



Research Article

Host preferences of the leafhopper *Scaphoideus titanus*, vector of “flavescence dorée” phytoplasma

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Abstract

The spread of “Flavescence dorée” (FD) phytoplasma among grapevines is made by the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae). Laboratory choice tests were performed to better understand insect vector preferences. The host preferences of nymphs and adults were compared between an experimental host plant, broadbean, and its natural host plant, grapevine, and between healthy and FD-infected plants. Grapevine was preferred to broadbean by nymphs and adults, but this preference was influenced by feeding experience. The presence of FD phytoplasma in plants made FD-infected grapevines more attractive than the healthy ones to both instar stages tested, while the preference for infected broadbean over healthy ones was only observed in adults. Plant yellowing due to the phytoplasma may increase the attractiveness of FD-infected plants. The experimental results showed that *S. titanus* was more attracted to yellow cues than to green, blue, or red ones. The attractiveness of FD-infected grapevines to the vector could be an efficient way to enhance disease spreading by increasing the proportion of infective vectors in the population.

Keywords: *Vitis vinifera*, *Scaphoideus titanus*, grapevine yellows; phytoplasma; feeding preference

Introduction

One of the challenges faced by modern agriculture is a dramatic increase in vector borne plant diseases (Almeida, 2008). Vector-pathogen relationships are important components of the epidemiology of vector-borne plant diseases, but the trophic interaction between plant and vector, *i.e.*, the ability of phytoplasmas to invade plants and to be transmitted by insects, are also critical steps in the evolution of a plant-phytoplasma-insect system. The transmission of a vectored pathogen from an infected to a healthy plant depends on a defined sequence of events mediated by stimuli from the host plant and inducing particular vector behaviors, like landing, leaving, and probing (Perring *et al.*, 1999). The transmission ability of an insect to propagate a disease, depends upon five main variables which are the vector density, the probability of the vector feeding on a host, including all behaviors related to feeding, the probability

of vector survival, the duration of the incubation period (IP), the vector competence, and the longevity of a vector life after incubation period (Chuche and Thiéry, 2014a; Cook *et al.*, 2008). Thus, knowledge of the probability of the vector feeding on a host, is critical to understanding the epidemiology of vector borne diseases and, therefore, to develop sound control strategies.

Since more than 50 years, European viticulture faces a serious yellows disease that is the “flavescence dorée” (FD), associated with the presence of phytoplasmas in the 16SrV-C and -D subgroups (Daire *et al.*, 1993; 1997a; Martini *et al.*, 1999; Angelini *et al.*, 2001). This disease is transmitted from and to grapevine by an insect vector only feeding on Vitaceae, the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Schvester *et al.*, 1961). Even though *Oncopsis alni* (Hemiptera: Cicadellidae) and *Dictyophara europaea* (Hemiptera: Dictyopharidae) have been recently shown to transmit FD-related phytoplasmas to grapevine from *Alnus*

glutinosa and *Clematis vitalba*, respectively (Filippin *et al.*, 2009; Maixner *et al.*, 2000), *S. titanus* remains the only known vector able to transmit the disease from one grapevine plant to another.

The often huge populations of *S. titanus* occurring in most European vineyards cause a severe epidemiologic concern (Boudon-Padieu, 2002; Chuche and Thiéry, 2009). The epidemic depends upon the movement of *S. titanus* from one host plant to another that is conditioned by several factors, including the life-history traits of the insect, its plant host range and host preferences, the availability and condition of these hosts, and their status as pathogen reservoirs (Carter, 1961). Previous studies on the spread of FD investigated life-history traits linked to the aggregative-like dispersal behavior of adults and nymphs (Bosco *et al.*, 1997; Chuche *et al.*, 2011; Lessio and Alma, 2006) and the flight activity of adults (Beanland *et al.*, 2006; Lessio and Alma, 2004a; 2004b; Pavan *et al.*, 2012). However, the importance of host plant characteristics on the vector's choice of a feeding site has received poor attention (Chuche and Thiéry, 2014a).

