POPULATION DYNAMICS, HOST PLANTS AND INFECTION RATE WITH STOLBUR PHYTOPLASMA OF *HYALESTHES OBSOLETUS* SIGNORET IN NORTH-WESTERN ITALY

F. Lessio, R. Tedeschi and A. Alma

Di.Va.P.R.A., Entomologia e Zoologia applicate all'Ambiente "C. Vidano", Università di Torino,
Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

SUMMARY

The planthopper *Hyalesthes obsoletus*, is the vector of grapevine bois noir disease, caused by stolbur phytoplasma. This hopper is widespread in Europe and the Middle East but it is not a grapevine specialist, and its host range is still controversial. This paper reports on the population dynamics, host range and infectivity of *H. obsoletus* in northwestern Italy. Field sampling of adults was done with yellow sticky traps and sweep nets on grapevines and in nettle beds, whereas nymphs were sought on nettle and bindweed roots. PCR and dot-blot analyses were used to detect stolbur phytoplasma. The life span of the planthopper was tested in the laboratory on broadbean, periwinkle, nettle and grapevine. Nymphs were found only on roots of *Urtica dioica* L., and many more adults were captured in nettle beds than on grapevine. Flight activity started in the middle of June and ended middle of September, with a flight peak at the end of July. In the laboratory, the longest life span was observed on nettle whereas broadbean and grapevine were not suitable for survival. Natural infectivity was 6-8% for nymphs and 9-50% for adults. These results confirm the strong links between *H. obsoletus*, Bois Noir, and *U. dioica* in northwestern Italy.

Key words: grapevine, *Urtica dioica*, planthopper, phytoplasma, bois noir, *Hyalesthes obsoletus*.

INTRODUCTION

Grapevine yellows associated with phytoplasmas is one of the main problems of viticulture worldwide. Currently, three types of yellows are present in Europe, and are caused by phytoplasmas belonging to two ribosomal groups: flavescence doreé (FD, subgroups -C and -D) and Palatinate grapevine yellows (PGY, subgroup -A) belonging to the Elm Yellow group, 16SrV; and Bois noir (BN) belonging to the stolbur group (16SrXII-A). The first two are less frequent, being limited to France, Spain, Switzerland, Italy, Serbia (FD) and Germany (PGY) (Boudon-Padieu, 2003). On the other hand, bois noir is widespread in Europe including Germany (Maixner *et al.*, 1995), Spain (Laviña *et al.*, 2006), France (Sforza *et al.*, 1998), Hungary (Palermo *et al.*, 2004), and former Yugoslavia (Petrovic *et al.*, 2003; Šeruga *et al.*, 2003) recently, it has also been reported in Ukraine (Milkus *et al.*, 2005). BN is present in Israel and Lebanon (Boudon-Padieu, 2003). In Italy, BN is currently reported in all regions including Sicily and Sardinia; although it is considered less epidemic than FD, it seems to be increasing in many regions.

At present, the planthopper *Hyalesthes obsoletus* Signoret (Homoptera Cixiidae) is considered the only vector of BN. It is widespread in Central and Southern Europe, in the Near East and in the Mediterranean area. Its importance as a pest for grapevine has been reported in Germany (Weber and Maixner, 1998), France (Sforza *et al.*, 1998), Slovenia (Petrovic *et al.*, 2003), Israel (Sharon *et al.*, 2005), and Hungary (Palermo *et al.*, 2004).

However, *H. obsoletus* is not a grapevine specialist: eggs are laid and nymphs live on the roots of herbaceous hosts, mainly weeds, and adults occasionally feed on grapevine leaves (Alma *et al.*, 1988; Sforza *et al.*, 1999). Its host range is quite controversial: in France adults live on a wide range of species, including *Convolvulus arvensis* L., *Calystegia sepium* L., *Lavandula angustifolia* Miller, and *Cardaria draba* L., and occasionally *Plantago cynops* L, *Linaria striata* L., *Galium verum* L., and *Satureia montana* L.; however, the life cycle can be completed only on *L. angustifolia, C. draba*, and *C. arvensis* (Sforza *et al.*, 1999). In Germany, reports of *H. obsoletus* are more frequent on *C. arvensis*, *Urtica dioica* L., *Ranunculus bulbosus* L., and *C. sepium* (Langer *et al.*, 2003). In Israel, *Vitex agnus-castus* L. has been recognized as a new occasional host for adults (Sharon *et al.*, 2005). In Italy, adults were found on *U. dioica, Tanacetum vulgare* L., *Artemisia vulgaris* L. and *C. arvensis* (Alma *et al.*, 1988); however, the life cycle can be completed only on *U. dioica* (Alma *et al.*, 2002). Population dynamics of *H. obsoletus* can also be different depending on geographical location. In Europe it is known to accomplish a single generation per year: adults occur at

