Insect Injection and Artificial Feeding Bioassays to Test the Vector Specificity of Flavescence Dorée Phytoplasma

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ABSTRACT

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The specificity of vector transmission of Flavescence dorée phytoplasma (FDP) was tested by injecting FDP, extracted from laboratory-reared infective *Euscelidius variegatus*, into specimens of 15 other hemipteran insect species collected in European vineyards. Concentrations of viable phytoplasma extracts and latency in vectors were monitored by injection of healthy-reared *E. variegatus* leafhoppers. Based on these preliminary results, insects were injected by using phytoplasma extracts that ensured the highest rate of FDP acquisition and transmission by *E. variegatus*. Transmission into an artificial diet through a Parafilm membrane about 3 weeks after insect injection was attempted. FDP-injected insects that

belonged to 15 hemipteran species were confined in cages and fed through the membrane for a 4- to 5-day inoculation access period. FDP DNA was detected by polymerase chain reaction (PCR) in the feeding buffer fed upon by *Anoplotettix fuscovenosus*, *Aphrodes makarovi*, *E. variegatus*, and *Euscelis incisus*. PCR amplification with specific primers detected FDP DNA in injected insects of all test insect species. Band intensity was positively correlated with the transmissibility of FDP. Transmission of FDP to plants by feeding was confirmed for *Anoplotettix fuscovenosus*, *E. variegatus*, and *Euscelis incisus*, but not for *Aphrodes makarovi*. Our results suggest that vector competency of FDP is restricted to specimens belonging to the family Cicadellidae, subfamily Deltocephalinae.

Additional keywords: grapevine yellows, Hemiptera, planthoppers.

Flavescence dorée (FD), a serious disease of the grapevine (*Vitis vinifera* L.) in temperate European areas, is associated with phytoplasmas that belong to the elm yellows (EY) group or 16SrV group (21,33,47) and are transmitted naturally by the leafhopper *Scaphoideus titanus* Ball (Hemiptera, Cicadomorpha, Cicadellidae) (45).

Flavescence dorée phytoplasma (FDP) seems to have a high degree of vector specificity similarly to other phytoplasmas clustered in the EY or 16SrV group (3,11,22,38). Nevertheless, Caudwell et al. (17) showed that leafhoppers *Euscelidius variegatus* Kirschbaum and *Euscelis plebejus* Fall (Hemiptera, Cicadomorpha, Cicadellidae) were able to experimentally transmit FDP to herbaceous host plants although they could not acquire the phytoplasma from infected grapevines. Later, the same author utilized *E. variegatus* as an alternative vector of FDP to maintain the phytoplasma in an experimental biological cycle (15).

The transmission capabilities of plant pathogens by arthropods may vary along a spectrum that ranges from complete incompetence to high efficiency (41). Commonly, approaches to determining vector specificity of phytoplasmas are based on transmission assays, by confining either wild insects or insects exposed for acquisition on diseased plants, to experimental host plants. The use of diagnostic methods, such as enzyme-linked immunosorbent assay or polymerase chain reaction (PCR), could help in detecting uncultivable pathogens in both vectors and experimentally infected plants (9,37). Transmission ability by one species must take into account experimental conditions that can affect

transmission results significantly (41). For example, host plant species used for acquisition and/or inoculation may greatly influence the success of pathogen transmission by vector species (7).

Injection is a potential useful tool for searching for insect vectors (12,16,53). This technique was first used by Black (5) to deliver infectious phytoplasma extracts into leafhoppers, using very thin glass needles. The method has some important advantages in studying vector transmissibility by prokaryotes if compared with feeding tests. In fact, injection into insect hemocoel increases the transmission efficiency by vectors by increasing the rate of phytoplasma acquisition and reducing latency (46,54). It is possible to test insects with different feeding habits or insects that have different host plant preferences and that could not feed well on the source plants used. Therefore, injection suppresses the effects of host plant–vector interactions in the acquisition process. Limitations of the technique are the requirement of a source of viable phytoplasmas, the high mortality that may occur of injected insects and a substantial amount of tedious work (29). Also, this technique essentially tests the ability of a pathogen to overcome the barriers of the salivary glands (23,40), although it does not provide information about the ability of the pathogen to overcome the gut barrier which is a first important step necessary for pathogen acquisition by feeding on source plants (23). Therefore, the injection technique should be associated to feeding acquisition assays. Nevertheless, if one insect species is not able to transmit a pathogen after injection, presumably it should not be able to transmit the pathogen after feeding acquisition on diseased plants.

Another interesting approach recently developed (49,57) and successfully applied utilizing several Hemiptera species (4,27,44) is the use of artificial feeding assays to test for the inoculative status of candidate insect vectors. This method allows a significant reduction of time if compared with transmission to host

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plants, considering the time necessary for symptom expression in inoculated plants (incubation period). In addition, the technique seems to increase the efficiency of vector transmission when compared with feeding transmission to host plants (27).

