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# DETECTION OF FLAVESCENCE DOREE PHYTOPLASMA STRAIN C ON DIFFERENT GRAPEVINE CULTIVARS IN SERBIAN VINEYARDS

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## Abstract

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The presence and distribution of grapevine phytoplasma Flavescence dorée (FD) was investigated in the eight important viticultural areas of Serbia from 2005 to 2007. Overall 272 vineyards were investigated in 67 vineyard localities. Results are presented through phytoplasma infection FD - C by locality and grapevine cultivars. The presence of FD stain C was detected in seven of nine investigated areas. Phytoplasma FD was detected in the six grapevine cultivars - Plovdina, Frankovka, Zupski bojadiser, Smederevka, Italian Riesling and Black Burgundy, of eight tested cultivars. The cultivar Plovdina appeared to be extremely sensitive to FD phytoplasma. Phytoplasma presence in grapevine is checked by PCR and RFLP. The RFLP patterns obtained from the Serbian grapevine samples confirmed that all detected FD strains bellong to 16SrV-C subgroupe of phytoplasma.

*Key words*: grapevine, Flavescence dorée phytoplasma, FD - C strain

## Introduction

Grapevine yellows (GY) is a complex of diseases of grapevine associated with phytoplasmas. Flavescence dorée (FD) and Bois noir (BN) of grapevine (*Vitis vinifera* L.) are the most important yellows in Europe. The two diseases display similar symptoms, but they considerably differ in their etiology and epidemiology. Nevertheless these disordes are caused by different phytoplasmas distinguished on the basis of molecular evidence (Boudon-Padieu, 2003, 2005). FD phytoplasma belong to the phylogenetic subgroups 16SrV-C and 16SrV-D of the phytoplasma taxonomy, but have not yet been officially established as a *Candidatus* Phytoplasma taxon. They are transmitted in nature by the leafhopper *Scaphoideus titanus* Ball. FD is the most economically important disease of grapevine in the principal wine-production areas in Europe (Boudon-Padieu, 2003). For these reasons FD is recognized by the European and Mediterranean Plant Protection Organization (EPPO) as

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a quarantine pest. FD is still a very dangerous disease, contributing to the significant reduction in yield across the European vineyards, over the last 20 years.

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 lignifications 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 and Martelli, 1992; Boudon-Padieu, 1999; Kuzmanovic et al., 2005; Duduk and Ivanovic, 2006; Martelli et Boudon-Padieu, 2006). It may occur in rootstocks too (Daire et al., 1993; Borgo et al., 2009). 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 (Battle, 2000), Switzerland (Stäubli, 2005; Gugerli, 2005), Portugal (De Sousa et al., 2003). Flavescence dorée strain C is present in France and Italy, while the FD strain D is present in France, Italy, Spain, Portugal and Switzerland.

Three phytoplasms are identified on the grapevine in Serbia up to now: »*Candidatus* Phytoplasma vitis« (causing – Flavescence dorée - FD), »*Candidatus* Phytoplasma solani« (causing blackening of the wood – Bois noir - BN) i »*Candidatus* Phytoplasma prunorum« (causing European Stone Fruit Yellows - ESFY) (Duduk et al., 2004; Kuzmanovic et al., 2004; Josic et al., 2005; 2006a; 2006b). These phytoplasmas are registered in almost all vineyards in Serbia and on all investigated cultivars (Jevremovic and Paunovic, 2005; Duduk et al., 2006; Kuzmanovic, 2007; Kuzmanovic et al., 2006; 2008).

More recently published the results of the presence of FD in Italy, in only three locality of Serbia and in four areas of France (Bertaccini et al., 2009) as well as throughout France (Salar et al., 2009).

In this paper we studed the molecular typing of FD strains in 272 affected vineyards in 67 most important vineyard locality of Serbia.

