Progress Report 2006-2008

I. Project Title: Assessing the potential of forage alfalfa crops to serve as *Xylella fastidiosa*, primary inoculum sources in the San Joaquin Valley.

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III. Objectives and Experiments to Accomplish Objectives:

Objective 1. Identify and quantify the potential of alfalfa to serve as reservoir hosts of *Xf* through field surveys to: (A) detect *Xf* in forage alfalfa adjacent to grape and almond with a history of *Xf*-infection, (B) characterize the seasonal abundance and dispersal of native sharpshooter vectors present within and emigrating from alfalfa.

Incidence of *Xylella fastidiosa* (*Xf*) is being monitored seasonally (winter, summer, fall, and spring) at multiple alfalfa sites. The number of sites monitored has varied due to rotation of study fields from alfalfa to alternate crops. Currently, 5 fields are being

monitored. Each field is broken into 9 quadrants (3 x 3) and 5 tillers are collected from each quadrant, for a total of 45 tillers per field per sampling event (Fig. 1). Each tiller is screened for the presence of *Xf* by DNA extraction followed by PCR using primer set RST 31/31 (Minsavage et al. 1994).

Seasonal abundance and dispersal of native sharpshooters is being evaluated at the same alfalfa sites as above. Two sampling methods are used: sticky traps and sweep nets. Sticky traps (approx.45 X 45 cm) are placed along 2 parallel, linear transects with 5 traps per transect. The first and last trap along each transect is located outside of the field. An additional 3 traps are placed outside each field on either end (Fig. 1). Sweep net samples are collected bi-monthly using a standard sweep net. Ten sets of 25 sweeps are collected by walking two parallel transects through the field (Fig. 1). Detailed records on irrigation, pesticide application, and cutting for each alfalfa field are maintained.

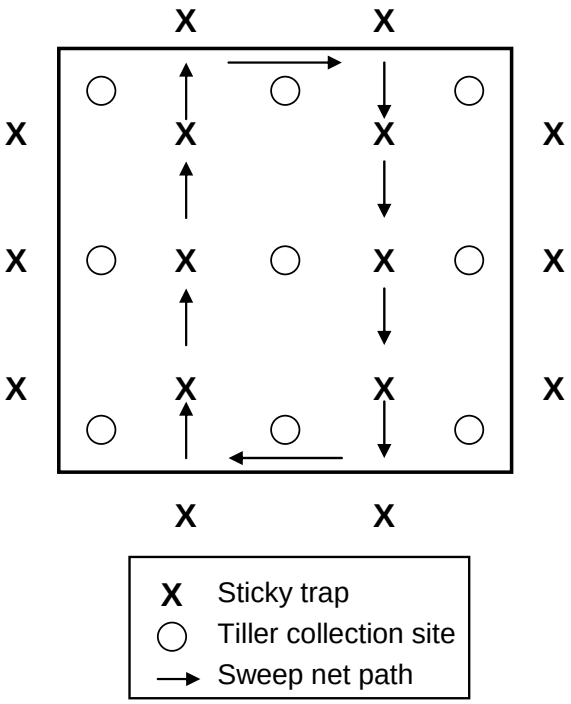

Fig. 1. Layout of field sampling plan. Location of sticky traps are indicated by a 'X'. The location of alfalfa collection sites are indicated by a circle. Fiver tillers are collected per collection site. Ten sets of twenty-five sweeps are collected. The paths followed while sweep netting are indicated by arrows.

Objective 2. Determine the comparative genetic structures of *Xf* populations obtained in reservoir alfalfa, dispersing vectors, and adjacent vineyards and orchards affected by Pierce's disease (PD) and almond leaf scorch (ALS) disease, respectively by simple sequence repeat (SSR) marker analysis.

