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The Role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the Occurrence of Bois noir of Grapevines in France

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With 5 figures

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Abstract

An epidemiological study on a grapevine yellows disease called bois noir was carried out for 3 years in the Rhône valley (France). This yellows is caused by a stolbur type phytoplasma. Vectors and alternative host plants were searched, and the inoculation period was determined. Detection of stolbur phytoplasma in insects and plants was obtained using primers STOL11 f2/r1. In addition, a nested polymerase chain reaction (PCR) was used with primers P1/P7 and fU5/rU3 for detection in stolbur-infected plants with low titre. Several thousand insects were captured and species of Hemiptera were listed. Fourteen wild or reared Hemiptera species were used in transmission trials. Thirty-four wild species were monitored for phytoplasma DNA by PCR. A planthopper, *Hyalesthes obsoletus* Sign., tested positive for stolbur at a level of 28% (98/343) in 1995 and 38% (205/529) in 1996. In 1995, two leafhoppers, *Mocydia crocea* (1/78) and *Euscelis lineolatus* (2/309), were infected at a much lower ratio. Successful experimental transmission to grapevine, periwinkle and thorn apple was only obtained with *H. obsoletus*. Among wild plant species, hoary cress (*Cardaria draba* L.), bindweed (*Convolvulus arvensis* L.), sweet cherry (*Prunus* sp.), plum (*Prunus domestica* L.), lilac (*Syringa vulgaris* L.), fig tree (*Ficus carica* L.) and elm (*Ulmus* sp.) were shown to be stolbur infected and hoary cress was identified as a new host plant for *H. obsoletus* in France. The role of some fallow lands close to vineyards as sites where the inoculum of stolbur phytoplasma was maintained in insect vector and reservoir plants, is discussed. The natural inoculation period to grapevine was shown to extend from June to August, corresponding to the adult activity of *H. obsoletus*. Together with the close relationships of the phytoplasmas involved in vergilbungskrankheit, a German grapevine disease, and bois noir, The results of this study suggest that the two yellows are very close.

Zusammenfassung

Bedeutung von *Hyalesthes obsoletus* (Hemiptera: *Cixiidae*) für das Auftreten der Bois-noir-Krankheit der Weinrebe in Frankreich

Im französischen Rhônetal wurde eine 3-jährige epidemiologische Studie über die Bois-noir-Vergilbungskrankheit der Weinrebe durchgeführt. Diese Krankheit wird von einem Phytoplasma des Stolbur-Typs ausgelöst. Es wurde nach Vektoren und alternativen Wirtspflanzen gesucht, und die Inokulationsperiode wurde bestimmt. Die Detektion von Stolbur-Phytoplasmen in Insekten und Pflanzen erfolgte mit Hilfe der Primer STOL 11f2/r1. Zudem wurde eine genestete PCR mit den Primern P1/P7 und fU5/rU3 zur Detektion in stolburinfizierten Pflanzen mit niedrigem Titer durchgeführt. Mehrere tausend Insekten wurden gefangen, und die Hemiptera-Arten wurden aufgelistet. 14 wilde oder gezüchtete Hemiptera-Arten wurden in Übertragungsversuchen verwendet, und 34 Wildarten wurden mittels PCR auf Phytoplasma-DNA untersucht. Der Fulgoride *Hyalesthes obsoletus* Sign. war 1995 mit 28% (98/343) und 1996 mit 38% (205/529) der getesteten Tiere stolburpositiv. 1995 waren die beiden Zwergzikaden *Mocydia crocea* (1/78) und *Euscelis lineolatus* (2/309) ebenfalls infiziert, jedoch in viel geringerem Ausmaß. Eine experimentelle Übertragung auf Weinrebe, Immergrün und Stechapfel war nur mit *H. obsoletus* erfolgreich. Unter den Wildpflanzenarten waren Pfeilkresse (*Cardaria draba* L.), Ackerwinde (*Convolvulus arvensis* L.), Süßkirsche (*Prunus* sp.), Pflaume (*Prunus domestica* L.), Flieder (*Syringa vulgaris* L.), Feigenbaum (*Ficus carica* L.) und Ulme (*Ulmus* sp.) stolburinfiziert. Die Pfeilkresse erwies sich in unseren Untersuchungen erstmals als Wirtspflanze von *H. obsoletus* in Frankreich. Die Bedeutung neben Weinanlagen liegender Brachflächen als Reservoir des Stolburphytoplasmas in Insektenvektoren und befallenen Pflanzen wird diskutiert. Die natürliche Inokulationsperiode der Weinrebe reicht von Juni bis August, was dem Aktivitätszeitraum der Imagines von *H. obsoletus* entspricht. Ebenso wie die

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engen Beziehungen zwischen den an der in Deutschland beobachteten Vergilbungskrankheit der Weinrebe beteiligten Phytoplasmen und den Erregern der Bois-noir-Krankheit legen unsere Ergebnisse nahe, daß die beiden Krankheiten eng miteinander verwandt sind.

