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FOREIGN EXPLORATION FOR NATURAL ENEMIES OF ASIAN CITRUS PSYLLID, *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE), IN THE PUNJAB OF PAKISTAN FOR USE IN A CLASSICAL BIOLOGICAL CONTROL PROGRAM IN CALIFORNIA USA

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ABSTRACT

Asian citrus psyllid, *Diaphornia citri*, is a global pest of citrus and also the vector of a bacterium that causes Huanglonbing (HLB), a lethal and incurable malady of citrus. This pest established populations in southern California USA in 2008. In 2012, HLB was discovered in California. As part of a much larger program targeting the control of *D. citri*, a classical biological control program was initiated with the intent of finding, importing and establishing in California host specific parasitoids that attack *D. citri* nymphs. Foreign exploration efforts were focused in the Punjab of Pakistan, part of the presumed natural range of this pest and also an area which has a strong climatic match to the major citrus production areas of Southern California. Five foreign exploration trips were completed over the period September 2010 to June 2012. Almost 2,000 parasitoids of *D. citri* were collected and returned to Quarantine at the University of California Riverside (UCR). The parasitoid guild was dominated by two primary parasitoids, *Tamarixia radiata* and *Diaphorencyrtus aligarhensis*. At least one hyperparasitoid, *Marietta leopardina*, was found attacking immature *T. radiata* and *D. aligarhensis*. By June 2012, almost 5,000 *T. radiata* had been released in Southern California and initial field surveys tentatively suggest establishment is likely at about 20% of release sites. Molecular analyses are underway to confirm that collected *T. radiata* are of Pakistani origin. The classical biological control program is now entering a more aggressive mass production phase for *T. radiata* and host specificity testing of *D. aligarhensis* is underway in Quarantine at UCR.

Keywords: California, Classical biological control, *Diaphorencyrtus aligarhensis*, *Diaphorina citri*, Foreign exploration, Hyperparasitoid, *Marietta leopardina,* Pakistan, *Tamarixia radiata*

INTRODUCTION

In September 2008, Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) was found in Southern California USA and it is presumed that founders may have invaded from Mexico where this pest is widespread and present in some Mexican cities (e.g., Tijuana) that border Southern California. Asian citrus psyllid (ACP) is considered to be one of the world's most serious threats to economic citrus production because it vectors a bacterium, *Candidatus Liberibacter asiaticus* (*C*Las), that causes Huanglongbing (HLB) (also known as citrus greening; HLB is Chinese and translates to yellow dragon disease), a malady that is lethal to most varieties of citrus (Halbert and Manjunath, 2004). The ACP-HLB combination is seen as a major threat to California's $1.2 billion (US) citrus industry (Grafton-Cardwell, 2008).

In countries where this psyllid-bacterium combination have successfully invaded, citrus production has dropped markedly because trees go into decline losing vigor, leaves drop from trees and the canopy becomes thin and developing fruit tends to become small, misshapen and bitter (Halbert and Manjunath, 2004; Halbert, 2006). The most dramatic impacts have been observed in Brazil and Florida the world's two largest producers of orange juice. In Florida, ACP was discovered in 1998, in 2005 HLB was detected for the first time, and by 2009, about 10% of citrus acreage (~60,000 acres) in Florida was unproductive because of HLB. The citrus industry in Florida is worth about $9 billion (US) and this reduction of citrus acreage in Florida corresponded with an estimated loss of 8,257 jobs, $2.7 billion (US) in lost revenues, and $1.8 billion (US) in lost economic activity

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associated with the citrus workforce in Florida (Hodges and Spreen, 2012).

In April 2012, the first detection of HLB in California was made in Hacienda Heights in Los Angeles County (Leavitt 2012). The California Department of Food and Agriculture (CDFA) detected *C*Las in an ACP found feeding on a backyard pumelo with a variety of citrus grafts, some of which may have originated from China, an area with widespread infestations of HLB. It is probable that these grafts were illegally introduced into California from an overseas source. Subsequent testing of the tree on which the ACP was found feeding confirmed the presence of *C*Las in this tree. The infected tree was eradicated and all citrus within an 800 meter radius of the infected tree were treated with insecticides by the CDFA to kill any ACP that may be carrying bacteria that cause HLB (Leavitt, 2012). Additionally, a 150 km^2 quarantine was established around the HLB find site. This quarantine prohibits the movement of all citrus nursery stock out of this area in case they are infected with HLB and these plants, if infected, could start new disease infestations in different areas of California. Any fruit from residential properties must not be moved to other areas in case ACP or HLB are accidentally moved into new areas (Leavitt, 2012).