Our goal in the present study was to determine the vector specificity of FDP in some hemipteran insect species. We selected those species that occur commonly in European vineyards and that could be potentially involved with spread of FDP, other species were selected because they are recognized vectors of phytopathogenic mollicutes different to FDP. We studied vector transmission ability by injecting FDP phytoplasma directly into the hemocoel of insects and vector inoculativity status using artificial feeding bioassays, attempting to minimize the effects of plant-insect interactions during both acquisition and transmission processes. We further confirmed FDP transmission ability by vector feeding. Our research suggests that vector competency of FDP is restricted to leafhopper species (Hemiptera, Cicadomorpha) belonging to the family Cicadellidae, subfamily Deltocephalinae.

MATERIALS AND METHODS

Host plants. Healthy plants were produced to supply suitable hosts for field-collected and reared leafhoppers and planthoppers. Maize (*Zea mays* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), broadbean (*Vicia faba* L.), nettle (*Urtica dioica* L.), sugar beet (*Beta vulgaris* L.), and wallflower (*Cheiranthus cheiri* L.) were produced as potted seedlings. Grapevine (*Vitis vinifera* L.), European privet (*Ligustrum vulgare* L.), and mugwort (*Artemisia vulgaris* L.) were propagated as healthy cuttings.

Collection of insects and establishment of colonies. Leafhopper and planthopper specimens (Table 1) used in this study were collected with a D-vac or a sweep-net in grape-growing areas located in the Mosel Region (Germany), Burgundy Region (France), and Veneto Region (Italy), or reared and provided by specialized laboratories (Table 1). Insects were caged on suitable host plants (Table 1), univoltine species were directly used for injection assays, while plurivoltine species were reared for at least one generation and the progeny was used in injection assays (Table 1). Healthy colonies of *E. variegatus* were maintained on maize inside cubical cages according to Caudwell and Larrue (18). They were periodically collected as fourth- and fifth-instar nymphs or young adults for injection assays and for feeding

acquisition and transmission to maintain phytoplasma strains. All the insect species were maintained in a growth chamber ($23 \pm 1^\circ\text{C}$, 16-h light/8-h dark).

FDP source and production of infective *E. variegatus*. The FDP strain FD92 (16SrV-D) was used for all the assays. The latter strain is widespread in France, it was also identified in Spain (21) and it cannot be distinguished from the Italian strain FD-D (2,21,39) which is also widespread in Italy. FD92 has been maintained in the greenhouse and climatic insectary through uninterrupted serial cycles of broadbean-to-broadbean transmission using *E. variegatus* as an alternative vector (17). To produce infective *E. variegatus*, young nymphs were allowed to feed on FD-infected broadbeans for an acquisition access period (AAP) of 2 weeks. Insects were successively transferred to healthy maize. This procedure ensured that almost the totality of insects were FDP-infected. Infective insects were used to inoculate 15-day-old broadbean seedlings after 35 to 40 days from the beginning of the AAP, when latency in most of the infected leafhoppers was over (9,34).

FDP transmission by insect injection and artificial feeding bioassays. *FDP extraction from E. variegatus.* FDP was extracted from infected adults of *E. variegatus* taken 42 to 56 days from the beginning of AAP. Groups of 30 insects were gently crushed in a tissue grinder with 900 μl of 0.3 M glycine buffer and 0.03 M MgCl_2 , pH 8 (53). The homogenate was clarified by centrifugation for 10 min at $800 \times g$. The collected supernatant was filtered through 0.45 μm sterile filters. All the above steps were performed at 4°C . The phytoplasma suspension was maintained on ice in 0.5-ml tubes and was used within 3 h of extraction.

Injection. FDP suspensions were delivered into insects by using glass needles extruded by a needle-puller device. The needle was connected to a 40-cm-long rubber tubing. FDP suspension was drawn up from the tubes by suction and expelled by gentle pressure using mouth aspiration. When nymphs or small size insects were injected, a procedure described by Purcell (42) was adopted to immobilize insects. Briefly, groups of 6 to 10 insects were anesthetized with carbon dioxide for about 50 s. Then they were put in an ice-cold plate positioned under a stereomicroscope, and they were gently held with a fine forceps to permit abdominal injection. Alternatively, fourth- and fifth-instar nymphs or young adults of *E. variegatus* were placed into a polypropylene shallow, flat-faced piston, over which was fitted a cylindrical sleeve closed at one end with thinly stretched Parafilm (13). Insects introduced

TABLE 1. Hemiptera species used for injection assays to study vector specificity of Flavescence dorée phytoplasma (FDP)

