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RESEARCH ARTICLE

Investigation on 'bois noir' epidemiology in north-eastern Italian vineyards through a multidisciplinary approach

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Abstract

A multidisciplinary approach, based on field surveys, molecular biology techniques, and spatial data analyses, was utilised to investigate the Bois noir (BN) epidemiology in north-eastern Italian vineyards during the years 2010–12. Symptomatic grapevines, weeds and specimens of the insect vector *Hyalesthes obsoletus* were monitored and mapped. Leaf samples from symptomatic grapevines and weeds, and captured insect specimens were analyzed by real-time PCR to identify BN phytoplasma (BNp; '*Candidatus* Phytoplasma solani' species), the etiological agent of BN. Data spatial distribution was analyzed using SADIE (Spatial Analysis by Distance IndicEs). Bois noir phytoplasma strains identified in weed candidates for an epidemiological role were characterised by RFLP-based analyses of *tuf* gene amplicons. Results highlighted that, in the examined areas, the host systems *Convolvulus arvensis* – *H. obsoletus* and *Urtica dioica* – *H. obsoletus* play the main role in BN diffusion. It was also evidenced that other weeds (i.e. *Chenopodium album* and *Malva sylvestris*) spatially associated with symptomatic grapevines and/or insect vectors and infected by the same *tuf* type identified in grapevines and insects, could play a role in BN diffusion. On the other hand, some weeds (i.e. *Trifolium repens*) were uninfected and not associated with symptomatic grapevines and/or insect vectors. The synergic application of our multidisciplinary approach improved the knowledge of BN epidemiology, and provided helpful indication for designing experimental plans to contain BN spreading in vineyards through weed management. The approach described in the present work could be used to investigate the complex epidemiology of other phytoplasma diseases.

Introduction

Bois noir (BN), a grapevine yellows (GY) disease caused by '*Candidatus* Phytoplasma solani' strains [taxonomic 16SrXII ('stolbur') group, subgroup 16SrXII-A] (Wei *et al.*, 2007; Quaglino *et al.*, 2013), is a major limiting factor for wine production in European countries (Laimer *et al.*, 2009). In almost all varieties of *Vitis vinifera* L., BN produces typical GY symptoms, including desiccation of inflorescences, berry shrivel, leaf discolorations, reduction of growth and irregular ripening of wood (Belli *et al.*, 2010). Bois noir phytoplasma (BNp) strains are transmitted by the planthopper *Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae), a polyphagous insect living preferentially on weeds (Maixner, 1994; Langer & Maixner, 2004; Berger *et al.*, 2009). Up to now, three natural ecologies of BNp have been described: (a) the host system *Convolvulus arvensis* – *H. obsoletus* – *V. vinifera*, related to BNp strains of type *tuf*-b (b) the host system *Urtica dioica* – *H. obsoletus* – *V. vinifera*, related to type *tuf*-a and (c) the host system *Calystegia sepium* – *H. obsoletus* – *V. vinifera*, related to type *tuf*-c (Langer & Maixner, 2004). In detail, *C. arvensis* and *U. dioica* have been reported as being the main host plants of *H. obsoletus* in Germany (Maixner *et al.*, 1995), northern Italy (Alma *et al.*, 2002; Lessio *et al.*, 2007; Mori *et al.*, 2008), Spain (Sabaté *et al.*, 2014) and Austria (Riedle-Bauer *et al.*, 2013). Alternative plant hosts of BNp

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include cultivated species as well as weeds found in or near vineyards (Marcone *et al.*, 1997; Škoric *et al.*, 1998; Batlle *et al.*, 2000; Lessio *et al.*, 2007; Borgo *et al.*, 2008). In several areas, a correlation was noticed between the incidence of BNp in grapevines and in other plant species. This suggests a direct role of the other host plants in the epidemiology of BN disease (Borgo *et al.*, 2008). *Cardaria draba* L., *Prunus* sp., *Prunus domestica* L., *Syringa vulgaris* L., *Ficus carica* L. and *Ulmus* sp. were shown to be susceptible to infection under experimental conditions; *C. draba* was identified as a favourable host plant for *H. obsoletus* (Sforza *et al.*, 1998). Furthermore, in vine-growing areas where *H. obsoletus* is absent, the presence of BNp implies the existence of alternative vectors. Recently, *Reptalus panzeri* has been reported as a natural vector of BNp in Serbian vineyards (Cvrković *et al.*, 2014). *Anaceratagallia ribauti* was also shown to carry the bindweed strain (type *tuf*-b) and to transmit it to some herbaceous hosts (Riedle-Bauer *et al.*, 2008). In addition, other studies reported that the Cixiidae *Reptalus quinquecostatus*, and the Cicadellidae *Adarrus taurus*, *Aphrodes bicinctus*, *Anaceratagallia laevis*, *Goniagnathus guttulinervis*, *Macrosteles sexnotatus*, *Neoaliturus fenestratus*, *Psammotettix striatus* and *Zyginidia scutellaris* have been captured within or near BN-diseased vineyards and found to contain BNp (Batlle *et al.*, 2000; Gatineau *et al.*, 2001; Garau *et al.*, 2004; Palermo *et al.*, 2004; Pinzauti *et al.*, 2008). Based on such information, it appears that, even if the role of these numerous hosts in BNp transmission has not been proven, it is probable that other host plants are involved in the epidemiology of BN disease, harbouring additional insect species capable of spreading the disease.

This complexity renders it difficult to design efficient BN control strategies. Insecticides applied to the grapevine canopy influence neither the disease nor the presence of *H. obsoletus* (Sforza & Boudon-Padieu, 1998; Maixner, 2007; Mori *et al.*, 2008). The management of *H. obsoletus* host plants in the vineyards and surrounding areas is therefore considered crucial for BN control. Indeed, in Europe, several studies showed that *H. obsoletus* host plants at the borders facilitate the spread of BNp (Maixner *et al.*, 2007; Riedle-Bauer, 2010; Mori *et al.*, 2012). Thus, preventive measures such as checking the sanitary status of propagation materials, and treating diseased mother plants through thermotherapy are applied to limit long distance dissemination and in-field spread of the disease (Belli *et al.*, 2010). Other strategies for reducing BN spread or incidence are based on (a) preventive removal of the grape suckers on which *H. obsoletus* could feed after grass mowing (Picciau *et al.*, 2010); (b) trunk cutting above the engagement point on the symptomatic grapevines (Credi *et al.*, 2011); and (c) treatments by resistance inducers (Romanazzi *et al.*, 2009).

Recently, several researches have focused on improving the knowledge of BN epidemiology in order to obtain information useful for developing efficient strategies of disease containment (Marchi *et al.*, 2011). Spatial Analysis by Distance IndicEs (SADIE) has been used to investigate the epidemiology of '*Ca*. Phytoplasma solani' strains infecting tomato, pepper and celery in the Czech Republic, highlighting that distributions of symptomatic crop plants and of *C. arvensis* and *Cirsium arvense* were associated, and suggesting the possible involvement of these weeds in the spread of stolbur (Navratil *et al.*, 2009). In the present study, we used a multidisciplinary approach based on field surveys, molecular biology techniques and spatial data analyses to investigate the role of weeds in BN epidemiology within vineyards in order to facilitate the development of novel field strategies for controlling the spread of BNp.

Materials and methods

The study was conducted in 2010–11 and was based on (a) monitoring and mapping symptomatic grapevines, weeds and *H. obsoletus* specimens for statistical analyses of data spatial distribution by means of SADIE (Perry *et al.*, 1999), (b) identification of BNp through real-time PCR analyses (Galetto *et al.*, 2005) performed on leaf samples collected from symptomatic grapevines and weeds and captured insect specimens. The monitoring and spatial distribution analysis of symptomatic grapevines was also done in 2012.

Characteristics of investigated vineyards

The investigation on BN epidemiology was conducted in two vineyards (1.20 ha/4995 vines – 1.32 ha/4976 vines, respectively) of the Chardonnay cultivar on SO4 rootstock in a grape-growing area of the Veneto Region (Northern Italy) (N 45,302104°; E 11,234721° Ronco all'Adige location; N 45.439782°; E 11.140831° San Pietro di Lavagno location). In both vineyards, rows were north-south oriented and grapevines were trained using the Guyot system (distance between rows 2.8 m in both vineyards; plant distance along the row 0.9 m in Ronco all'Adige and 1.0 m in San Pietro di Lavagno). Among the agricultural practices adopted in the vineyards, spring weeding on the row, mowing between rows and one insecticide treatment with the organophosphate Chlorpyriphos ethyl (applied at the end of June/beginning of July) against insect pests should be mentioned because they could have interfered with vineyard colonisation by *H. obsoletus*.

The Ronco all'Adige vineyard is composed of 29 rows, 150 m long. It is bordered by a cereal field to the north, other vineyards to the south, an orchard to the east

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and a *Platanus acerifolia* coppice to the west. The San Pietro di Lavagno vineyard is composed of 21 rows, 220 m long. It is bordered by buildings (houses and factories) to the north and east, other vineyards to the south and uncultivated meadows to the west.

Spatial distribution

A map was created of the vineyards and surrounding habitat, and the position of each grapevine plant was registered by a code comprising two numbers indicating the row and position on the row. In correspondence to each grapevine, the identified weed species were recorded to obtain a map of the flora present in the vineyards. In order to compare the spatial distribution of grapevines and weeds, the maps were referred to 200 block units in Ronco all'Adige (24 ± 2 plants per block; width 5.6 m, length 10.8 ± 0.9 m) and 225 block units in San Pietro di Lavagno (22 ± 2 plants per block; width 5.6 m, length 11.0 ± 1.0 m). Each block was geo-tagged with GPS spatial coordinates.

