Pakistan-U.S. Science and Technology Cooperation Program
Annual Report Form

Reports should be prepared jointly by the Pakistani and U.S. principal investigators and should cover all project-related activities carried out during the reporting period on both sides. Please expand the boxes below to accommodate all the information you need to include.

Project Title: **Management of greening by producing healthy plants, monitoring vectors and identification of tolerance**	
Pakistani Principal Investigator: **Dr. Iqrar A. Khan** **University of Agriculture, Faisalabad**	**U.S. Principal Investigator:** **Dr. Mikeal Roose** **Dept. of Botany and Plant Sciences,** **University of California, Riverside**
Reporting Period: 1 Jan 09 – 31 Dec 09	

This project has three main objectives: 1) develop an infrastructure to allow local germplasm in Pakistan to be therapied of graft transmissible pathogens; develop a protocol to have confidence that germplasm is therapied of greening, 2) evaluate germplasm of citrus and citrus relatives for resistance/tolerance/susceptibility to greening, and 3) apply quantitative real time PCR assays for detection of greening and tristeza pathogens in vectors and plants to obtain information on spread of these pathogens in field plantings under different management conditions.

USA

Research on objective 1 has resulted in exchange of information related to protocols to be followed for therapy of germplasm and indexing, both biological and laboratory based methods, to verify freedom from graft transmissible pathogens. A more rapid method is needed to eliminate HLB from citrus germplasm and to help raise confidence that HLB is in fact eliminated from propagation sources; the elimination of HLB from exposed germplasm is especially a problem in an area like Pakistan where existing propagation sources have not been under protected conditions to prevent exposure to vectors which transmit HLB, tristeza, and other vector borne graft transmissible diseases. Conventional thermotherapy of citrus has not been efficient for elimination of HLB, especially with sweet orange cultivars, and while shoot tip grafting works well, it is time consuming and requires a high level of expertise by the technician. Experiments have begun to study the use of antibiotics and/or heat therapy of budwood as a way to quickly eliminate HLB from budwood. In Riverside, the *Candidatus* Liberibacter associated with tomato psyllid yellows is being utilized to determine the effectiveness of various antibiotics to eliminate this pathogen in tomato, and citrus stubborn, a phloem-limited spiroplasma disease endemic to California, is being used as a model system to determine the tolerance of citrus to the antibiotics being tested and tolerance to different heat regimes and effectiveness in eliminating stubborn. The results from these preliminary trials are being transferred to cooperators in Pakistan for trials with citrus infected with HLB.

Pakistan

The first objective has resulted in exchange of information related to protocols to be used for therapy of germpalsm and indexing. For therapy biological indexing protocol was carried out. Biological indexing for HLB has been started on local germplasm including Kinnow mandarin, Musambi sweet orange and Rough Lemon rootstock so as to assess suitability of local germplasm as indicator plant for HLB. Leaf piece grafting and stick grafting were used with a reasonable number of replications from different HLB positive varieties of citrus collected from citrus zones of the country. This experiment is being conducted in the greenhouse conditions and results are expected to be available by the May, 2010. Conventional thermotherapy of citrus has not been efficient for elimination of HLB, especially with sweet orange cultivars, and while shoot tip grafting works well, it is time consuming and requires a high level of expertise.

USA

Research on objective 2 has been started. Seed from 92 citrus varieties and relatives was shipped and received by the project in Pakistan at the University of Faisalabad. Some of the varieties had over dry seed upon arrival (about 5%) and are being recollected at the present time for reshipment. The seed has been sown in a protected greenhouse in Faisalabad, and the first seedlings will be planted into the field this next quarter. The seedlings will be planted when they are at the 6-12 leaf stage. Five plants of each variety will be planted into the field in a replicated, random planting where they will be naturally exposed to huanglongbing (HLB) spread by the psyllid vector, *Diaphorina citri*. A 2 meter tall suction trap has been shipped to the University of Faisalabad and has been installed at the site to provide an unbiased estimate of vector populations. Arrangements have made to ship the psyllids from Faisalabad to Riverside where they will be tested for presence of the bacterium associated with HLB using real time PCR, however, following the training of personnel from Faisalabad in Riverside, now expected to occur in the first and second quarters of 2010, the testing of the psyllids may be split between the two research sites.

Pakistan

Seeds from 68 citrus verities and relatives have been planted. Some of the seeds were fungal infected and few of them had over dried during shipment. The seeds have been sown in controlled conditions under green house. The seedlings are ready to be planted in field at the 6-12 leaf stage. Five plants from that population will be transferred into the field conditions randomly in order to check the exposure of these cultivars against HLB by grafting local scion varieties to test HLB transmission or possible resistance.

