REARING METHODS FOR *AGENIASPIS CITRICOLA* (HYMENOPTERA: ENCYRTIDAE) AND *CIRROSPILUS QUADRISTRIATUS* (HYMENOPTERA: EULOPHIDAE) RELEASED IN A CLASSICAL BIOLOGICAL CONTROL PROGRAM FOR THE CITRUS LEAFMINER *PHYLLOCNISTIS CITRELLA* (LEPIDOPTERA: GRACILLARIIDAE)

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ABSTRACT

Rearing techniques for *Ageniaspis citricola* and *Cirrospilus quadristriatus* and their host, the citrus leafminer, *Phyllocnistis citrella*, are discussed as related to a classical biological control program. Methods for rearing the three trophic levels (citrus plants, leafminers, and parasitoids) are described in detail. An average of 1,155 adult citrus leafminers were produced in a cage filled with 60-72 young citrus trees. Between April and October 1994, a total of 15,230 *A. citricola* were produced, with an average of 750 adults per cage. Between late July and October 1994, a total of 1,328 *C. quadristriatus* were produced, with an average of 144 adults per cage. The two parasitoids were released into leafminer-infested citrus groves throughout Florida in a classical biological control project.

Key Words: Citrus leafminer, *Phyllocnistis citrella, Ageniaspis citricola, Cirrospilus quadristriatus*, biological control, rearing methods.

RESUMEN

Se discuten las técnicas para la cría del minador de los cítricos *Phyllocnistis citrella* y dos de sus parasitoides, *Ageniaspis citricola* y *Cirrospilus quadristriatus*, en relación a un programa de control biológico clásico. Son descritos en detalle los métodos para criar los tres niveles tróficos (plantas de cítricos, minadores y parasitoides). Se produjeron un promedio de 1,155 adultos del minador en una jaula provista con 60-72 plantas jóvenes. De abril a octubre de 1994 se produjeron un total de 15,230 *A. citricola*, con un promedio de 750 por jaula. De fines de julio a octubre de 1994 se produjeron un total de 1,328 *C. quadristriatus*, con un promedio de 144 adultos por jaula. Como parte de un proyecto de control biológico clásico, se liberaron los dos parasitoides en huertas de cítricos infestadas por el minador, en varios sitios del estado de Florida.

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton, (Lepidoptera: Gracillariidae) is an important pest of citrus. It affects production and can augment the severity of citrus canker (*Pseudomonas citri* Hasses) on damaged plants (Sohi & Sandhu 1968). The CLM originates from southeast Asia (Heppner 1993). Perhaps due to natural dispersal patterns and shipment of infested citrus, it has become established in other citrus production areas throughout the world.

The adult CLM is a minute moth, 2.5 mm long with folded wings and with a 4.5 mm wingspan. Adults emerge from their pupal chambers early in the morning (Beattie & Smith 1993). Mating, which lasts an average of 22 minutes (Pandey & Pandey 1964), occurs at dusk and early evening, 9 to 12 hours after emergence. Egg-laying begins 1 to 8 days later (Badawy 1967; Ba-Angood 1977; Beattie & Smith 1993). A single female can lay up to 20 eggs per night, for a total of more than 50 in her 5- to 20-day lifetime (Beattie & Smith 1993). The translucent oval eggs are typically laid near the midrib of young leaves on the under side of the leaf (Badawy 1967; Ba-Angood 1977; Beattie & Smith 1993). Egg eclosion occurs 1 to 10 days later (Pandey & Pandey 1964; Beattie & Smith 1993) with the young larva immediately burrowing under the waxy cuticle of the leaf (Sohi & Verma 1965). The larva feeds on the cells of the epidermis, creating silvery, serpentine mines along the leaf (Sohi & Verma 1965) often causing the leaf to curl (Heppner 1993). During warm weather, the small larva progresses through three feeding instars in 5 to 6 days and enters a fourth, non-feeding stage (prepupa) for one day before forming a pupal chamber by folding over a leaf edge. The pupal stage can last 6 to 22 days, depending on the time of year (Pandey & Pandey 1964). A generation is thus completed in 14 to 17 days during warm summer months (Beattie & Smith 1993), but can be as long as 52 days in winter (Pandey & Pandey 1964).