Considering the mobility of *H. obsoletus* (Bressan *et al.*, 2007; Mori *et al.*, 2011), its distribution inside the vineyards was studied referring the adult captures within a regular grid. In order to compare the spatial distribution of grapevines, weeds and vector, the plant maps and insect grid were overlapped. In detail, the insect grid was referred to 25 block units in Ronco all'Adige (197 ± 20 plants per block; width 15.4 ± 1.4 m, length 32.5 ± 2.9 m) and 24 block units in San Pietro di Lavagno (210 ± 23 plants per block; width 15.4 ± 1.4 m, length 38.5 ± 3.5 m). Each block was geo-tagged with GPS spatial coordinates.

Sampling of symptomatic grapevines

The grapevines were classified as symptomatic or asymptomatic depending on the presence of BN symptoms (partial or total lack of lignification of canes and shoots, rolling of leaves, sectorial discolorations of the blades). The inspection was made each year by the same two people. They inspected both sides of the plants in order to accurately check the presence of BN symptoms and, at the same time, exclude other causes of similar symptoms (e.g. partial broken canes, *Stictocephala bisonia* Kopp and Yonke activity). From 2010 to 2012, the incidence of symptomatic grapevines was evaluated in the vineyards. In 2011 and 2012, the incidence of new symptomatic grapevines was calculated in comparison with the asymptomatic grapevines in the previous years. In each block (used for insect grid) of both vineyards, five symptomatic leaves were collected from each of two symptomatic grapevine plants for BNp identification through molecular analyses. More precisely, in 2010–11 leaf samples were collected from 96 grapevines in Ronco all'Adige and from 100 grapevines in San Pietro di Lavagno. Collecting was carried out in September of each year, when the BN symptoms are evident on diseased plants.

Sampling of weeds

As spontaneous grasses (monocotyledonous species, except *Zea mays*) are not reported as BNp-host plants, investigations were exclusively on broadleaf species. In correspondence to each grapevine, the weed species observed at the two inter-row area sides were recorded and geo-tagged in order to obtain a map of the flora present in the vineyards. The incidence of each weed species was calculated as the percentage of grapevines (each vine was considered with its inter-row area sides) where the species was observed. In the middle of July, in each block used for the insect survey in both vineyards, five to 10 leaves were collected from at least one plant of the more frequent weed species for BNp identification through molecular analyses. Given that no symptomatic weeds were present, weed plants were sampled randomly within each block. More precisely, in 2010–11 leaf samples were collected from 231 weed plants in Ronco all'Adige and from 218 in San Pietro di Lavagno. Collecting was done in July of each year.

Sampling of *Hyalesthes obsoletus* specimens

In both vineyards, the presence of *H. obsoletus* was monitored every week from June to August by using yellow sticky traps (SuperColor Giallo®, Serbios, Badia Polesine (RO), Italy) placed in the center of each block unit. All the *H. obsoletus* specimens captured (182 in Ronco all'Adige, and 283 in San Pietro di Lavagno) were used for BNp identification through molecular analyses.

Bois noir phytoplasma identification

In each block of both vineyards, leaves collected from symptomatic grapevine plants and from the more frequent weed species were prepared for DNA extraction. Central leaf veins and petioles of a total of five leaves per plant were dissected, chopped into 3-mm-long pieces, and mixed together. DNA was extracted from 1 g of prepared leaf tissues following the phytoplasma enrichment procedure as described by Angelini *et al.* (2001), with some modifications. Briefly, plant tissues were placed in plastic bags (Bioreba, France) and ground by a mechanical pestle in 4 mL of CTAB (hexadecyl trimethyl-ammonium bromide)-based buffer plus ascorbic acid 0.5% pre-warmed to 60°C. Obtained homogenates (800 µL) were transferred

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to 2 mL tubes and held at 60°C for 30 min. After incubation, DNA was extracted with one volume of chloroform : iso-amylalcohol (24:1) and precipitated with one volume of isopropanol. Pellets were washed with 70% ethanol, air-dried, suspended in 400 µL of TE pH 8.0 buffer. DNAs were re-precipitated with two volumes of absolute ethanol and 10% volume of sodium acetate 3 M pH 5.2. Pellets were washed with ethanol 70% and 80%, air-dried, suspended in 100 µL of TE pH 8.0 buffer, and maintained at −30°C until use.

Total genomic DNA was extracted from individual insects following a protocol adapted from Marzachì *et al.* (1998). Briefly, the ethanol-preserved adults were dried on filter paper and homogenised in CTAB-based buffer plus ascorbic acid 0.5%. After incubation at 60°C for 30 min, DNA was extracted with one volume of chloroform : isoamylalchol 24:1 v/v solution and then precipitated with the addition of one volume of cold isopropanol. The DNA pellet was then washed with 70% ethanol, vacuum dried and resuspended in 100 µL TE pH 8.0.

Undiluted (insect) or 1:10 diluted (plants) extracted DNAs were used as templates (1 µL) for SYBR® Green real-time PCR amplification assays performed by means of 16SrXII-A subgroup-specific primer pairs StolFw/StolRev in the StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Monza, Italy), following the reaction condition described by Galetto *et al.* (2005). DNAs extracted from periwinkle plants separately infected by '*Ca*. Phytoplasma solani', reference strain STOL (taxonomic subgroup 16SrXII-A), and by '*Ca*. Phytoplasma asteris', strain SAY (taxonomic subgroup 16SrI-B), were included as controls. Reaction mixture devoid of DNA was used as negative control.

Spatial Analysis by Distance IndicEs

Data from vineyard monitoring, along with those from molecular identification of BNp in collected samples, were analyzed using the SADIE 'red-blue plots' methodology to detect spatial patterns in symptomatic grapevines, weeds, *H. obsoletus* cumulative captures, and BNp-infected *H. obsoletus* specimens within the season. This approach allows hypothesis testing for the presence of a spatial pattern in the form of clustering of a given variable into patches and gaps. Spatial Analysis by Distance IndicEs identifies neighbourhoods of consistently large or small counts by determining whether observed counts are arranged effectively at random or form clusters of similarly sized counts close to one another. The technique uses an algorithm to find the shortest distance to regularity within an observed matrix. Spatial Analysis by Distance IndicEs calculates for each sampling point the dimensionless indexes of clustering (v_i; v_j) that measure the local contribution to patch (i.e. a group of relatively high-density counts close one to another) or to gap (i.e. a group of zero or relatively small counts close one to another). An overall test of clustering is then provided by the comparison of the mean value of v_i and v_j with their corresponding values generated under the null hypothesis of a random distribution ($\alpha = 0.05$) (Perry *et al.*, 1999). Using linear kriging with 0 nugget variance with SURFER® (Golden Software Inc., Golden, CO, USA), indexes of local aggregation (v_i; v_j) and catches were interpolated and mapped on a two-dimensional map showing their spatial distribution. Datasets produced in the red-blue analysis are used to assess the similarity among spatial patterns detected for the different components (symptomatic grapevines, weeds and *H. obsoletus*). Spatial association analyses in SADIE package use a specific algorithm to determine the local spatial association and derive an overall index of spatial association (X). Randomisation test is used to calculate the significance (Px) of the association index and to test the null hypothesis of no association (Perry & Dixon, 2002). This is a two-tailed test that determines whether the clusters of the two species are associated ($Px < 0.025$), unassociated ($0.025 < Px < 0.975$) or dissociated ($P < 0.975$). As grapevine plants normally show BN symptoms from at least one year after the phytoplasma infection, spatial patterns of new symptomatic grapevines (plants showing BN symptoms for the first time) were compared with the spatial patterns of weeds and insects detected in the previous year (i.e. new symptomatic grapevines 2011 vs weeds 2010).

Bois noir phytoplasma typing

Bois noir phytoplasmas, identified by real-time PCR in symptomatic grapevines, insect specimens and weeds spatially associated with diseased grapevines and/or insects in at least one of the examined vineyards in 2010 and 2011, were typed by PCR-based amplifications of *tuf* gene, followed by restriction fragment length polymorphism (RFLP) analyses using the enzyme *Hpa*II. PCR and RFLP reaction conditions were as previously described (Langer & Maixner, 2004).

Results

Distribution of symptomatic grapevines, weeds and *Hyalesthes obsoletus*

In the years 2010–12, the incidence of symptomatic grapevines was stable in Ronco all'Adige and decreased in San Pietro di Lavagno (Table 1). Recovered plants were also observed in both vineyards in 2011–12 (Table 1). Interestingly, the incidence of newly symptomatic

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Table 1 Incidence of symptomatic and recovered grapevines, insects and weeds

	Incidence					
	Ronco all' Adige			S. Pietro di Lavagno		
	2010	2011	2012	2010	2011	2012
Grapevines						
Overall symptomatic grapevines	9.2	9.8	9.3	5.8	3.4	4.7
New symptomatic grapevines		3.5	3.9		1.9	2.6
Recovered symptomatic grapevines		0.4	1.0		0.8	3.5
Insects[a]						
Hyalesthes obsoletus Signoret	126	56		192	91	
Weeds[b]						
Amaranthus retroflexus L.	52.0	40.0		83.3	66.7	
Anagallis arvensis L.	24.0					
Artemisia vulgaris L.				95.8	79.2	
Chenopodium album L.	64.0	88.0		100.0	45.8	
Convolvulus arvensis L.	100.0	100.0		100.0	75.0	
Equisetum telmateia Ehrh.	8.0					
Erigeron canadensis L.	56.0				100.0	
Lactuca serriola L.	44.0	20.0				
Malva sylvestris L.	4.0	4.0		79.2	83.3	
Matricaria chamomilla L.					8.3	
Plantago lanceolata L.		52.0		100.0	83.3	
Plantago major L.	100.0	100.0				
Polygonum persicaria L.	16.0	36.0		95.8	95.8	
Portulaca oleracea L.	20.0	100.0			50.0	
Potentilla reptans L.	12.0	16.0		20.8	20.8	
Rumex acetosa L.	32.0	72.0		79.2	87.5	
Solanum nigrum L.	20.0					
Sonchus oleraceus L.	100.0	56.0		95.8	87.5	
Trifolium pratense L.				100.0	75.0	
Trifolium repens L.	96.0	100.0				
Taraxacum officinale (L.) Wiggers	100.0	100.0		83.3	83.3	
Urtica dioica L.	68.0	68.0		12.5	8.3	
Veronica arvensis L.		44.0				
Veronica persica Poir.	36.0	68.0				

[a]Incidence of *Hyalesthes obsoletus* is expressed as total number of specimens captured during the season.

