RAPD-PCR Fingerprinting and Southern Analysis of *Xanthomonas axonopodis* pv. *dieffenbachiae* Strains Isolated from Different Aroid Hosts and Locations

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ABSTRACT

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Anthurium blight, caused by *Xanthomonas axonopodis* pv. *dieffenbachiae*, is a systemic disease of *Anthurium* and other aroids. The aims of this work were to study the genetic diversity among *X. axonopodis* pv. *dieffenbachiae* strains and to identify, from the polymerase chain reaction (PCR) profiles, DNA probes that would be specific for the pathovar *dieffenbachiae*. Twenty-five *X. axonopodis* pv. *dieffenbachiae* strains, isolated from different hosts and geographical locations including Mauritius, were fingerprinted using the random amplified polymorphic DNA (RAPD)-PCR technique. The fingerprints were analyzed by the National Taxonomy System Software (NTSYS). The specificity of some of the RAPD fragments selected from PCR profiles was tested by Southern analyses of the PCR products. Ten arbitrary primers were chosen from an initial set of 111 decamers. Two hundred and nine RAPD markers were generated in eight individual DNA profiles. A correlation was found between the serotypes and the RAPD profiles for some groups of isolates. A possible link was also observed between the host range of the isolates tested and their RAPD profiles for strains isolated from *Dieffenbachia* and *Philodendron*. These results were confirmed by Southern analysis. Cluster analysis by the unweighted pair group method, arithmetic average (UPGMA) confirmed that the pathovar is genetically diverse with some strains that were clustered together showing similar host preferences. DNA probes with a potential use in molecular diagnostics of Anthurium blight were identified. This preliminary work could be used to develop PCR primers that will enable the sensitive detection of the pathogen in latently infected plants.

One of the most devastating *Xanthomonas* diseases affecting the cut flower industry worldwide is bacterial blight in aroids. Aroids include species and cultivars of *Anthurium*, *Aglaonema*, *Dieffenbachia*, *Epipremnum*, *Philodendron*, *Spathiphyllum*, and *Syngonium*. The disease is a persistent threat to *Anthurium* growers worldwide because latently infected plants, propagated by tissue-culture, harbor the pathogen for several months without exhibiting any biological symptoms (28,29) and also because of its quick spread to clean fields by aerosols (4,39). Tolerant varieties of *Anthurium* tend to act as permanent reservoirs of the pathogen, although they sustain the production of flowers in the presence of the disease (11,12). *Xanthomonas axonopodis* pv. *dieffenbachiae* (37) (= *X. campestris* pv. *dieffenbachiae* [23]) was first described as the causal agent of the disease of *Dieffenbachia maculata* (Lodd.) G. Don (syn. *D. picta*) in 1939. Several other aroid species

were reported to be susceptible to *Xanthomonas* blight (17,24–26). Production losses exceeding \$5 million have been reported in Hawaii (9). In spite of the use of strict sanitation to control the disease, there has been more than a 50% loss of plants in Hawaii (11). All Araceae taxa are potential sources of infection (30).

The pathovar *dieffenbachiae* is known to be serologically diverse and comprises 12 known serotypes (20). A biochemical diversity was also revealed by starch hydrolysis and oxidation of 95 substrates (BIOLOG, Hayward, CA) in a study by Alvarez et al. (3). The analysis of genomic DNA using PCR-based methods has proven to be a fast, sensitive, and reliable method for determining genetic relationships among pathogenic organisms. Several methodologies are currently available for studying genetic variability among strains of the same organism/pathovar. These include various modifications of the polymerase chain reaction (PCR), such as the random amplification of polymorphic DNA (RAPD) (40,41) and DNA amplification fingerprinting (8).

RAPD-PCR has the potential to detect polymorphism throughout the entire genome as compared to other techniques such as ribotyping (14). This technique can produce a spectrum of amplified products, characteristic of the template DNA due to

arbitrary priming, at a relatively low annealing temperature, at multiple locations. RAPD markers have been used for strain differentiation (27), identification, and other genetic analyses for several plant (10,13,42), fungal (5,15), and bacterial genera (1,14,16,22,31).

This work was carried out to investigate the use of the RAPD technique to generate polymorphic markers for *X. campestris* pv. *dieffenbachiae* and DNA probes for different groups of strains within the pathovar *dieffenbachiae*. The relationships among strains isolated from different hosts and from different geographical regions were also investigated.