The CLM was first recorded from Florida in May 1993 and has since dispersed throughout the state (Heppner 1993). It is now also recorded in Alabama, Louisiana, and Texas. The use of pesticides to control CLM is inefficient due to several reasons: price requirements for multiple applications, CLM larval stages are protected within their mines from topical applications, and the CLM may develop resistance to pesticides. Research to develop an integrated pest management program in citrus groves was initiated shortly after the CLM arrived. Classical biological control was identified as a high priority component of the IPM program. As part of the classical biological

control project for CLM in Florida, two parasitic wasps were imported from Australia: *Ageniaspis citricola* Logvinovskaya (Hymenoptera: Encyrtidae) and *Cirrospilus quadristriatus* Subba Rao & Ramamani (Hymenoptera: Eulophidae) in April 1994 (Hoy & Nguyen 1994a, b, c). These parasitoids are native to Asia and reported to be host specific to the CLM (Beattie 1992).

Synchronized rearing methods were developed in order to produce large numbers of the CLM and its parasitoids so inoculative releases could be made in Florida. The benefits of synchronized rearing for each species include: 1) the developmental stage and age of the colony is known; 2) the purity of the colony is easier to maintain; and 3) the likelihood of introducing pests and diseases into the colony is reduced. The purpose of this paper is to describe rearing methods for: 1) producing large numbers of citrus trees in flush suitable for rearing CLM in the greenhouse; 2) the CLM; and 3) the parasitoids *A. citricola* and *C. quadristriatus*.

Materials and Methods

Citrus

Citrus was either grown from seed or obtained as seedlings or grafted trees from local nurseries. Rough lemon (*Citrus jambhiri* Lushington) seeds were planted in a 1:1 mixture of potting soil and vermiculite in a cavity seedling tray (Hummert International, Earth City, MO) containing 96 2.5 × 2.5 × 7.5 cm cavities. Three 7- to 10-cm tall seedlings were transferred to each 3.8 liter plastic pot or 3.9 liter black plastic nursery bag (Poly-Cel, Hummert International, Earth City, MO). Two weeks after being transplanted, the seedlings were fertilized with a long-acting, slow-release fertilizer (19-10-10 plus iron, Once, Grace-Sierra, Horticultural Products Company, Milpitas, CA). Fertilizer was reapplied after 6 months. Seedlings were ready to use as hosts for the CLM when the stems were approximately 5 mm in diam and 30 to 50 cm tall. The time required to achieve this size depended upon the time of year the seeds were planted, ranging from 7 months if planted in fall/winter to 4 months if planted in spring/summer.

Grapefruit (*Citrus* × *paradisi* Macf.) and sour orange grafted on trifoliate orange (*Citrus trifoliata* (L.) Raf.) rootstock also are suitable as hosts for CLM. Both produce a large amount of flush from the nodes after pruning (1/4 to 1/2 of each branch removed) and leaf stripping. Grafted trees were obtained from nurseries when they were approximately 60 cm tall. Grafted trees were treated to reduce pest infestations by pruning and spraying them with oil (15 ml 97% petroleum oil, Ortho Volck Oil Spray, Chevron Chemical Company, CA in 3.8 liter water). The pruned plants produced sufficient flush for use after approximately 2 weeks at 30°C and 80% relative humidity. Other citrus varieties, including rough lemon, lime, trifoliate orange, and swingle, also were donated as seedlings and reared as above. All proved suitable for rearing the CLM.

Citrus plants were maintained either in a greenhouse or a shadehouse. Seedlings were grown in a 6.6 × 9.1 m greenhouse covered with a shadecloth that provided 35% shade. The average temperature in this greenhouse was 30°C (temperatures occasionally reached a maximum of 37.8°C and a minimum of 23.3°C) and the average relative humidity was 80% (90-100% for approximately 14 h per day, as low as 50% for a short time during the heat of the afternoon). Trees, including those recently pruned, donated material, and other extras, were also housed in a 6.1 × 24.4 m shadehouse constructed of 50% shadecloth. Recently-pruned plants were placed in the shadehouse within 61 × 61 × 61 cm mesh cages (BioQuip, Gardena, CA) until they had produced

new flush with no CLM. All trees were watered when needed, usually 2 to 3 times per week.