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the beginning of the summer, and eggs are laid at the base of host plants; incubation lasts 7 weeks, and is performed by third instar nymphs (Sforza *et al.*, 1999). On the other hand, in the Middle East two generations of *H. obsoletus* occur, adults appearing in spring and autumn (Sharon *et al.*, 2005). Given the great variability reported in different countries, more information is needed on the biology of this species. This research focuses on the population dynamics, host plant preference and natural infectivity of *H. obsoletus* in northwestern Italy.

MATERIALS AND METHODS

Field sampling. Insects were sampled during 2002-2005 in five different vine growing areas of Piedmont and Aosta Valley (NW Italy). The flight activity of *H. obsoletus* was studied in four vineyards using yellow sticky traps (Glutor®, 20 × 25 cm): three traps per vineyard were placed along a diagonal and three others were placed inside nettle beds nearby. On grapevines, traps were placed at 150 cm above the ground, to catch the planthoppers moving in the canopy. No differences in the density of nettle beds nor in soil and grapevine management were observed during different years. Traps were changed every ten days from the middle of June to the end of August, depending on the life cycle of the species. In the laboratory, captured *H. obsoletus* specimens were counted and divided by sex, using a stereomicroscope. Adults were further sampled using a sweep net, both on the grapevine canopy and in nettle beds, to collect living individuals for molecular analysis. The presence of *H. obsoletus* nymphs was checked by visual inspection on roots of *U. dioica* and *C. arvensis*. In each location, four plants of each species were sampled three times a year, at the beginning, middle and end of June thus a total of 240 plants per species were inspected. The insects caught were tested in the laboratory for the presence of Stolbur phytoplasma.

Laboratory rearing. In the laboratory, rearing of *H. obsoletus* adults was set up to investigate the life span on different host plants, and ability to transmit the BN phytoplasma. Adults collected in the field were placed in glass cages containing one of the following potted host plant seedlings: nettle (*U. dioica*), periwinkle (*Catharanthus roseus* L.), broadbean (*Vicia faba* L.) and grapevine (*Vitis vinifera* L.). Every cage held 5-10 planthoppers. Cages were maintained in a climatic chamber, with constant T = 25°C, RH = 70% and L:D = 16:8. Cages were inspected daily until no more insects were found alive; dead specimens where then put into labelled Eppendorf tubes and frozen, pending molecular analysis. Every five days, living insects were moved to a new plant of the same species. After removal of insects, plants were kept in a greenhouse (T = 25 ± 2°C, RH =

60%), and roots were inspected during the following year to detect the presence of nymphs.

Phytoplasma detection. To check the natural infectivity of *H. obsoletus* in the areas under investigation, total DNA was extracted from individual adults and from batches of 5 nymphs each, following a protocol specifically adapted to leafhopper phytoplasma vectors (Marzachì *et al.*, 1998).

The samples were then processed with direct PCR using M1/P8 specific primers (Marzachì *et al.*, 2000) and amplification products analysed by electrophoresis in 1% gel. In doubtful or negative cases, PCR amplicons were further analysed in Dot-Blots with the ribo-probe PTS1 224 (Marzachì *et al.*, 2000). Reaction and cycling conditions were as in the original papers.

Statistical analysis. Data were analysed using Sigma-Stat®2.0 software. Differences between captures in the vineyard and in nettle beds were analysed by a one-way ANOVA, summing captures on the same trap along different years and using each trap as a replication; data were square root transformed before analysis. Mean, standard error and confidence limits of life duration of *H. obsoletus* adults on different hosts were calculated. Differences of life span between different hosts was determined calculating the cumulative distribution frequencies of insects found alive per day and performing a chi square test. Molecular data were analysed by the Z test (Jandel Scientific Software, 1995) to compare proportions of BN-positive individuals in different years and also between males and females. The proportion of infected nymphs was estimated by maximum-likelihood estimator $\hat{p}$, calculated according to Swallow (1985): $\hat{p} = 1-H^{1/k}$, where H is the observed fraction of healthy groups and k is the number of insects per group, five in this case.

