DOCUMENTATION AND CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS IN LANDSCAPE HOSTS

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ABSTRACT
Strains of *Xylella fastidiosa* (*Xf*) were characterized using random amplified polymorphic (RAPD)-PCR and sequence analysis of the 16S-23S rDNA intergenic spacer regions (ISR). Results indicated that all mulberry leaf scorch (MLS) strains and the heavenly bamboo strain formed a cluster. Strains isolated from daylily, jacaranda and magnolia clustered with the *Xf* subspecies *sandyi*; strains isolated from spanish broom, a redbud strain, two new peach strains and several almond strains clustered with *Xf* subspecies *fastidiosa*, and the oak strains formed a separate cluster. Predicted members of the *Xf* subspecies *multiplex* were the strains isolated from purple leafed-plum, olive, peach, plum, some almond, sweet gum, maidenhair tree, crape myrtle and one redbud. Pathogenicity of MLS strains was demonstrated in glasshouse assays by stem inoculating mulberry, grape and oleander with *Xf* isolate morus059 grown for 7 days on PW. Three months post-inoculation, only inoculated mulberries exhibited leaf scorch symptoms and bacteria were recovered from most of the inoculated mulberries but not from oleander or grape. Given that strains isolated from magnolia (MG038), jacaranda (JM028), and daylily (HEM034) always grouped with oleander strains, we tested their ability to produce disease in oleander and grape. The three strains MG038, JM028, and HEM034 caused oleander leaf scorch (OLS) but not Pierce's disease (PD) symptoms and bacteria were re-isolated from oleanders that were diseased. This study served as a confirmation of our genetic results, as well as an indirect demonstration that the *Xf* subspecies *sandyi* might be found in other hosts than oleander. More cross inoculation experiments are underway.

INTRODUCTION
Xf multiplies and survives in a large number of plants species and its vectors, including the glassy-winged sharpshooter (GWSS), which feeds on a broad range of plants (Purcell and Saunders 1999, Wistrom and Purcell 2005). It has been shown that a single strain is able to infect and produce disease in different hosts (Almeida and Purcell 2003, Costa et al. 2004, Chen et al. 2005) and that the bacterium is able to infect and colonize a wide range of alternate hosts without causing disease (Purcell and Saunders 1999, Costa et al. 2004, Wistrom and Purcell 2005). The broad host range of *Xylella* and its ability to hide inside unaffected hosts make it a constant menace for agricultural crops. Very little was known previously about the fate of *Xylella* in ornamentals, the strains they are harboring and their ability to cause disease losses in plants of agronomic importance. To find some information in this subject, we characterized strains isolated from ornamental hosts using RAPD and 16S-23S rDNA Intergenic Spacer Region (ISR) analyses. Our results identified new hosts for the *Xf* subspecies *fastidiosa*, *Xf* subspecies *multiplex*, *Xf* subspecies *sandyi*, and for the MLS type strains.

Our knowledge of host range of *Xf* strains is still restricted. Some strains appear to have a very limited host range and some have a broader range of hosts, but for most strains the possible host-strain combination has not been extensively tested. Symptomless hosts harboring bacteria are potential inoculum sources for vectors to acquire *Xf* and spread the disease into economically important plants.

OBJECTIVES
1. Characterize genetically the strains of pathogen in landscape plant species.
2. Confirm pathogenic infection through inoculation studies with specific isolates.
3. Test the ability of new strains to infect agricultural crops including grape, and almond.

RESULTS
Objective 1. Characterization of Xf strains by analysis of the 16S-23S rDNA intergenic spacer region (ISR) Excluding Mulberry-VA (GeneBak accession number AY196794), a DNA region of 513 bases containing the 16S-23S rDNA ISR was amplified, cloned and sequenced from all the *Xf* strains listed in Table 1.

