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Survey of populations of the planthopper *Hyalesthes obsoletus* Sign. (Auchenorrhyncha, Cixiidae) for infection with the phytoplasma causing grapevine yellows in Germany

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Abstract: *H. obsoletus* is the vector of grapevine yellows (Vergilbungskrankheit, VK) in Germany. Monitoring the abundance of the vector populations and their infestation with the phytoplasma associated with VK is necessary to assess the risk of disease outbreaks and field-spread. We tested planthoppers collected in four different vineyards of the Mosel River valley in Germany for infection by VK using DAS-ELISA and PCR. ELISA readings of female planthoppers were significantly higher than those of males. The proportion of positive testing *H. obsoletus* was higher in PCR tests compared to ELISA. The trapping method, either sweep-net or sticky-trap, had no significant influence on the proportion of planthoppers that reacted positively in PCR tests. The infestation of vineyard populations estimated by PCR varied between 7% and 34%. Batch samples, prepared from mixtures of crude planthopper extracts or from groups of 25 planthoppers, proved to be reliable for the detection of a single infected vector. For routine monitoring, we suggest PCR tests of batch samples from sticky traps.

1 Introduction

Grapevine Yellows diseases (GY) affect viticulture in many areas all over the world (CAUDWELL, 1993). Different phytoplasmas have been found associated with various GY (CAUDWELL et al., 1971; BIANCO et al., 1993; CHEN et al., 1993; DAIRE et al., 1993a, b; PRINCE et al., 1993; MAIXNER et al., 1994; LAVINA et al., 1995; PADOVAN et al., 1995) although symptoms expressed by infected grapevines are almost indistinguishable. In spite of their considerable economic impact, the epidemiology of most GY is not well understood. Vectors and modes of spread are only known from two GY, Flavescence dorée (FD) in southern Europe and Vergilbungskrankheit (VK) in Germany. FD is transmitted by *Scaphoideus titanus* Ball (SCHVESTER et al., 1962), a nearctic, grape-feeding leafhopper (Auchenorrhyncha, Deltocephalidae) introduced into Europe (CAUDWELL, 1983), whereas the planthopper *Hyalesthes obsoletus* Sign. (Auchenorrhyncha, Cixiidae) has been identified as a vector of VK (MAIXNER, 1994). VK is not restricted to grapevine like FD, but infects herbaceous plants as well. Some of those alternative hosts are common weeds in vineyards, such as bindweed, *Convolvulus arvensis* L., and nightshade, *Solanum nigrum* L. (MAIXNER et al., 1995). They are also preferred hosts of *H. obsoletus* that feeds on grapevine only erroneously (VIDANO et al., 1987).

Spread and progress of plant diseases are influenced by the density of inoculum. Since phytoplasmas are transmitted by insects, epidemiological studies have to consider infected vectors as basic propagative units (CAMPBELL and MADDEN, 1990). Monitoring the population density or the activity of vectors and estimating the proportion of infected or inoculative vectors are therefore essential to predict the risk of infection and the disease progress (KRANZ, 1996). Furthermore, attempts to develop control measures rely on information about the presence of vectors and the infestation of their populations by the pathogen.

The incidence of phytoplasmas in vector populations has been determined either with bioassays by feeding insects on experimental hosts (BOUDON-PADIEU and LARRUE, 1986; OSLER et al., 1992; RICE MAHR et al., 1993) or through direct detection of the pathogens in insects. Serological techniques (BOUDON-PADIEU and LARRUE, 1986; BOUDON-PADIEU et al., 1989; FOS et al., 1992; OSLER et al., 1992) or nucleic acid hybridization (KIRKPATRICK et al., 1990; RAHARDJA et al., 1992; MAESO TOZZI et al., 1993) have been applied for various vectors and phytoplasmas. In this paper, we compare the results of surveys of *H. obsoletus* populations by ELISA and PCR with the objective to find a reliable procedure for monitoring field populations of this vector for infection with VK. Since sticky traps are widely used to monitor leafhoppers and planthoppers in vineyards, emphasis was placed upon the possibility to use insects caught by those traps for subsequent phytoplasma detection. Furthermore, the suitability of batch tests as a measure to screen large numbers of planthoppers has been investigated.

