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Full Length Research Paper

A study of suitability of grapevine cultivar Plovdina as a possible indicator plant for flavescence dorée disease

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Grapevine cv. Plovdina is a traditional local variety that is very sensitive to flavescence dorée (FD) disease. In this paper, we present the results of top-grafting FD-infected cv. Plovdina plants with scions originating from healthy mother plants of the same cultivar using the "green grafting" method. The first grapevine yellows (GY) symptoms, consisting of yellowing of the Plovdina leaves, have been observed on scions 20 days after grafting. Our results show that GY symptoms were due to phytoplasma and not the viral infection, since grapevine leafroll associated viruses-1, 2 and 3 (GLRaV-1, 2, 3) could not be detected in the scions. Twenty days after-grafting, phytoplasma FD was detected in all grafted scions. Our results indicate that grapevine cv. Plovdina is very suitable as an indicator plant for Flavescence dorée disease.

Key words: Grapevine, phytoplasma flavescence dorée, indicator plant.

INTRODUCTION

Phytoplasmas are cell wall-less, obligate parasitic, phloem-restricted bacteria, belonging to the class *Mollicutes,* responsible for severe diseases in grapevine. Phytoplasmas are transmitted by insect vectors and propagated by vegetative multiplication of plant material (Martelli and Boudon-Padieu, 2006). Grapevine yellows (GY) are a severe and worldwide disease complex caused by different phytoplasmas (Salar et al., 2009; Bertaccini and Duduk, 2009). Infected plants of *Vitis vinifera* show leaf rolling and curling, along with yellowing or reddening, rubberiness of the canes, and desiccated clusters.

Flavescence dorée (FD) is an economically important quarantine disease of grapevine in Europe. It is caused by different strains of phytoplasma belonging to 16SrV-C and 16SrV-D subgroups, with a proposed name of *Candidatus* Phytoplasma vitis (Firrao et al., 2005) and specifically transmitted by the ampelophagous leafhopper *Scaphoideus titanus* Ball (Schvester et al., 1961). Phytoplasma Flavescence dorée is widespread in many European countries: France (Descoins, 1995; Boudon-Padieu, 2003), Italy (Gotta and Morone, 2001; Belli et al., 2000), Spain (Batllle et al., 2000), Switzerland (Stäubli, 2005; Gugerli et al., 2002), Portugal (De Sousa et al., 2003) and Slovenia (Maixner, 2006).

FD can be recognized in the field by the following symptoms which develop mainly in summer (July onwards): leaves turn yellow or red depending on the cultivar, they roll downward, and become brittle; the interveinal areas of leaves may become necrotic. Shoots show incomplete lignification and rows of black pustules develop on the green bark along the diseased branches; they are thin, rubbery and hang pendulously. During winter they blacken and die (Caudweel et al., 1987; Bovey et Martelli, 1992; Boudon-Padieu, 1999; Kuzmanovic et al., 2008; Duduk and Ivanovic, 2006; Martelli and Boudon-Padieu, 2006). Symptoms may also occur in rootstocks (Daire et al., 1993, Borgo et al., 2009).

Phytoplasma can be detected in plants by fluorescence staining (Quaroni et al., 1991), serology (Chen et al., 1993), by polymerase chain reaction (Davise and Lee, 1993; Davise et al., 1993) and using indicator plants, mostly periwinkle (*Catharanthus roseus*) (Prince et al., 1993; Maixner et al., 1994; Tanne and Orenstein, 1997). The data in the literature on the indicator plants are very poor, and mainly relate to the suitability of periwinkle

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Figure 1. Cultivar Plovdina – "green grafting" method.

(*Catharanthus roseus*) for grafting (Tanne and Orenstein, 1997). According to the certification schemes of EPPO standards, recommended indicators are Hybrid Baco 22A and *V. vinifera* cv. Chardonnay and Aramon (OEPP, 2007). However, when using these indicator plants, symptoms like stunting, leaf yellowing and necrosis (white-berried varieties), leaf reddening and necrosis (red–berried varieties) are visible for only 2 to 3 months or more after inoculation (OEPP, 2008).

