Diversity of *Xanthomonas campestris* pv. *citrumelo* Strains Associated with Epidemics of Citrus Bacterial Spot in Florida Citrus Nurseries: Correlation of Detached Leaf, Monoclonal Antibody, and Restriction Fragment Length Polymorphism Assays

Tim R. Gottwald, A. M. Alvarez, J. S. Hartung, and A. A. Benedict

Research Plant Pathologist, USDA-ARS, Horticultural Research Laboratory, Orlando, FL; professor, Department of Plant Pathology, University of Hawaii, Honolulu; research plant pathologist, USDA-ARS, Fruit Laboratory, Beltsville, MD; and professor, Department of Microbiology, University of Hawaii, Honolulu.

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ABSTRACT

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The heterogeneity of 194 previously uncharacterized strains of *Xanthomonas campestris* pv. *citrumelo* isolated from citrus bacterial spot epidemics in four citrus nurseries in central Florida was evaluated by virulence reactions on detached leaves, reaction to a panel of monoclonal antibodies (MAbs), and reaction to a panel of restriction fragment length polymorphism (RFLP) probes. Detached-leaf assays were performed on the 194 strains that had been differentiated into five serological groups based on reactions with six MAbs. A subset of 27 strains, selected because of their diverse reactions to MAbs and the detached-leaf assay, were differentiated into five reaction types by RFLP analysis. There was good agreement between serological reaction patterns and RFLP results. Both assays were capable of distinguishing strongly aggressive from less aggressive strains as indicated by detached-leaf assay. In addition, MAb and RFLP assays often detected the same unique strains within the population of strains from individual nurseries. The assays also differentiated groups of strains originating from different foci of infection in one nursery.

Additional keywords: citrus canker, spatial arrangement.

Citrus bacterial spot (CBS) is caused by *Xanthomonas campestris* pv. *citrumelo* Gabriel pv. Nov. (syn = *Xanthomonas campestris* pv. *citri* (Hasse) dye strain E) (10). The disease originally was believed to be a novel form of citrus canker, which is caused by *Xanthomonas citri* ex Hasse (syn = *X. c. citri* dye pathotype A) (6,11,14); however, recent studies concerning the comparative pathogenic (virulence), physiological, genetic, and serological associations have clearly demonstrated the uniqueness of the nursery CBS strains (3,5,10,15,20,21,24).

Attempts to eradicate this new pathogen by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry, in conjunction with the USDA, Animal and Plant Health Introduction Service, have led to the destruction of more than 23 million nursery and young reset citrus trees and restricted both interstate and intrastate commerce of citrus (16,34). In spite of this effort, disease eradication continued to be unsuccessful and was curtailed in 1990. Thus, the impact of this disease and eradication effort on the citrus industry has been substantial.

The disease is characterized by flat necrotic lesions on leaves and young stems of susceptible citrus cultivars (34). Foliar lesions usually exhibit water-soaked margins and chlorotic halos when young. Stem lesions on some highly susceptible cultivars can be slightly erumpent in appearance and usually are not associated with chlorosis.

Detached-leaf assay has been demonstrated as a useful method to differentiate the relative aggressiveness (virulence) among isolates of *X. c. citrumelo* and *X. citri* (12,13,15,17,23). A rating scale of aggressiveness recently was developed to grade detached-leaf assay results in which 0 = no reaction, 1 = weakly aggressive, 2 = moderately aggressive, and 3 = strongly aggressive (13,17). Differences in aggressiveness also were reflected in field symptomatology (13,17).

Both monoclonal (MAb) and polyclonal antibodies have been used in enzyme-linked immunosorbent assay (ELISA) to differentiate between and among *X. citri* and *X. c. citrumelo* strains (1–5,7,28). Diversity within the *X. c. citrumelo* group has been demonstrated in preliminary studies by the use of MAbs (5) and by the companion study (3) in which there was some indication that reactivity with selected MAbs, namely CBS1 and Xct, was related to the degree of aggressiveness of 46 strains from the 1984–1985 and the 1987–1988 epidemics. To further examine this point, the detached-leaf assay was performed on all 194 strains isolated from four nurseries in the 1987–1988 epidemic.

