MAURITIUS RESEARCH COUNCIL

THE DEVELOPMENT OF A MOLECULAR DIAGNOSTIC TOOL FOR THE DETENTION OF XANTHOMONAS AXONOPODIS PV. DIEFFENBACHIAE, THE CASUAL AGENT OF ANTHURIUM BACTERIAL BLIGHT

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"THE DEVELOPMENT OF A MOLECULAR DIAGNOSTIC TOOL FOR THE DETECTION OF *XANTHOMONAS AXONOPODIS* PV. *DIEFFENBACHIAE*, THE CAUSAL AGENT OF *ANTHURIUMBACTERIAL* BLIGHT''

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1.

Summary

Production of *Anthurium andreanum*, a member of the *Araceae* family, is an important economic activity in many countries with tropical and sub-tropical climates. The spathe, which is the most attractive part of the plant, is sold as a flower because of its colour, shape and long-vase-life. A number of different varieties exhibit different colours, from dark red to white, green, orange and pink. The industry is worth about US$ 20 m annually and Mauritius is the third world producer.

Since early 1990's, the industry had been badly hit with a serious disease of bacterial blight, caused by the pathogen, *Xanthomonas axonopodis* pv *diffenbachiae*. Hawaii was amongst those countries, which had most severely suffered from this disease. As the organism can be propagated though latently infected plants, tissue cultured plantlets do not constitute a safeguard. Hence, many countries, including Mauritius, had to impose a ban on the import of Anthurium plants. Although the disease is known to be present in Reunion island, Mauritius has not so far been affected.

The main aim of this work was to develop molecular tools for the identification and characterization of the pathogen and to carry out genetic studies on strains of the organism from different geographical origins. In this report, the strategies used to do so are described and the results discussed. Specific DNA probes and primers for PCR were developed. These primers were tested in Turkey, where infected plants were available, and they were shown to positively assay for the presence of the bacterial cells with a good degree of sensitivity. DNA from a number of *X axonopodis* pv *diffenbachiae* isolates were obtained from other countries and used for testing the primers as well as for assessing the diversity of the isolates.

General Introduction

Tropical flowers constitute only about 3% of the world trade in fresh cut flowers. The *Anthurium* flower is a heart-shaped bract with a vase life of 14 to 28 days and is perceived as a luxury product. *Anthurium andreanum* is a slow growing perennial that requires shady humid conditions. The Netherlands is the leading world producer of *Anthurium* using high technology and modern facilities for an output of about 25 million stems on only 24 ha of glasshouses. The other major producers are Hawaii, Mauritius and Trinidad.

Higher production costs and the problem of bacterial blight have resulted in a decrease in production since 1988. Mauritius, which has an annual share of about US$4.4 million from the international market also experienced a decrease from about 15 million stems exported in 1996 to about 10 million stems in 2001. High freight charges and delicate packaging requirements also contribute to the difficulty in getting the products to the European market. In order to be on the competitive edge, some growers have invested in the breeding of new varieties for more unusual and attractive colours and shapes. In doing so, resistance against major pathogens such as the bacterial blight and *Ralstonia solnaceraum* is an important concern.

The causal organism, *Xanthomonas axonopodis pv. dieffenbachiae*

Xanthomonas axonopodis pv. dieffenbachiae is the causal agent of *Anthurium* bacterial blight. It was first reported on *Anthurium* in Brazil in 1960. The pathogen can survive in field crop residues such as leaves, petioles and roots for as long as four months, while retaining pathogenicity (Duffy, 2000). It is also infective on many members of the family Aracae but the most common hosts are *Anthurium, Dieffenbachia, Syngonium, Aglaonema, Philodendron, Xanthosoma, Caladium* and *Colocasia* (Sathyanarayana et al., 1997). Host specificity is observed for some strains and in other cases, strains isolated from one aroid can infect and multiply in another aroid without showing severe symptoms of blight (Chase et al. 1992).

Several detection methods are currently available for detection of *X axonopodis* pv *dieffenbachiae*. Most are adequate in that they can detect the presence of the organism when it is present at an appropriate concentration. However, they lack the sensitivity level required for detection of latent infection or when the number of cells is relatively low. Enzyme-linked immunosorbent assays (ELISA) with monoclonal antibodies (Norman and Alvarez, 1994b) has been optimized using an enrichment step. One monoclonal antibody (Xcd 108) is commonly used in commercial kits (AGDIA). Polyclonal antisera are also available for *Xad* (Lemattre et al., 1992).

Norman and Alvarez (1989) have used two media cellobiose-starch (CS) and esculin• trehalose (ET), simultaneously to enhance recovery and hence identification of *Xad* by inhibiting growth of non-target organisms by the antibiotics cycloheximide (150 mg/L) and cephalexin (50 mg/L). Modification of some essential nutrients also favoured the survival of xanthomonads. The nature of the thus isolated bacteria could be confirmed with monoclonal antibodies directed against the xanthomonads. (Norman and Alvarez, 1994b).

Molecular disease **diagnostic methods**

Nucleic acid-based methods have greatly contributed in assays for microbiological agents at a sensitivity, which cannot be attained by other methods. The most commonly used techniques are those based upon amplification of the microbial genome using primers specifically designed to do so. The specificity of the primers is assessed on related species to test their ability to clearly differentiate the target organism.

In addition to detection, such methods are very useful for genetic analysis to determine relatedness of strains of the same or different species. Hence, they have been widely applied to study phylogenetic relationships amongst a large number of organisms. Some regions of the genome are more likely to provide information on diversity than others and have been identified. The intergenic ribosomal sequences are a good example and are used routinely for organisms of differing complexities.

