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Interactive transmission of two phytoplasmas by the vector insect

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Running title: CYP and FDP competition in *E. variegatus*

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Abstract

Phytoplasmas are wall-less bacteria associated with many plant diseases of wild and cultivated plants, transmitted by hemipteran insects. In nature, vectors can acquire more than one phytoplasma by feeding on multiple-infected source plants or sequentially on plants infected by different phytoplasmas. The acquisition of multiple phytoplasmas may lead to their interaction in the insects. In this work, nymphs of the leafhopper *Euscelidius variegatus* were allowed to feed sequentially on Flavescence dorée (FDP)-infected and chrysanthemum yellows (CYP)-infected source plants and vice-versa. Following the acquisition feeding, the titre of the two phytoplasmas in the insect was measured over time. FDP and CYP transmission capability of the doubly-infected leafhoppers was studied by transmission to *Vicia faba* L., and to artificial feeding medium. Both phytoplasmas were acquired by the vector regardless of the feeding order and FDP titre, but not CYP titre, which was affected by double infection. FDP and CYP persisted in the insect for life. Only CYP was consistently and efficiently transmitted by doubly-infected leafhoppers. Consistently, FDP was seldom detected in the salivary glands and never in the artificial feeding medium of doubly-infected leafhoppers. In conclusion, competition between CYP and FDP affected only salivary gland colonization, while it had almost no effect on phytoplasma acquisition. Competition among phytoplasma strains in an evolutionary time scale may explain the greater ability of CYP to colonize the insect body and be transmitted.

Key words: *Euscelidius variegatus*, *Candidatus* Phytoplasma vitis, *Candidatus* Phytoplasma asteris, quantitative real time PCR, pathogen competition

Introduction

Phytoplasmas, are wall-less plant pathogenic bacteria of the Class Mollicutes infecting a wide variety of herbaceous and woody plants, and they cause important economic losses on crops worldwide (Hogenhout *et al.*, 2008). Phytoplasmas are transmitted by phloem-feeding hemipteran insects (plant, leafhoppers and psyllids) (Weintraub and Beanland, 2006) and transmission involves a latent period in the vector during which the ingested bacteria pass from the alimentary canal through the midgut into the haemocoel, and colonize salivary glands before being transmitted to a new host plant. These plant pathogens may have a

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broad or a restricted plant host range (Foissac and Wilson, 2010), and they can be transmitted by polyphagous or monophagous insects (Weintraub and Beanland, 2006). Once infected, vectors remain inoculative for life (Bosco and D'Amelio, 2010), and for these reasons transmission by the vector is by far the most important route of phytoplasma dispersion and a key element in the epidemiology of these vector-borne pathogens (Gratz, 1999; Bosco and D'Amelio, 2010).

In nature, insect vectors can potentially acquire more than one phytoplasma species by feeding sequentially on plants infected by different phytoplasmas or by feeding on multiply-infected plants. Mixed phytoplasma infections are known in several plant species (Lee *et al.*, 2000; Marzachi *et al.*, 2001; Roggia *et al.*, 2013), and competition between different phytoplasmas within the same plant has been described only for few closely related phytoplasma strains (Kuske and Kirkpatrick, 1992; Sinclair *et al.*, 2000; Seemuller *et al.*, 2010). In contrast, mixed phytoplasma infection in the vector have been only sporadically reported. Phytoplasma interactions in the vector insects may result in interactive (enhanced or suppressed) or non-interactive (independent) transmission of the two agents (reviewed in Bosco and D'Amelio, 2010). Generally, the interference is most pronounced between closely related strains of the same pathogen (Purcell, 1982). In this case the interaction results in lack of transmission of one of the strains or in a transmission pattern where the strain acquired first is also the first and most efficiently transmitted (Freitag,1967). Moreover, some suppressive interactions have been observed between phytoplasmas and other plant pathogens such as spiroplasmas (Maramorosch, 1958, De Oliveira *et al.*, 2007) and viruses (Hsu and Banttari, 1979). The interaction between aster yellows phytoplasma and oat blue dwarf virus (OBDV, Tymoviridae) by the vector *Macrosteles quadrilineatus* Forbes (= *fascifrons*) results in a decrease of the transmission rate of both pathogens (Hsu and Banttari, 1979), and the interaction between corn stunt spiroplasma and maize bushy stunt phytoplasma within the vector *Dalbulus maidis* results in the suppression of phytoplasma transmission ("unilateral cross-protection") when spiroplasma acquisition preceeds that of phytoplasma (Maramorosch, 1958; de Oliveira *et al.*, 2007).

The leafhopper *Euscelidius variegatus* (Kirschbaum) (Cicadellidae Deltocephalinae) is a natural vector of the chrysanthemum yellows strain (CYP) of the *Candidatus* Phytoplasma asteris (16SrI-B, Palermo *et al.*, 2001), and a well-known laboratory vector of the Flavescence dorée phytoplasma (FDP, 16SrV, Caudwell *et*

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al., 1970), and preliminary experiments have shown that it can acquire both phytoplasmas in mixed infections (D'Amelio *et al.*, 2007).