The lack of homogeneity was also demonstrated by restriction fragment length polymorphism (RFLP) analyses of a large collection of *X. c. citrumelo* strains from CBS outbreaks in Florida, from which several unique RFLP patterns were observed (19–21).

The purpose of this study was to compare CBS bacterial strains by detached-leaf assay, reactions to a panel of MAbs, and analysis of a panel of RFLP probes. Secondly, strain diversity, as determined by these three assays, was related to previously reported (13,17) spatial arrangement and strain aggressiveness of field epidemiological findings.

MATERIALS AND METHODS

Data collection. Four of the most significant outbreaks of CBS that occurred during September 1987–February 1988 were studied. The outbreaks in four different nurseries in the central Florida area are designated as Frostproof, Lake Wales, Ocoee, and Venice, and were described previously in detail (13). The Frostproof nursery consisted of about 5.7 ha of citrus, with mixed cultivars and rootstocks of different ages. Citrus bacterial spot was restricted to a single nursery bed in the southwest corner of the nursery. Cultivars contained within the bed were: Ruby Red grapefruit and Henderson red grapefruit, *Citrus paradisi* Macfady.; Parson Brown orange, *C. sinensis* (L) Osbeck; and Minneola

tangelo, *C. paradisi* × *C. reticulata* Blanco. All were budded onto sour orange rootstocks, *C. aurantium* L. The Lake Wales nursery consisted of about 520,000 trees primarily of Swingle citrumelo (*C. paradisi* × *Poncirus trifoliata* (L.) Raf.) rootstock in 28 nursery beds, of which six beds were affected by CBS. The Ocoee nursery consisted of about 0.9 ha and 95,000 trees in 58 north-south rows. Swingle rootstocks were planted on three different occasions which represented three different age groups and contiguous sections of a single bed within the nursery. Disease was restricted to the easternmost section of the nursery, in which Swingle rootstocks had been budded with red grapefruit and Hamlin and Pineapple orange, about 2 mo before disease assessment. Citrus bacterial spot in the Venice nursery was restricted to the three easternmost of five nursery beds of Swingle rootstock plants.

A total of 66, 61, 26, and 41 single-cell cultures were obtained from foliar lesions from the Frostproof, Ocoee, Lake Wales, and Venice nurseries, (indicated by FP, OC, LW, or VE followed by strain number) respectively. Each culture was maintained on a semiselective medium and tested for pathogenicity by detached-leaf assay as previously described with 10 replications per inoculation (17,27).

Detached-leaf serological and RFLP assays. Aggressiveness of some CBS strains was previously determined; however, the number of sample strains from any given nursery was relatively small. Thus, to determine how aggressiveness is related to serological reaction patterns, detached-leaf assays were performed on 194 strains that had been isolated from the four 1987–1988 epidemics. MAbs were prepared and assays conducted as described (1,3,4).

Analysis of RFLP was done with the isolation of DNA; electrophoresis and hybridization conditions were as described previously (20), except that electrophoresis was at 2 V/cm. The seven hybridization probes previously described (20) have average insert sizes of −25 kb, do not cross-hybridize, and were randomly selected from a genomic library of *X. citri* strain XC62. Biotinylation was by nick translation. Coefficients of similarity (F) between strains were calculated as described (20). All strain comparisons were made for samples on the same blots and were performed twice. Because it was impractical to analyze all 194 strains by RFLP, a subset of 27 strains was selected that represented the variation among strains, including unique strains, indicated by detached-leaf and MAb assays. These included eight Frostproof, five Lake Wales, eight Ocoee, and six Venice nursery strains.

Statistical analysis. Individual data, as well as all combinations of data from detached-leaf, serological, and RFLP assays, were converted to dissimilarity coefficient matrices by the average taxonomic distance method of the NTSYS-pc program (Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing, Ltd., Setauket, NY) as described previously (22,25,26,29,30–37). Cluster analysis was performed on each resulting dissimilarity matrix by the unweighted average pair group method (8,33–37). The results of the cluster analyses were utilized to prepare phenograms indicating the similarity (=1 dissimilarity) coefficients (relatedness) among clusters of strains (31). The matrices resulting from the cluster analysis were further converted to "cophenetic" matrices (30), which were used to test the goodness-of-fit of the data by comparing the original dissimilarity matrices that were clustered with the cophenetic value matrices via "cophenetic correlation" and the Mantel t test (22,26,30,32). The criteria used to assign observations to a given cluster depend on the strength of the statistical test and whether the cluster is circumscript for the reaction of bacterial strains to one type of assay (i.e., MAb) or the cluster is circumscript for the grouping of strains resulting from two assays taken together (i.e., MAb and detached-leaf assays).