PCR-based methods have largely superceded those based on hybridization for their convenience of application. Their success depends primarily on the choice of primers which have to be selected on the basis of how they are to be used. For example for detection purposes, the criteria for choosing the primers are that they are expected to amplify only the target organism and its variants. If however, the aim is to find polymorphism within a group, then the primers are chosen on their ability to target regions within the genome where the sequence variation is found.

Strategy adopted in this study

The general objective of the work was to identify regions of the *Xanthomonas axonopodis* pv *dieffenbachiae* genome which would be specific to the species. This was performed by using primers already available in the literature as well as those that can randomly amplify the genome. The primers used were those that target the following:

1. *IS* sequences have been found in *Xanthomonas spp.* (Berthier et al., 1994)
2. The hypersensitivity and pathogenicity genes *(hrp)* (Leite et al., 1994)
3. The intergenic transcribed spacer sequences (ITS) of 16S/23S ribosomal genes
4. The intergenic transcribed sequences (ITS) of tRNA genes
5. Random amplification of polymorphic DNA (RAPD)
6. Screening for fragments specific for the species *Xa.* pv *dieffenbachiae*
7. Testing specificity by hybridisation Cloning
8. and sequencing of the fragments Analysis of
9. sequences and design of primers
10. Testing of primers

METHODOLOGY

Study of local strains of *Xad* using new sets of ribosomal primers for 16S/23S rRNA intergenic spacer regions (ITS)

Preliminary results revealed that three local strains (HK.56, DK02 & DK04) that were isolated from aroid hosts had identical profiles as other *Xanthomonas campestris* pathovar including pv. *dieffenbachiae*. To verify the validity of this relationship four other sets of primers were tested in PCR amplifications in addition to the two sets of ribosomal primers [T3A/T5A (Pan *et al.* (1997) & 1493f/23r (Brosius *et al.*, 1980, Woese *et al.*, 1983)] used initially. The new primers were L1/G1 (Jensen *et al.*, 1993), 16Suni1330/23S uni322anti (Honeycutt *et al.*, 1995), OI2/23S1-rev (Jagoueix *et al.*, 1997), and FGPS6/FGPS1509' (Nesme, 1995). The PCR products of local *Xad* suspects were compared with those of reference *Xad* strains and control bacteria.

Southern analysis of ribosomal PCR products exhibiting polymorphism

When polymorphic patterns were produced by PCR amplification, DNA fragments, observed to be common to *Xad* strains but absent in control lanes after ethidium bromide staining, were isolated from the gel by the Freeze and Squeeze columns (Biorad), labelled using the Dig-High Prime Labelling kit (Boehringer Mannheim) and used in Southern hybridisation analysis of the PCR products using conventional techniques

25 mM disodium 3-(4-methoxyspiro{1, 2-dioxetane-3,2'- (5'-chloro) tricyclo[3.3.1.13, 7] decan}-4 yl) phenyl phosphate (CSPD) was used as substrate for chemiluminescent detections. The dephosphorylation of CSPD yields a chemiluminescent product that can be detected using an X-ray film exposed to the membrane for different periods of time depending on the intensity of the signals. After stringent washes, blocking and treatment with anti-dig antibody, the membrane was soaked in detection buffer for 10 min. The CSPD (Boehringer Mannheim) was evenly spread on its surface and the membrane was exposed to an X-ray film. The film was soaked in a developer solution (AGFA) for 2 min with constant agitation for development before being placed in a fixing bath for a further 2 min. The film was left under running water for 30 min and hung to dry.

Restriction endonuclease analysis (REA) of conserved ribosomal PCR products

A screening was performed using enzymes *RsaI, ndeII, TaqI* and *cfoI* to identify restriction endonuclease cut the respective ribosomal PCR products to yield visible bands to compare the different isolates and assess their relatedness by the conservation and/or deletion of restriction sites. Ten microliters of PCR products, 0.2 µl of bovine serum albumin (BSA), and 0.5 µl of enzyme (5U/µl) were mixed and topped up to 20 µl with sterile distilled water. The tubes were centrifuged briefly and incubated in a water bath for 4 h at 37 °C for all enzymes except *TaqI* (60°C). Products were visualized on a UV• transilluminator after agarose gel electrophoresis and ethidium bromide staining.

Cloning of probes and transformation of *E coli*

PCR fragments that were specific to all *Xad* strains or to those *Xad* strains known to cause blight on *Anthurium* were cloned in a pGEM®-T Easy plasmid vector (Promega).

Ligation of PCR product in plasmid vector

A pGEM®-T Easy plasmid vector (Promega) was used to clone PCR products directly after their isolation from agarose gels. The reaction was set up as in Table 2 and the ligation was done overnight at 4°C.

Study of genetic diversity using RAPD markers

One hundred and eleven ten-mers of 60-80% GC (Proligo) were screened for their ability to differentiate between different isolates of *Xanthomonas axonopodis* pv. *dieffenbachiae*. The optimal conditions for PCR parameters, obtained from the first fingerprinting study, were used to produce reproducible DNA fingerprints for 25 available *Xad* isolates. All the selected primers were individually tested under the same conditions. Amplifications were performed in a thermal cycler, programmed as follows: one cycle of 2 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C, and a final extension of 10 min at 72°C.

Details of methods used have been provided in the previous reports submitted to MRC.

Immunocapture PCR (IC-PCR)

PCR tubes were coated with monoclonal antibody MAb XII (Agdia) which is specific to *Xanthomonas campestris* pathovars (Alvarez et al., 1985). A 1: 400 dilution of the antibody in coating buffer (20 mM $NaCO_3$, 35mM $NaHCO_3$) was done and 25 µl were loaded in 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 the supernatant were loaded in 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 (singleplex or multiplex).

