Adv. Hort. Sci., 2018 32(2): 281-288 DOI: 10.13128/ahs-22565

AHS
Advances in Horticultural Science

The monitoring program of grapevine phytoplasmas in Tuscany (Italy): results of a four year survey

D. Rizzo [1], A. Materazzi [2], L. Stefani [1], A. Panattoni [2], R. Pierro [2], G. Marchi [3], T. Cinelli [3], L. De Bellis [4], A. Luvisi [2,4(*)]

[1] *Servizio Fitosanitario Regionale, Regione Toscana, Via Ciliegiole, 98, 51100 Pistoia, Italia.*

[2] *Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università di Pisa, Via del Borghetto, 80, 56124 Pisa, Italia.*

[3] *Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Università degli Studi di Firenze, Piazzale delle Cascine 28, 50144 Firenze, Italy.*

[4] *Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Via Provinciale Monteroni, 73100 Lecce, Italia.*

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(*) **Corresponding author:**
andrea.luvisi@unisalento.it

Citation:
RIZZO D., MATERAZZI A., STEFANI L., PANATTONI A., PIERRO R., MARCHI G., CINELLI T., DE BELLIS L., LUVISI A., 2018 - *The monitoring program of phytoplasmas in Tuscany (Italy): results of a four year survey.* - Adv. Hort. Sci., 32(2): 281-288

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 25 January 2018
Accepted for publication 18 April 2018

Key words: bois noir, flavescence dorée, grapevine yellows, phytoplasma.

Abstract: Quantitative PCR protocols for phytoplasma detection were used to monitor grapevine yellows (GY) in 373 vineyards located in nine Tuscan districts. Among more than 70,000 plants visually monitored, 1.867 plants were sampled and "flavescence dorée" phytoplasmas (FD) were detected in 122 plants and mainly identified as trains belonging to 16SrV-C subgroup. The "bois noir" (BN) phytoplasma was found in 734 samples, with prevalence of *tufB* type-b strains. The 2013–2015 monitoring program was strongly influenced by the first survey (2012) in which FD was found consistently in the North West (15 samples), whereas only a few cases were observed in the East territory (2 samples). Both areas were thoroughly monitored in the following years: few foci were found in the East (2 in 2014, 1 in 2015), while several infected areas were found in the North West (6, 10 and 22 foci in 2013, 2014 and 2015, respectively). Definitely, the novel FD foci detected in the survey (17, 6, 12 and 23 in each year of survey) and the widespread of BN, suggest a dangerous distribution of GY in Tuscany.

1. Introduction

"Flavescence dorée" (FD), the most harmful grapevine yellows (GY) in Europe, is present in the northern part of Italy and in some winegrowing areas it occurs simultaneously with "bois noir" (BN) (Bianco *et al.*, 2002; Baric and Dalla Via, 2007). Both diseaases are associated with the presence of phytoplasmas enclosed in 16SrV group, subgroups C and D (Martini *et al.*, 1999) and '*Candidatus* Phytoplasma solani' (Quaglino *et al.*, 2013), respectively. Disease control programs for FD are very expen-

sive in Italy because, due to mandatory uprooting of infected plants, growers must be refunded for yield losses and replanting (Belli *et al*., 2010), while BN control depend on management of wild plants Indeed, grapevines become dead-end hosts for this phytoplasma, therefore the spatial spread of BN most likely does not rely on transmission of the phytoplasma from vine to vine but on other plant species frequently observed in vineyards (Maixner, 1994; Marchi *et al*., 2015). Other phytoplasmas have also been found sporadically in grapevines in Italy, such as strains belonging to ribosomal subgroups 16SrI-B (Alma *et al*., 1996) and 16SrI-C (Landi *et al.*, 2013) of '*Ca*. P. asteris' (aster yellows, AY).

As reported by Belli *et al*. (2010), control measures against GY are being implemented in Italy, targeted mainly to FD because the economic importance of BN infections has emerged only recently, following the extensive use of molecular diagnostic assays. Moreover, the present knowledge of the epidemiology and control of BN (Mori *et al*., 2015; Chuche *et al*., 2016) and FD (Rashidi *et al*., 2014; Casati *et al*., 2017) is not completely defined. However, compulsory control measures involve uprooting and destruction of any vine with GY type symptoms in the area, even before confirmation of FD infection by laboratory tests.

