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RESEARCH ARTICLE

Transmission of '*Candidatus* Phytoplasma solani' by *Reptalus quinquecostatus* (*Hemiptera*: *Cixiidae*)

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Candidatus Phytoplasma solani'; Cixiidae; *Reptalus quinquecostatus*; Bois Noir; stolbur.

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Abstract

Transmission trials were carried out using *Reptalus quinquecostatus* (*Cixiidae*), a potential vector of '*Candidatus* Phytoplasma solani' ('*Ca.* P. solani'), to assess its ability to inoculate the phytoplasma to periwinkle (*Catharanthus roseus*), grapevine (*Vitis vinifera*), lavandin (*Lavandula* × *intermedia*) and tobacco (*Nicotiana tabacum*). Detection, genotyping and comparison of '*Ca.* P. solani' strains carried by *R. quinquecostatus* showed that *R. quinquecostatus* carried a higher diversity of '*Ca.* P. solani' than *Hyalesthes obsoletus*, major known vector of '*Ca.* P. solani' strains. Molecular analyses also showed the presence of a new strain only in grapevines and *R. quinquecostatus*. '*Ca.* P. solani' was successfully inoculated to periwinkles by *R. quinquecostatus*, but no transmission was achieved to the other tested plants. The ability to transmit '*Ca.* P. solani' to plants and observations of adults feeding on grapevines in vineyards consolidate the hypothesis that *R. quinquecostatus* is a specific vector of '*Ca.* P. solani' strains. Moreover, the discovery of a new genotype present in *R. quinquecostatus* and not in *H. obsoletus*, vector also present on grapes, suggests that *R. quinquecostatus* should have a direct role in '*Ca.* P. solani' epidemiology. Overall, even if *R. quinquecostatus* has a minor or no role in '*Ca.* P. solani' transmission from weeds to grapevines, it can have an indirect role in Bois Noir epidemiology. This planthopper can contribute to maintain an alternative '*Ca.* P. solani' cycle in weeds even in the absence of *H. obsoletus* preferentially by maintaining pathogen reservoirs in wild compartments neighbouring susceptible crops.

Introduction

Two serious phytoplasma grapevine diseases affect the European viticulture (Foissac & Maixner, 2013): the Flavescence dorée (FD) disease caused by a phytoplasma belonging to the elm group, subgroup 16SrV-C and -D (Martini *et al.*, 1999; IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004; Malembic-Maher *et al.*, 2011) and the Bois Noir disease caused by a phytoplasma belonging to the subgroup 16SrXII-A ['*Candidatus* Phytoplasma solani' ('*Ca.* P. solani')] (Quaglino *et al.*, 2013). The FD phytoplasma is transmitted by a unique insect vector, the leafhopper *Scaphoideus titanus* Ball essentially spreading across south-European vineyards (Chuche & Thiéry, 2014). '*Ca.* P. solani' epidemiology is more complex and involves an important range of host plants infected by several polyphagous vectors. Numerous insects were described as infected by this phytoplasma, while only a few were identified as true vectors, that is able to transmit the phytoplasma from one plant to another. Bois noir is a major grapevine disease affecting several grape production areas and probably underestimated in areas where FD is present. Bois noir disease is considered as an emergent problem in Europe with a recrudescence in Germany, Italy, Spain, Austria and some French regions associated with significant economic losses (Johannesen *et al.*, 2008; Belli *et al.*, 2010; Kuntzman *et al.*, 2014; Sabaté *et al.*, 2014).

Table 1 Insect vectors of '*Candidatus* Phytoplasma solani' other than *Hyalesthes obsoletus*

Family	Subfamily	Species	Plant/feeding medium
Cicadellidae	*Agalliinae*	*Anaceratagallia ribauti*	*Vicia faba*[1]
		Austroagallia (=*Peragallia*) *sinuata*	Feeding medium[2]
	Aphrodinae	*Aphrodes bicinctus*	*Vitis vinifera*[3]
			Callistephus chinensis[4,a]
			Trifolium hybridens[4,a]
			Trifolium repens[4,a]
	Deltocephalinae	*Cechenotettix quadrinotatus* (=*martini*)	*Lavandula* × *intermedia*[5,4,a]
		Euscelidius variegatus	*V. vinifera*[3]
		Euscelis lineolatus	Feeding medium[7]
		Euscelis obsoletus	*V. vinifera*[3]
		Euscelis plejebus[a]	*T. hybridens*[4,a]
			T. repens[4,a]
			Senecio vulgaris[4,a]
			Vinca rosea[4,a]
		Hardya tenuis	*Catharanthus roseus*[8]
		Macrosteles quadripunctulatus	*C. roseus*[9]
			Daucus carota[9]
			Solanum lycopersicum[9]
			V. vinifera[9]
		Psammotettix striatus	Feeding medium[8]
Cixiidae	*Cixiinae*	*Pentastiridius leporinus* (described as *Pentastiridius beieri*)	*Beta vulgaris*[10]
			C. roseus[10]
	Cixiinae	*Reptalus panzeri*	*V. vinifera*[11]
			Zea mays[12]
	Cixiinae	*Reptalus quinquecostatus*	Feeding medium[13]
Issidae	*Issinae*	*Issus* sp.	*V. vinifera*[3]
Triozidae		*Bactericera trigonica*	*D. carota*[14]

[1]Riedle-Bauer *et al.* (2008); [2]Sabaté *et al.* (2003); [3]Laviña *et al.* (2006); [4]Valenta *et al.* (1961); [5]Moreau & Leclant (1973); [6]Boudon-Padieu & Cousin (1999); [7]Landi *et al.* (2013); [8]Sabaté *et al.* (2003); [9]Batlle *et al.* (2008); [10]Gatineau *et al.* (2002); [11]Cvrković *et al.* (2014); [12]Jović *et al.* (2007); [13]Pinzauti *et al.* (2008); [14]Font *et al.* (1999).

