Characteristcs and identification of xylem-sap feeders

Workshop Manual

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1. SAMPLING FOR INSECT VECTORS

Our analysis of sampling data enables us to "see" the invisible: insect and microbial populations, their behaviour, and their changes over time. Proper sampling design, execution, and analysis does not guarantee success, but no or poor sampling or analysis will lead to failure every time.

Sampling is fundamentally quantitative. To both plan and interpret sampling, we need numerical methods, usually statistical. This is not within the scope of this workshop, but we are repeatedly exposed to descriptive statistics, and with guidance we all can use a bit of analytical or hypothesis testing statistics. Consult with a statistician as part of your creating a sampling plan and begin to analyze your results soon after starting a sampling plan rather than waiting after your entire sampling program has is completed.

Our choices of sampling methods should primarily be determined by our objectives. With vectors we are most interested to learn what plants they prefer for feeding and oviposition over time, the effects of habitat conditions such as soil moisture and plant condition (very critical for xylem sap feeders). Some sampling methods evaluate vector flights and movements. What are the effects of insecticidal treatments? Determining our sample size can be aided by (1) preliminary sampling to give us some idea of the amount of effort and sample size needed and the degree of variability in what we want to sample, and (2) our knowledge of the biology of the organism(s) we want to study.

1.1 ABSOLUTE AND RELATIVE SAMPLING METHODS

Absolute sampling estimates the number of individuals being sampling in a defined space or plant part. Absolute sampling is most useful for modeling populations and evaluating control methods. It can be very labor intensive to acquire accurate estimates of population density. It can also be expensive. For example, efficient vacuum traps to capture insects from the air are used to detect and estimate the numbers of flying aphids to study aphid flight dynamics and to determine the time to harvest seed potatoes to avoid virus infection of the potato crop by aphids. The equipment and the processing of the rap samples daily are very costly but justified by the value of the crop saved.

Another example of an absolute sample is using a vacuum collector (Fig. 1C) to collect all the vectors within a cylinder placed on the ground over low-growing plants. This method would not be accurate for vectors on a tree branch because too many insects would escape. On the other hand, counting nymphs on the last meter of a grapevine cane by simple inspection can be accurate if done so as to minimally alarm the insects. Spittlebug adults are difficult to count by direct observation of plants because the adults and highly mobile and evasive. On the other hand, spittlebug nymphs are not only sedentary, they also can be easily seen and counted because of the bubbles (spittle) surrounding their bodies.

A **D-Vac** (Fig. 1C) is a back-pack instrument powered by gasoline engines for complete freedom in the field. Insects are collected live in tissue bags and may be taken to the laboratory for separation. The advantage of D-VAC vacuum sampling is the more complete extraction of the tiny species and the immature forms of even the larger insects. A simpler

vacuum device can be made more inexpensively than a D-vac with a leaf blower that can be modified to suck insects into a collection bag (Fig. 1D).
The **mark-capture-release-recapture method** (Lincoln index) is an indirect method of sampling that attempts to provide absolute estimates of population density in a defined space. For leafhoppers, this has been done by capturing insects (typically with a sweep net in vegetation), marking them by dusting with a very fine fluorescent paint pigment powder, releasing them, then collecting the insects – all in the same defined space. The ratio of the number of recaptured marked insects divided by the total number of all insects captured after the release estimates should equal the percentage that the total number captured represents as a fraction of the population. For example, if 100 captured insects are marked and released, then the next day, 200 insects of the same species are collected and 20 (10%) of them are found to be marked, this estimates that 200 insects is 10% of the field population in the space evaluated. Disregarding mortality, immigration and emigration, the estimated field population would be 2000 insects (200/0.1). There is a large scientific literature on variations and use of this method.

1.2 RELATIVE METHODS

Relative methods are the most commonly used because they are simple and easy to use. Basically, relative sampling can show that the number of individuals in a defined area is different than on another date or in another location but does not estimate the actual number of individuals. A major problem with relative methods is mainly that they are strongly affected by many influences: temperature, wind, plant composition, sampler behavior, plant wetness, etc.

The most common sampling method is a sweep net (Fig. 1A). This sturdy net can endure use on plants with thorns. It is a reasonable relative sampling method for low vegetation unless the plants are wet or temperatures are so cold that the insects are mostly near the ground. Adults are usually collected much more commonly by sweeping than are nymphs of the same leafhopper species. Spittlebug nymphs surrounded by excrement bubbles are poorly captured by a sweep net. Sweep sampling jars insects on plants so that fall from the plants and fall into the passing net, Wet plants make this ineffective because the insects become caught up in water on the plant.

A beating sheet (Fig. 1B) operates much the same way as a sweep net. Fabric stretched over a square or circular frame is held under a branch or entire plant and the plant or branch is hit forcefully to dislodge the insects on it so that they fall onto the cloth. A problem is that the insects on the cloth can more easily escape than from a net.

1.3 TRAPS

Traps have the advantages of operating continuously and often capturing larger numbers of relatively rare insects that might be barely detected by sweeping. Traps may also reflect flight movements. Sticky traps are often one of the most useful methods of relative sampling for vector leafhoppers because in addition to population density they combine a way to assess flight movements.

Sticky traps are most commonly made of water resistant cardboard covered, brightly colored (usually yellow for most leafhoppers), and covered with a sticky, clear petroleum grease that ensnares insects that fly onto the trap. Sticky traps have many problems. Temperatures, rain, and debris are major influences. Some leafhoppers are not attracted to yellow. Traps require setting up and periodic counting and trap replacement. Even so, sticky traps are sometimes one of the most useful methods of relative sampling for vector leafhoppers. A variation of the sticky trap is a bright yellow tape that can be stretched along posts. This can be useful in pinpointing where insects most frequently fly.

Figure 1. (**A**) sweeping net; (**B**) beat tray; (**C, D**) vac aspirator; (**D**) yellow sticky trap.

Figure 2. With a continuous sticky tape the locations of insect catches can be recorded to determine the locations of the greatest number of an insect species.

