

Do spittlebugs feed on grape? Assessing transmission potential for *Xylella fastidiosa*

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Abstract The bacterium *Xylella fastidiosa* is a causal agent of Pierce's disease in grapevines and is considered to be vectored by several xylem-feeding insects. *Xylella fastidiosa* and its primary insect vector *Homalodisca vitripennis* are not currently present in New Zealand, but considered a biosecurity threat to the wine industry. Should it be introduced, infection and dispersal of *X. fastidiosa* within New Zealand vineyards could occur through extant vectors, especially spittlebugs. Electrical Penetration Graph technology was used to compare the feeding behaviour of three spittlebug species (*Philaenus spumarius*, *Carystoterpa fingsens* and *Carystoterpa minor*) on grape (*Vitis vinifera* Sauvignon blanc) and their original host plants. Results suggest that *Philaenus spumarius* feeds on grape more than the other two spittlebug species. As *Philaenus spumarius* has been reported as a vector of *X. fastidiosa*, their potential to transmit the bacterium into grapevines is discussed based on the real feeding times recorded by EPG.

Keywords *Xylella fastidiosa*, Pierce's disease, spittlebugs, Electrical Penetration Graph.

INTRODUCTION

Xylella fastidiosa is a causative agent of Pierce's disease of grape and numerous other scorch diseases that is vectored by xylem-sap feeding insect species (Cornara et al. 2016). It is a xylem-limited plant pathogenic bacterium that forms a biofilm believed to be responsible for disrupting the passage of water and nutrients. This causes symptoms similar to water stress, including leaf scorching, wilting, defoliation, chlorosis, and drying, resulting in plant death (De La Fuente et al. 2013). The bacterium has a rapidly expanding host range with over 300 plant species in 75 different plant families currently known to be affected. It presents a substantial risk to many important horticultural industries, including grapes, citrus, avocado, olives and

stone fruit. New Zealand natives (pōhutukawa, kauri, flax, and cabbage tree), ornamentals, grasses and forest trees are also at risk (Ministry for Primary Industries 2016). *Xylella fastidiosa* is known to be transmitted by about 50 species of Auchenorrhyncha belonging to the families Cercopidae (froghoppers), Aphrophoridae (spittlebugs), Cicadellidae (leafhoppers) and Membracidae (treehoppers) (de Coll et al. 2000; Redak et al. 2004). Overseas, the glassy-winged sharpshooter *Homalodisca vitripennis* (GWSS) is the primary vector of *X. fastidiosa* in grapes, causing Pierce's disease (Hopkins and Purcell 2002). However, other insects such as the meadow spittlebug *Philaenus spumarius* are also reported as vectors of *X. fastidiosa* (Redak

et al. 2004; Saponari et al. 2014; Cornara et al. 2016; Cornara et al. 2017). Although GWSS is not currently present in New Zealand, *Philaenus spumarius* and several other spittlebug species are common in New Zealand. If *X. fastidiosa* were accidentally introduced into this country, infection and dispersal within New Zealand vineyards could occur through New Zealand extant vectors, especially by spittlebugs. The accidental introduction of *X. fastidiosa* into Europe is an example of a pathogen invasion by endemic vectors (Saponari et al. 2013; Martelli et al. 2016). Pierce's disease is fatal to infected vines and there is no cure so it would pose a significant threat to New Zealand's wine industry. The total cost of research and management for Pierce's disease in California has been >US\$166 million for the period from 1999 to 2004 (Sandanyaka & Backus 2008).