[a]Symptomatic plant without pathogen characterisation.

The main insect vector of '*Ca.* P. solani' is the planthopper, *Hyalesthes obsoletus* Signoret (Sforza *et al.*, 1998), even though the incidence of '*Ca.* P. solani' is not always correlated to high densities of *H. obsoletus* populations (Batlle *et al.*, 2000). So far, more than 20 insect species have been demonstrated as '*Ca.* P. solani' vectors (Table 1), and above 50 additional species were found carrying '*Ca.* P. solani' without any evidence of plant inoculations (Table 2). Altogether, there is an increasing presumption that *H. obsoletus* may not be the only natural vector implicated in Bois Noir propagation in vineyards and several other vector species may contribute to the epidemiology of this disease.

In the vineyard agrosystem, different '*Ca.* P. solani' strains were found in wild plants, grapevines and insect vectors. The genetic characterisation of these strains helped not only to determine the reservoir of '*Ca.* P. solani' in crops and surroundings but also to associate vectors with such plant to plant transmissions (Langer & Maixner, 2004; Pacifico *et al.*, 2009; Fabre *et al.*, 2011*a*,*b*; Aryan *et al.*, 2014; Cvrković *et al.*, 2014). Besides *H. obsoletus*, three leafhopper and two planthopper species successfully transmit '*Ca.* P. solani' to grapevines (Table 1). Some other insects were found carrying '*Ca.* P. solani' in several vineyards indicating that these species are capable of feeding on infected plants. However, because of the specificity of the phytoplasma–vector relationship, only few insect species can transmit specific phytoplasma species. In order to be transmitted, phytoplasmas must pass through the intestinal barrier, penetrate cells of the salivary glands and multiply to high levels before being injected into plants with saliva during feeding (Weintraub & Beanland, 2006).

To demonstrate the ability of an insect species to be a vector of a specific phytoplasma without prior information on the host plant, an alternate identification method was developed by Zhang *et al.* (1998). This method is based on the detection of phytoplasma DNA in an artificial feeding medium in which a suspected vector previously fed in. This technique has the advantage to save time comparing with classical plant to plant transmission methods and to determine the seasonality of the infection when phytoplasmas are detected in the insect body all over the year (Tanne *et al.*, 2001). However, this method can also give false positives because phytoplasma DNA

Table 2 Insect bearing '*Candidatus* Phytoplasma solani' without transmission proof

Family	Subfamily	Species
Cicadellidae	*Agaliinae*	*Austragallia sinuata*[1]
		Dryodurgades reticulatus[2]
		Agallia laevis[3,4]
		Anaceratagallia venosa[4]
	Aphrodinae	*Aphrodes* sp.[3,5]
	Cicadellinae	*Cicadella viridis*[6,7]
	Deltocephalinae	*Adarrus taurus*[3]
		Anoplotettix fuscovenosus[2,7]
		Anoplotettix putoni[7]
		Balclutha sp.[2]
		Cicadula divaricata[1]
		Ciculifer haematoceps complex[8]
		Errastunus ocellaris[2]
		Euscelidius variegatus[1]
		Euscelis lineolatus[9]
		Euscelis sp.[5]
		Exitianus capicola[7]
		Goniagnathus guttulinervis[10]
		Macrosteles quadripunctulatus[8]
		Macrosteles sardus[2]
		Macrosteles sexnotatus[3]
		Macrosteles spp. (*M. cristatus*, *M. laevis*, *M. sexnotatus*)[2]
		Mocuellus collinus[2]
		Mocydia crocea[9]
		Neoaliturus fenestratus[3,8]
		Psammotettix alienus[4]
		Psammotettix confinis[4]
		Psammotettix spp. (*P. alienus*, *P. cephalothes*, *P. confinis*, *P. kolosvarensis*)[2]
		Thamnotettix zelleri[7]
	Typhlocybinae	*Emelyanoviana mollicula*[2]
		Eupteryx atropunctata[2]
		Zyginidia scutellaris[3,5]
Cixiidae	*Cixiinae*	*Hyalesthes luteipes*[7,11]
		Hyalesthes scotti[7]
		Reptalus cuspidatus[6,7]
Delphacidae	*Delphacinae*	*Laodelphax striatellus*[5,12]
		Toya propinqua[7]
Dictyopharidae	*Dictyopharinae*	*Dictyophara europaea*[7,13]
Membracidae	*Smiliinae*	*Stictocephala bisonia*[5]
Miridae	*Mirinae*	*Lygus* spp.[14]
Psyllidae		*Cacopsylla pyri*[15]
		Cacopsylla pyricola[15]
		Cacopsylla pyrisuga[15]

[1]Sabaté *et al.* (2003); [2]Riedle-Bauer *et al.* (2006); [3]Batlle *et al.* (2000); [4]Drobnjakovic *et al.* (2011); [5]Fos *et al.* (1992); [6]Mikec *et al.* (2006); [7]Alma *et al.* (2008); [8]Orenstein *et al.* (2003); [9]Sforza *et al.* (1998); [10]Garau *et al.* (2004); [11]Trivellone *et al.* (2005); [12]Sabaté *et al.* (2007); [13]Cvrković *et al.* (2011); [14]Březíková & Linhartová (2007); [15]Križanac *et al.* (2010).

can be detected in the feeding medium even if insects are not able to specifically transmit phytoplasmas to an host plant (Tanne *et al.*, 2001). In these cases, a possible multiplication of phytoplasmas in the midgut of those insects was suggested (Vega *et al.*, 1993). Importantly, this method does not consider any crucial interactions occurring between the vector, the phytoplasma and the plant during the establishment of a vector-borne disease (Alma *et al.*, 2001; Bressan *et al.*, 2005).

