Grapevine leafroll-associated virus 3 (GLRaV-3) transmission by three soft scale insect species (Hemiptera: Coccidae) with notes on their biology

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Several mealybug and two soft scale species have been identified as vectors of *Grapevine leafroll-associated virus 3* (GLRaV-3), the most abundant of the Closteroviridae associated with grapevine leafroll disease. To identify further soft scale vectors of GLRaV-3, the ability of three species, *Coccus longulus, Parasaissetia nigra* and a *Saissetia* sp., to transmit the virus to grapevine was determined under laboratory conditions. Using cultured soft scales, first-instar nymphs of *C. longulus* and *P. nigra* were given acquisition access to GLRaV-3-infected rootstock hybrid LN33. *Saissetia* sp. was reared on LN33 infected with GLRaV-3 and *Grapevine virus A* (GVA). Nymphs were transferred from virus source to virus-free grapevine plants (cv. Cabernet franc). Recipient plants were tested for GLRaV-3 and GVA with nested reverse transcription polymerase chain reaction (nested RT-PCR) and RT-PCR, respectively. The study shows for the first time that *C. longulus, P. nigra* and a *Saissetia* sp. are vectors of GLRaV-3. *Saissetia* sp. did not transmit GVA. The biology of *C. longulus* and *P. nigra* on grapevine was examined at different constant temperatures ranging between 18 and 35 °C, and at 25 and 30 °C, respectively. None of the nymphs survived past the second-instar stage except for one *C. longulus* female at 30 °C, which produced 117 offspring. The low survival rate could explain the low abundance and patchy distribution of soft scales in South African vineyards. However, outbreaks of soft scales in European vineyards have been reported and this study shows that more soft scale insect species than hitherto thought are able to transmit the virus.

Key words: Closteroviridae, grapevine leafroll disease, *Parasaissetia nigra, Coccus longulus, Saissetia* sp.

INTRODUCTION

Grapevine leafroll disease occurs in all major grapevine-growing regions throughout the world (Martelli 1986) and constitutes one of the most serious viral diseases of grapevine *Vitis vinifera* L. (Vitaceae), causing qualitative and quantitative yield losses (Goheen & Cook 1959; Credi & Babini 1997; Cabaleiro *et al.* 1999). *Grapevine leafroll-associated virus 3* (GLRaV-3, *Ampelovirus*) is one of the most widespread of the Closteroviridae associated with the disease (Martin *et al.* 2005; Pietersen 2006; Akbas *et al.* 2007). The phloem-limited virus has been shown to be transmitted through grafting, and by several mealybug (Hemiptera: Pseudococcidae) (*e.g.* Tanne *et al.* 1989; Engelbrecht & Kasdorf 1990a; Cabaleiro & Segura 1997; Petersen & Charles 1997; Golino *et al.* 2002; Sforza *et al.* 2003) and soft scale species (Hemiptera: Coccidae) (Belli *et al.* 1994; Mahfoudhi *et al.* 2009). The virus is thought to be transmitted in a semi-persistent manner (Martelli *et al.* 2002; Tsai *et al.* 2008). However, a persistent circulative transmission mechanism has been proposed by Cid *et al.* (2007).

Eighteen soft scale species have been recorded on grapevine (Ben-Dov *et al.* 2012) and several species have been reported to occur in vineyards where grapevine leafroll disease and GLRaV-3 were present and have been suggested as potential vectors, *e.g. Neopulvinaria innumerabilis* (Rathvon) and *Parthenolecanium corni* (Bouché) (Fortusini *et al.* 1996). Although studies have been undertaken to determine soft scale insect vectors of GLRaV-3, only two species have hitherto been identified as vectors, *Pulvinaria vitis* L. (Belli *et al.* 1994) and *Ceroplastes rusci* (L.) (Mahfoudhi *et al.* 2009). Transmission of GLRaV-3 by the soft scale *P. corni,* which has been reported to occur in GLRaV-3-infected vineyards in Europe, was not successful (Belli *et al.* 1994; Sforza *et al.* 2003; Hommay *et al.* 2008). In addition to GLRaV-3, scale insects have been reported as vectors of *Grapevine virus A* (GVA, Flexiviridae, *Vitivirus*) (Fortusini *et al.* 1997;

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Hommay *et al.* 2008). This virus frequently occurs together with *Grapevine leafroll-associated viruses 1* and *3* in grapevine plants (Zorloni *et al.* 2006, Hommay *et al.* 2008). In South Africa, GVA is found in combination with GLRaV-3 (Engelbrecht & Kasdorf 1990b).

