A genetic analysis reveals low prevalence of phytoplasma infection in *Hyalesthes obsoletus* Signoret, vector of 'bois noir', in SW-Germany

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Abstract

Bois Noir is a grapevine disease responsible for severe economic losses in wine production. Bois Noir is caused by *Candidatus* Phytoplasma solani, cell wall-less bacteria belonging to the taxonomic group 16SrXII-A. In Germany, they are known to be vectored from plant to plant by the cixiid *Hyalesthes obsoletus* (Signoret), but the prevalence of the disease in the vector population, as well as its spatio-temporal distribution is poorly understood. Here, we analyzed infections of *H. obsoletus* individuals collected in different vineyards in Baden (South-Western Germany) with quantitative real-time PCR. From the 125 collected analyzed individuals, only five were infected with *Ca*. P. solani. All infected individuals were colonized by *Ca*. P. solani tuf-type a, which is associated with the host plant *Urtica dioica* (stinging nettle). We conclude that more research is needed to understand the reasons of this surprisingly low prevalence of Bois Noir infections in the population of *H. obsoletus* in South-West Germany.

Key Words

body size, Bois noir, *Hyalesthes obsoletus*, infectious disease, Phytoplasma, planthopper, *Vitis Vinifera*

Introduction

Phytoplasma are cell-wall less prokaryotes. Lacking common metabolic pathways, they need host tissues for their survival and are therefore obligate parasites (Christensen *et al*., 2005). Phytoplasma have developed complex life cycles that involve replications in both insects and plants. In plants, they are usually found in phloem tissues, while in insects they need to cross the gut cells, replicate within internal tissues, and then reach salivary glands for transmission to new plants (Hogenhout *et al*., 2008). This peculiar life cycle allows them to easily reach new host plants taking advantage of the mobility provided by the insect hosts. In cultivated crops, they can also be transmitted through agricultural practices such as pruning and grafting (Hodgetts *et al*., 2007).

While the number of known insect vectors for phytoplasma is limited to the phloem feeders of the order Hemiptera (Weintraub & Beanland, 2006), there is a broad range of host plants. Phytoplasma infections have been found in over 700 plant species (Hoshi *et al*., 2009). Some of these infections cause severe damage to agricultural crops with serious economic impact. Some examples are: lethal yellowing of palms (Harrison *et al*., 2008), peach X-disease, apple proliferation (Tedeschi *et al*., 2003) and Bois Noir (BN), a grapevine phytoplasmosis. The latter is the focus of this study. Typical BN disease symptoms on *Vitis vinifera* include necrotic leaves with downward rolled margins, unlignified branches and shriveled berries (Alma, 2002). During winter season the shoots do not lignify and turn black, giving the disease its name of Bois Noir (Maixner, 1994). The infection usually leads to a significant decrease in yield (Garau *et al*., 2007). Due to the growing economic impact of the disease in European grapevine production, a better understanding of the transmission and dynamics of this phytoplasma is therefore of great importance.

According to current literature, the main vector of BN in Western Europe is the cixiid plant hopper *Hyalesthes obsoletus* Signoret 1865 (Alma *et al*., 1987, Maixner, 2006, Maixner *et al*., 1995, Sforza *et al*., 1998). Although *H. obsoletus* feeds on a wide

range of herbaceous plants, its life cycle can only be completed on few hosts. The two most relevant host plants in Germany are stinging nettle (*Urtica dioica* L.) and field bindweed (*Convolvulus arvensis* L.) (Maixner, 2005). According to a classification based on the genetic sequences of the elongation factor tu (*tuf),* two main genetic types can be distinguished: *Candidatus* Phytoplasma solani tuf-type a is related to *U. dioica,* while *Ca*. P. solani tuf-type b is associated to *C. arvensis* (Langer & Maixner, 2004). Both strains have grapevine as a "dead end host", which means that insects can infect a grape plant, but cannot acquire phytoplasma from infected grape plants (Kaul *et al*., 2009). Before the year 2000, *H. obsoletus* was considered a rare species in Germany, and *C. arvensis* was the major host plant for this epidemic cycle (Sergel, 1986). In the last 20 years, however, *H. obsoletus* was found more frequently on *U. dioica*. It has been speculated that the increasing mean temperatures could be connected to this host plant shift (Boudon-Padieu & Maixner, 2007). The new host allowed the insect to colonize new areas (that is where *U. dioica* is present and *C. arvensis* absent); but also increase its population density in areas where it was already present (Maixner *et al*., 2007). For these reasons, BN has become an increasing concern in Germany.

