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Transmission of *Grapevine virus A* and *Grapevine leafroll-associated viruses 1* and *3* by *Planococcus ficus* and *Pl. citri* fed on mixed-infected plants

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Abstract

The *Grapevine virus A* (GVA) and *Grapevine leafroll-associated viruses 1* and *3* (GLRaV-1 and GLRaV-3) are associated with grapevine diseases that induce severe reductions in yield and berry quality. These three viruses are known to coexist in both grapevine and insect vectors, but their co-transmission has been poorly characterized so far. This study investigates the acquisition and transmission of GLRaV-1, GLRaV-3 and GVA by *Planococcus ficus* and *Pl. citri* (Hemiptera: Pseudococcidae*)* following feeding on multiple-infected plants. The retention and load of each virus in insects were analysed, and a method for the quantification of GVA and GLRaVs in the vectors was set up for the first time. After feeding onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines, nymphs of *Pl. ficus* and *Pl. citri* showed similar virus acquisition rates and retained low quantities of viruses until the third post-acquisition day. Despite the similar acquisition patterns, the two vectors differed in transmission efficiency: *Pl. ficus* showed a higher efficiency in transmitting GVA and GLRaV-3, whereas *Pl. citri* transmitted more efficient in GLRaV-1. When focusing on the virus co-transmission, it appears that GVA could be transmitted to grapevine without GLRaV-1 and/or GLRaV-3 and that the GLRaVs transmission could take place in absence of GVA. This comparative study involving different viruses and vector species improves the current knowledge of the semi-persistent transmission of these three viruses and contribute to the understanding of the grapevine virus epidemiology.

Keywords

Mealybug, *Vitis vinifera*, Leafroll, Rugose wood.

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crop worldwide and represents a highly valuable commodity. The global grapevine production is threaten by many pests and pathogens, including viruses. Among the virus-induced diseases, the Grapevine Leafroll (GLD) and Rugose Wood (RW) are the most widespread and are responsible for severe reductions in yield and quality (Naidu *et al.*, 2014).

GLD and RW diseases are caused by positive single-stranded RNA viruses belonging to two distinct taxonomical groups and are frequently found in mixed infections. GLD is associated with a complex of virus species in the family *Closteroviridae* that are collectively referred to as *Grapevine leafroll-associated viruses* (GLRaV-1, -2, -3, -4, -7; Martelli *et al.*, 2012). RW includes different syndromes associated with virus species belonging to the family *Betaflexiviridae*, genus *Vitivirus* (*Grapevine virus A*, *B*, *D*, *E* and *F*; GVA, GVB, GVD, GVE and GVF) and *Foveavirus* (Grapevine rupestris stem pitting associated virus; GRSaV) (Martelli, 2014b). Both GLRaVs and RW-associated viruses are phloem-restricted and cause distinctive symptoms in grapevine, such as colour alteration and downward rolling of the leaves (GLD), stem grooving (RW), as well as delay in fruit ripening and alteration of final products (Martelli, 2014a; Martelli, 2014b).

Plant-to-plant transmission of GLRaVs and RW-associated viruses is mediated by phloem-sucking insects, mealybugs (Hemiptera: *Pseudococcidae*) and soft scales (Hemiptera: *Coccidae*). Compared to soft scales, mealybugs are likely to play a major role in virus spread because of their higher mobility and they are responsible for severe damages even at low infestation levels (Golino *et al.*, 2008; Cabaleiro *et al.*, 2008; Cabaleiro and Segura, 2006). Although all life stages of mealybugs are capable of transmission, first-instar nymphs are known to be the most efficient vectors (Tsai *et al.*, 2008; Petersen and Charles, 1997; Le Maguet *et al.*, 2012a).

The closteroviruses GLRaV-1 and GLRaV-3, known to be closely related within the genus

Ampelovirus (Maliogka *et al.*, 2008), are regarded as critical pathogens of grapevine because of severity of the associated symptoms and their wide distribution. The transmission of these two GLRaVs often occurs together with GVA, the vitivirus that has been associated with the Kolber stem grooving syndrome (a disease of the RW complex) and recently with the Shiraz disease. The distribution of the three viruses overlap in many regions across all the continents (Sforza *et al.*, 2003; Bertin *et al.*, 2010; Le Maguet *et al.*, 2012b) and their co-infections in grapevine are frequently reported (Pacifico *et al.*, 2011; Voncina *et al.*, 2011; Fuchs *et al.*, 2009).

GLRaV-1, GLRaV-3 and GVA also share insect vectors, among both mealybug and soft scale species (Fuchs *et al.*, 2009; Bertin *et al.*, 2010). *Planococcus ficus* Signoret and *Planococccus citri* Risso are involved in GLRaVs and GVA transmission and are regarded as a serious threat for viticulture (Cabaleiro and Segura, 1997a; Rosciglione and Castellano, 1985; Tsai *et al.*, 2010; Scotto *et al.*, 2009; Engelbrecht and Kasdorf, 1990; Golino *et al.*, 2002). These two mealybugs often coexist in several areas: Africa, Northern, Central and Southern America, Oriental Asia, Middle East and Mediterranean countries (Ben-Dov, 2001). The two species show similar ecological and biological features, and are known to easily hybridize in experimental conditions (Kol-Maimon *et al.*, 2014). Their morphology-based discrimination is awkward and it has been recently unravelled by the development of DNA markers (Cavalieri *et al.*, 2008; Daane *et al.*, 2011; Malausa *et al.*, 2011; Saccaggi *et al.*, 2008). The current data on virus acquisition, retention and inoculation indicate that both *Pl. ficus* and *Pl. citri* transmit the viruses in a semi-persistent manner (Cabaleiro and Segura, 1997b; Tsai *et al.*, 2008), although evidence of GLRaV-3 presence in the salivary glands of *Pl. citri* has been reported (Cid *et al.*, 2007).

Despite the occurrence of GLRaVs and RW-associated viruses mixed infections in vineyard, the co-transmission of these viruses has been poorly characterized. Indeed most of the studies focused on transmission of the sole GLRaV-3, that has emerged as the key virus of grapevine throughout the

world (Maree *et al.*, 2013). Moreover, the surveys of other grape viruses often did not go beyond the identification of new vector species and rarely investigated the relative efficiency of a vector in transmitting different viruses as well as the possible interaction among viruses during transmission (Tsai *et al.*, 2010; Le Maguet *et al.*, 2012a). Therefore, comparative studies involving different GLRaVs / RW-associated viruses and different vector species at a time would improve the current knowledge of transmission process and contribute to understand the epidemiology of diseases.