In order to understand factors leading host plant choice by *S. titanus*, role of host-plant species, infection status and color attractiveness were investigated. Nymphs and adults behaviors were compared to study potential shift in host preference due to developmental instar stage.

Materials and methods

Leafhoppers

Two-year-old grapevine woody canes of Pinot Noir cultivar carrying *S. titanus* eggs were collected after pruning during winter 2010, following Chuche and Thiéry methodology (2009) in an organic vineyard in Burgundy (46°45'27.31"N 4°41'23.95"E) where sizeable populations of the leafhopper had been observed for successive years and during the preceding summer. After collection, the canes bearing eggs were stored in a cold room at 5 ± 1°C and 85–90% relative humidity (RH) until use. Eggs were hatched by placing wood pieces (20–25 cm long) inside plastic hatching cages (50 × 38 × 36 cm) in a climatic chamber under a 16:8 L:D photoperiod, at 23 ± 1°C and 65–70% RH. To avoid desiccation of the eggs, the wood pieces were placed in a 1 cm layer of vermiculite (Efisol, Strasbourg, France) that was humidified with distilled water every 7 days. To harvest neonate nymphs, six leaves of healthy Cabernet-Sauvignon cultivar cuttings maintained in a glass tube with water, were added to the cages 20

days after the eggs were placed at 23 ± 1°C. Leaves were replaced as soon as they began to wither. Every day, nymphs were gently removed from the lower leaf surfaces with a mouth aspirator and either used in behavioral experiments or placed into cages with healthy Cabernet-Sauvignon cuttings until they reached the adult stage.

Euscelidius variegatus specimens were used to propagate the phytoplasma strain and to produce FD-infected broadbean. All insects were maintained in a growth chamber (23 ± 1°C, 16:8 hours, light/dark). Healthy colonies of *E. variegatus* were maintained on maize inside cubical cages according to Caudwell and Larrue (1977). They were periodically collected as fourth- and fifth-instar nymphs or young adults and fed on broadbean to acquire and transmit FD phytoplasma.

Test plants

Two plant species were used, grapevine, the host plant of FD phytoplasma and *S. titanus*, and broadbean (*Vicia faba* L.), the experimental host plant used to study FD phytoplasma (Caudwell *et al.*, 1972). Grapevine and broadbean were employed since both allow a complete development of *S. titanus*, both are hosts of FD phytoplasma and *S. titanus* can inoculate and acquire FD phytoplasma in these plants. The FD 92 strain (Angelini *et al.*, 2001) maintained in broadbean through serial transmission by the leafhopper *E. variegatus* (Caudwell and Larrue, 1977) was employed as phytoplasma source. Broadbean was grown as potted seedlings while the grapevine cultivar Baco 22 A was propagated as healthy cuttings. FD-phytoplasma was propagated from infected to healthy broadbean with infective *E. variegatus* (Caudwell *et al.*, 1972). FD grapevine was obtained using *S. titanus* that became infected on FD broadbean and transmitted the disease to healthy grapevine cuttings. Phytosanitary status of plants was both checked by presence of symptoms, and by PCR tests carried out as described below.

Host plant choice

Test choice to compare the attractiveness of grapevine with the one broadbean was carried out to verify the specificity of this leafhopper to *V. vinifera*. With the aim to determining if the choice made by *S. titanus* could be modulate by previous feeding experience, the preference between grapevine and broadbean of nymphs fed during 15 days on broadbean was also tested. To evaluate the effect of the presence of phytoplasma on *S. titanus* host plant choice, choices

between healthy and diseased plants, from the same species or not were proposed. Except for nymphs fed 15 days on broadbean, all the tests were made with neonate nymphs or adults reared on grapevine.

