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Investigation of the presence of two newly described grapevine viruses in grapevine stock collection at Pécs.

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I. INTRODUCTION

Grapevine (*Vitis vinifera L.*) is a valuable agricultural commodity that have an economical and cultural importance in Hungary where it is cultivated in various regions. It is a perennial crop which propagate vegetatively, making it more susceptible to pathogens. It is estimated that about 60 viruses are known to infect grapevine (Martelli, 2009), reducing both yield and quality of the fruit (Martelli and BoudonPadieu, 2006). It is therefore an essential investment to study the viruses infecting grapevine, by investigating the sanitary status of the vineyards and checking the presence of new viruses such as the ones found in the presented research.

Grapevine Syrah Virus-1 (GSyV-1) and Grapevine Pinot Gris Virus (GPGV) are newly identified viruses in Hungary etiology of which remains unclear. They were detected in different vit icultural regions infecting several non-related rootstock varieties (Czotter et al, 2015), however, their late detection does not indicate that they are new emerging viruses, but in fact they might have been always present but were not detected yet. There detection was carried out by using RT-PCR as a molecular test with virus specific primers. The potential for using such procedures for routine diagnosis of grapevine viruses offers new opportunity for understanding the disease complexes and open new paths for a better control of plat diseases.

Hence, more advance techniques are evolving providing more sensitive detection and precision but the woody structure of the grapevine made diagnostic methods more difficult, extraction protocol are long and complex, and multi-virus infections are very common in grapevine, which affect the accuracy of the detection.

Our survey was carried out in the Research Institute for Viticulture and Oenology at Pecs, where various rootstock varieties were investigated. We could detect positive results for the presence of both GSyV-1 and GPGV in several samples, these newly described viruses were detected in Hungary for the first time and that characterization points at the importance of regular diagnostics studies, which help as a preventive measure to spot the light on pathogen disseminations and infection rates that are not visible but present at a molecular level.

II. LITERATURE REVIEW

1. Historical origin of Grapevine

Grapevine is one of the earliest domesticated crop from the Vitaceae family where the most important genus is *Vitis vinifera* subsp. *vinifera*, originated from the Near East and was domesticated about 6,000-8,000 years ago. Its wild ancestor is *Vitis vinifera* subsp. *sylvestris. However, the Vitaceae family is composed of 60 inter-fertile species which are predominantly* distributed in Asia, North America and Europe under subtropical, Mediterranean and continental–temperate climatic conditions *(*Alleweldt and Possingham, 1988; This et al., 2006; Wan et al., 2008; Teral et al., 2009; Mylesa et al., 2010). The majority of the cultivated grapevine varieties arose from the Eurasian species Vitis vinifera L.

2. Botany and Morphology of Grapevine

Grapevine is a perennial, polycarpic, and deciduous species (Keller, 2010). As a woody procumbent plant, it uses its tendrils to climb and elongate to branches bearing lobed leaves and clusters of flowers (Pandey et al., 1993).

The upper structure of the plant is called canopy, which can be shaped by training the vine into specific arragement for a favorable growth and a better production depending on the seasons and vine varieties (Hellman, 2003).

The rootstock

The vine can grow on its own rootstock (self-rooted vines) (Hellman, 2003), but in most cases grafted vines are prefered for their combination of desirables features. The scion variety that consist of the shoot portion that have the desirable fruit properties, and the rootstock which is mostly used for their resistance against phylloxera and mildews diseases. The positionning of the graft union is crucial when it comes to graft compatibility, where the vascular cambium of the stock and scion should be connected to each other so that nutrient and water channels work properly (Hellman, 2003; This et al., 2009; Keller, 2010).

The rootstocks used nowadays derived principally from a hybridization of three native North American species: *V. riparia*, *V. rupestris*, and *V. berlandieri* (Galet, 1998), that were primarily

introduced to Europe for their disease-resistanceproperties against (phylloxera, mildews) (This et al., 2009).

Root System

The rooting system of the vine is generally multi-branching and extends either horizontally or vertically. Grape roots can be affected by certain soil fungi *"mycorrhizae*" which decrease their growth and influence their nutrient uptake (Hellman, 2003).

Trunk and Shoot

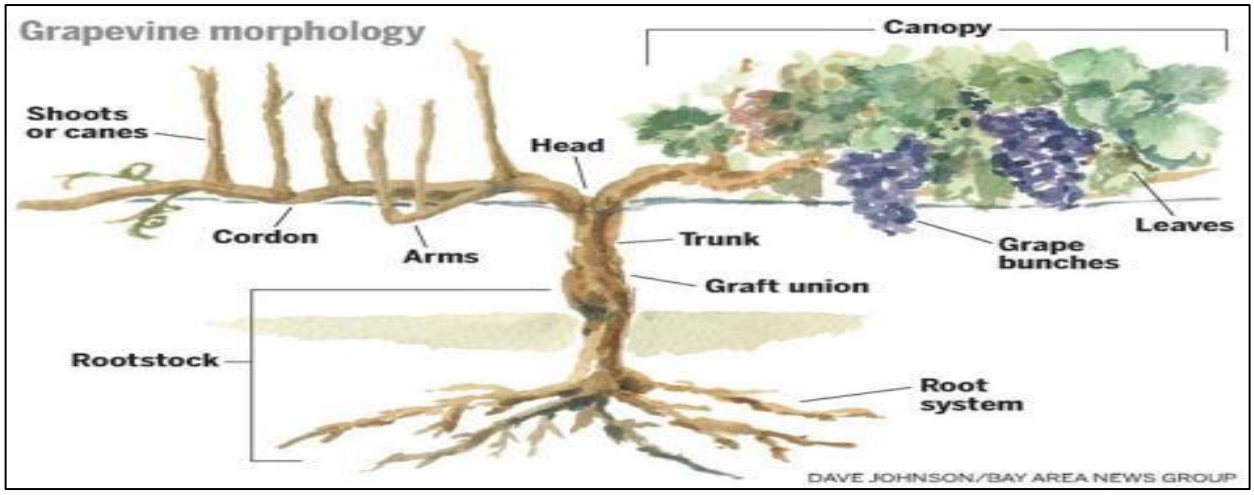
The aboveground section of the vine is formed by the trunk, the arms and shoots, they are termed "stem" by the botanists. Specific training systems are used to support shoot development; using a cordon and wire to support the trunk (Keller, 2010). Shoots generate tendrils that holds up other growing shoots (Mullins et al., 1992). Moreover, previous studies had suggested that tendrils are originally reproductive organs that have modified as climbing organs in the course of evolution (Calonje et al, 2004; Diaz-Requilme et al, 2009).

Leaf

The broad leaves of the vine plant are produced on the apical meristem. There are four categories of leaves: Cotyledons (embryonic leaves); Scales: grow around the buds; Bracts: small leaves found at branch points; foliage leaves (Keller, 2010).

Flowers and fruit

Most of *V. vinifera* cultivars embody perfect hermaphroditic flowers (Bourisquot et al., 1995) Depending on variety, a productive shoot generates about one to three flower clusters (Hellman, 2003).



