Potential role of the alien planthopper Ricania speculum as vector of Flavescence dorée phytoplasma

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Abstract

Ricania speculum (Walker) is an Asian planthopper, accidentally imported in Europe from Far East and officially recorded for the first time in Italy (Genoa province, Liguria) in 2014. Nowadays it is spread out in Eastern Liguria and in Tuscany. *Ricania speculum* is widely polyphagous on wild and cultivated plants, either herbaceous or woody crops. This phloem feeder frequently feeds on *Vitis* spp. (cultivated and wild grapevines) and *Clematis vitalba* plants, that are hosts of Flavescence dorée phytoplasma (FDp), a quarantine phloem-limited bacterial pathogen representing a major threat to viticulture in several European regions. The aim of this work was to assess if *R. speculum* could act as a vector of FDp, thus impacting on disease epidemiology. To explore the role of *R. speculum* in FDp transmission, nymphs were allowed to feed on FDp-infected *Vicia faba* (broad bean) plants to estimate acquisition efficiency and successively transferred onto grapevine, broad bean and *C. vitalba* test plants to determine transmission ability. *Ricania speculum* was unable to transmit FDp; some individuals could acquire the phytoplasma but supported a very low level of pathogen multiplication, compared with the competent vector. Consistently with transmission results, FDp was almost never detected in salivary glands. According to our results, the major damages that *R. speculum* may cause to plants derived from feeding and egg-laying and its contribution in FDp spread should be negligible.

Keywords

Vitis vinifera, *Clematis vitalba*, *Vicia faba*, insect vector, phytoplasma , qPCR.

Introduction

The exotic planthopper *Ricania speculum* (Hemiptera: Ricaniidae) was officially reported for the first time in Europe in 2014. Nymphs and adults were first observed in 2009 in different area of Genoa province (Liguria, Italy), but this species can now be considered established and spread also in provinces of La Spezia (Liguria), Massa Carrara, Pisa and Lucca (Tuscany). Ricaniidae species are mainly distributed in the tropics and sub-tropics of the eastern hemisphere, and *R. speculum* was reported in several Far East countries (Korea, Japan, China, Vietnam, Philippines and Indonesia) where it is a real pest for many crops (among others citrus, cocoa, cotton, sorghum and apple) due to its extreme polyphagy. The damages are mainly caused by sap suction and honeydew emission and to a lesser extent by egg-laying on thin shoots or thorns. The species is univoltine in Italy and overwinters as egg. Eggs are layered in linear clusters of about 20 units in such a typical manner that it can be used to describe the prevalence and diffusion. *Ricania speculum* resulted highly polyphagous in two field surveys conducted in Liguria region: typical egg clusters, nymphs and/or adults were collected from 49 different plant species, but host spectrum is suspected to be even larger. Beside many others, *Vitis* spp. and *Clematis vitalba* host both eggs and adults, and *C. vitalba* in particular, together with *Rubus* spp., turned out to be an excellent oviposition substrate (Rossi et al. 2015). Interestingly, *Vitis* spp. and *C. vitalba* are well-known plant hosts of the grapevine Flavescence dorée phytoplasma (FDp) (EFSA, 2016) and, since two unidentified Ricaniidae species were suspected to transmit Bogia coconut syndrome (BCS) phytoplasma in Papua New Guinea (Lu et al. 2016), concerns were raised on this alien species as a potential vector of FDp. Phytoplasmas are plant pathogenic bacteria belonging to class Mollicutes, associated to diseases affecting hundreds of plant species, with great impact on agricultural productions (Tomkins et al. 2018). In host plants, phytoplasmas are restricted to the phloem sieve tubes and are transmitted by leafhoppers, planthoppers or psyllids in a persistent-propagative manner (Hogenhout et al. 2008). Different phases took place during transmission process: insect vectors acquire phytoplasmas by feeding on phloem of infected plant, phytoplasmas from the insect gut lumen cross the intestinal epithelium and enter the haemocoel, multiply and circulate in the haemolymph and finally colonize the salivary glands and are transmitted with saliva. Indeed, phytoplasmas are wall-less obligate intracellular parasites able to survive and multiply both in plants and in insect vectors (Tomkins et al. 2018). Among the most destructive phytoplasma diseases, grapevine FD is a quarantine pest, epidemic in many economically important viticulture areas of Europe, including Italy. It causes strong yield reductions and is a very serious threat for European vine-growing regions. It is transmitted from grape to grape by the nearctict leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae), that was introduced in Europe by human intervention (Chuche and Thiéry, 2014). Other insect vectors are known and associated with alternative FD epidemiological cycles, namely *Dictyophara europaea* (Filippin et al. 2009) and *Orientus ishidae* (Matsumura) (Lessio et al. 2016), but are not directly associated with major epidemics of phytoplasma diseases of grapevine. Under controlled conditions, a standardized FDp transmission procedure is available relying on the laboratory vector *Euscelidius variegatus* Kirschbaum and *Vicia faba* (broad bean) as host plant (Caudwell et al. 1972). This controlled epidemiologic system is therefore useful to lab maintain FDp over time and it also allows to study infection mechanism in the natural hosts under controlled conditions, as *S. titanus* can acquire FDp from infected broad beans and back transmit it to grapevine (Eveillard et al. 2016).

