# New insights in phytoplasma-vector interaction: acquisition and inoculation of flavescence dorée phytoplasma by Scaphoideus titanus adults in a short window of time

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# New insights in phytoplasma-vector interaction: acquisition and inoculation of Flavescence dorée phytoplasma by *Scaphoideus titanus* adults in a short window of time

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**Running title:** Phytoplasma acquisition and transmission by *Scaphoideus titanus* adults

**Abstract:**

The leafhopper *Scaphoideus titanus* is able to transmit 16SrV phytoplasmas agents of grapevine's Flavescence dorée (FD) within 30-45 days, following an Acquisition Access Period (AAP) of a few days feeding on infected plants as a nymph, a Latency Period (LP) of 3-5 weeks becoming meanwhile an adult, and an Inoculation Access Period (IAP) of a few days on healthy plants. However, several aspects of FD epidemiology suggest how the whole transmission process may take less time, and may be start directly with adults of the insect vector. Transmission experiments have been set up under lab condition. Phytoplasma-free *S. titanus* adults were placed on broad bean (BB) plants (*Vicia faba*) infected by FD-C (16SrV-C) phytoplasmas for an AAP=7 days. Afterwards, they were immediately moved onto healthy BB for IAP, which were changed every 7 days, obtaining three timings of inoculation: IAP 1, IAP 2 and IAP 3, lasting 7, 14 and 21 days from the end of AAP, respectively. DNA was extracted from plants and insects, and PCR were performed to identify FD phytoplasmas. Insects were dissected and fluorescent in situ hybridization was made to detect the presence of phytoplasmas in midguts and salivary glands. The rate of infection in insects ranged 46-68% without significant differences among IAPs. Inoculation in plants succeeded in all IAPs, at a rate of 16-23% (no significant differences). Phytoplasma load was significantly higher in IAP3 than IAP 1-2 for both plants and insects. Phytoplasmas were identified both in midgut and salivary glands of *S. titanus* at all IAP times. The possible implications of these results in the epidemiology of Flavescence dorée are discussed.

**Key words:** Flavescence dorée, *Scaphoideus titanus*, *Vicia faba*, acquisition by adults, latency access period, transmission process

## Introduction

Flavescence dorée (FD) is a serious disease of grapevine caused by 16SrV phytoplasmas (subgroups C and D) (Arnaud *et al.*, 2007) transmitted by *Scaphoideus titanus* Ball (Chuche & Thiery, 2014), and to a lesser extent by *Dictyophara europaea* (L.) (Filippin *et al.*, 2009) and *Orientus ishidae* (Matsumura) (Lessio *et al.*, 2016). However, unlike these two last ones, *S. titanus* is able to accomplish its whole life cycle only on grapevine, both *Vitis vinifera* L. and American grapevines' and/or wild rootstocks (Maixner *et al.*, 1993; Vidano, 1966; Lessio *et al.*, 2007; Chuche & Thiery, 2014). *S. titanus* is a univoltine species, and overwinters as egg, laid under the bark of 2-yr old wood, although sometimes 1-yr old wood can be used as well (Bagnoli *et al.*, 2011; Lessio & Alma, 2013). During its postembryonic development, it undergoes five nymphal instars: N1, N2, N3, N4 and N5. The time elapsing between molts depends mostly on temperature (Falzoi *et al.*, 2014). Usually, eggs start hatching in the beginning of May. Hatching dynamic depends on the winter temperatures eggs are exposed to: the colder is the winter, the shorter will be the hatching period (Chuche & Thiery, 2009).

Transmission of phytoplasmas by insect vectors follows a typical sequence of events: Acquisition Access Period (AAP), Latency Access Period (LAP), or Latency Period (LP), an Inoculation Access Period (IAP) (Alma *et al.*, 2015). The transmission is therefore considered as "persistent-propagative". According to the state-of art, *S. titanus* nymphs (N3, N4 and N5) can acquire phytoplasmas by feeding on infected grapevines (both European and American), and during their LAP they become adults able to move from plant to plant and to transmit phytoplasmas to healthy plants (Chuche & Thiery, 2014; Lessio *et al.*, 2007). In *S. titanus,* firstly, the LAP was thought lasting about 28-35 days, with a minimum of 7 days for both AAP and IAP starting from N3 nymphs (Schvester *et al.*, 1961; 1969). Afterwards, Caudwell *et al.* (1970) demonstrated a possible inoculation to broad bean (BB) (*Vicia faba* L.) and grapevine after 21 days of AAP + LAP and 7 days of IAP (total days: 28).

Acquisition efficiency of phytoplasmas by *S. titanus* depends on a number of factors. The source of inoculum plays an important role: highly susceptible vine varieties are a better source than tolerant ones (Bressan *et al.*, 2005b; Galetto *et al.*, 2016); as well, a higher phytoplasma concentration increases acquisition efficiency (Galetto *et al.*, 2014; 2016), which on the other hand is lower in the case of recovered grapevines (Roggia *et al.*, 2014). Moreover, acquisition is more efficient in late summer because of a higher phytoplasma load in grapevines (Galetto *et al.*, 2014). However, in late summer there are no nymphs left, but only adults. If AAP is performed just by nymphs, this aspect would be biologically unrealistic, whereas it might be possible that adults acquire phytoplasmas too.

