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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/152811> since 2016-07-25T09:29:17Z

Published version:

DOI:10.1111/aab.12146

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UNIVERSITÀ DEGLI STUDI DI TORINO

This is the accepted version of the following article: [3. Rashidi M., D'Amelio R., Galetto L., Marzachì C., and Bosco D., 2014. Interactive transmission of two phytoplasmas by the vector insect. *Annals of Applied Biology*, 165: 404-413.],

which has been published in final form at [doi:10.1111/aab.12146]



Interactive transmission of two phytoplasmas by the vector insect

Journal:	<i>Annals of Applied Biology</i>
Manuscript ID:	AAB-2013-0321.R1
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Rashidi, Mahnaz; Consiglio Nazionale delle Ricerche, CNR, Istituto di Virologia Vegetale D'Amelio, Romina; Universita di Torino, DISAFA - Entomologia Galletto, Luciana; Consiglio Nazionale delle Ricerche, CNR, Istituto di Virologia Vegetale Marzachi, Cristina; Consiglio Nazionale delle Ricerche, CNR, Istituto di Virologia Vegetale Bosco, Domenico; Universita di Torino, DISAFA - Entomologia
Key Words:	Phytoplasmas, Transmission By Vectors, Entomology

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3 1 **Interactive transmission of two phytoplasmas by the vector insect**
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7 3 Rashidi M.^{1,2*}, D'Amelio R.^{1*}, Galetto L.², Marzachi C.², and Bosco D.^{1*}
8
9 4

10
11 5 ¹ University of Torino, DISAFA, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy.

12
13 6 ² Istituto di Virologia Vegetale, CNR, Strada delle Cacce 73, 10135 Torino, Italy.
14
15 7

16
17 8 *the two authors equally contributed to the paper
18
19 9

20
21 10 ***Corresponding author:** Domenico Bosco, University of Torino DISAFA, Largo Paolo Braccini 2,
22

23 11 Grugliasco (TO), Italy, tel. +39 011 6708529, fax +39 011 670853, domenico.bosco@unito.it
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26
27 13 **Running title:** CYP and FDP competition in *E. variegatus*
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15 Abstract

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

32
33 **Key words:** *Euscelidius variegatus*, *Candidatus* Phytoplasma vitis, *Candidatus* Phytoplasma asteris,
34 quantitative real time PCR, pathogen competition

36 Introduction

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

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3 43 broad or a restricted plant host range (Foissac and Wilson, 2010), and they can be transmitted by
4
5 44 polyphagous or monophagous insects (Weintraub and Beanland, 2006). Once infected, vectors remain
6
7 45 inoculative for life (Bosco and D'Amelio, 2010), and for these reasons transmission by the vector is by far
8
9 46 the most important route of phytoplasma dispersion and a key element in the epidemiology of these vector-
10
11 47 borne pathogens (Gratz, 1999; Bosco and D'Amelio, 2010).

12
13 48 In nature, insect vectors can potentially acquire more than one phytoplasma species by feeding sequentially
14
15 49 on plants infected by different phytoplasmas or by feeding on multiply-infected plants. Mixed phytoplasma
16
17 50 infections are known in several plant species (Lee *et al.*, 2000; Marzachi *et al.*, 2001; Roggia *et al.*, 2013),
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19 51 and competition between different phytoplasmas within the same plant has been described only for few
20
21 52 closely related phytoplasma strains (Kuske and Kirkpatrick, 1992; Sinclair *et al.*, 2000; Seemuller *et al.*,
22
23 53 2010). In contrast, mixed phytoplasma infection in the vector have been only sporadically reported.

24
25 54 Phytoplasma interactions in the vector insects may result in interactive (enhanced or suppressed) or non-
26
27 55 interactive (independent) transmission of the two agents (reviewed in Bosco and D'Amelio, 2010).

28
29 56 Generally, the interference is most pronounced between closely related strains of the same pathogen (Purcell,
30
31 57 1982). In this case the interaction results in lack of transmission of one of the strains or in a transmission
32
33 58 pattern where the strain acquired first is also the first and most efficiently transmitted (Freitag, 1967).

34
35 59 Moreover, some suppressive interactions have been observed between phytoplasmas and other plant
36
37 60 pathogens such as spiroplasmas (Maramorosch, 1958, De Oliveira *et al.*, 2007) and viruses (Hsu and
38
39 61 Bantari, 1979). The interaction between aster yellows phytoplasma and oat blue dwarf virus (OBDV,
40
41 62 Tymoviridae) by the vector *Macrostoteles quadrilineatus* Forbes (= *fascifrons*) results in a decrease of the
42
43 63 transmission rate of both pathogens (Hsu and Bantari, 1979), and the interaction between corn stunt
44
45 64 spiroplasma and maize bushy stunt phytoplasma within the vector *Dalbulus maidis* results in the suppression
46
47 65 of phytoplasma transmission (“unilateral cross-protection”) when spiroplasma acquisition precedes that of
48
49 66 phytoplasma (Maramorosch, 1958; de Oliveira *et al.*, 2007).