RESULTS

The 194 strains from four nurseries were separated into five serological groups (serogroups) based on differential positive reactions with six of the fifteen MAbs used in the assay (3). The distribution of these strains among the four nurseries is presented in Table 1. The greatest number of strains (98 of 194) corresponded to serogroup IV and were recovered from only two nurseries, Frostproof and Lake Wales, in 1987. The majority of these same strains were weakly aggressive or moderately aggressive in the detached-leaf assay (Table 1). A similar number of strains (87 of 194) were in serogroups II or III, in which all strains were classified as strongly aggressive by detached-leaf assay (Table 1). All eight less aggressive strains in serogroup V were recovered from Frostproof, and all of the aggressive strains in serogroup II were recovered from Ocoee and Venice nurseries (Table 1).

RFLP analysis of the 27-strain subset demonstrated six discrete clusters of strains of *X. c. citrumelo* (Fig. 1A). However, two of these clusters had similarity coefficients of 0.99 and thus were assumed to be homogeneous. Thirteen of the 14 strains from the Ocoee and Venice nurseries were indistinguishable by RFLP analysis (i.e., similarity coefficient of 1). One strain, OC16, was unique (0.91 similarity) compared with all other strains from these two nurseries. All strains from these two nurseries were more related to one another (0.91–1.00 similarity) than to strains from the other nurseries (0.72 similarity) (Fig. 1A). The bacterial strains from the Frostproof nursery were divided into two clusters of equal size by RFLP analysis (0.75 similarity). One of these clusters was highly related (0.99 similarity) to the majority of Lake Wales nursery strains tested. A single Lake Wales strain (LW37) was unique (0.75 similarity) among the other strains isolated from that nursery and was more closely related (0.89 similarity) to one cluster of the Frostproof strains (Fig. 1A).

MAb assay data and detached-leaf assay data were combined into a single dissimilarity matrix for cluster analysis (Fig. 1B). Strains of *X. c. citrumelo* isolated from the Ocoee and Venice nurseries formed a cluster (≥0.95 similarity) with only one unique strain (VE53). This cluster resembled the cluster of these strains in the RFLP dendrogram, indicating that strongly aggressive strains, as defined by detached-leaf assay (Table 1), can be dis-

TABLE 1. Relationship between virulence and reactivity to monoclonal antibodies among 194 strains of *Xanthomonas campestris* pv. *citrumelo* from four nursery epidemics of citrus bacterial spot

Nursery	Number of strains	Serogroup[a]					Detached-leaf assay reactions[b]			
		II	III	IV	V	VI	No reaction (0–0.4)	Weakly aggressive (0.5–1.4)	Moderately aggressive (1.5–2.4)	Strongly aggressive (2.5–3.0)
Frostproof	66	0	0	58	8	0	4	42	20	0
Lake Wales	41	0	0	40	0	1	6	35	0	0
Ocoee	26	23	3	0	0	0	0	0	0	26
Venice	61	60	1	0	0	0	0	0	0	61
Total	194	83	4	98	8	1	10	77	20	87

[a] See companion article for description of reaction patterns (5).

