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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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Bertin et al.: Virus transmission by
Heliococcus bohemicus

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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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15 **Abstract**

16 Mealybugs (Hemiptera: Pseudococcidae) represent a serious threat for viticulture as vectors

17 of phloem-restricted viruses associated with the grapevine rugose wood and leafroll diseases.

18 *Heliococcus bohemicus* (Šulc) is known to be involved in the spread of these two viral diseases,

19 being a vector of the *Grapevine virus A* (GVA) and the *Grapevine leafroll-associated virus 1* and *3*

20 (GLRaV-1 and -3). This study investigated the acquisition and transmission efficiency of *H.*

21 *bohemicus* fed on mixed-infected plants. Nymphs were field-collected onto GVA, GLRaV-1 and

22 GLRaV-3 multiple-infected grapevines in two vineyards in North-Western Italy, and were used in

23 transmission experiments under controlled conditions. Even if most of the collected nymphs were

24 positive to at least one virus, transmission occurred only to a low number of test grapevines. The

25 transmission frequency of GLRaV-3 was the highest whereas GVA was transmitted to few test

26 plants. The transmission of multiple viruses occurred at low rates, and nymphs that acquired all the

27 three viruses then failed to transmit them together. Statistical analyses showed that the three viruses

28 were independently acquired and transmitted by *H. bohemicus* and neither synergistic nor

29 antagonistic interactions occurred among them. GVA and GLRaVs transmission efficiencies by *H.*

30 *bohemicus* were lower than those reported for other mealybug vectors. This finding is consistent

31 with the slow spread of leafroll and rugose wood diseases observed in Northern Italy, where *H.*

32 *bohemicus* is the predominant vector species.

33

36   Mealybugs (Hemiptera: Pseudococcidae) are important pests that feed and reproduce on a

37   wide range of crops and ornamental plants worldwide. Grapevine is one of the most threatened host,

38   and reductions in plant vigor and yield can be noticed when mealybugs are abundant in the

39   vineyards. The impact on vine health is due to the phloem-feeding activity that takes away a lot of

40   sap and causes indirect damages, such as abundant excretion of honeydew that favors the

41   development of sooty mould, and transmission of phloem-inhabiting viruses. Actually, mealybugs

42   are known to transmit two different groups of positive single-stranded RNA viruses associated with

43   the grapevine leafroll and rugose wood diseases.

44   Leafroll disease is associated with a complex of virus species in the family *Closteroviridae*

45   collectively referred to as *Grapevine leafroll-associated viruses* (GLRaV-1, -2, -3, -4, -7) (Martelli

46   et al. 2012). The main leafroll symptoms are color alteration and downward rolling of the grapevine

47   leaves. The rugose wood complex includes different syndromes associated with virus species

48   belonging to the family *Betaflexiviridae*, genera *Vitivirus* (*Grapevine virus A*, *B*, *D*, *E*, and *F*; GVA,

49   GVB, GVD, GVE and GVF) and *Foveavirus* (Grapevine rupestris stem pitting associated virus;

50   GRSaV) (Martelli 2014b). Within this complex, the GVA-induced Kober stem grooving syndrome

51   is the most widespread and produces marked wood alteration. Both leafroll and rugose wood

52   diseases are responsible for delay in fruit maturation and severe reductions in quality and yield

53   (Martelli 2014a, b).

54   Besides mealybugs, also soft scales (Hemiptera: Coccidae) can transmit GLRaVs and rugose

55   wood-associated viruses to grapevine, but mealybugs are likely to play a major role in virus spread

56   because of their higher mobility. Although all life stages of mealybugs are capable of virus

57   transmission, first-instar nymphs are known to be the most efficient vectors (Petersen and Charles

58   1997, Tsai et al. 2008, Le Maguet et al. 2012). The current data on virus acquisition, retention and

59   inoculation indicate that GLRaV and vitivirus transmission occurs in a semi-persistent manner

60   (Cabaleiro and Segura 1997, Tsai et al. 2008), although the hypothesis of a circulative transmission

61   has been proposed, based on the evidence of GLRaV-3 presence in the mealybug salivary glands

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62   (Cid et al. 2007).