The genetic diversity of *Xf* isolated from alfalfa will be compared to that of *Xf* isolated from neighboring almond orchards and vineyards to determine the potential epidemiological significance of alfalfa as a reservoir host (i.e., important inoculum source) for PD and ALS. Thus, almond orchards and vineyards adjacent to alfalfa sites will be surveyed for the presence of infected grape and almond followed by isolation of *Xf* DNA from infected plants. The diversity of *Xf* genotypes isolated from almond and grape will be compared to those isolated from alfalfa and native vectors in Objective 1 via SSR fragment analyses (Lin et al. 2005).

Objective 3. Determine the relative susceptibility of selected alfalfa cultivars to infection by different genotypes of *Xf* and to determine the length of time infection(s) of *Xf* genotypes can persist in susceptible cultivars (overwintering).

The relative responses of 14 different alfalfa cultivars to infection by four strains (genotypes) of *Xf* will be evaluated. The following alfalfa cultivars will be evaluated: CUF 101, Moapa 69, WL 525 HQ, WL 625 HQ, WL 530 HQ, WL 342, Peruvian, African, Caliverde 65, California Common 49, UC Cibola, Japan, UC 2705, and Highline Foundation. Each cultivar will be tested against four strains of *Xf*: Temecula, Dixon, M23, and M12. Six replicates of each cultivar by *Xf* strain combination will be evaluated. Plants will be needle-inoculated by placing a 10 µl droplet of *Xf* on a young shoot (3 mm dia.) and then pricking the tissue with a 0.5-cc hypodermic needle. Each plant will be inoculated in three different locations. Mock inoculations with distilled water will serve as controls. Approximately 12 weeks post-inoculation, a sub-sample of alfalfa stems and petioles from all inoculated and non-inoculated test plants will be collected to confirm the presence of *Xf* using conventional PCR protocols.

The ability of *Xf* to overwinter in alfalfa will be evaluated for a subset of cultivars. Approximately, 20 plants of each cultivar will be inoculated in the summer. Half of the plants will be held in the greenhouse and the other half will be planted outdoors in a screen cage. Plants will be assayed every 8-10 weeks to determine if they are infected. Comparison of plants held indoors versus outdoors will elucidate the effects of cool winter temperature on *Xf* infections in alfalfa.

Objective 4. Determine the vector competence of a potentially new insect vector, the three-cornered alfalfa hopper, *Spissistilus festinus,* (Hemiptera: Membracidae).

Insect transmission assays will be conducted using a potentially new insect vector, the three-cornered alfalfa hopper, *Spissistilus festinus*. This species and other Membracid treehoppers have until recently been considered strict phloem feeders (Mitchell and Newsom 1984). Shugart (2004) recently documented phloem and xylem feeding in a related treehopper species, *Umbonia crassicornica*. In addition, fluorescently labeled *Xf* was observed in the foregut of adult *S. festinus* after a 36 h AAP on Gfp-labeled, *Xf*-infected *Vitis vinifera* var 'Chardonnay' (Groves unpublished data).

To test the vector competence of this species, field collected adults will be placed on *Xf*-infected alfalfa for a 4 day acquisition access period (AAP). After the 4 day AAP, insects will be transferred in groups of five to 12 week old greenhouse-grown alfalfa. These adults will be given a 4 day inoculation access period (IAP). Following the 4 day IAP, insects will be removed and held at -20 °C for PCR analyses. Recipient alfalfa plants will be held for 2 to 4 months and then assayed using conventional PCR methods to determine if transmission occurred. If infections are detected, bacteria will be recovered through isolation onto solid PW media and then re-inoculated into susceptible alfalfa to satisfy Koch's postulates.

IV. Summary of major research accomplishments and results for each objective

Objective 1. Identify and quantify the potential of alfalfa to serve as reservoir hosts of *Xf* through field surveys to: (A) detect *Xf* in forage alfalfa adjacent to grape and almond with a history of *Xf*-infection, (B) characterize the seasonal abundance and dispersal of native sharpshooter vectors present within and emigrating from alfalfa.