Species	Family	Voltinism ^a	Origin	Host plants	Developmental stage for injection ^b
<i>Agallia consobrina</i> Curtis	Cicadellidae	P	Germany	Broadbean	Nymphs
<i>Agalmatium flavescens</i> (Olivier)	Issidae	U	Italy	Mugwort	Adults
<i>Anoplotettix fuscovenosus</i> (Ferrari)	Cicadellidae	U	Italy	Broadbean, grapevine	Nymphs
<i>Aphrodes makarovi</i> Zachvatkin	Cicadellidae	U	Italy	Mugwort	Nymphs
<i>Circulifer haematoceps</i> (Mulsant & Rey) ^c	Cicadellidae	P	Reared	Wallflower	Nymphs-adults
<i>Euscelidius variegatus</i> Kirschbaum (wild type) ^d	Cicadellidae	P	Italy	Barley	Nymphs-adults
<i>Euscelis incisus</i> Kirschbaum ^c	Cicadellidae	P	Reared	Oat, barley broadbean	Nymphs
<i>Fieberiella florii</i> (Stål) ^c	Cicadellidae	P	Reared	European privet	Nymphs
<i>Hyalesthes obsoletus</i> Signoret	Cixiidae	U	France	Nettle	Nymphs-adults
<i>Laodelphax striatellus</i> (Fallén)	Delphacidae	P	Italy	Barley	Nymphs
<i>Metcalfa pruinosa</i> (Say)	Flatidae	U	Italy	Nettle, grapevine	Nymphs-adults
<i>Pentastiridius</i> sp.	Cixiidae	U	France	Sugar beet	Adults
<i>Phyllaenus spumarius</i> (Linneus)	Cercopidae	—	France	Grapevine	Adults
<i>Psammotettix</i> sp.	Cicadellidae	P	Italy	Barley, oat	Nymphs
<i>Stictocephala bisonia</i> (Kopp & Yonke)	Membracidae	U	Italy	Nettle	Adults

^a U = univoltine, Hemiptera species known to complete one generation per year. P = plurivoltine, Hemiptera species known to complete more than one generation per year.

^b Generally, fourth- or fifth-instar nymphs were used or young adults if necessary.

^c *C. haematoceps*, *Euscelis incisus*, and *F. florii* species were provided by X. Foissac (INRA Bordeaux, France), A. Alma (DIVAPRA, University of Torino-Italy), and M. Maixner (BBA, Bernkastel-Kues, Germany), respectively.

^d A new population of this leafhopper species was established. It is here referred to as *E. variegatus* "wild type" to distinguish from laboratory-reared population used to maintain the phytoplasma strains (control).

into the sleeve were immobilized between the piston and the Parafilm membrane by gently lowering the sleeve over the piston and they were then injected through the Parafilm. The latter device negated the effect of CO₂ on test insects. About 0.5 µl of FDP suspension was injected into each leafhopper. Injected insects were transferred to suitable healthy host plants (Table 1).

Viability of FDP extracts. To test the viability and concentration of phytoplasma cells on FDP extracts, those were serially tenfold diluted up 10⁻⁴ with 0.3 M glycine buffer and 0.03 M MgCl₂, pH 8. The undiluted extract, the four successive dilutions and extraction buffer only as a negative control, were injected into groups of 50 to 60 healthy young males of *E. variegatus* since they are more efficient than females in the acquisition of FDP by injection (32). Injected leafhoppers were caged in groups of 5 to 10 specimens on healthy broadbean seedlings and transferred weekly to new broadbean seedlings. After three serial passages on broadbean (21 days after injection), leafhoppers were caged individually for an inoculation access period (IAP) of 7 days on healthy broadbeans to test the rate of FDP transmission, and then they were collected and stored in tubes at -80°C until their use for phytoplasma detection. Because some groups of injected leafhoppers underwent a high mortality postinjection, the experiment was repeated three times to ensure a sufficient number of inoculative insects. Inoculated broadbeans were sprayed with Dichlorvos (0.5 g/liter) (Bayer) and placed in a greenhouse (25 ± 3°C and natural light) and maintained for symptom development.

Bioassays to test FDP transmissibility by Hemiptera species. On the basis of results of preliminary injection assays using *E. variegatus* as a model leafhopper vector (Table 2), we selected the undiluted FDP extract to ensure a high rate of pathogen acquisition by needle-injection of other Hemiptera species. When insect specimens were small we nevertheless injected at least 0.05 µl, which contained enough phytoplasma cells to ensure about 90% of FDP acquisition by the competent vector *E. variegatus* according to data presented in Table 2. Whenever possible, we allowed at least 20 days of incubation after injection before performing the inoculativity assays.

