I. Project Title: **RNA-interference and control of the glassy-winged sharpshooter (*Homalodisca coagulata*) and other leafhopper vectors of Pierces Disease**

II. Principal Investigators and Researchers:

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III. List of Objectives and Description of Activities conducted to accomplish each objective:

We proposed a new approach, based on RNA interference (RNAi) directed towards *H. vitripennis*. We originally proposed the three objectives given below and requested 2 years of funding. Our grant award was for only one year, therefore, we have focused our efforts only on objective 1 during the first 9 months of the grant.

Objectives:

1. To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of *Homalodisca vitripennis.*
2. To generate transgenic plants capable of expressing and delivering *Homalodisca vitripennis* deleterious RNAi molecules within their xylem.
3. To evaluate transgenic plants for their ability to generate RNAs capable of inducing RNAi vs. *Homalodisca vitripennis*.

Approaches and results for specific aspects of our research under objective 1 are given below.

Manipulating GWSS cells: For this effort we took two approaches to target GWSS. We cultured *H. vitripennis* cells as well as whole leafhoppers. We used the GWSS cell line developed by Drs. George Kamita and Bruce Hammock, UC Davis, Entomology. They generously supplied these cells (see Kamita et al., 2005, GWSS cell line Z15) and have greatly assisted us in learning how to maintain and manipulate them. We now have developed the skills to consistently manipulate these cell lines and we have performed a series of experiments to deliver constructs to these cells.

Rearing GWSS: We have also established reproducing colonies of GWSS in the UC Davis Contained Research Facility, and we are performing experiments using whole insects. We have had good help in establishing these colonies. We obtained GWSS from Dr. R. Almeida, as well as advice on rearing and maintaining these insects. We have also consulted other scientists, including Dr. E. Backus and their advice has been very helpful. We have established a regime that now allows us to maintain consistently reproducing populations of GWSS in growth chambers. We initially tried supplemental light in the greenhouse and failed to obtain continuous reproduction. But in growth chambers maintained at 24C and 16 hr days, we have cages which contain a mixture of basil, cotton and cowpea plants, and these have a continuous mixture of various age GWSS. Because when we first began our experiments we were not sure if we could rear enough GWSS for our experiments we also began rearing *Draeculacephala minerva* (the grass sharpshooter). This sharpshooter is a non-quarantined sharpshooter vector of *X. fastidiosa*, and much easier to rear in large numbers.

Delivering RNAs and DNAs to GWSS cells: In order to determine if we could deliver RNAs or DNAs to GWSS Z15cells, cells were first transfected with a plasmid expressing GFP under the control of an inducible insect promoter (kindly provided by Dr. Shou-wei Ding, UC Riverside). "DOTAP" and "FuGene HD" transfection reagents from Roche and "Cellfectin" transfection reagent from Invitrogen were compared for their ability to assist in transfecting GWSS cells. Manufacturer protocols were followed in all the transfection experiments. The maximum transfection efficiency (equal to 5%) was obtained using the Cellfectin transfection system (see results below). This level is lower than we initially hoped for and we are still assessing additional delivery and detection methods. We know that if we can efficiently deliver DNA plasmids to cells this also would prove useful for additional studies for inducing RNAi effects in cells. We would deliver recombinant plasmids engineered to, upon induction, express hairpin RNA constructs within transfected cells, and we believe this will still be useful in the long run. We also delivered dsRNA and siRNA constructs directly to GWSS cells. In order to monitor transfection efficiency, siRNAs were labeled with fluorescein (New England Biolabs) and transfection was performed using the Cellfectin and X-tremeGene transfection reagents.

Choosing dsRNA inducers for delivery to cells and whole insects: We initially chose to target GWSS, but later also included *D. minerva*. Fourteen *H. vitripennis* nucleotide sequences, derived from EST based nucleotide sequences available in GenBank and translatable in putative proteins, were used to design gene specific primers and to generate cDNAs from GWSS cell line Z15 and from *D. minerva* whole insects. Corresponding sequences were amplified by RT-PCR, cloned and sequenced to confirm their identity.