In South African vineyards soft scales are usually localized and occur on a few vines only. During a survey of scale insects (Hemiptera: Coccoidea) occurring on grapevine several soft scale species were recorded (Walton *et al.* 2009; Krüger *et al.*, in prep.). Of these, three species were selected to test their ability to transmit GLRaV-3.

The cosmopolitan long brown scale *Coccus longulus* (Douglas) is a polyphagous species that has been reported from *c.* 200 host plant species (Ben-Dov *et al.* 2012). Little information is available on the biology of this species. Its life-history was examined on pumpkin (*Cucurbita* spp., Cucurbitaceae) by El-Minshawy & Moursi (1976). Females reproduce parthenogenetically and are ovoviviparous. This species is usually not considered to be of economic importance on grapevine or other agricultural plants. During the 2001/2002 season it was widespread on grapevine in the Vredendal region in the Western Cape but not thereafter. However, *C. longulus* has been listed as a potential pest on grapevine in Australia (Buchanan 2008).

The nigra scale *Parasaissetia nigra* (Nietner) has more than 400 host plant records throughout the world (Ben-Dov *et al.* 2012). Although it has been recorded on a number of cultivated plant species, it is usually not considered a pest as it is kept under control by natural enemies (Ebeling 1959; Annecke & Moran 1982). Females reproduce parthenogenetically (Smith 1944; Le Pelley 1968) and males have not been recorded in South Africa (Annecke & Moran 1982).

Another scale insect species recorded locally on grapevine is a species of *Saissetia* Deplanche, which could not be identified to species level because the genus is in need of revision (I. Millar, pers. comm.). Two species belonging to this genus, the polyphagous *S. coffeae* (Walker) and *S. oleae* (Oliver), have been recorded on grapevine in other countries (Ben-Dov *et al.* 2012).

The objectives of the study were to determine whether the soft scales *C. longulus, P. nigra* and *Saissetia* sp. are able to transmit GLRaV-3 and GVA and to examine the survival of *C. longulus* and *P. nigra* on grapevine.

MATERIAL AND METHODS

Insects

Cultures of *C. longulus* and *Saissetia* sp. were established in Pretoria (Gauteng, South Africa) with specimens obtained from grapevine in the field at Vredendal and the rootstock hybrid LN33 in a greenhouse in Stellenbosch (Western Cape, South Africa), respectively. *Coccus longulus* was maintained on GLRaV-free grapevine (cv. Cabernet franc) and *Saissetia* sp. on LN33 infected with GLRaV-3 and GVA. Cultures were maintained in environment-controlled rooms at 25 °C, 16L:8D photoperiod and natural humidity. Nymphs of *C. longulus* and *Saissetia* sp. from cultures established for several generations were used for transmission experiments. Establishment of a culture of *P. nigra* on grapevine (cv. Cabernet franc) was not successful. Therefore, *P. nigra* crawlers collected from *Nepenthes* sp. (Nepenthaceae) and *Zantedeschia* sp. (Araceae) plants kept in a greenhouse at the University of Pretoria (Gauteng, South Africa) were used. Subsamples of cultured scale insects were tested for their virus status with nested reverse transcription polymerase chain reaction (nested RT-PCR, see below) before GLRaV-3 transmission experiments.

Insects were identified by I. Millar of the Biosystematics Division of the ARC-Plant Protection Research Institute (ARC-PPRI), Pretoria, South Africa. Voucher specimens were deposited in the National Collection of Insects (ARC-PPRI).

