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A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine

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Abstract

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, and two of its parasitoids, *Tamarixia radiata* (Waterston) [Hymenoptera: Eulophidae] and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal) [Hymenoptera: Encyrtidae], were reared under artificial light in a quarantine facility as part of a classical biological control program. *Diaphorina citri*, the vector of citrus greening disease, were reared on propagated orange jasmine (*Murraya paniculata* (L.) Jack) because it is not considered a host of the greening pathogen *Liberobacter asiaticum* and is more resistant to psyllid feeding damage than citrus. Each month, approximately 36,000 *D. citri* nymphs could be generated, yielding 6750 *T. radiata* and 1630 *D. aligarhensis* after 180 h of labor, and use of 150 *M. paniculata* plants, 20 m^2 of greenhouse space, and 18 m^2 of quarantine space. The developmental time from psyllid egg to the start of parasitoid emergence was 24 days for *T. radiata* and 28 days for *D. aligarhensis*. The sex ratio of laboratory-reared *T. radiata* was 1.8 ♀:1♂ ($n = 400$) in the Taiwanese population and 2.0 ♀:1♂ ($n = 400$) in the Vietnamese population. Both *T. radiata* and *D. aligarhensis* are long lived and could be stored for up to 30 days at 17 °C prior to field release, incurring less than 5% mortality. Ten percent of *T. radiata* (14 of 136 tested) and 10% of *D. aligarhensis* (22 of 209 tested) survived for 50 days when stored at 25 °C. Only females were observed from the population of *D. aligarhensis* obtained from Taiwan.

Keywords: *Diaphorina citri*; *Tamarixia radiata*; *Diaphorencyrtus aligarhensis*; Classical biological control; Parasitoids

1. Introduction

The Asian citrus psyllid (*Diaphorina citri* Kuwayama) is a vector of *Liberobacter asiaticum* now *Liberibacter asiaticus* (Garnier et al., 2000), which is the causative agent of greening disease in citrus (Huang et al., 1984). *Diaphorina citri* was discovered in three counties of south Florida in June 1998 (Knapp et al., 1998; Marais et al., 1998) and had spread to 12 counties in the principal citrus-growing region of Florida by 1999. By May 2000, *D. citri* could be found in 21 counties in south and central Florida (Halbert et al., 2000). By May 2001, the psyllid was discovered in Alachua County (Skelley, unpublished) and thus has colonized the entire citrus-growing region of Florida. No chemical control programs had been developed for this pest in Florida and, at the time of discovery, it had already spread past the geographic bounds for eradication efforts (Hoy and Nguyen, 1998).

Two potentially effective parasitoids, *Tamarixia radiata* (Waterston) and *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal), were identified from classical biological control projects which had dramatically reduced populations of *D. citri* in Reunion Island (Etienne and Aubert, 1980) and Taiwan (Chien and Chu, 1996). A classical biological control program for Florida was initiated (Hoy and Nguyen, 1998; Hoy and Nguyen, 2000; Hoy et al., 1999) and several small shipments of *T. radiata* and *D. aligarhensis* were obtained from Taiwan and Vietnam (Hoy and Nguyen, 2000). Colonies initially were established in the high-security quarantine at the Division of Plant Industry, Gainesville. Several hundred adult *T. radiata* from Taiwan and Vietnam were transferred to the quarantine facility at the University of Florida, Gainesville in May 1999. Laboratory tests were conducted to detect

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whether the parasitoids were contaminated by the greening bacterium, and all tested negative (Hoy et al., 1999, 2001).

Murraya paniculata (L.) Jack (Rutaceae) was chosen as the host plant to rear *D. citri* because it is not considered a host of *L. asiaticum* (Gottwald et al., 1989) and hence the likelihood of accidentally culturing the pathogen in the quarantined parasitoids and psyllids was reduced (Hoy et al., 1999, 2001). Greening disease is not yet present in Florida. Plants were propagated rather than purchased, because commercially available plants are commonly treated with systemic insecticides to control scale pests, which also could kill *D. citri*.

Tamarixia radiata (Hymenoptera: Eulophidae) is an arrhenotokous endoparasitoid that attacks fifth-instar *D. citri* (Chu and Chien, 1991). The adult females feed on younger nymphs and, by these combined actions, each can destroy up to 500 *D. citri* nymphs during her lifetime (Chien, 1995). *T. radiata* is not known to attack any psyllid other than *D. citri* (Aubert and Quilici, 1984).

The population of *D. aligarhensis* (Hymenoptera: Encyrtidae) from Taiwan is thelytokous, comprised only of females (Chien, 1995) and is infected with the intracellular endosymbiont *Wolbachia* (Jeyaprakash and Hoy, 2000), which could be the cause of its thelytoky. *Wolbachia* are able, in some cases, to transform arrhenotokous bisexual parasitoids into thelytokous populations (Stouthamer et al., 1990, 1999). *D. aligarhensis* are reported to use fourth-instar *D. citri* as hosts, and to destroy *D. citri* nymphs by host-feeding. By these combined actions, a single *D. aligarhensis* female can destroy 280 *D. citri* nymphs (Chien, 1995). Seventy *D. aligarhensis* females (Taiwan population) were received from the quarantine facility of the Division of Plant Industry in Gainesville Florida in June 1999.