RESULTS

Following identification of specific DNA fragments by PCR and hybridization, these were then cloned and sequenced. The sequences were checked manually and the primers designed using the known criteria for optimal annealing features. They were obtained from a commercial oligonucleotide synthesizer and first tested on DNA from *Xanthomonas axonopodis* pv *dieffenbachiae* isolates, both virulent and non-virulent strains, and on DNA of other control bacteria. (Table 1). Those which were specific for the amplification of the *X a.* pv *dieffenbachiae* isolates were selected and further tested.

PCR amplification using primers KJM11 and KJM12

Figure 1.1 shows the results of this reaction. A band of 805 bp was obtained from the anthurium isolates but no amplification was seen from isolates from *Dieffenbachia, Philodendron* and non-pathogenic strains from Mauritius (figure. 1.1 lanes 1-19). Up to 11 polymorphic bands (100 bp to 2.1 kb) were amplified from control bacteria (figure. 1.2)

With primers KJM73 and KJM74, a single product of 1250 bp was amplified from all reference strains of *X axonopodis* pv. *dieffenbachiae* that were isolated from *Anthurium* (figure. 1.3, lanes 1, 4, 6-9, 10-23, 26-37). No band was obtained from the non-pathogenic strains (lanes 23-25) or from strains that were isolated from other aroid hosts, namely *Philodendron, Dieffenbachia* and *Aglaonema* (lanes 2, 3, 5, 23, 25). *Dieffenbachia* strain ICMP576 (lane 9) showed a different profile with four bands (250, 280, 400 and 700 bp respectively). The non-pathogenic strain DK02 (lane 24) shared a band of 350 bp with strain D99 (lane 21). Up to 12 polymorphic bands ranging from 100 bp to 2.2 kb in size were amplified from the control bacteria (figure. 30,). The profile comprising only the band of 1250 bp was obtained in the positive control lane *(Xad* strain T60) only.

Table 1 Bacterial isolates used in this study

ISOLATE/ STRAIN	HOST	LOCATION
X axonopodis pv. *dieffenbachiae* virulent strains		
JV502	Anthurium	Venezuela
JV505	Anthurium	Hawaii
JV511	Anthurium	Puerto Rico
JV589	Anthurium	Reunion
JR579 (type strain LMG695)	Anthurium	Brazil
015-3, 093, 0108, 0147, 0194, XI, 099	Anthurium	Hawaii
ICMP 9564, ICMP 9565	Anthurium	USA
ICMP 9841	Anthurium	Tahiti
ICMP 7461, ICMP 4656	Anthurium	Brazil
ICMP 9569, ICMP 576	Oieffenbachia	USA
ICMP 9570	Epipremnum	USA
ICMP 9566, ICMP 9586	Philodendron	USA
T60, X210, X201, X204, X261, X263, X271	Anthurium	Turkey
Reference strains used as controls		
X axonopodis pv. *allii* BS6	Onion	Mauritius
X axonopodis pv. *begoniae* HK40	Begonia	Mauritius
X axonopodis pv. *carotae* HK46	Carrot	Mauritius
X axonopodis pv. *perlargonii* Sardunya	Geranium	Turkey
X axonopodis pv. *poinsettico/a* HK38	Poinsettia	Mauritius
X axonopodis pv. *phaseoli* HK50,	Bean	Mauritius
405	Bean	Turkey
X axonopodis pv. *phaseoli fuscans* 266	Bean	Turkey
X axonopodis pv. *vasculorum* RI,	Sugar cane	Mauritius
3301	Broom bamboo	Mauritius
X axonopodis pv. *malvacearum* 633,	Cotton	
397	Cotton	Turkey
X axonopodis pv. *vesicatoria* HK06,	Tomato	Mauritius
Kuyucu 3R	Pepper	Turkey
X axonopodis pv. *vignicola* GSPB 6A1	Cowpea	Turkey
X axonopodis pv. *vitians* 700	Lettuce	Turkey
X campestris pv. *campestris* HK01	Cabbage	Mauritius

X. campestris pv. *raphani* DC91-I		*R. sativus*	Turkey
X. campestris pv. *syngonii* HK6 I		Syngonium	Mauritius
X. hortorum pv. *perlargonii* 406		Perlargonium	Turkey
X. translucens pv. *translucens* 1943		Wheat	
X. vasicola pv. *holcicola* 1060		Sorghum	
X. albilineans 3303		Sugar cane	Mauritius
Stenotrophomonas maltophilia 418			Turkey
Pseudomonas rubrisubalbicans Prub		Sugar cane	
Pseudomonas chichorii MRX		Tomato	Turkey
Pseudomonas corrugata Pcorr		Tomato	Turkey
Pseudomonas syringae pv. Tomato	AAD-1b	Tomato	Turkey
Pseudomonas viridiflava Karun I		Melon	Turkey
Erwinia carotovora pv. *carotovora*	Dieff2b	Dieffenbachia	Turkey
Erwinia chrysanthemi 2835			
Erwinia amylovora 459			Turkey
Ralstonia solanacearum HK36		Potato	Mauritius
Pantoea agglomerans 441		Tomato	Turkey
Agrobacterium tumefaciens 27At2		Rose	Turkey
Clavibacter michiganensis subs. *Michiganensis* 1/2c		Tomato	Turkey
ISOLATE/ STRAIN		HOST	LOCATION
Strains isolated from aroids in Mauritius			
HK03, HK04, HK05, HK07, HK08, HK09, HKIO, HK57, DK02		Anthurium	Mauritius
HKI7, HK23, HK58		Caladium	
HK20		Colocasia	
HK56		Dieffenbachia	
DK04		Aglaonema	