Plants

Potted rooted stem cuttings of cv. Cabernet franc were used as recipient grapevine plants for virus transmission with *C. longulus, P. nigra* and *Saissetia* sp. Plants were grown under insect-free conditions and not treated with pesticides before experiments. Rooted plant cuttings were grown in 2-litre pots in a soil mixture comprising sandy soil, compost and vermiculite at a ratio of 2:2:1. The grapevine cuttings were watered twice a week. Liquid fertilizer (Wonder 3:2:1(22) Supranure Plus) was applied every fortnight during watering. Recipient plants were tested for their virus-free status with nested RT-PCR prior to transmission experiments.

Rooted stem cuttings infected with GLRaV-3 variant 623 (Jooste *et al.* 2010) obtained from the rootstock hybrid LN33 (1/5/2, ARC Infruitec-Nietvoorbij, South Africa) were used as a source for transmissions of GLRaV-3 with *C. longulus* and

Table 1. Transmission of GLRaV-3 and GVA from the rootstock hybrid LN33 to grapevine (cv. Cabernet franc) with first-instar nymphs of cultured soft scale species.

Soft scale species	Virus source in plant	Virus detected	Positive plants/ inoculated plants	Negative control: positive plants/ inoculated plants
Coccus longulus	GLRaV-3	GLRaV-3	1/2	0/2
Parasaissetia nigra	GLRaV-3	GLRaV-3	3/3	0/2
Saissetia sp.	GLRaV-3, GVA	GLRaV-3	1/4	0/2

P. nigra. The source plants were tested with PCR and/or immunosorbent electron microscopy (ISEM) for infection with GLRaV-1 to 4, 6 and 7, GVA and Grapevine fleck virus (GFkV) at the Virology section of the ARC-Plant Protection Research Institute, Pretoria (ARC-PPRI), South Africa. Plants that tested positive for GLRaV-3 only were used for transmission experiments with *C. longulus* and *P. nigra.* LN33 plants, from the same source as described above, on which *Saissetia* sp. was reared and which served as virus source, were infected with both GLRaV-3 and GVA.

GLRaV-3 transmission

Transmission experiments with cultured soft scales were carried out in the insectary at the University of Pretoria in environment-controlled rooms at 25 °C, 16L:8D photoperiod and natural humidity. *Coccus longulus* and *P. nigra* were given acquisition access periods (AAPs) of four days on virus source plants infected with GLRaV-3 variant 623 (rootstock hybrid LN33), and then transferred to rooted stem cuttings of virus-free Cabernet franc plants for AAPs of at least seven days. Small leaf cuttings with crawlers (newly-hatched first-instar nymphs) from the plants on which the insects were cultured were placed on virus source plants. As the leaf cuttings desiccated the crawlers moved across to the virus source plants. After the AAP of four days, crawlers moving on leaves were transferred from virus source plants to Cabernet franc plants using a fine paint brush. Crawlers of *Saissetia* sp. were directly transferred from LN33 plants, infected with GLRaV-3 variant 623 and GVA, on which they were reared to recipient plants with a fine paintbrush for AAPs of at least seven days. Thirty crawlers of each *P. nigra* and *Saissetia* sp. were transferred to each recipient plant, with the exception of one plant that received five *P. nigra* crawlers. Six and 21 *C. longulus* crawlers were transferred from virus source to recipient plants, respectively. For each transmission experiment between 30 moving crawlers of *C. longulus* and *P. nigra* collected from their original host plant were directly transferred to virus-free Cabernet franc plants as described above to serve as negative controls. Plants that were treated in the same manner as the recipient plants but without crawlers served as negative control for *Saissetia* sp. The number of plants per treatment (Table 1) was limited by the number of virus-, insect- and pesticide-free grapevine plants and crawlers available. Experiments for a given species were replicated by carrying out transmissions on different days with a negative control plant for each transmission day and each species. Where possible different GLRaV-3 source plants were used for virus transmissions. In addition, each recipient and control plant was kept singly in a separate ventilated glass cage ($40 \times 40 \times 40$ cm) placed on saucers with engine oil to avoid cross-contamination by movement of mealybugs between cages.

