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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

For Peer Review

**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 phytoplasmas 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*

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3 107 *al.*, 2013). Plants used for AAP were 30-40 cm high, and had 8-10 leaves; before use, phytoplasma  
4 108 infection was checked by qPCR (see below). AAP was performed by placing 1-2 infected plants  
5 109 into BugDorm® insect rearing cages (47.5 x 47.5 x 47.5 cm) along with 20-30 newly emerged  
6 110 adults of *S. titanus* for 7 days. The procedure was repeated several times until enough live infected  
7 111 adults to be used in IAPs were obtained.

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10 112 After AAP, insects were divided into batches of 5 individuals, and each batch was placed onto a  
11 113 healthy BB seedling (height 5 cm, 2 leaves), inside a Plexiglas cylinder (h=20 cm; diameter=12 cm)  
12 114 with the top covered by a fine mesh. Every 7 days, live insects were moved onto another plant up to  
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14 115 three times to perform a total of three IAPs, named IAP1-3 (Fig. 1). Cylinders were checked daily,  
15 116 and dead insects were removed and preserved into a freezer (-20°C) for molecular analyses to test  
16 117 for FDP (see later).

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19 118 The same set up was repeated for FISH analysis: at the end of each IAP five live leafhoppers from  
20 119 single batches (one for each of the IAPs) were collected and dissected to isolate midgut and salivary  
21 120 glands. Hence, a total of 28 batches with 140 specimens (25 batches with 125 specimens for qPCR,  
22 121 and 3 batches with 15 insects for FISH, Fig 1) started IAP 1, however the number of batches  
23 122 progressively decreased due to insect mortality or collection in IAPs 2 and 3. Inoculated plants were  
24 123 treated with an insecticide (Dichlorvos, 0.5 g/L) and kept in a climatic chamber, inside an insect-  
25 124 proof cage to avoid contamination, for three weeks from the beginning of phytoplasma inoculation  
26 125 before molecular analyses.

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35 127 DNA extraction and quantitative Real Time PCR analysis

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38 129 Total DNA extraction was performed from whole, single insects used in transmission experiments  
39 130 and from inoculated BB. Nucleic acids extraction from *S. titanus* was carried out by following a  
40 131 procedure previously described for leafhoppers (Marzachi *et al.*, 1998). Plant DNA was extracted  
41 132 from leaf tissue previously grounded with liquid nitrogen in a sterile mortar, according to the  
42 133 DNeasy Plant Mini Kit protocol instructions (Qiagen, Milan, Italy). Quantitative real-time PCR  
43 134 (qPCR) was carried out to measure the presence and concentration of phytoplasma genome units in  
44 135 insect and plant samples. A Chromo4 real-time detector (Bio-Rad, Milan, Italy) was used with  
45 136 PrecisionPlus™-SY Mastermix (Primerdesign, Chandler's Ford, UK). Reactions targeting the 16S  
46 137 rRNA gene of group 16SrV phytoplasmas were carried out on all samples by using the fAY/rEY  
47 138 primer pair (Marccone *et al.*, 1996; Marzachi *et al.*, 2001), with the conditions described by Galetto  
48 139 *et al.* (2005). To calculate the average FDP Genome Units (GU) / sample, 16S rRNA gene copy  
49 140 numbers were divided by two, because this gene is estimated to be in double copy in the genome of

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3 141 phytoplasmas (Schneider & Seemuller, 1994). Additionally, qPCR targeting the insect's 18S rRNA  
4 142 was performed on insect DNA gene to normalize the absolute phytoplasma density. Primers MqFw  
5 143 / MqRv were used according to Marzachi & Bosco (2005). Hence, normalized phytoplasma GU  
6 144 were calculated per pg of insect 18Sr RNA gene. On the other hand, qPCR results from BBs were  
7 145 expressed as FDp GU per 100 mg of leaf.