Positive controls were healthy *E. variegatus* injected in groups of 35 to 50 individuals with the same FDP extracts delivered into the tested insect species. Injected insects were transferred into cages with suitable host plants (Table 1) in the growth chamber and were supplied with fresh host plants whenever necessary. Transmission testing of injected insects was performed using an artificial feeding bioassay with a method similar to that described by Zhang et al. (57). Insects collected from rearing cages at the end of incubation were confined in groups of three in 3-cm-diameter cages consisting of two identical parts from two, 3-cm-diameter petri dishes. Two round holes were pierced in the first dish: the first hole (2.5-cm diameter) created on the lid of the dish

was overlaid with a 100 µm mesh nylon tissue to allow ventilation in the chamber; the second was a small lateral hole sealed with a sponge pad, allowing the release and collection of insects inside or outside the chamber. The external part of the second dish, built with a 2-mm-thick edge, was filled with 680 µl of TE (10 mM Tris and 1 mM EDTA, pH 8) enriched with 5% sucrose (TE-sucrose) and sealed with stretched Parafilm. The first dish was assembled on top of the second to create a cylindrical insect cage ventilated on one side and allowing access to the feeding diet on the other side. Insects were confined in the cages for an IAP of 4 days in the growth chamber. On completion of IAPs, dead insects were counted and all insects were stored in tubes at -80°C for further phytoplasma detection assays. The artificial feeding buffer was gently aspirated with a pipette after removal of the Parafilm membrane and immediately processed for FDP detection. For each transmission assay, in addition to the particular Hemiptera species tested and control injected *E. variegatus* batch, we considered as a control of transmission to the feeding buffer, groups of infective *E. variegatus* that had acquired FDP by feeding on diseased broadbeans and a group of healthy *E. variegatus*. All the latter control insects were confined in groups of three in artificial feeding cages, as were all other specimens.

FDP acquisition and transmission by feeding on broadbean.

We tested the ability of *Anoplotettix fuscovenosus* (Ferrari), *Aphrodes makarovi* Zachvatkin, *E. variegatus* (wild type), *Euscelis incisus* Kirschbaum, *Fieberiella florii* (Stål), and *Circulifer haematoceps* (Mulsant & Rey), previously tested by injection and artificial feeding assays, to acquire and transmit FDP by plant-feeding. Young nymphs of the above species were confined in groups of 60 to 130 insects on FDP-infected broadbeans for an AAP of 15 days. Insects were maintained on suitable host plants in rearing cages (Table 1) for an incubation period of at least 35 days. Leafhoppers were confined in groups of six to seven on 15-day-old broadbean seedlings for an IAP of 7 days. In addition, groups of FD-infective or healthy laboratory-reared *E. variegatus* were confined to other healthy broadbean seedlings as positive and negative controls, respectively. The leafhoppers were collected at the end of IAP and stored at -80°C in tubes until further phytoplasma detection. Inoculated broadbeans were sprayed with Dichlorvos as above and transported to a greenhouse and maintained for symptom development. About 45 to 50 days after inoculation, all broadbeans with or without symptoms used in the experiments were PCR-tested for FDP detection.

PCR detection of phytoplasma DNA in insects, plants, and feeding buffer. *DNA extraction.* DNA was extracted from the TE-sucrose buffer according to Zhang et al. (57). The sucrose solution was centrifuged at 12,000 × g for 15 min. Ten microliters of 0.5 N NaOH was added to the pellet followed by 20 µl of 1 M Tris-HCl buffer, pH 8, containing 1% sodium dodecyl sulfate and

TABLE 2. Flavescence dorée phytoplasma (FDP) transmission to broadbean seedlings by *Euscelidius variegatus* leafhoppers injected with five 10-fold dilutions of FDP extracts and rate of positive leafhoppers individually tested by polymerase chain reaction (PCR)

FDP extract dilutions	Symptomatic /insect exposed broadbeans								PCR positive/tested leafhoppers ^c		
	First week ^a		Second week ^a		Third week ^a		Fourth week ^b		Rate (%)		Density of PCR product ^d
	Rate	(%)	Rate	(%)	Rate	(%)	Rate	(%)			
Undiluted	0/9	(0)	0/9	(0)	6/6	(100)	21/35	(60)	33/35	(94)	+++++
10 ⁻¹	0/9	(0)	0/9	(0)	5/5	(100)	12/30	(40)	25/28	(89)	++++
10 ⁻²	0/9	(0)	0/9	(0)	3/6	(50)	2/42	(4.7)	19/45	(42)	++
10 ⁻³	0/9	(0)	0/9	(0)	0/6	(0)	0/46	(0)	12/46	(26)	+
10 ⁻⁴	0/9	(0)	0/9	(0)	0/7	(0)	0/45	(0)	3/40	(7.5)	+
Control ^e	0/3	(0)	0/3	(0)	0/3	(0)	0/9	(0)	0/9	(0)	-

^a Groups of 5 to 10 insects were confined per seedling for transmission.

^b One insect was confined per seedling for transmission.

^c The leafhoppers were individually assayed with PCR at the end of the fourth week of inoculation access period.