In the present study, the acquisition and transmission rate of GLRaV-1, GLRaV-3 and GVA by two different mealybugs, *Pl. ficus* and *Pl. citri*, were investigated under controlled conditions. Moreover, the virus retention was investigated and the virus load was measured by quantitative real-time RT-PCR (qRT-PCR) in the mixed-infected source grapevine as well as in the vectors. qRT-PCR was already applied to estimate the viral load in the host plants (Pacifico *et al.*, 2011; Osman *et al.*, 2012; Tsai *et al.*, 2012) as well as in insect vectors (Mason *et al.*, 2008; Debreczeni *et al.*, 2011), but this is the first application for the study of virus load in mealybug vectors.

Material and Methods

Insect and plant material

Specimens of *Pl. ficus* and *Pl. citri* were collected on vines in Liguria and Sicily regions (Italy) respectively, and were used to establish colonies in climatic chambers. The colonies were maintained on sprouted potatoes in the dark at 20-30°C. The two species were identified by means of morphological observations as well as amplification and sequence analysis of the *Planococcus* mitochondrial *cytochrome c oxidase subunit 1* gene (Saccaggi *et al.*, 2008).

Infected plant material was from an experimental vineyard planted with *Vitis vinifera* cv. Nebbiolo and situated in the Langhe, a traditional wine-producing area of Piemonte (North-western Italy). The sanitary status of the vineyard was regularly monitored for the presence of ampelovirus, vitivirus, maculovirus and nepovirus infection by serological (DAS-ELISA) and molecular analysis (PCR) (Gambino and Gribaudo, 2006; Gribaudo *et al.*, 2009). Based on these data, several grapevines carrying the GLRaV-1, -3 and GVA mixed-infection were identified. One of these plants served as "mother plant" and provided the source cuttings for the laboratory experiments. In detail, dormant shoots collected from this plant were rooted and maintained in a greenhouse, until they were about 50 cm tall and then used as source plants. Each sprouting cutting was further checked for GLRaV-1, GLRaV-3 and GVA infection before being used for the virus acquisition. Healthy grapevines obtained through micropropagation of clonal lines of *V. vinifera* of cv Barbera were used as test plants in the transmission assays.

Transmission experiments

Three repetitions of transmission trials were performed for *Pl. ficus* as well as for *Pl. citri*. In each experiment, a leaf-strewn branch from one of the virus-source cuttings generated from the mother plant was laid on the mealybug rearing. The mealybugs were allowed to move onto the branch for

four hours; then the branch was removed and maintained in a flask of water for 24 hours. After this 24h acquisition access period (AAP), the mealybugs were gently shaken off the source branch onto a dark paper. The first instar nymphs were isolated, and partly kept for RNA extraction and virus detection and partly transferred to the test plants for virus transmission. The transmission was carried out by caging five first-instar nymphs on the upper leaf surface of each test plant for 48h. Only the first-instar nymphs were used as they are known to be the most efficient vectors of grapevine viruses. Since the position of the leaves where the mealybugs fed during AAP (i.e. basal, intermediate or apical leaves) does not affect the transmission rate (Tsai *et al.*, 2011), the nymphs used for transmission were randomly chosen among the whole batch recovered on the dark paper. After the virus inoculation access period (IAP) of 48h, the first instars were removed from the test plants and these were drench-treated with a systemic insecticide (Actara, Syngenta Crop Protection). The test grapevines were maintained in a greenhouse and sprayed regularly with insecticide and fungicide for four-five months, until the RNA extraction and GLRaV-1, -3 and GVA detection. All plants were periodically pruned to avoid overgrowth.

Thirty, 18 and 10 test plants were inoculated by *Pl. ficus* in three transmission experiments and 27, 14 and 10 grapevines respectively lived through the growing period until the GVA, GLRaV-1 and GLRaV-3 detection. In the three experiments, a total of 71 first instar nymphs (8, 38 and 25 per trial) were assayed after 24h-AAP to estimate the virus acquisition rate. Three transmission trials were also performed for *Pl. citri*: 26, 19 and 10 test plants per experiment were inoculated and 25, 17 and 7 grapevines respectively were surviving for virus diagnosis. A total of 74 first instar nymphs (30, 29 and 15 per experiment) were analysed at the same time for GVA, GLRaV-1 and GLRaV-3 presence. Specimens from healthy colonies of *Pl. ficus* and *Pl. citri* were used as negative controls. To confirm the virus-free sanitary status of the test plants and to ensure that virus spread had not occurred within the greenhouse during experimental periods, *in vitro*-derived grapevines from the

same batch of test plants were not exposed to mealybug vectors and served as negative controls. Differences in virus acquisition and virus transmission frequencies within and between the two mealybug species were tested by chi-square (χ^2) analysis (SigmaPlot 11.0). Fisher Exact Test was used instead of χ^2 when the frequencies of one or more observations were less than five.

Retention experiments

The retention and the load of GLRaV-1, -3 and GVA were investigated in *Pl. citri* and *Pl. ficus*. Each mealybug species was caged to feed onto a virus-infected vine branch as described above. After an AAP of 24 hours, the first instar nymphs were partly collected for virus diagnosis (T_0) and partly transferred onto potatoes for post-acquisition feeding. The potato is known to be a non-host of grapevine viruses. Further nymphs were taken from potato sprouts after 24, 48, 72 and 96 hours from the end of the AAP (T_{24}, T_{48}, $T_{72,}$ T_{96}), and tested for virus presence. Samples that resulted positive to at least one of the three viruses at different post-acquisition times were further analysed by qRT-PCR to estimate the viral load.

RNA purification from insects and plants

The RNA for virus detection was purified from the *Pl. citri* and *Pl. ficus* nymphs collected after 24h-AAP in the transmission experiments and collected at T_0, T_{24}, T_{48}, $T_{72,}$ T_{96} in the retention experiments. Total RNA was extracted from single nymphs using the TRIzol® Reagent (Invitrogen) and following the manufacturer's instructions. Samples were treated with 2 units of RNase-Free DNase I (Applied Biosystems) in the supplied buffer to avoid residual DNA contamination. After DNA digestion, DNase was inactivated by phenol/chloroform extraction. RNA was finally resuspended in 20 µl of RNase-free water containing diethylpyrocarbonate (DEPC) 0.1 %.

Concentration and purity of extracts were evaluated using the ND-1000 Spectrophotometer (Nanodrop Technologies). The RNA was then diluted to 10 ng μl^{-1} and stored at -80 °C.