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2 Materials and methods

2.1 Collection of planthoppers

Adult *H. obsoletus* planthoppers were collected in four vineyards at different locations along the Mosel River valley in Germany with grapes affected by VK. The insects were either collected from their host plants using a sweep net or caught on yellow sticky traps (13 cm × 25 cm) which had been exposed in the vineyards for 1 week. The planthoppers were removed from the traps using a drop of benzene and air-dried at room temperature. All captured insects were stored in a freezer at −20°C. Laboratory bred *Fiberiella florii* (Stål) leafhoppers were used as healthy controls for ELISA tests. The proportions of PCR-positive individuals were compared between the groups planthoppers caught by sweep net or sticky trap by a 2 × 2 table χ^2 test (BORTZ et al., 1990).

2.2 Preparation of samples

Individual frozen planthoppers were cut longitudinally and the two halves were used separately. One half was always used to isolate DNA and to define the health status of the individual planthoppers by PCR. The second half of each insect was used either for ELISA or for PCR detection of the VK phytoplasma. DNA was isolated from *H. obsoletus* as described before (MAIXNER et al., 1995) and resuspended in 40 µl sterile water. Test antigens for ELISA were prepared by crushing half insects in 200 µl PBS. After addition of another 300 µl buffer, the debris was removed by centrifugation at 2000 *g* for 2 min and the supernatant was used for ELISA. Batch samples for PCR were prepared in two different ways. Crude extracts of crushed planthoppers whose other halves had been tested positively in a previous PCR were used either undiluted or mixed with extracts of healthy *H. obsoletus*. Extracts were blended in order to prepare defined dilutions of infected material (1:5, 1:10, 1:25, 1:50, 1:100, 1:500; v:v), and the mixtures were then subjected to the standard DNA extraction procedure. A second type of batch-samples was prepared from one half of an infected planthopper combined with 24 halves of PCR-negative insects. The insects were crushed together in a total volume of 7.5 ml extraction buffer, and an aliquot of 1 ml of the mixture was then used for standard DNA extraction.

2.3 Polymerase chain reaction

PCR was carried out in a total volume of 15 µl. Reaction mixtures consisted of 250 nm each of primers fStol and rStol (MAIXNER et al., 1995), 100 µm of each of four nucleotides, 0.38 U Replitherm DNA polymerase (Biozym Diagnostics, Hess. Oldendorf), 1 × reaction buffer supplied with the DNA polymerase, and 1 µl template DNA. Amplification was carried out with 35 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 58°C, and elongation for 30 s at 72°C. Amplification products were analyzed by electrophoresis of 5 µl of each reaction mixture in a 1.5% horizontal agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0; 5 V · cm^{-1}). DNA was stained with ethidium bromide and visualized in UV light.

2.4 ELISA

The Sanofi SMLO Test Kit (SANOFI Diagnostics, Marnes-la-Coquette, France; Ref. No. 51634) for the detection of stolbur phytoplasmas was used in a double antibody sandwich–ELISA (DAS–ELISA) procedure. All buffers except the sample buffer were used from the kit. Plates were coated with antibodies (1:200 in coating buffer) for 2 h at 37°C, and incubated with 100 µl sample antigen per well at 4 C overnight. Incubation with alkaline phosphatase–antibody conjugate (1:200 in conjugate buffer) was performed for 2 h at 37°C. 4-Nitrophenylphosphate was added as substrate (1 mg · ml^{-1}) and the optical density (OD_{405}) was recorded after 90 min with a Dynatech MR5000 reader (Dynatech, Denkendorf). Each step was followed by three washing cycles with PBS and all samples were tested in two repeats. The insects to be tested by ELISA were chosen according to the results of previous PCR tests of their second halves. Sixty-seven male (40 PCR-positive/27 PCR-negative) and female (39 PCR-positive/28-PCR-negative) planthoppers were used. An analysis of variance (ANOVA) was calculated using the GLM procedure (SAS INSTITUTE INC, 1989) to determine the significance of the effects of sex, phytoplasma infection, and the different test plates on the ELISA readings. The distributions of ELISA readings of male and female planthoppers were compared between the groups of PCR-negative and -positive insects by a Kolmogorov–Smirnov-omnibus test (KSO test, BORTZ et al., 1990).

Different positive–negative thresholds were calculated and applied to adjust the ELISA readings to the results of the previous PCR tests of the same insects. The average OD_{405} of four laboratory bred *F. florii* leafhoppers plus 2 × the standard deviation (s.d.) was defined as threshold A. This threshold was calculated separately for each test-plate. A second threshold (B) was calculated in the same way from the OD readings of all PCR-negative *H. obsoletus*. The third threshold (C) was calculated from PCR-negative male and female planthoppers separately, and applied to the readings of insects of the appropriate sex. Readings of different plates were combined to calculate the thresholds B and C.