Table 1. Strains used in this study and their host sources

Scientific name	Common name	Isolate designation	County of CA or state from which strain was isolated	Reference or source
Cercis occidentalis	Western redbud	Cercis050	Riverside	This study
Cercis occidentalis	Western redbud	Cercis049	Riverside	This study
Ginkgo biloba	Maidenhair tree	GB100	Riverside	This study
Hemerocallis sp.	Daylily	HEM034	Riverside	This study
Jacaranda mimosifolia	Jacaranda	JM028	Riverside	This study
Lagerstroemia indica	Crape Myrtle	LI021	San Bernardino	This study
Liquidambar styraciflua	Sweet gum	LS020	San Bernadino	This study
Liquidambar styraciflua	Sweet gum	LS022	San Bernadino	This study
Liquidambar styraciflua	Sweet gum	LS043	San Bernadino	This study
Magnolia grandiflora	Magnolia	MG038	San Bernadino	This study
Morus alba	White mulberry	MLS063	San Bernardino	This study
Morus alba	White mulberry	MLS059	San Bernardino	This study
Morus alba	White mulberry	MLS012	San Bernadino	This study
Morus alba	White mulberry	MLS024	Riverside	This study
Morus alba	White mulberry	Mulberry-VA	Virginia	(Huang and Sherald 2004)
Nandina domestica	Heavenly bamboo	NI065	San Bernardino	This study
Nerium oleander	Oleander	OLS012	Riverside	This study
Nerium oleander	Oleander	OLS028	Riverside	This study
Nerium oleander	Oleander	Ann1	Palm Springs	(Hendson et al. 2001)
Nerium oleander	Oleander	TR2	Orange	A. Purcell
Olea europaea	Olive	G12	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC057	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC086	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC045	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC052	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC053	Riverside	This study
Prunus cerasifera	Purple leafed-plum	PC076	San Bernardino	This study
Prunus cerasifera	Purple leafed-plum	PC012	Riverside	This study
Prunus domestica	Plum	Plum 2#4	Georgia	(Hendson et al. 2001)
Prunus dulcis	Almond	ALS2	San Joaquin	(Hendson et al. 2001)
Prunus dulcis	Almond	ALS1	San Joaquin	(Hendson et al. 2001)
Prunus dulcis	Almond	Tulare-ALS	Tulare	(Hendson et al. 2001)
Prunus dulcis	Almond	Fresno	Fresno	(Almeida and Purcell 2003)
Prunus dulcis	Almond	ALS6	San Joaquin	(Hendson et al. 2001)
Prunus dulcis	Almond	276	Temecula	(Costa et al. 2004)
Prunus dulcis	Almond	Dixon	Solano	(Hendson et al. 2001)
Prunus dulcis	Almond	Butte	Butte	(Hendson et al. 2001)
Prunus dulcis	Almond	Glenn	Glenn	(Almeida and Purcell 2003)
Prunus dulcis	Almond	ALS035	San Bernardino, CA	This study
Prunus dulcis	Almond	ALS036	San Bernardino, CA	This study
Prunus persica	Peach	Peach018	San Bernardino, CA	This study
Prunus persica	Peach	Peach.019	San Bernardino, CA	This study
Prunus persica	Peach	5R1	Georgia	(Hendson et al. 2001)
Quercus sp.	Oak	92-10	Florida	(Hendson et al. 2001)
Quercus sp.	Oak	92-3	Florida	(Hendson et al. 2001)
Spartium junceum	Spanish broom	N10	Temecula, CA	(Costa et al. 2004)
Spartium junceum	Spanish broom	SB-R	Riverside, CA	This study
Unknown	Bush	UK005	Riverside, CA	This study
Vitis vinifera	Grape	95-2	Florida	(Hendson et al. 2001)
Vitis vinifera	Grape	SJV1	Florida	A. Purcell
Vitis vinifera	Grape	Florida	Florida	Cooksey's lab collection
Vitis vinifera	Grape	STL	Napa. CA	(Hendson et al. 2001)
Vitis vinifera	Grape	Preston	Sonoma, CA	(Hendson et al. 2001)