No artificial diets exist to rear *T. radiata* and *D. aligarhensis*, so the psyllid host had to be reared. Both parasitoids are not known to attack hosts other than *D. citri* (Aubert and Quilici, 1984) and the rearing facility (Gainesville) initially was outside the infestation zone for *D. citri* in Florida. Hence, *D. citri*, *T. radiata*, and *D. aligarhensis* had to be reared in quarantine.

This paper describes the methods and resources required to propagate and rear plants, hosts, and parasitoids outside the *D. citri* infestation zone. In addition, experiments were conducted to determine if newly emerged (0- to 2-day-old) *D. aligarhensis* could develop on second-, third-, or fourth-instar *D. citri* equally well in no-choice tests, and an experiment was conducted to determine the reproduction rate through the lifetime of *D. aligarhensis* under quarantine conditions.

2. Materials and methods

2.1. Rearing *M. paniculata*

Murraya paniculata were grown in 3.8-liter plastic pots with a 4:2:1 mixture of potting soil (ProGro, The Scott's, Marysville, OH), perlite (Airlite Processing of Florida, Vero Beach, FL), and vermiculite (Verkite, Tampa, FL) in greenhouses or shade houses, then pruned to induce new growth, fertilized, and transferred into the quarantine facility. Temperatures in greenhouses were maintained at 15–35 °C. Fluorescent lamps suspended 1 m above the greenhouse benches provided a 16:8 (L:D) illumination cycle. Extra plants were housed on benches in unheated (range = 2–45 °C), roofed structures to control the amount of light and water the plants received.

The plants were watered approximately twice per week and fertilized monthly with Peter's 20–20–20 (N–P–K) water-soluble fertilizer (United Industries, St. Louis, MO) at a rate of 1 cc per liter of water (1 teaspoon/gallon). Greenhouses and roofed covers were either whitewashed or covered in shade cloth to provide 35% shade because *M. paniculata* scalded if kept in full sun.

2.2. Pest problems encountered in the greenhouses and the shade house

Nursery-grown *Murraya* had low levels of brown soft scale, *Coccus hesperidum* (Linnaeus) [Homoptera: Coccidae], and purple scale, *Lepidosaphes beckii* (Newman) [Homoptera: Diaspididae], when purchased in 1998, which became more numerous as the systemic pesticide titers in the plants dropped. Scales survived on greenwood cuttings for 6–10 weeks at 100% RH while the cuttings rooted. To control scale, the newly rooted cuttings were sprayed with 97% petroleum oil (Sunoco, Philadelphia, PA), at the rate of 3.95 ml per liter of water. In the third year of the program (2000), the parasitoid *Coccophagus lycimnia* (Walker) [Hymenoptera: Aphelinidae] was found attacking *C. hesperidum*, but oil applications were still needed monthly to control *L. beckii*. The oil spray and parasitoids were only partially effective, so heavily infested plants were discarded.

During the second year of the project (1999), the cowpea aphid, *Aphis craccivora* Koch [Homoptera: Aphididae], was routinely encountered on *Murraya* grown in greenhouses and shade houses. Cowpea aphids were controlled with a weekly spot treatment of 6% pyrethrins and 60% piperonyl butoxide, technical (Prentiss, Floral Park, NY) at a rate of 3.95 ml per liter water. In the third year of the program, *Lysiphlebus testaceipes* (Cresson) [Hymenoptera: Aphidiidae] heavily parasitized *A. craccivora* in the greenhouses, so pyrethrin treatments were infrequent.

Late in the third year (2000), the whitefly *Alueclava jasminii* (Takahashi) [Homoptera: Aleyrodidae] infested the greenhouse plants and were controlled with petroleum oil.

2.3. Murraya paniculata propagation

Murraya paniculata was obtained by rearing seedlings or rooted cuttings. Several hundred seedlings, 10- to 15-cm tall, were purchased from a commercial grower at the start of the project and kept uncovered on benches outdoors. They were slow to grow and establish a good root system and most were killed by root rot when the summer rains began. Thereafter, plants were reared outdoors under a roof so that water applications could be controlled. *M. paniculata* develops a strong root system on greenwood cuttings (Nguyen, personal communication), so 40 large (11.4-liter pot) shrubs (Lakeview var.) were purchased to generate cuttings. Growers commonly use the systemic pesticide avermectin (Novartis Crop Protection, Greensboro, NC) to control scale insects, so initially only the seedling plants were used to rear psyllids until the insecticide titer fell in the greenwood cuttings. Eventually, cuttings also were made from the seedling plants.

Greenwood cuttings were dipped in rooting hormone (indole-3-butyric acid, 0.1%, Schultz, St. Louis, MO) and potted, 10 per pot, into vermiculite. Pots were sealed in plastic bags and kept shaded in the greenhouse; approximately 90% successfully rooted after 6–10 weeks. Rooted cuttings were replanted singly into plastic pots (11-mm diameter × 9-mm high) and watered carefully. Propagated plants were repotted into 3.8-liter plastic pots when the roots filled the smaller pots. Plants were used to rear psyllids when they were at least 30 cm tall and stems were 7 mm in diameter, typically with 10–12 nodes.