Introduction

Grapevine yellows (GY) are severe diseases of grapevine (*Vitis vinifera* L.), and are associated with phytoplasmas which are specifically transmitted by phloem feeding insects. Leafhopper and planthopper species are main phytoplasma vectors, but other Hemiptera vectors, such as psyllids, have been reported (D'Arcy and Nault, 1982). In France and Germany, two GY have been known and studied for a long time, bois noir (BN) (Caudwell, 1961; Caudwell et al., 1972) and vergilbungskrankheit (VK) (Gärtel, 1965). Recently, the phytoplasmas associated to one and the other GY have been characterized and both shown to belong to the stolbur group (Daire et al., 1993; Maixner et al., 1995). Stolbur type phytoplasmas (SP) have also been detected in other GY occurring in several countries of Western Europe and Israel (Daire et al., 1993; Bertaccini et al., 1995; Laviña et al., 1995; Tanne et al., 1995). Stolbur was the name of an epidemic disease on solanaceous crop which was described in Eastern Europe in the 1940s and progressed to other countries (Suchov and Vovk, 1946; Valenta et al., 1961; Marchoux et al., 1970b). The SP is very ubiquitous and it can be detected in a wide range of plants (Garnier et al., 1990; Fos et al., 1992; Maixner et al., 1995).

SP seem to be very homogenous, although a few recent reports have shown a genomic variability in tomato isolates (Minucci and Boccardo, 1997; Vibio et al., 1997). However, a monoclonal antibody was reported (Garnier et al., 1990) which reacts with a number of reference strains and wild isolates (Fos et al., 1992). In addition, Daire et al. (1997a) could not demonstrate a genomic variability among stolbur phytoplasmas, including grapevine isolates from different countries. The SP associated to BN and VK and other related GY have not been differentiated up to now, although they occur under a range of different environmental conditions and in different cultivars (Daire et al., 1993; Bertaccini et al., 1995; Laviña et al., 1995; Maixner et al., 1995; Daire et al., 1997b).

In France, BN occurs in all viticultural areas (Daire et al., 1997b). Until now, the vector of BN was unknown in France. In the case of VK, Maixner (1994) showed that the planthopper *Hyalesthes obsoletus* Signoret 1865 (Hemiptera: Cixiidae) was an efficient vector of SP in Germany. In the present study, investigations were carried out to identify one or more vector species of SP in grapevine in south-eastern France. Particular attention was devoted to *H. obsoletus* and its potential vector role in BN disease. Moreover, when the months in which transmission occurs in the fields was determined and the biology of putative vectors according to their relationships with their host plants and grapevine were investigated.

Materials and Methods

Trapping of adults and nymphs

Trapping of leafhoppers and planthoppers were monitored in three vineyard blocks and adjacent fallow lands in 1995 and 1996; F1, F2 and F3 were fallow lands on the border of V1, V2 and V3, respectively. In 1996, a fourth fallow land, F3', situated 100 metres from V3 and F3 blocks, was included. Adults were captured by yellow traps and D-vac. In 1995, three yellow sticky traps, 60 cm by 30 cm (Biosystèmes, Cergy-Pontoise, France), and three yellow water-bowls were placed in each block. In 1996, only three yellow sticky traps were used in each block. The yellow traps were deposited on the earth, in a star-shaped array at 10 metres from each other. They were replaced weekly from 1 April to 1 October in 1995 and 1996. Insects were captured by D-vac (Ventura, CA, USA) suction every 15 days in the same period. In addition, the trapping of adults of *H. obsoletus* was extended to other viticultural areas in 1995 and 1996, in the south-eastern region (Languedoc) and north-eastern region (Burgundy). Nymphs of *H. obsoletus* were hand-picked from roots of different host plants in winter in 1995 and 1996.

Adults were identified (Ribaut, 1936; Ribaut, 1952; Della Giustina, 1989) and counted using a stereobinocular dissecting microscope (Nikon SMZ-U).

Insect rearing

Rearing of insects was carried out under controlled conditions (23 °C, photoperiod 16 h : 8 h, humidity 80%). Pathogen-free individuals of two leafhopper species, *Scaphoideus titanus* Ball, the vector of flavescence dorée, and *Euscelidius variegatus* Kbm, an experimental phytoplasma vector, were reared, respectively, on healthy grapevine and maize as previously described (Caudwell and Larrue, 1970). Rearing of the planthopper *H. obsoletus* was undertaken during this study. The adults were obtained in controlled conditions from eggs of wild females; a few adults were fed on stolbur-infected lavender (unpublished data).

Insect transmission

Transmission trials were performed with presumably naturally infected specimens of Hemiptera species, i.e. *Mocydia crocea*, *Euscelis lineolatus*, *Aphrodes* sp., *Psammotettix* sp., *Jassargus obtusivalvis*, *Fieberiella flori*, *Neoaliturus fenestratus*, *Asiraca clavicornis*, *Hyalesthes scotti* and *H. obsoletus*. Groups of 10–15 adults, captured by D-vac, were identified, fed on healthy plants until they died, then stored at −20 °C and analysed by polymerase chain reaction (PCR). The healthy plants used in transmission were cuttings of grapevine (cv Chardonnay), seedlings of periwinkle (*Catharanthus roseus* L.) and of thorn apple (*Datura stramonium* L.). Healthy cuttings of grapevine from hot-water treated material were provided by Etablissement National Technique pour l'Amelioration de la Viticulture (E.N.T.A.V.). Healthy laboratory-reared insects served as control. The duration of survival of wild *H. obsoletus* was checked on healthy bindweed (*Convolvulus arvensis* L.) and on grapevine under controlled conditions.

Diseased cuttings were collected from canes on BN-infected grapevines in the field. They were grown in the greenhouse and checked for SP by PCR. The ability of acquisition/transmission of SP was carried out with *S. titanus*. Reared healthy specimens were fed for 3 weeks on diseased cuttings, then transferred to healthy grapevine cuttings until they died. Reared specimens of *E. variegatus* were fed for 2 weeks on SP-infected periwinkle referred to as P3-I, then transferred to healthy periwinkle seedlings until they died. After exposure to insects, the plants were sprayed with Dichlorvos (Bayer, Puteaux, France) and transferred to a greenhouse. Plants with symptoms of yellows that were confirmed by PCR (see below) were considered to have been infected.