50
51 67 The leafhopper *Euscelidius variegatus* (Kirschbaum) (Cicadellidae Deltocephalinae) is a natural vector of
52
53 68 the chrysanthemum yellows strain (CYP) of the *Candidatus* Phytoplasma asteris (16SrI-B, Palermo *et al.*,
54
55 69 2001), and a well-known laboratory vector of the Flavescence dorée phytoplasma (FDP, 16SrV, Caudwell *et*

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3 70 *al.*, 1970), and preliminary experiments have shown that it can acquire both phytoplasmas in mixed
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5 71 infections (D'Amelio *et al.*, 2007).
6
7 72 The aims of the work were to describe the competition between two genetically unrelated phytoplasmas
8
9 73 (CYP and FDP) in the common vector *E. variegatus*, and provide details on the acquisition, multiplication
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11 74 and transmission pattern of the competing pathogens. For the first time, interactions between different
12
13 75 phytoplasmas in the same vector are studied in detail using molecular tools that allow detection and
14
15 76 quantification of phytoplasmas in the insect body. In competition experiments, transmission assays provided
16
17 77 information on the interaction between CYP and FDP, while molecular detection in the whole body and in
18
19 78 the dissected organs as well as in artificial feeding media explained some aspects of their competition.
20
21 79

22 23 80 **Materials and methods**

24 25 81 *Phytoplasma isolates, host plants and insect vector*

26
27 82 Two phytoplasmas were used in this study, chrysanthemum yellows phytoplasma (CYP, a strain of the
28
29 83 *Candidatus* Phytoplasma asteris, 16SrI-B genetic group originally found in the Italian Riviera (Conti *et al.*,
30
31 84 1988), and Flavescence dorée phytoplasma (FDP), 16SrV-D (Martini *et al.*, 2002), kindly provided by E.
32
33 85 Boudon-Padieu (INRA, Dijon, France). CYP and FDP were maintained by *Euscelidius variegatus*
34
35 86 (Kirschbaum) transmission in daisy (*Chrysanthemum carinatum* Schousboe) and broad bean (*Vicia faba* L.)
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37 87 plants, respectively.

38
39 88 Healthy colonies of *E. variegatus*, vector of both CYP (Bosco *et al.*, 1997; 2007) and FDP (Caudwell *et al.*
40
41 89 1970; Boudon-Padieu *et al.* 1989), were reared on oat (*Avena sativa* L.) inside plexiglas and nylon cages in
42
43 90 growth chambers at 20-25°C, photoperiod L16:D8. Healthy leafhopper colonies were never exposed to
44
45 91 infected plants and were checked by PCR to be phytoplasma-free.
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48 49 93 *Acquisition and transmission experiments*

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51 94 Two double infection experiments and two control experiments were carried out. In the first double-infection
52
53 95 experiment, about 150 3rd – 4th instar nymphs were first allowed to feed on CYP-source daisies for one week.
54
55 96 After that, all the insects were confined on FDP-source broad bean for another week. The second double-
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57 97 infection experiment was similar but nymphs were first fed on FDP-infected broad bean and then on CYP-
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3 98 infected daisies. In the two control experiments 3rd – 4th instar nymphs were allowed to feed for one week on
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5 99 CYP-source daisies only or on FDP-source broad bean only. Both CYP and FDP source plants were used for
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7 100 phytoplasma acquisition by *E. variegatus* soon after symptom appearance, two and five weeks post
8
9 101 inoculation, respectively. In all the experiments, at the end of the acquisition access period (AAP) on source
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11 102 plants, ten leafhoppers per plant were caged for serial 3-4 days inoculation access periods (IAP, two IAPs per
12
13 103 week) on 2-4 broad bean test plants in the same cage, for transmission until their death (60-80 dpa). All
14
15 104 experiments were carried out in climatic chambers with photoperiod 16:8 (l:d), 25-20°C (l-d). Acquisition
16
17 105 and inoculations were performed inside plexiglas and nylon cages (40x40x40 cm). All the transmission
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19 106 experiments were carried out twice, but the data of the two experimental repeats were combined because
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21 107 phytoplasma transmission over time was very similar and leafhoppers became infective at the same dates in
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23 108 the two repeats. Broad bean test plants were exposed to the vectors two weeks after sowing. At 14, 22, 33,
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25 109 40, 50 and 60 days post acquisition (dpa), 10-20 leafhopper adults from each treatment acquisition were
26
27 110 sampled and phytoplasmas were quantified in infected insects as described later. Also for phytoplasma
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29 111 quantification, the insects were sampled in two experiments but the data of the two experimental repeats
30
31 112 were combined for statistical analyses since a two-way ANOVA for dpa and repeat revealed no statistically
32
33 113 significant interaction between dpa and repeat for all the treatments (CYP only, FDP only, CYP first
34
35 114 acquisition, CYP second acquisition, FDP first acquisition, FDP second acquisition). . Test plants exposed to
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37 115 insects were then treated with insecticides and maintained in the greenhouse for 60 days. Broad beans
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39 116 exposed to healthy vectors were used as controls.

