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Spreading of diseases caused by phytoplasma on local and foreign grape varieties grown in Bulgaria from 2005 to 2018

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Abstract

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Viticulture and winemaking existed from time immemorial. This is determined by the favourable soil and climate conditions in this country. In an effort to promote the development of an ever-growing winegrowing sector and to avoid new quarantines and the spread of economically-relevant diseases caused by phytoplasma, the Bulgarian Food Safety Agency (BFSA) has developed a monitoring programme. The purpose of the study is to look into the spread of grape vine diseases caused by phytoplasma and then, on the one hand, to establish the specific non-autochthonous and autochthonous grape varieties prone to grapevine yellows and, on the other hand, to compare data on infections affecting wine, rootstock and dessert grapes used in Bulgaria.

Between 2005 and 2018, the study involved over 89 grape varieties of all grape growing regions and over 7 lines of rootstock material in Bulgaria. The tests were carried out in the Central Laboratory of Plant Quarantine (CLPQ) and 5519 plant samples of indigenous and foreign varieties in total were analysed for the entire period. Despite the tremendous efforts of the BFSA, the PCR tests only found Bois noir infections to different varieties. In total, there were 258 infected samples out of 37 varieties. Chardonnay, Merlot, Cabernet Sauvignon, Pinot Noir and Alicante Bouchet were found to be the most susceptible ones. The study found 25 infected samples out of 15 Bulgarian local varieties which is a considerably low percentage of infection. The findings are a good basis and will contribute to finding solutions in the selection of grape varieties and improving the health of new vineyards in Bulgaria.

Keywords: Bois noir, '*Ca*. Phytoplasma solani', grapevine, susceptible cultivars, Bulgaria

Introduction

Viticulture is one of the most important sectors of agriculture in Bulgaria. In the 1980s the country was ranked along line some major grape growing and winemaking nations such as France, Italy and Spain.

In the 1970s the grape cultivation areas peaked at 207000 ha; following the transition to market economy in the 1990s, the areas were considerably reduced; now the Ministry of Agriculture, Food and Forestry (MAFF) estimates that they were 63952 ha in 2017; however, there is an increase in the average grape yield from 3,349 t/ha in 2001 to 5818 kg/ha in 2017 (MAFF, Agrostatistics department, 2018). Following the accession of Bulgaria to the European Union in 2007, the imports of grape vines from countries outside the EU, such as Macedonia, Serbia, Moldova, etc., were stopped. Grape vines are only imported from EU countries and 9 million grape vines were planted in 2007, 4 million in 2008 and almost 420 thousand in 2009, over 1.9 million from 2010 to 2018. The major suppliers are France (over 7.2 million grapevine plants for planting), Germany (over 1 million grapevines). The grape varieties most in demand are Syrah, Cabernet Sauvignon, Merlot, Chardonnay, Sauvignon Blanc, Pinot Noir, Cabernet Franc and Traminer.

Bois noir (BN) was found in Eastern France in the 1960s. Grape vine yellows, Vergilbungskrankheit (VK), in the Rhein and Mosel valleys in Germany had similar symptoms (Caudwell, 1964; Gärtel, 1965). Such diseases appear in countries with moderate climate across Europe (Daire et al., 1993, Maixner et al., 1995, Daire et al., 1997 a). They are caused by a phytoplasma classified as '*Candidatus* Phytoplasma solani' (Firrao et al., 2005) from the 16SrXII –A Stolbur group. The Flavescence dorée (FD), quarantined in Bulgaria, is also found in all these countries importing to Bulgaria. Grape vine yellows are observed both in wild and cultivated grape vines.

Signs on the grapes can be seen on the non-infected ones. There are only few grape berries at the early stage of the infection, the flowers dry and fall (Caudwell, 1964). At the late stage of the infection the grape clusters become brown and shrivel while the rachis gets dry. In the case of the highly susceptible Bako 22A variety and a serious infection, the berries fall (Belli et al., 1973). All *Vitis vinifera* L. varieties are susceptible to varying degrees to the phytoplasma causing grape vine yellows. There are highly susceptible varieties such as Bako 22A, Chardonnay, Aramon, Alicante Bouchet, Pinot Noir, Sangiovese and Zweigelt (Refatti et al., 1992; OEPP/EPPO, 1994; Martelli and Walter, 1998). Others, such as Merlot, Cabernet Sauvignon and Syrah can recover completely from the phytoplasma disease depending on the climate and soil conditions (OEPP/EPPO, 1994a; Kölber et al., 2003). Studies indicate different rootstock varieties, such as Fercal 3309 line and the American Vitis varieties which are tolerant to various types of phytoplasma – in this case the infection is latent. Such rootstocks are a risk factor for the spread of the disease (Caudwell et al., 1994).

Hyalestes obsoletus (genus Cixiidae) is the main stalbur vector for vegetables from the family *Solanacea* (Fos et al., 1992) and BN and VK phytoplasma for grape vines in France, Germany and Italy (Caudwell, 1971; Sforza et al., 1998; Maixner et al., 1994,). It is very common on the Balkans and across the Mediterranean region. It is found most in the weeds near the grape vines and, in exceptional cases, feeds on the grape vines themselves. It spends the winter at a nymph stage on the roots of bindweed, nettle or other weeds. That is why it is important to eliminate them in order to reduce the pest population (Maixner, 1994). The adult insects can fly from mid-June to the end of July in Germany, Italy and France (Alma et al., 2002). It is only during this period that *H. obsoletus* might incidentally get into contact with the grape vines. This is the period of possible infection with yellows caused by phytoplasma. The weed plants (*Convolvulus arvensis, Urtica* spp., *Stelaria* spp., etc.) are infection reservoirs in the vineyards. Proof of this hypothesis was found when the *H. obsoletus* was identified as a vector of the VK and BN phytoplasma (Maixner et al., 1995; Sforza et al., 1998; Credi et al., 2006). The field bindweed (*C. arvensis*) is one of the major stolbur nests. The most important stolbur phytoplasma vectors in the vineyards are 4 types of Cixiidae, ranked in terms of their importance as follows: *H. obsoletus* – up to 38%, *R. quinquecostatus* – 15%, *Reptalus panzeri* – 8% (Cvrković et al., 2011) and *Dictyophara europaea* – below 8% (Lessio et al., 2008).

Grape vine yellows (GYs) were found in Roumania (Rafalia et al., 1970), Italy: in Sicily (Marcone et al., 1996), Emiglia-Romagna (Daire et al., 1993a), Tuscany (Daire et al., 1993b), Liguria (Bertaccini et al., 1995), Greece (Daire et al., 1997), Switzerland (Bourquin et al., 2000), Moldova (Marinesku et al., 1991), Spain (Batlle et al., 1995), Slovenia and Croatia (Škorić et al., 1998), Hungary (Varga et al., 2000), Macedonia (Segura et al., 2003), Bosnia and Hertzegovina (Delić et al., 2007), Ukraine (Milkus et al., 2005). On the American continent GY was found in the USA (Wolf et al., 1994), Canada (Rott et al., 2007), Chile (Gajardo et al., 2009). In Africa and Asia, it was found in Israel (Daire et al., 1977a), Lebanon (Verdin et al., 2002), Iran (Karimi et al., 2009), China (Duduk et al., 2011) and the South African Republic (Engelbrecht et al., 2010).

Dobrev described GY's in Bulgaria for the first time (1909, 1910). In 1952 Trifonova and others described three GY groups. The first one is the viral chlorosis, the second one is mosaic and the third one is stunting (court-noue). It is recommended to plant health grape vines as a measure to reduce the spread of the disease.

