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## Short Communication

### ILL'S MEALYBUG, *FERRISIA GILLI*, CAN TRANSMIT GRAPEVINE LEAFROLL-ASSOCIATED VIRUS-3 AFTER A 24-HOUR ACQUISITION TIME

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#### ABSTRACT

Grapevine leafroll disease (GLD) is a virus disease present in all grapevine-growing regions of the world. Mealybugs and scale insects have been reported as vectors of some *grapevine leafroll-associated viruses* belongs to the ampeloviruses (family *Closteroviridae*) in particular with *grapevine leafroll-associated virus-1* (GLRaV-1) and GLRaV-3. Both grape (*Pseudococcus maritimus*) and Gill's mealybugs are commonly present in Virginia vineyards, but we have limited information on acquisition and transmission of GLRaV-3 by Gill's mealybug (*Ferrisia Gilli*). We conducted acquisition and transmission assays in the greenhouse to examine the threshold for shorter acquisition time of GLRaV-3 with *F. gilli*. Approximately 67% and 58% rates of GLRaV-3 acquisition by *F. gilli* following 24 and 48 hours, respectively, of feeding period were documented. *F. gilli* first instars fed on a GLRaV-3-positive grapevine for 24 and 48 hours successfully transmitted GLRaV-3 to healthy grapevines after 24 hours of feeding/transmission time. The quick acquisition demonstrated in this study could be one of the factors that promoted the rapid expansion of GLRaV-3-infected vines in vineyards documented in previous studies.

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#### INTRODUCTION

Grapevine leafroll disease (GLD) is a virus disease present in all grapevine-growing regions of the world (Naidu *et al.*, 2014). In severely infected vineyards, direct yield loss can be as high as 40%, and indirect crop losses can be caused in the form of decreased wine quality due to uneven ripening of berries, reduced sugar levels, changes in pH and TA, and insufficient development of berry color (Cabaleiro *et al.*, 1999; Wolpert and Vilas, 1992).

Mealybugs and scale insects [order Hemiptera, suborder Sternorrhyncha, superfamily Coccoidea, families Coccidea (scale) and Pseudococcidae (soft scale/mealybug), have been reported as vectors of

grapevine-infecting viruses. The ampeloviruses (family *Closteroviridae*), *grapevine leafroll associated virus-1* (GLRaV-1) and GLRaV-3, which are known as causal agents of GLD, are known to be transmitted by these vectors (Tsai *et al.*, 2010). GLRaV-3 is more widespread across grapevine-growing regions than other GLRaVs (Naidu *et al.*, 2014).

The first mealybug documented to successfully transmit GLRaV-3 to a healthy grapevine in a laboratory setting was *Planococcus ficus* (vine mealybug) (Engelbrecht and Kasdorf, 1990). Recent studies have demonstrated that the grape (*Pseudococcus maritimus*), longtailed (*Pseudococcus longispinus*), citrophilus (*Pseudococcus calceolariae*), citrus (*Planococcus citri*), and obscure

(*Pseudococcus viburni*) mealybugs) are all capable of transmitting GLRaV-3 in semi-persistent manner (Cabaleiro and Segura, 1997; Golino *et al.*, 2002; Petersen and Charles, 1997; Tsai *et al.*, 2008; Tsai *et al.*, 2010).

Both *Pseudococcus maritimus* and the Gill's mealybugs (*Ferrisia gilli*) were commonly found in Virginia vineyards (Jones and Nita, 2019; Jones *et al.*, 2015). The grape mealybug is associated with GLRaV-3 and has a wide host range, including grapevines, figs, apples, and citrus crops (Wistrom *et al.*, 2016). Gill's mealybug is a hypothesized native to the southeastern United States and has been described as a pest of pistachios, almonds, and grapes in California (Wunderlich *et al.*, 2013). A study by Wistrom *et al.* (2016) demonstrated that *F. gilli* can transmit GLRaV-3, but not GLRaV-1, -2, and -4LV.

In our previous studies, we documented a widespread of GLRaV-3 among Virginia vineyards (Jones *et al.*, 2015) and a very rapid (up to 185% increase per year) spread of GLRaV-3-positive grapevines in a vineyard infested with both *F. gilli* and *P. maritimus* (Jones and Nita, 2016). However, information on *F. gilli* concerning the transmission of GLRaV-3 is limited, particularly their ability to transmit GLRaV-3 with short acquisition time (e.g., less than 48 hours). The rate of virus acquisition by a vector insect can affect the rate of infection, and thus, the management options (Madden *et al.*, 2000). The objective of this study is to examine the effect of shorter acquisition time on the ability of *F. gilli* to transmit GLRaV-3 to healthy grapevines. The information will help us understand the effectiveness of GLRaV-3 transmission by *F. gilli* in the field.