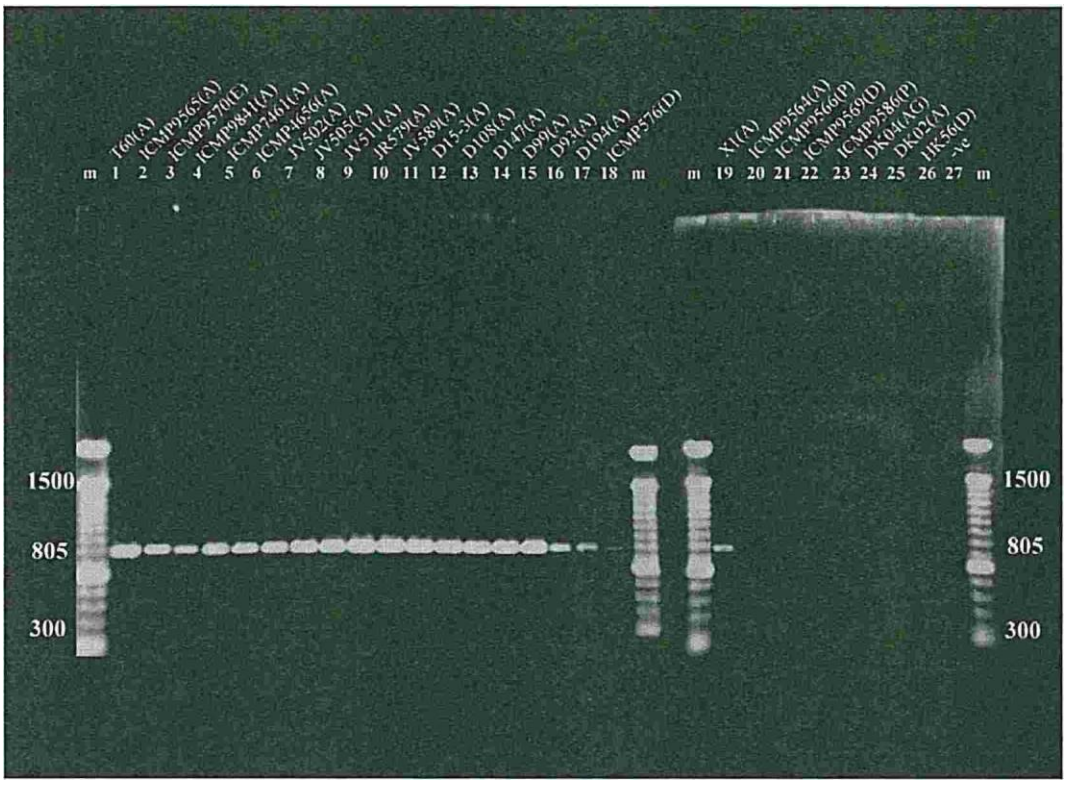

Figure 1.1 PCR amplification of strains of *Xanthomonas axonopodis* pv. *dieffenbachiae* with primers KJMI 1 and KJMI2. Lanes are labelled with strain numbers and hosts of origins: A, *Anthurium;* AG, *Aglaonema;* D, *Dieffenbachia;* E, *Epipremnum;* P, *Philodendron*