One of the most sensitive cultivars to GY disease appears to be cv. Plovdina, a traditional local variety (Kuzmanović, 2007; Kuzmanović et al., 2008). This cultivar is in our locality a host only to the FD phytoplasma. As previously reported (Kuzmanović et al., 2006), cv. Plovdina is very sensitive to Flavescence dorée (FD) disease. In this paper we further characterize susceptibility of cv. Plovdina to FD in order to investigate the suitability of this genotype as an indicator plant for early FD detection.

MATERIALS AND METHODS

Grapevine cv. Plovdina showing severe yellow symptoms served as source material for grafting and DNA extraction. Infected plants were selected in a vineyard in Nis region. Healthy plants of the same variety showing no symptoms of GY were collected from a vineyard in Sabac region as control plants.

Green grafting

Both GY diseased and healthy grapevines were top-grafted with scions originated from healthy mother plants of grapevine cv. Plovdina using the "green grafting" method (Figure 1). Graftings were performed in July 2004, 2005 and 2006. Two to five scions were grafted on each of the 10 diseased grapevines in 2004, while two scions were grafted on each of the 40 diseased grapevines in 2005 and 2006. Two scions were grafted on each of the three healthy grapevine plants every year as control plants and were grown under the greenhouse insect-proof conditions.

Scions grafted on diseased and control grapevines were monitored for growth and GY symptoms. The GY symptoms on the test scions of cv. Plovdina were evaluated 20, 30 and 45 days after grafting.

Phytoplasma detection by PCR

Before grafting, infected plants from Nis region (samples N - superscript year of sampling) and asymptomatic plants from Sabac region (samples S - superscript year of sampling) were tested for presence of phytoplasma using PCR. Leaves were collected during September 2004 and 2005 (the year before grafting) and tested by PCR for presence and identification of phytoplasmas (Table 1). After the onset of symptoms after grafting the same plants were re-tested for the presence of phytoplasma (samples GG). Control plants were also tested (samples GG_K, and superscript year of sampling). Total nucleic acids were extracted from 1 g of fresh plant tissue (leaf midribs) following DNA extraction protocol as described by Prince et al. (1993). Plant DNA was diluted in sterile deionized water and 20 ng of DNA was used for PCR assays. Nested PCR using two phytoplasma universal primer pairs, P1/P7 and R16F2n/R2 (1,200 bp) (Lee et al., 1995) was used for phytoplasma detection. Each reaction was performed in a total volume of 50 µl as described by Martini and Murari (1999), with standard procedure (annealing at 50°C) and modification for annealing at 55°C for R16F2n/R2 primers set. Amplification products were subjected to electrophoresis in a 1.2% agarose gels. To identify the type of phytoplasmas, five microliters of the PCR amplification products obtained using the R16F2n/R2 primer set in modified nested PCR with higher annealing temperature were digested with 2 U of *Alu*I and *Tru1*I (Fermentas, Lithuania). The RFLP fragments were visualized by electrophoresis in a 2.2% agarose gel in 0.5 × TBE buffer followed by staining with ethidium bromide and visualization under a UV transilluminator. Direct PCR analysis of FD infected samples with specific chromosomal primers FD9f/r (Daire et al., 1993) was also performed.

PCR detection of grapevine leafroll associated viruses-1, 2 and 3 (GLRaV-1, 2, 3)

One year old braches from infected (samples NN) and grafted plants (samples SS - superscript year of sampling) were tested by PCR test for detection of GLRaV -1, 2 and 3 using methods described by Minafra and Hadidi (1994). Viral RNA was reverse transcribed using random primers and then amplified by PCR with specific primer pairs LQV1-H47/LEVI-C447 for GLRaV-1, LR2U2/LR2L2 for GLRaV-2 and LC1/LC2 for GLRaV-3. GLRaV1, 2 and 3 infected samples of grapevine cv. Prokupac were used as positive control for grapevine leafroll associated viruses 1, 2 and 3 detection and to confirm sensitivity of the applied method.