^d The density of PCR products on agarose gels is indicative of the relative intensities of bands compared with one another. +++++ correspond to the intensity of positive controls; -, negative (as for negative control).

^e Insects that were injected with buffer.

10 mM EDTA. The mixture was incubated at 65°C for 15 min, precipitated with 2 volumes of ethanol, and kept at -20°C for at least 30 min. DNA was precipitated by centrifugation at 12,000 × g for 20 min and resuspended in 30 µl of TE.

The procedure described by Gatineau et al. (26) was used to detect DNA phytoplasma in insects. Insects were tested individually except for some specimens submitted to feeding acquisition that were tested in groups of two individuals. DNA was extracted from broadbean using the procedure described by Daire et al. (21).

PCR and electrophoresis. Amplification was performed with a nested-PCR on the nonribosomal DNA fragment called FD9, which is specific for phytoplasma strains in the 16SrV-group. The first pair of primers was FD9f2/r, and the second pair of primers was FD9f3/r2; the latter pair would amplify a fragment of about 1,160 bp. PCR conditions were those described by Boudon-Padiou et al. (8). Five microliters of the nested amplification product was evaluated with electrophoresis in 1.2% agarose gel, which was then stained with ethidium bromide and visualized under UV light. For each amplification, positive and negative controls were considered; the positive control was *E. variegatus* taken from an FDP-infected colony, and the negative control was *E. variegatus* collected from a healthy colony. The intensities of bands in each agarose gel were compared one to the other and to those obtained with positive controls to reveal differences in FDP DNA concentration between injected specimens. We assigned to band intensity discrete values starting from very faint bands (+) to very intense bands (+++++).

Statistical analysis. Data on proportion of PCR-positive FDP-injected *E. variegatus* leafhoppers and data on proportion of infected broadbeans inoculated by PCR-positive injected *E. variegatus* leafhoppers calculated per each injected phytoplasma extract were plotted as a function of dilutions of FDP extracts and fitted with a binomial (logit) distribution by using a maximum likelihood criterion provided by Statistica software (Statistica [2004], Statsoft, version 6.1, Italy). The fit of the model was additionally tested by calculating the coefficient of determination.

RESULTS

FDP transmission assays by insect injection and artificial feeding bioassays. *Viability of FDP extracts, latency, and transmission by injected E. variegatus.* Table 2 shows the results of FDP transmission and detection by PCR with laboratory-reared injected *E. variegatus* leafhoppers. Latency in injected *E. variegatus* leafhoppers was at least 14 days after injection since phytoplasma transmission occurred from the third week after injection, confirming previous reports (14). Transmission during the third week (with groups of 5 to 10 insects) occurred to six of six tested seedlings confined with insects injected with the undiluted and 10-fold diluted FDP extracts, to three of six tested seedlings confined with insects injected with a 10²-fold diluted phytoplasma extract, and no transmission occurred with insects injected with other lower dilutions. FDP transmission by leafhoppers that were confined individually during the fourth week after injection occurred except for 10²-fold and lower dilutions. Logistic curves obtained with data of the fourth week of *E. variegatus* leafhoppers confined individually on broadbean (as reported in Table 2) showed a reduction in the percentage of infected (PCR-positive) leafhoppers and in transmission rate along with increasing dilution of phytoplasma extracts injected into the leafhoppers (Fig. 1).

FDP inoculativity assays by 15 Hemiptera species. Table 3 reports the results of FDP detection by PCR using FD-specific primers in artificial feeding buffer and in FDP-injected insects for all 15 Hemiptera species tested. The length of incubation on host plants, survival by the end of the latter period, and survival after exposure to artificial feeding buffer are also reported for all 15 species tested and *E. variegatus* leafhoppers used as control. In

9 of 15 Hemiptera species tested, survival after 20 days postinjection ranged between 30 and 50%. For *Aphrodes makarovi*, *Euscelis incisus*, and *Phyllaenus spumarius* species, survival of injected insects was greater than 60%. Injected specimens of *Hyalesthes obsoletus* and *Pentastiridius* sp. underwent a high mortality; therefore, we began the transmission assays 16 days after injection of these species. Insect survival after 4 days of feeding on artificial buffer was greater than 60% for 12 of 15 test species. The froghopper *Phyllaenus spumarius* did not survive. Poor survival was observed for *Psammotettix* sp. and for *L. striatellus* specimens too.

Anoplotettix fuscovenosus, *Aphrodes makarovi*, *Euscelis incisus*, and *E. variegatus* (wild type) were able to transmit FDP to the feeding buffer. *Anoplotettix fuscovenosus*, *Euscelis incisus*, and *E. variegatus* leafhoppers acquired FDP very efficiently by injection as 89, 86, and 96%, respectively, of injected leafhoppers tested positive by PCR. A reduced acquisition rate occurred for *Aphrodes makarovi* leafhoppers (33%) and similarly a limited number of artificial feeding buffers (5/13) were inoculated by the latter species. In addition, PCR products obtained from DNA extracts of *Aphrodes makarovi* leafhoppers showed a reduced intensity on agarose gel when compared with PCR products obtained from leafhoppers (*Anoplotettix fuscovenosus*, *Euscelis incisus*, and *E. variegatus*) that transmitted FDP to the feeding buffer.

