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Transmission of Grapevine virus A and Grapevine leafroll-associated virus 1 and 3 by Heliococcus bohemicus (Hemiptera: Pseudococcidae) nymphs from plants with mixed infections

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Arthropod in Relation to Plant Disease

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3 **Transmission of *Grapevine virus A* and *Grapevine leafroll-associated virus 1* and *3* by**

4 ***Heliococcus bohemicus* (Hemiptera: Pseudococcidae) nymphs from plants with mixed**

5 **infections**

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Abstract

Mealybugs (Hemiptera: Pseudococcidae) represent a serious threat for viticulture as vectors of phloem-restricted viruses associated with the grapevine rugose wood and leafroll diseases. *Heliococcus bohemicus* (Šulc) is known to be involved in the spread of these two viral diseases, being a vector of the *Grapevine virus A* (GVA) and the *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1 and -3). This study investigated the acquisition and transmission efficiency of *H. bohemicus* fed on mixed-infected plants. Nymphs were field-collected onto GVA, GLRaV-1 and GLRaV-3 multiple-infected grapevines in two vineyards in North-Western Italy, and were used in transmission experiments under controlled conditions. Even if most of the collected nymphs were positive to at least one virus, transmission occurred only to a low number of test grapevines. The transmission frequency of GLRaV-3 was the highest whereas GVA was transmitted to few test plants. The transmission of multiple viruses occurred at low rates, and nymphs that acquired all the three viruses then failed to transmit them together. Statistical analyses showed that the three viruses were independently acquired and transmitted by *H. bohemicus* and neither synergistic nor antagonistic interactions occurred among them. GVA and GLRaVs transmission efficiencies by *H. bohemicus* were lower than those reported for other mealybug vectors. This finding is consistent with the slow spread of leafroll and rugose wood diseases observed in Northern Italy, where *H. bohemicus* is the predominant vector species.

Keywords

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

Mealybugs (Hemiptera: Pseudococcidae) are important pests that feed and reproduce on a wide range of crops and ornamental plants worldwide. Grapevine is one of the most threatened host, and reductions in plant vigor and yield can be noticed when mealybugs are abundant in the vineyards. The impact on vine health is due to the phloem-feeding activity that takes away a lot of sap and causes indirect damages, such as abundant excretion of honeydew that favors the development of sooty mould, and transmission of phloem-inhabiting viruses. Actually, mealybugs are known to transmit two different groups of positive single-stranded RNA viruses associated with the grapevine leafroll and rugose wood diseases.

Leafroll disease is associated with a complex of virus species in the family *Closteroviridae* collectively referred to as *Grapevine leafroll-associated viruses* (GLRaV-1, -2, -3, -4, -7) (Martelli et al. 2012). The main leafroll symptoms are color alteration and downward rolling of the grapevine leaves. The rugose wood complex includes different syndromes associated with virus species belonging to the family *Betaflexiviridae*, genera *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). Within this complex, the GVA-induced Kober stem grooving syndrome is the most widespread and produces marked wood alteration. Both leafroll and rugose wood diseases are responsible for delay in fruit maturation and severe reductions in quality and yield (Martelli 2014a, b).

Besides mealybugs, also soft scales (Hemiptera: Coccidae) can transmit GLRaVs and rugose wood-associated viruses to grapevine, but mealybugs are likely to play a major role in virus spread because of their higher mobility. Although all life stages of mealybugs are capable of virus transmission, first-instar nymphs are known to be the most efficient vectors (Petersen and Charles 1997, Tsai et al. 2008, Le Maguet et al. 2012). The current data on virus acquisition, retention and inoculation indicate that GLRaV and vitivirus transmission occurs in a semi-persistent manner (Cabaleiro and Segura 1997, Tsai et al. 2008), although the hypothesis of a circulative transmission has been proposed, based on the evidence of GLRaV-3 presence in the mealybug salivary glands

(Cid et al. 2007).

In Europe, several mealybug species are known to feed and breed on grapevine. The species distribution as well as the population abundance can vary, depending on the climatic and environmental conditions (Cabaleiro 2009). Some highly damaging vectors, such as *Planococcus ficus* (Signoret) and *Planococcus citri* (Risso), prefer mild temperatures and are mainly established in the Mediterranean basin. Other species are more tolerant to the severe continental climate and are spread across the Central Europe. Among these, the Palaearctic species *Heliococcus bohemicus* (Šulc) has been reported in vineyards in Northern France, Hungary, Germany and Northern Italy (Kosztarab and Kozár 1988, Jakab and Szendrey 1989, Dalla Montà et al. 2001, Sforza et al. 2003, Bertin et al. 2010). This species overwinters as nymph and can develop two generations per year, with peak population densities in early-July and September (Camporese 1994, Reggiani et al. 2003). The adult females of *H. bohemicus* can be easily recognized because of long and thin dorsal wax filaments. This distinctive trait is less evident in the three immature instars that lead up to adult females, and therefore the nymphs of *H. bohemicus* could be mistaken for other mealybug species co-existing on grapevine. Molecular taxonomic tools contribute to a reliable identification of these early stages and a PCR-based key is currently available for several grapevine mealybugs, including *H. bohemicus* (Bertin et al. 2010).

Heliococcus bohemicus was considered a minor pest of grapevine, but in the last two decades, it received a growing attention as vector of GLRaV-1, GLRaV-3 and GVA (Sforza et al. 2003, Zorloni et al. 2006). It was observed that, in case of mixed infections, GVA and GLRaVs can be acquired and transmitted together by *H. bohemicus*, and it was hypothesized that GLRaVs transmission would benefit from the presence of GVA (Zorloni et al. 2006, Bertin et al. 2010). The role of GVA as helper virus for GLRaVs transmission was suggested for other mealybug and soft scale species, but no conclusive evidence was provided so far (Fortusini et al. 1997, Tsai et al. 2010, Bertin et al. 2016).

