mauritius	A. Dookun-Saumtally[h]
3301	Broom bamboo	Mauritius	A. Dookun-Saumtally
X. axonopodis pv. *malvacearum* 633,	Cotton	–	Y. Jaufeerally-Fakim[i]
397	Cotton	Turkey	F. Sahin
X. axonopodis pv. *vesicatoria* HK06,	Tomato	Mauritius	This study
Kuyucu 3R	Pepper	Turkey	Y. Aysan
X. axonopodis pv. *vignicola* GSPB 6A1	Cowpea	Turkey	Y. Aysan
X. axonopodis pv. *vitians* 700	Lettuce	Turkey	F. Sahin
X.campestris pv. *campestris* HK01	Cabbage	Mauritius	This study
X. campestris pv. *syngonii* HK61	Syngonium	Mauritius	This study
X. hortorum 406	–	Turkey	F. Sahin
X. translucens pv. *translucens* 1943	Wheat	–	Y. Jaufeerally-Fakim
X. vasicola pv. *holcicola* 1060	Sorghum	–	Y. Jaufeerally-Fakim
X. albilineans 3303	Sugar cane	Mauritius	A. Dookun-Saumtally
Stentrophomaonas maltophilia 418	–		F. Sahin
Pseudomonas rubrisubalbicans Prub	Sugar cane	–	Y. Jaufeerally-Fakim
Pseudomonas chichorii MRX	Tomato	Turkey	Y. Aysan
Pseudomonas corrugata Pcorr	Tomato	Turkey	Y. Aysan
Pseudomonas syringae pv. Tomato	Tomato	Turkey	Y. Aysan
AAD-1b		Turkey	Y. Aysan
Pseudomonas viridiflava Karun1	Melon	Turkey	Y. Aysan
Erwinia carotovora pv. *carotovora*	Dieffenbachia	Turkey	Y. Aysan
Dieff 2b	Chrysanthemum	–	Y. Jaufeerally-Fakim
Erwinia chrysanthemi 2835	–	Turkey	F. Sahin
Erwinia amylovora 459	Potato	Mauritius	
Ralstonia solanacearum HK36	Tomato	Turkey	This study
Pantoea agglomerans 441	Rose	Turkey	F. Sahin
Agrobacterium tumefaciens 27At2	Tomato	Turkey	Y. Aysan
Clavibacter michiganensis subs. *Michiganensis* 1/2c			F. Sahin

Table 1. Continued

Isolate/strain	Host	Location	Source or reference
Unidentified strains isolated from aroids	*Anthurium*	Mauritius	This study
HK03, HK04, HK05, HK07, HK08	*Anthurium*		
HK09, HK10	*Caladium*		
HK17, HK23, HK58	*Colocasia*		
HK20	*Anthurium*		
HK57			

X. *Xanthomonas*
[a]I. Soustrade, CIRAD St Pierre, Reunion Island.
[b]A. Alvarez, University of Hawaii, Hawaii.
[c]J. Young, Landcare Research Ltd, Auckland, New-Zealand.
[d]M. H. R. Khoodoo, University of Mauritius, Réduit, Mauritius.
[e]S. Soomary, Agricultural Research and Extension Unit (AREU), Réduit, Mauritius.
[f]Y. Aysan, Çükürova University, Adana, Turkey.
[g]F. Sahin, Atatürk University, Erzurum, Turkey.
[h]A. Dookun-Saumtally, Mauritius Sugar Industry Research Institute (MSIRI), Réduit, Mauritius.
[i]Y. Jaufeerally-Fakim, University of Mauritius, Réduit, Mauritius.

thomonas strains, isolated from aroids in Mauritius were also included. All the work with the live cells was performed at Atatürk University and Çükürova University (Turkey). Re-inoculation of plants was performed with the bacterial suspension of *X. axonopodis* pv. *dieffenbachiae* strain X210 which was used to swab leaf surfaces.