Eleven Hemiptera species did not transmit FDP to the feeding buffer but tested positively for FDP by nested-PCR, confirming that injection of FDP into the insects had occurred. In general, the proportion of PCR-positive insects for nontransmitting species was much lower than the proportion of PCR-positive insects among species that inoculated FDP to the feeding buffer. In addition, PCR products in agarose gels were very faint for nontransmitting insect species (Table 3).

FDP acquisition and transmission by feeding on broadbean. Insects of *Aphrodes makarovi*, *C. haematoceps*, and *F. florii* species that fed for an AAP of 15 days on FDP-infected broadbeans were unable to transmit FDP to broadbean seedlings after 35 days of incubation (Table 4). *Anoplotettix fuscovenosus*, *Euscelis incisus*, and *E. variegatus* (wild type) insects transmitted FDP after acquisition on infected broadbeans. Inoculated broadbean seedlings showed typical symptoms of FDP infection. In addition, FDP was detected in symptomatic broadbeans inoculated with the latter species by PCR. FDP DNA detection was positive in leafhoppers *Anoplotettix fuscovenosus*, *Euscelis incisus*, and *E. variegatus*

Fig. 1. Logistic curves showing relationship between the proportion of *Euscelidius variegatus* retaining (solid box) and transmitting (open box) Flavescence dorée phytoplasma (FDP) following injection with serial 10-fold dilutions of FDP extracts. Equations and coefficient of determination values are as follows: $y = \exp(4.14 - 1.35 \times x) / [1 + \exp(4.14 - 1.35 \times x)]$, $R^2 = 0.97$, and $y = \exp(2.88 - 1.55 \times x) / [1 + \exp(2.88 - 1.55 \times x)]$, $R^2 = 0.90$, for the solid and dashed lines, respectively. Concentration 1, 2, 3, 4, and 5 in the figure correspond to the undiluted FDP extract, to the 10-fold, 100-fold, 1,000-fold, and 10,000-fold diluted extracts, respectively, that were injected into *E. variegatus*.

that transmitted FDP and in leafhoppers *C. haematoceps* and *F. florii* that did not transmit FDP. FDP DNA was not detected in *Aphrodes makarovi* (Table 4).

DISCUSSION

To our knowledge, this is the first time that insect injection and artificial feeding assays of candidate vectors are combined to test vector specificity of uncultivable phytopathogenic mollicutes. These procedures may help in screening potential vectors. In fact, we were able to eliminate most of the species from further consideration in vector screening and highlighted potential vectors for further testing by feeding acquisition.

Our results show that three leafhopper species (*Anoplotettix fuscovenosus*, *E. variegatus*, and *Euscelis incisus*) are potential vectors of FDP. These three Hemiptera species, as well as the natural vector of FDP, *S. titanus*, belong to the family Cicadellidae, subfamily Deltocephalinae. Also, we have shown that other Hemiptera species (*Agallia consobrina*, *Agalmatium flavescens*, *Aphrodes makarovi*, *C. haematoceps*, *F. florii*, *H. obsoletus*, *L. striatellus*, *Metcalfa pruinosa*, *Pentastiridius* sp., *Phyllaenus spumarius*, *Psammotettix* sp., and *Stictocephala bisonia*) were unable to transmit FDP after abdominal injection of phytoplasma suspensions. In addition, *Aphrodes makarovi*, *C. haematoceps*, and *F. florii* species were unable to transmit FDP after acquisition feeding on FDP-infected broadbeans. Therefore, our data indicate that all 12 latter species are unlikely vectors of FDP or are extremely inefficient in transmitting this mollicute.

To be transmitted by an insect vector under natural conditions, a phytopathogenic mollicute must traverse at least two physical barriers, namely gut and salivary glands (34,51). The combined assays of insect FDP acquisition by injection and feeding revealed that salivary glands and guts may act as independent barriers against the passage of phytoplasma cells. For example, *Aphrodes makarovi* specimens were able to transmit FDP after injection, although they were unable to acquire and retain the pathogen after feeding on diseased broadbean. In this instance, the gut seemed to be the primary obstacle to successful FDP transmission. Con-

versely, *C. haematoceps* and *F. florii* insects were unable to transmit FDP after abdominal injection, although they were able to retain FDP infection about 40 days after acquisition from diseased broadbean. Therefore, barriers to the salivary glands seemed to play an important role in excluding FDP transmission in these species, although we do not know the importance of gut barriers or whether FDP was able to reach the hemocoel following feeding acquisition. Detection of an AY phytoplasma strain in nonvector leafhopper *Dalbulus maidis* (De Long & Wolcott) by a PCR assay