After completion of transmission experiments, plants were treated with chlorpyrifos and imidacloprid to kill insects and prevent reinfestation. Thereafter plants were transferred to plant growth rooms at 24 °C, 16L:8D period and natural humidity. In addition to the negative control plants used in experiments, virus-free grapevine plants were kept in the plant growth rooms as additional negative controls throughout the study.

PCR

Recipient and control plants were tested before and at various time intervals for up to two years, where applicable, after transmission for the presence of GLRaV-3. The method described by La Notte *et al.* (1997) was adopted for virus extraction. Plants were tested for GLRaV-3 using the primers developed by Ling *et al.* (2001) and nested RT-PCR adapted by M. van der Merwe (ARC-PPRI) from

Ling *et al.* (2001) (see Douglas & Krüger 2008 for details).

RT-PCR for the detection of GVA was performed using the primers developed by MacKenzie (1997) and the protocol developed by M. van der Merwe (ARC-PPRI). The 50 μl reaction volume contained 5 μl 2 % Triton X-100, 5 μl of 10× NH_4 buffer, 1.5 mM $MgCl_2$, 10 mM DTT, 0.25 μM of each primer, 175 μM of each dNTP, 18 units HPRI RNase inhibitor, 40 units M-MLV reverse transcriptase, and 0.5 units BIOTAQ™ DNA Polymerase (BioLine, Luckenwalde, Germany) and 3 μl of the extraction. PCR thermal cycling conditions were: 37 °C for 45 min, 94 °C for 2 min, then 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min.

PCRs were performed using GenAmp PCR System 2400 (Perkin Elmer Applied Biosystems) and 2720 Thermal Cycler (Applied Biosystems) thermocyclers. PCR products were visualized under UV light on a 1.5 % agarose gel stained with ethidium bromide (EtBr).

Biology of Coccus longulus *and* Parasaissetia nigra *on grapevine*

During the transmission experiments it was observed that survival of first-instar nymphs was very low on grapevine. It was therefore decided to determine the survival of *C. longulus* and *P. nigra* on virus-free grapevine (cv. Cabernet franc) in controlled temperature experiments. Plants were not exposed to any pesticides throughout the study. Crawlers were obtained from the same sources as those used in transmission experiments from cultured insects. Development on grapevine by *C. longulus* and *P. nigra* was examined in incubators at constant temperatures of 18, 21, 27 and 30 °C, and 25 and 30 °C, respectively, and 16L:8D photoperiod and natural humidity. To avoid breaking the fragile mouthparts of nymphs, gravid females were transferred on small pieces of branches or leaves of plants to glass vials closed with gauze. Vials were checked daily for emergence of nymphs. First-instar nymphs crawling on the glass were carefully transferred with a fine paint brush from the vial to virus-free grapevine plants. A total of 30 to 57 newly hatched first-instar nymphs per temperature were transferred to plants. Survival and the developmental stage of nymphs were examined and recorded daily. Results per temperature were pooled because of the low survival of nymphs.

RESULTS

Virus transmission

In the controlled laboratory transmission experiments with cultured scale insects, all three species transmitted GLRaV-3 to healthy grapevine plants (Table 1). For *P. nigra* all three plants tested positive for GLRaV-3. One out of two and one out of four plants tested positive for GLRaV-3 transmission with *C. longulus* and *Saissetia* sp., respectively. None of the plants exposed to viruliferous nymphs of *Saissetia* sp. tested positive for GVA. Of the two plants that were exposed to a few nymphs only, the one exposed to five *P. nigra* nymphs tested positive for GLRaV-3 and the one exposed to six *C. longulus* nymphs tested negative.

A large number of crawlers had to be transferred to virus-source plants because survival of crawlers was very low. For example, out of more than 900 crawlers of *P. nigra* transferred to an LN33 plant only 10, approximately 1 %, had survived by the fourth day, or out of a batch of 44 crawlers of *C. longulus* transferred to LN33, six (14 %) survived the AAP of 4 days.