The *S. titanus* preferences for different host plants with similar traits were studied using choice tests between four plants (2 repeats 2 conditions) that were placed in each corner of an Altuglass® (Arkema, Nanterre, France) cage (60×60×60 cm). Plants about 30 cm height, and with similar architecture among the same species were selected for the experiment. From 30 to 75 nymphs or adults, depending on insect availability, were placed in the center of the cage equidistant from each plant. The number of *S. titanus* used in each test was low in order to avoid dispersal behaviors due to overcrowding. To determine the time required for an effective choice, *i.e.* when all insects had chosen a plant for feeding and there was very little movement between plants, preliminary observations of host-choice dynamics during a 24-h period were made. These experiments showed that most insects selected their feeding site within a few hours after they were released into the cage, and remained there for several hours. Thus, a 9-h period for feeding choice observations was selected to be able to conduct the experiment during the daytime. At the end of the experiment, the number of individuals on each plant was counted and the insects were removed. All experiments were performed in a climatized chamber with artificial light at 23 ± 1°C and 45–50% RH. After each choice test, plant positions were changed to avoid position effects. Six repetitions were made for each test, for a total number of insects ranging between 104 and 400.

Visual cues

Visual stimuli are generally important cues for Hemiptera. To test which colors attracted nymphs, one first-instar nymph was individually enclosed in a Petri dish (Ø8.5 cm) placed on a dark surface with four colored 2.2 cm squares — blue (Red Green Blue (RGB): 0, 0, 255), green (46, 208, 38), yellow (255, 255, 0), and red (255, 0, 0) — placed at equal distances from the center. Green was chosen as a generalist visual signal linked to plant, red and yellow as symptoms of FD on leaves, and blue that is not representative of grapevine, as a control. Nymphs that settled for at least 15 seconds on one colored surrogate were scored as having performed a choice. Individuals that did not express a choice after 15 minutes were counted as ‘no choice’. All experiments were performed in a single climate chamber under

controlled conditions as described in the leafhoppers section. After each trial, the colored squares were randomly rotated to avoid any position effect. One hundred twenty nine individual of *S. titanus* were tested.

Phytoplasma detection

The presence of phytoplasmas in symptomatic grapevine and broadbean plants was checked after choice tests by amplifying phytoplasma DNA with nested PCR. Primers and reaction conditions according to Clair *et al.* (2003) were used. Total DNA was extracted from vascular tissues of grapevine and broadbean lead veins. One gram of tissue was crushed in 10 mL of extraction buffer (3% cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, and 0.2% 2-mercaptoethanol) then heated in a water bath for 20 min at 65°C. An equal volume of chloroform was added, and the mixture was centrifuged before collecting the aqueous phase. The nucleic acids were pelleted using isopropanol precipitation and centrifugation. The pellet was washed with 70% ethanol, dried in vacuum, and dissolved in 150 µl of a buffer containing 10mM Tris, 1 mM EDTA, pH 8. The nested PCR took place in a 20 µL volume containing 0.375 µM each primer, 0.150 µM each dNTP, 1 µM MgCl₂, Taq buffer (Appligene, Strasbourg, France), and 1 U/100 µl of Taq DNA polymerase (Appligene). Five to ten nanograms of template DNA were added for the first amplification. The template was predenatured at 92°C for 90 seconds, followed by 30 cycles of denaturation at 92°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 72°C for 70 seconds. One microliter of the diluted (1/100) amplification product was amplified for 35 cycles with the second pair of primers in the same conditions.

For each set of amplifications, FD92-infected broadbean DNA was used as positive control and healthy broadbean DNA as a negative control. Two negative controls were used for each series of 24 specimens tested with PCR. Five microliters of the final product were electrophoresed in a 1.2% agarose gel, stained with Gelstar (Cambrex, East Rutherford, NJ, USA), and visualized under UV light.

Statistical analyses

Host-plant preferences of leafhoppers were compared using the Wilcoxon signed rank test for paired comparisons. The visual cue experiment provided binomial data. Thus, the choices among the four colors were assessed with the Cochran Q test. To determine which colors differed from each other in attractiveness,

all pairwise combinations of Wilcoxon signed rank test with Bonferroni correction was run. All statistical analyses were performed using R software version 3.0.0 (R Development Core Team, 2007).

