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

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ABSTRACT

To document the incidence of *Xylella. fastidiosa* (*Xf*) in landscape ornamental hosts, in 2003 and 2004 a survey of plants showing symptoms of scorch or dieback in urban locations in southern California was done. A total of 1,670 samples, representing 161 plant species were taken and analyzed at five locations: Fillmore, San Diego, Redlands, Riverside, and Tustin. From the total, 35% of plants tested (591), representing 102 identified species, gave positive results by *Xf*-specific enzyme linked immunosorbent assay (ELISA). Isolation of bacteria from ELISA-positive plants provided 39 isolates form 14 non-previously reported as *Xf*-hosts species: almond, crapemyrtle, daylily, gingko, jacaranda, grapevine (both *labrusca* and *vinifera*), magnolia, mulberry, oleander, cherry, purple-leaved plum, heavenly bamboo, olive, sweetgum, plum and western redbud. Random amplified polymorphic (RAPD)- polymerase chain reaction (PCR) and sequence analysis of the 16S-23S rDNA intergenic spacer regions (ISR) was used to genetically characterize the strains. Strains isolated from daylily, jacaranda and magnolia grouped with members of *Xf* subsp. *sandyi*. Some strains isolated from cherry, and one strain isolated from western redbud, grouped with *Xf* subsp. *fastidiosa* members and strains isolated from purple-leaved plum, olive, peach, plum, sweet gum, maidenhair tree, crape myrtle and another western redbud strain, clustered with members of the *Xf* subsp *multiplex*. All strains isolated from mulberry and one from heavenly bamboo formed a cluster that has not yet been defined as a subspecies. Koch's postulates were successfully tested for the strains isolated from sweet gum, purple-leaved plum, western redbud and mulberry. Cross-infectivity of those strains to grapevine, almond and oleander was also tested. This information contributed to better understand the role of these different strains in causing disease on plants in urban landscapes. However, the impact of *Xf* infections in landscape hosts and the diversity of strains still are far from being fully understood.

INTRODUCTION

Xylella fastidiosa (Xf) is a xylem-limited, insect-vectored, plant pathogen that can cause severe damage to a wide range of host plants including grape, almond and oleander. In addition to causing Pierce's disease (PD), Almond leaf scorch (ALS) and Oleander leaf scorch (OLS), *Xf* has been implicated in causing bacterial leaf scorch in a number of ornamentals and trees in the mid-Atlantic and southeastern U.S. Affected plants include oak, sycamore, elm, mulberry, maple and other shade trees in the landscape and urban forests (Sherald and Kostka 1992, McGovern and Hopkins 1994, McElrone et al. 1999). Multiple strains of *Xf* with different host ranges have been identified (Chen et al. 1992, da Costa et al. 2000, Hendson et al. 2001), but little is known about the diversity of these populations in the urban landscape and their ability to cause loss in plants of horticultural and agronomic importance. The arrival of a highly efficient vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*) in California has resulted in the rapid spread of this pathogen amongst both agricultural crops and landscape plants. Both PD and OLS are present in this area and recently, disease symptoms have been associated with the presence of partially characterized and potentially new strains of *Xf* in a number of landscape ornamentals including olive, liquidambar and purple-leaved plum. 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 isolate and characterized strains from ornamental hosts. Our studies identified new hosts for the *Xf* subspecies *fastidiosa*, *Xf* subspecies *multiplex*, *Xf* subspecies *sandyi*, and for the mulberry leaf scorch type strains. 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.

OBJECTIVES

1. Identification of landscape host species infected with *Xf*.
2. Genetic characterization of the strains of *Xf* isolated from landscape plant species.
3. Confirmation of pathogenic infection through inoculation studies with specific isolates.
4. Test ability of new strains to infect established host plants of *Xf* including grape, oleander and almond.

RESULTS

Objective 1. Identification of landscape host species infected with *Xf*.

In 2003 and 2004, a survey that expanded an area of approximately 15,000 km^2 in size was done. Typically, a single sampling run covered an approximate area of 10 km^2. Five urban locations were included: Fillmore (Ventura County), Redlands (San Bernardino County), Riverside (Riverside County), San Diego (San Diego County) and Tustin (Orange County). Starting from a central location in these cities, all plants with typical *Xf* symptoms (scorch, stunt, dieback, wilt, etc) were sampled. Samples were processed for ELISA using the PathoScreen Kit (Agdia Inc. Elkhart, IN). From plants testing ELISA positive attempts to isolate *Xf* were done in two media PD3 and PW and the identity of putative *Xf* colonies was confirmed by PCR using the RST31 and RST33 primer pair (Minsavage et al. 1994). Isolation of bacteria from ELISA-positive plants rendered 39 isolates obtained from almond, crapemyrtle, daylily, gingko, jacaranda, grapevine (both *labrusca* and *vinifera*), magnolia, mulberry, oleander, cherry, purple-leaved plum, heavenly bamboo, olive, sweetgum, plum and western redbud, 14 non-previously reported as *Xf*-hosts species in southern California (Tables 1 and 2)

Table 1. Strains isolated from novel landscape hosts in southern California and their genetic identity.