Plants tested positive for GLRaV-3 for the first time approximately one year after transmission and then in some instances only weakly (Fig. 1a, b). Plants that served as negative controls, i.e. plants exposed to non-viruliferous nymphs and additional virus-free plants maintained together with the plants used in the experiments in the same plant growth chamber, tested negative for GLRaV-3 throughout.

Biology of *Coccus longulus* and *Parasaissetia nigra* on grapevine

For both *P. nigra* and *C. longulus* survival of nymphs on grapevine was very low (Fig. 2a,b). Only one and two out of the 30 crawlers of *P. nigra* transferred from their original host plants to grapevine reached the second-instar stage at 25 and 30 °C, respectively. Three *C. longulus* nymphs reached the second-instar stage at 21 °C and one at 30 °C but not at any of the other temperatures tested. Two days after transfer of nymphs from the original host plant to grapevine, survival ranged between 5 % at 30 °C and 16 % at 21 °C for *C. longulus.* At 18 °C, the lowest temperature tested, none of the first-instar nymphs survived beyond 17 days on grapevine. Nymphs survived longer at higher temperatures; at 27 °C two first-instar nymphs survived for 36 days and one for 52 days.

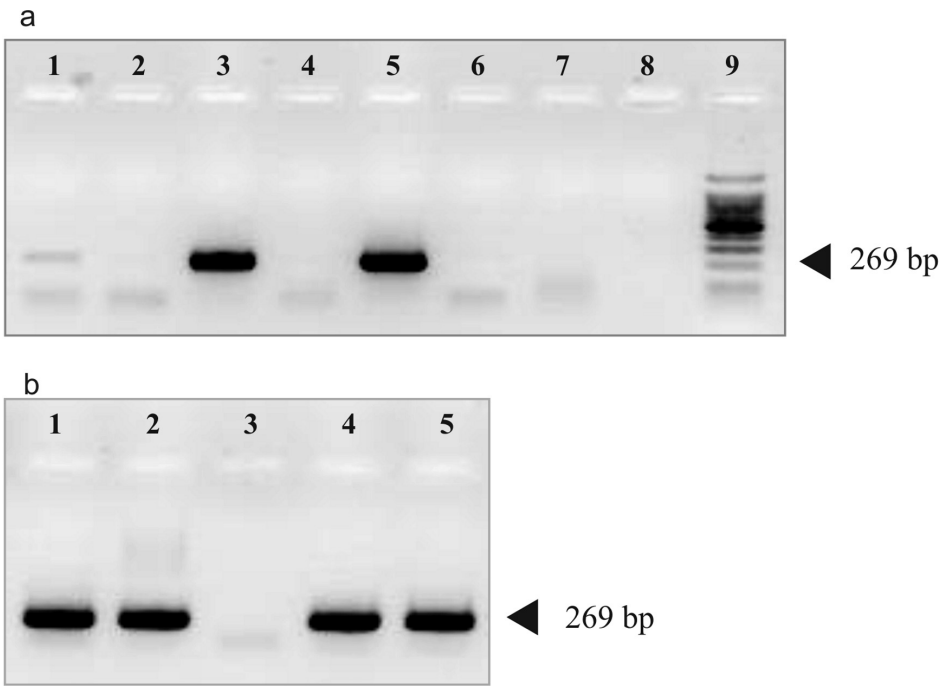

Fig. 1. Nested RT-PCR amplification of GLRaV-3 from grapevine cv. Cabernet franc after GLRaV-3 transmission with first-instar nymphs soft scale species. **a**, Lane 1: grapevine plant exposed to GLRaV-3 infected first-instar nymphs of *Saissetia* sp., lane 2: grapevine plant exposed to GLRaV-3-free nymphs (negative control), lane 3: positive plant control, lane 4: extraction control, lane 5: DNA control, lane 6: negative plant control, lane 7: no template control, lane 8: extraction buffer control, lane 9: 100 bp DNA size marker (Promega). **b**, Lane 1: positive plant control, lane 2: DNA control, lane 3: no template control, lanes 4 and 5: grapevine plants exposed to GLRaV-3 infected first-instar nymphs of *Parasaissetia nigra* and *Coccus longulus*, respectively. PCR products were visualized under UV light on 1.5 % agarose gel stained with ethidium bromide (EtBr).