In Tuscany, FD was not known since 2003 (Bertaccini *et al*., 2003) when it was detected in the North-western areas of the region. Thereafter, compulsory control measures against FD were enforced. But successful eradication of FD relies on accurate diagnosis, which should be considered when the monitoring is planned. A strategic role is played by effective sampling of plants to reduce the risk of false negatives, and disease recognition is easier in grapevines affected by single infections; conversely, discrimination between diseases is more difficult in abandoned vineyards or when plants are affected by mixed infections such as mixed virus infections, frequently observed in cv. Sangiovese (Rizzo *et al*., 2012, 2015). The major risks of FD spread may derive from vineyards that are poorly protected against vectors. Thus, badly managed vineyards may represent a good target for FD detection, however such vineyards are frequently characterized by symptomatic or poorly cultivated plants, which result in increasing difficulties in recognizing FD.

In this paper the identification of novel foci of FD in Tuscany during four years of monitoring is reported together with the estimation of the monitoring activity effectiveness.

2. Materials and Methods

Districts sampled

In 2012-2015, 373 vineyards were selected in nine Tuscan districts in the most important grape for wine production areas. Where available, small vineyards (<1 ha) were included in the monitoring, as well as poorly managed vineyards. GY symptomatic samples from 200 plants in each vineyard (20 plants in 10 rows, randomly selected) were collected. Thus, more than 70,000 plants were included in this symptoms survey. Districts were grouped in five areas: North-West (Massa-Carrara, Lucca, and Pistoia), North-East (Prato, Firenze), West (Livorno, Pisa), East (Arezzo, Siena), and South (Grosseto) (Fig. 1).

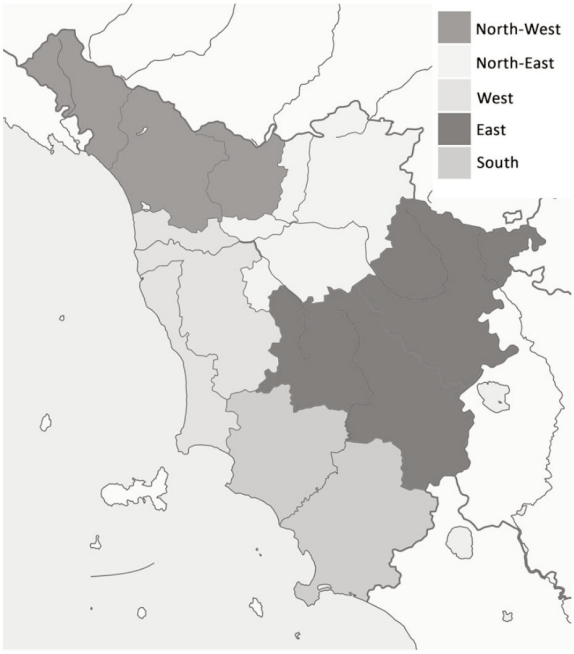

Fig. 1 - Tuscan districts were grouped in five territories: North-West (Massa-Carrara, Lucca, Pistoia), North-East (Prato, Firenze), West (Livorno, Pisa), East (Arezzo, Siena), South (Grosseto).

Sampling procedures

The overall number of vineyards and samples included in the GY monitoring was reported in Table 1. In view of the pre-2012 surveys (Bertaccini *et al*., 2013) the North West areas were considered the most susceptible to FD. In the following years, the number of samples and their distribution was determined based on the results of the previous year. Pathogen findings in North West in 2012 (Fig. 2 a), and first sporadic evidence of FD in Eastern areas (Fig. 2 b) led to increased monitoring activities in these areas, while monitoring in North East and East was drastically increased in 2013 (Table 1). Indeed, in

2013 North East sampling was increased by +574.1% in order to locate and eradicate any further infection site within a previously FD-free territory. Sampling in West was also increased (+125.0%) on the supposition that the disease would spread towards the Southern territories. In 2014, due to the results of the previous year, sample collection was globally similar (+2.3%). In 2015, sampling was concentrated in North West (+43.1%), where the presence of FD was alarming after three years of limited but constant findings (Fig. 2 a). Conversely, sampling in Western territory was reduced (-88.8%) (Fig. 3 a).

