CHAPTER VII-11

Application and Evaluation of Entomopathogens for Citrus Pest Control

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1 INTRODUCTION

From its origin in Southeast Asia, citrus has been moved to several arid or humid, subtropical to tropical regions of the world (Webber, 1948) to form widely diverse quasi-permanent tree crop plantings. These plantings range from small interplanted hillside patches to vast monocultural enterprises. Many arthropods infest citrus, some causing sufficient damage over a given area to constitute pest status while others are merely innocuous (Talhouk, 1975; Browning *et al.*, 1995). Among the chronic pests are several mite species, their importance to some degree, dictated by climate and to a lesser extent by horticultural practices. Homopterous pests are particularly common to citrus where they exhibit considerable biological diversity and specialization among groups (aphids, scales, whiteflies, etc.). By comparison, only a 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. Fruit flies are of major importance in many growing regions as direct pests of fruit.

Like other perennial tree crops grown in warm climates, a number of parasites, predators and pathogens inflict a high level of natural control on various citrus arthropods (McCoy, 1985; Browning *et al.*, 1995). Fungi are the most prevalent entomopathogens attacking citrus arthropods, particularly in humid regions such as shaded rainforest where citrus is planted as an under story crop. In monocultural plantings where tree canopies are hedged and topped regularly to control tree growth and cover cropping is not practical. Fungal survival appears to be less. Twenty-four mycopathogens, one virus, two protozoans, two species of nematodes, and no bacterial pathogens have been reported infecting citrus arthropods (McCoy, 1998).

Entomopathogens are widespread on citrus and some, such as *Aschersonia aleyrodes*, a fungal agent of citrus whitefly, have been isolated and used as a microbial control agent of mainly greenhouse pests (Landa and Jiramova, 1989); however, few have been used commercially and experimentally for citrus pest control. Major citrus pests and their entomopathogens with proven efficacy as microbial control agents are listed in Table 1.

L.A. Lacey and H.K. Kaya (eds.), Field Manual of Techniques in Invertebrate Pathology, 577–595.

TABLE 1. List of key arthropod pests of citrus and the entomopathogens utilized as microbial control agents.

Target host	Entomopathogen	Rate of application	Selected references
Phyllocoptruta oleivora	*Hirsutella thompsonii*	2.2 - 4.4 kg/ha (2.8×10^8 CFU/kg)	McCoy, 1985; 1996; 1998; McCoy and Couch, 1982
Panonychus citri	Non-inclusion virus	0.1% aqueous suspension	Reed, 1981; Shaw *et al.*, 1968; van der Geest, 1985
Toxoptera citricida	Hyphomycetes fungi	$2.5\text{-}5.0 \times 10^{13}$ conidia/ha	Poprawski *et al.*, 1999; Tsai, 1998
citrus root weevil *Diaprepes, Pachnaeus, Asynonychus*	*Beauveria bassiana*	53.8 ml/m^2 (2.1×10^{10} CFUs/ml)	Quintela and McCoy, 1998
	Metarhizium anisopliae	5 g/m^2 (mycelia)	Quintela and McCoy, 1998
	Entomopathogenic nematodes	4.94×10^6/ha 2×10^6/tree	Duncan *et al.*, 1996; McCoy, 1985; Morse and Lindegren, 1996

The fungal pathogen, *Hirsutella thompsonii,* is infectious to the mite at the conidial stage. Following conidial germination and cuticular entry to the host, hyphae ramify within the hemocoele subsequently killing the host. It has been mass-produced, formulated and applied in the field for the control of the citrus rust mite 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 of the host. 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 most arid citrus-growing regions causing premature defoliation and subsequent yield loss. A viral disease of the red mite was reported around 1955 from Florida and California (van der Geest, 1985). This rod-shaped, non-inclusion virus forms inside the nuclei of the midgut epithelial cells. The virus appears to cause epizootics in the field in California and Arizona (Reed, 1981), 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). Although the citrus red mite has potential as a microbial control agent, major drawbacks to its further development include the necessity of growing virus in living organisms and the instability of the virus.

