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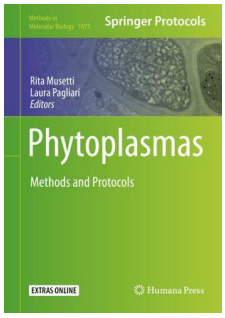

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Phytoplasma transmission: insect rearing and infection protocols

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Abstract

Phytoplasmas are obligate pathogens and thus they can be studied only in association with their plants or insect hosts. In this chapter, we present protocols for rearing some phytoplasma insect vectors, to obtain infected insects and plants under controlled environmental conditions. We focus on *Euscelidius variegatus* and *Macrosteles quadripunctulatus* that can infect *Arabidopsis thaliana*, and *Scaphoideus titanus*, that can infect grapevine.

1. Introduction

Since their discovery, the study of phytoplasmas has been hampered by the impossibility to culture them in vitro, although Contaldo et al ***(1)*** reported some positive results, so far not confirmed by

other studies. In fact, they lack several pathways for the synthesis of compounds considered to be necessary for the cell metabolism ***(2)***, exhibiting a strong host-specific correlation. This made necessary to study phytoplasmas associated with their hosts, plants or insect vectors. Pathosystems developed under controlled environmental conditions allow to focus on host-pathogen interaction, excluding unpredictable factors related to field condition. In this chapter we present protocols for rearing some phytoplasma vectors, to obtain infected insects and plants and, ultimately, to maintain phytoplasma strains by insect transmission. We focus on rearing protocols of two insects, *Euscelidius variegatus* and *Macrosteles quadripunctulatus*, that can infect *Arabidopsis thaliana* ***(3-5)***, that is a model plant of growing interest also for the studies of phytoplasma-plant interactions. Moreover, we present two important case studies for European viticulture, *Scaphoideus titanus* vector of FD phtoplasma ***(6)***, and *Hyalesthes obsoletus* vector of Bois noir ***(7)***. We provide a data sheet for each vector species with details on rearing and acquisition and transmission techniques.

2. Materials

2.1 Insect rearing and infection

1. Growth chambers. All phytoplasma-infected insects and plants should be kept in controlled growth rooms. Main requirements include sealed access, timer-controlled fluorescent lighting, temperature and humidity control.
2. Glasshouse for growing plants for maintenance of insect colonies. In some countries, contained facilities for quarantine phytoplasma-infected plants (for *S. titanus* insects infected by FD phytoplasma) are required.
3. Large plexiglass and net cages for insect colony development (in case of *E. variegatus* and *M. quadripunctulatus*, and *H. obsoletus*) or egg hatchings (for *S. titanus*) (Fig.1). To avoid fungi infection on herbaceous plants, cages should be properly cleaned with sanitizing products and should have large windows protected by insect-proof net.

4. Host plants (see 3.1.3. and 3.2.3 “Host plant cultivation” sections for further details), proper pots, sterilized with sodium hypochlorite, and proper soil for each kind of plant.
5. Aspirator for carefully handling the insects. A simple mouth aspirator can be built with a 50 ml-centrifuge tube, two pieces of 50 cm of transparent plastic tube (with a diameter of 5-7 mm) and a piece of gauze, to be used as a filter. Cut the tube lid, to create a hole where fixing one piece of the plastic transparent tube: it will be used for insect aspiration. At the same extremity of the tube, stick a small piece of gauze (which will avoid the accidental insect swallowing by the operator). Cut the bottom of the tube to create a hole where fixing the other piece of the plastic transparent tube: it will be inserted in the cage to capture the insects and to collect them inside the tube (*see* **Note 1**).
6. Insecticides and fungicide chemicals.

2.2. Host plant infection

1. For most of the host plants here described, a plexiglass cage can be used.
2. For Arabidopsis infection, plants should be exposed to infective insects (*see* **Note 2**). For this purpose, gauze cage or plexiglass tubes can be used.

2.3. Vector infection via abdominal microinjection

1. Autoclaved ceramic pestle and mortars.
2. Injection buffer. Prepare 30 µL of buffer per insect as follows: 300 mM glycine, 30 mM MgCl2, pH 8.0 ***(8)***.
3. 0.45 µm sterile filters, glass capillaries, needle-puller device or a Cell Tram Oil microinjector (Eppendorf)
4. CO_2 flush.

2.4. Artificial feeding

1. 1.5 ml-microcentrifuge tubes, cotton wool and Parafilm.
2. Feeding solution: 5% sucrose in TE ***(8, 9)***, or 5 % sucrose, 10 mM Tris/Cl, 1 mM EDTA, pH 8.0 ***(10, 11)***.

