GENETIC VARIATION IN *Xanthomonas axonopodis* pv. *dieffenbachiae*

By

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This document is dedicated to my grandparents, Doug and Valle Glover.

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Members of the Araceae are susceptible to the bacterial pathogen *Xanthomonas axonopodis* pv. *dieffenbachiae*, which is capable of causing crop losses up to 100 percent. When 187 strains isolated from nine aroid hosts were subjected to Rep-PCR, six genetic clusters were generated. One cluster was found to represent only strains isolated from *Syngonium* spp. Forty strains isolated from *Anthurium*, *Dieffenbachia*, and *Syngonium* were subjected to amplified fragment length polymorphism (AFLP) and tested for pathogenicity on five Aroid hosts. AFLP data correlated well with Rep-PCR data. Based on pathogenicity tests, the *Syngonium* strains were selectively pathogenic on *Syngonium*. None of the strains from other hosts caused significant disease on *Syngonium*. DNA from ten representative strains was amplified by PCR using primers to the ITS and *hrp*B. Phylogenetic analysis of sequenced PCR products reveals that the *syngonium* strains exist as a distinct group from other members of *X.a*.pv. *dieffenbachiae* and supports the use of pv. *syngonii* for strains isolated from *Syngonium*.

CHAPTER 1
INTRODUCTION

The United States tropical foliage plant industry has an annual wholesale value of more than 500 million dollars (Unites States Department of Agriculture, 2002), with approximately 61% of this production in Florida. Consistent growth has occurred in the tropical foliage industry due to the popularity of plants in the family Araceae. This family includes genera such as *Aglaonema, Anthurium, Dieffenbachia, Philodendron,* and *Syngonium* (www.aroid.org).

Aroids are native to the tropics and vary greatly in growth habit. Some are aquatic and totally submerged, others are hemi-epiphytic, and yet others are totally epiphytic growing on rocks and trees. Aroids can be found in their native habitats from coastal locations to higher elevations of several thousand feet. However, most of the commercially cultivated species are found growing at low elevations under humid tropical rainforest conditions. Thus, most commercial production of these crops is done either in tropical areas of the world or under greenhouse conditions. Costa Rica, Guatemala, and Honduras are primary suppliers of propagative material while the Netherlands, California, Florida, and Hawaii are the primary producers of finished plants.

Most aroids are known to be susceptible to bacterial blight caused by the bacterium *Xanthomonas axonopodis* pv. *dieffenbachiae*. Traditionally, it has been thought that the bacterium enters through the hydathodes. However, recent observations suggest that entry may also occur through the stomata. The hydathodes have been thought of as the main point of entry associated with this *Xanthomonas* due to the fact that these

specialized cells localized to the leaf margin secrete glutamine and/or glutamate, both which can serve as chemotaxic stimuli as well as a sole carbon source for *Xanthomonas dieffenbachiae.* The guttation fluid from infected plants serves as a source of inoculum, and is easily spread by overhead irrigation and/or worker's clothing. Since many of the aroids are propagated vegetatively, the disease is spread rapidly, on cutting utensils used in crop maintenance and propagation.

Once the bacteria have entered a susceptible host, whether via the stomata, hydathodes or wounds, the bacteria multiply at the point of entry, spreading through the xylem vessels. If the pathogen entry is via the hydathodes, water soaking develops at the leaf margin within a few days. As the disease progresses, the water soaking becomes chlorotic then necrotic, forming classical V-shaped lesions of dead tissue. If plants are grown in close proximity to each other and under warm and humid conditions, spread within and between plants can be rapid with crop losses up to 100%.

CHAPTER 2
LITERATURE REVIEW

History

Aroids, members of the plant family Araceae, constitute the majority of tropical foliage crops. McCulloch and Pirone published the first report of bacterial blight of *Dieffenbachia* (1939). They described an organism that caused leaf spots, blight, and in some cases death. They referred to the pathogen as *Bacterium dieffenbachiae.* During the 1920's and 1930's it was common practice to group all phytopathogenic bacteria in the genus *Bacterium* (Dowson 1943).

Over time, the necessity for additional genera was realized. Through the research efforts of Migula, Dowson and others in the early 1900's, new genera such as *Pseudomonas* and *Xanthomonas* were gradually accepted. In 1939, Bergey's Manual renamed the pathogen as *Phytomonas dieffenbachiae*. Dowson reclassified *Phytomonas dieffenbachiae* as *Xanthomonas dieffenbachiae*, the pathogen causing bacterial blight of *Dieffenbachia* (Dowson 1943). Roughly 20 years after Dowson's contribution to the nomenclature Young *et al*. transferred the species *dieffenbachiae* to a pathovar of *Xanthomonas campestris* designating it *X. campestris* pv. *dieffenbachiae* (*Xcd*) (Young *et al.* 1978). In 1980 Dye *et al.* formally described the use of the pathovar system and included *Xcd* as a member.

During the 1960's *Xcd* was described causing disease on other aroids including *Aglaonema* and *Philodendron* (McFadden 1962 and 1967). During the 1970's *Xcd* was also reported in *Anthurium* in Hawaii (Hayward 1972). In the 1980's, *Xcd* was described

on numerous aroid hosts throughout the United States (Chase and Poole 1986, Cooksey 1985, Porhonenzy *et al*. 1985, Chase *et al*. 1992).

In 1969 bacterial blight was described on *Syngonium podophyllum* (Wehlburg 1969). The pathogen was described as a *Xanthomonas*-like organism based on cultural and microscopic observations characterizing the pathogen as *X. vitians*. Only minor physiological differences between the two organisms were noted. In the 1980's when Dickey and Zumoff (1987) compared four strains from the *Syngonium* cultivar Cream (LX 103, LX105, LX106, LX114) and two strains from the cultivar White Butterfly (L 212, L215). These six strains were compared with two strains used in Wehlburg's study (XV29 = NCPPB2255 isolated from *Syngonium* ATCC19320) and type strains representing *X. c. vitians* (NCPPB 969), *X. c. campestris* (B-24), and strains of *Xanthomonas campestris* from aroid hosts with pathovar designations *aracearum* (XA-2), *dieffenbachiae* (XD-3), and *zantedeschiae* (XZ1). They noted that strains XA-2, XD-3, and ATCC 19320 produced disease symptoms on *Syngonium* when misted on plants at 10^8 CFU/ml. However it was also noted that the reactions produced by these strains were different from those produced by strains originally isolated from *Syngonium*. Dickey and Zumoff (1987) proposed designating the *Syngonium* organism as *Xanthomonas campestris* pv. *syngonii*. (Dickey and Zumoff 1987).

This pathovar designation *syngonii* is available from culture collections (LMG 9055 and ICMP 9152, 9153, 9154, 9155 all representing strains collected by Dickey) but does not appear anywhere in-approved lists of bacterial names or taxonomic lists. The xanthomonad that infects *Syngonium* is currently referred to as *X. campestris* pv. *dieffenbachiae* (Chase *et al.* 1992).

In 1995 the aroid pathogen was reclassified as *X. axonopodis* pv. *dieffenbachiae* based on DNA-DNA homology studies (Vauterin *et al.* 1995). In that study five *X. campestris* pv. *dieffenbachiae* strains were compared with 178 strains from 33 other pathovars of *X. campestris*. The five *X. campestris* pv. *dieffenbachiae* strains used, included three *Anthurium* strains from Brazil and two *Dieffenbachia* strains from the United States. The strains used in their study seem limited in regards to hosts of origin and excluded the incorporation of any strains from *Aglaonema, Philodendron, Syngonium,* or *Xanthosoma*.

Related Studies

The family Araceae is a large and diverse family of plants. Understanding this is important in recognizing the need to determine the diversity that exists in the many xanthomonads that infect and cause disease in these aroids. Traditionally, host range pathogenicity is a good starting point when describing bacterial plant pathogens. In recent years a number of PCR techniques have been developed and are used for investigating species, pathovars, strains, and races. The following is a brief review of the literature regarding host range, pathogenicity, and PCR-based phylogeny-type work.

