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RESEARCH PAPERS

Occurrence of different phytoplasma infections in wild herbaceous dicots growing in vineyards affected by bois noir in Tuscany (Italy)

Guido MARCHI[1], Tamara CINELLI[1], Domenico RIZZO[2], Luciana STEFANI[2] ,Emanuele GOTI[3], Michele DELLA BARTOLA[4], Andrea LUVISI[4], Alessandra PANATTONI[4] and Alberto MATERAZZI[4]

[1] Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Sezione di Patologia Vegetale ed Entomologia, Università degli Studi, Piazzale delle Cascine 28, 50144 Firenze, Italy
[2] Servizio Fitosanitario Regione Toscana, Laboratorio di Diagnostica Fitopatologica e di Biologia Molecolare, Via Ciliegiole 99, 51100, Pistoia, Italy
[3] Centro Interdipartimentale di Servizi per le Biotecnologie di Interesse Agrario, Chimico e Industriale, Università degli Studi, Via Romana 21, 50125 Firenze, Italy
[4] Dipartimento di Scienze Agrarie, Alimentari ed Agro-ambientali, Università di Pisa, Via del Borghetto 80, 56124 Pisa, Italy

Summary. Wild herbaceous dicotyledonous plants (dicots) showing symptoms ascribable to phytoplasma disorders were found to be widely distributed in organic vineyards in central Tuscany (Italy) affected by bois noir, a grapevine yellows disease caused by "*Candidatus* Phytoplasma solani". In 2010 symptomatic dicots were tentatively identified to species level and the incidence of symptoms estimated in two selected vineyards in the province of Florence. Incidence ranged from 2 to 77%, and was not related to the relative abundance of hosts since very common species as well as relatively rare ones were consistently found to be symptomatic. PCR indexing and 16S rRNA sequence analyses indicated that two phytoplasmas co-existed in the vineyards: "*Ca.* P. solani", infecting the root systems of 17 taxa, and a phytoplasma closely related to "*Ca.* P. phoenicium", infecting 11 taxa, and occasionally co-infecting the same plant. Regardless of the high frequency of both pathogens in the vineyards, only "*Ca.* P. solani" could be detected in the grapevines. Population screening by means of *tuf* sequence analyses revealed the presence of only the tuf-b "*Ca.* P. solani" type both in dicot hosts and grapevine. This supports current notions of bois noir epidemiology, indicating that some infected dicots act as sources of "*Ca.* P. solani" inoculum whereas others are dead-end hosts. When the same specimens were screened by sequence analysis of the *vmp1* gene, evidence was found that different phytoplasma genotypes may be predominant in grapevines and dicots.

Key words: wild plants, root infections, bois noir epidemiology, "*Candidatus* Phytoplasma phoenicium".

Introduction

In Tuscany (Italy), bois noir (BN) is a frequently occurring grapevine yellows (GY), a complex of diseases caused by phytoplasmas inducing similar symptoms, including leaf discoloration and roll, incomplete lignification of canes, reduced growth, and shriveled bunches (Musetti, 2008). Specifically, BN is caused by a phytoplasma belonging to the 16SrXII-A ribosomal subgroup currently classified as "*Candidatus* Phytoplasma solani" (Quaglino *et al*., 2013). This pathogen has a wide host range and is most commonly vectored to grapevine by the polyphagous cixids *Hyalesthes obsoletus* (Johannesen *et al*., 2008; Musetti, 2008) and, in the Balkans, *Reptalus panzeri* (Cvrković *et al*., 2014). Grapevines become dead-end hosts for "*Ca.* P. solani" as *H. obsoletus* larvae, which are responsible for acquiring this phytoplasma, cannot develop on *Vitis vinifera* (Johannesen *et al*., 2008). Therefore, the spatial spread of BN most likely does not rely on the diffusion of "*Ca.* P. solani" from vine to vine but on other plant species, both spontaneous and cultivated, some of which can host the vec-