In the case of GY there is a fall in the quality and quantity of yields. Grape clusters are smaller compared to the typical size for the given variety and have fewer berries. There is a high acidity level and low sugar level in the grapes. The wine made from such gapes is of low quality. Grape varieties for high quality wine (such as Riesling, Chardonnay, Cabernet Sauvignon, Pinot Noir) are highly susceptible to the organism causing GYs. The main damages to the grape vines include growth depression and malformation of plants and goes to maldevelopment of berries (Maixner et al., 1997). Phytoplasmas threaten traditional grape varieties in France (Faul Banc for cognac), Italy (Sangenovese and Nebiolo for Chianti in Tosany) and in Spain (the Malvasia and Tempranillo varieties in the Rioca regions) (Boudon–Padieu, 2006). The latest studies indicate cases of infections in other varieties such as Chardonnay, Frankovka, Reinishe Riesling, Župljanka in Serbia (Kuzmanovic et al., 2007); Pinot noir in Chile (Longone et al., 2011); Grk (Jezie et al., 2013) and Plovdina (Plavec, 2015) in Croatia; Frontenac, Seyval Noir and Geisenhem in Quebec CA, (Olivier et al., 2014); Chardonnay, Sauvignon

Blanc, Merlot, Cabernet Sauvignon, Sultani, Öküzgözü, Karal Karasl, Alicant Bouchet, A. Lavallée in Turkey (Ertunc et al., 2015); local cultivars in Iran (Mirchenari et al., 2015); Chardonnay, Rkatsiteli, Saperavi, Shavkapito, Tavkveri, Aladasturi, Kachichi, Ganjuri, Isabella, (*Vitis labrusca* L.) species hybrid cultivar Noah in Georgia (Chkhaidze et al., 2016); Sangiovese in Tuscany (Italy) (Rizzo et al., 2018).

In our country the data on diseases caused by phytoplasma are little and scarce and not in line with the latest knowledge (Dobrev, 1909, 1910; Trifonova et al., 1952; Abrasheva, 1977; Kovachevski et al., 1995; Genov et al., 2017; Avramov et al., 2018). This makes it necessary to research to gather new data on the topic.

Main goal

The purpose of the study is to look into the spread of grape vine diseases caused by phytoplasma and to establish the specific grape varieties prone to grapevine yellows.

We need to update the information because of the changes to the varieties in the new vineyards on the territory of Bulgaria and because of the increased imports of grapevines from the EU which are subject to checks to avoid the transmission of phytoplasma infections from areas which are known for the outbreak of such diseases.

Materials and Methods

The research was carried out in all the viticulture and winemaking regions of Bulgaria in autumn when the phytoplasma infection in the parts of the grapevines above the ground registers the highest levels. 5519 samples of 96 grape varieties were taken from the vineyards in the period from 2005 to 2018.

Plant total nucleic acids were extracted following previously described CTAB extraction protocol (Boudon-Padieu et al., 2003) or MiniPlant kit (Qiagene) according to the manufacturer's instructions.

Grapevine DNA samples were then analysed by Nested PCR assay according to Lee et al. (1994, 1995) with phytoplasma universal primers P1/P7 (Deng & Hiruki, 1991; Smart et al., 1995), followed by R16F2n/R2 (Daire et al., 1997) or fU5 и rU3 (Lorenz et al., 1995). As negative controls in all the experiments, DNA of symptomless grapevines was used. PCR reactions were performed with GeneAmpÒ S9700 thermocycler. To distinguish between the FD and BN phytoplasma, we applied the RFLP method – Restriction fragment length polymorphism – by using the *Alu*I or *Rsa*I (Amersham Biosciences) enzymes for all the positive samples and checks. We used the *Taq*I enzyme for the RFLP analysis concerning the subgroup classification (in the 16SrV group). Finally, reaction mixtures were analysed by electrophoresis. An electrophoresis, 1.5% (w/v) agarose in Tris-borate-EDTA (TBE) buffer or 10% polyacrilamide gel was used. Visualisation of DNA bands was done using a ultra-violet (UV) transilluminator (Angelini et al., 2001).

To confirm the appearance of the infection, we used a Real Time PCR with a FD and BN primers which amplify DNA fragments specific to the different types (Hren et al., 2007). We recorded the findings by using a MxPro (Stratagene) software product to detect the fluorescent signals with the degradation of the probe and to identify the fluorescent values of the different amplicons.

Results

Research of industrial-scale vineyards in Bulgaria

The findings of the study on the phytoplasma causing grape vine yellows concern grape vine samples from industrial-scale vineyards in the major viticulture and winemaking regions in Bulgaria and imported grape vines[1].

The results of the laboratory tests found that there was no *Grapevine Flavescence dorée* (FD). Table 1 show the number of test samples (over 5500) and their origin.

The stolbur phytoplasma causing GY, known as Boir noir disease, was found in the industrial-scale vineyards in Bulgaria (Tables 2, 3 and 4).

The research found the symptoms of a phytoplasma infection on the grape vines:

The leaves of the grape vines had the typical symptom of a phytoplasma infection. With the Chardonnay and Traminer white grape vines we found that the sun-exposed leaves had yellow spots with a metallic shine, and curled downwards while with the red grape vines the leaves were coloured in red. In the case of leaf curling, the leaves looked like a triangle and overlapped like roof tiles. There was necrosis and drying in the central part of the discoloured parts towards the end of October. In the case of the Cabernet Sauvignon and Grenache varieties, the red colour was limited to the leave veins while with the Merlot and Pinot Noir grapes; it affected the entire leaf surface and the veins. We also found changes to the leaf tissue which gave a cracking sound when touched as described by Caudwell in 1957 and 1971 (Figure 1). The green leaves of some grape vine shoots were next to red or yellow ones (Figure 2). We took the samples in terms of this symptom so as to identify the infection because it may be that the phytoplasma is concentrated in individual part of the grape vines where the damages are obvious (Caudwell, 1971; Gärtel, 1965).

[1]All the grape vine samples from the country had symptoms of a phytoplasma infection

Table 1. Results of the research of phytoplasma causing GY in Bulgaria

Years	Regions	No observ	PCR testing samples for			Established phytoplasma infection in regions
		Plants	Total	FD	BN	
2005	Bourgas, Stara Zagora, Pleven, Lovech, Varna, Blagoevgrad, Rousse, Plovdiv, Haskovo, Dobrich, V. Tarnovo	770	158	0	4	Sliven, Targovishte, Veliko Tarnovo
	France, Italy, Serbia, Germany, Austria (import materials)		111	0	1	Serbia
2006	Bourgas, Stara Zagora, Pleven, Lovech, Varna, Blagoevgrad, Rousse, Plovdiv, Haskovo, Dobrich, V. Tarnovo, Kyustendil, Vratsa, Vidin	930	537	0	33	Sliven,Targovishte, V. Tarnovo, Lovech, Blagoevgrad, Pazardzhik
2007	All grapevine-growing regions	1120	413	0	30	Bourgas, Vratsa, Rouse, Blagoevgrad, Yambol, Haskovo, Varna
2008	All grapevine-growing regions	1120	532	0	16	Targovishte, Pleven, Varna, Vidin, Bourgas, Vratsa, Plovdiv, Shumen
2009	All grapevine-growing regions	1260	649	0	51	Bourgas, Sliven, Shumen, Ruse, Plovdiv, Veliko Tarnovo, Targovishte, Lovech, Razgrad
2010	All grapevine-growing regions	650	274	0	19	All over Bulgaria
2011	All grapevine-growing regions	600	305	0	1	Bourgas
2012	All grapevine-growing regions	700	392	0	44	All grapevine-growing regions
2013	All grapevine-growing regions	596	435	0	17	Vidin, Vratsa, Montana, Pleven, Plovdiv, Rousse, Stara Zagora, Shumen.
2014	All grapevine-growing regions	690	396	0	22	Vidin, Vratsa, Montana, Plovdiv, Bourgas, Stara Zagora
2015	All grapevine-growing regions	650	320	0	7	Blagoevgrad, Vidin, Vratsa, Montana, Plovdiv, Bourgas, Stara Zagora
2016	All grapevine-growing regions	632	369	0	5	Stara Zagora, Vidin, Vratsa, Plovdiv, Bourgas.
2017	All grapevine-growing regions	732	361	0	4	Vidin, Montana, Vratsa
2018	All grapevine-growing regions	350	271	0	4	Vidin, Stara Zagora, Shumen, Rousse
All the grapevine and wine-growing regions of Bulgaria		10800	5523	0	258	All grapevine-growing regions of Bulgaria