The plant RNA was extracted from sprouting cuttings before each experiment, in order to select the source plants carrying GLRaV-1, GLRaV-3 and GVA, and from the test grapevines at the end of the transmission trials. The RNA was purified from a total of 0.1 g of midribs from both basal and apical leaves. The extraction was performed using the Concert™ Plant RNA Isolation Reagent (Invitrogen) and following the manufacturer's instructions. RNA was resuspended in 30 µl of DEPC 0.1 % RNase-free water, diluted to 10 ng μl^{-1} and stored at -80 °C.

Virus detection

The virus detection from both insect and plant RNA extracts was carried out by SYBR® Green real-time RT-PCR assays in the Chromo4 Real Time Thermal Cycler (Bio-Rad) supported by the OpticonMonitor 3.1.32 software (Bio-Rad). The GVA, GLRaV-1 and GLRaV-3 were detected with the following primer pairs, designed on the viral coat protein sequences: GVA-C7273 (5'-CATCGTCTGAGGTTTCTACTA-3') / GVA-H7038 (5'-AGGTCCACGTTTGCTAAG-3') (MacKenzie *et al.*, 1997); GLRaV-1fw (5'-CGTTTGAAAATCCTATGCGTCAG-3') / GLRaV-1rev (5'-GCAACTTTCTCGTTCGGCTTC-3') and GLRaV-3fw (5'-TTCGAGAAAGATCCAGACAAGTTC-3') / GLRaV-3rev (5'-ATAACCTTCTTACACAGCTCCATC-3') (Gribaudo *et al.*, 2009). The real-time RT-PCR was performed with the Iscript One-Step RT-PCR kit (Bio-Rad), using a final primer concentration of 300 nM. Ten nanograms of insect or plant total RNA were used as template. For all the primer pairs, the thermo-cycling conditions consisted of an initial cycle at 50°C for 10 min, followed by 5 min at 95°C and 40 cycles at 95°C for 10 s and 60°C for 60 s. Melting curves were produced at the end of the PCR to assess the reaction specificity: the PCR products were heated to 95°C for 1 min, cooled at 65°C for 1 min and then slowly heated back to 95°C at a rate of 0.5°C per cycle.

RNA of mealybugs or vines carrying GVA, GLRaV-1 and GLRaV-3 were used as positive controls. RNA of mealybugs from healthy colonies and from healthy *in vitro*-derived grapevines served as negative controls.

Virus quantification

Set up of a quantitative real-time RT-PCR assay. Viral load was measured in relation to the transcript copy number of a mealybug reference gene; this, rather than absolute quantitation, was chosen to avoid the influence of different yields during RNA extractions (Pacifico *et al.*, 2011). To identify the most stable insect gene upon mealybug development, the *actin β* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *18S ribosomal RNA* genes were selected among those suggested in the literature for gene expression studies in virus-vector insects (Maroniche *et al.*, 2011; Rotenberg *et al.*, 2009). The *18S rRNA* was amplified using the primers MqFw and MqRv, available from Marzachí and Bosco (2005) (Table 1). The sequences of the other two genes were not available for insects in the family *Pseudococcidae.* Therefore, the degenerated primers ActinDegF1 (5'-YGAYTGGARAARATCTGGC-3') / ActinDegR2 (5'-CGTCGTAYTCYTGYTTSGAG-3') and GAPDegF1 (5'-GGTATCAATGGWTTTGGC-3') / GAPCoccR2 (5'-CAWAYTCRTTGTCGTACC-3') were designed on the homologous genes of the aphid *Acyrthosiphon pisum*. The primers were tested in conventional PCR using 2 µl of *Pl. ficus* and *Pl. citri* cDNA as templates, under the following conditions: denaturation for 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 48°C and 1 min 30 s at 72°C; and a post-dwell period of 5 min at 72°C. PCR products were analysed by electrophoresis through a 1.5 % agarose gel in 1 × Tris-borate-EDTA (TBE) buffer along with a 1 kb plus DNA size marker (Gibco BRL), and visualised on a UV transilluminator. PCR products were isolated from the gel using the PureLink™ (Invitrogen), ligated into the pGEM-T easy vector (Promega) and transformed in *E. coli* DH5α. Plasmids were purified with the Fast Plasmid Mini kit

(Eppendorf AG) and sequenced (BioFab Research) with the universal primers M13F/R. Raw sequence data were manually edited with the DNAman program vers. 4.02 (Lynnon BioSoft) after at least twice the sequencing coverage for each nucleotide position. *ACTB* and *GAPDH* sequences of both *Pl. citri* and *Pl. ficus* were trimmed and aligned with the DNAman 4.02 program (Lynnon BioSoft). Finally, the specific primer pairs PfActFw2/PfActBRev1 and PfGapFw1/PfGapRev were designed (Primer Express tool; Applied Biosystems) (Table 1).

The primer specificity for the three target genes was tested in SYBR® Green Real-Time PCR assays. Two microliters of *Pl. citri* and *Pl. ficus* cDNA were added to a 2× iQ™ SYBR® Green Supermix (Bio-Rad) supplemented with 500 nM each primers and DEPC 0.1 % water to a final volume of 25 µl. Reaction conditions were as follows: 95°C for 5 min followed by 40 cycles of 30 s at 95°C and 90 s at 60°C (62°C for *GAPDH*). Melting curves were produced at the end of each specific real-time amplification.

To identify the most stable insect gene upon mealybug development, total RNA was singly extracted from three newly-hatched nymphs, three third instar nymphs, and three female adults of both *Pl. citri* and *Pl. ficus*, following the RNA extraction procedures detailed above. RNA extracts were then reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamers, according to the manufacturer's protocol.

For the calculation of reaction efficiencies (E), the cDNAs from nymphs and adults of the two species were pooled and diluted to be used as real-time PCR standards. Tenfold serial dilutions of standard cDNA (1:1, 1:10, 1:100 and 1:1000) in DEPC 0.1% RNase-free water were run in triplicate for each PCR system (ACTB, GAPDH and 18S rRNA). Standard curves were constructed by linear regression analysis of the threshold cycle (Ct) value of each of the standard dilution replicates versus the Log of arbitrary concentration values attributed to each cDNA dilution (1000, 100, 10, 1,

respectively). SYBR® Green Real-Time PCR conditions were the same described above. Data acquisition and analysis were handled by the OpticonMonitor 3.1.32 software (Bio-Rad).

To determine the best reference gene, the cDNA of each sample was run in duplicate under the same reaction conditions of the standards. The average Ct value of each sample was converted using the ΔCt method and the calculated PCR efficiency, then analysed with geNorm (Vandesompele *et al.*, 2002), Bestkeeper (Pfaffl *et al.*, 2004) and Normfinder (Andersen *et al.*, 2004) software, according to the authors' instructions.