MATERIALS AND METHODS

Bacterial strains. Table 1 indicates the origin of the strains used in this study. Because of phytosanitary measures, the DNA of foreign strains of *X. axonopodis* pv. *dieffenbachiae* and not live cells were obtained from collections in Hawaii and New Zealand. Three nonpathogenic *Xanthomonas* strains (HK56, DK02, and DK04), isolated from Mauritian aroids in a previous study (unpublished), were also included here.

DNA extraction. DNA was extracted from 50-ml overnight cultures in nutrient yeast extract glycerol broth (NYGB) by using a combination of protocols described by Ausubel et al. (6) and Lazo et al. (18). Cells were pelleted at 5,000 rpm and washed twice in 10 ml of STE buffer (50 mM Tris, 150 mM NaCl, 50 mM Na₂EDTA). After resuspension in STE and cell lysis, the lysate was treated with CTAB/NaCl mixture and chloroform extracted as described by Ausubel et al. (6). The CTAB/NaCl treatment and chloroform extraction were repeated until there was no precipitate between the aqueous and organic phases, and the nucleic acids were precipitated in isopropanol.

RAPD reaction. One hundred and eleven primers of 10 bp and 60 to 80% GC content (Proligo, Singapore) were screened for their ability to differentiate among isolates. Reaction parameters such as the concentration of MgCl₂, primer, and DNA template were sequentially varied until optimal conditions were found to produce reproducible RAPD fingerprints. All the other primers were then tested under the same conditions. DNA concentrations tested were 2, 5, 10, 15, 20, 25, 30, and 40 ng; MgCl₂ concentrations tested were 1.5,

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2.0, 2.5, 3.0, 3.5, and 4.0 mM; and primer amounts were 6, 8, 10, 12, 14, 16, 18, and 20 pmol. Reactions were set on ice to a final volume of 30 µl. Amplifications were performed in a thermal cycler (Techné Cyclogene, Cambridge, UK) programmed as follows: 1 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. The PCR products were kept at -25°C until further analysis. PCR products were run on a 2.0% agarose gel and the banding profiles recorded using the Advanced American Biotechnology (AAB) system comprising a camera placed on a UV-transilluminator (302 nm) linked to computer software allowing image capturing.

Screening and selection of primers for RAPD-PCR. Primers were first selected on the basis of the polymorphic bands common to three reference strains of *X. axonopodis* pv. *dieffenbachiae* (JV505, JR579, and JV589, respectively) but absent in the RAPD fingerprints of the other two isolates, *X. campestris* pv. *campestris* and *X. axonopodis* pv. *vesicatoria*.

The selected primers were then used in PCR reactions with a large number of

control bacterial DNA templates (Table 1). Primers yielding profiles common to *X. axonopodis* pv. *dieffenbachiae* strains and different from those of other pathogens/species of *Xanthomonas* were kept for further amplifications with the 22 *X. axonopodis* pv. *dieffenbachiae* strains and three nonpathogenic Mauritius strains (Table 1).

Scoring and statistical analysis of fingerprints. Clearly visible and reproducible RAPD products were scored from photographs of ethidium bromide stained gels. The presence or absence of bands was recorded, and the scores for each primer were merged in a Microsoft Excel spreadsheet.

NTSYS-pc V1.8 (33) computer software was used for cluster analysis of the independent markers. The generated pairwise similarity matrix was used to group strains by the unweighted pair group method, arithmetic average (UPGMA; 35). A dendrogram was derived from the similarity matrix.

Reproducibility testing. All amplification reactions with selected RAPD primers were repeated once again and loaded on agarose gels for Southern blotting. The

RAPD profiles were checked after electrophoresis for their identity to the previous respective reactions. The presence of a specific band in both sets of amplifications and the reproduction of the respective signals in Southern analyses served as evidence of reproducibility.

Southern analysis. DNA fragments specific to *X. axonopodis* pv. *dieffenbachiae* strains were isolated from agarose gels by the use of the Rapid Gel Extraction System (Life Technologies, Rockville, MD) and used as probes in Southern hybridizations. Digoxigenin (DIG) labeling was performed by the random-primed method using the Dig-high prime kit (Boehringer Mannheim, Indianapolis, IN) for the incorporation of DIG dUTP. Fifteen microliters of RAPD products were separated on a 0.8% agarose gel. The DNA fragments were transferred to a Hybond-N nylon hybridization membrane (Amersham, Buckinghamshire, England) overnight by Southern transfer. The DNA was fixed by cross linking after exposure to UV light. Hybridizations were allowed to take place overnight at 55°C.