[b] Each was assigned a reaction value of 0–3.0 by 0.1 increments as a result of the average reaction value of 10 inoculations per strain in detached-leaf assay. Reactions were graded as 0 = no reaction; 0.5–1.4 = ± indistinct water-soaking, with ± necrosis around inoculum wound; 1.5–2.4 = distinct water-soaking, but extending ≤1.0 mm completely around wound with distinct necrosis surrounding wound; and 2.5–3.0 = extensive water-soaking ≥1.0 mm around wound and extensive necrosis, but becoming indistinct from water-soaking.

tinguished by MAb and RFLP analysis (Fig. 1B). The remaining 14 strains from the Frostproof and Lake Wales nurseries fell into seven clusters (cluster being defined by >0.95 similarity among strains), demonstrating more variability of strains within these two nurseries as compared to the variability encountered in the two nurseries with aggressive strains. The strains within these seven clusters all were less aggressive, as determined by detached-leaf assay. Strain LW37 (serogroup VI) was unique among the Lake Wales strains in this combined data set, as it was with RFLP data alone (Fig. 1). The results of cluster analysis of detached-leaf bioassay, combined with reactions with MAbs, clearly separated all aggressive strains from other strains (0.0 similarity) (Fig. 1B).

The results of cluster analysis, including cophenetic correlation of all combinations of detached-leaf, MAb, and RFLP analyses of the 27 strains of *X. c. citrumelo*, are presented in Table 2.

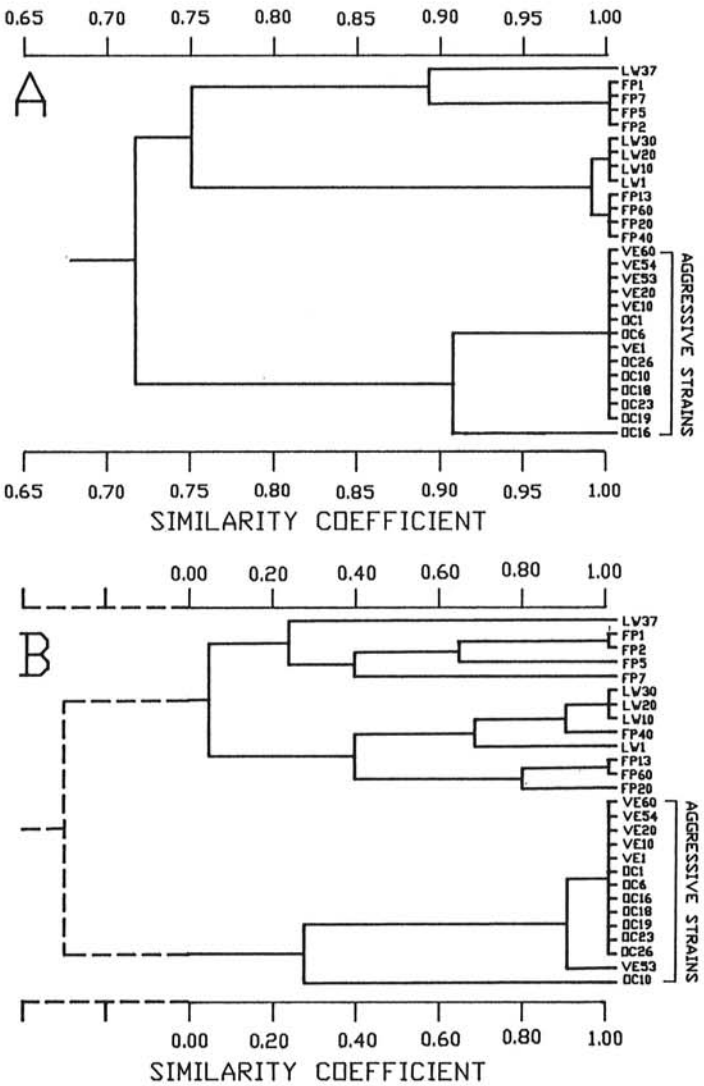

Fig. 1. A, Phenogram of the relative similarity of a 27-strain subset of *Xanthomonas campestris* pv. *citrumelo* as detected by RFLP analysis. Strain numbers from each of four nurseries are preceded by letters indicating the nursery from which the strain was isolated. FP = Frostproof, LW = Lake Wales, OC = Ocoee, and VE = Venice nurseries, respectively. The Venice and Ocoee strains are quite closely related, and all are considered aggressive by detached-leaf assay. Also the division of Frostproof strains into two groups was with only 0.75 similarity. These two groups correspond to strains isolated from two different foci within the Frostproof nursery. **B,** Phenogram of the same subset of 27 isolates based on a combination of similarity coefficients from detached-leaf assay and monoclonal antibody panel assay combined. Note the high degree of similarity among the strongly aggressive strains but the divergence of the remaining strains.