Plant material

In 1994, 1995 and 1996, groups of bait plants of different species known to be sensitive to SP were exposed for a 2-week period, beginning in April or May, and ending in October. In 1994, two sets of 10 groups of 20 plants (10 periwinkles and 10 tomato plants) were exposed in V1 and V2 (11 May–27 October). In 1995, three sets of 11 groups of 15 plants belonging to 14 species (two grapevines, thorn apple, periwinkle, lavender, clover, tomato, tobacco, courgette, celery, green pepper, thyme, petunia, sugar beet, broadbean), were exposed in V1, V2 and V3 (3 April–2 October), and in addition one group of 10 periwinkles were exposed in V2 (15 June–11 July). In 1996, three sets of nine groups of 15 plants (five grapevines, five thorn apples and five periwinkles) were exposed in V1, V2 and V3, and nine groups of six plants (three grapevines and three periwinkles) were exposed in F3′ (2 May–10 October).

Samples were also collected from plants showing yellows or decline symptoms, i.e. grapevines, bindweed and hoary cress (*Cardaria draba* L.) collected in V and F blocks, shrubs and trees collected in F blocks such as sweet cherry (*Prunus* sp.), plum (*Prunus domestica* L.), lilac (*Syringa vulgaris* L.), fig tree (*Ficus carica* L.) and elm (*Ulmus* sp.).

PCR detection of phytoplasmas

Procedures for plants

All the procedures for plant DNA extraction were carried out as previously described (Daire et al., 1992). PCR amplification was carried out with primers STOL11 f2/r1, specific for stolbur subgroup strains, according to Daire et al. (1997a). In addition, nested PCR was applied to plants with yellows symptoms which had tested negative with primers STOL11 f2/r1. Nested PCR amplification was performed with phytoplasma universal ribosomal primers, that is P1 (Deng and Hiruki, 1991) and P7 (Smart et al., 1996) for the first amplification and fU5/rU3 (Lorenz et al., 1995) as internal primers for the second amplification. The reaction mixture of 40 µl contained 50–100 ng of template DNA, 0.25 µM of each primer, 250 µM each dNTP, 2 units of *Taq* DNA polymerase (Appligene), *Taq* buffer (Appligene), and were overlaid with 40 µl of mineral oil. Amplification with P1/P7 was carried out for 30 cycles at 92°C for 45 s, 58°C for 45 s, and 72°C for 1 min 45 s. The PCR products were diluted 1/100 in water, and 1 µl was added to the same reaction mixture using primer fU5/rU3. Amplification with fU5/rU3 was carried out for 35 cycles at 92°C for 30 s, 57°C for 30 s, and 72°C for 45 s. The final PCR product was digested with *Tru*9I and submitted to RFLP analysis (Daire et al., 1997a). Agarose electrophoresis and polyacrylamide gel electrophoresis were carried out according to standard procedures.

Procedures for insects

The procedures for insect DNA extraction were as described by Maixner et al. (1995). Insect DNA extracts were obtained either from fresh insects or from insects dried, glued (stored 4 months on sticky traps) or stored 4 months in 70% ethanol. Wild insects were crushed by groups of two, five, 10, 15 or 20 of the same species. Specimens of *H. obsoletus* were analysed individually. Amplification was done with primers STOL11 f2/r1 for 40 cycles. Strain STOL C, isolated from tomato and maintained in periwinkle, kindly provided by M. T. Cousin, served as positive control and rearing of *H. obsoletus* provided controls.

Results

Survey for Hemiptera species

In 1995 and 1996 in the experimental blocks, 40 000 specimens of Hemiptera were captured, identified at genus or species level and listed in Table 1.

PCR results from wild insects

Out of the hundred species captured in the experimental blocks, one-third of taxa were tested by PCR (Table 1). But only specimens belonging to three species tested positive. The planthopper *H. obsoletus* was by far the species with the highest ratio of natural infection: 28.5% (98/343) of individuals in 1995 and 38.7% (205/529) in 1996. In addition, several specimens of *H. obsoletus* from different viticultural areas in France were also infected by SP, in Burgundy in 1995 (1/4) and in 1996 (8/58), and in Languedoc in 1996 (3/23).

Figure 1 shows the detection of SP by PCR on individuals from different species. Detection on *H. obsoletus* was possible independently of storage conditions (lane 1–4). SP could also be detected on nymphs of the fifth instar captured on roots of symptomatic bindweed (see below) (lane 5). Apart from *H. obsoletus*, only *M. crocea* (1/78; 1.3%) (lane 10) and *E. lineolatus* (2/309; 0.7%) (not shown) were found positive in 1995. All specimens tested in the other species were negative, including the two *Hyalesthes* species namely *H. scotti* and *Hyalesthes luteipes* (69 individuals of the two species were tested).