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42 117 In a third experiment, to confirm that transmission results were actually due to phytoplasma competition in
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44 118 the insect and not in the recipient plant, about 50 leafhoppers fed on both CYP- and FDP-source plants in
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46 119 both orders as previously described were singly caged for a 24 h IAP inside 1.5 ml Eppendorf tubes with
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48 120 caps filled with 200 µl of an artificial feeding medium (5% sucrose in TE buffer pH 8.0, 10mM Tris and
49
50 121 1mM EDTA) 35 days after the first acquisition period. At the end of the IAP, feeding medium was collected
51
52 122 and analysed for the presence of phytoplasmas as described below. At the same time, surviving insects were
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54 123 collected and salivary glands were dissected and analysed for the presence of phytoplasmas as described
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56 124 below.

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3 126 *DNA extraction and detection of CYP and FDP by conventional PCR*

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5 127 Leaves of all broad beans exposed to leafhoppers were sampled four weeks after inoculation and total DNA
6
7 128 was extracted from CYP and FDP inoculated broad bean leaves (0.1 g) with the PureLink Plant Total DNA
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9 129 Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol and eluted in 50 µL
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11 130 of kit elution buffer. Total DNA was extracted with the same protocol from healthy broad beans as control.

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13 131 Total DNA was also extracted from single leafhoppers sampled at 14, 22, 33, 40, 50 and 60 dpa following a
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15 132 procedure previously described by Marzachi *et al.* (1998). The same extraction procedure (volume was
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17 133 adjusted according to the smaller size of salivary glands compared with the whole body) was applied to
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19 134 extract DNA from the dissected salivary glands of leafhoppers caged inside Eppendorf tubes to feed on the
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21 135 sucrose feeding medium.

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23 136 Detection of CYP and FDP by conventional nested PCR was also performed on feeding media collected
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25 137 from Eppendorf tube caps after inoculation with infectious leafhoppers according to Tanne and co-workers
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27 138 (2001). Phytoplasma particles were pelleted out of the feeding solution by centrifugation at 12,000 g for 15
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29 139 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
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31 140 Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate and 20 mM EDTA. The mixture was incubated at
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33 141 65°C for 15 min and precipitated with 2 volumes of ethanol, and the pellet was dissolved in 30 µl of TE. 1.5
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35 142 µl of this preparation was used as PCR template.

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37 143 The presence of CYP and FDP in the broad beans, individual leafhoppers, individual salivary glands and
38
39 144 feeding media collected from Eppendorf tube caps following insect feeding was assessed by conventional
40
41 145 nested PCR using the primers R16F2/R2 followed by R16F1/R1 (I) or (V) (Lee *et al.*, 1993; Lee *et al.*,
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43 146 1994). Cycling conditions were as detailed in the original papers. The products were separated in a 1%
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45 147 (wt/vol) agarose gel, buffered in TBE (90 mM Tris borate and 2 mM EDTA, pH 8.3), stained with ethidium
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47 148 bromide, and visualized under UV light.

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52 150 *Quantification of phytoplasmas in the insect bodies*

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54 151 The concentration of DNA in preparations from single *E. variegatus* extracts was measured with a Nanodrop
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56 152 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

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3 153 Samples were then diluted in ddH₂O to a final concentration of 1 ng μl^{-1} , and 5 μl used as template for real-
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5 154 time assays in a Chromo4 Real-Time PCR (qPCR, Bio-Rad Laboratories, Hercules, CA, USA) thermal
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7 155 cycler, according to Marzachi & Bosco (2005). Phytoplasma and insect DNAs from the same sample were
8
9 156 separately quantified in each plate. The SecY DNA sequence was chosen as target for the amplification of
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11 157 phytoplasma DNAs (Roggia *et al.*, 2013).

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13 158 Absolute quantification of CYP was achieved by comparison of cycle thresholds (Cts) of the samples with
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15 159 those of four dilutions in distilled water of the plasmid pSecYCY (Galletto *et al.*, 2008), containing the CYP
16
17 160 *secY* target sequence ($1,09 \times 10^8$, $1,09 \times 10^6$, $1,09 \times 10^4$ copy number/ per μl). Absolute quantification of FDP
18
19 161 was achieved by comparison of Cts of the samples with those of four dilutions in distilled water of the
20
21 162 plasmid pSecYFD containing the FDP *secY* target sequence ($1,17 \times 10^8$, $1,17 \times 10^6$, $1,17 \times 10^4$ copy number/ per
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23 163 μl). For insect DNA amplification, the 18S rDNA sequence was chosen as target and amplified with primer
24
25 164 pair MqFw and MqRv (Marzachi and Bosco, 2005). Absolute quantification of total insect DNA was
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27 165 achieved by comparison of Cts of the samples with those of three dilutions (10 ng/ μl , 1 ng/ μl , 0.1 ng/ μl) of
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29 166 healthy insect DNA employed in the qPCR reactions for the standard curve construction.

30
31 167 CYP and FDP DNAs were measured as fg of phytoplasma DNA per ng of insect DNA and then transformed
32
33 168 into the number of phytoplasma cells per ng of vector DNA as described in the original method (Marzachi
34
35 169 and Bosco, 2005) with some modifications: one fg of pSecYFD and pSecYCY plasmids contained 234 and
36
37 170 219 molecules of plasmids, respectively, each containing a single copy of the *secY* gene. As this is a single
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39 171 copy gene in phytoplasma genomes (Oshima *et al.*, 2004), one femtogram of each plasmid corresponded to
40
41 172 234 and 219 FD or CY cells, respectively.