63   In Europe, several mealybug species are known to feed and breed on grapevine. The species

64   distribution as well as the population abundance can vary, depending on the climatic and

65   environmental conditions (Cabaleiro 2009). Some highly damaging vectors, such as *Planococcus*

66   *ficus* (Signoret) and *Planococcus citri* (Risso), prefer mild temperatures and are mainly established

67   in the Mediterranean basin. Other species are more tolerant to the severe continental climate and are

68   spread across the Central Europe. Among these, the Palaearctic species *Heliococcus bohemicus*

69   (Šulc) has been reported in vineyards in Northern France, Hungary, Germany and Northern Italy

70   (Kosztarab and Kozár 1988, Jakab and Szendrey 1989, Dalla Montà et al. 2001, Sforza et al. 2003,

71   Bertin et al. 2010). This species overwinters as nymph and can develop two generations per year,

72   with peak population densities in early-July and September (Camporese 1994, Reggiani et al. 2003).

73   The adult females of *H. bohemicus* can be easily recognized because of long and thin dorsal wax

74   filaments. This distinctive trait is less evident in the three immature instars that lead up to adult

75   females, and therefore the nymphs of *H. bohemicus* could be mistaken for other mealybug species

76   co-existing on grapevine. Molecular taxonomic tools contribute to a reliable identification of these

77   early stages and a PCR-based key is currently available for several grapevine mealybugs, including

78   *H. bohemicus* (Bertin et al. 2010).

79   *Heliococcus bohemicus* was considered a minor pest of grapevine, but in the last two

80   decades, it received a growing attention as vector of GLRaV-1, GLRaV-3 and GVA (Sforza et al.

81   2003, Zorloni et al. 2006). It was observed that, in case of mixed infections, GVA and GLRaVs can

82   be acquired and transmitted together by *H. bohemicus*, and it was hypothesized that GLRaVs

83   transmission would benefit from the presence of GVA (Zorloni et al. 2006, Bertin et al. 2010). The

84   role of GVA as helper virus for GLRaVs transmission was suggested for other mealybug and soft

85   scale species, but no conclusive evidence was provided so far (Fortusini et al. 1997, Tsai et al. 2010,

86   Bertin et al. 2016).

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87   Besides these preliminary observations, the virus transmission by *H. bohemicus* is still

88   poorly characterized. Therefore, we collected *H. bohemicus* nymphs fed onto GVA, GLRaV-1 and

89   GLRaV-3 mixed-infected grapevines in two vineyards in North-Western Italy and we tested the

90   ability of a subset of them to transmit the viruses under controlled conditions. The results provide

91   new insights into the acquisition and transmission efficiency of GVA and GLRaVs in case of mixed

92   infections, and on possible antagonistic or synergistic interactions among the three viruses. The

93   relevance of the results to the understanding of leafroll and rugose wood epidemiology is discussed.

94   **Materials and Methods**

95   **Insect Material.** Nymphs of *H. bohemicus* were collected in North-Western Italy in two

96   virus-infected vineyards located in Mango (Piemonte region) and Albenga (Liguria region). The

97   geographical location of the two vineyards is shown in Bertin et al. (2010). The experimental

98   vineyard in Mango, red-berried cv. Nebbiolo, consists of 19 rows of 45 plants each. Some non-

99   adjacent rows were originally planted with GVA + GLRaV-1 or GVA + GLRaV-3 infected vines,

100   while all the other rows were planted with healthy vines of the same clones. The sanitary status of

101   the vineyard was regularly monitored over several years by serological (DAS-ELISA) and

102   molecular analyses (PCR) to monitor the natural spread GVA, GLRaV-1 and GLRaV-3 (Gambino

103   and Gribaudo 2006, Gribaudo et al. 2009). Based on these data, two rows hosting GLRaV-1, -3 and

104   GVA mixed-infected grapevines were selected for the collection of *H. bohemicus* nymphs used for

105   estimating the acquisition efficiency. The selected plants tested negative to the *Grapevine fanleaf*

106   *virus* (GFLV) and the *Grapevine fleck virus* (GFkV), while the presence of other viruses was not

107   investigated. The presence of GVA, GLRaV-1 and GLRaV-3 in the selected plants were checked at

108   the beginning of each sampling season by real-time RT-PCR analysis.