3. Methods

3.1.Euscelidius variegatus **and** ***Macrosteles quadripunctulatus***

3.1.1. Insect description

E. variegatus is commonly found in weeds, lawns, pastures in Europe, Asia, northern Africa and the Western United States ***(12, 13)***. It has a light brown colour with numerous fuscous markings on body (Fig. 2a). Nymphs can be recognized by the absence of wing and marked transverse stripes (Fig. 2b-c). Adults are characterized by a medium size, ranging from 3.90-4.50 mm (male) to 4.10-5.50 mm (female). Nymph development requires five instar stages, each of them lasting approximately one week ***(14)***. *E. variegatus* is a known vector of phytoplasmas of clover phyllody ***(15)***, aster yellows ***(16)***, X-disease ***(17)***, Chrysanthemum yellows ***(18)*** and flavescence dorée (FD) ***(19)***, although it cannot acquire this phytoplasma from infected grapevines ***(8, 20)***.

M. quadripunctulatus is widely distributed from western Europe to central Russia, south to Cyprus, Iraq and Kashmir ***(21)***. It inhabits dry climatic regions, preferring scant, disperse vegetation and dry, well-drained soils. Among the plant species, it prefers *Medicago sativa* (L.), *Trifolium repens* (L.), *Agropyron repens* (L.) Beauv., *Poa pratensis* (L.) and *Digitaria sanguinalis* (L.) ***(22)***. It has a greenish yellow colour, with two pairs of black spots at the vertex and short black longitudinal bands between eyes and ocelli and ocelli reddish ***(21)***. Length (including tergmen) varies from 2.9–

3.3 mm (male) to 3.2–3.7 mm (female) ***(23)*** (Fig.2d-f). Its transmission capacity has been demonstrated for aster yellows (AY) phytoplasma in lettuce and carrot ***(24)***, Kok-saghyz yellows ***(25)*** and chrysanthemum yellows (CY) phytoplasma ***(18, 26, 27)***.

3.1.2. Rearing

Both leafhoppers can be reared in plexiglass cages on oat (*Avena sativa* L.) (*see* **Note 3**) at 20/22 °C and long-day conditions (16hL/8hD period). Since they are multivoltine insects and thus they breed continuously, an age-structured rearing is advised. An oviposition chamber where adults lay eggs on host plants for a short period (from few days to one week). After the oviposition, host plants are moved to new cages where the eggs give rise to the nymphs. Considering that, a complete egg hatching needs from 3 to 4 weeks ***(14)***, after a month oat plants should be replaced with fresh ones, to allow the nymphs to feed on good quality plants.

3.1.3. Host plant cultivation

Chrysanthemum carinatum (Schousboe) (daisy) plants are grown from seed in greenhouse at 20/22 °C, under long-day conditions (16hL: 8hD period), at 100 $\mu Em^{-2}s^{-1}$light intensity (with Plant Growth fluorescent lamps) and 50-70% humidity. After about 10 days from seedling emergence, plants are transplanted into 8-cm pots. Roughly 40 days after germination, plants at the 6-8 leaf stage are exposed to infective leafhoppers.

A. thaliana plants are grown at 20/22 °C, under short-day conditions (9hL:15hD period) (*see* **Note 4**), at 120-150 $\mu Em^{-2}s^{-1}$ light intensity (with Plant Growth fluorescent lamps) and 50-70% humidity. Seeds are hydrated on wet blotting paper at room temperature for roughly 3 hours. Soaked seeds are posed in soil and vermiculite mixture and pots are maintained at 4° C, for the so-called stratification period, to improve germination rate and synchrony. After three days, pots are placed in the growth room in high-humidity conditions, which can be easily reached covering the pots with a close-

fitting clear plastic dome. After five days, the dome is slightly displaced to reduce relative humidity gradually. After a few days of acclimation, the dome can be removed entirely. 20-day-old plants are than transplanted in single 8-cm pots (*see* **Note 5**).

3.1.4. Transmission protocol

As explained above, both leafhoppers can acquire and transmit different phytoplasmas. Here we present the protocol for the transmission of CY phytoplasma that can be easily transmitted to *A. thaliana*.

Late instar nymphs (Fig.2b,e) (*see* **Note 6**) are taken from healthy colonies grown on oats and transferred to CY-infected daisy plants (*see* **Note 7**) for a 7-day acquisition-feeding period. Twenty (for *M. quadripunctulatus*) or thirty days (for *E. variegatus*) after nymph transfer, 45-day-old *A. thaliana* plants [corresponding to the 3.50 growth stage ***(28)***] are individually exposed to 3 infective insects. Control plants are exposed to healthy insects. At the end of the 7-day inoculation feeding period, insects are manually removed and/or plants are treated with insecticide (*see* **Note 8**). Infected and control plants are maintained in two separated insect-proof plexiglass cages until symptom development [roughly 20 days after inoculation, corresponding to the 3.90 growth stage ***(28)***].

Purcell et al ***(12)*** noticed rod-shaped bacteria in the haemolymph of *E. variegatus*, designated as BEV. Even if the presence of endosymbionts and parasitic bacteria in insects may influence transmission ability ***(29)***, it was demonstrated that in *E. variegatus* BEV do not interfere with CY transmission ***(30)***.