Since the inception of this study more than 3,825 alfalfa samples have been screened for the presence of *Xf*. Of those samples, 6 were positive for *Xf*. Two of the positive samples were from a single collection in Fresno County during the summer of 2005 and the other 4 positives were from a single collection from a another site in Fresno County during the summer of 2007.

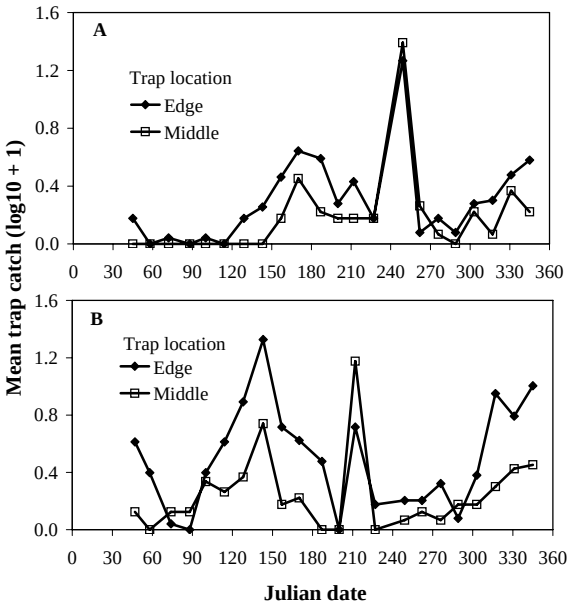

Fig. 2. Examples of green sharpshooter population dynamics in alfalfa at sites located in (A) Tulare and (B) Fresno counties. Sticky traps were located in the middle of fields or on edges. See Fig. 1 for trap locations.

Sticky trap and sweep net samples have been collected from multiple sites since January of 2006 (minimum number of sites monitored at any one time = 4). The predominate Xf-vector captured to date is the green sharpshooter (*Draeculacephala minerva*). No glassy-winged sharpshooters have been observed. Preliminary analysis of the distribution of green sharpshooter within alfalfa fields indicates some important trends. First, green sharpshooters are more abundant on field edges than in the middle of fields (Fig. 2). Second, in the fall of 2007 we scored the percentage of ground cover surrounding each sticky trap that was weeds. Analysis of the number of insects caught per trap and percent ground cover that was weeds indicates a positive relationship in all cases examined to date (Fig. 3). This indicates that green sharpshooter adults prefer weeds that are found along field margins. Insects collected via sweep net will be assayed for the presence of *Xf*.

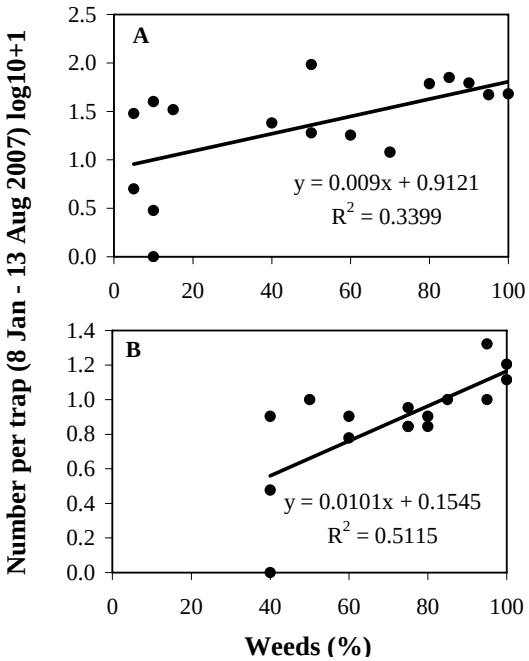

Fig. 3. Examples of the positive association between the percentage area covered by weeds surrounding a trap and trap catch. Results for two different alfalfa fields in Kern County are shown.

Sampling will continue through 2008.

Objective 2. Determine the comparative genetic structures of *Xf* populations obtained in reservoir alfalfa, dispersing vectors, and adjacent vineyards and orchards affected by PD and ALS, respectively by simple sequence repeat (SSR) marker analysis.