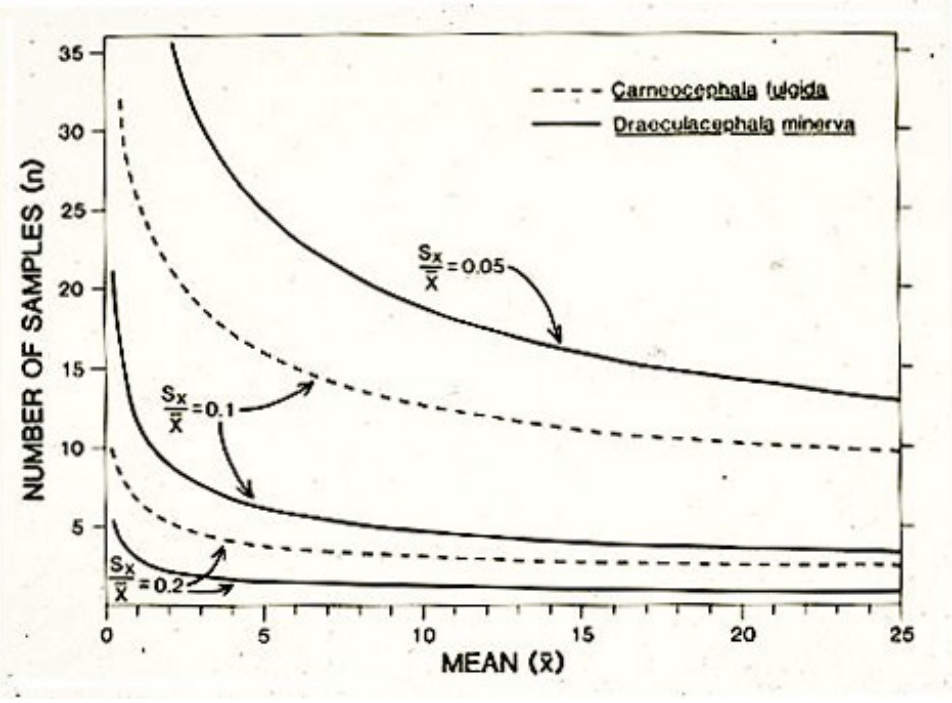


Figure 3. Increased precision requires a larger number of samples. The number of samples estimated to be required for a specified level of confidence (Sx/ x, where variance = Sx, x= average; in Hilgardia 53(4), 1985).

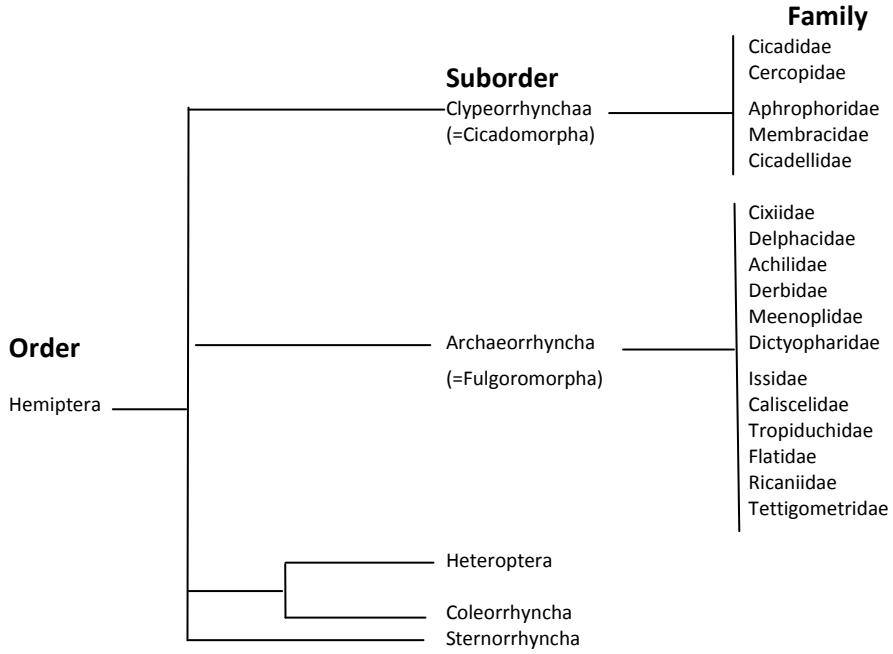
2. IDENTIFICATION OF THE EUROPEAN XYLEM-SAP FEEDER TAXA, POTENTIAL VECTORS OF *XYLELLA FASTIDIOSA*

2.1 SYSTEMATICS OF AUCHENORRHYNCHA

The Auchenorrhyncha are hemimetabolous insects member of the order Hemiptera (= Rhynchota) sometimes considered as superorder. Recent studies led to consider within the order four suborder Clypeorrhyncha (= Cicadomorpha), Archaeorrhyncha (= Fulgoromorpha), Heteroptera and Coleorrhyncha (Prosorrhyncha) and Sternorrhyncha. Both the suborders Clypeorrhyncha and Archaeorrhyncha were former considered as Auchenorrhyncha. The phylogenetic relationships among these four groups are not clarified yet.

2.2 CLASSIFICATION OF HEMIPTERA

Classification of Hemiptera, according to Campbell *et al.* 1995, Bourgoin & Campbell 2002, Sherbakov & Popov 2002, Sorensen *et al.* 1995, Yoshizawa & Saigusa 2001, with particular reference to the European families of Auchenorrhyncha, is shown in table belove. Known vectors of *Xylella fastidiosa* are xylem-sap feeder insects belonging to the families Cicadellidae, Aphrophoridae, Cercopidae and Cicadidae within the Cicadomorpha.



2.3 MORPHOLOGICAL CHARACTERISTICS

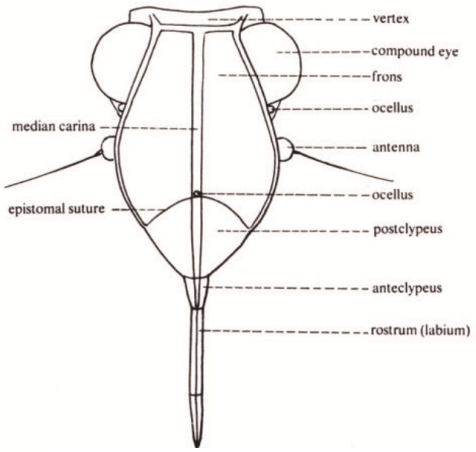
The external body structure of the Hemiptera is variable. Hemipterans differ, with few exceptions, from all other insects by their mouthparts. They have a piercing-sucking beak, the rostrum, from which the name Rhyncota, with maxillary and labial palps totally reduced, mandibles and maxillary laciniae modified into two pairs of stylets which at rest are more or less retracted in the head with their apical parts enclosed in the grooved, segmented labium. The fore wings are usually larger or at least longer than the hind wings. During flight the wings are usually held together by means of a wing-coupling apparatus. In most groups the anal field of the fore wings is demarcated from the rest of the wing by a straight suture, the claval suture. The presence of this so called clavus in both orders is, like the common structure of the mouthparts, strong evidence for a close relationship of the Homoptera and the Heteroptera. The wings may be normal or more or less reduced. Many Hemiptera species are wing-polymorphous, i. e. in one and the same species there are individuals with normal wings and others with more or less reduced wings. If there are only two alternatives in a species this is said to be wing-dimorphous.

FULGOROMORPHA: Insertions of the median coxae widely apart from each other. Bases of the fore wings each with a tegula.

