Infection of *'Candidatus* Phytoplasma Vitis' in a Hungarian Vineyard East of the Danube

'Candidatus Phytoplasma vitis' fertőzése egy Dunától keletre fekvő hazai szőlőültetvényben

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Abstract: In Hungary, two phytoplasma species are known to infect grapevines, the *'Candidatus* Phytoplasma vitis' and the *'Ca*. Phytoplasma solani'. The *'Ca.* Phytoplasma vitis' is one of Europe's economically most important grapevine pathogens, as its infection can cause significant yield losses and even the death of infected vines. The *'Ca*. Phytoplasma vitis' was first described in Hungary in 2013, and since then, it has been reported from grapevines and traveller's joys in several transdanubian counties, while east of the Danube, it has only been identified in a few cases in grapevines. In the summer of 2023, leaf yellows and rapid death of vine stocks were observed in Csongrád. After total nucleic acid extraction, nested PCR tests were carried out using universal phytoplasma primers (P1/P7 and fU5/rU3). All tested samples were positive for phytoplasma infection. Based on the results of the *in silico* RFLP analysis using *Tru*9I restriction endonuclease, the infection of '*Ca*. Phytoplasma vitis' were determined, which pathogen has not yet been reported from grapevines in Csongrád-Csanád County.

Keywords*: Vitis; grapevine; phytoplasma; PCR; Flavescence dorée*

Összefoglalás: Hazánkban szőlőn mind ez ideig két fitoplazma faj, a *'Candidatus* Phytoplasma vitis' és a *'Ca*. Phytoplasma solani' jelenlétét azonosították. A *'Ca*. Phytoplasma vitis' egyike a szőlő gazdaságilag legjelentősebb kórokozóinak. Fertőzése következtében a termés mennyisége és minősége jelentősen csökken, a fertőzött tőkék pedig akár néhány éven belül ki is pusztulhatnak. A *'Ca*. Phytoplasma vitis' hazai megjelenéséről elsőként 2013-ban számoltak be Zala vármegyéből, azóta a Dunántúl számos borvidékéről jelezték előfordulását szőlőről és erdei iszalagról, a Dunától keletre azonban szőlőről csupán néhány esetben azonosították. 2023 nyarán, egy csongrádi szőlőültetvényben, a tőkék levélzetének sárgulására és gyors tőkepusztulásra lettek figyelmesek. Össznukleinsav-kivonást követően, univerzális fitoplazma primerpárokat (P1/P7 and fU5/rU3) felhasználva, nested-PCR technikával teszteltük a minták fitoplazma-fertőzöttségét. Minden vizsgált minta fitoplazma fertőzöttnek bizonyult. A *Tru*9I restrikciós endonukleázzal végzett *in silico* RFLP vizsgálat eredményeképpen kapott hasítási mintázat alapján, a *'Ca*. Phytoplasma vitis' fajt azonosítottuk, mely kórokozó előfordulását Csongrád-Csanád vármegyéből, szőlőről eddig még nem jelezték.

Kulcsszavak*: Vitis; szőlő; fitoplazma; PCR; Flavescence dorée*

1. Introduction

Several phytoplasma species worldwide may cause the grapevine yellows (GY) disease. In Hungary, two phytoplasma species have been identified, causing leaf yellows on grapevine. The *'Ca.* Phytoplasma solani' (syn. *Stolbur phytoplasma*), the pathogen of Bois noir (BN) and the *'Ca.* Phytoplasma vitis' (syn. *Grapevine flavescence dorée phytoplasma*), causing Flavescens dorée (FD). The *'Ca*. Phytoplasma vitis', besides the bacterium *Xylella fastidiosa*, is currently considered the most dangerous grapevine pathogen as it can cause significant yield losses (up to 50%) and rapid death of grapevines (Benedek, 2014). *'Ca*. Phytoplasma vitis' is a quarantine pest in Europe. Its vector, the American grapevine leafhopper (*Scaphoideus titanus* Ball) - a pest that only feeds on grapevine species - can spread the pathogen rapidly (Mori et al., 2002).

The *'Ca*. Phytoplasma vitis' was first identified in Hungary in 2013, in Zala County, near the Slovenian border (Dancsházy and Szőnyegi, 2014; Kriston et al., 2013), and since then, it has been detected in many Transdanubian counties and wine districts, both in grapevines and traveller's joys (*Clematis vitalba*). In Hungary, east of the Danube, it was first reported in 2019 from a vineyard in the Hajós-Baja wine district. Since 2019, it has been spreading in vineyards in the east of the Danube as well (Kölber and Lázár, 2023), while previously, the pathogen was only identified from traveller's joys in this part of the country (Dancsházy, 2019; Tóth és Pableczki, 2015). According to Kölber and Lázár (2023), the occurrence of the pathogen in grapevines from the Szekszárd, Villány, Balatonfüred-Csopak, Nagy-Somló, Pannonhalma, Mátra, Eger, Tokaj and Csongrád wine districts is still not reported.