Table 2. Results of the tests grape vine root stock samples from 2005 to 2012 for phytoplasma infections

Rootstock	Total number of samples	2005		2006		2007		2008		2009		2010		2011		2012		Phytoplasma
		test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	test samples	Results PCR (+)	BN
Varieties	**318**	11	0	43	5	24	1	62	0	77	0	24	0	58	0	19	0	**6**
7		2005		2006		2007		2008		2009		2010		2011		2012		
Berlandieri x Riparia SO4 (SO4)	202	9		32	**5**	8		38		54		17		32		12		5
Chasselas x Berlandieri (41B)	40	2		10		6		1		11				10				**0**
BC1 x 333 EM (Fercal)	25			1				5		7				8		4		**0**
Berlandieri x Riparia (Kober 5 BB)	38					8	**1**	8		5		7		8		2		**1**
Berlandieri x Rupestris 1103 Paulsen	7					2		5										**0**
Berlandieri x Rupestris (Ruggeri 140)	5							5										**0**
Rupestris du Lot (Monticola)	1															1		**0**

Table 3. Results of the phytoplasma infection test of 2005 to 2018 of samples of grape vine varieties from other countries

Variety	Total number of samples	2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015		2016		2017		2018		Phyto plas-ma
		Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results of PCR (+)	number of samples	Results of PCR (+)	number of samples	Results of PCR (+)	BN
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Total number of varieties	**4489**	219	**5**	376	**23**	288	**23**	391	**15**	513	**49**	233	**18**	226	**1**	310	**39**	364	**16**	378	**21**	291	**6**	318	**4**	337	**3**	245	**4**	**227**
49		2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015		2016		2017		2018		
Chardonnay	926	12	**2**	68	**4**	33	**7**	53	**2**	134	**28**	80	**14**	54		55	**18**	91	**6**	84	**11**	77	**2**	88	**2**	73	**2**	24	**2**	**100**
Merlot	787	101	**3**	100	**4**	66	**5**	99	**4**	107	**6**	29	**1**	28		26	**2**	62	**3**	55	**4**	49	**1**	36	**1**	28	**1**	1		**35**
Cabernet Sauvignon	733	85		87	**9**	55	**3**	62	**4**	83	**3**	25	**2**	33		36	**2**	44	**3**	72	**3**	35	**1**	48	**1**	39		29		**31**
Pinot Noir	291	2		4		7	**2**	12		33	**1**	13		17		40	**8**	38	**2**	28	**2**	21	**1**	30		41		5		**16**
Alicante Bouchet	39			1		1				3	**3**	4	**1**	3	**1**	4	**1**	2	**1**	5		4		7		4		1		**7**
Sauvignon Blanc	191			8		9		16	**1**	5	**2**	14		21		15	**2**	19	**1**	14	**1**	16		17		13		24		**7**
Muscat Ottonel	238	9		26	**1**	12		13		29	**3**	7		9		20	**1**	17		19		8		8		21		40		**5**
Traminer	162	2		11	**2**	17	**1**	38	**2**	22		3		6		4		11		15		11		11		6		5		5
Syrah	261	2		14	**1**	33	**1**	35	1	27	**1**	9		12		14	**1**	13		13		23	**1**	29		19		18	**1**	**7**
Cabernet Franc	160	2		1		6	**1**	27		8		6		10		13	1	11		14		14		9		15		24		**2**
Riesling	138	1		8		18	**1**	4		4		2		8		17		14		10		5		5		18		24		1
Italy*	36	1		5	**1**	2		3		4	**1**	3		1		1		5		3		3		4		1				2
Sangiovese	26							3	**1**	5		1		2		3		4		2		1		2		3				1
Gamet	33			1	**1**	5				1		4				3		3		6		4		3		3				1
Grenache	35			1		1						1		2		4	**1**	3		2		1		3		4		13	**1**	**2**
Cot Malbec	33					1		4		5	**1**	1		3		3		4		3		2		2		3		2		1
Vinnier	36							1		1				3		6	**1**	4		1		3		2		6		9		**1**
Marselan	18													1		2	**1**	4		2		1		2		2		4		**1**
Muscat Hamburg*	46			11		4		2		7		4		1		2		3		4		2		2		2		2		0

Table 3. Continued

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Aligote'	22			7		3		1						3				1		3		3		1						0
Alphonse-lavallée*	6			2														1		2				1						0
Black Pearl*	32	2		5				1		7		3				6		1		3				1		3				0
Vranek	5			1		1														1		1		1						0
Grand Noir	13					1		6						1				1		3								1		0
Palieri*	25			9				3		4		2				2				4						1				0
Moldova*	18			6		5	**1**			1		1						1		2		1		1						1
Petit Verdot	27					1		1		6		3		1		5				1						6		3		0
Primitivo	3					1																1		1						0
Egio Dola	8					1								1		2										2		2		0
Dornfelder	9							1		2						2		1		1						2				0
Regent	10							2		2						3										3				0
Juni Blan	12							1		5						3										3				0
Victoria*	9									1		7								1										0
Carmenere	6									1		1		1				1										2		0
Pitor Gris	21									2		1				4		1				1		1		4		7		0
Petit Man-seng	11							1		1				1		3		1		1						3				0
Rebo	2									1				1																0
Tempranillo	1									1																				0
Nero D'avola	1									1																				0
Proseco	2															1										1				0
Nebiolo	10											1				3		1		2		1				1		1		0
Fetiasca Alba	4											3		1																0
Flame seedles*	6											5												1						0
Chenin	4													1				1		1		1								0
Grüner Velteline	7							1						1		2										3				0
Vostorg*	8					5	**1**											1		1				1						1
Cinsault*	4															2										2				0
Roussanne	9							1								2						1		1		2		2		0
Colombard	5															2						1						2		0

*- desert varietes

Table 4. Results of the phytoplasma infection tests of local Bulgarian and already known introduced grape varieties