Host scientific name	**Host common name**	**Isolate designation**	**County of CA from which strain was isolated**	**Genetic Identification**
Cercis occidentalis	Western redbud	Cercis050	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Cercis occidentalis	Western redbud	Cercis001	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Cercis occidentalis	Western redbud	Cercis049	Riverside	*X. fastidiosa* subsp. *multiplex*
Ginkgo biloba	Maidenhair tree	GB100	Riverside	*X. fastidiosa* subsp. *multiplex*
Hemerocallis sp.	Daylily	HEM034	Riverside	*X. fastidiosa* subsp. *sandyi*
Jacaranda mimosifolia	Jacaranda	JM028	Riverside	*X. fastidiosa* subsp. *sandyi*
Lagerstroemia indica	Crape Myrtle	LI021	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS020	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS022	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS043	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Magnolia grandiflora	Magnolia	MG038	San Bernadino	*X. fastidiosa* subsp. *sandyi*
Magnolia grandiflora	Magnolia	MG038	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Morus alba	White mulberry	MLS063	San Bernardino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS059	San Bernardino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS012	San Bernadino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS024	Riverside	*X. fastidiosa* subsp. *?*
Nandina domestica	Heavenly bamboo	NI065	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Olea europaea L.	Olive	G12	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC057	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC086	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC045	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC052	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC053	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC076	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PCAcl12	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus spp	Cherry	cherry018	San Bernardino	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	cherry019	San Bernardino	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	23Bing	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	37Rainier	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	17Bing	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	24Tulare	Riverside	*X. fastidiosa* subsp. *multiplex*
Unknown	Bush	UK005	Riverside	*X. fastidiosa* subsp. *multiplex*

Note: strain ID performed by sequence analysis of 16S-23S rDNA ISR sequences and RAPD-DNA (Hernandez-Martinez et al. 2006a, Hernandez-Martinez et al. 2006b)

Objective 2. Genetic characterization of the strains of *Xf* isolated from landscape plant species.
RAPD-PCR and sequence analysis of the 16S-23S rDNA ISR was used to genetically characterize the strains. Strains isolated from daylily, jacaranda and magnolia grouped with members of *Xf* subsp. *sandyi*. One strain isolated from western redbud, and one strain isolated from cherry grouped with *Xf* subsp. *fastidiosa* members. Strains isolated from purple-leaved plum, olive, peach, plum, sweet gum, maidenhair tree, crape myrtle and another western redbud strain, and three cherry strains clustered with members of the *Xf* subsp *multiplex*. Thus, the strains showed considerable diversity but belonged to previously described groups and subspecies and some hosts also can be infected with more than one subspecies, as shown here for the cherry and redbud strains (Tables 1 and 2).

Table 2. Current status of the identification of *Xf* strains isolated from landscape hosts.

Plant species	Common name of the host	Genetic Identification	Host symptoms	Current status
Ginkgo biloba	Maidenhair Tree or Ginkgo	*Xf* subsp. *multiplex*		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing.
Lagerstroemia indica	Crape Myrtle	*Xf* subsp. *multiplex*		
Olea europaea	Olive	*Xf* subsp. *multiplex*		
Prunus cerasifera	Cherry plum	*Xf* subsp. *multiplex*		Koch's postulates completed. We called the disease purple-leaved scorch. A strain PC045, infected almond but not grape or oleander.
Liquidambar styraciflua	Sweet gum	*Xf* subsp. *multiplex*		Koch's postulates completed. We called the disease sweet gum dieback. A strain LS022, did not infect almond, grape or oleander.
Cercis occidentalis	Western Redbud	Cercis049 strain of *Xf* subsp. *multiplex* Cercis001 strain *Xf* subsp. *fastidiosa*		Koch's postulates completed for two genotypically different strains. Cercis049 does not infect grape, almond or oleander and Cercis001 infected almond and grape but not oleander.
Morus alba	White Mulberry	Mulberry leaf scorch (maybe a new subspecies)		Koch's postulates completed; MLS definitely found in California. It does not infect oleander, grape or almond (Hernandez-Martinez et al. 2006b).
Nandina domestica	Heavenly Bamboo	Mulberry leaf scorch (maybe a new subspecies)		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing.