The aims of the work were to describe the competition between two genetically unrelated phytoplasmas (CYP and FDP) in the common vector *E. variegatus*, and provide details on the acquisition, multiplication and transmission pattern of the competing pathogens. For the first time, interactions between different phytoplasmas in the same vector are studied in detail using molecular tools that allow detection and quantification of phytoplasmas in the insect body. In competition experiments, transmission assays provided information on the interaction between CYP and FDP, while molecular detection in the whole body and in the dissected organs as well as in artificial feeding media explained some aspects of their competition.

Materials and methods

Phytoplasma isolates, host plants and insect vector

Two phytoplasmas were used in this study, chrysanthemum yellows phytoplasma (CYP, a strain of the *Candidatus* Phytoplasma asteris, 16SrI-B genetic group originally found in the Italian Riviera (Conti *et al.*, 1988), and Flavescence dorée phytoplasma (FDP), 16SrV-D (Martini *et al.*, 2002), kindly provided by E. Boudon-Padieu (INRA, Dijon, France). CYP and FDP were maintained by *Euscelidius variegatus* (Kirschbaum) transmission in daisy (*Chrysanthemum carinatum* Schousboe) and broad bean (*Vicia faba* L.) plants, respectively.

Healthy colonies of *E. variegatus*, vector of both CYP (Bosco *et al.*, 1997; 2007) and FDP (Caudwell *et al.* 1970; Boudon-Padieu *et al.* 1989), were reared on oat (*Avena sativa* L.) inside plexiglas and nylon cages in growth chambers at 20-25°C, photoperiod L16:D8. Healthy leafhopper colonies were never exposed to infected plants and were checked by PCR to be phytoplasma-free.

Acquisition and transmission experiments

Two double infection experiments and two control experiments were carried out. In the first double-infection experiment, about 150 3rd – 4th instar nymphs were first allowed to feed on CYP-source daisies for one week. After that, all the insects were confined on FDP-source broad bean for another week. The second double-infection experiment was similar but nymphs were first fed on FDP-infected broad bean and then on CYP-

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infected daisies. In the two control experiments 3rd – 4th instar nymphs were allowed to feed for one week on CYP-source daisies only or on FDP-source broad bean only. Both CYP and FDP source plants were used for phytoplasma acquisition by *E. variegatus* soon after symptom appearance, two and five weeks post inoculation, respectively. In all the experiments, at the end of the acquisition access period (AAP) on source plants, ten leafhoppers per plant were caged for serial 3-4 days inoculation access periods (IAP, two IAPs per week) on 2-4 broad bean test plants in the same cage, for transmission until their death (60-80 dpa). All experiments were carried out in climatic chambers with photoperiod 16:8 (l:d), 25-20°C (l-d). Acquisition and inoculations were performed inside plexigas and nylon cages (40x40x40 cm). All the transmission experiments were carried out twice, but the data of the two experimental repeats were combined because phytoplasma transmission over time was very similar and leafhoppers became infective at the same dates in the two repeats. Broad bean test plants were exposed to the vectors two weeks after sowing. At 14, 22, 33, 40, 50 and 60 days post acquisition (dpa), 10-20 leafhopper adults from each treatment acquisition were sampled and phytoplasmas were quantified in infected insects as described later. Also for phytoplasma quantification, the insects were sampled in two experiments but the data of the two experimental repeats were combined for statistical analyses since a two-way ANOVA for dpa and repeat revealed no statistically significant interaction between dpa and repeat for all the treatments (CYP only, FDP only, CYP first acquisition, CYP second acquisition, FDP first acquisition, FDP second acquisition). . Test plants exposed to insects were then treated with insecticides and maintained in the greenhouse for 60 days. Broad beans exposed to healthy vectors were used as controls.

In a third experiment, to confirm that transmission results were actually due to phytoplasma competition in the insect and not in the recipient plant, about 50 leafhoppers fed on both CYP- and FDP-source plants in both orders as previously described were singly caged for a 24 h IAP inside 1.5 ml Eppendorf tubes with caps filled with 200 µl of an artificial feeding medium (5% sucrose in TE buffer pH 8.0, 10mM Tris and 1mM EDTA) 35 days after the first acquisition period. At the end of the IAP, feeding medium was collected and analysed for the presence of phytoplasmas as described below. At the same time, surviving insects were collected and salivary glands were dissected and analysed for the presence of phytoplasmas as described below.

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DNA extraction and detection of CYP and FDP by conventional PCR

Leaves of all broad beans exposed to leafhoppers were sampled four weeks after inoculation and total DNA was extracted from CYP and FDP inoculated broad bean leaves (0.1 g) with the PureLink Plant Total DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol and eluted in 50 µL of kit elution buffer. Total DNA was extracted with the same protocol from healthy broad beans as control.

Total DNA was also extracted from single leafhoppers sampled at 14, 22, 33, 40, 50 and 60 dpa following a procedure previously described by Marzachì *et al.* (1998). The same extraction procedure (volume was adjusted according to the smaller size of salivary glands compared with the whole body) was applied to extract DNA from the dissected salivary glands of leafhoppers caged inside Eppendorf tubes to feed on the sucrose feeding medium.

