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Application and evaluation of entomopathogens for citrus pest control

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1 Introduction

Originally from Southeast Asia, citrus has been introduced into many arid or humid, subtropical to tropical regions around the world where it is cultivated in a diverse array of quasi-permanent tree-crop plantings that range from small interplanted hillside patches to vast monocultural enterprises (Webber, 1948). Numerous arthropods infest citrus and some are considered serious pests locally or more globally either because of the direct damage they cause or because they transmit or otherwise facilitate important plant pathogens (*e.g.*, the brown citrus aphid, *Toxoptera citricida*, vectors the citrus tristeza virus (CTV), Poprawski *et al.*, 1999). Among the most widespread and chronic pests are several mite species, and their importance is often dictated by climate and horticultural practices. Homopterous pests (*e.g.*, aphids, scales, whiteflies, psyllids, etc.) are also common and exhibit considerable diversity and specialization. By comparison, relatively few hemipteran and lepidopteran insects are found feeding on mature fruit and foliage, respectively. Coleopterans (mainly root weevils) and ants are the predominant soil pests whereas fruit flies are important direct pests of fruit (Talhouk, 1975; Browning *et al.*, 1995).

As with other perennial tree crops grown in warm climates, numerous parasites, predators and pathogens inflict a high level of natural control on citrus arthropods (McCoy, 1985; Browning *et al.*, 1995). Overall, fungi appear to be the most prevalent entomopathogens attacking citrus arthropods, especially in humid regions such as shaded rainforest where citrus is planted as an under story crop. However, in monocultural plantings where tree canopies are hedged and topped regularly and cover cropping is not practical, the incidence of fungal pathogens is much lower. To date, natural infections of at least twenty-four mycopathogens, one virus, one gregarine, one microsporidium, and several species of entomopathogenic nematodes have been reported from citrus arthropods (McCoy, 1998; Duncan *et al.*, 2003).

Entomopathogens are widespread on citrus and some, such as *Aschersonia aleyrodes*, a fungal pathogen of citrus whitefly, *Dialeurodes citri*, have been isolated and used as microbial control agents of greenhouse pests (Landa and Jiranova, 1989). However, relatively

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Table 1. Some arthropod pests of citrus and the entomopathogens known to be effective for their control

Target host	Entomopathogen	Rate of application	Selected references
Citrus rust mite, *Phyllocoptruta oleivora*	Fungus: *Hirsutella thompsonii*	2.2–4.4 kg/ha (2.8×10^8 CFUs/kg)	McCoy, 1985; 1996; 1998; McCoy and Couch, 1982
Citrus red mite, *Panonychus citri*	Non-occluded virus	0.1% aqueous suspension	Shaw *et al.*, 1968; van der Geest, 1985
Brown citrus aphid *Toxoptera citricida*	Hypocreales fungi	$2.5–5.0 \times 10^{13}$ conidia/ha	Poprawski *et al.*, 1999
Citrus root weevils, *Diaprepes, Pachnaeus, Asynonychus*	Fungus: *Beauveria bassiana*	53.8 ml/m^2 (2.1×10^{10} CFUs/ml)	Quintela and McCoy, 1998
	Fungus: *Metarhizium anisopliae*	5 g/m^2 (mycelia)	Quintela and McCoy, 1998
	Entomopathogenic nematodes: *Steinernema* spp., *Heterorhabditis* spp.	$2.0–4.0 \times 10^9$/ha; high rate for heavy soils[a]	McCoy, 1985; Duncan *et al.*, 1996, 2003; Morse and Lindegren, 1996; Shapiro-Ilan *et al.*, 2002

[a] Recommendation of Becker Underwood for control of *Diaprepes abbreviatus* with *Steinernema riobrave*.

few entomopathogens have been used either commercially or experimentally for citrus pest control (McCoy, 1998). Some major citrus pests and the entomopathogens known to be efficacious for their control are listed in Table 1.

The fungal pathogen, *Hirsutella thompsonii*, is infectious to mites at the conidial stage. Following conidial germination and cuticular entry into the host, hyphae ramify within the hemocoele and subsequently kill the host. This pathogen has been mass-produced, formulated and applied in the field for control of the citrus rust mite, *Phyllocoptruta oleivora*, and other acarine pests of turf, coconut, and greenhouse crops around the world (McCoy, 1996). Mite control can be achieved within 1–2 weeks when environmental conditions are optimal for fungal survival and infection. The first commercial formulation of *H. thompsonii* available in the USA was produced in 1976 by Abbott Laboratories (North Chicago, IL) as ABG 6065 and subsequently under the trade name Mycar®. An Experimental Use Permit was issued by the EPA in 1978 and registration granted in 1981, but this fungus is not available commercially today.

The citrus red mite, *Panonychus citri*, is a serious pest in California and other arid citrus-growing regions and causes premature defoliation and yield loss. A viral disease of this mite was reported around 1955 from Florida and California (van der Geest, 1985). This rod-shaped, non-occluded virus forms inside the nuclei of midgut epithelial cells. The virus causes epizootics in California and Arizona, and growers are advised to delay chemical sprays to allow natural epizootics to occur. Aqueous suspensions of titrated diseased mites sprayed on citrus trees gave satisfactory control in California (Shaw *et al.*, 1968). This virus has potential as a microbial control agent but instability and the necessity of growing the virus in living organisms are impediments to further development.

The brown citrus aphid, *Toxoptera citricida*, is considered the most efficient vector of citrus tristeza virus (CTV) and occurs in various regions around the world (Poprawski *et al.*, 1999). Citrus varieties grafted to a susceptible rootstock (*e.g.*, sour orange, *Citrus aurantium*) are subject to viral epidemics and subsequent destruction. Numerous hypocrealean (formerly classified within the class Hyphomycetes) fungi are pathogenic to the brown citrus aphid and commercial preparations of *Beauveria bassiana* (Mycotrol GHA®, Mycotrol ES9601, Mycotech Corp., Butte, MT), *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* have been field-tested for microbial control of this pest (Poprawski *et al.*, 1999).