Our study is concentrates on the region of Baden. Located in South-West Germany at the borders of France and Switzerland, Baden is one of the most important areas of Germany for wine production. Since BN is a relatively new challenge, only few studies have examined this epidemiological system in this area. Darimont & Maixner (2001) conducted an analysis of insect infestation in the year 1999 and 2000. Breuer *et al*. (2008) conducted a monitoring of the occurrence of *H. obsoletus* proving the insects' presence in all wine-growing districts, and Panassiti *et al*. (2013) reported the presence of the insect all over the region. From these studies, we have a basic understanding of the ecology of the insect vector and disease, but so far there is still very little known on disease prevalence in the insect population. It would also be desirable to better understand whether the phytoplasma infection has any consequences for the vector in terms of body size and thus implicates consequences for the vector fitness, e.g. on fecundity (Honěk, 1993).

To address these questions, we analyzed morphological traits and pathogen infection using real-time PCR of 125 *H. obsoletus* individuals collected in 45 vineyards across the region of Baden. The study objectives were i) to examine if morphological traits (body size and weight) of *H. obsoletus* differ between infected and non-infected individuals ii) to investigate the prevalence of *Ca*. P. solani in the vector population; and iii) to identify the predominant *Ca*. P. solani strain in the Baden region.

Materials and Methods:

In 2012, 85 vineyards in the Baden region (SW Germany) were selected for a bois noir (BN) monitoring (Panassiti *et al*., 2015). The selected vineyards were surveyed for soil-borne insect vector *Hyalesthes obsoletus* between June and August 2012 and 2013. Yellow sticky traps and the viticulture prediction tool "vitimeteo" (www.vitimeteo.de) were used to monitor and predict the flight activity of the insects, in order to guarantee optimal sampling conditions. Sampling of *H. obsoletus* individuals was performed as described in Panassiti, *et al*. (2013). In brief, in each of the locations, potential host plants were identified. If *U. dioica* was found, the sampling consisted of sweeping a sweep net (30 cm diameter) over the selected plants. If *C. arvensis* was found, suction sampling was performed. Stinging nettle

patches were swept 5 times per square meter. Suction sampling was applied for 3 minutes for every square meter of the patch.
The collected insects were transported in a cooling box and freeze dried in the laboratory with a freeze dryer "Christ Alpha 1-2 LDplus" (Martin Christ Freeze Dryers, Germany). We determined species and gender following the identification keys of Biedermann & Niedringhaus (2004) with a microscope "Zeiss Stereo LUMAR 1.2", (Carl Zeiss, Germany). We measured body length with the software "Axiovision Rel 4.8". The length of an individual was defined as the distance from head to the bottom of the fore-wings (Fig. 2). The samples were then frozen in liquid nitrogen and conserved at -80°C for further analyses.
DNA extraction and quantitative real-time PCR (qPCR) was carried out with the same procedure for extraction and the same primers, probes and cycle settings for qPCR as in Fahrentrapp *et al.* (2013). This method utilizes hydrolysis probes specific for selective DNA fragments of both, phytoplasma types I and II, as well as for insect DNA. The method allows to detect an infection and to distinguish the phytoplasma type. Based on the obtained results, we calculated the amount of phytoplasma DNA relative to the insect DNA, using the method $2^{-\Delta\Delta Ct}$ as described by Livak & Schmittgen (2001) for relative quantification of gene expression.

