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No evidence of transmission of grapevine leafroll-associated viruses by phylloxera (*Daktulosphaira vitifoliae*)

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Abstract Grapevine leafroll disease is associated with several species of phloem-limited grapevine leafroll-associated viruses (GLRaV), some of which are transmitted by mealybugs and scale insects. The grape phylloxera, *Daktulosphaira vitifoliae* (Fitch) Biotype A (Hemiptera: Phylloxeridae), is a common vineyard pest that feeds on the phloem of vine roots. There is concern that these insects may transmit one or more GLRaV species, particularly GLRaV-2, a species in the genus *Closterovirus*. A field survey was performed in vineyards with a high incidence of grapevine leafroll disease and *D. vitifoliae* was assessed for acquisition of GLRaV. In greenhouse experiments, the ability of *D. vitifoliae* to transmit GLRaV from infected root sections or vines to co-planted virus-free recipient vines was tested. There were no GLRaV-positive *D. vitifoliae* in the field survey, nor did *D. vitifoliae* transmit GLRaV-1, −2, −3, or -4LV in greenhouse transmission experiments. Some insects tested positive for GLRaV after feeding on infected source vines in the greenhouse, however there was no evidence of virus transmission to healthy plants. These findings, in combination with the sedentary behaviour of the soil biotype of *D. vitifoliae*, make it unlikely that *D. vitifoliae* is a vector of any GLRaV.

Keywords Closteroviridae · Grapevine leafroll-associated virus · Grapevine leafroll disease · *Daktulosphaira vitifoliae* · Phylloxera · Vector

Abbreviations

GLRaV grapevine leafroll-associated viruses
GLD grapevine leafroll disease

The species complex of grapevine leafroll-associated viruses (GLRaVs) (Closteroviridae) is associated with grapevine leafroll disease (GLD), an economically damaging disease of grapes (*Vitis spp.)* worldwide (Almeida et al. 2013). According to current taxonomy, there are four virus species associated with this disease (Maree et al. 2013). GLRaV-1, −3, and -4LV are in the genus *Ampelovirus* (Martelli et al. 2012) and can be transmitted by several species of mealybug and scale insects (Maree et al. 2013). Recent studies (e.g. Sharma et al. 2015; Wistrom et al. 2016) have collapsed several previously recognized GLRaV species associated with GLRaV-4 into GLRaV-4LV (LV denotes 'like viruses'), due to a lack of genetic diversity (Maree et al. 2013). There is no known vector of GLRaV-2, which is in the

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genus *Closterovirus*. Aphids (Hemiptera*:* Aphididae) are the only known group to transmit viruses in the genus *Closterovirus* (Ng and Falk 2006). A fifth species, GLRaV-7, is in the genus *Velarivirus*, has no known vector, and may not be associated with disease (Reynard et al. 2015).

Grape phylloxera, *Daktulosphaira vitifoliae* (Fitch) (Hemiptera: Phylloxeridae), is an economically damaging pest of *Vitis spp.* The insect is native in North America and invasive in other wine growing regions (Forneck et al. 2000; Granett et al. 2001). Each of two forms of *D. vitifoliae* feeds on the phloem of either leaves, where it forms galls, or roots, where it forms nodules. On commercial grape cultivars*,* the root life form is more common in Europe and North America (Granett et al. 2001) whereas the leaf form is more common in other regions, such as Brazil (Bao et al. 2015). Resistant rootstocks are therefore the primary management strategy to reduce the pest population and prevent crop losses in North America and Europe. The ability of *D. vitifoliae* to disperse is not well understood, but it is believed that insects stay on one vine for most of their life cycle.

Within the Hemiptera, the family Phylloxeridae is closely related to the family Aphididae (Blackman and Eastop 1994). Because *D. vitifoliae* is a common vineyard pest, a phloem feeder, and closely related to aphids, there is speculation that *D. vitifoliae* may be able to transmit GLRaV-2. We tested field-collected insects for the presence of GLRaVs, and used controlled greenhouse studies to test *D. vitifoliae* acquisition of GLRaVs from infected donor vines (*Vitis vinifera* L.) and to test *D. vitifoliae*-borne transmission of GLRaVs to uninfected recipient vines.

In November 2010 and June and August 2011, two vineyards in Amador County, CA USA were surveyed for GLRaV vine infection and GLRaV-positive *D. vitifoliae*. Both vineyards were mature own-rooted *V. vinifera* cv. Zinfandel, with a high incidence of GLD symptoms and severe *D. vitifoliae* infestations. Thirty-one symptomatic vines were excavated, using a backhoe; roots with *D. vitifoliae* nodules and above-ground shoot samples were collected for GLRaV testing. At the time of excavation in November and August three petioles were collected from each vine, and in June three suckers were collected from each vine. All samples were stored at −80 °C until testing for GLRaV.