A phylogenetic tree was constructed using those sequence data (Figure 1). The analysis of the tree revealed five groupings of strains. The first clade included two strains 92-10 and 92-3 isolated from oak. The second clade included strains from purple-leafed plum (PC086, PC045, PCAcl12, PC057, PC052, PC076 and PC053), olive, almond (Dixon, Butte, ALS6,

ALS2, 276 and Glenn), sweet gum (LQ020, LQ022, LQ043), peach (5R1), plum (Plum2#4), western redbud (cercis049), maidenhair tree and the UK005 strain. The third clade included mulberry strains (MLS024, MLS063, MLS012, MLS059, and Mulberry-VA) as well as the heavenly bamboo strain (NI065). The fourth clade included strains isolated from grape (Florida, STL, Preston, 92-5 and SJV1), almond (ALS1, Tulare, ALS035, ALS036 and Fresno), peach (Peach018 and Peach019), spanish broom (N10 and SB-R) and one western redbud strain (cercis050). And the last, included strains isolated from oleander (OLS028, OLS012, Ann1 and TR2), daylily (HEM034), magnolia (MG038) and Jacaranda (JM028).

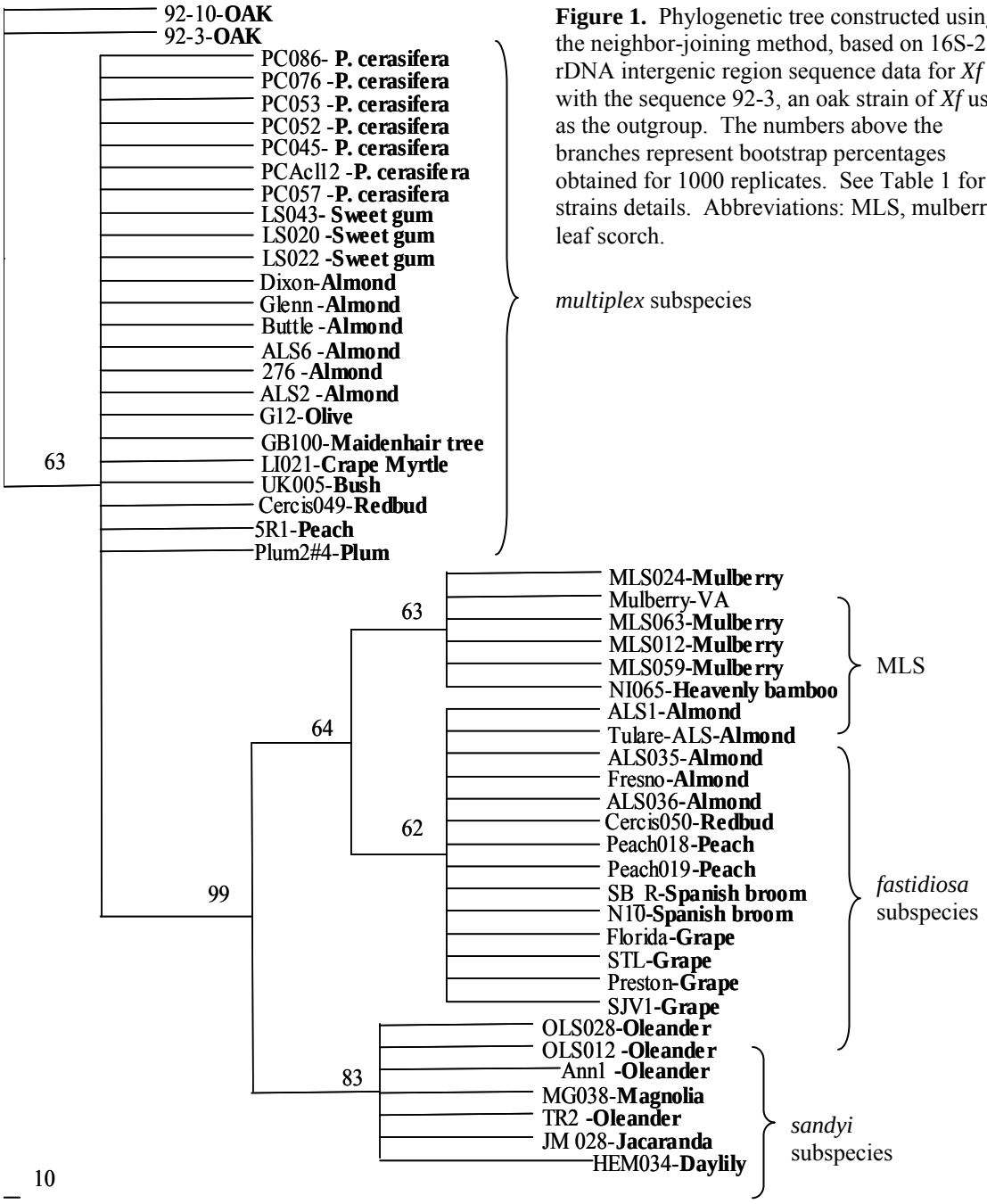

Figure 1. Phylogenetic tree constructed using the neighbor-joining method, based on 16S-23S rDNA intergenic region sequence data for *Xf* with the sequence 92-3, an oak strain of *Xf* used as the outgroup. The numbers above the branches represent bootstrap percentages obtained for 1000 replicates. See Table 1 for strains details. Abbreviations: MLS, mulberry leaf scorch.

Characterization of *Xf* strains by RAPD analysis

The PCR products were compared, and a distance matrix was constructed. Phylogenetic relationships based on 80 scorable RAPD characters were analyzed by the UPGMA method (Figure 2). Analysis of the phylogenetic tree revealed six main clades. The first clade comprised strains isolated from plum and peach (plum2#4 and 5R1). All members of the multiplex

subspecies clustered into the second clade. However, this clade seems to be subdivided into smaller ones. The most noticeable were: I) PC076, 276, AlS6, GB100 and LI021; II) LS043, LS022, LS020; III) ALS2 and Glenn; and IV) Butte and Dixon. The third clade included strains isolated from oak (92-10 and 92-3). The fourth included all the strains isolated from oleander (OLS028, OLS012, and Ann1), as well as the strains isolated from daylily (HEM034), Jacaranda (JM028) and Magnolia (MG038). The fifth included strains isolated from grape (92-5, Florida, STL, Preston and SJV1), almond (ALS036, ALS035, ALS1 and Fresno), peach (Peach019 and Peach018), spanish broom (N10 and SB-R) and redbud (Cercis50). The last clade was integrated from strains isolated from mulberry (MLS024, MLS063, MLS012 and MLS059) and heavenly bamboo (NI065).