Results

Preferences between healthy plants

Neonate nymphs did not exhibit a preference between broadbean and grapevine ($Z = 1.57$; $P = 0.12$; Figure 1), while adults preferred the grapevine ($Z = 2.20$; $P < 0.05$; Figure 2). Interestingly, nymphs that previously fed on broadbean for 15 days behaved like adults and preferred grapevine over broadbean ($Z = 2.20$; $P < 0.05$; Figure 1).

Preferences between healthy and FD plants

Phytoplasma symptom presence made both broadbean ($Z = 1.99$) and grapevine ($Z = 2.02$) plants more attractive to adults ($P < 0.05$; Figure 2). Although FD-infected grapevine was more attractive to nymphs than healthy grapevine ($Z = 2.20$; $P = 0.03$; Figure 1), FD-infected and healthy broadbeans were equally selected ($Z = 0.10$; $P > 0.5$; Fig. 1) by nymphs. However, in choice tests between broadbean and grapevine, healthy grapevine was preferred to diseased broadbean by both nymphs and adults (both $Z = 2.20$; $P < 0.05$; Figures 1 and 2).

Visual cues

Yellow was the most attractive color for *S. titanus* nymphs, followed by green, and then blue and red, which were similarly attractive (Table 1).

Table 1. Choice of *S. titanus* nymphs among visual cues.

		N	%	Q	P
Color choice	Tested individuals	129			
	No choice	16	12.40		
	Choice made	113	87.60		
	Yellow	64	56.64	a	77.761 < 0.001
	Green	34	30.09	b	
	Blue	11	9.73	c	
	Red	4	3.54	c	

Discussion

The experiments carried out demonstrated that the presence of FD symptoms, linked to phytoplasma presence, in a plant could modify the *S. titanus* preferences and that the choice between healthy or FD-infected plants depended on the developmental instar of the vector. Feeding experience on a plant also influenced preference suggesting that visual colored cues could be used in between or within host-plant choice by *S. titanus*.

Naive nymphs did not demonstrate a preference between broadbean and grapevine. However after

Figure 1. Choice of *S. titanus* nymphs between the two host plants. *: $P < 0.05$; #: *S. titanus* previously fed for 2 weeks on broadbean.