Trapping of *Hyalesthes obsoletus* and inoculation period to bait plants

Figure 2 shows the results of trapping of *H. obsoletus* in 1995 and 1996 by sticky traps and D-vac suction in vineyard blocks (V1, V2 and V3) and fallow land blocks (F1, F2, F3 and F3′). Altogether, the first adults were trapped in the middle of June (week 25 in 1995 and week 24 in

Table I
List of Hemiptera captured in experimental blocks in Rhône valley (France) in 1995 and 1996 by D-vac suction and yellow traps. Suborder classification follows Campbell et al. (1995). Bold figures give the ratio of the number of adults which tested positive for stolbur phytoplasma by PCR to the number of adults tested

Suborder (common name)	Family Subfamily	Genus and species	
NEOHEMIPTERA	FULGOROMORPHA		
(Planthoppers)	CIXIIDAE	*Cixius nervosus* (L. 1758)	*Hyalesthes scotti* Ferrari 1882
		Hyalesthes obsoletus Signoret 1865 (**303/872**)	*Oliarus* sp. Stäl 1862 (**0/2**)
		Hyalesthes luteipes Fieber 1876 (**0/69**)[a]	*Tachycixius pilosus* (Olivier 1791)
	DELPHACIDAE	*Asiraca clavicornis* (F. 1774) (**0/2**)	*Laodelphax striatellus* (Fallén 1826)
		Javesella pellucida (F. 1774)	Non-determined species
	DICTYOPHARIDAE	*Bursinia* sp. A. costa 1862	
		Dictyophara europea (L. 1758) (**0/5**)	
	ISSIDAE	*Caliscelis bonelli* (Latreille 1807) (**0/2**)	*Issus coleoptratus* (F. 1781) (**0/8**)
		Hysteropterum sp. Amyot & Serville 1843 (**0/1**)	
	TETTIGOMETRIDAE	*Tettigometra griseola* Fieber 1865	
	TROPIDUCHIDAE	*Trypetimorpha occidentalis* (Huang & Bourgoin 1993)	
EUHEMIPTERA	CICADOMORPHA		
(froghoppers)	CERCOPIDAE	*Aphrophora* sp.Germar 1821 (**0/1**)	*Cercopis vulnerata* Rossi 1807
		Cercopis intermedia (Kbm. 1868) (**0/3**)	*Philaenus spumarius* (L. 1758) (**0/5**)
		Cercopis sanguinolenta (Scopoli 1769)	
(treehoppers)	MEMBRACIDAE	*Centrotus cornutus* (L. 1758)	*Stictocephala bisonia* K. & Y. 1977
		Gargara genistae (F. 1775)	
(leafhoppers)	CICADELLIDAE		
	Typhlocybinae	*Arboridia parvula* (Boheman 1845)	*Eupteryx urticae* (F. 1803)
		Emelyanoviana mollicula (Boheman 1845)	*Hauptidia provincialis* (Ribaut 1931)
		Empoasca decipiens Paoli 1930	*Liguropia juniperi* (Lethierry 1876)
		Empoasca vitis (Göthe 1875) (**0/40**)	*Ribautiana cruciata* (Ribaut 1931)
		Erythria aureola ((Fallén 1806)	*Ribautiana tenerrima* (H-S 1834)
		Eupteryx atropunctata (Goeze 1778)	*Zyginidia scutellaris* (H. S. 1838)
		Eupteryx aurata (L. 1758)	*Zygina rhamni* Ferrari 1882
	Idiocerinae	*Balcanocerus pruni* (Ribaut, 1952)	*Viridicerus ustulatus* (M. & Rey 1855)
	Cicadellinae	*Cicadella viridis* (L. 1758)	
	Iassinae	*Iassus lanio* (L. 1761)	
	Macropsinae	*Macropsis fuscula* (Zetterstedt 1828) (**0/43**)	*Macropsis* sp. Lewis 1834
	Penthimiinae	*Penthimia nigra* (Goeze 1778) (**0/4**)	
	Dorycephalinae	*Eupelix cuspidata* (F. 1775) (**0/4**)	
	Agallinae	*Agallia consobrina* Curtis 1833	*Austroagallia sinuata* (M. & Rey 1855) (**0/17**)
		Anaceratagallia laevis (Ribaut 1935)	*Dryodurgades antonniae* (Melichar 1907)
		Anaceratagallia ribauti (Ossiannilsson 1838)	*Dryodurgades reticulatus* H-S (1834)
		Anaceratagallia. venosa (Fourcroy 1785) (**0/34**)	
	Aphrodinae	*Anoscopus limicola* (Edwards 1908)	*Aphrodes* sp. Curtis 1929 (**0/17**)[b]
		Anoscopus sp. Kbm. 1858	*Stroggylocephalus agrestis* (Fallén 1806)
		Aphrodes bicincta (Schrank 1776)	*Stroggylocephalus livens* (Zetterstedt 1840)
		Aphrodes makarovi Zachvatkin 1948	
	Evacanthinae	*Evacanthus interruptus* (L. 1758)	
	Megophthalminae	*Megophthalmus scabripennis* Edwards 1915 (**0/29**)	
	Deltocephalinae	*Adarrus multinotatus* (Boheman 1847) (**0/30**)	*Mocydia crocea* (H. S. 1836) (**1/78**)
		Adarrus taurus Ribaut (1952)	*Mocydiopsis* sp. Ribaut 1839
		Allygidius abbreviatus (Lethierry 1878) (**0/2**)	*Neoaliturus* fenestratus (H-S 1834) (**0/177**)
		Allygidius atomarius (F. 1794)	*Ophiola decumana* (Kontkanen 1868)
		Allygidius furcatus (Ferrari 1882)	*Phlepsius intricatus* (H-S 1838) (**0/10**)
		Araldus propinquus (Fieber 1869)	*Phlepsius spinulosus* Wagner 1963
		Arocephalus longiceps (Kbm. 1868) (**0/1**)	*Phlogotettix cyclops* (M. & Rey 1869)
		Arocephalus sagittarius Ribaut (1952)	*Placotettix taeniatifrons* (Kbm. 1868)
		Arthaldeus striifrons (Kbm. 1868)	*Platymetopius filigranus* (Scott 1876)
		Artianus interstitialis (Germar 1821)	*Platymetopius major* (Kbm. 1868)
		Cechenotettix quadrinotatus (M. & Rey 1855)	*Platymetopius rostratus* (H-S 1834)
		Cicadula placida var. *inornata* (Horvath 1897) (**0/3**)	*Platymetopius undatus* (de Geer 1773)
		Circulifer opacipennis (Lethierry 1876)	*Psammotettix alienus* (Dalhbom 1850)
		Conosanus obsoletus (Kbm. 1858)	*Psammotettix putoni* (Then 1898)
		Eohardya fraudulentus (Horvath 1903)	*Psammotettix striatus* (Linné 1758)
		Euscelidius variegatus (Kbm. 1858) (**0/31**)	*Psammotettix* sp. Haupt 1929 (**0/528**)[c]
		Euscelis incisus (Kbm. 1858) (**0/8**)	*Recilia schmidtgeni* (Wagner 1939)
		Euscelis lineolatus Brullé 1832 (**2/309**)	*Rhoananus hypochlorus* (Fieber 1869)
		Fieberiella flori (Stal 1864) (**0/13**)	*Rhopalopyx elongatus* (Wagner 1952) (**0/2**)
		Goldeus harpago (Ribaut 1925)	*Rhytistylus proceps* (Kbm. 1868)
		Goniagnathus brevis (H-S. 1836) (**0/8**)	*Scaphoideus titanus* Ball 1932 (**0/30**)
		Graphocraerus ventralis (Fallén 1806)	*Selenocephalus obsoletus* (Germar 1817)
		Grypotes staurus Ivanoff 1885	*Speudotettix subfusculus* (Fallén 1806)
		Jassargus obtusivalvis (Kbm. 1868) (**0/278**)	*Synophropsis lauri* (Horvath 1897)
		Laburrus quadratus (Forel 1864) (**0/1**)	*Thamnotettix dilutior* (Kbm. 1868) (**0/11**)
		Macrosteles quadripunctulatus (Kbm. 1868)	*Thamnotettix zelleri* (Kbm. 1868)
		Macrosteles sexnotatus (Fallén 1806)	

