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Trends in vector-borne transmission efficiency from coinfected hosts: *Grapevine leafroll-associated virus-3* and *Grapevine virus A*

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Abstract *Grapevine leafroll-associated virus 3* (GLRaV-3) is the most prevalent and destructive virus species that contributes to grapevine leafroll disease, an economically damaging disease that affects vineyards globally. *Grapevine virus A* (GVA) is a virus species in the rugose wood complex and is associated with several vineyard diseases. Both virus species are transmitted by several mealybug species. Transmission efficiency is a major facet of pathogen spread and may be influenced by virus species interactions in the vector or host. We tested transmission efficiency of GLRaV-3 and GVA from nine field-collected source vine samples of *Vitis vinifera* cv Chardonnay by first instars of *Planococcus ficus*. Transmission of GLRaV-3 was 22% greater than transmission of GVA. Establishment of new mixed GLRaV-3/GVA infections did not differ significantly from single GLRaV-3 infections following inoculation by *P. ficus*. These results suggest that GVA may have a higher likelihood of establishing new infections in concert with GLRaV-3 than in single infections.

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Abbreviations

AAP	Acquisition access period
GLRaV-3	Grapevine leafroll-associated virus 3
GVA	Grapevine virus A
IAP	Inoculation access period

The mealybug-transmitted *Grapevine leafroll-associated virus 3* (GLRaV-3; *Ampelovirus*, *Closteroviridae*) is the most prevalent and destructive virus species associated with grapevine leafroll disease globally (Almeida et al. 2013). As spread and prevalence of GLRaV-3 have increased in recent decades, its potential interactions with other mealybug-borne viruses that cause vineyard diseases may influence spread of those pathogens (Hommay et al. 2007; Blaisdell et al. 2015). *Grapevine virus A* (GVA; *Vitivirus*, *Betaflexiviridae*) is a virus species member of the rugose wood complex of grapevines, and has been associated with Kober stem grooving and Shiraz disease (Garau et al. 1994; Goszczynski and Jooste 2003). Both GLRaV-3 and GVA can be transmitted by several mealybug species (La Notte et al. 1997; Hommay et al. 2007; Bertin et al. 2016a, 2016b; Engelbrecht and Kasdorf 1990).

One major facet of the vector-virus relationship is transmission efficiency, which may be influenced by a

number of biological and environmental factors. For example, viruses coinfecting a source plant may affect each other during vector transmission and/or establishment in a new host plant (Blaisdell et al. 2015). Past research has indicated that grapes are often coinfected with GLRaV-3 and GVA (Preez et al. 2011). More recently, Rowhani et al. (2018) found an increased incidence of vitiviruses in coinfection with GLRaVs than in single infections, as well as elevated vitivirus populations in coinfections with GLRaVs relative to single infections. However, the interaction between these two economically damaging viruses is relatively unknown. Both viruses are transmitted by the vine mealybug species *Planococcus ficus* (Hemiptera, Pseudococcidae), which is itself an economically damaging pest, as well as other mealybug and soft scale species (Daane et al. 2018). The aim of this study was to compare the transmission efficiencies of GVA and GLRaV-3 from grapes with coinfections of the two virus species. We tested transmission efficiency of GLRaV-3 and GVA by *P. ficus* from field-collected source vine samples of *Vitis vinifera* cv Chardonnay that were coinfected with GLRaV-3 and GVA.

Virus-free *Vitis vinifera* cv Pinot noir grape cuttings from the Foundation Plant Services at the University of California, Davis were rooted on a greenhouse mist bench. The rooted cuttings were later potted and maintained in a greenhouse for use as recipient test plants. Concurrently, we obtained infected vine material from a commercial vineyard in Napa Valley, CA USA planted with *Vitis vinifera* cv Chardonnay that were heavily symptomatic for grapevine leafroll disease including misshaping and discoloration of leaves. The selected site corresponds to site 35 from Sharma et al. (2011), with a high incidence of GLRaV-3 infections. One cutting was collected from each of 10 infected vines; each field-collected cane was placed in a beaker of water, with the stem cut underwater to prevent interference with the xylem water column. Immediately following experimental manipulations, we removed three petioles from the infected cuttings, selecting leaves that were used as sources in inoculations, and stored them at −80 °C for future viral testing. Mealybugs (*P. ficus*) were used as vectors for virus transmission assays. The mealybug colonies were maintained on butternut squash in a growth chamber at 22 ± 2 °C, with a 12:12-h photoperiod. Only first instar individuals were used in the transmission experiments as this life stage is the most efficient vector of GLRaV-3 in laboratory transmission trials (Tsai et al. 2008).