DEVELOPING A CLASSICAL BIOLOGICAL CONTROL PROGRAM FOR ASIAN CITRUS PSYLLID IN SOUTHERN CALIFORNIA USA

In an attempt to manage ACP in California, Entomologists at the University of California Riverside have been working at the University of Agriculture in Faisalabad in Pakistan (formerly known as the Punjab Agricultural College and Research Institute in Lyallpur) to better understand the impacts natural enemies (predators and parasitoids) have on regulating ACP populations in the Punjab of Pakistan. The reason this area was selected for investigation is because the Punjab of Pakistan and India may be part of the area where ACP and HLB evolved. The reason for this assumption is that the first study on ACP was published by Mohammad Husain (an Imperial Entomologist based in Pusa India) and Dina Nath (an Assistant Entomologist with the Department of Agriculture in the Punjab of India) who studied ACP attacking citrus primarily in the Punjab region of Pakistan (Husain and Nath, 1927). Their research entitled "The Citrus Psylla (*Diaphorina citri* Kuw.) Psyllidae: Homoptera" was published in 1927 (Husain and Nath, 1927).

From the point of view of a biocontrol specialist, one of the most intriguing statements made in Husain and Nath's (1927) study is on page 24 of their 27 page publication. Here they state that nine species of parasitoid attack ACP in the Punjab, and several of these parasitoid species have their own parasitoids, or hyperparasitoids, attacking them. This high diversity of parasitoid species associated with ACP as reported by Husain and Nath (1927) also suggests that the Punjab may be an important part of the home region for ACP. Biological control theory states that natural enemy diversity should be highest at the evolutionary center of origin of a pest, because at this location, the greatest amount of time has been available for the evolution of a diverse guild of natural enemies to exploit the pest for food (Van Driesche *et al*., 2008).

Fig. 1
Mr. Zaman Shouket Khan, Mr. Muhammad Fakhar-ud-Din Razi, and Christina Hoddle processing parasitized ACP nymphs in a citrus orchard in Sargodha Pakistan.

Another aspect affecting the region from which natural enemies should be sourced for introduction against a pest in another area is consideration of climate. It is thought that searching for natural enemies in the part of the pest's native range that has a climate similar to the intended receiving range (i.e., the invaded area) may greatly increase the likelihood of parasitoids establishing and performing well as they are pre-adapted to the prevailing climatic conditions the pest is now living in (Van Driesche *et al.*, 2008). Consequently, climate matching can help focus and narrow down large geographical areas that need to be searched for natural enemies. In the case of California, CLIMEX, a climate modeling program was used to match the climate of the Central Valley of California (specifically Bakersfield) with an area within the presumed native range of ACP. The Punjab of Pakistan had about a 70% climate match with the major citrus production areas of California. This climate matching result when coupled with Husain and Nath's (1927) observations on parasitoids attacking ACP further reinforced the decision to prospect for natural enemies of this pest in the Punjab of Pakistan for use in a classical biological control program against ACP in California.

What is very curious about Husain and Nath's work is that today, we know of only two species of parasitoid that attack ACP, not nine species. One of these named parasitoids *Tamarixia radiata*, was reared from ACP living on lemon leaves collected in the Punjab of Pakistan in 1921 (Waterston, 1922). The second parasitoid, *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) was described in 1975 after it was reared from ACP collected in India (Shafee *et al*., 1975).

This lack of information on parasitoids associated with ACP in the Punjab of Pakistan raises several important and intriguing questions that need to be resolved: What are these other eight species of parasitoid attacking ACP in the Punjab of Pakistan? Is *Diaphorencyrtus aligarhensis* one of these eight species or is it an additional species that was not reared by Husain and Nath in Pakistan? What is the collective impact of this parasitoid guild on ACP in Pakistan? Is it great enough

to reduce the pest status of this insect in citrus? Could the performance of some of these ACP parasitoids be greatly improved if they were imported into other parts of the world, such as California, without their hyperparasitoids for classical biological control of ACP?

Fig. 2
Mr. Shouket Zaman Khan and Mark Hoddle with processed parasitized ACP nymphs that were moved under permit from Faisalabad to the Quarantine Facility at the University of California, Riverside.