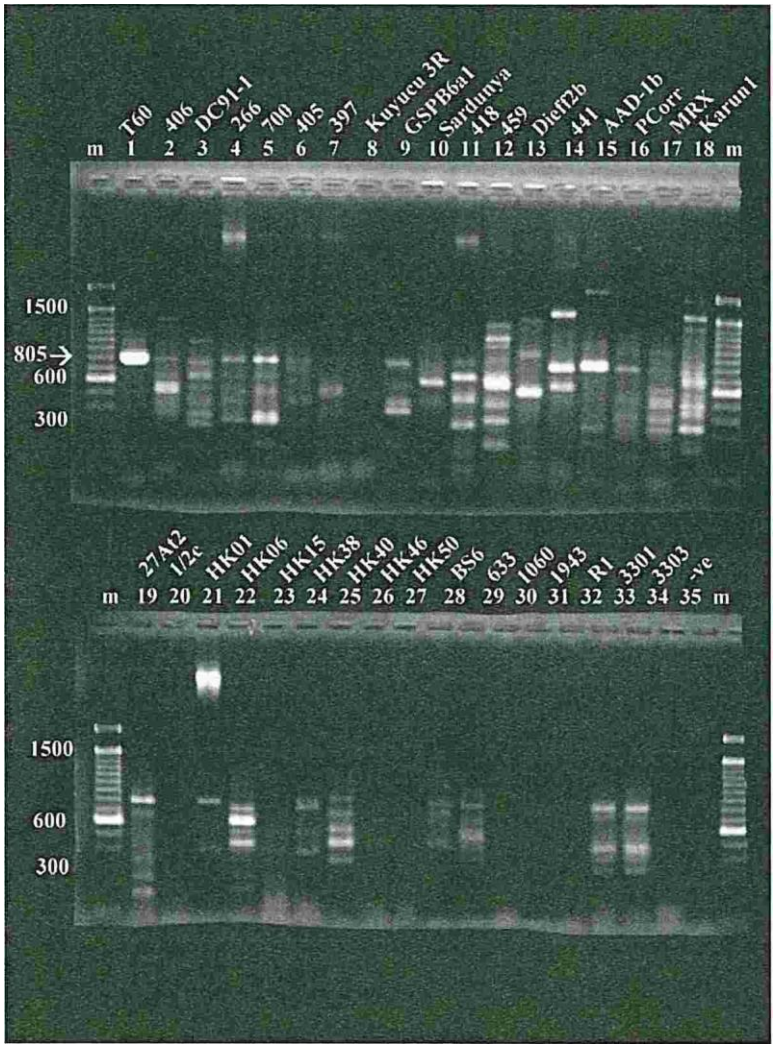

Figure 1.2. PCR amplification of control strains with primers KJMI 1 and KJM12. Lanes are labelled with strain numbers (**Table** 3, p33). m, 100 bp ladder; lane I, positive control; lanes 2-10: *X hortorum, X axonopodis* pv. *raphani, phaseoli-fuscans, vitians, phaseoli, malvacearum, vesicatoria, vignicola, 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.*

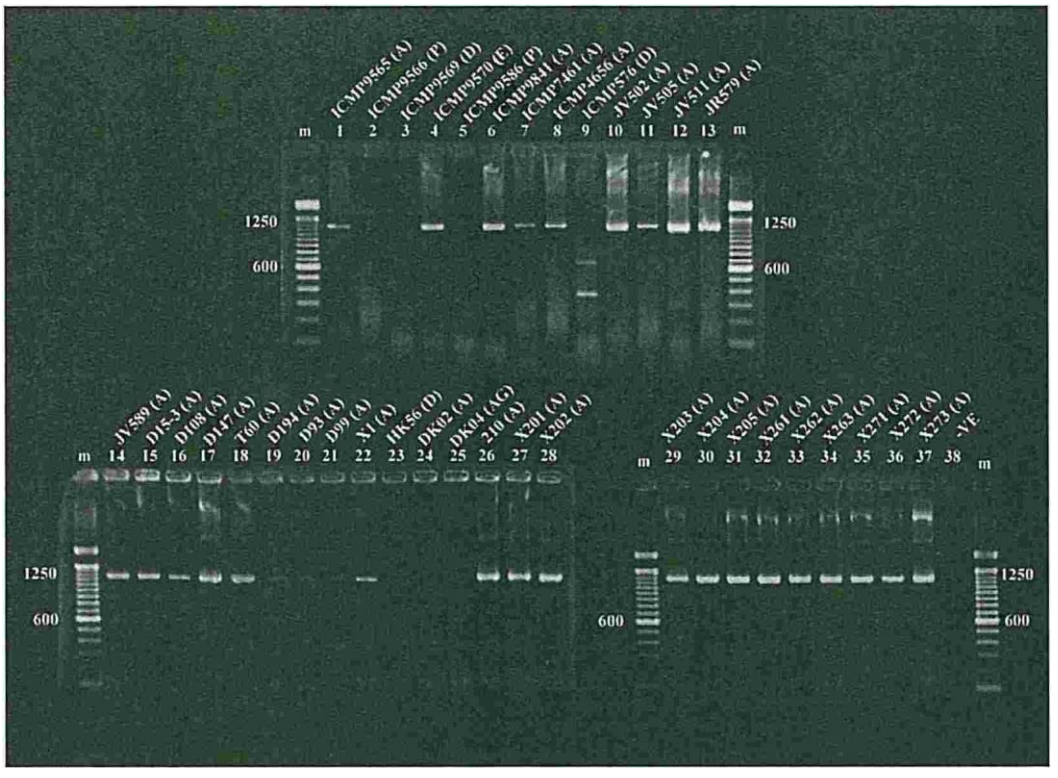

Figure 1.3 PCR amplification of strains of *Xanthomonas axonopodis* pv. *dieffenbachiae* with primers KJM73 and KJM74. Lanes are labelled with strain numbers and hosts of origins: A, *Anthurium;* AG, *Aglaonema;* D, *Dieffenbachia;* E, *Epipremnum;* P, *Philodendron.*

Diagnostic testing on infected plant material

This part of the work was carried by M. R. Khoodoo in Turkey, where a batch of in-vitro cultured plants had developed symptoms of the disease, and were available for testing. Extracts were made from the plants and were tested by direct PCR and by immuno• capture PCR. The sensitivity of the assay was measured.

Figure 2 shows the amplification of DNA from extracts of plant tissues with primers KJM73 and KJM74. The expected band of 1250 bp was obtained in most of the samples.

Figure 3 shows the amplification of DNA samples again from plant tissue extracts, with a combination of primers KJM11/KJM12, KJM34/KJM36 and KJM73/KJM74. This was an attempt to obtain an assay that would amplify a maximum number of strains in one reaction. The expected size of fragments were obtained.