3 Results

3.1 Comparison of PCR and ELISA results

The results of ELISA tests of *H. obsoletus* are summarized in table 1. ANOVA revealed that not only phytoplasma infection and the sex of the planthopper, but also the test plates had a significant effect on the OD_{405} readings. The means of female *H. obsoletus* responses were significantly higher then the average OD of males in both the PCR-positive and PCR-negative groups. Furthermore, the distributions of OD readings were significantly different between males and females (KSO test: $P < 0.001$). Consequently, the positive–negative thresholds A and B that did not differentiate between the sexes led to a weak correspondence between ELISA and PCR data. For example, only 15% of PCR-positive males and 67% of PCR-positive females were classified as ELISA-positive with threshold B, while 11% of PCR-negative females were rated falsely positive. The use of *F. florii* as negative controls was questioned by the observation that some of these insects led to high OD readings which could not be explained. Better results were achieved when the threshold was calculated separately for the two sexes (C). Still only two thirds of the PCR-positive planthoppers were rated ELISA-positive, but only one of 134 insects tested led to a false positive result. Despite the plate effects on OD readings, this threshold gave the best agreement between ELISA and PCR results.

Table 1. *Comparison of results of PCR and ELISA tests of* H. obsoletus

Parameter	PCR-negative hoppers			PCR-positive hoppers		
	Female	Male	Total	Female	Male	Total
Insects tested	28	27	55	39	40	79
Minimum OD_{405}	0.030	0.010	0.010	0.030	0.020	0.020
Maximum OD_{405}	0.440	0.080	0.440	1.910	0.700	1.910
Median OD_{405}	0.105	0.040	0.070	0.420	0.110	0.180
Mean OD_{405}	0.142	0.047	0.095	0.491	0.164	0.326
s.d. OD_{405}	0.089	0.018	0.080	0.397	0.164	0.342
% ELISA positive planthoppers with						
Threshold A	21.4	0.0	10.9	61.5	17.5	39.2
Threshold B	10.7	0.0	5.5	66.7	15.0	40.5
Threshold C	3.6	0.0	*1.8	61.5	65.0	*63.3

Thresholds: A: mean + 2 s.d. of OD_{405} readings from laboratory bred healthy *F. florii* leafhoppers; B: mean + 2 s.d. of OD_{405} readings from all PCR-negative *H. obsoletus* planthoppers; C: mean + 2 s.d. of OD_{405} readings from PCR-negative male or female *H. obsoletus* respectively; separate thresholds applied for male and female planthoppers.
* Threshold C levels used separately for male and female insects.

3.2 PCR analysis of samples collected by sweep-net and sticky-trap

The PCR tests of individual planthoppers revealed a high proportion of *H. obsoletus* infected by VK in three of the four vineyards. Approximately one third of the planthoppers caught by sweep net in Bernkastel, Kues and Enkirch tested positively (table 2a). The infestation was considerably lower in the population of Platten, where only 7% of the hoppers were PCR-positive. The infestation of female and male planthoppers did not differ significantly, although the fraction of positive female hoppers exceeded that of males in Bernkastel and Enkirch. The fraction of PCR-positive planthoppers was generally higher in the group of insects collected by sweep-net compared to the sticky-trap catches (table 2b). However, only the samples from Enkirch revealed a significant difference between the two trap types.

3.3 Batch samples

The results of PCR tests with samples prepared by diluting crude extracts of infected *H. obsoletus* with extracts from healthy planthoppers are summarized in table 3 and an example is given in fig. 1. Positive responses could be achieved in all samples with dilutions of infected extract up to 1:50. A clear signal was achieved

Table 2a. *Comparison of infestation rates of male and female* H. obsoletus *collected by sweep net at four locations of the Mosel Valley*

Origin of insects	Female		Male		Total planthoppers	
	No. positive/ no. tested	% Infestation	No. positive/ no. tested	% Infestation	No. positive/ no. tested	% Infestation
Bernkastel	26/68	38%	23/82	28%	49/150	32%
Kues	19/55	34%	23/68	34%	42/123	34%
Enkirch	30/82	37%	19/84	27%	49/166	30%
Platten	3/74	4%	8/91	9%	11/165	7%

Table 2b. *Comparison of infestation rates of* H. obsoletus *collected by sweep net or sticky trap at four locations of the Mosel valley*

Origin of insects	Sweep net		Sticky trap		Difference between trap types[1]
	No. positive/ no. tested	% Infestation	No. positive/ no. tested	% Infestation	P
Bernkastel	49/150	32%	11/50	22%	n.s.
Kues	42/123	34%	14/50	28%	n.s.
Enkirch	49/166	30%	7/50	14%	0.03
Platten	11/165	7%	4/50	8%	n.s.