For experimental inoculations, we collected a large number of mealybugs by placing moistened filter papers against butternut squash in the insect colonies, waiting for one hour, and then using forceps to remove the filter papers with mealybugs. We then pinned the filter papers to the leaf underside of source plant cuttings. As the paper dried, most mealybugs crawled onto the leaves of the source plants. The mealybugs were left on the leaves for a 24-h acquisition access period (AAP), after which we removed the leaves containing the mealybugs and shook the mealybugs onto a piece of black construction paper. With a paintbrush, we transferred mealybugs to clip cages (described in Tsai et al. 2008). Mealybugs from each source plant ($n = 10$) were transferred to 20 uninfected recipient test plants, using one cage with five mealybugs for each of the recipient test plants. The cages were positioned so that the mealybugs had access to the lower surface of one leaf of each test plant for a 24-h inoculation access period (IAP). After the IAP, the mealybugs were removed from the test plants, and then the recipient test plants were treated with a contact insecticide until all leaves were wet (Ace Hardware Insect Killer, active ingredients cypermethrin and prallethrin) to kill any remaining mealybugs. In addition to the 200 inoculated test plants used in the transmission experiments, 20 negative control test plants were used to confirm that no unintended mealybug transmissions of viruses occurred in the greenhouse. All test plants were kept in a light-supplemented greenhouse and were regularly treated with insecticide. Four months after inoculations, three petioles were collected from each test plant and stored at −80 °C for viral testing. Three of 200 recipient test plants died before petiole collection and were therefore excluded from analyses.

Source plant material was tested for a full panel of viruses and other known pathogens of grapes at Foundation Plant Services of University of California, Davis, using qPCR (Diaz-Lara et al. 2018; Rasool et al. 2019). Source plants were tested for presence of the following species and strains/variants: *Grapevine fleck virus*, *Grapevine fanleaf virus*, *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Tomato ringspot virus*, *Xylella fastidiosa,* Grapevine leafroll-associated viruses (GLRaV)-1, GLRaV-2, GLRaV-2RG, GLRaV-7, GLRaV-3 (with a general primer pair, and another pair specific to variant VI of GLRaV-3), GLRaV-4 (strains 5, 6, 9, 10, and Car), *Grapevine virus A*

(GVA), GVB, GVD, GVE, and *Tobacco ringspot virus*. In addition, samples were tested for 18S ribosomal RNA as an internal quality control.

One field-collected source cutting tested positive only for GLRaV-3. The remaining nine cuttings tested positive for both GLRaV-3 and GVA. Of the nine coinfected vines, two also tested positive for GRSPaV, and one tested positive for GVB (Table 1). Transmission efficiency of GLRaV-3 and GVA was tested from the nine cuttings that were coinfected with GLRaV-3 and GVA. Recipient vines were not tested for GVB or GRSPaV.

To test the experimentally-inoculated recipient vines for infection with GLRaV-3 and GVA, total RNA was extracted and denatured following Sharma et al. (2011), except that tissue of three petioles was pooled from each plant sampled. Recipient vines were tested for GLRaV-3 and GVA, using a 600 nM concentration for each primer. Primer pair CP (Sharma et al. 2011) was used for detection of GLRaV-3, and primer pair GVA (Minafra and Hadidi 1994) was used for detection of GVA. For all plant samples, multiplex one step RT-PCR was performed using Qiagen kits, followed by fragment analysis as described by Sharma et al. (2011).