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10 146 Standard curves were constructed by using dilutions of PCR-amplified 16S rRNA gene of FDP  
11 147 cloned with the pGEM T-easy Vector Cloning Kit (Promega, Milan, Italy). The detection limit was  
12 148 calculated as the highest dilution of cloned amplicons used for standard curves which was  
13 149 successfully amplified, and corresponded to  $1.0 \times 10^0$  FDP GU.  
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#### 18 19 151 Fluorescent *in situ* hybridization

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22 153 FISH analyses were performed on *S. titanus* midguts and salivary glands. All of the experiments  
23 154 were carried out using a fluorescent probe specifically targeting group 16SrV phytoplasmas, along  
24 155 with Mollicutes-specific and eubacterial probes. Specifically, the 16SrV phytoplasma-specific  
25 156 probe ph1298, labelled with Cy5 (indodicarbocyanine, absorption/emission at 650/670nm), the  
26 157 Mollicutes probe MCP52, labelled with fluorescein isothiocyanate (FITC, absorption/ emission at  
27 158 494/520 nm), and the eubacterial probe Eub338, labelled with Texas Red (absorption and emission  
28 159 at 595 nm and 620 nm, respectively) were used as described by Gonella *et al.* (2011) and Lessio *et*  
29 160 *al.* (2016).

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32 161 In order to perform whole mount FISH, salivary glands were dissected in a sterile saline solutions,  
33 162 then fixed for 2 min at 4°C in 4% paraformaldehyde and washed in PBS. All hybridization  
34 163 experiment steps were performed following Gonella *et al.* (2011). After hybridization, the samples  
35 164 were mounted in antifading medium and then observed in a laser scanning confocal microscope  
36 165 SP2- AOBS (Leica).  
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#### 40 41 42 167 Statistical analysis

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45 169 Statistical analyses were performed by mean of SPSS Statistics 24® (IBM Corp. Released 2016,  
46 170 Armonk, NY). A generalised linear model (GLM) was run for analysing infection rates (binomial:  
47 171 positive/negative) and quantitative PCR (qPCR) data, concerning both *S. titanus* adults and plants,  
48 172 as dependent variables, whereas the inoculation access period (IAP) was the categorical variable in  
49 173 both cases, counting three levels (IAP 1, IAP 2, and IAP 3). Concerning qPCR, negative values  
50 174 were excluded from GLM analyses. A binomial distribution with a Logit link function was used for  
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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  $1.46 \times 10^4$  to  $1.60 \times 10^5$  FDP GU / sample, with an average density of  $6.64 \times 10^4$  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 from 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  $1.30 \times 10^2$  to  $1.40 \times 10^3$  FDP GU / sample for leafhoppers and from  $5.74 \times 10^0$  to  $5.76 \times 10^2$  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).

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## 210 Discussion

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212 The results of the present study highlighted that, under laboratory conditions, *S. titanus* is able to  
213 acquire FD-C phytoplasma from infected BB as an adult too, and not only at the nymphal stage, as  
214 previously reported (Caudwell *et al.*, 1970; Bressan *et al.*, 2005b; Galetto *et al.*, 2014). Indeed,  
215 phytoplasmas were successfully acquired after seven days of AAP, blooming to detectable loads  
216 after only seven days of LAP. Additionally, the phytoplasma load in insects increased over a period  
217 of 28 days (7 days AAP + 21 days LAP), indicating that the pathogen multiplies inside the  
218 leafhopper's body, in agreement with a persistent-propagative transmission model (Alma *et al.*,  
219 2015). However, overall phytoplasma concentrations were lower compared to those reported about  
220 other vectors, such as *E. variegatus* and *Macrosteles quadripunctulatus* (Kirschbaum), in similar  
221 experiments (Rashidi *et al.*, 2014; Bosco *et al.*, 2007). Furthermore, the mortality of *S. titanus*  
222 adults, which may be related to experimental conditions, since BB is not the favorite plant host for  
223 *S. titanus* (Chuche *et al.*, 2016), suggests limited chance for adults to reach high levels of  
224 phytoplasma load, at least in laboratory. Under natural conditions, and especially on grapevine,  
225 adults' lifespan is longer, and acquisition of FDP from infected vines may therefore be more likely.  
226 This is in agreement with previous observations: *S. titanus* adults were thought to live up to 40 days  
227 (Vidano, 1964). Yet, recent (unpublished) results show how lifespan of adults is much longer under  
228 semi-natural conditions. If these data were confirmed, adults would be able to complete their  
229 transmission cycle (AAP+LAP+IAP) acquiring and transmitting phytoplasmas to vines, especially  
230 in the last part of the season, when no insecticides are used in vineyards. This is particularly  
231 threatening because of adults incoming from wild grapevine nearby (Lessio *et al.*, 2014): a similar  
232 pattern in FD transmission implies that adults could acquire phytoplasmas directly on infected  
233 grapevines in a vineyard, even if coming from outside. Yet, another issue which needs further  
234 investigation is the influence of FDP on the fitness of *S. titanus* when AAP occurs at the adult stage.  
235 It has been demonstrated that FDP have a negative influence on lifespan and fecundity of *S. titanus*  
236 when AAP occurs at the nymphal stage (Bressan *et al.*, 2005a). If confirmed for adults too, this  
237 could explain the low survival obtained in our experimental conditions.  
238 In addition, our results underlined that phytoplasma inoculation may occur after a shorter LAP than  
239 previously believed (Schvester *et al.*, 1969; Caudwell *et al.*, 1970), following an AAP performed  
240 by adults. In effect, transmission to BBs was detected as soon as seven days after the end of AAP,  
241 with no significant differences in transmission efficiency with respect to longer LAPs, in spite of  
242 the higher phytoplasma titer recorded at the last IAP. Since similar percentages of infected BBs