109   The vineyard in Albenga, white-berried cv. Vermentino, is irregularly shaped and made of

110   about 100 rows of 75-85 plants each. The presence of GVA, GLRaV-1 and GLRaV-3 in the vines

111   was monitored by real-time RT-PCR analysis between 2009 and 2011; the presence of other viruses

112   was not investigated. Many symptomatic grapevines repeatedly tested positive for GVA, GLRaV-1

113   and GLRaV-3 viruses in mixed infections. Fourteen of these latter vines were selected for sampling

114   *H. bohemicus*. The nymphs were partly tested by real-time RT-PCR to assess their acquisition

115   efficiency and partly used for transmission trials.

116   The *H. bohemicus* nymphs were identified by morphology. However, the morphology-based

117   identification of early life stages can be awkward. Therefore, a sub-set of insect material was kept at

118   each sampling time and analyzed for species identification by molecular assays. The mitochondrial

119   *cytochrome oxidase subunit 1* (*COI*) gene was amplified (Bertin et al. 2010): the 200 bp amplicon

120   provided evidence that only *H. bohemicus* was collected within the two vineyards, and the presence

121   of other mealybug species was excluded.

122   Specimens of *H. bohemicus* collected in the field were tentatively used to establish healthy

123   insect colonies to perform virus acquisition experiments under controlled conditions. The colonies

124   were maintained in climatic chambers on sprouted potatoes in the dark at 20-30°C, under the same

125   conditions we use to rear *P. ficus* and *P. citri* colonies. Three different rearings were attempted, and

126   a large batch of specimens including all nymphal stages as well as mature females were used. In

127   spite of all the efforts, we failed to establish a colony. Therefore, only acquisition data obtained in

128   the field were included in the study.

129   **Virus Acquisition.** In Mango vineyard, first- and second-instar nymphs of *H. bohemicus*

130   were collected from 2009 to 2011 at three different sampling times in July, August and September,

131   when early stages are known to be present (Bertin et al. 2010). A total of 45, 45 and seven nymphs

132   were collected in 2009, 2010 and 2011, respectively. The nymphs were singly assayed by real time

133   RT-PCR to estimate the rates of GVA, GLRaV-1 and GLRaV-3 acquisition.

134   In Albenga vineyard, first- and second-instar nymphs were collected in summer and either

135   tested by real time RT-PCR or used in transmission experiments. Eight, six and seven groups of five

136   nymphs were analyzed in June, July and August 2010, respectively. In 2011, the nymphs were

137   singly assayed and a total of 42 and 17 nymphs were analyzed in June and August, respectively.

138   **Transmission Experiments.** Fourteen GVA, GLRaV-1 and GLRaV-3 mixed-infected

139   grapevines were carefully inspected within the Albenga vineyard for the presence of early stages of

140   *H. bohemicus*. Leaves hosting *H. bohemicus* nymphs were collected from each plant and kept in

141   cool boxes for transport to the laboratory. Here, first- and second-instar nymphs were immediately

142   removed with a fine brush under a stereomicroscope and transferred on known uninfected recipient

143   grapevines for virus transmission. The transmission was performed by clip-caging groups of five

144   nymphs on the lower surface of one leaf of each test plant for a virus inoculation access period

145   (IAP) of 48 h. The healthy recipient grapevines were obtained through micropropagation and

146   acclimatization in greenhouse of clonal lines of *V. vinifera* cv. Barbera. 48h-IAP was chosen based

147   on the observations available for other GLRaV-1, -3 and GVA mealybug vectors (Tsai et al. 2008):

148   it was already known that the virus transmission rates may reach the maximum even with a 24h-IAP

149   and the insect infectivity was lost within four-days of post-acquisition feeding.

150   After 48h-IAP, the nymphs were removed from the test grapevines, and these were drench-

151   treated with a systemic insecticide (Actara, Syngenta Crop Protection, Basel, Switzerland). The

152   vines were maintained in a greenhouse and regularly sprayed with insecticide and fungicide for

153   four-five months, until the RNA extraction and GLRaV-1, -3 and GVA detection. All plants were

154   periodically pruned to avoid overgrowth.

155   Five transmission trials were performed in June, July and August 2010 (experiments 1-3)

156   and in June and August 2011 (experiments 4-5). About 15 - 25 test grapevines were exposed to

157   viruliferous mealybugs in each transmission test: 13, 14, 24, 18 and 16 plants were alive at the end

158   of the five experiments and assayed for virus presence. To confirm the virus-free sanitary status of

159   the test plants and to ensure that virus spread had not occurred within the greenhouse during

160   experimental periods, *in vitro*-derived grapevines (five per experiment) from the same batch of test

161   plants were not exposed to *H. bohemicus* nymphs and served as negative controls.