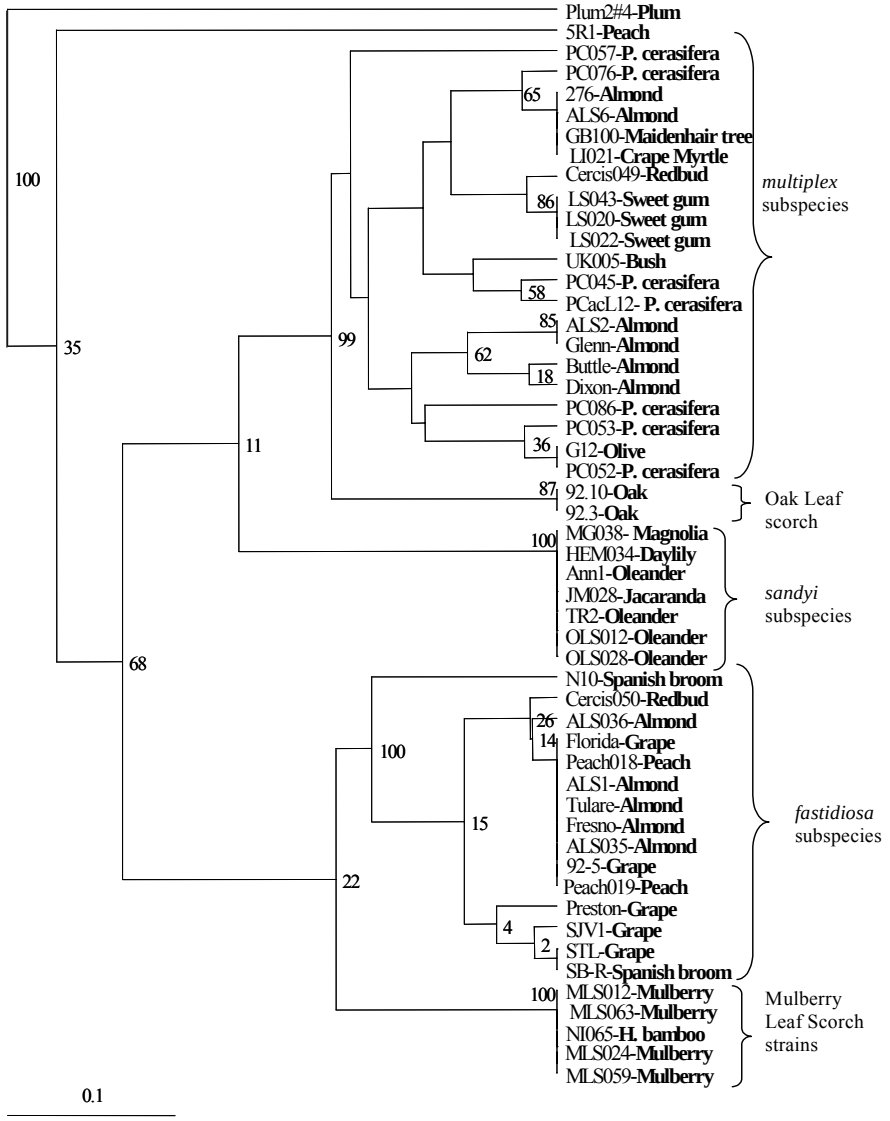

Figure 2. Phylogenetic tree constructed using the neighbor-joining method, based on RAPD data for *Xf* with the sequence 92-3, an oak strain of *Xf* used as the out-group. The numbers above the branches represent bootstrap percentages obtained from 1000 replicates. See Table 1 for strain details.

Objectives 2 and 3: Mechanical inoculation of novel strains into ornamental hosts, 12-month evaluation
In 2004, selected isolates of *Xf* from landscape host plants *Ginkgo biloba*, *Liquidambar styraciflua*, *Morus alba*, *Nandina domestica*, *Olea europea*, *Prunus cerasfiera* and *Prunus dulcis* were inoculated into their respective hosts of origin, grape and oleander to confirm pathogenicty and to see if any were also known PD or OLS types. Plants were tested at 3 month intervals by ELISA and for plants testing positive (at least two-times background), direct culturing of the pathogen was attempted (Table 2).

Table 2. Inoculation results for glasshouse tests (3, 6, 9 and 12--month evaluation)

