# STUDY OF PHYTOPLASMA-ASSOCIATED GRAPEVINE YELLOWS (GY) DISEASES IN GEORGIA

**Fabio Quaglino[1], David Maghradze[2], Paola Casati[1], Nona Chkhaidze[3], Mzagho Lobjanidze[3] Zurab Khidesheli[4], Osvaldo Failla[1], Piero Attilio Bianco[1]**

[1]Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy; [2]Institute of Horticulture, Viticulture and Oenology, Agricultural University of Georgia, Tbilisi, Georgia; [3]Laboratory of Plant Anatomy and Physiology, Agricultural University of Georgia, Tbilisi, Georgia; [4]Scientific-Research Center of Agriculture of Georgia, Saguramo, Georgia

## ABSTRACT

Flavescence dorée (FD) and Bois noir (BN), two phytoplasma-associated diseases belonging to the grapevine yellows (GY) complex, are responsible for serious crop losses in the Euro-Mediterranean area and in other continents. Even their symptoms are undistinguishable, FD and BN are associated with phytoplasmas distinct at both taxonomic/genetic and ecological/epidemiological level. Preliminary survey highlighted that BN affects grapevine varieties in Georgia, while FD was not reported. Further research was carried out to investigate the BN symptom severity in international and Georgian native varieties, and its epidemiology. Identification and characterization of BN phytoplasma was performed by analysis of multiple gene nucleotide sequences. During field surveys, moderate/mild and severe symptoms were observed on Georgian grapevine varieties and international cultivars, respectively. Molecular characterization revealed the presence of several genetically distinct BN phytoplasma types described for the first time. Molecular detection, supported by phylogenetic analyses, indicated that BN phytoplasma strains in Georgia are associated mainly with the bindweed-related host system. Moreover, the presence of the same phytpoplasma strains in grapevine cultivars showing a range of symptom intensity suggested a different susceptibility of Georgian local varieties to BN. To prevent the spread of GY diseases, further studies are needed to survey BN and FD phytoplasmas in Georgian vineyards and nurseries.

## INTRODUCTION

Phytoplasmas, cell wall-less plant pathogenic bacteria of the class *Mollicutes,* are associated with several hundred diseases affecting economically important crops (Bertaccini et al., 2014). In diseased plants, phytoplasmas are restricted to the phloem sieve tubes and are transmitted between plants by phloem-sap-feeding leafhoppers, planthoppers or psyllids in a persistent manner (Weintraub and Beanland, 2006). Based on unique molecular and biological features, phytoplasmas have been classified into species of the provisional genus '*Candidatus* Phytoplasma' and in taxonomic groupings (Marcone, 2014).

Flavescence dorée (FD) and Bois noir (BN) are two phytoplasma-associated diseases, belonging to the grapevine yellows (GY) complex, responsible for serious crop losses in the Euro-Mediterranean area and in other continents. Typical GY symptoms include berry shrivel, desiccation of inflorescences, color alterations and curling of the leaves, reduction of growth, and irregular ripening of wood. Even their

symptoms are undistinguishable, FD and BN are associated with phytoplasmas distinct at both taxonomic/genetic and ecological/epidemiological level (Belli et al., 2010).
FD phytoplasmas ('*Ca*. P. vitis', 16SrV-C/-D) are efficiently transmitted to grapevine by *Scaphoideus titanus*, a leafhopper sustaining its whole life cycle on *Vitis* spp., and by other insects (e.g., *Orienthus ishidae*). Consequently, geographic areas hosting large vector populations and FD phytoplasmas can be damaged by strong FD epidemics. Due to this aspect, FD phytoplasmas are quarantine pathogens to be controlled through mandatory measures (Casati et al., 2017). On the other hand, BN phytoplasmas ('*Ca*. P. solani', 16SrXII-A) are occasionally transmitted to grapevine by *Hyalesthes obsoletus*, a polyphagous planthopper living preferentially on nettle, bindweed, and chaste tree. Recent studies evidenced the presence of additional insect vectors of this phytoplasma in Europe (e.g., *Reptalus panzeri* and *R. quinquecostatus*) (Quaglino et al., 2013).
This scenario highlights the extreme complexity of the ecology of GY phytoplasmas and their insect vectors, underlying the difficulty in studying the epidemiology of diseases associated with this pathogen and in developing efficient control measures (Mori et al., 2015). An ambitious strategy is based on the selection of plant varieties as source of resistance-genes for plant breeding programs. Unfortunately, none of the examined *Vitis* species and *V. vinifera* varieties have been found resistant or tolerant to the GY phytoplasmas (Laimer et al., 2009). The Georgian native germplasm is composed by more than 500 cultivars constituting a very unique genetic pool (Imazio et al., 2013). Recent studies reported that grapevine varieties selected in domestication centers of *V. vinifera* L., such as Georgia, showed possible tolerance or resistance to plant pathogens, such as *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, associated with downy mildew (Toffolatti et al., 2016).
In the present study, field surveys and molecular analyses were carried out to study (i) the GY symptom severity in international and Georgian native varieties and (ii) the genetic diversity among GY phytoplasmas in Georgia.