[b]Incidence of each weed species is expressed as the percentage of grapevines (each vine was considered with its inter-row area sides) where the species was observed.

grapevines (plants showing GY symptoms for the first time in the studied year) increased in Ronco all'Adige (35% of overall symptomatic grapevines in 2011 and 41% in 2012), and was stable (56% of overall symptomatic grapevines in 2011 and 2012) in San Pietro di Lavagno (Table 1).

Twenty-one (10 perennial and 11 annual) and 16 (9 perennial and 7 annual) weed species were identified in the Ronco all'Adige and San Pietro di Lavagno vineyards, respectively (Table 1), reflecting a typical vineyard ground cover of Veneto region (north-eastern Italy). In detail, 15 and 13 weed species were present in both years in the Ronco all'Adige and San Pietro di Lavagno vineyards, respectively. On the other hand, in the Ronco all'Adige vineyard, four weeds (*Anagallis arvensis*, *Equisetum telmateia*, *Solanum nigrum* and *Erigeron canadensis*) were observed only in 2010, and two (*Plantago lanceolata* and *Veronica arvensis*) only in 2011. In the San Pietro di Lavagno vineyard, *Matricaria chamomilla*, *E. canadensis* and *Portulaca oleracea* were observed only in 2011. During the investigated period, seven and 10 weed species had an incidence > 50% in Ronco all'Adige and San Pietro di Lavagno, respectively (Table 1). The composition of weeds in the ground cover of the two vineyards was influenced by some agricultural practices such as irrigation, mowing and weeding.

On the basis of sticky trap captures, *H. obsoletus* was found in almost all the vineyard blocks during the investigated period (Table 1). Its flight period was 01 July–12 August in 2010 and 09 July–07 August in 2011 in Ronco all'Adige vineyard; 08 July–19 August in 2010 and 07 Jul–13 August in 2011 in San Pietro di Lavagno vineyard. The number of *H. obsoletus* captured specimens was higher in 2010 (126 and 192 in Ronco all'Adige and San Pietro di Lavagno, respectively) than in 2011 (56 and 91 in Ronco all'Adige and San Pietro di Lavagno, respectively) (Table 1).

Bois noir phytoplasma identification

SYBRGreen real-time PCR assay performed using 16SrXII-A subgroup-specific primer pair StolFw/StolRv amplified DNA from periwinkle plants infected by phytoplasma strain STOL (16SrXII-A), showing a Tm (Melting Temperature) of 81.5°C, and a Ct (cross threshold) of 14. No amplification was observed for periwinkle plants infected by phytoplasma strain SAY (16SrI-B) and reaction mixture devoid of DNA. Thus, only PCR products, amplified from grapevines, weeds and insect specimens, showing a Tm of 81.5 ± 0.2°C and a Ct < 37 were associated with the presence of BN phytoplasmas in analyzed plants and insects.

In the Ronco all'Adige vineyard, 96% and 98% of symptomatic grapevines analyzed were positive to real-time PCR amplification assays in 2010 and 2011, respectively (Table 2). In the San Pietro di Lavagno vineyard, 72% and 100% of symptomatic grapevines analyzed were positive to real-time PCR amplification assays in 2010 and 2011, respectively (Table 2).

Bois noir phytoplasmas were identified in 3% and 22% of insects captured in the Ronco all'Adige vineyard in 2010 and 2011, respectively, and in 23% of insects captured in the San Pietro di Lavagno vineyard in both 2010 and 2011 (Table 2).

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Table 2 Bois noir phytoplasma identification in grapevine samples and *Hyalesthes obsoletus* specimens

		2010		2011	
Vineyard	Host	Infected/Analyzed	% infection	Infected/Analyzed	% Infection
Ronco all'Adige	Grapevine	48/50	96	49/50	98
	Hyalesthes obsoletus	4/126	3	12/56	22
San Pietro di Lavagno	Grapevine	36/48	72	48/48	100
	Hyalesthes obsoletus	44/192	23	21/91	23

Table 3 Bois noir phytoplasmas identification in collected weeds

	Infected/Collected	
Species	Ronco all'Adige	San Pietro di Lavagno
Amaranthus retroflexus L.	0/3	6/19
Anagallis arvensis L.	0/3	
Artemisia vulgaris L.		5/20
Chenopodium album L.	4/11	7/23
Convolvulus arvensis L.	6/41	11/31
Erigeron canadensis L.	2/9	
Equisetum telmateia Ehrh.	0/1	
Lactuca serriola L.	0/3	
Malva sylvestris L.		3/10
Matricaria chamomilla L.		0/4
Plantago major L.	2/20	
Plantago lanceolata L.		3/16
Polygonum persicaria L.	0/2	7/23
Portulaca oleracea L.	0/3	1/5
Potentilla reptans L.	0/1	0/3
Rumex acetosa L.	0/1	3/9
Solanum nigrum L.	0/2	
Sonchus oleraceus L.	2/28	1/20
Taraxacum officinale (L.) Wiggers	4/26	0/19
Trifolium pratense L.		0/13
Trifolium repens L.	0/7	
Urtica dioica L.	7/64	0/2
Veronica arvensis L.	0/3	
Veronica persica Poir.	0/2	

In 2010–11, 11% and 21% of weed samples from Ronco all'Adige and San Pietro di Lavagno, respectively, were positive by real-time PCR. In detail, molecular analyses identified BNp in seven and ten weed species at Ronco all'Adige and San Pietro di Lavagno, respectively. On the other hand, 13 and six weed species in Ronco all'Adige and San Pietro di Lavagno, respectively, were found to be uninfected (Table 3).

Spatial Analysis by Distance IndicEs analyses

Spatial Analysis by Distance IndicEs detected significant clustering into patch/gap in the distributions of overall symptomatic grapevines and 12 weed species observed in Ronco all'Adige during 2010 (Table 4). During 2011, significant clustering into patch/gap was detected in the distribution of symptomatic grapevines and 12 weeds, while *P. lanceolata* and *H. obsoletus* BNp-infected specimens distribution was significantly clustered into gap only (Table 4). Moreover, distribution of grapevine plants showing yellows symptoms for the first time in 2011 was significantly clustered into patch (Table 4). In 2012, distribution of new symptomatic grapevines was significantly clustered into patch/gap (Table 4).

Spatial Analysis by Distance IndicEs evidenced that in 2010 spatial distributions of 10 weed species in San Pietro di Lavagno were characterised by significant clustering into patch/gap, while clustering into gap only was found in the distribution of *U. dioica* and *H. obsoletus* BNp-infected specimens (Table 5). During 2011, distributions of 13 weed species were significantly clustered into patch/gap (Table 5). Distribution of new symptomatic grapevines was significantly clustered into patch in 2011 and into patch/gap in 2012 (Table 5).

In the Ronco all'Adige vineyard in 2010, based on spatial association index (Table 6), distribution of overall symptomatic grapevines was found to be significantly associated with *U. dioica*, *E. telmateia*, *Polygonum persicaria*, *Potentilla reptans* and *Rumex acetosa*, and significantly dissociated from *Lactuca serriola*, *Malva sylvestris* and *P. oleracea*. Distribution of captured and BNp-infected *H. obsoletus* specimens was associated with *P. oleracea*. In contrast, distribution of captured *H. obsoletus* specimens was found dissociated from *P. persicaria*. Distribution of new symptomatic grapevines observed in 2011 was associated with *Veronica persica* in 2010, and was dissociated from *P. reptans* and *Sonchus oleraceus* in 2010. In the same vineyard in 2011 (Table 6), distribution of overall symptomatic grapevines was associated with *U. dioica*. No associations were found between distributions of captured and BNp-infected insect specimens and weeds. The distribution of new symptomatic grapevines observed in 2012 was associated with *M. sylvestris, V. persica* and *Amaranthus retroflexus* in 2011, and dissociated from *P. reptans* in 2011.