Detection of CYP and FDP by conventional nested PCR was also performed on feeding media collected from Eppendorf tube caps after inoculation with infectious leafhoppers according to Tanne and co-workers (2001). Phytoplasma particles were pelleted out of the feeding solution by centrifugation at 12,000 g for 15 min, at 4°C. DNA was extracted by adding 10 µl of 0.5 M NaOH, followed by the addition of 20 µl of 1M Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate and 20 mM EDTA. The mixture was incubated at 65°C for 15 min and precipitated with 2 volumes of ethanol, and the pellet was dissolved in 30 µl of TE. 1.5 µl of this preparation was used as PCR template.

The presence of CYP and FDP in the broad beans, individual leafhoppers, individual salivary glands and feeding media collected from Eppendorf tube caps following insect feeding was assessed by conventional nested PCR using the primers R16F2/R2 followed by R16F1/R1 (I) or (V) (Lee *et al.*, 1993; Lee *et al.*, 1994). Cycling conditions were as detailed in the original papers. The products were separated in a 1% (wt/vol) agarose gel, buffered in TBE (90 mM Tris borate and 2 mM EDTA, pH 8.3), stained with ethidium bromide, and visualized under UV light.

Quantification of phytoplasmas in the insect bodies

The concentration of DNA in preparations from single *E. variegatus* extracts was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

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Samples were then diluted in ddH2O to a final concentration of 1 ng μl^{-1}, and 5 µl used as template for real-time assays in a Chromo4 Real-Time PCR (qPCR, Bio-Rad Laboratories, Hercules, CA, USA) thermal cycler, according to Marzachì & Bosco (2005). Phytoplasma and insect DNAs from the same sample were separately quantified in each plate. The SecY DNA sequence was chosen as target for the amplification of phytoplasma DNAs (Roggia *et al.*, 2013).

Absolute quantification of CYP was achieved by comparison of cycle tresholds (Cts) of the samples with those of four dilutions in distilled water of the plasmid pSecYCY (Galetto *et al.*, 2008), containing the CYP *sec*Y target sequence ($1{,}09*10^8$ $1{,}09*10^6$, $1{,}09*10^4$ copy number/ per µl). Absolute quantification of FDP was achieved by comparison of Cts of the samples with those of four dilutions in distilled water of the plasmid pSecYFD containing the FDP *sec*Y target sequence ($1{,}17*10^8$, $1{,}17*10^6$, $1{,}17*10^4$ copy number/ per µl). For insect DNA amplification, the 18S rDNA sequence was chosen as target and amplified with primer pair MqFw and MqRv (Marzachi and Bosco, 2005). Absolute quantification of total insect DNA was achieved by comparison of Cts of the samples with those of three dilutions (10 ng/µl, 1 ng/µl, 0.1 ng/µl) of healthy insect DNA employed in the qPCR reactions for the standard curve construction.

CYP and FDP DNAs were measured as fg of phytoplasma DNA per ng of insect DNA and then transformed into the number of phytoplasma cells per ng of vector DNA as described in the original method (Marzachi and Bosco, 2005) with some modifications: one fg of pSecYFD and pSecYCY plasmids contained 234 and 219 molecules of plasmids, respectively, each containing a single copy of the *sec*Y gene. As this is a single copy gene in phytoplasma genomes (Oshima *et al.*, 2004), one femtogram of each plasmid corresponded to 234 and 219 FD or CY cells, respectively.

The amplification mix contained 0.3 µM of each primer (FD SecY Fw/Rev for FDP; CY SecY Fw/Rev for CYP; Mq Fw/Rev for vector DNA), 1X iQTM SYBR® Green Supermix (Bio-Rad) and templates in a final volume of 25 µl. Cycling conditions for CYP and insect 18S rDNA amplification were as follows: 95°C for 3 min, 63°C for 1 min repeated 45 times, 95°C for 1 min, 65°C for 1 min for 45 times. For FDP amplification were as follow: 95°C for 3 min, 61°C for 1 min repeated 45 times, 95°C for 1 min, 65°C for 1 min for 45 times. Specificity of the reaction was tested by running a melting curve analysis of the amplicons following each quantification reaction. In each qPCR plate, DNA from phytoplasma-free insects and water controls were also included. All samples were run in triplicate. CYP and FDP quantification by real time PCR was

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applied to the leafhoppers sampled at different times post acquisition that tested positive in nested PCR assays (see table 2). Therefore, sample sizes for phytoplasma titre estimation varied slightly but, on average, 10 insects per treatment per days after the start of AAP (dpa) were analised.

Data analysis

For the statistical analysis, raw data (number of phytoplasma cells/ ng of total insect DNA) were transformed into the logarithm. To compare the phytoplasma titres measured in individual leafhoppers following the same acquisition conditions at different dpa one-way ANOVA was performed. The same test was applied to compare the phytoplasma titres in the insects following single or mixed infection at different times post-acquisition (CYP in single and doubly infected insects at 14, 22, 33, 40 and 50 dpa, FDP in single and doubly infected insects at 14, 22, 33, 40 and 50 dpa). The t-test was applied to compare CYP and FDP titres at each sampling time. The proportion of phytoplasma-positive insects (as determined by PCR) following different acquisition conditions were compared with χ^2. Statistical tests were performed with Jandel SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA USA).