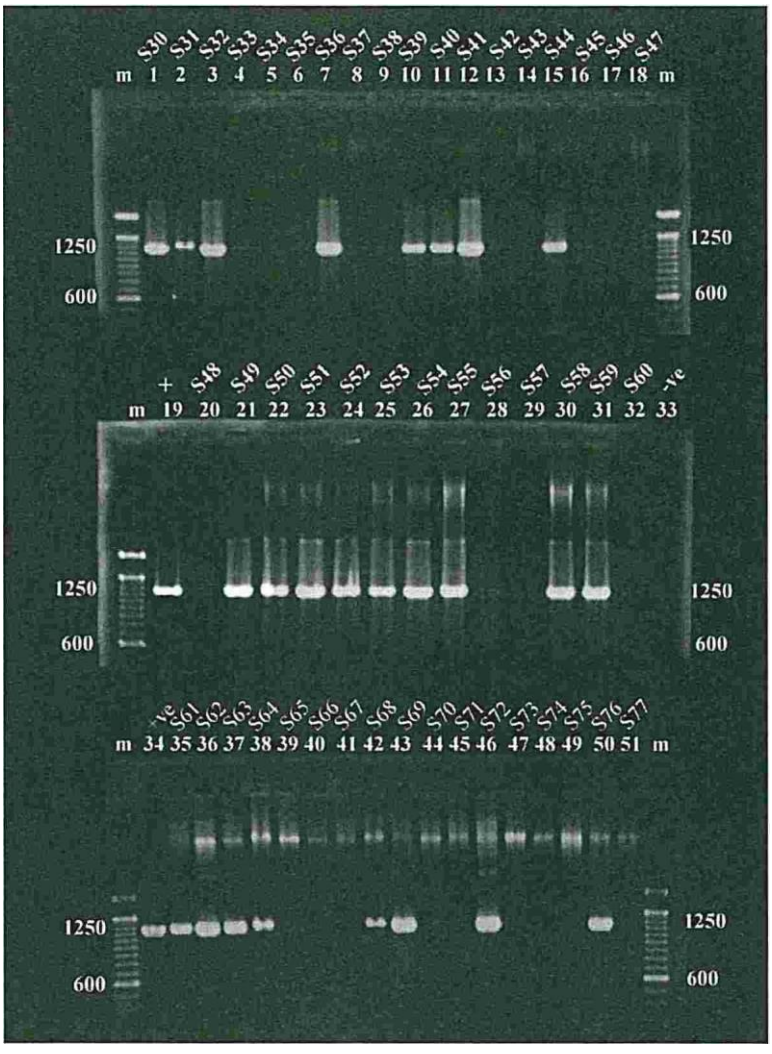

Fig. 2. Detection of the blight pathogen from DNA samples S30 to S77, extracted from infected leaves, in singleplex PCR with primers KJM73 and KJM74. m= 100 bp DNA ladder. Lanes are labelled with sample designations. Lanes 19 and 34, positive control.

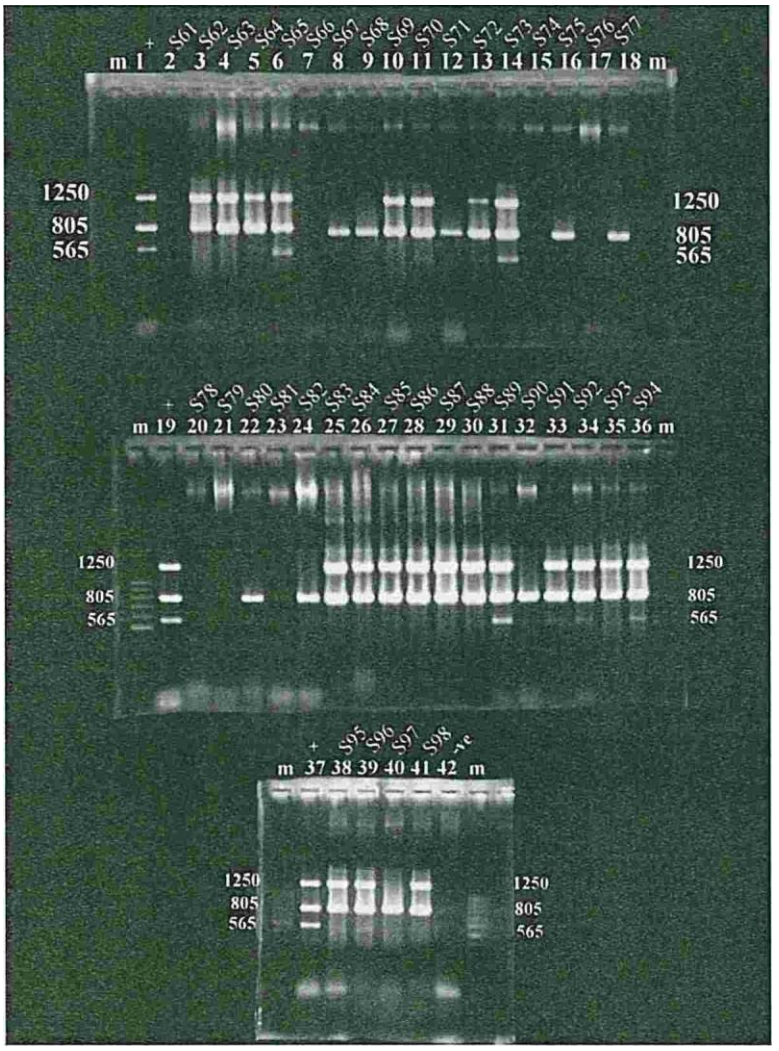

Fig. 3. Detection of the blight pathogen from DNA samples S61 to S99, extracted from infected leaves, in multiplex PCR with primers KJMI l/KJM12, KJM34/KJM36 and KJM73/KJM74. m= I 00 bp DNA ladder. Lanes are labelled with sample designations. Lanes I, l9 and 3 7, positive control.