Sangiovese was the predominant cultivar sampled (more than 80% of samples). Sampling was never redone in vineyards in which FD was found. Leaf samples were collected from symptomatic grapevine plants during September and October of each year. Each sample, consisted of 10-15 leaves showing typical sectorial reddening of the laminae processed independently (Fig. 4).

Detection methods

Total nucleic acid was extracted from grapevine leaf veins tissues using a variant of the CTAB method (Angelini *et al*., 2001) and a MM400 steel bead mixer

Table 1 - Tuscan vineyards (VY) and samples included in grapevine yellows monitoring

VY position	2012		2013		2014		2015	
	No. VY	No. Sample	No. VY	No. Sample	No. VY	No. Sample	No. VY	No. Sample
NorthWest	40	202	37	186	43	216	62	309
North East	5	26	21	106	20	98	6	32
Weast	5	27	36	182	29	145	17	83
East	6	32	14	72	23	116	3	13
South	0	1	4	18	0	2	0	1
Total	58	288	113	564	115	577	88	438

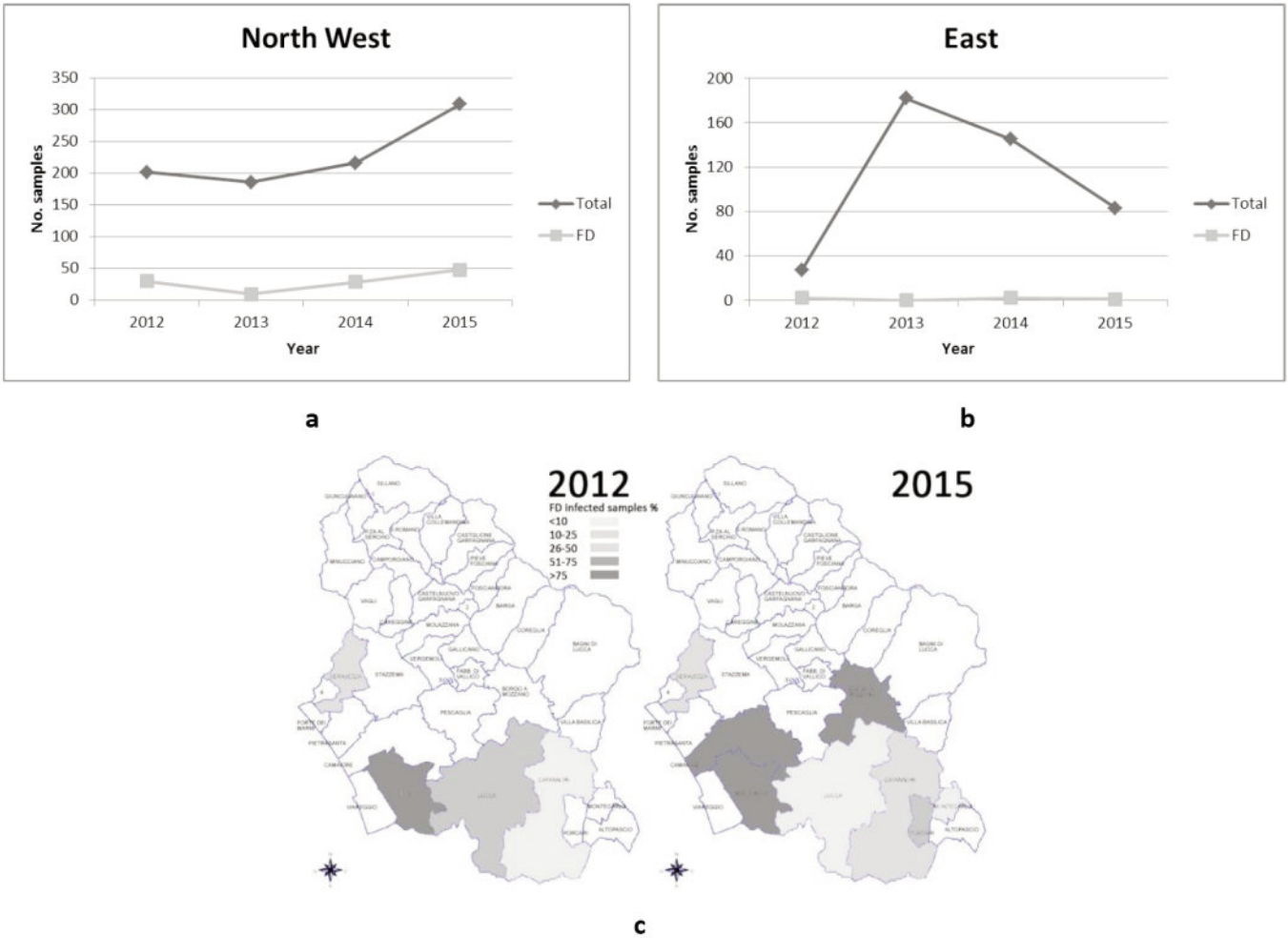