Cloning, sequencing of fragments, and design of specific primers

PCR products amplified by RAPD primers as recently reported (Khoodoo and Jaufeerally-Fakim, 2004) were purified from agarose gels by the Freeze and Squeeze® columns (Biorad) and were cloned in a pGEM®-T Easy plasmid vector (Promega, Madison, WI, USA). Recombinant *E. coli* cells were used for plasmid minipreps using the Wizard®Plus SV Miniprep kit (Promega) and sequencing was performed by the Spectrumedix SCE 2410 Genetic Analysis System and the BigDye Version 3.1 kit (ABI, Inqaba Biotechnical Industries, SA). BLAST searches of *GenBank* nucleotide (BLASTN) and protein (BLASTX) databases were also performed online (Altschul et al., 1997). Primers were designed to be 18–22 bases long and to have a melting temperature of 60–62 °C. The new primers were tested in PCR on all reference strains of the blight pathogen and bacterial controls from the available collection (Table 1). Only the primers that produced PCR profiles that could easily differentiate between strains of *X. axonopodis* pv. *dieffenbachiae* and control strains were selected for further use.

Specific amplifications and multiplex-PCR using designed primers

A 25 µl reaction was set up as follows: 30 ng of purified bacterial DNA, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each dNTP, one unit of *Taq* polymerase (Bioline), 1× enzyme buffer and the balance of water. Amplifications were performed in a thermal cycler (Biorad) programmed as follows: one cycle of 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were resolved after agarose gel electrophoresis and ethidium bromide staining. When using *Anthurium* DNA for the detection, 0.4 µg μl^{-1} of bovine serum albumin (BSA) were added to the master mix and about 5 µg of DNA, extracted from leaves, were used as template. The same reaction conditions were used for the multiplex PCR except that, instead of using only one pair of primers as for the specific amplification, the three primer pairs (Table 2) were used simultaneously.

Sampling of leaves

Anthurium plants were first sampled for DNA extraction and immunocapture-PCR one week after inoculation, before the appearance of blight symptoms and again after the appearance of

Table 2. Sequences of specific primers and the size of their amplicon

Selected PCR primer pairs	Sequence (5′-3′)	Size of amplicon (bp)	Genbank accession number of the sequenced fragment
KJM11[f]	GGGTGTGCGTAGTGCTCTG	805	AY923824
KJM12[r]	CTACGTCGTTCCGGTCAGC		
KJM34[f]	GACATCCGGGAGGACTTC	565	AY660605
KJM36[r]	TGAAGAACGTGGCCCATC		
KJM74[r]	CAGCAGTCCGGAGAAAGC	1250	AY923829
KJM73[f]	GGCGCGTTCTGATCCTTG		

The hybridisation temperature used for each primer pair was 62 °C.
[f]Forward primer.
[r]Reverse primer.

distinct foliar symptoms (marginal yellowing/ chlorosis/ water-soaked spots). For asymptomatic leaves, samples were taken from areas near the leaf margins while for symptomatic ones, samples were taken from the green area around the yellow halo. About 1 cm^2 of leaf area was kept for immunocapture PCR and the rest was weighed and frozen at −86 °C overnight for DNA extraction.

Extraction of DNA from Anthurium andreanum

About 200–250 mg of frozen leaves were crushed into a fine powder. A phenol-based protocol was used. Extractions were also performed from a few dried infected leaves. The crushed tissue was transferred to a 2 ml microfuge tube and 500 µl of extraction buffer (0.5 M NaCl, 0.2 M Tris, 50 mM EDTA, 2.5% PVP, 1% β-mercaptoethanol, 3% SDS and 20% EtOH) were added and vortexed for 10 s. Tubes were placed in a water bath set at 40 °C for 1 h with occasional vortexing. The extract was treated with phenol:chloroform:isoamylalcohol (25:24:1) for 20 min. The tubes were centrifuged at 16,000 rpm for 10 min and the aqueous phase was treated with an equal volume of chloroform:isoamylalcohol (24:1) for 20 min. Isopropanol was used to precipitate the DNA. The DNA was collected, washed in 70% ethanol and dissolved in a minimum volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The extracts were treated with DNAse-free RNAse at 37 °C for 30 min and stored at −86 °C until further use.