Pathogenicity Tests

The history of *Xanthomonas syngonii* raises questions that as of yet have not been answered, especially in regards to host range and pathogenicity. Dickey and Zumoff (1987) showed that strains XA-2, XD-3, and ATCC 19320 produced symptoms on *Syngonium* when misted at 10^8 CFU/ml. However, it was also noted that the reactions produced by these strains were different from those produced by strains originally isolated from *Syngonium.* It was also observed that xanthomonads isolated from *Syngonium* were weakly pathogenic on the *Dieffenbachia amoena* and not at all

pathogenic on *D. maculata.* Also, XD-3 (from *Dieffenbachia*) was capable of producing typical symptoms on *Dieffenbachia* yet only minor symptoms on *Syngonium.* The results from their study suggest *Syngonium* strains cause disease in *Syngonium* but appear to be less virulent when inoculated into *Dieffenbachia.* In addition, they concluded that there were typical and atypical *syngonii* strains based on pigmentation and growth on nutrient agar. It would appear that Dickey and Zumoff (1987) were suggesting that the *syngonium* strains are the only true pathogen of *Syngonium.*

Similar research on the *Syngonium* strains was conducted by Chase *et al*. (1992). In that study 149 *X. campestris dieffenbachiae* and *X. c.* pv. *syngonii* strains isolated from a variety of aroid hosts were subjected to physiological, pathological, and fatty acid analyses. They showed that various strains were not necessarily host-specific though they suggested that groups of strains have overlapping hosts. It was observed when monitoring populations in leaves infiltrated with bacterial suspensions adjusted to 10^4 CFU/ml that all strains (with the exception of the Syngonium strains) were capable of multiplying one log unit regardless of host tested. The *Syngonium* strains only increased in populations in *Syngonium* and *Aglaonema.* Chase *et al*. (1992) concluded that differences in host specificity were not significant enough to be used as a means to differentiate strains of *X. c.* pv. *dieffenbachiae* from those of *X. c.* pv. *syngonii.* It appears that due to this ambiguity in host range among these various strains there has been no pathovar designation for all of these strains aside from the all-encompassing pathovar *dieffenbachiae.*

Efforts by Lipp *et al*. (1992) to characterize strains isolated from *Dieffenbachia* and *Syngonium* employed a similar host range study. However, while the typical *syngonium*

strains were virulent on *Syngonium* and weakly virulent to avirulent on other host genera, the atypical strains were indistinguishable from other Xcd based on bacteriological tests (Lipp *et al,* 1992). They found no significant correlation between hosts of origin and host range, and concluded that this group was extremely heterogeneous.

PCR Based Analysis

Recently PCR has been used to amplify specific regions of an organism's genome for comparison with other organisms as a means to determine evolutionary relationships. Several of the PCR based techniques include Repetitive Element PCR (rep-PCR), Amplified Fragment Length Polymorphism (AFLP), and sequence comparisons of the *Hrp* region and a region corresponding to the ribosomal DNA (Louws *et al.* 1994, Janssen *et al.* 1996, Leite Jr. *et al.* 1995, and Hauben *et al.* 1997).

Rep-PCR is based on highly conserved regions of the genome. There are three regions that have been used: Repetitive Enterobacterial Palindrome (REP), (Gilson *et al*. 1984, Higgins *et al*. 1982) Enterobacterial Repetitive Intergenic Consensus (ERIC), (Hulton *et al*. 1991, Sharples and Lloyd 1990) and the BOX1A element from *E. coli* (BOX) (Martin *et al*. 1992). It has been shown that these elements are widely distributed in phytopathogenic bacteria including *Pseudomonas* and *Xanthomonas* and can be used for rapid molecular characterization especially at the pathovar level (Louws *et al.* 1994). Using rep-PCR Louws *et al.* (1994) were able to generate genomic "fingerprints" consisting of five to 20 bands ranging in size from 100 base pairs to 5 kbp. In comparing fingerprints from various *Pseudomonas* and *Xanthomonas* strains, they were able to distinguish *P. syringae* pv. *syringae* from *P. syringae* pv. tomato as well as *X. oryzae* pv. oryzae from *X. oryzae* pv. *oryzicola*. Using rep-PCR members from the same group they

were able to place *X. campestris* pv. *vesicatoria* strains into two groups, groups A and B (Louws *et al.*1995).

In 1995 the PCR based genomic "fingerprinting" method Amplified Fragment Length Polymorphism (AFLP) was developed. The basis of AFLP is to analyze the whole genome. First described by Vos *et al.* (1995), the process begins with fragmenting the whole genome with restriction endonucleases. Following the restriction or cutting of the genome, the second step employs the ligation of site-specific adapters to the restriction fragments. The adapted fragments are selectively amplified with primers designed to complement the adapters and allow specificity through 3' selective base(s) incorporated into the primers. This technique is useful with DNA of any origin and of any complexity (Vos *et al.* 1995). It has been suggested that this DNA fingerprinting technique is comparable in taxonomic value to that of DNA-DNA homology studies or fatty acid analyses (Janssen *et al.* 1996). That study demonstrated the usefulness of AFLP using *Xanthomonas* as a model. Various primer combinations were able to differentiate closely related bacterial strains. It was also observed that the placement of strains into groups deduced through this technique was in agreement with DNA homology studies as well as fatty acid data (Janssen *et al.* 1996). In a more recent study comparing AFLP and rep-PCR, *Xanthomonas* was used as the model and it was demonstrated that both fingerprinting methods were useful in determining taxonomic relationships (Rademaker *et al.* 2000).

DNA sequence analysis of genes followed by analysis for phylogenetic relationships is useful in determining relatedness of organisms. One region that has been examined for determining relatedness is the *hrp* (hypersensitive response and

pathogenicity). This region is commonly associated with the majority of plant pathogenic bacteria and is accountable for those organisms' ability to cause disease or a hypersensitive response. It has been suggested that by subjecting this region to PCR and subsequent analysis it is possible to differentiate xanthomonads as well as detect the presence of *X. c. vesicatoria* on pepper and tomato seed (Leite Jr. *et al.* 1995). Similarly, this technique has been used for detecting *X. fragariae* in nurseries (Roberts *et al.* 1996). That study found that the 49 strains of *X. fragariae* shared identical restriction profiles.

Another region used for taxonomic purposes is the ribosomal DNA. In the early 1990's, a study was conducted showing the high degree of variability that exists in ribosomal DNA restriction patterns of *X. campestris* pv. *dieffenbachiae* (Berthier *et al.*1993). This was supported by the data stating that 53 strains were characterized by five restriction patterns, and there were no patterns corresponding to geographic origin but clearly to host plant origin (Berthier *et al.*1993). In a later study Hauben *et al.* (1997) determined that the use of rDNA signatures to differentiate *Xanthomonas* species was not ideal due to the restricted variability. In that study only one strain of *X. axonopodis* pv. *dieffenbachiae* was used. However, a more recent study shows that rDNA analysis is sufficient in the identification of closely related xanthomonads such as *X. axonopodis* pv. *citri*, and *X. axonopodis* pv. *aurantifolii*, the causal agent of bacterial citrus canker types A and B, respectively (Cubero and Graham 2002).

CHAPTER 3
MATERIALS AND METHODS

This chapter includes all scientific and data collecting procedures. All experiments were conducted between May 2001 and May 2003. A majority of the experiments were conducted at the Mid Florida Research and Education Center-Apopka, including greenhouse studies, genomic comparisons, and storage of bacterial strains. All DNA sequencing work and growth chamber studies were conducted at the University of Florida in Gainesville.

Bacterial Strains Used

One hundred and eighty seven *X. a*. pv. *dieffenbachiae* strains were used in this study (Table 1). These strains were isolated over a 20-year period from ten aroid genera. Strains were revived from cryogenic storage when needed.

Genomic Comparisons

All strains in Table 1 were subjected to Repetitive Element PCR (rep-PCR) using BOX, ERIC, and REP primers. DNA from these strains was prepared using the GenomicPrep Kit, (Amersham Pharmacia Biotech, Pistcataway, NJ). Instead of using a master mix, PCR was done using RAPD Analysis Beads (Amersham). A modified program suggested by Amersham Pharmacia Biotech was used to simulate results obtained using a master mix. Template DNA was amplified using a PTC-100 thermocycler (M.J. Research Inc., Watertown, Mass). The thermocycler was programmed as follows. For BOX initial denaturation at 95º for five minutes, followed by 45 cycles denaturing at 95º for one minute and annealing at 53º for one minute. ERIC

and REP had annealing temperatures of 52º and 40º, respectively. Final extension was conducted at 72º for two minutes; upon completion the block was held at 4º. PCR products were separated on 1.5% gels, stained with ethidium bromide and photographed under a UV transluminator with a Kodak Digital Documentation System 120 (Rochester, NY). All gels photographed were stored as Tiff files.