Virus quantification. Within the retention experiments, a subset of *Pl. citri* and *Pl. ficus* nymphs carrying at least one virus among GVA, GLRaV-1 and GLRaV-3 was selected for virus quantification. The virus load was also assessed in the mother plant providing the source cuttings used for virus acquisition. The *RNA-dependent RNA polymerase* (*RdRp*) gene was chosen as target for virus RNA amplification. The *RdRp* gene of GVA, GLRaV-1 and GLRaV-3 was amplified by one-tube TaqMan® qRT-PCR, using primers and probes specific for each virus and described in Pacifico *et al.* (2011).

The insect *GAPDH* resulted to be the most stable reference gene (see Results) and was chosen as target for insect cDNA amplification. The *GAPDH* sequence was amplified by one-tube TaqMan® qRT-PCR, using the primer pair PfGapFw1/PfGapRev and the corresponding probe PfGapProbe (Table 1). Ten nanograms of total RNA extract from each insect were run in triplicate together with at least three dilutions of insect *GAPDH* and virus standard RNAs. Reaction conditions were the same reported by Pacifico *et al.* (2011). The standard RNAs for the absolute quantification of the viral genome copies were prepared as detailed in Pacifico *et al.* (2011). The insect standard RNAs were obtained by *in vitro* transcription of *Spe*I (Promega) linearized pGemGAPDHf with the MAXIscript® in vitro Transcription Kit (Applied Biosystems), following the manufacturer's instructions. To eliminate plasmid DNA, RNA was treated with two units of RNase-free DNase I (Applied Biosystems) in the supplied buffer. Following phenol/chloroform extraction, RNA was

dissolved in 30 µl of DEPC 0.1 % RNase-free water, and analysed using the ND-1000 Spectrophotometer. The number of RNA copies per microlitre was calculated according to Pacifico *et al.* (2011). RNA was diluted, distributed in aliquots and stored at –80°C. Tenfold serial dilutions of this RNA in DEPC 0.1% RNase-free water were run in triplicate under the following conditions: 52°C for 30 m, 5 min at 95°C, 45 cycles of 15 s at 95°C, 30 s at 52°C and 30 s at 60°C.

The viral load in mealybugs was expressed as viral Genome Units (GU) per insect *GAPDH*: the absolute quantity of the viral GU and *GAPDH* transcripts of each insect sample were derived from the raw qRT-PCR data, and the concentration of each virus was finally related to the *GAPDH* transcript copies, according to Pacifico *et al.* (2011). The GVA, GLRaV-1 and GLRaV-3 load was also measured in the mother plant by relating the viral GU with plant *GAPDH* transcript copy. The experimental protocol was according to Pacifico *et al.* (2011).

Results

Virus transmission

The results of GVA, GLRaV-1 and GLRaV-3 diagnosis in *Pl. ficus* and *Pl. citri* as well as in test plants are summarized in Table 2. The data of the three transmission experiments performed for each vector were cumulated as the results of the three repeats did not differ significantly. No viruses were detected in healthy mealybugs and *in vitro*-derived grapevines used as negative controls. Concerning the experiments carried out with *Pl. ficus*, at least one of the viruses was detected in 50 of the 71 (70%) first-instar nymphs assayed after 24h-AAP as well as in 27 of the 51 (53%) inoculated grapevines. Out of the positive *Pl. ficus* nymphs, the GVA rate (74%) was higher than GLRaVs rates ($\chi^2 = 9.90$, $df = 2$, $P < 0.01$). GLRaV-1 and GLRaV-3 showed similar acquisition frequencies (46% and 48%, respectively) but greatly differed in transmission frequencies ($\chi^2 = 17.26$, $df = 1$, $P < 0.001$), occurring in the test plants at the lowest (30%) and the highest (89%) rate

respectively. GLRaV-3 transmission efficiency was higher than acquisition efficiency, but the acquisition rate was assessed on single nymphs whereas the transmission experiments were performed with groups of five insects per plant. The maximum-likelihood estimator *P*s, calculated according to Swallow (1985), allows to infer the proportion of infected insects within each five-insect batch used for transmission. An estimated proportion of 0.36 *Pl. ficus* nymphs transmitted GLRaV-3. This value approaches the proportion of single nymphs that acquired this virus after 24h-AAP (0.48) and provides indications of the high GLRaV-3 transmission potential of *Pl. ficus*. GVA, GLRaV-1 and GLRaV-3 were all acquired and transmitted preferably as mixed rather than single viruses by *Pl. ficus* ($P < 0.05$ for the comparisons between single and mixed infections for all the three viruses at both acquisition and transmission steps). Within the mixed infections, the association of GVA + GLRaV-1 was found in the nymphs after 24h-AAP but not in the test plants exposed to the viruliferous mealybugs (Table 2).

Grapevine viruses were detected in 46 of the 74 (62%) tested *Pl. citri* first-instar nymphs, both in single and mixed infections. At least one virus was found in 34 of the 49 (69%) test plants inoculated by *Pl. citri*. The acquisition rate of GLRaV-1 and GLRaV-3 was the same (67% of the virus-positive nymphs; Table 2) and did not significantly differ from the GVA rate (83%; $\chi^2 = 2.09$, $df = 1$, $P = 0.149$). On the other hand, the frequency of GVA, GLRaV-1 and GLRaV-3 transmission (9%, 70% and 47% of the virus-positive test plants, respectively) significantly varied ($\chi^2 = 27.10$, $df = 2$, $P < 0.001$); this was mainly due to the low transmission of GVA, which was inoculated only to three grapevines, together with GLRaV-3 or with GLRaV-1 + GLRaV-3. The relative rates of GLRaV-1 and GLRaV-3 single and mixed infections differed in acquisition and transmission. Indeed, the two ampeloviruses were mainly acquired by *Pl. citri* in mixed infections (acquisition rate of single GLRaV-1 = 11%, acquisition rate of GLRaV-1 mixed infections = 56%, GLRaV-1 single *vs* mixed acquisitions: $\chi^2 = 19.46$, $df = 1$, $P < 0.001$; acquisition rate of single GLRaV-3 = 2%, acquisition rate

of GLRaV-3 mixed infections = 64%, GLRaV-3 single *vs* mixed acquisitions: $\chi^2 = 38.14$, *df* = 1, *P* < 0.001). However, the GLRaV-1 transmission occurred more frequently in single, 53%, than in mixed infections, 18%, (GLRaV-1 single *vs* mixed transmissions: $\chi^2 = 7.79$, *df* = 1, *P* < 0.01) and the transmission rates of GLRaV-3 single and mixed infections were the same (24%, Table 2). Within the mixed infections, the association GVA + GLRaV-1 + GLRaV-3 was acquired and transmitted by *Pl. citri* at different rates: the GVA + GLRaV-1 + GLRaV-3 frequency was the highest in nymphs (43%; *P* < 0.05 for all the comparisons: GVA + GLRaV-1 + GLRaV-3 *vs* GVA + GLRaV-1, GVA + GLRaV-1 + GLRaV-3 *vs* GVA + GLRaV-3 and GVA + GLRaV-1 + GLRaV-3 *vs* GLRaV-1 + GLRaV-3), but did not vary significantly in test plants (3%) compared to the other mixed infections (*P* > 0,05 for all the comparisons: GVA + GLRaV-1 + GLRaV-3 *vs* GVA + GLRaV-1, GVA + GLRaV-1 + GLRaV-3 *vs* GVA + GLRaV-3 and GVA + GLRaV-1 + GLRaV-3 *vs* GLRaV-1 + GLRaV-3).