All plant samples were extracted and tested for GLRaV −1, −2, −3 and -4LV as described in Osman et al. (2007). Field-collected samples were also tested for GLRaV-3 using the CP primer set (Sharma et al. 2011), which can detect some variants of GLRaV-3 not detected by the primers in Osman et al. (2007). Controls for GLRaV detection in plant material were roots and petioles of known GLRaV-positive or negative plants, and an extraction buffer blank with no plant material. RNA extractions of all insects were performed following Tsai et al. (2008) and tested for GLRaV −2, 3, and -4LV. Controls for GLRaV detection in insect samples were groups of five *D. vitifoliae* or *Planococcus ficus* (vine mealybug) nymphs from virus-free colonies, processed with 2 to 6 ul of extraction buffer from GLRaV-positive grape petioles. One-step RT-PCR kits were used for RNA amplification (Qiagen, Carlsbad, CA). Primers for GLRaV-1 and −3 were multiplexed (HSP70-149F/293R and LC1F/LC2R), and primers for GLRaV-2 and 4LV were multiplexed (L2F/U2R and HSP-26F/HSP-118R). RT-PCR products were visualized using agarose gel electrophoresis, ethidium bromide staining in TAE buffer, and a GelDoc XR imaging system (BioRad, Hercules CA).

From the field study, GLRaV-2 was detected in 14 shoot samples and 2 root samples. GLRaV-3 was detected in 2 of 31 vines with the CP primer set but not the LC1F/LC2R set. No shoot or root samples tested positive for GLRaV-1 or -4LV. *D. vitifoliae* insects ($n = 887$) of all life stages were collected from the roots and tested for GLRaV in groups of 5–50 insects; none of 645 collected from GLRaV-infected vines tested positive, nor did any of the 242 insects collected from adjacent GLRaV-negative vines. All *D. vitifoliae* were collected from roots; the foliar form was not observed. There was no evidence of field acquisition of GLRaV by *D. vitifoliae.*

For greenhouse studies, Foundation Plant Services (University of California, Davis, USA) kindly provided dormant cuttings of certified virus-free *V. vinifera* cv. Cabernet Sauvignon, as well as known GLRaV-infected accessions LV89–01, LR101, LR102, and LR109 (Table 1). Own-rooted cuttings were propagated as described in Blaisdell et al. (2015), then transplanted to Supersoil (Scott's Company, Marysville OH), and periodically fertilized with Osmocote 19–6-12 (Scott's Company). Vines were inoculated with *D. vitifoliae* biotype A, originating in Davis, CA USA. Insects were reared on virus-free *V. vinifera* cv. Cabernet Sauvignon root sections (2–4 mm diameter by 4–6 cm length). Each root section was first rinsed to remove adhering

Table 1 Test of GLRaV transmission from GLRaV-positive, *D. vitifoliae*-infested root sections to virus-free recipient test grapevines

Source vine accession [a]	GLRaV in source	Number of GLRaV+ recipient vines [c]			Number of GLRaV+ insects [c]
		Month 2	Month 3	Month 4	Month 4
LR102	1,2,4LV	0/6	0/8	0/1	0/30
LR109	2,3	0/6	0/7	0/1	0/5
LV89–01	2,3	0/6	0/5	0/2	0/100
Negative CS5 [b]	None	0/7	0/5	0/3	0/77

[a] Source plant material was from a collection at the University of California, Davis, USA

[b] Negative CS5 is virus-free Cabernet Sauvignon, Clone 5

[c] Number GLRaV+/Total number tested

potting media, wrapped with moist cotton at the proximal end, and the cotton was then covered with Parafilm. Thirty *D. vitifoliae* eggs were placed on the root section with a fine paintbrush, and the root section placed in a Petri dish on moistened filter paper. The Petri dish was sealed with Parafilm and maintained with no light at 20 ± 2 °C for one month, after which mature females and eggs were present and the root section was still in good condition. The infested root sections were then placed on roots of experimental vines, after which *D. vitifoliae* infestation and reproduction were monitored monthly by uprooting vines to record the presence or absence of root nodules, adults, nymphs, and eggs.

To test transmission by *D. vitifoliae* from root sections, insects were reared on either virus-free or GLRaV-positive root sections as described above. GLRaV-positive root sections were placed on roots of virus-free *V. vinifera* cv. Cabernet Sauvignon recipient test vines and covered with Weedblock brand polyethylene landscape fabric (Easy Gardener Products Inc., Waco TX) plus 1 cm of Supersoil, so that insects could colonize the roots of the potted recipient vines. Recipient vines were grown in 3.79 L containers, and hand-watered three times per week. In each of two trials, 15 vines each were infested with *D. vitifoliae* from virus source accessions LR102, LR109, and LV89–01, and, for negative controls, 15 vines infested from virus-free root sections. Recipient and negative control vines were tested for GLRaV infection four months post-infestation.