## MATERIAL AND METHODS

**Symptom observation and plant sampling**. In September 2013 and 2015, surveys on GY symptoms were carried out in vineyards and in field collections of international and native *Vitis vinifera* L. varieties in eastern Georgia. Grapevine varieties were classified in group I (mild symptoms), group II (moderate symptoms), and group III (severe symptoms). Leaf samples were collected from grapevine symptomatic plants of international and native Georgian varieties (Table 1), and from bindweed plants showing yellowing, reddening, dwarfism and leaf malformation.

**Phytoplasma detection.** Total DNA was extracted from examined plants and used for detecting phytoplasmas by nested-PCR amplification of 16S rDNA and subsequent RFLP assay (Quaglino et al., 2014). Total nucleic acids from periwinkle plants infected by phytoplasma strains EY1 ('*Ca.* P. ulmi'), STOL ('*Ca.* P. solani'), and AY1 ('*Ca.* P. asteris') were used as reference controls. Total nucleic acids from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls.
**Molecular characterization of '*Ca*. P. solani' strains and their association with symptom severity.** *Vmp1* and *stamp* genes of identified '*Ca*. P. solani' strains were amplified by nested PCR, as described in Fialova et al. (2009) and Fabre et al. (2011), sequenced (5x coverage), and deposited in the NCBI GenBank database. *Vmp1* nucleotide sequences were searched for mutations in *Rsa*I-recognition sites by virtual RFLP analyses using the software pDRAW32. The association between the *vmp1*-RFLP profiles and BN symptom severity was evaluated by $\chi^2$ test using SPSS statistical package for Windows, v. 22.0 (SPSS Inc.). *Vmp1* and *stamp* gene sequences, obtained in this study and retrieved from GenBank, were aligned and analyzed by the software BioEdit v.7.0.5. Based on sequence identities, BNp strains were grouped in *vmp1* and *stamp* genetic variants, and in collective *vmp*/*stamp* types. Strains of each variant/type shared 100% sequence identity. *Vmp1* and *stamp* gene sequences of '*Ca*. P. solani', from this and previous studies, were concatenated and used for phylogenetic analysis by the software MEGA6.

## RESULTS

**Symptoms observed on grapevine in Georgia.** Incidence of GY, based on symptom observation, was around 13% in Chardonnay and 1-3% in Georgian varieties, excepting Goruli Mtsvane (28% of vines with regular berry production) (Table 1). Severe symptoms were observed in international varieties (Chardonnay, Carignano, and Freisa) and in one local Georgian variety (Kisi); moderate symptoms were observed in four local Georgian varieties (Buera, Goruli Mtsvane, Saperavi, and Saperavi Pachkha); mild symptoms were observed in 22 local Georgian varieties and in one international variety (Moscato Bianco) (Fig. 1; Table 2).

**Phytoplasma identification.** PCR-RFLP detection revealed the presence of '*Ca*. P. solani' in 55 out of 81 examined grapevines, and in all bindweed samples (Table 2). In fact, all the phytoplasma strains had restriction patterns indistinguishable from one another and from the patterns characteristic of the reference strain STOL (data not shown).