The comparison between *Pl. ficus* and *Pl. citri* experimental data showed that the acquisition efficiency of the two species did not significantly vary for either GVA, or GLRaV-1, or GLRaV-3 (*P* > 0.05 for all the comparisons), though *Pl. citri* acquired the three viruses together with a higher efficiency ($\chi^2 = 12.26$, *df* = 1, *P* < 0.001). On the other hand, the two mealybugs differently transmitted the viruses to the test plants. Indeed, the overall GVA and GLRaV-3 transmission frequencies were higher for *Pl. ficus* than *Pl. citri* ($\chi^2 = 17.63$, *df* = 1, *P* < 0.001 for GVA; $\chi^2 = 9.88$, *df* = 1, *P* < 0.01 for GLRaV-3), whereas *Pl. citri* was significantly more efficient in transmitting GLRaV-1 ($\chi^2 = 8.55$, *df* = 1, *P* < 0.01), mainly as single infection ($\chi^2 = 14.79$, *df* = 1, *P* < 0.001). Looking at the transmission of virus mixed infections, it is worthy to note that *Pl. ficus* transmitted GVA + GLRaV-3 at a higher frequency than *Pl. citri* ($\chi^2 = 8.92$, *df* = 1, *P* < 0.01) and that both the vectors carried GVA + GLRaV-1 but they did not transmit this virus association to test plants.

Virus retention by *Pl. citri* and *Pl. ficus*

Both *Pl. citri* and *Pl. ficus* were daily assayed for virus retention up to four days after a 24h-AAP. A total of 218 individuals of *P. ficus* were analysed at T_0 (*n*=46), T_{24} (*n*=45), T_{48} (*n*=43), T_{72} (*n*=44) and T_{96} (*n*=40) by SYBR® Green real-time RT-PCR assays. The 72%, 46% and 11% of the samples tested at T_0 were positive to GVA, GLRaV-1 and GLRaV-3, respectively. The infection rate of GVA, GLRaV-1 and GLRaV-3 decreased from T_0 to T_{72} and none of the viruses was detected at T_{96} (Fig. 1a).

One hundred individuals of *Pl. citri* were analysed, twenty at each sampling point. In this species, the trends of GVA and GLRaV3 retention were similar to those observed in *Pl. ficus*. A slightly different retention trend of GLRaV-1 was observed (with a peak of virus presence at T_{72}), likely due to the small sample size (Fig. 1b).

Virus quantification

The absolute quantity of GLRaV-1, GLRaV-3 and GVA genomes in infected *Pl. ficus* and *Pl. citri* as well as in the source plant were measured by qRT-PCR. The *RdRp* gene of each virus was quantified and normalized on the expression level of insect/plant mRNA.

To choose the best insect reference gene, the expression stability of the *ACTB*, *GAPDH* and *18S rRNA* genes was verified. Based on the SYBR® Green Real-Time PCR efficiency calculated for each amplification system, Bestkeeper, GeNorm and Normfinder softwares ranked the *GAPDH* as the most stable gene (Table 1). Therefore the viral load in the mealybugs was expressed as viral GU per insect *GAPDH* transcript copy.

For the absolute quantification of viral GU and insect *GAPDH* transcripts, specific standard curves were obtained by running 10-fold serial dilutions of insect and virus standards RNAs. The *GAPDH* standard curve covered a range between 10^9 and 10^3 RNA copies, showing a correlation coefficient (R^2) = 0.996 and a reaction efficiency of 75%. For the GLRaV-1, GLRaV-3 and GVA absolute

quantification, the standard curves covered a range between 10^8 and 10^2 copies of transcript RNA, with the same R^2 and reaction efficiencies reported in Pacifico *et al.* (2011). The calculated *GAPDH* mRNA mean absolute quantities were 3.19×10^5 (SE = 1.06×10^5) in *Pl. ficus* and 1.67×10^5 (SE = 3.10×10^4) in *Pl. citri*, respectively.

GVA positive *Pl. ficus* (19 individuals) and *Pl. citri* (18 individuals) were analysed to quantify virus load. Virus load was above the quantification threshold in five *Pl. ficus* and four *Pl. citri* at T_0, and in two *Pl. ficus* at T_{48}, only (Table 3). GLRaV-1 positive *Pl. ficus* (23 individuals) and *Pl. citri* (7 individuals) were analysed to quantify virus load. As occurred for GVA, GLRaV-1 load was below the quantification threshold in most tested mealybugs (Table 3). GLRaV-3 load was below the quantification threshold in all the six *Pl. ficus* and six *Pl. citri* infected individuals. No amplification was obtained from water control devoid of template RNA or RT-PCR mix devoid of MuLV reverse transcriptase. Due to the low number of samples above the quantification threshold, a statistical comparison of virus accumulation between *Pl. ficus* and *Pl. citri* was not conducted.

Viral loads measured in the grapevine used as virus source were 0.52, 2.40 and 2.54 for GVA, GLRaV-1 and GLRaV-3 GU per 100 plant *GAPDH* transcript, respectively.

Discussion

This study investigates the co-transmission of GLRaVs and RW-associated viruses to grapevine by the mealybugs *Pl. ficus* and *Pl. citri*. After feeding onto GVA, GLRaV-1 and GLRaV-3 mixed-infected plants, the vectors were tested for their transmission efficiencies. The two species shared high rates of virus acquisition, being 70% and 62% of the tested first-instar nymphs positive to at least one virus for *Pl. ficus* and *Pl. citri* respectively. Looking at the overall virus transmission rates, *Pl. citri* was able to inoculate a higher number of test plants compared to *Pl. ficus* (% *vs* 53%). Anyway, both the species proved to be efficient vectors under our experimental conditions.