Reptalus quinquecostatus Dufour (*Cixiidae*) is commonly observed on grapevines in several European vineyards (Mazzoni, 2005; Mazzoni *et al.*, 2005; Alma *et al.*, 2008). Infected individuals were already collected on grapevines in Italy (Trivellone *et al.*, 2005; Alma *et al.*, 2008) and in Serbia (Cvrković *et al.*, 2014). No phytoplasma transmission to plants is yet achieved, but a successful demonstration of phytoplasma inoculation into a feeding medium has been reported (Pinzauti *et al.*, 2008). Furthermore, *R. quinquecostatus* specimens were also found in other crops infected by '*Ca.* P. solani' such as lavender (J.-L. Danet, personal communication).

Many '*Ca.* P. solani'-positive grapevines were found in a vineyard near Bordeaux area during a couple of years. Insect populations across the vineyard were frequently monitored and surprisingly, we recorded low population levels of *H. obsoletus* while *R. quinquecostatus* ones were abundant. Moreover, in other vineyards around Bordeaux area, specimens of *R. quinquecostatus* were also frequently found in the grapevine canopy during beating prospection (L. Delbac, personal communication).

The aim of this study is to determine the ability of *R. quinquecostatus* to be an efficient vector of '*Ca.* P. solani' and to evaluate its role in Bois Noir epidemic.

Materials and methods

Collection of insects

Insect were captured as adults in July 2012 and 2013 about 40 km east of Bordeaux (44°49′12″ N; 0°05′10″ W) in a 1.8 ha ground covered 'Cabernet-Sauvignon' plot surrounded with grapevine plots where many grapevines were found infected by '*Ca.* P. solani' the two previous years within a radius of 1 km (J. Chuche, unpublished data). *Convolvulus arvensis* was the predominant species in the ground vegetation, but *Poaceae* spp. and several dicotyledon plants, such as *Plantago* sp. were also present. Neither any *Urtica dioica* nor *Calystegia sepium* was observed. Neither insecticides nor herbicides were sprayed during the 2 years of sampling.

Reptalus sp. specimens caught were kept alive in cages with oat seedlings until further use (either, morphological identification, transmission assays or DNA extraction) within a quarantine greenhouse.

Morphological characterisation

Collected insects were individually identified according to Holzinger *et al.* (2003). External morphological features were observed with a stereomicroscope in order to determine family and genus. For species identification, male genitalia (aedeagus, paramere and anal tube) were placed in a 10% potassium hydroxide solution for 1 day to remove membranous soft tissues and conserve only chitinous tissues. Then, male genitalia were carefully dissected and observed under a stereomicroscope.

Insects DNAs extraction

Insect DNA was individually extracted from collected planthoppers, following the protocol described by Maixner *et al.* (1995). Briefly, whole insects were ground in 250 μL of cetyl-trimethyl-ammonium-bromide (CTAB)-based buffer: 2% w/v CTAB; 1.4 M NaCl; 20 mM EDTA pH 8.0; 100 mM Tris–HC1 pH 8.0; 0.2% β-mercaptoethanol. Male specimens were homogenised after removal of the aedeagus. After incubation at 65°C for 30 min, DNA was extracted with one volume of chloroform : isoamylalcohol (24:1) solution and then precipitated with ice-cold propan-2-ol. The DNA pellets were then washed with 70% ethanol and resuspended into 50 μL of sterile water.

Molecular identification of insects

To confirm morphological characterisations, a fragment of the mitochondrial cytochrome oxidase I gene (COI) was amplified using the primers C1-J-2195 (5′-TTGATTTT TTGGTCATCCAGAAGT-3′) and TL2-N-3014 (5′-TCCAA TGCACTAATCTGCCATATTA-3′) (Simon *et al.*, 1994). Polymerase chain reaction (PCR) amplifications were performed according to Bertin *et al.* (2010). Sequencing of PCR products were performed by Beckman Coulters Genomics (Takeley, UK). Raw sequence chromatograms were assembled and edited using GAP4 (Bonfield *et al.*, 1995). Multiple alignments were performed using ClustalW (Thompson *et al.*, 1994). The phylogenetic analyses were carried out with MEGA 4 using maximum of parsimony (Tamura *et al.*, 2007).

Plants DNA extraction

For plant DNA extractions, leaf midribs (0.5–1 g) were ground into 3 mL of CTAB buffer and DNA was extracted following the method described in Maixner *et al.* (1995). The DNA pellets were resuspended into 100 μL of sterile water.

Table 3 Number of plants and insects used in transmission trials

	Periwinkle	Grapevine	Lavandin	Tobacco
No. of plants used	10	10	7	3
No. of insect/plant	15	50	50	30
Total no. of insect used	150	500	350	90

'*Candidatus* P. solani' detection and genotyping

Detection and genotyping were carried out on the *Stamp* gene following (Fabre *et al.*, 2011*a*,*b*). Sequencing of PCR products and their subsequent analysis were performed as previously described in this study for molecular identifications of insects.