42
43 173 The amplification mix contained 0.3 μM of each primer (FD SecY Fw/Rev for FDP; CY SecY Fw/Rev for
44
45 174 CYP; Mq Fw/Rev for vector DNA), 1X iQTM SYBR® Green Supermix (Bio-Rad) and templates in a final
46
47 175 volume of 25 μl . Cycling conditions for CYP and insect 18S rDNA amplification were as follows: 95°C for 3
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49 176 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
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51 177 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
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53 178 times. Specificity of the reaction was tested by running a melting curve analysis of the amplicons following
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55 179 each quantification reaction. In each qPCR plate, DNA from phytoplasma-free insects and water controls
56
57 180 were also included. All samples were run in triplicate. CYP and FDP quantification by real time PCR was

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3 181 applied to the leafhoppers sampled at different times post acquisition that tested positive in nested PCR
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5 182 assays (see table 2). Therefore, sample sizes for phytoplasma titre estimation varied slightly but, on average,
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7 183 10 insects per treatment per days after the start of AAP (dpa) were analysed.
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10 11 185 *Data analysis*

12
13 186 For the statistical analysis, raw data (number of phytoplasma cells/ ng of total insect DNA) were transformed
14
15 187 into the logarithm. To compare the phytoplasma titres measured in individual leafhoppers following the same
16
17 188 acquisition conditions at different dpa one-way ANOVA was performed. The same test was applied to
18
19 189 compare the phytoplasma titres in the insects following single or mixed infection at different times post-
20
21 190 acquisition (CYP in single and doubly infected insects at 14, 22, 33, 40 and 50 dpa, FDP in single and
22
23 191 doubly infected insects at 14, 22, 33, 40 and 50 dpa). The t-test was applied to compare CYP and FDP titres
24
25 192 at each sampling time. The proportion of phytoplasma-positive insects (as determined by PCR) following
26
27 193 different acquisition conditions were compared with χ^2 . Statistical tests were performed with Jandel
28
29 194 SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA USA).
30

31 195

32 33 196 **Results**

34 35 197 *Symptoms of CYP- and FDP-infected broad beans*

36
37 198 Broad bean plants developed different symptoms following inoculations with CYP or FDP by the vector *E.*
38
39 199 *variegatus*. CYP induced thickening and vein yellowing of the basal leaves while FDP induced upward leaf
40
41 200 roll of younger leaves with a variable level of yellowing of the same leaves (Supplementary Figure S1A and
42
43 201 B). Symptoms induced by the two phytoplasmas were the same both in single- and doubly-infected plants
44
45 202 and these latter showed CYP symptoms on the basal leaves and FDP symptoms on the apical ones.
46
47 203 Symptoms of CYP infection appeared consistently earlier than symptoms of FDP: 15-18 versus 28-30 dpi.
48

49 204

50 51 205 *CYP and FDP transmission*

52
53 206 Infection of all the test plants was established by species-specific PCR assays for both phytoplasmas.
54
55 207 Symptom development was in agreement with results of PCR detection. The total number of plants infected
56
57 208 by CYP and FDP, following single and double acquisitions, are summarised in table 1. Only transmission
58
59
60

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

219

220 *CYP and FDP acquisition*

221 The numbers of leafhoppers that acquired CYP and/or FDP following single and double AAPs are
222 summarized in Table 2. *E. variegatus* acquired CYP with higher efficiency than it acquired FDP, both
223 following a single AAP ($\chi^2 = 11.48$ with 1 d.f.; $P = 0.003$) and two subsequent AAPs ($\chi^2 = 40.952$ with 2 d.f.;
224 $P < 0.001$). The proportion of leafhoppers that became CYP-infected was significantly lower in treatments
225 providing exposure to both phytoplasmas (FDP+CYP) than treatments containing only CYP. ($\chi^2 = 11.480$
226 with 2 d.f.; $P = 0.003$). A similar phenomenon seemed to occur for FDP, though the difference was not
227 significant ($\chi^2 = 5.022$ with 2 d.f.; $P = 0.081$). *E. variegatus* acquired CYP with higher efficiency if it fed
228 first on FDP and then CYP than if it was exposed to the two phytoplasmas in the opposite order. ($\chi^2 = 4.891$
229 with 1 d.f.; $P = 0,027$). On the contrary, acquisition of FDP was not influenced by feeding order ($\chi^2 = 0.153$
230 with 1 d.f.; $P = 0.696$).