RESULTS

Hosts and flight activity. Nymphs of *H. obsoletus* were found only on nettle roots, and never on bindweed. Adults were more abundant in nettle beds, where 62.6 ± 20.9 planthoppers per trap were captured against the 6.3 ± 2.3 caught on grapevine (F = 15.31, P = 0.017). Flight activity began in mid June and stopped in mid September; more males than females were captured (Fig. 1).

Lifespan. *H. obsoletus* adults had different life spans when reared on different host plants. The hoppers lived for up to 3 weeks on *U. dioica*, although the mean was 5.5 days; however, they survived for much shorter times on other host plants: 3 days on periwinkle and 1 day on grapevine; confidence limits showed that lifespan dura-
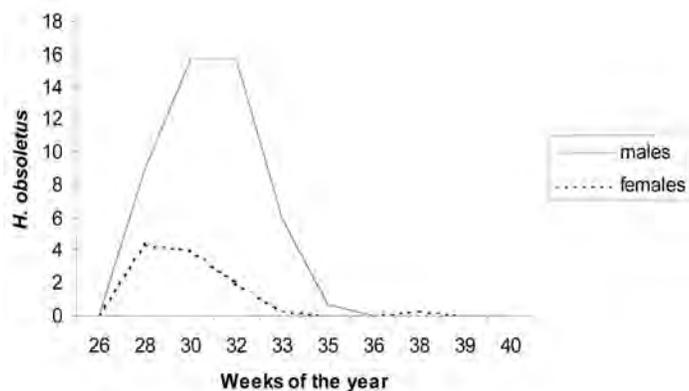
Fig. 1. Seasonal flight activity of *Hyalesthes obsoletus* Signoret in northwestern Italy, detected by means of yellow sticky traps placed in nettle beds (mean of individuals caught per trap, year and location).
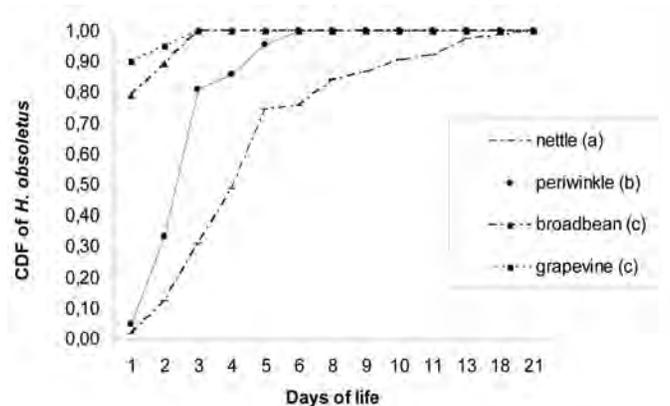

Fig. 2. Cumulative distribution frequencies (CDF) of the life span of *Hyalesthes obsoletus* Signoret on different host plants in the laboratory. Different letters indicate significant differences in life duration (chi square test, P<0.05).

tion in laboratory conditions varied between 4 and 6 days on nettle, 2 and 4 on periwinkle, 1 and 3 on broadbean and only 1 day on grapevine (Table 1). The cumulative frequency distribution showed that the probability of living less than five days was 0.50 on nettle, 0.80 on periwinkle and 1.00 on broadbean and grapevine (Fig. 2); significant differences were detected between nettle and periwinkle (chi-square 32.70, P=0.001), nettle and broadbean (chi-square 137.68, P<0.001), nettle and grapevine (chi-square 149.57, P<0.001), periwinkle and broadbean (chi-square 82.57, P<0.001); periwinkle and grapevine (chi-square 95.49, P<0.001); on the other

hand, no differences were detected between broadbean and grapevine (chi-square 0.80, P=1.00).

Phytoplasma detection. In all years, *H. obsoletus* specimens positive to "*Ca.* Phytoplasma solani" were recorded in direct PCRs or dot-blots (Table 2). Natural infection was similar in 2002 and 2003 in *H. obsoletus* collected in Piedmont, whereas it was much lower in 2004; an intermediate rate of infection was recorded in 2005, but only 6 specimens were tested. The infection rate of the specimens collected in the Aosta Valley was very high in 2003 reaching almost 82%, strongly de-

Table 1. Lifespan of adults of *Hyalesthes obsoletus* Signoret on different host plants under laboratory conditions. M: sample mean; SD: sample standard deviation; D: 95% confidence interval; M-X: lower limit of population mean; M+X: upper limit of population mean.