Corresponding author: G. Marchi
E-mail: guido.marchi@unifi.it

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tor for its reproduction, and therefore be sources of the pathogen. In European vineyards two major epidemiological cycles have been described for BN according to the plant species acting as the primary inoculum source for vines, and the subtype of "*Ca.* P. solani" infecting grapevine as defined by a Single Nucleotide Polymorphism (SNP) on the constitutive EF Tu encoding (*tuf*) gene (Langer and Maixner, 2004; Pacifico *et al.*, 2009). In the first cycle *Urtica dioica* (stinging nettle) and the narrow host range tuf-a sub-population of "*Ca.* P. solani" are involved. The second cycle involves the wide host range tuf-b subpopulation and various herbaceous species, among which *Convolvulus arvensis* (field bindweed) is most commonly infected. Very recently, the presence of another *tuf* subpopulation that can infect vines as well as *Urtica* spp. has been demonstrated (Aryan *et al.*, 2014). According to nucleotide sequence analyses, this new *tuf* sub-population, assigned as tuf-b2, is currently widespread in Austrian vineyards as well as in Macedonia and Montenegro (Atanasova *et al.*, 2015; Kosovac *et al.*, 2015). This new population shares 99% identity with "*Ca.* P. solani" *tuf* sequences, commonly infects stinging nettle but displays a PCR-RFLP pattern that is identical to tuf-b from bindweed (Aryan *et al.*, 2014). Nevertheless, spatial pattern analysis has shown that BN incidence may be greater near vineyard borders, strongly suggesting inwards movements of the vectors from spontaneous plants growing nearby, as well as a within vineyard association between spontaneous plants, *H. obsoletus* and diseased vines (Maixner *et al.*, 2007; Mori *et al.*, 2008, Mori *et al.*, 2015). The presence in Italian vineyards of wild herbaceous dicotyledonous plants (dicots), other than stinging nettle and bindweed, showing symptoms typical of those caused by phytoplasmas (Schneider *et al.*, 1997) has long been observed (Vidano, 1988). However, in many circumstances their role in the epidemiology of BN within vineyards has remained uncertain, either because the specific presence of "*Ca.* P. solani" could not be detected (Angelini *et al.*, 2004; Tolu *et al.*, 2006; Berger *et al.*, 2009), or because the *tuf* sub-population type that was found did not consistently match that present in vines (Pasquini *et al.*, 2007; Mori *et al.*, 2008; Berger *et al.*, 2009; Mori *et al.*, 2015).

In Tuscany, one of the most important grapevine growing regions of Italy, the role of dicots as potential reservoirs of phytoplasma inoculum for grapevines is currently being investigated. To improve knowledge of epidemiology and management of grapevine yellows, we selected two organic vineyards with previous history of BN (Braccini *et al.*, 2010) and, for the first time, quantified the relative distribution of the different dicots showing symptoms ascribable to phytoplasma disorders. Composition and comparison of diversities of phytoplasma populations infecting dicots and grapevines were determined using nested and real-time PCR indexing targeting the 16S rRNA gene as well as by sequencing a portion of the 16S rRNA, *tuf* and *vmp1* genes. Previous studies have shown that *vmp1* is specific for "*Ca.* P. solani", is highly polymorphic, and is undergoing positive selection. The encoded putative membrane protein VMP1 also contains domain repeats that may vary in number according to the isolate (Cimerman *et al.*, 2009; Pacifico *et al.*, 2009; Johannesen *et al.*, 2012). Recently, the existence of a characteristic pentapeptide possibly acting as a signature sequence for the "*Ca.* P. solani" tuf-a genotypes has been described (Johansen *et al.*, 2012). Thus, with respect to the highly conserved 16S rRNA and *tuf* genes, *vmp1* represents a far more variable marker for differentiating isolates by PCR-RFLP (Murolo *et al.*, 2014) or, more accurately, by sequence analysis (Cimerman *et al.*, 2009; Murolo *et al.*, 2010; Murolo *et al.*, 2013).

Materials and methods

Experimental vineyards and disease incidence data collection

Vineyards, Cs and Fd, were established, respectively, in 2001 and 2002, in the province of Florence (Tuscany, Italy), approx. 8 km from each other. Vines of cv. Sangiovese (red berry) were planted at spacings of 0.6 m between vines in rows 3 m apart (Cs) and 0.6 m between vines in rows 2.5 m apart (Fd), in calcareous loam-clay soils. Both vineyards were maintained according to organic principles (Council Regulation (EC) No 834/2007 and Commission Regulation (EC) No 889/2008), and weed control was only by mechanical mowing. In vineyard Cs mowing was performed two to three times a year, both between and within rows, while in vineyard Fd mowing was done once a year and only within the rows. Grapevine yellows disease incidence data were collected during October 2010 in plots of 2,539 (Cs) and 472 (Fd) vines. Bidimensional maps with locations of all symptomatic and asymptomatic vines

were created, and the total annual disease incidence of each plot was calculated as the number of plants that were visibly diseased relative to the total number of plants assessed.

In 2010, several dicot taxa that were part of the vineyard flora showed symptoms that presumptively could be ascribed to phytoplasma infections, including yellowing or reddening of the leaves and stems, stunted growth, virescence, phyllody and other abnormalities of the flowers. In order to estimate their relative abundance within each vineyard plot, a matrix with cells of four × four vines was overlaid on the map of each vineyard, and arranged so that one axis of the matrix was parallel to the direction of plant rows and the other axis was perpendicular, obtaining 160 cells in vineyard Cs, and 30 cells in vineyard Fd. A vine was selected randomly within each cell, and the dicots growing in its surroundings (over rectangular cell areas of 3.6 m^2 in plot Cs and 3 m^2 in plot Fd, centered on the selected plant) were identified to genus or species level according to dichotomous keys (Tutin, 1976; Pignatti, 1982). The occurrence of symptoms was always recorded. Presence/absence data were compiled for all identified taxa per matrix cell, and the frequency of each taxon in the vineyard floor was estimated as the percentage of cells in which each taxon was found (Greig-Smith, 1982). Incidence of symptoms was estimated for each taxon as the percentage of cells in which symptomatic specimens were present.