At present, the whole literature about *S. titanus* and transmission of FD phytoplasma (FDP) involves the nymphs for AAP, and adults for IAP, after a LAP of 28- 35 days. It is therefore necessary to ascertain if the whole transmission period could be completed within adults' lifespan. The aim of this research was to investigate if *S. titanus* adults are able to complete the transmission process starting from AAP and finishing with IAP. Moreover, since adults have never been used for AAP before, we reviewed the LAP in order to state if the whole transmission process could be completed within a shorter period.

## Materials and methods

### Insect source

*S. titanus* was reared under lab conditions in order to obtain healthy individuals. Grapevine canes containing eggs (two-year old wood, or older) were collected during winter in 2014 and 2015 in several Piedmontese grapevine growing areas, in particular Asti Province and Canavese district, where *S. titanus* was detected in great number during the previous summer, by means of yellow sticky traps. Canes were cut in pieces of approximately 20 cm length, placed in plastic bags, periodically sprinkled to avoid desiccation of eggs, and preserved into a cool chamber (+4°C) before use. The twigs were placed into BugDorm® insect rearing cages (47.5 x 47.5 x 138 cm) made of mesh and polyethylene at springtime, along with potted plants of healthy BB. Broad beans were used to rear *S. titanus* through its whole life cycle to avoid any phytoplamas contamination of the insects before the transmission experiments, as 16SrV phytoplasmas are thought not being transmitted through seeds (Duduk and Bertaccini, 2009). The cages were kept either outdoors, under a shelter against rain and sunlight, from May 15th to September 1st, or into a greenhouse from March 15th to May 15th and from September 1st to November 15th, and periodically sprinkled. Insects were reared up to the adult stage, and then used for transmission experiments. Adults from such rearing were also directly collected and dissected for fluorescence in situ hybridization (FISH) analysis, as a negative control.

### Transmission experiments

Transmission experiments were performed in a climatic chamber (T=25°C, RH=75%). Acquisition (AAP) of phytoplasmas by *S. titanus* adults was made on BBs previously infected with FD-C phytoplasmas by means of *Euscelidius variegatus* (Kirschbaum) (Caudwell *et al.*, 1972; Salar *et* *al.*, 2013). Plants used for AAP were 30-40 cm high, and had 8-10 leaves; before use, phytoplasma infection was checked by qPCR (see below). AAP was performed by placing 1-2 infected plants into BugDorm® insect rearing cages (47.5 x 47.5 x 47.5 cm) along with 20-30 newly emerged adults of *S. titanus* for 7 days. The procedure was repeated several times until enough live infected adults to be used in IAPs were obtained.

After AAP, insects were divided into batches of 5 individuals, and each batch was placed onto a healthy BB seedling (height 5 cm, 2 leaves), inside a Plexiglas cylinder (h=20 cm; diameter=12 cm) with the top covered by a fine mesh. Every 7 days, live insects were moved onto another plant up to three times to perform a total of three IAPs, named IAP1-3 (Fig. 1). Cylinders were checked daily, and dead insects were removed and preserved into a freezer (-20°C) for molecular analyses to test for FDP (see later).

The same set up was repeated for FISH analysis: at the end of each IAP five live leafhoppers from single batches (one for each of the IAPs) were collected and dissected to isolate midgut and salivary glands. Hence, a total of 28 batches with 140 specimens (25 batches with 125 specimens for qPCR, and 3 batches with 15 insects for FISH, Fig 1) started IAP 1, however the number of batches progressively decreased due to insect mortality or collection in IAPs 2 and 3. Inoculated plants were treated with an insecticide (Dichlorvos, 0.5 g/L) and kept in a climatic chamber, inside an insect-proof cage to avoid contamination, for three weeks from the beginning of phytoplasma inoculation before molecular analyses.

DNA extraction and quantitative Real Time PCR analysis

Total DNA extraction was performed from whole, single insects used in transmission experiments and from inoculated BB. Nucleic acids extraction from *S. titanus* was carried out by following a procedure previously described for leafhoppers (Marzachì *et al.*, 1998). Plant DNA was extracted from leaf tissue previously grounded with liquid nitrogen in a sterile mortar, according to the DNeasy Plant Mini Kit protocol instructions (Qiagen, Milan, Italy). Quantitative real-time PCR (qPCR) was carried out to measure the presence and concentration of phytoplasma genome units in insect and plant samples. A Chromo4 real-time detector (Bio-Rad, Milan, Italy) was used with PrecisionPlus$^{TM}$ -SY Mastermix (Primerdesign, Chandler's Ford, UK). Reactions targeting the 16S rRNA gene of group 16SrV phytoplasmas were carried out on all samples by using the fAY/rEY primer pair (Marcone *et al.*, 1996; Marzachì *et al.*, 2001), with the conditions described by Galetto *et al.* (2005). To calculate the average FDP Genome Units (GU) / sample, 16S rRNA gene copy numbers were divided by two, because this gene is estimated to be in double copy in the genome of phytoplasmas (Schneider & Seemuller, 1994). Additionally, qPCR targeting the insect's 18S rRNA was performed on insect DNA gene to normalize the absolute phytoplasma density. Primers MqFw / MqRv were used according to Marzachì & Bosco (2005). Hence, normalized phytoplasma GU were calculated per pg of insect 18Sr RNA gene. On the other hand, qPCR results from BBs were expressed as FDp GU per 100 mg of leaf.

Standard curves were constructed by using dilutions of PCR-amplified 16S rRNA gene of FDP cloned with the pGEM T-easy Vector Cloning Kit (Promega, Milan, Italy). The detection limit was calculated as the highest dilution of cloned amplicons used for standard curves which was successfully amplified, and corresponded to $1.0\times10^{0}$ FDP GU.