## Materials and Methods

***Presence and distribution of GY in vineyards of Serbia***: **Sixty-seven vineyards** located in six different viticultural regions of Serbia (Figure1) – Zapadnomoravski (1), Nisavsko-juznomoravski (2), Juznobanatski (3), Timocki (4), Sumadijsko-velikomoravski (5) i Sremski (6) were inspected visually for three years (2005-2007) in order to register the symptoms of GY presence. The sur-

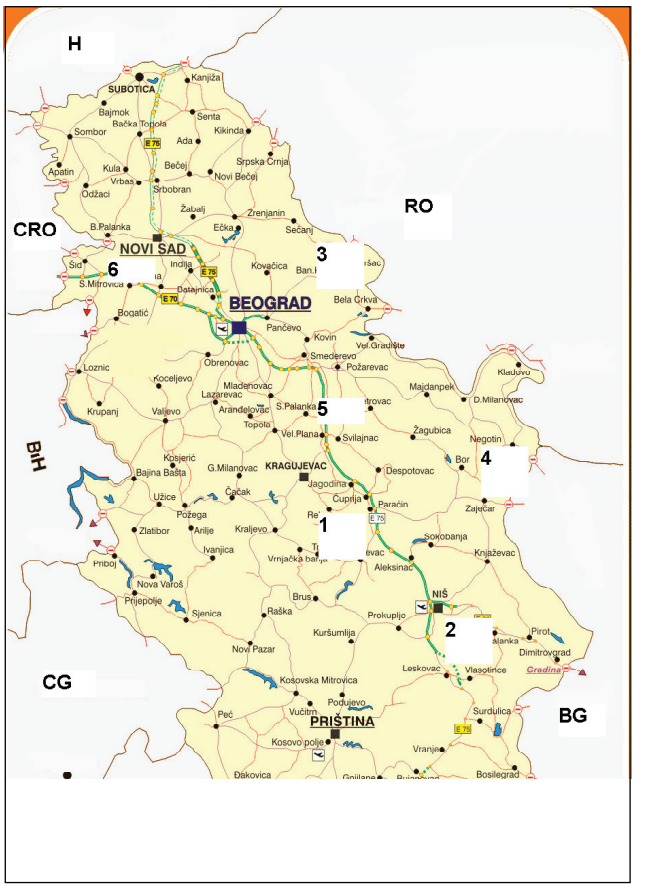


**Fig. 1. Viticultural regions in Serbia: 1 – Zapadnomoravski; 2 - Nisavsko-juznomoravski; 3 – Juznobanatski; 4 – Timocki; 5 - Sumadijsko-velikomoravski; 6 - Sremski**

veys were carried out three times a year in July, August and September. Typical simptoms od GY are discoloration and necrosis of leaves, downward curling of leaves, shrinking of berries, incomplete lignification of canes and stunted growth and necrosis of the shoots.

***Sources of infected plant material for molecular analyses***: A total of 272 samples of grapevines showing the symptoms described, were collected in September 2005, 2006 and 2007 from 67 vineyard locality. For molecular investigations grapevine shoots and leaves were collected from eight grapevine cultivars: Italian Riesling, Black Burgundy, Plovdina, Smederevka, Zupski bojadiser, Frankovka, Rhine Riesling and Chardonnay.

***DNA extraction and PCR / RFLP analyses for phytoplasma identification and characterisation*:** Phytoplasma presence in symptomatic plants was tested with the PCR molecular technique. Total nucleic acid was extracted using leaf midribs or other tissues from grapevine according Prince et al. (1993). Nearly full-length 16S rDNA sequences were prepared by using a nested PCR with universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by the universal primer pair R16F2n/R16R2 (Lee et al., 2003). Amplification product of R16F2n/R16R2 primers (1.2 kb) were digested with restriction enzymes *Mse*I and *Alu*I. Partial 16S rDNA (about 1.8 kb) amplified using primer pair P1/P7 was subjected to restriction with *Taq*I according to the manufacturers' instructions (Fermentas, Vilnius, Lithuania). Amplified products were electrophoresed through a 1.5% agarose gels and the digested products through a 2.5% agarose or 5% polyacrylamide gels. After ethidium bromide staining, obtained products were visualised on UV transilluminator and photographed. Fragment size markers used: ϕX174 digest with *HaeIII* (Fermentas, Vilnius, Lithuania); 1Kb DNA Ladder (Amersham Biosciences); 1Kb (Gibco BRL); Gene Ruler DNA (Ladder Mix, Fermentas, Vilnius, Lithuania).