[a]: Total ratio for *H. scotti* and *H. luteipes*; [b]: Total ratio for all *Aphrodes* species; [c]: Total ratio for all *Psammotettix* species.

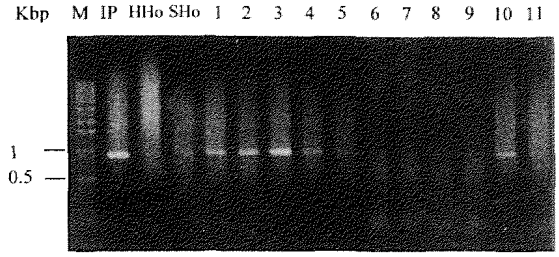

Fig. 1 Results of DNA amplification with primers STOL11 f2/r1 using template DNA prepared from wild specimens of *Hyalesthes obsoletus* captured in 1996 and leafhoppers either from laboratory rearings or collected in experimental blocks in Rhone valley in 1995. M, molecular weight marker (1kb ladder BRL); IP, periwinkle infected with strain STOL C; HHo, laboratory-reared healthy *H. obsoletus*; SHo, laboratory-reared stolbur-infected *H. obsoletus*; 1–4, stolbur-infected adults of *H. obsoletus* from fields stored in different conditions; 1, fresh; 2, glued and stored 4 months on sticky trap; 3, stored 1 month on the bench; 4, stored 4 months in 70% ethanol; 5, fresh nymph of fifth instar of *H. obsoletus* captured on root of symptomatic bindweed; 6–9; laboratory-reared leafhoppers used for experimental stolbur transmissions trials; 6, healthy *Scaphoideus titanus*; 7, *S. titanus* after a 3-week acquisition period on Chardonnay with bois noir symptoms and a 2-week inoculation period on healthy vine; 8, healthy *Euscelidius variegatus*; 9, *E. variegatus* after a 2-week acquisition period on P3-1 periwinkle (see Results) and 2-week inoculation period on healthy periwinkle; 10 and 11, wild leafhopper *Mocydia crocea* individuals