3.1.5. Symptom development

Within 20 days from inoculation, common phytoplasma disease symptoms appear on plants exposed to infective insects. Considering that CY is transmitted by single *M. quadripunctulatus* and *E. variegatus* with high efficiency (on daisy plants with transmission rate of 100% and 82%, respectively), almost all the plants exposed to infective insect should develop symptoms ***(31)***.

In contrast to healthy plants (Fig. 3a), infected daisies are characterized by leaf yellowing and reduced growth (Fig. 3b). The apical part of the plant shows clear curvature (of roughly 30°) in comparison to the plant growth vertical axis, with shorten internodes and short and thick leaves.

In Arabidospis, yellowing and general stunting are accompanied by a decrease of ~40% in growth ***(5)***. In contrast to healthy plants (Fig. 2c), leaves having emerged after phytoplasma inoculation were shorter, with a thick main vein and a smaller petiolar area (Fig. 2d).

3.1.6. Vector infection via abdominal microinjection

Microinjection is a useful tool for studying transmission mechanisms by vectors as it allows to overcome the barrier of salivary glands, reduce latency period and is highly efficient ***(30)***. Moreover, with this technique it is possible to test for transmission insects with different feeding habits or insects that could not feed properly on the source plants used. This technique, first set by Black ***(32)*** to deliver phytoplasma extracts into leafhoppers, was used by ***(8)*** to assess vector specificity of FD phytoplasma in several species of Cicadellidae ***(10)*** and to assess the role of midgut barrier in phytoplasma colonization of the insect ***(30)***. The same technique is applied to leafhopper vectors of spiroplasmas ***(33)***.

Phytoplasma suspension for microinjection is prepared by crushing infected *E. variegatus* in ice cold filter sterilized injection buffer. The extract is centrifugated (10 min, 800 g, 4 °C) and the supernatant filtered through 0.45 μm sterile filters. Newly emerged healthy *E. variegatus* adults are anaesthetized by CO_2 flushing for few seconds and, immediately, about 0.2 μL of solution is injected between two abdominal segments under a stereomicroscope. The phytoplasma suspension

must be maintained on ice and used within 4 h (Galetto et al, 2009; Rashidi et al, 2015) This technique, requires some skills from the operator to avoid high mortality rates.

3.1.7. Artificial feeding: a test for the transmission ability of candidate insect vectors

The **transmission ability** of candidate insect vectors is normally tested by PCR only after the inoculation of the target plant, because of the destructive feature of this technique. A rapid and non-destructive method is the use of artificial feeding assays, that is successfully applied with several Hemiptera species and was developed also for leafhoppers and planthoppers ***(8, 9, 11, 21, 34)***. Moreover, this technique can be adopted when the host plant species is unknown or test plants are poor hosts for the potential vector.

1.5-2 ml-microcentrifuge tubes can be used as insect chambers. Caps (*see* **Note 9**) are filled with 200µl of 5% sucrose in TE ***(8, 9)*** and sealed with Parafilm. The bottom ends of tubes are cut, to introduce an insect. Finally, the cut-ends are sealed with cotton wool and each tube is kept at 23 to 25°C for 48 to 72 h in a vertical position with the cap facing a light source to attract the insects to the feeding medium. At the end of the inoculation period (*see* **Note 10**), the artificial feeding buffer is gently aspirated and processed for phytoplasma detection (*see* **Note 11**).

3.2 Scaphoideus titanus* and *Hyalesthes obsoletus

3.2.1. Insect description

Scaphoideus titanus (Hemiptera: Cicadellidae) (Fig. 4) is the main vector of FD phytoplasma and widespread in most European vineyards and was reported in most American states and Canadian provinces ***(6)***. Nymph colour varies according to the nymphal stage. At hatching they are almost translucent, go through a milky white, then become ivory white at the end of the 2nd instar. In the 3rd instar, the nymph become an ivory yellow more and more accentuated as they age. Finally, the

4th and 5th instars are characterized by the appearance of light to dark brown irregular spots and the appearance of wing and elytral drafts ***(35)***. Nymphs have two black spots arranged symmetrically in the dorso-lateral position at the posterior end of the abdomen ***(35)***. Adult females are larger (5.5 - 5.8 mm) than males (4.8 -5 mm) and there are three brown transverse bands at the vertex level for females, compared to only one for males ***(35)***. Nymph development requires five instar stages, each of them lasting approximately ten day ***(36)***. This leafhopper is mostly recorded on *Vitis vinifera* in Europe, while in North America, *V. labrusca* and *V. riparia* are reported as the preferred host plant ***(37, 38)***. Five nymphal instars lead to adults in about 50 days in laboratory conditions ***(36)***. Nymphal instars can be distinguished using the key of Della Giustina et al ***(39)***. Longevity of males can reach 55 days while females can live up to 80 days, but infected individuals have a reduced lifespan ***(40)***. *Scaphoideus titanus* is the main vector of phytoplasmas responsible of FD ***(35)***. However, *S. titanus* can also transmit phytoplasma from the 16SrI-C ***(41, 42)*** and 16SrI-B groups ***(43)*** and it was introduced in Europe probably as a consequence of the massive importation of American rootstocks after the phylloxera crisis ***(6)***.

