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Characterisation of bois noir phytoplasmas from Bosnia and Herzegovina

The Short Term Scientific Mission (STSM) was conducted in DISTA University of Bologna, Faculty of Agriculture, Phytoplasmology laboratory under the supervision of Prof. Dr Assunta BERTACCINI from 16/03/2010 to 26/03/2010.

Background

The presence of grapevine yellows phytoplasmas in Bosnia and Herzegovina has been described since 2005. Since then, the presence of 'bois noir' (BN) phytoplasma has been detected in the main grapevine growing areas (Mostar, Trebinje, Gradiska). Results from laboratory testing indicate the presence of this phytoplasma in B&H vineyards with commercial production of vine in cultivars such Žilavka, Blatina, Vranac, Smederevka. Moreover, the Flavescence dorée phytoplasma vector, *Scaphoideus titanus* Ball has been identified in vineyards of the Trebinje region. Molecular identification of 'bois noir' phytoplasmas is performed by specific PCR on the 16S rRNA gene of grapevine samples.

Purpose of visit

The main objective of this work was to verify the presence of genetic variability in selected strains of 'bois noir' phytoplasmas detected in heavily BN-infected vineyards in the Srpska region of Bosnia and Herzegovina. Polymorphisms were studied in the 16S rRNAI, *Tuf* and ribosomal protein genes of BN – infected samples.

Description of the work

DNAs and PCR products were used as templates to study BN phytoplasma polymorphisms in B&H. DNAs were extracted from the leaf midribs of grapevine samples using the DNeasy Plant Mini kit protocol with slight modifications. Samples were collected during monitoring of the presence of grapevine yellows phytoplasmas in 2008 and were collected in several location of the Srpska grapevine growing region. Thirty-five DNA samples of grapevines that were shown to be positive by PCR testing of the 16S rRNA gene were selected for the study. In addition, PCR products of the same DNA samples amplified with P1/P7 and R16F2n/R16R2 primers were also included.

Table 1: Grapevine varieties and locations of the positive BN phytoplasma samples employed

Number	Grapevine variety	Location
1.	Frankovka	Galicic/Popovo polje
2.	Smederevka	Galicic/Popovo polje
3.	Smederevka	Galicic/Popovo polje
4.	Smederevka	Galicic/Popovo polje
5.	Vranac	Galicic/Popovo polje
6.	Vranac	Galicic/Popovo polje
7.	Vranac	Galicic/Popovo polje
8.	Cardinal	Popovo polje
9.	unknown	Popovo polje
10.	unknow red variety	Popovo polje
11.	Afus ali	Popovo polje
12.	Afus ali	Popovo polje
13.	Afus ali	Popovo polje
14.	Afus ali	Popovo polje
15.	Vranac	Popovo polje
16.	Vranac	Popovo polje
17.	Vranac	Trebinje
18.	Vranac	Petrovo polje
19.	Vranac	Petrovo polje
20.	Vranac	Petrovo polje
21.	Vranac	Petrovo polje
22.	Vranac	Mokro polje
23.	Vranac	Mokro polje
24.	Vranac	Mokro polje
25.	Vranac	Mokro polje
26.	Vranac	Mokro polje
27.	Vranac	Mokro polje
28.	Vranac	Mokro polje
29.	Vranac	Mokro polje
30.	Vranac	Mokro polje
31.	Vranac	Mokro polje
32.	Vranac	Mokro polje
33.	Smederevka	Mokro polje
34.	Frankovka	Gradiska
35.	Frankovka	Gradiska

P1/P7 and R16F2n/R16R2 PCR products of positive BN samples employed in the study were previously amplified in the plant pathology laboratory at the Faculty of Agriculture, University of Banjaluka. Variability among BN phytoplasmas was checked by RFLP analyses. Some of the P1/P7 PCR amplicons (visible on agarose gel) were restricted with *Mbo*II RE. The variability of R16F2n/R16R2 PCR products (from nested PCR) was studied using *Rsa*I, *Mbo*II and *Hpy*188I RE enzymes.

All PCR reactions were performed in a total mixture of 25 µL (24 µL PCR mixture and 1 µL of DNA samples). PCR results were analyzed on 1% agarose gels and visualized by staining with ethidium bromide under UV transillumination. RFLP analyses were conducted according to the vendors' instructions and products were analyzed on 5 % polyacrylamide gels and visualized by staining with ethidium bromide under UV transillumination

DNA of phytoplasma samples were also subjected to molecular characterization using *Tuf* gene primers: Tuf1f/r in direct PCR, and primers TufAYf/r, and TufINT1f/TufINT4r in nested PCR reactions. RFLP analyses of all obtained amplicons were performed with *Hpa*II restriction enzyme (RE).

The same DNA samples were also submitted to the nested PCR analyses on ribosomal protein genes using rp/STOL primers, after which positive samples were submitted to the RFLP analyses with *Tai*I and *Ssp*I restriction enzymes.

Finally all DNA samples were amplified again using direct PCR with universal phytoplasma primers P1/P7 in a direct PCR reaction. Amplicons produced were diluted (1:30) in sterile water and R16F2n/R16R2 primers were used for nested PCR.

Table 2: Primer pairs and RE enzymes employed for analyses of DNA samples

Type of samples	Amplified phytoplasma genes	Restriction endonuclease enzymes
DNA	*Tuf* *rp* 16S rRNA	*Hpa* II *Tai, Ssp*I See table 3

Table 3: RE enzymes used for RFLP analyses of 16S rRNA and 16S rRNA plus spacer region PCR amplicons

Type of samples	Restriction endonuclease enzymes
P1/P7 PCR products (from direct PCR)	*Mbo*II
R16F2n/R16R2 PCR products (from nested PCR)	*Rsa*I, *Mbo*II, *Hpy*188I

Restriction profiles of the *Tuf* amplicons restricted with *Hpa*II show the presence of the Tuf type-I of the BN phytoplasmas. On the other hand some grapevine samples restricted with other enzymes showed different restriction profiles that were compared with some of those recently published. The samples showing 16Sr or 16Sr/spacer region polymorphism will be further studied by sequence analyses.

These DNA and PCR product samples will be maintained at DISTA, University of Bologna for further studies on BN phytoplasma diversity under framework of WG1 COST FA0807 action. Further analyses based on the sequences analyses of B&H and of samples form other countries will give clearer insights into the BN phytoplasma diversity, polymorphisms and they role in the disease epidemiology.