A complex of tropical root weevils including the genera *Diaprepes* and *Exophthalmus* are

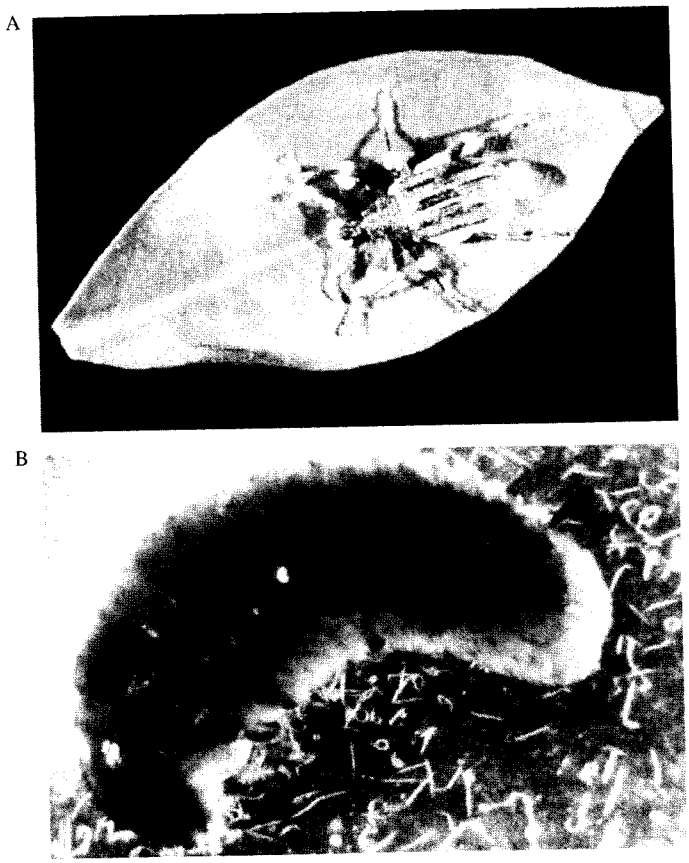

Figure 1. Diagnostic characteristics of cadavers infected with entomopathogens of selected citrus arthropods: A. Adult *Diaprepes abbreviatus* root weevil with fungal hyphae of *Beauveria bassiana* protruding from intersegmental sites of the exoskeleton; B. Infective juvenile nematodes of *Heterorhabditis bacteriophora* dispersing from the body of a late instar larva of *D. abbreviatus*

serious pests of citrus and other agricultural crops worldwide (McCoy, 1998; McCoy *et al.*, 2003). In citrus, the arboreal adults are of minor economic importance but larvae feeding on the roots reduce productivity and can cause tree death either directly or by facilitating infection by soil-borne pathogens (*e.g.*, *Phytophthora* spp., Graham *et al.*, 2003). Both indigenous entomopathogenic fungi and nematodes infect adults and larvae in the soil (Figure 1). Experimental studies show that the fungus, *B. bassiana*, as a commercial product formulated in oil (Mycotrol ES9601) or a combination of fungus and a sublethal concentration of imidacloprid (a chloronicotinyl insecticide) are efficacious against neonate larvae and teneral adults when applied as a soil barrier (Quintela and McCoy, 1998). However, the efficacy of Mycotrol as a weevil control agent is hindered by its poor persistence in soil.

Numerous laboratory and field studies have been conducted in Florida citrus on the use of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of citrus root weevils, particularly *Diaprepes abbreviatus* (McCoy *et al.*, 2002; Shapiro-Ilan *et al.*, 2002; Duncan *et al.*, 2003; Stuart *et al.*, 2004). In Florida, entomopathogenic nematodes have been marketed to growers for weevil control for over 15 years with two species, *Steinernema riobrave* (BioVector®, Becker Underwood, Ames, IA) and *Heterorhabditis indica* (Symbion-South®, BioControl Systems, Aurora, IN), currently available. These nematodes appear to be most effective at high temperatures (27 ± 2°C) in sandy loam soils. Larval mortality rates of over 90% have been reported for field trials with

S. riobrave when applied at a rate of 1.2×10^{10} infective juveniles/ha (Duncan *et al.*, 1996). In California citrus, larvae of the Fuller rose weevil, *Asynonychus godmanni*, were suppressed by 80% using *S. carpocapsae* at 50–500 infective juveniles/cm^2 (Morse and Lindegren, 1996).

This chapter provides a description of field protocols developed to evaluate the performance of some of the above entomopathogens when applied to the fruit and foliage of citrus or to the soil beneath the tree canopy to suppress specific pests.

2 Protocols for application and evaluation of selected microbial control agents

A Selection of test site

In the selection of a citrus planting for the application of entomopathogens, numerous factors must be considered including the plant system (arrangement of trees and tree density, age, and health), target and non-target pests (abundance, distribution, natural enemies) and the microbe being applied (natural prevalence, abiotic factors influencing survival). One or more of these factors can influence the outcome of an experiment. For example, the selection of immature or atypical trees with small or weak canopies is inappropriate because less canopy shading will result in greater solar radiation that can influence the survival of entomopathogens applied to either the foliage or the soil beneath the tree (Duncan *et al.*, 1996).

Entomopathogens such as fungi and nematodes require irrigation to maximize survival and efficacy in the soil. Microsprinkler irrigation under the tree canopy supplies necessary moisture and offers a good way to apply microbes. Other horticultural practices such as fertilizer, pesticide, hedging, and weed control schedules can be of importance in selecting a test site. Information regarding these factors should be obtained from the cooperator. A close relationship between the scientist and grower is imperative to a successful experiment because an unexpected fungicide application can doom a mycopathogen field test.

The final step in the selection of a test site is the preparation of a detailed plot diagram with each tree within the experimental site designated on a map. A copy of the diagram should be retained by the grower cooperator.

B Plot design

Different plot designs have been used for comparative testing of entomopathogens as foliar or soil treatments on citrus. Both completely randomized and randomized block designs have been used for both small and large-scale tests. Plot size can vary widely from single-tree to multiple-tree plots centrally located with surrounding untreated or treated buffer rows. Replication can differ widely and is influenced by plot size or by quantity of entomopathogen available for testing. Buffer zones and plot sizes can also be influenced by motility of target insects and rates of infestation (continual, intermittent).