INITIATING A CLASSICAL BIOLOGICAL CONTROL PROGRAM FOR ASIAN CITRUS PYSLLID WITH SUPPORT FROM PAKISTANI COLLEAGUES

To develop a better understanding of the ACP-natural enemy situation in Pakistan, the author and his wife, Christina Hoddle, visited the University of Agriculture in Faisalabad (UAF) in September 2010 to meet with collaborators and to identify potential field sites for the collection of ACP natural enemies and for initiating medium term phenology studies on ACP and associated natural enemies. Vice Chancellor Dr. Iqrar Khan, a University of California Riverside (UCR) Alumnus and HLB researcher enthusiastically endorsed this potential collaborative project between UCR and UAF. Consequently, during this visit to assess logistics it was determined that UAF would be an excellent base in which to commence this biocontrol project. It also provided the opportunity to recruit and train a M.Sc. student (Mr. Shouket Zaman Khan under the tutelage of Dr. Mohammad Jalal Arif in the Department of AgriEntomology, UAF) to run a two year phenology study on ACP and its associated predators and parasitoids on Kinnow and sweet orange at two different study sites at UAF, Square 9 and PARS (Hoddle, 2010).

Since this initial "scouting" trip, four additional visits to UAF were completed: (1) March 10 to April 10, 2011. During this period, 5,000 ACP were field collected from sites around Faisalabad, Toba Tek Singh, and Sargodha. About 150 parasitoids were reared and returned to Quarantine at UCR to establish colonies for host specificity testing (i.e., safety tests against non-target psyllids in California). During this time, a Malaise trap was set up in Square 9 to monitor ACP and natural enemy phenology, and to collect other insect species associated with citrus in Pakistan. Knowing the identity of these species may help identify potential new invasive pest species that could become problematic in the future. The MS student was recruited and trained to work on ACP with these specific research goals: (A) monitor ACP and natural enemy phenology on Kinnow and sweet orange; (B) monitor flush growth phenology of kinnow and sweet orange for comparison to ACP population phenology, (C) measure percentage parasitism and rearing of ACP parasitoids, (D) find other species of native psyllids and rear natural enemies from these species. (2) June 4-13, 2011, about 400 ACP parasitoids were collected and returned to UCR for rearing in Quarantine. (3) October 23-28, 2011, more than 1,000 ACP parasitoids were returned to Quarantine at UCR. (4) June 2-5, 2012 more than 400 parasitoids were returned to the UCR Quarantine Facility.

Two ACP parasitoid species dominated the collections from Pakistan during these collection trips; *T. radiata* and *D. aligarhensis* were the most common parasitoids collected. Three additional parasitoid species were reared from the March 10 to April 10, 2011 collections: (1) *Sympiesis* sp. (Eulophidae), a possible leafminer parasitoid, 52 specimens were reared out in Quarantine but did not reproduce on ACP. (2) C*irrospilus* sp. (Eulophidae), a leafminer parasitoid, 5 specimens were reared but were not tested on ACP. (3) *Citrostichus phyllocnistoides* (Eulophidae), a leafminer parasitoid, 15 specimens reared but were not tested on ACP. Collection of these contaminants was very instructive because they suggest that Husain and Nath (1927) probably overestimated the number of parasitoids attacking ACP. It is very possible that these other parasitoid species were accidentally reared from other pests that were unobserved in their collections. Consequently, they were recorded as ACP parasitoids when in fact they were not. A hyperparasitoid, *Marietta leopardina*, was reared from < 5% of parasitized ACP collected in October 2011 and June 2012.

ESTABLISHING PAKISTANI PARASITOIDS IN CALIFORNIA FOR CLASSICAL BIOLOGICAL CONTROL OF ASIAN CITRUS PSYLLID

ACP is established in two major but widely separated areas in southern California: (1) along the US-Mexico border in San Diego and Imperial Counties, and (2) Los Angeles County with satellite populations established in neighboring San Bernardino and Riverside Counties. All of the major infestations are in urban areas and citrus growing in gardens in residential areas is the major reservoir for this pest. At this time there are no known ACP infestations in commercial citrus in California. The CDFA had been aggressively treating ACP in these areas with pesticides. Imidacloprid, a slow

acting systemic pesticide, was applied as a drench to the soil, while cyfluthrin, a contact insecticide, was sprayed on foliage to quickly kill nymphs and adults. The ACP infestation has been well controlled along the California-Mexico border, but less than satisfactory suppression has occurred in Los Angeles. Consequently, the CDFA modified its spray program in February 2012 once it became obvious that attempts to eradicate this pest in Los Angeles County were unlikely to succeed.