Immunocapture PCR (IC-PCR)

PCR tubes were coated with monoclonal antibody MAb XII (Agdia) which is specific to the genus *Xanthomonas* (Alvarez et al., 1985). A 1:400 dilution of the antibody in coating buffer (1.59 gl^{-1} $NaCO_3$, 2.93 g l^{-1} $NaHCO_3$) was done and 25 µl were added to each tube. Tubes were left at 37 °C for 3 h. Three washes were performed with 100 µl of phosphate buffer saline containing Tween 20 (PBST). About 1 cm^2 of leaf sample was macerated in 250 µl of PBST buffer and 25 µl of this mix were added to the coated PCR tubes and incubated at 37 °C for 3 h. Five washes were performed with PBST buffer before adding aliquots of the appropriate PCR mix (single or multiplex PCR).

Diagnosis of blight by indirect ELISA

The sampling of infected leaves was done as described earlier except that for ELISA, samples were macerated in coating buffer instead of PBST. Symptomatic samples were labelled E1–E12 and 100 µl of the suspension were placed in individual wells of the ELISA microtiter plates. Coating was done overnight at 4 °C. Standard procedures were followed as described by Lipp et al. (1992). The primary antibody (MAb XII) was used in a dilution of 1:400, the secondary antibody (anti-mouse IgG) was used in a dilution of 1:200 and *p*-nitrophenyl phosphate (PNP) was used for the detection. All tests were conducted twice. DNA extracts from samples E1–E12 were tested by single and multiplex IC-PCR.

Sensitivity assays for ELISA, IC-PCR and ICM-PCR

Dilutions of cells (reference *Xad* strain X210) from a concentration of 1×10^8 CFU ml^{-1} to about 40 CFU ml^{-1} were done using either the coating

buffer when performing the ELISA or sterile distilled water for IC-PCR. The concentration of cells was monitored by measuring the absorbance of the bacterial suspension at 600 nm ($0.1\ A_{600} = 1 \times 10^8$ CFU ml^{-1}). The volumes of the samples used were 100 µl for ELISA and 25 µl for PCR. Primers KJM11 and KJM12 were used in the single PCR reaction and the other two primer pairs were added for testing the multiplex reaction.

Results

BLAST analysis

Eight DNA fragments, M1–M8, were sequenced and the sequences are accessible in *Genbank* (AY923824, AY923825, AY660605, and AY923826 to AY923830 respectively). No significant matches were found for RAPD fragments M1 and M7 by BLASTN (E-values of 0.13 and 0.18, respectively). BLASTX did not significantly match M1 with any protein while M7 was found to be homologous to a glycosyltransferase from *Burkholderia fungorum*. The remaining sequences (M2, M3, M4, M5, M6 and M8) matched conserved sequences in bacterial genomes. However, BLASTX analysis revealed a high homology of the translated sequence of M4 (AY923826) with a VirB4 protein of *Xanthomonas campestris* pv. *campestris*.

Specificity of designed primers

Three pairs of primers were designed from each sequence (M1 to M8) and were individually tested against all *Xad* strains and control bacteria in the available collection (Table 1). Three pairs of primers were selected from the initial set of 48 primers because they could differentiate between strains of *X. axonopodis* pv. *dieffenbachiae* and other xanthomonads by the PCR profiles that they generated. The results obtained with the selected primers are summarised in Table 3. Primers KJM11 and KJM12, which were designed from sequence M1, amplified a product of 805 bp from all strains of *X. axonopodis* pv. *dieffenbachiae* except those isolated from *Dieffenbachia*, and *Philodendron* and the non-pathogenic strains from Mauritius (for example in Figure 1, lanes 2, 5, 7–9, 11–23, 27–38). Up to 11 polymorphic bands (100 bp to 2.1 kb) were amplified from control bacteria. Similarly primers KJM73 and KJM74, which were designed from sequence M7, amplified a single product of 1250 bp from all reference strains of *X. axonopodis* pv. *dieffenbachiae* that were isolated from *Anthurium* but not from non-pathogenic strains and strains that were isolated

Table 3. Table of results for the amplification of strains of *Xanthomonas axonopodis* pv. *dieffenbachiae* using the selected primers