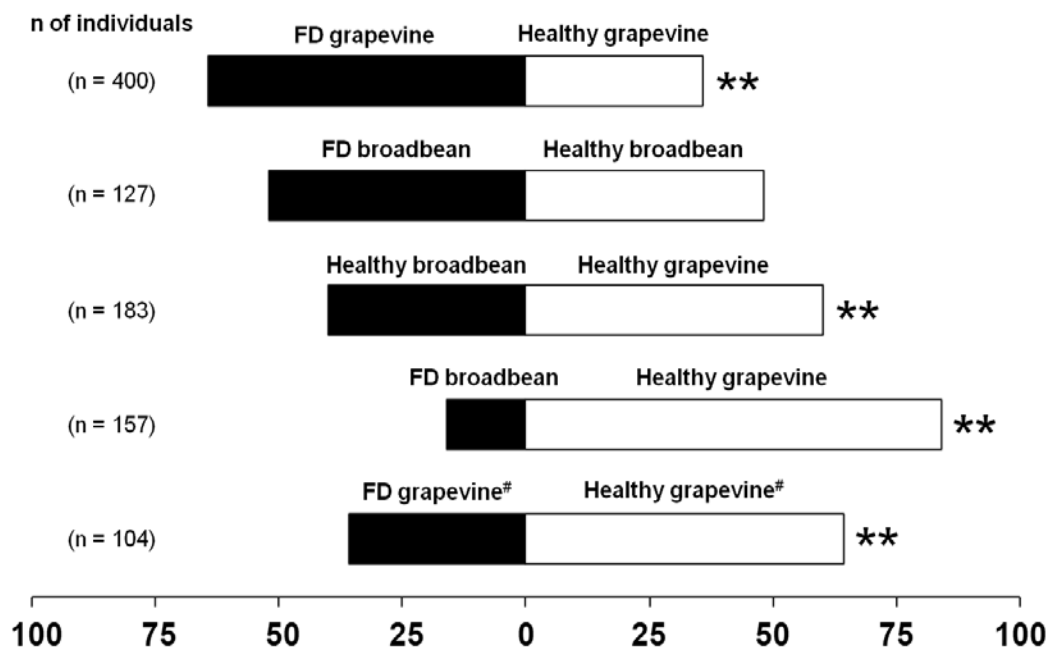
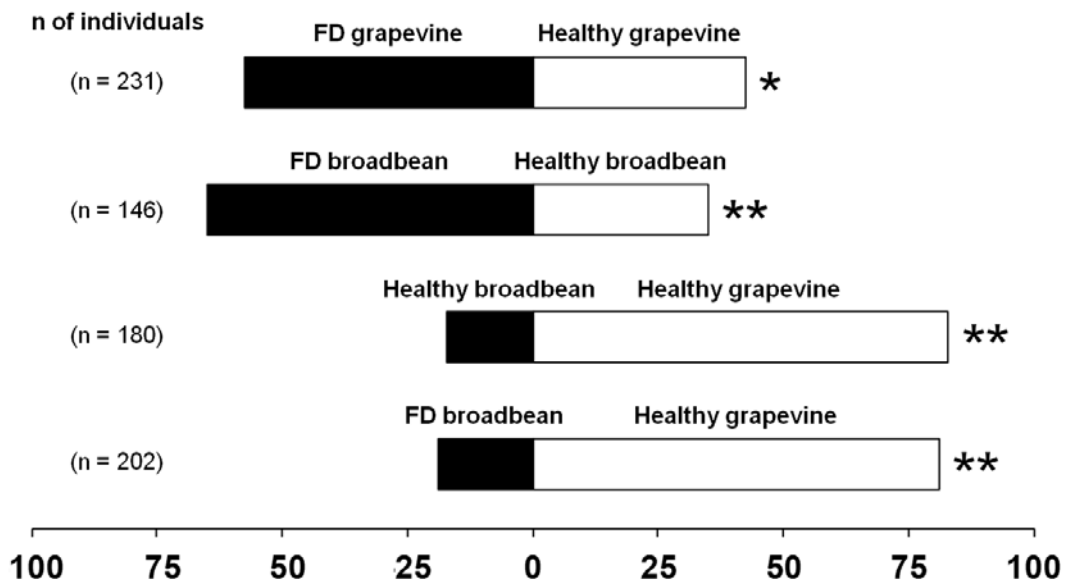


Figure 2. Choice of *S. titanus* adults between the two host plants. *: $P < 0.05$.



feeding on broadbean for 15 days, they preferred grapevine, their natural host plant, rather than broadbean. Nymphs made a choice between the two plants only after experiencing broadbean, which they then avoided. The results obtained could indicate that learning may play a role in *S. titanus* host-plant choice. This could represent a critical point for the FD spread by also affecting host plant preference (*e.g.* grapevine cultivar, infected/uninfected vine) and feeding behavior of vectors. Such capacity for learning was described in several mosquito vectors and could lead to increased disease transmission due to the avoidance of less suitable hosts (Chilaka *et al.*, 2012; McCall and Kelly, 2002). By choosing where they oviposit, females select where their offspring feed and can influence the host-plant choices of future generations, phenomenon described as Hopkins effect or natal host plant induction (Davis and Stamps, 2004; Moreau *et al.*, 2008). Another explanation for this difference in plant preference could be the consequence of a lack in the diet of nymphs fed on broadbean. Broadbean induces a high nymph mortality rate (J. Chuche, personal observation), so this plant is not optimal for *S. titanus* development and maybe do not provides all the required nutrients and/or not in sufficient amounts. Polyphagous and oligophagous insect herbivores can regulate their nutrient intake by feeding on different plants (Behmer, 2009). *S. titanus* is often described as a specialist of *Vitis* species. This genus is the only one on which eggs were found and constitute the host plant (Chuche and Thiéry, 2014a). But *Vitis* species is not the

only plant genus on which *S. titanus* can feed. This leafhopper can diversify its diet by probing both in phloem and xylem. Some studies revealed that *S. titanus* can be found on other plants outside the *Vitis* genus that supported its survival or its development (Schvester *et al.*, 1962; Caudwell *et al.*, 1970; Chuche and Thiéry, 2014b; Trivellone *et al.*, 2013). Thus broadbean could not provide the optimal amount and blend of nutrients for the leafhopper and individuals reared on this species turned to grapevine to improve their nutrient intake.