Phytoplasmas are devastating plant pathogens. Protection of our plants against them is complicated and is based on preventing infection. The most essential is producing and using healthy, phytoplasma-free propagating material. Other solutions are controlling the vectors that spread the pathogen and the destruction of infected vines during cultivation. Removing potential host plants in neglected vineyards is also crucial (Benedek, 2014; Ember et al., 2012).

The primary vector of the pathogen, the American grape leafhopper (*Scaphoideus titanus*), was first detected in Hungary in 2006 (Dér et al., 2007), and currently, it is widespread throughout the country (Orosz and Zsolnai, 2010; Szalárdi et al., 2014). The leafhopper feeds exclusively on grapes, primarily on cultivated species (*Vitis vinifera*) in Europe (Mori et al., 2002).

Different symptoms can appear on grapevines after the infection of *'Ca*. Phytoplasma vitis'. The leaves roll downward, forming a triangular shape. Its tissue becomes hard and brittle, and the colour turns yellow or red depending on the variety. The shoots become thin and rubbery. The lignification of the shoots is incomplete. During winter, they blacken and die. The fruit set is reduced. The berries become shrivelled and turn brown, and their sugar content decreases while the acidity increases compared to healthy grapes (OEPP/EPPO, 2007). The symptoms listed above are almost identical in the case of the infection of both phytoplasma species causing grapevine yellows in Hungary. Thus, visual identification is impossible. Molecular diagnostic methods are the only reliable way to determine the infecting phytoplasma species (Dancsházy and Kriston, 2012).

In July 2023, in a small family vineyard in Csongrád, leaf yellowing and rapid death of many vine stockes were noticed. These symptoms could be caused by phytoplasma infection. Therefore, the testing of the infection of grapevine samples was aimed at using the PCR technique.

2. Materials and Methods

In the summer of 2023, leafy grapevine shoots of vines of the 'Chardonnay' variety showing symptoms collected in a 4-year-old plantation in Csongrád (Csongrád wine district, Csongrád-Csanád County) were sent to the laboratory of the Plant Pathology Department of the Hungarian University of Agriculture and Life Sciences. Two-two approximately 20 cm long symptoms showing leafy shoots were collected from four randomly selected vines. The collected plant parts were stored at -70 ºC until use.

Total nucleic acid extraction was performed using a simplified CTAB method (Xu et al., 2004). 0.5 g of plant tissue was used per sample for total nucleic acid extraction. Extracts from the mixture of central veins and petioles and extracts from the phloem tissue of the shoots were prepared (Table 1). The nucleic acid extracts were visualised on a 1% TBE agarose gel containing a fluorescent dye and stored at -70 °C until further use.

Table 1. Grapevine samples

Vines	Collected plant parts	Plant parts used for total nucleic extraction	Samples
Sz1	leafy grapevine shoot	mixture of main vein + petiole	H-FD1
		phloem tissue	H-FD5
Sz2	leafy grapevine shoot	mixture of main vein + petiole	H-FD2
		phloem tissue	H-FD6
Sz3	leafy grapevine shoot	mixture of main vein + petiole	H-FD3
		phloem tissue	H-FD7
Sz4	leafy grapevine shoot	mixture of main vein + petiole	H-FD4

Nested PCR was carried out for the molecular detection. Universal phytoplasma primer pairs, P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and fU5/rU3 (Lorenz et al., 1995) were used in the first and second PCR rounds for testing phytoplasma infection of all seven grapevine samples. The P1/P7 primer pair amplifies an approx. 1800 bp long DNA fragment that encompasses the 16S rRNA–IGS–5' 23S rRNA region, the fU5/rU3 primers amplify an approx. 880 bp long PCR product. Sterile distilled water was used as a negative control for all PCRs. The following parameters were set for the first PCR round: 94 °C for 5 min, for 35 cycles 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min, and finally 72 °C for 10 min. The following parameters were set for the second PCR round: 94 °C for 2 min, for 35 cycles 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. The PCR amplicons were visualised on 1% TBE agarose gel staining with fluorescent dye.

The PCR fragments were isolated from the 1% agarose gel with the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions, and two nucleotide sequences were determined (Biomi Kft., Gödöllő). The obtained sequences were aligned and joined using the CLC Sequence Viewer 8.0 program package and compared to the sequences available in the NCBI database using the BLAST program. An *in silico* RFLP analysis was also performed, mapping the cleavage sites of the *Tru*9I restriction endonuclease.

3. Results and Discussion

15-20% of the previously homogeneous, healthy-looking vines finished growing in the second half of the spring of 2023, and rapid grapevine deaths occurred in the vineyard in Csongrád. The leaves on the vines rolled downward and became light in colour (Figure 1). The set bunches dried up. Vines that showed the leaf rolling and yellowing symptoms but did not die developed stunted shoots that broke easily.

