Chapter 6

Capturing Insect Vectors of Phytoplasmas

Phyllis Weintraub and Jürgen Gross

Abstract

Insect vectors of phytoplasmas are limited to leafhoppers, planthoppers, and psyllids. While populations can be monitored by a number of passive techniques in the field, the capture of live insects is necessary for manipulation and study. A number of physical methods for capturing these insects already exist, but more innovative traps equipped with infochemical lures for species-specific monitoring and mass trapping are being developed.

Key words: Beat sheet, Emergence cages, Infochemicals, Kairomones, Mass trapping, Monitoring, Pheromones, Push-pull method, Sticky traps, Vacuuming, Volatile organic compounds

1. Introduction

There are three mechanisms for phytoplasmas to infect plants: insect feeding, vegetative propagation/grafting, or connection between infected and noninfected plants by parasitic plants such as *Cuscuta* spp. Insect feeding is the most important means of transmission at a local level. All known vectors are species of Hemiptera, which has important consequences in the epidemiology of the pathogen: both immature and adults feed on the same plants; they feed specifically in certain plant tissues such as the phloem where phytoplasmas reside; and feeding is nondestructive and does not elicit plant defense responses. The vectors are limited to the Cicadellidae, Fulgoromorpha, and Psyllidae (1). In the competent vector, the phytoplasma must be able to penetrate the midgut cells and migrate to and replicate in specific cells in the salivary gland to be transmitted. The period of time from acquisition to transmission competence is known as the latent period and can be days to months in length. There are documented cases of insects acquiring phytoplasmas but being unable

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to transmit them (2). For this reason, to determine the ability of any species to transmit phytoplasmas, transmission trials with live insects must be performed.

As symptoms of phytoplasma infection in a plant take some time to develop, the association of insects with a plant at the time symptoms are observed may be irrelevant because the vector may no longer be associated with the plant. Therefore, long-term monitoring must be conducted to determine when the potential vectors arrive. Some vectors, such as *Scaphoideus titanus*, are limited to feeding on a single genus of plants (3) whereas others, such as *Cacopsylla pruni*, are found on two entirely different hosts in different localities (4), thus requiring different forms of monitoring. Psyllids are usually captured live by using beating/beat sheets (5) although sweep nets are also used. Leafhoppers are usually captured live by the use of light traps (6), sweep nets (7), or vacuum sampling (8). Malaise traps are used for determining the directional movement of vectors (9). Emergence cages are used to determine plant hosts for nymphal Fulgoromorpha (10). All vectors can be monitored by yellow or clear sticky traps.

Current findings on the chemical ecology of Psyllidae and Fulgoromorpha may help to design new and innovative methods for trapping these insects (Fig. 1). It was shown recently that these insects use chemical cues for orientation and host identification. These so-called infochemicals (or semiochemicals) can influence target organisms in general (e.g., green leaf volatiles), may be specific for a specific group of insects (e.g., many kairomones), or may even be species-specific (e.g., pheromones). For instance, the psyllid species *Cacopsylla picta* and *C. melanoneura* are able to distinguish their specific reproduction and overwintering hosts by means of chemical signals produced by their host plants (11). Analyzing the chemically mediated interactions between *C. picta*, the main vector of the phytoplasma causing apple proliferation disease, ('*Candidatus* Phytoplasma mali'), and its host plants (reproduction and overwintering hosts), this phytoplasma was found to lure its highly adapted vector *C. picta* to infected apple trees by changing their odor (12, 13). The phytoplasma stimulates apple trees to produce higher amounts of a sesquiterpene (β-caryophyllene; Fig. 2b1) that preferentially attracts newly hatched adults of *C. picta* (emigrants) just before they start their emigration to their overwintering host plants (12, 14). By intensified feeding on infected plants, the probability of phytoplasma acquisition increases. In contrast, nonvector species like the hawthorn psyllid, *C. melanoneura*, or the plum psyllid, *C. pruni*, did not react to this sesquiterpene. After overwintering, *C. picta* remigrates to apple plants (remigrants), but prefers to oviposit mainly on uninfected plants (15).