To provide a more accurate description of transmission patterns, the overall transmission data were detailed for each single- and mixed-virus association and the retention and load of each virus in insects were analysed as well. To measure the virus load, a method for the quantification of GVA and GLRaVs in the vectors was set up for the first time. In this assay the absolute quantity of viral genomes was related to the copy numbers of a suitable insect reference gene to reduce the experimental bias due to RNA extraction and PCR set up. To this aim, the expression stability of three candidate reference genes proposed for gene expression studies of planthopper and thrip vectors was evaluated and the *GAPDH* gene was selected as the best insect reference gene in our experimental conditions. The virus RNA quantification was performed by using the *RdRp* gene, already used as amplification target in grapevine (Pacifico *et al.*, 2011; Tsai *et al.*, 2012; Velasco *et al.*, 2014). This target avoids the overestimation of viral loads as *RdRp* copy numbers are directly related to the number of viral genomes.

For the transmission experiments, insects were fed on source cuttings derived from a mother plant with a known amount of the three viruses. The virus load in plant may vary as a consequence of specific interaction virus-host and may influence the acquisition and transmission by the same vector (Martin and Elena, 2009). For example, different studies reported that crinivirus transmission efficiency by whitefly vectors to herbaceous plants was directly influenced by the virus concentration in the acquisition source (Ng *et al.*, 2004; Wintermantel *et al.*, 2008). In this study, the qRT-PCR assay performed on the mother plant showed that the quantities of the three viruses were similar: the load of GLRaV-1 and GLRaV-3 was almost the same (2.40 - 2.54 GU / 100 *GAPDH*) and GVA was slightly less concentrated (0.52 GU / 100 *GAPDH*). These can be reasonably considered as similar loads, especially when compared with the GVA, GLRaV-1 and GLRaV-3 amounts measured in grapevines by Giribaldi *et al.* (2011) and Pacifico *et al.* (2011).

Besides having the same load in plant, GLRaV-1 and GLRaV-3 also showed similar acquisition

patterns. Indeed the acquisition frequencies of the two viruses were very close in the experiments carried out with *Pl. ficus* and *Pl. citri* and their uptake preferably occurred together with GVA. However, their transmission patterns diverged in the two species. The GLRaV-3 transmission by *Pl. ficus* occurred at very high frequencies. The percentage of GLRaV-3 infected plants (89%) as well as the proportion of single infecting nymphs (*P*s = 0.36) were higher than the estimates reported in previous studies dealing with GLRaV-3 transmission by first- and second-instar nymphs of *Pl. ficus* (Douglas and Krüger, 2008; Tsai *et al.*, 2008; Tsai *et al.*, 2010; Tsai *et al.*, 2011; Mahfoudhi *et al.*, 2009). Almeida *et al.* (2013) matched up all these transmission data and provided an overall *P*s interval covering the information from several studies: these *P*s values just ranged from 0.04 to 0.2 and therefore did not include our value. The high transmission efficiency observed for *Pl. ficus* nymphs is not associated with a high virus load in the insect, as GLRaV-3 load never reached the quantification threshold and was detected only up to 48h after AAP. These data suggest that *Pl. ficus* may have a high capability to transmit GLRaV-3, in spite of the accumulation of a very low virus load.

Planococcus citri is known as GLRaV-3 vector in vineyards, and high percentages of *Pl. citri* individuals carrying the virus can be trapped in the field (Cid *et al.*, 2010). However, only Cabaleiro and Segura (1997b) performed laboratory experiments to estimate the GLRaV-3 transmission efficiency by this mealybug. The authors reported low GLRaV-3 transmission rates even against high acquisition rates (10% of test plants resulted positive to GLRaV-3 following exposure to groups of *Pl. citri* nymphs that were expected to have 80% of GLRaV-3 viruliferous individuals) and showed that nymphs quickly lost their infectivity (1h after leaving the infected grapevine). In our study, the GLRaV-3 transmission efficiency was higher and virus retention lasted longer. Therefore, this species proved to be an efficient vector, consistently with the fast GLRaV-3 spread that was recorded even in case of low field-population densities of *Pl. citri*. As occurred for *Pl. ficus*, *Pl. citri*

nymphs hosted a GLRaV-3 load below the quantification threshold. This confirms that the GLRaV-3 transmission to plants could efficiently occur even if the vector nymphs carry a very low virus load. Anyway, the comparison between the transmission rates performed by the two mealybug species showed that *Pl. ficus* is a more efficient GLRaV-3 vector than *Pl. citri*, as previously suggested by Tsai *et al.* (2008).

A large genetic variability was described for GLRaV-3 and different virus variants were found within single plants (Sharma *et al.*, 2011). It is known that these variants can follow different patterns of vector transmission and plant infection and that the disease spread and severity can be affected by the virus genotype (Almeida *et al.*, 2013; Blaisdell *et al.*, 2015). Therefore, future studies should be addressed to re-assess the GLRaV-3 transmission efficiency of *Pl. ficus* and *Pl. citri* in the presence of different virus isolates.

GLRaV-1 is less widespread than GLRaV-3, and the virus transmission mechanisms received little attention. So far, only few soft scale and mealybug species are recognized as competent vectors, including *Pl. ficus* (Sforza *et al.*, 2003; Tsai *et al.*, 2010). In this study, *Pl. ficus* nymphs transmitted GLRaV-1 but the rates were significantly lower than those observed for GLRaV-3. The low transmission performance occurred although the nymphs retained relatively high quantities of virus throughout the 48h-IAP.

Planococcus citri was initially excluded from the list of GLRaV-1 vectors (Golino *et al.*, 2002), but later (Scotto *et al.*, 2009) proved the ability of *Pl. citri* nymphs and adults to acquire and transmit GLRaV-1. Our study confirms these results and indicates that both acquisition and transmission occurred with a high efficiency. In detail, GLRaV-1 was acquired mainly together with GLRaV-3 and GVA but was then inoculated to grapevine preferably as single infection, and the number of test plants infected by the sole GLRaV-1 was significantly higher than the number of plants carrying the other two viruses, either alone or in associations. *Planococcus citri* was a more efficient vector of

GLRaV-1 than *Pl. ficus* (70% *vs* 30% inoculated plants), thus suggesting that it could contribute to the GLRaV-1 spread in vineyard more efficiently than the most common vine mealybug, *Pl. ficus*. GVA infections are widespread in all major wine-producing regions and the virus is often detected in grapevine together with GLRaVs. Indeed, GVA and GLRaVs share several vectors among mealybugs and soft scales, including *Pl. ficus* and *Pl. citri* (Rosciglione and Castellano, 1985). This study shows that both insects acquired GVA very efficiently, and the GVA rate in *Pl. ficus* nymphs was even significantly higher than GLRaV-1 and -3 rates, although the three viruses had similar loads in the source plant. The two mealybugs also showed common patterns of GVA retention, since the virus was detectable until 72h after AAP in both species and was accumulated at similar load after acquisition. *Planococcus ficus* kept high level of efficiency also in transmitting GVA, especially when the vitivirus was inoculated to grapevine together with GLRaV-3 and GLRaV-1 + GLRaV-3. On the contrary, *Pl. citri* poorly transmitted GVA: no single infections were recorded in test plants and the mixed infections were limited to few plants. The lack of transmission was even more evident looking at the trend of the GVA + GLRaV-1 + GLRaV-3 association: the rate of *Pl. citri* nymphs carrying all the three viruses prevailed against the other virus combinations, but this predominance could not be observed in test plants after inoculation. This leads to suppose that the nymphs carrying GVA in mixed infections may lose the ability to inoculate GVA to grapevine but maintain the ability to transmit GLRaVs.