The proportions of 20 recipient test plants that became infected with GLRaV-3, GVA, both GLRaV-3 and GVA, or did not become infected, were compared among the nine source plants that were coinfected with GLRaV-3 and GVA. The tenth source plant, in which GVA was not detected, was excluded from the analysis of establishment in recipient test plants. The proportions of established infections differed among the nine source plants ($\chi^2 = 64$, $P < 0.0001$, df = 24). Therefore, each source plant was treated as one replicate group for each analysis comparing establishment of each virus combination. A t-test was used to compare the overall establishment of GLRaV-3 to GVA, regardless of coinfection status. We used ANOVA to compare the establishment of each possible virus combination, GLRaV-3, GVA, or coinfection. A follow-up Tukey's HSD was performed to compare these three possible infection outcomes. The proportions of recipient plants that became infected with each possible virus combination from each of the nine source plants were arcsine-transformed prior to analyses to better meet the assumptions of ANOVA. Untransformed values are presented for ease of interpretation. All analyses were performed using version 3.2.0 of R and results are presented as mean ± SE.

Overall, 58 ± 6% of all vines became infected with at least one of the two viruses tested. Both GVA and GLRaV-3 were successfully transmitted from all nine coinfected source vines. A t-test comparing GLRaV-3 and GVA regardless of coinfections revealed that infection with GLRaV-3 (55 ± 7%) was greater than infection with GVA (33 ± 6%) (t = 2.39, $P = 0.0293$, df = 16). The proportion of recipient plants that became infected with each of three possible combinations differed significantly (F = 8.59, $P = 0.00153$, df = 2, 24). Tukey's HSD revealed that

Table 1 Transmission results from nine source vines coinfected with GLRaV-3 and GVA. Row and vine number are indicated for each source vine. Recipient test plant status shows number positive/total tested, and percent in parentheses

		Recipient test plant status[a]		
Source plant	Viruses in source plant	GLRaV-3	GVA	GLRaV-3 and GVA
R3V5	GLRaV-3, GVA	2/20 (10.0)	0/20 (0)	11/20 (55.0)
R4V7	GLRaV-3, GVA	3/20 (15.0)	1/20 (5.0)	9/20 (45.0)
R2V3	GLRaV-3, GVA	3/20 (15.0)	0/20 (0)	9/20 (45.0)
R6V8	GLRaV-3, GVA	5/20 (25.0)	0/20 (0)	8/20 (40.0)
R8V2	GLRaV-3, GVA, GRSPaV	4/19 (21.5)	0/19 (0)	3/19 (15.8)
R9V9	GLRaV-3, GVA	2/20 (10.0)	1/20 (5.0)	4/20 (20.0)
R11V4	GLRaV-3, GVA, GRSPaV	2/19 (10.5)	2/19 (10.5)	2/19 (10.5)
R7V7	GLRaV-3, GVA, GVB	9/19 (47.4)	0/19 (0)	8/19 (42.1)
R10V11	GLRaV-3, GVA	13/20 (65.0)	0/20 (0)	1/20 (5.0)

[a] The transmission rate is calculated as the number of test plants positive for the given virus or viruses out of the total number of test plants that were inoculated with viruses from the source plant; dead test plants were excluded from the total number of test plants