Variety	Total number of samples	2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015		2016		2017		2018		Phy-to-plas-ma
		Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results PCR (+)	Number of samples	Results of PCR (+)	BN
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Total number of varieties	**712**	39	**0**	118	**5**	101	**6**	79	**1**	59	**2**	17	**1**	21	**0**	59	**5**	71	**1**	18	**1**	29	**1**	51	**1**	24	**1**	26	**0**	**25**
40		2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015		2016		2017		2018		
Evmolpia	29			6				1		4	**2**	3		2		3	**1**	4		2				3		1				**3**
Misket Cherven	35	2		6	**1**			9		1						13	**1**	2						2						**2**
Mavrud	85	3		7		14		15		1				5		18	**1**	5						11		6				1
Velika*	88	1		12		9		15		9		3		1		2	**1**	11	**1**	4	**1**	7	**1**	13	**1**	1	**1**			**6**
Melnik 55	35	14		7	**2**	3								2		1		2						1		2		3		**2**
Nadezda*	30	6		2	**1**	7				1		3		1		1		1		3		2		1		1		1		**1**
Slivenska Shevka	10			6	**1**																	1		3						**1**
Misket Vrachanski	14					2				3		1	**1**	1						1		3		1		1		1		**1**
Buket	7			1				1	**1**							3		1				1								**1**
Misket Kailushki	15			1		5	**1**	1		2						1	**1**	1				1		3						**2**
Ryahovo*	6					2	**1**			2												2								**1**
Bolgar*	50	1		17		11		6		4		1		1		3		3		1		1				1				**0**
Brestovitsa*	37	1		6		4		5		5		3		1				3		3		3		1		2				**0**
Pamid	24	1		5				7		3				1				2				3		1		1				**0**
Cardinal*	34			8		5	**1**	4				1				1		8		1		1		5						1
Melnishki Rubin	21			5		10										1		5												**0**

Table 4. Continued

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Rubin	17							6		2						5						1		1				2		**0**
Shiroka Mel-nishka Loza	13					9		2		1														1						**0**
Gamza	18							4		3				4		1										3		3		**0**
Dimyat	15					2		2		1		1		2		1				1		1		1		2		1		0
Dunav*	21	1		6		5	**1**					1						4		1		1		2						**1**
Hybrid VI-4*	8	6				1										1														**0**
Tamyanka	12					2				5						2								1				2		**0**
Maritsa	8			6														2												**0**
Rusalka*	14			2		5	**1**	1		1						1		2		1		1								**1**
Armira*	8									6																1		1		**0**
Rusensko edro*	10			5														5												**0**
Siyana*	11			5														5								1				**0**
Melnik 1300	4			1						1								1								1				**0**
Drujba	2	1								1																				**0**
Storgozia	2									2																				**0**
Ruen	3			1												1		1												**0**
Naslada*	1	1																												**0**
Misket Karlovo	1	1																												**0**
Prista*	2			1														1												**0**
Misket Sandan-ski	2			1														1												**0**
Sultanina*	12																											12		**0**
Hybrid 537 Zornitsa*	3			1						1								1												**0**
Trakijska perla*	5					5	1																							**1**
Wild vines	4															4														**0**

* – desert varieties

Fig. 1. General view of the grape vine infected with phytoplasma

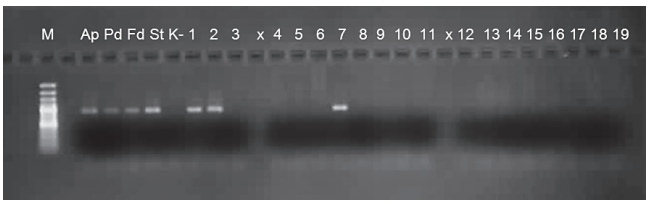
Fig. 2. Symptoms of phytoplasma infection on the shoots

We found that some parts of shoots of affected branches exhibit rows of black pustules that develop along the internodes. Due to incomplete lignification, these shoots were more flexible than normal. They were hanging and were visibly different from the healthy woody and brown shoots. We cut them and found that there was an excessive amount of phoema compared to the amount of wood. The non-woody parts were located on the nodes. The shoots were thinner, did not give a cracking sound when curved, the plant tissue was like rubber and the internodes were shorter: symptoms described by Belli et al. (1973) and Maixner et al. (1995b).

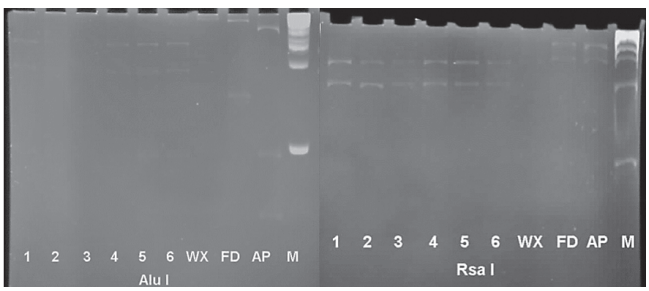
Findings of the molecular diagnostics – PCR test of plant samples

The infection of *stolbur phytoplasma* was found for the first time in this country by means of a PCR test in 2005 when there were massive scale tests of grape vine samples (230) from different viticulture and winemaking regions of Bulgaria. 111 samples of grape vines imported from France, Italy and Serbia were also tested because of the risks associated to their origin from country where phytoplasma infections were proven to have appeared (Table 1, Figure 2).

PCR test for *Flavescence dorée* were negative (Figure 3).



A) PCR with universal primers



B) PAGE – *Alu*I and *Rsa*I

Fig. 3. *A*: PCR amplification of 16S rDNA of phytoplasma isolates with universal primers (P1/P7 and U3/U5). Lines 1 to 20 – grape vine samples; Lines x – empty; Lines 1, 2 and 7 – positive samples; K – negative checks; Lines Ap, Pd, Fd, St, – positive checks; M – marker 10kb (EUROGENETEC); *B*: RLFP AluI / RSA enzymes: Lines 1-6 – grape vine samples; Lines WX, FD and AP – positive checks; Line M – marker 10kb (EUROGENETEC).

BN phytoplasma was found for the first time in Sliven – 3 samples, Merlot variety, in the village of Sadijsko pole (Figure 4); Targovishte, 1 sample, Chardonnay variety in the village of Venchan, and in the region of Veliko Tarnovo, 1 sample, Chardonnay variety in the village of Sovata.

Figure 3 shows the results of a Nested PCR test (Lorenz et al., 1995), confirming the BN infection in the grape vine samples, Merlot variety from the region of Sliven. The DNA fragment BN amplification is 876 bp (Figure 3A).

In 2006 there were tests of 581 samples of grape vines grown in Bulgaria and 38 of them were infected. The appearance of BN was confirmed in the region of Sliven, 8 samples, Merlot variety, in the village of Sadijsko pole (8 samples); Chardonnay (1 sample), Red Misket (1 sample) and Sefkat (1 sample); Targovishte 5 samples, Merlot variety (1)village of Venchan, Cabernet Sauvignon (1), Traminer variety (2), Muscat Ottonel variety (1) and Veliko Tarnovo 5 samples, (2 in Merlot, Gamet (1) and 2 samples, Chardonnay variety, in

Fig. 4. Symptoms of the phytoplasma infection in the region of Sliven (village of Sadijsko pole, 2006)

the village of Sovata; Lovech – 1 sample, Merlot variety, in the village of Yoglav; Blagoevgrad – 5 samples in the Melnik 55 variety (2), SO4 (2) and Nadezhda variety; Haskovo – 1 sample, Merlot variety; Pleven – 3 samples, Cabernet Sauvignon variety (2) and Merlot; Pazardzhik – 6 samples, Syrah" (2), SO4 (3) and Cabernet Sauvignon.