Table 2. (continued).

Plant species	Common name of the host	Genetic Identification	Host symptoms	Current status
Hemerocallis	Day Lily	*Xf* subsp. *sandyi*		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing. Isolates from Day Lily, Jacaranda and Magnolia caused scorch symptoms when inoculated into Oleander test plants.
Jacaranda mimosifolia	Jacaranda	*Xf* subsp. *sandyi*		
Magnolia grandiflora	Southern Magnolia	*Xf* subsp. *sandyi*		
		Xf subsp. *multiplex*		
Nerium oleander	Oleander	*Xf* subsp. *sandyi*		Previously established as a host.
Prunus spp.	Cherry	*Xf* subsp. *fastidiosa*		Koch's postulates not completed using mechanical inoculation. But two strains (17Bing and cherry018) diseased grape and almond plants. Another strain (24Tulare) produce mild disease symptoms in almond but do not disease grape.
		Xf subsp. *multiplex*		
Prunus	Plum	*Xf* subsp. *multiplex*		Koch's postulates not completed using mechanical inoculation.

Note: strain ID performed by sequence analysis of 16S-23S rDNA ISR sequences and RAPD-DNA (Hernandez-Martinez et al. 2006a, Hernandez-Martinez et al. 2006b).

Objectives 2 and 3. Mechanical incoculation of novel strains into ornamental hosts.
In 2005, selected isolates of *Xf* from landscape host plants *Liquidambar styraciflua*, *Nandina domestica*, *Olea europea*, *Prunus cerasfiera, lagerstroemia indica* and *Prunus sp.* were inoculated into their respective hosts of origin, grape, almond and oleander to confirm pathogenicty and to see if any were also known PD, ALS or OLS genotypes. Plants were tested at three month intervals by ELISA and for plants testing positive (at least two-times background), direct culturing of the pathogen was attempted. Mechanical inoculation technique (Hill and Purcell 1995) worked on grape, oleander and almond, as well as for the new hosts, liquidambar, mulberry, redbud, and purple-leaved plum. Bacteria were able to cause systemic infections and produce disease symptoms.

Mechanical inoculations of a strain isolated from liquidambar.
Xf was isolated from trees showing progressive dieback and decline in southern California. Three isolates were recovered from trees testing positive by ELISA and confirmed as *Xf* using the specific PCR primer set RST31-33. Isolated strains were further characterized as members of the *Xf* subsp. *multiplex* by sequencing of their 16S-23S rDNA ISR and random amplified polymorphic DNA-PCR analysis. The pathogenicity of one strain, LS022, was confirmed by inoculating glasshouse-grown sweetgum plants. Nine months after inoculation, the pathogen was recovered from five of 25 inoculated plants showing dieback symptoms. Inoculation of grapevines, oleanders and almonds with the LS022 strain or inoculation of sweetgum plants with PD, OLS, or ALS-strains did not result in any disease or recovery of the pathogen up to one year later.

Inoculation results are shown in Table 3 and the aspect of diseased plants are seen in Figure 1. These experiments completed Koch's postulates for this disease and indicate that this strain lacks cross-infectivity to grapevine, almond or oleander.

Table 3. Evaluation of sweet gum, almond, grape and oleander plants inoculated with *Xf* isolated from sweet gum.

Xf strain/	Inoculum source plant	Tested plant	Number inoculated	No. of plants positive (a)		
				ELISA	Culture	PCR
LS022	Sweetgum	Sweetgum	25	7	3	3
LS022	Sweetgum	Almond	15	0	0	0
LS022	Sweetgum	Grape	15	0	0	0
LS022	Sweetgum	Oleander	15	0	0	0
A05	Grape	Grape	15	15	15	15
Riverside3	Oleander	Oleander	15	15	15	15
276	Almond	Almond	15	10	10	10
A05	Grape	Sweetgum	10	0	0	0
276	Almond	Sweetgum	10	0	0	0
Riverside3	Oleander	Sweetgum	10	0	0	0

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

Figure 1. Sweet gum plants mechanically inoculated with *Xf* strain LS022 showing chlorosis and tip dieback (lefft) as compared to a healthy non-diseased plant (right).

Mechanical inoculations of *Xf* strain PC045 isolated from Purple leaved plum (*Prunus cerasifera*) into the original host grape, oleander and oleander plants.