The brown citrus aphid, *Toxoptera citricida*, is the most efficient vector of citrus tristeza virus throughout the world (Tsai, 1998). Citrus varieties grafted to susceptible rootstock such as sour orange, *Citrus aurantium,* are susceptible to viral epidemics and subsequent destruction. Numerous hyphomycetes fungi are pathogenic to the brown citrus aphid and related aphid species. A number of commercial preparations of *Beauveria bassiana* (Mycotrol GHA®, Mycotrol ES9601, Mycotech Corp., Butte, MT), *Metarhizium*

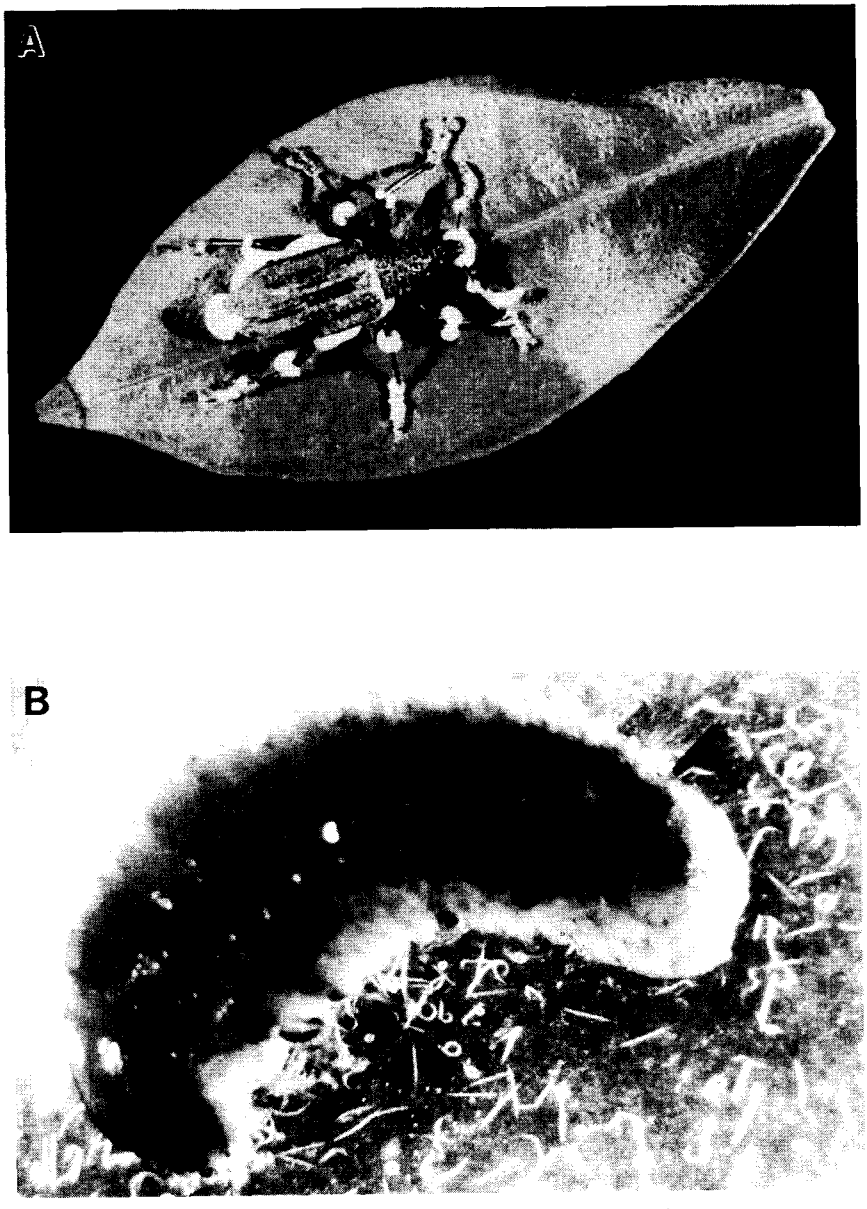

Figure 1. Diagnostic characteristics of cadavers infected with entomopathogens of selected citrus arthropods: A. Adult citrus root weevil with fungal hyphae of *Beauveria bassiana* protruding from intersegmental sites of the exoskeleton; B. Infective juvenile nematodes of *Heterorhabditis bacteriophora*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

anisopliae and *Paecilomyces fumosoroseus* isolates are being field-tested as microbial control agents of the aphid (Poprawski *et al.*, 1999).