Results

We collected 125 *Hyalesthes obsoletus* individuals from the 45 vineyards (Fig. 1). 52 of those were identified as males, 70 as females and 3 were not identifiable due to the lack of the final abdominal segments. The observed female ratio of 56% is not significantly different from an even sex ratio (p=0.18 with a binomial test against H_0 = 50%; the 95% confidence interval spans 47% - 65%). Of all 125 individuals, five (four females and one male) were infected with *Ca.* P. solani, tuf-type a. This corresponds to a disease prevalence of 4%, with the 95% confidence interval from a binomial model ranging from 1.3% to 9.2%. The five infected individuals were caught in different locations spread all over Baden (Fig. 1), with no discernable spatial pattern. The amount of phytoplasma DNA detected in the samples varied substantially. The sample with the highest amount of phytoplasma DNA has roughly 17 times more phytoplasma-DNA than the sample with the lowest amount (Table 1).
We observed a difference in weight and length between males and females. Females were in general longer and heavier than males. The average length and dry weight for females and males was 4.95 (+/-0.31), 4.01 (+/-0.28) mm as well as 1.7 (+/-0.57) and 0.67 (+/-0.31) mg, respectively. Our results for sex ratio, weight and length are also displayed table 2 and in figures 3 and 4. Due to the low number of infected insects, it was not possible to draw any conclusions about the influence of infections on insect length and body mass. The five infected individuals show values that are well within the range found for non-infected individuals.

Discussion and Conclusions

The prevalence of *Ca.* P. solani infection in *H. obsoletus* determined in this study was surprisingly low. Although we have evidence of infected plants in studied vineyards (Panassiti, *et al.*, 2015), we only found five infected individuals in 125 samples analyzed (4%). Previous studies in different areas showed higher infection rates. Langer & Maixner (2004) observed insect infection rates of 28% and 54% (host plant *U. dioica*) in two different viticultural areas in Germany. In a study conducted in the

north of Italy, Lessio *et al*. (2007) reported a variable infection rate during different years, reaching up to 80%. Darimont & Maixner (2001), who also sampled in Baden, but over different years, reported an average infection rate of 23% over several years. We can only speculate about the reasons for the low disease prevalence in the present study. The sample size of this study is relatively low, but as the 95% confidence interval extended only up to 9.2%, the explanation that random variation alone is causing this low prevalence seems incompatible with the assumption that the true disease prevalence is around 20%. A plausible explanation is that the sampling methods used in our study were different from previous studies. In our study, sampling locations were chosen randomly. In the previously mentioned studies the site selection criteria were sometimes not clearly described and it could be that sampling locations were chosen close to vineyards in which the disease has already been observed. In this case, a higher prevalence would be expected. We see a need for further studies to resolve the question of the average disease prevalence in the Baden region.

The length measurements of *H. obsoletus* are in agreement with values from the literature. Alma (2002) described body length of 3.7 - 4 mm and around 5 mm for males and females, respectively. To our knowledge, no previous studies reported on the dry body weight of the insects that could be compared to our results. No significant bias of the sex-ratio was observed in this study, although this could have been expected for our sampling method: using yellow sticky-traps, the gender ratio may be biased (Lessio, *et al*., 2007), because in many Homoptera species males have a greater flight activity and a higher dispersal rate compared to females (Lessio & Alma, 2004).

In conclusion, our study showed an unexpectedly low prevalence of BN-causing phytoplasma in individuals of the insect vector *H. obsoletus* caught in Baden. We were not able to draw any conclusion on differences in size of the individuals due to the low number of infected individuals.

Further surveys with a higher number of specimen, sampling locations and an analysis of plant material for *U. dioica*, *C. arvensis* and *V. vinifera*, will help to understand if this low prevalence reflects a true decline of the disease in the vector population, or whether it can be explained with systematic differences with previous studies.

Acknowledgments

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204 **Tables**
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208
209 Table 1. Morphological measurements qPCR results (DNA folds) for infected
210 *Hyalesthes obsoletus*.