Transmission trials

Transmission trials were conducted with *R. quinquecostatus* captured in 2013. Collected adults were kept in polyglass cages at a constant temperature (23–25°C) and a photoperiod of 16:8 h. Transmission trials were carried out on healthy periwinkle (*Catharanthus roseus*), grapevine (*Vitis vinivera* cv. Chardonnay), lavandin (*Lavandula* × *intermedia* cv. Abrial) and tobacco plants (*Nicotiana tabacum* cv. ITB 683) (Table 3) for as long as the insects survived. The DNA was then extracted on each individual and tested for the presence of '*Ca.* P. solani.' Plants used in transmission experiments were treated with insecticides (Pyrevert, Valagro, France) and kept in an insect-proof greenhouse until symptoms appeared. Six months after the end of the trial, symptomatic and asymptomatic plants were tested for '*Ca.* P. solani' presence using PCR analysis.

Results

Field samplings

The majority of *R. quinquecostatus* adults were collected on vineyard ground cover whereas *H. obsoletus* specimens were only found in a mixed nettle and bindweed vegetation from a ditch near the plot. Within the 2 years of prospection, planthopper populations were variable with almost 200 *H. obsoletus* and 100 *R. quinquecostatus* collected in 2012 but only 10 *H. obsoletus* and more than 1000 *R. quinquecostatus* collected in 2013 (Table 4).

'*Ca.* P. solani' detection

Reptalus quinquecostatus harboured the highest diversity of '*Ca.* P. solani' strains (4), including one new genotype, whereas *H. obsoletus* carried only two different strains (Fig. 1). Three different '*Ca.* P. solani' genotypes were

Table 4 Numbers of planthoppers infected by '*Ca.* P. solani'

	Positive/tested		Infection rate (%)	
	Reptalus quinquecostatus	*Hyalesthes obsoletus*	*R. quinquecostatus*	*H. obsoletus*
2012	1/40	11/29	2.50	37.93
2013	54/623	0/10	8.67	0
Total	55/663	11/39	8.30	28.20

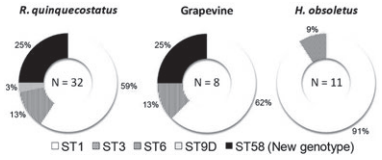

Figure 1 '*Ca.* P. solani' genotypes (gene *Stamp*) present in grapevine and plant-hoppers. ST9D is identical to Rqg31 (Cvrković *et al.*, 2014) and St_At9 (Aryan *et al.*, 2014). The new genotype, ST58 according to the nomenclature of the network SEE-ERANET Phytoplasma epidemiology in Southeastern Europe (Foissac *et al.*, 2013), is a one single nucleotide polymorphism variant of the main European ST1 genotype.

found in surrounding grapevines, two of them only carried by *R. quinquecostatus*. Most of the positive samples obtained from both planthopper and plant samples contained the ST1 genotype. Interestingly, the proportion of ST1, ST6 and the new genotype ST58 in grapevines and *R. quinquecostatus* were very similar.

The '*Ca.* P. solani' isolate RQ161 sequence, corresponding to the newly identified ST58 genotype, has been deposited in the European Nucleotide Archive database (GenBank accession number LN823951).

Transmission trials

Almost 1000 insects were used in the different transmission assays. Survival of planthoppers on tested plants was assessed and two groups were identified. First, grapevine, lavandin and periwinkle plants allowed more than 50% of the insects to be still alive 4 days after the beginning of the trial, and tobacco plants on the other side with 90% of the insects dead during the first 24 h (Fig. 2).

The percentage of infective insects was low (8.67%, 54/623), and there was similar for males and females (8.11%, 33/407 vs. 9.72%, 21/216). The PCR analysis performed on insects showed there was a high variability in insect infection rates between trials conducted with each tested plant. The highest rate for '*Ca.* P. solani' transmission was observed for the trial looking at tobacco inoculations and the lowest was recorded during grapevine transmissions (almost five times less) (Table 5).