A comprehensive survey was conducted in the citrus growing areas of the Punjab with main emphasis on Sargodha District. Some of the citrus groves were found to be free of psyllid whereas others were highly infested. The insect population and activity was also found to be varying with seasonal conditions and different climatic conditions. The fruit symptoms for HLB were found to be the most prominent in the Kinnow mandarin, whereas other varieties could not show such symptoms of similar severity. It is usually thought that during the high temperatures of the summer months the insect population is highly decreased however, we observed that there is no big decrease or environmental demise in the populations rather the insects slow down their activities to avoid the deadly hot temperatures. Yellow tags with 4 × 6 inches dimensions were found to be more useful than the bigger ones. These tags attracted

the insects whenever the temperature, humidity and young foliage were suitable.

Status of rootstock material originated from seed provided by NCGRCD:

Total number of accessions available: **33**

Total number of seedlings available: **356** (*For details please see annexure I*)

USA

Research on objective 3 has focused on application of real time PCR to monitor psyllids for presence of HLB and to detect the presence of HLB in plant tissue. Field caught psyllids are sometimes stored in non-optimal conditions to preserve DNA, and in Riverside research has examined modifications to extraction procedures to better recover the DNA from samples caught on sticky traps and stored for a long period of time in the ethylene glycol/sodium chloride solution used in the suction traps. Taqman probes for regions of the citrus genome and the psyllid genome enable quantitative evaluation of the amount of DNA extracted. Research has also centered on how to process a large amount of samples (both plant and psyllid) in a short period of time at the lowest possible cost per sample. To increase confidence that procedures developed under objective 1 have been effective at eliminating HLB from propagation sources, we are evaluating modifications of the real time PCR assays to include additional target areas of the HLB genome other than the 16sRNA gene for detection. This is possible now as more genome sequence information is available for HLB.

Pakistan

It has focused on use of real time PCR for detection of HLB in plant tissues and to monitor psyllids. More than 150 adult and nymph samples were collected and preserved for pathogen detection in the vector. These samples are to be studied in Riverside, California, USA in the second quarter of 2010. The molecular identification of the citrus relatives and the understanding of phylogenetic relationship will be carried out by using PCR, which will be amplified, cloned, sequenced and analyzed.

Citrus and citrus relatives collected from the field for testing for presence of HLB and tristeza are often of unknown species and variety or are misidentified. To aid in molecular identification of the citrus relatives and to understand the phylogenetic relationships among members of Aurantioideae, a 1.6 Kb fragment of a nuclear gene, malate dehydrogenase, was PCR amplified, cloned, sequenced and analyzed by the post doctoral researcher working in Riverside. The study included taxa belonging to seventy-six species and thirty-eight genera. Twenty-nine genera belonged to Aurantioideae and nine genera were from closely related sub-families. Taxa with heterozygous bases were resolved into two haplotypes. The sequences were analyzed using Phred, Phrap, Consed, Sequencher and Contig Express programs. The data set consisted of about 400 parsimony-informative characters. The sequences were aligned and used to construct phylogenetic trees using Maximum Parsimony and Mr. Bayes software. Interestingly, the general pattern of clustering of the accessions was in agreement with the traditional classification of the sub-family Aurantioideae proposed by Swingle and Reece in 1967 based on morphological characters, with a few interesting exceptions. Preliminary results were presented at the annual meeting of the American Phytopathological Society in July and at the Plant and Animal Genome XVIII Conference, San Diego, CA Jan 9-13, 2010.

Educational Impacts: Please provide information on the numbers of students and other junior collaborators (graduate and undergraduate students, healthcare workers, laboratory technicians, data collectors, etc.) involved in the projects, and discuss new courses or degree programs created (if any) or changes to existing course curricula as a result of your project.

PhD Students: Two synopses have been finalized and one under preparation.

M.Sc. Students: One synopsis has been finalized and two under preparation.

Infrastructure Development: Please list any equipment acquired during this reporting period with grant funds and discuss the impact the new equipment will have on research and educational activities.

A 2 meter high insect suction trap was purchased from Riverside funds and shipped to the project in Pakistan.