The single *C. longulus* nymph that survived at 30 °C developed into an adult female and produced 117 offspring, approximately 90 days after the experiment commenced.

DISCUSSION

Results of the current study show for the first time that *C. longulus, P. nigra* and a *Saissetia* sp. are vectors of GLRaV-3. All three species transmitted GLRaV-3 from the rootstock hybrid LN33 to grapevine plants (cv. Cabernet franc) under controlled laboratory conditions, whereas none of the negative control plants tested positive. The number of positive plants is in accordance with Belli *et al.* (1994), where two out of five plants tested positive for GLRaV-3 with *P. vitis* as vector. In contrast, only one out of 60 plants tested positive with *C. rusci* as vector (Mahfoudhi *et al.* 2009).

The negative results obtained in previous studies identifying soft scale vectors could be due to the inability of the species tested to transmit GLRaV-3. However, other possible reasons include low virus concentrations in plant tissue and the insufficient sensitivity of the detection methods used (Belli *et al.* 1994). For example, ELISA, a method that is less sensitive than PCR, has frequently been used for detection of GLRaV-3 in mealybug and scale insect transmission experiments. Belli *et al.* (1994) were unable to detect GLRaV-3 with ELISA after transmission tests with *P. vitis* but detected the virus when using PCR.

Another problem could be the time elapsed after virus transmission and tests performed (Cabaleiro & Segura 1997). After GLRaV-3 transmission to grapevine by *Planococcus ficus* (Hemiptera: Pseudococcidae) nymphs, plants tested positive as early as two months after transmission (Tsai *et al.* 2008). However, plants in the current study only tested positive more than one year after infection, and then in some instances only weakly. This could be a reflection of soft scale insects being poor

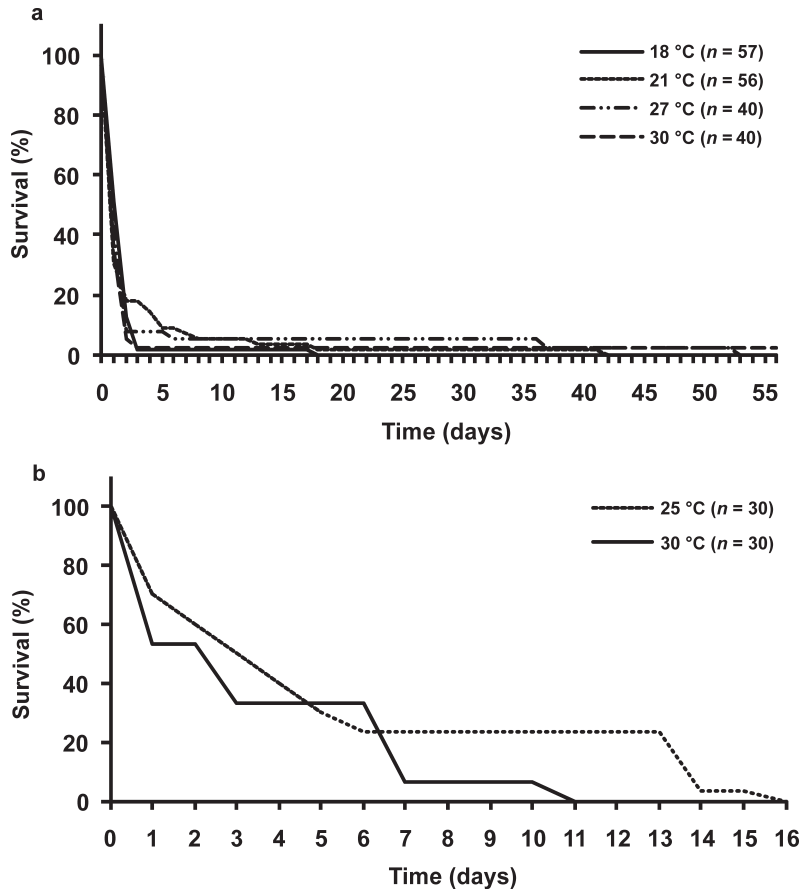

Fig. 2. Survival of first-instar nymphs of (**a**) *Coccus longulus* and (**b**) *Parasaissetia nigra* on grapevine at different constant temperatures.