[1] χ^2 test for difference between infestation data of samples collected by the two trap types.

Table 3. *Results of PCR test of samples derived from mixtures of extracts of VK-infected and healthy* H. obsoletus *planthoppers*

Sample	Sex of infected hopper	Dilution of crude extract of infected *H. obsoletus* with extract from healthy insect						
		1:1	1:5	1:10	1:25	1:50	1:100	1:500
1	Male	++	++	++	+	+	+	+
2	Female	+	++	++	++	++	—	o
3	Male	++	++	++	++	+	o	o
4	Female	++	++	++	+	o	—	o

Intensity of bands after agarose gel-electrophoresis of 5 µl amplification products: ++, high intensity; +, low intensity; o, faint band; —, no amplification product.
See fig. 1 as reference for sample 4.

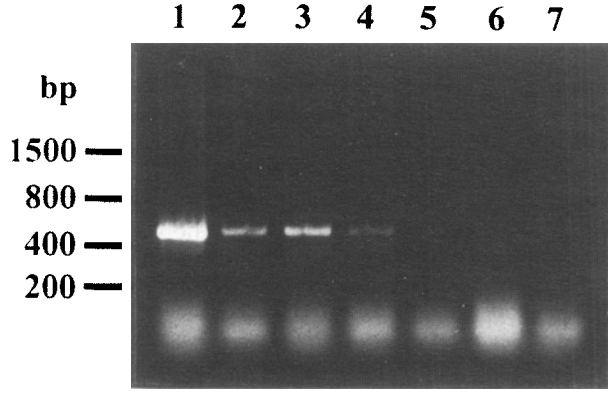

Fig. 1. *Results of PCR amplification of DNA from samples prepared from mixtures of VK infected and healthy* Hyalesthes obsoletus *(sample 4 in table 3). Row 1: undiluted sample of a PCR-positive male. Rows 2–7: samples prepared from mixtures of the infected planthopper from row 1 and healthy planthoppers. Dilution of infected extracts (v:v): 1:5, 1:10, 1:25, 1:50, 1:100, 1:500*

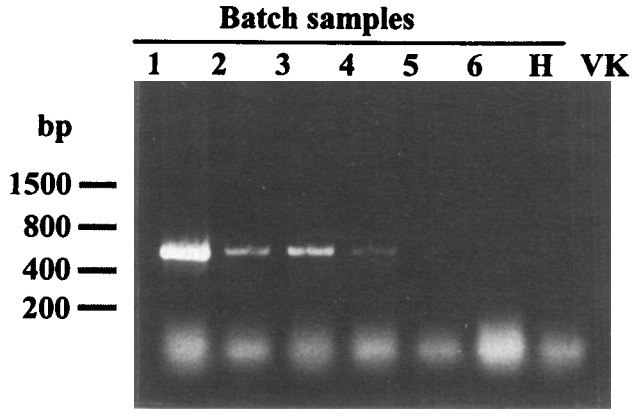

Fig. 2. *Results of PCR amplification of planthopper samples with stolbur specific primers. VK: positive control — periwinkle isolate of Vergilbungskrankheit. Batch samples: 1, 2, 3: Three different samples, each prepared from one half of a PCR-positive male and 24 halves of PCR-negative* Hyalesthes obsoletus *(12 male, 12 female); 4, 5, 6: three different batch samples, each prepared from one half of a PCR-positive female and 24 halves of PCR-negative* H. obsoletus *(12 male, 12 female). H: healthy control — batch sample of halves of 12 male and 13 female PCR-negative* H. obsoletus

from one planthopper, even with a dilution of 1:500. Clearly positive results were achieved in all samples in a dilution of 1:25. Consequently, all of six samples prepared from batches of one infected and 24 healthy *H. obsoletus* were clearly positive in PCR while no amplification was achieved from preparations of healthy insects (fig. 2).