This behavior results in a high probability of transmission of the phytoplasmas to uninfected plants. The identity of the infochemical(s) that regulate this egg-laying behavior still remains unknown. Other psyllid species, such as *C. pruni* (J. Gross,

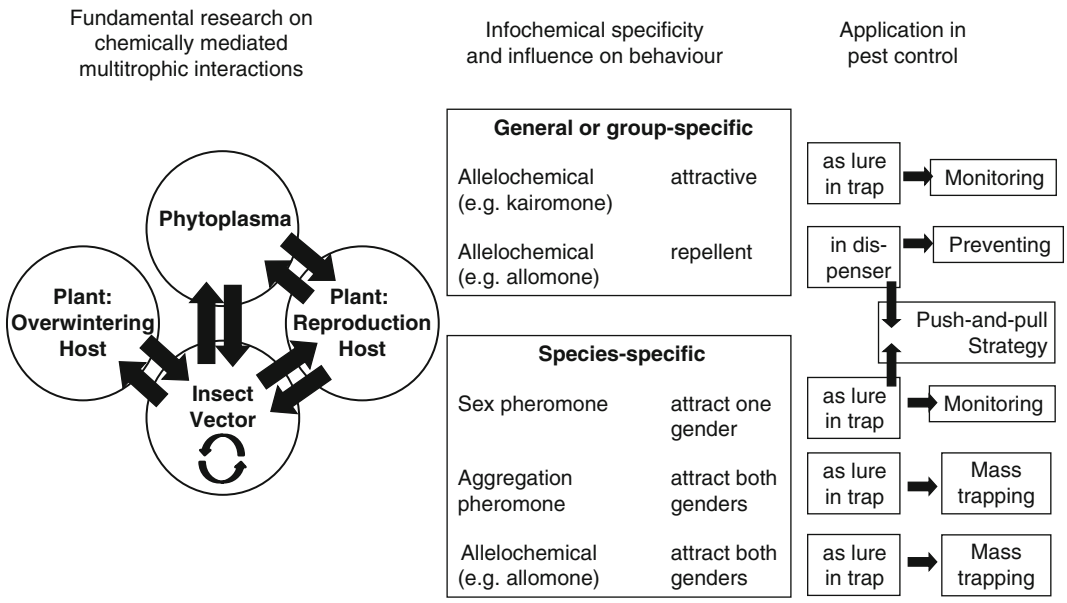

Fig. 1. The research on chemically mediated multitrophic interactions between phytoplasmas, their host plants, and insect vectors reveals the possibility to identify infochemicals influencing the behavior of insect vectors. Such behavior-modifying compounds may be used in biotechnical pest control. Methods of application are the use of attractive components as a lure in traps for monitoring or, in cases where both genders are attracted, for mass trapping. Repellent compounds could be used as a spray or in dispensers for making the crop unattractive or unsuitable for the vector. Attractive and repellent compounds can be combined in so-called push-and-pull strategies (see Note 16).

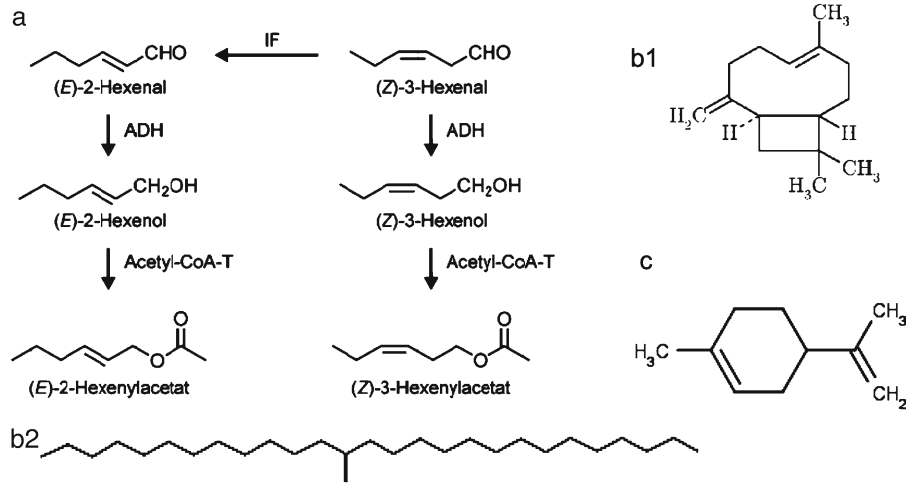

Fig. 2. Examples of volatile organic compounds which modify the behavior of phytoplasma transmitting insects. (**a**) General attractive compounds (green leaf volatiles); (**b**) species-specific attractive compounds, 1:β-caryophyllene (allomone, attractive for *Cacopsylla picta*) (12), 2:13-methylheptacosane (sex pheromone, attractive for *Cacopsylla pyricola* males) (25); (**c**) limonene (repellent for *Trioza apicalis*) (33).

unpublished results), *Diaphorina citri* (16, 17), or the planthopper *Hyalesthes obsoletus* (10), also use chemical cues for host recognition.