Hyalesthes obsoletus (Hemiptera: Cixiidae) (Fig. 5) is a Paleartic species and the main natural vector of '*Candidatus* Phytoplasma solani', (16SrXII-A genetic group) which is associated with diseases of several crops such as grapevine, lavender, maize and potato. Nymphs are white to brown, with red eyes, and the abdomen is bearing a lot of wax secreted by wax plates. 1 to 3rd larval instars do not have compound eyes ***(44)***. Adults are dark with red eyes two and females have wax glands and are often bearing wax on the end of their abdomen. Length is about 4 mm with female that are bigger than males and host plant having an effect on the adult size. This mesophilic species is monovoltin in Europe but bivoltin in Israel ***(45)***. Females lay their eggs in the soil near the basis of different host plants species. After hatching, the five nymphal instars live underground and feed on the roots. Acquisition of '*Ca*. P. solani' can be achieved by nymphs feeding on infected root phloem, while transmission to cultivated and wild plants is done by flying adults during summer. Because most of the crops affected by '*Ca.* P. solani' are dead-end hosts phytoplasma acquisition

occurs on wild plants. This insect is found on a great diversity of plants, mainly wild. About 19 species belonging to 10 different plant families are known to harbor both nymphs and adults but adults can be observed on more species ***(46)***. Multiple hosts enhance the opportunity for genetic local adaptations and led for *H. obsoletus* to the existence of host races which specialize on different host plants ***(47)***. As consequence, a *H. obsoletus* from a host plant cannot be reared on another host plant.

3.2.2. Rearing

Scaphoideus titanus is a univoltine species that is difficult to rear in captivity from egg to egg. It is possible to make all the life cycle in controlled conditions, but it is time consuming and no one has succeeded yet in obtaining more than one generation a year. Nymphs can be obtained from eggs laid in the field by collecting two-year-old (or older) grapevine woody canes during winter ***(48)***. Woody canes should be collected in vineyards with high populations of the leafhopper (yellow sticky traps can estimate the population level during summer). After collection, the woody canes can be checked to see if they are bearing eggs, and then are kept in a cold room at 5 ± 1 °C and 85–90% humidity until egg incubation. Egg hatchings are obtained by placing woody canes inside plastic hatching cages (50 × 38 × 36 cm) in a climatic chamber under a 16h:8h L:D photoperiod, at 23 ± 1 °C, and 65–70 % humidity. To avoid eggs desiccation a 1 cm layer of vermiculite is placed below the eggs and is kept humid. To harvest neonate nymphs, food is provided by placing grapevine leaves maintained in a glass tube with water, ca. 20 days after the canes with eggs are removed from the cold room. Leaves must be replaced when they began to wither. Nymphs can be reared individually or in group on grapevine cutting ***(49)***. Nymphs and adults can also be kept alive by placing them in a small container in which a grapevine leaf disk was laid over a 1-cm layer of technical agar solution [0.8% (wt/vol)] at the bottom and is replaced twice a week ***(50)***. Alternatively, woody canes can be placed in larger cages together with small potted grapevine and

broad bean plants: hatched nymphs can survive very well under these conditions and can be collected for experiments when needed. In order to extend the period of egg hatchings and perform transmission experiments over the year, grapevine wood with eggs can be stored in cold chamber at 4-6 °C for some months.

Hyalesthes obsoletus can be reared on different species, e.g. *Lavandula angustifolia* (lavender), *Urtica dioica* (nettle) and *Salvia sclarea* (clary sage) ***(44, 51, 52)***. Because it is quite impossible to collect eggs in the soil, rearing should start with nymphs from uprooted plants or adults collected in the field. Females lay their eggs at the basis of seedlings or just under the soil surface. It is impossible to know the sanitary status of captured nymphs and adults. Thus, after egg laying and before first hatchings, eggs should be gently transferred close to the roots of a new plant obtained from seed to initiate a phytoplasma-free rearing. This step can be avoided to obtain diseased plants further used for phytoplasma acquisition.

Hereafter we describe the rearing on nettle. Plants are kept at 25 ± 1°C under a 16h:8h L:D photoperiod and 80% relative humidity. The soil used in the pots should be loose to allow nymphs movements and access to roots, for example a mixture of 50% peat and 50% small gravel, ca. 0.5 mm diameter ***(44)***. Pots are watered every two days by pouring water into the saucers in which each pot was standing. Care is taken to prevent overwatering to avoid egg and nymphal drowning. After about 1 month, nymphs hatched and developed above ground at the base of the plant. Adult emergence starts about 2 months later, and they are gradually transferred to a new cage to initiate the next generation. Only a small proportion of eggs give rise to adults due to a high nymphal mortality rate.