162   **RNA Purification From Insects and Plants.** RNA of *H. bohemicus* was purified from

163   groups of five nymphs as well as from single nymphs using the TRIzol® Reagent (Invitrogen -

164   Thermo Fisher Scientific, Waltham, MA) and following the manufacturer's instructions. Samples

165   were treated with two units of RNase-Free DNase I (Applied Biosystems - Thermo Fisher

166   Scientific) to avoid residual DNA contamination. After DNA digestion, DNase was inactivated by

167   phenol/chloroform extraction. RNA was finally resuspended in 20 µl of RNase-free water

168   containing diethylpyrocarbonate (DEPC) 0.1%. Concentration and purity of extracts were evaluated

169   using the ND-1000 Spectrophotometer (Nanodrop - Thermo Fisher Scientific). RNA was then

170   diluted to 10 ng µl^{-1} and stored at -80 °C.

171   Plant RNA was extracted from the grapevines selected as sources of GLRaV-1, GLRaV-3

172   and GVA in Mango and Albenga vineyards. Total RNA was also extracted from test grapevines at

173   the end of the transmission trials. RNA was extracted from a total of 0.1 g of midribs from both

174   basal and apical leaves of each source /test plant. The extraction was performed using the Concert™

175   Plant RNA Isolation Reagent (Invitrogen) and following the manufacturer's instructions. RNA was

176   resuspended in 30 μl of DEPC 0.1% RNase-free water, diluted to 10 ng μl^{-1} and stored at -80 °C.

177   **Virus Detection.** Virus detection from both insect and plant RNA extracts was carried out

178   by SYBR® Green real-time RT-PCR assays in a Chromo4 Real Time Thermal Cycler (Bio-Rad, Life

179   Science Research, Hercules, California) supported by the OpticonMonitor 3.1.32 software (Bio-

180   Rad). GVA, GLRaV-1 and GLRaV-3 were detected with the following primer pairs, designed on the

181   appropriate viral coat protein sequences: GVA-C7273 (5′-CATCGTCTGAGGTTTCTACTA-3′) /

182   GVA-H7038 (5′-AGGTCCACGTTTGCTAAG-3′) (MacKenzie et al. 1997); GLRaV-1fw (5′-

183   CGTTTGAAAATCCTATGCGTCAG-3′) / GLRaV-1rev (5′-GCAACTTTCTCGTTCGGCTTC-3′)

184   and   GLRaV-3fw   (5′-TTCGAGAAAGATCCAGACAAGTTC-3′)   /   GLRaV-3rev   (5′-

185   ATAACCTTCTTACACAGCTCCATC-3′) (Gribaudo et al. 2009). Real-time RT-PCR was

186   performed with the iScript One-Step RT-PCR kit (Bio-Rad), using a final primer concentration of

187   300 nM. Ten nanograms of insect or plant total RNA were used as templates. For all the primer

188   pairs, thermo-cycling conditions consisted of an initial cycle at 50°C for 10 min, followed by 5 min

189   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

190   of the PCR to assess the reaction specificity: the PCR products were heated to 95°C for 1 min,

191   cooled at 65°C for 1 min and then slowly heated back to 95°C at a rate of 0.5°C per cycle.

192   RNA of *H. bohemicus* carrying GVA, GLRaV-1 and GLRaV-3 and RNA of healthy *P. ficus*

193   from laboratory colony on sprouted potatoes were served as positive and negative controls,

194   respectively. RNA extracts from GVA, GLRaV-1 and GLRaV-3 infected vines as well as from

195   healthy *in vitro*-generated grapevines were used as controls for virus detection in plant.

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

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197   RNA (100 ng) was reverse transcribed into cDNA using High Capacity cDNA Reverse

198   Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The full-length

199   GLRaV-3 coat protein gene was then amplified using KSL95-5 / KSL95-6 primer pair (Ling et al.

200   1997). PCR was carried out as suggested by Gouveia et al. (2011), except for the cycling conditions

201   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

202   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

203   for 30 s, 52°C for 30 s, 68°C for 5 min. PCR fragments were cloned and sequenced. Sequences

204   were aligned with the coat protein genes from representative GLRaV-3 isolates available in

205   GenBank and assigned to the corresponding phylogenetic group (I-VI) according to Maree et al.

206   (2013).