The aim of this study was to investigate the feeding ability of three New Zealand extant spittlebug species collected near vineyards in Auckland. These species were chosen as they are common in other wine-growing areas in the country. The probing and real feeding time of the spittlebugs on grape (*Vitis vinifera* Sauvignon blanc (Sb)) was quantified and compared with the plants from which they were collected, using Electrical Penetration Graph technology (EPG). The EPG technique was used here because it allows the quantification of stylet penetration activities and real feeding (ingestion) (McLean & Kinsey 1964; Tjallingii 1978, Tjallingii & Hogen Esch 1993) of xylem-sap feeding insects on host plant (Almeida & Backus 2004; Backus et al. 2005b; Sandanyaka et al. 2007; Sandanyaka & Backus 2008). Electrical Penetration Graph technology is designed to monitor the stylet penetration behaviour of individual insects on individual plants where the insects are not given a choice of more than one plant.

MATERIALS AND METHODS

Insects and plants: A survey was carried out around 10 vineyards in Auckland, Waiheke Island, Hawkes Bay, Gisborne and Tauranga during October and November 2016 to identify

xylem-sap feeding insect species. Three spittlebug species were identified from around Auckland vineyards: *Philaenus spumarius*, *Carystoterpa fingens*, and *Carystoterpa minor*. These spittlebugs were collected from kikuyu grass (*Pennisetum clandestinum*), *Coprosma robusta* and *celery* (*Apium graveolens*) respectively, and identification of the spittlebugs was carried out by experienced staff at Invertebrate Diagnostic Services, Landcare Research, Auckland, New Zealand). Nymphs and adults were collected on the shoots of their original host plants and the shoots were kept fresh by placing the stems in water. Insects on plant shoots were maintained under high humidity (ca 70%) at 19±1°C and a 16:8 h light:dark cycle inside transparent plastic containers (20 × 20 × 22 cm) fitted with a vented lid. Males and female adults were identified. Potted *V. vinifera* Sb grapevines (ca 1-m tall, free from insecticide spray) were maintained in a glasshouse at 22±1°C and 16:8 h light:dark cycle.

Protocol: Fresh shoots were excised from *V. vinifera* Sb plants (from the glasshouse) and from original hosts (from the field) on the day of the experiments. Each of the three spittlebug species was tested on shoots of *V. vinifera* Sb and its field host, making a total of six treatments. The experiments took place in a temperature-controlled room at 20±1°C, under fluorescent lights on for the duration of testing. Tests were conducted inside Faraday cages to reduce electrical interference. Ten replicates of female and male spittlebugs from each treatment were planned, using a fresh insect and fresh plant material for each replicate. However, due to issues with the electrical noise caused by poor wire connection on some insects tested, the number of replicates varied between treatments (Table 1).

EPG tests: Adult spittlebugs were immobilised by exposing them to carbon dioxide for 2–3 s. Once immobile, a gold wire (18.5 µm in diameter) was attached to the dorsum of the insect with a drop of silver conductive paint (n-butyl acetate solvent; Ladd Research Industries, Williston, VT, USA). This 3-cm-long gold wire was attached to a 3-cm-long copper wire that was attached to the head of a 3-mm-diameter copper nail which was

used as the insect electrode. This nail attached to the gold wire served as a handle to move the wired spittlebugs and to tightly fit into the input probe of the EPG monitor. Wired insects were left for a 30-min recovery period before being placed either on the stems or the leaves of the test plant material, depending on how they were observed on leaves or young stems of their host plants. The plant electrode (a thick copper wire) was inserted into the tube filled with water around the excised plant stem. The stylet penetration of each insect was monitored using Giga-8 DC-EPG system (WF Tjallingii, Wageningen, The Netherlands). Data from the EPG monitor were acquired and stored using Stylet+d software (EPG Systems, Wageningen, Netherlands). Individual recordings ranged from 1.5–12 h. The durations of the main EPG waveforms representing main stylet penetration activities (Sandanyaka et al. 2013) of each individual insect were measured. The main EPG waveform parameters total non-probing/probing periods, number of non-probing events, total feeding time and number of feeding events were measured.