Results

Symptoms of CYP- and FDP-infected broad beans

Broad bean plants developed different symptoms following inoculations with CYP or FDP by the vector *E. variegatus*. CYP induced thickening and vein yellowing of the basal leaves while FDP induced upward leaf roll of younger leaves with a variable level of yellowing of the same leaves (Supplementary Figure S1A and B). Symptoms induced by the two phytoplasmas were the same both in single- and doubly-infected plants and these latter showed CYP symptoms on the basal leaves and FDP symptoms on the apical ones. Symptoms of CYP infection appeared consistently earlier than symptoms of FDP: 15-18 versus 28-30 dpi.

CYP and FDP transmission

Infection of all the test plants was established by species-specific PCR assays for both phytoplasmas. Symptom development was in agreement with results of PCR detection. The total number of plants infected by CYP and FDP, following single and double acquisitions, are summarised in table 1. Only transmission

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experiments carried out from 28 days post acquisition (dpa) onwards were considered in table 1 as most of the leafhoppers were not yet infectious (latent period was not completed) in earlier transmission tests (Supplementary table S2). Figure 1 describes the transmission of CYP (A) and FDP (B) over time by *E. variegatus* following different acquisition conditions. *E. variegatus* transmitted CYP from 17-21 dpa and FDP from 28 dpa onwards. The vector transmitted both phytoplasmas until its death. When *E. variegatus* was allowed to acquire both phytoplasmas, CYP transmission was not affected by the concurrent acquisition of FDP (χ^2= 0,764 with 2 d.f., P=0,682) while FDP transmission was suppressed in the presence of CYP (χ^2=71,222 with 2 d.f., P<0,001) (Table 1). The rate of CYP transmission to broad bean was always greater than 75%, but the rate of FDP transmission was significantly lower after FDP-only acquisition (7-16%) than after FDP+CYP acquisition (62%)..

CYP and FDP acquisition

The numbers of leafhoppers that acquired CYP and/or FDP following single and double AAPs are summarized in Table 2. *E. variegatus* acquired CYP with higher efficiency than it acquired FDP, both following a single AAP (χ^2 = 11.48 with 1 d.f.; P = 0.003) and two subsequent AAPs (χ^2 = 40.952 with 2 d.f.; P < 0.001). The proportion of leafhoppers that became CYP-infected was significantly lower in treatments providing exposure to both phytoplasmas (FDP+CYP) than treatments containing only CYP. (χ^2 = 11.480 with 2 d.f.; P = 0.003). A similar phenomenon seemed to occur for FDP, though the difference was not significant (χ^2 = 5.022 with 2 d.f.; P = 0.081). *E. variegatus* acquired CYP with higher efficiency if it fed first on FDP and then CYP than if it was exposed to the two phytoplasmas in the opposite order. ($\chi2$ = 4.891 with 1 d.f.; P = 0,027). On the contrary, acquisition of FDP was not influenced by feeding order ($\chi2$ = 0.153 with 1 d.f.; P = 0.696).

CYP and FDP titres in infected Euscelidius variegatus

CYP titre in *E. variegatus*, as determined in qPCR, ranged from about three thousand cells per ng of insect DNA at 14 dpa, up to a few tens of thousands of cells at later stages in the infection process (Figure 2). One-way ANOVA for CYP titre over time showed a significant increase (P <0.001). CYP concentration was not influenced by the acquisition conditions. One-way ANOVA for CYP titre (14, 22, 33, 40 and 50 dpa) always

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indicated that CYP concentration was the same, irrespective of the acquisition conditions (single or double). FDP titre in *E. variegatus* ranged from one thousand cells per ng of insect DNA at 14 dpa to a few hundred thousand cells later in the infection process. One-way ANOVA for FDP titre over time showed a significant increase (P <0.001). FDP titre was influenced by the acquisition conditions (single or double)- Following double acquisition, at 14 and 22 dpa an increase in FDP titre was recorded (P=0.045 and P=0.047, respectively), while at 33 dpa a decrease was recorded (P=0.040). Later in the infection process FDP titres measured in single or doubly-infected insects did not differ significantly. Leafhoppers infected by FDP hosted a higher phytoplasma titre compared to those infected by CYP. This higher FDP titre was recorded both in singly (22 dpa, P=0.004; 33 dpa, P<0.001; 40 dpa, P=0.017; 50 dpa, P=0.005) and doubly-infected (22 dpa, P=0.015; 33 dpa, P=0.004; 40 dpa, P<0.001; 50 dpa, P=0.048) insects from 22 dpa onwards (Figure 2, Supplementary table S3).