The number of clusters ranged from 6 to 11 (>0.90 similarity of strains within cluster), with aggressive strains usually demonstrating cohesive relationships among themselves. More heterogeneity was found associated with less aggressive strains.

Results of detached-leaf assay and reactions with MAbs also were compared for all strains collected from each nursery and for a combination of all 194 strains from all four nurseries (Table 2). The number of clusters resulting from matrix comparisons was greater for the Frostproof and Lake Wales nurseries with less aggressive strains, and fewer for the Ocoee and Venice nurseries, which had strongly aggressive strains. This further demonstrates greater variability among less aggressive strains.

The relative similarity of clusters of bacterial strains from matrix comparison of RFLP, MAb, and detached-leaf assay was examined by three-dimensional graphic representation (Fig. 2). Relationships among clusters of bacterial isolates could be detected visually and were assigned to four groups based on their spatial proximity to each other, resulting in groupings generally with ≥0.40 similarity among isolates. Two groupings, group 1 (cube) and group 4 (cylinder), contained individual bacterial strains (OC10 and LW37, respectively) that appeared to be unique. These two isolates were indistinguishable by detached-leaf assay; however, OC10 (group 1) was unique by MAb analysis (serogroup III), and LW37 (group 4) was unique by both MAb (serogroup VI) and RFLP analysis. The latter groups thus were spatially separated from cluster groupings 2 and 3 by some distance (Fig. 2). Group 1 was made up entirely of strongly aggressive strains from Ocoee and Venice nurseries.

Two discrete foci of infection, located about 40 m apart in the same row of grapefruit trees, were found in the Frostproof nursery (14,18). Isolates from the first focus of infection, represented by FP1, FP2, FP5, and FP7, were distinct from strains from the second focus, represented by isolates FP13, FP20, FP40, and FP60 by RFLP analysis (0.75 similarity) (Fig. 1A). In addition, strains from the first focus were included in serogroup V, whereas isolates from the second focus were included in serogroup IV (Table 1). Detached-leaf ratings for isolates from the first focus ranged from 0.9 to 2.0, whereas those from the second focus ranged from 0.3 to 2.0. These values were not statistically different by Student's *t* test.

TABLE 2. Goodness-of-fit comparisons of similarity coefficient matrices from detached-leaf, monoclonal antibody, and RFLP assays.

Matrices compared[a]	Number of strains	Matrix correlation (r^2)[b]	Number of cluster groups identified[c] 75%	90%
DL vs MAb				
Frostproof	66	0.854	7	13
Lake Wales	41	0.924	5	5
Ocoee	26	0.996	3	4
Venice	61	0.999	2	3
All four nurseries	194		11	17
Assay combinations (strain subset)				
DL × MAb	27	0.947	9	10
DL × RFLP	27	0.924	10	11
MAb × RFLP	27	0.875	6	6
DL × MAb × RFLP	27	0.921	10	11

[a] DL = detached-leaf assay, MAb = monoclonal antibody assay, RLFP = restriction fragment length polymorphism assay. Each designator represents a matrix of similarity coefficients for the same 27 *Xanthomonas campestris* pv. *citrumelo* strains. In addition, detached-leaf and monoclonal antibody similarity coefficient matrices were compared for data from each nursery independently.

[b] r = The cophenetic correlation coefficient or the matrix correlation coefficient of the comparison of the similarity matrices indicated; considering 27 strains. $r \leq 0.7$ (very poor fit); $0.7 \leq r < 0.8$ (poor fit); $0.8 \leq r < 0.9$ (good fit); $0.9 \leq r \leq 1.0$ (very good fit).

[c] Clusters are discrete groups of bacterial strains statistically similar at the indicated percentages.