4 Discussion

The development of *H. obsoletus* in the soil is a big obstacle for culturing this species in the laboratory. Therefore, we were obliged to collect all planthoppers for our experiments from the field. The only way to work with material of well-defined health status was to check one half of each insect for phytoplasma infection by PCR and to use only the second half for further experiments. In our experiment, it was no problem to use halves of *H. obsoletus* for PCR and ELISA testing. The data obtained from four different field populations show that both ELISA and PCR techniques can be applied to detect planthoppers bearing the phytoplasma associated with VK. A problem with ELISA was the lack of true negative controls of *H. obsoletus*. The use of laboratory reared *F. florii* as healthy controls was not satisfactory due to strong reactions of some of these leafhoppers which we could not explain sufficiently. Although *F. florii* were used on each plate as internal standards, the positive–negative thresholds calculated from their OD readings did not allow us to classify the tested planthoppers according to the PCR results. To overcome this problem, all PCR-negative *H. obsoletus* were regarded as healthy, although this method could not be evaluated with laboratory bred healthy and infected planthoppers. Furthermore, the readings of different plates were combined to calculate thresholds B and C. Satisfactory results were only achieved with threshold C, calculated for the two sexes separately. With this threshold, 63% of the PCR-positive planthoppers were classified positive and only one false-positive result was observed. However, this threshold is not applicable for monitoring programs where insects of unknown health status need to be tested. Fos et al. (1992) tested *H. obsoletus* by ELISA for tomato stolbur, a phytoplasma closely related to VK (SEEMÜLLER et

al., 1994) which is transmitted by this planthopper in France. They reported OD readings of positive planthoppers between 0.14 and 2.0 which is comparable to our experiments where positive insects responded with ODs between 0.09 and 1.9. However, no information about the discrimination of positive and negative values is given in their paper.

The OD readings of male and female *H. obsoletus* were considerably different. Female planthoppers exhibited a significantly stronger response than males in both the PCR-positive and -negative groups. Differences between sexes in transmission efficiency or in serological assays are known from different phytoplasma vectors. Healthy and infected males of *Euscelidius variegatus* (Kirschbaum) exhibited stronger ELISA responses with antibodies to Flavescence dorée than females, probably due to a different sensibility to phytoplasma infection (BOUDON-PADIEU et al., 1989). CHIYKOWSKI and SINHA (1970) reported a difference in transmission efficiency of clover proliferation between male and female *Macrosteles fascifrons* (Stål) and hypothesized a more intensive acquisition of phytoplasmas by females due to a higher feeding activity. One reason for the variation in ELISA responses reported in this paper could be the significant difference in size between male and female *H. obsoletus*. The average length of females was 4.9 mm compared to 4.0 mm for males ($n = 460$). Thus, the probably higher concentration of planthopper proteins as well as phytoplasma infected tissues (LEFOL et al., 1994) in extracts of females could have led to higher unspecific and specific ELISA readings. More detailed investigations on VK phytoplasma–planthoppers interactions with laboratory cultures of *H. obsoletus* would be necessary to answer this question.

The epidemiological significance of PCR and ELISA results deserves discussion. Due to the high sensitivity of the PCR technique, ingested phytoplasmas can be detected even in nonvector species (VEGA et al., 1993; HARRISON et al., 1996) while ELISA readings do not increase before the pathogens have multiplied in various organs of the vector (BOUDON-PADIEU et al., 1989; LEFOL et al., 1994). Therefore, the variation between ELISA and PCR results probably represents the difference between the proportions of inoculative and inoculated planthoppers. However, this question is relevant for epidemiological studies rather than for monitoring in the field.