The coexistence of GVA and GLRaVs in source plants offers the chance to investigate possible interactions among the viruses during transmission. To date, both competition or facilitation among GLRaVs and vitiviruses were hypothesized without coming to any conclusive evidences (Almeida *et al.*, 2013). Some authors suggested that GVA may require the presence of GLRaVs in the source plant to be transmitted by mealybugs and soft scales and establish infection in a susceptible plant (Hommay *et al.*, 2008; Engelbrecht and Kasdorf, 1990), whereas other studies

indicated that GLRaVs would benefit from GVA for transmissibility (Fortusini *et al.*, 1997; Zorloni *et al.*, 2006; Tsai *et al.*, 2010). Our study confirms the finding of Blaisdell *et al.* (2012), who reported that *Pl. ficus* can transmit GVA from infected to susceptible grapevines without simultaneous transmission of GLRaVs. However, this was not confirmed by the transmission experiments with *Pl. citri*. At the same time, we did not find evidences that GVA would mediate the GLRaVs transmission: GLRaV-1 and GLRaV-3 can be acquired and transmitted without GVA by *Pl. ficus* and *Pl. citri*. Namely, GLRaV-1 did not benefit from the presence of GVA, since the association GVA + GLRaV-1 was acquired but never transmitted by both vectors. Therefore, a clear picture about virus interactions is still lacking and further experiments are needed to better characterize the biology of GVA and GLRaVs associations in mealybugs as well as in grapevine. For example, transmission experiments from GVA singly infected plants would indicate whether the known patterns are due to mechanisms of interaction or are merely circumstantial.

This study also provides information about the mode of GLRaV-1, GLRaV-3 and GVA transmission by mealybugs. The vitiviruses GVA and GVB are known to be semi-persistently transmitted by their vectors (Adams *et al.*, 2004) as well as all closteroviruses studied so far (Ng and Falk, 2006). However, less information are available for the GLRaVs and concern GLRaV-3 only: it was reported that *Pl. ficus* need less than 24h to acquire GLRaV-3 and that both *Pl. ficus* and *Pl. citri* quickly lose the ability to transmit the virus (GLRaV-3 retention time of *Pl. ficus* = 3 days; GLRaV-3 retention time of *Pl. citri* = 24h; Tsai *et al.*, 2008; Cabaleiro and Segura, 1997b). These data are consistent with the characteristics of the semi-persistent transmission (Ng and Falk, 2006), even though the presence of GLRaV-3 particles observed in the *Pl. citri* salivary glands raised the question that the virus might circulate in the vector body (Cid *et al.*, 2007). Our study confirms that a 24h feeding period is enough for efficient acquisition of GLRaV-1 and GLRaV-3 by *Pl. ficus* and *Pl. citri,* and shows that the retention of the two viruses gradually decreased over time, and at the fourth post-

acquisition day viruses cannot be detected anymore in both vectors. This supports the hypothesis that the GLRaVs transmission by mealybugs is semi-persistent rather than circulative. Moreover, circulative viruses are known to have a relatively high load in their vectors because they colonize the haemocoel and several organs, whereas our data from qRT-PCR suggest that the amount of GLRaVs and GVA is low in both *Pl. ficus* and *Pl. citri*. Indeed, GLRaV-3 was below the detection threshold in all our tested insects, and most of the mealybugs that were positive to GVA and GLRaV-1 in real-time RT-PCR assay (that targeted the coat protein gene) did not provide detectable signals in the qRT-PCR assay. This latter is less sensitive because it targets the single copy *RdRp* gene, that has no subgenomic RNAs unlike coat protein gene.

In conclusion, this study provides new insights into the mealybug-mediated transmission of GLRaVs and RW- associated viruses. It is already known that this transmission lacks of vector-virus specificity, since a same insect species can transmit different viruses and a same virus can be transmitted by many vector species (Tsai *et al.*, 2010; Le Maguet *et al.*, 2012a). However, the relative efficiency of mealybugs towards GLRaVs and RW-associated viruses remained largely unknown because of the low number of comparative transmission studies. This study steps forward the question and deals with different sides of the story at a time: the “virus side”, by comparing transmission patterns of different co-infecting viruses, and the “vector side”, by comparing the transmission efficiency of two different mealybug species. Looking at the vectors, nymphs of *Pl. ficus* and *Pl. citri* that fed on the same source plant showed similar acquisition but different transmission patterns of GVA, GLRaV-1 as well as of GLRaV-3. Indeed *Pl. ficus* was mainly efficient in GLRaV-3 and GVA transmission, whereas *Pl. citri* was efficient in GLRaV-1 transmission and inefficient in GVA transmission. The “virus side” would provide information about possible antagonisms or synergisms between GLRaVs and vitiviruses. We observed that GVA could be transmitted to grapevine without GLRaV-1 and/or GLRaV-3 by *Pl. ficus* but not by *Pl. citri* and that

the GLRaVs transmission could take place also in absence of GVA.

Virus-vector relationships, together with new insights into the genetic characterization of the virus strains, may allow a better interpretation of GLD and RW epidemiology, and contribute to the development of control strategies against these virus-associated diseases.

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Figure 1 Pattern of virus retention in *Planococcus ficus* (a) and *Pl. citri* (b) after a 24h acquisition access period (AAP). Proportion of nymphs positive to *Grapevine virus A* (GVA), *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) at different post-acquisition times (T_0, T_{24}, T_{48}, T_{72}, T_{96}).

Table 1 *Actin β* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *18S ribosomal RNA* (*18S rRNA*) genes of *Planococcus ficus* and *Pl. citri*: amplification systems and expression stability. Sequences of the primers used for *ACTB*, *GAPDH* and *18S rRNA* amplification in SYBR® Green Real-Time PCR; sequence of the probe used for *GAPDH* amplification in TaqMan® qRT-PCR. Ranking of gene stability derived from GeNorm, Bestkeeper and Normfinder analysis. GeNorm: M values; Bestkeeper: Correlation coefficient, p = 0.001; Normfinder: Stability value and Standard error (SE).