TABLE 4. Transmission and polymerase chain reaction (PCR) results on leafhoppers that fed as early nymphs on Flavescente dorée phytoplasma (FDP)-infected broadbeans and underwent an incubation period of about 35 days after inoculation of broadbean seedlings

Species	Symptomatic broadbeans/insect exposed ^a	FDP PCR detection on insects	
		Positive/ tested leafhoppers ^b	Density of PCR product ^c
<i>Anoplotettix fuscovenosus</i>	10/10	9/12	+++++
<i>Aphrodes makarovi</i>	0/10	0/12	—
<i>Circulifer haematoceps</i> ^d	0/4	2/4	++++
<i>Euscelis incisus</i>	7/8	18/18	+++++
<i>Euscelidius variegatus</i> (wild type)	9/9	15/15	+++++
<i>Fieberiella florii</i> ^d	0/4	11/11	+++++
<i>Euscelidius variegatus</i> (healthy control) ^e	0/4	0/6	—
<i>Euscelidius variegatus</i> (positive control)	4/4	4/4	+++++

^a The presence of FDP in leafhopper-exposed broadbeans was confirmed by nested-PCR.

^b About 20 to 50% of insects were tested for each species, except for *F. florii* leafhoppers that were all tested.

^c The density of PCR products on agarose gels is quoted as relative intensities of bands compared to one another. +++++ correspond to the intensity of positive controls; —, negative (as for negative control).

^d Leafhoppers were tested in groups of two insects per sample.

^e Healthy *E. variegatus* leafhoppers were collected from healthy colonies and confined directly to broadbean seedlings.

TABLE 3. Results of polymerase chain reaction (PCR) detection of Flavescente dorée phytoplasma (FDP) DNA in Tris-EDTA (TE)-sucrose feeding buffer of 15 Hemiptera species injected with FDP extracts^a

Species	Incubation (days) ^b	Survival after incubation		PCR-positive/ tested diets ^{c,d}		Survival after inoculation		PCR positive/tested leafhoppers ^d		
		Rate	(%)	Rate	(%)	Rate	(%)	Rate	(%)	Density of PCR product ^e
<i>Agallia consobrina</i>	22	23/45	51	0/8	0	22/23	96	6/18	33	+
<i>Agalmatium flavescens</i>	22	13/40	32	0/4	0	10/13	77	2/8	25	+
<i>Anoplotettix fuscovenosus</i>	24	21/50	42	5/7	71	17/24	71	16/18	89	+++++
<i>Aphrodes makarovi</i>	24	38/45	84	5/13	38	36/38	95	9/27	33	++++
<i>Circulifer haematoceps</i>	21	18/50	36	0/6	0	11/18	61	14/15	93	+
<i>Euscelidius variegatus</i> (wild type)	21	22/47	47	4/7	57	15/22	68	12/14	86	+++++
<i>Euscelis incisus</i>	21	51/56	91	14/17	82	48/51	94	47/49	96	+++++
<i>Fieberiella florii</i>	22	12/45	26	0/4	0	7/12	58	3/7	43	++
<i>Hyalesthes obsoletus</i>	16	9/65	14	0/3	0	6/9	66	4/6	66	+
<i>Laodelphax striatellus</i> ^f	21	12/40	30	0/4	0	3/12	16	2/4	50	+
<i>Metcalfa pruinosa</i>	22	13/40	32	0/4	0	10/13	77	1/13	7	+
<i>Pentastiridius</i> sp.	16	13/50	26	0/4	0	8/13	61	5/16	31	+
<i>Phyllaenus spumarius</i>	25	21/35	60	0/9	0	0/21	0	18/21	86	+
<i>Psammotettix</i> sp.	21	15/40	37	0/5	0	5/15	33	3/13	23	+
<i>Stictocephala bisonia</i>	22	7/20	35	0/3	0	7/7	100	1/7	14	++
<i>Euscelidius variegatus</i> (noninjected control) ^g	—	—	—	0/18	0	40/54	74	0/26	0	—
<i>Euscelidius variegatus</i> (positive control)	21–24	60/220	27	16/20	80	37/56	66	56/60	93	+++++

^a Length of allowed incubation, survival at the end of incubation, survival at the end of the inoculation period (IAP) on feeding buffer and PCR detection of FDP in individual leafhoppers at the end of IAP are reported for each species.

^b Incubation was the time (days) allowed on suitable host plants after injection before transmission assays on TE-sucrose.

^c Positive diets/total tested; three insects per cage were commonly used.

^d Nested-PCR was performed with two FDP-specific primer pairs.

^e The density of PCR products on agarose gels is quoted as relative intensities of bands compared with one another. +++++ correspond to the intensity of positive controls; —, negative (as for negative control).