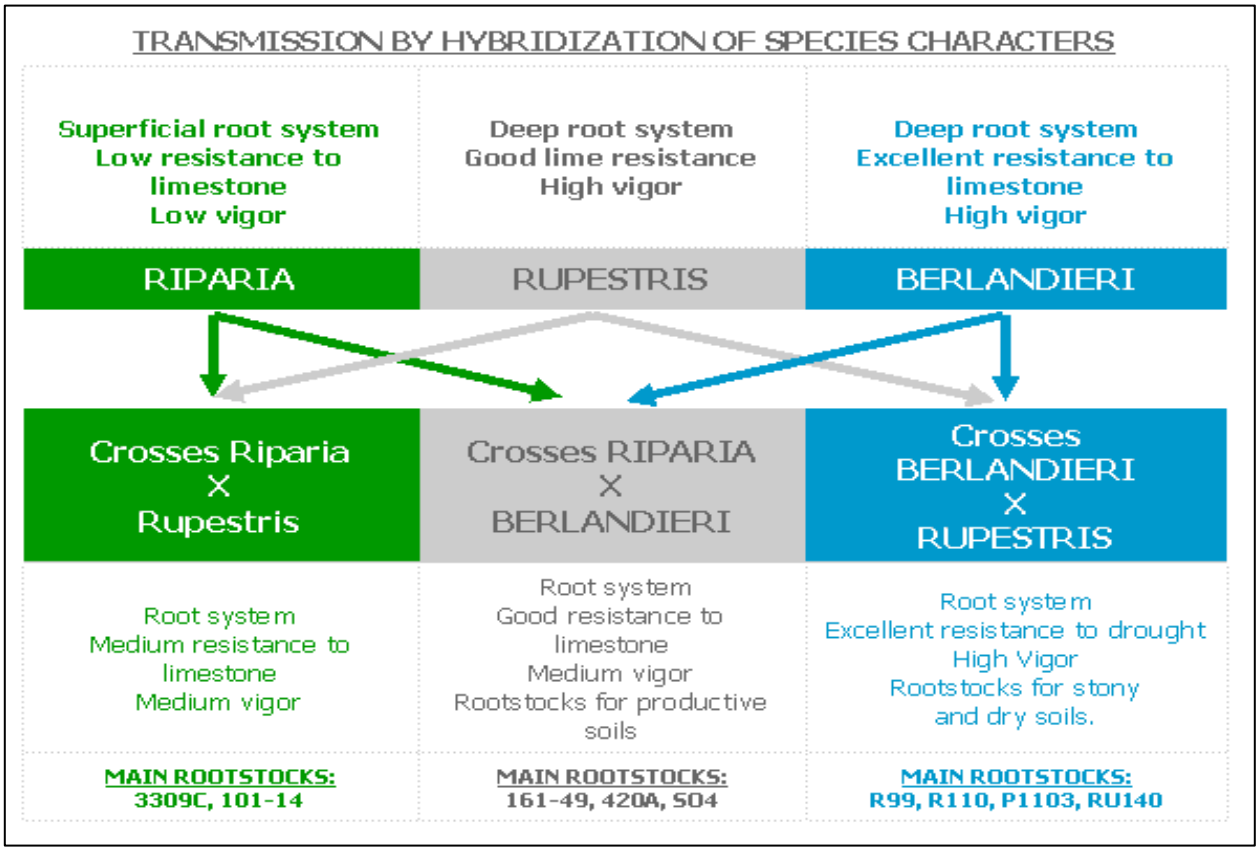
Source: http://www.mercurynews.com/2015/04/07/wine-trivia-anatomy-lesson-from-rootstalk-to-tendrils/

Figure 1: Grapevine morphology structure

3. Grapevine rootstock breeding and varieties

The main reason behind using rootstock in viticulture was to confer resistance against soil borne pests, the most importantly against phylloxera, . which was a key point in rootstock breeding programs (Cousins et al., 2007).

Rootstock breeding started when three breeders from France searched for *Vitis* wild species in North America that are resistant to phylloxera. *Vitis berlandieri*, *Vitis riparia*, *Vitis rupestris* were introduced to Europe but the ecological conditions were not favorable for the American wild species. Therefore, hybridization was initiated with the Eurasian varieties (*Vitis vinifera L.*) to decrease unfavorable traits (Reynolds, 2015). According to Keller (2015) "the genetic basis of the world is extremely narrow because as many as 90% of all *V. vinifera* vines are grafted to fewer than 10 different rootstock varieties, which threatens the vines from mutant strains of soil pests"



Source : http://www.richter.fr/en/rootstocks-vine.html

Figure 2: Principal hybridization strategies of North American rootstocks and their specific features

4. Hungarian rootstock breeding

The pioneer of Hungarian rootstock breeding Zsigmond Teleki (1854-1910) was the first who produced new rootstock varieties by crossing. He selected hybrid seedlings of hybrid seeds according to their resistance to phylloxera, lime, vine size and affinity between the rootstock and the scions (Reynolds, 2015). His varieties were spread around the world which are well known as:

- Teleki-Fuhr SO4,
- Teleki-Kober 5BB,
- Teleki 5C,
- Teleki-Kober 125 AA,
- Teleki 8B
- Teleki 10A (Csepregi and Zilai, 1955).

Following Teleki's footsteps, Bakonyi founded a rootstock variety collection from foreign and national rootstocks with the aim of identifying the unknown characteristics of potential rootstocks (Reynolds, 2015).

5. Description of new grapevine rootstock viruses

Among more than 60 grapevine infecting viruses, a widespread distribution of two newly described viruses was identified for the first time in Hungary named: Grapevine Syrah virus-1 and Grapevine Pinot Gris virus (Czotter et al., 2015).

5.1. Grapevine Syrah Virus 1 (GSyV-1)

Grapevine Syrah virus-1 is a member of the genus *Marafivirus* within the family *Tymoviridae*, and was firstly identified in 2009 in the United States. From that time, it was reported in several countries such as Chile, Brazil, France, Italy, and most recently Slovakia and the Czech Republic (Al Rwahnih et al., 2009; Glasa et al., 2015).

The symptoms caused by Syrah Decline were: swollen graft unions, cracking and pitting of the wood, stem necrosis, red discoloration of the leaves and scorching, vine decline, and death of the vines (Monis, 2009) as shown in Figure 3 and 4.

Figure 3: Symptom of Syrah decline (Reduced vigour and premature red discoloration of leaves)

Figure 4: Symptom of Syrah decline (Swelling at the graft union)

Adapted from (Coetzee.B, 2010)

Al Rwahnih and colleagues (2009) suggested that Grapevine rupestris stem pittingassociated virus (GRSPaV), Grapevine rupestris vein-feathering virus (GRVFV) and the recently described Grapevine Syrah virus-1 (GSyV-1) were the main causal agents of Shiraz decline.