Chemiluminescent detection. Chemiluminescent detection was performed on

Table 1. Origins of bacterial strains used in the study

Isolate/strain	Host	Location	Source or reference
<i>Xanthomonas axonopodis</i> pv. <i>dieffenbachiae</i> JV502,	<i>Anthurium</i>	Venezuela	I. Soustrade ^a
JV505,	<i>Anthurium</i>	Hawaii	I. Soustrade
JV511,	<i>Anthurium</i>	Puerto Rico	I. Soustrade
JV589,	<i>Anthurium</i>	Reunion	I. Soustrade
JR579 (type strain LMG695),	<i>Anthurium</i>	Brazil	I. Soustrade
D15-3, D108, D147 (all of serotype 1), D93 and D194 (serotype 8),	<i>Anthurium</i>	Hawaii	A. Alvarez ^b
X1 (serotype 10), D99 (serotype 12),	<i>Anthurium</i>	Hawaii	A. Alvarez
ICMP 9564, ICMP 9565,	<i>Anthurium</i>	South Florida	J. Young ^c
ICMP 9841,	<i>Anthurium</i>	Tahiti	J. Young
ICMP 7461 (LMG8664), ICMP 4656,	<i>Anthurium</i>	Brazil	J. Young
ICMP 9569, ICMP 576,	<i>Dieffenbachia</i>	Florida	J. Young
ICMP 9570,	<i>Epipremnum</i>	Florida	J. Young
ICMP 9566, ICMP 9586,	<i>Philodendron</i>	Florida	J. Young
HK56 (nonpathogenic on <i>Anthurium</i>),	<i>Dieffenbachia</i>	Mauritius	M. H. R. Khoodoo ^d
DK02 (nonpathogenic on <i>Anthurium</i>),	<i>Anthurium</i>	Mauritius	M. H. R. Khoodoo
DK04 (nonpathogenic on <i>Anthurium</i>)	<i>Aglaonema</i>	Mauritius	M. H. R. Khoodoo
<i>X. campestris</i> pv. <i>campestris</i>	Cabbage	Mauritius	This study
<i>X. campestris</i> pv. <i>syngonii</i>	Syngonium	Mauritius	This study
<i>X. axonopodis</i> pv. <i>begoniae</i>	Begonia	Mauritius	This study
<i>X. axonopodis</i> pv. <i>carotae</i>	Carrot	Mauritius	This study
<i>X. axonopodis</i> pv. <i>poinsetticola</i>	Poinsettia	Mauritius	This study
<i>X. axonopodis</i> pv. <i>phaseoli</i>	Bean	Mauritius	This study
<i>X. axonopodis</i> pv. <i>vasculorum</i>	Sugar cane	Mauritius	A. Dookun-Saumtally ^e
<i>X. axonopodis</i> pv. <i>vasculorum</i>	Broom bamboo	Mauritius	A. Dookun-Saumtally
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	Tomato	Mauritius	This study
<i>X. axonopodis</i> pv. <i>allii</i>	Onion	Mauritius	S. Soomary ^f
<i>X. axonopodis</i> pv. <i>malvacearum</i>	Cotton	...	Y. Jaufeerally-Fakim ^g
<i>X. translucens</i> pv. <i>translucens</i>	Wheat	...	Y. Jaufeerally-Fakim
<i>X. vasicola</i> pv. <i>holcicola</i>	Sorghum	...	Y. Jaufeerally-Fakim
<i>X. albilineans</i>	Sugar cane	Mauritius	A. Dookun-Saumtally
<i>Pseudomonas rubrisubalbicans</i>	Sugar cane	...	Y. Jaufeerally-Fakim
<i>Erwinia chrysanthemi</i>	Potato	...	Y. Jaufeerally-Fakim
<i>Ralstonia solanacearum</i>	Tomato	Mauritius	This study

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membranes using 25 mM disodium 3-(4-methoxyspiro[1, 2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}] decan]-4 yl) phenyl phosphate (CSPD) as substrate. All procedures were followed according to the manufacturer's instructions (Boehringer Mannheim). An X-ray film (AGFA) was exposed to the membrane for 30 min (longer for faint signals) before development.