**'*Ca*. P. solani' strain characterization by *vmp1* and *stamp* gene sequence analysis**. *Vmp1* gene fragment was amplified from 43 out of 55 infected grapevines, and from all the infected bindweeds (Table 2). Virtual RFLP-based comparison of *vmp1* RFLP profiles evidenced that Georgian BN phytoplasma strains showed previously described (V1, V14, V15) and new [V-G1, -G2, -G3] restriction patterns (Fig. 2). Strains showing profiles V1, V14 and V-G2 were prevalent and were identified, with significantly different distribution, in grapevine varieties showing severe, moderate and mild symptoms ($\chi^2$ = 16.671; d.f. = 10; $P$ = 0.029). Nucleotide sequence analysis revealed the presence of 12 and 7 genetic variants of *vmp1* (here designated as VmGe1 to VmGe12) and *stamp* (here designated as StGe1 to StGe7) genes, respectively. Eleven Georgian BNp *vmp*/*stamp* types were described as the combination of *vmp1* and *stamp* genetic variants.

Comparison with *vmp1* and *stamp* genetic variants from GenBank evidenced that BN phytoplasma strains from Georgia showed 11 *vmp1* (VmGe1 to VmGe7, VmGe9 to VmGe12) and 6 *stamp* (StGe1 to StGe6) novel genetic variants, previously unreported. Only BN phytoplasma strains Tsol89 and Kiqu94 (VmGe12/StGe7) shared 100% sequence identity with '*Ca*. P. solani' strain P7 (Vm53/St15), identified in periwinkle in Lebanon (Cimerman et al., 2009).

**Table 1.** Symptom severity observed in Georgian vineyards

| CULTIVAR | SYMTPOM SEVERITY | VINES | % SYMPTOMATIC |
|---|---|---|---|
| Chardonnay | +++ | 870 | 14,4 |
| Kisi | +++ | 713 | 2,8 |
| Goruli Mtsvane | ++ | 237 | 28,3 |
| Alexandrouli | + | 374 | 2,1 |
| Mtsvane Kakhuri | + | 336 | 3,3 |
| Rkatsiteli | + | 1313 | 2,7 |
| Saperavi | + | 870 | 1,1 |
| Shavkapito | + | 191 | 1,0 |
| Tavkveri | + | 463 | 2,2 |
| Tsolikouri | + | 355 | 2,8 |
| Usakhelauri | + | 320 | 2,2 |

**Table 2.** Symptom severity and phytoplasmas in Georgian vineyards

| PLANT HOST | SYMPTOM | NO. OF | PCR-RFLP | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | severity | samples | 16SrXII-A | | *vmp1* | | | | | |
| | | | | | V1 | V14 | V15 | V-G1 | V-G2 | V-G3 |
| Chardonnay | +++ | 20 | 20 | | 8 | 5 | | 2 | 3 | |
| Moscato Bianco | + | 3 | 3 | | | 1 | | | | |
| Carignano | +++ | 1 | 1 | | | | | | 1 | |
| Freisa | +++ | 1 | 1 | | | | | | 1 | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Adznizhi | + | 1 | | | | | | | | |
| Amlakhu | + | 1 | 1 | | | | | | | 1 |
| Asuretuli Shavi | + | 1 | | | | | | | | |
| Buera | ++ | 1 | 1 | | 1 | | | | | |
| Chinuri | + | 1 | | | | | | | | |
| Chkhaverii | + | 1 | | | | | | | | |
| Chuberi | + | 2 | | | | | | | | |
| Goruli Mtsvane | ++ | 4 | 1 | | | 1 | | | | |
| Grdzelmtevana | + | 1 | | | | | | | | |
| Khikhvi | + | 1 | 1 | | | | | | 1 | |
| Khikhvi variation | + | 1 | | | | | | | | |
| Kikhvi Loladzis | + | 1 | 1 | | | | 1 | | | |
| Kisi | +++ | 3 | 3 | | 1 | | | | 1 | |
| Korkaula | + | 3 | 2 | | | | 1 | | | |
| Mtredisphekha | + | 1 | | | | | | | | |
| Mtsvane Kakhuri | + | 1 | | | | | | | | |
| Mujuretuli | + | 1 | 1 | | | | | | | |
| Rkatsiteli | + | 9 | 4 | | 2 | | 1 | | | |
| Saperavi | ++ | 13 | 9 | | 4 | 1 | | | 2 | |
| Saperavi Budeshuri | + | 2 | 1 | | | | | | 1 | |
| Saperavi Pachkha | ++ | 1 | | | | | | | | |
| Tavkveni Saperaviseburi | + | 1 | 1 | | | | | | | |
| Tavkveri | + | 1 | 1 | | | | 1 | | | |
| Tshnoris Tetri | + | 1 | | | | | | | | |
| Tsitska | + | 1 | 1 | | | | | | 1 | |
| Tsolikouri | + | 1 | 1 | | | | | | | |
| Usakhelouri | + | 1 | 1 | | | | 1 | | | |
| *Convolvulus arvensis* | +++ | 6 | 6 | | | 4 | | 2 | | |
| | | 87 | 61 | | 16 | 12 | 5 | 4 | 11 | 1 |