Field sample collection and nucleic acids extraction

Leaf samples were collected from each symptomatic vine during October 2010. Each sample, usually consisting of between ten and 15 leaves, was processed independently. Overall, 65 (plot Cs) and 10 (plot Fd) samples were collected from symptomatic vines. In the same month, roots were excised from 83 (Cs) and 52 (Fd) symptomatic dicots. The roots were thoroughly washed under running tap water, and air-dried. Total nucleic acids were extracted from grapevine leaf veins or from the roots of dicots collected from single plants using a variant of the CTAB method (Angelini *et al.*, 2001) and a MM400 steel bead mixer mill (Retsch). The nucleic acids were re-suspended in TE solution (10 mM Tris; 0.1 mM EDTA; pH 8.0), aliquoted, and stored at -21°C until further use. DNAs extracted from periwinkle plants infected with "*Ca.* Phytoplasma solani" reference strains 1925 (tuf-a) or GGY (tuf-b) were included as controls in the PCR reactions.

PCR based detection of phytoplasmas

Three independent real-time PCR protocols targeting the 16S ribosomal RNA (16S rRNA) gene were used to determine the presence of the phytoplasmas belonging to each of the ribosomal groups 16SrI ("*Ca*. P. asteris"), 16SrXII-A ("*Ca*. P. solani") and 16SrV ("*Ca*. P. vitis") (Angelini *et al.*, 2007). The presence of phytoplasmas other than those mentioned above was also ascertained by nested PCR targeting the 16S rRNA gene. Universal primers P1/P7 were always used as the external pair, while the universal pair R16F2n/R2 (Schneider *et al.*, 1995; Gundersen and Lee, 1996; Tolu *et al.*, 2006) or pair D7f2/D7r2, specific for members of the 16SrIX ribosomal group, were used as nested primers (Verdin *et al.*, 2003; Texeira *et al.*, 2008). The concentrations of the reagents in the PCR mix, as well as the cycling conditions, were as originally described, except that the High Fidelity PCR enzyme mix (Thermo-Scientific) was used and the annealing temperature of primers D7f2/D7r2 was 68°C. Based on the expected size, amplicons were visualized after electrophoresis in 1 or 2% agarose gels in 1× Tris-borate-EDTA buffer and staining with ethidium bromide (0.5 $\mu g\ mL^{-1}$). When required, amplicons were purified with an Exonuclease I and Thermosensitive Alkaline Phosphatase mix (Thermo-Scientific) and both strands sequenced on an ABI prism 310 CE system (Applied Biosystems). Sense and antisense nucleotide sequences were visualized and checked for quality using CHROMAS LITE 2.01 (Technelysium Pty, Ltd), aligned using MUSCLE as implemented in MEGA 5.2 (Tamura *et al.*, 2011) and assembled manually to obtain single consensus sequences. After removing primer oligonucleotides, identity searches were performed on the INSDC database (http://www.insdc.org/). To establish the possible relationships among representative strains of the 16SrI, 16SrV, 16SrIX and 16SrXII ribosomal groups and the phytoplasmas that were detected in the vineyard plots, nucleotide sequences of a selection of 14 R16F2n/R2 amplicons were aligned with MUSCLE and a Maximum Likelihood phylogenetic tree (approx. 1126 bp of the 16S rRNA gene in the final dataset) constructed using MEGA version 5.2 (Tamura *et al.*, 2011).

"*Ca.* P. solani" characterization

To characterize the "*Ca.* P. solani" populations, fragments of the *tuf* and of the *vmp1* genes were amplified from a selection of 20 nucleic acids samples per plot: 12 extracted from dicots and eight from grapevines, chosen among those found positive for "*Ca.* P. solani". Nested-PCR reaction conditions and primers were as originally reported by Langer and Maixner (2004) and Fialova *et al.* (2009) for *tuf* and *vmp1* genes, respectively, with the only exception that the High Fidelity PCR enzyme mix (ThermoScientific) was used. Amplicons obtained were purified from 1% agarose gels using the Wizard SV Gel and PCR Clean-Up System kit (Promega), ligated into the pGEM-T Easy vector (Promega) and cloned in *Escherichia coli* strain DH5α. For each sample and gene fragment type, one recombinant colony was selected and lysed by boiling for 10 min. Lysates were directly added to the PCR mix and each insert amplified with the plasmid primer pair T7/SP6. PCR conditions were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, preceded by 3 min at 95°C for initial denaturation, and followed by 5 min at 72°C for final extension. PCR products were purified, sequenced using T7/SP6 primers, and analyzed as described above. The partial *vmp1* gene sequences of "*Ca.* P. solani" strains ARSIA1 and SFRT1 were deposited in GenBank under the respective accession numbers KJ129605 and KJ129606.