Banding patterns were compared between strains using Pearson Correlation and Unweighted Pair Group Means Analysis (UPGMA) with the BioNumerics program ver. 2.1 (Applied Maths, Kortrijk, Belgium). For strains with unique banding patterns DNA was extracted again, PCR was redone, and the analysis was repeated. Using the BioNumerics program, a combined comparison was made of banding patterns from all three rep-PCR primers. Cluster cut-off values were also calculated using the BioNumerics software in each comparison. The ATCC *X.a.d* Type strain 23379 was used as a standard.

Amplified Fragment Length Polymorphism (AFLP)

Based on rep-PCR clusters, 40 strains were selected from seven distinct genetic clusters. Due to the large number of *Anthurium* strains an emphasis was placed on these as well as those representing strains from *Syngonium* and *Dieffenbachia*. These strains were compared using AFLP. DNA was prepared as above using the kit obtained from Amersham. Template was prepared using LI-COR (Lincoln, NE) template preparation kit according to the provided protocol. The protocol was modified only for the selective amplification step by following a procedure according to Janssen *et al*., (1996) LI-COR's template preparation kit uses the *Eco*RI and *Mse*I restriction enzymes and a two-dye system for labeling rather than the traditional radiolabelling. An *Mse*I primer containing 3'cytosine was obtained from Genomechanics (Alachua, FL). Following the

nomenclature provided by Janssen *et al.* (1996) this primer will be referred to as M02. Using the LI-COR system, the *Eco*RI primers were labeled with an IR-dye. Two dyes were obtained from LI-COR: one 700 *Eco*RI primer with no selective base (E00) and one 800 dye with cytosine as a selective base (E02). Another 700 dye was obtained for (E02) as the 800 dye gave less than ideal products. Acrylamide gels were prepared using KB plus gel matrix (LI-COR), temed and ammonium persulfate according to manufacturer's specifications. Gels were run 4 hours at 1500 volts on a Global LI-COR IR^2 System. Images were recorded using the SAGA software (LI-COR). Tiff files were analyzed using the BioNumerics program.

Sequence Comparisons

Two regions were chosen for sequence comparison, the *HrpB* (for hypersensitivity response and pathogenicity) and the intergenic transcribed sequence (ITS). DNA for the 12 selected strains was prepared as previously mentioned. The hrp region was selected to determine whether or not this group of organisms contained *hrp* genes. PCR products were obtained using "Hrp" (RST65-RST69) primers used for amplification of a 420bp product in *X. campestris* pv. *vesicatoria*. Products obtained from strains isolated from various hosts were subjected to restriction digests using *Cfo*I, and *Hae*III. Different profiles were generated and observed on NuSieve 3:1 agarose gels. Based on the variation in restriction profiles, PCR was repeated, products were cleaned using a spin column (Qiagen, Germantown, MD) and submitted for sequencing at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) sequencing lab in Gainesville. The ITS region was not subjected to restriction digest, but was amplified using (J13-J14) primers and sequenced in the same manner. Sequences were assembled

using the Sequencher software and alignments were performed using the European Bioinformatics Institute's CLUSTALW (www.ebi.ac.uk/clustalw/).

Pathogenicity and Host Range

Using the 40 pre-selected strains, a limited host range was conducted using both mist and leaf infiltration techniques. Strains used in the pathogenicity tests were grown on Nutrient Agar (Difco) amended with 5% sucrose at 28º C for 24-48 hours prior to use. Bacteria were harvested from the petri plates and suspended in 0.1M NaCl solution. Bacterial suspensions were adjusted with a spectrophotometer to 1 X 10^8 CFU/ml with an optical density 0.3 (A_{600}). This suspension was applied to leaf surfaces using hand-pump sprayers. Immediately after misting, plants were placed in plastic bags for 24 hours to maintain high relative humidity. The inoculated plants were observed for progression of disease weekly for two months. Plant species utilized were aglonema (*Aglonema commutatum*, Schott 'Maria'), dieffenbachia (*Dieffenbachia maculata* (Lodd) G. Donn., 'compacta'), philodendron, (*Philodendron scandens oxycardium*), syngonium (*Syngonium podophyllum*, 'White Butterfly'), and three species of anthurium (*Anthurium crystalinum* Linda Andre, 'Crystal Hope'; a complex interspecific hybrid of *A. amnicola* Dressler x *A. andreaneum* Linden ex André, 'Red Hot'; and *A. andraeanum* 'Hearts Desire'). Plants were kept under identical cultural conditions in a fiberglass shadehouse with maximum irradiance of 125 μmol s^{-1} m^{-2}, temperature range of 21 to 37 ºC with natural photoperiod, and high relative humidity.

For leaf infiltrations, bacterial suspensions were serially diluted to 1 X 10^5 CFU/ml. The same host plant species mentioned above were utilized with the exception of only using one anthurium cultivar ('Red Hot'). Leaf tissue, approximately 4 $cm^2$was infiltrated on two leaves on each of two plants of each host plant species using a 25gauge

hypodermic needle. Younger leaves were preferentially used. The infiltrated areas were observed for symptom expression over a two-month period. For both methods of inoculation a saline buffer and a strain of *X. campestris* pv. c*ampestris* (ATCC 33913) were used as negative controls. All greenhouse tests were conducted between June and September 2002.

Table1. Sources and hosts of *Xanthomonas axonopodis* pv. *dieffenbachiae* strains utilized in this study.

Strain ID	Original source ID	Host	Geographic origin
01-156	MREC	*Anthurium*	Florida
13	MREC	*Anthurium*	Florida
14	MREC	*Anthurium*	Florida
82	A990-5	*Anthurium*	Hawaii
84	A844-1	*Anthurium*	Hawaii
158	MREC	*Syngonium*	Florida
159	MREC	*Syngonium*	Florida
161	MREC	*Syngonium*	Florida
162	MREC	*Syngonium*	Florida
166	MREC	*Syngonium*	Florida
170	MREC	*Philodendron*	Florida
172	MREC	*Syngonium*	Unknown?
173	MREC	*Syngonium*	Unknown?
175	MREC	*Dieffenbachia*	Florida
176	MREC	*Epipremnum*	Florida
178	MREC	*Syngonium*	Florida
181	MREC	*Syngonium*	Florida
183	MREC	*Dieffenbachia*	Florida
187	MREC	*Syngonium*	Florida
191	MREC	*Syngonium*	Florida
192	MREC	*Syngonium*	Florida
195	GWS 2218-83	*Dieffenbachia*	Florida
265	ICMP 9586	*Philodendron*	Florida
268	D 150	*Anthurium*	Hawaii
269	D 158	*Anthurium*	Hawaii
271	D 36.12	*Syngonium*	Hawaii
272	D 129.12	*Syngonium*	Hawaii
326	PDD – 1145-87	*Anthurium*	Florida
328	DPI – P87-2307	*Anthurium*	Florida
376	PDD – 1399-87	*Aglaonema*	Florida
422	PDD – 21-88	*Anthurium*	Florida
423	PDD – 20-88	*Anthurium*	Florida
430	D129.1M	*Syngonium*	Hawaii
431	D129.1D	*Syngonium*	Hawaii
451	D61.11	*Anthurium*	Hawaii
452	D68.00	*Anthurium*	Hawaii
453	D184.00	*Aglaonema*	Hawaii
454	D182.00	*Anthurium*	Hawaii
455	D183.00	*Anthurium*	Hawaii
456	D185.00	*Aglaonema*	Hawaii
457	D206.00	*Colocasia*	Hawaii
458	D191.00	*Epipremnum*	Hawaii
460	D228.00	*Colocasia*	Hawaii

Table 1 (Continued).