Following equipments has been purchased in UAF-Pakistan

1) Gel Electrophoresis System
2) PCR machine
3) Genetic Analyzer
4) Gel Doc System
5) Centrifuge (Temperature Controlled)
6) Freezer -20^{0}C
7) Micro-Centrifuge
8) Micro Pipettes
9) Incubator
10) Computer
11) Vortexer

The procurement of following instruments is still in process

1- Spectrophotometer
2- Water purification system
3- Freezer -80^{0}C

Publications: Please provide citations for any papers published or conference presentations made as a result of your project.

Ramadugu,C., K. L. Manjunath, R. F. Lee and M. Roose. 2010. Phylogenetic Analysis of Aurantioideae plants based on Sequence Information obtained from a Nuclear Gene. P 183Plant and Animal Genomics Workshop, San Diego, CA. http://www.intl-pag.org/18/abstracts/P03e_PAGXVIII_183.html.

Ramadugu, C., K. L. Manjunath, S. Halbert, M. L. Roose, and R. F. Lee. 2009. Aurantioideae: Phylogeny and susceptibility to HLB. Phytopathology 99:S107.

Ramadugu, C., K. L. Manjunath, C. Ramos, S. Halbert, S. Webb, R. F. Lee. 2008. Role of garden centers and retail nurseries in spreading citrus huanglongbing disease. Phytopathology 98:S129.

Miller, S., and R. Lee. 2009. Invasive bacterial pathogens with vectors: Management success and failure. Phytopathology 99:S160.

Razi M. F., M. M. Khan., M. J. Jaskani and I. A. Khan. 2009. Citrus Greening: An extremely lethal disease of citrus groves. Agricultural Digest. 44 (1-2): 58-59.

Additional Funding: Please list any additional funding applied for or received to help support your project during this reporting period.

USA

Information obtained from research on this US-Pakistan Project has contributed to the development of additional projects which were funded by the Florida Citrus Production Research Advisory Council, scientifically coordinated by the National Academy of Science. These projects (PI, title, amount to Riverside location for current year) are: Lee PI, Recovery of citrus germplasm from Florida, $36,970; Mizell, PI, An efficient trap for Asian citrus psyllid that can be used to monitor groves and plants for sale, $32,625; Stansley, PI, Evaluation of systemic acquired resistance inducers combined with psyllid control to manage greening in infected groves, $5,000; Roberts, PI, Assessment of *Zanthoxylum fagara* as a natural host for citrus greening, $11,500; and Roberts, PI, Spatial and temporal incidence of Ca Liberibacter in citrus and psyllids detected using real time PCR, $31,032.

Pakistan

UAF provided additional funds for purchase of additional equipments, lab supplies and stipends.

Linkages with Government or Private Industry: Please describe any linkages developed with government agencies or companies interested in implementing the results of your project.

Two PhD and one M.Sc. research projects include sample collections from farmer fields.

Problems Encountered: Please provide information on any problems you may have encountered in making progress on your project objectives and describe steps you are taking to resolve the problems.

USA

Face to face communication is a major problem. While video conferencing is technically feasible, both sides rely on technical help to set up the system, and the time difference is such that it does not work well. We are looking into Webinar type systems which may work better as they may be set up on personal computers and don't require IT people.

Pakistan

- Seed viability
- Visa process

Plans for Activities During the Coming Year: Please provide details on project activities during the next year, including planned exchange visits, training events, and ongoing research efforts.

USA

A Ph.D student from Pakistan is scheduled to visit Riverside for six months beginning in the first quarter of 2010. He will received training in the molecular characterization of HLB and detection of HLB in psyllids and plants. A second student from Pakistan will visit Riverside later in the year to received training on shoot tip grafting, thermotherapy of germplasm, and will work in the research area of antibiotic/temperature treatments to eliminate greening from budwood and development of multiplex real time PCR for detection of multiple prokaryotic pathogens in citrus. Lee, Riverside, will visit Pakistan for 2-3 weeks if travel restrictions are lifted.

Research will continue in Riverside and Pakistan on the use of antibiotics and variations of temperature treatments of budwood to eliminate HLB. Germplasm in Pakistan that has been "cleaned" by antibiotic, thermotherapy, or shoot tip grafting, will be maintained under protected conditions where vectors are excluded, and repeatedly tested at intervals by real time PCR using present protocols, and protocols being developed for more sensitive detection of HLB as outlined under objective 3, to build up data whereby in the future we could have a high confidence level that if a source, for example, tests negative for four bimonthly test trials, the source is, in fact, free of HLB.