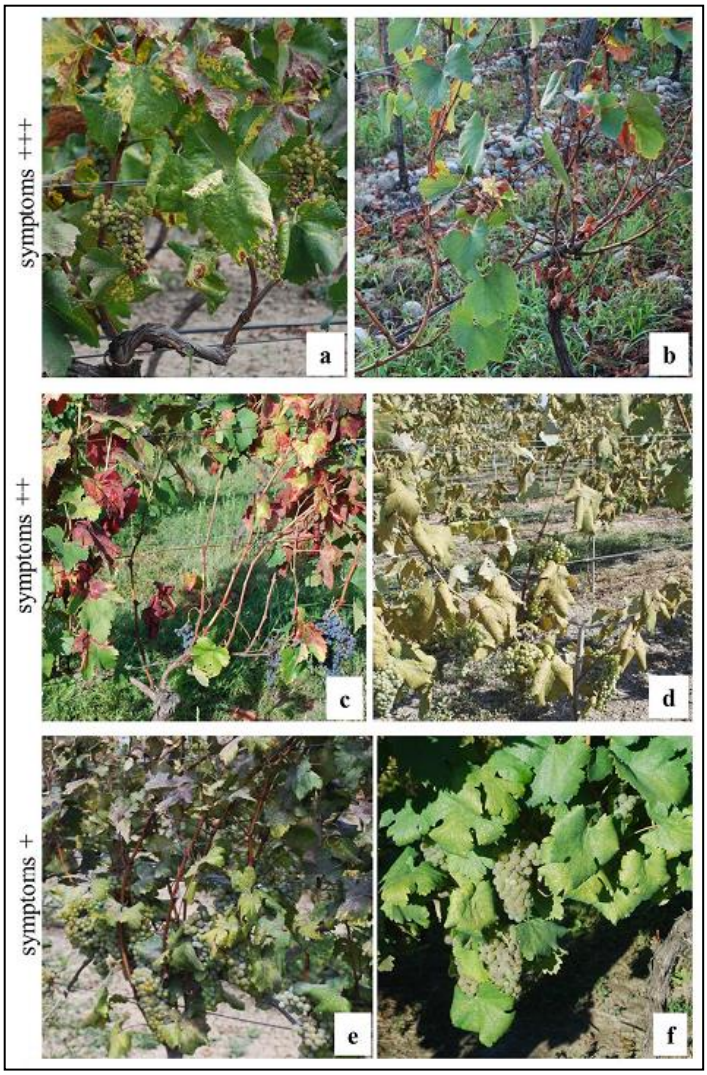


**Figure 1**. Intensity of grapevine yellows symptoms observed in Georgian vineyards. Severe symptoms in cultivars Chardonnay (a) and Kisi (b); moderate symptoms in cultivars Saperavi (c) and Goruli Mtsvane (d); mild symptoms in cultivars Rkatsiteli (e) and Tsiska (f).