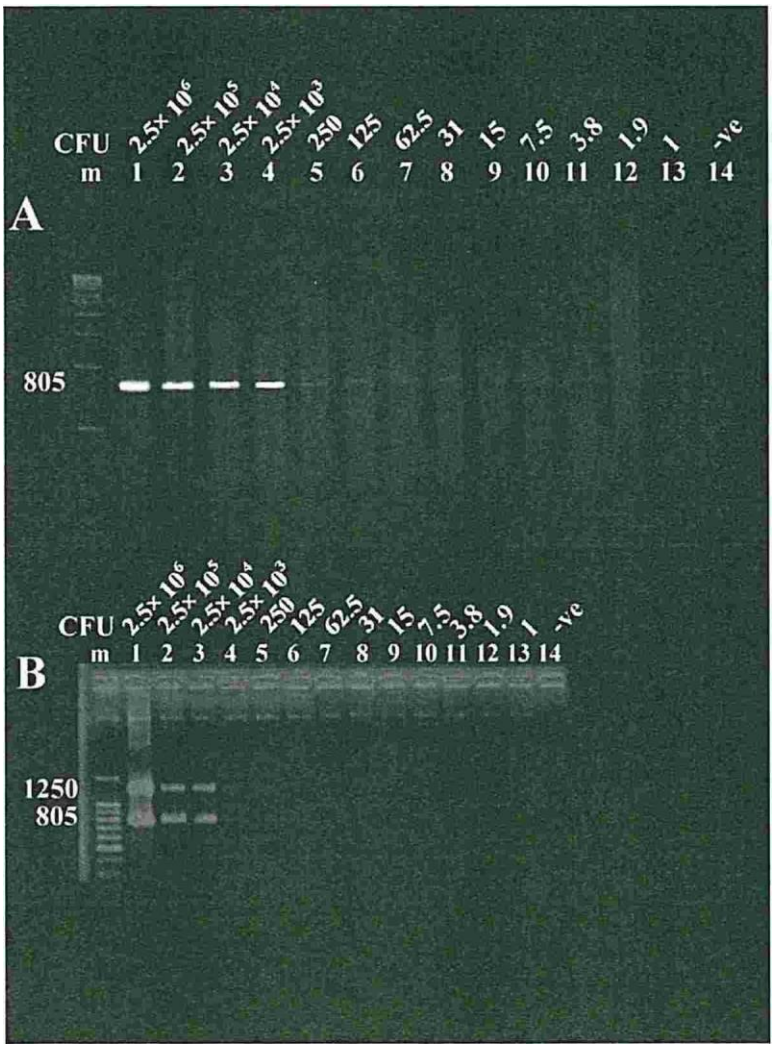

Fig. 4 Results of sensitivity tests for (A) Immunocapture-PCR with primers KJMI I and KJM12 and **(B)** Immunocapture Multiplex PCR with the six primers. Lanes are labelled with the number of CFU per reaction. m= DNA size marker.

Sensitivity of detection

The assays were done with known number of CFU after a dilution of bacterial cells. The sensitivity limit of the indirect ELISA was estimated to be 1×10^5 CFU in the 100 µl used (10^6 CFU/ml). However, when the MAb XII was used for immunocpature prior to an amplification by primers KJM11 and KJM12, the band of 805 bp, was obtained with the sample diluted to 2 CFU per reaction (80 CFU/ml). In the case of immunocapture• multiplex PCR, two bands (805 and 1250 bp) were visible in lanes 1-5 only **(fig. 4B,),** representing 250 CFU per reaction (10^4 CFU/ml). The band of 805 bp only was detected in lane 6 (125 CFU per reaction or 5000 CFU/ml).

Discussion and Major Outcomes

In the above section, the use of the primers designed in this study is shown in the form of amplification products of PCR reactions. Several sets of primers had been chosen on the basis of the sequences of DNA fragments obtained previously and which were shown to be specific for the organism, *Xanthomonas axonopodis* pv. *dieffenbachiae* . They were first tested on pure DNA of the pathogen as well on a number of other control bacterial DNA and a preliminary selection was made.

The selected primer pairs were then tested on samples from infected plants at the University of Erzurum, in Turkey. They could amplify the same bands as when the pure DNA was used, indicating that the bacterial DNA was being successfully targeted. In addition, an immunocapture- PCR assay was also performed and was found to work.

In this study, several methods have been optimized which allow the differentiation of *Xad* from other control bacteria. These were based on PCR amplification and hybridization techniques and proved to work accurately and reproducibly. They were then optimized to be used as a detection method for the pathogen.

The tools thus obtained allowed deeper study into the genetic variability of the strains of the same species. Hence, it was possible to detect differences among the isolates of different aroid hosts. It was also shown that even when the disease is not reported, isolates of the same pathogen can be found on the anthurium plants without them being virulent.

This study describes the combination of six pnmers m a multiplex reaction for the profiling of *X axonopodis* pv. *dieffenbachiae* in a high stringency PCR. It was demonstrated that the multiplex PCR could differentiate among the various strains tested within the group of strains of *X axonopodis* pv. *dieffenbachiae* and more importantly the profiling could differentiate between strains of the blight pathogen and the control strains. Distinct profiles were obtained from most of the strains that were isolated from *Anthurium* or *Philodendron* or *Dieffenbachia.* The products targeted by primers KJMI 1 and KJM12 and primers KJM73 and KJM74 were not amplified from the non-pathogenic strains of *Xanthomonas* and 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 hypothesized.