Strain ID	Original source ID	Host	Geographic origin
462	D36.10	*Syngonium*	Hawaii
463	D18.00	*Anthurium*	Hawaii
464	D139.00	*Anthurium*	Hawaii
465	D227.00	*Colocasia*	Hawaii
466	D135.00	*Anthurium*	Hawaii
467	D122.00	*Colocasia*	Hawaii
468	D206.00	*Colocasia*	Hawaii
469	D204.00	*Colocasia*	Hawaii
471	D162.00	*Anthurium*	Hawaii
472	D170.00	*Anthurium*	Hawaii
473	D17.10	*Anthurium*	Hawaii
474	D145.00	*Anthurium*	Hawaii
475	D147.00	*Anthurium*	Hawaii
476	D150.00	*Anthurium*	Hawaii
477	D145.00	*Anthurium*	Hawaii
478	D1.21	*Anthurium*	Hawaii
479	D30.00	*Spathiphyllum*	Hawaii
480	D55.1	*Anthurium*	Hawaii
481	D110.00	*Anthurium*	Hawaii
482	D120.00	*Anthurium*	Hawaii
483	D131.00	*Anthurium*	Hawaii
484	D93.00	*Spathiphyllum*	Hawaii
485	D94.40	*Anthurium*	Hawaii
487	D101.00	*Anthurium*	Hawaii
488	D160.00	*Anthurium*	Hawaii
489	D46.20	*Anthurium*	Hawaii
490	D52.00	*Anthurium*	Hawaii
491	D69.10	*Anthurium*	Hawaii
492	D70.00	*Anthurium*	Hawaii
493	D71.50	*Anthurium*	Hawaii
494	D38.10	*Anthurium*	Hawaii
495	135.00	*Anthurium*	Hawaii
497	D40.10	*Anthurium*	Hawaii
585	MREC	*Anthurium*	Florida
586	MREC	*Anthurium*	Florida
606	DPI – 072-743	*Anthurium*	Florida
628	DPI – 076-953	*Philodendron*	Florida
641	DPI – 072-745	*Anthurium*	Florida
642	MREC	*Philodendron*	Florida
661	PDD – 1772-88	*Aglaonema*	Florida
696	DPI – P88-3370	*Philodendron*	Florida
697	DPI – P87-2081	*Dieffenbachia*	Florida
746	MREC	*Anthurium*	Florida
747	MREC	*Anthurium*	Florida

Table 1 (Continued).

Strain ID	Original source ID	Host	Geographic origin
757	PDD – 15017-89	*Anthurium*	Florida
758	PDD – 1500-89	*Anthurium*	Florida
764	PDD – 2033-89	*Aglaonema*	Florida
765	PDD – 2025-89	*Anthurium*	California
766	PDD – 2050-89	*Anthurium*	Florida
767	PDD – 2050-89	*Anthurium*	Florida
768	PDD – 2050-89	*Anthurium*	Florida
785	PDD – 2129-89	*Anthurium*	Florida
786	PDD – 2129-89	*Anthurium*	Florida
788	MREC	*Anthurium*	Florida
790	PDD – 2444-89	*Anthurium*	Florida
805	PDD – 2507A-89	*Dieffenbachia*	Florida
807	MREC	*Caladium*	Florida
811	MREC	*Aglaonema*	Florida
813	MREC	*Caladium*	Florida
818	DPI – 89-3142-1	*Colocasia*	Florida
830	PDD – 2912-89	*Anthurium*	Florida
831	PDD – 2927-89	*Anthurium*	Florida
834	MREC	*Anthurium*	Florida
840	MREC	*Syngonium*	Florida
841	MREC	*Anthurium*	Florida
851	PDD – 3523-89	*Philodendron*	Florida
868	DPI – P89-4119-1	*Dieffenbachia*	Florida
875	DPI – P89-4526-10	*Caladium*	Florida
979	ATCC33913	Cabbage	United Kingdom
1176	MREC	*Anthurium*	Florida
1181	MREC	*Philodendron*	Florida
1185	MREC	*Anthurium*	Florida
1186	MREC	*Anthurium*	Florida
1188	MREC	*Caladium*	Florida
1268	MREC	*Aglaonema*	Florida
1272	Cooksey	*Anthurium*	California
1277	DPI – P90-5399	*Anthurium*	Florida
1278	DPI – P90-3705	*Philodendron*	Florida
1279	DPI – P90-3919	*Philodendron*	Florida
1283	DPI – P90-3731-1	*Syngonium*	Florida
1341	PDD – 3466-91	*Syngonium*	Florida
1343	DPI – P91-2557	*Anthurium*	Florida
1353	MREC	*Anthurium*	Florida
1354	MREC	*Anthurium*	Florida
1390	DPI – P91-3902	*Epipremnum*	Florida
1420	MREC	*Philodendron*	Florida
1474	MREC	*Philodendron*	Florida
1476	PDD – 359A-92	*Syngonium*	Florida

Table 1 (Continued).

Strain ID	Original source ID	Host	Geographic origin
1477	PDD – 359B-92	*Syngonium*	Florida
1559	MREC	*Aglaonema*	Florida
1564	MREC	*Aglaonema*	Florida
1567	MREC	*Anthurium*	Florida
1568	MREC	*Anthurium*	Florida
1610	MREC	*Aglaonema*	Florida
1617	M 101	*Xanthosoma*	Florida
1618	M 102	*Xanthosoma*	Florida
1619	M 103	*Xanthosoma*	Florida
1620	M 105	*Xanthosoma*	Florida
1621	M 106	*Xanthosoma*	Florida
1622	M 110	*Xanthosoma*	Florida
1623	M 113	*Xanthosoma*	Florida
1624	M 114	*Xanthosoma*	Florida
1625	M 115	*Xanthosoma*	Florida
1626	M 116	*Xanthosoma*	Florida
1627	M 117	*Xanthosoma*	Florida
1628	M 123	*Xanthosoma*	Florida
1629	M 124	*Xanthosoma*	Florida
1630	M 126	*Xanthosoma*	Florida
1631	M 127	*Xanthosoma*	Florida
1671	MREC	*Anthurium*	Florida
1672	MREC	*Anthurium*	Florida
1673	MREC	*Anthurium*	Florida
1674	MREC	*Syngonium*	Florida
1688	Z 27	*Anthurium*	Puerto Rico
1689	Z 23	*Anthurium*	Puerto Rico
1694	MREC	*Difffenbachia*	Florida
1697	MREC	*Syngonium*	Florida
1698	MREC	*Syngonium*	Florida
1699	MREC	*Epipremnum*	Guatemala
1701	MREC	*Syngonium*	Florida
1702	MREC	*Syngonium*	Florida
1703	MREC	*Dieffenbachia*	Florida
1704	MREC	*Syngonium*	Florida
1705	MREC	*Anthurium*	Florida
1706	MREC	*Caladium*	Florida
1707	MREC	*Syngonium*	Florida
1708	MREC	*Dieffenbachia*	Florida
1709	MREC	*Dieffenbachia*	Florida
1711	MREC	*Syngonium*	Florida
1713	MREC	*Aglaonema*	Florida
1718	MREC	*Dieffenbachia*	Florida
1750	LMG7399[Z]	*Dieffenbachia*	USA

Table 1 (Continued).

Strain ID	Original source ID	Host	Geographic origin
1751	LMG695	*Anthurium*	Brazil
1752	LMG7400	*Dieffenbachia*	USA
1753	LMG7484	*Anthurium*	Brazil
1754	LMG8664	*Anthurium*	Brazil

[y] Strains were obtained from the following laboratories: (A or D) A. Alvarez, Department of Plant Pathology, University of Hawaii at Manoa, Honolulu, HI, 96822. (ATCC) American Type Culture Collection, Manassas, VA 20108. (BCCM/LMG) Belgian Coordinated Collections of Micro-organisms, Brussels, Belgium. (ICMP) International Collection of Micro-Organisms from Plants, Mt Albert, Auckland, New Zealand. (M) K. Pernezny, Everglades Research and Education Center, Belle Glade, FL 33430. (MREC) Mid-Florida Research and Education Center, University of Florida, Apopka, FL 32703. D. Cooksey, University of California – Riverside. (DPI) J. Miller, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL 32602. (GWS) G. Simone, Department of Plant Pathology, University of Florida, Gainesville, FL 32611. (NZTCC) New Zealand Type Culture Collection. (PDD) D. Brunk, Plant Disease Diagnostics, Inc., Apopka, FL 32703. (NCPPB) National Collection of Plant Pathogenic Bacteria, York, UK. (Z) M. Zapata, University of Puerto Rico, Mayasuez, PR.