Besides these preliminary observations, the virus transmission by *H. bohemicus* is still poorly characterized. Therefore, we collected *H. bohemicus* nymphs fed onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines in two vineyards in North-Western Italy and we tested the ability of a subset of them to transmit the viruses under controlled conditions. The results provide new insights into the acquisition and transmission efficiency of GVA and GLRaVs in case of mixed infections, and on possible antagonistic or synergistic interactions among the three viruses. The relevance of the results to the understanding of leafroll and rugose wood epidemiology is discussed.

Materials and Methods

Insect Material. Nymphs of *H. bohemicus* were collected in North-Western Italy in two virus-infected vineyards located in Mango (Piemonte region) and Albenga (Liguria region). The geographical location of the two vineyards is shown in Bertin et al. (2010). The experimental vineyard in Mango, red-berried cv. Nebbiolo, consists of 19 rows of 45 plants each. Some non-adjacent rows were originally planted with GVA + GLRaV-1 or GVA + GLRaV-3 infected vines, while all the other rows were planted with healthy vines of the same clones. The sanitary status of the vineyard was regularly monitored over several years by serological (DAS-ELISA) and molecular analyses (PCR) to monitor the natural spread GVA, GLRaV-1 and GLRaV-3 (Gambino and Gribaudo 2006, Gribaudo et al. 2009). Based on these data, two rows hosting GLRaV-1, -3 and GVA mixed-infected grapevines were selected for the collection of *H. bohemicus* nymphs used for estimating the acquisition efficiency. The selected plants tested negative to the *Grapevine fanleaf virus* (GFLV) and the *Grapevine fleck virus* (GFkV), while the presence of other viruses was not investigated. The presence of GVA, GLRaV-1 and GLRaV-3 in the selected plants were checked at the beginning of each sampling season by real-time RT-PCR analysis.

The vineyard in Albenga, white-berried cv. Vermentino, is irregularly shaped and made of about 100 rows of 75-85 plants each. The presence of GVA, GLRaV-1 and GLRaV-3 in the vines was monitored by real-time RT-PCR analysis between 2009 and 2011; the presence of other viruses was not investigated. Many symptomatic grapevines repeatedly tested positive for GVA, GLRaV-1 and GLRaV-3 viruses in mixed infections. Fourteen of these latter vines were selected for sampling *H. bohemicus*. The nymphs were partly tested by real-time RT-PCR to assess their acquisition efficiency and partly used for transmission trials.

The *H. bohemicus* nymphs were identified by morphology. However, the morphology-based identification of early life stages can be awkward. Therefore, a sub-set of insect material was kept at each sampling time and analyzed for species identification by molecular assays. The mitochondrial *cytochrome oxidase subunit 1* (*COI*) gene was amplified (Bertin et al. 2010): the 200 bp amplicon

provided evidence that only *H. bohemicus* was collected within the two vineyards, and the presence of other mealybug species was excluded.

Specimens of *H. bohemicus* collected in the field were tentatively used to establish healthy insect colonies to perform virus acquisition experiments under controlled conditions. The colonies were maintained in climatic chambers on sprouted potatoes in the dark at 20-30°C, under the same conditions we use to rear *P. ficus* and *P. citri* colonies. Three different rearings were attempted, and a large batch of specimens including all nymphal stages as well as mature females were used. In spite of all the efforts, we failed to establish a colony. Therefore, only acquisition data obtained in the field were included in the study.

Virus Acquisition. In Mango vineyard, first- and second-instar nymphs of *H. bohemicus* were collected from 2009 to 2011 at three different sampling times in July, August and September, when early stages are known to be present (Bertin et al. 2010). A total of 45, 45 and seven nymphs were collected in 2009, 2010 and 2011, respectively. The nymphs were singly assayed by real time RT-PCR to estimate the rates of GVA, GLRaV-1 and GLRaV-3 acquisition.

In Albenga vineyard, first- and second-instar nymphs were collected in summer and either tested by real time RT-PCR or used in transmission experiments. Eight, six and seven groups of five nymphs were analyzed in June, July and August 2010, respectively. In 2011, the nymphs were singly assayed and a total of 42 and 17 nymphs were analyzed in June and August, respectively.

Transmission Experiments. Fourteen GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines were carefully inspected within the Albenga vineyard for the presence of early stages of *H. bohemicus*. Leaves hosting *H. bohemicus* nymphs were collected from each plant and kept in cool boxes for transport to the laboratory. Here, first- and second-instar nymphs were immediately removed with a fine brush under a stereomicroscope and transferred on known uninfected recipient grapevines for virus transmission. The transmission was performed by clip-caging groups of five nymphs on the lower surface of one leaf of each test plant for a virus inoculation access period (IAP) of 48 h. The healthy recipient grapevines were obtained through micropropagation and

acclimatization in greenhouse of clonal lines of *V. vinifera* cv. Barbera. 48h-IAP was chosen based on the observations available for other GLRaV-1, -3 and GVA mealybug vectors (Tsai et al. 2008): it was already known that the virus transmission rates may reach the maximum even with a 24h-IAP and the insect infectivity was lost within four-days of post-acquisition feeding.

After 48h-IAP, the nymphs were removed from the test grapevines, and these were drench-treated with a systemic insecticide (Actara, Syngenta Crop Protection, Basel, Switzerland). The vines were maintained in a greenhouse and regularly sprayed 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.

Five transmission trials were performed in June, July and August 2010 (experiments 1-3) and in June and August 2011 (experiments 4-5). About 15 - 25 test grapevines were exposed to viruliferous mealybugs in each transmission test: 13, 14, 24, 18 and 16 plants were alive at the end of the five experiments and assayed for virus presence. 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 (five per experiment) from the same batch of test plants were not exposed to *H. bohemicus* nymphs and served as negative controls.