vectors. A further reason could be the low transmission success of some scale insects compared to mealybugs. For example, after controlled transmission experiments with GLRaV-3 in the same study, 30 % and 1.7 % of recipient plants were infected when using the mealybug *P. ficus* and the soft scale *C. rusci* as vectors, respectively (Mahfoudhi *et al.* 2009). Tsai *et al.* (2008), who determined transmission characteristics of GLRaV-3 by *P. ficus,* observed that the number of positive plants did not increase after two months, using RT-PCR. The current study, however, highlights the importance of testing plants for longer periods after transmission.

So far, GVA transmission by soft scale species has only been reported when grapevine plants were infected with GLRaV-1 and GVA (Fortusini *et al.* 1997; Hommay *et al.* 2008), but not when infected with GLRaV-3 and GVA (Hommay *et al.* 2008). The negative results for GVA transmission with *Saissetia* sp. could be due to the absence of GLRaV-1 in the virus source plants used for transmission in the current study.

None of the nymphs of *C. longulus* of *P. nigra* developed into adults at any of the temperatures tested with the exception of one female of *C. longulus.* The life cycle of *C. longulus* on pumpkin ranged between 125 to 151 days at 19 to 25 °C (El-Minshawy & Moursi 1976), whereas it was shorter on grapevine with approximately 90 days at 30 °C for a single female in the current study. Although the low survival of crawlers could be due to low humidity, Smith (1944) observed *P. nigra* thriving under both humid and arid conditions. The low survival of crawlers of the two soft scales observed in this study of 10 % or less is in agreement with Smith (1944), who observed in laboratory and field trials that more than 90 % of

crawlers of *P. nigra* died before settling on *Pittosporum undulatum* (Pittosporaceae).

Our study has shown for the first time that the cosmopolitan species *C. longulus* and *P. nigra,* and a *Saissetia* species, are vectors of GLRaV-3. Semi-persistent viruses (non-circulative transmission) have a wider range of insect vector species than persistent viruses (circulative transmission) (Fereres & Moreno 2011). The addition of scale insects of three different genera to the long list of vectors of GLRaV-3 supports findings that transmission of this virus is not restricted to a few vectors only. Almost all mealybug and, to a lesser extent, soft scale species tested in laboratory studies were found to be able to transmit GLRaV-3. This provides further evidence that GLRaV-3 transmission occurs in a semi-persistent rather than persistent manner.

Although soft scales are not common and have a very patchy distribution on grapevine in South Africa, outbreaks of soft scale insects have been reported in European vineyards (*e.g.* Pavan *et al.* 1996; Masten-Milek *et al.* 2007). In addition, scale insects have been observed in vineyards where grapevine leafroll disease was recorded spreading in the absence of mealybugs (*e.g.* Fortusini *et al.* 1996). However, further studies are needed to show whether the three species are efficient natural vectors of GLRaV-3 in vineyards.

ACKNOWLEDGEMENTS

We thank W. Strümpfer (University of Pretoria) for assistance with the study on the immature survival, E. Jooste (ARC-PPRI) for determining the GLRaV-3 isolate, M. van der Merwe, K. Kasdorf (ARC-PPRI) and G. Malherbe (UP) for assistance with GLRaV-3 diagnostics, R. Carstens (ARC-Infruitec/Nietvoorbij), Vititec for providing plants, and anonymous referees for constructive comments. This project was supported by Winetech, THRIP (Technology and Human Resources for Industry Programme) and the University of Pretoria.

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Accepted 21 August 2012