The infestation of *H. obsoletus* populations is high compared to the data reported for other phytoplasma diseases, which spread much faster than VK. More than 30% of the planthoppers carried the VK phytoplasma in three of the four vineyards under investigation while the ratio of infected planthoppers was only 7% in a forth vineyard (Platten). BOUDON-PADIEU and LARRUE (1986) reported 16–33% of infected *S. titanus* from areas where FD was endemic in France, and OSLER et al. (1992) found 29% of *S. titanus* infected in Italian vineyards. The infestation of different vectors of X-disease collected in and around affected orchards varied between 1% and 2.5% for *Scaphytopius acutus* (Say) and *Fiberiella florii* (KIRKPATRICK et al., 1990) and 0–46% in populations of *Paraphlepsius irroratus* (Say) (RAHARDJA et al., 1992). MAESO TOZZI et al. (1993) reported between 3 and 15% infestation of *Scaphytopius* spp. populations in blueberry plantings with a high level of field infection by blueberry stunt disease. The contradiction between high levels of infestation of *H. obsoletus* and a relatively slow spread of VK in the respective vineyards can be explained by the biology of *H. obsoletus*. Unlike most of the vectors mentioned above which feed on cultivated plants, *H. obsoletus* is usually not feeding on grapevines to which it transmits the disease. We could show by feeding experiments that this planthopper survives on grapevine for a few days only and is strongly restricted to herbaceous plants (WEBER and MAIXNER, unpublished data). This situation is comparable to tomato stolbur in France, another phytoplasma transmitted by *H. obsoletus*. Although the infestation of the vector population was $\approx 25\%$ the incidence of stolbur in tomato fields was only around 5% (FOS et al., 1993). In accordance with the situation in our vineyards, the vectors were found rarely on tomato but widespread on bindweed. *Convolvulus arvensis* is an important factor for the epidemiology of VK because it serves as a reservoir for the pathogen and is a preferred host of *H. obsoletus* (MAIXNER et al., 1995; WEBER, unpublished data). The importance of infected herbaceous hosts for the contamination of vectors is emphasized by a comparison of the infestation levels in the four vineyards we investigated: *Ranunculus bulbosus* L., which we never found to be infected with VK, was the main host of *H. obsoletus* in Platten, where only 7% of the planthoppers carried the pathogen. *C. arvensis*, on the other hand, was common in the other vineyards, and a considerable proportion of this weed exhibited symptoms of phytoplasma infection and tested positive for VK. As a result, a considerable proportion of *H. obsoletus* was infected in these vineyards.

Sticky traps are reliable and convenient for the monitoring of leafhopper and planthopper activity in vineyards. Surveys of vector populations for VK would be facilitated if insects from sticky traps could be used for phytoplasma detection. We tested the suitability of those insects, which are exposed to potentially adverse climatic conditions before they are removed from the traps and stored, by a comparison of PCR results of *H. obsoletus* collected by sticky traps and by sweep net. Except for the planthoppers from Enkirch, the infestation of the two groups of planthoppers was not significantly different. This is in accordance with the results of KIRKPATRICK et al. (1990), who recorded only a weak decrease of the proportion of positive-testing vectors of X-disease after an exposure on sticky traps for 2 weeks. The reliability of the detection of blueberry stunt phytoplasma in *Scaphytopius* spp. decreased more quickly. MAESO TOZZI et al. (1993) found 15% of the leafhoppers infected on traps exposed for 1 week compared to only 3% on 2-week-old traps. We suggest removal of sticky traps after 1 week if the planthoppers should be used for PCR. The difference between the trap types observed in Enkirch may be explained by high average temperatures in this vineyard, which is exposed to the south on a steep hill (WEBER, unpublished data).

Although batch samples of groups of planthoppers

provide less information about the precise level of infestation, they are useful where large numbers of insects need to be tested. During our study, for example, we collected more than 3000 planthoppers in the four vineyards. These could not be tested individually in a reasonable time and with rational costs. It was no problem to detect one infected *H. obsoletus* among 24 healthy insects. When mixtures of crude extracts were combined, a positive response could be obtained in all samples in a dilution of 1:50. One of the planthoppers could be continuously detected up to a dilution of 1:500. Similar results were obtained by WITHCOMB and COAN (1982) who found ≈ 50% of *Macrosteles fascifrons* able to transmit aster yellows after they had been injected with a mixture of infected and healthy leafhoppers in a dilution of 1:1000. High titres of FD phytoplasma in inoculative vectors were also reported by LEFOL et al. (1994). We expect most of adult *H. obsoletus* emerging from the soil in summer to be inoculative already because they acquire the VK phytoplasma by feeding as nymphs on the roots of infected bindweed (MAIXNER, unpublished data).

Our results show that it is possible to monitor field populations of *H. obsoletus* for the presence and abundance of the VK phytoplasma. PCR tests of hoppers collected by sweep net are appropriate if information about the exact proportions of infected insects is required. However, it is often sufficient to estimate the approximate proportions of infected vectors, for example to predict the risk of spread of the disease or to monitor the presence of VK in a particular region. In this case, we suggest collection of the insects by sticky traps and batch testing by PCR. The survey methods described here are useful if the risk of new infections and the spread of VK need to be predicted. As an example, in areas like the valleys of the Mosel and the Middle-Rhine River in Germany where VK is of reasonable importance, growers are especially concerned about the infection of young vineyards. A survey for the presence, abundance and infestation of *H. obsoletus* provides information whether actions such as management of green cover and the control of bindweed are necessary in advance of new plantings.

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