[z] Pathovar reference strain for *X. a* pv. *dieffenbachiae*.

CHAPTER 4
RESULTS

Genomic Comparisons

Rep-PCR

The profiles for BOX, REP, and ERIC were combined and used in computer analysis. The results of this analysis yielded six distinct clusters, shown in Figure 1. Cluster III contained 81 strains and was comprised primarily of strains originally isolated from *Anthurium spp.* This cluster consists of two sub-clusters of 37 and 42 strains. The two sub-clusters represent those strains isolated from *Anthurium spp.* with 86% and 93% of the strains originating from *Anthurium spp.,* respectively. There were a couple of outliers from this cluster containing two strains isolated from *Syngonium spp.* Cluster II contained 33 strains and was very heterogeneous in regards to host of origin, with nine of the ten host genera being represented. Cluster I consisted of 15 strains sharing 75% similarity, which was isolated from *Xanthosoma spp.* Three outliers representing three strains isolated from *Aglaonema, Dieffenbachia,* and *Epiprenum* also were grouped between cluster II and cluster III. Strains originally isolated exclusively from *Syngonium spp.* comprised cluster IV containing 19 strains. The fifth cluster was relatively small and mixed in regards to host of origin. Cluster VI was composed of 15 strains in which ten or 67% of the strains were isolated from *Philodendron spp.*.

Amplified Fragment Length Polymorphism

Forty representative strains based on the results from the rep-PCR with an emphasis on strains originating from *Anthurium*, *Dieffenbachia*, and *Syngonium spp.*

were used in AFLP analysis. Strains isolated from *Aglaonema*, *Caladium*, *Colocasia*, and *Philodendron spp.* were also represented in AFLP analysis. Results using the M02 and E00 primer combination generated clusters for 38 strains and are shown in Figure 2. The cluster analysis yielded similar results as that of the rep-PCR technique. There were five distinct clusters. Cluster I contained strains showing 53% similarity and contained both type strains (LMG 7399, 7400) originating from *Dieffenbachia spp.* as well as two of the three strains isolated from *Aglaonema spp*. There was also a strain isolated from *Caladium spp.* within this cluster. Cluster II consisted of nine strains showing 66% similarity, of which six were strains from *Anthurium*. Two representing the *Anthurium* type strains (LMG 7484, 8664), and three from *Dieffenbachia spp.* Cluster III contained six strains all of which were isolated from *Anthurium spp.* one of which represented the *Anthurium* type strain (LMG 695). Cluster IV contained six strains all of which were isolated from *Syngonium spp.* and shared 54% similarity. Between clusters IV and V, there was an outlier, X-457, isolated from *Colocasia spp.* Cluster V had slightly lower similarity of 44%, and contained six strains isolated from four different host genera. Three of these strains were isolated from *Dieffenbachia* and the other three representing strains isolated from *Anthurium*, *Syngonium*, and *Philodendron spp.* Another outlier, X-271, was below cluster V, isolated from *Syngonium spp.* Strain X-805 isolated from *Dieffenbachia spp* was most distantly related to all other strains.

DNA Sequencing

Sequence comparisons were done using the European Bioinformatics Internet CLUSTALW program. The results for the ribosomal DNA placed all strains tested within two groups with which one group was composed of strains originating from *Syngonium.* Two strains, X-430 and X-1674, grouped together but were clearly different

from other strains. Most of the variation between strains was observed in the ITS region. The *Syngonium* strains varied primarily in positions in between the 16S and 23S known as the ITS region, and were two nucleotides shorter in overall length. The *hrp* sequences revealed similar results placing *Syngonium* strains in one group distinct from others.

Host Range Tests

The pathogenicity tests proved the heterogeneity of *X. axonopodis* pv. *dieffenbachiae* with strains varying immensely in their ability to produce symptoms on the hosts tested. Host range tests did not show distinct relationships between strains based on host of origin as was predicted by preliminary rep-PCR data. Although many of the strains isolated from their host of origin showed pathogenicity on other aroids, there were some important trends observed. Results from representative strains originally isolated from *Dieffenbachia*, *Anthurium*, and *Syngonium* are presented in Figures 3,4, and 5 respectively.

Dieffenbachia Strains

In pathogenicity tests with ten strains originally isolated from *Dieffenbachia* (Figure 3), disease on *Dieffenbachia* was 60% and 72% when misted at 10^8 cfu/ml and infiltrated at 10^5 cfu/ml, respectively. Four strains failed to produce symptoms on *Dieffenbachia maculata* after mist inoculations (X-183, X-697, X-805, and LMG 7399). Whereas they produced some symptoms on *D. maculata* in the infiltration inoculations, X-805 failed to produce symptoms on any of the hosts tested. In addition to causing disease in *D. maculata,* a number of these *Dieffenbachia* strains produced symptoms on the *Anthurium spp.* in both tests. Of the strains tested, X-1709 and X-1718 appeared the most aggressive towards *Anthurium* even resulting in systemic necrosis. Strains producing symptoms in *Anthurium* also produced symptoms on *Aglaonema commutatum*.

Only two strains (X-1703 and LMG 7400) produced symptoms on *Syngonium podophyllum* and on all hosts tested with the exception of *Philodendron scandens oxycardium*. Only X-1718 consistently produced symptoms on the *Philodendron spp.* tested.

Anthurium Strains

Figure 4 illustrates the results obtained from 12 strains originally isolated from *Anthurium*. *Anthurium* strains produced symptoms in a similar manner to that of the *Dieffenbachia* strains. A majority of the strains (greater than 70%) produced symptoms on *Dieffenbachia* whether infiltrated or misted. Symptom production was greater in *D. maculata* than that of any of the *Anthurium* cultivars tested. Nine of the twelve strains reliably produced symptoms in misting tests. The exceptions (X-14, X-84, X-1705) failed to produce symptoms on *Anthurium* in either test and failed to produce consistent symptoms in any of the hosts tested. It was observed during the infiltration tests that leaf age plays a role in susceptibility particularly when working with the cultivar Red Hot. This may account for the low number of strains producing symptoms when using the infiltration technique. The results for the misting yielded 50%, 56%, and 62% disease on the cultivars 'Red Hot', 'Crystal Hope', 'Heart's Desire' respectively. The strains producing symptoms in *Anthurium spp.* did not consistently produce symptoms in *A. commutatum* as was observed with the strains isolated from *Dieffenbachia*. None of the strains originating from *Anthurium* produced any symptoms on *S. podophyllum* nor the *Philodendron spp.* tested. This exclusiveness in pathogenicity is noteworthy.

Syngonium Strains

Results from strains isolated from *Syngonium* are shown in Figure 5. The *Syngonium* strain inoculations appeared considerably different from those with strains

isolated from *Anthurium* or *Dieffenbachia.* Of these strains 25% produced symptoms when misted and 68% produced symptoms on *D. maculata* when infiltrated. Only one of these strains (X-191) produced symptoms on the 'Red Hot' and 'Heart's Desire' cultivars of *Anthurium* and failed to produce symptoms on 'Red Hot' in the infiltration inoculations. *Syngonium* appears to be the most susceptible host for these strains. Three (X-159, X-187, X-271) of the ten strains failed to produce symptoms on *S. podophyllum* in the misting test. Strains X-159 and X-187 expressed partial symptoms when infiltrated. Strain X-271 failed to produce symptoms on any of the hosts tested. The *Aglaonema* tests were similar to those of the *Anthurium* isolates except, that fewer strains induced symptoms. In both the infiltration and mist inoculations no *Syngonium* strains produced symptoms on *Anthurium spp.*