As said, beside grapevine, very few other plant species were described as naturally infected with FDp in field, basically *C. vitalba*, *Alnus* spp. and *Ailanthus* spp. (EFSA, 2016). Being *C. vitalba* one of the most visited plants by *R. speculum*, and considering this planthopper lays eggs on both American and European grapevines, we aimed at exploring the potential role of this new alien species in FDp transmission. To this purpose, FDp acquisition and transmission trials were performed with *R. speculum* as potential vector and *V. faba*, *V. vinifera* and *C. vitalba* as test

plants, along a period of two consecutive years, under lab containment facility. Genotype of the FDp strain detected in *R. speculum* was confirmed and the amount of FDp was compared with that measured in the vector *E. variegatus*. The potential impact of *R. speculum* on FD epidemiological cycle is discussed.

Materials and methods

Phytoplasma isolate, insects and host plants.

The FDp strain, namely "FD-D CRA AT", used for acquisition and transmission trials was originally isolated in 2015 from infective adults of *S. titanus* collected in an experimental vineyard of the CREA research Institute (Asti, Piedmont) and allowed to feed on *V. faba* (broad bean) plants under laboratory conditions. This FDp isolate was genetically identified as D strain on *dna*K gene and mixed profile on *mal*G gene, according to (Rossi et al. submitted). FDp was then routinely maintained under controlled conditions by sequential transmissions from broad bean to broad bean by the experimental vector *Euscelidius variegatus*, continuously reared under laboratory conditions on *Avena sativa* (oats) as described in (Rashidi et al. 2014).

For acquisition and transmission experiments, *R. speculum* individuals were obtained from eggs laid on twigs of *Clematis vitalba*, *Ligustrum lucidum* and *Rubus* spp. collected in winter in infested areas (Liguria and Tuscany). Eggs were allowed to hatch under laboratory conditions (21 ± 1 °C, 60 ± 10% RH, and a photoperiod of 16:8 L:D h) on healthy broad beans and hatched nymphs were therefore phytoplasma-free, as confirmed by PCR. *Vicia faba*, *V. vinifera* and *C. vitalba* were used as test plants: broad beans were grown from seed, potted grapevines derived from commercial cuttings of different Piedmont typical cultivars grafted onto Kober 5BB rootstock, and *C. vitalba* were obtained by cuttings of field plants checked to be PCR-negative for phytoplasma presence.

Acquisition and transmission experiments

Two set of experiments were carried out with *R. speculum* in 2016 and 2017.