RESULTS

RAPD fingerprinting. The conditions for reproducible fingerprints were 25 ng of DNA, 0.2 mM of each dNTP, 0.5 μ M of primer, 2.5 mM MgCl₂, and 1 unit of *Taq* polymerase (Boehringer Mannheim). The modification of the initial cycle parameters did not result in improved fingerprints; therefore the initial parameters were used for the whole study. Eight primers (Table 2) out of 111 allowed a comparison among *X. axonopodis* pv. *dieffenbachiae* strains and were selected for the study of genetic diversity. RAPD reactions were performed

several times over a number of months, and the results were found to be reproducible. The descriptions for four primers are illustrated below.

Amplification polymorphism among *X. axonopodis* pv. *dieffenbachiae* strains was obtained with the four primers P27, P49, P54, and P59. With primer P27, a band of 1.3 kb was amplified from 16 out of 18 *Anthurium* strains (Fig. 1, lanes 1, 2, 6–9, 11–19, and 23) and one *Epipremnum* strain (lane 5). Bands ranging in size from about 400 to 2,300 bp were amplified. Isolates JV502, JV505, JV589, D108, D194, and D93 (lanes 11, 12, 15, 17, 19, and 20) were distinguished by a fragment of 1,500 bp not amplified from other strains. Strains JV502, JV511, JV579, and D99 (lanes 11, 13, 14, and 21) shared a fragment of 900 bp. Two *Dieffenbachia* strains (ICMP 9569 and 576; lanes 4 and 10) shared four bands (approximately 550, 600, 750, and 900 bp), but strain ICMP 576 also shared a 1,400-bp fragment with *Anthurium* strains ICMP 9841 and DK02 (lanes 7 and 25).

The profiles obtained with primer P49 are shown in Figure 2. A fragment of 840 bp was amplified from 17 out of 18 *Anthurium* strains (Fig. 2 lanes 1, 2, 7–9, 11–19, 21, 22, and 24) and the *Epipremnum* strain (lane 5). However, a band of 1 kb was amplified from four strains (lanes 3, 4, 6, and 10) that were isolated from either *Dieffenbachia* (ICMP 9569 and 576) or *Philodendron* (ICMP 9566 and 9586). Different banding patterns were produced for strains ICMP 9564, D194, D93, D99, X1, HK56, DK04, and DK02 between 500 bp and 2 kb (lanes 1, 19–25).

Primer P54 also related the strains with respect to their host of origin and gave rise to relatively more complex profiles than the primers described above (Fig. 3). A fragment of 800 bp was amplified from ICMP strains 9565, 9570, 9841, 7461, and 4656 (lanes 2, 5, 7–9) and strains JV502, JV505, JV511, JR579, JV589, D15-3, D108, and D147 (lanes 11–18). Strains ICMP 9566 and 9586 (both isolated from *Philodendron*) had an identical profile (lanes 3 and 6). *Anthurium* strains ICMP 7461, 4656, and JR579 (all isolated from Brazil) showed different profiles and shared only two bands of 700 and 1,250 bp (lanes 8, 9, and 14). Strains D194 and D93 (same serotype) shared four bands of 750, 1,250, 1,400, and 1,500 bp (lanes 19 and 20).

Additional RAPD markers were generated with primer P59. A product of 790 bp was amplified from all isolates of *X. axonopodis* pv. *dieffenbachiae* except those of serotype 8 (D194 and D93; Fig. 4, lanes 19 and 20). Strains D93 and D194 were distinguished by a 1,050-bp band not present in other strains. A fragment of 1 kb

Table 2. Sequences of selected random amplified polymorphic DNA (RAPD) primers for the study of diversity

Primer	Nucleotide sequence (5'-3')	Name of probe	Size ^a (bp)
P27	GGCAGGCTGT	P27-1300	1,300
P49	GAAACGGGTG	P49-840	840
		P49-1000	1,000
P54	CAGCACCCAC	P54-800	800
P59	AGGTGACCGT		...
P69	GTCCACACGG	P69-1300	1,300
P81	GTGAGGCGTC		...
P86	TGGACCGGTG		...
P102	TGAGCGGACA		...

^a Size of probe demonstrated to be specific to *Xanthomonas axonopodis* pv. *dieffenbachiae*.