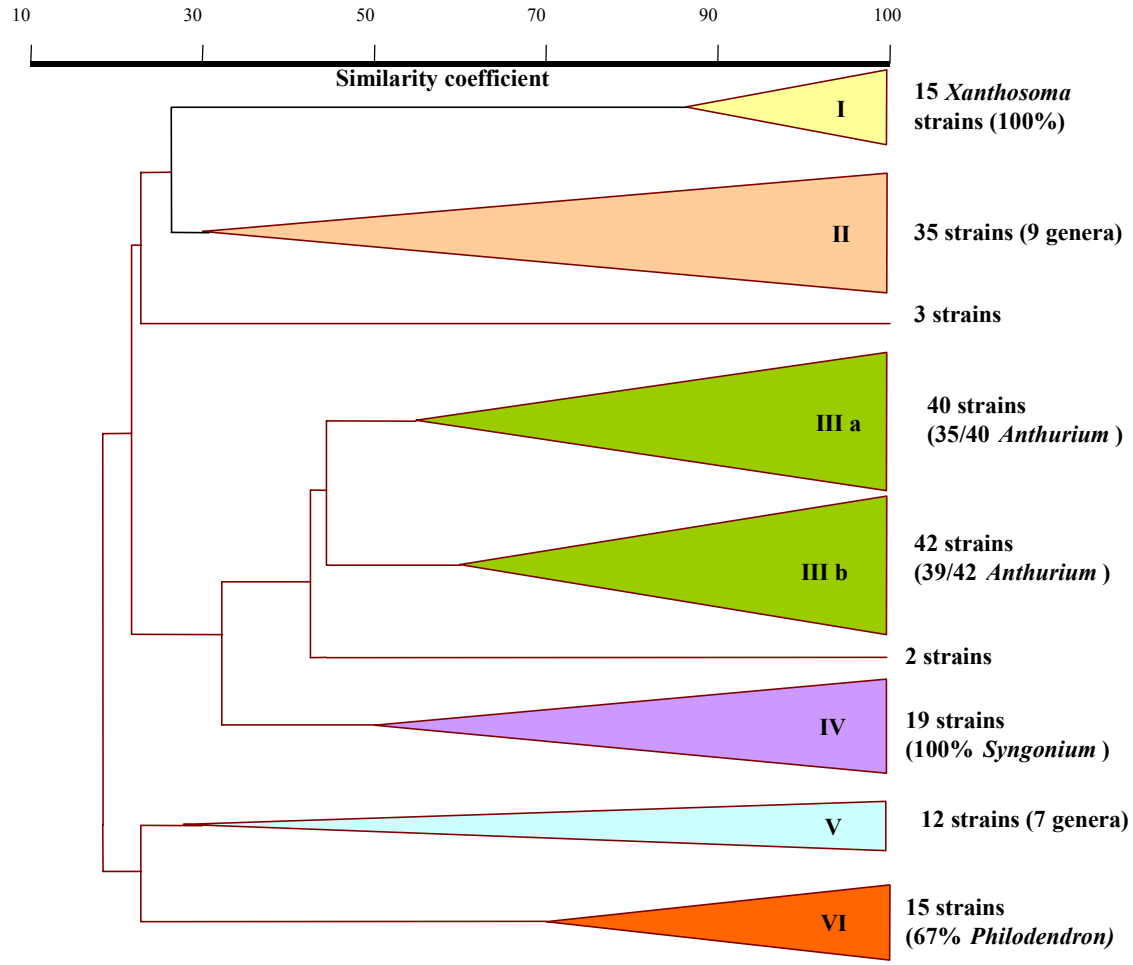

Figure 1. Cluster analysis of 183 *X.a.d* strains isolated from ten host genera over a 20-year period based on rep-PCR using BOX, ERIC and REP primers. Banding patterns were compared between strains using Pearson Correlation and Unweighted Pair Group Means Analysis (UPGMA) with the BioNumerics program ver. 2.1

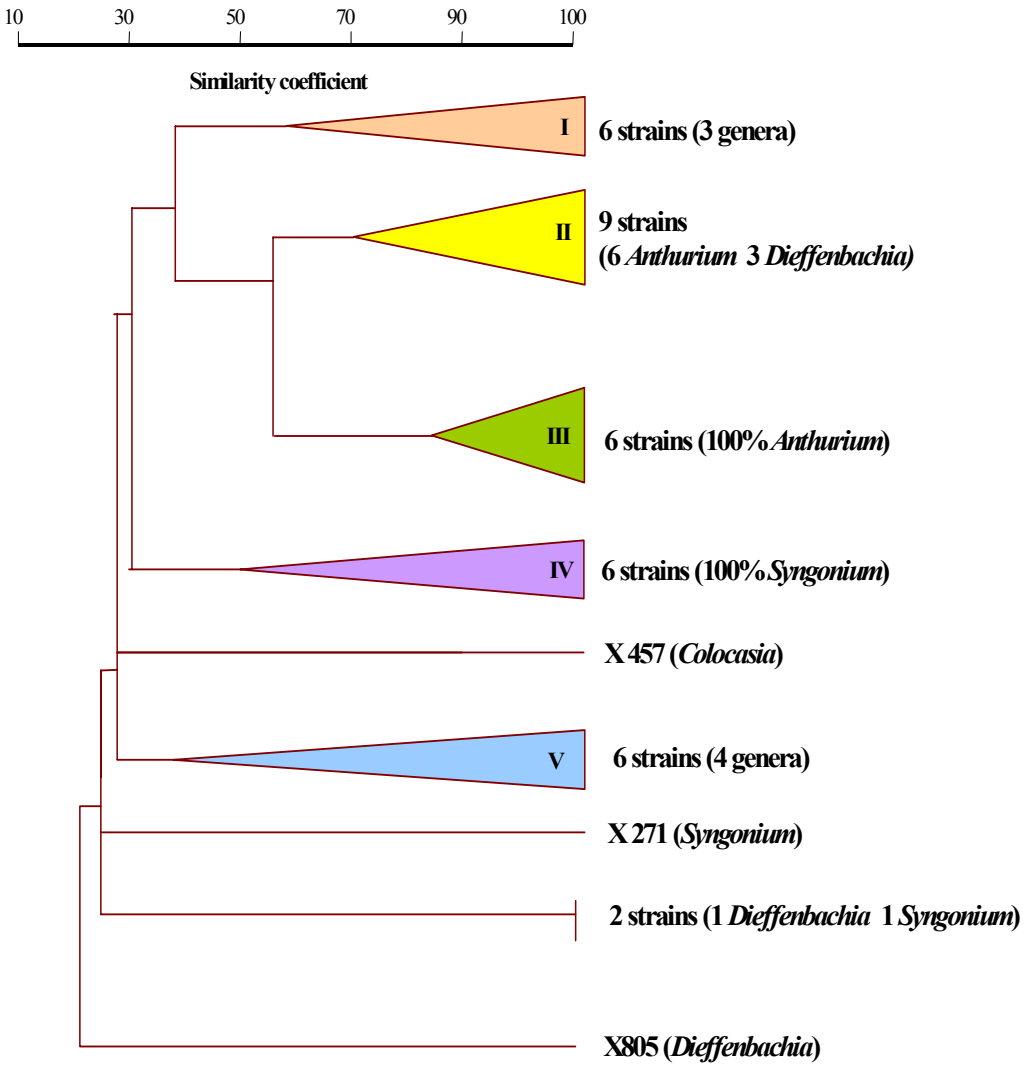

Figure 2. Cluster analysis of 38*X.a.d* strains isolated from six host genera over a 20-year period based on AFLP using M02, and E00 primers combinations. Banding patterns were compared between strains using Pearson Correlation and Unweighted Pair Group Means Analysis (UPGMA) with the BioNumerics program ver. 2.1