^f The planthopper *L. striatellus* was tested by PCR in groups of four insects per sample.

^g Healthy *E. variegatus* leafhoppers were collected from healthy colonies and confined directly into artificial feeding cages.

was possible 21 days after exposure of the insects to infected source plants according to Vega et al. (50). The authors suggested that a possible multiplication of the phytoplasma in the midgut of the insect may have occurred. This could be the case in our study of *C. haematoceps* and *F. florii* insects and FDP. The use of quantitative tests in combination with injection assays or in situ detection methods could clarify whether multiplication and eventual passage into the hemocoel of FDP may have occurred in *C. haematoceps* and *F. florii* after feeding acquisition.

Some hemipterans tested for FDP transmission are known vectors of phytopathogenic mollicutes. *C. haematoceps* is a natural vector of *Spiroplasma citri* Saglio (24), of sesame phyllody phytoplasma (16SrII) (30), and according to Weintraub et al. (52), this species transmits phytoplasmas in EY (16SrV) and pigeon pea witches'-broom (16SrIX) groups. *F. florii* is a vector of X-disease phytoplasma (16SrIII) in California (55), *H. obsoletus* and *Pentastiridius* sp. are European vectors of stolbur phytoplasmas (16SrXII-A) (26,36), and *Pentastiridius* sp. also transmits a γ -3 proteobacteria (bacteriumlike organism), the causal agent of "Basses richesses" syndrome of sugar beet in France (25). *Euscelis incisus* is known to transmit several phytoplasma strains in the AY clade (16SrI) (7,20). *E. variegatus* experimentally transmits X-disease phytoplasma (16SrIII) (28) and several phytoplasma strains in the AY clade (16SrI) (7,19,20). As not all these species were able to transmit FDP after injection, it seems plausible to assume that different routes or recognition systems are utilized by phytopathogenic mollicutes to traverse the barrier of salivary glands in different insect vectors. In the case of cultivable mollicutes, such as spiroplasmas (31,56) and mycoplasmas (58), surface proteins were recognized as receptors that mediate recognition and invasion of their host and some evidence suggested that this might be the case of FDP with the experimental vector *E. variegatus* (35).

The PCR procedure used in our research helped in understanding when one tested hemipteran species was potentially able to transmit FDP or not, according to the intensity of bands observed in agarose gels. Although we did not use a quantitative PCR procedure, we assumed that faint bands on agarose gels were obtained from a very small amount of target DNA in the samples and that intense bands were associated to higher titers of target DNA. Phytoplasma DNA was also detected by nested-PCR in nonvector species 20 to 25 days after injection. However, the intensity of bands on gels was faint and the proportions of positive nonvector insects were low, when compared with species that transmitted FDP, suggesting that the titer of viable phytoplasma in nonvectors decreased over time after injection. If this assumption is correct, we could rely on quantitative assays such as real-time PCR to recognize if an insect is a vector of a given phytoplasma strain. Nevertheless, Purcell et al. (43) showed that X-disease phytoplasma multiplies in the hemocoel of injected nonvector *Macrostelus* leafhoppers.

In addition, we observed that the titer of phytoplasma delivered by injection into a competent vector is fundamental to successful phytoplasma transmission. As observed in injected *E. variegatus*, the probability of FDP transmission by FDP-infected leafhoppers (verified by PCR) decreased with the logarithm of the dilution of injected FDP. This fact was probably associated with a requirement for a longer latency in insects injected with a lower titer of FDP. Actually, Whitcomb et al. (54) reported that the median latent period in X-disease phytoplasma-injected *Colladonus montanus* (Van Duzee) was correlated with the degree of dilution of injected phytoplasma extracts.

In this study, we attempted to identify leafhopper species that might play a role in maintaining FDP in vineyards or their vicinity. Both *E. variegatus* and *Euscelis incisus* are polyphagous and plurivoltine leafhoppers to several herbaceous host plant species, and they may occur abundantly in vineyards. Although grapevine seems not to be a preferred host plant for these leafhopper

species, earlier studies indicated that they can feed on the phloem of grapevine (10). Conversely, *Anoplotettix fuscovenosus* is univoltine and it can spend part of the life cycle on grapevine, actually adults may feed on grapevine and lay eggs in the bark of this plant species (1). Several Hemiptera species may occur in vineyards because of the presence of weed hosts growing in interrows or on the borders (6,48). Nevertheless, our research suggests that vector competency of FDP is probably restricted to leafhoppers in the subfamily Deltocephalinae, reducing significantly the number of potential candidate vectors.

Further research is needed to address whether newly identified potential vectors are unequivocally involved in the spread of FDP in vineyards by testing whether field leafhopper species are naturally infected by FDP, and by testing the ability of such leafhoppers to inoculate FDP to grapevines.

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