231

232 *CYP and FDP titres in infected Euscelidius variegatus*

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

1
2
3 237 indicated that CYP concentration was the same, irrespective of the acquisition conditions (single or double).
4
5 238 FDP titre in *E. variegatus* ranged from one thousand cells per ng of insect DNA at 14 dpa to a few hundred
6
7 239 thousand cells later in the infection process. One-way ANOVA for FDP titre over time showed a significant
8
9 240 increase ($P < 0.001$). FDP titre was influenced by the acquisition conditions (single or double)- Following
10
11 241 double acquisition, at 14 and 22 dpa an increase in FDP titre was recorded ($P=0.045$ and $P=0.047$,
12
13 242 respectively), while at 33 dpa a decrease was recorded ($P=0.040$). Later in the infection process FDP titres
14
15 243 measured in single or doubly-infected insects did not differ significantly. Leafhoppers infected by FDP
16
17 244 hosted a higher phytoplasma titre compared to those infected by CYP. This higher FDP titre was recorded
18
19 245 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
20
21 246 (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
22
23 247 2, Supplementary table S3).
24
25 248

27 249 *CYP and FDP detection in salivary glands and artificial feeding media*

29 250 The two phytoplasmas colonized the salivary glands with with significantly different efficiencies. Following
30
31 251 CYP + FDP acquisitions, 23/24 and 3/24 leafhoppers had CYP- and FDP-positive salivary glands,
32
33 252 respectively. Following FDP + CYP acquisitions, 15/16 and 3/16 leafhoppers had phytoplasma-positive
34
35 253 salivary glands to CYP and FDP, respectively. Consistently, CYP was detected by PCR in 11/24 feeding
36
37 254 media following CYP + FDP acquisitions and in 7/16 feeding media following the FDP + CYP acquisition,
38
39 255 while FDP was never detected in feeding media (Table 3).
40
41 256

43 257 **Discussion**

45 258 The competition between two unrelated phytoplasma species within the leafhopper vector *E. variegatus* was
46
47 259 studied following serial acquisition of the vector on plants singly infected by the chrysanthemum yellows
48
49 260 strain of the '*Ca. P. asteris*' (CYP) and the Flavescence dorée phytoplasma (FDP). The two phytoplasmas
50
51 261 were chosen as they can be both transmitted by *E. variegatus* to broad bean plants, although, as this
52
53 262 leafhopper is not the natural vector of FDP, the possibility that interactions between naturally occurring
54
55 263 phytoplasmas might be different cannot be excluded. In particular, CYP and FDP induced distinctive
56
57 264 symptoms in the basal and apical parts of the infected plants, respectively, and doubly infected broad beans
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59
60