## Fluorescent *in situ* hybridization

FISH analyses were performed on *S. titanus* midguts and salivary glands. All of the experiments were carried out using a fluorescent probe specifically targeting group 16SrV phytoplasmas, along with Mollicutes-specific and eubacterial probes. Specifically, the 16SrV phytoplasma-specific probe ph1298, labelled with Cy5 (indodicarbocyanine, absorption/emission at 650/670nm), the Mollicutes probe MCP52, labelled with fluorescein isothiocyanate (FITC, absorption/ emission at 494/520 nm), and the eubacterial probe Eub338, labelled with Texas Red (absorption and emission at 595 nm and 620 nm, respectively) were used as described by Gonella *et al.* (2011) and Lessio *et al.* (2016).

In order to perform whole mount FISH, salivary glands were dissected in a sterile saline solutions, then fixed for 2 min at 4°C in 4% paraformaldehyde and washed in PBS. All hybridization experiment steps were performed following Gonella *et al.* (2011). After hybridization, the samples were mounted in antifading medium and then observed in a laser scanning confocal microscope SP2- AOBS (Leica).

## Statistical analysis

Statistical analyses were performed by mean of SPSS Statistics 24® (IBM Corp. Released 2016, Armonk, NY). A generalised linear model (GLM) was run for analysing infection rates (binomial: positive/negative) and quantitative PCR (qPCR) data, concerning both *S. titanus* adults and plants, as dependent variables, whereas the inoculation access period (IAP) was the categorical variable in both cases, counting three levels (IAP 1, IAP 2, and IAP 3). Concerning qPCR, negative values were excluded from GLM analyses. A binomial distribution with a Logit link function was used for infection rates, whereas a normal distribution and a logarithm link function was chosen for qPCR data. When IAP effects were significant, Helmert contrasts were performed to identify differences in infection rates and qPCR data (for both plants and insects), as follows: IAP1 vs. mean (IAP2, IAP3) and IAP2 vs. IAP3.

## Results

### FDP transmission by adult *S. titanus*

The acquisition of FDP by adult leafhoppers was performed on a total of eight experimentally infected BBs. The phytoplasma concentration in those plants was stable, ranging from $1.46\times10^4$ to $1.60\times10^5$ FDP GU / sample, with an average density of $6.64\times10^4$ FDP GU / sample.

Quantitative PCR analysis on insects and BBs showed that transmission of FDP did actually occur, and started from the first week after AAP (IAP1). The percentage of FDP-infected *S. titanus* adults and their corresponding BBs obtained at the end of each IAP are presented in Table 1, along with the average concentration of phytoplasma cells found in positive samples. FISH experiments confirmed the results of qPCR analyses, as at the end of all IAPs, 16SrV phytoplasmas were found not only in the midgut of leafhoppers (Fig. 2 A-C), but also in salivary glands (Fig. 2 D-F). Phytoplasma- specific hybridization was not observed in insects directly collected form the mass rearing, where the eubacterial signal was only detected (Fig. 2 G-I).

Considering *S. titanus* individuals, the infection rate reached 70% of the total, whereas infected BBs were up to 25%. For both insects and plants, infection rates peaked at the end of IAP2, while the lowest percentages of positive samples were recorded at the end of IAP1 as expected; however, according to binomial GLM analysis, no significant differences between IAP levels were found (*S. titanus*: $\chi^2 = 5.359$; df = 2; P = 0.07; BBs: $\chi^2 = 0.634$; df = 2; P = 0.73). Symptoms (such as yellowing and curling, especially of apical leaves) were observed on 9 BBs out of 13 which resulted FD-positive after molecular analyses (69%). Symptomatic plants were evenly distributed among IAP times (2 plants in IAP 1 and 3, 3 plants in IAP 2). On the other hand, none of the 49 FD-negative plants have shown any symptom.

The average concentration of phytoplasma cells ranged from $1.30\times10^2$ to $1.40\times10^3$ FDP GU / sample for leafhoppers and from $5.74\times10^0$ to $5.76\times10^2$ FDP GU / sample for inoculated plants, and in both cases IAP factor was significant (GLM, *S. titanus*: $\chi^2 = 16.60$, df = 2; P < 0.001; BBs: $\chi^2 = 29.76$, df = 2; P < 0.001). Helmert tests showed significant differences between IAP levels, except from IAP 1 vs. mean (IAP 2, IAP 3) in broad beans (Table 2).