Strain	Host	Location	PCR amplicons		
			1250[1]	805[2]	565[3]
ICMP 9564	*Anthurium*	USA	–	–	–
ICMP 9565	*Anthurium*	USA	+	+	–
ICMP 9566	*Philodendron*	USA	–	–	+
ICMP 9569	*Dieffenbachia*	USA	–	–	+
ICMP 9570	*Epipremnum*	USA	+	+	–
ICMP 9586	*Philodendron*	USA	–	–	+
ICMP 9841	*Anthurium*	Tahiti	+	+	–
ICMP 7461	*Anthurium*	Brazil	+	+	–
ICMP 4656	*Anthurium*	Brazil	+	+	–
ICMP 576	*Dieffenbachia*	USA	–	–	+
JV502	*Anthurium*	Venezuela	+	+	–
JV505	*Anthurium*	Hawaii	+	+	–
JV511	*Anthurium*	Puerto Rico	+	+	–
JR579	*Anthurium*	Brazil	+	+	–
JV589	*Anthurium*	Reunion	+	+	–
D15-3	*Anthurium*	Hawaii	+	+	–
D108	*Anthurium*	Hawaii	+	+	–
D147	*Anthurium*	Hawaii	+	+	–
T60	*Anthurium*	Turkey	+	+	–
D194	*Anthurium*	Hawaii	+	+	–
D93	*Anthurium*	Hawaii	+	+	–
D99	*Anthurium*	Hawaii	–	+	±
X1	*Anthurium*	Hawaii	–	+	+
HK56	*Dieffenbachia*	Mauritius	–	–	+
DK02	*Anthurium*	Mauritius	–	–	+
DK04	*Aglaonema*	Mauritius	–	–	+
X210	*Anthurium*	Turkey	+	+	–
X201	*Anthurium*	Turkey	+	+	–
X202	*Anthurium*	Turkey	+	+	–
X203	*Anthurium*	Turkey	+	+	–
X204	*Anthurium*	Turkey	+	+	–
X205	*Anthurium*	Turkey	+	+	–
X261	*Anthurium*	Turkey	+	+	–
X262	*Anthurium*	Turkey	+	+	–
X263	*Anthurium*	Turkey	+	+	–
X271	*Anthurium*	Turkey	+	+	–
X272	*Anthurium*	Turkey	+	+	–
X273	*Anthurium*	Turkey	+	+	–

[1]Amplicon amplified by primer pair KJM73/KJM74.
[2]Amplicon amplified by primer pair KJM11/KJM12.
[3]Amplicon amplified by primer pair KJM34/KJM36.
+: band amplified.
–: band not present.
±: faint positive band.