Plot size can be an especially important factor. For example, tests have shown that both healthy and diseased mites are readily dispersed among trees and even among groves by the wind (Bergh and McCoy, 1997). Thus, when a fungus or virus is applied as a foliar spray to control phytophagous mites in a comparative study on citrus, small plots are ineffective because untreated controls are readily contaminated by other treatments (Shaw *et al.*, 1968; McCoy and Couch, 1982). For these studies, plot size should be no less than 1 ha and only a small central area of the plot (*e.g.*, 4 trees) utilized for sampling. Larger plots (> 0.5 ha) also tend to be better for assessing treatment effects on certain natural enemies or various other non-target organisms.

C Plot modifications

Larvae and teneral adults of root weevils appear to aggregate within the rhizosphere of the citrus tree at relatively low population densities and their distribution from tree to tree can be highly variable and unpredictable (*e.g.*, McCoy *et al.*, 2003). Therefore, artificial seeding of laboratory-reared larvae has been used to increase larval numbers in plots for experimental purposes. The procedure for artificial infestation is as follows:

1. Pre-irrigate the grove for about 2 h (5-cm depth).
2. Flag designated trees for infestation and record them in the plot diagram.
3. Place 400 neonate larvae (~ 48 h old) obtained from a greenhouse weevil colony into snap cap micro-centrifuge tubes (volumetric estimate or visual count). Micro-centrifuge tubes should contain about 1 g of moistened soil to prevent larval injury.
4. Place larvae in a cooler (10–12 °C) for transport to the field.
5. Scatter contents of each vial on the soil beneath the tree within 10–20 cm of the trunk.
6. Perform larval infestation late in the day or under cloudy skies.
7. Assess experimental results by adult trapping at emergence or destructive tree sampling and counting larvae recovered from the rhizosphere via soil sieving.

D Preparation of the field inoculum

The foliage of citrus trees is tolerant of a wide range of spray adjuvants (spreader-stickers, surfactants and anti-transparents) used to enhance the efficacy and persistence of sprayable mixtures. Spray mixtures have been tested in the field in combination with fungal inocula in free form as adjuvants or with conidial preparations commercially formulated as wettable powder (*e.g.*, Mycar®, Abbott Laboratories) or as oil mixtures (*e.g.*, Mycotrol ES, Mycotech Corp.) to improve fungal deposition on the leaf (McCoy and Couch, 1982). Petroleum-based spray oils are widely used as combination spreader-sticker/pesticides. When the adjuvant is not included in the formulated product, it can be added to the tank mix after the fungus has been thoroughly mixed with water under vigorous agitation. Generally, adjuvants are applied at 0.05–5.0% (v/v) without affecting the inoculum or causing phytotoxicity to the plant.

Prior to application of unformulated or formulated fungal preparations with and without adjuvants, the viability of the conidial suspension should be determined. Plating techniques are the preferred method to measure viable propagules per unit volume, but at least 3 days must elapse before a reading can be taken. This delay is generally unacceptable. Therefore, quantification methods using a hemacytometer have been used with some success (Goettel and Inglis, 1997). Conidial mixtures (20–30 ml) should be placed in nutrient broth in a test tube and agitated for 30 sec. The suspension is then incubated on a shaker at 27 °C at a moderate speed (100–120 rpm) for 12 h to allow for germination. Ten random samples/treatment are taken via pipette and transferred to a hemacytometer for counting with phase microscopy (420X). Conidia are considered viable if germ tube lengths are twice the diameter of the propagule in question and a count of 50 to 100 conidia/sample is considered appropriate. With some adjuvants, visibility of the conidia in the hemacytometer may be limited by its properties. In such cases, the suspension should be diluted with water at the time of the count.

Prior to the application of nematodes in the field, viability should be determined by examining a sample of 50–100 nematodes via a dissecting microscope and counting the live and dead nematodes (Kaya and Stock, 1997). When probed with a needle, live nematodes that are merely immobile will respond to the stimulus. Ideally, viability should be checked before transport to the field, at the spray tank, and at the nozzle to assure viability during the entire application process. The spray tank should be triple rinsed if chemicals have been used previously because nematodes and fungi are not compatible with some agri-chemicals (Table 2). However, in some cases, pesticides act synergistically with entomopathogens and could improve the efficacy of applications (Quintela and McCoy, 1998; Grewal, 2002).

Fungal and nematode preparations should always be stored out of direct sunlight and kept cool (12–25 °C) prior to use in the field. When tank mixing nematode preparations in the field, an estimate of the number of nematodes per container can be obtained by counting the number volumetrically. With the sprayer agitator running, the nematodes are added to the holding tank containing a known amount of water (50% of capacity). Then, the remaining water is added to bring the tank mix to a designated concentration. The pH of the water should always fall between 4 and 8. Water dispersible granule formulations of nematodes can be premixed in water before being added

Table 2. Nematodes can be used simultaneously with most citrus-registered pesticides and fertilizers at standard rates with the following exceptions

Schedule	Chemical name	Trade name(s)
Do not use 1 week before / after nematode application	**HERBICIDES**	
	2, 4-D	Various names
	Triclopyr	Turflon®, Confront®
	INSECTICIDES	
	azinphosmethyl	Guthion®
	bendiocarb	Rotate®
	chlorphyrifos	Dursban®
	methomyl	(non-encapsulated)
		Lannate®
Do not use 2 weeks before/after nematode application	**INSECTICIDES**	
	carbofuran	Furadan®
	ethoprop	Mocap®
	NEMATICIDES	
	aldicarb	Temik®
	fenaminphos	Nemacur®
	oxamyl	Vydate®

Recommendation of Becker Underwood for *Steinernema riobrave*.

to the spray tank. When this is not possible, the agitator should be run fast enough to thoroughly dissolve the dry preparation almost immediately as it is slowly poured into the tank. Fungi require similar procedures when tank mixing. Many different formulations exist (see Grewal, 2002), and the manufacturer's instructions should be followed closely for mixing and application.