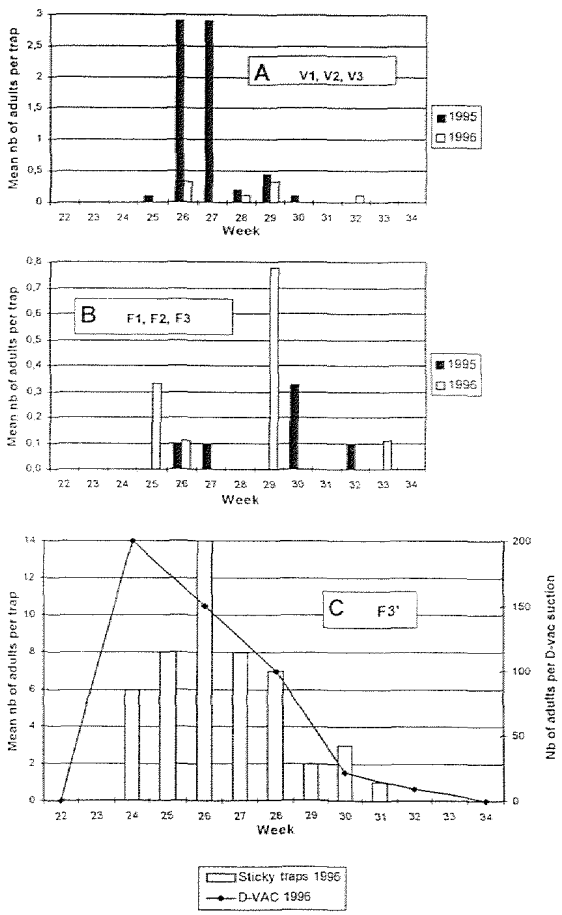

Fig. 2 Trapping of *Hyalesthes obsoletus* in 1995 and 1996 in vineyard blocks (V1, V2 and V3) and in fallow land blocks (F1, F2, F3 and F3′) in the Rhône valley, with two different methods of capture. **A**: total weekly capture by yellow sticky traps (nine traps per week) in V blocks in 1995 and 1996; **B**: total weekly capture by yellow sticky traps (nine traps per week) in F blocks in 1995 and 1996; **C**: comparative captures in 1996 in F3′ between D-vac suction (5 min trapping every 2 weeks) and sticky traps (three traps per week). No adults were captured from week 14 to week 24 (week 23 for C) and from week 33 (week 34 for B) to week 40

1996) and the last adults were captured in August (week 32 in 1995 and week 33 in 1996). Thus, adults were present for at least 7–9 weeks. The same results were obtained with yellow water-bowls (data not shown). A striking contrast in the regularity of captures and in the number of individuals appeared between F3′ (Fig. 2C) and the other blocks (Fig. 2A, 2B). In 1996, the captures were erratic in V and F blocks (Fig. 2A, 2B). In F3′, adults were captured weekly. Numerous nymphs of fourth and fifth instar could also be observed in F3′ in May and June on bindweed and hoary cress which were frequent on the block; during the same period much smaller numbers of specimens could be found also on these plants in V3.

The inoculation period was determined by bait plant experiment. All the bait plants exhibiting phytoplasma symptoms in 1994, 1995 and 1996, turned out to be stolbur positive in the PCR assays. Neither symptoms nor phytoplasmas were detected in the control plants. Examples of PCR results on bait plants are given in Fig. 3 (lane 8 and 10) and Fig. 4 (lane 9 and 10). In 1994, none of the 100 tomato plants became infected; out of the 100 periwinkles, two plants which had been exposed from 21 June to 7 July (weeks 25–27) were infected and referred to as P3-1 and P8-1. In 1995, out of 506 bait plants, one thorn apple exposed from 29 May to 12 June (weeks 22–24) and four periwinkles exposed from 15 June to 11 July (weeks 24–28) were infected. In 1996, out of the 453 bait plants, 17 were infected by a SP, i.e. four thorn apples, 10 periwinkles and three grapevines (Chardonnay and Pinot noir).

Figure 5 shows both the results in 1996 of stolbur infection in bait plants from week 24 to week 31, and the presence of *H. obsoletus* expressed as the total number of adults captured by sticky traps in the experimental V and F blocks during the same period. Percentages of stolbur-infected bait plants were 14% (7 infected/51 exposed) for week 24 and 25, 8% (4/51) for week 26 and 27, 6% (3/51) for week 28 and 29 and 6% (3/51) from week 30–31.

Transmission trials

Experimental transmission of SP to grapevine, periwinkle and thorn apple was obtained only with wild *H. obsoletus*. All transmission trials with all other wild Hemiptera species failed. The acquisition process of SP by reared leafhoppers, *S. titanus* (0/69) on grapevine with symptoms which had tested SP positive, or by *E. variegatus* (0/25) on infected periwinkle, did not result in infected insects (Fig. 1: lane 6–9).

Hyalesthes obsoletus transmitted SP in 1995 to grapevine (8/31) and to periwinkle (1/1), and in 1996 to grape-

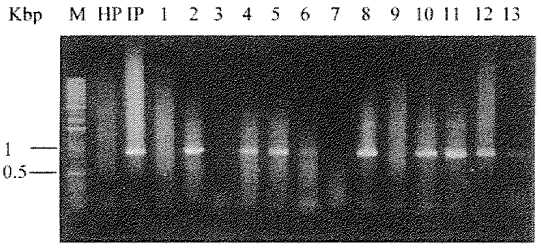

Fig. 3 Results of DNA amplification with primers STOL11 f2/r1 using template DNA from wild plants collected in experimental blocks in the Rh–ne valley, bait plant and plants used for experimental transmission with wild *Hyalesthes obsoletus*. M, molecular weight marker (1kb ladder BRL); HP, healthy periwinkle (*Catharanthus roseus*); IP, periwinkle infected with strain STOL C; 1, symptomless bindweed (*Convolvulus arvensis*) collected in fallow land; 2, bindweed with symptoms collected in fallow land with nymphs of *H. obsoletus* on roots; 3, healthy Chardonnay; 4–7: vines exhibiting phytoplasma symptoms; 4, Chardonnay; 5, Grenache; 6, Pinot noir; 7, Cinsaut (weak band); 8, bait plant (periwinkle) with symptoms of yellows exposed in a vineyard block from 26 June to 7 July in 1996; 9, healthy seedling of thorn apple (*Datura stramonium*); 10, bait plant (thorn apple) with symptoms exposed in a vineyard block from 10 June to 26 June in 1996; 11–13: plants exhibiting symptoms after experimental transmission of stolbur phytoplasma by newly wild-emerged *H. obsoletus*; 11, periwinkle; 12, thorn apple; 13, Chardonnay