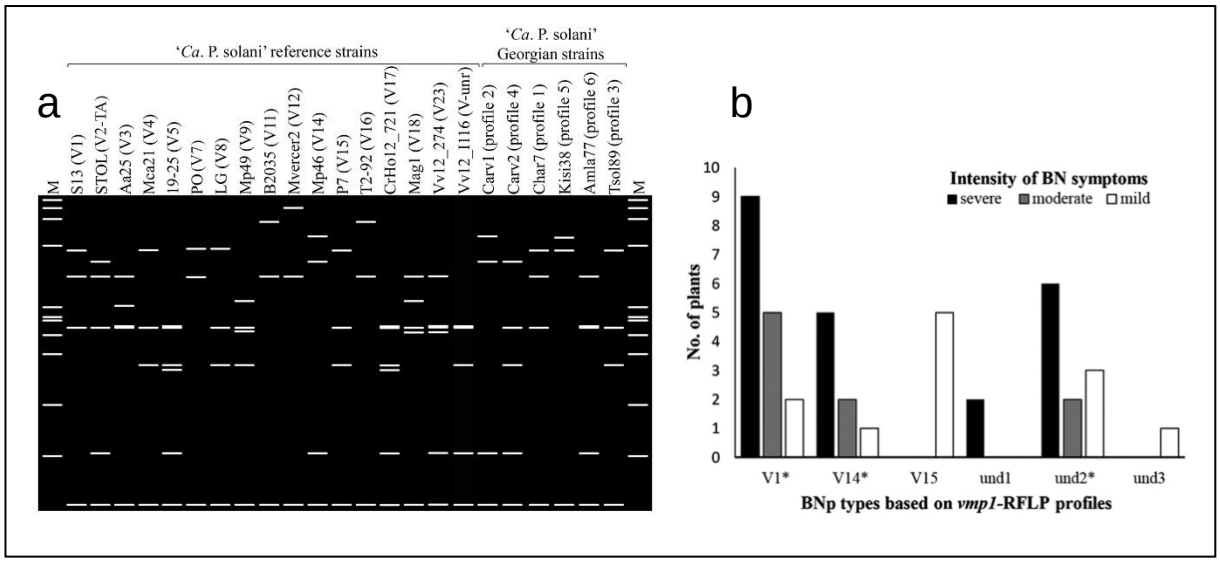


**Figure 2.** Virtual *Rsa*I-RFLP profiles of *vmp1* amplicons obtained from BN phytoplasma strain populations in Georgia (a) and their association with symptom severity (b).

**Phylogenetic analysis of '*Ca*. P. solani' strains from Georgia and other geographical regions.** Based on phylogenetic analysis of *vmp1* and *stamp* concatenated sequences, five *vmp*/*stamp* clusters were identified. The cluster *vmp*/*stamp*-4 included BN phytoplasma strains associated with nettle, while the other four clusters (*vmp*/*stamp*-1, -2, -3, -5) included BN phytoplasma strains associated with bindweed. The majority of Georgian BN phytoplasma strains grouped within bindweed-related clusters *vmp*/*stamp*-3 and *vmp*/*stamp*-5, while only the strain Amla77 grouped within nettle-related cluster *vmp*/*stamp*-4 (data not shown). The majority of Georgian BN phytoplasma strains, grouped within cluster *vmp*/*stamp*-3, were found to be closely related to strain P7, previously identified in naturally infected periwinkle plant in Lebanon.

## DISCUSSION

The results obtained in this study highlighted the presence of GY in Georgia. In fact, molecular analyses evidenced the strong association between specific GY disease symptoms and grapevine plant infection by BN phytoplasma ('*Ca*. P. solani' strains) within the examined vineyards; FD phytoplasma was never detected (Quaglino et al., 2014). In order to gain an insight into the genetic diversity among BN phytoplasma strains in Georgia, nucleotide sequence analysis was performed on two genes (*vmp1* and *stamp*) coding for membrane proteins putatively involved in the recognition and interaction of phytoplasmas with its hosts (Cimerman et al., 2009; Fabre et al., 2011). Based on *Rsa*I-RFLP digestions of *vmp1* gene amplicons, the profiles V1, V14 and V-G2 were prevalent. This data confirmed the specific association of pattern V14 with East Europe (Foissac et al., 2013), and highlighted an unexpected diffusion of type V1, reported as the prevalent type in Italy, France and Germany (Foissac et al., 2013), in the Caucasian geographic regions. This evidence, along with the prevalence of type V1 in the international cultivar Chardonnay, could suggest the non-indigenous origin of this type, possibly introduced in Georgia through import of planting material. The majority of autochthonous Georgian grapevine cultivars were found mildly symptomatic, maintaining complete berry production. Intriguingly, the different distribution of BN phytoplasma strains showing *Rsa*I-RFLP profiles V1, V14 and V-G2 of the gene *vmp1* in grapevine cultivars exhibiting severe, moderate and mild symptoms suggested a different susceptibility of such cultivars.