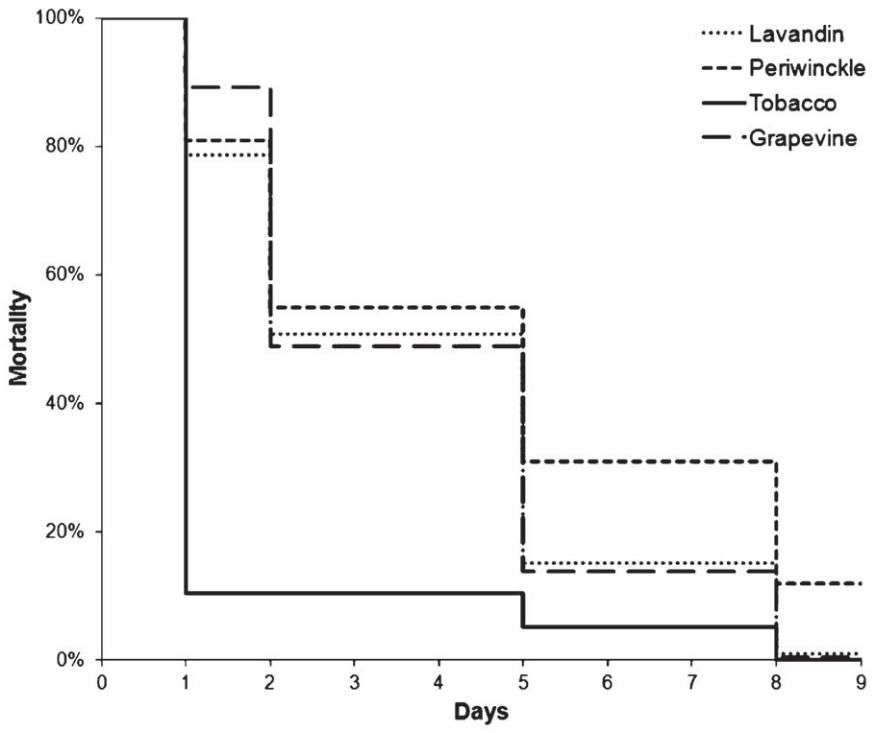

Figure 2 Survival of *Reptalus quinquecostatus* on different plants.

Table 5 Infection of *Reptalus quinquecostatus* used for transmission trials and transmission efficiency to different plants

	Insects		Plants	
	Positive/ tested	Infection rate (%)	Positive/ tested	Inoculation efficiency (%)
Periwinkle	8/84	9.52	2/10	20
1	0/10	0.00		
2	0/12	0.00		
3	0/8	0.00		
4	0/4	0.00		
5	0/10	0.00		
6[a]	2/10	20.00		
7[a]	5/8	62.50		
8	0/9	0.00		
9	1/8	12.50		
10	0/5	0.00		
Grapevine	17/285	5.96	0/10	0
1	2/15	13.33		
2	2/30	6.67		
3	1/28	3.57		
4	0/33	0.00		
5	1/27	3.70		
6	1/37	2.70		
7	2/35	5.71		
8	1/34	2.94		
9	4/29	13.79		
10	3/17	17.65		
Lavandin	23/225	10.22	0/7	0
1	9/34	26.47		
2	5/8	2.38		
3	1/42	10.34		
4	3/29	21.74		
5	5/23	6.67		
6	2/30	6.90		
7	1/38	2.63		
Tobacco	10/39	25.64	0/3	0
1	6/13	46.15		
2	1/14	7.14		
3	3/12	25.00		

[a]Transmission success.

None of the crop was infected by '*Ca.* P. solani' and only two periwinkles sorted out to be positives for '*Ca.* P. solani' after transmission with *R. quinquecostatus* (Table 5). Both ST1 and ST6 genotypes were transmitted to periwinkles.

Discussion

This study conducted on wild specimens of *R. quinquecostatus* naturally infected with '*Ca.* P. solani' and collected in a production vineyard, demonstrates that *R. quinquecostatus* can successfully inoculate '*Ca.* P. solani' to plants. This confirms previous results showing the presence of phytoplasma in sucrose medium in which *R. quinquecostatus* had been fed (Pinzauti *et al.*, 2008).

The rate of infected insects was found rather low (8.30%) compared with those commonly observed in other European vineyards, that is between 15% and 76% (Pinzauti *et al.*, 2008; Berger *et al.*, 2009; Cvrković *et al.*, 2011; Sabaté *et al.*, 2014). Characterisation of the '*Ca.* P. solani' isolates carried by *R. quinquecostatus* showed that this cixiid appears to visit the same plant species as *H. obsoletus*. Some specimens were infected by isolates typical of nettles (ST6), some others infected with bindweed isolates (ST1) (Foissac *et al.*, 2013). Moreover, a new '*Ca.* P. solani' strain (ST58) was detected only in *R. quinquecostatus*. There was no evidence of strain specificity for an insect species vector of '*Ca.* P. solani' before the specificity observed here for the strain ST58. The similar composition of '*Ca.* P. solani' genotypes in *R. quinquecostatus* and grapevines, particularly the presence of ST6 and the new ST58 genotype, associated with the absence of this genotype in *H. obsoletus*, appears to be an indirect proof of the ability of *R. quinquecostatus* to transmit '*Ca.* P. solani' to grapevines. However, as found by Cvrković *et al.* (2014), no transmission to grapevines by *R. quinquecostatus* could be achieved in a greenhouse assay.

The discovery of a new genotype (ST58) present in *R. quinquecostatus* and not in *H. obsoletus*, insect but present in grapevines, questions the origin of the *R. quinquecostatus*/ST58 association. Assuming that the difference observed between *R. quinquecostatus* and *H. obsoletus* is not because of sampling, the relationship between former and new phytoplasma strains could be the result of: (a) a differential feeding behaviour of *R. quinquecostatus* on wild host plants harbouring this strain and not visited by *H. obsoletus*, or (b) a better acquisition of this strain by *R. quinquecostatus*, or (c) the incapacity to *H. obsoletus* to acquire this strain. In the first case, we could hypothesise that there is an unidentified plant species in this specific vineyard that could constitute a reservoir for '*Ca.* P. solani'. *R. quinquecostatus* would do its life cycle on this plant and would acquire phytoplasmas as nymphal instars by feeding on root phloem. Once adults and infective, individuals would disseminate phytoplasmas to other plants, such as grapevines. *R. quinquecostatus* could also transfer the new strain to plant where *H. obsoletus* can acquire it, creating new epidemic cycles. It cannot be excluded that the ST58 strain could be transmitted to grapevine by another vector species while *R. quinquecostatus* propagated this strain only to wild plants. This possibility appears to be unlikely because we did not observed any other putative planthopper and leafhopper vector species during our samplings.