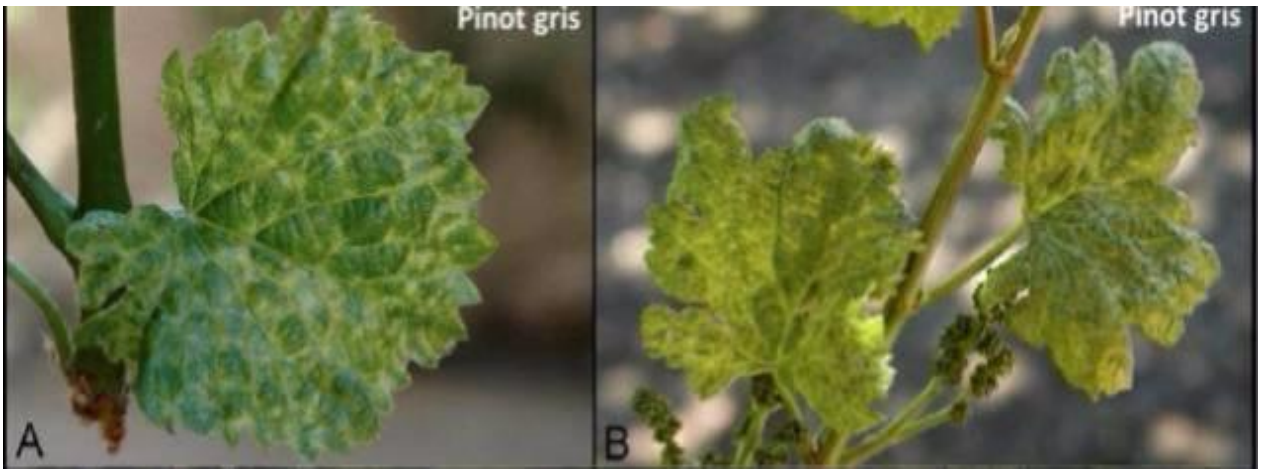
The presence of (GsyV-1) in Hungary was investigated for the first time by *NARIC, "*Agricultural Biotechnology Institute, Gödöllo", Hungary, and validated by RT-PCR using primers DetF and DetR (Al Rwahnih et al., 2009) and in 10 samples, originating from five grapevines the result was positive proven by the amplification of a 296 bp product (Czotter et al., 2015).

Furthermore, when the Hungarian GSyV-1 strain was compared with Slovakian and Czech strains; it revealed a 94-97% and 70-98% identity and, respectively, proving that there is a high variability in the European GSyV-1 strains (Glasa et al., 2015). In that context, twelve GSyV-1-derived PCR products were purified sequenced and the sequences were deposited in GenBank (Czotter et al., 2015).

5.2. Grapevine Pinot Gris Virus (GPGV)

Grapevine Pinot Gris Virus (GPGV) is a single stranded RNA (ssRNA) virus of the genus Trichovirus from the Betaflexiviridae family (Giampetruzzi et al., 2012).

The symptoms displayed by GPGV are similar to other viral diseases, chlorotic mottling, puckering and deformation of leaves which cause reducing in yield and decrease the quality of grapes, it was first observed in 2003, in Pinot Gris variety in Trentino vineyards (Italy) (Gualandri et al., 2016).



Source: http://www.vitisphere.com/actualite-83023-LInra-a-identifie-deux-nouveaux-virus-de-la-vigne-en-2015.htm

Figure 5: Symptoms of GPGV on Pinot Gris variety

However, GPGV is not restrained to Pinot Gris cultivar. It has been detected in several other varieties in Italy, France (Beuve et al., 2015), Slovenia (Mavrič Pleško et al., 2014), Slovakia (Glasa et al., 2014), South Korea (Cho et al., 2013) and many other countries were GPGV has been identified.

Some hypothesis suspected that GPGV can be transmitted from vine to vine by the eriophyid mite *Colomerus vitis* (Malagnini et al., 2015; Beuve et al., 2015) but still, not much research has been done on GPGV to ascertain its mode of transmission.

GPGV infected vine can be categorized in two genetic groups, a group which displays symptoms related to the disease, whereas the other group is symptomless, yet, the risks of the last one are less predictable and more exposed to attract combined infections. Through proteomics and molecular researches, it was found that the symptomless group has six extra amino acids in their movement protein, a distinction which l has no explanation yet (Habili, 2016).

6. Virus detection, prevention and novel virus technologies

Grapevine viruses are mostly disseminated in the vineyards by insect vectors like mealybugs, aphids, nematodes, or by infected propagating materials used by workers. Consequently, a proper sanitation of the vineyard has to be maintained by using insecticide to prevent the spread, including the usage of sterile materials during propagation operations, and discarding the infected vines in an appropriate quarantine (Coetzee, 2010).

It is therefore primordial to integrate efficient and sensitive detection methods to assess the most prevalent viruses (Martelli and BoudonPadieu, 2006). Although, it is more efficient to use new techniques to identify new emergent viruses to preserve the vineyards and varieties from infection and death.

6.1. Conventional diagnostic techniques

The routine methods used in grapevine virus detection are based on bioassays, serological tests such as enzyme-linked immunosorbent assay (ELISA) which rely on the interaction of the viral antigen and specific antibodies (Monis, 2011; 2012).

Molecular testing techniques are PCR based-protocols, the most used PCR is the reverse-transcription-polymerase chain reaction (RT-PCR), it targets the genetic material of the virus and relies on the amplification of a region of the viral genome using specific primers (Coetzee, 2010).

Although bioassays are widely used, ELISA test is a time-consuming process that does not represent a sensitive detection when there is a low virus concentration in the host tissues. RT-PCR assays are limited by inhibition of the reverse transcriptase or polymerase activity by compounds that were co-extracted with the nucleic acids leading to false negative results (Gambino and Gribaudo, 2006; Constable et al., 2010).

It is known that viral diseases are usually caused by a viral complex leading to a multiple infection in a single plant (Coetzee, 2010), which aggravates disease severity and intensifies symptom expression (Prosser et al., 2007). Therefore, several advanced procedures have been established to solve the inconveniences that could not been resolved by the basic methods.

6.2. Innovative diagnostic techniques

- **Deep (Next generation) sequencing**

Next -generation sequencing is a powerful diagnostic tool that can identify a plant virus with no prior information of the virus, by analyzing small interfering RNA (siRNAs) (Kreuze et al., 2009), or fragmented double-stranded RNAs (dsRNAs) from sequenced libraries sampled from viral infected plant tissue (Al Rwahnih et al., 2009; Coetzee et al., 2010). It was firstly introduced in 2005, when the initial NGS platform was commercially available, the FLX Genome Sequencer by 454 Life Sciences (Margulies et al., 2005)

Different platforms were developed and used in grapevine virus diagnostics, where they have shown important results, like, identifying new grapevine viruses: Roche 454 for Grapevine Syrah-1 virus (Al Rwahnih et al., 2009),

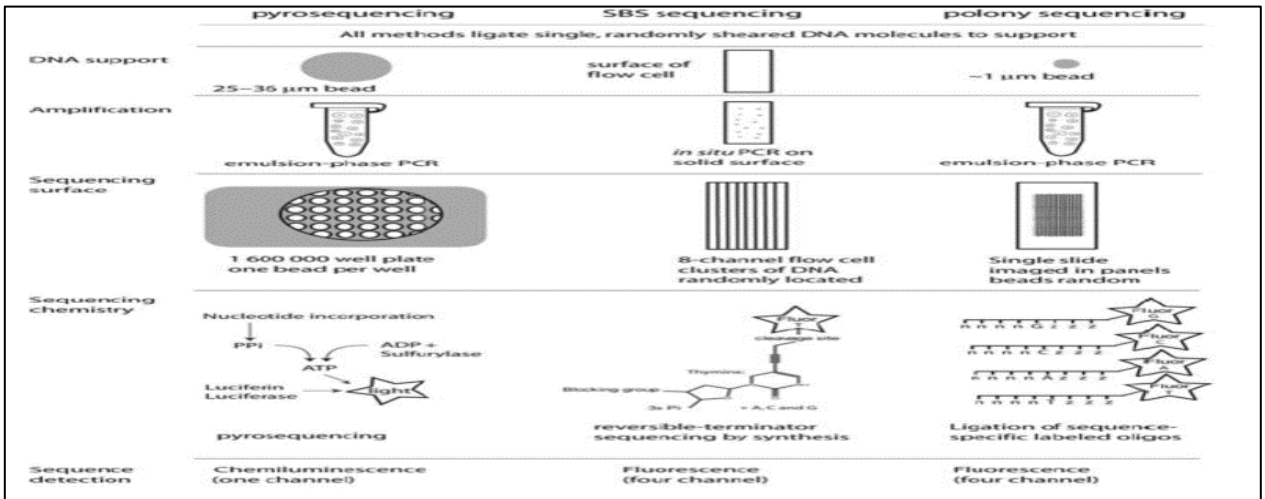


Figure 6: General comparison of the sequencing technologies from the three next-generation sequencing platforms: 454/Roche, Illumina and ABI SOLiD (Adapted from Hudson, 2008).