Sample	Sex	Length (mm)	Dry weight (mg)	DNA Folds*	St. Dev.
1114	Female	4.74	1.7	2.6	0.3
1109	Female	4.03	1.7	17.2	0.5
1123	Female	5.19	2.3	3	0.7
1041	Female	4.9	2.1	13.3	0.7
1134	Male	3.48	1.1	1	1

211 *The DNA folds are calculated using the ratio phytoplasma DNA/insect DNA,
212 relative to the sample with the lowest amount of DNA (1134). Relative titers of DNA
213 normalized to sample 1134.
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216
217 Table 2. Maximum, minimum and average values of morphological observations
218 grouped by sex of all specimens collected in this study.
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Measurement	Average value (Std. Dev.)	Max.	Min
Sex ratio (%)	56 (9)		
Male length (mm)	4,01 (0.28)	4.49	3.18
Female length (mm)	4.95 (0.42)	5.7	3.73
Male body mass (mg)	0.67	1.4	0.1
Female body mass (mg)	1.7 (0.57)	2.7	0.2

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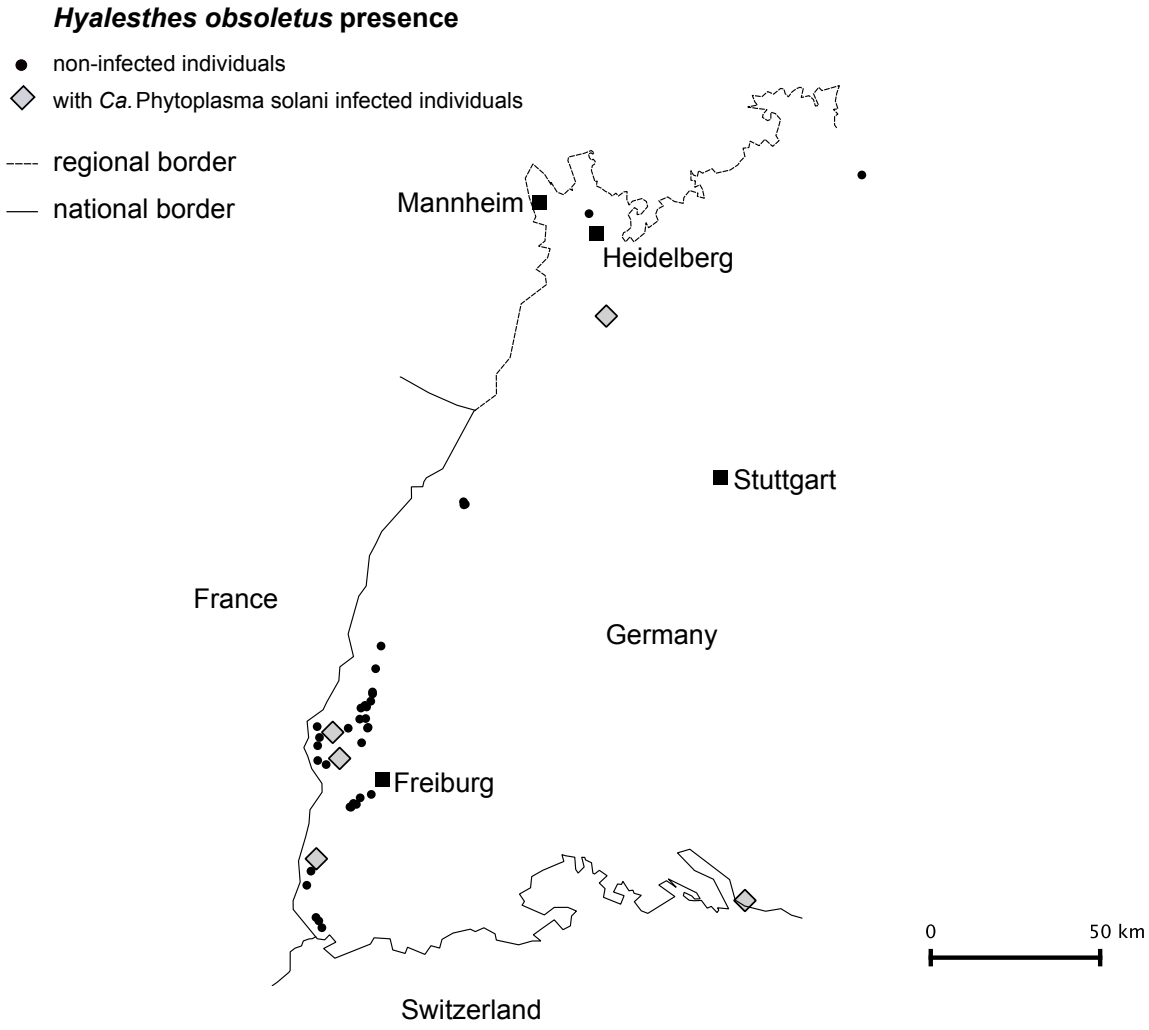
Figures