The 2007 results show that – out of 437 samples from Bulgaria – the positive samples were as follows: out of 29 sample in the region of Bourgas 9 stolbur (BN) Chardonnay (2), Trakijska perla (1), Cabernet Sauvignon (1) from Pomorie, Syrah (1) in the village of Ognene and Merlot (1) from Karnobat, Vostorg variety (1), Rusalka (1) and Riahovo (1); Vratsa a sample Cabernet Franc variety; Blagoevgrad 2 samples, Merlot variety from the region of Petrich; Ruse 7

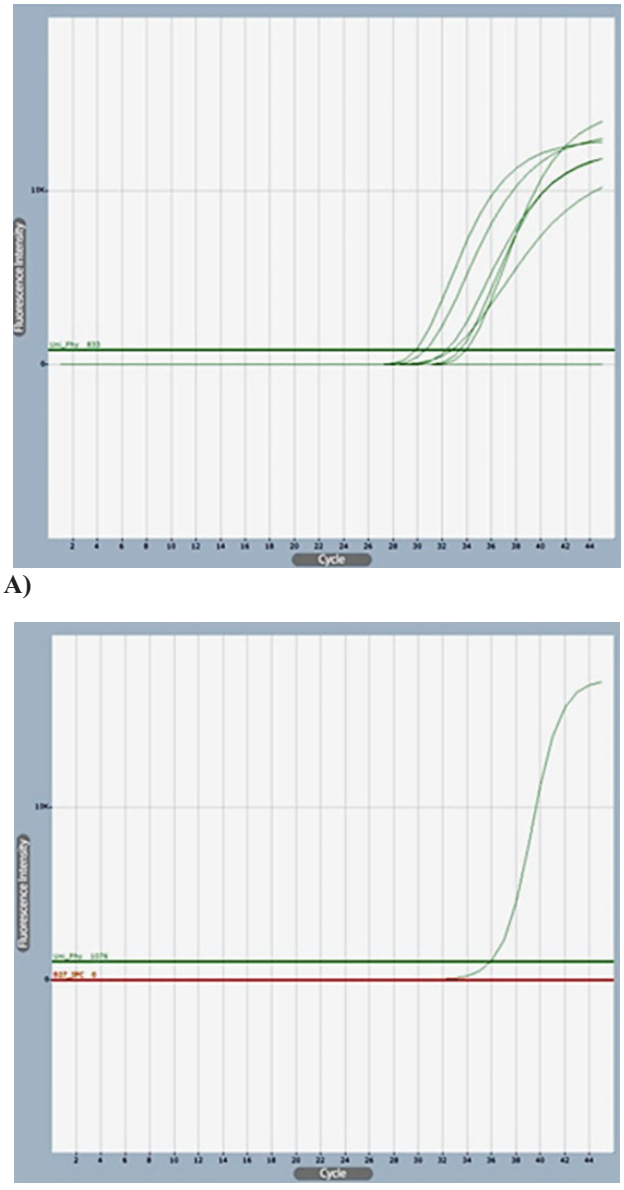


Fig. 5. Results of the Real Time PCR.
***A*: BN positive grape vine samples (extential BN curves);**
***B*: Chardonnay sample**

samples of grapevine nursery, Cabernet Sauvignon (2), Merlot, and also in Moldova, Kardinal, Pleven and Dunav varieties from the region of Biala; Yambol a sample, Cabernet Sauvignon variety; Haskovo Merlot variety from Harmanli; Veliko Tarnovo 2 samples Pinot Noir varifety and Varna 6 samples, Chardonnay (5) and Riesling.

There were 16 positive PCR tests for BN phytoplasma in 2008 (Tables 1, 3, 4) from the regions of Targovishte, 4

samples, Cabernet Sauvignon (2), Sauvignon Blanc and Chardonnay; Pleven, 4 samples, Cabernet Sauvignon (2), Merlot and Chardonnay; Varna, 2 samples, Merlot and Sangiovese; Vidin, 2 samples, Merlot and Traminer; Bourgas, a sample of Traminer variety; Vratsa, a sample of Buket variety; Plovdiv, a sample of Syrah variety; Shumen, a sample of Merlot variety.

To confirm the results, all the positive samples were tested by using a Real Time PCT this year (Hren et al., 2007). The results confirmed only the appearance of BN (Figure 5).

In 2009 BN phytoplasma infection was found in 51 samples out of 726; the 51 cases were from the following regions (Tables 3, 4): Bourgas – 18 samples, varieties: Chardonnay (11), Muscat Ottonel (2), Alicante Bouchet (3), Cabernet Sauvignon and Merlot; Sliven – 9 samples, Chardonnay (4), Merlot (3), Pinot Noir and Evmolpia; Shumen – 3 samples, Chardonnay; Ruse – 4 samples, Chardonnay, Italy, Cabernet Sauvignon and Thracian Mavrud; Plovdiv – 3 samples, Chardonnay (2) and Syrah; Veliko Tarnovo – 4 samples, Chardonnay; Targovishte – 8 samples, Chardonnay (3), Sauvignon Blanc (2), Muscat Ottonel, Malbec and Cabernet Sauvignon; Lovech – 1 sample of Merlot; Razgrad – 1 sample, Merlot.

In 2010 BN infection was found in 19 samples out of a total of 298 samples; the infected samples were from the following regions: Shumen – 5 samples, Chardonnay; Ruse – 4 samples, Cabernet Sauvignon (2), Chardonnay, Merlot; Bourgas – 4 samples, Chardonnay (3) and Alicante Bouchet; Vratsa – 3 samples, Chardonnay; Plovdiv – 2 samples, Chardonnay; Varna – a sample, Vrachanski Misket.

The findings of the 2011 tests of 363 samples showed a positive result for BN in one sample of the Alicante Bouchet variety from the region of Burgas. In 2012 there were 44 BN infected samples out of 411 test samples, the infected samples were from the following regions: Ruse – 11 samples, Chardonnay (3) (Figure 6), Cabernet Sauvignon (Figure 6), „Sauvignon Blanc“, Thracian Mavrud, Kailashki Misket, Syrah, Pinot Noir, Merlot and Velika; Bourgas – 8 samples, Chardonnay (5), Pinot Noir (3); Yambol – 7 samples, Chardonnay (3), Pinot Noir, Alicante Bouchet, Cabernet Sauvignon, Merlot; Montana – 4 samples, Pinot Noir (2), Chardonnay and Cabernet Sauvignon; Targovishte – 4 samples, Chardonnay (2), Grenache, Muscat Ottonel; Shumen – 2 samples, Chardonnay; Plovdiv – 2 samples, Mavrud and Red

Table 5. Spread of the phytoplasma infection by regions and grape varieties in Bulgaria

Region	Grape varieties most commonly affected by BN			
Blagoevgrad	Chardonnay	Merlot	Melnik 55	SO4
Bourgas	Chardonnay	Alicante Bouschet	Cabernet Sauvignon	Traminer
V. Turnovo	Chardonnay	Pinot Noir	Merlot	Gamay Noir
Varna	Chardonnay	Misket Vrachanski	Merlot	Cabernet Sauvignon
Vidin	Chardonnay	Cabernet Sauvignon	Traminer	Syrah
Vratca	Chardonnay	Merlot	Marselan	Cabernet Franc
Montana	Chardonnay	Pinot Noir	Merlot	Cabernet Sauvignon
Plovdiv	Chardonnay	Cabernet Sauvignon	Velika	Pinot Noir
Rousse	Cabernet Sauvignon	Chardonnay	Merlot	Velika
Sliven	Chardonnay	Merlot	Pinot Noir	Evmolpia
Pazardjik	Merlot	Cabernet Sauvignon	Pinot Noir	
Pleven	Chardonnay	Merlot	Syrah	
Dobrich	Chardonnay	Merlot	Vionnier	
Silistra	Cabernet Sauvignon	Chardonnay		
St. Zagora	Chardonnay	Merlot		
Targoviste	Chardonnay	Traminer		
Shumen	Chardonnay	Rizling		
Haskovo	Chardonnay			
Yambol	Chardonnay			
Kiustendil	Chardonnay			

Fig. 6. Symptoms of Bois noir phytoplasma infection in grape vines, Cabernet Sauvignon and Chardonnay from the region of Ruse

Misket; one sample from the region of Sliven Pinot Noir; one sample from the region of Pazardzhik, Chardonnay; one sample from Dobrich, Vionnier; one sample from Vratsa, Marselan; one sample from Varna, Cabernet Franc and one sample from Veliko Tarnovo, Chardonnay.