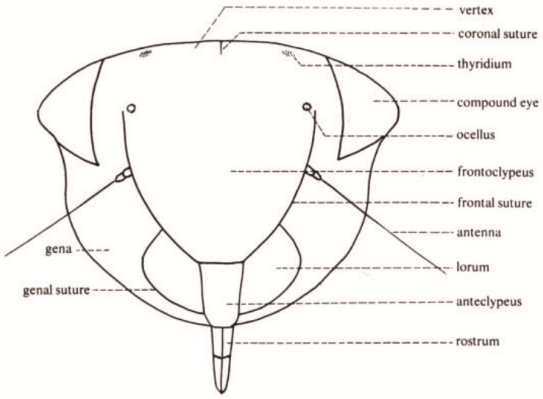
CICADOMORPHA: Insertions of the median coxae close to each other. Fore wing bases without tegulae

Morphology and diagnostic characters of the Auchenorrhyncha

Head



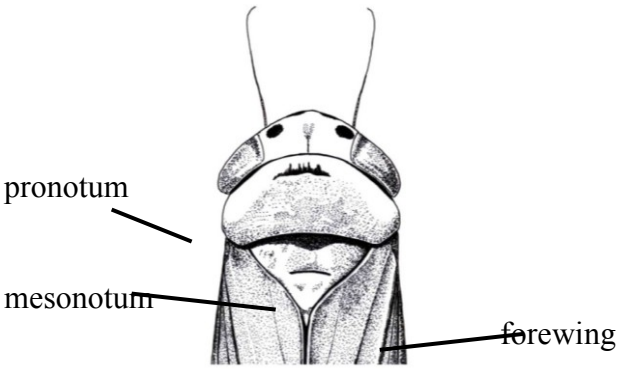
***Cixius similis* Kirschbaum (Cixiidae), face**



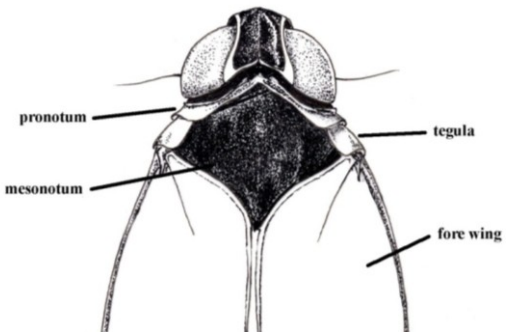
***Hesius domino* (Reuter) (Cicadellidae), face**

Thorax

The prothorax is the foremost segment of the thorax. The pronotum, its tergal part, is a transverse plate. The mesothorax, the second segment, carries the fore wings. The mesonotum, the tergum of the mesothorax, consists of four more or less distinct parts arranged in order from the front: prescutum, scutum, scutellum, and postscutellum. The metathorax, the third thoracic segment, carries the hind wings, and metanotum, its tergal part, is entirely concealed by the wings in repose.



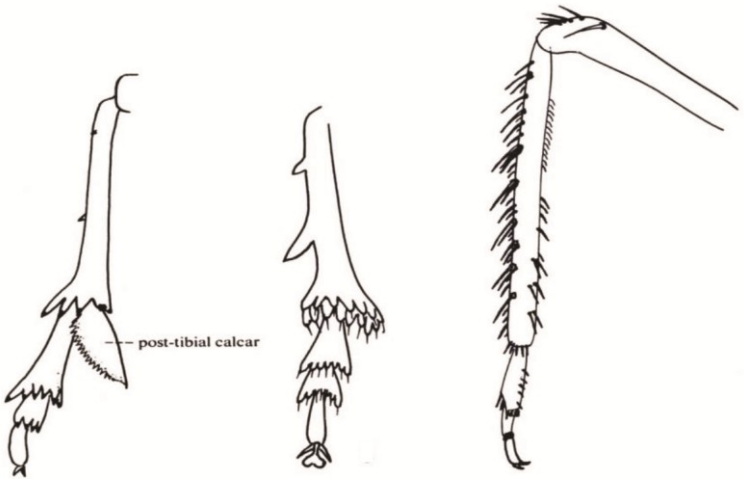
Cicadomorpha



Fulgoromorpha

Legs

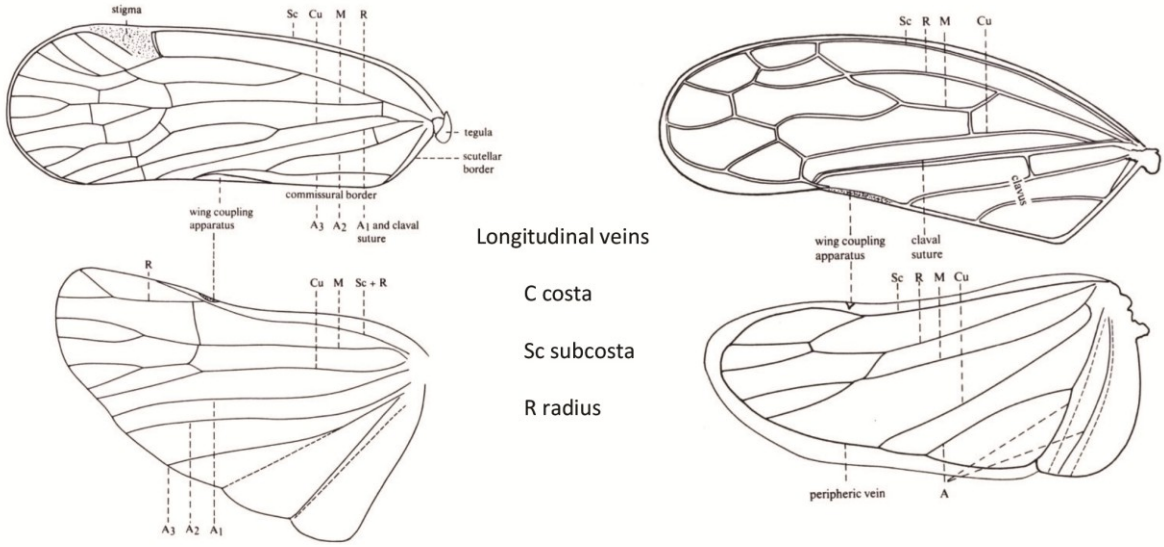
These consist of the same elements as those of other insects. The tibiae are often armed with fixed spines and/or movable setae, the latter usually arranged in longitudinal rows. The hind femora in the Cicadellidae are apically armed with a few strong setae, the number of which is constant within the various taxa, giving a character useful in keys. Also the chaetation of the dorsal surface of the anterior and median tibiae furnishes characters for the separation of genera, especially in the Deltocephalinae. Characteristic of the Delphacidae is the so called post-tibial calcar, which varies within the family in shape, size, and number of marginal teeth as well as in the degree of development of its apical tooth.