Molecular characterization by *vmp1* and *stamp* gene sequence analysis evidenced that BN phytoplasma populations in Georgia are constituted mainly by previously unreported strains. Only Georgian strains Tsol89 and Kiqu94 shared 100% sequence identity with the sequences of the '*Ca*. P. solani' strain P7 (*vmp*/*stamp* type Vm53/St15), identified in naturally-infected periwinkle in Lebanon in 2001 (Cimerman et al., 2009). Phylogenetic analysis revealed that the majority of BN phytoplasma Georgian strains, identified both in grapevine and bindweed, grouped along with the Lebanese strain P7 within the cluster *vmp*/*stamp*-3. Interestingly, this cluster is clearly distinct from other *vmp*/*stamp* clusters including bindweed- and nettle-related BN phytoplasma strains previously identified in Central and Southern Europe. Only one strain (Amla77) grouped with nettle-related cluster *vmp*/*stamp*-4. In conclusion, results from the present study evidenced that BN phytoplasma strain populations in Georgia is constituted mainly by new unreported '*Ca*. P. solani' strains associated with both nettle- and bindweed-related BN host systems. Moreover, the distribution of BN phytoplasma strains among grapevine cultivars showing a variable range of symptoms intensity suggests a different susceptibility of such local cultivars to BN disease (Quaglino et al., 2016). Further studies are in progress to evaluate this important topic in the perspective of improving breeding programs for the production of novel grapevine cultivars tolerant and/or resistant to phytoplasma diseases.

## REFERENCES

Belli G, Bianco PA, Conti M, 2010. Grapevine yellows: past, present and future. Journal of Plant Pathology 92: 303-326.

Bertaccini A, Duduk B, Paltrinieri S, Contaldo N, 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. American Journal of Plant Science 5: 1763-1788.

Casati P, Jermini M, Quaglino F, Corbani G, Schaerer S, Passera A, Bianco PA, Rigamonti IE, 2017. New insights on “flavescence dorée” phytoplasma ecology in the vineyard agro-ecosystem in southern Switzerland. Annals of Applied Biology 171: 37-51.

Cimerman A, Pacifico D, Salar P, Marzachì C, Foissac X, 2009. Striking diversity of *vmp1*, a variable gene encoding a putative membrane protein of the stolbur phytoplasma. Applied and Environmental Microbiology 75: 2951-2957.

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

Fialová R, Válová P, Balakishiyeva G, Danet J-L, Šafářová D, Foissac X, Navrátil M, 2009. Genetic variability of stolbur phytoplasma in annual crop and wild plant species in South Moravia (Czech Republic). Journal of Plant Pathology 91: 411-416.

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. Proceedings of the 3rd European Bois Noir Workshop, 2013. Barcelona, Spain. E. Torres, A. Laviña and A. Batlle, eds., 11-13.

Imazio S, Maghradze D, De Lorenzis G, Bacilieri R, Laucou V, This P, Scienza A, Failla O, 2013. From the cradle of grapevine domestication: molecular overview and description of Georgian grapevine (*Vitis vinifera* L.) germplasm. Tree Genetics and Genomes 9: 641-658.

Laimer M, Lemaire O, Herrbach E, Goldschmidt V, Minafra A, Bianco PA, Wetzel T, 2009. Resistance to viruses, phytoplasmas and their vectors in the grapevine in Europe: a review. Journal of Plant Pathology 91: 7-23.

Marcone C, 2014. Molecular biology and pathogenicity of phytoplasmas. Annals of Applied Biology 165: 199-221.

Mori N, Quaglino F, Tessari F, Pozzebon A, Bulgari D, Casati P, Bianco PA, 2015. Investigation on ‘bois noir’ epidemiology in north-eastern Italian vineyards through a multidisciplinary approach. Annals of Applied Biology 166: 75-89.

Quaglino F, Maghradze D, Chkhaidze N, Failla O, Casati P, Bianco PA, 2014. First report of '*Candidatus* Phytoplasma solani' and '*Candidatus* Phytoplasma convolvuli' associated with grapevine bois noir and bindweed yellows, respectively, in Georgia. Plant Disease 98: 1151.

Quaglino F, Maghradze D, Casati P, Chkhaidze N, Lobjanidze M, Ravasio A, Passera A, Venturini G, Failla O, Bianco PA, 2016. Identification and characterization of new ‘*Candidatus* Phytoplasma solani’ strains associated with Bois Noir disease in *Vitis vinifera* L. cultivars showing a range of symptom severity in Georgia, the Caucasus Region. Plant Disease 100: 904-915.

Quaglino F, Zhao Y, Casati P, Bulgari D, Bianco PA, Wei W, Davis RE, 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.

Toffolatti SL, Maddalena G, Salomoni D, Maghradze D, Bianco PA, Failla O, 2016. Evidence of resistance to the downy mildew agent *Plasmopara viticola* in the Georgian *Vitis vinifera* germplasm. Vitis 55: 121-128.