In 2013 there were laboratory tests of 435 samples and BN was found in 17 of them, from 6 grape varieties: 6 wine varieties – Chardonnay (6), Merlot (3), Cabernet Sauvignon (3), Pinot Noir (2) and Alicante Bouchet, Sauvignon Blanc – and one Bulgarian dessert variety – Velika (Table 3).The regions where the infection was found were: Bourgas (4 infected samples), Vidin (2), Plovdiv (2), Shumen (2), Vratsa (2), Stara Zagora (2), Montana (2) and Blagoevgrad.

Following the laboratory tests of 396 grape vine samples in 2014, 22 infected samples were found in eight regions of the country, with the varieties most affected being Chardonnay (11), Merlot (4), Cabernet Sauvignon (3), Pinot Noir (2) and one of Sauvignon Blanc. It was only the Velika variety that was infected out of all the local grape varieties (Table 4).

In the next four-year period (from 2015 to 2018) over 1300 samples were tested and only 19 infected samples were found (Tables 3 and 4). The varieties most affected by the phytoplasma were Chardonnay (8) and the local Bulgarian variety Velika (2). The low infection level might be the result of better awareness on the part of wine makers and the measures to prevent the infections transmitted by leafhoppers and planthoppers in the Bulgarian vineyards.

Results

Based on the results we have found that the phytoplasma infection varies according to years when the temperatures are different and according to region in Bulgaria and according to grape variety as described in the literature (Caudwell, 1964, Belli et al., 1973; Martelli, 1993; Credi, 1994; Walter, 1997; Boudon-Padieu et al., 2000). In Bulgaria the total number of samples infected with BN is 258.

The first BN outbreaks were found to be in Sliven, Targovishte and Veliko Tarnovo in 2005 and affected Chardonnay and Merlot grape varieties (Table 1, Figure 5). We found that there were more and more affected regions in the following years. In 2006 we found proof of the BN infection in Lovech, Blagoevgrad and Pazardzhik. Based on the research and the analysis of samples, we identified phytoplasma infection in Bourgas, Vratsa, Ruse, Yambol, Haskovo and Varna in 2007 while in 2008 we found it in other regions too – Shumen, Plovdiv, Vidin. In 2009 there was BN infection in all the viticulture and winemaking region of Bulgaria (Table 1).

Tables 1, 2 and 3 show the results of the test samples over the years and the grape varieties. We tested seven grape rootstock varieties in total and found the BN infection in only two: SO4 (1.9%) and Cober BB (0.43%). This indicates a low incidence of phytoplasma infection which confirms the information from the literature concerning the transmission level of the phytoplasma infection in nursery grapes (Osler et al., 1997). Out of the wine grapes, the highest incidence of

phytoplasma infection was found with Chardonnay (38.8%), Merlot (13.6%), Cabernet Sauvignon (12.0%), Pinot Noir (6.2%), Alicante Bouchet (2.7%), Sauvignon Blanc (2.7%), Syrah (2.7%), Muscat Ottonel (over 1.9%), Traminer (1.9%), Cabernet Franc (0.8%), Grenache (0.8%) and Riesling (over 0.4%). The spreading of the phytoplasma infection by regions and grape varieties in Bulgaria are shown in Table 5.

The results of the tests of 96 grape varities indicate that there is a lower incidence of phytoplasma infection with dessert varieties. Out of 28 tested varieties, there were only individual positive BN tests (up to 6.6%) from the Italy and Cardinal varieties and the newly created Bulgarian varieties of Velika, Nadezhda, Dunav, Rusalka and Riahovo.

These results confirm the data on grape sensitivity and susceptibility to the phytoplasma infection (Walter, 1997) and the Flavescence dorée is likely to be found in these varieties. We found a total of 100 BN infected samples of the Chardonnay variety across the country (Table 3).

Table 4 shows the results of the tests of Bulgarian or varieties known in Bulgaria. We found BN in 15 grape varieties[2] but, in general, the incidence is lower which probably resulted from the fact that there were no risk factors (origin of the plants, specifics of the variety, age of the planted grape vines, agrotechnical measures and pest control). The BN infection was most commonly found in the following Bulgarian grape varieties: Velika (24%), Evmolpia (12%), Red Misket (8%), Melnik 55 (8%), Kaylashki Misket (8%), Nadezhda, Buket, Slivenska perla, Vrachanski Misket (more than 4% for each variety).

The tests of 96 grape varieties showed that the phytoplasma BN infection was less common with dessert grape varieties (35%) than with the wine varieties (39.7 %). Out of 10 dessert varieties there were individual infected samples from the varieties of Velika, Italy, Cardinal, Nadezhda, Dunav, Rusalka and Thracian pearl, around 6.2% in total (Tables 3 and 4).

This is likely to be the result of the difference between the phenological stages of wine and dessert grape varieties and the phenological development of the insect vectors *H.obsoletus* and *Reptalus* spp. This hypothesis might be the subject of future research and might be confirmed or rejected.

Concusions

We have not found the Grapevine Flavescence doree phytoplasma which is put in quarantine for Bulgaria. All the infections and all the isolates found in the research belonged to the stolbur grop of `*Candidatus* Phytoplasma solani`.

Based on the test results, we have found that the phytoplasma infection varies according to years when the temperatures are different, according to region in Bulgaria and according to grape variety as described in the literature (Caudwell, 1964, Belli et al., 1973; Martelli, 1993; Credi, 1994; Walter, 1997; Boudon-Padieu et al., 2000).

The test of 96 grapevine varieties showed that there is lower incidence of phytoplasma infection with dessert grape varieties.

Out of 28 tested varieties, there were only individual positive BN tests (around 6%) from the Italy and Cardinal varieties and the newly created Bulgarian varieties of Velika, Nadezhda, Dunav, Rusalka and Riahovo.

Merlot, Cabernet Sauvignon and Syrah can recover completely from the phytoplasma disease depending on the climate and soil conditions.

It is proven that the following white grape varieties are highly susceptible to the BN: Chardonnay, Merlot, Cabernet Sauvignon, Pinot Noir, Alicante Bouchet, Sauvignon Blanc, Syrah, Muscat Ottonel, Traminer, Cabernet Franc, Grenache and Riesling.

It is proven that the phytoplasma causing BN is less common with the dessert varieties of Italy and Cardinal and the newly created Bulgarian varieties of Velika, Nadezhda, Dunav, Rusalka and Riahovo.

Recommendation on preventive treatments needs to be prepared, in particular for the years with favourable climate and the government offices needs to step up its control measures and the implementation of the guidelines on control for grapevine production for planting and vineyards.

The preservation of the characteristics of the autochthonous grape varieties and the purity in grape varieties and rootstock varieties in Bulgaria show that there is opportunity to increase the cultivation of grape vines of high quality and to create new grape varieties in Bulgaria.

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References

Abrasheva, P. (1977). Grapevine Flavescence doree. In: Plant Viruses and Mycoplasm Diseases, *Zemizdat*, 292 – 293 (Bg).

[2]Statistic data for all varieties

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

Avramov, Zh., Laginova, M., Panayotova, D., Ivanova, I. & Mihaylov, M. (2018). Monitoring of the quarantine phytoplasmic diseases on the vine in Bulgaria in the period 2012–2018. *Acta Oecologica Carpatica, X. I,* 85-94.

Batlle, A., Larrue, J., Clari, D., Daire, X., Boudon-Padieu, E. & Laviña, A. (1995). Identification of the phytoplasma associated with bois noir in Spain. *Phytoma-España, 68,* 40 – 44.