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3 243 were found at the end of IAPs 1-3, it can be assumed that the presence of positive leafhoppers at the  
4 244 end of IAP1, although bearing a low titer, is not merely due to transient ingested phytoplasma cells,  
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6 245 but more likely to effective colonization of insects, which rapidly become infective. The  
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8 246 transmission of a phloem-restricted plant pathogen by adult vectors has been previously  
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10 247 demonstrated only for *Diaphorina citri* Kuwayama, vectoring ‘*Candidatus Liberibacter asiaticus*’.  
11 248 However, diverging results were obtained by different studies: while Pelz-Stelinski *et al.* (2010) and  
12 249 Wu *et al.* (2016) reported successful adult transmission, even though with lower efficiency than in  
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14 250 nymphs, Inoue *et al.* (2009) found no adults transmitting the pathogen. Moreover, Pelz-Stelinski *et al.*  
15 251 *al.* (2010) demonstrated that a one day long IAP is enough for *D. citri* to transmit liberibacter, but  
16 252 with an efficiency lower than 10%. Our results show that *S. titanus* adults are much more efficient  
17 253 in transmitting FDP, especially with short IAP, suggesting that different insect-pathogen dynamics  
18 254 occur for liberibacters and phytoplasmas, causing divergent transmission pathways.  
19 255 Temperature may have played a role concerning multiplication of FDP both in insects and in plants.  
20 256 It has been (partially) demonstrated that, under lab conditions, multiplication of FDP in *S. titanus*  
21 257 was faster at low temperatures and CO<sub>2</sub> concentrations, whereas this trend was inverted in plants  
22 258 (Galetto *et al.*, 2011). Our experiments took place at 25 °C, but under field conditions temperatures  
23 259 change along with the day and the season. It is possible therefore that the multiplication of  
24 260 phytoplasmas occur faster in the last part of summer, increasing the threat caused by long-living  
25 261 adults.  
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27 262 Although successful transmission was observed, the average phytoplasma concentration in infected  
28 263 BB samples was always lower if compared to densities reported about phytoplasmas in wooden  
29 264 plants (Raddadi *et al.*, 2011; Galetto *et al.*, 2016; Jawhari *et al.*, 2015), possibly because of the  
30 265 herbaceous nature of BB. Moreover, higher FDP loads were recorded in BBs experimentally  
31 266 infected by *E. variegatus* (Salar *et al.*, 2013); low phytoplasma concentration found in inoculated  
32 267 plants may be due to the limited number of infected leafhoppers hosted by each plant during our  
33 268 experiments and the reduced pathogen load in inoculating insects.  
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35 269 Even though it must be pointed out that our results were obtained with BB, where *S. titanus* was  
36 270 reported to acquire the phytoplasma more rapidly than from grapevine in laboratory conditions  
37 271 (Chuche & Thiery, 2014), this work suggests a potential role of *S. titanus* adults in acquiring FDP  
38 272 from infected grapevines in vineyards, and a consequent inoculation to healthy plants within  
39 273 insects’ lifespan. This evidence opens to concerning scenarios for viticulture, explaining at least  
40 274 partially the epidemic development of FD which is observed in some cases, in spite of continuous  
41 275 control. Indeed, insecticide sprays are generally limited with respect to the occurrence of adults in  
42 276 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.