Almond orchards and vineyards neighboring monitored alfalfa fields were surveyed for the presence of *Xf* infected trees or vines. Infected vines or trees were detected at four sites and a total of 37 and 40 infected grape and almond samples were collected, respectively. These samples, along with the six positive samples collected from alfalfa fields were subjected to SSR marker analysis (Fig. 4). To date, these samples have been screened with seven different SSR markers. Preliminary analyses using GenAlEx.6 software (Peakall and Smouse 2006) to calculate Nei's genetic identity indicates that samples collected from the two alfalfa sites are more different from one another than from samples collected from grape or almond (Table. 1). Additional grape, almond, and alfalfa samples will be collected during fall 2008 and subjected to further analysis.

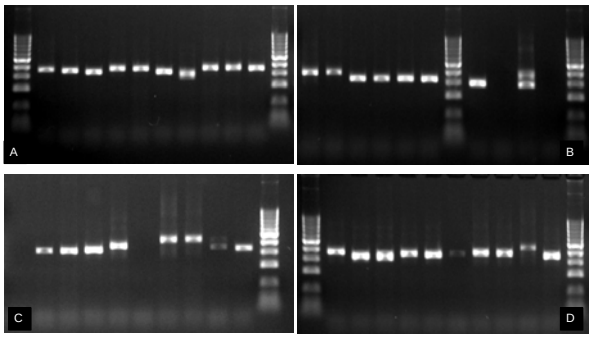

Fig. 4. Example of SSR alleles for the locus ASSR 20 for samples collected from grape (A), alfalfa (B), and almond (C and D). Products range in size from 308-370 bp.

Table. 1. Nei's genetic identity for pairwise comparisons of samples collected from different crops and sites. "Site A, almonds" bordered "Site A, alfalfa" and "Site B, almonds" bordered "site B alfalfa".

Site A Almonds	Site B Almonds	Site A Alfalfa	Site B Alfalfa	Site C Grape	Site D Grape	
1.00						Site A, Almonds
0.83	1.00					Site B, Almonds
0.63	0.58	1.00				Site A, Alfalfa
0.69	0.75	0.38	1.00			Site B, Alfalfa
0.86	0.72	0.50	0.72	1.0		Site C, Grape
0.83	0.73	0.64	0.74	0.86	1.0	Site D, Grape

Objective 3. Determine the relative susceptibility of selected alfalfa cultivars to infection by different genotypes of *Xf* and to determine the length of time infection(s) of *Xf* genotypes can persist in susceptible cultivars (overwintering).

Fourteen alfalfa cultivars were screened to determine their relative susceptibility to infection by four different *Xf* strains (Temecula, Dixon, M12, and M23). Plants were screened for infection using conventional PCR methods 12 weeks after inoculation. *Xf* was detected in at least three out of 24 plants for each cultivar and the percentage of plants infected averaged across the four *Xf* strains varied from 13 to 48% (Fig. 5).

For 5 cultivars (CUF 101, Moapa 69, WL 530 HQ, WL 625 HQ, and WL 342 HQ) a more detailed experiment was completed to determine the seasonal fate of *Xf* in alfalfa. Approximately twenty plants of each cultivar were needle inoculated in July/August of 2007. Six weeks after the 1st inoculation event, plants were screened for the presence of *Xf* using conventional PCR methods. For each plant, two samples were evaluated. One was collected near an inoculation site (referred to as "inoculation site") and the other was collected away from the inoculation site (referred to as "away from inoculation site"; Fig. 6). In October, plants were randomly assigned to one of two groups. The first group was planted outside in a screen cage. The second group was moved into a greenhouse. Screening of samples collected "away from inoculation sites" in October indicated no differences between the plants held outdoors versus those moved into the greenhouse (Fig. 6). However, by January few samples from plants held outdoors were positive for *Xf* whereas most samples from plants held in the greenhouse were positive (Fig. 6). This suggests that cold winter temperatures may eliminate or reduce *Xf* titers in alfalfa plants. These plants will be screened approximately every eight weeks until the fall of 2008 to determine if *Xf* titers in plants held outdoors increase during the summer.