Next-generation sequencing is a time-cost saving technology (Harismendy et al., 2009; Mardis, 2008) that makes multiple viruses sequence detection possible by using universal adaptors instead of sequence-specific primers (Coetzee, 2010).

All the three systems have shown reliable results, however, each of them has specific qualities and inconvenience that differently contributes to the resulted output (Coetzee, 2010).

Recently, the NGS bears witness to a remarkable expansion by providing good sequence quality, read length and total data output, making next-generation sequencing specifically suitable for metagenomic sequencing (Adams et al., 2009; Coetzee, 2010).

- **Metagenomic sequencing**

Metagenomics is an approach that was first used to analyze microbial populations in a sample by examining the nucleotide sequence content (Edwards et al., 2006). It was found that metagenomics can open new possibilities to pathogen detection in the diagnostic plant virology field, by overcoming parallel screening methods and the non-specificity of traditional techniques (Adams et al., 2009).

Because there are no universal viral genes or sequences, metagenomic methods should use non-specific-sequences, in that context, several methods have been developed to improve the viral sequences in collected samples which will be sequence analyzed directly or after an amplification step (Roossinck et al., 2015).

Roossinck et al., (2015) emphasized the key feature of implementing plant virus metagenomics studies is that it can trace specific geographical locations of the virus and provide further characterizations within their eventual original host.

Through viral metagenomics, unpredictable putative mode of plant-virus transmission has been disclosed, out of the common plant-virus-insect synergy, where many secondary other vectors should be taken in consideration such as Large herbivores (Ng et al., 2014), bats (Donaldson et al., 2010), rodents (Phan et al., 2011), or irrigation water (Hamza et al., 2011).

II. MATERIALS AND METHODS

In this survey, samples of 34 rootstock varieties of grapevine (Table 1) were collected at the vinicultural region of Pécs, and the presence of two newly discovered grapevine viruses were investigated. RNA was extracted from the samples; cDNA pools were synthesized from RNA samples for the detection of viruses using PCR with virus-specific primers that specifically hybridize to the target sequence. The amplification products were visualized by gel electrophoresis.

1. Sample collection and origin

The samples were collected on 27 July of 2015 in the Research Institute of Viticulture and Enology of Pécs. Samples represented different parts of the plants: shoot tip, old leaf, young leaf, flower, and tendril of each vine rootstock.

Figure 7: Picture of the investigated vineyard at the Research Institute of Viticulture and Enology of Pécs

Table 1: Names of the rootstock varieties of the collection of Pécs.

n°	Rootstock variety name		
1.	Teleki 8B	18.	Teleki-Fuhr S. O.4
2.	Teleki 5C I	19.	Teleki 5C Gm. 6
3.	Teleki-Kober 5BB	20.	Teleki 5C Gm. 10
4.	Szilagyi 157 Pécs	21.	Teleki-Kober 5BB Gm. 13
5.	Riparia portalis	22.	Teleki-Kober 5BB Wei.48
6.	Rupestris du Lot	23.	Teleki 5C wed.
7.	Rupesris metallica	24.	Teleki-Kober 5BB Fr. 148
8.	Chasselas x Berlandieri 41 B M. et de G	25.	Teleki-Kober 5 BB
9.	Aramon x Rupestris G.1	26.	Teleki 5C II
10.	Aramon x Riparia 143 B M. et de G.	27.	Teleki 5C P
11.	Mourvédre x Rupestris 1202 C.	28.	Teleki-Kober 5 BB P XII.4
12.	Rupestris x Berandieri T .10A	29.	Teleki-Kober 5 BB P XVIII.37
13.	Solonis x Riparia 1616 C	30.	Teleki-Kober 5BB Cr 2.
14.	Golia	31.	Borner
15.	Galiardo	32.	Fercal
16.	Riparia x Rupestris 101 – 14 M et de G.	33.	Richter 110
17.	Riparia Martin de Perrier	34.	Richter 140

2. RNA extraction

2.1. CTAB protocol

For RNA isolation Cetyltrimethylammonium bromide (CTAB) based protocol of Gambino and coworkers (2008) was used.

The solutions used in the CTAB protocol consisted of:

Extraction buffer:

- 2% CTAB (hexadecyltrimethylammonium-bromide),
- 2.5% PVP (polyvinylpyrrolidone),
- 100 mM Tris- HCL (pH = 8.0),
- 25 mM EDTA,
- 2 M NaCl.

SSTE:

- 1 M NaCl,
- 0.5% SDS,
- 10 mM Tris- HCL (pH= 8.0)
- 1 mM EDTA

- The extraction buffer was heated at 65°C in a water bath.
- 850 µl extraction buffer was measured to labelled 2 ml microcentrifuge tubes, the tubes were placed into Thermo block at 65°C.
- Plant samples (old leaf, young leaf, flower, and tendril) of each rootstock variety (about 150/200 mg tissue) were homogenized in a mortar with the extraction buffer and 17 µl ß-mercaptoethanol.

Then the homogenized tissues were transferred to microcentrifuge tubes and vortexed thoroughly.

- Samples were incubated at 65°C in water bath for 10 minutes, and vortexed at least once.
- 850 µl chloroform-isoamyl alcohol was added to the samples and inverted for few times.
- The tubes were centrifuged at 10,.000 rpm for 10 minutes at 4°C.
- The upper phase (supernatant) of the solution was transferred to new labelled tubes containing 800 µl chloroform-isoamyl alcohol and centrifuged at 10,000 rpm for 10 minutes at 4°C
- Meanwhile new 1.5 ml micro centrifuge tubes were labelled and 250 µl LiCl was added into them.
- After centrifugation, the upper phase was transferred to the LiCl containing 1.5 ml micro centrifuge tubes, followed by a few inversions.
- Tubes were kept on ice for 30 minutes.
- Than samples were centrifuged at 13,000 rpm for 20 minutes at 4°C and the supernatant was discarded.
- The pellet was resuspended in 450 µl SSTE solution preheated to 65°C. Than equal volume of chloroform-isoamyl alcohol was added, with a brief inversion.
- The samples were centrifuged at 10,000 rpm for 10 minutes at 4°C.

- New 1, 5 ml Eppendorf tubes were labelled we measured 280 µl izopropanol and 30 µl 4 M Na acetate into them.
- The supernatant of the previous centrifugation was transferred into them, and centrifuged at 13,000 rpm for 20 minutes at 4°C.
- The supernatant was discarded and pellets were washed with 1 ml 70% cold ethanol and centrifuged at 13,000 rpm for 5 minutes at 4°C or room temperature
- The supernatant was discarded, and the pellet was dried for 10 minutes in speed vac.
- The pellet was resuspended in 25 µl sterile water and vortexed gently.