E Application of inoculum

1 Foliar

Fungal and viral spray mixtures applied as foliar sprays to citrus trees have been delivered successfully using hydraulic and air blast systems designed for chemical pesticide application. Two basic sprayers have been used. For small plot work, a hydraulic hand sprayer with a single nozzle is normally used. The amount of liquid applied per tree can be estimated by simply measuring the discharge in the holding tank. From this information and a knowledge of the concentration of the spray mix, an estimate of foliar coverage can be calculated.

The general procedures for applying a microbial using a hydraulic system are as follows:

1. Apply water to 3 or more trees to spray runoff after setting spray pattern for hand applicator. Spray applicator should always apply the finished product at a fixed distance from the tree (approximately 3 m) and travel clockwise around the tree during application.
2. Calculate the average volume of spray required to treat a whole tree to runoff.
3. Based on the number of trees/treatment, tank mix the total finished spray needed to complete the spray operation.
4. For mature canopy trees, set pump pressure at 3100–3450 kPa (450–500 psi) to assure penetration of the tree canopy.
5. Always apply the lowest spray concentration first and continue to the highest.
6. Monitor weather conditions, and never spray when the wind speed exceeds 16 km/h.

The conventional sprayer for large plot studies is the air blast type. Tractor-drawn air blast sprayers function on the principle of air displacement within the tree canopy. Coverage is more precise and application is less labor intensive than for a hydraulic hand sprayer. Nozzles are attached to the sprayer at fixed locations and travel past the trees at a constant speed (3–5 km/h). Normally one-half the spray volume is directed to the upper half of the tree and one-half to the lower by arranging the nozzles and air deflectors. Both high and low volumes (preferred for microbials) can be applied with an air blast sprayer. Smaller trees can be sprayed with the same concentration of microbial control agent by using fewer nozzles.

Generally, microbials have been applied in the range of 2,366–4,733 liters/ha. The pH of the tank mixture should be maintained in a range of 4.0–8.5 and application made late in the day to reduce solar effect. The general procedures for application using an air blast system are as follows:

1. Select intended application rate by:
   a. Determining nozzle arrangement (based on tree height).
   b. Measuring spray discharge for nozzle arrangement.
   c. Calibrating sprayer ground speed (distance traveled in time).
2. Prepare tank mix based on plot size and ensure proper agitation and spray mixture compatibility.
3. Monitor weather conditions (wind speed, rainfall, temperature) before and during application.
4. Maintain a two-row buffer between plots.

2 Soil

Both fungal and nematode mixtures have been applied beneath the tree canopy for control of weevil larvae by using herbicide applicators and chemigation systems (injection via low volume irrigation systems). Using a tractor mounted herbicide spray unit with a 1.5–2.5 m boom, a treated band can be uniformly applied to the soil surface beneath the tree between the trunk and the canopy edge. Fungal conidial preparations are applied in 285–475 liters water/ha at 2.4 km/h. For uniform coverage #10 flood jets at 30 cm spacing with a #80C outside nozzle assures coverage of both sides of the tree. Nematodes should be mixed in a minimum of 300 liters of water allowing for the extra volume remaining in the tank after spraying. Apply under moderate agitation to assure thorough distribution of the tank mix and adequate aeration during application. For best results, nematodes should be applied at 900–950 liters/ha and operating pressures not exceeding 2000 kPa (290 psi). However, for at least one nematode species (*H. megidis*), operating pressures should not exceed 1380 kPa (200 psi) (Fife *et al.*, 2003). The pH of the water should be maintained in the range of 4–8. Pre-irrigate the grove to assure soil moisture to a depth of 5 cm.

The application of fungi and nematodes via under tree microsprinkler irrigation is a feasible strategy, which offers the advantage of the treated area being limited to the soil surface beneath the tree. This can result in cost savings in material used in application, especially for young trees. Sprinkler emitters should be positioned about 30 cm from the tree trunk and be of a type to assure about 80% coverage of the root zone. Ideally, they are equipped with 360° nozzles (Fan-Jet, Bowsmith Inc., Avon Park, FL) capable of covering a 1.8–2.4 m radius with a volume output of 38–76 liters/h (10–20 gal/h). Volume output at the injection pump should be adjusted to a rate based on tree number and type of irrigation system. By knowing the volume of the holding tank and rate of output, the inoculum concentration can be determined. Add sufficient nematodes to the tank to achieve the desired inoculum concentration and inject all during a 30–60 min period. Whether applying nematodes or fungi, the content of the holding tank must be agitated throughout the application. Applications should be carried out in late afternoon or evening to reduce exposure to UV radiation and increase the number of hours of high humidity following application. In line variability at the emitters and variation in coverage beneath the tree can be determined during application by collecting spray mix in collecting pans placed on the ground beneath the tree. Viability of the inoculum also should be checked at the holding tank and at the nozzle to ensure effective application. Inoculum concentration is often reduced near the ends of irrigation lines (Duncan, unpublished).

The following stepwise procedure is advised:

1. Pre-irrigate the grove if necessary to achieve uniform soil moisture to at least 5 cm (deeper for sandy soils).
2. Prepare the irrigation system by removing screens and filters if possible. If screens must remain, a U.S. mesh screen size of 50 or coarser should be used.
3. Do not subject nematodes to excessive pump pressures (usually < 2000 kPa or 290 psi).
4. Calibrate the injection pump to deliver the concentrate over a 30–60 min period.
5. Irrigate for 15–30 min immediately prior to starting injection to cool the lines and establish a wetting pattern under the trees.
6. Water dispersible granules can be poured directly into the tank containing water or a slurry can be

pre-mixed and then added to the tank water. The water temperature should be between 10 and 30 °C.
7. The nematode suspension can be held in the agitated tank for up to 24 h, but the tank must be kept cool (under 27 °C).
8. Irrigate the grove after application to a depth of 1.5–2.5 cm of water.