Test Strain	Inoculum Source Plant	Test Plant	# Inoculated	ELISA Positive Samples								Recovered Strain ID
				3-month		6-month		9-month		12-month		
				+	tested	+	tested	+	tested	+	tested	
276	Almond	Almond	25	7	23	4	23	1	24	0	24	ALS[b]
276	Almond	Grape	25	3	21	0	25	0	25	1	24	
276	Almond	Oleander	15	2	15	0	15	0	15	2	15	
GB100	Ginkgo	Ginkgo	25	2	25	1	24	4	24	6	24	
GB100	Ginkgo	Grape	25	1	24	0	24	0	24	0	14	
GrapeA05	Grape	Grape	25	10	10	25	25	2	2[a]	0	0[a]	PD[b]
LI021	Crapemyrtle	Crapemyrtle	25	8	24	6	25	3	25	2	25	
LI021	Crapemyrtle	Grape	25	4	25	1	24	0	20	1	20	
LI021	Crapemyrtle	Oleander	15	1	14	4	14	2	12	1	13	OLS[b]
LiquidambarUI12	Liquidambar	Grape	25	2	22	0	24	0	24	0	24	
LiquidambarUI12	Liquidambar	Liquidambar	25	0	24	0	25	2	25	0	25	
LiquidambarUI12	Liquidambar	Oleander	15	1	14	1	13	0	10	0	10	
NI065	Nandina	Grape	25	3	23	2	23	0	19	0	15	
NI065	Nandina	Nandina	25	2	25	0	25	2	25	0	25	
NI065	Nandina	Oleander	15	0	14	0	14	0	15	0	14	
G12	Olive	Grape	25	3	17	1	25	0	24	0	20	
G12	Olive	Oleander	10	1	9	0	10	0	10	0	10	
G12	Olive	Olive	25	1	25	4	25	23	24	21	24	
PC076	Plum	Grape	25	3	24	2	25	0	18	0	15	
PC076	Plum	Oleander	10	0	10	0	10	0	10	0	10	
PC076	Plum	Plum	25	1	25	0	25	1	25	1	25	
Riverside3	Oleander	Oleander	25	9	10	24	25	21	21	10[a]	10[a]	OLS[b]
Control	PBS buffer	Almond	10	0	2	0	3	0	3	1	3	
Control	PBS buffer	Crape Myrtle	10	0	4	2	9	1	10	2	10	
Control	PBS buffer	Gingko	15	0	4	0	10	0	8	0	8	
Control	PBS buffer	Grape	15	2	10	0	10	0	10	0	10	
Control	PBS buffer	Liquid amber	10	0	4	0	10	0	10	0	10	
Control	PBS buffer	Mulberry	10	4	10	0	10	0	10	0	10	
Control	PBS buffer	Nandina	10	0	4	0	10	0	10	0	10	
Control	PBS buffer	Oleander	10	0	2	1	10	4	9	0	9	
Control	PBS buffer	Olive	10	1	4	3	10	9	10	9	10	
Control	PBS buffer	Plum	10	0	4	0	10	0	10	0	10	

[a] after 9- and 12-month incubation periods after infection, the majority of the test plants were dead from *Xf* infection

[b] when *Xf* was successfully cultured from plants testing positive from ELISA, isolates were characterized by 16S-23S rDNA sequencing into known strain-types

Generally, the mechanical inoculation technique described by Hill and Purcell (1995) worked extremely well on grape, oleander and almond, but had mixed results for the other isolates and host species tested. With the exception of mulberry (discussed below), none of the new isolates from liquidambar, crape myrtle, gingko, nandina, olive or plum were able to cause systemic infections from which the bacteria could be isolated. Interestingly, olive plants tested consistently positive by ELISA for *Xf*, even for the PBS-inoculated plants, indicating perhaps that for this plant type, there may be a high number of false positives or that the plants may have been already infected before testing, with a long latent period that prevented us from detecting the presence of the bacteria in initial experiments. Almond leaf scorch (ALS), PD, and OLS strains were easily recovered from inoculated almond, grape and oleander (respectively) indicating that the general inoculation protocol was successful, yet for liquidambar, crape myrtle, gingko, nandina, olive or plum, we will continue to test at 3-month intervals. For isolate LI021, from crape myrtle, it was found to cause symptoms on oleander and further characterization indicated it was of OLS-type, suggesting that OLS-strains are capable of using crape myrtle as an alternate host, although we have not yet completed Koch's postulates for this isolate in crape myrtle.