Host plant	N. insects	M + SD	Range	D	M-X	M+X
Nettle	74	5.5 + 3.7	1-21	0.85	4.69	6.39
Periwinkle	21	3.0 + 1.2	1-6	0.50	2.50	3.50
Broadbean	19	1.3 + 0.7	1-3	0.30	1.00	1.60
Grapevine	20	1.2 + 0.5	1-3	0.31	0.94	1.36

Table 2. Results of PCR and Dot-Blot analyses on *Hyalesthes obsoletus* Signoret adults collected in the Aosta Valley and in Piedmont to detect Stolbur phytoplasma. Different letters show significant differences between years (Z test, P<0.05).

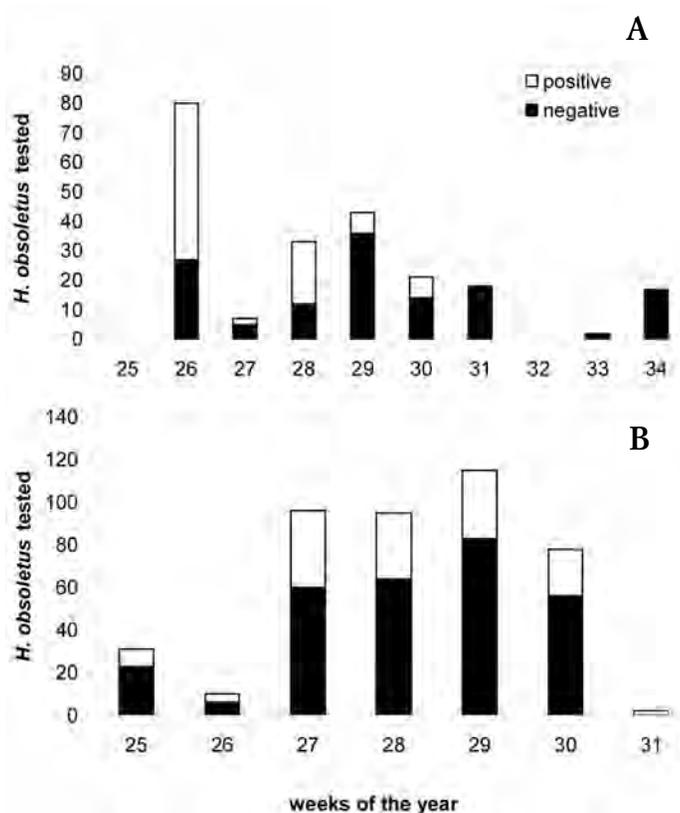| Year	Aosta Valley		Piedmont	
	N. positive/tested adults	%	N. positive/tested adults	%
2002	-	-	66/186	35.48 b
2003	63/77	81.82 c	93/239	38.91 b
2004	11/101	10.89 a	7/94	7.45 a
2005	16/43	37.20 b	1/6	16.67 ab

Fig. 3. Seasonal stolbur infection of *Hyalesthes obsoletus* Signoret adults in the years 2002-2005 in the Aosta Valley (**A**) and in Piedmont (**B**).

creased in 2004 and rose again in 2005 (Table 2). Bois noir-infected insects were randomly distributed between males and females ($Z = 0.217$; $P = 0.828$). The proportion of infected nymphs was calculated in the years 2002, 2003 and 2005 in Piedmont and was 8.00%, 5.96%, and 0%, respectively. In the Aosta Valley it was calculated only in 2005 and was 8.25%.

The seasonal infectivity of *H. obsoletus* adults in the years 2002-2005 is shown in Fig. 3. In the Aosta Valley, the highest infection rate was recorded at the beginning of the season during weeks 26 (end of June, 66.25%) and 28 (second week of July, 63.64%). A lower rate was seen in week 29, whereas all insects collected in August were negative. In Piedmont, the highest infection rate was found in weeks 26 (40.00%) and 27 (37.50%), while it ranged between 27.83% and 32.63% in the following weeks.