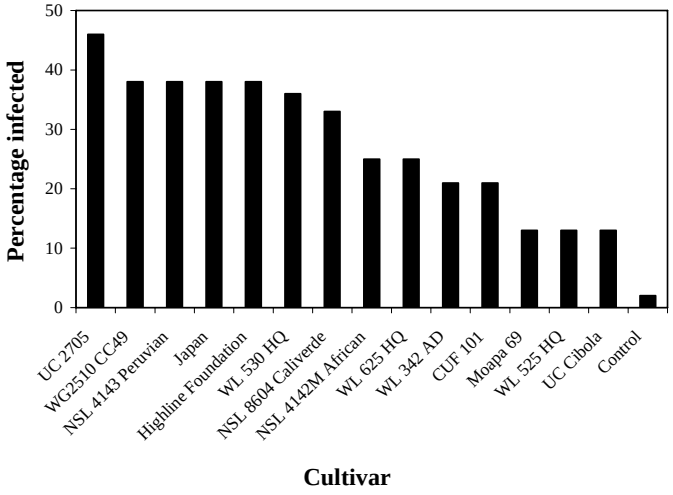

Fig. 5. Percentage of each alfalfa cultivar succesfully needle incoulated with *Xf* across all *Xf* strains tested.

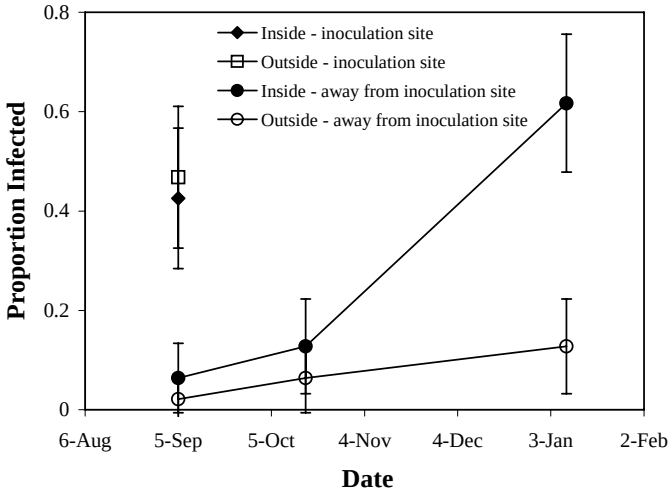

Fig. 6. Detection of *Xf* in needle inoculated plants held inside (e.g., greenhouse) verus outdoors. On the first sampling date, two samples were collected from each inoculated plant: inoculated tillers (referred to as "inoculation site") and non-inoculated tillers (referred to as "away from inoculation site"). On subsequent sampling dates only samples away from the inocultion site were collected. Results are from a pooled analysis of 5 cultivars. Means and 95% confidence intervals are shown.

Objective 4. Determine the vector competence of a potentially new insect vector, the three-cornered alfalfa hopper, *Spissistilus festinus*, (Hemiptera: Membracidae).

Transmission assays were conducted in the summer of 2006 and 2007 in Parlier, CA. Needle inoculated alfalfa plants were used as acquisition hosts. In most cases a four day acquisition access period was used (AAP), although a limited set of tests were done using a 9 day AAP. For all tests, a four day inoculation access period was used (IAP) and each test plant received 5 insects. Over two years 630 insects were evaluated (126 plants x 5 insects per plant = 630). Competent transmission was not documented in any of these tests.

Complementary tests were conducted in Berkeley, CA during 2007 with similar results. In these tests, grape was the acquisition host and grape, almond, and alfalfa were used as recipient hosts. Approximately 100 three cornered alfalfa hoppers were tested during the course of this study and none were documented to transmit *Xf.* Similarly, PCR analysis of insect heads did not detect the presence of *Xf.* The combined results of the tests conducted in Parlier and in Berkeley lead us to conclude that the three cornered alfalfa hopper is not a competent vector.