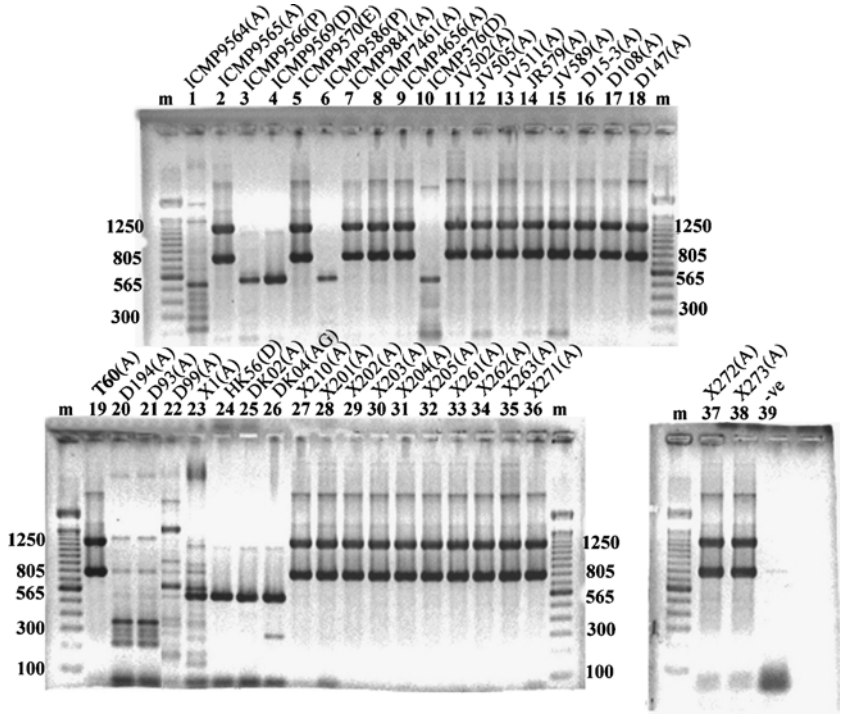

Figure 1. Inverted photograph of a gel showing the results of multiplex PCR for strains of *Xanthomonas axonopodis* pv. *dieffenbachiae*. Lanes are labelled with strain numbers (Table 1) and hosts of origins; A, *Anthurium*; D, *Dieffenbachia*; E, *Epipremnum*; AG, *Aglaonema*; P, *Philodendron*.

from other aroid hosts (for example in Figure 1, lanes 2, 5, 7–9, 11–21, 27–38). Up to 12 bands ranging from 100 bp to 2.2 kb in size were amplified from the control bacteria. Primers KJM34 and KJM36, which were designed from sequence M3, amplified a single PCR product of 565 bp from strains of *X. axonopodis* pv. *dieffenbachiae* isolated from *Dieffenbachia*, and *Philodendron* and all the non-pathogenic strains from Mauritius (for example in Figure 1, lanes 3, 4, 6, 10, 22–26). Among the control bacteria only *Xanthomonas axonopodis* pv. *raphani* produced the band of 565 bp.

Multiplex PCR

The use of equimolar (0.5 μM) concentrations of each primer in the multiplex reaction gave satisfactory amplifications of all the expected size fragments at levels comparable to the single PCR reactions. The fragment of 565 bp was amplified from 11 strains, the fragment of 805 bp from 30 strains and the fragment of 1250 bp from 28 strains (Figure 1). Different profiles were observed for strains D93 and D194 (lanes 20 and 21), D99 (lane 22), X1 (lane 23) and ICMP9564 (lane 1). Apart from strain ICMP9564, all strains of *X. axonopodis* pv. *dieffenbachiae* showed SCAR profiles that comprised either the product of 565 bp (3, 4, 6, 10, 24–26), the two products of 805 and 1250 bp (lanes 2, 5, 7–9, 11–21, 27–38), or the two products of 565 and 805 bp (lanes 22 and 23). When tested against control bacteria (Figure 2), complex profiles comprising one to 17 polymorphic bands ranging from about 100 bp to 2 kb were amplified. The positive control (Figure 2, lane 1) consisted of the PCR products amplified from a mixture of DNA of strains T60 (a strain isolated from *Anthurium* in Turkey) and HK56 (a non-pathogenic strain isolated from *Dieffenbachia*). As expected *Xanthomonas axonopodis* pv. *raphani* produced a band of 565 bp (Figure 2, lane 3). All the other control strains produced a profile that was markedly different from that of strains of *X. axonopodis* pv. *dieffenbachiae* (Figure 2, lane 1).