The pathogenicity of one strain, PC045 was tested inoculating glasshouse grown purple-leaved plum, oleander, grapevine and almond plants. Three months after inoculation, purple-leaved plum and almonds started showing typical leaf scorch symptoms and the pathogen was recovered from all inoculated plants. Inoculation of grapevine and oleander plants with the same strain did not result in any disease or recovery of the pathogen up to six months later. This indicates that this strain was cross-infective to almond but not to oleander or grape. Inoculation results are shown in Table 4 and the aspect of diseased plants are seen in Figure 2. The fulfillment of Koch's postulates established that *Xf* caused purple-leaved plum leaf scorch increasing the host range for this bacterium.

Table 4. Evaluation of purple-leaved plum, almond, grape and oleander plants inoculated with *Xf* isolated from purple-leaved plum.

Xf strain	Inoculum source plant	Tested Plant	Number inoculated	No. of plants positive (a)			No of sick plants
				ELISA	Culture	PCR	
PC045	Purple leafed-plum	Purple leafed-plum	15	15	14	14	15
PC045	Purple leafed-plum	Almond	15	15	15	15	15
PC045	Purple leafed-plum	Grape	15	0	0	0	0
PC045	Purple leafed-plum	Oleander	15	0	0	0	0
STL	Grape	Grape	15	15	15	0	15
Riverside3	Oleander	Oleander	15	15	15	0	15

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

Mechanical inoculations two strains: cercis049 and cercis001 of *Xf* isolated from redbud (*Cercis occidentallis*).

The pathogenicity of two strains isolated from redbud was tested inoculating glasshouse redbud, oleander, grapevine and almond plants. Six months after inoculation, plants are starting to show leaf scorch and stunting symptoms and the pathogen has been recovered from few plants. The strain cercis001 inoculated in grapevines and almonds produced typical PD and ALS symptoms respectively and bacteria have been recovered from diseased plants. The strain cercis049 did not produce disease symptoms in grape but it seems to infect almonds producing mild symptoms. Inoculation of oleander plants with both strains did not result in any disease or recovery of the pathogen up to six months later (Table 5, Figure 3). This indicates that the strain cercis001 is a PD strain or a member of the *Xf* subsp. *fastidiosa*, able to infect almond and grape. A PD strain, STL was able to disease redbud plants, which indicated cross-infectivity of the strains. The infectivity of the strain cercis049 remains under evaluation.

Figure 2. Purple-leafed plum (left) and almond (right) plants mechanically inoculated with *Xf* strain PC045 showing leaf scorch as compared to a healthy non-diseased plant.

Table 5. Evaluation of grape, almond and oleander plants inoculated with *Xf* isolated from Redbud (*Cercis occidentallis*).

***Xf* strain/ subspecies**	**Inoculum source plant**	**Tested plant**	**Number inoculated**	**No. of plants positive (a)**	
				ELISA	**Culture***
Cercis049	*C. occidentalis*	Red bud	20	4	0
Cercis001	*C. occidentalis*	Red bud	20	3	3
Cercis049	*C. occidentalis*	Oleander	10	0	0
Cercis001	*C. occidentalis*	Oleander	10	0	0
Cercis049	*C. occidentalis*	Almond	7	4	0
Cercis001	*C. occidentalis*	Almond	10	4	3
Cercis049	*C. occidentalis*	Grape	10	0	0
Cercis001	*C. occidentalis*	Grape	15	11	3
STL	Grape	Red bud	10	6	0
Buffer		Red bud	10	0	0

(a) Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial ELISA kits, media culturing methods.

CONCLUSIONS

Ornamental hosts harbor different strains of *Xf.* Members of four groups of *Xf* were isolated. *Xf* subsp. *fastidiosa* from cherry and western redbud; *Xf* subsp *multiplex* from crape myrtle, maidenhair tree, olive, sweetgum, purple-leaved plum and western redbud; *Xf* subsp *sandyi* from daylily, magnolia and jacaranda; and the mulberry leaf scorch group from heavenly bamboo and mulberry. We have the first report of Mulberry leaf scorch (MLS) in California (Hernandez-Martinez et al. 2006b), expanding the number of strains present in this state, we also found evidences that MLS strains are likely non-pathogenic to grape or oleander. We showed that strains isolated from jacaranda, daylily, and magnolia are able to produce disease in oleander but not in grape. We tested the Koch's postulates for purple-leaved plum and found that a strain (PC045), cross-infected almond but not grape or oleander. On the other hand, strains isolated from sweetgum seems to form a new pathovar since a strain inoculated in grape, oleander and almond did not produce disease symptoms. Two different strains were isolated from redbud, one a *Xf* subsp. *fastidiosa* member (cercis001) infected redbud, almond and grape while cercis049,a member of the *Xf* subsp. *multiplex* does not seem to be pathogenic towards almond or grape. We found out that cherries can be affected by two genetically different strains of *Xf* , however Koch's postulates has not been successfully