Fig. 1. Random amplified polymorphic DNA profiles obtained with primer P27. Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control.

was observed in 14 strains isolated from *Anthurium* (lanes 2, 7–9, 11–19, and 25) and the *Epipremnum* strain (lane 5). However, *Anthurium* strains ICMP 9564, D93, D99, and X1 (lanes 1, 20–22) and strains ICMP 9566, 9569, 9586, and 576 (isolated from *Philodendron* and *Dieffenbachia*) did not produce this fragment. Different RAPD profiles could be clearly distinguished. Strains in lanes 2, 5, 12, 16–18 had an identical profile with four bands (790 bp, 1.0, 1.5, and 1.7 kb).

Statistical analysis. Analysis of the genetic coefficient matrix derived from the

scores of RAPD profiles (Fig. 5) showed that the minimum and maximum percent similarities among the isolates studied were in the range of 10 and 65%, respectively. Results of cluster analysis are represented by the UPGMA dendrogram (Fig. 6). The isolates were clustered in four main groups (GP1, GP2, GP3, and GP4). GP1 consisted of two strains (ICMP 9564 and DK04) that were isolated from *Anthurium*. Twelve out of 18 *Anthurium* strains and one *Epipremnum* strain formed GP2. *Philodendron* and *Dieffenbachia* strains were segregated into two subgroups

of GP3 except for the nonpathogenic strain HK56, which was clustered in GP4. All remaining strains belonged to GP4, which also comprised a subgroup for two Hawaiian strains isolated from *Anthurium* (D93 and D194).

Southern hybridization analysis. The Southern analysis was useful in identifying RAPD products with sequence similarity to the one used as probe. After hybridization of PCR products in Figure 1 with probe P27-1300 (from *Anthurium* strain D108, amplified with primer P27), a strong signal of 1.3 kb was observed for 13 out of

Fig. 2. Random amplified polymorphic DNA profiles obtained with primer P49. Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control.

Fig. 3. Random amplified polymorphic DNA profiles obtained with primer P54. Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control.

18 *Anthurium* strains (ICMP 9564, 9565, 9841, 7461, 4656, JV502, JV505, JV511, JR579, JV589, D15-3, D108, and D147; Fig. 7, lanes 1, 2, 7–9, 11–18). The *Epipremnum* strain (ICMP 9570) and a *Philodendron* strain (ICMP 9586) also showed a signal of 1.3 kb (lanes 5 and 6). No signal was observed from control bacteria.

Figure 8 shows the results of Southern analysis of PCR products in Figure 2, using probe P49-840 (from *Anthurium* strain D108, amplified with primer P49). The expected signal of 840 bp was observed for 17 *Anthurium* strains (ICMP 9564, 9565, 9841, 7461, 4656, JV502, JV505, JV511, JR579, JV589, D15-3, D108, D147, D194, D99, and X1; lanes 11–19, 21, and 22), one *Aglaonema* strain (DK04; lane 24),

and the *Epipremnum* strain (lane 5). *Dieffenbachia* and *Philodendron* strains as well as *Anthurium* strains D93 and DK02 did not produce this signal. No signal was observed from control bacteria.

When PCR products in Figure 2 were hybridized to probe P49-1000 (from *Dieffenbachia* strain ICMP 9569, amplified with primer P49), signals of 1 kb were observed for strains ICMP 9566 and 9586 (from *Philodendron*; Fig. 9, lanes 3 and 6) and strains ICMP 9569 and 576 (from *Dieffenbachia*; lanes 4 and 10). The expected signal was not observed from *Anthurium* strains except strains D99 and X1 (lanes 21 and 22). No signal was observed from control bacteria.

Figure 10 shows results of Southern analysis of PCR products in Figure 3 when

hybridized to probe P54-800 (from *Anthurium* strain D108, amplified with primer P54). *Anthurium* strains ICMP 9565, 9841, 7461, 4656, JV502, JV505, JV511, JR579, JV589, D15-3, D108, and D147 (lanes 2, 7–9, 11–18) produced the expected signal of 800 bp. The *Epipremnum* strain ICMP 9570 (lane 5) also produced the signal. No signal was observed from strains isolated from other hosts and from control bacteria.

DISCUSSION

RAPD profiling has been widely used for the genetic differentiation of plant pathogens including pathovars of *Xanthomonas campestris* other than the pathovar *dieffenbachiae* (21,36). The characterization of strains of the pathovar *dieffenba-*

Fig. 4. Random amplified polymorphic DNA profiles obtained with primer P59. Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 19, D147; lane 20, D194; lane 21, D93; lane 22, D99; lane 23, X1; lane 24, HK56; lane 25, DK04; lane 26, DK02. Lanes 18 and 27, negative control.