Mechanical inoculations of putative (MLS) strains intro mulberry, grape, and oleander plants

For inoculation proposes, three strains were chosen; Morus063, isolated from a mulberry with MLS symptoms; A05, isolated from a PD-affected grapevine in Temecula Valley area (Costa et al. 2004) and Riverside3 isolated from an oleander-affected plant in Riverside, CA. A 7 days old culture of *Xf* was grown on PW medium and resuspended to get a turbid solution in phosphate-buffered saline (PBS) solution. Plants were inoculated by pipetting a small drop of the bacterial solution onto a stem and probing the drop with a #1 insect pin until observed uptake from the drop. Morus063 strain was inoculated into 25 mulberries, 25 grapevines and 15 oleanders plants. A05 and Riverside3 strains were needle-inoculated into 25 grapevine or oleander plants respectively and served as positive controls. Inoculations with PBS served as negative controls. After three months of the inoculation, only inoculated mulberries exhibited leaf scorch symptoms and bacteria were recovered from most of the inoculated mulberries but not from oleander or grape (Table 3).

Table 3. Evaluation of mulberry, grape and oleander plants inoculated with *Xylella fastidiosa* isolated from mulberry plant.

Xf strain/ subspecies	Inoculum source plant	Tested Plant	Number inoculated	No. of positive plants [a]			No. of plants with[b]		
				ELISA	Culture	PCR	PD	OLS	MLS
Morus059*	Mulberry	Mulberry	25	21	21	21	0	0	21
Morus059*	Mulberry	Grape	25	1	0	0	0	0	0
Morus059*	Mulberry	Oleander	15	1	0	0	0	0	0
AO5	Grape	Grape	25	25	16	16	25	0	0
Riverside3	Oleander	Oleander	25	25	16	16	0	25	0
Control	PBS Buffer	Mulberry	10	0	0	0	0	0	0
Control	PBS Buffer	Grape	10	0	0	0	0	0	0
Control	PBS Buffer	Oleander	10	0	0	0	0	0	0

* Putative MLS strains

[a] Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial enzyme-linked inmunosorbent assay (ELISA) kits, media culturing methods, and RST31-33 primers for polymerase chain reaction (PCR) analysis (Minsavage et al. 1994)

[b] Number of plants exhibiting symptoms out of total of inoculated plants. Abbreviations: PD, Pierce's disease; OLS, oleander leaf scorch; MLS, mulberry leaf scorch

Mechanical inoculations of putative Xf subspecies sandyi strains intro grape and oleander plants

Since the strains from jacaranda, magnolia and daylily (JM028, MG038 and HM034) always clustered with OLS strains, our hypothesis was that they should be able to infect oleander but not grape (Purcell et al. 1999). To probe this hypothesis, they were inoculated into oleanders and grape plants. We also inoculated the OLS strain Riverside3 and the PD strain A05 as positive controls. All oleanders inoculated with the strains Riverside3, JM028, MG038 and HM034 showed symptoms after two months of inoculation (Table 4). On the other hand, only the grapes inoculated with the A05 strain presented disease symptoms. At that point ELISA gave strong positive results from positive plants (≤ 2X the positive control) and bacteria were recovered from all infected plants. Colonies did not differ morphologically (light microscope), serologically (ELISA), or by growth in PD3 medium with the original strains used as inoculum. For confirmation of identity, reisolated bacteria were tested with primers RST31 and RST33, and all of them produced a band of 733 bp as expected (data not shown). Strains JM028, MG038, HM034 and Riverside3 inoculated into grapes were unable to produce disease symptoms or give ELISA-positive results, and the same was true when the A05 strain was inoculated into oleanders. No control plants gave positive ELISA or PCR reactions, and *Xf* was never isolated from any control plants.

Table 4. Evaluation of grape and oleander plants inoculated with *Xf* isolated from oleander, grape, jacaranda, magnolia, and daylily.