Figure 3. Redbud (*Cercis occidentallis*) plants mechanically inoculated with *Xf* strain cercis001 showing leaf scorch and stunting (left) as compared to a healthy non-diseased plant (right).

tested. Some studies still are underway to fulfill the Koch's postulates as well as to reveal their fate on grape, almond and oleander. 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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PLASMID ADDICTION AS A NOVEL APPROACH FOR DEVELOPING A STABLE PLASMID VECTOR FOR *XYLELLA FASTIDIOSA*

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ABSTRACT

The lack of genetic and molecular tools that can be used to study the biology of *Xylella fastidiosa* (*Xf*) has made it extremely difficult for researchers to use genetic methods to establish the importance of a particular gene in the development of Pierce's disease (PD). During the period under review, we have focused on developing plasmid vectors that are stably maintained in *Xf* throughout the infection cycle. To increase the stability of autonomously replicating plasmid vectors, we have introduced two different types of stabilizing elements into plasmid vectors pXF004, pRL1342, and pBBR1MCS-5. These stabilizing elements include the plasmid addiction systems, *hok/sok* and *parDE*, and the active partitioning system, *parA*. We are currently examining how addition of these stability elements affects plasmid maintenance both *in vitro* and *en planta*. We have also developed two integration vectors, which will allow researchers to introduce genes into two different nonessential regions of the *Xf* chromosome. We are currently evaluating the properties of the insertion strains *en planta* to make sure that these strains still exhibit the normal PD infectious cycle and have begun to examine the usefulness of both of these vectors for complementation analysis in *Xf*.

INTRODUCTION

Xylella fastidiosa (Xf) is the causative agent of numerous economically important plant diseases, including Pierce's disease (PD) of grapevine (Hopkins and Purcell 2002). An important feature of the *Xf* infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of plants and the foregut of insect vectors. Successful colonization of these hosts is dependent on the ability of *Xf* to subvert host defense networks and to acquire essential nutrients. The virulence determinants of *Xf* include proteins involved in adhesion and biofilm formation, extracellular enzymes, and toxins.

A fundamental strategy for investigating virulence in bacterial pathogens is to generate mutations and examine the impact of the absence of these gene products on pathogenicity. Over the past five years, many research laboratories have been generating insertion mutations in specific *Xf* genes and examining the impact of these mutations on the development of PD (Guilhabert and Kirkpatrick 2003, Feil *et al*. 2003, Reddy *et al*. 2004, Roper *et al*. 2004, Meng *et al*. 2005, Hernandez-Martinez *et al*. 2006). These studies have led to the identification of a number of mutant strains that do not show the normal PD infection cycle. Although the simplest explanation for these phenotypes is that the gene containing the insertion mutation is required for the normal development of PD, it is also possible that a secondary mutation was acquired during the construction of the original mutation and that the secondary mutation is responsible for the phenotype.

The classic approach to overcoming this type of objection is to perform complementation analysis. If the reintroduction of a wild-type copy of the gene into the mutant strain restores the normal PD infection cycle *en planta*, the researcher can conclude that the specific gene is important for the development of PD. One common strategy used to reintroduce the wild-type copy of a gene in Gram-negative bacteria involves the use of autonomously replicating plasmid vectors that carry antibiotic resistance genes and multiple cloning sites. Plasmid vectors with these features have been developed that are capable of replicating in *Xf* and that are stably maintained in the presence of antibiotics. These plasmids have been extremely useful for introducing genes into *Xf* and for *in vitro* complementation studies. Unfortunately, most of these plasmids are quickly lost from *Xf* in the absence of selective pressure, which limits the usefulness of these plasmids for studies *en planta*. Therefore, a major goal of this study is to develop a set of plasmid vectors that will allow researchers to perform complementation analysis *en planta*.

OBJECTIVES

1. Develop a stable plasmid vector for *Xf*
   a. Evaluate the potential of various plasmid addiction systems for ability to convert plasmids known to replicate in *Xf* into stable vectors.
   b. Evaluate how plasmid maintenance by *Xf* is affected by other genetic mechanisms known to affect plasmid stability, such as systems for multimer resolution and active partitioning systems.
2. Evaluate the stability of the newly development plasmid vectors when propagate in *Xf en planta*.