STRAIN	
ICMP 9564	1.000
ICMP 9565	0.137 1.000
ICMP 9566	0.068 0.204 1.000
ICMP 9569	0.106 0.169 0.254 1.000
ICMP 9570	0.153 0.524 0.169 0.162 1.000
ICMP 9586	0.094 0.158 0.365 0.174 0.188 1.000
ICMP 9841	0.182 0.386 0.158 0.152 0.479 0.177 1.000
ICMP 7461	0.125 0.553 0.280 0.156 0.533 0.268 0.535 1.000
ICMP 4656	0.140 0.452 0.179 0.152 0.543 0.237 0.511 0.650 1.000
ICMP 576	0.115 0.123 0.278 0.295 0.175 0.206 0.203 0.255 0.203 1.000
JV502	0.123 0.277 0.140 0.172 0.346 0.108 0.367 0.300 0.340 0.129 1.000
JV505	0.104 0.400 0.149 0.123 0.405 0.130 0.366 0.385 0.366 0.157 0.375 1.000
JV511	0.119 0.378 0.155 0.167 0.440 0.175 0.353 0.396 0.468 0.161 0.581 0.390 1.000
JR579	0.125 0.405 0.208 0.156 0.468 0.183 0.375 0.455 0.435 0.169 0.413 0.459 0.558 1.000
JV589	0.113 0.310 0.091 0.077 0.413 0.098 0.409 0.333 0.348 0.083 0.386 0.471 0.465 0.622 1.000
D15-3	0.153 0.455 0.190 0.179 0.510 0.188 0.340 0.380 0.392 0.121 0.321 0.341 0.333 0.408 0.354 1.000
D108	0.154 0.449 0.188 0.162 0.500 0.186 0.368 0.407 0.472 0.141 0.426 0.347 0.549 0.407 0.358 0.500 1.000
D147	0.117 0.400 0.214 0.147 0.404 0.210 0.273 0.388 0.373 0.177 0.353 0.349 0.479 0.388 0.333 0.460 0.569 1.000
D194	0.189 0.180 0.085 0.072 0.113 0.127 0.119 0.123 0.119 0.045 0.161 0.125 0.155 0.143 0.154 0.169 0.206 0.236 1.000
D93	0.098 0.212 0.097 0.083 0.177 0.071 0.094 0.115 0.129 0.058 0.113 0.160 0.145 0.097 0.103 0.197 0.250 0.220 0.333 1.000
D99	0.175 0.186 0.136 0.133 0.231 0.139 0.203 0.230 0.167 0.231 0.206 0.161 0.200 0.172 0.145 0.176 0.176 0.215 0.103 0.113 1.000
X1	0.063 0.105 0.172 0.130 0.106 0.210 0.077 0.115 0.129 0.123 0.078 0.074 0.145 0.079 0.049 0.090 0.127 0.143 0.063 0.059 0.113 1.000
HK56	0.138 0.094 0.087 0.118 0.127 0.093 0.132 0.103 0.132 0.096 0.101 0.083 0.083 0.119 0.076 0.194 0.115 0.145 0.071 0.082 0.117 0.068 1.000
DK04	0.167 0.135 0.123 0.121 0.131 0.127 0.158 0.185 0.138 0.190 0.121 0.125 0.117 0.164 0.132 0.190 0.134 0.193 0.067 0.097 0.154 0.079 0.119 1.000
DK02	0.069 0.137 0.145 0.090 0.153 0.129 0.161 0.167 0.140 0.172 0.085 0.128 0.082 0.105 0.073 0.193 0.172 0.155 0.033 0.031 0.138 0.063 0.072 0.105 1.000

Fig. 5. Genetic similarity coefficient matrix for 25 *Xanthomonas axonopodis* pv. *dieffenbachiae* isolates based on 209 polymerase chain reaction fragment patterns obtained from eight random amplified polymorphic DNA primers. Values were calculated by Jaccard's coefficient.

chiae has been reported using various techniques such as the physiological and fatty acid profiling (9), pathogenicity testing (9,12), and biochemical analyses (3,34). However, reports specifically mentioning the use of molecular techniques for analysis of the *Anthurium* blight pathogen mostly include serological methods (19,20,29), while those concerning DNA analyses are rare. In the absence of sequence information about the genome of the pathogen for DNA analysis, RAPD was the ideal technique to use, but because it is so sensitive to reaction conditions for the

identification of genetic relatedness, an optimization of reaction parameters was required to achieve reproducibility.