2.4. Pruning M. paniculata to induce new growth

New growth was produced by removing the apical meristem and 5–10 of the topmost shoots and compound leaves, comprising as much as half of the leaves. Immediately after pruning, plants were sprayed with a mixture of 97% petroleum oil, 6% pyrethrins, and 60% piperonyl butoxide, technical, at a rate of 15 ml of each in 3.8 liters water, to control aphids and other unwanted insects. Any aphids subsequently found on developing shoots were removed by hand. Plants were fertilized with 20–20–20 (N–P–K) (Peter's) water-soluble fertilizer immediately after pruning, at a rate of 4 cc per liter water, then watered carefully until the new growth was 1–3 mm long, typically within 3–14 days. At this point, aphids, scale insects, and spiders were removed by hand and plants were moved into the quarantine facility.

2.5. Rearing D. citri

The quarantine facility had several physical limitations: there was no natural light, the three rearing rooms were small (ca. 2 m × 3 m), and more than one species had to be reared in each room. Rearing rooms (Fig. 1) were outfitted with standard cool white fluorescent fixtures (1.46-m long) hung approximately 2.5 cm above each rearing cage, which produced 5000–6000 lux (Digital Light Meter, Control, Friendswood, TX). A light regimen of 18L:6D was provided to stimulate maximum oviposition by *D. citri* (Yubin, 1989).

The quarantine rooms were maintained at 27 ± 2.5 °C, except once when the controls failed and temperatures reached 34 °C for five days. During this time *D. citri* females stopped laying eggs, but once temperatures were reduced they gradually resumed ovipositing over 2–3 weeks. The relative humidity (RH) in the room ranged from 30 to 65% (winter–summer). Relative humidity in the cage ranged from 80% just above the pot soil to 70% within the plant canopy (Airguide Humidity Indicator, Airguide Instrument, Chicago, IL). The RH at the top of cage was the same as that in the room. Psyllids produced fewer eggs when the room RH dropped below 40%. This was remedied by humidifying the

Fig. 1. Organization of cages and lights in a quarantine rearing room.

room to 45–55% RH with a cool-vapor humidifier (Duracraft, Southborough, MA). Airflow in the rearing rooms was provided by the HEPA-filtered heating, ventilation, and air conditioning system, and a small fan placed at floor level provided additional air movement. Workers wore gloves, a dust mask, and safety glasses when working in the rearing rooms or collecting psyllids or wasps to avoid contact with the waxy exudates and honeydew produced by *D. citri* nymphs and to prevent startled adult psyllids or wasps from jumping or flying into workers' eyes.

Cages (74.5 mm wide, 46.5 mm deep, and 61.5 mm tall) were constructed of 1.27-cm outer diameter (OD) (0.5 in. OD) polyvinylchloride (PVC) tubing and fine polyester voile covers. A flexible, clear plastic window was sewn into the fabric front of the cage to view insect colonies and plant conditions. Plastic trays (35.3 cm × 17.75 cm) rested on the PVC tubing to form the cage floors. The mesh cover allowed good air flow and prevented mold from developing on psyllid wax and honeydew, which had been a problem when psyllids were housed initially in wooden or plastic cages. Plants could be watered directly through the fabric mesh, thereby reducing the likelihood that psyllids or parasitoids could escape or cross-contaminate the colonies.

The cages were cleaned and sanitized with a dilute bleach solution (0.05% sodium hypochlorite) followed by a water rinse, or by wiping with 70% ethanol. Each rearing room contained nine rearing cages: eight placed on shelving units, and the ninth on a counter-top.

Colonies were reared in separate quarantine rooms to reduce contamination. Because the *T. radiata* colonies from Taiwan and Vietnam respond differently to temperature and humidity (McFarland and Hoy, 2001), we wanted to release them separately. The first room contained cages of the Vietnam colony of *T. radiata* reared on psyllid nymphs; room two held cages of psyllids only; and room three held cages of the Taiwan colony of *T. radiata* and *D. aligarhensis*.

Plants with young vegetative shoots (flush) were placed into a cage with egg-laying adults in the psyllid-rearing room. Reproductively mature *D. citri*, as indicated when the abdomen of males and females changed color from tan or green to orange (Husain and Nath, 1927), were aspirated from a holding cage and released onto the new plants at a rate of three per young shoot (Fig. 2). After 24 h, plants with more than three psyllids (♀ or ♂) per young shoot were tapped with the thumb and forefinger to redistribute adults more evenly, which produced a more uniform distribution of eggs.

Murraya paniculata plants were left in the cage with egg-laying adults for 2–4 days until *D. citri* eggs had been deposited at the base and folds of developing leaflets, or until the flush was 18 mm long, dark green, and hardened off. The adult psyllids were then removed from the plants by placing a slit paper towel around the base of the plant and tapping the stem and branches firmly to dislodge the adults. Most psyllids flew off or dropped onto the paper towel, which was immediately removed from the plant. The plant was removed quickly from the cage, and the few psyllids remaining on the plant were aspirated off. This tapping technique eliminated the need to aspirate hundreds of adult psyllids from the plants and allowed synchronous production of nymphs. A new set of plants with new shoots was then placed into the cage to allow *D. citri* females to oviposit again. Plants with eggs were held in separate cages until nymphs developed to the fourth or fifth instar, which was usually by day 10 or 12 after eggs were deposited.