CYP and FDP detection in salivary glands and artificial feeding media

The two phytoplasmas colonized the salivary glands with with significantly different efficiencies. Following CYP + FDP acquisitions, 23/24 and 3/24 leafhoppers had CYP- and FDP-positive salivary glands, respectively. Following FDP + CYP acquisitions, 15/16 and 3/16 leafhoppers had phytoplasma-positive salivary glands to CYP and FDP, respectively. Consistently, CYP was detected by PCR in 11/24 feeding media following CYP + FDP acquisitions and in 7/16 feeding media following the FDP + CYP acquisition, while FDP was never detected in feeding media (Table 3).

Discussion

The competition between two unrelated phytoplasma species within the leafhopper vector *E. variegatus* was studied following serial acquisition of the vector on plants singly infected by the chrysanthemum yellows strain of the '*Ca*. P. asteris' (CYP) and the Flavescence dorée phytoplasma (FDP). The two phytoplasmas were chosen as they can be both transmitted by *E. variegatus* to broad bean plants, although, as this leafhopper is not the natural vector of FDP, the possibility that interactions between naturally occurring phytoplasmas might be different cannot be excluded. In particular, CYP and FDP induced distinctive symptoms in the basal and apical parts of the infected plants, respectively, and doubly infected broad beans

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showed both symptoms. Symptom expression in each phytoplasma – plant combination may differ due to the presence of different phytoplasma effector proteins (Sugio *et al.*, 2011) and different plant responses to them. *E. variegatus* transmitted CYP after a shorter LP than FDP, in line with previous experiments (this group, unpublished). Also, *E. variegatus* transmitted CYP more efficiently than FDP after exposure to singly infected plants, and CYP transmission was not influenced by FDP, irrespective of the sequence of acquisition. On the other hand, FDP transmission was severely reduced by the acquisition of CYP, irrespective of the order of exposure to both sources. A similar situation was reported for *Dalbulus maidis* where prolonged acquisition of corn stunt spiroplasma suppressed maize bushy stunt phytoplasma transmission (Maramorosch, 1958), and for the vector *Macrosteles quadrilineatus* (= *fascifrons*) where the majority of the leafhoppers transmitted only the first phytoplasma to which they had access when allowed to acquire different strains of aster yellows phytoplasmas (Freitag, 1967). In these previous studies, the absence of molecular tools hampered the analyses of other analyses of aspects of the competition between mollicutes other than transmission. In this study, competition was not recorded at the acquisition level, as FDP acquisition efficiency was not affected by CYP in double acquisition trials, while a slight decrease was observed in CYP acquisition when *E. variegatus* was also exposed to FDP. When the titre of both phytoplasmas was measured within the vector body, both microorganisms actively multiplied in singly infected vectors, and FDP reached a much higher concentration than CYP. In the presence of CYP, FDP titre seemed to be suppressed but this effect was significant only at 33 dpa. In contrast, CYP multiplication over time was not affected by the presence of FDP and also CYP titre was not influenced by co-acquisition of FDP. These results indicate an unilateral competition of the two phytoplasmas within the insect body, where the presence of CYP seemed to hamper the multiplication of FDP and limit the number of FDP cells in the vector. Despite this competition, FDP reached higher concentration compared to CYP at later stages of infection (from 22 dpa onwards) in doubly infected vectors. However, it is important to note that FDP was unable to colonize the salivary glands and reach the saliva of doubly infected vectors as it was only sporadically detected in dissected salivary glands of *E. variegatus* following exposure to CYP and FDP-infected plants, in both orders, and it was never detected in the artificial medium following inoculation. This suggests that salivary glands are the barrier where competition between CYP and FDP prevents efficient FDP transmission by *E. variegatus*. It can be suggested that the phytoplasma having the shorter latent period

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in the vector, CYP, is the first to colonize salivary glands and therefore is the most competitive for transmission. In this case, salivary glands already colonized by a phytoplasma might not be available for further invasion by a second one. FDP showed a more active multiplication in the insect body, but it did not suppress CYP multiplication. Therefore, the speed of salivary glands colonization, rather than the level of multiplication, is likely to be the key factor for successful transmission. This hypothesis may also explain the suppression of maize bushy stunt phytoplasma (MBSP) by corn stunt spiroplasma (CSS) in the vector *D. maidis*: CSS may move more rapidly in the insect body, thus suppressing transmission of MBSP (Maramorosch, 1958). CYP transmission depends on phytoplasma titre in the salivary glands of *E. variegatus,* as non-transmitter individuals host only a few hundred CYP cells per ng of insect DNA in their salivary glands while transmitters host thousands of cells (Galetto *et al.*, 2009), therefore a high titre of phytoplasma in the salivary glands seems to be a prerequisite for efficient transmission. The erratic presence of FDP in the salivary glands of *E. variegatus* exposed subsequently to CYP and FDP and the absence from saliva (feeding media) also ruled out phytoplasma competition in the inoculated plants as possible cause of the failure of FDP transmission to broad bean. A correlation between phytoplasma titre and transmission efficiency occurs for CYP and two of its natural vectors: *Macrosteles quadripunctulatus* sustains a higher phytoplasma multiplication compared to *E. variegatus*, and it is also the most efficient vector (Bosco *et al.*, 2007). The same correlation does not apply to FDP and CYP transmission by *E. variegatus*, since this vector sustains a higher FDP multiplication compared to CYP but transmits the latter more efficiently. This apparent contradiction may be explained by the competition for the colonization of the salivary glands when two phytoplasmas co-infect the same individual. From an evolutionary perspective, the phytoplasma colonizing salivary glands first would obtain a fitness gain compared to a slower one (the faster, the fitter). The different behaviour of the two phytoplasmas may also be explained by the interactions with the insect host, besides competition between the phytoplasmas. The host immune system may recognize and suppress the CYP, or the CYP may have developed mechanisms to invade the salivary glands during the long-term relationship of the insect host and the phytoplasma.