RESULTS AND DISCUSSION

Green grafting (2004)

Fifteen out of the 32 (46.8%) graftings of healthy Plovdina scions on the 10 diseased plants of the same cv. were

Table 1. List of samples analyzed by PCR phytoplasma detection.

Substrates and scions		Grafts	
Year of sampling	**Sample index**	**Year of sampling**	**Sample index**
2004	N_5^{04} [a]	2005	GG_5^{05} [c]
	N_6^{04}		GG_6^{05}
	N_8^{04}		GG_8^{05}
	N_9^{04}		GG_9^{05}
	N_{12}^{04}		GG_{12}^{05}
	S_1^{04} [b]		GG_K^{05} [d]
	S_2^{04}		
2005	N_{16}^{05}	2006	GG_{16}^{06}
	N_{21}^{05}		GG_{21}^{06}
	N_2^{05}		GG_2^{06}
	N_3^{05}		GG_3^{06}
	S_{10}^{05} [b]		GG_K^{06} [d]
	S_{11}^{05}		

[a-] N -Samples from diseased plants from Nis, [b-] S -samples from helty plants from Sabac; [c-] GG – samples of graft, [d-] GG_K – samples of control graft, [06] superscript denotes a year of sampling.

Table 2. GY symptoms appearance on scions of healthy cv Plovdina grafted on FD infected vines of the same variety and PCR confirmation in 2004, 2005 and 2006.

Plovdina FD infected plant	Grafting taking	Symptoms appearance 20 days after	Positive for FD by PCR
Grafting taking (2004)	15/32	14/15	14
Grafting taking (2005)	34/80	32/34	32
Grafting taking (2006)	41/80	39/41	39

Figure 2. GY symptoms of the Plovdina leaves -20 days after grafting.

successful in 2004, 34(42.5%) in 2005 and 41(51.3%) on 40 plants in 2006 (Table 2). All graftings on control plants performed in greenhouse were successful.

The first GY symptoms consisting of yellowing of the Plovdina leaves have been observed 20 days after grafting (Figure 2). Thirty days after grafting, symptoms typical for FD, consisting of yellowing and reddening followed by downward rolling of the laminate could be observed (Figure 3). Forty-five days after grafting the leaves turned completely red (Figure 4). All the taken top grafted scions showed GY symptoms with the exception of one scion in 2004, three in 2005 and two in year 2006. No symptoms were apparent on leaves developed on scions grafted on control plants in any investigation year.

Phytoplasma detection

Nested PCR using universal primers P1/P7 and R16F2n/R2 (Figure 5) detected the presence of phytoplasmas in 40 grapevine samples of cv Plovdina before grafting in 2005 and 2006. No phytoplasmas were detected in cv. Plovdina from Sabac region.

On the basis of RFLP analysis (Figure 6) the infected samples collected in Nis vineyards showed the presence

Figure 3. GY symptoms of the Plovdina leaves - 30 days after grafting.

Figure 4. GY symptoms of the Plovdina leaves - 40 days after grafting.

of FD phytoplasmas (16SrV group). No phytoplasma presence was detected in Sabac vineyards (Figure 5).

The presence of FD type phytoplasma in the infected samples was confirmed by the 1300 bp amplicon obtained with specific chromosomal primer FD9f/r (Figure 7). Control sample used in all analyses was PI 27, previously confirmed as 16SrV-C on cv. Plovdina (Kuzmanovic, 2007; Kuzmanovic et al., 2008). After the appearance of GY symptoms on scions grafted on FD infected vines we confirmed presence of FD phytoplasma in the scions using the same protocol.

PCR detection of GLRaV-1, 2, 3

GY symptoms observed on scions after grafting due to the infection with FD phytoplasma are very similar to symptoms that can be caused by GLRaV viruses. In order to confirm these symptoms were indeed caused by FD phytoplasma all the investigated plants were also tested for presence of GLRaV. Results of PCR amplification with primers specific for GLRaV have shown that grapevine leafroll associated viruses 1, 2, and 3 were not present in the investigated samples. Figure 8 showed the results of cv. Plovdina grafts (line 3 to 7), tested for GLRaV-3.