2.6. Pest problems in quarantine

Aphids (*Aphis craccivora*) could out-compete the *D. citri* on young shoots, causing loss of the shoot and psyllid nymphs. *A. craccivora* were brushed or aspirated from plants every 2–3 days as the psyllid nymphs developed until parasitoids were introduced; thereafter, the plants were not disturbed. Aphid populations built up slowly over the next 12 or 18 days in the rearing cycles of *T. radiata* or *D. aligarhensis*, respectively. Plants were thoroughly sprayed with petroleum oil after the psyllid adults and parasitoids were collected from them. Eventually, because the aphids were successfully controlled by the parasitoid *L. testaceipes* in the greenhouses, the only infested plants left were in quarantine. These quarantine plants were autoclaved and the aphid problem was resolved.

Occasionally, crazy ants (*Paratrechina longicornis* (Latreille) [Hymenoptera: Formicidae] were brought into quarantine in *Murraya* pots. They were controlled by standing the shelving units in containers filled with soapy water and by maintaining boric acid/sugar solution bait (1 g boric acid/10 g sucrose/100 ml water) in the rooms. The entire potted plant was bagged and autoclaved when an ant colony was discovered in quarantine.

2.7. Rearing T. radiata

Room conditions for rearing *T. radiata* were the same as for *D. citri*, except the room temperature was maintained at 25 ± 2 °C for workers' comfort. *Murraya* plants were transferred to parasitoid rooms when the first individuals in the psyllid population reached the fifth instar (Fig. 2). Plants were placed on pebble-filled trays in the cages and the trays were watered to increase the RH. Paper strips (Kimwipe, Kimberly-Clark Corporation, Roswell, GA) impregnated with a mixture of honey and yeast extract (5% by weight, Difco Laboratories, Detroit, MI) were hung from the plants to provide food for adult wasps (Chien et al., 1994). Fifty *T. radiata*, in a ratio of approximately 2 ♀:1♂, were introduced into the

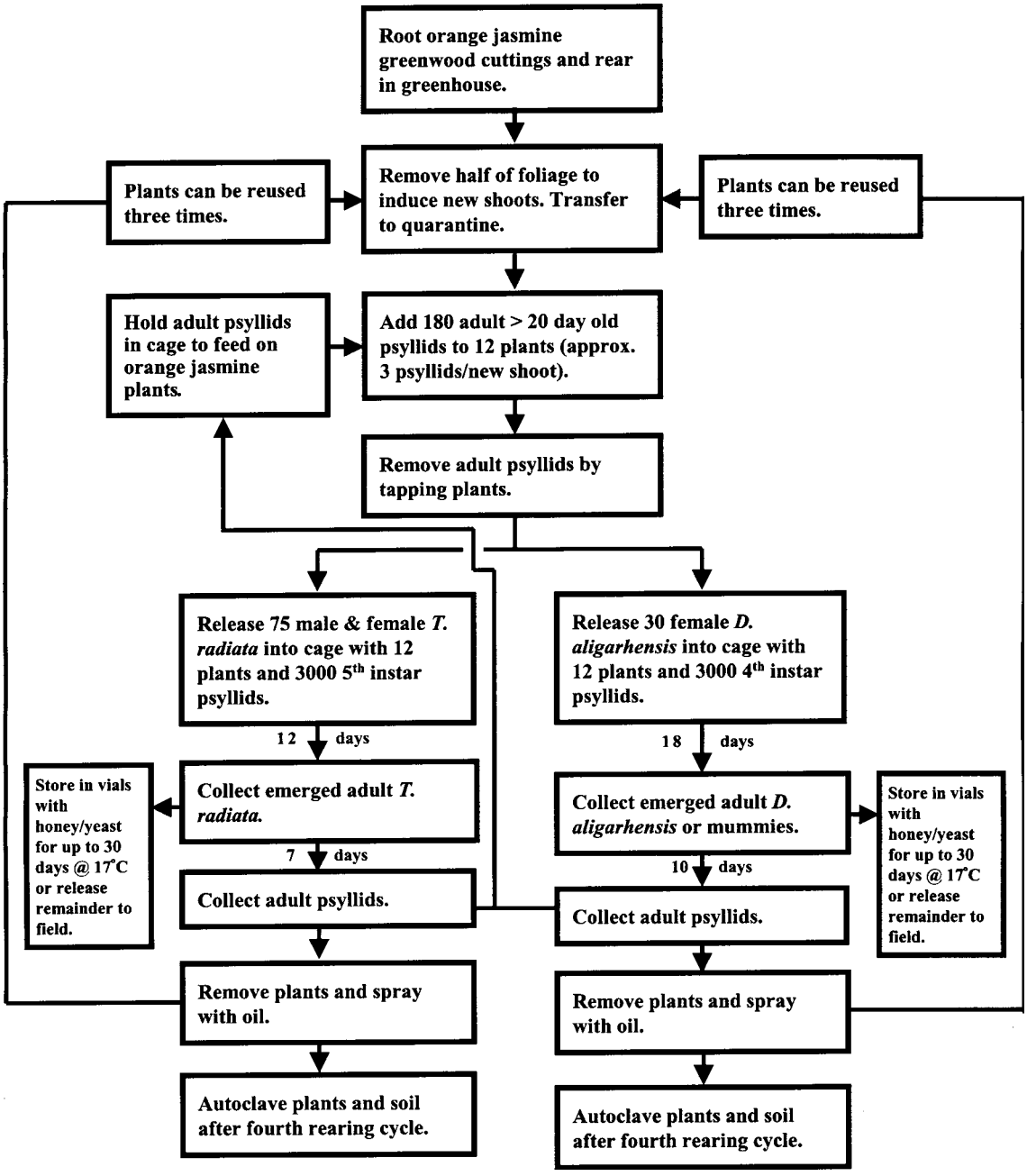

Fig. 2. Flow chart of steps and approximate time required to rear *Murraya paniculata*, *Diaphorina citri*, *Tamarixia radiata*, and *Diaphorencyrtus aligarhensis* in a synchronous, tritrophic quarantine system.