The PCR technique (singleplex and multiplex) using infected plant DNA was more efficient in the detection of the pathogen when compared to indirect ELISA in which two out of 12 infected plants were tested negative in both replicates **(Table 14,** p136). Immunocapture PCR (IC-PCR) was shown to be twice more sensitive than conventional PCR for the detection of the pathogen. This can be attributed to the fact that the cell capturing by the monoclonal antibody circumvents the problem of PCR with contaminated DNA. In latently infected *Anthurium* 85.2% of plants were tested positive by IC-PCR whereas only 44.4% of plants were positive by conventional PCR. In plants showing symptoms, the number of samples in which the pathogen was detected varied for primers KJM11/KJM12, KJM73/KJM74 and the multiplex reaction using these two sets of primers and primers KJM34/KJM36 for conventional PCR. When using primers KJM11 and KJM12 in conventional PCR, only 44.3% of the plants were positive for the blight pathogen. However, the pathogen was detected in 74.3% of the samples when using primers KJM73 and KJM74 and in 82.6% of the samples in the multiplex PCR. The variations in the number of positive samples using the same DNA extracts and the occurrence of false negatives when using *Anthurium* DNA as template for the PCR could be attributed to the presence of PCR inhibitors that might have contaminated the DNA during extraction. Direct amplification 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 modifications of DNA extraction procedures could resolve these problems, but the protocols normally employed involve the use of numerous and complex reagents (Klotz and Zimm, 1972; Maniatis et al., 1982; Schneider et al., 1993). The high risk of contamination related to the steps necessary for preparing the samples and the use of toxic compounds such as phenol and chloroform, make these procedures difficult for processing a high number of samples. The probability of detecting the pathogen could be increased by increasing the number of samples from each plant.

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 singleplex IC-PCR for primers KJM11 and KJM12 was about 60-fold more sensitive than the multiplex IC-PCR and concentration as low as 2 CFU per reaction could be detected (fig. 42, p147). This could be explained by inhibition of the PCR due to the high primer concentration in the multiplex reaction. The singleplex IC-PCR technique is therefore recommended for each of the primer sets described for the diagnostic assays using *Anthurium* extracts directly. The multiplex PCR using the six primers could be useful for the purpose of strain differentiation when using purified bacterial DNA in evolutionary and phylogenetic studies of the pathovars.

Current assays to identify pathogenic bacteria in *Anthurium* tissue by means of plating on selective or conventional media, immunoassays, host inoculation, etc., are labour-intensive and in some cases not sensitive enough. The need for rapid, reliable, sensitive and specific methods for detection of plant pathogenic bacteria is especially worthy in the case of those pathogens that are responsible of important economic losses and when plant material is subject to quarantine. The PCR method is used for the detection of several plant quarantine bacteria as a number of primers are available (Bereswill et al., 1992; Seal et al., 1992, 1993; Hartung et al., 1993; Henson and French, 1993; Schneider et al., 1993; Bereswill et al., 1995; Li and De Boer, 1995; Hartung et al., 1996). This study showed that the application of molecular techniques, such as IC-PCR, could resolve most of the difficulties that the other methods present when a rapid and accurate analysis is

essential 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 as it is the case for the "mother" *Anthurium* plants imported in Mauritius.

The main outcome of this study is the development of diagnostic tools for the pathogen *Xaxonopodis* pv. *dieffenbachiae*. This is the first reported molecular tool for this pathogen, apart from the immunoassays using monoclonal antibodies. The tools consist of specific primers, which were obtained from the specific DNA fragments of the organism. Such diagnostic methods are crucial in any disease management strategy and the ones described here will be available for plant indexing purposes and for use in quarantine control. They can also be applied for screening in vitro cultured plantlets. The latter can harbour the pathogen in a latent phase, leading to the disease manifesting once the plants are in the fields. Several countries have recently experienced such problems when importing tissue-cultured materials. With the availability of a sensitive detection method, the disease will be better managed, thus leading to fewer losses by the growers.

The DNA sequence of the various fragments have been submitted to GenBank.

All the DNA extracts, recombinant clones and probes are stored at -go ° C and available from the principal investigator. Attached is a copy of the paper which emanated from this work and which appeared in Plant Disease.

Berthier, Y., Thierry, D., Lemattre, M., and Guesdon, J-L (1994) Isolation of an Insertion Sequence *(IS1051) from Xanthomonas campestris* pv. *dieffenbachiae* with Potential Use for Strain Identification and Characterization. *Applied and Environmental Microbiology*. 60, No. 1: 377-384.

Chase, A. R., Stall, R. E., Hodge, N. C., Jones, J.B. (1992) Characterization of *Xanthomonas campestris* strains from aroids using physiological, pathological, and fatty acid analyses. *Phytopathology*. 82: 754-759.

Duffy, B. (2000) Survival of the anthurium blight pathogen, *Xanthomonas axonopodis* pv. *dieffenbachiae*, in field crop residues. *European Journal of Plant Pathoogyl*. 106: 291-295.

Leite Jr., R. P., Minsavage, G. V., Bonas, U. and Stall, R. E. (1994) Detection and Identification of Phytopathogenic *Xanthomonas* Strains by Amplification of DNA sequences related to the *hrp* Genes of *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Environ. Microbial*. 60, No 4: 1068-1077.

Norman, D., and Alvarez, A. M. (1989) A rapid method for the presumptive identification of *Xanthomonas campestris* pv. *dieffenbachiae* and other xanthomonads. *Plant Dis*. 73: 654-658.

Norman, D. J. and Alvarez, A. M. (1994a) Latent infections of in vitro Anthurium caused by *Xanthomonas axonopodis* pv. *dieffenbachiae*. *Plant Cell, Tissue and Organ Culture* 39: 55-61.

Norman, D. J. and Alvarez, A. M. (1994b) Rapid detection of *Xanthomonas campestris* pv. *dieffenbachiae* in Anthurium plants with a miniplate-enrichment/ELISA system. *Plant Diease*. 78: 954-958.

Sathyanarayana, N., Reddy, O. R., and Latha, S. (1997) Interception of *Xanthomonas campestris* pv. *dieffenbachiae* on Anthurium Plants from the Netherlands. *Plant Disease*. 82: 262.