A complex of tropical root weevils inhabits citrus worldwide. Numerous species within the genera *Diaprepes* and *Exophthalmus* are very serious pests of numerous agricultural crops including citrus. The arboreal adults are of minor economic importance to the tree. Larval feeding on tree roots, however, results in a loss of vitality, productivity, and even tree survival and creates invasive sites for soil-borne pathogens. Both indigenous entomopathgenic fungi and nematodes infect adults and larvae during their time in the soil (Fig. 1). Experimental laboratory and field studies show that the fungus, *B. bassiana*, as a commercial product formulated in oil (Mycotrol ES9601) or a combination of fungus and a sublethal dose of imidacloprid (a chloronicotinyl insecticide) are efficacious against neonates and tenereal adults when applied as a soil barrier (Quintela and McCoy, 1998). Efficacy of Mycotrol as a weevil control agent is hindered by its poor persistence in the soil.

Numerous laboratory and field studies have been conducted in the Caribbean region including Florida and California, using different species of nematodes as biological control agents of citrus root weevils (Duncan *et al.*, 1996). In Florida, *Steinernema carpocapsae, S. riobrave* and *Heterorhabditis bacteriophora* are or have been sold commercially for about 15 years for larval control. Currently, *S. riobrave* (BioVector 355®, Thermo-Trilogy Corp., Columbia, MD) appears to be most effective at high temperatures ($27 \pm 2\,^{\circ}C$) in sandy loam soils killing over 90% of the larvae when applied at a rate of 4×10^8 juveniles/ha. In California, Fuller rose weevil larvae were suppressed by 80% using *S. carpocapsae* at 50-500 juveniles/cm^2 in citrus (Morse and Lindegren, 1996).

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

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 foliar canopies is inappropriate. Less canopy shading will result in greater solar radiation that can influence the survival of entomopathogens applied to either foliage or the soil beneath the tree (Duncan *et al.*, 1996).

The planting system best suited for field plot research is a rectangle in which trees are placed at the intersection of lines that cross at right angles. This system is easiest to use when dividing a grove into square or oblong plots of uniform dimensions.

Tree density varies greatly from grove to grove and is determined by the original setting pattern. High density plantings (≥ 280 trees/ha) in a hedgerow system are superior to low density plantings (≤ 200 trees/ha) because the former supplies the greatest biological diversity and moderate microclimate in a monocultural setting and offers the best conditions for entomopathogen survival. Tree age is also important, with mature trees (≥ 8 yr) being preferred for field plot research for the above reasons.

Entomopathogens such as fungi and nematodes require irrigation to maximize survival and efficacy in the soil. Under-trees microsprinkler irrigation 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. For example, 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, usually influenced by plot size or the 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).

When a fungus or virus has been applied in the past as a foliar spray to control phytophagous mites on citrus, small plots appeared to be ineffective because untreated controls were frequently contaminated by other treatments (Shaw *et al.*, 1968; McCoy and Couch, 1982). Both healthy and diseased mites are readily dispersed from tree to tree and grove to grove by wind (Bergh and McCoy, 1997). Therefore, plot size should be no less than 1 ha and only the center 4 trees of the plot utilized for sampling. In addition, large plots (≥ 0.5 ha) are required to obtain valid yield and tree health data. Large plots are more effective in the determination of treatment effects on non-target organisms and natural enemies.

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 clumped. Therefore, soil sampling methods are ineffective in the recovery of these stages, even when sample size is high. Artificial seeding using laboratory-reared larvae has been used to increase larval numbers among plots.