## Discussion

The results of the present study highlighted that, under laboratory conditions, *S. titanus* is able to acquire FD-C phytoplasma from infected BB as an adult too, and not only at the nymphal stage, as previously reported (Caudwell *et al.*, 1970; Bressan *et al.*, 2005b; Galetto *et al.*, 2014). Indeed, phytoplasmas were successfully acquired after seven days of AAP, blooming to detectable loads after only seven days of LAP. Additionally, the phytoplasma load in insects increased over a period of 28 days (7 days AAP + 21 days LAP), indicating that the pathogen multiplies inside the leafhopper's body, in agreement with a persistent-propagative transmission model (Alma *et al.*, 2015). However, overall phytoplasma concentrations were lower compared to those reported about other vectors, such as *E. variegatus* and *Macrosteles quadripunctulatus* (Kirschbaum), in similar experiments (Rashidi *et al.*, 2014; Bosco *et al.*, 2007). Furthermore, the mortality of *S. titanus* adults, which may be related to experimental conditions, since BB is not the favorite plant host for *S. titanus* (Chuche *et al.*, 2016), suggests limited chance for adults to reach high levels of phytoplasma load, at least in laboratory. Under natural conditions, and especially on grapevine, adults' lifespan is longer, and acquisition of FDP from infected vines may therefore be more likely. This is in agreement with previous observations: *S. titanus* adults were thought to live up to 40 days (Vidano, 1964). Yet, recent (unpublished) results show how lifespan of adults is much longer under semi-natural conditions. If these data were confirmed, adults would be able to complete their transmission cycle (AAP+LAP+IAP) acquiring and transmitting phytoplasmas to vines, especially in the last part of the season, when no insecticides are used in vineyards. This is particularly threatening because of adults incoming from wild grapevine nearby (Lessio *et al.*, 2014): a similar pattern in FD transmission implies that adults could acquire phytoplasmas directly on infected grapevines in a vineyard, even if coming from outside. Yet, another issue which needs further investigation is the influence of FDP on the fitness of *S. titanus* when AAP occurs at the adult stage. It has been demonstrated that FDP have a negative influence on lifespan and fecundity of *S. titanus* when AAP occurs at the nymphal stage (Bressan *et al.*, 2005a). If confirmed for adults too, this could explain the low survival obtained in our experimental conditions.

In addition, our results underlined that phytoplasma inoculation may occur after a shorter LAP than previously believed (Schvester *et al.*, 1969; Caudwell *et al.*, 1970), following an AAP performed by adults. In effect, transmission to BBs was detected as soon as seven days after the end of AAP, with no significant differences in transmission efficiency with respect to longer LAPs, in spite of the higher phytoplasma titer recorded at the last IAP. Since similar percentages of infected BBs were found at the end of IAPs 1-3, it can be assumed that the presence of positive leafhoppers at the end of IAP1, although bearing a low titer, is not merely due to transient ingested phytoplasma cells, but more likely to effective colonization of insects, which rapidly become infective. The transmission of a phloem-restricted plant pathogen by adult vectors has been previously demonstrated only for *Diaphorina citri* Kuwayama, vectoring '*Candidatus* Liberibacter asiaticus'. However, diverging results were obtained by different studies: while Pelz-Stelinski *et al.* (2010) and Wu *et al.* (2016) reported successful adult transmission, even though with lower efficiency than in nymphs, Inoue *et al.* (2009) found no adults transmitting the pathogen. Moreover, Pelz-Stelinski *et al.* (2010) demonstrated that a one day long IAP is enough for *D. citri* to transmit liberibacter, but with an efficiency lower than 10%. Our results show that *S. titanus* adults are much more efficient in transmitting FDP, especially with short IAP, suggesting that different insect-pathogen dynamics occur for liberibacters and phytoplasmas, causing divergent transmission pathways.

Temperature may have played a role concerning multiplication of FDP both in insects and in plants. It has been (partially) demonstrated that, under lab conditions, multiplication of FDP in *S. titanus* was faster at low temperatures and $CO_2$ concentrations, whereas this trend was inverted in plants (Galetto *et al.*, 2011). Our experiments took place at 25 °C, but under field conditions temperatures change along with the day and the season. It is possible therefore that the multiplication of phytoplasmas occur faster in the last part of summer, increasing the threat caused by long-living adults.

Although successful transmission was observed, the average phytoplasma concentration in infected BB samples was always lower if compared to densities reported about phytoplasmas in wooden plants (Raddadi *et al.*, 2011; Galetto *et al.*, 2016; Jawhari *et al.*, 2015), possibly because of the herbaceous nature of BB. Moreover, higher FDP loads were recorded in BBs experimentally infected by *E. variegatus* (Salar *et al.*, 2013); low phytoplasma concentration found in inoculated plants may be due to the limited number of infected leafhoppers hosted by each plant during our experiments and the reduced pathogen load in inoculating insects.

Even though it must be pointed out that our results were obtained with BB, where *S. titanus* was reported to acquire the phytoplasma more rapidly than from grapevine in laboratory conditions (Chuche & Thiery, 2014), this work suggests a potential role of *S. titanus* adults in acquiring FDP from infected grapevines in vineyards, and a consequent inoculation to healthy plants within insects' lifespan. This evidence opens to concerning scenarios for viticulture, explaining at least partially the epidemic development of FD which is observed in some cases, in spite of continuous control. Indeed, insecticide sprays are generally limited with respect to the occurrence of adults in the vineyard, which may be prolonged until autumn (Lessio *et al.*, 2011) due to global increase of temperatures, therefore the total contribution of adults to FDP transmission can be relevant. Moreover, additional insecticide treatments in late summer, targeting adults, may be difficult to apply due to food safety issues. In addition, the presence of hotbeds with overgrown grapevine's rootstocks is a serious threat for vineyards due to incoming adults during late summer (Lessio *et al.*, 2014; Lessio *et al.*, 2015; Pavan *et al.*, 2012).

The results of this work, by showing that *S. titanus* is able to acquire and transmit FDP at the adult stage with reduced LAP in laboratory, represent the necessary scientific background for a deeper study of interactions involving adult leafhoppers, pathogens and host plants in different agricultural models, including the grapevine-FD pathosystem. Further researches are needed to shed light on such relationships, in order to improve the control of phytoplasma diseases.