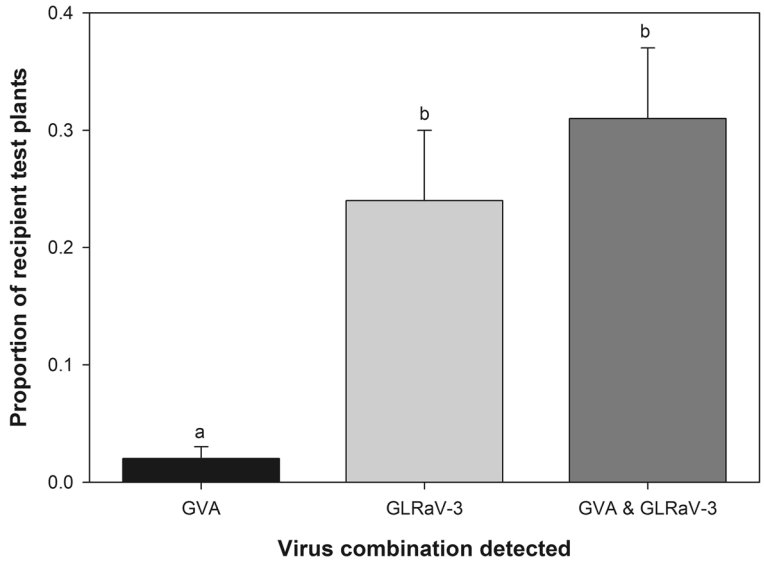

Fig. 1 Proportion of recipient test plants in which new infections of *Grapevine leafroll-associated virus-3*, *Grapevine virus A*, or coinfections were detected, mean + standard error. There were significantly fewer single infections with *Grapevine virus A* than single infections with *Grapevine leafroll-associated virus-3* and coinfections ($P < 0.05$), which did not significantly differ from each other

establishment of only GVA (2 ± 1%) was significantly lower than establishment of only GLRaV-3 (24 ± 6%) and coinfections (31 ± 6%), which did not significantly differ from each other ($P < 0.05$) (Fig. 1).

In conclusion, we found evidence that the mealybug-borne transmission of GVA may be augmented by the presence of GLRaV-3. Recently, however, tests of acquisition and transmission of GLRaV-1, GLRaV-3, and GVA by *H. bohemicus*, *P. citri*, and *P. ficus* did not find support for synergy among viruses during transmission (Bertin et al. 2016a, 2016b), perhaps related to lower overall transmission rates or weaker statistical power. In support of our findings, molecular analysis of material collected from multiple wine regions globally over 10 years combined with inoculation trials found increased incidence and disease severity, as well as larger relative viral populations of vitiviruses in coinfections with GLRaV-1 and -3 than when GLRaVs were absent (Rowhani et al. 2018). We did not measure viral population size in this study. Field inoculations of *V. vinifera* cv Pinot noir using *P. maritimus* provided tentative support for the influence of GLRaV-3 on the establishment of *Grapevine virus B* (Vitivirus, Betaflexiviridae) (Blaisdell et al. 2016). It is important to consider that vector-borne virus transmission and plant infection may be influenced by virus interactions within the vector or within the susceptible host during establishment of infection (e.g. Blaisdell et al. 2015). We did not attempt to isolate factors driving the findings of this study.

We note that even with testing for a full panel of known viruses, as yet undiscovered viruses that could influence transmission of GVA and GLRaV-3 may have been present in our source vines. Virus-virus interactions may be related to vector feeding behavior, the timing of inoculations, host defenses, and other factors (Julve et al. 2013; Gutiérrez et al. 2013). Although virus-virus interactions may be difficult to characterize, they play important roles in the epidemiology of their associated plant diseases. Knowledge of these interactions will be useful in the development of better management approaches for grapevine leafroll disease as well as diseases associated with GVA.

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Compliance with ethical standards

Conflict of interest The authors certify that we do not have any actual or potential conflict of interest, the study described is original, and has not been published previously. All authors have reviewed the manuscript and approved the final version of manuscript before submission.

Human participants and/or animals Not applicable.

Informed consent The authors certify that this research followed the principles of ethical and professional conduct.

References

Almeida, R. P. P., Daane, K. M., Bell, V. A., Blaisdell, G. K., Cooper, M. L., Herrbach, E., & Pietersen, G. (2013). Ecology and management of grapevine leafroll disease. *Frontiers in Virology, 4*, 94. https://doi.org/10.3389/fmicb.2013.00094.

Bertin, S., Cavalieri, V., Gribaudo, I., Sacco, D., Marzachì, C., & Bosco, D. (2016a). Transmission of *Grapevine virus A* and *Grapevine leafroll-associated virus 1* and *3* by *Heliococcus bohemicus* (Hemiptera: Pseudococcidae) nymphs from plants with mixed infections. *Journal of Economic Entomology, 109*, 1504–1511.

Bertin, S., Pacifico, D., Cavalieri, V., Marzachì, C., & Bosco, D. (2016b). Transmission of *Grapevine virus A* and *Grapevine leafroll-associated viruses 1* and *3* by *Planococcus ficus* and *Planococcus citri* fed on mixed-infected plants. *Annals of Applied Biology, 169*, 53–63.