F Pre- and post-treatment sampling of target insects

Knowledge of the seasonal population dynamics of an arthropod pest is basic to designing an appropriate sampling and application plan for microbial control. Each year, citrus produces new leaves that have a life expectancy on the tree of about 1.5 years. For foliar and soil pests, seasonal population dynamics are often governed by abiotic and biotic factors, and diseases are often involved. Insect diapause is virtually non-existent in citrus growing areas, and seasonal sampling for pests should begin when the rate of increase in the abundance of the pest on feeding sites such as new citrus flush or fruit begins to rise. Many entomopathogens such as the fungus, *H. thompsonii*, cause predictable natural epizootics at high host densities during the summer (McCoy, 1996), and knowledge of when epizootics occur is an important consideration for the sampling plan and the timing of applications of microbial control agents. If the microbial control agent is being applied as a "prophylactic" treatment prior to the onset of a natural epizootic then it is likely that cadavers resulting from the treatment will be mixed with those dying from the natural inoculum.

Methods for estimating mite and aphid populations involve counting the numbers of one or more developmental stages of the arthropod on fruit or leaves in the field or removing the arthropod stage(s) from the fruit or leaves and counting a known fraction in the laboratory. Because of their small size, mite counts are frequently performed with the aid of a hand lens in the field or a stereoscopic microscope in the laboratory. Counts can involve the whole leaf or fruit or most often just a fixed amount of the surface area.

Sampling of the diminutive citrus rust mite and its fungal pathogen, *H. thompsonii*, involves different methods in the field and laboratory. For large field trials, an estimate of mite population density and disease prevalence on fruit or leaves before and after a spray application is made using a gridded hand lens (20X). Live, dead and dead diseased adult mites observed within a $1-cm^2$ area are easily distinguished: live mites are clear and mobile, dead mites are deformed, and dead diseased mites are deformed with filamentous hyphae protruding from their collapsed bodies. However, live mites with disease cannot be distinguished from those without disease using this method.

1 Sampling procedures for citrus rust mites and its fungal pathogen

a Field (direct method)

1. Sample four adjacent trees from the center of each plot at any time of the day.
2. Select at random 5 fruit/tree or 20 fruit/4 tree plot.
3. Using a hand lens (20X) with a $1\,cm^2$ grid attachment, count the number of live, dead and diseased mites.
4. Count the mite forms at 2 random sites/fruit. A minimum sample/treatment should be in the range of 100–120 fruit.
5. Perform 2 pre-treatment samplings one week apart and post-treatment sampling at weekly intervals.

Using this sampling frequency, the immediate effect of the microbial application and its persistence can be determined with a declining mite population and increasing prevalence of infection in the field. The fact that this sampling procedure can be performed quickly in the field is an important advantage and makes this the preferred method.

An indirect sampling procedure also has been used with good results in estimating more accurately the prevalence of diseased mites in a population. This procedure is as follows:

b Laboratory (indirect method)

1. Perform 2 pre-treatment samplings one week apart and post-treatment sampling at weekly intervals.
2. Sample four adjacent trees from the center of each plot at anytime of the day.
3. Collect at random 10 leaves/tree from the inner and outer canopy at 1.5 m above the ground.
4. Place leaves for each plot in separate paper bags. Keep leaves cool during transport to the laboratory.

5. In the laboratory, count the number of live, dead and visibly diseased mites in 2 microscope fields selected from the upper and lower leaf surface at 100X magnification using a stereoscopic microscope.
6. Select randomly no more than 50 live and dead mites from a given treatment, clear separately in 1.0% lactic acid or Nesbitt's clearing reagent by placing the mite in a droplet of clearing agent and heating for 30 sec.
7. After mounting cleared mites in Hoyer's solution, examine with a phase contrast microscope to confirm infection.

The accumulated percentage of diseased mites for each treatment is obtained by dividing the accumulated total of diseased mites by the total number sampled. Although more time consuming, this method is more precise than the field method.

2 Sampling citrus red mite and its virus disease

To estimate the effect of a foliar spray of an aqueous suspension of titrated diseased mites with a non-occluded virus on citrus red mite populations, an indirect sampling procedure has been used in the field. The procedure is as follows:

1. Count adult female citrus red mites twice per week for 2 weeks pre-treatment and weekly thereafter.
2. Collect 20 leaves randomly per treatment in paper bags. Keep leaves cool during transport to the laboratory.
3. Mites are removed from leaves manually or with the aid of a mite brushing machine (Morgan *et al.*, 1955).
4. If available, 20 live and 20 dead mites (or any combination equaling 40 individuals) are mounted in Hoyer's medium and examined microscopically.
5. Virus infection is confirmed by the presence of birefringent crystals.

3 Sampling brown citrus aphid and its fungal agents

The brown citrus aphid presents different circumstances in terms of sampling. Aphids develop on the newly-formed leaf flush of growing terminals forming on the tree particularly in the spring. Therefore, the sampling unit is the terminal. Pre-treatment and post-treatment population estimates are made by counting the live and dead aphids on each terminal with a hand lens (10X). According to Poprawski *et al.* (1999), fine mesh sleeve cages are needed to exclude predatory insects. Cages are placed carefully over the treated and untreated terminals immediately after treatment.

4 Sampling entomopathogens of citrus root weevils

Numerous sampling methods have been employed in citrus groves to measure directly or indirectly the effect of either fungi or nematodes on adult root weevils on the tree and larvae in the soil. For adult weevil control on the tree with a fungal agent, trees are generally sprayed with an air blast sprayer. The following procedures are used to assess efficacy:

1. Immediately after spray application, 3–5 adult weevils are placed in a standard screened limb bag (30 × 46 cm).
2. For treated and untreated foliage, infested bags are placed over clumps of shoots with new leaves (preferred food source) and attached to the limb with cord to prevent weevil escape.
3. After one week, the bags are removed and weevil mortality recorded.
4. Samples from treatments are replicated a minimum of 5 times (15–25 weevils/treatment).
5. Dead weevils are returned to the laboratory and placed in a humidity chamber at 95–100% RH for 3–4 days to confirm fungal mycosis.
6. The procedure is repeated weekly on selected terminals to measure fungal contact effect and persistence.