RNA Purification From Insects and Plants. RNA of *H. bohemicus* was purified from groups of five nymphs as well as from single nymphs using the TRIzol® Reagent (Invitrogen - Thermo Fisher Scientific, Waltham, MA) and following the manufacturer's instructions. Samples were treated with two units of RNase-Free DNase I (Applied Biosystems - Thermo Fisher Scientific) 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 - Thermo Fisher Scientific). RNA was then diluted to 10 ng μl^{-1} and stored at -80 °C.

Plant RNA was extracted from the grapevines selected as sources of GLRaV-1, GLRaV-3 and GVA in Mango and Albenga vineyards. Total RNA was also extracted from test grapevines at the end of the transmission trials. RNA was extracted from a total of 0.1 g of midribs from both basal and apical leaves of each source /test plant. 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. Virus detection from both insect and plant RNA extracts was carried out by SYBR® Green real-time RT-PCR assays in a Chromo4 Real Time Thermal Cycler (Bio-Rad, Life Science Research, Hercules, California) supported by the OpticonMonitor 3.1.32 software (Bio-Rad). GVA, GLRaV-1 and GLRaV-3 were detected with the following primer pairs, designed on the appropriate 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). 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 templates. For all the primer pairs, 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 *H. bohemicus* carrying GVA, GLRaV-1 and GLRaV-3 and RNA of healthy *P. ficus* from laboratory colony on sprouted potatoes were served as positive and negative controls, respectively. RNA extracts from GVA, GLRaV-1 and GLRaV-3 infected vines as well as from healthy *in vitro*-generated grapevines were used as controls for virus detection in plant.

RNA extracted from source grapevines was used to identify the GLRaV-3 genetic variant.

RNA (100 ng) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The full-length GLRaV-3 coat protein gene was then amplified using KSL95-5 / KSL95-6 primer pair (Ling et al. 1997). PCR was carried out as suggested by Gouveia et al. (2011), except for the cycling conditions that were as follows: a denaturation cycle at 94°C for 5 min, 5 cycles at 94°C for 30 s, 42°C for 30 s, 68°C for 60 s, 34 cycles at 94°C for 30 s, 52°C for 30 s, 68°C for 60 s, and a final cycle at 94°C for 30 s, 52°C for 30 s, 68°C for 5 min. PCR fragments were cloned and sequenced. Sequences were aligned with the coat protein genes from representative GLRaV-3 isolates available in GenBank and assigned to the corresponding phylogenetic group (I-VI) according to Maree et al. (2013).

Data analysis.

The data from virus acquisition experiments carried out in Mango and Albenga vineyards and from transmission experiments were analyzed through generalized linear model (GLM) using a binomial distribution and logit as link function (SPSS version 22). This analysis allowed to infer the combination prevailing among the three viruses in each experiment, the effect of the time among the experimental replicates, and the possible interaction between these two factors. Since transmission experiments were conducted with five insects per plant, while the acquisition rate was assessed on single nymphs, the maximum-likelihood estimator, *P*s (Swallow, 1985), was applied to estimate the actual proportion of infected insects within each five-insect batch used for transmission.

Then, acquisition frequencies of single nymphs collected in the Mango and Albenga vineyards and transmission frequencies to inoculated plants were analyzed to infer possible antagonistic or synergistic interactions, among GVA, GLRaV-1 and GLRaV-3. The interactions were tested by comparing the observed against the expected frequencies, through Fisher Exact Test for Count Data (Fisher 1934). Fisher Exact Test was used instead of χ^2 because expected frequencies were less than five in some classes, that is a limit in χ^2 application. The analyses were

performed using R version 3.1.3. Virus acquisition/transmission frequencies were distributed among eight (2^3) classes that correspond to every possible combination of presence/absence of GVA, GLRaV-1 and GLRaV-3 (absence of viruses, presence of one virus, presence of two viruses and presence of three viruses). Expected class frequency distribution was calculated under the null hypothesis that antagonism or synergism does not exist among GVA, GLRaV-1 and GLRaV-3 in both acquisition and transmission. Expected frequency for each class (F_i) was calculated as follow:

$$F_i = N*P_i$$

where:

N = number of analyzed nymphs

P_i = probability of each class.

The probability of each class was calculated combining the probability of each acquisition/transmission according to binomial distribution, as follow:

$$P_i = (P \text{ or } Q)_{GVA} * (P \text{ or } Q)_{GLRaV\text{-}1} * (P \text{ or } Q)_{GLRaV\text{-}3}$$

where $(P \text{ or } Q)_{virus}$ is the probability of acquisition/transmission or non-acquisition/non-transmission associated to each virus in that class. Obviously, P + Q = 1 for each virus. As information on probability of acquisition/transmission associated to each virus were not available *a priori*, the *P*/*Q* values were obtained by fitting from least square procedure the GVA, GLRaV-1 and GLRaV-3 frequencies observed in single nymphs from Mango and Albenga and in inoculated plants. The Root Mean Square Error (RMSE) of each fitting was calculated (Loague and Green 1991).

Results

Virus Acquisition. Table 1 reports the results of GVA, GLRaV-1 and GLRaV-3 detection in *H. bohemicus* nymphs collected in Mango vineyard in 2009, 2010 and 2011. GLM results showed a significant effect of the virus combination, while no significant effect were recorded for time or interaction combination x time (Table 2). All the specimens tested positive for at least one virus. Considering the total number of nymphs collected in Mango from 2009 to 2011, 63% acquired all the three viruses together and this rate was higher than all the other single and mixed virus combination rates. Among the single and double virus acquisitions, GVA + GLRaV-1 combination showed the highest frequency (24% tested nymphs).