In 2016, about 200 nymphs (3rd and 4th instar) of *R. speculum* were isolated on FDp-infected broad beans for an acquisition access period (AAP) of three weeks and transferred onto healthy broad beans to complete the latent period (LP) for one extra week. FDp exposed adults were then used to inoculate test plants (groups of 5 insects per plant) in three sequential inoculation access periods (IAP) each of one week. In detail, 24 broad beans were inoculated in the first round IAP, 21 grapevines in the second one and 3 *C. vitalba* plants in the third IAP. After inoculation of broad beans, insects in the same group of 5 were, whenever possible, grouped again altogether on grapevines for the successive IAP. Plastic cylinders were used to isolate insects on broad beans, net cages were used to isolate insects on branches of grapevines and *C. vitalba* plants (Fig. 1). Following all IAPs, insects were collected, DNAs were extracted, analyzed by PCR, characterized by sequencing and FDp loads were measured by qPCR. Inoculated plants were periodically inspected for eventual symptom appearance, and DNAs were extracted from broad beans 1 month post inoculum (mpi) and from grapevines and *C. vitalba* at 3 and 12 mpi.

In 2017, AAP with *R. speculum* was repeated as described, and FDp exposed insects were used to investigate the presence and the amount of pathogen in dissected salivary glands.

Healthy *R. speculum* insects that were not exposed to phytoplasma were used as negative controls.

To evaluate the potential risk related with the FDp amount recorded in *R. speculum*, the phytoplasma load measured in the experimental FDp vector *E. variegatus* was used as basis for comparison. Healthy 3rd and 4th instar *E. variegatus* nymphs were allowed to acquire phytoplasma from infected broad beans as described above, and at 40 days post acquisition (dpa) FDp exposed adults were collected, DNAs were extracted and analyzed in qPCR for phytoplasma quantification.

DNA extraction

Samples of *R. speculum* DNAs were extracted from whole insect devoid of wings (Exp. 2016) or from salivary glands dissected from single insect (Exp. 2017) with cethyl-trimethyl-ammonium bromide (CTAB) buffer, as described in (Rashidi et al. 2014). In detail, salivary glands were dissected with sterile forceps under a stereomicroscope from anaesthetized *R. speculum* adults, either FDp exposed or healthy as negative control, washed four times in sterile phosphate buffered saline (PBS) 1x and singly stored in clean tube at -20 °C prior DNA extraction.

Total DNAs were also extracted from single whole *E. variegatus* specimen (Rashidi et al. 2014) and from plant samples (1 g of leaf tissues) with CTAB buffer, as described in (Pelletier et al. 2009). Insect and plant samples were resuspended in 50 and 100 µl of 10 mM Tris-Cl pH 8.0, respectively. Concentration and purity of extracted total DNAs were checked at UV-visible spectrophotometer Nanodrop 2000 (Thermofisher).

Phytoplasma detection, characterization and relative quantification

DNAKf/DNAKr primers, designed on FDp *dna*K gene, were used to detect FDp presence in *R. speculum* samples, by conventional PCR as described in the original paper (Rossi et al. submitted). The corresponding *dna*K PCR products obtained in positive samples were purified with DNA Clean & Concentrator kit (Zymo Research) and Sanger sequenced by BMR Genomics (Padova, Italia), to confirm the identity of FDp strains. Clustal Omega was used to align the obtained sequences with the reference *dna*K gene of FD-D strain (xxx accession number da Rossi et al. submitted).

Primers mapFD-F/mapFD-R and TaqMan probe mapFD-FAM (Pelletier et al. 2009) were used to detect FDp presence in inoculated plants by qPCR, using 1x iTaq Universal Probe Supermix (Bio-Rad) in a reaction mix of 10 µl volume. Final concentrations were 300 and 200 nM for primers and probe, repectively, and cycling conditions were as indicated in the original paper (Pelletier et al. 2009). Samples were run in duplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad).