Results

Analyses of disease incidence

In 2010, incidence of GY was 2.1% in the Fd plot and 2.5% in the Cs plot. When each of the vines that were symptomatic was independently analyzed by means of the three 16S rRNA gene real-time PCR assays specific for "*Ca.* P. asteris", "*Ca.* P. solani" and "*Ca.* P. vitis" and of the nested PCR protocol specific for members of the 16SrIX ribosomal group, "*Ca.* P. solani" was the only phytoplasma that could be detected (Figure 1). Fifteen of the dicot taxa spontane-

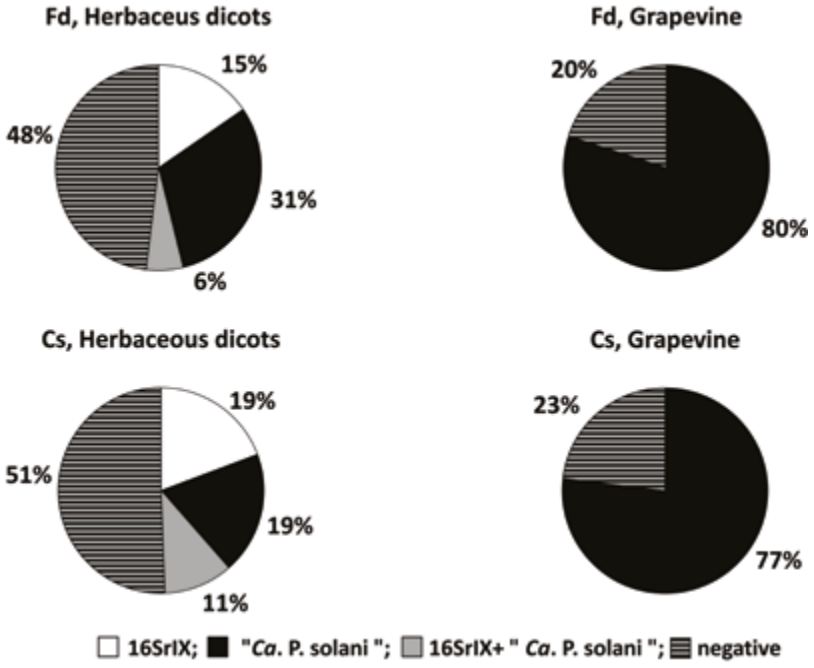

Figure 1. Overall results of PCR indexing for "*Ca.* P. solani", "*Ca.* P. vitis" "*Ca.* P. asteris" (real-time PCR) and for the phytoplasmas of the ribosomal group 16SrIX (nested PCR) carried out in October 2010 on single symptomatic grapevines and wild herbaceous dicot plants growing in vineyard plots Fd and Cs. All specimens tested negative for "*Ca.* P. vitis" and "*Ca.* P. asteris".

Table 1. Within vineyards frequency of the wild herbaceous dicot taxa that in October 2010 were showing symptoms resembling those induced by phytoplasmas, and overall presence of "*Ca*. P. solani" and 16SrIX phytoplasmas in the collected specimens, according to real-time PCR and nested PCR results, respectively. All specimens tested negative for "*Ca*. P. vitis" and "*Ca*. P. asteris".

Vineyard plot[a]	Host	Taxon frequency (%)[b]	Major symptoms observed	Symptoms incidence (%)[c]	PCR indexing results[d]	
					16SrIX	"*Ca*. P. solani"
Cs	*Bupleurum tenuissimum*	11.8	Flower abnormalities	11.3	+	+
	Cephalaria transsylvanica	66.2	Reddening	22.5	-	+
	Cichorium intybus	3.1	Flower proliferation	2.5	-	+
	Cirsium arvense	20.6	Yellowing	18.1	+	-
	Convolvulus spp.	79.3	Stunting	19.4	+	+
	Daucus carota	73.7	Reddening	36.9	+	+
	Lactuca saligna	66.2	Phylloidy	30.6	+	+
	Medicago sp.	96.2	Yellowing	20.4	+	+
	Picris echioides	77.5	Virescence and phyllody	29.4	+	+
	Picris hieracioides	12.5	Virescence and phyllody	4.4	+	+
	Tussilago farfara	3.1	Reddening	1.9	-	+
Fd	*Atriplex* sp.	3.3	Reddening	3.3	-	-
	Chenopodium sp.	6.0	Reddening	3.3	-	-
	Cirsium arvense	56.6	Yellowing	43.3	-	+
	Convolvulus spp.	80.0	Stunting	33.3	-	+
	Daucus carota	66.6	Reddening	26.7	-	+
	Galium sp.	53.3	Reddening	36.7	+	+
	Lactuca saligna	13.3	Rosetting	3.3	+	+
	Linaria vulgaris	36.6	Reddening	33.3	-	+
	Medicago sp.	53.3	Reddening	6.7	-	+
	Mercurialis annua	76.6	Yellowing	76.6	-	+
	Picris echioides	60.0	Virescence and phyllody	13.3	+	-
	Picris hieracioides	43.3	Virescence and phyllody	23.3	+	-
	Potentilla reptans	50.0	Reddening	6.7	-	+
	Rubia peregrina	3.3	Reddening	3.3	+	+
	Sonchus sp.	56.6	Reddening	6.7	+	+

[a] A total of 160 and 30 cells were inspected in plots Cs and Fd, respectively.
[b] Percentage of plot cells in which one or more specimens were found.
[c] Percentage of plot cells in which one or more symptomatic specimens were found.
[d] + and - indicate the presence and absence of "*Ca*. P. solani" and/or 16SrIX ribosomal group phytoplasmas based on the overall results of PCR indexing analysis (see Material and Methods for details).