CYP, as the other aster yellows strains, has a broad host range and therefore it is very likely that in an evolutionary time scale it interacted with several different vector species. On the contrary, FDP has a very narrow host range (grapevine and very few alternative hosts) and it is not likely to be co-evolved with

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different vector species. This may contribute to explain why CYP is more competitive for transmission in *E. variegatus*.

In summary, *E. variegatus* transmitted CYP with high efficiency and it was not influenced by the presence of FDP, and CYP was able to colonize the salivary glands and reach saliva, consistent with the high efficient transmission results. On the other hand, the good efficacy of *E. variegatus* as vector of FDP following single acquisition was significantly lower when competition with CYP was introduced, although FDP persisted for life within the vector. *E. variegatus* is a natural vector of CYP (Bosco *et al.*, 1997; Palermo *et al.*, 2001), while it transmits FDP under laboratory conditions only, as it is not able to feed on grape, the natural host plant of FDP. In the case of *E. variegatus*-CYP association, both partners are of Palaearctic origin and therefore co-evolved for a long time so that phytoplasma multiplication is mitigated and no pathogenic effects are recorded (D'Amelio *et al.*, 2008). In contrast, the association between FDP and *E. variegatus* is artificial and this can explain the phytoplasma low ability to colonize the salivary glands (needed for transmission) and also its high multiplication in the vector, which causes severe pathogenic effects (Bressan *et al.*, 2005). Nevertheless, even the "natural" vector of FDP, *Scaphoideus titanus* Ball, is not long co-evolved with FDP because the leafhopper is of Nearctic origin (Bertin et al., 2007) while FDP is very likely of Palaearctic origin (Arnaud et al., 2007). Therefore, it is possible that the same phenomenon also occurs in *S. titanus*, but further studies are needed to demonstrate this.

In nature, polyphagous vectors have chances of acquiring different phytoplasmas by visiting and feeding on different host plants. This work, together with those of Freitag (1967) on related strains of aster yellows phytoplasmas infecting *M. quadrilineatus*, Maramorosch (1958) and de Oliveira and co-workers (2007) on different mollicutes within *D. maidis*, indicates that interactive transmission (up to unilateral suppression) is more common than non-interactive transmission, as this latter has not been reported so far for phytoplasmas. Interestingly, unilateral suppression occurs between both genetically related (Freitag, 1967) and un-related phytoplasmas (this work). This is the first time that interaction / competition of different phytoplasmas has been described and dissected at the acquisition, multiplication, body colonization and transmission levels.

The role of interactive transmission in nature must be taken into account for the description and prediction of phytoplasma epidemiology, as different phytoplasmas are likely to be present in different environments and

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interactive transmission may contribute to explain differential spread of the same phytoplasma in the same crop under different environmental conditions.

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Table and Figure legends

Table 1. Results of transmission experiments of chrysanthemum yellows (CYP) and Flavescence dorée (FDP) phytoplasmas with *Euscelidius variegatus* to broad bean plants. Number of infected/exposed plants as determined by species-specific nested PCR are reported, following single (CYP only; FDP only) and double (CYP + FDP; FDP + CYP) acquisitions. Group transmissions for 3-4 day IAPs from 28 dpa onwards. AAP, acquisition access period. Within columns, figures followed by the same letter do not differ significantly (χ^2 test).

Table 2. Results of acquisition experiments of *Euscelidius variegatus* following AAP on chrysanthemum yellows (CYP) and Flavescence dorée (FDP) -infected broad bean plants. Number of infected leafhoppers are reported following single (CYP only; FDP only) and double (CYP +

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FDP; FDP + CYP) acquisitions as determined by species-specific nested-PCR tests. AAP, acquisition access period. Within columns, figures followed by the same letter do not differ significantly (χ^2 test).

Table 3. PCR detection (presence: +; absence: -) of chrysanthemum yellows phytoplasma (CYP) and Flavescence dorée phytoplasma (FDP) in salivary glands of single *Euscelidius variegatus* and in corresponding sucrose feeding media following feeding on CYP- and FDP-infected source plants in both orders. The analysis was carried out 35 days post acquisition. Each line corresponds to a single insect.

Figure 1. Results of phytoplasma transmission experiments with *Euscelidius variegatus*. A: chrysanthemum yellows phytoplasma (CYP) transmission following acquisition on CYP-infected plants or on CYP- and Flavescence dorée phytoplasma (FDP)- infected source plants. B: FDP transmission following acquisition on FDP-infected plants or on CYP- and FDP- infected source plants. AAP, acquisition access period.