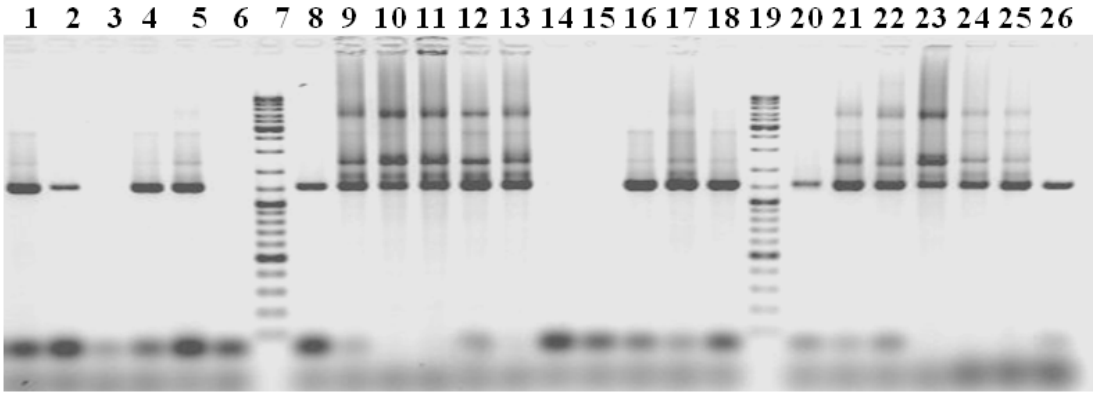

Figure 5. Nested PCR products obtained by two phytoplasma universal primer pairs, P1/P7 and R16F2n/R2 (1200 bp) from infected and control grapevine plants and positive control with 16SrV-C (+K); grapevine cv. Plovdina from Nis (1, 2, 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 20, 21, 22, 23, 24, 25, 26) and Sabac (3, 6, 14, 15) *1*. N_5^{04}; *2*. N_6^{04}; *3*. S_1^{04}; *4*. N_8^{04}; *5*. N_9^{04}; *6*. S_2^{04}; *7*.M; *8*. +K; *9*.GG_5^{05}; *10*. GG_6^{05}; *11*. GG_8^{05}; *12*. GG_9^{05}; *13*. GG_{12}^{05}; *14*. S_{10}^{05}; *15*.S_{11}^{05}; *16*. N_{16}^{05}; *17*. N_{21}^{05}; *18*. +K; *19*. M: GeneRuler DNA Ladder Mix SM0331 Fermentas, Lithuania; *20*. N_2^{05}; *21*. N_3^{05}; *22*. GG_{16}^{06}; *23*. GG_{21}^{06}; *24*. GG_{22}^{06}; *25*. GG_{23}^{06}; *26*. +K.