Genes	Amplification systems		Expression stability		
			GeNorm	Bestkeeper	Normfinder
ACTB	PfActFw2	5'-TGAYTTAACCGACTACTTGA-3'	1.287	0.965	0.203 (SE = 0.060)
	PfActBRev1	5'-TCCAAAGCGACATAGCAC-3'			
GAPDH	PfGapFw1	5'-AAGAAAGTYATCATCTCAGC-3'	1.035	0.985	0.148 (SE = 0.069)
	PfGapRev	5'-GCGTGTACGGTGGTCATTA-3'			
	PfGapProbe	FAM-ATGTACGTCTGCGGTGTCAA-BHQ1			
18S rRNA	MqFw*	5'-AACGGCTACCACATCCAAGG-3'	1.312	0.982	0.251 (SE = 0.059)
	MqRv*	5'-GCCTCGGATGAGTCCCG-3'			

*from Marzachì and Bosco (2005)

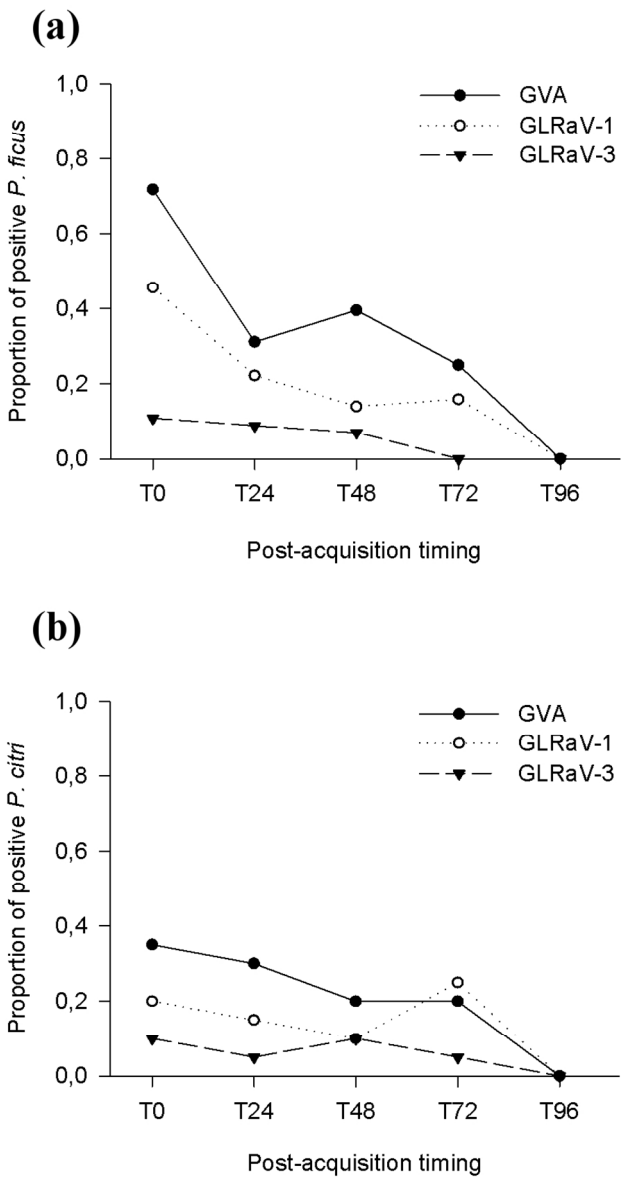
Table 2 Rate of *Planococcus ficus* and *Pl. citri* first-instar nymphs positive to *Grapevine virus A* (GVA), *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) in single and mixed infections after a 24h-AAP on triple-infected grapevine; rate of test grapevines positive to GVA, GLRaV-1 and GLRaV-3 in single and mixed infections after a 48h-IAP by *Pl. ficus* and *Pl. citri*. Virus detection was performed by SYBR® Green real-time RT-PCR.

	Pl. ficus		*Pl. citri*	
	Positive nymphs (n = 50[a])	Positive grapevines (n = 27[b])	Positive nymphs (n = 46[c])	Positive grapevines (n = 34[d])
GVA	0.20	0.07	0.13	-
GLRaV-1	0.10	0.04	0.11	0.53
GLRaV-3	0.12	0.22	0.02	0.24
GVA + GLRaV-1	0.22	-	0.09	-
GVA + GLRaV-3	0.22	0.41	0.17	0.06
GLRaV-1 + GLRaV-3	0.04	0.11	0.04	0.15
GVA + GLRaV-1 + GLRaV-3	0.10	0.15	0.43	0.03
Total GVA	0.74	0.63	0.83	0.09
Total GLRaV-1	0.46	0.30	0.67	0.70
Total GLRaV-3	0.48	0.89	0.67	0.47

[a] Total number of *Pl. ficus* nymphs positive to at least one virus, out of the 71 tested samples.
[b] Total number of grapevines positive to at least one virus after inoculation by *Pl. ficus*, out of the 51 tested samples.
[c] Total number of *Pl. citri* nymphs positive to at least one virus, out of the 74 tested samples.
[d] Total number of grapevines positive to at least one virus after inoculation by *Pl. citri*, out of the 49 tested samples.

Table 3 Mean loads of *Grapevine virus A* (GVA) and *Grapevine leafroll-associated virus 1* (GLRaV-1) in *Planococcus ficus* and *Pl. citri* at different post-acquisition times. The mean loads are expressed as viral genome units per 100,000 insect *GAPDH* transcripts ± the Standard Error. *Grapevine leafroll-associated virus 3* (GLRaV-3) titre was below the quantification threshold in all the 6 *Pl. ficus* and 6 *Pl. citri* individuals that tested positive in SYBR® Green real-time RT-PCR. *n*: number of quantified samples.

Post-acquisition times	Total samples	GVA mean loads (viral GU/100,000 *GAPDH* ± SE)		GLRaV1 mean loads (viral GU/100,000 *GAPDH* ± SE)	
		Pl. ficus	*Pl. citri*	*Pl. ficus*	*Pl. citri*
T_0	16	1.07 (± 1.5) *n* = 5	2.34 (± 1.1) *n* = 4	400 (± 521.0) *n* = 7	
T_{24}	4			1,170 (± 893.0) *n* = 2	84.00 (± 69.0) *n* = 2
T_{48}	3	2.91 (± 0.69) *n* = 2		8,670 *n* = 1	
T_{72}	1				83.1 *n* = 1



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