In Albenga, the estimate of virus acquisition was carried out in 2010 and 2011, at three and two sampling dates respectively. Due to the huge number of *H. bohemicus* specimens infesting the vineyard, the first-year analyses were performed with batches of five nymphs; all the tested batches were positive to the three viruses. Such a result was not informative for an accurate estimate of the incidence of single and mixed GVA, GLRaV-1 and GLRaV-3 combinations in each viruliferous nymph. Therefore, the further two analyses carried out in 2011 were performed with single nymphs. Most of the singly-assayed nymphs acquired at least one virus, being the total rate of positive samples between 88 and 100% (Table 3), and no significant effect of the time was observed between the two experiments (Table 2). Both the virus combination and the interaction combination x time resulted to be significant by GLM analysis (Table 2), indicating that the number of nymphs positive to single GVA, GLRaV-1 and GLRaV-3 as well as to the different virus combinations significantly differed between the two samplings performed in 2011. In any case, the GVA + GLRaV-1 + GLRaV-3 acquisition was prevailing against all the other single and mixed virus combinations also in singly-tested nymphs at both samplings.

GLRaV-3 isolate infecting the source grapevines in both Mango and Albenga vineyards belonged to the phylogenetic group I, according to Maree et al. (2013).

Virus Transmission. Five transmission trials were performed with nymphs collected in

2010 and 2011 in the Albenga vineyard. Following 48h-IAP, the virus transmission occurred in all the experiments (Table 4). No viruses were detected in *in vitro*-generated grapevines used as negative controls in each experiment. Percentages of plants positive to at least one virus ranged between 7 and 39% and no plants infected by all the three viruses together and by GVA + GLRaV-1 were found. No significant effect of the time alone or in interaction with combination was recorded (Table 2).

The GLM analysis revealed a significant effect of the virus combination on the transmission results. Considering the total number of positive plants over the five transmission experiments (Table 4), it is evident that GVA was poorly transmitted: it was detected in three grapevines only, alone or together with GLRaV-3. GLRaV-1 and GLRaV-3 were transmitted to a total of seven and 16 plants respectively, and their transmission mainly occurred as single infection. Indeed, the rate of both GLRaV-1 and GLRaV-3 single infections was higher than the rate of mixed infections. Overall incidences of GVA, GLRaV-1 and GLRaV-3 on the 22 virus-positive test grapevines were 14, 32 and 73%, respectively. The estimated proportion of infecting *H. bohemicus* nymphs was: *P*s = 0.03 for GVA, *P*s = 0.07 for GLRaV-1 and *P*s = 0.23 for GLRaV-3. GLRaV-3 transmission rate was the highest.

Inferences on Virus Interaction. Possible antagonisms or synergisms among GVA, GLRaV-1 and GLRaV-3 during acquisition and transmission were inferred by comparing observed *vs* expected virus frequencies in single nymphs from Mango and Albenga vineyards and in test grapevines. Expected frequency values depend on the probability of acquisition/transmission associated to each virus (P_{GVA}, $P_{GLRaV\text{-}1}$ and $P_{GLRaV\text{-}3}$). *P* values of virus acquisition in Mango and Albenga vineyards as well as of virus transmission were calculated (Table 5); the values always fitted with an error of estimation (RMSE) that was lower than one unit of response (one acquisition or transmission), indicating that the estimate procedure is very good. Acquisition values of P_{GVA}, $P_{GLRaV\text{-}1}$ and $P_{GLRaV\text{-}3}$ were high for both Mango and Albenga vineyards, because most of the single nymphs from both sampling sites tested positives to all the three viruses. On the contrary, the low

number of plants inoculated after 48h-IAP made the transmission probabilities low for all the three viruses, with an upward trend from P_{GVA} to $P_{GLRaV\text{-}1}$ and to $P_{GLRaV\text{-}3}$.

Probability estimates allowed to calculate the expected acquisition and transmission frequencies for each of the eight classes generated by all possible combinations of presence/absence of GVA, GLRaV-1 and GLRaV-3 (Table 6) under the null hypothesis that antagonistic nor synergistic interactions exist between acquisition or transmission of different viruses. At each class, the distribution of the expected frequencies were compared with the observed ones. No significant differences were recorded between expected and observed acquisition frequencies in single nymphs from both Mango and Albenga vineyards. Expected and observed transmission frequencies in test plants did not differ as well. This leads to accept the null hypothesis that the frequency class distribution is generated by random processes and neither antagonistic nor synergistic interactions among GVA, GLRaV-1 and GLRaV-3 could be identified at both acquisition and transmission.

Discussion

Heliococcus bohemicus is gaining a growing economical relevance for its role in the spread of leafroll and rugose wood diseases, and this study improves the current knowledge in its virus acquisition and transmission efficiency. The vector competence of *H. bohemicus* has been ascertained only for GVA, GLRaV-1 and GLRaV-3 so far. These three viruses are also the most common grapevine viruses in the regions where *H. bohemicus* is present. Other vector-transmissible closteroviruses and vitiviruses, such as GLRaV-4 and GVB, are infrequent in Central Europe (CABI 2016), and were therefore not included in this study.

The insect material was from two different vineyards in North-Western Italy, which have been monitored over several years for both mealybug infestation and GVA, GLRaV-1 and GLRaV-3 infection. The experimental vineyard in Mango was originally planted with healthy vines together with GVA + GLRaV-1 or GVA + GLRaV-3 infected vines, in alternating rows. After several years, few grapevines became infected by all the three viruses, as a result of natural virus spread. *Heliococcus bohemicus* is the only mealybug species found within the vineyard and occurs at low population density (Bertin et al. 2010). Its low density is probably a side effect of the compulsory insecticide treatments targeted against *Scaphoideus titanus* Ball, the leafhopper vector of Flavescence dorée. In Albenga vineyard, the density of *H. bohemicus* population is noticeably high (Bertin et al. 2010), perhaps thanks to the Mediterranean mild climate of the Liguria region and the absence of compulsory treatments until 2014. Moreover, spot diagnoses were performed on vines to estimate the virus spread within the vineyard and high rates of infection were observed: only few sampled grapevines resulted to be virus-free and many plants tested positive to all the three viruses (C.M., unpublished data).