Delphacidae ***Cercopidae*** ***Cicadellidae***

Wings

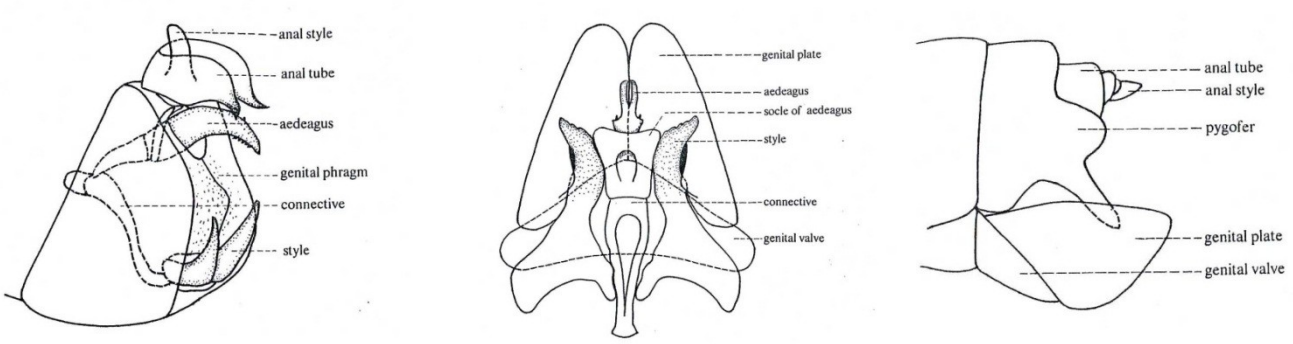
The fore wings may be leathery or membranous. Even in the latter case they are usually somewhat firmer than the hind wings. The wing-polymorphism is usually a dimorphism. Brachypterous: short-winged, Macropterous: long-winged.



Fulgoromorpha **Cicadomorpha**

Abdomen

The abdomen shape in most Auchenorrhyncha is usually longish, cylindrical, conical, or with a triangular transverse section. The first abdominal segment or segments I and II together contains a sound-producing apparatus, the so called tymbal organ. The most important part is the apex which contains the genitalia. The specific identification mainly rely on male genitalia



3. INSECT VECTOR REARING

Insect cultures are useful and sometimes necessary for experiments on pathogen transmission, insect morphology studies, etc. Having reared insects instead of those collected from the field facilitate these types of experiments and allow control of the age and numbers of insects to be used.

Plants for rearing leafhoppers and spittlebugs should be of the appropriate species (sometimes even the variety is important) and condition. The temperatures needed or tolerated vary with the insect species. Most insects in temperate climates have reproductive diapause (arrested reproduction until a more desirable season) beginning in the autumn. During short days of late autumn through winter, supplemental light is needed to continue the insects' reproduction. Most leafhoppers will continue to reproduce if temperatures are warm enough and daylight periods are at least 14 hours per day, although shorter lighted periods may work for some species. For insects that have only a single generation per year (example: *Philaenus spumarius*), keeping the dormant eggs alive until spring can be difficult.

Three important requirements for rearing are:

1. Suitable plants of the ideal age and condition when they are needed for replacing depleted plants and to set up egg laying.
2. Cages that prevent insect escape and keep out unwanted insects and pests.
3. A protected space with adequate temperatures and light.
4. A place to change the plants or cages while preventing the reared insects from escaping. This should be separated from where the reared insects are kept.
5. Regular or scheduled changes of plants or arranging for egg laying or hatching.

3.1 SCHEDULING AND PLANNING

Rearing requires regular changing of plants on which the insects are kept. Insects colonies that are too crowded, kept for too long a period without changing plants, or having plants in a poor condition will produce insects that die sooner than you may have planned. Xylem sap-feeding insects are especially sensitive to being overcrowded because they may produce liquid excrement that can be 100 to 1000 times their own weight per day! These sharpshooter leafhoppers and spittlebugs require plants with succulent growth. Nymphs are especially sensitive to plants that are too old or in a declining condition. For this reason, having surplus plants on hand allows for changing plants if they decline unexpectedly.

For example, if a weekly change of plants is desired, plants must be planted weekly to provide plants of the proper size and age at the time they are needed. Fast growing plants such as barley can be produced on as quickly as 3-4 weeks (Fig. 4). Setting up a schedule on a calendar is one way to be sure all persons involved have the same information. After planting, the plants should be watered and fertilized on a regular basis to keep them in good condition until they are used. It is often the case that some persons are responsible for normal operations to raise or maintain plants, often different from the persons suing the plants for rearing or experiments.

In this case the greenhouse (glasshouse) personnel need to be carefully informed of what to do and what to avoid (pest and pesticides, overwatering, etc) to avoid catastrophes.

Figure 4. Barley from 10 to 30 days after planting by seed.

3.2 PLANTS

These should be planted from seed or propagated plants in suitably sized containers. Selecting the plants to be used may require research or tests to determine the best plants to be used. Be careful that the plants have not been treated with pesticides that might kill the reared insects. Water, fertilizer, light and temperature conditions should be adjusted to produce the best plants to rear your insects. If you are using the plants or reared insects for transmission experiments that require noninfectious insects, the rearing plants must be protected from contamination with specific pathogens.

Rearing plants should be protected from pests such as aphids, thrips, mites, etc. so that the pests do not interfere with the reared insects. This is a common and can be a serious problem that requires a protected greenhouse space, planning, and vigilance. If a pesticide is required to control pests, the type of pesticide should have a short residual activity and not threaten the survival and health of the reared insects.

3.3 CAGES

There are many varieties of cages that may be used. In general, these can be two categories of cages: large cages that can contain many insects and plants, or smaller cages that contain a single plant. The larger cages generally require less effort per week but have the disadvantages that the colony of insects in the cage will have a wide range of adult ages. For species with very long-lived adults, this may not be a problem. The use of smaller cages take less space and allow for easy control of age by confining egg laying adults on the plants for a short period (ex.: one week), and then keeping the plants after removing the adults for a suitable time for most of the eggs to hatch. Cages should be durable, well ventilated (to prevent free moisture on the plants and cage walls), and easy to handle.

An example at UC Berkeley of a cage type that has served well for raising leafhoppers, psyllids and aphids for over 75 years is a wood frame cylinder (typically 20-25 cm diameter and 30 to 50 cm height) with one glass side and the top and remaining walls covered with a fine mesh cloth of clear or white Dacron glued to the frame. This type of cage rests on a circular gasket of foam rubber that is a seal between the plant pot and the cage (Fig. 5). To change the plants periodically, the cages are put on a counter with the heavy glass side of the cage facing a window in a small room compartment. The cage is tilted towards the window and then carefully lifted to shake and blow (by mouth) the insects into the cage. The cage is then placed atop a replacement plant with a new foam rubber gasket already in place. An aspirator or vacuum machine hose with a collecting device can be used to capture the desired number of escaped insects to return them to the cage. The surplus insects are then disposed of by vacuuming with the aid of a brush. Because these cages can be easy to tip over, a tightly stretched wire can be placed over the cage and anchored on each side of the cage to the table holding the cages.

Figure 5. Wood frame cages covered with Dacron fabric and with a glass side. The wooded frames here have lasted 60 years.