1
2
3 265 showed both symptoms. Symptom expression in each phytoplasma – plant combination may differ due to the
4
5 266 presence of different phytoplasma effector proteins (Sugio *et al.*, 2011) and different plant responses to them.
6
7 267 *E. variegatus* transmitted CYP after a shorter LP than FDP, in line with previous experiments (this group,
8
9 268 unpublished). Also, *E. variegatus* transmitted CYP more efficiently than FDP after exposure to singly
10
11 269 infected plants, and CYP transmission was not influenced by FDP, irrespective of the sequence of
12
13 270 acquisition. On the other hand, FDP transmission was severely reduced by the acquisition of CYP,
14
15 271 irrespective of the order of exposure to both sources. A similar situation was reported for *Dalbulus maidis*
16
17 272 where prolonged acquisition of corn stunt spiroplasma suppressed maize bushy stunt phytoplasma
18
19 273 transmission (Maramorosch, 1958), and for the vector *Macrostelus quadrilineatus* (= *fascifrons*) where the
20
21 274 majority of the leafhoppers transmitted only the first phytoplasma to which they had access when allowed to
22
23 275 acquire different strains of aster yellows phytoplasmas (Freitag, 1967). In these previous studies, the absence
24
25 276 of molecular tools hampered the analyses of other analyses of aspects of the competition between mollicutes
26
27 277 other than transmission. In this study, competition was not recorded at the acquisition level, as FDP
28
29 278 acquisition efficiency was not affected by CYP in double acquisition trials, while a slight decrease was
30
31 279 observed in CYP acquisition when *E. variegatus* was also exposed to FDP. When the titre of both
32
33 280 phytoplasmas was measured within the vector body, both microorganisms actively multiplied in singly
34
35 281 infected vectors, and FDP reached a much higher concentration than CYP. In the presence of CYP, FDP titre
36
37 282 seemed to be suppressed but this effect was significant only at 33 dpa. In contrast, CYP multiplication over
38
39 283 time was not affected by the presence of FDP and also CYP titre was not influenced by co-acquisition of
40
41 284 FDP. These results indicate an unilateral competition of the two phytoplasmas within the insect body, where
42
43 285 the presence of CYP seemed to hamper the multiplication of FDP and limit the number of FDP cells in the
44
45 286 vector. Despite this competition, FDP reached higher concentration compared to CYP at later stages of
46
47 287 infection (from 22 dpa onwards) in doubly infected vectors. However, it is important to note that FDP was
48
49 288 unable to colonize the salivary glands and reach the saliva of doubly infected vectors as it was only
50
51 289 sporadically detected in dissected salivary glands of *E. variegatus* following exposure to CYP and FDP-
52
53 290 infected plants, in both orders, and it was never detected in the artificial medium following inoculation. This
54
55 291 suggests that salivary glands are the barrier where competition between CYP and FDP prevents efficient
56
57 292 FDP transmission by *E. variegatus*. It can be suggested that the phytoplasma having the shorter latent period
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3 293 in the vector, CYP, is the first to colonize salivary glands and therefore is the most competitive for
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5 294 transmission. In this case, salivary glands already colonized by a phytoplasma might not be available for
6
7 295 further invasion by a second one. FDP showed a more active multiplication in the insect body, but it did not
8
9 296 suppress CYP multiplication. Therefore, the speed of salivary glands colonization, rather than the level of
10
11 297 multiplication, is likely to be the key factor for successful transmission. This hypothesis may also explain the
12
13 298 suppression of maize bushy stunt phytoplasma (MBSP) by corn stunt spiroplasma (CSS) in the vector *D.*
14
15 299 *maidis*: CSS may move more rapidly in the insect body, thus suppressing transmission of MBSP
16
17 300 (Maramorosch, 1958). CYP transmission depends on phytoplasma titre in the salivary glands of *E.*
18
19 301 *variegatus*, as non-transmitter individuals host only a few hundred CYP cells per ng of insect DNA in their
20
21 302 salivary glands while transmitters host thousands of cells (Galletto *et al.*, 2009), therefore a high titre of
22
23 303 phytoplasma in the salivary glands seems to be a prerequisite for efficient transmission. The erratic presence
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25 304 of FDP in the salivary glands of *E. variegatus* exposed subsequently to CYP and FDP and the absence from
26
27 305 saliva (feeding media) also ruled out phytoplasma competition in the inoculated plants as possible cause of
28
29 306 the failure of FDP transmission to broad bean. A correlation between phytoplasma titre and transmission
30
31 307 efficiency occurs for CYP and two of its natural vectors: *Macrostes quadripunctulatus* sustains a higher
32
33 308 phytoplasma multiplication compared to *E. variegatus*, and it is also the most efficient vector (Bosco *et al.*,
34
35 309 2007). The same correlation does not apply to FDP and CYP transmission by *E. variegatus*, since this vector
36
37 310 sustains a higher FDP multiplication compared to CYP but transmits the latter more efficiently. This
38
39 311 apparent contradiction may be explained by the competition for the colonization of the salivary glands when
40
41 312 two phytoplasmas co-infect the same individual. From an evolutionary perspective, the phytoplasma
42
43 313 colonizing salivary glands first would obtain a fitness gain compared to a slower one (the faster, the fitter).
44
45 314 The different behaviour of the two phytoplasmas may also be explained by the interactions with the insect
46
47 315 host, besides competition between the phytoplasmas. The host immune system may recognize and suppress
48
49 316 the CYP, or the CYP may have developed mechanisms to invade the salivary glands during the long-term
50
51 317 relationship of the insect host and the phytoplasma.
52
53 318 CYP, as the other aster yellows strains, has a broad host range and therefore it is very likely that in an
54
55 319 evolutionary time scale it interacted with several different vector species. On the contrary, FDP has a very
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57 320 narrow host range (grapevine and very few alternative hosts) and it is not likely to be co-evolved with
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2
3 321 different vector species. This may contribute to explain why CYP is more competitive for transmission in *E.*
4
5 322 *variegatus*.
6
7 323 In summary, *E. variegatus* transmitted CYP with high efficiency and it was not influenced by the presence of
8
9 324 FDP, and CYP was able to colonize the salivary glands and reach saliva, consistent with the high efficient
10
11 325 transmission results. On the other hand, the good efficacy of *E. variegatus* as vector of FDP following single
12
13 326 acquisition was significantly lower when competition with CYP was introduced, although FDP persisted for
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15 327 life within the vector. *E. variegatus* is a natural vector of CYP (Bosco *et al.*, 1997; Palermo *et al.*, 2001),
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17 328 while it transmits FDP under laboratory conditions only, as it is not able to feed on grape, the natural host
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19 329 plant of FDP. In the case of *E. variegatus*-CYP association, both partners are of Palaearctic origin and
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21 330 therefore co-evolved for a long time so that phytoplasma multiplication is mitigated and no pathogenic
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23 331 effects are recorded (D'Amelio *et al.*, 2008). In contrast, the association between FDP and *E. variegatus* is
24
25 332 artificial and this can explain the phytoplasma low ability to colonize the salivary glands (needed for
26
27 333 transmission) and also its high multiplication in the vector, which causes severe pathogenic effects (Bressan
28
29 334 *et al.*, 2005). Nevertheless, even the “natural” vector of FDP, *Scaphoideus titanus* Ball, is not long co-
30
31 335 evolved with FDP because the leafhopper is of Nearctic origin (Bertin *et al.*, 2007) while FDP is very likely
32
33 336 of Palaearctic origin (Arnaud *et al.*, 2007). Therefore, it is possible that the same phenomenon also occurs in
34
35 337 *S. titanus*, but further studies are needed to demonstrate this.
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37 338 In nature, polyphagous vectors have chances of acquiring different phytoplasmas by visiting and feeding on
38
39 339 different host plants. This work, together with those of Freitag (1967) on related strains of aster yellows
40
41 340 phytoplasmas infecting *M. quadrilineatus*, Maramorosch (1958) and de Oliveira and co-workers (2007) on
42
43 341 different mollicutes within *D. maidis*, indicates that interactive transmission (up to unilateral suppression) is
44
45 342 more common than non-interactive transmission, as this latter has not been reported so far for phytoplasmas.
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47 343 Interestingly, unilateral suppression occurs between both genetically related (Freitag, 1967) and un-related
48
49 344 phytoplasmas (this work). This is the first time that interaction / competition of different phytoplasmas has
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51 345 been described and dissected at the acquisition, multiplication, body colonization and transmission levels.
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53 346 The role of interactive transmission in nature must be taken into account for the description and prediction of
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55 347 phytoplasma epidemiology, as different phytoplasmas are likely to be present in different environments and
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3 348 interactive transmission may contribute to explain differential spread of the same phytoplasma in the same
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5 349 crop under different environmental conditions.