Delivering dsRNAs, siRNAs and DNAs to whole GWSS. We used two approaches in attempts to deliver nucleic acids to whole GWSS. We learned to micro-inject GWSS intra-thoracically from Dr. A. H. Purcell. We have used micro-injections with specific dsRNAs as noted below. We modified a micro-pipettor to hold capillary glass pulled needles (we use a needle puller on the UCD campus to make our own needles) so we can inject GWSS with exact volumes (1 – 2 ul). We also have attempted to deliver nucleic acids orally. Being able to do this will be important as it will more accurately reflect delivery that we hope to obtain by using transgenic plants. So far our oral acquisition experiments for GWSS have been done by inserting plant stems (cotton or basil) with one or two leaves, into a solution containing the nucleic acid

solution. We are planning to work with collaborators to develop a way to feed GWSS nymphs directly on artificial membranes containing specific RNA or DNA constructs. We will use GWSS nymphs for these experiments, but also *D. minerva*.

Monitoring RNAi effects: Our long-term goal is of course to be able to monitor phenotype, insect or cell death as an effect of RNAi. However, because this may not be possible with all treatments we chose two approaches to monitor RNAi effects, one based on cytology effects in GWSS cells and the other based on target RNA abundance. For target RNA abundance, we used quantitative and semi-quantitative RT-PCR-based approaches. Realtime RT-PCR and primers/ probe sets were designed for our target genes. These were tested initially using real time RT-PCR assays of GWSS cell line-derived RNA. This system will now be used to measure the amount of SAR1 and actin mRNAs in transfected cells and whole *H. vitripennis* and *D. minerva* insects, following RNAi delivery. We also have used these targets and semi-quantitative RT-PCR in *H. vitripennis* injection studies. Since the assays are performed using only one gene per experiment, the second gene can be used as an endogenous control.

We also developed cytological means to examine actin filaments in GWSS cells. GWSS cells were grown on microscope cover slips and then stained using phalloidin. Using transmitted light, actin filaments are white. However when examined with a UV filter, actin filaments are green (see Fig. 1). As one of the targets identified above is actin, we will deliver actin interfering RNAs and assess effects by staining cells and examining for actin filament disruption.

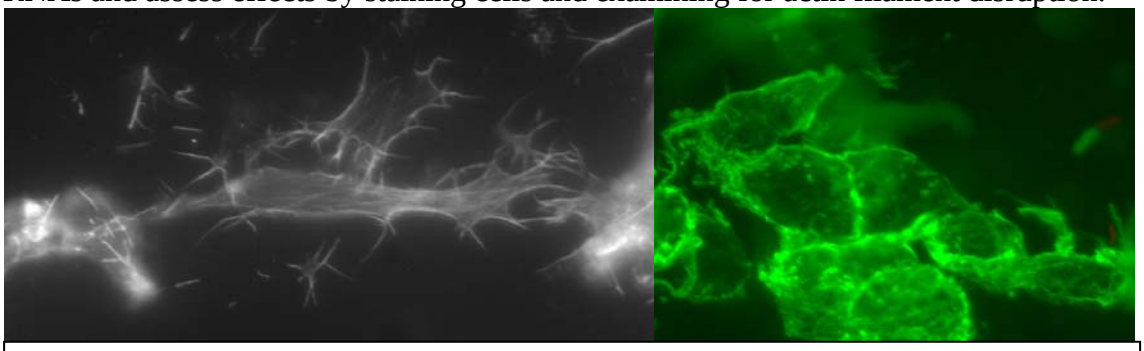


Fig. 1. Image of GWSS cells grown on a glass cover and stained with phalloidin. Using transmitted light, actin filaments are white. However when examind with a UV filter, actin filaments are green. Fibroblast like cell is visible in the middle.

IV. Summary of Major Research Accomplishments and Results for Objective 1:

Two major accomplishments are that we have established reproducing colonies of GWSS, and we have stable GWSS cell lines, and both can be used for our experiments. Neither of these is trivial, GWSS are difficult to maintain. We can do it and have used them for initial experiments. Our experiments so far have shown that we can deliver and express genes and siRNAs in GWSS cells, we can deliver nucleic acids via oral delivery into GWSS whole insects, and dsRNAs via injection.