cage. For the first 5 days, tap water was sprayed through the fabric cage top with a squeeze bottle to form fine water droplets on the plant foliage from which the parasitoids were observed to drink. The water spray was withheld for the next 6 days to reduce mold growth on psyllid wax and honeydew, commenced on day 12, and continued until all progeny had been collected. Fresh honey/yeast strips were provided every 5 days.

Tamarixia radiata mummies were observed after 7 days, often clumped in groups as large as 50, typically along the midveins of leaflets and on the rachis and green twigs. *T. radiata* adults began to emerge 13 days after introducing the parents and were aspirated from the top of the cage in late afternoon because they moved from the plant canopy toward the fluorescent lights at that time. Progeny were collected every day for 7 days. On day 23, all remaining *T. radiata* and adult psyllids were collected. The plants were sprayed with petroleum oil, then pruned, fertilized, and placed in clean cages for 6–9 days to resprout. After a total of four pruning/rearing cycles, plants were autoclaved and replaced with fresh plants from the greenhouses.

To evaluate the sex ratio of progeny, *T. radiata* were collected from a rearing cage at the beginning, middle, and end of the emergence period (a total of 100 wasps), in four separate replicates for each colony over a period of 4 months. Each specimen was examined under a dissecting microscope and sexed on the basis of antennal and abdominal characters (Waterston, 1922).

2.8. Rearing D. aligarhensis

Diaphorencyrtus aligarhensis was reared in the same room as the Taiwanese colony of *T. radiata*. All cultural conditions were the same except plants bearing psyllid nymphs were transferred to the parasitoid rearing room when the first individuals in the nymph population reached the fourth instar (Fig. 2).

Thirty thelytokous *D. aligarhensis* females were introduced into the cage and *D. aligarhensis* mummies were observed 10 days later, typically in the upper half of the canopy, singly or in groups of two or three. Mummies are found along the outer margins, on the upper or lower surface of leaflets. In cages that went undisturbed for 7 days after emergence began, *D. aligarhensis* adults were observed to rest along the edges of the sewn-in window panel and preferentially on the plants closest to the window panel. *D. aligarhensis* adults were collected for 10 days, then the adult psyllids were collected and the plants were sprayed with petroleum oil. Two thousand *D. aligarhensis* from four rearing cages were examined and sexed on the basis of antennal and abdominal characters (Shafee et al., 1973).

2.9. No-choice and serial transfer tests with D. aligarhensis

Experiment one was a no-choice test designed to determine if *D. aligarhensis* could oviposit and develop equally well on second-, third-, or fourth-instar *D. citri*. Sixteen days after parents were introduced into a rearing cage, *D. aligarhensis* mummies were collected into a polystyrene tube outfitted with a blotted honey/yeast strip, and held at 25 °C and 14L:10D until adults emerged. Eighteen newly eclosed *D. aligarhensis* were collected individually into glass vials provisioned with honey/yeast strips and held for 24 h at 25 °C.

Concurrently, 50 *D. citri* at the second, third, or fourth instar were individually transferred with a 5/0 red sable-hair artists' brush onto each of 18 pruned and flushed plants. A double layer of paper coffee filters was placed around the plant stem to cover the soil surface and permit detection of fallen psyllids or dead parasitoids. A single parasitoid was introduced by pressing the base of the uncapped vial into the soil and quickly covering the plant with a plastic cylinder. Plants were held in the rearing room at 25 °C under 18L:6D conditions. After 24 h, each parasitoid was individually collected into a glass vial, labeled, frozen, and its hind tibia length (HTL) measured. Plants were kept in cylinders for 30 days and the resultant *D. aligarhensis* progeny were collected, labeled, counted, frozen, and HTL measured.

Because nine of the 18 wasps failed to leave the vials in the first experiment, and it was thought that the 24-h exposure time may have been too short, a second no-choice experiment was conducted with a cohort of 24 *D. aligarhensis*. Plants were prepared as before, but to discourage the parasitiod from staying in the vials, the individual vials did not contain honey/yeast strips. The parasitoids were 4- to 6-days-old when released into the cylinders and allowed 48-h of exposure to the psyllid nymphs.

In experiment three, 7 newly eclosed *D. aligarhensis*, prepared as in experiment one, were individually introduced onto 7 *M. paniculata* plants, each infested with approximately 150 *D. citri* (eggs through second instars). After 10 days, each parasitoid was located if possible, and the live wasps transferred onto new, psyllid-infested plants to host-feed and oviposit. This was repeated until the parasitoids were either dead or could not be found. All plants were kept covered with plastic cylinders. Thirty days after the parent was introduced, their progeny were collected, labeled, counted, frozen, and their HTLs measured. The experiment was replicated with 13 newly eclosed *D. aligarhensis*.