Pest Problems. A variety of pests had to be managed in the greenhouse where trees were reared, including citrus whitefly (*Dialeurodes citri* Ashmead), broad mite (*Polyphagotarsonemus latus* Banks), and citrus red mite (*Panonychus citri* McGregor). Trees in the greenhouse and shadehouse were monitored at least weekly for pests. Broad mites were controlled by lightly hand dusting only the new flush with sulfur (90% sulfur, Southern Agricultural Insecticide, Inc., Hendersonville, NC) semiweekly or when needed. Citrus whiteflies and citrus red mite were controlled by spraying with 5% insecticidal soap solution (Safer, Inc. Eden Prairie, MN) semiweekly. The sulfur and insecticidal soap were applied on alternate weeks.

Citrus mealybug (*Planococcus citri* Risso) was an occasional pest in the shadehouse. Mealybugs were physically removed from plants when detected. Some trees also became infested with scale insects, primarily Caribbean black scale (*Saissetia neglecta* DeLotta) and cottony cushion scale (*Icerya purchasi* Mask.). When scales of any type were discovered, the adult scales were removed by hand and the plant was sprayed with insecticidal soap, or the plant was discarded if the infestation was severe.

Citrus Leafminer

CLM-infested foliage was initially obtained from citrus groves around Lake Alfred, Florida in February 1994. Infested foliage was also obtained occasionally from trees in Gainesville to supplement the colony. Occasionally, mines were found with dead larvae (<5%), but no bacterial, fungal, or viral diseases were observed in CLM larvae or pupae although detailed observations were not made.

Initially, isolation of adult moths from the infested foliage was difficult. Several standard methods produced few adults or were very labor-intensive. Placing infested leaves on a water-soaked cotton pad in glass petri dishes was attempted; moth emergence rates were high, but collection was slow and it was a space- and labor-consuming procedure. Infested foliage was placed in several dark containers of different sizes fitted with one or more glass emergence tubes at the top, streaked with honey. The moths preferred to rest on the leaves and did not fly toward the light unless disturbed. The few that flew into the tubes often did not stay for a long period of time but returned to the foliage to rest.

The most efficient emergence method tested involved placing infested leaves with pupal chambers in clear plastic bags. Approximately 120 leaves were placed in each 30.4×25.2 cm bag. If only a few leaves were placed in a bag, a pad of moistened cotton was added to prevent the leaves from drying out. The bags were then inflated by blowing into them, and the end of the bag was twisted and secured. The bags were placed under a fluorescent light in the laboratory. A high rate (averaging 81%) of adult CLM emerged, usually early in the morning. Adult moths could be removed from the bags every other day in late morning or early afternoon using a vacuum aspirator. The vacuum pump aspirated the moths through 5 mm plastic tubing into a 29.6-ml plastic cup. Mouth aspiration is unsafe because the moth scales are allergenic. The bags were wiped with paper towels after each aspiration to reduce condensation, although the CLM adults did not seem to be adversely affected by free moisture.

After the moths were aspirated into a cup, they were fed honey by adding a honey-soaked tissue (Kimwipe, Kimberly-Clark, Roswell, GA) or by streaking thin lines of honey on the lid or sides of the cup. Adult CLM were allowed to feed for 1 to 2 h before being placed in a large cage to mate and to oviposit on potted citrus trees in flush.

Citrus trees suitable for oviposition by CLM were those with young flush 1 to 2 cm long. We placed 20 to 24 pots into a 76.2 × 114.3 × 91.4 cm screened cage. Honey was streaked in fine lines on two 5 × 8 cm clear plastic sheets which were taped to the inside top of the cage frame as a food source for CLM adults. We added 175 to 250 adult moths (sex ratio unknown) to each cage by placing the opened plastic cups on the floor of the cage.