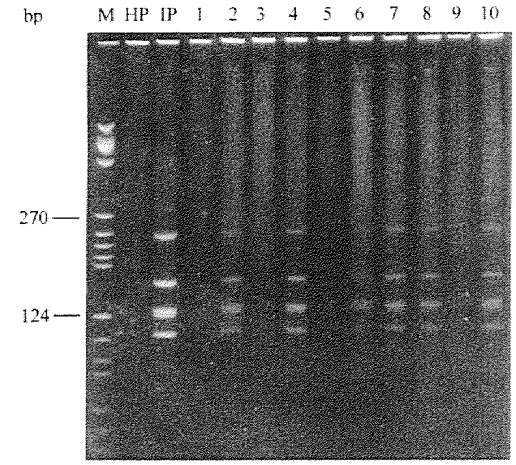

Fig. 4 *Tru*91 digestion of the nested PCR products with primers P1/P7 and fU5/rU3 using template DNA from wild plants collected in experimental blocks and from bait plants in Rhône valley in 1996. M, molecular weight marker (1kb ladder BRL); HP, healthy periwinkle (*Catharanthus roseus*); IP, periwinkle infected with strain STOL C; 1, symptomless hoary cress (*Cardaria draba*); 2, symptomatic hoary cress; 3–10: wild shrubs and trees collected in fallow land blocks; 3, symptomless cherry (*Prunus* sp.); 4, diseased sweet cherry (*Prunus* sp.); 5, symptomless fig tree (*Ficus carica*); 6, symptomatic fig tree; 7, symptomatic lilac (*Syringa vulgaris*); 8, symptomatic elm (*Ulmus* sp.); 9–10: bait plants exposed in vineyard blocks from 10 June to 26 June; 9, symptomless Chardonnay; 10, Chardonnay with symptoms of yellows

vine (17/61), to periwinkle (16/27) and to thorn apple (3/4). Successful transmissions were realized as well with the first adults emerging in June as with specimens captured in July and August (data not shown). The SP infection was confirmed by PCR and examples of assays are given in Fig. 3 (lane 11–13).

The survival of adults of wild *H. obsoletus* caged on grapevine or bindweed were compared. Starting with 53 adults on the grapevine, 80% of mortality was obtained in the first 3 days and none exceeded 7 days. However, survival on bindweed was much longer, starting with 80 adults, 75% (60) were still alive after 7 days, 25% (20) after 21 days, 12% (10) after 35 days, and the last one was observed at day 56.

PCR assays on plant material with symptoms from the fields

Figures 3 and 4 present some results of PCR assays on grapevine, bait plants, and naturally infected plants collected in the fields (Fig. 3, lane 1–12; Fig. 4, lane 1–8). In grapevine, positive assays with STOL 11 f2/r1 (Fig. 3, lane 4–7) were obtained from Chardonnay (20 infected/29 tested), Grenache (1/2), Pinot noir (1/2) and Cinsaut (3/3) collected in the experimental blocks and surrounding vineyards. This showed that BN was prevalent in the region, in agreement with other reports (Daire et al., 1997b).

SP was detected in bindweed with STOL 11 f2/r1 (31 infected/61 tested) (Fig. 3, lane 1–2). Nested PCR was sometimes necessary to detect SP in other plants, i.e. hoary cress (3/7), sweet cherry (1/6), plum (1/1), lilac (1/1), fig tree (1/3) and elm (1/2) as shown in Fig. 4 (lane 1–8). Symptoms on hoary cress were dwarfism, reddening of the leaves and an absence of flowers; a general decline and a yellowing of leaves were noticed on sweet cherry and elm, a decline and a shrivelling of leaves on plum and lilac, and decline on fig tree.

Discussion

The results of this study show for the first time the major role of *H. obsoletus* in BN disease occurrence in France. In this study, it was also found to be an efficient vector of SP to grapevine in spite of its poor survival when it was caged on this plant. Results for the percentage of infected *H. obsoletus* among the populations were in agreement with previous studies (Fos et al., 1992; Maixner et al., 1995). As BN and VK have closely related, if not similar, pathogenic agents and have the same insect vector, it appears that they are similar diseases occurring in different geographical areas on different cultivars.

Hyalesthes obsoletus was the major species found to be stolbur-infected. Although wild leafhoppers, namely *M. crocea* and *E. lineolatus*, were occasionally found to be carrying SP, no transmission was obtained with these species. In France, Fos et al. (1992) detected SP in three other leafhopper genera, namely *Aphrodes* sp., *Neoaliturus* sp., and *Psammotettix* sp. The latter species was also found infected by a phytoplasma in Italy (Albanese et al., 1997). In the present study high numbers of adults among these genera were tested without detecting SP. This might suggest an occasional acquisition by these genera. Their ability to transmit should be demonstrated. In addition, the inability of feeding access acquisition by reared *S. titanus* and *E. variegatus* strongly suggested that SP transmission is specific.