V. Publications or reports resulting from the project:

Sisterson, M., Groves, R., Daane, K., and S. Thammiraju. 2007. Assessing the potential of forage alfalfa crops to serve as *Xylella fastidiosa* primary inoculum sources in the San Joaquin Valley, pp. 279-280. *In,* Proceedings of the Pierce's Disease Research Symposium, 12-14 Dec 2007, San Diego, CA.

VI. Presentations on research:

Sisterson, M. S., S. R. Thammiraju, K. Daane, and R. L. Groves. Alfalfa as an important inoculum source of *Xylella fastidiosa.* Poster presented at the American Phytopathological Society Meeting in San Diego, CA. August, 2007.

Thammiraju, S. R., M. Sisterson, R. Groves, and K. Daane. Evaluation of *Xylella fastidiosa* strain diversity in multiple crops. Pierce's Disease Research Symposium. December 2007.

Sisterson, M. S., S. R. Thammiraju, K. Daane, and R. L. Groves. Epidemiology of xylellae diseases in the San Joaquin Valley of California: the role of alfalfa. Oral presentation, Pacific Branch of the Entomological Society of America. Napa, CA. To be presented April 2008.

VII. Research Relevance Statement:

In recent years, Pierce's disease of grape and almond leaf scorch disease have emerged as serious disease threats throughout California's San Joaquin Valley. Limited information exists to document where potential insect vectors acquire the pathogen, when they move into orchards, and when they spread the pathogen. To address these questions, this project will document and quantify the potential of alfalfa forage crops to serve as

sources of either inoculum or vectors which contribute to the incidence of important xylellae diseases in the San Joaquin Valley.

VIII. Summary of current year's results: Max 500 words

Laboratory inoculation of alfalfa plants indicates that all cultivars tested are suitable hosts for *Xf* (Fig. 5). Comparison of inoculated plants held in the greenhouse versus plants held outdoors indicates that titers of *Xf* appear to decline to undetectable levels when plants are exposed to cool winter temperatures (Fig. 6). Screening of field collected alfalfa corroborates this result as positive samples have only been collected in the summer. Incidence of *Xf* in field collected alfalfa during the summer was low, 0.5% (6 out of 1,125 samples). Low incidence of *Xf* in alfalfa is partially explained by the behavior of the main *Xf* vector found in alfalfa fields. Analysis of trapping data on the distribution of green sharpshooter within alfalfa fields indicates that they prefer weedy field margins (Fig. 2, 3). Such behavior is likely to limit the spread of *Xf* within alfalfa fields. The results demonstrate that alfalfa has the potential to be an important source of inoculum, but this potential is limited because of seasonal changes in *Xf* titer and vector behavior. As green sharpshooter is often abundant along field margins, alfalfa may serve as an important source of the vector.

IX. Status of funds:

Seventy percent of the budget has been directed to supporting a post-doctoral research to coordinate and implement the molecular portions of this project. The remaining budget is directed towards technical support to help collect and screen samples from this labor intensive project.

X. Summary of intellectual property

NONE

Literature Cited:

Lin, H., E.L. Civerolo, R. Hu, S. Barros, M. Francis, and M.A. Walker. 2005. Multilocus simple sequence repeat markers for differentiating strains and evaluating genetic diversity of *Xylella fastidiosa*. Appl. & Environ. Microbiol. 71: 4888-4892.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L, Leite, R.M.V.B.C., and Stall, R.E. 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84: 456-461.

Mitchell, P.L., and Newsom, L.D. 1984. Histological and behavioral studies of three cornered alfalfa hopper feeding on soybean. Ann. Entomol. Soc. Am. 77:174-181.

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Shugart, H. 2004. Ontogeny of feeding behavior of the subsocial treehopper, *Umbonia crassicornica*. Masters thesis. University of Missouri, Columbia, MO.