2.2. Examination of the extracted RNA

RNA products were detected by 1.2% agarose gel electrophoresis in TE buffer, stained with ethidium bromide and visualized under UV light.

1. RNA samples were prepared by mixing 3 µl of the extracted RNA with 5 µl FDE loading dye and 2 µl sterile water.
2. Denaturation was made at 65°C for 5 min
3. Samples were separated by gel-electrophoresis testing 10 µl from each RNA sample on 1.2% agarose gel.

Quantification of the samples was determined using NanoDrop spectrophotometer.

3. cDNA synthesis

3.1. Conception of gene pools

RNA samples from different organs (old leaf, young leaf, flower, and tendril) of the same rootstock variety were assembled into one pool, creating 34 RNA pools.

In order to have a proper starting material for cDNA synthesis we selected only the best samples, using only RNA extracted from young leaves and flower for their higher RNA content. The prepared RNA pools were centrifuged and vortexed and their RNA concentration was measured using NanoDrop spectrophotometer.

3.2. First strand cDNA synthesis protocol

The “Revert Aid™ First Strand cDNA Synthesis Kit” was used to synthesize first strand cDNA from total RNA template.

1. Starting by adding the following reagents into a sterile nuclease free tube on ice:
   - 0.25 µl random hexamer (RH) primer
   - 0.5 µg template RNA
   - Adding water until 3, 12 µl
   -Chilling on ice and spinning down
2. Incubation at 65°C for 5 minutes

   Chilling on ice, spinning down and placing the vial back on ice
3. Prepare reaction mixture by adding the following components for each vial:

   - 1 µl 5X Reaction buffer
   - 0.5 µl 10 mM dNTP
   - 0.13 µl ribolock RNase inhibitor (protecting RNA templates from degradation)
   - 0.25 µl Revert aid reverse transcription enzyme (a recombinant M-MuLV RT which maintains activity at 42-50°C and is suitable for synthesizing cDNA up to 13 kb)

The reaction was mixed gently and centrifuged briefly.

4. Incubation of the reaction mix was carried out as follows:

   - 25°C for 10 min
   - 42°C for 50 min
   - 45°C for 10 min
   - 70°C for 5 min

The resulted cDNA was stored at -20 ºC.

4. Control PCR amplification

To test the quality of the cDNA product a control amplification of a grapevine endogen gene: actin was performed using "Phire Green Hot start II DNA polymerase" as follows:

- Diluting an aliquot of the cDNA generated by 10x,

- Gentle vortexing and brief centrifugation of all PCR reagents after thawing.

- Preparing reaction mixture by adding the following reagents in each tube:

- 6.1 µl water
- 2 µl 5X Phire Green Reaction Buffer
- 0.5 µl primer A (Vv actin 601 s)
- 0.5 µl primer B (Vv actin 1200 as)
- 0.2 µl 10 Mm dNTP
- 0.2 µl Phire Hot Start DNA Polymerase
- 0.5 µl template 10x RT

PCR program was performed in a thermal cycler according to the following steps (Table 2):

Table 2: Control PCR amplification program

Step	Temperature °C	Time	Number of Cycle
Initial Denaturation	98	30 s	1
Denaturation	98	10 s	35
Annealing	55	10 s	
Extension	72	20 s	
Final extension	72	1 min	1
Hold	4	∞	-

The cDNA product was screened by gel electrophoresis using 1.2% agarose gel.

5. Virus diagnostics of Grapevine Syrah Virus1 (GSyV1) and Grapevine Pinot Gris (GPGV)

5.1. Reverse transcription PCR Procedure

The two viruses Grapevine Syrah virus-1 (GSyV-1) and Grapevine Pinot Gris (GPGV) were investigated by RT-PCR reaction using the same "Phire Green Hot start II DNA polymerase protocol" as for the previous "Actin test". Accordingly, two pairs of virus-specific primers (forward and reverse) were added to the PCR master mix and measured out into each of the 34 cDNA pools.

Table 3: Virus-specific primers of Grapevine Syrah virus1 (GSyV1) and Grapevine Pinot Gris GPGV used for the RT-PCR diagnostics

Virus	Primer name	Primer sequence (5'-3')	At/Tm (C°)	Fragment Length (nt)	Gene	Reference
GSyV-1	Det-F Det-R	CAAGCCATCCGTGCATCTGG GCCGATTTGGAACCCGATGG	nd	296	putative movement protein	Al Rwahnih et al. 2009
GPGV	GPG6609F GPG7020R	GAGATCAACAGTCAGGAGAG GACTTCTGGTGCCTTATCAC	56	412	coat protein	Glasa et al. 2014

5.2. Virus detection procedure

Electrophoresis technique was applied to analyse and separate the different DNA product of RT-PCR reaction in order to detect virus specific products.

a. Preparing and running agarose gel

The equipment and supplies necessary for conducting agarose gel electrophoresis include:

- An electrophoresis chamber and power supply
- Gel casting trays
- Sample combs
- Electrophoresis buffer Tris-borate-EDTA (TBE).
- Loading buffer
- Ethidium bromide, a fluorescent dye used for staining nucleic acids.
- Trans illuminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels.

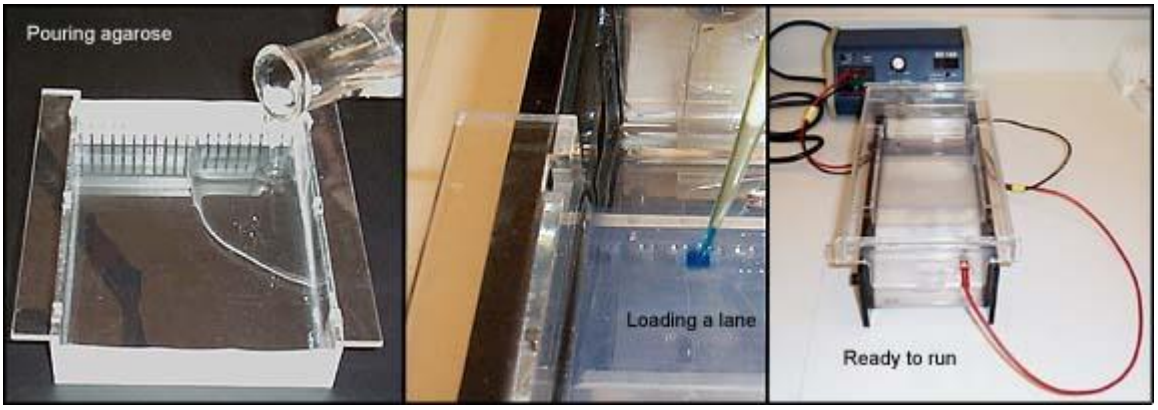
b. Preparation of the agarose gel

A quantity of 3.6 g agarose was incorporated to 300 ml of 1x TBE (Tris base, boric acid, EDTA), and heated in a microwave oven at 30s intervals until completely melted. 0.7 µl (10 µg/µl) of ethidium bromide is added to 25 ml of agarose gel to facilitate the visualization of DNA after electrophoresis. After cooling down the gel is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

c. Running the agarose gel

After the gel, has solidified, it was inserted into electrophoresis chamber and covered with 1xTBE buffer. Samples containing DNA mixed with 1µl loading buffer are then pipetted into the sample wells. In the first lane of gel a molecular weight ladder was applied which later indicated the corresponding molecular weight of the RT-PCR product. While the others wells were loaded with DNA samples to be investigated. The gel was run at 80-150 V.