RNAi inducers: Fourteen sequences were amplified by RT-PCR (Fig. 2). Two of our original sequence targets, corresponding to the vitellogenin and rhodopsin genes, could not be amplified from the *H. vitripennis* cell line mRNA pool, thus we obtained 12 target sequences. Comparison

of the nucleotide sequences for GWSS and *D. minerva* cloned cDNAs showed that identities of different fragments ranged from 85 – 92% for given genes. cDNAs of 3 actin mRNAs and 1 SAR mRNA expressed in the *H. vitripennis* cell line were cloned in the pGMTeasy vector in both orientations downstream of the T7 RNA polymerase promoter, and sequenced. These plasmids were directly used for T7 RNA polymerase-mediated *in vitro* transcription to generate specific dsRNAs (Ambion, dsRNA MaxiScript; see Fig. 3) which were used as described below.

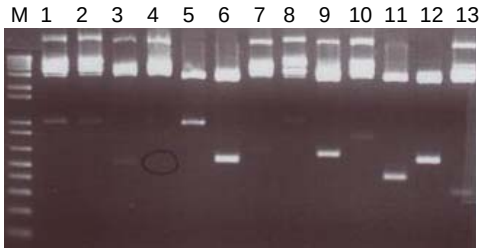


Fig. 2. Digestion products for selected *D. minerva* mRNA cDNAs cloned in pGEMT vector (Promega). Lanes 1–14 show products for actin (1), arginine kinase (2), ferritin (3), rhodopsin (4-5), Fructose 1,6 biphosphate (6-7), delta 9 desaturase (8), RAB1-1 (9), tropomyosin (10), ubiquitin conjugating enzyme (11), Sar1 (12), histone 3 (13).

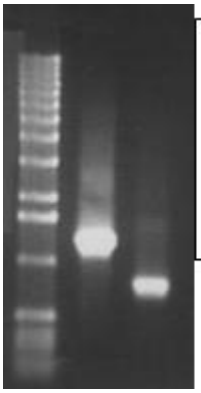


Fig. 3. *In vitro* transcripts were annealed to give dsRNAs and analyzed by agarose gel electrophoresis. Lane 1 = DNA markers, Lanes 2 and 3 are actin and SAR 1 dsRNAs, respectively.

Cell transfection: GWSS line Z15 cells were transfected with a plasmid expressing GFP under the control of a copper inducible promoter (kindly provided by Dr. Shou-wei Ding, UC Riverside). Upon induction only transfected cells will show GFP. In our experiments with this plasmid system, the maximum transfection efficiency (equal to 5%) obtained by us so far was by using the Cellfectin transfection system (see Fig. 4). We have continued to evaluate and improve cell transfection conditions and our most recent experiments show that we can transfect siRNAs directly into cells and obtain much higher transfection efficiencies.

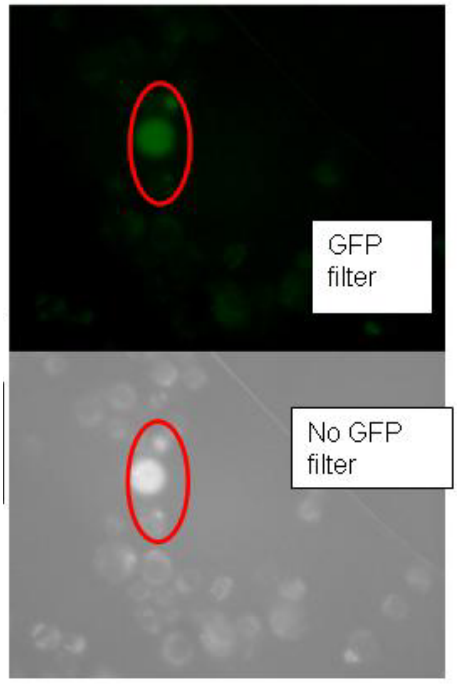


Fig.4. GWSS cells were transfected with a GFP-expressing plasmid. Top shows fluorescent cells, bottom shows light visualization. Maximum transfection efficiency was 5%.