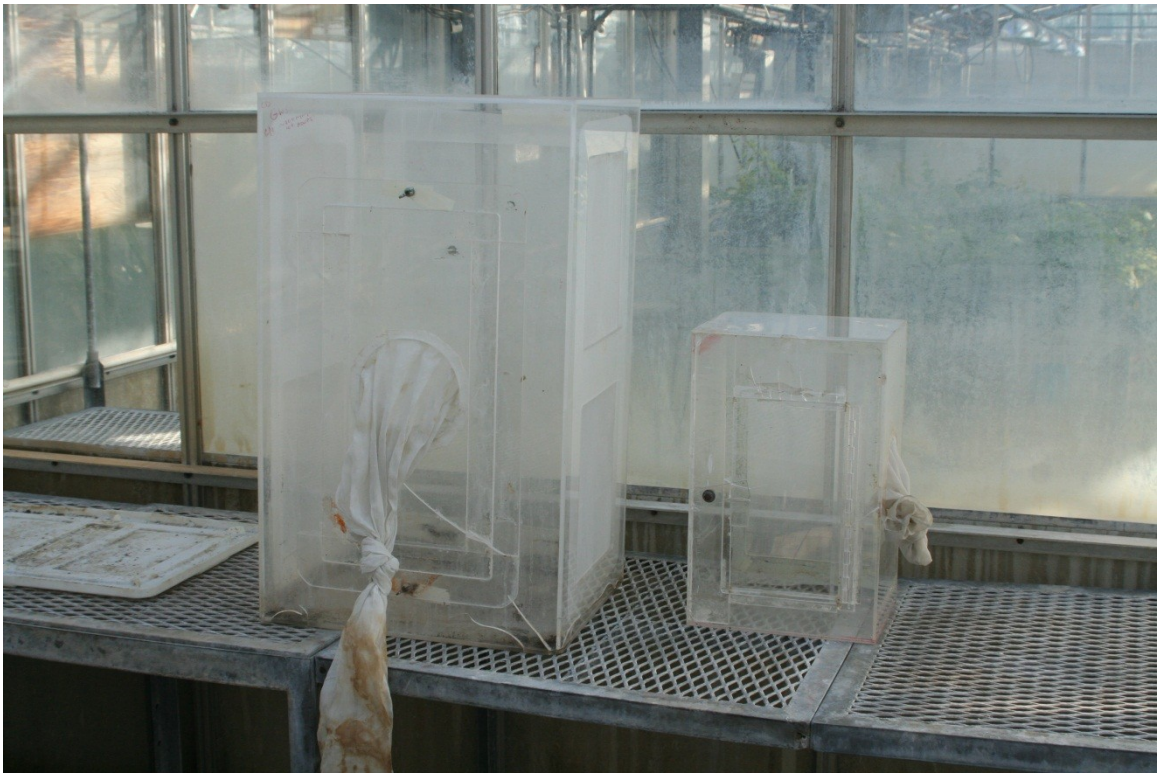


Figure 6. Large cages (Plexiglass and fabric) that can contain several plants.

3.4 FACILITIES

Greenhouse (or glasshouse). Screens or filters to keep out unwanted pests and prevent the escape of experimental insects are a necessity for work with insect vectors of pathogens. An insectary (insect rearing space or facility) for vectors may have special barriers to prevent infectious insects from escaping from the greenhouse or other type of facility. Shade cloths or whitewashing glass roofs and walls may be needed to prevent overheating colony cages exposed to direct sunlight. Fertilizer is often applied in water via a greenhouse hose. Otherwise, slow release pellets may be useful for fertilization.

Changing rooms. A darkened room with a small bench or table against a sun-facing window is useful to attract any escaped insects to the glass window. The sides and ceiling surrounding the bench minimize escapes into the room. A bright lamp outside the window can supplement sunlight (or replace the sun at night) in attracting insects to the window. An industrial dry/wet vacuum cleaning machine is very useful in collecting escaped insects.

Figure 7. Bench for changing insect colonies or adding or removing insects from test plants in transmission experiments. Sunlight in the window and fluorescent light (top) attract the insects. The rear of the bench can be shielded with cloth to reduce insect escapes.

Environmental cabinets (or chambers) provide programmable temperatures and light/dark periods, as well as protection from pest invasions. Some disadvantages are expenses, requirement for frequent repairs, and size limitations. Light intensity should be suitable to maintain plants in a healthy growing condition.

4. INSECT VECTOR TRANSMISSION OPERATIONS

There is no other way to demonstrate that an insect species is a vector of a plant pathogen than to confine a putative vector on a plant for some period of time and then keep the plant to later confirm that the pathogen has colonized the test plant. The presence of a pathogen, regardless of how abundant it is - in or on an insect - is not proof that the insect is a vector. There are many examples of detecting a pathogen in insects that never transmit the pathogen to a plant or only transmit under special circumstances. For example, sharpshooters can be fed on a suspension of *Xylella fastidiosa* cells through an artificial membrane. If the suspension has a high number of cells (>108/ml) the insects can consume over 50,000 cells but never transmit it to a sensitive plant host. However, if the cells are cultured in the presence of pectin or related chemicals, the insects not only acquire *Xylella fastidiosa* cells but also deliver them to plants.

In addition to knowing what vectors transmit a pathogen to plants, what other characteristics of vector transmission are important to know?

First it is helpful to understand what we mean when we use the following terms, even there is disagreement about their definitions. Some transmission characteristics of *Xylella fastidiosa* make controlling *Xylella fastidiosa* diseases by insecticidal control of the vectors.

Transmission: the process by which one organism acquires and delivers (inoculates) a pathogen to a host.

Acquisition: The uptake (acquisition) of a pathogen by a vector may be occur from transovarial passage of the pathogen from a parent (usually from the mother) or by feeding on an infected host.

Acquisition access period (AAP): the time allowed on an infected host for a vector to acquire the pathogen.

Inoculation: the delivery of an organism to another organism.

Inoculation access period (IAP): the time allowed on a host for a vector to inoculate the pathogen.

Infection: a pathogen's occupation of a host, usually involving pathogen multiplication A pathogen in some hosts may not cause disease in other hosts. Colonization is the multiplication and spread of a pathogen within a host. *Xylella fastidiosa* can colonize many host plants without causing disease.

Infective (Infectious): able to inoculate another host with a parasite. Infectivity with *Xylella fastidiosa* is estimated by testing insects collected to determine if they transmit *Xylella fastidiosa* to plants. The frequency that an infective insect transmits depends on its transmission efficiency.

Transmission efficiency: percentage of insects that transmit when given a set of specified conditions. The environment (time of day, temperature, etc.), pathogen strain, test plant, etc. can affect transmission efficiency.

Incubation period: the period during which an organism grows and may also circulate within a host to the point when the organism can then be transmitted to other hosts.