Figure 8: Major steps of electrophoresis technique



Source: http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/gelrun.jpg

d. Visualization of DNA fragments migration

When the dye line was about at 75-80% of the way down the gel, the electrophoresis was completed, power supply turned off and the lid of the gel box was removed.

Finally, for screening and observing the migration of the DNA fragments "Bio-RAD chemidoc MP imaging system" was used.

IV. RESULTS AND DISCUSSION

1. Analysis of the results

1.1. RNA extraction screening

The results of the RNA extraction were screened and only the ones with high amount of intact RNA were selected for the virus diagnostics. These are supposed to have a higher cellular activity like young leaves, flower and tendrils.

All the 34 rootstock samples were screened, and RNA concentrations were measured with Nano Drop Spectrophotometer. Plant n°15 namely variety Galiardo was taken as an example in Figure 9, where we can observe clear appearance of the rRNAs. In table 4 RNA concentration of extracts (measured by Nano Drop Spectrophotometer) of the same plant is listed.

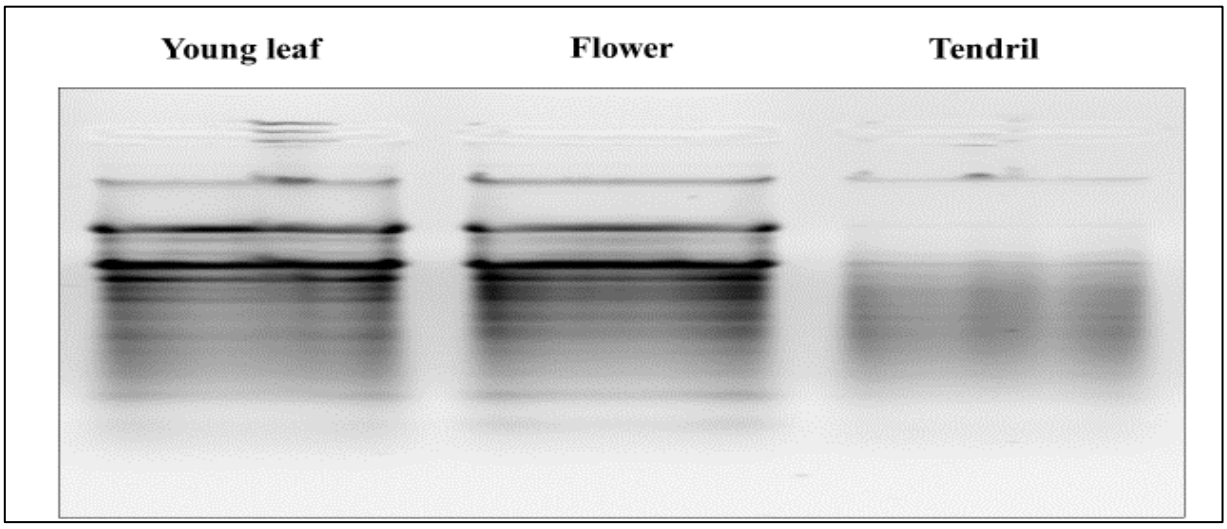


Figure 9: Screening of the RNA extraction results of plant n°15 (Galiardo)

Table 4: RNA concentration of Plant n°22 measured by NanoDrop Spectrophotometer

Rootstock Sample		RNA concentration (ng/ml)
Rootstock n°15 (Galiardo)	Young leaf (YgL)	404.8
	Flower (Flw)	706.7
	Tendril (Tdr)	245.3

1.2. The cDNA synthesis and quality test

The cDNA synthesis was carried out using a random hexamer primer to produce complementary DNA from RNA of the 34 rootstock samples.

To test how successful the cDNA synthesis was, an RT-PCR was carried out with actin specific primers amplifying a 599 bp product from the endogenous grapevine actin gene. The Figure 3 shows that a distinct 599 bp PCR product is present in all the investigated samples and a negative response in the control (C-) which signify that the cDNA synthesis is successfully achieved.

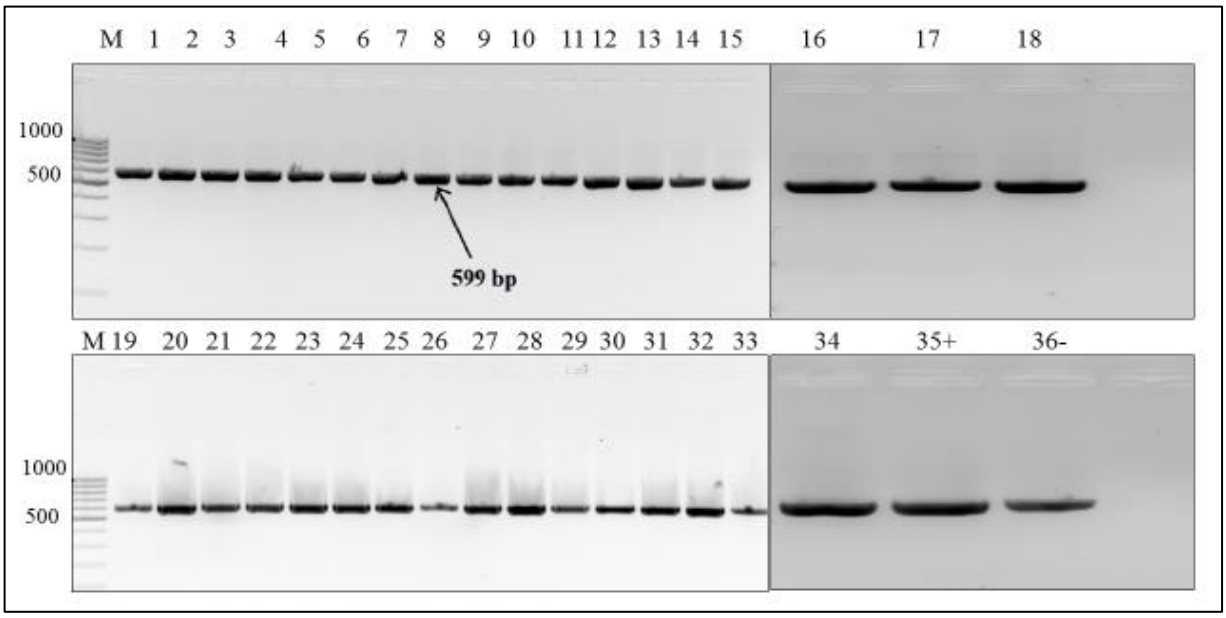


Figure 10: Results of the cDNA test (Actin test)

After completing cDNA synthesis, the presence of viruses was investigated separately, widespread viruses were covered by Fakhreddine Houhou in his thesis "Virus diagnostic survey of grapevine rootstock varieties from the stock collection of Pécs".

On the other hand, newly described viruses in Hungary which are Grapevine Syrah virus 1 (GSyV1) and Grapevine Pinot Gris virus (GPGV) were invastigated next in my thesis.