Fig. 2 - Number of samples positive to "flavescence dorée" (FD) out of total samples tested in 2012-2015 in (a) North West and (b) East areas of Tuscany; (c) comparison of incidence of FD in Lucca district in North Western Tuscany in 2012 and 2015.

mill (Retsch, Bergamo, Italy). TNA was re-suspended in TE solution (10 mM Tris; 0.1 mM EDTA; pH 8.0) and aliquots stored at -18°C until further use.

Quantitative PCR protocols targeting the 16S ribosomal RNA (16Sr rRNA) gene were used to determine the presence of the phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII-A and 16SrV (Angelini *et al*., 2007).

Leaves collected by *V. vinifera* plants, previously found infected by 'Ca. P. solani' (subgroup 16SrXII-A), Flavescence dorée phytoplasmas (subgroups 16SrV-C or -D) and '*Ca*. P. asteris' (subgroups 16SrI-B or -C) were used as infected controls (ICs). The infected controls were characterized following Angelini *et al*. (2007) or Berger *et al*. (2009) and conserved by Phytosanitary Service of the Tuscany Region.

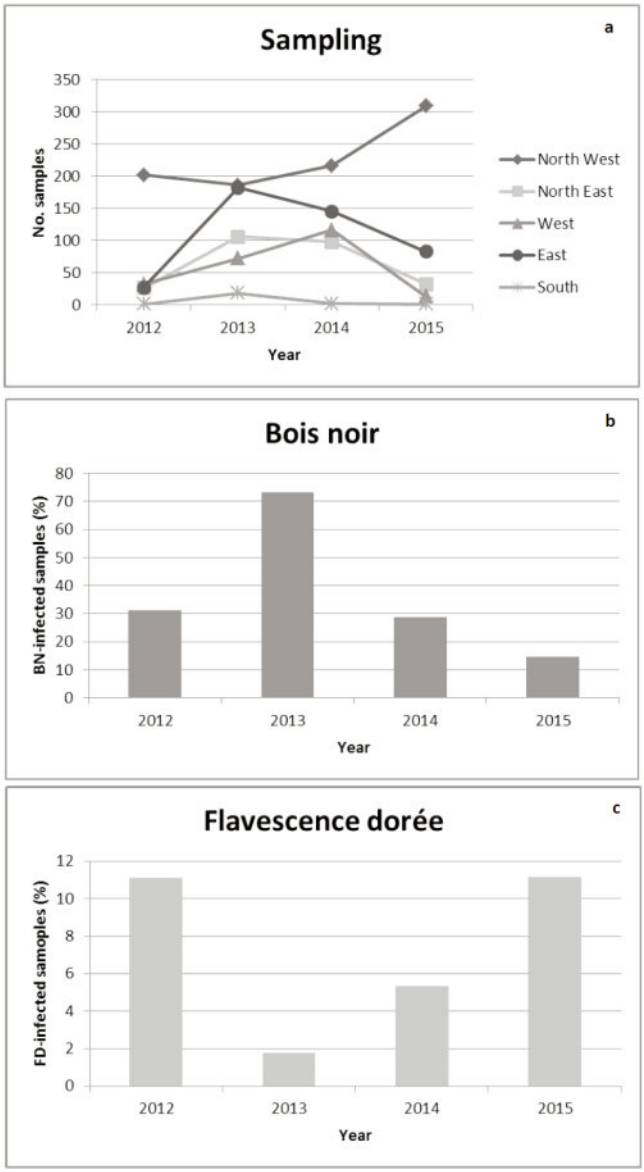

Fig. 3 - Distribution of (a) sampling among Tuscan provinces in 2012-2015. Samples infected by (b) "bois noir" and (c) "flavescence dorée".