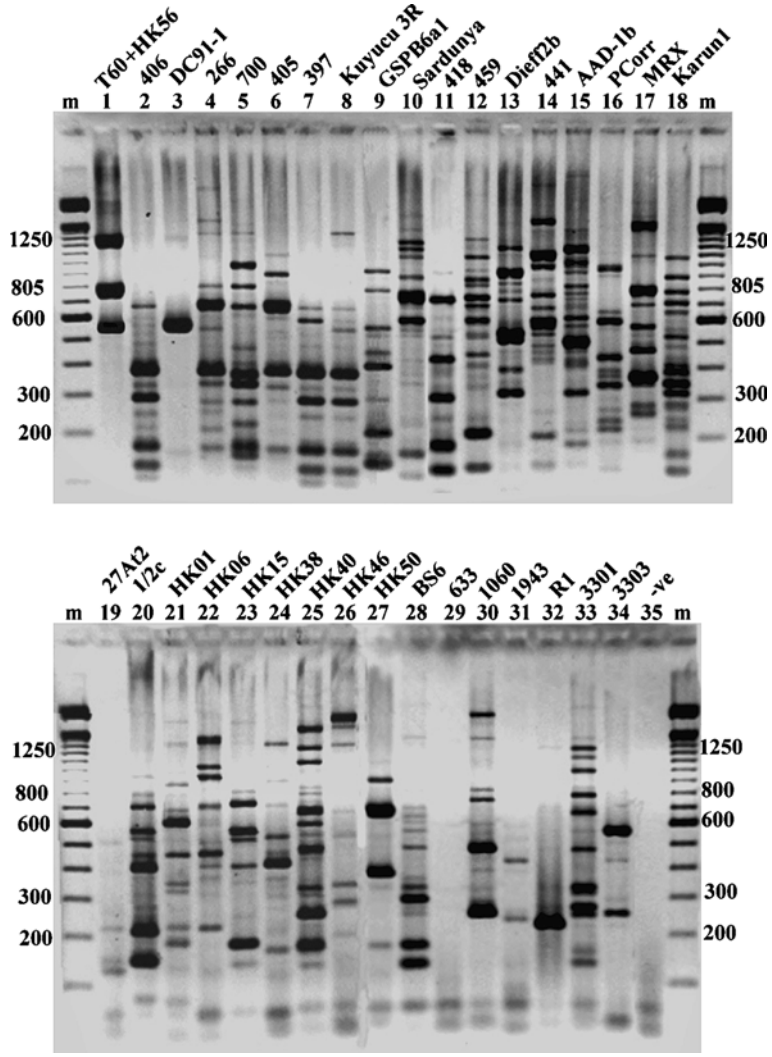

Figure 2. Inverted photograph of a gel showing the results of multiplex PCR for control bacterial strains. Lanes are labelled with strain numbers (Table 1). m, 100 bp ladder; lane 1, positive control; lanes 2–10: *X. hortorum*, *X. axonopodis* pv. *raphani*, *phaseolifuscans*, *vitians*, *phaseoli*, *malvacearum*, *vesicatoria*, *vignicola*, *perlargonii*; lanes 11–20: *Stenotrophomonas maltophilia*, *Erwinia amylovora*, *E. carotovora*, *Pantoea agglomerans*, *Pseudomonas syringae*, *P. corrugata*, *P. chichorii*, *P. viridiflava*, *Agrobacterium tumefaciens*, *Clavibacter michiganensis*; lanes 21–34: *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, *X. campestris* pv. *syngonii*, *X. axonopodis* pv. *poinsetticola*, *begoniae*, *carotae*, *phaseoli*, *allii*, *malvacearum*, *X. vasicola* pv. *holcicola*, *X. translucens* pv. *translucens*, *X. axonopodis* pv. *vasculorum* (sugar cane), *X. axonopodis* pv. *vasculorum* (broom bamboo), *X. albilineans*; lane 35: negative control.

Comparison between the detection of the pathogen by indirect ELISA and immunocapture PCR (IC-PCR)

Infected plants that showed clear symptoms of *Anthurium* blight were used. ELISA assays were positive for 10 out of 12 samples when the results of the two replicates were combined (Table 4). Two samples (E6 and E10) tested negative in both replicates. All 12 samples tested positive in IC-PCR using primers KJM11 and KJM12 and in the immunocapture-multiplex PCR using the three sets of primers simultaneously.

Table 4. Summary of the results of the different detection assays

Plant sample	Number of samples positive					
	Number tested	ELISA	PCR	IC-PCR[1]	mPCR[2]	IC-mPCR[3]
Symptomatic	12	10	–	12	–	12
Asymptomatic	27	–	12	22	–	–
Symptomatic	36	–	17	32	–	–
Symptomatic	69	–	–	–	57	–

[1]IC-PCR: single PCR following immunocapture using leaf extracts.
[2]mPCR: multiplex PCR using plant DNA extracts.
[3]IC-mPCR: multiplex PCR following immunocapture using leaf extracts.
–: not applicable.