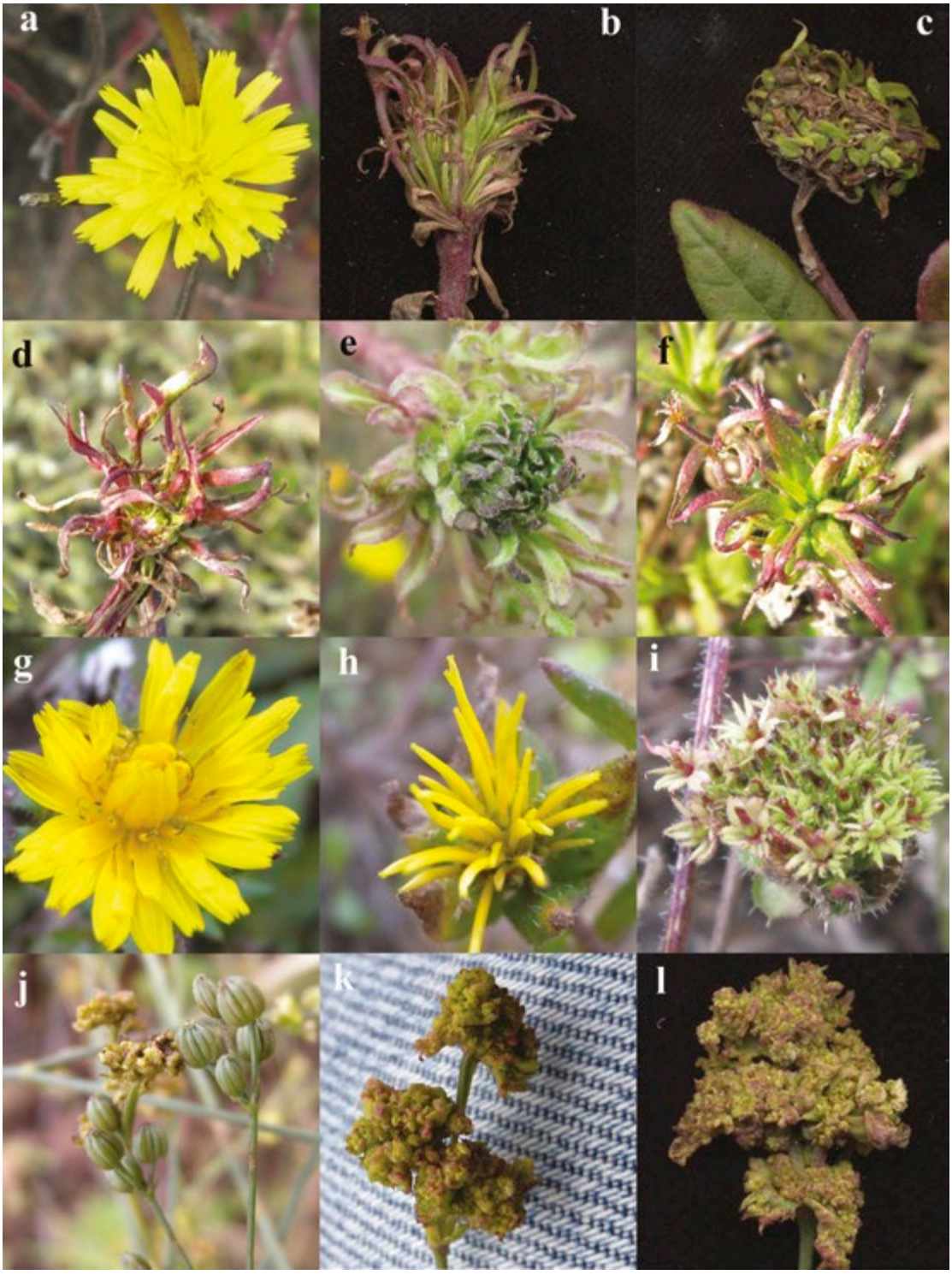

Figure 2. Examples of common symptoms shown by some of the wild herbaceous dicot taxa that were growing in the organic vineyards Cs and Fd in October 2010: b to f, *Picris hieracioides*; h and i *P. echioides*; k and l, *Bupleurum tenuissimum*. For comparison purposes the healthy organs of *P. hieracioides*, *P. echioides* and *B. tenuissimum* are shown in figures a, g and j, respectively.