1.3. Virus diagnostics results of Grapevine Syrah virus 1 (GSyV1) and Grapevine Pinot Gris virus (GPGV)

To identify the presence of the two new viruses, virus specific primers were used in the RT-PCR. Reliable cDNA sequence from previous RTs were used as positive control

1.3.1. Grapevine Syrah virus (GSyV-1) detection

GSyV-1 was detected as a 296 bp product shown in Figure 11, samples n° 3,4, 5, 8, 11, 12, 16, 18, 19, 20, 22, 23, 25, 26 showed its presence, whereas, the negative control (36-) is clean.

This case confirms the presence of this newly described virus in Hungarian grapevines at a high rate, touching 14 of the 34 rootstock varieties.

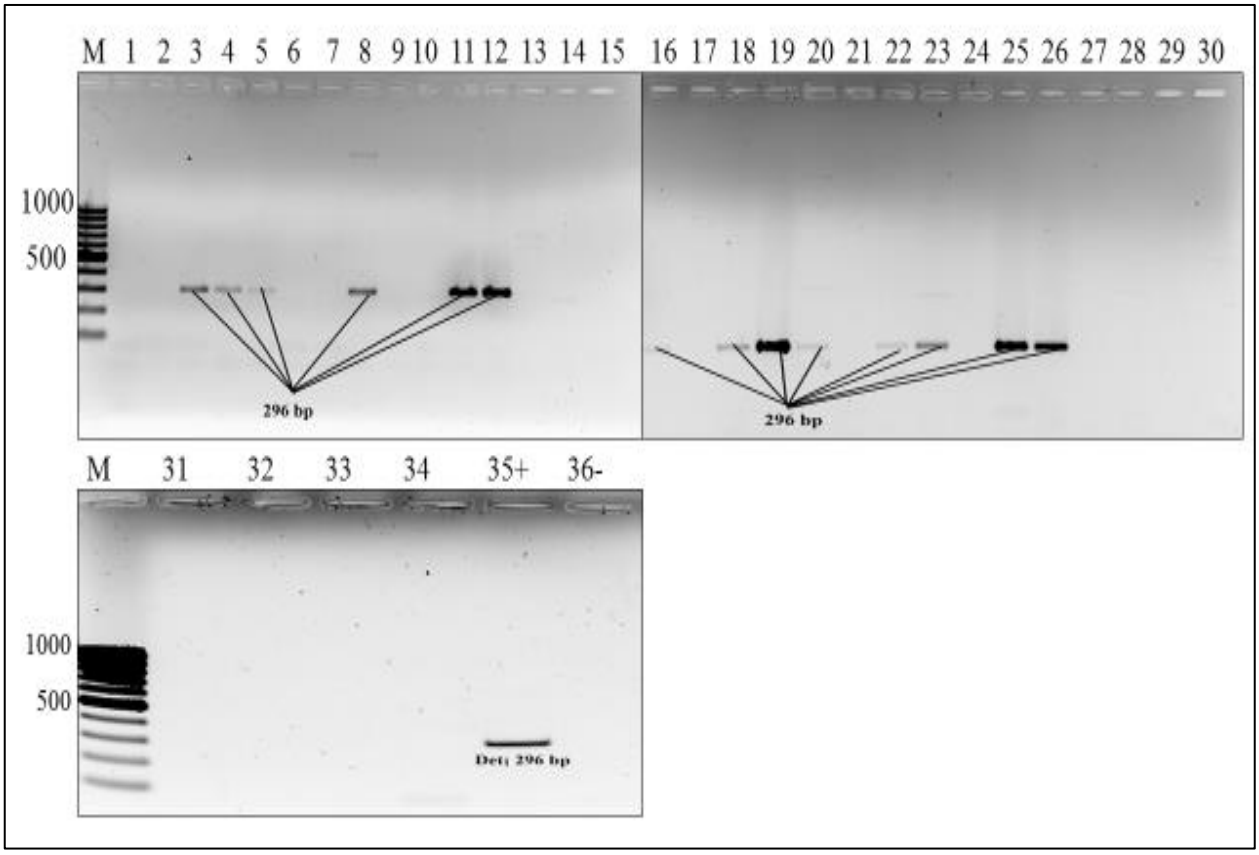


Figure 11: Screening of GSyV-1 detection in rootstock samples

1.3.2. Grapevine Pinot Gris (GPGV) detection

The presence of the virus infection is clearly visible in most of the samples (Figure 12 except for n°2, 5, 20, 33, 34. We can observe that a 411 bp fragment is present in the positive control while the negative control is clear from the viral fragment, however an insignificant lower weight fragment is visible which does not interfere with the accuracy of the results.

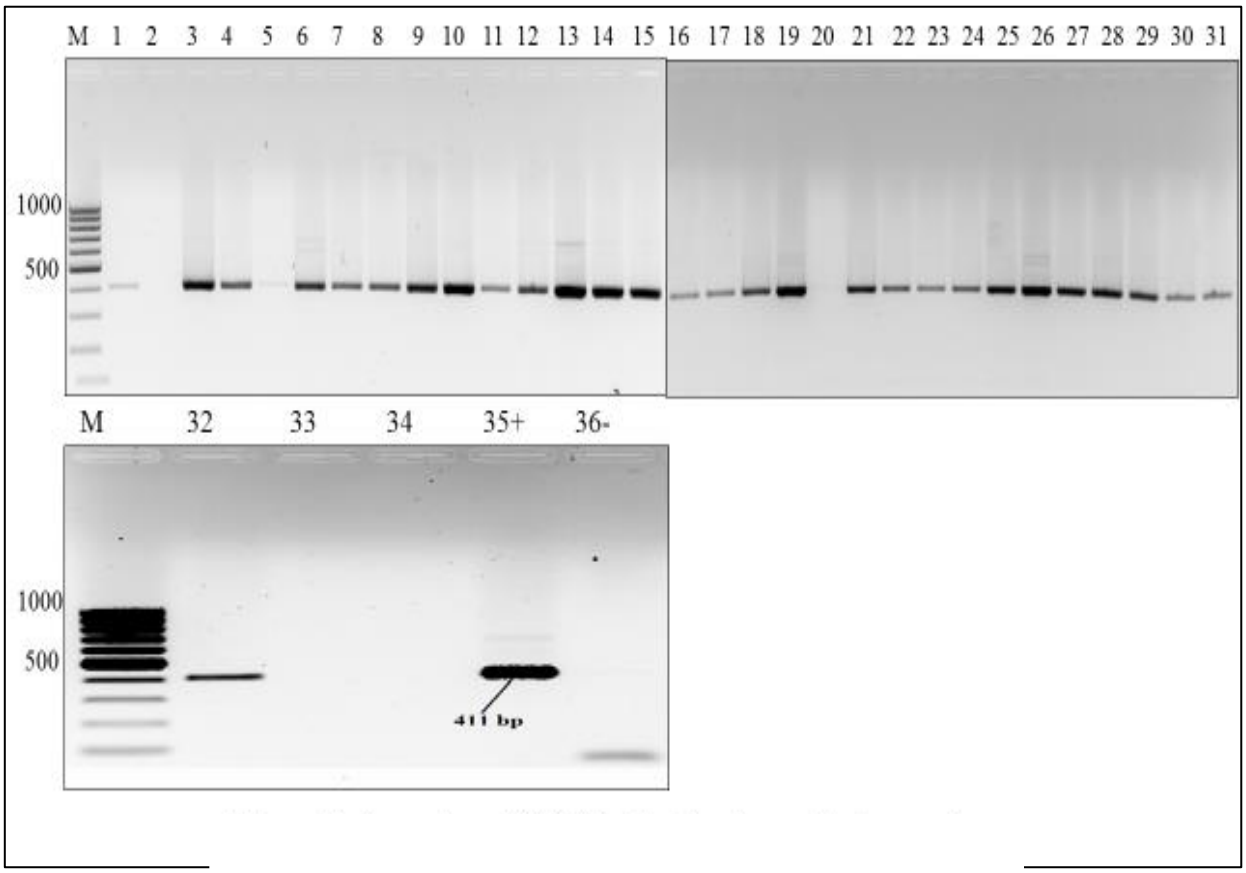


Figure 12: Screening of GPGV detection in rootstock samples

2. Conclusions

To start the diagnosis, RNA was extracted and only the young leaves and flowers were used for further investigation. Gene pools were created to facilitate the analysis of each rootstock variety.