### Acknowledgements

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**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.

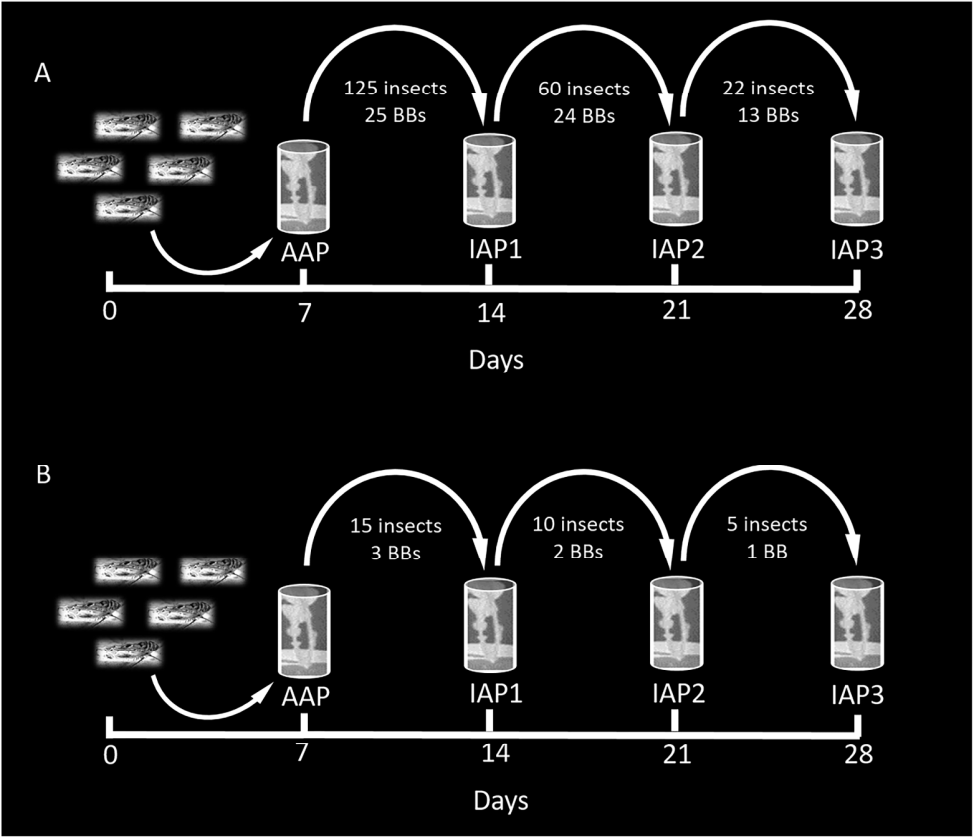
**Figure 2.** FISH on *S. titanus* midgut and salivary glands. Exemplificative micrographs showing results from FISH of: midgut of a specimen at the end of IAP2 (A-C), salivary glands of an adult at the end of IAP3 (D-F), and midgut of a mass-reared individual (negative control) (G-I). A, D and G show 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  $\mu$ m. The phytoplasmas were effectively detected in the organs of leafhoppers after transmission experiments, while they were absent in the negative control.