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# References

Alma A., Tedeschi R., Lessio F., Picciau L., Gonella E., Ferracini C. (2015) Insect vectors of plant pathogenic Mollicutes in the European-Mediterranean region. *Phytopathogenic Mollicutes,* **5**, 53-73.

Arnaud G., Malembic-Maher S., Salar P., Bonnet P., Maixner M., Marcone C., Boudon-Padieu E., Foissac X. (2007) Multilocus sequence typing confirms the close genetic interrelatedness of three distinct flavescence doree phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology,* **73**, 4001-4010.

Bagnoli B., Angelini E., Borgo M., Ferretti L., Gargani E., Pasquini G. (2011) Valutazione del rischio di diffusione di *Scaphoideus titanus* mediante il materiale di propagazione della vite. *Rivista di viticoltura e di enologia,* **64**, 25-26.

Bosco D., Galetto L., Leoncini P., Saracco P., Raccah B., Marzachi C. (2007) Interrelationships between "*Candidatus* Phytoplasma asteris" and its leafhopper vectors (Homoptera : Cicadellidae). *Journal of Economic Entomology,* **100**, 1504-1511.

Bressan A., Girolami V., Boudon-Padieu E. (2005a) Reduced fitness of the leafhopper vector *Scaphoideus titanus* exposed to Flavescence doree phytoplasma. *Entomologia Experimentalis Et Applicata,* **115**, 283-290.

Bressan A., Spiazzi S., Girolami V., Boudon-Padieu E. (2005b) Acquisition efficiency of Flavescence doree phytoplasma by *Scaphoideus titanus* Ball from infected tolerant or susceptible grapevine cultivars or experimental host plants. *Vitis,* **44**, 143-146.

Caudwell A., Kuszala C., Bachelier J. C., Larrue J. (1970) Transmission of the golden flavescence of vines to herbaceous plants by the prolongation of the time during which *S. littoralis* can be used and the study of its survival on a large number of plant species. *Annalés de Phytopathologie,* **2**, 415-428.

Caudwell A., Kuszala C., Larrue J., Bachelier J. C. (1972) Transmission de la Flavescence dorée de la fève à la fève par des cicadelles des genres *Euscelis* et *Euscelidius.* Intervention possible de ces insectes dans l'épidémiologie du Bois noir en Bourgogne. *Annales de Phytopathologie,* **Hors série**, 181-189.

Chuche J., Boudon-Padieu E., Thiéry D. (2016) Host preferences of the leafhopper *Scaphoideus titanus*, vector of "flavescence dorée" phytoplasma. *Phytopathogenic Mollicutes,* **6**, 38-45.

Chuche J., Thiery D. (2009) Cold winter temperatures condition the egg-hatching dynamics of a grape disease vector. *Naturwissenschaften,* **96**, 827-834.

Chuche J., Thiery D. (2014) Biology and ecology of the Flavescence doree vector *Scaphoideus titanus*: a review. *Agronomy for Sustainable Development,* **34**, 381-403.

Duduk B., Bertaccini A. (2009) Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathologia Mediterranea,* **48**, 355-378.

Falzoi S., Lessio F., Spanna F., Alma A. (2014) Influence of temperature on the embryonic and post-embryonic development of *Scaphoideus titanus* (Hemiptera: Cicadellidae), vector of grapevine Flavescence doree. *International Journal of Pest Management,* **60**, 246-257.

Filippin L., Jovic J., Cvrkovic T., Forte V., Clair D., Tosevski I., Boudon-Padieu E., Borgo M., Angelini E. (2009) Molecular characteristics of phytoplasmas associated with Flavescence doree in clematis and grapevine and preliminary results on the role of *Dictyophara europaea* as a vector. *Plant Pathology,* **58**, 826-837.

Galetto L., Bosco D., Marzachì C. (2005) Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insec vectors. *Annals of Applied Biology,* **147**, 191-201.

Galetto L., Marzachì C., Marques R., Graziano C., Bosco D. (2011) Effects of temperature and $CO_2$ on phytoplasma multiplication pattern in vector and plant. *Bulletin of Insectology,* **64**, 151-152.

Galetto L., Miliordos D., Pegoraro M., Sacco D., Veratti F., Marzachì C., Bosco D. (2016) Acquisition of Flavescence dorée phytoplasma by *Scaphoideus titanus* Ball from different grapevine varieties. *Internationa Journal of Molecular Sciences,* **17**, 1563.

Galetto L., Miliordos D., Roggia C., Rashidi M., Sacco D., Marzachi C., Bosco D. (2014) Acquisition capability of the grapevine Flavescence doree by the leafhopper vector *Scaphoideus titanus* Ball correlates with phytoplasma titre in the source plant. *Journal of Pest Science,* **87**, 671-679.

Gonella E., Negri I., Marzorati M., Mandrioli M., Sacchi L., Pajoro M., Crotti E., Rizzi A., Clementi E., Tedeschi R., Bandi C., Alma A., Daffonchio D. (2011) Bacterial endosymbiont localization in *Hyalesthes obsoletus*, the insect vector of Bois noir in *Vitis vinifera*. *Applied and Environmental Microbiology,* **77**, 1423-1435.

Inoue H., Ohnishi J., Ito T., Tomimura K., Miyata S., Iwanami T., Ashihara W. (2009) Enhanced proliferation and efficient transmission of Candidatus Liberibacter asiaticus by adult Diaphorina citri after acquisition feeding in the nymphal stage. *Annals of Applied Biology,* **155**, 29-36.