The first- and second-instar nymphs of *H. bohemicus* were collected in Mango and Albenga vineyards onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines. The nymphs were infected by all the three viruses in a very high proportion. Therefore, *H. bohemicus* showed high efficiency of GVA, GLRaV-1 and GLRaV-3 acquisition in the field, independently of the climatic

and agricultural conditions. Such acquisition rates allowed to predict a high infective potential for all the three viruses. However, it has already been observed for other mealybugs that differences between acquisition and transmission rates can considerably lower the actual rate of infected plants (Cabaleiro and Segura 1997; Bertin et al., 2016).

The insects used in transmission trials were only from Albenga vineyard, where the abundance of source grapevines and the high density of *H. bohemicus* population ensured an adequate number of insects needed for plant inoculation. Experiments were carried out with nymphs of the same age of those used for testing virus acquisition, and collected on the same leaves. Although the nymphs showed high acquisition efficiency, the virus transmission occurred at relatively low rates: only 26% of the inoculated test grapevines resulted positive to at least one virus. However, much lower rates of virus transmission were observed in the few studies dealing with vector competence of *H. bohemicus*. Sforza et al. (2003) reported that large groups of individuals carrying GLRaV-1 and GLRaV-3 were able to transmit at least one virus to 23% of test plants. This rate is similar to the transmission frequency that we observed under our experimental conditions, but was obtained with a higher number of insects per plant (30 – 50 individuals of all stages *vs* five nymphs). Moreover, when Zorloni et al. (2006) tested the co-transmission of GVA, GLRaV-1 and GLRaV-3 by *H. bohemicus*, they found one GLRaV-3 positive plant and one GLRaV-3 + GVA positive plant out of 77 test grapevines only. Therefore, our estimate of virus transmission efficiency is higher than previously reported for *H. bohemicus*. The different life stages employed in the transmission experiments could contribute to explain these different efficiencies. Sforza et al. (2003) used individual of all stages, and Zorloni et al. (2006) employed AAPs ranging from 4 to 21 days, thus using in the inoculation phase late instar nymphs and probably adult females that lost their infectivity after molting (Tsai et al. 2008). Now it is known that early instar nymphs are much more efficient vectors compared to older nymphs and adults (Tsai et al. 2008, Mahfoudhi et al. 2009, Le Maguet et al. 2012), and our experiments were carried out accordingly. This probably increased the transmission rates. Moreover, the different sensitivity of

359 the methods used for virus detection in test grapevines (ELISA vs Real-Time PCR) may also partly
360 explain the gap between the results.

361 The transmission of GVA, GLRaV-1 and GLRaV-3 occurred at different rates. GLRaV-3
362 showed the highest transmission frequency, being found in 73% of the virus-positive plants, mainly
363 as single virus. We estimated that a proportion of 0.23 *H. bohemicus* nymphs transmitted GLRaV-3
364 under our experimental conditions, and this value was within the *P*s range calculated for other
365 mealybug and soft scale species (Almeida et al. 2013). For example, the vine mealybug *P. ficus*, one
366 of the most efficient vectors of GLRaV-3, showed closed *P*s values and, similarly to *H. bohemicus*,
367 its GLRaV-3 transmission rate is higher than GVA and GLRaV-1 rates (Bertin et al. 2016). Such
368 transmission efficiencies, together with the high number of different competent vectors, would
369 explain why GLRaV-3 is the most abundant and widespread leafroll-associated virus (Maree et al.
370 2013). This broad distribution favored the differentiation of at least six GLRaV-3 genetic variants,
371 consecutively numbered I-VI (Maree et al. 2013). It is known that these variants can follow
372 different patterns of vector transmission and plant infection and that the disease spread and severity
373 can be affected by the virus genotype (Almeida et al. 2013, Blaisdell et al. 2015). In this study, only
374 the group I was identified in the source plants in both Mango and Albenga vineyards. Therefore,
375 further research should be performed to study possible interactions between different GLRaV-3
376 isolates during transmission and plant infection by *H. bohemicus*.

377 GVA widely spread within the Albenga vineyard and was detected together with GLRaVs in
378 most of the field-collected *H. bohemicus* nymphs. However, the GVA transmission only occurred in
379 three of the 22 virus-infected test grapevines. Such a transmission pattern, consisting of low rates
380 of GVA transmission and concurrent high rates of single GLRaVs transmission, was also observed
381 for *P. citri* (Bertin et al. 2016). Besides vector efficiency, the interaction between virus and host
382 plant can be responsible for the observed infection rates. It is known that co-infecting viruses can
383 differently establish in the plant after inoculation, as a consequence of diverse mechanisms such as
384 the competition for nutrients or the different ability to overcome the plant defenses. The pattern of

385 establishment of GLRaVs and vitiviruses in grapevine is still largely unknown. It was recently
386 observed that two genetic variants of GLRaV-3 were equally transmitted by the same vector but
387 were established at different rates in a new host plant (Blaisdell et al. 2015). This study opens new
388 perspectives in the interpretation of infection dynamic and encourages further experiments with
389 different GLRaVs or GLRaVs + vitiviruses mixed infections.

390 The detection data obtained from single nymphs collected in Mango and Albenga vineyards
391 and from the inoculated plants offered the chance to infer some possible interactions among GVA,
392 GLRaV-1 and GLRaV-3 during transmission and plant infection. For this purpose, a statistical
393 analysis was appropriately set up and the observed virus acquisition and transmission frequencies
394 were tested under the null hypothesis that no interactions are present. This approach can contribute
395 to investigate mechanisms that are still controversial: to date, both synergisms or antagonisms were
396 hypothesized for GLRaVs and vitiviruses transmitted by different mealybug species but no
397 conclusive evidences were provided (Almeida et al. 2013). Some authors suggested that GVA may
398 require the presence of GLRaVs in the source plant to be transmitted by mealybugs and soft scales
399 and establish infection in a susceptible plant (Engelbrecht and Kasdorf 1990, Hommay et al. 2008),
400 whereas other studies indicated that GLRaVs would benefit from GVA for transmissibility
401 (Fortusini et al. 1997, Zorloni et al. 2006, Tsai et al. 2010). Previous observations on *H. bohemicus*
402 were in line with the second hypothesis and suggested that GVA may act as helper virus for the
403 GLRaVs transmission when insects fed onto mixed-infected grapevines (Zorloni et al. 2006). Our
404 statistical analyses showed that the rates of single and mixed GVA, GLRaV-1 and GLRaV-3
405 infections observed in both nymphs and test plants were generated by random processes. This
406 suggests that virus acquisition and transmission by *H. bohemicus* were not influenced by neither
407 competition nor facilitation among the three viruses. The role of GVA as helper virus seems
408 therefore unlikely, also considering that the transmission of both GLRaV-1 and GLRaV-3 occurred
409 even without GVA in our experiments. Especially GLRaV-1 did not benefit from the presence of
410 this vitivirus, since the combinations GVA + GLRaV-1 and GVA + GLRaV-1 + GLRaV-3 were