Figure 2. Mean ± SE quantities of chrysanthemum yellows (CYP) and Flavescence dorée (FDP) phytoplasmas (log of cells per ng of insect DNA) measured at different days post acquisition in *Euscelidius variegatus* leafhoppers fed on CYP source only, FDP source only, CYP followed by FDP (CYP + FDP) and FDP followed by CYP (FDP + CYP).

Supplementary Figure S1. Symptoms of chrysanthemum yellows (A) and Flavescence dorée (B) phytoplasmas on broad bean plants.

Supplementary TableS2. Results of chrysanthemum yellows (CYP) and Flavescence dorée (FDP) phytoplasma transmission experiments with *Euscelidius variegatus* following acquisition on CYP-

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infected plants, on FDP-infected plants, or on both source plants. Number of phytoplasma infected plants over exposed ones are reported, as determined by species-specific nested-PCR tests. AAP, acquisition access period.

Supplementary TableS3. Summary of the statistical analyses performed on the phytoplasma titres measured by R-PCR in *Euscelidius variegatus* individual leafhoppers following different phytoplasma acquisition conditions. Raw data (phytoplasma cells/ ng of total insect DNA) were transformed into the logarithm for the analyses.

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Table 1. Transmission of chrysanthemum yellows (CYP) and Flavescence dorée (FDP) phytoplasmas. Numbers of infected/exposed plants as determined by species-specific nested PCR are reported, following single (CYP only; FDP only) and double (CYP + FDP; FDP + CYP) acquisitions. Group transmissions for 3-4 day IAPs from 28 dpa onwards. AAP, acquisition access period. Within columns, figures followed by the same letter do not differ significantly ($\chi 2$ test).

Acquisition source	CYP transmission positive/exposed plants (% of infected plants)	FDP transmission positive/exposed plants (% of infected plants)
CYP only (7-day AAP)	51/64 (79.7%)a	-
FDP only (7-day AAP)	-	67/108 (62.0%)a
CYP + FDP (two 7-day AAPs)	53/68 (77.9%)a	5/68 (7.4%)b
FDP + CYP (two 7-day AAPs)	56/76 (76.3%)a	12/76 (15.8%)b

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Table 2. Acquisition of phytoplasmas by *Euscelidius variegatus* following AAP on chrysanthemum yellows (CYP) and Flavescence dorée (FDP) -infected broad bean plants. Number of infected leafhoppers are reported following single (CYP only; FDP only) and double (CYP + FDP; FDP + CYP) acquisitions as determined by species-specific nested-PCR tests. AAP, acquisition access period. PCR assays were carried out on insects sampled from 35 to 60 days post acquisition. Within columns, figures followed by the same letter do not differ significantly (χ2 test).

Acquisition source	CYP acquisition positive/tested (% of infected leafhoppers)	FDP acquisition positive/tested (% of infected leafhoppers)
CYP only (7-day AAP)	54/59 (91.5%)a	-
FDP only (7-day AAP)	-	46/69 (66.7%)a
CYP + FDP (two 7-day AAPs)	75/105 (71.4%)b	44/89 (49.4%)a
FDP + CYP (two 7-day AAPs)	103/122 (84.4%)c	57/107 (53.3%)a

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Table 3. PCR detection (presence: +; absence: -) of chrysanthemum yellows phytoplasma (CYP) and Flavescence dorée phytoplasma (FDP) in salivary glands of single *Euscelidius variegatus* and in corresponding sucrose feeding medium following feeding on CYP- and FDP-infected source plants in both sequences. The analysis was carried out 35 days post acquisition. Each line corresponds to a single insect.

Acquisition access on CYP followed by FDP					Acquisition access on FDP followed by CYP				
Insect number	Salivary gland		Feeding medium		Insect number	Salivary gland		Feeding medium	
	CYP	FDP	CYP	FDP		CYP	FDP	CYP	FDP
1	+	+	-	-	1	+	-	-	-
2	+	-	-	-	2	+	-	+	-
3	+	-	+	-	3	+	-	+	-
4	+	-	-	-	4	+	-	-	-
5	+	-	+	-	5	+	-	-	-
6	+	-	+	-	6	+	-	+	-
7	+	-	-	-	7	+	-	+	-
8	+	-	-	-	8	+	+	-	-
9	+	-	-	-	9	+	-	-	-
10	+	-	+	-	10	+	+	-	-
11	+	-	+	-	11	+	-	+	-
12	+	+	-	-	12	-	+	-	-
13	+	+	-	-	13	+	-	-	-
14	+	-	+	-	14	+	-	+	-
15	+	-	+	-	15	+	-	-	-
16	+	-	-	-	16	+	-	+	-
17	+	-	-	-					
18	+	-	+	-					
19	+	-	-	-					
20	-	-	-	-					
21	+	-	+	-					
22	+	-	+	-					
23	+	-	+	-					
24	+	-	-	-					

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Figure 1.