Jawhari M., Abrahamian P., Sater A. A., Sobh H., Tawidian P., Abou-Jawdah Y. (2015) Specific PCR and real-time PCR assays for detection and quantitation of 'Candidatus Phytoplasma phoenicium'. *Molecular and Cellular Probes,* **29**, 63-70.

Lessio F., Alma A. (2013) Influenza dello sviluppo del ritidoma e della termoterapia sulle uova di *Scaphoideus titanus* Ball. *Petria,* **23**, 157-160.

Lessio F., Mondino E. B., Alma A. (2011) Spatial patterns of *Scaphoideus titanus* (Hemiptera: Cicadellidae): a geostatistical and neural network approach. *International Journal of Pest Management,* **57**, 205-216.

Lessio F., Picciau L., Gonella E., Mandrioli M., Tota F., Alma A. (2016) The mosaic leafhopper *Orientus ishidae*: host plants, spatial distribution, infectivity, and transmission of 16SrV phytoplasmas to vines. *Bulletin of Insectology,* **69**, 277-289.

Lessio F., Portaluri A., Paparella F., Alma A. (2015) A mathematical model of flavescence dorée epidemiology. *Ecological Modelling,* **312**, 41-53.

Lessio F., Tedeschi R., Alma A. (2007) Presence of *Scaphoideus titanus* on American grapevine in woodlands, and infection with "flavescence doree" phytoplasmas. *Bulletin of Insectology,* **60**, 373-374.

Lessio F., Tota F., Alma A. (2014) Tracking the dispersion of *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) from wild to cultivated grapevine: use of a novel mark-capture technique. *Bulletin of Entomological Research,* **104**, 432-443.

Maixner M., Pearson R. C., Boudonpadieu E., Caudwell A. (1993) *Scaphoideus titanus*, a possible vector of grapevine yellows in New York. *Plant Disease,* **77**, 408-413.

Marcone C., Ragozzino A., Schneider B., Lauer U., Smart C. D., Seemuller E. (1996) Genetic characterization and classification of two phytoplasmas associated with spartium witches'-broom disease. *Plant Disease,* **80**, 365-371.

Marzachi C., Bosco D. (2005) Relative quantification of chrysanthemum yellows (16Sr I) phytoplasma in its plant and insect host using real-time polymerase chain reaction. *Molecular Biotechnology,* **30**, 117-127.

Marzachi C., Palermo S., Boarino A., Veratti F., d'Aquilio M., Loria A., Boccardo G. (2001) Optimisation of a one-step PCR assay for the diagnosis of Flavescence dorée related phytoplasmas in field-grown grapevines and vector populations. *Vitis,* **40**, 213-217.

Marzachi C., Veratti F., Bosco D. (1998) Direct PCR detection of phytoplasmas in experimentally infected insects. *Annals of Applied Biology,* **133**, 45-54.

Pavan F., Mori N., Bigot G., Zandigiacomo P. (2012) Border effect in spatial distribution of Flavescence dorée affected grapevines and outside source of *Scaphoideus titanus* vectors. *Bulletin of Insectology,* **65**, 281-290.

Pelz-Stelinski K. S., Brlansky R. H., Ebert T. A., Rogers M. E. (2010) Transmission Parameters for Candidatus Liberibacter asiaticus by Asian Citrus Psyllid (Hemiptera: Psyllidae). *Journal of Economic Entomology,* **103**, 1531-1541.

Raddadi N., Gonella E., Camerota C., Pizzinat A., Tedeschi R., Crotti E., Mandrioli M., Bianco P.A., Daffonchio D., Alma A. (2011) '*Candidatus* Liberibacter europaeus' sp nov that is associated with and transmitted by the psyllid *Cacopsylla pyri* apparently behaves as an endophyte rather than a pathogen. *Environmental Microbiology,* **13**, 414-426.

Rashidi M., D'Amelio R., Galetto L., Marzachi C., Bosco D. (2014) Interactive transmission of two phytoplasmas by the vector insect. *Annals of Applied Biology,* **165**, 404-413.

Roggia C., Caciagli P., Galetto L., Pacifico D., Veratti F., Bosco D., Marzachi C. (2014) Flavescence doree phytoplasma titre in field-infected Barbera and Nebbiolo grapevines. *Plant Pathology,* **63**, 31-41.

Salar P., Charenton C., Foissac X., Malembic-Maher S. (2013) Multiplication kinetics of Flavescence dorée phytoplasma in broad bean. Effect of phytoplasma strain and temperature. *European Journal of Plant Pathology,* **135**, 371-381.

Schneider B., Seemuller E. (1994) Presence of 2 sets of ribosomal genes in phytopathogenic Mollicutes. *Applied and Environmental Microbiology,* **60**, 3409-3412.

Schvester D., Carle P., Moutous G. (1961) Transmission de la Flavescence dorée de la vigne par *Scaphoideus littoralis* Ball, (*Homopt. Jassidae*). *Annales Epiphyties,* **14**, 175-198.

Schvester D., Carle P., Moutous G. (1969) Nouvelles données sur la transmission de la Flavescence dorée de la vigne par *Scaphoideus littoralis* Ball. *Annale de Zoologie et écologie animale,* **1**, 445-465.

Vidano C. (1964) Scoperta in Italia dello *Scaphoideus littoralis* Ball, cicalina americana collegata alla *Flavescence dorée* della vite. *L'Italia Agricola,* **10**.