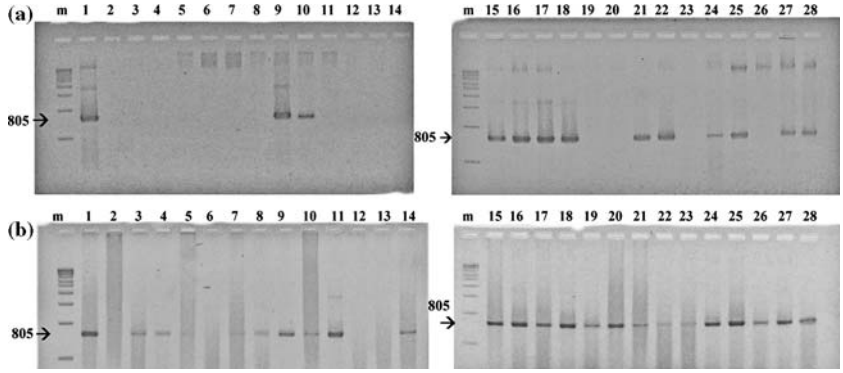

Figure 3. Inverted photograph of a gel showing the comparison between the detection of the pathogen by (a) conventional PCR using DNA samples from latently infected plants as template and (b) Immunocapture PCR using the leaf samples in (A) and primers KJM11 and KJM12. m = 1 kb DNA ladder. Lane 1, positive control; lanes 2–28, asymptomatic samples AS2–AS28.

Detection of Xanthomonas axonopodis *pv.* dieffenbachiae *from infected* Anthurium *using different PCR strategies*

In the assay comparing the detection of the pathogen in latently infected plants (asymptomatic) by conventional PCR with the detection by IC-PCR, all 27 plants that were inoculated eventually developed typical symptoms of *Anthurium* blight and were expected to be positive in the PCR tests. However, only 12 out of the 27 plants were tested positive when PCR was used to test the plant DNA extracts (Table 4; Figure 3a, lanes 9, 10, 15–18, 21, 22, 24, 25, 27, 28) whereas 22 out of the 27 plants were tested positive when IC-PCR was used to test the leaf extracts (Figure 3b, lanes 3–4, 7–11, 14–28).

The assay comparing PCR and IC-PCR for the detection from symptomatic plants involved the use of primers KJM11 and KJM12. The pathogen was detected in 17 out of 36 samples by the direct amplification of the 805 bp product from plant DNA extracts whereas 32 out of 36 samples were found to be positive for the blight pathogen when using IC-PCR on the same leaf samples.

The detection of the pathogen from *Anthurium* by multiplex PCR was performed on 69 DNA extracts from individual symptomatic leaves that were inoculated beforehand. The pathogen was detected in 57 samples by the amplification of the products of 805 or 1250 bp or both. There were 12 false negatives. The band of 565 bp amplified by primers KJM34 and KJM36 was present in 18 samples.

Sensitivity tests

The sensitivity limit of the indirect ELISA was estimated to be 1×10^5 CFU in the 100 µl used (10^6 CFU ml^{-1}). The band of 805 bp amplified

by primers KJM11 and KJM12, after immunocapture by MAb XII, was visible in the sample that was diluted to 3.8 CFU per reaction (152 CFU ml^{-1}). In the case of immunocapture-multiplex PCR, bands were visible only up to a cell concentration of 250 CFU per reaction (10^4 CFU ml^{-1}).

Discussion

In this study, several DNA fragments were isolated from *Xanthomonas axonopodis* pv. *dieffenbachiae* and sequenced in order to design primers that could be used in the molecular diagnostics of this serious disease of aroids. The pairwise alignments by BLASTN revealed that two sequences (M1 and M7) had no significant match in the *GenBank* nucleotide database. One set of primers from each of the three fragments M1, M3 and M7 allowed a differentiation between *Xad* and the control bacteria by producing a markedly different SCAR profile for strains of *X. axonopodis* pv. *dieffenbachiae*. However, none of the primer pairs amplified its target locus from all the strains of *X. axonopodis* pv. *dieffenbachiae*. This can be explained by the fact that different DNA groups are known to be present within the pathovar *dieffenbachiae* as shown in a recent study where some probes reacted only with strains that were specific to certain hosts (Khoodoo and Jaufeerally-Fakim, 2004). Interestingly the amplification using primers developed from sequence M3 complemented the results of the amplifications using primers developed from sequences M1 and M7. Hence a multiplex PCR reaction could be used to detect all the strains by the amplification of at least one of the three loci.