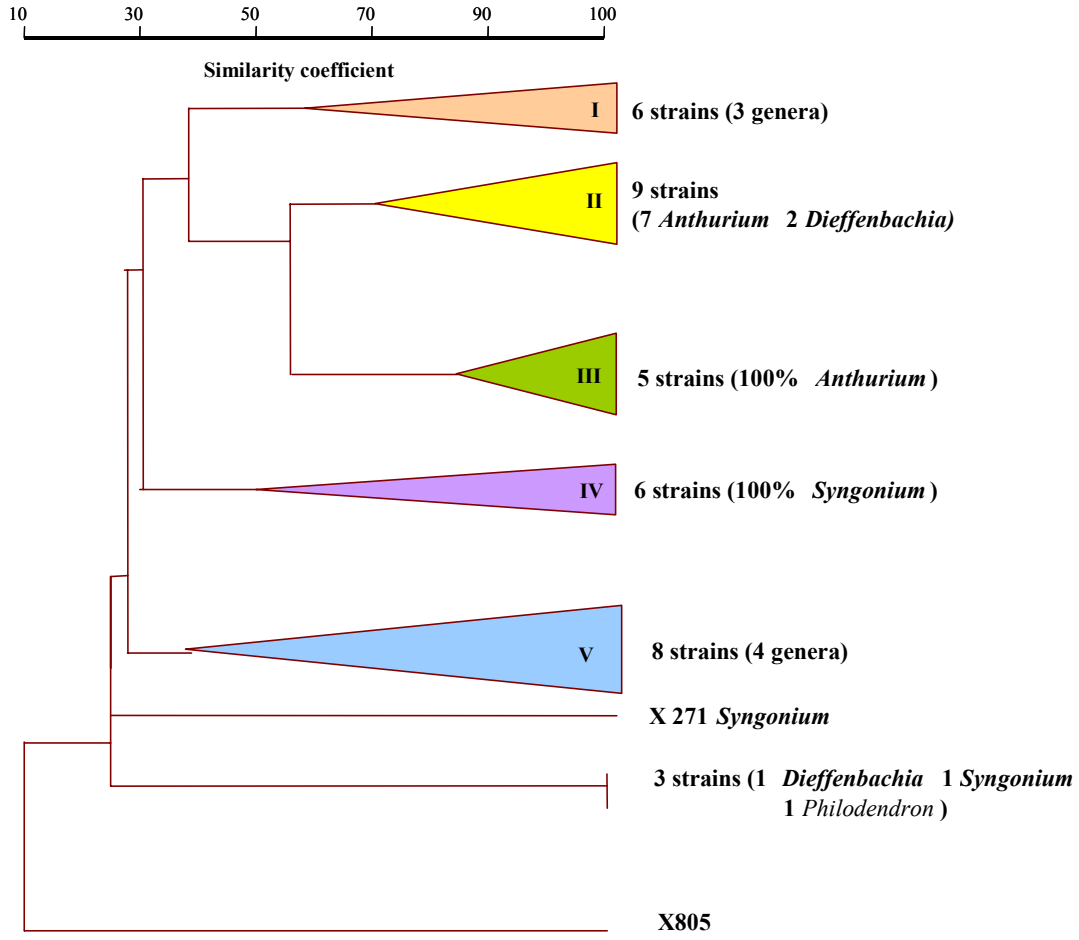

Figure 3. Cluster analysis of 39*X.a.d* strains isolated from six host genera over a 20-year period based on AFLP using M02, and E02 primers combinations. Banding patterns were compared between strains using Pearson Correlation and Unweighted Pair Group Means Analysis (UPGMA) with the BioNumerics program ver. 2.1

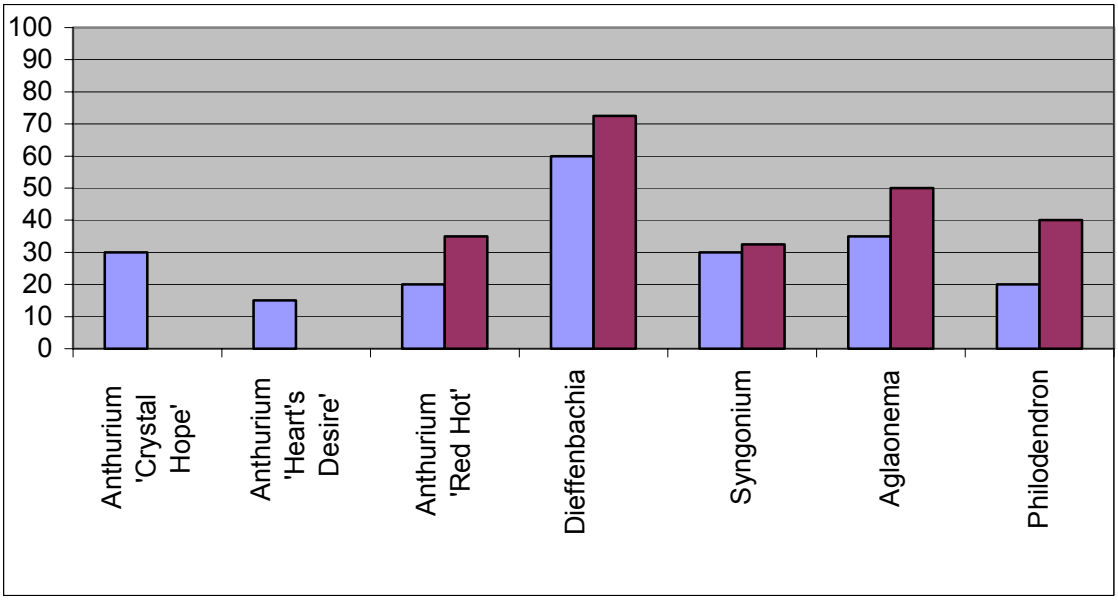

Figure 4. Percent of *X. a. dieffenbachiae* strains isolated from *Dieffenbachia spp.* producing disease when inoculated into various aroid hosts. Blue bars represent mist inoculations of bacterial suspension adjusted to 1.0 X 10^8 CFU/ml. Red bars represent infiltration inoculations using bacterial suspensions adjusted to 1.0 X 10^5 CFU/ml. Results based on two mistings and four infiltrations.

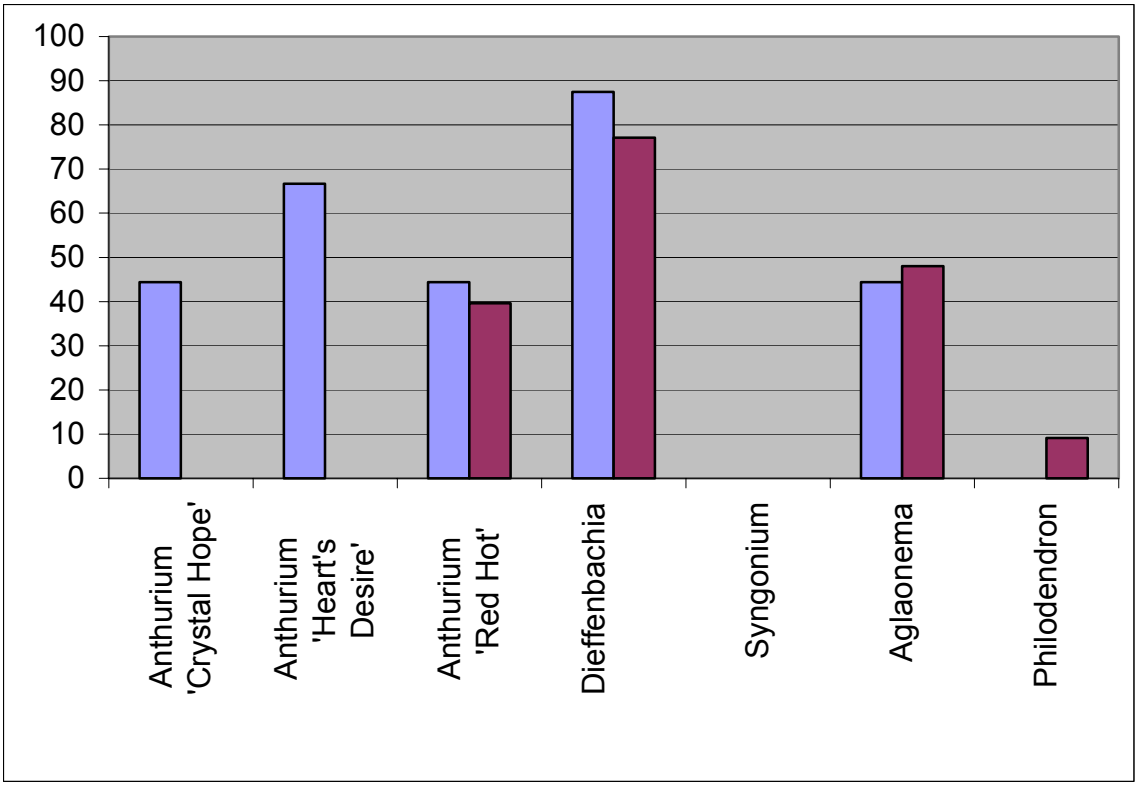

Figure 5. Percent of *X. a. dieffenbachiae* strains isolated from *Anthurium spp.* producing disease when inoculated into various aroid hosts. Blue bars represent mist inoculations of bacterial suspension adjusted to 1.0 X 10^8 CFU/ml. Red bars represent infiltration inoculations using bacterial suspensions adjusted to 1.0 X 10^5 CFU/ml. Results based on two mistings and four infiltrations.