In San Pietro di Lavagno during 2010, the distribution of overall symptomatic grapevines was associated with five weeds (*A. retroflexus*, *U. dioica*, *C. arvensis*, *P. reptans*, and *P. lanceolata*) (Table 7). Moreover, distribution

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Table 4 Average indexes of clustering into patch (mean v_i) and into gap (mean v_j) with associated probability (P) from randomisation test in Ronco all'Adige vineyard[a]

	2010				2011			
Folder	Mean v_i	P (mean v_i)	Mean v_j	P (mean v_j)	Mean v_i	P (mean v_i)	Mean v_j	P (mean v_j)
Overall symptomatic grapevines	**1.652**	**0.0085**	**−1.796**	**0.0085**	**1.59**	**0.0158**	**−1.399**	**0.0429**
2011-new symptomatic grapevines	**1.396**	**0.0427**	−1.25	0.1197				
2012-new symptomatic grapevines					**1.829**	**0.0023**	**−2.124**	**0.0005**
Hyalesthes obsoletus captured specimens	0.865	0.6239	−0.855	0.7179	1.179	0.1849	−1.472	0.0546
Hyalesthes obsoletus BNp-infected specimens	0.987	0.4615	−0.948	0.5556	1.232	0.1612	**−1.606**	**0.0261**
Amaranthus retroflexus L.	**1.523**	**0.0256**	**−1.515**	**0.0342**	**1.565**	**0.025**	**−1.794**	**0.0052**
Anagallis arvensis L.	1.14	0.1966	−1.336	0.0513				
Chenopodium album L.	**2.569**	**<0.0001**	**−2.554**	**<0.0001**	**1.892**	**0.0023**	**−1.662**	**0.0102**
Convolvulus arvensis L.	**1.536**	**0.0171**	**−1.573**	**0.0085**	**1.6**	**0.0134**	**−1.634**	**0.0109**
Equisetum telmateia Ehrh.	**1.951**	**<0.0001**	**−2.3**	**<0.0001**				
Erigeron canadensis L.	**1.377**	**0.0427**	−1.28	0.094				
Lactuca serriola L.	**1.455**	**0.0427**	**−1.597**	**0.0085**	**1.528**	**0.0317**	**−1.456**	**0.0407**
Malva sylvestris L.	1.347	0.1197	−1.266	0.1624	**1.72**	**0.0153**	−1.439	0.0571
Plantago lanceolata L.					1.354	0.0556	**−1.376**	**0.0496**
Plantago major L.	**1.619**	**0.0171**	**−1.987**	**<0.0001**	1.312	0.0798	−1.373	0.0571
Polygonum persicaria L.	1.122	0.2821	−0.956	0.4786	**1.603**	**0.0159**	**−1.532**	**0.022**
Portulaca oleracea L.	**1.93**	**0.0085**	**−1.797**	**0.0085**	**2.733**	**<0.0001**	**−2.917**	**<0.0001**
Potentilla reptans L.	**2.737**	**<0.0001**	**−2.551**	**<0.0001**	**2.669**	**<0.0001**	**−2.488**	**<0.0001**
Rumex acetosa L.	1.267	0.1197	**−1.522**	**0.0171**	**1.615**	**0.0117**	**−1.914**	**0.0022**
Solanum nigrum L.	0.983	0.3504	−1.086	0.2479				
Sonchus oleraceus L.	**2.193**	**<0.0001**	**−2.414**	**<0.0001**	1.155	0.1793	−1.251	0.1158
Taraxacum officinale (L.) Wiggers	1.017	0.3333	−1.003	0.3504	**3.134**	**<0.0001**	**−3.203**	**<0.0001**
Trifolium repens L.	**1.449**	**<0.0001**	**−1.707**	**<0.0001**	**1.531**	**0.0191**	**−1.681**	**0.0087**
Urtica dioica L.	**3.025**	**<0.0001**	**−2.753**	**<0.0001**	**2.369**	**<0.0001**	**−2.288**	**<0.0001**
Veronica arvensis L.					1.333	0.0724	−1.34	0.0727
Veronica persica Poir.	1.14	0.2137	−1.199	0.1453	**2.091**	**0.0008**	**−2.272**	**0.0003**

[a]Numbers in bold indicate significant results at randomisation test ($\alpha = 0.05$).

of captured *H. obsoletus* specimens was associated with *A. retroflexus*, *C. arvensis*, *M. sylvestris*, *R. acetosa* and *P. reptans*; and it was dissociated from *Artemisia vulgaris*. Distribution of BNp-infected *H. obsoletus* specimens was associated with *Chenopodium album*, *U. dioica* and *P. reptans*. Furthermore, distribution of new symptomatic grapevines observed in 2011 was associated with *U. dioica* and BNp-infected *H. obsoletus* specimens in 2010. In the same vineyard in 2011 (Table 7), distribution of overall symptomatic grapevines was associated with *C. arvensis*, *Trifolium pratense* and *Taraxacum officinale*; on the other hand, it was dissociated from *C. album*. No associations were found between distributions of captured and BNp-infected insect specimens and weeds. Moreover, distribution of new symptomatic grapevines observed in 2012 was associated with *C. album*, *Matricaria chamomilla* and *E. canadensis* in 2011; and it was dissociated from six weeds (*A. vulgaris*, *C. arvensis*, *P. persicaria*, *P. oleracea*, *S. oleraceus* and *T. officinale*) in 2011.

Bois noir phytoplasma typing

On the basis of *Hpa*II-RFLP profiles obtained from *tuf* gene amplicons, BNp types *tuf*-a and *tuf*-b were identified in the examined vineyards. In the Ronco all'Adige vineyard, BNp type *tuf*-a was identified in all grapevines (97 plants), *H. obsoletus* (16 specimens) and nettle (seven plants); on the other hand, BNp type *tuf*-b was identified in bindweed (6 plants) and *C. album* (four plants) (data not shown). In the San Pietro di Lavagno vineyard, BNp type *tuf*-a was identified in grapevines (46 plants) and *H. obsoletus* (26 specimens); on the other hand, BNp type *tuf*-b was identified in grapevines (38 plants), *H. obsoletus* (37 specimens), bindweed (11 plants), *C. album* (seven plants) and *M. sylvestris* (three plants) (data not shown).

Discussion

Bois noir and other plant diseases associated with phytoplasmas have a complex biological cycle, involving different host plants and/or insect vectors. Owing to a lack of knowledge about the epidemiology of such diseases, it is difficult to develop efficient strategies to manage their transmission (Weintraub & Beanland, 2006). Recently, data spatial analysis was successfully used for an in-depth investigation on the possible role of host plants and insect vectors in the spread of phytoplasma diseases (Navratil

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Table 5 Average indexes of clustering into patch (mean v_i) and into gap (mean v_j) with associated probability (P) from randomisation test in San Pietro di Lavagno vineyard[a]

	2010				2011			
Folder	Mean v_i	P (mean v_i)	P (mean v_j)	Mean v_j	Mean v_i	P (mean v_i)	Mean v_j	P (mean v_j)
Overall symptomatic grapevines	0.895	0.5849	−0.898	0.5785	1.266	0.1309	−1.109	0.2554
2011-new symptomatic grapevines	**1.662**	**0.0426**	−1.52	0.0689				
2012-new symptomatic grapevines					**1.607**	**0.0335**	**−1.652**	**0.0308**
Hyalesthes obsoletus captured specimens	1.334	1.1218	−1.555	0.0568	1.115	0.2656	−1.556	0.0516
Hyalesthes obsoletus BNp-infected specimens	1.561	0.0545	**−1.916**	**0.0173**	0.99	0.3945	−1.145	0.2609
Amaranthus retroflexus L.	**2.896**	**<0.0001**	**−2.837**	**<0.0001**	**3.629**	**<0.0001**	**−3.947**	**<0.0001**
Artemisia vulgaris L.	**3.738**	**<0.0001**	**−3.259**	**<0.0001**	**3.876**	**<0.0001**	**−3.358**	**<0.0001**
Chenopodium album L.	**2.451**	**0.0005**	**−2.548**	**0.0002**	**1.915**	**0.0140**	**−1.967**	**0.0099**
Convolvulus arvensis L.	**4.678**	**<0.0001**	**−4.772**	**<0.0001**	**3.194**	**<0.0001**	**−3.061**	**0.0003**
Erigeron canadensis L.					**1.945**	**0.0106**	**−2.156**	**0.0034**
Malva sylvestris L.	**2.542**	**0.001**	**−2.709**	**0.0005**	**1.966**	**0.0109**	**−2.182**	**0.0031**
Matricaria chamomilla L.					1.592	0.0564	−1.502	0.0742
Plantago lanceolata L.	0.941	0.4694	−1.016	0.3712	**2.049**	**0.0048**	**−2.303**	**0.0024**
Polygonum persicaria L.	**1.602**	**0.0396**	**−1.588**	**0.0389**	**1.849**	**0.0137**	**−1.585**	**0.0386**
Portulaca oleracea L.					**3.209**	**0.0003**	**−2.347**	**0.0007**
Potentilla reptans L.	**3.424**	**<0.0001**	**−3.45**	**<0.0001**	**3.353**	**<0.0001**	**−3.066**	**<0.0001**
Rumex acetosa L.	1.081	0.2886	−1.268	0.148	**2.106**	**0.0041**	**−2.148**	**0.0024**
Sonchus oleraceus L.	**1.912**	**0.007**	**−1.826**	**0.0124**	**1.996**	**0.0051**	**−2.183**	**0.0014**
Taraxacum officinale (L.) Wiggers	**2.839**	**<0.0001**	**−3.186**	**<0.0001**	1.422	0.0752	−1.437	0.0694
Trifolium pratense L.	**1.995**	**0.006**	**−2.393**	**0.0008**	**3.003**	**<0.0001**	**−3.342**	**<0.0001**
Urtica dioica L.	1.6	0.053	**−2.084**	**0.0022**	0.837	0.6338	−0.896	0.5309

[a]Numbers in bold indicate significant results at randomisation test ($\alpha = 0.05$).

et al., 2009; Bonnot *et al.*, 2010; Rappussi *et al.*, 2012). This approach can help to identify weed candidates whose role has to be determined by further research including phytoplasma detection and typing as well as investigating their link to potential vectors and their role as host/reservoir plants. Thus, in this research, we used a multidisciplinary approach to study the epidemiology of BN disease, based on synergic application of field surveys, data spatial analyses and molecular biology techniques on the three main components involved in BN diffusion in vineyards: grapevines (cultivated crop host), *H. obsoletus* (vector) and weeds (*inoculum* source).