DISCUSSION

Our data confirmed that in Italy the main host plant of *H. obsoletus* nymphs is *U. dioica*. However, Milanesi *et al.* (2005) showed that in laboratory conditions it also developed on *C. arvensis, C. arvense, Plantago major* L.

and *Medicago sativa* L.; in Germany, the life cycle can also be completed on *R. bulbosus* and *C. arvensis* (Langer *et al.*, 2003). Due to this great variability between different regions, it is possible that soil type (e.g. consistency, water content etc.) may play a role in development of nymphs on different host plants: poor development of nymphs in heavy soil was already suggested (Orestein *et al.*, 2003), and organic mulch seems to improve nymphal development of *Myndus crudus* Van Duzee, a vector for coconut lethal yellowing (Howard and Oropeza, 1998). This could explain the higher survival and developmental rate on many more host plants in the laboratory than in the field.

Adults seem to be more adaptable, since they survived on many host plants besides nettle: this is in agreement with other findings, that adult *H. obsoletus* can feed on many weed and tree species (Alma *et al.*, 1988; Sforza *et al.*, 1999; Sharon *et al.*, 2005). However, many species are just an occasional host for *H. obsoletus*, and, in particular, this hopper does not last long on grapevine, although a short time is enough for transmitting BN (Bressan *et al.*, 2006). The importance of the presence of herbaceous host plants for the spread of *H. obsoletus* in vineyards is already clear (Maixner *et al.*, 2001; Bressan *et al.*, 2006) but more information is needed about its dispersal patterns, considering that correct management of ground cover in vineyards might well reduce the hopper populations, whereas chemical control of this polyphagous insect would be problematic.

In northern Italy *H. obsoletus* produced one generation per year, with the flight peak at the end of July, except in 2003 when high temperatures accelerated the population dynamics. This agrees with other data obtained in Europe (Sforza *et al.*, 1998), whereas in the Middle East this hopper can complete two generations per year. It was also shown that in the Middle East adults shift to grapevine mainly when weeds lose their leaves in dry weather (Sharon *et al.*, 2005); this supports the idea that grapevine is an occasional host. The male-biased sex ratio is not surprising, since in many homopteran species males have greater flight activity (Lessio and Alma, 2004).

We found that natural infection of *H. obsoletus* with Stolbur phytoplasma was variable over the four years of study. Apart from 2004, the infectivity of *H. obsoletus* was high compared with that of other phytoplasma vectors, a result also obtained by Weber and Maixner (1998). In fact more than one third of the specimens carried the Stolbur phytoplasma. The low infectivity observed during 2004 may have been caused by the high temperature and low rainfall of 2003, and the possible effect of this on its main wild host, the nettle.

The highest natural infection of *H. obsoletus* was recorded at the beginning of the season in both regions. This could be explained by the fact that phytoplasmas are acquired mainly by nymphs feeding on infected net-

tle roots, organs in which phytoplasmas are concentrated during winter. For this reason, further acquisition from nettle aerial parts by *H. obsoletus* adults are less efficient. Some of the Stolbur-positive specimens were also tested for strains other than the BN isolate (Pacifico *et al.*, 2005), but we found (data not shown) only the BN isolate known as VK-Type I, associated with nettle plants (Langer and Maixner, 2004), confirming the close association between the vector and nettle as its natural host in northwestern Italy (Alma *et al.*, 2002).

Although few specimens of *H. obsoletus* were captured on grapevine, it is likely that their high infectivity is enough to assure a constant rate of BN transmission throughout the season. However, other BN vectors might be present: many planthopper species have been found BN-positive, including *Reptalus panzeri* (Löw) (Palermo *et al.*, 2004), *Reptalus quinquecostatus* (Dufour) and *Hyalesthes luteipes* Fieber (Trivellone *et al.*, 2005), *Pentastiridius beieri* Wagner (Gatineau *et al.*, 2001), *Mocydia crocea* Van Duzee and *Euscelis lineolatus* (Kirschbaum) (Sforza *et al.*, 1998), *Euscelis obsoletus* (Kirschbaum) (Laviña *et al.*, 2006), and *Circulifer haematoceps* Mulsant *et* Rey (Orestein *et al.*, 2003). For some species, the transmission of Stolbur phytoplasmas to artificial nutrient media and, in the case of *E. obsoletus*, to *in vitro* grapevine plants was obtained (Laviña *et al.*, 2006), however to date there is no evidence that any of them can vector BN to grapevine in natural conditions.

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