SiRNAs were labeled with Fluorescein in vitro (New England Biolabs) and used to transfect GWSS cells, using the Cellfectin (Invitrogen) and X-tremeGene (Roche) transfection reagents, following the manufacturer protocols. Both transfection systems gave an efficiency of transfection of 60% (see Fig.5). We can make siRNAs in vitro (by RNase III digestion of dsRNAs) and deliver these to GWSS cells to assess RNAi effects.

Oral delivery of RNAi inducers: So far we have attempted oral delivery of nucleic acids to GWSS by a modified stem infusion technique. Our initial experiments have been so far just to optimize conditions, but results are encouraging. In one experiment, cotton stems with one or two leaves were placed in a vial containing a solution of linear dsDNA in water, and the stem-vial interface was

covered with parafilm (Fig 6). The vial and plant were placed inside a cylindrical cage and GWSS nymphs were added and allowed to feed on the stem. Twenty four hours later nymphs were removed and the stems and nymphs were tested by PCR. The DNA was readily detected in

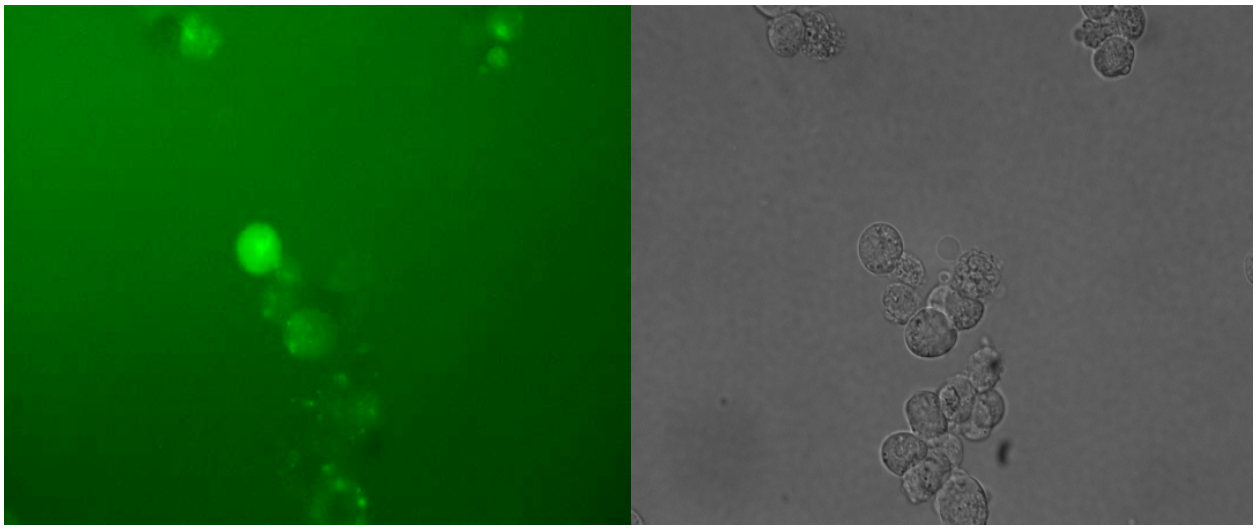


Fig.5. SiRNA labeled with Fluorescein (New England Biolabs) were used to transfect GWSS cells. On the left: cells transfected with siRNA shows fluorescence under UV light using a GFP filter. Fluorescence is visible as bright speckles in the cell cytoplasm. On the right: the same cells were photographed using bright field light.microscopy.