207   **Data analysis.**

208   The data from virus acquisition experiments carried out in Mango and Albenga vineyards

209   and from transmission experiments were analyzed through generalized linear model (GLM) using a

210   binomial distribution and logit as link function (SPSS version 22). This analysis allowed to infer the

211   combination prevailing among the three viruses in each experiment, the effect of the time among

212   the experimental replicates, and the possible interaction between these two factors. Since

213   transmission experiments were conducted with five insects per plant, while the acquisition rate was

214   assessed on single nymphs, the maximum-likelihood estimator, Ps (Swallow, 1985), was applied to

215   estimate the actual proportion of infected insects within each five-insect batch used for

216   transmission.

217   Then, acquisition frequencies of single nymphs collected in the Mango and Albenga

218   vineyards and transmission frequencies to inoculated plants were analyzed to infer possible

219   antagonistic or synergistic interactions, among GVA, GLRaV-1 and GLRaV-3. The interactions

220   were tested by comparing the observed against the expected frequencies, through Fisher Exact Test

221   for Count Data (Fisher 1934). Fisher Exact Test was used instead of χ^2 because expected

222   frequencies were less than five in some classes, that is a limit in χ^2 application. The analyses were

223   performed using R version 3.1.3. Virus acquisition/transmission frequencies were distributed among

224   eight (2^3) classes that correspond to every possible combination of presence/absence of GVA,

225   GLRaV-1 and GLRaV-3 (absence of viruses, presence of one virus, presence of two viruses and

226   presence of three viruses). Expected class frequency distribution was calculated under the null

227   hypothesis that antagonism or synergism does not exist among GVA, GLRaV-1 and GLRaV-3 in

228   both acquisition and transmission. Expected frequency for each class (F_i) was calculated as follow:

229          $F_i = N*P_i$

230   where:

231   N = number of analyzed nymphs

232   P_i = probability of each class.

233   The   probability   of   each   class   was   calculated   combining   the   probability   of   each

234   acquisition/transmission according to binomial distribution, as follow:

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

236   where $(P \text{ or } Q)_{virus}$ is the probability of acquisition/transmission or non-acquisition/non-

237   transmission associated to each virus in that class. Obviously, $P + Q = 1$ for each virus. As

238   information on probability of acquisition/transmission associated to each virus were not available *a*

239   *priori*, the P/Q values were obtained by fitting from least square procedure the GVA, GLRaV-1 and

240   GLRaV-3 frequencies observed in single nymphs from Mango and Albenga and in inoculated

241   plants. The Root Mean Square Error (RMSE) of each fitting was calculated (Loague and Green

242   1991).

243 **Results**

244 **Virus Acquisition.** Table 1 reports the results of GVA, GLRaV-1 and GLRaV-3 detection in

245 *H. bohemicus* nymphs collected in Mango vineyard in 2009, 2010 and 2011. GLM results showed a

246 significant effect of the virus combination, while no significant effect were recorded for time or

247 interaction combination x time (Table 2). All the specimens tested positive for at least one virus.

248 Considering the total number of nymphs collected in Mango from 2009 to 2011, 63% acquired all

249 the three viruses together and this rate was higher than all the other single and mixed virus

250 combination rates. Among the single and double virus acquisitions, GVA + GLRaV-1 combination

251 showed the highest frequency (24% tested nymphs).

252 In Albenga, the estimate of virus acquisition was carried out in 2010 and 2011, at three and

253 two sampling dates respectively. Due to the huge number of *H. bohemicus* specimens infesting the

254 vineyard, the first-year analyses were performed with batches of five nymphs; all the tested batches

255 were positive to the three viruses. Such a result was not informative for an accurate estimate of the

256 incidence of single and mixed GVA, GLRaV-1 and GLRaV-3 combinations in each viruliferous

257 nymph. Therefore, the further two analyses carried out in 2011 were performed with single nymphs.

258 Most of the singly-assayed nymphs acquired at least one virus, being the total rate of positive

259 samples between 88 and 100% (Table 3), and no significant effect of the time was observed

260 between the two experiments (Table 2). Both the virus combination and the interaction combination

261 x time resulted to be significant by GLM analysis (Table 2), indicating that the number of nymphs

262 positive to single GVA, GLRaV-1 and GLRaV-3 as well as to the different virus combinations

263 significantly differed between the two samplings performed in 2011. In any case, the GVA +

264 GLRaV-1 + GLRaV-3 acquisition was prevailing against all the other single and mixed virus

265 combinations also in singly-tested nymphs at both samplings.