3.2.3. Host plant cultivation

Vitis vinifera (grapevine) can be obtained from cuttings or *in vitro* plantlets. Cutting are grown in a potting compost mix (Substrate 5; Klasmann-Deilmann, Geeste, Germany) at 22 ± 2 °C, under

long-day conditions (16hL:8hD period) and irrigated twice a week. Plantlets are planted into 12 cm pots filled with same compost mix as cuttings (Eveillard et al 2016). Plantlets are grown at 25 ± 2°C, and 50–80% humidity, under illumination of 50 $\mu Em^{-2}s^{-1}$ for a 16 h period (Osram, Lumilux), and under 400 W high pressure sodium lamps for a 14 h period, *in vitro* and in the greenhouse, respectively. Since no *Vitis* plant is known to be immune to FDP, in theory all *Vitis* species/cv can be used in transmission experiments. However, since infected rootstocks are generally non-symptomatic and cultivated varieties differ a lot in FDP susceptibility ***(53, 54)***, the use of a very susceptible variety is advised to obtain a better estimation of the vector infectivity (*see* **Note 12**).

Vicia faba (broadbean) plants are grown from seed under the same conditions than grapevine cuttings.

Lavandula angustifolia, *S. sclarea* and *U. dioica* plants are grown from seeds in pots (12 cm diameter) filled with a peat-based standard substrate containing a slow-release fertilizer in a heated greenhouse at 25 ± 1°C under a 16:8 L:D photoperiod and 80% relative humidity.

3.2.4. Transmission protocol

Because FD is listed in Quarantine Pests for Europe (*see* **Note 13**), all experiments using FD phytoplasma (FDP), grapevine and *S. titanus* should be done in confined greenhouse and under safety rules. FDP can be maintained by continuous broadbean to broadbean transmissions, using *E. variegatus* as an alternative vector ***(20)***. When needed, *S.titanus* can be fed on FDP-infected broad beans, that ensure a higher acquisition rate compared to infected grapevines ***(48)***. Third to fifth instar nymphs are caged on FD—broadbeans to allow FDP acquisition. After one week of acquisition access period, insects are transferred onto grapevine cuttings for a 3-4 weeks latency period. Then, a variable number of adults are caged on a grapevine plant and removed after 1 week. Plants are treated with insecticide (*see* **Note 8**) and maintained in confined greenhouse at 25°C constant, which is the optimum temperature for the multiplication of FDP ***(55)***. The transmission

rate of FDP is higher in males than in females ***(56)***, probably due to the different feeding behaviour observed between both sexes ***(57)***. Test plants for FDP transmission assays can be cuttings (grafted or not), that generally requires one year to be scored for infection, or herbaceous *ex-vitro* plantlets, that are very susceptible to phytoplasma infections and develop disease symptoms and can be scored by infection by PCR in some weeks ***(53)***.

Due to the existence of host plant races, phytoplasma acquisition by *H. obsoletus* should not be done on any plant species. One possible way is to rear two distinct strains, a healthy and an infected one. The main difficulty is avoiding cross-contamination, by keeping the rearings in separate chambers/greenhouses but this system can produce infected vectors anytime. It is also possible to transfer third and fourth instar nymphs from a healthy plant to an infected one and collect the adult that would be infective 4-5 weeks later. In laboratory/greenhouse conditions, nymphs can develop on the aerial parts of the plants.

3.2.5. Symptom development

At 5 and 10 weeks post-transmission, the FD symptoms and the number of contaminated grapes can easily be scored, when using *ex-vitro* plantlets ***(53)***. Since evaluation by symptoms is not reliable enough on grapevine plantlets, PCR assays of leave and/or root tissues for FDP presence are needed. Test cuttings (grafted or not) generally requires one year to be scored for infection. The first visible symptoms on grapevine cuttings are on leaves that roll downwards and become yellowish for white cultivars or reddish for red ones. The new shoots then become weeping shaped due to a lack of lignification and some black punctuations can be observed on the petioles. Hydric stress increase the symptoms expression ***(58)*** (Fig. 6a,b).

Symptomatic broadbeans are stunted and had small leaves with upward-curled edges. Flowers are not affected (Fig. 6c,d).

Symptoms of lavender decline, the diseased caused by '*Ca.* P. solani', are yellowing and either standing up or rolling down of the leaves, and reduction and abortion of inflorescences ***(59)***. Infected clary sages show typical symptoms of stolbur, such as stunted and very small leaves ***(52)*** (Fig. 6e).