Molecular analyses revealed a high percentage of PCR-positive symptomatic grapevine samples, confirming the strong association between specific GY disease symptoms and infection by BNp ('*Ca*. Phytoplasma solani' strains) within the examined vineyards, where no other phytoplasmas associated with GY diseases (i.e. Flavescence dorée, '*Ca*. Phytoplasma vitis') (IRPCM, 2004; according to rule 28b of the Bacteriological Code, '*Ca*. Phytoplasma vitis' is an incidental citation and does not constitute prior citation) have been reported, even in previous years (Quaglino *et al.*, 2009). On the other hand, the presence of PCR-negative symptomatic grapevines could be connected with the low titre and/or sporadic distribution of phytoplasmas in symptomatic plant tissues (Constable *et al.*, 2003). The number of symptomatic grapevines was quite stable in Ronco all'Adige (around 9.5% of the total) and in San Pietro di Lavagno (around 5%) over the years, even if numerous newly symptomatic grapevines were observed in both vineyards in each year. Moreover, several recovered grapevines (symptomatic plants that spontaneously regain a healthy condition) (Osler *et al.*, 1993; Belli *et al.*, 2010) were reported. Interestingly, this evidence highlighted that the impact of BN on the examined vineyards and on the distribution of symptomatic grapevines is influenced by two main driving forces: (a) the transmission of BNp from infected source plant(s) to grapevines and (b) the spontaneous recovery of diseased grapevines. These two components influenced the aggregation pattern of total symptomatic grapevines in the examined vineyards during the considered period, placing more epidemiological significance on the distribution of the new symptomatic plants.

BNp-infection percentage (23%) among captured *H. obsoletus* specimens, except for the Ronco all'Adige vineyard in 2010 (3% BNp-infected insects), is in agreement with evidence from previous studies (Sforza *et al.*, 1998; Bressan *et al.*, 2007; Mori *et al.*, 2008). Distribution of *H. obsoletus*, significantly clustered into patch/gap, was found to be associated only with new symptomatic grapevines and its weed hosts in 2010 in the San Pietro di Lavagno vineyard. This reinforces the idea

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Table 6 Probability associated to spatial association index in Ronco all'Adige[a]

	2010				2011			
Folder	Overall Symptomatic Grapevines	*Hyalesthes obsoletus* Captured	*Hyalesthes obsoletus* BNp-infected	2011-new Symptomatic Grapevines	Overall Symptomatic Grapevines	*Hyalesthes obsoletus* Captured	*Hyalesthes obsoletus* BNp-infected	2012-new Symptomatic Grapevines
Overall symptomatic grapevines		0.6401	0.5775	0.1942		0.7541	0.7611	0.8027
2011-new symptomatic grapevines	0.1942	0.5564	0.4097					
2012-new symptomatic grapevines					0.8027	0.4268	0.4056	
Hyalesthes obsoletus captured	0.6401		0.1501	0.5564	0.7541		**0.0191**	0.4268
Hyalesthes obsoletus BNp-infected	0.5775	0.1501		0.4097	0.7611	**0.0191**		0.4056
Amaranthus retroflexus L.	0.3897	0.2903	0.3327	0.518	0.8325	0.1202	0.2291	**<0.0001**
Anagallis arvensis L.	0.2862	0.4383	0.2787	0.7697				
Chenopodium album L.	0.9742	0.4143	0.1207	0.6805	0.4824	0.5008	0.4047	0.0811
Convolvulus arvensis L.	0.0847	0.5634	0.5221	0.6691	0.4533	0.7645	0.6426	0.8885
Equisetum telmateia Ehrh.	**0.008**	0.9263	0.9711	0.8212				
Erigeron canadensis L.	0.9511	0.91	0.9646	0.8078				
Lactuca serriola L.	*0.9957*	0.4038	0.4314	0.1163	0.8749	0.4701	0.2939	0.0831
Malva sylvestris L.	*0.998*	0.1943	0.0739	0.5877	0.8749	0.088	0.1042	**0.001**
Plantago lanceolata L.					0.8746	0.5043	0.5291	0.4037
Plantago major L.	0.6315	0.8389	0.7605	0.8658	0.3603	0.5163	0.335	0.4526
Polygonum persicaria L.	**0.0047**	*0.9799*	0.9188	0.8749	0.0745	0.6729	0.6004	0.9169
Portulaca oleracea L.	*0.9757*	**0.0184**	**0.0131**	0.2784	0.7083	0.0715	0.0465	0.0372
Potentilla reptans L.	**0.0012**	0.6658	0.6744	*0.9813*	**0.0106**	0.9402	0.8843	*0.9996*
Rumex acetosa L.	**0.0174**	0.6679	0.599	0.9371	0.743	0.6527	0.4487	0.3331
Solanum nigrum L.	0.9707	0.3676	0.3414	0.7219				
Sonchus oleraceus L.	0.9416	0.5846	0.2721	*0.9829*	0.4714	0.86	0.5956	0.9231
Taraxacum officinale (L.) Wiggers	0.7748	0.7441	0.8346	0.201	0.2866	0.9031	0.766	0.3626
Trifolium repens L.	0.1644	0.7968	0.7713	0.9225	0.7039	0.4868	0.1575	0.8127
Urtica dioica L.	**0.0162**	0.9377	0.9449	0.7177	**0.002**	0.6903	0.422	0.8992
Veronica arvensis L.					0.7394	0.7673	0.8207	0.8977
Veronica persica Poir.	0.0294	0.6696	0.6346	**0.0216**	0.8716	0.1074	0.0287	**<0.0001**

[a]Numbers in bold indicate associations ($P < 0.025$), while numbers in italics indicate dissociations ($P > 0.975$) (38).

that *H. obsoletus* is a BNp-vector (Gatineau *et al.*, 2001; Palermo *et al.*, 2004; Cvrković *et al.*, 2014).

The results from PCR analyses showed that *C. arvensis*, *U. dioica*, *P. persicaria*, *T. officinale*, *P. lanceolata*, *C. album*, *A. retroflexus*, *M. sylvestris*, *A. vulgaris* and *S. oleracea*, previously reported as BNp-host plants (Langer & Maixner, 2004; Berger *et al.*, 2009; Kessler *et al.*, 2011), were frequently infected by BNp. Furthermore, BNp was identified for the first time in *E. canadensis, R. acetosa* and *P. oleracea*. Interestingly, *E. canadensis* and *P. oleracea* were previously reported as host plants of '*Ca*. Phytoplasma pruni' and '*Ca*. Phytoplasma asteris' strains, respectively (Schneider *et al.*, 1997). On the other hand, the species *A. arvensis*, *M. chamomilla*, *P. reptans*, *S. nigrum*, *T. pratense*, *E. telmateia*, *L. serriola*, *V. arvensis* and *V. persica* were found uninfected in the examined vineyards. In other studies performed in diverse geographic regions, *P. reptans*, *S. nigrum* and *T. pratense* were found as host plants of BNp (Batlle *et al.*, 2000; Langer & Maixner, 2004; Credi *et al.*, 2006; Franova *et al.*, 2009; Sabaté *et al.*, 2014).

On the basis of BNp-positivity and statistically significant association with overall and/or new symptomatic grapevines and/or insect vector, we propose to rank the weeds identified in the examined vineyards in four epidemiological groups: weeds BNp-infected and associated with symptomatic grapevines and/or *H. obsoletus* captures in 2010 and 2011 in at least one of the studied vineyards (group 1); weeds BNp-infected and associated with symptomatic grapevines and/or *H. obsoletus* captures in 2010 or 2011 in at least one of the studied vineyards (group 2); BNp-infected weeds, not associated with symptomatic grapevines and/or *H. obsoletus* captures, or uninfected but associated with symptomatic grapevines and/or *H. obsoletus* captures in 2010 or 2011 in at least one of the studied vineyards (group 3); uninfected weeds and not associated with symptomatic grapevines and/or *H. obsoletus* captures (group 4).

On the basis of this epidemiological classification, group 1 includes *C. arvensis* and *U. dioica*, widely reported as BNp and *H. obsoletus* host plants involved in the

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Table 7 Probability associated to spatial association index in San Pietro di Lavagno[a]

	2010				2011			
Folder	Overall Symptomatic Grapevines	*Hyalesthes obsoletus* Captured	*Hyalesthes obsoletus* BNp-Infected	2011-new Symptomatic Grapevines	Overall Symptomatic Grapevines	*Hyalesthes. obsoletus* Captured	*Hyalesthes obsoletus* BNp-Infected	2012-new Symptomatic Grapevines
Overall symptomatic grapevines		0.0253	0.1509	0.0532		0.8921	0.8129	0.3267
2011-new symptomatic grapevines	0.0532	0.5309	**0.0089**					
2012-new symptomatic grapevines					0.3267	0.9873	0.2799	
Hyalesthes obsoletus captured	0.0253		**0.0039**	0.5309	0.8921		0.2088	0.9873
Hyalesthes obsoletus BNp-infected	0.1509	**0.0039**		**0.0089**	0.8129	0.2088		0.2799
Amaranthus retroflexus L.	**0.0047**	**0.0073**	0.0384	0.0864	0.0265	0.1881	0.4358	0.9227
Artemisia vulgaris L.	0.7129	*0.9966*	*0.9886*	0.5444	0.0267	0.8550	0.5493	*0.9999*
Chenopodium album L.	0.3711	0.0345	**0.0207**	0.2801	*0.9923*	0.0355	0.1494	**<0.0001**
Convolvulus arvensis L.	**0.0059**	**0.0181**	0.1076	0.06	**0.0003**	0.3112	0.5864	*0.9982*
Erigeron canadensis L.					0.7963	0.1749	0.2215	**0.0008**
Malva sylvestris L.	0.4424	**0.0115**	0.1074	0.8251	0.8697	0.6083	0.3721	**0.0001**
Matricaria chamomilla L.					0.9050	0.4645	0.4883	**<0.0001**
Plantago lanceolata L.	**0.0069**	0.3693	0.5637	0.0686	0.0438	0.7471	0.3126	0.474
Polygonum persicaria L.	0.4543	0.5912	0.4954	0.2222	0.0345	0.7619	0.6205	*0.9908*
Portulaca oleracea L.					0.2708	0.7856	0.4129	*0.9987*
Potentilla reptans L.	**0.0249**	**<0.0001**	**0.0019**	0.056	0.2698	0.2005	0.1682	0.4638
Rumex acetosa L.	0.1342	**0.005**	0.0589	0.5948	0.1095	0.7102	0.7122	*0.985*
Sonchus oleraceus L.	0.7761	0.5371	0.6566	0.9523	0.0584	0.23	0.7063	*0.9968*
Taraxacum officinale (L.) Wiggers	0.2829	0.0588	0.1947	0.3201	**0.0074**	0.5029	0.3761	*0.9999*
Trifolium pratense L.	0.5931	0.5459	0.911	0.9145	**0.0154**	0.7828	*0.9968*	0.5923
Urtica dioica L.	**0.0122**	0.0272	**0.0071**	**0.0059**	0.7016	0.3824	0.3912	0.9756

[a]Numbers in bold indicate associations ($P < 0.025$), while numbers in italics indicate dissociations ($P > 0.975$) (38).