266 GLRaV-3 isolate infecting the source grapevines in both Mango and Albenga vineyards

267 belonged to the phylogenetic group I, according to Maree et al. (2013).

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

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269   2010 and 2011 in the Albenga vineyard. Following 48h-IAP, the virus transmission occurred in all

270   the experiments (Table 4). No viruses were detected in *in vitro*-generated grapevines used as

271   negative controls in each experiment. Percentages of plants positive to at least one virus ranged

272   between 7 and 39% and no plants infected by all the three viruses together and by GVA + GLRaV-1

273   were found. No significant effect of the time alone or in interaction with combination was recorded

274   (Table 2).

275   The GLM analysis revealed a significant effect of the virus combination on the transmission

276   results. Considering the total number of positive plants over the five transmission experiments

277   (Table 4), it is evident that GVA was poorly transmitted: it was detected in three grapevines only,

278   alone or together with GLRaV-3. GLRaV-1 and GLRaV-3 were transmitted to a total of seven and

279   16 plants respectively, and their transmission mainly occurred as single infection. Indeed, the rate of

280   both GLRaV-1 and GLRaV-3 single infections was higher than the rate of mixed infections. Overall

281   incidences of GVA, GLRaV-1 and GLRaV-3 on the 22 virus-positive test grapevines were 14, 32

282   and 73%, respectively. The estimated proportion of infecting *H. bohemicus* nymphs was: Ps = 0.03

283   for GVA, Ps = 0.07 for GLRaV-1 and Ps = 0.23 for GLRaV-3. GLRaV-3 transmission rate was the

284   highest.

285   **Inferences on Virus Interaction.** Possible antagonisms or synergisms among GVA,

286   GLRaV-1 and GLRaV-3 during acquisition and transmission were inferred by comparing observed

287   *vs* expected virus frequencies in single nymphs from Mango and Albenga vineyards and in test

288   grapevines. Expected frequency values depend on the probability of acquisition/transmission

289   associated to each virus (P_{GVA}, $P_{GLRaV-1}$ and $P_{GLRaV-3}$). P values of virus acquisition in Mango and

290   Albenga vineyards as well as of virus transmission were calculated (Table 5); the values always

291   fitted with an error of estimation (RMSE) that was lower than one unit of response (one acquisition

292   or transmission), indicating that the estimate procedure is very good. Acquisition values of P_{GVA},

293    $P_{GLRaV-1}$ and $P_{GLRaV-3}$ were high for both Mango and Albenga vineyards, because most of the single

294   nymphs from both sampling sites tested positives to all the three viruses. On the contrary, the low

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295　number of plants inoculated after 48h-IAP made the transmission probabilities low for all the three

296　viruses, with an upward trend from P_{GVA} to $P_{GLRaV-1}$ and to $P_{GLRaV-3}$.

297　　　　Probability estimates allowed to calculate the expected acquisition and transmission

298　frequencies for each of the eight classes generated by all possible combinations of presence/absence

299　of GVA, GLRaV-1 and GLRaV-3 (Table 6) under the null hypothesis that antagonistic nor

300　synergistic interactions exist between acquisition or transmission of different viruses. At each class,

301　the distribution of the expected frequencies were compared with the observed ones. No significant

302　differences were recorded between expected and observed acquisition frequencies in single nymphs

303　from both Mango and Albenga vineyards. Expected and observed transmission frequencies in test

304　plants did not differ as well. This leads to accept the null hypothesis that the frequency class

305　distribution is generated by random processes and neither antagonistic nor synergistic interactions

306　among GVA, GLRaV-1 and GLRaV-3 could be identified at both acquisition and transmission.

307   **Discussion**

308   *Heliococcus bohemicus* is gaining a growing economical relevance for its role in the spread

309   of leafroll and rugose wood diseases, and this study improves the current knowledge in its virus

310   acquisition and transmission efficiency. The vector competence of *H. bohemicus* has been

311   ascertained only for GVA, GLRaV-1 and GLRaV-3 so far. These three viruses are also the most

312   common grapevine viruses in the regions where *H. bohemicus* is present. Other vector-transmissible

313   closteroviruses and vitiviruses, such as GLRaV-4 and GVB, are infrequent in Central Europe

314   (CABI 2016), and were therefore not included in this study.