The load of FDp was quantified in insects by qPCR using two primer pairs: FdSecyFw/FdSecyRv, targeting FDp *sec*Y gene (Roggia et al. 2014), and MqFw/MqRv, targeting insect 18S ribosomal gene (Marzachí and Bosco 2005). Insect DNA samples were diluted to 10 ng/µl and 1 µl was added to a qPCR reaction mix of 10 µl volume, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 300 nM of each primer. Samples were run in triplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Cycling conditions were as detailed in the original paper (Roggia et al. 2014). For each primer pair a standard curve, based on serial dilutions of either a plasmid harboring FDp *sec*Y gene or total DNA of a healthy insect sample, was always run together with analyzed samples. Mean starting quantities were automatically calculated by CFX Maestro™ Software (Bio-Rad) and used to express FDp load as Genome Unite (GU)/ng insect DNA. SigmaPlot version 13 (Systat Software) was used to compare FDp loads in *R. speculum* and *E. variegatus* samples, by Mann-Whitney test.

To exclude the presence of phytoplasmas potentially present in *C. vitalba* cuttings collected in field and then used to propagate test plants, P1/P7 and 16SF2/R2 primer pairs were used in direct and nested PCR, respectively, as described in (Lee et al. 1993) for universal phytoplasma detection.

Results and discussion

All the broad beans, grapevines and *C. vitalba* plants inoculated by FDp exposed *R. speculum* did not show any symptom and always resulted negative in diagnostic qPCR (Table 1). To exclude a latent infection, inoculated grapevines and *C. vitalba* plants were monitored for symptom appearance and PCR tested not only in 2016, the same season of inoculation (first diagnosis at 3 months post inoculation, mpi), but also in summer 2017 (at 12 mpi) (Table 1). Under our experimental condition *R. speculum* individuals were unable to transmit FDp to test plants.

Interestingly, phytoplasma detection on whole insect revealed that *R. speculum* is able to acquire FDp, as more than 50% of the 15 tested insects were PCR positive (Table 2). As expected, sequencing of the obtained PCR amplicons (targeting FDp *dna*K gene) confirmed a 100% identity with the same "FD-D CRA AT" phytoplasma strain used for acquisition. Nevertheless, FDp load in *R. speculum* adults was generally low, ranging from 3.2 to 130.4 FDp GU/ng insect DNA (Table 2). Only 6 out of 8 positive insect samples resulted quantifiable, being the remaining two below the sensitivity threshold of the quantification method (Table 2).

Consistently with lack of transmission, FDp was detected only in one salivary gland, dissected from over 50 *R. speculum* individuals, fed on infected broad beans (Table 2). In this unique positive sample FDp load was low, in line with mean phytoplasma GU measured in whole insect samples of *R. speculum* (Table 2).

The amount of FDp, measured in the vector *E. variegatus* as a comparison, ranged from 837.9 to 11640.5 FDp GU/ng insect DNA, with a mean value of 3780.1 ± 1151.6 (SEM) calculated on 10 individuals (Fig. 2). The mean FDp load detected in *R. speculum* insects was significantly lower than the phytoplasma amount measured in the vector species *E. variegatus* (Fig. 2, $p = 0.00138$).

The main concern about *R. speculum* was the possible role of this planthopper in opening new epidemiological cycles of FD between alternative woody host plants and grapevine, thus representing a serious threat for Italian and European viticulture. Its high polyphagy, its frequent association with *Vitis* spp. and *C. vitalba* and its establishment in regions like Tuscany, which is a world-renowned wine producing area, made this hypothesis frightening.