Figure 1. Collection sites in the Baden region. Black dots are the sampling locations. Grey squares represent the sampling locations where infected *H. obsoletus* were found.



228 Figure 2. Magnified photographs of (A) *H. obsoletus*, (B) male genital capsule, and
229 it's (c) female ovipositor.
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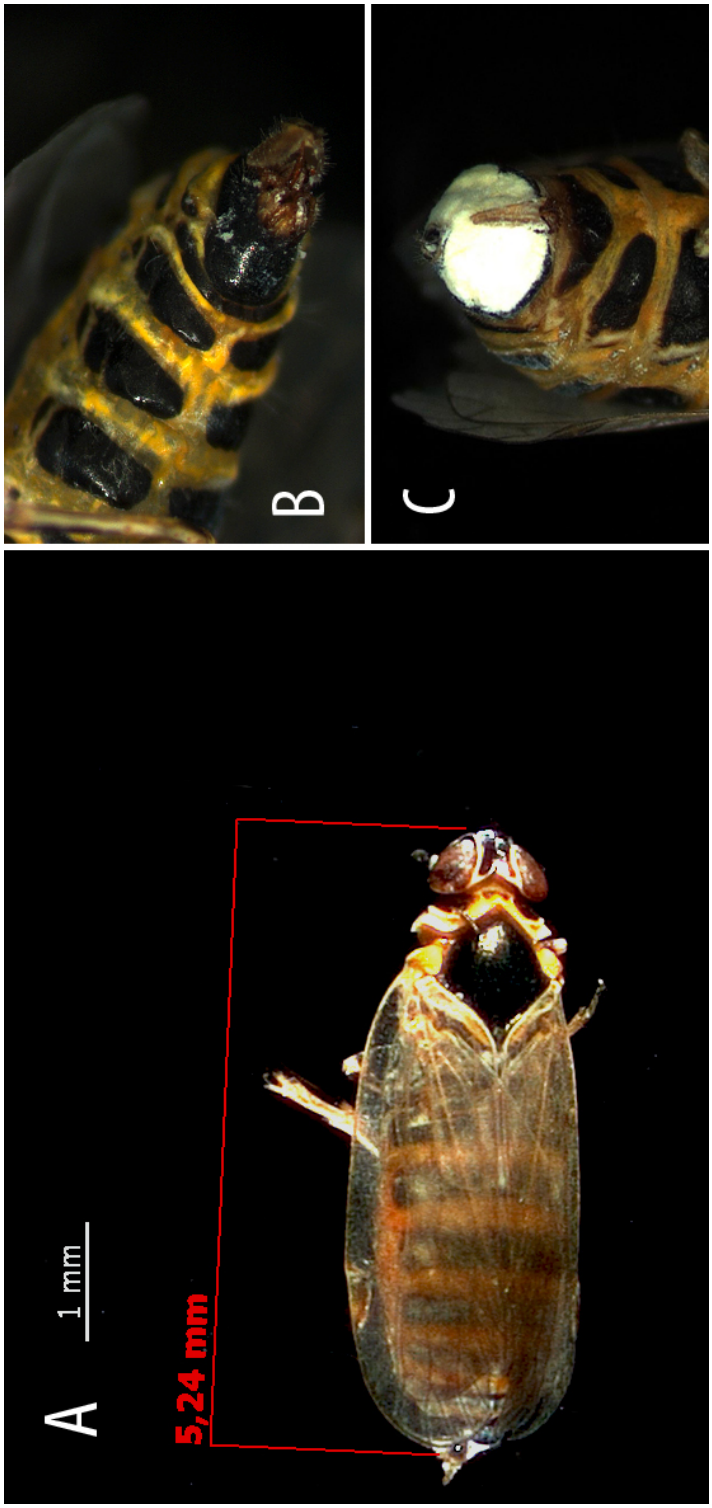