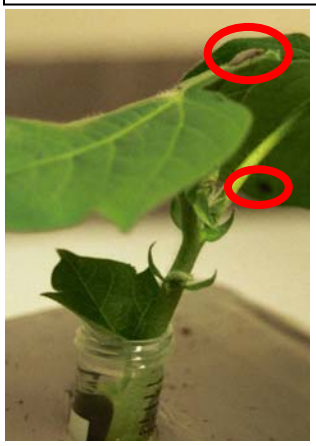
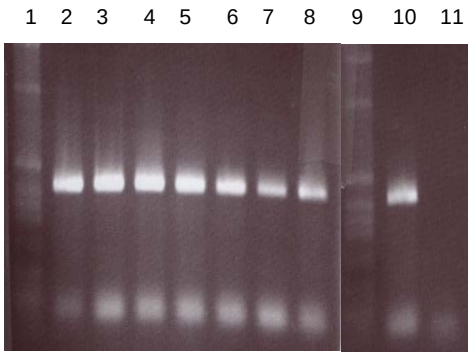
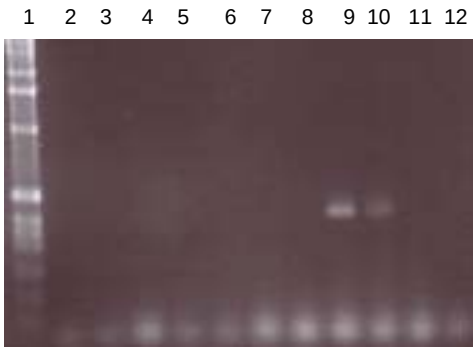


Fig. 6. GWSS nymphs (in circle) were fed on young basil and/or cotton plants infused with a 1µg/ml solution of exogenous dsDNA for 24 hours. Plants and insects were tested by PCR and results are shown below. At lower left gel lanes 2-8 show the presence of PCR products from cotton leaves (lanes 2-4) petioles (lanes 5-7) and stem (lanes 8 and 10) infused with dsDNA. Lane 11 is a negative control. Lanes 1 and 9 were loaded with 1Kb plus marker (Invitrogen). At lower right gel shows PCR products for GWSS that fed on infused basil. Lanes 2 to 6 show PCR products from GWSS fed on control basil. Lanes 7 to 11 show PCR products from GWSS fed on dsDNA-infused basil. Two out of five are positive (see arrow). Lane 12 is a negative control. Lane 1 was loaded with 1Kb plus marker (Invitrogen).





the cut stems, PCR results were positive for all segments tested (see Fig. 6). We also found 2 of 5 GWSS to be PCR-positive in this first experiment (Fig. 6). This demonstrates that we can deliver DNAs to GWSS, and likely other sharpshooters, by feeding them on cuttings immersed in a solution of the test material. We hoped this would be the case as the xylem should pick up and transport materials in the solution containing the cut stems. We attempted this approach because of difficulties that we and others have had in using GWSS for acquiring

specific treatments via artificial diet membrane feeding. However we have not given up on this approach and will attempt more in the future, especially as we now have sufficient GWSS nymphs to perform artificial diet acquisitions.

Monitoring RNAi effects: We used semi-quantitative RT-PCR to compare relative levels of target mRNAs in GWSS after injection. Two of our GWSS dsRNAs, corresponding to the Actin and Sar1 transcripts, were delivered via injection into *H. vitripennis* insects. One ul was injected intra-thoracically using a concentration of 1.3 ug/uL and 1.7 ug/uL for actin and SAR1 dsRNAs, respectively. Twenty nymphs were injected with each treatment, and 20 nymphs with the dsRNA purification buffer as a control. Nymphs were transferred to basil plants and survival was monitored over time. In this first experiment, the majority of nymphs injected with dsRNAs were dead 24 hour after injection, while 16/20 injected with buffer survived (Table 1). This was