BN epidemiology throughout Europe (Johannesen *et al.*, 2012), *C. album* and *M. sylvestris*; group 2 includes *A. retroflexus* and *P. lanceolata*; group 3 includes *A. vulgaris*, *E. telmateia*, *E. canadensis*, *M. chamomilla*, *P. major*, *P. persicaria*, *P. oleracea*, *P. reptans*, *S. oleraceus*, *R. acetosa*, *T. officinale*, *T. pratense* and *V. persica*; group 4 includes *A. arvensis*, *L. serriola*, *S. nigrum*, *T. repens* and *V. arvensis*.

Within group 1, *U. dioica* showed association with symptomatic grapevines and insects in 2010 and 2011 in the Ronco all'Adige vineyard and only in 2010 in the San Pietro di Lavagno vineyard (Fig. 1). On the other hand, *C. album*, *C. arvensis* and *M. sylvestris* showed association with symptomatic grapevines and *H. obsoletus* in 2010 and 2011 only in the San Pietro di Lavagno vineyard. Interestingly, association of such weeds with symptomatic grapevines and insects significantly clustered into patch/gap indicated the strict relationships among these different epidemiological components. In order to validate the combined data from spatial analyses and BNp detection, suggesting that plant species of epidemiological group 1 (*U. dioica*, *C. arvensis*, *C. album* and *M. sylvestris*) are weed candidates as BNp *inoculum* source and could play a role in its transmission, *tuf* gene typing was carried out on BNp strains identified in grapevines, *H. obsoletus*, and these weeds. In the Ronco all'Adige vineyard, where nettle, bindweed and *C. album* were found BNp-infected, but only the distribution of nettle was associated with symptomatic grapevines, BNp type *tuf*-a was detected in nettles and in all grapevines and *H. obsoletus* specimens analyzed. In the same vineyard, bindweed and *C. album* were infected by BNp type *tuf*-b. Distribution and prevalence of BNp *tuf* types in the analyzed hosts confirmed that only nettle (BNp type *tuf*-a), spatially associated with diseased grapevines and infected by the same BNp *tuf* type (*tuf*-a) identified in grapevines and vector specimens, play a role in the diffusion of BNp in the Ronco all'Adige vineyard. Moreover, in the same vineyard, the scarce presence of BNp-infected *H. obsoletus* and its distribution not associated with nettle suggested the possibility that additional vector(s) of the BNp type *tuf*-a could be present. Additionally, in the San Pietro di Lavagno vineyard, where bindweed, *C. album* and *M. sylvestris* were found infected by BNp type *tuf*-b and spatially associated with symptomatic grapevines and *H. obsoletus*, both BNp types *tuf*-a and *tuf*-b were identified in symptomatic grapevines and in insect vector specimens. The spatial analysis and the co-presence of BNp type *tuf*-b in grapevines, insects and weeds highlighted the role of

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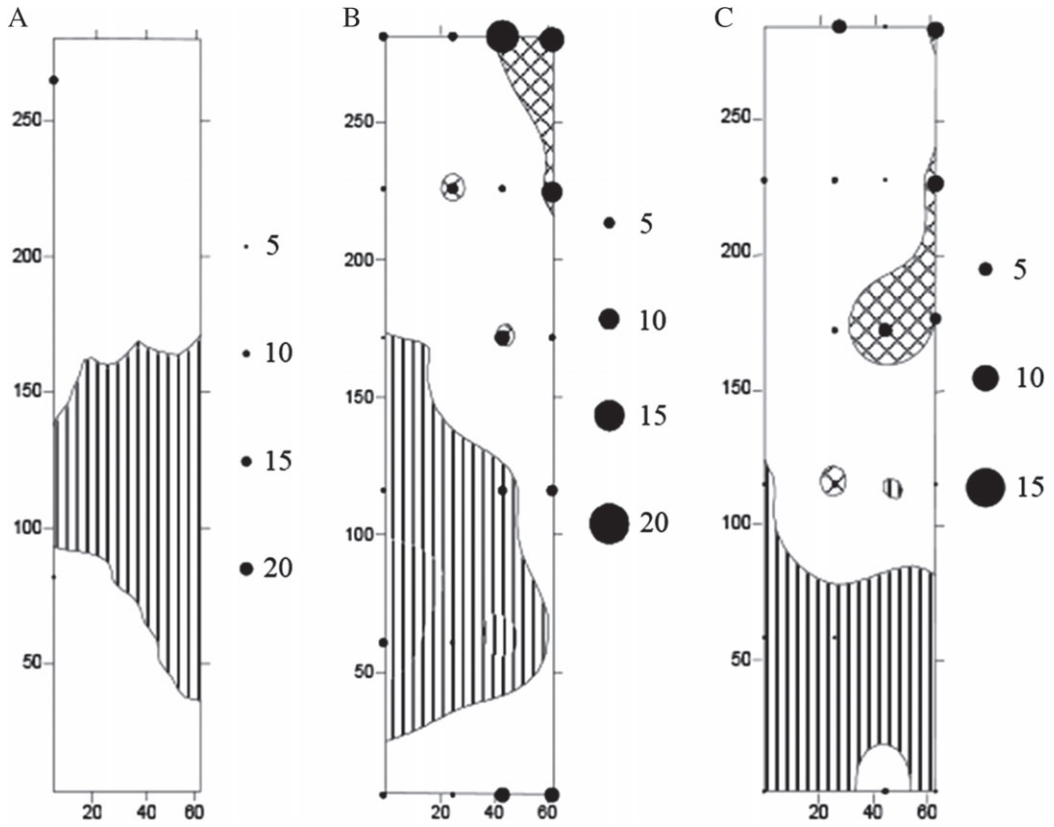

Figure 1 Map of counts and clustering indexes of *Urtica dioica* (A), BNp-infected *Hyalesthes obsoletus* (B) and new symptomatic grapevines (C) in San Pietro di Lavagno in 2010. The maps show an example of statistically significant association of a weed (*U. dioica*) of epidemiological group 1 with grapevines and insect vector. Dots represent number of plants or insects observed in each plot. Gridded areas represent patches with interpolated cluster index $v_j > 1.5$. Vertically lined areas are gaps with interpolated cluster index $v_j < -1.5$. Values on axis indicate coordinates in metres.

bindweed, *C. album* and *M. sylvestris* as potential *inoculum* source of BNp. On the other hand, identification of BNp type *tuf*-a in grapevines and insects and their spatial association with nettle (reported as exclusive weed host of BNp type *tuf*-a) suggested that nettle, even present in low density (only two plants randomly collected were uninfected), play a role in BNp type *tuf*-a transmission in this vineyard. Such evidence, obtained by comparing data of spatial and molecular analyses (identification and typing of BNp), revealed that SADIE spatial analyses provided a strong indication on determining the role of weed candidates as *inoculum* source of BNp.

Association of *U. dioica* and *C. arvensis* with symptomatic grapevines and insect vector captures, and their different distribution clustering within the examined vineyards are fully in agreement with the results reported in previous studies on BN epidemiology in Europe (Sforza *et al.*, 1999; Maixner *et al.*, 2007; Kessler *et al.*, 2011; Mori *et al.*, 2012). On the other hand, further studies should be carried out to confirm the role of *C. album* and *M. sylvestris* in BN diffusion. In particular, additional research should be performed to investigate the association of the perennial species *M. sylvestris* with the larval stage of *H. obsoletus* and with other vector(s). Moreover, considering that perennial plants are the main phytoplasma reservoirs and hosts of the vectors (Weintraub & Beanland, 2006), it is interesting to report the presence of one annual weed (*C. album*) within this group 1. This could be explained by different hypotheses. Firstly, this weed could favour the BNp diffusion over the years by means of seeds, as reported for other annual plants (Olivier *et al.*, 2010; Calari *et al.*, 2011). Secondly, some infections in the weed flora might result from alternative epidemiological cycles with alternative vectors and with or without relation to grapevines. *H. obsoletus* becomes infected during its larval stage (Maixner, 2011). As its larval development is not possible on *C. album* because it is an annual species, *H. obsoletus* cannot acquire BN phytoplasma from this weed. Considering the six weeks activity period of adult *H. obsoletus* observed in the present study, feeding of infective adult vectors on *C. album* could explain the occurrence of infected plants. On the other hand, *C. album*