Latent period (LP): the time required for a pathogen within or on a vector until the vector becomes capable of delivering the pathogen that infects another host. Some transmission processes do not require any latency after acquisition. An AAP of 15-20 seconds after probing stars is ideal for some aphid-borne viruses classified as "non-persistently" transmitted. Some viruses must circulate from the gut into the body cavity (hemocoel), then enter the salivary glands in order to be transmitted. This process can require less than a day or as much as several days. Pathogens such as phytoplasmas, require a long latent period, such as from about a week until over a month. The latent period often is affected by temperature.

** There is no such thing as THE latent period or THE MINIMUM latent period, but all vector transmission experiments will result in showing a VIRTUAL latent period simply because not every vector insect capable of transmitting actually transmits at every opportunity. When overall transmission efficiency is very low (example: 3%), the calculated LP can be several days when there actually is no required LP. Increasing the number of insect vectors tested and the frequency of changing test plants can affect the accuracy of calculating the average or median LP. Using 25 to 30 insects tested individually AND 10 to 20 insects per plant on at least several plants for each treatment and an equal number of controls is a good place to start if transmission efficiency is unknown.

Average latent period: is the mathematical average of the time taken for each tested vector to transmit for the first time.

Median latent period (LP50): is the estimated time required for half of the tested insects to transmit for the first time. The calculation of an LP50 can be analogous to estimating the median lethal dosage (LD50; kills 50% of text insects) in dosage-mortality essays of an insecticide.

Parasite: (1) an organism or virus that depends on another host for food or shelter. The parasite is usually much smaller than the host. (2) Most people consider that the term "parasite" pertains to an organism is harmful to its host in addition to the characteristics in (1) above. [Purcell opinion: not all organisms in the first definition cause harm to the host – in fact they may be required for the host. Obligate symbiotic bacteria of aphids fit this description].

5. TRANSMISSION OF *XYLELLA FASTIDIOSA*

1. All known *Xylella fastidiosa* vectors are specialist feeders on xylem sap. Because of the low nutrient concentration of xylem sap, from about 100 to 1000 times the body weight can be consumed in warm conditions by these insects.

2. Many factors can affect transmission efficiency. The most important is the host plant species and vectors species, including the specific combination of the two. For example, Graphocephala atropunctata (blue-green sharpshooter) and Philaenus spumarius (meadow spittlebug) are very efficient on grapevine, with transmission rates commonly greater than 90% per day. In contrast, Homalodisca vitripennis usually transmits to grape at less than 6% per day. G. atropunctata is a poor transmitter of *Xylella fastidiosa* to alfalfa, whereas Draeculacepahala minerva (green sharpshooter) a much more efficient vector of *Xylella fastidiosa* to alfalfa but a poor vector to grape.

3. There is no measureable latent period for vector transmission of *Xylella fastidiosa*. Experiments using one hour for AAP and a one hour IAP resulted in 9 % transmission. This was the product of a ~30% acquisition in one hour followed immediately by a one hour IAP with ~30% efficiency. The contingency probability of any single insect both acquiring *Xylella fastidiosa* from a plant in one hour and then inoculating *Xylella fastidiosa* into a test plant in one hour is 0.3 X 0.3 = 0.09 (or 9%). This is what you would predict if there was not latent period. This experiment concluded that there was no evidence that a latent period was required for transmission.

4. Vector transmission of *Xylella fastidiosa* is persistent. Vectors that acquire *Xylella fastidiosa* as adults remain infective for life. We know of no other pathogen that has no latent period but is also transmitted persistently. This implies that *Xylella fastidiosa* must multiply in vectors to continue to be transmitted for months.

5. Vectors cannot transmit after molting but can immediately reacquire the bacterium by feeding on an infected plant. When nymphs that transmit *Xylella fastidiosa* molt, they stop transmitting *Xylella fastidiosa*. However, they can immediately transmit after re-acquiring *Xylella fastidiosa* from plants. The shedding of the foregut exoskeleton during molting implies that the foregut is the location from which vectors deliver *Xylella fastidiosa* cells to a plant.

6. Only a very small estimated number of live *Xylella fastidiosa* cells in the foregut of a vector is needed for efficient transmission. In general, the higher the concentration of live cells in a plant, the higher the probability of acquisition by a vector feeding on the plant. However, the numbers of live cells (or PCR estimates of cell numbers) in the head of a vector do not correlate with transmission efficiency. In 2 vector species tested, insects from which no *Xylella fastidiosa* were cultured transmitted *Xylella fastidiosa* about as well as insects that had under 100 cells per head. Scanning electron microscopy of the foregut (the cibarial pump chamber and precibarium canal) showed that populations in the foregut can grow steadily in number, eventually forming a “lawn” or “carpet” of bacteria. This does not seem to increase transmission by insects with many fewer bacteria. A major implication of these findings is that a

small area of the foregut is the key location from which *Xylella fastidiosa* is transmitted.

7. Numerous *Xylella fastidiosa* genes affect vector transmission, implying that vector transmission is not a simple mechanical process, although mechanical inoculation of plants with a simple needle easily infects plants.

5.1 TRANSMISSION METHODS

Vector transmission experiments are very simple but need careful attention to all aspects.

- The main concern should be avoiding accidental infection of plants by vectors. Under continuously ideal (25o to 28o C) greenhouse conditions, grapevines have been infected with *Xylella fastidiosa* by contamination of pruning tools. Briefly swishing pruning tools in a bucket with 10-20 liters of water between pruning cuts should avoid this contamination danger.

- Insure vectors used in experiments are free of *Xylella fastidiosa* (not infectious). If you can only collect insects to be tested from the field, test groups of insects on sensitive plants before using them in transmission jexperiments. Of course, the results from these screening plants may not be available for months. Those groups that proved to have transmitted will have to have their subsequent transmission results set aside. Nymphs reared on plants that are not hosts of *Xylella fastidiosa* can be placed on a new plant as soon after molting as possible to enable the nymphs or adults after molting to be free of *Xylella fastidiosa*. Once a colony free of *Xylella fastidiosa* is established, these insects' progeny should be free of *Xylella fastidiosa* as long as the plants provided to them are always free of *Xylella fastidiosa*.

- If you want to determine the natural infectiousness of vectors in the field, test 100 insects with 20 per plant (on 5 plants for 4 days), then select at random 20 individuals from the 100 and test them singly on test plants (also 4 days). If natural infectivity of vectors is low (<10%), testing 100 insects can detect this with confidence. If vector infectivity is high (>30%), using 20 insects/plant will probably infect all the plants whether infectivity is 30% or 100%. A 2 to 4 day IAP should maximize chances of transmission by infective insects. Testing of vectors with very low transmission efficiency on your chosen test plants is a problem for accurate estimates of natural infectivity because by definition there is a low probability that such vectors will transmit to test plants even if all of them are infectious.