All GLRaVs (−1, −2, −3 and -4LV) were found in infested root sections one month after placement in Petri dishes, and those positive root sections were then used to infest recipient test vines. In accession LR102, root samples were positive in 15/15 samples for GLRaV-1, 7/15 for GLRaV-2, and 14/15 for GLRaV-4LV; in accession LR109, 15/15 were positive for both GLRaV-2 and −3; and in accession LV89–01, 14/15 were positive for GLRaV-2 and 15/15 for GLRaV-3. Insects were found on 42 %, 42 %, and 7 % of the inoculated experimental vines after two, three, and four months, respectively. At the conclusion of the experiment, many vines had died due to the *D. vitifoliae* infestation. Vine survival and infestation incidence did not differ among virus source or virus-free accessions (df = 3, χ^2 = 0.2–1.9, P = 0.60–0.98). Only results from the infested recipient test vines are reported (Table 1); no recipient test vines tested positive for GLRaV. No insects tested positive for GLRaV at the conclusion of the experiment.

Table 2 Test of GLRaV transmission from GLRaV-positive, *D. vitifoliae*-infested source vines to co-planted, virus-free recipient test grapevines

Source vine accession [a]	GLRaV in source	Number of GLRaV+ recipient vines [cd]	Number of GLRaV+ insects [ce]
LR101	3	0/3	0/130
LR102	1,2,4LV	0/12	0/200
LR109	2,3	0/8	0/162
LV89–01	2,3	0/7	0/121
Negative CS5 [b]	None	0/8	0/208

[a] Source plant material was from a collection at the University of California, Davis, USA

[b] Negative CS5 is virus-free Cabernet Sauvignon, Clone 5

[c] Number GLRaV+/Total number tested

[d] Recipient test vines were tested four months after co-planting

[e] Insects were tested after a minimum of two months in the presence of a GLRaV+ source vine. Insects were collected from roots of recipient test vine

We further tested the possibility of *D. vitifoliae* transmission by planting infected and clean grape plants together in the same small pots. Infected source vines were grown in one side of 11.4 L containers partitioned in half using Weedblock fabric. Fifty GLRaV-positive (15 each of FPS LR102, LR109 and LV89–01, and 5 of LR101) and 15 virus-negative grapevines were infested with *D. vitifoliae* in September. One virus-negative root section with approximately 20 mature female insects and 200 eggs was placed on the roots of each potted GLRaV-positive donor vine, and covered with 1 cm of soil. Two months later, the weed block was removed and a virus-negative grapevine was planted into the same container with the *D. vitifoliae*-infested, GLRaV-positive donor vine. Two and four months after infestation, insects, roots, and petioles were collected from all vines for GLRaV testing. Following infestation with *D. vitifoliae*, watering was restricted to minimize disturbance of the insects: vines were drip-irrigated with 100 ml water daily September–December, then every two days from January through April. The experiment was halted four months after co-planting because many vines had died due to *D. vitifoliae* infestation and root diseases.

Insects were found on 49 % of virus source vines two months post-infestation; additional insects were added at co-planting to augment infestation. Two and four months after co-planting, insects were found on 74 % and 50 % of GLRaV donor vines, respectively, having multiple generations and life stages of insects. Virus accessions did not differ in successful *D. vitifoliae* infestation rates (df = 4, $\chi^2 = 0.4.1$, $P = 0.40$). Two and four months post-infestation, insects were collected from GLRaV source vines and pooled in groups of 5 for GLRaV testing. At two months, all 25 groups tested negative for GLRaV −2, −3, and -4LV. At four months, one of 16 groups tested positive for GLRaV-4LV. Six months post-infestation, up to 50 insects were collected from each source vine, and tested in groups of five or 20; GLRaV-3 was detected in one of 11 groups (254 insects tested). No GLRaV was detected in any of the insect groups, comprising 222 individuals, collected from roots of recipient (virus-free) test vines. GLRaV was detected more consistently in petioles than roots of GLRaV source vines at the conclusion of the experiment: 94 % of petiole and 57 % of root samples tested positive for GLRaV. Four months after co-planting, no roots or petioles from the 30 remaining recipient test vines, or the groups comprising 613 insects collected from the roots of recipient vines, tested positive for GLRaVs (Table 2). None of the 8 negative control vines, co-planted with infested virus-free vines, tested positive for GLRaVs, and none of the 208 insects collected from these infested control vines tested positive for GLRaVs.

In conclusion, we found no evidence that the root life form of *D. vitifoliae* would pose a threat with respect to transmission of GLRaVs. To our knowledge, these experiments provide the first comprehensive testing of GLRaV acquisition and transmission by *D. vitifoliae*. While in some cases insects tested positive for GLRaV, there was no evidence of virus transmission. Our lower detection rates of GLRaVs in root compared with shoot samples may have been caused by methodological factors such as longer time between sample collection and storage at −80 °C, the presence of soil, or sampling unhealthy roots. Using ELISA, Teliz et al. (1987) found similar frequency and populations of virus in root and shoot samples of vines with grapevine leafroll disease. In these greenhouse experiments and field surveys, *D. vitifoliae* either was not a vector of GLRaV, or its transmission efficiency was so low that it was not detected in our experiments.

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