The procedure for artificial infestation is as follows:

1. Pre-irrigate grove for about 2 h (5cm depth).
2. Flag designated trees for infestation and record them in the plot diagram.
3. Place 400 neonates (≥ 48 h old) obtained from a wild adult greenhouse colony into snap cap micro-centrifuge tubes (volumetric estimate or visual count) in laboratory. Micro-centrifuge tubes should contain about 1 g moistened soil to prevent larval injury.
4. Place larvae in cooler (10-12 °C) for transport to the field.
5. Scatter content 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 (Mycar®, Abbott Laboratories) or as oil mixtures (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 two times 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 using standard procedures (Kaya and Stock, 1997) by examining a sample of 50-100 nematodes via dissecting microscope. When probed with a needle, live nematodes that are 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. Nematodes and fungi are not compatible with some agri-chemicals (Table 2).

TABLE 2. Nematodes may be used simultaneously with citrus-registered pesticides and fertilizers, except for the following.

Schedule	Chemical name	Trade name(s)
Do not use 1 week before/after nematode application	**HERBICIDES**	
	2, 4-D	Various
	INSECTICIDES	
	azinphosmethyl	Guthion®
	bendiocarb	Rotate®
	chlorphyrifos	Lorsban®
	methomyl	Lannate®
Do not use 2 weeks before/after nematode application	**NEMATICIDES**	
	aldicarb	Temik®
	fenaminphos	Nemacur®
	oxamyl	Vydate®

Recommendation of Thermo-Triology Corporation

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 quantified 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 a 19-liter pail of water before adding to the spray tank. When this is not possible, the agitator should be run at a speed 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.

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 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 kmh.

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 supplied by a fan. 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 kph). 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 volume (preferred for microbials) are 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 pH 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 emphasizing 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 canopy of the tree for control of weevil larvae using herbicide applicators and chemigation systems (injection via

low volume irrigation systems) (Table 3). 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 kph. 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. 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.

TABLE 3. Nematode (*Steinernema riobrave*) field rates for Florida citrus.

Microsprinkler application		
Diameter of area covered by emitter (m)	Treated area/tree (m^2)	Nematodes/m^2 [a]
1.8	2.6	150,000
2.4	4.7	85,000
3.1	7.3	55,000
3.7	10.5	45,000
Band Application		
Boom length (m)	Area treated (m^2)	Nematodes/m^2 [a]
0.9	1011.7	46,000
1.8	2023.4	23,000
2.7	3035.0	11,500

[a] Recommended rate: 4.94×10^6 nematodes/ha or 1.7×10^6 nematodes/tree (4.94×10^6/ha x 290 trees/ ha)

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 (Table 3). This can result in cost savings in material used in application. 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 95 liters/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. 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. In addition, viability of the inoculum should be checked at the holding tank and at the nozzle to ensure effective application.

The following stepwise procedure is advised:

1. Pre-irrigate grove if necessary to achieve uniform soil moisture to at least 5 cm (deeper for sandy soils).
2. Prepare irrigation system by removing screens and filter 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 pump pressures exceeding 300 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 trees.
6. Water dispersible granules can be poured directly into the tank containing water or a slurry may be pre-mixed and then added to the tank water. The water temperature should be between 10 and 30°C.
7. The nematode suspension may be held in the agitated tank for up to 24 h, but keep the tank cool (under 27°C).
8. Irrigate grove after application to a depth of 1.5-2.5 cm of water.

F Pre- and post-treatment sampling of target insects

Each year, citrus produces new leaves which have a life expectancy on the tree of about 1.5 years. Generally, the seasonal population dynamics of foliar and soil pests are governed by abiotic and biotic factors that often involve a disease. Insect diapause is virtually non-existent.

Knowledge of the seasonal population dynamics of foliar pests of citrus is basic to designing a sampling plan for a target arthropod pest. Seasonal sampling begins when the rate of increase of a pest begins on new citrus flush and fruit. Many entomopathogens such as the fungus, *H. thompsonii,* cause predictable natural epizootics at high host densities during the summer (McCoy, 1996). Knowledge of when epizootics occur is basic to the sampling plan and timing of the application of a given microbial control agent. If the microbial control agent is being applied as a "prophylactic" treatment prior to the onset of a natural epizootic, it is likely that cadavers resulting from treatment will be mixed with those dying from the natural inoculum.