Detection in plants from the field showed that bindweed and hoary cress, but also fig tree, lilac, elm, and

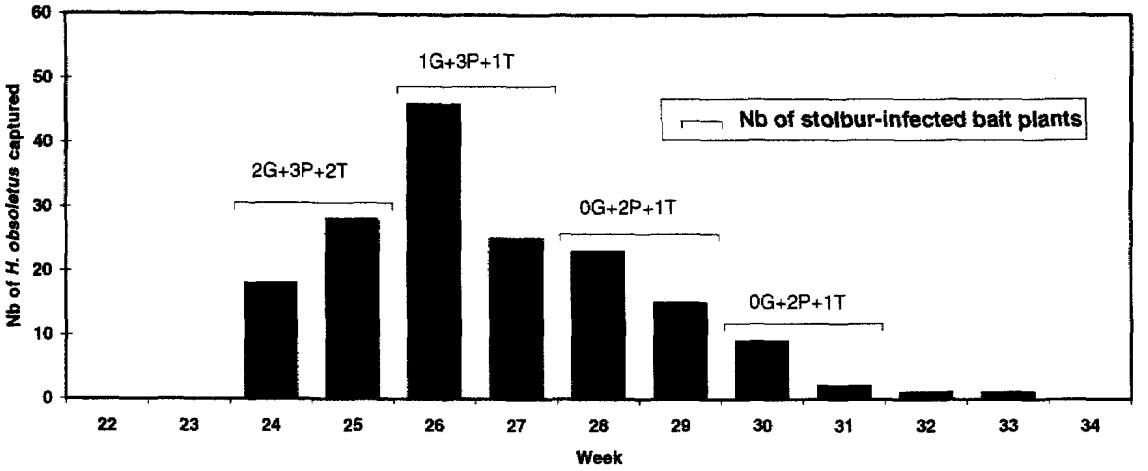

Fig. 5 Comparison between the number of stolbur-infected bait plants and the number of *Hyalesthes obsoletus* captured in the experimental blocks in Rhone valley in 1996. The bars give the total number of adults captured during 1 week by 21 sticky traps in all the blocks. The stolbur-infected bait plants were obtained during a 2-week period among the 51 bait plants exposed in all the blocks. All plants with symptoms were PCR tested and turned out to be stolbur positive. No adults were captured from week 14 to week 23 and from week 34 to week 40; no stolbur-infected bait plants were obtained in the same period. G, grapevine; P, periwinkle; T, thorn apple

sweet cherry could be infected by SP. The presence of SP in bindweed was suspected by Cousin et al. (1969) and confirmed by Fos et al. (1992) and Maixner et al. (1995). In the present study, symptomatic bindweed were often found in vineyards and fallow lands, and SP was detected in a high proportion of samples, using direct PCR with STOL11 f2/r1. The results on hoary cress are new interesting data, as this plant was known to be a host of *H. obsoletus* in Eastern Europe (Guclu and Ozbek, 1988). Thus, hoary cress might be a reservoir for the SP inoculum. As detection on this species was obtained only by nested PCR, it might be a low titre host for the SP, which could explain why it was not reported in previous studies using other less sensitive detection methods. The results of SP infection in lilac, elm, and fig tree were unexpected data. However, the DNA fragments amplified were clearly visible and negative results were obtained from a number of samples from the same species. The occurrence of SP in such plant species could result from occasional inoculation during feeding probing.

The study has shown the role of hoary cress and confirmed the role of bindweed as host-plants of *H. obsoletus* in France. Thus, together with lavender, three plant species are now known in France both as host plants and stolbur reservoirs (Leclant, 1968; Fos et al., 1992). Hoary cress and bindweed were numerous in fallow lands, such as F3'. As *H. obsoletus* was regularly captured in F3', the latter site should be considered as a source of inoculum for the disease. From such a site, adults could transport SP from these hosts and occasionally inoculate grapevine, shrubs and trees. The poor survival on grapevine supports the assumption that feeding on this plant is occasional. Effective transmission was nevertheless possible on grapevine cuttings as well in controlled conditions as in the open air. In order to control BN disease, special attention should be devoted to fallow lands presenting characteristics of stolbur inoculum seats near the vineyards.

It was shown that the inoculation period of bait plants occurred during the activity of adults. The threat to a grapevine would be constant during a 3-month period in the Rhône valley and in other viticultural areas where positive *H. obsoletus* were found. In a previous study on tomato stolbur, Garnier et al. (1990) used only periwinkle as bait plants. To ensure a qualitative approach of plant species susceptible to SP inoculation, a greater diversity of plants was used in the present study. It was shown that inoculation could occur from beginning of June to August.

This is the first report of the experimental inoculation of SP by *H. obsoletus* to thorn apple. The species had already been reported as naturally infected (Valenta et al., 1961) and dodder inoculated (Marchoux et al., 1970a). At the earliest period of inoculation (beginning of June), SP could be inoculated to grapevine, periwinkle and to thorn apple with wild *H. obsoletus* from the first captures. Thus, It can be assumed that an acquisition process takes place at nymphal stage on roots of infected host plants. This is supported by SP detection on the fifth instar found on diseased bindweed. No studies have been conducted up to now on the route and multiplication of phytoplasmas in planthoppers. On the basis of the first data obtained on experimental acquisition of SP by *H. obsoletus* in the laboratory, it can be assumed that SP have a similar cycle in the insect body as described in leafhopper vectors for other phytoplasmas which persist and multiply in the organs of the vector (Maillet and Gouranton, 1971; Lherminier et al., 1989; Lefol et al., 1994).

Further data on the ethology of *H. obsoletus* and SP transmission will be obtained with the use of exper-

imentally reared colonies of this species, in studies currently being undertaken.

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