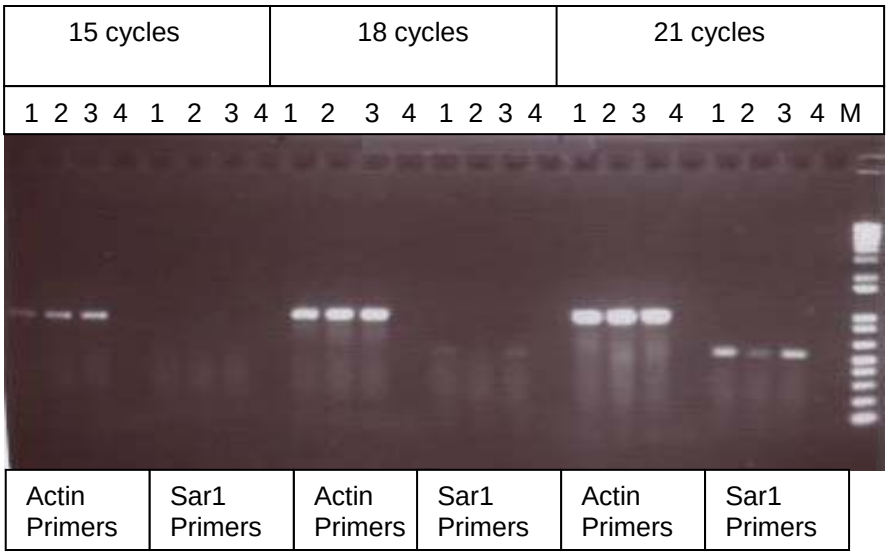


Fig. 7. Semi-quantitative RT-PCR results, showing the RNA level in insects injected with 1.3 µg of actin dsRNA (lanes 1), 1.7 µg of Sar1 dsRNA (lanes 2) or with injection buffer (lanes 3). Each RT-PCR was performed using 100 ng total RNA. Lanes 4 are negative RT-PCR controls. The RT-PCR was performed for 15, 18 and 21 cycles. Results show that insects treated with actin dsRNA (lane 1 cycle 15) have less mRNA than insects treated with Sar1 dsRNA (lane 2 cycle 15) or buffer (lane 3 cycle 15).
Furthermore, insects treated with Sar1 dsRNA (lanes 2 cycle 18 and 21) have less mRNA than insects treated with actin dsRNA (lanes 1 cycle 18 and 21) or buffer (lanes 3 cycle 18 and 21).

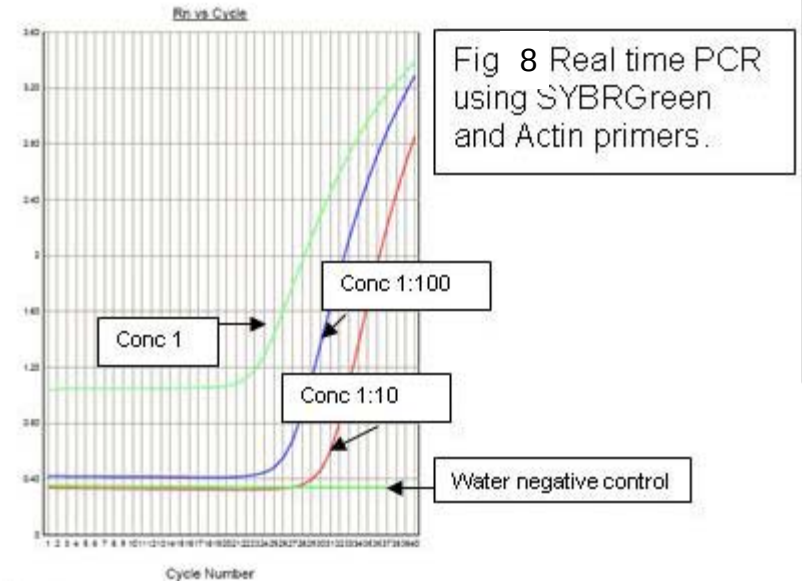
unexpected, we did not anticipate seeing such rapid mortality and even though 80% of the buffer control injected GWSS survived in this first experiment we cannot be sure if mortality in the dsRNA injected GWSS was due to specific RNAi effects. Therefore to attempt to obtain supporting data in this regard, we performed semi-quantitative RT-PCR for the actin and SAR1 mRNAs in these insects. Total RNAs were extracted from five (three dead and 2 alive combined together for the given treatment) GWSS collected 24 hr post injection. Results of these analyses showed that the

Table 1	Buffer	1.3 µg Actin dsRNA	1.7 µg Sar dsRNA
Day 0	20/20	20/20	20/20
Day 1	16	6	4
Day 10	10	1	0

Table 1. GWSS nymphs (20 each) were injected at Day 0 with the treatments shown in the top row. Numbers in columns below indicate survivors at the given days post-injection.