4. Notes

1. When sucking insects with a mouth aspirator, the operator should shake the plants to push the insects out of the plants and collect only those ones that are along the cage walls: insects set on plants could have their stylet inserted in the leaf tissue, and thus being damaged by aspiration. Moreover, leaves can be contaminated by thrips that can be transferred from one cage to another together with vectors.
2. Efficient transmission can be obtained with just one insect, nevertheless, when one wishes to maximise transmission, the use of batches of infective insects per plant is advisable.
3. *E. variegatus* and *M. quadripunctulatus* can be reared also on barley (*Hordeum vulgare*), wheat (*Triticum* spp.) and perennial ryegrass (*Lolium perennae*) ***(12, Bosco, personal observation)***. *M. quadripunctulatus* can be reared also on barley (*Hordeum vulgare*) and perennial ryegrass (*Lolium perennae*)
4. Short-day conditions enhance vegetative growth, necessary for the following steps of the infection protocol.
5. Extensive information about *A. thaliana* cultivation can be found in "101 ways to try to grow Arabidopsis" in Horticulture Department of Purdue University website (https://ag.purdue.edu/hla/Hort/Greenhouse/Pages/101-Ways-to-Grow-Arabidopsis.aspx), which describes various aspects of Arabidopsis growing.

6. While in *M. quadripunctulatus* the acquisition efficiency of CYP is not influenced by nymph life stage, in *E. variegatus* late instar nymphs are more efficient in acquiring CY phytoplasma when compared to younger stages ***(18)***.
7. *Catharantus roseus* (L.) can be used as an alternative CY-source plants, even if both leafhoppers show better acquisition efficiency on daisy and suffer high mortality on this plant species ***(18)***.
8. Different insecticides can be used, but in case the inoculated plants have to be further used as inoculum source for insects, non-residual insecticides should be used.
9. White microcentrifuge tubes are indicated to guarantee maximum transparency, nevertheless, a plastic yellow transparent film can be placed on top of the Eppendorf tubes to attract insect to the feeding medium
10. Despite insects are handled with the maximum care, some of them don't survive to this treatment. Because of the sucrose-TE diet, survival of *E. variegatus* (in the fourth and fifth week after the beginning of acquisition) is lower than 50 % ***(9)***. Dead insects can be collected and PCR-tested for phytoplasma presence.
11. Briefly, phytoplasma cells are pelleted by centrifugation at 12,000 × g for 15 min and diluted in 10 μL of 0.5 M NaOH and 20 μL of 1 M Tris–HCl (pH 8.0), containing 1% sodium dodecyl sulfate and 20 mM EDTA. After a 15-min incubation at 65°C, genomic DNA is precipitate with 2 volumes of ethanol and dissolved in 30 μ L of TE. For other detail, please refer to ***(11)***.
12. Highly susceptible cultivars, as Baco 22A, Chardonnay, Barbera, Cabernet Sauvignon, are suggested.
13. For any information about quarantine pests in Europe please refer to EPPO website (https://www.eppo.int/QUARANTINE/quarantine.html). EPPO is an intergovernmental organization responsible for cooperation and harmonization in plant protection within the European and Mediterranean region. EPPO lists are reviewed every year by the Working Party on Phytosanitary Regulations and approved by Council.

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Figure 1. Example of a plastic cage for insect colony development (in case of *E. variegatus* and *M. quadripunctulatus*) and egg hatchings (for *S. titanus* and *H. obsoletus*).

Figure 2. *E. variegatus* development stages: adult(a), late instar nymphs (b), early instar nymphs (c). *M. quadripunctulatus* development stages: adult(d), late instar nymphs (e), early instar nymphs (f). Late instar nymphs (b,e) are transferred to CY-infected daisy plants.

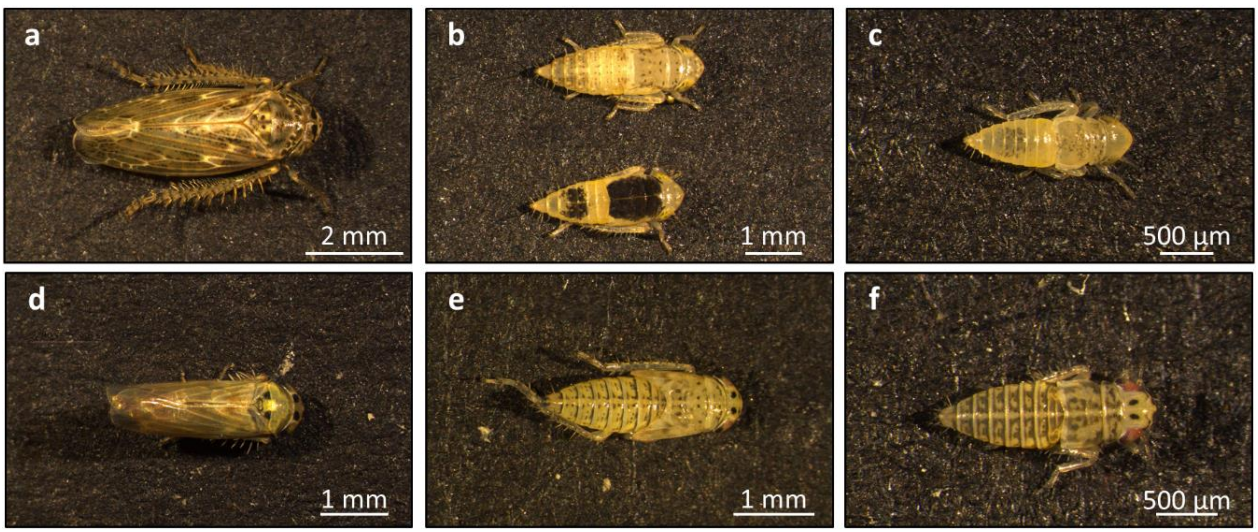


Figure 3. Healthy (a) and infected (b) daisies. Infected daisies are characterized by leaf yellowing, reduced growth and a clear curvature of the apical part. Healthy (c) and infected (d) Arabidospis. Infected Arabidopsis are yellowing and stunted, with shorter leaves, with a thick main vein and a smaller petiolar area.