In addition to allelochemicals, which are attractive for some vector insect species, sex pheromones may play a role in the courtship behavior of psyllids. For a long time, it was thought that intraspecific communication within planthoppers, leafhoppers, and psyllids was only through sound and substrate-borne vibrational signaling (18–20). The first behavioral evidence for the existence of sex pheromones in psyllids was shown for *Cacopsylla bidens* (21), *Cacopsylla pyricola* (22, 23), and *D. citri* (24). The first psyllid sex pheromone (13-methylheptacosane; Fig. 2b2) was recently identified in the pear psyllid *C. pyricola* (25).

Because *Cacopsylla* are very small insects and morphologically similar, they are difficult for nonexperts to identify. Thus, the development and use of species-specific traps could simplify correct identification by farmers and further help reduce the amount of chemical insecticides applied by determining the ideal date for spraying. Based on these facts, it is possible to create group- or species-specific traps, lured with attractive components for capturing psyllids for monitoring purposes (14, 26) (Fig. 2). The cost of chemical baits, their attraction radius (effectiveness), and chemical longevity in the field are important considerations when developing new trapping systems (27). When allelochemicals produced by infected plants are attractive to both genders of vectoring psyllids, as is the case in apple proliferation-infected trees (12, 14), it could also be possible to develop mass trapping systems for a more sustainable control of these insects in the near future (Fig. 1) (see Note 1). This chapter presents a variety of passive and active traps for the capture and monitoring of potential vectors or phytoplasmas which can be used in a variety of situations.

2. Materials

Equipment (such as beating sheets, emergence cages, light traps, malaise traps, sticky traps, sweep nets, and vacuum samplers) can be purchased through entomological and/or scientific suppliers. Some allelochemicals are available for purchase, whereas others need to be synthesized by specialized laboratories or in conjunction with other researchers.

2.1. Mechanical Traps

1. Beat sheet. Prepare a square sheet (0.5–1 m^2) of sturdy material, such as muslin, and reinforce the corners to create triangular pockets that are attached on either side of each corner at a length of ~10 cm. Purchase two lengths of dowelling (~1 cm diameter)

to create a criss-crossed frame fitting the corners of the sheeting. Any similar device, such as a light-colored umbrella, light-weight light-colored tray, or light-colored ground cloth, can be substituted.

2. Vacuum trap. Modify a gas-driven portable leaf blower by switching the air intake and exhaust ports. Attach a 1–1.5 m long, 15–30 cm diameter flexible or rigid hose to the air intake port. Put 3–4 pop rivets evenly spaced around the distal opening. Purchase or make collection bags of fine nylon mesh to fit the diameter of the hose. Secure collection bags in the hose with rubber bands over the pop rivets.
3. Emergence cages. Square or rectangular cages can be constructed of any size with a metal, plastic, or wooden frame, keeping it lightweight to be easily transported. Five sides should be covered with insect exclusion screening (50-mesh) to prevent vectors from escaping but allow some air movement to prevent overheating. A sticky trap may be inserted to capture emerging insects.
4. Sweep nets. For use with low vegetation, a 1 m handle is suitable; for trees use a net with a handle extendable up to 4 m.

2.2. Trapping Using Visual or Chemical Signals

1. Traps equipped with visual signals. Traps can be made of yellow or clear plastic with enough rigidity to not fold in on itself. Trap size may vary up to ~50 cm square, but should be consistent throughout monitoring period. Water-soluble or oil-based glues can be purchased or cooking oil can be used.
2. Traps baited with attractive chemicals. Known attractive chemicals can be purchased if commercially available (some volatile organic compounds (VOCs) emitted by plants) or may have to be synthesized in adequate amounts by a chemical laboratory (pheromones). The release of chemical lures can be assured by passive substrates (filter paper, membrane dispensers, rubber septum dispensers, tape dispensers, sol–gel formulations, etc.), or active systems like micro-pumps or piezoelectric microsprayers (28, 29). They can be used for the release of any mixture or single substances of attractive infochemicals or odors at constant ratios and rates; there are many different types available, which should be adjusted to the requirements necessary for trapping the target species. Release of very volatile compounds can be delayed by adding paraffin oil to the active compounds. Many different trap types are commercially available and can be purchased, or must be developed, depending on the species-specific behavior of the target vector species. They have to be equipped additionally with the appropriate system for releasing the attractants and the specific mixture of compounds attractive for the target species.

3. Methods

Monitoring of insect populations must be carried out on a regular basis: weekly, bi-weekly, monthly, and preferably before the first insects are observed in the field until after their population has declined. There must be replicate sampling in each field, usually a minimum of ten traps or plants to obtain accurate data (see Note 2).

3.1. Beating

For use on woody bushes and trees to collect live vectors, primarily psyllids (see Note 3).