Fresh honey was streaked on the plastic sheets after 2 days. The trees were watered as needed, typically every three days. Cages were maintained in a 2.8 × 6 m greenhouse covered with shadecloth which provided 35% shade. The greenhouse for rearing CLM averaged 30°C (but occasionally reached a maximum of 37.8°C for a brief duration and a minimum of 23°C) and 80% relative humidity (with a minimum of 50% for approximately 1 h during the middle of the day). Because CLM adults survive best in >85% RH (J. Villanueva-Jiménez, unpublished), we attempted to maintain high humidity (around 80-90%) by flooding the floor of the greenhouse once or twice a day and/or by running portable humidifiers.

Young mines (1-3 mm long) were observed on the foliage after 4 to 6 days. Plants were then used to rear the 2 parasitoid colonies or for maintaining the CLM colony (Fig. 1). If the infested foliage was used for colony maintenance, leaves with pupal chambers, which developed 9 to 12 days after adding adults to the cage, were removed from the trees and placed in plastic bags in the laboratory for adult emergence as described above. Adults emerged in bags 4 to 21 days after the first pupal chambers formed.

To estimate productivity of the rearing, 3 large cages were selected at random between June and July and the number of CLM produced from 20 to 24 pots (containing 57 to 70 trees) per cage was recorded.

Pest Problems in the Citrus Greenhouse. To manage ants in the greenhouse, sticky barriers (The Tanglefoot Company, Grands Rapid, MI) were applied to the legs of the greenhouse benches. Additionally, commercial ant baits (Combat Insect Control Systems, Oakland, CA) were placed on the benches and in the cages. One species, *Tapinoma melanocephalum*, was especially difficult to control because they were not controlled with commercial baits. To reduce infestation by *T. melanocephalum*, plants were thoroughly watered in an attempt to flush any ant colonies from the pots before they were placed in cages with the CLM and again before the CLM-infested trees were transferred to cages containing the parasitoids.

Ageniaspis citricola

A. citricola is an endoparasitoid, parasitizing eggs and early instar larvae (Logvinovskaya 1983; Hoy & Nguyen 1994b; O. R. Edwards, personal communication) and producing 1 to 10 individuals per single host. Both males and females are found, contrary to previous reports of thelytoky (Evans 1995). Unmated females produce only male progeny, suggesting that this species is arrhenotokous (O. Edwards, personal communication). When first instar CLM larvae were visible on foliage, typically 4 to 6 days after CLM adults were introduced, the trees were ready to be used for *A. citricola* colonies. At this stage, foliage will have both eggs and mines containing first instar larvae. Prior to placing the trees in cages with *A. citricola*, they were thoroughly watered to reduce ant densities.

Parasitoid cages were maintained in the same greenhouse where citrus plants were housed. Fifteen to twenty pots, usually containing three infested citrus trees each, were added to each cage and 50 to 75 *A. citricola* adults were then introduced. The sex ratio of the introduced adults was unknown, although the average sex ratio