The seedlings from the variety trial to detect tolerant/resistant/susceptible varieties for use by citrus breeders and molecular bioengineers to ultimately incorporate resistance/tolerance into commercial citrus, will be planted out this next quarter. They will be tested at regular intervals using quantitative real time PCR assays to determine ingression of HLB into the plants, relative titer, and effect on growth of the trees. Psyllids will be hand captured and the population in the area of the seedling trial will be monitored by the insect suction trap. All psyllids will be preserved in alcohol and shipped to Riverside for testing for HLB until training has been done in Pakistan for these procedure, then psyllids will either be split with some being tested in Riverside and some in Pakistan, or all DNA extracted in Pakistan with aliquots of the DNA being shipped and tested in Riverside and aliquots being tested also in Pakistan. The cooperators in Pakistan will be responsible for obtaining the proper permits

for shipment of insect specimens to Riverside.

Scientists in Riverside, with the help of visiting scientists from Pakistan, will work on development of more sensitive real time PCR assays for detection of HLB based on areas that appear to be conserved in the HLB genome. Research will continue on the development of better methods to extract DNA from a large number of samples at the lowest possible cost per sample. This will be monitored by using Taqman probes for low copy genes in the plant and/or psyllid genome.

Pakistan

1. About 30% seedlings are at 4-5 leaf stage and 70% seedlings are at 2-3 leaf stage. They would be transferred in field, when they will reach at 6-12 leaf stage. Land has been allocated and being prepared for plantation.

2. Development of capability to allow local germplasm in Pakistan to be therapied of graft transmissible pathogen of HLB.

3. Evaluation of citrus and citrus relatives' germplasm under greenhouse conditions for resistance/tolerance/susceptibility to greening using graft transmission.

4. Visit of one PhD candidate in second quarter of 2010.

5. Application of quantitative real time PCR assays for detection of Greening.

Supplementary Information: If applicable, please attach copies of project-related workshop or conference agendas, course curricula developed, summaries of research data collected in the course of the project, or articles about the project appearing in newspapers, journals, or Web sites. **Please note that your report will be posted on the program Web site, so please do not include any data you do not wish to make publicly available at this point in your research.**

Indicators	Reporting Period:
1. Number of higher education partnerships between Pakistani and U.S. institutions (see note below)	
2. Number of journal articles, technical reports, books, or book chapters (published or accepted for publication) resulting from your project during the reporting period	4 (abstracts) 1 (Pakistan)
3. Number conference presentations resulting from your project during the reporting period	4
4. Number of training events (courses, workshops, seminars, conferences, stakeholders' meetings) conducted on your project during the reporting period	
5. Total number of Pakistanis making exchange visits on your project during the reporting period	0
Number of women	0
Number of men	0
6. Total number of Americans making exchange visits on your project during the reporting period	0
Number of women	0
Number of men	0
7. Total number of exchange visits overall during the reporting period	0
8. Total number of Pakistani PhD students involved in the project	2
Number of women	0
Number of men	2
9. Total number of American PhD students involved in the project	0
Number of women	0
Number of men	0
10. Total number of all other Pakistanis not listed above who participated in your project during the reporting period (Include in this total those who were involved as researchers, MS or undergraduate students, technicians, or data collectors, as well as those who received formal training in workshops or courses or participated in conferences or stakeholders' meetings organized as part of the project.)	3**
Number of women	1
Number of men	2
11. Total number of all other Americans not listed above who participated in your project during the reporting period (Include in this total those who were involved as researchers, MS or undergraduate students, technicians, or data collectors, as well as those who received formal training in workshops or courses or participated in conferences or stakeholders' meetings organized as part of the project.)	5*
Number of women	3
Number of men	2

*****Students in a career internship program at California State San Bernardino, required to work 100 hours/quarter in a research laboratory.

** M.Sc. Students

Note on Question 1: For the number of higher education partnerships, please count the partnership between your institution and your Pakistani counterpart's institution as <u>one</u>. If your project also involves collaboration with other Pakistani institutions / US institutions (universities, research institutes, government agencies, or non-governmental organizations), please add each such additional institution to your total.