411 acquired but never transmitted. These results increase the knowledge of multiple virus transmission
412 by *H. bohemicus*, but further transmission experiments, by feeding insects on grapevines singly and
413 mixed infected by GVA and GLRaVs are needed to obtain fully conclusive results on GVA and
414 GLRaVs interactions for transmission.

415 Our estimates of GVA, GLRaV-1 and GLRaV-3 transmission contribute to a better
416 knowledge of leafroll and rugose wood epidemiology in those grape-growing regions where *H.*
417 *bohemicus* is the predominant vector species. The observed rates of GVA and GLRaVs transmission
418 are lower than the ones reported for other mealybug species, such as *P. ficus* and *P. citri* (Douglas
419 and Krüger 2008, Tsai et al. 2008, Bertin et al. 2016). These species are actually recognized as
420 efficient virus vectors and are responsible for wide and fast virus spread in the field even at low
421 population densities (Cabaleiro and Segura 2006, Cabaleiro et al. 2008, Golino et al. 2008). Such a
422 virus spread cannot be predicted when *H. bohemicus* is the predominant vector species, as it occurs
423 in the temperate regions of Europe. Actually in the vineyards of the Langhe and Roero areas (like
424 Mango), where *H. bohemicus* is the only mealybug species and the population density is low
425 because of the insecticide treatments against *S. titanus* (Bertin et al. 2010), the spread of GLRaV-1,
426 GLRaV-3 and GVA within the vineyards is slow (Gribaudo et al. 2009). However, it is worthy to
427 note that a possible reduction of insecticide treatments might result in *H. bohemicus* population
428 increase even in these areas, thus accelerating the disease spread. The scenario of the Albenga
429 vineyard confirms this hypothesis: in the presence of the same vector species, but at higher
430 population level, much higher rates of plant infection were recorded. Thus, the disease management
431 programs should always include monitoring of vector populations, even in the presence of a poorly
432 efficient vector such as *H. bohemicus*. Moreover, transmission experiments with different virus
433 genetic variants and from single-infected source grapevines might improve the knowledge on the
434 epidemiology of leafroll and rugose wood diseases and provide further indications for their
435 management.
436

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References Cited

Almeida, R. P. P., K. M. Daane, V. A. Bell, G. K. Blaisdell, M. L. Cooper, E. Herrbach, and G. Pietersen. 2013. Ecology and management of grapevine leafroll disease. Frontiers in Microbiology 4: 94.

Bertin, S., V. Cavalieri, C. Graziano, and D. Bosco. 2010. Survey of mealybug (Hemiptera: Pseudococcidae) vectors of *Ampelovirus* and *Vitivirus* in vineyards of northwestern Italy. Phytoparasitica 38: 401-409.

Bertin, S., D. Pacifico, V. Cavalieri, C. Marzachì, and D. Bosco. 2016. Transmission of *Grapevine virus A* and *Grapevine leafroll-associated viruses 1* and *3* by *Planococcus ficus* and *P. citri* fed on mixed-infected plants. Annals of Applied Biology Early View DOI: 10.1111/aab.12279.

Blaisdell, G. K., S. Zhang, J. R. Bratburd, K. M. Daane, M. L. Cooper, and R. P. P. Almeida. 2015. Interactions within susceptible hosts drive establishment of genetically distinct variants of an insect-borne pathogen. Journal of Economic Entomology 108: 1531-1539.

Cabaleiro, C. 2009. Current advances in the epidemiology of grapevine leafroll disease, pp. 264-268. *In* E. Boudon-Padieu (ed.), Proceedings, 16th Meeting of ICVG, 31 August - 4 September 2009, Dijon, France. Le progrès agricole et viticole Montpellier France.

Cabaleiro, C., and A. Segura. 1997. Some characteristics of the transmission of *Grapevine leafroll associated virus 3* by *Planococcus citri* Risso. European Journal of Plant Pathology 103: 373-378.

Cabaleiro, C., and A. Segura. 2006. Temporal analysis of *Grapevine leafroll associated virus 3* epidemics. European Journal of Plant Pathology 114: 441-446.

Cabaleiro, C., C. Couceiro, S. Pereira, M. Cid, M. Barrasa, and A. Segura. 2008. Spatial analysis of epidemics of *Grapevine leafroll associated virus-3*. European Journal of Plant Pathology 121: 121-130.

CABI. 2016. Invasive Species Compendium. CAB International, Wallingford, UK. www.cabi.org/isc.

Camporese, P. 1994. First biological notes on *Heliococcus bohemicus* Sulc in the vineyards of Veneto. Memorie della Società Entomologica Italiana No. 72: 195-200.

Cid, M., S. Pereira, C. Cabaleiro, F. Faoro, and A. Segura. 2007. Presence of *Grapevine leafroll-associated virus 3* in primary salivary glands of the mealybug vector *Planococcus citri* suggests a circulative transmission mechanism. European Journal of Plant Pathology 118: 23-30.