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9
10 352 **Acknowledgements**

11
12 353 MR was supported by a grant from Piedmont Region within the project “FLADO”, RD’A was supported by
13
14 354 a grant from Piedmont Region within the project “Valutazione dell’azione di microrganismi rizosferici ed
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16 355 elicitori di resistenza sull’infezione da fitoplasmi in un sistema modello (CIPE 2006).

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36 466 **Table and Figure legends**

37
38 467 Table 1. Results of transmission experiments of chrysanthemum yellows (CYP) and Flavescence
39
40 468 dorée (FDP) phytoplasmas with *Euscelidius variegatus* to broad bean plants. Number of
41
42 469 infected/exposed plants as determined by species-specific nested PCR are reported, following single
43
44 470 (CYP only; FDP only) and double (CYP + FDP; FDP + CYP) acquisitions. Group transmissions for
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46 471 3-4 day IAPs from 28 dpa onwards. AAP, acquisition access period. Within columns, figures
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48 472 followed by the same letter do not differ significantly (χ^2 test).
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53
54 474 Table 2. Results of acquisition experiments of *Euscelidius variegatus* following AAP on
55
56 475 chrysanthemum yellows (CYP) and Flavescence dorée (FDP) -infected broad bean plants. Number
57
58 476 of infected leafhoppers are reported following single (CYP only; FDP only) and double (CYP +
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3 477 FDP; FDP + CYP) acquisitions as determined by species-specific nested-PCR tests. AAP,
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5 478 acquisition access period. Within columns, figures followed by the same letter do not differ
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7 479 significantly (χ^2 test).
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11 481 Table 3. PCR detection (presence: +; absence: -) of chrysanthemum yellows phytoplasma (CYP)
12 and Flavescence dorée phytoplasma (FDP) in salivary glands of single *Euscelidius variegatus* and
13
14 482 in corresponding sucrose feeding media following feeding on CYP- and FDP-infected source plants
15
16 483 in both orders. The analysis was carried out 35 days post acquisition. Each line corresponds to a
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18 484 single insect.
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25 487 Figure 1. Results of phytoplasma transmission experiments with *Euscelidius variegatus*. A:
26
27 488 chrysanthemum yellows phytoplasma (CYP) transmission following acquisition on CYP-infected
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29 489 plants or on CYP- and Flavescence dorée phytoplasma (FDP)- infected source plants. B: FDP
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31 490 transmission following acquisition on FDP-infected plants or on CYP- and FDP- infected source
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33 491 plants. AAP, acquisition access period.
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37
38 493 Figure 2. Mean \pm SE quantities of chrysanthemum yellows (CYP) and Flavescence dorée (FDP)
39
40 494 phytoplasmas (log of cells per ng of insect DNA) measured at different days post acquisition in
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42 495 *Euscelidius variegatus* leafhoppers fed on CYP source only, FDP source only, CYP followed by
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44 496 FDP (CYP + FDP) and FDP followed by CYP (FDP + CYP).
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49 498 Supplementary Figure S1. Symptoms of chrysanthemum yellows (A) and Flavescence dorée (B)
50
51 499 phytoplasmas on broad bean plants.
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56 501 Supplementary TableS2. Results of chrysanthemum yellows (CYP) and Flavescence dorée (FDP)
57
58 502 phytoplasma transmission experiments with *Euscelidius variegatus* following acquisition on CYP-
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3 503 infected plants, on FDP-infected plants, or on both source plants. Number of phytoplasma infected
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5 504 plants over exposed ones are reported, as determined by species-specific nested-PCR tests. AAP,
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7 505 acquisition access period.
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11 507 Supplementary TableS3. Summary of the statistical analyses performed on the phytoplasma titres
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13 508 measured by R-PCR in *Euscelidius variegatus* individual leafhoppers following different
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15 509 phytoplasma acquisition conditions. Raw data (phytoplasma cells/ ng of total insect DNA) were
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17 510 transformed into the logarithm for the analyses.
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3 513 Table 1. Transmission of chrysanthemum yellows (CYP) and Flavescence dorée (FDP)
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5 514 phytoplasmas. Numbers of infected/exposed plants as determined by species-specific nested PCR
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7 515 are reported, following single (CYP only; FDP only) and double (CYP + FDP; FDP + CYP)
8
9 516 acquisitions. Group transmissions for 3-4 day IAPs from 28 dpa onwards. AAP, acquisition access
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11 517 period. Within columns, figures followed by the same letter do not differ significantly (χ^2 test).
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518

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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520

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

527

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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529

530 Table 3. PCR detection (presence: +; absence: -) of chrysanthemum yellows phytoplasma (CYP)
 531 and Flavescence dorée phytoplasma (FDP) in salivary glands of single *Euscelidius variegatus* and
 532 in corresponding sucrose feeding medium following feeding on CYP- and FDP-infected source
 533 plants in both sequences. The analysis was carried out 35 days post acquisition. Each line
 534 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.