Under our experimental conditions, *R. speculum* was not able to transmit FDp to none of the three tested host species (*V. faba*, *V. vinifera*, *C. vitalba*), despite a quite high acquisition rate. This finding can be explained by the fact that acquired phytoplasma cells could probably be retained in the alimentary canal, eventually cross gut epithelium, but were unable to colonize salivary glands to be transmitted. Indeed *R. speculum* supported a very limited FDp multiplication rate, the phytoplasma load being nearly 60 times lower than in the vector species *E. variegatus*. Transmission ability has been indeed correlated with phytoplasma load in insect body, and FDp amount in *R. speculum* samples was well below that measured in the non-transmitter individuals of *E. variegatus* exposed to 'Candidatus Phytoplasma asteris' (approximately 1800 phytoplasma GU/ng insect DNA) (Galetto et al. 2009). Consistently, a very low frequency of FDp detection in *R. speculum* salivary glands was observed (1 case out of 51). Several insect species are able to acquire but not transmit phytoplasmas and there are many reports of field-collected insects found positive for phytoplasma presence, but then unable to transmit. Considering for example the two *Ricanidae* species suspected to transmit BCS phytoplasma in Papua New Guinea, the pathogen was detected in both species and also in artificial media used to feed insects of one of the two species, indicating that acquisition occurred but transmission did not (Lu et al. 2016). Similar situations were reported also for species even phylogenetically distant, such as the case of aphids: *Aphis craccivora* Koch and *Myzus persicae* (Sulzer) were able to acquire but not to transmit 'Ca. P. asteris' (Galetto et al. 2011), as well as three apple aphid species acquired but did not transmit 'Ca. P. mali' (Cainelli et al. 2007). Indeed, vector competency strongly depends on the capability of phytoplasmas to colonize salivary glands (Hogenhout et al. 2008). As an example, among 15 tested Hemiptera species, *Anoplotettix fuscovenosus* (Ferrari), *E. variegatus*, and *Euscelis incisus* Kirschbaum were the only three able to transmit FDp to broad beans, following abdominal microinjection with phytoplasma suspension, that allows phytoplasmas to avoid the gut barrier but not the salivary gland one (Bressan et al. 2006). All the previous 15 species confirmed to harbor phytoplasma cells in the body, but transmission occurred only in the few pathogen/vector compatible combinations, probably based on a molecular

specific mechanism of host recognition (Bressan et al. 2006). Lacking of phytoplasma vector-recognition may explain the inability of phytoplasma transmission also in the case of *R. speculum*. Several specific recognition factors were demonstrated to be the bases of an effective pathogen/vector combination. Considering the molecular recognition mechanism between phytoplasma and vector, it is noteworthy to mention that some insect proteins (namely actin, myosin and ATP synthase alpha and beta subunits) showed specific *in vitro* interaction with the major antigenic membrane protein (amp) of different strains of the '*Ca*. P. asteris' (Suzuki et al. 2006; Galetto et al. 2011). Moreover these specific interactions are *in vivo* required for insect colonization by phytoplasma, as '*Ca*. P. asteris' amp protein mediated bot the pathogen crossing of vector gut epithelium and colonization of salivary glands (Rashidi et al. 2015), showing the real involvement of these specific molecular interactions in a compatible phytoplasma/vector combination. A similar specific recognition mechanism was recently demonstrated also for FDp and its vector *E. variegatus*: the variable membrane protein A (vmpA) expressed on the surface of pathogen cells mediated the phytoplasma binding to insect cell culture and the pathogen retention in the perimicrovillar membrane of vector midgut epithelium (Arricau-Bouvery et al. 2018). Lacking of such molecular recognition between *R. speculum* tissues and FDp membrane proteins could explain the low multiplication rate in this host, the lack of salivary gland colonization and the consequent inability of transmission. Some preliminary experiments to investigate the *in vitro* interaction between *R. speculum* organs and an FDp recombinant membrane protein confirmed this hypothesis (Valeria Trivellone, personal communication).

The three plant species chosen for transmission trials, when infected by FDp, normally all show very clear and evident symptoms, as described in (Salar et al. 2013) for broad beans, in (Roggia et al. 2014) for grapevines and in (Filippin et al. 2009) for *C. vitalba* plants. Such symptoms were never observed in our cases. Broad beans were assayed for phytoplasma presence one month after the inoculation, as it is an herbaceous host and it is not very long lasting when potted. However this period of time is long enough for this species to develop clear symptoms, as constantly demonstrated in our routine maintainment of FDp strain by insect transmission from broad bean to broad bean. Consistently, FDp multiplication rate measured in broad beans reached its maximum at 30 days post inoculation (Salar et al. 2013). Inoculated grapevines and *C. vitalba* plants were instead assayed three months after inoculation and the following season too, to allow the occurrence of an eventually slow pathogen multiplication, which is known to happen in perennial hosts as demonstrated for field (Roggia et al. 2014) and potted grapevines (Eveillard et al. 2016). Moreover, FD symptoms frequently appear during the vegetative season following the year of the inoculation, and therefore, grapevine and *C. vitalba* plants were further assayed in the following season. The diagnosis also in this case confirmed that the plants were negative for phytoplasma presence.