## MATERIALS AND METHODS

### GLRaV-3 detection from mealybugs and grapevines

Mealybug nucleic acid extraction was conducted with the QIAGEN DNeasy Blood and Tissue kit following the manufacturer's instruction. Nucleotide extraction from grapevines was conducted as described in Jones *et al.* 2015 and Rowhani *et al.* 2000. The detection of GLRaV-3 from these extracts was using a one-tube one-step RT-PCR method (hereafter referred to as the LR3 RT-PCR) (Jones *et al.*, 2015; Rowhani *et al.*, 2000).

### Grapevine preparation

Grapevines used in the study were harvested from a section of a 'Cabernet franc' vineyard at the AHS AREC, Winchester, Virginia, USA (latitude 39.112873, longitude -78.284048). Vines were tested for GLRaV-3 in early

August and again in late September of 2014 and validated as GLRaV-3 free. A total of 100 cuttings were taken in February 2015 from these GLRaV-3-tested vines, to propagate in 1.5-gallon pots (20 cm in diameters (both top and bottom), and 21 cm in height). In addition, canes from GLRaV-3-positive Cabernet franc vines in another vineyard were used to create 50 propagated vines in 1.5-gallon pots. All vines used in this study were trained to single shoots to ensure that mealybugs to feed on a specific area of the plant, since grapevine viruses are not evenly distributed throughout all shoots. Vines were sprayed with potassium bicarbonate (Kaligreen applied at 3.4 kg/ha, Toagosei Co., LTD, Tokyo, Japan) to manage powdery mildew. Three ultra-fine-mesh cages (1.83m x 1.83m x 1.83m in dimension, BioQuip Products Inc., Rancho Dominguez, CA) were placed in a greenhouse to avoid contact with other insects. Hereafter, we refer to these cages as insect cages. Both GLRaV-3-negative and -positive vines were re-tested with the LR3 RT-PCR one day before the experiment to confirm the absence and presence of GLRaV-3, respectively.

### *Ferrisia gilli* preparation

Colonies of *F. gilli* were started with 54 adult female *F. gilli* collected from a vineyard in Orange County, Virginia (latitude 38.1670391, longitude -78.2616859) in May 2015., Mealybugs were gently transferred to sprouted potatoes using a fine-point paintbrush from vines in the field for transportation. In the greenhouse, mealybugs were gently moved to a GLRaV-3-negative Cabernet franc vine in an insect cage for rearing.

### Validation of the lack of transovarial passing of GLRaV-3 by *F. gilli*

Prior to the experiment, a study was conducted to validate the viruliferous nature of *F. gilli* with respect to GLRaV-3. A total of eleven adult females were fed on a GLRaV-3-positive Cabernet franc vine for several days, and eleven first instars born from these females were tested for GLRaV-3 as soon as they emerge. Nine of eleven adult females were positive for GLRaV-3. However, all eleven first instars were negative for GLRaV-3, indicating the lack of transovarial passing of GLRaV-3 with *F. gilli*. Since other mealybug species transmit other *Amperoviruses* in non-circulative, semi-persistent manner, this result was expected (Douglas and Krüger, 2008; Petersen and Charles, 1997). In addition, the species of these instars was confirmed as *F. gilli* using a previously published

vineyard mealybug multiplex PCR assay (Cooper *et al.*, 2011; Jones *et al.*, 2015).

#### GLRaV-3 acquisition assay with *F. gilli*

A total of 72 *F. gilli* first instars reared on GLRaV-3-negative vines were placed onto a GLRaV-3-positive vine and allowed to feed for 1, 2, 6, 12, 24, and 48 hours. At the end of each acquisition time, 12 instars were sampled and immediately tested for GLRaV-3. The experiment was conducted in May 2015. A three-parameter logistic regression model was fit to the data using JMP Pro (ver. 15, SAS Institute, Cary, NC, USA) to describe the relationship between feeding time and the rate of acquisition.

#### GLRaV-3 transmission assay with *F. gilli*

A total of 60 *F. gilli* first instars from a GLRaV-3-negative vine in an insect cage were placed onto the GLRaV-3-positive vine in a separate insect cage and allowed to feed 1, 2, 6, 12, 24, and 48 hours. Then transferred to GLRaV-3-negative vines in another insect cage to fed for 24 hours. There were two GLRaV-3-negative vines per each time group, and each vine received five mealybugs. In addition, ten first instars that were fed on two GLRaV-3-negative vines to serve as the negative control. The experiment was conducted from June to October 2015.