DISCUSSION

There was good agreement between the results obtained by the MAb and RFLP assays. Both assays were capable of distinguishing the strongly aggressive from the less aggressive strains of the bacteria and, in one case, the difference between foci within the Frostproof nursery. In addition, both assays often recognized the same strains within the bacterial populations of each nursery as unique. The detached-leaf assay was utilized as a method to determine the relative aggressiveness of individual bacterial strains within and among nurseries. This aggressiveness rating agreed well with field spatial analysis of each nursery studied (13,17). The bacterial strains that were recognized as more aggressive by detached-leaf assay were more virulent in field epidemics (13,17). Relative aggressiveness was based on the average of host reactions of individual detached leaves. Some variation occurred from leaf to leaf, although the range of reactions for each strain was small. However, individual strains from a single nursery, which were unique by MAb reactions and/or RFLP assay, often were not distinguishable by the detached-leaf assay. Thus, the detached-leaf assay alone was not as sensitive an assay and did not detect strain diversity or heterogeneity, but was quite useful as an indicator of field virulence.

Of the four nurseries studied, natural spatial spread of the CBS pathogen occurred only in the Venice nursery (13). Bacterial strains from this nursery were all rated as strongly aggressive by detached-leaf assay; all but one strain, VE15, reacted to MAb CBS1, and the majority were indistinguishable by RFLP. However, strongly aggressive strains are rare, having been found in only four of the more than 55 nurseries where CBS has been detected in Florida through 1989 (17). In this study, 83 of 87 aggressive strains were in serogroup II.

Similarly, the Ocoee nursery consisted of 26 strains, all of which were strongly aggressive by detached-leaf assay, and all were included in serogroup II. Strain OC16 was unique by RFLP but not by MAb assay. Strains OC10, OC15, and OC17 were distinguished by their MAb reactions (serogroup III); only OC10 was analyzed by RFLP, but it also was unique by this assay. In neither the Venice nor Ocoee nurseries were the unique strains in novel spatial locations. Rather, the lesion from which isolations were made was merely one of a population at that location. Thus, the heterogeneity encountered in these two nurseries with strongly aggressive isolates of *X. c. citrumelo* was not associated with any particular location or circumstance. It should be noted that strains included in serogroup II are not always aggressive, even though it would appear so from this study (Table 1). In the companion paper (3), serogroup II included six moderately aggressive strains from six locations. However, all the aggressive strains were restricted to serogroups II and III.

A single strain, LW37, of the 41 analyzed from the Lake Wales nursery was unique by both RFLP and MAb assays, and was more aggressive than 88% of the rest of the strains from that nursery. As with unique strains of the Ocoee and Venice nurseries, strain LW37 was not recovered from any novel spatial location.

Considering the relatively few strains (194) isolated from the nurseries studied, a considerable amount of heterogeneity was detected by both RFLP and MAb assays. A great deal of heterogeneity was found among *X. c. citrumelo* strains isolated from different nurseries (9,10,19–21). Heterogeneity has been described previously for strains at a single location via RFLP analysis (20,21). However, this is the first time intralocation heterogeneity has been described for the same strains by diverse methodologies. Heterogeneity of CBS strains also was characteristic of their fatty acid profiles, multilocus isozyme analysis, genomic fingerprints, and RFLP analysis (9,10,20,21,34). Gabriel et al (9) presented evidence that some CBS strains varied in aggressiveness and belonged to different clonal groups. Their observations are consistent with our serological data in that strains included in clonal groups E1 and E2 were delineated by the MAb reaction patterns of serogroups I and II (3). Such a high amount of detectable heterogeneity raises some questions. Are CBS bacteria sufficiently unstable under field conditions to account for this high level of diversity? Or could a diverse, yet related, population of xanthomonads exist naturally in which all are capable of causing a similar disease syndrome? The stability of the bacteria subjected to numerous serial transfers on media in the laboratory suggests that the latter may be more plausible.

Strains from the Frostproof nursery were recovered from two different foci of infection with centers about 45 m apart. In a previous study where fewer strains were examined, these two foci were distinguishable by significantly different detached-leaf assay results (19). However, in this study, when a larger number of strains from each focus were considered, strains from these two foci had overlapping detached-leaf assay ratings, and strains isolated from the two foci formed two distinct groups by reactions with MAb and RFLP assays. Numerous infected plants resulted from spread of the pathogen in the vicinity of each focus, although one focus was considerably more extensive. Three possible scenarios exist to explain this distribution. First, the two foci could have developed from independent introductions. Second, early in the epidemic, a variant of the bacteria from one focus could have arisen and become established at a distance from the original focus. Third, the initial introduction of bacteria into the nursery was not as a pure population, but rather as a mixture of strains. Different strains could have become predominant at the different foci as a result of random events. The second scenario is unlikely because such malleability of *X. c. citrumelo* in the field is inconsistent with stability in the laboratory. Because the first scenario would require separate introductions into the nursery of pure populations (which are not expected in nature), the third scenario is the simplest and the preferred explanation.