In conclusion, although *R. speculum* can damage plants by sup suction, honeydew emission and egg-laying, it should not be regarded as a threat for FDp spread.

Author Contributions

Conceived and designed the experiments: D. Bosco, C. Marzachì and A. Lucchi. Performed the experiments: M. Pegoraro, L. Galetto and E. Rossi. Analyzed the data: M. Pegoraro, L. Galetto and D. Bosco. Wrote the paper: L. Galetto. Critical revision of the manuscript: all authors.

Compliance with ethical standards

Conflict of interest

The authors have declared that no competing interests exist.

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Tables

Table 1. Evaluation of *Ricania speculum* capability to transmit Flavescence Dorée phytoplasma (FDp) to different plant host species.

| Inoculated plants species	Plant sampling dates (months post inoculation, mpi)	Experimental year	PCR positive/total analysed samples
Vicia faba	1 mpi	2016	0/24
Vitis vinifera	3 mpi	2016	0/21
	12 mpi	2017	0/21
Clematis vitalba	3 mpi	2016	0/3
	12 mpi	2017	0/3

Table 2. Evaluation of *Ricania speculum* capability to acquire Flavescence Dorée phytoplasma (FDp) and quantification of FDp loads in whole insects or in salivary glands expressed as mean FDp Genome Units (GU)/ ng of insect DNA ± standard error of the mean (SEM) with sample size indicated in parenthesis (N).

Sample type	Insect sampling dates (days post acquisition, dpa)	Experimental year	PCR positive/total analysed samples	Mean FDp GU/ng insect DNA ± SEM (N)
Whole insects	50 dpa	2016	8/15	65.4 ± 27.3 (6)
Salivary glands	40 dpa	2017	1/51	317.6 (1)