Figure 3. Distribution of length and weight for the individuals analyzed. Females are colored in yellow diamonds, males are represented in red dots and the samples with the abdomen missing (unrecognisable) are shown in blue squares.

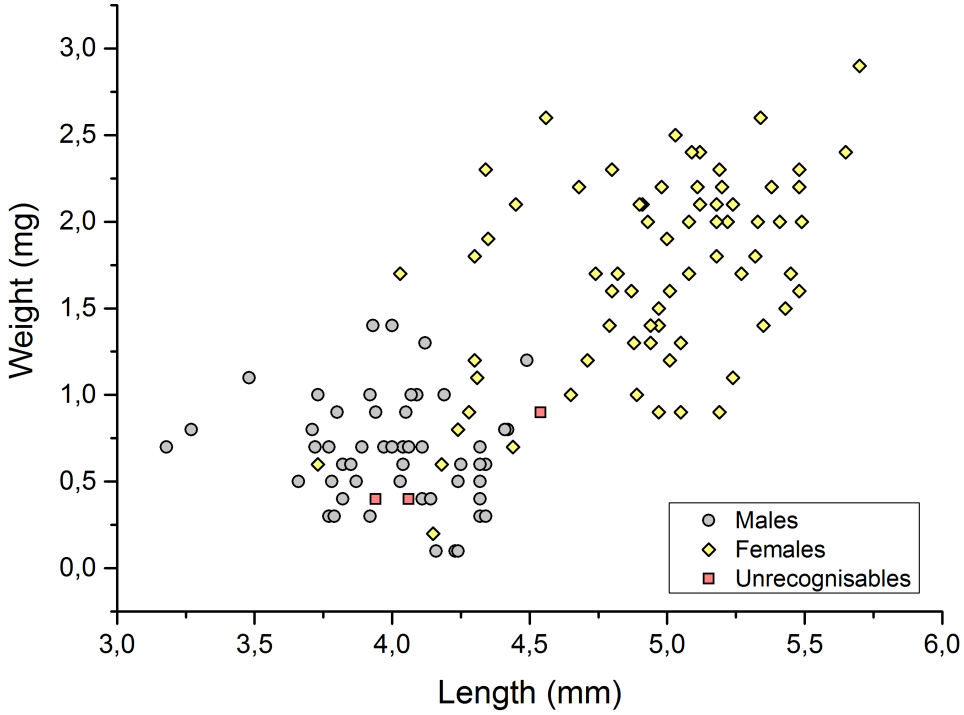
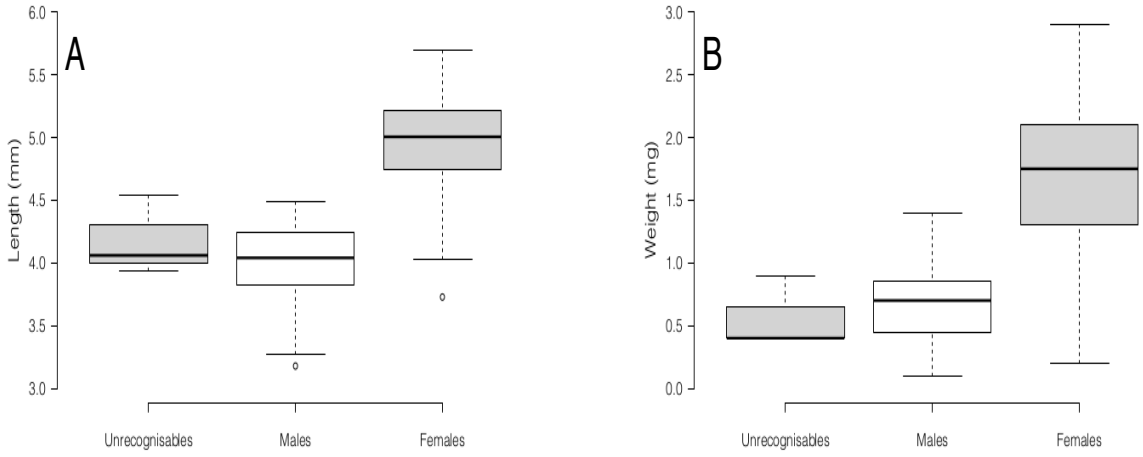