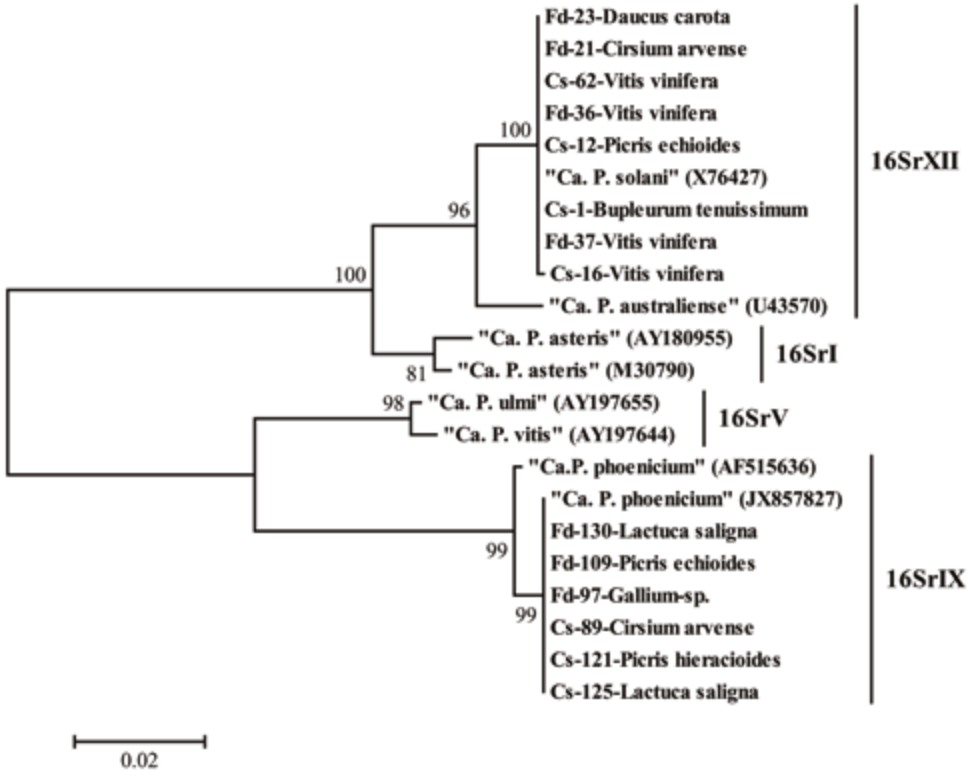

Figure 3. Maximum likelihood tree showing the phylogenetic relatedness between phytoplasmas found in vineyard plots Cs and Fd, and representative members of the ribosomal groups 16SrI, V, IX and "*Ca*. P. solani" (16SrXII-A). GenBank accession numbers are shown in parentheses. The tree was built using the sequences of a fragment of the 16S rRNA gene (1126 positions in the final dataset). Numbers above or below branches are bootstrap values based on 500 pseudoreplicates under the Tamura 3-parameter model of evolution with gamma rate of variation across sites. Nucleotide sequences determined in this study are indicated by Cs or Fd according to the vineyard plot of origin.

ously growing in the Fd plot, and 11 in the Cs plot, showed symptoms ascribable to phytoplasmas (Table 1, Figure 2). Seven taxa, namely *Cirsium arvense*, *Convolvulus* spp., *Daucus carota*, *Lactuca saligna*, *Medicago* sp., *Picris echioides*, and *Picris hieracioides* were common to both environments. *Convolvulus* spp., *Medicago* sp., *P. echioides* and *D. carota* were widespread in both plots, being present in more than 50% of the cells sampled, while taxa like *Galium* sp., *Sonchus* sp., *Mercurialis annua* and *Potentilla reptans* (plot Fd) or *Cephalaria transylvanica* (plot Cs) were detected in only one vineyard plot, some being very common. Symptom incidence within each taxon varied from a minimum of 3.3% (*Atriplex* sp., *Chenopodium* sp., *L. saligna* and *Rubia peregrina)* to 76.6% (*M. annua*) in plot Fd, and from a minimum of 1.9% (*Tussilago farfara*) to a maximum of 36.9% (*D. carota*) in plot Cs (Table 1). In general, symptom incidence was not related to taxon frequency, since very common species, such as *M. annua* (plot Fd), as well as relatively rare ones, such as *T. farfara*, *Cichorium intybus* or *Bupleurum tenuissimum* (plot Cs), recurrently showed symptoms ascribable to phytoplasma disorders.

Phytoplasmas belonging to the 16SrI and 16SrV ribosomal groups were never detected by real-time PCR, while "*Ca.* P. solani" (16SrXII-A) was found to infect approx.one out of three symptomatic specimens in both plots (Figure 1). Nevertheless, according to sequence and phylogenetic analyses of a portion of the 16S rRNA gene, phytoplasmas belonging to the 16SrIX ribosomal group also occurred (Figure 3). Based on D7f2/D7r2 PCR results these latter phytoplasmas were present in approx. 21% (Fd) and 30% (Cs) of the samples (Figure 1). When the results obtained from each specimen by each independent PCR based assay were compared, 6% (Fd) and 11 %

Table 2. Characterization of the '*Ca*. P. solani' population found in the different plant hosts growing in vineyards Cs and Fd in October 2010, according to *vmp1* gene sequence analysis.