The attractiveness of FD plants may be correlated to the leaf yellowing induced by the disease. Indeed, in these experiments, yellow was the most attractive color for *S. titanus*. This color is also well known to be very attractive for many insect species, especially for sap suckers like leafhoppers (Prokopy and Owens, 1983; Todd *et al.*, 1990a). As previously shown (Chuche *et al.*, 2011; Mazzoni *et al.*, 2011), yellow was the most attractive to nymphs and adults of *S. titanus* in the field, while red was most attractive to females in a laboratory experiment (Mazzoni *et al.*, 2011) and for males in a vineyard study (Lessio and Alma, 2004a). Thus, color perception depends on environmental conditions, especially light exposure, but color attractiveness to *S. titanus* is not fully understood and would require further investigation.

Phytoplasma diseases not only alter plant color, but also modify plant physiology (Christensen *et al.*, 2005) and could affect their attractiveness to insect vectors. This was the case with '*Candidatus* Phytoplasma mali', which changed the odor, in particular the amount of

the volatile organic compound β -caryophyllene, of its host plant, *Malus domestica* (Mayer *et al.*, 2008). These modifications made the infected plant more attractive to one of the phytoplasma vectors, the psyllid *Cacopsylla picta*. Even if *S. titanus* can detect *Vitis* leaves and shoot odours (Mazzoni *et al.*, 2009), this insect has a reduced number of olfactory receptors and sensory neurons *per sensillum* as compared with other leafhoppers (Stacconi *et al.*, 2014) which is contradictory with the hypothesis of a strong role of the odors in host choice. Greater attractiveness of infected plants – the pathogen source – could result in more infective insect vectors within the population. FD spreads very rapidly in the absence of vector control (Chuche and Thiéry, 2014a). One explanation for the epidemic spread could be an alteration of this vector behavior by phytoplasma. Indeed, parasites often alter the behaviors of their hosts and vectors to benefit themselves (Hurd, 2003). If uninfected vectors are attracted to diseased plants, thus the proportion of infective vectors in the population would increase. However, the incidence of such attraction on the epidemic spread would also depend on the displacement capacities of infective vectors. However, because of quarantine constraints, the dispersion capacities of infective leafhoppers could not be tested.

Plant color is a basic cue in leafhopper and aphid host-plant selection (Prokopy and Owens, 1983; Todd *et al.*, 1990b; Bullas-Appleton *et al.*, 2004; Powell *et al.*, 2006). However, yellowing broadbeans resulted less attractive than healthy grapevine to adults and nymphs *S. titanus*. Moreover, FD infected broadbeans were not preferred to the healthy ones by nymphs. These results reinforce the hypothesis that, in addition to color changes, phytoplasmas can induce metabolic modifications that affect vector attraction. Phytoplasmas alter the expression of genes implicated in stress responses and in metabolic pathways, and host plant-phytoplasma interactions vary with plant and phytoplasma species (Carginale *et al.*, 2004; Hren *et al.*, 2009). These interactions could explain the differences in *S. titanus* attraction to FD-infected grapevines and FD-infected broadbeans. Recent studies began to provide data on the grapevine metabolic response to phytoplasma occurrence that could help to better understand the relationship between the vector and infected/uninfected plants (Abbà *et al.*, 2014; Margaria and Palmano, 2009; 2011).

Increased knowledge on the relationships between *S. titanus* and its host plant is needed. Future studies should explore the link between vector feeding

behaviour and grapevine cultivars to understand the mechanisms of plant susceptibility to FD disease as an important clue in controlling this disease.

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