Economics were a major reason behind the decision to suspend the ACP spray program in Los Angeles. In 2000, US Census data estimated that there were 3,270,909 housing units in Los Angeles. A crude visual survey of houses in the cities of Compton and Bell Gardens in Los Angeles County indicated that at least 63% of residences had 1 or more citrus growing in gardens. This translates to about 2,044,318 residences in Los Angeles County with at least one citrus tree. By October 2011, the CDFA had treated citrus with pesticides in 67,863 residences, or about 3% of Los Angeles properties with citrus. This treatment program cost $10,722,657 (US) or about $158 per residence (treatment data taken from the CPDPC 2011/12 Budget.) Clearly this was unsustainable, and this program was drawing resources away from dealing with the leading edge of the ACP invasion out of Los Angeles that were moving towards major production areas in Ventura County, and the Central and Coachella Valleys. The spray program was re-organized to concentrate on containing ACP at the leading edges of the spread from residential areas and this meant that urban areas behind the leading edge of ACP spread needed an alternative treatment program. The alternative to spraying that was decided upon was classical biological control, in particular, the use of parasitoids sourced from the Punjab of Pakistan for ACP suppression. These parasitoids came from the project detailed here. ACP surveys conducted in Los Angeles up to this point had failed to detect any significant natural enemy activity. This absence of natural enemy mortality, especially parasitism, may have facilitated the development of widespread and high density populations in Los Angeles; ACP was living in and exploiting natural enemy-free space. The classical biocontrol program had one major goal, re-associate host specific parasitoids from the home range of ACP with this pest in Los Angeles thereby re-constituting this specialized upper trophic level for top-down suppression of the target pest.

The first Pakistani parasitoid released in southern California for classical biological control of ACP was *T. radiata*. However, before this natural enemy could be released from Quarantine safety testing had to be completed. Psyllid species selected for host specificity testing of the Pakistani strain of *T. radiata* sourced from the Punjab in Quarantine were: *Dichlidophlebia fremontiae* (host plant = *Fremontodendron californicum*), *Euphyllura olivina* (a pest species of olives that is closely related to ACP because of its placement in the Euphyllurinae, to which ACP belongs), *Calophya californica* (*Rhus ovata*), two *Heteropsylla* spp. (host plants are *Acacia* sp. and *Prosopis* sp.), *Bactericera cockerelli* (a native pest attacking potatoes, tomatoes, and peppers), and *Arytainilla spartiophylla* (a self-introduced psyllid that attacks a noxious weed, *Cytisus scoparius*.) Results clearly demonstrated that ACP was the preferred host for *T. radiata*. A 60 page Environment Assessment Report detailing the results of these safety tests was prepared and submitted to USDA-APHIS on November 15 2011, at 6:00pm for review. On December 7 2011, USDA-APHIS issued a release permit clearing *T. radiata* from Quarantine for release in southern California (Hoddle 2011).

By June 2012, over 5,000 *T. radiata* had been released in about 30 different sites in Los Angeles County. Releases commenced in December 2011 (2 releases), and were repeated in January 2012 (2 releases), February 2012 (1 release), March 2012 (2 releases), April 2012 (1 release), May 2012 (1 release), and June 2012 (1 release). About 20% of total release sites (recoveries have been made in Azusa and Bell Gardens) have evidence of parasitism by *T. radiata*. Molecular analyses have commenced to confirm that *T. radiata* recovered from field sites is of Pakistani origin. There is evidence from at least one field site that suggests *T. radiata* has of its own volition moved about 100 m onto new ACP infestations. These initial results are encouraging because *T. radiata* releases began during winter when ACP densities and temperatures were low, and relatively few parasitoids have been released so far.

Fig. 3
Mark Hoddle examining an ACP nymph for evidence of parasitism at PARS, University of Agriculture, Faisalabad.

Fig. 4
Many farmers in Sargodha and Toba Tek Singh provided unrestricted access to their citrus orchards for the collection of parasitized ACP nymphs.

FUTURE PLANS

A major program is now underway at UCR to mass rear *T. radiata* for release in southern California. A significant effort has been made to preserve as much genetic diversity in the *T. radiata* populations collected from Pakistan as possible. To do this, about 13-18 isocage lines of *T. radiata* were established in Quarantine with parasitoids collected from different places and times in Pakistan. These cages have not been interbred, so the "genetic snapshot" represented in each cage has been preserved and this has theoretically prevented genetic homogenization and adaptation to prevailing conditions in Quarantine. Prior to release, material from each of the isocages is collected, commingled, and liberated at preselected release sites. This approach helps ensure that as much genetic diversity as possible is distributed across sites. This approach should allow natural selection to favor amongst the various *T. radiata* genotypes released those best adapted to prevailing environmental conditions at particular release sites. Additionally, host specificity testing is underway in Quarantine for *D. aligarhensis*. It is envisioned that this Pakistani guild of ACP parasitoids from the Punjab, *T. radiata* and *D. aligarhensis*, will be reconstructed in southern California for the classical biological control of ACP in citrus.

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