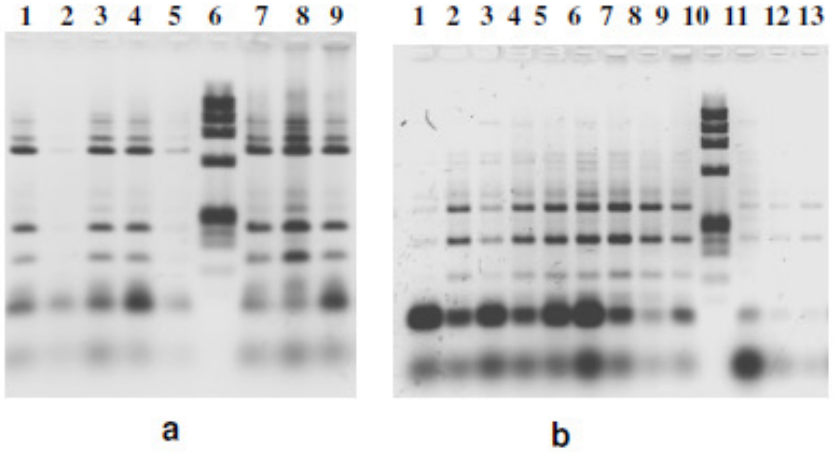

Figure 6. Detection of flavescence dorée (FD) in different samples of grapevine cv. Plovdina using RLFP analysis. Agarose gel electrophoresis of 16SrDNA fragments of 1200bp PCR product obtained with R16F2n/R2: a) *Alu* I patterns: *1*. N_5^{04}; *2*. N_6^{04}; *3*. N_2^{05}; *4*. N_3^{05}; *5*. GG_5^{05}; *6*. M: ϕX174 digested with *BsuR*I, SM0251 Fermentas, Lithuania; *7*. + K; *8*. GG_6^{05}; *9*. GG_{16}^{06}; b) *Tru1*I patterns: *1*. N_5^{04}; *2*. N_6^{04}; *3*. N_2^{05}; *4*. N_3^{05}; *5*. GG_5^{05}; *6*. GG_6^{05}; *7*. GG_8^{05}; *8*. GG_{21}^{06}; *9*. + K; *10*. M ; *11*. GG_2^{06}; *12*. GG_2^{06}; *13*. GG_{16}^{06}.

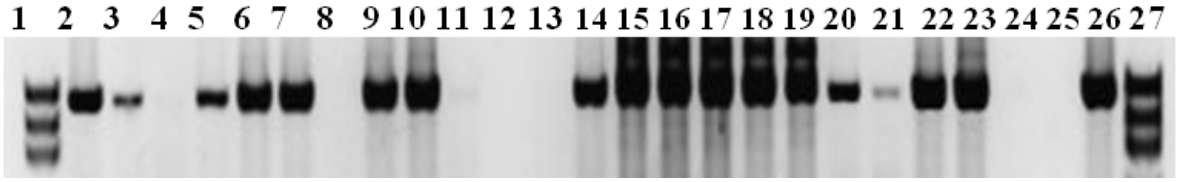

Figure 7. Detection of flavescence dorée (FD) in different samples of grapevine cv. Plovdina using specific chromosomal primer FD9f/r (fragments of 1300bp). *1*.M: GeneRuler DNA Ladder Plus SM0321 Fermentas, Lithuania; *2*. N_5^{04}; *3*. N_3^{05}; *4*. S_1^{04}; *5*. N_6^{04}; *6*. N_2^{05}; *7*. GG_5^{05}; *8*. S_{11}^{05}; *9*. N_8^{04}; *10*. +K; *11*.GG_K^{05}; *12*. GG_K^{05}; *13*. blanck; *14*. GG_6^{05}; *15*. GG_8^{05}; *16*. GG_{21}^{06}; *17*. GG_2^{06}; *18*. GG_2^{06}; *19*. + K; *20*. N_9^{04}; *21*. N_{12}^{04}; *22*. N_{16}^{06}; *23*. N_{21}^{05}; *24*. GG_K^{06}; *25*. GG_K^{06}; *26*. GG_4^{05}; *27*.M.

Figure 8. Detection of GLRaV-3 in different samples using specific primer pairs LC1/LC2 (fragment 550 bp), Positive control-grapevine cv.Prokupac (Pr 10 and Pr 11). 1. Pr 10, 2. Pr 11 3. NN^{05}, 4. NN^{06}, 5. SS^{05}, 6.SS^{06} (line 3-6), 7-M: pBR322/AluI, SM0121 Fermentas, Lithuania.

Several research groups (Constable et al., 2003; Laimer et al., 2009; Osler et al., 2003; Curkovic-Perica et al., 2003) have shown that there is a great variability among grapevine cultivars and rootstocks regarding their susceptibility to GY infection. Our results confirm that grapevine cv. Plovdina is very sensitive to Flavescence dorée disease. Green grafting showed to be a practical and reliable grafting method. The symptoms developed very quickly on grafted scions, three to four weeks after the green grafting, which is significantly faster (by one to two months) compared to indicator plants that are recommended in the EPPO Standards Certifications schemes (EPPO, 2007, 2008). We have confirmed that the observed symptoms were indeed caused by FD phytoplasma and not by GLRaV viruses that could potentially cause similar symptoms. Therefore, we suggest that cv. Plovdina can be used as a suitable indicator plant for FD detection.

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