Statistical analyses: The percentage of insects completing the 12-h recording period (i.e. not escaped) was analysed with a Bernoulli generalised linear model (GLM, McCullagh & Nelder 1989) with logit link (with each insect scoring 1 for completed or 0 if escaped). The total probing and feeding times as percentages of recording time were analysed with a binomial GLM with a logit link, with the binomial totals being the recording period. The numbers of non-probing and feeding events were analysed with a Poisson GLM with a logarithmic link, with an 'offset' (McCullagh & Nelder 1989) of log (recording period) included to adjust results to the equivalent number for a full 12-hour period. The mean time per feeding event was calculated as total time feeding/number of feeding events. This was analysed with analysis of variance, after first log-transforming the data. All results are presented as means along with 95% confidence limits: these were obtained on the link or transformed scale, and back-transformed for presentation. The analyses were all carried out with Genstat (Payne et al. 2015).

Table 1 The percentage of spittlebugs (*Carystoterpa fingsens*, *Philaenus spumarius*, and *Carystoterpa minor*) completed 12-h recording period and the Electrical Penetration Graph measurements of their probing and feeding on *Vitis vinifera* Sb shoots and shoots from their respective field host plants over a 1.5–12 h recording period. Confidence limits of 95% are in parentheses.

Spittlebug species	Plant type	No. of replicates	Insects completed 12 h recording period (%)	Probing time as a percentage of recording time	Feeding time as a percentage of recording time	Total non-probing events	Feeding time per event (min)
<i>Carystoterpa fingsens</i>	<i>Vitis vinifera</i> Sb	10	80.0 (45.9,95.0)	43.7 (34.1,53.7)	57.5 (41.1,72.3)	10.3 (7.5,14.2)	10.7 (6.4,17.9)
	<i>Coprosma robusta</i>	9	89.9 (50.0,98.5)	95.2 (88.4,98.1)	79.4 (68.7,87.1)	4.9 (3.0,7.8)	54.9 (31.8,97.8)
<i>Philaenus spumarius</i>	<i>Vitis vinifera</i> Sb	10	70.0 (37.6,90.0)	79.2 (69.5,86.5)	79.8 (67.8,88.1)	7.1 (4.8,10.6)	50.1 (29.9,84.1)
	<i>Pennisetum clandestinum</i>	10	60.0 (29.7,84.2)	76.1 (65.7,84.0)	79.9 (67.2,88.6)	9.9 (7.0,14.1)	52.8 (31.5,88.6)
<i>Carystoterpa minor</i>	<i>Vitis vinifera</i> Sb	7	0 (0.0)	54.4 (38.9,69.1)	41.6 (22.0,64.3)	10.8 (6.7,17.6)	7.8 (4.2,14.4)
	<i>Apium graveolens</i>	8	75.0 (37.7,93.7)	81.9 (71.7,89.0)	74.1 (60.9,84.0)	8.4 (5.7,12.4)	28.9 (16.2,51.6)

RESULTS

The stylet penetration activities of individual insects started with a non-probing baseline at 0 voltage level on the EPG output. The initial probe had the highest amplitude waveform indicating the first electrical contact between stylet tip and plant tissue. That continued with a mixture of waveforms with different frequencies representing several stylet pathway activities including saliva secretion. Xylem-ingestion waveforms appeared within pathway waveforms, and started with a distinctive, repetitive waveform pattern at the same voltage level. Watery excreta from the spittlebugs were observed during the time that ingestion waveforms were recorded.

Out of 54 insects tested, 35 completed a full (12 h) recording period on the test plant (Table 1). The variation between *V. vinifera* Sb and original host in the percentage of insects that completed the full 12-h recording period varied, (and thus, the percentage that escaped from the plant in the middle of the recording), with a differential effect between the species ($P=0.012$ for the host type by species interaction). For *Philaenus spumarius* and *Carystoterpa fingsens*, the percentage completing a 12-h recording period was fairly similar for the two hosts ($P=0.639$ and $P=0.592$; *V. vinifera* Sb/original host comparison for the two species respectively), with about 84% of *Carystoterpa fingsens* and 65% of *Philaenus spumarius* completing the full recording period. In contrast, no *Carystoterpa minor* completed a 12-h recording period on *V. vinifera* Sb, whereas 75% of this species completed on the original host ($P<0.001$) (Table 1).