5.2 CAGES AND TRANSFER METHODS.

Cages

You need to use cages that are reliable in keeping vectors alive and well (and NOT escaping) while on test plants and do not damage the plants inside the cage (Fig. 9). Use a cage size that is suited to the type and size of the plant and pot. For a plant in a 10 cm square or circular pot, cages should be small enough to just fit into the pot. A strip of tape on each side of the cage connecting it to the pot can help to prevent accidental separation of the cage from the pot during handling. A small cage can be used to confine insects to feeding on part of a leaf (Fig. 9).

The easiest cages to make are plastic bottles with the top and bottom cut off and mesh glued to the top. Finding a suitable glue or adhesive is difficult. A hole in the side of cage to introduce the insects can be plugged with cotton or a cork after use. It is better to have at least one mesh-covered window on the side of the cage to improve ventilation. Having free moisture on the inside of the cage or on the plants can trap insects in the water. Avoid using styrene plastic or any plastic that is easily distorted by heat. Most beverage bottles are made of Polyethylene Terephthalate (PET or PETE) and are stable up to 93 C. Polypropylene (PP) is stable to 104oC. Perspex (Plexiglass) is too thick, rigid, and expensive to use for most cages.

Fabric bags are very useful for transmission studies in the field. They are easy to make and take little room to transport to the field. Rain is a potential problem for all cages, but especially problems for fabric cages. One can simply sew the size and shape of bag desired. Use doubly sewn seams. Fasten the bottom of the cage with a twist-tie wire. A major problem for bag cages is that the insects can become trapped in a fold or seam and die. A simple wire frame inside can be inserted into the cage to keep it from collapsing or folding.

Transfers

A simple tubular aspirator is the most useful type of aspirator. Use a 30 to 40 cm long clear, rigid plastic tube with an inside diameter of about 5 mm. Surround one end of the tube with a small piece of moistened plastic mesh cloth and slide a 40 to 50 cm long flexible vinyl tubing or latex tubing that fits tightly over the rigid tube. The length of both pieces of tubing depends on the use and size of the person using it. Expelling the insects from the aspirator will damage the insects if yu blow too hard to expel them. With practice, this simple tool is very easy to use. Puff gently so as to not damage the insects you are expelling. The main problemfor this type of aspirator is if you are allergic to small particles that you might suck in.

A transfer room is very helpful to maintain colonies and to transfer insects into and out of cages. Some experiments require dozens or even hundreds of transfer in a day. You should be able to make the room completely dark except for one window. The edges of doors should be insulated with strips that prevent insects from leaving or entering the room. The window with a benchtop should have 2 walls and a ceiling that enclose 5 sides of the transfer space. Painting the sides and ceiling yellow attracts many leafhoppers and makes them easy to see. The insects should be attracted to the window. Placing a tubular fluorescent lamp at the top and outside of the window adds light to attract insects if the space is used at night or on cloudy days. The rearing colonies should be transferred before working with infective insects or colonies (even better another day).

Figure 8. Cages useful for transmission experiments with small plants.

Figure 9. A small clip cage can confine vectors to a small area (2 cm). The cage glued to a hair pin and rests on foam rubber glued to one side of the pin. The cage has fabric on both sides but allows insects in the cage to feed through the mesh.

5.3 PLANTS

Plants with *Xylella fastidiosa*-caused disease and have advanced symptoms are poor feeding sources for xylem sap-feeders. Don't use acquisition plants with only severe symptoms or most insects will die in a day or two. Test plants should be a size that is compatible with the cages used. They should be in succulent condition that is suitable for xylem sap-feeders.

After removing the insects from plants, take precautions that any eggs that might have been laid by the insects kept in the cage do not produce nymphs that could then spread the pathogen to other plants. Test plants can be treated with systemic insecticides (neonicotinoids) to quickly kill emerged nymphs. Keep inoculated test plants isolated from rearing plants and future test plants. During winter months in cool climate regions, vector transmission may be difficult even with environmental chambers for insect rearing and transmission tests and heated greenhouses with supplemental lights.

Feeding insects on artificial diet can be done with a liquid diet (20 ml in Fig. 10) containing Xylella fastidiosa. The construction is depicted below (Fig. 10). This is illustrated below in Fig. 10. See details for diet and bacterial preparation in Proceedings of the National Academy of Sciences USA 2009, 106:22416-22420.

Figure 10. Sachets containing suspensions of bacteria between two layers of finely stretched ParafilmR. Insects confined in a small cage under the sachet can feed on the suspension. The Parafilm (top) is stretched in two directions to make it as thin as possible without breaking (third from top). One layer of Parafilm is stretched over a small cage. The diet (red in this figure) is pipetted onto the bottom layer and carefully covered with a top layer of Parafilm. Insects usually feed best when the cage is inverted sachet side down towards a light.

6. INSECT PREPARATION FOR MOLECULAR ANALYSIS (PCR)

Field–collected insects can be analyzed for the presence of X. fastidiosa by PCR. ELISA test is not sensitive enough, as the bacterium only colonizes the insect foregut (precibarium) where, in spite of its multiplication, it is generally present at low titer. Insect samples may originate from collection of living samples (obtained with sweeping net or with aspirators such as D-Vac or simple hand aspirator) or trap collection (yellow sticky traps or malaise traps). Living samples are obviously good quality material for DNA extraction and PCR, while dead insects from sticky traps are of low quality. However, it has been demonstrated that very often dead insects from sticky traps are still suitable for PCR detection of plant pathogens. Quality of the dead insects from sticky traps mainly depends on the period of time the traps have been hanging in the field (the shorter the period, the better the sample), temperature conditions and amount of rain during the trap collection period. If the living or dead samples cannot be processed immediately after the capture, they should be stored at – 20 °C for short periods or under 95-99% ethanol or acetone for longer periods. Also, sticky traps can be stored at – 20 °C if the operator has no time to tear the insects out of the trap and transfer them in a storage tube, under ethanol if required by the storage condition.

Living insects for analysis can be killed by freezing or by exposure to carbon dioxide or ethyl acetate. Please be aware that CO2, when applied for one or few minutes can anesthetized, rather than kill, the insects.

Insects from sticky traps can be removed from the traps using small forceps/pincers with the help of a proper solvent (xylene works well but its use is not advisable as it is carcinogenic, while products such as vegetal xylene, Bio-Clear (Bio-Optica, Milano, Italy), kerosene or regular fuel can be used (handling of these reagents should be done under a hood). After removal from the traps, insects should be rinsed in ethanol/acetone and stored under ethanol/acetone.

Before DNA extraction, it is imperative to remove the solvent (ethanol/acetone). To achieve this, the insects can be transferred for few minutes on a dry filter paper and eventually further dried in a speed-vac centrifuge, to facilitate evaporation of the solvent.

Since X. fastidiosa only colonizes the foregut and does not systemically spread into the body, only the head of the insect should be used for DNA extraction, thus avoiding the extraction of several contaminants that may inhibit Taq Polimerase-dependent amplification. For this very simple dissection, the insect should be placed under a stereo microscope and observed ventrally, then the head can be separated from the rest of the body by simply tearing apart the head with fine needles (Fig. 11).