Plot	Specimen number/ plant host [a]	*vmp1* sequence similarity [b]		
		Sequence length, bp [c]	Closest relatives (Acc. n°, bp)	Identities (%)
Cs	1/*Bupleurum tenuissimum*	1394	B7 (HM008608, 1329)	1329/1329 (100)
	2/*Convolvulus* sp.	1394	B7 (HM008608, 1329)	1329/1329 (100)
	3/*Picris echioides*	1394	B7 (HM008608, 1329)	1329/1329 (100)
	4/*Bupleurum tenuissimum*	1391	C6 (HM008618, 1326)	1326/1326 (100)
	5/*Cephalaria transylvanica*	1391	C6 (HM008618, 1326)	1326/1326 (100)
	6/*Daucus carota*	1391	C6 (HM008618, 1326)	1326/1326 (100)
	7/*Vitis vinifera*	1391	C6 (HM008618, 1326)	1326/1326 (100)
	8/*Convolvulus* sp. x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	9/*Daucus carota* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	10-11/*Vitis vinifera* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	12/*Convolvulus* sp.	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	13-14/*Lactuca saligna*	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	15/*Medicago* sp.	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	16-17/*Vitis vinifera*	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	18-20/*Vitis vinifera*	1400	B2035 (HM008611, 1335)	1335/1335 (100)
Fd	21/*Convolvulus* sp. x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	22-23/*Daucus carota* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	24/*Lactuca saligna* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	25-26/*Linaria vulgaris* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	27/*Medicago* sp. x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	28/*Mercurialis annua* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	29/*Sonchus* sp. x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	30/*Vitis vinifera* x	1388	T2-92 (AM992106, 1784)	1385/1399 (99)
	31/*Cirsium arvense*	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	32/*Linaria vulgaris*	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	33-38/*Vitis vinifera*	1649	400-05 (EF655660, 2071)	1649/1649 (100)
	39/*Sonchus* sp.	1400	B2035 (HM008611, 1335)	1335/1335 (100)
	40/*Vitis vinifera* y	1151	LG (AM992097, 1536)	1119/1151 (97)

[a] Specimens that were found to be infected by the novel *vmp1* genotypes ARSIA1 and SFRT1, are indicated by an x and a y, respectively.
[b] Characterization of stolbur populations found in vineyards plots Cs and Fd on the basis of *vmp1* gene sequence.The closest relative by sequence comparison according to the INSDC database (http://www.insdc.org/), accession numbers and sequence length of the closest related database entries, nucleotide identities and similarity percentages are given.
[c] Length of the amplified sequence upstream and downstream of the primers TYPH10F and TYPH10R binding sites (Fialova *et al*., 2009).

(Cs) of the dicots specimens were found to be co-infected by members of the two ribosomal groups. Out of 19 dicot taxa considered in this study, phytoplasma DNA was detected in at least one symptomatic specimen of each taxon with the exception of *Atriplex* sp. and *Chenopodium* sp. Six taxa, including *C. transylvanica*, *C. intybus*, *T. farfara*, *Linaria vulgaris*, *M. annua* and *P. reptans,* were infected by "*Ca.* P. solani" only, while 11 taxa may host the phytoplasmas of either of the two ribosomal groups in their root systems (Table 1). To the best of our knowledge, "*Ca.* P. solani" infections have not been previously reported in *B. tenuissimum, C. transylvanica, L. saligna*, *L. vulgaris* and *R. peregrine*, and 16SrIX phytoplasmas have not been reported in *B. tenuissimum*, *C. arvense*, *Convolvulus* spp., *D. carota*, *L. saligna*, *Medicago* sp., *P. hieracioides*, *Galium* sp., *R. peregrina* and *Sonchus* sp.

Heterogenicity of "*Ca.* P. solani" populations

According to comparative sequence analysis of the *tuf* gene fragment (906 bp), all clones shared 100% nucleotide identity with one another and with the tuf-b strain STOL11 (JQ797670). This indicates an exact correspondence between "*Ca.* P. solani" populations infecting grapevine and dicots (data not shown). On the other hand, when further characterization was carried out considering the *vmp1* gene, sequences that varied between 1151 bp and 1649 bp were obtained (approx. 70% of the complete gene sequence), and four *vmp1* types were found from plot Fd and five were found from plot Cs (Table 2). Three genotypes, namely B2035, 400-05 and ARSIA1, were common to both vineyards and all but two, SFRT1 and ARSIA1, were identical to some of the homologous sequences deposited in the INSDC database (Table 2). The novel SFRT1 genotype shared closest identity (97% over 1151bp) with strain LG (AM992097). ARSIA1 was genetically close (99% identity over 1388 bp) to strain T2-92 (AM992106), from which it could be differentiated by three consecutive non-synonymous nucleotide changes spanning two codons, and by the deletion of an 11 bp repeated element (GCAAAAAGTAAC). The deletion restored the translation frame of the gene. According to sequencing results, SFRT1 was restricted to a single grapevine plant in plot Fd, while ARSIA1, which infected *Vitis vinifera* as well as seven dicot taxa, was the most frequent genotype found in this study (100% identity between sequences). Nevertheless, distribution of ARSIA1 was different between the two plots. In Fd, ARSIA1 was most frequent in dicots (nine out of 12 of the specimens tested), but only sporadic in symptomatic vines (one out of eight in which "*Ca.* P. solani" was found), which were mostly infected by the 400-05 genotype (six out of eight) (Table 2). Conversely in Cs, only two out of 12 dicot specimens and two out of eight vines were infected by this genotype. In agreement with sequence analysis of the *tuf* fragment none of the VMP1 sequences were characterized by the DVANN pentapeptide reported as diagnostic for "*Ca.* P. solani" tuf-a subpopulation (Johansen *et al*., 2012).