Citrus root weevil females deposit their eggs in masses sealed between leaves in the tree canopy. Hatching neonate larvae drop from the canopy and enter the soil to feed on roots and will emerge from the soil as adults mainly in the spring and summer months of the next year (McCoy *et al.*, 2003). Two types of ground traps have been used to monitor adults that emerge from soil in the year following treatments to control larvae (Stansly *et al.*, 1997; Duncan *et al.*, 2001; McCoy *et al.*, 2003). These traps are referred to as cone traps and Tedders traps (Figure 2). For both types of traps, the trap top or "collector" generally consists of the top portion of a cotton

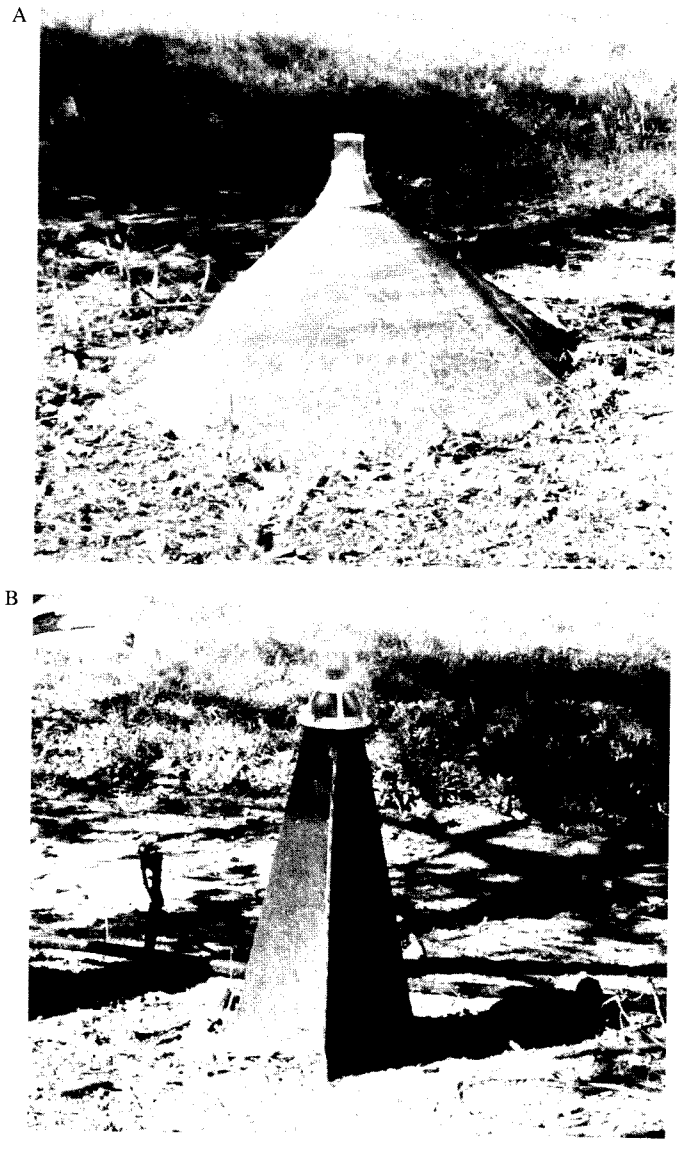

Figure 2. Ground traps used to catch adult root weevils beneath the tree canopy: A. cone trap; B. Tedders trap (Photos by Ian W. Jackson)

boll weevil trap (Great Lakes IPM, Vestaburgh, MI). The collector consists of a small screen cone (10 cm high × 11.5 cm dia) inside a plastic frame that has a removable ventilated plastic cup fitted on top. The collector is secured to the trap base with screws. The cup holds the weevils after they enter the collector and pass through a 1-cm dia. hole at the top of the screen cone.

The bottom portion of the cone trap is constructed of standard galvanized hardware cloth (0.3-cm mesh size) with a base of 0.9 m in diameter, and covers about 0.6 m^2 of soil surface. The cone trap is placed beneath the tree as close to the trunk as possible with the bottom rim of the cone buried in the soil (Figure 2A). Adults emerging from the soil beneath the cone trap crawl up the wire mesh, pass through a 1-cm dia hole in the top of the cone, and are captured in the collector.

A modified Tedders trap (or citrus Tedders trap) has a pyramid-shaped base 61 cm high. The base is formed from two roughly triangular-

shaped pieces of black masonite or corrugated polyethylene (PBE Graphics, West Palm Beach, FL) that are slotted in the middle to half their lengths in opposite directions so that they fit together and thereby form a pyramid with four flanges extending in different directions (Figure 2B). The Tedders trap is placed just inside the margin of the tree canopy and is anchored tightly to the soil with wire loops or hooks running over or through the flanges (Wireco, Winter Haven, FL). Newly emerged weevils tend to crawl up the trunks of trees and the Tedders trap catches adults exhibiting this behavior. It has been estimated that 100 traps/ha are generally necessary to collect meaningful data on weevil emergence (Bullock *et al.*, 1999). A statistical comparison of Tedders trap catches from various sites indicates that the optimal number of traps depends on weevil abundance and the degree of precision desired in the results (Duncan *et al.*, 2001).

A Tedders trap usually catches more adults than a cone trap because, unlike the cone trap, it is not restricted to catching weevils emerging from the soil directly beneath the trap. Moreover, weevil catches in cone traps and Tedders traps are not always correlated, and Tedders trap catches might be a better indication of general weevil activity than weevil emergence *per se* (McCoy *et al.*, 2003). Because Tedders traps capture weevils from a broad area of unknown size, they should be used to monitor weevils only in relatively large experimental plots (*e.g.*, 0.5–1.0 ha), whereas cone traps are appropriate for either small or large-plot research. Because of lengthy larval developmental times, both types of traps should be monitored weekly throughout the year of a larval treatment and through the following spring (late June) to properly assess the efficacy of a larval treatment. Adult catches should be examined in the laboratory for microbial infection. For fungi, adults should be incubated in a humidity chamber at 95–100% RH for a week after being captured.