Vidano C. (1966) Scoperta della ecologia ampelofila del cicadellide *Scaphoideus littoralis* Ball nella regione neartica originaria. *Annali della Facoltà di Scienze Agrarie della Università degli Studi di Torino,* **3**.

Wu T. Y., Luo X. Z., Xu C. B., Wu F. N., Qureshi J. A., Cen Y. J. (2016) Feeding behavior of Diaphorina citri and its transmission of "Candidatus Liberibacter asiaticus' to citrus. *Entomologia Experimentalis Et Applicata,* **161**, 104-111.

**Table 1**. Results of inoculation trials of FDP to broad beans (BB) by means of *S. titanus* adults. Data of qPCR analyses on leafhoppers ("*S. titanus*" columns) and inoculated plants ("BB" columns) are indicated.

**Table 2.** Results of Helmert contrasts between levels of the categorical variable "Inoculation Access Period (IAP)", for the dependent variable "quantitative PCR (qPCR)" concerning both *S. titanus* (ST) and broad beans (BB).

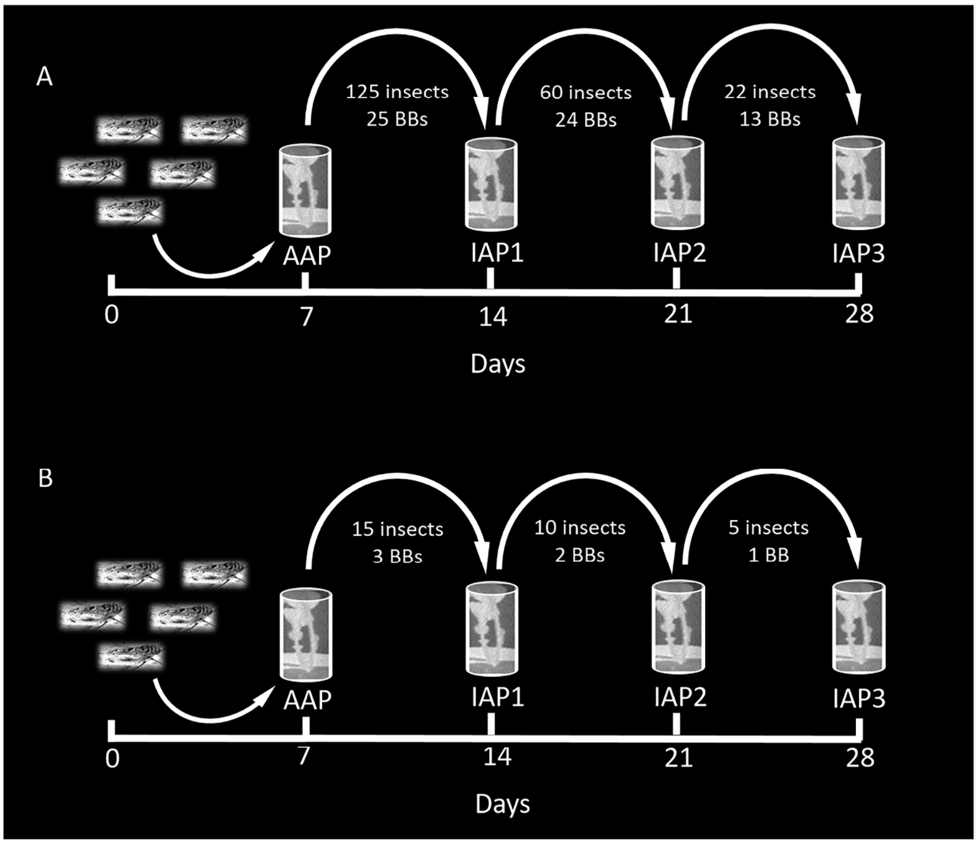
**Figure 1**. Experimental design of FDP transmission trials. Adult *S. titanus* [25 batches with 125 insects for qPCR analysis (A) and three batches with 15 insects for FISH analysis (B)] were caged for phytoplasma acquisition at day 0, and then from day 7 they were maintained on BB seedlings in groups of five. Every seven days live specimens were moved onto a new plant to perform up to three IAPs: 14 days (IAP1), 21 days (IAP2), and 28 days (IAP3) after the beginning of AAP, respectively.

**Figure 2**. FISH on *S. titanus* midgut and salivary glands. Exemplificative micrographs showing results from FISH of: midgut of a specimen at the end of IAP2 (A-C), salivary glands of an adult at the end of IAP3 (D-F), and midgut of a mass-reared individual (negative control) (G-I). A, D and G show interferential contrast micrographs of the organs. Hybridizations with Mollicutes probe (green signal in B) and eubacterial probe (green signal in E and H) are presented; moreover, hybridization with 16SrV phytoplasma probe is shown in C (cyan signal), F (red signal), and I. Bars = 75 µm. The phytoplasmas were effectively detected in the organs of leafhoppers after transmission experiments, while they were absent in the negative control.

| | ***S. titanus*** | | | | **BB** | | | |
|---|---|---|---|---|---|---|---|---|
| **Rank** | N | Positive samples | Infection rate[1] ± SE | Titer[2] ± SE | N | Positive samples | Infection rate [1] ± SE | Titer ± SE |
| IAP1 | 65 | 30 | 0.46 ± 0.06 | $1.30\times10^{2} \pm 3.99\times10^{1}$ | 25 | 4 | 0.16 ± 0.07 | $6.57\times10^{1} \pm 5.43\times10^{1}$ |
| IAP2 | 38 | 26 | 0.68 ± 0.08 | $5.05\times10^{2} \pm 1.39\times10^{2}$ | 24 | 6 | 0.25. ± 0.09 | $5.74\times10^{0} \pm 1.33\times10^{0}$ |
| IAP3 | 22 | 14 | 0.64 ± 0.10 | $1.40\times10^{3} \pm 6.42\times10^{2}$ | 13 | 3 | 0.23 ± 0.12 | $5.76\times10^{2} \pm 1.98\times10^{2}$ |