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

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 spray application is determined using a gridded hand lens (20X). Healthy and diseased adult mites observed within a 1-cm^2 area are easily recognized: healthy mites appearing clear and mobile, dead mites deformed, and diseased mites deformed with filamentous hyphae

protruding from a collapsed body. Live mites with diseases cannot be detected with 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 anytime 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 healthy, 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 is 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 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, the method is more precise.

2. Sampling citrus red mite and its virus disease

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

1. Count adult female citrus red mites twice per week for 2 weeks post-treatment and weekly thereafter.
2. Collect 20 leaves randomly per treatment in paper bags. Keep leaves cool during transport to laboratory.
3. Mites are removed from leaves manually or with the aid of mite brushing machine (Morgan *et al.*, 1955).
4. If available, 40 mites, 20 live and 20 dead or a combination equaling 40 individuals are mounted in Hoyer's medium and examined microscopically.
5. Virus infection confirmed by the presence of bire fringent 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. In the case of 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 wild adult weevils are placed in a standard screened limb bag (30 x 46 cm).
2. The infested bag is placed over a clump of shoots with new leaves (preferred food source) and attached to the limb with cord to prevent weevil escape.
3. After one week, the bag is removed from the treated and untreated foliage and weevil mortality recorded.
4. 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. Procedure is repeated weekly on selected terminals to measure fungal contact effect and persistence.

Citrus root weevil females deposit their eggs in masses between two leaves within the tree canopy. Neonates entering the soil from the tree in one year emerge from the soil as adults mainly in the spring, and summer months of the following year. Various ground traps (Fig. 2) have been devised to catch emerging adults in the year following soil treatments for larvae. Cone traps constructed of screen are placed randomly on the ground beneath the tree as close to the trunk as possible. A trap 0.9 m in diameter covers about 0.6 m^2. Generally, one trap is placed/tree with the number of traps/treatment varying according to plot size. Although the optimum number is unknown, it has been estimated that 100 traps/ha are necessary to collect meaningful data (Bullock *et al.*, 1999). A pyramid trap with low light reflectance has been compared to the cone trap in recent years (Stansly *et al.*, 1997). The pyramid trap can be used in the same manner as the cone trap, however, it should be placed just inside the tree canopy margin to maximize efficiency.

A pyramid trap will catch more adults/tree than cone traps, because it is not restricted to catching only weevils emerging from the soil beneath the trap. Pyramidal traps should be placed in large experimental plot (0.5-1.0 ha) while cone traps are better for small plot research. Both traps should be monitored weekly throughout the year of treatment and through the following spring (late June). Weekly adult catches should be examined in the laboratory for microbe infection. In the case of fungi, they should be incubated in a humidity chamber at 95-100% RH for another week.

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 of hyphomycetes fungi at the soil surface beneath the tree can be obtained by collecting of 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 out and 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 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 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.

Figure 2. Different ground traps used to catch adult root weevils found beneath the tree canopy: A. Cone ground trap; B. pyramid cone trap.

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 neonates. 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 (Fig. 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 and missing.
6. Dead larvae are held at 27°C at 100% RH for 10 days to assess larval mycosis.