One-way analysis of variance was conducted on the datasets collected for each of the three experiments using StatView (Third edition) software for Macintosh (SAS, 1999).

2.10. Insect collecting, handling, and storage

Psyllids were collected with an aspirator and vacuum pump into padded plastic containers. Adult *D. citri* were difficult to collect until their behavior was characterized into three distinct types. Newly emerged adults do not fly or drop readily when the plant is tapped; they must be aspirated from the undersides of leaves. Reproductively mature *D. citri* appear to exhibit a dispersal behavior; they fly or drop when the plant is tapped, and hence the plants can be removed from the cage, leaving most of the psyllids behind. Actively ovipositing, gravid *D. citri* (on flush) do not fly or drop when the plant is tapped. Instead, they hide in the young leaflets and grasp the plant tightly. They can be coaxed into the aspirator collection tube by gently brushing them out of the foliage with a small sable-hair brush.

Adult *D. citri* were collected as a byproduct from the parasitoid colonies and transferred to a holding cage to feed on *M. paniculata* until they reached reproductive maturity (approximately 20 days after emerging as adults) (Fig. 2). They were then aspirated from the holding cage and released into a new cage of flushed plants to oviposit. The aspirator inlet tube used to col-

lect wasps was comprised of a 10-cm clear PVC tubing (Nalge, Rochester, NY) because both *T. radiata* and *D. aligarhensis* hopped or flew away from opaque tubing. Adult *D. aligarhensis*, unlike *T. radiata*, are not strongly attracted to bright fluorescent light (at the top of cages), nor were they attracted to ultraviolet lamps, or to yellow-colored surfaces (Samways, 1987). They usually were found resting on the undersides of the lowest leaflets of the plants, and searching the plants to collect them consumed 1 h per cage each day.

A less time-consuming method to collect *D. aligarhensis* involves aspirating the mummies into collection tubes supplied with honey/yeast strips and held at 75% RH in a small ($\leqslant 1$ liter) bell jar containing a pan of saturated $NaCl_2$ solution (Winston and Bates, 1960). The bell jar is kept in a growth chamber at 25 °C, under a 14L:10D light cycle. Overnight, *D. aligarhensis* adults emerge and can be collected into fresh tubes supplied with blotted honey/yeast strips. Ninety-five percent of mummies held in this manner emerged over a 7-day interval.

Parasitoids were collected, 50–100/vial, into 50 ml polystyrene tubes with blotted honey/yeast strips. After collection, a piece of polyester voile was placed over the open end of the vial and secured with a rubber band, and the vials were held overnight at 25 °C and 75% RH to humidify the wasps and honey. The fabric mesh then was replaced with a solid cap and Parafilm (American National Can, Greenwich, CT) was applied to ensure a vapor-tight seal. The sealed vials were held at 17 °C (Chien et al., 1993) in a growth chamber and removed to room temperature (ca. 25 °C) for several hours every other day to allow the wasps to feed. The stored wasps could be held until used to start a new colony or until released into the field (Fig. 2). Both *T. radiata* and *D. aligarhensis* could be held for up to 1 month with less than 5% mortality prior to release into the field.

2.11. Shipping

Parasitoids in 50-ml collection vials were shipped in styrofoam coolers. A frozen ice pack was placed into the cooler and approximately 2.5 cm of packing material (paper, cardboard, or foam sheet) placed on top of the ice pack to keep the parasitoids from freezing. Vials were sealed in a plastic bag, placed in the cooler, and additional packing was added to immobilize the vials. The styrofoam cover was then taped and the package shipped overnight for release in the field.

3. Results

3.1. Murraya paniculata propagation

Plants grew to a useable size 4 months after greenwood cuttings were planted. Of the 1800 plants propagated in the first year of the program, 1000 were used for rearing insects, while 800 were retained in the greenhouses for later use. Of the 1000 plants used for rearing insects, 700 were autoclaved and 300 were reused in quarantine during the winter months to conserve resources.

Plants could be reused through three cycles (Fig. 2) but each successive pruning/growth cycle produced smaller shoots, which resulted in fewer *D. citri* eggs deposited on them. By the fifth pruning, shoots grew only 25 mm on average and often shriveled and dried. By contrast, fresh plants produced shoots that grew as much as 150 mm, averaging 100 mm in length. Each plant produced an average of 5 shoots. Each cage in quarantine held 12 plants in 3.8-liter pots, with an average of 60 shoots. We found no advantage in using smaller plants or plants in small pots. Although more small pots fit in quarantine cages, small plants produced smaller and fewer shoots which supported fewer *D. citri* nymphs.

Approximately 225 liters of potting mix were needed to produce the 150 plants used each month in the program. About 5 m^2 of bench space was used for each 150 plants, so that 20 m^2 of covered bench space was in continuous use for plant propagation.