CBS strains in serogroups IV and V were not found before the 1987–1988 epidemics. The occurrence of these strains may indicate a transition in CBS strains. However, all serogroup IV strains were weakly to moderately aggressive and very unlike *X. c. citri* strains, which are also MAb A1-positive. In addition, MAb CBS2 reacted with only a small subset of strains (serogroup V) from the Frostproof nursery, and no other strains from either the four nurseries in this study or from any previous epidemic reacted. Therefore, rather than a transition of previously encountered CBS strains toward MAb A1 reactivity, it is probably more

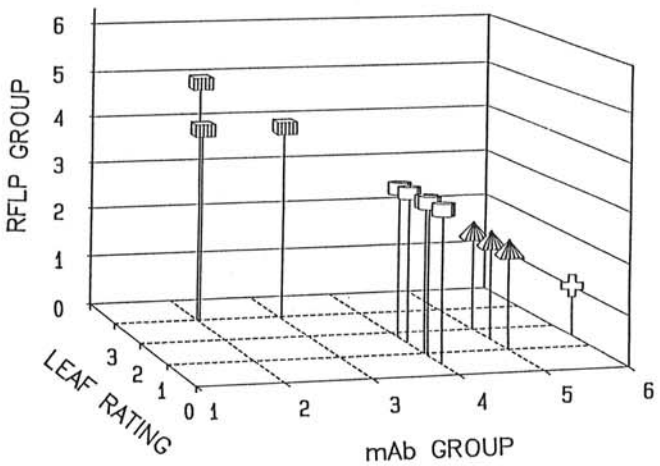

Fig. 2. Three-dimensional spatial representation of the relative position of clusters of strains of *Xanthomonas campestris* pv. *citrumelo* from four citrus nurseries based on detached-leaf assay, a panel of six monoclonal antibody (MAb) probes, and restriction fragment length polymorphism (RFLP) assay results. Each cluster of associated strains is represented by a different shape. The clusters were further associated with groups based on visual assessment. Groupings of cube, cylinder, pyramid, and cross consisted of 14, 8, 5, and 1 strains, respectively. The group of cube shapes represents those isolates with strongly aggressive detached-leaf reactions. Coefficients of similarity for groups are: cubes >90% similarity (strain OC10 not considered); >27% (strain OC10 considered); cylinders >40% similarity; pyramid >40% similarity. MAb group 3, with leaf rating 3, and RFLP group 4 are represented by single strain OC10. MAb group 6, leaf rating 1.5, and RFLP group 1 (cross) are represented by a single strain LW37.

likely that these exact strains had not been previously encountered. This further substantiates the diversity of *Xanthomonas* strains capable of causing the CBS syndrome.

It is most likely that the diversity of strains is the result of a population of similar opportunistic organisms, all capable of causing leaf spots on citrus and citrus relatives. Only those strains identified as strongly aggressive by detached-leaf assay appear capable of multiplying in leaf tissue and, even then, only in certain citrus species (18). The type strain for *X. c. citrumelo* is strongly aggressive by detached-leaf assay. Strongly aggressive strains were more similar to each other than less aggressive *X. c. citrumelo* strains (10,21). This diversity of less aggressive strains, combined with their lack of multiplication in citrus, is under consideration as justification for further revision of the designation of *X. c. citrumelo* that presently is given to all bacteria isolated from citrus bacterial-infected tissues (18,19).

Previous epidemiological studies have shown that strains with a stronger aggressiveness in the detached-leaf assay were also more virulent in nursery environments (13,17). The concept that the detached-leaf assay reliably measures real differences among bacterial strains has been reinforced by correlations between aggressiveness in the detached-leaf assay and particular RFLP patterns (J. S. Hartung and E. L. Civerolo, *unpublished*) and RFLP patterns and MAb profiles in the present work.

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