The combination of six primers in a multiplex reaction for the profiling of *X. axonopodis* pv. *dieffenbachiae* in a high stringency PCR is described. It was demonstrated that the multiplex PCR could differentiate among the various strains tested within the group of *X. axonopodis* pv. *dieffenbachiae* and also between strains of the blight pathogen and the control strains. Distinct profiles were obtained for most of the strains that were isolated from *Anthurium* or *Philodendron* or *Dieffenbachia*. The products targeted by primers KJM11 and KJM12 and primers KJM73 and KJM74 were not amplified from the non-pathogenic strains of *Xanthomonas* or strains that were isolated from *Philodendron* and *Dieffenbachia*. However since the number of such strains was limited in this study, a correlation between their pathogenicity on *Anthurium* and their SCAR profiles could only be hypothesised.

It has been shown in this study that the IC-PCR (single and multiplex PCR) on plant extracts is a reliable diagnostic assay when compared to indirect ELISA. When 12 infected plants were tested by indirect ELISA, two false negatives were obtained in both replicates while all were positive by IC-PCR. Multiplex PCR applied to plant DNA extracts from inoculated plants gave 57 positives out of 69 symptomatic plants tested. Twelve test results were therefore false negatives. In latently infected *Anthurium* 81.5% of plants were tested positive by IC-PCR whereas only 44.4% of samples were positive by direct PCR. Immunocapture PCR was demonstrated to be almost two times more sensitive than direct PCR for the detection of the pathogen. In symptomatic plants direct PCR detected the pathogen in 47.2% of the samples whereas IC-PCR detected the pathogen in 88.9% of the samples. The probability of detecting the pathogen could be increased by increasing the number of samples tested from each plant. The false negatives produced when using *Anthurium* DNA as template for the PCR were probably due to inhibitors that contaminated the DNA samples during the extraction. Direct amplification using crude DNA extracts is often not successful because of the presence of phenolic compounds and other components that bind to the DNA after cell lysis (Sarkar et al., 1990; John, 1992; Henson and French, 1993; Pich and Schubert, 1993). Further DNA purification procedures could probably resolve these problems.

The immunocapture technique eliminates the need for DNA extraction by selectively binding bacterial cells from the plant extract. The use of the genus-specific monoclonal antibody MAb XII (Alvarez et al., 1985) ensured that only xanthomonads were trapped leaving behind other common plant pathogens and epiphytic bacteria. This antibody reacted with all strains of *X. axonopodis* pv. *dieffenbachiae*, both virulent and non-pathogenic strains. Another commercial antibody, MAb Xcd 108 (Lipp et al., 1992), which is specific to *Xad* strains, was not used because it was reported that 5% of pathogenic strains of *X. axonopodis* pv. *dieffenbachiae* do not react with this antibody

whereas all pathogenic strains react with the genus-specific antibody MAb XII (Lipp et al., 1992).

The sensitivity assay using cell dilutions showed that the single IC-PCR for primers KJM11 and KJM12 was much more sensitive than the multiplex IC-PCR and concentrations as low as about 4 CFU per reaction could be detected. This could be explained by inhibition of the PCR due to the high primer concentration in the multiplex reaction when low concentrations of cells were used. Therefore a single IC-PCR assay for each of the primer sets described could be used for the diagnosis of blight using plant extracts directly. On the other hand, the multiplex assay with the six primers could be used for strain differentiation when using purified bacterial DNA as the template. This approach might be a good one for evolutionary and phylogenetic studies of the pathovar.

Current assays to identify pathogenic bacteria in *Anthurium* by means of plating on selective or conventional media, immunoassays and host inoculation are labour-intensive and in some cases not sensitive enough. There is a need for a rapid, reliable, sensitive and specific method of detection of plant pathogenic bacteria that are responsible for important economic losses and when plant material is subject to quarantine. This study showed that IC-PCR using primers KJM11 and KJM12 is an attractive solution to most of the difficulties that the other methods have when a rapid and accurate analysis is needed for phytosanitary control. The use of such a sensitive tool as IC-PCR could help to increase the reliability of actual quarantine techniques in Mauritius and other countries that import tissue-cultured *Anthurium* plants for breeding and propagation. Although it would be difficult to conceive the testing of every single plant in a field, this tool could find applications for indexing smaller numbers of plants such as the 'mother' plants imported in Mauritius.

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