315   The insect material was from two different vineyards in North-Western Italy, which have

316   been monitored over several years for both mealybug infestation and GVA, GLRaV-1 and GLRaV-

317   3 infection. The experimental vineyard in Mango was originally planted with healthy vines together

318   with GVA + GLRaV-1 or GVA + GLRaV-3 infected vines, in alternating rows. After several years,

319   few grapevines became infected by all the three viruses, as a result of natural virus spread.

320   *Heliococcus bohemicus* is the only mealybug species found within the vineyard and occurs at low

321   population density (Bertin et al. 2010). Its low density is probably a side effect of the compulsory

322   insecticide treatments targeted against *Scaphoideus titanus* Ball, the leafhopper vector of

323   Flavescence dorée. In Albenga vineyard, the density of *H. bohemicus* population is noticeably high

324   (Bertin et al. 2010), perhaps thanks to the Mediterranean mild climate of the Liguria region and the

325   absence of compulsory treatments until 2014. Moreover, spot diagnoses were performed on vines to

326   estimate the virus spread within the vineyard and high rates of infection were observed: only few

327   sampled grapevines resulted to be virus-free and many plants tested positive to all the three viruses

328   (C.M., unpublished data).

329   The first- and second-instar nymphs of *H. bohemicus* were collected in Mango and Albenga

330   vineyards onto GVA, GLRaV-1 and GLRaV-3 mixed-infected grapevines. The nymphs were

331   infected by all the three viruses in a very high proportion. Therefore, *H. bohemicus* showed high

332   efficiency of GVA, GLRaV-1 and GLRaV-3 acquisition in the field, independently of the climatic

333   and agricultural conditions. Such acquisition rates allowed to predict a high infective potential for

334   all the three viruses. However, it has already been observed for other mealybugs that differences

335   between acquisition and transmission rates can considerably lower the actual rate of infected plants

336   (Cabaleiro and Segura 1997; Bertin et al., 2016).

337   The insects used in transmission trials were only from Albenga vineyard, where the

338   abundance of source grapevines and the high density of *H. bohemicus* population ensured an

339   adequate number of insects needed for plant inoculation. Experiments were carried out with nymphs

340   of the same age of those used for testing virus acquisition, and collected on the same leaves.

341   Although the nymphs showed high acquisition efficiency, the virus transmission occurred at

342   relatively low rates: only 26% of the inoculated test grapevines resulted positive to at least one

343   virus. However, much lower rates of virus transmission were observed in the few studies dealing

344   with vector competence of *H. bohemicus*. Sforza et al. (2003) reported that large groups of

345   individuals carrying GLRaV-1 and GLRaV-3 were able to transmit at least one virus to 23% of test

346   plants. This rate is similar to the transmission frequency that we observed under our experimental

347   conditions, but was obtained with a higher number of insects per plant (30 – 50 individuals of all

348   stages *vs* five nymphs). Moreover, when Zorloni et al. (2006) tested the co-transmission of GVA,

349   GLRaV-1 and GLRaV-3 by *H. bohemicus*, they found one GLRaV-3 positive plant and one

350   GLRaV-3 + GVA positive plant out of 77 test grapevines only. Therefore, our estimate of virus

351   transmission efficiency is higher than previously reported for *H. bohemicus*. The different life

352   stages employed in the transmission experiments could contribute to explain these different

353   efficiencies. Sforza et al. (2003) used individual of all stages, and Zorloni et al. (2006) employed

354   AAPs ranging from 4 to 21 days, thus using in the inoculation phase late instar nymphs and

355   probably adult females that lost their infectivity after molting (Tsai et al. 2008). Now it is known

356   that early instar nymphs are much more efficient vectors compared to older nymphs and adults (Tsai

357   et al. 2008, Mahfoudhi et al. 2009, Le Maguet et al. 2012), and our experiments were carried out

358   accordingly. This probably increased the transmission rates. Moreover, the different sensitivity of

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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

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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.

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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.

Single viruses and virus combinations	No of positive/tested single nymphs			
	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.

Single viruses and virus combinations	No of positive/tested single nymphs		
	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.

Single viruses and virus combinations	No of positive/tested grapevines					
	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%
$P_{GLRaV-1}$	94.7%	93.0%	8.2%
$P_{GLRaV-3}$	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.

Frequency classes	Acquisition Mango		Acquisition Albenga		Transmission	
	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.