1. Place beating sheet beneath the branch to be sampled.
2. Use a short piece of rubber or plastic hose to hit the branch without causing injury to the tree or bush.
3. Collect samples by aspiration.

3.2. Vacuum Traps

For use with all types of plants and vectors.

1. Vacuum a section of the plant.
2. Secure the collection bag and place in a cooler for transportation to the laboratory for specimen identification and manipulation.

3.3. Emergence Cages

For use in determining suitable host plants, primarily for Fulgoromorpha.

1. Place trap beneath target plant and secure it so that emerging vectors cannot escape.
2. Sticky traps may be placed in cage to collect specimens.

3.4. Sweep Nets

For use with all plants, for live specimens.

1. For monitoring purposes, use a fixed number of swings in at least four locations in a field (7).
2. Aspirate desired specimens.

3.5. Light Traps

For night-time monitoring of vector species, for live or dead specimens.

1. Specimens can be collected in a bucket or bottle beneath the light source.
2. Collection vessel may be filled with 70% ethanol or polyethylene glycol to preserve specimens.

3.6. Malaise Traps

For monitoring directional movement of vectors, for live or dead specimens.

1. Traps may contain collection bottles for two-, four-, or nondirectional movement of vectors.
2. Collection vessel may be filled with 70% ethanol or polyethylene glycol to preserve specimens.

3.7. Sticky Traps

For general monitoring over long periods of time, dead specimens only.

1. Place trap in desired location for as long as required (see Note 4).
2. Specimens may be removed with hexanes, butane, or other solubilizing chemicals.

3.8. Traps Baited with Attractive Chemicals

3.8.1. Identification of Potential Infochemical Lures

1. Put the organism (plant or insect) in an adequate glass container, or wrap branches of larger plants in polyethylene terephthalate (PET) bags (Ø 20 cm).
2. Pump commercially available purified air or charcoal-cleaned air (granulated charcoal, 4–8 mm) through the container or bag for an adequate time period. Control the air flow by a flowmeter.
3. Collect the VOCs from the plant or insect headspace, choosing a method which is appropriate for your experimental setting (see Note 5).
   - Dynamic headspace sampling technique: collect VOCs in collection filters (e.g., 5 mg charcoal, Gränicher + Quartero, Daumazan, France) and elute the extract containing the VOCs by rinsing the filter with a solvent containing an internal standard (IS) for later quantification, or collect VOCs on thermodesorption tubes (glass or metal) filled with an appropriate adsorbent material (see Note 6).
   - Static headspace sampling technique: solid phase microextraction (SPME) (see Note 7).
4. To analyze the VOCs collected from plant or insect headspace, samples are either injected as solvent extracts (see above) into a gas chromatograph (GC), or are thermally desorbed from their adsorbent (thermodesorption tube or SPME fiber) by heating the matrix to 250–300°C (see Note 8).
5. Choose a GC-column for separation with properties belonging to the chemical structures of the collected VOCs. For volatile analysis, use a fused silica capillary column with an appropriate stationary phase, such as nonpolar dimethyl polysiloxanes (e.g., DB-5, DB-1), or more polar polyethylene glycol polymers (e.g., DB-Wax, Carbowax®). Use Helium 6.0 as the mobile phase.
6. Use an appropriate temperature programme for optimum separation of the collected compounds.

7. Use a mass spectrometer (MS) coupled with the GC for the identification of the separated compounds by comparing the characteristic ion fragmentation pattern (mass spectrum) with data from mass spectra libraries (see Note 9).
8. Confirm the identification by injection of synthetic samples on the same column, or preferably on two columns with different polarities, by comparing retention times and mass spectra (see Note 10).

3.8.2. Evaluating Potential Behavior-Modifying Compounds

1. Use gas chromatography coupled with electro-antennographic detection (GC-EAD) to identify compounds detected by the insect antenna (see Note 11).
2. Behavioral tests can be carried out by using dynamic or static olfactometers, wind tunnel, or a Kramer sphere, depending on the specific behavior of target organisms (see Note 12).
3. Pump purified air through two containers containing the test VOC and attach each outlet with a test arm of the olfactometer. Control the air flow with a flow meter (see Note 13).
4. Count every individual that passes a final mark on one of the test arms within a specific period of time (normally 5–15 min) (see Note 14).
5. Analyze the data statistically (e.g., by dependent paired *t*-test or *G*-test, if more than one psyllid was tested simultaneously).
6. Choose an attractive compound or mixture as a lure for infochemical traps.