Figure 11. Dissection of the head from a leafhopper body.

Total DNA can be extracted from single insect heads following different procedures, often based on the CTAB buffer, or using a number of DNA extraction kits. Several commercial kits for insect DNA extraction are available (e.g., prepGEM™ Insect, ZyGEM, Solana Beach, CA, USA; E.Z.N.A.® Insect DNA Kit, Omega Bio-Tek, Norcross, GA, USA; EZgeneTM Insect gDNA Kit). These options are more expensive than traditional methods but yield greater DNA purity. Nevertheless, attention must be paid in verifying that the insect specific kits are effective also for bacterial DNA. Commercial DNA extraction kits specific for small sample volumes (e.g., QIAamp®DNA Investigator Kit, QIAGEN) may ensure acceptable DNA quality also when the insect sample is in poor conditions and badly preserved.

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ANNEX: EQUIPMENT, SUPPLIES AND INSECT STORAGE

The equipment necessary to conduct surveys and monitoring of Auchenorryncha are relatively simple. In addition to those described below, the following items are usually included into the collector's kit:

- **Forceps**. Fine, lightweight forceps are recommended; if sharp-pointed forceps are used, care must be taken not to puncture specimens. If possible, grasp specimens with the part of the forceps slightly behind the points.
- **Vials** containing alcohol or other preservatives.
- **Small boxes or containers** for storing specimens after their removal from killing bottles. These may be made of cardboard, plastic, or metal and should be partly filled with soft tissue or cloth to keep specimens from rolling about. Do not use cotton because specimens become entangled in the fibers and may become difficult impossible to extricate without damage.
- A small, fine **brush** (marten's hair is best) for picking up minute specimens. Moisten the tip (even by saliva); tiny specimens will adhere to it and may be transferred to a killing bottle or vial.
- **Plastic bags** for storing material.
- A **hand lens**.
- **Sweeping net** (Fig. 12): this tool is similar to a butterfly net, but have strong handle and a durable bag to withstand been dragged through dense vegetation; one of the problem could be the sweeping on prickly Mediterranean vegetation, like bramble, that can easily damage the tissue. Standard sweeping net has a diameter of the rim of 38 cm; beyond this dimension, the use of the net for sampling became uncomfortable. Efficient use of a net is gained only with experience. The collection from weeds have to be performed with a pendulum-like movement, with the rim of the net as close as possible the soil, to prevent the specimen from escaping. The same movement can be apply to the canopy of the plant that has to be sampled. Swing the net rapidly to capture the specimen, then follow through to force the insect into the very bottom of the bag. Twist the wrist as you follow through so that the bottom of the bag hangs over the rim (Fig.); this will entrap the specimen. Hold the tip of the bag up with one hand. The content of the bag can be empty into a plastic bag, for further counting and identification of the collected material. Remember to write on each plastic bag date, locality, and swept site (for example: Olive canopy, Weeds, ext.). If the aim of the sweep is collecting alive insects, after the sweeping the bag can be shacked to concentrate them on the bottom; collection can be performed with an aspirator.
- **Aspirator** (Fig. 13): it is an effective device to collect small and highly mobile insects able to crawl and fly quickly. There are two types of aspirator that can be used for our aim: mouth aspirator or motorized suction device. The former is composed by a vial, closed with a cap or a cork equipped with two tubes, one for suction, the other for sample collection. One of the end of the suction tube is closed with a fine mesh to prevent the user from ingesting captured materials. After the suction, the cork can be replaced by a cap aerated trough anti-aphid net. To keep the insects alive, it could be useful to fill the vial before the use with part of weeds; this allow to maintain moisture inside the vial. Some workers prefer for suction to use plastic tube instead of the

described one. Note that there is some danger of inhaling harmful substances or allergens when using a mouth aspirator. To prevent this, motorized aspirators, available from special biological and naturalistic supplies houses, can be used.

- **Sticky traps:** In this type of trap, a board, piece of tape, pane of glass, piece of wire net, cylinder, or other object, often yellow, is coated with a sticky substance and suspended from a tree branch or other convenient support. Insects landing on the sticky surface are glued on and unable to escape. The sticky material is later dissolved with a suitable solvent, usually toluene, xylene, ethyl acetate, or various combinations of these, and the insects are washed first in Cellosolve and then in xylene. Toluene for five minutes then absolute EtOH for five minutes give good results. However, use caution in selecting a sticky substance because some are difficult to dissolve. For molecular analysis, is strongly suggest to detach the insects gently with forceps, avoiding the use of chemicals.
- **Frappage:** insects can be collected from the canopy by shacking branches on a white sheet, stretched below the sampling site. The samples dropped on the sheets can be picked up with the aspirator.
- **Storage:** Insects can be killed and preserved directly in EtOH 75-80 %. Cover the samples with an EtOH volume thirty times the volume of the insect. Another possibility is to use smaller amount of EtOH, with meniscus just above the sample, changing it at least three times in the following two days. The other way is to kill them in a killing jar with ether or ethyl acetate, or carbon dioxide. Auchenorryncha can be prepared gluing them with a small drop of entomological glue on the tip of a point card, placed below the first abdominal sternites. Collected samples for molecular analysis must be preserved in coolbox until the arrivals to the lab. Once in the lab, they must be stored in -20°C, or in -80°C for long period. Insects can be preserved also in EtOH 95%.

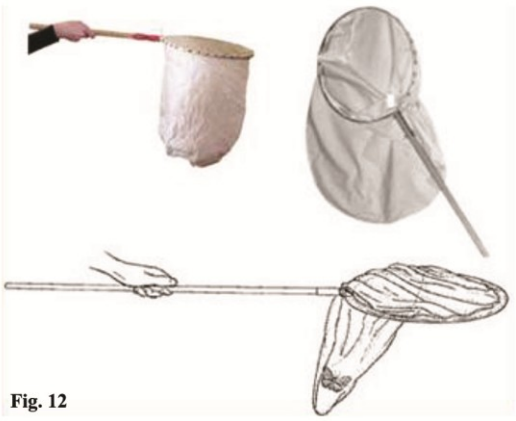

Fig. 12

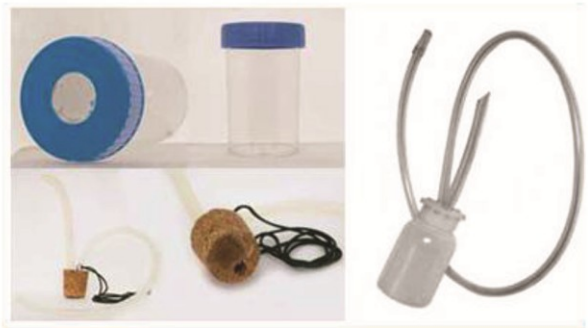

Fig. 13

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