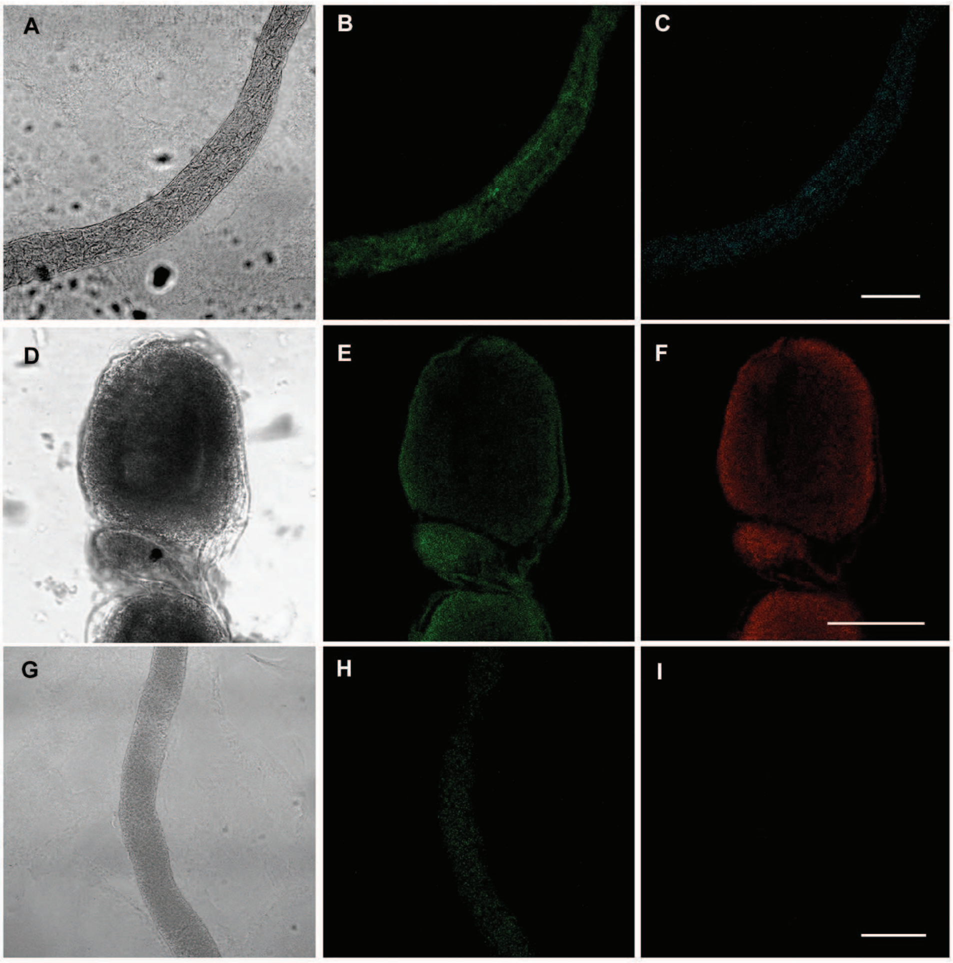
[1] Rate of 16SrV phytoplasma-positive individuals related to the total tested samples. [2] 16SrV phytoplasma GU per sample (single insect or 100 mg of plant tissue). Values below the detection limit ($1.00\times10^{0}$ GU /sample) were considered negative (cut-off value). SE: Standard Error.

| | IAP 1 vs. mean (IAP 2, IAP 3) | | | IAP 2 vs. IAP 3 | | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | df | P | $\chi^2$ | df | P |
| ST qPCR | 12.957 | 1 | 0.00 | 6.736 | 1 | 0.01 |
| BB qPCR | 2.078 | 1 | 0.15 | 29.597 | 1 | 0.00 |



Experimental design of FDP transmission trials. Adult *S. titanus* [25 batches with 125 insects for qPCR analysis (A) and three batches with 15 insects for FISH analysis (B)] were caged for phytoplasma acquisition at day 0, and then from day 7 they were maintained on BB seedlings in groups of five. Every seven days live specimens were moved onto a new plant to perform up to three IAPs: 14 days (IAP1), 21 days (IAP2), and 28 days (IAP3) after the beginning of AAP, respectively.

209x180mm (300 x 300 DPI)



FISH on *S. titanus* midgut and salivary glands. Exemplificative micrographs showing results from FISH of: midgut of a specimen at the end of IAP2 (A-C), salivary glands of an adult at the end of IAP3 (D-F), and midgut of a mass-reared individual (negative control) (G-I). A, D and G show interferential contrast micrographs of the organs. Hybridizations with Mollicutes probe (green signal in B) and eubacterial probe (green signal in E and H) are presented; moreover, hybridization with 16SrV phytoplasma probe is shown in C (cyan signal), F (red signal), and I. Bars = 75µm. The phytoplasmas were effectively detected in the organs of leafhoppers after transmission experiments, while they were absent in the negative control.

180x181mm (300 x 300 DPI)