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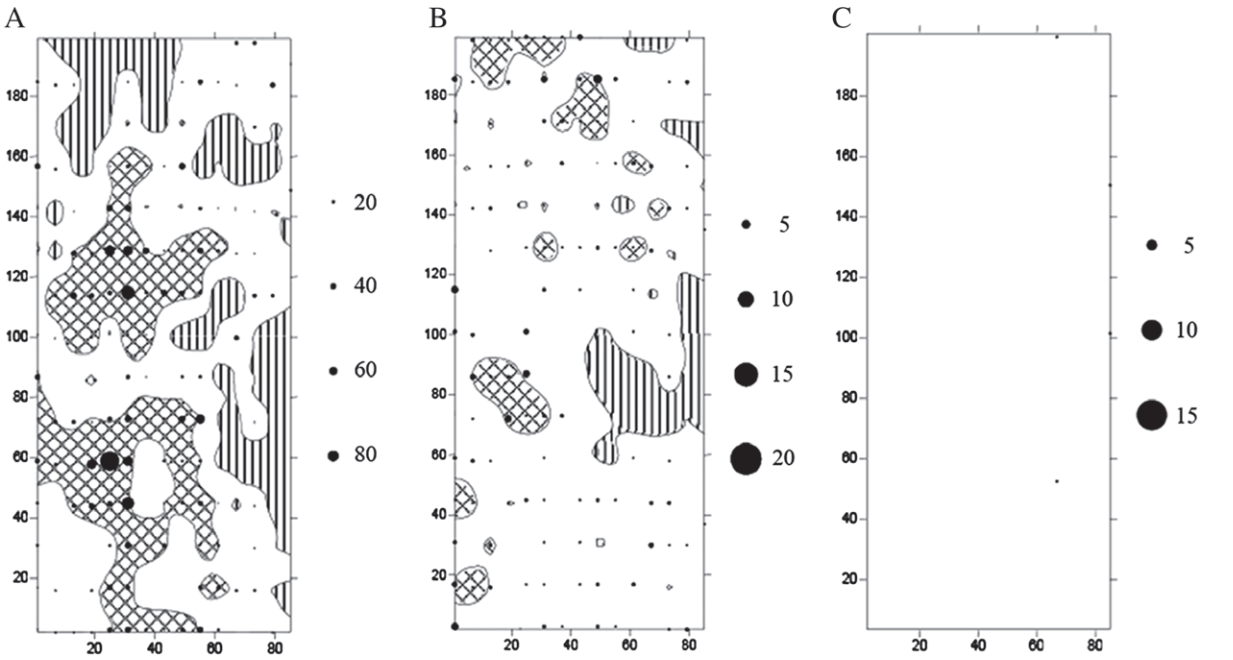

Figure 2 Map of counts and clustering indexes of *Trifolium repens* (A), new symptomatic grapevines (B) and BNp-infected *Hyalesthes obsoletus* (C) in Ronco all'Adige in 2010. The maps show an example of the distribution of a weed (*T. repens*) of epidemiological group 4 in comparison with grapevines and insect vector. Dots represent number of plants or insects observed in each plot. Gridded areas represent patches with interpolated cluster index $v_i > 1.5$. Vertically lined areas are gaps with interpolated cluster index $v_j < -1.5$. Values on axis indicate coordinates in metres.

could constitute the inoculation target and the acquisition source of alternative vector(s), probably present in the vineyard as adults for a longer period, during the same vegetative season.

Within group 2, *A. retroflexus* and *P. lanceolata* were associated with symptomatic grapevines significantly clustered into patch/gap only in 2010, indicating a temporally limited strict relationship among the different epidemiological components. Within group 3, BNp-infected weeds, not associated with symptomatic grapevines and/or vector captures in the same vineyard, were *A. vulgaris*, *P. major*, *P. persicaria*, *P. oleracea* and *S. arvensis*; uninfected weeds associated with symptomatic grapevines and/or vector captures were *E. telmateia*, *M. chamomilla*, *P. reptans*, *T. pretense* and *V. persica*. Based on this evidence, all the weeds of epidemiological groups 2 and 3 have no clear role in spreading the BN in the examined vineyards, but given their BNp-infection or associations and data from previous studies they could have a role in BN diffusion. For example, *A. vulgaris* was reported as host plant of *H. obsoletus* (Alma *et al.*, 1988) and *P. reptans* was found as host plant of BNp (Credi *et al.*, 2006).

Weeds belonging to group 4 do not play a role in BN epidemiology because of their scarce ground cover, except for *T. repens* (Fig. 2), and the absence of phytoplasma infection and associations.

Data obtained in this and previous studies indicate that BN epidemiology is influenced by several weed species and their distribution patterns inside and outside vineyards (Maixner *et al.*, 2007; Maixner & Johannesen, 2013), and could provide helpful indications for designing experimental plans to contain BN spreading in vineyards through weed management (Riedle-Bauer *et al.*, 2010). Given that BNp-infected and associated weeds are dicotyledonous, it could be important to favour the vineyard ground cover with grass instead of broadleaves by sowing selected grass species at transplanting, applying selective chemical treatments and frequent cutting. In conclusion, this work highlighted that in the examined areas (a) the host systems *C. arvensis* – *H. obsoletus* and *U. dioica* – *H. obsoletus* have a role in BN diffusion; (b) other weeds could play a role in BN diffusion; (c) new wild plants have been found as BNp hosts; (d) the synergic application of multidisciplinary methods improved the knowledge of BN epidemiology. As pedo-climatic conditions and agricultural practices influence the vineyard ecosystems, further studies should be conducted in different grape-growing areas over more years to investigate BN epidemiology more accurately. In particular, the role of additional potential vectors, suggested here by the association of some weeds with symptomatic grapevines but not with *H. obsoletus*, and the genotyping

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of BNp strains infecting symptomatic grapevines, insects and weeds should be considered. Furthermore, the experimental approach used in the present study could be a suitable tool for obtaining accurate information about the epidemiology of other diseases associated with phytoplasmas characterised by complex ecological cycles.

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References

Alma A., Arnò C., Arzone A., Vidano C. (1988) New biological reports on Auchenorrhyncha in vineyards. In *Proceedings of 6th Auchenorrhyncha Meeting, Turin, Italy, 1987*, pp. 509–516. Eds C. Vidano and A. Arzone. Turin, Italy: University of Turin.

Alma A., Soldi G., Tedeschi R., Marzachì C. (2002) Ruolo di *Hyalesthes obsoletus* Signoret (Homoptera, Cixiidae) nella trasmissione del legno nero della vite in Italia. *Petria*, **12**, 411–412.

Angelini E., Clair D., Borgo M., Bertaccini A., Boudon-Padieu E. (2001) Flavescence dorée in France and Italy: occurrence of closely related phytoplasma isolates and their near relationships to palatinate grapevine yellows and an alder yellows phytoplasma. *Vitis*, **40**, 79–86.

Batlle A., Martinez M.A., Lavina A. (2000) Occurrence, distribution and epidemiology of grapevine yellows in Spain. *European Journal of Plant Pathology*, **106**, 811–816.

Belli G., Bianco P.A., Conti M. (2010) Grapevine yellows: past, present and future. *Journal of Plant Pathology*, **92**, 303–326.

Berger J., Schweigkofler W., Kerschbamer C., Roschatt C., Dalla Via J., Baric S. (2009) Occurrence of Stolbur phytoplasma in the vector *Hyalesthes obsoletus*, herbaceous host plants and grapevine in South Tyrol (Northern Italy). *Vitis*, **48**, 185–192.

Bonnot F., de Franqueville H., Lourenço E. (2010) Spatial and spatiotemporal pattern analysis of coconut lethal yellowing in Mozambique. *Phytopathology*, **100**, 300–312.

Borgo M., Albanese G., Quaglino F., Casati P., Ermacora P., Ferretti L., Ferrini F., Filippin L., Pasquini G., Angelini E. (2008) Ruolo di altre piante nell'epidemiologia dei fitoplasmi agenti di Flavescenza dorata e Legno nero. *Petria*, **18**, 261–263.

Bressan A., Turata R., Maixner M., Spiazzi S., Boudon-Padieu E., Girolami V. (2007) Vector activity of *Hyalesthes obsoletus* living on nettles and transmitting a stolbur phytoplasma to grapevines: a case study. *Annals of Applied Biology*, **150**, 331–339.

Calari A., Paltrinieri S., Contaldo N., Sakalieva D., Mori N., Duduk B., Bertaccini A. (2011) Molecular evidence of phytoplasmas in winter oilseed rape, tomato and corn seedlings. *Bulletin of Insectology*, **64**, S157–S158.

Constable F.E., Gibb K.S., Symons R.H. (2003) Seasonal distribution of phytoplasmas in Australian grapevines. *Plant Pathology*, **52**, 267–276.

Credi R., Terlizzi F., Milanesi L., Bondavalli R., Cavallini G., Montermini A., Dradi D. (2006) Wild host plants of stolbur phytoplasma and its vector, *Hyalesthes obsoletus*, at sites of grapevine Bois noir occurrence in Emilia-Romagna, Italy. In *Extended Abstracts 15th Meeting ICVG, Stellenbosch, South Africa*, pp. 182–184.

Credi R., Terlizzi F., Reggiani N., Bacchiavini M. (2011) Vines healed from black wood with topping. *Informatore Agrario*, **67**, 60–63.

Cvrković T., Jović J., Mitrović M., Krstić O., Toševski I. (2014) Experimental and molecular evidence of *Reptalus panzeri* as a natural vector of bois noir. *Plant Pathology*, **63**, 42–53.

Franova J., Navratil M., Jakesova H. (2009) Molecular identification of stolbur phytoplasma associated with red clover dwarf disease symptoms. *Journal of Phytopathology*, **157**, 52–56.

Galetto L., Bosco D., Marzachì C. (2005) Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. *Annals of Applied Biology*, **147**, 191–201.