corresponding target mRNA levels were specifically reduced for each treatment (Fig. 7). GWSS injected with actin dsRNA (lane 1 cycle 15) show less RT-PCR product than GWSS injected with Sar1 dsRNA (lane 2 cycle 15) or buffer (lane 3 cycle 15). Similarly, GWSS injected with Sar1 dsRNA (lanes 2 cycles 18 and 21) have less RT-PCR product than GWSS injected with

actin dsRNA (lanes 1 cycle 18 and 21) or buffer (lanes 3 cycles 18 and 21). These results are very intriguing, but only from one experiment so far and must be interpreted with caution. Still, because of the differential detection from the dsRNA injected GWSS, this is encouraging and we are repeating this now, with some experimental modifications.



We also developed real time RT-PCR as a very sensitive and quantitative means to assess target mRNA levels in GWSS insects and cells. Figure 8 shows quantitative results for dilutions of GWSS total RNAs and real time RT-PCR analysis for actin mRNAs. This will be used to monitor actin mRNAs in GWSS cells and whole insects to give us reliable data as to the efficiency of our RNA interference assays.

V. Publications or Reports from Project: No refereed journal publications yet, but see VI. Presentations of Research for publication in the PD Research Symposium proceedings.

VI. Presentations of Research:

2007 Falk, B. W., Sudarshana, M. R., and Parrella, M. RNA interference and control of the glassy-winged sharpshooter and other leafhopper vectors of Pierce's Disease. Symposium Proceedings, Pierce's Disease Research Symposium, Dec. 12 – 14, 2008. Pages 86 – 89. Oral presentation and poster by Bryce Falk.

VII. Research Relevance Statement: Our research is very relevant and timely. It is unique, not overlapping the work of others with GWSS, and our work will complement other efforts on controlling GWSS and PD. Efforts at using RNA interference strategies to control insect pests of plants are becoming much more common. One example is that the November 2007 issue of Bio/technology had three articles on RNA interference towards insect pests. Clearly RNA interference approaches offer new and important opportunities for insect control and continuing research will identify even more applications. We also have established important biological resources for GWSS research. Our GWSS colonies are continuously reproducing, something that has not been easy for others to achieve. This allows us to do research year round, and because we have a good supply of various nymph stages and adults, we can use specific stages for different experiments. The GWSS cell lines also are now well established. Both our whole

GWSS colonies and GWSS cell lines offer opportunities for us and others working with in the area of GWSS and Pierce's Disease.

VIII. Lay Summary of Current Year's Results: During the first nine months of this project we have made important discoveries, established necessary infrastructure and materials for our research effort, and we have positioned ourselves to move rapidly to make important progress in using RNA interference strategies against GWSS. During this period we were able to successfully rear *H. vitripennis* insects and continuously grow cell lines. We now have a continuous supply of both and are not limited as to when we must do our experiments. We have identified sharpshooter genes as potential targets for RNA interference and we have cloned cDNAs for these targets. We have done this for both GWSS and *D. minerva*, and based on nucleotide sequence homology we know that these genes are highly conserved between these two sharpshooter vectors of *X. fastidiosa*, and thus are good potential targets for these and likely other sharpshooters. We have used the GWSS cloned cDNAs to make dsRNAs and siRNAs *in vitro* for use in RNA interference experiments. Preliminary experiments showed that intra-thoracic injection of dsRNAs into GWSS nymphs is a good approach to determine if specific dsRNAs can induce RNAi effects in whole insects. We are working still to optimize oral acquisition approaches. Also, siRNAs can be directly delivered into cultured GWSS cells, this also will provide an important and rapid means to identify potential targets for RNA interference strategies. We have developed sensitive and specific assays to monitor effects in whole insects and cultured cells. Thus, we are in an excellent position to move on to further experiments (see our original objectives 2 and 3 above) aimed at developing effective RNA interference strategies against GWSS and other sharpshooter vectors of *X. fastidiosa*.

IX. Status of Funds: Funds have been used to support Dr. Cristina Rosa, who has done the majority of work on this project so far, and HoChuen Hsui, a lab assistant. Funds were used to pay recharge fees for CRF space, and for routine laboratory work. We anticipate that all funds will be expended by June 30, 2008.