A set of ribosomal primer pairs for universal detection of phytoplasma associated to FD was used in nested-PCR: the direct was performed with P1/P7 (Smart *et al.,* 1996) followed by the nested PCR with 16r758f/M23Sr primers (Gibb *et al.,* 1995; Padovan *et al.,* 1995). The nested amplicons obtained were digested with *Taq*I (New England Biolabs, USA), according to the manufacturer's instructions, and digestion fragments were separated through electrophoresis on 3% agarose gel in Tris-borate-EDTA (TBE) buffer. PCR conditions and protocols were as described by Angelini *et al*., 2007.

TaqMan allelic discrimination assay were performed following the protocol as described by Berger et al 2009, using *tufB* type-specific probes carrying different fluorescent dyes. The concentrations of the reagents in the PCR mix, as well as the cycling conditions, were as originally described. Reactions were performed in a CFX96 Real-Time thermocycler (Biorad, USA). Data were analyzed by measuring the threshold cycles (Ct).

Once FD was detected in a vineyard, that vineyard was not further included in the monitoring and PPS started the plant uprooting program.

3. Results

GY monitoring activity in 2012

Among collected samples, 14.9% and 7.4% were positive for FD in North West and Eastern samples respectively (Fig. 2 a, b). A total of 17 novel FD foci were detected, 15 in the North West (mainly in Massa-Carrara and Lucca districts) and 2 in both of the districts in the East (Table 2). No FD was detected in samples from the North East, West or South. In 2012, the highest level of BN presence was found in the Eastern samples (62.9%), while the lowest level of infection was observed in the North West (22.8%) (Fig. 5 a-e). At the regional level, FD was found in 11.1% of the samples, while BN was found in 31.3% (Fig. 3 b, c). AY was found at 0.3%.

GY monitoring activity in 2013

In 2013, sampling was increased in the North East, East and West (+95.8%, globally) (Fig. 3 a). North Eastern vineyards were thoroughly investigated (+307.7% of sampling) because they are situated between the historically infected territory (North West) and the newly infected one (East) (Fig. 2 a, b). No FD-infected sample was found in North East territory, while FD was found only in North West (5.4%).

Globally, 6 novel FD foci were identified, 5 of them in the Lucca district (Table 2). In 2013, infection rate of BN was generally high, reaching more than 80% of collected samples in North East and East territories, while the rate was lower in West (43.1%) (Fig. 5 a-e). At regional level, FD was set at 1.8% and while BN was found in 73.4% of samples (Fig. 3 b, c).

GY monitoring activity in 2014

Sample distribution was also similar to 2013, but more sampling was carried out in West (+61.1%) (Fig. 3 a). FD was still found in North West (13.4%) and few samples was infected in East (1.4%) (Fig. 2 a, b), confirming widespread infection sites in North West and sporadic (but difficult to eradicate) FD presence in Eastern Tuscany. In fact, 12 new foci were detected, 10 in Lucca district and 2 in Siena district (Table 2). With regard to BN, disease rates were lower in 2014 compared to the previous year. More than 50% of samples were positive only in North East or South, while very low infection rate was observed in North West (15.2%) and West (10.3%) (Fig. 5 a-e). At regional level, BN infection rate was quite low (28.8%), while FD apparently (5.4%) increased (Fig. 3 b, c).

GY monitoring activity in 2015

In 2015, the largest number of FD-infected samples was detected since this survey was started, with 15.5% of positive samples in North West and 1.2% in East territory (Fig. 2 a, b). Unfortunately, infected samples were found in many different vineyards, thus 23 novel foci were detected, most of them in Lucca (15), but a consistent number (6) in Pistoia, the eastern district of North West territory (Table 2). Further decrease in BN detection was observed in North East, East and West, where about 25% of samples were BN-positive, while a lower level was recorded elsewhere (Fig. 5 a-e). At regional level, a further increase in FD-positive samples was observed (11.1%), while BN infection was very low (14.6%) (Fig. 3 b, c).