As the phytoplasma references the following strains were used: EY1, belonging to the Elm yellows (16SrV-A), PL27, belonging to the FD-C (16SrV-C), and P-TV, belonging to the Stolbur (16SrXII-A) group, provided by M.Martini, Italy.

## Results and Discussion

***Presence and distribution of grapevine yellows on different grapevine cultivars in grape-growing regions of Serbia.*** During field surveys, typical symptoms of GY were observed on the whole plant or on a few shoots. The data obtained from field surveys are summarized in Table 1.

The presence of FD strain C was detected in seven of nine investigated areas – Zupsko, Trstenicko, Matejevacko, Sicevacko, Rajacko, Oreovacko and Fruskogorsko, in 51 of 67 investigated localities, and in 231of 272 investigated vineyards. The incidence (%) of FD-C symptomatic plants was the higest in Zupsko area (6-90%), then in Sicevacko (2-90%), Trstenicko (5-50%), Fruskogorsko (6-18%) and finally Rajacko (<5) and Oreovacko (3%). The presence of the phytoplasma FD was not confirmed in the Vrsacko area.

The analysis of the rate of infections demonstrates that six out of eight vinegrape cultivars were infected. The cultivars Rhine Riesling and Chardonnay were not infected. The most sensitive clutivar was Plovdina, which was infected in 90% of the 50 localities investigated, followed by Frankovka (which was infected in 35% cases in 4 localities); Zupski bojadiser (30% in one locality); Smederevka (27% in four localities); Black Burgundy (20% in two localities) and Italian Riesling (9% in one locality).

The results on the presence of the phytoplasma in six regions and 67 vineyards locality in Serbia, between 2005-7, confirm that the phytoplasma FD is identified in all investigated vineyards except Vrsacko. Duduk et al. (2004) and Duduk et al. (2006) proved the the phytoplasma were present in 14 vineyards, and Krnjajic et al. (2007) confirmed

**Table 1**
**Incidence (%) of phytoplasma disease on the grapevine cultivars in some of the most important viticultural regions of Serbia in the period 2005-7**

| Region | Vineyard area | Cultivar | No of localities | No of vineyards | Incidence (%) 2005-2007 | Type of phytoplasma |
|---|---|---|---|---|---|---|
| **1** | Zupsko | Italian Riesling | 1 | 1 | 9 | FD-C |
| | | Black Burgundy | 2 | 3 | 10-20 | FD-C |
| | | Plovdina | 8 | 58 | 12-90 | FD-C |
| | | Smederevka | 4 | 18 | 6-27 | FD-C |
| | | Z. bojadiser | 1 | 1 | 30 | FD-C |
| | | Frankovka | 2 | 6 | 6-29 | FD-C,BN |
| | | Rhine Riesling | 2 | 4 | 11-20 | BN |
| | | Chardone | 1 | 3 | <5 | BN |
| | Trstenicko | Plovdina | 4 | 21 | 5->50 | FD-C |
| **2** | Matejevacko | Plovdina | 4 | 28 | <5 | FD-C |
| | | Chardone | 1 | 1 | 20 | BN |
| | Sicevacko | Plovdina | 6 | 42 | 2-90 | FD-C |
| | | Frankovka | 4 | 14 | 6-35 | FD-C |
| **3** | Vrsacko | Chardone | 7 | 11 | 6-30 | BN |
| | | Frankovka | 3 | 4 | 6-34 | BN |
| **4** | Rajacko | Plovdina | 5 | 31 | <5 | FD-C |
| | | Chardone | 3 | 8 | 6-67 | BN |
| **5** | Oreovacko | Plovdina | 2 | 4 | 3 | FD-C |
| | Levacko | Chardone | 1 | 4 | 11-63 | BN |
| **6** | Fruskogorsko | Plovdina | 2 | 4 | 6-18 | FD-C |
| | | Chardone | 4 | 6 | <5-43 | BN |