Dalla Montà, L., C. Duso, and V. Malagnini. 2001. Current status of scale insects (Hemiptera: Coccoidea) in the Italian vineyards. Bollettino di Zoologia agraria e di Bachicoltura, Ser II 33: 343–350.

Douglas, N., and K. Krüger. 2008. Transmission efficiency of *Grapevine leafroll-associated virus 3* (GLRaV-3) by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae). European Journal of Plant Pathology 122: 207-212.

Engelbrecht, D. J., and G. G. F. Kasdorf. 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus*. Phytophylactica 22: 341–346.

Fisher, R. A. 1934. Statistical methods for research workers, 5th ed. Oliver and Boyd, London and Edimburgh.

Fortusini, A. G., G. Scattini, S. Prati, S. Cinquanta, and G. Belli. 1997. Transmission of *Grapevine leafroll virus-1* (GLRaV-1) and *Grapevine virus A* (GVA) by scale insects., pp. 121–122. *In* Proceedings, 12th Meeting of ICVG, 28 September - 2 October 1997, Lisbon, Portugal. Department of Plant Pathology, EAN/INIA, Oeiras, Portugal.

Gambino, G., and I. Gribaudo. 2006. Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant

RNA as internal control. Phytopathology 96: 1223-1229.

Golino, D. A., E. Weber, S. Sim, and A. Rowhani. 2008. Leafroll disease is spreading rapidly in a Napa Valley vineyard. California Agriculture 62: 156-160.

Gouveia, P., M. T. Santos, J. E. Eiras-Dias, and G. Nolasco. 2011. Five phylogenetic groups identified in the coat protein gene of *Grapevine leafroll-associated virus 3* obtained from Portuguese grapevine varieties. Archives of Virology 156: 413-420.

Gribaudo, I., G. Gambino, S. Bertin, D. Bosco, A. Cotroneo, and F. Mannini. 2009. Monitoring the spread of viruses after vineyard replanting with heat-treated clones of *Vitis vinifera* 'Nebbiolo'. Journal of Plant Pathology 91: 741-744.

Hommay, G., V. Komar, O. Lemaire, and E. Herrbach. 2008. *Grapevine virus A* transmission by larvae of *Parthenolecanium corni*. European Journal of Plant Pathology 121: 185-188.

Jakab, J., and L. Szendrey. 1989. The appearance of *Heliococcus bohemicus* Šulc in the vineyards of Heves county. Növényvédelem 25: 216.

Kosztarab, M., and F. Kozár. 1988. Scale insects of Central Europe, Kluwer Academic Publishers, Dordrecht, Netherlands.

Le Maguet, J., M. Beuve, E. Herrbach, and O. Lemaire. 2012. Transmission of six ampeloviruses and two vitiviruses to grapevine by *Phenacoccus aceris*. Phytopathology 102: 717-723.

Ling, K. S., H. Y. Zhu, H. Alvizo, J. S. Hu, R. F. Drong, J. L. Slightom, and D. Gonsalves. 1997. The coat protein gene of *Grapevine leafroll associated closterovirus-3*: cloning, nucleotide sequencing and expression in transgenic plants. Archives of Virology 142: 1101-1116.

Loague, K., and R. E. Green. 1991. Statistical and graphical methods for evaluating solute transport models: overview and application. Journal of Contaminant Hydrology 7: 51-73.

MacKenzie, D. J., M. A. McLean, S. Mukerji, and M. Green. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease 81: 222-226.

Mahfoudhi, N., M. Digiaro, and M.H. Dhouibi. 2009. Transmission of grapevine leafroll viruses by *Planococcus ficus* (Hemiptera: Pseudococcidae) and *Ceroplastes rusci* (Hemiptera: Coccidae). Plant Disease 93: 999–1002.

Maree, H. J., R. P. P. Almeida, R. Bester, K. M. Chooi, D. Cohen, V. V. Dolja, M. F. Fuchs, D. A. Golino, A. E. C. Jooste, G. P. Martelli, R. A. Naidu, A. Rowhani, P. Saldarelli, and J. T. Burger. 2013. *Grapevine leafroll-associated virus 3*. Frontiers in Microbiology 4.

Martelli, G. P. 2014a. Grapevine leafroll. Journal of Plant Pathology 96: S51-S70.

Martelli, G. P. 2014b. Rugose wood complex. Journal of Plant Pathology 96: S73-S88.

Martelli, G. P., N. Abou Ghanem-Sabanadzovic, A. A. Agranovsky, M. Al Rwahnih, V. V. Dolja, C. I. Dovas, M. Fuchs, P. Gugerli, J. S. Hu, W. Jelkmann, N. I. Katis, V. I. Maliogka, M. J. Melzer, W. Menzel, A. Minafra, M. E. Rott, A. Rowhani, S. Sabanadzovic, and P. Saldarelli. 2012. Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus *Ampelovirus* and the putative species unassigned to the family. Journal of Plant Pathology 94: 7-19.

Petersen, C. L., and J. G. Charles. 1997. Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinus* and *P. calceolariae*. Plant Pathology 46: 509-515.

Reggiani, A., R. Cornale, S. Maini, and G. Pellizzari. 2003. Observations on biology and distribution of *Heliococcus bohemicus* Sulc (Rhynchota: Pseudococcidae) in the vineyards of Emilia-Romagna Region (Italy). Informatore Fitopatologico 53: 42-45.

Sforza, R., E. Boudon-Padieu, and C. Greif. 2003. New mealybug species vectoring *Grapevine leafroll-associated viruses-1* and *-3* (GLRaV-1 and -3). European Journal of Plant Pathology 109: 975-981.

Swallow, W.H. 1985. Group testing for estimating infection rates and probabilities of disease transmission. Phytopathology 75: 882–889.

Tsai, C. W., A. Rowhani, D. A. Golino, K. M. Daane, and R. P. P. Almeida. 2010. Mealybug transmission of grapevine leafroll viruses: an analysis of virus-vector specificity. Phytopathology 100: 830-834.