Garau R., Sechi A., Tolu G., Prota V.A., Lentini A., Prota U. (2004) *Goniagnathus guttulinervis* (Kirschbaum), new natural host of the Stolbur subgroup 16SrXII-A phytoplasmas in Sardinia. *Journal of Plant Pathology*, **86**, 179.

Gatineau F., Larrue J., Clair D., Lorton F., Richard-Molard M., Boudon-Padieu E. (2001) A new natural planthopper vector of stolbur phytoplasma in the genus *Pentastiridius* (Hemiptera: Cixiidae). *European Journal of Plant Pathology*, **107**, 263–271.

IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004) '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 1243–1255.

Johannesen J., Foissac X., Kehrli P., Maixner M. (2012) Impact of vector dispersal and host-plant fidelity on the dissemination of an emerging plant pathogen. *PLoS ONE*, **7**, e51809.

Kessler S., Schaerer S., Delabays N., Turlings T.C.J., Trivellone V., Kehrli P. (2011) Host plant preferences of *Hyalesthes obsoletus*, the vector of the grapevine yellows disease 'bois noir', in Switzerland. *Entomologia Experimentalis et Applicata*, **139**, 60–67.

Laimer M., Lemaire O., Herrbach E., Goldschmidt V., Minafra A., Bianco P., Wetzel T. (2009) Resistance to viruses,

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phytoplasmas and their vectors in the grapevine in Europe: a review. *Journal of Plant Pathology*, **91**, 7–23.

Langer M., Maixner M. (2004) Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. *Vitis*, **43**, 191–200.

Lessio F., Tedeschi R., Alma A. (2007) Population dynamics, host plants and infection rate with stolbur phytoplasma of *Hyalesthes obsoletus* Signoret in North-Western Italy. *Journal of Plant Pathology*, **89**, 97–102.

Maixner M. (1994) Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis*, **33**, 103–104.

Maixner M. (2007) Biology of *Hyalesthes obsoletus* and approaches to control this soilborne vector of Bois noir disease. *Bulletin OILB/SROP*, **30**, 3–9.

Maixner M. (2011) Recent advances in Bois noir research. *Petria*, **21**, 17–32.

Maixner M., Ahrens U., Seemüller E. (1995) Detection of the grapevine yellows MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology*, **101**, 241–250.

Maixner M., Johannesen J. (2013) The spatiotemporal distribution of *Hyalesthes obsoletus*, nettle host plants and stolbur phytoplasma in a fallow vineyard. In Proceedings of the 3rd European Bois Noir Workshop, Spain, pp. 37–39. Eds E. Torres, A. Laviña and A. Batlle. Cabrils, Spain: Institut de Recerca i Tecnologia Agroalimentáries.

Maixner M., Johannesen J., Michel K., Lux B., Seitz A. (2007) Host plant specificity of *Hyalesthes obsoletus* and consequences for "bois noir" epidemiology. *Bulletin of Insectology*, **60**, 399–400.

Marchi G., Contaldo N., Braccini P., Paltrinieri S., Rizzo D., Cinelli T., Bertaccini A. (2011) Epidemiology of 'bois noir' disease and molecular variability of associated phytoplasmas in organic vineyards in Tuscany (Italy). *Bulletin of Insectology*, **64**, S193–S194.

Marcone C., Ragozzino A., Seemüller E. (1997) Detection and identification of phytoplasmas in yellows-diseased weeds in Italy. *Plant Pathology*, **46**, 530–537.

Marzachì C., Veratti F., Bosco D. (1998) Direct PCR detection of phytoplasmas in experimentally infected insects. *Annals of Applied Biology*, **133**, 45–54.

Mori N., Pavan F., Bondavalli R., Reggiani N., Paltrinieri S., Bertaccini A. (2008) Factors affecting the spread of "Bois Noir" disease in north Italy vineyards. *Vitis*, **47**, 65–72.

Mori N., Pavan F., Reggiani N., Bacchiavini M., Mazzon L., Paltrinieri S., Bertaccini A. (2012) Correlation of bois noir disease with nettle and vector abundance in northern Italy vineyards. *Journal of Pest Science*, **85**, 23–28.

Mori N., Reggiani N., Pozzebon A., Duso C., Pavan F. (2011) Influence of nettle control along a ditch on spatial distribution of *Hyalesthes obsoletus* Signoret in a neighbouring vineyard. *IOBC/WPRS Bulletin*, **67**, 295–298.

Navratil M., Valova P., Fialova R., Lauterer P., Safarova D., Stary M. (2009) The incidence of stolbur disease and associated yield losses in vegetable crops in South Moravia (Czech Republic). *Crop Protection*, **28**, 898–904.

Olivier C.Y., Galka B., Seguin-Swartz G. (2010) Detection of aster yellows phytoplasma DNA in seed and seedlings of canola (*Brassica napus* and *B. rapa*) and AY strain identification. *Canadian Journal of Plant Pathology*, **32**, 298–305.

Osler R., Carraro L., Loi N., Refatti E. (1993) Symptom expression and disease occurrence of a Yellows disease of grapevine in Northeastern Italy. *Plant Disease*, **77**, 496–498.

Palermo S., Elekes M., Botti S., Ember I., Alma A., Orosz A., Bertaccini A., Kölber M. (2004) Presence of Stolbur phytoplasma in Cixiidae in Hungarian vineyards. *Vitis*, **43**, 201–203.

Perry J.N., Dixon P.M. (2002) A new method to measure spatial association for ecological count data. *Ecoscience*, **9**, 133–141.

Perry J.N., Winder L., Holland J.M., Alston R.D. (1999) Red-blue plots for detecting clusters in count data. *Ecology Letters*, **2**, 106–113.

Picciau L., Lavezzaro S., Morando A., Cesano A., Cuttini D., Saladini M.A., Alma A. (2010) Controlling the vector limits the black wood incidence in grapevines. *Informatore Agrario*, **25**, 57–59.

Pinzauti F., Trivellone V., Bagnoli B. (2008) Ability of *Reptalus quinquecostatus* (Hemiptera: Cixiidae) to inoculate stolbur phytoplasma to artificial feeding medium. *Annals of Applied Biology*, **153**, 299–305.

Quaglino F., Mori N., Casati P., Zorloni A., Zanini G., Bianco P.A. (2009) Further data on occurrence of grapevine yellows-associated phytoplasmas in vineyards of Veneto region (north-eastern Italy). *Progrès Agricole et Viticole*, **Horse Série**, 204–205.

Quaglino F., Zhao Y., Casati P., Bulgari D., Bianco P.A., Wei W., Davis R.E. (2013) '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology*, **63**, 2879–2894.

Rappussi M.C.C., Eckstein B., Flôres D., Haas I.C.R., Amorim L., Bedendo I.P. (2012) Cauliflower stunt associated with a phytoplasma of subgroup 16SrIII-J and the spatial pattern of disease. *European Journal of Plant Pathology*, **133**, 829–840.

Riedle-Bauer M. (2010) Stolbur phytoplasma (black wood disease) of grape: Current knowledge and new research results. *Mitteilungen Klosterneuburg*, **60**, 69–73.

Riedle-Bauer M., Hanak K., Sára A., Bauer H. (2010) Control of Bois noir and practices increasing biodiversity – a contradiction? *Mitteilungen Klosterneuburg*, **60**, 376–381.

Riedle-Bauer M., Mörtel J., Pastar M., Aryan A., Brader G. (2013) Mass occurrence of *Hyalesthes obsoletus* on *Urtica dioica* in Austria and sole presence of *tuf*-type b stolbur phytoplasma on stinging nettles, grapevine and in the transmitting insects. In Proceedings of the 3er European

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Bois Noir Workshop, Barcelona, Spain, 2013, pp. 26–27. Eds E. Torres, A. Laviña and A. Batlle. Cabrils, Spain: Institut de Recerca i Tecnologia Agroalimentáries.

Riedle-Bauer M., Sára A., Regner F. (2008) Transmission of a stolbur phytoplasma by the Agalliinae leafhopper *Anaceratagallia ribauti* (Hemiptera, Auchenorrhyncha, Cicadellidae). *Journal of Phytopathology*, **156**, 687–690.

Romanazzi G., D'Ascenzo D., Murolo S. (2009) Field treatment with resistance inducers for the control of grapevine bois noir. *Journal of Plant Pathology*, **91**, 677–682.

Sabaté J., Laviña A., Batlle A. (2014) Incidence of Bois Noir phytoplasma in different viticulture regions of Spain and Stolbur isolates distribution in plants and vectors. *European Journal of Plant Pathology*, **139**, 185–193.

Schneider B., Marcone C., Kampmann M., Ragozzino A., Lederer W., Cousin M.T., Seemüller E. (1997) Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA. *European Journal of Plant Pathology*, **103**, 675–686.

Sforza R., Boudon-Padieu E. (1998) The main vector of bois noir disease. *Phytoma*, **510**, 33–37.

Sforza R., Bourgoin T., Wilson S.W., Boudon-Padieu E. (1999) Field observations, laboratory rearing and descriptions of immatures of the planthopper *Hyalesthes obsoletus* (Hemiptera : Cixiidae). *European Journal of Entomology*, **96**, 409–418.

Sforza R., Clair D., Daire X., Larrue J., Boudon-Padieu E. (1998) The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of Bois noir of grapevine in France. *Journal of Phytopathology*, **146**, 549–556.

Škoric D., Šaric A., Vibio M., Murari E., Krajacic M., Bertaccini A. (1998) Molecular identification and seasonal monitoring of phytoplasmas infecting Croatian grapevines. *Vitis*, **37**, 171–175.

Wei W., Davis R.E., Lee I.-M., Zhao Y. (2007) Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 1855–1867.

Weintraub P.G., Beanland L. (2006) Insect vectors of phytoplasmas. *Annual Review of Entomology*, **51**, 91–111.

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