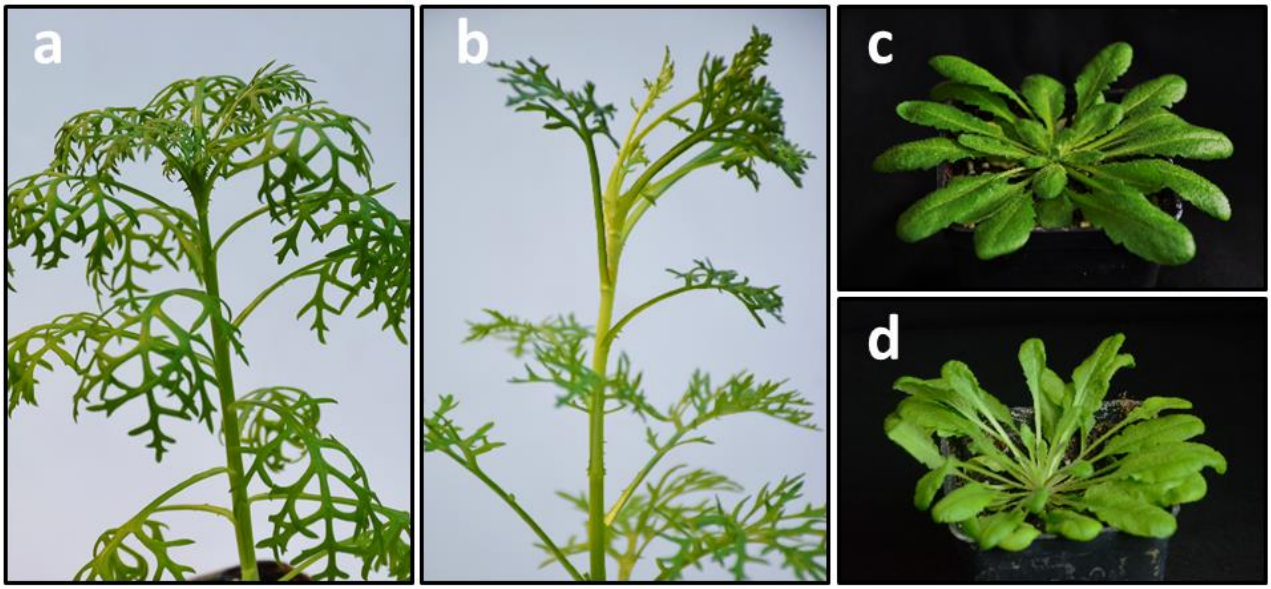


Figure 4. Nymphs (a) and adult (b) of *Scaphoideus titanus*.