3.8.3. Development of Infochemical Traps

1. Choose a passive substrate or active system (e.g., dispenser) for providing the chemical compound/mixture in the trap over the flight period of the target organism.
2. Check the release rate of the dispensers by weighing them over the test period under natural conditions (see Note 15).
3. Adjust the release rates of chemical compounds according to appropriate requirements of the target species.

3.8.4. Modifying Specialized Traps

1. Purchase a commercially available trap and modify it with the system developed above for providing the infochemical.
2. Add sticky surface, insecticide, or fluid (70% ethanol or polyethylene glycol to preserve specimens).
3. Inspect the traps at regular intervals for caught specimens.
4. Monitor the charging level of the dispenser during the flight period of the target species and refill when necessary.

4. Notes

1. Besides monitoring of insect vectors by traps baited with attractive chemicals, mass trapping for control purposes may also be possible when the chemical lure is attractive for both genders of the target species. This can be the case when aggregation pheromones, plant kairomones, or allomones are used as the chemical lure (Fig. 1). The efficacy of mass trapping methods can be explored through field experiments and by computer simulations. The costs of chemical baits, their attraction radius, and chemical longevity in the field must be taken into consideration when developing a mass trapping system. For further reading, the Web site of Byers (27) provides software, Java applets, and other tools regarding this method.
2. Certain types of monitoring, sweep net sampling, vacuum sampling must be carried out after dew has evaporated, as insects caught by these methods will adhere to the bags and are difficult to dislodge. Conversely, under conditions of moderate to strong wind, insects move deep into plants and are difficult to dislodge. Monitoring by beating, sweep net and vacuuming should be avoided during these times.
3. For receiving quantitatively comparable results, the monitoring of population dynamics of vector insects can be carried out according to (30). For taking one sample, ten comparable branches of each host plant are strongly tapped with a pole (about 1 m long) three times per branch/plant. The dislodged insects can be collected in a beating sheet held beneath, equipped with a removable polyethylene collection bag. After sampling, the collection bag can be placed in a cooler for transportation to the laboratory where collected insects can be counted and stored in vials for later identification or experiments.
4. The length of time in the field is determined by the amount of dust that accumulates on the trap.
5. Further information on practical approaches to plant volatile analysis for researchers inexperienced with analytical techniques can be found described in detail in (31).
6. For an overview on commonly used adsorbents, see ref. (31). The closed loop stripping analysis (CLSA) can be used when only very small amounts of volatiles will be emitted by the organism.
7. SPME is a fast and simple method for collecting volatiles at very low detection limits, but due to its specific characteristics it is suitable for sampling VOCs for qualitative rather than quantitative analysis.

8. When using a two-stage thermal desorber, the thermally released volatiles will be concentrated in a cold trap prior to their injection into the gas chromatograph, while the SPME fiber can be directly desorbed within the injector.
9. Suitable libraries include NIST/EPA/NIH Mass Spectral Library, National Institute of Standards and Technology, Wiley.
10. In case of resulting complex volatile patterns, bioinformatics like multivariate analysis should be implemented in the statistical interpretation of differences between plant VOC or insect pheromone mixtures (32).
11. This will reduce the number of VOCs to those which may be responsible for vector attraction to the host plant, and so also reduce the amount of the effort needed for identification of behavior-modifying compounds.
12. For some psyllid and planthopper species, dynamic Y-shaped olfactometers have been proven to work (10, 12). They consist mostly of a Y-shaped glass tube mounted on an angular board.
13. According to the target species-specific behavior in the olfactometer, test either one, five (10), or ten specimens (12, 21) of the same gender together. Do not test different genders simultaneously.
14. In case the infection status of the vector needs to be determined later, place each specimen after the behavioral trial into a separate vial filled with ethanol (70–96%). Extract the DNA and identify the phytoplasma as required.
15. Ideally, the release rate of the attractants should be relatively constant and remain effective during the whole flight period of the target species. However, during periods of higher ambient temperatures, the release rate may increase depending on the volatility of the components. Thus, it is important to monitor the charging level of the dispenser during the flight period of the target organism often and refill when necessary. In order to reduce the release of volatile compounds provided by passive substrate, dilute them in paraffin oil. To avoid temperature effects in general, use an active system such as a micro-pump or a piezoelectric micro-sprayer with constant release rates.
16. The so-called push-pull strategies consist of a behavioral manipulation of an insect vector by the integration of two different stimuli. A repellent makes the protected resource unattractive or unsuitable to the vector (push stimulus), while luring it toward an attractive source or attractant (pull stimulus), e.g., located in a sticky trap. By doing fundamental research on multitrophic interactions between plants, phytoplasma and insect vectors, the isolation and identification of attractive and repellent infochemicals may also open the path to this very innovative control strategy.

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