Additional observations on FD monitoring

A comparison between FD findings in 2012 and 2015 in Lucca district of North West territory (where FD findings were numerous) were reported (Fig. 2 c) and pathogen spread seems to be directed towards the South Eastern territories. Moreover, the FD eradication was not achieved in the North West and East territories, despite application of intense monitoring

Fig. 4 - Symptoms of GY on cv. Sangiovese.

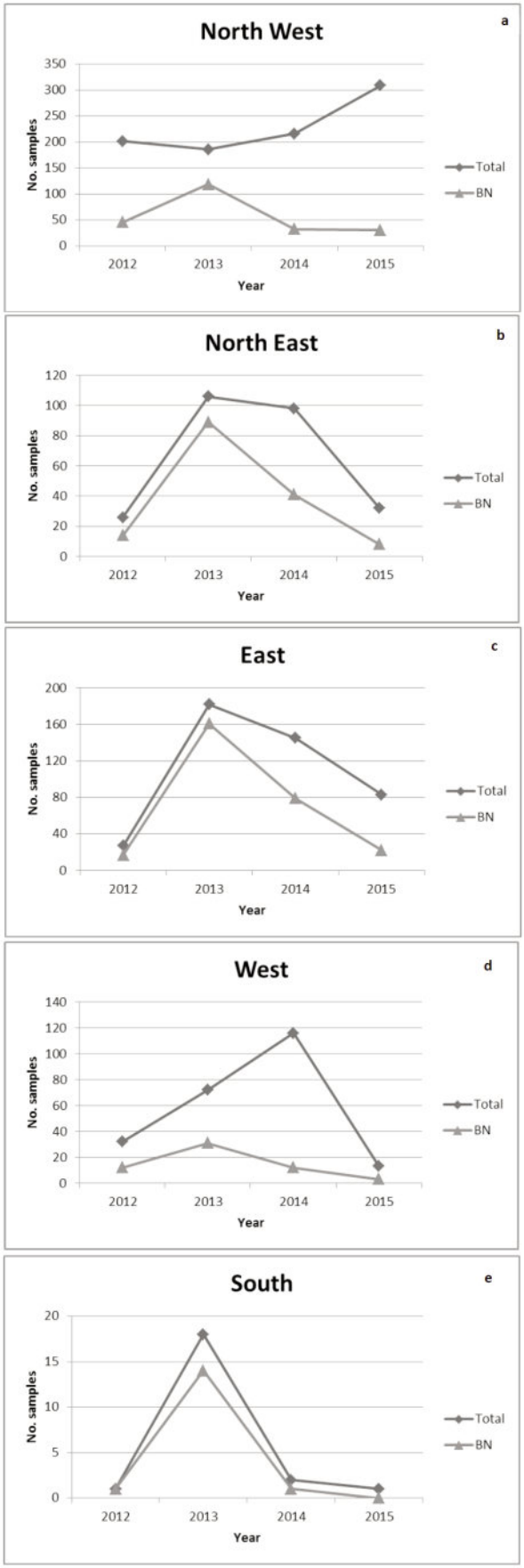

Fig. 5 - Samples positive to "bois noir" in Tuscan areas in 2012-2015.

Table 2 - Findings of novel flavescence dorée foci

Territory/districts	Novel flavescence dorée foci			
	2012	2013	2014	2015
North West	15	6	10	22
Massa-Carrara	*8*	*0*	*0*	*1*
Lucca	*6*	*5*	*10*	*15*
Pistoia	*1*	*1*	*0*	*6*
North East	0	0	0	0
West	0	0	0	0
East	2	0	2	1
Siena	*1*	*0*	*2*	*1*
Arezzo	*1*	*0*	*0*	*0*
South	0	0	0	0
Total	17	6	12	23

programs. With regard to GY characterization, FD-C was most frequently found (Fig. 6 a). Few samples of FD-D were found in North West (Lucca district). Among BN, *tufB* type-b strains were significantly more frequent in all territories except South, were only *tufB* type-a was found (Fig. 6 b).