The role of *R. quinquecostatus* in '*Ca.* P. solani' epidemiology still needs to be clarified in agrosystems and especially during the transmission from weeds to grapevines. It could have a direct or an indirect role in Bois Noir epidemiology. This planthopper can contribute to maintain an alternative '*Ca.* P. solani' cycle in weeds

even in the absence of *H. obsoletus* by maintaining a pathogen reservoir near crops. Transmission trials succeeded to inoculate '*Ca.* P. solani' phytoplasma to *C. roseus*, but not to the crop plants tested (tobacco, grapevine and lavandin). Many reasons can explain this difference in inoculation success. *R. quinquecostatus* has its best survival rate on *C. roseus* compared with the other tested plants allowing an extended feeding period. Because inoculation efficiency depends on the period that an infective vector has access to a host plant (Purcell, 1982), the transmission capability of *R. quinquecostatus* on crop plants could have been under-evaluated. *R. quinquecostatus* insects used for transmissions to tobacco plants had the highest infection rate but most of the vectors died within the first 24 h limiting or preventing any inoculation events of '*Ca.* P. solani' to occur. Thus, the low infection rates observed associated with a poor survival of *R. quinquecostatus* on grapevines (or crops or plants) could explain the low inoculation rates obtained. But other factors can affect the transmission efficiency of a phytoplasma by its vector. Indeed, transmission success depends greatly on the relationship between the insect vector, the host plant and pathogen strains (Alma *et al.*, 2001; Bressan *et al.*, 2005; Lopes *et al.*, 2009). The lack of phytoplasma inoculations to grapevines, lavandin and tobacco could also be because of a poor suitability of these plants to *R. quinquecostatus* feeding. Hemipteran sap-feeders feeding on plants resistant to insects see their probing and feeding behaviours modified probing less into phloem vessels and more into the xylem sap (Lopes *et al.*, 2009). In this way, phytoplasma vectors feeding on non-suitable plants spend less time feeding into phloem vessels and thus, are not capable of injecting enough phytoplasmas into phloem to initiate an infection.

Rates of infection for males and females were similar (8.11% and 9.72%, respectively) as previously reported by Pinzauti *et al.* (2008). This contrasts with the vector of the FD, *S. titanus*, having males generally found more often infected than females (Maixner *et al.*, 1993; Lessio *et al.*, 2009). This difference in phytoplasma infections between males and females was partly explained as a consequence of different feeding behaviours (Chuche & Thiery, 2014). Because the dynamics of phytoplasma acquisitions and transmissions by a vector are closely linked to its feeding behaviour, especially the time spent feeding into phloem sap (Purcell, 1982), we hypothesised that males and females have close feeding behaviours and should contribute with the same proportions to '*Ca.* P. solani' epidemiology.

Our study demonstrated for the first time that *R. quinquecostatus* is an effective vector of '*Ca.* P. solani' to plants, and could contribute to the epidemic of Bois noir in vineyards, at least by maintaining pathogen reservoirs in wild plants. The ability of this insect to inoculate the '*Ca.* P. solani' to susceptible crops, such as grapevines, remains to be determined before considering a possible direct role of *R. quinquecostatus* in '*Ca.* P. solani' epidemiology.

'*Candidatus* P. solani'-highly infected *R. quinquecostatus* adults are frequently found in vineyards and are often observed feeding on grapevines. Because *R. quinquecostatus* can transmit '*Ca.* P. solani' to plants and was found carrying the same genotypes than those found in grapevine, its role in Bois Noir epidemiology deserves to be further investigated.

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References

Alma A., Palermo S., Boccardo G., Conti M. (2001) Transmission of Chrysanthemum yellows, a subgroup 16SrI-B phytoplasma, to grapevine by four leafhopper species. *Journal of Plant Pathology*, **83**, 181–187.

Alma A., Lessio F., Picciau L., Tota F., Forte V., Borgo M., Bagnoli B., Pinzauti F., Trivellone V., Rapisarda C., Cavalieri V., D'Urso V. (2008) Rapporti tra cicaline, fitoplasmi e piante ospiti nell'agroecosistema vigneto. *Petria*, **18**, 257–260.

Aryan A., Brader G., Mörtel J., Pastar M., Riedle-Bauer M. (2014) An abundant '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus*. *European Journal of Plant Pathology*, **140**, 213–227.

Batlle A., Martínez M.A., Laviña A. (2000) Occurrence, distribution and epidemiology of Grapevine Yellows in Spain. *European Journal of Plant Pathology*, **106**, 811–816.

Batlle A., Altabella N., Sabaté J., Laviña A. (2008) Study of the transmission of stolbur phytoplasma to different crop species, by *Macrosteles quadripunctulatus*. *Annals of Applied Biology*, **152**, 235–242.

Belli G., Bianco P.A., Conti M. (2010) Grapevine yellows in Italy: 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.

Bertin S., Picciau L., Ács Z., Alma A., Bosco D. (2010) Molecular differentiation of four *Reptalus* species (Hemiptera: Cixiidae). *Bulletin of Entomological Research*, **100**, 551–558.

Bonfield J.K., Smith K.F., Staden R. (1995) A new DNA sequence assembly program. *Nucleic Acids Research*, **23**, 4992–4999.