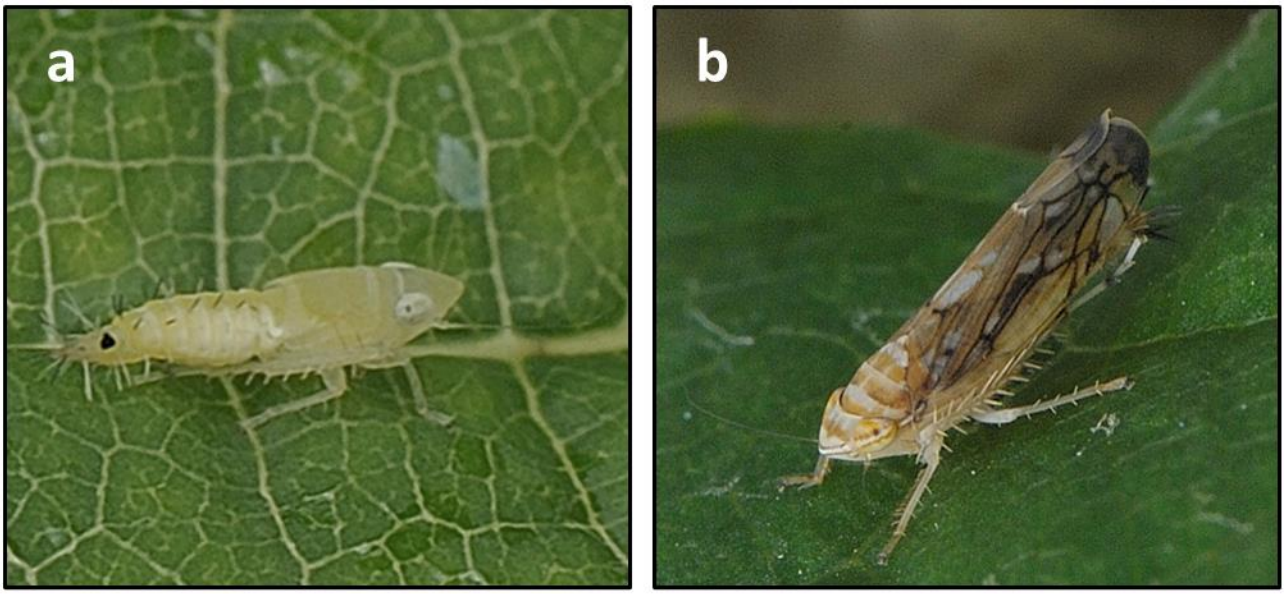


Figure 5. Nymphs (a) and adult (b) *Hyalesthes obsoletus* on *Urtica dioica* in a rearing cage.

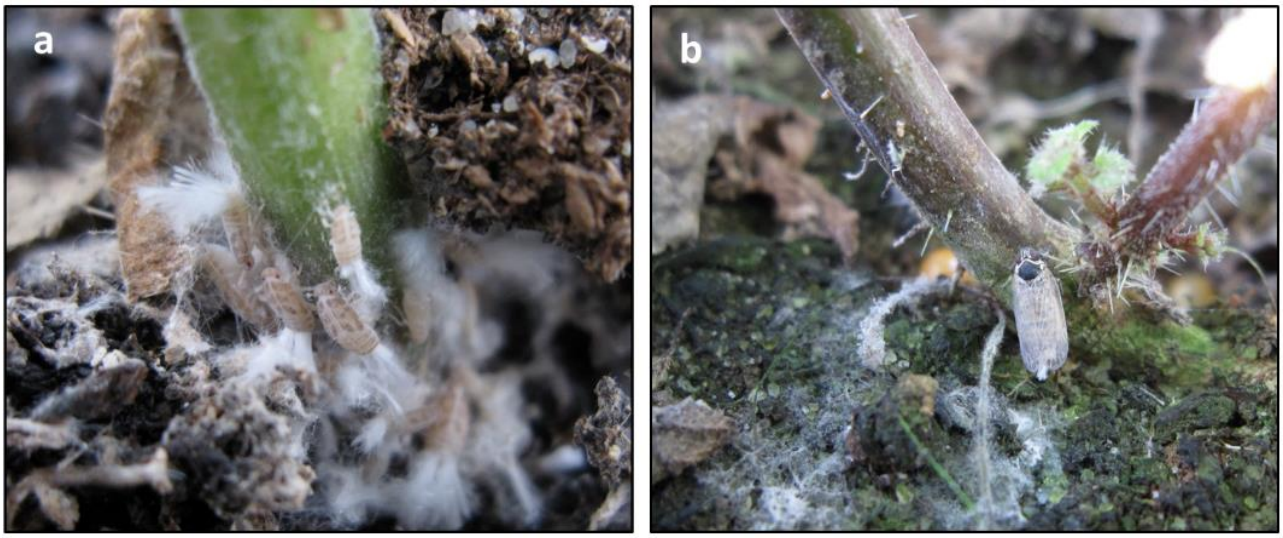
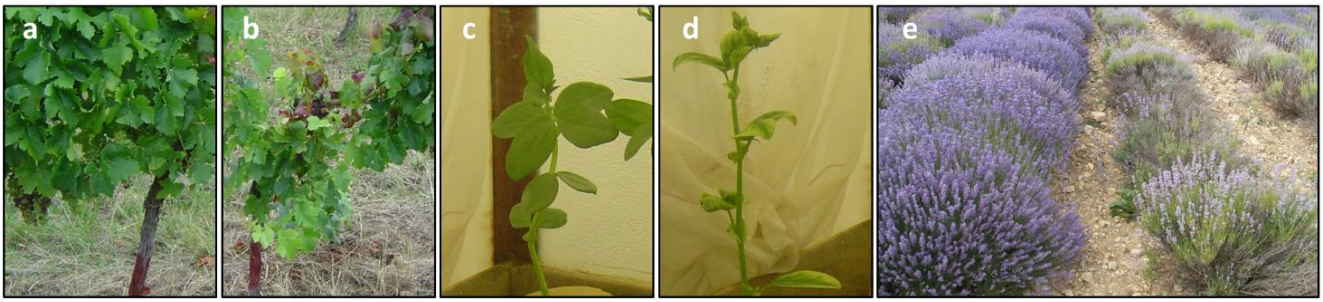


Figure 6. Healthy (a) and Flavescence doree symptomatic (b) *Vitis vinifera* cv. Cabernet Sauvignon in a vineyard. Healthy (c) and Flavescence doree symptomatic (d) *Vicia faba.* Healthy (left) and lavender decline symptomatic (right) *Lavandula angustifolia* in the field (e).



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