The most common method for quantifying fungal inoculum on or in the soil is based on dilution plating (Goettel and Inglis, 1997). The abundance and persistence hypocrealean fungi at the soil surface beneath the tree can be obtained by collecting a minimum of 10 g of soil from randomly selected sites with a spatula. If soil depth is a consideration, a #9 cork borer is used to cut soil columns to a depth of 15 cm. Usually 5 cm increments are separated and then pooled. A pooled soil sample of about 30 g is adequate to estimate propagule density using the soil dilution plate method. The procedure for estimating fungal colony-forming units (CFUs) is as follows:

1. In the laboratory, the soil sample contained in a plastic bag is thoroughly mixed by hand shaking.
2. A 10 g sample is placed in a 50 ml conical centrifuge tube containing 30 ml of sterile distilled water.
3. After vigorously shaking the tube for 30 sec, serial dilutions are performed using a 100 μl inoculum. Additional dilutions (10^{-2}, 10^{-3}, and 10^{-4}) can be made based on an estimate of propagule density in the soil.
4. Two-hundred microliters of diluted suspension is spread on divided Petri plates (two inoculations/plate) containing oatmeal-dodine selective media.
5. After 7–10 days incubation at 27–28 °C, the number of fungal propagules per 200 μl is determined per plate.
6. Plate values are then converted to CFUs/g of soil.

In addition to quantifying fungal inoculum in treated and untreated field soil, soil column bioassays can be performed simultaneously to confirm pathogenicity using laboratory-reared neonate larvae. The procedures are as follows:

1. Sample all treatments at 0, 7, 14, 21, and 30 days post-treatment.
2. Collect 20 soil cores using a #9 cork borer (area 1.27 cm^2) to a depth of 2.5 cm randomly from an area halfway between the trunk and tree skirt beneath the tree.
3. Upon collection, each intact soil core is carefully placed in a plastic tube with a screen base (20 mm mesh) to allow for larval escape into the well of a plastic tissue culture plate (Figure 3).
4. In the laboratory, 10 vigorous neonate larvae (48-h-old) are placed on the soil surface of each column.
5. After 72 h at 25 °C, the number of larvae capable of moving through the soil column to the catch well of the bioassay unit are counted as live, dead or missing.
6. Dead larvae are held at 27 °C at 95–100% RH for 10 days to assess fungal mycosis.

Generally, nematode density in the soil after an inoculative application is correlated with host infection in time (Duncan *et al.*, 1996).

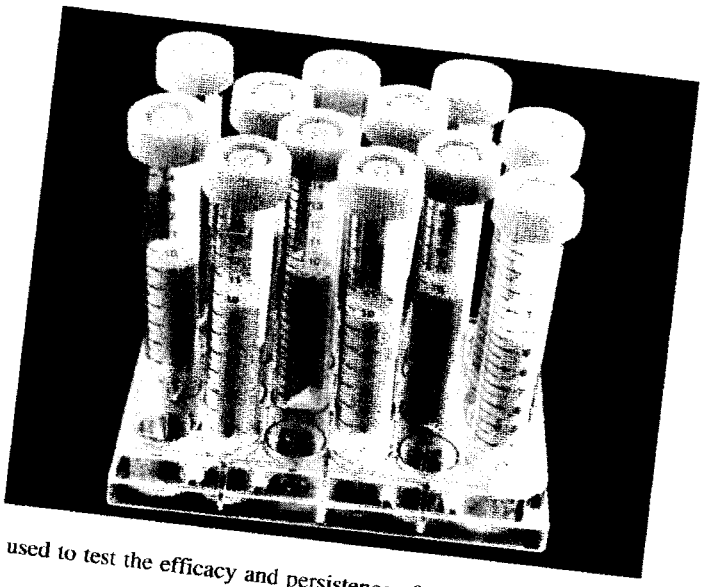

Figure 3. The bioassay unit used to test the efficacy and persistence of fungal conidial inocula applied to the soil surface

Nematode density and persistence can be readily measured in time using the following procedures:

1. At predetermined intervals after application, 10–15 soil probe samples to a depth of 15 cm are taken from beneath the tree canopies in each plot and pooled to form a single sample.
2. Samples are obtained from the same location relative to the irrigation emitter about halfway between the trunk and the dripline of the tree.
3. The soil samples are collected in a plastic bag for transport to the laboratory in a cooler.
4. In the laboratory, soil is thoroughly mixed, and then a 60 ml aliquot is placed in a Baermann funnel (Kaya and Stock, 1997).
5. After 72 h, nematodes trapped in the water are poured into test tubes (100 ml) and allowed to settle overnight.
6. After aspirating excess water, the infective juvenile (dauer stage) nematodes collected from the bottoms of the tubes are counted microscopically using a gridded Petri dish.
7. Taxonomic keys and known samples of live *Heterorhabditis* or *Steinernema* are used as a reference in counting.

The biological activity of nematodes applied to the soil surface for suppressing weevil larvae in the citrus rhizosphere can be estimated using a tree removal/soil sampling procedure (Duncan *et al.*, 1996). Briefly, trees are uprooted using a tractor-mounted front-end loader with a fork head attachment. By vigorously shaking the tree *in situ*, most of the soil adhering to the roots is removed along with any larvae. The tree is then placed on a plastic tarpaulin in the row middle and the soil lodged in the tree crown area is removed by flushing the root zone with high-pressure water delivered by a sprayer applicator. Larvae from the crown area are then collected from the tarp. To estimate the number of larvae remaining in the upper soil dislodged from roots (30 cm depth) and lower rhizosphere (30–60 cm depth), a soil sample of about 0.25 m^3 is collected using a shovel and then sieved through a coarse screen to separate the larvae from the soil.