3.2. Rearing D. citri

Adult *D. citri* oviposited for 2–4 days on fresh shoots (Fig. 2) and utilized up to 95% of the shoots, producing 25–100 nymphs per shoot. Approximately 3000 [range = 1500–4500] nymphs could be produced in a cage on 60 shoots (12 plants/cage $\times$ 5 shoots/plant). Very high nymph densities (75–100/2.5 cm) caused the shoot tips to whither, killing the youngest nymphs before they could move down the shoot. Dwarfed shoots on reused plants supported an average of only 10 nymphs per shoot. Over- or under-watering plants had a negative effect on shoot growth and therefore on the population size and age-structure of the psyllid nymphs.

Under these quarantine conditions, the developmental time for *D. citri* from egg to adult was 15 days. Adult psyllids fed on *Murraya* an additional 20 days before reaching reproductive maturity. Adult psyllids are long-lived (Husain and Nath, 1927), and the psyllid colony could be over-wintered in quarantine for several months simply by keeping adults on healthy *Murraya* plants, where they fed on the mature hardened foliage.

3.3. Rearing T. radiata

During June to December 1999, 18 quarantine cages were initiated, each of which produced an average of 658 [standard deviation (SD) = 534, range = 40–2231] *T. radiata* (Taiwan) from 11 plants per cage. Between January and September 2000, after rearing methods

were further developed, each of 13 cages produced an average of 1085 wasps (SD = 618, range = 102–2296) on 13 plants per cage.

The productivity also doubled when rearing the Vietnam colony of *T. radiata*. Each of 13 cages initiated in 1999 produced an average of 402 wasps (SD = 399, range = 30–1207) from 15 plants per cage. In 2000, 15 initiated cages produced an average of 844 wasps (SD = 561, range = 49–1943) from 11 plants per cage. From the two colonies of *T. radiata*, a total of 17,000 were produced and 12,000 were released in 1999; 26,700 produced and 16,800 released in 2000; and 16,000 produced and 8000 released in 2001.

The sex ratio for a field population of *T. radiata* from Taiwan is reported to be 3.2 ♀:1 ♂ (Chien, 1995). The sex ratio in our laboratory was 1.8 ♀:1.0 ♂ ($n = 400$) for the Taiwan population, and 2.0 ♀:1.0 ♂ ($n = 400$) in the Vietnam population, and these ratios did not vary throughout the emergence period.

In both *T. radiata* populations, most parasitoids emerged on days 13–20 after parents were introduced into cages containing fifth-instar hosts (Fig. 2). Each *T. radiata* cage typically yielded 200 'unused' adult psyllids, which were returned to the holding cage in the psyllid room until they were used to produce eggs.

3.4. Rearing D. aligarhensis

In 1999, each of 5 *D. aligarhensis* cages initiated produced an average of 214 (SD = 80, range = 81–275) wasps from 18 plants per cage. In 2000, after rearing methods were further developed, each of 16 cages produced an average of 408 wasps (SD = 323, range = 82–1098) on 11 plants per cage. A total of 6500 *D. aligarhensis* females were produced and 5800 were released in 2000; 3400 were produced and 400 released in 2001.

Diaphorencyrtus aligarhensis began to emerge 18 days (Fig. 2) after females were introduced into the cage, and were collected over a 10-day period. Only females were observed in this population, confirming the observations of Chien (1995). Cages were dismantled 28 days after introducing females into cages containing fourth-instar psyllids. Each *D. aligarhensis* cage yielded 500–800 adult psyllids, indicating that host feeding and parasitism failed to kill many of the hosts.

3.5. No-choice and serial transfer tests in D. aligarhensis

Two experiments were conducted to determine whether female *D. aligarhensis* produced more progeny from second-, third-, or fourth-instar *D. citri* hosts. The first experiment allowed females access to hosts for 24 h, and the second for 48 h. ANOVA indicated there was no significant difference in the mean number of progeny per female per 24-h exposure so data from the two experiments were pooled. ANOVA of pooled data indicates there were no significant differences in the mean number of progeny produced per *D. aligarhensis* female when given second-, third-, or fourth-instar *D. citri* hosts ($F = 1.369$, $P = 0.26$) (Table 1). There were no significant differences (ANOVA, $P = 0.13$) in hind tibia length in *D. aligarhensis* progeny developed from second-, third-, or fourth- instar *D. citri* hosts (Table 2). In the serial transfer experiment, the number of adult progeny produced by *D. aligarhensis* females did not differ in the first 2 weeks after eclosion, when compared to 3–4, or 5–6 weeks posteclosion (ANOVA, $F = 1.870$, $n = 22$, $P = 0.17$), but sample sizes were low and standard deviations were high ($\bar{X} = 6.6$, SD = 8.9, range = 0–31) (Table 3).

3.6. Labor

Approximately 180 h of labor were required each month to rear *M. paniculata*, *D. citri*, *T. radiata*, and *D. aligarhensis*: 52 h were used to propagate and prepare

Table 1
Mean number of adult female progeny produced (per day of exposure time) by *Diaphorencyrtus aligarhensis* females offered second-, third-, or fourth-instar *Diaphorina citri* hosts in no-choice tests[a]

Instar provided	No. of *D. aligarhensis* females tested	Range of progeny produced	Mean no. of progeny/female	SD
Second	12	0–7	1.8	1.7
Third	13	0–12	3.0	2.1
Fourth	9	0–12	2.7	2.0

[a] Progeny from 24- to 48-h no-choice tests were combined because no significant differences were found between them in mean number of progeny per female.