Discussion

Several studies have shown that herbaceous dicots are potentially involved in BN epidemics as primary inoculum sources. Eradication of the hosts that are most commonly found to be infected, *C. arvensis* and *U. dioica,* is strongly recommended, in order to limit the spread of "*Ca.* P. solani" into vineyards (Vidano, 1988; Langer *et al*., 2003; Musetti, 2008). In this respect, vineyards in which rows and between row spaces were left covered by diverse, dense, and nearly permanent communities of herbaceous plants, as is often intentionally done in the organic vineyards in Tuscany, would be expected to be at the highest risk of BN. Keeping in mind these aspects of BN, to get insights on its epidemiology, we selected two organic vineyards in which "*Ca.* P. solani" appeared to be the only agent of grapevine yellows. In both plots we observed that many annual and perennial dicot species (besides *Convolvulus* spp.) showed symptoms that could be caused by phytoplasma infections. These symptoms included reddening or yellowing of the leaves, reduction of internode growth, greening of tissue, and phyllody. Combining our results we found that at least 17 dicots, several of which were present in both vineyards, can be infected by "*Ca.* P. solani". Further infection/transmission trials are needed to determine if these plants have a role as inoculum sources for grapevine, or if they are merely dead end hosts in vector/BN cycles.

We found evidence that the relationship between the presence of "*Ca.* P. solani" within the dicot species growing on the vineyard floor and BN of grapevine may not be straightforward, even when

only the tuf-b type is present. Considering the same specimens, we found , six "*Ca.* P. solani" genotypes, three of which were detected in both vineyards, when we indexed "*Ca.* P. solani" variation by typing a fragment of the *vmp1* gene. In the case of plot Cs, three out of five *vmp1* genotypes infected dicots and grapevine, supporting the hypotheses that the dicots may serve as phytoplasma inoculum sources. On the contrary, in the case of vineyard Fd, different genotypes dominated the two "*Ca.* P. solani" sub-populations, suggesting that within stand inoculum movements from dicots to vines were only occasional. Nevertheless, we cannot discount the possibility that the results obtained in plot Fd were due to the temporal shift between "*Ca*. P. solani" strains occurring in annual weeds and perennial grapevine hosts, that can incubate BN for long periods. A strain found at year n, results from inoculation at least at year n-1.

There is evidence that species other than grapevine, some of which are considered non-hosts for "*Ca.* P. solani", may represent more attractive food sources for *H. obsoletus* (Maixner *et al.*, 2001; Sharon *et al.*, 2005). If this is the case, their presence as cover crops within vineyards could possibly act by limiting the movements of polyphagous infective vectors towards grapes. In Germany, reduced incidence of BN has been recorded in organic vineyards compared to close conventionally managed plots, although *H. obsoletus* population densities were similar (Langer *et al.*, 2003). Further research is required to establish if the consistent characteristic fluctuations in BN incidence (Maixner, 2006) can be related to the shifts in the abundance, homogeneity, or specific composition of the dicot cover between years. It will also be of interest to verify if perennial dicots that grow in the same environments as grapevines go through the phenomenon of recovery, and if the temporal shifts in the incidence of "*Ca.* P. solani" that are commonly observed on grapevines also occur on the herbaceous hosts.

Apart from its usefulness as a molecular marker with a high degree of variability, nothing is known about VMP1 functions, and various hypotheses have been made to possibly explain the existence of considerably different alleles, including a specific association with the insect vector or the plant host (Cimerman *et al.*, 2009). We did not find evidence that the different *vmp1* types are host species specific. We observed that the same *vmp1* genotype can infect different host taxa in the same environment, such as ARSIA1 in vineyard Fd, while the same host can be infected by different genotypes, as was common in vineyard Cs. We have also found that different phytoplasmas are commonly associated with dicots that grow in the vineyard floor. In both plots, two ribosomal groups, 16SrIX and 16SrXII-A ("*Ca.* P. solani"), occurred at comparable frequencies and occasionally in the same host specimen. Although pathogenicity trials are required to confirm that the symptoms we have observed are caused by phytoplasma, at present our findings suggest that "*Ca*. P. solani" and the 16SrIX phytoplasma have wide and nearly coincident host ranges, as well as the potential capability to preserve their inoculum from one year to the next by colonizing the root systems of perennials such as *Cirsium arvense* or *Potentilla reptans*. At the same time, their epidemiology in relation to grapevine is different, since on this host, which is reported as susceptible to both phytoplasmas (Canik *et al.*, 2011), we were able to find only "*Ca.* P. solani". The existence of different vectors cannot be ruled out.

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