the presence of the vectors of the FD *S. titanus* in all vineyards of Serbia, even those where the presence of FD has not been confirmed, such as Vrsacko (3). This indicates the likelyhood that the phytoplasma are present in the regions where the vectors are found. Salar et al. (2009) strongly suggests that FD strain D has been extensively diffused in France by human trading of infected plant material.

As it has already been noted (Kuzmanovic et al., 2003; 2005**)** our investigations confirm that the most susceptible cultivar is Plovdina, followed by Frankovka. Medium sensitivity has been found in Zupski bojadiser, Smederevka, Black Burgundy and Italian Riesling; and the most resistant cultivar were Chardonnny and Rhiene Riesling. That two cultivars in our condition are hight sensitive to the Phytoplasma Bois Noir, but to resistant to the FD. Similar results are cited by Boudon-Padieu (2005), Martelli et Boudon-Padieu (2006) who quote Chardonnay and Black Burgnudy as the two cultivars most susceptible to the yellowing disease en general. In our regions cultivars Chardonnay and Rhiene Riesling are very sensitive to the yellowing causing by phytoplasma BN, but they are not the host plants for the phytoplasma FD.

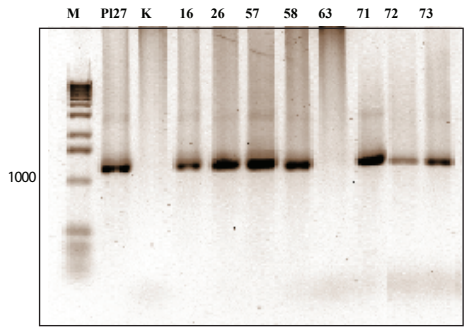


**Fig. 2. Electrophoresis on 125% agarose gel of nested-PCR products amplified by P1/P7 primers, according R16F2n/R16R2 primers of grapevine samples from different vineyards in Serbia (table 1) and the reference strain of FD-C: Pl27. K negative control; M-Marker, 1kb DNA Ladder (Amersham Biosciences)**

about 1800bp and 1200bp, respectively. Amplicon of 1200bp were detected in all symptomatic investigated samples and in referent strains (Figure 2). RFLP analysis of R16F2n/R16R2 amplification products digested with restriction enzymes *Mse*I and *Alu*I showed two types of phytoplasmas (Figure 3) detected as FD and BN.

P1/P7 amplicon of infected samples detected as FD phytoplasma were digested with *TaqI,* to distinguish FD-C and FD-D strain of phytoplasma (Figure 4). Comparing it with restriction patterns of P1/P7 amplicons, obtained with *Taq*I restriction enzyme on FD-C and FD-D reference strains, we confirmed that all detected FD strains bellonging to 16SrV-C subgroupe.

Molecular identification and geographic distribution of Flavescence dorée insuficient number of samples originated from three Serbian area during 2006-2008, was investigated by Bertaccini et al. (2009). They also determined only FD-C