A cDNA synthesis was necessary to amplify virus specific primers from the plant samples using the RNA as a template, random hexamer primers, and a DNA reverse transcriptase.

The presence of two new viruses was investigated on 34 rootstock varieties, to have a complete diagnosis on the rate of infection and the sensitivity of the rootstock toward the viruses.

n°	Specific virus / Rootstock variety	GSyV-1	GPGV
1	Teleki 8B	-	X
2	Teleki 5C I	-	-
3	Teleki-Kober 5BB	X	X
4	Szilagyi 157 Pécs	X	X
5	Riparia portalis	X	-
6	Rupestris du Lot	-	X
7	Rupesris metallica	-	X
8	Chasselas x Berlandieri 41 B M. et de G	X	X
9	Aramon x Rupestris G.1	-	X
10	Aramon x Riparia 143 B M. et de G.	-	X
11	Mourvédre x Rupestris 1202 C.	X	X
12	Rupestris x Berandieri T .10A	X	X
13	Solonis x Riparia 1616 C	-	X
14	Golia	-	X
15	Galiardo	-	X
16	Riparia x Rupestris 101-14 M et de G	X	X
17	Riparia Martin de Perrier	-	X
18	Teleki-Fuhr S. O.4	X	X
19	Teleki 5C Gm. 6	X	X
20	Teleki 5C Gm. 10	X	-
21	Teleki-Kober 5BB Gm. 13	-	X
22	Teleki-Kober 5BB Wei.48	X	X
23	Teleki 5C wed.	X	X
24	Teleki-Kober 5BB Fr. 148	-	X
25	Teleki-Kober 5 BB	X	X
26	Teleki 5C II	X	X
27	Teleki 5C P	-	X
28	Teleki-Kober 5 BB P XII.4	-	X
29	Teleki-Kober 5 BB P XVIII.37	-	X
30	Teleki-Kober 5BB Cr 2.	-	X
31	Borner	-	X
32	Fercal	-	X
33	Richter 110	-	-
34	Richter 140	-	-

Table 5: The rate of infection of the rootstock varieties invastigated

Table 5 summarizes the results found in the rootstock collection. We can observe significant rate of infection where 94% of the rootstocks were touched by either a single or a double virus infection. For all that, only two rootstocks survived the virus epidemic that are both Richter variety (110 and 140), which can demonstrate a kind of resistance toward both GSyV-1 and GPGV.

Grapevine Syrah Virus -1

The disease was detected in 14 varieties which are not related to each other, meaning that the spreading did not take place on the field but the plants were already infected at the place of origin which is unknown.

Grapevine Pinot Gris Virus

As it is firstly identified in Hungary by the ABC diagnostics laboratory GPGV was found in different grapevine plantations in several parts of the country.

The samples investigated in the region of Pécs confirm the alarming situation and the rapid spread of this virus which touched 29 individuals from the collection.

The transmission was probably conducted using infected propagated materials which can further spread if the use of sterile material will not be managed. In this case, it is an open question whether the appearance of GPGV can be connected to specific virus symptoms or not.

However, the high rate of GPGV in the rootstock plantations can cause further issues on new varieties that might evolve into new strains with severe symptoms.

V. SUMMARY

The grapevine is one of the most cultivated horticultural crops that is permanently targeted by viruses due to its continuous vegetative propagation. However, visual detection of a prospective infection is not always noticeable, depending on the virus strain and the host plant, the infection may occur showing symptomless hosts, which can make great damages without having an idea on the cause. Therefore, early and regular detection surveys are essential for sustaining virus free vineyard.

The investigation was performed on 34 collection of grapevine rootstocks with the aim of finding a prospective presence of two viruses named Grapevine Syrah virus 1 (GSyV1) and Grapevine Pinot Gris virus (GPGV) that are investigated for the first time in Hungary.

After extracting RNA from all the samples using CTAB protocol (Gambino et al., 2008), RNA was converted to cDNA libraries for each variety and then, amplified by RT-PCR as the main diagnostic tool, virus-specific primers were utilized for each of the two viruses to detect the viral fragments and screened by gel electrophoresis.

The final results were set out, and positive results were found in most of the samples, Grapevine Syrah Virus -1 was detected in 14 varieties and Grapevine Pinot Gris Virus in 29 varieties out of 34 rootstock collection. The numbers presented are alarming and exhibit the severity of the dissemination in the vineyard.

The cause of the infections is still unknown but we can suggest that for GSyV-1 the plants were infected from the place of origin because the plants were planted far from each other, whereas for GPGV the spreading might have been caused mechanically by infected materials used for propagation.

From these case study, we can perceive the importance of strict plant sanitary regulation and sterile materials, lack of which can cause severe damages.

As for the detection procedure, every step of the protocol was detrimental to the final output, including the quality of the plant samples, the sterility level undertaken during laboratory work as well as the efficiency of the detection method chosen. RT-PCR have provided relatively accurate and clear results on the level of infection.

RECOMMENDATION

Conventional detection methods such as RT-PCR present a broad screen when investigating known viruses, thus these techniques suffer from significant drawbacks, especially when used in diagnostics of new uncharacterized viruses. In that framework, advanced techniques were developed such as Next-generation sequencing which steps up the efficiency in virus diagnostic, combining metagenomic analysis and deep sequencing that has successfully identified known and unknown viruses from long or short reads (Capobianchi et al., 2013).

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Summary

Thesis title: Investigation of the presence of two newly described grapevine viruses in grapevine stock collection at Pécs.

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1. **Primary thesis adviser** (Erzsébet Kiss, Professor, Institute of Genetics and Biotechnology, Szent István University, Faculty of Agricultural and Environmental sciences, Gödöllő)

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Among the 60 viruses and viroids that can infect grapevine, only a portion of them has been detected and put under investigation. When regular and proper diagnostic methods are lacking virus infections can occur without prior knowledge of the causal agent resulting in an uncontrollable dissemination of the virus in the vineyard.

The purpose of our survey was to investigate, newly described viruses in a rootstock collection located in the Research Institute of Viticulture and Enology at Pécs using a molecular detection method. The analysis covered 34 rootstock varieties, which were sampled and then proceeded to RNA extraction by CTAB protocol. After that the samples were converted to cDNA pools for each rootstock variety, and amplification of a virus specific product was carried out using RT-PCR. The final results validated the widespread presence of two viruses Grapevine *Pinot Gris virus and Grapevine Syrah virus1* never described before in Hungary only by our group. We could conclude that the investigation as a complete detection method provided accurate and sensitive results, which affirm the importance of molecular tests in virus diagnostics.