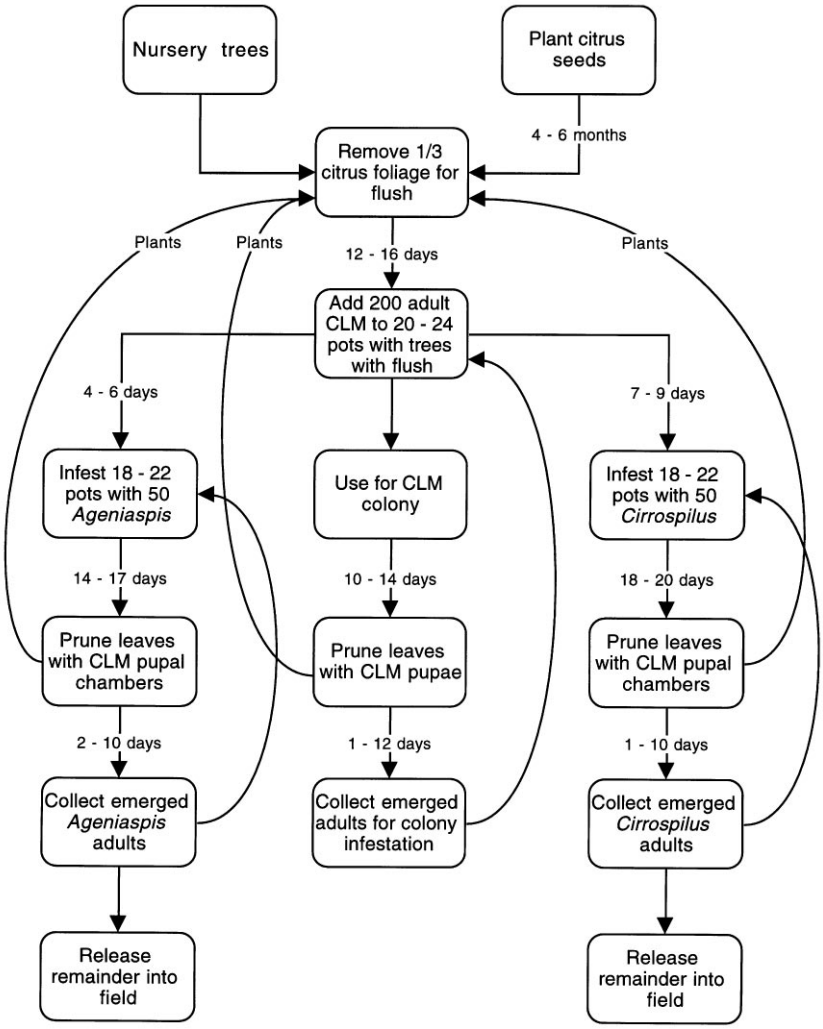

Figure 1. A flow diagram indicating the steps and approximate time involved in rearing citrus, the CLM and the two parasitoids. See text for more detail.

was 1 male:1.8 females based on 204 individuals sexed from random samples from 7 different cages between October and November. Honey was streaked on several pieces of plastic suspended from the top of the cage to provide food for parasitoid adults. Honey was reapplied after 48 hours and the plants were watered as needed.

Between April and October, new *A. citricola* adults emerged 16 to 18 days after adults were introduced into the cages. The parasitoids were collected by pruning foliage containing CLM pupal chambers from the trees after 15 to 17 days. The exact time was judged by opening a few pupal chambers to determine if the majority of *A. citricola* pupae were dark brown. The leaves were then placed in inflated plastic bags with

paper towels, and the bags were checked daily to collect wasps and to eliminate condensation on the bags. Adult parasitoids were collected once a day via mouth aspiration into a 50 ml vial containing tissues in the bottom to provide a soft surface. A honey-soaked strip of tissue was placed in the vial to provide food for the adult parasitoids.

If the parasitoids were to be released into citrus groves, the vials were placed in a growth chamber at 19°C until they were shipped. Parasitoids were delivered by automobile or shipped via overnight mail in styrofoam containers with blue ice packs to maintain temperatures at approximately 17°C. If the parasitoids were used to maintain the colony, they were allowed to feed and then were introduced into a new cage with trees infested with CLM eggs and early instar larvae. Adult *A. citricola* only live for 2 to 5 days, so they must be used for colonies or shipped to growers as soon as they are collected.

Problems Encountered. We encountered several problems in rearing *A. citricola*. Initial attempts to rear the parasitoids in a rearing room or in a shaded alcove failed to produce progeny, possibly because light intensity was low. Cages were then held within a greenhouse with a relative humidity below 60%. In an attempt to increase relative humidity, plastic sheeting was placed over the cages and a humidifier was placed under the greenhouse bench. These cages also did not produce wasps, possibly due to the high temperatures (>38°C) that were reached under the plastic.

Adult *A. citricola* are small (<2 mm) and difficult to collect from the cages because they tend to rest on the foliage and do not go to the top of the cage. High rates of adult *A. citricola* emergence were achieved by placing leaves with parasitized CLM pupae into inflated plastic bags, in a manner similar to that used to obtain adult CLM emergence. Adult parasitoids could easily be aspirated from the bags. One problem with plastic bags is the amount of condensation that develops on the inside. *A. citricola* are easily trapped and die in free moisture, so the bags must be wiped dry at least once a day.