4. Conclusions

Even though a few FD infected plants were detected in the four years of monitoring, novel foci

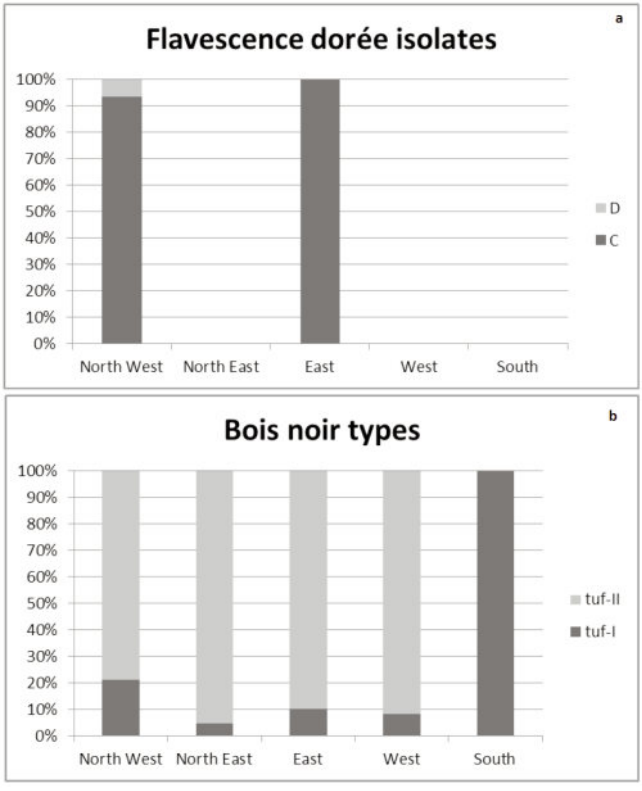

Fig. 6 - Distribution of (a) "flavescence dorée" and (b) "bois noir" subgroups and tufB types respectively in Tuscany.

continued to appear (17, 6, 12 and 23 in each year of survey), suggesting a more severe health condition of Tuscan vineyards and a dangerous distribution of FD in Tuscany.

Indeed, GY constitutes a serious concern for Tuscan viticulture, considering the repeated finding of novel FD foci in the northern part of Tuscany, the first detection of FD in the Eastern territories and the frequent presence of BN in all districts. Comparison between the 2012 and 2015 data in Lucca district indicate how the disease is spreading in the North West of Tuscany despite monitoring. In Lucca district, novel foci were observed each year, whereas the consistent findings of novel FD foci in the eastern district of North West in 2015 was also worrisome. Fortunately, no FD infected samples were found in North East, besides novel FD findings in the East.

With regard to GY characterization, the prevalence of FD-C confirms the results of surveys in North-Eastern territories of Italy (Veneto) (Borgo *et al*., 2001) and North-Western territories of Italy (Piedmont) (Marzachì *et al*., 2001). In Tuscany, *tufB* type-b was predominant over *tufB* type-a in most districts. This evidence is in accordance with data reported by Pierro *et al*. (2018), where the presence of the only *tufB* type-b was identified in a case vineyard in the Chianti Classico area (Tuscany). This strain has, as main host plant of the phytoplasma and of the vector, *Convolvulus arvensis* which high abundance was also reported in Tuscan vineyards (Marchi *et al*., 2015).

True positive rate of GY was overestimated in 2012, 2014 and 2015, probably due to simultaneous foliar symptoms caused by virus and fungal disease. Viruses, which are frequently found in Tuscany (Rizzo *et al*., 2012, 2015), as well as damage due to leafhopper, may mistake sampler. Nevertheless, the percentage of infection was comparable to those obtained in Northern Italy (Marzachì *et al*., 2001; Marzachì and Pacifico, 2006).

Eradication of FD from Tuscany seems a difficult task even in recently colonized territories, probably due to the jeopardized distribution of the pathogen. That may lead, in the subsequent years, to the discovery of many further foci characterized by only a few plants.

Acknowledgements

The authors wishes to thank the Inspectors of Phytosanitary Service of the Tuscany Region.

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