Annexure I

Sr.No.	Group No.	Cultivar Name	Accession No.	Seedling Nos./Remarks
1	1 Citrus relative	Aeglopsis chevailieri	P1 539143	Dry seeds
2		Atalantia ceylanica	P1 539146	Dry seeds
3		Balsamocitrus dawei	P1 539147	Rotten/dead seeds
4		Bergera koenigii	P1 539745	Watery seeds
5		Citropsis daweana	P1 247137	*(Dry seeds)
6		**Clausena lansium**	**P1 539716**	NP
7		Clausena harmandiana	P1 600640	02(Fungus)
8		Clausena excavata	P1 235419	18(Fungus)
9		Glycosmis pentaphylla	P1 127866	11 (Watery)
10		**Glycosmis perakensis**	**P1 600638**	NP
11		**Glycosmis tricanthera**	**RRUT 12**	NP
12		Hawaiian Mock Orange	P1 539747	06(Testa split in some seeds)
13		Hesperethusa crenulata	P1539748	Dry seeds
14		**Orange Jessamine**	**P1539283**	NP
15		Sevirinia disticha	P1 607467	Dry seeds
16		**Triphasia trifolia**	**P1 539800**	NP
17	2 Mandarin	Citrus amblicarpa	P1 93601	10
18		Citrus leiocarpa	P1 539276	01(Dry seeds)
19		Citrus lycopersicaformis	P1 539347	Dry seeds
20		Citrus nippokoreana	P1 53945	Sunken seeds/Dry
21		Citrus sunki	P1 539678	12(Testa Open in some/Dry)
22		Parson s special	P1 539497	32
23		Scarlet emperor	P1 539505	33
24		Soh niamtra	P1 254779	Dry seeds
25		Sun Chu Sha	P1 539544	39
26	3 Sour Orange hybrid	Citrus intermedia	P1 539255	Half Shrivelled seeds
27		Citrus maderaspatana	P1 539348	
28		Gabbuchinee	P1 539225	6
29	4 Sour Orange	Bouquet des Fleurs	P1 539174	Dry seeds

30		Goutoucheng	P1 539170	02
31		Nansho daidai	P1 539680	03
32		Standard	P1 539176	NG
33		Zhuluan	P1 539171	6
34	5 Papeda	Citrus ichangensis	P1 539253	Shrivelled Seeds
35		Hanayu	P1 539198	NG
36		Citrus latipes	P1 230987	18
37	6 Papeda Hybrid	Citrus macrophylla	P1 539182	12
38	7 Citron Hybrid	Citrus halimii	P1 539196	Dry seeds
39	8 Citrumello	African shaddock x Rubidoux trif	P1 539830	16 (Half shrivelled seeds)
40		Swingle	P1 539828	11(Fungus)
41	9 Lime	Davaoensis	P1 539187	11(Half shrivelled)
42		Limon real	P1539193	04 (Shrivelled /Dry seeds)
43		Maxican	P1 600629	Half dry seeds
44		Winged lime	P1 539346	13(Half shrivelled seeds)
45	10 Citron	Diamante	P1 539424	08
46	11 Microcitrus hybrid	Faustrimedin	P1 539855	Dry small sized seeds
47		Sydney Hybrid	P1 539740	09 (Some seeds dry)
48	12 Microcitrus	Microcitrus australis	P1 312881	Dry seeds
49		**Microcitrus inodora**	**P1 539742**	NP
50		Var.sanguinea	P1 539734	Dry seeds
51	13 Rough lemon	Florida	P1 539268	01
52	14 kumquat	Fortunella hindsii	P1 539723	Dry seeds
53		Meiwa	P1 539721	Dry seeds
54		Nagami	P1 539729	Dry seeds
55	15 Kumquat Hybrid	Procimequat	P1 539805	Dry small sized seeds
56	16 Lemon- eureka	Fros eureka	P1 539318	09
57	17 Pummelo Hybrid	Hassaku	P1 539223	3
58	18 Trifoliate	Little-leaf	P1 600648	6 (Half Shrivelled seeds/Fungus)
59		Simmons	P1 539780	15 (Half dry Seeds/Fungus)
60	19 Trifoliate Hybrid	S-281 citrangelo	P1 539842	04(Half dry Seeds)

61		X 639	P1 539847	13
62	20 Pummelo	Reinking	P1 539391	NG
63		Mato buntan	P1 539398	NG
64	21 Sweet Lime	Palestine	P1 539283	02(Dryseeds)
65	22 Sweet Orange	Pineapple	P1 539622	13 (Some seeds Shrivelled)
66	23 Citrange	Rusk	P1 13002	Fungus
67	24 Lemon Hybrid	Unnamed	P1 539804	09
68	25 Tanger	Citrus benikoji	P1 539178	Some seeds Shrivelled

NP = highlighted were not available.
* Total seeds = 02
NG = Not germinated
Fungus = Fungus attack on seed at the time of sowing