Rank	<i>S. titanus</i>				BB			
	N	Positive samples	Infection rate <sup>1</sup> ± SE	Titer <sup>2</sup> ± SE	N	Positive samples	Infection rate <sup>1</sup> ± SE	Titer ± SE
IAP1	65	30	0.46 ± 0.06	1.30×10 <sup>2</sup> ± 3.99×10 <sup>1</sup>	25	4	0.16 ± 0.07	6.57×10 <sup>1</sup> ± 5.43×10 <sup>1</sup>
IAP2	38	26	0.68 ± 0.08	5.05×10 <sup>2</sup> ± 1.39×10 <sup>2</sup>	24	6	0.25 ± 0.09	5.74×10 <sup>0</sup> ± 1.33×10 <sup>0</sup>
IAP3	22	14	0.64 ± 0.10	1.40×10 <sup>3</sup> ± 6.42×10 <sup>2</sup>	13	3	0.23 ± 0.12	5.76×10 <sup>2</sup> ± 1.98×10 <sup>2</sup>

<sup>1</sup> Rate of 16SrV phytoplasma-positive individuals related to the total tested samples. <sup>2</sup> 16SrV phytoplasma GU per sample (single insect or 100 mg of plant tissue). Values below the detection limit (1.00×10<sup>0</sup> GU /sample) were considered negative (cut-off value). SE: Standard Error.

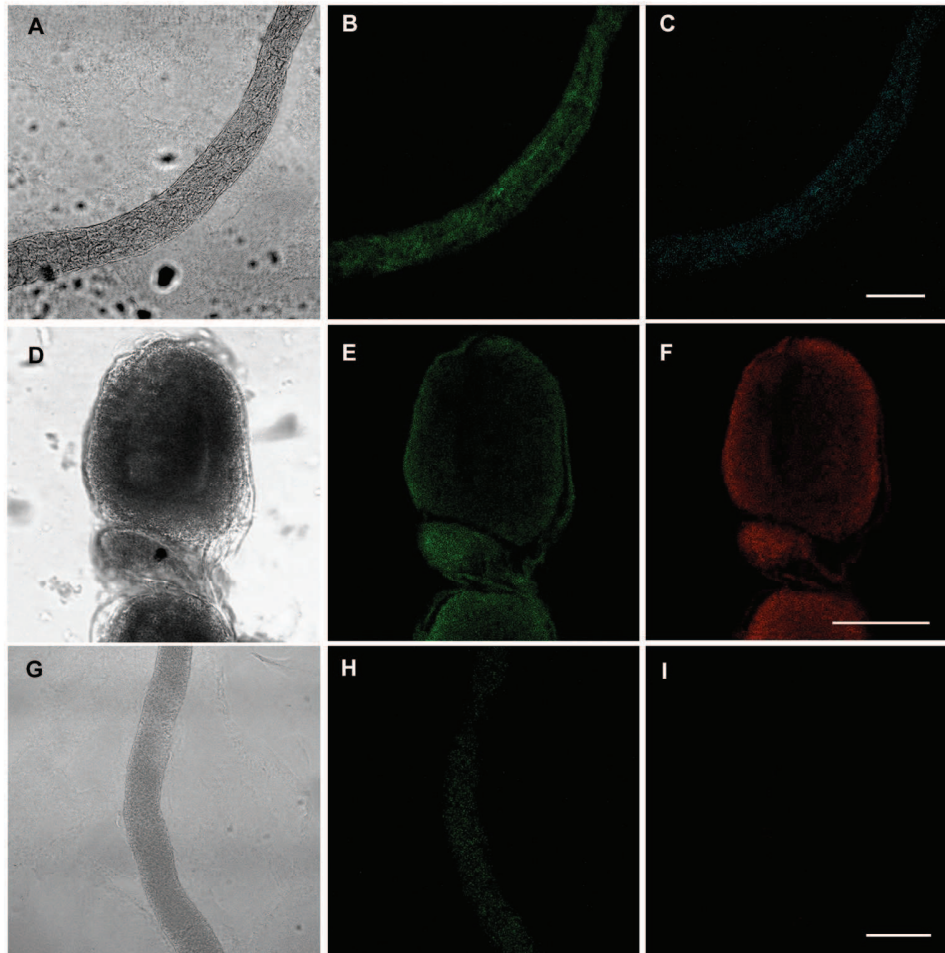
	IAP 1 vs. mean (IAP 2, IAP 3)			IAP 2 vs. IAP 3		
	$\chi^2$	df	P	$\chi^2$	df	P
ST qPCR	12.957	1	0.00	6.736	1	0.01
BB qPCR	2.078	1	0.15	29.597	1	0.00

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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)