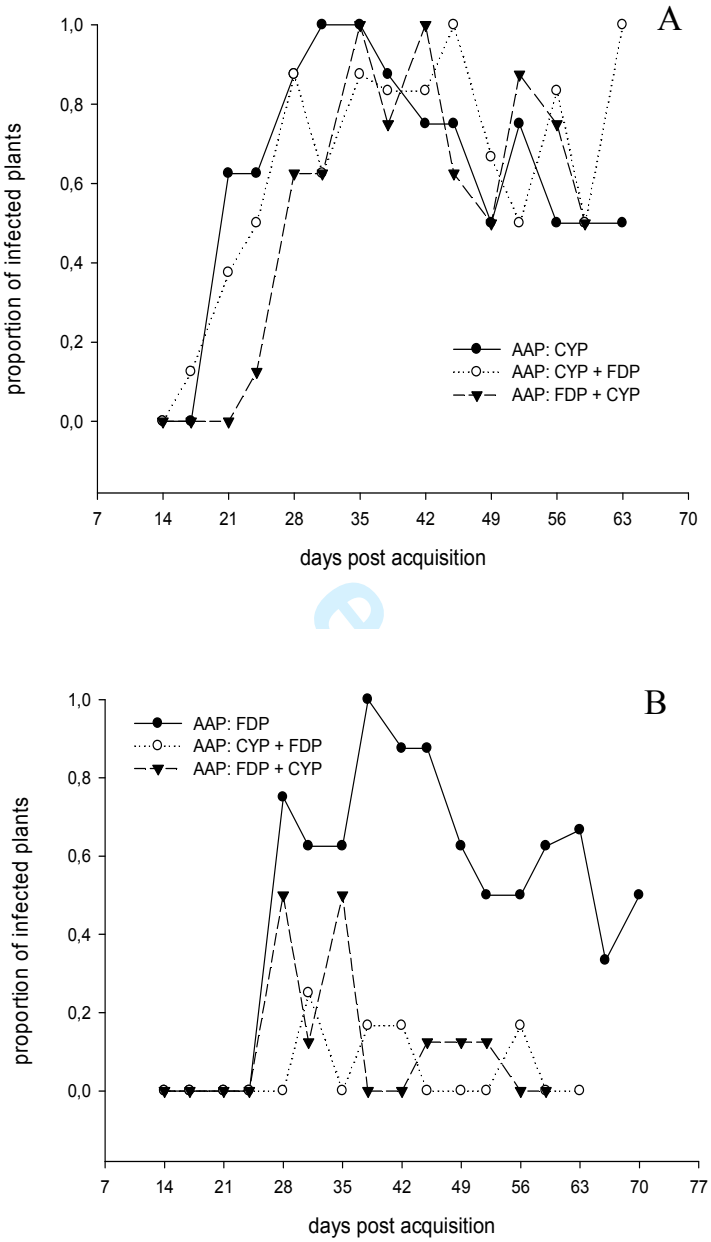
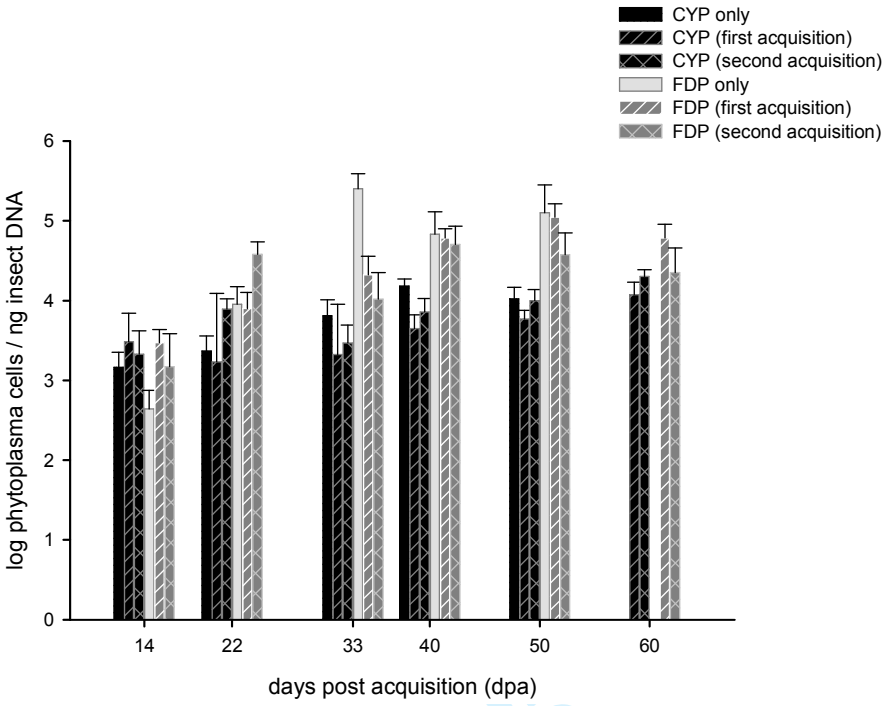

A
proportion of infected plants
days post acquisition
AAP: CYP
AAP: CYP + FDP
AAP: FDP + CYP
B
proportion of infected plants
days post acquisition
AAP: FDP
AAP: CYP + FDP
AAP: FDP + CYP

Figure 2



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