Figure 1. *Vines of 'Chardonnay' variety showing symptoms of grapevine yellows in Csongrád. (Photo: Merkely)*

Nested-PCR gave positive results for all seven tested samples prepared from the leafy shoots originating from four vines showing yellowing symptoms. In the cases of all samples, the approx. 880 bp long DNA fragments were amplified. Negative results were obtained in the control water samples (Figure 2).

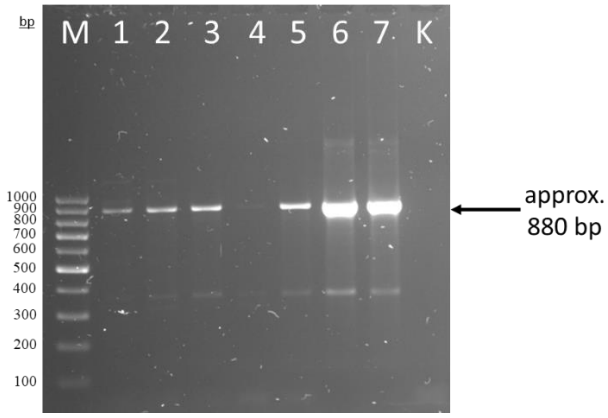

Figure 2. *Nested-PCR analysis of grapevine samples*

Legend: M: DNA marker (ThermoFischer Scientific GeneRuler 100 bp DNA Ladder), K: Negative control, 1: H-FD1, 2: H-FD2, 3: H-FD3, 4: H-FD4, 5: H-FD5, 6: H-FD6, 7: H-FD7 PCR products amplified with primers fU5 and rU3. The approx. 880 bp phytoplasma-specific PCR-fragment (arrow) shows the phytoplasma infection of the sample.

Sequence comparisons were made after determining the nucleotide sequences of two PCR products. When comparing the obtained nucleotide sequences (842 nt) with the nucleotide sequences of the corresponding region of isolates available in the international database, 100% similarity values were obtained with isolates belonging to different phytoplasma species thus both isolates (H-FD1, H-FD6) could be identified as a phytoplasma. H-FD1 and H-FD6 isolates

showed complete identity (100%) with phytoplasma sequences from the database and with each other at the nucleic acid level.

Phytoplasma species can be differentiated by restriction analysis of the PCR-amplified part of the 16S rDNA region. Based on the restriction pattern of this region, phytoplasmas are classified into different groups (Lee et al., 1993, 2000; Schneider et al., 1993; Seemüller et al., 1994), out of which *'Ca*. Phytoplasma vitis' belongs to the 16SrV (Elm yellows, EY; OEPP/EPPO, 2007), while *'Ca*. Phytoplasma solani' to the 16SrXII group (Stolbur, STOL; Quaglino, 2013). RFLP analysis of the PCR product amplified by the fU5/rU3 primer pair results in different cleavage patterns using *Tru*9I restriction endonuclease in the case of phytoplasma species belonging to 16SrV or 16SrXII groups (OEPP/EPPO, 2007). The cleavage sites of *Tru*9I were mapped on the nucleotide sequences of H-FD1 and H-FD6 isolates to determine either *'Ca*. Phytoplasma vitis' or *'Ca*. Phytoplasma solani' caused the leaf yellowing on grapevines in Csongrád. The restriction patterns obtained by the *in silico* RFLP tests were the same as the cleavage pattern typical to phytoplasma species belonging to the 16SrV group according to the data of the diagnostic protocol for the detection of '*Ca.* Phytoplasma vitis' published by the EPPO (OEPP/EPPO, 2007). Based on this result, it was concluded that the samples were infected with *'Ca*. Phytoplasma vitis' belonging to the 16SrV group. To our knowledge, this is the first report of the infection of *'Ca*. Phytoplasma vitis' on grapevines from Csongrád-Csanád County, Hungary.

The pathogen could have entered the vineyard in Csongrád, either with the propagating material used to establish the plantation or via its leafhopper vectors. It spreads over long distances, mainly with infected propagating material, but its vectors, especially the American grape leafhopper, also play a significant role at the local level. Although the size of the population of leafhopper species capable of transmitting the pathogen may change from year to year in a given area (Kutas, 2022), their presence and, thus, the possibility of the infection can not be excluded even though the most effective plant protection treatments, due to individuals flying in from far and potentially carrying the pathogen.

Unfortunately, the infected propagating material is not always recognisable. It is often symptomless, so that the pathogen can be spread unintentionally. Therefore, grapevine propagating material from infected countries must be imported only after careful phytosanitary control, and only certified, pathogen-free grafted plants are purchased from domestic nurseries.

Despite strict phytosanitary regulations, limiting the pathogen's spread is challenging. The Danube, previously referred to as a natural defence line, could not stop the pathogen's spread to wine districts east of the Danube. Viticulturists and home garden grapevine growers are responsible for overcoming the transmission and infection of *'Ca*. Phytoplasma vitis'.

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