Boudon-Padieu E., Cousin M.T. (1999) Yellow decline of *Lavandula hybrida* Rev and *L. vera* DC. *International Journal of Tropical Plant Diseases*, **17**, 1–34.

Bressan A., Spiazzi S., Girolami V., Boudon-Padieu E. (2005) Acquisition efficiency of Flavescence dorée phytoplasma by *Scaphoideus titanus* Ball from infected tolerant or susceptible grapevine cultivars or experimental host plants. *Vitis*, **44**, 143–146.

Březíková M., Linhartová S. (2007) First report of potato stolbur phytoplasma in hemipterans in southern Moravia. *Plant Protection Science*, **43**, 73–76.

Chuche J., Thiéry D. (2014) Can differences in feeding behaviour between *Scaphoideus titanus* males and females be related to phytoplasma transmission efficiency? *IOBC-WPRS Bulletin*, **105**, 177–183.

Chuche J., Thiéry D. (2014) Biology and ecology of the Flavescence dorée vector *Scaphoideus titanus*: a review. *Agronomy for Sustainable Development*, **34**, 381–403.

Cvrković T., Jović J., Mitrović M., Krstić O., Krnjajić S., Toševski I. (2011) Potential new hemipteran vectors of stolbur phytoplasma in Serbian vineyards. *Bulletin of Insectology*, **64**, S129–S130.

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.

Drobnjaković T., Perić P., Marčić D., Picciau L., Alma A., Mitrović J., Duduk B., Bertaccini A. (2011) Leafhoppers and cixiids in phytoplasma-infected carrot fields: species composition and potential phytoplasma vectors. *Pesticidi i Fitomedicina*, **25**, 311–318.

Fabre A., Balakishiyeva G., Ember I., Omar A., Acs Z., Kölber M., Kauzner L., Della Bartola M., Danet J.-L., Foissac X. (2011a) *StAMP* encoding the antigenic membrane protein of stolbur phytoplasma is useful for molecular epidemiology. *Bulletin of Insectology*, **64**, S21–S22.

Fabre A., Danet J.-L., Foissac X. (2011b) The stolbur phytoplasma antigenic membrane protein gene *stamp* is submitted to diversifying positive selection. *Gene*, **472**, 37–41.

Foissac X., Maixner M. (2013) Spread of grapevine phytoplasma diseases in Europe. *Phytopathogenic Mollicutes*, **3**, 47–50.

Foissac X., Carle P., Fabre A., Salar P., Danet J.L., STOLBUR-EUROMED consortium. (2013) '*Candidatus* Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. Invited conference. In E. Torres, Lavina A., Batlle A. (ed.), Third European Bois Noir Workshop, Barcelona, Spain, pp. 11–13.

Font I., Abad P., Albiñana M., Espino A.I., Dally E.L., Davis R.E., Jorda C. (1999) Amarilleos y enrojecimientos en zanahoria: una enfermedad a diagnóstico. *Boletin de Sanidad Vegetal – Plagas*, **25**, 405–416.

Fos A., Danet J.L., Zreik L., Garnier M., Bove J.M. (1992) Use of a monoclonal antibody to detect the stolbur mycoplasmalike organism in plants and insects and to identify a vector in France. *Plant Disease*, **76**, 1092–1096.

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 phytoplasma in Sardinia. *Journal of Plant Pathology*, **86**, 179–179.

Gatineau F., Jacob N., Vautrin S., Larrue J., Lherminier J., Richard-Molard M., Boudon-Padieu E. (2002) Association with the syndrome "basses richesses" of sugar beet of a phytoplasma and a bacterium-like organism transmitted by a *Pentastiridius* sp. *Phytopathology*, **384**, 384–392.

Holzinger W.E., Kammerlander I., Nickel H. (2003) *The Auchenorrhyncha of Central Europe. Volume 1: Fulgoromorpha, Cicadomorpha excl. Cicadellidae*. Leiden, the Netherlands: Brill.

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., Lux B., Michel K., Seitz A., Maixner M. (2008) Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe. *Entomologia Experimentalis et Applicata*, **126**, 217–227.

Jović J., Cvrković T., Mitrović M., Krnjajić S., Redinbaugh M.G., Pratt R.C., Gingery R.E., Hogenhout S.A., Toševski I. (2007) Roles of stolbur phytoplasma and *Reptalus panzeri* (Cixiinae, Auchenorrhyncha) in the epidemiology of Maize redness in Serbia. *European Journal of Plant Pathology*, **118**, 85–89.

Križanac I., Mikec I., Budinščak Ž., Musić M.Š., Škorić D. (2010) Diversity of phytoplasmas infecting fruit trees and their vectors in Croatia. *Journal of Plant Diseases and Protection*, **117**, 206–213.

Kuntzman P., Foissac X., Beccavin I., Chambin C., Choloux S., Coarer M., Delorme G., Méjean I., Molot B., Mourot F., Renard I., Rouchaud E., Savarit P., Viguès V. (2014) Bois noir de la vigne: synthèse des dernières observations. *Phytoma*, **679**, 31–36.

Landi L., Isidoro N., Riolo P. (2013) Natural phytoplasma infection of four phloem-feeding Auchenorrhyncha across vineyard agroecosystems in Central-Eastern Italy. *Journal of Economic Entomology*, **106**, 604–613.

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

Laviña A., Sabaté J., Batlle A. (2006) Spread and transmission of Bois noir phytoplasma in two regions of Spain. In Extended Abstracts 15th Meeting ICVG, Stellenbosch, South Africa, pp. 218–220.