Xf strain/ subspecies	Inoculum source plant	Tested Plant	Number inoculated	No. of positive plants [a]			No. of plants with[b]	
				ELISA	Culture	PCR	PD	OLS
A05/*fastidiosa*	Grape	Oleander	15	0	0	0	0	0
A05/*fastidiosa*	Grape	Grape	10	10	10	10	10	0
Riverside3/*sandyi*	Oleander	Oleander	15	15	15	15	0	15
Riverside3/*sandyi*	Oleander	Grape	10	0	0	0	0	0
JM028/*sandyi**	Jacaranda	Oleander	15	15	15	15	0	15
JM028/*sandyi**	Jacaranda	Grape	10	0	0	0	0	0
MG038/*sandyi**	Magnolia	Oleander	15	15	15	15	0	15
MG038/*sandyi**	Magnolia	Grape	10	0	0	0	0	0
HEM034/*sandyi**	Daylily	Oleander	15	15	15	15	0	15
HM034/*sandyi**	Daylily	Grape	10	0	0	0	0	0
Control	PBS Buffer	Oleander	15	0	0	0	0	0
Control	PBS Buffer	Grape	10	0	0	0	0	0

* Putative members of the *sandyi* subspecies

[a] Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial enzyme-linked inmunosorbent assay (ELISA) kits, media culturing methods, and RST31-33 primers for polymerase chain reaction (PCR) analysis (Minsavage et al. 1994)

[b] Number of plants exhibiting symptoms out of total of inoculated plants. Abbreviations: PD, Pierce's disease; OLS, oleander leaf scorch.

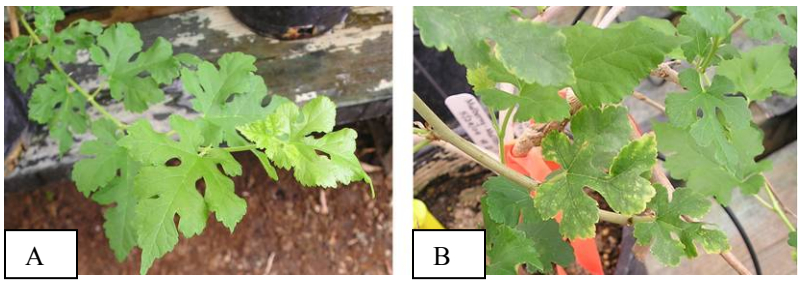

Figure 3. A. Mulberries inoculated with PBS buffer. **B.** Mulberries inoculated strain Morus059 of *Xylella fastidiosa.*

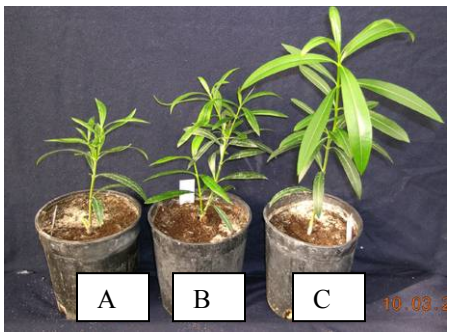

Figure 4. A. Oleander infected with a strain isolated from magnolia. **B**. oleander infected with a strain isolated from jacaranda. **C.** Oleander inoculated with PBS buffer.

CONCLUSIONS

The methods used here indicated that ornamental hosts are able to harbor different strains of *Xylella.* Our findings revealed new hosts for the subspecies *fastidiosa* (peach and redbud) *multiplex* (crape myrtle, maidenhair tree, olive, liquidambar, purple-leaf plum and redbud), *sandyi* (daylily, magnolia and jacaranda) and MLS (heavenly bamboo). We have the first report of MLS in California, expanding the number of strains present in this state, and found evidences that MLS strains are likely non-pathogenic to grape or oleander. We also showed that strains isolated from jacaranda, daylily, and magnolia are able to produce disease in oleander but not in grape. More studies are underway to fulfill the Koch's postulates of the strains characterized here as well as to reveal their fate on grape, almond and oleander plants. Since knowledge of the source of inoculum is essential in developing effective disease management strategies, additional studies must be done to elucidate the full host range of *Xf*. For now, the results of this work increased our information about the hosts range spectrum of the pathogen and their latent risk in ornamentals.

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