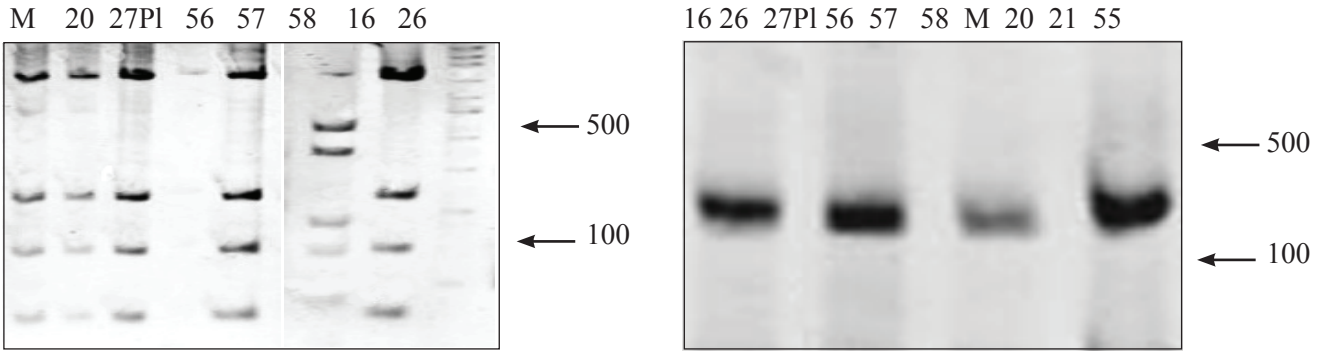


**Fig. 3. RFLP analysis of nested-PCR products amplified by P1/P7 following by R16F2n/R16R2 of grapevine samples from different vineyards in Serbia, (table 1). PCR products digested with *Alu*I (a) and *MseI* (b) and separated by electrophoresis through 5% polyacrylamid gel. Pl 27– Plovdina, positive control (FD-C). M - GeneRuler DNA Ladder Mix, Fermentas, Lithuania. Isolates 20, 21 and 55 represent Stolbur phytoplasma presence**

***Phytoplasma identification and characterisation***

Detection of phytoplasma in symptomatic grapevine plants were performed by nested PCR assay. First step PCR were caried out using P1/P7 primers par and second using the R16F2n/R16R2 primers. Thes primers gave the amplicons sizes of

subgroup of phytoplasma in FD infected Serbian grapevine. Analysis of rp and secY genes of FD infected samples of grapevine in Serbia previously investigated by Kuzmanovic et al. (2008) showed only FD-C phytoplasma strain, confirming results of RFLP analysis.

This paper presents the results of the molecular

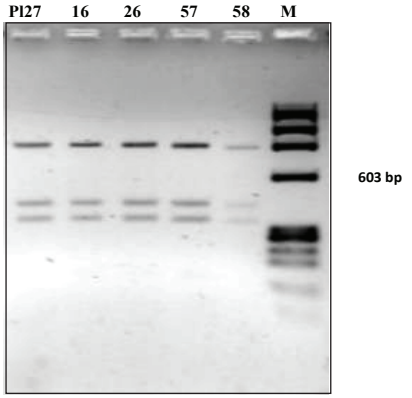


**Fig. 4. *Taq*I RFLP analysis of P1/P7 amplicon of FD detected grapevine samples from Serbia (table 1) separated on 2.5% agarose gel. M- ϕX174 digest with** Hae**III (Fermentas, Vilnius, Lithuania); Pl 27– Plovdina, positive control**

identification tests undertaken on a large sample of grapevines with the symptomes of phytoplasma infection. This is therefore the most thorough analysis of the distribution of th phytoplasma FD in the vineyards and cultivars in Serbia.

We suggest that future investigations need to focus on the susceptibility or the resistance of different cultivars to the known phytoplasmas. It would be also worth extending the number of samples in order to confirm the finding that the cultivar Plovdina is infected only with the phytoplasma FD, while Chardonnay and Rhine Riesling only with the phytoplasma BN.

## Conclusion

The analysis of the spread of the phytoplasma in Serbia in the period between 2005-7 indicates that the the incidence of infection is the highest in the Zapadnomoravski region (1). Phytoplasma strain FD is confirmed in all investigated vineyards, except Vrsacko (3). FD – C was the only strain found. Phytoplasma FD was detected in the six grapevine cultivars - Plovdina, Frankovka, Zupski bojadiser, Smederevka, Italian Riesling and Black Burgundy, of eight tested cultivars. Plovdina cultivar was found to be the most susceptible cultivar. The cultivars Chardonnay and Rhine Riesling in our condition don't contain the phytoplasma FD.

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