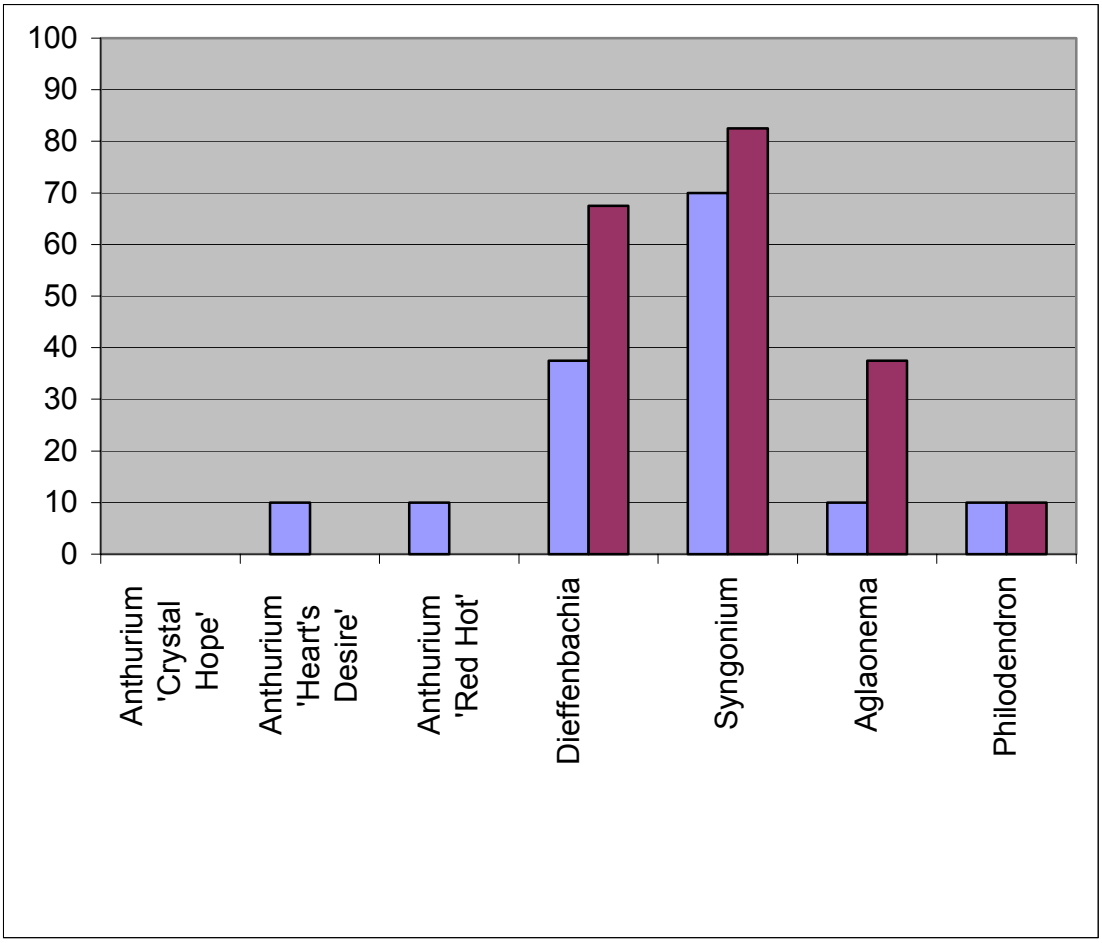

Figure 6. Percent of *X. a. dieffenbachiae* strains isolated from *Syngonium spp.* producing disease when inoculated into various aroid hosts. Blue bars represent mist inoculations of bacterial suspension adjusted to 1.0 X 10^8 CFU/ml. Red bars represent infiltration inoculations using bacterial suspensions adjusted to 1.0 X 10^5 CFU/ml Results based on two mistings and four infiltrations.

CHAPTER 5
DISCUSSION

In this study I addressed the variation that exists within *Xanthomonas* affecting aroid hosts. Using rep-PCR it was observed that six genetic clusters were generated by the 183 strains tested. Four of these clusters appeared to cluster based on host of origin. Clusters clearly represented strains originating *Philodendron, Xanthosoma, Anthurium,* and *Syngonium.* These results suggest that each of the four clusters may represent a different pathovars as previously described by Louws et al. (1994).

When representative strains were subjected to AFLP, similar results were observed to that of the rep-PCR. Distinct clusters representing strains virulent on *Anthurium* and *Syngonium* were observed. Genetic differences between these two clusters and between the others provide considerable evidence for the variation within *X. a. dieffenbachiae*, and particularly for the *Syngonium* strains. These results when combined with the rep-PCR data may reveal that these are significant species since the techniques provide information close to the resolution of DNA-DNA hybridization (Rademaker *et al.* 2000).

Upon phylogenetic analysis of rDNA, the same differentiation was observed between *X. a. dieffenbachiae* and those strains isolated from *Syngonium*. A great degree of variability had been observed in restriction patterns within pathovar *dieffenbachiae* (Berthier *et al.* 1993). These results also elicited some minor differences within strains from *Anthurium* and *Dieffenbachia*. However, quite significant variation was observed between these strains and those from *Syngonium*, particularly in the ITS region. The same variation was found upon investigating the *hrp*B region. Individually or in

combination these two techniques were useful in differentiating *X. a. dieffenbachia* from pathovar *syngonii*.

Many of the strains tested exhibited overlapping pathogenicity on host plants, which was observed in other studies (Lipp *et al.* 1992, Chase *et al.* 1992). This was especially the case when looking at the data from those strains isolated from *Anthurium* and *Dieffenbachia*. Interestingly, the *Syngonium* strains did not exhibit pathogenicity in a manner similar to the *X. a. dieffenbachiae* strains. Strains from *Philodendron* and *Xanthosoma* were found in distinct genetic clusters based on rep-PCR data and might also exhibit specialization in pathogenicity.

The pathovar, *syngonii*, was described earlier based on NaCl tolerance, pH optima, gelatin hydrolysis, and growth on SX media (Dickey and Zumoff 1987). However, there were many reports on the inadequacy of the tests used. Hodge *et al.* (1990) concluded that there was not enough variation in fatty acid profiles to differentiate *X. a. dieffenbachiae* from *X. c. syngonii*, and strains from *Syngonium* and should be included in *X. a. dieffenbachiae*. In an attempt to differentiate the two with the use of monoclonal antibodies, Lipp *et al.* (1992) came to a similar conclusion stating that typical *Syngonium* strains are most similar to *Anthurium* strains while the atypical *Syngonium* strains are more similar to those strains isolated from *Dieffenbachia*. Even when using physiological, pathological and fatty acid analysis, Chase *et al.* (1992) concluded that none of these tests were useful in differentiating *X. a. dieffenbachiae* from *X. c. syngonii*. The *Syngonium* cluster (cluster V in figures 4 and 5) represents fastidious xanthomonads, which are specialized in pathogenicity in *Syngonium*. The pathovar, *syngonii*, previously

described by Dickey and Zurmoff would be an appropriate designation for these strains (1987).

This work has provided genetic evidence for the pathovar designation *syngonii* when referring to those strains specialized in pathogenicity on *Syngonium spp*. The genetic data is convincingly reinforced by host range tests. These results illustrate the usefulness of molecular tools in bacterial taxonomy. In order to be absolute in this matter it may still be necessary to conduct DNA-DNA hybridization experiments with additional strains representing pathovar syngonii. The strains comprising *X.a.d.* used by Vauterin *et* al. (1995) proved to be very similar upon rep-PCR and AFLP analysis though they varied in their pathogenicity. None of these strains were representative of those strains representing pathovar *syngonii* and further justifies the need for them to be included in a DNA-DNA homology study.

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BIOGRAPHICAL SKETCH

Ryan Donahoo is a Florida native with a vested interest in the tropical foliage industry. He received his B.S. in plant science-plant pathology with a minor in plant molecular and cellular biology from the University of Florida in 2001. While completing this undergraduate degree, Mr. Donahoo worked with various microorganisms in laboratory research studies. This experience fostered an interest in bacterial pathogens, which was expanded upon in this study. Mr. Donahoo plans to continue his work in plant pathology and microbiology in a professional research setting.