Cirrospilus quadristriatus

C. quadristriatus is an ectoparasitoid of late instar larvae of the CLM (Beattie 1992; Hoy & Nguyen 1994c) producing a single individual per host. Both males and females are produced. Under our conditions, foliage inoculated with CLM reached the suitable host stage in 7 to 10 days. Trees with third and fourth instar larvae were watered and transferred into a new cage. Because adults live for almost 2 weeks, a mixed age class of trees was added to the cages (same size as used with the CLM). One third of the cage was filled with trees that had been infested with CLM for 7 to 10 days. We then added 50 to 75 adult wasps (unknown sex ratio, extremely difficult to sex) to the cage. After 2 days, another third of the cage was filled with new trees that were 7 to 10 days old. The last third was filled after another 2 days. Honey was streaked every other day on plastic sheets suspended in the cages and the plants were watered when needed. These cages were held in the citrus greenhouse.

Adult *C. quadristriatus* began emerging 11 to 13 days after they were introduced into the cages. Adults were aspirated from the cage every afternoon, when the wasps were most active. *C. quadristriatus* are easy to locate in cages due to their large size, orange color, and because they typically rest on the top of the leaves. After allowing the wasps to emerge in the cage for approximately one week, leaves with intact pupal chambers were pruned off each plant and placed in plastic bags to allow additional *C. quadristriatus* adults to emerge. This procedure was adopted to allow early wasps to emerge while allowing later larvae to continue to develop. Adult parasitoids were fed with a honey-soaked piece of tissue or by streaking thin lines of honey in the vial.

Adult *C. quadristriatus* can be held longer before being supplied to growers or used in colony maintenance because they live for approximately 2 weeks. The adults were placed in a growth chamber held at 19°C until they were used or shipped in the same manner as *A. citricola*. If held for a longer period of time, the adults were supplied with fresh honey every 48 hours.

Results and Discussion

Citrus Leafminer

A mean of 1426 ± 168 (± SD) intact pupal chambers were produced in each cage. The average number of leaves infested per tree was 10.2 ± 7.9, with a range from 0 to 35 leaves. The average number of CLM pupae produced per leaf was 2.8 ± 1.8. The maximum number of pupal chambers on one leaf was 13. Approximately the same number of pupal chambers were located on the lower surface of the leaf (1.8 ± 1.1) as compared to the upper surface (1.1 ± 1.0).

Ageniaspis citricola

Between April and October, the productivity of cages (n=21) used to rear *A. citricola* was evaluated. An average of 750 adults (± 410) was produced from 18 to 24 pots containing an average number of 60 trees in each cage. The maximum number of adults from a single cage was 1491, while the minimum was 109. One to ten *A. citricola* develop from a single CLM pupa; the average number of *A. citricola* individuals emerging per CLM pupal chamber in our greenhouse cages was 2.8 ± 1.1.

Cirrospilus quadristriatus

Since their release from quarantine in late July, an average of 144 (± 25.8) parasitoids have been reared from each of 9 cages, each containing approximately 60 trees. The maximum number of adults obtained from a single cage was 171 while the minimum was 101. Reasons for low rate of production of these parasitoids are unknown. We do not know how many eggs are laid by each female or the preferred relative humidity. Also, as already stated, only one *C. quadristriatus* is produced per host.

Conclusions

The methods described provide parasitoids for inoculative releases, but do not allow large scale augmentative releases. Rearing is time consuming and expensive because 3 trophic levels must be maintained. A total of 15,230 *A. citricola* were reared between April and October 1994, and 1328 *C. quadristriatus* were reared between late July and October 1994. This required 225.7 m^2 in greenhouse and shadehouse space, approximately 2,500 citrus trees, and one full-time employee devoted solely to this project (plus some hours performed by other employees). The potential for augmentative releases would be improved if an artificial diet was available either for the CLM or the parasitoids.

Acknowledgments

We thank Ru Nguyen for his advice on rearing and his efforts in rearing the parasitoids in the quarantine facilities. We thank Owain Edwards for advice and assis-

tance and Juan Villanueva-Jiménez for the spanish translation of the abstract. Ayyamperumal Jeyaprakash, Denise Johanowicz, Mark Pomerinke, Jim Presnail, Shawn Rogers, and three anonymous reviewers provided advice on the manuscript. This work was funded in part by the Citrus Production Research Advisory Council. This is journal series number R-04482.

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