Figure 4. A: Box plots of distribution of length (A) and weight (B) of specimen collected in this study.



Literature

Alma, A., 2002. Auchenorrhyncha as pests on grapevine. Denisia. 4(176): 531-538.

Alma, A., Arnò, C., Arzone, A. Vidano, C., 1987.- New biological reports on Auchenorrhyncha in vineyards pp. 509-516. (Vidano, C. and Arzone, A., Eds) Proceedings of 6th Auchenorrhyncha Meeting, Torino, Italy.

Biedermann, R. Niedringhaus, R., 2004.- The planthoppers and leafhoppers of Germany - Identification key to all species.- WABV-Verlag, Scheeßel, Germany.

Boudon-Padieu, E. Maixner, M., 2007.- Potential effects of climate change on distribution and activity of insect vectors of grapevine pathogens pp. 1-8. Global warming, which potential impacts on the vineyards?, Dijon, France.

Breuer, M., Fahrentrapp, J. Michl, G., 2008.- Geographical distribution of “bois noir” and Hyalesthes obsoletus in Baden (SW Germany). 1st International Bois Noir Workshop 2008,

Christensen, N. M., Axelsen, K. B., Nicolaisen, M. Schulz, A., 2005. Phytoplasmas and their interactions with hosts. Trends in Plant Science. 10(11): 526-535.

Darimont, H. Maixner, M., 2001. Actual distribution of *Hyalesthes obsoletus* Signoret (Auchenorrhyncha: Cixiidae) in German viticulture and its significance as a vector of Bois noir. IOBC/WPRS Bulletin. 24: 199-202.

Fahrentrapp, J., Michel, G. Breuer, M., 2013. Quantitative PCR assay for detection of Bois noir phytoplasmas in grape and insect tissue. Vitis. 52(2).

Garau, R., Sechi, A., Prota, V. A. Moro, G., 2007. Productive parameters in Chardonnay and Vermentino grapevines infected with``bois noir"and recovered in Sardinia. Bulletin of Insectology. 60(2): 233.

Harrison, N. A., Helmick, E. E. Elliott, M. L., 2008. Lethal yellowing-type diseases of palms associated with phytoplasmas newly identified in Florida, USA. Annals of Applied Biology. 153(1): 85-94.

Hodgetts, J., Ball, T., Boonham, N., Mumford, R. Dickinson, M., 2007. Use of terminal restriction fragment length polymorphism (T-RFLP) for identification of phytoplasmas in plants. Plant Pathology. 56: 357-365.

Hogenhout, S. A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H. N. Namba, S., 2008. Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology. 9(4): 403-423.

Honěk, A., 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos. 483-492.