Belli, G., Fortusini, A., Osler, R. & Amici, A. (1973). Presence of flavescence dorée-like symptoms in the vineyards of Oltrepépavese. *Rivista di Patologia Vegetale, 9,* 50 – 56.

Bertaccini, A., Vibio, M. & Stefani, E. (1995). Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). *Phytopatologica Mediteranea*, *34,* 137 – 141.

Boudon-Padieu, E. (2006). *ICPM* – First Internet Conference on Phytopathogenic Mollicutes – Grapevine phytoplasmas.

Boudon-Padieu, E., Ridé, M. & Walter, B. (2000). Maladies à virus, bactéries et phytoplasmes de la vigne. *Éditions Féret, Bordeaux, France*. ISBN 2-902416-58-X.

Boudon-Padieu, E., Béjat, A., Clair, D., Larrue, J., Borgo, M., Bertotto, L. & Angelini, A. (2003). Grapevine yellows: Comparison of different procedures for DNA extraction and amplification with PCR for routine diagnosis of phytoplasma in grapevine. *Vitis, 42 (3),* 141 – 149.

Bourquin, L., Schmid, A., De Meyer, J., Cazelles, O., Ramel, M.-E. & Gugerli, P. (2000). Confirmation of the presence of stolbur type yellows in Swiss vineyards by molecular diagnostics of grapevine. *13th Meeting of ICVG, Australia, The University of Adelaide and CSIRO publishers*, 111 – 112.

Caudwell, A. (1957). Deux annees d'etudes sur la flavescence doree, nouvelle maladie grave de la vigne. *Annual Amel. Plantes*, *4,* 359 – 393.

Caudwell, A. (1964). Identification et étude d'une nouvelle maladie à virus de la vigne, la flavescence dorée. *Annales des Epiphyties, 15 (1),* 193.

Caudwell, A., Larrue, J., Kuszala, C. & Bachelier, J. C. (1971). Pluralité des Jaunisses de la vigne. *Annales de Phytopathologie, 3 (1)*, 95 – 105.

Caudwell, A., Larrue, J., Tassart, V., Boidron, R., Grenan, S., Leguay, M. & Bernard, P. (1994). Caractère porteur de la Flavescence dorée chez les vignes porte-greffes en particulier le 3309 C et le Fercal. *Agronomie*, *14,* 83-94.

Chkhaidze, N. M., Lobjanidze, Mz. I., Dzmanashvili, I. I., Barjadze, Sh. Z. & Maghradze, D. N. (2016). Grapevine phytoplasma disease in Georgia. *Annals of Agrarian Science, 14 (3)*, 153-162.

Credi, R., Terlizzi, F., Milanezi, L., Bondavalli, R., Cavallini, G., Montermini, A. & Dradi, D. (2006). Wild host plant of stolbur phytoplasma and its vectot *Hyalesthes obsoletus* at sites of grapevine bois noir occurance in Emilia – Romagna, Italy. *Abstract 15th Meeting ICVG South Africa,* 182 – 183.

Cvrković, T., Jović, J., Mitrović, M., Petrović, A., Krstić, O., Krnjajić, S. & Toševski, I. (2011). Potential new hemipteran vectors of stolbur phytoplasma in Serbian vineyards. *Bulletin of Insectology*, *64* (Suppl.), 129 – 130.

Daire, X., Clair, D., Larrue, J., Boudon-Padieu, E., Alma, A., Arzone, A., Carraro, L., Osler, R., Refatti, E., Granata, G., Credi, R., Tanne, E., Pearson, R. & Caudwell, A. (1993b). Occurrence of diverse MLOs in tissues of grapevine affected by grapevine yellows in different countries. *Vitis*, *32,* 247 – 248.

Daire, X., Clair, D., Larrue, J., Boudon-Padieu, E. & Caudwell, A. (1993a). Diversity among Mycoplasma – like organisms inducing grapevine yellows in France. *Vitis*, *32,* 159 – 163.

Daire, X., Clair, D., Reinert, W. & Boudon-Padieu, E. (1997). Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and the stolbur subgroup by PCR amplification of nonribosomal DNA. *European Journal of Plant Pathology, 103,* 507 – 514.

Delić, D., Seljak, G., Martini, M., Ermacora, P., Carraro, L., Myrta, A. & Duric, G. (2007). Surveys for grapevine yellows phytoplasmas in Bosnia and Herzegovina. *Bulletin of Insectology, 60*, 369 – 370.

Deng, S. & Hiruki, C. (1991). Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of Microbiological Methods, 14,* 53 – 61.

Dobrev, I. (1909). Distortion of leaves or court-noue on Grapevwine. *Zhemledelie, 15 (19)*, 6 – 9, (Bg).

Dobrev, I. (1910). Leafleavss Yellowing on Grapewine. *Zhemledelie, 16 (13 – 14),* 202 – 203, (Bg).

Engelbrecht, M., Joubert, J. & Burger, J. T. (2010). First Report of Aster Yellows Phytoplasma in Grapevines in South Africa. *Plant Disease*, *94,* 373 – 373.

Ertunc, F., Cakir, A., Söylemezoglu, G., Canik, D., Topkayaq, S. & Bayram, S. (2015). Reactions of some grapevine cultivars to "bois noir" phytoplasma. *Phytopathogenic Mollicutes,* doi: 10.5958/2249-4677.2015.00046.8, *5* (1-Supplement), 109-110.

Firrao, G., Andersen, M., Bertaccini, A., Boudon, E., Bove, J. M., Daire, X., Davis, R. E., Fletcher, J., Garnier, M., Gibb, K., Gundersen-Rindal, D., Harrison, N., Hiruki, C., Kirkpatrick, B. C., Jones, P., Kuske, C. R., Lee, I. M., Liefting, L., Marcone, C., Namba, S., Schneider, B., Sears, B. B., Seemuller, E., Smart, C. D., Streten, C. & Wang, K. (2004). The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group – ‚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.

Firrao, G., Gibb, K. & Streten, C. (2005). Short taxonomic guide to the genus „*Candidatus* Phytoplasma". *Journal of Plant Pathology, 87,* 249 – 263.

Gajardo, A., Fiore, N., Prodan, S., Paltrinieri, S., Botti, S., Pino, A. M., Zamorano, A., Montealegre, J. & Bertaccini, A. (2009). Phytoplasmas Associated with Grapevine Yellows Disease in Chile. *Plant Disease*, *93,* 789 – 796.

Gärtel, L. (1965). Untersuchungen über das Auftreten und das Verhalten der flavescence dareé in den Weinbaugebieten an Mosel und Rhein. *Weinberg und Keller*, *12,* 347 – 376.

Genov, N., Filippin, L. & Angelini, E. (2017). Occurrence and diagnostic of grapevine yellows on Chardonnay variety in the

region of Pleven, Bulgaria. *Journal of Mountain Agriculture on the Balkans*, *20 (3),* 289-299.

Hren, M., Boben, J., Rotter, A., Kralj, P., Gruden, K. & Ravnikar, M. (2007). Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics. *Plant Pathology, 56*, 785 – 796.

IALV (2009) Web site: http://www.eavw.com/bg/zak_drugi/Doklad-IALV-2009-FIN

Jezie, M., Kontic, J. K., Preiner, D., Maletic, E. & Curkovic-Perica, M. (2013). Grapevine yellows affecting the Croatian indigenous grapevine cultivar Grk. *Acta Botanica Croatica, 72 (2),* 287-294.

Karimi, M., Contaldo, N., Mahmoudi, B., Duduk, B. & Bertaccini, A. (2009). Identification of stolbur-related phytoplasmas in grapevine showing decline symptoms in Iran. *Le Progrès agricole et viticole HS,* 16 th Meeting ICVG: 208 – 209.