After the 24-hour transmission times had expired, mealybugs were removed from the vines, placed into 0.5-ml tubes containing 95% ethanol, and stored at -20°C, then subjected to RNA extraction and the LR3 RT-PCR described above. In addition, all instars used in the acquisition and transmission assays were subjected to species confirmation using the vineyard mealybug multiplex PCR assay (Cooper *et al.*, 2011; Jones *et al.*, 2015). Five petioles were arbitrarily collected at 75 and 135 days after feeding then subjected to the LR3 RT-PCR (Rowhani *et al.*, 2000).

## RESULTS

#### GLRaV-3 acquisition assay with *F. gilli*

The presence of GLRaV-3 was confirmed with one, eight, and seven out of 12 *F. gilli* instars for the acquisition time of 12, 24, and 48 hours, respectively (Figure 1). The logistic model showed a good fit to the data with  $R^2 = 0.992$ . Although the sample size was small, these results demonstrated *F. gilli* was able to acquire GLRaV-3 after 12 hours of feeding, with an approximately 67% and 58% rate of acquisition per mealybug after 24 hours and 48 hours, respectively, of the feeding period. The negative check vines which were fed with *F. gilli* that were never fed on GLRaV-3-positive grapevine remained GLRaV-3-negative.

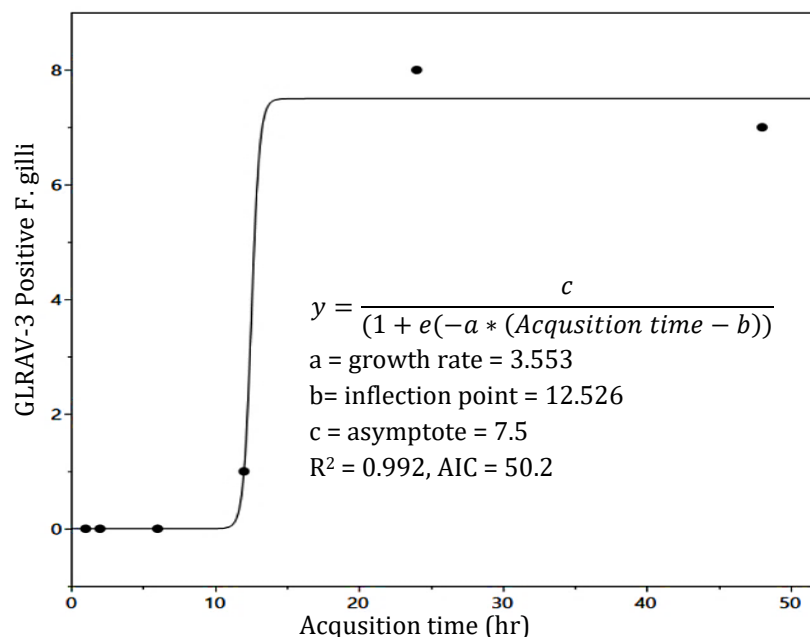


Figure 1. Number of GLRaV-3-positive *F. gilli* at 1-, 2-, 6-, 12-, and 48-hour acquisition time. The LR3 RT-PCR was conducted immediately after the end of each acquisition time (Rowhani *et al.* 2000). A green line shows a result of three parameter logistic model. The equation, parameters,  $R^2$ , and AIC (Akaike's Index of Criterion for the model fit) values are shown in the figure (JMP Pro ver. 15, SAS Institute).

### GLRaV-3 transmission assay with *F. gilli*

When the LR3 RT-PCR was conducted after 24-hour feeding/transmission time, one, seven, and six out of ten first instars, for 12-, 24-, and 48-hour acquisition feeding, respectively, were positive with GLRaV-3 (Table 1). At 75 days after transmission feeding, three vines were positive for GLRaV-3. One vine with a 24-hour

acquisition feeding period and two vines with a 48-hour acquisition feeding period were GLRaV-3-positive. The negative check vines remained free of GLRaV-3. The result did not change at the second test on the same vines at 135 days after transmission feeding. The transmission efficiency based on Swallow (1985) is 0.03. All instars used in the assay were confirmed as *F. gilli*.

Table 2. Numbers of GLRaV-3-positive grapevines after 75- and 135-day post-transmission feeding and *F. gilli* immediately after feeding/transmission time.

Days after feeding <sup>a</sup>	Acquisition time (in hours)					
	1	2	6	12	24	48
75	0	0	0	0	1	2
135	0	0	0	0	1	2
<i>Number of GLRaV-3-positive F. gilli</i> <sup>b</sup>	0	0	0	1	7	6

- a. For each acquisition time, two GLRaV-3-negative Cabernet franc vines were used. For each vine, five *F. gilli* first instars were placed. The same vines were assessed at 75- and 135-day after feeding.
- b. Number of GLRaV-3-positive *F. gilli* out of ten individuals per acquisition time. The LR3 RT-PCR (Rowhani et al. 2000) was conducted immediately after transmission feeding (i.e., at the end of transmission time).