Tsai, C. W., J. Chau, L. Fernandez, D. Bosco, K. M. Daane, and R. P. P. Almeida. 2008. Transmission of *Grapevine leafroll-associated virus 3* by the vine mealybug (*Planococcus ficus*). Phytopathology 98: 1093-1098.

Zorloni, A., S. Prati, P. A. Bianco, and G. Belli. 2006. Transmission of *Grapevine virus A* and *Grapevine leafroll-associated virus 3* by *Heliococcus bohemicus*. Journal of Plant Pathology 88: 325-328.

Table 1. Virus acquisition: results of virus detection in first- and second-instar nymphs of *H. bohemicus* collected within the Mango vineyard onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines in 2009, 2010 and 2011.

	No of positive/tested single nymphs			
Single viruses and virus combinations	2009	2010	2011	Total[a]
GVA	1/45	1/45	0/7	2/97
GLRaV-1	1/45	0/45	0/7	1/97
GLRaV-3	2/45	2/45	0/7	4/97
GVA + GLRaV-1	12/45	9/45	2/7	23/97
GVA + GLRaV-3	2/45	0/45	0/7	2/97
GLRaV-1 + GLRaV-3	4/45	0/45	0/7	4/97
GVA + GLRaV-1 + GLRaV-3	23/45	33/45	5/7	61/97
Total positive samples[b]	45/45	45/45	7/7	97/97

[a] Number of positive/tested single nymphs over the three sampling years.
[b] Number of nymphs resulted positive to at least one virus.

Table 2. Significance values obtained from application of the GLM to the two acquisition experiments and one transmission experiment.

Source	Acquisition Mango	Acquisition Albenga	Trasmission Albenga
Combinations	0.020	0.000	0.032
Time	n.s.	n.s.	n.s.
Combinations x Time	n.s.	0.000	n.s.

Table 3. Virus acquisition: results of virus detection in first- and second-instar nymphs of *H. bohemicus* collected in the Albenga vineyard onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines in 2011.

	No of positive/tested single nymphs		
Single viruses and virus combinations	June 2011	Aug. 2011	Total
GVA	1/42	0/17	1/59
GLRaV-1	3/42	0/17	3/59
GLRaV-3	1/42	2/17	3/59
GVA + GLRaV-1	3/42	2/17	5/59
GVA + GLRaV-3	1/42	0/17	1/59
GLRaV-1 + GLRaV-3	2/42	2/17	4/59
GVA + GLRaV-1 + GLRaV-3	31/42	9/17	40/59
Total positive samples[a]	42/42	15/17	57/59

[a] Number of nymphs resulted positive to at least one virus.

Table 4. Virus transmission: results of five experiments carried out with first- and second-instar nymphs of *H. bohemicus* (five nymphs per plant; 48h-IAP). The nymphs were field-collected onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines.

	No of positive/tested grapevines					
Single viruses and virus combinations	Exp 1 June 2010	Exp 2 July 2010	Exp 3 Aug. 2010	Exp 4 June 2011	Exp 5 Aug. 2011	Total[a]
GVA	0/13	0/14	0/24	1/18	0/16	1/85
GLRaV-1	0/13	0/14	0/24	3/18	2/16	5/85
GLRaV-3	1/13	1/14	5/24	2/18	3/16	12/85
GVA + GLRaV-1	0/13	0/14	0/24	0/18	0/16	0/85
GVA + GLRaV-3	1/13	0/14	1/24	0/18	0/16	2/85
GLRaV-1 + GLRaV-3	1/13	0/14	0/24	1/18	0/16	2/85
GVA + GLRaV-1 + GLRaV-3	0/13	0/14	0/24	0/18	0/16	0/85
Total positive samples[b]	3/13	1/14	6/24	7/18	5/16	22/85

[a] Number of positive/tested grapevines over the five transmission experiments.
[b] Number of test grapevines resulted positive to at least one virus.

Table 5. Probability estimates (*P*) of GVA, GLRaV-1 and GLRaV-3 acquisition by single nymphs of *H. bohemicus* in Mango and Albenga vineyards; probability estimates (*P*) of GVA, GLRaV-1 and GLRaV-3 transmission to test grapevines.

Virus probabilities	Acquisition Mango	Acquisition Albenga	Transmission
P_{GVA}	92.8%	87.2%	2.8%
$P_{GLRaV\text{-}1}$	94.7%	93.0%	8.2%
$P_{GLRaV\text{-}3}$	72.2%	85.8%	16.5%
RMSE[a]	0.44	0.68	0.24

The probability values are fitted from least square procedure.
[a] RMSE: Root Mean Square Error; RMSE is in unit of response

Table 6. Expected, under the null hypothesis of no antagonistic nor synergistic interactions between viruses, and observed frequencies, of acquisition and transmission of GVA, GLRaV-1 and GLRaV-3. The frequencies are distributed among the eight classes corresponding to the possible combinations of presence/absence of the three viruses.

	Acquisition Mango		Acquisition Albenga		Transmission	
Frequency classes	Expected frequencies	Observed frequencies	Expected frequencies	Observed frequencies	Expected frequencies	Observed frequencies
Absence of viruses	0.1	0	0.1	2	63.3	63
GVA	1.3	2	0.5	1	1.8	1
GLRaV-1	1.8	1	1.0	3	5.6	5
GLRaV-3	0.3	4	0.5	3	12.5	12
GVA + GLRaV-1	23.7	23	6.8	5	0.2	0
GVA + GLRaV-3	3.5	2	3.1	1	0.4	2
GLRaV-1 + GLRaV-3	4.8	4	6.0	4	1.1	2
GVA + GLRaV-1 + GLRaV-3	61.6	61	41.0	40	0.0	0
Sign.[a]	0.570 (NS)		0.414 (NS)		0.840 (NS)	

NS, not significant.
[a] Fisher Exact Test performed between expected and observed frequencies testing the distribution in the different frequency class.