Xylem ingestion occurred on all the plants tested by insects from all three species. The time spent probing as a percentage of recording time varied between *V. vinifera* Sb and the original host, but differentially for the three species ($P<0.001$ for the species by host type interaction). For *Philaenus spumarius*, percentage probing was fairly similar for the two hosts ($P=0.614$) with about 78% of time spent probing on average. In contrast, *Carystoterpa fingsens* spent a significantly lower proportion of time probing when on *V. vinifera* Sb than when on the original host ($P=0.019$), with only half the probing time

on *V. vinifera* Sb compared to the original host. For *Carystoterpa minor*, the percentage time probing on *V. vinifera* Sb was also significantly lower than that on the original host ($P=0.003$), but the effect was smaller than observed for *Carystoterpa fingsens*, with about a third less time spent probing on *V. vinifera* Sb.

As a percentage of total recording time, the patterns in time spent feeding were reasonably similar to those for time spent probing, except that for *Carystoterpa fingsens* the percentages on the *V. vinifera* Sb and original host were much closer, and the species by host interaction was weaker ($P=0.119$). On average, the percentage time spent feeding was greatest for *Philaenus spumarius* (62%), and quite similar for *Carystoterpa fingsens* and *Carystoterpa minor* (50% and 48%; $P=0.096$ for the species main effect). However, the percentage time feeding on *V. vinifera* Sb was less than that for the original host for both *Carystoterpa fingsens* and *Carystoterpa minor* ($P=0.019$ and $P=0.014$), but similar for the original host and *V. vinifera* Sb for *Philaenus spumarius* ($P=0.988$).

The number of non-probing events per 12 h varied between the *V. vinifera* Sb and the original hosts, with the change varying between the species ($P<0.001$ for the species by host type interaction). For *Carystoterpa fingsens*, the number of non-probing events was more than double on *V. vinifera* Sb compared to on the original host ($P=0.008$). However, for both *Philaenus spumarius* and *Carystoterpa minor*, the numbers of non-probing events were fairly similar on the two host types ($P=0.205$ and $P=0.409$ respectively) (Table 1).

The mean time per feeding event varied between the host types, but the change between these varied with species ($P<0.001$ for the species by host type interaction). For *Carystoterpa fingsens* and *Carystoterpa minor*, time feeding on *V. vinifera* Sb per event was substantially lower than that for the original host, at only 20% and 27% of the time spent on per event feeding on the original host. In contrast, feeding time per event were fairly similar for *V. vinifera* Sb and the field host for *Philaenus spumarius* ($P=0.866$) (Table 1).

DISCUSSION

In this study, all three of the tested spittlebug species showed that they accept *V. vinifera* Sb as a food source and that xylem ingestion occurred to some degree, suggesting that they all have the potential to transmit *X. fastidiosa* in grapevines. The percentage of probing or feeding time of *Philaenus spumarius* on *V. vinifera* Sb was not different from that on its field host *Pennisetum clandestinum*, suggesting that *V. vinifera* Sb could be a potential food plant of *Philaenus spumarius*. Some of these feeding behaviours also may relate to transmission activities of *X. fastidiosa*. The feeding duration per event demonstrates the possibility of uninterrupted transmission events and longer feeding events could result in a successful transmission activity by the vector (Backus et al. 2005a).