### DISCUSSION

An approximately 67% rate of virus acquisition by *F. gilli* following 24 hours of feeding period was documented, and results from 48 hours of feeding did not differ greatly (58%). Our results suggested although *F. gilli* was able to acquire GLRaV-3 after 12 hours of feeding, the rate of acquisition was meager (1/12 or 8.3%). The transmission assay used the same set of feeding times to acquire GLRaV-3 from an infected vine, and the combined rate of acquisition with a 24 and 48-hour feeding period was 68.1%, and 59.1%, respectively. A study by Wistrom *et al.* (2016) tested acquisition time of 48 and 96 hours, and reported after 48 hours, 64% of *F. gilli* instars were able to acquire GLRaV-3. Our assays examined shorter acquisition times from 1 to 48 hours, and the mean rate of acquisition at 24 hours were numerically higher than that of 48 hours, and similar to the rate at 48 hours of feeding reported by Wistrom *et al.* (2016). Thus, *F. gilli* instars are more likely to acquire GLRaV-3 after 24 hours.

The vine mealybug, *Planococcus ficus*, is considered to be the most efficient at the transmission of GLRaV-3, and it can successfully acquire the virus after 1 hour of plant-feeding with the success rate steadily increasing up to the maximum of around 60-70% with 24 hours of feeding (Tsai *et al.*, 2008). Our results showed *F. gilli* was not able to acquire GLRaV-3 with less than 24 hours of feeding, suggesting that the maximum rate of acquisition

between *F. gilli* and *P. ficus* can be similar, but the time required for the acquisition is probably longer with *F. gilli*. In our study, trans-ovarial passing of GLRaV-3 was not found in *F. gilli*. A study on *P. ficus* also found the lack of GLRaV-3 in the first instars (Tsai *et al.*, 2008).

The results of our transmission assay supported the semi-persistent nature of mealybug transmission of closteroviruses (Martelli *et al.*, 2002). *F. gilli* instars fed on GLRaV-3-positive grapevine for 24 hours could transmit GLRaV-3 to the healthy vine after 24 hours of feeding, and we were able to detect GLRaV-3 from *F. gilli* after the 24-hour transmission time period. The transmission efficiency of 0.03 (Swallow, 1985) was less than what Wistrom *et al.* (2016) reported, which was 0.085. With vine mealybugs (*P. ficus*), the transmission efficiency of GLRaV-3 was 0.11 to 0.18 (Tsai *et al.*, 2008). However, our sample size was too small to develop a solid conclusion. A recent study with *P. maritimus*, which is another commonly found mealybug species in Virginia, used a one-week inoculation period and reported a 65% transmission rate (O'Hearn and Walsh, 2020).

Gill's mealybugs are reported to have two in-season generations in California, where they overwinter as second and third instars under bark along the cordon and trunks (Wunderlich *et al.*, 2013). The first instars, which were used in this study, appear in June and then late-August/early-September in California. First instars

can be disseminated by their own mobility, wind, and other causes; therefore, considered as the most efficient phase of transmission (Petersen and Charles, 1997). This study demonstrated that first instars of *F. gilli*'s can acquire GLRaV-3 in 24 hours and transmit GLRaV-3 to the healthy grapevines. This relatively short acquiring time could be one of factors of a very rapid spread of GLRaV-3-positive vines documented in our previous study (Jones and Nita, 2016). In the same study, we documented the effectiveness of an insecticide spirotetramat in reducing number of mealybugs, which was a mix population of *F. gilli* and *P. maritimus*, with two field trials. However, a study by O'Hearn and Walsh (2020) indicated that even with the application of spirotetramat, *P. maritimus* was able to transmit GLRaV-3 with a 40% transmission rate, which is very alarming. With its wide host ranges and ability to transmit GLRaV-3, vineyard managers need to be aware of the presence of *F. gilli* and take actions to help manage this pest. In addition, the use of clean (virus-tested) plant materials has to be incorporated into Grape Leafroll Disease management in the field (Jones and Nita, 2019; Maliogka *et al.*, 2015).

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#### CONFLICT OF INTEREST

Mizuho Nita and Taylor Jones declare that we do not have any conflicts of interest.

#### AUTHORS CONTRIBUTIONS

Both authors contributed to experiments, data collection, data analysis, writing, and editing of this manuscript.

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