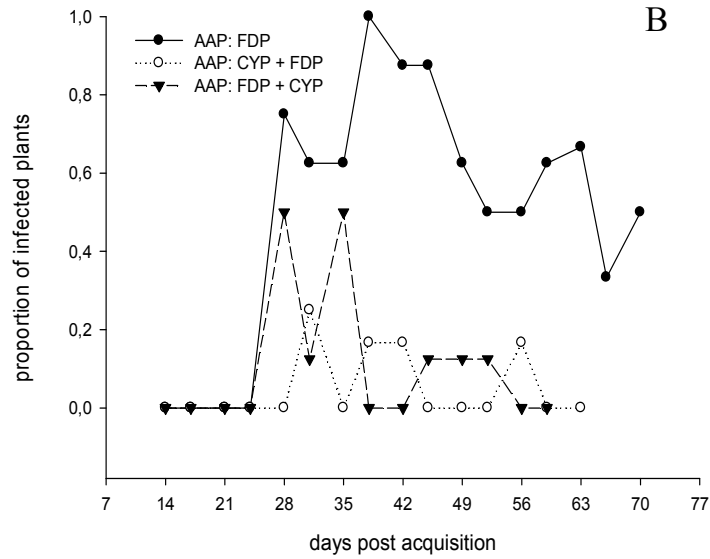
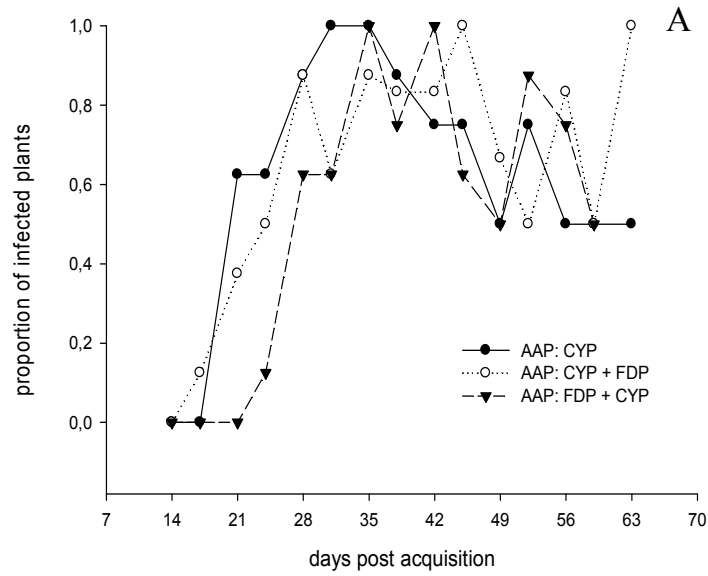
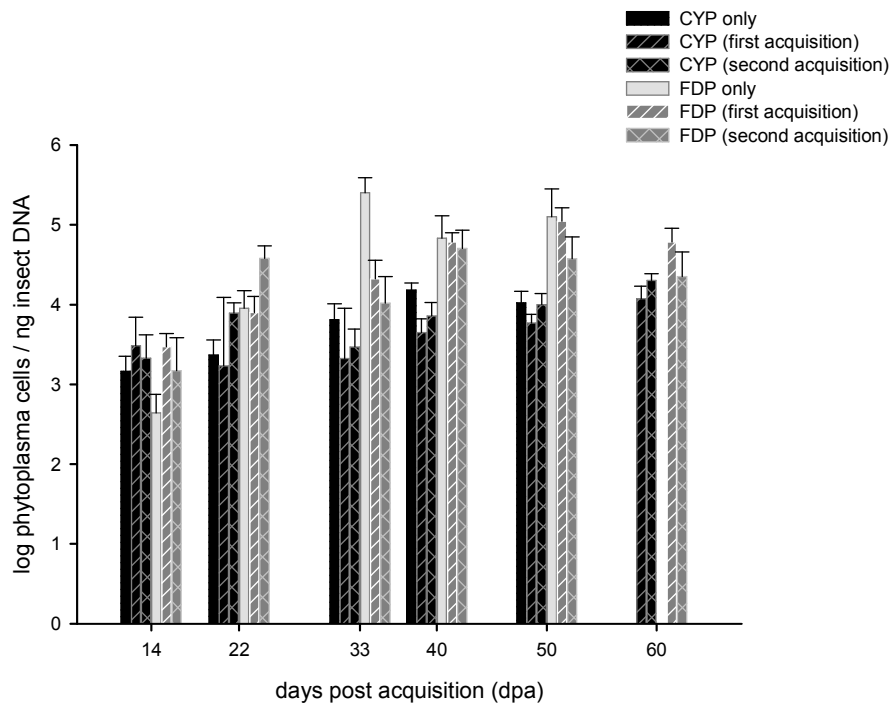


Figure 2



Peer Review

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