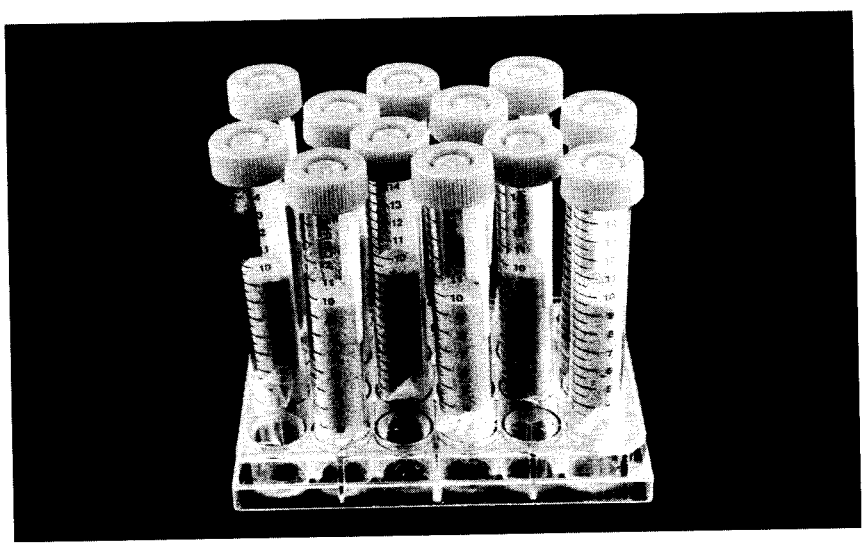

Figure 3. The bioassay unit used to test the efficacy and persistence of fungal conidial inocula applied to the soil surface.

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

An estimate of the biological activity of nematodes applied to the soil surface for suppressing larvae in the citrus rhizosphere can be determined most quickly and probably with greater precision using a tree removal–soil sampling procedure (Duncan *et al.*, 1996). Simply, 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 are removed along with any larvae. The tree is then removed into the row middle and the soil lodged in the tree crown area removed by flushing the root zone with high-pressure water delivered by a sprayer applicator. Larvae from the crown area are then collected on a plastic tarp placed on the ground. To estimate the number of larvae remaining in the upper soil dislodged from roots (30 cm depth) and lower rhizosphere (30-60 cm depth) about 0.25 m^3 of soil is collected using a shovel and sieved through a coarse screen to separate the larvae from the soil.

Two field methods using cages with and without a host plant have been devised to estimate nematode parasitism. A cage without a plant was designed for burial in the soil at different depths. This cylindrical cage (7.6 cm length and 1.9 cm dia) was constructed from a 6.5 ml vial and 2 mm mesh galvanized steel screen. A vial was simply cut in two parts to form the ends of the cage with the screen forming the sides. The above described cages may leave the contained larvae open to predation (*e.g.*, ants). A predator proof cage has also been designed using a 225 mesh stainless steel in-line sprayer filter (7 cm in length x 3 cm dia.). The procedure for use of the cages is as follows:

1. Place one 6^{th} instar larva in each cage containing field soil taken from the approximate depth of cage placement.
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 to treated and untreated soil.
5. Perform this process at 0, 1, 2, 3, and 4 week post-treatment.
6. Record the number of healthy and dead larvae in the laboratory; place each dead larvae in a Petri dish containing a moist filter paper and a wax moth larva to detect nematode parasitism.

The second field cage was designed for burial to a depth of 150 mm with the top (165 mm diameter) open to the surface at the soil line. The cage was made of polyvinyl chloride pipe (165 mm 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 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 is then placed on the upper edge of the cage to prevent invasion by predators.
3. Remove 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 (tree decline) as influenced by citrus pests and their biological control agents. Measurements should be taken for a minimum of 3 years.

1. Fibrous root density

Root mass is directly related to shoot growth of a citrus tree. Procedures for measurement are as follows:

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 16-20.
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 via wet sieving (100 mm mesh).
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 are as follows:

1. Perform sampling in June and November annually.
2. Measure photosynthetically active radiation (PAR) for given number of trees using a Accupar (Decagan Devices, Inc., Seattle, WA). Perform 2 readings/tree beneath tree canopy at a fixed location at mid-day with full sunlight.
3. Determine size (volume) by measuring tree height from top to skirt, skirt to soil surface, and soil surface to widest part of canopy. Also, measure canopy diameter.
4. Calculate canopy volume and percent PAR transmittance.

3. Tree trunk growth

Citrus trees in decline will show little or no increase in trunk growth from year to year. Procedure for measurements are as follows:

1. Perform sampling in June and November annually.
2. Select at random a given number of trees and flag for identification.
3. Measure the diameter of the tree trunk at approximately 5 cm above the budunion with calipers.

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 which 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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