Hoshi, A., Oshima, K., Kakizawa, S., Ishii, Y., Ozeki, J., Hashimoto, M. Namba, S., 2009. A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proceedings of the National Academy of Sciences. 106(15): 6416-6421.

Kaul, C., Seitz, A., Maixner, M. Johannesen, J., 2009. Infection of Bois-Noir tuf-type-I stolbur phytoplasma in *Hyalesthes obsoletus* (Hemiptera: Cixiidae) larvae and influence on larval size. Journal of Applied Entomology - Zeitschrift Fur Angewandte Entomologie. 133(8): 596-601.

Langer, M. Maixner, M., 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RELP-analysis of non-ribosomal DNA. Vitis. 43(4): 191-199.

Lessio, F. Alma, A., 2004. Seasonal and daily movement of Scaphoideus titanus ball (Homoptera : Cicadellidae). Environmental Entomology. 33(6): 1689-1694.

292 Lessio, F., Tedeschi, R. Alma, A., 2007. Population dynamics, host plants and
293 infection rate with stolbur phytoplasma of *Hyalesthes obsoletus* Signoret in north-
294 western Italy. Journal of Plant Pathology. 89(1): 97-102.
295 Livak, K. J. Schmittgen, T. D., 2001. Analysis of relative gene expression data using
296 Real-Time Quantitative PCR and the 2^{-ddcT} method. Methods. 25: 402–408.
297 Maixner, M., 2006.- Grapevine yellows – current developments and unsolved
298 questions pp. 86-88. 15th Meeting of ICVG, Stellenbosch, South Africa.
299 Maixner, M., 2005.- Risks posed by the spread and dissemination of grapevine
300 pathogens and their vectors pp. 141-146. Introduction and Spread of Invasive Species,
301 Symposium Proceedings,
302 Maixner, M., 1994. Transmission of German grapevine yellows (Vergilbungskrankheit)
303 by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). Vitis. 33(2):
304 103-104.
305 Maixner, M., Ahrens, U. Seemüller, E., 1995. Detection of the German grapevine
306 yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a
307 specific PCR procedure. European Journal of Plant Pathology. 101: 241-250.
308 Maixner, M., Johannesen, J., Michel, K., Lux, B. Seitz, A., 2007. Host plant specificity
309 of *Hyalesthes obsoletus* and consequences for "bois noir" epidemiology. Bulletin of
310 Insectology. 60(2): 399-400.
311 Panassiti, B., Breuer, M., Marquardt, S. Biedermann, R., 2013. Influence of
312 environment and climate on occurrence of the cixiid planthopper *Hyalesthes obsoletus*,
313 the vector of the grapevine disease 'bois noir'. Bulletin of Entomological Research.
314 103: 621–633.
315 Panassiti, B., Hartig, F., Breuer, M. Biedermann, R., 2015. Bayesian inference of
316 environmental and biotic factors determining the occurrence of the grapevine disease
317 'bois noir'. Ecosphere. in press.
318 Sergel, R., 1986. Ein weiterer Nachweis der Cixiide *Hyalesthes obsoletus* Signoret in
319 Franken (Homoptera: Auchenorrhyncha: Fulgoroidea). Abhandlugen des
320 Naturwissenschaftlichen Vereins Würzburg. 25: 81-82.
321 Sforza, R., Clair, D., Daire, X., Larrue, J. Boudon-Padieu, E., 1998. The role of
322 *Hyalesthes obsoletus* (Hemiptera: Cixiidae) on the occurrence of bois noir of
323 grapevines in France. Journal of Phytopathology. 146: 549-556.
324 Tedeschi, R., Visentin, C., Alma, A. Bosco, D., 2003. Epidemiology of apple
325 proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive
326 psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera:
327 Psyllidae). Annals of Applied Biology. 142(3): 285–290.
328 Weintraub, P. G. Beanland, L., 2006. Insect vectors of phytoplasmas. Annual Review
329 of Entomology. 51: 91-111.
330

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