Figures
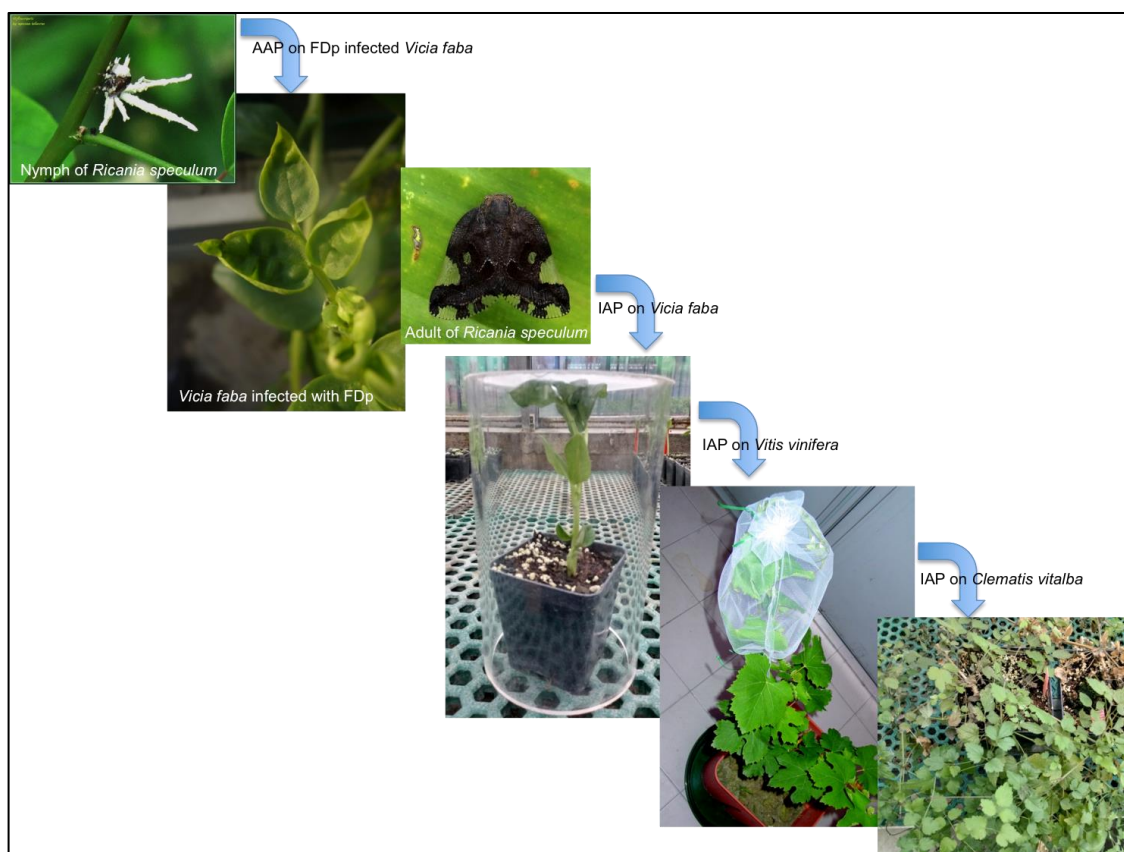
Figure 1. Experimental workflow of transmission trials of Flavescence dorée phytoplasma (FDp) with the alien planthopper *Ricania speculum*. Following an acquisition access period (AAP) of three weeks on FDp infected *Vicia faba* (beoad beans), and a latent period of one week on healthy broad bean (not shown), *R. speculum* individuals were isolated for three consecutive inoculation access periods (IAP) of one week on broad beans, *Vitis vinifera* and *Clematis vitalba* plants.
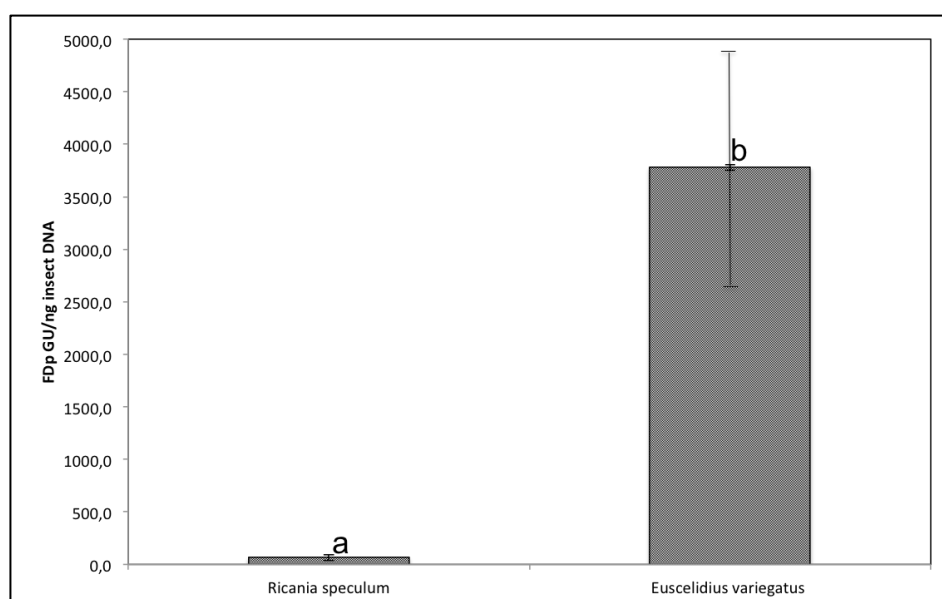
Figure 2. Amount of Flavescence dorée phytoplasma (FDp), expressed as mean FDp Genome Unit (GU) per ng of insect DNA, measured in the alien planthopper *Ricania speculum* and in the vector *Euscelidius variegatus*.