Table 2
Mean number of adult progeny produced by *Diaphorencyrtus aligarhensis* females 1–2, 3–4, or 5–6 weeks after eclosion

D. aligarhensis age after eclosion (weeks)	No. of *D. aligarhensis* females tested	Range in number of progeny collected	Mean no. of progeny/female	SD
1–2	22	0–31	6.6	8.9
3–4	13	0–14	2.0	4.2
5–6	4	1–4	2.3	1.5

Table 3
Mean hind tibia lengths of *Diaphorencyrtus aligarhensis* females reared in second-, third-, or fourth-instar *Diaphorina citri* hosts[a]

Host instar	No. of *D. aligarhensis* measured	Mean hind tibia length (mm)	SD
Second	25	0.338	0.026
Third	56	0.350	0.024
Fourth	46	0.344	0.024

[a] Progeny from 24- to 48-h no-choice tests were combined because no significant differences were found between them.

150 plants; 25 h to rear 35,000 *D. citri* nymphs; 65 h to rear, collect, and store 6750 *T. radiata*; and 35 h to rear, collect, and store 1630 *D. aligarhensis*. The labor required to produce these parasitoids can be expressed as: 1.4 h per 100 *T. radiata* (♀ and ♂), and 3.7 h per 100 *D. aligarhensis* (♀).

4. Discussion

The key to successful production of large quantities of parasitoids in a tritrophic system is to synchronize the life cycles of *M. paniculata*, *D. citri*, and *T. radiata* (or *D. aligarhensis*) (Fig. 2). Regulating the RH of the rearing facility and cages also was important to prevent mold from growing on psyllid exuvia, honeydew, and parasitoid mummies. *Murraya paniculata* plants must be healthy to produce uniform and robust shoots attractive to ovipositing *D. citri*, which oviposit only on tender new growth. *D. citri* adults must be maintained under a bright light and long daylength regimen to synchronously produce many eggs in a short time. The youngest *D. citri* nymphs died if the plant shoots shriveled under heavy feeding pressure. In the third trophic level, both parasitoids were long-lived and the risk of losing the colonies was low, but each parasitoid had its unique cultural requirements.

Tamarixia radiata oviposits on fifth-instar *D. citri* and host feeds on all instars (Chien, 1995) so adequate quantities of nymphs had to be supplied for both purposes. A ratio of approximately 45 *D. citri* nymphs for each *T. radiata* female was adequate to satisfy these requirements and yielded several hundred 'unused' adult psyllids to produce the next host generation (Fig. 2). Under these rearing conditions *T. radiata* expresses many traits desirable in a biological control agent; *T. radiata* has a higher reproductive rate with ca. 36% of psyllids parasitized, 57% killed by host feeding, and 7% that survive. In contrast, *D. aligarhensis* parasitized 7%, host-fed on 66%, and allowed 27% of *D. citri* to survive. *T. radiata* also has a shorter generation time than *D. aligarhensis* (12 vs. 18 days). When its higher productivity became apparent, we concentrated efforts on rearing both populations of *T. radiata* for field releases during 1999, 2000, and 2001.

Diaphorencyrtus aligarhensis is reported to parasitize fourth-instar *D. citri* and to host-feed on first through fourth instars (Chien, 1995). When *D. citri* reach the fifth instar they are no longer suitable for oviposition or host-feeding by *D. aligarhensis* and thus are 'unused,' although they can be recovered as adults to produce the next generation of psyllid nymphs. In no-choice tests, we found no significant difference in the number of adult progeny produced by *D. aligarhensis* offered second-, third-, or fourth-instar *D. citri* when wasps were exposed to hosts for 24 or 48 h (Table 1). The data on hind tibia length indicate that development in second- or third-instar *D. citri* hosts should not adversely affect the size and fecundity of *D. aligarhensis* reared for field release. This suggests that rearing efficiency may be improved without a loss in reproductive fitness by introducing *D. aligarhensis* into a rearing cage when the first individuals in the *D. citri* population reach the second instar, thus allowing them longer exposure time to suitable hosts.

We concentrated our rearing efforts on producing *T. radiata* for field release so the *D. aligarhensis* colony cages were set up less frequently and with older females. This may account for some of the low productivity seen in colony rearing. *D. aligarhensis* showed a trend to produce more progeny in the first 2 weeks after eclosion, although the difference was not significant, probably due to the small sample sizes used and the high standard deviations obtained. Therefore, even though they can be stored for 30 days with little effect on survivability, *D. aligarhensis* should be released into the field or used in rearing soon after eclosion to achieve the maximum reproductive rate.

The low reproductive rate in *D. aligarhensis* also may be due to the presence of *Wolbachia*, which is known to exact a fitness cost in some parasitoids (Stouthamer et al., 1999; Werren, 1997). A small shipment of an arrhentokous population of *D. aligarhensis* from Vietnam failed to establish in quarantine, so it is not possible to compare the fertility of the thelytokous and arrhenotokous populations at this time.

Tamarixia radiata established and overwintered in southeastern Florida in 1999–2000 (Hoy and Nguyen, 2000). Thus, the first steps in a classical biological control program with *T. radiata* have been completed. Additional releases of *D. aligarhensis* were made in 2001 and 2002 and of *T. radiata* during 2001 in additional counties.

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