Eight primers produced polymorphic banding patterns that provided information about the relatedness of *X. axonopodis* pv. *dieffenbachiae* strains. A few strains of known serotypes could be distinguished by their RAPD profiles. For instance, with primer P27, strains of serotype 8 (D93 and D94) shared a band of 1,550 bp (Fig. 1, lanes 19 and 20), with primer P54 four bands (Fig. 3, lanes 19 and 20), and with primer P59 a band of 1.1 kb (Fig. 4, lanes

19 and 20). Similarly, strains of serotype 1 (D15-3, D108, and D147) had an identical profile of four bands with primer P59 (Fig. 4, lanes 16–18), and it was observed that at least one of these bands was amplified from every strain tested. Strains D99 (serotype 12; lane 21) and X1 (serotype 10; lane 22) could be distinguished from those of serotype 1 (lanes 16–18) and serotype 8 (lanes 19 and 20) by the different profiles obtained with each of the primers used. However, since the serological information was not available for many of the strains used, one can only hypothesize a correla-

Fig. 6. Unweighted pair group method, arithmetic average dendrogram constructed from random amplified polymorphic DNA data indicating the relationship among the 25 *Xanthomonas axonopodis* pv. *dieffenbachiae* isolates of the study. The dendrogram was generated from the similarity matrix in Figure 5. Hosts are indicated as Ant = *Anthurium*, Die = *Dieffenbachia*, Agl = *Aglaonema*, Phi = *Philodendron*, Epi = *Epiphyllum*. Geographical origins are indicated as USA = U.S. mainland, Haw = Hawaii, Mau = Mauritius, Bra = Brazil, Tah = Tahiti, Ven = Venezuela, Pue = Puerto Rico.

tion between RAPD profiling and serotyping. The UPGMA analysis confirmed that strains of serotype 1 and serotype 8 were respectively genetically similar since the reference strains for each of these serotypes were clustered together (Fig. 6).

A relationship between the RAPD profiling and the plant host was observed for some strains. For instance, primer P49 produces an 840-bp band for all reference strains isolated from *Anthurium*, while a 1-kb band was amplified for reference strains that were isolated from *Philodendron* and *Dieffenbachia* (Fig. 2). These results correlate with the dendrogram (Fig. 6) where most of the reference *Anthurium* strains were clustered in GP2, while GP3 comprised four strains isolated from *Philoden-*

dron and *Dieffenbachia*. The strain isolated from *Epipremnum* (ICMP 9570) was grouped with *Anthurium* strains in GP2 and was therefore expected to be genetically closer to these strains than to strains isolated from other aroids. No geographical correlation could be found, as *X. axonopodis* pv. *dieffenbachiae* strains isolated from the same region were often found in different groups, except for two Brazil strains (ICMP 7461 and 4656) and two Hawaii strains (D108 and D147). A significantly high percent similarity (60%) was observed between a strain isolated in Reunion (JV589) and the type strain from Brazil (JR579), suggesting a common origin.

Southern hybridization patterns correlated with the UPGMA clustering. The

probe of 1.3 kb isolated from the profile of an *Anthurium* strain amplified with primer P27 gave a strong signal for one strain in GP1 (Fig. 7, lane 1) and all strains in GP2 (Fig. 7, lanes 2, 5–9, 11–18) except strain D99 (lane 21). Strains belonging to GP3 and GP4 did not produce a signal with this probe. Similarly, the 800-bp probe isolated from profiles amplified with primer P54 gave a signal for all strains belonging to GP2 only (Fig. 10). The probe of 840 bp isolated from profiles amplified with primer P49 hybridized strongly to all strains clustered in GP1 (Fig. 8, lanes 1 and 24) and GP2 (Fig. 8, lanes 2, 7–9, 11–18, and 21), and two strains from GP4 (Fig. 8, lanes 19 and 22). Strains clustered in GP3 did not give a signal. The second

Fig. 7. Southern hybridization of polymerase chain reaction products from Figure 1 with probe P27-1300 (1.3 kb, isolated from *Anthurium* strain D108). Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control. Hybridization profiles support the clustering in Figure 6.