Kovachevski, I., Markov, M., Yankulova, M., Trifonov, D., Stoyanov, D. & Kacharmazov, V. (1995). Virus and virus-like diseases of crop plants. ISBN 054-14-0003-5. *PENSOFT & PSSA.*

Kölber, M., Ember, I., Varga, K., Botti, S., Martini, M., Lázár, L. & Bertaccini, **A**. (2003). Six-year survey of grapevine yellows distribution in Hungary. Extended Abstracts 14th Meeting of ICVG, Locorotondo 2003, 99 – 100. http://www.agr.uniba.it/ICVG2003/

Kuzmanovic, Sl., Martini, M., Ivanovic, Z., Josic, D., Živkovic**, S. & Starovic, M.** (2007). Detection and incidence of FD and BN phytoplasmas in vineyards of different grapevine cultivars in Serbia. *Bulletin of Insectology, 60 (2),* 371-372.

Lessio, F. & Alma, A. (2008). Host plants and seasonal presence of *Dictyophara europaea* in the vineyard agro-ecosystem. *Bulletin of Insectology, 61 (1),* 199 – 200.

Longone, V., González, F., Zamorano, A., Pino, A.-M., Araya, J., Díaz, V., Paltrinieri, S., Calari, A., Bertaccini, A., Picciau, L., Alma, A. & Fiore, N. (2011). Epidemiological aspects of phytoplasmas in Chilean grapevines. *Bulletin of Insectology, 64* (Suppl.), 91 – 92.

Lorenz, K-H., Schneider, B., Ahrens, U. & Seemüller, E. (1995). Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. *Phytopathol, 85,* 771 – 776.

Maixner, M. & Reinert, W. (1997). Spatio-temporal analysis of the distribution of grapevine yellows in Germany. *Proceedings 12th Meeting ofICVG*, Lisbon, 75 – 76.

Maixner, M., Aherns, U. & Seemuller, E. (1994). Detection of mycoplasmalike organisms associated with a yellows disease of grapevine in Germany. *Journal of Phytopathology*, *142,* 1 – 10.

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.

Maixner, M., Dair, X., Boudon-Padieu, E., Laviňa, A., Batlle, A. & Reinert, W. (1997). Colmar, Sanitary selection of the grapevine. *Phytoplasmas*, *86*, 183 – 195.

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

Maixner, M., Rüdel, M., Daire, X. & Boudon-Padieu, E. (1995b). Diversity of grapevine yellows in Germany. *Vitis*, *34,* 235 – 236.

Marcone, C., Ragozzino, A., Credi, R. & Seemüller, E. (1996). Detection and haracterization of phytoplasmas infecting grapevine in Southern Italy and their genetic relatedness to other grapevine yellows phytoplasmas. *Phytopathologica Mediterranea, 35,* 207 – 213.

Marinesku, V. G., Kalashyan, Y. A. & Verderevskaya, T. D. (1991). Grapevine yellows in MoldavianSSR. In: Proceedings of the 10th meeting of ICVG, Volos, Greece: *ORES Publishing*, 218.

Martelli, G. P. & Wagner, B. (1998). Virus Certification of Grapevines. *Plant Virus Disease Control*, Hadidi, A., Khetarpal, R.K., Koganezawa, H. (ed), 261 – 276.

Milkus, B., Clair, D., Idir, S., Habili, N. & Boudon-Padieu, E. (2005). First detection of stolbur phytoplasma in grapevines (*Vitis vinifera*, cv Chardonnay) affected with grapevine yellows in the Ukraine. *Plant Pathology*, *54*, 236.

Ministry of Agriculture, Food and Forestry (2018). agrostatisticalresults2000-2017 http://www.mzh.government.bg/media/filer_public/2018/04/30/ra342-vinewine2017.pdf

Mirchenari, S. M., Massah, A. & Zirak, L. (2015). 'Bois noir': new phytoplasma disease of grapevine in Iran. *Journal of Plant Protection Research*, *55 (1),* 88- 93.

OEPP/EPPO (1994a). Phytosanitary procedure. No.57. Mycoplasm-like organisms in fruit trees and grapevine. Inspection and test methods. Bulletin OEPP/EPPO *Bulletin, 24***,** 339-342.

OEPP/EPPO (1994b). Certification schemes. No. 8. Pathogen-tested material of grapevine varieties and rootstocks. Bulletin OEPP/EPPO *Bulletin, 24*, 347-368.

Olivier, Ch., Vincent, Ch., Saguez, J., Lasnier, J., Hammond, Ch., & Dumonceaux, **T.** (2014). Phytoplasma biodiversity in grapevine and leafhoppers sampled in vineyards of Quebec, *Entomological Society of America Annual Meeting,* November 2014.

Plavec, J. (2015). Molecular epidemiology of grapevine phytoplasma pathosystems: Multilocus sequence typing approach, Doctoral thesis, Department of Biology, Faculty of Science, University of Zagreb, Croatia.

Rafalia, C. & Costache, M. (1970). The golden flavescence (*Flavescence dorée*), a new disease of grape-vine in Roumania. *An. Inst. Cercet. Protectia Plant, 6,* 151 – 156.

Refatti, E., Osler, R., Carraro, L. & Pavan F. (1992). Natural spread of a flavescence dorée-like disease in north-east Italy. *Proceedings of the 10th Meeting of ICVG*. Volos, Greece.

Rizzo, D., Materazzi, Al., Stefani, L., Panattoni, Al., Roberto, Marchi, G., Cinelli, T., De Bellis, L. & Luvisi, A. (2018). The monitoring program of grapevine phytoplasmas in Tuscany (Italy): results of a four year survey. *Advances in Horticultural Science*, *32(2),* 281-288.

Rott, M., Johnson, R., Masters, C. & Green, M. (2007). First report of Bois Noir phytoplasma in grapevine in Canada. *Plant Diseas, 91,* 1682.

Schneider, B., Seemüller, E., Smart, C. D. & Kirkpatrick, B. C.

(1995). Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Razin, S. & Tully, J.G. Molecular and diagnostic procedures in mycoplasmology. *Academic Press, New York, USA,* 369 – 380.

Segura, M., Skoric, D., Kozina, B., Mitrev, S., Krajacic, M., & Curkovic Peruka, M. (2003). Molecular identification of a phytoplasma infecting grapevine in the Republic of Macedonia. *Vitis, 42,* 181 – 184.

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

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

Trifonova, V., Tscalev, M., Hristov, Al., Kovachevski, I., Martinov, S., Krasteva, L. & Kaytazov, A. (1952). More important plant quarantine pests and diseases and methods for detection. *Zhemizdat*. (Bg).

Varga, K., Kolber, M., Martini, M., Pondrelli, M., Ember, I., Tokes, G., Lazar, J., Mikulas, J., Papp, E., Szendery, G., Schweigert, A. & Bertaccini, A. (2000). Phytoplasma identification in Hungarian grapevines by two nested-PCR systems*, 13th Meeting of ICVG, Australia,* 113 – 115.

Verdin, E., Salar, P., Danet, J-L., Choueiri, E., Jreijiri, F., El Zammar, S., Gélie, B., Bové, J. & Garnier, M. (2002). „*Candidatus* phytoplasma phoeniceum", a new phytoplasma associated with an emerging lethal disease of almound trees in Libanon and Iran. *International Journal of Systematic and Evolutionary Microbiology, 53,* 833 – 838.

Wolf, T. K., Prince, J. P. & Davis, R. E. (1994). Occurrence of grapevine yellows in Virginia vineyards. *American Journal of Enology and Viticulture, 44,* 474.

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