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

Blaisdell, G. K., Cooper, M. L., Kuhn, E. J., Taylor, K. A., Daane, K. M., & Almeida, R. P. P. (2016). Disease progression of vector-mediated *Grapevine leafroll-associated virus 3* infection of mature plants under commercial vineyard conditions. *European Journal of Plant Pathology, 146*, 105–116.

Daane, K. M., Vincent, C., Isaacs, R., & Ioriatti, C. (2018). Entomological opportunities and challenges for sustainable viticulture in a global market. *Annual Review of Entomology, 63*, 193–214.

Diaz-Lara, A., Klaassen, V., Stevens, K., Sudarshana, M. R., Rowhani, A., Maree, H. J., Chooi, K. M., Blouin, A. G., Habili, N., Song, Y., Aram, K., Arnold, K., Cooper, M. L., Wunderlich, L., Battany, M. C., Bettiga, L. J., Bester, R., Xiao, H., Meng, B., Preece, J. E., Golino, D., & Al Rwahnih, M. (2018). Characterization of grapevine leafroll-associated virus 3 genetic variants and application towards RT-qPCR assay design. *PLoS One, 13*(12), e0208862.

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

Garau, R., Prota, V. A., Piredda, R., Boscia, D., & Prota, U. (1994). On the possible relationship between Kober stem grooving and *Grapevine virus A*. *Vitis, 33*, 161–163.

Goszczynski, D. E., & Jooste, A. E. (2003). Identification of divergent variants of *Grapevine virus A*. *European Journal of Plant Pathology, 109*, 397–403.

Gutiérrez, S., Michalakis, Y., Van Munster, M., & Blanc, S. (2013). Plant feeding by insect vectors can affect life cycle, population genetics and evolution of plant viruses. *Functional Ecology, 27*, 610–622.

Hommay, G., Komar, V., Lemaire, O., & Herrbach, E. (2007). *Grapevine virus A* transmission by larvae of *Parthenolecanium corni*. *European Journal of Plant Pathology, 121*, 185–188.

Julve, J. M., Gandía, A., Fernández-del-Carmen, A., Sarrion-Perdigones, A., Castelijns, B., Granell, A., & Orzaez, D. (2013). A coat-independent superinfection exclusion rapidly imposed in *Nicotiana benthamiana* cells by tobacco mosaic virus is not prevented by depletion of the movement protein. *Plant Molecular Biology, 81*, 553–564.

La Notte, P., Buzkan, N., Choueiri, E., Minafra, A., & Martelli, G. P. (1997). Acquisition and transmission of grapevine virus a by the mealybug *Pseudococcus longispinus*. *Journal of Plant Pathology, 79*, 79–85.

Minafra, A., & Hadidi, A. (1994). Sensitive detection of *Grapevine virus A, B*, or *leafroll-associated III* from viruliferous mealybugs and infected tissue by cDNA amplification. *Journal of Virological Methods, 47*, 175–187.

Preez, J., Stephan, D., Mawassi, M., & Burger, J. T. (2011). The grapevine-infecting vitiviruses, with particular reference to *Grapevine virus A*. *Archives of Virology, 156*, 1495–1503.

Rasool, S., Naz, S., Rowhani, A., Diaz-Lara, A., Golino, D. A., Farrar, K. D., & Al Rwahnih, M. (2019). Survey of grapevine pathogens in Pakistan. *Journal of Plant Pathology, 101*, 725–732.

Rowhani, A., Daubert, S., Arnold, K., Al, R. M., Klaassen, V., Golino, D., & Uyemoto, J. K. (2018). Synergy between grapevine vitiviruses and grapevine leafroll viruses. *European Journal of Plant Pathology, 4*, 919–925.

Sharma, A. M., Wang, J., Duffy, S., Zhang, S., Wong, M. K., Rashed, A., Cooper, M. L., Daane, K. M., & Almeida, R. P. P. (2011). Occurrence of grapevine leafroll-associated virus complex in Napa Valley. *PLoS One, 6*(10), e26227.

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