Two field methods using caged weevil larvae, either with or without a host plant, have been devised to estimate nematode efficacy. A buried cage without a plant was designed for use in the soil at various depths. This cylindrical cage (7.6 cm length and 1.9 cm dia) was constructed from a 6.5 ml plastic vial and galvanized steel screen (2-mm mesh). The vial was cut into two parts to form the ends of the cage and the screen formed a cylindrical portion in the middle that was glued to both ends. Unfortunately, this cage leaves the larva open to predation by ants, which is problematic for interpreting experimental results. Consequently, a predator proof cage was designed using a 225 mesh stainless steel in-line sprayer filter (7 cm length × 3 cm dia.) (Chemical Container, Lake Wales, FL). The cylindrical filter is capped on both ends using polyethylene snap caps (3.0 cm outside

diameter). For both types of buried cages, it is useful to secure a 40 cm wire through one or both ends of the cage. When the cage is buried vertically, the wire protrudes through the soil surface and can be flagged so that the cage is more easily located and excavated after field exposure.

Larval instars of *D. abbreviatus* vary considerably in their susceptibility to *S. riobrave* with younger instars being much more susceptible than older instars (Stuart and McCoy, unpublished). Thus, the use of a particular instar (*e.g.*, 6th instar) as indicated by head width measurements (see Quintela *et al.*, 1998) is essential for larval assays of nematode efficacy to produce consistent results. The procedure for use of the cages is as follows:

1. Place one 6th instar larva in each cage and fill the cage with sterilized sand at 8% moisture by weight.
2. Place the cage in the soil beneath the tree using a probe to cut a hole to a given depth (15–20 cm). Fill the open hole with soil.
3. Bury at least 4 cages per plot about 1 m inside the margin of the tree canopy.
4. Remove the cage from the soil after 7 days exposure in treated and untreated plots.
5. Repeat this process at 0, 1, 2, 3, and 4 weeks post-treatment.
6. Record the number of healthy and dead larvae in the laboratory; place each dead larva in a Petri dish containing moist filter paper and a wax moth (*Galleria mellonella*) larva to detect nematode parasitism. *G. mellonella* larvae are extremely susceptible to entomopathogenic nematodes (Kaya and Stock, 1997).

A different field cage was designed for burial to a depth of 15 cm with the top (16.5 cm dia) open to the surface at the soil line. This cage was made of polyvinyl chloride pipe (16.5 cm diameter) with a fine mesh screen base (225 mm mesh) on the bottom to allow for water leaching and predator exclusion. This pot-like cage can be used to support a citrus plant as food for weevil larvae added to the trap. The procedure for use of the cage is as follows:

1. Inoculate each soil-filled cage with 15, 6th instar larvae a few days before placing the cages in the field.
2. Bury larval infested cages, 2/plot, in the field in a row, one week prior to nematode application. At this time, cut the top off the seedling to expose the soil surface to the treatment. A bead of Tanglefoot (The Tanglefoot Company, Grand Rapids, MI) is then placed on the upper edge of the cage to prevent invasion by predators.
3. Remove the cages from the soil at one week post-treatment.
4. Separate the plant from the soil and sieve all soil to recover live and dead larvae.
5. In the laboratory, diagnose nematode parasitism using the procedure described above.

G *Assessing tree health*

The following methods have been developed for assessing tree health (or tree decline) as influenced by citrus pests and their biological control agents.

1 *Fibrous root density*

Root mass is directly related to shoot growth of a citrus tree. Procedures for measurement are as follows (*e.g.*, Duncan *et al.*, 1994):

1. Perform sampling in June and November annually.
2. Using a standard soil probe, take 1 sample/tree to a depth of 25 cm halfway between trunk and canopy dripline from 16–20 trees/plot or increase the number of cores/tree to result in 20–30 samples.
3. Pool soil from each plot into one plastic bag.
4. Store soil samples in cold room at 4 °C until processed.
5. Separate citrus fibrous roots from soil and debris via wet sieving (100 mm mesh) and hand picking with forceps.
6. Determine dry root weight/sample.

2 *Tree canopy size and density*

The foliage of a citrus tree canopy generates the photosynthate that in turn determines fruit yield. Procedures for measurement of photosynthetically active radiation (PAR) transmittance and citrus canopy volume are as follows (Albrigo *et al.*, 1975; McCoy *et al.*, 2004):

1. Perform sampling in June and November annually.
2. Measure photosynthetically active radiation (PAR) for a given number of trees using a Accupar (Decagan Devices, Inc., Seattle, WA). Perform 2 readings/tree beneath the tree canopy at a fixed

location at mid-day with full sunlight. Calculate percent PAR transmittance.

3. Determine canopy volume for a given number of trees by taking the measurements necessary for use of the following formula. The canopy of a citrus tree can approximated as being a half prolate spheroid above a cylinder, and the volume of the tree canopy (vc) can be calculated using the formula: $vc = \partial R^2(2X/3+Y)$ where $X = HT–HD$ and $Y = HD–HS$ with R=half the tree diameter at the widest point, HT=the overall tree height, HS=the skirt height, and HD=the height from the ground to the widest point of the tree.

3 Tree trunk growth

Citrus trees in decline will show little or no increase in trunk growth from year to year. Procedures for measurements are as follows (McCoy *et al.*, 2004):

1. Perform sampling in June and November annually.
2. Select at random a given number of control and treatment trees and flag for identification.
3. Measure the diameter of the tree trunk at approximately 5 cm above the budunion with calipers.
4. Compare the relative growth rate of the cross sectional area of the trunk between control and treatment trees over time.

4 Fruit yield

Yield is the most difficult measurement of tree health to be collected mainly because of labor requirements, yet it is the best measure of treatment profitability. Procedures for measurements are as follows:

1. Harvest a minimum 100 trees/ha or 40% of a given plot.
2. Weigh the total fruit/tree.

5 Fruit quality

The citrus rust mite inflicts a peel injury to the fruit that is visible to the naked eye. Fruit quality in experimental plots can be determined for various treatments using the following procedure:

1. Flag a given number of trees selected randomly per plot (4–8).
2. Insert a frame representative of $0.37\,m^2$ at 4 cardinal directional sites within the tree canopy (Stout, 1962).
3. Examine each fruit within an imaginary tunnel extending from the frame to the center of the tree for the presence or absence of mite injury.
4. Calculate percent fruit injury.

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