Lessio F., Tedeschi R., Pajoro M., Alma A. (2009) Seasonal progression of sex ratio and phytoplasma infection in *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). *Bulletin of Entomological Research*, **99**, 377–383.

Lopes J.R.S., Daugherty M.P., Almeida R.P.P. (2009) Context-dependent transmission of a generalist plant pathogen: host species and pathogen strain mediate insect vector competence. *Entomologia Experimentalis et Applicata*, **131**, 216–224.

Maixner M., Pearson R.C., Boudon-Padieu E., Caudwell A. (1993) *Scaphoideus titanus*, a possible vector of Grapevine Yellows in New York. *Plant Disease*, **77**, 408–413.

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

Malembic-Maher S., Salar P., Filippin L., Carle P., Angelini E., Foissac X. (2011) Genetic diversity of European phytoplasmas of the 16SrV taxonomic group and proposal of 'Candidatus Phytoplasma rubi'. *International Journal of Systematic and Evolutionary Microbiology*, **61**, 2129–2134.

Martini M., Murari E., Mori N., Bertaccini A. (1999) Identification and epidemic distribution of two *flavescence doree*-related phytoplasmas in Veneto (Italy). *Plant Disease*, **83**, 925–930.

Mazzoni V. (2005) Contribution to the knowledge of the Auchenorrhyncha (Hemiptera Fulgoromorpha and Cicadomorpha) of Tuscany (Italy). *Redia*, **88**, 85–102.

Mazzoni V., Santinelli C., Lucchi A., Santoni M., Porcacchia C., Santini L. (2005) Le cicaline del vigneto in Umbria. *L'Informatore Agrario*, **61**, 73–76.

Mikec I., Križanac I., Budinščak Ž., Musič M.Š., Krajačić M., Škorić D. (2006) Phytoplasmas and their potential vectors in vineyards of indigenous Croatian varieties. In 15th Meeting ICVG, Stellenbosch, South Africa, pp. 255–257.

Moreau J.P., Leclant F. (1973) Contribution a l'étude de deux insectes du lavandin, *Hyalesthes obsoletus* Sign. et *Cechenotettix martini* Leth. (Hom. Auchenorrh.) *Annales de Zoologie, Ecologie Animale*, **5**, 361–364.

Orenstein S., Zahavi T., Nestel D., Sharon R., Barkalifa M., Weintraub P.G. (2003) Spatial dispersion patterns of potential leafhopper and planthopper (Homoptera) vectors of phytoplasma in wine vineyards. *Annals of Applied Biology*, **142**, 341–348.

Pacifico D., Alma A., Bagnoli B., Foissac X., Pasquini G., Tessitori M., Marzachi C. (2009) Characterization of Bois Noir isolates by restriction fragment length polymorphism of a Stolbur-specific putative membrane protein gene. *Phytopathology*, **99**, 711–715.

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.

Purcell A.H. (1982) Insect vector relationships with procaryotic plant pathogens. *Annual Review of Phytopathology*, **20**, 397–417.

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.

Riedle-Bauer M., Tiefenbrunner W., Otreba J., Hanak K., Schildberger B., Regner F. (2006) Epidemiological observations on Bois Noir in Austrian vineyards. *Mitteilungen Klosterneuburg, Rebe und Wein, Obstbau und Fruchteverwertung*, **56**, 166–170.

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.

Sabaté J., Laviña A., Batlle A. (2003) Potential vectors of grapevine Bois noir phytoplasma in Spain and evaluation of their transmission capacity. In 14th Meeting ICVG, Locorotondo, Italy, 113 pp.

Sabaté J., Laviña A., Legorburu J., Fortanete J., Pérez de Obanos J.J., Pérez Marín J.L., Reyes J., Batlle A. (2007) Incidence of "Bois Noir" phytoplasma in different wine-growing regions of Spain and its relation to *Hyalesthes obsoletus*. *Bulletin of Insectology*, **60**, 367–368.

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.

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 grapevines in France. *Journal of Phytopathology*, **146**, 549–556.

Simon C., Frati F., Beckenbach A., Crespi B., Liu H., Flook P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. *Annals of the Entomological Society of America*, **87**, 651–701.

Tamura K., Dudley J., Nei M., Kumar S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596–1599.

Tanne E., Boudon-Padieu E., Clair D., Davidovich M., Melamed S., Klein M. (2001) Detection of phytoplasma by polymerase chain reaction of insect feeding medium and its use in determining vectoring ability. *Phytopathology*, **91**, 741–746.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,

position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.

Trivellone V., Pinzauti F., Bagnoli B. (2005) *Reptalus quinquecostatus* (Dufour) (Auchenorrhyncha Cixiidae) as a possible vector of stolbur-phytoplasma in a vineyard in Tuscany. *Redia*, **LXXXVIII**, 103–108.

Valenta V., Musil M., Mišiga S. (1961) Investigation on European yellows-type viruses. I. The stolbur virus. *Phytopathologische Zeitschrift*, **42**, 1–38.

Vega F.E., Davis R.E., Barbosa P., Dally E.L., Purcell A.H., Lee I.-M. (1993) Detection of a plant pathogen in a nonvector insect species by the polymerase chain reaction. *Phytopathology*, **83**, 621–624.

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

Zhang J., Miller S., Hoy C., Zhou X., Nault L. (1998) A rapid method for detection and differentiation of aster-yellows phytoplasma-infected and inoculative leafhoppers. *Phytopathology*, **88**, S84.