For the three species tested, the acceptations (i.e. feeding time) order from good to poor of *V. vinifera* Sb was *Philaenus spumarius* > *Carystoterpa fingsens* > *Carystoterpa minor*. The percentage time feeding on *V. vinifera* Sb was less than that for the original host for both *Carystoterpa fingsens* and *Carystoterpa minor* ($P=0.019$ and $P=0.014$) but similar for the two hosts for *Philaenus spumarius* ($p=0.988$). The feeding times per event for *Philaenus spumarius* were similar for the *V. vinifera* Sb and its field host *Pennisetum clandestinum* ($P=0.866$) while for *Carystoterpa fingsens* and *Carystoterpa minor*, time feeding on *V. vinifera* Sb per event was lower than that for the original host. Normally short probes were terminated after only a short duration of trial ingestion, indicating as test probes for acceptability of xylem. Long-duration probes usually have longer pathway phases including continuous xylem ingestion. The wire attachment to the insect could restrict its plant access area and the deprivation could cause feeding on poor host plants.

In the current study, a 12-h total recording period was used because previous studies revealed that EPG recordings of about this length provide reliable data on sustained feeding to identify food plants of the test insects (Sandanayaka et al. 2013). Overall, 65% of insects completed 12-h

recording period; however, all the test insects showed feeding even for a short period. None of the *Carystoterpa minor* individuals tested on *V. vinifera* Sb completed a 12-h recording period while 75% completed 12-h recording period on *A. graveolens* (Table 1). The settlement of spittlebugs on the plants was indicated by continuous probing with fewer non-probing events. The percentage of recording time spent for probing and feeding would provide a reasonable estimate of the feeding acceptance by the insect. The duration per feeding event would indicate the length of uninterrupted feeding on the host plant. Typically, feeding events are longer in more acceptable plants (Tjallingii & Hogen Esch 1993), with a higher percentage of time spent for ingesting (Sandanayaka & Backus 2008).

Results of this study confirmed previous records of *Carystoterpa fingsens* feeding on *V. vinifera* Cabernet Sauvignon (Sandanayaka et al. 2013). In this study, the total feeding period of *Carystoterpa fingsens* was longer on its field host *Coprosma robusta* than on *V. vinifera* Sb whereas Sandanayaka et al. (2013) reported shorter feeding periods of *Carystoterpa fingsens* on *Coprosma robusta* than on *V. vinifera* Cabernet Sauvignon. The difference in *V. vinifera* varieties may have resulted in feeding differences. In addition, the insects for the previous study were mostly collected from *Hebe azure* and the insects for the current study were exclusively collected from *Coprosma robusta*.

Host plant acceptance by spittlebugs may depend on several factors including the change of plant nutrition and phytochemicals in spring. Spittlebugs emerge from overwintering eggs in the spring and the nymphs and adults are found in wild only during the 3- to 4-month period starting from late September. Most grape varieties have young growth during spring months which may be favourable to adult spittlebugs as an alternate food source.

Only adult spittle bugs were tested in this study because they were more robust for long recording periods than early life stages. Nymphs normally feed in groups covered with copious amounts of excreted fluid. The main function

of those spittle masses is to protect nymphs from desiccation, which would cause mortality under dry conditions, and attaching a wire to their tender bodies through spittle masses is impossible. In addition to that, *X. fastidiosa* multiplies in the precibarium and cibarium of the vector and is apparently limited to this area of the foregut (Almeida & Purcell 2006), therefore nymphs lose infectivity after moulting when the cuticular lining of the foregut is shed (Purcell & Finlay 1979). Hence adults are more efficient vectors than early life stages.

Results suggest that *Philaenus spumarius* accept *V. vinifera* Sb as a food source more than the other two spittlebug species tested. There is evidence for *Philaenus spumarius* being a competent vector of *X. fastidiosa* (Saponari et al. 2014; Cornara et al. 2016, 2017). Even in the absence of *X. fastidiosa*, the feeding of spittlebugs by itself could cause a significant damage to grapevines and other plant species where they are found. Future research will determine *X. fastidiosa* inoculation success of these spittlebug species. Also, real feeding (ingesting) times recorded by EPG may potentially provide an indication of host plant acceptance and preferences of sucking insects.

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