Fig. 8. Southern hybridization of polymerase chain reaction products from Figure 2 with probe P49-840 (840 bp, isolated from *Anthurium* strain D108). Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control. Hybridization profiles support the clustering in Figure 6.

probe (1 kb) isolated from the profile of a *Dieffenbachia* strain amplified with primer P49 complemented the results of the first probe (840 bp) by hybridizing to the respective RAPD fragment for strains clustered in GP3 (isolated from *Philodendron* and *Dieffenbachia*; Fig. 9). Apart from GP3 strains, only one strain from GP2 (Fig. 9, lane 21) and one from GP4 (Fig. 9, lane 20) produced a signal with this probe. None of the probes tested produced signals with the RAPD products amplified from control bacteria used in this study and were therefore considered to be specific to *X. axonopodis* pv. *dieffenbachiae*. The hybridization patterns further confirm the existence of subpopulations in the pathovar *dieffenbachiae*. It is therefore expected that since the pathovar is extensively diverse,

more than one DNA probe or primer pair might be needed to identify all *X. axonopodis* pv. *dieffenbachiae* strains.

In the present study, RAPD fingerprinting confirmed the heterogeneity of the pathovar *dieffenbachiae*, as previously reported by Berthier et al. (7), with genetic similarities among clusters ranging from about 10 to 65%. Jaccard's coefficient (35), used in the analysis, does not consider the joint absence of a marker as an indication of genetic similarity (2); therefore, the reliability of the analysis is enhanced with respect to the presence of clearly amplified RAPD markers. Although the analysis involved a small number of strains of the blight pathogen, the results obtained showed that RAPD markers are valuable tools for the study of the genetic relatedness among *X. axonopodis*

pv. *dieffenbachiae* strains. However, more strains would need to be studied to confirm the correlation among RAPD profiling, serotyping, and host range. The DNA probes that were developed from RAPD fragments complemented each other in the Southern analysis to give their respective signals for the range of reference strains used. As previously reported for *Xanthomonas axonopodis* pv. *pelargonii*, RAPD markers can be used to develop specific DNA primers for the detection of the pathogen in infected plants (21). The RAPD markers that were used in this study are presently being sequenced to develop a sensitive diagnostic tool for Anthurium blight.

The current classification of xanthomonads is based on DNA homology, repetitive

Fig. 9. Southern hybridization of polymerase chain reaction products from Figure 2 with probe P49-1000 (1 kb, isolated from the *Dieffenbachia* strain ICMP 9569). Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control. Hybridization profiles support the clustering in Figure 6.

Fig. 10. Southern hybridization of polymerase chain reaction products from Figure 3 with probe P54-800 (800 bp, isolated from *Anthurium* strain D108). Lanes m, DNA size marker VI; lanes 1–25, *Xanthomonas axonopodis* pv. *dieffenbachiae* strains: lane 1, ICMP 9564; lane 2, ICMP 9565; lane 3, ICMP 9566; lane 4, ICMP 9569; lane 5, ICMP 9570; lane 6, ICMP 9586; lane 7, ICMP 9841; lane 8, ICMP 7461; lane 9, ICMP 4656; lane 10, ICMP 576; lane 11, JV502; lane 12, JV505; lane 13, JV511; lane 14, JR579; lane 15, JV589; lane 16, D15-3; lane 17, D108; lane 18, D147; lane 19, D194; lane 20, D93; lane 21, D99; lane 22, X1; lane 23, HK56; lane 24, DK04; lane 25, DK02. Lane 26, negative control. Hybridization profiles support the clustering in Figure 6.

extragenic palindromic (REP)-PCR, and amplified fragment length polymorphism (AFLP) (32,37,38). However, for many groups it has not yet been possible to classify pathovars using genomic information based on these fingerprints (38). The probes used in this study indicate that *Xanthomonas* isolates from aroids have undergone evolutionary changes, possibly as part of the speciation process. Three out of four probes that were isolated from *Anthurium* strains did not hybridize to strains that were isolated from *Philodendron* and *Dieffenbachia*. However, the fourth probe (P49-1000, which was isolated from a *Dieffenbachia* strain) hybridized to *Philodendron* and *Dieffenbachia* strains as well as to two *Anthurium* strains. Since the probes did not hybridize to the controls and were specific to *X. axonopodis* pv. *dieffenbachiae*, it could be speculated that the isolates from aroids might have originated from a common ancestor. However, a larger number of strains from different aroids should be screened to confirm this observation.

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